Chronic Lung Allograft Dysfunction After Lung Transplantation

Novel insights into immunological mechanisms

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Chronic Lung Allograft Dysfunction After Lung Transplantation

Novel insights into immunological mechanisms

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(met een samenvatting in het Nederlands)

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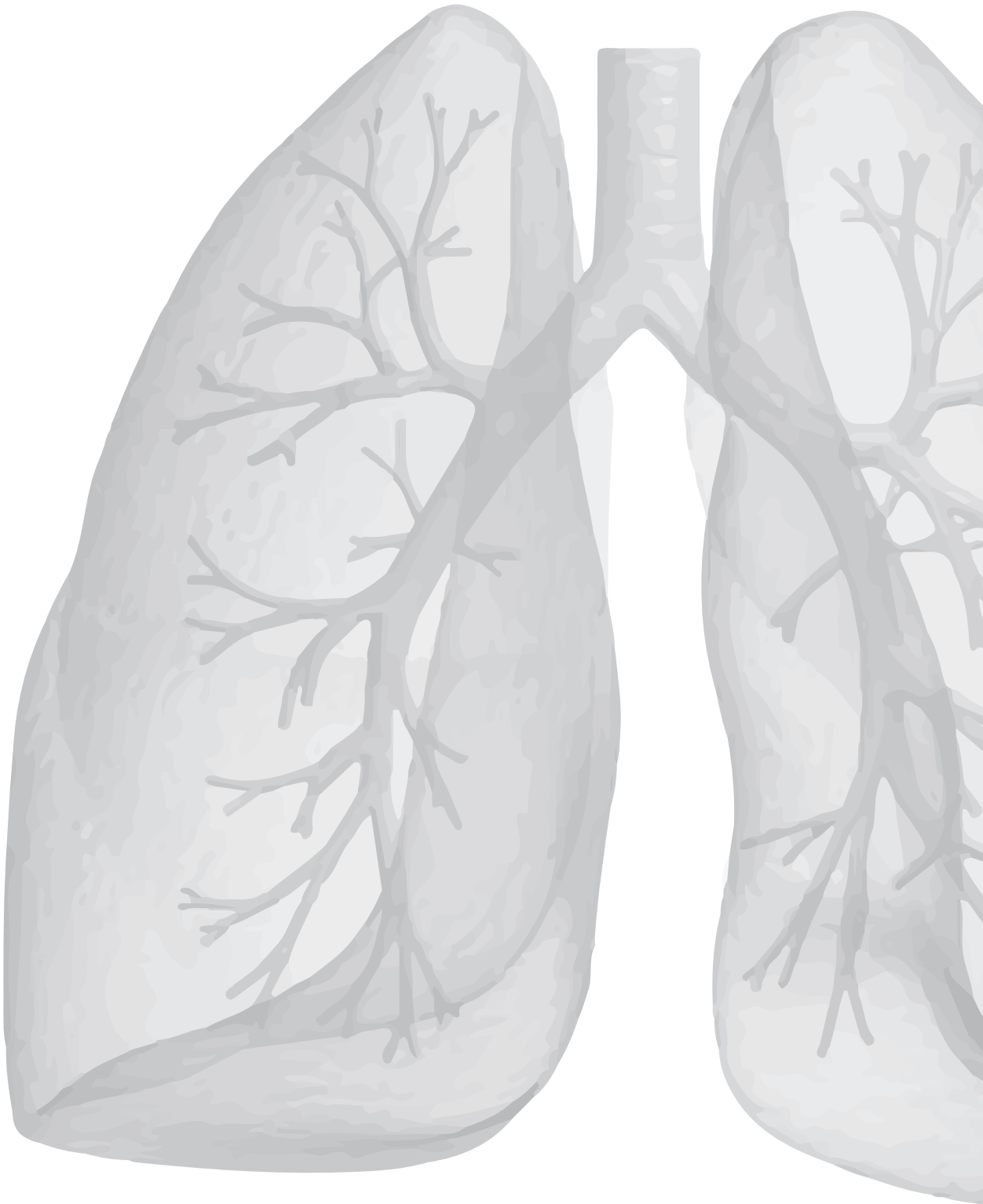

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General introduction

Since the first lung transplantation (LTx) was conducted in 1963, this procedure has become the major clinical intervention for patients suffering from end-stage lung diseases, including chronic obstructive pulmonary disease, cystic fibrosis, and interstitial lung disease[1,2]. Both surgical advancements, progress in immune surveillance and understanding of rejection pathogenesis, has resulted in an increase in patient survival. Despite these improvements, long term survival is mainly hampered by chronic rejection and over the years studies have shown that 5 year survival after LTx is only 53% and 10 year survival 31%, making LTx the least successful solid organ transplantation[3,4].

Chronic rejection after lung transplantation

Three forms of rejection are described. Hyper acute rejection arises within minutes after transplantation due to pre-existing donor specific human leukocyte antigen (HLA) antibodies that induce complement-mediated cell damage in the grafted organ. However, case reports on hyper acute rejection after LTx are limited, especially compared to kidney transplantation, likely reflecting differences in HLA sensitization rate in both conditions[5]. The risk for developing acute rejection is the highest during the first few months after transplantation. Acute rejection is predominantly mediated by cellular responses, in which antigen presenting cells recognize foreign MHC molecules or processed self-peptides of the graft, and subsequently induces an immune response against the grafted organ[6,7]. This type of rejection is well-controlled by current immune suppressive regimens.

The third form of rejection is chronic lung allograft dysfunction (CLAD), which is a main determinant of long-term survival after LTx. Since 2010 two major forms of CLAD are discriminated, obstructive CLAD (bronchiolitis obliterans syndrome, BOS) and a restrictive CLAD (restrictive allograft syndrome, RAS). Clinically BOS is characterized by obstructive pulmonary function tests (Forced Expiratory Volume in 1 second, $FEV_{1,}$ <80% from baseline FEV_1) persisting longer than 3 weeks, whereas RAS is characterized by restrictive pulmonary function tests (Forced Vital Capacity, FVC, <80% from baseline FVC) also persisting longer than 3 weeks[8].

BOS following LTx was first described in 1984 by Burke *et al.* from Stanford University[9]. BOS is hallmarked by excessive fibrosis, extracellular matrix deposition, bronchial epithelial cell loss, and formation of scar tissue which all lead to small airway obliteration resulting in decreased lung function and eventual lung failure[10] (Figure 1). Bronchiolitis obliterans is a focal process within the lung and due to the easily induced tissue damage biopsies are not often taken. Therefore, a decline >20% in the FEV_1 in the absence of any other disease etiology has become the clinical accepted surrogate marker for BOS

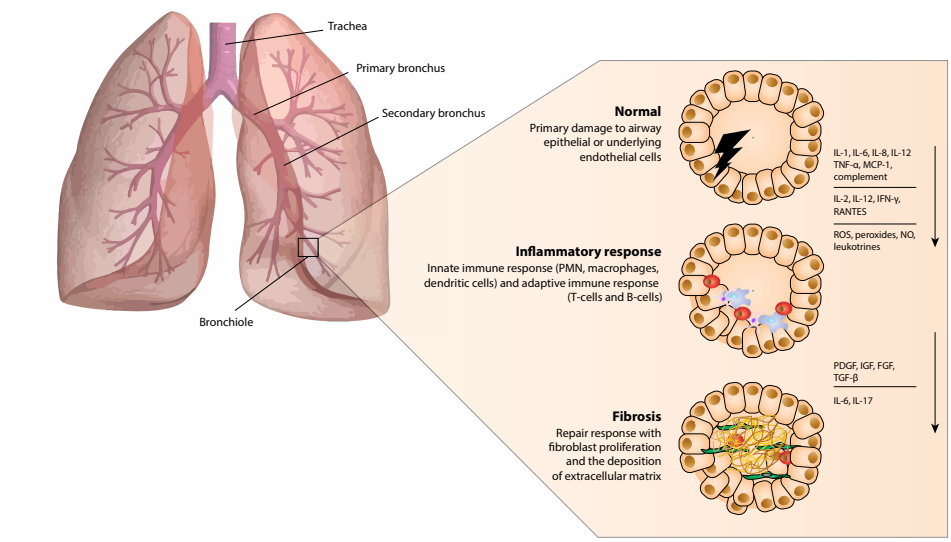

Figure 1: Bronchiolitis obliterans syndrome pathology after lung transplantation

Primary damage to the airway epithelium or underlying endothelial cells leads to the activation of the innate and adaptive immune system and a subsequent inflammatory response. Cytokine secretion of the activated epithelium or endothelium leads to the influx of polymorphonuclear leukocytes (PMNs), macrophages, dendritic cells, T cells and B cells. Epithelial cell damage leads to repair responses, the secretion of profibrotic cytokines, fibroblast proliferation and extracellular matrix deposition. These extensive chronic repair responses could lead to airway obstructions and decrease in pulmonary function, which clinically manifests as the bronchiolitis obliterans syndrome (adapted from Boehler A., and Estenne M. Eur Respir J. 2003).

diagnosis[11].

The RAS was first described by Sato *et al*[12]. The clinical symptoms of this syndrome are consolidations on HRCT scans of the thorax and concurrent fibrosis of the upper lung zones[13] which can be confirmed histologically in autopsies from patients with RAS[17]. The pathogenesis of RAS remains to be clarified, but it is likely to be caused by rejection[8]. Generally, the survival of patients with RAS is worse than that of patients with BOS[14]. This thesis focuses on the immune pathophysiology of BOS.

Though several risk factors have been associated with BOS development e.g. episodes of acute rejection, primary graft dysfunction, HLA mismatches, viral infections but also autoimmunity in the form of auto-reactive antibodies[15,16], a dominant immune mechanism has not been identified. Probably, BOS best can be regarded as a final common pathway in which both cell-mediated and humoral immunity play an important role[17].

Anti-HLA antibodies and mismatching

Involvement of humoral immunity in chronic lung allograft rejection has been well established. Various studies have shown a correlation between the presence of anti-HLA antibodies in the circulation and BOS development. These anti-HLA antibodies can be pre-existing[18] or formed post-LTx via *de novo*[19] synthesis. The first clinical correlation between the degree of HLA-A locus matching and BOS incidence has been published in 1986[20], and confirmed by subsequent studies. Single as well as combined HLA-A and HLA-B mismatches have been found to correlate with BOS progression[21].

Following HLA locus mismatched LTx, anti-HLA antibodies arising *de novo* predispose to the severity of BOS development after LTx[19,22,23]. Interestingly, therapy that decreased anti-HLA antibodies was found to be associated with a lower risk of BOS incidence[24]. Besides clinical correlations, results from functional experiments support the concept of the importance of anti-HLA antibodies in chronic rejection development.

Allospecific HLA antibodies induce the activation of NF-κB, a transcription factor involved in the activation of various proinflammatory cytokines, in human endothelial cells[25]. These cytokines could than contribute to the immunogenic environment which preludes BOS development. Furthermore, crosslinking of HLA class 1 molecules via anti-HLA antibodies triggers cytoskeleton remodeling and the formation of F-actin stress fibers in endothelial cells[26]. A proteomic study on anti-HLA induced actin remodeling identified the protein eIF4A1, which is associated with the promotion of translation and endothelial cell proliferation, as a key factor[27].

Besides cytoskeleton remodeling, crosslinking of HLA molecules also leads to the activation of Rho GTPase and the phosphorylation of Src and FAK. Src is involved in the PI3K/AKT signaling cascade, which is a key pathway in both cell proliferation and survival[28,29]. Since HLA class I molecules do not possess a signaling motif, other receptors are involved in intracellular signaling upon crosslinking. One of the identified cell adhesion molecules is integrin β4. Not surprisingly, integrin β4 signaling results in cell proliferation and survival, and especially via FAK and Src. These effector mechanisms can well be placed within a model of chronic rejection and can thus contribute to BOS development.

Finally, human airway epithelial cells subjected to anti-HLA class 1 antibodies are activated, induce fibroblast growth, and become apoptotic. In mice, exposure to anti-HLA class 1 antibodies resulted in a BOS like phenotype, including fibrosis and small

airway obstruction[30,31]. Interestingly, despite these observations, not all BOS patients have evidence for anti-HLA immunity, which led to the idea that non-HLA antibodies could be of importance in these patients.

Non-HLA antibodies and autoimmunity

The first evidence for the occurrence of non-HLA antibodies involved in BOS was published in 1995, when anti-epithelial cell antibodies detected before LTx were associated with a decrease in 1-year graft survival[32]. Furthermore, Jaramillo and colleagues have shown that non-HLA antibodies can also induce the production of profibrotic cytokines and epithelial cell activation, and the authors speculate that non-HLA antibodies might play a role in BOS pathogenesis in the absence of HLA antibodies[33].

The focus of non-HLA antibodies shifted towards anti-endothelial cell antibodies following a study by Magro *et al.* from 2002, in which the authors observed antibody-mediated like rejection in patients that were negative for panel reactive antibodies, but were positive for anti-endothelial cell antibodies after screening[34]. This has stimulated other studies[16,35], and the role of antibody-mediated rejection involving non-HLA antibodies on BOS development is being studied intensively.

The first discovery of autoimmunity involvement on the advancement of BOS stems from the late '70s in a paper by Ende *et al*[36]. These authors detected circulating lung-specific autoantibodies in patients with BOS following LTx. More recently, the first auto-antigens for such antibodies were identified, in particular type V collagen (col(V)), a minor fibrillar collagen has been studied extensively[37]. Based on these and other findings, Sumpter and Wiles put forward the concept that rejection is biphasic, with the first phase involving tissue damage and the second phase representing autoimmunity against self-antigens exposed upon cell damage[38]. This autoimmunity involves auto-reactive T cell proliferation and autoantibody production. In the normal situation col(V) resides within the lung interstitium, hidden from the immune system, though it is also expressed by epithelial cells to a lesser extent[39]. Upon interstitial remodeling after transplantation, col(V) becomes more exposed, particularly ischemia reperfusion injury. Moreover, matrix metalloproteases, activated due to both ischemia reperfusion injury and interstitial remodeling, cleave collagen, resulting in the exposure of col(V) antigenic fragments[40]. Graft reactive autoantibodies against both col(V) and K-alpha tubulin appearing *de novo* after LTx are associated with BOS[37]. Similar to col(V), K-alpha tubulin is expressed by airway epithelial cells, and upon autoantibody binding, profibrotic cytokines, which have been implicated in BOS, are expressed by the airway epithelium in vitro[41]. Interestingly,

studies in a rat model have shown that tolerance induction to col(V) improves outcome of minor histoincompatible lung allografts[42]. Also, mice exposed to anti-MHC class 1 antibodies show BOS like phenotypes and detectable levels of autoantibodies against both col(V) and K-alpha tubulin[31], possibly providing a link between both humoral autoimmunity and anti-HLA antibodies.

Other studies have also addressed the occurrence of autoantibodies after LTx. Hagedorn *et al.* have shown, using antigen microarray profiling, that BOS patients, with different disease severity, have characteristic autoantibody profiles, despite the fact that only 1% of all the proteins of the lung proteome were analyzed[43]. We have also shown that antibodies against non-HLA antigens are present post-LTx and can add to early BOS prediction, and diagnosis[44].

Autoantibodies against autoantigens have been described in other solid organ transplantation settings as well. In cardiac transplantation, cardiac myosin[45,46], and vimentin[47] are relevant in allograft rejection, but also col(V) and K-alpha tubulin[48]. Most studies on autoantibodies in solid organ transplantation have focused on kidney transplantation. Angiotensin II type-1 receptor autoantibodies contribute to refractory vascular rejection[49], as is the case for ET_AR[50], and MICA[51], though data on the latter is inconsistent[52]. Other studies have identified various non-HLA antibodies, though extensive clinical studies are necessary to validate these observations[53,54]. Also, anti-endothelial cell antibodies arising *de novo*[55] and pre-Tx[56] are found to be predictive with rejection, though the data is conflicting[57].

Interestingly, both these allo- and autoantibodies seem organ specific since they have not been identified in a LTx setting and the relation between humoral immunity and BOS progression, is not a black and white phenomenon. Though allo- and autoantibodies are associated with BOS incidence, they are not a requirement for disease pathogenesis, emphasizing the heterogeneity of the syndrome.

Complement effector mechanisms

The role of the complement system in protection against pathogens is widely recognized. This sophisticated cascade compromises both membrane-bound and fluid-phase proteins that all interact to finally form, among others, the membrane attack complex (MAC) which binds the surface of pathogens and initiates cell lysis. Both auto- and alloantibodies can initiate activation of the complement system via the classical pathway in which the binding of C1q to antigen-bound antibodies initiates the reaction. In the

early half of the nineties, the initial discovery was made that would eventually lead to the consensus that C4d deposition is indicative for antibody-mediated rejection (AMR) after kidney transplantation[58,59]. C4d is a split-product from the complement factor C4, which is generated during activation of both the classical and the lectin pathways[60,61]. More specifically, C4d is part of C4b, which becomes covalently bound to the activator as well as to the antibodies fixed to it, amongst others via OH and NH_2-groups[62]. Fixed C4b becomes degraded leaving C4d bound to the activator. The covalent link explains the long-lasting presence of C4d at the site of complement activation[63]. Subsequent studies into C4d, endothelial activation, and graft injury after kidney transplantation have confirmed the diagnostic value of C4d as a clinical marker for AMR[64-66]. Although that in its initial discovery C4d deposition was associated with hyper acute and acute rejection episodes, studies have shown C4d staining patterns years after transplantation, prompting the hypothesis that AMR can also be involved in chronic rejection.

AMR has been widely accepted as a significant cause of graft failure in other solid organ transplants, including heart- and pancreas transplantation. This does not hold for chronic rejection after LTx[20], though more studies concerning AMR as a diagnostic tool for BOS diagnosis are required, if not essential[10]. In the current literature there are indications that C4d deposition in the lungs may have diagnostic value, but the data is conflicting. Magro *et al.* have shown in biopsy material from 13 BOS patients that C5b-9 (MAC complex), C4d, and C1q depositions were present along the bronchial epithelium. They also detected anti-endothelial antibodies in these patients, indicating the presence of AMR[35]. A subsequent study by this group also presented data on C4d deposition in LTx patients who developed early BOS[67]. These data suggest that humoral immunity precedes BOS development and has potential as a clinical marker for BOS diagnosis. However, immunological staining of C4d in lung tissue may yield some technical difficulties. Indeed, an extensive study in 68 lung allograft biopsies could not identify specific staining profiles correlating to either acute or chronic episodes of rejection[68]. Finally, C3d deposition, which can be indicative for complement activation in acute renal allograft rejection[69], has been examined in biopsies of lung allo-graft. Results indicate that more extensive depositions of C3d correlate with chronic graft dysfunction. In another study C3d deposition was associated with bronchial wall fibrosis[70].

Although current literature favors a role for complement-mediated rejection in the pathogenesis of bronchiolitis obliterans, patients with clinical features of BOS do not always have increased C4d/C3d depositions and vice versa. Extensive C4d depositions without clinical complications has been described in ABO incompatible kidney transplantation. Prior to transplantation, patients are treated with plasmapheresis which

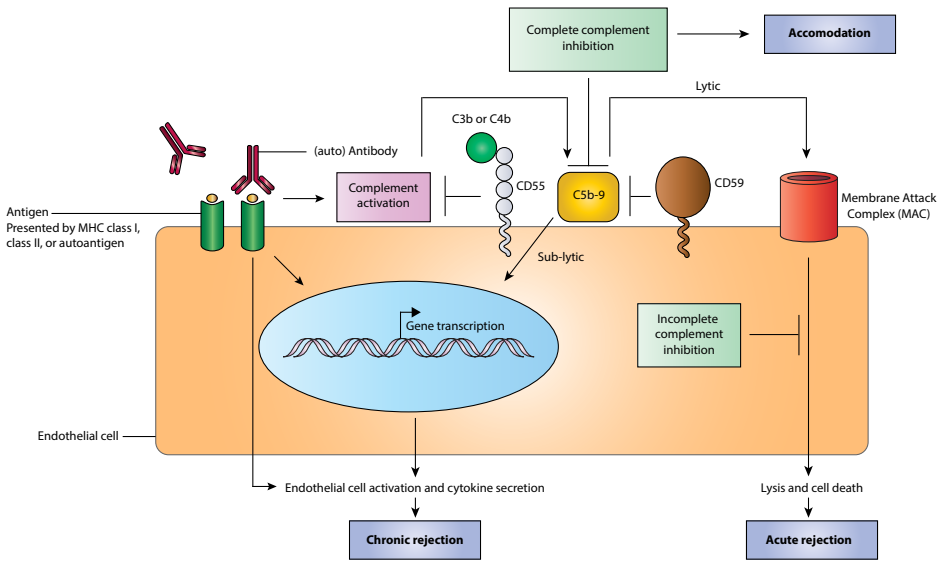

Figure 2: Complement-fixing antibody binding to graft endothelial cells

Complement-fixing antibodies (either auto- or alloantibodies) can bind to their respective antigens on the cellular surface of endothelial cells. This leads to complement activation and subsequent target cell lysis, which is observed clinically as acute rejection after transplantation. Complete inhibition of complement, as observed in ABO incompatible kidney transplantation, leads to accommodation, a process where complement is inhibited via complement regulatory proteins expressed by the target cell. Incomplete inhibition is sufficient to prevent cell lysis, but can lead to complement-mediated activation and secretion of cytokines associated with chronic rejection (adapted from Colvin R.B., and Smith N. Nat Rev Immunol. 2005).

removes pre-existing immunoglobulin's, including anti-blood antibodies[71]. However, these patients may develop anti-blood group antibodies due to the incompatible graft, which raises concerns of AMR. Although these patients presented with increased C4d deposition during protocol biopsies within the first year of transplantation, none showed signs of histological tissue injury or impaired graft function[72-74]. This process is termed accommodation, in which the graft protects itself from complement mediated cell damage induced by anti-blood group antigens via hyper expression of membrane attached complement regulatory proteins (mCRPs), including CD55 (decay accelerating factor), which accelerates the disintegration of the C3 convertase, and/or CD59 (protectin), which inhibits the formation of the membrane attack complex via binding to the C5b-8 complex[75] (Figure 2). Indeed, Iwasaka and colleagues have shown that upon endothelial cell activation, CD55 and CD59 are upregulated, which enhances resistance to antibody induced complement mediated cell damage or activation[76].

Endothelial cells, upon activation, are known to secrete cytokines associated with BOS development, including IL-8, CCL5, MCP-1[77], CXCL9, CXCL10[78,79], and IL-6[80]. Moreover, cytokines secreted by activated endothelial cells are also known to be both proinflammatory and profibrotic. Sublytic MAC deposition on endothelial cells leads to production of IL-8, MCP-1[81], bFGF, and PDGF[82]. The increased secretion of Weibel-Palade bodies upon stimulation by proinflammatory cytokines such as IL1β[83,84], or by anti-HLA antibodies[85], can enhance the immunogenic properties of the endothelial environment which can further contribute to BOS progression.

Aim and outline of this thesis

Obstructive CLAD is considered to be a multifactorial process in which both humoral and cellular processes are involved. Due to the heterogeneity of the syndrome, the exact pathogenic mechanisms remain unknown. Previous research on obstructive chronic lung allograft dysfunction has primarily been conducted from a recipient's perspective. In this thesis, we take a more donor-oriented approach regarding the immunological processes leading to chronic rejection. As aforementioned, antibody formation, which can initiate complement and cellular activation, is associated with BOS development. We hypothesize that complement regulation, or other factors within the transplanted graft, could contribute to organ protection and therefore play a role in the development of chronic rejection.

Since the diagnosis of BOS is based upon a surrogate marker which becomes manifest late in the disease process of BOS development, there is a high medical need for novel biomarkers to identify patients at risk for BOS development early after transplantation. Therefore, in this thesis, several novel potential biomarkers are evaluated.

This thesis is divided into three separate sections in which both BOS pathogenesis, as well as novel potential diagnostic tools for BOS diagnosis will be addressed. Due to accumulating evidence of a role for humoral immunity in BOS development, the scope of **Part I**, *Chapter 2* till *6*, is focused on complement and (auto) antibody formation. In **Part II**, *Chapter 7* and *8*, we shift gears towards cellular immunity. In **Part III**, *Chapter 9* till *11*, we describe the results from studies focused on genetics and gene regulation in both lung transplant donors and recipients.

Finally, in *Chapter 12* we will summarize the abovementioned chapters and discuss the respective findings to put them in a broader perspective.

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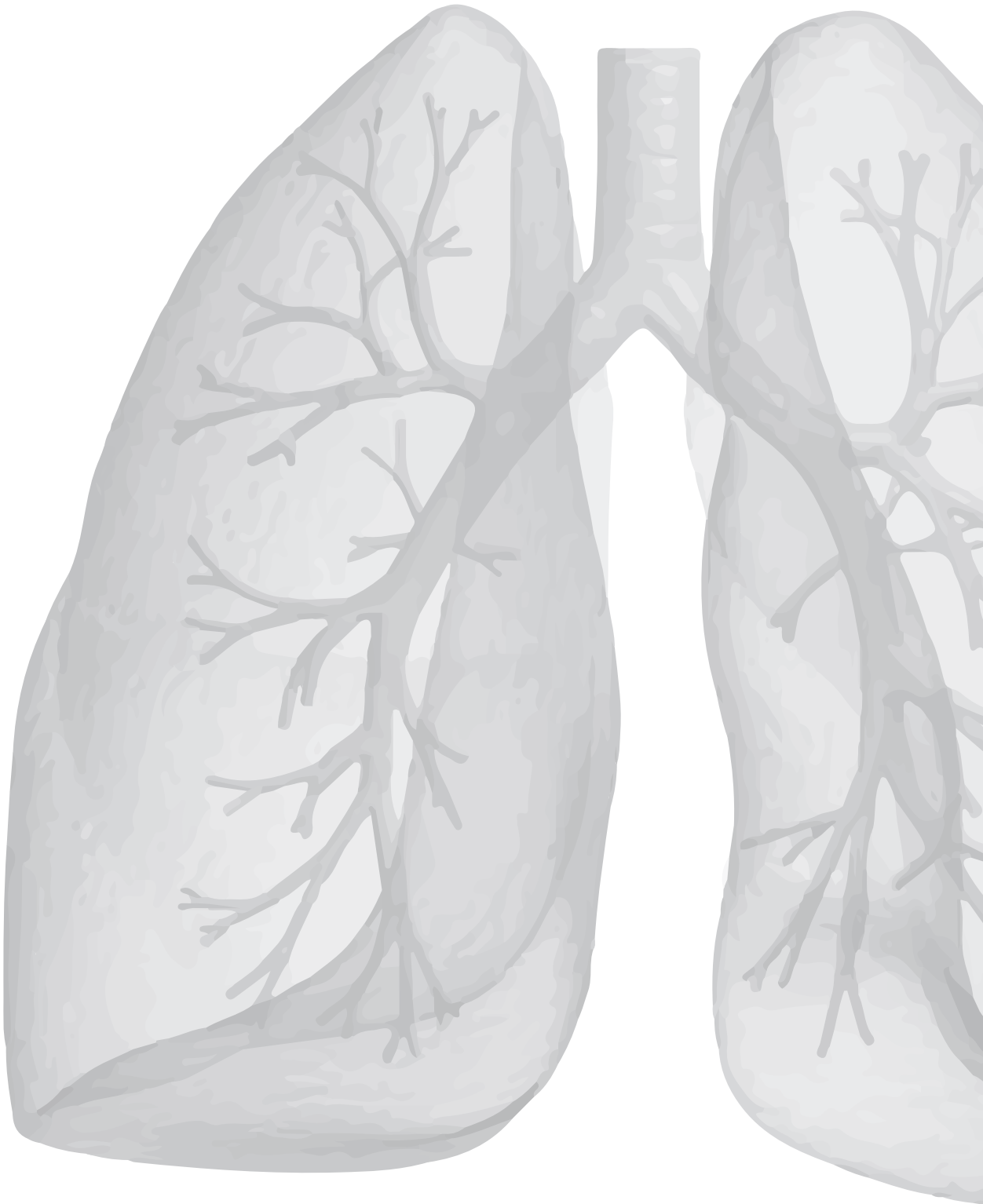
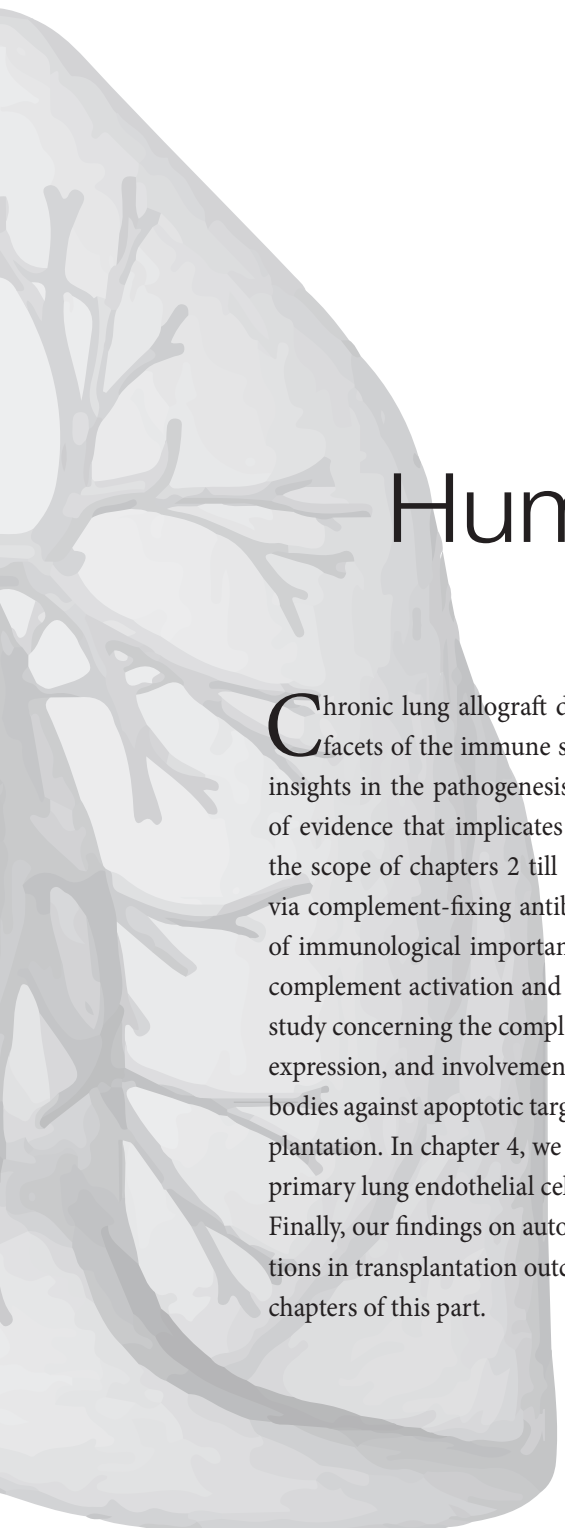
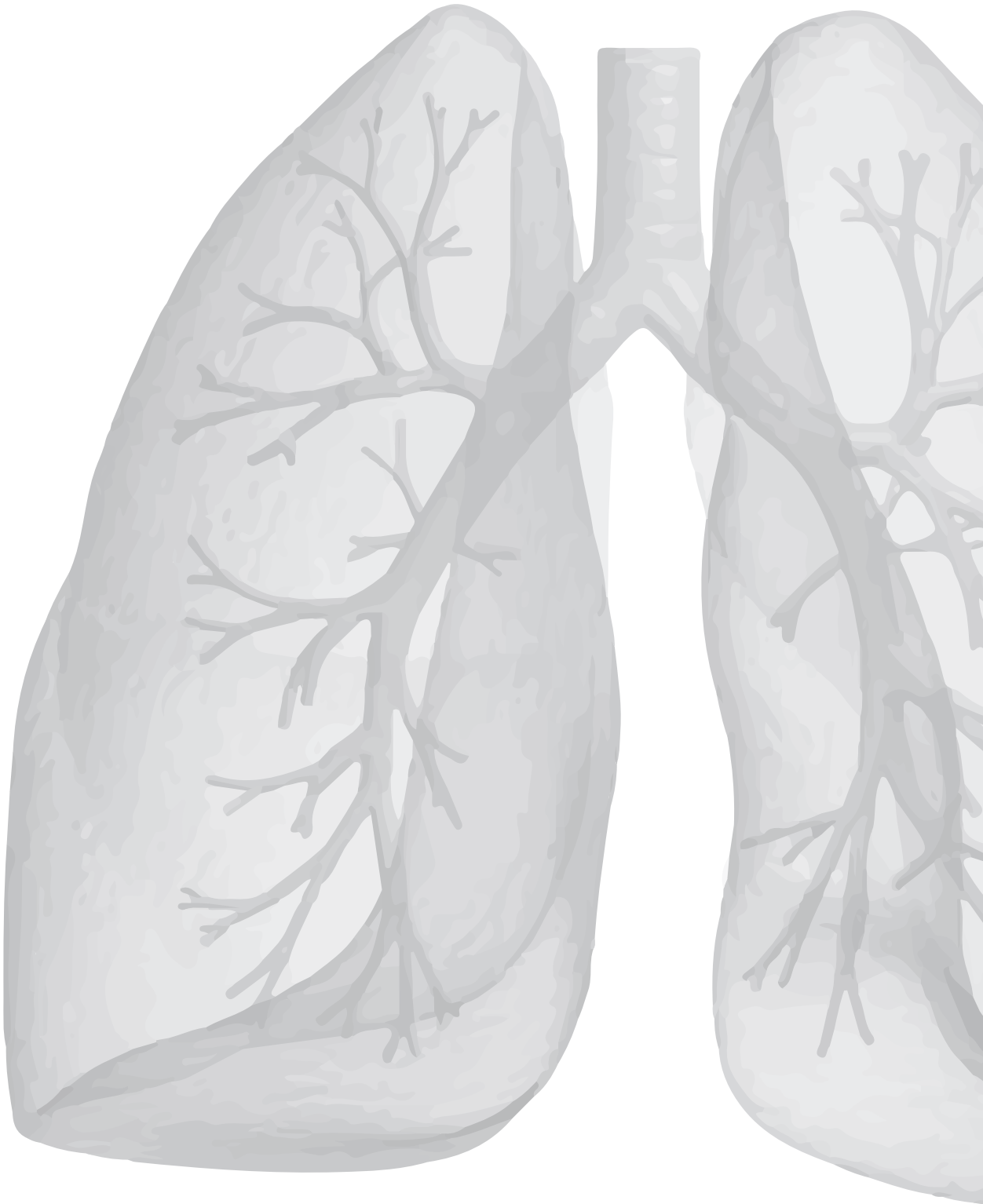




Part I

Humoral Immunity

Chronic lung allograft dysfunction is a heterogeneous complication in which many facets of the immune system are involved. In this first part, we aimed to gain more insights in the pathogenesis of chronic rejection after LTx. Due to the increasing body of evidence that implicates antibody formation to be important in BOS development, the scope of chapters 2 till 6 is focused on humoral immunity. Complement activation via complement-fixing antibody binding to cellular surfaces can initiate a broad variety of immunological important pathways. Therefore, we investigated the relation between complement activation and regulation in more detail. In chapter 2 and 3 we present our study concerning the complement regulatory protein CD59 and discuss protein function, expression, and involvement in the development of rejection after transplantation. Antibodies against apoptotic targets are found to be associated within the field of kidney transplantation. In chapter 4, we discuss the findings on antibodies directed against apoptotic primary lung endothelial cells and describe the culture method used to obtain these cells. Finally, our findings on autoantibodies directed against various targets and their implications in transplantation outcome and end-stage lung diseases will be addressed in the last chapters of this part.



2

A promotor polymorphism in the *CD59* complement regulatory protein gene in donor lungs correlates with a higher risk for chronic rejection after lung transplantation

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2

Abstract

Complement activation primarily leads to membrane attack complex formation and subsequent target cell lysis. Protection against self-damage is regulated by complement regulatory proteins, including CD46, CD55, and CD59. Within their promotor regions, single nucleotide polymorphisms (SNPs) are present that could influence transcription. We analyzed these SNPs and investigated their influence on protein expression levels. A single SNP configuration in the promotor region of CD59 was found correlating with lower CD59 expression on lung endothelial cells (p=0.016) and monocytes (p=0.013). Lung endothelial cells with this SNP configuration secreted more pro-fibrotic cytokines IL-6 (p=0.047) and FGF-β (p=0.036) upon exposure to sublytical complement activation than cells with the opposing configuration, whereas monocytes were more susceptible to antibody-mediated complement lysis (p<0.0001). Analysis of 137 lung transplant donors indicated that this CD59 SNP configuration correlates with impaired long-term survival (p=0.094) and a significantly higher incidence of the bronchiolitis obliterans syndrome (p=0.046) in the recipient. These findings support a role for complement in the pathogenesis of this post-transplant complication and are the first to show a deleterious association of a donor CD59 promotor polymorphism in lung transplantation.

Introduction

The complement system primarily functions in the detection and destruction of pathogens and comprises both fluid-phase and membrane-bound proteins. Complement activation leads to C3 convertase formation and, among others, membrane attack complex (MAC) deposition, resulting in target cell lysis. Cells are protected from unwanted effects of complement activation by expression of membrane complement regulatory proteins (mCRPs), CD46 (membrane cofactor protein), CD55, (decay accelerating factor) and CD59 (homologous restriction factor). CD46 regulates alternative and classical pathway activation by facilitating Factor I-mediated cleavage of C3b and C4b. CD55 inhibits complement via C3 convertase decay. CD59 inhibits MAC formation via blocking the binding of C9 to C5b-C8 complexes[1].

CD59 expression is controlled by regulatory sequences identified upstream of intron 1. Within the first intron, an enhancer is identified which exerts a strong effect on transcription[2-5]. Furthermore, single nucleotide polymorphisms (SNPs) are present in the CD59 promoter region that might influence gene transcription.

Expression of mCRPs is important in resistance against complement-mediated cell damage and/or activation. Observations in patients with antibodies against blood group antigens following ABO-incompatible kidney transplantation indeed suggested that endothelial cells (ECs) become resistant to complement-mediated cell damage by increased expression of CD55 and CD59, a process termed accommodation[1,6,7].

High CD59 expression on ECs in donor organs could explain why some transplants remain free from antibody-mediated rejection despite the presence of circulating antibodies against donor HLA. In lung transplantation (LTx) donor-specific HLA antibodies and graft-reactive *de novo* auto-antibodies are associated with bronchiolitis obliterans syndrome (BOS) development[8], a form of chronic lung allograft dysfunction (CLAD)[9], limiting long-term graft survival[10]. These graft-reactive antibodies lead to *in situ* complement activation as is suggested by depositions of complement component C4d and immunoglobulins in allografts[11]. Recently, specific HLA-G alleles and haplotypes are found to be associated with outcome after LTx[12].

Here, we sequenced the promotor and adjacent regions of CD59, analyzed promotor polymorphisms and determined haplotypes. Identified SNP configurations and haplotypes were tested for their influence on CD59 expression, protection against complement-mediated cell lysis, and cytokine production upon complement-mediated EC activation.

Furthermore, we analyzed identified SNP configurations in a death-censored survival analysis and found a significant correlation between a genetic configuration in the *CD59* promoter region of the donor and BOS development post-LTx in the recipient.

Patients and Methods

Patients and donors

We included 137 patients undergoing LTx in our center between January 2004 and March 2012, based on material availability. Informed consent was obtained and the medical ethical committee approved the study (METC 06-144). BOS was defined according to ISHLT criteria[13]. PBMCs were isolated from heparin blood or spleen specimens collected from the donor during surgery, using Ficoll-Paque Plus (GE Healthcare). Samples were frozen in RPMI (Lonza), with 20% fetal bovine serum (FBS; Bodinco) and 10% DMSO (Sigma-Aldrich) final concentration and preserved in liquid nitrogen until tested.

Sequencing of the *CD59*, *CD46*, and *CD55* promotor and adjacent regions

DNA was isolated from PBMCs obtained from donor spleen specimens using the MagnaPure Compact System (Roche Diagnostics). Sequence analysis and haplotyping was performed using BioEdit (Ibis Biosciences) and SeqScape® (Life Technologies). For details see Supplementary Methods.

Complement-mediated cytotoxicity assay

Both PBMCs and donor derived vascular endothelial cells were used in a complement-mediated cytotoxicity assay. Further details can be found in the Supplementary Methods.

Expression of CD59 on PBMCs and ECs

PBMCs were thawed in RPMI 20%FBS, centrifuged, and dissolved in FACS buffer (PBS, 0.1% bovine serum albumin, 0.1% sodium azide). Lymphocytes were gated according to CD45+ and side-scatter. Due to high expression of CD59[14], ECs were incubated with a mixture of anti-CD59-FITC and non-CD59-FITC antibodies with identical binding epitopes to reduce the fluorescent signal, or the respective isotype control antibody. ECs were gated according to typical forward- and sideward scatter. Cellular subsets and antibodies are depicted in supplementary Table S2. Data were acquired and analyzed using a BD FACS Canto II and FACS DIVA software (BD).

Monocyte isolation

Donor PBMCs were thawed, diluted in 1ml FBS and centrifuged for 10min at 1300rpm. The pellet was dissolved in 1ml RPMI 20%FBS and loaded on 1ml Ficoll-Paque. The isolated interphase was washed in 2ml RPMI 20%FBS, and a total of 10-15x10^6 cells was incubated for 15min at 4°C with magnetic Protein A beads (Miltenyi-Biotec) pre-coated with mouse-anti-human CD14-IgG2a (SouthernBiotech). Labeled cells were separated using MS columns and a MiniMACS™ separator (Miltenyi-Biotec).

EC activation and cytokine profiling

SNP genotyped vascular ECs were cultured O/N at 37°C in 24-wells plates (ThermoScientific) at 5.0x10^4 cells/well in specific medium. Once confluent, cells were incubated with 50µl Thymoglobulin (1250µg/ml), which binds ECs[15], for 30min RT. Cells were washed and human serum was diluted 1:1 in veronal buffer (Lonza) which was then added 1:3 to culture medium, yielding a 1:6 final serum dilution. Incubation took place O/N at 37°C. Culture supernatant was obtained after activation and stored in aliquots at -80°C. Samples were analyzed for cytokine concentrations using a multiplec analyzer[16].

Details on statistical analyses can be found in the Supplementary Methods.

Results

Sequence results and SNP analysis

Sequencing of the CD59 promotor and adjacent regions, from position -704 till +57, of 137 LTx donors revealed 11 SNPs. These SNPs were in linkage disequilibrium yielding 13 different haplotypes. The frequency of individual SNP configurations range from 1% till 50% and that of the haplotypes from 0.7% till 41%. As control, we sequenced promoter regions of CD46 and CD55. CD46 promotor region sequencing from position -718 till +38 resulted in the identification of 11 SNPs in 11 different haplotypes. Among identified SNPs was a SNP, heterozygosity (A/G) at position -659, that does not appear in the Ensembl genome browser. CD55 promotor region sequencing from position -695 till +6 revealed 12 SNPs distributed over 8 haplotypes. Here, we also identified an unknown SNP, heterozygosity (C/T) at position -244. Haplotypes and SNP configurations are summarized in Supplementary Table S3.

2 Sensitivity of A/x genotyped PBMCs to complement-mediated lysis

SNP and haplotype variants found in at least 10% of the cohort were analyzed for functional impact on complement-mediated cell lysis. Donors with the most frequent homozygosity (e.g. G/G) at a distinct position were compared to the heterozygous (C/G) or homozygous (C/C) variant. We did not compare each homozygous- and heterozygous variants separately because of low numbers. W6/32 and ATG were used to sensitize PBMCs genotyped for the identified mCRP promotor polymorphisms. Incubation with dose-response curves of antibodies and human serum, revealed the suboptimal dose for W6/32, 7.8-15.6µg/

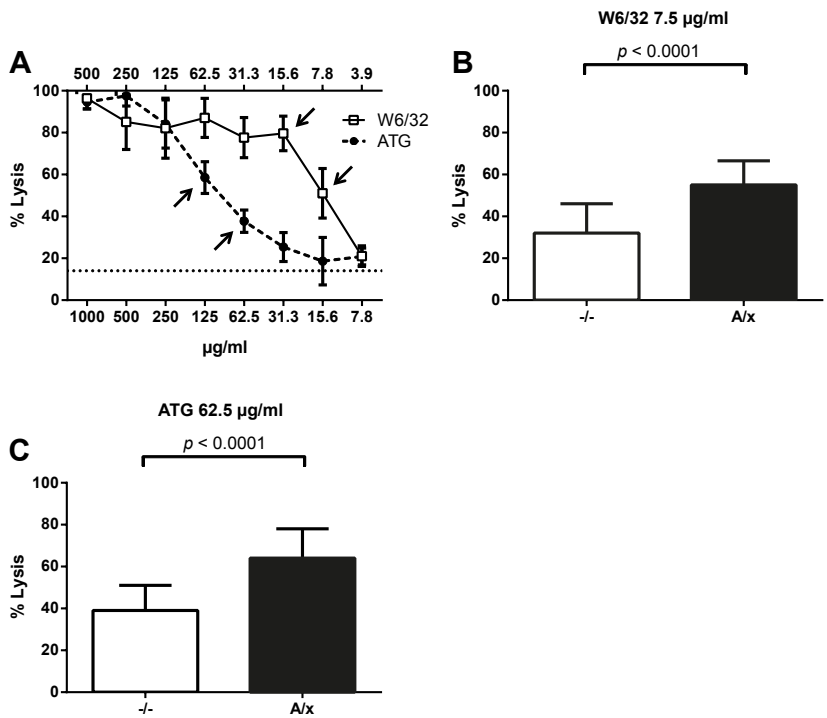

Figure 1: A/x genotyped PBMCs are more susceptible to complement mediated cell lysis compared to -/- genotyped PBMCs

A Dose response curve of W6/32 (open squares, upper x-axis) and ATG (closed circles, lower x-axis). Suboptimal lytic concentration used in further experiments are indicated by arrows. Each point represents the mean and SD of 6 measurements. **B** Complement mediated cell lysis using W6/32 at 7.5 µg/ml and **C** ATG at 62,5 µg/ml to sensitize cells. Depicted are the results of 18 different A/x donor PBMCs, 14 -/- donor PBMCs each tested with 10 different complement sources. Data represent median ± interquartile range. Data were analyzed with Mann-Whitney test.

ml, and of ATG, 62.5-125µg/ml (Figure 1A). Upon incubation of PBMCs with 15µg/ml W6/32 and human serum 61% of cells from A/x genotyped donors, the SNP configuration for rs147788946, were lysed, versus 43% of -/-, the wildtype variant. This difference was confirmed with a lower antibody concentration (Figure 1B). Incubation with 125µg/ml ATG yielded a mean lysis of 77% versus 54% for donors with the A/x and -/- genotype, respectively. A similar difference was noted with lower ATG concentrations (Figure 1C). Comparison of cell lysis between other haplotypes of CD59 or other mCRPs did not reveal significant differences (data not shown).

A/x genotyped monocytes have a lower CD59 expression and are more susceptible to complement-mediated cell lysis

CD59 expression is highest on monocytes and $CD4^+$ T cells (data not shown). Lymphocytes had similar CD59 expression when stratified by SNP configuration (Figure 2A), as did neutrophils (data not shown). A/x genotyped monocytes had lower CD59 expression than cells with the alternative genotype (p=0.013, Figure 2B). We observed no differences in CD55 (Figure 2C,2D) or CD46 (Figure 2E,2F) expression when stratified for SNP genotype. These results were confirmed in an independent cohort of 5 A/x and 5 -/- genotyped PBMCs from healthy controls. Difference in sensitivity to complement-mediated lysis of PBMCs of either genotype were also observed when purified monocytes were tested: either sensitized with W6/32 or with ATG, monocytes with A/x genotype were lysed more than monocytes with -/- genotype (Figure 3). No differences in sensitivity to complement-mediated cell lysis were observed with monocyte-depleted cells of either genotype (Figure 3).

SNP rs147788946 influences CD59 expression on ECs

A/x genotyped primary ECs expressed less CD59 (p=0.016), measured with anti-CD59-FITC, than cells with the other genotype (Figure 4A). CD59 expression measured with anti-CD59-APC and anti-CD59-PB staining confirmed this (data not shown). Additionally, quantification of CD59 expression was attempted by measuring soluble CD59 upon PL-C treatment of ECs[17]. However, because of limited cell numbers we could not quantify soluble CD59 using a commercially available ELISA.

ECs are resistant against complement-mediated cell lysis

To assess functional effects of the identified *CD59* SNP on the capacity of ECs to resist complement-mediated cell lysis we used an ATG-mediated cytotoxicity assay. Incubation

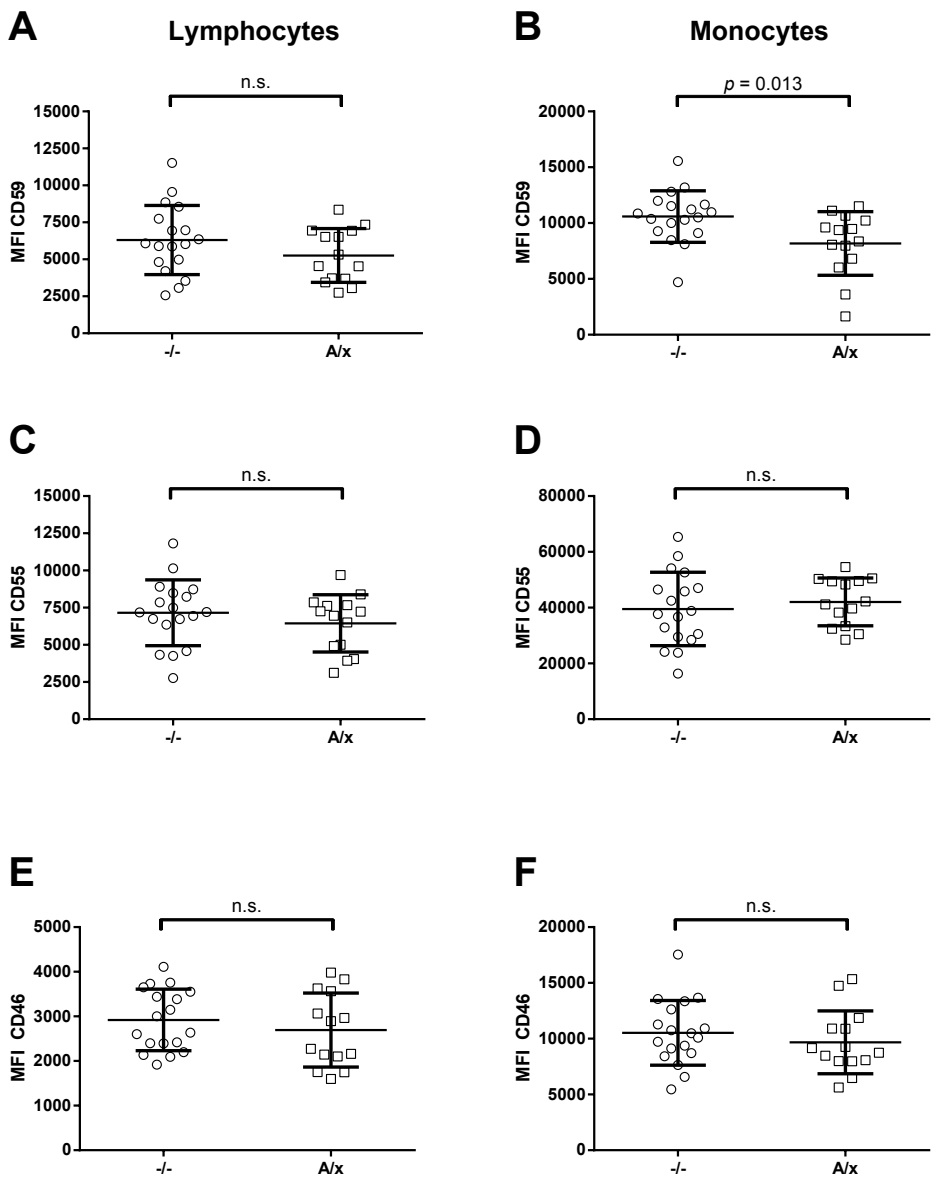

Figure 2: CD59 expression by PBMCs from donors with different *CD59* genotype

Lymphocytes (selected as CD45+CD14-) show no difference in CD59 expression based on SNP stratification (**A**). A/x genotyped monocytes (selected as CD45+CD14+) exhibit a lower expression of CD59 than monocytes of donors with -/- genotype (p=0.013, **B**). No differences in expression of CD55 (**C** and **D**) or CD46 (**E** or **F**) were observed when stratified per SNP configuration. Data represent mean ± SD, symbols indicate data for individuals. Data were analyzed with unpaired t-test. -/-, n=18; A/x, n=14.

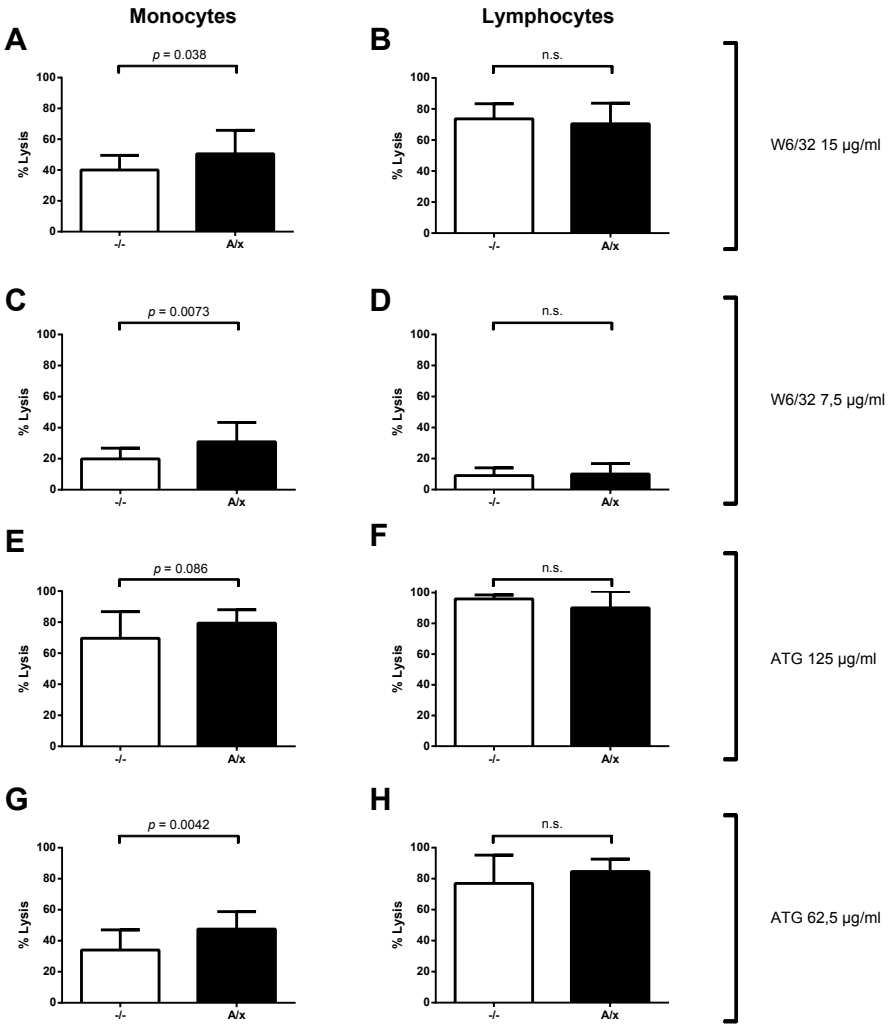

Figure 3: Complement mediated cell lysis on monocytes and lymphocytes stratified for SNP configuration

CD14- and CD14+ cell fractions were suspended at 4-6x10^6/ml and both monocyte depleted and monocyte enriched fractions were used in a complement-mediated cytotoxicity assay. Monocytes with different genotypes show a different sensitivity to complement mediated cell lysis when incubated with 15 µg/ml (**A**) or 7.5 µg/ml (**C**) W6/32 or 125 µg/ml (**E**) or 62.5 µg/ml (**G**) ATG, whereas lymphocytes do not (**B**, **D**, **F**, **H**). Data are mean ± SD and were analyzed with unpaired *t*-test. -/-, *n*=15; A/x, *n*=12.

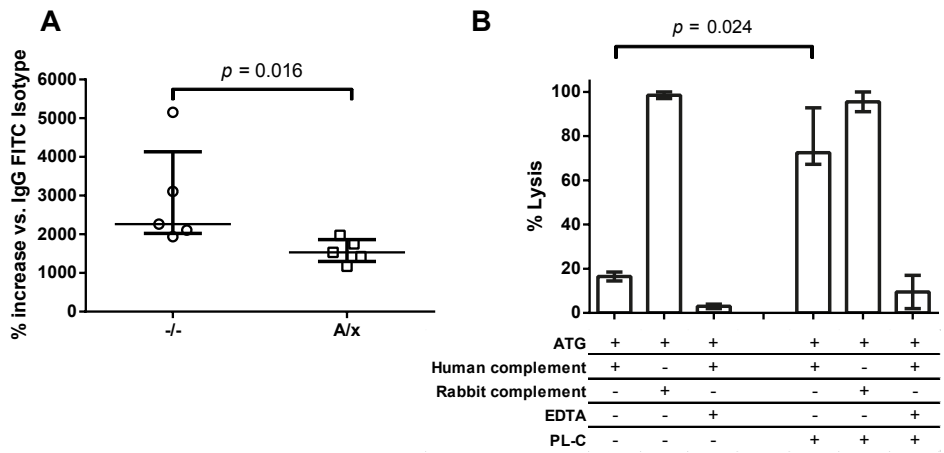

Figure 4: CD59 expression on primary endothelial cells with A/x or -/- genotype and sensitivity to complement mediated cell lysis

A CD59 expression measured by FACS analysis and corrected for a-specific binding using the respective isotype control, differs between A/x and -/- genotyped endothelial cell samples ($p = 0.016$, Mann-Whitney test). Data are median and interquartile range. **B** SNP genotyped endothelial cells (-/-), pre-treated with PL-C or not, were incubated with ATG and human serum as source of complement. Lysis was measured. Rabbit complement, which bypasses human complement regulation by CD59, was used as a positive control for complement dependent lysis and EDTA added to human serum as a negative control. Data represents median ± interquartile range of 3 pooled serum samples on 4 endothelial cell sources, and were analyzed with the Mann-Whitney test.

of ECs with ATG and human serum resulted in minimal cellular lysis (±18%) above the EDTA control (2-3%, Figure 4B) or above the control with heat-inactivated serum (10%). Resistance of ECs to substantial lysis by complement reflected high mCRPs expression since pre-incubation of the cells with phospholipase C (PL-C), which cleaves off GPI anchored proteins including CD59, resulted in increased lysis (Figure 4B). However, as lysis of cells by ATG and human serum was minimal, we did not further study the effect of the SNP on sensitivity to complement in this assay.

Cytokine expression after EC activation

The results discussed previously suggest that exposure of ECs to antibodies and human serum mainly resulted in sublytical MAC levels. These can activate ECs and induce cytokine production[18]. Therefore, we incubated ECs with ATG and human serum and investigated cytokine secretion. No differences in cytokine expression upon activation were observed

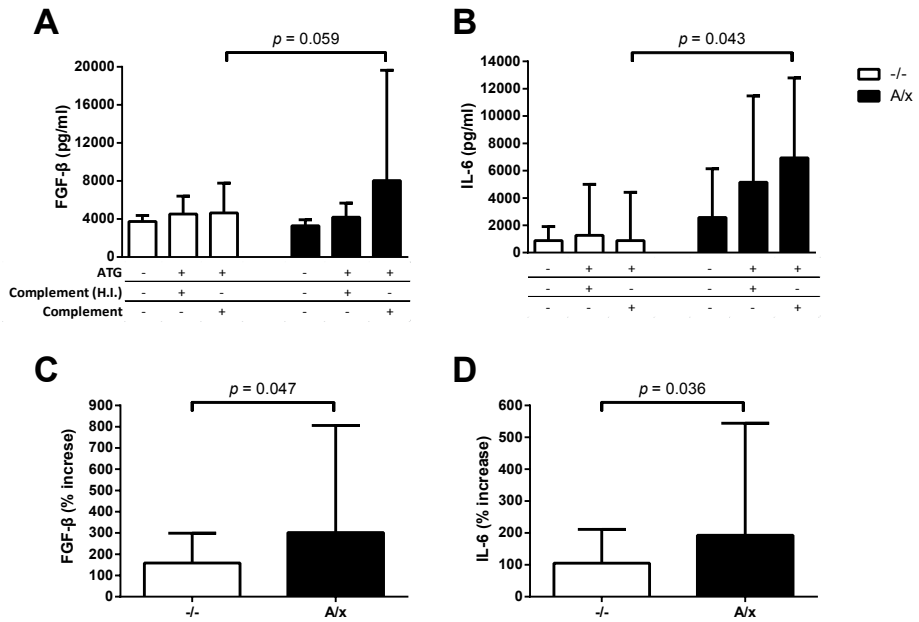

Figure 5: Sublytical complement activation induces enhanced cytokine production by endothelial cells with A/x genotype

-/- endothelial cells (open columns) and A/x endothelial cells (filled columns) were incubated with ATG and human serum as complement source overnight. FGF-β (**A** and **C**) and IL-6 (**B** and **D**) levels in the supernatants were measured. Panels **A** and **B** are absolute levels measured, **C** and **D** are relative levels with levels produced in absence of complement and ATG set at 100%. H.I. = heat inactivated. Data represent median ± interquartile range and were analyzed with Mann-Whitney test. -/-, *n*=12; A/x, *n*=10.

for IL-8, MCP-1, RANTES or CXCL10 (data not shown). However, we observed a trend (p=0.059) in increased secretion of FGF-β and a significantly higher secretion (p=0.043) of IL-6, compared to heat-inactivated human serum (Figure 5A and 5B respectively). Incubation with human serum in the absence of antibody did not result in significant differences between genotyped ECs. To correct for differences in basal expression levels of both cytokines, we calculated the cytokine production as relative concentrations. Using this approach, both FGF-β and IL-6 were secreted at higher concentrations by A/x ECs compared to -/- ECs, (p=0.047 and p=0.036, respectively, Figures 5C and 5D).

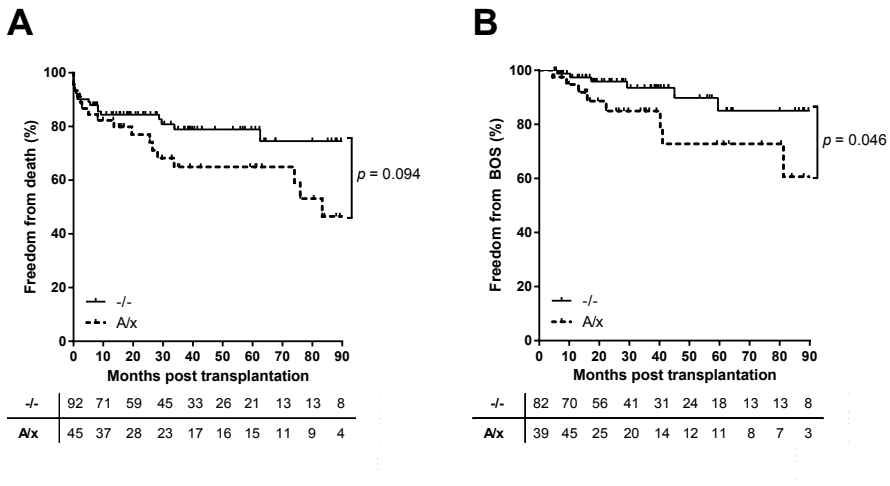

Figure 6: Comparison of survival and BOS-free survival in relation to the *CD59* SNP rs147788946

Patients were stratified by *CD59* SNP rs147788946, which determines whether or not an extra adenine (A/- or A/A, together indicated as A/x; dotted line) is present at position -314 in the region upstream of the *CD59* promotor, or not (-/-; solid line). Patients with lung allografts of the -/- genotype show a trend towards higher survival (**A**, p=0.094) and a significant longer BOS-free survival (**B**, p=0.046) than patients with lung transplants of the A/x genotype.

rs147788946 identifies patients at risk for the development of chronic rejection post-LTx

Since our previous observations indicated that A/x genotyped ECs express less CD59 and secrete more fibrosis-related cytokines upon sublytical complement activation, we hypothesized that this could influence the capacity of a donor organ to protect itself from the recipients' complement system. Therefore, we analyzed the effect of an A/x genotyped graft on the development of chronic rejection post-LTx.

From all 137 patients and donors the clinical and demographic parameters are depicted in Table 1. Patients received standard immunosuppressive therapy consisting of basiliximab, tacrolimus, mycophenolate mofetil and prednisone. Fourteen patients developed BOS, the obstructive form of CLAD. None of the patients developed restrictive allograft syndrome[9].

Patients who received a graft from donors with an insertion of an adenine at SNP

rs147788946 at position -314 in the promotor region of *CD59* showed a trend towards lower 5-year survival (p=0.094, Figure 6A). Moreover, the incidence of BOS, excluding patients who deceased within the first 3 months post-LTx (n=121), was significantly higher in patients who received a lung transplant with this genotype (p=0.046, Figure 6B). We found no significant difference in BOS incidence when patients were stratified by the other SNPs or haplotypes in the *CD46*, *CD55*, and *CD59* promotor regions (data not shown). A multivariate Cox proportional-hazards model for BOS incidence, including known (donor) risk factors[19] identified SNP rs147788946 in the promotor region of *CD59* as an independent predictor for BOS with a trend towards significance (hazard ratio 3.1, 95% CI 1.0-9.9,p=0.058, Table 2).

Discussion

In this study we show that a polymorphism in the *CD59* promotor region affects CD59 expression, susceptibility to complement-mediated cell lysis and EC cytokine secretion upon complement activation. Also, we demonstrate that this polymorphism is associated with a risk for BOS development. CD59 expression is important in various diseases, including age related macular degeneration[20]. Furthermore, hyperexpression of CD59 on ECs is suggested to be crucial in graft-accommodation following transplantation[1,5,7].

Despite the novelty, there are several limitations with respect to this study. Firstly, our cohort of LTx patients is relatively small for a SNP association study. This is also noted when conducting a multivariate analysis. Furthermore, certain p-values were only marginal significant and we therefore would like to stress that validation of our findings and further research is desired to strengthen our observations.

We identified 2 unknown SNPs with a low frequency which may explain why they were not described before. All other SNPs identified in mCRPs promotor regions had comparable frequencies compared to Ensembl[21]. Only a few SNPs had a frequency high enough for use in association studies. In the *CD46* promotor region we identified 2, in the *CD55* promotor region 2, and in the CD59 region 3 SNPs with suitable frequencies. Only one SNP, rs147788946 in the CD59 promotor region, was associated with BOS development.

The exact promotor region of CD59 is unknown. Two SP1 binding sites upstream from exon 1 in the promotor region have been described, which, as most household genes, does not contain TATA or CAAT boxes[2,3]. Furthermore, positive regulatory sequences between positions -35 and -70, position -70 and -151, and -151 and -2000 upstream of exon 1 have been identified[4]. The CD59 gene consists of 4 exons of which the first is

	All	Non - BOS	BOS	*p* -value
Patients				
Total number	*137*	*123*	*14*	
Gender				
Male	69 (50%)	64 (52%)	5 (36%)	0.247
Female	68 (50%)	59 (48%)	9 (64%)	
Mean age (years)	46 (17-64)	46 (17-64)	45 (17-63)	0.820
Mean follow-up (months)	35 (0-105)	36 (0-105)	28 (5-81)	0.392
Primary disease				
CF	43 (31%)	38 (31%)	5 (36%)	0.912
COPD	55 (40%)	49 (40%)	6 (43%)	
ILD	37 (27%)	34 (27%)	3 (21%)	
PH	2 (2%)	2 (2%)	0	
Infection				
EBV high risk	14 (10%)	10 (8%)	4 (29%)	0.061
CMV high risk	31 (23%)	28 (23%)	3 (21%)	0.910
Genotype				
A/x	45 (33%)	37 (30%)	8 (57%)	**0.032*
-/-	92 (67%)	86 (70%)	6 (43%)	
Type of graft				
Bilateral	106 (77%)	94 (76%)	12 (86%)	0.431
Single	31 (23%)	29 (24%)	2 (14%)	
Episode of acute rejection	24 (18%)	17 (14%)	7 (50%)	*0.001*
Ischemic times (min)				
Bilateral	319 (82-1770)	323 (82-1770)	287 (120-416)	0.775
Single	242 (161-340)	246 (161-340)	215 (165-265)	0.392
Donors				
Gender				
Male	63 (46%)	59 (48%)	4 (29%)	0.168
Female	74 (54%)	64 (52%)	10 (71%)	
Donor age (years)				
Mean age	45 (11-67)	44 (11-67)	53 (31-66)	*0.020*
> 60	13 (9%)	10 (8%)	3 (21%)	0.160
Donor type				
HB	116 (85%)	102 (83%)	14 (100%)	0.093
non HB	21 (15%)	21 (17%)	0 (0%)	
Smoking				
Yes	49 (36%)	47 (38%)	2 (14%)	0.077
No	88 (64%)	76 (62%)	12 (86%)	

Table 1: Clinical and demographic parameters of lung transplant donors and recipients

Statistical significant values are in italics. * *p*-value calculated in a Kaplan-Meier survival analysis. BOS = bronchiolitis obliterans syndrome, CF = cystic fibrosis, COPD = chronic obstructive pulmonary disease, ILD = interstitial lung diseases, PH = pulmonary hypertension, EBV = Epstein-Barr virus, CMV = cytomegalovirus, HB = heart beating, NHB = non-heart beating. EBV or CMV high risk: EBV/CMV negative recipient + EBV/CMV positive donor. Continuous variables are depicted as mean + min/max.

untranslated. Within the first intron an enhancer with a strong effect on transcription has been identified[5]. This enhancer contains two SNPs, rs831628 and rs831629, that are frequent in the population. Correlation analysis between these SNPs and rs147788946 resulted in a positive correlation with rs831628 and a negative correlation with rs831629, indicating linkage equilibrium between these SNPs and the configuration of rs147788946.

We identified one SNP within the -35 to -70 region, but due to low population frequency we could not assess the effect of this SNP on CD59 expression. SNP rs147788946 lies within the -151 to -2000 region identified previously. Our results confirm the importance of this region for CD59 expression. Moreover, our data showing this SNP is correlated with expression in monocytes and ECs, but not lymphocytes suggest a tissue-specific influence on CD59 expression, which was proposed previously[4].

The ENCODE project[22] provides insights into gene regulatory marks and ChIP-seq established transcription factor binding sites throughout the whole genome. This data strongly confirms the presence of the abovementioned regulatory region (supplementary Figure S3) in a novel promotor region of CD59 and sheds light on possible functional implications of rs147788946. Intriguingly, this SNP is located at an RNA polymerase 2 (POL2) and Max-interacting-1 (MXI1) binding site, hence possibly directly influences transcription initiation in this region.

Our complement-mediated cell lysis data support our hypothesis that promotor polymorphisms influence protection against complement-mediated cell damage. Both the usage of ATG and W6/32 resulted in a significant difference in complement-mediated cell lysis between A/x or -/- genotyped donor PBMCs in a dose-dependent manner.

	Hazard ratio (95% CI)	*p*-value
SNP configuration A/x	3.1 (1.0 - 9.9)	0.058
Donor age (≥60)	7.0 (1.4 - 34.6)	0.017
Donor smoking state	0.2 (0.05 - 1.2)	0.084
CMV reactivation	0.7 (0.2 - 2.8)	0.613
EBV reactivation	2.7 (0.8 - 9.0)	0.114
Recipient age (≥60)	4.2 (1.0 - 17.3)	0.047
Episode of acute rejection	1.7 (0.5 - 5.9)	0.404

Table 2: Multivariate analysis on BOS incidence in 121 patients treated with lung transplantation

As potential confounders a high donor age (≥60), the smoking status of the donor (ever smoker vs. never smoker), CMV and EBV reactivation, episode of acute rejection, and recipient age (≥60) were included. Cox regression, 100 months post-LTx.

Furthermore, we confirmed that this difference is due to CD59 expression levels, since we observed both differences in expression of CD59 and sensitivity to complement-mediated cell lysis on monocytes but not on lymphocytes. Monocytes genotyped A/x for SNP rs147788946 appear to be less resistant to the MAC, consequently lowering the threshold for cell lysis and/or activation.

Decreased CD59 function due to glycation has been identified to lead to increased growth factor release by ECs exposed to low-grade complement activation, which is suggested to explain the proliferative vasculopathy in human diabetes mellitus[23]. Hence, we studied the effect of the CD59 SNP on cytokine production by ECs exposed to sublytic complement activation. Activated ECs upregulate the secretion of proinflammatory cytokines, including IL-6, and the chemokine IL-8[24]. IL-6, with TGF-β, promotes Th17 proliferation from naïve Th progenitor cells[25]. IL-17 release from Th17 cells induces fibroblast activation, proliferation and EC activation[26]. IL-8, IL-6, MCP-1 and GROα are stored in secretory organelles, including Weibel-Palade bodies, of ECs and can be secreted upon cell activation[27]. Antibodies against HLA can activate ECs and act as stimulator for Weibel-Palade bodies exocytosis[28]. Furthermore, exogenous complement is important for the release of secretory bodies[29]. Sublytic MAC exposure, resulting in extracellular Ca^{2+} influx through pore formation, indeed can activate ECs (17) leading to exocytosis of Weibel-Palade bodies, and the release of IL-8, IL-6, and fibroblast growth factor[30]. We found that A/x genotyped ECs indeed secrete higher amounts of both IL-6 and FGF-β upon exposure to sublytical complement activation, indicating their threshold for activation upon exposure to activated complement is indeed reduced, as compared to cells with the other genotype.

Since lung ECs are the premier cells encountered by recipient's antibodies and complement and could well be a source of growth factors involved in BOS development, we postulate that this model is relevant for BOS progression. BOS is the main cause of graft failure post-LTx. Several studies have suggested that HLA-specific and graft reactive auto-antibodies play a role in the development of this syndrome[8,13]. CD59 regulates complement activation downstream of C4 and due to accommodation, complement activation in lung allografts may not always lead to chronic rejection because of mCRPs expressed by ECs in the graft.

In summary, we show that a specific nucleotide affects CD59 expression and sensitivity of cells to complement-mediated cell lysis and subsequently lowers the threshold for production of proinflammatory cytokines that promote fibroblast proliferation and fibrosis. Furthermore, we identified a clinical association of this SNP with a risk for BOS development following LTx. Though further studies should reveal the molecular

mechanisms explaining the influence of SNP rs147788946 on CD59 translation and expression, our study points to an important role of the complement system in the development of BOS following LTx. We suggest that increased production of cytokines and growth factors by donor lung ECs upon exposure to activated complement is key to this new role of complement in BOS development.

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Authorship and Disclosures

Author contributions: K.B., E.v.d.G., C.E.H., and H.G.O. designed research; K.B. and T.K.H. performed research; E.v.d.G., J.K-v.E., E-J.D.O, and D.A.v.K. contributed patient material; K.B., E.v.d.G., T.K.H., J.C.A.B., C.E.H., and H.G.O analyzed and interpreted data; and K.B., E.v.d.G., J.C.A.B., C.E.H., and H.G.O. wrote the paper. All authors critically revised and approved the manuscript. The authors declare to have no conflicts of interests to disclose as described by the American Journal of Transplantation.

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Supplementary Methods

Sequencing of the CD59, CD46, and CD55 promotor and adjacent regions

Primers and protocols are summarized in Supplementary Table S1. A schematic overview of promotor and adjacent regions of mCRPs including PCR and sequence primers and SNP positioning is shown in Supplementary Figure S1.

Complement-mediated cytotoxicity assay

Donor PBMCs were thawed, diluted in 1ml FBS and centrifuged for 10min at 1300rpm. The pellet was dissolved in 1ml RPMI 20%FBS and loaded on 1ml Ficoll-Paque (Axis shield PoC). The interphase was isolated, washed in 2ml RPMI 20%FBS, and dissolved in 100µl RPMI 20%FBS at 4-6x106/ml. Terasaki plates (Greiner Bio-One) were loaded with 1µl cell suspension/well and incubated with 1µl 15µg/ml or 7,5µg/ml of W6/32 (anti-human HLA-A,B,C BioLegend) or 1µl 125µg/ml or 62,5µg/ml Thymoglobulin (Genzyme), and incubated for 30min at room temperature (RT). Human serum, from 10 healthy volunteers, was diluted 1:4 in veronal buffered saline (Lonza). Ten µl of the mixture was added per well followed by 1hr incubation at 37°C. Pooled human serum diluted 1:1 in 0.1 M EDTA, pH 7.5 was used as negative, and rabbit complement (Cedarlane) as positive control.

Human vascular ECs were cultured until confluence in T75 flasks (Greiner Bio-One) in EBM-2 culture medium with additives for vascular ECs (Lonza). These donor ECs were obtained during surgical procedure and stored in liquid nitrogen until tested. Cells were analyzed for expression of CD31 (PECAM-1), CD13, and the intracellular presence of Von Willebrand factor (data not shown). These ECs were washed with PBS and detached using AccutaseTM Cell Detachment Solution (BD). ECs were further sub-cultured in Terasaki plates until confluency and washed with PBS followed by 1hr incubation at 37°C with 1µl 5 U/ml PL-C (Sigma-Alderich), or EBM-2 culture medium. After washing with PBS cells were incubated with 1µl 1250µg/ml Thymoglobulin for 30min RT followed by 1hr incubation at 37°C with human serum either or not supplemented with EDTA or with rabbit complement. Cells were stained with FluoroQuence (Ethidium Bromide/Acridine Orange and fixatives, One Lambda) for 30min in the dark. Percentage of cell lysis, with the negative- and positive control set at 0% and 100% respectively, was measured using an automated fluorescence microscope and custom designed software (Leica Microsystems), see Supplementary Figure S2.

2 Statistics

Categorical data were analyzed by the Fisher's exact test or Pearson's $\chi 2$ test, continuous variables by the ANOVA test and survival via Kaplan-Meyer analysis. Multivariate testing was conducted using a Cox Regression proportional hazards model and results are displayed as hazard ratios with 95% confidence intervals and respective p-values. Data were analyzed for normality using the D'Agostino-Pearson omnibus normality test, and, in the case of normal distribution subsequently analyzed via t-test or, if not, Mann-Whitney rank-sum test. Normally distributed data is displayed by mean+SD, non-Gaussian distributed data is displayed as median+interquartile range, indicated in the figure legend. Statistical analysis was performed using GraphPad Prism 6.02 (GraphPad Software Inc.) and SPSS 21 (IBM Corp.).

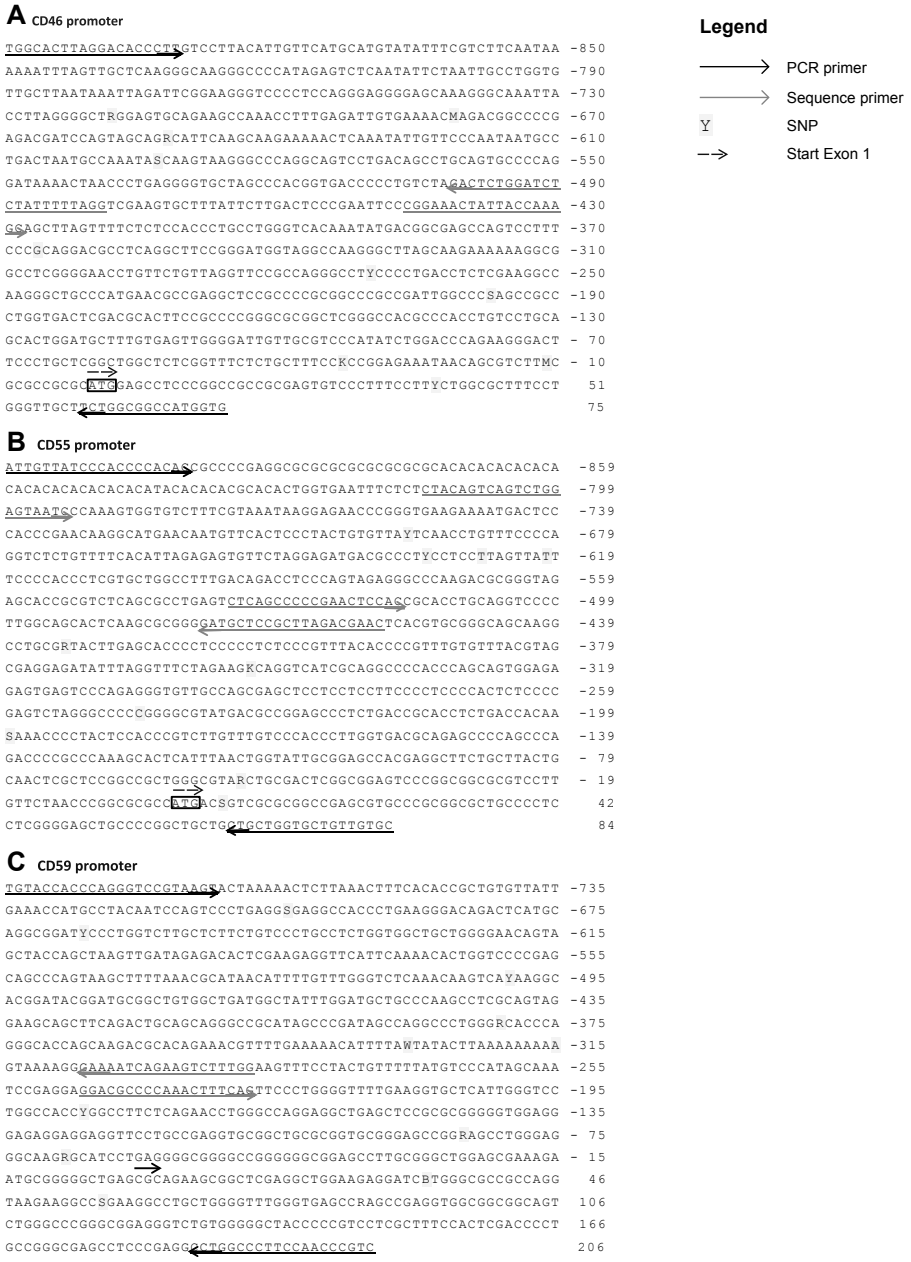
	CD46	CD55	CD59
PCR primer			
Forward	5'-TGGCACTTAGGACACCCTT-3'	5'-ATTGTTATCCCACCCCACAC-3'	5'-TGTACCACCCAGGGTCCGTAAGT-3'
Reverse	5'-CACCATGGCCGCCAGA-3'	5'-GCACAACAGCACCAGCAG-3'	5'-GACGGGTTGGAAGGGCCAGG-3'
PCR protocol	3 min 95°C 15 sec 95°C 23 sec 63°C | 32 cycles 1.5 min 72°C 10 min 72°C	3 min 95°C 15 sec 95°C 23 sec 60°C | 32 cycles 1.5 min 72°C 10 min 72°C	3 min 95°C 15 sec 95°C 25 sec 62°C | 32 cycles 5 min 72°C 10 min 72°C
Sequence primer			
Forward	5' CGGAAACTATTACCAAAGC 3'	5' CTACAGTCAGTCTGGAGTAATC 3' 5' CTCAGCCCCCGAACTCCAC 3'	5' GGACGCCCCAAACTTTCAG 3'
Reverse	5' CCTAAAAATAGAGATCCAGAGTC 3'	5' GTTCGTCTAAGCGGAGCATC 3'	5' CCAAAGACTTCTGATTTTC 3'
Sequence protocol	10 sec 96°C 10 sec 96°C 10 sec 50°C | 25 cycles 2 min 60°C	10 sec 96°C 10 sec 96°C 10 sec 50°C | 25 cycles 2 min 60°C	10 sec 96°C 10 sec 96°C 10 sec 50°C | 25 cycles 2 min 60°C

Supplementary Table S1: PCR and sequence primers and protocols

The different PCR primers and sequence primers, both forward and reverse, are depicted for each mCRP promotor region. Also, per PCR the exact protocol is indicated.

Supplementary Figure S1: Overview of mCRP promotor regions and primer design.

Promotor region of CD46 (**A**), CD55 (**B**), and CD59 (**C**). Black arrows indicate the used PCR primers for DNA amplification. Red arrows indicate additional sequence primers. Various SNPS and their respective NC-IUB codes are highlighted in yellow. The dashed arrow identifies the start of Exon 1, while the box indicates the start codon. The ATG region in the CD59 gene is present further downstream of the transcription initiation site and is therefore not depicted in **C**. These adjusted images were obtained from the Ensembl database (21).


A CD46 promoter
TGGCACTTAGGACACCCTTGTCCTTACATTGTTCATGCATGTATATTTCGTCTTCAATAA -850
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TTGCTTAATAAATTAGATTCGGAAGGGTCCCCTCCAGGGAGGGGAGCAAAGGGCAAATTA -730
CCTTAGGGGCTRGGAGTGCAGAAGCCAAACCTTTGAGATTGTGAAAACMAGACGGCCCCG -670
AGACGATCCAGTAGCAGRCATTCAAGCAAGAAAAACTCAAATATTGTTCCCAATAATGCC -610
TGACTAATGCCAAATASCAAGTAAGGGCCCAGGCAGTCCTGACAGCCTGCAGTGCCCCAG -550
GATAAAACTAACCCTGAGGGGTGCTAGCCCACGGTGACCCCCTGTCTAGACTCTGGATCT -490
CTATTTTTAGGTCGAAGTGCTTTATTCTTGACTCCCGAATTCCCGGAAACTATTACCAAA -430
GSAGCTTAGTTTTCTCTCCACCCTGCCTGGGTCACAAATATGACGGCGAGCCAGTCCTTT -370
CCCGCAGGACGCCTCAGGCTTCCGGGATGGTAGGCCAAGGGCTTAGCAAGAAAAAAGGCG -310
GCCTCGGGGAACCTGTTCTGTTAGGTTCCGCCAGGGCCTYCCCCTGACCTCTCGAAGGCC -250
AAGGGCTGCCCATGAACGCCGAGGCTCCGCCCCGCGGCCCGCCGATTGGCCCSAGCCGCC -190
CTGGTGACTCGACGCACTTCCGCCCCGGGCGCGGCTCGGGCCACGCCCACCTGTCCTGCA -130
GCACTGGATGCTTTGTGAGTTGGGGATTGTTGCGTCCCATATCTGGACCCAGAAGGGACT - 70
TCCCTGCTCGGCTGGCTCTCGGTTTCTCTGCTTTCCKCCGGAGAAATAACAGCGTCTTMC - 10
GCGCCGCGATGAGCCTCCCGGCCGCCCGCGAGTGTCCCTTTCCTTYCTGGCGCTTTCCT 51
GGGTTGCTTCTGGCGGCCATGGTG 75
B CD55 promoter
ATTGTTATCCCACCCCACAGCGCCCCGAGGCGCGCGCGCGCGCGCACACACACACACA -859
CACACACACACACACATACACACGCACACTGGTGAATTTCTCTCTACAGTCAGTCTGG -799
AGTAATGCCAAAGTGGTGTCTTTCGTAAATAAGGAGAACCCGGGTGAAGAAAATGACTCC -739
CACCCGAACAAGGCATGAACAATGTTCACTCCCTACTGTGTTAYTCAACCTGTTTCCCCA -679
GGTCTCTGTTTTCACATTAGAGAGTGTTCTAGGAGATGACGCCCTYCCTCCTTAGTTATT -619
TCCCCACCCTCGTGCTGGCCTTTGACAGACCTCCCAGTAGAGGGCCCAAGACGCGGGTAG -559
AGCACCGCGTCTCAGCGCCTGAGTCTCAGCCCCCGAACTCCAGCGCACCTGCAGGTCCCC -499
TTGGCAGCACTCAAGCGCGGGGATGCTCCGCTTAGACGAACTCACGTGCGGGCAGCAAGG -439
CCTGCGRTACTTGAGCACCCCTCCCCCTCTCCCGTTTACACCCCGTTTGTGTTTACGTAG -379
CGAGGAGATATTTAGGTTTCTAGAAGKCAGGTCATCGCAGGCCCCACCCAGCAGTGGAGA -319
GAGTGAGTCCCAGAGGGTGTTGCCAGCGAGCTCCTCCTCCTTCCCCTCCCCACTCTCCCC -259
GAGTCTAGGGCCCCCGGGGGCGTATGACGCCGGAGCCCTCTGACCGCACCTCTGACCACAA -199
SAAACCCCTACTCCACCCGTCTTGTTTGTCCCACCCTTGGTGACGCAGAGCCCCAGCCCA -139
GACCCCGCCCAAAGCACTCATTTAACTGGTATTGCGGAGCCACGAGGCTTCTGCTTACTG - 79
CAACTCGCTCCGGCCGCTGGGCGTARCTGCGACTCGGCGGAGTCCCGGCGGCGCGTCCTT - 19
GTTCTAACCCGGCGCGCCATGCSGTCGCGCGGCCGAGCGTGCCCGCGGCGCTGCCCCTC 42
CTCGGGGAGCTGCCCCGGCTGCTGCTGGTGCTGTTGTGC 84
C CD59 promoter
TGTACCACCCAGGGTCCGTAAGTACTAAAAACTCTTAAACTTTCACACCGCTGTGTTATT -735
GAAACCATGCCTACAATCCAGTCCCTGAGGSGAGGCCACCCTGAAGGGACAGACTCATGC -675
AGGCGGATYCCCTGGTCTTGCTCTTCTGTCCCTGCCTCTGGTGGCTGCTGGGGAACAGTA -615
GCTACCAGCTAAGTTGATAGAGACACTCGAAGAGGTTCATTCAAAACACTGGTCCCCGAG -555
CAGCCCAGTAAGCTTTTAAACGCATAACATTTTGTTTGGGTCTCAAACAAGTCAYAAGGC -495
ACGGATACGGATGCGGCTGTGGCTGATGGCTATTTGGATGCTGCCCAAGCCTCGCAGTAG -435
GAAGCAGCTTCAGACTGCAGCAGGGCCGCATAGCCCGATAGCCAGGCCCTGGGRCACCCA -375
GGGCACCAGCAAGACGCACAGAAACGTTTTGAAAAACATTTTAWTATACTTAAAAAAAAA -315
GTAAAAGGAAAATCAGAAGTCTTTGGAAGTTTCCTACTGTTTTTATGTCCCATAGCAAA -255
TCCGAGGAGGACGCCCCAAACTTTCAGTTCCCTGGGGTTTTGAAGGTGCTCATTGGGTCC -195
TGGCCACCYGGCCTTCTCAGAACCTGGGCCAGGAGGCTGAGCTCCGCGCGGGGGTGGAGG -135
GAGAGGAGGAGGTTCCTGCCGAGGTGCGGCTGCGCGGTGCGGGAGCCGGRAGCCTGGGAG - 75
GGCAAGRGCATCCTGAGGGGCGGGGCCGGGGGGCGGAGCCTTGCGGGCTGGAGCGAAAGA - 15
ATGCGGGGGCTGAGCGCAGAAGCGGCTCGAGGCTGGAAGAGGATCBTGGGCGCCGCCAGG 46
TAAGAAGGCCSGAAGGCCTGCTGGGGTTTGGGTGAGCCRAGCCGAGGTGGCGGCGGCAGT 106
CTGGGCCCGGGCGGAGGGTCTGTGGGGGCTACCCCCGTCCTCGCTTTCCACTCGACCCCT 166
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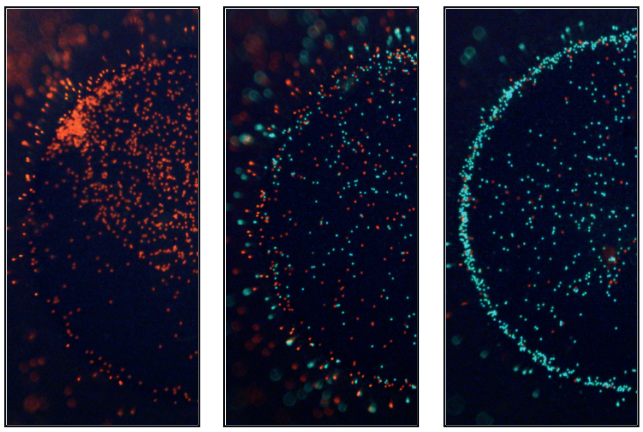
Legend
PCR primer
Sequence primer
Y SNP
Start Exon 1



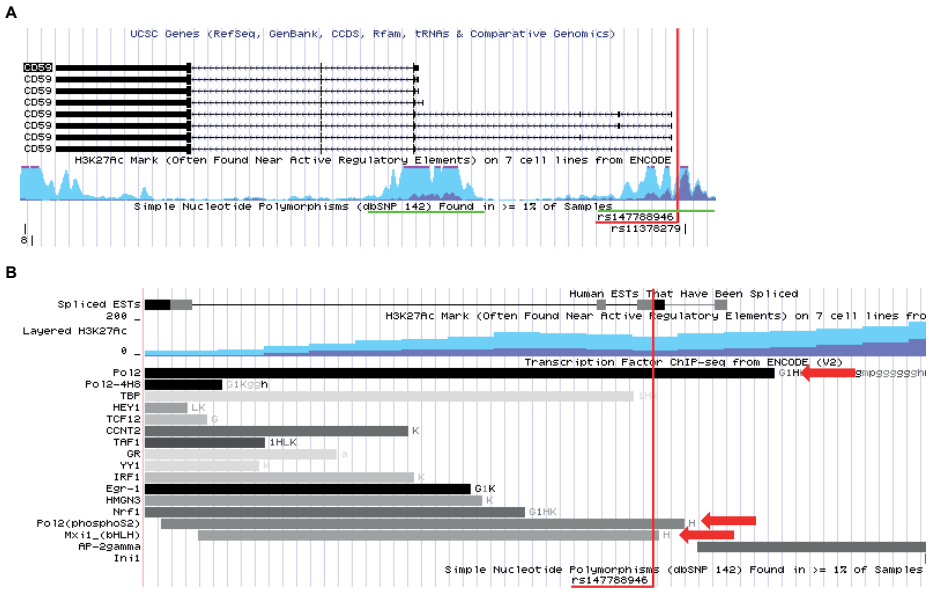
Supplementary Figure S2: Visualization of the complement-mediated cytotoxicity assay

Visualization of the complement-mediated cytotoxicity assay used, left panel 100% lysis (positive control with rabbit complement), center panel ~50% cell lysis and right panel, 0% cell lysis (negative control, EDTA pre-incubation).

Subset	Antibody	Fluorochrome	Company
Hematopoietic cells	CD45	PO	Invitrogen
T cells	CD3	PB	Biolegend
	CD4	APC	Imm Tech
	CD8	PECy7	BD
B cells	CD19	PerCP	Biolegend
Monocytes	CD14	APC H7	BD
CD59 expression	CD59	FITC	eBioscience
	CD59	APC	eBioscience
	CD59	PE	Exbio

Supplementary Table S2: Cellular subsets and antibodies

Per cellular subset the used antibody, fluorochrome and company are depicted. All 3 CD59 antibodies recognize the exact same epitope. Respective isotype controls were supplied by Invitrogen, Biolegend, and BD.



Supplementary Figure S3: ENCODE database of the CD59 promotor region

A Overview of the two promotor regions, identified by ChIP-sequencing of active regulatory elements underlined in green, of CD59 according to the ENCODE database (22). Both the long and short transcript variants are depicted in black. SNP rs147788946, underlined in red, lies in the middle of a DNA region to which transcriptional elements can potentially bind. **B** An enlargement of element A. SNP rs147788946, underlined in red, lies in the binding region of pol2 (upper red arrow) and its phosphos2 subunit (center red arrow). Also, transcription factor MXI1 (bottom red arrow) has a potential binding site in this regulatory region. Both images were obtained from the ENCODE database, accessed on the 30th of June 2015.

Supplementary Table S3: Haplotype and SNP configuration of the promotor region of CD46, CD55, and CD59

SNP configurations, NC-IUB code and frequencies are displayed vertically according to their relative position to the transcription initiation site. Different haplotypes and frequencies are displayed in linkage disequilibrium horizontally. We identified two previously unknown SNPs in both the CD46 promotor region, at position -659, and the CD55 promotor region, at position -244. Observed SNP frequencies are in concordance with the Ensemble database (next page).

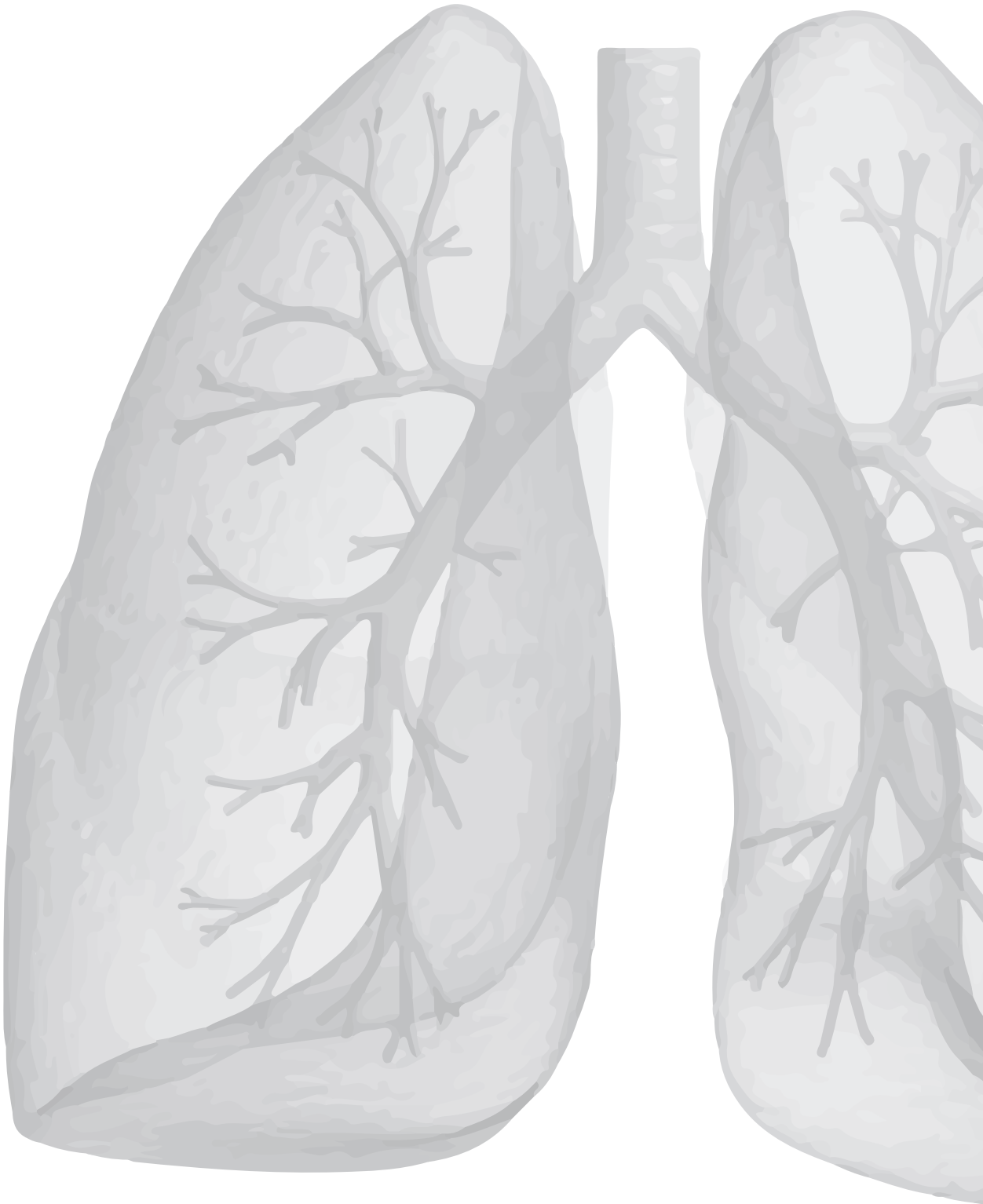
CD46 SNP	rs150315189	rs186440169	rs not defined	rs2796267	rs41266393	rs2796268	rs41266395	rs41266397	rs200947813	rs74477032	rs138843816		
Position	-718	-681	-659	-652	-593	-366	-270	-197	-33	-11	+38		Haplotype
NC-IUB	R	M	R	R	S	G	Y	S	K	M	Y	# found	frequence
Haplotype	G	A	G	A/G	C	A/G	C	C	T	C	C	56	.41
	G	A	G	A	C	A	C	C	T	C	C	35	.26
	G	A	G	G	C	G	C	C	T	C	C	20	.15
	G	A	G	G	C	A/G	C	C	T	C	C	9	.07
	G	A	G	A/G	C	A	C	C	T	C	C	6	.04
	G	A	G	A	C	A/G	C	C	T	C	C	4	.03
	G	A	G	G	C	A	C	C	T	C	C	2	.01
	G	A	G	A	C	G	C	G	T	C	C	2	.01
	G	A	G	A/G	C	G	C	C	T	C	C	1	.007
	G	A	A/G	A	C	A	C	C	T	C	C	1	.007
	A/G	A	G	G	C	G	C	C	T	C	C	1	.007
												137	
Configuration	A/G: .01	A: 1	A/G: .01	A/G: .46	C: 1	A/G: .50	C: 1	C: .99	T: 1	C: 1	C: 1		
frequence	G: .99		G: .99	G: .23		A: .32		G: .01					
				A: .3		G: .18							

NC-IUB:	R = GA
	Y = TC
	W = AT
	S = GC
	M = AC
	K = GT
	B = GTC

CD55 SNP	rs2564978	rs75511334	rs150046210	rs3841376	rs12078463	rs28371583	rs180745130	rs not defined	rs28371585	rs28371586	rs144950436	rs7542430		
Position	-695	-633	-627	-627	-620	-432	-352	-244	-244	-198	-53	+6		Haplotype
NC-IUB	Y	Y	Insertion*	T	Y	R	K	Y	Insertion A	S	R	S	# found	frequence
Haplotype	C/T	T	+/-	T	T	A	G	C	-	C	G	C	40	.29
	C	T	+	T	T	A	G	C	-	C	G	C	29	.21
	C	T	+	T	T	A/G	G	C	-	C	G	C	23	.17
	C/T	T	+/-	T	T	A/G	G	C	-	C	G	C	22	.16
	T	T	-	T	T	A	G	C	-	C	G	C	12	.09
	C	T	+	T	T	G	G	C	-	C	G	C	9	.07
	C/T	T	+/-	T	T	A	G	C/T	-	C	G	C	1	.007
	C/T	T	+/-	T	T	A	G/T	C	-	C	G	C	1	.007
													137	
Configuration	C/T: .39	T: 1	+: .92	T: 1	T: 1	A/G: .46	G/T: .01	C/T: .01	-/-: 1	C: 1	G: 1	C: 1		
frequence	C: .45		-: .08			A: .47	G: .99	C: .99						
	T: .16					G: .07								

* = + insertion of TAGTTACTTCCCCTCCTTCCC

CD59 SNP	rs74932292	rs831603	rs141014570	rs831602	rs76100475	rs147788946	rs151050218	rs142803201	rs831601	rs13614	rs80285764		
Position	-704	-666	-500	-381	-331	-315	-186	-85	-68	+32	+57		Haplotype
NC-IUB	S	Y	Y	R	W	Insertion A	Y	R	R	B	S	# found	frequence
Haplotype	C	T/C	C	G	A	-/-	C	G	G	C/T	C	35	.26
	C	C	C	G	A	-/-	C	G	G	T	C	29	.21
	C	T	C	G	A	-/A	C	G	G	C	C	22	.16
	C	T/C	C	G	A	-/A	C	G	G	C/T	C	15	.11
	C	T	C	G	A	-/-	C	G	G	C	C	14	.10
	C	T/C	C	G	A	-/-	C	G	G	C	C	9	.07
	C	T	C	G	A	A/A	C	G	G	C	C	4	.03
	C	T/C	C	G	A	-/A	C	G	G	C	C	4	.03
	C	C	C	G	A	-/-	C	G	G	C/T	C	2	.01
	C	T	C	A/G	A	-/-	C	G	A/G	C	C	1	.007
	C	C	C	G	A	-/-	C	G	G	C	C	1	.007
	C	T/C	C	A/G	A	-/-	C	G	A/G	C/T	C	1	.007
												137	
Configuration	C: 1	T/C: .47	C: 1	A/G: .01	A: 1	-/-: .67	C: 1	G: 1	A/G: .001	C/T: .39	C: 1		
frequence		T: .30		G: .99		-/A: .30			G: .99	C: .40			
		C: .23				A/A: .03				T: .21			



3

Soluble CD59 is a novel biomarker for the prediction of obstructive chronic lung allograft dysfunction after lung transplantation

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Manuscript submitted

Abstract

CD59 is a complement regulatory protein that inhibits membrane attack complex formation. A soluble form of CD59 (sCD59) is present in various body fluids and is associated with cellular damage after acute myocardial infarction. Lung transplantation (LTx) is the final treatment for end-stage lung diseases but overall survival is severely hampered by the development of chronic lung allograft dysfunction, which presents itself in an obstructive form as the bronchiolitis obliterans syndrome (BOS). We hypothesized that, due to cellular damage and activation during chronic inflammation, serum levels of sCD59 can be used as biomarker preceding BOS development. We analyzed serum concentrations of sCD59 in 90 LTx patients, of whom 20 developed BOS. BOS patients exhibited higher sCD59 serum concentrations at the time of diagnosis compared to clinically matched non-BOS patients (p=0.018). Furthermore, sCD59 titers were elevated at 6 months post-LTx (p=0.0020), at which time patients had no symptoms of BOS or a decline of FEV_1. Survival-analysis showed that LTx patients with sCD59 titers above 400pg/ml 6 months post-LTx have a significant (p<0.0001) lower chance of BOS-free survival than patients with titers below 400pg/ml, 32% vs. 80% respectively, which was confirmed by multivariate analysis (hazard ratio 6.2, p<0.0001). We propose that circulating levels of sCD59 constitute a novel biomarker to identify patients at risk for BOS following LTx.

Introduction

CD59 is a membrane anchored complement regulatory protein that inhibits membrane attack complex (MAC) formation, thereby preventing complement mediated cell lysis[1,2]. Regulation of CD59 expression plays a pivotal role in various diseases. Hyperexpression of CD59 on endothelial cells increases resistance to complement mediated cell damage, which has been proposed to explain the process known as graft accommodation to circulating pathogenic allo- or autoantibodies following organ transplantation[3-5]. In contrast, age-related macular degeneration is associated with lower CD59 expression on monocytes[6], whereas paroxysmal nocturnal hemoglobinuria is caused by CD59 deficiency[7].

Different processes, including cell damage and activation, induce the release of membrane anchored proteins from the cell surface, a process designated as shedding[8]. CD59 can detach from the cell membrane to be released into the circulation or the interstitial fluid in a soluble form (sCD59). Indeed, sCD59 can be detected in various body fluids including urine, milk, serum, and plasma[9-11]. Various studies have designated sCD59 as biomarker for disease activity. Elevated circulating sCD59 concentrations have been found in acute myocardial infarction[10], and higher serum titers of glycated sCD59 have been described in diabetes mellitus[12]. Despite these findings, the role of sCD59 and its association with disease is still largely unidentified.

Lung transplantation (LTx) is the final treatment for selected patients with end-stage lung diseases. Long-term outcome after LTx is hampered by chronic lung allograft dysfunction (CLAD) which can be divided into an obstructive CLAD (bronchiolitis obliterans syndrome, BOS) and a restrictive CLAD (restrictive allograft syndrome, RAS). Clinical characterization of BOS involves obstructive pulmonary function tests (forced expiratory volume in one second (FEV_1) below 80% from baseline FEV_1) enduring for more than three weeks. The second form of CLAD, RAS, is identified by restrictive pulmonary functions tests (forced vital capacity (FVC) below 80% baseline FVC) presenting more than 3 weeks[13]. BOS is considered to be caused by chronic rejection which will lead to obliterative bronchiolitis and lung damage. In the clinical setting the FEV_1 decrease is used as surrogate marker and will occur after obliterative bronchiolitis has already developed. The recognition of this damage and therefore the diagnosis obliterative bronchiolitis prior to BOS development may be of help in designing therapies for BOS prevention. Five years after LTx the BOS-free survival is 50%, though most patients who survive short-term complications will eventually develop BOS[14].

Considering high CD59 expression by bronchial epithelial cells and sCD59 release following

cellular damage[2,10], we hypothesized that sCD59 may be a marker for inflammatory lung tissue damage predicting BOS incidence and progression. Therefore, we measured sCD59 in serum samples of LTx patients and assessed the correlation of sCD59 titers with BOS incidence. We show, that sCD59 levels measured six months post-LTx are strongly associated with BOS development and thus may be used as clinical marker for chronic rejection after LTx.

Patients and Methods

Patients

Eighty-nine patients that underwent LTx between September 2003 and May 2011 at the Heart Lung Center of the Utrecht Medical Center were included in the study, based upon serum availability, drawn at month 6 post-LTx (55% of the transplanted patients within this time period, Supplementary Figure 2). From all patients informed consent was obtained and the study was approved by the medical ethical committee (METC 06-144). Blood samples were collected several hours prior to transplantation, monthly during the first year of follow up, and every 3 months thereafter. Blood samples were processed and stored as serum aliquots at -80°C. Patients were treated with an immunosuppressive regime, consisting of tacrolimus, basiliximab, prednisone, and mycophenolate mofetil. Patients at risk for CMV reactivation (defined as CMV- recipient/CMV+ donor) were treated with valganciclovir until 6 months post-LTx.

Assay for soluble CD59

sCD59 concentrations in serum were determined via ELISA (USCN Life Science Inc., China) according to manufacturer's instructions. Serum samples were thawed, diluted 1:400 in PBS, and incubated on NUNC maxisorp plates (NUNC, Roskilde, Denmark) coated with a mouse anti-human CD59 monoclonal antibody for 2 hours at 37°C. The plates were washed and incubated with biotin-conjugated rabbit anti-human CD59 polyclonal antibodies for 1 hour at 37°C. Plates were washed again and incubated for 30 minutes with streptavidin-horseradish peroxidase. Bound biotinylated antibodies were visualized with TMB substrate for 15 minutes at 37°C. The reaction was terminated with H_2SO_4. Optical density of the wells was measured at 450 nm with a Multiskan EX Microplate photometer (ThermoScientific, IL). OD450 values were compared to standard concentrations of recombinant CD59 and are expressed in pg/ml. The minimal detectable dose of human sCD59 is 6.7 pg/ml. Intra- and inter-assay coefficients of variation are <10% and <12% respectively. Furtheremore, the recovery rate of the ELISA in our hands

was 87% (range 83%-91%), which was in concordance with the manufacturers description (avaerage recovery rate in serum 87%, range 80%-94%). All samples were measured in duplicate.

Statistics

Statistical analysis was performed using GraphPad Prism version 5.03 (GraphPad Software Inc., San Diego, CA) and SPSS version 20 (IBM Corp., Armonk, NY). Distribution of data was tested for normality via the D'Agostino & Pearson omnibus normality test. Data following a Gaussian distribution are represented as mean value ± SEM, whereas data not normally distributed are displayed as median and interquartile range, indicated in the respective results section and the corresponding figure legend. Categorical data such as age, gender, and primary disease were analyzed using the Fischer's exact test, type of transplantation using the Pearson's χ^2 test. Differences between continuous variables were tested via ANOVA. Differences between 2 groups were tested with unpaired *t* test in case the values followed a Gaussian distribution, and with the Mann-Whitney test otherwise. BOS-free survival was analyzed using a death censored, i.e. graft failure definition excludes patient death with a functioning graft, Kaplan-Meier analysis. Finally, we used a Cox proportional hazards model to identify sCD59 as an independent predictor for BOS incidence. A p-value < 0.05 was considered to be statistical significant.

Results

Patient demographics

Eighty-nine patients treated with LTx because of chronic obstructive pulmonary disease (COPD, n=40), cystic fibrosis (CF, n=30), interstitial lung disease (ILD, n=18);including extrinsic allergic alveolitis (n=1), idiopathic pulmonary fibrosis (n=6), lymphangioleiomyomatosis (n=3), non-specific interstitial pneumonia (n=1), pulmonary Langerhans cell histiocytosis (n=2), progressive systemic sclerosis (n=2) and sarcoidosis (n=3) or pulmonary hypertension (n=1), were included in the study. Further demographic details are given in Table 1. Twenty patients developed BOS, diagnosed according to international guidelines[15] and RAS was not observed. No differences were found between BOS and non-BOS patients, except mean follow-up time, which was shorter in the BOS group due to the death of 9 BOS patients during follow-up and episodes of acute rejection.

	Total LTx patients		
	BOS	**Non BOS**	***p*- value**
Total number	20	69	
BOS grade			
I	8	N.A.	
II	5	N.A.	
III	7	N.A.	
Onset of BOS (month)	28 (5 - 81)	N.A.	
Mean follow up (months)	48 (10 - 105)	50 (7 - 118)	0.72
Type of transplantation			0.38
Single	3	15	
Bilateral	17	54	
Mean age (years)	45 (16 - 63)	45 (17 - 63)	0.91
Gender			0.053
Male	6	37	
Female	14	32	
Primary disease			0.72
COPD	10	30	
CF	7	23	
ILD	3	15	
PH	0	1	
Episode of acute rejection	9	13	*0.021*

Table 1: Clinical and demographic profile of lung transplantation patients

Patients are clustered by diagnosis, which are COPD (chronic obstructive pulmonary disease), CF (cystic fibrosis), ILD (interstitial lung disease), and PH (pulmonary hypertension). Significant inter-group differences are given in italics.

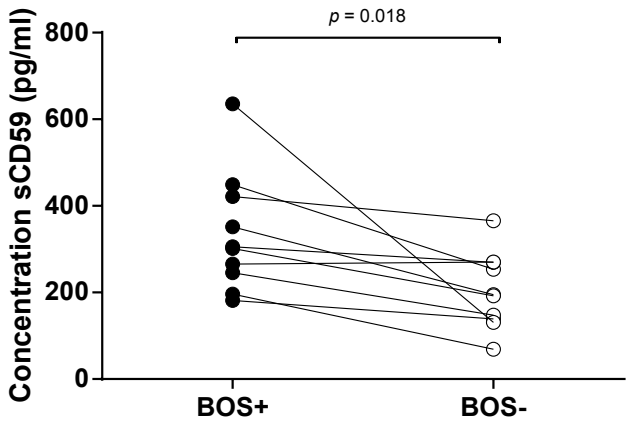

Figure 1: Serum sCD59 concentrations are elevated at the time of BOS diagnosis

From 10 BOS (filled circles) and 10 non-BOS (open circles) patients serum sCD59 concentrations were measured (matched pairs indicated by lines). BOS patients show significant higher sCD59 titers at the time of BOS diagnosis (335.5 ± 43.31 pg/ml vs. 203.3 ± 27.59 pg/ml) compared to their matched counterparts (p=0.018, Gaussian distribution, paired t-test).

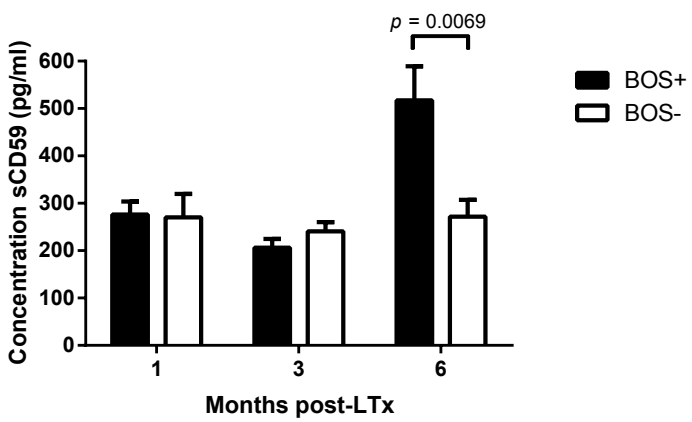

Figure 2: Serum sCD59 concentrations are elevated before the clinical diagnosis of BOS

sCD59 serum concentrations were measured at fixed time points after LTx in 10 matched BOS (black bars) and non-BOS (white bars) patients, the normally distributed data are depicted as mean ± SEM. No differences were observed either at 1 month or 3 months post-LTx. BOS patients show significant higher serum concentrations sCD59 (517.0 ± 72.22 pg/ml vs. 271.8 ± 35.22 pg/ml) 6 months post-LTx compared to their matched counterparts, before the clinical diagnosis of BOS (p = 0.0069, Unpaired t-test).

Concentrations of serum sCD59 are elevated at the time of BOS diagnosis

We first examined, in a pilot experiment, whether sCD59 levels differed between BOS and non-BOS patients. To this end we measured sCD59 in serum of 10 BOS patients at the month of BOS diagnosis, and compared these levels with those in 10 non-BOS patients matched for gender, age, primary disease, and month post-LTx. BOS was diagnosed at month 35 post-LTx on average (Supplementary Table 1). BOS patients have significant higher sCD9 concentrations compared to their matched counterpart (p=0.018, data normally distributed, paired t-test) at the time of diagnosis (Figure 1). In total, 80% of the BOS patients have elevated sCD59 serum concentrations compared to the median of matched controls.

Serum sCD59 concentrations are elevated before BOS diagnosis

To study potential sCD59 concentration differences before BOS diagnosis we analyzed sCD59 concentrations in the same matched cohort of ten patients at fixed time points after transplantation, and examined the course of sCD59 levels in either group. At month 1 and 3 post-LTx serum concentrations of sCD59 were comparable in both patients groups. However, a significant difference (p=0.0069) was observed at month 6 post-LTx (Figure 2, Gaussian distribution, mean ± SEM, unpaired t-test). These elevated sCD59 titers persisted over time until BOS diagnosis (data not shown).

sCD59 levels at 6 months post-LTx are indicative for BOS incidence

To verify that sCD59 titers are elevated at month 6 post-LTx in BOS compared to non-BOS patients, we examined serum concentrations of sCD59 in an additional patient cohort. Analysis of sera taken 6 months post-LTx from 10 additional BOS and 59 non-BOS patients confirmed the prior observation (data not shown). Taken these results together, total analysis of 89 LTx patients, 20 BOS and 69 non-BOS resulted in a significant difference (p=0.0020, non-Gaussian distribution, median + interquartile range, Mann-Whitney test) in sCD59 titers 6 months post-LTx (Figure 3A). A ROC curve was used to determine the possibility of predicting BOS 6 months post-LTx. This analysis demonstrated the optimal cut-off value for positivity at 400 pg/ml, AUC 0.72 (0.58–0.86) with a sensitivity of 60% and a specificity of 84% (Supplementary Figure 1). A Kaplan-Meier analysis showed that a sCD59 titer above 400 pg/ml significantly (p<0.0001) reduced likelihood of BOS-free survival compared to a sCD59 titer below 400 pg/ml, 32% vs. 80% respectively (Figure 3B). Furthermore, we assessed the possibility of confounding by usage of a multivariate Cox

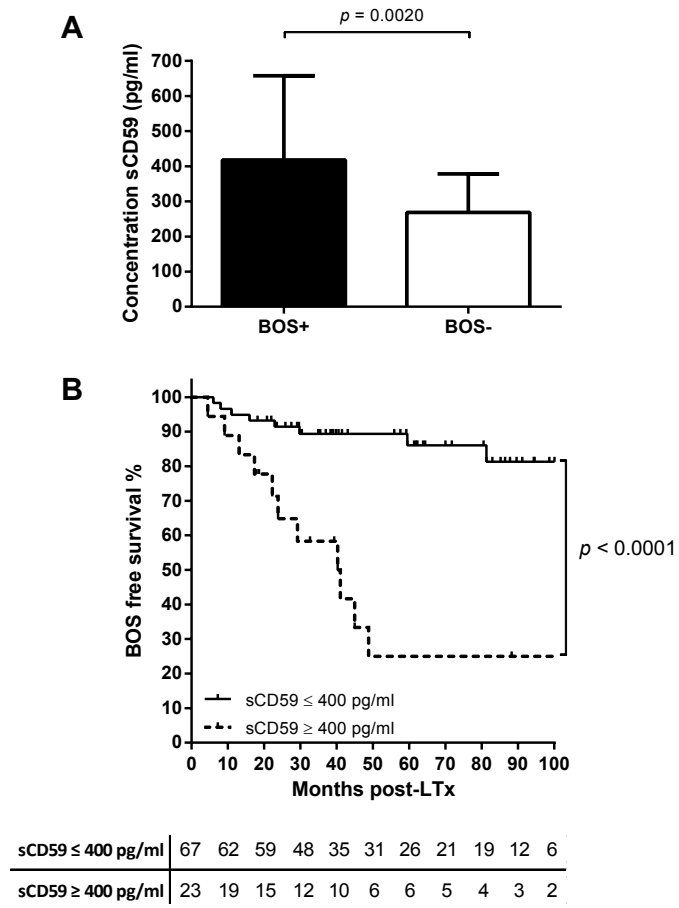

Figure 3: sCD59 serum titers 6 months post-LTx are indicative for BOS incidence

A Whole cohort analysis shows that sCD59 titers, displayed in pg/ml, 6 months post-LTx are elevated in BOS patients (*n*=20, black bar) compared to non-BOS patients (*n*=70, white bar), *p*=0.0020, Mann Whitney test. Both median and interquartile range are depicted due to the fact that the non-BOS serum titers follow a non-Gaussian distribution. **B** Death-censored Kaplan-Meier analysis for BOS incidence after LTx. LTx patients who present serum sCD59 titers above 400 pg/ml 6 months post-LTx (dashed line) have a significant lower chance of BOS free survival than patients with titers below 400 pg/ml (closed line), 25% vs. 81% respectively, $p < 0.0001$.

proportional hazard model including known risk factors as episodes of acute rejection, defined as a decline in lung function in absence of other causes of lung function decline treated with methylprednisolone pulse, high donor age and as patient being at high risk for CMV reactivation [16]. This model identified sCD59 titers ≥400 pg/ml 6 months post-LTx as an independent predictor for BOS development after transplantation (hazard ratio 5.7, 95% CI 2.3 – 14.3, $p<0.0001$), see Table 2.

We also assessed the relation between sCD59 and other parameters. The tye of transplantation (single vs. bilateral) did not correlate with sCD59 titers or LTx outcome. Club cell secretory protein 16 (CC16), an epithelial cell damage and leakage marker[17], measured 6 months post-LTx, did not correlate with sCD59 titers. Also, we found no

	Hazard ratio (95% CI)	*p*- value
sCD59 ≥ 400 pg/ml	6.2 (2.4-15.8)	*<0.0001*
Donor age (≥ 60)	4.6 (1.4-15.0)	*0.011*
CMV reactivation	0.7 (0.3-2.1)	0.572
Episode of acute rejection	1.8 (0.7-4.4)	0.230

Table 2: Multivariate analysis on BOS incidence after LTx

Within our multivariate analysis we included potential confounders for BOS incidence after LTx (see results section 3.4). In our cohort both a sCD59 titer ≥400 pg/ml and donor age ≥60 were identified as independent predictors for BOS development. Significant intergroup differences are given in italics.

increase of general markers of inflammation such as C reactive protein (CRP) or neutrophil counts specifically in the BOS group. Finally, since sCD59 can be secreted via glomerular filtration, we investigated whether patients with increased sCD59 levels had a worse renal function. No differences in creatinine levels were found between patients with elevated or normal sCD59 (data not shown).

Discussion

Long-term survival post-LTx is severally hampered by BOS development. Current diagnostic tools for BOS upon LTx are limited to FEV_1 measurement and there is a clinical need for biomarkers to early identify patients at risk for developing BOS. We evaluated serum concentrations of a soluble form of the membrane anchored complement regulatory protein CD59, sCD59, as a risk marker for BOS in a cohort of LTx patients. We show that serum levels of sCD59 in LTx patients are increasing prior to BOS diagnosis. This indicates that sCD59 titers may be used to identify patients at risk for BOS development earlier.

This study is limited with respect to the number of patients diagnosed with BOS after LTx. Therefore, we could not correlate sCD59 titers with different stages of BOS. Furthermore, the number of cases presented, limits the inclusion of covariates in our multivariate analysis. Hence, validation of these results is desired using a multicenter approach to increase patient numbers.

Absolute serum levels of sCD59 do differ comparatively with previous studies. Landi *et al.* measured serum concentrations of sCD59 in adults and children and found mean titers of approximately 70 ng/ml[18]. Väkevä *et al.* found sCD59 concentrations in healthy controls to

be around 8 pg/ml, though these measurements have been performed in plasma[10]. Ghosh *et al.* quantified sCD59 in standard peptide units, so no comparison could be made with our data[12]. Reported differences in sCD59 serum titers between the previous and our studies are probably reflecting differences in antibodies and standards used for the assays. Standardization of methods to measure sCD59 is urgently needed. We measured sCD59 with a commercial ELISA which is widely available to the medical community.

sCD59 is detectable in body fluids including plasma, urine, tears, sweat and human milk[1,2,19]. sCD59 exists with or without the phospholipid tail, and both variants differ in complement inhibiting capacities[9]. Our assay could not discriminate the two isoforms. Several mechanisms have been proposed to explain the occurrence of circulatory sCD59, including shedding via secretion of membrane vesicles containing phospholipid-tailed sCD59, or enzymatic cleavage via phospholipases C and/or D. The latter cut GPI anchored proteins at their phospholipid tail resulting in anchor-less sCD59. Other mechanisms claim that a substantial MAC deposition may lead to CD59 release, or that lipid secretion results in CD59 shedding from the cell surface[9,18]. Zangh *et al.* have shown that activation of vascular endothelial cells leads to an increase in phospholipase C secretion, which mediates sCD59 release[20]. This mechanism has also been proposed for diabetes mellitus, where enzymatic shedding in chronically activated endothelial cells is supposed to explain increased glycated sCD59 concentrations in serum[12,21]. Our data do not allow conclusions regarding the mechanisms underlying increased sCD59 levels in BOS or non-BOS LTx patients. However, diabetes was no confounder for sCD59 levels in our transplantation cohort.

CD59 is a GPI anchored membrane protein that suppresses complement mediated cell lysis via MAC inhibition[1,21-23]. CD59 is highly expressed on vascular endothelial cells. Also, expression is observed in the ductal epithelia of salivary systems and the bronchi[2]. Human respiratory tract tissue, including bronchi and alveolar tissue, expresses high levels of complement regulatory proteins, and most predominantly CD59. In healthy tissue, CD59 expression is observed at the apical and luminal side of the epithelium, whereas during inflammation a more diffuse and basolateral distribution is observed[24]. Interestingly, evidence suggests increased complement activation in bronchoalveolar lavage fluid (BALF) of patients with various lung diseases including cystic fibrosis[25] and sarcoidosis[26]. Infections and local inflammatory reactions may lead to increased complement activation and upregulation of complement regulatory proteins in order to protect self-tissue from complement mediated cell damage. Indeed, CD59 expression is increased in diseased compared to healthy tissue and CD59 is also distributed along the extracellular matrix of the respiratory tract[24]. The same pattern of increased complement regulation during

inflammation has been observed in the gut. CD59 is upregulated in patients with gastritis, coeliac disease and inflammatory bowel disease. It is proposed that cytokines secreted in inflammatory lesions can induce CD59 expression on the apical surface of colonic epithelium[27].

We demonstrate that sCD59 increases at the time BOS becomes clinically manifest, and that sCD59 levels at month 6 post-LTx discriminate patients with BOS from those without. These elevated sCD59 levels do not seem to be protective against aggravated complement activity, presumably due to decreased functionality[9]. The explanation that sCD59 reflected renal clearance was ruled out since we found no correlation with creatinine levels. We therefore assume that sCD59 reflects the inflammatory processes ensuing in the transplanted lungs preceding the clinical manifestations of BOS. To discriminate whether cellular damage or activation underlies increased sCD59, we conducted a correlation analysis between CC16, a lung epithelial injury marker[17], and sCD59, but no correlation was observed. We found no relation between sCD59 and general markers of infection, including CRP and neutrophil counts. Generally, these results suggest that sCD59 reflects a local inflammatory process that is linked to the development of BOS rather than a general state of immune activation.

We hypothesize a model in which chronic inflammation, a hallmark of BOS development, results in cellular activation, presumably of endothelial or epithelial cells, and increased expression of CD59 which is then shed from the cell surface and detectable at increased levels in the circulation. Our observations are in concordance with previous results, where decreased membrane attached CD59 and increased sCD59 was observed in patients after acute myocardial infarction[10]. Interestingly, both endothelial and epithelial cells when activated secrete IL-8, CCL-5 and CCL2, measured in BALF, cytokines associated with BOS development[28], strengthening the concept of the role of cellular activation in the BOS pathogenesis. Unfortunately, in our study we did not have access to BALF since surveillance bronchoscopy was not performed. Therefore, we could not quantify sCD59 in BALF or correlate any changes in cytokine secretion or cellular subset composition in BALF to sCD59 titers.

Recently, novel biomarkers have been identified to predict BOS development. Also, auto- and alloantibodies have been associated with BOS incidence[4]. It is therefore reasonable to assume that humoral rejection and complement activation plays a pivotal role in BOS pathogenesis, as shown by Magro *et al.*[5]. Furthermore, soluble CD30 and TARC are found to have a predictive value on BOS development[29,30]. Further studies are needed to establish the predictive value of each of these markers for BOS.

To summarize, we report a significant increase of sCD59 in patients with BOS following LTx. This increase occurred at 6 months post-LTx and preceded clinical manifestations of BOS. Further studies are needed to elucidate the molecular mechanisms underlying increased sCD59 in BOS. We propose that sCD59 may provide a suitable biomarker to identify patients at risk for BOS development after LTx.

Acknowledgments

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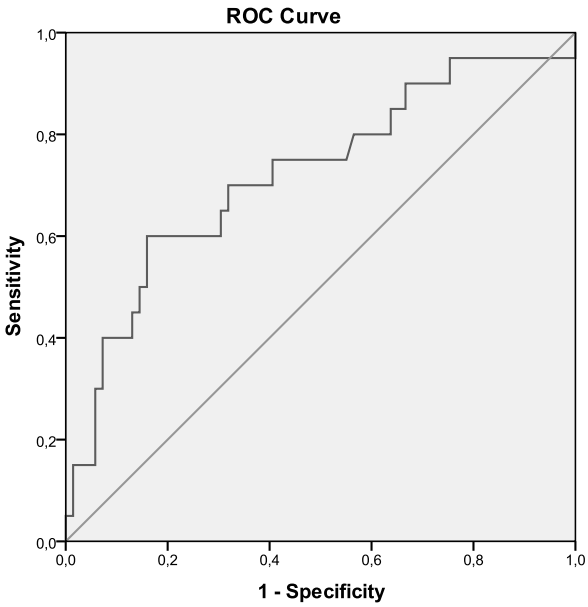
Supplementary Information



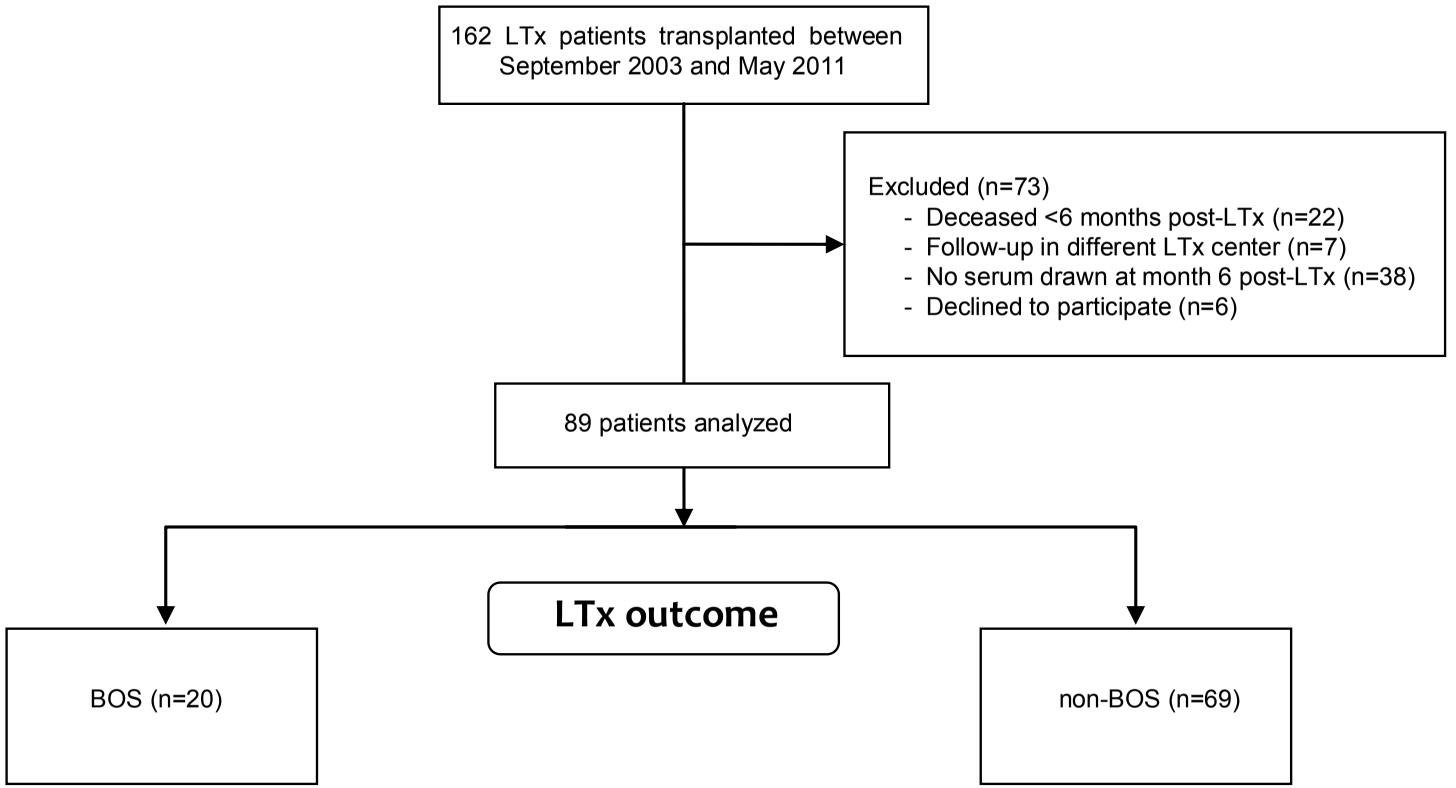
Supplementary Figure 1: ROC curve analysis of sCD59 concentrations

A ROC curve was generated to identify the optimal concentration of sCD59 as prognostic marker for BOS development. This analysis demonstrated the optimal cut-off value for positivity at 400 pg/ml, AUC 0.72 (0.58-0.86) with a sensitivity of 60% and a specificity of 84%.

	Matched BOS and non-BOS patients		
	Total LTx patients		
	BOS	**Non BOS**	***p* value**
Total number	10	10	
Gender			0.709
Male	2	2	
Female	8	8	
Mean age (years)	42 (16 - 61)	43 (21 - 61)	0.957
Onset of BOS (months)	35 (23 - 59)	n.a.	n.a.
Primary disease			
COPD	6	6	0.675
CF	4	4	

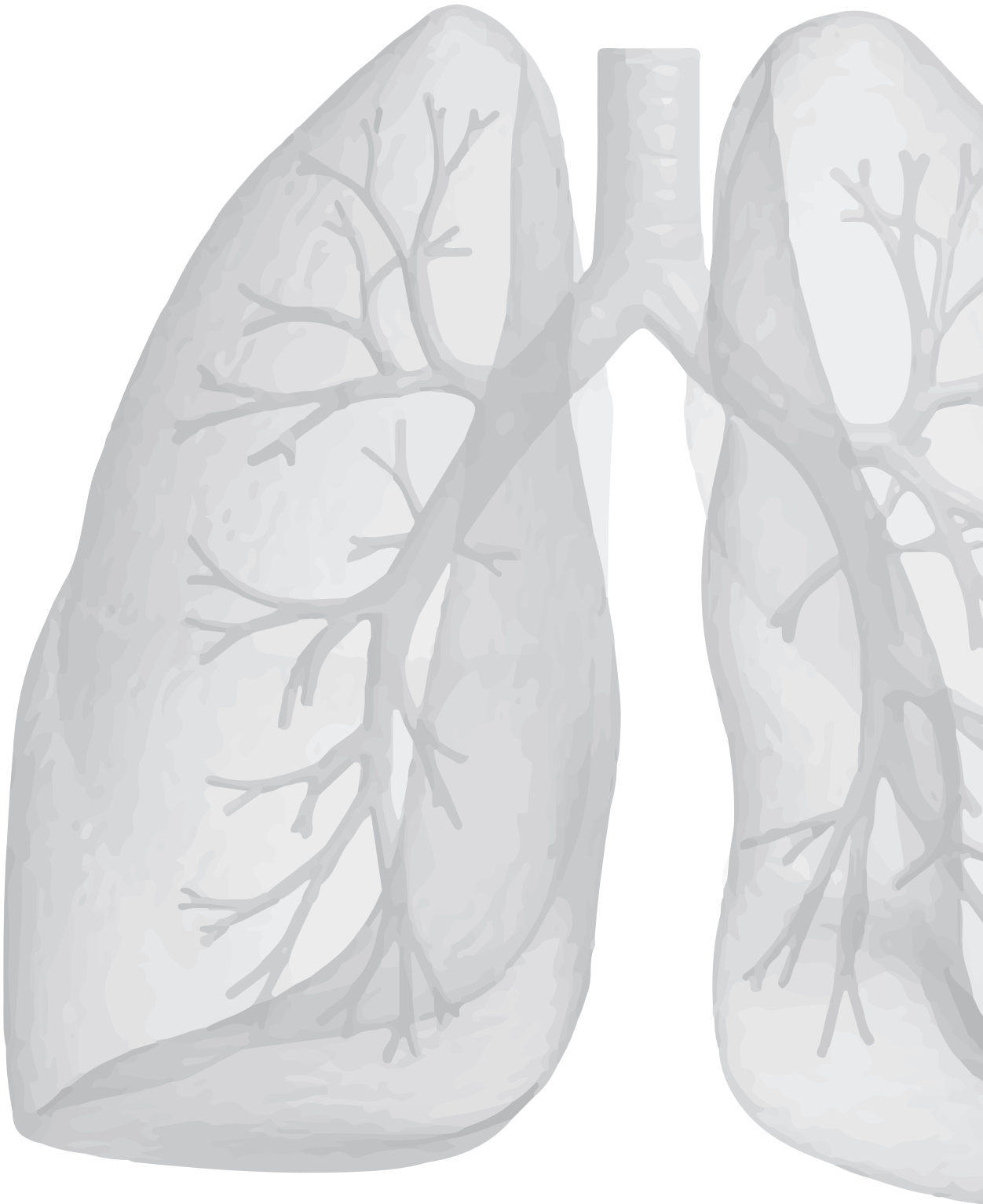
Supplementary Table 1: Clinical and demographic profile of matched BOS and non-BOS patients

Patients are divided in BOS and non-BOS groups. Patients were matched for gender, age, onset of BOS, and primary disease. No significant differences were observed between the two patient groups.



Supplementary Figure 2: FLOW chart of patient inclusion

A total of 162 LTx patients were transplanted between September 2003 and May 2011. Seventy-three patients were excluded, leaving 89 patients for analysis.



4

Anti-apoptotic lung endothelial cell antibodies present in end-stage lung disease patients do not correlate with clinical outcome after lung transplantation

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Abstract

Antibodies directed against both HLA and non-HLA targets are associated with transplantation outcome in multiple organs. Recently, pre-transplant serum IgG antibody levels directed against apoptotic Jurkat cells were found to correlate with kidney allograft loss. We investigated the presence of these antibodies in a cohort of lung transplantation (LTx) patients and evaluated the correlation of pre-LTx serum levels of IgG antibodies against apoptotic Jurkat cells (anti-AJC) with LTx outcome. Furthermore, primary donor lung endothelial cells (EC) obtained from donor lung perfusion fluid collected during LTx procedure, were isolated, expanded *in vitro* and analyzed as targets for anti-apoptotic reactivity. Cultured cells exhibited characteristic EC morphology and were CD31+, CD13+ and vWF+ , shown by FACS analysis. End-stage lung disease patients showed elevated serum IgG levels directed against apoptotic lung EC (anti-AEC; p=0.0018) and of anti-AJC (p<0.0001) compared to healthy controls. Interestingly, levels did not correlate with each other, hinting at target cell specificity. We observed no correlation between the incidence of chronic or acute rejection and pre-LTx serum levels of anti-AJC or anti-AEC. Also, levels of antibodies directed against apoptotic targets did not differ between matched patients developing chronic rejection or not during post-LTx follow-up or at the time of diagnosis, as they remained as high as prior to transplantation. Thus, circulating levels of anti-apoptotic cell antibodies are elevated in end-stage lung disease patients but our data do not support a role for these antibodies in the outcome of LTx.

Introduction

Chronic lung allograft dysfunction (CLAD) is the main complication after lung transplantation (LTx). In 2014, the nomenclature for chronic rejection after LTx has been revised and CLAD is divided in two different clinical phenotypes, an obstructive form, or bronchiolitis obliterans (BOS), and a restrictive from, or restrictive allograft syndrome (RAS)[1]. Currently, BOS is the dominant type of CLAD. According to recent reports, the worldwide 5-year BOS free survival rate is 50% and mean survival post-LTx is around 10 years[2].

The pathogenesis of BOS following LTx is a multifactorial process. Risk factors for BOS include viral infections, primary graft dysfunction, lymphocytic factors, air pollution, genetic factors and episodes of acute rejection[3,4]. Also, the development of donor-specific antibodies and *de novo* autoantibodies may contribute to the development of BOS, though their role is not fully understood. Some studies have underlined the importance of alloimmune reactions against the transplant, particularly donor-specific HLA-antibodies on transplantation outcome[5,6], although their exact role is debated[7]. In addition to alloimmunity, autoimmunity, especially in the form of non-HLA specific autoantibodies against collagen type V and k-alpha-tubulin, are thought to contribute to an increased risk of BOS development[8,9]. Thus, humoral immunity against the transplant may be important in BOS pathogenesis and progression.

A major limiting factor in kidney transplantation is pre-transplant allosensitization, due to blood transfusions, pregnancies or previous allografts[10]. In line with observations in LTx, also pre-existing non-HLA antibodies have been associated with an increased risk of rejection. For example, pre-existing anti-angiotensin type 1 receptor antibodies and anti-endothelin-1 type A receptor antibodies constitute an independent risk factor for graft loss[11,12]. Also, vimentin, an intra-endothelial cell (EC) protein that can be exposed to the immune system after EC damage and can act as a target for antibody formation[13]. Interestingly, Gao *et al.* have shown that pre-transplant antibodies against apoptotic Jurkat T cells (anti-AJC) predict antibody-mediated rejection and graft failure of kidney transplants[14]. The antigens of anti-apoptotic antibodies have been partly elucidated. Polyreactive anti-AJC may react with phospholipids, phosphatidylserine and lysophosphatidylcholine, which during apoptosis become exposed on the cell-membrane upon membrane flip-flop[15,16].

Given the similarities in pathogenic mechanisms induced by graft-reactive antibodies in kidney- and lung transplantation, we hypothesized that these antibodies against apoptotic targets pre-existing or induced upon transplantation, may correlate with outcome

following LTx. To test this hypothesis we evaluated the presence of circulating anti-AJC in a cohort of LTx patients, and assessed their correlation to outcome. Since EC are the primary cells encountered by the recipient's immune system we also assessed the role of antibodies directed against apoptotic primary lung EC (anti-EC) in this respect. These cells were obtained from the donor during transplantation procedure. Our results indicate that antibodies against both apoptotic EC and Jurkat cells are present in patient serum prior to transplantation, but that these antibody levels do not correlate with transplantation outcome.

Patients and Methods

Patients and sampling

We included 80 LTx patients that underwent LTx within our center between September 2003 and November 2012, and of whom pre-transplantation serum was available. Twenty-nine of these patients were diagnosed with BOS based upon ISHLT criteria[17], whereas 22 patients deceased. Seventeen patients developed one or more episodes of acute rejection (AR) during follow-up. Further clinical and demographic parameters are depicted in Table 1. All patients were treated with standardized immunosuppressive regime consisting of tacrolimus, basiliximab, prednisolone and mofetil mycophenolate. Patients depicted as being at risk for CMV or EBV reactivation (defined as a CMV-/EBV- patients receiving a graft from a CMV+/EBV+ donor) were prophylactically treated with valganciclovir up until 6 months after transplantation. Serum samples from 20 healthy controls (HC) were obtained, processed, and stored at -80°C until further usage.

Perfusate analysis, lung endothelial cell collection, and cell culturing

To reduce the risk for thromboembolic complication shortly after LTx[18], the grafted lungs were flushed antegradely via the pulmonary artery with Perfadex® solution. During this procedure, the lungs were ventilated at tidal volume and topically cooled. After explantation, the lungs were flushed for a second time, but now via the pulmonary vein, until the fluid became clear and upon inspection was free of blood cloths. This flush fluid was collected and centrifuged for 10 min at 1800 RPM. The cell pellet was dissolved in phosphate buffered saline (PBS, Sigma-Aldrich, St. Louis, MO) and subsequently loaded on Ficoll-Paque (GE Healthcare, Milwaukee, WI) for cell separation. The cells were isolated from the interphase, put in aliquots of 1 ml in RPMI-1640 (Gibco, Waltham, MA) supplemented with DMSO (Sigma-Alderich) and 20% v/v fetal bovine serum (FBS, Bodinco, Alkmaar, The Netherlands) and stored in liquid nitrogen until further processing.

Parameters	Values
Gender, n (%)	
Male	39 (48.8%)
Female	41 (51.2%)
Age in year (mean ± SD)	44.4 ± 13.2
Follow-up in months (mean ± SD)	78.7 ± 36.7
End-stage lung disease, n (%)	
COPD	37 (46.2%)
CF	27 (33.8%)
ILD	16 (20.0%)
BOS, n (%)	29 (36.3%)
AR, n (%)	17 (21.3%)
Deceased, n (%)	22 (27.5%)

Table 1: Clinical and demographic profile of lung transplanation patients.

COPD: chronic obtructive pulmonary disease, CF: cystic fibrosis, ILD: interstitial lung disease, BOS: bronchiolitis obliterans syndrome

Both whole perfusate and frozen cell aliquots were inspected for the presence of lung EC via FACS analysis using a FACSCanto™ and FACSDiva™ software (BD Biosciences, Durham, NC) and FlowJo v.10 (FlowJo, LLC, Ashland, OR). Either whole perfusate cell samples or thawed frozen cell aliquots were stained with a combination of CD45-PO (Thermo Fisher Scientific, Rockford, IL), CD31-FITC (BD Biosciences), CD13-PE (BD Biosciences), CD90-BV421 (BioLegend, San Diego, CA), VEGFR-2-AF647 (BD Pharmingen, San Diego, CA), or respective isotyes (Biolegend and R&D Systems, Minneapolis, MN). Intracellular staining for Von Willebrand Factor (vWF) was conducted using vWF-APC (R&D systems) and Intrastain kit (DAKO, Glostrup, Denmark).

For cell culturing, frozen cell samples were thawed at 37°C, washed with RPMI-1640 20% FBS, centrifuged for 10 min at 1800 RPM and the pellet was dissolved in endothelial cell specific growth medium (EBM-2, Lonza, Basel, Switzerland) and additives for vascular EC (EGM-2 SingleQuots™, Lonza). Cells were cultured in gelatin (Sigma-Aldrich) pre-coated Nunclon™ T-25 culture flasks (Sigma-Aldrich) in EBM-2 supplemented with Primocin™ (Invivogen, San Diego, CA) until confluency. Confluent flasks were treated with Accutase™ (BD Biosciences) and cells were sub-cultured (50.000 per flask) in Nunclon™ T-75 culture flasks. Cell morphology was assessed via light microscopy (Leica DM IL, Leica Microsystems, Wetzlar, Germany), and cell numbers analyzed via Bürker-Türk cell counting chambers (Laboroptik Ltx. Lancing, UK).

Jurkat cells (ATCC, Manassas, VA) were cultured in T-75 culture flasks in RPMI-1640 10% FCS supplemented with Primocin™ and sub-cultured 1:6 twice a week.

IgG purification

IgG was purified from frozen serum samples using Magne™ Protein G Beads (Promega, Madison, WI) according to manufacturer's instructions. Briefly, 50 µl beads were incubated with 50 µl pre-centrifuged thawed serum samples for 30-60 min at room temperature while shaking. Supernatant was removed via magnetic separation of the beads which were subsequently washed multiple times. Purified IgG was diluted from the beads and the purified IgG quality and concentration was obtained using the NanoDrop® ND-1000 system (Thermo Fisher Scientific). Purified IgG fractions were stored at 4°C until further usage.

Antibodies against apoptotic Jurkat and primary lung endothelial cells

Anti-AJC and anti-AEC antibodies were assessed via flow cytometry. Jurkat cells were co-incubated with 5 µl 1 µM staurosporine (Sigma-Aldrich) O/N at 37°C to induce apoptosis. Cells were centrifuged for 5 min at 1800 RPM, the supernatant discarded and the cell pellet was dissolved in 5 ml culture medium. One-hundred µl cell suspension was added to a 96-wells round bottom plate (Sigma-Aldrich), centrifuged (5 min, 1500 rpm) and subsequently, were incubated with 60 µl purified IgG for 30 min at 37°C. Each well was washed twice with 150 µl PBS and centrifuged (5 min, 1500 RPM). The supernatant was discarded and the cells were incubated with mouse anti human IgG PE (BioLegend), diluted 1:12.5 in PBS for 30 min at 4°C. Cells were washed once with 150 µl PBS and once with 150 µl annexin V binding buffer (1L H_2O+10 mM Hepes+140 mM NaCL+2.5 mM $CaCl_2 \cdot 2H_2O$; pH 7.4). Cells were centrifuged, the supernatant discarded and 100 µl binding buffer supplemented with 4 µl 7AAD (BD Pharmingen) and annexin V FITC (ITK Diagnostics, Uithoorn, The Netherlands), diluted 1:2, was added followed by 10 min incubation at room temperature and fluorescence measurement.

In order to induce apoptosis, lung EC were subjected to serum starvation for 48-72 hours at 37°C, culturing with staurosporine O/N or exposed to UV light using an UV stratalinker (Stratagene, Santa Clara, CA). Serum starvation yielded the largest amount of apoptotic EC (35%-40%). After serum starvation, cells were washed with PBS and detached with 4 ml Accutase™ for 5 min at 37°C. Cell detachment was determined via light microscopy. Six ml of culture medium was added to the culture flask and centrifuged for 5 min at 1500 RPM. The supernatant was discarded and the cell pellet dissolved in PBS. One-hundred µl cell suspension was added per well (V bottom 96-wells plate, Sigma-Aldrich) and incubated with 60 µl purified IgG (mean concentration 548 ± 154 µg/ml),

diluted 1:5 in PBS for 30 min at 37°C. Subsequent antibody incubations were identical to the previous described Jurkat protocol. All measurements for either Jurkat or primary EC were conducted on the same day. To determine the inter- and intra-assay variation, cells were seeded in duplicate and identical sera measured on multiple days. The inter- and intra-assay variability for this assay were 18% and 4.1% respectively.

Statistics

The D'Agostino & Pearson omnibus normality test was used to test the data for Gaussian distribution. Differences in non-normally distributed data was analyzed via the Mann-Whitney test, whereas student *t*-tests were used to asses differences in normally distributed data. Correlation analyses on non-normally distributed data were performed with the Pearson correlation coefficient. Statistical analyses were performed using GraphPad Prism version 6.02 (GraphPad Software Inc., San Diego, CA) and SPSS version 20 (IBM Corp., Armonk, NY). Information on statistical testing and graph display is depicted in the respective figure legend. A p-value<0.05 was considered to be statistically significant.

Results

Lung endothelial cell culturing and characterization

First, we analyzed perfusate samples from 6 patients for the presence of vascular EC as determined by FACS. Vascular lung EC were identified as being CD45-/VEGFR-2+/CD13+, and confirmed by intracellular staining of vWF. The percentage of EC in each perfusate was assessed by analyzing 1.000.000 cells. This percentage ranged from 0.0002% and 0.0256% (see Figure 1A). For further analysis of EC, cells in the perfusate were first expanded in primary cell cultures. Individual EC were observed within the first week after initial cell seeding. Cell numbers sufficient for sub-culturing or cell marker analysis were reached in 2-5 weeks (Figure 1B). EC were cultured for a maximum of nine passages.

EC were characterized via cell morphology, cell surface-, and intracellular markers. Cells in culture presented typical EC morphologic characteristics. Cell density per passage differed. Furthermore monolayers were observed in later culture passages, and cell morphology remained constant during culturing (Figure 1C-1,-2,-3,-4). EC were non-lymphoid (CD45-). Cell could be cultured without any morphological indications of cell death until passage nine. FACS analysis showed that cultured EC expressed both EC specific markers CD13 and CD31 on their cell surface. Furthermore, intracellular staining revealed these cells to be positive for vWF (Figure 1D).

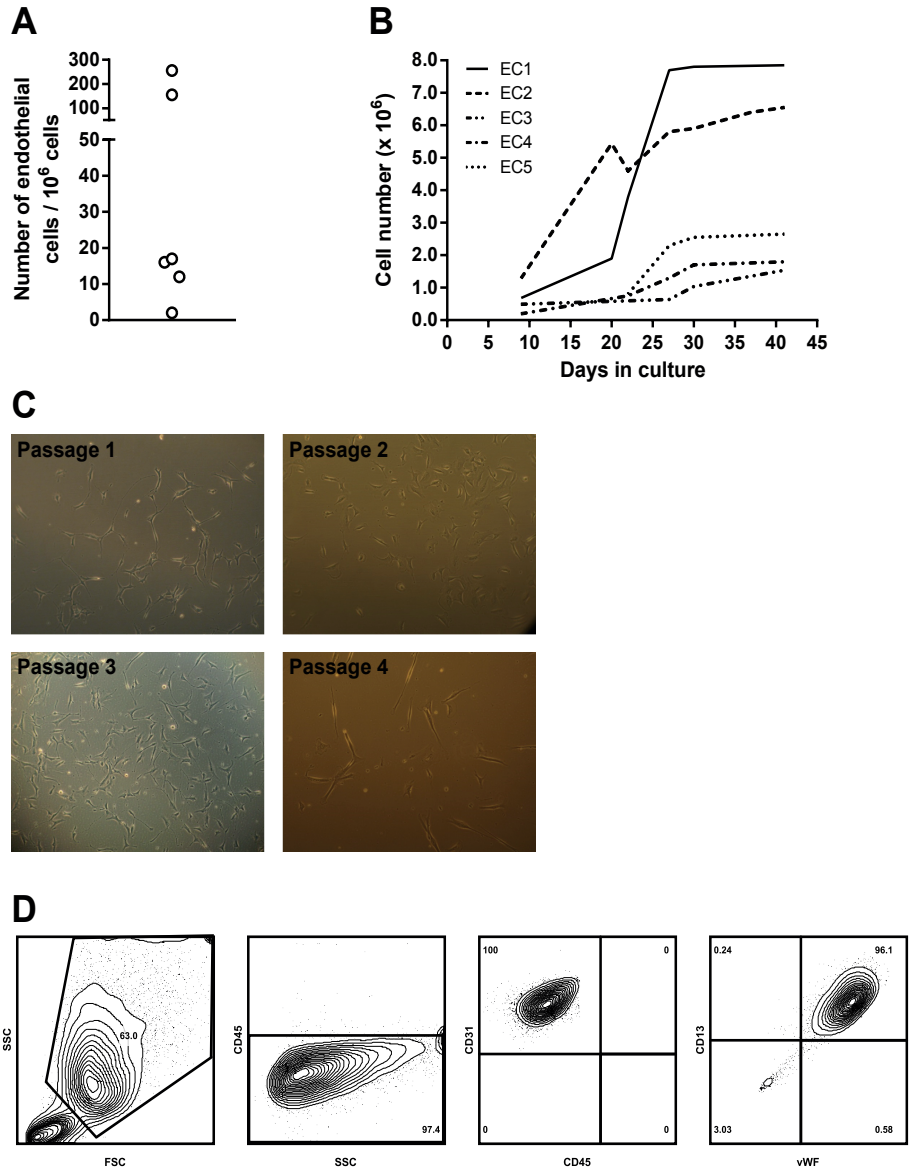

Figure 1: Endothelial cell culture characteristics

A Schematic representation of the numbers of EC per 1.000.000 cells in a single lung perfusate sample. Cell numbers were quantified via flow cytometry. **B** Overview of primary lung EC cultures. EC from different donor perfusate samples were cultured in EBM-2 EC specific medium. Five different perfusate samples were seeded in gelatin coated culture flasks and cell numbers were assessed on different culture days, when cell density was high enough to further passage the cell cultures. Large difference were observed in cell growth rates between individual cell cultures. **C** Light

microscopy images of different primary EC cultures. Typical EC morphology could be observed, as well as clustering of EC at higher cell densities. Passage 1, 100x, Passage 2, 400x, Passage 3, 100x, Passage 4, 100x. **D** EC characterization via flow cytometry. Cell cultures were detached using Accutase™ cell detachment solution. EC were gated as large cells based on forward/sideward scatter. Gated cells were from the non-lymphoid lineage (CD45-) and expressed both CD31 and CD13, characteristic for EC. Furthermore, intracellular staining revealed these cells to be vWF positive. Density plots were created using FlowJo analysis software.

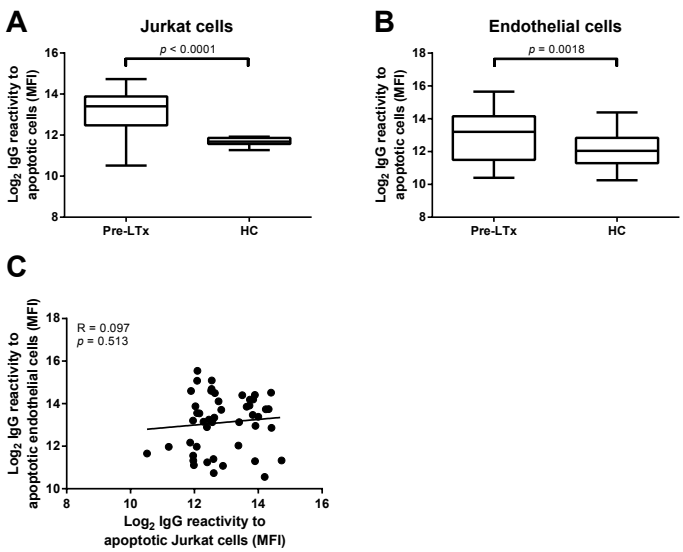

Figure 2: End-stage LTx patients present with elevated levels of serum IgG against apoptotic targets

Pre-LTx serum IgG reactive against both apoptotic primary EC (**A**) and Jurkat cells (**B**) were measured and compared to HC. End-stage lung disease patients present with significant higher levels of antibodies against apoptotic EC and apoptotic Jurkat cells compared to HC (p<0.0001 and p=0.0018, respectively). The y-axis depicts the Log_2 values of the measured mean fluorescent intensity (MFI). The data is represented as box and whiskers plot where the horizontal bar depicts the median value, the box both 25th and 75th percentiles and the whiskers the minimum and maximum of the observed values. The data follows a non-Gaussian distribution, Mann-Whitney test, pre-LTx n=80, HC n=20. **C** No correlation was found between serum levels of antibodies directed at apoptotic Jurkat or apoptotic EC. The data is normally distributed, Pearson correlation coefficient.

End-stage lung disease patients exhibit increased circulating levels of antibodies against apoptotic cells

As these cells have been used frequently as a source of apoptotic cells, we first tested the presence of antibodies against apoptotic Jurkat cells. Apoptosis was induced as described in the methods section. Overall apoptosis rates in Jurkat cells were around 60%-70% of total cell numbers. Higher levels of antibodies against apoptotic Jurkat cells were observed in LTx patients before transplantation as compared to HC (p<0.001, Figure 2A).

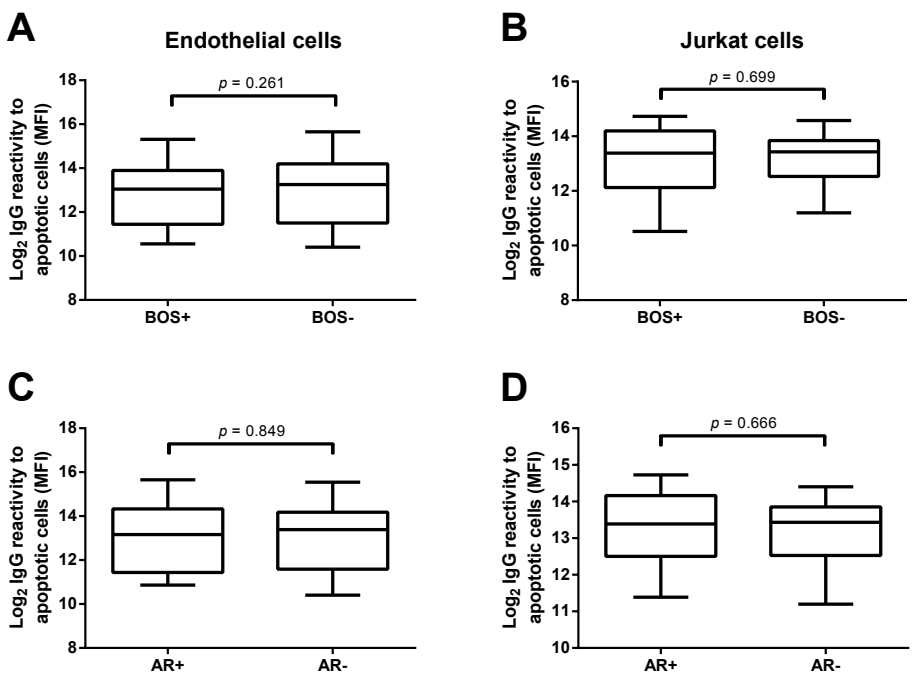

Figure 3: Pre-LTx IgG antibodies to apoptotic cells do not correlate with clinical outcome after LTx

Pre-LTx purified IgG levels against apoptotic EC (**A** and **C**) and Jurkat cells (**B** and **D**) were stratified according to either BOS diagnosis (**A** and **B**) or the incidence of AR (**C** and **D**). Neither BOS+ or AR+ patients presented with different purified pre-LTx IgG levels. Therefore, no correlation between IgG reactivity against apoptotic cells and clinical outcome after LTx was observed. The data is represented as box and whiskers plot where the horizontal bar depicts the median value, the box both 25th and 75th percentiles and the whiskers the minimum and maximum of the observed values. **3B** data normally distributed, unpaired t test. **3A**, **3C**, **3D**, data not normally distributed, Mann-Whitney test. BOS+ n=29 BOS- n=51, AR+ n=17, AR- n=63.

EC induced to apoptosis were analyzed with flow cytometry. Annexin V+/7AAD- cells were considered to be early apoptotic cells and Annexin V+/7AAD+ cells late apoptotic. Overall apoptosis rates in EC was around 20% to 30% of total cell numbers. Apoptosis-induction via exposure to UV radiation did not result in sufficient apoptotic EC numbers (<5%, data not shown). Purified pre-LTx serum IgG of 80 end-stage lung disease patients was analyzed for binding to apoptotic EC, from two different lung donors, together with serum IgG of 20 healthy controls (HC). We observed a higher IgG reactivity to apoptotic ECs in pre-LTx serum samples compared to HC (p=0.0018, Figure 2B).

The difference between apoptotic specific IgG levels between end-stage lung disease patients and HCs was higher on apoptotic Jurkat cells. We did not observer differences in IgG antibody reactivity to apoptotic targets when early and late apoptotic EC or Jurkat cells were differentiated. Stratification of serum IgG titers per end-stage lung disease, clustered as COPD, CF and ILD did not show any significant differences. Also, we observed no correlation between anti-AJC and anti-AEC (Figure 2C).

Pre-LTx serum IgG reactivity to apoptotic targets does not correlate with clinical outcome after LTx

We analyzed the relation of pre-LTx levels of anti-AJC and anti-AEC and outcome after LTx. First, we stratified patients by development of BOS during follow-up. Twenty-nine patients were diagnosed with BOS. No differences were observed between serum IgG levels of anti-AJC or anti-AEC in BOS+ and BOS- patients (Figure 3).

Second, we investigated the predictive value of pre-LTx serum IgG levels on the incidence of acute rejection (AR) post-LTx. In total 17 patients were diagnosed with one or more episodes of AR. We observed no significant differences between serum levels of anti-AJC or anti-AEC in LTx patients with or without AR (Figure 3C and 3D respectively).

Serum IgG reactivity during BOS development

Since we did not observe any predictive value concerning pre-transplant levels of serum IgG reactivity to apoptotic cells, we investigated levels of these antibodies at the time of BOS diagnosis. We isolated serum IgG from serum samples obtained from 10 patients within a month that BOS was diagnosed. In parallel, we selected serum samples from 10 patients who did not develop BOS and were matched for gender, age, primary lung disease and month post-LTx. IgG was purified from these samples as described in methods. No increase in IgG reactivity was observed against either apoptotic lung EC (Figure 4A) or apoptotic Jurkat cells (Figure 4B).

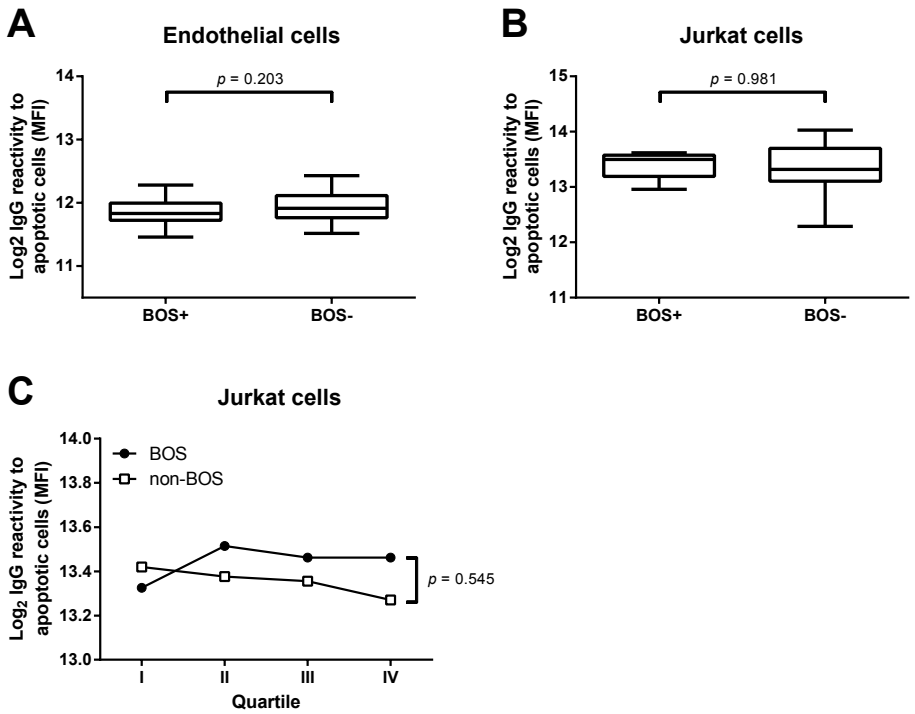

Figure 4: Purified serum IgG against apoptotic cells at BOS diagnosis and progress during transplantation follow-up

Levels of purified serum IgG, obtained at BOS diagnosis, were analyzed in a cohort of 10 BOS+ patients and 10 BOS- patients matched for age, gender, month after transplantation and primary lung disease. No differences between these two groups were observed using either apoptotic ECs (**A**) or Jurkat cells (**B**) or EC. Data normally distributed, unpaired *t*-test, BOS+ *n*=10 BOS- *n*=10.

In order to gain insight into the course of IgG antibodies against apoptotic targets during follow-up after LTx we used a quartile-based selection method. For this end, we divided the time from transplantation to the moment of BOS diagnosis in 4 equal quartiles and purified IgG from serum taken at each quartile. Parallel to 10 BOS+ patients, we analyzed 10 matched BOS- patients, mentioned above, via this approach. BOS+ patients present with slightly elevated IgG levels specific for apoptotic targets, although this difference is not significant. Each dot represents the mean of 10 analyzed individuals. Two-way ANOVA on Log_2 transformed MFI, with BOS as independent source of variation.

In order to gain insight into the course of IgG antibodies against apoptotic cells we divided the time from transplantation to BOS diagnosis in 4 even time frames (quartile based selection). Subsequently, we isolated IgG from serum samples obtained during each quartile and analyzed these samples for IgG reactivity. This procedure was also conducted for the matched BOS- patients. Overall, we observed an increased level of IgG antibodies against apoptotic Jurkat cells in BOS+ patient compared to BOS- patients during follow-up. However, this difference is not significant, and does not seem to be discriminative at time points prior to clinical manifestations of chronic rejection (Figure 4C).

Discussion

The prediction and diagnosis of chronic rejection after LTx remains challenging. In this research, we analyzed the levels of antibodies against apoptotic EC and Jurkat cells in a cohort of end-stage LTx patients. Our results indicate that end-stage lung disease patients have elevated levels of anti-apoptotic antibodies against both cell types. However, these titers do not seem to be predictive for the clinical outcome after LTx. Furthermore we describe the technique to culture primary lung vascular endothelial cells from perfusate obtained during LTx procedure.

Antibodies against apoptotic targets have been identified in previous studies, with Jurkat cells as model for cellular apoptosis[15,19,20]. In our initial observations on antibodies against apoptotic Jurkat cell we observed Log_2 IgG reactivity levels in concordance to the results published by Gao *et al*[15]. Furthermore, the levels of antibodies directed at apoptotic EC were also within this range.

The primary EC culturing method presented in this paper is novel, and has potential implications for LTx research. Donor lung EC are the first cells recognized by the recipient's immune cells, initiating, amongst others, direct allorecognition via MHC antigens which are expressed on these cells[21]. Also, EC are suggested to play a major role in the pathogenesis of chronic rejection, due to the secretion of pro-inflammatory cytokines upon activation[22,23]. Therefore, research has been conducted into the interplay of transplantation patient's immune cells or serum components, serially collected post-transplantation and endothelial cell lines, such as human umbilical vein EC[24,25]. Since our method allows to culture these EC directly from the donor, this *in vitro* model enables us to investigate the interface of immune activation between donor EC and patient PBMCs in a biologically more relevant way. This opens new perspectives for LTx research.

Pre-transplant antibodies against apoptotic Jurkat cells in kidney transplantation patients

have been found to correlate with late kidney allograft loss[14]. We did not confirm these observations in our cohort of LTx patients. Neither pre-LTx anti-apoptotic IgG serum levels directed against apoptotic EC or Jurkat cells correlated with the incidence of acute- or chronic rejection. Furthermore, levels of these antibodies during follow-up after LTx were not discriminative for differences in clinical outcome. We also distinguished between early- and late apoptotic cells, but no differences could be observed regarding end-stage lung disease or outcome after LTx. These results suggest differences in the contribution of these antibodies on the underlying pathological process in the development of rejection in both kidney and lung transplantation. Synergistic effects of anti-HLA antibodies and non-HLA antibodies on graft survival have been described for different non-HLA antibodies, including antibodies against apoptotic cells, in kidney transplantation[26,27]. The impact of anti-HLA antibodies on LTx outcome is less well established as compared to kidney transplantation. Hence, our findings may imply that observations on the impact of various antibodies on graft survival in kidney transplantation cannot be simply extrapolated to LTx. For example, immunosuppressive regimens or tissue-specificity of involved anti-apoptotic antibodies, hamper such extrapolation[28,29].

Clearance of apoptotic cells is facilitated by various immune-involved processes, including IgM natural antibodies, speculated to be specific for lysophospholipids[15,20]. Furthermore, it is postulated that, in the autoimmune disease systemic lupus erythematosus, class-switching of the natural IgM antibodies to the IgG isotype occurs[30]. Whereas their epitopes are unknown, antibodies of the IgG isotype against apoptotic Jurkat cells are believed to be polyreactive against multiple epitopes on the membranes of apoptotic cells, including phospholipids[16]. Interestingly, we found no correlation between the levels of antibodies reactive against apoptotic Jurkat cells or apoptotic lung EC, which suggests different epitope specificity of these IgG antibodies. The nature of these epitopes, in particular EC, remain speculative.

The importance of antibodies in end-stage lung diseases has been stressed by previous research from our group and others. Autoantibodies have been identified in end-stage CF patients[31,32], and the levels of various autoantibodies are associated with disease progression[33]. Autoantibodies, including antibodies against various tissue epitopes, are present in COPD patients and relate to lung function[34,35], Indeed, indications for autoimmunity have been observed in specific subgroups of ILD patients[36].

To our knowledge, we are the first to report on antibodies against apoptotic cells in end-stage lung disease patients. We speculate that extensive tissue damage in the diseased lungs of LTx patients has led to the presentation of novel antigens which have triggered antibody

formation. In conclusion, pre-existing antibodies against apoptotic lung EC or apoptotic Jurkat cells do not correlate with outcome after LTx which is in marked contrast to observations in other transplantation settings. Hence, one should be careful to extrapolate observations on immune mechanisms from one transplantation setting to another.

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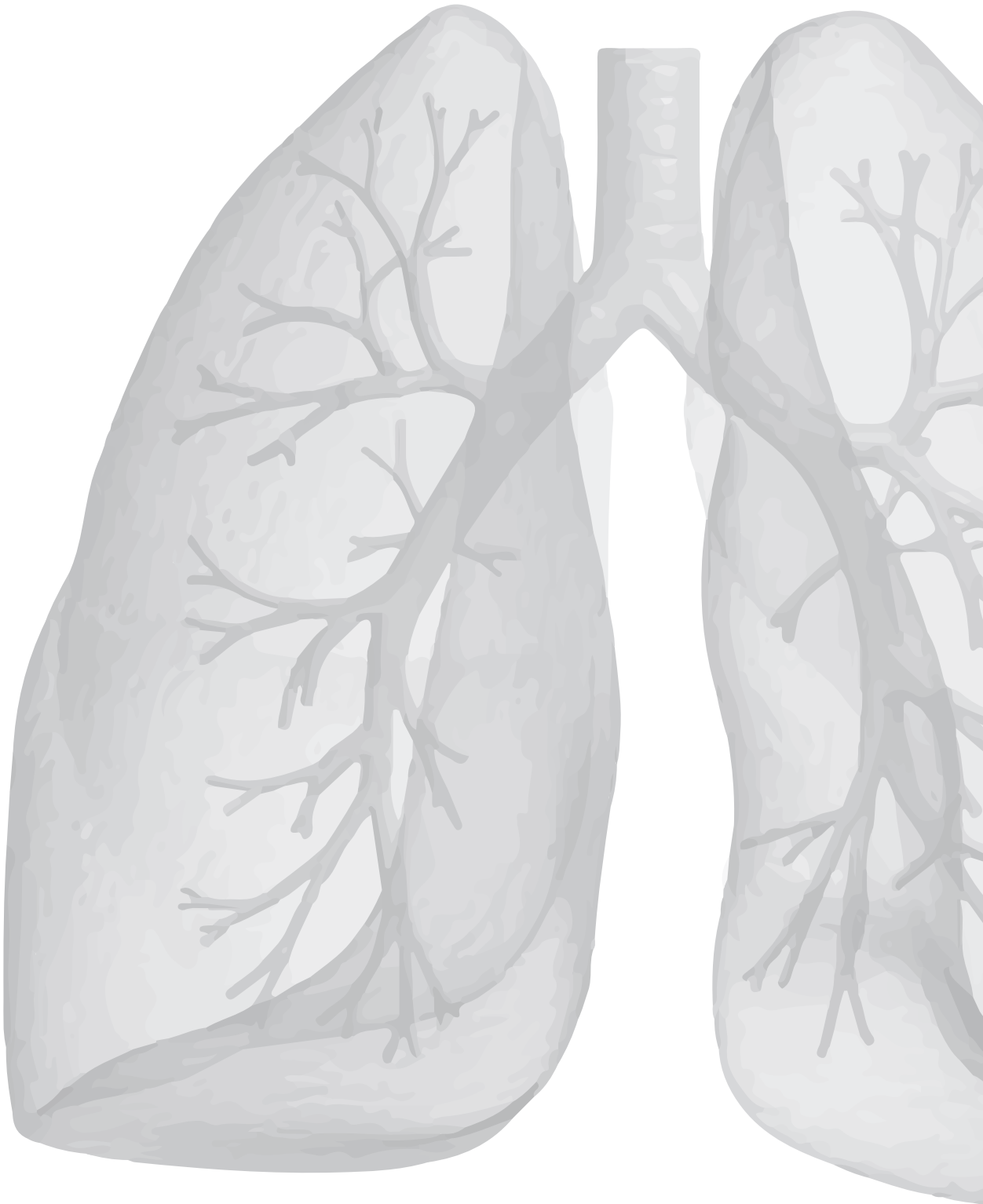

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Anti-BPIFA1/SPLUNC1: a new autoanitbody prevalent in patients with end-stage cystic fibrosis

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Abstract

Bactericidal/permeability increasing protein fold containing family A (BPIFA) 1, is a secreted protein of the upper airways that shares structural homology with BPI and exhibits comparable antimicrobial capacities. We hypothesized that CF patients have circulating IgG or IgA anti-BPIFA1 autoantibodies, similarly as reported for BPI autoantibodies. We analyzed pre- and post-transplantation sera from 67 end-stage lung disease patients who underwent lung transplantation (LTx) because of COPD (n=27), CF (n=25), and ILD (n=15). Anti-BPIFA1 (48%) and anti-BPI (92%) were elevated in CF patients compared to healthy controls, with anti-BPIFA1 IgG isotype being most prevalent, whereas anti-BPI is of the IgA isotype. Levels of anti-BPI autoantibodies significantly declined post-LTx, whereas anti-BPIFA1 did not. No relation was found between autoantibodies against BPIFA1 and BPI. Our results indicate that BPIFA1 is a novel target for autoantibodies in CF. The function of these autoantibodies needed to be investigated in future studies.

Introduction

Cystic fibrosis (CF) is an autosomal recessive disorder, which mainly occurs in the Caucasian population. Morbidity and mortality in patients with CF mostly relates to respiratory failure resulting from chronic inflammation of the bronchi. Chronic infections by airway pathogens, particularly *Pseudomonas aeruginosa*, contribute to progressive impairment of pulmonary function[1]. For end-stage CF patients lung transplantation (LTx) is the final treatment modality[1,2].

Autoantibodies against bactericidal/permeability increasing protein (BPI) have been described in previous studies in patients with both CF[3-6], and vasculitis[7]. BPI is a defense protein of the host's innate immune system against gram-negative bacteria, such as *P. aeruginosa*, and is expressed in neutrophils where it is stored in acidic granules[8]. Epithelial cells, the first line of defense against bacteria, also may express the protein[9]. The presence of anti-BPI autoantibodies correlate with pulmonary infections and poor prognosis in CF patients[6-10]. However, to what extent autoantibodies against BPI in other end-stage lung diseases, including chronic obstructive pulmonary disease (COPD) and interstitial lung diseases (ILD), occur, is unknown.

In a previous study, we reported the presence of autoantibodies against a protein expressed by epithelial cells in 4 patients six months after LTx[11]. Sequence reanalysis of the cloned gene showed that these autoantibodies were actually recognizing the PLUNC family variant SPLUNC1, a protein first described in the trachea and bronchi of adult mice[12]. Because of the structural homology of SPLUNC1 with BPI, the protein has recently been renamed bactericidal/permeability increasing protein fold containing family A (BPIFA)1, due to the presence of a single structural BPI fold domain[13-14]. Although there is structural homology between BPI and BPIFA1, this is exclusively based on the N-terminal region of BPI, a highly variant region. Sequence alignments show that the structural homology is primarily based on the secondary rather than the primary structure of the protein[15].

BPIFA1 is mainly expressed in the upper regions of the respiratory tract, accounting for 1% of the total protein load in the airway surface liquid[16]. Specifically, BPIFA1 is expressed highest by epithelial cells in the lung and tracheobronchial region, the submucosal glands of the trachea, and the bronchi[17]. More recently, neutrophils were also found to express PLUNC family proteins, suggesting a more widespread distribution than previously considered[18]. Various antimicrobial activities have been attributed to BPIFA1, especially against *P. aeruginosa*[19-22]. The structural homology between BPI and BPIFA1, the antimicrobial effects of both BPI and BPIFA1, and the clinical implications

of bacterial infections in CF patients, led to the hypothesis that CF patients also may have autoantibodies against BPIFA1.

The aim of this study was to investigate whether anti-BPIFA1 occurs not only after, but also prior to LTx in patients with end-stage lung disease. We examined both the prevalence and possible clinical relevance of BPIFA1 and BPI autoantibodies in CF, COPD, and ILD. We also addressed the potential cross reactivity between both autoantibodies since they recognize structurally related proteins. As a control for autoantibodies, we measured class-specific rheumatoid factors (RF), as these are completely unrelated to infection with *P. aeruginosa*.

We describe, for the first time, the presence of autoantibodies against BPIFA1 and that these autoantibodies are predominantly found in patients with CF, and that autoantibody titers, contrary to RF and BPI, are not affected by transplantation procedure or immunosuppressive regime.

Patients and Methods

Patients

A total of 67 patients undergoing lung transplantation (LTx) between July 2002 and May 2011 at the Heart Lung Centre in the Utrecht Medical Centre were included in this study. From all patients informed consent was obtained and the study was approved by the medical ethical committee. Blood was obtained several hours prior to LTx and 6 months post-LTx, processed and stored as serum aliquots at -80°C. All patients received standard immunosuppressive therapy consisting of basiliximab, tacrolimus, mycophenolate mofetil and prednisone. Patients at risk for CMV were treated with valganciclovir until 6 months post-transplantation. During follow-up, 14 patients developed BOS, defined as a decline of the forced expiratory volume in the first second of expiration (FEV_1) from the post-operative baseline at two distinctive time-points by >-20% in the absence of apparent causes such as infection[23]. Serum from 20 healthy volunteers, which was also stored at -80°C prior to analysis, was used as control group.

Detection of autoantibodies against BPI, BPIFA1, and rheumatoid factors

Antibody levels were quantified with ELISAs using purified native BPI (Athens Research & Technology, Inc., Athens, GA), purified recombinant BPIFA1 (OriGene Technologies,

Inc., Rockville, MD), as antigens, respectively. Human serum albumin (HSA, Albuman, Sanquin, The Netherlands) was used as a background control. NUNC Maxisorp 96-well ELISA plates (NUNC, Denmark) were coated with 1 μg/ml of the protein in PBS for 3 hours at room temperature. Final volume of this step as well as all subsequent steps was 100 μl. After each step, plates were washed three times with PBS 0.05%, w/v, Tween-20 (Bio-Rad Laboratories, Hercules, CA). Wells were blocked with PBS containing 5%, w/v, HSA, 5%, w/v, sucrose (Sigma-Aldrich, St. Louis, MO), and 0.005%, w/v, sodium azide (Sigma-Aldrich, St. Louis, MO). Serum samples were diluted 1:10 and 1:50 in dilution buffer, which was PBS containing 0.5% HSA and 0.05% Tween-20. The plates were then incubated with either horse radish peroxidase (HRP)-conjugated rabbit anti-human IgG (DakoCytomation, Denmark) or, HRP-conjugated rabbit anti-human IgA (DakoCytomation, Denmark). Bound conjugated antibodies were visualized using a TMB substrate kit (ThermoScientific, Rockford, IL) according to manufacturer's protocol. The OD450 was measured using a Multiskan EX Microplate Photometer (ThermoScientific, Rockford, IL). The inter- and intra-coefficients of variation for the IgG anti-BPI and IgA anti-BPI ELISA were 4.1% and 6,4%, and 7.8% and 5.9%, respectively. For the IgG anti BPIFA1 the inter- and intra-coefficients of variation were 3.6% and 10.2% and for IgA anti BPIFA1 1.1% and 13.4%.

In each assay, OD values obtained with individual serum samples were corrected for background OD450 values obtained with HSA coated plates. Dilutions of sera yielding a strong reaction in the ELISA of IgG or IgA anti-BPI or anti-BPIFA1 were used as a standard in the respective ELISA, and were set at 100 Arbitrary Units per ml (AU/ml). Concentrations of anti-BPI or anti-BPIFA1 levels in each serum sample were calculated by comparing the corrected OD450 values to those of the standard curves and expressed as AU/ml. Although BPI and BPIFA1 autoantibody titers are both expressed in AU, these titers are not quantitatively comparable, since we used different sera as calibration standards.

IgG1 and IgG2 subclass of the autoantibodies were measured with the same ELISA procedures as described above except that goat anti-human IgG1 or IgG2 was used (both from The Binding Site, Birmingham UK) followed by HRP-conjugated mouse anti-goat/sheep (Sigma-Aldrich, St. Louis, MO). IgG3 and IgG4 subclasses were measured using HRP-conjugated mouse anti-human IgG3 or IgG4 (both from SouthernBiotech, Birminhgam, AL). The presence of IgA1 or IgA2 subclasses was determined using mouse HRP-conjugated anti-human IgA1 or IgA2, (both obtained from SouthernBiotech, Birmingham, AL). Reagents needed to assess IgG-, and IgA-RF were kindly provided by

Thermofischer Scientific and concentrations were measured on the Phadia ImmunoCAP 250 according to manufacturer's protocol (Thermofischer Scientific, Nieuwegein, Netherlands).

Statistics

Categorical data (gender, risk for CMV/EBV, BOS) were analyzed by the Fisher's exact test, continuous variables (mean age, follow up, and the onset of BOS) by the ANOVA test, and influence of the type of graft (bilateral or single) by the Pearson's χ^2 test. Autoantibody titers pre- and post-LTx were compared using the Wilcoxon signed-rank test, whereas the Mann-Whitney rank-sum test was used to compare titers between CF, COPD, ILD and healthy controls. Correlations between IgA and IgG autoantibodies against BPI, BPIFA1 and RF were determined using the Spearman's rank correlation. A p-value≤0.05 was considered to be statistically significant.

	All	COPD	CF	ILD	*p*-value
Total Number	67	27	25	15	
Gender					
Male	31	8	15	8	0.074
Female	36	19	10	7	
Mean age	44 (16-63)	53 (32-63)	31 (16-53)	49 (24-62)	*< 0.0001*
Mean follow up (months)	54 (5-124)	49 (7-124)	56 (5-105)	62 (9-95)	0.155
Type of graft					
Bilateral	54	17	25	12	*0.003*
Single	13	10	0	3	
Infection					
EBV high risk	6	2	4	0	0.226
CMV high risk	17	2	12	3	*0.003*
BOS					
No	53	20	20	13	0.624
Yes	14	7	5	2	
Onset of BOS (months)	31 (5-81)	35 (16-81)	33 (5-49)	13 (9-17)	0.409
Ischaemic time (minutes)					
Bilateral	297 (124-1460)	255 (124-405)	288 (145-765)	375 (165-1460)	0.053
Single	223 (161-337)	210 (182-265)		252 (161-337)	0.272

Table 1: Clinical and demographic profile of lung transplant patients

Patients are divided in three diagnostic clusters, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), and interstitial lung disease (ILD). Significant intergroup variations are displayed in italics.

Results

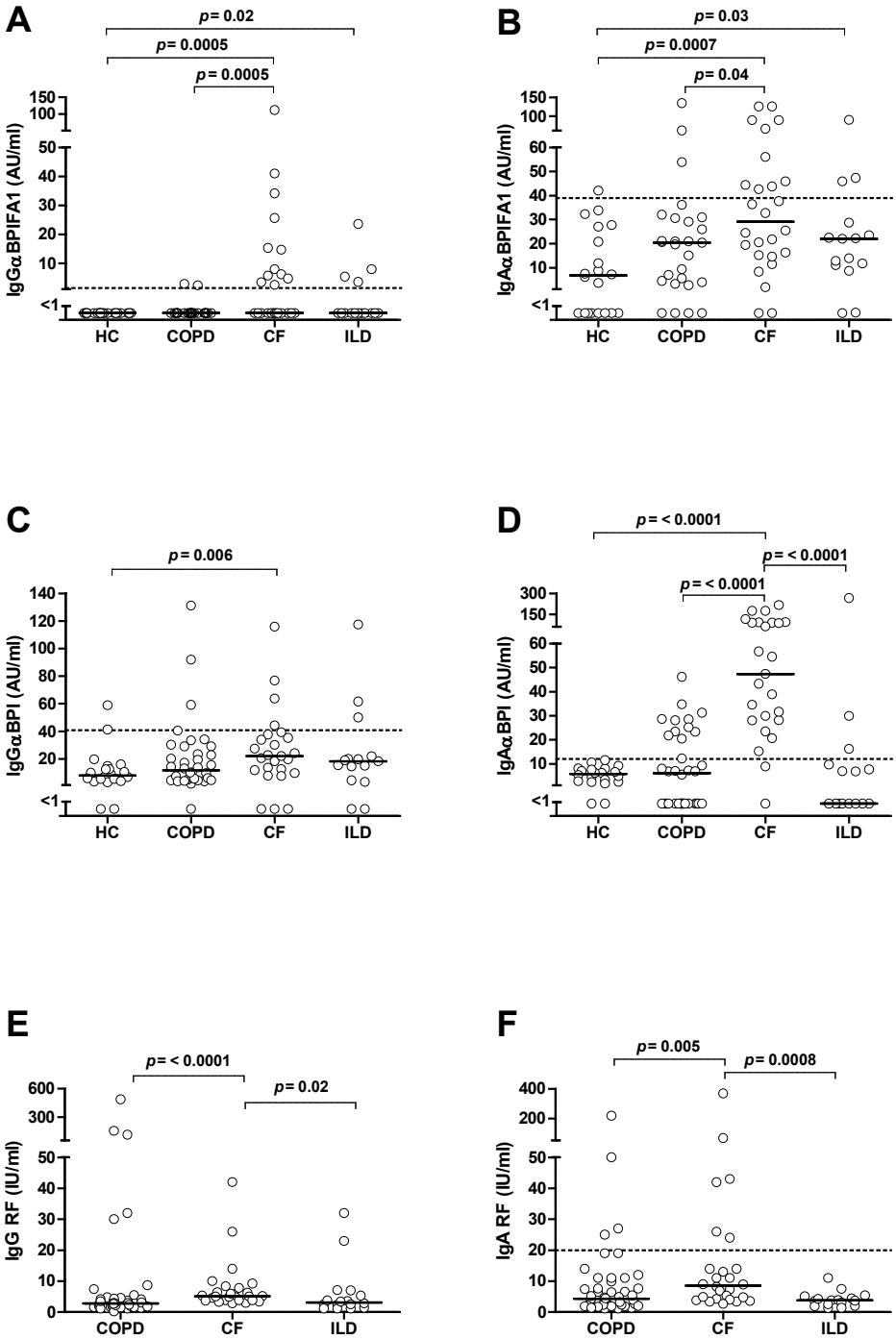
Patient characteristics

The cohort included in this study consisted of 67 LTx patients, of whom 27 suffered from COPD, 25 from CF and 15 from ILD. The mean age of the patients at inclusion was 44 years (range 16-63 years). Fourteen patients developed BOS post-LTx. Further demographic and clinical details by subgroup are summarized in Table 1. CF patients were younger than the other patients, and received a bilateral transplantation in 100% of the cases. Probably because of their younger age at the time of transplantation, CF patients had less frequently positive serology for CMV than COPD or ILD patients. All other demographic parameters were similar in each diagnostic cluster.

IgG anti-BPIFA1 is increased in patients with Cystic Fibrosis

All sera taken prior to transplantation were screened for the presence of anti-BPIFA1 (IgG and IgA), anti-BPI (IgG and IgA) and rheumatoid factors (IgG and IgA). IgG anti-BPIFA1 autoantibody titers were significantly higher in CF patients than in healthy controls (HC) and COPD patients (p = 0.0005) whereas ILD patients only had significantly higher IgG anti-BPIFA1 autoantibody titers than HC (figure 1A). IgG anti-BPIFA1 had a specificity of 88% for CF. A similar pattern was found for IgA anti-BPIFA1 autoantibody titers. Autoantibody levels in CF patients were higher than in HC (p=0.007) or COPD patients (p=0.04). ILD patients only had significantly higher IgA anti-BPIFA1 levels when compared to HC (p=0.03, see Figure 1B), but titers are elevated in multiple disease states. In the CF diagnostic subgroup, an IgG anti-BPIFA1 positive and negative group was found. We observed a trend (p=0.09) for the association of IgG anti-BPIFA1 and diabetes mellitus (see Figure 2). We did not observe significant differences between pre- and post-LTx levels of both IgG- and IgA anti-BPIFA1 autoantibody titers. These levels also did not correlate to any demographic or clinical parameters.

IgG anti-BPI autoantibody levels were higher in CF patients than in HC (p=0.006, Figure 1C), whereas levels in COPD and ILD were similar as in HC. However, IgA anti-BPI autoantibody levels in CF patients were significantly higher than those found in HC and in COPD and ILD patients (p<0.0001, figure 1D), and showed a specificity of 73% for CF. Subclass analysis of IgGαBPI showed a heterogeneous presence of IgG1, IgG2, IgG3, and IgG4, whereas IgAαBPI autoantibodies all consisted of the IgA1 subtype (data not shown). Post-LTx measurement of IgG and IgA autoantibody titers did not show any significant differences among subgroups.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p= 0.0008
IgA RF (IU/ml)
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CF
ILD

Figure 1: Pre-transplant autoantibody levels against BPIFA1, BPI and RF

Autoantibody titers are displayed in arbitrary unit / ml (AU/ml) for IgG anti-BPIFA1 (**A**), IgA anti-BPIFA1 (**B**), IgG anti-BPI (**C**), IgA anti-BPI (**D**), IgG RF (**E**), and IgA RF (**F**). A minimal detection level of 1 AU/ml was used for both BPIFA1 and BPI autoantibodies. Patients are represented by open circles and the median by a horizontal bar. For BPI and BPIFA1 autoantibodies, the cut off was determined as the mean titer of HC + 2 x SD . The cut off for IgA RF positivity was set according to manufacturer's instructions, but was not available for IgG RF. Measurements were performed as described in section 2.2 of the materials and methods.

IgG and IgA RF were significantly higher in CF patients than in ILD and COPD, although the median level of IgA RF was below the cut-off level for positivity. The cut-off level was not available from the manufacturer for IgG RF. IgG RF was elevated in 11% of the COPD patients, 15% of the CF patients and, 12% of the ILD patients. For IgA RF was elevated in 9% of the COPD patients and 22% of the CF patients (Figure 1E and 1F). Similarly to anti-BPIFA1 and anti-BPI post-LTx autoantibody titers, we found no significant differences in both IgG and IgA RF. Also, no patients had RF titers above cut-off value for positivity.

LTx affects levels of autoantibodies, except IgG anti-BPIFA1

To examine the influence of lung transplantation on autoantibody levels, titers were expressed as ratio of autoantibody levels to total Ig isotype levels (Figure 3). No significant change in IgG anti-BPIFA1 or IgA anti-BPIFA1 autoantibody ratio was found, neither in the whole cohort, nor in the individual CF population (Figure 3A and 3B). However, a small but significant decline was observed in the ratio of IgG anti-BPI/total IgG in

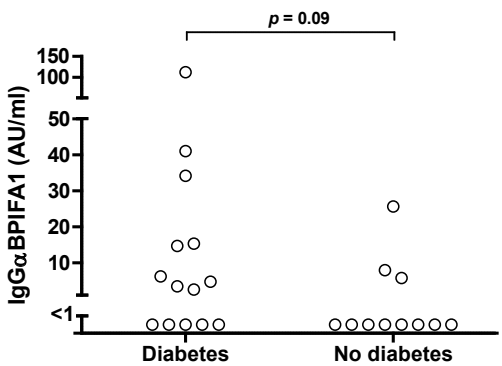

Figure 2: Cystic fibrosis related diabetes mellitus and IgG anti-BPIFA1

CF patients have been divided into two groups, with or without CF related diabetes mellitus and compared for their respective IgG anti-BPIFA1 autoantibody titers.

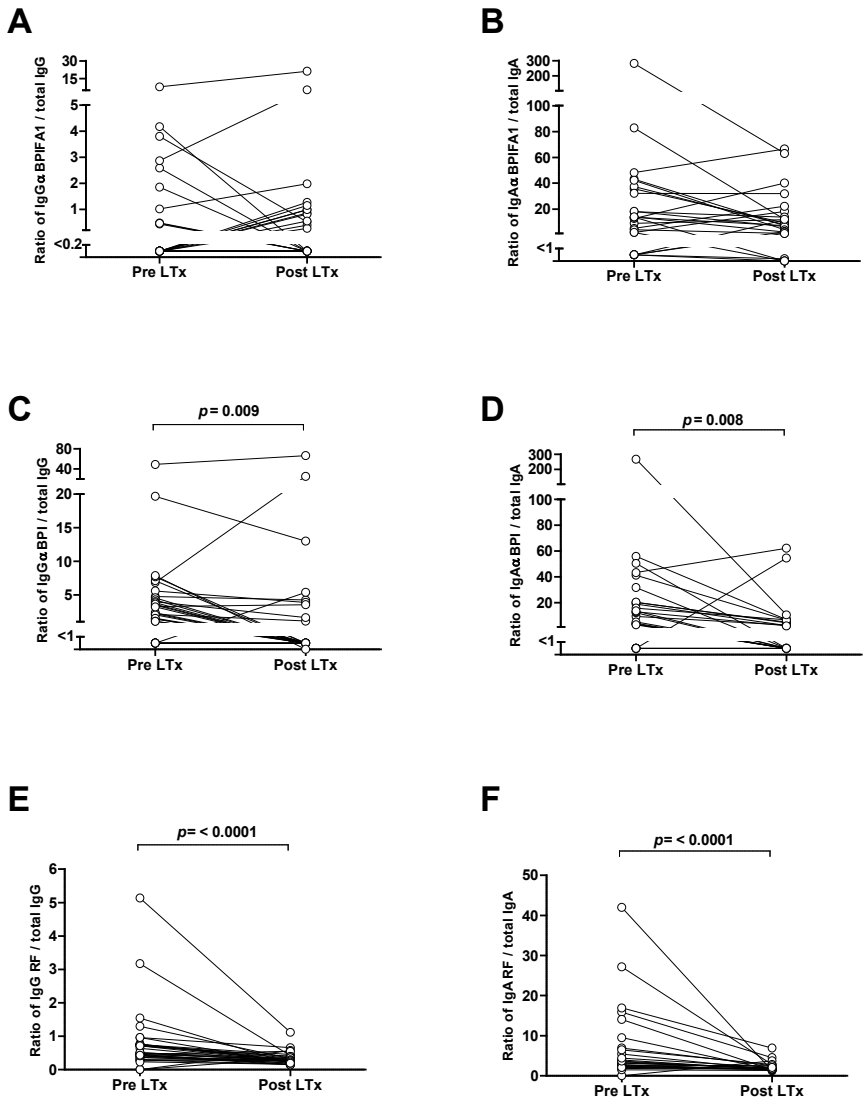

Figure 3: Autoantibody titers pre and post lung transplantation

We measured autoantibody titers pre and post LTx for IgG anti-BPIFA1 (n=31, **A**), IgA anti-BPIFA1 (n=24, **B**), IgG anti-BPI (n=32, **C**), IgA anti-BPI (n=25, **D**), IgG RF (n=32, **E**), and IgA RF (n=25, **F**). Pre- and post-LTx IgG and IgA autoantibody titers, measure in arbitrary units / ml (AU/ml) were corrected for the total level of IgG or IgA, in μg/ml, measured at the same time point, and the ratio is displayed on the y-axis. A minimal ratio of 0.2 was used for IgG anti-BPIFA1 and 1 for IgA anti-BPIFA1 and both isotypes of anti-BPI. Patients in this paired comparison are depicted by open circles. Measurements were performed as described in section 2.2 of the materials and methods.

pre- and post-LTx sera (4.94, versus 4,26, $p = 0.009$, see Figure 3C). More pronounced differences were found for pre- and post-LTx IgA anti-BPI (25.35 versus 6.80, $p = 0.008$, see Figure 3D). This decrease was also found for RF. Ratios of total IgG-RF / total IgG decreased on average by 2.4 upon LTx (Figure 3E). A similar reduction was observed for IgA RF titers, decreasing by 3.4 (Figure 3F). Thus, the lung transplantation procedure did not influence levels of anti-BPIFA1, but significantly reduced levels of anti-BPI and RF. Changes in autoantibody titers did not correlate with any demographic or clinical parameter, including BOS development, age, sex, ischemic time or risk for CMV or EBV reactivation. Donor characteristics such as age and smoking history also did not correlate with the change in autoantibody levels against BPIFA1 and BPI either.

Discussion

In this study, we have identified a novel autoantibody against BPIFA1, an antimicrobial protein which shares structural homology with bactericidal/permeability increasing protein (BPI) and exhibits similar antimicrobial capacities. Anti-BPI and anti-BPIFA1 autoantibodies were the highest in CF patients, although their main isotypes were different, as was their responses to LTx or immunosuppressive therapy.

Autoantibodies against BPI correlate with pulmonary infections and poor prognosis in patients with CF. Carlsson *et al.* observed a decrease in IgA BPI-ANCA in seven CF patients who underwent LTx, although both pre- and post-LTx measurement were at large time intervals[6]. A decline in post LTx autoantibody titers in CF patients who underwent LTx or extensive sinus surgery was also observed by Aanaes *et al.*, and they speculated that surgical eradication of the infectious focus and the reduction in mucosal inflammation reduces levels of autoantibodies against BPI[24]. We also observed a decrease of autoantibodies against BPI in the majority of CF patients upon LTx. However, decreasing autoantibody titers in our cohort were not only found in CF, but also COPD and ILD patients. These decreases cannot be due to a general effect of LTx and immunosuppression since titers of another autoantibody, i.e. anti-BPIFA1, did not show a similar course. Therefore, one may speculate that IgA anti-BPI, when elevated in patients with infectious-inflammatory disease, will decrease in case of effective removal of the infectious-inflammatory lesions. However, in each subgroup we also observed increases of IgA anti-BPI in individual patients. Such increases did not correlate with re-infection, or any other clinical parameter.

In the past years, various functions have been attributed to BPIFA1. The structural homology between BPIFA1 and BPI suggests antimicrobial activity and several studies have demonstrated this. In a paper by Chu *et al.* it was shown that growth of *Mycoplasma*

pneumoniae was significantly reduced in the presence of recombinant murine BPIFA1 (19). Zhou *et al*. demonstrated that BPIFA1 binds lipopolysaccharide and inhibits growth of *P. aeruginosa in vitro* in a dose-dependent manner[20]. Lukinskiene *et al.* showed that transgenic mice in which human BPIFA1 was expressed by the airway epithelium, displayed enhanced bacterial clearance after *in vivo P. aeruginosa* challenge. Also, overexpression of this construct led to a higher survival rate of mice after *P. aeruginosa* infection[21]. Finally, Gakhar *et al*. suggest that BPIFA1 contributes to the surfactant properties of airway secretions thereby possibly interfering with the biofilm formation of airway pathogens[22].

Autoantibodies against BPI in CF patients are directed at both C-terminal and N-terminal epitopes[25], and we cannot rule out the possibility of cross-reactivity between anti-BPI and anti-BPIFA1. However, because both autoantibodies are of a different isotype, and there is a different influence of LTx on autoantibody titers, we conclude that it is most likely that autoantibodies against BPIFA1 and BPI recognize a different epitope, and are exclusively directed against a single protein.

We describe here the presence of both IgG and IgA autoantibodies against BPIFA1 in end-stage CF patients. We identified these autoantibodies in CF patients with advanced disease progression, and our findings are therefore not necessarily representative of less advanced disease stages. It is reasonable to expect that advanced disease progression is associated with more tissue damage and more autoantibody production. Therefore, it would be highly interesting to analyze autoantibody production development during disease progression in a longitudinal cohort of CF patients.

IgG and IgA anti-BPIFA1 autoantibody levels did not decrease upon LTx and immunosuppressive therapy. This is in contrary to the antibody titers of anti BPI and RF. We reason that there is a different mechanism of autoantibody production underlying this observation. Also, we did not find a correlation between the change of autoantibody titers and clinical or demographic parameters.

We can only speculate on the potential pathogenic role of anti-BPIFA1. Along with anti-BPI, multiple other autoantibodies are prevalent in CF, such as anti-Saccharomyces cerevisiae antibodies, anti-Kα1 tubulin, anti-collagen-V, rheumatoid factors and antinuclear antibodies. Besides the fact that a delineation of autoimmunity of CF may provide biomarkers used to identify patients at risk for developing lung damage, autoantibodies have potential significant clinical impact in airways disease, as was shown for autoelastin antibodies in COPD[26]. Interestingly, autoantibodies against BPI directly inhibit the antibiotic function mediated by the N-terminal region of the protein[27]. It is

reasonable to assume that BPIFA1 autoantibodies operate in a similar way, resulting in a less functional part of the innate immune system that might have an impact on disease progression.

Whereas an anti BPI autoantibody is, in ANCA immunofluorescence terminology, an atypical c-ANCA, or p-ANCA without nuclear extension, we did not observe any fluorescent staining when we analyzed anti-BPIFA1 positive sera using the diagnostic ANCA protocol (Euroimmun, Lübeck, Germany), despite the fact that PLUNC is a secreted product of neutrophil granules[18]. However, the study of Bartlett *et al.* was conducted before the differentiation of PLUNC variants based on BPI homology. It could therefore be possible that autoantibodies against BPIFA1 do not recognize the PLUNC variant expressed by neutrophils.

BPIFA1 is highly expressed in the upper respiratory tract and interestingly, BPIFA1 expression is increased in the epithelium of small airways in advanced CF patients compared to healthy controls, patients with emphysema or patients with pneumonia[28]. Taking the antimicrobial capacities of BPIFA1 and the chronic state of infection of these patients into consideration, we reason that this highly immunoactive region could contribute to autoantibody production. Bingle *et al.* showed no expression of BPIFA1 by either neutrophils or macrophages, making the literature on the expression of BPIFA1 by neutrophils not conclusive[18,28]. We therefore conclude that the source for the auto antigen BPIFA1 is the epithelial cells of small airways. Although the antigenic source for anti-BPIFA1 autoantibodies is replaced after LTx procedure, we did not observe a decline in autoantibody levels. Activation of donor lung epithelial cells after transplantation due to the recipient's alloimmune response could lead to an increased expression of BPIFA1, thereby maintaining antigen levels. This could explain the difference in autoantibody titers between anti-BPI and anti-BPIFA1 post-LTx since immunosuppression could have a different impact on the source of the antigens.

The identified autoantibodies against BPIFA1 did not correlate with demographic parameters, though we observed a trend between IgG anti-BPIFA1 positivity and the incidence of CF related diabetes mellitus. The development of CF related diabetes is associated to chronic infection in CF patients, and the development of these autoantibodies could be used as a tool to identify patients at risk for this clinical outcome. Considering the antimicrobial capacities of BPIFA1, we propose a model wherein autoantibody production against BPIFA1 leads to a deregulated innate immune response and a failed clearance of *P. aeruginosa.* However, both the clinical and diagnostic implications of anti-BPIFA1 need to be established in future studies.

RF may develop upon chronic exposure to cigarette smoke, and COPD patients, both smokers and as well as ex-smokers, are 100% positive for IgA RF[29]. We did not confirm this data in our LTx cohort, since we detected significantly higher levels of IgG and IgA RF in CF patients, compared to both COPD and ILD, though a number of patients had undetectable levels. All LTx patients ceased smoking half a year prior to entering the waiting list. Also, there are no ever-smokers in the CF patient group. Therefore, we found no relation between the presence of IgG and IgA RF and smoke exposure. This difference with published literature may be due to the use of a different assay, though we used a commercial validated IgA RF kit. We found a correlation between IgA RF and IgA anti-BPI. Moreover, both isotypes of RF showed a similar course upon LTx and immune suppression. Considering the fact that production of RF is T cell dependent[30], one may speculate that decreasing RF titers simply reflected the use of immunosuppressive therapy. However, as stated above, levels of anti-BPIFA1 did not show such course, making this interpretation less likely. Therefore we suggest that RF titers, similarly to anti-BPI titers, decreased because of reduced infectious and inflammatory burden in the patients. Indeed, infections can trigger RF production in humans[31].

In summary, we have identified a novel autoantibody against BPIFA1 in CF patients. Isotype distribution and response to LTx and immunosuppressive therapy of these autoantibodies seem different to those of anti-BPI autoantibodies. Further studies are needed to elucidate the origin, the pathogenicity and the potential role of biomarkers for lung damage of these autoantibodies in CF.

Conflict of Interest

The authors declare no competing financial interests.

Acknowledgments

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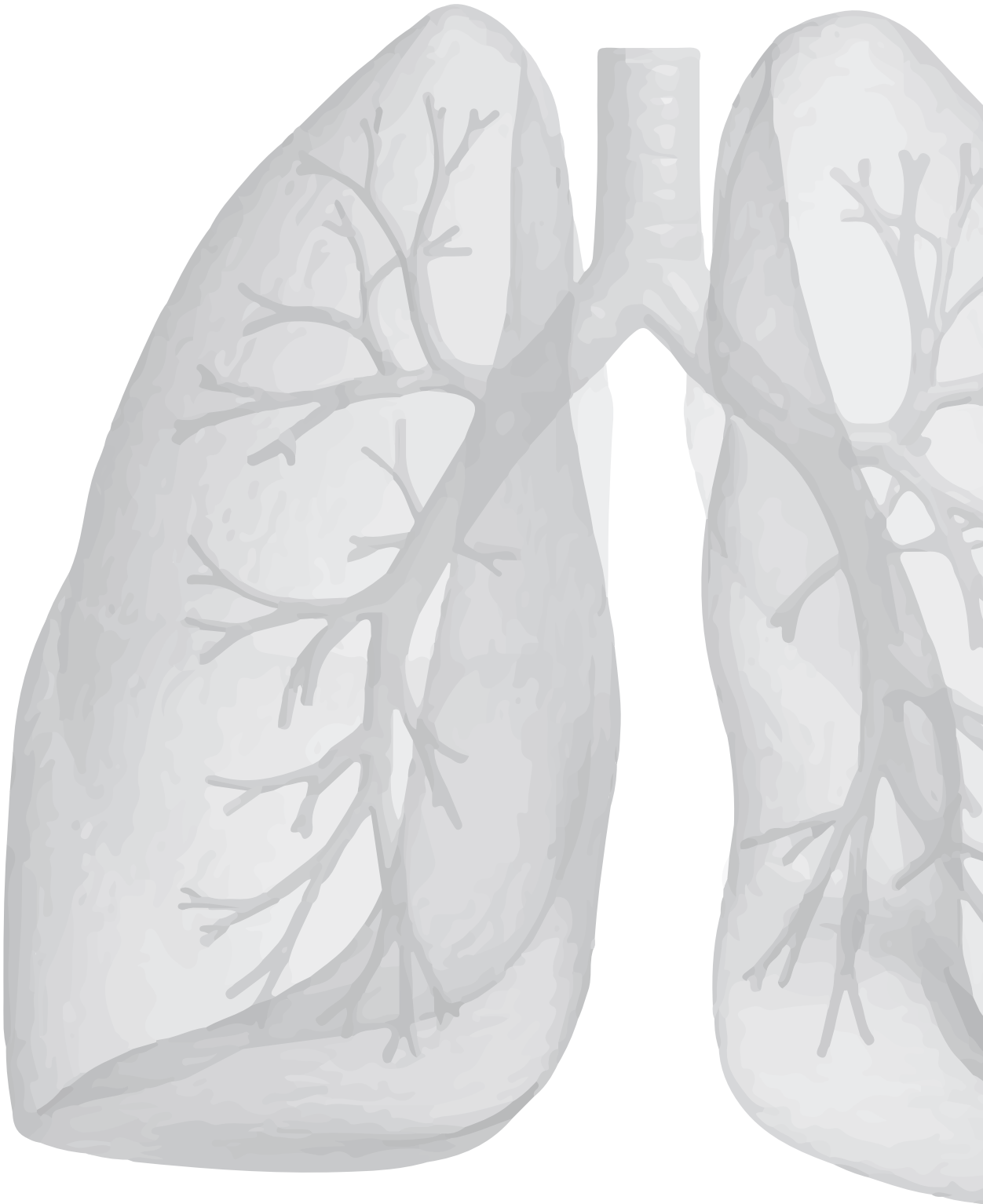

6

Anti-ET_AR and Anti-AT_1R autoantibodies are elevated in patients with end-stage cystic fibrosis

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Abstract

Autoantibodies against endothelin-1 type A receptor (ET_AR) are present in systemic sclerosis complicated by lung fibrosis and pulmonary hypertension. As increased serum levels and local overproduction of endothelin-1 in the airways is reported in cystic fibrosis (CF) patients, we reasoned that anti-ET_AR antibodies could be prevalent in end-stage CF patients prior to lung transplantation (LTx). Also, ET_AR autoantibodies are frequently associated with autoantibodies against the angiotensin II type 1 receptor (AT_1R). We analyzed the presence of anti-ET_AR and anti-AT_1R autoantibodies in 43 LTx patients (chronic obstructive pulmonary disease (COPD), *n*=20; CF, *n*=13; interstitial lung disease (ILD), *n*=1. We observed overall higher anti-ETAR and anti-AT1R autoantibody titers in sera taken prior to LTx in the CF patient group compared to COPD. No difference was found in autoantibody levels between patients with CF versus ILD. In sera taken post-LTx we found the same difference in anti-ETAR and anti-AT1R autoantibody titers between patients with CF versus COPD. No difference was found in antibody titers between sera taken prior to or 6 months after LTx. There was no association between autoantibody levels and other relevant demographic parameters, and we found no association between autoantibody titers and the development of the bronchiolitis obliterans syndrome. Both autoantibody titers were strongly correlated. We hypothesize that due to prolonged exposure to bacterial infection, increased levels of AT_1R and ET_AR result in a deregulated immune response causing autoantibody formation. Further research is expedient to elucidate the occurrence of autoantibodies against ET_AR and AT_1R and their role in disease progression.

Introduction

Cystic fibrosis (CF) is an autosomal recessive disorder, which mainly occurs in the Caucasian population. Patients are often confronted with chronic infections of Gram-negative bacteria in the lungs, in particular *Pseudomonas aeruginosa*, which results in the influx of both neutrophils and macrophages to the site of infection[1]. In addition, CF is associated with manifestations of autoimmunity including primary sclerosing cholangitis, arthropathy and vasculitis. Multiple autoantibodies are prevalent in CF, such as anti-bactericidal/permeability-increasing protein (BPI), anti-Saccharomyces cerevisiae antibodies, anti-Kα1 tubulin, anti-collagen-V, rheumatoid factors and antinuclear antibodies[2,3], but not anti-proline-glycine-proline or antielastin autoantibodies[4]. A delineation of autoimmunity of CF may provide biomarkers used to identify patients at risk for developing lung damage, as has been shown for anti-BPI which is associated with a poor prognosis of CF disease[5].

Endothelin-1 is a protein secreted primarily by both lung endothelial and epithelial cells and, in addition to its vasoconstrictor activity, has proinflammatory capacities such as priming and stimulating neutrophils, activating mast cells, and macrophage stimulation that results in proinflammatory cytokine production. Increased serum levels and local overproduction of endothelin-1 in the airways is reported in CF patients[6]. In addition, overexpression of endothelin-1 is associated with the bronchiolitis obliterans syndrome and bacterial infection in lung transplant recipients[7,8]. Endothelin-1 acts via binding to its type A or type B receptor and autoantibodies against endothelin-1 type A receptor (ET_AR) have already been identified in systemic sclerosis commonly complicated by lung fibrosis and pulmonary hypertension. ET_AR autoantibodies are frequently associated with autoantibodies against the angiotensin II type 1 receptor (AT_1R)[9]. We reasoned that, beside increased levels of endothelin-1, anti-ET_AR and/or AT_1R autoantibodies could be prevalent in patients with end-stage CF.

Patients and Methods

Patients

We included 43 patients in this pilot study that underwent LTx between January 2004 and May 2011 at our center. This study was approved by the medical ethics committee and from all patients informed consent was obtained. Several hours prior to and monthly after LTx blood was obtained from each patient, processed, and stored as serum aliquots at -80°C until analysis.

Determination of anti-ET_AR and anti-AT_1R concentrations in serum

A solid phase sandwich ELISA kit (CellTrend GmbH, Luckenwalde, Germany) was used to measure both anti-ET_AR and anti-AT_1R antibody levels in serum according to protocol[8]. The inter- and intra-assay CVs are 8.3% and 6.3% for the ET_AR- and 11.5 % and 6.9% for the AT_1R ELISA kit. The lower limit of detection was 1 U/ml. Serum samples were thawed and centrifuged for 10 minutes at 1800 rpm prior to analysis. All samples were subjected to identical freeze-thaw cycles and analyzed in the same experiment. The OD450 measurement was conducted using a Multiskan EX Microplate Photometer (ThermoScientific, Rockford, IL). Data analysis was performed using AT_1R / ET_AR analysis software provided by the manufacturer.

Results

Patient demographics

The analyzed patient cohort consisted of 43 LTx patients of which 20 suffered from chronic obstructive pulmonary disease (COPD), 13 from CF, and 10 from interstitial lung disease (ILD), including idiopathic pulmonary fibrosis (n=3), lymphangioleiomyomatosis (n=2), non-specific interstitial pneumonia (n=1), pulmonary Langerhans cell histiocytosis (n=2), progressive systemic sclerosis (n=1) and sarcoidosis (n=1). Nineteen of these patients developed the bronchiolitis obliterans syndrome (BOS), among which 6 had CF as a primary disease, during follow-up, defined according to ISHLT criteria[10]. After transplantation immunosuppression consisted of basiliximab, tacrolimus, mycofenolate and prednisolone. Treatment with valganciclovir was initiated when a patient was considered to be at risk for CMV. Further demographic parameters are summarized in Table 1. Both significant differences, mean age of inclusion and high risk for CMV reactivation, can be explained by the younger age of CF patients at the time of transplantation.

Anti-ET_AR and AT_1R autoantibody titers are elevated in CF patients

We observed overall higher anti-ET_AR and anti-AT_1R autoantibody titers pre-LTx in CF patients compared to COPD (Figures 1A and 1C). No significant difference was found in autoantibody levels between patients with CF versus ILD. In post-LTx sera we found the same difference in anti-ET_AR and anti-AT_1R autoantibody titers between patients with CF versus COPD (Figures 1B and 1D). Unfortunately, no post-LTx serum samples were available from three COPD, one ILD, and on CF patient, and could thus not be analyzed. No significant difference was found in antibody titers between sera taken prior to or post-LTx.

	All	COPD	CF	ILD	*p*-value
Total Number	43	20	13	10	
Gender					
Male	20	6	5	4	0.129
Female	23	14	8	6	
Mean age	44 (16-63)	53 (32-63)	27 (16-53)	49 (24-62)	*< 0.0001*
Mean follow up (months)	55 (5-105)	53 (5-102)	56 (5-105)	47 (9-95)	0.534
Type of graft					
Bilateral	34	13	13	8	0.054
Single	9	7	0	2	
Infection					
EBV high risk	3	1	2	0	0.321
CMV high risk	12	2	7	3	*0.023*
BOS					
No	24	10	7	7	0.574
Yes	19	10	6	3	
Onset of BOS (months)	27 (5-81)	30 (6-82)	30 (5-49)	12 (10-18)	0.339
Ischaemic time (minutes)					
Bilateral	305 (120-1460)	245 (124-405)	282 (145-433)	438 (120-1460)	0.069
Single	238 (161-337)	226 (187-265)		249 (161-337)	0.833

Table 1: Clinical and demographic profile of lung transplant patients

Patients are divided in three diagnostic clusters, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), and interstitial lung disease (ILD). Categorical data (gender, risk for CMV/EBV, BOS) were analyzed by the Fisher's exact test, continuous variables (mean age, follow up, and the onset of BOS) by the ANOVA test, and influence of the type of graft (bilateral or single) by the Pearson's χ^2 test (SPSS version 20, IBM Corp., Armon, NY). Significant intergroup variants are displayed in italics. Eleven out of the 13 CF patients were colonized with *P. aeruginosa*. One patient was colonized with *S. aureus* and one patient was colonized with *S. maltophilia*. Before transplantation infections were treated with antibiotics according to the sputum cultures. Eleven out of 13 patients were analyzed for CFTR mutation status. Nine patients were genotyped with ΔF508, one with a homozygous 1998+5GT, and one with a G542x/R347P mutation. Two CF patients were diagnosed by sweat test. Five patients were diagnosed with cystic fibrosis related diabetes before transplantation. Six patients were on steroids because of a diagnosed allergic bronchopulmonary aspergillosis or to reduce inflammation. All patients were given best clinical care, had a BMI higher than 18.5, and had a lung function <35% (range 13%-34%) prior to transplantation.

There was no association between autoantibody levels and other relevant demographic parameters: age, gender, type of graft, BOS incidence, ischemic time, lung function, *Pseudomonas* infection, CFTR mutation, or CF related diabetes mellitus. Anti-ET_AR and anti-AT_1R autoantibody titers were strongly correlated ($p < 0.0001$, $r = 0.756$, data not shown).

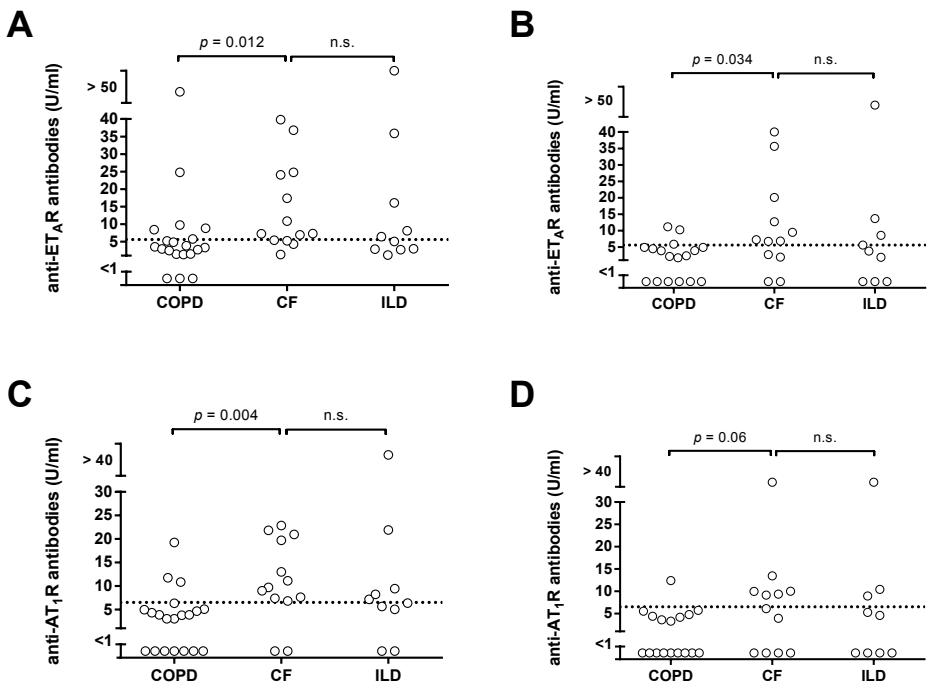

Figure 1: ET_AR and AT_1R autoantibody titers pre-and post-lung transplantation

Pre- (Figures **A** and **C**) and post-transplantation (**B** and **D**) antibody levels against ET_AR (Figures **A** and **B**) and AT_1R (Figures **C** and **D**) are displayed in U/ml. Cut-off levels to define positivity were set according to previously described healthy control values (see materials and methods). Each dot represents the result from 1 serum sample. No significant differences between CF and ILD were found between pre- and post-LTx anti-ET_AR, p=0.31 and p=0.38, and pre- and post-LTx anti-AT_1R autoantibodies, p=0.28 and p=0.58 respectively. A Mann-Whitney rank-sum test was used for statistical analysis (GraphPad Prism version 5.03, GraphPad Software Inc., San Diego, CA).

Discussion

In this pilot experiment we show that autoantibodies against both ET_AR and AT_1R are present in end-stage lung diseases, particularly CF. Like AT_1R, ET_AR is a G-protein coupled receptor to which both an endogenous ligand, endothelin-1, and autoantibodies may simultaneously bind resulting in a more prolonged and detrimental biologic response.

Levels of endothelin-1 are elevated in CF patients sputum, compared to healthy controls and COPD patients[11], and endothelin-1 is detectable in both the peripheral blood and

bronchoalveolar lavage (BAL) fluid of COPD patients[12]. In our study we did not have access to BAL fluid, since surveillance bronchoscopy was not performed, which would allow simultaneous analysis of endothelin-1 and autoantibody levels, and their correlation with disease progression.

Besides being elevated in the sputum of CF patients, endothelin-1 is also a biomarker for the detection of BOS in serum[13]. The authors postulate that high endothelin-1 levels arise due to an inflammatory response after lung tissue damage. Autoantibodies against ET_AR and AT_1R are associated with systemic sclerosis with fibrotic complications[9]. In this study we could not find a correlation between autoantibody titers and development of BOS, which is hallmarked by fibrotic development in the lungs[10]. Also, both anti-ET_AR and anti-AT_1R autoantibodies have been identified in episodes of cellular and antibody-mediated rejection after heart transplantation, and anti-AT_1R autoantibodies are associated with antibody mediated rejection after kidney transplantation[14]. However in our study we were unable to distinguish cellular and antibody mediated rejection due to the absence of lung biopsy material.

We show for the first time that autoantibodies against both ET_AR and AT_1R are elevated in end-stage CF patients. Although both receptors are involved in a common vasocontrictive pathway, their natural ligands differ in functional capacities. AT_1R autoantibodies are agonistic[15], but we can only speculate on the role of both ET_AR and AT_1R autoantibodies and their part in CF pathology. We hypothesize that due to prolonged exposure to bacterial infection, increased levels of AT_1R and ET_AR result in a deregulated immune response causing autoantibody formation. The development of these autoantibodies would then be a consequence of disease progression. Anti-ET_AR and anti-AT_1R autoantibodies could thus have an additive value in disease prognosis for young CF patients, which is desired, since besides genetic background, more factors can contribute to their respective clinical outcome[5]. However, we only analyzed end-stage lung disease patients, indicating that further research is expedient in a follow-up cohort of different lung diseases, to elucidate the occurrence of autoantibodies against ET_AR and AT_1R and their role in disease progression.

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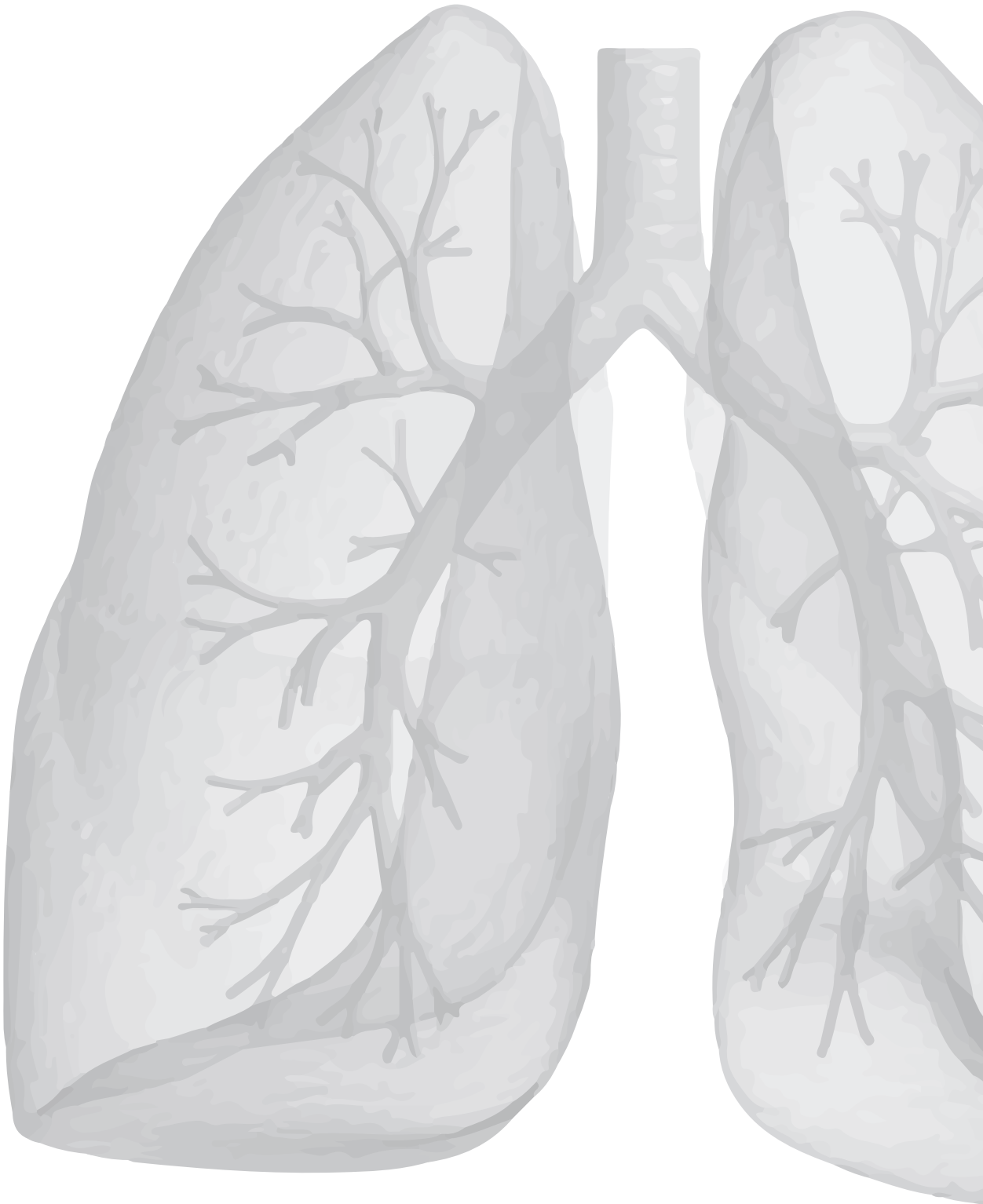
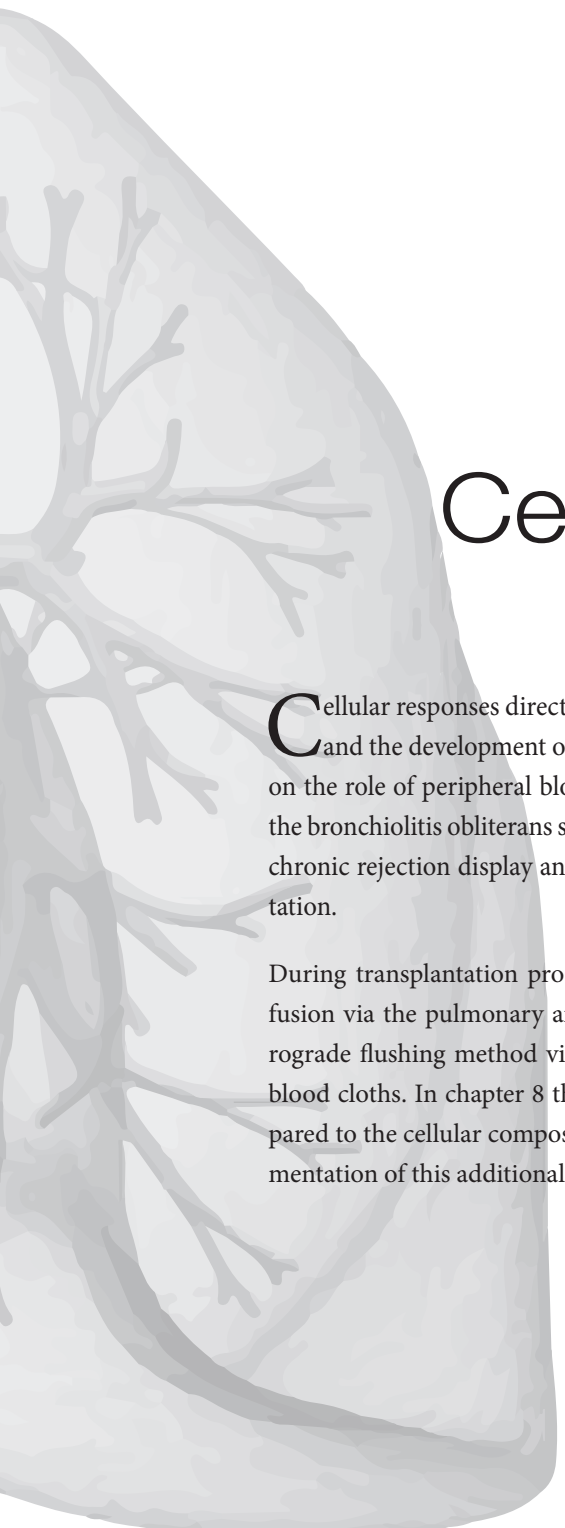


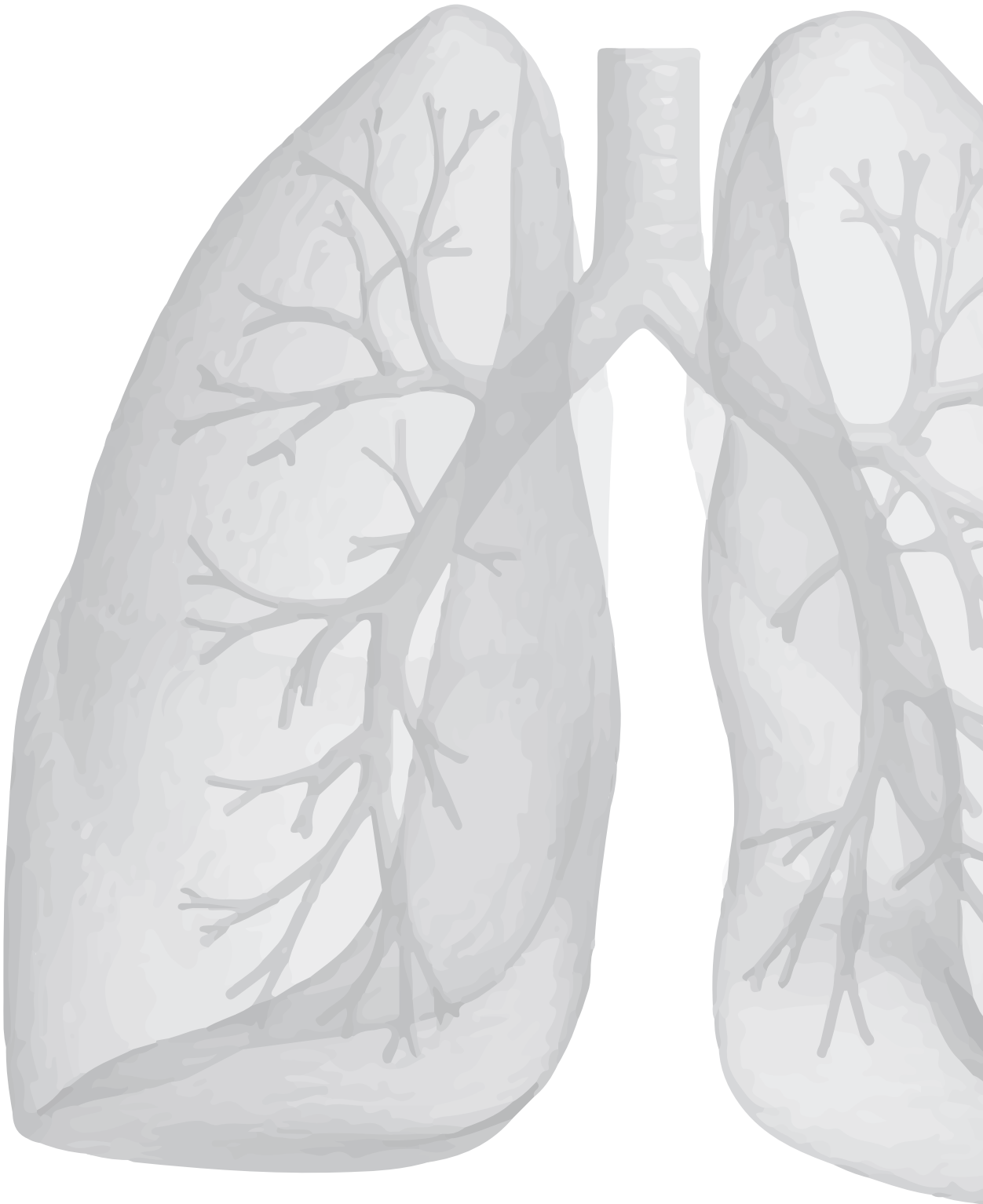


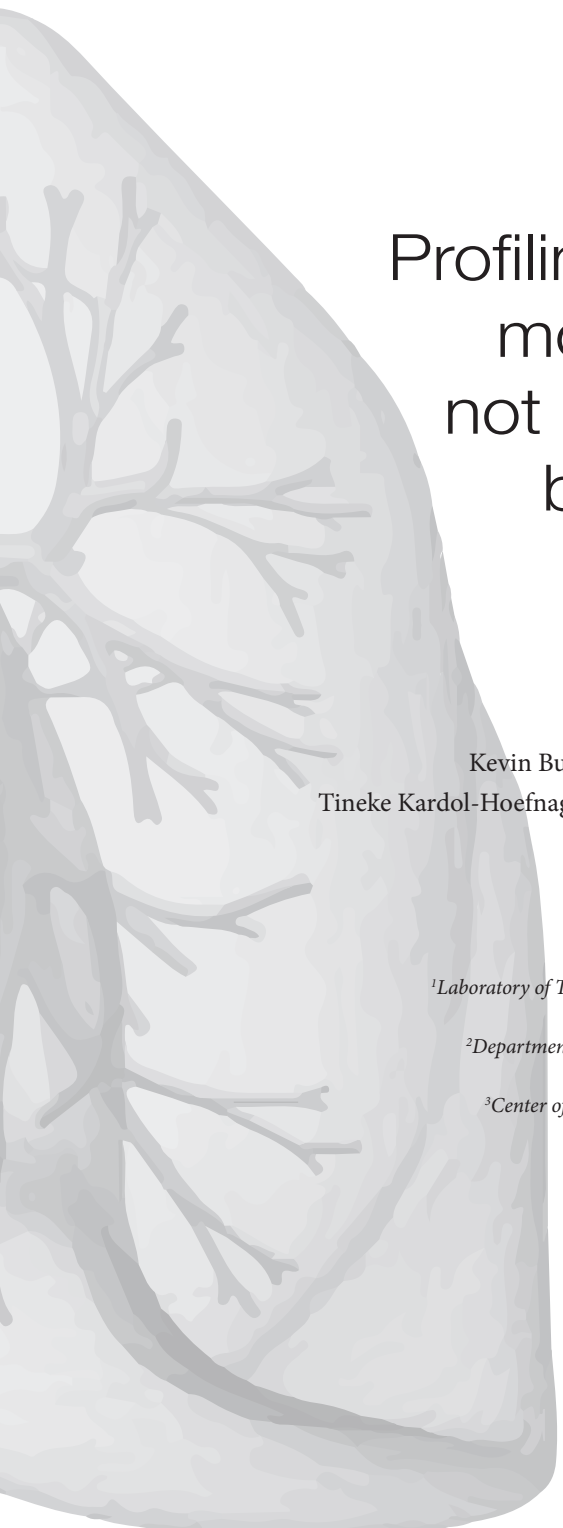
Part II

Cellular Immunity

Cellular responses directed from the patient are known to contribute to graft rejection and the development of chronic lung allograft dysfunction. In this part, we elaborate on the role of peripheral blood mononuclear cells and subset composition in relation to the bronchiolitis obliterans syndrome. In chapter 7 we show that patients who will develop chronic rejection display an altered composition of cellular subsets early after transplantation.

During transplantation procedure, the explanted lungs are subjected to antegrade perfusion via the pulmonary artery. Since August 2008, our center has implemented a retrograde flushing method via the pulmonary vein in order to remove potential harmful blood cloths. In chapter 8 the cellular composition of this fluid is investigated and compared to the cellular composition in the circulation. Furthermore, the effect of the implementation of this additional flush procedure on lung transplantation outcome is assessed.





7

Profiling of peripheral blood mononuclear cells does not accurately predict the bronchiolitis obliterans syndrome after lung transplantation

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Abstract

After lung transplantation (LTx), circulating mononuclear cell composition and their subsets may be predictive for the bronchiolitis obliterans syndrome (BOS). We investigated the cellular composition in patients developing BOS, or not, by analyzing peripheral blood taken at multiple time points after transplantation. PBMCs of 11 BOS and 39 non-BOS patients were analyzed by FACS for monocytes, dendritic cells, NK-, NKT-, B- and T cells as well as B- and T cell subsets. Analysis of blood samples taken monthly during the first year post-LTx showed that circulating NK, NKT and dendritic cell percentages were not indicative for BOS development, whereas increases in T cells, monocytes and lowered fractions of B cells were related to BOS development. B- and T cell subset analysis at month 5 post-LTx indicated that IgM+IgD- memory B cells and central memory CD8+ T cells were decreased, whereas NKT cells were increased in BOS patients compared to non-BOS patients. Prior to BOS diagnosis, the composition of specific mononuclear cells on a group level differs from patients remaining BOS free. However, given the overlap in percentages of cellular frequencies between the patient groups investigated, this analysis does not allow prediction or risk stratification for development of BOS in individual patients.

Introduction

Lung transplantation (LTx) is the final treatment option for several selected end-stage lung diseases[1,2]. The outcome after LTx is severely hampered by the development of chronic lung allograft dysfunction (CLAD), which manifests either as an obstructive CLAD (bronchiolitis obliterans syndrome, BOS) or as a restrictive CLAD (restrictive allograft syndrome, RAS). The two forms of CLAD are diagnosed either by obstructive pulmonary function tests in the case of BOS or restrictive pulmonary function tests in the case of RAS[3].

Approximately 50% of patients survive five years after LTx[4,5]. Although the pathogenesis of BOS is mainly unknown, the immune system probably contributes to chronic graft rejection. Prior to BOS development infiltration of immune cells into the allograft has been shown. Accumulation of dendritic cells has been reported as well as submucosal lymphocyte and plasma cell infiltrates[6,7]. These submucosal lymphocyte infiltrates consisted mainly of T cells, both CD4+ and CD8+ T cells, but also NK cells have sometimes been identified[8-11]. In bronchoalveolar lavage fluid (BALF) the alveolar macrophage count is decreased from patients with BOS versus those without[12,13], and differences were reported in numbers of neutrophils, CD4+ and CD8+ T cells between patients with and without BOS[12-15].

Only a few studies have been published on the composition of peripheral blood mononuclear cells (PBMC) after lung transplantation. CD19+ B cells were described to be dramatically decreased and hardly present in the peripheral blood of patients with BOS while CD8+ T cells were increased[16]. NK cells were found to be activated and decreased in the peripheral blood of patients with BOS compared to patients without BOS, although others reported this decrease of NK cells in all LTx patient[11,17]. More detailed research was performed on T regulatory cells showing that levels of CD4+CD25+CD69- and CD4+CD25+ were decreased in patients with BOS[18]. In addition, the frequency and phenotype of peripheral NK cells drastically changes after LTx, with immature NK cells being more prominent while mature NK cells are being more activated, but overall less cytotoxic (CD16-CD56dim)[17].

In this study we have focused on the mononuclear cell composition of multiple peripheral blood samples sequentially collected after LTx. Cells characterized include monocytes, dendritic cell, NK cells, NKT cells, and also T/B cells and their subsets. The primary objective was to examine whether this shows differences between patients eventually developing BOS versus patients remaining BOS-free, and if so whether differences found early after transplantation can be used as predictive biomarkers to identify patients at risk

for BOS development.

Patients and Methods

Patients

Fifty LTx patients transplanted between September 2003 and March 2008 at the Heart Lung Center in Utrecht, the Netherlands, were included in this study, based on patient material availability. BOS was defined as a decline of the FEV_1 from the post-operative baseline at two distinctive time-points of more than 20% in the absence of infection or other etiology according to international guidelines[19]. All patients suspected for BOS had a bronchoscopy and CT scanning to exclude large airway complications and infections. BOS grade was assessed at BOS onset. Standard immunosuppressive therapy consisted of tacrolimus, mycophenolate-mofetil and prednisone. Upon suspicion of BOS patients were treated with azithromycin. Directly after lung transplantation induction therapy was given at day 0 and day 4 with basiliximab. No other induction therapy was applied. Clinically there were no signs of severe reflux and all patients were treated with protein pump inhibitors to decrease the effect of acid reflux.

Blood sampling

Patient follow-up started in September 2003, after approval by the medical-ethical committee and informed consent was obtained from each patient. Blood samples were collected routinely once prior to, once every month during the first year after transplantation, and every quarter thereafter. From 40 ml heparinized whole blood PBMC were isolated by Ficoll-Paque Plus (GE healthcare, Sweden). All samples were frozen in RPMI 20% FCS 10% DMSO and preserved in liquid nitrogen until measurement. In a cross-sectional study blood samples at approximately 5 months after LTx (range 4.2-6.1 months) were used.

Flow cytometry

Cryopreserved PBMC were rapidly thawed in a 37°C water bath and added to medium containing 10% fetal calf serum (FCS) after which the cells were centrifuged and resuspended in PBS with 0.1% BSA and 0.1% Natrium-azide. Two million PBMC were incubated with either relevant antibodies or isotype controls for 30 min on ice in the dark followed by washing and measurement. Data acquisition and analysis was performed on a BD FACS Canto II with 8 color detection (BD Biosciences, CA). Each recipient sample

that was measured had approximately 194,000 events (range 69,000– 573,000 events) in the lymphocyte gate, which was defined by $CD45^+$ and side-scatter (SSC), and analyzed with FACS DIVA software (BD Bioscience, CA).

Antibodies

Lymphocyte subsets were distinguished by the use of CD45-PE Cy7 (BioLegend) for lymphocytes, CD3-eFluor 450 (eBioscience) for T cells, CD19-PerCP (BioLegend) for B cells, CD14-APC-H7 (Becton Dickinson) for monocytes, and CD16/CD56-PE (BioLegend) for NK cells. NKT cells were defined as CD3+ and CD16/56+. T cell subsets were identified by CD3-eFluor 450 (eBioscience), CD4-APC H7 (Becton Dickinson), CD8a-PE (BioLegend), CD45RO-PE Cy7 (Becton Dickinson), CD27-PerCP (BioLegend), CD127-PE (Bioscience) and CD25-PB (BioLegend). B cell subsets were identified by CD38-PE Cy7 (Bioscience), CD10-APC Cy7 (BioLegend), CD27-PerCP (BioLegend), IgM-APC (BioLegend), and IgD-PE (DAKO). NK cells were identified as CD45+CD3-CD16/56+, NKT cells as CD3+CD16/56+ while T cells were defined as CD45+CD3+CD16+/56-. CD4+ and CD8+ T cells were further identified by CD27 and CD45RO expression. CD27+CD45RO- are naïve, CD27+CD45RO+ are central memory, CD27-CD45RO+ are effector memory and CD27-CD45RO- are terminally differentiated effector T cells. Regulatory T cells were distinguished by CD25 and CD127 expression. NKG2D-APC (BD Pharmingem) expression was analyzed on NK cells, NKT cells and T cells. CD19+ B cell subsets were defined as recent bone marrow emigrants (RBE; CD38+CD10+IgM+IgD+CD27-), naïve (CD38-CD10-IgM+IgD+CD27-), and memory (CD27+) cells which were further separated according to IgM and IgD expression. An overview of the gating strategy can be found in Supplementary Figure 1. DC and subsequent subtypes were identified according to previous experiments[20]. Each sample was incubated with specific antibodies as well as isotype-matched control monoclonal antibodies in order to determine the MFI cut-off discriminating signal from background fluorescence.

Statistics

A p-value of <0.05 was considered to be significant. Mann-Whitney rank-sum tests were used to study the differences between patients with BOS and without BOS. Graphpad Prism software version 6.02 (GraphPad Software Inc., San Diego, CA) was used to perform statistical analyses.

	BOS	Non-BOS
Total number (n=50)	11 (20%)	39 (78%)
Age		
Years (range)	46.4 (17-62)	43.8 (18-64)
Follow-up time		
Months (range)	47.5 (9-80)	57.5 (31-82)
Gender		
Male	4 (36%)	23 (59%)
Female	7 (64%)	16 (41%)
Primary disease		
Cystic fibrosis	3 (27%)	18 (46%)
Emphysema	5 (46%)	10 (26%)
Fibrotic disease	3 (27%)	11 (28%)
Type of graft		
Single	2 (18%)	4 (10%)
Double	9 (82%)	35 (90%)
CMV		
Reactivation	4 (36%)	14 (36%)
No reactivation	7 (64%)	25 (64%)
CMV IgG antibodies prior to transplantation (donor/recipient)		
Negative/negative	1 (9%)	11 (28%)
Negative/positive	5 (46%)	11 (28%)
Positive/negative	2 (18%)	9 (24%)
Positive/positive	1 (9%)	4 (10%)
Unknown/positive	2 (18%)	4 (10%)
BOS onset		
Months (range)	22.5 (5-50)	Not applicable
BOS grade		
I	3 (27%)	
II	3 (27%)	
III	5 (46%)	

Table 1: Clinical and demographic profile of lung transplant patients

BOS: bronchiolitis obliterans syndrome, CMV: cytomegalovirus

Results

BOS and patient demographics

Eleven out of 50 patients included in this study eventually developed BOS and no RAS was observed. Patient characteristics are presented in Table 1. No differences exist between the patient group developing BOS and the patients without BOS based on age, gender or primary disease, type of graft, CMV reactivation or antibodies against CMV prior to transplantation. Also, we found no potentially confounding factors that could have affected PBMC profiles (e.g. active infection, acute cellular rejection, or lymphocytic bronchiolitis) present at the time points of peripheral blood sampling. The average time to develop BOS was 22.5 months after LTx (range 8-49 months). The average follow-up time of BOS patients was shorter than patients without BOS (47.5 months (range 9-80) and 57.5 months (range 31-82) respectively). More patients with BOS than patients without BOS died during follow-up (p=0.01). HLA antibodies did not influence the development

of BOS or the survival outcome, as only low titers (MFI<500) of Luminex-defined IgG anti-HLA antibodies are present in our cohort, both pre- and post-transplant[21].

Stability of circulating mononuclear cell composition after LTx

From 10 patients (BOS n=5, non-BOS n=5) we had samples available taken each month within the 1st year after transplantation and analyzed them for T, NK, and NKT cells (Figure 1A, B, and C). From 11 patients (BOS n=4. Non-BOS n=7) sufficient samples were present for analysis of B cells, monocytes and dendritic cells (Figure 1D, E, and F). No differences were found in total leukocyte or lymphocyte numbers between the BOS and non-BOS patient groups (data not shown). Also, we could not identify any correlation between % of cellular subsets and underlying disease. In Figure 1, each box represents the combined cell numbers of the monthly taken samples during the first year after transplantation for one patient. Analysis of the mononuclear cell composition over time showed that the percentage of T cells, monocytes and dendritic cells is relatively stable in all patients, whereas B cells, NK cells and NKT cells appear to display a more variable bandwidth in some patients after transplantation, indicated by the larger box-and-whisker plots (Figure 1). Taken the data from al BOS and non-BOS patients together, patients developing BOS have a higher percentage of B cells (p=0.02) and a lower percentage of T cells (p = 0.008) and monocytes (p=0.04) compared to patients not developing BOS. However, the overlap in these percentages does not allow identification of patients at risk for developing BOS (Figure 1). Furthermore, the changes over time in the percentage of any mononuclear cell type investigated is not related to development of BOS in individual patients.

In these samples, NKG2D$^+$ expression (data not shown) was found on average on 62% NK cells (range 45%-70%, standard deviation (SD) range 7.4%-20.5%); NKT cells on average displayed 50% expression of NKG2D$^+$ (range 30%-74%, SD range 1.5%-20.9%). Analysis of NKG2D$^+$ expression on T cells showed an average of 31% positive T-cells (range 20%-41%, SD range 4.8%-13.1%). A comparison showed that no differences were found between the patient group developing BOS or not with respect to NKG2D$^+$ expression on NK-, NKT-, or T cells. No changes in NKG2D$^+$ expression on the cells were noticed preceding BOS diagnosis or CMV infections.

Since the circulating mononuclear cell composition is relatively stable during the first year after transplantation, lung function stabilizes around month 5 post-LTx, and immunosuppressive therapy is reduced after 5 months, we conducted a cross-sectional analysis of the circulating mononuclear cell composition in 50 LTx patients at month 5 after transplantation.

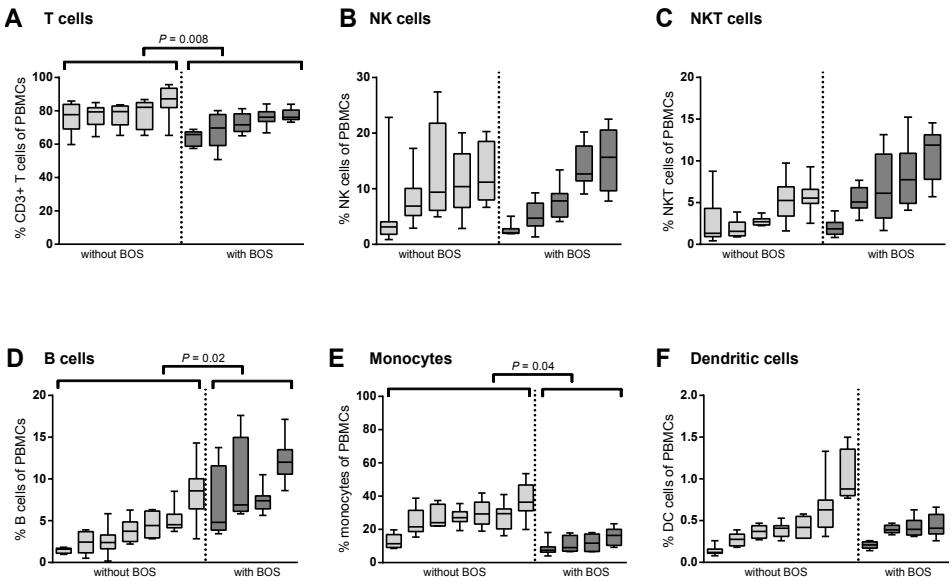
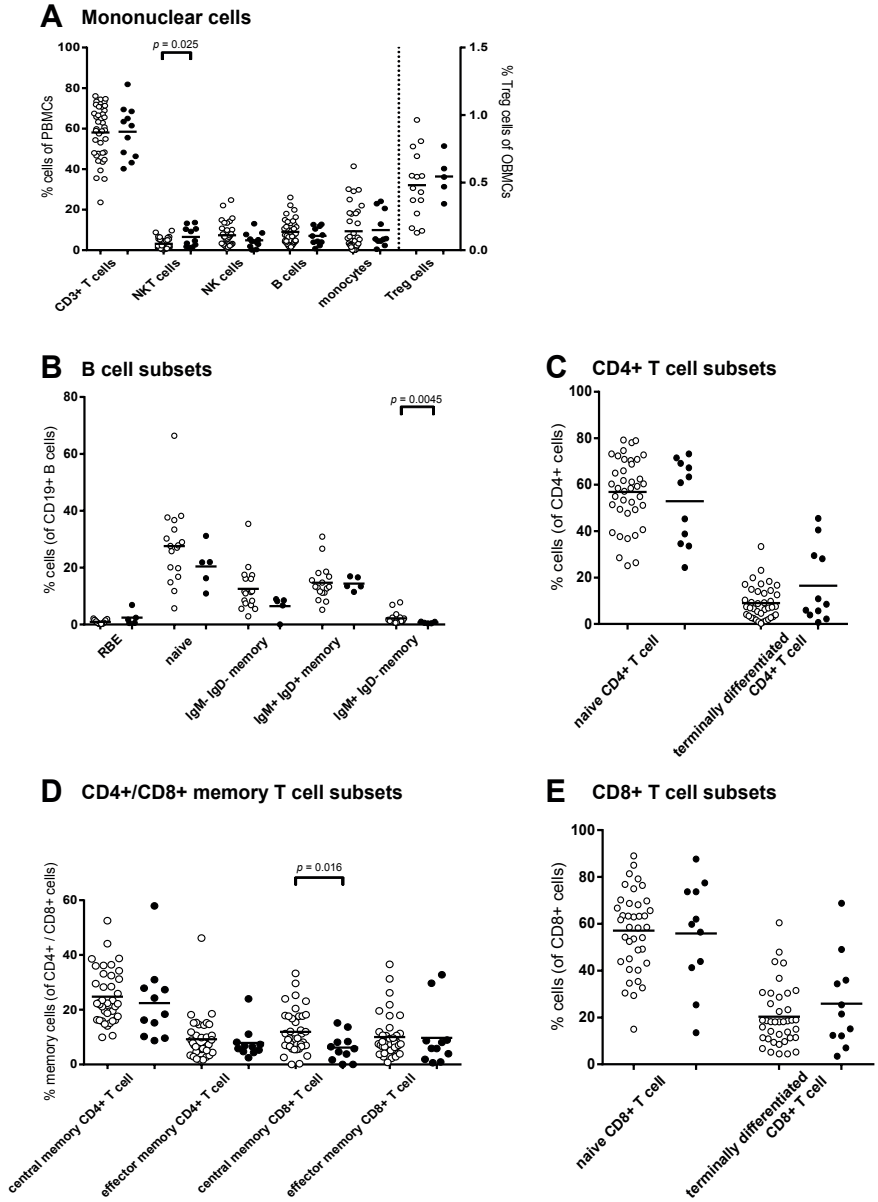


Figure 1: Composition of mononuclear cells over time after transplantation

Peripheral blood mononuclear cells were obtained monthly after lung transplantation and stored in liquid nitrogen. Since the samples were subjected to Ficoll-Paque separation and one freeze-thaw cycle, the CD45+ population only consists of lymphocytes and monocytes, as is also shown in supplementary figure 1. A total of 162 samples with an average of 9 samples per patients (range 5-12) could be analyzed within the first year after transplantation. Results are displayed as box-and-whisker plots with the boxes covering the 75% interval and the median displayed within, and the whiskers displaying the 91st percentile. Each box represents the combined cell numbers of the monthly taken samples during the first year after transplantation for one patient. For T cells, NK cells and NKT cells sufficient material from 5 BOS and 5 non-BOS patients was available, whereas sufficient samples from 4 BOS and 7 non-BOS patient where available for the B cells, monocytes, and DC analyses. Results of patients not developing BOS are displayed on the left side of the figures in boxes filled with light grey whereas those from patients developing BOS are displayed in darker shaded boxes. Significant differences between the total BOS and non-BOS patients cell percentages are indicated in the respective figures. FACS analysis was performed as indicated in Patients and Methods in order to determine the percentage of T cells, B cells, monocytes, NK cells, NKT cells, and dendritic cells as indicated in **A**-**F**.

Figure 2: Composition of lymphoid subsets after lung transplantation

Peripheral blood mononuclear cells obtained 5 months after lung transplantation were analyzed by FACS as indicated in Patients and Methods. Percentages of monocytes, T, NKT, NK, B, and Treg cells were determined (**A**). Results of T regulatory cells are displayed on the right y-axis in a scale adapted to display their relatively low frequency, indicated by the dashed vertical line. B cell subsets,



including RBE, naïve and memory subsets, discriminated via IgM/IgD expression are depicted in **B**. **C** shows naïve and terminally differentiated CD4+ T cells, whereas **E** depicts these subsets for CD8+ T cells. Finally, both CD4+ and CD8+ memory T cell subsets, consisting of central memory and effector memory, are displayed in **D**. Patients who will eventually develop BOS are displayed by black circles, whereas patients who remain BOS free are indicated by open circles. Significant differences are indicated in the respective figures.

Specific T cell and B cell subsets are associated with, but not predictive for BOS

B and T cell subset analysis was performed on blood taken at month 5 after LTx from 50 patients of whom 11 developed BOS (Figure 2A-2E). For Treg cells analysis 15 non-BOS and 5 BOS samples were available, for B cell subset analysis sufficient material was available from 17 non-BOS and 5 BOS patients. No differences were observed between patients developing BOS or not in percentages of specific circulating B cell subsets (recent bone marrow emigrants and naïve B cells, separated according to IgM/IgD expression, Figure 2B), T regulatory cells (Figure 2A, right y-axis), or specific CD4+/CD8 T cell subsets (naïve, terminally differentiated, Figure 2C and Figure 2E respectively). However, NKT cells (p=0.025, Figure 2A) were increased, whereas IgM+IgD- memory B cells (p=0.0045, Figure 2B) and central memory CD8+ T cells were decreased (p=0.016, Figure 2D) in patients developing BOS versus those remaining BOS-free. In contrast to Figure 1C, the cross-sectional analysis at month 5 post-LTx did not show any differences in the percentage of monocytes. To investigate whether the levels of these cells might be an early predictor of BOS, a ROC analysis was performed. For NKT cells an area under the curve (AUC) of 0.72 was obtained in a ROC analysis with 3.2% (percentage of CD45+ cells) as best discriminative cut-off value at month 5 after LTx. However, a Kaplan-Meyer (log rank) analysis showed no significant difference (p=0.19) between patients with high or low levels NKT cells. Central memory $CD8^+$ T cells had an AUC of 0.74 with 7.7% as best discriminative cut-off level at month 5 after LTx. Also, using this cut-off value did not result in significant differences (p=0.17). IgM+IgD- memory B cells had an AUC of 0.094 with 0.94% as best discriminative cut-off level at month 5 after LTx. A Kaplan-Meyer (log rank) analysis showed a significant difference (p=0.04), but due to low specificity (0.2) and sensitivity (0.24), indicated by ROC analysis of the cut-off level, usage of this subset is limited for predictive diagnosis. Therefore, measurement of NKT cells, central memory $CD8^+$ T cells, and IgM+IgD- memory B cells at 5 months after LTx are not a predictive marker for BOS.

Discussion

This study is novel with regard to the analysis of the mononuclear cell composition and T/B cell subsets in peripheral blood taken monthly up to 1 year after lung transplantation. Furthermore, this is the first study to show significant differences in levels of various specific lymphoid subsets in peripheral blood between patients who eventually developed BOS versus patients who did not.

Despite several optimistic early reports from multicenter studies it now has become clear that patients with BOS respond poorly to augmented immunosuppressive therapy[2,22]. Although augmented therapy applied preceding the onset of the disease maybe more likely to be successful in delaying, preventing or slowing down the progression of BOS, at present it is not feasible to engage such studies as it is not possible to predict BOS. This indicates that there is a need for sensitive markers that predict the fall in graft performance. In addition, markers that are directly correlated with the degree of functional immunosuppression, can be used to guide immunosuppressant dosage which will be of benefit in treating patients with a lung transplant. Differences found in blood between LTx patients in time developing BOS versus those who do not can result in powerful diagnostic tools for the prediction of patients at risk of BOS development[23]. Cellular differences found in this study are present relatively early after lung transplantation and might be an indication of ongoing inflammatory responses. These processes might be 'silent rejection' and go unnoticed until deterioration of lung function is measured when there is no return for BOS. Previous studies in peripheral blood after lung transplantation have been mainly focused on analysis of a certain hematopoietic lineage and its activation status, and/or some subsets analysis in peripheral blood, after the diagnosis BOS has been made. For instance, in a small study using 2 color FACS technology the composition of lymphocytes after LTx was reported, showing a decrease in B cells and $CD4^+$ T cells and an increase in $CD8^+$ T cells in patients diagnosed with BOS[24]. Also, differences were present between previous studies with regard to sampling methods and immune suppressive regime employed, which hampers data comparison. However, no elaborate study has been reported on the composition of all mononuclear cells present in blood prior to the diagnosis BOS[11,17].

Despite the fact that bronchoalveolar lavage fluid (BALF) might reflect the local environment of inflammation in the lung more truly than peripheral blood, several studies made clear that the procedures for analysis of BALF are hard to standardize and results did not reach conformity[12-15,25] A small study investigated the differences between BALF and blood after LTx. CD3+ and CD4+ T cells were decreased while CD8+ T cells were increased in BALF compared to peripheral blood[15]. However, differences between patients developing BOS and patients without BOS were not conclusive as the number of available samples was limited for the measurements in peripheral blood (subjects with BOS = 4). Fluctuations over time of especially the amount of T cells are documented in BALF[24]. The composition of PBMC may also change in time after lung transplantation, hence it is important that our cross-sectional study results are further verified in longitudinal studies

We have used an 8 color staining on a larger patient cohort resulting in an overview of

mononuclear cell composition shortly after transplantation. We have observed interesting differences in PBMC composition, however these differences do overlap to some extent and were not predictive for BOS development early after transplantation. Further analyses in larger patient cohorts are needed to both confirm and strengthen our observations. The increase in NKT cells after LTx in patients developing BOS is interesting. Although NKT cells represent only a small population (0.5%) of the total $CD3^+$ T cells, they produce both Th1 and Th2 cytokines and interact with cells from the innate immune system. In case of *Staphylococcus pneumonia*, NKT cells were able to attract neutrophils to the lungs and an increase in neutrophils has been detected in patients whom developed BOS after LTx[26-28]. NKT cells have been shown to contribute to renal and hepatic ischemia-reperfusion injury[29], a process which has been assigned a risk factor for development of BOS[19]. Additionally, increased peripheral blood NK and NKT cells are associated with BOS. The authors postulate that these cells migrate to the lungs and can induce airway damage via the secretion of perforin, granzymes and pro-inflammatory cytokines[30] Also, a decrease in memory B cells has been associated with a higher susceptibility to infection in patients suffering from chronic graft-versus host disease, and infections are a known risk factor for BOS development[23,31]. Furthermore, CD8+ central memory cells have been shown to suppress allograft rejection in mice. A decrease in CD8+ central memory cells such as we have observed at month 5 post-LTx could well lead to a decrease in the suppression of graft rejection[32].

NKT cells, central memory $CD8^+$ T cells and IgM+IgD- memory B cells were found to be different between patients whom would develop BOS and patients remaining BOS free. However, according to ROC analysis the sensitivity and specificity of the optimal cut-off point is limited with regard to prediction of BOS. The overlap in circulating percentages of these cells between the 2 patient groups investigated is the reason that the differences found are not predictive for individual patients. Nevertheless, the predictive strength for BOS may be increased by further subset analysis of the 3 cell types reported in this study.

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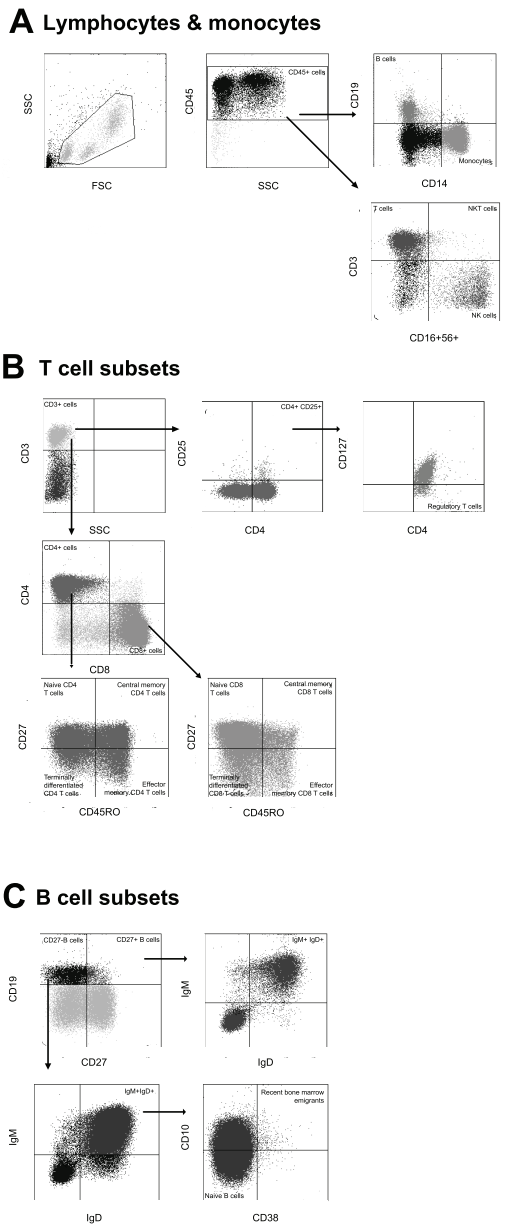
Supplementary Information



Supplementary Figure 1: Gating strategy

Different cell types (**A**) are identified based on FSC/SSC and CD45 expression, and are further characterized as CD19+ (B cells), CD14+ (monocytes), CD3+ (T cells), CD3+CD16+CD56+ (NKT cells), and CD3-CD16+CD56+ (NK cells). From the CD45+ gate CD3+ T cells are selected (**B**) and from either CD4+ T cells and CD8+ T cells, naïve (CD45RO-CD27+), central memory (CD45RO+CD27+), effector memory (CD45RO+CD27-), and terminally differentiated T cells (CD45RO-CD27-) are distinguished. Regulatory T cells are identified as CD4+CD25+CD127-. From the CD45+ gate, CD19+ B cell subsets are identified (**C**). Memory B cells were selected as CD19+CD27+ and were further characterized based upon IgM and IgD expression. Recent bone marrow emigrants were classified as CD19+CD27-IgM+IgD+CD10+CD38- and naïve B cells as CD19+CD27-IgM+IgD+CD10-CD38-.

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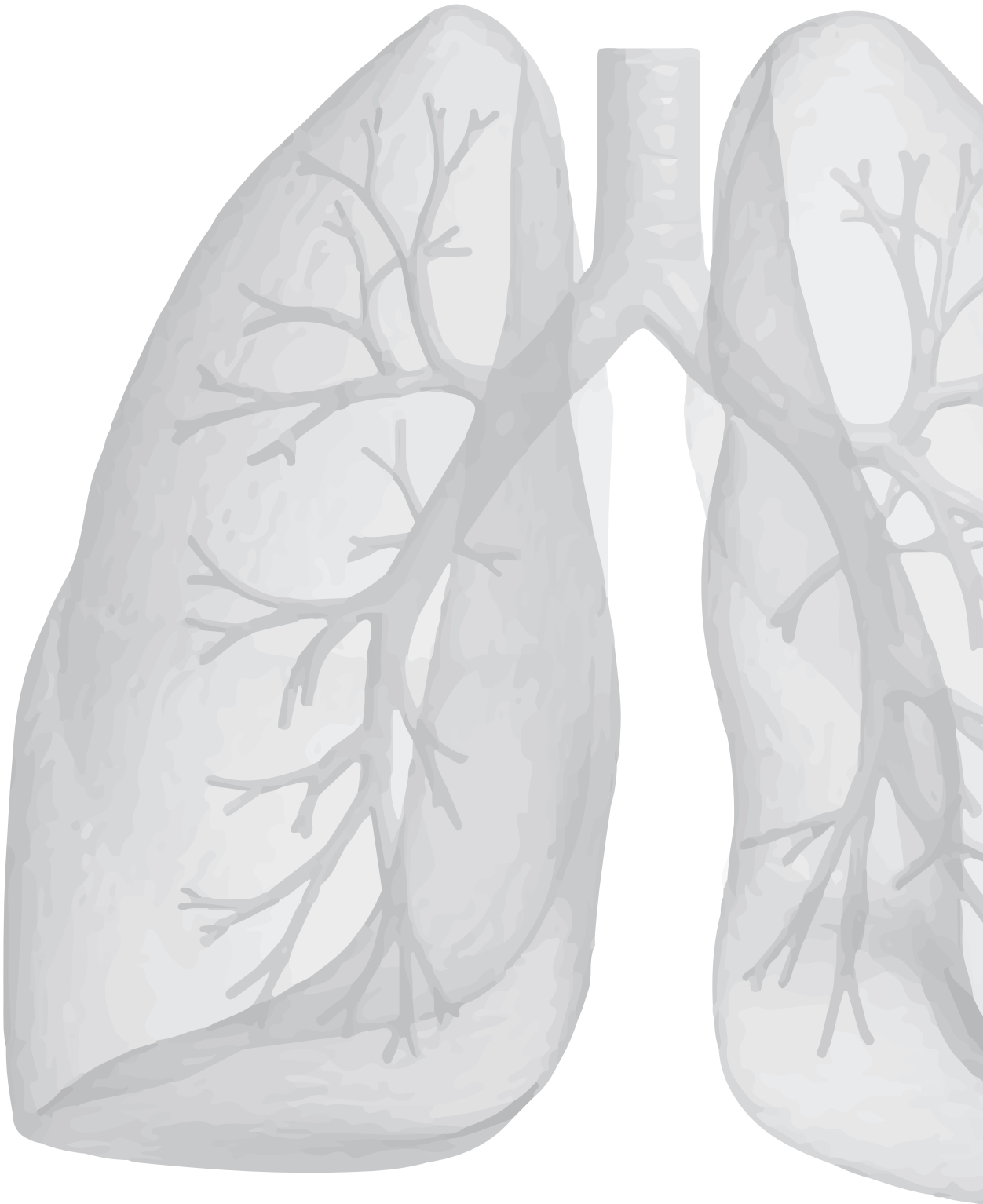

8

Retrograde flushing of the pulmonary vein during explantation: lymphocyte composition in the perfusate and impact on clinical outcome after lung transplantation

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Manuscript submitted

To the editor:

Lung transplantation (LTx) remains the sole treatment option for patients suffering from end-stage lung diseases. However, survival after LTx is limited, due to the development of chronic lung allograft dysfunction (CLAD) which can present itself obstructively, as the bronchiolitis obliterans syndrome (BOS), or in a restrictive form (restrictive allograft syndrome, RAS)[1]. BOS is diagnosed in circa 50% of LTx patients in the first 5 years after transplantation[2]. One of the factors essential for successful transplantation outcome is the technique of lung preservation. Studies have shown a beneficial effect of retrograde flushing on early graft dysfunction and bronchial complications, but there is no concordance in literature. Consequently, antegrade lung perfusion is still the standard method of practice in most centers for LTx procedure[3,4].

In our center, in order to reduce the risk for thromboembolic complications early after transplantation[3], lungs are flushed antegradely via the main pulmonary artery with Perfadex® solution while the lungs are topically cooled and ventilated at tidal volume (F_1O_2<1). After lung harvesting, the extracted lung-bloc is subjected to retrograde perfusion via the pulmonary vein, also with Perfadex® solution, until the perfusate seems clean from blood cloths and thrombocytes. During the transplantation procedure, this flush is collected in a tray and further processed for various research purposes.

Here, we investigated the total number of peripheral blood mononuclear cells (PBMCs) and the cellular composition in the collected perfusate after retrograde flushing. Furthermore, we studied the influence of the combined antegrade and retrograde flushing procedure on outcome after LTx.

Total retrograde perfusate volumes used during transplantation procedure ranged from 500-2,000 ml. First, we analyzed the total cell number in the perfusate collected during flushing of the explanted graft. Since the analyzed samples were only fractions of the total perfusate volume, subsequent cell numbers are quantified as cellular percentages instead of total cell numbers. The obtained flush was centrifuged (1800 RPM, 10 min) and the pooled cell fraction was loaded on Ficoll-Paque (GE Healthcare) to isolate PBMCs. The total cell yield was counted using a Coulter Cell Counter and isolated cells were stored as aliquots in liquid nitrogen until further analysis. Unfortunately, flush samples were only available for 47 out of 58 LTx procedures. Total cell numbers largely varied between analyzed samples, but nonetheless showed a significant correlation, p=0.0005, between the total amount of PBMCs in the obtained flush and the total amount of perfusion fluid that was analyzed. These numbers reached a total of $8.0x10^8$ cells in single individuals,

	Antegrade lung preservation (n=72)	Antegrade + retrograde lung preservation (n=58)	Total cohort (n=130)	*p* -value
Recipient gender				0.457
Male	37 (51%)	26 (45%)	63 (48%)	
Female	35 (49%)	32 (55%)	67 (52%)	
Gender mismatch	11 (15%)	2 (3%)	13	0.023
Recipient age (years)	44 ± 13	46 ± 13	45 ± 13	0.525
Type of transplantation				0.518
Single	14 (19%)	14 (24%)	28 (22%)	
Bilateral	58 (81%)	44 (76%)	102 (78%)	
Primairy disease				0.270
COPD	24 (33%)	28 (48%)	52 (40%)	
CF	25 (35%)	18 (31%)	43 (33%)	
ILD	22 (31%)	12 (21%)	34 (26%)	
Other	1 (1%)	0 (0%)	1 (1%)	
Infection				
CMV high risk	15 (21%)	17 (29%)	32 (25%)	0.265
EBV high risk	7 (10%)	9 (13%)	16 (12%)	0.347
Clinical complications				
BOS	26 (36%)	19 (33%)	45 (35%)	0.690
Patient death	27 (38%)	19 (33%)	46 (35%)	0.574
Episode of AR	21 (29%)	6 (10%)	27 (21%)	*0.009*
Donor gender				0.162
Male	26 (36%)	28 (48%)	54 (42%)	
Female	46 (64%)	30 (52%)	76 (58%)	
Donor age (years)	46 ± 12	45 ± 16	45 ± 14	0.762
Donor oxygenation ratio	50 ± 14	60 ± 13	54 ± 13	0.384
Graft ischaemic time				
Right lung	226 ± 71	293 ± 250	255 ± 176	*0.038*
Left lung	310 ± 75	404 ± 273	351 ± 194	*0.010*

Table 1: Clinical and demographic parameters of LTx patients and donors

Both pre-flush and post-flush patient groups are compared for various clinical and demographic parameters. Patients were defined as being high-risk for CMV or EBV reactivation when a CMV- or EBV- patient received a graft from a CMV+ or EBV+ donor. Categorical data were analyzed by the Fischer's exact or Pearson's χ^2 test and continuous variables by the ANOVA test. Both right lung and left lung ischemic times were longer in the antegrade + retrograde flush patient group. Also, more episodes of AR were observed in the antegrade lung preservation group. COPD: chronic obstructive pulmonary disease, CF: cystic fibrosis, ILD: interstitial lung disease, CMV: cytomegalovirus, EBV: Epstein-Barr virus, BOS: bronchiolitis obliterans syndrome, AR: acute rejection. Statistical analyses were performed using GraphPad Prism version 6.02 (GraphPad Software Inc.) and SPSS version 20 (IBM Corp.).

donor PBMCs which, in case of antegrade perfusion only, would be transplanted in the respective recipient (data not shown).

Second, to investigate the representation of peripheral blood samples for perfusate composition, we analyzed the cell composition of 15 donor flush samples and compared cell frequencies with paired donor blood samples using FACS analysis. We observed large differences in PBMC subset composition between the collected retrograde perfusate and the circulation of the donor. Both T cells and B cells were decreased in the lung flush, p=0.0027 and p=0.0010 respectively, whereas the percentage of NK cells was significantly higher compared to the circulation (p=0.0043, Figure 1A). No differences in percentage were found between flush and blood in dendritic cells (DCs) and respective subsets mDC1s, mDC2s or pDCs (data not shown).

The procedure of retrograde flushing prior to the implantation of the graft was implemented in August 2008. We therefore decided to analyze the impact of retrograde flushing on survival, BOS development, diagnosed via ISHLT criteria, and episodes of acute rejection (AR) in the first 4 years after transplantation. We included a total of 130 patients of which 72 were transplanted prior to August 2008 and 58 thereafter. Both left and right lung ischemic times were longer in the antegrade + retrograde lung preservation group. Further details can be found in Table 1. Within the first 4-year time period after transplantation 38 patients deceased, 27 patients developed the obstructive form of CLAD, BOS and 24 patients presented at least a single episode of AR. Since biopsies were not performed, for this study AR was defined as a spontaneous decline of $FEV_{1,}$ in the absence of other disease manifestations, which increased after treatment with steroid pulses (3x1000 mg). No RAS was observed.

Kaplan-Meier survival analyses showed no difference on the incidence of obstructive chronic rejection or on survival post-LTx in the first 4 years after transplantation between the pre- and post- retrograde flush patient groups (Figure 1B and C). However, we did observe a significant higher number of episodes of AR in patients transplanted prior to the implementation of retrograde flushing during transplantation procedure (p=0.039, Figure 1D). Unfortunately, due to the lack of biopsies, we could not distinguish the patients on severe or mild AR. Also, due to low patient numbers no associations could be made between time to AR in AR+ patients. In 2008, no other changes in clinical protocol were enforced and we therefore conclude that the implementation of retrograde flushing is primary responsiblefor the observed differences in clinical outcome after LTx.

The total amount of perfusion fluid used for retrograde perfusion varies per transplantation

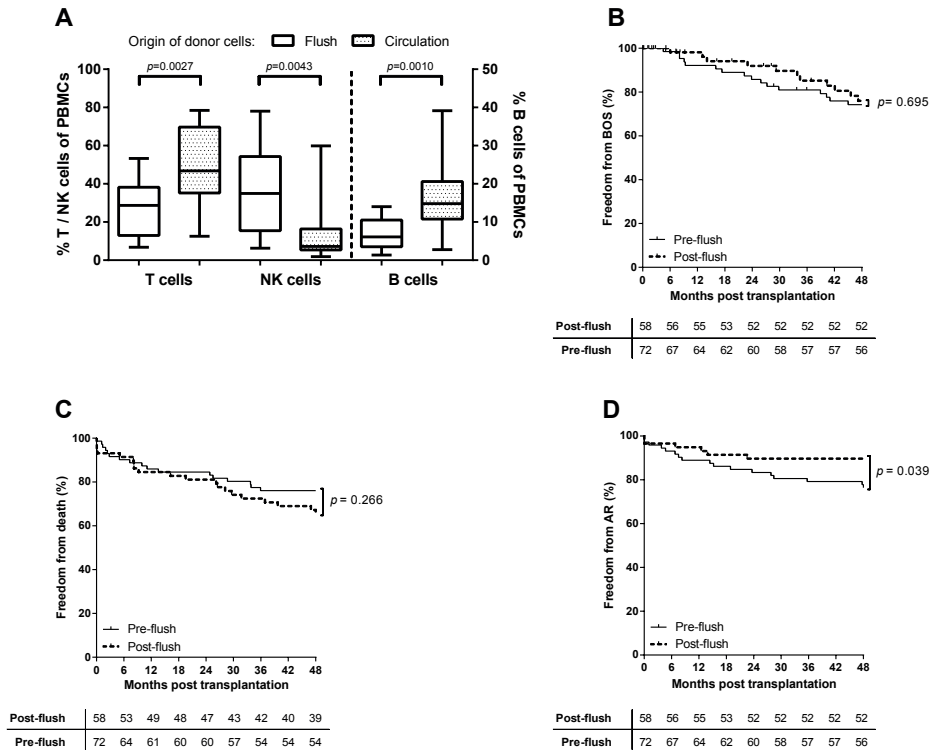

Figure 1: Cellular composition of lung perfusate and the impact on retrograde flushing on LTx outcome.

A The cellular composition of the lung perfusate was analyzed using a FACS Canto and accompanying FACS Diva software (BD). Prior to analysis cells where thawed, washed, and incubated with the respective antibodies (PBMCs: CD45-PE Cy 7 (BioLegend); T cells: CD3-eFluor 450 (eBioscience); B cells: CD19-PerCP (BioLegend); NK cells: CD16/CD56-PE (BioLegend)). Large differences are observed when comparing the cellular compositions of the donor circulation (shaded box plots) and lung perfusate (open box plots) in 15 matched donor samples. Percentages of T cells and NK cells are depicted in the left y-axis whereas B cells are depicted on the right y-axis. The box indicates the median and the 25th to 75th percentiles and the whiskers the min and max values. Data was tested for Gaussian distribution via the D'Agostino and Pearson omnibus test and differences between groups was analyzed via Mann-Whitney test. **B**, **C**, **D** Kaplan-Meier survival analyses on BOS development, survival and episodes of AR between groups subjected to retrograde flushing (post-flush, dotted line) or not (pre-flush, striped line). A significant higher number of LTx patients was diagnosed with episode(s) of AR in the first 4 years after transplantation in the pre-flush patient group. Numbers at risk are depicted for each 6-month interval. Statistical analyses were performed using GraphPad Prism version 6.02 (GraphPad Software Inc.) and SPSS version 20 (IBM Corp.).

since the procedure is stopped when the perfusate becomes clear and colorless. Therefore, we could not asses potential correlations between the amount of perfusate fluid used for retrograde flushing and LTx outcome. Nevertheless, without retrograde flushing large numbers of passenger leukocytes would be transferred from the donor lung into the patient. The exact role of passenger leukocytes after LTx remains unclear. Both beneficial and disadvantageous effects of these leukocytes have been described in literature[4-6].

Our results indicate that, as a result of retrograde back flushing, high numbers of donor PBMCs are captivated from the graft which apparently has a beneficial impact on outcome after LTx. This study has limitations with respect to the amount of included patients. Also, the presented results would benefit from validation in an external cohort since they are obtained in a single center study setting.

We have observed that the cellular composition of the lung perfusate highly varies when compared to the circulation of matched donors, especially when focused on NK cells. Our data indicate that high numbers of NK cells are present in the lung vascular bed which are removed from the graft via retrograde flushing. NK cells can secrete a large repertoire of pro-inflammatory cytokines and are thought to play a role in rejection after LTx[7]. Therefore, removal of these cells could have a beneficial effect on LTx outcome.

Other centers have investigated the beneficial effects of different forms of organ perfusion, though with different results. Positive effects have been observed on gas-exchange and the removal of emboli and fat clots[8]. To our knowledge, our results are the first to report a positive effect of retrograde flushing on AR after lung transplantation, but not on overall survival or BOS development. Since episodes of AR are considered to be a major risk factor for BOS development, one could expect a beneficial influence of retrograde flushing on BOS incidence during a longer follow-up period. Since our study focusses on the first 4-years after transplantation, due to cohort constraints, we can only speculate on this topic.

Although further clinical validation of our observations is essential, the addition of retrograde flushing during organ preservations can be beneficial for LTx outcome.

Disclosure Statement

The authors have no conflict of interest to disclose.

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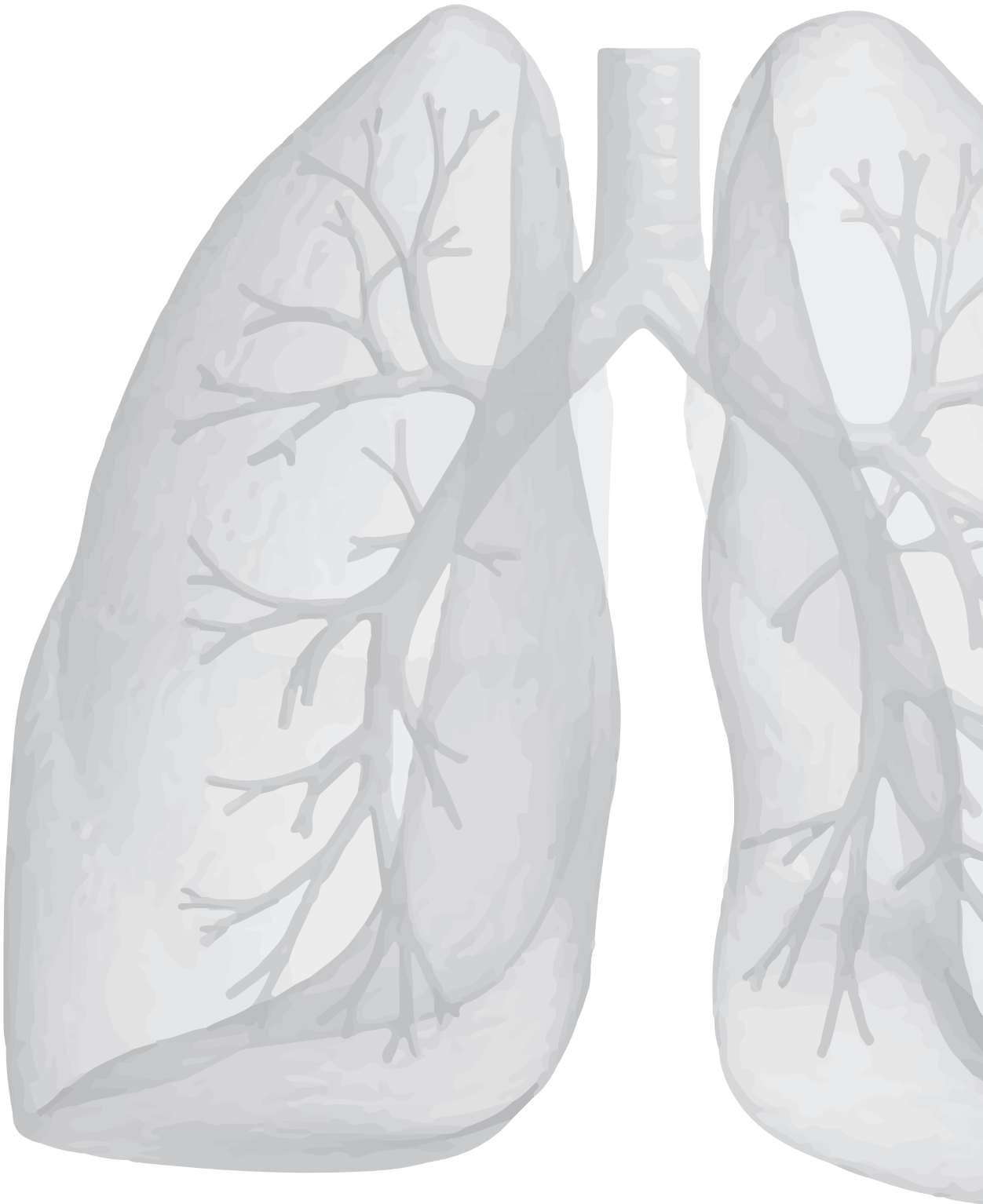
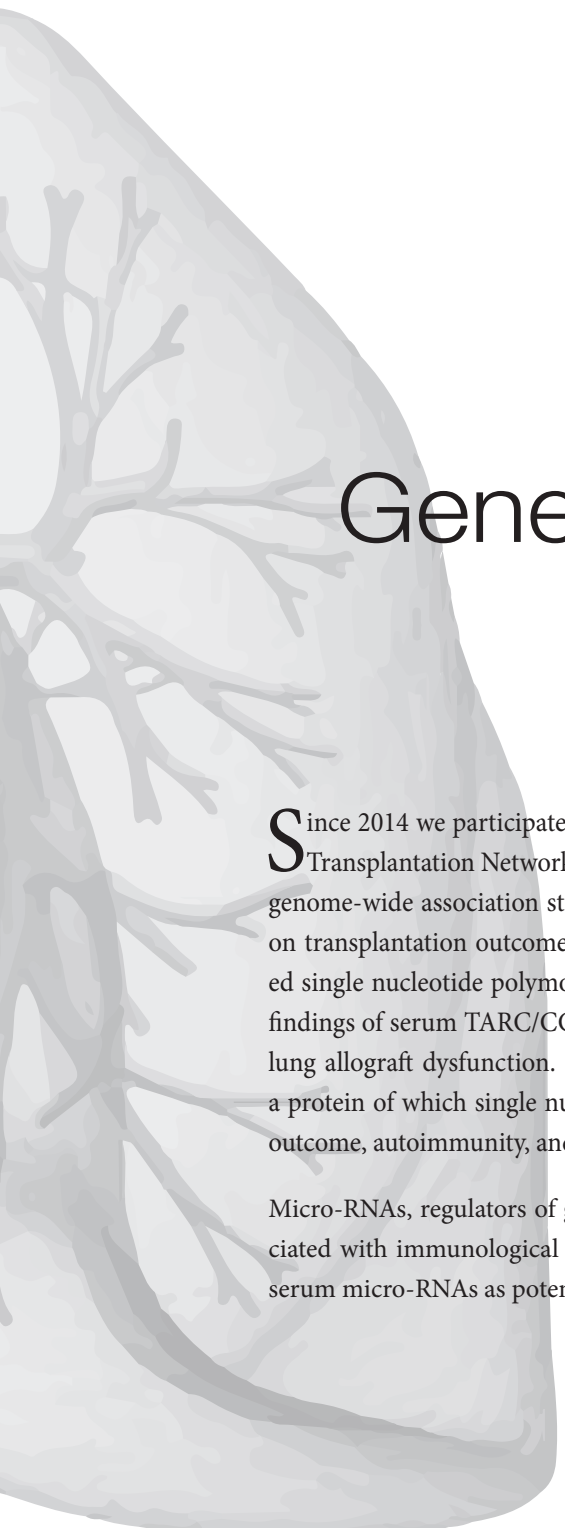


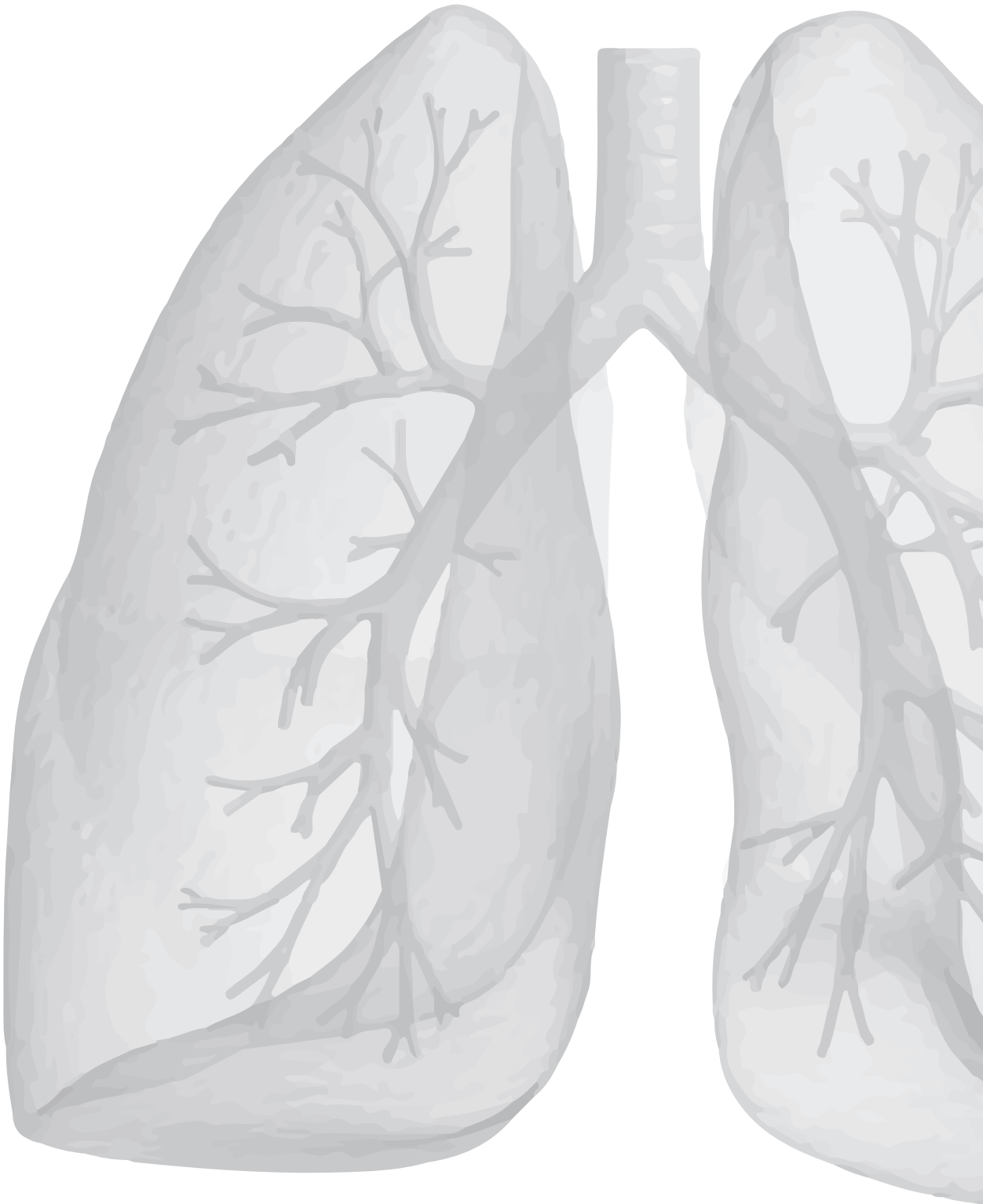


Part III

Genetics and Gene Regulation

Since 2014 we participate in The International Genetics and Translational Research in Transplantation Network (iGeneTRAiN). This consortium is initiated in order to start genome-wide association studies and targeted single nucleotide polymorphism analyses on transplantation outcome in a multi-organ setting. In chapter 9 the presented targeted single nucleotide polymorphism analyses shines new light on the already established findings of serum TARC/CCL17 as predictor for the development of obstructive chronic lung allograft dysfunction. In chapter 10 we present our findings concerning PTPN22, a protein of which single nucleotide polymorphisms are associated with transplantation outcome, autoimmunity, and respiratory syncytial virus-induced bronchiolitis.

Micro-RNAs, regulators of gene expression and protein synthesis, are found to be associated with immunological disease phenotypes. In chapter 11 we investigate the role of serum micro-RNAs as potential biomarkers for chronic rejection.



9

A single donor *TARC/CCL17* promotor polymorphism correlates with serum TARC/CCL17 levels and is associated with impaired clinical outcome after lung transplantation

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Abstract

Lung transplantation (LTx) outcome is hampered by development of chronic rejection, often manifested as the bronchiolitis obliterans syndrome (BOS). TARC/CCL17 is a chemo-attractant of which serum levels measured during the first month post-LTx are predictive for BOS development. Since *TARC/CCL17* promotor polymorphisms correlate with serum TARC/CCL17 levels, we investigated seven selected single nucleotide polymorphisms (SNPs) present in this region and their potential association with LTx outcome. We analyzed donor and patient SNP configurations and haplotypes and identified a single SNP (rs223899) in the donor correlating with patient TARC/CCL17 serum levels post-transplantation (p=0.066). Interestingly, this SNP configuration in patients did not show any correlation with pre-LTx TARC/CCL17 serum levels (p=0.776). Survival analysis showed that receiving a graft from a donor heterozygous for rs223899 has a disadvantageous impact on transplantation outcome. When stratified per donor SNP genotype, patients receiving a transplant from a heterozygous donor showed a significant lower BOS-free survival (50% vs. 75%, p=0.023) and lower survival rate (50% vs 80%, p=0.0079). Since rs223899 is located within a NFκB binding site, heterozygosity at this position could result in a reduced expression of TARC/CCL17. Our data indicate that a single SNP in the promotor region of *TARC/CCL17* in the donor correlates with lower serum TARC/CCL17 levels measured one month after LTx and affects clinical outcome after LTx.

Introduction

For patients suffering from end-stage lung disease, lung transplantation (LTx) can be the final treatment modality. Currently, 5 year survival after LTx is 50%, predominantly due to the development of chronic lung allograft dysfunction (CLAD)[1]. CLAD can present an obstructive (bronchiolitis obliterans syndrome, BOS) and a restrictive form (restrictive allograft syndrome, RAS)[2]. CLAD pathogenesis is poorly understood, however various donor and patient risk factors associated with disease development have been identified, particularly regarding development of BOS[3,4]. A clinical diagnosis of BOS is often made using a 20% decline of the forced expiratory volume in 1 second compared to baseline in the absence of any other disease etiology[5]. Thus, a clinical diagnosis is made at the time that obliterative bronchiolitis has fully developed. To prevent BOS, novel biomarkers reflecting pre-clinical development identifying patients at risk early after transplantation, are urgently needed[6].

Thymus and activation regulated chemokine (TARC/CCL17) is a chemo attractant, which is secreted by a broad variety of cell types, including endothelial cells, dendritic cells, keratinocytes, bronchial epithelial cells and fibroblasts[7-10]. It mainly functions as a chemo attractant for Th2 cells via the interaction with its receptor CCR4[11,12]. TARC/CCL17 serum levels are associated with various types of lung diseases including idiopathic pulmonary fibrosis[13] and eosinophilic pneumonia[14], and as risk marker for lung cancer[15]. Interestingly, previous results from our group have shown that serum levels of TARC/CCL17 in the first month post-transplantation are predictive for BOS development after LTx[16].

The *TARC/CCL17* gene is located on chromosome 16q13, in near proximity of the CCR4 interacting chemokine *CCL22* and *CX3CL1*[17]. TARC/CCL17 expression is controlled by multiple pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-1 and IL-4[18]. The transcriptional regulation of the TARC/CCL17 gene has partly been elucidated. Both the transcription factors STAT6 and NFκB have binding sites in the promotor region of *TARC/CCL17*[18,19]. Several single nucleotide polymorphisms (SNPs) in the *TARC/CCL17* promotor region correlate with serum levels of TARC/CCL17 and are associated with a risk for Kawasaki disease, and different allergic diseases[20-22].

As serum levels of TARC/CCL17 predict a risk for post-LTx BOS, we hypothesized that *TARC/CCL17* polymorphisms may be correlated to outcome after LTx. In the present study we genotyped and analyzed several SNPs in the TARC promotor region of patients undergoing LTx as well as in that of the donor. We show that a single donor

SNP configuration in the promotor region of *TARC/CC17* of the donor correlates with recipient TARC/CCL17 serum levels and relates to BOS development and overall survival after LTx.

Patients and Methods

Patients

A total of 144 patients undergoing LTx between January 2004 and March 2013 in the Heart Lung Center of the University Medical Center Utrecht, The Netherlands, were included in this study. Informed consent was obtained from all study participants and the study was approved by the medical ethical committee. Post-transplantation follow-up therapy was standardized and consisted of tacrolimus, prednisolone and mofetil mycophenolate. Patients at high risk for CMV or EBV activation, i.e. CMV- or EBV-negative patients transplanted with a EBV- or CMV-positive donor, were treated with valganciclovir for 6 months after transplantation. A clinical diagnosis of BOS was made when FEV_1 had declined by 20% of more compared to baseline[5]. Since surveillance biopsies were not performed, acute rejection (AR) was defined as a spontaneous decline of lung function which was reversed after steroid pulse treatment and for which other causes of lung function decline were included

Prior to transplantation, blood was obtained from donor and patient, as well as a spleen samples from the donor. Mononuclear cells from patient and donor samples were isolated using Ficoll-Paque (GE Healthcare, Little Chalfont, UK), which were then aliquoted and stored in liquid nitrogen until further use. In addition, serum from the patient was collected and stored at -80°C.

DNA extraction

Frozen mononuclear cells were used for DNA isolation via the MagnaPure Compact System (Roche Diagnostics, Switzerland). Cell samples were thawed at 37°C, dissolved in 9 ml RPMI-1640 (Lonza, Basel, Switzerland) supplemented with 20%, v/v, fetal bovine serum (Bodinco, Alkmaar, The Netherlands) and centrifuged for 10 min at 1800 RPM. Prior to DNA extraction, cells ware dissolved in phosphate buffered saline (PBS, Sigma-Aldrich, USA) at a concentration of $5x10^6$ cells/ml.

SNP selection and genotyping

Six SNPs (rs223895, rs223897, rs223898, rs223899, rs223900, and rs229827) in the

promotor region of *TARC/CCL17* that are frequent in the western European population were selected from the HapMap (http://hapmap.ncbi.nlm.nih.gov/) and the Ensemble databases[23]. We also analyzed the configuration of rs229828, which configuration has previously been associated with CCCL17/TARC serum levels[20]. Samples were genotyped using the Affymetrix "TxArray"[24,25] containing 767,203 variants, and stringent quality control (QC) was conducted to remove low-quality SNPs and samples. Samples with a missing rate >3% were removed. We created a subset of high-quality, independent SNPs with missing rate <1%, Hardy-Weinberg P>0.001, minor allele frequency >0.1 and LD pruning leaving no SNP-pairs with r^2>0.2. Using this subset, we removed samples with heterozygosity >2SD from the mean of all samples, related samples (keeping only one samples of each pair with proportion of IBD>0.2), and samples of non-European ancestry (based on principle component analysis using the 1000 Genomes Project (Phase 1) populations as reference[26]. SNPs were removed if they had a missing rate >5%, Hardy-Weinberg P<0.01, or if they were monomorphic. After QC, 543,637 SNPs and 133 patients and 131 donor samples remained. Untyped SNPs were imputed using a combined reference panel of the 1000 Genomes Project (Phase 3)[27] and the Genomes of the Netherlands (v5)[28]. Samples were first phased with SHAPEIT[29] and then imputed with IMPUTE v2[30].

Measurement of serum TARC/CCL17 concentrations

Serum concentrations of TARC/CCL17 were determined via a solid-phase ELISA kit (R&D systems, Minneapolis, MN) according to protocol. Briefly, wells were first incubated with serum samples for 2 hours, then with conjugate for one hour, and finally with substrate. From OD450 values levels were calculated by reference to a standard curve. Serum samples were briefly centrifuged prior to analysis. All samples where measured in duplicate. Inter- and intra-assay variabilities of the assay were 8.3% and 4.4%, respectively.

Statistics

All statistical analyses were performed using GraphPad Prism version 6.02 (GraphPad Software Inc., San Diego, CA) and SPSS version 21 (IBM Corp., Armonk, NY). Data were tested for Gaussian distribution via the D'Agostino & Pearson omnibus normality test. Normally distributed data is represented as mean value ± SEM whereas data not following a Gaussian distribution is represented as median ± interquartile range. Depending on the distribution of the data, differences between groups were analyzed with the unpaired *t* test or the Mann-Whitney test, indicated in the respective figure legend. Differences in categorical data were analyzed using the Fischer's exact test and in continuous variables via ANOVA. Survival analyses were conducted using Kaplan-Meier analysis with both

BOS incidence as overall survival as endpoint parameters. A p-value <0.05 was considered to be statistical significant.

Results

Patient demographics

From the total cohort of 144 patients transplanted in our center, 65 were treated with LTx because of chronic obstructive pulmonary disease (COPD), 42 because of cystic fibrosis (CF), 36 because of interstitial lung disease and one patient was diagnosed with pulmonary vascular disease prior to transplantation. Besides the fact that BOS+ patients were slightly older at the time of transplantation, no significant demographic and clinical differences were observed between BOS+ and BOS- groups (Table 1). During transplantation follow-up, 44 patients developed BOS. No RAS was observed. In total, 44 patients deceased during the study period, whereas 20 patients presented with one or more AR episodes.

TARC/CCL17 promotor polymorphisms

All extracted DNA samples from patient/donor couples were analyzed on the Affymetrix-based TxArray and selected SNPs were imputed as described in the patients and methods section. After stringent pre- and post-imputation quality control, including deviation from Hardy-Weinberg equilibrium, sample and SNP missingness, heterozygosity checks, and principle component analyses (data not shown)[31], 133 patients and 131 donor could be genotyped for the selected *TARC/CCL17* promotor SNPs (92.4% and 91.7% of the total cohort, respectively). From all samples identified SNP genotypes were stratified per haplotype. Table 2 describes these results as well as haplotype and genotype frequencies of the individual SNPs. We observed no significant differences in either SNP or haplotype distribution between patients and donors. Also, the genotype frequencies of the selected *TARC/CCL17* SNPs were in concordance with frequencies found in the HapMap and the Ensemble databases.

rs223899 influences serum TARC/CCL17 concentrations post-transplantation

From a subset of 67 representative patients, serum samples were obtained during the first month after transplantation and analyzed for TARC/CCL17 levels. The relation between identified both donor and patient haplotypes and SNP genotypes and serum TARC/CCL17 levels was then analyzed. The strongest association was observed between

	All	Non - BOS	BOS	*p*-value
Patients				
Total number	144	100	44	
Gender				
Male	69	52	21	0.560
Female	75	48	23	
Mean age (years)	46 ± 13	44 ± 14	50 ± 11	*0.026*
Mean follow-up (months)	61.2 ± 36.8	59.2 ± 39.4	65.6 ± 30.2	0.341
Primary disease				
CF	65	40	25	0.247
COPD	42	33	9	
ILD	36	26	10	
PVD	1	1	0	
Infection				
EBV high risk	14	7	7	0.115
CMV high risk	32	21	11	0.456
Type of graft				
Bilateral	112	81	31	0.119
Single	32	19	13	
Episode of acute rejection	20	14	6	0.495
Ischemic times (min)				
Bilateral	312.3 ± 188.9	321.4 ± 216.9	288.6 ± 73.8	0.426
Single	244.1 ± 53.5	238.2 ± 48.8	238.7 ± 73.0	0.314
Donors				
Gender				
Male	65	46	19	0.449
Female	79	54	25	
Donor age (years)				
Mean age	45 ± 14	44 ± 15	47 ± 14	0.184
> 60	17	12	5	0.579
Donor type				
HB	116	20	8	0.497
non HB	28	80	36	
Smoking				
Yes	52	35	17	0.407
No	92	65	27	

Table 1: Clinical and demographic parameters of lung transplant patients and donors

Cohort overview of both patients and donors subdivided for the incidence of BOS after LTx. BOS: bronchiolitis obliterans syndrome, CF: cystic fibrosis, COPD: chronic obstructive pulmonary disease, ILD: interstitial lung disease, PVD: pulmonary vascular disease, EBV: Epstein-Barr virus, CMV: cytomegalovirus, HB: heart beating.

Haplotypes							Patients		Donors	
rs223895	rs223897	rs223898	rs223899	rs223900	rs223827	rs223828				
Y	Y	K	K	Y	Y	Y	*n*	%	*n*	%
C	C	T	G	C	T	C	42	0.316	41	0.318
Y	Y	K	K	Y	Y	C	26	0.195	32	0.242
Y	C	T	G	C	Y	C	17	0.128	15	0.114
C	C	T	G	C	Y	C	12	0.090	13	0.098
Y	Y	K	K	Y	Y	Y	8	0.060	7	0.053
T	Y	K	K	Y	C	C	7	0.053	9	0.068
Y	Y	K	K	Y	C	C	4	0.030	3	0.023
T	T	G	T	T	C	Y	3	0.023	4	0.030
Y	Y	K	G	Y	Y	C	3	0.023	2	0.015
T	T	G	T	T	C	C	2	0.015	1	0.008
T	T	G	K	T	C	C	2	0.015	n.o	n.o
T	T	G	T	T	C	T	2	0.015	n.o	n.o
T	Y	K	G	Y	C	C	1	0.008	n.o	n.o
T	Y	K	K	Y	C	Y	1	0.008	n.o	n.o
T	C	T	G	C	C	C	1	0.008	n.o	n.o
Y	Y	K	G	Y	C	C	1	0.008	n.o	n.o
Y	Y	K	K	Y	C	Y	1	0.008	2	0.015
C	C	T	G	C	C	C	n.o	n.o	1	0.008
T	Y	K	K	Y	Y	C	n.o	n.o	1	0.008
C: 0.413	C: 0.538	G: 0.053	G: 0.564	C: 0.538	C: 0.170	C: 0.892	133		131	
T: 0.129	T: 0.053	T: 0.538	T: 0.045	T: 0.053	T: 0.314	T: 0.008				
Y: 0.458	Y: 0.406	K: 0.409	K: 0.391	Y: 0.409	Y: 0.515	Y: 0.10				

Table 2: Genetic configuration of selected *TARC/CCL17* promotor polymorphisms

Overview of incidence and frequency of SNP configurations (vertically) and observed haplotypes (horizontally) for both patients and donors. SNPs are displayed according to their relative distance from the transcription initiation site. All observed SNP frequencies were in concordance with the Ensemble database.

donor SNP rs223899 and serum levels. Serum TARC/CCL17 levels in patients with lungs from donors with the homozygous SNP configuration of rs223899, which leads to the replacement of a guanine by a thymine, tended to be higher than those in patients with a lung from a donor with heterozygous configuration (p=0.066, Figure 1A). Notably, serum TARC/CCL17 levels before transplantation were not different in these patient groups (n=38, p=0.776, Figure 1B).

Donor SNP rs223899 influences the clinical outcome after LTx

Since we observed that patient serum TARC/CCL17 levels correlate with the configuration of donor SNP rs223899, and decreased serum levels of TARC/CCL17 predict a higher risk for BOS development after transplantation (16), we analyzed the genotyped donor haplotypes and individual donor SNP configurations in a Kaplan-Meier survival analysis. For overall survival, all 131 patients for which the imputed donor SNP passed QC, were included. For the analyses of BOS development, we excluded patients who had deceased within the first 4 months after transplantation or from whom SNP analysis did not pass

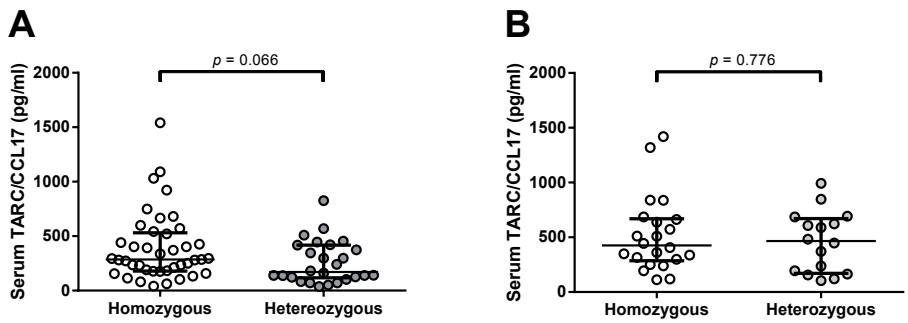

Figure 1: Donor rs223899 correlates with serum TARC/CCL17 concentrations post-LTx but not pre-LTx

A Serum TARC/CCL17 levels were measured in LTx patients one month after LTx stratified for the configuration of donor SNP rs223899 (homozygous vs. heterozygous). A trend towards significance (p=0.066) was observed with increased TARC/CCL17 serum concentrations in patients who received a graft genotyped homozygous for SNP position rs223899. Non-Gaussian distribution, Mann-Whitney test, homozygous n=41, heterozygous n=26. **B** Serum TARC/CCL17 levels were also assessed pre-LTx in patients based upon serum availability. No differences in serum TARC/CCL17 could be observed when patients were stratified for the respective rs223899 SNP genotype. Non-Gaussian distribution, Mann-Whitney test, homozygous n=22, heterozygous n=16.

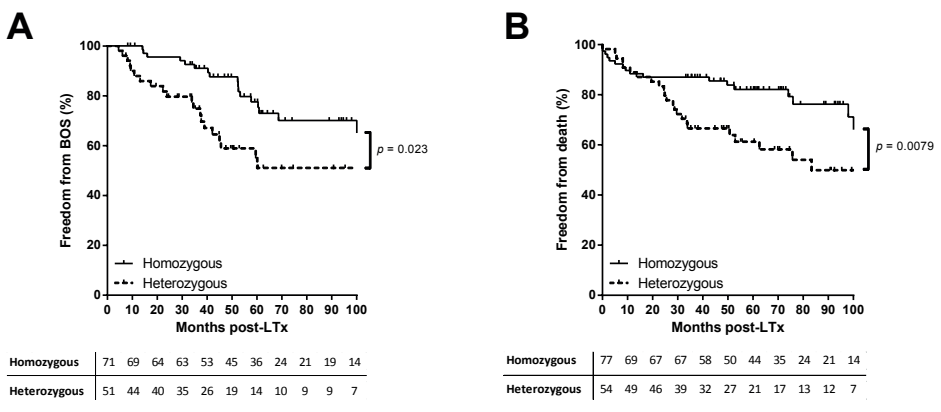

Figure 2: Donor rs22399 affects clinical outcome after LTx

A Kaplan-Meier analysis on BOS incidence after LTx. Patients were stratified according to the SNP configuration of rs223899 in the received allograft. Patients who received a graft genotyped as heterozygous for this specific SNP have a lower BOS-free survival rate measured over the first 100 months after transplantation (p=0.023). **B** Kaplan-Meier analysis on survival after LTx. Patients were stratified as mentioned previously. Additional to an increase of chronic rejection after LTx, stratification of LTx patients for receiving a grafted organ genotyped heterozygous at SNP position rs223899 resulted in a lower survival rate post-LTx (p=0.0079). Log-rank test used in both analyses.

quality control, resulting in the inclusion of 122 patients.

In total 6 different donor haplotypes had a frequency above 5% and were analyzed for correlation to outcome after LTx. None of the donor of patient haplotypes of the 7 selected *TARC/CCL17* SNPs showed a correlation with either AR episodes, BOS incidence, or survival after LTx (data not shown). In contrast we observed a significant difference in the development of chronic rejection when patients were stratified by donor SNP rs223899 genotype. Of the patients that received a transplant from a donor heterozygous at position rs223899, 50% remained free from BOS within the first 100 months after transplantation. This percentage was significantly higher, 75%, in the patients that had received a graft from a homozygous donor (p=0.023, Figure 2A). Furthermore, this nucleotide substitution in the promotor region of *TARC/CCL17* in the donor correlated with a lower survival rate of recipients post transplantation (50% vs. 80% respectively, p=0.0079, Figure 2B). Moreover, none of the other individual donor SNP correlated with BOS development or survival after LTx.

Discussion

In this study, we analyzed the relation of both donor as well as patient *TARC/CCL17* genotypes to clinical parameters, but only found one single donor SNP to be correlated with BOS development. Interestingly, this specific promotor SNP configuration, heterozygosity for rs223899, also correlated with lower serum TARC/CCL17 levels, which is in concordance with previous observations that low serum TARC/CCL17 levels in the first month after transplantation are predictive for BOS development[16]. We have also assessed patient-donor combinations stratified by rs223899 genotype. We observed higher concentrations of serum TARC/CCL17 in the first month after LTx when patients heterozygous for SNP rs223899 were transplanted with a homozygous donor. This was not observed in the other 3 combinations (patient/donor homozygous, patient/donor heterozygous, and patient homozygous/donor heterozygous).

We only analyzed SNPs that are frequent in the European population. Therefore, validation in an external cohort, and re-analysis of our findings in populations with other genetic backgrounds is expedient.

Most studies on the role of genetics in lung transplant complications have focused on the obstructive form of CLAD, BOS[32]. These results are mainly obtained using patient DNA, illustrated by studies of Awad *et al.* concerning SNPs in IFN-γ and TGF-β1, in which the authors correlate gene polymorphisms with increased allograft fibrosis[33,34]. Also,

an association between an IL-6 polymorphism and BOS development was observed[35]. However, these findings could not be validated in independent cohorts[36]. Recently, our group has shown that a SNP in the promotor region of complement regulatory protein CD59 in the donor correlates with a higher risk for chronic rejection after lung transplantation[37]. Furthermore, a specific donor MBL promotor haplotype has been associated with graft survival and BOS development after transplantation[38]. Taken together, these data stress the potential importance of both patient and donor SNPs on the clinical outcome after lung transplantation.

The correlation between rs223828, another *TARC/CCL17* promotor polymorphism, and protein serum levels has been described previously in a cohort of Japanese patients[10]. This polymorphism was also found to be associated with atopy and asthma in children, as well as with higher circulating levels of TARC/CCL17[21]. We could not confirm the correlation of this SNP with serum TARC/CCL17 levels in our cohort of western European LTx patients, presumably due to low minor allele frequency in our patient cohort. Observations in patients suffering from Kawasaki disease have shown that rs223899 is associated with disease progression. Furthermore, these patients presented increased serum levels of TARC/CCL17 compared to healthy controls. However, individual SNPs, including rs223899, did not correlate with serum levels when stratified by genotype[22].

The genetic regulation of the *TARC/CCL17* gene has partly been elucidated. Two STAT6 binding sites have been identified at position -213/-223 and -177/-187 relatively to ATG upstream of Exon 1. Furthermore, a binding-motif for NFκB is present upstream of the two STAT6 binding sites[18]. Interestingly, using a RSV-inducible mice epithelial cell model, Monick *et al*., have shown that optimal TARC expression is achieved via the combined activation of both transcription factors, which would involve the recruitment of CREB-binding protein/p300 via NFκB and is essential for STAT mediated transcription[19]. The identified SNP rs223899 lies within consensus binding sequence for NFκB[39]. Thus, the heterozygous configuration of rs223899 could result in a less optimal NFκB binding, which would lead to a reduced expression of TARC/CCL17.

The role of serum levels of TARC/CCL17 in LTx outcome remains speculative. Bronchial epithelial cells have the potency to secrete large amounts of TARC/CCL17 when activated[7] and considering the small size of TARC/CCL17 (10.5 kD), it seems logical to assume leakage from the allograft into the circulation which can subsequently be quantified in serum. Immunoregulatory functions have been attributed to TARC/CCL17 due to the presence of its receptor, CCR4, on a specific subset of regulatory T cells[40]. A plausible explanation could be that increased TARC/CCL17 secretion hints towards a more

Th2 driven immune response, needed to balance the deleterious Th1 response, often associated with transplant rejection[41,42]. Thus, increased amounts of TARC/CCL17 shortly after transplantation could result in dampening the inflammatory response against the allograft, which apparently seems beneficial for long-term outcome.

In summary, our data indicate that heterozygosity for a single SNP in the promotor region of *TARC/CCL17* located within the consensus sequence of the binding site of transcription factor NFκB correlates with serum levels of the TARC/CCL17 protein. Low serum TARC/CCL17 levels are predictive for BOS development following LTx. In line with these observations, we show that patients who receive a heterozygous allograft for SNP rs223899 present with a higher BOS incidence and impaired survival after LTx.

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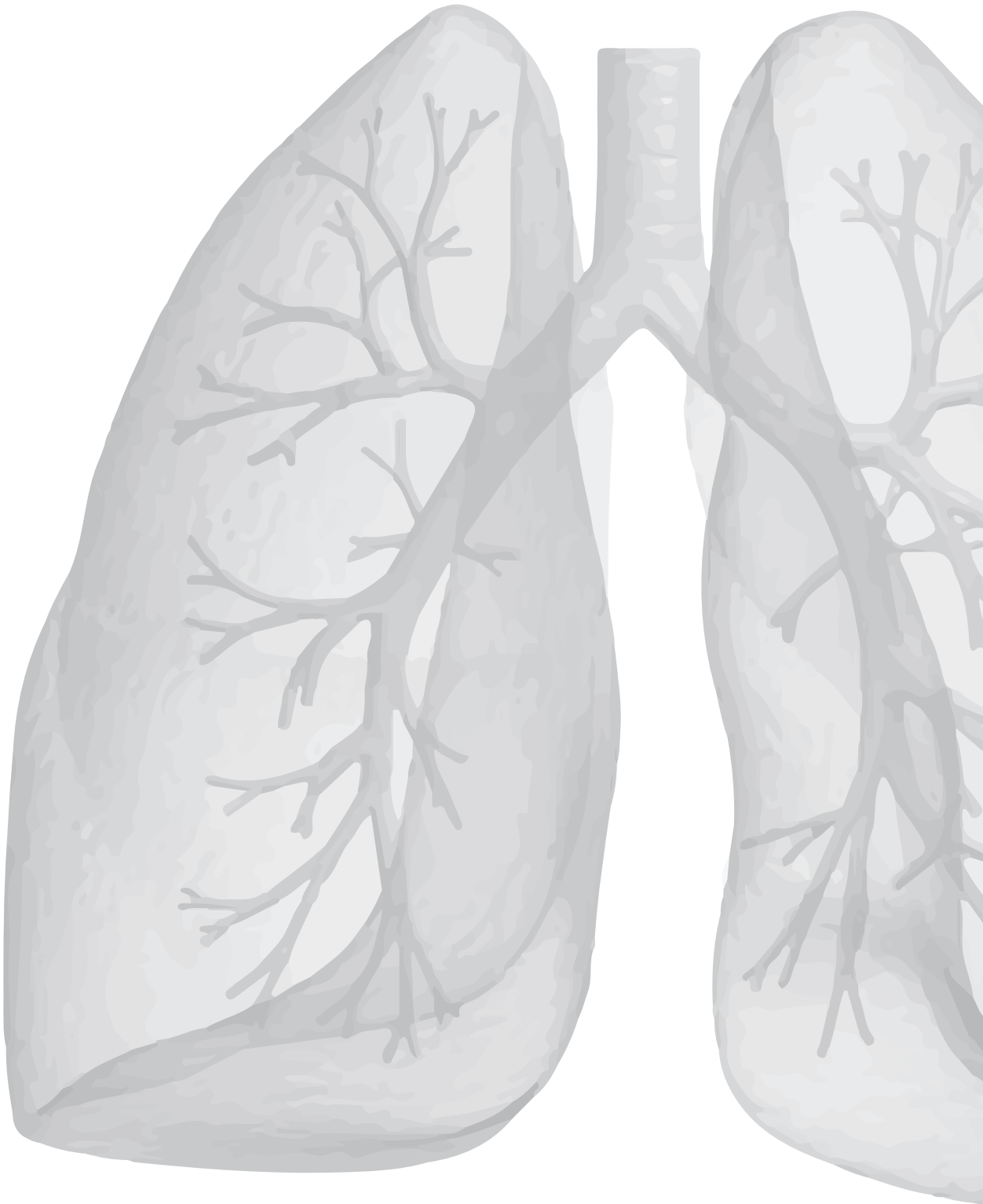

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The autoimmune-associated single nucleotide polymorphism within *PTPN22* correlates with clinical outcome after lung transplantation

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Abstract

Obstructive chronic lung allograft dysfunction (BOS) is the major limiting factor for lung transplantation (LTx) outcome. Both T cell an B cell mediated autoimmunity contribute to the development of autoantibodies associated with the development of chronic rejection. *PTPN22* is described as the hallmark autoimmunity gene, and one specific single nucleotide polymorphism (SNP), rs2476601, is associated with multiple autoimmune diseases, impaired T cell regulation and autoantibody formation. Taking into consideration the contribution of autoimmunity to LTx outcome, we hypothesized that polymorphisms in the *PTPN22* gene could be correlated to BOS incidence. Therefore, we identified six selected SNPs within *PTPN22* and analyzed both patient and donor genotypes on BOS development post-LTx. A total of 144 patients and matched donors were included, and individual SNPs and haplotype configurations were analyzed. We found a significant association between patients carrying the heterozygous configuration of rs2476601 and a higher risk for BOS development (p=0.005, OR: 4.400, 95%CI: 1.563–12.390). This was confirmed via Kaplan-Meier analysis which showed that heterozygous patients exhibit a lower BOS-free survival compared to patients homozygous for rs2476601 (p=0.0047). Furthermore, one haplotype, which solely contained the heterozygous risk variant, was associated with BOS development (p=0.015, OR: 7.029, 95%CI: 1.352–36.543). Our results show that LTx patients that are heterozygous for SNP rs2476601 are more susceptible for BOS development and indicate a deleterious effect of the autoimmune-related risk factor of *PTPN22* in patients on LTx outcome.

Introduction

Lung transplantation (LTx) is the last line of treatment for patients suffering from end-stage lung diseases including cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), and interstitial lung disease (ILD). LTx is the least successful solid organ transplantation due to the high incidence of chronic lung allograft dysfunction (CLAD). CLAD is clinically defined as obstructive (bronchiolitis obliterans syndrome, BOS), the most observed form of CLAD, or restrictive (restrictive allograft syndrome, RAS)[1]. Latest numbers show that 5 year BOS-free survival is 50%, whereas only 25% of the transplanted patients remain free from BOS within the first 10 years after LTx[2].

BOS is a heterogeneous complication in which multiple parts of the immune system appear to play a role. Evidence exist for the involvement of humoral immunity, including complement and antibody formation, and features of autoimmunity[3]. Also, cellular mediated processes, in which T cell reactivity seems to play a pivotal role, have been observed in patients diagnosed with BOS[4]. A remarkable example of antibody formation is the association between autoantibodies directed against K-alpha 1 tubulin and collagen V and the development of BOS, also in the absence of donor specific anti-HLA antibodies[5]. Furthermore, auto reactive CD4+ T cells directed against collagen V are observed in LTx patients, indicating that both B cell and T cell mediated autoimmunity plays a pivotal role in BOS development[6].

Upon T cell receptor-ligand interaction, T cell reactivity is controlled, among others, by intracellular proteases and phosphatases. One important protein tyrosine phosphatase (PTP) involved in T cell regulation is encoded by the *PTPN22* gene, which is located on chromosome 1. The *PTPN22* gene is considered to be one of the most important autoimmunity risk genes. Within the *PTPN22* gene different single nucleotide polymorphisms (SNPs) are present, of which rs2476601 is the most striking clinically relevant example. Autoimmunity-related associations are, among others, rheumatoid arthritis (RA)[7], systemic lupus erythematosus (SLE)[8], vitiligo[9], and progressive systemic sclerosis[10,11]. The SNP rs2476601 is a missense mutation leading to an arginine tryptophan substitution within the first proline-rich sequence of the C-terminal domain, resulting in a disrupted interaction with CSK and enhanced enzyme activity[12]. Currently, different functional models for the PTPN22–R620W substitution are described in literature[13].

Besides investigations on associations in autoimmunity and functional implications on T cell receptor signaling, *PTPN22* SNPs are also studied in the field of transplantation. In kidney transplantation, *PTPN22* gene polymorphisms, were studied in two

independent cohorts, but no associations with kidney function could be observed after transplantation[14,15]. Interestingly, Dullin *et al.* investigated selected SNPs within the *PTPN22* gene and observed that rs2476601 is associated with multiple episodes of acute rejection after liver transplantation[16].

Taking into consideration the results obtained in liver transplantation, the associations of PTPN22 with autoimmunity and the observed features of autoimmunity in LTx, we hypothesized that SNPs in the *PTPN22* gene could be associated to outcomes after LTx. For this end, we selected 6 SNPs within the *PTPN22* gene and used both genotype and haplotype analysis on patient and donor samples and assessed correlations with transplantation outcome.

Patients and Methods

Patients

For this study, we included 144 patients and their respective donors that were treated with LTx between January 2004 and March 2013. From al study participants informed consent was obtained and this study was approved by the medical ethical committee of our center. All patients received standardized immunosuppressive therapy consisting of tacrolimus, prednisolone, and mofetil mycophenolate. Furthermore, patients received treatment with valganciclovir for up to 6 months when categorized as at high risk for cytomegalovirus (CMV) or Epstein-Barr virus (EBV), defined as a CMV- or EBV-negative patient receiving a graft from a CMV- or EBV-positive donor. BOS was diagnosed according to international guidelines as a decline of the forced expiratory volume in 1 second in absence of any other cause of disease of 20% compared to baseline level[17]. Samples of donor spleen, donor blood and patient blood was collected prior to or during transplantation procedure. From each respective sample, mononuclear cells were isolated using Ficoll-Paque gradient centrifugation (20 min, 2400 RPM, break/acceleration 2) and stored in liquid nitrogen until further analysis.

DNA isolation, genotyping, and SNP selection

The MagnaPure Compact System (Roche Diagnostics, Basel, Switzerland) was used for DNA isolation from frozen mononuclear cell samples, according to manufacturer's instructions. In addition, cell samples were thawed at 37°C and dissolved in RPMI-1640 (Lonza, Basel, Switzerland) 20% fetal bovine serum (Bodinco, Alkmaar, The Netherlands), followed by 10 min centrifuging at 1800 RPM. The obtained cell pellet was dissolved at a

concentration of 5 x 10^6 cells/ml in phosphate buffered saline, and used for DNA isolation. Samples were genotyped within the *i*GeneTRA*i*N network using the specifically designed and developed Affymetrix "TxArray", which contains 767,203 variants[18,19]. Subsequently, samples were subjected to stringent quality control (QC) in order to remove both low-quality genotyped SNPs and samples. We removed samples with a missing rate >3%. Subsequently, we generated a subset of high-quality independent SNPs according to the following conditions: missing rate <1%, Hardy-Weinberg P>0.001, minor allele frequency >0.1 and LD pruning leaving no SNP-pairs with r^2>0.2. Subsequently, samples were removed with heterozygosity >2SD from the mean of all samples, related samples (keeping only one samples of each pair with proportion of IBD>0.2), and samples of non-European ancestry (based on principle component analysis using the 1000 Genomes Project (Phase 1) populations as reference[20]. SNPs were removed if they presented a missing rate >5%, Hardy-Weinberg P<0.01, or when they were monomorphic. After QC, 543,637 SNPs, 132 patients and 131 donor samples remained. We used the method of imputation[21,22] for SNP genotyping with the 1000 Genomes Project (v3)[23] and the Genomes of the Netherlands (v5)[24] as reference panels. Samples were phased with SHAPEIT[25] and imputed with IMPUTE v2[26].

Six SNPs were selected from the *PTPN22* gene that were frequent in the Western European population. Furthermore, these SNPs were selected according to published literature on *PTPN22*-related transplantation research[14-16], and their associations with other disease phenotypes. SNP rs2488457 lies in the promotor region of *PTPN22* within the binding site for the transcription factor activator protein 4, and is associated with protein expression levels[27]. SNP rs33996649 , located in exon 10, is protective in SLE[28], whereas rs2476601, is one of the most important associated genetic polymorphism associated with autoimmunity[29]. Both rs1310182 and rs1217388 are located within intronic transcription factor binding sites, and rs3789604 is a downstream variant, which is located in a transcription factor binding site implicated in protein expression[30].

Statistics

We used SPSS version 21 (IBM Corp., Armonk, NY), GraphPad Prism version 6.02 (GraphPad Software Inc., San Diego, CA) and R version 3.0.3 (The R Foundation for Statistical Computing, Vienna, Austria) for statistical analyses. Categorical data were analyzed via the Fischer's exact test, whereas differences in continuous variables were assessed via ANOVA. Odds ratios (OR) and 95% confidence intervals (CI) were generated via logistic regression and used to estimate strengths of associations. Kaplan-Meier analysis was used for survival analyses and differences were analyzed via log-rank test. A

p-value <0.05 was considered to be statistically significant.

Results

Patient and donor demographics

A total of 144 LTx patient and donor couples were included in this study, based upon material availability. Sixty-five of these patients were transplanted because of chronic obstructive pulmonary disease, 42 because of cystic fibrosis, 36 due to interstitial lung disease and one patient was diagnosed with pulmonary vascular disease as primary lung disease. Forty-four patients developed BOS during follow-up and RAS was not observed. Patient and donor clinical and demographical parameters are depicted in Table 1. No significant differences were observed between BOS+ and BOS- patients or respective donors, except for the mean age at the time of transplantation, which was slightly higher in

	All	No BOS	BOS	*p*
Patients				
Total number	144	100	44	
Gender				
Male	69	52	21	0.560
Female	75	48	23	
Mean age (years)	46 ± 13	44 ± 14	50 ± 11	*0.026*
Mean follow-up (months)	61.2 ± 36.8	59.2 ± 39.4	65.6 ± 30.2	0.341
Primary disease				
COPD	65	40	25	0.247
CF	42	33	9	
ILD	36	26	10	
PVD	1	1	0	
Infection				
EBV high risk	14	7	7	0.115
CMV high risk	32	21	11	0.456
Type of graft				
Bilateral	112	81	31	0.119
Single	32	19	13	
Episode of acute rejection	20	14	6	0.495
Ischemic times (min)				
Bilateral	312.3 ± 188.9	321.4 ± 216.9	288.6 ± 73.8	0.426
Single	244.1 ± 53.5	238.2 ± 48.8	238.7 ± 73.0	0.314
Donors				
Gender				
Male	65	46	19	0.449
Female	79	54	25	
Donor age (years)				
Mean age	45 ± 14	44 ± 15	47 ± 14	0.184
> 60	17	12	5	0.579
Smoking				
Yes	52	35	17	0.407
No	92	65	27	
Donor type				
HB	116	20	8	0.497
non HB	28	80	36	

Table 1: Clinical and demographic parameters of lung transplant patients and donors

Cohort overview of both patients and donors subdivided for the incidence of BOS after LTx. BOS: bronchiolitis obliterans syndrome, CF: cystic fibrosis, COPD: chronic obstructive pulmonary disease, ILD: interstitial lung disease, PVD: pulmonary vascular disease, EBV: Epstein-Barr virus, CMV: cytomegalovirus, HB: heart beating.

SNP		Patient No BOS n = 91	Patient BOS n = 41	p	OR		Donor No BOS n = 92	Donor BOS n = 39	p
rs2488457	GG	5 (5%)	5 (12%)	NS		GG	6 (7%)	2 (5%)	NS
	GC	26 (29%)	12 (29%)			GC	32 (35%)	11 (28%)	
	CC	60 (66%)	24 (59%)			CC	54 (59%)	26 (67%)	
rs33996649	CC	88 (97%)	40 (98%)	NS		CC	85 (92%)	36 (92%)	NS
	CT	3 (3%)	1 (2%)			CT	7 (8%)	3 (8%)	
	TT	0 (0%)	0 (0%)			TT	0 (0%)	0 (0%)	
rs2476601	GG	82 (90%)	30 (73%)	0.005	4.400 (1.563 - 12.390)	GG	74 (80%)	31 (79%)	NS
	GA	7 (8%)	11 (27%)			GA	15 (16%)	7 (18%)	
	AA	2 (2%)	0 (0%)			AA	3 (3%)	1 (3%)	
rs1310182	AA	20 (21%)	9 (22%)	NS		AA	19 (21%)	3 (8%)	NS
	AG	44 (48%)	19 (46%)			AG	43 (47%)	21 (54%)	
	GG	27 (30%)	13 (32%)			GG	30 (33%)	15 (38%)	
rs1217388	GG	6 (7%)	0 (0%)	NS		GG	8 (9%)	2 (5%)	NS
	GA	27 (30%)	32 (78%)			GA	34 (37%)	13 (33%)	
	AA	58 (64%)	9 (22%)			AA	50 (54%)	24 (62%)	
rs3789604	TT	52 (57%)	28 (68%)	NS		TT	62 (67%)	30 (77%)	NS
	TG	32 (35%)	13 (32%)			TG	28 (30%)	8 (21%)	
	GG	7 (8%)	0 (0%)			GG	2 (2%)	1 (3%)	

Table 2: Genotype frequency per PTPN22 SNP for both lung transplantation recipients and donors.

BOS: bronchiolitis obliterans syndrome, OR: odds ratio + 95% confidence interval, obtained via logistic regression analysis, NS: not significant. Percentages approximated.

the BOS+ patient group. During follow-up, 44 patients deceased and a total of 20 patients presented with either one or more episodes of acute rejection.

PTPN22 single nucleotide polymorphism distribution

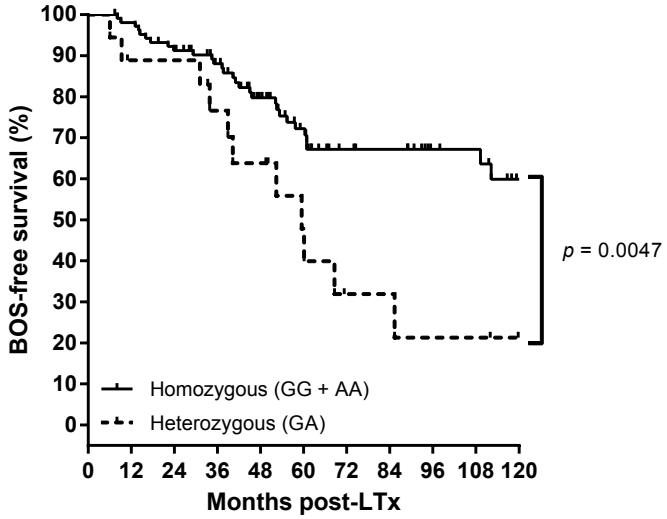
The selected *PTPN22* SNPs, see Patients and Methods, were genotyped via imputation and both SNPs and samples were subjected to stringent QC steps, including deviation from Hardy-Weinberg equilibrium, sample and SNP missingness, heterozygosity checks, and principle component analyses (data not shown)[31]. A total of 132 patients, consisting of 41 patients who did develop BOS and 91 patients who did not, passed the sample quality control steps. For the donors these numbers were slightly different; 131 samples passed quality control check and 39 coupled patients developed BOS during follow-up and 92 did not. Total genotyping results and SNP frequencies are depicted in Table 2 for both patients and donors, stratified per diagnosis of chronic rejection. Neither rs2488457, rs33996649, rs1310182, rs1217388 nor rs3789604 was significantly associated with chronic rejection in either patients or donors. However we did observe an association between the SNP configuration of rs2476601 in patients, but not in donors, and the incidence of BOS during follow-up after LTx. The frequency distribution of the heterozygous variant (GA) was increased in BOS+ compared to BOS- patients (27% vs. 8% respectively). This association was significant and patients carrying this heterozygous genotype present with an increased risk for BOS development (p=0.005, OR: 4.400, 95% CI: 1.563–12.390). No association between either genotype in patients or donors was observed with episodes of acute rejection.

Heterozygosity for rs2476601 is associated with lower BOS-free survival post-LTx

The primary observation that LTx patients genotyped heterozygous for SNP rs2476601 are at higher risk for BOS development post-LTx was confirmed via Kaplan-Meier and subsequent log-rank analysis. After the exclusion of patients who deceased within the first six months post-LTx, 125 patients were included. Kaplan-Meier analysis showed that patients heterozygous for rs2476601 have a significant lower BOS-free survival rate compared to homozygous patients (Figure 1, p=0.0047).

Haplotype analyses on *PTPN22* SNPs

Stratification of the identified configurations of the selected *PTPN22* SNPs resulted in the generation of 19 different haplotypes (frequencies from 0.8% till 29%) in the patient group



Homozygous (GG + AA)	107	101	92	79	60	45	31	27	20	19	13
Heterozygous (GA)	18	15	15	12	10	6	3	3	2	2	0

Figure 1: Survival analysis on BOS incidence in LTx patients stratified per rs2476601 genotype

A total of 125 patients were stratified according to rs2476601 genotype, either as heterozygous (GA) or homozygous (GG and AA). Patients who deceased within the first 6 months after transplantation were excluded from analysis. Patients genotyped as heterozygous (dashed line) present a lower BOS-free survival rate compared to homozygous patients (solid line), p=0.0047, log-rank test.

	Haplotype	1	2	3	4	5
	rs2488457	C	C	S	S	C
	rs33996649	C	C	C	C	C
	rs2476601	G	G	G	R	G
	rs1310182	G	R	R	R	A
	rs1217388	A	A	R	R	A
	rs3789604	T	K	T	T	G
Patient	**Frequency**	38 (29%)	31 (23%)	17 (13%)	8 (6%)	7 (5%)
	No BOS	26 (29%)	22 (24%)	14 (15%)	2 (2%)	7 (8%)
	BOS	12 (29%)	9 (22%)	3 (7%)	6 (15%)	0 (0%)
	p	NS	NS	NS	0.015	NS
Donor	**Frequency**	39 (30%)	24 (18%)	15 (12%)	15 (12%)	
	No BOS	26 (28%)	16 (17%)	11 (12%)	9 (10%)	
	BOS	13 (33%)	8 (21%)	4 (10%)	6 (15%)	
	p	NS	NS	NS	NS	

Table 3: Patient and donor haplotype analysis on BOS incidence after LTx

BOS: bronchiolitis obliterans syndrome, NS: not significant. Percentages approximated.

and 17 different haplotypes (frequencies from 0.8% till 30%) in the donor group (Table 3). Five patient and 4 donor haplotypes were included , which occurred more than 5% in our cohort. The high-risk heterozygous configuration of rs2476601 was present in only one haplotype which was also the only one associated with BOS (p=0.015, OR: 7.029, 95% CI: 1.352–36.543).

Discussion

The *PTPN22* gene is considered to be the most important non-HLA autoimmunity gene, of which the genetic variant rs2476601, 1858 C>T leading to an arginine–tryptophan substitution, is associated with multiple indications of autoimmunity, including RA, SLE, and systemic sclerosis. Given the reported associations between autoimmunity and outcome after LTx we assessed the relation of the *PTPN22* risk variant in both patients and donors and the incidence of chronic rejection after LTx.

Features of autoimmunity are often observed in or associated with chronic rejection after LTx[32]. Type V collagen and K-α 1 tubulin are antigens for autoantibodies, presumably due to tissue remodeling and the activity of matrix metalloproteases that are induced by ischemia reperfusion injury and cleave collagen, thereby releasing antigenic fragments. Binding of autoantibodies to their respective targets on airway epithelial cells results in increases of profibrotic growth factors and proinflammatory cytokines[5,33]. Linked-recognition requires antigen recognition by T cells, and indeed auto reactive T cells directed at Type V collagen are present in LTx patients[6].

The non-receptor PTP encoded by *PTPN22* consists of different domains, including the N-terminal catalytic active domain involved in dephosphorylation and a C-terminal domain which consists of proline-rich regions that facilitate binding of intracellular adapter proteins[29]. PTPN22 is exclusively expressed by hematopoietic cells[34], and distinctive roles have been described in the regulation of immune cell signaling[13,35]. In T cell signaling, PTPN22 inhibits T cell activation via inhibiting downstream T-cell receptor signaling. In more detail, T cell activation after T cell receptor binding requires a cascade of tyrosine phosphorylation steps involving Src- (LCK, Fyn), and Syk family (ZAP-70) member kinases, that phosphorylate immunoreceptor tyrosine-based activation motifs located on the intracellular CD3-ζ chain[36]. These all function as substrates for PTPN22, resulting in the dephosphorylation of their respective activation, and inhibition of T cell signaling[37]. Furthermore, in T cells, the adapter protein binding domains on PTPN22 mediate the binding of tyrosine-protein kinase CSK, which also inhibits intracellular T cell receptor signaling[38,39].

PTPN22 rs2476601 is associated with the occurrence of autoantibodies in RA, including rheumatoid factor and anti-citrullinated peptide[7], presumably due to the contributive effect on the generation on autoreactive B cells. Dai *et al*. have demonstrated spontaneous autoimmunity with infiltrations in both lungs and livers in a C57BL/6x129 mouse model homologous to the human PTPN22 risk variant[40]. Also, deregulation in the deletion of clonal B cells and escape of autoreactive B cells from deletion stimuli have been observed in studies concerning the PTPN22 risk variant[41]. These observations suggest a contributing factor of rs2476601 in autoantibody production, associated with impaired clinical outcome after LTx.

T regulatory cells are known to induce tolerance to self-antigens and suppress Th-1 autoimmunity, which has also been shown in human LTx[6]. An altered T cell differentiation model is introduced as explanation for the pathogenesis towards autoimmunity. Disbalance between T cells and T regulatory cells is observed in *PTPN22* knockout mice, suggesting tight regulation between effector and regulatory T cells via PTPN22. This has been strengthened by research of Vang *et al.* showing that T cells from individuals homozygous for the *PTPN22* risk variant depicted increased Th-1 induced IFN-γ secretion and a decreased suppression of Th1 cells via regulatory T cells[42]. Translating these observations to the field of LTx suggests that a decreased suppression of Th1 immune responses in patients genotyped with the risk variant of *PTPN22* could contribute to autoimmunity and the pathogenesis of BOS development.

Here, we analyzed six selected *PTPN22* gene polymorphisms in a cohort of patients who underwent LTx. Also, coupled donors were analyzed for these specific SNP configurations. Our analysis shows that a single missense SNP in the *PTPN22* gene in patients is associated with outcome after LTx and the incidence of obstructive chronic lung allograft dysfunction. From our Kaplan-Meier analysis we can conclude that this association is predominantly with late chronic allograft dysfunction, indicated by the difference in inclination after month 30 post-LTx. Of the analyzed haplotypes only one haplotype showed a significant association with BOS incidence. Since the risk allele for BOS after LTx, heterozygosity at position rs2476601, was present only in this haplotype, the observation that this SNP configuration appears to be important in LTx outcome is further strengthened. Previously, our group identified autoantibodies in pre- and post-LTx sera of the investigated cohort[43,44]. However, no correlation was found between autoantibody titers or stratified rs2476601 SNP configuration (data not shown). Within our cohort, we observed differences between the age at the time of LTx procedure between BOS+ and BOS- patients. Patients developing BOS were significantly older compared to non-BOS patients. This differs from other studies, were the reverse was observed, explained by the fact that younger patients have a

more active immune system, although this remains speculative[45,46]. After Cox regression modelling in our cohort, including both the risk allele for *PTPN22* and age at time of LTx, SNP rs2476601 was still an independent risk factor for BOS development (p=0.004, OR: 2.748, 95% CI: 1.369–5.516, data not shown).

Targeted SNP analyses and genetic studies focused on solid organ transplantation and especially LTx outcome have increased over the past few years[47]. Our group previously reported a genetic promotor polymorphism in complement regulation to be associated with chronic rejection after LTx, with the rationale that after binding to the graft, antibodies are less functional[48]. Furthermore, the LTx research group from Leuven recently published findings on a polymorphism in the IL-17 receptor that predisposes to primary graft dysfunction[49], and correlations between SNP genotypes and immunosuppressive therapy efficacy have been described[50]. These results are encouraging and indicate the importance of this emerging field, despite the need of prospective multicenter and validation studies. The recently installed aforementioned *i*GeneTRA*i*N consortium could facilitate these needs[18,19].

Studies concerning the influence of rs2476601 on transplantation outcome have been conducted on other solid organ transplantations, albeit with different results. In liver transplantation, associations between rs2476601 and multiple episodes of acute rejection were observed, although chronic rejection associations were not assessed[16]. No associations with outcome after kidney transplantation and *PTPN22* gene polymorphisms were found by two independent groups[14,15]. Inter-organ differences could be explained by variations in either humoral or cellular immunologic mechanisms predisposing chronic rejection pathology, or differences in immunosuppressive regimens[51].

To summarize, our results are the first to show a deleterious effect of the autoimmune-related risk factor of *PTPN22* in patients on LTx outcome. These results could be used for the further optimization of risk stratification algorithms to identify LTx outcome prior to, or earlier after transplantation. More research is expedient to elucidate the specific pathogenic pathways affected by PTPN22 in the development of chronic rejection after LTx. Importantly, larger study cohorts are needed to replicate our findings and to incorporate other genetic polymorphisms, in both patients and donors, associated with the development of rejection after transplantation. Our results could serve to further optimize patient treatment and follow-up therapy, and increase the long-term efficacy of LTx treatment for selected end-stage lung disease patients.

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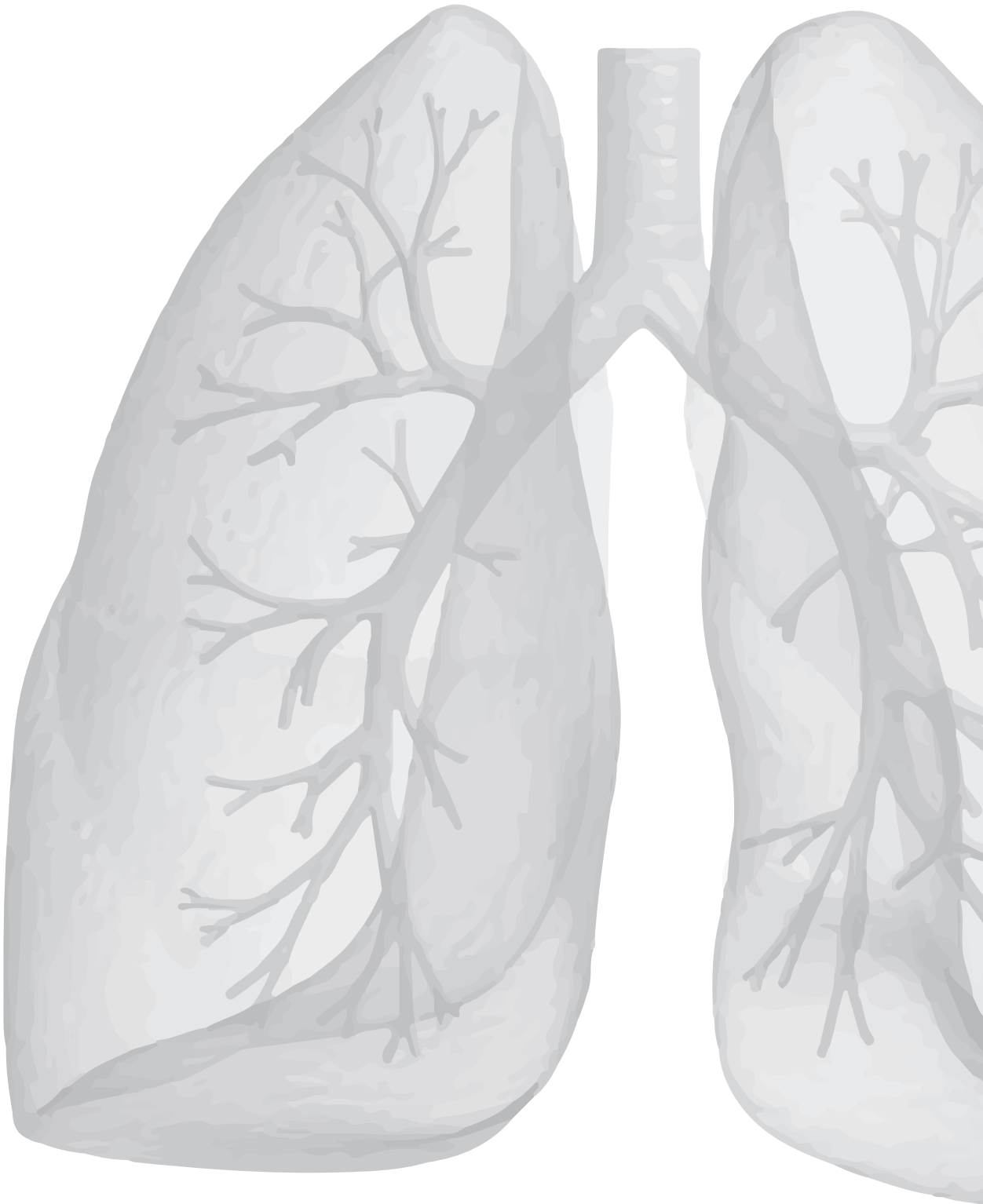

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Serum miRNAs as potential biomarkers for the bronchiolitis obliterans syndrome after lung transplantation

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Abstract

Lung transplantation (LTx) is the last treatment for patients suffering from end-stage lung diseases. Survival post-LTx is hampered by the development of the bronchiolitis obliterans syndrome (BOS) whose diagnosis is often late. Given the urgent clinical need to recognize BOS patients at an early stage, we analyzed circulating miRNAs to identify possible stratification markers for BOS development post-transplant. Therefore, selected pro-fibrotic (miR-21, miR-155), anti-fibrotic (miR-29a), and fibrosis-unrelated (miR-103, miR-191) miRNAs were analyzed in serum of end-stage lung disease patients and during LTx follow-up. Significant elevated levels of serum miRNAs were observed for all investigated miRNAs in both chronic obstructive pulmonary disease and interstitial lung disease patients compared to healthy controls. The same miRNAs were also significantly increased in serum of BOS+ vs. BOS- patients. Most importantly, miR-21, miR-29a, miR-103, and miR-191 levels were significantly higher in BOS+ patients prior to clinical BOS diagnosis. We demonstrated that a selected group of miRNAs investigated is elevated in end-stage lung disease and BOS+ patients, prior to clinical BOS diagnosis. Even if further research is expedient on the prognostic value of circulating miRNAs in BOS and lung conditions in general, these results strongly suggest that circulating miRNAs could be used as potential biomarkers for BOS development.

Introduction

Lung transplantation (LTx) is the last treatment option for patients suffering from end-stage lung diseases. Survival after LTx is severely hampered by the development of chronic lung allograft dysfunction, which can manifest in a restrictive form, restrictive allograft syndrome (RAS), or an obstructive form. The latter is defined as bronchiolitis obliterans (BO), and occurs in approximately 50% of LTx patients within 5 years after the transplantation[1]. BO is diagnosed via a surrogate marker, i.e. decline of the FEV_1 of 80% compared to baseline levels and is referred to as bronchiolitis obliterans syndrome (BOS). This diagnosis is often late and therefore there is urgent clinical need for novel biomarkers to identify patients at risk for BOS development at an earlier stage[2].

Micro-RNAs (miRNAs) are short non-coding RNAs, that inhibit gene expression at the post-transcriptional level by binding to the 3'UTR of target messenger-RNAs, thereby promoting their degradation or inhibiting translation[3]. Besides representing crucial endogenous regulators of gene expression within the cell, miRNAs can be found in biological fluids, including plasma, serum, breast milk etc., although their role in circulation is still largely unknown. The levels of circulating miRNAs were found to be either elevated or decreased in various transplantation settings, including kidney, heart, liver, and small intestine transplantation[4]. Interestingly, it is still unknown whether clinical parameters in LTx are also associated with levels of circulating miRNAs.

In this study we hypothesized that selected miRNAs could serve as stratification markers for patients who do or do not develop BOS after LTx and thereby function as novel diagnostic biomarker to identify patients at risk for BOS development. For this end, we analyzed the levels of different miRNAs in a cohort of LTx patients that did or did not develop BOS post-LTx. Furthermore, we investigated the levels of these selected miRNAs in patients suffering from end-stage lung diseases. Our results show that a panel of selected miRNAs might prove to be beneficial to early identify patients who develop BOS post-LTx.

Results

Patients and miRNA selection

The cohort of end-stage lung disease patients consisted of patients suffering from chronic obstructive pulmonary disease (COPD, *n*=5), cystic fibrosis (CF, *n*=5), and interstitial lung disease (ILD, *n*=5). All patients were treated with standardized immunosuppressive therapy consisting of tacrolimus, basiliximab, prednisone, and mycophenolate mofetil.

Incidentally, patients that were defined as being at high risk for either CMV or EBV reactivation were treated with valganciclovir up until 6 months after transplantation. For follow-up analyses we included 10 BOS+ and 10 BOS- patients matched for underlying disease prior to transplantation, age, and gender (Table 1), resulting in a total of 80 serum miRNA level determinations. None of the patients analyzed presented with episodes of acute rejection or infections at the time of sampling. Also, no RAS was observed.

We hypothesized that ILD patients (diagnosis for lung allocation score: other pulmonary fibrosis) might present higher pro-fibrotic miRNA levels because prolonged ILD is often associated with pulmonary fibrosis. Additionally, BOS is associated with extensive pulmonary fibrosis[2]. Therefore, we selected two pro-fibrotic miRNAs, miR-21 and miR-155, given their association with multiple fibrotic conditions in literature[6-8]. Based on previous knowledge on these conditions, we also selected an anti-fibrotic miRNA (miR-29a) and control miRNAs unrelated to fibrosis, i.e. miR-103 and miR-191[9].

Levels of selected serum miRNAs are elevated in end-stage lung disease patients compared to healthy controls

The qPCR analysis revealed that all the selected miRNAs were significantly increased in the pre-LTx serum of patients suffering from end-stage lung diseases as compared to

	BOS+	BOS-	*p*-value
Total number	10	10	
BOS grade			
I	6	N.A.	
II	3	N.A.	
III	1	N.A.	
Onset of BOS (month)	35 (23 - 59)	N.A.	
Mean follow up (months)	43 (24 - 104)	60 (26 - 103)	0.105
Quartiles (months)			
I	9 (6 - 17)	9 (5 - 15)	0.861
II	18 (12 - 30)	18 (11 - 29)	0.986
III	27 (18 - 45)	28 (16 - 48)	0.832
IV	38 (24 - 61)	38 (24 - 63)	0.905
Type of transplantation			
Single	4	2	0.329
Bilateral	6	8	
Mean age (years)	43 (16 - 61)	43 (21 -61)	0.957
Gender			
Male	2	2	1.000
Female	8	8	
Primary disease			
COPD	6	6	1.000
CF	4	4	

Table 1: Clinical and demographic profile of lung transplantation patients

Overview of selected BOS+ and BOS- patients. No differences were observed between the matched BOS+ and BOS- patients as indicated by the respective *p*-values. BOS: bronchiolitis obliterans syndrome, COPD: chronic obstructive pulmonary disease, CF: cystic fibrosis.

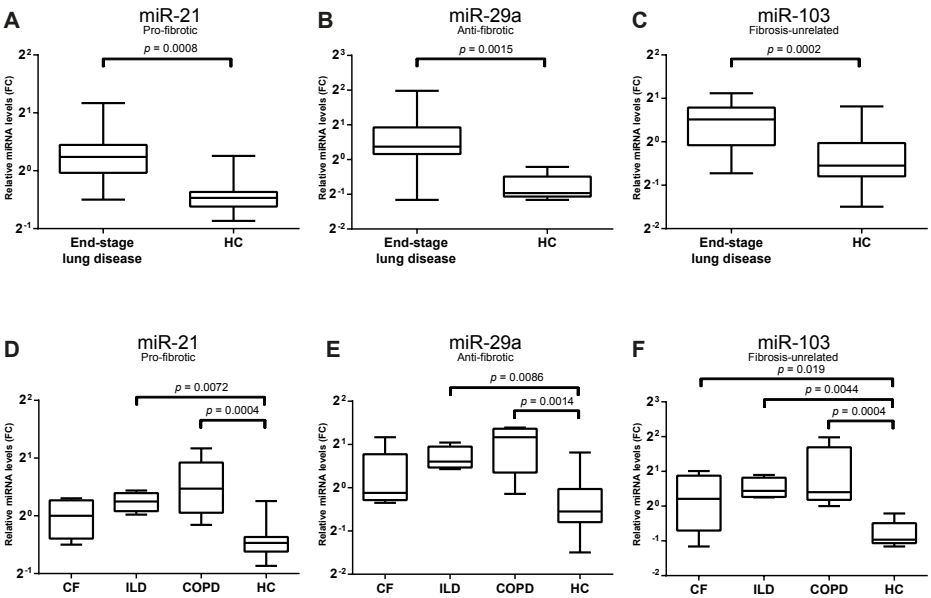

Figure 1: Levels of serum miRNAs are elevated in end-stage lung disease patients compared to healthy controls

The levels of serum miRNAs were analyzed by RTqPCR as described in the text in the serum of patients after lung transplantation and represented as mean FC ± SEM. Micro-RNAs were quantified in the serum of patients with end-stage lung diseases that did not undergo transplantation and were depicted as box and whisker plots, where boxes are indicating the median and the 25th to 75th percentiles and the whisker the min and max values within each diagnostic group. Differences between HC and end-stage lung disease patients were analyzed via the Mann-Whitney test (**A**,**B**,**C**). To compare the means of group pairs per diagnostic cluster, we analyzed the data using the one-way ANOVA while the Holm-Sidak's test was used for multiple comparisons (**D**,**E**,**F**). CF: cystic fibrosis, ILD: interstitial lung disease, COPD: chronic obstructive pulmonary disease, HC: healthy controls, FC: fold change.

healthy controls (HC), see Figure 1A, B, C. Moreover, when stratified per type of lung disease, all the serum miRNAs investigated were significant elevated in both COPD and ILD patients compared to HC. For CF patients, only miR-103 was significantly increased compared to HC. No differences between the diagnostic clusters CF, COPD, and ILD were observed (Figure 1D, E, F). Similar results were obtained for miR-155 and miR-191 (data not shown).

Selected serum miRNAs are elevated in BOS+ patients compared to matched BOS- patients

We next investigated whether BOS+ patients had consistently high serum levels of miRNAs post-transplant or whether these levels increased further at the time of BOS development. To this end, serially obtained post-transplant sera were analyzed for miRNA levels. The time from lung transplant until the onset of BOS of each patient was divided into four equal quadrants and one sample taken in the middle of each quadrant was analyzed. Analysis of these serum samples demonstrated that the levels of all analyzed miRNAs showed a similar pattern, and were overall significantly increased in the serum of BOS+ vs. BOS- patients. In addition, the levels of miR-21, miR-103, and miR-191 were significantly higher in BOS+ at the 2nd quartile (p=0.036, p=0.049, p=0.021 respectively,

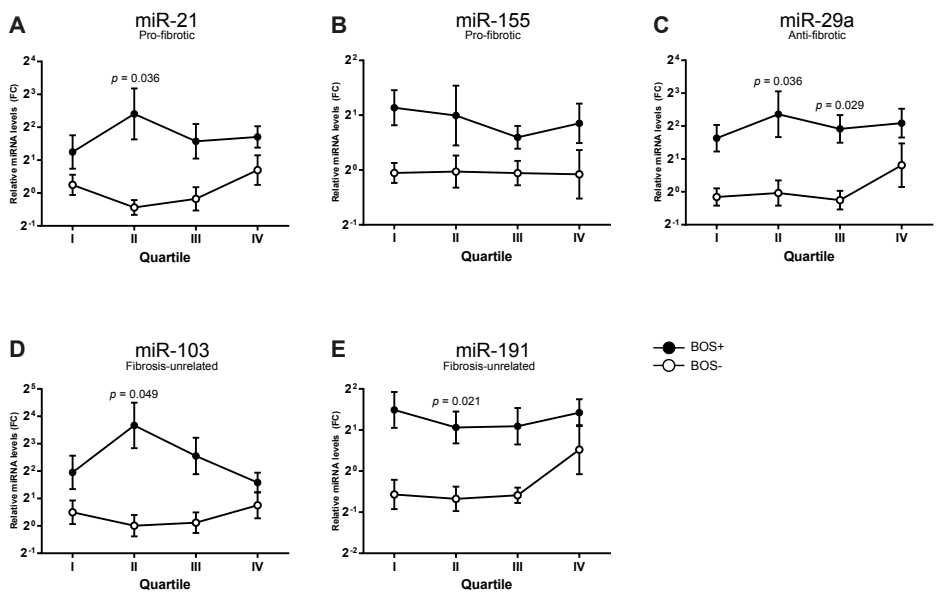

Figure 2: BOS+ patients present higher levels of selected serum miRNAs compared to matched BOS- patients

The impact of time and BOS development of the level of circulating miRNAs was analyzed by using the two-way ANOVA, setting time and disease as variables. Taken together, all different miRNAs were significant elevated in BOS+ compared to BOS- groups (p<0.04). Multiple T-tests were used to evaluate the significance of differences in miRNA levels at each quartile, BOS+, n=10, BOS-, n=10 (**A**-**E**).

Figure 2A, D, E), and miR-29a at the 2nd and 3rd quartile post-LTx (p=0.036 and p=0.029, respectively, Figure 2C). These results suggest that patients developing BOS have higher levels of circulating miRNAs in serum as compared to BOS-.

Discussion

In this study, we investigated the presence of selected miRNAs in serum of both end-stage lung disease patients prior to LTx as well as in serum of a follow-up cohort consisting of both BOS+ and BOS- patients. Our results show that all selected pro-fibrotic, miR-21 and miR-155, anti-fibrotic, miR-29a, and fibrosis-unrelated, miR-103 and miR-191, miRNAs were elevated in end-stage lung disease patients. Given that the levels of circulating miRNAs are consistently higher in patients with end-stage lung diseases, we investigated whether this was maintained after transplantation and/or exacerbated in case of BOS development. We show that these miRNAs are elevated in patients who develop BOS and that these elevated levels are measurable prior to any clinical manifestations.

Despite the relatively small cohort sizes, we showed that the presence of an inflammatory lung disease is associated with increased levels of circulating miRNAs. Interestingly, the ILD patients investigated did not exhibit elevated levels of pro-fibrotic or diminished levels of anti-fibrotic miRNAs compared to other end-stage lung diseases, despite the pulmonary fibrosis which is often observed in these patients.

Intriguingly, both pro- and anti-fibrotic miRNAs, as well as fibrosis-unrelated miRNAs, were found increased in the circulation of BOS+ patients and in patients suffering from other forms of inflammatory lung diseases not related to transplant complications. Even if the discriminative value of either pro- or anti-fibrotic miRNAs is limited, the combination of selected miRNAs could perhaps improve this distinction.

The overall increase of serum miRNAs in BOS+ patients could be detected long before the actual (18.1 ± 1.4 months post-LTx) diagnosis of BOS, suggesting that the detection of elevated circulating miRNAs might serve as early biomarkers for BOS development. Further supporting this concept, serum miRNAs have been proposed as potential prognostic biomarkers in different transplantation settings[4,10,11]. In addition, dysregulated expression of other miRNAs was observed in peripheral mononuclear cells of BOS patients with donor specific-HLA antibodies, further stressing the importance of miRNA regulation in this transplant complication[12] and their potential value as biomarkers for this condition validated. LTx patients identified as being at risk for BOS development by using such miRNA-biomarkers could therefore undergo alternative treatment regimens,

which could potentially restrain disease progression and delay clinical manifestations. Furthermore, as circulatory miRNAs are easily accessible and highly stable in human blood, they would represent profoundly good biomarkers for routine diagnostic follow-up after transplantation procedure[9].

In summary, we demonstrated that a selected group of miRNAs is elevated in BOS+ patients compared to BOS- patients and this difference is present prior to the clinical diagnosis of BOS. This work opens new perspectives for future research aimed to further elucidate the prognostic value of circulating miRNAs in BOS and lung conditions in general.

Patients and Methods

Patients and sampling

All selected patients included in this study were transplanted at the Heart and Lung Center of the University Medical Center Utrecht in The Netherlands between May 2003 and September 2010. All patients gave informed consent and the study was approved by the medical ethical committee. Furthermore, all methods were carried out in accordance with the approved guidelines within our center.

For our analyses, we selected serum samples collected at 4 time points (quartiles) equally-distributed from the time of transplant until BOS diagnosis (quadrant-based selection). For clarification, the time between the transplantation date and the clinical diagnosis of BOS was divided by 4 to generate quartiles for each patient. Subsequently, serum obtained at each quartile was selected for further analysis. For each BOS+ patient, a matched BOS- patient with similar follow-up length was selected in parallel.

miRNA isolation and quantification

Serum RNA, including miRNAs, was extracted from 200 µl of serum isolated from patients and previously stored at -80°C, by using the miRCURY™ RNA Isolation Kit for Biofluids (Exiqon, Denmark) according to the manufacturer's instructions. cDNA was synthesized from 2,5 µl of serum-RNA by using individual miRNA-specific RT primers contained in the TaqMan Human miRNA assay in the presence of 3.3 U/µl MultiScribe RT enzyme (Lifetechnologies, USA), by using the following thermal cycler conditions: 10 min, 4°C; 30 min, 16°C; 20 min, 42°C; 5 min, 85°C. Circulating miRNA levels were quantified in duplicate from 3 µl cDNA, with TaqMan Fast Advance Master Mix and specific primers of the TaqMan Human miRNA assay, using the following amplification

condition on the Quantstudio 12k flex Real-Time PCR system (Lifetechnologies, USA): 2 min, 50°C; 20 sec,m95°C; 40 cycles of 1 sec, 95°C; 20 sec, 60°C. RTqPCR data were analyzed via the comparative threshold cycle method[5]. The abundance of each circulating miRNA was expressed as relative fold change (FC) as compared to the median level detected among all patients set as 1.

Statistics

Statistical analysis was performed using GraphPad Prism software version 6.02 (GraphPad Software, USA) and SPSS version 20 (IBM Corp., Armonk, NY). The normally distributed log2-transformed FC results were analyzed via usage of the one-way and two-way ANOVA and the Holm-Sidak's method for multiple testing comparison with power of test set at α=0.05. Values for end-stage lung disease patients and healthy controls (HC) were tested for Gaussian distribution via the D'Agostino-Pearson omnibus normality test and subsequently analyzed via the Mann-Whitney test. A p <0.05 was considered to be statistically significant.

Author Contributions and Disclosure Statement

KB performed the research; MR, EAG, and HGO participated in data analysis; EAG contributed patient serum; KB, MR, EAG, and HGO participated in research design; KB, MR, EAG, TRDJR and HGO wrote the paper. All authors provided final approval of the version to be published and declare to have no conflict of interest.

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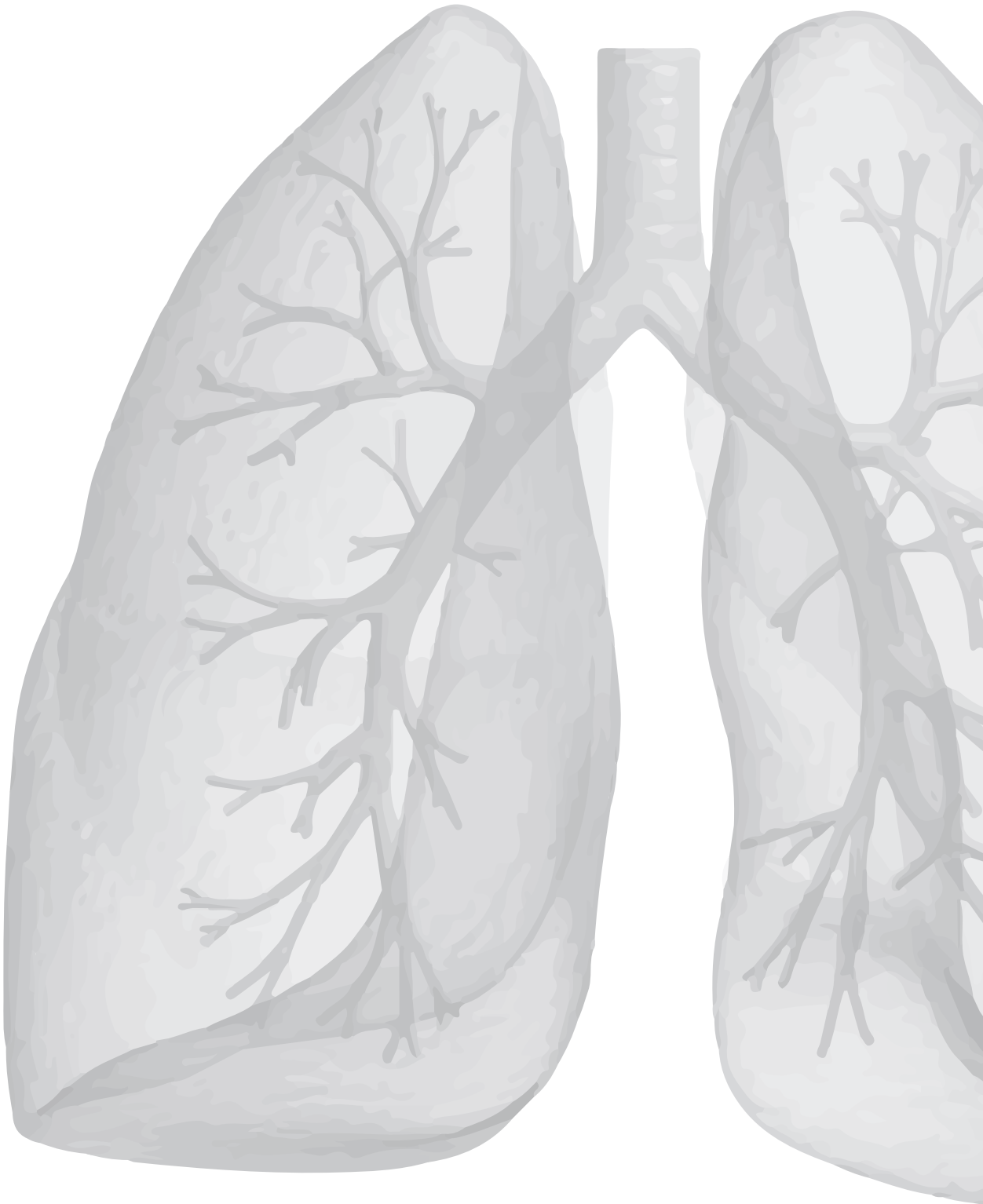

12

General discussion

Chronic lung allograft dysfunction (CLAD) remains the major limiting factor for successful long-term clinical outcome after lung transplantation (LTx)[1]. Current numbers show that worldwide 5 year survival after LTx is 50% and only 25% of the LTx treated patients survive for more than 10 years after LTx[2] (Figure 1). In a clinical setting, CLAD predominantly results from chronic rejection. The main forms of chronic rejection are BOS and RAS, which are diagnosed using surrogate markers. Therefore, they are often diagnosed at a stage that extensive lung damage and fibrosis have already taken place and are irreversible. Recent studies have shown a beneficial effect of azithromycin on a subset of CLAD patients characterized by the presence of excess (≥15%) bronchoalveolar lavage neutrophils, although limited data is available on the efficacy of this treatment on BOS[3,4]. Therefore, there is a high clinical need for novel biomarkers to identify patients at risk for CLAD development earlier after LTx.

The exact mechanisms that will eventually lead to BOS or RAS are not clear[5]. Several risk factors are found that contribute to BOS development, including previous episodes of acute rejection, HLA mismatching or the presence of anti-HLA antibodies, and also infections including those by cytomegalovirus[6], *Pseudomonas aeruginosa*, or *Staphylococcus aureus*, or *Aspergillus fuminatus*[7].

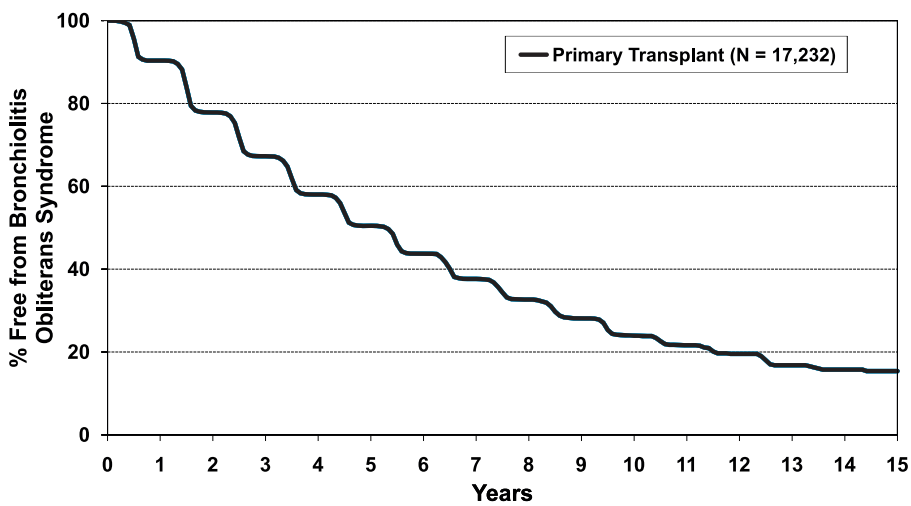

Figure 1: Freedom from BOS after lung transplantation

Worldwide numbers on BOS incidence after LTx show that 5-year freedom from BOS is currently around 50%. Ten years after LTx the BOS-free survival is decreased to only 25%. Figure adapted from the ISHLT registry report by Yusen, *et al*[2].

The goal of this thesis was to identify novel markers to identify patients at risk for the development of chronic rejection, before or early after LTx. In particular, we aimed to investigate novel markers for BOS development prior to the clinical manifestations of the syndrome.

Allo- and autoimmunity in lung transplantation

The first part of this thesis consists of studies on the role of humoral immunity and complement on LTx outcome. Alloimmunity and pre-transplant sensitization as contributing factors for allograft rejection are well established in the field of kidney transplantation. Stringent HLA matching is common clinical practice in bone marrow and kidney transplantation, but not in LTx or heart transplantation, mainly because of practical considerations. Lung grafts are very sensitive to ischemia, which leaves no time for extensive screening of potential recipients, which puts LTx procedures often under time pressure[8]. The impact of pre-sensitization on LTx outcome remains under debate. In a recent study, pre-transplant allosensitization was not associated with adverse effects after transplantation[9], contradicting previous observations[10,11]. Anti-HLA antibodies appearing *de novo* during transplantation follow up precede BOS development according to the results of Jaramillo *et al*[12]. Furthermore, these antibodies can activate endothelial cells which subsequently leads to pro-fibrotic cytokine secretion[13,14]. Interestingly, in our cohort, *de novo* anti-HLA antibody formation was not observed[15], which probably reflects different immunosuppression strategies[16,17]. Recently, the impact of autoimmunity on LTx outcome has become clear. Most notably, autoantibodies against collagen V and K α-1 tubulin are associated with BOS development[18].

In **chapter 4** we show that extensive tissue damage in end-stage lung disease patients is associated with increased levels of autoantibodies directed against apoptotic targets. This is in agreement with findings in kidney transplantation, were these antibody titers were found increased in end-stage kidney disease patients prior to transplantation[19]. However, the finding in kidney transplantation that these titers correlate with post-transplant graft loss and antibody-mediated rejection apparently does not hold for LTx. Furthermore, the course of anti-apoptotic antibody titers post-LTx, did not discriminate patients that did or did not develop BOS. These results suggest differences in the underlying pathological processes between different forms of rejection in kidney- and lung transplantation. In addition to the study by Gao *et al.*, not only apoptotic Jurkat cells but also apoptotic primary lung endothelial cells were used as antigens for antibodies against apoptotic cells. Interestingly, we found no correlation between antibody levels directed against apoptotic Jurkat or apoptotic lung endothelial cells which suggests cell antigen specificity of these

antibodies. Cell specificity of these antibodies could explain the observed differences between kidney- and lung transplantation. Furthermore, the primary endothelial cell culture model described in **chapter 4** has potential implications for LTx research. Both HLA and non-HLA antibodies can stimulate endothelial cells to secrete pro-inflammatory cytokines[20,21]. Also, endothelial cells are the first cells encountered by the recipient's immune system, and can activate human T cells[22]. Our culture model allows for *in vitro* analyses of the direct interaction between donor endothelial cells, patient serum, and patient peripheral blood mononuclear cells (PBMCs), and therefore may be helpful in studies on the immune mechanisms triggered by LTx. As such, this system with donor derived primary lung endothelial cells may be complementary to human umbilical vein endothelial cell systems, which are often used in the field of LTx research.

In **chapter 5** we describe a novel autoantibody against BPIFA1 in a cohort of 67 end-stage lung disease patients. BPIFA1 shares structural homology with the anti-microbial peptide BPI and also has anti-microbial activities. We show that serum levels of anti-BPIFA1 are elevated in end-stage lung disease patients, and in particular in patients suffering from end-stage cystic fibrosis. These observations correspond with previous findings concerning autoantibodies against the homologue BPI, which are correlated to lung function deterioration in these patients[23]. Levels of anti-BPIFA1 did, however, not correlate with the development of chronic rejection after LTx. In **chapter 6** we analyzed the presence of autoantibodies against angiotensin II type 1 receptor (AT_1R) and endothelin-1 type A (ET_AR) in our LTx cohort. The presence of these autoantibodies is associated with episodes of cellular and antibody-mediated rejection in both heart- and kidney transplantation[24,25]. Furthermore, both autoantibodies are associated with systemic sclerosis, particularly with fibrotic complications[26]. Although BOS is hallmarked by fibrotic complications in the lung, we did not detect an association between the presence of either autoantibodies or BOS development in our study cohort. Moreover, serum levels of anti-BPIFA1, anti-ET_AR, and anti-AT_1R autoantibodies were not affected by LTx treatment and subsequent immunosuppressive treatment. This differed from the observations on anti-BPI autoantibodies and rheumatoid factor, of which titers decreased post-LTx. Decreasing anti-BPI levels following LTx have been reported previously, and are considered to reflect lower infection burden following LTx, particularly by *P. aeruginosa* in CF patients. Infections by these bacteria lead to an overproduction of anti-microbial proteins, including BPI, which triggers autoantibody formation[27]. The effect of immunosuppressive therapy on levels of antibodies post-LTx is illustrated by the pharmacodynamics of mycophenolate mofetil. This drug blocks *de novo* purine synthesis and interferes with in B cell proliferation and subsequent formation of (auto) antibodies. Why anti-BPIFA1,

anti-ET_AR, and anti-AT_1R, are not affected due to LTx treatment remains questionable. Different mechanisms underlying autoantibody formation could explain this observation.

Complement regulation: chronic rejection from a donor's perspective

Previous research has mainly focused on the process of rejection from a patients perspective. This is understandable since the immune reaction against the grafted organ is initiated and mediated by the patient's immune response. However, some of the risk factors for rejection, such as both auto- and alloantibodies, display an interesting interplay with the grafted organ. The fact that allo- and autoimmunity contribute to BOS development, but mechanisms of action of these immune responses can be counteracted by donor immune regulation, opens new perspectives for research into chronic allograft rejection.

Antibody binding to membrane antigens primarily leads to the activation of the classical pathway of the complement system. Complement activation results, among others, in the formation of the membrane attack complex (MAC) which can insert into the cell membrane and initiate cell lysis. In order to protect from unwanted complement activation, cells are equipped with various membrane-attached complement regulatory proteins to inhibit complement effector mechanisms. The involvement of complement regulation can have great impact on disease progression. Downregulation of CD59 has been observed in *Escheria coli*-induced hemolytic syndrome, correlating with severe disease outcome[28]. Decreased function or expression, or even the absence of CD55 and CD59 contributes to the development of paroxysmal nocturnal hemoglobinuria[29]. Also, hyperglycation of CD59 in patients with diabetes mellitus affects the complement inhibitory activity of CD59, resulting in exposure of endothelial cells to sublytic concentrations of MAC and subsequent stimulation of production cytokines and growth factors leading to proliferation, increased tissue growth and vascular complications[30]. Concluding, these studies indicate that loss of complement regulation can have severe clinical impact on disease progression.

In **chapter 2** we investigated the role of complement regulation on LTx outcome, with the hypothesis that differences in the expression of complement regulatory proteins could influence the susceptibility of donor lung endothelial cells to complement-mediated cell lysis or activation. We show that a SNP in the promotor region of the donor *CD59* gene correlates with CD59 expression on monocytes, endothelial cells, and the incidence of chronic rejection after LTx. Following ABO-incompatible kidney transplantation, patients often develop antibodies against blood group antigens. Intriguingly, these do not necessarily affect graft function, which suggests resistance against complement-mediated

cell damage via the expression of complement regulatory proteins. This process is termed accommodation[31-33]. These observations are in line with our results, that are the first to show that intrinsic properties of the lung graft contribute to LTx outcome. A diminished expression of CD59 could well result in a lowering of the threshold for endothelial cell activation by complement, and subsequent increase of pro-fibrotic cytokine secretion. Taking these results into consideration together with the phenomenon of accommodation via which complement regulation has a profound effect on ABO incompatible kidney transplantation, donor factors can potentially have a significant outcome on patient survival. This has been shown previously in kidney transplantation, where the different expression of donor C3 alleles has a differential effect on late allograft outcome[34]. In the coming years these insights need to be further investigated to elucidate the mechanisms via which the grafted organ can protect itself against the recipient's immune system. The challenge remains to translate these findings into novel therapies to expand the possibilities to treat or prevent the progression of chronic rejection, and to increase patient survival after LTx.

Immunological risk stratification for BOS development

Current diagnostic tools for the diagnosis of BOS are limited and often late, which implicates that novel markers for this complication are urgently needed. Several biomarker studies have been published over the past few years. Due to ease of accessibility, blood, plasma, or serum biomarkers would be ideal candidates. Indeed, plasma sRAGE and serum sCD30 levels are associated with BOS development[35,36]. Furthermore, our group has previously shown that TARC/CCL17 serum levels in the first month after LTx are indicative for BOS incidence[37]. In **chapter 3** we analyzed soluble CD59 (sCD59) as early novel biomarker for BOS development. Circulating levels of sCD59 at 6 months after LTx can be used to identify patients at risk for the development of chronic rejection. Furthermore these levels remain elevated when BOS becomes clinically manifest. Unfortunately, since surveillance bronchoscopy is not performed at our center, we could not quantify sCD59 levels in bronchoalveolar lavage fluid. CD59 is a GPI anchored protein which can be enzymatically cleaved of the cellular surface via the activity of phospholipases[38]. Activated vascular endothelial cells are known to secrete increased amounts of phospholipase-C, which could further add to a decreased protection of these cells by membrane-attached CD59, crucial in maintaining accommodation[39]. This has also been observed in patients suffering from acute myocardial infarction[40]. We assume that the increased levels of serum sCD59 following LTx originate from lung endothelial cells. However further studies are needed to elucidate the molecular mechanisms underlying our observations into sCD59

as a biomarker for BOS development.

Further biomarker studies are presented in **chapter 7** and **chapter 11**. In **chapter 7** we have assessed the composition of mononuclear cells in the blood of patients who underwent LTx in our center. Data shows that BOS is characterized by the infiltration of a variety of immune cells into the allograft, including lymphocytes, dendritic cells, natural killer cells and plasma cells[41-43]. Furthermore, differences have been observed in the mononuclear cell composition in bronchoalveolar lavage fluid of patients developing BOS and those who do not[44-46]. We observed significant differences between B cell, T cell and monocyte numbers in the circulation of LTx patients with or without BOS. However, these differences were too small for risk stratification analyses. Interestingly, our group previously reported on the level of microchimerism of dendritic cell subsets in peripheral blood of patients after LTx. Although the numbers of patients studied were too small to draw conclusions on the correlation with BOS onset[47] and the effect of stable microchimerism on LTx outcome remains unclear[48,49], the investigation on PBMC composition in relation to transplant outcome remains relevant.

In **chapter 11**, we explored levels of serum micro-RNAs (miRNAs) in relation to the development of chronic lung allograft dysfunction after LTx. Micro-RNAs are short non-coding RNAs, that can inhibit post-transcriptional gene expression, via binding to the 3'UTR of target messenger RNA which will lead to RNA degradation or the inhibition of translation[50]. Intracellular differences in miRNA expression patterns were observed in LTx patients, especially in patients who had antibodies to donor HLA. These different expression patterns were observed in PBMCs obtained from LTx patients either negative, single, or double positive for DSA and BOS post-LTx[51], and in a murine-model for BOS development[52]. Besides intracellular, miRNAs can also be present in micro-vesicles in various body fluids, including human serum, the ideal biomarker candidate. Not surprisingly, research has been conducted in other solid-organ transplantation settings into the potential of these serum miRNAs as biomarkers for transplantation outcome. In liver-, kidney-, small intestine-, and heart transplantation levels of various miRNAs were found to be changed and correlated to clinical outcome[53]. Our results are the first to show that a specific subset of selected pro-fibrotic miRNAs is elevated in the serum of patients who develop BOS following LTx compared to matched patients who do not. Intriguingly, these differences in miRNA levels are already present long before the clinical diagnosis of BOS. Furthermore, analysis of these selected miRNAs in serum obtained from end-stage lung disease patients prior to LTx showed an increase compared to healthy controls, stressing their importance in (prolonged) lung damage. Further studies are needed to confirm the diagnostic value of serum miRNA profiles to predict clinical course and

outcome after LTx.

Genetics and transplantation

Over the past few years genome wide association studies (GWAS) have yielded novel insights into the understanding of pathogenic mechanisms of a broad variety of diseases[54]. Associations of transplantation outcome and polymorphisms are well known for HLA whereas genetic studies focusing on non-HLA polymorphisms are limited in literature, presumably, presumably reflecting that GWAS require larger numbers of patients. A more suitable approach would be hypothesis-driven candidate gene studies, of which **chapter 2** is an example. In order to facilitate these studies, the international genetics and translational research in transplantation network (*i*GeneTRA*i*N) has been established and unites researchers in different solid-organ transplantation settings[55]. In **chapter 9** and **chapter 10** we present results of candidate gene studies conducted with data generated within the *i*GeneTRA*i*N consortium with the usage of a genome-wide association genotyping array tailored for transplantation-specific studies[56]. The observation that a single donor *TARC/CCL17* promotor polymorphism correlates with serum TARC/CCL17 levels and is associated with clinical outcome after LTx is based on the previous observation that circulating levels of TARC/CCL17 can be used as a biomarker for chronic rejection[37]. The *PTPN22* gene is the archetypical non-HLA autoimmunity gene of which the risk variant is associated with a broad spectrum of autoimmune disorders[57], and is found to be associated with RSV-induced bronchiolitis (unpublished observation). As discussed previously, autoimmunity is observed in BOS development after LTx. Furthermore, the selected *PTPN22* SNPs researched, did also show to be associated with episodes of rejection after liver transplantation[58], and have been investigated in kidney transplantation[59,60]. Despite the fact that large patient numbers are lacking from our studies, the presented observations are in line with data on other solid-organ transplantations or previous results in LTx-related research. Although there appears to be a difference in the underlying disease mechanisms driving chronic rejection in different solid-organ transplantations, the *i*GeneTRA*i*N consortium is highly suitable to easily validate and implement genetic observations from different research groups. Within our group we are currently investigating other SNPs in complement-related proteins, and SNPs associated with either lung fibrosis or lung diseases in relation to chronic rejection. Most research conducted on transplantation outcome after LTx has focused on the general form of CLAD, with no further characterization of BOS or RAS phenotypes. The first studies that found a relation between CLAD and specific genotypes were conducted by Awad *et al*. and focused on the inflammatory cytokine IFN-γ and the pro-fibrotic cytokine TGF-β1[61,62].

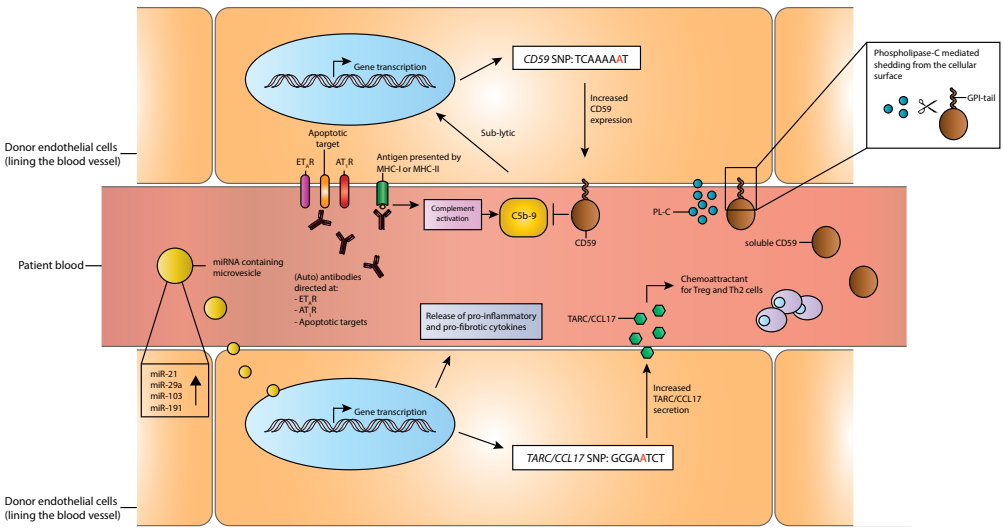

Figure 2: Schematic overview of endothelial cell activation and consequential cellular processes

Donor lung endothelial cells lining the blood vessel come in close contact with patient blood. Herein, complement-fixing (auto) antibodies (**chapters 4-6**) might be present that lead to complement activation and, in the case of partial complement inhibition, complement-mediated endothelial cell activation. Differences in CD59 expression are observed when donors are stratified per CD59 genotype, which could result in lower CD59 expression and a lowered threshold for cellular activation (**chapter 2**). CD59 can be cleaved from the cellular surface by PL-C, which results in an increase in soluble CD59, which is a biomarker for BOS development (**chapter 3**). Also, endothelial cell activation can lead to an increase in miRNA-containing microvesicles, of which selected miRNAs are associated with BOS (**chapter 11**). Furthermore, both pro-inflammatory and pro-fibrotic cytokine secretion is observed in activated vascular endothelial cells (**chapter 2**). Finally, the chemo-attractant TARC/CCL17 can be secreted by vascular endothelial cells to attract T regulatory and Th2 cells, to counteract the immune response. Increased serum levels of TARC/CCL17 are found to be associated with a reduced risk for BOS development. Our results have indicated that a *TARC/CCL17* promotor polymorphism correlates with both lower serum TARC/CCL17 levels and a higher risk for BOS development after LTx (**chapter 9**).

Later studies failed to confirm these observations, but showed that a polymorphism within IL-6 was associated with CLAD development[63]. Our group has previously shown that also LTx patients with specific SNP configurations in TLR genes[64] and a SNP in the protein matrix metalloproteinase-7[65], involved in lung tissue repair, have an increase risk to develop CLAD after transplantation. More recently, the research group from Leuven has shown that a polymorphism in the IL-17R did not only associated with acute, but also with chronic rejection[66]. Above-mentioned results implicate the potential importance of genetic studies on LTx outcome. Importantly, our studies contribute to the increasing body of evidence concerning genetic polymorphisms and BOS development, with the addition that also donor SNPs are taken into consideration, illustrated by our observations on CD59 and TARC/CCL17. The challenge remains to translate these observations into clinical applications and to develop risk-stratification models which can subsequently be used for identification and personalized treatment of patients at risk for BOS development after LTx.

Concluding remarks

Since the first performed lung transplantation in 1963, thousands of patients have benefited from this surgical procedure. Post-transplantation survival numbers have increased over the years, but due to surgical complications and the incidence of chronic rejection, lung transplantation remains the least successful solid-organ transplantation. In this thesis, we describe novel insights into immunological mechanisms involved in chronic lung allograft dysfunction after LTx.

Our genetic studies could well be implemented within a risk-stratification model for BOS development. Early after LTx both patient and donor DNA, which is already available from the LTx procedure, can be used to genotype the high-risk genetic polymorphisms, including *CD59*, *PTPN22*, and *TARC/CCL17*, identified in this thesis. During follow-up, LTx patients could subsequently be monitored for TARC/CCL17 and sCD59 serum titers, as well as levels of selected serum miRNAs, when further validated (see Figure 2).

In conclusion, the research conducted in this thesis has led to novel findings which contribute to a better understanding of underlying disease mechanisms, and may lead to improved treatment or prevention of post-transplantation complications, resulting in better transplantation outcomes and an improved survival of LTx-treated patients.

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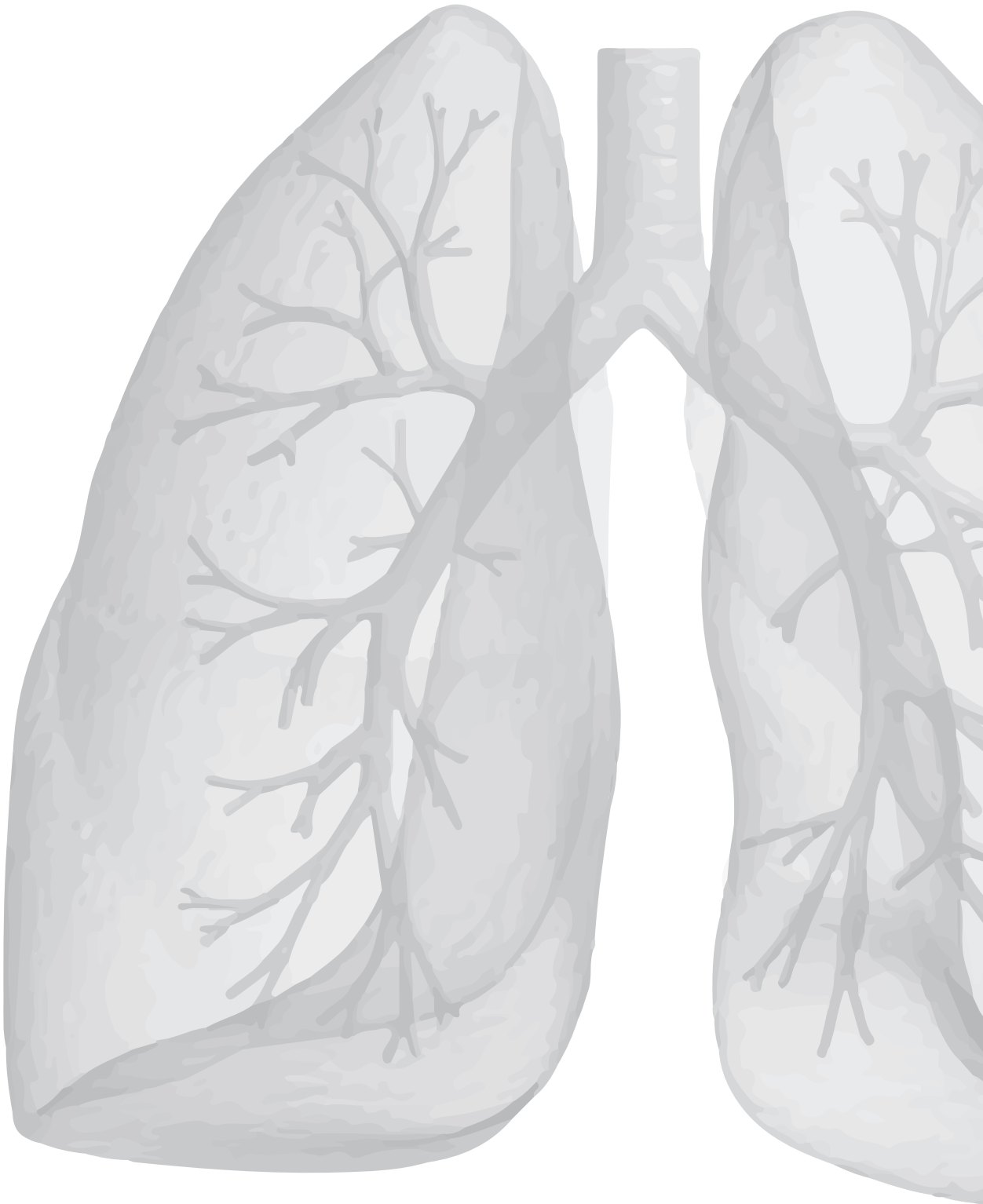

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Appendices

Summary

Lung transplantation (LTx) is the final treatment option for patients suffering from end-stage lung diseases. Survival after LTx is severely hampered by the development of chronic lung allograft dysfunction which presents itself in an obstructive form as the bronchiolitis obliterans syndrome (BOS) or in a restrictive form as the restrictive allograft syndrome. BOS is hallmarked by excessive fibrosis and scar tissue formation leading to small airway obliteration, decrease in lung function and eventually organ failure. BOS is diagnosed via a surrogate marker, a decline in lung function >20% compared to baseline levels in the absence of any other disease etiology. This diagnosis is often late, and therefore there is a high clinical need for biomarkers to identify patients at risk for BOS development earlier after transplantation. The main purpose of this thesis was to gain insight into the pathological mechanisms preceding BOS development and to identify novel biomarkers for chronic rejection after LTx.

Chapter 1 serves as a general introduction which describes the different forms of rejection that are observed in LTx patients. We review known pathological mechanisms involved in BOS development, including alloimmunity, autoimmunity, but also elaborate on subsequent complement effector mechanisms. Furthermore, the general outline of this thesis is discussed.

The first part of this thesis is focused on humoral immunity and starts with our observations on the role of the complement system on transplantation outcome. **Chapter 2** discusses complement activation, which primarily leads to membrane attack complex formation and subsequent target cell lysis. We show that the capacity of the grafted organ to protect itself from the recipient's complement system correlates with BOS incidence after LTx.

In **chapter 3** we describe our findings regarding the soluble form of CD59 and its potential role as biomarker for BOS development. For this study we hypothesized that serum of levels of sCD59 can be used as biomarker. We show that BOS patients exhibited higher sCD59 serum concentrations at the time of diagnosis compared to clinically matched non-BOS patients. Furthermore, we found that these titers were already elevated at 6 months post-LTx, at which time patients had no symptoms of BOS or a decline of lung function. We therefore propose that these circulating levels of sCD59 constitute a novel biomarker for chronic rejection after LTx.

In kidney transplantation it is shown that antibodies directed at apoptotic targets correlate with kidney allograft loss. In **chapter 4** we investigated the presence of these antibodies in

the serum of LTx patients and assessed levels of IgG directed against apoptotic Jurkat cells with LTx outcome. Furthermore, we describe a method to obtain primary lung endothelial cells from the donor, which were expanded and subjected to apoptosis induction.

Chapters 5 and 6 discuss autoantibodies against BPIFA1, a secreted protein of the upper airways that shares structural homology with BPI and exhibits comparable antimicrobial capacities, and ET_AR and AT_1R in a cohort of end-stage lung disease patients and during follow-up after LTx.

In the second part of this thesis we shift gears to cellular immunity. In **chapter 7** we analyzed if the cellular mononuclear cell composition and their subsets are predictive for BOS. We found that increases in T cells and monocytes and lowered fractions of B cells were related to BOS development. Prior to BOS diagnosis the composition of specific mononuclear cells on a group level differs from patients remaining BOS free.

In **chapter 8** we investigated the cell numbers and cell composition of mononuclear cells in the collected retrograde perfusion fluid during LTx procedure. We found that large numbers of cells are present in the retrograde flush and that the cellular composition largely varies from the circulation. Both T cells and B cells are decreased and percentages of NK cells are increased. Since the method of retrograde flushing was implemented in august 2008 we also analyzed whether this procedure influences outcome after transplantation.

The final part of this thesis describes the first results obtained in within the *i*GeneTRA*i*N network. Serum levels of the chemo-attractant protein TARC/CCL17 measured during the first month post-LTx are predictive for BOS development. Since *TARC/CCL17* promotor polymorphisms correlate with serum TARC/CCL17 levels we investigated, in **chapter 9**, selected SNPs present in this region and their potential association with LTx outcome. We identified that a single SNP in this region in the donor correlates with TARC/CCL17 serum levels in the patient. Furthermore, survival analysis showed that receiving a graft from a donor heterozygous for this SNP has a disadvantageous impact on transplantation outcome.

In **Chapter 10** we identified six selected SNPs within *PTPN22*, the archetypal autoimmunity gene, and analyzed both patient and donor genotypes on BOS development post-LTx. Our results show that LTx patients that are heterozygous for a *PTPN22* SNP are more susceptible for BOS development and indicate a deleterious effect of the autoimmune-related risk factor of *PTPN22* in patients on LTx outcome.

In **Chapter 11**, we explore the novel research filed of micro-RNAs (miRNAs). We

conducted a pilot experiment in which we investigated selected pro-fibrotic, anti-fibrotic, and fibrosis-unrelated, miRNAs in the serum of end-stage lung disease patients who underwent LTx. Our suggest that circulating miRNAs could be used as potential biomarkers for BOS development.

In the final chapter of this thesis, **chapter 12**, we discuss the abovementioned observations in a broader perspective. With this thesis, we want to stress the importance of cellular and genetic donor factors, which have been largely neglected in literature, on the development of chronic rejection after LTx, but presumably also in other solid-organ transplantation settings. In conclusion, with this thesis we contribute to a better understanding of the different immunological mechanisms involved in BOS pathogenesis. Also, the biomarker and genetic association studies presented in this thesis could contribute to the generation of a risk-stratification model to earlier identify patients at risk for complications after LTx. Clinical and diagnostic implementations of our findings could lead to novel treatment or prevention modalities and subsequently improve lung transplantation outcome.

Nederlandse Samenvatting

Chronische Long Allograft Dysfunctie na een Longtransplantatie

Nieuwe inzichten in immunologische mechanismen

Voor patiënten met een ernstige longaandoening, zoals chronische obstructieve longziekte (COPD), cystic fibrose (CF) of interstitiële pulmonaire fibrose (IPF), kan een longtransplantatie (LTx) de laatste behandelmethode zijn. Wereldwijde cijfers laten zien dat de gemiddelde levensverwachting voor patiënten na een LTx ongeveer 10 jaar is. De grootste complicatie na een LTx is het optreden van chronische afstoting, wat chronische long allograft dysfunctie (CLAD) wordt genoemd. CLAD kan zich uiten in twee verschillende vormen, gebaseerd op specifieke klinische verschijnselen. Een obstructieve vorm, die het bronchiolitis obliterans syndroom (BOS) wordt genoemd, of een restrictieve vorm, het restrictieve allograft syndroom (RAS). Binnen de patiëntengroep die onderzocht is in dit proefschrift hebben we ons beperkt tot patiënten die BOS ontwikkelen na een LTx omdat RAS in ons cohort maar zelden voorkomt.

Het exacte mechanisme waardoor BOS ontstaat is (nog) niet helemaal opgehelderd. Het huidige ziekte-model gaat uit van het ontstaan van beschadigingen in de getransplanteerde longen. Dit leidt vervolgens tot de vorming van littekenweefsel en ontstekingen, waar cellen van het immuunsysteem bij zijn betrokken. Hierdoor raken de kleine luchtwegen in de longen verstopt en neemt de longfunctie af. Dit kan uiteindelijk leiden tot longfalen en het overlijden van de getransplanteerde patiënt.

Een bijkomende complicerende factor is dat het proces van afstoting moeilijk te diagnosticeren is. In tegenstelling tot niertransplantaties zijn routinematige biopsieën (weefselafnames) na een longtransplantatie niet gebruikelijk, omdat deze ingreep erg zwaar is voor de patiënt. Ook kan het proces van afstoting zeer lokaal plaatsvinden. Omdat de long een erg groot orgaan is, kan een biopsie in een niet aangetast gebied een vals negatieve uitslag geven. Momenteel kan BOS pas gediagnosticeerd worden op het moment dat er klinische verschijnselen zijn. Dit gebeurt via een functioneel longonderzoek waarbij het maximale volume wordt gemeten wat de patiënt in één seconde kan uitademen. De gemeten waarde wordt vervolgens vergeleken met de hoogste twee waarden gemeten

tijdens het nabehandelingstraject. Wanneer er sprake is van een afname van meer dan 20% zonder dat een andere klinische factor, zoals bijvoorbeeld een verkoudheid of griep, daar de oorzaak van kan zijn wordt de diagnose BOS gesteld. BOS is dan al dermate vergevorderd dat het onomkeerbaar is. Er kan alleen met medicijnen geprobeerd worden om verdere longfunctiedaling tegen te gaan. Het is dus van groot belang dat er nieuwe diagnostische markers worden gevonden zodat het proces van chronische afstoting in een eerder stadium kan worden vastgesteld.

Het immuunsysteem van de mens

Het immuunsysteem van de mens omvat twee componenten, het aangeboren en het adaptieve afweersysteem. Het aangeboren immuunsysteem is er met name op gericht om zeer snel ziekteverwekkers te herkennen en ze te vernietigen. Bepaalde moleculaire patronen op ziekteverwekkers (ook wel pathogenen genoemd) kunnen door dit systeem herkend worden, waarna de verwijdering plaats kan vinden. Het aangeboren immuunsysteem omvat onder andere het complement systeem. Dit is een complex netwerk van verschillende eiwitten die nauw met elkaar samen werken om bacteriële en lichaamsvreemde cellen te herkennen en ze te doden. Ook speelt dit systeem een belangrijke rol bij de rekrutering van andere immuun cellen, zoals neutrofielen en macrofagen, die ondersteuning bieden bij het opruimen van deze pathogenen.

Het adaptieve immuunsysteem heeft als grote voordeel ten opzichte van het aangeboren immuunsysteem dat het over een geheugen beschikt. Echter, dit zorgt er ook voor dat een eerste herkenning van een potentiele ziekteverwekker langer duurt dan bij het aangeboren afweersysteem. Eén onderdeel van het immunologisch geheugen is de vorming van antistoffen door B-cellen. Deze antistoffen zijn in staat om zeer specifiek lichaamsvreemde cellulaire structuren te herkennen, ook wel antigenen genoemd. Ook T-cellen zijn betrokken bij het ontwikkelen en het in stand houden van het adaptieve immuunsysteem. Daarnaast is er sprake van samenwerking van de twee componenten van het immuunsysteem. Een voorbeeld hiervan is de activatie van het complement systeem nadat antistoffen gebonden hebben aan hun herkenningsstructuur. Hierdoor kan celdood geïnitieerd worden.

Wetenschappelijk onderzoek heeft aangetoond dat beide takken van het immuunsysteem betrokken kunnen zijn bij de ontwikkeling van chronische afstoting.

Doel van dit proefschrift

Het doel van het onderzoek beschreven in dit proefschrift is om meer inzicht te krijgen

in de pathogenese (ontwikkeling van een ziektebeeld) van BOS. Wij hebben gekeken naar het immuunsysteem van de ontvanger, maar ook naar wat het donororgaan zou kunnen doen om zich tegen het immuunsysteem van de ontvanger te beschermen. Verder hebben wij onderzoek gedaan naar nieuwe voorspellers (ook wel biomarkers genoemd) van BOS die ervoor kunnen zorgen dat complicaties in de long in een eerder stadium gediagnosticeerd kunnen worden. Dit zou kunnen betekenen dat de ziekte in een eerder stadium bestreden kan worden met medicijnen. Aangezien longtransplantatiepatiënten vaak voor routineonderzoek naar het ziekenhuis komen en er hierbij bloed wordt geprikt, hebben wij ons in dit onderzoek met name gericht op biomarkers in het bloed van de patient.

Humorale immuniteit en het ontstaan van BOS

Het eerste gedeelte van dit proefschrift kijkt met name naar de rol van het aangeboren immuunsysteem en de vorming van antistoffen in relatie tot het ontstaan van BOS. In **hoofdstuk 2** bespreek ik de resultaten van het onderzoek naar complement regulatoire eiwitten, die lichaamseigen cellen kunnen beschermen tegen de activiteit van het complement systeem. Deze eiwitten zijn van groot belang aangezien dit systeem relatief aspecifiek is waardoor overactiviteit kan leiden tot ongewenste bijeffecten. Wij hebben gekeken naar de hoeveelheid van deze eiwitten op het celoppervlak van donorlongen. Daarmee hebben we onderzocht of een verminderde hoeveelheid, en dus een verminderde bescherming tegen het complement systeem van de ontvanger, invloed heeft op de overleving na een LTx. In dit hoofdstuk laat ik zien dat door een genetische mutatie sommige donorlongen minder CD59, één van de onderzochte complement regulatoire eiwitten, op hun celoppervlak tot expressie brengen. Dit zorgt er vervolgens voor dat de ontvangers van deze donorlongen eerder chronische afstoting ontwikkelen.

In **hoofdstuk 3** staan de resultaten van het onderzoek of de niet-membraangebonden variant van CD59, ook wel sCD59 genoemd, gebruikt kan worden om het proces van chronische afstoting vóór de diagnose BOS te voorspellen. Van dit eiwit is bekend dat het in grotere hoeveelheden in het bloed voorkomt na weefselschade, iets wat ook optreedt tijdens het proces van chronische afstoting. Ons onderzoek laat zien dat een verhoogde hoeveelheid sCD59 in het bloed, gemeten op 6 maanden na een LTx, de ontwikkeling van BOS kan voorspellen. Dit verschil was meetbaar voordat de patiënt symptomen van BOS en een verminderde longfunctie liet zien.

Het proces van gereguleerde celdood wordt ook wel apoptose genoemd. Apoptose is van groot belang om ervoor te zorgen dat ongewenste of overbodige cellen op een gecontroleerde

manier verwijderd worden, een voorbeeld hiervan is het verdwijnen van de vliezen tussen de vingers tijdens de embryonale ontwikkeling. Onderzoek naar niertransplantaties heeft laten zien dat antistoffen die apoptotische cellen herkennen in verhoogde concentraties voorkomen in het bloed van patiënten die na een niertransplantatie te maken krijgen met afstotingsverschijnselen. In **hoofdstuk 4** beschrijf ik ons onderzoek naar het voorkomen van deze antistoffen bij LTx-patiënten. Daarnaast presenteer ik in dit hoofdstuk ook een nieuwe methode die het mogelijk maakt om longcellen van de donor te isoleren uit restmateriaal van de transplantatieprocedure en deze in het lab te kweken. Deze cellen zijn vervolgens ook gebruikt in onze apoptose experimenten om antistoffen tegen apoptotische longcellen te meten. Deze cellen zijn namelijk een perfect modelsysteem voor wat er in de getransplanteerde long plaatsvindt.

In **hoofdstuk 5 en 6** staan de resultaten die zijn voortgekomen uit ons onderzoek naar nieuwe autoantistoffen die eventueel voorspelbaar zouden kunnen zijn voor de uitkomst na een LTx.

Cellulaire immuniteit en chronische afstoting

Het tweede gedeelte van dit proefschrift behandelt onderzoek gericht op de cellulaire afweer na een LTx. In **hoofdstuk 7** hebben we samenstelling van verschillende celtypen in het bloed van LTx patiënten bekeken in de eerste maanden na de operatie. We ontdekten dat deze samenstelling anders is in patiënten die BOS ontwikkelden, in vergelijking tot patiënten die geen chronische afstoting ontwikkelden. Het percentage T-cellen en monocyten was in BOS-patiënten verhoogd in de eerste zes maanden na transplantatie, terwijl het percentage B-cellen juist verminderd was. Helaas was het verschil dermate klein dat de samenstelling niet voor voorspellende doeleinden kan worden gebruikt.

Tijdens de LTx-procedure worden de donorlongen doorgespoeld (geflushed) met zogenaamde perfusie vloeistof. Dit wordt gedaan om eventuele bloedpropjes, die kunnen zorgen voor complicaties, te verwijderen voordat de long in de patiënt wordt geplaatst. Sinds augustus 2008 worden de longen in ons ziekenhuis zowel antegraad (via de longslagader) als retrograad (via de longader) geflushed. We hebben geanalyseerd of deze additionele flush methode invloed heeft op de uitkomst na een LTx. Beide groepen, getransplanteerd voor en na 2008, zijn vergeleken op drie uitkomst criteria: het voorkomen van BOS, het voorkomen van acute afstoting en algehele overleving. Ook hebben we gekeken naar de compositie van de flush en hebben we de verschillende immunologische celtypen in kaart gebracht. De resultaten van dit onderzoek worden besproken in **hoofdstuk 8**.

Genetica en LTx

Het laatste gedeelte van dit proefschrift omvat een aantal genetische studies die zijn uitgevoerd binnen het *i*GeneTRA*i*N transplantatieonderzoek netwerk. Dit is een samenwerking opgezet om wereldwijd genetische studies te kunnen uitvoeren naar verschillende soorten orgaantransplantaties. Van zowel patiënten als donoren is DNA geanalyseerd en onderzocht of er bepaalde genetische veranderingen zijn die wellicht van invloed kunnen zijn op de uitkomst na een transplantatie. Eerder onderzoek van onze groep heeft laten zien dat het gehalte TARC/CCL17, een eiwit o.a. betrokken bij de regulering van het immuunsysteem, gemeten in het bloed op maand één na een LTx, voorspellend is voor het ontstaan van chronische afstoting. Ook is bekend dat er bepaalde genetische mutaties gerelateerd zijn aan de hoeveelheid TARC/CCL17 in het bloed. In het onderzoek beschreven in **hoofdstuk 9** hebben wij gekeken of deze genetische mutaties ook geassocieerd zijn met chronische afstoting na een LTx. Via een overlevingsanalyse hebben we laten zien dat één enkele genetische mutatie in de donor, welke geassocieerd is met de hoeveelheid TARC/CCL17 in het bloed, een nadelige invloed heeft op de uitkomst na LTx.

In **hoofdstuk 10** bespreek ik de resultaten van het onderzoek naar mutaties in het DNA van het eiwit PTPN22. Van deze mutaties is bekend dat ze vaker voorkomen bij een aantal auto-immuunziektes, waaronder reumatoïde artritis en systemische lupus erythematodes. Aangezien verschijnselen van auto-immuniteit ook worden gezien bij patiënten die met BOS zijn gediagnosticeerd, hebben we deze mutaties onderzocht in ons cohort van LTx patiënten. Ook hierbij hebben we door het gebruik van een overlevingsanalyse een genetische mutatie geïdentificeerd die vaker voorkomt bij patiënten die BOS ontwikkelden dan bij patiënten waarbij dit niet het geval was.

In **hoofdstuk 11** bespreek ik de eerste experimenten en resultaten van het onderzoek naar micro-RNA's. Micro-RNA's zijn kleine RNA-strengen die kunnen binden aan zogenaamde messenger-RNA-strengen. Op deze manier zijn micro-RNA's betrokken bij de regulatie van translatie, het omzetten van de genetische code van het DNA naar een eiwit. Een aantal van de micro-RNA's zijn geassocieerd met fibrose vorming (kenmerkend voor BOS), terwijl andere een anti-fibrotisch effect hebben. Wij hebben gekeken naar het voorkomen van deze micro-RNA's in het bloed van LTx patiënten. Onze resultaten suggereren dat micro-RNA's gebruikt kunnen worden als potentiele biomarker voor het ontwikkelen van BOS.

In het laatste hoofdstuk van dit proefschrift, **hoofdstuk 12**, worden de hierboven

genoemde resultaten in een breder perspectief geplaatst. De resultaten worden vergeleken met wat er momenteel in de literatuur bekend is.

Conclusie

Dit proefschrift laat zien dat zowel cellulaire als genetische donorfactoren een cruciale rol kunnen spelen voor het ontstaan van BOS in de donor-ontvangende patiënt. Op dit moment is er in de literatuur weinig bekend over deze donorfactoren en hun invloed op transplantatie uitkomst. Dit proefschrift draagt bij aan een beter begrip van de verschillende immunologische mechanismen die betrokken zijn bij de pathogenese van chronische afstoting. Verder kunnen de verschillende biomarker en genetische studies die zijn gepresenteerd in dit proefschrift bijdragen aan het ontwikkelen van een risico-stratificatie model om patiënten die een hoog risico hebben op het ontwikkelen van chronische afstoting eerder te identificeren. Zowel klinische als diagnostische implementatie van onze bevindingen zou kunnen leiden tot nieuwe behandelmethodes of preventieve therapieën die de uitkomst van een longtransplantatie kunnen verbeteren.

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Curriculum Vitae

Kevin Budding was born on the 17th of December 1985 in Almere, The Netherlands. In 2004 he completed his secondary education at the Sint-Joriscollege in Eindhoven (VWO-atheneum). In the same year he started with the bachelor program "Biomedical Sciences" at Utrecht University. He wrote his bachelor thesis on HIV replication under supervision of dr. Monique Nijhuis and, after graduation, enrolled the master program "Infection and Immunity" at Utrecht University. As part of this program he did his 9-month research internship at the department of Medical Microbiology in the group of prof. dr. Jos van Strijp under supervision of dr. Carla de Haas. Here, he studied immune evasion proteins secreted by *Staphylococcus aureus*, with a focus on G-protein coupled receptors. Hereafter, Kevin enrolled a 6-month minor program on "Fundamentals of Business and Economics" organized by both the Medical Faculty of the Utrecht University as well as the Utrecht University School of Economics.

In 2011, he started his PhD training at the "Eijkman Graduate School for Infection and Immunity", part of the University Medical Center Utrecht, The Netherlands. His research was embedded in the Laboratory of Translational Immunology under the supervision of dr. Henny Otten and dr. Ed van de Graaf (copromotors) and prof. dr. Erik Hack and prof. dr. Jan-Willem Lammers (promotors). During his PhD, he investigated the pathological mechanisms preceding the development of the bronchiolitis obliterans syndrome and identified novel biomarkers for this form of chronic rejection after lung transplantation. The results of this work are described in this thesis.

List of Publications

Budding K., van de Graaf E.A., Kardol-Hoefnagel T., Broen J.C., Kwakkel-van Erp J.M., Oudijk E-J. D., van Kessel D.A., Hack C.E., Otten H.G. A Promotor Polymorphism in the *CD59* Complement Regulatory Protein Gene in Donor Lungs Correlates With a Higher Risk for Chronic Rejection After Lung Transplantation. *Am J Transplant*. 2015 Oct 30. Doi: 10.1111/ajt.13497

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