Organ Transplantation Research Horizons

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Lung Transplantation

Therapies, Complications and Outcomes

Richard D. Ferguson
Craig A. Holmer
Editors

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LUNG TRANSPLANTATION: THERAPIES, COMPLICATIONS AND OUTCOMES

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Preface

Lung transplantation (LT) is the only definitive treatment for many forms of end-stage pulmonary diseases. However, its success is limited by several factors including organ infection/disease, acute rejection and chronic allograft dysfunction. Progresses made in patient selection, surgical techniques as well as in therapeutic management (immunosuppressive regimes) have led to a growing increase in the one-year survival rates up to 75%, however, the 5-year survival rate following LT remains only approximately 50%. This book presents research in the study of lung transplantation, including primary graft dysfunction in lung transplantation; the impact of viral pathogens in lung transplant patients; neurologic complications of lung transplantation; intensive care management of the lung transplantation patient and the surgical issues facing lung transplant surgeons.

Chapter 1 - Primary graft dysfunction (PGD) after lung transplantation is a major source of morbidity and mortality in the post operative patient. Clinical features of this early graft dysfunction include reduced gas exchange, reduced pulmonary compliance, and patchy pulmonary infiltrates seen on chest X-ray. PGD has also been found to be an independent predictor for the development and progression of bronchiolitis obliterans.

Multiple strategies have been developed to reduce primary graft dysfunction in lung transplantation. These include ventilatory techniques to reduce airway pressures both during procurement and implantation, development of improved organ preservation solutions, use of leukocyte filters on the cardiopulmonary bypass circuits, controlled reperfusion of the implanted organ, pharmacological agents to attenuate the inflammatory component of the ischemia reperfusion injury (IRI), antioxidants, surfactant, nitric oxide, prostacyclin and a variety of other agents.

In this chapter we will review the pathophysiology of PGD in lung transplantation, examine the evidence for the currently used measures and discuss the currently available management options.

Chapter 2 - Advances in patient and donor selection, ventilatory management, and improvements in the treatment of rejection and infections have made human lung transplantation an effective and acceptable option for patients with end-stage lung disease. However, many important factors, related both to an increasing "marginality" of the implanted graft and unexpected perioperative complications make immediate postoperative management still challenging and the early outcome unpredictable.

Intensive care treatment following lung transplant is focused on cardiovascular stabilization, respiratory assistance, adequate fluid management, infection prophylaxis, immunosuppression, active physiotherapy, and treatment of any organ dysfunction.

Early postoperative management is highly demanding as dramatic changes may occur on both the allograft and the "distant" organs. While satisfactory rates of survival have been obtained from multidisciplinary, collaborative efforts, significant hurdles have yet to be overcome, including issues of delayed postoperative hemodynamic recovery, severe hypoxia, acute allograft dysfunction, acute rejection, disseminate infections, adverse effects of multiple drugs, and surgical complications.

Even though the outcome of lung transplantation lags behind that of other solid organ transplants, an aggressive postoperative care is indispensable to treat allograft failure and prevent dysfunction of nonpulmonary organ systems. Skillful vigilance, a thorough knowledge of pathophysiologic characteristics of the transplanted lung, and early recognition of life-threatening clinical problems are fundamental for a successful ICU treatment.

Chapter 3 - Lung transplantation is the only definitive mode of treatment for many forms of end-stage pulmonary diseases; however, its success may be limited by several factors, including infections, acute rejection (AR), and chronic graft dysfunction termed bronchiolitis obliterans syndrome (BOS).

Viral infections of the graft (including those from community acquired respiratory viruses and from persistently infecting viruses, such as herpesviruses) are responsible for organ infection/disease; in addition to direct sequelae, accumulating data suggest that viruses may be triggers for a cascade of events, including upregulation of allo-reactive cells, potentially leading to AR or chronic graft dysfunction.

Community acquired respiratory viruses (CARV) have been increasingly recognized as common pathogens in lung transplantation (LT) and include the *paramyxoviridae* (respiratory syncytial virus, parainfluenza virus, human metapneumovirus), the *orthomyxoviridae* (influenza A and B), the *picornaviridae* (rhinovirus, enterovirus), the *coronaviridae* (coronavirus) and the *adenoviridae* (adenovirus). It has been suggested that LT recipients infected with CARV exhibit a high rate of progression to severe viral pneumonitis. Moreover, previous studies have evidenced that patients with CARV infection of the lower respiratory tract are predisposed to AR and high-grade BOS development, and, conversely, that patients with BOS are predisposed to CARV infections.

Herpesviruses, mainly human cytomegalovirus (HCMV), are highly seroprevalent and are considered as potential pathogens causing direct and indirect effects in transplant recipients and establishing latency in various tissue, including lung. Whereas HCMV represents the main viral pathogen responsible for organ infection and disease, the role of other herpesviruses, including human herpesviruses 6 and 7 (HHV-6 and HHV-7) and Epstein-Barr virus (EBV) is less defined. The role of herpesviruses reactivation in LT in relation to the development of AR and chronic graft dysfunction remains controversial, as it seems that despite high viral loads detected in bronchoalveolar lavages (BAL), virus replication results not associated with the development of rejection, however data are conflicting and few studies have specifically investigated this issue. In this Chapter, the impact of viral pathogens, including CARV and persistently infecting viruses, on the clinical course and the onset of rejection and graft dysfunction will be analyzed reporting the results of the main studies published in literature and the experience of our Laboratory of Virology.

Chapter 4 - The bronchiolitis obliterans syndrome (BOS) is considered to be the consequence of chronic lung allograft rejection, characterized histologically by airway epithelial cell (AEC) apoptosis and luminal fibrosis in the respiratory bronchioles causing airflow obstruction. Although the detailed etiology and pathogenesis of BOS are not clear, it has become evident that both the humoral and the cellular allogeneic immune response against AEC and endothelial cells, contributes significantly to the pathogenesis of BOS. It was demonstrated that the presence of allo-antibodies reacting with HLA and non-HLA antigens expressed on AEC may precede BOS development, suggesting that non-HLA antigenic systems may also play a role in chronic lung allograft rejection. These data are in line with results obtained in kidney transplantation, in which it was demonstrated that endothelial cell-reactive non-HLA antibodies could be found in sera of patients, which have suffered from hyperacute or acute kidney allograft rejection.

Identification of non-HLA antigens recognized by the patients' humoral immune system after lung transplantation provides insight in the immunopathogenesis of rejection and may lead to tailor-made immune suppression. Therefore, research has focussed towards new methods identifying non-HLA antibodies after solid organ transplantation. In literature, 3 methods have been described for identification of previously unknown antigens recognized by antibodies in the sera of patients after transplantation. One method is based on protein arrays. A second, recently described technique, uses SIMT which is an immunoprecipitation followed by Matrix-assisted laser desorption/ionization-Time-of-flight mass spectrometry (MALDI-TOF). The third method is the serologic analysis of antigens by recombinant expression cloning (SEREX), which has been applied on lung transplantation and is able to screen a very large spectrum of antigens expressed by a target tissue like the bronchus in a single screening. Here, we review the advantages and disadvantages of these large-scale screening techniques which can be used to identify antigens recognized by the immune system after lung transplantation (LTx), and provide a comprehensive overview of the antigens identified so far. In addition, the possibilities of identifying patients at risk for rejection using antibody-based screening procedures will be discussed.

Chapter 5 - The main surgical issues facing lung transplant surgeons today are access to the thorax, anastomotic problems and size mismatch of the lungs. As double lung transplantation becomes more popular, with survival advantage being demonstrated for more conditions, the clam shell incision is being increasingly utilized. However, problems with healing of the transverse sternotomy, particularly in immunocompromised patients, is a significant source of post operative morbidity. This chapter will review various techniques to improve sternal apposition and healing and discuss alternatives to the clam shell incision.

Dealing with anastomotic size discrepancy, and avoiding problems intra-operatively are of paramount importance when performing lung transplantation. This chapter will review techniques for dealing with inadequate cuffs at the venous and arterial anastomsoses and techniques for performing the bronchial anastomosis.

Size mismatch between donor and recipient is an important issue with paediatric and small adult recipients being disadvantaged on the waiting list. The use of lobar transplantation, non anatomical cut down and split lung transplantation has allowed larger donor lungs to be downsized for use in smaller recipients. There are also instances during surgery when donor lungs are larger than expected for the recipient and size reduction is required for an ideal fit. This chapter discusses the sizing issues that impact on outcomes in this group of patients.

Chapter 6 - Lung transplantation has proven to be an effective treatment for end-stage respiratory failure, but the post-transplant clinical course is still impeded by surgical and medical complications, and neurologic complications have been reported in up to 68% of lung transplant recipients.(1, 2) Complex pretransplant course and high immunosuppression requirements create an environment that increases the risk of neurologic morbidity after lung transplantation. Higher incidence of rejection with lung allografts than with most other solid organ allografts, generally requires greater chronic immunosuppression and persistent risk of opportunistic infections and immunosuppressant neurotoxicity. Increased frequency of neurologic complications has been reported in lung transplant recipients with cystic fibrosis.

Neurologic complications are a significant source of morbidity after lung transplantation, but the presence of neurologic complications is usually not associated with decreased survival. Most common etiologies include calcineurin inhibitor (CNI) neurotoxicity and opportunistic infections. Early onset of CNI neurotoxicity is attributable to high dosing needed to prevent early rejection and chronic immunosuppression increases the risk of systemic and CNS infections.

We will review clinical spectrum of neurologic complications after lung transplantation and diagnostic and treatment strategies.

Chapter 7 - The strong allo-immune response to the transplanted lung necessitates combined pharmacological immunosuppression to prevent graft rejection. Immunosuppressants used to prevent and treat rejection involve several classes of drugs and many target the production of pro-inflammatory cytokines by T cells, monocytes and other immune cells. Although most effective transplantation immunosuppressive strategies are based on interruption of IL-2 signaling by the calcineurin inhibitors cyclosporin A and tacrolimus, intensification of immunosupressive therapies has not led to any improvement in graft survival. Treatment with these drugs is also associated with serious adverse effects including specific organ toxicities, increased risk of developing a range of malignancies and susceptibility to infections. High inter- and intra-individual pharmacokinetic variability of both drugs may mean some patients do not require the high levels of drugs and associated adverse side effects for effective therapeutics. While current assessment of therapeutic drug levels simply involves the empirical measurement of plasma drug concentrations, there is a need for more physiological assessment of combined immunosuppression strategies, particularly at the site of action. Recent research has identified measurement of inflammatory cytokines at the cellular level using novel flow cytometric techniques as a strategy to assess the physiological response to treatment. Intracellular cytokine levels in both peripheral blood and in the airways have been investigated and have highlighted important differences in responses seen at the transplant site versus systemically. While Th1 pro-inflammatory cytokines were significantly reduced in blood T cells from transplant patients, levels of these cytokines in T cells from the airways were significantly greater in transplant patients compared with healthy control subjects. Furthermore, patients undergoing infection or rejection episodes were characterised by significantly decreased or increased Th1 intracellular T cell cytokines in the airways respectively, compared with stable lung transplant patients. To overcome patient inter-individual variability of leucocyte cytokine production, longitudinal monitoring of patient cytokines may be useful in predicting adverse episodes of rejection and/or infection. These techniques may complement or ultimately replace current standard approaches to therapeutic drug monitoring and monitoring by invasive biopsy and have the

potential to improve current immunosuppression protocols, optimise individual therapy and possibly provide new therapeutic options to improve the morbidity of lung transplant patients.

Chapter 8 - Lung transplantation has become established therapy in the treatment of selected patients with end stage lung diseases. However, five year survival after lung transplantation is little better than 50%, largely due to chronic graft failure. The basis of this failure is poorly understood but chronic rejection is probably a major factor. At the cellular level, graft rejection is associated with an increase in graft T-cell infiltration, alveolar macrophages, and pro-inflammatory cytokine expression. Although most effective transplantation immunosuppressive strategies are based on interruption of IL-2 signaling by calcineurin inhibitors, Cyclosporin A (CsA) and Tacrolimus (Tac), intensification of immuno-supressive therapies has not lead to any improvement in chronic graft failure. In addition, treatment with these drugs is associated with serious adverse side effects including specific organ toxicities, susceptibility to infections and an increased risk of developing a range of malignancies. Pharmacokinetic properties of both drugs show high inter- and intra-individual variability which may mean some patients do not require the high levels of drugs (that cause adverse side effects) for effective therapeutics. With the availability of novel flow cytometric techniques, recent research has focused on the measurement of inflammatory cytokines at the cellular level as a strategy to assess the physiological response to treatment. Importantly, cytokine levels in both peripheral blood and in the airways have been investigated, which has highlighted important differences in responses seen locally versus systemically. These techniques may complement or ultimately replace current standard approaches which rely on the measurement of plasma drug levels and monitoring by invasive biopsy. The application of these techniques has the potential to improve current immunosuppression protocols, optimise individual therapy and possibly provide new therapeutic options to improve the morbidity of lung transplant patients.

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Chapter 1

Primary Graft Dysfunction in Lung Transplantation

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Abstract

Primary graft dysfunction (PGD) after lung transplantation is a major source of morbidity and mortality in the post operative patient. Clinical features of this early graft dysfunction include reduced gas exchange, reduced pulmonary compliance, and patchy pulmonary infiltrates seen on chest X-ray. PGD has also been found to be an independent predictor for the development and progression of bronchiolitis obliterans.

Multiple strategies have been developed to reduce primary graft dysfunction in lung transplantation. These include ventilatory techniques to reduce airway pressures both during procurement and implantation, development of improved organ preservation solutions, use of leukocyte filters on the cardiopulmonary bypass circuits, controlled reperfusion of the implanted organ, pharmacological agents to attenuate the inflammatory component of the ischemia reperfusion injury (IRI), antioxidants, surfactant, nitric oxide, prostacyclin and a variety of other agents.

In this chapter we will review the pathophysiology of PGD in lung transplantation, examine the evidence for the currently used measures and discuss the currently available management options.

Introduction

Primary graft dysfunction (PGD) is the term used to describe the injury that occurs to an implanted organ as the result of ischemia-reperfusion injury(IRI). Primary graft dysfunction

after lung transplantation occurs within hours to days of the implantation. It manifests as hypoxaemia, pulmonary oedema and produces infiltrates on chest radiography. It is essentially, a diagnosis of exclusion. The definition and diagnosis were standardised in a consensus statement six years ago which should allow easier communication between centres regarding incidences and outcomes[1]. PGD has been the leading cause of early morbidity and mortality after lung transplantation. The injury is best managed by avoidance and most research is focussing on measures which are taken at the time of donor management, organ preservation and implantation. Once PGD occurs, the mainstay of management is supportive to allow the lung to recover from the reperfusion injury. This chapter will outline the current definition and diagnostic criteria, the pathogenesis and identified risk factors as well as the currently used preventative and management modalities.

Definition

Primary graft dysfunction (PGD) occurs to a variable degree after lung transplantation as a result of ischemia-reperfusion injury (IRI). IRI occurs when the restoration of blood supply to an ischemic organ (or cell) results in damage greater than expected due to ischemia alone. Endothelial cell damage, neutrophil sequestration, oxidative stress, complement activation and cellular pump failure contribute in differing degrees to graft dysfunction. The clinical impact seen is a failure of oxygenation, which can lead to both early and late morbidity as well as increasing mortality risk [2-6]. The current PGD definition was proposed by the International Society for Heart and Lung Transplantation (ISHLT) as a way of standardising the definition and providing a framework for future research [1]. The definition is based on the PaO_2/FiO_2 (P/F) ratio and infiltrates on CXR taken at several time points in the first 72 hours post transplant. PGD occurs in the absence of other causative factors, such as pulmonary vein anastomotic obstruction, cardiogenic oedema, hyperacute rejection and infection.

Pathogenesis

The mechanism of injury in IRI is a combination of oxidative stress, neutrophil sequestration, complement activation, endothelial cell damage, mitochondrial damage and cell membrane ion transport pump failure[7].

Role of Oxidative Stress

Two known mechanisms of oxidative stress in the lungs which lead to the production of reactive oxygen species are the accumulation of xanthine oxidase leading to the formation of superoxide radical after reoxygenation, and the NADPH dependent system (present mainly on the membrane surface of neutrophils, monocytes and macrophages) which produces hydrogen peroxide and superoxide anion from the ionic reduction of oxygen[7]. In lung, these oxygen reactive species also have a deleterious effect on lung surfactant production leading to

atelectasis and edema. When the production of oxygen radical species overwhelms endogenous antioxidant defences, cellular and tissue injury occurs.

Inhibitors of xanthine oxidase can block the superoxide radical production which occurs in the presence of anoxia[8]. The interaction between bioactive factors released during oxidative stress and cellular ischemia further up-regulates adhesion molecules of the pulmonary endothelium[8]. Such increase in density and avidity of adhesion molecules results in an "unwanted" accumulation of leukocytes (neutrophils and monocytes) in the lungs. During reperfusion, it is believed that the reactive oxygen species and proteases generated by leukocytes can initiate and amplify inflammatory cascades thus increasing lung damage[9]. This can be a direct ionizing effect of oxidative radical injury on cellular membrane and damage to the cellular ionic exchanger which maintains cellular homeostasis.

High intracellular calcium is a consequence of IRI[10] and hypothermic ischemic storage[8]. High cellular calcium enhances the conversion of xanthine dehydrogenase to xanthine oxidase, and in the process increases the damaging effect of free radicals on cellular organelles including mitochondria. Other effects of high intracellular calcium include the disruption of homeostatic intracellular processes. It has been demonstrated that the addition of a calcium channel blocker (verapamil, nifedepine or diltiazem) is protective against IRI (by the reduction of lipid peroxidation and endothelial damage)[8].

Role of Cytokines and Complement

Cytokines and complement also play an important role in the development of IRI[11]. This collective group of peptides from cell plasma and membranes modulate and play a key role in the body's defence. There are those that amplify inflammation such as tumour necrosis factor (TNF)-alpha and interleukins (IL-1, IL-6), those that are anti-inflammatory (eg. IL-10), those that activate other cells (eg. IL-12, IL-18) and those that induce proliferation of other cells (eg. IL-2)[8].

Equally complex is the role of complement in cellular injury in the "classic and alternate" pathway of injury and its interaction with cytokines[7,11]. Ischemia-reperfusion favours the release of pro-inflammatory peptides which has both local and systemic effects. Well known complement activation peptides include C3a and C5a; these are known as anaphylatoxins. There are also complement factors C5b-C9 which as a collective group, are known as the "membrane attack complex" capable of causing cell lysis by formation of pore channels via cellular membrane. These then lead to up-regulation of adhesion molecules and stimulate release of cytokines and platelet aggregating factors[7,11]. By understanding the role of complement, specific therapies (eg. complement receptor 1 agonist) have been used with some clinical success [11].

Cellular lipid injury as a result of oxidative stress releases bioactive factors in the process of cellular repair. These bioactive factors such as phospholipases function as inflammatory mediators by inducing the release of inflammatory peptides and lipids in other cells (eg. platelet activating factor) which in turn favours the production of other inflammatory and vasoactive agents. Examples of these include the production of thromboxane A_2 (vasoconstrictor, bronchoconstrictor), leukotriene B, C, D and E which can increase capillary permeability. In short this complex cascade affects not only local cellular responses, but also

leads to a systemic effect with further amplification and recruitment of other cellular defences such as leukocytes, macrophages and lymphocytes.

Role of Mitochondria

The mitochondrion is a sub-cellular organelle responsible for the generation of adenosine tri-phosphate (ATP). There is a paucity of mitochondria in lung, compared to other organs such as the heart, and as a result, little attention has been directed to investigate their role in lung I/R injury in the past. Detry and co-workers studied the respiratory function of isolated pulmonary mitochondria in a lung IRI model [12]. They demonstrated that prolonged hypothermic (4 degrees C) ischemia followed by normothermic reperfusion induces significant "lesions of the mitochondrial respiratory chain" [12,13]. Fukuse and co-workers studied the relationship between energy metabolism and mitochondrial damage during lung preservation [14]. Firstly, they noted that lungs preserved in an aerobically inflated cold preservation state outperformed lungs preserved in an anaerobic environment. Secondly they demonstrated increasing mitochondrial damage (ultrastructural examination) in lungs subjected to increasing ischemic stress. This was characterized by the degree of swelling or loss of cristae in the mitochondria. Thirdly, the increasing severity of IRI had a negative correlation with the total adenine nucleotide and ATP levels. Total adenine nucleotide and ATP levels were negatively correlated with impaired pulmonary function (shunt fractions). In summary, they placed a high value on functioning mitochondria in attenuating IRI [14].

Thus, IRI in the lung impairs the ability of the mitochondria to generate ATP. This leads to a further post-ischemic depletion of energy, and ATP-dependent cellular homeostasis fails leading to cellular necrosis and apoptosis.

Incidence and Diagnosis

The reported incidence of PGD post lung transplantation varies widely from 10-57% [2-6]. The reason for this is the wide variation in definition used before the standardised ISHLT definition was proposed. As a result it is difficult to compare reported incidences and outcomes to more recent publications.

This pathological change usually develops within the first three days following lung transplantation in absence of bacterial infection or rejection. One important physiological feature of early primary graft dysfunction is the impairment of oxygen transfer, characterized by the ratio of $Pa0_2$ to $Fi0_2$ [1,15]. This (P/F) ratio describes the ratio of arterial blood gas oxygen tension to the fraction of inspired oxygen. In clinical analysis, $Pa0_2$ / $Fi0_2$ taken between 6 and 12 hours after lung transplantation were useful indicators of transplantation outcomes. There was a moderate correlation (P/F ratio) with the duration of intubation ($r = -0.44$, $r^2 = 0.19$), length of intensive care unit stay ($r= -0.38$) and a thirty-day mortality [15].

Early (within 72 hours) dysfunction of transplanted lungs should alert the clinician to the possibility of PGD. Prior to the standardisation of nomenclature in 2004 by the International Society of Heart Lung Transplantation [1], the syndrome was known by various other names, such as reimplantation oedema, reimplantation response, reperfusion injury, reperfusion

oedema, primary graft failure and early graft dysfunction. Currently, the standardised definition is based on the measurement of the P/F ratio and presence of CXR infiltrates at time point 0-6 hours, 24, 48 and 72 hours post transplantation. The diagnosis of primary graft dysfunction following lung transplantation is made primarily on two factors – hypoxaemia and infiltrates on chest X ray.

Hypoxaemia

The hypoxaemia of PGD is quantified by the use of the ratio of arterial partial pressure of oxygen to the fraction of inspired oxygen (the P/F ratio). Generally, a P/ F ratio of greater than 300 is considered acceptable, and less than 200 is considered severely dysfunctional.

The measurement of PaO_2 is done at multiple time points following the lung transplant, however ideally the first measurement ("T zero") is made within 6 hours of lung reperfusion, with FiO_2 1.0 and peak end expiratory pressure (PEEP) of 5 cm H_2O. At the extremes of the P/F ratio the measurement may be less useful as there may be confounding factors such as the use of PEEP. Additionally, at the low end of FiO_2, an otherwise acceptable PaO2 may still result in a borderline P/F ratio. Thus it has been suggested that all extubated patients should be graded as severity grade 0 or 1[1,16]. The other caveat is that any patient on extracorporeal membrane oxygenation is automatically considered Grade 3.

Radiographic Changes

The other criteria in the diagnosis is the presence of infiltrates on plain chest radiography. There have been attempts to grade the severity of infiltrates on X ray to correlate with clinical outcomes, such as by Kahn et. al, who devised a grading system based on the number of radiographic "zones" involved in the infiltrative process [6]. They found that increasing radiological severity (as "pulmonary reimplantation response") correlated with lower P/F ratio and an increased ICU stay and duration of ventilation. However in their analysis, the presence of pulmonary reimplantation response did not appear to have any impact on long term survival.

The difficulty with using a grading system for pulmonary infiltrates is the inherent subjectiveness of the system. Previous trials looking at ARDS demonstrated significant interobserver variability between clinicians when grading alveolar consolidation in different quadrants of the CXR [17]. However, others have shown that there can be moderate agreement between observers in the grading of diffuse alveolar infiltrates, which can be improved with training [18].

Grading System of PGD

Thus, there is a spectrum of PGD which ranges from mild disease through to severe. The consensus definition has classified the severity into 4 grades from 0 to 3, depending on the P/ F ratio and the presence or absence of pulmonary infiltrates on CXR (Table 1)[1].

Table 1. Grading of Primary Graft Dysfunction in Lung Transplant [1]

Grade	P/F ratio	Chest Infiltrate
0	> 300	Absent
1	> 300	Present
2	200-300	Present
3	< 200	Present

Exclusion Criteria

Factors that are important to exclude prior to making the diagnosis of PGD are the absence of factors like cardiogenic oedema, venous anastomotic obstruction, hyperacute rejection and infection.

Refinements

As PGD is a clinical syndrome, prior to the ISHLT consensus, various groups used several criteria additional to the ones mentioned above in their diagnosis. Several refinements have also been suggested after the consensus was issued, aiming to improve the discriminatory ability of the grading system in identifying patients at risk for poor outcome.

Oto et al., recognised that there was much higher prevalence of PGD grade 3 in single lung transplantation compared to bilateral lung transplantation (up to 26% at 24hrs for single lung transplant vs 7% for bilateral) [19]. The authors suggest that due to ventilation perfusion mismatch in the native lung, the P/F ratio in single lung transplantation is usually lower than in bilateral. Thus the severity of PGD was higher in the single lung transplants, despite a significant number of single lung transplant patients being extubated within 6 hours of their surgery. The conclusion was that single and double lung transplants should be considered for PGD grade separately.

Prior to the development of the standardised definition, use of the oxygenation index was proposed as a predictor of post operative graft dysfunction. Calculated by the equation

$$\frac{\text{Mean airway pressure x percentage of inspired oxygen}}{\text{Partial pressure of arterial oxygen}}$$

an index of > 10 indicates severe pulmonary dysfunction and > 30 indicates need for ECMO.

Such an index takes into account variables such as extubation and peak end expiratory pressure used in invasive ventilatory settings [20].

Risk Factors

The risk factors for development of PGD can broadly be broken down into three categories – factors related to the donor, factors related to the recipient and the factors related to the operation (both the procurement and the implant).

Donor Factors

Due to the lack of organ donors, there has been an increasing push to use organs from older donord. The extension of previously established criteria for donors has resulted in an increased donor pool, of "marginal donor" organs [21]. In view of static or even declining organ donation rates, the use of marginal donor organs has been important in maximising lung transplant opportunities [22]. Factors such as increasing age and smoking history which previously would have excluded donors have been investigated as to their impact on the development of PGD.

Whitson et al., identified increasing donor age as a risk factor for development of PGD [23]. That analysis of 402 consecutive lung transplant patients found that donor age greater than 32 years was associated with a higher incidence of grade 3 PGD (42% vs. 29%). Organs from donors greater than 55 years of age were almost 4 times more likely to develop severe PGD, with a suggestion of a linear relationship between age and likelihood of severe PGD starting from donor age 35 years and upward. This concurs with the experience at the Alfred Hospital, Australia, which found a correlation between increasing donor age and recipient P/F ratio [24].

Christie and colleagues analysed 255 lung transplant procedures and could not find an association between donor smoking history and PGD [25]. However the study was limited in that they analysed smoking history as a dichotomous variable. In contrast to this finding, the group at the Alfred Hospital, Australia performed a more in depth analysis, looking at 161 transplant patients and quantifying the donor smoking history in terms of current versus former smoker, and number of cigarettes per day or pack year smoking history [26]. They were able to demonstrate that the recipients who received smokers' organs had a lower P/F ratio, and increased time on invasive mechanical ventilation and intensive care unit stay. Additionally, they were able to show that the effects were dose dependant.

The presence of bacteria on bronchoalveolar lavage may indicate ongoing pneumonia, and is correlated with lower P/F ratio and longer duration on mechanical ventilation [27]. Not surprisingly, aspiration and prolonged mechanical ventilation have also been identified as potential risk factors for PGD.

Donor female gender has been correlated with PGD, with the potential explanation relating to size mismatch between donor and recipient. Donor African American status has also been shown to be associated with higher rate of PGD [25].

An additional useful tool in determining the risk of PGD based on the donor is donor pulmonary vein gas. At the time of organ procurement, with the donor being ventilated with 100% oxygen, blood from the superior and inferior pulmonary veins is aspirated. A pulmonary vein gas of less than 300mmHg was 2.3 times more likely to suffer from PGD in a study from the UK [28].

Haemodynamic instability during donor procurement, although not studied directly with relation to lung transplantation, has been shown to adversely affect kidney and liver grafts. It would be logical to assume that a similar deleterious effect would be present with the pulmonary graft.

There are also various biological markers on the horizon that may be useful in determining organs which are at risk of developing PGD. Interleukin 8 (IL-8) has been shown to be upregulated in donor lung tissue and bronchoalveolar lavage sample and is correlated with increased incidence of PGD [29]. Additionally the IL-6 to IL-10 ratio has been shown to correlate with increased PGD [30].

Recipient Risk Factors

Various risk factors in relation to the recipient and PGD have been proposed, however none have been conclusively shown to be related. These include:

Obesity

A report from the UK cardiothoracic transplant audit found that a high recipient BMI was modestly associated with an increased risk of death from PGD (odds ratio 1.10) [31]. Two other studies looked at recipient body weight and identified them as risk factors for mortality and prolonged ventilation, however they did not look specifically at the development of PGD [32,33].

Hepatic Impairment (Including Cystic Fibrosis Related Liver Disease)

Whether hepatic impairment impacts on PGD specifically is yet to be studied. There does seem to be a correlation between liver dysfunction and early post transplant mortality [34]. The impact appears to be dependant on the aetiology of the liver dysfunction whether from right heart failure secondary to pulmonary hypertension or from cystic fibrosis liver disease, which may not be as significant[35].

There is little data to support whether other co-morbid diseases such as renal impairment, left ventricular dysfunction or diabetes increases the incidence or severity of primary graft dysfunction [36]. The ISHLT registry studies have correlated these factors to early mortality, however the impact on PGD remains unclear.

ABO compatible graft versus ABO identical graft - Yu and colleagues studied whether there was a difference in outcome, including primary graft dysfunction, in patients who received an ABO identical graft, versus those who received an ABO compatible graft [37]. Of the 100 patients studied, 64 were ABO identical and 36 ABO compatible. No difference in PGD incidence was observed.

Medications

Although medications such as steroids may increase the risk of airway anastomotic complications [38,39], no link has been made between any particular medication and the development of PGD.

The effect of prior thoracic surgery, pleural adhesions or mechanical ventilation again has not been specifically proven to increase PGD, however they may increase the risk of

development of PGD via secondary means such as increased operative bleeding and transfusion requirement. Interestingly, a report by Hadjiliadis et al looking at patients undergoing lung transplant with pulmonary mycetomas found that three of the nine patients reported died of PGD [40]. The pleural reaction from mycetoma may explain this occurrence [36].

Level of Inotropic Support

Pilcher et al., from the Alfred Hospital, Melbourne Australia, found that a high recipient inotropic requirement was associated with poorer P/F ratio [24]. The explanation of this finding was a combination of mechanisms including that deteriorating oxygenation prompted the use of fluid restriction, diuretics and a consequent need for vasopressors. Patients who were on inotropes for poor LV function may have had a high pulmonary wedge pressure and subsequent poor oxygenation. Finally patients who may have had a severe systemic inflammatory response and increased pulmonary capillary permeability would require high inotropes and have poor oxygenation. This correlates with the UK data also demonstrating a 1.92 fold increase in death from PGD in recipients who required inotropic support [31].

Chronic obstructive airways disease versus cystic fibrosis - Studies in this area are inconclusive, however there seems to be a suggestion that patients who undergo transplantation for chronic obstructive pulmonary disease have the lowest risk of PGD, with rates reported as low as 3% [25,42]. Patients who are transplanted for cystic fibrosis have an intermediate risk, with an odds ratio of 2.3 of developing PGD [25].

The strongest recipient related variable appears to be lung transplantation for primary pulmonary hypertension. The retrospective analysis by Christie et al indicated a 4.4 fold increase in the rate of PGD in recipients with primary pulmonary hypertension [25]. Raised pulmonary artery pressures have also been identified by other groups as increasing the risk of post operative PGD [23]. Several mechanisms have been postulated including the requirement for blood products to reverse anticoagulation preoperatively, or an intrinsic inability to handle the stress of lung transplantation perhaps relating to the underlying pathophysiology of primary pulmonary hypertension.

Operative Risk Factors

Graft ischaemic time has been proposed as a significant risk factor for development of PGD and subsequent mortality. Thabut et al., recently completed a multicenter analysis of 752 patients who underwent single or bilateral lung transplantation [43]. After adjustment for confounding variables, a 1 hour increase in graft ischemic time correlated to an absolute decrease in P/F ratio of 13.3 in single lung transplant, and 20.8 in bilateral lung transplant. There was also a marked decrease in overall long term survival in patients who had transplantation with ischemic time organs greater than 330 minutes. This confirms the findings of others whereby an ischemic time of greater than five hours was associated with decreased long term survival [44].

There may also be an association between the use of cardiopulmonary bypass during transplantation and PGD. However the effect is unclear as it may be that the results are confounded by the patient's severity of illness and operative difficulty. There is conflicting evidence as to whether in itself CPB contributes to PGD [24,41]. Of the 128 patients analysed

in the Alfred hospital data from Pilcher et al, 11 required cardiopulmonary bypass with a mean bypass time of 172 minutes [24]. They did not find that cardiopulmonary bypass correlated with poor recipient oxygenation. This is in conflict with the findings of other groups [45]. They report on 100 lung transplant patients of which 55 patients were done with cardiopulmonary bypass, with a mean bypass time of 186 minutes. They found that patients who underwent bypass had poorer oxygenation (measured using the alveolar/arterial oxygen tension ratio) as well as more severe pulmonary infiltrates, and worse 1 month survival. There is also a suggestion that cardiopulmonary bypass may actually be protective in certain circumstances. A group from the Netherlands reported on 62 patients who underwent lung transplant, 35 of whom were done with cardiopulmonary bypass [46]. In a subgroup of 28 patients with a primary diagnosis of emphysema, who when transplanted had two HLA-DR mismatches between donor and recipient, 14 out of 19 who were alive at 2 years had their transplant on bypass. The authors suggest a protective effect due to the immunosuppression on bypass, stating that the patients who died did so from graft failure, due to a combination of rejection, infection or chronic rejection.

Prevention

Understanding the pathological processes that contribute to primary graft dysfunction, allows us to tailor therapies to either prevent or ameliorate the impact of such injury. There are many potential approaches to minimizing lung dysfunction after transplantation. However, although many of these techniques yield good results in laboratory experiments, it has been more difficult to demonstrate significant improvements clinically. Attempts to minimize IRI in lung transplantation have focused on donor management, lung preservation, anti-oxidants and other pharmacological agents [47], surfactant [48,49], leukocyte depletion [50-51] and interventions during surgical implantation such as low pressure reperfusion [52-54, combined protective ventilation and perfusion [55], and other pharmacological agents [56,57].

Donor Management

Specific donor management protocols are able to increase the available donor pool without compromising survival of recipients [58]. Controlled studies on humans involving donor management to prevent PGD are scarce. Alvarez and colleagues undertook a stepwise analysis of 476 human lung donors and concluded that little has been done in improving donor management at the early stages of brain death [60]. They identified 3 stages involved in assessment of donor lungs for transplantation:

Stage 1: Assessment of donor suitability prior to operative inspection
Stage 2: Assessment of donor lungs at operative inspection prior to procurement
Stage 3: Assessment of donor lungs following transport just prior to implantation.

It was recommended that interventions for the optimal management of brain dead donors should be started at the time of brain death declaration rather than at the time of donor offer [60]. Various factors may contribute to lung injury in the donor even before brain death occurs including pneumonia, traumatic lung injury, aspiration, ventilator-induced injury and pulmonary embolism. Management of these can extend the pool of donors [61-63].

The physiological changes associated with donor brain death and their management have important implications for prevention of PGD of lungs. In a pig lung model, injury to the donor lung was more apparent in brain-dead donors compared with cardiac-death donors [64]. Brain death leads to significant changes in the cardiovascular, respiratory and endocrine systems.

Blood Pressure Management

Following brain death, neurogenic vasoplegia occurs due to sympathetic overdrive followed by a prolonged period of hypotension. The rise in systemic vascular resistance leads to a decrease in left ventricular output and increased left atrial pressure, but concomitant increased venous return will increase right ventricular output [65]. Due to the differing implications for the right and left heart, it is prudent to monitor not only the central venous pressure but also place a Swan-Ganz catheter for monitoring pulmonary artery pressures [66].The increased blood flow to the lungs eventually leads to neurogenic pulmonary oedema [67] which may be exacerbated by excessive fluid administration. This process of hemodynamic instability is an important contributor to IRI and PGD in the recipient. Extravascular lung water index measurements are useful in assessing pulmonary oedema and its measurement and manipulation could be important in the management of lung donors [68]. Normal alveolar fluid clearance helps to resolve pulmonary oedema post-transplantation [69]. At the same time, a systemic inflammatory state develops with neutrophilic infiltration and increased IL-8 levels in bronchoalveolar lavage which has been shown to correlate with acute lung injury [70].

Experimental techniques in rats have revealed the need to aggressively treat hypertension following donor brain death and that the use of alpha adrenergic blockers lead to less IRI [71]. Catecholamine use may also decrease IRI by modulating adhesion of leukocytes [72]. However, Mukadam and coworkers performed a retrospective review of 60 human lung transplants and found that the exogenous administration of catecholamines led to worse oxygenation and graft function for the recipient [73]. Although noradrenaline lessens the inflammatory response following brain death it has unfavourable effects on the donor heart. A favourable vasopressor in terms of correcting neurogenic hypotension appears to be low-dose vasopressin. Rostron et al. found that arginine vasopressin was as efficacious as noradrenaline in reduced pulmonary capillary leak, and pulmonary oedema [74].

Ventilation

Pilcher and colleagues at the Alfred Hospital, Melbourne, Australia reviewed 128 human lung transplants and found that low donor oxygenation was predictive of primary graft dysfunction [24]. The commonest physiological mechanisms leading to hypoxemia in the

donor include ventilation-perfusion mismatch, abnormal oxygen diffusion and hypoventilation [75].

Hypoxemia can be treated by adjusting FiO_2 or airway pressures during mechanical ventilation [76], but more importantly by addressing the underlying cause. Therefore treatment of pneumonia, bronchospasm, and mucus plugging are important as are routine supportive measures such as suctioning and side-to-side turning. Expectorants administered orally are thought not to act rapidly enough to be of use to the donor, especially if there is humidification of inspired gas [75]. Altering the position of the donor to use gravity to promote airway clearance is often difficult in the presence of hemodynamic instability. High-frequency chest wall oscillation has been documented as a strategy in donor management to promote removal of secretions, but its effectiveness has not been found to be statistically significant [77].

Inspiratory manoeuvres to recruit closed airways arising from lung collapse due to ventilation in a supine position are an important ventilator strategy in optimal donor management. To prevent alveolar collapse, an appropriate level of PEEP should be used [78]. An example of a recruitment strategy would be to use pressure-controlled ventilation with inspiratory pressures of 25 cm H_2O and PEEP of 15 cm H_2O for 2 hours then changing to volume-controlled ventilation with a tidal volume of 10 ml/kg and PEEP of 5 cm H_2O [79]. Excessively high tidal volumes could lead to barotrauma and injured lung. There are no prospective randomised controlled trials in humans comparing the optimal ventilator strategy.

Treatment of oxygen diffusion abnormalities involves improving cardiac output, using diuretics and administering colloids to support plasma oncotic pressure [75]. Furthermore, normal alveolar fluid clearance improves gas exchange. Ware and colleagues demonstrated that aerosolized β_2-adrenergic agonists improve alveolar fluid clearance in a study involving 31 human donor lungs previously deemed unsuitable for procurement [80]. Donor treatment with low-dose dopamine was associated with faster clearance of alveolar fluid [80]. Administration of diuretics was associated with lower extravascular lung water in explanted lungs.

Bronchoscopy

Bronchoscopy is an important tool in diagnosing and also treating potential causes of respiratory problems in the donor. Bronchoscopy can detect malposition of an endotracheal tube and allows for removal of sputum or blood clots causing obstruction, directly from the larger airways [81]. Bronchoscopy should be routinely performed on all potential lung donors to assess for airway damage and visible signs of infection. The utility of bronchoalvelor lavage during bronchoscopy has been debated [8,23]. Whitson et al., state that there was no difference in development of grade 3 PGD in recipients' lungs from donors with positive versus negative gram-stain results [23]. However, bronchoalveolar lavage of the donor lung can help to tailor antibiotic therapy in the donor and later the recipient.

Hormonal Resuscitation

Another consequence of brain death is the reduction in levels of cortisol, insulin, thyroid hormones and antidiuretic hormones. Aggressive hormonal replacement with a regimen involving a methylprednisolone bolus and vasopressin infusion followed by either triiodothyronine or L-thyroxine has been shown to improve donor availability [66]. However, whether or not this correlates with improved graft function of the lungs is still contentious. Follette et al. conducted a retrospective review of 118 consecutive lung donors of whom 80 patients received high dose steroids (approximately 15mg/kg of methylprednisolone)[82]. The donors treated with steroids had significantly better PF ratios than the control group. Venkateswaran and coworkers performed a placebo-controlled randomised controlled trial on 182 lung donors using various combinations of methylprednisolone and triiodothyronine [81]. They reported no benefit of hormonal administration of either agent alone or in combination on the PF ratio in the recipients.

Preservation Solutions

Current recommended lung preservation techniques include infusion of the cold preservation solution via the pulmonary artery followed by excision and immersion of the "inflated lung" (with approximately 50% oxygen in the alveoli) in an appropriate preservation solution with a storage temperature of 4- 8 ^{0}C [8]. The alveolar oxygen allows the lungs to maintain aerobic cellular metabolism. Therefore during this preservation process, the lung is largely subjected to ischemic insult (lack of blood flow) rather than pure hypoxia (lack of oxygen). Traditionally intracellular type solutions such as Euro-Collins and University of Wisconsin preservation solutions have been used as lung transplantation has evolved. Euro-Collins solution was however originally developed for kidney transplantation. Papworth preservation solution is an extra-cellular type solution comprising Ringers solution (500ml/l), albumin (200ml/l), Mannitol (100ml/l), donor blood (300ml/l) and heparin (8000IU/l) [83]. This solution has been shown to be effective in preserving lung tissue to a satisfactory level for up to four hours after harvest [84,85]. More recently, a low potassium dextran extracellular type solution (Perfadex [Vitrolife, Gothenburg, Sweden]) has been specifically developed for lung transplantation and is rapidly superseding other solutions in transplant centres around the world [86]. However, despite promising experimental data, its use has failed to convincingly improve results [87]. A recent national cohort study from the United Kingdom showed no differences in death due to PGD between three groups using Euro-Collins, low potassium dextran solution (Perfadex) and blood albumin preservation [31]. That study also showed no significant differences in early or mid-term survival between the three groups. A review of five clinical studies comparing Perfadex to Euro-Collins concluded that Perfadex does improve early graft function after lung transplantation (as measured by oxygenation, duration of mechanical ventilation, improved reperfusion scores and improved compliance)[87]. However, all of the studies included were non randomized, four of the five were retrospective and all had very small numbers. More clinical data is required to further assess this particular solution.

Intraoperative Anaesthetic Management

As the pulmonary allograft is very sensitive to pulmonary oedema, fluid restriction and protective lung ventilation strategies should be implemented. These include use of a smaller tidal volume, at 6–8ml/kg, use of low FiO2 compatible with adequate oxygenation, and PEEP 5–15cmH2O. Optimal fluid management can be guided by judicious fluid replacement aiming for a central venous pressure of less than 7 cmH2O, with systemic perfusion supported by vasoactive infusions. Excessive bleeding and a need for blood transfusion may also threaten the new lung allograft. There is some evidence that blood transfusion may increase the risk of infection and other complications. There should be a low threshold for the use of intra-operative cell salvage [88].

McIlroy and colleagues, in a recent observational study explored potential anaesthetic factors that could influence the incidence of PGD after lung transplantation [88]. In 107 consecutive patients undergoing lung transplantation, anaesthetic factors such as epidural catheterisation, fluid administration, blood and blood product administration and central venous pressure were analysed using multivariate regression. Increasing volume of intra-operative colloid was independently associated factor with lower P/F ratio [88]. Currey and colleagues developed an evidence-based guideline for the ICU management of patients after lung transplantation [89]. The guidelines involve the use of respiratory and haemodynamic management algorithms targeting a central venous pressure < 7 mmHg and early extubation if P/F ratios were >200 mmHg during the first 72 hours. The implementation of these guidelines resulted in improved P/F ratios, lower PGD grade, lower post-operative fluid balances and vasopressor doses when compared with historical controls at their institution [90].

Operative Techniques

Delivery of Preservation Solution

Antegrade Flush

The dual blood supply of the lung has implications for delivery of preservation solution (pneumoplegia) to the donor lungs. It is common practice to deliver cold pneumoplegia in an antegrade fashion by flushing the pulmonary arteries through the main pulmonary trunk [91]. This may require donor pre-treatment with a prostaglandin analogue (PGE1 or prostacyclin) to overcome pulmonary vasoconstriction resulting from hypothermia and hyperkalemia [92]. Pulmonary vasoconstriction may result in unequal distribution of preservation solution to the donor lung. A major drawback of the antegrade flush technique is a failure to adequately perfuse the bronchial circulation [93]. Unexpected donor pulmonary arterial emboli have been highlighted as a contributive factor to PGD [62,94,98] and the presence of these hinders complete antegrade flushing of the pulmonary arteries.

Retrograde Flush

The first report of pneumoplegia delivered solely through a retrograde route via the left atrium and pulmonary veins at the time of donor procurement was in 1993 [93]. This technique not only delivers solution to the pulmonary vasculature but also to the bronchial

vessels as evidenced by flush pouring out of the bronchial ostia in the descending aorta [93]. Retrograde flush should provide more complete protection to the tracheobronchial mucosa thereby promoting bronchial healing and provides more flushing to the poorly ventilated areas of the lung such as the posterior segments of the lower lobes [79]. Retrograde flush is also reported to be more protective of surfactant function [95]. By limiting the amount of pulmonary vasoconstriction, this method of preservation solution delivery also obviates the need for concomitant administration of pulmonary artery vasodilators [96]. However, treatment with PGE1 in the setting of retrograde flush has been shown to be beneficial for dynamic pulmonary compliance following reperfusion [97]. Retrograde flush can also help to clear out pulmonary emboli and can further protect damaged endothelium which may predispose to later emboli [94].

The optimal timing and strategy for delivery of the retrograde flush remains controversial. It may be administered at the time of donor lung procurement instead of the antegrade flush or in addition to the antegrade flush. Furthermore, it may be delivered at the time of preimplantation, "on the backtable". A late flush with Carolina rinse solute has been shown to limit cell death in pulmonary allografts [99]. This preimplantation late retrograde flush can be performed to further optimise detection of unexpected pulmonary emboli (fat, blood clot, brain tissue emboli) which have not been detected in the pre-operative phase or time of procurement [36,62]. Although there may be no macroscopic evidence of thromboemboli or fat emboli from a retrograde flush prior to implantation, analysis of supernatant of the flush, for example, may reveal fat droplets which could potentially cause PGD [94].

Retrograde flush instead of antegrade flush at the time of procurement has been deemed superior in various animal studies [95,100-102]. Kofidis et al 2003 performed single lung transplantation in 12 pigs, with 6 donor pigs receiving antegrade perfusion at the time of procurement, and the other 6 donor pigs receiving retrograde perfusion [102]. The respiratory function outcomes were better in the group that received retrograde flush perfusion. It has been proposed that the mechanism is due to greater resistance during antegrade perfusion and a more uniform distribution during retrograde perfusion [93]. More recently experiments in pigs to simulate non-beating heart donors demonstrated the superiority of retrograde flush in terms of less microthrombi, lower pulmonary vascular resistance and more complete clearance of residual blood [103,104].

A few trials have examined the use of a late retrograde flush at the time of implantation as an adjunctive strategy to antegrade flush at the time of procurement. Venuta et al. 1999 performed a prospective randomised controlled trial of 14 patients to investigate the additional benefit of a preimplantation retrograde flush in donor lungs that had been antegradely flushed at the time of procurement [105]. In that study, there was no use of retrograde flushing at the time of procurement. The preimplantation retrograde flush led to lower mean airway pressures, improved early oxygenation, faster extubation and improved CXR appearances. Ferraro et al. 2008 performed a retrospective review involving 153 patients, with 23 patients receiving a retrograde flush preimplantation [106]. In contradistinction to the findings of Venuta et al., this group demonstrated that retrograde perfusion at the time of implantation does not decrease the severity of PGD. The Alfred Hospital, Melbourne, Australia, previously reported utilising an exploratory late retrograde flush with Cold Ringer's solution in 74 human donor lungs to detect unexpected pulmonary embolism in 38% with 28% being clot and 9 % fat [62]. Those with such emboli were

reported to have significantly worse oxygenation, worse CXR appearances, increased pulmonary vascular resistance, prolonged intubation, increased ICU stay and decreased 1year transplant survival. Multivariate analysis revealed pulmonary embolism as an independent factor for prolonged intubaton [62].

A strategy which involves a combination of antegrade and retrograde flush at the time of donor procurement is becoming routine in many transplant units despite the lack of supportive evidence from prospective randomised controlled trials in humans [79]. In some institutions, this may be followed by a second exploratory retrograde flush prior to implantation. Interestingly, experiments in pigs have demonstrated a preservation advantage using a combined antegrade flush of the pulmonary and bronchial arteries over strategies involving retrograde flush [107,108].

Volume and Pressure of Flush

One reason put forth for the favourable results of retrograde flushing over antegrade flushing in animal studies is the inherent pressure differences between the two routes [101,109]. The retrograde pulmonary venous compartment is more distensible which could allow for more vascular recruitment compared with the high vascular resistance in the arterial side of the vascular bed.

Consideration of the actual pressure generated during delivery of the flush solution is also important in minimising lung tissue damage. Haverich et al. 1986 reported that a large volume of perfusate flushed at a high flow rate (60ml/kg over 4 minutes) in mongrel dog lungs resulted in better lung function following reperfusion [52]. There was no monitoring of pressures obtained in the pulmonary artery by this method. Yamazaki et al. 1990 in their investigation of preservation solution using donor rabbit lungs maintained a flushing pressure which was less than 25 mmHg, whilst Bresticker et al. 1992 safely used pressures of 15mmHg in mongrel dogs [53,54].

Sasaki et al. reported reperfusion injury in preserved rabbit lungs that were antegradely flushed with preservation solution at pressures greater than 20mmHg [110]. This was later explained by the interference of endogenous nitric oxide producing ability of the donor lung [111]. The optimal flush pressure was found to be 10 to 15mmHg with lower pressure of 5mmHg leading to inadequate flushing of the capillary bed and worse lung function. Schumann et al. used a porcine model to demonstrate that high antegrade flush perfusion pressures are the most important determinant of lung oedema formation in their comparison of 41 mmHg versus 27mmHg [112]. Despite these various experiments there is yet to be consensus on the optimal flushing pressure for organ preservation. It is proposed that the flushing pressure should be no more than 20mmHg [113].

Temperature of Flush

Flushing with solutions at temperatures of 10^{o}C or lower has been shown in multiple small animal models to be detrimental to graft function when compared with flush temperatures between 15 and 23^{o}C [114-116]. It is hypothesised that hypothermic solutions can injure the endothelial-epithelial gas exchange barrier [117]. However, it has been argued that the contribution of hypothermia to this injury is minimal when compared with the effect of ischemia [118] and therefore the recommendation is for a hypothermic preservation solution of 4 to 8^{o}C [119].

Storage Considerations

Ventilation and Inflation of Lung Prior to Storage

Atelectasis results in maldistribution of lung preservation solutions which emphasises the importance of inflation of the lung prior to delivery of pneumoplegia. Inflated lungs have better compliance and secretion of surfactant as well as improved fluid clearance. Donor lung hyperinflation prior to storage or high FiO_2 can lead to pulmonary vascular injury and increased capillary permeability [120]. It is important to avoid barotrauma induced by high tidal volumes and high PEEP. Therefore it has been recommended that the degree of lung inflation should be no more than 50% of the total lung capacity [121] or to an airway pressure of 10 mmHg [122].

Ventilation of the lungs with oxygen during the ischemic period is thought to be beneficial by providing some degree of aerobic metabolism, preventing alveolar collapse and maintaining cyclic "stretch" of the alveolar structures [123]. Furthermore it protects the integrity of the pulmonary surfactant and preserves epithelial fluid transport [119]. Kao et al. 2004 studied 96 male rats and concluded that static inflation attenuated IRI, irrespective of ventilation with oxygen or nitrogen, thereby postulating improved graft function from a mechanical mechanism and not an aerobic mechanism [124]. In fact, ventilation alone without supplying oxygen has been shown to be even more protective than static inflation [125]. During storage, an FiO2 of greater than 50% can actually be harmful by resulting in lipid peroxidation [122,126].

Temperature of Storage

There are mixed results from experimental studies regarding the optimal temperature for storage of donor lungs. Although storage at 10°C has been shown to be superior to 4°C [122,126], another study found no difference in outcomes between these two temperatures [127]. Storage at 10°C is better than at 15°C [122], however the risk of free radical-mediated vascular injury increases as temperature increases to moderate hypothermia above 10°C [128].

Technique of Implantation

Protective Reperfusion and Ventilation

Bharbra and co-workers [128] evaluated the impact and minimum duration of low pressure reperfusion of rat lungs and found that 10 minutes of low pressure reperfusion (50% of physiological pulmonary pressure) resulted in significant improvements in pulmonary function. The significance of lowering reperfusion pressure was confirmed by Halldorsson and co-workers [129] in porcine studies looking at different reperfusion pressures. They found that the group with lower reperfusion pressures (20-30 mmHg) had outperformed the group with reperfusion pressures of 40-50 mmHg.

To date there has been a few clinical trials in humans with promising results. Two studies conducted initially demonstrated the feasibility of setting up an extra corporeal circuit and reperfusing the transplanted lungs under low pressure for 10 minutes with modified leukodepleted contents [130-131]. These trials subsequently led to a larger human trial conducted with retrospective controls (23 patients). In the larger trial by Ardehali and co-workers, the combination of all modalities reduced the incidence of reperfusion injury, shortened the duration of ventilation and hence the duration of intensive care stay [132].

De Perrot et al. investigated two strategies of mechanical ventilation in rats receiving a single lung transplant and showed that those who had higher tidal volumes at low PEEP had worse lung function compared to those who had lower tidal volumes and PEEP adjusted to minimise pulmonary stress [133]. Hyperoxic ventilation also worsens lung reperfusion injury indicating that minimal FiO_2 should be employed [134].

Singh et al. used a rabbit lung model and investigated a combination of controlled ventilation and perfusion by gradually increasing perfusion and ventilation over 5 minutes to 60ml/minute and 1.8 Litres/minute respectively [55]. In the conventional reperfusion and ventilation group the respective flows were immediately instituted at 60 ml/minute and 1.8 Litres/minute. The protective approach led to significant improvement in lung function.

The Toronto Group gradually reinflate the implanted lung at an airway pressure of 20cmH_2O prior to reperfusion and then use pressure-controlled ventilation with peak airway pressures up to 25 cmH_2O with FiO2 of 0.5 and PEEP 5 cmH_2O (Table 2)[8].

Table 2. Summary of Operative Techniques in Donor Procurement and Transport for Lung Preservation: Toronto Lung Transplant Group Recommendations [8]

Volume of flush solution	50 - 60 ml/kg
Pulmonary arterial pressure during flush	10 - 15 mmHg
Temperature of flush	4 - 8 ^oC
Lung ventilation	Tidal volume 10ml/kg and PEEP: 5cmH_2O
Oxygenation	≤50% FiO_2
Lung inflation airway pressure	15 - 20 cmH_2O
Storage temperature	4 – 8 ^oC

Pharmacological Agents

Aprotinin has been shown to be beneficial in a number of studies looking at its effect on lung PGD in animal models. It has been incorporated into the Euro-Collins flush solution and shown to improve arterial oxygenation in an isolated lung model after 18 hours of cold storage[135], and after 6 and 12 hours cold storage[136]. It has also been shown to improve lung compliance, decrease capillary permeability[136], and reduce peak airway pressure after reperfusion[137]. However, results of the use of aprotinin to attenuate PGD in the clinical setting have been conflicting [57,138-140]. Interestingly, investigation of the impact of adding aprotinin to newer preservation solutions has shown no improvement over Perfadex alone and a significant deterioration in postischaemic lung performance when used with

Celsior[138]. Systemic use of aprotinin in the recipient during the implantation has also been shown to have a detrimental effect on post operative PGD [57], and a recent study in a brain death rat model showed no attenuation of IRI with aprotinin treatment [140].

Some studies have used a variety of compounds with antioxidant properties and demonstrated protection of lungs against IRI [47,141 – 147] However, these benefits were not universal [47]. N-acetyl-l-cysteine has been used and found to attenuate the inflammatory changes seen in IRI [148]. Other pharmacological agents tested include the lipid lowering agent simvastatin which has antioxidant effects and is also thought to modulate endothelial nitric oxide synthase [141]. Captopril, and enalapril, both angiotensin converting enzyme inhibitors, have been shown to be protective in animal models of I/R injury in organs other than lungs by acting as free radical scavengers [142,143]. Captopril has also been shown to ameliorate I/R injury in a rat model of lung transplantation after extended cold storage when added to the preservation solution [144]. Human thioredoxin and recombinant Kunitz protease inhibitor have also been used in animal models with success [145,146].

Soluble complement receptor 1 inhibitor has been used in a small clinical study whereby infusion of the drug prior to reperfusion of the lungs achieved close to 90% complement inhibition for 24 hours [11]. They postulated that by using an antagonist to CR1, the downstream effects of C5b-C9 would be suppressed, hence not only reducing cellular injury but also reduce the pro-inflammatory cascades of the cytokines, cellular adhesion molecules and platelet activating factor. The treated patients demonstrated earlier extubation, most noticeable in those patients who required intraoperative cardiopulmonary bypass. .

Platelet activating factor (PAF) plays a role in the initiation and amplification of ischemia reperfusion injury. It is a glycerophospholipid which interacts with leukocytes and promotes a pro-inflammatory response via increasing leukocyte activation, release of inflammatory cytokines and factors that contribute to increased vascular permeability. Given encouraging results in a laboratory setting, Wittwer et al., conducted a prospective randomised control trial utilising a PAF antagonist [149]. Manufactured PAF antagonist competes with native PAF in binding receptor sites on cells causing the native PAF to be metabolised. The clinical trial involving 24 patients with double lung transplantation were divided into control groups, low and high dose PAF antagonist group. The treatment groups had PAF antagonist administered to the preservation fluid and intra-operatively to the recipient. Irrespective of dose, the treatment groups had better oxygenation (alveolo-arterial difference) during the early recovery phases (3 to 8 hours and 8 to 12 hours post transplantation). There was also a better trend in gas exchange within the first 32 hours post transplantation[149].

Surfactant

I/R injury affects lung surfactant. In normal lung, surfactant consists of 90% lipids mostly in the form of phosphatidylcholine and 10% proteins such as surfactant apoproteins A-D. Surfactant is produced by Type II pneumocytes and acts to lower the surface tension between air and fluid. In the lungs, it prevents atelectasis and maintains the balance of fluid movement between the alveoli and the capillaries [150]. Reduction in surfactant leads to alveolar collapse, reduced lung compliance, ventilation-perfusion mismatch, pulmonary oedema and hypoxaemia.

Struber et al evaluated the role of surfactant in lung preservation [48]. In a clinical trial involving 30 patients, the treatment group had bovine surfactant administered (100mg/kg body weight donor) prior to preservation and harvesting. Although there were no differences in gas exchange and dynamic lung compliance in the immediate post operative phase, the authors noted that the protein content in the bronchoalveolar lavage was lower, indicating less fluid leakage across the alveolocapillary membrane [48]. In addition, lung function at 4 weeks post operatively as judged by forced expiratory volume in 1 second (FEV1) was significantly better in patients who received surfactant instillation. Such recovery in dynamic lung function was seen in the control group only after 1 year. The recovery of FEV1 in the control group after one year was from 62% (at 4 weeks) to 82% (at 1 year). This is in comparison to the modest increase achieved in the treatment group from 82% to 90% in the comparative period [48].

Amital el al conducted a prospective randomised clinical trial of 42 lung transplant recipients involving the use of bovine surfactant [49]. This was instilled via bronchoscopy after completion of the bronchial anastomosis, and prior to completion of the vascular anastomosis and reperfusion of the graft. The authors noted that recipients of surfactant had improved oxygenation, better chest radiographic appearances by the fourth post operative day with earlier trends towards improvement from the first operative day. There were fewer patients with severe primary graft dysfunction and shorter duration spent on the ventilator. In the first month post transplant, the forced vital capacity in the surfactant treated group was better (61% vs 50%, $p = 0.022$) [49]. I/R is a vicious cycle of diminished quality and production of surfactant which leads to further cellular injury by liberalisation of injurious agents. The authors reasoned that exogenous surfactant may dampen the vicious cycle indirectly.

Leukocyte Filtration

The important role of leukocytes in PGD has led to many strategies aimed at ameliorating such damage. Although promising in many laboratory settings, the practicality of leukodepletion or filtration has been difficult in clinical lung transplantation. These strategies include decreasing leukocyte-endothelial adherence [50,51], inactivating pulmonary/ alveolar macrophages (with gadolinium chloride [151] or reduction of leukocytes by use of a leukocyte removing filter [9,50,152].

Animal studies suggest that leukocyte depletion in combination with low pressure reperfusion and modified reperfusate improves the post operative outcomes in lung transplantation [9,50]. These well documented measures ameliorate reperfusion injury.

In an early laboratory study, Levine and co-workers evaluated the role of leukocyte depletion in lung I/R injury after a short duration (4 hours) of cold ischemia (4 degrees Celsius) [50]. The benefits noted included lower capillary filtration (less reperfusion induced hyper-permeability) and lower myeloperoxidase activity which indicates less neutrophil sequestration. However the functional outcomes in this group (gas exchange, airway pressures and pulmonary artery pressures) although had improved trends, were not significant [50]. An important point of interest was that the same benefits were seen in the two different protocols of duration of leukocyte filtration (10 minutes vs 30 minutes). This highlights the importance of achieving leukocyte depletion early during reperfusion. In the discussion, the

author acknowledged the limitation of leukocyte filters used in an animal circuit (sanguinous circuit connected to a support animal) in that the efficiency of the leukocyte filter reduces significantly.

In an earlier laboratory study by Halldorsson et al., leukocyte filtration was one component of three that ameliorated I/R injury in porcine lungs subjected to prolonged cold ischemia (4 degrees Celcius for 24 hours)[9]. These ischemic lungs were then transplanted to new recipients thus mimicking clinical lung transplantation. The benefits observed in the treatment group included improvement in pulmonary compliance, reduction in pulmonary vascular resistance, improved alveolar gaseous exchange and lower myeloperoxidase activity [9].

Clinical studies utilising leukocyte depletion in lung transplantation as an isolated modality of treatment are lacking. This is not because of the diminished role of leukocytes in lung reperfusion injury but the technical difficulty in achieving a sustainable and clinicially significant leukodepletion in the clinical setting owing to the fact that the body has a large capacity to overwhelm the filter. The only clinical study (prospective case series of 23 patients with a matched historical control) was performed by Ardehali and co-workers [152]. In that study, leukocyte filtration was one component out of three used in attenuating post transplantation injury. The other two components included modification of reperfusate and low pressure reperfusion. Compared to the matched historical group, the treated group had a lower incidence of IRI which impacted on the duration of intensive care stay.

Filters can be divided into two classes, depth filters and surface filters, by the manner in which they achieve separation. In a depth filter, particles become attached and removed from a fluid as it flows through its long tortuous passages. Additional circuit characteristics that aid the removal of particles include the use of materials that have different surface charge, and increasing the surface area in contact with particles. Materials which have been used as depth filters include wool, paper, glass fibre or asbestos. Surface filters are also known as membrane filters. These retain particles on the upstream whilst allowing fluid to flow through the membrane. Particles smaller than the pore size of the membrane can flow through the membrane. Surface filters are typically used in critical applications such as sterilisation and dialysis. Leukocyte filters are typically depth filters. Depth filters have the ability to handle larger flow rates but at the expense of efficiency. As a result of its shortfall, the clinical application of leukocyte filters (depth filters) in lung transplantation are restricted. In most cases, leukocyte depletion form a component of a multi-modal approach in attenuating reperfusion injury.

This explains the current limitation of leukocyte filters in the clinical situation. If leukocyte filtration is to be used clinically, it would need to be strategic as these filters can become exhausted and overwhelmed by the body's recruitment. As seen in a clinical study by Salamonsen and co-workers, by the time patients were at ICU, the leukocyte count was almost equal [153]. Not surprisingly the benefits were not evident.

Nitric Oxide

Nitric Oxide (NO) is an integral component of the pulmonary vascular endothelium. NO has a number of actions via its induction of intracellular cGMP production. These actions include pulmonary vasodilation, and prevention of platelet aggregation and leukocyte

adhesion to the endothelium. which occur via the induction of intracellular cGMP production. I/R in transplanted lungs disrupts the production of NO leading to increased pulmonary vascular resistance, increased leukocyte adhesion to the endothelium and sequestration, and platelet aggregation. The resulting oxidant injury further damages the endopthelium and propagates the inflammatory process. The production of endothelin-1, a potent vasoconstrictor, is also inhibited by NO. As NO production is disrupted, endothelin-1 production increases subjecting the pulmonary vasculature to its vasoconstrictor actions which also include apoptotic and mitogenic effects. The administration of NO has been shown to reverse all of these unwanted effects in experimental studies [154-161]. Studies of inhaled NO administration in clinical lung transplantation have yielded conflicting results, probably because of the wide variations in protocol of administration, particularly duration [162-167]. The most recent study, has however, shown a significant decrease in PGD from 40% to 29% [162](Table 3). Use of NO is not without risks with a side effect profile that includes methemoglobinemia, and rebound pulmonary hypertension. Therefore NO must be used with caution, but can be used safely with appropriate monitoring.

Table 3. Summary of clinical trials on use of inhaled nitric oxide in prevention of PGD

Author/ year	Inhaled nitric oxide protocol	Outcomes
Moreno [162] 2008 (n=32)	10ppm for 48hrs from reperfusion	Lower incidence of PGD (29% vs 40%) Lower IL-6 in BAL and serum Lower IL-8 in BAL and serum
Botha [163] 2007 (n=20)	20ppm for 30 mins from reperfusion	No difference in PGD No difference in gas exchange No difference in neutrophil sequestration No difference in IL-8, myeloperoxidase, nitrotyrosine
Perrin [164] 2006 (n=30)	20ppm for 12hrs from reperfusion	No difference in lung water content No difference in gas exchange
Cardella [165] 2004 (n=20)	20ppm starting 10mins from reperfusion and weaned according to protocol	nNOS protein higher in treatment group (2 hrs post reperfusion) No difference in iNOS and eNOS
Meade [166] 2003 (n=84)	20ppm starting 10mins post reperfusion and weaned according to protocol	No difference in IRI, gas exchange, ventilation duration or inpatient stay.
Ardehali [167] 2001 (n=28)	20ppm from reperfusion and weaned according to protocol	Does not prevent IRI Improves gas exchange and reduces pulmonary artery pressure in those with IRI

ppm – parts per million; BAL - bronchoalveolar lavage; iNOS - inducible nitric oxide synthase; eNOS - endothelial nitric oxide synthase; nNOS - neuronal nitric oxide synthase; IRI - ischemia reperfusion injury; PGD – primary graft dysfunction.

Preconditioning in Lung Transplantation

Ischaemic Conditioning and Remote Ischaemic Conditioning

Ischaemic preconditioning (IPC) was first demonstrated in 1986 where it was shown that brief periods of ischaemia induced in the myocardium could reduce the size of the subsequent infarct when the myocardium was exposed to more prolonged ischaemia, thereby attenuating the degree of I/R injury[168] However, this clinical intervention involves the application of ischaemia in an already diseased organ and furthermore is limited to situations where the timing of ischaemic insult can be readily anticipated.

Remote ischaemic preconditioning is a phenomenon whereby transient ischaemia in a remote tissue (such as skeletal muscle) is able to confer similar protection as IPC from IRI in another tissue (such as the heart) [169]. In humans, using a simple blood pressure cuff on the arm and 3 cycles of inflation and deflation, remote ischaemic preconditioning can be initiated and was shown to protect against endothelial IRI in healthy volunteers[170] and in patients with stable coronary artery disease[171]. Importantly, this same group of investigators went on to show that a remote ischaemic conditioning stimulus applied after the onset of ischaemia but prior to reperfusion could similarly reduce endothelial IRI, introducing the concept of remote ischaemic post-conditioning in humans.

The mechanisms underlying remote preconditioning are currently unclear. The link between the remote organ and the target organ is also unclear however there appears to be strong evidence for a humoral factor as evidenced by the requirement for a reperfusion period to 'washout' a humoral factor that is then transported to the target organ[172,173]. Once the message is conveyed to the target organ, protection from IRI occurs through reduced oxidative stress and preservation of mitochondrial function[174]. It is thought that the mitochondrial potassium ATP pump (K_{ATP} pump) and a mitochondrial permeability transition pore (mPTP) represent a final common intracellular pathway in conditioning responses from ischaemic preconditioning, remote ischaemic preconditioning to post-conditioning [175]. The mitochondrial ATP pump is a potassium pump on the inner mitochondrial membrane. Opening of the K_{ATP} pump leads to the generation of reactive oxygen species and activation of pro-survival protein kinases [176] which then interacts with the mPTP to close the pore, preserving oxidative phosphorylation and preventing cellular swelling and apoptosis [175,177]. Furthermore, pharmacological inhibitors of the K_{ATP} pump such as the oral hypoglycaemic agent glibenclamide [172,178] effectively abolishes ischaemic preconditioning, whilst mPTP openers such as κ-opioid agonists [174] and cyclosporine can induce myocardial protection [179].

Evidence in Humans

Recently human randomised controlled trials have demonstrated the ability of remote ischaemic preconditioning to protect against myocardial enzyme leakage after coronary bypass surgery [180,181], and protects and the heart and kidneys during open abdominal aortic aneurysm repair [182]. Similarly, in a randomised controlled trial of paediatric patients undergoing cardiac surgery for congenital heart disease, Cheung and colleagues, in addition

to demonstrating a significant reduction in myocardial enzyme leakage were able to show a significant reduction in airway resistance in treated patients [183].

Evidence in Lung Transplantation

There is increasing evidence for the role of remote ischaemic conditioning in the attenuation of IRI in different organ systems [184]. Preliminary data exists in animals supporting the potential for IPC and remote pre- and post-conditioning to protect the lungs from IRI. A number of investigators have demonstrated that lungs in various animal models can be protected from IRI by direct IPC [185-188]. Most importantly, Waldow and colleagues showed that remote preconditioning by hind limb ischaemia could similarly reduce porcine lung IRI as measured by pulmonary vascular resistance, pulmonary artery pressure, pulmonary venous and arterial oxygenation [189].

Outcomes

Early mortality from severe lung I/R injury has been estimated to be in the range of 40% to 60% [2,3.190] There is an associated impairment in residual pulmonary function in the longer term for patients who recover from the initial assault [190]. It has also been shown that of patients who have severe PGD early in their transplant, the trend of improvement is also predictive of outcome. Patients who have an improvement in their P/F ratio of less than 20% in their first 12 hours post transplant had a 6.8 odds ratio of early mortality as compared to those who demonstrated more favourable trends [191]. There have been attempts to correlate the severity of PGD to clinical outcome. Prekker et al demonstrated that patients with PGD grade 3 had a significantly higher early mortality as compared to grade 1 (90 day mortality 33% vs. 7%), as well as longer ICU and hospital stays, and lower long term FEV1 [192].

Whitson et al in a retrospective review of 402 lung transplant patients found the prevalence of severe PGD decreased over the first 48 hours post transplant but the 90 day death rate associated with the occurrence of severe PGD at each time point increased with time [23]. Furthermore, at all time points, grade 3 PGD was associated with a higher perioperative mortality. The occurrence of grade 3 PGD was also associated with a poorer long term survival [23] (Figure 1). Other independent predictors of poorer survival were raised preoperative pulmonary artery pressures, use of cardiopulmonary bypass and single lung transplantation.

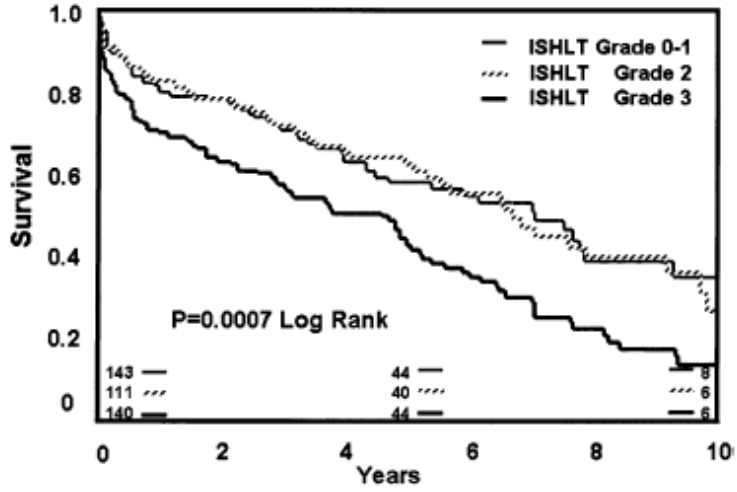

Figure 1. Association of grade of PGD with long term survival [23].

King and co-workers conducted a retrospective review (100 patients) of the consequences of reperfusion injury in lung transplantation [2]. In patients with ischemia-reperfusion injury, there was associated increase in hospital mortality (41% vs 12%), prolonged mechanical ventilation (393 hours vs 57 hours) and increased length of stay (49 days vs 26 days).

However, more recent publications are reporting a marked improvement in survival rates in patients who sustain PGD [193]. In particular, a review of 291 lung transplants performed at the University of Virginia between 1990 and 2006 compared results from the early era (before 2000) and the current era (after March 2000) and showed no significant difference in the incidence of IRI or in severe IRI [193]. The 30 day survival rates between the two eras were, however, significantly improved with time (11.8% versus 3.9%;p=0.003). IRI still remained the most important cause of early mortality however.

IRI has also been shown to be an independent predictor for the development and progression of bronchiolitis obliterans syndrome [151,194]. Bronchiolitis obliterans syndrome is the most common cause of long term morbidity and mortality post lung transplantation [151,195]. Huang et al have confirmed these findings, reporting that PGD of all grades at all post operative time points is an independent risk factor for the development of bronchiolitis obliterans syndrome, with increasing severity of PGD increasing the risk [194].

Treatment

The treatment of PGD is essentially supportive while waiting for time to improve the condition of the lungs. In essence, this means that treatment is very much like that offered to patients with Acute Respiratory Distress Syndrome (ARDS). The mainstays of management are the avoidance of excessive fluid administration while maintaining optimal circulation to all organs. This is usually achieved by restricting intravenous fluid replacement which minimises the exacerbation of pulmonary oedema in the setting of a leaky capillary syndrome which occurs during PGD.

Supportive Management

Circulatory management ensures adequate cardiac output and perfusion of all organs. Furthermore, it is important that the newly formed bronchial anastomoses are not subjected to prolonged periods of hypotension and poor tissue perfusion. Low dose systemic vasoconstrictors are often used, in conjunction with fluid restriction. If positive inotropy is required, then either adrenaline (epinephrine) or milrinone (phosphodiesterase inhibitor) can be used. Milrinone has the added benefit of pulmonary vasodilatation.

Ventilatory management is obviously paramount and follows many of the recommendations developed for ARDS. Over distension of already damaged alveoli is to avoided leading to the development of a 'protective ventilation' developed for ARDS [196,197]. This approach uses smaller tidal volumes (6-8ml per kg of body weight), elevated positive end-expiratory pressure, lower plateau pressures and higher frequency ventilation, usually with a pressure-controlled ventilator mode [198]. Other ventilator management algorithms may include permissive hypercapnia, inverse ratio ventilation and high frequency (oscillatory) ventilation, however there is very little data on these management options in lung transplant patients with almost all data extrapolated from non transplant populations. Independent lung ventilation may be required in single lung transplant patients, particularly in those with emphysema where the high tidal volumes and PEEP required to improve a dysfunctional transplanted lung, cause overinflation in the native lung. This can lead to barotrauma, pneumothorax, haemodynamic instability and reoperation. Furthermore, as a result of the overdistension of alveoli in the transplanted lung, pulmonary vascular resistance increases in the native lung, shunting blood into the allograft and exacerbating the already oedematous lung. Double lumen intubation with a ventilator for each lung on different settings is the best way to manage this problem.

Pulmonary Vasodilators

Pharmacological agents may also be used to assist in the management of PGD post lung transplantation. NO is used in many centres performing lung transplantation. Its prophylactic use has been discussed above and outlined in Table 3 [162-167] There is little clinical data available on its efficacy if commenced after PGD has manifested. There may be some benefit to its use in this manner if results extrapolated from treatment of ARDS can be applied to the pathophysiological changes seen in PGD.

Prostaglandin E_1, is also a vasodilator and exerts its actions via an increased production of cyclic-3'5' adenosine monophosphate (cAMP). Other effects include reduced neutrophil adhesion, platelet aggregation and capillary permeability. PGE_1 is used in many centres as an additive to preservation fluid where its vasodilatory action is thought to improve the distribution of the preservation fluid. In the treatment of PGD it is used either intravenously or aerosolised. Again experimental evidence has shown improved oxygenation which has been attributable to the vasodilatory effects and subsequent reduction in sheer stress injury to the graft endothelium [199-203]. More recent studies have shown anti-apoptotic effects of PGE_1 as well as downregulation of pro-inflammatory cytokines [204]. However, again there is little clinical data of its use for treatment of PGD after lung transplantation, with most of the available data coming from studies in patients with primary pulmonary hypertension. One

recent clinical study, using aerosolised prostaglandin E_1 intraoperatively in lung transplantation found significant reductions in pulmonary artery pressures, pulmonary resistance and shunt fraction with improvements in oxygenation in a non randomised study of 18 patients [205]. The improvements were dependent on the baseline values in the patients, indicating that this therapy may only be useful in a subset of patients.

Surfactant

The use of surfactant in the prevention of PGD has been discussed above. Evidence for the use of surfactant in the treatment of established PGD is lacking with only a few case reports in the literature [206-207]. The most recent reports on five patients with life threatening PGD after lung transplantation who had bronchoscopic instillation of surfactant at 3 or 7 days post transplant. There were measurable improvements in oxygenation within hours, with all patients being discharged home and demonstrating satisfactory FEV_1 at 6 months follow up [206]. A study from an Australian group reports on 6 patients who were treated in the same way in the setting of established PGD, with improvements on CXR and oxygenation evident within 24 to 48 hours. They also report a 100% survival with mean follow up of 19 months [207].

ECMO

Extra corporeal membrane oxygenation provides support for the failing donor lung when the above measures are unsuccessful. With improvements in reliability of circuits and oxygenators and smaller calibre cannulae which can be inserted peripherally, the results of ECMO have improved considerably [208-216]. The details of cannulation, institution, conduct and weaning of ECMO have been described elsewhere [217]. In brief, ECMO in lung transplantation can be instituted as veno-venous or, if there is haemodynamic instability, as veno-arterial support. The patient can be cannulated peripherally via the femoral vessels, or centrally. Peripheral cannulation usually does not afford as high flows because of the smaller cannulae. If this is an issue, improved venous drainage can usually be achieved by splicing a second venous drainage cannula into the circuit. This second cannula is usually inserted into the internal jugular vein.

Over the last ten years, the use of ECMO for pulmonary support post lung transplant has increased rapidly with improving familiarity and results. In fact, its use as a ‘prophylactic’ measure has even been reported with some groups placing the patient on ECMO pre-operatively, using it as support intra-operatively and then weaning it slowly post operatively in the intensive care unit[209, 212]. This technique is in contradiction to studies which have found the use of cardiopulmonary bypass as a risk factor for post operative PGD. However, a rational explanation for such an approach can be identified when considering that a slow wean from ECMO post transplant is essentially a version of low pressure reperfusion of the implanted organ. This technique also avoids aggressive mechanical ventilation and thus further protects the allograft in the early post operative period.

Conclusion

In conclusion, PGD remains an important cause of morbidity and mortality post lung transplantation. This chapter has reviewed the pathophysiology of this condition as well as its treatment. Prevention of PGD by attention to donor management, organ preservation and the implantation appear to have more scope for success than treatment of the manifestations of the injury. Further prospective randomised studies are required to provide clinicians with a sound evidence base for many of the current treatment modalities, which have evolved based on experimental work with minimal human trials in this condition.

However, results are improving markedly over time with regards to both incidence and outcomes which is encouraging.

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Chapter 2

Intensive Care Management of Lung Transplant Recipient

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Abstract

Advances in patient and donor selection, ventilatory management, and improvements in the treatment of rejection and infections have made human lung transplantation an effective and acceptable option for patients with end-stage lung disease. However, many important factors, related both to an increasing "marginality" of the implanted graft and unexpected perioperative complications make immediate postoperative management still challenging and the early outcome unpredictable.

Intensive care treatment following lung transplant is focused on cardiovascular stabilization, respiratory assistance, adequate fluid management, infection prophylaxis, immunosuppression, active physiotherapy, and treatment of any organ dysfunction.

Early postoperative management is highly demanding as dramatic changes may occur on both the allograft and the "distant" organs. While satisfactory rates of survival have been obtained from multidisciplinary, collaborative efforts, significant hurdles have yet to be overcome, including issues of delayed postoperative hemodynamic recovery, severe hypoxia, acute allograft dysfunction, acute rejection, disseminate infections, adverse effects of multiple drugs, and surgical complications.

Even though the outcome of lung transplantation lags behind that of other solid organ transplants, an aggressive postoperative care is indispensable to treat allograft failure and prevent dysfunction of nonpulmonary organ systems. Skillful vigilance, a thorough knowledge of pathophysiologic characteristics of the transplanted lung, and

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early recognition of life-threatening clinical problems are fundamental for a successful ICU treatment.

Introduction

Lung transplantation (LTx) has become a widely accepted treatment for a variety of end-stage pulmonary diseases. As a result of refinements of harvesting and preservation techniques, improved understanding of transplant immunology, and better critical care management of complications, lung transplanted patients survive longer.

Generally, single lung and double sequential LTxs bring about a remarkable improvement of respiratory function, gas exchange, and exercise tolerance. However, several major challenges such as the use of marginal donors and impaired recovery of the implanted organ, along with some postoperative adverse events may negatively affect the evolution of the procedure, resulting in a limited success on an individual basis and in an unsatisfactory long-term outcome.

A crucial time in the LTx course is the early postoperative period when careful evaluation of graft functional capacity, strict monitoring and support of cardiorespiratory function, timely recognition of unexpected complications and prompt treatment of dysfunction of nonpulmonary organ system are mandatory.

Because of poor preoperative clinical conditions, a particularly complicated intraoperative course and graft cell damage secondary to preservation procedures and reperfusion, lung transplant recipients are expecially susceptible to an increased intensive care unit (ICU) morbidity and mortality.

Intensive care management of lung transplanted patient centers on rapid hemodynamic stabilization, proper fluid administration, appropriate ventilatory management, adequate postoperative pain control, infection prophylaxis, physiotherapy and rehabilitation.

Early supportive strategies of implanted allografts aim at maintaining adequate perfusion and gas exchange while minimizing intravenous fluid administration, cardiac work, and ventilatory barotrauma.

Before focusing on early postoperative management of lung transplant recipients a brief description of some particular features of transplanted lung deserves reporting.

Physiology of the Transplanted Graft

Bilateral lung transplantation (BLTx) is the elective procedure for patients with chronic pulmonary infections such as cystic fibrosis and bronchiectasis. Both septic native lungs must, in fact, be excised. Single-lung transplantation (SLTx) is usually performed on patients with emphysema and interstitial pulmonary fibrosis. Significant differences exist between BLTx and SLTx with respect to blood flow, pulmonary artery pressure, and immediate cardiac index.

After SLTx the majority of cardiac output passes through the allograft while ventilation remains evenly distributed. This relies on the different compliance of the native and the implanted lung and intrinsic characteristics of pulmonary circulation. As allograft

implantation does not consistently alter ventilation/perfusion (Va/Q) relationship. [1] SLTx is usually tolerated and does not limit physical performance. However, in the face of reperfusion injury, infection or rejection the hypoxic pulmonary vasoconstriction reflex may be attenuated and important gas exchange impairment may result from increased ventilation/perfusion mismatch [2][3]. In fact, in cases of graft dysfunction these recipients have a very limited functional reserve from their native lung as pulmonary blood flow continues to be preferentially shunted through the allograft despite a significant impairment of ventilation.

DLTx do not usually present marked differences in compliance between the lungs, and therefore ventilation-perfusion mismatches are only minimal.

Both SLTx and BLTx are highly susceptible to fluid overload since tracheal or main bronchi resection during harvesting interrupts lymphatic drainage. Ischemia-reperfusion injury, along with disrupted pulmonary lymphatics predispose extra vascular lung water (EVLW) accumulation and may be responsible for pulmonary edema development following even the most minimal fluid overloads [4].

Denervation of the lungs results in a diminished cough reflex and impairment of mucociliary clearance mechanisms. The bactericidal activity of alveolar macrophages is reduced as well. These features make the recipients vulnerable to pulmonary infections and necessitate aggressive pulmonary toilet.

Airway tone, primarily mediated via parasympatic efferents on bronchial smooth muscles, is not particularly affected by the denervation determined by harvesting, and airway response to $\beta 2$ agonists is preserved.

Sometimes transplanted patients for emphysema may display persistent hypercapnia due to a reduced central response to CO_2. The return to normal $PaCO_2$ values in patients with preoperative hypercapnia takes about 15 days.

Postoperative Hemodynamic Assessment and Stabilization

Hemodynamic instability due to hypovolemia, depressed myocardial contractility, right ventricular hypoperfusion and pulmonary hypertension may frequently occur in the immediate postoperative period [5]. Cardiac output may transiently decrease secondary to the prolonged effects of anesthesia and/or intraoperative cardiopulmonary bypass. Left or right ventricular failure secondary to myocardial ischemia or infarction should be considered in the differential diagnosis. Postoperative supraventricular dysrhythmias are among the various causes of hemodynamic deteriorarion. They may occur because of electrolyte abnormalities, hyper-hypovolemia, inotropic drugs, and intraoperative manipulation of the heart. It is hypothesized that each atrial cuff suture line provides an electrophysiologic substrate for atrial flutter by creating a zone of conduction block around which circus electrical movement can occur [6]. Older patients are at increased risk of arrhythmias as well as the recipients with pulmonary idiopathic fibrosis related coronary artery disease and those with an enlarged atrium on an echocardiogram. Arrhythmias respond to routine management with anti-arrhythmic drugs, calcium channel blockers, and, at times, they require electrical cardioversion if severe enough to determine hemodynamic collapse. LTx recipients suffering from prolonged supraventricular dysrhythmias often have a longer intensive care unit stay and

hospital stay [7]. Because of an almost constant cardiocirculatory instability hemodynamic monitoring must be strictly carried out in the immediate postoperative period. Continuous knowledge of the mean and transpulmonary pressure, pulmonary vascular resistance (PVR), preload and afterload indexes and both right (RV) and left ventricular (LV) function is mandatory in managing pharmacologic interventions, volume therapy, vasoactive drug administration and ventilatory adjustment. Pulmonary artery catheter (PAC) equipped with a fast response thermistor capable of assessing RV ejection fraction (RVEF%) and ventricular filling through RV end diastolic volume calculation (RVEDV), the PiCCO System (Pulsion Medical System, Munich, Germany) and transesophageal echocardography (TEE) are always adopted in our institution. Perivascular and interstitial edema from ischemia, cardiopulmonary bypass, and increased levels of circulating catecholamines may increase PVR. A transient increase in pulmonary arterial pressure may persist in the postoperative period of LTx recipients for pulmonary hypertension. Episodes of systemic hypertension or hypotension arising from fluctuations in allograft PVR are common in these individuals as well. Maintenance of chronotropic and inotropic function, careful fluid infusion, reduction of both PVR and intrathoracic pressure along with avoidance of systemic hypotension and the administration of systemic vasopressors are useful to counterbalance a decreased RV contractility. Beta adrenergic agonists in continuous infusion are also needed when both ventricles are failing. Postoperative administration of inhaled nitric oxide (NO) may be beneficial in patients undergoing SLTx, as well as in recipients with persistent pulmonary arterial hypertension. NO modulates pulmonary vascular tone via smooth muscle relaxation and can improve ventilation/perfusion matching and oxygenation. NO has been demonstrated to reduce pulmonary vascular resistance, mean pulmonary artery pressure, and improve RV performance. Pulmonary vasodilators such as intravenous or inhaled prostacyclin and prostaglandin E_1 have also been used to reduce right ventricular afterload and pulmonary hypertension. In the presence of a hemodynamically unstable LTx recipient the assessment of cardiac preload is of primary importance in guiding volume therapy and vasoactive drug administration in order to optimize organ perfusion and avoid fluid overload.

Fluid Management and Volumetric Monitoring

Alterations in pulmonary capillary permeability from cardiopulmonary bypass, graft ischemia, and disruption of lymphatics lead to extravasation of fluid to the interstitial and intra-alveolar spaces.

Appropriate fluid management is essential in the early postoperative period and a negative fluid balance is often attempted during the first 24 to 48 hours to prevent the occurrence of pulmonary edema. The goal of fluid management after LTx is to minimize edema formation in the transplanted lung while maintaining adequate cardiac function. Cardiac filling pressures, pulmonary artery pressure and pulmonary capillary wedge pressure have been kept lower to minimize the formation of low-pressure pulmonary edema. However, keeping pulmonary capillary wedge pressure as low as possible after surgery may compromise ventricular preload and cardiac output. It has been demonstrated that cardiac and pulmonary pressures can only serve as indirect indicators of filling volume, especially in mechanically ventilated patients with positive end expiratory pressure [8]. The application of

the transpulmonary thermodilution single indicator technique (PiCCO System) allows the determination of continuous cardiac output, based on the pulse contour method, along with estimation of preload index (intrathoracic blood volume, ITBVI) and "lung edema " index (extravascular lung water, EVLWI)[9]. Indicator dilution-derived ITBVI has been considered a sensitive indicator of cardiac preload because volume changes preferentially alter the volume in the intrathoracic compartment, which serves as the primary reservoir for the left ventricle [10]. Della Rocca et al. [11] demonstrated that ITBVI is a parameter of cardiac preload superior to pulmonary capillary pressure in patients undergoing LTx.

The Stroke volume variation (SVV) and pulse pressure variation (PPV) variables are also continuously obtained with the transpulmonary thermodilution single indicator technique, and nowadays considered to be the best parameters (dynamic parameters) in predicting fluid responsiveness in mechanically ventilated patients after various surgical procedures [12]. In the early postoperative period, a careful balance between colloids and cristalloids, under the continuous guidance of dynamic parameters should be considered both to prevent peripheral organ hypoperfusion from an unrecognized hypovolemia and to avoid excessive i.v. fluid. Loop diuretics are usually required to maintain a negative fluid balance. However, the liberal use of diuretics must sometimes be countered by fluid volume replacement. Renal insufficiency from aggressive diuresis should be avoided. Indirect evidence of the severity of alveolar damage and increased capillary permeability, which allow leakage of crystalloid and colloid solutions into the extravascular lung space, may be detected with the continuous monitoring of EVLW. Due to the complete absence of lymphatic drainage in the transplanted lung an increase in EVLW is commonly seen following LTx; however, a persistent abnormal elevation of EVLW in spite of a fluid restriction regimen and with a normal intravascular oncotic pressure, indirectly attests to serious capillary dysfunction, as is likely to occur after severe ischemia-reperfusion injury.

Primary Graft Dysfunction

Primary graft dysfunction is the major complication following LTx and one of the most important cause of increased morbility in the early postoperative period [13]. According to Christie et al LTx patients with primary graft failure have a greater than fivefold increase in the risk of death during hospitalization. Due to sustained impairment of gas exchange, a severe lung dysfunction leads to prolonged mechanical ventilator support and an increased length of ICU stay [14].

Despite continuous improvements in graft preservation techniques, intraoperative hemodynamic optimization and fluid management, incidence of primary graft failure has not changed significantly in recent years. It seems to affect 12-25% of lung recipients [15]. Primary graft dysfunction (PGD) or failure after LTx has been also defined as post-reimplantation edema, reperfusion edema, and post-LTx non cardiogenic pulmonary edema. It usually begins by the first day after the transplant and is always present by day 3. It frequently progresses over the first few days but peaks by day 4 or 5.

PGD presents a wide spectrum of disease severity characterized by varying degrees of impairment of gas exchange, and is associated with delayed extubation, prolonged intensive

care and hospital length of stay (LOS), increased early mortality, and worse long-term outcome among survivors.

The presence of the following criteria has been advocated to define PGD: 1) diffuse radiographic infiltrates in the graft that develop within the first three days following LTx, 2) PaO2/fraction of inspired oxygen (FiO2) ratio <200 persisting beyond the initial 48 h postoperatively, 3) no obstruction of pulmonary venous outflow, 4) absence of cardiogenic pulmonary edema (pulmonary artery occlusion pressure <18 mmHg and resolution of infiltrates with diuretics), and 5) no evidence of bacterial infection, rejection, or atelectasis [16]. Virtually all LTx recipients have some degree of mild reperfusion edema; PGD due to reperfusion edema is only defined after exclusion of secondary causes such as volume overload, rejection, pneumonia, or venous anastomotic obstruction [17]. PGD may be the consequence of many factors such as post-ischemia-reperfusion lung injury, prolonged cold ischemia, hypothermia, inadequate graft preservation, prolonged intraoperative cardiopulmonary by-pass, etc. It is characterized by a profound endothelial dysfunction, interstitial and alveolar edema, hypoxemia and diffuse pulmonary infiltrates on chest x-ray. While most cases are mild and resolve with appropriate and correct care, some progress to become primary graft failure. Worsening of PGD can evolve into severe diffuse alveolar damage associated with hemodynamic impairment requiring full ventilatory and cardiocirculatory support. Minor clinical signs of PGD or a "latent" PGD, may be further impaired by the stress of excessively traumatic postoperative mechanical ventilation and some Authors emphasize that an under-recognized ventilator–induced lung injury may be potentially involved in the negative evolution of PGD [18]. Early lung graft dysfunction mainly benefits from aggressive supportive measures. The edematous lung is managed by keeping the patient as dry as possible without compromising blood pressure or renal function. Judicious administration of loop diuretics, ventilatory support, along with inotropes, is usually effective in non severe post-reperfusion syndromes. In less responsive clinical pictures, non-invasive positive pressure ventilation (NIV) or tracheal intubation becomes necessary. At our center, one trial of NIV is nearly always attempted before intubation in the case of PGD, and the prone position is often adopted in order to promote faster healing of diseased lung areas. Experience with these techniques has prompted us to combine both procedures in an effort to avoid a more invasive approach [19]. Since pulmonary blood flow in the supine position remains distributed primarily in the dorsal regions, applying a NIV by facial mask or helmet in the prone position can obtain a better ventilation/perfusion match along the antero-posterior axis. The improvement in oxygenation after turning the recipient prone while he/she is noninvasively ventilated may be a consequence of the combined effects of extravascular fluid redistribution, recruitment of non-aerated alveoli, and redirection of pulmonary blood flow [20]. In case of severe PGD refractory to a non-invasive approach, mechanical ventilation should be instituted. Avoidance of high peak inflation and plateau pressure is the main goal of the ventilatory management of the injuried allograft. Due to shear, mechanical stress, conventional ventilation may provide the "second hit" in a two-hit injury model, the first hit being lung transplantation injury [21]. A "minimal mechanical stress" mode may be beneficial in the prevention of overdistension, with the associated risk of volutrauma and barotrauma. Low peak inspiratory pressures and mean pressure are also recommended to prevent ventilatory trauma on bronchial anastomosis. Excessive airway pressure can, in fact, compromise bronchial mucosal flow and cause stress on the suture line. Based on the ARDS Network Study [22] the "protective ventilation" approach, which is now

widely recommended for patients with acute lung injury (ALI) and adult respiratory distress syndrome (ARDS), should be recommended for severe PGD as well. Low tidal volume with moderate-to-high PEEP and relatively high respiratory rate must be applied to limit alveolar overdistension while maintaining the small airways open. Lowering the plateau pressure seems to be associated with a reduced release of proinflammatory mediators and cytochines [23]; with this care satisfactory oxygenation may be obtained with fewer morphologic signs of injury. A progressive impairment of lung injury may be responsible for a sustained hypoxemia unresponsive to protective ventilation with high PEEP, inverse ratio ventilation and maximal fraction of inspired oxygen; in this circumstance, administration of inhaled NO can be an option to try to improve gas exchange. NO has demonstrated to significantly improve the PaO_2/FiO_2 ratio in severe postoperative allograft dysfunction [24].

NO can modulate ischemia/reperfusion injury by limiting the generation of superoxide anions, interfering with the neutrophil function and protecting against reactive oxygen species. Date et al found that 20 to 60 ppm of inhaled NO significantly decreased pulmonary artery pressure and improved the PaO_2/FiO_2 ratio during the extended time of therapy [25]. Inhalation of NO at the time of reperfusion and in the following period has been prophylactically proposed to prevent postoperative graft failure; however, in many cases it has been associated with disappointing results. Ardehali et al administered NO at 20 ppm during reperfusion in 28 lung recipients and observed an incidence of primary graft failure comparable to that previously reported (18%)[26]. Perrin et al performed serial measurements of extravascular lung water after LTx and demonstrated that a dose of inhaled NO of 20 ppm preventively administered at the time of reperfusion has no effect on reducing the amount of extravascular lung water nor on preventing pulmonary edema formation [27]. At the moment prophylactic use of NO is no longer recommended; in case of established primary graft failure it appears to be effective for improving gas exchange, sometimes transiently, but, currently there are no randomized studies to support its use for survival benefit [21].

Based on the reported altered surfactant composition and activity following graft ischemia-reperfusion injury [28], surfactant replacement therapy has been proposed to protect the lung from harmful effects; in a selected group of recipients with primary graft dysfunction it has been associated with an improvement in the early outcome [29]. Direct intrabronchial instillation or nebulized administration of surfactant has proven useful in reducing pulmonary edema following allograft dysfunction [30]. The immediate instillation of surfactant led to improvement in the oxygenation and compliance of the allograft and faster extubation. The experimental findings and clinical experience suggest that exogenous surfactant therapy can be a promising treatment for PGD, even though prospective and randomized studies are still missing. Low dose of prostaglandin E_1 (PGE_1) infusion has also been proposed for severe PGD, as this treatment proved helpful in animal studies. In humans, however, there are currently no available data to support a prophylactic or therapeutic approach with this drug. Aerosolized PGE_1 has also been administered to improve ventilation/perfusion mismatch and oxygenation in patients with different types of lung injury, including PGD following LTx [31]. Even though a shift from proinflammatory cytokines to anti-inflammatory cytokines was detected after the infusion of PGE_1 during the reperfusion period in experimental models [32] further clinical trials are required to confirm its benefit in the clinical setting. Protective ventilation associated with inhaled, aerosolized or infusion drugs is not always sufficient to prevent PGD worsening, which manifests in chest x-rays with patchy diffuse multilobar infiltrates. In the presence of a significantly decreased

lung compliance, or when the infiltrates are distributed preferentially on the dorsal areas on a CT-scan, the rotation of recipient in the prone position while ventilated with "protective strategies" can be useful in correcting a life-threatening hypoxemia. Following a prolonged supine position, alveolar ventilation is shifted preferentially to the nondependent part of the lung and the dependent lung regions tend to collapse (atelectases development) also under moderate-to-high PEEP. As previously reported, the prevalent distribution of perfusion along the dorsal areas, due to the gravitational effect and to the loss of efficacy of hypoxic vasoconstriction, contributes significantly to the progressive impairment of gas exchange. Even though the effects on the final outcome of ventilation in the prone position are still under debate, turning the recipient prone while invasively ventilated may aim at the following benefits: 1) improvement in oxygenation, due to redistribution of perfusion and more homogeneous ventilation of dorsal regions, 2) increase in lung volumes and decrease of atelectatic regions (alveolar recruitment), 3) increased drainage of secretions, and 4) reduction of ventilator-associated lung injury [33]. In spite of all best efforts with aggressive and multimodal treatment, a severe PGD may still determine a serious impairment of gas exchange under full ventilatory assistance. In these circumstances extracorporeal membrane oxygenation (ECMO) is a suitable treatment option to provide life-saving temporary support until spontaneous graft recovery from reperfusion injury. An early ECMO institution is generally recommended, before extremely severe oxygenation deficit and ventilatory-induced barotrauma have been developed. Fiser et al demonstrated that ECMO started within 2 hours of not achieving adequate oxygenation had an 80% survival compared with 20% survival in the group receiving delayed or no ECMO assistance. Besides providing cardiocirculatory support in case of hemodynamic instability, well-protective ventilation strategies with very low tidal volume are easily permitted under ECMO, thus minimizing mechanical stress injury on the alveoli [34]. Allowing a reduction in the fraction of inspired oxygen ECMO may also reduce the risk of post-reperfusion oxygen toxicity. ECMO support must be considered when sub-optimal in size grafts (small grafts or lobar transplant) are significantly exposed to a temporary hyperafflux syndrome; "small for size" grafts have, infact, to "accommodate" the entire right ventricular output after reperfusion. Generally, older recipients and those receiving SLTx do not benefit from ECMO as well as younger patients or those undergoing BLTx [35]. Caution is required when appliying ECMO in the early course of LTx in order not to completely decompress the right ventricle. As the allograft parenchyma and stroma survive solely on pulmonary blood flow, ECMO flow should be adjusted to maintain a sufficient pulmonary flow; for this reason a pulsatile pulmonary blood flow should always be detected in the pulmonary arterial trace.

Postoperative Management of Ventilation

After changing the double-lumen tube to a single lumen endotracheal tube in the operating room, the recipient is transferred and ventilated in ICU until hemodynamic stabilization and the criteria for a safe weaning have been obtained. There are various reports of successful early tracheal extubation in a selected population of recipients undergoing lung implantation with a prompt recovery of allograft function [36]. Della Rocca et al. [37] reported on a very early extubation after LTx by adopting an anesthetic technique based on

short-acting anesthetic drugs, postoperative pain control with epidural and early ventilatory assistance through a full face mask. The prevention of ventilatory-induced graft injury and the adoption of the best strategies to promote a faster weaning from mechanical ventilation are the main goals of postoperative respiratory management. Rapid recovery of allograft function, along with withdrawal of vasoactive drugs and adequate postoperative analgesia allow for rapid weaning from mechanical ventilation. In LTx for pulmonary hypertension sedation and mechanical ventilation are recommended for at least 24 hours. This strategy may help avoid the persistence of high PVR with dangerous consequences on RV function. A smooth weaning process is necessary because an abrupt avoidance of sedation and an incomplete analgesia may lead to recurrent pulmonary hypertension and coronary ischemia.Even in the absence of PGD ventilation modes should always be adjusted to limit peak airway pressures and prevent barotrauma to the parenchyma and bronchial anastomosis. Pressure control ventilation is used in some Institutions to limit peak and plateau pressure but volume control ventilation with relatively low tidal volume and respiratory rate of 12 to 16 breathes per minute is also extensively applied. No major differences on the recovery of graft function have been observed by the selection of a pressure-or volume cycled mode of postoperative mechanical ventilation. Patients should be maintained on a nontoxic fraction of inspired oxygen (FiO2); to minimize the risk of oxygen toxicity the FiO_2 should be set at the minimum value that guarantees a peripheral saturation > 92-93% . Application of positive end expiratory pressure (PEEP) may be guided by the underlying disease and the type of transplant performed. BLTx recipients may safely receive up to 8-10 cm H2O of PEEP, provided a reduced tidal volume is contemporarily delivered. Moderate to high PEEP is also beneficial in cases of SLTx for pulmonary hypertension since the increased blood flow to the graft may predispose pulmonary edema development. In SLTx for emphysema, zero or minimal PEEP along with an adequate expiratory time, is fundamental to prevent air trapping in the native lung (7). Expiratory hold maneuvers should be intermittently applied to detect air trapping in these recipients. Postoperative mechanical ventilation of SLTx for chronic obstructive pulmonary disease may be challenging, especially when the graft is affected by severe reperfusion injury, pneumonia or atelectasis. In these circumstances the variable reduction in the graft compliance makes the overly compliant native lung prone to hyperinflation under the effect of positive pressure ventilation. An excessive native lung overdistension determines a mediastinal displacement toward the transplanted lung with an increased risk of further impairment of graft ventilation. In addition, as a result of hyperinflation, the native lung's pulmonary vascular resistance increases and blood is shunted to the diseased graft, with a worsening of the ventilation/perfusion mismatch (7). Avoiding PEEP, minimizing tidal volume and accepting mild respiratory acidosis may result as beneficial in overcoming the critical period of graft dysfunction. However, a conservative treatment in some individuals may increase the risk of a permanent loss of variable pulmonary units in the allograft, as the native lung overdistension may take longer to regress. Lateral positioning with the transplant side up and aggressive chest physiotherapy are useful maneuvers to favor the delivery of inspiratory flow to the graft. When the compression of the allograft becomes significant and/or the mediastinal shift determines an impairment of venous return, a conservative treatment is no longer recommended and independent lung ventilation should be instituted.

Differential ventilation via double lumen endotracheal tube [38] and two mechanical ventilators is necessary to prevent hypoventilation of the graft and possible hemodynamic

derangement. Different settings of ventilation should be applied: low tidal volumes with minimal or no PEEP, high flows and low respiratory rate for the native emphysematous lung; low tidal volume with high PEEP and respiratory rate ("protective ventilation") plus recruitment maneuvers to the graft. Even if differential ventilation may provide a better graft expansion, the previously "compressed" implanted lung may take longer to reinflate the peripheral atelectatic areas.

Weaning from Mechanical Ventilation

Prolonged endotracheal intubation carries a significant risk for respiratory infections. Therefore fast weaning and an early postoperative extubation should be promoted. In many LTx patients the intrinsic ventilator response to carbon dioxide may be reduced or consistently attenuated both as a consequence of previous respiratory disease and by postoperative narcotics [39]. This condition is associated with low respiratory rate and small tidal volumes which sometimes hinder rapid weaning.

Most patients can be weaned and extubated within the first days following transplantation. In patients with significant pulmonary hypertension who undergo SLTx, a risk exists for the development of pulmonary edema in the donor lung. In these instance weaning is started once the hemodynamic and oxygenation status are stable. Weaning is usually accomplished through successive decrements in the intermittent mandatory ventilation rate, followed by a trial of continuous positive airway pressure. A valid adjunct in accelerating the extubation process might be a non-invasive method of ventilation (NIV) applied to assist the patient's spontaneous ventilation soon after an early extubation [40]. NIV is increasingly being used in the postoperative care of LTx patients who do not completely fulfill the criteria for safe extubation. Unloading respiratory muscles, decreasing respiratory rate and sensation of dyspnea, improving ventilation/perfusion abnormalities, and improving hemodynamics are among the recognized benefits of NIV. Advantages of NIV in assisting a difficult-to-wean patient or in treating impending muscle fatigue after apparently successful extubation have been demonstrated in several trials [41].

In our practice, the helmet system has emerged as the preferred interface for applying NIV; in cases of dyshomogeneous dorsobasal lung infiltrates, it allows effective ventilatory support in the prone position as well [40].

Besides the persistence of PGD, infectious disease and atelectases, the presence of pleural effusion should also be considered among the various causes of gas exchange impairment during a difficult weaning from mechanical ventilation. Pleural effusions are quite common in the early postoperative period and are usually small to moderate in size. These effusions are usually bloody, exudative, neutrophil predominant and tend to resolve spontaneously [42]. They are mainly due to the increased permeability of the alveolar capillaries in the first few days after LTx resulting from allograft ischemia, denervation and subsequent reperfusion. As previously mentioned peribronchial allograft lymphatics are severed during transplantation so that lymphatic flow is severely disrupted. Herridge et al found a higher rate of complicated pleural effusions in BLTx compared with SLTx recipients [43]. It should be recognized that pleural effusions are a common finding among primary graft dysfunctions and are often associated with acute lung rejection, an event that occurs at least once in almost all lung-

transplant recipients [42]. Intraoperative phrenic nerve injury is another cause of prolonged weaning from mechanical ventilation. Unilateral phrenic nerve paralysis may compromise ventilator autonomy to some extent, while bilateral phrenic nerve injury certainly would result in dyspnea and prolonged mechanical ventilation. In most patients, phrenic nerve palsy is transient and generally improves over the following weeks to months.Fundamental supportive measures in order to not only facilitate the weaning process but also to maintain satisfactory gas exchange after extubation and prevent respiratory complications, are vigorous chest physiotherapy, postural drainage, inhaled bronchodilators administration and frequent clearance of secretions [7]. Maintenance of spontaneous ventilatory autonomy following tracheal extubation mainly relies on aggressive physiotherapy, absence or regression of any graft dysfunction or rejection and avoidance of superinfection. Surveillance fiberoptic bronchoscopy should be done as needed during the initial posttransplant period; it is recommended to perform a sterile suctioning with saline lavage, to assess airway secretions, healing of anastomoses, the condition of the bronchial mucous membrane and to screen the allograft for infection.

In some recipients, however, due to pre-existing lung donor's disease or infection, or as a consequence of a slow-resolving pneumonia, bronchial secretions become copious and the recipient becomes less independent in clearing the airways with an almost ineffective cough. In these circumstances a repeated and frequent bronchoscopic clearance becomes intolerable and no longer sufficient for an effective toilet. To manage this problem an attempt with minitracheotomy may first be done, consisting of an indwelling narrow-bore endotracheal tube inserted under local anesthesia via the cricothroid membrane, before performing a percutaneous or a surgical tracheostomy in case of failure of the "semiinvasive" approach. In our Institution percutaneous tracheotomy is almost always preferred because of the faster and better healing of tracheo-cutaneous structures once the cannula has been removed. Our policy is to perform an early percutaneous tracheostomy not only to manage an allograft dysfunction or pneumonia which are supposed to require a prolonged intubation but also to provide better patient comfort and cooperation, oral hygiene and pulmonary secretions removal.

Postoperative Pain Control

Post-LTx thoracic pain may not become apparent until the 2^{nd}-3^{rd} postoperative day, when a complete recovery of consciousness and the progression of patient mobilization may unmask an insufficient blockade of nociceptive afferent fibers. Active control of postoperative pain is of paramount importance to promote definitive weaning from the ventilator, facilitate coughing, and prevent atelectases and respiratory infections. Uncontrolled pain impedes repeated powerful coughs and hinders valid respiratory excursions and graft expansion. Optimal pain relief is essential to ensure satisfactory cooperation with the physiotherapist. Due to frequent renal impairment induced by chronic immunosuppression, non-steroidal anti-inflammatory drugs should be avoided as they can significantly deteriorate glomerular filtration rate. Caution must also be taken in administering opioids. Their beneficial effects might, in fact, be counterbalanced by the risk of respiratory depression, mild attenuation of the cough reflex, and diaphragm elevation due to bowel distension. Delayed and prolonged postoperative sedation may be determined by the

metabolites morphine-3-glucuronide and morphine-6-glucuronide accumulation. Tramadol in continuous infusion, usually associated with rescue doses of paracetamol, represents a valid alternative to opioids. This association is almost always devoid of outright sedation and it is generally applied without strict respiratory monitoring. Local anesthesia may be useful on cutaneous areas close to the emergence of chest tubes, but it requires frequent repetition. Thoracic epidural analgesia (TEA) is now considered the first choice for pain control after LTx. Besides enabling an optimal modulation of analgesia, TEA has been shown to avoid excessive sedation associated with systemic opioids, and in other surgical settings to reduce pulmonary complications and overall patient mortality [44]. In our institution a thoracic epidural catheter is inserted at the level of vertebral interspaces 4 to 6 (T4-T6) before induction of anesthesia in patients not expected to require intraoperative cardiopulmonary by-pass. In the immediate postoperative period the delivery of repeated epidural boluses or continuous infusion of fentanyl or sufentanil alone is given to prevent any sympathetic blockade in recipients with cardiovascular instability and under vasoactive treatment. Once hemodynamic stability is reached, and well before planning for tracheal extubation, local anesthetics in epidural solution guarantee better afferent segmental sensory block with minimal side effects [45]. Local anesthetics in relatively low concentrations are very effective in dynamic pain control thus allowing the patient to "exercise" and cooperate with the physiotherapist. Low dose opioid plus low concentrations of local anesthetics in epidural infusion preserve cough and maintain resting tidal volumes, respiratory rate, minute ventilation and lung volumes. In our practice, once postoperative pain intensity diminishes, opioids are withdrawn and only local anesthetics are epidurally administered; this contributes to reducing intestinal distension which represents an important obstacle to diaphragm descent.

Acute Postoperative Physical Rehabilitation

Early chest physiotherapy and exercising while still intubated are extremely useful in accelerating the recovery but are only possible with constant involvement of a physical therapy team.

Fortunately, most patients undergoing transplantation are not hospitalized prior to surgical procedure; they thus have an adequate time to participate in pulmonary rehabilitation. Postoperative rehabilitation is an essential component of the LTx program and should begin as early as feasible following surgical procedure, in particular during the ICU stay [46].

Ineffective airway clearance and breathing pattern occur very frequently after LTx; it is related both to denervation of the transplanted graft which leads to impaired cough and slowing of mucociliary clearance, and/or altered chest wall musculoskeletal function. Mucus plugging can lead to volume loss of the transplanted lung(s) and consolidation. In addition, it increases the susceptibility to bacterial superinfections. The patient might not be able to sense secretions that have moved below the suture lines.

Adequate inhaled bronchodilator therapy (beta-agonist, anticholinergic, or combination) must be considered to prevent bronchial hyperreactivity.

An aggressive pulmonary physiotherapy with vibropercussion and postural drainage should be applied several times a day. In some patients, due to incisional and chest tube discomfort, postural drainage with shaking or vibration is better tolerated than percussion

[47]. Trendelenburg and high Fowler's or sitting positions can be used once the patient is hemodynamically stable.

LTx recipients benefit from training in breathing strategies that decrease the sensation of dyspnea and improve ventilation efficiency. Normal breathing patterns with retraining of the diaphragm muscle should be instituted. Thoracic mobility may be improved by instructing the patient in chest and upper-extremities mobilization [48]. Incentive spirometry for deep breathing and trials of positive expiratory pressure therapy are highly recommended after extubation. Directed cough techniques should also be taught. The upright sitting position should be encouraged for coughing, as it has been shown to produce the greatest expiratory flow rates [49]. In patients who are unable to generate substantial airflow the technique of stacking breaths before the expulsion phase can increase the effectiveness of a cough [50]. A decreased mobility, due to various equipment in the ICU that limits movement in bed, may decrease the patient's attitude to exercising despite his/her best effort at preoperative rehabilitation. While ICU ambulation is very labor intensive, a progressive move to transfer out of bed to chair and then to ambulation should be promptly promoted. Changing the patient's position from supine to side laying or upright has been noted to increase output from chest tubes and augment the drainage of pulmonary secretions. In the event of an increase in bronchial secretions the frequency of chest physiotherapy and postural drainage should be increased as well. Maintenance of joint range of motion and muscle strength must be encouraged. If the patient complains of pain and is reluctant to move an adequate incisional pain control must be provided. Early ambulation to begin walking should be performed as soon as feasible. In many instances, a stationary bicycle or treadmill is moved to the patient's room in the ICU to facilitate such exercise in a controlled environment.

Positioning

Both SLTx and BLTx benefit from turning position; many advantages of lateral or prone positioning are not only related to the potential improvement in ventilation and perfusion but also to the reduction of the risk of graft infection, pressure ulcers and thrombophlebitis [51].

BLTx with unilateral graft disease are better treated by positioning the patients with the most involved graft non-dependent. In this situation the increased perfusion in the dependent lung is better matched by a well-preserved ventilation of the dependent graft, with a reasonable improvement in PaO_2. Because of the gravitational decrease in blood flow, the non-dependent lung may theoretically become more compliant to inspiratory gas flow with a greater likelihood that the collapsed alveoli re-expand under both mechanical and spontaneous ventilation. Patient undergoing BLTx and without unilateral graft dysfunction should be turned side-to-side (beginning gradually at 20-30 degrees, while assessing for changes in blood pressure and oxygen saturation), every 2-4 hours. The patient is slowly progressed to full 90-degree turns and prone or semi-prone positions. In both SLTx and BLTx a prolonged mechanical ventilation is always characterized by pooling of interstitial fluid in the dependent lung regions, which, in a permanent supine recipient, are the more dependent posterior areas of the lung. Since these areas normally receive more perfusion, an increase in interstitial fluid may cause a reduction in lung compliance, with an increase in resistance to inspiratory flow, alveolar instability, alveolar collapse and significant deterioration of gas

exchange. Prolonged periods in the supine position must also be avoided to minimize retention of pulmonary secretions. Repeated turning by an experienced staff member in the immediate postoperative period is a rather safe procedure; recipients tolerate lateral positioning with minimal sedation and without the compromising of oxygenation or hemodynamic status. Positioning a SLTx in the early postoperative period in the lateral decubitus with the transplanted lung facing up not only decreases the likelihood of reperfusion edema formation but also allows a better spontaneous clearance of airway secretions. As previously reported, in order not to promote an overexpansion of the native lung during mechanical ventilation, a single lung recipient with emphysema should be positioned with the transplanted lung up. This maneuver theoretically favors the delivery of a greater amount of inspiratory tidal volume to the allograft thus preventing its compression by the more compliant native lung while in supine position. In SLTx for pulmonary fibrosis, both ventilation and perfusion favor the allograft, therefore there are no strict recommendations for proper positioning; however, a better spontaneous pulmonary toilet can be obtained with the transplanted graft up. Unlike the majority of observations [52] George et al. [53] reported that during the immediate postoperative period, in recipients of a SLTx, changes in oxygenation, ventilation, and blood flow were similar regardless of whether the patient was positioned supine, lateral with the allograft down, or lateral with the native lung down. Furthermore, oxygenation, ventilatory and blood flow variables did not differ significantly between patients with fibrosis and patients with emphysema. According to a recognized good clinical practice both LTx recipients under mechanical ventilation and those spontaneously breathing should also be turned in the prone position. As previously underlined, by turning the recipient in the prone position ventilation-perfusion matching may improve as better aerated areas of the graft are dependent where most of pulmonary blood flow tends to go [54]. Furthermore pleural pressure may become more negative thereby enhancing alveolar recruitment and allowing for the reduction of PEEP. Prone position has also demonstrated to favor a downward displacement of the diaphragm and improve lymphatic drainage [54].

Infection Prophylaxys

Infectious complications remain one of the most important causes of morbidity and mortality in LTx recipients. The allograft is particularly susceptible to infectious disease due to: 1) the direct exposure to airway colonization and aspiration, 2) denervation, which implies an impaired cough reflex and abnormal mucociliary clearance, 3) the impaired lymphatic drainage, 4) anastomosis associated complications, 5) transmission of infections from the donor lungs, 6) infections from the native lung in SLTx, and 7) the obligatory immunosuppression [55]. Postoperative infections can result in prolonged mechanical ventilation or sepsis and indirectly increase the risk of allograft rejection. Almost two-third of patients experience an infective complication during the first month after transplantation. Early infections are commonly bacterial and manifest as pneumonia, mediastinitis, urinary tract infections, catheter-related sepsis, and skin infections. Posttransplant invasive infections are frequently caused by organisms cultured from the donor. Conversely, bacterial infections developing in patients with septic lung disease, particularly cystic fibrosis, most commonly

originate from the recipient's airways and sinuses. Bacterial pneumonia, mainly due to retained secretions, and ventilator-associated pneumonia are caused by both gram-positive (Staphylococcus aureus) and gram-negative organisms (Pseudomonas spp, Klebsiella, and Haemophilus influenzae). Cytomegalovirus (CMV) infection remains a significant problem following lung transplantation. The incidence of CMV infection is related to the preoperative CMV status of both the donor and the recipient. Primary CMV infection develops in CMV-seronegative recipients, i.e., those who have not been exposed to CMV prior to transplantation, but receive a graft from a CMV-seropositive donor. Primary infections occur in the blood, lungs, gastrointestinal tract, and retina. Secondary infection occurs as a result of reactivation or reinfection in the patient who was CMV-seropositive at the time of transplantation; it tends to be less serious than primary infection. Fungal infections usually develop from the interaction between net host defense depression and environmental exposure. The risk of fungal infections goes up with increasing immunosuppression, especially when acute rejection episodes are treated with high dose of corticosteroids or antilymphocyte agents. Fungal colonization detected within 3 days significantly predicts a highly invasive and dangerous disease. Both Aspergillus and Candida can be found after Ltx. Mortality of up to 60% has been noted following invasive infection with aspergillus pneumonia. The bronchial anastomosis is a particularly vulnerable site for the development of these infections, and careful attention to these areas must be given during all bronchoscopic evaluations. Treatment of bacterial infections generally involves characterization of the infective agent (e.g., cultures and antibiotic sensitivities), source control (e.g., catheter removal and debridement), and appropriate antibiotic regimens, based on the individual hospital antimicrobial susceptibility patterns. Prophylactic antibiotics are primarily tailored to cover for gram negative (Pseudomonas sp, Haemophilus influenza and Klebsiella sp) and gram-positive organisms (Staphylococcus aureus). In patients with cystic fibrosis, the most recent culture results and sensitivities dictate the choice of antibiotic administered. Although perioperative antibiotic regimens vary widely between transplant centers, third or fourth generation cephalosporins with anti pseudomonas activity (e.g., ceftazidime or cefepime) and vancomycin are commonly used. Prophylactic intravenous antibiotics are given for approximately 7 to 14 days. Preemptive therapy with ganciclovir in recipients who are perceived to be at high risk of CMV infection (CMV-positive recipient or CMV-negative recipient receiving an allograft from a CMV-positive donor) may prevent reactivation or attenuate the clinical course of infection. Full dose of ganciclovir for 4 weeks is a reasonable prophylaxis for CMV disease. Additional administration of intravenous CMV-directed immunoglobulins is also recommended [56]. Other drugs with anti-CMV activity such as foscarnet and cidofovir, proposed for ganciclovir-resistant CMV strains, should be used cautiously, because they significantly affect renal function. Various preventive strategies are being used against fungal infections, but there is still uncertainty to which approach and duration are appropriate. The use of systemic antifungals is sometimes limited by toxicity and interactions with the various types of immunosuppressive agents. Fungal prophylaxis against mucosal *Candida* infection may include use of daily nystatin swish and swallow. In our institute, and similarly to the practice of other institutions [57], aerosolized amphotericin B lipid formulation is always administered, as it results safe and effective to inhibit *Aspergillus* colonization. Both aerosolized and intravenous lipid preparations of amphotericin are given in high risk LTx recipients. Husain et al [58] examined the efficacy and toxicity of prophylactic use of voriconazole. Comparing 30 patients receiving a targeted prophylaxis

with fluconazole alone or in combination with itraconazole, and/or inhaled amphotericin B, the rate of invasive aspergillosis at 1 year following LTx in the recipients with voriconazole was 1.5% versus 23% of the targeted prophylaxis group. However, in the voriconazole group the incidence of Candida colonization, particularly non-albicans species, was significantly higher.

Acute Rejection

Most patients develop at least one episode of rejection within the first 3 weeks following transplantation, typically in the first 5-15 days. Acute rejection is the host's response in the recognition the graft as foreign. DeVitoDabbs et al. [59] reported that 85% of their patients had at least 1 episode of acute rejection within the first year ; the median onset of the first episode was day 20 (range 3-359 days) after LTx, and the first acute episodes occurred within 6 weeks following LTx. Diagnosis of acute rejection in the early posttransplant period is often based on clinical parameters. Symptoms and signs of rejection include fever, dyspnea, a widened alveolar-arterial oxygen gradient (manifested as a decrease in arterial PO_2), a diminished forced expiratory volume in 1 second (FEV_1) a fall in vital capacity, and the development of characteristic bilateral interstitial infiltrates on chest x-ray. A significant number of patients with mild rejection, however, can be asymptomatic and rejection episodes are only diagnosed by surveillance transbronchial biopsy. Persistent infiltrates beyond the first week suggest infection or acute rejection. In approximately half the cases of rejection, the findings on chest radiograph are nonspecific, such as new, worsening, or persistent perihilar infiltrates, ground-glass infiltrate with interstitial fluid, and rapidly developing pleural effusion. CT-scan imaging shows ground-glass opacities, septal thickening, nodules, and consolidations. Acute rejection must be mainly differentiated from bronchitis and pneumonia, but it is often difficult to distinguish the primary disease based on clinical findings alone. Nowadays fiberoptic bronchoscopy (with transbronchial parenchymal lung biopsy and bronchoalveolar lavage) is the gold standard for the differentiation of acute lung rejection and pulmonary infection [60]. Acute rejection is classified into 5 grades based on the severity and extent of the perivascular lymphocytic infiltration. The range is from no significant abnormality (grade A0) to severe abnormality (grade A4). Pathologically, acute rejection initially manifests as a perivascular lymphocytic infiltrate with increased lymphocyte count in the bronchoalveolar lavage fluid, moderate rejection is characterized by perivascular mononuclear cell infiltrates with extension into the adjacent alveolar septa, and severe rejection by an extensive involvement of the interstitium and air space, pneumocyte damage, vasculitis and even parenchymal infarction [61]. LTx recipients who experience an early onset of acute rejection or have a greater severity grade for the first acute episode are more likely than other recipients to have serious rejection within the first year.

Hyperacute Rejection

Hyperacute rejection arises within minutes after the newly transplanted organ begins to be perfused. Hyperacute rejection is mediated through preexisting antibodies against ABO

blood groups, HLAs, or other antigens that interact with vascular endothelium. These cause activation of complement and other cytokines, and results in acute diffuse alveolar damage. Hyperacute rejection is characterized by an abrupt severe graft dysfunction and almost always leads to loss of graft. Intravascular thrombosis, necrosis of vessel walls, and infiltration with mononuclear and polymorphonuclear cells are the common hystopathological findings.

ABO blood group matching and preoperative screening for antibodies against common antigens has largely eliminated this problem.

Acute rejection, particularly when recurrent or severe, has recently been shown to be a major risk factor for the development of chronic rejection or obliterative bronchiolitis [62]. Prevention, early diagnosis, and treatment of acute rejection are important for optimizing short- and long-term outcomes. Episodes of acute rejection are prevented by maintenance of satisfactory immunosuppression. The mainstay of therapy is pulse intravenous methylprednisolone, usually in a dose of 500-1000 mg/d intravenously. Cyclosporin A and azathioprine are also maximized. Often, a dramatic response to treatment with corticosteroids and increased immunosuppression is observed within 24 hours. The treatment of resistant, persistent, or recurrent rejection is sometimes challenging as repeated course of corticosteroids may not be effective. Some authors suggest switching from cyclosporine to tacrolimus [63] while others use pulse treatments with a polyclonal antithymocyte globulin (ATG), anti-interleukin-2 receptor (IL2R) antagonists or muromonab-CD3 (OKT3)[64]. Other therapies that have been considered in the management of severe or persistent rejection include alemtuzumab (antiCD52 antibody), extracorporeal photoferesis and total lymphoid irradiation [65] .

Survival of LTx recipients is enhanced when acute rejection is detected early and appropriate treatments are implemented [66].

Postoperative Renal Complications

Acute renal failure is a common complication of the immediate postoperative period of LTx. The incidence of acute renal failure, its effect on survival and the subsequent changes in chronic renal function are not well defined yet. Castro et al. [67] described a 56% postoperative incidence of acute renal failure, with only 8% of LTx recipients requiring dialysis. Long-term renal dysfunction has been reported with an incidence of 25.5% at 1 year after transplant and 37.8 at 5 years after transplant. By 6 months after transplant, 91% of lung transplant recipients undergo some degree of renal decline from their baseline pretransplant level [68][69]. Several factors may contribute to the development of renal complications. Preoperative decrease of glomerular filtration rate is well-recognized in patients with respiratory failure [67]. Intraoperative hemodynamic instability and high dose vasoconstrictors might be responsible for renal hypoperfusion, thereby favoring the development of postoperative renal dysfunction. Other important causes include the use of diuretics to manage pulmonary edema, fluid restriction and reduction of circulating blood volume in the case of severe PGD, postoperative use of calcineurin inhibitors, and sometimes nephrotoxic antimicrobials [70]. Treatment with parenteral amphotericin B increases the risk of developing renal failure (liposomial and lipid formulations to a lesser extent), especially when it is combined with cyclosporine and/or aminoglycosides. Rocha et al. [71] reported a

direct causality between length of postoperative mechanical ventilation and acute renal dysfunction. Nephrotoxicity due to calcineurin inhibitor is caused by reversible vasoconstriction of afferent and efferent glomerular arterioles, resulting in increase of renovascular resistance by increased levels of the vasoconstrictor endothelin, and impairing the production of the vasodilatory nitric oxide [72]. Broekroelofs et al.[73] demonstrated that the loss in glomerular filtration rate after LTx was greater for patients affected by cystic fibrosis compared with patients with primary pulmonary hypertension or emphysema. The high incidence of renal failure in cystic fibrosis recipients is likely due to the combination of higher need for immunosuppressors, the frequent administration of nephrotoxic antibiotics (i.e., aminoglycosides or colistin) and the recurrent use of non-steroidal anti-inflammatory drugs such as ibuprofen, sometimes prescribed to reduce the intense airway inflammation. Evolution of postoperative renal failure can be variable. Most renal insults in this setting are mild and perhaps caused by reversible hemodynamic-mediated reductions in glomerular filtration rate. Rocha et al. [71] observed that the majority of episodes did not require dialysis and had a small influence on perioperative morbidity and mortality. Conversely, severe renal dysfunction requiring dialysis was much less common, but greatly affected all clinical outcomes studied, including mortality. Sixteen of these patients died during the initial hospitalization for LTx, for an in-hospital mortality of 70%. Castro et al. [67] did not find any association between 1-month mortality and the degree of renal failure in the immediate postoperative period. There was a positive correlation between the degree of kidney failure at 1-month and that observed 6 and 12 months after the procedure. Even though the occurrence of kidney dysfunction after LTx may not be prevented, some prophylactic and therapeutic interventions are worth doing. Continuous assessment of volume status by dynamic volumetric parameters may avoid an excessively negative fluid balance and a larger requirement for vasoactive agents; an almost normal volemic status contributes to prevent renal vasoconstriction and tubular hypoperfusion better than a frank dehydration. Caution should be taken in analgesic prescription as adverse effects of many drugs and some diagnostic procedures on the glomerular filtration rate are exacerbated by nonsteroidal anti-inflammatory drugs. Lowering the levels of calcineurin inhibitors, when feasible, is always a beneficial strategy. Strict monitoring of their blood levels in the postoperative phase may prevent prolonged peaks of overtreatment. Ishani et al reported that use of tacrolimus within the first 6 [69].

Months after LTx is associated with less renal dysfunction than cyclosporine. Alternative immunosuppressive agents such as sirolimus and everolimus, and/or mycofenolate mofetil (MMF) to replace or minimize calcineurin inhibitor use can certainly be considered [74]. Using amphotericin B may not be devoid of renal consequences; agents such as voriconazole and/or echinicandins, when acceptable, should be preferred. Prophylactic administration of fenoldopam after major cardiac surgery has been proposed to reduce the risk of acute renal failure in patients in whom endogenous and exogenous cathecolamines may induce a renal vascular constrictive condition [75]. Even though a preservation of creatinine clearance by counterbalancing the renal vasoconstrictive effect of cyclosporine and the maintainance of perioperative renal vasodilation in patients undergoing liver transplantation has been reported [76][77] there are still no data that confirm a beneficial effect on renal protection by prophylactic fenoldopam in the perioperatiive setting of LTx. Because of the major risk of fluid overload associated with kidney dysfunction renal replacement therapy must be instituted as soon as diuretic treatment is no longer effective. Continuous veno-venous

techniques (CVVH) rather than conventional dialysis (HD) are diffusely applied to control water volume, electrolyte imbalance, and acid-base balance. In the early period following LTx routinary utilization of intermittent hemodialysis is not recommended because of severely compromised hemodynamics; CVVH is better tolerated to remove excess body fluid and to eliminate uremic metabolites [78]. In the presence of acute renal failure complicating a severe PGD continuous renal replacement therapy has to be started early to prevent further allograft edema. Even though the extracorporeal techniques are associated with an increased mortality [71] if renal function is not swiftly restored severe gas exchange impairment and multi-organ damage can result.

Immunosuppression

Aggressive immunosuppressive therapy after LTx is needed due to both intrinsic immunologic functions of the lung and permanent exposure to environmental antigens. All transplant patients are placed on an antirejection protocol; immunosuppressive protocols may be different among LTx centers. An adequate immunosuppression plays a pivotal role in the early and long term success of LTx. Induction of immunosuppression usually takes place intraoperatively during graft implantation. Upon the completion of pulmonary artery anastomosis, SLTx recipients are given a bolus of methylprednisolone (1000 mg), whereas BLTx recipients receive half-dose (500 mg) before the first reperfusion and the second half-dose (500 mg) before the second graft reperfusion. Most centers for maintenance use a 3-drug regimen: a combination of cyclosporin A, azathioprine, and glucocorticoids.

Cyclosporine A(CsA), a calcineurin inhibitor (CNI), acts by suppressing the T mediated cytotoxic response and B-cell function. It is metabolized in the liver through the citochrome P-450 system. Serum levels of 300-400 ng/ml are usually maintained for the first month and, thereafter, levels of 150-200 ng/ml are considered therapeutic. Renal toxicity, hypertension, and, to a minor extent, liver dysfunction are the major side effects. Azathioprine is an anti-metabolite which sets a block in DNA synthesis in white cells series and prevents the immune cell proliferation as a response to antigenic stimuli. Azathioprine is begun at a dosage of 1.5-2 mg/kg/d, and the dose is adjusted to maintain a WBC count of no less than 4000 cells/mm^3. Relevant side effects are thrombocytopenia, leucopenia and anemia (dose-dependent myelosuppression), and sometimes hepatic dysfunction. Tacrolimus, an antibiotic belonging to the macrolid class, acts similarly to CsA. Nephrotoxicity and its systemic complications, electrolyte disturbances, a lowered threshold for generalized major motor seizures, and a higher risk for peripheral neuropathy or psychiatric symptoms should all be considered during the postoperative period. Tacrolimus is the primary immunosuppressive agent for LTx recipients with lymphangioleiomyomatosis and, in recent years, has replaced cyclosporine in many centers. CsA may be a reasonable choice in patients with diabetes, neurotoxicity, and gastrointestinal complications, while tacrolimus is better tolerated in patients with lipid metabolism disorders, hypertension or other cardiovascular risk factors, hirsutism, gingival hyperplasia. Conversion to oral forms of both CsA and tacrolimus is done when patients are capable of eating and dosing is adjusted according to blood trough levels.

Corticosteroids are still a mainstay of immunosuppressive protocols for lung transplants [79].

Low doses of intravenous corticosteroids (0.5 mg/kg/d usually) are given for maintenance in combination with cyclosporin A and azathioprine. An oral dose of prednisone is begun 5-7 days postoperatively. Steroids are associated with multiple side effects, including the development of cushingoid features, hypertension, diabetes, osteoporosis, and peptic ulcer disease.

Mycophenolate mofetil or enteric-coated mycophenolate sodium is increasingly being used instead of azathioprine: selective block of purine synthesis, a preliminary step for lymphocyte T and B proliferation, makes mycophenolate more effective and less toxic than azathioprine [80].

Induction therapy with antilymphocyte globulin and monoclonal anti-CD3 antibodies has been advocated for years but is no longer recommended by many physicians. The more specific and less toxic antibodies (IL-2RA basiliximab [simulect], daclizumab [zenapax], and campath-1H [alemtuzumab]) are under investigation in LTx. Reported side effects with these agents are rashes, fever, and gastrointestinal symptoms.

A mammalian target of rapamycin (mTOR) inhibitors (sirolimus, everolimus) may be introduced as a substitute for calcineurin-inibitors and anti-metabolites, or when major complications arise [79].

Gastrointestinal (GI) Complications

Early GI complications (within thirty postoperative days) are frequent after LTx and have an important impact on morbidity. According to Lubetkin et al (ART 121 di art LAU) they may occur in as many as 50% of recipients. GI complications are related to recipient pulmonary disease, surgical procedure and immunosuppressive therapy. Chronic obstructive pulmonary disease, cystic fibrosis, and idiopathic pulmonary fibrosis are the disorders at increased risk of postoperative GI complications. Esophagitis, pancreatitis, gastrointestinal reflux, gastric atony, gastritis, peptic ulcer disease, gastric bezoar, gastrointestinal bleeding, cholecystitis, diverticulitis, adynamic colonic ileus, CMV hepatitis and colitis are the most frequently reported [81]. Surgical complications, such as intra-abdominal bleeding, small bowel obstruction, bowel perforation, colonic perforation, appendicitis, etc. have been reported in 4 to 17% of LTx recipients [82] . They can occur at any time and may be masked by the side effects of immunosuppression. Cystic fibrosis recipients are the most involved in the development of GI complications; long term steroid therapy, malnutrition from chronic infection, and reduced bowel motility are certainly involved in determining the high incidence. Diagnosis of GI disease is sometimes difficult due to the presence of many confounding factors and the unavoidable coexistence of GI adverse effects of multiple medications. However, because of their severity, early recognition and prompt investigation are essential to proper management. Most abdominal complications respond to conservative treatment but when surgery is required it can be performed with an acceptable morbidity.

Coagulation Disorders

Coagulation abnormalities are common in the early period following LTx. They may occur as a result of intraoperative cardiopulmonary bypass, heparinization, and perioperative excessive bleeding requiring massive blood product replacement. Cystic fibrosis patients often have liver disease and vitamin K deficiency. A reduced synthesis of procoagulant factors is also due to pancreatic insufficiency, low oral intake, and gut flora suppression by antibiotics [83]. Pleural and pericardial adhesions due to chronic infections predispose these patients to an increased perioperative haemorrhagic risk. Lung recipients with alpha-1-antitrypsin deficiency and pulmonary hypertension may also be affected by chronic hepatic disease and coagulopathy. Prolonged postoperative blood loss from chest drainages may be observed in patients with pleural adhesions or previous thoracic surgery. Pleural and mediastinal bleeding may require rethoracotomy. Platelets, fresh frozen plasma, and cryoprecipitate are given as needed to restore hemostasis. Bronchial mucosal bleeding is a rare complication of repeated bronchoscopies, even in the absence of frank coagulopathy. Vulnerability of the mucosal capillaries due to immunosuppression is probably accentuated by increased intrathoracic pressure induced by coughing in a closed airway system. High pressure in the intrathoracic vessels may be transferred to the bronchial vascular system. Besides requiring an obligatory heparinization, LTx recipients on ECMO, may develop complex coagulation disorders which can result in persistent bleeding from surgical and non-surgical sites. Type II heparin-induced thrombocytopenia (HIT) is a rare but very severe complication of heparin treatment in patients under ECMO. Frequent measurement of platelet count, fibrinogen level, fibrin degradation products, prothrombin time, and activated thromboplastin times is mandatory for monitoring the severity of a refractory coagulopathy. Thromboelastography (TEG), a method that enables a more global assessment of coagulation, is almost always required to assess and manage the serious disorders of clot initiation, amplification, propagation, and fibrinolysis, disorders that cannot be assessed by other current clinically available methods. In the early posttransplant period a prothrombotic condition can also be present in some individuals. An increased risk of venous thromboembolism (VTE), which includes deep vein thrombosis (DVT) and pulmonary embolism (PE) has been reported in LTx recipients with pulmonary hypertension, idiopathic pulmonary fibrosis systemic lupus erythematosus and antiphospholipid antibodies [84]. VTE is often associated with an increased amount of vascular trauma, higher levels of cyclosporine, longer ischemic time, older age, cytomegalovirus disease, rejection, and central venous catheters [85]. Low molecular weight heparin, in the absence of significant risk of surgical bleeding, should always be administered to prevent VTE.

Conclusion

Advances in anesthesia and surgical techniques, perioperative ventilatory management, respiratory and renal extracorporeal supportive measures, and improvement in the treatment of both short- and long-term complications have made human lung transplantation an effective and acceptable option for patients with end-stage lung disease. However, the ICU morbidity and mortality associated with a delayed postoperative hemodynamic recovery,

acute allograft dysfunction, adverse effects of many drugs, and surgical complications are still substantial. Early postoperative management is highly demanding as dramatic changes may occur on both allograft and "distant" organs, as a consequence of the progressive increase in "marginality" of the implanted graft, the extremely debilitated recipient and difficult intraoperative course. While satisfactory rates of survival have been obtained from multidisciplinary, collaborative efforts, significant hurdles have yet to be overcome, including issues of severe hypoxia, acute rejection, disseminate infections, unexpected severe complications, and systemic organs dysfunction. Skillful vigilance, a thorough knowledge of the type of procedure performed, an extensive understanding of pathophysiologic characteristics of transplanted lung, and early recognition of life-threatening clinical problems are fundamental for a successful ICU treatment.

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Chapter 3

Impact of Viral Pathogens in Lung Transplant Recipients

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Abstract

Lung transplantation is the only definitive mode of treatment for many forms of end-stage pulmonary diseases; however, its success may be limited by several factors, including infections, acute rejection (AR), and chronic graft dysfunction termed bronchiolitis obliterans syndrome (BOS).

Viral infections of the graft (including those from community acquired respiratory viruses and from persistently infecting viruses, such as herpesviruses) are responsible for organ infection/disease; in addition to direct sequelae, accumulating data suggest that viruses may be triggers for a cascade of events, including upregulation of allo-reactive cells, potentially leading to AR or chronic graft dysfunction.

Community acquired respiratory viruses (CARV) have been increasingly recognized as common pathogens in lung transplantation (LT) and include the *paramyxoviridae* (respiratory syncytial virus, parainfluenza virus, human metapneumovirus), the *orthomyxoviridae* (influenza A and B), the *picornaviridae* (rhinovirus, enterovirus), the *coronaviridae* (coronavirus) and the *adenoviridae* (adenovirus). It has been suggested that LT recipients infected with CARV exhibit a high rate of progression to severe viral pneumonitis. Moreover, previous studies have evidenced that patients with CARV infection of the lower respiratory tract are predisposed to AR and high-grade BOS

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development, and, conversely, that patients with BOS are predisposed to CARV infections.

Herpesviruses, mainly human cytomegalovirus (HCMV), are highly seroprevalent and are considered as potential pathogens causing direct and indirect effects in transplant recipients and establishing latency in various tissue, including lung. Whereas HCMV represents the main viral pathogen responsible for organ infection and disease, the role of other herpesviruses, including human herpesviruses 6 and 7 (HHV-6 and HHV-7) and Epstein-Barr virus (EBV) is less defined. The role of herpesviruses reactivation in LT in relation to the development of AR and chronic graft dysfunction remains controversial, as it seems that despite high viral loads detected in bronchoalveolar lavages (BAL), virus replication results not associated with the development of rejection, however data are conflicting and few studies have specifically investigated this issue. In this Chapter, the impact of viral pathogens, including CARV and persistently infecting viruses, on the clinical course and the onset of rejection and graft dysfunction will be analyzed reporting the results of the main studies published in literature and the experience of our Laboratory of Virology.

Introduction

Lung transplantation (LT) is the only definitive treatment for many forms of end-stage pulmonary diseases. However, its success is limited by several factors, including: organ infection/disease, acute rejection (AR), and chronic allograft dysfunction [1,2]. Progresses made in patient selection, surgical techniques as well as in therapeutic management (immunosuppressive regimens) have led to a growing increase in the one-year survival rates up to 75%; however, the 5-year survival rate following LT remains only approximately 50%, with the most significant impact on long-term survival being represented by the onset of chronic graft dysfunction [3]. Chronic rejection has been reported as high as 60% to 80% at 5 to 10 years after LT [4-6].

In the context of transplantation, organ infection/disease is defined by the evidence of local infection with positivity to laboratory assays together with clinical symptoms and histopathological evidence of tissue injury (i.e. symptomatic infection).

Acute rejection is defined by the presence of perivascular and interstitial mononuclear infiltrates, with small airway inflammation forming a lesion know as lymphocytic bronchiolitis; while chronic allograft dysfunction is termed bronchiolitis obliterans syndrome (BOS) and can be diagnosed clinically based on sustained declines in lung function, whereas it is histologically defined as bronchiolitis obliterans (BO)[7]. Acute cellular rejection was once thought to be a major risk factors for BOS, although new data support an important role for lymphocytic bronchiolitis, independent of so-called vascular AR [8].

Lung transplant recipients present specific risk factors for viral infections, including potent immunosuppression regimens, direct exposure of the transplanted organ to the environment with airborne viruses, impaired mucociliary clearance, poor cough reflex due to denervation of the allograft, and abnormal lymphatic drainage. In addition to direct consequences of viral infections with progression to organ disease, accumulating data, primarily from retrospective studies, indicate that viruses may determine severe indirect effects. In particular, it has been hypothesized that viruses may act as triggers for a cascade of

immunological events, including up-regulation of allo-reactive cells, leading to the development of acute and chronic rejection.

Community acquired respiratory viruses (CARV) are frequently responsible for acute respiratory illness in the general population [9,10] and have been increasingly recognized as common pathogens causing in allograft patients, particularly in lung transplant recipients [11-16]. On the other hand, in a recent study on pediatric LT recipients, although respiratory viral infections occurred in the majority of study population, these were not associated with mortality or chronic allograft rejection [17]. Considering the possible association between viral infection and subsequent acute and chronic rejection, it should be investigated whether specific antiviral treatment at the time of infection could have an impact on preventing these indirect effects. Apart influenza virus for which agents are available, proven antiviral therapy for respiratory viral infections is limited. The most common CARVs include those belonging to the *Paramyxoviridae* family (respiratory syncytial virus, parainfluenza virus, human metapneumovirus), the *Orthomyxoviridae* family (influenza A and B), the *Picornaviridae* family (rhinovirus, enterovirus), the *Coronaviridae* family (coronavirus) and the *Adenoviridae* family (adenovirus).

Persistently infecting viruses, such as herpesviruses (mainly human cytomegalovirus [HCMV[), are highly seroprevalent and are considered as potential pathogens causing direct and indirect effects in transplant recipients and establishing latency in various tissue, including lung. Whereas HCMV represents the main viral pathogen responsible for organ infection and disease, the role of other herpesviruses, including human herpesvirus 6 [HHV-6] and 7 and Epstein-Barr virus [EBV] is less defined. The role of herpesviruses reactivation in LT in relation to the development of AR and chronic graft dysfunction remains controversial, as it seems that despite high viral loads detected in bronchoalveolar lavage (BAL), virus replication results not associated with the development of rejection, however data are conflicting and few studies have specifically investigated this issue and almost always retrospectively.

In the following paragraphs the impact of viral pathogens in LT recipients in terms of organ infection/disease and association with acute and chronic graft rejection will be described singularly.

Community Acquired Respiratory Viruses

Paramyxoviridae (Parainfluenzaviruses, Respiratory Syncytial Virus, Human Metapneumovirus)

The *Paramyxoviridae* family is constituted by relatively large RNA viruses with 150-300 nm in diameter and spherical or pleomorphic shape. Paramyxoviruses causing upper and lower respiratory infections and hospitalization in adults and children are classified in two subfamilies: *Paramyxovirus* and *Pneumovirinae*. The *Paramyxovirus* subfamily includes the genera *Paramyxovirus* (parainfluenza virus 1 [PIV1] and parainfluenzavirus 3 [PIV3]), Rubulavirus (parainfluenza virus 2 [PIV2] and parainfluenzavirus 4 [PIV4], mumps virus), and *Morbillivirus* (measles virus), while the *Pneumovirinae* subfamily includes the genera *Pneumovirus* (respiratory syncytial virus [RSV]) and *Metapneumovirus* (human

metapneumovirus [hMPV]). From a generic structural point of view, paramyxoviruses present a nucleocapsid core containing negative sense, unsegmented, single stranded RNA, along with RNA-dependent polymerase complex constituted by three non-structural proteins closely associated with RNA (i.e. nucleoprotein NP, phosphoprotein P, large protein L). Paramyxoviruses are enveloped viruses with a lipid bi-layer associated to two virus-specific glucoproteins (hemagglutinin-neuraminidase [HN] being a viral attachment protein also responsible for hemadsorption and hemagglutination; and fusion protein [F] promoting the fusion of viral and host cell membranes, thus participating into the initial steps of infection. Among physical properties, paramyxoviridae are labile, although highly infectious, sensitive to heat and detergents, and antigenically stable.

Parainfluenza viruses are ubiquitous and common causes of respiratory infections, including rhinitis, pharyngitis, cough, croup, bronchiolitis, pneumonia, occurring as epidemic as well as sporadically. Transmission occurs via the respiratory route (large droplets transmitted person to person through close contact, aerosol of respiratory secretions) or by fomites (up to 10 hours of stability on surfaces). In the otherwise healthy host, parainfluenzavirus infection is usually mild and self-limiting with the virus being shed for approximately one week.

Respiratory syncytial virus is the most important worldwide cause of respiratory tract infection in infants and children, infecting almost all by the age of two years [18] and having a great impact in pediatric hospitalizations and mortality [19], with a disease spectrum including a wide array of symptoms from rhinitis and otitis media to pneumonia and bronchiolitis. Although traditionally considered as a pediatric pathogen, RSV can also cause severe pulmonary disease in immunocompromised and the elderly.

Human metapneumovirus was first identified in 2001 from archived respiratory cultures collected from infants and young children in which no other pathogen was isolated [20]. Infection has been widely reported in wintertime in pediatric patients presenting with clinical manifestations similar to RSV and characterized by wheezing and bronchiolitis. However, as immunity induced by hMPV is incomplete, reinfections may occur in adults of all ages and, although frequently asymptomatic, it may sometimes determine severe infections requiring hospitalization in the elderly [21].

Studies indicate that paramyxoviruses play a role in the etiology of respiratory tract infections in LT recipients; these patients appear to be more susceptible to paramyxoviral infections in comparison to other solid organ recipients. A cumulative incidence of infection of 6% to 21% has been described over periods ranging from 5 to 7 years [22-25]. The effects of paramyxoviral infections in LT recipients include upper and lower respiratory tract signs and symptoms, decline in lung function as measured by spirometry (a median decrease of FEV_1 of 25% has been described [24,26]), and impaired oxygenation. The reported mortality of paramyxoviral infection in LT patients is as high as 20%, being lower that that described in bone marrow transplantation, but higher than that reported in other solid organ transplant recipients. In a study by McCurdy and coll. [24], although most of patients returned to baseline lung function within 3 months, RSV and PIVs contributed to either long-term pulmonary dysfunction or death in 33% of cases.

Studies have suggested that paramyxoviruses may increase the risk of BO in LT recipients, with rates of occurrence in up to 20% of cases [27]. Paramyxoviridae are able to cause long-term pulmonary inflammation potentially leading to significant obstructive disease of the airways. However, other studies have been unable to evidence an association between

viral infection and the development of BO [24]. In particular, among the recently described viruses, hMPV has been described as a risk factor for the development of BOS and in a study 33% of LT patients infected by hMPV developed BOS [15]. As regards progression from early BOS to higher stages, while two studies found an increased risk of progression [11,28], this was not confirmed by others [15], and controversy remains.

Diagnosis is made on upper respiratory tract specimens, such as nasopharyngeal swabs, or following bronchoscopy and BAL evaluation, as appropriate, by rapid antigen detection, viral culture or molecular methods.

The administration of aerosolized and systemic ribavirin is usually well tolerated and may be temporally associated with an improvement in symptoms in most patients [29]. Although it is likely that early treatment of viral infections may play a preventive effect on the subsequent development of BO, this should be further evaluated given the small number of treated patients in available studies [15].

Influenza Viruses

The term influenza indicates an acute febrile illness with systemic symptoms, usually occurring in the wintertime, that may be caused by several different bacterial and viral agents; however, true influenza is caused by a member of the *orthomyxoviridae* family. Influenza viruses are negative-sense, segmented single stranded RNA viruses. Virions are pleomorphic and contain RNA polymerase packaged within the virus particle. The internal antigens (M1 and NP) are the type specific antigens used to determine the virus type; A, B, or C. There are eight segments of RNA in influenza A. Influenza A is most extensively studied; A and B are the most important in human disease; type B is usually associated with milder symptoms. The viruses have an envelope and two membrane glycoproteins: hemagglutinin (HA), the attachment and fusion protein; and neuraminidase (NA), important for the release. These are used to determine the specific strain of influenza. Transmission is via aerosols (particles with diameter <10 μm), the virus may also survive briefly on surfaces and subsequently infects the epithelial cells of the respiratory tract. After a short incubation period (18-72 hours), infection becomes symptomatic with the well-known "flu" pattern: uncomplicated (fever, myalgias, headache, cough, nasal discharge); pulmonary complications (croup, primary influenza virus pneumonia, secondary bacterial infection); non-pulmonary complications (myositis, cardiac complications, encephalopathy, Reye's syndrome, Guillain-Barrè syndrome). Major cause of death are secondary bacterial pneumonia and cardiac failure, with up to 95% of deaths occurring in people over 65 years of age. HA and NA proteins undergo antigenic drift with cumulating mutations so that immunity to the original strain does not mean immunity to the drifted one. Antigenic drift results in sporadic outbreacks and limited epidemics. Influenza A periodically presents an apparently new HA and/or NA, therefore scant pre-existing immunity is present and an epidemic/pandemic is seen.

Diagnosis is made by viral isolation or molecular tests; rapid antigen tests have been recently approved. A new vaccine is developed annually using the types and strains of influenza predicted to circulate for that year based on the worldwide monitoring of influenza and is recommended in persons at risk for medical complications or more likely to require medical care (aged 50 years and older, children aged 6-59 months, children and adolescents on long-term aspirin therapy, women who will be pregnant during the influenza season, adults

and children with chronic medical condition, immunocompromised adults and children). Antiviral agents (rimantadine and amantadine and neuraminidase inhibitors) are available and are used for prevention. Rimantadine and amantadine can reduce the duration of influenza A if early administered.

Transplant recipients present significant morbidity and mortality as a result of influenza infection. Several factors may impact on clinical course of influenza infection in the transplant setting, such as patient age, exposure, level of specific immunity, degree of immunosuppression, and type of epidemics. In organ transplant recipients influenza infection may be associated with higher rate of pulmonary involvement, in particular prolonged shedding of influenza virus, interstitial pneumonia and BO (in LT patients) [30]. Annual trivalent inactivated vaccine is recommended following transplantation by the guidelines of the American Society of Transplantation [31]. In early 2009, a new strain of influenza A H1N1 was described and resulted in a worldwide pandemic. This newly identified strain was a reassortant virus with genes from swine, avian, and human viruses. Pandemic H1N1, as influenza virus in general, is more likely to cause severe disease in transplant recipients in comparison to general population. In a multicentre cohort study recently published, it has been evidenced that influenza A H1N1 causes a substantial morbidity in recipients of solid organ transplants and that starting antiviral therapy early is associated with clinical benefit as measured by need for admission to intensive care unit and mechanical ventilation [32].

Rhinoviruses

Rhinoviruses belong to the *Picornaviridae* family and consist in small, positive-sense, single stranded RNA viruses, with a naked nucleocapsid. More than 100 serotypes on rhinoviruses are known, thus explaining the failure of developing vaccines. Rhinoviruses are sensitive to low pH and temperature. They are spread by aerosols and also by fomites such as hands and other direct contacts. Rhinoviruses account for 30% to 50% of all the cases of common cold and related upper respiratory tract complications, such as otitis and sinusitis. Although generally associated to mild disease, the epidemiologic and economic impact of rhinoviruses is very relevant. More recently, a pathogenic role for rhinoviruses in the lower respiratory tract has been reported, particularly in immunocompromised patients, although data are controversial and few studies have been performed on adults [33-35]. Although rhinoviruses are generally temperature restricted in replication with optimal growth at 33–35°C, as in the upper respiratory tract, the temperatures in the tracheobronchial tree are often lower than body core temperatures, also in relation to external temperature and frequency of ventilation, thus being permissive for replication and many serotypes can replicate efficiently at core body temperature [36,37]. In contrast to immunocompetent subjects, the clearance of rhinoviruses in immunocompromised patients may be delayed with prolonged shedding. In a study on rhinoviruses detection in BAL from 68 LT recipients, Kaiser and coll. described three patients with lower respiratory symptoms and graft dysfunction, two of which with AR and persistent infection over a period of 12 months [38]. In a cohort study on LT recipients, rhinoviruses and coronaviruses together accounted for 52% of confirmed viral infections, although all of these infections were relatively mild and self-limited. However, in a subset of patients, serious indirect sequelae were found: for example, among eight patients with rejection, four had prior rhinovirus infection, one coronavirus infection, one influenza and

one RSV [14]. Further studies on the clinical impact of rhinoviruses in LT recipients are needed to better elucidate the possible involvement in the development of rejection. It could be hypothesized that rhinoviruses, similarly to other CARVs, contribute to the onset of a cascade of events potentially leading to acute and chronic graft dysfunction. Pleconaril has antiviral activity for rhinoviruses, however remains an investigational drug.

Adenoviruses

Adenoviruses are medium-sized, non-enveloped double stranded DNA viruses initially grown from adenoidal tissue. The virus is able to infect several cells, including respiratory, bladder, and intestinal epithelium, conjunctiva, and the central nervous system, therefore adenoviral infections may be associated with respiratory, ocular, or gastrointestinal diseases. At least seven human adenoviruses species (from A through G), including 52 serotypes, have been described, with different organ tropism. Transmission is person to person, through water, fomites, and instruments; adenoviruses are highly stable in the environment. Seasonal variation occurs as regards respiratory infections (late winter through early summer)[39]. Immunocompromised hosts are at increased risk of adnoviral infections [40] and the incidence of adenoviral infection in solid organ transplant recipients has been found to be 5-10% [30]. Several antiviral therapies have been used in transplant recipients with adenoviral disease, including cidofovir, ribavirin, and ganciclovir [30]; although no randomized trial is available.

Persistently Infecting Viruses: The Herpesviridae Family

Herpesviruses are enveloped double stranded DNA viruses, with genome consisting of long and short fragments. Herpesviruses are divided in three subfamilies: alpha-herpesvirinae (including herpes simplex virus type 1 and 2, varicella-zoster virus), beta-herpesvirinae (including HCMV, HHV-6 and 7), and gamma-herpesvirinae (including EBV and human herpesvirus 8). Following primary infections, herpesviruses establish a life-long relationship with their host by setting up latent or persistent infections and periodically reactivating, particularly during periods of immunosuppression. Both primary infection and reactivation are likely to be more severe in the immunocompromised host. The capability of viral reactivation in immunocompromised patients, such as solid organ transplant recipients, remains a major problem complicating the care of these patients, sometimes even leading to the loss of the transplanted organ or death. Apart for human herpesvirus 8, herpesviruses are characterized by high seroprevalence in adults, with primary infections usually occurring early in the childhood in the majority of cases; latency in several sites, including lung; and reactivation in immunocompromised conditions leading to direct and indirect effects impacting on the outcome of transplant patients. Lung transplant recipients may be predisposed to lower respiratory tract viral infection/reactivation by herpesviruses. At this regards, in an epidemiological analysis performed on 165 BAL specimens from LT recipients and 412 samples from other patients (both immunocompromised and immunocompetent) by our laboratory, the detection of at least one herpesvirus (including β-herpesviruses and EBV) was quite frequent (>70%) in LT recipients and significantly higher in comparison to other

transplant patients, immunocompromised and immunocompetent individuals, in particular as regards HCMV and HHV-6 (unpublished data).

The prevalence of β-herpesviruses and EBV in BAL fluid from LT patients has been investigated in some studies. In particular, HCMV has been reported in 13% to >50% of BALs from LT patients [41-43]. Several studies have evidenced that persistent DNAemia is associated with a reduced long-term survival of the patient. Human cytomegalovirus is a clinically relevant agent of organ disease (pneumonia), with a viral load > 10^5 genome copies/ml BAL suggested as cut-off level for preemptive therapy in LT recipients [44]. Human cytomegalovirus is the most important viral agent implicated as a potential trigger for acute and chronic allograft injury. Human cytomegalovirus is well recognized as an immunomodulatory viral agent, although in clinical studies the role of its replication in the graft environment in terms of pathogenesis remains controversial. Among the 165 BAL specimens from LT recipients evaluated at our laboratory (as described in the following paragraph), only HCMV (among herpesviruses) was significantly associated with the occurrence of AR, although without relation to viral load; the association between HCMV and AR was confirmed by the time-dependent Cox proportional-hazards regression model, with a hazard ratio of AR occurrence in the presence of previous detection of HCMV increased by a factor 3.85 for 1 month.

As regards the conflicting results obtained by different studies in terms of possible association between HCMV and chronic graft dysfunction, these discrepancies could be due to differences in the definition of infection and disease, as well as immunosuppressive regimens and antiviral strategies [45-49]. Despite of these conflicting results, it is likely that a severe and symptomatic HCMV infection predisposes to the development of chronic graft dysfunction, in particular by increasing the predisposition to AR [50-54].

The occurrence of HHV-6 in BAL samples from our series (165 specimens) was 24.2% and resulted not different from those found in other studies on LT recipients, in which viral DNA was detected in 20% to 31% of cases (patients or specimens) [42,44]. Similarly, the prevalence of HHV-7 DNA found in our and other studies ranges between 20% up to 40% [55].

Few studies have investigated the occurrence of EBV DNA in the lower respiratory tract. In a study by Neurohr and coll. [41], polymerase chain reaction for EBV in BAL fluid resulted positive in 19 of 64 LT patients (30%); while in another study by Bauer and coll. [56], the prevalence of EBV DNA was 43.6% (34/78 specimens). Overall, these data evidenced that, independently from the administration of antiviral prophylaxis, the reactivation of β-herpesviruses and EBV in the graft is common following LT, although this has to be considered taking into account the biology of the viruses and viral load, as lung may represent a latency site.

Few studies have been published on β-herpesviruses, others than HCMV, and EBV and the association with AR in LT. Studies on solid organ transplant recipients other than lung have found a relation between HHV-6 and -7 and AR, as well as a potential for indirect effects such as favouring HCMV disease [57-59]. In a study on 87 LT patients [41], univariate and multivariate analysis revealed that HHV-6 DNA detection in BAL specimens (but not HCMV and HHV-7) was associated with an increased risk to develop BOS, separate from the risk attributable to AR; while only AR was a distinct risk factor for BOS at a Cox regression analysis. However, it is to note that a limitation of this study was that beyond the first 3 months after transplantation, bronchoscopy was performed only for clinical indications,

thus representing a selection bias in favour of detecting viral pathogens. Another study on 19 LT patients suggested an association between HHV-7 detection in BAL and bronchiolitis obliterans organizing pneumonia (BOOP) [60], an important complication in pulmonary transplantation. As regards EBV, very few studies have investigated the role of EBV in the development of AR in LT patients. Two studies have evaluated the detection of EBV DNA in blood: Bakker and coll., by monitoring EBV load in blood, found no association with AR [61]; while in a large prospective cohort study on lung and heart-lung transplant recipients, Engelmann and coll. [62] evidenced that repeated EBV DNA detection in blood, possibly reflecting chronic EBV replication, was associated with the development of chronic graft dysfunction, as well as AR.

Role of B-Herpesviruses and Epstein-Barr Virus in the Lower Respiratory Tract From Lung Transplant Patients: Results of a Study Performed at the Virology Unit of the University Hospital San Giovanni Battista, Turin, Italy

The University Hospital San Giovanni Battista of Turin, Italy, is a large tertiary-care center IN North-Western Italy (Piemonte region); approximately 350-400 BAL specimens are referred for virological investigation over a 1-year period. Herein we reported the epidemiological and clinical data obtained by investigating the occurrence of β-herpesviruses and EBV reactivation in BAL specimens from LT recipients in comparison to other immunocompromised or immunocompetent patients and to assess the association with AR, lymphocytic bronchiolitis, BO, and interstitial pneumonia.

Table 1. Clinical features of lung transplant recipients

Features	**N = 33 (%)**
Age (yr)	
Mean ± SD	49.2 ± 16.6
Range	19-67
Sex (M/F)	22/11
Underlying disease	
Cystic fibrosis	11 (33.3%)
COPD/emphysema	10 (30.3%)
Pulmonary fibrosis	8 (24.2%)
Hyaline membrane disease	1 (3.0%)
Alpha-1-antitrypsin deficiency	1 (3.0%)
Sarcoidosis	1 (3.0%)
Bronchiectasis	1 (3.0%)

SD, standard deviation; COPD, chronic obstructive pulmonary disease.

Methods

Over a two-year period (March 2007-2009), all the 165 consecutive BAL specimens from 33 patients receiving a LT at the University Hospital San Giovanni Battista of Turin were evaluated. Patients' clinical features are summarized in Table 1.

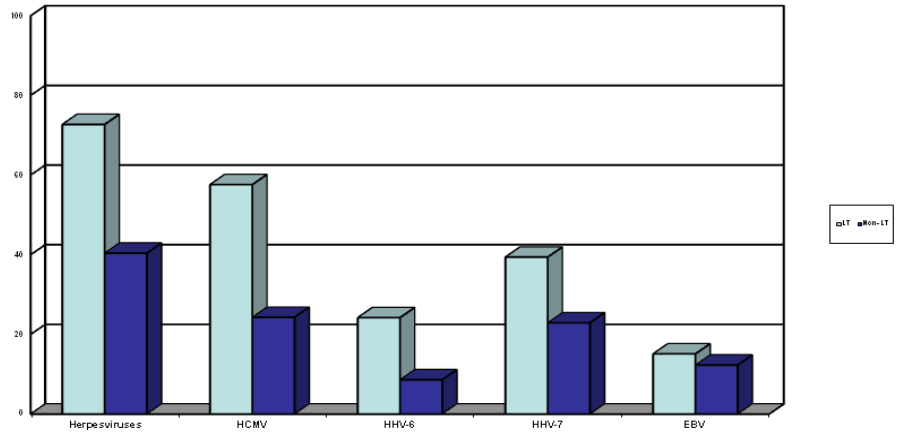
Samples were collected as routine follow-up (to each BAL a transbronchial biopsy [TBB] was obtained during bronchoscopy procedure) at month 1 post-transplantation and subsequently at three-month intervals (150 samples, 90.9%) or in addition for investigating the cause of unexplained fever and/or respiratory symptoms and/or new infiltrates on chest X-ray (15 samples, 9.1%). According to centre's practice, a standard therapeutic regimen was given to all patients: cyclosporine or tacrolimus (in patients with cystic fibrosis), mycophenolate mofetil and prednisone (to be tapered at low dosage or discontinued) for long-term immunosuppression; valganciclovir (450 mg twice daily) from day 21 after transplantation for 3 weeks associated with HCMV-Ig (Cytotect Biotest) at days 1, 4, 8, 15, and 30 (1.5 ml/kg body weight) and every month for 1 year (1 ml/kg body weight), irrespective of HCMV mismatching; trimethoprim-sulfamethoxazole; and voriconazole for chronic infection prophylaxis [63,64]. Ganciclovir or valganciclovir were further administered in case of HCMV positive rapid shell vial culture and/or HCMV DNA load $>10^4$ genome equivalents (GEq)/ml in the presence of symptoms or $>10^5$ in any case [65]. Bronchoalveolar lavage procedures were performed as previously described [10]. Acute rejection, lymphocytic bronchiolitis, and BO were diagnosed by surveillance TBB using the criteria defined in the Revision of the 1996 Working Formulation for the Standardization of Nomenclature in the Diagnosis of Lung Rejection by the International Society for Heart and Lung Transplantation (ISHLT)[7]. Moreover, in the same time period, 412 BAL samples obtained from 292 patients, either immunocompromised (218 specimens from 162 patients, including 109 specimens from 80 transplant patients other than LT and 109 specimens from 82 otherwise immunocompromised individuals: immunosuppression due to chemotherapy, HIV infection or long-term use of corticosteroids) or immunocompetent (194 specimens from 130 patients) were investigated. Informed consent was obtained from all the patients or the nearest relative. Automated total nucleic acid extraction from BAL was performed using the automated Nuclisens EasyMAG platform (Biomerieux, Marcy l'Etoile, France), according to the manufacturer's instructions. Real time PCR assays were performed with the 7300 Real Time PCR System (Applied Biosystems, Monza, Italy) by using TaqMan platform and LUX technology, in particular for the detection of HCMV- and EBV-DNA commercial kits were used (Q-CMV Real Time Complete Kit, EBV Q-PCR Alert kit; Nanogen Advanced Diagnostics, Milano, Italy) and for the detection of HHV-6 and -7 DNA two home-made protocols of real time TaqMan and LUX™ (Light Upon eXtension) PCR, respectively, as previously described [66,67]. Amplifications were set up in a reaction volume of 25 µl, containing 5 µl of the extracted specimen, or negative control (sterile double-distilled H_2O), or standard plasmid dilutions, and 20 µl of the corresponding reaction master (ampliprobe + amplimaster Q-CMV or EBV Q-PCR Kit [Nanogen], for HCMV and EBV; master mix with ROX [Invitrogen, Carlsbad, CA], 200 nM of primer sense, 200 nM of primer antisense, 100 nM of probe, and H_2O, for HHV-6; master mix with ROX [Invitrogen], 400 nM of primer sense LUX [Invitrogen], with a fluorophore attached to the 3' end in a harpin structure, and 400 nM of primer antisense, and H_2O, for HHV-7), and processed at the following conditions: 50 °C for 2 min., initial denaturation step at 95°C for 10 min., followed by 45 cycles at 95°C

for 15 sec. (denaturation) and 60°C for 1 min. (annealing and extension). Standard curves for the quantification of DNA were constructing by plotting the threshold cycle against the logarithm of serial 10-fold dilutions of the corresponding plasmid. Amplifications data were analyzed by the Sequence Detection System software (Applied Biosystem). Specimens were subjected to simultaneous TaqMan PCR for the housekeeping gene glycerin-aldehyde-3-phosphate-dehydrogenase (GAPDH), as internal control. Results were considered acceptable only in the presence of GAPDH with a threshold cycle (Ct) lower than 39. The assays have a detection limit of 1760 GEq/ml for HCMV and 880 for HHV-6, -7 and EBV. A "nucleotide-nucleotide blast" search for short nucleotide sequences performed at the National Centre of Biotechnology Information and the National Library of Medicine web site (available at: http://www.ncbi.nlm.nih.gov) confirmed that the primers used should not amplify other viruses pathogenic to humans.

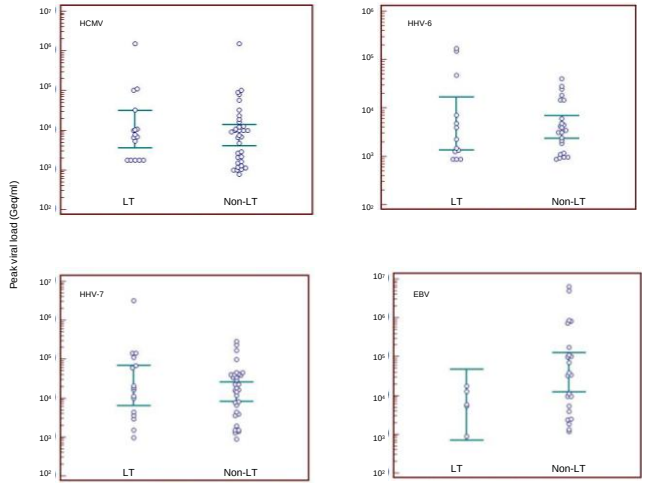
Statistical analysis was performed using the chi square test, the t-test, and the ANOVA, as appropriate. A time-dependent Cox proportional-hazards regression model was used to evaluate for an association between viral positivity and the development of AR. For Cox proportional-hazards regression analysis, a forward method by which the independent variables are entered into the model was chosen (i.e. significant variables are entered sequentially; a variable is entered into the model if its associated significance level is less than p-value 0.05, while a variable is removed from the model if its associated significance level is greater than p-value 0.1). All analyses were performed employing a commercially available software (MedCalc; version 9.2.1.0). A p-value <0.05 was considered statistically significant.

Results

Overall, 24/33 LT recipients (72.7%) had at least one positive result for at least one herpesvirus vs 112/292 of other patients (40.4%)($p < 0.0001$)(Figure 1a): in particular, vs 36/80 (45%) transplant patients other than LT ($p = 0.013$), vs 31/82 (37.8%) otherwise immunocompromised individuals ($p <0.001$), and vs 45/130 (34.6%) immunocompetent patients ($p <0.0001$). HCMV was the most frequently detected β-herpesvirus, being positive in at least one BAL specimen from 19/33 (57.6%) LT recipients vs 71/292 other patients (24.3%; $p <0.0001$); HHV-6 in 8/33 (24.2%) vs 25/292 (8.6%; $p = 0.012$); HHV-7 in 13/33 (39.4%) vs 67/292 (22.9%; p = n.s.); EBV in 5/33 (15.1%) vs 36/292 (12.3%; p = n.s.)(Figure 1a). A co-infection by at least two β-herpesviruses was detected in 12/33 (36.4%) LT patients vs 28/292 other patients (9.6%; $p <0.0001$). Among the 15 specimens from nine patients collected because of clinical symptoms, six from three patients resulted positive to HCMV. Rapid shell vial culture for HCMV resulted positive in at least one specimen in 13/33 (39.4%) patients, always positive also to HCMV-DNA. Based on HCMV serostatus before transplantation as abstracted from clinical charts, three of these patients presented a primary infection. Considering the highest value in each patient, median peak viral load was 6768 GEq/ml (range 1760-1500000) for HCMV; 1853 (range 880-171015) for HHV-6; 15932 (range 940-3212899) for HHV-7; and 8930 (range 880-17296) for EBV. Mean peak viral loads of each virus did not differ between LT recipients and other patients (Figure 1b).



(A)



(B)

Figure 1. (A) Prevalence of herpesviruses HCMV, HHV-6, HHV-7, and EBV DNA in bronchoalveolar lavage specimens from lung transplant patients (LT = 33) and non-lung-transplant patients (non-LT = 292). p <0.0001 for herpesviruses (HCMV+HHV-6+HHV-7+EBV) and HCMV; p = 0.012 for HHV-6; p = n.s. for HHV-7 and EBV. (B) Peak viral load for HCMV, HHV-6, HHV-7 and EBV in bronchoalveolar lavage specimens from lung transplant patients (LT = 33) and non-lung-transplant patients (non-LT = 292). GEq, genome equivalents. p = n.s. for each virus.

Acute rejection was diagnosed on TBB in 14 cases from 10 LT patients: the corresponding BAL specimens resulted positive to HCMV in 10/14 vs 25/151 cases without AR (p <0.0001); to HHV-6 in 2/14 vs 19/151 (p = n.s.); to HHV-7 in 4/14 vs 16/151 (p = n.s.)(Figure 2a); with no significant difference in mean viral loads (Figure 2b). No specimen obtained from a patient with AR resulted positive to EBV. Small airway inflammation was found in four cases from as many patients: the corresponding BAL specimens resulted positive to HCMV in 3/4 vs 32/161 cases without small airway inflammation (p = 0.041); to HHV-6 in 1/4 vs 20/161 (p = n.s.); to HHV-7 in 2/4 vs 18/143 (p = n.s.); and none positive to EBV.

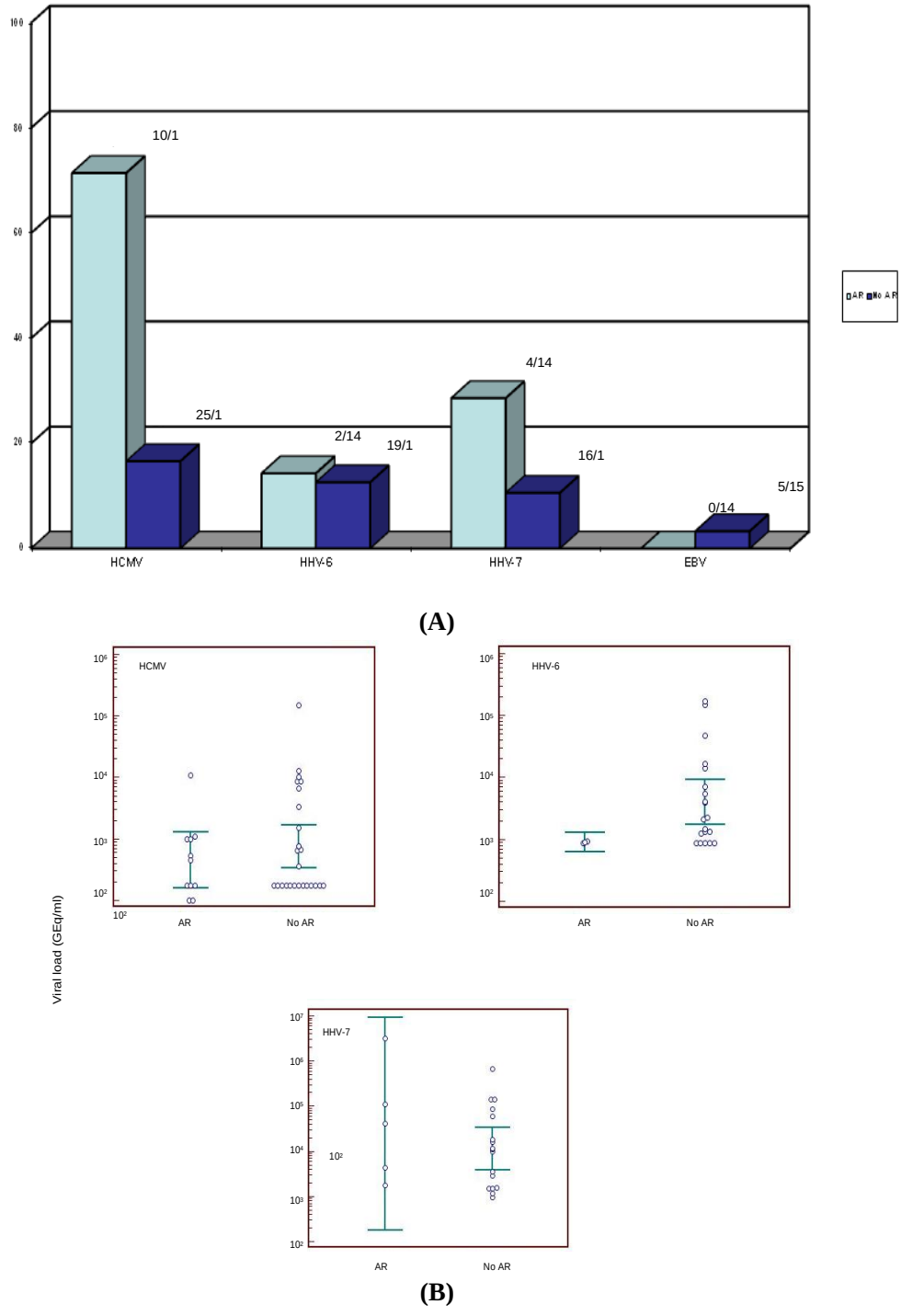


Figure 2. (a) Prevalence of HCMV, HHV-6, HHV-7 and EBV DNA in 165 bronchoalveolar specimens from lung transplant patients with (AR, N = 14) or without (No AR, N = 151) histologically confirmed acute rejection. $p < 0.0001$ for HCMV; p = n.s. for HHV-6, HHV-7 and EBV. (b) Viral load for HCMV, HHV-6, HHV-7 and EBV in bronchoalveolar lavage specimens from lung transplant patients with (AR, N = 14) or without (No AR, N = 151) histologically confirmed acute rejection. p = n.s. for each virus.

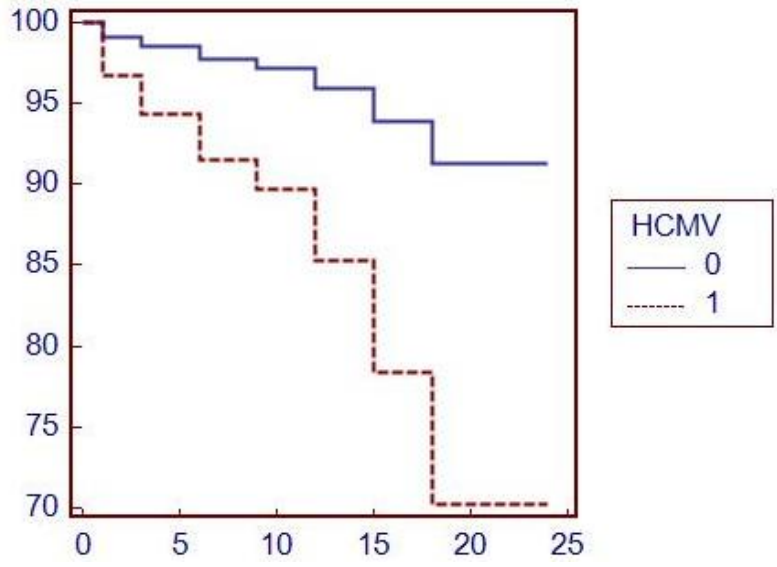
Two cases of BO were diagnosed, both HCMV positive. The occurrence of organizing pneumonia was found in three TBB specimens from the same patient at three different time points (at 6, 9, and 12 months post-transplantation): the corresponding BAL specimens resulted positive to HHV-6 and -7 (viral load, 47107 and 940 GEq/ml, respectively) and to HCMV (6563 GEq/ml) at 12 months. The occurrence of histologically confirmed interstitial pneumonia was evidenced in four cases, three positive to HCMV (viral load ranging from 1760 to 129456 GEq/ml) in corresponding BAL specimens. At univariate analysis, no variable (including age, sex, underlying disease, and type of LT) resulted significantly associated to AR, lymphocytic bronchiolitis, BO, organizing pneumonia, interstitial pneumonia, although patients presenting AR tended to be older (mean ± standard deviation; 50.4 ± 16.8 years vs 41 ± 17.7).

The time-dependent Cox proportional-hazards regression model evidenced a significant association between HCMV and the development of AR (Table 2 and Figure 3), while no other association between herpesviruses and AR was found.

Table 2. Time-dependent Cox proportional-hazards regression model for evaluating the risk associated with the development of acute rejection

Overall Model fit - ACUTE REJECTION					
Significance level p = 0.0153					
Coefficients and Standard Errors					
Covariate	b	SE	P	Exp(b)	95% CI of Exp(b)
HCMV	1.3481	0.5921	0.02279	3.8501	1.2135 to 12.2147
Variables not included in the model HHV-6, HHV-7, EBV					
Baseline cumulative hazard function					
Time (months)	Baseline cumulative hazard	At mean of Covariates			
		Cumulative Hazard		% cases free of acute rejection	
1	0.009	0.014		98.6	
3	0.015	0.025		97.5	
6	0.023	0.039		96.2	
9	0.028	0.047		95.4	
12	0.041	0.069		93.3	
15	0.063	0.106		90.0	
18	0.092	0.154		85.8	

b, coefficient for months; Exp(b), factor of increase of hazard ratio for an increase of 1 month; SE, standard error; 95% CI, 95% confidence interval.



% cases free of acute rejection at different time points (months from transplantation).

Figure 3. Time-dependent Cox proportional-hazards regression: curve describing the percentage of cases free of event (acute rejection) at different time points in relation to the occurrence of the covariate. Development of acute rejection in relation to HCMV DNA positivity (absent, HCMV 0; present, HCMV 1).

Discussion

Lung transplant recipients may be predisposed to lower respiratory tract viral infection/reactivation, given the dysfunctional pulmonary background and the altered local immunity due to impaired ciliary clearance, poor cough reflex, and abnormal lymphatic drainage. In this study, the detection of at least one herpesvirus (including β-herpesviruses and EBV) was quite frequent (72.27%) in LT and significantly higher in comparison to other transplant patients, immunocompromised and immunocompetent individuals, in particular as regards HCMV and HHV-6. These results evidenced that, despite of the universal antiviral prophylaxis, the reactivation of β-herpesviruses and EBV in the graft was common following LT, although this has to be considered taking into account the biology of the viruses and viral load, as lung may represent a latency site.

Despite of the relatively high detection rate of β-herpesviruses and EBV in BAL specimens, only HCMV detection in BAL fluid was significantly associated with the occurrence of AR, although without relation to viral load; the association between HCMV and AR was confirmed by the time-dependent Cox proportional-hazards regression model, with a hazard ratio of AR occurrence in the presence of previous detection of HCMV increased by a factor 3.85 for 1 month. Moreover, HCMV resulted a relevant viral pathogen responsible for interstitial pneumonia. The findings of this study do not support a relation between EBV detection in BAL and AR. However, we cannot exclude that limitation in study population may impact on these issues and further studies are needed to evaluate also the potential role of variables other than virus detection.

Conclusion

In the context of LT, viral infections and reactivations may cause tissue-invasive disease and have been hypothesized to be involved in the pathogenesis of acute and chronic allograft rejection. Both community-acquired and persistently infecting viruses have been investigated in this context, although results of studies remain controversial as regards the relation to rejection, while it is recognized that the transplanted lung represents an environment favouring long-lasting viral replication and persistence. In particular, infections with CARVs may result in prolonged viral shedding and increased involvement of the lower respiratory tract; while herpesviruses are frequently detected in specimens from the lower airways; given the biological behaviour of the members of the *Herpesviridae* family, that is characterized by latency, it could be difficult to discriminate between latency and productive infection; at this regards, results of virological assays (in particular, quantitative molecular methods) could be useful and should be carefully evaluated together with clinical presentation, thus making important a multidisciplinary approach involving virologists, pathologists, thoracic surgeons, and physicians. Future studies should be oriented to the evaluation of the potential impact of timely administration of antiviral prophylaxis/therapy on the subsequent development of acute and chronic graft rejection.

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Chapter 4

Identification of Allo- and Auto-Antibodies after Lung Transplantation

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Abstract

The bronchiolitis obliterans syndrome (BOS) is considered to be the consequence of chronic lung allograft rejection, characterized histologically by airway epithelial cell (AEC) apoptosis and luminal fibrosis in the respiratory bronchioles causing airflow obstruction. Although the detailed etiology and pathogenesis of BOS are not clear, it has become evident that both the humoral and the cellular allogeneic immune response against AEC and endothelial cells, contributes significantly to the pathogenesis of BOS. It was demonstrated that the presence of allo-antibodies reacting with HLA and non-HLA antigens expressed on AEC may precede BOS development, suggesting that non-HLA antigenic systems may also play a role in chronic lung allograft rejection. These data are in line with results obtained in kidney transplantation, in which it was demonstrated that

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endothelial cell-reactive non-HLA antibodies could be found in sera of patients, which have suffered from hyperacute or acute kidney allograft rejection.

Identification of non-HLA antigens recognized by the patients' humoral immune system after lung transplantation provides insight in the immunopathogenesis of rejection and may lead to tailor-made immune suppression. Therefore, research has focussed towards new methods identifying non-HLA antibodies after solid organ transplantation. In literature, 3 methods have been described for identification of previously unknown antigens recognized by antibodies in the sera of patients after transplantation. One method is based on protein arrays. A second, recently described technique, uses SIMT which is an immunoprecipitation followed by Matrix-assisted laser desorption/ionization-Time-of-flight mass spectrometry (MALDI-TOF). The third method is the serologic analysis of antigens by recombinant expression cloning (SEREX), which has been applied on lung transplantation and is able to screen a very large spectrum of antigens expressed by a target tissue like the bronchus in a single screening. Here, we review the advantages and disadvantages of these large-scale screening techniques which can be used to identify antigens recognized by the immune system after lung transplantation (LTx), and provide a comprehensive overview of the antigens identified so far. In addition, the possibilities of identifying patients at risk for rejection using antibody-based screening procedures will be discussed.

Keywords: antibody mediated rejection, lung transplantation, antigen screening, SEREX.

The Bronchiolitis Obliterans Syndrome After Lung Transplantation

For end stage lung diseases lung transplantation (LTx) is the only treatment option. Although, through the years many successful immune suppression regimens were introduced, the overall survival of lung transplant recipients is severely hampered by development of chronic rejection, also known as the bronchiolitis obliterans syndrome (BOS). The overall BOS free survival is 50% in 5 years after LTx [1]. The exact processes leading to development of BOS are unknown, but several risk factors have been described, like primary graft dysfunction (PGD), infections, gastro-oesophageal reflux disease (GERD) and HLA antibodies [2-6].

Antibody Mediated Rejection

Antibodies playing a role in humoral chronic rejection of kidneys have been reported as early as 1970 [7] Nowadays, this role is confirmed and widely studied. After antibodies bind to the graft they have the ability to fix and activate complement, and thereby damaging the graft [8-10]. In the classic pathway of complement activation, after C1q interacts with antibodies, C4d is a product of complement activation, which is covalently bound to the graft and is a marker for antibody mediated rejection (AMR) [11], before C5b-C9 can be fixed in the membrane and form pores. The reactivity against epithelium cells activated the production of growth factors and stress proteins which in turn activates fibroproliferation [12]. The

damage done by complement to the allograft leads to graft dysfunction and the bronchiolitis syndrome, although this route has not been documented extensively in lung transplantation and some conflicts between studies are present [13-18]. Deposits of C4d and C1q were found on the bronchial wall in patients with BOS, in addition C4d deposits and immunoglobulins have been detected on the bronchial epithelium as well providing the proof that the complement cascade is activated via the classical pathway [13, 19, 20].

HLA Antibodies

Antibody mediated rejection (AMR) mainly is ascribed to the presence of HLA antibodies. Evidence was provided by studies showing that HLA antibodies reactive with donor cells prior to renal- and later also lung-transplantation correlated with hyperacute rejection [21-23]. Many studies describe the relation between HLA antibodies prior to or after LTx and rejection either acute or chronic and the HLA antibodies are considered to be a major predisposing factor of BOS [6, 24-28]. It is beyond the scope of this review to describe these relations in detail. More recently the focus has been on donor specific antibodies (DSA), and it was shown that these donor-specific HLA alloantibodies can initiate rejection through complement-mediated and antibody-dependent, cell-mediated cytotoxicity [29, 30]. In lung transplantation the immunosuppressive regime might be of major influence as recently described in two studies analyzing HLA antibodies after lung transplantation by luminex. Under a regime consisting of tacrolimus, mycophenolate mofetil and prednisone HLA antibodies were present at low titers and did not correlate with BOS, while under another regime a correlation between DSA HLA antibodies and BOS was described [31, 32], hence HLA antibodies cannot always serve as marker for patients at risk of development of BOS.

Non-HLA Antibodies

From studies on renal transplantation it became apparent that not only HLA antibodies contribute to AMR. Associations between non-HLA antibodies directed against endothelial cells were found in some patients whom had a kidney transplantation that rejected their allograft and later also confirmed for heart and lung transplantation [33-38]. In lung transplantation anti-epithelial cell (anti-AEC) antibodies were detected in patients without HLA-reactivity prior to transplantation, and the presence of these anti-AEC antibodies was related to a poorer graft survival, indicating non-HLA antibodies are important in BOS [39, 40]. But this became really apparent with a study on renal transplantation between HLA-identical siblings also lead to chronic rejection via the antibody mediated pathway, and in 2005 Opelz et al. revealed that non-HLA immunity plays an important role in chronic rejection in kidney transplant recipients from HLA-identical siblings [41]. Furthermore, it was reported that only 18% of renal graft failure could be contributed to HLA antibodies, while 38% was due to non-HLA antibodies and 43% were associated to non-immunologic factors [42].

Extensive research on non-HLA antibodies has concentrated to the MHC class I polypeptide related chain A (MICA). Zwirner et al was the first to report the non-HLA

antibody against MICA to be present in kidney transplantation [43]. In lung transplantation elaborate studies on MICA antibodies and BOS are absent. We were able to show in a longitudinal study of 50 lung transplant recipients, with an immunosuppressive regime consisting of mycophenolate mofetil, tacrolimus and prednisone that MICA antibodies are present and increased after lung transplantation but they are not related to the bronchiolitis obliterans syndrome. Furthermore, the MICA antibodies detected in 7 lung transplant recipients with BOS after lung transplantation were not donor specific [31]. However, recently it was shown that patients under a different immune suppressive regime (cyclosporine, azathioprine and prednisone) did have MICA antibodies which correlated with the development of BOS [32]. Therefore, the immunosuppressive regime applied is of importance as well whether certain antibodies can be applied as biomarkers as described above for HLA antibodies.

Testing both HLA and non-HLA antibodies is of clinical importance as both HLA and non HLA antibodies, rise early after transplantation and their appearance was reported well before the rise in serum creatinin, an indication for rejection in kidney transplantation [44]. Another non-HLA antibody found in renal transplant patients with allograft rejection but not in other patients is against the angiotensin II type 1 (AT1) receptor [45], and in heart transplantation antibodies recognizing vimentin, myosin and phospholipids have been detected after transplantation [46-48]. Although testing for (donor specific) HLA antibodies is routine prior to and post transplantation, methodological options for identification and characterization of non-HLA antigens targeted during rejection after transplantation are scarce.

Screening Strategies

Growing evidence that next to HLA antibodies non-HLA antibodies might be part of processes leading to chronic rejection called for techniques to identify such autoantibodies. Therefore, measurements of antibodies post transplantation might provide an early biomarker for detection of patients at risk of rejection. The prognostic significance of HLA antibody detection of transplantation is somewhat limited as they appear in patients after solid organ transplantation who reject this organ but also some patients who do not experience rejection episodes. In addition, patients without HLA antibodies still can develop chronic rejection. This indicates that there is a need to find relevant biomarkers that are highly sensitive and very specific for early diagnosis and prognosis of chronic rejection by humoral mechanisms.

Using antibodies, against yet to be determined non-HLA antigens, as possible biomarkers for chronic rejection after lung transplantation has some practical advantages. They are relatively easy accessible by a non invasive approach using sera from patients Moreover, autoantibodies are naturally stable and persist in the serum for a relatively long period of time because they are not subjected to the types of proteolysis observed for other polypeptides [49]. A disadvantage that needs to be considered is probably the heterogenicity of antigenic targets. A high variety of antibodies against different antigens is found between patients. Therefore, lacks a single antibody test both sensitivity and specificity and need the test to be repeated or the combination of several autoantibodies can be used [50].

When researchers in the transplantation field became more interested in the role and possibilities of autoantibodies, as it became apparent they might contribute to the development of rejection, new techniques had to be developed. Systematic screening methods were needed to be able to identify possible new antigens in a high throughput manner which was not labor intensive. For that reason different techniques have been developed and amended. One of the first studies in renal transplantation describing the usefulness of such techniques showed that harvesting endothelial cells from peripheral blood using TIE-2 antibodies on dynabeads used for easy patient anti-endothelial cell crossmatch [51]. Here some of the techniques will be briefly reviewed and their contribution to the field of human (lung) transplantation will be enlighted.

Serological Proteome Analysis

Serological proteome analysis (SERPA) also known as Proteomex is a combination of 2-DE gels and western blotting. The technique was originally developed for the identification of tumor antigens in kidney cancer [52, 53]. And was one of the first techniques allowing identification of proteins/antigens in a high throughput manner. SERPA has been widely used in identification of tumor antigens, antigens in autoimmune diseases and possible vaccine strategies of infectious diseases [54, 55]. Although, it has been very successful identifying several possible markers for different lung cancers it has not been used in the research to identify antigens for autoantibodies in lung transplantation or other forms of solid organ transplantation [56-58].

Every technique have advantages and disadvantages compared to other techniques. For SERPA one major advantage is the use of isolated protein as starting material which makes it possible to identify post-translational modifications and protein isoforms. Additionally as no library has to be constructed, SERPA is less time consuming than SEREX (described later) which uses a cDNA library. On the other hand, the techniques has its limitations, for instance it has a bias to abundant proteins because of the sensitivity of the staining methods. Furthermore hydrophobic and insoluble transmembrane proteins as well as small proteins (<10kDA) or proteins with extreme isoelectric points are difficult to detect [59-61]. Due to the use of western blotting as staining method only linear epitodes are detected [50].

The SERPA technique has proved the possibilities of identifying new target antigens, and based on this technique protein arrays were designed.

Protein Arrays

Protein arrays are based in antigen immobilization on a support where sera of patients can react. Commercially there are arrays available coated with over 5000 different proteins developed as screening for (ovarian) cancer and therefore at present probably not the best starting point to screen for (organ) specific allograft rejection related antigens. However, these arrays have been used in pediatric kidney transplantation studies, and proven the feasibility of using protein arrays to detect new target antigens during rejection [62]. Antigens detected in these studies include MICA and antibodies against the renal pelvis area and cortex

specific antigens. One of the antigens related to renal area was Protein kinase Cζ (PKCζ) which was shown to be related to development of acute rejection [63].

An advantage of using protein arrays to identify unknown antigenic structures is that they are not labor intensive as well as they allow analysis of a large number of targets in one step and therefore are high-throughput [64-66]. Another advantage of the small arrays is that only little material is needed to perform one array [67].

On the other side, protein arrays are limited by the availability of commercial or home made proteins on the arrays. They do not cover the whole proteome. Furthermore with protein arrays it is not possible to differentiate between intracellular and surface expressed proteins, while especially surface expressed antigens are of interest in the context of allograft rejection [68]. In addition, the protein arrays themselves have some disadvantages as well. It is difficult to produce and purify native protein targets and once bound to the array they have a short shelf-life [69, 70].

SIMT

Recently a new technique was developed called Sequential analysis of Immunoprecipitation followed by Matrix-assisted laser desorption/ionization-Time-of-flight mass spectrometry (SIMT) [71]. SIMT is a combination between immunoprecipitation and mass spectrometry to identify possible new target antigens for allograft rejection. An advantage of this method over the previously described methods is the usage of native human cells and indirectly measuring the clinically more relevant antigens expressed on the surface of these cells.

However, as the technique has only been used as prove of principle it still has to prove its benefits for transplantation research. In a HLA-B27 or -B7 setting the technique was able to pickup these antigens as targets of an immune response, other HLA molecules and even non-HLA targets need to be investigated. The technique has only been used in a setup phase where samples with proven reactivity in lymphocytotoxic assays were used opposed to autologous material. As SIMT is a relatively new technique it might be optimized. At present, there are problems with detecting bands of non specific serum antigens binding to the beads. And a major drawback is the use of material as much serum is needed for one screening, as well as the relatively laborious steps of which the method is composed.

SEREX

The technique Serological identification of antigens by recombinant expression cloning (SEREX) using a cDNA expression library has been developed to explore the humoral response in sera from patients with their own tumor as source in 1995 [59, 72]. Since the development of the technique numerous tumor associated antigens reactive with many cancer types have been identified by SEREX and over 2300 are documented in a database [73-75]. As the technique has proven its strength in the field of cancer research it had been utilized in other fields like auto-immune diseases and transplantation.

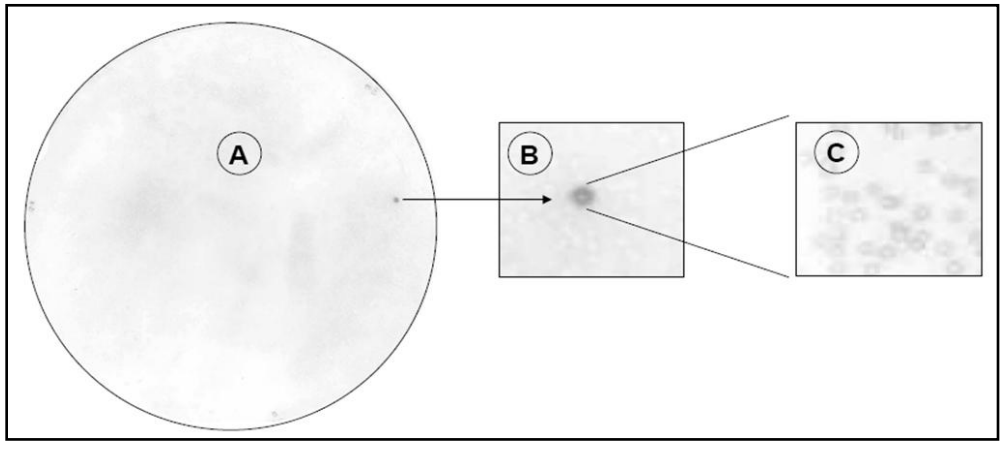
One of the strengths of the SEREX technique is the use of target tissue, like a specific tumor or a specific organ as source of the mRNA from which a cDNA library is constructed. The SEREX techniques knows some disadvantages as well, the work needed to construct a cDNA library is quite laborious. It uses an artificial expression system and therefore potentially representing denatured or improperly glycosylated proteins. It cannot discriminate between intracellular and surface proteins like SIMT does. SEREX might produce numerous false positives due to the identification of a humoral response specific to patient but unrelated to tumor [59]. This is in concert with our study after lung transplantation (data shown below), however using patient and respective donor material the false positive results might be decreased [76]. There might be a bias towards antigens that are highly expressed in the target tissues used to generate the cDNA libraries [77]. And small proteins (<120 aa) are less well incorporated in the expression system [78].

We made use of the SEREX system in the setting of stem cell, kidney and lung transplantation. The results obtained are briefly summarized below.

Lung Transplantation

For the identification of antigens after lung transplantation several screenings have been performed: 3 months after lung transplantation, 6 months after lung transplantation and less than three months before development of BOS.

An epithelial-cell cDNA expression library was made from trachea of 15 lung donors. In the first pilot sera of 11 random patients from the cohort were taken and each screened against 3000 plaques from the library [79]. In a later stage the screening was elaborated and sera derived from 7 patients, taken 3 months after LTx or $\leq$3 months prior to BOS was screened against $4x10^4$ plaques from the library. Recognized plaques were isolated, further seeded and rescreened until 100% of the reseeded plaques were recognized by the serum (Figure 1).



Legends to Figure 1: Bacteriophages were seeded out using E-coli plates as described in patients & methods, yielding approximately 4000 plaques per plate. The nitrocellulose filters were screened with patient serum diluted 1:500. Shown are A) an example of a first screening result from 1 patient serum on 1 plate in which 1 positive plaque was detected B) an optical enlargement of that plaque marked by the arrow and C) the results of reseeding and rescreening of the recognized plaque.

Rescreening as shown above in Figure 1, resulted in 28 plaques recognized, and alignment of all inserts showed several identical amino-acid sequence motifs occurring in different inserts. Next to identification of the inserts in the plaques 15 amino acid long peptides were designed on the possible motifs and tested on ELISA. This however did not result in positive signals above background, and therefore it was concluded that these peptide were no possible epitopes.

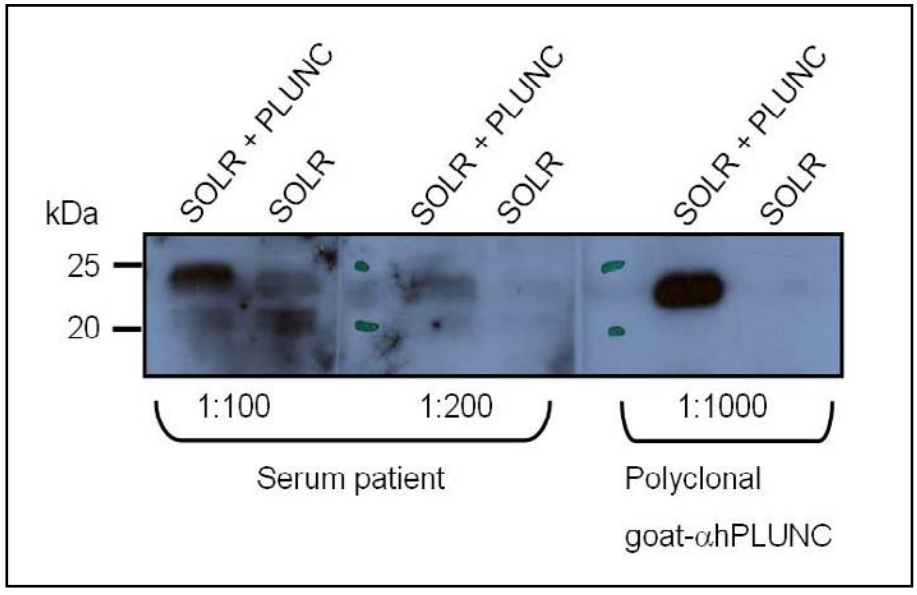
After sequencing some of the plaques were identified as non coding regions (n=22) and 3 were found to hold genes without start codon and 3 plaques contained genes with start codon.

Table 1. SEREX screenings after lung transplantation

	3 months post LTx	**6 months post LTx**	**< 3 months prior to BOS**
Number of patients	7 patients with BOS	11 random patients	7 patients with BOS and 1 patient with rejection problems
Plaques per patient	40 000	3 000	40 000
Positive plaques	7	11	10
Identification	7 non coding regions	3 genes with start codon 3 genes without start codon 5 non coding regions	10 non coding regions

Legends to Table 1
SEREX screenings were performed at 3 different time points.

The genes without start codon were identified as XP_931864, LOC 284058 and PSMC 4, and the genes with start codon were PLUNC, F3, and ZNF 33A. These possible antigens are all internally expressed with the exception of PLUNC and F3. Because this first protein palate lung nasal clone (PLUNC) is expressed in the upper respiratory tract and oral cavity it was of special interest regarding a possible antigen target after lung transplantation [80-82]. PLUNC's putative function is thought to be as an immune defence protein of epithelial surfaces against pathogenic microorganisms because its sequence is homologue to lipopolysaccharidebinding protein (LBP) and bacteriocidal/permeability-increasing protein (BPI) [83-85]. The cDNA of PLUNC was excreted from the plaque and cloned into SOLR bacteria. Lysates from these bacteria were tested on Western Blot with different sera of lung transplantation patients and a commercially available antibody. As shown in **Figure 2**, patient serum was able to detect PLUNC in the lysate of SOLR bacteria and therefore antibodies were directed against PLUNC. From sera of 12 lung transplantation tested 4 were capable to positively recognize PLUNC.



Legends to Figure 2:

Lysate of SOLR bacteria or SOLR bacteria with PLUNC were separated on a 12% SDS-PAGE gel before blotted onto a nitrocellulose membrane and incubated with either serum of a lung transplantation patient or a polyclonal antibody against PLUNC. The protein is detected by both the patient's serum and antibody at approximately 24 kDa only in the lysate of bacteria with PLUNC.

Another study by *Goers et al.* has also detected non-HLA antigens after lung transplantation [12]. This was not after elaborate screenings but a quick scan in a few individuals. A combination of PRA and western blotting on airway epithelial cells, followed by protein isolation and sequencing identified K-α1 tubulin as a possible target antigen. Further analysis revealed that 36 BOS and 36 non BOS together with 10 HC had no reactivity against HLA, but 12 out of 36 BOS had specific reactivity against AEC via K-α1 tubulin.

Kidney Transplantation

For kidney transplantation non-HLA antibodies have been reported to influence allograft survival. One of the non-HLA antigens identified is the angiotensin II type 1 (AT1) receptor and in a recent study, the presence of activating IgG antibodies targeting this receptor was examined in 33 kidney-transplant recipients with refractory vascular rejection [45, 86]. Activating IgG anti-AT1 receptor antibodies were detected in serum from all 16 patients with malignant hypertension - in absence of anti-HLA antibodies - but not in the other patients.

Antibodies have also been described against MHC class-I related A antigens (MICA), which are expressed on endothelial and epithelial cells, monocytes and fibroblasts [87]. In a large scale multicenter study on 1329 patients with functioning kidney transplants, it was shown that the presence of anti-MICA antibodies in post-transplant sera is significant

correlated to kidney allograft loss [88]. In this study, donor graft survival was 72% vs 81% in patients which had or did not have anti-MICA antibodies, respectively.

Here, the main purpose of was to identify non-HLA antigens recognized by antibodies from patients awaiting kidney retransplantation with SEREX. To this end, 7 patients (M/F = 3/4; median age = 46; range = 29-60 years) were selected having rejected their kidney. Serum was taken after nephrectomy and analyzed for reactivity against an epithelial cell protein-expression library SEREX. Serum of every individual was screened against approximately 3x10^4 plaques. A total of 8 plaques were recognized by the sera of 4 patients. After rescreening as shown above in **Figure** 1, five inserts were very small (5-67 aa) of which the original encoding gene could not be identified. The other inserts consisted of 3 different non-HLA antigens: tetraspanin 8, LPLUNC1 and BSCv (also known as C20ORF3 or adipocyte plasma membrane-associated protein). These were recognized by 4/7, 5/7 and 3/7 patient sera tested respectively, but not by sera from 3 healthy controls.

Stem Cell Transplantation

Treatment with rituximab, a B-cell inhibitor, has a positive effect on the disease and in several studies allo- or autoantibodies have been detected in chronic Graft versus Host Disease (cGVHD) patients. The aim of this study was to examine if auto- and alloantigens in cGVHD can be identified by SEREX. To this end 10 sera derived from patients with cGVHD were examined by SEREX with a cDNA bank from epithelial cells of a lung. Furthermore, it was also determined whether the identified antigens were also recognized by other cGVHD patient sera and a healthy control.

Nineteen positive cloned inserts were found. Nine of those were identified as interferon gamma inducible protein 16 (IFI16), one was identified as trophoblast glycoprotein (TPBG), another as syndecan binding protein (SDCBP), and two were unknown, one was an artifact and the other five are unidentified. IFI 16 was of special interest because it was identified by screening the sera of 3 out of 10 different patients.

Overall the SEREX technique is powerful enough to detect various possible antigens after stem cell and solid organ transplantation. Antigens identified were recognized by several different patients but not healthy controls. However, as for antigens found for GvHD and kidney transplantation, the antigens detected after lung transplantation could not be cloned into fusion proteins to be purified and used on ELISA for high trough put screening of a patient cohort. Due to instability proteins were not fused to a tag and analysis of the other possible targets as bacterial lysate on Western Blot revealed cross reactivity in the samples, multiple bands on different heights were detected, which were not multimers of the predicted size.

Table 2. Result of screening 10 patient sera by SEREX

Patient	nr of total plaques	nr of positive plaques	nr of IgG detected in 1st screening	nr of IgG detected in 2nd screening	nr of plaques recognized by control serum	final nr of positive plaques
1	67.000	98	80	11	1	6
2	39.000	51	37	8	1	5
3	45.000	53	43	8	2	0
4	36.000	22	17	2	2	1
5	28.000	17	11	1	3	2
6	14.000	nd	nd	nd	nd	0
7	13.000	nd	nd	nd	nd	0
8	11.500	nd	nd	nd	nd	3
9	10.000	nd	nd	nd	nd	2
10	16.000	nd	nd	nd	nd	0
Total	279.500	246	188	30	9	19

Legends to Table 2

Abbreviations: nr, number; nd, not determined. Patients 1, 2, 3, 4 and 5 were screened differently from patients 6, 7, 8, 9 and 10 therefore not all results are available as prescreening was not performed. The number of initial positive plaques is the total amount of positives that were observed on the filter after the first screening. The number of IgG in the first screening is the number of initial positive plaques that were identified as false positives during the prescreening. More IgG was seen when the total number of plaques was higher. The number of positive plaques in the first screening where isolated and underwent a second screening. During this second screening the filters were also incubated with patient serum and with TBS. A lot of the plaques were IgG despite of the prescreening. Plaques that were found positive during the second screening were isolated and screened again; the final screening. During this screening the filter was also incubated with normal serum and when positive plaques were seen on this filter they were considered negative. Identification of the isolated plaques after the second screening was performed if they were found to be positive after the final screening.

Conclusion

Delineation of the non-HLA antibody spectrum after lung transplantation may facilitate our understanding of both allo- and autoimmune responses in chronic allograft rejection. The screening for possible antigens after transplantation is relatively new, but examples proving the strengths of the high through put wide technologies in the cancer research field are abundant. However, many of the antigens discovered via one of the above mentioned techniques still need to prove their power as biomarkers in the clinic [89]. Many antibodies against these new antigens have a low frequency and are present in approximately 20-30% of patients of specific tumors [59]. The combination of detection of several of the newly discovered non-HLA antibodies or biomarkers seems to allow the uncovering of tumors with higher efficiency than isolated biomarkers [65, 66, 90-94].

Next to the clinical relevance and low frequency that the antibodies are found in patients, it should be explained why screenings result in antigens mainly originating from intracellular proteins [95]. This might be circumvented by the introduction of SIMT as a new screening technique, which is able to screen only surface expressed proteins.

At the moment all screening techniques focus on finding antibodies of IgG isotype. Recently it has become clear that IgM antibodies might contribute more to rejection after

transplantation than previously thought. Both IgM HLA and non-HLA antibodies were described to be deleterious for the overall survival of transplant recipients [96, 97].

Overall, high trough put screening technologies might contribute to the detection of possible antigens after lung transplantation. However the clinical significance as well as the labor intensive character are major aspects to be considered before employing these methods.

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Chapter 5

Surgical Aspects of Lung Transplantation

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Abstract

The main surgical issues facing lung transplant surgeons today are access to the thorax, anastomotic problems and size mismatch of the lungs. As double lung transplantation becomes more popular, with survival advantage being demonstrated for more conditions, the clam shell incision is being increasingly utilized. However, problems with healing of the transverse sternotomy, particularly in immunocompromised patients, is a significant source of post operative morbidity. This chapter will review various techniques to improve sternal apposition and healing and discuss alternatives to the clam shell incision.

Dealing with anastomotic size discrepancy, and avoiding problems intra-operatively are of paramount importance when performing lung transplantation. This chapter will review techniques for dealing with inadequate cuffs at the venous and arterial anastomsoses and techniques for performing the bronchial anastomosis.

Size mismatch between donor and recipient is an important issue with paediatric and small adult recipients being disadvantaged on the waiting list. The use of lobar transplantation, non anatomical cut down and split lung transplantation has allowed larger donor lungs to be downsized for use in smaller recipients. There are also instances during surgery when donor lungs are larger than expected for the recipient and size reduction is required for an ideal fit. This chapter discusses the sizing issues that impact on outcomes in this group of patients.

Introduction

Lung transplantation has evolved rapidly over the last 27 years with major modifications and improvements to the surgical technique as well as significant advances in organ preservation and immunosuppression. The first successful lung transplant was performed as a single lung. Later heart lung 'block' transplant was devised to extend the procedure to patients with septic lung disease bringing with it the option of a domino procedure whereby the recipients' heart (previously discarded) was used to perform an orthotopic heart transplant in another patient. Double lung transplant followed but was soon abandoned due to high complication rates particularly at the tracheal anastomosis [1,2]. Single lung transplant continued to develop as a surgical option for non infective lung disease in the early 1990s and bilateral sequential lung transplant evolved in the mid 1990s [3] remaining today the most commonly performed type of lung transplant [4]. Initially indicated for suppurative lung conditions only, it has now become standard management for non suppurative lung conditions such as pulmonary hypertension and obstructive airways disease.

This chapter will focus on the technical surgical issues inherent in the single and bilateral sequential lung transplant procedure.

Access to the Thorax

Sternotomy

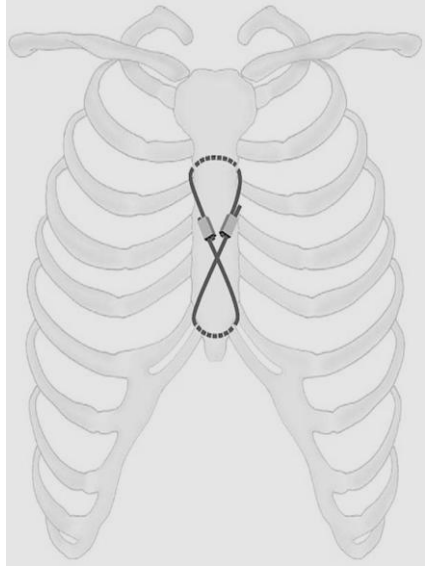
Because lung transplantation evolved as a heart lung block transplant, initial thoracic access was via a median sternotomy. Hilar access is however limited and in general the approach is now reserved for concomitant lung transplant and cardiac surgery in most centres.

Clam-Shell Incision

As bilateral sequential lung transplant developed, use of the clam shell incision became popular, allowing incomparable access to both pleural cavities[3]. This incision also gives excellent exposure to the ascending aorta and right atrium to allow cannulation and initiation of cardiopulmonary bypass if required[5,6]. Unfortunately the transverse sternotomy developed a reputation for being prone to poor healing and, high incidences of sternal wound complications have been reported (up to 46%) [7-10]. Division of both internal thoracic arteries potentially devascularizes the bone but more importantly, the group of patients undergoing lung transplantation has many risk factors for poor wound healing such as poor nutrition status, steroid use, obesity, osteoporosis, diabetes and the requirement for immunosuppression. Recent improvements in immunosuppression have to a large extent reduced this complication and when carefully titrated in combination with prolonged antibiotic cover and meticulous surgical technique, the clam shell incision remains safe and the most versatile exposure for double lung transplantation.

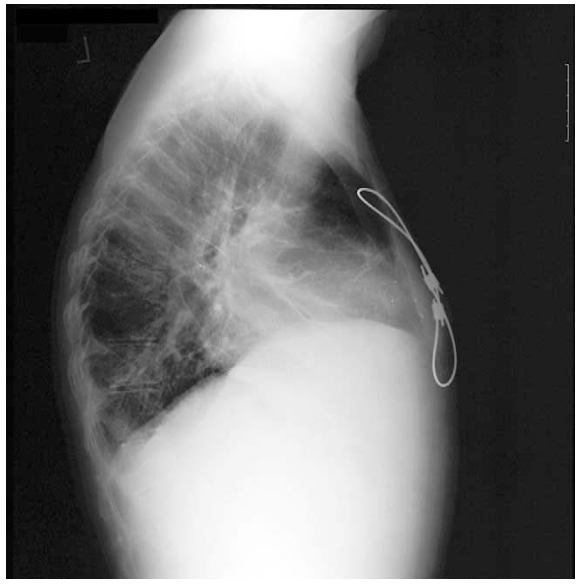
Techniques for closing the transverse sternotomy include simple apposition of the bone ends with stainless steel wires in either a figure of eight or simple longitudinal configuration.

In osteoporotic bone, however, cutting through or loosening can result in either over-riding bone ends or widely separated bones ends with movement, which can lead to pain and or infection as a consequence. Various techniques have been developed to improve the transverse sternotomy closure such as plates [11,12] and cables [13] (Figures 1 & 2).



McGiffin et al., 2005.

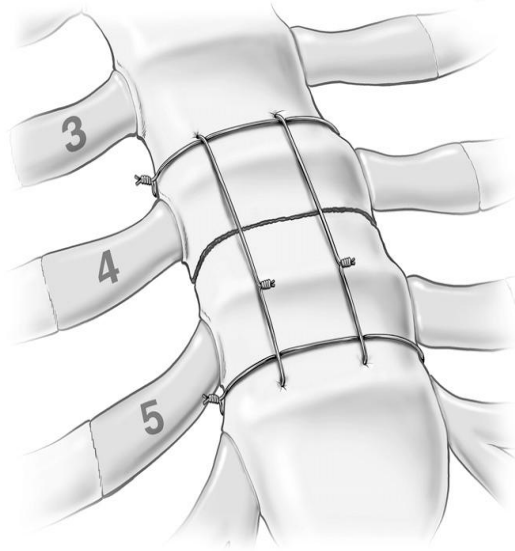
Figure 1. Peristernal cables are passed behind the sternum through intercostal spaces above and below the sternal division, and then passed through the sleeves, tensioned, the sleeves crimped, and the excess cable excised [13].



McGiffin et al., 2005.

Figure 2. Lateral chest roentgenogram demonstrating satisfactory alignment of the sternal fragments [13].

Both anterior cortical and bicortical plates have been used and appear to give very good results[11,12]. Removal of plates at redo transplantation can prove troublesome however. A reinforced wiring technique has also been reported whereby transverse peristernal wires are placed first at the third and fifth intercostal spaces prior to the placement of the conventional longitudinal wires[14] (Figure 3).



Oto et al., 2007.

Figure 3. Two peristernal stainless-steel wires are placed on the each side of the sternum at the level of the third and fifth intercostal spaces inside the conventional 2 longitudinal wires, which cross the sternotomy line [14].

The purpose of the transverse wires is to prevent cut through of the longitudinal wires. This technique has the benefits of technical familiarity, cost effectiveness and has been shown to be clinically effective in a retrospective review [14]. However, there is a concern with this technique that bone ends distal to the wire encirclage may be additionally devascularised.

Bilateral Anterolateral Thoracotomy

Perhaps the best method to deal with transverse sternotomy complications is to avoid the sternotomy altogether. The incision follows the inframammary crease, enters the fourth intercostal space and extends from lateral sternal edge to anterior axillary line. Where possible, bilateral internal mammary arteries can be preserved. Rotation of the operating table optimizes exposure for each side. Numerous groups reporting their results claim that an incidence of sternal wound complication approaching 0% can be achieved [9,15]. Others have reported shorter operative times with this technique, possibly due to quicker closure of the chest [10]. Improved chest wall mechanics, reduced pain, better mobilization and spirometry results have also been demonstrated [10]. The bilateral anterior thoracotomy approach also offers a cosmetic benefit which may be important particularly to younger transplant recipients. Further it retains the clam shell as an option for retransplantation with the potential for fewer adhesions at reoperation.

This technique is not without risks however. Access to the mediastinal structures for the institution of cardiopulmonary bypass is limited and emergency conversion to a clamshell incision for either institution of cardiopulmonary bypass or to control life threatening bleeding has been reported in 19% [9]. The technique also provides limited access for patients requiring concomitant cardiac procedures, patients with cardiomegaly and in those with dense pleural adhesions. Some of these limitations can be overcome by the use of cardiopulmonary bypass using either a transthoracic wall cannulation technique or femoral cannulation [16].

Anterior Axillary Muscle Sparing Thoracotomy

In selected thin patients this may offer the optimal cosmetic result with preserved chest wall and shoulder girdle mechanics, reduced post operative pain and facilitate faster recovery. Access is limited however, increasing the technical demands on the surgeon.

Posterolateral Thoracotomy

This may retain a place for left single lung transplantation where cardiomegaly compromises anterior access.

Recipient Pneumonectomy

When performing bilateral sequential lung transplant the lung with the least physiologic contribution is approached first. In the event that the contralateral lung will not support oxygenation, cardiopulmonary bypass is established. Hilar dissection is performed taking care to avoid injury to the phrenic, vagus and recurrent laryngeal nerves. The inferior pulmonary ligament is divided, and the pulmonary arteries and veins dissected to their bifurcations. On the right side, the superior vena cava is dissected off the main pulmonary artery to allow proximal clamping. Sondegaards groove is developed, allowing placement of the clamp on the right pulmonary cuff to be placed well proximal to the venous bifurcation. The clamp is essentially placed on a cuff of left atrium at the junction with the pulmonary veins. Heparin 5000U is administered intravenously prior to the application of the vascular clamps on the pulmonary artery and venous cuff.

Once vascular isolation is achieved the pneumonectomy can proceed. Placement of additional clamps or ties to the structures on the side of the resected lung avoids blood spillage but is not essential. All structures are divided with sharp dissection, avoiding in particular the use of any electrocautery to the transected bronchial edge. Hilar haemostasis is established with a combination of electrocautery and clips, again avoiding any devascularisation of the cut bronchial edge. The recipient bronchus should be kept as long as possible given its established blood supply. Thus it is divided just proximal to the take off of the upper lobe branch. The pulmonary vein is prepared by communicating any branches and maximizing the size of the pulmonary vein sleeve.

Implantation

The bronchial anastomosis is performed . The technical issues associated with the bronchial anastomoses are outlined below. Our preference is to use a continuous 4/0 polypropylene suture to anastomose the bronchus end to end. Suturing commences at the posterior membranous portion. Where size discrepancy exists, a degree of overlap can be accommodated while still securing end to end. In states of significant size discrepancy the two ends can be telescoped with a preference for placing the most viable (recipient) bronchus inside the donor. Bronchoscopy is performed by the anaesthetist immediately upon completion to confirm technical excellence.

Next a 5/0 polypropylene suture is used to facilitate the pulmonary venous anastomosis. Any size mismatch can be made up gradually along the entire circumference of the suture line. Attention must be paid to orientation. Despite being a large orifice and a low pressure system, torsion can occur. On completion, trans oesophageal echocardiography should be used to confirm patency. Turbulence or increased flow may indicate stenosis or obstruction (Myles). On completion, suture ends are left untied to allow for later deairing.

The pulmonary artery anastomosis is then performed. Again orientation is critical to avoid torsion, length must be ideal to avoid kinking and size mismatch best accommodated along the entire suture line. On completion, ends of the suture are also left untied for deairing.

We place the patient in a trendelenburg position prior to declamping and use transosophageal echocardiography to assist with assessment of adequacy of deairing. We use retrograde reperfusion over a number of minutes and if not on cardiopulmonary bypass, use a cell saver to scavenge blood spilt during the deairing procedure. Carbon dioxide insufflation throughout the procedure is optional but remains to be shown to achieve clinical benefit in neurocognitive outcomes. The pulmonary arterial suture line is then tied and antegrade perfusion allowed to occur gradually by slowly opening the pulmonary arterial clamp. Further deairing occurs through the pulmonary venous suture line and once the cardiac chamber microbubbles clear on echocardiography, the pulmonary venous suture is tied and the clamp removed. At this time a period of transient hypotension often follows due to the systemic vasodilatory effects of the organ preservation solution circulating from the newly perfused lungs. This is treated with vasopressors in the first instance with minimal use of intravenous fluids.

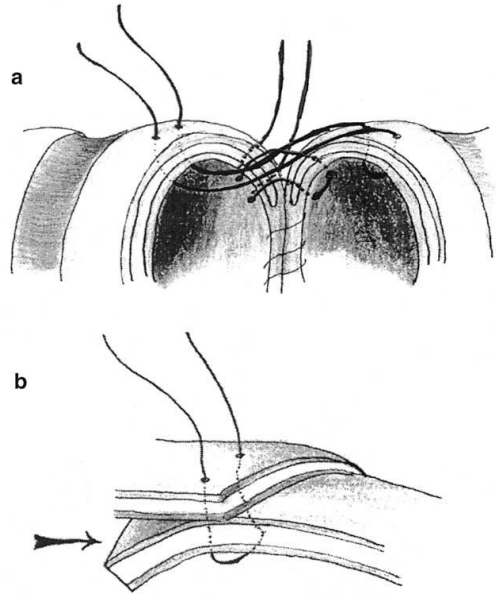
Bronchial Anastomotic Techniques

The bronchial anastomosis is perhaps one of the surgical steps in the lung transplant operation which has evolved the most over the last couple of decades. Problems with the anastomosis such as dehiscence, necrosis and stenosis were a significant factor in the early evolution of the operation. The main reason for this is the relative ischaemia at the bronchial anastomosis which relies on retrograde blood flow through the pulmonary circulation via collaterals. Studies in animals have shown that the bronchial arterial circulation can take up to four weeks to reestablish and this is likely to be longer in the setting of post operative factors such as haemodynamic instability and steroid administration [17,18]. Initially, end to end bronchial anastomoses with omental wrapping were performed in an effort to protect the

anastomosis and enhance the bronchial microcirculation [19]. However, as evidence emerged showing no significant impact of this technique on anastomotic complication rates [20], the technique was abandoned. Further, there were complications with this technique such as diaphragmatic herniation [21] and as a better understanding of the vascular anatomy of the area was gained, other techniques developed. Direct bronchial artery revascularization has also been advocated to reduce airway complications [22]. However, this technique never became popular, due to the increased operative time, technical difficulty and failure to show significantly better results.

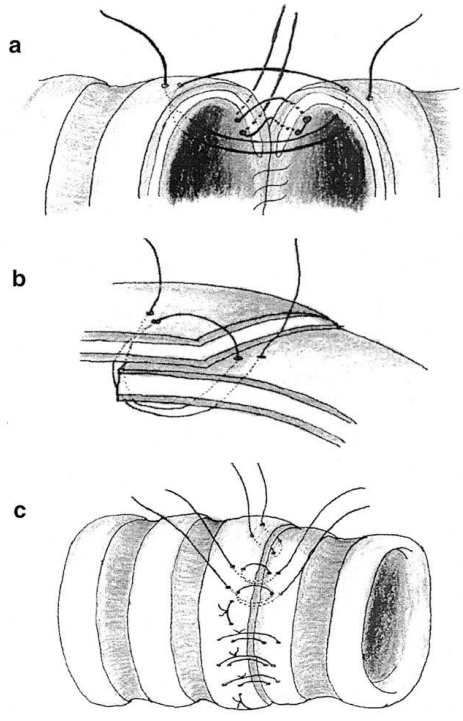
Many different types of bronchial anastomotic techniques have been tried over the years. Most of them have been discarded due to problems with anastomotic stenosis. The problem of stenosis is multifactorial although ischaemia of the proximal recipient bronchus is the most prominent causative factor. Avoidance of this problem is best commenced at the time of explant of the recipient lung. The recipient bronchus should be left as long as possible with minimal disruption to its blood supply. Thus, judicious use of cautery is advocated, and lymph nodes and other peribronchial tissue should be left undisturbed as much as possible. The division of the bronchus should be performed using a knife without using any cautery to the cut edge of the bronchus. The donor bronchus should be trimmed quite short to minimize the length of devascularised tissue forming the new bronchus. Leaving only one or two cartilaginous rings proximal to the secondary carina (take off of the upper lobe branch) appears to be a reliable technique. Some authors have advocated dividing the donor bronchus even shorter, at an oblique angle from the origin of the upper lobe bronchus along the medial aspect of the bronchus intermedius [23].

Telescopic and reverse telescopic bronchial anastomoses were popular initially but have been associated with an increased incidence of bronchial stricture and have largely been abandoned [24 – 28]. In one review of 32 telescoped bronchial anastomsoses, the complications of ischaemia, dehiscence, and severe stenosis, were observed in 11 (34%), 8(25%), and 11 (34%) respectively [29]. These incidences were significantly higher than in the comparator group of 44 end-to-end anastomoses where the rates were 4 (9%), 1 (2%), and 2 (5%) of 44 end-to-end anastomoses (P = .0087,P = .0034, and P = .0012, respectively). Thus the relative risk of ischemia, dehiscence, and severe stenosis in telescoped anastomoses was 2.1, 2.5, and 2.5, respectively, compared with end-to-end anastomoses [29]. Admittedly that study had limitations in that the choice of anastomotic technique was individual surgeon preference, so there could well have been different surgical techniques other than the anastomotic technique which impacted on outcomes. However, the authors did try to match groups as much as possible by including only single lung transplants for emphysema in the study cohort. No prospective study has looked specifically at this issue and the retrospective studies, even those published recently, have all been somewhat conflicting in their results [23,30]. Some authors have reported improved results with modifications of the telescopic technique whereby the invaginated cartilage of the bronchial edge (Figure 4) is tacked down with figure of eight stitches to avoid the presence of a devascularised flap within the bronchial lumen [31] (Figure 5).



Schroder et al., 2003.

Figure 4. The classic telescoping technique (a) leaves a devascularized shelf of bronchial tissue protruding into the lumen (b, arrow) using just U stitches. A running suture is used for the membranous posterior wall [31].



Schroder et al., 2003.

Figure 5. The modified telescoping technique (c) uses just three U stitches (a) at 0, 90 and 180 degrees and two or three figure-of-eight sutures in between (b) to coapt the walls properly. A running suture is used for the membranous posterior wall [31].

Comparison of complication rates between centres has been difficult because of differences in definition and grading of anastomotic healing. The system devised by Couraud et al assesses early anstomotic healing by bronchoscopic evaluation whereas the system of Shennib et al has been expanded to include late complications [32,33]. The rates of these complications will also vary depending on how they are identified i.e. at routine bronchoscopic examination or at presentation with a clinical syndrome. Thus incidences and outcomes vary widely between centres with anastomotic complication rates of 0-48% reported [34-37]. Incidences also vary with the underlying recipient lung pathology, with suppurative lung diseases such as cystic fibrosis having higher reported rates than other lung conditions without as much bacterial or fungal colonization [33,38,39]. Other factors which have been shown to be associated with anastomotic complications include post operative pneumonia (although whether this is a consequence or cause is unknown) and longer donor intubation time (inferring the likelihood of increased colonization of the donor lungs) [40]. The use of post operative steroids was also thought to be a risk factor initially [41] but has since been shown to be safe, and important for the prevention of rejection [42]. Reperfusion oedema and early rejection have been shown to be associated with anastomotic complications [43] . A strong association between the presence of Aspergillus in the bronchus, bronchial necrosis and airway complications has also been demonstrated [44].

Most groups worldwide appear to have moved to an end to end technique. The two main variations are a running suture to the membranous portion of the bronchus and interrupted sutures to the cartilaginous portion, or a continuous running suture to the entire circumference of the anastomosis [45].

There does not seem to be any significant differences in outcomes between these two techniques. Criticisms which could be raised regarding these techniques are the increased time required to tie multiple knots using the interrupted suture technique, and the increased amount of foreign material these knots represent at the anastomosis. In contrast, the risk of ‘purse-stringing’ a circumferential anastomosis (although this seems unlikely to occur at the cartilaginous portion), and the risk of the entire anastomosis dehiscing if one part of the suture pulls through are potential risks of the continuous suture technique. However, the continuous suture technique does facilitate the take up of minor discrepancies in luminal diameter with ease, reduces operative time and may be an easier technique to use through smaller access incisions.

The incidence of bronchial complications appears to be reducing worldwide as techniques improve. Improvements in organ preservation, immunosuppression and post operative management are also thought to be implicated in these improved results, although the surgical technique seems to be the most significant factor. The mortality rate associated with airway complications has also decreased markedly over the last two decades with a recent review reporting a mortality rate of 2.6% [40].

Pulmonary Vascular Anastomoses

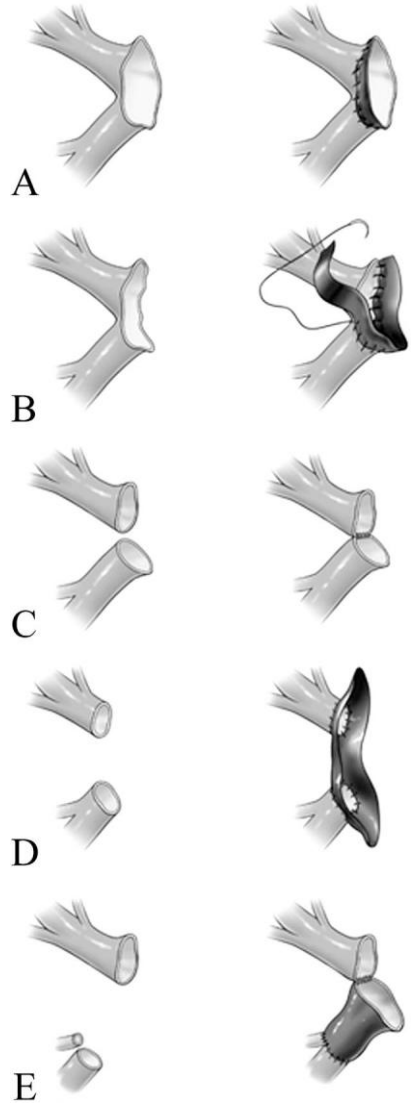
The types of difficulties encountered when undertaking the pulmonary left atrial and arterial anastomoses are inadequate tissue on the donor lung, size discrepancy, and torsion. If these are not dealt with adequately, the donor lung may be unusable or post operative

problems of stenosis, thrombosis, or graft failure may occur. It is vital that the transplant surgeon can deal with these technical issues as they present to ensure that the lung transplant can proceed and that donor organs are not wasted. Furthermore, the recipient may be already under anaesthesia and with their chest opened awaiting the arrival of the donor organs. To abort a transplant at this stage is likely to put the recipient at grave risk of prolonged ventilation and even death.

Usually the donor lung will arrive in the recipient theatre and be inspected and the hilum prepared, prior to explanting the recipient lung. This gives the surgeon the opportunity to identify any potential technical issues with the donor lung and alter the hilar preparation and explant of the recipient lung accordingly. On the right side, Sondegaards groove should be dissected out as far as practical to provide space for the atrial clamp and allow more atrial tissue to be available for the anastomosis. Similarly, this interatrial groove can be dissected out in the donor prior to division of the heart lung block to facilitate division with adequate cuffs. Other issues which may be identified at this time include a small donor pulmonary artery. This may prompt the surgeon to tie off the upper lobe branch of the pulmonary artery in the recipient and prepare the smaller interlobar portion of the pulmonary artery for anastomosis. A short left atrial cuff might be able to be dealt with by dividing the pulmonary veins in the recipient more distal than usual and using that extra tissue to elongate the recipient cuff once the bridging tissue between the superior and inferior pulmonary veins is opened. Extra length can also be obtained when preparing the donor organ by dissecting free the pulmonary veins from the lung parenchyma at the hilum.

The issue of inadequate left atrial cuff arises at the time of division of the heart lung block at which time either technical error or pulmonary venous anatomical variation result in a reduced amount of left atrial tissue at the lung hilum. A number of strategies have been developed to deal with this potentially critical problem. The degree of ‘tissue loss’ can be classified as outlined in figure 6 [46].

Generally the donor lung is procured with a large amount of pericardium still attached to the hilum. This can be easily used to augment the anterior and posterior wall of the left atrial cuff using a continuous running suture to tailor the patch to size [46](Figure 7). The use of autologous pericardium to augment inadequate donor atrial cuff has also been described using a ‘sutureless’ technique whereby no suture line is used to stabilize the venous structures within the pericardial skirt. A single suture line only is used to suture the pericardial cuff to the recipient atrium [47].

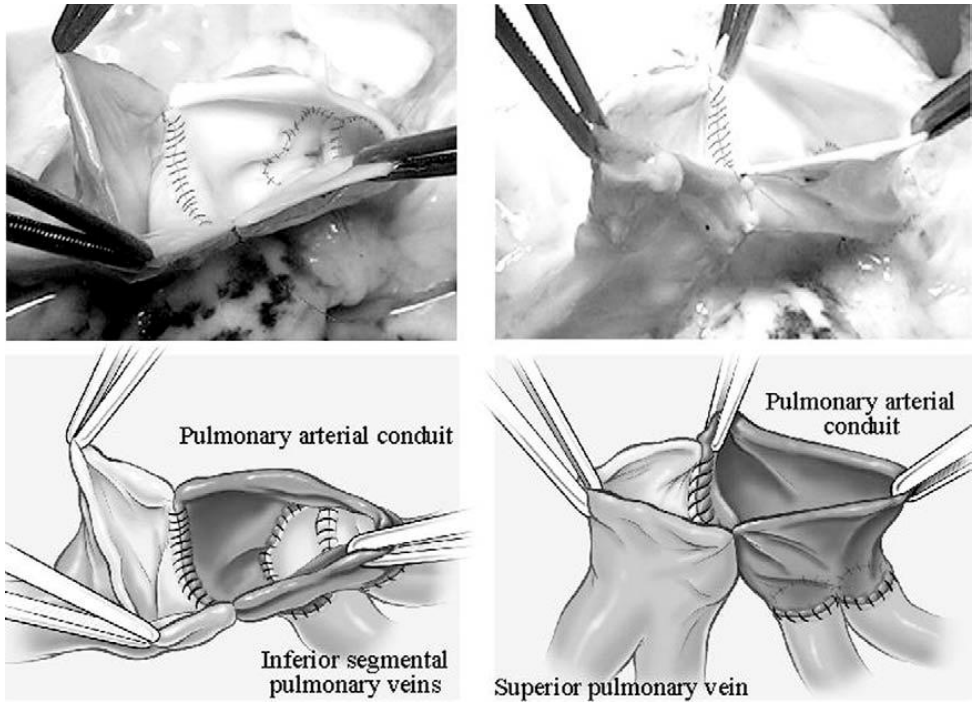


Oto et al., 2006.

Figure 6. Reconstruction techniques for an inadequate left atrial cuff. (A) For a short length of the left atrial cuff limited in its anterior wall, a partial patch repair with donor pericardium was performed. (B) For a short length of the left atrial cuff seen both in its anterior and posterior wall, a donor pericardial patch repair for both the anterior and posterior walls was performed. (C) For separated superior and inferior pulmonary veins, the two separated veins were directly sutured back together with a wide septum to create an oval crosssectional cuff. (D) For separated superior and inferior pulmonary veins that were too short to be directly sutured back together, the divided edges of the two veins were sutured with pericardium around each of the vein orifices, and a new cuff was created. (E) When the inferior pulmonary vein was discontinued at the level of a segmental branch, a cylinder of donor pulmonary artery was used as a conduit between segmental veins that had been sutured back together. This cylinder was sutured to the superior pulmonary vein to create a new atrial cuff [46].

If the superior and inferior pulmonary veins have been separated in the donor, they may be able to be reopposed with a running suture along their adjacent walls to create a single ‘double barreled’ orifice. This could in turn be augmented with an autologous pericardial

patch or cuff. The situation of separation and loss of length of either donor pulmonary vein requires careful reconstruction. Usually there is more than adequate pulmonary artery, particularly on the right donor lung and this can be procured as a cylinder to perform an end to end reconstruction of the short pulmonary vein (Figures 6 & 7) [46].



Oto et al., 2006.

Figure 7. Operative photographs (top panels) and corresponding line diagrams (bottom panels) showing the technique for reconstructing a new left atrial cuff using a segment of donor pulmonary artery as a conduit [46].

The use of donor iliac vein [48] and donor superior vena cava [49] has also been described. Care needs to be taken with all these extra anastomoses to ensure that there is no torsion, stenosis or risk of thrombosis. In particular, care must be taken when using pericardial patch material that any fat is excluded from the endovascular surface as this will increase the risk of thrombosis.

A review of left atrial cuff reconstruction from a single institution report showed an incidence of 2.7% with no increase in post operative complications compared to the historically comparable cohort of patients who did not require cuff reconstruction [46]. In contrast, failure to identify inadequate tissue could lead to an anstomosis under tension which is likely to cause problems healing, or in the more immediate term, dehiscence and bleeding. Both the atrial cuff and the pulmonary artery can tear in a ‘postage stamp’ type pattern where the suturing further weakens the tissue by virtue of the perforations caused by the needle. Further, these patients often have fragile tissues due to long term steroid use, or pulmonary hypertension which can cause atheromatous disease in the pulmonary arteries, resulting in very fragile tissues.

The most common problem encountered with the pulmonary arterial anastomosis is size discrepancy. This is usually because the recipient pulmonary artery is larger than the donor and this can be addressed by anastomosing to the interlobar portion of the recipient pulmonary artery as mentioned above. Care must be taken not to stenose the artery at the anastomosis, particularly when using a continuous running suture technique for the entire circumference. The other risk is torsion, usually due to incorrect apposition of the donor and recipient artery. The upper lobe branch is a useful marker to ensure the arteries are aligned correctly.

Lung Size Reduction

The amount of time spent on the waiting list due to graft size incompatibility issues can often be prolonged and detrimental for paediatric patients and small adults in urgent need of transplantation[50]. There are also instances during surgery when donor lungs are larger than expected for the recipient and size reduction is required for an ideal fit. The use of lobar transplantation, non anatomical cut down and split lung transplantation has allowed larger donor lungs to be downsized for use in smaller recipients[51]. However, despite these advantages, the techniques have not been widely adopted.

The use of lobar transplantation was first described in living lobar donors in 1994[52]. Several years later Wisser et al., described their results with lung tailoring (non anatomical peripheral segmental resections) to overcome size discrepancies in cadaveric donors[53]. Since then, there has been a rapid expansion of reports of living related lobar transplantation but a much smaller number of published experiences with cadaveric lobar transplantation[52,54-60]. Much of our current knowledge of cadaveric lobar transplantation has stemmed from international experience with this operation. Numerous studies have reported acceptable early operative outcomes, functional outcomes and survival in recipients of living related lung transplantation[61,62].

Another innovative type of size reduced transplant is the split lung transplantation initially described by Couetil and colleagues[63]. Inspired by the success of liver partitioning for paediatric transplantation, Couetil developed the procedure of lung partitioning in dogs and then applied that to a series of pediatric recipients in the early 1990's. Procuring the heart lung block in a standard fashion, they then divided the left lung into upper and lower lobes, taking advantage of donors with complete oblique fissures. The pulmonary artery is divided between the apical branch of the lower lobe and the lingular artery; the two pulmonary veins are separated from each other at the level of their drainage into the pulmonary cuff and the upper and lower lobe bronchi are transected at their origin. The left upper lobe is implanted into the right hemithorax of the recipient after being rotated 180° and the left lower lobe is implanted into the left hemithorax. Clearly this procedure is quite technically demanding and very few centres have published results using this operation.

Another method to optimize the use of available organs which must be considered is the bipartitioning of donor lungs to be used in two recipients. This has been described using the left lung [56]. The presence of a complete oblique fissure facilitates the procedure. The pulmonary artery is divided in the fissure between the apical branch of the lower lobe and the lingular branch. The pulmonary veins are separated from each other at the hilum. The upper

and lower lobe bronchi are transected at their origin. The left upper lobe is implanted into the right chest, having been rotated 180 degrees on the vertical axis. Thus when performing the bronchial anastomosis, the membranous portion of the recipient is sutured to the cartilaginous portion of the donor and vice versa. The venous structures are well aligned for a straight forward anastomosis. The donor pulmonary artery lies posterior to the bronchus although it is heading anteriorly. Dissection of sufficient length of the recipient pulmonary artery allows it to come forward to meet the donor artery anterior to the bronchial anastomsosis. The left lower lobe implantation into the left chest is then more straightforward with the structures being well aligned. There will often be some size discrepancy in the hilar structures however which needs to be addressed.

Back Table versus In Situ Lobectomy

The lobectomy can be performed on the back table before implantation of the lung, or after implantation of the entire lung (Figures 8 & 9). There are advantages and disadvantages to both techniques. The advantage of performing the lobectomy on the back table is that the lobectomy can be performed by a second surgeon, minimizing cold ischaemic time. It is also useful in very small patients where implantation of the whole lung in the first instance would obscure the view of the hilum because of the gross size mismatch. The disadvantage of this technique is that it can be technically difficult. None of the vessels are distended by blood and all the structures appear white making it difficult to identify and separate veins from arteries. Because the lung is 'free' it does tend to move around more making it necessary to have an assistant to stabilise the organ. Lung and vascular stapling devices are used to facilitate the procedure and ensure good haemostasis. The lobectomy performed is anatomical, leaving the hilar structures intact so that the hilar anastomoses are completed in the same fashion as is done when the entire lung is implanted.

Lobectomy after implantation of the entire right or left lung poses different technical difficulties. Because the lung has been implanted into a chest cavity that is too small, there is a lot of lung parenchyma in the way which can obscure access to the hilum. This requires careful retraction by an assistant and / or packing with large sponges. The hilar structures are then anastomosed in the usual manner. We perform retrograde reperfusion and deairing as a routine prior to removal of the pulmonary arterial clamp. The entire lung is then perfused and ventilated at low peak airway pressures to avoid injury. It may be evident at this stage that the lung is too big but with the clamshell incision open, there is plenty of room for the lung to expand. We do not perform the lobectomy at this time however, but rather proceed to the implantation of the contralateral lung. The reason for this is because the pulmonary artery to the contralateral lung will be clamped during the next stage of the transplant, sending the entire cardiac output through the newly implanted lung. To send the entire cardiac output through a newly implanted lobe alone, would be more likely to precipitate reperfusion injury and should be avoided at all costs. Once the second lung is implanted, both lungs can be observed fully ventilated and a decision to proceed with the lobectomy made at that time. The risk of reperfusion oedema due to high flow is less in the paediatric patients because of their smaller circulating volume and thus a back table lobectomy is appropriate in those patients.

In contrast to the back table lobectomy, a lobar size reduction once the entire lung is implanted can be easier as it is relatively simple to identify the vascular structures now that

they are distended. The lung is also fixed by its attachment to the hilum making it easier to work on it with minimal assistance. The difficulties at this stage can be oedema if there is some reperfusion injury developing, making the tissues fragile and the lung parenchyma in particular, prone to tearing. Complete fissures at this stage are a most welcome anatomical variant.

Choosing which Lobe to Resect

The choice of which lobe to resect is based on many factors. Technical issues such as the amount of volume to be removed and the area in the chest cavity where the volume is excessive need to be considered. A middle lobectomy for example will reduce lung volume in the antero-posterior diameter and is useful in slender patients with narrow antero-posterior diameters. A lower lobectomy is useful in patients with a short chest cavity or relatively raised diaphragm such as pulmonary fibrosis patients. An upper lobectomy will also reduce volume especially in the vertical aspect but this leaves the vast majority of the lung parenchyma below the hilum with a potential apical space. Leaving a potential basal space seems to be easier to deal with as the diaphragm will rise to fill that space, similar to what is seen after lower lobectomy in other indications such as lung cancer.

Pulmonary tailoring has been used by a number of groups and is technically much easier than removing an entire lobe. Whereas lobectomy can reduce the lung size by half, non anatomical segmental resections can only safely remove approximately 15% of lung parenchyma. It could be argued that most thoraces would accept a 15% increase in lung parenchyma without major sequelae. However, if the clinical situation requires it, this amount of lung tissue can easily be removed using a lung stapler. Removal of segments such as the lingula, or tailoring of the entire upper lobe with stapled resection have been described [53,54].

Choosing How Much Lung to Resect

When the need to reduce lung volume is unexpected and the decision is made once surgery has commenced, then the decision making about which lobe or how much lung to remove is generally based on size and shape issues as outlined in the above section. However, if there is a planned lobar reduction because of a known large size mismatch, or in the event of an adult donor lung going to a pediatric recipient, then the decision about lobar reduction is made preoperatively and the predicted donor and actual recipient total lung capacities (TLC) are taken into account. The use of TLC to match donor and recipient has been previously described [64]. Donor TLC (liters) is estimated by the formula 7.99 x H – 7.08 in males and 6.6 x H – 5.79 in female donors, where H = height^2 (m). The prediction of post operative TLC in size reduced lung transplantation can be predicted by the donor TLC which is corrected for the number of segments actually transplanted [65]. Thus, based on the presence of 19 segments in both right and left lungs combined, the following formula can be used:

$TLC_{\text{size reduction}} = (TLC/19) \cdot n$ transplanted segments

Using this formula has been shown to give good correlation to the post operative best TLC in the recipient [65].

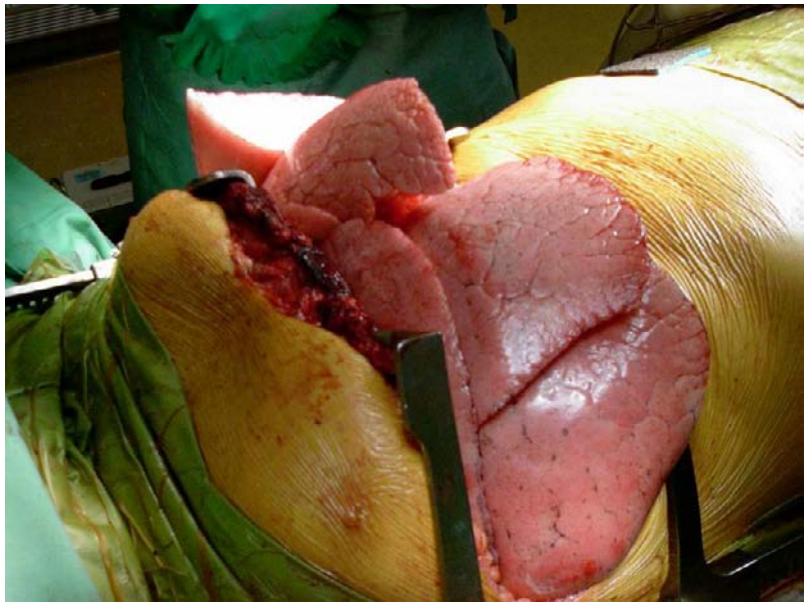


Figure 8. Operative photograph. Bilateral sequential lung transplant showing excessive size of inflated lungs.

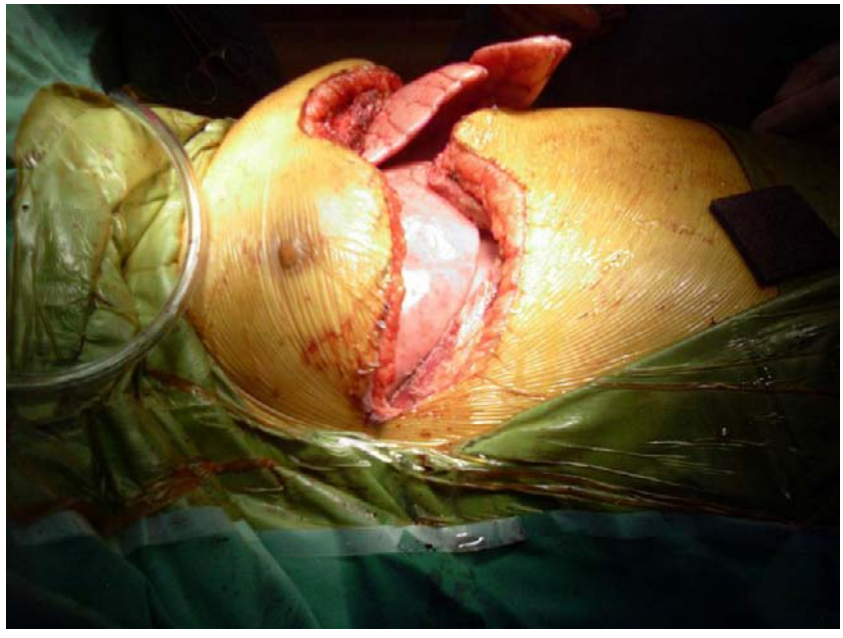


Figure 9. Operative photograph. After in situ right middle lobectomy, the remaining right lung fits well into the recipient chest cavity. A left lower lobectomy was also required in this patient.

Conclusion

In over a quarter of a century, techniques for lung transplantation have evolved to keep pace with advances in organ preservation and immunosuppression. Bilateral sequential lung transplantation remains the procedure of choice for most patients and work continues to refine techniques that will reduce risk, speed recovery and increase the total donor pool available for the procedure. At all stages careful planning, communication and meticulous surgical technique remain the cornerstones of success.

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Chapter 6

Neurologic Complications of Lung Transplantation

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Abstract

Lung transplantation has proven to be an effective treatment for end-stage respiratory failure, but the post-transplant clinical course is still impeded by surgical and medical complications, and neurologic complications have been reported in up to 68% of lung transplant recipients.[1, 2] Complex pretransplant course and high immunosuppression requirements create an environment that increases the risk of neurologic morbidity after lung transplantation. Higher incidence of rejection with lung allografts than with most other solid organ allografts, generally requires greater chronic immunosuppression and persistent risk of opportunistic infections and immunosuppressant neurotoxicity. Increased frequency of neurologic complications has been reported in lung transplant recipients with cystic fibrosis.

Neurologic complications are a significant source of morbidity after lung transplantation, but the presence of neurologic complications is usually not associated with decreased survival. Most common etiologies include calcineurin inhibitor (CNI) neurotoxicity and opportunistic infections. Early onset of CNI neurotoxicity is attributable to high dosing needed to prevent early rejection and chronic immunosuppression increases the risk of systemic and CNS infections.

We will review clinical spectrum of neurologic complications after lung transplantation and diagnostic and treatment strategies.

Abbreviations

CIM:	Critical illness myopathy
CIP:	Critical illness polyneuropathy

CNI: Calcineurin inhibitor
CNS: Central nervous system
PML: Progressive multifocal leukoencephalopathy
PTLD: Post-transplant lymphoproliferative disorder
PRES: Posterior reversible encephalopathy syndrome

1. Lung Transplantation

Lung transplantation has evolved into important life-saving treatment for patients with end-stage lung disease most frequently caused by cystic fibrosis, COPD/ emphysema, and idiopathic pulmonary fibrosis.[3] Refinement of surgical and immunosuppression protocols allowed improved outcomes and 1- and 5-year post-transplant survival of 84% and 47%, respectively.[4] However this remains significantly lower than 5-year survival of 74% and 85% after liver or kidney transplantation, respectively.[4] Chronic rejection of the lung allograft remains a major obstacle to long-term survival and frequently dictates higher immunosuppression requirements. Intense immunosuppression increases the risk of opportrunistic infections and immunosuppressant toxicity. Primary illnesses which led to initial respiratory failure may also progress or recur and lead to a wide spectrum of medical complications. Additionally, postoperative transplant course is still associated with a wide spectrum of medical complications not directly related to allograft function, including neurologic complications.[2].

2. Neurologic Complications of Organ Transplantation

Neurologic complications are a common source of morbidity after transplantation and overall affect 30-60% of transplant recipients (Table 1).[5,6] Clinical spectrum of post-transplant complications gradually evolves following the transplantation. In the early phase after solid organ transplantation, postsurgical complications, metabolic disorders, anoxic encephalopathy and toxic effects of medications, are the dominant causes of neurologic disorders. Bone marrow transplant recipients are at highest risk of infection immediately after transplantation, before immune system is reconstituted. The risk of opportunistic infections is determined by the intensity of epidemiologic exposure to pathogens and the characteristics of immunosuppression regimen. Chronic immunosuppression maintains long-term risk of opportunistic infections and immunosuppressant neurotoxicity. Most neurologic complications are common to all types of transplantation but some are more frequent with different types of allografts.

Early post-transplant deaths may be associated with neurologic complications in individual cases, but neurologic complications overall do not impact the rate of success of transplantation. However, in individual patients clinical course may be significantly affected, so increased alertness is required for prompt and accurate diagnosis and treatment.

3. Neurologic Complications of Lung Transplantation

Neurologic complications after lung transplantation are frequent and may affect up to 68% of allograft recipients (Table 2).[1, 7-9] Underlying etiology is usually related to complex toxic-metabolic disturbances, immunosuppressant neurotoxicity and opportunistic infections. Additionally, primary illnesses which led to initial lung failure may be also associated neurologic complications (e.g. sarcoidosis, connective tissue diseases). Most common complications include alterations of consciousness, seizures and headaches.[1, 7-9]

Due to clinical complexity of lung transplant recipients and multiple concurrent potential etiologies, extensive investigations are frequently needed to establish underlying etiology of transplant complications. Detailed evaluation should include a thorough review of history of present illness and consideration of type and timing of transplantation and cause of primary organ failure. Additionally, we have to consider a possible relevance of an opportunistic infection or neurotoxicity of immunosuppressive medications. If central nervous system (CNS) infections are suspected, cerebrospinal fluid studies may be needed to identify the cause, but this should not delay treatment. MRI imaging of brain or spine is generally preferred to CT, although CT may be an initial study in an acute setting.

3.1. Disorders of Consciousness and Behavior

Disorders of consciousness and behavior are relatively common in posttransplant clinical course and are usually related to toxic-metabolic disturbances, systemic and CNS infections and adverse effects of medications.[10]

Hypoxic-ischemic encephalopathy is relatively common after lung transplantation and may occur in the context of respiratory failure, systemic hypotension or cardiorespiratory arrest. [1, 7, 9] Patients with primary graft failure requiring extracorporeal membrane oxygenation (ECMO) may suffer catastrophic hypoxic-ischemic brain injury.[11]

Impaired function of liver and kidney may precipitate neurotoxicity of hepatically- and renally-metabolized medications including antidepressants, benzodiazepines, acyclovir or cephalosporins.[12]

Fluctuating encephalopathy consistent with delirium usually manifests in patients with various toxic and metabolic abnormalities and should not be mistaken for non-convulsive seizures. Abnormal function of urea cycle enzymes may precipitate hyperammonemic coma which carries high mortality. Successful treatment was reported with a combination of alternate nitrogen waste agents (e.g. sodium benzoate) and hemodialysis. [13] Opportunistic CNS infections are frequenty associated with encephalopathy, but systemic infections may also cause septic encephalopathy, without spreading of infection to CNS.[14]

Post-transplant psychiatric disorders range from depressed mood to mania and psychosis, and are commonly precipitated by use of various medications, including corticosteroids and tacrolimus, complex drug-drug interactions, post-traumatic stress related to transplantation or exacerbation of pre-existing psychiatric conditions. Transplant recipients and their family members may also develop anxiety and posttraumatic stress disorder, especially in early post-transplant period.[15] Mood disorders and psychosis may also affect patient's compliance

with medications resulting in inadequate immunosuppression which may lead to organ rejection.

3.2. Seizures

In transplant recipients, seizures are relatively common and frequent causes include metabolic disturbance, hypoxic-ischemic brain injury and drug toxicity (especially CNI).[1, 9, 16]

Most frequent clinical manifestations are secondarily generalized complex partial seizures and subtle symptoms of partial onset may be easily overlooked. Occurrence of complex partial seizures suggests focal brain lesions, and workup may reveal brain hemorrhages, abscesses, viral encephalitides or ischemic strokes.[1, 9, 16] As in non-transplant patients, postanoxic myoclonic status epilepticus is associated with very poor prognosis.[17]

EEG is indispensable in the evaluation of stuporous and unresponsive patients, especially since it may be very difficult to distinguish toxic-metabolic encephalopathy from non-convulsive status without EEG.[18] Furthermore, focal EEG abnormalities may prompt neuroimaging studies which may demonstrate underlying pathology (e.g. stroke, brain abscess).

Treatment of status epilepticus in transplant recipients does not differ significantly from treatment of non-transplant patients, and standard protocols are based on initial use of benzodiazepines followed by phenytoin.[19] Protocols for use of maintenance therapy to prevent recurrence of seizures are less standardized and depend more on individual clinical features of patients. Long-term use of phenytoin is limited by its side-effects and interactions with immunosuppressants and other newer antieplieptics with better pharmacologic profile are frequently preferred, including levetiracetam, gabapentin and lacosamide.[10, 20]

Lacosamide, levetiracetam and gabapentin are not hepatically metabolized, and do not affect CNI pharmacokinetics with limited drug-drug interactions.

Approach to maintenance therapy with antiepileptic medications is more individualized taking into consideration individual patient features. If the seizure was related to an underlying transient metabolic disturbance, long-term treatment is not needed unless metabolic disturbance was severe enough to precipitate a brain injury. At this time there is no consensus on maintenance therapy after seizures related to CNI toxicity as following the resolution of neurotoxicity many patients with have subsequent normal EEG and neuroimaging studies. While it is not clear what is the duration of an increased risk of seizures after posterior reversible encephalopathy syndrome (PRES), it is a common practice to continue antiepileptic medications for another 2 or 3 months before tapering them off.[21]

3.3. Cerebrovascular Complications

Cerebrovascular complications are an important source of morbidity after lung transplantation and are almost as common as in heart transplantation recipients.[1, 9, 22] Brain infarcts have been reported in 3-7% of lung transplant recipients.[1, 7-9] While etiologies mostly do not differ from non-transplant stroke patients, some pathophysiologic

mechanisms may be quite unique for this patient population (Table 3).[23, 24] Frequent occurrence of atrial fibrillation after lung transplantation carries an increased risk of cardiac embolism and these patients may need long-term anticoagulation.[25] Formation of thrombus at the atrial anastomosis is rare and may also precipitate embolic stroke, and transesophageal echocardiography may be needed to confirm presence of the embolic source.[24] Increased risk of deep venous thrombosis and pulmonary embolism was reported in lung transplant recipients (up to 18% in first year after transplantation)[26], raising the issue of possible hypercoagulable state. Rarely, air embolism has been reported as a cause of stroke.[23] Thrombotic microangiopathy associated with CNI toxicity may also manifest with neurologic complications including strokes and seizures.[27]

The use of ECMO in patients with allograft rejection is associated with an increased risk of neurologic complications including hemorrhagic and ischemic stroke.[28] Risk of seizure recurrence after embolic strokes may mandate long-term maintenance therapy with antiepileptic medications in some transplant recipients, but antiepileptics are not routinely used after embolic or hemorrhagic strokes.

Long-term use of CNI is associated with an increased prevalence of hyperlipidemia and hypertension, increasing the long-term risk of cardiovascular and cerebrovascular morbidity.

3.4. Neuromuscular Complications

Neuromuscular complications after organ transplantation are frequently related to entrapment neuropathies and critical illness myopathy/polyneuropathy.[29-31] Additionally, metabolic deficiencies, frequent drug-drug interactions and use of some potentially neurotoxic antibiotics may precipitate toxic neuropathy. Interestingly, despite a high risk of diabetes in CF patients, there is no evidence of an increased risk of diabetic neuropathy in this population of lung transplant recipients.[1]

Phrenic neuropathy is relatively common complication following lung transplantation and is related to the mechanical injury of the phrenic nerve and thermal injury related to cold-packing. It is usually found ipsilaterally to the side of surgical access, and associated respiratory dysfunction results in prolonged hospital stay and longer ventilation requirements.[32] Noticeably higher prevalence of phrenic neuropathy was reported after heart-lung transplantation when compared to isolated lung transplantation, 42.8% vs. 9.3%. [32] As different studies used various methodologies to define phrenic nerve dysfunction reported frequencies vary and another group reported the overall prevalence of phrenic neuropathy after lung transplantation to be as high as 29.6%.[33]

Posttransplant flaccid weakness is frequently related to critical illness myopathy (CIM) and polyneuropathy (CIP). Clinically, CIM manifests with weakness in the absence of sensory symptioms and it has been described in up to 7% of liver transplant recipients, but less is known about its prevalence with other types of allografts.[29] Onset of weakness can be often described as acute (“acute quadriplegic myopathy”), but symptoms are commonly masked by prior sedation. Symptoms of CIM and CIP frequently overlap and commonly prolong hospitalization and ventilatory dependence.[34] Main risk factors for CIM/CIP include sepsis, systemic inflammatory response syndrome (SIRS) and multiple organ failure.[34] The role of intravenous corticosteroids and paralyzing neuromuscular blocking agents in development of CIM/CIP remains somewhat controversial.[34, 35] While diagnosis

of CIM can be firmly established only with biopsy, muscle or nerve biopsies are rarely pursued now that risk factors have been recognized.

Treatment-resistant bacterial infections may require use of potentially neurotoxic antibiotics increasing the risk of toxic neuropathy. Similarly to toxic neuropathy, complex pharmacologic regimens with combinations of statins and other potentially myotoxic medications and cyclosporine or tacrolimus may precipitate rhabdomyolysis.[36]

Opportunistic infections are less commonly associated with neuromuscular complications. Prevalence of post-transplant herpes zoster has not been studied systematically in lung allograft recipients, but post-transplant immunosuppression would increase its risk similarly as after other types of transplants.[31] Cytomegalovirus-associated polyradicultis has been reported in patients with AIDS and after bone marrow transplantation, but so far there are no reports of it in solid organ transplant recipients.[37]

3.5 Immunosuppressant Neurotoxicity

High immunosuppression requirements after lung transplantation are associated with an increased risk of adverse effects including opportunistic infections and neurotoxicity of cyclosporin and tacrolimus (Table 4). Clinical manifestations of cyclosporin and tacrolimus neurotoxicity overlap and are frequently categorized as "calcineurin inhibitor neurotoxicity" as both of these medications inhibit protein phosphatase calcineurin.

Neurotoxicity of calcineurin inhibitors (CNI) is more common with intravenous dosing and high serum levels of medication, but it can also occur with "normal" levels.[38] Different factors were described as associated with an increased risk of CNI neurotoxicity including hypomagnesemia and hypocholesterolemia, although the clinical significance remains uncertain. Typically, CNI neurotoxicity presents with tremor, encephalopathy and some patients develop PRES. Clinical features of PRES related to CNI neurotoxicity are indistinguishable from eclampsia and hypertensive encephalopathy, but the underlying pathophysiology is still not well understood.

Neuroimaging findings are relatively specific in PRES and include hyperintense areas in posterior white matter on T2-weighted images with some involvement of the overlying cortex. These regions are usually hypointense or isointense on diffusion-weighted images, with an increase of the apparent diffusion coefficient, indicating vasogenic edema.[39] Histopatologic studies showed evidence of endothelial injury with vasogenic edema in the white matter in the absence of demyelination.[40] Clinically, these patients present with confusion, headache and sometimes cortical blindness. Occurrence of cortical blindness after transplantation is suggestive of CNI neurotoxicity and it may be also accompanied by seizures originating from occipital lobe.[41]

Headaches are also common with CNI neurotoxicity and may be related to exacerbation of pre-existing migraines, but we have to remain careful not to miss an underlying mass lesion or CNS infection. Tremor is very common with use of CNI but it's usually not severe enough to warrant dose adjustments and does not limit patient's activity.

Other immunosuppressive medications may also cause neurologic complications (Table 4). Monoclonal antibody muromonab (also known as OKT3) is used for induction therapy after transplantation and may precipitate aseptic meningitis.[42] Corticosteroids can be associated with mood disorders, hyperglycemia and diabetes, and intravenous high dosing may increase the risk of CIM.[35] Use of mycophenolate may precipitate headaches, and

these are usually not very severe. Infrequently, sirolimus toxicity may precipitate PRES, but severe neurologic complications are generally very uncommon with this medication.[43, 44].

3.6. Opportunistic Infections

Opportunistic infections are a major source of morbidity in immunocompromised transplant recipients and involve central nervous system usually in the context of disseminated infection.[45] Prompt and accurate diagnosis is imperative for successful treatment and good outcome and early identification of pathogens is assisted by estimating the extent of immunosuppression, determining epidemiologic exposures and establishing features of clinical presentation.[45] Opportunistic CNS infections usually occur in the setting of more widespread systemic disease, but signs and symptoms of systemic infection may not be obvious from the onset. In the early postoperative course after solid organ transplantation (first 30 days), infection is usually related to previous colonization and nosocomial exposures, followed by activation of latent infections between first and sixth month and community acquired infections after 6 months. Opportunistic infections after transplantation are usually caused by fungal and viral pathogens, while bacterial and parasitic infections are less common. Infectious complications are common after lung transplantation and specific risk factors in this population include direct exposure to infectious agents via inhalation and impaired clearance in transplanted lung.[46] Different preventive strategies have been used to limit and prevent posttransplant infections, mostly with variable success.

Allograft recipients with cystic fibrosis typically have longstanding history of chronic infections and colonization, but so far there are no reports of increased prevalence of opportunistic CNS infections in this population.

Viral CNS infections may present as encephalitides or meningoencephalitis (more common) and are most commonly associated with HSV, VZV, EBV, CMV or HHV6 viruses. Recent epidemics of West Nile virus in US was also associated with severe infections in transplant recipients.[47] Another viral CNS infection in transplant recipients caused by JC virus is progressive multifocal leukoencephalopathy. (PML). PML is almost invariably fatal despite treatment, but fortunately remains very rare. Imaging findings with PML infection may also resemble PRES,[48] but it usually occurs late and does not improve after CNI are reduced or withdrawn.

Fungal infections have been reported in 15-35% of lung transplant recipients.[46] Most common pathogens are Aspergillus and Candida species. Early diagnosis is important for successful treatment, but mortality remains very high.

CNS involvement usually occurs in the context of systemic infection with fungemia, but rare cases of isolated opportunistic CNS infections without evidence of pulmonary or other organ involvement have been reported as well. Chronic fungal sinusitis may lead to fungal meningitis by direct extension to nearby structures. Fungal meningitis carries very high mortality and early and accurate diagnosis is crucial to determine effective therapy.[49]

As with other types of allografts, bacterial meningitis is uncommon. Environenmental exposure to Listeria may lead to rhombencephalitis. Mycobacterial CNS infections are also uncommon and mostly limited to patients with pretransplant history of mycobacterial infections.[46].

3.7. Other Neurologic Complications

Other common neurologic post-transplant complications include headaches, abnormal movements and post-transplant CNS malignancies. Headaches are frequently overshadowed by other systemic or neurologic complaints and symptoms in transplant recipients. However, new onset of severe headache may rarely present as an early manifestation of a brain mass (abscess, intracranial hemorrhage), opportunistic infection or CNI neurotoxicity, and CNI may also aggravate pre-existing migraine disorder.[50, 51] Therefore prompt attention and workup are needed in transplant recipients with new onset of headache. Rarely, use of CNI may trigger severe pain related to calcineurin inhibitor pain-associated syndrome.[52] Pathophysiologic mechanisms of this syndrome remain uncertain and pain may be quite disabling. One of the most common complications of CNI use is tremor, but it is usually self-limited and does not significantly impact patient's quality of life. Stroke affecting basal ganglia may also lead to parkinsonian symptoms, including tremor. Nonepileptic myoclonus in transplant recipients is usually drug-induced, particularly with opiates and antidepressants, and this is usually self-limited and stops shortly after offending medication is discontinued.

Higher prevalence of solid brain tumors has been reported in transplant recipients, but the magnitude of risk increase remains uncertain. In addition to solid tumors, transplant recipients are also at increased risk of developing hematologic malignancies, especially lymphoproliferative which may be associated with EBV infection. Posttransplant lymphoproliferative disorder (PTLD) has been reported in 2.5% of lung transplant patients, and older patients were more frequently affected (>55 years).[53] Overall, CNS involvement has been reported in 15% of transplant patients with PTLD, and indicates worse prognosis.[54].

Conclusion

Neurologic complications frequently occur after lung transplantation, but only rarely determine the outcome of transplantation. Complex toxic-metabolic disturbances, frequent opportunistic infections and high immunosuppression requirement predispose lung allograft recipients to a variety of posttransplant medical complications, including a wide spectrum of neurologic disorders. Early and accurate identification of underlying etiologies allow timely treatment and facilitate more effective clinical care leading to improved outcomes and decreased morbidity.

Table 1. Neurologic complications of organ transplantation [1,9, 54-58]

Ref.	Allograft	(n)	Neurologic complications (%)	Seizure (%)	CNS infection (%)	Neuromuscular (%)	Encephalopathy (%)	Stroke (%)
[1]	Lung	132	68	8	1	21	25	7
[9]	Lung	132	45	27	1.5	n.d.	6.7	3.7
[22]	Heart	261	28	6.5	3.1	10.3	2.3	7.7
[55]	Liver	463	20	8.2	1.2	n.d.	11.8	2.1
[56]	Liver	657	27	6	1.1	4	11	4
[57]	Intestine	54	85	17	7	7	43	4
[58]	Bone marrow	361	16	5	4.2	3.3	2.8	1.7

n.d. – no data

Table 2. Neurologic complications of lung transplantation [1, 7-9]

Ref.	% (n)	Seizures (%)	CNS inf (%)	NM (%)	Encephalopathy (%)	Stroke (%)
[1]	68% (132)	8	1	21	25	7
[7]	26% (100)	10	3	n.d.	3	5
[8]	21% (278)	4	n.d.	n.d.	8	3
[9]	45% (135)*	27	2	n.d.	7	4

* - pediatric patients; CNS inf - CNS infections; NM – neuromuscular; n.d. - no data

Table 3. Cerebrovascular complications associated with lung transplantation

Cardioembolic ischemic stroke	atrial fibrillation, endocarditis, air embolism
Hemorrhagic stroke	hemorrhagic transformation of ischemic stroke, intracranial bleeding, opportunistic vasoinvasive fungal infections
Cerebral venous sinus thrombosis	dehydration, hypercoagulable state
Thrombotic microangiopathy	similar to thrombocytopenic thrombotic purpura (TTP), skin and kidney involvement

Table 4. Clinical manifestations of immunosuppressant neurotoxicity

Calcineurin inhibitors	PRES, seizure, encephalopathy, tremor,
Muromonab	aseptic meningitis
Corticosteroids	hyperglycemia, steroid psychosis/mood disorders, diabetic neuropathy, steroid myopathy
Mycophenolate	headache
Sirolimus	PRES (rare)

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Chapter 7

Leucocyte Intracellular Cytokines in Lung Transplant Patients - A More Physiological Indicator of Immunosuppression than Plasma Drug Levels

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Abstract

The strong allo-immune response to the transplanted lung necessitates combined pharmacological immunosuppression to prevent graft rejection. Immunosuppressants used to prevent and treat rejection involve several classes of drugs and many target the production of pro-inflammatory cytokines by T cells, monocytes and other immune cells. Although most effective transplantation immunosuppressive strategies are based on interruption of IL-2 signaling by the calcineurin inhibitors cyclosporin A and tacrolimus, intensification of immunosupressive therapies has not led to any improvement in graft survival. Treatment with these drugs is also associated with serious adverse effects including specific organ toxicities, increased risk of developing a range of malignancies and susceptibility to infections. High inter- and intra-individual pharmacokinetic variability of both drugs may mean some patients do not require the high levels of drugs and associated adverse side effects for effective therapeutics. While current assessment of therapeutic drug levels simply involves the empirical measurement of plasma drug

concentrations, there is a need for more physiological assessment of combined immunosuppression strategies, particularly at the site of action. Recent research has identified measurement of inflammatory cytokines at the cellular level using novel flow cytometric techniques as a strategy to assess the physiological response to treatment. Intracellular cytokine levels in both peripheral blood and in the airways have been investigated and have highlighted important differences in responses seen at the transplant site versus systemically. While Th1 pro-inflammatory cytokines were significantly reduced in blood T cells from transplant patients, levels of these cytokines in T cells from the airways were significantly greater in transplant patients compared with healthy control subjects. Furthermore, patients undergoing infection or rejection episodes were characterised by significantly decreased or increased Th1 intracellular T cell cytokines in the airways respectively, compared with stable lung transplant patients. To overcome patient inter-individual variability of leucocyte cytokine production, longitudinal monitoring of patient cytokines may be useful in predicting adverse episodes of rejection and/or infection. These techniques may complement or ultimately replace current standard approaches to therapeutic drug monitoring and monitoring by invasive biopsy and have the potential to improve current immunosuppression protocols, optimise individual therapy and possibly provide new therapeutic options to improve the morbidity of lung transplant patients.

Introduction

Lung transplantation is the only definitive therapy for many forms of end-stage lung disease. However, five year survival after lung transplantation is only approximately 50%. This is a disappointing outcome considering the huge expenses involved in lung transplantation and post-transplant care and the acute shortage of donor organs. Acute graft failure is more common with lungs than any other solid organ resulting in poorer short term survival after lung transplant compared with recipients of other organs. In the longer term, the principle reason for graft failure is believed to be chronic allograft dysfunction. Chronic graft failure is manifested by progressive airways obstruction and worsening shortness of breath. The clinical syndrome is referred to as "bronchiolitis obliterans syndrome" (BOS). In many cases, there is a defined pathological correlate, "obliterative bronchiolitis" (OB).

Although the precise pathogenesis of chronic graft failure is unclear, there are certain strong associations such as acute rejection; with early and repeated episodes of acute rejection significantly increasing the risk of subsequent OB. This suggests that immune mechanisms may in part underly OB and have thus lead to the hypothesis that the disorder is a form of chronic rejection. However, many patients with acute rejection do not develop OB, and some patients with OB have never experienced clinically detected acute rejection. OB may also be associated with pulmonary infections including CMV infection, primary graft dysfunction and gastro-esophageal reflux disease.

Acute and Chronic Transplant Rejection

At the cellular level, acute graft rejection is associated with a marked increase in graft T-cell infiltration, alveolar macrophages, and pro-inflammatory cytokine expression including

IL-2, IFN-γ and TNF-α [2]. Acute cellular rejection involves recipient T cell recognition of HLA molecules expressed on donor-derived, antigen presenting cells (direct allorecognition) or presentation of donor derived peptides by recipient antigen-presenting cells to recipient T cells (indirect allorecognition). Once the alloantigens are recognised as foreign, the activation and production of cytokines by T lymphocytes, monocytes and other immune cells lead to the amplification of the alloimmune response.

OB is also associated with a moderate increase in graft airway T-cell infiltration, alveolar macrophages, and pro-inflammatory cytokine expression [3, 4]. Airway fibrosis is also associated with OB, which may be due to the presence of pro-fibrotic chemokines/cytokines such as MCP-1, IL-4 and TGFβ [4]. The strong alloimmune response to the transplanted lung necessitates combined pharmacological immunosuppression to prevent graft rejection. Immunosuppression has improved graft survival but leaves patients susceptible to infectious complications and malignancies. Pulmonary infections are the leading cause of morbidity and mortality in this patient group. Immunosuppressants used to prevent and treat rejection involve several classes of drugs and many target the production of pro-inflammatory cytokine by T cells, monocytes and other immune cells.

Immunosuppression

Corticosteroids

Glucocorticoids were the first immunosuppressants used in transplantation. They are the least selective agents and affect multiple cell lines, including T and B cells, monocytes, macrophages and neutrophils. In lymphocytes and monocytes, glucocorticoids exert negative regulatory effects on cytokine gene expression [5], although one study showed upregulation of IL-10 production by monocytes in the presence of low concentrations of prednisolone in vitro [6].

Calcineurin Inhibitors: Cyclosporin A (CsA) and Tacrolimus (Tac)

Calcineurin inhibitors CsA and Tac have greatly decreased the incidence of allograft rejection and are the mainstay of current immunosuppression regimes. They are more T-cell selective and their use has helped preserve other cell lines by reducing the overall incidence of infection [7, 8]. Both CsA and Tac inhibit production of IL-1β, IL-2, IL-6, IL-8, IFNγ and TNFα while Tac preferentially suppresses T-helper-type-1 (Th1) cells over Th2 cells [5].

Both agents do however upregulate TGFβ, which although having several immunosuppressive properties on pro-inflammatory cytokines, promotes matrix formation and may contribute to allograft fibrosis as observed in chronic rejection.

Antimetabolites: Azathioprine and Mycophenolate Mofetil

Azathioprine and Mycophenolate Mofetil are antimetabolites that inhibit the production of purine which is required for T and B-cell activation and proliferation. Azathioprine also

acts by inhibiting CD28 costimulation [9] while Mycophenolate Mofetil reduces cytokine production through inhibition of clonal expansion [9, 10].

Other agents such as sirolimus and everolimus inhibit mRNA responsible for cell cycle progression thus blocking IL-2 postreceptor signaling and preventing T-cell proliferation [10].

Anti-lymphocyte Antibodies

Polyclonal anti-lymphocyte antibodies have a long history of use for induction of immuno-suppression and in the treatment of acute rejection, targeting different cell lines such as lymphocytes, thymocytes or specific cell lines. For example, OKT3 is a murine monoclonal anti-lymphocyte antibody directed against the T-cell receptor causing T-cell opsinisation and removal by mononuclear phagocytes [8].

Anti-cytokine Receptor Antibodies

Anti-cytokine receptor monoclonal antibodies directed against the α-chains of the CD25 molecule, a key unit of the IL-2 receptor, have also been used successfully in the transplant setting [10] due to the central role of IL-2 in regulating T-cell activation.

"Triple immunosuppression" is the common form of immunosuppression used in lung transplantation and consists of a calcineurin antagonist (cyclosporin A or tacrolimus), an antimetabolite (Azathioprine and Mycophenolate Mofetil) and corticosteroids [9]. Trough plasma drug levels of either CsA A or Tac are kept within recommended therapeutic ranges (range for CsA (80-250 μg/L) and Tac (5-20 μg/L)). However, therapeutic drug plasma concentrations are broad and may vary according to the clinical situation and the choice of immunosuppressive agents being given.

Therapeutic monitoring of single drug levels has certain limitations as the combined therapeutic effect of all drugs used should be assessed for each patient. An alternative more physiological approach has recently been suggested [11].

Cytokine Measurement in Lung Transplant Patients

There are three sites from which cells or fluid can be assessed for cytokine analysis in recipients of pulmonary allografts; blood, the bronchial epithelium and the alveolar region.

Previous methodology to measure cytokine levels in transplant patients include ELISA quantification from serum and peripheral blood mononuclear cell culture [12, 13] and RTPCR of cytokine mRNA levels [14]. Measurement of soluble cytokines by ELISA and more recently by cytometric bead arrays have proven useful for diagnosis in several disease states [15, 16]. However, cytokines bind to proteoglycan components of the cell surface or extracellular matrix [17] and have a very limited availability within the blood, effectively limiting the prognostic value of assay of soluble cytokines. Standard techniques such as ELISA are time consuming and expensive if several cytokines are quantified and give no

indication of cytokine-producing cell types. Cell purification techniques lead to loss of specific cell subsets [18] and increased apoptosis of cells [19]. While RTPCR is a sensitive technique, results depend on purification of cells from heterogeneous cell populations and are subject to technical error.

The production of cytokines by cells can be assessed using a range of techniques. Several of these assays require cell activation prior to analysis. In the case of T cells this is accomplished by polyclonal stimulation with mitogens active on the cell surface or within the cell or by antigenic stimulation. Multiparameter flow cytometry offers a powerful tool to analyse multiple pro- and anti-inflammatory intracellular cytokines following polyclonal stimulation in thousands of cells by individual cell types. This technique provides information regarding not only the phenotype but also the activation state of each individual immune effector cell.

Assessment of the activation state of T-cells by measurement of cell-specific cytokine production has also been used to investigate differences in unstimulated cells in several clinical situations such as between cord and adult blood in the context of graft versus host disease [20] and effects of other therapeutics [21]. Recently, this technique has been used in preliminary studies in lung transplant patients.

Effect of Immunosuppression Protocols on Intracellular Cytokines in Blood T Cells

Until very recently it was unknown if levels of immunosuppressive drugs correlate with pro-inflammatory cytokine expression in peripheral blood T cells. As lymphocytes traffic from the blood stream to the lung and later rejoin the peripheral circulation [22], measurement of blood T cell cytokines may be reflective of graft infiltrating T-cell cytokine profiles.

The immunomodulatory effects of currently used immunosuppressive regimes on T cell cytokine production from a group of stable, non-infected lung transplant patients and control volunteers was recently assessed using the technique of intracellular cytokine analysis and multiparameter flow cytometry [11]. This study provided evidence that current immunosuppression protocols have a significant immunosuppressive effect on pro-inflammatory cytokine production by peripheral blood CD4+ T cells consistent with therapeutic intention. However, a limited effect on peripheral blood CD8+ T-cells was noted, particularly IFNγ inflammatory cytokine production in lung transplant patients (Figure 1).

All transplant patients in this study had plasma levels of CsA or Tac within their therapeutic ranges suggesting that analysis of intracellular T cell cytokine production may provide a more accurate assessment of immunosuppression than drug levels.

This study showed that immunosuppression protocols are adequate for CD8- (CD4+) but not CD8+ T-cell inflammatory cytokines, particularly IFNγ. Results from this study also showed that current immunosuppression agents increased anti-inflammatory cytokine production of IL-4 and TGFβ by T cells from lung transplant patients compared to controls (Table 1).

IL-4 negatively regulates IFNγ and IL-2 [23], pro-inflammatory cytokines that have been reportedly increased during acute rejection episodes [14]. TGFβ has also been shown to

inhibit IL-2 and IFNγ production by T cells [24] and may partially explain the significantly reduced levels of IFNγ and IL-2 in peripheral blood T cells noted in this study. TGFβ has been shown to inhibit CD4+ Th1 responses compared to CD8+ T cells [25] which may help explain the observation of increased inhibition of these cytokines in CD4+ cells compared to CD8+ T cells.

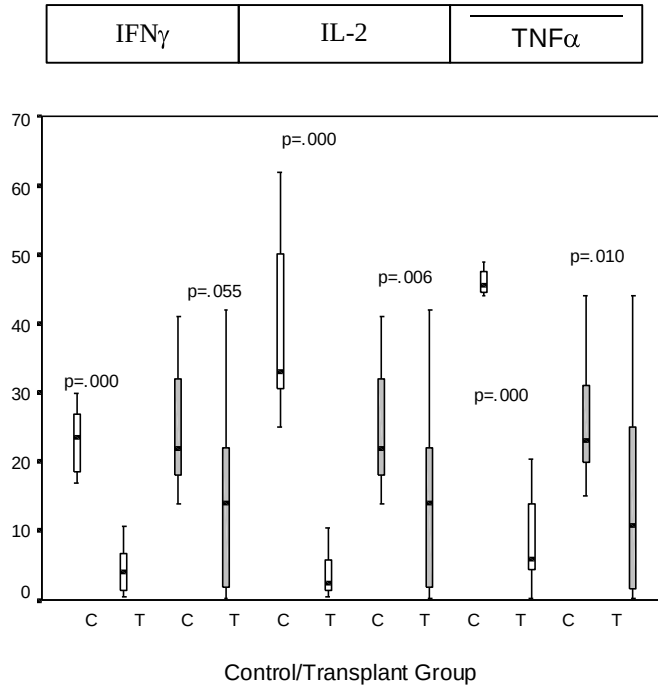

Figure 1. Box plot graphs showing the production of pro-inflammatory cytokines by CD4+ ☐ and CD8+ ■ T-cells from the blood of lung transplant (T) and control (C) subjects following *in vitro* stimulation (mean ± SD and range). The percentage of CD4+ and CD8+ T-cells producing IL-2 and TNFα was significantly reduced from lung transplant patients compared to control. The percentage of CD4+ T-cells producing IFNγ was also significantly reduced from lung transplant patients but not the percentage of CD8+ T cells producing IFNγ. Note the marked inhibition of inflammatory T-cell cytokines in CD4+ cells compared with CD8+ cells in transplant patients compared to control group.

Table 1. The percentage of T cell subsets producing anti-inflammatory cytokines, IL-4 and TGFβ from 9 lung transplant patients (T) compared with 15 control subjects (C) (mean ± SD). There was a significant increase in IL-4 by both T-cell subsets and TGFβ by CD8+ T-cell subsets by transplant patients compared with control subjects (bold)

	IL-4		**TGFβ**	
	CD4	**CD8**	**CD4**	**CD8**
C	0.4±0.3	0.0±0.0	5.3±3.2	3.0±2.6
T	1.0±0.8	1.4±0.0	4.9±3.1	4.6±2.4
P	.019	.010	.890	.017

Increased absolute numbers of CD8+ T cells were also noted in transplant patients which is consistent with a previous report [11]. TGFβ has been shown to be co-stimulatory for CD8+ T cells but not CD4+ T cells [26]. IL-4 enhances the proliferation of precursors of cytotoxic lymphocytes and their differentiation into active cytotoxic CD8+ T cells [28]. In CsA or Tac treated mice, T-cell proliferation was shown to be suppressed in CD4+ but not CD8+ subsets [28]. These findings of increased TGFβ by CD8+T cells and increased IL-4 production by both CD4+ and CD8+ T-cell subsets may therefore be causative factors in the significant increase in cytotoxic T cells in these patients.

In contrast to acute rejection, chronic graft failure is associated with increased fibrosis in the lung. Both IL-4 [29] and TGFβ [30] have been shown to promote fibroblast proliferation in the lung. Therefore, although TGFβ and IL4 are anti-inflammatory cytokines, their increased production by cytotoxic lymphocytes that migrate to the lung may contribute to increased fibrosis noted in chronic graft rejection, hence predisposing patients receiving current immunosuppression protocols to BOS.

In an attempt to minimise the significant toxic side effects of current immunosuppression protocols [31], a number of low toxicity protocols have been developed [32]. One could hypothesise that if a transplant patient was showing signs of drug toxicity and levels of intracellular T-cell inflammatory cytokines were markedly reduced compared to control (eg., Patient B in Figure 2), the dose of drug could be reduced and therapeutic effects monitored or tailored to suit the individual patient. Alternately, if intracellular T-cell inflammatory cytokines were not reduced (eg., CD8 T cells in Patient A, Figure 2), other immunosuppressive drugs should be considered.

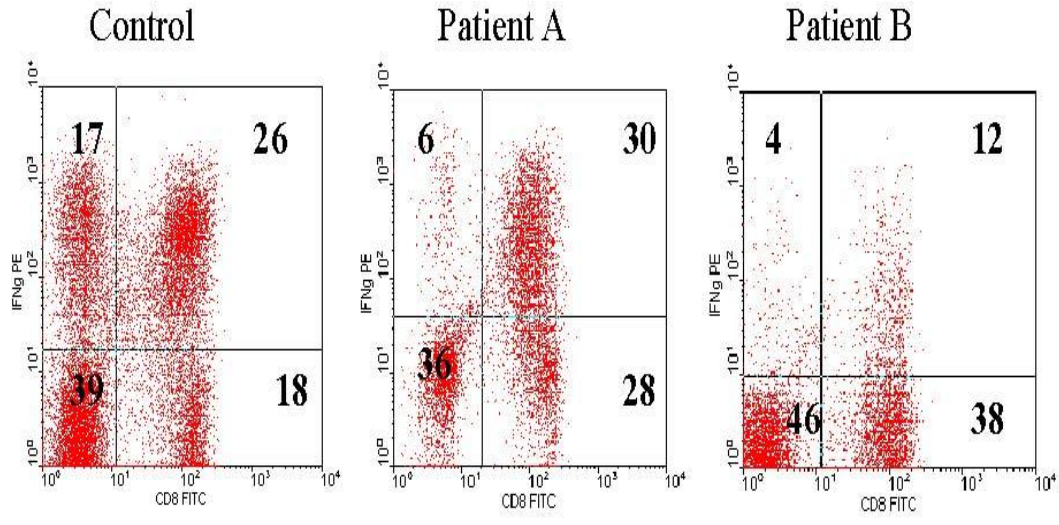

Figure 2. Representative dot plots showing the effect of immunosuppression therapy on IFNγ production by CD8+ and CD8- (CD4+) T cells from the blood of 2 lung transplant patients and control. T cells were identified by CD3 PC5 versus side scatter characteristics. Patient A shows immunosuppression of IFNγ in CD8- (CD4+) T cells but not CD8+ T cells. Patient B shows immunosuppression of IFNγ in both T-cell subsets. Note the reduced percentage of CD4 T cells and increased CD8 T cells in transplant patients compared to control.

This study showed that current immunosuppression protocols have limited effect on peripheral blood IFNγ production by CD8+ T-cells but do upregulate T-cell anti-inflammatory cytokines, TGFβ and IL4. Current protocols for reducing graft rejection in these patients may include drugs that effectively reduce IFNγ production by CD8+ T cells. Intracellular cytokine analysis using flow cytometry may be a more appropriate indicator of immunosuppression of all therapeutic drugs used than measurement of plasma CsA or Tac levels in these patients.

Effect of Immunosuppression Protocols on Blood Monocyte Cytokine and Chemokine Production

Blood monocytes that migrate to the lung form alveolar macrophages which are a major source of inflammatory cytokines/chemokines involved in the pathogenesis of lung transplant rejection. Chemokines are inflammatory mediators that specifically stimulate the directional migration of T cells and monocytes and play an important role in immune cell recruitment into sites of antigenic challenge [33] such as in the pulmonary allograft. Chemokines such as IL-8 and MCP-1 have been reportedly increased in BAL of transplant patients undergoing rejection [34]. MCP-1 induces monocyte migration and differentiation to macrophages and plays a pivotal role in BOS [35]. A recent report investigated monocyte production of cytokine/chemokine mediators in peripheral blood of lung transplant patients [36]. Intracellular cytokine/chemokine production by peripheral blood monocytes following stimulation with LPS from a group of stable lung transplant patients and control volunteers was investigated. There was a significant increase in the percentage of monocytes producing chemokines MCP-1, MCP-3 and IL-8 and anti-inflammatory cytokine IL-10, but no change in the percentage of monocytes producing IL-6, TNFα, IL-1α, IL-12, MIP-1α, MIP-1β and TGFβ (Table 2). Representative histograms showing the increase in the percentage of monocytes producing chemokines MCP-1, MCP-3 from a transplant patient is shown in figure 3.

Current immunosuppression protocols were shown to have limited effect on peripheral blood monocyte inflammatory cytokine production and were inadequate at suppressing monocyte chemokine production in lung transplant patients. This was the first report of increased MCP-3 in lung transplant patients. MCP-3 has been shown to be a mediator in the activation of extracellular matrix gene expression in addition to promoting leucocyte trafficking in systemic sclerosis [37]. Monocytes migrating to the lung may be acting similarly in lung transplant patients. Interestingly, it has been shown that the effects of MCP-3 can be diminished by neutralising antibody to TGFβ [38]. As TGFβ has been reported to play a major role in OB, the possible role of TGFβ – MCP-3 mediated effects clearly warrants further study.

Table 2. Monocyte cytokine/chemokine production in 9 lung transplant and 15 control subjects (% positive cells) following LPS stimulation. The percentage of blood monocytes producing IL-8, IL-10 and MCP-1 and MCP-3 was significantly increased in lung transplant patients (bold) but levels of IL-6, TNFα, IL-1α, IL-12, MIP-1α, MIP-1β and TGFβ were unchanged

		IL-8	**IL-6**	**TNFα**	**IL-10**	**IL-1α**	**IL-12**	**MCP-1**	**MCP-3**	**MIP1α**	**MIP1β**	**TGFβ**
Controls	Mean	82.4	72.9	82.7	4.3	79.5	24.7	11.4	5.5	13.6	71.6	6.2
	SD	7.6	20.2	7.2	1.5	13.1	9.3	5.7	3.1	11.4	15.9	3.1
Patients	Mean	92.3	65.1	82.1	8.8	73.9	20.1	28.8	18.9	13.9	72.2	5.5
	SD	6.6	21.8	8.2	3.7	16.6	12.3	16.9	16.3	9.1	23.1	2.6
	P =	.039	.731	.612	.042	.714	.542	.006	.010	.875	.788	.468

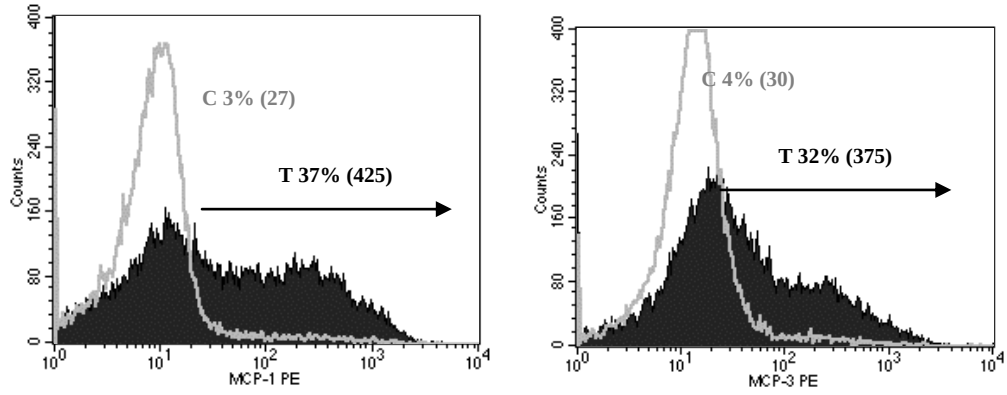

Figure 3. Representative histograms showing intracellular staining of MCP-1 and MCP-3 in peripheral blood monocytes from a transplant patient (T) and control subject (C) following LPS stimulation. The percentage of monocytes producing MCP-1 and MCP-3 was significantly increased in the lung transplant patient compared to control (marker set on negative control-not shown). The amount of MCP-1 and MCP-3 (as indicated by MFI) was also significantly increased in the lung transplant patient compared to control.

MCP-1 levels have been reported to be increased in lung allograft rejection, especially in OB [35]. Alveolar macrophages were identified as the major source of MCP-1. Blood monocytes migrating to the lung are the probable source of these high MCP-1 producers, indicating that these cells are producing MCP-1 before entering the lung. Interestingly MCP-3 has been shown to be a functional ligand for MCP-1 receptor [38]. The increased levels of monocyte MCP-3 identified in transplant patients may enhance the chemoattractant effects of MCP-1, leading to further increases in lymphocyte and monocyte recruitment in the lung.

IL-10 is a regulatory Th2 cytokine that has been shown to prevent acute rejection and OB in the animal models [39]. IL-10 and IL-4 negatively regulate Th1 cytokines IFNγ and IL-2 [23], two cytokines that are increased during acute rejection episodes [2]. MCP-1 has also been shown to upregulate IL-4 [40] and enhance Th2 polarisation [41]. Monocyte production of IL10 and MCP-1 was increased in the present study. There have been previous reports of increased T-cell production of IL-4 in lung transplant patients [11]. Taken together, these findings may explain the previous findings of significantly reduced levels of IFNγ and IL-2 in peripheral blood T cells of lung transplant patients. Increased production of IL-10 may be partially caused by treatment with immunosuppressive drugs as it has previously been shown that monocyte IL-10 production is upregulated in the presence of methylprednisolone [6]. IL-12, a Th1 cytokine that has been shown to be downregulated in the presence of methylprednisolone [6], was unchanged in monocytes from transplant patients compared with control. IL-12 synthesis has been shown to be unaltered in the presence of CsA [42]. As IL-12 is a potent inducer of cell-mediated immunity and IFNγ in T cells [23], drugs that help reduce this important regulatory cytokine may be of benefit for transplant patients. Upregulation of MCP-1 and IL-8 in BAL has recently been shown to be a predictive marker of post-transplant airway obliteration [34] which is consistent with these findings.

As described for T cells, detection of intracellular cytokines in blood monocytes may help to provide a more accurate assessment of immunosuppression than systemic therapeutic drug levels as all patients had drug plasma trough levels within their therapeutic range. The technique is relatively rapid, easy to perform and provides a detailed analysis of

cytokine/chemokine levels in specific leucocyte subtypes in individual patients. The relatively large standard deviation for chemokines MCP-1 and MCP-3 in the transplant patients indicate these patients are a heterogeneous group. It would thus be of interest to serially monitor intracellular cytokines/chemokines from transplant patients with a view to detect shifts in cytokine/chemokine profiles that are indicative of transplant rejection status. These studies may reveal that patients with elevated MCP-1 and MCP-3 progress to transplant rejection earlier than patients with "normal" levels. Drugs that modulate these cytokines/chemokines may improve current protocols for reducing graft rejection in these patients.

Effect of Current Immunosuppression Protocols on Intracellular Pro- and Anti-inflammatory Cytokines in Bronchoalveolar Lavage T Cells of Stable Lung Transplant Patients

Analysis of cells and cytokines in bronchoalveolar lavage (BAL) has previously been used as an indicator of transplant rejection in humans [43, 44] and the canine model [45]. While changes in CD4/CD8 in BAL have been associated with acute and chronic rejection [43, 44], surface phenotyping gives little information regarding the activation state, as assessed by cell-specific cytokine production, of an individual cell. It has previously been shown that analysis of inflammatory cytokines in BAL and blood using ELISA may not be as reliable as analysis of intracellular cytokines using flow cytometry [46].

To overcome these problems and to investigate the immunomodulatory effects of currently used immunosuppressive regimens on BAL T-cell cytokine production, whole blood and BAL from a group of stable lung transplant patients and control volunteers was determined [47]. Results showed that there was no difference in T-cell pro-inflammatory cytokines between blood and BAL compartments in stable lung transplant patients (Table 3). In contrast, the control group showed significantly less pro-inflammatory T-cell cytokine production in BAL compared with blood (Table 4). Although there was no difference in T-cell pro-inflammatory cytokine production between blood and BAL compartments in lung transplant patients there was a significant increase in Th1 cytokines by T cells in the BAL compared to the control group (Figure 4).

Table 3. The percentage of T-cells producing intracellular cytokines in blood and BAL of the 9 stable lung transplant patients (mean ± SD). There was a significant increase in the percentage of T cells producing IL-4 and TGFβ in BAL compared to blood (bold)

	CD3		IFNγ		IL-2		IL-4		TGFβ		TNFα	
	CD4	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**
Blood	55±18	44±18	7±5	17±15	6±5	5±4	1±.1	1±1	4.9±2	5±2.5	12±7	14±12
BAL	44±16	55±16	6±5	13±8	4±3	5±4	5±2	9±6	2±2	2.3±1	9±7	14±11
P	.251	.251	.965	.965	.289	.935	.001	.000	.001	.018	.479	.989

Table 4. The percentage of T-cells producing intracellular cytokines in blood and BAL of the Control group (mean ± SD). There was a significant decrease in the percentage of CD4+ and CD8+ T cells producing IFNγ, IL-2, TGFβ and TNFα and a significant increase in the percentage of CD4+ and CD8+ T cells producing IL-4 in BAL compared to blood (bold)

	CD3		IFNγ		IL-2		IL-4		TGFβ		TNFα	
	CD4	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**
Blood	62±7	37±8	19±5	21±8	36±16	8±4	0.4±.3	.5±.3	5±3	3±2	43±11	24±11
BAL	70±15	29±15	2±2	1±.8	1±1	.5±.4	6±6	3±2.6	1±1	.8±.7	8±5	6±6
P	.141	.141	.000	.000	.000	.010	.003	.024	.008	.006	.000	.000

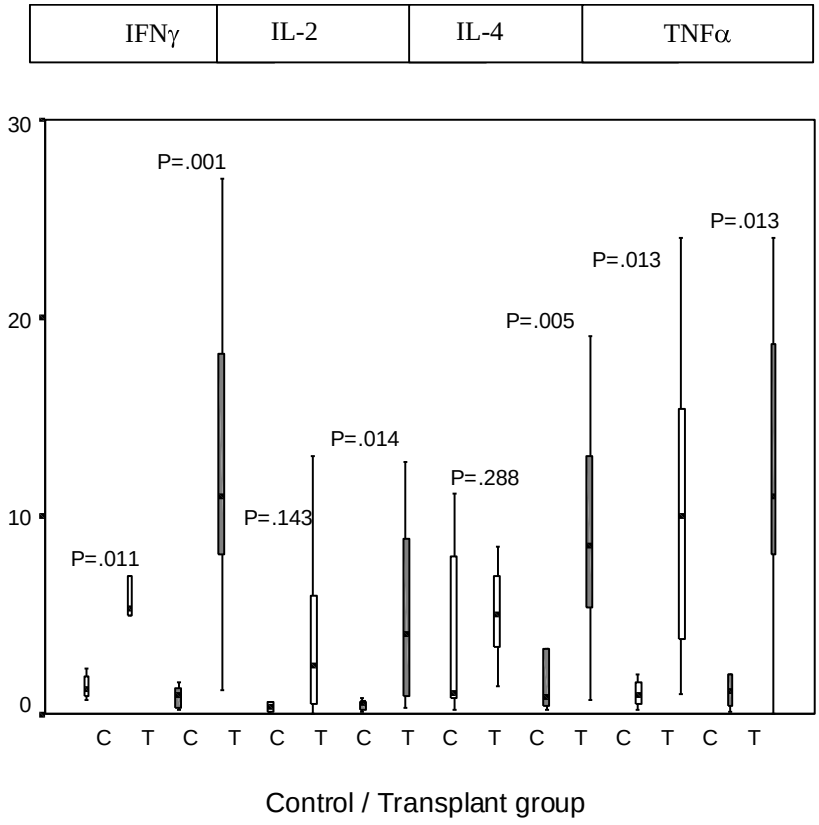

Figure 4. Box plot graphs showing the production of cytokines by BAL CD4+ □and CD8+ ■ T-cells from 9 lung transplant (T) and 10 control (C) subjects following *in vitro* stimulation (mean ± 2SD and range). The percentage of CD8+ T-cells producing IFNγ, IL-2 and IL-4 and the percentage of CD4+ cells producing IFNγ and TNFα was significantly increased in lung transplant patients. The percentage of CD4+ T-cells producing IL2 and IL4 was unchanged in lung transplant patients compared to control.

It has previously been shown that there was a good correlation between the percentage of CD8+ T cells in blood of stable lung transplant patients and IFNγ production by these cells [11]. In contrast, the present study showed that there is a poor correlation between the percentage of CD8+ T cells in BAL and IFNγ production suggesting that previous methods to quantify cytotoxic cells in BAL by immunophenotyping [44, 48] do not give accurate results of functional characteristics of these cytotoxic CD8+ T cells. There was also a significant increase in the anti-inflammatory cytokine, IL-4 in the BAL compared to blood of both the transplant and control group. Representative dot plots showing IL-4 and IFNγ production by BAL CD8+ and CD8- (CD4+) T cells from 2 lung transplant patients and controls are shown in Figure 5.

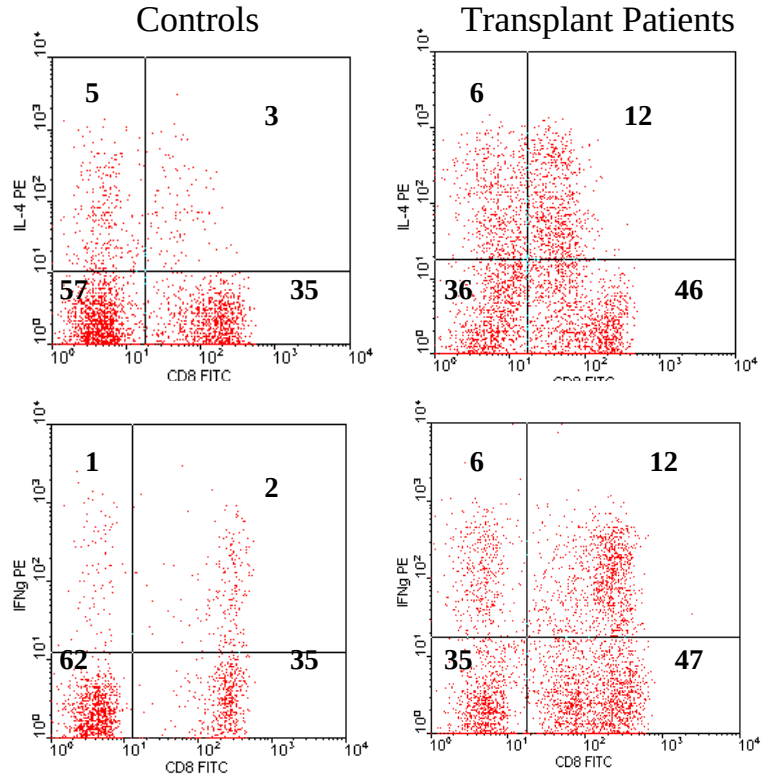

Figure 5. Representative dot plots showing IL-4 and IFNγ production by BAL CD8+ and CD8- (CD4+) T cells from 2 lung transplant patients and controls. T cells were identified by CD3 PC5 versus side scatter characteristics. Transplant patients showed an increase in IL-4 in CD8 dim but not CD8- (CD4+) T cells. Transplant patients showed an increase in IFNγ in both CD4+ and CD8 bright T cells. Note the reduced percentage of CD4+ T cells and increased CD8+ T cells in transplant patients compared to control.

IL-4 is a Th2 cytokine that provides a negative feedback on Th1 cytokine production [23], thus its reduced expression by CD8+ T cells suggests that this regulatory mechanism may be ineffective in stable transplant patients. The Th2 response may be systemic, as the same authors have recently shown increased levels of IL-10, another Th2 cytokine, by monocytes from stable lung transplant patients [36]. The anti-inflammatory cytokine, TGFβ, was also increased in CD8+ T cells in BAL of transplant patients compared with control. TGFβ has previously been shown to inhibit T-cell production of IFNγ and IL-2 [24] suggesting that the function of this regulatory mechanism is also altered in stable transplant patients. The increased sensitivity of CD4+ T cells to TGFβ in reducing Th1 responses compared to CD8+ T cells [25] may help explain the observation of increased inhibition of these cytokines in CD4+ cells compared to CD8+ T cells.

Chronic graft rejection is associated with increased fibrosis in the lung. Both IL-4 [29] and TGFβ [4] have been shown to promote fibroblast proliferation in the lung. Therefore, although TGFβ and IL4 are anti-inflammatory cytokines, their increased production by cytotoxic lymphocytes in the lung may contribute to increased fibrosis (as observed in chronic graft rejection).

This study shows that measurement of blood T-cell cytokine production in lung transplant recipients is reflective of BAL T-cell cytokine production. However, comparison of T-cell cytokine balance between the two compartments may give a more accurate indication of rejection episodes. All transplant patients in this study had plasma levels of CsA or Tac within their therapeutic range, again suggesting that analysis of cytokine production may provide a more accurate assessment of the level of immunosuppression in an individual patient This study also confirms reports that monitoring intracellular T-cell cytokine profiles may be a more appropriate indicator of patient immunosuppression and transplant status than cell phenotypes. Transplant patients were a very heterogeneous group and exhibited a broad range of inflammatory cytokines compared with controls (Figure 1). Effective reduction of T-cell pro-inflammatory cytokines in BAL through targeted use of immunosuppression may improve current protocols for prolonging graft survival in these patients. One such targeted intervention is the use of CsA in aerosolised form. Aerosolised CsA treatment has been used successfully and safely in reducing inflammatory cytokines in refractory acute rejection [49] and this therapy may be of benefit in treating lung transplant patients identified with high percentages of inflammatory cytokine producing T cells whilst minimising systemic side effects of immunosuppressive agents.

Effect of Current Immunosuppression Protocols on Intracellular pro-Inflammatory Cytokines in Bronchial intra-epithelial T Cells of Stable Lung Transplant Patients

Analysis of inflammatory cytokine profiles of intra-epithelial T cells in bronchial brushing (BB) has not previously been performed and may provide additional information to assess immune graft status in lung transplant patients.

A recent study provides the first report of the use of flow cytometry to measure intracellular pro- and anti-inflammatory cytokines in BB-derived intra-epithelial T cells [50]. Results show that although there was a decrease in T-cell pro-inflammatory cytokine production in blood of transplant patients, this was not found in BAL or bronchial intra-epithelial CD8 T-cell subsets, suggesting that the same level of immunosuppression may not occur in the lung of transplant recipients (Table 5 and 6). This study also demonstrated no difference in the percentage of bronchial intra-epithelial CD8 T-cells that produce pro-inflammatory cytokines between stable lung transplant patients and control subjects (Tables 5-8) indicating that current immunosuppression protocols are ineffective at reducing pro-inflammatory T-cell cytokines in the airways of pulmonary allografts.

Table 5. The percentage of T-cells producing intracellular cytokines in blood and BB of 13 lung transplant patients (mean ± SD). There was a significant decrease in the percentage of CD4+ and a significant increase in the percentage of CD8+ T cells in BB (bold). There was a significant increase in the percentage of CD8+ T cells producing IFNγ, IL-4 and TNFα, a significant decrease in the percentage of CD4+ T cells producing IL-2 and CD4+ and CD8+ T cells producing TGFβ in BB compared to blood

	CD3		IFNγ		IL-2		IL-4		TGFβ		TNFα	
	CD4	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**
Blood	53±11	47±11	13±7	26±15	18±13	4±4	1±.8	.9±.4	3.2±2	3±1.7	18±14	16±12
BB	37±11	63±11	17±7	40±13	8±4	3±1.6	1.2±.6	2.3±.8	2±.9	1.7±1	18±7	30±11
P	.002	.002	.085	.026	.042	.300	.179	.043	.039	.028	.738	.002

Table 6. The percentage of T-cells producing intracellular cytokines in BAL and BB of the 13 lung transplant patients (mean ± SD). There was a significant decrease in the percentage of CD4+ and a significant increase in the percentage of CD8+ T cells in BB (bold). There was a significant increase in the percentage of CD4 and CD8+ T cells producing IFNγ and CD8 T cells producing TNFα, and a significant decrease in the percentage of CD4+ and CD8+ T cells producing IL-4 and TGFβ in BB compared to BAL

	CD3		IFNγ		IL-2		IL-4		TGFβ		TNFα	
	CD4	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**
BAL	50±11	50±11	10±9	17±15	5±5	3±3	5±4	5±4	4.6±2	5±2.1	19±10	12±11
BB	37±11	63±11	17±7	40±13	8±4	3±1.6	1.2±1	2.3±2	2±.9	1.7±1	18±7	30±11
P	.006	.006	.029	.001	.100	.210	.002	.025	.035	.028	.657	.002

Table 7.The percentage of T-cells producing intracellular cytokines in blood and BB of 10 control volunteers (mean ± SD). There was a significant decrease in the percentage of CD4+ and a significant increase in the percentage of CD8+ T cells in BB (bold). There was a significant increase in the percentage of CD8+ T cells producing IFNγ and TNFα, a significant decrease in the percentage of CD4+ and CD8+ T cells producing IL-2 in BB compared to blood

	CD3		IFNγ		IL-2		IL-4		TGFβ		TNFα	
	CD4	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**
Blood	69±4.4	31±6.9	18±7.8	20±5	40±17	6±4	.8±.4	.5±.3	4±2	3±2	43±12	20±6
BB	36±19	64±19	15±6	45±17	10±8	3±.2	.5±.3	.9±.7	3±1	1±.3	20±6	37±17
P	.003	.003	.065	.019	.001	.038	.560	.189	.185	.064	.017	.025

Table 8. The percentage of T-cells producing intracellular cytokines in BAL and BB of 10 control volunteers (mean ± SD). There was a significant decrease in the percentage of CD4+ and a significant increase in the percentage of CD8+ T cells in BB (bold). There was a significant increase in the percentage of CD4+ and CD8+ T cells producing IFNγ and IL-2 and CD8+ T cells producing TNFα in BB. There was a significant decrease in the percentage of CD4+ and CD8+ T cells producing IL-4 in BB compared to BAL

	CD3		IFNγ		IL-2		IL-4		TGFβ		TNFα	
	CD4	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**
BAL	71±11	29±11	4±2.7	6±5	3±.8	.8±.7	8.5±2.4	5±2	2±2	.5±.5	14±8	7.5±6
BB	36±19	64±19	15±6	45±17	10±8	3±.2	.5±.3	.9±.7	3±1	1±.3	20±6	37±17
P	.002	.003	.041	.000	.028	.042	.001	.002	.198	.358	.530	.000

Transplant patients showed a CD4:CD8 inversion in BAL and BB consistent with a previous report [51] in contrast to control subjects who showed a CD4:CD8 inversion only in the BB compartment. The relative increase in CD8 T cells in BB may be due to an increase in proliferation of these cytotoxic cells and/or an increase in migration of these cells via specific Th1 chemokine receptors [52]. The percentage of CD8+ T cells producing IFNγ and TNFα in BB was increased compared with blood and BAL.

Representative dot plots showing IFNγ production by CD8+ and CD8- (CD4+) T cells from blood, BAL and BB in a lung transplant patient are shown in Figure 6.

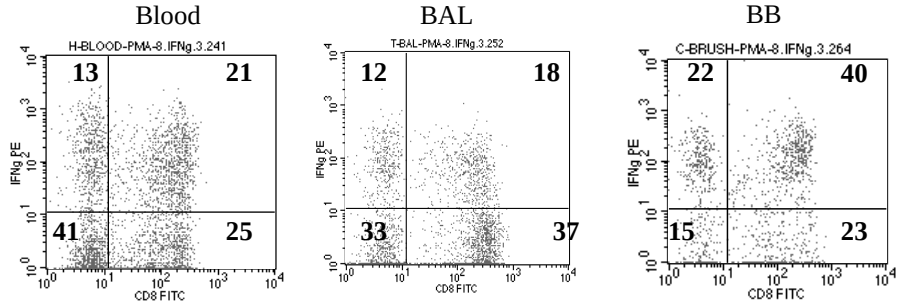

Figure 6. Representative dot plots showing IFNγ production by CD8+ and CD8- (CD4+) T cells from blood, BAL and BB in a lung transplant patient. T cells were identified by CD3 PC5 versus side scatter characteristics. Transplant patients showed an increase in the percentage of CD8+ T cells producing IFNγ in BB compared with blood. Transplant patients showed an increase in IFNγ in both CD4+ and CD8 T cells in BB compared with BAL. Note the decrease in CD4+ and increase in CD8+ T cells in BB compared with blood and BAL.

As IL-4 and TGFβ are negative regulators of Th1 inflammatory cytokines [23, 24], the findings of decreased IL-4 and TGFβ in some bronchial intra-epithelial T-cell subsets compared with blood and BAL may help explain these latter findings. Conversely, chronic rejection or OB is associated with an increase in TGF-β production [4], indicating that current immunosuppression protocols may be effective in reducing OB in lung transplant patients via this pathway.

Others have suggested that studies of intragraft immune cells may be more relevant in terms of effecting graft injury than analysis of peripheral circulating cells [53]. All transplant patients in this study had plasma levels of CsA or Tac within their therapeutic range. Our findings therefore suggest that analysis of T-cell cytokine production may provide a more accurate assessment of immunosuppression in the various compartments than systemic drug levels and show that these patients are inadequately immunosuppressed especially in the lung compartment.

A longitudinal surveillance of cell phenotypes in individuals has been suggested to identify a preclinical state of rejection [48]. Monitoring intracellular T-cell cytokine profiles may be a more appropriate indicator of patient immunosuppression and transplant status than cell phenotypes. This study demonstrates that there is compartmentalisation of pro-and anti-inflammatory CD8+ T-cell cytokine production in BB compared with blood and BAL. Drugs that effectively reduce bronchial intra-epithelial CD8+ T-cell pro-inflammatory cytokines may improve current protocols for prolonging graft survival in these patients.

Airway Infection is Associated with Increased Immunosuppression of Intracellular Th1 Cytokines in Bronchoalveolar Lavage CD8+ T Cells Compared with Stable Lung Transplant Patients

Current immunosuppression protocols to reduce lung transplant rejection include drugs to reduce pro-inflammatory Th1 cytokines. However, Th1 cytokine production is important in host defense against microbial infection in the lungs, particularly *Aspergillus* and *Pseudomonas spp*., organisms shown to be leading causes of mortality in immunocompromised patients [54, 55] Excessive immunosuppression of these cytokines may leave patients susceptible to infection.

Intracellular Th1/Th2 cytokines in BAL and blood T cells from clinically stable lung transplant patients in whom potentially pathogenic organisms were isolated from BAL were compared with a culture-negative group. The predisposing pathology, cultured organism and plasma drug levels in these patients is shown in Table 9.

Table 9. Predisposing pathology and organisms isolated from "culture positive" transplant patient group

Patient	Predisposing pathology	Cultured organism	CsA / Tac levels
1	Bronchiecstasis	*Asp. Pseud.*	Tac 14.5
2	Congenital bronchial webbs	*Pseud.*	Tac 11.4
3	Cystic fibrosis	MRSA. *Pseud*	Tac 9
4	Pulmonary hypertension	*Asp. Pseud.*	CsA 276
5	Cystic Fibrosis	*Asp. Pseud.*	CsA 258
6	Emphysema	*Pseud.*	CsA 300
7	Emphysema	*Pseud.*	CsA 260
8	Emphysema	*Pseud.*	Tac 6
9	Pulmonary fibrosis	*Asp.*	CsA 349
10	Pulmonary hypertension	*E. coli*	CsA 152
11	Cystic fibrosis	*Asp.*	CsA 205
12	Emphysema	*Asp.* MRSA	CsA 235
13	Agammaglobulinaemia	*Pseud.*	CsA 185

Therapeutic range for CsA (80-250 μg/L) and Tac (5-20 μg/L).

Asp. (*Aspergillus spp*.), *Pseud.* (*Pseudomonas spp*.), MRSA (Methicillin resistant *Staphylococcus aureus*), *E. coli* (*Escherichia coli*).

Th1 cytokines were significantly higher in BAL from stable, non-infected transplant recipients compared with culture-positive patients. All transplant patients in this study had plasma levels of CsA or Tac within therapeutic range (Table 9). There was no change in T cell cytokines in the blood of infected and non-infected patients (Table 10). Importantly, the majority of patients with the greatest degree of immunosuppression, as judged by intracellular Th1 cytokine production in BAL, were infected with pathogenic microorganisms (Table 11).

Table 10. The percentage of T-cells producing intracellular cytokines in blood of the 13 BAL culture-negative (N) and 13 culture-positive (P) lung transplant groups (mean ± SD). There were no significant differences in intracellular cytokine production by T-cell subsets between either patient group

	IFNγ			IL-2			IL-4			TNFα		
	CD4	**CD8**	**CD3**	**CD4**	**CD8**	**CD3**	**CD4**	**CD8**	**CD3**	**CD4**	**CD8**	**CD3**
N	10±6	24±17	34±12	14±12	6±5	20±9	1.4±.1	.9±.6	2±4	20±15	15±10	35±12
P	9±6	16±11	25±8	15±14	2.4±2	17±8	.9±.7	1.4±.1	2±4	15±10	18±16	33±12
P	.965	.265	.348	.935	.284	.728	.765	.785	.820	.690	.560	.889

Table 11. The percentage of T-cells producing intracellular cytokines in BAL of the 13 BAL culture-negative (N) and 13 culture-positive (P) lung transplant groups (mean ± SD). There was a significant decrease in the percentage of CD3+ T cells producing IL-2 and CD8+ T cells producing TNFα in BAL of the culture-negative compared to the culture-positive transplant group (bold)

	IFNγ			IL-2			IL-4			TNFα		
	CD4	**CD8**	**CD3**	**CD4**	**CD8**	**CD3**	**CD4**	**CD8**	**CD3**	**CD4**	**CD8**	**CD3**
N	9±6	22±12	31±9	5±4	5±4	10±4	6±4	11±4	17±4	12±9	15±11	27±9
P	7±6	11±8	18±8	2±2	1.7±1.1	3.7±2	6±2	6±5	12±4	12±8	8±6	20±8
P	.765	.165	.248	.218	.104	.031	.898	.157	.657	.890	.038	.200

These organisms have been shown to be the leading cause of mortality in immunocompromised patients [54, 55]. In the mouse model, lung challenge with *P. aeruginosa* resulted in significantly less severe lung pathology, bacterial loads and mortality in mice that responded with a Th1-like response [56]. Transient over-expression of IFNγ within the lungs augmented host immunity against *Aspergillus* [54]. Hence excessive suppression of Th1 cytokines may leave patients susceptible to infection. Although levels of BAL Th1 cytokines differed between patient groups, there was no difference in Th1 cytokines in blood, suggesting that reduced BAL Th1 cytokines were only associated with localised lung infection and not systemic disease.

The data shows that a cut-off value of greater than 8% CD3+ T cells producing IL-2 or 20% CD8+ T cells producing TNFα is associated with culture negative results and hence would be protective of infection in the lungs of these patients. Potent immunosuppressive drugs such as Tac and CsA cause significant toxic side effects [32]. Reducing levels of these drugs in culture-positive patients that have low Th1 cytokines would also have benefits associated with reduced organ toxicity. However, a reduction of immunosuppression due to infection must be balanced with appropriate immunosuppression of proinflammatory Th1 cytokines that have been reportedly increased in the lungs of patients undergoing graft rejection [1-3]. The degree to which transplant recipients are immunosuppressed influences their risk of infection and rejection [58]. The restoration of Th1 responses has been shown to be an important predictor of fungal infection outcome in stem cell transplantation patients [59]. Monitoring the balance of intracellular Th1 cytokines between levels associated with infection and rejection may improve morbidity in our patient group.

In conclusion, this study demonstrates that lung infection is associated with decreased intracellular Th1 cytokines in BAL T cell subsets of stable lung transplant patients. Modifying immunosuppression by monitoring intracellular Th1 cytokines in BAL T cells may improve morbidity and infection rates in this patient group.

Longitudinal Monitoring of Immunosuppression in Transplant Patients by Measurement of Intracellular T Cell Cytokines

Analysis of intracellular T cell cytokines in blood from lung transplant patients and controls showed a broad range of cells producing individual cytokines (Figure 1). To determine longitudinal changes in intracellular cytokines/chemokines in patients and controls, whole blood from several stable, non-infected lung transplant patients and control volunteers was stimulated *in vitro* and intracellular T cell cytokine production determined on several occasions over a three year period. Samples from transplant patients were collected following routine surveillance assessment and histology of bronchial biopsies showed no evidence of acute or chronic rejection. All BAL samples were negative for bacteria, fungi and viruses.

The percentage of T cell subsets producing intracellular cytokines from blood of 5 healthy control (C) volunteers on 3-8 occasions (N) (mean ± SD) are shown in Table 12. The SD of cytokines from individual subjects was significantly less compared with the overall SD of cytokines from 14 control subjects (*overall mean ± SD*), suggesting that intracellular T cell cytokines are relatively stable over time from healthy individual subjects.

Table 12. The percentage of T cell subsets producing intracellular cytokines from blood of 5 healthy control (C) volunteers on 3-8 occasions (N) (mean ± SD). The SD of cytokines from individual subjects was significantly less compared with the overall SD of cytokines from 14 control subjects (*overall mean ± SD*), suggesting that intracellular T cell cytokines are relatively stable over time from healthy individual subjects.

		CD3		IFNγ		IL-2		IL-4		TGFβ		TNFα	
	N	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**
C1	8	61±2	39±2	17±2	25±2	38±3	6±1	.6±.1	.4±.1	4±.8	3±1.1	44±4	26±2
C2	4	64±3	36±2	19±2	20±2	36±3	10±1	.4±.1	.6±.1	6±1.9	2±1.2	42±4	22±3
C3	3	58±2	42±2	23±3	15±2	28±2	8±1	.3±.1	.5±.1	3±.1.1	2±1.0	36±2	24±2
C4	5	60±3	40±2	22±2	22±3	25±2	9±2	.5±.1	.3±.1	7±.1.2	4±1.4	38±3	18±2
C5	3	56±2	34±2	18±2	24±2	37±3	5±2	.4±.1	.4±.1	4±.1.4	5±1.5	50±4	29±2
14C		*62±7*	*37±8*	*19±5*	*21±8*	*36±16*	*8±4*	*0.4±.3*	*.5±.3*	*5±3*	*3±2*	*43±11*	*24±11*

Table 13. The percentage of T cell subsets producing intracellular cytokines from blood of 4 stable non-infected transplant patients (P) on 3-4 occasions (mean ± SD). The SD of cytokines from individual patients was significantly less compared with the overall SD of cytokines from 12 transplant patients (*12P*) (*overall mean ± SD*), suggesting that intracellular T cell cytokines from individual stable transplant patients are relatively stable over time.

		CD3		IFNγ		IL-2		IL-4		TGFβ		TNFα	
	N	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**
P1	4	36±4	64±4	12±3	25±8	8±4	6±2	.6±.2	.9±.2	5±.1.5	6±1.2	9±5	9±6
P2	3	53±2	47±2	10±4	23±11	9±5	5±2	.8±.3	.6±.3	6±2.1	5±1.3	11±6	15±7
P3	3	64±3	36±3	9±1	17±5	6±3	3±1	.5±.3	.8±.1	4±.1.2	7±1.8	15±4	14±5
P4	3	48±2	42±2	7±2	16±6	25±2	7±2	.5±.1	.7±.3	7±.1.9	4±1.1	12±5	18±4
12P		*55±18*	*44±18*	*7±5*	*17±15*	*6±5*	*5±4*	*1±.1*	*1±1*	*4.9±2*	*5±2.5*	*12±7*	*14±12*

Table 14. T cell subsets and the percentage of intracellular blood T cell cytokines from a stable transplant patient (S) on three occasions (mean ± SD) and on one occasion during an episode of acute rejection (AR). There was no significant change in CD4+ or CD8+ T cell subsets or cytokine production during the acute rejection episode

	CD3		IFNγ		IL-2		IL-4		TNFα	
	CD4	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**	**CD4**	**CD8**
S	53±2	47±2	12±4	23±8	9±4	3±1	.8±.3	1.1±.2	11±6	15±8
AR	60	40	10	15	2	2	.6	.9	2	2

The percentage of T cell subsets producing intracellular cytokines from blood of 4 stable non-infected transplant patients (P) on 3-4 occasions (mean ± SD) are shown in Table 13. The SD of cytokines from individual patients was significantly less compared with the overall SD of cytokines from 12 transplant patients (*12P*) (*overall mean ± SD*), suggesting that intracellular T cell cytokines from individual stable transplant patients are relatively stable over time.

Analysis of intracellular T cell cytokines in BAL and BB from lung transplant patients and controls also showed a broad range of cells producing individual cytokines (Table 3 and 4). Intracellular cytokine analyses were performed on one stable, non-infected transplant patient on three occasions and again during one occasion when the patient was undergoing acute rejection as determined by bronchial biopsy histology (A2B0). Results of the intracellular T cell cytokines from blood, BAL and BB from this patient are shown in Table 14, Figure 7 and 8 respectively.

The results from this study show that during an episode of acute rejection, there was a change in T cell subsets and intracellular cytokines in the blood of patients. There were significant increases in pro-inflammatory Th1 cytokines in BAL T cells and IL-2 production by T cells in BB. These results suggest that immunosuppression therapy may be effective in the blood compartment but not in the lungs during an episode of rejection. These data suggest that analysis of intracellular cytokines in the lung compartment, particularly in BAL T cells may be an effective, relatively non-invasive technique in the diagnosis of acute rejection episodes in lung transplant patients. Although caution must be taken when interpreting results from one patient, it will be of great interest to follow the results of these longitudinal studies on a larger cohort of lung transplant patients.

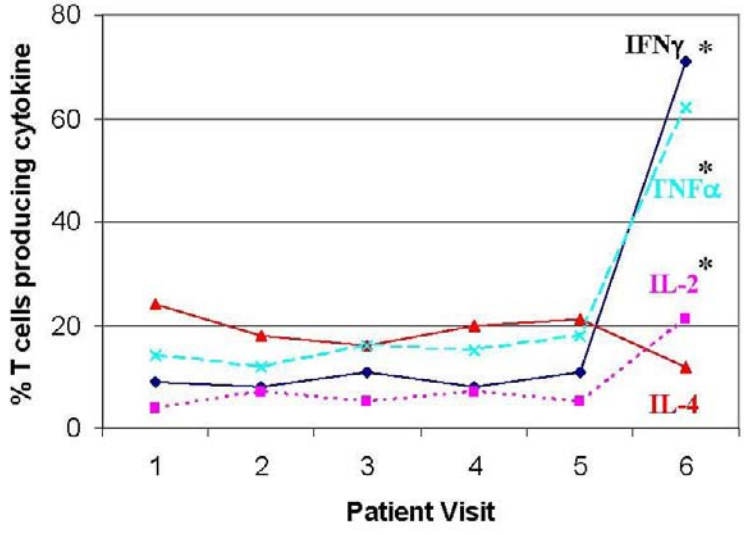

Figure 7. T cell subsets and the percentage of intracellular BAL T cell cytokines from a stable transplant patient (S) on five occasions and on one occasion during an episode of acute rejection (AR). There was a significant increase in IFNγ, IL-2 and TNFα by T cells during the acute rejection episode* (patient visit 6) but no change in IL-4 production.

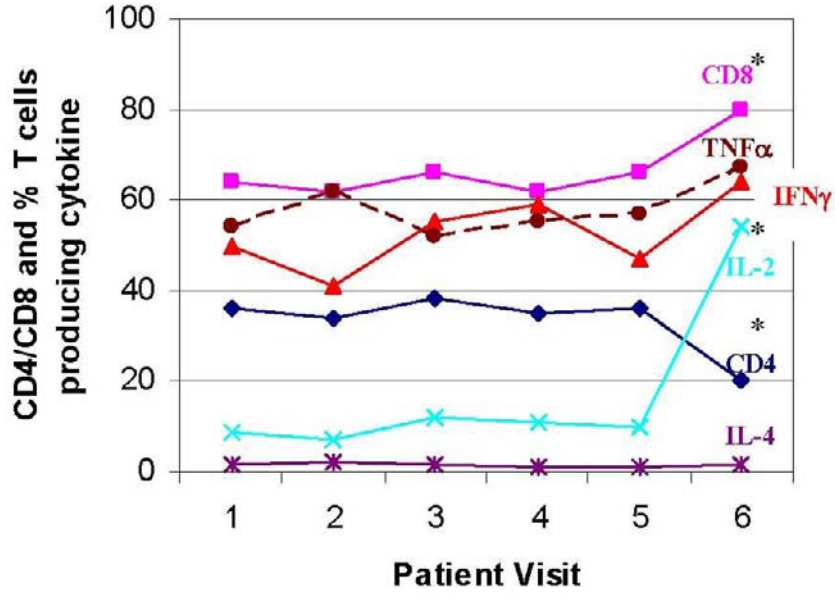

Figure 8. T cell subsets and the percentage of intracellular BB T cell cytokines from a stable transplant patient (S) on five occasions and on one occasion during an episode of acute rejection (AR). There was a significant decrease in CD4, increase in CD8 and a significant increase in IL-2 by T cells during the acute rejection episode* (patient visit 6).

Analysis of Intracellular Cytokines to Improve Therapeutic Immunosuppression Monitoring Following Lung Transplantation Now and in the Future

Assessment of immune effector cell activation state by measurement of cell-specific cytokine production across multiple immune cell subsets within the blood may provide physiological evidence of systemic levels of immunosuppression that may be more relevant than traditional therapeutic drug monitoring. Although direct examination of biopsy tissue still provides the "gold-standard" measure of allograft rejection, it is likely that analysis of intracellular cytokine expression within (assessed using bronchial brushing) or immediately adjacent to the graft (BAL) will also be of diagnostic and prognostic value. In future the clinical utility of flow cytometric evaluation of the pulmonary allograft may even extend beyond assessment of immune effector cell activation state and cell-specific cytokine production. In preliminary studies we have been able to assess protein expression by allograft epithelial cells obtained from bronchial brushings [60]. This technique may provide early information regarding impending airway fibrosis and obliteration associated with BOS. It may even be possible to adapt this technique to better assess the poorly defined entity of pulmonary allograft humoral rejection by looking for the footprint of complement activation – C4d staining [61].

Conclusion

Longitudinal monitoring of immune effector cell activation state in BAL, bronchial brushing and blood in individual patients by measuring cell-specific cytokine production may provide an early warning of impending rejection or infection. Maintaining immune effector intracellular cytokines within stable (non-infection, non-rejection) levels by regulating doses of immunosuppressant drugs may lead to reduced drug toxicity without compromising allograft function. Additionally, a considerable morbidity advantage and cost savings may be found in reduced drug costs and reduced costs associated with treatment of infection due to unnecessary immunosuppression. Although individualized monitoring of immunosuppression using intracellular cytokines as opposed to therapeutic drug monitoring is very promising, further research is required before it is likely to become of proven diagnostic utility in clinical lung transplantation.

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Chapter 8

Measurement of Intracellular Cytokines to Improve Therapeutic Monitoring of Immunosuppressive Drugs Following Lung Transplantation

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Abstract

Lung transplantation has become established therapy in the treatment of selected patients with end stage lung diseases. However, five year survival after lung transplantation is little better than 50%, largely due to chronic graft failure. The basis of this failure is poorly understood but chronic rejection is probably a major factor. At the cellular level, graft rejection is associated with an increase in graft T-cell infiltration, alveolar macrophages, and pro-inflammatory cytokine expression. Although most effective transplantation immunosuppressive strategies are based on interruption of IL-2 signaling by calcineurin inhibitors, Cyclosporin A (CsA) and Tacrolimus (Tac), intensification of immuno-supressive therapies has not lead to any improvement in chronic graft failure. In addition, treatment with these drugs is associated with serious adverse side effects including specific organ toxicities, susceptibility to infections and an increased risk of developing a range of malignancies. Pharmacokinetic properties of both drugs show high inter- and intra-individual variability which may mean some patients do not require the high levels of drugs (that cause adverse side effects) for effective therapeutics. With the availability of novel flow cytometric techniques, recent research has focused on the measurement of inflammatory cytokines at the cellular level as a strategy to assess the physiological response to treatment. Importantly, cytokine levels in both peripheral blood and in the airways have been investigated, which has highlighted important differences in responses seen locally versus systemically. These techniques

may complement or ultimately replace current standard approaches which rely on the measurement of plasma drug levels and monitoring by invasive biopsy. The application of these techniques has the potential to improve current immunosuppression protocols, optimise individual therapy and possibly provide new therapeutic options to improve the morbidity of lung transplant patients.

Introduction

Lung transplantation has become established therapy in the treatment of selected patients with end stage lung disease due to a variety of causes. However, five year survival after lung transplantation in many series is little better than 50%, clearly a dissapointing statistic given the huge expenses involved in lung transplatation and post-transplant care, and the critical shortage of donor organs. The principle reason for graft failure is believed to be chronic rejection, a problem which is seen much more frequently in transplanted lungs than in other solid organ transplants. Clinically, chronic graft failure is manifested by progressive airways obstruction (fall in forced expiratory volume in one second, FEV1) and worsening shortness of breath. The clinical syndrome is referred to as "bronchiolitis obliterans syndrome" (BOS). In many cases, there is a defined pathological correlate, "obliterative bronchiolitis" (OB), although this pattern is not seen in all subjects with the clinical syndrome. Histologically, OB is characterised by fibro-proliferative obstruction and obliteration of small airways [1].

Although the precise pathogenesis of chronic graft failure is unclear, there are certain recognised associations. The strongest risk factor is acute rejection; with early and repeated episodes of acute rejection significantly increasing the risk of subsequent OB. This association clearly suggests that imuune mechanisms may in part underly OB and have thus lead to the hypothesis that the disorder is a form of chronic rejection. However, no clear association with, for example, HLA mismatching has been proven. Further, many patients with acute rejection do not develop OB, and some patients with OB have never experienced clinically detected acute rejection. Several non-immune associations also exist, including variable associations with pulmonary infection. In some studies the closest association seems to be with CMV infection, and there is also evidence that CMV prophylaxis reduces subsequent OB. However, this association remains controversial. Other factors that have been proposed are pulmonary ischemia, gastro-oesophageal reflux disease (and micro-aspiration) and several less well-supported associations including donor asthma and when primary pulmonary hypertension is the recipient disease. In summary, OB appears to represent a final common pathway of response to a variety of noxious insults. This heterogeneity of associations has thus made it difficult to determine a single treatment strategy that will benefit all patients.

Allograft Rejection

Allograft rejection is mediated primarily by T cells and antigen presenting cells, with B cells playing a role via antibody production. Acute cellular rejection involves recipient T cell recognition of HLA molecules expressed on donor-derived, antigen presenting cells (direct

allorecognition) or presentation of donor derived peptides by recipient antigen-presenting cells to recipient T cells (indirect allorecognition). Once the alloantigens are recognised as foreign, the activation and production of cytokines by T lymphocytes, monocytes and other immune cells lead to the amplification of the alloimmune response.

At the cellular level, acute graft rejection is associated with a marked increase in graft T-cell infiltration, alveolar macrophages, and pro-inflammatory cytokine expression including IL-2, IFN-γ and TNF-α [2].

OB is also associated with a moderate increase in graft airway T-cell infiltration, alveolar macrophages, and pro-inflammatory cytokine expression [3, 4]. An additional feature is airway fibrosis, which may be due to the presence of pro-fibrotic chemokines/cytokines such as MCP-1, IL-4 and TGFβ [4]. Pharmacological immunosuppression is required to prevent the allo-immune response to the transplanted lungs. Immunosuppression has improved graft survival but leaves patients susceptible to infectious complications, of which pulmonary infections are the leading cause of morbidity and mortality. Although immunosuppressants used to prevent and treat rejection involve several classes of drugs, many target the production of pro-inflammatory cytokine by T cells, monocytes and other immune cells.

Immunosuppressants

Corticosteroids

Glucocorticoids were the first immunosuppressants used in transplantation. Although glucocorticoids are potent, they are the least selective agents and affect multiple cell lines, including T and B cells, monocytes, macrophages and neutrophils. In lymphocytes and monocytes, glucocorticoids exert negative regulatory effects on cytokine gene expression by directly inhibiting two transcription factors: activator protein-1 and nuclear factor-κB [5].

Calcineurin Inhibitors: Cyclosporin A (CsA) and Tacrolimus (Tac)

The calcineurin inhibitors CsA and Tac have greatly decreased the incidence of allograft rejection. As they are more T-cell selective, their use has helped preserve other cell lines and has reduced the overall incidence of infection by facilitating the lowering of corticosteroid doses [6]. These immunosuppressants inhibit T-cell activation by binding to intracellular immunophilins. Both CsA and Tac inhibit production of IL-1β, IL-2, IL-6, IL-8, IFNγ and TNFα. Tac preferentially suppresses T-helper-type-1 (Th1) cells over Th2 cells [5].

Both agents have been shown to upregulate TGFβ, which although having several immunosuppressive properties on pro-inflammatory cytokines, promotes matrix formation and may contribute to allograft fibrosis. This effect may play a role in chronic rejection. Thus by inhibiting T-cell activation, proliferation and cytokine production, the calcineurin inhibitors are potent immunosuppressants.

Antimetabolites: Azathioprine (AZA) and Mycophenolate Mofetil (MMF)

AZA and MMF are antimetabolites that inhibit the production of purine which is required for T and B-cell activation and proliferation. AZA also acts by inhibiting CD28 costimulation [7] while MMF inhibits glycostlation of leucocyte adhesion molecules, thereby decreasing recruitment of lymphocytes and monocytes to areas of inflammation and reduces cytokine production through inhibition of clonal expansion [7, 8].

Other agents such as sirolomis and everolomis inhibit mRNA responsible for cell cycle progression thus blocking IL-2 postreceptor signalling and preventing T-cell proliferation [7].

Anti-Lymphocyte Antibodies

Polyclonal anti-lymphocyte antibodies have a long history of use for induction of immuno-suppression and in the treatment of acute rejection, targeting different cell lines such as lymphocytes, thymocytes or specific cell lines. For example, OKT3 or Muromonab-CD3 is a murine monoclonal anti-lymphocyte antibody directed against the epsilon unit of the T-cell receptor. Binding causes T-cell opsinisation and removal by mononuclear phagocytes [7].

Anti-Cytokine Receptor Antibodies

Anti-cytokine receptor monclonal antibodies directed against the α-chains of the CD25 molecule, a key unit of the IL-2 receptor, have also been used successfully in the transplant setting due to the central role of IL-2 in regulating T-cell activation, differentiation and apoptosis.

Standard immunosuppression therapy usually comprises combinations of either cyclosporin A (CsA) or tacrolimus (Tac) with prednisolone and azathioprine. Trough plasma drug levels of either CsA or Tac are kept within recommended therapeutic ranges (range for CsA (80-250 μg/L) and Tac (5-20 μg/L)). Therapeutic drug plasma concentrations however are broad and may vary according to the clinical situation and the choice of immunosuppressive agents being given. Recent data supports the use of therapeutic monitoring of CsA by measurment of the blood concentration two hours after administration of the dose with data showing better prediction of clinical effects [9]. Nevertheless, therapeutic drug monitoring has certain limitations with blood concentrations not necessarily reflecting the concentration of drug at the site of action. In addition, the combined therapeutic effect of all drugs used should be assessed for each patient. An alternative approach has recently been suggested [10].

Measurement of Inflammatory Cytokines in Transplant Patients

There are three potential sites from which cells or fluid can can be assessed for cytokine analysis in transplant patients; blood, the lung airways and within the allograft itself. For routine diagnostic purposes, assays based on peripheral blood would be advantageous.

Previous methodology to measure cytokine levels in transplant patients include ELISA quantification from serum and peripheral blood mononuclear cell culture [11, 12] and RTPCR of cytokine mRNA levels [13]. Measurement of soluble cytokines by ELISA or more recently by cytometric bead arrays have proven useful for diagnosis in several disease states [14, 15]. However, cytokines bind to proteoglycan components of the cell surface or extramedullary matrix [16] and have a very limited availability within the blood, effectively limiting the prognostic value of assay of soluble cytokines. Standard techniques such as ELISA give no indication of cytokine-producing cell types and is time consuming and expensive if several cytokines are quantified. Cell purification techniques lead to loss of specific cell subsets [17] and increased apoptosis of cells [18]. While RTPCR is a sensitive technique, results depend on purification of cells from heterogeneous cell populations and are subject to technical error.

The production of cytokines by individual immune cell types can be assessed using a range of techniques. These assays require cell activation proir to analysis. In the case of T cells this is accomplished by polyclonal stimulation with mitogens active on the cell surface or within the cell or by antigenic stimulation. In the case of alloreactive cells, when the frequency of the responding cells is relatively high, activation by alloantigen presentation can provide a useful technique to assess the potential to mount an antigraft immune response using ELISPOT [19] or limiting dilution analysis [20]. Following polyclonal stimulation, multiparameter flow cytometry offers a powerful tool to analyse multiple pro- and anti-inflammatory intracellular cytokines in thousands of cells by individual cell types.

Measurement of T-cell intracellular cytokines has been used to investigate differences in several clinical situations such as between cord and adult blood in the context of graft versus host disease [21] and effects of other therapeutics [22]. This approach has also been used in preliminary studies in lung transplant patients.

Effect of Immunosuppression Protocols on Intracellular Cytokines in Blood T Cells

It is currently unknown if levels of immunosuppressive drugs correlate with pro-inflammatory cytokine expression in peripheral blood T cells. Lymphocytes are known to traffic from the blood stream to the lung and later rejoin the peripheral circulation [23] suggesting that measurement of blood T cell cytokines may be reflective of graft infiltrating T-cell cytokine profiles.

To investigate the immunomodulatory effects of currently used immunosuppressive regimes, whole blood from a group of 9 stable, non-infected lung transplant patients (9 patients, 13 episodes) and 15 control volunteers was stimulated *in vitro* and intracellular T cell cytokine production determined using multiparameter flow cytometry [10]. This is the

first report of intracellular pro- and anti-inflammatory cytokines in peripheral blood T cells from lung transplant patients and provides important new information regarding the immunosuppressive effect of current drug protocols in these patients. We found evidence that current immunosuppression protocols have a significant immunosuppressive effect on pro-inflammatory cytokine production by peripheral blood CD4+ T cells consistent with therapeutic intention. However, there was a limited effect on peripheral blood CD8+ T-cells, particularly IFNγ inflammatory cytokine production in lung transplant patients (figure 1).

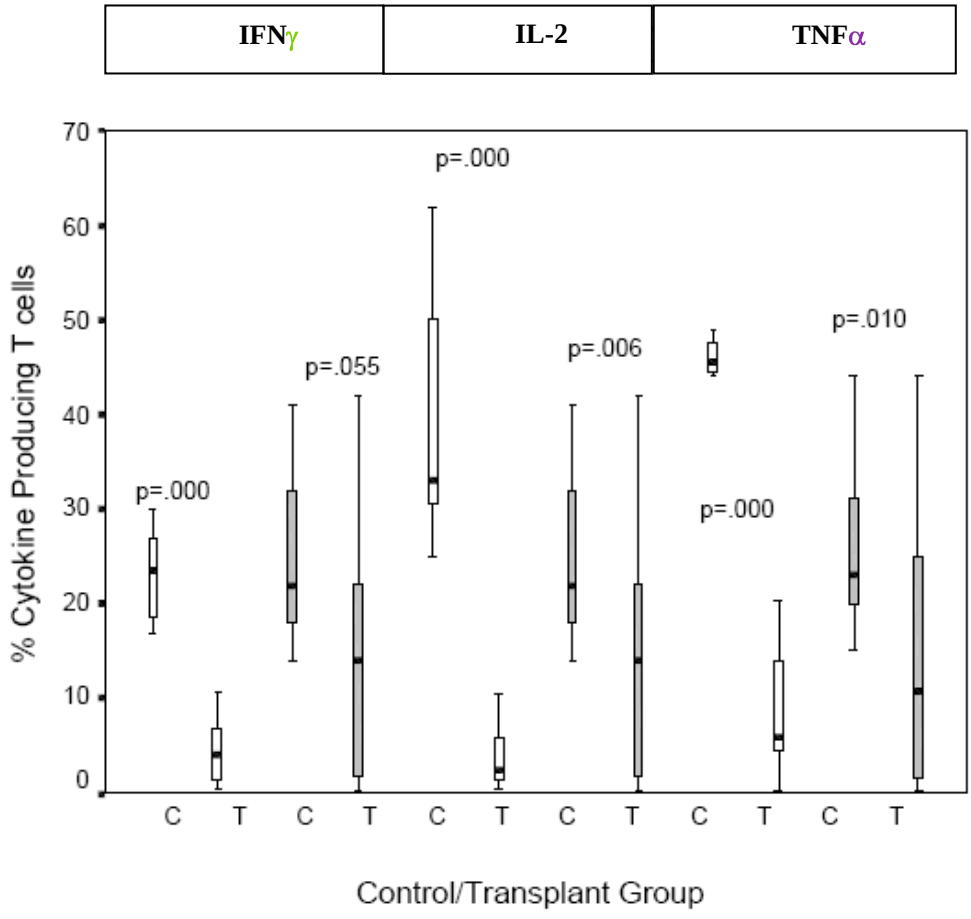


Figure 1. Box plot graphs showing the production of pro-inflammatory cytokines by CD4+ (clear bars) and CD8+ T-cells (grey bars) from lung transplant (T) and control (C) subjects following *in vitro* stimulation (mean ± SD and range). The percentage of CD4+ and CD8+ T-cells producing IL-2 and TNFα was significantly reduced from lung transplant patients compared to control. The percentage of CD4+ T-cells producing IFNγ was also significantly reduced from lung transplant patients but not the percentage of CD8+ T cells producing IFNγ. Note the marked inhibition of inflammatory T-cell cytokines in CD4+ cells compared with CD8+ cells in transplant patients compared to control group.

All transplant patients in this study had plasma levels of CsA or Tac within their therapeutic ranges suggesting that analysis of cytokine production may provide a more accurate assessment of immunosuppression than drug levels.

In contrast to these results [10] a recent report failed to show any differences between inflammatory T-cell cytokines in stable renal transplant patients and controls [24]. However, this study did not distinguish between CD8+ and CD8- T-cell cytokine production while our results showed that immunosuppression protocols are adequate for CD8- (CD4+) but not CD8+ T-cell inflammatory cytokines, particularly IFNγ. We also showed that current immunosuppression agents increased anti-inflammatory cytokine production of IL-4 and TGFβ by T cells from lung transplant patients compared to controls (table 1).

Table 1. The percentage of T cell subsets producing anti-inflammatory cytokines, IL-4 and TGFβ from 9 lung transplant patients (T) compared with 15 control subjects (C) (mean ± SD). There was a significant increase in IL-4 by both T-cell subsets and TGFβ by CD8+ T-cell subsets by tranplant patients compared with control subjects (bold)

	IL-4		TGFβ	
	CD4	CD8	CD4	CD8
C	0.4±0.3	0.0±0.0	5.3±3.2	3.0±2.6
T	1.0±0.8	1.4±0.0	4.9±3.1	4.6±2.4
P	**.019**	**.010**	.890	**.017**

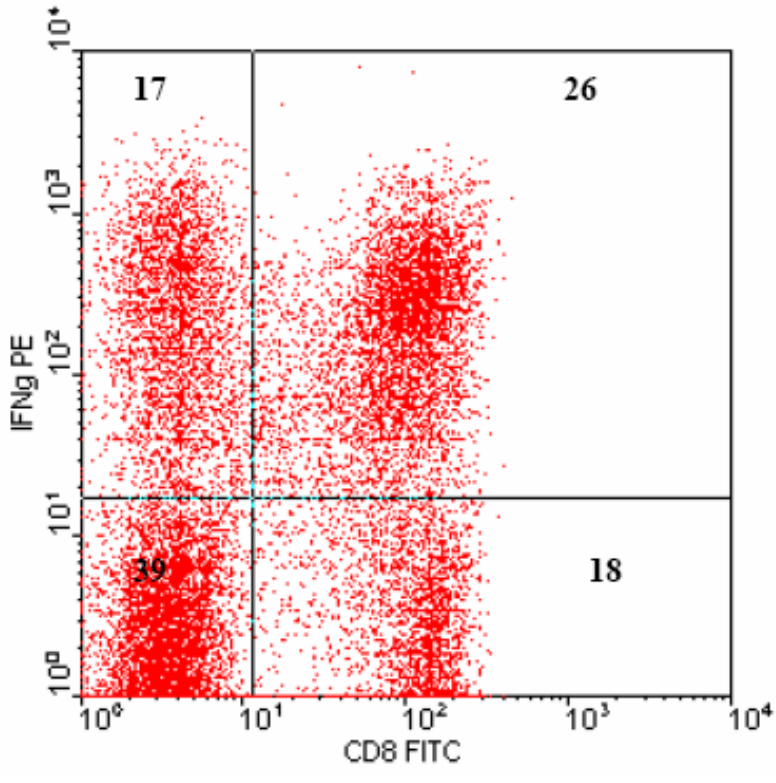
IL-4 negatively regulates Th1 cytokines IFNγ and IL-2 [25] that have been reportedly increased during acute rejection episodes [13]. TGFβ has also been shown to inhibit IL-2 and IFNγ production by T cells [26]. Thus, these findings of increased IL-4 and TGFβ production by T-cells in lung transplant patients may partially explain the significantly reduced levels of IFNγ and IL-2 in peripheral blood T cells. The increased sensitivity of CD4+ T cells to TGFβ in reducing Th1 responses compared to CD8+ T cells [27] may help explain the observation of increased inhibition of these cytokines in CD4+ cells compared to CD8+ T cells.

The previous reports that IL-2 and IFNγ are inhibited in the presence of methylprednisolone [22, 28] are similar to the reported effects of CsA and Tac [29]. However, although Tac and CsA have been shown to inhibit T-cell IL-4 production in vitro [29], low levels of corticosteroids have previously been shown to be stimulatory for T-cell IL-4 production [30] and may be acting similarly in transplant patients. Nevertheless the net combined effect of CsA or Tac, methylprednisolone and AZA may probably accounts for the significant reduction in these pro-inflammatory T-cell cytokines in transplant patients.

We also found increased absolute numbers of CD8+ T cells in transplant patients which is consistent with a previous report [31]. TGFβ has been shown to be co-stimulatory for CD8+ T cells but not CD4+ T cells [32]. IL-4 enhances the proliferation of precursors of cytotoxic lymphocytes and their differentiation into active cytotoxic CD8+ T cells [33]. In CsA or Tac treated mice, T-cell proliferation was shown to be suppressed in CD4+ but not CD8+ subsets [34]. These findings of increased TGFβ by CD8+T cells and increased IL-4 production by both CD4+ and CD8+ T-cell subsets may therefore be causative factors in the significant increase in cytotoxic T cells in these patients. The relative increase in absolute numbers of CD8+ T cells and excellent correlation between the percentage of CD8+ T cells and the amount of IFNγ being produced by these cytotoxic cells suggests that current immunosuppressive protocols are ineffective at reducing this inflammatory cytokine.

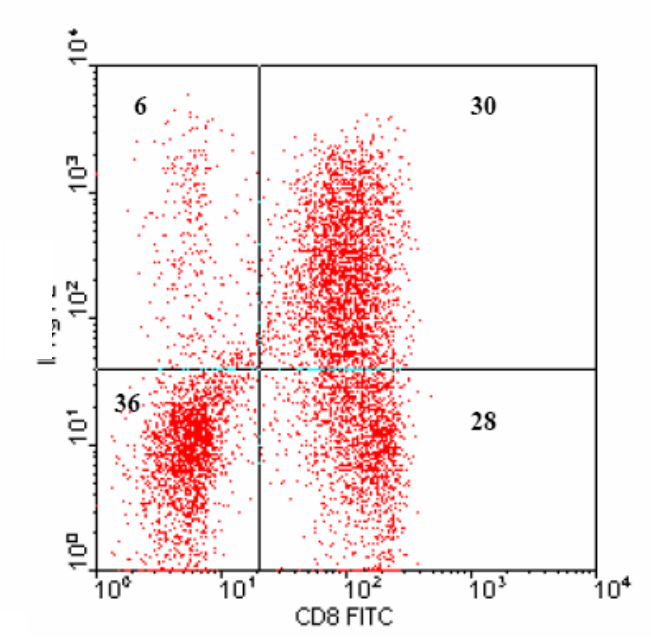
In contrast to acute rejection, chronic graft failure is associated with increased fibrosis in the lung. Both IL-4 [35] and TGFβ [36] have been shown to promote fibroblast proliferation in the lung. Therefore, although TGFβ and IL4 are anti-inflammatory cytokines, their increased production by cytotoxic lymphocytes that migrate to the lung may contribute to increased fibrosis in chronic graft rejection.

Current immunosuppression protocols are not without significant toxic side effects [37]. In an attempt to minimise these side effects a number of low toxicity protocols have been developed [38]. One could hypothesise that if a transplant patient was showing signs of drug toxicity and levels of intracellular T-cell inflammatory cytokines were markedly reduced compared to control (eg., Patient B in figure 2), the dose of drug could be reduced and therapeutic effects monitored or tailored to suit the individual patient. Alternately, if intracellular T-cell inflammatory cytokines were not reduced (eg., CD8 T cells in Patient A, figure 2), other immunosuppressive drugs should be considered.

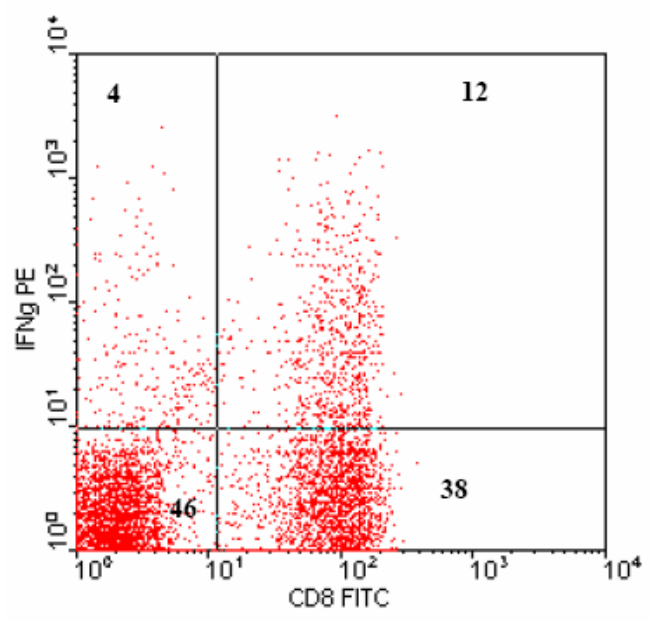


Control

Figure 2. Continued on next page



Patient A



Patient B

Figure 2. Representative dot plots showing the effect of immunosuppression therapy on IFNγ production by CD8+ and CD8- (CD4+) T cells from 2 lung transplant patients and control. T cells were identified by CD3 PC5 versus side scatter characteristics. Patient A shows immunosuppression of IFNγ in CD8- (CD4+) T cells but not CD8+ T cells. Patient B shows immunosuppression of IFNγ in both T-cell subsets. Note the reduced percentage of CD4 T cells and increased CD8 T cells in transplant patients compared to control.

The conclusions drawn from this study were that current immunosuppression protocols have limited effect on peripheral blood IFNγ production by CD8+ T-cells but do upregulate T-cell anti-inflammatory cytokines, TGFβ and IL4. Drugs that effectively reduce IFNγ production by CD8+ T cells may improve current protocols for reducing graft rejection in these patients. Intracellular cytokine analysis using flow cytometry may be a more appropriate indicator of immunosuppression of all therapeutic drugs used than measurement of plasma CsA or Tac levels in these patients. This technique may prove useful in optimising therapy for individual patients.

Effect of Immunosuppression Protocols on Blood Monocyte Intracellular Cytokine and Chemokine Production

Alveolar macrophages are a major source of inflammatory cytokines/chemokines involved in the pathogenesis of lung transplant rejection and are derived from blood monocytes that migrate to the lung. Chemokines are inflammatory mediators that specifically stimulate the directional migration of T cells and monocytes and play an important role in immune cell recruitment into sites of antigenic challenge [39] such as in lung transplant. Chemokines such as IL-8 and MCP-1 have been reportedly increased in BAL of transplant patients undergoing rejection [40]. MCP-1 induces monocyte migration and differentiation to macrophages and plays a pivotal role in BOS [41]. MCP-3 has also been shown to be chemotactic for T cells and monocytes [39] and may also play a role in the pathogenesis of transplant rejection. Thus we investigated monocyte production of cytokine/chemokine mediators in peripheral blood of lung transplant patients [42]. We studied intracellular cytokine/chemokine production by peripheral blood monocytes following stimulation with LPS from a group of 9 stable lung transplant patients and 15 control volunteers using multiparameter flow cytometry. There was a significant increase in the percentage of monocytes producing chemokines MCP-1, MCP-3 and IL-8 and anti-inflammatory cytokine IL-10, but no change in the percentage of monocytes producing IL-6, TNFα, IL-1α, IL-12, MIP-1α, MIP-1β and TGFβ (table 2). Representative histograms showing the increase in the percentage of monocytes producing chemokines MCP-1, MCP-3 from a transplant patient is shown in figure 3.

We found that current immunosuppression protocols have limited effect on peripheral blood monocyte inflammatory cytokine production and are inadequate at suppressing monocyte chemokine production in lung transplant patients. This was the first report of increased MCP-3 in lung transplant patients. MCP-3 has been shown to be a mediator in the activation of extracellular matrix gene expression in addition to promoting leucocyte trafficking in systemic sclerosis [43]. Monocytes migrating to the lung may be acting similarly in lung transplant patients. Interestingly, it has been shown that the effects of MCP-3 can be diminished by neutralising antibody to TGFβ [43]. As TGFβ has been reported to play a major role in OB, the possible role of TGFβ – MCP-3 mediated effects clearly warrants further study.

Table 2. Monocyte cytokine/chemokine production in 9 lung transplant and 15 control subjects (% positive cells) following LPS stimulation. The percentage of monocytes producing IL-8, IL-10 and MCP-1 and MCP-3 was significantly increased in lung transplant patients (bold) but levels of IL-6, TNFα, IL-1α, IL-12, MIP-1α, MIP-1β and TGFβ were unchanged

		IL-8	IL-6	TNFα	IL-10	IL-1α	IL-12	MCP-1	MCP-3	MIP1α	MIP1β	TGFβ
Controls	Mean	82.4	72.9	82.7	4.3	79.5	24.7	11.4	5.5	13.6	71.6	6.2
	SD	7.6	20.2	7.2	1.5	13.1	9.3	5.7	3.1	11.4	15.9	3.1
Patients	Mean	92.3	65.1	82.1	8.8	73.9	20.1	28.8	18.9	13.9	72.2	5.5
	SD	6.6	21.8	8.2	3.7	16.6	12.3	16.9	16.3	9.1	23.1	2.6
	***P* =**	**.039**	.731	.612	**.042**	.714	.542	**.006**	**.010**	.875	.788	.468

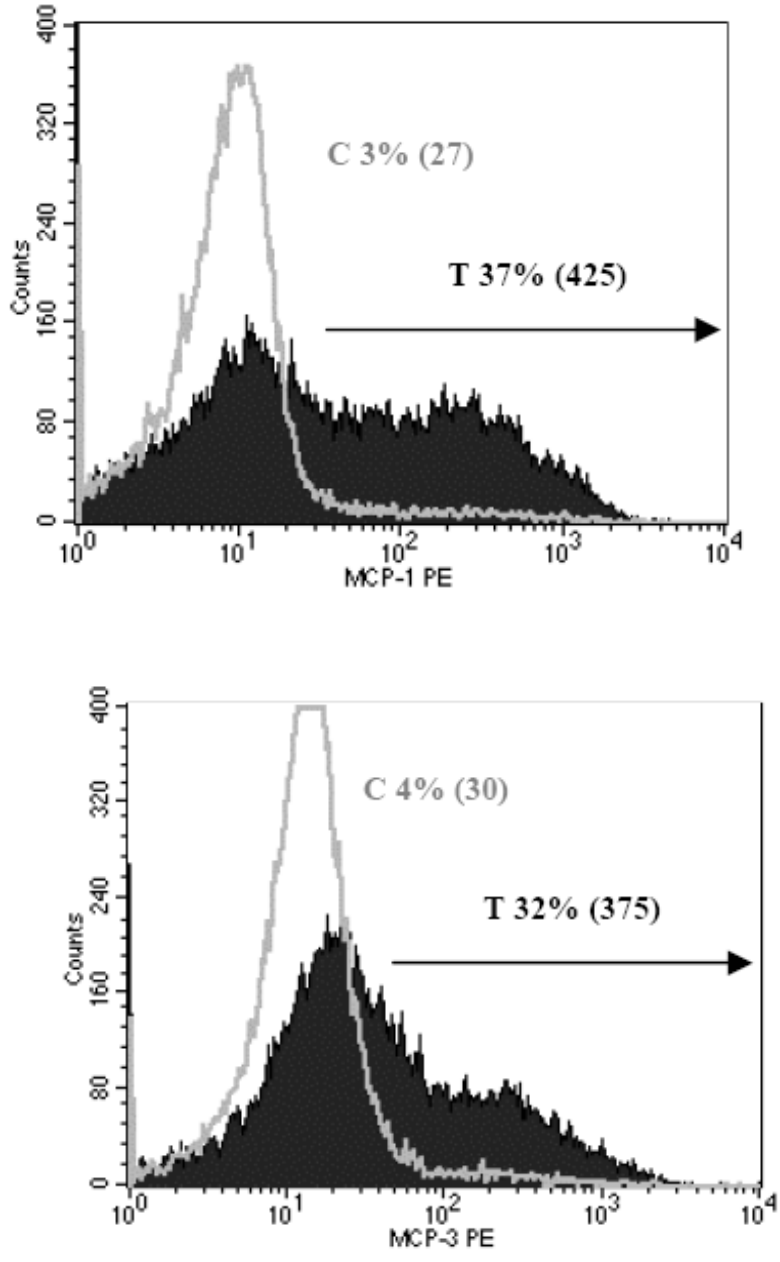


Figure 3. Representative histograms showing intracellular staining of MCP-1 and MCP-3 in peripheral blood monocytes from a transplant patient (T) and control subject (C) following LPS stimulation. The percentage of monocytes producing MCP-1 and MCP-3 was significantly increased in the lung transplant patient compared to control (marker set on negative control-not shown). The amount of MCP-1 and MCP-3 (as indicated by MFI) was also significantly increased in the lung transplant patient compared to control.

MCP-1 levels have been reported to be increased in lung allograft rejection, especially in OB [41]. Alveolar macrophages were identified as the major source of MCP-1. Blood monocytes migrating to the lung are the probable source of these high MCP-1 producers,

indicating that these cells are producing MCP-1 before entering the lung. Interestingly MCP-3 has been shown to be a functional ligand for MCP-1 receptor [44]. The increased levels of monocyte MCP-3 identified in transplant patients may enhance the chemoattractant effects of MCP-1, leading to further increases in lymphocyte and monocyte recruitment in the lung. In the mouse model, treatment with neutralising antibody to MCP-1 reduced mononuclear phagocyte recruitment to the lung and led to an attenuation of OB [41]. These findings suggest that treatments aimed at reducing MCP-3 may also be of benefit at reducing monocyte recruitment in lung transplant patients.

IL-10 is a regulatory Th2 cytokine that has been shown to prevent acute rejection and OB in the animal models [45]. IL-10 and IL-4 negatively regulate Th1 cytokines IFNγ and IL-2 [25], two cytokines that are increased during acute rejection episodes [2]. MCP-1 has also been shown to upregulate IL-4 [46] and enhance Th2 polarisation [47]. Monocyte production of IL10 and MCP-1 was increased in the present study. We have previously reported increased T-cell production of IL-4 in lung transplant patients [10]. Taken together, these findings may explain the previous findings of significantly reduced levels of IFNγ and IL-2 in peripheral blood T cells of lung transplant patients. Increased production of IL-10 may be partially caused by treatment with immunosuppressive drugs as it has previously been shown that monocyte IL-10 production is upregulated in the presence of methylprednisolone [22, 28]. IL-12, a Th1 cytokine that has been shown to be downregulated in the presence of methylprednisolone [28], was unchanged in monocytes from transplant patients compared with control. IL-12 synthesis has been shown to be unaltered in the presence of CsA [48] and downregulated by tacrolimus but only when combined with rhG-CSF treatment [49]. As IL-12 is a potent inducer of cell-mediated immunity and IFNγ in T cells [25], drugs that help reduce this important regulatory cytokine may be of benefit for transplant patients. Upregulation of MCP-1 and IL-8 in BAL has recently been shown to be a predictive marker of post-transplant airway obliteration [40] which is consistent with these findings. Treatment with dexamethasone [22] and pulse methylprednisolone [50] has been shown to be associated with a substantial decrease in monocyte MCP-1 synthesis and may be of benefit in the treatment of lung transplant patients.

As described for T cells, detection of intracellular cytokines in blood monocytes may help to provide a more accurate assessment of immunosuppression than systemic therapeutic drug levels as all patients had drug plasma trough levels within their therapeutic range. The technique is relatively rapid, easy to perform and provides a detailed analysis of cytokine/chemokine levels in specific leucocyte subtypes in individual patients. The relatively large standard deviation for chemokines MCP-1 and MCP-3 in the transplant patients indicate these patients are a heterogeneous group. It would thus be of interest to serially monitor intracellular cytokines/chemokines from transplant patients with a view to detect shifts in cytokine/chemokine profiles that are indicative of transplant rejection status. These studies may reveal that patients with elevated MCP-1 and MCP-3 progress to transplant rejection earlier than patients with “normal” levels. Our studies show that current immunosuppression protocols have limited effect on peripheral blood monocyte inflammatory cytokine production and are inadequate at suppressing monocyte chemokine production. Drugs that modulate these cytokines/chemokines may improve current protocols for reducing graft rejection in these patients.

Increased Intracellular Pro- and Anti-Inflammatory Cytokines in Bronchoalveolar Lavage T Cells of Stable Lung Transplant Patients

Analysis of cells and cytokines in bronchoalveolar lavage (BAL) has previously been used as an indicator of transplant rejection in humans [52, 53] and the canine model [54]. While changes in CD4/CD8 in BAL have been associated with acute and chronic rejection [52, 53], surface phenotyping gives little information regarding cytokine production by cells. It has previously been shown that analysis of inflammatory cytokines in BAL and blood using ELISA may not be as reliable as analysis of intracellular cytokines using flow cytometry [55].

To overcome these problems and to investigate the immunomodulatory effects of currently used immunosuppressive regimens on BAL T-cell cytokine production, whole blood and BAL from 9 stable lung transplant patients and 10 control volunteers were stimulated *in vitro* and cytokine production by CD8+ and CD8- (CD4+) T-cell subsets determined using multiparameter flow cytometry [56]. We showed that there was no difference in T-cell pro-inflammatory cytokines between blood and BAL compartments in stable lung transplant patients (table 3). In contrast, the control group showed significantly less pro-inflammatory T-cell cytokine production in BAL compared with blood (table 4). Although there was no difference in T-cell pro-inflammatory cytokine production between blood and BAL compartments in lung transplant patients there was a significant increase in Th1 cytokines by T cells in the BAL compared to the control group (figure 4).

It has previously been shown that there was a good correlation between the percentage of CD8+ T cells in blood of stable lung transplant patients and IFNγ production by these cells [10]. In contrast, the present study showed that there is a poor correlation between the percentage of CD8+ T cells in BAL and IFNγ production suggesting that previous methods to quantify cytotoxic cells in BAL by immunophenotyping [53, 57] do not give accurate results of functional characteristics of these cytotoxic CD8+ T cells. Interestingly, there was a significant increase in the anti-inflammatory cytokine, IL-4 in the BAL compared to blood of both the transplant and control group. Representative dot plots showing IL-4 and IFNγ production by BAL CD8+ and CD8- (CD4+) T cells from 2 lung transplant patients and controls are shown in figure 5.

Table 3. The percentage of T-cells producing intracellular cytokines in blood and BAL of the 9 stable lung transplant patients (mean ± SD). There was a significant increase in the percentage of T cells producing IL-4 and TGFβ in BAL compared to blood (bold)

	CD3		IFNγ		IL-2		IL-4		TGFβ		TNFα	
	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8
Blood	55±18	44±18	7±5	17±15	6±5	5±4	1±.1	1±1	4.9±2	5±2.5	12±7	14±12
BAL	44±16	55±16	6±5	13±8	4±3	5±4	5±2	9±6	2±2	2.3±1	9±7	14±11
P	.251	.251	.965	.965	.289	.935	**.001**	**.000**	**.001**	**.018**	.479	.989

Table 4.The percentage of T-cells producing intracellular cytokines in blood and BAL of the Control group(mean ± SD). There was a significant decrease in the percentage of CD4+ and CD8+ T cells producing IFNγ, IL-2, TGFβ and TNFα and a significant increase in the percentage of CD4+ and CD8+ T cells producing IL-4 in BAL compared to blood (bold)

	CD3		IFNγ		IL-2		IL-4		TGFβ		TNFα	
	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8
Blood	62±7	37±8	19±5	21±8	36±16	8±4	0.4±..3	.5±.3	5±3	3±2	43±11	24±11
BAL	70±15	29±15	2±2	1±.8	1±1	.5±.4	6±6	3±2.6	1±1	.8±.7	8±5	6±6
P	.141	.141	**.000**	**.000**	**.000**	**.010**	**.003**	**.024**	**.008**	**.006**	**.000**	**.000**

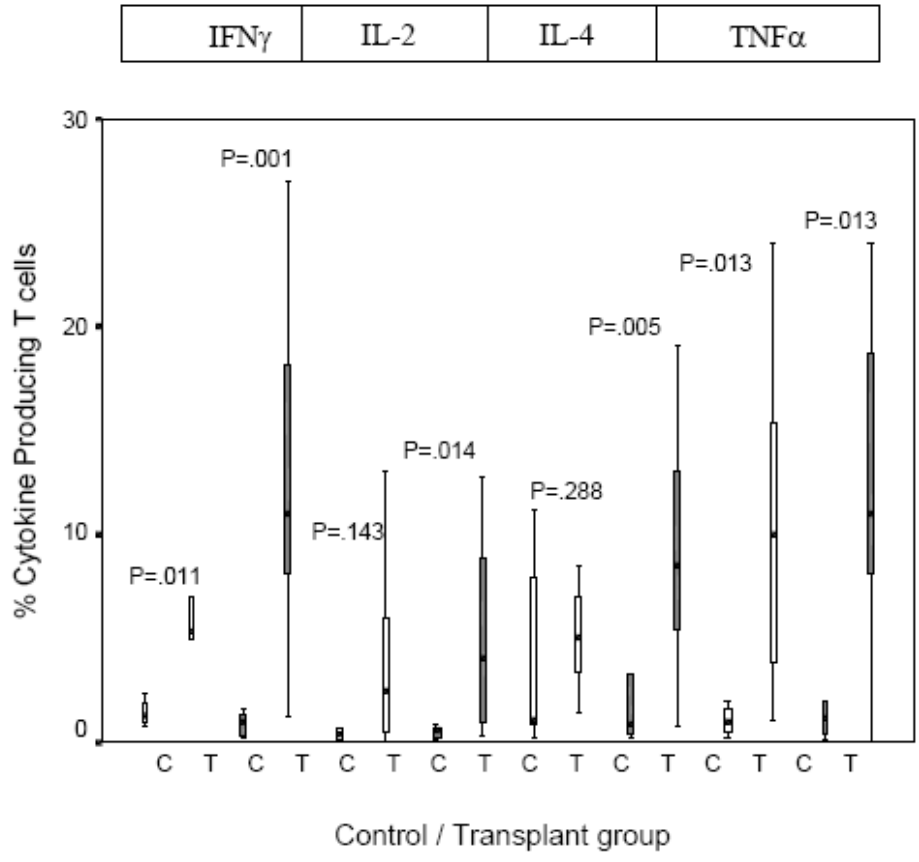
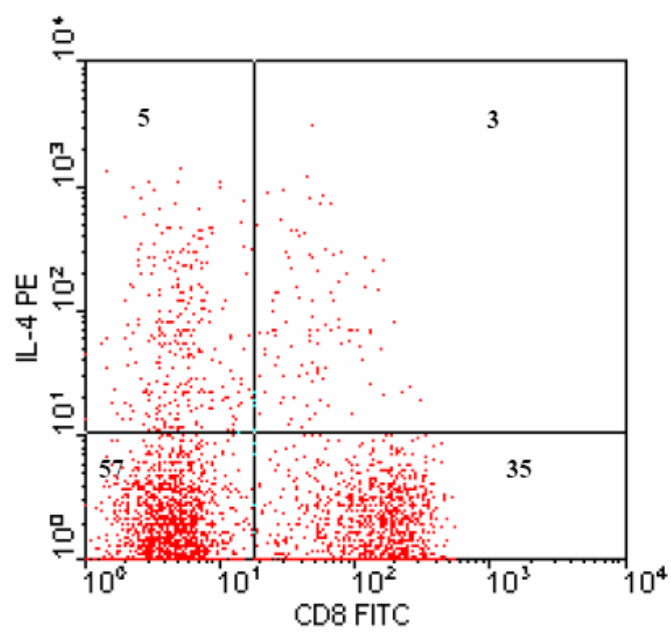
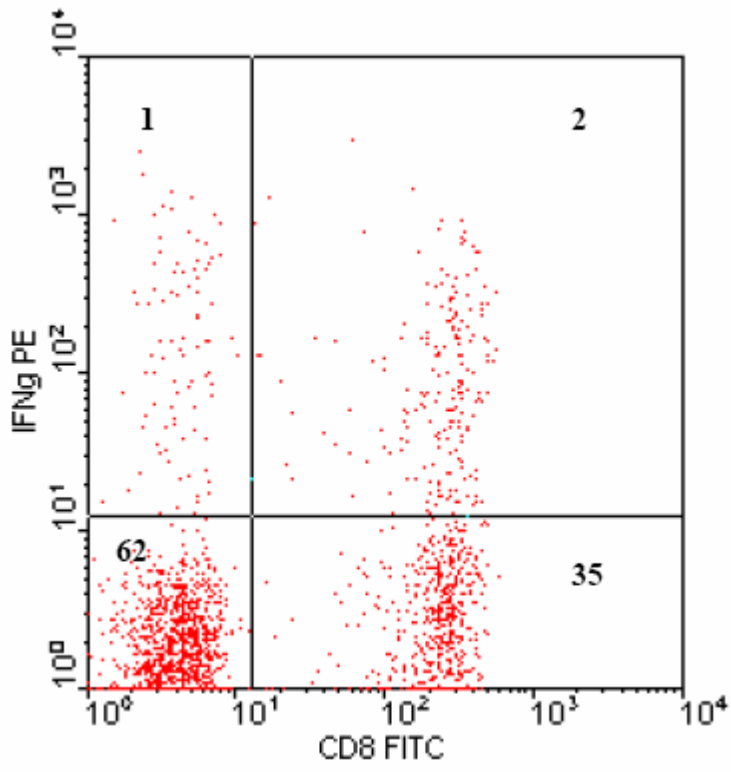


Figure 4. Box plot graphs showing the production of cytokines by BAL CD4+ and CD8+ T-cells from 9 lung transplant (T) and 10 control (C) subjects following *in vitro* stimulation (mean ± 2SD and range). The percentage of CD8+ T-cells producing IFNγ, IL-2 and IL-4 and the percentage of CD4+ cells producing IFNγ and TNFα was significantly increased in lung transplant patients. The percentage of CD4+ T-cells producing IL2 and IL4 was unchanged in lung transplant patients compared to control.

IL-4 is a Th2 cytokine that provides a negative feedback on Th1 cytokine production [25], thus its reduced expression by CD8+ T cells suggests that this regulatory mechanism may be ineffective in stable transplant patients. The Th2 response may be systemic, as the same authors have recently shown increased levels of IL-10, another Th2 cytokine, by monocytes from stable lung transplant patients [42]. The anti-inflammatory cytokine, TGFβ, was also increased in CD8+ T cells in BAL of transplant patients compared with control. TGFβ has previously been shown to inhibit T-cell production of IFNγ and IL-2 [26] suggesting that the function of this regulatory mechanism is also altered in stable transplant patients. The increased sensitivity of CD4+ T cells to TGFβ in reducing Th1 responses compared to CD8+ T cells [27] may help explain the observation of increased inhibition of these cytokines in CD4+ cells compared to CD8+ T cells.

Controls





Transplant

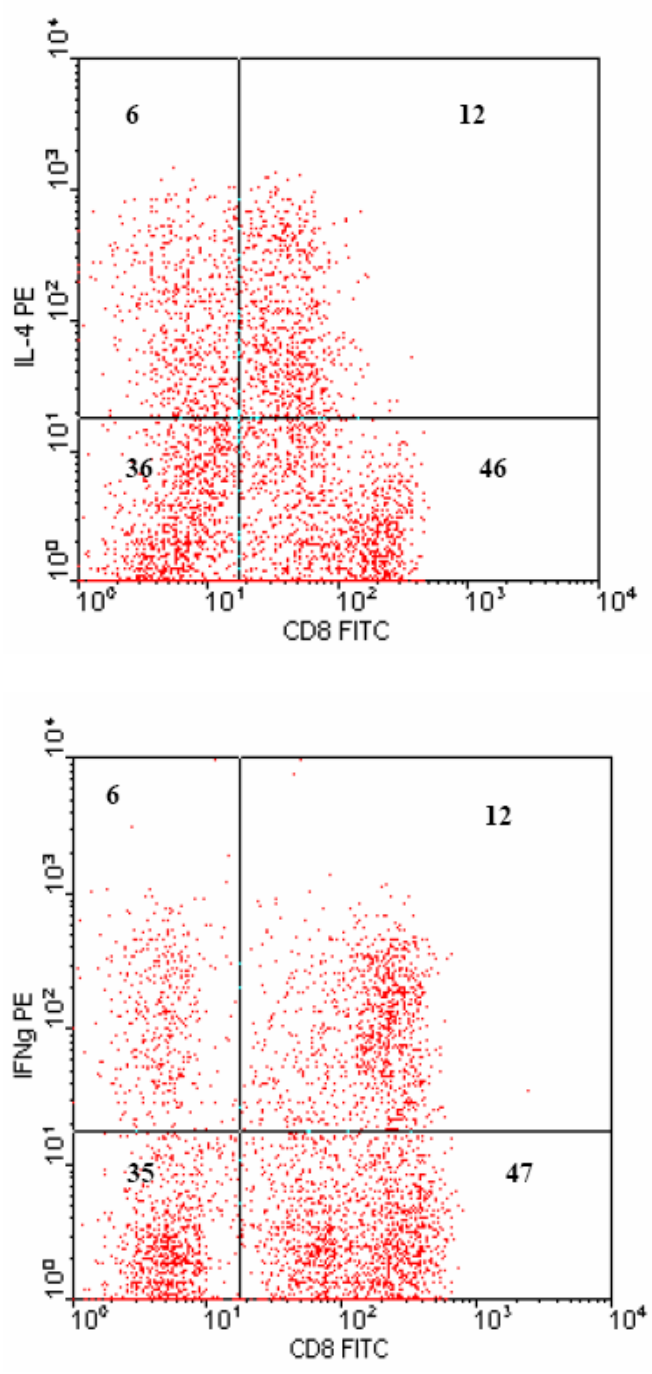


Figure 5. Representative dot plots showing IL-4 and IFNγ production by BAL CD8+ and CD8- (CD4+) T cells from 2 lung transplant patients and controls. T cells were identified by CD3 PC5 versus side scatter characteristics. Transplant patients showed an increase in IL-4 in CD8 dim but not CD8- (CD4+) T cells. Transplant patients showed an increase in IFNγ in both CD4+ and CD8 bright T cells. Note the reduced percentage of CD4+ T cells and increased CD8+ T cells in transplant patients compared to control.

TGFβ has been shown to be co-stimulatory for CD8+ T cells but not CD4+ T cells [32]. IL-4 enhances the proliferation of precursors of cytotoxic lymphocytes and their differentiation into active cytotoxic CD8+ T cells [33]. In CsA or Tac treated mice, T-cell proliferation was shown to be suppressed in CD4+ but not CD8+ subsets [58]. Increased TGFβ and IL-4 by CD8+T cells in BAL and IL-4 and TGFβ by CD4+ and CD8+T cells respectively in blood, may therefore be causative factors in the significant increase in cytotoxic T cells in both blood and BAL in these patients. Chronic graft rejection is associated with increased fibrosis in the lung. Both IL-4 [35] and TGFβ [4] have been shown to promote fibroblast proliferation in the lung. Therefore, although TGFβ and IL4 are anti-inflammatory cytokines, their increased production by cytotoxic lymphocytes in the lung may contribute to increased fibrosis (as observed in chronic graft rejection).

Although this study shows that measurement of blood T-cell cytokine production in lung transplant recipients is reflective of BAL T-cell cytokine production, comparison of T-cell cytokine balance between the two compartments may give a more acurate indication of rejection episodes. All transplant patients in this study had plasma levels of CsA or Tac within their therapeutic range, again suggesting that analysis of cytokine production may provide a more accurate assessment of immunosuppression than drug levels and show that these patients are inadequately immunosuppressed. This study confirms previous findings that current immunosuppression protocols have a limited effect on peripheral blood CD8+ T-cell pro-inflammatory cytokine production in stable lung transplant patients [10]. A longitudinal surveillance of BAL cell phenotypes in individuals has been suggested to identify a preclinical state of rejection [40]. Monitoring intracellular T-cell cytokine profiles may be more appropriate indicator of patient immunosuppression and transplant status than cell phenotypes and we are currently undertaking such a study to investigate this. One could hypothesise that an increase in longitudinal IL-4 and TGFβ may be predictive of chronic rejection whereas an increase in IFNγ, IL-2 and TNFα may be predictive of acute rejection episodes [2-4]. Transplant patients were a very heterogeneous group and exhibited a broad range of inflammatory cytokines compared with controls (figure 1). Aerosolised CsA treatment has been used successfully and safely in reducing inflammatory cytokines in refractory acute rejection [59]. This therapy may be of benefit in treating lung transplant patients identified with high percentages of inflammatory cytokine producing T cells whilst minimising systemic side effects of immunosuppressive agents.

In conclusion, this study demonstrates that it is possible to monitor intracellular T-cell cytokine production in BAL. The study showed decreased T-cell pro- and anti-inflammatory cytokine production in BAL compared with blood in control subjects but not in stable lung transplant patients. Current immunosuppression protocols have limited effect on pro-inflammatory cytokine production by T cells in BAL, especially CD8+ T-cells, but do upregulate T-cell anti-inflammatory cytokines IL-4 and TGFβ. Drugs that effectively reduce T-cell pro-inflammatory cytokines in BAL may improve current protocols for prolonging graft survival in these patients.

Table 5. The percentage of T-cells producing intracellular cytokines in blood and BB of 13 lung transplant patients (mean ± SD). There was a significant decrease in the percentage of CD4+ and a significant increase in the percentage of CD8+ T cells in BB (bold). There was a significant increase in the percentage of CD8+ T cells producing IFNγ, IL-4 and TNFα, a significant decrease in the percentage of CD4+ T cells producing IL-2 and CD4+ and CD8+ T cells producing TGFβ in BB compared to blood

	CD3		IFNγ		IL-2		IL-4		TGFβ		TNFα	
	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8
Blood	53±11	47±11	13±7	26±15	18±13	4±4	1±.8	.9±.4	3.2±2	3±1.7	18±14	16±12
BB	37±11	63±11	17±7	40±13	8±4	3±1.6	1.2±.6	2.3±.8	2±.9	1.7±1	18±7	30±11
P	**.002**	**.002**	.085	**.026**	**.042**	.300	.179	**.043**	**.039**	**.028**	.738	**.002**

Table 6. The percentage of T-cells producing intracellular cytokines in BAL and BB of the 13 lung transplant patients (mean ± SD). There was a significant decrease in the percentage of CD4+ and a significant increase in the percentage of CD8+ T cells in BB (bold). There was a significant increase in the percentage of CD4 and CD8+ T cells producing IFNγ and CD8 T cells producing TNFα, and a significant decrease in the percentage of CD4+ and CD8+ T cells producing IL-4 and TGFβ in BB compared to BAL

	CD3		IFNγ		IL-2		IL-4		TGFβ		TNFα	
	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8
BAL	50±11	50±11	10±9	17±15	5±5	3±3	5±4	5±4	4.6±2	5±2.1	19±10	12±11
BB	37±11	63±11	17±7	40±13	8±4	3±1.6	1.2±1	2.3±2	2±.9	1.7±1	18±7	30±11
P	**.006**	**.006**	**.029**	**.001**	.100	.210	**.002**	**.025**	**.035**	**.028**	.657	**.002**

Table 7.The percentage of T-cells producing intracellular cytokines in blood and BB of 10 control volunteers (mean ± SD). There was a significant decrease in the percentage of CD4+ and a significant increase in the percentage of CD8+ T cells in BB (bold). There was a significant increase in the percentage of CD8+ T cells producing IFNγ and TNFα, a significant decrease in the percentage of CD4+ and CD8+ T cells producing IL-2 in BB compared to blood.

	CD3		IFNγ		IL-2		IL-4		TGFβ		TNFα	
	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8
Blood	69±4.4	31±6.9	18±7.8	20±5	40±17	6±4	.8±.4	.5±.3	4±2	3±2	43±12	20±6
BB	36±19	64±19	15±6	45±17	10±8	3±.2	.5±.3	.9±.7	3±1	1±.3	20±6	37±17
P	**.003**	**.003**	.065	**.019**	**.001**	**.038**	.560	.189	.185	.064	**.017**	**.025**

Table 8. The percentage of T-cells producing intracellular cytokines in BAL and BB of 10 control volunteers (mean ± SD). There was a significant decrease in the percentage of CD4+ and a significant increase in the percentage of CD8+ T cells in BB (bold). There was a significant increase in the percentage of CD4+ and CD8+ T cells producing IFNγ and IL-2 and CD8+ T cells producing TNFα in BB. There was a significant decrease in the percentage of CD4+ and CD8+ T cells producing IL-4 in BB compared to BAL.

	CD3		IFNγ		IL-2		IL-4		TGFβ		TNFα	
	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8
BAL	71±11	29±11	4±2.7	6±5	3±.8	.8±.7	8.5±2.4	5±2	2±2	.5±.5	14±8	7.5±6
BB	36±19	64±19	15±6	45±17	10±8	3±.2	.5±.3	.9±.7	3±1	1±.3	20±6	37±17
P	**.002**	**.003**	**.041**	**.000**	**.028**	**.042**	**.001**	**.002**	.198	.358	.530	**.000**

Compartmentalisation of Intracellular Pro-Inflammatory Cytokines in Bronchial Intra-Epithelial T Cells of Stable Lung Transplant Patients

Analysis of inflammatory cytokine profiles of intra-epithelial T cells in bronchial brushing (BB) may provide additional information to assess immune graft status in lung transplant patients. To investigate the immunomodulatory effects of currently used immunosuppressive regimens on bronchial intra-epithelial T-cell cytokine production, whole blood, BAL and BB from 13 stable lung transplant patients and 10 control volunteers were stimulated *in vitro* and cytokine production by T-cell subsets quantified [60].

This is the first report of the use of flow cytometry to measure intracellular pro- and anti-inflammatory cytokines in BB-derived intra-epithelial T cells. Although there was a decrease in T-cell pro-inflammatory cytokine production in blood of transplant patients, this was not found in BAL or bronchial intra-epithelial CD8 T-cell subsets, suggesting that the same level of immunosuppression may not occur in the lung of transplant recipients (table 5 and 6). This study shows that there is no difference in the percentage of bronchial intra-epithelial CD8 T-cells that produce pro-inflammatory cytokines between stable lung transplant patients and control subjects (tables 5-8) indicating that current immunosuppression protocols are ineffective at reducing pro-inflammatory T-cell cytokines in transplant grafts.

In contrast, the same authors have previously shown a decrease in CD4+ T-cell pro-inflammatory cytokine production in blood of stable transplant patients compared with control subjects consistent with immunosuppression protocol strategy [10] and the current study confirms these findings. They have also previously shown non-compartmentalisation of inflammatory T cells cytokines between blood and BAL in stable lung transplant patients compared with control group [56], results also consistent with the current findings. However, both studies showed a failure to suppress CD8+T-cell pro-inflammatory cytokine production, results consistent with the current findings in BB T cells from stable transplant patients. Transplant patients showed a CD4:CD8 inversion in BAL and BB consistent with a previous report [61] in contrast to control subjects who showed a CD4:CD8 inversion only in the BB compartment. The relative increase in CD8 T cells in BB may be due to an increase in proliferation of these cytotoxic cells and/or an increase in migration of these cells via specific Th1 chemokine receptors [62]. The percentage of CD8+ T cells producing IFNγ and TNFα in BB was increased compared with blood and BAL.

Representative dot plots showing IFNγ production by CD8+ and CD8- (CD4+) T cells from blood, BAL and BB in a lung transplant patient are shown in figure 6.

As IL-4 and TGFβ are negative regulators of Th1 inflammatory cytokines [25, 26], the findings of decreased IL-4 and TGFβ in some bronchial intra-epithelial T-cell subsets compared with blood and BAL may help explain these latter findings. Conversely, chronic rejection or OB is associated with an increase in TGF-β production [4], indicating that current immunosuppression protocols may be effective in reducing OB in lung transplant patients via this pathway.

Others have suggested that studies of intragraft immune cells may be more relevant in terms of effecting graft injury than analysis of peripheral circulating cells [63]. All transplant

patients in this study had plasma levels of CsA or Tac within their therapeutic range. Our findings therefore suggest that analysis of T-cell cytokine production may provide a more accurate assessment of immunosuppression in the various compartments than systemic drug levels and show that these patients are inadequately immunosuppressed especially in the lung compartment.

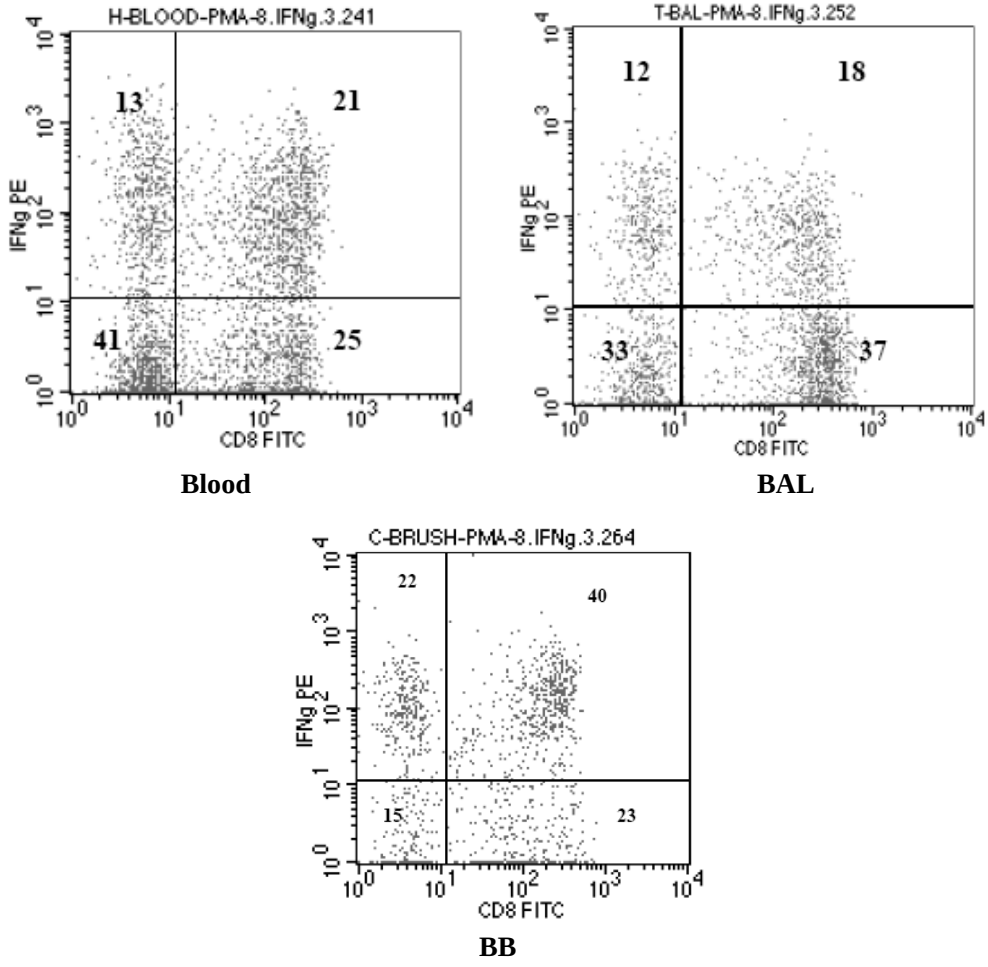


Figure 6. Representative dot plots showing IFNγ production by CD8+ and CD8- (CD4+) T cells from blood, BAL and BB in a lung transplant patient. T cells were identified by CD3 PC5 versus side scatter characteristics. Transplant patients showed an increase in the percentage of CD8+ T cells producing IFNγ in BB compared with blood. Transplant patients showed an increase in IFNγ in both CD4+ and CD8 T cells in BB compared with BAL. Note the decrease in CD4+ and increase in CD8+ T cells in BB compared with blood and BAL.

A longitudinal surveillance of cell phenotypes in individuals has been suggested to identify a preclinical state of rejection [57]. Monitoring intracellular T-cell cytokine profiles may be a more appropriate indicator of patient immunosuppression and transplant status than cell phenotypes. This study demonstrates that it is possible to monitor intracellular cytokines in bronchial intra-epithelial T cells and that there is compartmentalisation of pro-and anti-

inflammatory CD8+ T-cell cytokine production in BB compared with blood and BAL. Drugs that effectively reduce bronchial intra-epithelial CD8+ T-cell pro-inflammatory cytokines may improve current protocols for prolonging graft survival in these patients. The clinical relevance of this work is being further pursued with longitudinal follow-up of this patient group as comparison of T-cell cytokine levels between the three compartments may show important changes during rejection episodes.

Airway Infection Is Associated with Decreased Intracellular Th1 Cytokines in Bronchoalveolar Lavage CD8+ T Cells of Stable Lung Transplant Patients

Current immunosuppression protocols to reduce lung transplant rejection include drugs to reduce pro-inflammatory Th1 cytokines. However, Th1 cytokine production is important in host defense against microbial infection in the lungs, particularly *Aspergillus* and *Pseudomonas spp*., organisms shown to be leading causes of mortality in immunocompromised patients [64, 65] Excessive immunosuppression of these cytokines may leave patients susceptible to infection. To investigate whether infection is associated with reduced Th1 cytokines, whole blood and BAL from a group of 13 lung transplant patients with "culture negative" BAL, and a group of 13 lung transplant patients with "culture positive" BAL were stimulated *in vitro* and cytokine production by T cell subsets studied.

Table 9. Predisposing pathology and organisms isolated from "culture positive" transplant patient group

Patient	Predisposing pathology	Cultured organism	CsA / Tac levels
1	Bronchiecstasis	*Asp. Pseud.*	Tac 14.5
2	Congenital bronchial webbs	*Pseud.*	Tac 11.4
3	Cystic fibrosis	MRSA. *Pseud*	Tac 9
4	Pulmonary hypertension	*Asp. Pseud.*	CsA 276
5	Cystic Fibrosis	*Asp. Pseud.*	CsA 258
6	Emphysema	*Pseud.*	CsA 300
7	Emphysema	*Pseud.*	CsA 260
8	Emphysema	*Pseud.*	Tac 6
9	Pulmonary fibrosis	*Asp.*	CsA 349
10	Pulmonary hypertension	*E. coli*	CsA 152
11	Cystic fibrosis	*Asp.*	CsA 205
12	Emphysema	*Asp.* MRSA	CsA 235
13	Agammaglobulinaemia	*Pseud.*	CsA 185

Therapeutic range for CsA (80-250 μg/L) and Tac (5-20 μg/L).
Asp. (*Aspergillus spp*.), *Pseud.* (*Pseudomonas spp*.), MRSA (Methicillin resistant *Staphylococcus aureus*), *E. coli* (*Escherichia coli*).

Table 10. The percentage of T-cells producing intracellular cytokines in blood of the 13 BAL culture-negative (N) and 13 culture-positive (P) lung transplant groups (mean ± SD). There were no significant differences in intracellular cytokine production by T-cell subsets between either patient group

	IFNγ			IL-2			IL-4			TNFα		
	CD4	CD8	CD3	CD4	CD8	CD3	CD4	CD8	CD3	CD4	CD8	CD3
N	10±6	24±17	34±12	14±12	6±5	20±9	1.4±.1	.9±.6	2±4	20±15	15±10	35±12
P	9±6	16±11	25±8	15±14	2.4±2	17±8	.9±.7	1.4±.1	2±4	15±10	18±16	33±12
P	.965	.265	.348	.935	.284	.728	.765	.785	.820	.690	.560	.889

Table 11. The percentage of T-cells producing intracellular cytokines in BAL of the 13 BAL culture-negative (N) and 13 culture-positive (P) lung transplant groups (mean ± SD) . There was a significant decrease in the percentage of CD3+ T cells producing IL-2 and CD8+ T cells producing TNFα in BAL of the culture-negative compared to the culture-positive transplant group (bold)

	IFNγ			IL-2			IL-4			TNFα		
	CD4	CD8	CD3	CD4	CD8	CD3	CD4	CD8	CD3	CD4	CD8	CD3
N	9±6	22±12	31±9	5±4	5±4	10±4	6±4	11±4	17±4	12±9	15±11	27±9
P	7±6	11±8	18±8	2±2	1.7±1.1	3.7±2	6±2	6±5	12±4	12±8	8±6	20±8
P	.765	.165	.248	.218	.104	**.031**	.898	.157	.657	.890	**.038**	.200

Intracellular Th1/Th2 cytokines in BAL and blood T cells from clinically stable lung transplant patients in whom potentially pathogenic organisms were isolated from BAL were compared with a culture-negative group. The predisposing pathology, cultured organsim and plasma drug levels in these patients is shown in table 9.

We showed that Th1 cytokines were significantly higher in BAL from stable, non-infected transplant recipients compared with culture-positive patients. All transplant patients in this study had plasma levels of CsA or Tac within therapeutic range (table 9). There was no change in T cell cytokines in the blood of infected and non-infected patients (table 10). Importantly the investigations showed that the majority of patients with the greatest degree of immunosuppression, as judged by intracellular Th1 cytokine production in BAL, were infected with pathogenic microorganisms (table 11).

These organisms have been shown to be the leading cause of mortality in immunocompromised patients [64, 65]. In the mouse model, lung challenge with *P. aeriginosa* resulted in significantly less severe lung pathology, bacterial loads and mortality in mice that responded with a Th1-like response [66]. Transient over-expression of IFNγ within the lungs augmented host immunity against *Aspergillus* [64]. Hence excessive suppression of Th1 cytokines may leave patients susceptible to infection. There was no correlation between type of infective organism cultured, predisposing patient pathology and BAL or blood cytokines, although these findings need confirmation in a larger study. Although levels of BAL Th1 cytokines differed between patient groups, there was no difference in Th1 cytokines in blood, suggesting that reduced BAL Th1 cytokines were only associated with localised lung infection and not systemic disease. There has been a report of cytokine modulatory activity of CD4+ splenic T cells in the mouse model by Pseudomonas aeruginosa quorum-sensing signal molecules [67] suggesting that infection with this organism may inhibit the patient's immune response. However, there have been no reports of cytokine modulatory activity of CD8+ T cells or by other organisms isolated from the BAL of these patients other than immunostimulatory, [64, 66, 67] suggesting that the reduced Th1 response by these patients is more likely due to treatment with immunosuppressants.

The data shows that a cut-off value of greater than 8% CD3+ T cells producing IL-2 or 20% CD8+ T cells producing TNFα is associated with culture negative results and hence would be protective of infection in the lungs of these patients. In the ongoing study it would be of interest to investigate whether patients with the lowest levels of these BAL Th1 cytokines have the highest morbidity. Whether reducing immunosuppression in the culture-positive group would improve morbidity and mortality rates also remains to be investigated. Of further interest is whether protection from infection is afforded by subsets of T cells that produce combinations of Th1 cytokines eg., TNFα+IL2. The percentages of BAL T cells producing IL-4, an important Th2 cytokine that negatively regulates Th1 response [25] was unchanged between patient groups in this study, suggesting that the differences in Th1 cytokines observed was not due to altered levels of this regulatory cytokine. Potent immunosuppressive drugs such as tacrolimus and cylclosporin A cause significant toxic side effects [38]. Reducing levels of these drugs in culture-positive patients that have low Th1 cytokines would also have benefits associated with reduced organ toxicity. However, a reduction of immunosuppression due to infection must be balanced with appropriate immunosuppression of proinflammatory Th1 cytokines that have been reportedly increased in the lungs of patients undergoing graft rejection [1-3]. The degree to which transplant

recipients are immunosuppressed influences their risk of infection and rejection [68]. The restoration of Th1 responses has been shown to be an important predictor of fungal infection outcome in stem cell transplantation patients [69]. Monitoring the balance of intracellular Th1 cytokines between levels associated with infection and rejection may improve morbidity in our patient group.

In conclusion, this study demonstrates that lung infection is associated with decreased intracellular Th1 cytokines in BAL T cell subsets of stable lung transplant patients. Modifying immunosuppression by monitoring intracellular Th1 cytokines in BAL T cells may improve morbidity and infection rates in this patient group. The clinical relevance of this work is being further pursued with longitudinal follow-up of this group and a much larger patient cohort.

Longitudinal Monitoring of Intracellular T Cell Cytokines in Transplant Patients

Analysis of intracellular T cell cytokines in blood from lung transplant patients and controls showed a broad range of cells producing individual cytokines (figure 1). To determine longitudinal changes in intracellular cytokines/chemokines in patients and controls, whole blood from several stable, non-infected lung transplant patients and control volunteers was stimulated *in vitro* and intracellular T cell cytokine production determined on several occasions over a three year period. Samples from transplant patients were collected following routine surveillance assessment and histology of bronchial biopsies showed no evidence of acute or chronic rejection. All BAL cultures were negative, as were serology for mycoplasma and CMV.

The percentage of T cell subsets producing intracellular cytokines from 5 healthy control (C) volunteers on 3-8 occasions (N) (mean ± SD) are shown in table 12. The SD of cytokines from individual subjects was significantly less compared with the overall SD of cytokines from 14 control subjects (*overall mean ± SD*), suggesting that intracellular T cell cytokines are relatively stable over time from healthy individual subjects.

The percentage of T cell subsets producing intracellular cytokines from 4 stable non-infected transplant patients (P) on 3-4 occasions (mean ± SD) are shown in table 13. The SD of cytokines from individual patients was significantly less compared with the overall SD of cytokines from 12 transplant patients (*12P*) (*overall mean ± SD*), suggesting that intracellular T cell cytokines from individual stable transplant patients are relatively stable over time.

Analysis of intracellular T cell cytokines in BAL and BB from lung transplant patients and controls also showed a broad range of cells producing individual cytokines (table 3 and 4). Intracellular cytokine analyses were performed on one stable, non-infected transplant patient on three occasions and again during one occasion when the patient was undergoing acute rejection as determined by bronchial biopsy histology (A2B0). Results of the intracellular T cell cytokines from blood, BAL and BB from this patient are shown in table 14, 15 and 16 respectively.

Table 12. The percentage of T cell subsets producing intracellular cytokines from 5 healthy control (C) volunteers on 3-8 occasions (N) (mean ± SD). The SD of cytokines from individual subjects was significantly less compared with the overall SD of cytokines from 14 control subjects (*overall mean ± SD*), suggesting that intracellular T cell cytokines are relatively stable over time from healthy individual subjects

		CD3		IFNγ		IL-2		IL-4		TGFβ		TNFα	
	N	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8
C1	8	61±2	39±2	17±2	25±2	38±3	6±1	.6±..1	.4±.1	4±..8	3±1.1	44±4	26±2
C2	4	64±3	36±2	19±2	20±2	36±3	10±1	.4±..1	.6±.1	6±1.9	2±1.2	42±4	22±3
C3	3	58±2	42±2	23±3	15±2	28±2	8±1	.3±..1	.5±.1	3±.1.1	2±1.0	36±2	24±2
C4	5	60±3	40±2	22±2	22±3	25±2	9±2	.5±..1	.3±.1	7±.1.2	4±1.4	38±3	18±2
C5	3	56±2	34±2	18±2	24±2	37±3	5±2	.4±..1	.4±.1	4±.1.4	5±1.5	50±4	29±2
14C		*62±7*	*37±8*	*19±5*	*21±8*	*36±16*	*8±4*	*0.4±.3*	*.5±.3*	*5±3*	*3±2*	*43±11*	*24±11*

Table 13. The percentage of T cell subsets producing intracellular cytokines from 4 stable non-infected transplant patients (P) on 3-4 occasions (mean ± SD). The SD of cytokines from individual patients was significantly less compared with the overall SD of cytokines from 12 transplant patients (*12P*) (*overall mean ± SD*), suggesting that intracellular T cell cytokines from individual stable transplant patients are relatively stable over time

		CD3		IFNγ		IL-2		IL-4		TGFβ		TNFα	
	N	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8
P1	4	36±4	64±4	12±3	25±8	8±4	6±2	.6±.2	.9±.2	5±.1.5	6±1.2	9±5	9±6
P2	3	53±2	47±2	10±4	23±11	9±5	5±2	.8±.3	.6±.3	6±2.1	5±1.3	11±6	15±7
P3	3	64±3	36±3	9±1	17±5	6±3	3±1	.5±.3	.8±.1	4±.1.2	7±1.8	15±4	14±5
P4	3	48±2	42±2	7±2	16±6	25±2	7±2	.5±.1	.7±..3	7±.1.9	4±1.1	12±5	18±4
12P		*55±18*	*44±18*	*7±5*	*17±15*	*6±5*	*5±4*	*1±.1*	*1±1*	*4.9±2*	*5±2.5*	*12±7*	*14±12*

Table 14. T cell subsets and the percentage of intracellular blood T cell cytokines from a stable transplant patient (S) on three occasions (mean ± SD) and on one occasion during an episode of acute rejection (AR). There was no significant change in CD4+ or CD8+ T cell subsets or cytokine production during the acute rejection episode

	CD3		IFNγ		IL-2		IL-4		TNFα	
	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8
S	53±2	47±2	12±4	23±8	9±4	3±1	.8±.3	1.1±.2	11±6	15±8
AR	60	40	10	15	2	2	.6	.9	2	2

Table 15. T cell subsets and the percentage of intracellular BAL T cell cytokines from a stable transplant patient (S) on three occasions (mean ± SD) and on one occasion during an episode of acute rejection (AR). There was a significant increase in IFNγ, IL-2 and TNFα by both CD4+ and CD8+ T cell subsets during the acute rejection episode (bold)

	CD3		IFNγ		IL-2		IL-4		TNFα	
	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8
S	55±8	45±8	5±4	4±3	3±2	1±1	12±.4	12±.7	9±6	5±3
AR	60	40	40	31	17	4	4	6	38	24

Table 16. T cell subsets and the percentage of intracellular BB T cell cytokines from a stable transplant patient (S) on three occasions (mean ± SD) and on one occasion during an episode of acute rejection (AR). There was a significant decrease in CD4:CD8 and a significant increase in IL-2 by CD8+ T cells during the acute rejection episode (bold)

	CD3		IFNγ		IL-2		IL-4		TNFα	
	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8
S	36±5	64±5	14±6	36±13	5±4	4±2	.3±.3	1.5±1.4	18±4	36±11
AR	20	80	14	50	4	50	.3	1.5	13	54

The results from this study show that during an episode of acute rejection, there was no discernable change in blood T cell subsets or intracellular cytokines. Although plasma levels of CsA and Tac were within therapeutic range, there were significant increases in pro-inflammatory Th1 cytokines in BAL T cells and CD8 T cells and IL-2 production by CD8+ T cells in BB. These results suggest that immunosuppression therapy may be effective in the blood compartment but not in the lungs during an episode of rejection. Whether the drugs are not reaching the lungs or do enter the lungs but are ineffective in reducing pro-inflammatory T cell cytokines remains to be determined. These data suggest that analysis of intracellular cytokines in the lung compartment, particularly in BAL T cells may be an effective, relatively non-invasive technique in the diagnosis of acute rejection episodes in lung transplant patients. Although these are results from one case of acute rejection in one patient and must be viewed with caution, they are nonetheless exciting and it will be of great interest to follow the results of these longitudinal studies on a larger cohort of lung transplant patients. It will also be of interest to observe changes associated with chronic rejection as these have been reported to be associated with moderate increases in pro-inflammatory cytokines and the profibrotic cytokines TGFβ and IL-4 [1-4].

Future of Intracellular Cytokines to Improve Therapeutic Monitoring Following Lung Transplantation

It is clear that no single compartmental approach to intracellular cytokine analysis is sufficient to produce simple valid diagnostic or prognostic data at every stage during rejection of lung transplant. Results of intracellular cytokine analysis of multiple immune cell subsets within the blood may provide physiological evidence of systemic levels of immunosuppression that may be more relevant than drug plasma levels. Using these techniques, identification of specific cell subsets producing cytokines/chemokines associated with graft rejection may allow targeting of these subsets or mediators to improve the morbidity of these patients. However, direct examination of biopsy tissue still provides the "gold-standard" measure of allograft rejection, it is likely that analysis of intracellular cytokine expression within or immediately adjacent to the graft (BAL) will be of diagnostic and prognostic value. Longitudinal monitoring of cytokines in both BAL and blood in individual patients may offer early signs of episodes associated with infection (and possibly rejection). Maintaining intracellular cytokines within stable (non-infection, non-rejection) levels by regulating doses of immunosuppression drugs may lead to less adverse drug toxicity. Targeting local perturbations in pro-inflammatory cytokines/chemokines within the lung compartment may also further reduce systemic effects of therapeutics and reduce morbidity in this patient group.

Although the potential role of monitoring immunosuppression using intracellular cytokines as opposed the pharmacokinetic dose is very promising, further research is required before it is likely to become of practical value in clinical lung transplantation.

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