The role of donor CD4 T lymphocyte chimerism in lung transplant recipients

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Declaration

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text. It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my thesis has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. It does not exceed the prescribed word limit for the relevant Degree Committee.

Abstract

The role of donor CD4 T lymphocyte chimerism in lung transplant recipients **Olivera Gjorgjimajkoska**

Long-term outcomes for lung transplantation remain disappointing; as many as 50% of lung transplant recipients develop progressive Bronchiolitis Obliterans Syndrome (BOS) by 5 years after transplant. In the last decade, several experimental and clinical studies have demonstrated that cellular and humoral autoimmune responses to 'self' antigens may play a causative role in the onset of BOS. Work from our research group has highlighted a novel mechanism for triggering autoimmunity following transplantation. In a murine model of chronic heart rejection, transplant-induced autoimmunity - characterised by production of class-switched anti-nuclear effector autoantibody responses was dependent upon provision of help from donor CD4 T cells that were passengers within the graft. However, the fate of the donor passenger CD4 T cells in clinical organ transplantation is unclear.

To assess whether a similar mechanism may occur in human transplant recipients, I have studied the presence and the impact of passenger donor CD4 T cells on the development of humoral auto- and alloimmune responses in a prospective cohort of primary lung transplant recipients (*n*=21). The development of autoantibody and, in particular, the specificity of target autoantigens was also evaluated in a lung transplant patients with either established grade 2-3 BOS (*n*=10), or without BOS (*n*=10).

My work suggests that the presence of donor CD4 T cell chimerism in the peripheral blood of lung transplant recipients is a uniform phenomenon. Donor CD4 T lymphocytes were consistently detected in the recipients' peripheral blood during the first post-operative month; however, the number of detectable donor CD4 T cells fluctuated over time, and varied between individual lung transplant recipients. In a follow-up period of one year after transplantation, three distinct patterns of donor CD4 T cell chimerism were

observed: short (donor CD4 T cells detectable for up to six weeks after transplantation, n=13), intermediate (donor CD4 T cells detectable between three to six months after transplantation, n=3) and long-lasting chimerism (donor CD4 T cells detectable for more than six months after transplantation, n=5). Surprisingly, the degree of HLA mismatching and the predicted NK cell alloreactivity did not correlate with the longevity of donor CD4 T cell chimerism, and did not influence the development of BOS. Furthermore, transcriptomic analysis of the donor CD4 T cell population consisted of a heterogenous mixture of different CD4 T cell sub-types, with no consistent pattern evident in patients with short and long lasting donor CD4 T cell chimerism.

The assessment of the humoral autoimmune responses revealed unexpected findings. Anti-nuclear IgG autoantibody levels were greater in the recipients' sera before the transplant, when compared to sera sampled at one and 12 months after transplantation, but the titre of pre-transplant anti-nuclear autoantibody did not correlate with the subsequent development of BOS. Similar findings were observed in the retrospective cohort of 24 lung and 18 heart and lung transplant recipients. Assessment of the repertoire of pretransplant autoantibody did however suggest that a unique set of autoantigens were targeted in those patients that subsequently developed BOS.

The involvement of donor CD4 T cell in the development of transplant-induced autoimmunity and augmentation of humoral alloimmunity was not confirmed in human lung transplant recipients and the role of donor CD4 T chimerism remains unclear. However, solid organ transplant recipients are subjected to highly potent T-cell depleting immunosuppressive drugs that can alter the number and function of T cells. This may obviate the impact of donor CD4 T cell chimerism in the development of transplant-induced autoimmunity. Understanding the clinical relevance of the autoantibody repertoire present at the time of transplant may, however, hold potential in identifying recipients with predisposition to develop BOS, and may therefore possibly provide a window of opportunity for targeted immunosuppression.

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Publications and Presentations

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3. Reid A.W., Harper S., Jackson C.H., Wells A.C., Summers D.M., **Gjorgjimajkoska O**., Sharples L.D., Bradley J.A., Pettigrew G.J. Expansion of the kidney donor pool by using cardiac death donors with prolonged time to cardiorespiratory arrest. *American Journal of Transplantation.* 11(5): 995-1005: 2011.

Published Abstracts

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Oral Presentations

1. 25th British Society for Histocompatibility & Immunogenetics Conference, Manchester, UK (2014)
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Abbreviations

7-AAD	7-amino-actinomycin
μL	Microliter (s)
°C	Degrees Celsius
ΔC_T	Delta C_T
Ab	Antibody
ACR	Acute cellular rejection
ACTB	β-actin
ADCC	Antibody dependent cell cytotoxicity
Ag	Antigen
AMR	Antibody mediated rejection
ANA	Antinuclear antibody
APC (1)	Allophycocyanin
APC (2)	Antigen presenting cell
AR	Acute rejection
B6	C57BL/6 mouse strain
BCL	B cell receptor
BD	Becton Dickinson
BOS	Bronchiolitis Obliterans Syndrome
Breg	Regulatory B cell
BSA	Bovine serum albumin
CAV	Chronic allograft vasculopathy
CCR7	C-C chemokine receptor type 7
CD	Cluster of differentiation
cDNA	Complementary DNA
CD62L	L-selectin
CR	Chronic rejection
CR3	Complement receptor 3
C_T	Threshold cycle
CXCR5	C-X-C chemokine receptor type 5

DCs	Dendritic cells
DNA	Deoxyribonucleic acid
DTH	Delayed-type hypersensitivity
ELISA	Enzyme-Linked Immunosorbent Assay
EVLP	Ex Vivo Lung Perfusion
FACS	Fluorescence activated cell sorting
FCS	Foetal Calf Serum
FcR	Fc receptor
FDR	Follicular dendritic cell
FITC	Flourexceine isothiocynanate
FO	Follicular
FSC	Forward scatter
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GvHD	Graft-versus-host disease
GvL	Graft-versus-leukaemia
HLA	Human Leukocyte Antigen
ICAM-1	Intercellular Adhesion Molecule-1
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IIF	Indirect Immunofluorescence
ISHLT	International Society for Heart and Lung Trasnsplantation
IRBP	Immune Response Biomarker Profiling
KIRs	Killer-cell immunoglobulin-like receptors
LFA-1	Lymphocyte function-associated antigen-1
mAb	monoclonal Antibody
MBL	Mannose binding lectin
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
Min	Minute (s)
MLR	Mixt lymphocyte reaction
MM	Mismatch
MMF	Mycophenolate Mofetil

mRNA	Messenger RNA
MZ	Marginal zone
NK sells	Natural killer cells
NOD-SKID mice	Non-obese diabetic-immunedeficient mice
OD	Optical density
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR-SSP	Polymerase-Chain Reaction Sequence-Specific Primers
PE	Phyocoerythrin
qPCR	Quantitative Polymerase Chain Reaction
r-ATG	Rabbit-antithymocyte globulins
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RvD	Recipient versus Donor
RT	Reverse Transcription
RT-PCR	Real Time – Polymerase Chin Reaction
RQ	Relative quantification
SD	Standard deviation
SLO	Secondary lymphoid organ
SSC	Side scatter
STATs	Signal Transducers and Activators of Transcription
TCR	T cell receptor
Th	Helper T cell
Tfh	Helper follicular T cell
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor – alpha
Treg	Regulatory T cell
VLA-4	Very Late Antigen-4
vWf	von Willebrand factor
SAB	Single Antigen Beads

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1 Chapter 1

Introduction

1.1 A brief history of lung transplantation

The earliest attempts to transplant heart and lungs in the experimental animal model date from the beginning of the 20th century. In 1905, Alexis Carrel performed the first successful heterotopic heart-lung block transplantation into the neck of a recipient cat, reviewed by Stolf, 2017 [1]. It took over 50 years of extensive experimental research before lung transplantation moved from animal models to humans.

The pioneering work of Dr James Hardy laid the foundation of lung transplantation in humans. After seven years of experimental work Dr Hardy and colleagues obtained the first ethical permission to perform lung transplantation in a human recipient. On the 11th of June in 1963 at the University of Mississippi Medical Centre they performed the first single-lung transplant. The patient was later identified as a convicted prisoner who died 18 days after the transplant due to renal failure and infection with good lung function and no evidence of rejection [2].

During the 1970s and early 1980s lung transplantation suffered its own setbacks and proved more challenging since most allografts were lost due to post-operative surgical complications and allograft rejection [3]. During this period less than 40 lung transplants were performed worldwide without any long-term survivors.

After years of additional experimental animal work in 1981, Bruce Reitz and Norman Shumway performed the first successful heart and lung transplant in a patient with primary pulmonary hypertension who survived for many years after transplantation. The patient was a newspaper executive who later wrote a book detailing his story of this amazing medical accomplishment [4]. During the mid-1980s the Toronto lung transplant group published their experience with successful single-lung transplants in patients with pulmonary fibrosis [5] reviving the hope that lung transplantation could become a visible therapeutic option.

The number of human lung transplants performed grew dramatically during the late 1980s to the late 1990s; however, since then lung transplant activity has plateaued, mainly due to the limited number of suitable lung donors. The limited number of available donors has led to the development of living donor lung transplant programmes and increased use of lungs previously deemed unsuitable for transplantation; including the use of donors after circulatory death (DCD) and use of lungs after prior assessment and reconditioning during ex vivo lung perfusion (EVLP).

Over the last four decades, improved surgical techniques, development of immunosuppression regimens and highly sensitive antibody screening strategies have significantly improved the early clinical outcome; however, development of progressive lung allograft dysfunction manifesting as bronchiolitis obliterans syndrome (BOS) limits survival to only 50% at 5 years after lung transplantation [6].

1.2 Pathogenesis and risk factors for development of bronchiolitis obliterans syndrome

BOS is a disease of multifactorial etiology; it is believed that it represents the end result of repeated immune and non-immune related injury to the allograft. Factors that contribute to the development of BOS have been classified as: alloimmune-independent and immune-dependent [7] including both alloimmune and autoimmune responses.

1.2.1 Alloimmune-independent contributing factors for development of BOS

The lung is a vulnerable organ due to the constant exposure to environmental agents, such as inhaled dust, toxins, infectious material and irritants, which when inhaled could promote local inflammation and tissue damage. Alloantigen-independent factors such as infections, gastroesophageal reflux disease and primary graft dysfunction are the main factors that increase the risk for development of BOS.

1.2.1.1 Infections as a contributing factor for development of BOS

Infections are the leading cause of morbidity and mortality in lung transplant recipients (LTR). Due to the highly potent immunosuppression regimens all solid organ transplant recipients suffer increased incidence of infections [8]; nevertheless, lungs are more prone to infections, and this largely contributes to several factors unique to lung allografts. Firstly, lungs are in constant contact with environmental antigens, second, exposure of the allograft to previously colonised native airways, and lastly, denervation of the lung during the surgical procedure leading to impaired cough reflex [9]. These factors together with the immunosuppressed state of the recipients provide an ideal environment for development of opportunistic infections.

Lung allografts are the most susceptible to infections during the first post-operative months. In a retrospective study of 51 lung transplant recipients, a total of 71 infection episodes were reported, of which 42% occurred within the first 3 months and 75% during the first year after transplantation [10]. Bacterial pneumonia is the most common post-transplant complication [11], followed by infection with cytomegalovirus (CMV) with incidence ranging from 50% to 75% in comparison to the incidence of CMV infections in kidney transplant patients ranging between 8 and 32% [12]. Recipients of donor CMV seropositive are at highest risk for CMV infection [12]. The CMV infects various cells and elicits innate and adaptive immune responses, both cellular and humoral.

Post-transplant Infections with human respiratory syncytial virus (RSV) have been associated with acute allograft rejection and development of BOS [13]. The respiratory epithelial cell infection with RSV induces innate immune mechanism via TLR4, a potent activator of Th1 driven adaptive immunity, characterised by increased production of IFN-γ, TNF-α and IL-2 production and cytotoxic T cell responses [14]. Activation of the adaptive immune responses leads to viral clearance. However, due to the immunosuppression regimens LTRs have impaired production of IL-2 resulting in inadequate viral

clearance and epithelial cell damage, thus, subsequently contributing to allograft dysfunction [15].

Another common post-transplant complication in LTRs is the development of posttransplant lymphoproliferative disorder (PTLD) due to infection with the Epstein-Barr virus. The incidence of PTLD in LTRs ranges between 6.2 and 9.4% and is in correlation with the level of immunosuppression and time after lung transplantation [16].

Bacterial pneumonia is the most common infection in LTRs in the early post-operative period. The most common bacterial isolates are *Staphylococcus aureus* and *Acinetobacter* [17]. Amongst the fungal infections with an incidence of 32% are the infections with the *Aspergillus* species [17]. Post-transplant complication with fungal infections usually occurs at the site of anastomosis as a result of transmitted infection from the donor or the native lung may serve as a reservoir in the case of single lung transplantation.

1.2.1.2 Gastroesophageal reflux disease and BOS

Gastroesophageal reflux disease (GERD) is a mucosal inflammatory diseases caused by the stomach acids reflux into the esophagus. It occurs in variety of lung diseases prior to transplantation especially in patients with idiopathic pulmonary disease (IPF) [18] and cystic fibrosis (CF) [19]. The incidence of GERD is even more common after lung transplantation, presumably due to an intraoperative vagal nerve injury and continuous use of immunosuppression such as cyclosporine A and prednisolone. It is not clear whether GERD directly contributes to the pathogenesis of BOS; nevertheless, several reports have shown that GERD was associated with worse pulmonary function after lung transplantation [20]. Patients treated with surgical fundoplication (a surgical procedure that prevents stomach acid reflux into the esophagus) had improved lung function, suggesting the involvement of GERD in the development of BOS [21]. Experimental studies in a rat lung transplant model,

in which bile acid was directly aspirated into the transplanted lung, led to lymphocytic lung inflammation and allograft rejection [22].

It is possible that prolonged contact of the airways with the gastric content may lead to epithelial lung injury, thus creating a local up-regulated inflammatory milieu. Increased levels of bile acids in bronchoalveolar lavage (BAL) has been associated with alveolar neutrophilia [23]. These findings support the role of GERD in the development of BOS, presumably by augmenting the non-alloimmune and alloimmune responses.

1.2.1.3 Ischemia-reperfusion-induced injury and BOS

Ischemia-reperfusion-induced (IRI) injury is characterised by non-specific alveolar damage, lung edema and hypoxemia occurring within the first 72 hours after lung transplantation [24]. IRI represents a main cause of primary graft dysfunction that in its most severe form is a leading cause of morbidity and mortality after lung transplantation. Over the years, improvements in lung preservation techniques and development of a new preservation solution specifically designed to support the lungs have reduced the incidence of primary graft dysfunction from 30% to less than 15% [24, 25]. IRI is a two-stage process, a period of cold ischemic storage and reperfusion following revascularisation of the graft. The allograft cold storage is kept as short as possible and usually for lung transplantation ranges between 4 and 8 hours.

Although, cold storage is essential for organ preservation, it is associated with a number of events such as oxidative stress, sodium pump inactivation, intracellular calcium overload, iron release and cell death. These processes may lead to up-regulation of cell surface molecules and release of inflammatory mediators that can activate the passenger donor cells and recipient leukocytes following reperfusion [25].

The pro-inflammatory conditioning of the lung is further amplified by a number of physiological changes that occur in brain-death donors; including

hemodynamic instability, endocrine abnormalities, hypothermia, coagulopathy, pulmonary dysfunction, and electrolyte imbalances [26]. As a consequence, these events lead to upregulation of cell adhesion molecules and activation of passenger macrophages.

Macrophages are antigen-presenting cells of the innate immune system; their activation leads to release of proinflammatory cytokines such as IL-1, IL-6, IL-8, IL-12, IL-18, TNF-α and INF-γ [27]. In human lung transplantation, IL-8 has been shown to be upregulated in the BAL and lung tissue of DCD donors [28]. The level of IL-8 has been associated with the increase incidence of primary graft dysfunction [28, 29]. Moreover, release of pro-inflammatory cytokines initiates recruitment and activation of neutrophils and T lymphocytes that further amplify the inflammatory process resulting in tissue damage. Thus, as a consequence at the time of implantation the allograft is already armed with vigorously active immune responses that may further augment alloimmune responses.

1.2.2 Immune-dependent contributing factors for development of BOS

Immune recognition of antigenic differences between the recipient-donor pair plays a major role in the development of BOS and the strength of alloimmune responses is influenced by the degree of HLA mismatching, episodes of acute rejection and its underlining cellular and/or humoral mechanisms involved and pre-existence of donor specific HLA antibodies. In the following Sections I will describe the role of HLA mismatching, allorecognition pathways and types of allograft rejection as contributing factors for development of BOS.

1.2.2.1 HLA mismatching

The immunological events that initiate allograft rejection occur due to the recognition of antigenic differences in the Major Histocompatibility Complex (MHC) molecules between the host and transplanted organ. The main

function of the MHC molecules is presentation of self and foreign (non-self) antigens in the form of short peptides to T lymphocytes.

The MHC region comprises of group of genes found on the short arm of chromosome six and it represents the most studied region of the human genome. It spans over four megabases and contains more than 250 expressed genes, making it the most polymorphic and diverse region of the human genome [30].

The MHC region contains three clusters of genes: class I, class II and class III. The genes found within class I and class II region encode for proteins also know as Human Leukocyte Antigens (HLA) whereas the genes in the class III portion encode for immune related proteins such as complement factors (C2, C4A, C4B), lymphotoxin alpha and beta, heat-shock proteins (HSP70), and others; and none of these proteins are involved in antigen presentation [31].

MHC class I molecules are encoded by three genetic loci HLA-A, -B and Cw, consisting of two non-covalently linked polypeptide chains. The alpha heavy chain is a highly polymorphic glycoprotein anchored to the cell membrane and associated with β2-microglobulin, which is encoded by a non-polymorphic gene located on chromosome 15. The heavy chain α1 and α2 domains fold together creating a cleft, known as peptide-binding site [32]. Thus, polymorphism within these two domains determines the type of peptides that bind to the HLA class I antigens. In contrast, the α3 domain is highly conserved and acts as a ligand for CD8 co-receptor expressed on T lymphocytes. MHC class I molecules are expressed on all nucleated cells and present short (8-10 amino acids in length) endogenous peptides to CD8 T cells [32, 33].

There are other class I loci, however, knowledge about their function is only beginning to emerge. Amongst these is HLA-G expressed on placental trophoblast cells and it represents the only known ligand for activating KIR2DL4 receptor expressed on all natural killer (NK) cells [34], implicating

their role in fetal development during early pregnancy [35]. HLA-E, -F and -G, termed as non-classical HLA class I antigens serve as a ligands for NK cell receptors [36, 37]. Their role in solid organ transplantation is unclear.

The genes that encode class II molecules HLA-DR, -DQ and -DP are clustered within the class II region of the MHC. The MHC class II molecules consist of two polypeptide chains, α and β both anchored to the cell membrane. In the MHC class II molecules the $\alpha 1$ and $\beta 1$ domain form the peptide-binding site and the most polymorphic part of the molecule lies within the $\beta 1$ domain [38]. In contrast to MHC class I molecules, the MHC class II molecules are mainly expressed on professional antigen presenting cells (APC) including macrophages, dendritic cells (DCs) and B cells and present peptides (13 to 25 amino acids in length) derived from exogenous antigens [38, 39]. The $\beta 2$ domain is highly conserved and associates with CD4 co-receptor on T lymphocytes, making them restricted to HLA class II interaction. In addition, HLA class II expression can be induced in other cell types such as endothelial and epithelial cells by IFN-γ and TNF-α [40].

The extensive polymorphism of HLA has evolved due to evolutionary pressure to ensure efficient binding and presentation of a vast array of peptides derived from potentially pathogenic organisms, thus provoking strong immune responses and eradication of pathogenic organisms. Therefore, particular HLA genes have evolved differently according to geographic regions and ethnic groups. Contrary to this, the differences within the HLA class I and class II genes between recipient-donor pair represent a limiting factor for successful solid organ and bone marrow transplantation.

Evidence for their involvement as the major transplantation antigens arose from transplants performed between genetically related individuals [41]. In the early 1970s and 1980s graft survival correlates with the number of HLA mismatched antigens between donor and recipient, with 90% of graft survival in transplants performed between HLA identical siblings compared to 60% of patients who received a full HLA mismatched graft [41, 42].

Nevertheless, 10% of kidney transplant patients who received graft from HLA identical sibling reject the graft and up to 40% of HLA identical bone marrow patients experience acute graft-versus-host disease (GvHD), most likely due to polymorphism in proteins other than the MHC molecules itself, termed as minor histocompatibility (mH) antigens [43, 44]. These antigens are presented by the MHC class I and class II molecules and represent targets for allorecognition. There are several mH antigens recognised, in bone marrow transplantation the male HY antigen has been associated with severe GvHD in an HLA identical sibling pair [45]. The role of mH antigens in solid organ transplantation is not clear.

In a hope to improve graft survival in many countries across the world HLA matching has been incorporated in the organ allocation scoring criteria. In addition, minimising the number of HLA mismatched antigens between the recipient and donor limits the potential for patient sensitisation to HLA antigens, which is of great importance for patients requiring more than one transplant.

However, since the introduction of sophisticated immunosuppression regiments incorporating calcineurine inhibitors (cyclosporine, tacrolimus), anti-proliferative agents (azathioprine, mycophenolate mofetil (MMF), sirolimus, everolimus) and both polyclonal and monoclonal antibodies (anti-thymocyte globulin, Basiliximab, Daclizumab, Almtuzumab) the short-term graft survival of all solid organ transplants has improved significantly [46]. Nowadays, the United Kingdom national average one-year kidney graft survival is around 96% vs 98% for deceased and living kidney transplants, respectively [47].

Historically, in renal transplantation, HLA matching played an important role in organ allocation; patients with 000-mis-matched kidneys (HLA-A/B/DR) have significantly improved graft survival in comparison to poorly matched grafts [48]. Nevertheless, in the current era of modern immunosuppression the impact of HLA matching has been rendered [49], which in the United Kingdom has resulted in changes in the kidney offering and matching criteria [47, 50].

In comparison to kidney transplantation, HLA matching has not been taken into consideration for allocation of thoracic organs, due to shorter ischemia time and a smaller donor pool. Tendrich et al., and others have demonstrated that HLA matching is not associated with prolonged graft survival in heart transplant recipients [51, 52]. In contrast, analysis from the UNOS/ISHLT Thoracic Registry showed that HLA matching has a beneficial impact on graft survival in heart and single-lung transplant patients; primarily matching at HLA-A and DR loci [53]. In other studies, matching for HLA-A [54] or HLA-DR loci [55] has been associated with reduced incidence of BOS; whereas Yamada et al., have showed that the number of HLA mismatches is an independent risk factor for development of BOS [56]

Although the data available is conflicting, it is important to bear in mind that allorecognition of non-self-donor HLA antigens is the basis for initiation of alloimmune responses [57].

1.2.2.2 Allorecognition

Allorecognition refers to detection of same-species, non-self antigens by the host immune system. It is primarily driven by the ability of the recipient T cells to recognise both intact donor MHC molecules on donor antigen presenting cells, known as direct pathway of allorecognition; and processed donor derived MHC peptides presented in the form of self-MHC peptide complexes, an indirect pathway [58]. The two pathways of allorecognition differ in their cellular mechanism.

1.2.2.2.1 Direct pathway of allorecognition

Direct pathway of allorecognition is unique to transplantation in a sense that it differs from the conventional rule of self-MHC restriction. It has been proposed that the direct allorecognition may result from cross-reactivity of T cell receptor (TcR) specific for self-MHC molecules with an allogeneic MHC molecule. Lombardi et al., have shown that high a proportion of T cell primary

alloresponses are the result of previously primed T cells, implying that the cells have previously been primed against foreign antigens in the context of self-MHC molecules [59].

The high precursor frequency of T cells with reactivity to allogeneic MHC molecules can be measured *in vitro* in a mixed leukocyte reaction (MLR) and represents *in vivo* analogue to acute allograft rejection [60]. Two hypotheses have been proposed to explain this phenomenon; “high determinant hypothesis” where the allogeneic MHC molecules itself, independent of the peptide bound plays a major ligand for alloreactive T cells [61], and “multiple binary complex hypotheses” primarily driven by the peptide bound on the MHC molecule [62, 63].

A single MHC molecule can display a diverse array of peptides; this would mean that a single allogeneic MHC molecule could stimulate a large number of alloreactive T cells with specificity for an individual peptide-MHC complex.
The priming of recipient T cells with direct allospecificity depends on interaction with stimulated passenger donor dendritic cells (DCs) in the secondary lymphoid organs [64]. Removal of donor DCs from the graft by “parking” the graft in the intermediate recipient before re-transplantation into a second recipient leads to loss of immunogenicity and prolonged graft survival [65]. Whereas transfer of donor DCs restores the immunogenicity [65] and provokes allograft rejection. This experimental evidence suggests that acute allograft rejection occurring early after transplantation is dependent on the presence of donor derived DCs passenger within the allograft.

Activated donor DCs express both MHC class I and class II molecules and other co-stimulatory molecules that can facilitate priming and activation of the recipient CD4 and CD8 T cell with direct allospecificity in a 3-cell cluster, allowing a conventional CD8 T cell activation [66]. Activated direct pathway CD8 T cells can mediate cell killing by inducing apoptosis via Fas activation [67], or cell lysis in an IFN-γ dependent manner [68]. Moreover, Kreisel et al., have showed that naïve cytotoxic CD8 T cells with direct allospecificity can

mediate cell killing via direct interaction with the graft endothelial cells and independently of CD4 T cell help [69].

Evidence that CD4 T cells are involved in direct allorecognition comes from the experimental work where reconstitution of Rag1 -/- mice (mice do not have mature CD4 or CD8 T cells) with syngeneic CD4 T cells leads to rejection of MHC class II expressing cardiac grafts but not MHC class II deficient grafts [70]. Moreover, reconstitution of Rag1 -/- MHC class II -/- mice with CD4 T cells lead to graft rejection, suggesting that CD4 T cell with direct allospecificity are sufficient to mediate allograft rejection since these mice are unable to mediate MHC class II restricted indirect allorecognition and do not have CD8 T cells [70].

Furthermore, recipient CD4 T cells with direct allospecificity can also mediate allograft rejection by initiating a Delayed Type IV hypersensitivity (DTH) response [70], thus resulting in recruitment and activation of macrophages, neutrophils and natural killer (NK) cells.

It is believed that the direct pathway is important in acute rejection but its contribution to chronic rejection is debatable due to the short lifespan of passenger donor DCs. Thus, absence of donor DCs diminishes the effector capacity of recipient CD4 and CD8 T cells with direct allospecificity. Reduction in the frequency of both CD4 and CD8 T cells with direct allospecificity has been demonstrated in heart transplant recipients with progressive coronary artery disease [71], suggesting that an alternative pathway is responsible for the ongoing alloimmune responses.

1.2.2.2.2 Indirect pathway of allorecognition

Indirect pathway of allorecognition refers to recognition of allogeneic MHC molecules after they have been internalised, processed and presented in the context of self-MHC peptide complexes, analogue to conventional T cell response to foreign antigens [72]. The term “indirect allorecognition” refers exclusively to recognition of allogeneic MHC peptides, although this pathway

can allow recognition of other donor derived polymorphic peptides such as minor histocompatibility antigens (mHA).

The frequency of CD4 T cells with indirect allospecificity accounts for a very small proportion (1-5%) of the T cell repertoire in comparison to the T cells with direct allospecificity [73]; however, following clonal expansion of indirect alloreactive T cells and a rapid decline of direct alloresponses following loss of donor DCs this deference becomes insignificant. Inaba et al., have shown donor DCs within the lymph nodes are rapidly phagocytosed and presented by the recipient DCs [74], thus donor DCs migratory to secondary lymphoid organs provide a source of donor antigens for indirect allorecognition [75].

In MHC class II deficient mice donors, Auchincloss et al. have demonstrated the involvement of CD4 T cells with indirect allospecificity in allograft rejection [76]. They showed that class II deficient skin grafts were rapidly rejected by recipients that were previously depleted of CD8 T cells and since donor grafts lacked class II antigens, their results indicated that CD4 T cells with indirect allospecificity were responsible for skin graft rejection. Furthermore, they showed that in the absence of direct alloresponses the indirect pathway alone could result in a rapid acute allograft rejection [76].

The indirect pathway is likely to be predominant in the later post-transplant period and probably plays a predominant role in the progression of chronic allograft vasculopathy (CAV), months or years after transplantation [72, 77]. Clinically, activation of the indirect pathway has been linked with development of chronic rejection, as evidenced by increased frequency of T cells with indirect allospecificity in patients with chronic heart, kidney and liver transplant rejection [77, 78], whereas no difference in the frequencies of direct pathway CD4 T cells was observed between patients with chronic rejection and patients with stable allograft function.

Indirect pathway CD4 T cells play a central role in mediating chronic allograft rejection by providing help for initiation of humoral responses and production of class-switched alloantibodies. Help for B cell activation is provided via

“cognate” interaction between CD4 T cell-receptor and the MHC class II/peptide complex expressed on B cells [79, 80]. This interaction leads to generation of alloantigen-specific B cells and production of alloantibodies. Indirect CD4 T cells can differentiate into a subpopulation of follicular T cells that are able to enter the B cell follicles and sustain the germinal center reaction [80]. A subset of B cells differentiates into long-lived antibody producing plasma cells that produce alloantibody for many years, making them the main source of donor specific antibody (DSA) responsible for recipient sensitisation and chronic rejection. The role of B cells is further discussed in Section 1.2.3.

Conventional CD4 T cells are also able to provide help for CD8 T cell activation via APCs that co-express both CD4 and CD8 T cell epitopes [81, 82] in the same fashion as direct pathway allorecognition, where donor APCs expressing class I and class II antigens are able simultaneously to engage with CD4 and CD8 T cells in a 3-cell cluster. In the context of transplantation, the mechanism of how indirect CD4 T cells provide help for activation of direct CD8 T cells [83] is not clear. An alternative pathway called semi-direct pathway of allorecognition has been most recently described, and is based on the ability of dendritic cells to acquire intact MHC-peptide complexes from other dendritic cells or endothelial cells [84]. The semi-direct pathway postulates that recipient DCs acquire donor MHC-peptide complexes by membrane capture, resulting in chimeric recipient DCs that co-express intact donor class I and class II molecules as well as self-MHC molecules armed with processed donor MHC peptides. Under these circumstances, the recipient DCs are able to simultaneously activate both the recipient CD4 and CD8 T cells with direct allospecificity and the recipient CD4 T cells with indirect allospecificity, thus allowing delivery of help for direct CD8 T cell activation [58, 85]. The significance of the semi-direct pathway in allograft rejection is not established, but it could provide sustained stimulation of directly reactive CD4 and CD8 T cells, even after donor APC have diminished. Nevertheless, the evidence of direct CD8 T cell involvement in the development of chronic rejection is limited [86].

1.2.2.3 Lung allograft rejection

Based on the time of onset, allograft rejection is classified as hyperacute, acute and chronic (Figure 1.1). Hyperacute rejection develops within minutes to hours after transplantation, as a result of recipient pre-sensitisation to donor tissue antigens [87] or as a result of preformed antibodies against ABO blood group antigens [88]. Binding of the preformed antibodies to ABO and/or MHC antigens expressed on the graft endothelial cells activate the classical complement pathway, resulting in edema, graft cell death, recruitment of inflammatory cells, platelet accumulation and microvascular thrombosis. The characteristic features of hyperacute lung rejection include small vessel vasculitis, intra-alveolar hemorrhage, platelet and fibrin thrombi, capillary congestion with neutrophils and macrophages and antibody deposition [89]. These processes lead to graft ischemia, necrosis and graft failure; in some cases fatal outcomes have been reported. Historically, hyperacute rejection has mainly been observed in kidney [90] and heart transplant recipients [91]. Nevertheless, to date seven cases of hyperacute rejection have been reported in lung transplant recipients of whom 6 have resulted in death occurring within 4 to 77 hours after transplantation [89, 92-94]. Nowadays, hyperacute rejection occurs rarely due to widespread use of very sensitive antibody screening methodologies that can detect and identify recipients preformed circulating DSA and confirmatory ABO compatibility testing.

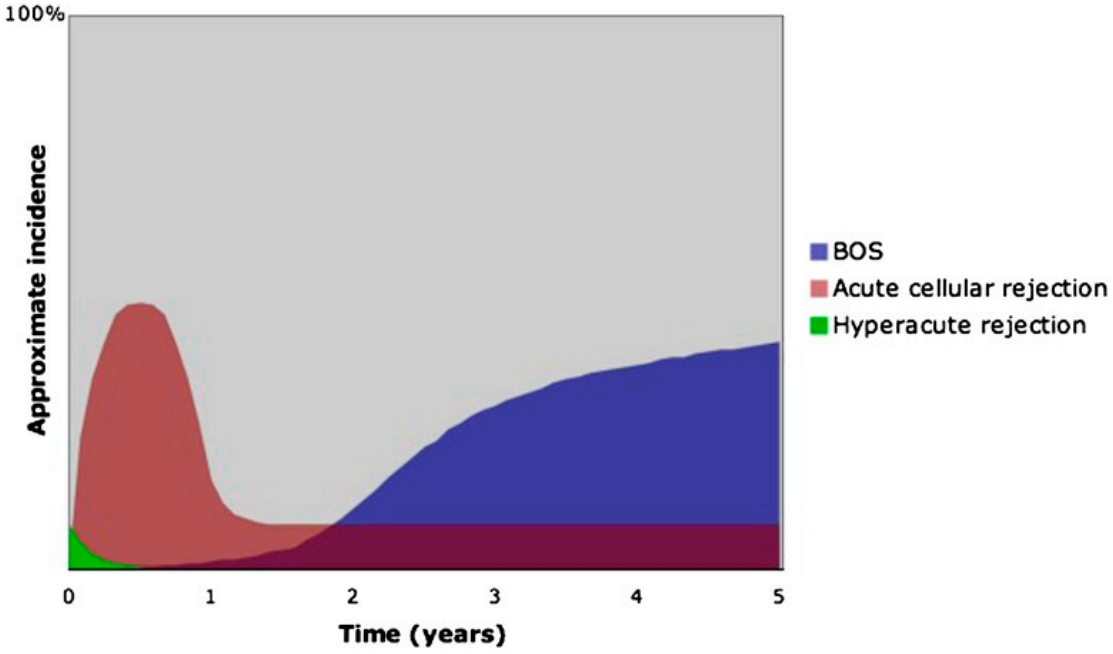


Figure 1.1 Time of onset and type of rejection in lung transplant recipients. (Image adapted from Martinu et al., 2009)

1.2.2.3.1 Characterisation of acute rejection in lung transplant recipients

Despite the significant advances in the development of highly potent immunosuppression regimens, acute allograft rejection affects up to 55% of lung transplant recipients within the first year after transplantation [6]; and, represents the most significant risk factor for development of BOS.

Acute rejection can occur within a few days to years after transplantation, mainly as a consequence of T cell alloresponse to foreign MHC antigens (acute cellular rejection (ACR)) and/or activation of humoral immune responses and production of alloantibody with specificity to donor HLA antigens (acute antibody-mediated rejection (AAMR)). In clinical settings usually a mixture of both effector arms (cellular and humoral) are observed in recipients experiencing acute rejection.

In lung transplantation, acute cellular rejection is characterised by lymphocytic perivascular or peribronchiolar mononuclear cell infiltrates in the lung tissue [95]. Due to the non-specific nature of the symptoms diagnosis of AR in lung transplant recipients is not always clear, thus firstly it is important to exclude symptoms associated with infection rather than rejection. Amongst various tests available bronchoscopy with transbronchial biopsy has proven to be the most accurate test for diagnosis of acute cellular rejection [95]; its significance has been highlighted by the fact that a relatively high percentage of Grade A2 acute rejection has been detected in asymptomatic patients during surveillance bronchoscopies [96]. The clinical significance of asymptomatic low-grade acute rejection is considered as not harmful in long-term outcomes. In a prospective study of 184 lung transplant recipients Hopkins et al., have confirmed A1 histological lesions in 279 biopsies sampled from 128 recipients; of those only 24 A1 biopsies were symptomatic. Detailed histopathologic features for diagnosis and grading of acute lung rejection are presented in Table 1.1.

The pathophysiological changes associated with humoral rejection are less clear and in some cases inconclusive due to the fact that not all features of

AMR may be present at one time point making diagnosis very challenging. Numerous studies evaluating the immunoglobulin and complement deposition in the subendothelial space have shown that detection of complement products C1q, C3d and/or C4d are associated with presence of HLA alloantibodies, allograft dysfunction and development of BOS [97, 98]. However, evidence of C3d and C4d deposition without the presence of HLA antibodies has been reported in lung transplant recipients with non-alloimmune lung injury including infection and primary graft dysfunction [98]; thus making their appearance not very conclusive biomarker for AMR. In general, pathologic finding of small vessel intimitis or endothelialitis together with immunohistochemical staining for complement deposition, presence of DSA and allograft dysfunction is strong evidence for antibody-mediated rejection requiring treatment [99]. The classification of AMR is presented in Figure 1.2.

Category	Grade	Meaning	Appearance
A: acute rejection	0	None	Normal lung parenchyma
	1	Minimal	Inconspicuous small mononuclear perivascular infiltrates
	2	Mild	More frequent, more obvious, perivascular infiltrates, eosinophils may be present
	3	Moderate	Dense perivascular infiltrates, extension into interstitial space, can involve endothelialitis, eosinophils, and neutrophils
	4	Severe	Diffuse perivascular, interstitial, and air-space infiltrates with lung injury. Neutrophils may be present.
B: airway inflammation	0	None	No evidence of bronchiolar inflammation
	1R	Low grade	Infrequent, scattered or single layer mononuclear cells in bronchiolar submucosa
	2R	High grade	Larger infiltrates of larger and activated lymphocytes in bronchiolar submucosa. Can involve eosinophils and plasmacytoid cells.
	X	Ungradable	No bronchiolar tissue available
C: Chronic airway rejection – obliterative bronchiolitis	0	Absent	If present describes intraluminal airway obliteration with fibrous connective tissue
	1	Present	
D: Chronic vascular rejection – accelerated graft vascular sclerosis		Not graded	Fibrointimal thickening of arteries and poorly cellular hyaline sclerosis of veins. Usually requires open lung biopsy for diagnosis.

Table 1.1 Histopathological grading of lung rejection. (Table adapted from Martinu et al., 2009)

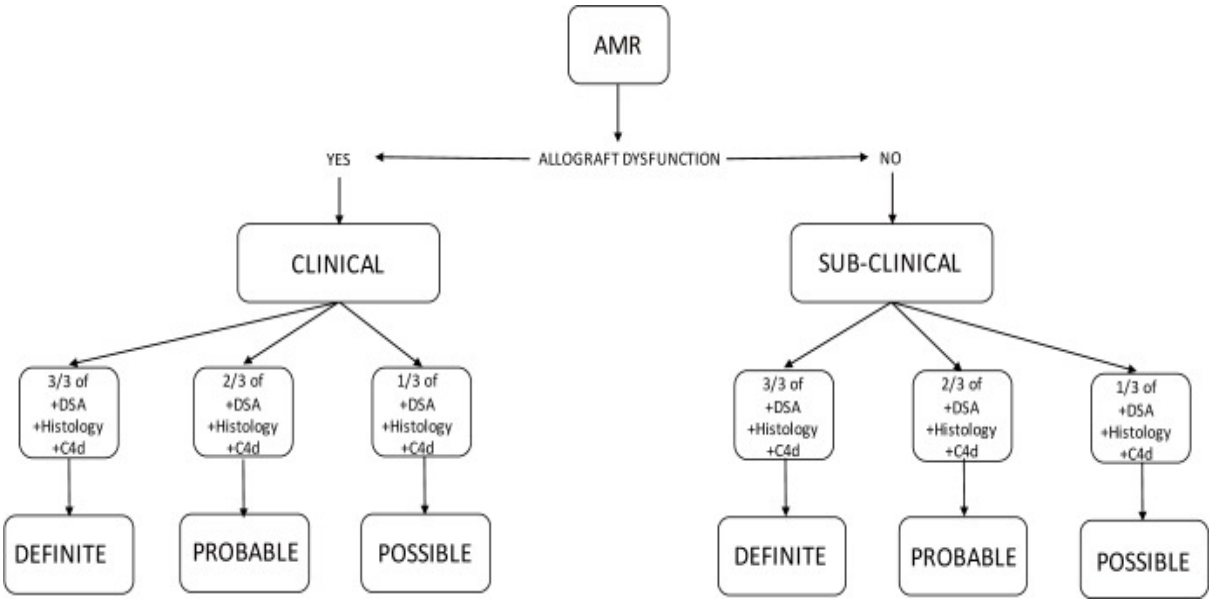


Figure 1.2 Antibody-mediated rejection (AMR) classification according to the presence or absence of diagnostic features and presence (clinical) or absence (sub-clinical) of allograft dysfunction. (Image adapted from Benzimra et al., 2017)

Treatment of lung acute cellular rejection consists of increased immunosuppression with pulse-steroids and in some transplant centres, in addition to steroid use treatment for acute cellular rejection incorporates monoclonal and/or polyclonal antibody therapy such as antithymocyte globulin (ATG), Basiliximab (anti-CD25 monoclonal antibody) and alemtuzumab (anti-CD52 monoclonal antibody) [100]. In comparison, humoral rejection is non-responsive to steroids or T cell depleting reagents [101]. Current treatment regimens for AMR incorporate plasmapheresis and intravenous immunoglobulin (IVIG) and possibly monoclonal antibodies such as rituximab [100]. Rituximab is an anti-CD20 monoclonal antibody that depletes B cell from the peripheral circulation but it has no effect on plasma cells due to the luck of CD20 cell surface expression. The clinical use of rituximab has been previously reviewed [102]. In lung transplantation, rituximab has demonstrated efficacy at reducing the development of HLA antibody post-transplant in patients that undergo humoral rejection [103]. Contrary to this, in a randomized controlled study the use of rituximab, as an induction therapy in

renal transplant recipients was associated with increased incidence of acute cellular rejection in comparison to the renal transplant control group that received daclizumab (anti-CD25 monoclonal antibody), (83% vs. 14%), possibly as a result of pan-B cell depletion including depletion of regulatory B cells at the time of transplantation [104].

1.2.2.3.2 Chronic allograft vasculopathy

Chronic rejection (CR) also known as chronic allograft vasculopathy (CAV) is the main factor that limits long-term graft survival. Chronic rejection develops within the relatively short period after transplantation (months to years) and typically is characterised by the development of severe intimal hyperplastic lesions that consist of smooth muscle cells, macrophages, monocytes and T lymphocytes [105, 106]. The progressive luminal narrowing affects the vascular flow resulting in ischemic damage of the graft with organ-specific pathology. CR affects all allografts and its development is manifested as a gradual deterioration in function. CR affects up to 30% - 40% of heart allografts [107], 20% of kidney, up to 17% liver [108] and up to 55% of lung allografts [109] within 5 years after transplantation.

In lung transplantation chronic rejection is characterised by the development of obliterative bronchiolitis (OB) leading to progressive airway obstruction and deterioration in pulmonary function, a condition known as Bronchiolitis Obliterans Syndrome (BOS) [110]. BOS was first described at Stanford University in heart-lung transplant recipients who developed a progressive deterioration in forced expiratory volume in 1 s (FEV1) [111]. Development of BOS is a major post-transplant complication in lung transplant recipients and is a leading cause of death. The median time of diagnosis is 16 to 20 months after transplantation. BOS affects more than 20% of recipients one year after transplantation, and up to 60% of recipients who survive 5 years after transplantation. It accounts for 30% of all deaths [112] and survival at 5 years after transplantation is 20% to 40% lower in recipients that develop BOS in comparison to recipients that remain free from the disease [112].

BOS is a disease of multifactorial etiology; it is believed that it represents the end result of repeated immune and non-immune related injury to the allograft. Table 1.2 summarises the probable and potential risk factors associated with development of BOS [113].

Probable	Potential
Acute rejection	CMV infection (without pneumonitis)
CMV pneumonitis	Donor antigen-specific activity
HLA mismatching	Epstein-Barr virus reactivation
Lymphocytic bronchitis/bronchiolitis	Etiology of native lung disease
Noncompliance with medications	Gastroesophageal reflux
Primary graft dysfunction	Older donor age
	Pneumonia (bacterial, viral, fungal)
	Ischemia-reperfusion injury
	Recurrent infections

Table 1.2 Probable and potential risk factors associated with development of bronchiolitis obliterans syndrome. (Table adapted from Hayes, D., 2011)

Diagnosis of BOS is based on histopathologic features and pulmonary function tests. Nevertheless, due to the patchy nature of the disease, BOS is difficult to diagnose using transbronchial biopsy, thus BOS is defined as irreversible decline in FEV1 of at least 20% of the baseline FEV1 levels, according to the 1993 classification grading system of the International Society of Heart and Lung Transplantation (ISHLT) [110]. The 1993 ISHLT classification system was modified in 2002 and incorporated measurement of midexpiratory flow (FEF_{25-75}) of > 70% of baseline to be more sensitive for early detection of BOS [114]. In the latest revision of ISHLT classification system the ISHLT, American Thoracic Society and European Respiratory Society committee members have acknowledged that a substantial cohort of patients and histopathological changes do not fit the previous definition of BOS. It is expected that a broad definition of chronic lung allograft dysfunction (CLAD) together with the recognition of different clinical entities may clarify the pathophysiologic mechanisms that lead to CLAD. These may lead to new strategies for patient management, which may improve long-term survival

after lung transplantation. However, this classification has not been approved yet, therefore, in this thesis patient graft dysfunction is defined as BOS according to the ISHLT classification system of 2002 (Table 1.3) [113].

BOS Stage	Classification
0	FEV1 > 90% of baseline & FEF25-75% > 75% of baseline
0-p*	FEV1 81-90% of baseline &/or FEF25-75% ≤ 75% of baseline
1	FEV1 66-80% of baseline
2	FEV1 51-65% of baseline
3	FEV1 ≤ 50% of baseline

*0-p = potential BOS,

Table 1.3 Bronchiolitis obliterans syndrome classification. (Table adapted from Hayes, D., 2011)

1.2.3 Humoral responses

The contribution of humoral responses in the development of BOS is increasingly recognised. B cells play a central role in orchestrating the humoral immune response in multiple ways: alloantibody production and differentiation into antibody-producing plasma cells, maintenance of long-term humoral memory, antigen presentation, formation of tertiary lymphoid organs (TLOs) and secretion of pro- and anti-inflammatory cytokines. A brief description of the B cell subsets, B cell development, B cell activation and antibody production will be discussed next. The clinical significance of allo- and autoantibody in solid organ transplantation and the mechanism of antibody-mediated injury are described in Chapter 6.

1.2.3.1 B cell subsets

B cells originate from the pluripotent hematopoietic stem cells in the bone marrow where they undergo several developmental stages involving rearrangement of immunoglobulin gene region and production of functional membrane bound B-cell receptor (BCR) [115]. Immature B cells characterised by cell surface expression of $CD21^{low}$ $CD23^{low}$ IgD^{low} IgM^{high} HSA^{+} [116, 117] are released from the bone marrow and join the transitional B cell compartment in the spleen where they continue their maturation process and differentiate into two distinct populations; follicular (FO) B cells and marginal zone (MZ) B cells that mediate T-dependent and T-independent responses [118], respectively, both constitute B2 lineage. MZ B cells are retained in the spleen, whereas FO B cells recirculate through the blood and lymphatic system [119].

To ensure generation of mature B cells that are non-responsive to self-antigens, developing B cells undergo positive and negative selection via antigen-independent and antigen-dependent signaling pathway, respectively. There are four possible fates for developing B cells bearing BCR that strongly binds to self-antigens: clonal deletion (cell death by apoptosis), receptor

editing (process that initiates further light chain gene rearrangement and replacement of self-reactive BCR with new non-self reactive BCR), cells enter the state of anergy or unresponsiveness (self reactive but unresponsive BCR) and immunological ignorance [115], Figure 1.3.

Another B cell subpopulation derived from B2 lineage has been described, called regulatory B cells or Bregs. These cells have been described as high IL-10 producers and may act in an immunosuppressive fashion to provide control in autoimmune disease [120, 121], regulation of germinal centre reaction and in transplant settings they may modulate allograft immune responses [120].

The second lineage of B cells, called B1 cells, derive from B1 progenitors in fetal livers and reside in the peritoneal and pleural cavity. B1 cells produce polyreactive natural IgM antibodies directed against T-independent antigens and they do not require T cell help to elicit antibody production [122]. B1 cells secrete antibodies against ABO blood group antigens [123], and polyreactive IgA antibodies that contribute to mucosal immunity [124].

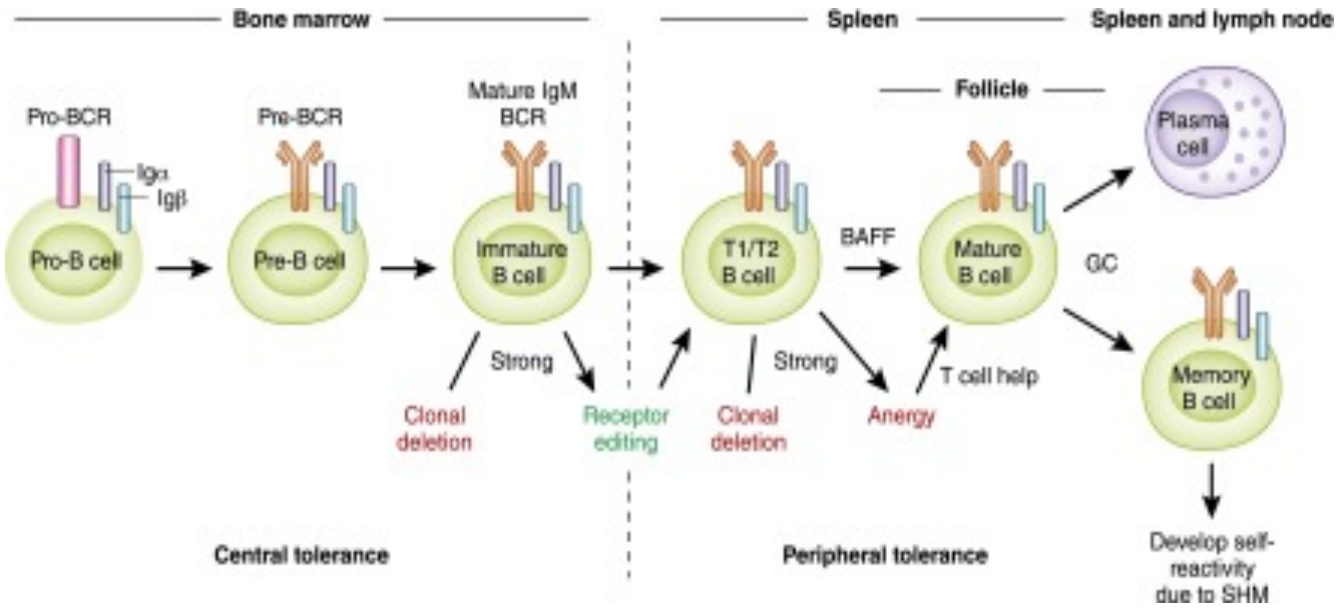


Figure 1.3 B cell development and mechanisms of tolerance to self-antigens.

B cells originate in the bone marrow (BM) where they undergo three developmental stages (pro-B, pre-B and immature B cell). In the BM immature B cell with strong reactivity to self-antigens undergo clonal deletion or receptor editing (process that initiates further light chain gene rearrangement and replacement of self-reactive BCR with new non-self reactive BCR). Immature B cells expressing B-cell receptor (BCR) that are not self-reactive are released from the BM to enter the transitional B-cell pool in the spleen where they undergo further differentiation into mature B cells. In the periphery transitional 1 and 2 (T1/T2) B cells with strong self-reactivity are clonally deleted or enter the state of anergy or unresponsiveness (self reactive but unresponsive BCR). Non self-reactive B cells develop into mature B cells that can recirculate through the blood and lymphatic system where they can encounter antigen and enter the secondary lymphoid organs (SLOs) through the T cell-rich area of the spleen known as periarteriolar lymphoid sheath (PALS) to eventually reside in the germinal center (GC) B cell follicle. The GC reaction gives rise to isotype-switched memory B cells and long-lived antibody producing plasma cells. (Image adapted from Hoffman, W. et al., 2016)

1.2.3.2 B cell activation and antibody production

Mature naïve B cells continuously recirculate through the peripheral blood and lymphatic system and enter secondary lymphoid organs (SLOs) through the T cell-rich area of the spleen, known as periarteriolar lymphoid sheath (PALS), to gain access into B cell follicle where they can encounter antigen and interact with follicular DCs (FDCs) and sinus-associated macrophages [119].

Naïve B cell activation requires two signals: the first signal is received via BCR signaling pathway that is initiated by receptor aggregation due to antigen binding and the second signal is received through cognate interaction with follicular CD4 T cells at the T-B cell border. Upon activation B cells can differentiation into extrafollicular or germinal center (GC) driven memory B cells and long lived plasma cells (LLPCs) [125].

Signaling through the BCR activates members of the receptor associated Src family of protein tyrosine kinases such as Blk, Fyn and Lyn [126]. Activated kinases phosphorylate the BCR Ig-α and Ig-β cytoplasmic tail carrying tyrosine-based activation motifs (ITAMs), which allows recruitment and activation of cytosolic protein kinase Syk [127]. Syk phosphorylates target proteins to initiate a cascade of intracellular signaling pathways leading to calcineurin activation and NFAT translocation to the nucleus, activation of MAPK/ERK pathway and activation of Ca2+-dependent protein kinase that activates NF-κB1 pathway [127].

These signals initiate metabolic and transcriptional changes resulting in BCR-antigen complex internalisation and delivery into the endosome, endosomal antigen processing (antigen is degraded into peptides and loaded onto MHC class II molecules), upregulation of MHC class II/peptide complexes expressed on the cell surface and gene transcription leading to induction of co-stimulatory molecules that enable B cell to receive stimulatory signals through cognate interaction with Tfh [125].

T-cell help to B cells is provided via engagement of B cell MHC class II-peptide complex with TCR and ligation of co-stimulatory molecules such as CD40L-CD40, B7 (CD86)-CD28, ICOSL-ICOS, PDL-1PD-1 and IL-21R-IL21 which leads to formation of immunological synapse. Upregulation of the transcription factor B-cell lymphoma 6 (Bcl-6) directs B cell migration back to the follicle where B cells rapidly proliferate and produce activation-induced cytidine deaminase (AID), an enzyme which is essential for somatic hypermutation (SHM), class-switch recombination, affinity maturation and generation of high-affinity class-switched memory B cells and antibody producing plasma cells [128].

The clinical importance of allo- and autoantibody in solid organ transplantation and the mechanisms of antibody-mediated injury are described in Chapter 6.

1.3 Chimerism in solid organ transplantation

Significant numbers of donor-derived haematopoietic cells that are not flushed from the graft during retrieval are transferred to the recipient at the time of transplantation. The presence and co-existence of these cells in the blood and tissues of allograft recipients forms a state called chimerism. Thus, chimerism refers to co-existence of two or more genetically distinct cell populations. Depending on the number of chimeric cells present; the terms micro- and macrochimerism have been used to describe chimeric population present at less than 1% or greater than 1% of the total number of cells, respectively [129].

Evidence for presence of donor chimerism in the circulation of solid organ allograft recipients was first demonstrated with karyotyping studies in female liver transplant recipients who have received an organ from a male cadaveric donor [130, 131]. Donor leukocyte microchimerism has been documented in human liver [130], kidney [132, 133], lung and heart [134] transplant recipients, intestinal and multivisceral transplantation [135].

In solid organ transplantation presence of donor microchimerism includes a population of donor T cells [132]; their possible existence is supported by the recent demonstration that T cells migrate through non-lymphoid organs [136]. Functionally and phenotypically, these cells comprise a mixture of naïve, effector and memory T cells [136].

The number and type of lymphocyte distribution present in non-lymphoid organs has been studied in discarded human hearts and lungs. Analysis of mononuclear cell distribution in donor heart tissue has demonstrated that CD4 T cells are the most common cell type of all leukocytes present in the tissue; and the majority of this cell population expressed high levels of CD45RO cell surface molecule [137], which represents a marker of memory T cells [138].

Furthermore, Richter et al., have studied passenger mononuclear cells in both the human donor lung tissue itself and the lung associated regional lymph nodes [139]. They have identified two main sources of lymphocytes; one derived from the lung associated lymphoid tissue (LALT) mainly comprising of resting T and B cells and the second cell populations derived from the lung tissue itself, comprising of activated lymphocyte and monocytes/macrophage population expressing high levels of HLA-DR and CD45RO markers; and adhesion molecules including LFA-1, VLA-4 and ICAM-1 [139], suggesting that these cells can migrate throughout the tissue.

Since the first evidence of donor chimerism in liver transplant recipients in 1969 [131], numerous studies have reported the presence of chimerism following solid organ transplantation; however, the precise role of its existence is unclear. In 1992, Starzl et al*.,* postulated that transfer of leukocytes from the donor can create a state of systemic mixed allogeneic chimerism that, if persistent, promotes graft acceptance and resistance to cellular and humoral rejection [131]. Despite these observations, the role of passenger leukocytes in solid organ transplantation remains unclear; and conflicting evidence has been reported that in some settings donor-derived passenger leukocytes can initiate graft rejection [140] whereas in others they can contribute to graft acceptance [130, 131]. Furthermore, donor chimerism can occasionally trigger

onset of graft-vs-host disease (GvHD) [141]. In solid organ transplantation, GvHD is a rare post-transplant complication; nevertheless, detrimental outcomes have been documented in liver and lung transplant patients [142, 143].

Win et al., in our department used a murine model of chronic heart graft rejection to study how donor CD4 T cells that were passengers within the heart graft influenced the host response to the graft. In Win's model, the donor bm12 strain varies from the C57BL/6 (B6) recipient by three amino acid residues in the MHC class II I-A antigen (human HLA-DQ). Bm12 heart allografts are rejected slowly (median survival time 50 days), and this rejection was associated with the development of graft vascular pathology consisting of progressive intimal thickening and luminal narrowing. In addition, there was histologic evidence of humoral vascular rejection, characterised by C4d complement and IgG endothelial deposition. No evidence of rejection was observed in syngeneic transplants or the bm12 allografts transplanted into either MHC class II gene knockout mice or B cell-deficient recipients. Further experiments revealed that the B6 recipients did not develop circulating IgG alloantibodies but instead developed long-lasting IgG antinuclear autoantibody [144].

To investigate the involvement of T-cell-dependent responses; B6 recipients were primed with synthetic allopeptide corresponding to the disparate region of the I-A^{bm12} antigen 14 days prior to transplantation with a bm12 heart allograft. Bm12 heart grafts were rejected more rapidly than in unprimed, control recipients, but without evidence of alloantibody development. Moreover, despite the rapid rejection, the levels of circulating IgG autoantibodies were not augmented. These experiments suggested that development of autoantibody is independent of indirect pathway T cell responses. Given that indirect-pathway is thought to be the only means by which CD4 T cell help for alloantibody production is provided [79, 80], this result was surprising and suggested that an alternative mode of help was responsible for the development of autoantibody in the bm12-B6 model. On the basis that adoptive transfer of bm12 splenocytes triggers lupus-like

humoral autoimmunity in B6 recipient mice [145], the potential role of passenger donor CD4 T cells in the provision of help to host B cells was investigated. Surprisingly, transplantation with heart allografts from donor bm12 mice that either were depleted of CD4 T cells prior to heart allograft procurement or were genetically deficient in T and B cells did not prompt autoantibody production and heart allograft survival was prolonged [144].

Win et al., concluded that in their model, help for autoantibody production was provided via a direct cognate interaction between the donor CD4 T cells and the recipient auto-reactive B cells, thereby replicating the normal interaction between a self-restricted host helper CD4 T cell and B cell [144]. Win's studies further demonstrated that the autoantibody contributed to the development of chronic allograft vasculopathy.

The aim of my thesis was therefore to address whether a similar mechanism may occur in human transplant recipients. We chose to examine the role of donor CD4 T cells in lung transplant recipients, because the lung graft is a large and leucocyte rich organ. In assessing the potential contribution of donor CD4 T cells to human allograft rejection, it was first necessary to demonstrate the presence of donor CD4 T cells in the lung recipient's circulation following transplantation.

The question raised by our previous study is whether a similar mechanism is responsible for the development of autoantibody in human transplant recipients. To our knowledge, specifically the role of donor passenger CD4 T cells has not been formally studied in clinical transplantation.

1.4 Aims and objectives

Previous work in our department has highlighted a novel mechanism for how help for development of *de novo* autoimmunity following transplantation is delivered to recipient auto-reactive B cells. In Win et al., model help for autoantibody production was provided via a direct cognate interaction between the donor CD4 T cells passenger within the graft and the recipient auto-reactive B cells. Thus, the overall objective of my work was to assess whether a similar mechanism may occur in human lung transplant recipients.

My first aim was to assess the presence and longevity of donor CD4 T cell chimerism in the peripheral blood of primary lung transplant recipients by targeting the donor HLA class I (HLA-A and/or HLA-B) mismatched antigens with human monoclonal HLA antibodies.

Furthermore, I wanted to investigate whether the number of HLA mismatched antigens between the recipient and donor pairs and the recipient-*vs*-donor NK cell alloreactivity affects the donor CD4 T cell dynamics. Along with this, I wanted to also characterise the donor-derived CD4 T cell subsets using RT-PCR gene expression analysis of flow-sorted donor CD4 T cells.

My third aim was to investigate the humoral allo- and auto-immune responses in lung transplant recipients and their possible correlation with the presence of donor CD4 T cell chimerism; and, in turn, how this influences the development of BOS. In parallel to this, I aimed to evaluate clinically relevant autoantibody profile in long-term survivors of lung and heart and lung transplant recipients with functioning allograft and free from BOS and recipients with established BOS by utilizing high-density protein arrays for autoantibody profiling. The protein arrays have the potential to serve as a tool to identify new biological targets that can be used to identify patients that are more likely to undergo chronic rejection.

2 Chapter 2

Materials and Methods

2.1 Study design and participants

This is a cohort observational study. Prior to commencement the Cambridgeshire 4 Ethics Committee, Papworth Hospital Research and Development Department and the University of Cambridge Research and Development Department ethically approved the study. In addition, this study was registered with the NIHR Clinical Research Network Portfolio.

All patients waiting for primary deceased donor lung or heart and lung transplantation at Papworth Hospital, Papworth Everard, Cambridge, United Kingdom who fulfilled the study participant inclusion criteria were given written and verbal information about the study. In addition, patients that had undergone lung or heart and lung transplantation prior to commencement of the study were also informed.

Study Participant Inclusion Criteria:

- Participant is willing and able to give informed consent for participation in the study.
- Male or Female, age 16 years or above.
- Diagnosed with lung or heart and lung failure, and listed for primary lung or heart and lung transplantation; or have undergone primary lung or heart and lung transplantation prior to commencement of the study.

Patients who had already had two or more transplants were not eligible for participation in this study.

All participants that took part in this study gave full, informed written consent for the clinical intervention, experimental investigations and collection of demographic and clinical information from patients' medical records and electronic databases. A copy of the Participant Information Sheet and Consent Form is attached as Appendix 1.

The study cohort consists of two groups of patients:

- Pre-transplant group - patients were recruited whilst waiting for deceased donor lung or heart and lung transplantation at Papworth Hospital.

- Post-transplant group - patients were recruited after primary lung or heart and lung transplantation. All patients were transplanted at Papworth Hospital between 1986 and 2011.

2.2 Blood collection

Patients' peripheral blood was obtained by venipuncture. Blood samples (40 ml) for cell separation were collected in 10 ml Coagulation Sodium Citrate S-Monovette® (0.106 molar solution) tubes (SARSTEDT, S-Monovette®, Germany). Blood samples (10 ml) for serum separation were collected in 9 ml serum sample tubes (SARSTEDT Natural S-Monovette®, Germany).

2.3 Cell and serum preparation

All cell preparation techniques were performed using Lympholyte®-H density gradient (1.0770 ± 0.001 g/cm3 at 22°C) separation medium (Cedarlane® Laboratories Ltd., Burlington, Canada), referred to as "separation medium" unless otherwise stated. Centrifugation was performed in a Megafuge 1.0R (Heraeus Instrument, DJB Labcare Ltd., UK). Following separation the cells were re-suspended in RPMI 1640 (Roswell Park Memorial Institute) tissue culture medium (Gibco^{TM}, Invitrogen, Paisley, UK) with 10% fetal calf serum (FCS) (Biowest Ltd., Ringmer, East Sussex, UK), referred to as "culture medium" unless otherwise stated. Cells were counted in Neubauer cell counting chambers (Hawksley, Lancing, Sussex, UK), Section 2.3.5.

2.3.1 Density gradient separation of human peripheral blood mononuclear cells (PBMC)

PBMC were separated using separation medium specifically designed for the isolation of viable lymphocytes from human peripheral blood. The blood was diluted with phosphate buffered saline (PBS) (ratio 1:1) and layered over 10 ml of cell separation medium in a 50 ml centrifuge tube. Centrifugation was performed for 20 minutes (mins) at 800g. After centrifugation, the lymphocyte layer at the interphase was aspirated and transferred into a 15 ml centrifuge tube. The cells were washed with PBS and further centrifuged at 1000g for 10 mins. Supernatant was discarded and the cells were re-suspended and washed in PBS at 600g for 5 mins. Finally, the supernatant was discarded and the cells were re-suspended in 5 ml culture medium for further analysis.

2.3.2 Splenocytes separation

Surplus donor spleen samples were obtained from the Tissue Typing Laboratory, Addenbrooke's Hospital, Cambridge, UK (Addenbrooke's Tissue Typing Laboratory). The spleen was cut into pieces and flushed with 40 ml culture medium using a 20 ml syringe with needle. The cell suspension was collected and the splenocytes were separated in the same manner as PBMC separation protocol (Section 2.3.1).

2.3.3 Lymph node cell separation

Surplus donor lymph node samples were obtained from Addenbrooke's Tissue Typing Laboratory. The lymph nodes were flushed with 40 ml culture medium using a 20 ml syringe. The cell suspension was collected into 15 ml centrifugation tubes and centrifuged for 10 min at 800g. After centrifugation, the supernatant was discarded and the cells were re-suspended and washed with PBS for 5 min at 600g. Finally, the supernatant was discarded and the cells were re-suspended in 5 ml culture medium for further analysis.

2.3.4 Separation of passenger mononuclear cells from the *Ex Vivo* Lung Perfusion (EVLP) leucocyte filter

The EVLP leucocyte filter is attached to the EVLP circuit during the whole procedure; at the end of the procedure, the leukocyte filter was removed and stored overnight in RPMI media at 4°C. Mononuclear cells were flushed out of the filter with 40 ml cold culture medium (RPMI) using a 20 ml syringe. The cell suspension was collected into 15 ml centrifugation tubes and centrifuged for 10 min at 800g. After centrifugation, the supernatant was discarded and the cells were re-suspended and washed with PBS for 5 min at 600g. Following the washing step the mononuclear cells were re-suspended in 5 ml culture medium and cell count was performed (Section 2.3.5). Flow cytometric analysis was performed to characterise the mononuclear cells (Section 2.4.4).

2.3.5 Cell count

Cells were counted in Neubauer cell counting chambers (Hawksley, Lancing, Sussex, UK). 5 µl of cell suspension was diluted in 95 µl of 0.4% Trypan Blue cell counting solution (ThermoFisher Scientific, CA, USA; live cells do not take Trypan Blue), dilution factor 1:20. Using a pipette, 10 µl of Trypan Blue-treated cell suspension was applied to the Neubauer chamber counting grid and the Neubauer chamber was placed on the microscope stage. Unstained live cells were counted in 5 big squares. The number of cells in the original cell suspension was calculated by the following formula: Cell/ml = Number of cells counted x 10,000 (grid volume) x 20 (dilution factor).

2.3.6 Serum separation

Blood samples obtained for serum separation were centrifuged for 10 mins at 400g. Without disturbing the cell layer the serum was aspirated, transferred into 2 ml micro tubes (SARSTEDT Micro tube 2ml, Germany) and stored at -80°C until further analysis.

2.4 Flow cytometry

Flow cytometric analysis was carried out using 5 x 10^5 target cells per flow cytometry tube (BD Falcon, Franklin Lakes, NJ, USA unless otherwise stated). Cells were washed with 1 ml FACS buffer (PBS + 0.1% sodium azide + 0.1 % bovine serum albumin (BSA), Sigma-Aldrich, St. Louis, MO, USA). Staining with monoclonal antibodies (mAb) was carried out at 4°C for 30 mins, in the dark. After staining was completed, cells were re-suspended in FACS buffer for flow cytometric analysis. Cells were analysed fresh (without fixatives) using BD FACSCantoTM flow cytometer with BD FACSDiva software (both Becton Dickinson, San Jose, CA, USA unless otherwise stated). Specific flow cytometry protocols are described below.

2.4.1 Selection of human monoclonal HLA antibody using flow cytometric analysis

Donor HLA class I mismatched antigens were used as target antigens to distinguish between recipient and donor cells using biotin-conjugated human monoclonal HLA antibody (Table 2.1); a kind gift of Prof Frans H.J. Claas and Dr Arend Mulder (Leiden University Medical Center, Leiden, Netherlands). The monoclonal HLA antibodies were supplied as purified biotinylated immunoglobulins. In brief, human hybridomas were established from B-lymphocytes of HLA antibody-seropositive, multiparous women by EBV transformation. The HLA specificities of mAbs were determined by complement-dependent cytotoxicity against a panel (n > 240) of serologically HLA typed PBMCs. The specificity of HLA mAb was also tested using recipient peripheral blood samples obtained prior to transplantation and donor lymphocytes obtained from spleen or lymph nodes at the time of donation.

In brief, cells were stained with 10 µl of biotin-conjugated human HLA mAbs, followed by APC-conjugated streptavidin (Table 2.1). Cells were washed twice with FACS buffer before and after staining with APC-conjugated streptavidin.

Flow cytometric analysis was performed using BD FACSCantoTM flow cytometer with BD FACSDiva software. Antibodies that bind to more than 95% of donor lymphocytes, but did not bind to the patient lymphocytes obtained before transplantation, were selected, and used for detection of donor CD4 T lymphocytes in the patients' peripheral blood after transplantation (Figure 2.1).

Table 2.1 Panel of biotin-conjugated human HLA monoclonal antibodies used for detection of donor CD4 T lymphocytes in recipients' peripheral blood after transplantation.

Clone-	HLA-Specificity	Lot No.	Concentration µg/ml
BVK1F9	B8	6482	50µg/ml
DK7C11	B12	6484	50µg/ml
GV5D1	A1/A9 (not A*24:03, A80)	6910	50µg/ml
MUS4H4	Bw4/A24/A25/A32	6483	50µg/ml
SN230G6	A2/B17	6877	50µg/ml
SN607D8	A2/A28	6876	50µg/ml
BVK5C4	A9	6743	50µg/ml
JOK3H5	B40/B21/B13/B12/B41/B70	6746	50µg/ml
BRO11F6	A11/A3/A24	6874	50µg/ml
HDG8D9	B51/B35	6875	50µg/ml
VTM1F11	B27/B7/B60	6911	50µg/ml
IND2D12	B15/B35/B21/B70	6747	50µg/ml
OK2F3	A3	6748	50µg/ml
OK6H10	B15/B21/B56/B35/B72	6744	50µg/ml

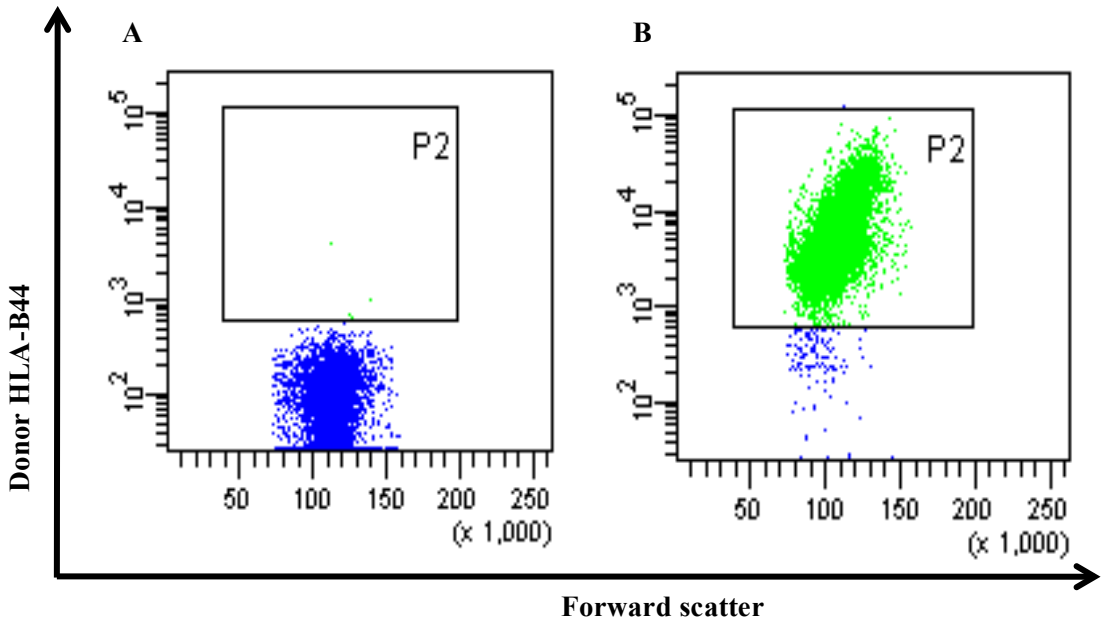


Figure 2.1 A representative graph of human HLA monoclonal antibody testing using flow cytometry. A) Recipient and B) donor lymphocytes obtained prior to transplantation were stained with biotin-conjugated human HLA mAbs with specificity to donor HLA-B44 antigen. HLA-B44 expressing cells were detected with APC-conjugated streptavidin.

2.4.2 Flow cytometric analysis for detection of donor CD4 T lymphocytes in patients' peripheral blood after transplantation (Chimerism analysis)

Patients' PBMC were separated as described previously (Section 2.3.1). Cellular Fc receptor non-specific antibody binding was blocked with purified anti-FcγR antibody; cells were incubated in the dark at 4°C for 15 mins (1:10 dilution). Following Fc receptor blocking step, cells were washed and re-suspended in FACS buffer at 5 x 10^5 cells per tube for each control test sample; the remaining cells were used for detection of donor CD4 T lymphocytes. Each control sample was incubated with: CD3 FITC-labeled, CD4 PE-labeled and double positive CD3-FITC/CD4-PE labeled primary anti-human monoclonal antibodies (Table 2.2). Patients' test samples obtained after transplantation, together with the patients' and respective donor cells obtained prior to transplantation, were incubated with primary biotin-conjugated human monoclonal HLA antibodies (Table 2.1); followed by APC-conjugated streptavidin (Table 2.2). The recipients' and respective donor cells

obtained prior to transplantation served as a negative and positive control, respectively. Lastly, the cells were incubated with CD3 FITC-labeled and CD4 PE-labeled mAbs. Cells were washed twice with FACS buffer before and after staining. The control samples were re-suspended in 300 µl of FACS buffer, while the test samples were re-suspended in FACS buffer at 1 x 10^7 cell per ml.

Table 2.2 Anti-human monoclonal antibodies used for detection of CD4 T lymphocytes using flow cytometric analysis.

Antibody	Conjugate	Clone	Working dilution	Source
FcγR	-	130-059-901	1: 10	Miltenyi Biotec Inc.
CD3	FITC	HIT3a	1:100	BD Pharmingen™
CD4	PE	RPA-T4	1:100	BD Pharmingen™
Streptavidin-	APC	-	1:1000	Invitrogen

The flow cytometric plot was set to include all PBMC; based on the forward and side scatter parameters lymphocyte population was gated and 20,000 cells were recorded for all control samples. Doublets were excluded. An additional gate was set to include cells stained positive for expression of CD3 and CD4 cell surface marker. Donor CD4 T lymphocytes were detected as a subpopulation of double positive (CD3/CD4) recipient T lymphocytes. Depending on the blood sample size, whenever possible 1,000,000 recipient CD4 T lymphocytes were recorded. The number of donor CD4 T lymphocytes detected in recipients' peripheral blood after transplantation is presented as a percentage of the total number of CD4 T cells analysed (Figure 2.2).

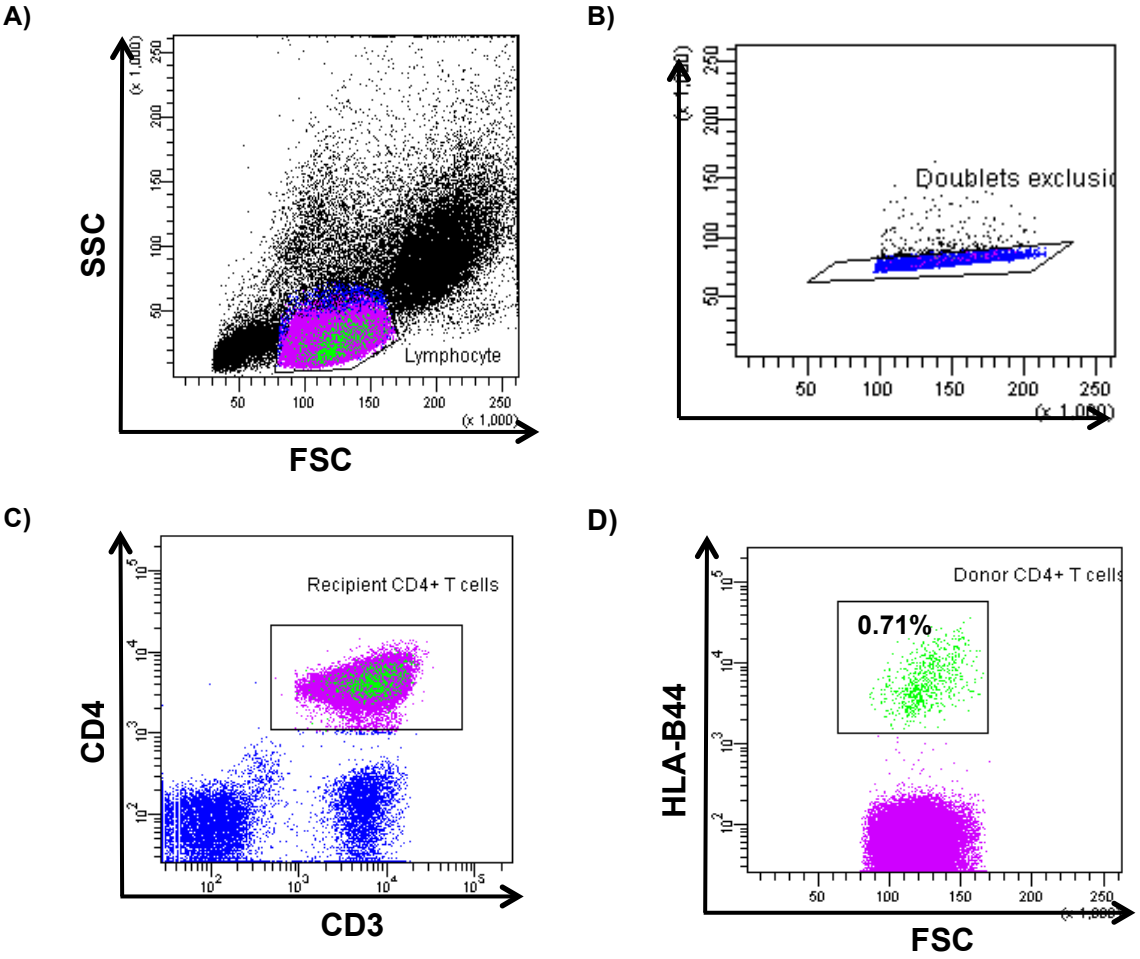


Figure 2.2 A representative graph of flow cytometric analysis used for detection of donor CD4 T lymphocytes in recipients' peripheral blood following primary lung transplantation.

Recipient PBMCs obtained after transplantation were stained with CD3-FITC, CD4-PE and donor specific biotin-conjugated human monoclonal HLA antibodies followed by APC-conjugated streptavidin and assessed by flow cytometry. A) Lymphocyte cell population was gated; B) Doublet cells were excluded from further analysis. C) Subpopulation of lymphocytes expressing cell surface CD3 and CD4. D) Donor CD4 T lymphocytes were detected as a subpopulation of double positive (CD3/CD4) recipient T lymphocytes. Depending on the blood sample size, whenever possible 1,000,000 recipient CD4 T lymphocytes were recorded. The number of donor CD4 T lymphocytes detected in recipients' peripheral blood after transplantation is presented as a percentage of the total number of CD4 T cells analysed.

2.4.3 Isolation of donor CD4 T lymphocytes from recipients' peripheral blood following transplantation

Recipients' PBMC obtained at regular time intervals after transplantation were processed as previously described in Section 2.4.2. Following cell preparation donor CD4 T lymphocytes were isolated using BD FACSAria™ III cell sorter and analysis was performed with BD FACSDiva software (both Becton Dickinson, San Jose, CA, USA). To discriminate dead cells, prior to donor CD4 T lymphocyte isolation the cell suspension was incubated with 10 µl of 7-aminoactinomycin D ((7-AAD), Invitrogen, CA, USA)) for 10 mins on ice, in the dark. Donor CD4 T lymphocytes that did not stain for 7-AAD (live cells) were collected in 500 µl RNA*later*® Solution (Ambion, Life Technologies, CA, USA) and stored at -20°C until further analysis.

2.4.4 Flow cytometric analysis of passenger mononuclear cells mechanically removed from the donor lungs by *Ex Vivo* Lung Perfusion procedure

Mononuclear cells isolated from the EVLP leucocyte filter, previously described in Section 2.3.4 were characterised using flow cytometry. In brief, mononuclear cells were simultaneously stained with seven fluorescent-labeled primary anti-human monoclonal antibodies specific for CD3-PE, CD4-FITC, CD8-APC, CD19-APC-Cy7, CD11b-Alexa Fluor-647, CD14-Brilliant Violet-421 and CD16-Brilliant Violet-510 (Table 2.3 and Table 2.5). In a separate test sample CD4 T lymphocyte population was characterised by simultaneous staining with seven fluorescent-labeled primary anti-human monoclonal antibodies specific for CD3-PE, CD4-Brilliant Violot-510, CD45RA-Brilliant Violet-650, CD45RO-APC-H7, CD62L-PE-CF-594, CXCR5-Alexa Fluor-488 and CCR7-Brilliant Violet-421 (Table 2.4 and Table 2.5). Cells were washed twice with FACS buffer and test samples were re-suspended in 300 µl of FACS buffer. Prior to analysis the test samples were incubated with 10 µl 7-AAD for 10 mins on ice, in the dark. LSR-Fortessa Flow Cytometer and BD FACSDiva software (both Becton Dickinson, San Jose,

CA, USA) were used for analysis of the mononuclear cell populations and CD4 T cell subsets.

Table 2.3 Anti-human monoclonal antibodies used for characterisation of mononuclear cells isolated from the EVLP leucocyte filter using flow cytometric analysis.

Antibody	Conjugate	Clone	Working dilution	Source
FcγR	-	130-059-901	1:10	Miltenyi Biotec Inc.
CD3	PE	HIT3a	1:100	BD Pharmingen™
CD4	FITC	RPA-T4	1:100	BD Pharmingen™
CD8	APC	RPA-T8	1:200	BD Pharmingen™
CD19	APC-Cy7	HIB19	1:200	BioLegend
CD14	Brilliant Violet-421	M5E2	1:50	BioLegend
CD16	Brilliant Violet-510	3G8	1:100	BioLegend
CD11b	Alexa Fluor-647	M1/70	1:200	BD Pharmingen™
7-AAD	PerCP-Cy5.5	-	-	BD Pharmingen™

Table 2.4 Anti-human monoclonal antibodies used for characterisation of CD4 T lymphocytes isolated from the EVLP leucocyte filter using flow cytometric analysis.

Antibody	Conjugate	Clone	Working dilution	Source
FcγR	-	130-059-901	1:10	Miltenyi Biotec Inc.
CD3	PE	HIT3a	1:50	BD Pharmingen™
CD4	Brilliant Violot-510	OKT4	1:50	BioLegend
CD45RA	Brilliant Violet-650	HI100	1:500	BioLegend
CD45RO	APC-H7	UCHL1	1:100	BD Pharmingen™
CD62L	PE-CF-594	DREG-56	1:500	BD Horizon™
CXCR5	Alexa Fluor-488	RF8B2	1:50	BD Pharmingen™
CCR7	Brilliant Violet-421	G043H7	1:50	BioLegend
7-AAD	PerCP-Cy5.5	-	-	BD Pharmingen™

Table 2.5 Function of the cluster of differentiation (CD) cell surface markers used for characterisation of mononuclear cells and CD4 T lymphocytes isolated from the EVLP leucocyte filter.

Cluster of Differentiation (CD3)	Function of the cluster of differentiation cell surface markers
CD3	T cell co-receptor associated with T-cell receptor; helps to activate naïve CD4 and CD8 T cells.
CD4	Glycoprotein predominantly found on the surface of T helper cells that serves as a co-receptor; interacts with β_2-domain of MHC class II molecules.
CD8	Glycoprotein predominantly found on the surface of cytotoxic T cells that serves as a co-receptor; interacts with α_3-domain of the MHC class I molecules.
CD19	Cell surface molecule expressed on B cells; acts as an adaptor protein to recruit cytoplasmic signaling proteins and is part of B cell receptor signaling complex.
CD14	Expressed mainly on monocytes/macrophages and neutrophils; acts as a co-receptor for detection of bacterial LPS.
CD16	Also know as FcyRIII expressed on the surface of NK cells, neutrophils, monocytes and macrophages; involved in ADCC.
CD11b	Protein subunit that forms heterodimeric integrin alpha-M beta 2 molecule also known as complement receptor 3. Expressed on monocytes, macrophages, granulocytes and NK cells.
CD45RA	Transmembrane signaling molecules that regulate cellular processes; expressed on naïve T cells.
CD45RO	Transmembrane signaling molecules that regulate cellular porcesses expressed on activated and memory T cells.
CD62L	Also know as L-selectin, a cell adhesion/homing receptor expressed on T cells; plays important role in T cell entry to secondary lymphoid tissue via high endothelial venules.
CXCR5	Expressed on T follicular helper cells; enables their migration to B cell follicles.
CCR7	T cell homing receptor to secondary lymphoid tissue such as lymph node and spleen.

2.4.5 Artificial chimerism

Artificial chimerism was created by mixing a known number of carrier cells (lymphocyte main cell population) and a known number of "chimeric cells". The HLA mismatched antigens were used as a target to detect chimeric cell (positive selection) or carrier cells (negative selection) using flow cytometric analysis, as previously described in Section 2.4.2. In the positive selection approach CD4 T cell chimerism was detected by targeting the HLA mismatched antigen expressed on the chimeric cell; and, in contrast, in the negative selection approach CD4 T cell chimerism was detected by targeting the HLA mismatched antigen expressed on the carrier cells. Table 2.6 shows the percentage of artificial CD4 T cell chimerism, number of chimeric cells per $1x10^6$ lymphocytes and percentage of chimeric CD4 T cell detected by both the positive and negative selection approach.

Table 2.6 Artificial chimerism.

Test tube No.	**Percentage of CD4 T cell chimerism in $1x10^6$ lymphocyte**	**Number of chimeric CD4 T cells in $1x10^6$ lymphocyte**	**Percentage of chimeric CD4 T cells detected (+ve selection)**	**Percentage of chimeric CD4 T cells detected (-ve selection)**
1	5%	50,000	4.8	4.89
2	2.5%	25,000	2.6	2.58
3	1.25%	12,500	1.3	1.35
4	0.625%	6250	0.7	0.73
5	0.316%	3125	0.41	0.39
6	0.156%	1562	0.21	0.22
7	0.078%	781	0.096	0.082
8	0.039%	390	0.067	0.053
9	0.019%	195	0.04	0.04
10	0.009%	97	0.001	0.005

2.5 Antibody detection

Recipients' sera were used for detection and characterisation of HLA and non-HLA antibodies. Specific protocols used are described bellow.

2.5.1 HLA alloantibody detection and characterisation using Luminex LABScreen® Mixed and LABScreen® Single Antigen HLA Class I and Class II Antibody detection beads

Patients' sera obtained prior to transplantation and at regular time points after transplantation were thawed and centrifuged at 13.000 rpm for 3 mins to remove any aggregates, before the start of the assay. A 96-well filter plate (Multiscreen HTSTM, Millipore Co, MA) was pre-wetted with 300 µl of wash buffer (LABScreen® Wash Buffer, One Lambda Inc., Canoga Park, CA, USA) and incubated at 22°C on a rotating platform (100 rpm) for 10 mins. Twenty µl of negative control serum (One Lambda Inc., Canoga Park, CA, USA) and patient serum sample was added to the appropriate wells of the plate. For detection and characterisation of HLA antibodies, 3 µl of LABScreen® Mixed beads or LABScreen® Single Antigen Class I or Class II beads, respectively, (both One Lambda Inc., Canoga Park, CA, USA) was added to each well and the plate was incubated at 22°C on a rotating platform (100 rpm) for 30 mins, in the dark. Following incubation, to each well 270 µl of wash buffer was added and aspirated using the vacuum manifold. Washing was repeated five times. To each well 60 µl of PE-conjugated goat anti-human IgG (One Lambda Inc., Canoga Park, CA, USA) was added; the plate was further incubated at 22°C on a rotating platform (100 rpm) for 30 mins, in the dark. Washing was repeated five times, as previously described. Eighty µl of PBS was added to each well. The beads were re-suspended on a rotating platform (100 rpm) for five mins. Data were acquired and analysed using Luminex LifeMatch flow analyser (LABScan TM 100) and Luminex XY platform (both Luminex Co, Austin, TX, USA).

2.5.1.1 Luminex data analysis

The patients' serum reactivity, i.e. the level of IgG antibody binding to each HLA coated bead was assessed using the normalised fluorescent intensity (MFI) signal obtained during the analysis. The MFI values for each bead were normalised and corrected for non-specific binding in relation to the negative control (NC) bead (#001) MFI value. For LABScreen® Mixed the normalised fluorescent signal equals the value of the Class I or Class II coated bead minus the value of the NC bead. For LABScreen® Single Antigen Class I and Class II the normalised fluorescent value for each HLA coated bead equals the value of each bead divided by the value of the NC bead.

For the purpose of this study MFI values ≥ 2,000 were considered positive. The MFI cut-off value was chosen based on the clinical experience of the Addenbrooke's Tissue Typing Laboratory over the past ten years of Luminex based HLA antibody screening. MFI values ≥ 2,000 are considered clinically relevant.

2.5.2 Detection of anti-nuclear antibodies (ANA) using HEp-2 Indirect Immunofluorescence (IIF) Assay

NOVA Lite® HEp-2 ANA Indirect Immunofluorescence (IIF) assay (INOVA Diagnostics, Inc., San Diego, CA, USA) was used for detection of anti-nuclear antibodies (ANA); all reagents required were supplied with the test kit. The assay was carried out according to the manufacturer protocol. In brief, patients' sera were thawed just before the assay and diluted (1:40) with PBS. Twenty µl of diluted patient serum sample, positive and negative control serum were added to the appropriate well of the HEp-2 slide and incubated for 30 mins in a moist chamber at room temperature. Following incubation, the slides were washed with PBS. Twenty µl of FITC-conjugated goat anti-human IgG was added to each well and incubated as previously described. The slides were washed again and one drop of mounting medium was applied to

each well. The slides were covered with a coverslip and for each test sera; three images were photographed using an ORCA-ER digital Camera (Hamamatsu Photonics, Japan) and IX81 Microscope with a 20x 0,70 UplanApo objective lens (Olympus, Japan). The fluorescent intensity was acquired with CellR 2.6 software (Olympus Soft Imaging Solutions GmbH, Germany). The mean fluorescent value was established for each test sera; and the MFI value is expressed as a percentage of IgG ANA binding to the HEp-2 cells in comparison to the MFI value of the positive control sample. The positive cut-off value was established as a MFI value of the negative control samples ± 2 x standard deviation (SD).

2.5.3 Detection of anti-nuclear antibodies (ANA) using HEp-2 Enzyme-Linked Immunosorbent assay (ELISA)

ANA-Hep2 ELISA microplate (AESKU Diagnostics, Wendelsheim, Germany) was also used for detection of ANA. The assay was carried out according to the manufacturer protocol. In brief, 100 µl of each test sera (diluted 1:101 in sample buffer), cut-off calibrator; negative and positive controls (in duplicates) were incubated in a 96-well ELISA microplate coated with lysed HEp-2 cells for 30 mins at room temperature, in the dark. All incubations were carried out under the same conditions. Following incubation, the microplate was washed three times with 300 µl wash buffer. To each well 100 µl anti-human IgG conjugated to horseradish peroxidise was added and incubated. The plate was washed as previously described and incubated with 100 µl TMB (3,3',5,5'-tetramethylbenzidine) substrate. After incubation, 100 µl of 0.16M sulfuric acid (stop solution) was added to each well and absorption was read in a FLUOStar OPTIMA ELISA plate reader (BMG Labtech, UK) at 450 nm. The mean optical density (OD) value was calculated for each test sera and the level of IgG ANA binding is expressed as an ELISA ratio (ER).

$$\mathrm{ER} = \frac{\text{OD value of the test sample} - \text{OD value of the negative control}}{\text{OD of the positive control} - \text{OD value of the negative control}}$$

The positive cut-off value was established as a MFI value of the negative control sample ± 2SD.

2.5.4 Autoimmune response profiling using Protein Microarrays for Autoantibody Characterisation

Invitrogen Human ProtoArray® v5.0 protoarray (Invitrogen, CA, USA) were used for characterisation of autoantibody profile in lung transplant recipients. Patients' serum samples were processed as previously described. Twenty patients were selected based on the clinical diagnosis for the presence or absence of Bronchiolitis Obliterans Syndrome (BOS) according to the International Society of Heart & Lung Transplantation (ISHLT) scoring criteria (10 patients free from BOS and 10 patients with established BOS grade 2 and grade 3). For each patient the autoantibody profile was established at the time of transplantation and at a single time point after transplantation, reflecting the patients' clinical status, i.e. presence or absence of BOS.

2.5.4.1 Protein microarray composition

The Invitrogen Human ProtoArray® v5.0 contains 9480 human proteins derived from Ultimate^{TM} ORF (open reading frame) collection. The proteins have been expressed in insect cells as glutathione-S-Trasferase (GTS) fusion proteins; and purified under native conditions. Each protein is spotted in duplicates onto a 1" x 3" glass slide coated with a layer of nitrocellulose. The proteins are printed in 110µm spots arrayed in 48 sub-arrays (4400-μm^2 each) and equally spaced in vertical and horizontal directions with 22 columns and 22 rows per sub-array. Each sub-array contains control proteins. Control proteins provide reference points for data acquisition and analysis.

1. Alexa Fluor® conjugated antibody. The fluorescent antibody signal indicates that the array has been properly scanned; and, used as a reference spots to orient the microarray.
2. Human IgG Signal. A protein gradient of purified human IgG is printed on each sub-array and serves as a positive control when anti-human IgG is used for detection. The human IgG signal is used to verify proper probing and detection reagents.

3. Anti-human IgG signal. A protein gradient of goat anti-human IgG is printed on each sub-array. The IgG from the human serum binds to the anti-human IgG on the array and is used to verify proper probing and detection reagents.

2.5.4.2 Protein microarray: Immune Response Biomarker Profiling (IRBP) – probing and scanning

The protein microarrays were used according to the manufacturer's recommendation. In brief, the slides were equilibrated at 4°C for 30 min. Upon removal from the mailer the arrays were placed in a 4-chamber incubation tray (Greiner Bio-One, Germany) and incubated with 5 ml blocking buffer (Invitrogen, CA, USA) for 1 hour at 4°C on a circular shaker at 50 rpm, in the dark. All incubations were carried out under the same conditions unless otherwise stated. After incubation, the blocking buffer was aspirated. The slides were washed with 5 ml of wash buffer for 5 min at 4°C on a circular shaker set at 50 rpm. The wash buffer was aspirated and the slides were incubated with 5 ml of patient serum sample (diluted 1:400 in wash buffer) for 90 min. After incubation the sample was aspirated and slides were washed with 5 ml wash buffer five times; as previously described. Human bound antibodies were detected after incubation with Alexa Fluor® 647 Goat Anti-Human IgG (diluted 1:2000 in wash buffer) for 90 min at 4°C. After incubation, the arrays were washed with 5 ml of wash buffer five times, as previously described and dried by centrifugation in a 50 ml conical tube at 200g for 1 min at room temperature. After drying, the slides were placed in a slide box protected from light. The scanning was performed immediately. Two negative control assays were performed in an identical manner to the experimental assays, except that the slides were incubated with buffer containing no serum, prior to incubation with Alexa Fluor® 647 Goat Anti-Human IgG detection antibody. To minimize variations between slides all assays were performed using arrays with the same lot specific number.

The scanning was performed using GenPix® 4000B (Molecular Devices Corporation) scanner at a wavelength: 635nm, PTM Gain: 800, Laser Power: 100%, Pixel Size: 10µM, lines to Average: 1.0 and Focus Position: 0µM. An image was acquired for each slide and saved as a .tiff file for data acquisition and analysis.

2.5.4.3 Protein array data acquisition

The acquired images with the GenPix® 4000B scanner required analysis prior to the data interpretation. GenPIx Pro 6.0 acquisition and analysis microarray software (Molecular Devices Corporation) were used for image analysis. Prior to the image analysis the .GAL (GenePix Array List) files describing the location and identity of all spots on the protein microarray was downloaded from the Invitrogen Online Tools www.invitrogen.com/protoarray containing the Lot Specific Information. The .GAL file allows a grid of spots to be overlaid onto the image. Once the grid is aligned for each spot, the array pixel intensity is acquired for each array. The GenPIx Pro 6.0 software generates a .gpr file that is required for data analysis using ProtoArray® Prospector v 5.2 software (free software for analysis of ProtoArray, Invitrogen, CA, USA).

2.5.4.4 ProtoArray® Prospector software data analysis

ProtoArray® Prospector v5.2 software is a data analysis tool that provides rapid interpretation of numerical data generated using Invitrogen Human ProtoArray® used for IRBP assay (Invitrogen, CA, USA; now provided by ThermoFisher Scientific, CA, USA).

The software is designed to identify proteins on the microarray (human protein features) that are bound to exogenous antibody (antibody present in the sera). Identification of proteins (positive targets) involves a 3-step process:

1. Single array analysis: for each protein on the array the software calculates the relative fluorescent signal. The fluorescent signal is then corrected for the background staining (protein fluorescent value minus the background fluorescent value). The corrected protein fluorescent value is expressed as Z-Factor. Z-Factor represents a confidence value that the signal for each protein on the array is significantly different from the negative control (cut-off value is 0.4).
2. Group characterisation: signals for each individual protein across all samples from a given population (patients with established BOS group and patients without BOS group) are aligned for downstream analysis.
3. Two groups comparison: identifies differences between two populations (BOS vs No BOS) using M-statistics. The M-statistics incorporates data normalisation analysis using Robust Linear Model (RLM). The RLM has been specifically developed for functional protein analysis [146].

2.5.5 Detection of RUNX1T1 antibody using RUNX1T1 Enzyme-Linked Immunosorbent assay (ELISA)

96-well ELISA plates (Immunlon^{TM} 2HBX, Thermo Fisher Scientific Inc.) were coated with RUNX1T1 (Runt-related transcription factor 1; translocated to, 1 (cyclin D-related)) protein diluted in 1 M Na_2CO_3-$NaHCO_3$ buffer (pH 9.6; concentration at 1 μg / 50μl) at 4°C overnight. Each plate was washed six times with 300 μl wash buffer (0.05% Tween in PBS) and non-specific binding sites were blocked with 5% Marvel dried skimmed milk powder (Premier International Foods, UK) in 1% PBS and 0.1% Tween (blocking solution) for 2 hours at 37°C.

Serum samples were diluted in blocking solution (1:100) and incubated on the plate in duplicates for 1 hour at 37°C. Serial dilutions of RUNX1T1 monoclonal mouse anti-human antibody (Novus Biological, Bio-Techne Ltd., UK, clone 5A12) diluted 1:1000 in blocking solution were also incubated on the plate

(positive control standard curve). Known positive and negative control serum and blocking solution only (control for background binging) were also incubated on the plate. Following incubation the plates were washed six times with 300 μl wash buffer. Biotinylated anti-mouse and anti-human IgG antibody (Sigma-Aldrich Inc.) diluted 1:1000 in blocking solution were used for establishment of the positive control standard curve and detection of RUNX1T1 antibodies in test samples, respectively, followed by streptavidin-HRP (Sigma-Aldrich Inc.) diluted 1:1000 in blocking solution. ELISA incubations were carried out in a 50 μl volume for 1 hour at 37°C followed by six washes with wash buffer. To generate a colorimetric signal 50 µl TMB substrate was added to each well and plates were incubated for 5 mins at room temperature. The reaction was stopped with 0.2M H_2SO_4 (stopping solution). The absorption was read in a FLUOStar OPTIMA ELISA plate reader (BMG Labtech, UK) at 450 nm.

2.5.5.1 Analysis of RUNX1T1 ELISA

Standard curve was generated using the absorbance value from the serial dilutions of the RUNX1T1 monoclonal mouse anti-human antibody (positive control); the concentration of the unknown samples was determined by interpolation from the standard curve. RUNX1T1 antibody levels are expressed as arbitrary units; 1000 is the highest point on the standard curve.

2.6 Molecular methodologies

2.6.1 DNA extraction

Blood samples obtained from the patients prior to transplantation and their respective donors was used for extraction of genomic DNA using QIAamp® DNA Blood Kit (Qiagen Ltd., Sussex, UK). All reagents required for DNA extraction were supplied with the kit. The DNA extraction was carried out according to the manufacturer's recommendations. In brief, 200 µl of whole blood was mixed with 20 µl Protease and 200 µl buffer AL in a 1.5 ml microcentrifuge tube, and incubated in a water bath at 56°C for 10 mins. 200 µl of ethanol was added to the mixture, and the mixture was transferred into a QIAamp Spin Column. The spin column was centrifuged at 8000 rpm for 1 minute. The column was washed with 500 µl of buffer AW1 and centrifuged as previously described. Washing was repeated using buffer AW2 and further centrifuged at 14,000 rpm for 3 mins. The spin column was transferred into a new 2 ml collection tube and centrifuged at 14,000 for 1 minute. Finally, the column was transferred into a 1.5 ml microcentrifuge and 200 µl of distilled water was added to the column. To elute the DNA from the spin column, the column was incubated at room temperature for 5 mins and centrifuged at 8000 rpm for 1 minute. The DNA concentration was established using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, CA, USA). The ratio of absorbance at 260 nm and 280 nm was used to assess the DNA purity. A ratio of ~1.8 (1.7 – 1.9) was accepted as "pure" DNA.

2.6.2 HLA genotyping

The HLA genotype was established in order to assess the immunological compatibility between the patients and their respective donors. HLA genotyping was performed by low/medium resolution in-house PCR-SSP (Polymerase Chain Reaction - Sequence Specific Primers) methodology, previously described by Bunce et al. [147]. Genomic DNA was used as a template for the PCR-SSP reaction. The DNA was extracted as previously described in Section 2.6.1. PCR-SSP utilises a panel of oligonucleotide primer

mixes able to amplify HLA-A, -B, -Cw, -DRB1, -DRB3, -DRB4, -DRB5 and -DQB1 alleles [147]. The PCR-SSP method is based on repeated cycles of denaturing, oligonucleotide primer annealing and extension, which generate exponential amplification of a targeted DNA sequence. Each PCR reaction contains an additional pair of primers that amplify "housekeeping" genes (genes present in all samples e.g. human growth hormone). The amplification of "housekeeping" genes allows identification of a negative and/or a failed reaction. In brief, 425.3 µl deionized water, 875 µl PCR buffer (PCR buffer IV (750mM Tris HCL pH 8.8, 200mM $(NH_4)_2SO_2$, 0.1% Tween 20), 2mM M_gCl_2, 5% glycerol, 100 µg/ml cresol red, 200 µM dNTP), 87.5 µl DNA at concentration of 225 ng/µl and 8.8 µl Taq DNA polymerase (Bioline Ltd., London, UK) were mixed by vortexing and 8 µl of the mixture was pipetted into each well of a 96-well pate containing 5 µl of sequence specific primers. The PCR was carried out in a GeneAmp 9700 Thermal Cycler (Applied Biosystems, Life Technologies, USA). Table 2.7 shows the PCR-SSP thermal cycler conditions.

Table 2.7 PCR-SSP Thermal Cycler Settings

	Hold	**5 Cycle**			**21 Cycle**		
Temperature	96°C	96°C	70°C	72°C	96°C	65°C	72°C
Time	1 min	20 sec	45 sec	25 sec	25 sec	50 sec	30 sec
	4 Cycles			**Hold**	**Hold**		
Temperature	96°C	55°C	72°C	20°C	4°C		
Time	30 sec	60 sec	90 sec	60 sec	10 min		

The PCR amplified products were loaded onto a 1.5% agarose gel containing Ethidium Bromide (10mg/ml) followed by electrophoresis at 180V for 40 mins. The gel electrophoresis was performed in a Helena Bioscience HU-25 gel tray (Helena Bioscience Europe, UK) containing 0.5 x TBE (Tris Boric acid EDTA) buffer. The PCR products were visualised under the UV light (Uvitec, Cambridge, UK). An example of recipient HLA class I genotyping is presented in Figure 2.3.

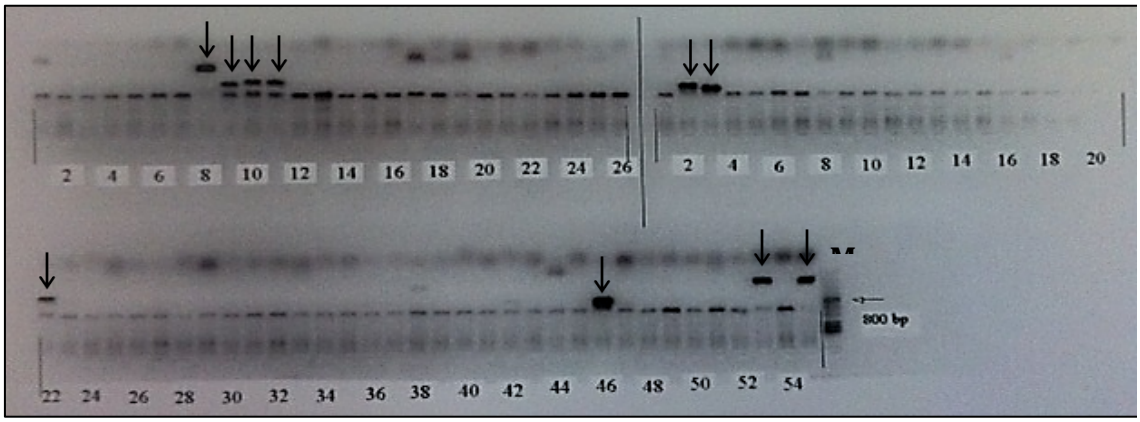


Figure 2.3 Human leucocyte antigen (HLA) A and B loci and Bw epitope genotyping of a lung transplant recipient. Genomic DNA was isolated from recipient peripheral blood collected prior to transplantation. HLA-A and –B loci and Bw epitope genotyping was performed by polymerase chain reaction using sequence-specific primers (PCR-SSP). The arrows represent positive reaction i.e. presence of specific alleles. The recipient HLA genotype was identified as HLA-A25, -A26; -B8, -B57; Bw4 and Bw6.

2.6.3 Killer Immunoglobulin-like receptor (KIR) genotyping using quantitative KIR automated typing (qKAT) methodology

KIR genotyping was carried out by the KIR typing service at the Department of Immunology, Cambridge University. Genomic DNA obtained as previously described in Section 2.6.1 was genotyped for KIR gene content and copy number, using a recently developed high-throughput technology known as qKAT, as described in detail by Jiang et al., [148, 149]. Briefly, a set of 10 multiplex quantitative PCR (qPCR) assays was carried out in quadruplicate for each patient. Each assay detected two different KIR genes and one reference gene, using sequence specific primers and probes labeled with different fluorophores. Monitoring of fluorescence signal for each probe, in each reaction, and subsequent Ct determination, allowed relative quantification of copy number for the 17 KIR genes (*2DL1-5, 2DS1-3, 2DS4 (2DS4f and 2DS4v), 2DS5, 3DL1-3, 3DS1, 2DP1,* and *3DP1*) and their major variants.

2.6.3.1 Natural killer (NK) cell alloreactivity mismatched definition

NK cell activation is controlled by inhibitory and activating KIRs. The NK cell inhibitory KIRs have the ability to respond to HLA class I antigens; and absence of HLA class I ligands leads to loss of inhibition, thus resulting in NK cell activation. NK cell alloreactivity was predicted based on the presence or absence of the inhibitory KIR genes (*2DL1-3 and 3DL1*) and their corresponding HLA class I ligands (C1, C2 and Bw4) Table 2.8. Thus, NK cell alloreactivity was expected if there was at least one KIR-ligand mismatch between the recipient and donor pair. For example, NK cell alloreactivity is expected if an inhibitory *KIR2DL1* gene and its corresponding HLA ligand (C2) was identified on the recipient's cells, but the HLA ligand (C2) is absent on the donor cells.

Table 2.8 KIR-ligand mismatch definitions. (Table adapted from van Bergen et al., 2011)

Inhibitory KIR-ligand	Recipient		Donor
	KIR	HLA	HLA
KIR2DL1/C2	KIR2DL1 +	C2 +	C2 –
KIR2DL2/C1	KIR2DL2 +	C1 +	C1 –
KIR2DL3/C1	KIR2DL3 +	C1 +	C1 –
KIR3DL1/Bw4	KIR3DL1 +	Bw4 +	Bw4 –

2.6.4 Gene expression analysis using reverse transcription – polymerase chain reaction (RT-PCR) methodology

RT-PCR methodology is commonly used to detect RNA expression levels in biological samples. Traditionally, the first step for RT-PCR requires availability of pure RNA; extracted from the experimental sample. Due to the limited amount of experimental biological sample available we were not able to obtain sufficient amounts of RNA for gene expression analysis; therefore, an alternative method was utilised. TaqMan® Gene Expression Cells-to-C_T™ (Ambion, Life Technologies, CA, USA) technology enables RNA reverse-

transcription into complementary DNA (cDNA) directly from cell lysates without the requirements to isolate and purify the RNA. The manufacturer's protocol was modified and adapted specifically for this study. The RT-PCR gene expression protocol used for gene expression profiling is described below.

2.6.4.1 Donor CD4 T cell lysis

Donor CD4 T lymphocytes isolated from the recipients' peripheral blood samples (Section 2.4.3) were used as a substrate for reverse-transcription (RT) reaction. Cells were washed with 500 µl of sterile cold PBS and centrifuged for 10 min at 2 x g. The supernatant was aspirated and discarded without disturbing the cell pellet. The cell pellet was re-suspended in 1000 µl cold PBS and washed twice, as previously described. Following washing steps, the cell pellet was lysed in 20 µl of Lysis Solution and 0.2 µl DNase I by pipetting up and down 10 times. The lysed cells were incubated for 5 min at room temperature. The reaction was stopped with 5 µl of Stop Solution; mixed and further incubated for 2 min at room temperature.

2.6.4.2 Reverse Transcription

RT reaction was carried out in 50 µl reaction consisting of: 25 µl 2x RT Buffer, 2.5 µl 20x RT Enzyme, 2.5 µl nuclease-free water and 20 µl of cell lysate. The reaction was mixed and centrifuged briefly to collect the content at the bottom of the 96-well plate (Thermo Scientific, CA, USA) and placed in a Thermal Cycler (Mx3005P, Stratagene, CA, USA). Table 2.9 shows the RT thermal cycler conditions.

Table 2.9 Reverse Transcription Thermal Cycler Settings

	Stage	Repeated cycles	Temperature	Time
Reverse transcription	1	1	37°C	60 min
RT inactivation	2	1	95°C	5 min
Hold	3	1	4°C	5 min

The RT product i.e. cDNA was used as a template for the next reaction step. Firstly, in order to increase the amount of cDNA available for detection of the genes of interest, the cDNA for each gene was pre-amplified using gene expression assays.

2.6.4.3 Pooling TaqMan® gene expression assays and Preamplification of genes of interest

Equal volume of 20x TaqMan® gene expression assays (Table 2.10), (Applied Biosystems, Life Technologies, CA, USA) were combined and diluted with 1 x TE Buffer to a final volume of 0.2x. The pooled gene expression assays were used for preamplification of the genes of interest.

Each preamplification reaction consisted of 25 µl TaqMan PreAmp Master Mix (2x) (Applied Biosystems, Life Technologies, CA, USA), 12.5 µl pooled gene expression assays, 2.5 µl nuclease-free water and 10 µl cDNA. The reaction was mixed and centrifuged briefly to collect the content to the bottom of the 96-well plate and placed in a Thermal Cycler (Mx3005P, Stratagene, CA, USA) Table 2.10 shows the pre-amplification thermal cycler settings and Table 2.11 contains a list of gene expression assays used for characterisation of donor CD4 T lymphocyte subsets. A representative image of gene preamplification is presented in Figure 2.4.

Table 2.10 Pre-amplification Thermal Cycler Settings

	Hold	**14 Cycles**	
Temperature	95°C	95°C	60°C
Time	10 min	15 sec	4 min

Table 2.11 List of gene expression assays used for characterisation of donor CD4 T lymphocyte subsets.

Gene expression assays – manufacture catalogue ID	Brief description of gene function
GAPDH – Hs99999905_m1	**Endogenous control.** Involved in glycolysis (breakdown of glucose).
SELL (CD62L) – Hs00174151_m1	Adhesion molecule expressed on naïve and central memory T cell subset.
STAT5B – Hs00273500_m1	Transcription factor regulates differentiation of CD4 T cells into regulatory T cells.
CD27 – Hs00386811_m1	Marker for regulatory T cells.
CD44 – Hs01075861_m1	Marker for effector memory T cells involved in cell-cell interaction, cell adhesion and migration.
RORC – Hs01076122_m1	Transcription factor regulates differentiation of CD4 T cells into Th17 cell subset.
IL21 – Hs00222327_m1	Involved in differentiation of naïve T cells into Th17 cell subset.
CCR6 – Hs00171121_m1	Regulates migration of Th17 cells to the inflammatory tissue.
GATA-3 – Hs00231122_m1	Transcription factor regulates differentiation of CD4 T cells into Th2 cell subset.
STAT6 – Hs00598625_m1	Transcription factor regulates differentiation of CD4 T cells into Th2 cell subset.
BCL6 – Hs00153368_m1	Regulates CD4 T cell differentiation into follicular T cell subset.
T-bet - Hs00203436_m1	Transcription factor regulates differentiation of CD4 T cells into Th1 cell subset.
STAT3 - Hs00374280_m1	Promotes differentiation of CD4 T cells into Th17 subset.
STAT4 - Hs01028017_m1	Transcription factor regulates differentiation of CD4 T cells into Th1 cell subset.
FOXP3 – Hs01085834_m1	Transcription regulator for development of regulatory T cells.

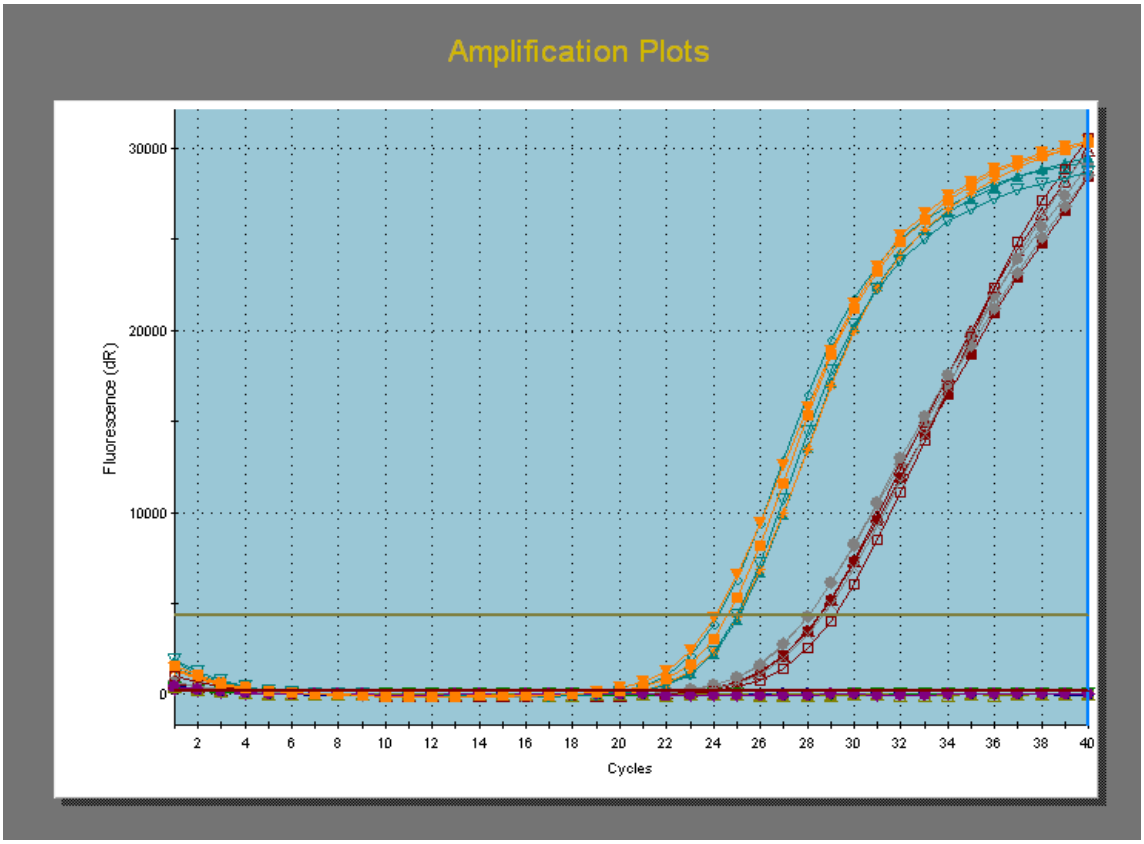


Figure 2.4 A representative image of CD44 gene preamplification using TaqMan® gene expression assay. Five cDNA samples were selected for CD44 gene expression analysis using cDNA without a preamplification step (dark red and gray amplification curves) and cDNA with previous preamplification step (orange and blue amplification curves). The preamplification step increased the signal for CD44 gene expression on average by four Ct cycles, which is equivalent to a 16-fold increase in the amount of cDNA available for the targeted gene. All samples tested were first subjected to preamplification of the gene of interest. The pre-amplified cDNA product was used as a template for gene expression analysis using Real Time - PCR (RT-PCR).

2.6.4.4 Real-time – PCR

RT-PCR was carried out in MicroAmp® Optical 384-well reaction plates (Applied Biosystems, Life Technologies, CA, USA) in a final volume of 5 µl. The RT-PCR reaction contained 2.5 µl TaqMan gene expression Master Mix (Applied Biosystems, Life Technologies, CA, USA), 0.25 µl 20x gene expression assay, 1 µl nuclease-free water and 1.25 µl pre-amplified cDNA. Each reaction was carried out in duplicates. The plate was vortexed and centrifuged briefly to collect the content at the bottom of the 384-well plate and placed in a 7900HT Fast Real-Time PCR instrument (Applied

Biosystems, Life Technologies, CA, USA), Table 2.12 shows the RT-PCR thermal cycler settings. A representative image of CD44 gene expression analysis using RT-PCR methodology is presented in Figure 2.5.

Table 2.12 Real-time PCR Cycling Conditions

	Hold	Hold	40 cycles	
Temperature	50°C	95°C	95°C	60°C
Time	2 min	10 min	15 sec	1 min

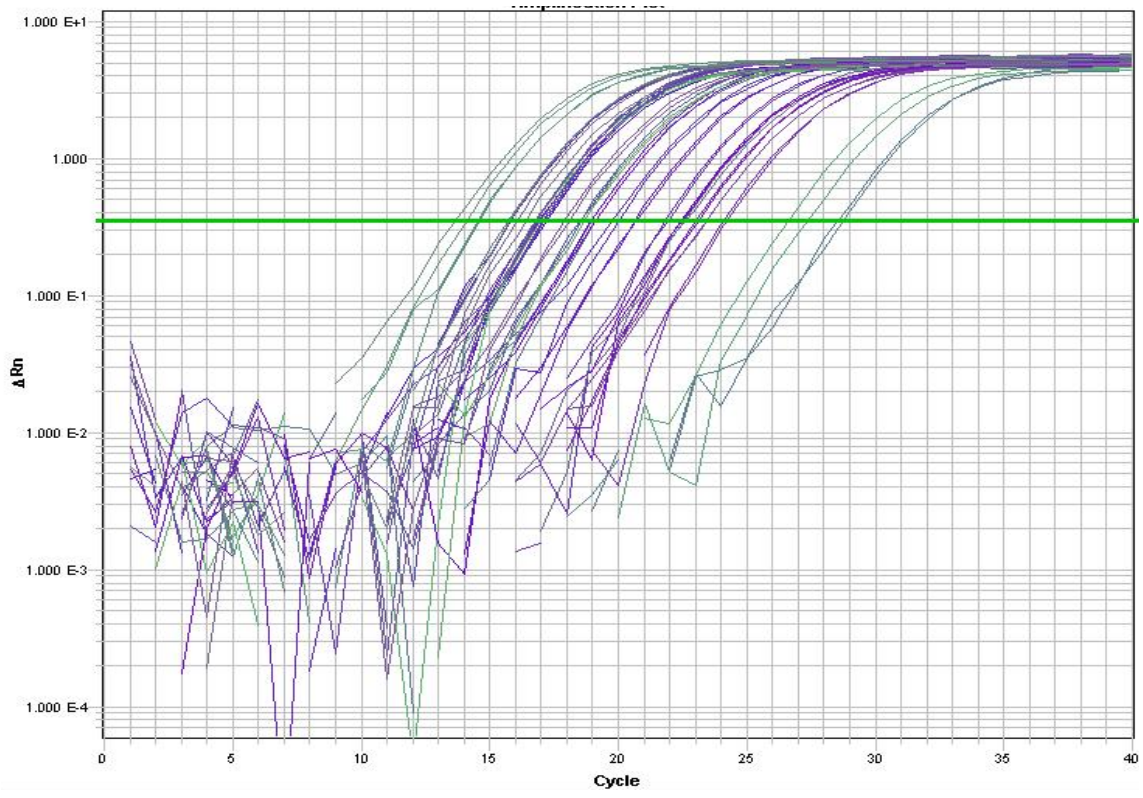


Figure 2.5 A representative image of CD44 gene expression analysis using RT-PCR methodology. RT-CPR amplification plot representing CD44 gene expression levels recipients' peripheral blood isolated donor CD4 T cells. The RT-PCR was performed as previously described in Section 2.4.6. Each sample was tested in duplicates.

2.6.5 Relative quantification of the gene expression

The RT-PCR gene expression levels were quantified using the relative quantification (RQ) method [150]. RQ is a widely applied method for gene expression analysis where the aim is to describe a particular cell type based on the gene expression levels or to study differences in the level of gene expression between two groups of patients. In addition, the RQ method is not dependent on the starting sample size, but instead the expression of the target gene i.e. gene of interest is quantified in relation to the endogenous control gene, also known as a reference gene [150]. Endogenous control represents a gene with a stable level of expression. In this study GAPDH was used as a reference internal control gene. DataAssist^{TM} v3.01 software (ThermoFisher Scientific, CA, USA) was used to calculate the relative quantification (fold-change) of the gene expression.

DataAssist^{TM} v3.01 software performs the following step analysis:

1. Performs mean Ct analysis.
2. Performs sample Ct normalization known as delta Ct (ΔCt) relative to the mean Ct value of the endogenous internal control gene (GAPDH). ΔCt equals the Ct of the target gene minus the Ct of the reference gene (ΔCt = Ct (target gene) – Ct (reference gene - GAPDH)).
3. Performs ΔCt normalization known as delta delta Ct ($\Delta\Delta$Ct) between two groups of patients or two samples obtained at different time points $\Delta\Delta$Ct equals ΔCt of the patient group/test samples minus the ΔCt of the calibrator. The calibrator represents patient group/test sample used for comparison analysis ($\Delta\Delta$Ct = ΔCt (test) – ΔCt (calibrator).
4. Performs relative quantification (fold-difference) for sample comparison and t-test for biological group (patient group) comparison ($2^{-(\Delta\Delta Ct)}$ = Normalised relative gene expression).

2.7 *Ex Vivo* Lung Perfusion

In the United Kingdom about 80% of potential donor lungs are deemed unsuitable for clinical lung transplantation. Thus, the shortage of donor lungs has impacted the donor selection criteria; which has resulted in an increased use of donor after cardiac death (DCD) and more “marginal” donors. Ex-Vivo Lung Perfusion (EVLP) is a novel technique originally described by Steen et al., in which unusable donor lungs can be assessed and potentially reconditioned for safe use in clinical transplantation [151, 152].

In 2013, the United Kingdom launched a multi-centre EVLP clinical trial (DEVELOP-UK) to evaluate the effectiveness, safety and cost-effectiveness of EVLP. The EVLP was performed using Vivoline® system, which is a semi-automated EVLP circuit consisting of bypass pump, oxygenator / deoxygenator, perfusion reservoir, organ contained unit and leucocyte filter.

In brief, donor lungs were connected to a “modified” heart-lung bypass circuit to which a leucocyte filter is attached; two-litres of acellular Steen Solution^{TM} and two-units of packed red blood cells (RBCs) were continuously pumped through the lungs for four hours which warms the lungs to body temperature. After slow rewarming the lungs are ventilated with oxygen by connecting them to a standard ITU ventilator. This is followed by one-hour cooling perfusion phase during which lungs are cooled-down to 6°C. During the whole EVLP procedure the leukocyte filter is attached to the circuit. At the end of the procedure, the leukocyte filter is removed and stored overnight in RPMI media at 4°C for further analysis.

2.8 Statistical analysis

Recipient and donor demographics are presented as a median ± SD. Statistical analyses were performed using GraphPad Prism, GraftPad Software, Inc., San Diego, CA, USA. Comparison between two groups was assessed by chi-square or Fisher's exact test where appropriate. Paired t-test, Mann-Whitney t-test or Wilcoxon matched pairs test were used to assess the change in the autoantibody levels in sera obtained at two different time points. The effects of HLA and KIR-ligand mismatching on both, duration of donor CD4 T cell chimerism and development of BOS were assessed using Kaplan-Meier method. Nonparametric correlation Spearman r test was used for correlation analysis. A p value of <0.05 was considered significant. DataAssist™ v3.01 software (ThermoFisher Scientific, CA, USA) was used for gene expression relative quantification (fold-change). ProtoArray® Prospector v5.2 software provided by Invitrogen (Invitrogen, CA, USA; now provided by ThermoFisher Scientific, CA, USA) was used for protein microarray data analysis.

Prior to commencement of this study the incidence of donor CD4 T cell chimerism and the development of autoantibody in lung transplant recipients were unknown entities. Power calculations will be applied whenever relevant. There is little data on which to base the number of human patients required for study, but assuming that approximately more than 30% of recipients develop autoantibody [153], and a similar percentage have detectable donor lymphocyte chimerism [132], 70 patients will allow a clinically-relevant proportional association of 70:30 to be statistically demonstrable. Papworth Hospital perform around 25 to 30 lung transplants each year, so it will take approximately 2.5 years to recruit 70 patients to the study. From previous publications [134], donor lymphocyte chimerism that persists for more than a year is likely to be physiologically relevant. We thus anticipate that if an association between donor lymphocyte chimerism persistence and autoantibody exists, it will be visible to demonstrate statistical significance within the study period of four years.

3 Chapter 3

Detection of donor CD4 T lymphocyte chimerism in lung transplant recipients

3.1 Introduction

Donor-derived haematopoietic cells that are not flushed from the graft during retrieval will be transferred to the recipient at the time of transplantation. The presence and co-existence of these cells in the blood and tissues of the recipients results in a chimeric state - the co-existence of two or more genetically distinct cell populations. Depending on the proportions of chimeric cells present; the terms micro- and macrochimerism have been used to describe chimeric populations present at less than 1%, or greater than 1% of the total number of cells, respectively [129].

Chimerism has also been defined according to the mechanism of its development: induced chimerism and spontaneously occurring chimerism. Induced chimerism has been used as a therapeutic approach in living renal transplant recipients, where transplantation of the allograft is accompanied by donor HSC infusion, with the aim of inducing donor-specific tolerance. In contrast, spontaneous chimerism occurs due to migration of donor leukocyte passengers from within the allograft into recipient peripheral blood and tissues, a phenomenon typically observed in recipients of solid organ transplants. Consequently, there is a fundamental difference between induced and spontaneous chimerism in terms of the type of cells transferred, their maturity, and presumably their role in solid organ transplantation.

3.1.1 Historic overview of the immunologic implications of chimerism and induction of donor-specific tolerance

From the beginning of the modern transplant era in the 1960s, chimerism has been considered to likely influence transplant outcomes. However, several decades later, the role of microchimerism in tolerance and/or rejection remains controversial. The immunological relevance of chimerism was first demonstrated by Owen's observation that cattle twins contain a mixture of two distinct types of erythrocytes at birth, and in some cases, the chimeric state persists into adulthood [154]. Furthermore, Medawar et al., observed that

chimeric cattle twins were fully tolerant to skin grafts of each other, but that, as expected, skin transplanted from a third party was rejected [155].

Billingham, Brent and Medawar recognised the significance of these observations and designed an experimental model to demonstrate “how to make tissue homografts immunologically acceptable to hosts which would normally react against them” [156]. They showed that inoculation of living cells from an adult mouse of one strain into allogeneic neonatal recipients led to chimerism that persisted into adulthood and that these mice were specifically tolerant to donor strain skin grafts, yet retained the ability to react to third-party skin grafts [156]; a process described as “active acquired tolerance”.

Strober et al. reported the first cases of acquired tolerance in humans, when three patients with end-stage renal disease were treated with total lymphoid irradiation and subsequently transplanted with a kidney transplant from a deceased donor [157]. Similar findings were observed in two patients who had received bone marrow transplants from HLA-identical donors. Both patients subsequently received a kidney from the same living donor and neither received immunosuppression [158].

However, reluctance to expose patients to radiation has hampered the wider clinical application of this approach. Instead, administration of anti-lymphocyte serum and sirolimus, together with donor bone marrow has been investigated as an alternative approach for induction of donor-specific tolerance [159]. Using this approach Hale et al., in a completely mismatched murine model, have shown long-lasting donor-specific tolerance, which surprisingly was dependent on the presence of B cells [159]. These observations were further confirmed in T cell-knockout experimental model [160]; nevertheless, using the same conditioning protocol, Umemura et al., have reported prolonged skin allograft survival in a completely MHC-mismatched mouse model, even in the absence of B cell chimerism [160], suggesting that neither T nor B cells are essential for allograft tolerance induction. In contrast, Tomita et al., showed that long-term donor-specific tolerance is dependent on donor T cells [161].

Umemura et al., have further reported that expression of MHC class II antigen on bone marrow cells is essential for induction of tolerance [162].

3.1.2 The role of spontaneous chimerism in solid organ recipients

The phenomenon of spontaneously occurring chimerism has been observed in solid organ transplant recipients. The first evidence for the presence of chimerism following solid organ transplantation was obtained in 1969, with karyotyping studies in female liver transplant recipients identifying persistent cells of male donor origin [131, 163].

It was not until 1992 when Starzl et al*.,* postulated that transfer of leukocytes from the donor can create a state of systemic mixed allogeneic chimerism, that if persistent, promotes graft acceptance and resistance to cellular and humoral rejection. This hypothesis was initially tested in female liver transplant recipients of male donors 10 to 19 years after transplantation, using *in-situ* hybridisation of Y chromosome. Evidence of Y chromosome was detected in the majority of patients' blood and lymph node samples. In addition, donor cells were detected in skin biopsy samples in all patients [131, 164].

In support that persistent chimerism leads to allograft acceptance, Starzl et al. made similar observations in long-term living kidney transplant survivors. Interestingly, in this small cohort (n=5), chimerism was detected even three decades after transplantation [165]. In all patients, donor chimerism was detected in freshly obtained kidney and skin biopsy samples and/or lymph nodes obtained from the groin; using both immunocytochemistry and PCR methodology. In four out of the five patients, chimerism was detected by targeting donor mismatched HLA antigens and in one patient whose donor had died (father to daughter), chimerism was confirmed by identifying the sex-mismatched Y chromosome. [165]. In addition, four patients, whose donors were still alive, underwent further *in vitro* analyses to assess the responsiveness to their donor, using unidirectional mixed lymphocyte reaction

(MLR) culture and cell-mediated lymphocytotoxicity. Non-responsiveness to irradiated donor cells was observed in two of the four kidney recipients, and a modest response was detected in the other two, whilst in all cases the recipients retained their ability to respond to third party lymphocytes. Similarly, the donor lymphocyte fraction in the chimeric recipients was not reactive to the recipients' lymphocytes, but was responsive to third party lymphocytes [165].

The phenomenon of spontaneously occurring chimerism has also been demonstrated in experimental models [166, 167], and human transplant recipients of multivisceral [168], and heart and lung [169] transplants.

Despite these observations, the role of passenger leukocytes in solid organ transplantation remains unclear; and conflicting evidence has been reported that in some settings donor-derived passenger leukocytes can initiate graft rejection [140], whereas in others they can contribute to graft acceptance [131, 164, 165]. For example, experimental work in a rat heart transplant model of cyclosporine-induced tolerance demonstrated that depletion of donor-derived leukocytes at the time of transplantation resulted in acute rejection of the grafts by day 23 post-transplantation, whereas depletion of donor leukocytes at day 18 post-transplantation resulted in long-term allograft acceptance [170]; suggesting a beneficial immunemodulatory effect.

These observations have raised the question of whether the immuno-modulatory role of donor chimerism and its influence on transplant outcome is time-dependent. Two phases of donor chimerism have been proposed an early and late phase. In the early post-transplant stage, the donor chimerism comprises mature T, B and DC cells that have been "washed out" from the graft [171, 172]. In contrast, donor chimerism that can be detected in the late post-transplant phase may result from bone marrow engraftment of donor-derived haematopoietic stem cells (HSC) originating from the graft itself [173].

Several clinical and experimental studies have reported the presence of HSCs in the liver. Taniguchi et al., identified the existence of HSCs expressing c-kit$^+$

Sca^{+} $Lin^{lo/-}$ in adult mouse liver: interestingly, adoptive transfer of these cells rescued lethally irradiated mice and reconstituted all linage blood cells [174]. Moreover, multilineage haematopoietic reconstitution was achieved in supralethally irradiated rat model following syngeneic liver transplantation. A similar approach in syngeneic heart transplantation significantly prolonged graft survival, but failed to fully reconstitute the haematopoietic compartment [175], presumably because the heart contains relatively few HSCs.

Similar findings have been reported in long-term survivors of liver transplants, where donor $CD34^{+}$ HSCs have been detected in patients' bone marrow and peripheral blood [173].

These studies suggest that long-term graft survival and donor-specific tolerance might be achieved via engraftment of graft passenger HSCs. Nevertheless, it is not clear whether other organs such as kidney, pancreas, heart and lungs contain HSCs and if they do, whether the numbers of resident HSCs are sufficient to maintain donor-specific tolerance.

In contrast to these observations, donor chimerism can occasionally trigger onset of graft vs host disease (GvHD). In solid organ transplantation, GvHD is a rare post-transplant complication; nevertheless detrimental outcomes have been reported in liver [176, 177] and lung transplant patients [178, 179].

Based on the experimental and clinical evidence available, it is likely that graft outcomes depend upon a complex interplay between the donor-derived passenger leukocytes and recipients' own immune responses The effect of this interplay will likely differ according to the different organs transplanted. Understanding the role of microchimerism and the underlying mechanisms involved may provide fundamental insights that ultimately inform the development of new strategies for improving transplant outcomes.

3.1.3 The role of donor CD4 T cell chimerism in the development of *de novo* autoimmunity following transplantation

Win et al., in our department used a murine model of chronic heart graft rejection to study how donor CD4 T cells that were passengers within the heart graft influenced the host response to the graft. In Win's model, the donor bm12 strain varies from the C57BL/6 (B6) recipient by three amino acid residues in the MHC class II I-A antigen (human HLA-DQ). Bm12 heart allografts are rejected slowly (median survival time 50 days), and this rejection was associated with the development of graft vascular pathology consisting of progressive intimal thickening and luminal narrowing. In addition, there was histologic evidence of humoral vascular rejection, characterised by C4d complement and IgG endothelial deposition. No evidence of rejection was observed in syngeneic transplants or the bm12 allografts transplanted into either MHC class II gene knockout mice or B cell-deficient recipients. . Further experiments revealed that the B6 recipients did not develop circulating IgG alloantibodies but instead developed long-lasting IgG antinuclear autoantibody [144].

To investigate the involvement of T-cell-dependent responses; B6 recipients were primed with synthetic allopeptide corresponding to the disparate region of the I-A^{bm12} antigen 14 days prior to transplantation with a bm12 heart allograft. Bm12 heart grafts were rejected more rapidly than in unprimed, control recipients, but without evidence of alloantibody development. Moreover, despite the rapid rejection, the levels of circulating IgG autoantibodies were not augmented. These experiments suggested that development of autoantibody is independent of indirect pathway T cell responses. Given that indirect-pathway is thought to be the only means by which CD4 T cell help for alloantibody production is provided [79, 80], this result was surprising and suggested that an alternative mode of help was responsible for the development of autoantibody in the bm12-B6 model. On the basis that adoptive transfer of bm12 splenocytes triggers lupus-like humoral autoimmunity in B6 recipient mice [144, 145], the potential role of

passenger donor CD4 T cells in the provision of help to host B cells was investigated. Surprisingly, transplantation with heart allografts from donor bm12 mice that either were depleted of CD4 T cells prior to heart allograft procurement or were genetically deficient in T and B cells did not prompt autoantibody production and heart allograft survival was prolonged [144].

Win et al., concluded that in their model, help for autoantibody production was provided via a direct cognate interaction between the donor CD4 T cells and the recipient auto-reactive B cells, thereby replicating the normal interaction between a self-restricted host helper CD4 T cell and B cell [144]. Win's studies further demonstrated that the autoantibody contributed to the development of chronic allograft vasculopathy.

The aim of my thesis was therefore to address whether a similar mechanism may occur in human transplant recipients. We chose to examine the role of donor CD4 T cells in lung transplant recipients, because the lung graft is a large and leucocyte rich organ. In assessing the potential contribution of donor CD4 T cells to human allograft rejection, it was first necessary to demonstrate the presence of donor CD4 T cells in the lung recipient's circulation following transplantation.

Two questions will be addressed in this chapter: are donor CD4 T cells detectable in patients' peripheral blood following lung transplantation; and if so, for how long do these donor CD4 T cells remain detectable in the recipient? The donor CD4 T cells were detected by targeting the donor HLA class I (HLA- A and/or HLA-B) mismatched antigens with human monoclonal HLA antibodies.

3.2 Results

3.2.1 Patient cohort

Informed written consent was obtained from 63 patients awaiting diseased donor lung and heart and lung transplantation at Papworth Hospital. Twenty-nine patients were transplanted during the study. Immediately after their transplant, eight patients were excluded from the study: five due to the lack of appropriate human monoclonal anti-HLA antibody to detect donor-mismatched HLA class I antigens; and three patients were deemed unsuitable for participation in the study due to post-operative transplant-related complication.

In total, 21 patients were clinically stable for participation in this study; 19 patients received bilateral lung and two patients received single lung transplant. The presence of donor CD4 T cells and their persistence in the recipients' peripheral blood was assessed for 12 months after lung transplantation.

The recipient age varied between 18 and 66 years. Of the 21 patients who underwent lung transplantation, 8 were male and 13 female. Seven patients were sensitised to HLA antigens prior to transplantation. All patients received triple drug immunosuppression; in addition, two patients received induction therapy with Basiliximab, a humanised monoclonal antibody to α chain (CD25) of the IL-2 receptor expressed on T cells. A detailed representation of the recipients' and donors' demographic characteristics is presented in Table 3.1.

Table 3.1 Lung transplant recipients and donors characteristics

Recipient age (Median ± SD)	56 ± 13.6 (18-66 years)
Recipient sex (M:F)	8:13
Transplant type	
SLT	2
BLT	19
Donor age (Median ± SD)	48 ± 11.2 (19-60 years)
Donor sex (M:F)	10:11
Donor type	
DBD	20
DCD	1
Indication for transplantation	
Cystic fibrosis	3
Emphysema	4
COPD	7
Pulmonary fibrosis	2
Bronchiectasis	2
A1AD	1
Emphysema + A1AD	2
HLA Sensitisation	7
Immunosuppression therapy	Tacrolimus/MMF/Prednisolone

SLT – Single Lung Transplant; BLT – Bilateral Lung Transplant; DBD – Donation after brain steam death; DCD – Donation after cardiac death; COPD - Chronic obstructive pulmonary disease; A1AD- α1 antitrypsin deficiency; HLA – Human Leukocyte Antigen;
MMF – Mycophenolate Mofetil.

3.2.2 Identification of donor HLA class I mismatched antigens

To detect the presence of donor CD4 T cell chimerism in patients' peripheral blood following primary lung transplantation, donor HLA class I mismatched antigens were used as targets to distinguish between patient and donor cells. Recipient and donor blood samples and spleen and/or lymph node cells, respectively, were obtained prior to transplantation and used as a source for DNA extraction and HLA genotyping.

Recipient and donor HLA class I mismatched antigens were identified using molecular PCR methodology. Genomic DNA obtained prior to transplantation was used as a template for PCR-SSP, previously described in Section 2.6.1 and Section 2.6.2.

Table 3.2 shows the genotype of the recipients and corresponding donors for HLA-A and HLA-B antigens and Bw epitope; donor HLA mismatched antigens are highlighted in red. All recipient-donor pairs had at least one HLA class I mismatched antigen that was used as a target for detection of donor CD4 T lymphocytes with human monoclonal HLA antibodies using flow cytometric analysis.

Table 3.2 Human leucocyte antigen (HLA) A and B loci and Bw epitope genotype of lung transplant recipients and their corresponding donors using PCR-SSP. Donor HLA mismatched antigens are highlighted in red.

Patient No.	Recipient HLA-A; -B; -Bw	Donor HLA-A; -B; Bw (mismatched antigens highlighted in red)
1	HLA-A1,32; -B8; Bw6	HLA-A2,3; -B44,62; Bw4,6
2	HLA-A25,26; -B8,57; Bw4,6	HLA-A2; -B8,35; Bw6
3	HLA-A1,3; -B7,57; Bw4,6	HLA-A2; -B51,27; Bw4
4	HLA-A24,25; -B62,37; Bw4,6	HLA-A3,24; -B51,7; Bw4,6
5	HLA-A2; -B44,61; Bw4,6	HLA-A3,33; -B7,65; Bw6
6	HLA-A3; -B51,65; Bw4,6	HLA-A1,11; -B7,56; Bw6
7	HLA-A1,68; -B8,65; Bw6	HLA-A2,3; -B7,44; Bw4,6
8	HLA-A2,24; -B7,44; Bw4,6	HLA-A1,11; -B8,62; Bw6
9	HLA-A2,30; -B7,44; Bw4,6	HLA-A1,23; -B44,42; Bw4,6
10	HLA-A24,68; -B8,62; Bw6	HLA-A1,3; -B7,57; Bw4,6
11	HLA-A2,24; -B65,57; Bw4,6	HLA-A1,29; -B52,44; Bw4
12	HLA-A1,32; -B7,55; Bw6	HLA-A1,3; -B7,8; Bw6
13	HLA-A1,2; -B51,8; Bw4,6	HLA-A2; -B7,56; Bw6
14	HLA-A2,23; -B8,44; Bw4,6	HLA-A2; -B51,44; Bw4
15	HLA-A2,24; -B7,44; Bw4,6	HLA-A2,24; -B60,62; Bw6
16	HLA-A3,32; -B51,62; Bw4,6	HLA-A2; -B44; Bw4
17	HLA-A1,2; -B7; Bw6	HLA-A1,11; -B7,8; Bw6
18	HLA-A24,31; -B62,51; Bw4,6	HLA-A2; -B44,18; Bw4,6
19	HLA-A1,3; -B7,57; Bw4,6	HLA-A2,3; -B44,35; Bw4,6
20	HLA-A1,3; -B8,27; Bw4,6	HLA-A2; -B27,60; Bw4,6
21	HLA-A2,3; -B7,62; Bw6	HLA-A68,36; -B57,71; Bw4,6

3.2.3 Selection of human HLA monoclonal antibody for detection of donor CD4 T cells in peripheral blood of lung transplant recipients

A limited set of 14 human HLA monoclonal antibodies (mAbs) was used for detection of donor CD4 T cell chimerism (Table 2.1). We estimated that this panel enabled discrimination between recipient and donor lymphocytes in the majority of recipient-donor pairs. Human HLA mAbs with specificity to donor HLA class I mismatched antigens were not available for five recipient-donor pairs; subsequently, these patients were excluded from the study.

The specificity of each mAb was initially tested using recipients' PBMCs obtained prior to transplantation and donor lymphocytes obtained from spleen or lymph nodes at the time of donation. The HLA mAb selection was based on the binding capacity to the recipient and donor lymphocytes; HLA mAb that did not bind to recipient lymphocytes obtained prior to transplantation and at the same time showed >95% binding to the donor HLA antigens were selected for detection and isolation of donor CD4 T cells from the recipients' peripheral blood at regular time intervals following transplantation. Figure 3.1 is an example of flow cytometric analysis used for selection of human mAbs and detection of donor CD4 T lymphocytes.

Recipients' PBMCs obtained prior to transplantation served as a control to identify patients with pre-existing chimerism. In this patient cohort, pre-existing chimerism was not detected. Table 3.3 shows the recipient-donor pair HLA mismatched antigens and the specificity of the HLA mAbs used to target the donor CD4 T cells.

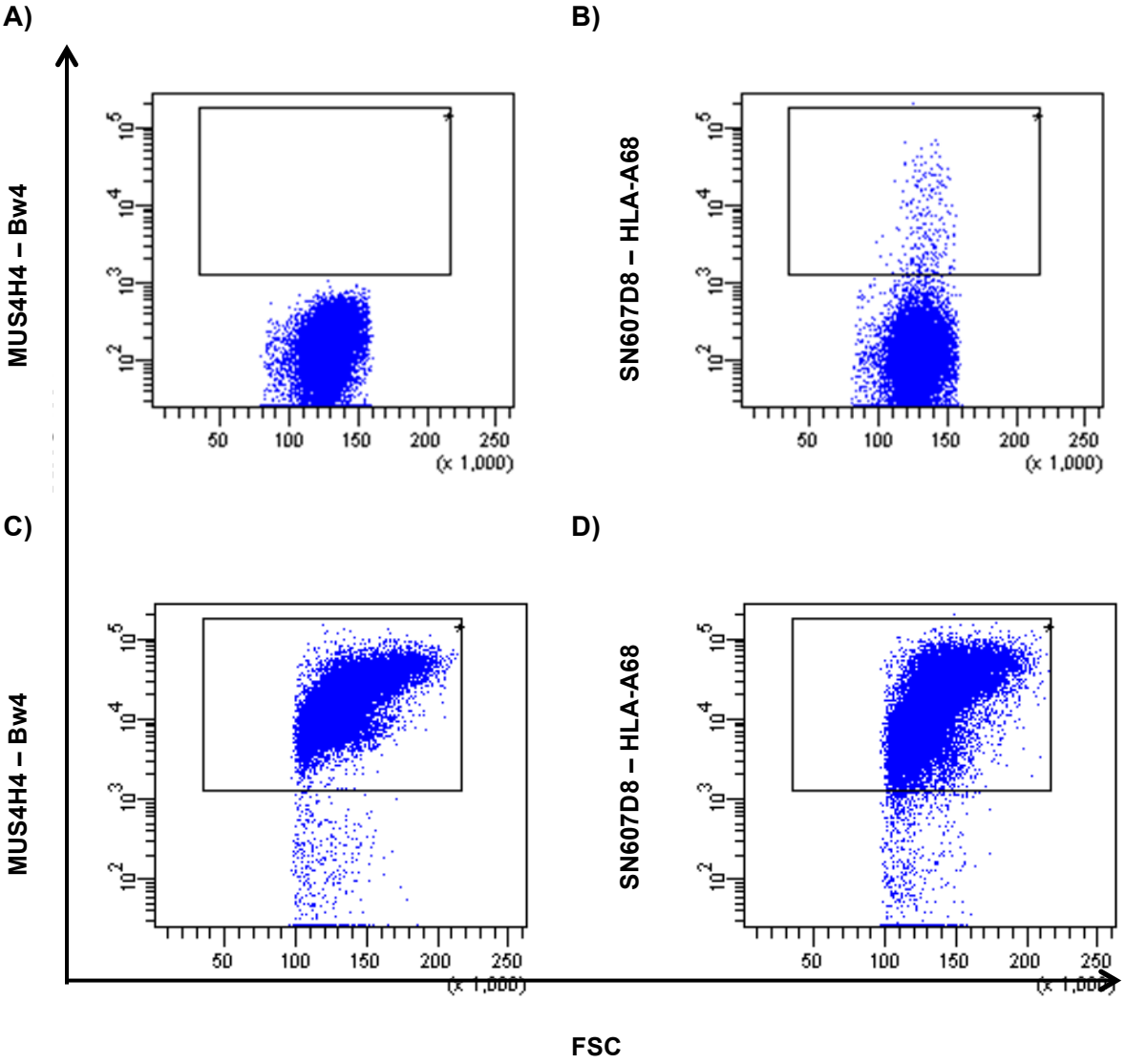


Figure 3.1 A representative image of flow cytometric analysis. Selection of human HLA monoclonal antibodies (mAbs) for detection of donor CD4 T cell chimerism in the peripheral blood of lung transplant recipients.

Recipient and donor pair was mismatched for HLA-A68, 36; -B57, 71 and Bw4 epitope. Two biotin-conjugated HLA mAbs were tested, MUS4H4 and SN607D8 with specificity for Bw4 epitope and HLA-68 antigen, respectively, using recipient and donor lymphocytes obtained prior to transplantation. Biotin-conjugated human HLA mAbs were detected with APC-conjugated streptavidin. A) MUS4H4 with specificity for Bw4 epitope did not stain recipient cells obtained prior to transplantation, C), but it stained >95% of donor lymphocytes. B) SN607D8 with specificity for HLA-A68 antigen stained ~ 5% of recipient lymphocytes; and, D) >95% of donor lymphocytes. The MUS4H4 with specificity for Bw4 epitope was selected for detection of donor CD4 T lymphocytes chimerism in samples obtained at regular time intervals after transplantation.

Table 3.3 Human monoclonal HLA antibodies used for detection of donor HLA mismatched antigens. Targeted donor HLA mismatched antigens are highlighted in red.

Recipient No.	Donor HLA-A, -B and Bw mismatched antigens	Human monoclonal HLA antibody specificity used to target the donor HLA mismatched antigens
1	HLA- A2,3; -B44,62; Bw4	DK7C11- B12(44,45)
2	HLA-A2, -B35	SN607D8– A2/A28(68,69)
3	HLA-A2; -B51,27	SN607D8– A2/A28(68,69)
4	HLA-A3, -B51,7	OK2F3– A3
5	HLA-A3,33; -B7,65	OK2F3– A3
6	HLA-1,11; -B7,56	VTM1F11– B27/B7/B60
7	HLA-A3, 2; -B7,44, Bw4	OK2F3– A3
8	HLA-A1,11; -B8,62	BVK1F9– B8
9	HLA-A1,23; -B42	GV5D1– A1/A9(23, 24, 2403)
10	HLA-A1,3; -B57,7; Bw4	SN230G6– A2/B17(57,58)
11	HLA-A1,29; -B44,52	DK7C11– B12(44, 45)
12	HLA-A3; -B8	BVK1F9– B8
13	HLA-B7,B56	OK6H10– B15/B21(49,50)/B56/B35/B72
14	HLA-B51	HDG8D9– B51/B35
15	HLA-B60,B62	IND2D12– B15(62,63,75,76)/B35/B21/B70
16	HLA-A2, -B44	SN607D8– A2/A28(68,69)
17	HLA-A11; -B8	BVK1F9– B8
18	HLA-A2, -B44,18	SN230G6– A2/B17(57,58)
19	HLA-A2; -B44,35	DK7C11– B12(44,45) & HDG8D9-B51/B35
20	HLA-A2; -B60	JOK3H5- B40(60,61)/B21/B13/B12/B41
21	HLA-A68,36; -B57,71; Bw4	MUS4H4– Bw4

3.2.4 Detection and longevity of donor CD4 T cell chimerism in recipients' peripheral blood following primary lung transplantation

To investigate the presence of donor CD4 T lymphocytes in the peripheral blood of lung transplant recipients (n=21), multiple blood samples were obtained in the first post-operative month and, where possible, three-monthly blood samples were obtained thereafter during the first year after transplantation. Donor CD4 T lymphocytes were detected and isolated as described in Sections 2.4.2 and 2.4.3.

Donor CD4 T cell chimerism was detected in all recipients in the first post-operative month as a proportion of the recipient total number of peripheral blood CD4 T cells. In all patients, the highest percentage of donor CD4 T cell chimerism detected was observed in the blood sample obtained within the first week after transplantation. In addition, we observed considerable variation in the percentage of donor CD4 T lymphocytes detected between patients, ranging from 0.06 to 6% of the circulating CD4 T cell population (donor and recipient). In addition to donor-derived CD4 T cells, we also assessed the total number of donor leucocytes in the recipients' peripheral blood. The percentage of donor leucocytes detected ranged from 1 to 8%, suggesting that other cell types were also present in the recipients' peripheral blood. Although, we did not characterise the whole donor leucocyte population, it is likely that these cells were B cell, NK cells and/or DCs. Interestingly, I observed that at least 50% of donor lymphocyte population were CD4 T cells ranging between 50 and 65%.

The duration of donor CD4 T cells detectable in the recipient circulation was then assessed. Three patterns of donor CD4 T cell chimerism were observed:

- Short donor CD4 T cell chimerism – donor CD4 T lymphocytes were detectable for six weeks after transplantation (n=13),

- Intermediate donor CD4 T cell chimerism – donor CD4 T lymphocytes were detectable between three and five months after transplantation (n=3); and,
- Long-lasting donor CD4 T cell chimerism – donor CD4 T lymphocytes were detectable for more than six months after transplantation (n=5).

Figure 3.2 represents one-year post-transplant follow-up for the presence and duration of donor CD4 T cell chimerism detectable in recipients' peripheral blood after transplantation; where each dot represents the time point at which patients' peripheral blood was obtained and tested for the presence of donor CD4 T lymphocytes. The green dot represents patients' samples that were tested and donor CD4 T cells were detected, the red dot represents samples tested but donor CD4 T cells were not detected and the black dot shows time of death (n=3).

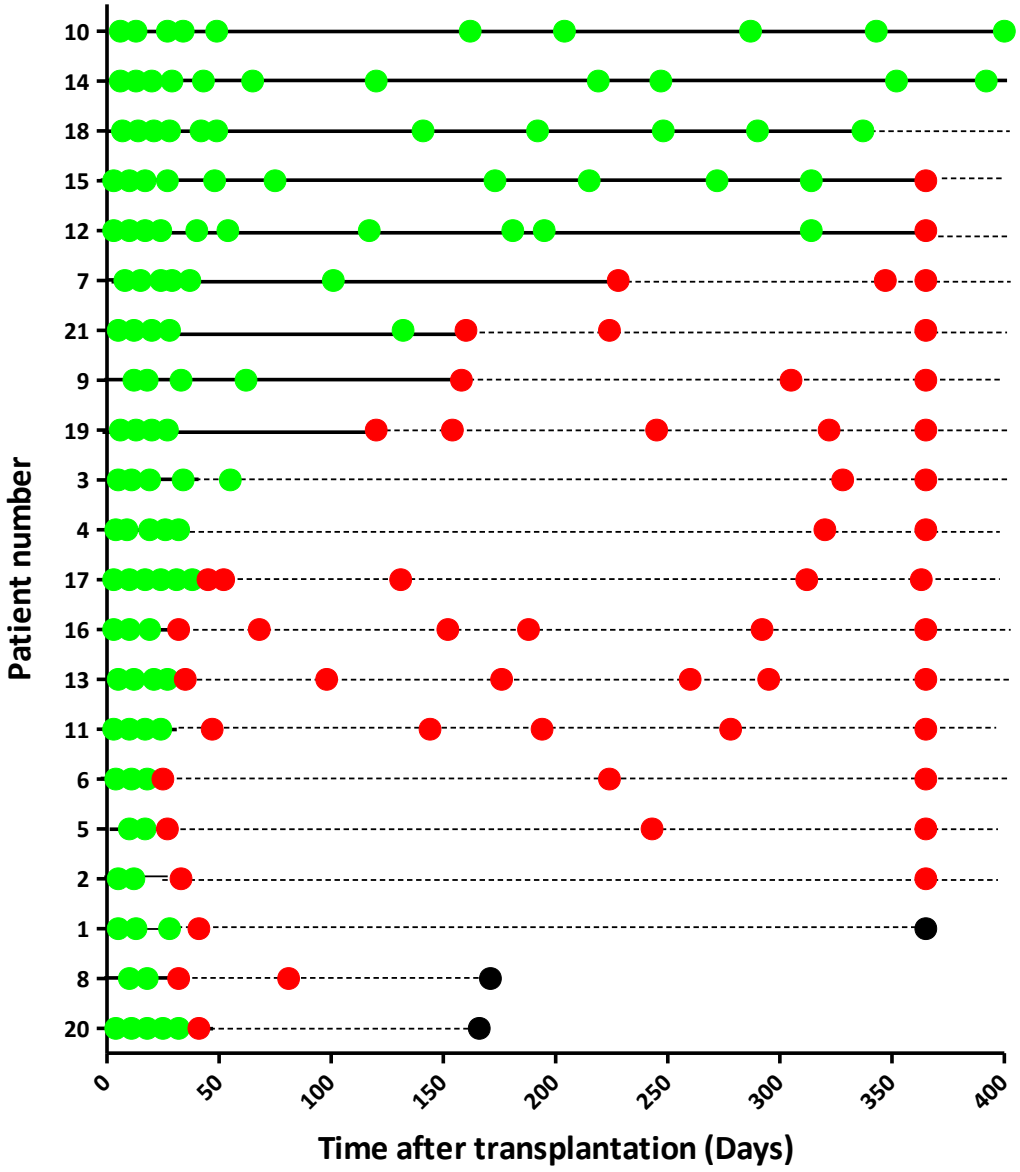


Figure 3.2 Detection and duration of donor CD4 T cell chimerism in lung transplant recipients, one-year post-transplant follow-up.

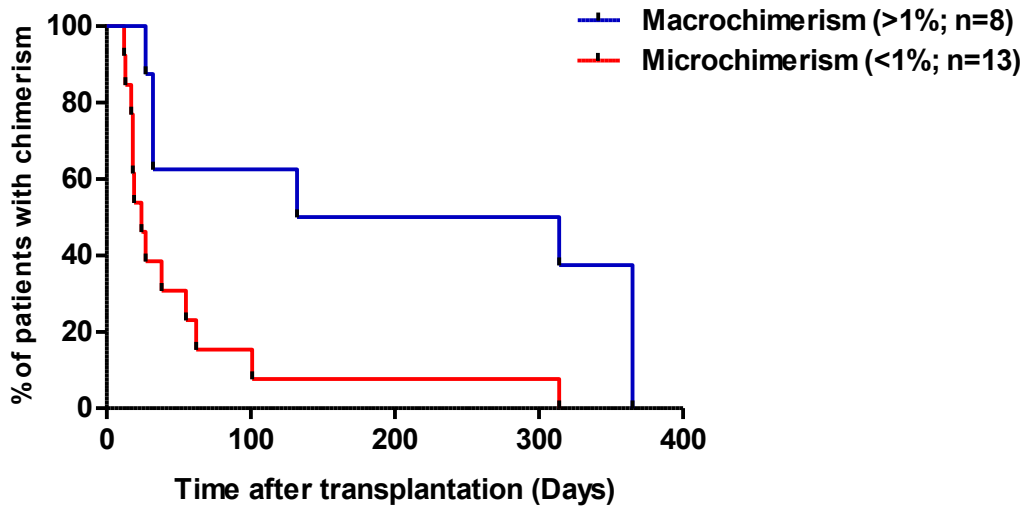
Donor HLA class I mismatched antigens were used as a target for detection of donor CD4 T cell chimerism in lung transplant recipients using flow cytometry. Three patterns of donor CD4 T cell chimerism were observed: short chimerism (donor CD4 T cells were detectable for six weeks after transplantation, patient 20, 8, 1, 2, 5, 6, 11, 13, 16, 17, 3, 4 and 19), intermediate chimerism (donor CD4 T cells were detectable between 3 and 6 months after transplantation, patient 9, 21 and 7), and long chimerism (donor CD4 T cells were detectable for more than six months after transplantation, patient 12, 15, 18, 14 and 10). Green dot - blood sample tested and donor CD4 T cells detected. Red dot – blood sample tested, but donor CD4 T cells were not detected. Black dot – patient died.

Interestingly, the initial proportion (detected in test samples obtained within the first week after transplantation) of donor CD4 T cells appeared to correlate with the length of time that the cells were detectable in the recipient circulation. Depending on the blood sample size (usually 40ml of recipient peripheral blood was obtained for chimerism analysis) whenever possible 1,000,000 recipient CD4 T lymphocytes were recorded. Donor CD4 T lymphocytes were detected as a subpopulation of double positive (CD3/CD4) recipient T lymphocytes, Table 3.4 shows the initial percentage and duration of donor CD4 T cell chimerism in the recipients' peripheral blood.

Table 3.4 Initial proportion and duration of donor CD4 T cell chimerism in lung transplant recipients.

Patient No.	Initial proportion of donor CD4 T cell (%)	Duration of donor CD4 T cell (days after transplant)
1	0.059	13
2	0.421	12
3	0.469	55
4	3.856	32
5	0.767	17
6	0.367	18
7	0.332	101
8	0.318	18
9	0.544	62
10	1.019	365
11	0.534	24
12	0.712	314
13	0.167	27
14	1.025	365
15	0.927	314
16	0.939	19
17	0.821	38
18	1.048	365
19	5.972	27
20	1.103	32
21	2.821	132

Depending on the proportions of chimeric cells present; the terms micro- and macrochimerism have been used to describe chimeric populations present at less than 1%, or greater than 1% of the total number of cells, respectively [129]. Based on the percentage of donor CD4 T cell chimerism detected I have assessed whether the type of chimerism (macrochimerism vs microchimerism) has an effect on the longevity of donor CD4 T cell chimerism, Figure 3.3. In my study cohort donor CD4 T cell macrochimerism was detected in 8 patients and microchimerism was observed in 13 patients. Univariate analysis revealed significant association between the type of chimerism (macrochimerism vs microchimerism) and the longevity of donor CD4 T cells detectable in the recipients' peripheral blood (log-rank; p=0.008). In majority of patients where macrochimerism was observed donor CD4 T cell were detectable for longer than 100 days post-transplant. However, the type of donor CD4 T cell present (macrochimerism vs microchimerism) was not associated with development of BOS during the first post-operative year (Fisher's exact test, *p*=1.000).



p=0.008

Figure 3.3 Kaplan-Meier plot showing the percentage of lung transplant recipients with macrochimerism and microchimerism described as donor CD4 T cell chimerism detected in greater than 1% and less than 1%, respectively.

3.2.5 The levels and duration of donor CD4 T cell chimerism does not affect the clinical outcome of lung transplant recipients

To investigate whether the percentage and the longevity of donor CD4 T cell chimerism was related to the incidence of rejection and the development of BOS, a nonparametric correlation analysis were performed.

3.2.5.1 Incidence of BOS in lung transplant recipients

In this study cohort, a sizable number of lung transplant recipients (33.34%) developed BOS grade 1 and 2 within the first postoperative year. In total, seven recipients developed BOS; of these, four recipients developed grade 2 and three recipients developed BOS grade 1. At one year after the lung transplant, 14 recipients were free from BOS. The correlation analysis did not reveal an association between withers the levels or the longevity of donor CD4 T cell chimerism and the incidence of BOS (Spearman r, p=0.692 and p=0.188). Figure 3.4, shows the percentage of lung transplant recipients with established BOS (n=7) and recipients free from BOS (n=14) at one-year after transplantation.

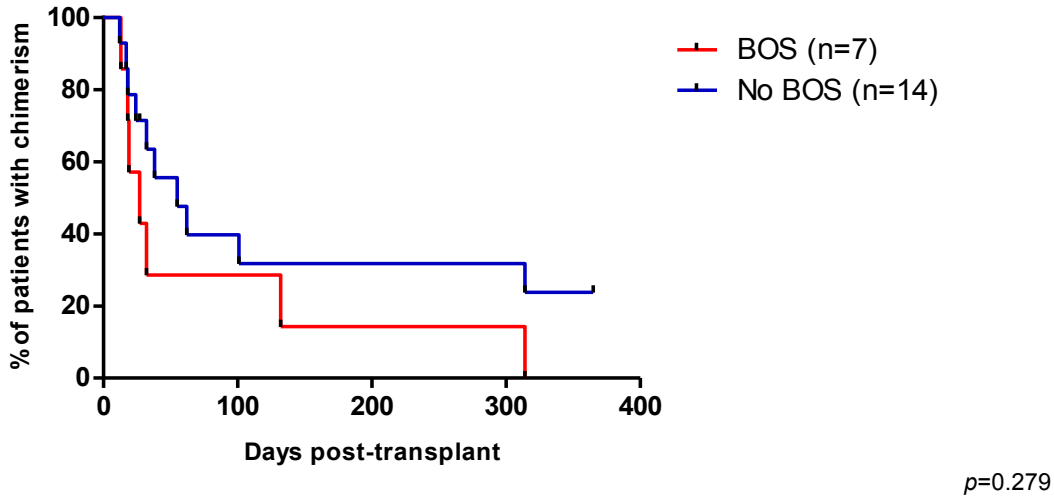


Figure 3.4 Kaplan-Meier plot showing the percentage of lung transplant recipients with donor CD4 T cell chimerism who within one-year after the transplant developed BOS and recipients that remained free from BOS.

3.2.5.2 Incidence of rejection in lung transplant recipients

During the first three months after transplantation, six patients (28.6%) had biopsy proven acute cellular rejection. Two patients had two episodes and four patients had one episode of acute cellular rejection. All patients were treated with intravenous steroids and responded well. Antibody mediated rejection was not observed in any of the patients. The correlation analysis revealed that the incidence of acute cellular rejection did not correlate with either the level of donor CD4 T cells detected in the recipients' peripheral blood or the longevity of donor CD4 T cell chimerism (Spearman r, p=0.319 and p=0.169, respectively).

The incidence of acute rejection episodes is considered the most significant risk factor for development of BOS. In this cohort, only two recipients who experienced acute cellular rejection during the first three months post-transplant developed BOS. One patient had two episodes and one recipient had one episode of acute cellular rejection; both recipients developed BOS grade 2. Nevertheless, the incidence of rejection did not correlate with the development of BOS during the first post-operative year (Spearman r, p=0.712). This reflects that five patients developed BOS without early acute rejection episode.

3.2.5.3 Patient survival

During the study period, two lung transplant recipients died (day 166 and day 171 following lung transplantation), both from non-transplant related complications. Both were free from BOS and had a well functioning allograft at the time of death. One further recipient has died as a consequence of BOS after the post-transplant follow-up period (day 461). Figure 3.5, shows the percentage of patient survival at one-year after lung transplantation.

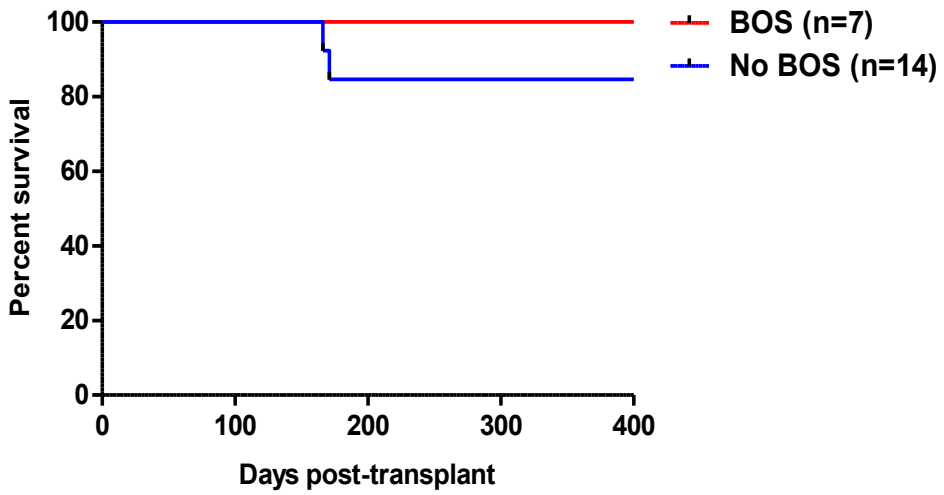


Figure 3.5 Kaplan-Meier survival curve for overall survival of lung transplant recipients with established BOS and recipients free from BOS. Vertical bars indicate censored events. *P*-values were based on the log-rank test on Kaplan-Meier plots. *P*-value of <0.05 was considered statistically significant.

3.3 Discussion

Two main findings emerged from the experiments described in this chapter. Firstly, it appears that the presence of donor CD4 T cell chimerism in the peripheral blood of lung transplant recipients is a uniform phenomenon. During the first post-operative month, donor CD4 T lymphocytes were consistently detectable in the recipients' peripheral blood. Secondly, distinct patterns of donor CD4 T cell chimerism were evident the first post-operative year: short, intermediate and long-lasting. My findings are similar to other studies that have demonstrated that the dynamics of chimerism in solid organ transplantation fluctuates over time and vary between individual patients [171, 172, 180].

To ensure that detected CD4 T cells were of donor origin, I tested patients' peripheral blood obtained prior to transplantation for the presence of pre-existing chimerism. CD4 T cells expressing donor HLA antigens were not detected in the recipients' peripheral blood prior to transplantation nor in the blood samples stained with third-party human HLA monoclonal antibodies, i.e. an HLA monoclonal antibody with specificity to an HLA antigen that is not expressed on both recipient and donor lymphocytes. These observations confirmed that detected CD4 T cells were of donor origin.

To ensure clear separation between both lymphocyte populations the sensitivity (limit of detection) of the flow cytometric analysis was adjusted for each transplant pair. For this reason, the flow cytomertic analysis included negative control test sample (patient PBMCs obtained prior to transplantation), which identifies the "background staining" i.e. non-specific binding of the mAb used; and positive control test sample (donor lymphocytes obtained at the time of donation) that detects the binding capacity of the mAbs to the donor HLA antigens. The selection of mAbs for each patient is described in Chapter 2, Section 3.2.3. Tailoring of the flow cytometric analysis for each patient individually allowed precise detection of donor CD4 T lymphocytes.

Detection of CD4 T lymphocytes that express donor HLA antigens that are not of donor origin might be possible if recipient CD4 T cells have somehow acquired donor-derived HLA class I antigens onto their cell surface. This phenomenon, termed trogocytosis, has been described for dendritic cells (DCs) [84]. Whether CD4 T lymphocytes possess this ability is unclear. To test, it would be necessary to assess for expression of both donor and recipient HLA on the same CD4 T cell. Unfortunately, due to the limited amount of blood samples and the limited set of mAbs available this approach was not possible. Instead, I created an artificial microchimerism containing a known number of “chimeric cells” and flow cytometric analysis was performed. The “chimerism cells” were detected by two methods: positive selection where “chimeric cells” were targeted directly and negative selection where the main cell population was targeted instead of “chimeric cells”, previously described in Chapter 2, Section 2.4.5. The same number of “chimeric cells” were observed in both the positive and the negative selection methods; thus, uptake of soluble HLA class I antigens by donor CD4 T lymphocytes was not observed. These observations provide additional support that donor CD4 T lymphocytes detected in the recipients’ peripheral blood were truly derived from the donor.

Although, donor CD4 T cell chimerism was detected in all patients, the percentage and the longevity of donor CD4 T cells detectable in the patients’ peripheral blood after lung transplantation varied between patients. The variation in the percentage of donor CD4 T cells was within the range of 0.06% and 6%; whereas, the time-interval for which the donor CD4 T cells were detectable in the recipients’ peripheral blood varied between six weeks and one-year after transplantation. The level and the duration of donor chimerism detected in the first month after transplantation in lung transplant recipients was comparable to the study by Richter et al. [171], However, in their study, the donor lymphocyte population was detectable for only two to four weeks after transplantation. Similar findings have been reported for liver transplant recipients [181]. The reasons for the observed variations in both the levels and the longevity of donor CD4 T cells in the recipients’ peripheral

blood following lung transplantation in spite of the same immunosuppressive protocol are not apparent.

I observed that 50% of donor lymphocytes detectable in the recipient circulation were CD4 T cells. This accords with Richter et al.'s, study [171]. Richter further reported detectable populations of donor–derived B and NK cells, whereas I did not characterise the cell types of the non-T cell donor lymphocyte population that was present in our cohort.

In a separate study, Richter et al. have assessed the number and the type of lymphocytes present in discarded human lungs [139]. They have identified two main sources of lymphocytes; one derived from the lung associated lymphoid tissue (LALT) mainly comprising of resting T and B cells and the second cell populations derived from the lung tissue itself, comprising of activated lymphocyte and monocytes/macrophage population. In addition they observed considerable difference in the number of lymphocytes present between different human donor lungs [139].

Taking into consideration the Richter et al. findings, it is possible that the observed variation in the percentage of the donor CD4 T cell chimerism detected is due to the nature of the donor lungs itself and, possibly related to both the donor history and to allograft conditioning. It has been reported that factors such as donor gender, age, days on ventilation and type of donor can affect the cell population transferred with the allograft. Increased levels of pro-inflammatory cytokines in donors after brain-death (DBD) (in comparison to donors after circulatory death (DCD)) have been associated with increased number of lymphocytes in heart and lung allografts [182].

In this cohort, 20 of the 21 patients received lungs from a DBD and only one patient received lung from a DCD; thus, due to the small numbers it was not possible to investigate whether there is a difference between both groups.

Interestingly, I observed association between the donor CD4 T cells macrochimerism and the duration of donor CD4 T cells detectable in the

recipients' peripheral blood following lung transplantation. This raises the question whether the longevity of donor CD4 T cell chimerism is largely a consequence of the level of CD4 T cells present within the lung at the time of transplantation or whether other factors, such as HLA mismatch, also influence the longevity of donor CD4 T cell chimerism in lung transplant recipients.

Several factors that might influence the donor CD4 T cell longevity in the recipients' peripheral blood have been taken into consideration. As previously discussed, presence of donor HSCs have been observed in the bone marrow of long-term liver transplant survivors [173]; nevertheless, Nerhoff et al., have not investigated the ability of the bone marrow derived donor HSCs to differentiate into different cell subsets; a phenomenon observed in mice models [174]. Thus, if similar mechanisms do exist in human allografts it is possible that the long lasting donor chimerism is associated with migration of passenger HSCs from within the allograft into the recipient bone marrow, where they can give rise to various cell types of donor origin.

Previous studies have demonstrated that T cells have the ability to recirculate through non-lymphoid organs [136]; functionally and phenotypically these cells are a mixture of naïve, effector and memory T cells that express cell-surface markers such as CCR7, LFA-1, VLA-4 and ICAM-1, that govern the homing to secondary lymphoid tissue and other peripheral tissues [136, 137, 139, 183]. Migration of allograft passenger cells to non-lymphoid tissues such as the skin has been observed in liver [131] and kidney [165] transplant recipients even decades after transplantation. Thus, it is possible that rapid migration of donor CD4 T lymphocytes from within the allograft into other tissue sites might explain why, in the majority of lung transplant recipients (n=13), donor CD4 T lymphocytes were detectable in the recipients' peripheral blood for only a few weeks after transplantation.

Unfortunately, due to the nature of this study it was not possible to assess whether donor lungs contained HSCs and if present whether these cells have migrated to the recipients' bone marrow. Similarly, I was unable to assess

donor CD4 T lymphocytes tissue distribution. To elucidate whether these factors affect the longevity of donor CD4 T cell chimerism in lung transplant recipients, will require further study.

Donor chimerism can occasionally trigger GvHD, a condition that results as a consequence of immune responses of donor immunocompetent cells against the recipient cells. In solid organ transplantation, GvHD is a rare post-transplant complication; nevertheless detrimental outcomes have been reported in liver [176, 177] and lung transplant patients [178, 179]. Immunogenetic discrepancies within the major and minor histocompatibility antigens are the main targets for allorecognition, and this response is bidirectional: graft *vs* host or host *vs* graft.

In this cohort, all patients received HLA mismatched allografts. This raises the question whether the elimination of donor CD4 T lymphocytes from the recipients' peripheral blood is due to recipient T and/or NK cells alloreactivity against donor passenger lymphocytes.

The impact of the HLA and NK cell alloreactivity on the duration of donor CD4 T cell chimerism in the recipients' peripheral blood following primary lung transplantation is addressed in the following Chapter; Chapter 4.

3.4 Key points

- Donor CD4 T cell chimerism is a uniform phenomenon that occurs in all lung transplant patients as a result of cell migration from within the allograft into the recipient peripheral blood following transplantation.

- The percentage of donor CD4 T cells detected in recipients' peripheral blood is extremely variable between patients.

- The dynamics of donor CD4 T cell persistence in the recipients' peripheral blood is different between patients, despite the fact that all patients were subjected to the same immunosuppressive regiment.

- The longevity of donor CD4 T cells detectable in recipients' peripheral blood was associated with the presence of donor CD4 T cell macrochimerism.

4 Chapter 4

Determinants of longevity of donor CD4 T cell chimerism in lung transplant recipients

4.1 Introduction

The immunological events that initiate allograft rejection, both cellular and humoral immune responses, occur due to allorecognition of the antigenic differences between the recipient and the donor major histocompatibility complex (MHC) proteins and minor histocompatibility antigens (mHA).

Thus, allorecognition refers to detection of same-species, non-self-antigens by the host immune system. It is primarily driven by the ability of the recipient T cells to recognise both intact donor MHC molecules expressed on donor antigen presenting cells, known as a direct pathway of allorecognition, and processed donor derived MHC peptides presented in the form of self-MHC peptide complexes, an indirect pathway. A semi-direct pathway of allorecognition occurs when the recipient T cells recognise intact donor MHC:peptide complexes that have been acquired by the trafficking recipient dendritic cells (DCs) [58].

In contrast to T cell allorecognition, NK cell allorecognition occurs due to lack of interaction between the NK cell inhibitory KIRs and a subset of MHC class I molecules that act as ligands for the NK cell KIRs; rather than due to the antigenic differences within the major histocompatibility antigens [184]. The main MHC class I molecules that act as ligands for NK cell inhibitory KIRs are HLAs, including HLA-Cw and some HLA-A and HLA-B antigens that express Bw4 epitope [184].

4.1.1 Alloantigens

Allorecognition of foreign antigens represents the main barrier for successful organ transplantation; the degree of alloantigen discrepancy between the recipient and donor pair determines the magnitude of acceptance or rejection of transplanted tissue and/or allograft. Broadly speaking, alloantigens are divided into two groups: major and minor histocompatibility antigens.

4.1.1.1 Major histocompatibility antigens

The genes that encode for MHC proteins, known as HLA, are clustered within the class I and class II MHC region located on the short arm of chromosome six [185].

The MHC class I molecules are encoded by three genetic loci: HLA-A, HLA-B and HLA-Cw. They consist of two non-covalently linked polypeptide chains, alpha heavy chains anchored to the cell membrane and associated with β2-microglobulin. The heavy chain α1 and α2 domains fold together creating a cleft, known as a peptide-binding site [32]. They are constitutively expressed on the surface of the all nucleated cells and present endogenous short (8-10 amino acids) peptides to CD8 T cells [32, 33].

The MHC class II molecules are encoded by HLA-DR, HLA-DQ and HLA-DP loci; they consist of two polypeptide chains, α and β both anchored to the cell membrane. The α1 and β1 domain form the peptide-binding site and present exogenous peptides (13 to 25 amino acids in length) to CD4 T cells. The class II molecules are constitutively expressed only on dendritic cells, macrophages and B cells, also known as professional antigen presenting cells [38, 39]; nevertheless, their expression can be induced on other cells in the presence of IFN-γ and TNF-α [40].

The most polymorphic region of the HLA class I and class II antigens is found within the α2 domain and β1 domain, respectively [39]. The extensive polymorphism of the HLA genes allows presentation of vast array of peptides derived from potentially pathogenic organisms in order to ensure efficient presentation and eradication of pathogens by CD8 and CD4 T cells.

In transplant settings the differences within the HLA class I and class II genes between the recipient and donor pair limits the success of the transplant outcome due to the potential of triggering vigorous alloimmune responses, ultimately resulting in allograft rejection.

4.1.1.2 Minor histocompatibility antigens

Any polymorphic non-MHC protein able to initiate immune responses in an HLA-matched recipient and donor pair is known as a minor histocompatibility (mH) antigen and represents a potential target for allorecognition [186]. The mH antigens are presented to T cells by both MHC class I and class II molecules.

Several mH antigens have been identified that are able to elicit immune responses resulting in transplant rejection and/or GvHD following haematopoietic stem cell transplantation (HSCT); their role in solid organ transplantation is less convincing.

The mHAs are peptides derived by genes encoded by: sex chromosome, autosomes and genes encoded by mitochondrial DNA (miDNA); including male H-Y antigens (encoded on the short arm of the Y chromosome), autosomal antigens such as HA-1, HA-2, HA-3, HA-8, myosin 1G, LCB oncogene and other; and, polymorphism in mitochondrial genes encoding for enzyme mt-ND1. These are the most studied mHA that have been associated with GvHD and graft rejection in HSCT [43, 187].

4.1.2 T cell mediated alloresponses to major and minor histocompatibility antigens

The term alloresponse is used to describe the effector arm of the immune system that is initiated due to allorecognition of immunogenic major and/or minor alloantigens. In an alloresponse the innate and adaptive immune system function together to reject the allograft.

This Chapter focuses only on the T-cell and NK–cell mediated alloimmune responses. Humoral alloimmune responses are discussed in Chapter 6. Recognition of alloantigens by the recipient T cells; and, cognate interaction between the T-cell receptor (TCR) and its co-receptors CD8 or CD4 with MHC

class I and class II/peptide complexes, respectively, by itself does not lead to T cell activation. Naïve T cell activation and proliferation is dependent on the delivery of secondary co-stimulatory signals provided by the activated antigen presenting cells, manly DCs [188]. These signals are delivered via interactions between co-stimulatory molecules present on activated DCs such as B7.1 (CD80), B7.2 (CD86), CD40 and OX40 ligand and their corresponding receptors CD28, CD40 ligand (CD154) and OX40 expressed on the T cell membrane [188, 189]. These cell-cell interactions, and as a consequence T cell activation, take place in the T cell area of the secondary lymphoid organs (SLOs) such as lymph nodes or spleen [190], although there is increasing evidence suggesting that T cell activation can occur in the tertiary lymphoid organs (TLOs) [191, 192]. TLOs are non-capsulated ectopic lymphoid formations that display characteristics of SLOs [192].

Activated CD4 helper T cells undergo proliferation, a process that is dependent on T cell growth factors (TCGF) such as IL-2, IL-4 and IL-5 and differentiation into various T cell subsets including T-helper 1 (Th1), T-helper 2 (Th2), T-helper 17 (Th17), T follicular helper (Tfh) cell and regulatory T cell (Tregs); whose effector function is characterised by the type of cytokines they produce. CD4 T cell differentiation is described in more detail in Chapter 5.

In the context of allograft rejection, the effector Th1 cells trigger activation of cytotoxic CD8 T cells, activation of macrophages and they provide help for B cell activation and synthesis of complement-fixing antibodies. In addition, CD4 T cells may mediate cytotoxic activity via Fas-ligand/Fas receptor death pathway [193]. Conversely, the cytokines produced by effector Th2 cells trigger eosinophil activation and provide help for B cell activation and antibody production [194, 195].

In contrast, activated effector cytotoxic CD8 T cells have the ability to directly kill the target cell via perforin/granzyme-mediated and Fas-mediated cell death pathways [196, 197]. Recognition and ligation of cytotoxic CD8 TCR with allo-MHC class I molecules triggers activation process, which induces formation of intracellular cytotoxic granules and expression of perforin and

granzymes. CD8 cell killing is achieved by formation of a tight junction with the target cells and release of the cytotoxic granules to the region of cell contact [198]. The perforin forms channels onto the target cell membrane, allowing insertion of granzyme A and B into the cell cytoplasm, leading to activation of a cascade of intracellular caspases resulting in DNA fragmentation and ultimately cell death by apoptosis [197]. These complex immune responses can result in allograft rejection and/or GvHD following HSCT.

In direct and semi-direct allorecognition pathways, the CD4 or C8 T cells with direct allospecificity recognise conformationally intact allo-MHC molecules. The frequency of T cells with direct allospecificity responding to allogeneic MHC molecules is around 10% [59, 60]. Two hypotheses have been proposed to explain this phenomenon; “high determinant hypothesis” where the allogeneic MHC molecule itself, independent of the peptide bound, plays a major ligand for alloreactive T cells [61], and “multiple binary complex hypotheses” process driven exclusively by the peptide bound on the MHC molecule [62, 63].

In contrast, allorecognition of donor derived MHC peptides by the recipient T cells with indirect allospecificity mimics the conventional T cell response to foreign antigens. Thus, donor MHC molecules are first internalised, processed and presented as peptides bound within the self-MHC/peptide complexes [58].

The degree of antigenic discrepancy between the donor and recipient is a major factor in determining the strength of the alloresponse. Thus, it has long been recognised that HLA matching between donor and recipient is associated with better allograft and patient survival; and in the case of HSCT, sex matching to avoid responses against H-Y mHA antigen results in better outcomes [199]. Nevertheless, finding non-related HLA matched tissue and/or organ donor has proven difficult and is not always possible and some degree of mismatching is typical.

4.1.3 HLA matching

The Human Leukocyte Antigens represent the main targets for allorecognition in HLA mismatched transplants and the HLA discrepancies between the recipient and donor pair represent a limiting factor for successful solid organ and HSCT.

In HSCT HLA matching at HLA-A, -B -Cw, -DR and -DQ loci is fundamental for successful clinical outcome [200]. Similarly, in renal transplantation, HLA matching plays an important role in organ allocation; patients who have received 0.0.0-mismatched kidneys referring to HLA-A, -B and -DR loci i.e. a fully matched kidney, have much better outcomes [201]. One-year graft survival rate varies from 98% vs 80% for patients that received 0.0.0 vs 2.2.2 mismatched kidney transplant [201]. In more recent years, increased use of more potent immunosuppressive regiments has rendered the impact of HLA compatibility in renal transplantation [202]; hence, the new National kidney allocation criteria is not heavily based on HLA matching [50].

Contrary to this, conflicting evidence has been generated supporting the significance of HLA matching in cardiothoracic transplantation; and, currently HLA matching is not taken into consideration for allocation of cardiothoracic organs, due to the smaller donor pool and to the requirements to limit organ cold ischemia.

Tendrich et al., and others have demonstrated that HLA matching is not associated with prolonged graft survival in heart transplant recipients [51, 52]. In contrast, analysis from the UNOS/ISHLT Thoracic Registry showed that HLA matching has a beneficial impact on graft survival in heart and single-lung transplant patients; primarily matching at HLA-A and HLA-DR loci [53]. In other studies, matching for HLA-A [54] or HLA-DR loci [55] has been associated with reduced incidence of BOS in lung transplant recipients.

4.1.4 Natural Killer cell mediated alloresponses

NK cells are cells of the innate immune system: they mediate their effector function without prior exposure to antigens via a range of germline-encoded stimulatory and inhibiting killer-cell immunoglobulin-like (KIRs) receptors expressed at their cell surface [184]. The NK cell surface receptors interact with specific alleles of HLA class I antigens. This allows them to distinguish between self and non-self [184]. Two different NK cell subsets have been described $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$, with predominately cytokine producing and cytotoxic effector functions, respectively [203].

The NK cell activation is primarily controlled by a number of activating (*KIR2DS2, KIR2DS1*) and inhibitory (*KIR2DL2, KIR2DL3, KIR2DL1* and *KIR3DL1*) KIRs that bind to HLA class I antigens, primarily to HLA-Cw and some HLA-A and HLA-B antigens that express Bw4 epitope [204]. The HLA class I KIR ligands have been divided into two groups based on the dimorphism at position 80 of the α helix within the HLA-Cw antigens: C1 group (Cw1, 3, 7, 8, 12, 14, 1507 & 1601) ligands that contain asparagine at position 80 and C2 group (Cw2, 4, 5, 6, 1204, 1205, 15, 1602, 17 & 18) ligands containing lysine at the same position. Similarly, Bw4, a public epitope found on some HLA class I antigens (HLA-A24, HLA-B13, 27, 44, 51, 52, 53, 57 and 58), contains either isoleucine or threonine at the same position [204, 205].

The inhibitory *KIR2DL2* and *KIR2DL3* receptors bind to HLA-Cw antigens that belong to the C1 group, *KIR2DL1* binds to antigens belonging to the C2 group, and *KIR3DL1* is an inhibitory receptor that binds to HLA class I antigens that express the Bw4 epitope [204].

Absence of interaction between the NK cell inhibitory KIRs and its corresponding HLA ligands leads to loss of NK cell inhibition, thus resulting in NK cell activation and target cell killing, a process termed as “missing self-recognition” [206].

In the transplant setting, NK cell alloreactivity is bidirectional: graft-*versus*-host (GvH) and host-*versus*-graft (HvG) [207, 208]. The NK cell GvH alloreactivity occurs when the NK cells of the donor origin have a missing ligand for its inhibitory KIR receptors on the recipient cells; whereas HvG NK cell allorecognition occurs in the opposite manner, due to lack of engagement between the recipient NK cell inhibitory receptors and the appropriate ligands present on donor cells.

Graft-*versus*-host NK cell alloreactivity can be beneficial, and is a strategy frequently adopted in HLA haplotype-mismatched (haploidentical) HSCT for patients with high-risk acute myeloid leukaemia (AML) [208]. In haploidentical HSCT, NK-alloreactive donors are selected based on the "missing-self" recognition, where donor NK cells express both the inhibitory KIR and its corresponding HLA class I ligand, but this HLA class I ligand is absent on recipient cells. Transplantation from NK-alloreactive donors has been associated with reduced leukaemia relapse rate, lack of GvHD and improved event free survival [209].

The role NK cell alloreactivity in solid organ transplantation is less clear. The effect of NK cell alloreactivity on graft survival rates was examined in a study of 2,757 kidney transplant recipients [210], where KIR ligand incompatibility was determined based on the presence or absence of KIR ligand epitopes for C1 group, C2 group and Bw4 between the donor and recipients. The 10-year graft survival rates were comparable between all patient groups [210]. However, in this study the degree of HLA mismatching between the donor and recipients was not examined.

Van Bergen et al. undertook a similar study where the role of KIR ligand mismatching was assessed in an HLA compatible (HLA-A, -B & -DR) kidney transplants and transplants compatible for HLA-DR but not for HLA-A and HLA-B antigens [211]. They have showed that the presence of one or more KIR ligand mismatches in HLA compatible kidney transplants was associated with reduced 10-year graft survival rates from 81% to 59%; whereas, KIR ligand mismatching did not affect the graft survival rates in HLA-A and/or

HLA-B incompatible (mismatched) transplants [211]. This is the first clinical study that showed that KIR ligand incompatibility might hamper the success of HLA compatible solid organ transplants.

Moreover, in murine transplant models several studies have reported the role of NK cells in the induction of tolerance [212] and rejection [213]. Yu et al., have shown that long-term skin allograft survival was achieved in mouse model by rapid clearance of donor passenger APCs by the host NK cells; while, depletion of host NK cells prior to skin transplantation led to prolonged survival of donor APCs and rapid skin allograft rejection [212].

Recent evidence suggests that NK cells contribute to rejection of human lung transplants. The NK cell frequency in patients' peripheral blood at 3 months after lung transplantation was shown to be significantly lower in comparison to the NK cell frequency present at the time of transplantation and in healthy controls [214]. Furthermore, the number of $CD56^{bright}$ NK cell phenotypes, cytokine-producing NK cells, has been observed to be increased in the bronchoalveolar lavage (BAL) of patients experiencing acute rejection and/or CMV reactivation [214]. It remains unclear, however, whether NK cells play a direct causative role and/or amplify the proinflammatory local environment in the lungs.

Moreover, Fildes et al., have shown that patients with bronchiolitis obliterans syndrome have predominantly activated NK cell phenotype in the peripheral blood when compared with stable patients; and they have observed a significant increase in the number of NK cells in the lung tissue itself [215].

In contrast, in a separate study of 48 lung transplant patients, the development of BOS was not associated with KIR ligand mismatching [216]; but instead with the *KIR* haplotype. Patients carrying a *KIR* haplotype A were more likely to develop BOS in comparison to patients carrying *KIR* haplotype B or haplotype AB [216].

In lung transplantation, recipient-versus-donor (RvD) NK cell alloreactivity has been studied in relation to the incidence of allograft rejection and the incidence of BOS, with conflicting results. The impact of NK cell alloreactivity on the development and duration of circulating donor chimerism has not been examined.

Having identified considerable variation in the extent and duration of circulating donor CD4 T cell chimerism between different lung transplant recipients (Chapter 3), the experimental work in this chapter addresses whether the duration of donor CD4 T cell chimerism is determined by the degree of HLA mismatching or by RvD NK cell alloreactivity.

4.2 Results

4.2.1 Does HLA mismatching affect the duration of donor CD4 T cell chimerism detectable in the peripheral blood of primary lung transplant recipients?

To address the question of whether the duration of donor CD4 T cell chimerism detectable in the recipients' peripheral blood following lung transplantation is influenced by the HLA incompatibility, the degree of HLA mismatching for HLA-A, -B and -DR loci between the recipients and their donors was assessed.

Genomic DNA was extracted and used as a template for HLA genotyping for each patient and their respective donor prior to transplantation. The HLA genotyping was performed using low/medium resolution in-house PCR-SSP methodology which utilises a panel of oligonucleotide primer mixes specific for amplification of HLA-A, -B, -Cw, -DRB1, -DRB3, -DRB4, -DRB5 and –DQB1 alleles, previously described in Section 2.6.1 and Section 2.6.2.

Following the assessment of the amplified PCR products the HLA genotype was identified and the degree of the HLA mismatched grade was established for each recipient and donor pair in this cohort (n=21). Table 4.1 represents the HLA genotype of each recipient and donor pair for HLA-A, -B and -DR antigens, highlighting the donor mismatched HLA antigens.

Table 4.1 Human leucocyte antigen (HLA) A, B and DR genotype (presented as serological equivalent) of lung transplant recipients and their corresponding donors using PCR-SSP methodology. Donor HLA mismatched antigens are highlighted in red.

Patient No.	Recipient HLA-A; -B; -DR	Donor HLA-A; -B; DR
1	HLA-A1, 32; -B8; DR103, 17	HLA-A2, 3; -B44, 62; DR13, 15
2	HLA-A25, 26; -B8, 57; DR17, 7	HLA-A2; -B8, 35; DR17, 11
3	HLA-A1, 3; -B7, 57; DR1, 7	HLA-A2; -B51, 27; DR11, 16
4	HLA-A24, 25; -B62, 37; DR4, 10	HLA-A3, 24; -B51, 7; DR15
5	HLA-A2; -B44, 61; DR7, 13	HLA-A3, 33; -B7, 65; DR1, 17
6	HLA-A3; -B51, 65; DR17, 4	HLA-A1, 11; -B7, 56; DR1, 4
7	HLA-A1, 68; -B8, 65; DR15, 17	HLA-A2, 3; -B7, 44; DR7, 15
8	HLA-A2, 24; -B7, 44; DR15, 16	HLA-A1, 11; -B8, 62; DR17, 13
9	HLA-A2, 30; -B7, 44; DR15, 4	HLA-A1, 23; -B44, 42; DR18, 14
10	HLA-A24, 68; -B8, 62; DR103, 7	HLA-A1, 3; -B7, 57; DR7, 15
11	HLA-A2, 24; -B65, 57; DR13, 7	HLA-A1, 29; -B52, 44; DR15, 7
12	HLA-A1, 32; -B7, 55; DR4	HLA-A1, 3; -B7, 8; DR17, 4
13	HLA-A1, 2; -B51, 8; DR1, 17	HLA-A2; -B7, 56; DR1, 15
14	HLA-A2, 23; -B8, 44; DR13, 10	HLA-A2; -B51, 44; DR4, 11
15	HLA-A2, 24; -B7, 44; DR103, 13	HLA-A2, 24; -B60, 62; DR4, 13
16	HLA-A3, 32; -B51, 62; DR1, 13	HLA-A2; -B44; DR11, 15
17	HLA-A1, 2; -B7; DR1, 13	HLA-A1, 11; -B7, 8; DR15, 17
18	HLA-A24, 31; -B62, 51; DR15,13	HLA-A2; -B44, 18; DR7, 11
19	HLA-A1, 3; -B7, 57; DR1, 15	HLA-A2, 3; -B44, 35; DR4, 11
20	HLA-A1, 3; -B8, 27; DR1, 17	HLA-A2; -B27, 60; DR4, 13
21	HLA-A2, 3; -B7, 62; DR8	HLA-A68, 36; -B57, 71; DR17, 13

The majority of patients in this study group received poorly matched grafts; none of the patients received 0.0.0 mismatched primary lung transplant for HLA-A, -B and -DR. Of 21 patients, three patients were matched for HLA-A, 9 patients had one and 9 patients had two HLA-A mismatched antigens.

In contrast, all patients in this study group were mismatched at a minimum one allele for HLA-B and HLA-DR. Seven patients had one mismatched HLA-B antigen and 14 patients had two HLA-B mismatched antigens. The number of patients mismatched for HLA-DR was similar: 8 patients had one and 15 patients had two HLA-DR mismatched antigens, Table 4.2 represents a summary of the number and the percentage of patients that received donor HLA mismatched primary lung transplant for HLA-A, HLA-B and HLA-DR antigens.

Table 4.2 Number and percentage of patients that received donor HLA mismatched primary lung transplant for HLA-A, -B and -DR antigens.

Donor HLA mismatched antigens (HLA-A, -B, -DR)	Number and percentage of patients with mismatched donor HLA antigens *n* = 21 (%)
HLA-A	
0	3 (14.3)
1	9 (42.8)
2	9 (42.8)
HLA-B	
0	0 (0)
1	7 (33.4)
2	14 (66.4)
HLA-DR	
0	0 (0)
1	8 (38.1)
2	13 (61.9)

All patients (n=21) received allografts mismatched at minimum three out of six HLA antigens for HLA-A, -B and -DR. Only three patients were matched for HLA-A and none of the patients were fully matched for HLA-B or HLA-DR. Nearly half of the patients in this cohort received fully mismatched lung transplants; 8 patients were mismatched at six out of six HLA antigens and 4 patients were mismatched at five out of six HLA antigens (Figure 4.1).

In this study the relationship between HLA mismatching, and the duration of donor CD4 T cell chimerism was analysed separately for each locus (HLA-A, -B and -DR). Based on the number of HLA mismatched antigens (0, 1 or 2) for each locus, patients were separated into three groups: patients with zero, patients with one and patients with two mismatched HLA antigens individually for HLA-A, -B and -DR.

Univariate analysis did not reveal an association between the number of HLA mismatched antigens for HLA-A (p=0.493), (Figure 4.1, A) -B (p=0.839) (Figure 4.1, B) and -DR (p=0.841) (Figure 4.1, C) locus and the duration of donor CD4 T cell chimerism in the peripheral blood of lung transplant recipients following transplantation.

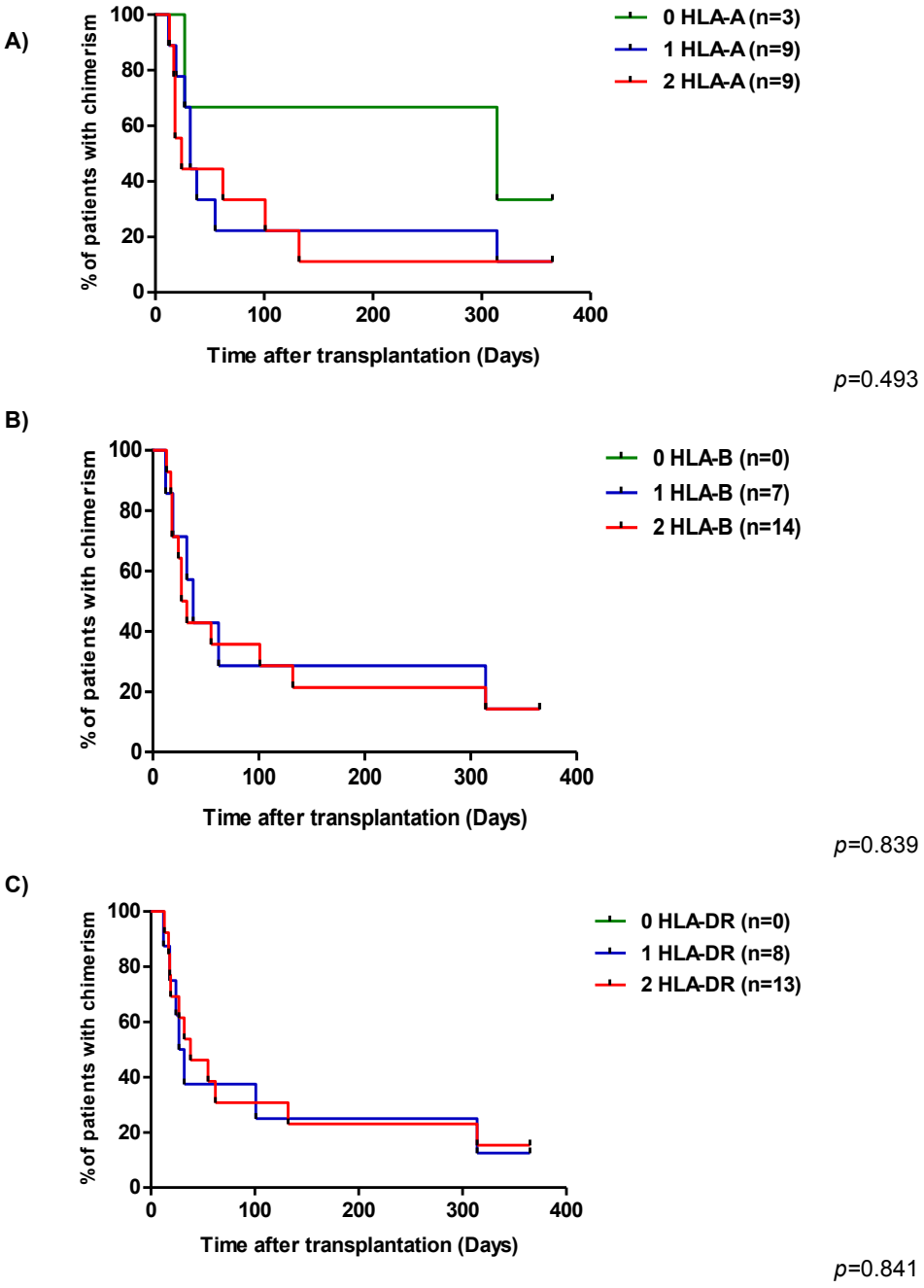


Figure 4.1 Impact of human leukocyte antigen (HLA); HLA-A, HLA-B and HLA-DR mismatching on the duration of donor CD4 T cell chimerism detectable in the recipients' peripheral blood following primary lung transplantation. A) Number of HLA-A mismatched antigens, B) Number of HLA-B mismatched antigens and C) Number of HLA-DR mismatched antigens. *P*-values were based on the log-rank test on Kaplan-Meier plots. *P*-value of <0.05 was considered statistically significant.

The cumulative effect of HLA mismatched antigens for HLA-A, -B and -DR was also assessed. Based on the total number of HLA mismatched antigens patients were separated into three groups: patients with zero to two mismatched antigens (n=0), patients with three to four mismatches (n=9) and patients with five and six mismatched antigens (n=12). Univariate analysis did not reveal an association between the total number of HLA mismatched antigens for HLA-A, -B and -DR and the duration of donor CD4 T cells detectable in the recipients' peripheral blood following lung transplantation (p=0.867), Figure 4.2. In addition, the number of HLA mismatched antigens was not associated with the incidence of acute cellular rejection and development of BOS within the first year after transplantation (Fisher's exact test, p=1.000 and p=1.000, respectively).

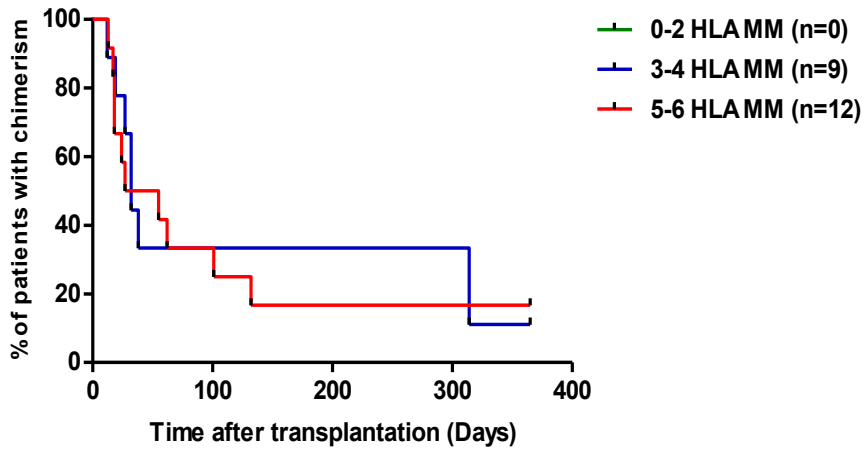


Figure 4.2 Cumulative effect of the human leukocyte antigen (HLA) (HLA-A, -B and –DR) mismatching on the duration of donor CD4 T cell chimerism detectable in the recipients' peripheral blood following primary lung transplantation. *P*-values were based on the log-rank test on Kaplan-Meier plots. *P*-value of <0.05 was considered statistically significant.

4.2.2 Does NK cell alloreactivity affect the duration of donor CD4 T cell chimerism detectable in the peripheral blood of primary lung transplant recipients?

To address the question of whether the duration of donor CD4 T cell chimerism detectable in the peripheral blood of primary lung transplant recipients is influenced by host NK cell alloreactivity, the presence and/or absence of RvD NK cell alloreactivity for each recipient and donor pair was predicted based on the "missing-self" hypothesis, previously described in Section 2.6.3.1.

To predict the RvD NK cell alloreactivity the recipients and their donors were genotyped for HLA-Cw alleles and the Bw4 epitope using genomic DNA as a template for PCR-SSP, as described in Section 2.6.1 and Section 2.6.2. In addition, the recipients were also genotyped for presence of the inhibitory KIR genes including *KIR2DL1, KIR2DL2, KIR2DL3* and *KIR3DL1*. The KIR gene content was analysed using high-throughput technology known as quantitative qKAT, described in Section 2.6.3.

Recipient-versus-donor NK cell alloreactivity was expected if there was at least one KIR-ligand mismatch between the recipient and donor pair. Thus, KIR-ligand mismatch signifies the presence of a minimum one inhibitory KIR (*KIR2DL1, KIR2DL2, KIR2DL3* and/or *KIR3DL1)* that during NK cell development has been educated to interact with "self" HLA ligand and presence of "self" HLA ligand (C1, C2 and/or Bw4) on the recipient cells; and, absence of the HLA ligand (C1, C2 and/or Bw4) on the donor cells. Under these conditions NK cell expressing an inhibitory KIR biding "self" HLA can be activated when challenged will allogeneic cells lacking an HLA ligand for the inhibitory receptor. Table 4.3 demonstrates a schematic description of the KIR-ligand mismatch definition.

Table 4.3 KIR-ligand mismatch definition. (Table adapted from van Bergen et al., 2011)

Inhibitory KIR-ligand	Recipient		Donor
	KIR	HLA	HLA
KIR2DL1/C2	KIR2DL1 +	C2 +	C2 –
KIR2DL2/C1	KIR2DL2 +	C1 +	C1 –
KIR2DL3/C1	KIR2DL3 +	C1 +	C1 –
KIR3DL1/Bw4	KIR3DL1 +	Bw4 +	Bw4 –

KIR-ligand mismatch definition applies only under the circumstances when a particular inhibitory KIR (*KIR2DL1, KIR2DL2, KIR2DL3 and/or KIR3DL1*) and its corresponding ligands are present on the recipient NK cells and at the same time the ligand is absent on the donor cells. It is essentially four examples where KIR inhibitory ligand mismatching occurs and is functional for recipient against donor, Table 4.3. Absence of inhibitory signalling will allow activating NK cell receptors to cause NK cell degranulation and target cell killing.

Based on the KIR-ligand mismatch definition for each recipient and donor pair in this study (n=21), RvD NK cell alloreactivity was assessed. Table 4.4 shows the recipient and donor HLA class I genotype and its segregation into three groups based on the dimorphism at position 80 of the α helix within the HLA-Cw antigens and the Bw4 epitope ((C1, C2 and Bw4), HLA class I segregation is described in Section 4.1.4), recipients' inhibitory KIR genotype and predicted RvD NK cell alloreactivity for each recipient and donor pair. Recipient KIR gene content highlighted in red represents presence of KIR genes that were expected to have been educated during the NK cells development i.e. licenced to recognise "self" (recipient cells contain both the KIR gene and its corresponding ligand).

Table 4.4 Predicted recipient *versus* donor (RvD) NK cell alloreactivity based on the "missing-self" theory. This Table shows the recipient and donor HLA class I genotype its segregation into C1 group, C2 group and Bw4, recipients' inhibitory KIR genotype and predicted RvD NK cell alloreactivity.

Patient No.	Recipient HLA-Cw / Bw4 epitope	Recipient HLA-Cw groups (C1*, C2**) and Bw4***	Recipient KIR^ gene content (*2DL1, 2DL2, 2DL3, 3DL1)*^^	Donor HLA-Cw / Bw4 epitope	Donor HLA-Cw groups (C1*, C2**) and Bw4***	Predicted RvD' NK" cell allo-reactivity (Yes / No)
1	Cw7, - / -	C1	*2DL1,* ***2DL3,*** *3DL1*	Cw5, 6 / Bw4	C2, C2, Bw4	Yes
2	Cw6, 7 / Bw4	C1, C2, Bw4	***2DL1, 2DL2, 2DL3, 3DL1***	Cw7, 4 / -	C1, C2	No
3	Cw7, 6 / Bw4	C1, C2, Bw4	***2DL1, 2DL2, 2DL3, 3DL1***	Cw15, 1/ Bw4	C2, C1, Bw4	No
4	Cw9, 6 / Bw4	C1, C2, Bw4	***2DL2, 3DL1***	Cw16, 7/ Bw4	C2, C1, Bw4	No
5	Cw16, 2 / Bw4	C1, C2, Bw4	***2DL1, 2DL2, 2DL3, 3DL1***	Cw7, 8 / -	C1, C1	No
6	Cw15, 8 / Bw4	C2, C1, Bw4	***2DL1, 2DL2, 2DL3, 3DL1***	Cw15, 1 / -	C2, C1	No
7	Cw7, 8 / -	C1, C1	*2DL1,* ***2DL3,*** *3DL1*	Cw7, 5 / Bw4	C1, C2, Bw4	No
8	Cw7, - / Bw4	C1, Bw4	*2DL1,* ***2DL3, 3DL1***	Cw9, 7 / -	C1, C1	No
9	Cw7, 5 / Bw4	C1, C2, Bw4	***2DL1, 2DL2, 2DL3, 3DL1***	Cw7, 17/ Bw4	C1, C2, Bw4	No
10	Cw7, 9 / -	C1, C1	*2DL1,* ***2DL3,*** *3DL1*	Cw7, 6 / Bw4	C1, C2, Bw4	No
11	Cw8, 6 / Bw4	C1, C2, Bw4	***2DL1, 2DL2, 2DL3, 3DL1***	Cw12, 16/Bw4	C1, Bw4	No
12	Cw7, 9 / -	C1, C1	*2DL1,* ***2DL3,*** *3DL1*	Cw7, - / -	C1	No
13	Cw1, 7 / Bw4	C1, C1, Bw4	*2DL1,* ***2DL2, 2DL3, 3DL1***	Cw1, 7 / -	C1, C1	No
14	Cw7, 4 / Bw4	C1, C2, Bw4	***2DL1, 2DL2, 2DL3, 3DL1***	Cw15, 5 / Bw4	C2, C2, Bw4	No
15	Cw7, 5 / Bw4	C1, C2, Bw4	***2DL2,*** *3DL1*	Cw10, 9 / -	C1, C1	No
16	Cw1, - / Bw4	C1, Bw4	*2DL1,* ***2DL2, 2DL3, 3DL1***	Cw5, 7 / Bw4	C2, C1, Bw4	No
17	Cw7, - / -	C1	*2DL1,* ***2DL2,*** *3DL1*	Cw7, - / -	C1	No
18	Cw9, 15 / Bw4	C1, C2, Bw4	***2DL1, 2DL3, 3DL1***	Cw7, 16 / Bw4	C1, C1, Bw4	No
19	Cw7, 6 / Bw4	C1, C2, Bw4	***2DL1, 2DL3, 3DL1***	Cw5, 4 / Bw4	C2, C2, Bw4	No
20	Cw7, 1 / Bw4	C1, C1, Bw4	***2DL1, 2DL2, 2DL3, 3DL1***	Cw2, 10 / Bw4	C2, C1, Bw4	No
21	Cw7, 1 / -	C1, C1	*2DL1,* ***2DL2, 2DL3,*** *3DL1*	Cw2, 10 / Bw4	C2, C1, Bw4	No

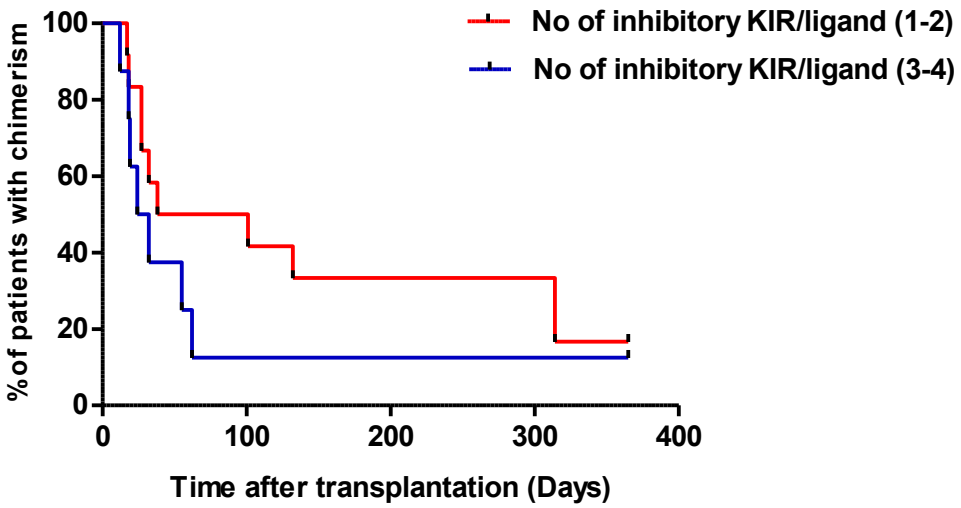
KIR^ – Killer-cell Immunoglobulin-like Receptors.
2DL1, 2DL2, 2DL3, 3DL1^^ – inhibitory killer-cell immunoglobulin-like receptors.
C1* group: HLA-Cw antigens that contain asparagine at position 80 of the α helix includes: Cw1, 3, 7, 8, 12, 14, 1507 and 1601, ligands for *KIR2DL2* and *KIR2DL3.*
C2** group: HLA-Cw antigens that contain lysine at position 80 of the α helix includes: Cw2, 4, 5, 6, 1204, 1205, 15, 1602, 17 and 18, ligands for *KIR2DL1.*
Bw4*:** public epitope found on some HLA class I antigens including: HLA-A24, B13, B27, B44, B51, B52, B53, B57 and B58 containing either isoleucine or threonine at 80 of the α helix, ligands for *KIR3DL1.*
RvD' – Recipient-*versus*-Donor.
NK" – Natural killer cells.
Dash (-) – presence of gene and/or epitope not detected.
KIR genes highlighted in red – represent KIRs that were expected to have been educated during NK cell development i.e. licenced to recognise "self" (recipient cells contain both KIR gene and its corresponding ligand).

Twenty of 21 patients were matched for KIR-ligand, and only one patient was KIR-ligand mismatched. Thus, RvD NK cell alloreactivity was expected only in one patient that received a primary lung transplant from a donor that did not carry a ligand for the recipient inhibitory KIR.

Patient one was genotyped as Cw7 (HLA-Cw antigen that belongs to the C1 group) and was a carrier of the inhibitory *KIR2DL1*, *KIR2DL3 and* KIR3DL1, that ligate with HLA-Cw antigens belonging to C2 group, C1 group and Bw4 epitope, respectively. However, the recipient contains an HLA ligand only for *KIR2DL3* gene; thus *KIR2DL3* is the only gene that is assumed to have been educated during NK cell development i.e. licensed to recognise "self", Table 4.4. The donor for this recipient was genotyped as Cw5 and Cw6: both belong to the C2 group and the Bw4 epitope. Thus, RvD NK cell alloreactivity was expected due to the absence of inhibitory *KIR2DL3* ligand (HLA C1 ligand) on the donor cells; instead, the donor HLA-Cw antigens (Cw5 and Cw6) are ligands for *KIR2DL1* and *KIR3DL1* receptors, respectively. Under these circumstances absence of signalling through the inhibitory NK cell receptors is expected to trigger NK cell activation and donor CD4 T cell killing.

In my study cohort RvD NK cell alloreactivity was expected only in the recipient-donor pair; thus, it was not possible to separate the patients into a group where NK cell alloreactivity is expected (n=1) and a group where NK cell alloreactivity was not expected (n=20).

To investigate whether the strength of inhibition has an effect on the chimerism longevity, I separated the patient into two groups based on number of inhibitory KIR-ligands present in the RvD direction; group one contained between 1 and 2 inhibitory KIR-ligands (n=12) and group two contained 3 or 4 inhibitory KIR-ligands (n=8). Univariate analysis revealed that the number of inhibitory KIR-ligands was not associated with the longevity of donor CD4 T cell chimerism in the recipients' peripheral blood after primary lung transplantation, (log-rank: p=0.290, Figure 4.3).



p=0.397

Figure 4.3 Impact of the number of inhibitory KIR-ligand on the duration of donor CD4 T cell chimerism detectable in the recipients' peripheral blood following primary lung transplantation. For definition of KIR-ligand mismatches see Table 4.3. Vertical bars indicate censored events. *P*-values were based on the log-rank test on Kaplan-Meier plots. *P*-value of <0.05 was considered statistically significant.

4.3 Discussion

Clinical evidence showing the profound impact of HLA matching in renal transplantation and HSCT has influenced organ and tissue allocation; consequently, HLA matching for HLA-A, B- and -DR and HLA-A, -B, Cw, -DR and -DQ is a critical aspect determining kidney and HSCT allocation, respectively [200, 201]. In contrast, HLA matching in lung transplantation has not become clinical practice mainly due to the smaller donor pool, shorter ischemia time and patients' clinical urgency for transplantation. Instead, matching of the lungs is based on ABO compatibility and the size of the organ irrespective of the clinical evidence showing that HLA mismatching has a beneficial impact on graft survival, incidence of rejection and development of BOS [53-55]; consequently, most lung transplant recipients receive poorly matched organs.

Similar findings were observed in this study cohort: 12 out of 21 patients received lungs mismatched at minimum five loci for HLA-A, -B and -DR and none of the patient in this cohort received fully matched lungs (Table 4.1 and Figure 4.2). In addition, my analysis did not reveal an association between the degree of HLA mismatching and the longevity of donor CD4 T cells chimerism, either when the HLA antigens were assessed (HLA-A, HLA-B and HLA-DR) individually or as well as for the cumulative effect of all HLA mismatched antigens. To my knowledge, this is the first study that has assessed the impact of HLA mismatching on the development and duration of donor CD4 T cell chimerism following lung transplantation.

In solid organ transplantation, matching for HLA-Cw has been overlooked due the lack of evidence supporting the impact of HLA-Cw matching on the clinical outcome [217, 218]; presumably due to the lower cell surface expression in comparison to HLA-A and HLA-B [219].

Nevertheless, taking into consideration that HLA-B and HLA-Cw antigens are in strong *linkage disequilibrium* (a non-random association between alleles that are encoded at neighbouring loci), it is expected that most transplant

pairs that are mismatched for HLA-B will be mismatched for HLA-Cw antigens, as it has been reported to occur in approximately 50% of kidney transplant pairs [211]. Comparable observation was made in this study cohort where more than half of the patients (n=12) were mismatched at both HLA-Cw alleles.

In addition to antigen presentation, the main role of the HLA-Cw antigens is to provide protection against cell lysis by the NK cells [220] via interaction with the inhibitory KIR receptors expressed on the NK cell membrane. HLA-Cw antigens represent the main ligands for KIRs and regulate NK cell activation. Extensive work has been undertaken to characterise the specificity of KIR-ligands interactions; and, currently KIR-ligand mismatching has become a widely used approach to treat patients with high-risk AML in an HLA haplotype-mismatched HSCT in the hope of achieving graft-*vs*-leukaemia effect [207, 208].

I adopted the inhibitory KIR-ligand mismatching approach to assess possible associations between the predicted NK cell alloreactivity (Table 4.3) and longevity of donor CD4 T cell chimerism in the recipients' peripheral blood following primary lung transplantation. The analysis revealed that in my study cohort NK cell alloreactivity was expected only in transplant pair (patient No 1, Table 4.4); thus, based on a small number further analysis were not possible. Instead, I have assessed whether the cumulative number of inhibitory KIR-ligands are more efficient at prolonging the survival of donor CD4 T cells in recipients' peripheral blood (Figure 4.3). My data did not reveal differences in the duration of donor CD4 T cell chimerism between the two groups of patients.

In kidney transplant patients, KIR-ligand mismatching is associated with reduced long-term graft survival only in HLA compatible transplant pairs, but does not have any effect in patients mismatched for HLA-A, -B and/or -DR [211]. It is believed that the impact of KIR-ligand mismatching is "unmasked" in the absence of HLA incompatibility between the transplant pair. In comparison, considering that HLA matching is not part of the lung allocation

criteria; majority of lung transplant recipients receive poorly matched lungs. As a consequence it would be very difficult to construct large enough cohort to assess the KIR-ligand mismatching in isolation of HLA mismatches. Unfortunately, due to the small number of patients in my study cohort I was not able to assess the impact of NK cell alloreactivity on the duration of donor CD4 T cell chimerism in HLA mismatched lung transplant recipients.

It should also be noted that although predicting NK cell alloreactivity based on inhibitory KIR-ligand mismatching (as adopted in this study) is widely used in HSC transplantation, this approach has its own limitations. Firstly, in this cohort, recipient-versus-host NK cell alloreactivity was predicted by HLA genotyping the recipient and their donor, and by genotyping the recipient's KIR ligands. This approach is indicative of *in vivo* NK alloreactivity but does not prove that the repertoire of each KIR encoded is also expressed on the NK cell membrane. It has been reported that KIR cell surface expression is in direct correlation with the encoded KIR gene content [221], but not all NK cells express the encoded KIR genes. It is now known that only a fraction of the total number of NK cells express a set of inhibitory KIR receptors [209, 221, 222].

Furthermore, the number and percentage of NK cell subsets ($CD56^{dim}$ – cytotoxic NK cells *vs* $CD56^{bright}$ – cytokine producing NK cells) vary between individuals [203]; and, the number and function of both can be affected by the use of current immunosuppressive therapy [223-225]. It has been reported that patients treated with anti-thymocyte globulin have an altered fraction of NK cells expressing KIRs; however, *de novo* KIR receptor expression has not been observed to occur in renal transplant recipients [224].

Lastly, another feature that NK cells exhibit is the requirement for inhibitory KIR receptor "licensing". Development of functionally competent NK cell is dependent on the inhibitory KIR "licensing" by self-MHC class I molecules which involves interaction between NK cell inhibitory KIRs and its corresponding self-MHC class I molecule, a process that enables them to discriminate self from non-self [226, 227]. NK cells that do not undergo this

process are self-tolerant but functionally incompetent [228]; such cells will therefore not necessarily kill target cells that lack expression of the inhibitory MHC class I ligand. In addition, the level of KIR gene expression varies between different NK cell subsets ($CD56^{dim}$ – cytotoxic NK cells *vs* $CD56^{bright}$ cytokine producing NK cells); and, it has been implicated that $CD56^{bright}$ do not express KIRs. Analysis of peripheral blood of 20 healthy individuals have revealed that inhibitory KIRs such as CD158a (*KIR2DL1*), CD158B (*KIR2DL2/L3*) NKB1 (*KIR3DL1*) are almost exclusively expressed on $CD56^{dim}$ NK cells but not on $CD56^{bright}$ NK cells [229]; whereas expression of activating receptors (CD94/NKG1 and CD161) were found on both NK cell subsets and higher expression of CD94/NKG1 has been observed in $CD56^{bright}$ NK cells [229]. In my study the analysis of KIR gene content showed that all patients (n=21) carry different combination of inhibitory KIRs (*KIR2DL1, KIR2DL2, KIR2DL3 and KIR2DL1*), Table 4.4; suggesting presence of $CD56^{dim}$ NK cell population. Nevertheless, presence of $CD56^{bright}$ NK cells population cannot be excluded. Considering the frequency of the inhibitory KIR genes in different populations [148, 230, 231], it is not surprising that in my cohort all recipients contained at least two inhibitory KIR genes and at least one KIR gene was expected to have been licenced to recognise “self”; presence of *KIR3DL1* gene was detected in all patients (Table 4.4).

Unfortunately, due to insufficient samples available the NK cell numbers, NK cell subsets and inhibitory KIR receptor expression pre- and post-transplant were not studied. Nevertheless, none of these NK cell features can be ruled out as potential factors that may influence the threshold for NK cell alloreactivity and consequently its impact on the duration of donor CD4 T cell chimerism in lung transplant recipients. In addition, it has to be stressed that the number of participants in this study is very small to reach firm conclusion including negative findings.

My observations did not reveal an association between both the NK cell alloreactivity and the HLA mismatching and the longevity of donor CD4 T cell chimerism detectable in the peripheral blood of primary lung transplant recipients. Nevertheless, these observations have raised additional questions

regarding the type of donor CD4 T cell present in the recipients' peripheral blood as well as their state of maturation.

These questions are addressed in the following Chapter 5. Using a molecular gene expression profiling approach, I investigated whether there was a difference between the type of donor CD4 T cells present in the peripheral blood of patients with short chimerism (defined as donor CD4 T cells detectable for less than six weeks after transplantation) and patients with long chimerism (defined as donor CD4 T cells detectable for more than six months after transplantation).

4.4 Key points

- The degree of HLA mismatching does not affect the duration of donor CD4 T cell chimerism detectable in the recipients' peripheral blood following primary lung transplantation.

- NK cell alloreactivity (based on the presence of KIR-ligand mismatches) was expected only in one transplant pair; thus, I was not able to assess the impact of NK cell alloreactivity on the duration of donor CD4 T cell chimerism in HLA mismatched lung transplant recipients.

- The cumulative number of inhibitory KIR-ligand was not associated with the longevity of donor CD4 T cell chimerism detectable in the peripheral blood of lung transplant recipients.

5 Chapter 5

Characterisation of donor CD4 T cell subsets

5.1 Introduction

Common lymphoid progenitors generated from hematopoietic stem cells in the bone marrow that migrate to the thymus give rise to ether CD4 helper T cells or CD8 cytotoxic T cells. At point of entry into the thymus, lymphoid progenitors are characterised by cell surface expression of CD44 (thymus homing cell adhesion molecule that regulates early T-cell development) [232] and lack expression of T cell receptor complex (α/βTCR/CD3-), CD25 CD8 and CD4 [233], termed as double negative thymocytes. Four key processes characterise thymic T cell development: death by neglect; negative selection; positive selection; and lineage-specific differentiation into mature CD4 or CD8 T cells that are then released into the circulation (reviewed in Germain, 2002 [233] and Singer et.al., 2008 [234]). In addition, a small fraction of natural regulatory T cells (nTregs) and Natural killer T cells (NKT) are also generated in the thymus [235]. Once released into the blood stream, they recirculate through peripheral lymphoid organs, where they can encounter target ligand and undergo antigen-mediated effector differentiation. Activation of naïve T cells is reviewed in Chapter 1 and Chapter 4.

Effector CD4 T cell subtypes can be defined by their composition of cell surface markers, the transcription factors they express, and the type of cytokines they produce. Thus, CD4 T cells have been characterised into five distinct subtypes: T-helper 1 (Th1); T-helper 2 (Th2); T-helper 17 (Th17); T follicular helper (Tfh); and regulatory T cells (Tregs).

5.1.1 CD4 T cell differentiation

CD4 T cell lineage-specific differentiation mainly depends on the cytokine milieu present at the time of TCR cognate interaction with MHC class II peptide complex presented by APCs (DCs, B cells and/or macrophages). The cytokine milieu triggers a cascade of signaling transducer and activator of transcription (STAT) protein activation, upregulation of transcription factors

and production of cytokines that polarize CD4 T cells into different subpopulations.

The existence of CD4 T cell subtypes was initially described in a murine model by Mosmann et al., [236]; mouse helper T cells were characterised as Th1 and Th2 cells based on the pattern of lymphokines they produce. Th1 produce IFN-γ and IL-2 whereas Th2 produce IL-4, IL-5, IL-13, TNF-α and a small amount of IL-9, later distinguished as a separate Th9 subpopulation [237].

Binding of IFN-γ to its receptor on naïve CD4 T cells triggers dimerisation of the IFNGR [238], phosphorylation of intracellular domains, and recruitment of STAT1 that is further phosphorylated and activated by JAK kinase intracellular signaling pathway. These events result in translocation of STAT1 to the nucleus where it activates gene transcription of T-bet (T-box transcription factor) and together promote IFN-γ production [238, 239]; thus, securing commitment of CD4 T cells into a Th1 subpopulation. T-bet is considered to be a major factor for induction of IFN-γ production and Th1 cell differentiation; animal work in T-bet knockout experiments have shown severe defects in Th1 cell differentiation [239]. Another transcription regulator that promotes Th1 differentiation and IFN-γ production is STAT4. STAT4 is activated by IL-12 [240] and acts as a negative regulator of GATA3 [241], transcription factor that induces Th2 cell differentiation and production of IL-4, IL-5 and IL-13, Th2 signature cytokine profile. On the other hand GATA3 suppresses the STAT4 signaling pathway [194], thus preventing development of Th1 subpopulation.

GATA3 is a major transcription factor of the Th2 subpopulation, regulated by IL-4 and STAT6 intracellular signaling pathway [242]. STAT6 indirectly regulates IL-4 production [242]. Another signaling transducer and activator of transcription protein that promotes Th2 differentiation of CD4 T cells is STAT5.

STAT5 driven Th2 differentiation is independent of GATA3 activation [243]; instead, it binds directly to the DNase I hypersensitive site II and III in the second intron of *IL4* locus [243]. STAT5 deficient cells do not respond to IL-2 but instead they are hypersensitive to IL-12, which leads to Th1 subpopulation [244].

Moreover, STAT5 activation by IL-2 is vital for development of peripheral Treg. It has been suggested that STAT5 activation also regulates the expression of forkhead box P3 (Foxp3) by direct binding to its promoter in a dose dependent manner [245]. STAT5 activation suppresses Th1 and Th17 subpopulations while promoting Th2 and Treg differentiation [246] presumably due to enhanced expression of Foxp3.

Foxp3 is a master transcription regulator for development of Tregs cells that produce TGF-β. Binding of TGF-β to its receptor triggers activation of a JAK-STAT signaling pathway, which regulates gene expression, including upregulation of Foxp3 and downregulation of STAT1 and STAT3, thus preventing CD4 T cell differentiation onto Th1 and Th17, respectively [247].

The existence of Th17 cells was recognised following identification of IL-12 congener IL-23 [248]; a heterodimer molecule that shares one subunit (p40) with IL-12, but that is coupled with a unique p19 subunit. Gene targeting experiments showed absence of IL-17 producing CD4 T cells in mice lacking the expression of IL-23 p19 subunit but normal IFN-γ production by Th1 cells. In contrast, IL-12 p35 deficient mice have an increased number of IL-17 producing CD4 T cells [248, 249]. Further analysis revealed that TGF-β, IL-6, IL-21 and IL-23 are even more efficient at driving CD4 T cell differentiation onto Th17 subpopulation [250], via activation of STAT3. Deletion and/or mutation of STAT3 results in the loss of IL-17 producing CD4 T cells [251, 252]. STAT3 regulates IL-17 production in conjugation with receptor-related orphan receptor gamma (RORγτ); both bind directly to *IL17* gene [253].

STAT3 activation by TGF-β and IL-6 leads to downregulation of Foxp3 expression, thus preventing CD4 T cell differentiation into periphery induced regulatory T cell (iTreg) subset [254].

Another subset of CD4 T cells that have been identified as effector T cells that provide help for selection and affinity maturation of B cells are Tfh cells. B-cell lymphoma 6 (Bcl-6) transcription factor, whose expression is regulated by STAT5, is vital for Tfh differentiation [255]; and, together with the C-X-C chemokine receptor 5 (CXCR5) permits homing of Tfh cells into the B cell follicles [256].

It is important to note that cytokines produced by each Th subpopulation play a positive feedback role in promoting further differentiation of a particular subtype, e.g. IFN-γ for Th1, IL-4 for Th2, IL-12 for Th17 and TGF-β for iTregs [257].

5.1.2 CD4 T cell surface markers

During CD4 T cell development, maturation and differentiation CD4 T cell subsets express wide repertoire of cell surface receptors that can be used as markers for their classification. Mature CD4 T cells can be classified as naïve, central memory and effector memory T cells.

In humans, memory T cells are characterised by the expression of CD45RO isoform and by the lack of expression of CD45RA isoform. Thus, $CD45RO^{+}CD45RA^{-}$ T cells comprise of heterogeneous populations of memory T cell subsets that co-express CD44 cell surface protein involved in cell-cell interaction and migration of memory CD4 T cells [258]. Naïve CD4 T cells lack expression of CD44.

Furthermore, migration of CD4 T cell subsets is dependent on cell surface expression of homing receptors. CC-chemokine receptor 7 (CCR7) is a lymph node-homing receptor uniformly expressed on naïve T cells, reflecting their

predominant residence in the lymphoid tissue [259], whereas based on the co-expression of CCR7 and CD45RO memory T cells are divided into $CD45RA^{-}CCR7^{+}$ central memory T (T_{CM}) which can migrate through the lymphoid tissue and $CD45RA^{-}CCR7^{-}$ effector memory T (T_{EM}), which migrate through peripheral tissue sites and display effector function [260]. Migration of naïve and $CCR7^{+}$ memory T cells through the lymph nodes is enabled by co-expression of CD62L adhesion molecule that allows T cell to role on the high endothelial venules [260]. This interaction slows down the T cells thus allowing firm $CCR7^{-}$SLC interaction and transmigration of the T cells into the lymph node. Co-expression of CCR7 and CD62L is essential for naïve and T_{CM} cells migration to lymph nodes [260].

CCR6 is another homing receptor that plays a crucial role in trafficking of Th17 cells to the intestine and mucosal tissues [261]. TGF-β is required for its expression and in most instances CCR6 is co-expressed with STAT3, ROR$\gamma\tau$ and IL-21 [261], whole mark features of Th17 subpopulation.

Another cell surface marker used to differentiate between naïve and activated CD4 T cells is CD27. Activation of T cells leads to CD27 cell surface expression; and, when co-expressed with Foxp3 has been used a cell surface marker for characterisation of Treg subpopulation [262].

As previously discussed in Chapter 3 and Chapter 4, I observed substantial difference in the dynamics of donor CD4 T cell persistence in the recipients' peripheral blood following lung transplantation. Interestingly, these variations were not affected by the degree of HLA mismatching between the recipients and their respective donors. Furthermore, my data showed that predicted NK cell alloreactivity due to inhibitory KIR-ligand mismatching did not influence the longevity of donor CD4 T cell chimerism.

These observations have raised the question whether donor CD4 T cell subsets and/or activation state are responsible for the observed variation between patients with short and patients with long-lasting chimerism.

To address this question I have used gene expression analysis assay to characterise the donor CD4 T cell subpopulations based on the expression of genes encoding cell surface markers and transcription factors specific for different CD4 T cell subtypes.

In addition, flow cytometric analyses were used to investigate the composition of migrating donor CD4 T cell subsets that have been immobilised within the leukocyte filter during *ex-vivo* lung perfusion. The *Ex vivo* Lung Perfusion procedure is described in Chapter 2, Section 2.7.

5.2 Results

Gene expression profiling was used to characterise isolated donor CD4 T cells, by adopting standard RT-PCR methodology (Section 2.6.4.4.), with the level of gene expression quantified using relative quantification (RQ) (as described in Section 2.6.5). The RQ gene expression level for the genes targeted was measured in reference to the level of expression of the internal control gene, also known as “internal endogenous control” or “reference gene”. The reference gene represents a gene with a stable level of expression.

To ensure precise analysis and interpretation of the data generated by expression profiling, the assay was first validated. Based on the longevity of donor CD4 T cell chimerism, patients were categorised into two groups: those with short (n=5) and those with long-lasting (n=5) chimerism (defined as donor CD4 T cells detectable in the recipients’ peripheral blood for less than six weeks and more than six months after transplantation, respectively). Recipients’ peripheral blood isolated donor CD4 T cells were characterised using gene expression profiling, having identified an internal endogenous control gene, and the gene expression profile was compared between the two groups of patients.

In addition, to assess the type of cells that are migrating from the human lungs following revascularisation of the allograft I examined human lungs subjected to the *Ex vivo* Lung Perfusion procedure (previously described in Section 2.7) prior to revascularisation. Cells migrating out of the lungs during the EVLP procedure are immobilised on a leucocyte filter that is attached to the EVLP circuit. Characterisation of mononuclear cell populations was performed by flow cytometry, previously described in Section 2.4.4. Unfortunately, due to unforeseen circumstances the DEVLOP UK study was prematurely terminated and for the duration of my study lungs harvested from only one DCD donor were subjected to analysis.

5.2.1 Identification of stable internal endogenous control gene

To identify an internal endogenous control gene with stable expression, the gene expression transcripts of three genes was measured: glyceraldehyde 3-phosphate dehydrogenase (GAPDH), beta-actin (ACTB) and cluster of differentiation 4 (CD4). GAPDH is an enzyme encoded by the *GAPDH* gene and is involved in glycolysis (breakdown of glucose) and other non-metabolic processes such as transcription and apoptosis [263]. ACTB is a non-muscle cytoskeletal protein involved in cell motility, structure and integrity and is encoded by *β-actin* gene [264]. CD4 is a glycoprotein encoded by *CD4* gene found on the surface of T helper cells [265].

Nine samples containing equal numbers of isolated donor CD4 T cells (20,000 cells; previously described in Section 2.4.3), were selected and used for detection of stable internal endogenous control gene including (GAPDH, ACTB and CD4) using qRT-PCR methodology (Section 2.6.4.4). Each assay was carried out in duplicate. The results of the gene expression analysis for GAPDH, ACTB and CD4 are presented as average Ct value of the duplicates. Ct value is the point (PCR cycle number) where the PCR curve crosses the threshold in the linear part of the exponential phase of the PCR reaction (Section 2.6.5). Figure 5.1 A, represents the mean Ct value for each sample tested for the level of expression for GAPDH, ACTB and CD4. Massive variation was observed in the gene expression levels for ACTB and CD4 ranging between Ct 15.8 to 17.4 and Ct 16.8 to 18.2, respectively. One Ct difference between two samples is equivalent to 2-fold change, which means that 4-fold difference existed between samples for both ACTB and CD4 gene expression.

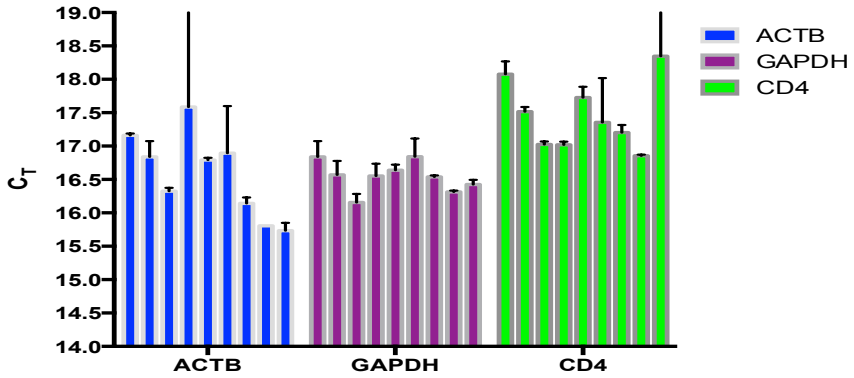
In comparison, the variation between the samples for GAPDH level of expression was less than one Ct cycle; the Ct value was ranging between Ct 16.1 and Ct 16.8. Variation of less than one Ct cycle is considered not significant; thus, GAPDH was identified as a good candidate for internal endogenous control gene.

To further assess the validity of the assay, an additional test was performed to confirm the stability of the GAPDH gene expression. In this case the GAPDH level of expression was re-assessed using nine samples containing 20,000 isolated donor CD4 T cells and an additional two samples containing 10,000 cells. The average Ct value obtained for the samples containing 20,000 cells was Ct 16.5 and for the samples containing 10,000 cells the Ct value was 17.5. Thus, the observed difference between samples containing 20,000 and 10,000 CD4 T cells was one Ct cycle that is equivalent to 2-fold difference, which corresponds to the number of cells present in each test group; 20,000 vs 10,000 donor CD4 T cells. These observations have confirmed the validity of the assay and the stability of the GAPDH gene expression, Figure 5.1 B.

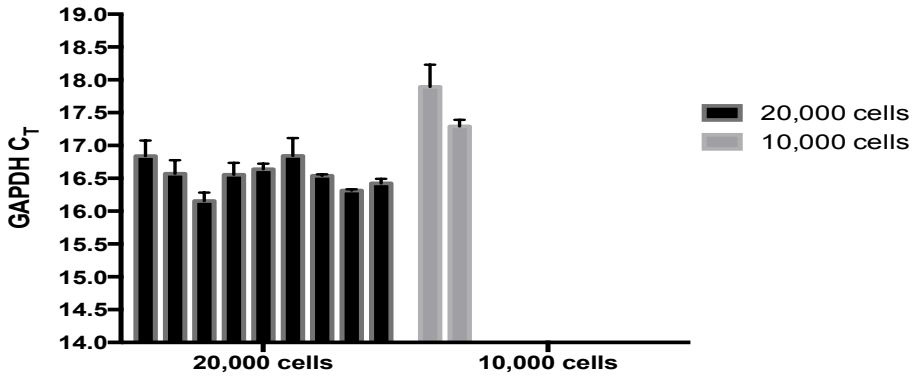
Lastly, serial dilutions of isolated donor CD4 T cells were prepared to test whether GAPDH level of expression correlates with the number of donor CD4 T cells used as a starting amount of biological material. Six samples containing 100, 200, 500, 2,000, 10,000 and 20,000 isolated CD4 T cells were tested in duplicates. Figure 5.1 C represents the Ct values for each test sample Ct 25.5, Ct 24.5, Ct 23, Ct 21, Ct 18.5 and Ct 17.3, respectively. The analysis revealed correlation between the Ct values and the number of cells present in each test sample. For example, one Ct cycle difference was observed between the samples containing 100 and 200 cells (Ct 25,5 *vs* 24.5) and samples containing 10,000 and 20,000 cells (Ct 18.5 *vs* 17.3), confirming that serial dilutions were equivalent to a 2-fold difference.

The analysis revealed that the expression of GAPDH is stable, with minimal variation between duplicate samples. Thus, GAPDH was selected as an internal endogenous control. The level of expression of all target genes assessed for characterisation of the passenger donor CD4 T cells were normalised in reference to the GAPDH level of expression (Section 2.6.5).

A)



B)



C)

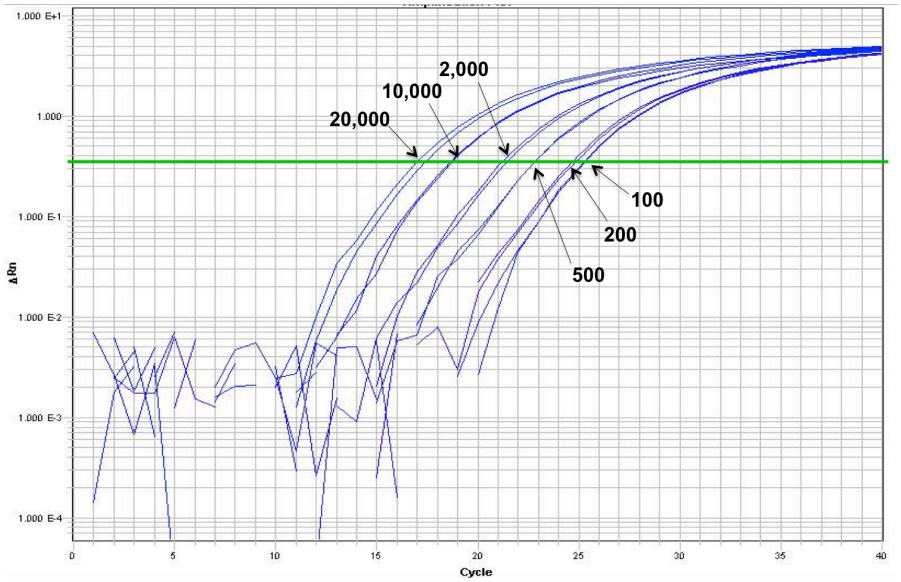


Figure 5.1 Identification of stable internal endogenous control gene. A) Identification of the level of expression of GAPDH, ACTB and CD4 in nine test samples containing 20,000 isolated donor CD4 T cells; Ct value is the point (PCR cycle number) where the PCR curve crosses the threshold. B) GAPDH level of expression tested in nine test samples containing 20,000 and two samples containing 10,000 isolated donor CD4 T cells. C) RT-PCR amplification plot representing GAPDH level of expression in samples containing 100, 200, 500, 2,000, 10,000 and 20,000 isolated donor CD4 T cells. Each sample was tested in duplicates.

5.2.2 Patient cohort for characterising donor CD4 T cell gene expression profiling.

Of 21 patients with donor CD4 T cell chimerism detectable in the recipients' peripheral blood following primary lung transplantation, 10 patients were selected for characterisation of donor CD4 T cells using gene expression profiling. Based on the longevity of donor CD4 T cell chimerism, patients were categorised into two groups: patients with short chimerism (n=5) and patients with long-lasting chimerism (n=5), defined as donor CD4 T cell detectable in the recipients' peripheral blood for less than six weeks and more than six months after transplantation, respectively.

To my knowledge donor CD4 T cell gene composition has not been previously studies and at this stage the type of donor CD4 T cell chimerism is completely unknown entity. The number of patients in both groups is relatively small; nevertheless undertaking a small pilot experiment to evaluate the presence of donor CD4 T cell subpopulation in the recipients' peripheral blood following lung transplantation may provide interesting findings that could drive further experimental directions. Recipient and donor demographics are presented in Table 5.1.

Table 5.1 Recipients and donor characteristics.

Recipient and donor demographics	Short donor CD4 T cell chimerism (n=5)	Long donor CD4 T cell chimerism (n=5)	*p* Value
Recipient age (Median ± SD)	58.1 (± 6.6)	55.8 (± 17.5)	*ns*
Recipient sex (M:F)	2:3	2:3	*ns*
Donor age (Median ± SD)	56 (± 6.8)	46 (± 14.9)	*ns*
Donor sex (M:F)	3:2	3:2	*ns*
Transplant type			
SLT	0	0	*ns*
DLT	5	5	
Indication for transplantation			
Cystic fibrosis	0	1	*ns*
Emphysema	1	0	
COPD	4	2	
A1AD	0	1	
Emphysema + A1AD	0	1	
Pre-transplant HLA sensitisation	3/5	1/5	*ns*
HLA mismatch grade			
≤ 3	0	2	*ns*
≥ 4	5	3	
Immunosuppression			
Tac/MMF/Pred	3*	3	
Tac/MM/Aza	1	1	*ns*
Tac/Pred	1*	1	

SLT – Single lung transplant; DLT – Bilateral lung transplant; COPD - Chronic obstructive pulmonary disease; A1AD- α1 antitrypsin deficiency; HLA – Human Leukocyte Antigen; Tac – Tacrolimus; Aza – Azathioprine. Pred – Prednisolone; MMF – Mycophenolate Mofetil; *Induction therapy with Basiliximab.

5.2.3 Characterisation of isolated donor CD4 T cells using gene expression profiling

To investigate whether the observed inconsistency in the longevity of donor CD4 T cells chimerism between patients with short (n=5) and patients with long chimerism (n=5) is due to the presence of different donor CD4 T cell subsets and/or their maturity state, we aimed to characterise the donor CD4 T cells isolated (Section 2.4.3) from the recipients' peripheral blood following primary lung transplantation. Fourteen commercially available gene expression assays (Table 2.10) were used to investigate their level of expression including: CD44, CD27, CD62L, CCR6, FoxP3, ROR-γc, Bcl6, GATA3, T-bet (TBX21), STAT4, STAT6, STAT3, STAT5 and IL-21.

In brief, CD44 is a marker for effector-memory T cells. Co-expression of CD27 and FoxP3 represent a marker of regulatory T cell subset [266]. STAT5 is a transcription factor that regulates T cell development and their differentiation into regulatory T cell subset [245, 246]. CD62L is an adhesion molecule expressed on naïve and central memory CD4 T cells [260]. CCR6 regulates migration of Th17 cells to the inflammatory tissue [261] and is usually co-expressed with STAT3, ROR-γc and IL-21 [253]. Naïve CD4 T cell differentiation into T follicular helper cell subset is regulated by the transcription factor Bcl6. T-bet, STAT4, STAT6 and GATA3 are transcription factors that regulate naïve T helper (Th) cell differentiation; T-bet and STAT4 commits cell differentiation into Th1 lineage [239, 240], whereas STAT6 and GATA3 drive differentiation of naïve Th cells into Th2 subset [194, 242].

Isolated donor CD4 T cells were lysed (Section 2.6.4.1) and used as a substrate for reverse-transcription and production of cDNA (Section 2.6.4.2). The cDNA was then used as a template for preamplification of the genes of interest, a method that allows an increase in the amount of cDNA available only for the targeted genes (Section 2.6.4.3); and, the pre-amplified product was used as a template for gene expression analysis by RT-PCR (Section

2.6.4.4). The gene expression levels were determined by relative quantification (RQ) method, Section 2.6.5.

Donor CD4 T cell gene expression levels were assessed in donor samples obtained at the time of donation, identified as time zero for all patients in this cohort. In addition, for patients with short chimerism; gene expression levels were tested in donor derived CD4 T cells, previously isolated from the recipients' peripheral blood samples at around two weeks (ranging from between 10 and 13 days) after transplantation; whereas, for patients with long chimerism the level of gene expression was tested in donor derived CD4 T cell isolated at around two weeks (ranging from 13 to 17 days); and six months after transplantation. Table 5.2 represents the RT-PCR average Ct value for each test sample.

Furthermore, in order to establish the level of expression of targeted genes; the Ct value of each gene tested was normalised relative to the Ct value of the endogenous internal control gene (GAPDH); the normalised Ct values are presented as delta CT (ΔCt). The ΔCt equals the Ct of the target gene minus the Ct of the reference gene (ΔCt = Ct (target gene) – Ct (reference gene - GAPDH)). Table 5.3 and Figure 5.2 represent the gene expression levels for genes targeted in test samples obtained at time zero, two weeks and six months after transplantation.

Table 5.2 represents the RT-PCR average Ct value.

Target genes av. Ct	SCM P1		SCM P2		SCM P3		SCM P4		SCM P5		LCM P1			LCM P2			LCM P3			LCM P4			LCM P5		
	D0	D11	D0	D12	D0	D13	D0	D10	D0	D10	D0	D14	M6	D0	D17	M6	D0	D14	M7	D0	D17	M6	D0	D13	M6
GAPDH	20	20	31	16	17	16	21	18	24	17	20	22	23	18	17	25	17	14	20	24	24	24	24	22	17
CD44	18	18	28	14	16	14	19	16	26	16	18	20	21	16	16	21	15	13	18	22	22	23	23	17	15
CD27	21	22	34	17	19	19	22	20	31	20	21	23	25	20	20	27	19	17	24	24	27	26	25	22	18
CD62L	20	20	29	15	20	18	21	18	29	18	22	21	23	19	17	23	17	16	20	23	24	24	25	18	17
CCR6	23	25	-	20	22	21	22	23	32	22	25	26	27	23	23	31	21	19	26	31	31	31	29	24	22
FoxP3	21	22	33	17	21	19	25	20	30	19	22	25	25	22	20	27	19	17	25	27	26	27	28	22	19
ROR-γc	23	24	-	18	21	21	28	21	34	20	24	25	28	21	21	28	20	18	25	30	28	33	30	25	21
Bcl6	21	24	-	20	19	18	22	21	-	21	21	25	25	20	20	31	18	18	22	25	32	34	26	24	21
GATA3	21	21	35	17	18	17	24	20	-	19	20	24	24	19	18	25	18	16	21	23	28	26	26	21	18
T-bet	22	24	31	20	17	17	24	22	32	21	21	25	27	20	19	28	26	16	20	27	26	30	25	22	20
STAT4	19	19	30	16	17	15	22	18	31	17	19	21	22	17	16	23	16	14	19	23	23	25	24	20	17
STAT6	20	20	30	16	17	16	21	19	33	18	20	21	23	18	17	25	18	15	20	24	25	25	24	21	17
STAT3	21	21	30	17	18	18	23	21	-	19	21	23	24	19	19	25	18	16	21	24	26	28	25	20	19
STAT5	19	20	31	16	18	17	23	18	34	18	20	22	24	18	18	25	18	16	20	24	25	26	25	20	18
IL21	26	28	-	22	22	26	29	24	-	25	24	-	-	26	-	-	22	23	-	33	34	-	34	28	26

Av. Ct – average Ct value of duplicate samples tested for the genes listed above; SCM – patients with short donor CD4 T cell chimerism; LCM – patients with long donor CD4 T cell chimerism; P – patient; D0 – time zero (samples obtained prior to transplantation); D – days after transplantation; M – months after transplantation; " – " not amplified; Description of the targeted genes is presented in Table 2.10.

Table 5.3 represents gene expression levels for genes targeted in test samples obtained at time zero (prior to transplantation); two weeks and six months after transplantation.

Target genes ΔCt	SCM P1 ΔCt		SCM P2 ΔCt		SCM P3 ΔCt		SCM P4 ΔCt		SCM P5 ΔCt		LCM P1 ΔCt			LCM P2 ΔCt			LCM P3 ΔCt			LCM P4 ΔCt			LCM P5 ΔCt		
	D0	D11	D0	D12	D0	D13	D0	D10	D0	D10	D0	D14	M6	D0	D17	M6	D0	D14	M7	D0	D17	M6	D0	D13	M6
GAPDH	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CD44	-2.5	-1.8	-3.1	-2.2	-1.4	-1.1	-2.2	-1.5	2.0	-1.7	-2.0	-2.0	-2.1	-2.7	-1.7	-3.2	-2.2	-1.4	-2.1	-2.1	-2.0	-0.8	-2.0	-5.1	-1.9
CD27	0.7	1.7	3.3	1.4	1.9	3.8	1.3	2.4	7.3	2.2	0.5	1.5	1.6	1.2	2.4	2.2	1.6	2.6	3.7	0.4	2.5	2.1	0.9	0.0	0.8
CD62L	0.0	-0.3	-2.3	-0.6	2.4	2.4	-0.7	0.3	5.5	0.2	2.0	-0.3	-0.5	0.6	0.1	-1.4	-0.2	1.1	0.3	-1.1	-0.3	0.3	0.2	-4.0	-0.1
CCR6	3.2	4.9		3.9	5.2	5.2	1.0	4.7	8.1	4.3	4.8	4.0	4.1	4.5	6.1	6.6	3.5	4.6	6.3	7.6	6.7	6.7	5.0	1.9	4.6
FoxP3	0.9	1.8	2.2	1.5	3.4	3.3	4.2	1.7	6.1	1.8	1.6	2.8	1.6	3.1	2.6	2.7	1.3	2.8	4.6	3.5	2.2	2.5	3.0	0.2	2.2
ROR-γc	3.0	4.0		2.1	3.4	5.1	6.7	2.8	10.1	2.4	3.8	3.5	4.8	3.0	3.8	4.0	2.9	3.5	4.8	6.5	4.1	9.2	5.8	2.5	3.7
Bcl6	1.1	3.5		3.6	1.6	2.7	1.0	3.5		3.5	0.6	3.0	2.0	1.6	3.1	6.5	1.0	3.8	2.0	1.5	7.7	9.5	1.8	1.8	3.8
GATA3	0.4	1.4	3.6	0.9	0.7	1.4	2.9	1.7		1.1	0.2	1.8	1.3	0.6	1.0	0.9	0.6	1.8	1.2	-0.9	3.3	1.4	1.2	-0.8	0.9
T-bet	1.9	4.3	0.1	3.6	-0.2	1.4	2.9	4.3	8.6	3.8	1.0	3.0	3.5	1.4	1.6	3.8	8.3	1.8	0.5	2.9	1.9	5.5	1.0	0.0	2.7
STAT4	-1.5	-0.6	-0.9	-0.4	-0.3	-0.9	1.1	-0.1	7.0	-0.3	-1.5	-1.0	-0.8	-1.7	-0.9	-1.5	-1.5	0.0	-1.3	-1.1	-1.1	0.8	-0.3	-2.7	0.0
STAT6	-0.3	0.3	-1.0	0.1	0.2	0.4	-0.6	1.3	9.3	0.5	0.1	-0.4	0.1	-0.1	-0.4	0.1	0.4	0.5	0.3	0.3	0.6	0.9	-0.5	-1.1	-0.2
STAT3	1.1	1.3	-0.7	1.5	0.9	1.8	1.4	2.8		1.8	0.9	1.2	1.1	0.8	1.5	0.8	0.8	1.9	1.4	0.4	1.7	3.6	0.7	-1.7	2.1
STAT5	-1.0	0.5	-0.1	0.1	0.5	1.7	1.3	0.6	9.9	0.3	-0.1	0.1	0.5	0.1	0.4	0.1	0.4	1.6	0.6	0.4	0.8	1.6	0.5	-1.8	0.3
IL21	6.0	7.9			5.3	10.0	7.6	6.2		7.5	4.2			7.7			4.6	8.2		9.0	9.6		9.3	6.1	8.3

ΔCt – normalized gene expression levels of targeted genes in reference to the expression level of the endogenous internal control gene GAPDH; SCM – patients with short donor CD4 T cell chimerism; LCM – patients with long donor CD4 T cell chimerism; P – patient; D0 – time zero (samples obtained prior to transplantation); D – days after transplantation; M – months after transplantation, Description of the targeted genes is presented in Table 2.10.

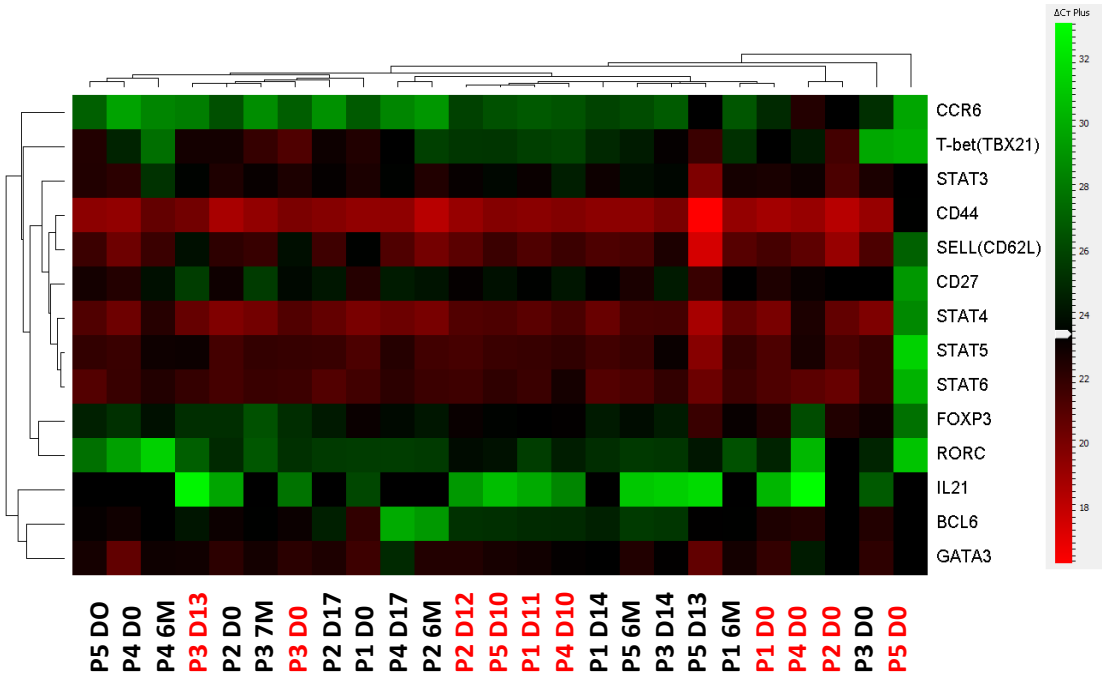


Figure 5.2, heat map represents gene expression levels. The level of gene expression was evaluated in reference to the endogenous internal control gene GAPDH expression levels in all patients (red colour; patients with short chimerism - P1, P2, P3, P4 and P5; and black colour; patients with long chimerism - P1, P2, P3, P4 and P5). Test samples obtained at three different time points: day 0 (prior to transplantation), two weeks (ranging between day 10 and day 17) after transplantation and at six months after transplantation. Each square on the heat map represents an individual sample. Red colour indicates upregulated gene expression level in relation to the internal control GAPDH gene, black colour represents no change in the level of expression and green colour represents downregulation in relation to the internal control GAPDH gene.

ΔCt – normalized gene expression levels of targeted genes in reference to the expression level of the endogenous internal control gene GAPDH; P – patient; D0 – time zero (samples obtained prior to transplantation); D – days after transplantation; M – months after transplantation; Description of the targeted genes is presented in Table 2.10.

I observed three different patterns in the level of gene expression: overall the level of gene expression was increased in five target genes (CD44, CD62L, STAT4, STAT5 and STAT6) indicative of red colour (Figure 5.2); there was a minimal change in the level of expression in CD27, STAT3, FOXP3 and GATA3 (black colour, Figure 5.2); and the level of gene expression was decreased for RORC, T-bet, CCR6, IL-21 and BCL6 (green colour, Figure 5.2).

The highest increase in the level of gene expression was observed in CD44, suggesting a predominant memory donor CD4 T cell phenotype. In the majority of test samples the level of gene expression was also increased for CD62L, STAT4, STAT5 and STAT6. Expression of CD62L was decreased in two samples and in six samples change was not observed. The expression of STAT5 and STAT6 was decreased in one patient with short chimerism in the test sample obtained prior to transplantation (Figure 5.2, P5 D0). In the remaining test samples there was a little increase in the expression of STAT5 and STAT6. In contrast, the expression of STAT4 was increased in 23 out of 25 samples tested.

In contrast, expression of RORC, T-bet, CCR6, IL-21 and BCL6 was decreased in the majority of samples tested, with the exception of a few test samples where there was no difference in the expression levels; and, the expression of T-bet was increased in only one patient with short chimerism in the test sample obtained prior to transplantation (Figure 5.2, P3 D0).

Expression of CD27, STAT3, FOXP3 and GATA3 was largely unchanged, with the exception of a few test samples where expression levels of FOXP3 were decreased.

5.2.3.1 Comparison of CD4 T cell gene expression profile between patients with short and patients with long donor CD4 T cell chimerism

DataAssist^{TM} v3.01 software was used to calculate the relative quantification (fold-change) for the level of gene expression between both groups of patients at two different time points. The software also performs the following analysis: mean Ct, ΔCt, $\Delta\Delta$Ct between two groups of patients, RQ (fold-difference) and *t*-test for patient group comparison (Section 2.6.5).

Firstly, we compared the level of gene expression between the patients with short and long chimerism at the time of donation. The analysis revealed that the expression levels of CD27 (1.4 fold), CD44 (1.1 fold), GATA3 (1.6 fold), IL21 (2.7 fold), RORC (1.1 fold), STAT4 (1.8 fold) and STAT5 (1.1 fold) were higher in the patients with long lasting chimerism.

When we compared the level of gene expression in the test samples obtained at two weeks after transplantation we observed a different donor CD4 T cell profile. The fold-chance in the level of expression was higher in all genes tested with the exception of IL21 and RORC in the patients with long lasting chimerism than in the patients with short chimerism. The highest increase was observed in CCR6 (2.4 fold), CD44 (2.9 fold), CD62L (4.5 fold), STAT3 (3.5 fold) and T-bet (2.9 fold); suggesting the presence of effector Th1 and Th17 donor CD4 T cell subsets.

However, despite the observed differences in the level of gene expression between the patients with short and patients with long chimerism, the fold-change was not statistically significant (log10 fold-change >2, Benjamini-Hochberg False Discovery Rate); presumably due to massive variations in the level of gene expression between test samples within the same biological group and between the two biological groups. Figure 5.3 shows the overall range of Ct distribution sorted by assay for each test sample.

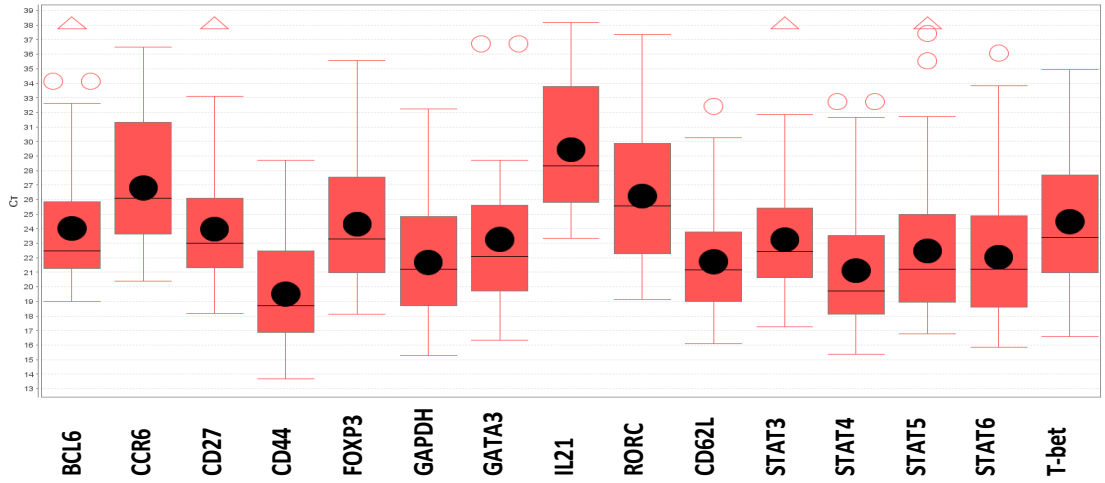


Figure 5.3 shows the overall range of Ct distribution arranged by gene expression assay. The ends of the vertical lines (whiskers) indicate the minimum and maximum Ct value. The points outside the end of the whiskers are outliers or suspected outliers and are excluded from statistical analysis.

Similar observations were made when we compared the level of gene expression between test samples obtained at different time points for both patient groups.

For patients with short chimerism, the fold change for RORC expression (2.5-fold) was higher in the donor CD4 T cells obtained at week two post-transplant in comparison to donor CD4 T cells obtained prior to transplantation, while the expression of all other genes was lower.

Contrary to this, in patients with long chimerism the expression of 10 out of 14 genes tested was increased in the donor CD4 T cells obtained at two weeks in comparison to test samples obtained prior to transplantation. The highest increase was observed in CCR6 (2.2-fold), CD44 (2-fold), and CD62L (4-fold); whereas, the expression of BCL6 and IL21 was lower, 0.25-fold and 0.04-fold, respectively. In addition, in this group of patients, with the exception of CD62L, the expression of all other genes was lower in the cells obtained at six months post-transplant in comparison to the cells obtained prior to transplantation.

Due to the huge variation between the test samples, the observed difference in the level of gene expression between samples obtained at different time points for both groups of patients were not statistically significant.

5.2.4 Characterisation of passenger mononuclear cells mechanically removed from donor lungs by *Ex Vivo* Lung Perfusion procedure

Ex vivo lung perfusion (EVLP); previously described in Section 2.7) is an alternative method of organ preservation, allowing prolonged time for careful evaluation and reconditioning of donor lungs that would otherwise be clinically rejected for transplantation. In addition, it provides a unique opportunity to study the immunological contribution and immune cell migration that donor lungs deliver at the time of transplantation.

Five lung transplant centers in the United Kingdom undertook a National Clinical Study known as A Study of Donor Ex-Vivo Lung Perfusion in UK Lung Transplantation (DEVELOP-UK) to assess the potential usability of lungs deemed to be unsuitable for transplantation. As part of the existing study, I was granted additional ethical approval for the use of discarded leucocyte filters following the EVLP procedures performed at Papworth Hospital. Unfortunately, due to unforeseen technical difficulties the DEVELOP-UK study was suspended and for the duration of the study the composition of leucocytes trapped within the filter was assessed only for one EVLP filter.

The EVLP procedure was performed on human lungs harvested from a 59 year-old DCD donor. In brief, the lungs were connected to a modified heart-lung bypass circuit to which a leucocyte filter is attached; two-litres of nutrient Steen solution and two-units of packed RBCs were continuously pumped through the lungs for 4 hours which warms the lungs to body temperature; followed by one-hour cooling perfusion phase during which lungs are cooled-down to 6°C. At the end of the EVLP procedure, the leukocyte filter was removed and stored overnight in RPMI media at 4°C for further analysis. Mononuclear cells were isolated from the filter and characterised using flow cytometric analysis, previously described in Section 2.3.4 and Section 2.4.4, respectively.

5.2.4.1 Evaluation of passenger mononuclear cells mechanically removed with *Ex Vivo* Lung Perfusion procedure from DCD lungs

The total number of mononuclear cells isolated from the EVLP leucocyte filter was at least 25-30 x 10^9 (counted in three test samples), previously described in Section 2.3.4 and Section 2.3.5. The mononuclear cells were analysed by flow cytometry (Section 2.4.4). The flow cytometric plot was set to display all mononuclear cells; then, based on the forward and side scatter (which measures the cell size and granularity respectively), three major cell populations were identified: lymphocytes; monocyte/macrophages; and granulocytes. Of the total number of mononuclear cells analysed, 59% were identified as lymphocytes, 22.0% as monocytes/macrophages and 18.5% as granulocytes.

5.2.4.2 Immunophenotyping of lymphocyte population using flow cytometry

Cell types present in each population were characterised by targeting cell surface markers with monoclonal antibodies using flow cytometry (Section 2.4.4). Of the total number of mononuclear cells, 59% were lymphocytes. The percentage of dead cells (characterised as 7-AAD positive) was less than 1%; these were excluded from further analysis. Interestingly, 58% of the lymphocyte population were B cells, characterised by the expression of B cell surface marker $CD19^+$. Of the remaining lymphocyte population, only 3.7% were identified as T cells expressing $CD3^+$ cell surface marker; of which, 1% were $CD4^+$ cells and 2.7% were $CD8^+$ T cells, Table 5.4.

The remaining 38.5% of the leucocyte population did not express $CD3^-$, $CD4^-$, $CD8^-$ nor $CD19^-$ cell surface markers. Further analyses of these cells revealed that 23.3% expressed a high level of $CD11b^+$ cell surface marker, but were negative for $CD14^-$ and $CD16^-$, suggesting presence of monocyte/macrophages and/or dendritic cells (DCs). The remaining cells (11.7%) lacked $CD14^-$, $CD11b^-$ and $CD16^-$ cell surface expression; these cells were not further characterised, Table 5.4.

Table 5.4 Immunophenotyping of leucocyte population mechanically removed form donor lungs using EVLP.

Cell surface markers (Immunophenotype)	Percentage (%)	Cell types
$CD3^+$	3.7%	T cells
$CD4^+$	1%	CD4 T cells
$CD8^+$	2.7%	CD8 T cells
$CD19^+$	58%	B cells
$CD11b^+$	23.3%	Dendritic cells
$CD3^- CD4^- CD8^- CD19^- CD14^- CD11b^- CD16^-$	11.7%	Uncharacterised

5.2.4.3 Immunophenotyping of leukocyte population using flow cytometry

The flow cytomertic analysis of the monocyte/macrophage cell population revealed two main cell subsets; cells that expressed $CD14^+$ cell surface marker (88.1%) and cells that did not express $CD14^-$ surface marker (11.9%). Of the $CD14^+$ cells, 99.3% co-expressed $CD11b^+$, but not CD16; a phenotype consistent with the monocyte population. Cells that did not express $CD14^-$ surface marker expressed both $CD11b^+$ and $CD16^+$ surface markers, a hallmark feature of macrophages, Table 5.5.

In contrast, the analysis of the granulocyte population revealed that the majority of these cells were $CD14^-CD11b^+CD16^{+/-}$, whereas cells that were $CD14^+$ also co-expressed $CD11b^+$ and $CD16^{+/-}$, suggesting the presence of neutrophils Table 5.5.

Table 5.5 Immunophenotyping of leucocytes population mechanically removed form donor lungs using EVLP.

Cell surface markers (Immunophenotype)	Percentage (%)	Cell types
Monocyte/macrophage population		
$CD14^+ CD11b^+ CD16^-$	88.1%	Monocytes
$CD14^- CD11b^+ CD16^+$	11.9%	Macrophages
Granulocyte population		
$CD14^- CD11b^+ CD16^{+/-}$	79.3%	Unknown/Macrophages
$CD14^+ CD11b^+ CD16^{+/-}$	20.7%	Neutrophils

5.2.4.4 Characterisation of donor CD4 T cells mechanically removed from donor lungs using EVLP

Immunophenotyping of donor CD4 T cells was performed using simultaneous seven-colour flow cytometric analysis, previously described in Section 2.4.4. Of the total number of lymphocytes, only 1.7% of the cells analysed expressed $CD4^+$ cell surface marker, Figure 5.4 A c). Of the CD4 T cell population, 45.1% expressed CD45RA, whereas 54.9% expressed CD45RO, consistent with naïve / resting and activated / memory CD4 T cells, respectively (Figure 5.4 A d).

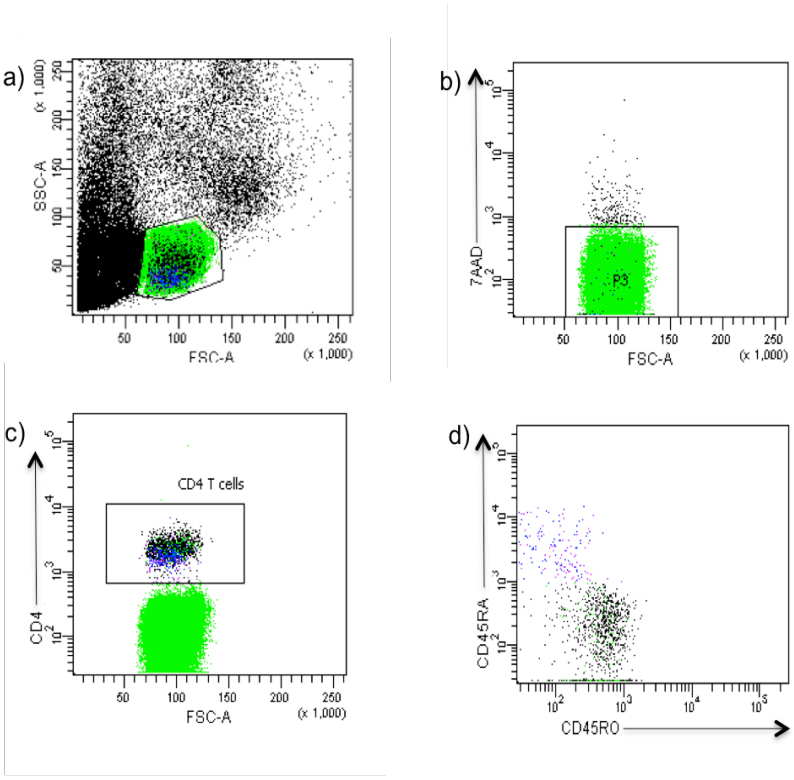
97% of the naïve CD4 T cells ($CD45RA^+$ $CD45RO^-$) expressed lymph node homing receptor L-selectin (CD62L); and, in addition, the majority of these cells (93.2%) also expressed CCR7 cell surface marker, another lymph-node homing receptor expressed on naïve and memory T and B cells. Interestingly, 68.2% of the $CD62L^+$ $CCR7^+$ cells co-expressed the cell surface chemokine receptor CXCR5, suggesting the presence of naïve follicular T (Tfh) cells. Whereas, 25% of the $CD62L^+$ $CCR7^+$ did not express CXCR5 cell surface chemokine receptor, Table 5.6 and Figure 5.4 B a), b) and c).

In comparison, 36.3% of the $CD45RA^-$ $CD45RO^+$ activated/memory phenotype CD4 T cells also expressed CD62L; the remaining 63.7% were $CD62L^{low}$, compatible with central memory and effector memory CD4 T cells, respectively (Table 5.6). Further analysis revealed that the $CD45RO^+$ cells expressed neither CXCR5 nor CCR7 receptor. lymph node homing receptor, suggesting that these cells are unable to recirculate through the lymph nodes, Figure 5.4 B d), e) and f). Table 5.6 shows subpopulations and surface marker expression of passenger CD4 T cells in human donor lungs mechanically removed from the lungs with EVLP.

Table 5.6 Immunophenotyping of donor CD4 T cell subpopulations mechanically removed from donor lungs using EVLP.

Cell surface markers	Percentage (%) and type of CD4 T cell subpopulations
$CD45RA^{+}$ $CD62L^{+}$ $CXCR5^{-}$ $CCR7^{+}$	25% Naïve CD4 T cells
$CD45RA^{+}$ $CD62L^{+}$ $CXCR5^{+}$ $CCR7^{+}$	68.2% Follicular helper T cells
$CD45RO^{+}$ $CD62L^{+}$ $CXCR5^{-}$ $CCR7^{-}$	36.3 Central memory CD4 T cells
$CD45RO^{+}$ $CD62L^{-}$ $CXCR5^{-}$ $CCR7^{-}$	63.7 Effector memory CD4 T cells

A)


a)
SSC-A
(x 1,000)
FSC-A
(x 1,000)
b)
7AAD
P3
FSC-A
(x 1,000)
c)
CD4
CD4 T cells
FSC-A
(x 1,000)
d)
CD45RA
CD45RO

B)

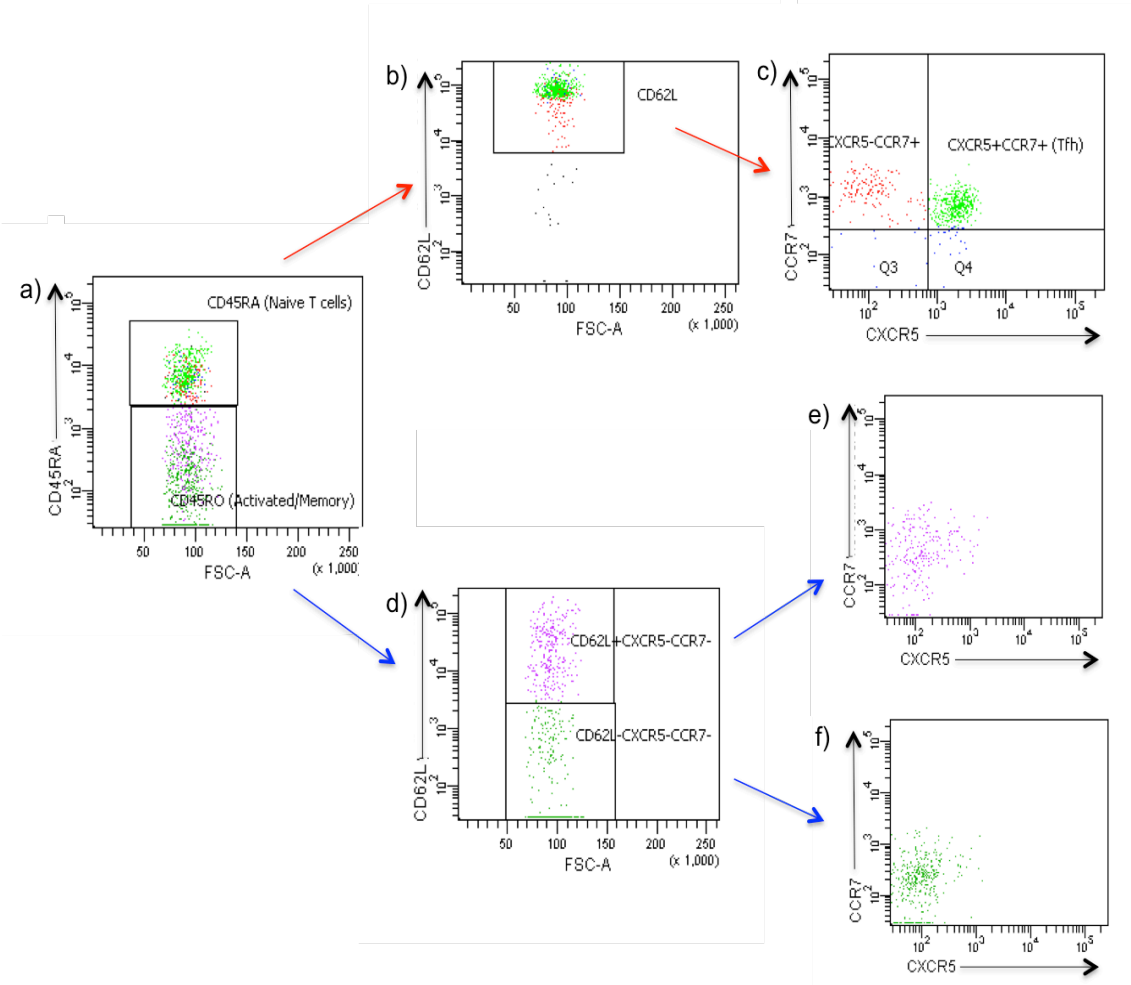


Figure 5.4 Immunophenotyping of donor CD4 T cells mechanically removed from donor lungs using EVLP. A) Isolated donor CD4 T cells were incubated with fluorescence labeled primary anti-human monoclonal antibodies specific for CD4, CD45RA, CD45RO, CD62L, CXCR5 and CCR7 and 7-AAD (7-aminoactinomycin D, death cell exclusion marker) and analysed by flow cytometer (Section 2.4.4); a) Side and forward scatter were set to detect lymphocyte population; b) 5µl of 7-AAD were added to the cells for exclusion of death cells; c) CD4 expressing cells were selected and identified as a subpopulation; d) Cytometric plot showing $CD4^+$ $CD45RA^+$ and $CD4^+$ CD45RO+ expressing cells. B) a) $CD45RA^+$ and $CD45RA^-$ population was further characterised; b) Cytometric plot showing $CD45RA^+$ cell population expressing CD62L surface marker, c) Two subpopulations of $CD62L^+$ cells were identified $CXCR5^+CCR7^+$ and $CXCR5^-CCR7^+$. Flow-cytometric plot d, e and f showing $CD45RA^-$ cell subpopulation analysis for the expression of CD62L, CXCR5 and CCR7; d) Showing cell subpopulation expressing CD62L and cell subpopulation lacking CD62L expression; e) and f) Cytometric plots showing $CD62L^+$ and $CD62L^-$ subpopulations; both lack co-expression of CXCR5 and CCR7 cell surface marker.

5.3 Discussion

To evaluate whether different donor CD4 T cell subpopulations may account for the observed variation in the longevity of donor CD4 T cell chimerism in our lung transplant patient study group, I attempted to characterise the isolated donor CD4 T cells using the RT-PCR relative quantification method. The expression of multiple gene transcripts that are thought to characterise different CD4 T cell subpopulations were assessed in patients with short and patients with long lasting donor CD4 T cell chimerism.

Previous studies have showed the presence of different cell populations, including donor CD4 T cells, in patients' peripheral blood within the first four weeks after transplantation [139, 267]. Nevertheless, to my knowledge, our analysis is the first study that attempted to characterise the donor CD4 T cell subpopulations.

I observed massive variations in the level of GAPDH gene expression in the patient samples (ranging between Ct 14 and Ct 31, Table 5.2), irrespective of the stable expression of the GAPDH internal control gene (Figure 5.1 B and C). The most likely explanation for the observed variation is the utilisation of different numbers of isolated donor CD4 T cells. For patients with short CD4 T cell chimerism the number of isolated CD4 T cells used ranged between 167 and 40,000 cells and for patients with long chimerism the number of isolated CD4 T cells used ranged between 345 and 51,000 cells.

In order to establish the gene expression levels, I calculated the delta Ct value for each gene tested. Delta Ct represents the level of gene expression in relation to the expression of GAPDH, Table 5.3. Heat map was generated to visualise the overall gene expression levels for each sample tested at different time points.

The analysis did not reveal a unique pattern of genes being expressed at one particular time nor between patient groups. Overall, an increase in the gene expression levels was observed for CD62L, CD44, STAT4, STAT5 and

STAT6 (Figure 5.2, red colour), with minimal change in level of expression for CD27, STST3 and Foxp3 and decreased level of expression for RORC, CCR6, IL21 and Bcl6 (Figure 5.2; black and green colour, respectively).

Of all genes tested the highest increase in the gene expression levels were observed in CD44, CD62L and STAT4 which may indicate the presence of memory Th1 phenotype; however, the observed differences were statistically not significant and inconclusive. It has to be stressed, the level of gene expression does not signify an absolute quantification; therefore, it is unknown whether these levels are sufficient and represent “true” effector memory Th1 subpopulation.

Ultimately, my aim was to determine whether different donor CD4 T cell subpopulations account for the observed variation in the longevity of donor CD4 T cell chimerism between the patients with short *vs* patients with long chimerism.

In spite of the small difference in the level of gene expression between the two groups of patients including both, samples obtained prior to and after the transplant due to the massive variation in the gene transcripts, the comparison analyses did not reveal unique gene expression pattern and I was unable to characterise the donor CD4 T cell subpopulations.

The number of cells utilised for the donor CD4 T cell characterisation and the size of study cohort limited this study. Unfortunately, due to the nature of the study it was not possible to correct for these limitations.

Furthermore, I have assessed the immune cell composition of donor lungs that undergo EVLP prior to revascularisation. This procedure mimics the cardiovascular conditions encountered following revascularization of the transplanted lungs; thus, the procedure provides a unique setting to study leukocyte migration from within the lungs into the recipient. Under EVLP conditions, migrating leukocytes are immobilised in the leukocyte filler attached to the EVLP circuit.

Another limitation of this study is the number of utilised ELVP leukocyte filters. It was unfortunate that the DEVELOP-UK study was prematurely terminated; thus, for the duration of the study I was able to assess only one EVLP leukocyte filter. Assessment of one EVLP filter is not sufficient to reach any conclusions; however, it does demonstrate that such studies are feasible and further investigation is required to assess whether removal of leukocyte population has an effect on clinical outcome in lung transplant recipients.

The first study that assessed the cell composition of human DBD lungs was reported by Richter et al.; they observed the presence of different cell populations localised in two different compartments of the lungs: the lymph nodes and the tissue of the lung itself [139]. Similarly, I have assessed the type of cells immobilised within the EVLP leukocyte filter; and to my knowledge this is the first study that attempted to characterise the EVLP isolated presenter mononuclear cells.

My data revealed that migrating mononuclear cells comprise of three distinct cell populations: lymphocytes, monocyte/macrophage and granulocyte population. The lymphocyte population was dominant cell population (59%) in comparison to monocyte/macrophage (22%) and granulocyte (18.5%).

Further analyses of monocyte/macrophage cell population revealed that the majority of these cells are monocytes (88.1%), characterised by the expression of $CD14^+$ cell surface marker and lack of $CD16^-$ expression. $CD14^+CD16^-$ cells have been described as classical blood monocytes [268]; suggesting that most likely these cells originate from the lung vasculature. Interestingly, these cells also expressed CD11b cell surface marker. CD11b is a β-chain integrin that associates with CD18 and forms complement receptor 3 (CR3) heterodimer [269]. CD11b is predominantly expressed on macrophages, dendritic cells, neutrophils and other leukocytes including monocytes; and, play a role in leukocyte adhesion and migration to mediate inflammatory responses [269]; thus, it is possible that cells with $CD14^+CD11b^+CD16^-$ phenotype represent classical monocytes that are

undergoing differentiation into either macrophages and/or dendritic cells. The remaining 11% of the cells were identified as mature macrophage population, phenotypically characterised as $CD14^{-}CD11b^{+}CD16^{+}$.

Analysis of the EVLP leukocyte filter isolated lymphocyte population comprised of a mixture of lymphocytic cells that proportionally differed from the lymphocytic cell prevalence in the peripheral blood, suggesting that the lymphocyte population does not originate from the lung vasculature. Surprisingly, my data showed that the majority of lymphocytes isolated from the EVLP filter were B cells (58%; Table 5.4). Furthermore, only 3.7% of lymphocytes were T cells, of which 2.7% were identified as cytotoxic CD8 T cells and 1% of the cells were helper CD4 T cells. The percentage of DCs characterised as lymphocytes that express CD11b cell surface marker was 23.3%.

My findings are comparable with the Richter et al., observations in terms of the cell populations present in the human lungs. They showed that the majority of mononuclear cells present in the tissue of the lung itself were lymphocytes and monocyte/macrophage population; and, around 50% of these cells were lymphocytes [139]. Nevertheless, I have observed significant difference in the percentage of B cells. Richter et al., showed variable number of B cells, 19% *vs* 45% present in the lung parenchyma and lymph nodes, respectively. In comparison, my observation showed higher number of B cells (58% of the lymphocyte population were identified as B cells), which could suggest that these cells derive form the lymph nodes rather than from the lung parenchyma itself. Furthermore, B cell population in Richter et al., study was identified as $CD20^{+}$ expressing cells, whereas I described the B cell population as $CD19^{+}$ lymphocytes, Table 5.4 [139]. Another difference that I observed was the percentage of CD3, CD4 and CD8 expressing lymphocytes. It is rather unexpected that in my study the percentage of $CD3^{+}$ T cells detected was very low (3.7%) in comparison to Richter's study (55% in parenchyma vs 52% in lymph nodes) [139]; the reasons for these differences are not obvious. Furthermore, the number of mononuclear cells they detected varied between 20-30 x 10^{9}, which is also comparable to my study; however, I

assessed only one EVLP leukocyte filter and it is possible that the number and percentage of various cell populations if variable between different donors. Assessment of more EVLP leukocytes filer is necessary to reach firm conclusion.

The remaining 11.7% of the lymphocyte population that did not express any of the targeted cell surface markers ($CD3^-$ $CD4^-$ $CD8^-$ $CD19^-$ $CD14^-$ $CD11b^-$ $CD16^-$) were not further characterised; however, it is possible that these cells represent NK cell subsets. Two different subsets of NK cells have been previously described $CD56^{bright}CD16^-$ and $CD56^{dim}CD16+$ [270], with predominately cytokine producing and cytotoxic effector function, respectively [270]. In comparison, Richter et al., observations showed that 15% of the lung tissue lymphocytes were NK cells expressing $CD56^+$ cell surface marker [139]. Unfortunately, due to the limitation of the sample size I did not use monoclonal antibody for detection of CD56 cell surface marker; thus, I am unable to confirm presence of NK cells, but the numbers are comparable to Richter's study.

Furthermore, Richter et al., have showed that the lymphocyte population varied between the lymph nodes and the lung tissue in regards to their cell surface marker expression and their activation state [139]; the lymph nodes cells consisted exclusively of naïve T and B cells, where most of the CD4 T cells were $CD45RA^+CD45RO^-$, whereas, majority of lung tissue T cells were activated $CD45RA^-CD45RO^+$ expressing cells [139].

In comparison to my data, the CD4 T cell immunophenotyping analysis revealed four subpopulations including naïve T cells, T follicular helper cells, central memory and effector memory T cells. Firstly, based on the expression of CD45RA or CD45RO CD4 T cells we separated them into two populations' naïve (45.1%) and effector/memory (54.9%) T cells, respectively.

The majority of naïve $CD45RA^+$ expressing cells (93.2%) co-expressed CD62L and CCR7 cell surface markers. CD62L is an adhesion molecule that plays an important role in lymphocyte-endothelial cell interactions [260],

whereas, CCR7 is a lymph-node homing receptor expressed on naïve and memory T and B cells [259]; implicating the ability of these cells to recirculate through the secondary lymphoid tissue. Interestingly, 68.2% of the $CD62L^+$ $CCR7^+$ cells co-expressed cell surface chemokine receptor CXCR5, defined as a hallmark feature of T follicular helper cells (Tfh), a CD4 Th cell subset that promotes germinal center reactions, selection and affinity maturation of B cells [255, 256].

In comparison, $CD45RO^+$ expressing CD4 T cells did not express CCR7 and CXCR5 cell surface markers, suggesting that these cells do not have the ability to recirculate through the secondary lymphoid organs. In addition, 36.3% of these cells expressed CD62L and the remaining 63.7% lacked CD62L expression, suggesting presence of two CD4T cell subsets: central memory CD4 T cells and effector memory cells, respectively. In theory, if antigen challenged these cells may trigger a vigorous immune response.

Although, only one EVLP filter was utilised in this study, our findings showed that the lung is a mononuclear cell rich organ and considerable number of immunocompetent CD4 T cell subsets bearing the ability to recirculate through the secondary lymphoid organs are transferred with the allograft at the time of transplantation.

Removal of leukocytes by EVLP procedure has generated conflicting data in animal model. Stone et al., in a porcine model of EVLP, have demonstrated that depletion of passenger leukocytes reduces the incidence of acute rejection in lung transplantation [271]; whereas in a separate study the same group have showed no effect on leukocyte immobilisation in a kidney transplant porcine model [272]. Whether immobilisation of passenger mononuclear cells using the EVLP procedure will affect the clinical outcome in human lung transplantation remains to be seen and is beyond the scope of this study.

Irrespective of the limited data set, my findings have increased our knowledge about the type of mononuclear cells that are transferred from within the lung

allograft into the recipient at the time of transplantation; and, in particular, the type of CD4 T cell subpopulations. A further work is required to confirm whether the observed cell populations are uniform between different lung donors or the lung cell composition depends on the type of donor and/or cause of donors' death.

In summary, due to the massive heterogeneity in the level of gene expression I was unable to characterise the peripheral blood isolated donor CD4 T cells; thus, it remains unclear whether the observed variation in the longevity of donor CD4 T cell chimerism in our lung transplant patient study group is due to the presence of different CD4 T cell subsets. At this particular stage, we are limited in gaining further knowledge to increase our understanding about factors that may influence the longevity of donor CD4 T cells following primary lung transplantation.

5.4 Key points

- Due to the variation in the levels of gene expression I was not able to characterise the isolated donor CD4 T cells; thus, I cannot compare whether there is a difference in the CD4 T cell subpopulation between the patients with short and patients with long donor CD4 T cell chimerism.

- Donor lungs contain large number of mononuclear cells consisting of three cell populations including lymphocyte, monocyte/macrophage and granulocyte population.

- Donor lungs contain a mixture of naïve and activated/memory CD4 T cells that presumably originate from both the local lymph nodes and the tissue of the lung itself.

- Immunophenotypically human donor lung CD4 T cells can be classified into four subpopulations: naïve, follicular helper T cells, effector memory and central memory T cells.

6 Chapter 6

Humoral allo- and auto-immunity in lung transplant recipients

6.1 Introduction

In solid organ transplantation, humoral immune responses are characterised by the production of alloantibody. The alloantibodies are generated predominantly against mismatched donor MHC class I and class II antigens [87], and occasionally against mHA [186]. In lung transplantation, histopathological evidence of small vessel intimitis or endothelialitis, together with immunohistochemical staining for complement deposition and presence of anti-HLA alloantibody is considered strongly suggestive of a humoral effector response, and is termed antibody-mediated rejection (AMR) [273]. Depending on the time of onset, AMR can occur within minutes to years after allograft transplantation. In more recent years, accumulating evidence suggests that in addition to alloantibody responses, transplantation may also generate humoral autoimmune responses against a diverse array of autoantigens [144].

6.1.1 Clinical significance of allo- and autoantibody in solid organ transplantation

Sensitisation to HLA antigens is acquired through prior pregnancy, transfusion or transplantation; and about 10% to 15% of patients awaiting lung transplant are sensitised to HLA antigens.

Transplantation of organs into patients with pre-existing IgG alloantibodies directed against mismatched donor HLA class I (donor specific antibodies, DSA) and ABO blood group antigens is often associated with hyperacute or accelerated acute allograft rejection [87, 89, 274]. Consequently, transplantation of organs into recipients with existing donor specific anti-MHC class I alloantibody is avoided, particularly in the presence of a positive, complement-dependent cytotoxicity (CDC) crossmatch, unless desensitisation protocols are applied prior to transplantation [275]. There is a lack of evidence supporting the association of preformed HLA class II DSAs with hyperacute or

accelerated allograft rejection [276]; nonetheless, patients with class II DSA have poor re-graft survival.

Clinical studies of kidney transplant recipients have revealed that development of *de novo* DSA following transplantation is more commonly directed against donor MHC class II, than MHC class I alloantigens. Such responses are associated with reduced graft survival and development of chronic allograft rejection [277, 278].

Likewise, in a small cohort of lung transplant recipients, it was reported that 8 out of 9 patients developed *de novo* anti-MHC class II DSA and only one patient developed anti-MHC class I DSA. The development of anti class II DSA has been associated with early onset of BOS [279]. For patients with pre-existing DSA, these are associated with an increased incidence of primary graft dysfunction and acute rejection episodes, with worse patient survival rates reported [280]. In addition, poor outcome has also been observed in lung transplant recipients with preformed HLA antibodies without specificity for donor mismatched antigens.

Humoral rejection has been observed in lung transplant patients who developed antibodies to non-HLA self-antigen, even when anti HLA alloantibody is not detectable [281]. For, example, *de novo* autoimmunity to type V collagen [282] and K-alpha 1 tubulin (K-α1 tubulin) [283] has been implicated as an independent predictor for development of BOS. Interestingly, Hachem *et al*., in a large cohort of 108 lung transplant recipients, showed that patients who developed both anti-MHC alloantibody and autoantibodies to collagen V and K-α1 tubulin had increased risk of developing BOS. They further showed that patients that responded to antibody mediated therapy and cleared the autoantibodies were less likely to develop BOS in comparison to patients with persistent autoantibodies, irrespective of the presence or absence of alloantibodies [284].

The involvement of cellular and humoral immunity to type V collagen and its association with primary graft dysfunction (PGD) has also been confirmed in experimental rat models; passive transfer of collagen V immune serum resulted in PGD and increased levels of pro-inflammatory cytokines [285]. In a mouse models of lung transplantation, *de novo* autoimmune responses to collagen V and K-α1 tubulin were induced following injection of anti-donor HLA antibody directly into the lung [286], suggesting that both allo- and auto-immune responses contribute to the development of BOS.

Several other autoantigens have been recognised as a target for the development of autoimmune responses in organ transplant recipients. These include: cardiac myosin [287, 288]; vimentin [289]; angiotensin II type 1-receptor (AT1R) [290]; renal pelvis antigens [291]; smooth muscle and nuclear antigens [292]. All have been associated with increased risk of rejection and poorer outcomes.

The pathophysiology of how allo- and autoantibody cause graft injury and as a consequence allograft dysfunction is not fully understood and remains the most challenging immunological barrier in human transplantation. AMR is often unresponsive to current immunosuppressive regimens; and, it has been recognised as a major cause of allograft loss. Thus, understanding the exact mechanisms by which alloantibodies (including both DSA and non-DSA) and autoantibody mediate allograft rejection is crucial for developing strategies specifically designed to ameliorate AMR and prolong allograft survival.

6.1.2 Mechanisms of antibody-mediated allograft injury

The mechanisms underlining antibody mediated allograft rejection involve: activation of the complement system via components of C1q-dependent and mannose-binding lectin (MBL) – dependent pathway; direct antibody ligation to the endothelial cells; and recruitment and activation of innate immune system effector cells, including monocytes, macrophages, neutrophils and NK

cells. These innate immune cells exert their effector function via Fcγ receptors (FcγRs) and complement receptors (CR).

6.1.2.1 Complement-dependent alloantibody mediated allograft injury

The complement system is part of the innate immune system that can result in non-specific tissue injury via three main pathways: classical, MBL and alternative pathway. Each pathway produces C3 convertase (C4bC2a is produced by the classical and lectin pathway and C3bBb is produced by the alternative pathway) that cleaves C3 protein. In humans, IgG1 and IgG3 complement-fixing donor specific alloantibodies (DSA) activate the complement system primarily via the classical pathway initiating complement cascade by binding and activating the complement component 1 (C1). C1 is composed of C1q, an antibody binding component and two proteases, C1r and C1s. Activated C1 enzymatically cleaves C4 and C2 protein into two fragments C4a and C4b and C2a and C2b, respectively. The larger C4b fragment binds covalently to surrounding tissue and with C2a to form enzyme complex C3 convertase (C4bC2a). MBL pathway also generates C3 convertase (C4bC2a) via activation of MASP-1, 2 and 3 associated serine proteases through binding to carbohydrates on microorganisms, IgM, some isotypes of IgG and injured or apoptotic cells; although the significance of MBL complement pathway in allograft rejection is not clear.

The C4b component of C3 convertase is inactivated by Factor I and generates C4d fragments, which is the end product of C4 activation. C4d binds covalently to surrounding tissue and serves as a marker of antibody-mediated rejection [293].

C3 convertase cleaves C3 protein into C3b and C3a fragments. The C3b component then binds to C3 convertase to generate C5 convertase (C4bC2aC3b), which cleaves C5 protein into two biologically active products C5a and C5b. The split product C5b initiates the terminal phase of the complement system via binding to C6, C7, C8 and C9 proteins to form a

tubular structure known as the membrane attack complex (MAC; C5b-C9), which forms pores on the target cell membrane leading to cell lysis [294].

Interestingly, lysis of allograft endothelial cells is not a prominent feature of AMR, possibly due to regulation of MAC formation by decay accelerating factor (DAF) and protectin (CD59) [295]. These are complement regulatory molecules that are also observed to be upregulated in allografts undergoing accommodation, for example, in biopsies of ABO incompatible heart transplant recipients [296]. However, *in vitro* studies have revealed that small amounts of MAC can activate endothelial cells to upregulate the expression of adhesion molecules such as E-selectin, intracellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 [297]; and promote production of chemokine mediators such as IL-1α [298], monocyte chemotactic factor (MCP)-1 and IL-8 [299, 300]. These pro-inflammatory mediators trigger recruitment and activation of leukocytes into the allograft. Similarly, in human heart transplant recipients, AMR has been associated with increased expression of P-selectin and von Willebrand factor (vWf) on the endothelium [301]; which promotes adhesion of platelets and monocytes to the endothelium and production of pro-inflammatory mediators [302]. Thus, endothelial activation alters endothelial cells from an anti-inflammatory protective barrier into a pro-coagulant, adhesive and chemoattractive layer that promotes inflammation; resulting in recruitment of platelets, neutrophils, monocytes, macrophages and NK cells to the site of injury.

The importance of MAC induced endothelial cell activation and antibody-initiated cell injury has been highlighted in experiments incorporating C6-deficient rats. C6-deficient hearts and lungs transplanted into C6-deficient recipients had moderate expression of endothelial cell adhesion molecules, minimal platelet aggregation and showed no evidence of alveolar hemorrhage and edema [303].

Other components of the complement system are able to sustain allograft endothelial cell inflammation by recruitment of proinflammatory cells. C3b and

C4b are ligands for complement receptor 1 (CR1) and promote extravasation of neutrophils, macrophages and leukocytes into the graft [295]. C3b can trigger the alternative pathway of complement activation, thus amplifying the complement response. C3a and C5a split products promote chemotaxis and inflammation. In particular, C5a has a pivotal impact in AMR. C5a is a strong chemoattractant for neutrophils and macrophages, and triggers macrophage activation via upregulation of stimulatory FcγRII and downregulation of the inhibitory FcγRIIB [304]: macrophages thus become more responsive to antibodies and complement split products. Avoidance of MAC formation by targeting C5 complement fragment and generation of C5a with monoclonal antibodies such as Eculizumab have proved effective at reducing the requirements for dialysis after transplantation in kidney transplant recipients diagnosed with atypical hemolytic uremic syndrome (aHUS) [295, 305]. Siedlecki et al., have showed that at six mounts after transplantation graft function is significantly better in patients with aHUS that received Eculizumab before and after transplantation in comparison to patients that received Eculizumab after transplantation only [305]. Furthermore, in more recent years Eculizumab has been successfully used as a treatment for ameliorating AMR [306] and prevention of AMR in blood group incompatible kidney transplant recipients [307].

6.1.2.2 Complement-independent alloantibody mediated allograft injury

Several studies have shown that alloantibodies can mediate complement-independent endothelial cell activation by cross-linking MHC molecules. This results in phosphorylation of tyrosine proteins, production of growth factors and increased cell proliferation [308]. The mechanism underlining this response is not clear. However, several studies have reported involvement of mTOR signaling pathway. Association of HLA class I with intergrin β4 subunit permits intracellular mTOR-signaling pathway; knockdown of intergrin β4 subunit abrogates the ability of HLA class I to activate endothelial cells [309]. In further support, lung epithelial carcinoma cell line cultured *in vitro* with pooled serum from highly sensitised patients with BOS showed greater

tyrosine phosphorylation and cell proliferation, suggesting a direct action of HLA class I alloantibody on allograft endothelium [310]. Similarly, mouse endothelial cells undergo activation, characterised by exocytosis of vWf and cell surface expression of P-selectin, when cultured with monoclonal antibodies directed against their surface MHC class I antigen [311]. In cardiac transplant patients with active AMR, the endothelial cell activation correlates with activation of mTOR-signaling pathway [312].

Alloantibody may also augment allograft injury mediated by monocytes, macrophages and NK cells through interaction with their surface Fc receptors and the Fc portion of IgG1, as classically occurs with antibody-dependent cell-mediated cytotoxicity (ADCC) [313]. Engagement of IgG1 alloantibody with stimulatory Fcγ receptors causes recruitment and accumulation of neutrophils and macrophages, a key characteristic feature of AMR. In a mouse experimental model, Hirohashi et al., have demonstrated that passive transfer of non-complement fixing DSA in both B6.RAG$^{-/-}$ KO recipients and complement-deficient (RAG$^{-/-}$C3$^{-/-}$) models caused tissue injury and development of CAV that was dependent on NK cell and macrophage infiltration into the graft [314].

6.1.2.3 Autoantibody mediated allograft injury

In more recent years accumulating evidence suggests that, in parallel to alloimmune responses and production of alloantibody, transplantation also triggers humoral autoimmune responses against a diverse array of autoantigens [144]. These may be organ specific such as cardiac myosin [287], or ubiquitously expressed, such as smooth muscle and vimentin antigens found in liver [292] and heart transplant recipients [289], respectively. Autoantibody development in transplant patients without pre-existing autoimmune disease suggests that autoimmunity develops *de novo* after organ transplantation; these autoantibodies are mainly class switched IgG [144, 287], implying that their generation is T-cell dependent.

Despite the peripheral and central mechanisms of clonal deletion and receptor editing involved in regulation of B cell development to prevent self-reactivity, occasionally these mechanisms fail to eliminate all autoreactive B cells. Small populations of autoreactive B cells and the presence of IgM and/or low titre of IgG autoantibodies are thus found in healthy individuals [315]. These autoantibodies differ from the high-affinity somatically mutated IgG autoantibodies found in transplant recipients and patients with autoimmune diseases; they are mainly polyclonal and reactive to self and infectious antigens [315].

In autoimmune diseases, multiple pathways lead to autoantibody-induced tissue injury (previously reviewed by Ludwig et al., [316]). Similar mechanisms may be involved in triggering autoimmune responses following transplantation. These include: early allograft damage (tissue injury) that may trigger release of cryptic self-antigens that have not been previously encountered by developing B and T cells [317]; formation and exposure of neoantigens generated by alteration of cellular proteins [318]; epitope spreading to encompass autoreactivity, secondary to prolonged alloimmune responses [319]; immune responses to polymorphic non-self mHAgs [320]; and cross-reactivity due to molecular mimicry of autoantigens with infectious agents [321]. All of these pathways could potentially result in autoantibody-mediated complement-dependent and non-complement-dependent allograft injury. However, the exact mechanism by which autoantibody causes tissue injury is not established, and it has proven difficult to distinguish between the auto- and allo-immune contribution to allograft rejection.

Lastly, work from our group has highlighted a unique transplant related mechanism for triggering autoimmunity following transplantation, previously described in Chapter 3, Section 3.1.3. In brief, Win et al., used a murine model of chronic heart graft rejection to study how donor CD4 T cells that were passengers within the heart graft influenced the host response to the graft. In this model, bm12 strain mouse heart allografts were transplanted into C56BL/6 strain recipients. These strains differ only by three amino acid residues within the MHC class II I-A antigens (HLA-DQ in humans);

consequently, bm12 hearts are rejected slowly (MST > 50 days). Win's work demonstrated that rejection was associated with histologic evidence of antibody-mediated rejection, characterised by C4d complement and IgG endothelial deposition.

Surprisingly, C56BL/6 recipients did not develop alloantibody, but instead graft vascular injury was associated with development of long-lasting IgG antinuclear autoantibody [144]. Further experiments revealed that development of autoantibody was independent of indirect pathway T cell responses; suggesting that an alternative mode of help was responsible for the development of autoantibody in the bm12-B6 model. Win thus investigated the potential role of passenger donor CD4 T cells in the provision of help to host B cells. Transplantation of heart allografts from donor bm12 mice that lacked T and B cells or were depleted of CD4 T cells prior to heart allograft procurement did not prompt autoantibody production in the recipient, and heart allograft survival was prolonged [144]

Win concluded that in her model, help for autoantibody production was provided via a direct cognate interaction between the donor passenger CD4 T cells and the recipient auto-reactive B cells [144]; highlighting a novel mechanism for how help for autoantibody production is delivered to recipient auto-reactive B cells. Further experiments provided evidence for a contributory role of autoantibody in the development and progression of chronic allograft vasculopathy.

The work in my thesis was undertaken to investigate whether a similar mechanism may occur in human lung transplant recipients. In Chapter 3, I showed that donor CD4 T cell passengers within the allograft are readily detectable in the peripheral blood of primary lung transplant recipients. Surprisingly, the percentage and dynamics of donor CD4 T cell persistence in the recipients' peripheral blood was variable between patients. Based on these observations, patients were characterised into three groups: patients with short donor CD4 T cell chimerism (donor CD4 T cells detectable for six weeks after transplantation, n=13), patients with intermediate donor CD4 T

cell chimerism (donor CD4 T cells detectable between three to six months after transplantation, n=3) and patients with long lasting donor CD4 T cell chimerism (donor CD4 T cells detectable for more than six months after transplantation, n=5). As detailed in the previous chapters, the variable patterns of survival of donor CD4 T cells in the recipient circulation neither correlated with the degree of donor / recipient HLA mismatching nor the gene expression profile of the donor CD4 T cells.

To address this question, the following experiments had two aims. The first aim was to assess the humoral allo- and autoimmune responses in my study cohort of primary lung transplant recipients (n-21). Serum samples obtained at the time of transplant and at regular time intervals after transplantation were examined for the presence of IgG alloantibody and IgG autoantibody using Luminex methodology and HEp-2 Indirect Immunofluorescence (IIF) assay, respectively.

Due to the small number of participants in my study cohort, an additional 43 lung and heart and lung transplant recipients (retrospective cohort) were recruited retrospectively for participation in this study. The retrospective study cohort consisted of 23 bilateral lung, 2 single lung and 18 heart and lung transplant recipients. Humoral allo- and autoimmune responses (either pre-existing or those that developed *de* novo after transplantation) were assessed by evaluating the presence of allo- and autoantibody using the same methodologies.

The second aim was to evaluate the humoral autoimmune response in my cohort of transplant recipients, by using high-density protein microarrays to profile the autoantibody generated in long-term surviving lung transplant recipients with either well functioning allografts and free from BOS (n=10) or with established BOS (n=10). The reasoning behind this set of experiments was to explore the potential for characterisation of a novel set of “biomarkers” which could prompt new screening strategies to monitor and manage recipients in the hope of improving graft and patient survival.

6.2 Results

Two study cohorts were subjected for assessment of humoral allo- and autoimmune responses in lung transplant recipients: a prospective cohort (lung transplant recipients recruited for participation in this study prior to transplantation); and a retrospective cohort (lung and heart and lung transplant recipients recruited for participation in this study at different time points after transplantation).

The anti-HLA alloantibody response was characterized by testing recipient sera obtained at the time of transplant and at regular times thereafter, using Luminex LABScreen® Mixed and LABScreen® Single Antigen HLA Class I and Class II Antibody detection beads (detailed in Chapter 2, Section 2.5.1.)

Reactivity to anti-nuclear antibody (ANA) was assessed using HEp-2 Indirect Immunofluorescence (IIF) assay and HEp-2 Enzyme-linked Immunosorbent assay (Chapter 2, Section 2.5.2 and 2.5.3, respectively).

The autoantibody profiling was evaluated in pre- and post-transplant sera of 20 lung and heart and lung transplant recipients, 10 with established BOS and 10 without BOS, using Invitrogen Human ProtoArray® v5.0 protoarray, (Chapter 2, Section 2.5.4.).

6.2.1 Prospective study cohort of lung transplant recipients

The prospective cohort consisted of 21 lung transplant recipients; informed written consent was obtained prior to transplantation from each participant in this study cohort. All patients underwent lung transplantation at Papworth Hospital, Papworth Everard, Cambridge, UK; 19 patients received bilateral lung and two patients received single lung transplants.

The recipient age varied between 18 and 66 years. Of the 21 patients who underwent lung transplantation, 8 were male and 13 female. All patients

received triple drug immunosuppression; in addition, two patients received induction therapy with Basiliximab, a humanised monoclonal antibody to α chain (CD25) of the IL-2 receptor expressed on T cells. Detailed representations of the recipient and donor demographic characteristics are presented in Table 6.1 (Table 6.1 is same as Table 3.1; considering that multiple cohorts are presented in this Chapter (Chapter 6), for clarity Table 6.1 is presented again).

Previously, in each participant in this study cohort donor CD4 T cell chimerism was detected in the peripheral blood obtained at various time points after lung transplantation. All patients were followed for 12 months after transplantation.

Table 6.1 Prospective study cohort. Lung transplant recipients and donor characteristics (Table same as Table 3.1)

Recipient age (Median ± SD)	56 ± 13.6 (18-66 years)
Recipient sex (M:F)	8:13
Transplant type	
SLT	2
BLT	19
Donor age (Median ± SD)	48 ± 11.2 (19-60 years)
Donor sex (M:F)	10:11
Donor type	
DBD	20
DCD	1
Indication for transplantation	
Cystic fibrosis	3
Emphysema	4
COPD	7
Pulmonary fibrosis	2
Bronchiectasis	2
A1AD	1
Emphysema + A1AD	2
Immunosuppression therapy*	Tacrolimus/MMF/Prednisolone

SLT – Single Lung Transplant; BLT – Bilateral Lung Transplant; DBD – Donation after brain steam death; DCD – Donation after cardiac death; COPD - Chronic obstructive pulmonary disease; A1AD- α1 antitrypsin deficiency; MMF – Mycophenolate Mofetil.
*Two patients received induction therapy with Basiliximab.

6.2.1.1 Incidence of pre- and post-transplant HLA alloantibody in lung transplant recipients

To assess the recipients' sensitisation to HLA alloantigen, sera obtained at the time of transplant were screened for the presence of HLA alloantibody using Luminex LABScreen® Mixed detection beads (Chapter 2, Section 2.5.1). All sera that tested positive for the presence of HLA alloantibody were further characterised using LABScreen® Single Antigen HLA Class I and Class II antibody beads (detailed in Chapter 2, Section 2.5.1) to establish the precise specificity of the anti-HLA. The same methodology was used for assessment of *de novo* humoral alloimmune responses using test serum samples obtained at regular time intervals after the transplant (1, 3, 6, 9 and 12 months). Two patients died during the study follow-up period (one patient died at day 166 and one at day 171 after the transplant, due to non-transplant related complications); all other patients were followed for one year after the transplant.

Of the 21 patients, 7 patients had detectable HLA class I antibody prior to transplantation and 14 were not sensitised to HLA antigens. None of these patients had DSA and the retrospective CDC crossmatch using sera obtained prior to transplantation was negative for all patients. Table 6.2 represents the patients' HLA antibody profile in sera tested before the transplant and after transplantation transplant at 1, 3, 6, 9 and 12 months.

In summary, all patients that were not sensitised to HLA antigens prior to transplantation tested negative for presence of HLA antibody during the first year after the transplant; except for one patient (P18, Table 6.2) that developed transient *de novo* class I and class II DSA with specificity to B8, DR52 and DQ2 antigens.

Table 6.2 represents the patients' HLA antibody profile (DSA/non-DSA) in sera tested pre-transplant and post-transplant at 1, 3, 6, 9 and 12 months.

Patient ID	Pre-Tx	Post-Tx 1 month	Post-Tx 3 months	Post-Tx 6 months	Post-Tx 9 months	Post-Tx 12 months
P1	NS	No Ab	No Ab	No Ab	No Ab	No Ab
P2	NS	No Ab	No Ab	No Ab	No Ab	No Ab
P3	NS	No Ab	No Ab	No Ab	No Ab	No Ab
P4	non-DSA	non-DSA	non-DSA	non-DSA	No Ab	No Ab
P5	NS	No Ab	No Ab	No Ab	No Ab	No Ab
P6	NS	No Ab	No Ab	No Ab	No Ab	No Ab
P7	NS	No Ab	No Ab	No Ab	No Ab	No Ab
P8	non-DSA	No Ab	No Ab	No Ab	-	-
P9	NS	No Ab	No Ab	No Ab	No Ab	No Ab
P10	NS	No Ab	No Ab	No Ab	No Ab	No Ab
P11	NS	No Ab	No Ab	No Ab	No Ab	No Ab
P12	non-DSA	non-DSA	non-DSA	No Ab	**DSA / non-DSA**	**DSA / non-DSA**
P13	non-DSA	non-DSA	non-DSA	non-DSA	non-DSA	**DSA / non-DSA**
P14	NS	No Ab	No Ab	No Ab	No Ab	No Ab
P15	NS	No Ab	No Ab	No Ab	No Ab	No Ab
P16	non-DSA	**DSA / non-DSA**	**DSA / non-DSA**	**DSA / non-DSA**	**DSA / non-DSA**	**DSA / non-DSA**
P17	non-DSA	non-DSA	**DSA/non-DSA**	**DSA/non-DSA**	**DSA/non-DSA**	**DSA/non-DSA**
P18	NS	**DSA / non-DSA**	**DSA / non-DSA**	No Ab	No Ab	No Ab
P19	NS	No Ab	No Ab	No Ab	No Ab	No Ab
P20	NS	No Ab	No Ab	No Ab	-	-
P21	non-DSA	No Ab	No Ab	No Ab	No Ab	No Ab

P – patient
Pre-Tx – prior to transplantation
Post-Tx – after transplantation
NS – non-sensitised patient prior to transplantation
DSA – Donor Specific Antibody
Non-DSA – non-donor specific antibody
No-Ab – HLA antibody not detected

Interestingly, sera obtained at around 12 months after transplantation tested positive for the presence of both DSA and non-DSA antibody in four patients (P12, P13, P16 and P17, Table 6.2); all of which were sensitised to HLA antigens before the transplant. One patient had anti-class I DSA, one patient had class II DSA only, and two patients had both class I and class II DSA. HLA antibodies were not detectable in three patients that were sensitised before the transplant (P4, P8 and P21, Table 6.2). All other patients tested were negative for the presence of HLA alloantibody. Figure 6.1 represents the number of patients sensitised to HLA antigens prior to transplantation and the presence of both DSA and/or non-DSA antibody at 1, 3, 6, 9 and 12 months after transplantation.

Surprisingly, the presence of pre-existing HLA antibodies was not associated with the onset of BOS. Of the 21 recipients, seven recipients had HLA antibodies at the time of transplant; of these four developed BOS within the first post-operative year and three remained free from BOS. In comparison, three out of 14 recipients who did not have HLA antibody prior to transplantation also developed BOS (Fisher's exact test, p=0.156), Table 6.3. Likewise, the incidence of acute cellular rejection did not correlate with the presence of HLA antibody at the time of transplant. Only four out of seven patients with pre-existing HLA antibodies experienced acute cellular rejection (ACR); whereas, two out of the 14 recipients without HLA antibody prior to transplantation had ACR during the first post-operative year (Fisher's exact test, p=0.119), Table 6.3. Antibody mediated rejection was not diagnosed in any of the recipients in this cohort. Table 6.3 details the patients' HLA antibody profile, incidence of BOS and acute cellular rejection.

In addition, in this study cohort we did not observe correlation between the onset of BOS and the development of *de novo* DSA. In total, seven recipients developed BOS within the first post-operative year; of these two recipients developed DSA and in five recipients DSA were not detected, (Fisher's exact test, p=0.574), Table 6.3.

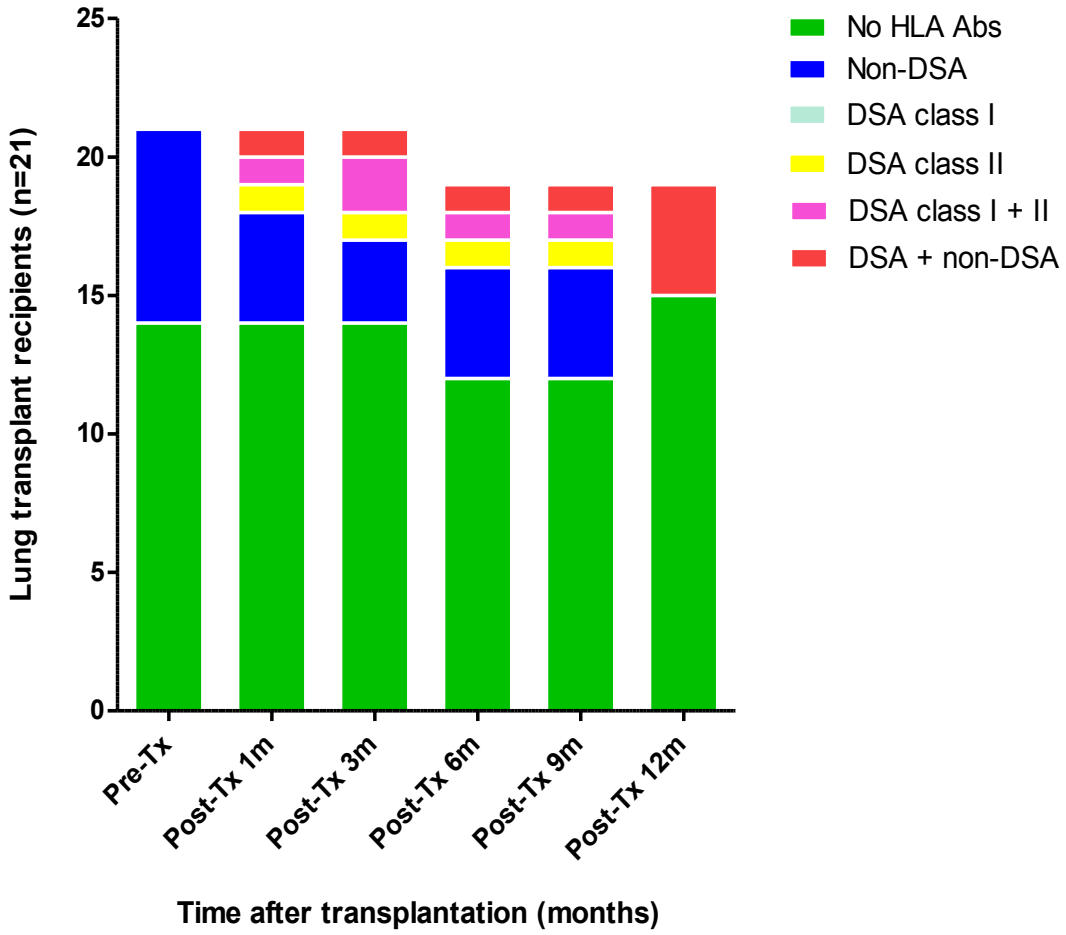


Figure 6.1 HLA sensitisation in lung transplant recipients before the transplant and development of *de novo* HLA antibody after transplantation. The graph shows the number of lung transplant recipients sanitised to HLA antigens prior to transplantation and development of *de novo* HLA antibody after transplantation. HLA antibody development was assessed in the recipients' sera obtained at regular time intervals after transplantation (1, 3, 6, 9, and 12 months). Luminex LABScreen® Mixed beads and LABScreen® Single Antigen HLA Class I and Class II antibody beads were used for detection and characterisation of HLA antibody including both, donor specific antibody (DSA) and non-donor specific antibody (non-DSA).

Table 6.3 Recipients' HLA antibody profile, incidence of BOS and acute cellular rejection episodes.

Patient ID	Pre-Tx	Post-Tx (12 months)	BOS (Grade)	Acute cellular rejection (No of episodes)
P1	NS	No Ab	**Yes (2)**	No
P2	NS	No Ab	No	No
P3	NS	No Ab	No	No
P4	non-DSA	No Ab	**Yes (2)**	**Yes (1)**
P5	NS	No Ab	No	**Yes (1)**
P6	NS	No Ab	**Yes (2)**	**Yes (1)**
P7	NS	No Ab	No	No
P8	non-DSA	-	No	**Yes (2)**
P9	NS	No Ab	No	No
P10	NS	No Ab	No	No
P11	NS	No Ab	No	No
P12	non-DSA	DSA / non-DSA	**Yes (1)**	No
P13	non-DSA	DSA / non-DSA	No	**Yes**
P14	NS	No Ab	No	No
P15	NS	No Ab	No	No
P16	non-DSA	DSA / non-DSA	**Yes (2)**	No
P17	non-DSA	DSA / non-DSA	No	**Yes**
P18	NS	No Ab	No	No
P19	NS	No Ab	**Yes (1)**	No
P20	NS	-	No	No
P21	non-DSA	No Ab	**Yes (1)**	No

P – patient
Pre-Tx – prior to transplantation
Post-Tx – after transplantation
NS – non-sensitised patient prior to transplantation
DSA – Donor Specific Antibody
Non-DSA – non-donor specific antibody
No-Ab – HLA antibody not detected
BOS – Bronchiolitis Obliterans Syndrome

6.2.1.2 Incidence of pre- and post-transplant anti-nuclear (ANA) autoantibody in recipients of primary lung transplants

To assess the incidence of antinuclear autoantibody present in recipients' sera, serum samples obtained before the transplant and at two time points after transplantation (one and twelve months) were tested using HEp-2 Indirect Immunofluorescence (IIF) assay, Chapter 2, Section 2.5.2.

HEp-2 cells provide a non-specific, but highly sensitive test for detection of antinuclear autoantibody. Because they grow as a monolayer on the microscopic slide, cells at different stages of the cell cycle are present on the same slide; this allows exposure of nuclear antigens expressed only at a particular stage of the cell cycle.

The HEp-2 indirect immunofluorescence assay revealed test sera reactivity to various anti-nuclear antigens in all test samples, Figure 6.2. Interestingly, the anti-nuclear IgG autoantibody titre was highest in the patients' sera obtained at the time of transplant in comparison to the sera obtained after transplantation. In comparison to the sera obtained at the time of transplant a significant decrease in the anti-nuclear IgG autoantibody titre was observed in the post-transplant test samples obtained at one-month (Paired t test, p=0.002) and at 12 months (Paired t test,, p=0.006) after transplantation, Figure 6.2. Difference in the anti-nuclear IgG autoantibody titre between the post-transplant serum samples obtained at one and 12 months after transplantation was not observed (Paired t test, p=0.372).

Nonparametric correlation analysis revealed that the level of anti-nuclear IgG autoantibody present at the time of transplantation neither correlated with the onset of BOS nor with the recipient age at the time of transplant (Spearman r, p=0.645 and p=0.385, respectively).

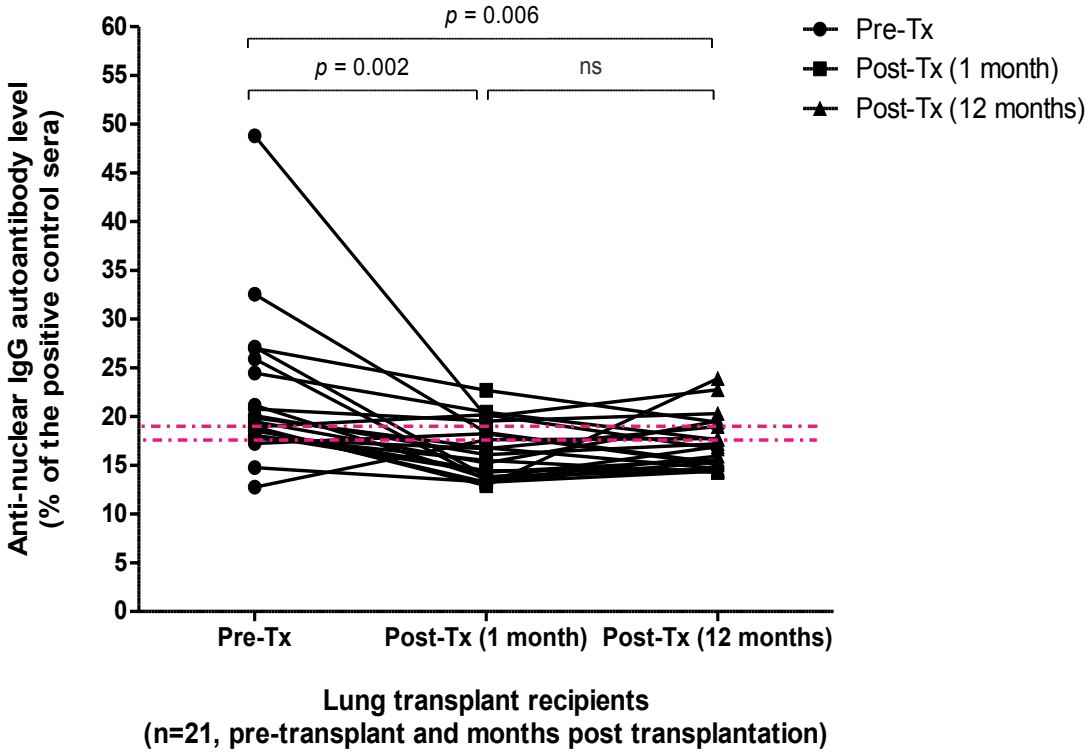


Figure 6.2 Anti-nuclear IgG autoantibody level in lung transplant recipients detected using HEp-2 Indirect Immunofluorescence assay. The graph shows the anti-nuclear IgG autoantibody titres present in the recipients' pre-transplant sera and sera obtained at one-month and 12 months after transplantation. The data is presented as a percentage of the positive control sera (human positive control serum supplied by the manufacture). Recipients' sera reactivity to anti-nuclear antigens was strongest in the sera obtained prior to transplantation. A significant decrease in IgG anti-nuclear autoantibody titres was observed in the test sera obtained at one month (p=0.002; paired t test) and 12 months after transplantation (p=0.002; paired t test) in comparison to the sera obtained at the time of transplant. There was no significant change in the IgG anti-nuclear autoantibody titres between the sera obtained at one month and 12 months after transplantation (p=*ns*; paired t test). Paired t test was used to calculate significance. *P*-value of <0.05 was considered statistically significant. Red broken-lines show negative control sera cut-off values (pooled human serum from seven healthy individuals); represented as a mean ± 2SD.

6.2.2 Retrospective study cohort of lung and heart and lung transplant recipients

Humoral allo- and autoimmune responses were assessed in another study cohort (retrospective cohort) of 43 lung and heart and lung transplant recipients. 23 patients received bilateral lung, 2 single lung and 18 heart and lung transplants at Papworth Hospital, Papworth Everard, Cambridge, UK. All patients gave written consent for participation in this study. Pre-transplant serum samples were not available for three patients (1 lung and 2 heart and lung transplant recipients), subsequently these patients were excluded from the study.

The patients were divided into two groups: lung transplant only (n=24) and heart and lung transplant group (n=16). The recipient age, recipient gender, donor gender and time after transplantation were significantly different between the two groups, Table 6.4.

Recipients' serum samples were obtained at a single time point after transplantation and the timing was variable between patients in each group. The average time between transplantation and blood sampling was significantly shorter in the lung transplant group in comparison to heart and lung transplant groups (4.2 vs 9 years, respectively, p=0.023). All transplant recipients received comparable immunosuppression. However, the choice of immunosuppressive agents reflects a shift towards tacrolimus in more recent years. Table 6.4 shows detailed presentation of the recipient and donor demographics.

Table 6.4 Retrospective study cohort recipient and donor characteristics

	Lung transplant recipients (n=24)	Heart and lung transplant recipients (n=16)	*p* Value
Recipient age (Median ± SD)	54 ± 11.7	31 ± 10.7	0.001
Recipient sex (M:F)	17:7	5:11	<0.001
Donor age (Median ± SD)	42 ± 14.2	41 ± 13.9	*ns*
Donor sex (M:F)	14:10	5:11	0.045
Donor type			
DBD	22	16	*ns*
DCD	2	0	
Recipient-donor age difference (Median ± SD)	10.5 ± 15.1	16.5 ± 8.2	*ns*
Indication for transplantation			
Cystic fibrosis	5	8	
Histocytosis	1	-	
Emphysema	8	-	
Pulmonary fibrosis	4	-	N/A
Bronchiectasis	1	1	
A1AD	2	-	
Pulmonary hypertension	3	3	
Congenital heart disease	-	2	
Eisenmenger syndrome	-	2	
Years after transplantation			
Mean	4.2	9.0	0.023
Range (years)	(1-12 years)	(1-21 years)	
HLA mismatches			
≤3	8	4	*ns*
≥4	16	12	

DBD – Donation after brain steam death; DCD – Donation after cardiac death; M – male; F – female; A1AD – α1 antitrypsin deficiency; HLA – Human leukocyte Antigen.

6.2.2.1 Incidence of pre- and post-transplant HLA alloantibody in lung and heart and lung transplant recipients

The recipients' pre-transplant and post-transplant serum samples were assayed for detection and characterisation of HLA alloantibody using Luminex LABScreen® Mixed detection beads and LABScreen® Single Antigen HLA Class I and Class II antibody beads, respectively, Chapter 2, Section 2.5.1.

Of the 25 patients that received lung transplant; pre-transplant serum sample was available for 24 patients; one patient was excluded from the analysis. Six patients (25%) were sensitised to HLA antigens prior to transplantation; 5 patients had pre-transplant class I HLA alloantibody and one patient had class II HLA alloantibody; all HLA alloantibodies detected were non-DSA.

Analysis of the sera obtained after transplantation did not indicate development of *de novo* HLA alloantibody. Only 4 patients with pre-existing non-DSA remained positive after transplantation. Additional specificities to HLA antigens were detected in all patients. Only one patient developed *de novo* class II non-DSA alloantibody and one patient developed class I and class II DSA with specificity to donor mismatched B8 and DQ2 antigens. In two patients with pre-existing HLA alloantibodies, the alloantibodies were no longer detectable in the post-transplant serum sample. Circulating HLA alloantibodies were not detected in the pre- and post-transplant sera of eighteen lung transplant recipients, Figure 6.3.

In this cohort eighteen patients underwent heart and lung transplantation. Pre-transplant serum samples were available for 16 patients; subsequently, two patients were excluded from further analysis. Of these, only one patient had pre-transplant class II non-DSA alloantibody, a change in the HLA specificity was not observed in the test samples obtained after transplantation. The remaining 15 patients did not have detectable HLA alloantibody in either the pre- or post-transplant serum samples, Figure 6.3.

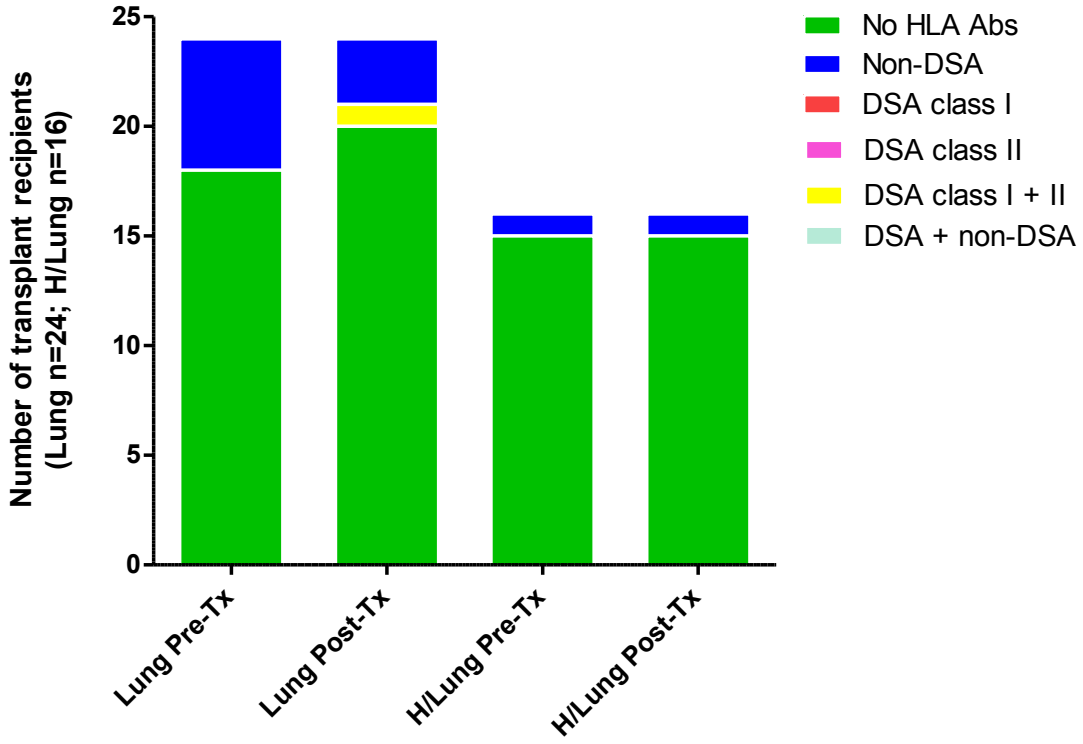


Figure 6.3 HLA sensitisation in lung and heart and lung transplant recipients before the transplant and after transplantation. The graph shows the number of lung and lung and heart (H/Lung) transplant recipients sanitised to HLA antigens prior to transplantation. Development of *de novo* HLA antibody was assessed in the recipients' sera obtained at one time point after transplantation. Sera were tested for the presence of both donor specific antibody (DSA) and non-donor specific antibody (non-DSA).

6.2.2.2 Incidence of pre- and post-transplant anti-nuclear autoantibody in lung and heart and lung transplant recipients

Autoantibody responses were assessed by testing the recipients' serum for the presence of IgG autoantibody with specificity for nuclear antigens using two commercially available methodologies, HEp-2 Indirect Immunofluorescence Assay (IIF) and HEp-2 Enzyme-Linked Immunosorbent Assay (ELISA), previously described in Chapter 2, Section 2.5.2. HEp-2 cells are a non-specific but highly sensitive substrate for detection of anti-nuclear autoantibody.

The advantage of the HEp-2 cells IIF assay is that the cells are grown as a monolayer on microscopic slides, thus allowing detection of autoantibody with specificity to nuclear antigens that are expressed only at a particular stage of the HEp-2 cell cycle. In comparison, the HEp-2 ELISA contains HEp-2 cell extract (lysed cells) as a substrate; this methodology offers availability of total cellular components.

Indirect immunofluorescent staining of the HEp-2 cells revealed weak sera reactivity to anti-nuclear antigens in the majority of patients in both groups, Figure 6.4. Interestingly, overall the anti-nuclear IgG autoantibody levels were higher in the pre-transplant sera in comparison to the sera obtained after transplantation. The difference between the pre- and post-transplant sera was not significant in the recipients that received lung transplant only (Paired t test, p=0.541). In this test group a massive increase in the IgG autoantibody titres in the sera obtained after transplantation was observed in one patient, Figure 6.4 A. In the heart and lung transplant group the anti-nuclear IgG autoantibody levels were significantly decreased in the sera obtained after transplantation in comparison to the pre-transplant sera (Paired t test, p=0.031), Figure 6.4 B.

In addition, HEp-2 IIF assay revealed various nuclear and cellular staining patterns, suggesting that the IgG autoantibody were directed to multiple nuclear and/or cellular targets. The staining patterns were different between patients and in several patients, different staining patterns were detected in the pre- and post-transplant sera; suggesting epitope spreading, development of *de novo* IgG autoantibody or increase in the IgG titre. In some patients IgG autoantibody with specificity to multiple nuclear and/or cellular antigens was observed, characterised by the presence of mixed staining pattern.

Similar findings were observed in the HEp-2 ELISA assay; overall the IgG alloantibody titres were lower in the sera obtained after transplantation in comparison to the pre-transplant serum samples. The IgG autoantibody levels were significantly decreased in the sera obtained after transplantation in both groups; lung transplant recipients (Figure 6.5 A) and lung and heart transplant recipients (Figure 6.5 B), $p<0.001$ and $p=0.004$, respectively.

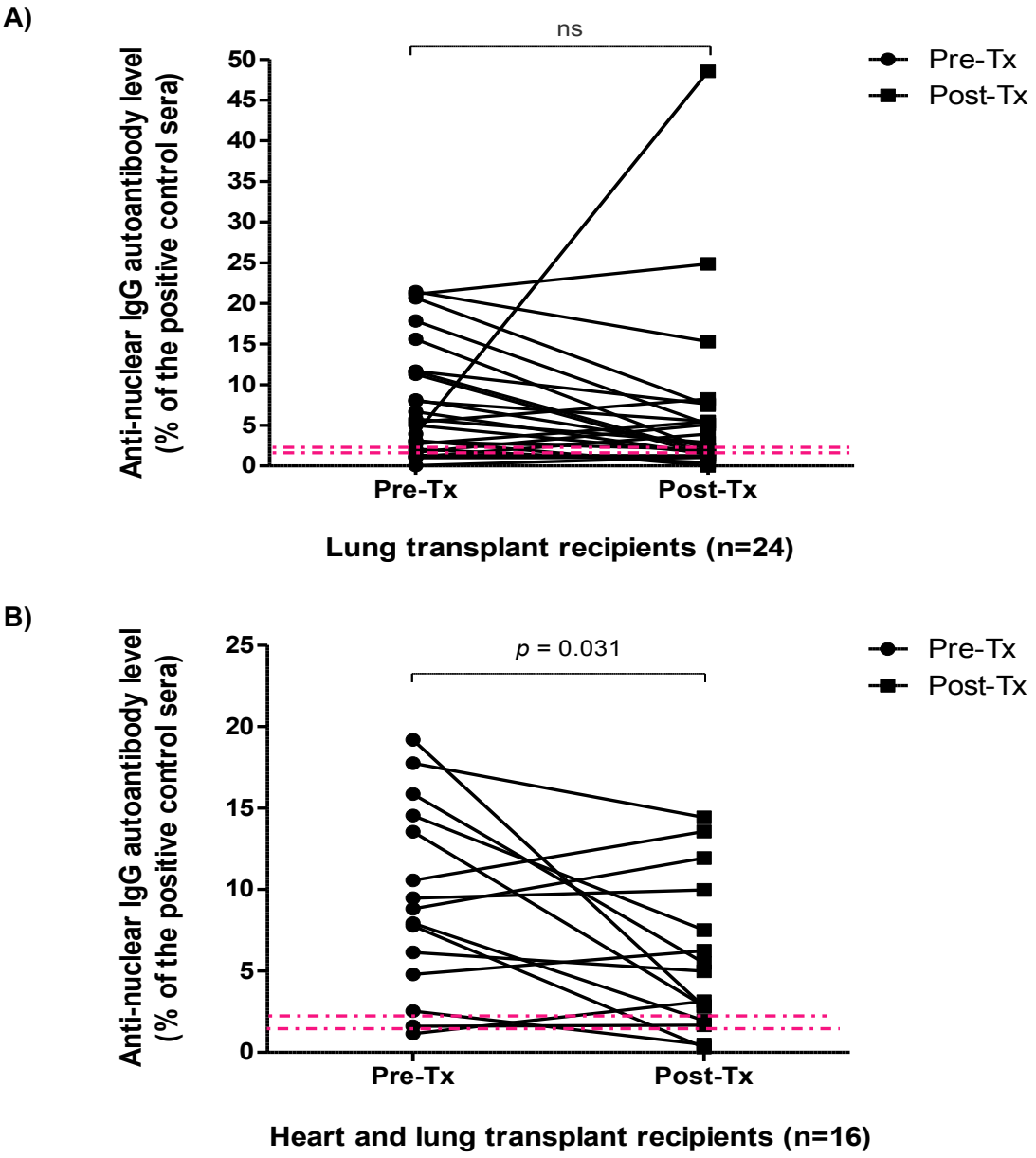


Figure 6.4 Anti-nuclear IgG autoantibody level in lung and heart and lung transplant recipients detected using HEp-2 Indirect Immunofluorescence assay. The graphs show the anti-nuclear IgG autoantibody titres present in the recipients' pre-transplant and post-transplant sera obtained at one time point after transplantation (variable between patients and groups). The data is presented as a percentage of the positive control sera (human positive control serum supplied by the manufacture) Recipients' sera reactivity to anti-nuclear antigens was stronger in the sera obtained prior to transplantation. A) Significant decreases in the anti-nuclear IgG autoantibody titres were not observed between the pre- and post-transplant sera (p=*ns*; paired t test) in the lung transplant recipients; B) Significant decreases in IgG anti-nuclear autoantibody titres was observed between the pre- and post-transplant serum samples in the lung and heart transplant recipients (p=0.031; paired t test). Paired t test was used to calculate significance. *P*-value of <0.05 was considered statistically significant. Red broken-lines show negative control sera cut-off values (pooled human serum from seven healthy individuals); represented as a mean ± 2SD.

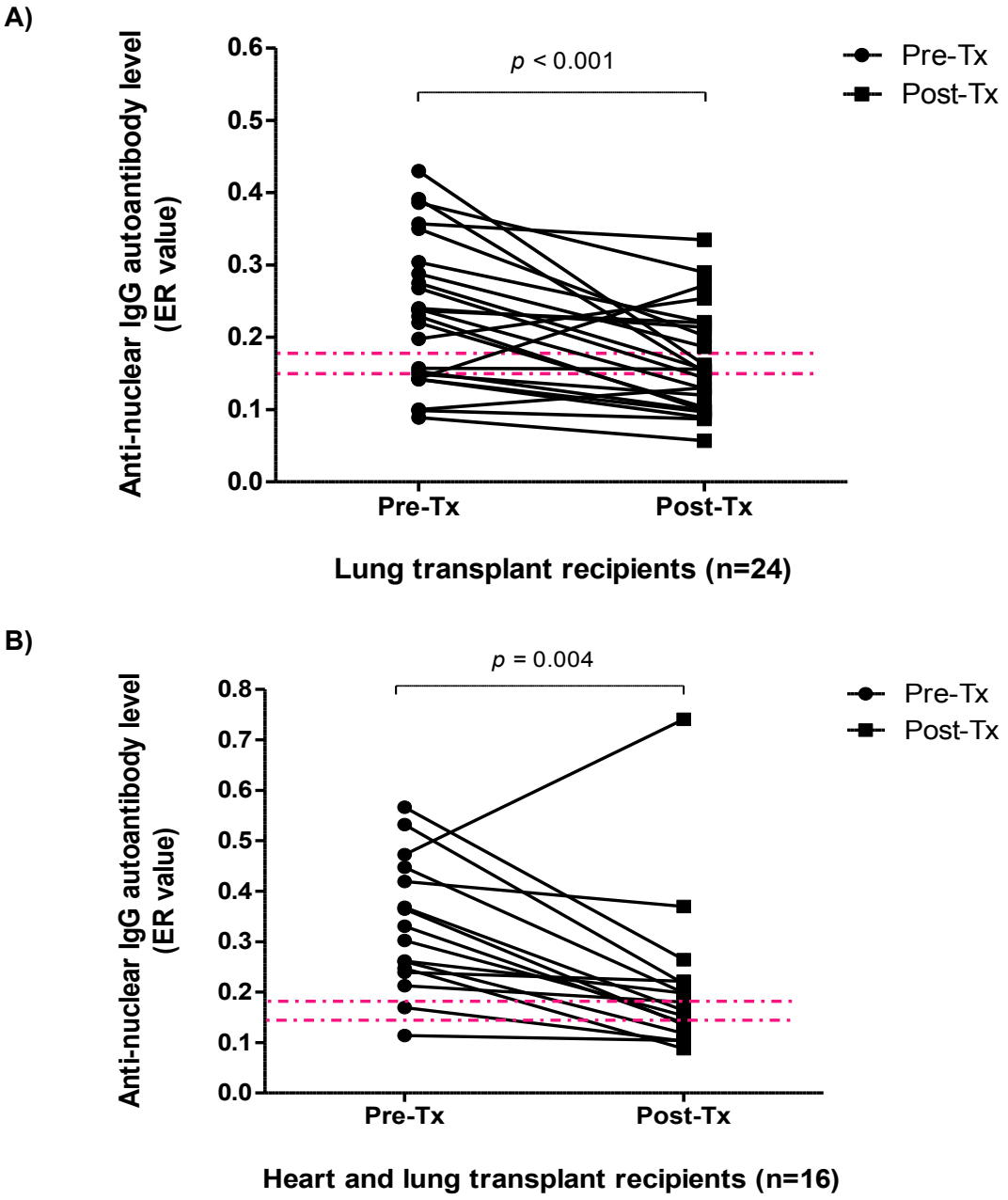


Figure 6.5 Anti-nuclear IgG autoantibody level in lung and heart and lung transplant recipients detected using HEp-2 Enzyme-Linked Immunosorbent (ELISA) assay. The graphs show the anti-nuclear IgG autoantibody titres present in the recipients' pre-transplant and post-transplant sera obtained at one time point after transplantation (variable between patients and groups). The data is presented as an ELISA ratio (ER). ER equals the OD* value of the test sample minus the OD* value of the negative control divided by the OD* value of the positive control minus the OD* value of the negative control. Significant decreases in the anti-nuclear IgG autoantibody titres were observed between the pre- and post-transplant sera A) Lung transplant recipients (p<0.001; paired t test); and, B) Heart and lung transplant recipients (p=0.004; paired t test). Paired t test was used to calculate significance. *P*-value of <0.05 was considered statistically significant. Red broken-lines show negative control sera cut-off values (pooled human serum from seven healthy individuals); represented as a mean ± 2SD. *OD – optical density.

6.2.2.3 Correlation between anti-nuclear autoantibody and BOS development

Nonparametric correlation analysis revealed that the presence of anti-nuclear IgG autoantibody detected by both HEp-2 IIF (Figure 6.6 A) and ELISA Figure 6.6 B) did not correlate with development of BOS in lung and heart and lung transplant recipients (n=40). Spearman r test was used for correlation analysis.

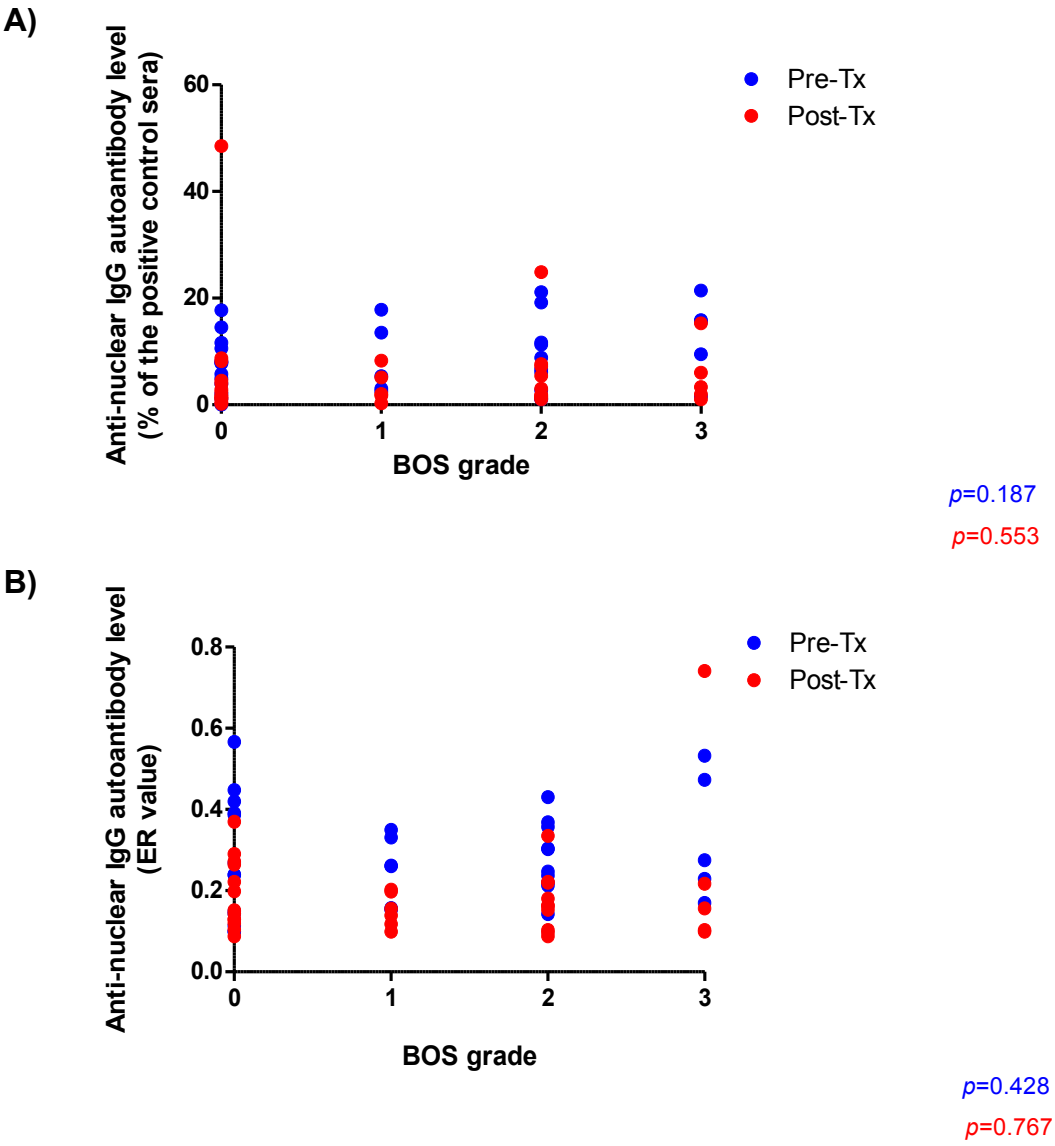


Figure 6.6 Correlation between the level of anti-nuclear IgG autoantibody and development of BOS in lung and heart and lung transplant recipients. A) Level of anti-nuclear IgG autoantibody detected by Hep-2 IIF assay in pre- and post-transplant sera of lung and heart and lung transplant recipients (n=40); B) Level of anti-nuclear IgG autoantibody detected by ELISA assay in pre- and post-transplant sera of lung and heart and lung transplant recipients (n=40). Spearman r test was used for correlation analysis. *P*-value of <0.05 was considered statistically significant.

6.2.2.4 Correlation between anti-nuclear autoantibody and recipient age

Nonparametric analyses revealed that the level of anti-nuclear IgG autoantibody detected by ELISA correlated with the recipient age at the time of transplant (6.7 A, *p*=0.023). This correlation was not observed when anti-nuclear IgG autoantibody was measured using HEp-2 IIF (6.7 B, *p*=0.509).

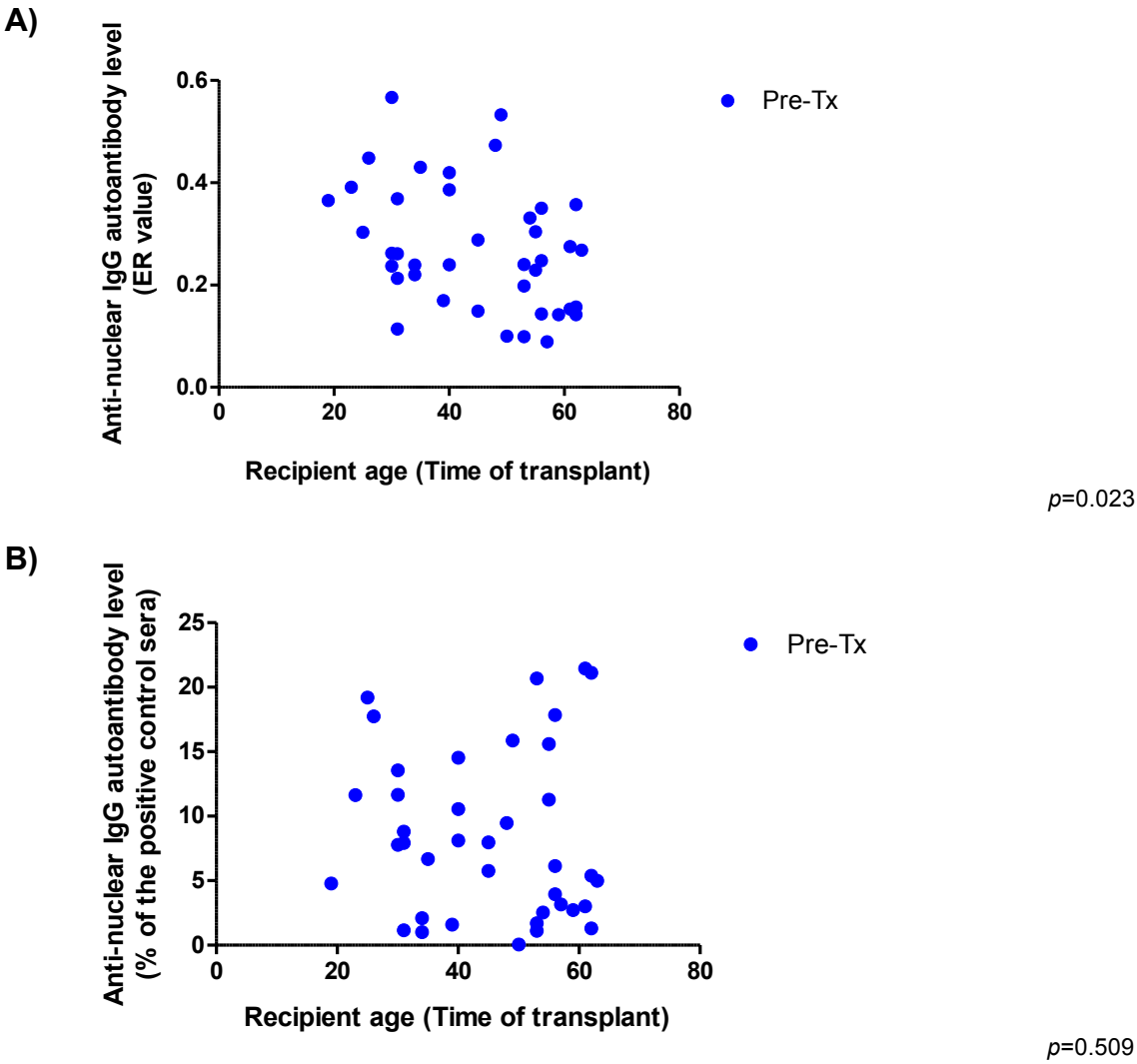


Figure 6.7 Correlation between the level of anti-nuclear IgG autoantibody and recipient age at the time of lung or heart and lung transplant. A) Level of anti-nuclear IgG autoantibody detected by Hep-2 IIF assay (n=40); B) Level of anti-nuclear IgG autoantibody detected by ELISA assay (n=40). Spearman r test was used for correlation analysis. *P*-value of <0.05 was considered statistically significant.

6.2.3 Evaluation of the HEp-2 Indirect Immunofluorescence assay

The HEp-2 IIF and HEp-2 ELISA were performed according to the manufacturer's recommendation using serum dilutions of 1:40 and 1:100, respectively, Chapter 2, Section 2.5.2 and Section 2.5.3. However, according to the guidelines for laboratory use of IIF tests to aid diagnosis of systemic autoimmune diseases, it is recommended that titres lower or equal to 1:40 should be considered as negative, titres between 1:40 and 1:80 are weak positive and titres greater than 1:160 are considered positive.

To comply with these recommendations [322], five serum samples with strong positive HEp-2 staining pattern were re-tested using serum serial dilutions of 1:40, 1:80 and 1:160. Interestingly, all serum samples lost the reactivity to HEp-2 cells with dilution factor of 1:160.

6.2.4 Identification of autoantibody signature profile in lung plus heart and lung transplant recipients using protein microarray

To expand the screening strategy, autoimmune responses were evaluated in lung plus heart and lung transplant recipients using a protein microarray platform (Invitrogen Human ProtoArray® v5.0 protoarray). This platform contains 9480 human recombinant proteins, previously described in Chapter 2, Section 2.5.4. I reasoned that the application of a large protein panel to unveil autoantibody specificities may lead to characterisation of novel sets of "biomarkers", which could prompt new screening strategies to monitor and improve allograft survival in lung transplant recipients.

The humoral autoimmune responses were assessed in a sub-cohort of 20 lung and heart and lung transplant recipients with (n=10 Grade 2 and 3 BOS: ISHLT scoring criteria, Table 1.4) and without (n=10, No BOS group) established BOS (the sub-cohort was selected from the primary retrospective cohort of 24 lung and 16 heart and lung transplant recipients). Humoral autoimmune responses were assessed at two time points: at the time of transplantation and after transplantation. The post-transplant serum sample was obtained at different time points after transplantation, ranging from 1 to 11 years for patients free from BOS and from 2 to 20 years for patients with established BOS.

Table 6.5 shows the recipients' demographic characteristics; recipient and donor age, gender, and type of organ transplanted were comparable between the two groups. Circulating HLA alloantibody was not detected in the pre- and post-transplant sera in either group. Four patients in the BOS group had experienced a single episode of acute cellular rejection in the first six months after transplantation in comparison to only two patients in the No BOS group. Time of blood samples (years after transplantation), the number of HLA mismatched antigens and indication for transplantation were comparable between the two groups (Table 6.5).

Table 6.5 Recipient and donor characteristics (Sub-cohort for identification of autoantibody profile in lung transplant recipients with established BOS and recipients without BOS)

	BOS (n=10)	No BOS (n=10)	*p* Value
Recipient age (Median ± SD)	43.5 ± 11.7	40 ± 10.6	*ns*
Recipient sex (M:F)	5:5	4:6	*ns*
Donor age (Median ± SD)	48.5 ± 12.5	35.5 ± 14.8	*ns*
Donor sex (M:F)	4:6	4:6	*ns*
Transplanted organ			
Lung	5	6	*ns*
Heart and lung	5	4	
Indication for transplantation			
COPD	3	3	
Cystic Fibrosis	3	4	
Idiopathic Pulmonary Hypertension	2	1	*ns*
Idiopathic Pulmonary Disease	1	0	
Congenital Heart Disease	1	2	
Pre-transplant HLA alloantibody	0/10	0/10	*ns*
Post-transplant HLA alloantibody	0/10	0/10	*ns*
Rejection episodes*	4/10	2/10	*ns*
Years after transplantation			
Mean	7.6	6	*ns*
Range	(2-15 years)	(1-20 years)	
HLA mismatch antigens			
≤3	2	3	*ns*
≥4	8	7	*ns*
BOS Grade			
0	0	10	
1	0	0	<0.001
2	7	0	
3	3	0	

M – male; F – female; COPD – Chronic Obstructive Pulmonary Disease; HLA – Human Leukocyte Antigen; BOS – Bronchiolitis Obliterans Syndrome; * Cell-mediated rejection.

6.2.4.1 Optimisation of the protein microarray scanning settings

In order to maximise the protein microarray fluorescent signal (discovery of "true" positive reactions), while minimising the false protein discovery rate, the protein microarray scanning setting were first optimised using negative control arrays.

Two arrays were probed with buffer containing no serum, followed by the incubation with secondary Alexa Fluor® 647 Goat Anti-Human IgG detection antibody. Scanning was performed using GenPix® 4000B scanner photomultiplier power (PMT) at three different optimization settings: PMT 600, PMT 700 and PMT 800 V (Figure 6.8) (detailed in Chapter 2, Section 2.5.4.2.).

The Alexa Fluor® conjugated antibody imprinted onto the arrays was used as a guide for correct positioning of each spot (imprinted protein) and indicates that the array has been scanned correctly.

The protein microarrays contain internal control proteins that serve as a guide for correct assignment of positive signals. A protein gradient of goat anti-human IgG is printed onto each sub-array; binding of the human IgG to the goat anti-human IgG generates a fluorescent signal that serves as a reference to verify correct probing. As recommended by the manufacturer, the signal obtained from the goat anti-human IgG imprinted at a highest concentration has to be just below the maximum saturation, Figure 6.9. The optimum near to saturation signal for the goat anti-human IgG was obtained with PMT of 800 V, Figure 6.8 C. Subsequently, the experimental microarrays probed with patients' serum samples were scanned under the same scanning conditions.

In the negative control arrays positive signal was obtained for 95 proteins (<1%) spotted onto the microarrays. These proteins were excluded from subsequent analysis.

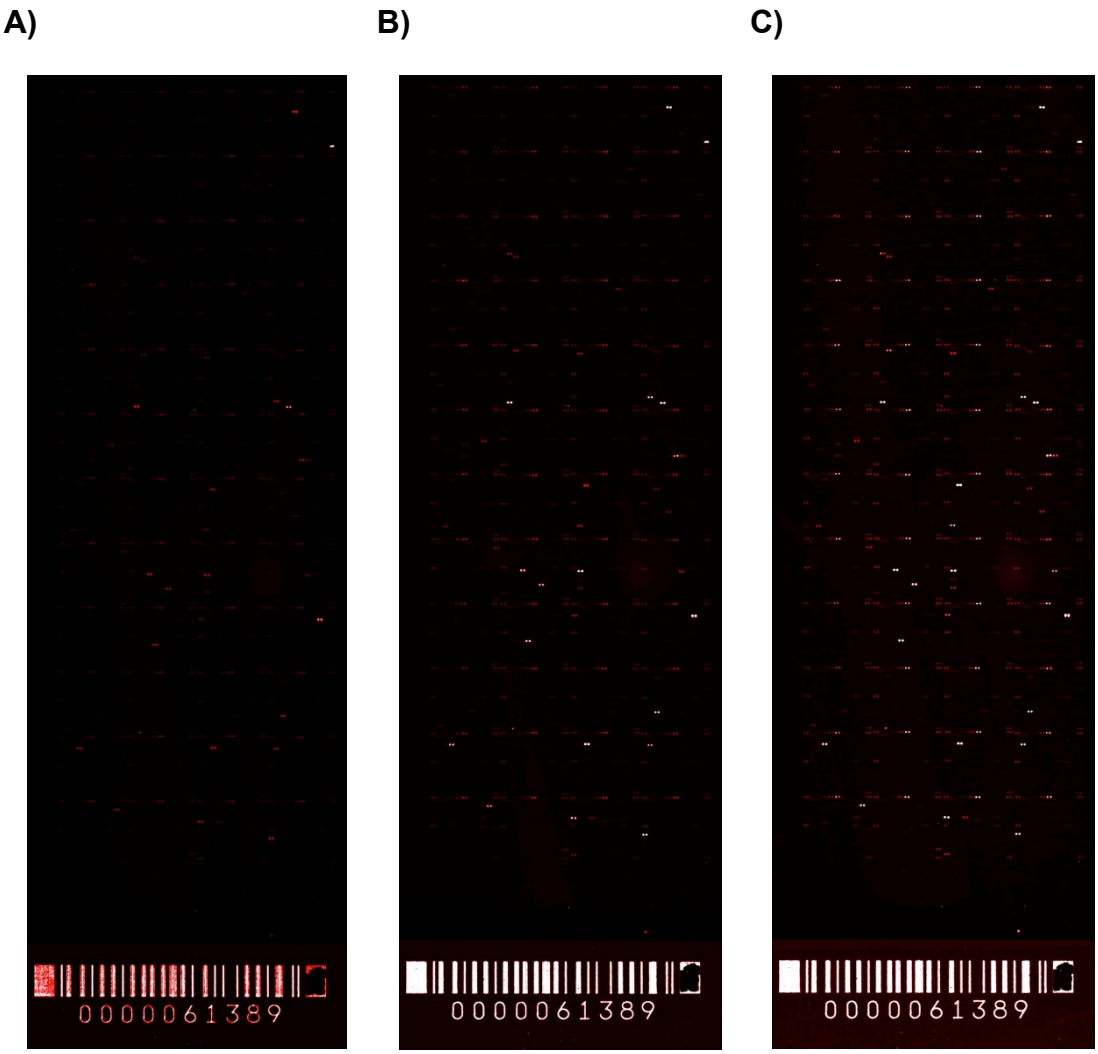


Figure 6.8 Optimisation of the protein array scanning settings. Two negative control protoarrays were tested for optimisation of GenPix® 4000B scanner photomultiplier power (PMT) performance settings. The protoarrays were probed with buffer containing no serum, prior to the incubation with Alexa Fluor® 647 Goat Anti-Human IgG detection antibody. Scanning was performed using three different settings: A) PMT 600 V, B) PMT 700 V, C) PMT 800 V. To maximise the signal intensity recorded, the human IgG signal printed on the protoarray at a highest concentration has to be near to saturation. An optimum near to saturation signal was obtained with PMT of 800 V as showed in C. The experimental protoarrays probed with human test sera were scanned under same scanning conditions.

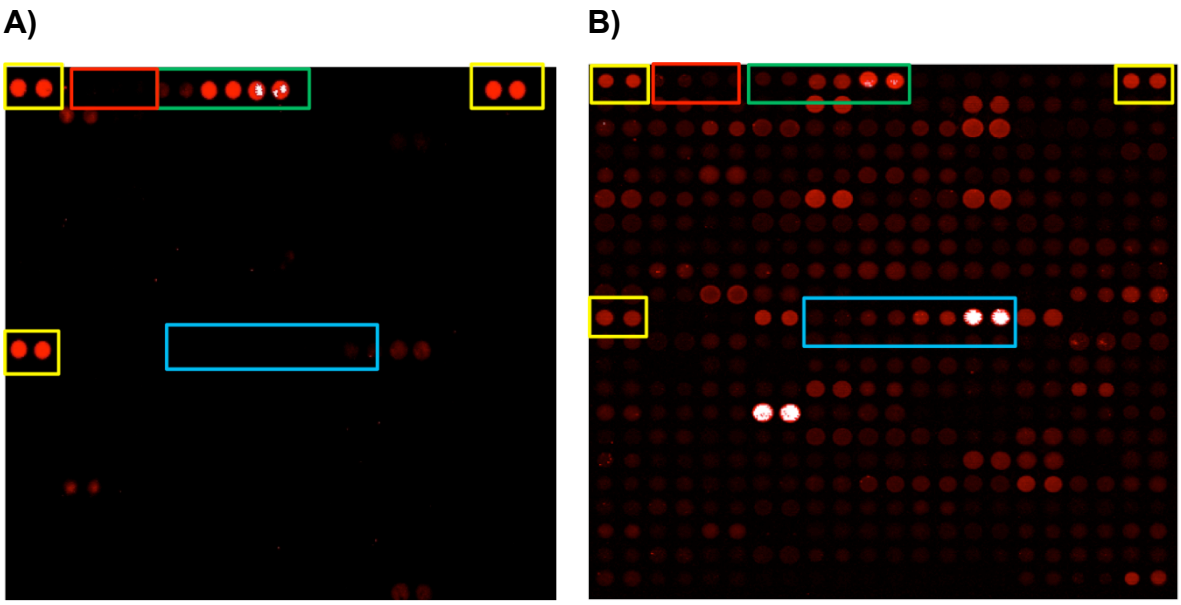


Figure 6.9 Visual schematic representative image of sub-array internal control proteins. The protoarray contain 9480 proteins imprinted in duplicates. The proteins are printed in 110μm spots arrayed in 48 sub-array (4400-μm^2 each) and equally spaced in vertical and horizontal directions with 22 columns and 22 rows per sub-array. Each sub-array contains control proteins. Control proteins provide reference points for data acquisition and analysis. This graph shows one sub-array containing 22 columns and 22 rows and the internal control proteins. A) Negative control assay, the protoarray was probed with buffer containing no serum, B) Experimental assay; the protoarray was probed with patient serum sample. Protoarray positive controls are highlighted: *yellow,* Alexa Fluor® conjugated antibody signal indicates that the protoarray has been properly scanned and serves as an indicator for correct positioning of the protoarray; *green*, Anti-human IgG signal used for verification of proper probing and detection reagents; *blue*, Human IgG Signal serves as a positive control when anti-human IgG is used for detection and *red,* protoarray negative controls (BSA and Buffer).

6.2.4.2 Characterisation of autoantibody signature profile in lung transplant recipients with established BOS and recipients free from BOS

To characterise the IgG autoantibody signature profile present at the time of transplant and after transplantation in patients with established BOS (n=10) and patients free from BOS (n=10) the data obtained from the protein microarray assay was analysed with the ProtoArray® Prospector software provided by Invitrogen (detailed in Chapter 2, Section 2.5.4.4). Figure 6.10 shows a representative image of the protein array probed with pre- transplant A) and post-transplant B) sera form a patient with established BOS.

Data analysis is a 3-step process and involves single array analysis, group characterisation and two-group comparison analysis. Single array analysis was performed individually for each test sample using the manufacture recommendations; positive probes or hits were setup to a cutoff value of Z-Score > 3 and Z-Factor ≥ 0.4. The relative fluorescent unit (RFU) for each protein is normalised against the background staining, and positive hits are identified based on the significant difference between the RFU value of individual protein and the negative controls.

The number of proteins identified as positive hits varied between patients in both groups. In the BOS group, the number of positive hits ranged from 333 hits (min., 3.55%) to 2892 hits (max., 30.82%) and 383 hits (min., 4.08%) to 2819 hits (max., 30.04%), for the pre- and post-transplant sera, respectively, Table 6.6. Similar findings were observed in the patient group free from BOS; the number of positive hits in the pre-transplant sera ranged from 716 hits (min., 7.63%) to 3098 hits (max., 33.01%); and, in the post-transplant sera the number of positive hits ranged from 823 (min., 8.77%) to 2302 hits (max., 24.53%). Table 6.6 and Table 6.7 show the number and percentage of proteins that were identified as positive in the recipients' pre- and post-transplant sera.

Table 6.6 Number and percentage of proteins identified as positive hits in lung transplant recipients with established BOS

Patient ID (BOS Group)	No. of positive hits (pre-transplant sera)	% of protoarray positive hits* (pre-transplant sera)	No. of positive hits (post-transplant sera)	% of protoarray positive hits* (post-transplant sera)
P1	333	3.55	2819	30.04
P2	829	8.83	1557	16.50
P3	960	10.23	1290	13.75
P4	980	10.44	383	4.08
P5	1113	11.86	1396	14.87
P6	1420	15.13	1830	19.50
P7	1617	17.23	1225	13.05
P8	2640	28.13	2059	21.94
P9	2745	29.25	1317	14.03
P10	2892	30.82	2024	21.57

*Single array contain 9480 proteins, 95 proteins show reactivity with the negative control assay and were excluded from the analysis; and recipients' IgG autoantibody specificity was assessed for 9385 proteins.

Table 6.7 Number and percentage of proteins identified as positive hits in lung transplant recipients without BOS

Patient ID (No-BOS Group)	No. of positive hits (pre-transplant sera)	% of protoarray positive hits* (pre-transplant sera)	No. of positive hits (post-transplant sera)	% of protoarray positive hits* (post-transplant sera)
P1	716	7.63	1653	17.61
P2	1079	11.50	2059	21.94
P3	1315	14.01	1055	11.24
P4	1360	14.49	823	8.77
P5	1625	17.31	1869	19.91
P6	1971	21.00	1424	15.17
P7	2343	24.97	1964	20.93
P8	2426	25.85	1329	14.16
P9	2821	30.06	2302	25.53
P10	3098	33.01	1334	14.21

*Single array contain 9480 proteins, 95 proteins show reactivity with the negative control assay and were excluded from the analysis; and recipients' IgG autoantibody specificity was assessed for 9385 proteins.

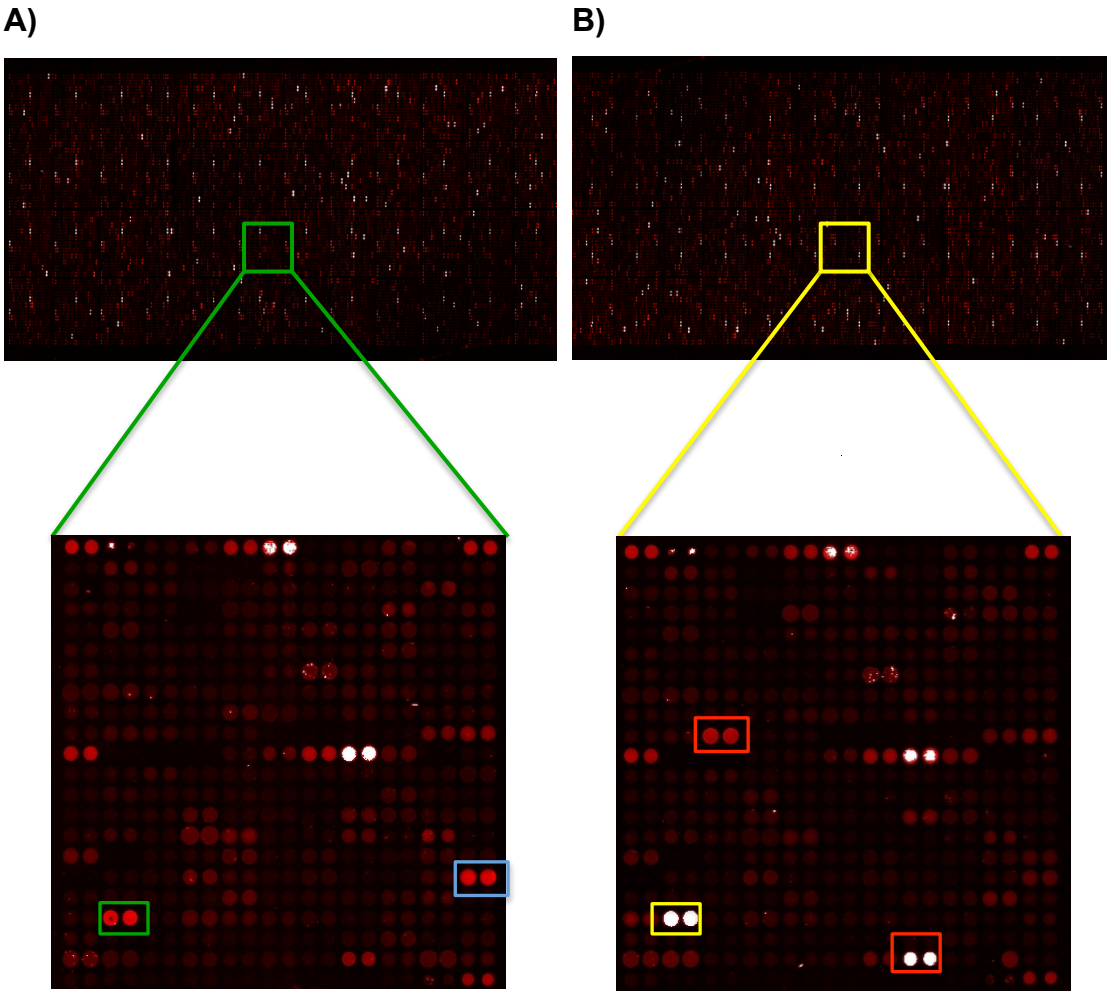


Figure 6.10 Characterisation of IgG autoantibody specificity using protein array methodology. Protoarrays containing 9480 human proteins were probed with pre- and post-transplant sera from patients with established BOS (n=10) and patients free from BOS (n=10). This figure shows representative image of IgG autoantibody reactivity of patient with established BOS. The array was probed with A) sera obtained at the time of transplant and B) sera obtained after transplantation. In both arrays a representative sub-array is enlarged showing the recipient IgG autoantibody reactivity: protein boxed in A) *green* and B) *yellow* show the same protein identified as positive hits in the pre- and post-transplant sera, except that the protein boxed in yellow show stronger reactivity. A) *Blue* boxed protein represents protein that was identified as positive hit in the pre-transplant sera, but is negative in the post-transplant sera; and, B) *Red* boxed proteins identified as positive hits in the post-transplant sera only. Each protein is spotted onto the array in duplicates. ProtoArray® Prospector software (cutoff value of Z-Score > 3 and Z-Factor ≥ 0.4) was used to identify positive proteins or hits. Proteins are scored as positive hits if there is a significant difference in the mean fluorescent intensity (MFI) between the target protein and the negative controls.

Group characterisation was performed according to the manufacturer's recommendations; four groups were characterised: No BOS pre-transplant (n=10), No BOS post-transplant (n=10), BOS pre-transplant (n=10) and BOS post-transplant (n=10). In this analysis step the RFU value for each protein across all samples in each study group are aligned together for downstream analysis using Linear Model with internal reference protein features (positive controls used for normalisation are human IgG and anti-human IgG gradients printed in each sub-array) normalisation method.

Lastly, two-group comparison analyses were performed; this step identifies differences between two groups using M-statistics (data normalisation analysis using Robust Linear Model), counts the proteins more prevalent in the test groups and generates P-Value (P-value of <0.05 was considered statistically significant), previously described in Chapter 2, Section 2.5.4.4.

Two-group comparison analysis was performed between the BOS pre-transplant and No BOS pre-transplant groups and between the BOS post-transplant and No BOS post-transplant groups. One-hundred and thirty eight proteins were identified as more prevalent positive hits in the BOS pre-transplant sera in comparison to the No BOS pre-transplant sera, ($p < 0.5$), whereas in the No BOS pre-transplant sera 787 proteins were identified as more prevalent targets when compared to BOS pre-transplant sera, ($p < 0.5$); interestingly, none of these proteins overlapped between both groups, Figure 6.11.

Comparison analysis of the autoantibody profile in the sera obtained after transplantation between the patients with BOS and patients without BOS revealed 316 protein positive hits were more prevalent in patients with BOS; and, 303 proteins were more prevalent in the No BOS group, ($p < 0.5$), Figure 6.11.

To illustrate the number of proteins identified as positive hits in both groups of patients for the pre- and post-transplant sera, a Venn diagram was generated using R 2.9.2 software for statistical computing and graphics; in addition, we assessed whether the protein positive hits were present specifically in one test group or whether they were shared between the patient groups and/or test samples.

The results revealed that IgG autoantibodies with specificity to 170 proteins were present at the time of transplantation in the patients free from BOS (No BOS pre vs No BOS post) and remained positive in the test sample obtained after transplantation, Figure 6.11. Autoantibody specific for eight proteins were found to be present in the pre-transplant sera of patients without BOS and the post-transplant sera of the patients with established BOS. Analysis of the patients with established BOS revealed the presence of IgG autoantibody with specificity to 44 proteins in both sera obtained at time of transplant and after transplantation.

Interestingly, shared IgG autoantibody profile was not observed between the patients with BOS and patients free from BOS in the sera obtained at the time of transplant and after transplantation (Figure 6.11), suggesting a unique autoantibody signature profile may influence the development of BOS after transplantation.

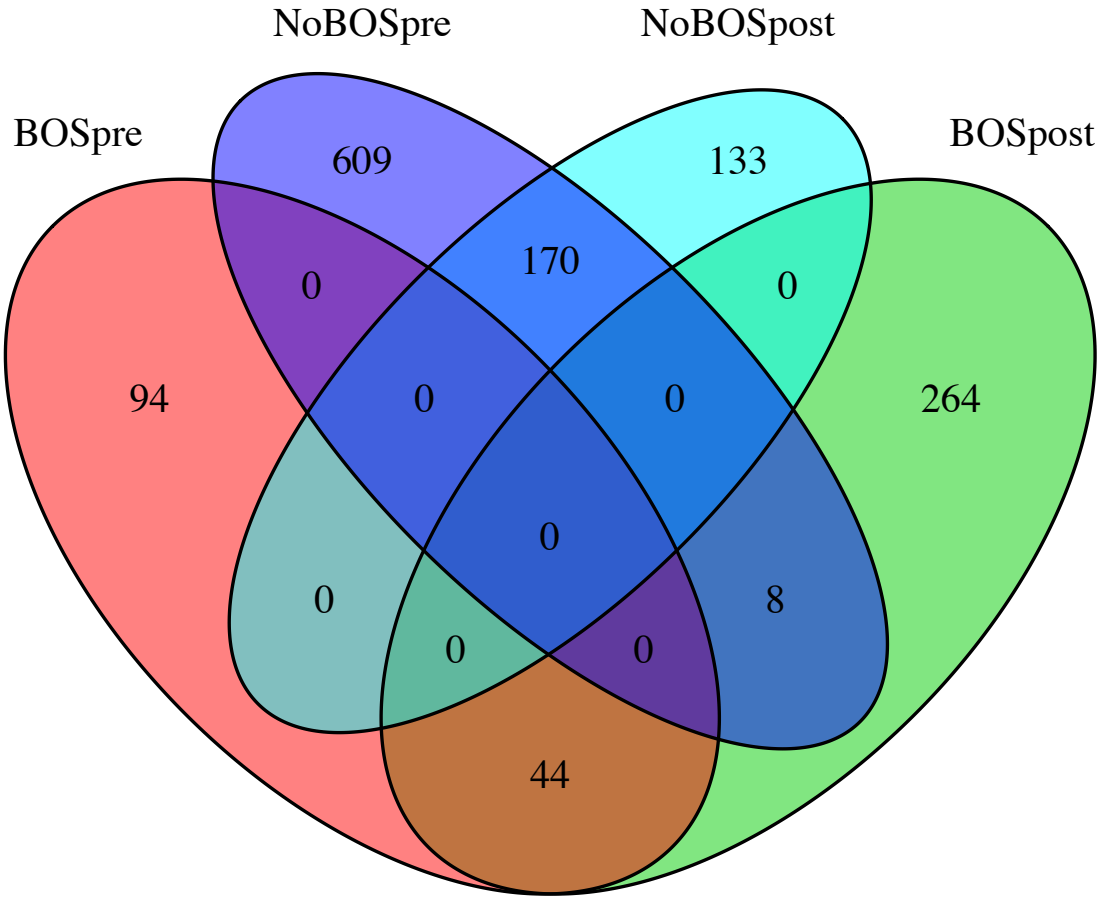


Figure 6.11 Venn diagram illustrating the number of proteins identified as positive hits in sera obtained at the time of transplant and after transplantation in patients with established BOS and patients free from BOS. Two-group comparison analysis was performed using ProtoArray® Prospector software. The autoantibody profile present in sera obtained at the time of transplant and after transplantation was compared between patients with BOS and patients without BOS (BOSpre vs NoBOSpre and BOSpost vs NoBOSpost). 138 proteins were more prevalent in the BOSpre group, 787 proteins were more prevalent in the NoBOSpre, 316 proteins were more prevalent in the BOSpost and 303 proteins were more prevalent in the NoBOSpost group, p-value < 0.05. The Venn diagram was generated using R 2.9.2 software for statistical comuting and graphics. P-Value of < 0.05 was considered statistically significant.

In addition, a protein clustering analysis for the proteins identified as positive hits (n=1322, p-value of < 0.05) was performed to assess the autoantibody repertoire in the patients with established BOS and patients without BOS. Protein clustering is used to to construct group of similar proteins to be used for analysis. Heat maps with dendograms graphical representation was generated for the protein clustering analysis using R 2.9.2 software for statistical computing and graphics.

The clustering analyses highlighted distinct patterns between the patients with established BOS and patients without BOS. Figure 6.12 A and B shows the cluster analysis of patients with BOS and patients without BOS for both sera obtained at the time of transplant (Figure 6.12 A) and sera obtained after transplantation (Figure 6.12 B).

The autoantibody repertoire present at the time of transplantation revealed two distinct clusters between the patients that developed BOS and patients that remained free from BOS after transplantation, suggesting that the autoantibody repertoire present at the time of transplantation may influence development of BOS. Conversely, a “unique” autoantibody repertoire was observed in the sera obtained after transplantation in both groups of patients, Figure 6.12.

In addition, we assessed the autoantibody repertoire in the pre- and post-transplant sera in the patients with BOS alone. The clustering analyses show two distinct autoantibody patterns and most of the proteins identified as positive targets were present at the time of transplantation, signifying that the pre-transplant autoantibody repertoire may correlate with the development of BOS after transplantation, Figure 6.13.

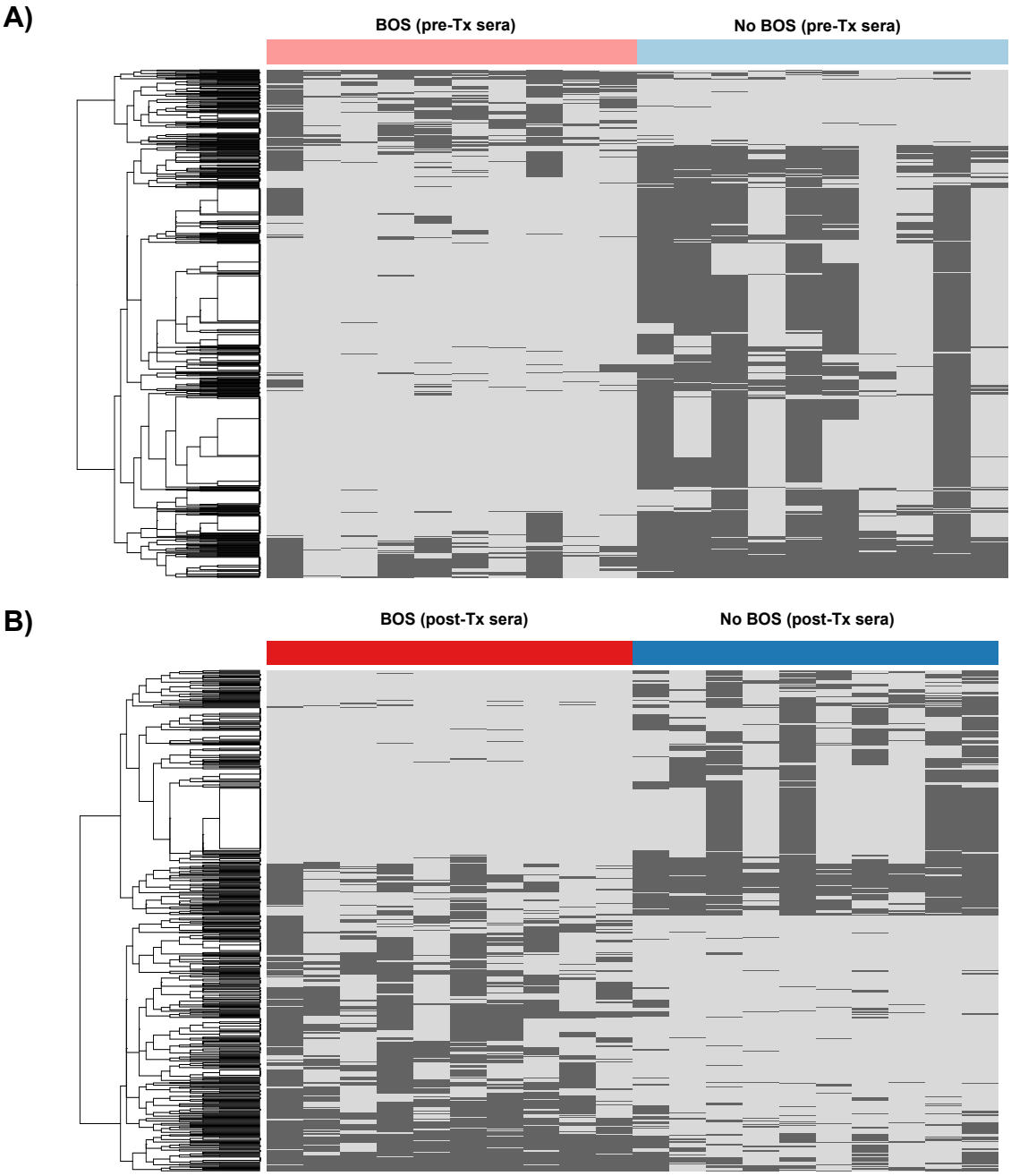


Figure 6.12 Heat maps of autoantibody repertoire detected in patients with BOS and patients without BOS at the time of transplant and after transplantation. The heat maps show the total number of proteins identified as positive according to calculated prevalence with p-value of < 0.05. Each column represents individual serum sample and the rows are protein targets that were identified as positive in each serum sample. A) Autoantibody repertoire present at the time of transplantation in patients with established BOS (BOS pre-Tx, n=10; pale red) and patients without BOS (No BOS post-Tx, n=10; pale blue); B) Autoantibody repertoire present after transplantation in patients with established BOS (BOS post-Tx, n=10; dark red) and patients without BOS (No BOS post-Tx, n=10; dark blue). Cluster analysis was performed using R 2.9.2 software for statistical computing and graphics. P-Value of < 0.05 was considered statistically significant.

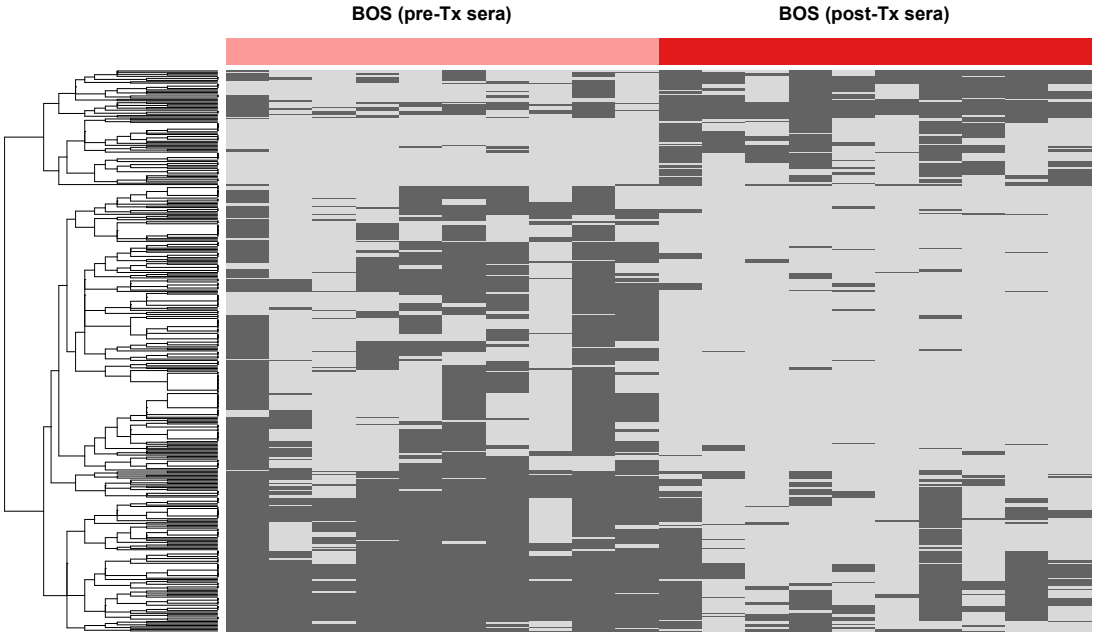


Figure 6.13 Heat map of autoantibody repertoire present in patients with BOS at the time of transplant and after transplantation. The heat map shows the total number of proteins identified as positive according to calculated prevalence with p-value of < 0.05. Each column represents individual serum sample and the rows are protein targets that were identified as positive in each serum sample. The heat map shows the autoantibody repertoire in patients with BOS present at the time of transplantation (BOS pre-Tx, n=10; pale red); and, the autoantibody repertoire present after transplantation (BOS post-Tx, n=10; dark red). Cluster analysis was performed using R 2.9.2 software for statistical computing and graphics. P-Value of < 0.05 was considered statistically significant.

6.2.4.3 Classification of the protein of interest

The purpose of utilising a large number of protein targets for characterising the autoantibody repertoire was to identifying specific protein targets that may serve as novel sets of “biomarkers” to aid and improve patient management.

The clustering analyses highlighted two distinct patterns between test patients with established BOS and patients without BOS, and the presence of “unique” autoantibody patterns in the test sera obtained at the time of transplantation. These autoantibody repertoires appear to indicate patients that will either subsequently develop BOS or that will remain free from BOS.

To narrow down the number of proteins identified as targets for autoantibody development, I selected the proteins with the highest prevalence in patients with established BOS. At the time of transplantation, eight proteins were identified as positive in the BOS group, with a prevalence of >65%, such that at least 7/10 patients in the BOS group had autoantibody with specificity to these proteins; in contrast only one patient in the No BOS group exhibited these autoantibody specificities. Analysis of the protein relative fluorescence units (RFU) revealed that the difference in the RFU was statistically significant ($p<0.05$) only for five proteins, Figure 6.14.

Table 6.8 lists the proteins identified as positive with highest prevalence in patients with established BOS (database ID, description of the protein and protein function). Figure 6.14 shows the IgG autoantibody levels in the patients with established BOS and patients without BOS.

Table 6.8 List of proteins identified as positive with highest prevalence in patients with established BOS.

No	Database ID	Description of the protein	Protein function
1	NM_001659.1	ADP-ribosylation factor 3 (ARF3)	Involved in protein trafficking; may modulate vesicle budding and un-coating within the Golgi apparatus.
2	NM_175634.1	Protein CBFA2T1	Protein CBFA2T1 is a transcriptional co-repressor, which facilitates transcriptional repression via its association with DNA-binding transcription factors and recruitment of other co-repressors and histone-modifying enzymes.
3	NM_006949.1	Syntaxin-binding protein 2	Involved in intracellular vesicle trafficking and vesicle fusion with membranes. Regulates cytotoxic granule exocytosis in natural killer (NK) cells.
4	NM_004349.2	Runt-related transcription factor 1; translocated to, 1 (cyclin D-related) (RUNX1T1), transcript variant 1	DNA binding transcription factor that regulates expression of numerous genes. Chromosomal translocation results in a fusion protein that down-regulated the expression of another transcription factor called RUNX1. RUNX1 is a main transcription factor that regulates naïve T cell differentiation into either Th17 or regulatory T cell subsets.
5	NM_007194.2	CHK2 checkpoint homolog (S. pombe) (CHEK2), transcript variant 1	Checkpoint kinases (Chks) are serine/threonine kinases that are involved in the control of the cell cycle; in response to double strand breaks.

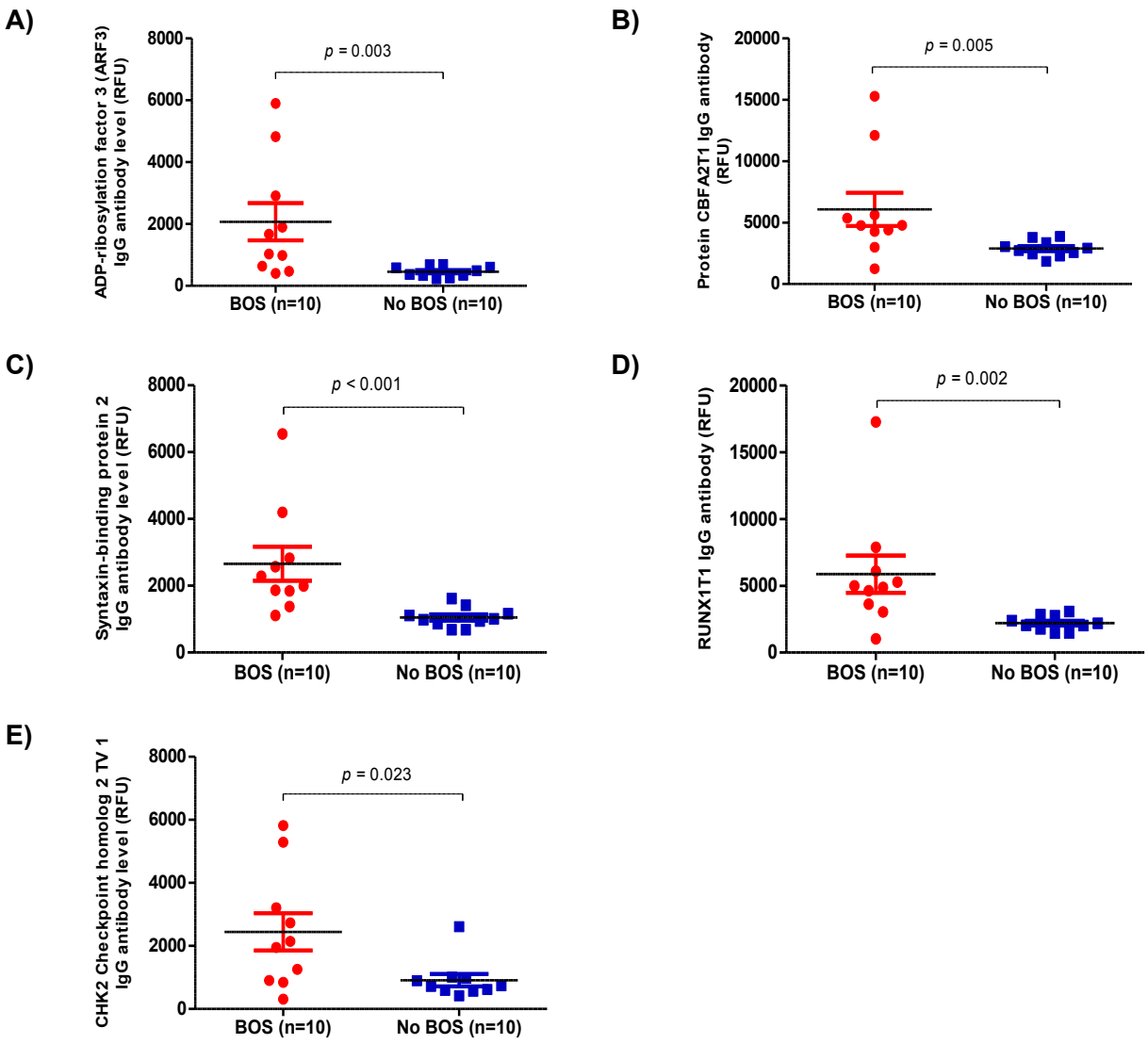


Figure 6.14 Autoantibody levels present at the time of transplant in patients with established BOS and patients without BOS. The graph shows IgG autoantibody level to five proteins present in the recipients' sera at the time of transplant. Recipients' serum reactivity was assessed using protein microarray comprising of 9840 proteins. ProtoArray® Prospector software was used for data analysis. A) ADP-ribosylation factor 3 (ARF3); B) Protein CBFA2T1; C) Syntaxin-binding protein 2; D) Runt-related transcription factor 1; translocated to, 1 (cyclin D-related) (RUNX1T1), transcript variant 1; and E) CHK2 checkpoint homolog (S. pombe) (CHEK2), transcript variant 1 were identified as positive target proteins with highest prevalence in recipients with established BOS (BOS group) in comparison to recipients without BOS (No BOS group). The autoantibody levels were significantly higher in the patients with BOS in comparison to patients without BOS. The autoantibody levels are presented as mean Relative Fluorescent Units (RFU). Mann-Whitney t-test was used to calculate significance. *P*-value of <0.05 was considered statistically significant.

In theory, each protein identified as a potential candidate “biomarker” should be evaluated on a confirmatory study cohort, using a different detection methodology. I therefore assessed the validity of RUNX1T1 as a potential candidate protein for autoantibody development, because, given its potential role in influencing Th17 differentiation (see Table 6.6 (4)) and the likely contribution of Th17 T cells to autoimmune responses against Col (V) and K-α1 tubulin [284], RUNX1T1 appeared as an attractive candidate “biomarker” for lung transplantation.

I therefore developed a RUNX1T1 specific ELISA (Chapter 2, Section 2.5.5) to assess the presence of autoantibody against RUNX1T1 in the “discovery” cohort (BOS group, n=10 and No BOS group, n=10), and in another, unrelated study cohort (confirmatory study cohort; n=41) of lung and heart and lung transplant recipients, that included 24 patients with BOS grade 1, 2 and 3 and 17 patients free from BOS, Table 6.9 and Figure 6.16. Initially, I screened the discovery study cohort for RUNX1T1 specific autoantibody by ELISA, Figure 6.15 A. The IgG antibody level is presented as arbitrary units interpolated from a standard curve. The results obtained by ELISA methodology did not highlight differences in the RUNX1T1 IgG antibody level between the two groups of patients in comparison to the RUNX1T1 IgG antibody level detected by protein microarray methodology, Figure 6.15 B. In addition, I have also included a control group of seven healthy volunteers; we observed significant difference between the test groups and the controls, but there was no difference between the patient groups, Figure 6.15 C.

Likewise, we did not observe differences in the RUNX1T1 antibody level between the patients with BOS and patients without BOS in the confirmatory study cohort, Figure 6.16. Table 6.9 shows the confirmatory study cohort recipient and donor characteristics.

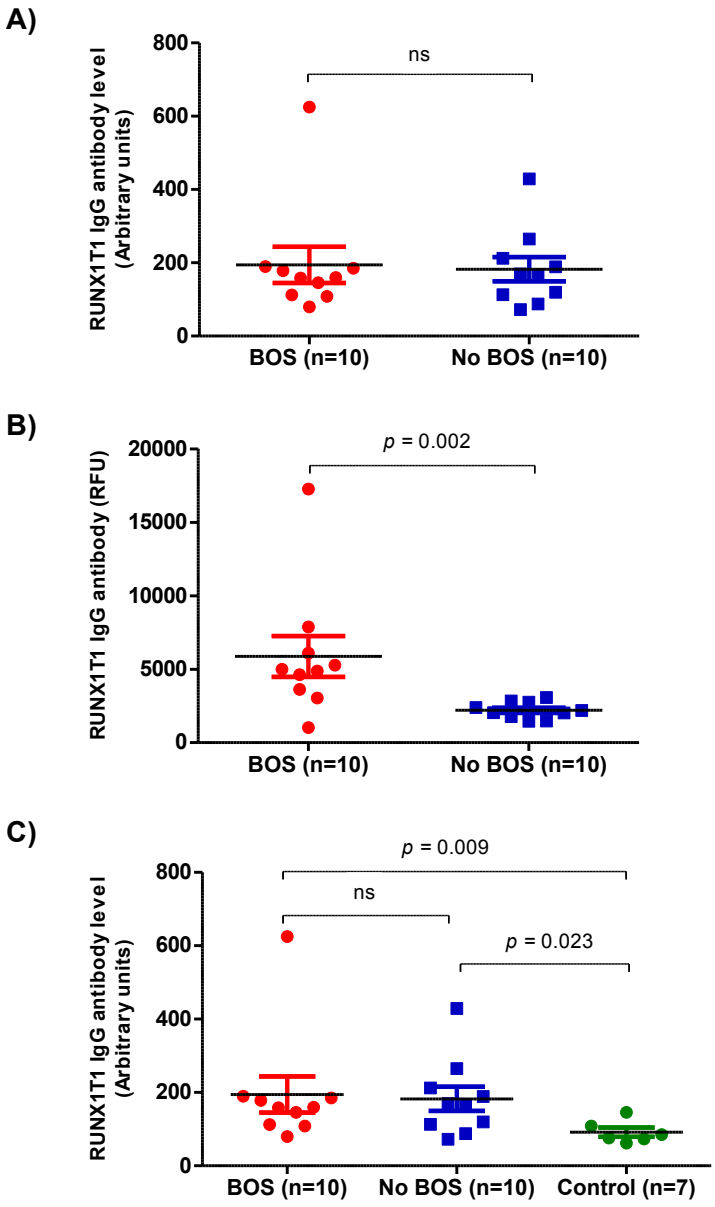


Figure 6.15 RUNX1T1 antibody level present at the time of transplant in patients with established BOS and patients without BOS. The graph shows RUNX1T1 IgG autoantibody level tested using ELISA (A and C) and protein microarray methodology (B). ProtoArray® Prospector software was used for protein microarray data analysis; B) autoantibody levels are presented as mean Relative Fluorescent Units (RFU); A) and C) autoantibody levels are expressed as arbitrary units interpolated from a standard curve. The standard curve was constructed using serial dilution of RUNX1T1 monoclonal antibody (1000 is the highest point on the curve). A) Significant difference in the RUNX1T1 antibody level was not observed between the patient groups (BOS vs No BOS) when tested by ELISA; B) The RUNX1T1 antibody level was significantly higher in patients with BOS when tested by protein microarray; C) Significant difference in the RUNX1T1 antibody level was observed between the test groups and the controls (healthy volunteers), but there was no difference between the patient groups when tested by ELISA. Mann-Whitney t-test was used to calculate significance. *P*-value of <0.05 was considered statistically significant.

Table 6.9 Confirmatory study cohort recipient and donor characteristics

	BOS (n=24)	No BOS (n=17)	*P* value
Recipient age (Median ± SD)	56 ± 15.8	56 ± 12.2	*ns*
Recipient sex (M:F)	13:11	10:7	*ns*
Transplanted organ			
SLT	3	1	
BLT	15	15	*ns*
HLT	6	1	
Donor age (Median ± SD)	46 ± 14.7	45 ± 11.8	*ns*
Donor sex (M:F)	12:12	12:5	*ns*
Indication for transplantation			
COPD	3	5	
Cystic fibrosis	5	3	*ns*
Emphysema	7	5	
Other	9	4	
HLA sensitisation (pre-transplant)	9	3	*ns*
Years after transplantation			
Mean	3.9	2.1	*ns*
Range (years)	(1-13 years)	(1-21 years)	
BOS grade			
BOS 0	0	17	
BOS 1	10	0	*<0.0001*
BOS 2	9	0	
BOS 3	5	0	

BOS – Bronchiolitis Obliterans Syndrome; M – male; F – female; COPD - Chronic obstructive pulmonary disease; SLT – Single Lung Transplant; BLT – Bilateral Lung Transplant; HLT – Heart and Lung Transplant; HLA – Human Leukocyte Antigen.

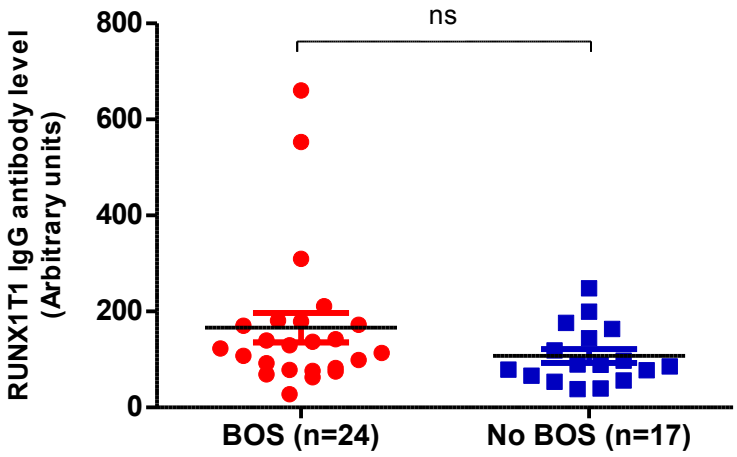


Figure 6.16 RUNX1T1 antibody level present at the time of transplantation in patients with established BOS and patients without BOS. The graph shows RUNX1T1 IgG autoantibody level in patients with established BOS (n=24) and patients without BOS (n=17). The autoantibodies were detected using RUNX1T1 specific ELISA and the autoantibody levels are expressed as arbitrary units interpolated from a standard curve. The standard curve was constructed using serial dilution of RUNX1T1 monoclonal antibody (1000 is the highest point on the curve). Significant difference in the RUNX1T1 antibody levels was not observed between patients with BOS and patients without BOS. Mann-Whitney t-test was used to calculate significance. *P*-value of <0.05 was considered statistically significant.

6.3 Discussion

To evaluate the humoral allo- and autoimmune responses in lung transplant recipients', sera obtained at the time of transplant and after transplantation were tested for presence of HLA alloantibody and anti-nuclear autoantibody. HLA alloantibodies were detected and characterised using Luminex methodology, whereas, presence of autoantibody with specificity for anti-nuclear antigens was tested using HEp-2 IF and HEp-2 ELISA assay.

The analysis of humoral alloimmune responses revealed that 33.33% of the patients in the prospective study cohort (n=21) were sensitised to HLA antigens prior to transplantation (Figure 6.1) in comparison to 17.5% of patients in the retrospective study cohort (n=40, Figure 6.3). These observations are slightly higher than the average percentage of sensitised patients awaiting lung transplantation ranging between 10% and 15% [6].

In the prospective cohort development of *de novo* humoral alloimmune responses to donor mismatched antigens was observed in 4 patients (21%); these patients were also sensitised to HLA antigens prior to the transplant. Interestingly, humoral alloimmune responses did not develop during follow up in patients that were not sensitised prior to transplantation, except for one patient that developed transient HLA antibodies (P18, Table 6.2).

In comparison, only one patient in the retrospective group (2.5%) developed *de novo* DSA. In this cohort, humoral alloimmune responses were assessed at different time points after transplantation, ranging from 1 to 21 years; and approximately 50% of the patients were long-term survivors (more than 6 years after transplantation). Previous studies have reported that patients that do not develop HLA alloantibody (including both DSA and/or non-DSA) have better clinical outcome [323].

Assessment of the humoral autoimmune responses revealed that the majority of patients in both study groups (prospective and retrospective study group) had IgG autoantibody directed to various cellular and nuclear antigens at the time of transplant. Interestingly, significant reduction in the autoantibody reactivity was observed in the test samples obtained after transplantation (Figure 6.2, Figure 6.4 and Figure 6.5), irrespective of the time of serum sample collection. In the prospective study cohort significant reduction in the autoantibody profile was observed in the serum samples obtained at one month after the transplant, Figure 6.2, and the autoantibody level remained unchanged at 12 months after transplantation. There was no significant difference in the autoantibody levels between sera obtained at one and 12 months after transplantation.

Similar findings were observed in the retrospective cohort, Figure 6.4; however, there was a difference between the HEp-2 IIF and the HEp-2 ELISA assay. The reduction of the autoantibody levels was more prominent in the post-transplant serum samples tested by ELISA (Figure 6.5) in comparison to the HEp-2 IIF assay (Figure 6.4). Although direct comparison between two different methodologies cannot be made, it is tempting to speculate that the observed difference is probably due to the higher sensitivity of the ELISA assay. The HEp-2 ELISA uses lysed HEp-2 cells as a substrate allowing exposure of the total cellular components; however, the autoantibody specificity cannot be characterised with the HEp-2 ELISA; whereas, in contrast HEp-2 IIF assay offers higher specificity allowing detection of autoantibody to specific antigens characterised by unique staining patterns.

The HEp-2 IIF assay revealed various nuclear and cellular staining patterns; suggesting that the IgG autoantibody were directed to multiple nuclear and/or cellular targets. In addition, several patients' had different staining patterns in the pre- and post-transplant sera, suggesting development of *de novo* IgG autoantibody, epitope spreading and/or increase in the autoantibody titres. In some patients IgG autoantibodies with specificity to multiple nuclear and/or

cellular antigens were observed, characterised by the presence of mixed staining pattern.

This study demonstrated the presence of autoantibody with specificity to multiple anti-nuclear antigens at the time of transplant in the majority of lung transplant recipients; interestingly, a significant decrease in the autoantibody levels was observed after transplantation. In contrast, Porcheray et al, have reported increased reactivity to HEp-2 cells in kidney transplant patients with chronic humoral rejection in comparison to patients without chronic humoral rejection in the serum samples obtained after transplantation, but not at the time of transplant [324].

HEp-2 IIF and HEp-2 ELISA are widely used screening assays for systemic autoimmune diseases [325, 326], many of which are characterised by the presence of anti-nuclear antibody to a specific antigen; however, these tests are not diagnostic tests. Therefore, positive HEp-2 screening tests are reported as a titre; and disease differential diagnosis is dependent on a confirmatory antigen specific immunoassay. Additionally, clinical usefulness of HEp-2 assay is only considered when interpreted in conjugation with patient clinical symptoms. Therefore, it has been recommended that the assay should not be used as a screening test for individuals without any clinical symptoms, due to the observed weak serum reactivity of healthy individuals, older people, pregnant woman, cancer patients, patients with chronic infections, and other diseases [322].

My findings have raised questions regarding the clinical significance of autoantibody titres present in the recipients' serum at the time of transplant and the adequacy for utilisation of HEp-2 IIF and HEp-2 ELISA assays in lung transplantation. It is quite possible, for example, that the autoantibody levels observed are below a cut-off of what is deemed clinically relevant, irrespective of the significant difference between the pre- and post-transplant test samples.

Assessment of the patients' sera with strong positive HEp-2 staining pattern using serial dilutions revealed absence of sera reactivity to HEp-2 cells (dilution factor 1:160), Section 6.3. These finding suggest that the autoantibody titres present in the patients' sera at the time of transplant may not represent "true" positive findings. It is questionable whether the use of serial dilutions is suitable for interpretation of our findings. Unfortunately, at this stage the application of HEp-2 assays for assessment of humoral autoimmune responses in lung transplantation is not obvious.

It is important to stress that a control population of a random collection of disease-free, non-transplanted patients was not included in the study. Thus, it is extremely difficult to know whether the autoantibody responses that I have observed are real findings or simply represent non-specific binding. It is quite possible that autoantibody responses observed in BOS free lung transplant recipients are due to the nature the underlining chronic lung disease. Therefore, assessment of autoantibody responses in an appropriate reference population would be crucial to be able to draw a firm conclusion.

In addition, it is possible that the observed reduction in the autoantibody IgG titres might be due to the reduction of the total IgG levels, a known post-transplant complication in solid organ transplantation [327] observed in kidney [328], liver [329], heart and lung transplant recipients [330]. The total IgG levels present at the time of transplant and after transplantation were not measured in my study and I cannot confirm whether the observed reduction in the autoantibody levels is due to reduction of the total IgG levels.

To widen my screening strategy, the humoral autoimmune responses were assessed in a sub-cohort of lung transplant recipients in whom alloimmune responses were not detected. Thus, in the absence of alloimmune responses I aimed to evaluate the autoantibody repertoire in lung transplant recipients with established BOS (n=10) and recipients free from BOS (n=10) using protein microarray methodology. The platform allows simultaneous

characterisation of IgG autoantibody specificity to 9480 human recombinant proteins.

This study examined the variations in serum reactivity between samples collected at two different time points in predefined study groups (BOS pre vs No BOS pre and BOS post vs No BOS post). Several observations can be made from my set of data. Firstly, a significant proportion of the proteins imprinted onto the array were identified as positive hits; ranging between 3.5% and 30.8% and 4.08% and 30.04% in the pre- and post-transplant serum obtained from patients with established BOS (Table 6.6). Surprisingly, the percentage of proteins identified as positive in patients without BOS was slightly higher in comparison to patients with established BOS for both, the pre- and post-transplant serum ranging from 7.6% to 33% and 8.7% to 25.5%, respectively (Table 6.7).

Secondly, group comparison analysis showed a unique set of autoantibody profile present at the time of transplant in both groups of patients. Interestingly, serum reactivity among patients free from BOS was five times higher in comparison to patients with BOS (Figure 6.11); suggesting that the autoantibody repertoire present at the time of transplant may impact whether the patients will develop BOS after the transplant or whether they will remain free from BOS. Overall, the serum reactivity was more prominent in patients without BOS at the time of transplant in comparison to the serum obtained after transplantation; and, in contrast, in patients with BOS the autoantibody repertoire was more diverse in the sera obtained after transplantation, Figure 6.11.

Lastly, the clustering analyses have highlighted two distinct autoantibody patterns between the patients with BOS and patients without BOS for both test serum samples (pre- and post-transplant), Figure 6.12. Considering the extent of the graft damage in the patients with established BOS perhaps it is not surprising that there is a variation in the autoantibody specificity between the patients with BOS and patients without BOS. In this cohort the post-

transplant autoantibody repertoires reflect only a single time point after transplantation; thus, it is not possible to assess whether these autoantibody developed prior to the onset of BOS and have a causative effect in the development of BOS or their appearance is a consequence of BOS.

Clustering analysis of the autoantibody profile in the patients with BOS alone have highlighted a distinct autoantibody pattern present at the time of transplantation (Figure 6.13); these findings agree with the HEp-2 analysis.

The obvious question raised by these observations is whether the autoantibodies to these protein targets are clinically relevant; in other words are any of these pre-existing autoantibody potential “biomarkers” that can predict which patients are more likely to develop BOS after transplantation?

To address this question I looked at the top ranked proteins with the highest prevalence (>65%) in patients with established BOS in the serum samples obtained prior to transplantation. Eight proteins were identified; of these the autoantibody levels were significantly higher in the patients with BOS in comparison to patients without BOS only for 5 protein targets, Figure 6.14. Of these, the validity of the RUNX1T1 protein as a potential candidate protein was assessed using RUNX1T1 ELISA assay in a confirmatory study cohort (n=41) consisting of 24 patients with established BOS grade 1, 2 and 3 and 17 patients without BOS, Table 6.9.

The RUNX1T1 ELISA did not highlight differences between the two groups of patients in the “discovery” cohort, Figure 6.15 A; significant difference was observed between the test group and the controls; but there was no difference between the patient groups (Figure 6.15 C). Similarly, the RUNX1T1 autoantibody levels were not different between both groups of patients in the confirmatory study cohort, Figure 6.16.

Overall the difference observed by protein microarray was not so apparent by the RUNX1T1 ELISA; nevertheless, the pattern is similar between the protein microarray and the ELISA (Figure 6.15 A and B), suggesting that we might be looking at slightly different epitope. The ELISA does not offer the conformational target as the protein on the microarray. Furthermore, it is possible that the protein microarray are more sensitive in comparison to the ELISA methodology; thus, the level of autoantibody detected by protein microarray may not be clinically relevant. In addition, the possibility for presence of IgG autoantibody that may cross react with different protein targets cannot be excluded.

It has to be acknowledged that this study has its own drawbacks; firstly, the numbers are small; perhaps screening a larger cohort for presence of RUNX1T1 autoantibodies may provide a more definite conclusion regarding its validity as a potential “biomarker”.

The Prospector software used for data analysis has its own integrated characteristics in the sense that it can only assess the data between pre-defined study groups and at this stage we don’t fully understand what this level of autoantibody means in clinical settings. Thus, exploring an alternative way to analyse the data sets may prove more useful at eliminating the background noise and characterisation of “true” positive protein targets.

Ultimately, characterisation of clinically relevant protein targets requires further data analysis and assessment of other protein targets that were identified as potential candidate proteins. Lastly, perhaps an in depth assessment of IgG autoantibody subclasses may provide better understanding of the pathogenicity to these antibodies and their contribution to development of BOS after transplantation.

6.4 Key points

- My data suggest that the pre-existing autoantibody signature may correlate with development of BOS.

- Distinct autoantibody signature patterns were identified in the patients with established BOS and patients without BOS; thus, the protein microarray may prove to be a useful tool that can discriminate between patients with established BOS and patients without BOS.

- The clinical relevance of these protein targets has to be evaluated on a separate cohort.

- Protein targets identified using protein microarray may prove difficult to evaluate using different methodology.

7 Chapter 7

Discussion

7.1 Final discussion

Chronic allograft rejection remains the main obstacle that limits the success of transplantation; and in particular, long-term outcomes for lung transplantation remain disappointing. As many as 50% of lung transplant recipients develop progressive BOS by 5 years after the transplant [6]. Traditionally, transplantation research has focused on the role of T-cell mediated cellular immunity, as highlighted by the development of highly potent T-cell depleting immunosuppressant regiments. Although these have led to significant improvements in short-term outcomes, long-term allograft survival has not altered dramatically.

The development of extremely sensitive antibody screening methodologies, allied to the ability to precisely identify alloantibody specificity, has, however, highlighted the contribution of humoral alloimmunity to chronic allograft rejection. In addition, in the last decade, several experimental and clinical studies have demonstrated that cellular and humoral responses to autologous 'self' antigens may play a causative role in the onset of BOS [144, 282].

Work from our research group has highlighted a novel mechanism for triggering autoimmunity following transplantation [144]. Win et al., in a murine model of chronic heart rejection (Bm12 to B6) showed that transplant-induced autoimmunity - characterised by production of class-switched anti-nuclear autoantibodies and development of chronic graft rejection - was dependent upon provision of help from donor CD4 T cells that were passengers within the graft. Surprisingly, donor CD4 T cell help was provided via a direct cognate interaction with recipient B cells [144].

I have designed a pilot experimental work firstly to evaluate both the incidence of donor CD4 T cell chimerism and the autoantibody responses following lung transplantation. Secondly, to address the question whether a similar mechanism is responsible for the development of autoantibody in human transplantation, I have studied the presence and the impact of passenger

donor CD4 T cells on the humoral auto- and alloimmune responses in a prospective cohort of primary lung transplant recipients (n=21).

My work suggests that the presence of donor CD4 T cell chimerism in the peripheral blood of lung transplant recipients is a uniform phenomenon. Donor CD4 T lymphocytes were consistently detected in the recipients' peripheral blood during the first post-operative month; however, the number of detectable donor CD4 T cells fluctuated over time, and varied between individual lung transplant recipients. In a follow-up period of one year after transplantation, three distinct patterns of donor CD4 T cell chimerism were observed: short (donor CD4 T cells detectable for up to six weeks after transplantation, n=13), intermediate (donor CD4 T cells detectable between three to six months after transplantation, n=3) and long-lasting chimerism (donor CD4 T cells detectable for more than six months after transplantation, n=5).

Immunogenic discrepancies within the HLA antigens between the recipient and donor pair are the main targets for allorecognition and rapid cell killing of passenger DCs within the first few weeks after transplantation [212, 331]. Similarly it may be possible that donor passenger CD4 T cells are also subjected to rapid killing soon after transplantation. My work suggests that the degree of HLA mismatching does not correlate with the longevity of donor CD4 T cell chimerism. Furthermore, the HLA mismatching did not affect the incidence of acute cellular rejection episodes and the development of BOS during the first post-operative year.

In kidney transplant recipients, KIR-ligand mismatching is associated with reduced long-term graft survival only in HLA compatible transplant pairs (n=137), but does not have an effect in patients mismatched for HLA-A, -B and/or -DR [211]. It is believed that the impact of KIR-ligand mismatching is "unmasked" in the absence of HLA incompatibility between the transplant pair. In my study RvD NK cell alloreactivity was predicted only in one recipient; thus, association between NK cell alloreactivity and longevity of donor chimerism was not assessed. In addition, taking into an account the number

of lung transplants performed each year and that fact that all lung transplant recipients receive poorly matched lungs it will be very difficult to assess the role of KIR-ligand mismatching in a large cohort of HLA matched lung transplants.

Predicting NK cell alloreactivity based on KIR-ligand mismatching is a widely used approach in HSCT. NK cell alloreactivity is dependent on the absence of an interaction between the NK cell inhibitory KIRs and its corresponding HLA ligands; the loss of inhibition results in NK cell activation and target cell killing [206, 208]. NK cell alloreactivity was similarly assessed in my study, but it should be acknowledge that this approach is not without limitations. Predicting NK cell alloreactivity based on HLA genotyping of both recipients and their corresponding donors and recipients' KIR genotype in a recipient-*versus*-donor direction is indicative of *in vivo* NK alloreactivity but does not prove that the repertoire of each KIR encoded is also expressed on the NK cell membrane. It has been reported that KIR cell surface expression is in direct correlation with the encoded KIR gene content [221] but not all NK cells express the encoded KIR genes. It is now known that only a fraction of the total number of NK cells express a set of inhibitory KIR receptors [209, 221, 222]. Furthermore, development of a functionally competent NK cell is dependent on the inhibitory KIR "licensing" by self-MHC class I molecules. This involves interaction between NK cell inhibitory KIRs and their corresponding self-MHC class I molecules, a process that enables them to discriminate self from non-self [226, 227]. NK cells that do not undergo this process are self-tolerant but functionally incompetent [228].

Considering the frequency of the inhibitory KIR genes in different populations [148, 230, 231], it is not surprising that in my cohort all recipients contained a combination of at least two inhibitory KIR genes; presence of *KIR3DL1* gene was detected in each participant. In addition, based on the recipient HLA-C and Bw4 epitope genotyping all patients had at least one KIR gene that was expected to be "licenced" to recognise self-MHC class I molecules. Five patients (patient 1, 7, 10, 12 and 17; Table 4.4) had one KIR gene; four patients had 2 KIR genes, four patients had 3 KIR genes and eight patients

had 4 KIR genes that were expected to be “licenced” to recognise “self”. Of the 20 transplant pairs that were matched for KIR ligands, 12 donors contained between 1 and 2 inhibitory KIR ligands in the RvD direction and 8 donors contained 3 or 4 inhibitory KIR ligands. Univariate analysis showed no association between the cumulative number of inhibitory KIR ligands and the longevity of donor CD4 T cell chimerism.

Due to insufficient test samples available, the NK cell numbers, NK cell subsets, inhibitory KIR receptor expression pre- and post-transplant and the strength of inhibition between different KIR-ligands were not studied. Precise determination of the NK cell alloreactivity between each recipient and donor pair would require adoption of an *ex vivo* NK cell assay. Notwithstanding, my work suggests that the duration of donor CD4 T cell chimerism is neither dependent upon the degree of HLA mismatching nor the degree of KIR-ligand inhibition.

A separate study from our research group investigating the impact of donor CD4 T cells on humoral alloimmune responses has shown that provision of help by donor CD4 T cell to host B cells is dependent upon avoidance of host NK cell recognition and donor CD4 T cell survival early after transplantation [332]. Interestingly, once donor CD4 T cells have provided efficient help to recipient B cells (resulting in generation of allo- and autoantibody), the donor CD4 T cells were rapidly cleared (within the first week after transfer). This would imply that long lasting donor CD4 T cell chimerism represents not only avoidance of NK cell allorecognition early after transplantation, but also a lack of cognate interaction between the donor CD4 T cells and recipient B cells, because otherwise alloantibody responses would be generated that would result in donor cell lysis. In my study only five recipients had long lasting donor CD4 T cells chimerism and NK cell alloreactivity was not expected in any of the recipients with long lasting chimerism; interestingly only one of these patients developed anti-HLA antibodies, suggesting that the donor CD4 T cells have avoided NK cell allorecognition and that they had been ineffective at interacting with recipient B cells. The findings in my study do not mirror Harper et al., observations [332]; possibly because the lung transplant

recipients receive an immunosuppression regimen that affects cell behaviour. In comparison, in Harper's et al., and Win's et al., work the experimental models were not subjected to immunosuppression and their experimental work were carried out in a completely sterile germ free environment.

In addition, the mice strains used in their work were inbred strains. Mating of brother-sister mice for at least 20 generations generates inbred strains; eventually they become almost entirely homozygous and with each generation half of the pre-existing heterozygosity is lost. The sex difference is the only heterogeneity remaining [333].

In Harper's work most experiments were carried out on different mice models that have a C57BL/6 (B6) genetic background H-2K^{b}, D^{b}, I-A^{b} [332]; in humans these antigens are equivalent to HLA-A, HLA-B and HLA-DQ antigen; and most importantly this strain lacks the H-2L gene (human HLA-C antigen).

In my work the NK cell alloreactivity was predicted based on the KIR ligand mismatching which mainly relies on the mismatching at HLA-C antigens (H-2L gene in mice); a gene that is not encoded in Harper's mice model. Instead, in her model the role of NK cells was studied either by transfer of CD4 T cells purified from a completely mismatched BALB/c (H-2K^{d}, D^{d}, I-A^{d}, I-E^{d}; equivalent to human HLA-A, -B, -DQ and -DR) donor strain into B6 mice model that lacked T and B cells because of deficiency of the Recombinase Activating Gene 2 (B6 *Rag-2* $^{-/-}$) or by depletion of NK cells by administration of anti-NK1.1 antibody in B6 *Rag-2* $^{-/-}$ recipients [332].

Although, Harper's work has been carefully designed and showed that provision of help by donor CD4 T cell to host B cells is dependent upon avoidance of host NK cell recognition and donor CD4 T cell survival early after transplantation [332]; it has proven difficult to relate the same experimental design to human studies. My findings have not mirrored previous mice findings but considering the differences between the mice and human perhaps my observations are not surprising. A similar mechanism may exists

in humans; however, in human studies it is almost impossible to tease-out individual effector arms of the immune system and to study them separately.

Another important aspect of donor chimerism that was not addressed in my study is the migration of donor CD4 T cells. Previous studies have demonstrated that T cells have the ability to recirculate through non-lymphoid organs [136]. Functionally and phenotypically these cells are a mixture of naïve, effector and memory T cells that express cell-surface markers such as CCR7, LFA-1, VLA-4 and ICAM-1, that govern the homing to secondary lymphoid tissue and other peripheral tissues [136, 137, 139, 183]. Migration of allograft passenger cells to non-lymphoid tissues such as the skin has been observed in liver [131] and kidney [165] transplant recipients even decades after transplantation. Thus, it is possible that rapid migration of donor CD4 T lymphocytes from within the allograft into other tissue sites might explain why, in the majority of lung transplant recipients (n=13), donor CD4 T lymphocytes were detectable in the recipients' peripheral blood for only a few weeks after transplantation.

Using a molecular gene expression profiling approach, I investigated whether the type of donor CD4 T cell present in the recipients' peripheral blood is a contributing factor for the observed variation in the longevity of donor CD4 T cell chimerism. Unfortunately, in this small pilot study cohort a unique pattern of donor CD4 T cell subpopulation was not observed due to massive variation in the level of gene expression between the test samples and the test groups.

I also characterised the immune cell composition of donor lungs that undergo reconditioning with the EVLP procedure. The procedure provides a unique setting to study leukocyte migration from the lungs into the recipient where, under EVLP conditions, migrating leukocytes are immobilised in the leukocyte filter on the EVLP circuit. I showed that migratory leukocytes consist of a mixture of lymphocytes, monocyte/macrophage and granulocyte population and only 1% of lymphocytes were characterised as CD4 T cells.

Most importantly, I showed that the CD4 T cells comprise of four subpopulations including naïve T cells, T follicular helper cells, central memory and effector memory T cells; and, the majority of naïve CD4 T cells co-expressed CD62L and CCR7. A fraction of these cells also expressed CXCR5, consistent with the presence of follicular helper T cells. These findings imply that donor CD4 T cells have the ability to recirculate through secondary lymphoid tissue, and potentially, if engaged in cognate interaction with recipient B cells, provide help for B cell activation and antibody production, as previously suggested by the work generated from our laboratory [144, 332].

The lung is a mononuclear cell-rich organ and considerable numbers of immunocompetent cells bearing the ability to recirculate through the secondary lymphoid organs are transferred with the graft at the time of transplantation. Whether immobilisation of passenger mononuclear cells using the EVLP procedure will affect the clinical outcome in human lung transplantation remains to be seen and is beyond the scope of this study. With regards to the role of donor CD4 T cell chimerism it would be intriguing to investigate the presence and duration of donor CD4 T cells in another study cohort of lung transplant recipients that have received lungs subjected to EVLP conditioning prior to transplantation.

It has to be stressed that my observations were made based on one EVLP filter and I cannot generalised and reach firm conclusion regarding the cell composition of the EVLP filters; however, as a prove of principle I have shown that this studies are feasible. Considering that the ELVP filter cell composition is completely unknown entity; I cannot envisage how many filters have to be assessed in order to reach any conclusion. Assessment of at least 10 filters is necessary to give us better understanding of the EVLP filter cell composition.

I also investigated whether donor CD4 T cell chimerism is associated with development of transplant-induced autoimmunity and whether the donor CD4 T cell affects humoral alloimmune responses. The premise for this examination was the previous murine work performed in our laboratory, which

demonstrated in a murine model of chronic heart allograft rejection that donor CD4 T cells that are passengers within the heart grafts provide help to host B cells through donor CD4 T-cell allorecognition of MHC class II on host B cells. The donor (bm12) and recipient (B6) strains differ by three amino acids within the MHC class II I-A antigens (HLA-DQ in humans); thus, irrespective of bound peptide donor CD4 T cells provide equal non-specific help to all host B cells, resulting in production of autoantibodies with specificity to multiple anti-nuclear antigens. The antibody mediated graft injury was associated with development of long-lasting IgG anti-nuclear autoantibody [144]. Subsequently, in a more disparate MHC mismatched model it was reported that passenger donor CD4 T cells significantly augment recipient cellular and humoral alloimmunity resulting in severe allograft vasculopathy and accelerated graft failure. The impact of donor CD4 T cells was dependent upon avoidance of host NK cell recognition and class-switched alloantibody production was dependent on simultaneous ligation of BCR with the target antigen and interaction with host CD4 T cells with indirect allospecificity; thus, resulting in production of auto- and alloantibodies [332]. If the same mechanism applies to human lung transplant recipients, one could expect augmented humoral alloimmune responses in the recipients with short donor CD4 T cell chimerism in which NK cell alloreactivity was not expected.

Contrary to this, in my cohort (n=21) 13 patients had short-lasting donor CD4 T cell chimerism and NK cell alloreactivity was not expected in 12 of these recipients, suggesting that donor CD4 T cell can avoid NK-cell mediated killing at least early after transplantation. Nevertheless, none of these recipients developed HLA antibodies after the transplant.

My work showed that at the time of transplant seven recipients were sensitised to HLA antigens, none of which were donor specific. At one month after the transplant, in addition to the pre-existing non-DSA, two recipients developed *de novo* DSA and interestingly in the sera of two recipients that were previously sensitised to HLA antigens, HLA antibodies were no longer detectable. Sera tested at one-year post-transplant revealed that only four

recipients with pre-existing non-DSA developed *de novo* DSA and non-DSA, all other recipients tested negative for HLA antibodies. Of these, two recipients had long-lasting donor CD4 T cell chimerism and NK cell alloreactivity was not expected in any of these recipients. The other two recipients that developed DSA had short donor CD4 T cell chimerism and NK cell alloreactivity was also not expected in both patients. Considering the size of this cohort and the fact that recipients are subjected to a highly potent immunosuppressive regimen, it is not possible to firmly conclude that passenger donor CD4 T cells have a similar impact at augmenting host humoral alloimmune responses, as observed in our previous murine studies. At this stage, the role of donor CD4 T cell chimerism in human transplantation is not apparent.

In comparison, only one patient in the retrospective group developed *de novo* DSA. In this cohort humoral alloimmune responses were assessed at different time points after transplantation ranging from 1 to 21 years; and approximately 50% of the patients were long-term survivors (more than 6 years after transplantation). Numerous clinical studies have reported that patients that do not develop HLA alloantibody, including both DSA and/or non-DSA, have better clinical outcome [323]; thus, this cohort may mirror previous clinical findings and perhaps it is not surprising that development of humoral alloimmune responses were observed only in a small fraction of patients. Donor CD4 T cell chimerism studies were not applied to this cohort.

In comparison to Win et al., murine model observations, in my study the assessment of the humoral autoimmune responses revealed unexpected findings. Anti-nuclear IgG autoantibody levels were more profound in the recipients' sera before the transplant in comparison to sera obtained at one and 12 months after transplantation. The reduction of autoantibody titre was statistically significant and there was no change in the autoantibody titre between the test samples obtained at one month and 12 months post-transplant. Similar findings were observed in the retrospective cohort of 24 lung and 18 heart and lung transplant recipients. Contrary to the previous

findings in a murine model studies, this would apply that irrespective of donor CD4 T cell dynamics, NK cell allorecognition and presumably interaction between the donor CD4 T cells with recipients B cells, the donor CD4 T cells have not been efficient at providing adequate help to recipient B cells; thus, as a consequence transplant-induced autoimmunity and augmentation of humoral alloimmune responses was not demonstrated.

In Win et al., work the effector role for autoantibody was confirmed by the early rejection and development of sever vasculopathy in bm12 hearts transplanted into animals primed for humoral autoimmunity by transfer of donor CD4 T cells. If this is the case than passenger donor CD4 T cell should augment the humoral autoimmune responses in recipients with pre-existing IgG anti-nuclear autoantibody [144]. In contrast, in my study the level of anti-nuclear IgG autoantibodies was much higher before the transplant in comparison to the levels in test sera obtained after transplantation.

The obvious explanation for the lack of evidence that donor CD4 T cells are involved in the development of transplant-induced autoimmunity and augmentation of humoral alloimmune responses is that solid organ transplant recipients are highly immunosuppressed and most of the immunosuppressive drugs are T cell depleting reagents which can alter the number and the function of T cells.

Furthermore, it is possible that the observed reduction in the autoantibody IgG titres might be due to the reduction of the total IgG levels instead, a known post-transplant complication in solid organ transplantation [327] observed in kidney [328], liver [329], heart and lung transplant recipients [330]. The total IgG levels present at the time of transplant and after transplantation were not measured in my study and I cannot confirm whether the observed reduction in the autoantibody levels is due to reduction of the total IgG levels.

Win et al., used two inbred strain experimental models; bm12 mice (C57BL/6 B6 H-2K^{b}, D^{b}, I-A^{bm12}) as a donor and B6 mice (C57BL/6 B6 H-2K^{b}, D^{b}, I-A^{b}) as a recipient. The bm12 mice has three non-consecutive nucleotide changes, resulting in three amino acid substitutions in the β1 exon [334]. Thus, both strains differ by only three amino acids within the MHC class II I-A antigens (HLA-DQ in humans). In addition, bm12 model has number of impaired physiological systems including: abnormal T cell physiology, abnormal levels of surface class II molecules, decreased susceptibility to autoimmune disorders and increased anti-double stranded and single-stranded DNA antibody levels [334]. Perhaps the most striking feature of this model is that adoptive transfer of bm12 splenocytes or purified bm12 CD4 T cells into B6 results in development of common characteristics with human systemic lupus erythematosus (SLE) [335]. This model has been used as an inducible model to study SLE, an autoimmune disease that is characterised by production of anti-nuclear antibodies.

Furthermore, Win et al., showed that irrespective of bound peptide donor bm12 CD4 T cells provide equal non-specific help to all host B cells, resulting in production of autoantibodies with specificity to multiple anti-nuclear antigens [144]. Accordingly, bearing in mind the physiological features of bm12 experimental model, perhaps Win's observations are not surprising. However, this raises the question whether Win's murine model observations are suitable for comparison to human studies of transplant-induced autoimmunity.

One important aspect of transplant-induced autoimmunity and its contribution to development of BOS might depend on the nature of the target antigen. In lung transplantation humoral allograft rejection has been observed in patients who developed antibodies to other non-HLA associated antigens without presence of HLA antibodies [281]. *De novo* autoimmunity to type V collagen [282] and K-alpha 1 tubulin (K-α1 tubulin) [283] has been implicated as an independent predictor for development of BOS. Interestingly, Hachem et al., in a large cohort of 108 lung transplant recipients, showed that patients who

developed both allo- and autoantibodies to collagen V and K-α1 tubulin had increased risk of developing BOS; furthermore, they showed that patients that responded to antibody mediated therapy and cleared the autoantibodies were less likely to develop BOS in comparison to patients with persistent autoantibodies, irrespective of the presence or absence of alloantibodies [284].

My work suggests that presence of anti-nuclear autoantibodies at the time of transplant do not correlate with development of BOS; however, it is possible that these target autoantigens may not have clinical relevance in lung transplantation. Porcheray et al. have reported increased reactivity to HEp-2 cells in kidney transplant patients with chronic humoral rejection in comparison to patients without chronic humoral rejection in the serum samples obtained after transplantation, but not at the time of transplant [324]; which might not be the case in lung transplantation.

In addition, clinical usefulness of HEp-2 assay is only considered when interpreted in conjugation with patient clinical symptoms. It has been recommended that the assay should not be used as a screening test for individuals without any clinical symptoms, due to the observed weak serum reactivity of healthy individuals, older people, pregnant woman, cancer patients, patients with chronic infections, and other diseases [322, 331]; thus, raising questions regarding the clinical significance of autoantibody titres detected in the recipients' serum at the time of transplant and the adequacy for utilisation of HEp-2 IIF and HEp-2 ELISA assays in lung transplantation.

Lastly, despite the fact that in the prospective cohort 7 lung transplant recipients developed BOS within the first year after transplantation, it has to be emphasised that BOS is a disease that develops between 2 and 5 years after transplantation; therefore, a longer post-transplant period and larger study cohort might be necessary in order to assess the association between the longevity of donor CD4 T cell chimerism and development of transplant-induced autoimmunity.

From a clinical prospective, discovering markers of early graft damage is essential to help identify the cohort of recipients who may be more susceptible to developing of BOS and therefore, require more aggressive surveillance and treatment for mitigating the harmful effects of autoantibodies.

In a pilot experiment a sub-cohort of lung transplant recipients with established BOS (n=10) and recipients free from BOS (n=10) in whom humoral alloimmune responses were not detected, I also performed simultaneous characterisation of IgG autoantibody specificity to 9480 human recombinant proteins. My results suggest that a significant proportion of lung transplant recipients display autoreactivity to a large number of protein antigens at the time of transplant. Furthermore, in the sera obtained at the time of transplant a unique set of autoantibody patterns were identified in both groups of patients. Interestingly, serum reactivity among patients free from BOS was five times higher in comparison to patients with BOS; suggesting that the autoantibody repertoire present at the time of transplant may impact whether the patients will develop BOS after the transplant or whether they will remain free from BOS.

The autoantibody levels against five protein targets were significantly different between both groups of patients. The clinical relevance was assessed only for RUNX1T1 protein using RUNX1T1 specific ELISA in a confirmatory study cohort of 41 lung and heart and lung transplant recipients (24 patients with established BOS grade 1, 2 and 3 and 17 patients without BOS). The findings did not reveal differences in the RUNX1T1 autoantibody titres between recipients with BOS and recipients without BOS; thus, its usefulness as a potential “biomarker” was not established.

The Prospector software used for the protein microarray data analysis has its own integrated characteristics in a sense that it can only assess the data between pre-defined study groups and at this stage we don’t fully understand what these level mean in clinical settings. Thus, exploring an alternative way to analyse the data sets may prove more useful at eliminating the background

noise and characterisation of “true” positive protein targets. Characterisation of clinically relevant protein targets requires further data analysis and assessment of other proteins targets that were identified as potential candidate proteins.

The obvious questions raised by these observations is whether the autoantibodies with specificity to these protein targets are clinically relevant and if any of these pre-existing autoantibodies represent potential “biomarkers” that can predict which patients are more likely to develop BOS after transplantation? Clarification of these questions requires further data analysis, conformation using different methodology and utilisation of a larger study cohort.

One potential approach to utilize larger study cohort for autoantibody profiling in lung transplant recipients is through combining data with other research groups. Nayak et al., have recently undertaken similar study where autoantibody profiling was performed using protein microarrays in lung transplant recipients with developed CLAD (n=12) and recipients free from CLAD (n=11), (Abstract, [336]). Interestingly, they also observed a unique autoantibody repertoire in patients that subsequently developed CLAD [336]. My intensions are to contact Dr Mohanakumar, a principal leader of the same research group to express my interest for sharing my findings with them; and the possibility to combine and re-analyse the protein microarray data. It would be interesting to assess whether similar autoantibody profiling patterns will be observed in the combined cohort of 43 patients in which the patients will not be segregated into pre-defined groups.

In conclusion, although the presence of donor lymphocytes in the circulation of recipients of solid organ allografts was first demonstrated more than two decades ago, the extent to which they affect the recipients’ auto- and alloimmunity has remained unclear and this study has not reflected previous murine model findings. Nevertheless, consideration the physiological nature of the murine models perhaps it is not surprising that I was not able to reproduce

the same findings. The question is whether a similar mechanism of transplant-induced autoimmunity does exist in humans or the proposed mechanisms is dependent on the type of model used? If, a similar mechanism does exist in humans, it is tempting to speculate that the mechanism may not be so apparent in the presence of the current immunosuppression regiments. However, prior to addressing these questions I believe that it is inevitable to study larger cohort of primary lung transplant recipients (at least 70 lung transplant recipients) for longer post-transplant follow-up period. Nevertheless, at present the role of donor CD4 T cell chimerism in the development of transplant-induced autoimmunity and augmentation of humoral alloimmune responses in lung transplantation was not established.

7.2 Summary

In summary, my work confirmed that the donor chimerism is a uniform phenomenon in lung transplantation. However, the longevity of donor CD4 T cell in the recipients' peripheral blood is variable between patients and independent of the degree of HLA mismatching and degree of KIR-ligand inhibition. The involvement of donor CD4 T cell in the development of transplant-induced autoimmunity and augmentation of humoral alloimmunity previously observed in a murine model in our research group was not confirmed in human lung transplant recipients and the role of donor CD4 T cells remains unclear. Understanding the clinical relevance of the autoantibody repertoire present at the time of transplant may prove particularly relevant at identifying recipients with predisposition to develop BOS and improve their clinical management.

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9 Appendix 1

9.1 Participant Information Sheet

Date: 1 st February 2011
Version No: 3

Participant Information Sheet

UNIVERSITY OF CAMBRIDGE
Papworth Hospital
NHS Foundation Trust

Study Title: Humoral autoimmunity and allograft vasculopathy

Chief Investigator: Mr Gavin Pettigrew
Investigator: Miss Olivera Gjorgjimajkoska
Sponsor: University of Cambridge and Papworth Hospital NHS Foundation Trust
Host organisation: University of Cambridge
Funder: British Heart Foundation

Brief description:

We would like to invite you to take part in our research study.

- This study is an observational study and is being undertaken as part of an educational (PhD) project.
- We aim to study the development of autoimmune responses (immune responses directed against the recipient's own tissues) following heart and/or lung transplantation.
- The research will involve multiple blood sampling during your post-transplant follow-up visits.
- Before you decide we would like you to understand why the research is being done and what it would involve for you.
- One of our team will go through the information sheet and answer any questions you may have. Please ask about anything you do not fully understand or wish to have explained in more detail.
- If you would like this information in another format or language, please ask a member of our staff.
- We suggest that reading this information sheet is likely to take about 10 minutes of your time.

1. What is the purpose of the study?

Transplantation is the only treatment option for end-stage heart and lung failure. However, transplants do not last indefinitely and almost all transplants suffer a slow deterioration in function, through a process termed chronic rejection; characterised by thickening of the graft arteries, know as allograft vasculopathy.

In the last few years, it has become clear that 'auto-immune responses (immune responses directed against the recipient's own tissues) can develop following transplantation and can contribute to allograft vasculopathy. We have recently demonstrated in a mouse heart transplant model that specialised immune cells of the donor, that are passengers within the graft at the time of transplant, can migrate to the recipient and trigger an antibody response that is directed against the recipient's own proteins.

The reason we are doing this research is to formally study whether the presence of donor cells in the recipient's blood is associated with the development of autoantibody and whether the autoantibody has a damaging effect on the graft and contributes to development of allograft vasculopathy. If this were the case, this would lead to evaluation of new strategies to remove donor cells from the grafts prior to transplantation, in the hope that this would minimise the autoantibody responses and lead to improved graft survival.

2. Why have I been invited?

This study is purely observational. We are inviting you because we aim to observe 70 prospective and 100 retrospective heart or lung and heart and lung transplant patients and study the following:

- Presence of donor cells in the recipients' blood circulation after transplantation;
- Development of autoantibody;
- To see whether there is an association between the presence of donor cells and autoantibody development; and
- The contribution of autoantibody to allograft vasculopathy.

3. What will happen to me if I take part?

If you decide to take part in this study you will be asked to give a small blood sample for our research. This will be requested at different time points:

- Immediately before transplantation prospective transplant patients only: 60mls blood sample (4 tablespoons) will be obtained to test for autoantibody.
- After transplantation: 60mls blood sample (4 tablespoons; prospective transplant patients only) or 10mls (2 teaspoons; retrospective transplant patients only) will be obtained at regular intervals to test for the presence of donor cells and/or development of autoantibody. To test for donor cells in your blood we will use either cells or DNA extracted from your blood sample.
- During the study, you will have to have your blood taken up to a maximum of 18 times over a three-year study period. In most cases, this will happen at your regular post-transplant follow-up clinics; or we will send you a pre-labelled envelope with blood tube containers so you can have your blood taken at your local GP surgery.

- In order to analyse the study results we will have to compare the results with your medical records, therefore sections of your medical notes may be looked at by the individuals involved in the study.

4. Do I have to take part in this study?

It is up to you to decide whether to join the study. We will describe the study and go through this information sheet. If you agree to take part, we will then ask you to sign a consent form. Also, it up to you whether you want your GP to be informed of your participation in this study. Not taking part in this study will not affect your medical care.

5. What are the possible benefits of taking part?

This is an observational study. You will not benefit from this study directly, but your participation and the information we obtain from the investigations may contribute to advancing our understanding of the immune responses involved in the development of allograft vasulopathy.

6. What are the possible risks of taking part?

The risks of taking part in this study are minimal. You will simply have blood samples taken from your arm with a needle and syringe.

7. What happens when the research study stops?

The duration of this study is three years. On completion of the study, you and your GP will receive written confirmation of the end of study. Your transplant follow-up will continue at your normal post-transplant clinic according to departmental protocols. Any blood samples collected during the study will be discarded in accordance with the Human Tissue Authority's Code of Practice.

8. Will my taking part in the study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence.

9. What will happen if I do not want to carry on with the study?

You are free to withdraw at any time, without giving reason. This would not affect the standard of care you receive. However, any data collected up to the point of consent withdrawal will be included for analysis.

10. What will happen to the results of the research study?

Because this is an observational study and we are not at this stage sure what the results mean for an individual patient until they are fully analysed, you will not be informed directly about your results. The study results will be disseminated in the form of peer review publications; alternatively you may find some information about the progress of our research on the British Heart Foundation website (www.bhf.org.uk) or the NIHR Clinical Research Network Portfolio website (www.ukcrm.org.uk).

11. Who is organising and funding the research?

The study is taking place in the Department of Surgery, University of Cambridge and in collaboration with Papworth Hospital. The British Heart Foundation is the funder of this study and the study is being undertaken as part of a PhD project.

12. Who has reviewed the study?

The project has been independently reviewed; scientifically by the British Heart Foundation, and ethically by the Cambridgeshire 4 Research Ethics Committee. The sponsors of this study are the University of Cambridge and Papworth Hospital Research & Development Department.

13. Further information and contact details.

If you have a concern about any aspect of this study, you should ask to speak to the researchers and we will do our best to answer your questions. For further information please contact Mr Gavin Pettigrew or Miss Olivera Gjorgjimajkoska at the Department of Surgery, Box 202, Level E9, Addenbrooke's Hospital, Hills Road, Cambridge, CB2 0QQ; Tel: 01223 763 103; E-mail: gjp25@cam.ac.uk or og241@cam.ac.uk. Alternatively, you can contact Dr Clive Lewis, Consultant Cardiologist, Department of Surgery, Box 76 , Transplant Unit, Papworth Hospital, Papworth Everard, Cambridge, CB23 3RE; Tel: 01480 830 541; E-mail: clive.lewis@papworth .nhs.uk.

Please ask if you require this information in another format or language.

14. What do I need to do to take part in this study?

If you decide that you wish to participate in this study, you need to sign the consent form. Once you have signed the consent form, you do not have any responsibilities until you are called for transplantation. When you are called for transplantation a health care professional will collect pre-transplant blood sample (prospective transplant patients only). Further blood sampling will take place at regular intervals over a three-year study period (For your information please see the tables below). Please do not worry about the blood collection times, each time you will be remained by a member of the health care team. We are very grateful for your consideration.

Table 1: Post-transplant blood sample collection times (prospective transplant patients):

1st year post-transplant	Day 3; Day 7; Week 2; Week 4; Week 6; Week 8; Month 4; Month 6; Month 8; Month 10; Month 12.
2nd and 3rd year post-transplant	During the second and third year post-transplant blood samples will be collected every four months.

Table 2: Post-transplant blood sample collection times (retrospective transplant patients):

Post-transplant blood sample	During the post-transplant period maximum of 3 blood samples will be collected every 6 months.

9.2 Consent Form

Date: 1 st February 2011
Version No: 3

Consent Form

UNIVERSITY OF CAMBRIDGE
Papworth Hospital
NHS Foundation Trust

Study Number:

Participant Identification Number for this study:

CONSENT FORM

Study Title: **Humoral autoimmunity and allograft vasculopathy**

Name of Chief Investigator: Mr Gavin Pettigrew

Name of Investigator: Miss Olivera Gjorgjimajkoska

Please tick box

1. I confirm that I have read and understood the information sheet dated 01/02/2011, Version No: 3 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals involved in this study from the NHS Trust. I give permission for these individuals to have access to my records. ☐
4. I agree to my GP being informed of my participation in this study. ☐
5. I agree to take part in the above study. ☐

Name of Participant	Date	Signature
Name of Person taking consent	Date	Signature

When completed: 1 for participant; 1 for research site file; 1 (original) to be kept in medical notes.

10 Appendix 2

10.1 Published manuscripts

10.1.1 Prolongation of allograft survival by passenger donor regulatory T cell.

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ORIGINAL ARTICLE

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Prolongation of allograft survival by passenger donor regulatory T cells

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Tissue resident lymphocytes are present within many organs, and are presumably transferred at transplantation, but their impact on host immunity is unclear. Here, we examine whether transferred donor natural regulatory CD4 T cells (nT-regs) inhibit host alloimmunity and prolong allograft survival. Transfer of donor-strain lymphocytes was first assessed by identifying circulating donor-derived CD4 T cells in 21 consecutive human lung transplant recipients, with 3 patterns of chimerism apparent: transient, intermediate, and persistent (detectable for up to 6 weeks, 6 months, and beyond 1 year, respectively). The potential for transfer of donor nT-regs was then confirmed by analysis of leukocyte filters recovered from ex vivo normothermic perfusion circuits of human kidneys retrieved for transplantation. Finally, in a murine model of cardiac allograft vasculopathy, depletion of donor CD4 nT-regs before organ recovery resulted in markedly accelerated heart allograft rejection and augmented host effector antibody responses. Conversely, adoptive transfer or purified donor-strain nT-regs inhibited host humoral immunity and prolonged allograft survival, and more effectively so than following administration of recipient nT-regs. In summary, following transplantation, passenger donor-strain nT-regs can inhibit host adaptive immune responses and prolong allograft survival. Isolated donor-derived nT-regs may hold potential as a cellular therapy to improve transplant outcomes.

KEYWORDS
basic (laboratory) research/science, cellular biology, immune regulation, immunobiology, lymphocyte biology, organ transplantation in general, T cell biology, tolerance: experimental, tolerance: mechanisms, translational research/science

Abbreviations: ANOVA, analysis of variance; APC, allophycocyanin; CAV, chronic allograft vasculopathy; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EVG, elastin van Gieson; FCS, fetal calf serum; MST, median survival time; nT-regs, natural regulatory CD4 T cells; PBMCs, peripheral blood mononuclear cells; PE, phycoerythrin; PBS, phosphate-buffered saline; RT-PCR, real time-polymerase chain reaction; SD, standard deviation.

1 | INTRODUCTION

Although still considered a novel technology, ex vivo perfusion of recovered organs from deceased donors is likely to become widely adopted in the near future.[1,2] Ex vivo perfusion offers the potential to assess the viability of organs before transplantation, and to extend the acceptable period between recovery and implantation. It may also enable targeting of the isolated organs with specific therapies aimed at prolonging allograft survival.[3] One particular focus of such strategies is likely to be donor-derived T cell populations (naïve or memory) that are resident within the graft.[4,5]

We have recently reported that passenger T cells are present within human donor organs recovered for transplantation and, using murine transplant models, have demonstrated that donor T effector cells can augment host alloimmune responses directed against the allograft.[6] Thus, although seemingly counterintuitive, these passenger lymphocytes contribute to rejection of the organ. Here, we examine whether donor-derived natural regulatory CD4 T cells (nT-regs) can, conversely, prolong allograft survival.

2 | MATERIALS AND METHODS

2.1 | Identification of circulating donor CD4 T lymphocytes in human lung transplant recipients

Following adult deceased-donor lung or heart plus lung transplantation, blood from consenting recipients was sampled at predetermined time points (initially weekly for the first 2 months after transplantation, and monthly/bimonthly thereafter) and donor CD4 T lymphocytes was identified by flow cytometry, on the basis of the expression of MHC alloantigen. Briefly, peripheral blood mononuclear cells (PBMCs) were labeled with anti-CD3-FITC (fluorescein isothiocyanate, clone HIT3a) and anti-CD4 PE (phycoerythrin, clone RPA-T4) monoclonal antibodies (both BD Biosciences, Oxford, UK) and with the relevant MHC class I HLA-specific biotinylated antibody that were selected to bind exclusively to donor (but not recipient) HLA class I MHC alloantigen (see Table S1; kindly gifted by Prof. Frans Claas, Leiden University Medical Center, Leiden, the Netherlands). Cells were further labeled with allophycocyanin (APC)–conjugated streptavidin (Invitrogen, Paisley, UK) and donor cells were identified using BD FACSCantoTM flow cytometer with BD FACSDiva software (BD Pharmingen, Berkshire UK). Pure populations of donor and recipient CD4 T cells (obtained from donor spleen/lymph nodes and recipient blood before transplantation, respectively) were used as positive and negative controls for donor lymphocyte identification. Positive identification of donor CD4 T cells in test samples was based on relative intensity of staining of control donor to recipient cells (Figure S1).

The human lung study received a favorable ethical opinion by the Cambridgeshire 4 Research Ethics Committee and was approval by the Health Research Authority. The study was registered with the National Institute of Health Research (NIHR) Clinical Research Network Portfolio.

2.2 | Characterization of lymphocyte subsets released during ex vivo normothermic perfusion

Kidneys underwent 1 hour of normothermic machine perfusion, as described previously,[7] with a leukocyte filter, RS1VAE (Haemonetics, Coventry, UK), in the circuit. After 1 hour, the filter was removed and flushed in an antegrade direction with 400 mL of sterile phosphate-buffered saline (PBS). The filters were then incubated with 20 mL of trypsin-ethylenediaminetetraacetic acid (EDTA) at 37°C for 10 minutes, and cells were recovered by flushing in a retrograde direction with 400 mL of sterile PBS. Cell pellets were cryopreserved with 10% DMSO (dimethyl sulfoxide) in fetal calf serum (FCS), and stored at −80°C. For flow cytometry characterization, cells were quickly thawed in Dulbecco′s Modified Eagle′s Medium (Gibco, D5030, ThermoFisher Scientific, UK) with 2% FCS and resuspended in FACS buffer (PBS, 1% FCS, 0.02% sodium azide). Cells were stained in FACS buffer for 30 minutes on ice with the following antibodies: PE anti-human CD127 (clone eBioRDR5, ThermoFisher Scientific), Brilliant Blue 515 anti-human CD25 (clone 2A3, BD Pharmingen), APC Cy7 anti-human CD3 (clone SK7, BioLegend, London, UK), PE Cy7 anti-human CD4 (clone SK3, BD Pharmingen), and dead cell exclusion dye 7-aminoactinomycin D (BD Pharmingen). Cells were washed twice with FACS buffer after antibody staining, and cell events were collected on FACSCanto II analyzers (BD Pharmingen) and analyzed with FlowJo software (FlowJo, LLC, Ashland, OR). The human kidney study had received favorable ethical approval from Newcastle & North Tyneside 2 Research Ethics Committee REC (15/NE/0408).

2.3 | Animals

C57BL/6J (H-2^b; B6) were purchased from Charles River Laboratories (Margate, UK). Bm12 mice (B6(C)-H2-Ab1bm12/KhEgJ [H-2bm12]) and H-2^b T cell receptor-deficient mice (*Tcrbd*$^{-/-}$ [B6.129P2-*Tcrb*^{tm1Mom}*Tcrd*^{tm1Mom}/J][8]) were purchased from the Jackson Laboratory (Bar Harbor, ME).

2.4 | Heterotopic heart transplantation

Vascularized cardiac allografts were transplanted intra-abdominally as described previously.[9,10] Heart graft survival was monitored by daily abdominal palpation, with rejection defined as cessation of a detectable beat. Grafts were excised at predetermined time points after transplantation and stored at −80°C or fixed in 10% buffered formalin. In certain experiments, recipient B6 mice were depleted of CD4 T-regs by treatment with 0.5 mg of anti-CD25 mAb (PC-61, Bio X Cell, West Lebanon, NH), i.p., on day -1 followed by 0.25 mg, i.p., on days 1, 3, 5, and 7, in relation to bm12 heart graft transplantation. Donor T-reg depletion was achieved by administering 0.5 mg of anti-CD25 mAb (PC-61), i.p., on days -6 and -2 before recovery of heart allograft. Pilot experiments confirmed that this treatment resulted in depletion of typically 85%-90% of *FoxP3*$^{+ve}$ splenic CD4 T cells.

2.5 | Adoptive transfer of donor/recipient–derived nT-regs

Recipient B6 mice were adoptively transferred by tail-vein intravenous injection with 1×10^6 nT-regs derived from B6 or bm12 animals on the first postoperative day after bm12 cardiac transplantation. nT-regs were purified from spleens of naïve B6 or bm12 animals using the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec, Auburn, CA) and an autoMACS separator (Miltenyi); cell purity (typically >90% $CD25^{+ve}$ $CD4^{+ve}$) was analyzed by flow cytometry prior to injection.

2.6 | Quantification of humoral autoantibody responses

Antinuclear autoantibody responses were determined by HEp-2 indirect immunofluorescence (The Binding Site, Birmingham, UK) as described previously,[11] by incubating test sera on slides coated with HEp-2 cells and detecting bound antibody with FITC-conjugated goat anti-mouse IgG (STAR 70; Serotec, Oxford, UK). For each test serum, photomicrographs were taken, and the intensity of staining was determined by integrated morphometric analysis using MetaMorph software. The fluorescence value was then derived by comparison with a standard curve obtained for each assay by serial dilutions of a pooled hyperimmune serum that was assigned an arbitrary value of 1000 fluorescence units.

2.7 | Histopathology

Cardiac allograft vasculopathy was assessed on elastin van Gieson – stained paraffin sections by morphometric analysis as described previously.[11] Luminal stenosis [percentage cross-sectional area luminal stenosis = (area within internal elastic lamina - area of lumen)/area within internal elastic lamina × 100]. All elastin-positive vessels in each section were evaluated, with approximately 10 vessels/heart analyzed.

2.8 | Statistics

Data were presented as mean ± standard deviation (SD) where appropriate. Mann-Whitney tests were used for analysis of nonparametric data. Two-way analysis of variance (ANOVA) was employed for comparison of antinuclear and anti-vimentin autoantibody responses. Graft survival was depicted using Kaplan-Meier analysis and groups compared by log-rank (Mantel-Cox) testing. Analysis was conducted using GraphPad 4 (GraphPad Software, San Diego, CA). Values of $P < .05$ were considered significant.

3 | RESULTS

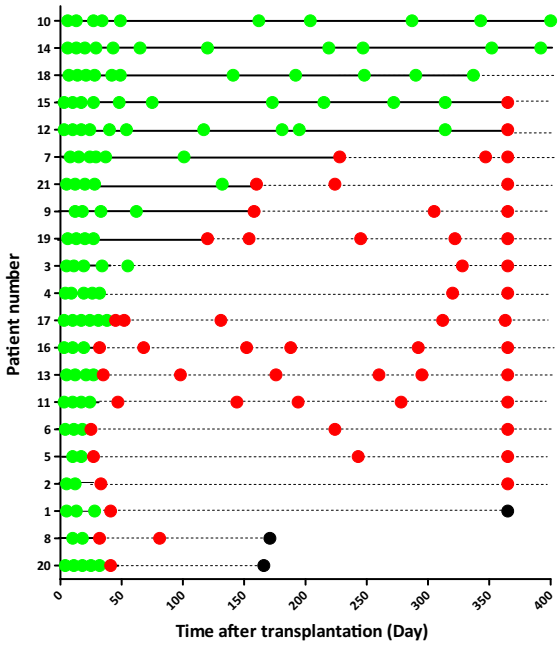
3.1 | Different CD4 T cell lineages are released from human allografts

Having previously demonstrated the presence of CD4 T effector cells within human organs recovered for transplantation,[6,12] we sought to determine whether donor CD4 T cells, and specifically, donor T-regs, could potentially also be released into the recipient's circulation following transplantation. Human lung transplant recipients (n = 21) were therefore followed for the first year following transplantation, and the presence of circulating donor-derived CD4 T cells determined by surface expression of mismatched HLA donor antigen (Figure S1). As shown in Figure 1, donor-derived CD4 T cells were detectable immediately following transplantation in all patients, representing between 0.06% and 6% of the total CD4 T cell population detectable in the recipient (mean chimerism at 1 week; 1.54 ± 1.41%). Numbers of cells recovered were too small to definitely assess different T cell lineages, but real-time polymerase chain reaction (RT-PCR) gene expression analysis of flow sorted donor CD4 T cells (not shown) revealed profiles consistent with naïve and $CD44^{hi}$ memory CD4 T cells, albeit samples from the same patient varied markedly at different time points, with no consistent phenotype observed. Notwithstanding, 3 different patterns of chimerism were evident (Figure 1A): transient (detectable for up to 6 weeks); intermediate (detectable for up to 6 months); or persistent (lasting for over a year).

The release of donor T-regs was then assessed by analysis of leukocyte filters recovered from human kidneys that had been obtained using standard recovery techniques, but then perfused normothermically ex vivo using leukocyte-depleted blood.[2] Hence leukocytes captured by the filter in the circuit reflect those cells that would be released into the recipient circulation had the organ been transplanted without first being subject to ex vivo perfusion. CD4 T cells were readily recovered from the filters and represented 6.57 ± 1.30% of the total lymphocyte population (Figure 1B). A small, but consistently present, population of CD4 T cells with surface T-reg phenotype ($CD25^{pos}CD127^{lo}$; 6.74 ± 4.73% of CD4 T cells) was also recovered (Figure 1B). T cells were not evident on analysis of the stored leukocyte-depleted blood used in the circuit (not shown), suggesting that the T-reg population had been released on reperfusion of the retrieved kidneys.

3.2 | T-reg depletion results in augmented humoral immunity and accelerated allograft rejection

The influence of donor and recipient T-regs on allograft outcomes was then examined using an MHC class II–mismatched murine model of chronic heart allograft rejection. Our previous work has highlighted that chronic allograft vasculopathy (CAV) in this model is associated with the development of effector autoantibody responses that are triggered by graft-versus-host recognition of MHC class II on host B cells by passenger donor CD4 T lymphocytes.[6,12,13] In comparison to unmodified WT C57BL/6 recipients, depletion of the T-reg population by administration of anti-CD25 mAb to C57BL/6 mice at, and following, transplantation with bm12 (B6(C)-H2-Ab1bm12/KhEgJ) heart allografts resulted in much more rapid heart graft rejection, and was associated with markedly augmented host autoantibody responses (Figure 2A,B). This accelerated rejection was nevertheless dependent on adoptive transfer of donor CD4 T cells, because heart


Patient number
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14
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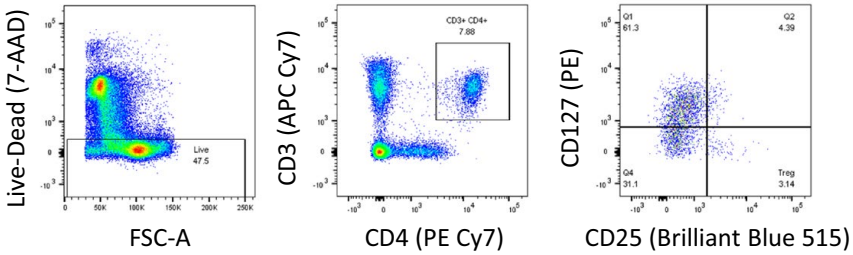
Time after transplantation (Day)


Live-Dead (7-AAD)
FSC-A
CD3 (APC Cy7)
CD4 (PE Cy7)
CD127 (PE)
CD25 (Brilliant Blue 515)

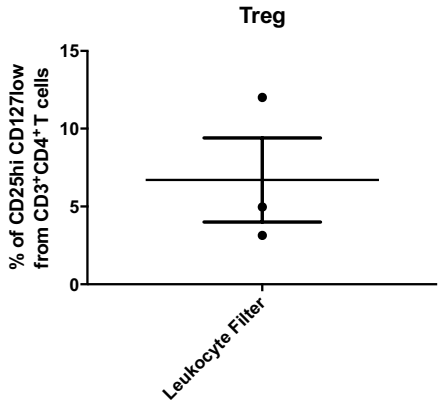

Treg
% of CD25hi CD127low from CD3+CD4+T cells
15
10
5
0
Leukocyte Filter

FIGURE 1 Solid organ human transplants contain passenger CD4 T lymphocyte subsets. A, Donor HLA class I mismatched antigens were used as a target for detection of donor CD4 T cell chimerism in lung transplant recipients using flow cytometry. Three patterns of donor CD4 T cell chimerism were observed: short-term chimerism (donor CD4 T cells detectable for up to six weeks after transplantation [patients 20, 8, 1, 2, 5, 6, 11, 13, 16, 17, 3, 4 and 19]); intermediate-term chimerism (donor CD4 T cells detectable up to 6 months after transplantation [patients 9, 21 and 7]), and long-term chimerism (donor CD4 T cells detectable for longer than one year after transplantation [patients 12, 15, 18, 14 and 10]). Green dot, blood sample tested and donor CD4 T cells detected. Red dot, blood sample tested, donor CD4 T cells not detected. Black dot, patient died. B, Representative flow cytometry plots for analysis of live CD4 T cells recovered from leukocyte filters of human kidney organs undergoing ex vivo normothermic perfusion. Histogram depicts the proportion of CD3+ve CD4+ve T lymphocytes that expressed $CD25^{hi}CD127^{lo}$ T regulatory cell surface phenotype (n = 3)

allografts from T cell–deficient bm12.$TCR^{-/-}$ donors did not trigger host autoantibody responses and survived indefinitely, without developing CAV (Figure 2C), even following recipient T-reg depletion (Figure 2A). This suggests that the T-regs were principally influencing the donor T cell/host B cell axis.

3.3 | Donor-derived T-regs prolong allograft survival more effectively than recipient T-regs

In the preceding experiments, anti-CD25 treatment of the recipient was continued after transplantation, raising the possibility that

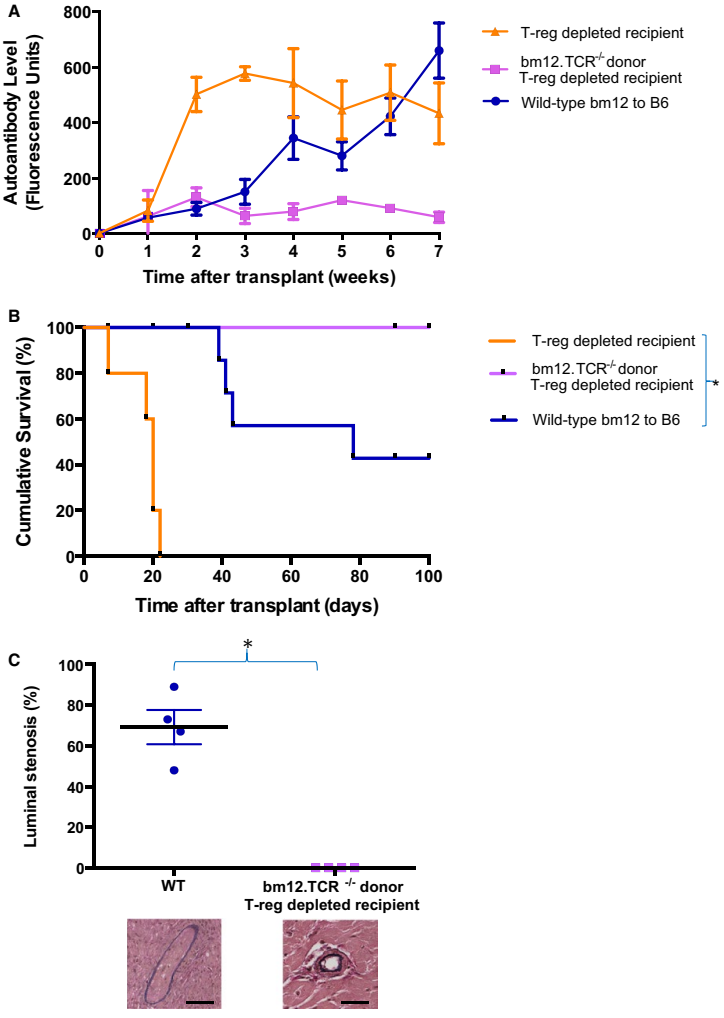


FIGURE 2 T-reg depletion augments donor T cell–dependent effector autoantibody responses and accelerates allograft rejection. MHC-class II mismatched cardiac allografts from WT or T cell–deficient ($TCR^{-/-}$) bm12 donor mice were transplanted into unmodified WT C57BL/6 (B6) or T-reg–depleted recipients and effector autoantibody responses (A), allograft survival (B), and allograft vasculopathy at explant on day 100 (C) was assessed (allograft vasculopathy for T-reg–depleted recipients of WT bm12 heart allografts were not analyzed because of rapid graft destruction). T-reg depletion results in augmented autoantibody responses ($P = .04$, Kruskal-Wallis test) and rapid allograft rejection (*$P < .0001$, log-rank test), but this impact is dependent on transfer of passenger donor T cells. Representative elastin van Gieson staining showing allograft vasculopathy in WT recipients compared to nondiseased vessels in ($TCR^{-/-}$) bm12 hearts transplanted into T-reg–depleted recipients (scale bars 100 μM). *$P = .03$, Mann-Whitney test. Data are expressed as mean ± SD and represents a minimum of 4 animals per group

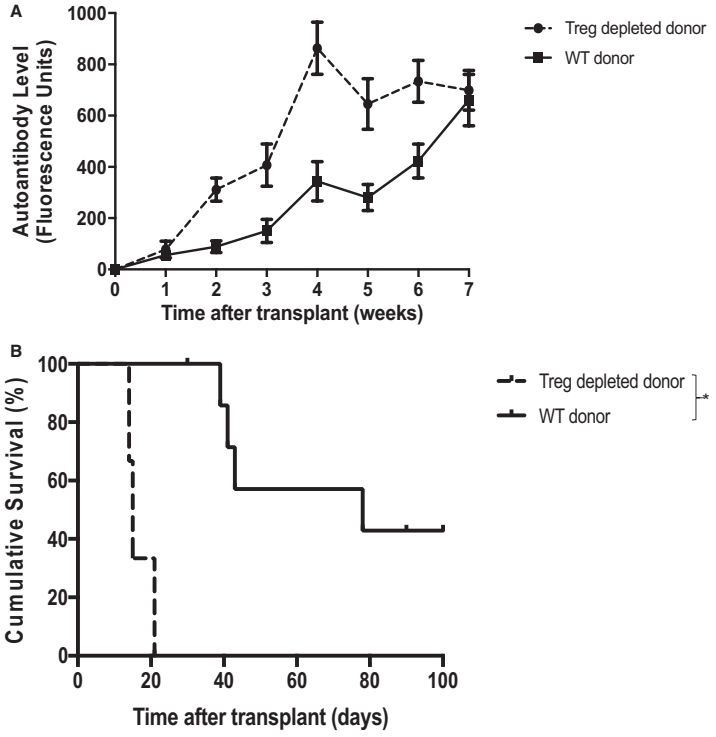


FIGURE 3 Donor T-reg depletion results in exacerbated autoantibody production and accelerated graft loss. Heart allografts from unmodified (WT) or T-reg–depleted bm12 donor mice were transplanted into WT C57BL/6 mice and effector autoantibody responses (A) and allograft rejection (B) were assessed. Compared to unmodified donor hearts, donor T-reg depletion results in acute allograft rejection (median survival time [MST] 14 days vs 78 days; $^{*}P < .01$, log-rank test), with markedly augmented recipient autoantibody responses ($^{**}P < .001$, 2-way ANOVA). Data expressed as mean ± SD, n = 4

transferred donor T-regs were also targeted. Notably, transplantation of heart allografts from donor bm12 mice that had received anti-CD25 treatment before organ recovery also triggered markedly augmented autoantibody responses in WT C57BL/6 recipient mice, and heart allografts were rejected at least as rapidly as following recipient T-reg depletion (Figure 3A,B). To test the comparative efficacy of donor versus recipient-derived T-regs in preventing allograft rejection, WT C57BL/6 recipients of unmodified bm12 heart grafts were additionally transferred with nT-regs,[14,15] purified from either the recipient or donor strains. Of interest, whereas transfer of recipient-strain nT-regs had little discernible impact on transplant outcome, transfer of donor-strain nT-regs was associated with abrogation of recipient autoantibody responses, a reduction in the severity of CAV, and prolonged allograft survival (Figure 4A-C).

4 | DISCUSSION

Our results demonstrate that following solid organ transplantation, donor-derived CD4 T cells are released into the recipient circulation, and, at least following lung transplantation, may persist for some time. Within a larger population of conventional CD4 T effector cells, smaller numbers of regulatory T cells can be identified, and our murine studies confirm that these can inhibit host adaptive immune responses. These findings may hold particular pertinence to ex vivo organ perfusion strategies currently being developed; they highlight that rather than blanket depletion, preservation of select passenger lymphocyte subsets within the allograft may be beneficial.

It is perhaps surprising that donor-derived nT-regs were more effective than recipient-derived nT-regs at blocking host humoral responses. Although the precise target epitopes remain ill-defined,[16,17] nT-regs are thought to recognize specific, self-restricted peptide epitopes (typically autoantigens[18]). Donor-derived nT-regs therefore presumably recognize intact host MHC class II complexes on recipient cells via the direct pathway,[19] and in which case, do so with a much greater precursor frequency than for a self-restricted response, with approximately 5% of the clonal repertoire responding.[20] We have recently demonstrated that this enables naïve donor T cells to provide promiscuous, "peptide-degenerate" help to all host B cells, with plasma cell differentiation dictated by simultaneous B cell receptor ligation.[6,21] By extension, recognition of MHC class II alloantigen on host B cells by passenger T-regs within the allograft is likely to provide broad inhibition of host humoral immunity. Whether this inhibition is the result of direct killing of the B cell by the T-reg,[22-25] or delivery of inhibitory signals to the B cell,[26,27] or blockade of delivery of essential help from CD4 T effector cells is as yet unknown and is the subject of ongoing investigation in our laboratory. Of particular interest, our recent work has highlighted a critical role for germinal center autoantibody[28] and alloantibody[29,30] reactions in the progression of allograft vasculopathy, and our ongoing

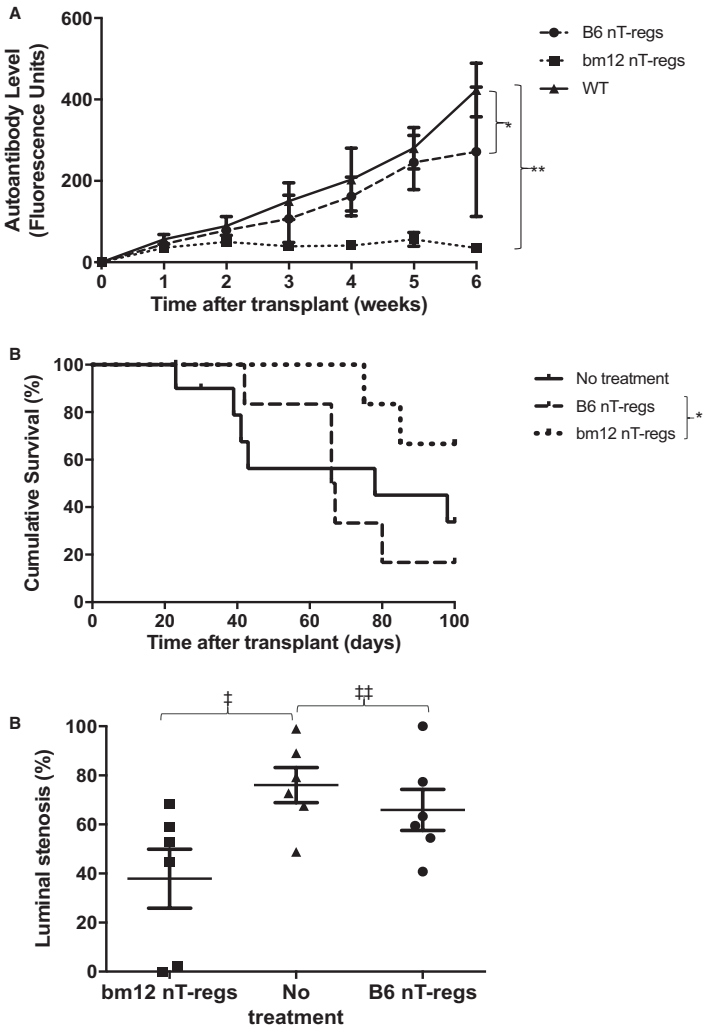


FIGURE 4 Adoptive transfer of donor nT-reg inhibits recipient autoantibody responses and prolongs allograft survival. C57BL/6 (B6) recipients of bm12 heart allografts were adoptively transferred the day after transplantation with natural T-regs (nT-regs) purified from a donor (bm12) or recipient (B6) strain, and recipient autoantibody responses (A), allograft survival (B), and allograft vasculopathy (C) were assessed as detailed in Figure 2 legend. Control recipients received no treatment. Whereas administration of recipient-strain nT-regs had little impact on rejection responses or rejection kinetics, administration of donor-strain nT-regs inhibited effector autoantibody responses (*P = .27, **P < .001, 2-way ANOVA), prolonged allograft survival (MST 91 vs 67 days; *P = .03. log-rank test) and was associated with reduction in the severity of allograft vasculopathy (‡P = .02, ‡‡P = .38; Mann-Whitney test). Data are representative of 6 animals per group, and expressed as mean ± SD, n = 6

investigations are examining the impact of donor-derived T-regs on host germinal center B cell/T follicular helper cell interactions.

In addition to providing support for strategies that selectively retain donor T-regs within the allograft, our results suggest that donor-derived T-regs may hold potential as a cellular therapy for prolonging allograft survival. This would differ from strategies that are currently under evaluation clinically, and that typically employ recipient-derived CD4 T-regs that are either polyclonal or exhibit direct allospecificity for the donor.[31] In a similar fashion to donor effector CD4 T cells (that provide promiscuous help to all B cells engaging target antigen), transferred donor-derived T-regs would be expected to inhibit host B cell responses against concurrently encountered alloantigen, even those alloantigens that are expressed on the T-reg surface.[6] Thus, it seems probable that donor-derived T-regs will be effective in transplant models incorporating donor-recipient strain combinations that are more MHC-mismatched; certainly, direct-pathway allorecognition of host MHC class II by donor-derived T-regs is likely to be at least as robust in more mismatched strain combinations as in the bm12 to B6 model. For the same reasoning, we would anticipate that bm12 nT-regs could be used as a cellular therapy to block host B cell alloresponses against a variety of different donor-strain transplants into B6 recipients. Potency of this approach could be enhanced by either increasing the proportion of T-regs within the transferred population that exhibit direct-pathway allospecificity, or by first generating memory T-regs directed against intact host MHC class II.[32] In this regard, it is notable that heart allografts that contain memory CD4 T cells specific for host MHC class II (by priming the

donor with recipient alloantigen 6 weeks prior to heart donation) are rejected much more rapidly than hearts from unmodified donors, with greatly augmented autoantibody responses.[6,28]

Such a use of third-party T-regs to block host humoral alloimmunity would be distinctly different from proposed strategies that differentiate/expand T-regs with self-restricted specificity for alloantigen from the individual's endogenous T cell population,[25,33] and may offer a particular advantage. T cell help for alloantibody production can only be provided by host CD4 T cells with indirect allospecificity.[34-36] Thus, for maximum effectiveness, recipient-derived T-regs would need to recognize the relevant allopeptide epitope presented by host MHC class II. Prediction of these peptides is, however, challenging, not least because the repertoire of presented allopeptide peptides may change with time.[37] In contrast, third-party T-regs with direct allospecificity would be expected to interact with the individual's B cells in a peptide-degenerate fashion, and would therefore potentially block all concurrently active B cell responses. The crucial attribute in enabling donor-derived T-regs to inhibit host B cell responses is avoidance of recognition and killing by host Natural Killer cells.[6] Thus, only third-party donors that are minimally MHC mismatched against the individual are likely to be effective. This limitation could be overcome by transduction of an individual's purified nT-reg population with T cell receptor (*TCR*) genes,[38] which encode direct-pathway reactivity to that individual's own MHC class II, with the relevant *Tcrα* and *Tcrβ* sequences first established by identifying dividing clones in standard mixed leukocyte reactions using third-party cells as responders against recipient stimulators.[39] This would generate autologous CD4 T-regs with heightened specificity for self.

This approach may have wider uses beyond transplantation. It could, for example, be refined as a potential treatment for humoral autoimmunity, wherein nT-regs from a third-party donor that have direct-pathway allospecificity for the individual's (recipient's) MHC class II antigens would be expected to block cognate interactions between autoreactive B and T helper cells in the host, thereby inhibiting autoantibody production.

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DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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10.1.2 Impact of donor mismatches at individual HLA-A, -B, -C, -DR, and -DQ loci on the development of HLA-specific antibodies in patients listed for repeat renal transplantation.

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Impact of donor mismatches at individual HLA-A, -B, -C, -DR, and -DQ loci on the development of HLA-specific antibodies in patients listed for repeat renal transplantation

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We have analyzed the relationship between donor mismatches at each HLA locus and development of HLA locus–specific antibodies in patients listed for repeat transplantation. HLA antibody screening was undertaken using single-antigen beads in 131 kidney transplant recipients returning to the transplant waiting list following first graft failure. The number of HLA mismatches and the calculated reaction frequency of antibody reactivity against 10,000 consecutive deceased organ donors were determined for each HLA locus. Two-thirds of patients awaiting repeat transplantation were sensitized (calculated reaction frequency over 15%) and half were highly sensitized (calculated reaction frequency of 85% and greater). Antibody levels peaked after re-listing for repeat transplantation, were independent of graft nephrectomy and were associated with length of time on the waiting list (odds ratio 8.4) and with maintenance on dual immunosuppression (odds ratio 0.2). Sensitization was independently associated with increasing number of donor HLA mismatches (odds ratio 1.4). All mismatched HLA loci contributed to the development of HLA locus–specific antibodies (HLA-A: odds ratio 3.2, HLA-B: odds ratio 3.4, HLA-C: odds ratio 2.5, HLA-DRB1: odds ratio 3.5, HLA-DRB3/4/5: odds ratio 3.9, and HLA-DQ: odds ratio 3.0 (all significant)). Thus, the risk of allosensitization following failure of a first renal transplant increases incrementally with the number of mismatches at all HLA loci assessed. Maintenance of re-listed patients on dual immunosuppression was associated with a reduced risk of sensitization.

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KEYWORDS: HLA; immunosuppression; matching; nephrectomy; re-transplantation; sensitization

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The immune response to a kidney allograft is directed principally against mismatched human leukocyte antigen (HLA) glycoproteins expressed on donor tissue. Historically, deceased donor kidney allocation policies aimed to minimize the number of HLA mismatches between donors and recipients in order to help reduce the incidence of acute rejection and to improve graft survival.[1] In the context of modern immunosuppressive therapy, the beneficial effect of HLA matching on graft survival has diminished and kidney allocation algorithms now place considerably less emphasis on HLA matching.[2–4] HLA matching, however, remains important for those patients who have developed HLA-specific antibodies after prior exposure to HLA alloantigens, because of pregnancy, blood transfusion, or a failed renal transplant.[5] It is important in such patients to avoid donor HLA mismatches to which they are sensitized as this may result in antibody-mediated rejection that is refractory to treatment. For highly sensitized patients who have antibodies to the majority of the potential donor population, the need to identify an HLA antibody–compatible (cross-match negative) donor kidney markedly prolongs the waiting time to transplantation and may make transplantation almost impossible.

Recipients of a deceased donor kidney transplant can expect, on average, to maintain a functioning transplant for approximately 12–15 years, after which repeat transplantation is required, particularly in younger patients with a longer life

expectancy. Around 23% of patients listed for renal transplantation in the United Kingdom are awaiting a repeat transplant and most have developed donor HLA–specific antibodies. Such antibodies are frequently cross-reactive and bind a wide range of HLA specificities with shared epitopes commonly expressed on many different HLA alleles,[6] limiting the opportunity for repeat transplantation.[7–9]

It is generally assumed that recipients of a kidney allograft mismatched for multiple HLA alleles are more likely to develop donor HLA–specific antibodies, although the evidence to support this assumption is very limited and is based on an old technology that is unable to resolve complex alloantibody profiles.[10–13] The relative influence of HLA mismatches at individual loci on alloantibody responses in recipients returning to the transplant waiting list after a failed first transplant has not been reported previously.

We have used Luminex single-antigen bead technology to assess the influence of an HLA-mismatched first kidney transplant on development of alloantibodies in patients returning to the transplant waiting list following graft failure.

RESULTS

The demographic and clinical details of the 131 patients studied are shown in Table 1. The UK allocation policy for deceased donor kidneys favors HLA-DR matching over HLA-A and -B matching and, as a consequence, the cohort generally received first kidney transplants that were moderately well HLA matched, particularly for HLA-DR. In the case of HLA class I (HLA-A, -B, and -C), there were 0–1 mismatches in 14 (11%) patients, 2–4 mismatches in 93 (71%) patients, and 5–6 mismatches in 24 (18%) patients. In the case of HLA class II (HLA-DRB1, -DRB3/4/5, and -DQB1), there were 0–1 mismatches in 64 (49%) patients, 2–4 mismatches in 61 (46%) patients, and 5–6 mismatches in 6 (5%) patients.

HLA mismatch and calculated reaction frequency (cRF) determined using Luminex single-antigen HLA antibody-detection beads

Of the 131 patients studied, 17 (13%) were sensitized to HLA class I (>15% cRF) before their first transplant; 12 of these patients (71%) received kidneys with 0–4 HLA class I mismatches, reflecting the requirement to avoid a positive cross-match (Figure 1a). At the time of return to the transplant waiting list, 44 (34%) patients were sensitized to HLA class I; when serum samples with peak cRF were considered, 80 (61%) patients were sensitized, of whom 50 (38% of the total) were highly sensitized (cRF ⩾85%). Patients who received kidneys with the greatest number of HLA-A, -B, and -C mismatches more commonly developed antibodies to HLA class I while on the waiting list for re-transplantation: it was notable that none of the 24 patients who received a kidney with 5–6 class I mismatches were highly sensitized at the time of transplantation, whereas 13 of the 24 (54% of the total) became highly sensitized while awaiting a second transplant.

Table 1 | Study population characteristics

Patient cohort characteristics	*N* = 131 (%)
Primary transplant donor type[a]	
DBD donor	76 (58)
DCD donor	25 (19)
Live donor	25 (19)
Simultaneous kidney and pancreas transplant (DBD)	4 (3)
Recipient ethnicity	
White Caucasian	117 (89)
Black African	3 (2)
Asian (Indian/Pakistani)	8 (6)
Oriental	3 (2)
Recipient gender	
Male	87 (66)
Female	44 (34)
Donor age (years), median (range)	49 (10–78)
Recipient age (years), median (range)	38 (14–73)
Number of HLA-A, -B, -C mismatches[b]	
0–1	14 (11)
2-4	93 (71)
5-6	24 (18)
Number of HLA-DRB1, -DRB3/4/5, -DQB1 mismatches	
0–1	64 (49)
2-4	61 (46)
5-6	6 (5)
Primary diagnosis	
Glomerulonephritis	22 (17)
Vasculitis	10 (7)
Polycystic disease	8 (6)
Reflux nephropathy	8 (6)
Diabetes	14 (11)
Hypertension	6 (5)
IgA nephropathy	19 (15)
Other	44 (33)
Time from 1st transplant to re-listing (days), median (range)	1298 (3–5132)
Time from re-listing to end of follow-up (days), median (range)	937 (14–4809)

Abbreviations: DBD, donation after brain death; DCD, donation after circulatory death; HLA, human leukocyte antigen; IgA, immunoglobulin A.
[a]Donor type (DBD or DCD) was not known for one deceased donor kidney transplant.
[b]HLA-C typing information was not available for six cases. When HLA-C type was missing, HLA class I mismatch grade was calculated based on HLA-A and -B types.

Donor mismatches at each of the HLA-A, -B, and -C loci all contributed to an increasing likelihood of developing sensitization to HLA class I, but HLA-C less than HLA-A and -B (Figure 1b–d and Table 2, column 1). Donor mismatches at HLA-A and -B were also associated with development of antibodies to an increasing number of HLA-A and -B specificities, as defined by increasing cRF (Figure 1 and Table 2, column 2; odds ratio (OR): 1.4, 95% confidence interval (CI): 1.2–1.8 per mismatch for HLA-A, $P = 0.002$ and OR: 1.3, 95% CI: 1.1–1.6 per mismatch for HLA-B, $P = 0.006$).

We also considered whether mismatches at 'public' HLA class I epitopes had a disproportionally strong effect on the development of high cRF levels. We examined the effect of

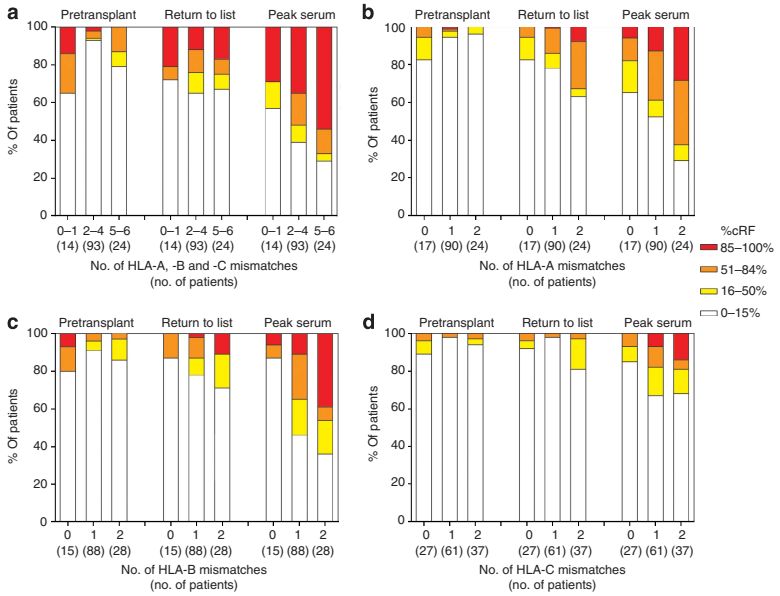

Figure 1 | Association between first transplant human leukocyte antigen (HLA) class I (HLA-A, -B, and -C) mismatches and HLA-specific sensitization expressed as calculated reaction frequency (cRF). HLA class I–specific alloantibodies were detected using single-antigen HLA beads (with a median fluorescence intensity (MFI) cutoff threshold of 2000); the likelihood of identifying an antibody-compatible organ donor (cRF) was determined by comparing individual patient HLA-specific antibody profiles with the HLA types of 10,000 consecutive UK deceased organ donors. Panel (**a**) shows cRF levels attributable to antibodies against HLA-A, -B, and -C considered collectively according to the total number of donor–recipient HLA-A, -B, and -C mismatches (0–6) at the three different time points: pretransplant, on return to the transplant waiting list, and at peak cRF while on the waiting list. Panels (**b**–**d**) show the cRF attributable to antibodies against HLA-A, HLA-B, and HLA-C, respectively, according to the number of HLA specificities (0, 1, or 2) mismatched at the individual HLA loci. Patients were categorized according to the likelihood of identifying an antibody-compatible organ donor as cRF 0–15%, cRF 16–50%, cRF 51–84%, and cRF 85–100%. cRF levels attributable to antibodies against each of the HLA class I loci and for HLA class I loci considered collectively increased between the three different time points (Stuart–Maxwell test $P<0.01$), with the exception of the comparison between pretransplant and return to the transplant waiting list for HLA-C ($P=0.427$). The number of patients within each cRF category at the three different time points is depicted in Supplementary Table S1A online stratified according to the number of HLA mismatches.

Table 2 | Influence of HLA mismatches on the likelihood of developing HLA-specific allosensitization after re-listing for repeat transplantation

	Likelihood of developing sensitization to individual HLA loci per mismatch		Likelihood of increasing cRF for individual HLA loci per mismatch	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
HLA-A	3.2 (2.0, 4.7)	<0.001	1.4 (1.2, 1.8)	0.002
HLA-B	3.4 (2.2, 4.9)	<0.001	1.3 (1.1, 1.6)	0.006
HLA-C	2.5 (1.5, 3.5)	<0.001	1.2 (1.0, 1.5)	0.074
HLA-DRB1	3.5 (2.3, 5.5)	<0.001	1.3 (1.0, 1.6)	0.015
HLA-DRB3/4/5	3.9 (2.4, 7.8)	<0.001	1.3 (1.1, 1.7)	0.011
HLA-DQ	3.0 (2.0, 4.3)	<0.001	1.4 (1.1, 1.8)	0.003

Abbreviations: CI, confidence interval; cRF, calculated reaction frequency; HLA, human leukocyte antigen; MFI, median fluorescence intensity; OR, odds ratio.
The influence of mismatches at individual HLA loci on the likelihood of developing HLA locus-specific alloantibodies is shown in column 1, using an MFI threshold of >2000. Column 2 shows the influence of mismatches at individual HLA loci on the likelihood of developing HLA locus-specific alloantibodies to an increasing number of HLA specificities, as defined by increasing cRF, using an MFI threshold of >2000.

mismatches at the most common 'public' epitopes Bw4 and Bw6 and found that the impact of a mismatch at Bw4 or Bw6 on cRF was no greater than that observed for HLA-B mismatches when there was no mismatch at either Bw4 or Bw6 (Supplementary Figure S1 online).

In the case of HLA class II (Figure 2a), only 7 (5%) of the 131 patients were sensitized to HLA class II (>15% cRF) at the time of their first transplant and none were highly sensitized (cRF ⩾85%). On return to the waiting list, 29 (22%) patients were sensitized, and when serum samples with peak cRF were considered this number rose to 56 (43%) patients, of whom 29 (22% of the total) became highly sensitized. Patients who received kidneys with the greatest number of HLA-DRB1, -DRB3/4/5, and -DQ mismatches more commonly developed alloantibodies to HLA class II while on the transplant waiting list (Figure 2a), although few ($n=6$) patients received a kidney with 5–6 class II mismatches.

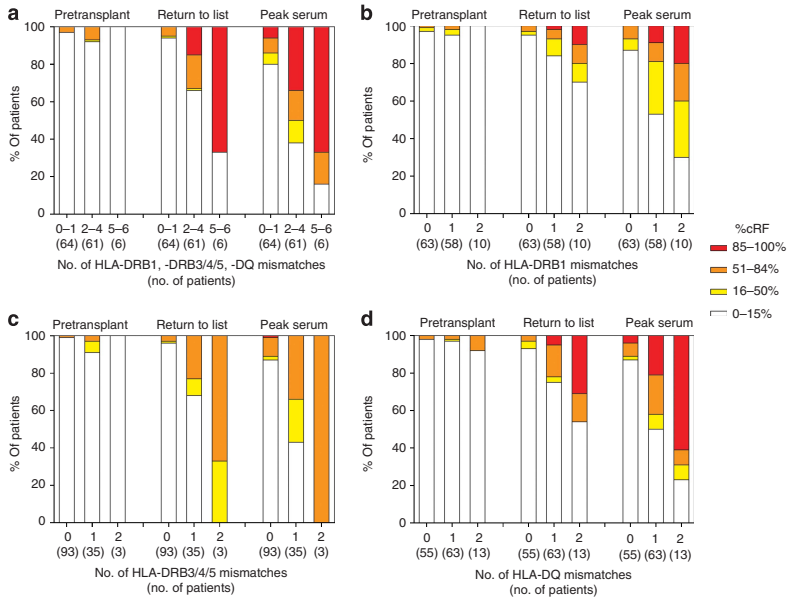

Figure 2 | Association between first transplant human leukocyte antigen (HLA) class II (HLA-DRB1, -DRB3/4/5, and -DQ) mismatches and HLA-specific sensitization expressed as calculated reaction frequency (cRF). HLA class II–specific alloantibodies were detected using single-antigen HLA beads (with a median fluorescence intensity (MFI) cutoff threshold of 2000); donor organ incompatibility (cRF) was determined by comparing individual patient HLA-specific antibody profiles with the HLA types of 10,000 consecutive UK deceased organ donors. Panel (**a**) shows cRF levels attributable to antibodies against HLA-DRB1, -DRB3/4/5, and -DQ considered collectively, according to the total number of donor–recipient HLA-DRB1, -DRB3/4/5, and -DQ mismatches (0–6) at the three different time points: pretransplant, on return to the transplant waiting list and at peak cRF while on the waiting list. Panels (**b**–**d**) show the cRF attributable to antibodies against HLA-DRB1, HLA-DRB3/4/5, and HLA-DQ, respectively, according to the number of HLA specificities (0, 1, or 2) mismatched at the individual HLA loci. Patients were categorized according to the likelihood of identifying an antibody-compatible organ donor as cRF 0–15%, cRF 16–50%, cRF 51–84%, and cRF 85–100%. cRF levels attributable to antibodies against each of the HLA class II loci and for HLA class II loci considered collectively increased between the three different time points (Stuart–Maxwell test $P < 0.01$). The number of patients within each cRF category at the three different time points is depicted in Supplementary Table S1A online stratified according to the number of HLA mismatches.

Donor mismatches at each of the HLA-DRB1, -DRB3/4/5, and -DQ loci all contributed to sensitization to HLA class II, which became most apparent after return to the waiting list for re-transplantation (Figure 2b–d and Table 2, column 1). Donor mismatches at each HLA class II locus were associated with development of antibodies to an increasing number of HLA class II specificities as defined by increasing cRF (Figure 2 and Table 2, column 2; OR: 1.3, 95% CI: 1.0–1.6 per mismatch for HLA-DRB1, $P = 0.015$, OR: 1.3, 95% CI: 1.1–1.7 per mismatch for HLA-DRB3/4/5, $P = 0.011$ and OR: 1.4, 95% CI: 1.1–1.8 per mismatch for HLA-DQ, $P = 0.003$).

When HLA class I and class II were considered collectively (HLA-A, -B, -C, -DRB1, -DRB3/4/5, and -DQ combined), analysis of peak reactive sera while on the list for re-transplantation showed that the percentage of sensitized patients with a cRF $>15\%$ was 47% for first grafts mismatched for 0–2 HLA specificities, rising to 65% for 3–5 mismatched specificities, and 80% for 6–12 mismatched specificities (Figure 3). The percentage of sensitized patients with a cRF $\geqslant 85\%$ for 0–2, 3–5, and 6–12 HLA mismatches was 32%, 44%, and 63%, respectively. This and all previous calculations of cRF are based on an median fluorescence intensity (MFI) cutoff threshold of 2000, the widely used threshold above which donor-specific antibodies are considered to have potential clinical relevance. For MFI cutoff levels at higher thresholds (>5000 and >8000), indicating higher levels of circulating alloantibody, cRF values also increased markedly with the total number of HLA class I and II mismatches, and even for an MFI cutoff of >8000, 41% of patients transplanted with a poorly matched first graft (6–12 mismatches) were classified as highly sensitized (Figure 3).

As expected, mismatches at individual HLA class I loci were commonly associated with the development of alloantibodies to epitopes shared by serologically cross-reactive specificities but there was no evidence for development of alloantibodies against alleles of a different HLA class (donor mismatches at HLA class II did not influence the development of alloantibodies against HLA class I, and vice versa).

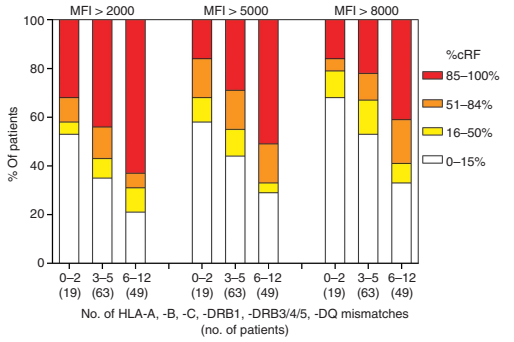

Figure 3 | Association between first transplant human leukocyte antigen (HLA) class I and II mismatches and peak HLA-specific sensitization according to different levels (median fluorescence intensity (MFI)) of circulating alloantibodies. HLA-specific alloantibodies were identified in sera from patients re-listed for repeat transplantation, using HLA single-antigen beads. Donor organ incompatibility (calculated reaction frequency (cRF)) was determined by comparing individual patient HLA-specific antibody profiles with the HLA types of 10,000 consecutive UK deceased organ donors. The figure shows cRF levels in peak reactive sera attributable to antibodies against HLA-A, -B, -C, -DRB1, -DRB3/4/5, and -DQ considered collectively, according to the total number of first transplant donor HLA mismatches (0–12). Alloantibodies were analyzed at the three different MFI cutoff thresholds (2000, 5000, and 8000), where alloantibodies present at increasing thresholds indicate increasing immunological risk for repeat kidney transplantation. Patients were categorized according to the likelihood of identifying an antibody-compatible organ donor as cRF 0–15%, cRF 16–50%, cRF 51–84%, and cRF 85–100%. The number of patients within each cRF category is depicted in Supplementary Table S1B online stratified according to the number of HLA class I and class II mismatches.

Although the focus of our analysis was on the use of Luminex-based single-antigen beads to determine the effect of HLA mismatch at individual loci on allosensitization, sera were also tested for the presence of clinically relevant immunoglobulin G lymphocytotoxic antibodies to lymphocyte panels (peripheral blood lymphocyte and B-cell chronic lymphocytic leukemia) incorporating a broad range of HLA types. The results are summarized in Supplementary Figure S2 online and show that an increasing number of HLA class I and class II mismatches results in increasing levels of immunoglobulin G lymphocytotoxic panel-reactive antibodies (PRAs).

Analysis of factors associated with development and levels of HLA-specific antibodies after return to the kidney transplant waiting list

Table 3 shows a univariate analysis of the association of donor–recipient HLA mismatch and other clinical variables with the development of sensitization after re-listing for repeat transplantation. First graft HLA mismatch was strongly associated with sensitization and each additional HLA class I and class II donor mismatch increased the likelihood of developing HLA-specific alloantibodies incrementally (OR 1.41, 95% CI: 1.15–1.74 per mismatch, $P = 0.001$). An additional clinical variable associated with

Table 3 | Influence of donor and patient factors on the likelihood of developing HLA-specific alloantibodies (adjusted for pretransplant sensitization, MFI threshold of >2000)

Variable	Value	Adjusted odds ratio	95% CI	Wald *P*
Donor type[a]	Deceased ($n = 106$)	1.79	0.70, 4.58	0.224
	DBD ($n = 79$)	1.64	0.62, 4.37	0.315
	DCD ($n = 26$)	2.25	0.70, 7.69	0.183
	Live ($n = 25$)	—	—	—
Donor age[b]	0–17 ($n = 3$)			
	18-49 ($n = 59$)	0.87	0.48, 1.89	0.731
	50+ ($n = 53$)	—	—	—
Recipient age	0–17 ($n = 4$)			
	18-49 ($n = 97$)	0.65	0.24, 1.61	0.357
	50+ ($n = 30$)	—	—	—
HLA class I + II mismatches[c]	Per mismatch	1.41	1.15, 1.74	**0.001**
Immunosuppression at time of re-listing[d]	None ($n = 48$)	—	—	—
	Single ($n = 27$)	1.08	0.38, 3.31	0.871
	Dual ($n = 53$)	0.68	0.28, 1.59	0.377
Immunosuppression while on the waiting list[e]	None ($n = 61$)	—	—	—
	Single ($n = 36$)	0.90	0.33, 2.51	0.841
	Dual ($n = 30$)	0.15	0.05, 0.40	**<0.001**
Nephrectomy	Yes ($n = 56$)	3.42	1.51, 8.30	**0.004**
	No ($n = 75$)	—	—	—
Blood transfusion after transplantation	Yes ($n = 118$)	2.01	0.61, 6.64	0.253
	No ($n = 13$)	—	—	—
Blood transfusion after listing for re-transplantation	Yes ($n = 41$)	1.91	0.73, 4.26	0.225
	No ($n = 90$)	—		
Recipient gender	Female ($n = 44$)	1.61	0.67, 4.09	0.292
	Male ($n = 87$)	—	—	—
Pregnancy	Yes ($n = 31$)	1.44	0.88, 2.74	0.185
	No ($n = 13$)	—	—	—
Time from first transplant to re-listing	⩽1 year ($n = 45$)	—	—	—
	1–5 years ($n = 40$)	0.77	0.29, 2.02	0.595
	>5 years ($n = 46$)	0.49	0.19, 1.22	0.133
Time from re-listing to end of follow-up	⩽1 year ($n = 27$)	—	—	—
	1–5 years ($n = 68$)	3.53	1.38, 9.43	**0.010**
	>5 years ($n = 36$)	8.36	2.58, 31.16	**<0.001**

Abbreviations: CI, confidence interval; DBD, donation after brain death; DCD, donation after circulatory death; HLA, human leukocyte antigen; MFI, median fluorescence intensity.
[a]Donor type (DBD or DCD) was not known for one deceased donor kidney transplant.
[b]Donor age information was missing for 16 cases.
[c]HLA-C typing information was not available for six cases.
[d]Immunosuppression information was not available for three patients at the time of re-listing.
[e]Immunosuppression information was not available for four patients while on the waiting list.
Bold values indicate greater significance than $P < 0.05$.

Table 4 | Multiple variable analysis: influence of donor and patient factors on the development of HLA-specific alloantibodies (adjusted for pretransplant sensitization, MFI threshold of >2000)

Variable	Value	Adjusted odds ratio	95% CI	Wald *P*
Likelihood of developing HLA-specific alloantibodies				
HLA class I + II mismatches	Per mismatch	1.40	1.10, 1.79	**0.009**
Immunosuppression while on the waiting list				
	None ($n=61$)	—	—	—
	Single ($n=36$)	1.19	0.35, 3.05	0.975
	Dual ($n=30$)	0.24	0.06, 0.61	**0.005**
Time from re-listing to end of follow-up				
	⩽1 year ($n=27$)	—	—	—
	1–5 years ($n=68$)	2.80	0.80, 7.28	0.119
	>5 years ($n=36$)	6.33	1.28, 20.4	**0.024**
Likelihood of developing increasing levels of HLA-specific sensitization (increasing cRF)[a]				
HLA class I + II mismatches	Per mismatch	1.13	0.97, 1.30	0.115
Immunosuppression while on the waiting list				
	None ($n=61$)	—	—	—
	Single ($n=36$)	1.11	0.53, 2.05	0.899
	Dual ($n=30$)	0.43	0.14, 0.96	**0.048**
Time from re-listing to end of follow-up				
	⩽1 year ($n=27$)	—	—	—
	1–5 years ($n=68$)	3.25	1.35, 6.74	**0.008**
	>5 years ($n=36$)	4.68	1.76, 10.35	**0.002**

Abbreviations: CI, confidence interval; cRF, calculated reaction frequency; HLA, human leukocyte antigen; MFI, median fluorescence intensity.
[a]This analysis included those patients from the initial cohort that developed HLA-specific alloantibodies following allograft failure (cRF>0).
Bold values indicate greater significance than $P<0.05$.

development of HLA-specific antibodies was the length of time on the waiting list (>5 years vs. <1 year after re-listing, OR 8.36, 95% CI: 2.58–31.16, $P<0.001$). Continuation of two or more immunosuppressive agents (but not single-agent immunosuppression) following re-listing for transplantation was associated with a reduced likelihood of sensitization (OR 0.15, 95% CI: 0.05–0.40, $P<0.001$). Of the 36 patients maintained on single-agent immunosuppression, 32 (89%) were given prednisolone. Of the 30 patients receiving multiple agents, 28 (93%) were on prednisolone, 21 (70%) on a calcineurin inhibitor, 16 (53%) on an antiproliferative agent (mycophenolic acid or azathioprine), and 2 (7%) on a mammalian target of rapamycin inhibitor. Multivariate analysis confirmed that HLA mismatch, length of time on the waiting list following re-listing, and use of dual-agent immunosuppression while on the waiting list remained significantly associated with the likelihood of sensitization (Table 4). Graft nephrectomy was associated with HLA-specific sensitization on univariate analysis (OR 3.42, 95% CI: 1.51–8.30, $P=0.004$), but this effect was not apparent when withdrawal of immunosuppression was taken into account (79% of patients who underwent graft nephrectomy had immunosuppression completely withdrawn).

Table 5 shows a univariate analysis of the association of HLA mismatch and other variables with increasing cRF levels in the 92 patients who became sensitized while listed for repeat transplantation. When donor HLA mismatches for all loci (0–12 mismatches) were considered collectively, each additional HLA mismatch was associated with an incremental increase in cRF level (OR 1.16, 95% CI: 1.00–1.36, $P=0.065$), although the average effect size of each additional HLA mismatch was reduced by the inclusion of HLA-C (where there was no effect, Table 2, column 2), and because the effect of increasing number of HLA mismatches became less marked as the total number of mismatches rose. Multivariable analysis showed that maintenance of dual-agent immunosuppression and length of time on the waiting list after re-listing were independently associated with increasing cRF levels (Table 4).

DISCUSSION

The results of renal transplantation have improved progressively over the last two decades, but many recipients are likely to require repeat transplantation during their lifetime. The chances of a suitable HLA-compatible kidney being available for patients who require repeat transplantation are markedly reduced by the presence of HLA-specific antibodies that arise during or following failure of their first transplant. The results of this study show that the extent to which a failed first graft was mismatched for HLA is strongly associated with the development of HLA-specific antibodies while awaiting a repeat transplant. A strength of our study is that it is the most comprehensive analysis of this type to date and the first to use Luminex single-antigen bead technology to examine the effect of mismatches at each HLA locus on both the range and level of HLA-specific alloantibodies developing posttransplant, and their influence on likely access to re-transplantation. A weakness of the study is that HLA-DP typing data were not available for the study cohort and so we

Table 5 | Influence of donor and patient factors on the likelihood of developing HLA-specific alloantibodies to an increasing number of HLA specificities (increasing cRF) (adjusted for pretransplant sensitization, MFI threshold of >2000)[a]

Variable	Value	Adjusted odds ratio	95% CI	Wald *P*
Donor type[b]	Deceased ($n=106$)	0.79	0.36, 1.91	0.600
	DBD ($n=79$)	0.86	0.40, 2.00	0.737
	DCD ($n=26$)	0.61	0.24, 1.57	0.305
	Live ($n=25$)	—		
Donor age[c]	0–17 ($n=3$)			
	18–49 ($n=59$)	0.75	0.40, 1.39	0.355
	50+ ($n=53$)	—	—	—
Recipient age	0–17 ($n=4$)			
	18–49 ($n=97$)	1.64	0.87, 3.18	0.133
	50+ ($n=30$)	—	—	
HLA class I + II mismatches[d]	Per mismatch	1.16	1.00, 1.36	0.065
Immunosuppression at time of re-listing[e]	None ($n=48$)	—	—	—
	Single ($n=27$)	1.78	0.78, 3.82	0.154
	Dual ($n=53$)	1.12	0.56, 2.17	0.759
Immunosuppression while on the waiting list[f]	None ($n=61$)	—	—	—
	Single ($n=36$)	1.22	0.61, 2.39	0.565
	Dual ($n=30$)	0.39	0.14, 0.99	0.058
Nephrectomy	Yes ($n=56$)	0.99	0.54, 1.85	0.985
	No ($n=75$)	—	—	—
Blood transfusion after transplantation	Yes ($n=118$)	1.01	0.98, 1.03	0.624
	No ($n=13$)	—	—	—
Blood transfusion after listing for re-transplantation	Yes ($n=41$)	1.65	0.84, 2.86	0.155
	No ($n=90$)			
Recipient gender	Female ($n=44$)	2.24	1.20, 4.14	**0.010**
	Male ($n=87$)	—	—	—
Pregnancy	Yes ($n=31$)	1.38	1.04, 1.76	**0.016**
	No ($n=13$)	—	—	—
Time from first transplant to re-listing	≤1 year ($n=45$)	—	—	—
	1–5 years ($n=40$)	2.97	1.38, 5.99	**0.004**
	>5 years ($n=46$)	0.90	0.44, 1.79	0.760
Time from re-listing to end of follow-up	≤1 year ($n=27$)	—	—	—
	1–5 years ($n=68$)	2.81	1.29, 6.59	**0.012**
	>5 years ($n=36$)	4.00	1.63, 10.32	**0.003**

Abbreviations: CI, confidence interval; cRF, calculated reaction frequency; DBD, donation after brain death; DCD, donation after circulatory death; HLA, human leukocyte antigen.
[a]This analysis included those patients from the initial cohort who developed HLA-specific alloantibodies following allograft failure (cRF > 0).
[b]Donor type (DBD or DCD) was not known for one deceased donor kidney transplant.
[c]Donor age information was missing for 16 cases.
[d]HLA-C typing information was not available for six cases.
[e]Immunosuppression information was not available for three patients at the time of re-listing.
[f]Immunosuppression information was not available for four patients while on the waiting list.
Bold values indicate greater significance than $P<0.05$.

are not able to comment on the potential role of mismatches at HLA-DP to sensitization after graft failure.

There is a surprising paucity of published studies seeking to clarify the relationship between HLA matching for renal transplantation and subsequent sensitization following graft failure.[10–12,14,15] Previous studies have been limited to assessment of allosensitization using cytotoxicity against a peripheral blood lymphocyte panel (PRA) that primarily detects only HLA-A- and -B-specific antibodies. The use of PRA alone does not provide information about antibodies directed against individual HLA loci, nor sensitization to HLA class II. Moreover, PRA is subject to wide inter-laboratory variation, and may detect clinically irrelevant immunoglobulin M non-HLA-specific lymphocytotoxic antibodies.[13] A large registry analysis from the United States of patients re-listed for transplantation after loss of their first kidney transplant[14] demonstrated a combined effect of HLA-A and -B mismatches on PRA levels at the time of listing for repeat transplantation and, notwithstanding the limitations of PRA, suggested the importance of HLA-A and -B matching of first grafts for patients who may subsequently require repeat transplantation.

In this study, Luminex single-antigen beads were used to enable precise antibody specification against individual HLA loci.[13,16] The results show for the first time that an increasing number of mismatches at both HLA class I and class II is associated with higher likelihood of allosensitization after re-listing for repeat transplantation, and mismatches at HLA-A, -B, -DR, and -DQ all contribute to higher levels of sensitization and donor incompatibility (increased cRF). Importantly, the association between donor HLA mismatch and likelihood of HLA-specific sensitization was apparent for the three MFI cutoff thresholds (≥2000, ≥5000, and ≥8000) that reflect increasing immunologic risk for repeat kidney transplantation.[17,18] The detection of alloantibodies at MFI values below 2000 is subject to technical artifact and the clinical significance of antibodies detected at such low threshold is controversial[19–21] and we did not, therefore, formally evaluate the impact of alloantibodies with an MFI below 2000 on cRF. However, we found that using an MFI cutoff value for antibody detection of 1000 resulted in only three additional donor-specific antibodies becoming apparent.

The majority of patients who developed HLA-specific antibodies in this study only did so >12 months after they had been re-listed for repeat transplantation. This reflects the fact that patients were often still being maintained on immunosuppression at the time of re-listing, and immunosuppression was then typically reduced or withdrawn to obviate potential adverse effects. This highlights the importance of continuing immunosuppression to prevent development of alloantibodies when early re-transplantation is likely, usually because of the availability of a potential live kidney donor, but unless prompt re-transplantation is anticipated, continued immunosuppression is more difficult to justify in view of the side effects. Maintenance on steroids

alone did not protect from the development of sensitization, whereas those maintained on two or more agents while on the waiting list were much less likely to develop HLA-specific antibodies. Previous studies have reported that transplant nephrectomy is associated with the development of allosensitization, although the timing of nephrectomy and maintenance of immunosuppression influence the temporal relationship between nephrectomy and emergence of alloantibody making direct comparisons between studies difficult.[22,23] In this study, less than half of the recipients underwent graft nephrectomy and after the effect of first transplant HLA mismatch and withdrawal of immunosuppression were taken into account, nephrectomy was not an independent risk factor for allosensitization.

Our analysis shows that mismatches at HLA-A, -B, -C, -DR, and -DQ all contribute independently to allosensitization and that, for each locus, two mismatches contribute significantly more than one mismatch. Given that no mismatch (except HLA-C) is worse than any other mismatch, there are two potential approaches to HLA matching to minimize the subsequent risk of sensitization. One is to focus on avoiding more than one mismatch at any given loci, and another is to focus on reducing the total number of HLA mismatches with no regard to the number of mismatches within a given locus. Our data do not support one approach over the other and a practicable clinical strategy would be to avoid six or more antigen mismatches across all loci when possible, irrespective of the number of mismatches within any given locus. Although our data provide a convincing argument for improved HLA matching when repeat transplantation is a likely future possibility, the degree of HLA matching likely to be achievable through most kidney allocation schemes is such that many patients will still receive grafts that lead to some degree of sensitization when they fail. A further practical approach to preventing sensitization is to continue immunosuppression after graft failure with the aim of early re-transplantation, but the additional risks of maintaining immunosuppression after return to dialysis have to be balanced carefully against the chance of early re-transplantation. In patients who have already developed high levels of allosensitization despite immunosuppression, there is no advantage of maintaining them on immunosuppression when listed for re-transplantation. In patients with an HLA-mismatched graft, who have little or no allosensitization, a careful risk benefit assessment should be made to decide on whether continuation of maintenance immunosuppression can be justified. Although the additional risks of immunosuppression, in terms of infection, cardiovascular and metabolic complications, and increased risk of malignancy, seem justifiable if early re-transplantation is facilitated, they are more difficult to justify for those who do not proceed to re-transplantation in a short timescale.

In this study, we focussed on the traditional approach to HLA matching that assigns equal weight to all HLA mismatches within a given HLA locus. This strategy is widely used to inform most national kidney allocation schemes but does not take into account variation in donor immunogenicity according to recipient HLA type. Analysis of HLA alloantibody profiles based on mismatches at broadly reactive public epitopes (Bw4 and Bw6) did not provide any additional insight into HLA immunogenicity. However, there is increasing interest in the concept of predicting HLA immunogenicity on the basis of mismatched HLA epitopes using programs such as HLAMatchmaker that defines immunogenicity according to exposed epitopes (triplets/eplets) on the surface of HLA molecules.[24–26] Although adopting such an approach was beyond the scope of this study, the ability to define and avoid donor kidneys expressing epitopes that are particularly immunogenic for a given individual is very attractive and should be the focus of future studies.[27]

In summary, the results of this study show a clear relationship between the level of mismatch at the HLA-A, -B, -C, -DR, and -DQ loci of a first kidney transplant and the development of HLA-specific alloantibodies after subsequent graft failure and listing for repeat transplantation, and highlight the role of continued immunosuppression in preventing alloantibody production. Allosensitization to HLA markedly restricts the number of antibody-compatible kidney donors, severely limiting opportunities for repeat transplantation. This problem could be reduced by placing more emphasis on HLA matching in national kidney allocation schemes for those recipients who are likely to require repeat transplantation. Existing matching schemes are predicated on graft survival rather than sensitization and often place most emphasis on HLA-DR matching rather than on HLA class I matching or HLA-DQ matching. However, because all mismatched HLA loci contribute to sensitization, existing allocation schemes are unlikely to solve the problem of allosensitization. A more practical approach may be to continue immunosuppression and aim for early re-transplantation, but the risks of immunosuppression will have to be balanced carefully against the chance of early re-transplantation.

MATERIALS AND METHODS

Patients

The study population comprised 131 consecutive patients who received a first kidney transplant between September 1995 and April 2010 (inclusive) and returned to the Cambridge kidney transplant waiting list following failure of their graft during this time period. On 31 January 2012 (study census date), 26 patients were active on the waiting list, 3 were transferred to a different transplant center, 68 had undergone repeat transplantation (for these patients and for the purpose of this study, the follow-up was terminated at the time of repeat transplantation), 13 had died on the waiting list, and 21 had been permanently suspended for clinical reasons. Transplant nephrectomy was performed in 56 (43%) patients.

HLA-specific antibody screening

Serum samples for antibody screening were obtained at the time of first kidney transplant, on return to the transplant waiting list, and 3 monthly thereafter. Sera were screened using unmodified and dithiothreitol-modified complement-dependent lymphocytotoxicity

against a panel of HLA-typed peripheral blood lymphocytes from healthy blood donors (expressing HLA-A, -B, and -C specificities), and a B-cell panel comprising HLA-typed patients with B-cell chronic lymphocytic leukemia–expressing HLA-A, -B, -C, -DRB1, -DRB3/4/5, -DQ, and -DP specificities.[13] Immunoglobulin G PRAs (% IgG-PRA) were calculated for the peripheral blood lymphocyte and chronic lymphocytic leukemia panels to reflect HLA class I and class I/II lymphocytotoxic alloreactivity, respectively.

In addition, sera were screened for HLA-specific antibodies using solid-phase Luminex HLA antibody-detection beads and selected HLA-specific antibody-positive samples were analyzed using Luminex single-antigen HLA class I and class II antibody-detection beads (One Lambda, Canoga Park, CA). HLA single-antigen bead–defined antibody reactivity was determined using MFI with cutoff thresholds of 2000, 5000, and 8000 to reflect increasing antibody levels.

Calculated reaction frequency

The HLA antibody specificities identified in sera using Luminex single-antigen beads were used to determine the cRF. cRF defines the percentage of a standardized panel of 10,000 consecutive HLA-typed UK deceased organ donors (identified by NHSBT) that is incompatible with the alloantibody profile of a potential transplant recipient and therefore reflects the chance of identifying a suitable donor organ for a given recipient.[16,28] It is dependent on the individual antibody specificities present in the sera and the frequency of the corresponding HLA alleles in the donor population. At each MFI threshold, the cRF was determined for individual loci (HLA-A, -B, -C, -DRB1, -DRB3/4/5, and -DQ), HLA class I (HLA-A, -B, and -C combined), HLA class II (HLA-DRB1, -DRB3/4/5, and -DQ combined), and HLA class I and class II combined.

The 'peak' reactive serum for each patient re-listed for transplantation was identified as that showing the highest cRF. Although a low cRF ($\leqslant$15%) is considered antibody compatible with the large majority of the organ donor pool, patients with $>$15% cRF are defined as 'sensitized' and expected to be antibody incompatible with a proportion of the organ donor pool; patients with $\geqslant$85% cRF are defined as 'highly sensitized', being antibody incompatible with the majority of the organ donor pool.[13,28]

Statistical methods

For descriptive purposes, patient characteristics are summarized as the number (%) in each category or the median (range) for continuous variables. Simple comparisons in the frequencies in each of these categories between pre- and posttransplantation used the Stuart–Maxwell marginal homogeneity test.[29] As cRF is zero for a large proportion of cases, and is bounded above by one (i.e., 100% is the maximum possible cRF), we use a (hurdle jump) zero-inflated beta model for the associations between cRF and covariates.[30] These models have two parts, one to assess the relationship between covariates and non-zero cRF and one to assess the relationship between covariates and each unit increase in the level of cRF in those for whom it is non-zero. Results are summarized as ORs and their 95% CIs for the effects of covariates on the probability of having non-zero cRF. The relationship between covariates and cRF level (for non-zero CRF) is modeled as a proportion using a logit link, and summarized as the OR per percentage increase in cRF, with 95% CI. The *P*-values were taken from the Wald tests. These analyses included baseline sensitization as a covariate. Variables that had a significant association with sensitization were entered into a multiple-variable model to assess independence of relationships. Missing covariates were imputed multiple times in order that the full data set could be used, but the uncertainty associated with estimation of the covariates was fully incorporated. This analysis was completed in Stata/IC version 12.0 (StataCorpLP, College Station, TX) and in WinBUGS version 1.4 (MRC Biostatistics Unit, Cambridge University, Cambridge, UK), with non-informative prior distributions for all parameters. As some similarity between the mismatches at different loci was expected, we used random effects models for the relationship between the number of mismatches within a locus and the locus-specific cRF response and these results are given in Table 2. These results were similar to analyses that assumed that all loci were independent (data not presented).

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Figure S1. Association between first transplant mismatches at the 'public' epitopes HLA-Bw4 and -Bw6 and HLA-B specific sensitisation expressed as calculated reaction frequency (cRF).
Figure S2. Association between first transplant HLA class I and class II mismatches and lymphocytotoxic HLA-specific alloantibody responses expressed as %IgG PBL-PRA and %IgG B-CLL-PRA.
Table S1. Number of patients with HLA-specific alloantibody levels within each cRF category at the three specified study time points stratified according to the number of HLA mismatches (MFI threshold of $>$2000).
Table S2. Number of patients with HLA-specific alloantibody levels within each cRF category at peak sensitisation while on the waiting list (depicted for MFI thresholds of $>$2000, $>$5000 and $>$8000).
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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10.1.3 Expansion of the kidney donor pool by using cardiac death donors with prolonged time to cardiorespiratory arrest.

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Expansion of the Kidney Donor Pool by Using Cardiac Death Donors with Prolonged Time to Cardiorespiratory Arrest

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Donation after Cardiac Death (DCD) is an increasingly important source of kidney transplants, but because of concerns of ischemic injury during the agonal phase, many centers abandon donation if cardiorespiratory arrest has not occurred within 1 h of controlled withdrawal of life-supporting treatment (WLST). We report the impact on donor numbers and transplant function using instead a minimum 'cut-off' time of 4 h. The agonal phase of 173 potential DCD donors was characterized according to the presence or absence of: acidemia; lactic acidosis; prolonged (>30 min) hypotension, hypoxia or oliguria, and the impact of these characteristics on 3- and 12-month transplant outcome evaluated by multivariable regression analysis. Of the 117 referrals who became donors, 27 (23.1%) arrested more than 1 h after WLST. Longer agonal-phase times were associated with greater donor instability, but surprisingly neither agonal-phase instability nor its duration influenced transplant outcome. In contrast, 3- and 12-month eGFR in the 190 transplanted kidneys was influenced independently by donor age, and 3-month eGFR by cold ischemic time. DCD kidney numbers are increased by 30%, without compromising transplant outcome, by lengthening the minimum waiting time after WLST from 1 to 4 h.

Key words: DCD, expanding pool, kidney donation, organ recovery

Abbreviations: CI, confidence interval; CIT, cold ischaemic time; DBD, donation after brain-stem death; DCD, donation after cardiac death; DGF, delayed graft function; eGFR, estimate of glomerular filtration rate; PNF, primary nonfunction; SD, standard deviation; UK, United Kingdom; US, United States; WLST, withdrawal of life-supporting treatment.

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Introduction

The major problem facing transplantation worldwide is unavailability of organs. In the United States over 85 000 people are now waiting for a kidney transplant but there were less than 7300 deceased donors in 2009 (1). Similarly, the UK kidney waiting list has doubled in the last decade to over 7000, whereas deceased donor rates have remained relatively constant, at approximately 800 per year (2). Organ scarcity has prompted medical and political initiatives to improve donor rates (3).

One initiative has been re-appraisal of the use of organs from potential donors, who do not fulfill brain-stem death criteria, but whose clinical condition is futile; upon cardiorespiratory arrest following withdrawal of life-supporting treatment (WLST), organ recovery is possible, if performed promptly. Organs retrieved from such 'donation after cardiac death' (DCD) donors differ from conventional 'donation after brain-stem-death' (DBD) donors in that they are subject to warm ischemia during the period from cardiorespiratory arrest to organ perfusion with cold preservative solution. Nevertheless, despite an increased incidence of delayed graft function (DGF), transplant survival rates of DCD kidneys are broadly comparable to those of DBD kidneys (4–12) and they have become an important resource, providing 32% of deceased kidney allografts in the United Kingdom in the 2008–2009 financial year (2).

However, concerns persist regarding additional ischemic damage; incurred not only after cardiorespiratory arrest, but also from prolonged preterminal hypotension during the period from WLST to cardiorespiratory arrest [termed hereafter the agonal phase (13)]. Consequently, extended criteria donors, such as donors older than 60, are often not considered for DCD kidney donation, because of fears that the additional ischemic insult, when allied to an already-marginal kidney, will result in very poor transplant outcomes. Perhaps more tellingly, very few centers pursue 'uncontrolled' (Maastricht category 1 or 2) DCD donors (14) and instead only use kidneys from Maastricht category 3 donors, whereby WLST is performed in a controlled

fashion, with the recovery team scrubbed awaiting donor cardiorespiratory arrest.

For logistical reasons and to minimize the perceived potential for ischemic kidney injury, several consensus documents recommend a cut-off time of 1 h from WLST to cardiorespiratory arrest, after which organ donation is abandoned (13,15). Surprisingly however, this cut-off time has not been validated, in that no publication has addressed the association between length of agonal phase and kidney transplant outcome. To maximize donor numbers, when we established our recovery policy for potential DCD donors, we chose a minimum cut-off period from WLST of 4 h before the organ recovery team stands down. We report here our experience applying this policy for all controlled DCD donors referred to our center and demonstrate that the number of potential donors from whom kidneys are retrieved is increased substantially. Whereas some donors remained relatively stable for an extended period after WLST before quickly deteriorating and undergoing cardiorespiratory arrest, others had extended periods of instability in the agonal phase characterized, for example, by prolonged hypotension, hypoxia or oliguria. Such extended donor instability might be expected to have a detrimental effect on the quality of the kidneys retrieved, and this report aims to analyze how such instability influenced outcome of the transplanted kidneys.

Materials and Methods

Donor selection, recovery operation and recipient management

Between 1 June 2004 and 31 May 2009, 425 potential controlled DCD donors (Maastricht category 3) were referred to the Cambridge Transplant Centre. If there were no contraindications to donation, treatment was withdrawn either in the Intensive Care Unit or in the theatre anesthetic room, vital signs monitored regularly and upon cardiorespiratory arrest, death certified by an independent medical practitioner, with a minimum 'stand-off' time of 5 min observed between arrest and commencement of organ recovery. Data on the agonal phase characteristics were recorded prospectively by donor coordinators and held within a central library of donor records.

Donation was pursued for a minimum of 4 h after WLST from a potential DCD donor. For logistical reasons, donation was abandoned at this point, unless arrest thought imminent. Donation at later time-points was however possible for selected referrals from our base hospital; Cambridge University Hospitals NHS Trust, when the team could re-attend rapidly (within 30 min) once the potential donor became unstable.

Kidneys were assessed visually following recovery and discarded if poorly perfused, damaged, or gross macroscopic disease, such as severe aortic atherosclerosis, was present. Preimplantation histopathological scoring [from 0 to 12, according to Remuzzi et al. (16)] of wedge biopsies was performed when deemed clinically necessary, and kidneys also discarded if chronic disease, as indicated by a score >4, was revealed. These were performed more frequently as the DCD program developed and more elderly donors, with an increased incidence of cardiac comorbidities, were considered. DCD kidneys were used locally and allocated according to a national algorithm that awards points to potential recipients depending on: age matching to the donor; HLA mismatch; and time spent on the waiting list (17). Immunosuppression was administered postoperatively according to standard protocols, as described previously (6).

Analysis

The impact of donor characteristics and agonal-phase and recovery/implantation variables on kidney transplant function [the presence or absence of DGF and the estimated glomerular filtratation rate (eGFR) at 3 and 12 months] was examined. DGF was defined as the provision of postoperative dialysis in the first week and onwards after transplantation, except when required for hyperkalemia in the first 24 h after surgery. eGFR was calculated using the four variable Modification of Diet in Renal Disease formula 7 [MDRD7, Ref. (18)] and is expressed in milliliters per minute, adjusted for body surface-area. Primary nonfunction (PNF) was defined as a graft that never achieved sufficient function to allow discontinuation of dialysis, excluding acute vascular thrombosis.

Donors were scored for the presence of hypotension, hypoxia, oliguria, acidosis and lactic acidemia using predetermined cut-off values (see Table 1, chosen empirically as representing a clinically relevant deterioration). The impact of these variables and also donor characteristics on transplant outcome was assessed by multivariable regression analysis [WinBUGS software Ref. (19)] using linear and logistic regression for analyzing relationship to 3- and 12-month eGFR and DGF. HLA mismatch between donor and recipient was categorized according to differences at the HLA-A, HLA-B and HLA-DR loci: with 0–1 of 6 possible mismatches categorized as level 1; 2–4 mismatches as level 2 and 5–6 as level 3. The statistical dependence between the pair of kidneys from each donor was incorporated using a random effect on the intercept for each regression.

Two regression models were fitted to each of these outcome measures: in model 1, the agonal phase variables were evaluated as separate predictors; whereas in model 2, each donor was scored (0–5) according to the presence or absence of each variable and the overall agonal-phase score assessed against outcome. When possible, arterial blood gasses were sampled immediately prior to WLST and hourly thereafter, but because of either absence of arterial line in the donor or inability to assay lactic acid on the available blood gas analyzer, acidemia and lactic acidosis were only assayed in 114 (65.9%) and 78 (45.0%) of patients, respectively. Missing data for these variables are included by performing Bayesian multiple imputation (19,20). A paired *t*-test was used to compare 3-month eGFR values in kidneys transplanted from the same donor. Proportional-odds ordinal regression was used to assess the impact of agonal phase duration on agonal phase

Table 1: Input donor, agonal phase and implantation variables for multivariable regression analysis on renal transplantation outcomes

	Mean (SD, range)/frequency
Donor characteristics	
Age (years)	45.5 (19.2, 17–74)
Female gender	34%
Serum creatinine (μmol/L)	97.6 (67.4, 36–504)
Trauma death	31%
Recipient characteristics	
Age (years)	49.0 (14.4, 19–73)
HLA mismatch	Level I 11.6% Level II 84.9% Level III 3.5%
Agonal phase factors	
Hypotension (systolic BP < 85 mmHg for >30 min)	19%
Oliguria (<30 mL/h)	24%
Hypoxia (SaO_2 < 70% for > 30 min or arterial pO_2 < 6 kPa)	26%
Acidemia (arterial pH < 7.30)	35%
Lactatemia (arterial lactate >2.0 mmol/L)	34%
Agonal phase duration (h)	1 h 25 (0–31 h)
Recovery/implantation variables	
Warm ischemic time (>15 min)	43%
Cold ischemic time (h)	13 h 40 (3 h 49, 5 h 46–23 h 07)

score. The relationship between age and eGFR was assessed using linear regression.

A contemporaneous group of kidneys from DBD donors was selected for comparison by choosing, for the first 4 years of study, the DBD kidney transplants undertaken immediately before and after each pair of transplants from the DCD donor. When only one DCD kidney was transplanted, the DBD kidney transplant that preceded it was selected. For the last year of the study all DBD kidneys were included, because by then, numbers of DCD kidney transplants exceeded that of DBD kidneys. The *t*-test was used to compare 3-month eGFR in the kidney transplants from the DBD and DCD groups.

Kaplan–Meier curves were plotted to depict kidney transplant outcome. Graft survival was censored for patient death. Curve comparison was performed with the Mantel–Cox test.

Data was analyzed following discussion with our Local Research and Ethics Committee as appropriate service evaluation.

Results

Referrals for potential DCD donation.

This study reports the Cambridge Transplant Centre's experience of controlled DCD kidney donation from 2004 to 2009 (Figure 1A); a period characterized by marked expansion in the DCD program, with increasing numbers of referrals (Figure 1B) of increasingly elderly donors (Figure 1C). In the final year, approximately twice as many DCD as DBD kidney transplants were performed (Figure 1B) and this represents the most active DCD program in the United Kingdom (Figure 1D). In total, 425 patients (Figure 1E) were referred for consideration as potential DCD donors (52.0% males, median age 60 [range 16–92]). The majority (55.5%) were not pursued either because of medical unsuitability or relative refusal (Figure 1A). Of the 173 who underwent WLST, 117 (67.6%) became donors; donation was abandoned in the remaining 56 (32·4%), because a minimum of 4 h had elapsed and cardiorespiratory arrest had not occurred (51, 29.5%) or, in a small number of cases (5, 2.9%), because of concerns of protracted agonal-phase instability within the initial 4 h wait period.

Our practice of waiting 4 h following WLST differs from the commonly observed standard waiting time of a 1 h maximum (13,15). The impact of our protocol on the conversion of potential DCD donors to actual donors is depicted in Figure 2, which demonstrates that although the majority of donors arrested within the first hour (90 of 117, 76.9%), many additional donors (27, an increase of 30%) were realized by a longer minimum wait time of 4 h. Of these, eight occurred between 1 and 2 h following WLST and 11 between 2 and 4 h. A further eight potential donors in our base hospital were converted successfully after more than 4 h had elapsed because WLST; the longest time from withdrawal to cardiorespiratory arrest was 31 h.

The principal reason for 'standing-down' in the 56 potential donors that were abandoned after WLST had occurred was the logistical inability to pursue donation for longer than 4 h. Thus the 27 donors with agonal phases of greater than 1 h that were successfully pursued were not preferentially selected according to favorable donor characteristics.

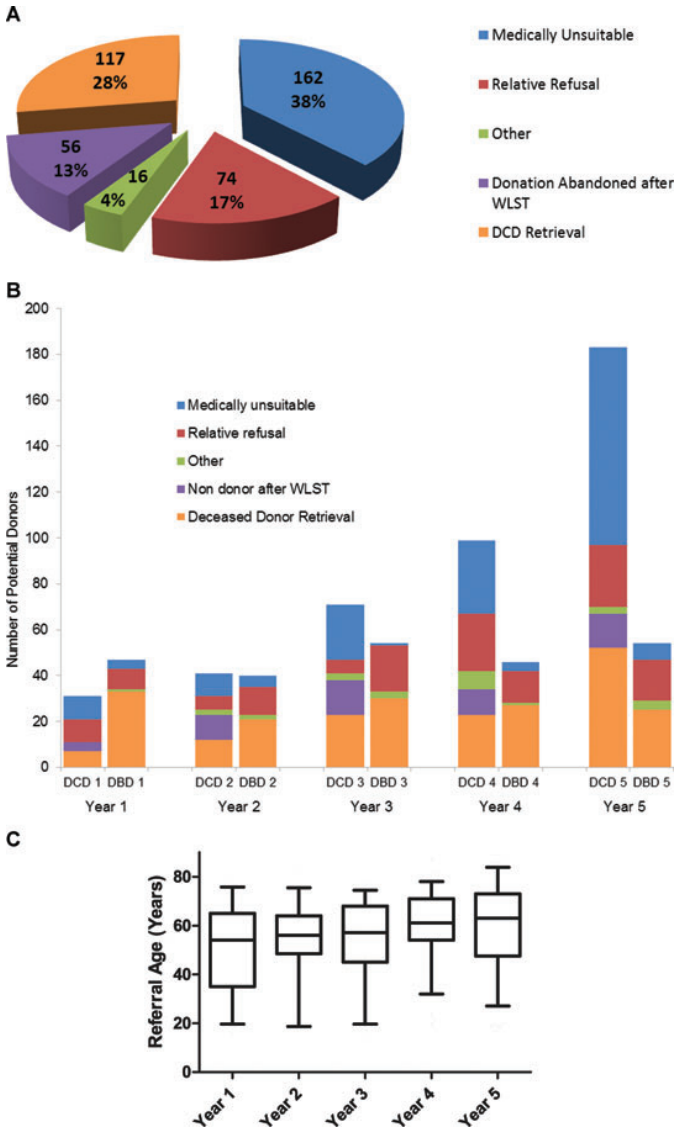
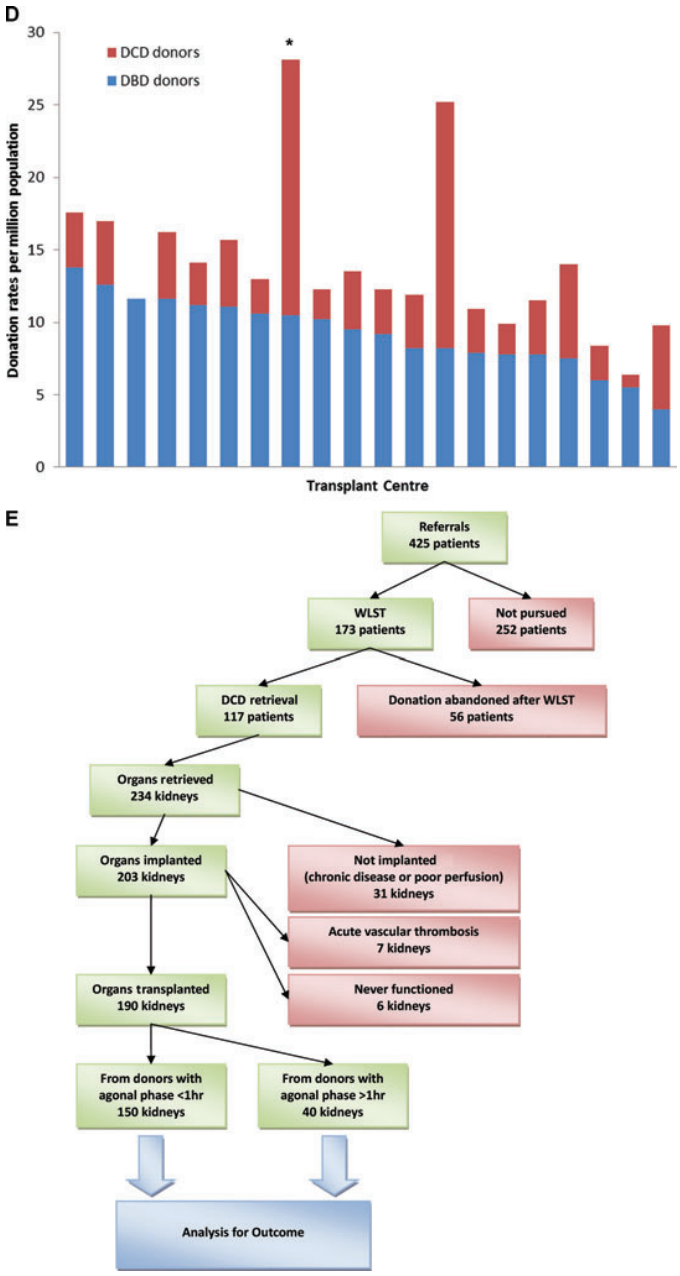


Figure 1: Development of Cambridge DCD program 2004–2009. (A) Outcomes of referrals for consideration as potential DCD donors. (B) Yearly development of DBD and DCD programs. The majority of DCD referrals did not proceed to donation. 'Nondonors after WLST' refers to potential donors in whom donation was abandoned after controlled withdrawal of life-supporting treatment, but before cardiorespiratory arrest had occurred. (C) Boxplot depiction of age of potential DCD donors for each year of program. Potential donors were, on average, 2.5 years older in each successive year (linear regression, $p < 0.001$). (D) Donor rates per million population for each of the 20, anonymized UK renal transplant centers for the year 2008 to 2009. *Cambridge Transplant Centre. Data from NHS BT (2). (E) Outcome of referrals for consideration of DCD donation.

Indeed, these 27 donors were older (mean 57.1 years, range 21–75 years) than the 90 donors that arrested less than 1 h after WLST (mean 48.6 years, range 17–74 years, $p = 0.01$) and a similar age to those patients in whom donation was abandoned (mean 56.3 years, range 17–78 years, $p = 0.79$).

Agonal phase characteristics

The agonal phase of those donors proceeding to WLST was characterized according to the presence or absence of the hemodynamic and biochemical indices listed in Table 1. Agonal phase instability occurred frequently, with donors suffering prolonged periods of hypotension (19%), hypoxia



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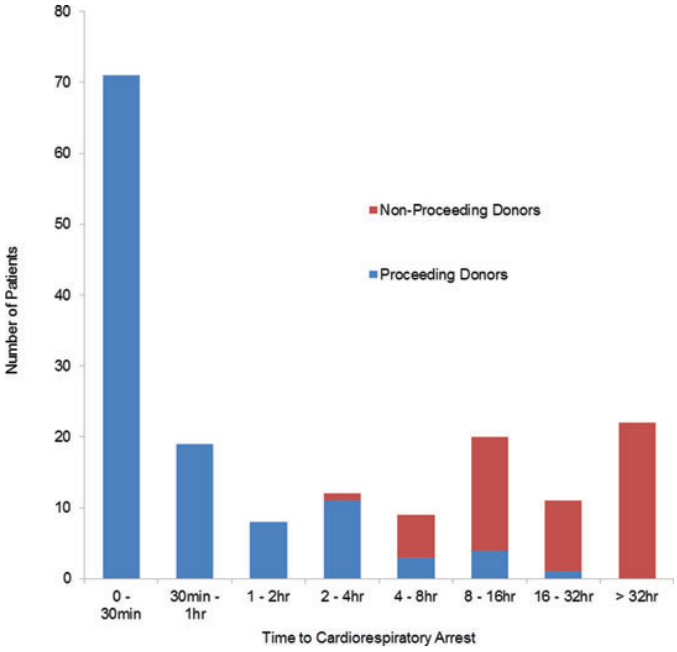

Figure 2: Time to cardiorespiratory arrest following WLST in potential DCD donors. The time to cardiorespiratory arrest from WLST is depicted for proceeding donors (blue) and non-proceeding donors (red). Although five potential donors were abandoned within 4 h after withdrawal of life-supporting treatment due to concerns regarding prolonged agonal phase instability, all but one died after more than 4 h had elapsed.

(26%), oliguria or anuria (24%), acidemia (35%) and lactic acidosis (34%) after WLST. Compared to agonal phases of less than 1 h, longer agonal phases were associated with a higher incidence of hypotension, hypoxia, oliguria, acidosis and lactic acidemia (Figure 3A). Similarly, higher global agonal phase scores were associated with longer agonal phases (Figure 3B), although a number of donors with agonal phases of less than 1 h had become unstable during assessment prior to WLST and thus also scored highly (Figure 3B).

The influence of agonal phase on transplant outcome

The impact of a prolonged agonal phase on immediate and long-term function of the retrieved kidneys has not been reported previously, and without consensus from the literature, the decision to abandon the recovery process before 4 h had elapsed in five patients with unfavorable agonal-phase characteristics was made on an individual basis by the recovery surgeon. Nevertheless, agonal-phase instability developed in a number of donors (and not just those with agonal phases greater than 1 h) that was seemingly at least as severe (Figure 3B) and donation was still pursued. We thus sought to clarify how such instability influences kidney transplant outcomes. Of 234 kidneys retrieved, 31 (13.2%) were not implanted due to poor perfusion with preservative fluid (7, 3.0%) or, more frequently, because chronic disease, apparent macroscopically or on preimplantation biopsy analysis, was present (24, 10.3%). The proportion of kidneys discarded from donors with agonal phases greater than 1 h (10 of 54, 18.5%) was not different from those discarded from donors with agonal phases less than 1 h (21 of 180, 11.7%, $p = 0.9$). PNF occurred in a further six grafts (2.6% incidence, including 1 anastomotic dehiscence following *Escherichia coli* septicemia) and seven (3.0%) developed acute arterial or venous thrombosis, but neither PNF nor vascular thrombosis were related to agonal phase duration. The mean 3-month eGFR in those kidneys transplanted was 45.5 mL/min (SD, 19.2), which is comparable to the group of contemporaneously-transplanted DBD kidneys (47.3 ± 20.2, $p = 0.4$). Patient and graft survival curves for the first three years for all transplanted kidneys are depicted in Figure 4.

The impact of unfavorable agonal phase characteristics and agonal phase duration on transplant outcome (development of DGF and 3- and 12-month eGFR values) was assessed by multivariable regression analysis. Donor factors (gender, age, mode of death and creatinine), demonstrated previously to have an association with transplant outcome (9,21,22) were included in the analysis, as were: degree of sensitization; and the cold ischemic and warm ischemic

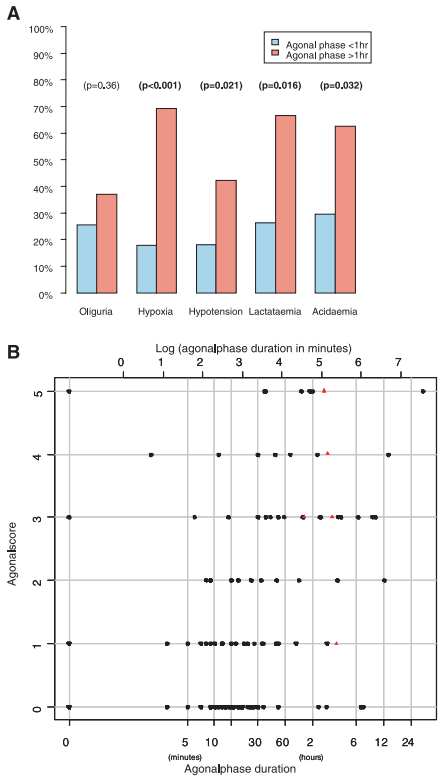

Figure 3: Donor agonal phase duration and the development of hemodynamic/biochemical instability. Instability during the agonal phase (from WLST to cardiorespiratory arrest) in each donor (n = 117) was characterized according to the presence of five abnormal physiological/biochemical parameters (Table 1). (A) Incidence of each parameter in donors with agonal phase <1 h vs. >1 h. Chi-squared test p values as indicated (B) Global agonal phase instability was assessed by scoring for the presence of each positive parameter (total out of five) and plotted against Log of agonal phase duration. One unit of log (duration in min) is associated with a 43% (CI = 21%–70%) increase in the odds of higher agonal phase score. Those donors with high agonal phase scores, but with short agonal phase durations (<30 min) represent donors who had developed instability prior to WLST. The points in red represent the five potential DCD donors in whom donation was abandoned before 4 h had elapsed because of unfavorable agonal phase characteristics and depict the agonal phase score at the time donation was abandoned.

times. Recipient age and degree of HLA mismatch were not included in this multivariate model as they showed no univariate associations with 12-month eGFR. Mean eGFR was 44.4 mL/min (SE 3.2) for 0–1 HLA mismatches and mean 43.7 mL/min (SE 1.4) for 2 or more mismatches (p = 0.86). Pearson correlation of 12-month eGFR with recipient age was –0.12 (p = 0.09).

As depicted in Table 2(A), multivariable regression analysis did not identify a statistically significant association between any of the agonal phase characteristics, or its duration, and eGFR at 3 or 12 months. Instead, the only identifiable variables that impacted upon eGFR were donor age [with a 10-year increase associated with a decline in recipient 12-month eGFR of 4.24 mL/min (95% CI 2.49, 6.02)] and CIT [with each hour's increase associated with a decline in 3-month eGFR of 1.27 mL/min (95% CI 0.58, 1.95)]. However, by 12 months the association between CIT and eGFR was no longer statistically significant. The detrimental impact of CIT on early graft function is also reflected by paired analysis of outcomes for kidneys from the same donor, with the 3-month eGFR of the kidney transplanted second on average 6·91 mL/min (95% CI 1.61, 12.22, p = 0·012) lower than the kidney transplanted first. By 12 months this difference has lessened to 2.67 mL/min and was no longer significant (95% CI –4.78, 5.66, p = 0.87). Figure 5 depicts the relationship between eGFR and donor age, CIT and agonal phase duration as scatter plots. The impact of agonal phase duration on graft outcome is depicted in the multivariate analysis in Table 2, comparing agonal phase duration <1 h to >1 h. Agonal phase duration was also assessed as a continuous variable following log transformation to normalize the distribution (data not shown). Again this showed no correlation with outcome.

Although not apparent on analysis of 3- and 12-month eGFR values, the presence of agonal phase instability, such as hypotension and oliguria, might have been expected to have a more discernible impact on the development of acute tubular necrosis (ATN) and DGF. DGF occurred in 94 (49.5%) of 190 DCD kidneys, but logistic regression analysis revealed a surprising lack of association with the agonal phase characteristics. Confidence intervals were however, very wide and we cannot rule out clinically important effects (Table 2A). In comparison, elderly donors and prolonged CIT were both associated with an increased incidence of DGF, although this did not reach statistical significance. Linear regression analysis of the days to dialysis independence was also performed, in the expectation that more profound agonal phase instability might lead to more severe ATN and hence more prolonged DGF; again there was no association with agonal phase characteristics (not shown).

To assess whether the presence of a combination of unfavorable characteristics (such as hypotension plus hypoxia plus oliguria) had a detrimental impact on graft outcome, the analysis was repeated using the global agonal-phase score. This again demonstrated donor age and CIT as independent predictors of 3-month eGFR, but there was no significant association with the agonal-phase score (Table 2B).

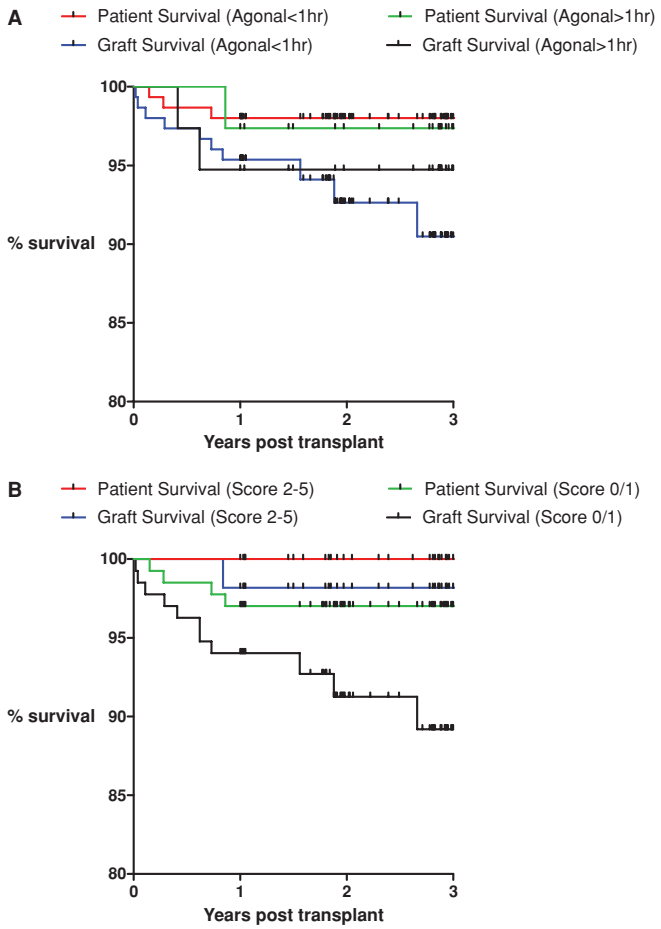

Figure 4: Kaplan-Meier plot for patient and graft survival. (A) Kaplan–Meier graft and patient survival curves for kidneys with agonal phase duration >1 h are comparable to those with agonal phase duration of <1 h to those with agonal phase duration >1 h at both 1 and 3 years posttransplant ($p = 0.22$). (B) Graft and patient survival of kidneys with agonal phase scores of 2–5 (98.2%, 100.0%) were no worse than those with low agonal phase scores (0–1) at 1 year (94.0%, 97.0%). Similarly, at 3 years graft and patient survival of kidneys with high agonal phase scores were no worse than those with low scores and, if anything, graft survival was significantly better ($p = 0.032$).

This analysis also revealed terminal donor creatinine as an independent predictor of DGF. This presumably reflects our policy of not declining potential DCD donors with acute renal impairment; donor terminal creatinine ranged widely (Table 2A), and was greater than 200 μmol/L in 10 donors.

Kaplan–Meier plots (Figure 4) for kidneys from donors with agonal phase durations >1 h demonstrated comparable survival at 1 and 3 years posttransplant to those with agonal phase durations <1 h. Similarly, patient and graft survival for kidneys from donors with higher agonal phase scores (2–5) was no worse than from donors with lower agonal phase scores and, if anything, was surprisingly better in the former group (Figure 4B).

Discussion

The finding that agonal-phase instability did not impact upon 3-month eGFR values for controlled DCD kidneys is surprising. However, although consensus documents advise abandoning DCD kidney donation 1 h after WLST (13,15), these recommendations are based on earlier studies that adopted a cut-off time of 1 h as standard protocol at the onset of their programs; a formal examination of the relationship between the agonal phase and DCD kidney transplant outcomes has not been performed previously. Poor results have certainly been reported for liver transplants from controlled DCD donors with prolonged agonal phases (23,24), and the reason why similarly severe hypotensive/hypoxic insults do not influence kidney

Table 2: Predictors of kidney allograft outcomes

	Estimated difference in 3-month eGFR (95% CI)	Estimated difference in 12-month eGFR (95% CI)	Odds ratio for DGF (95% CI)
(A) Agonal phase variables analyzed independently			
Age*	–4.24 (–6.02, –2.49)	–3.99 (–5.68, –2.30)	1.65 (1.04, 3.11)
Female sex	–1.24 (–7.30, 4.90)	–3.55 (–9.05, 2.00)	3.85 (0.80, 32.46)
Trauma death	3.77 (–2.44, 10.14)	–4.47 (–10.42, 1.50)	1.11 (0.23, 6.83)
Creatinine** (μmol/L)	–0.32 (–7.05, 6.37)	–0.49 (–6.95, 5.95)	5.17 (0.93, 47.70)
Hypotension	3.21 (–5.39, 11.93)	3.52 (–4.84, 11.66)	0.42 (0.035, 3.93)
Oliguria	–5.18 (–13.10, 2.80)	–3.54 (–10.96, 3.88)	1.51 (0.20, 14.01)
Hypoxia	8.47 (–1.10, 18.06)	4.31 (–4.77, 13.59)	0.51 (0.033, 6.77)
Acidemia	–1.63 (–9.46, 6.28)	1.60 (–5.64, 8.83)	0.87 (0.078, 8.93)
Lactatemia	–8.27 (–16.78, 0.52)	–4.80 (–13.21, 3.77)	2.61 (0.23, 53.25)
Agonal phase duration >1 h	–6.99 (–14.63, 0.59)	–6.07 (–13.38, 1.17)	2.08 (0.29, 19.91)
Warm Ischemic time >15 min	–5.05 (–11.01, 0.75)	–1.58 (–7.12, 3.94)	2.94 (0.62, 19.85)
Cold ischemic time (h) †	–1.27 (–1.95, –0.58)	–0.54 (–1.18, 0.10)	1.08 (0.95, 1.25)
PRA > 20%	–1.24 (–7.30, 4.90)	–3.55 (–9.05, 2.00)	3.85 (0.80, 32.46)
(B) Agonal phase variables analyzed combined as global score (0–5)			
Age*	–4.52 (–6.24, –2.79)	–4.21 (–5.86, –2.56)	1.51 (1.05, 2.38)
Female sex	–2.05 (–7.92, 3.72)	–3.72 (–9.32, 1.85)	2.60 (0.75, 10.89)
Trauma death	3.50 (–2.66, 9.66)	–4.10 (–10.02, 1.83)	1.12 (0.30, 4.45)
Creatinine**	–4.64 (–10.20, 0.92)	–2.75 (–8.05, 2.59)	5.09 (1.49, 24.85)
Agonal score (2/3 vs. 0/1)	0.35 (–6.48, 7.11)	0.67 (–5.79, 7.18)	0.41 (0.080, 1.73)
Agonal score (4/5 vs. 0/1)	1.16 (–8.41, 10.59)	3.33 (–5.93, 12.42)	2.24 (0.25, 26.74)
Agonal phase duration > 1 h	–5.79 (–12.90, 1.43)	–5.35 (–12.26, 1.52)	1.73 (0.37, 8.93)
Warm ischemic time > 15min	–5.91 (–11.68, –0.17)	–2.06 (–7.52, 3.45)	1.96 (0.57, 7.81)
Cold ischemic time†	–1.22 (–1.89, –0.53)	–0.48 (–1.12, 0.16)	1.07 (0.95, 1.21)
PRA > 20%	–2.05 (–7.92, 3.72)	–3.72 (–9.32, 1.85)	2.60 (0.75, 10.89)

Multivariable regression analysis of the impact of donor and agonal phase and recovery/implantation variables on the outcome (eGFR at 3-months and the development of DGF) of the 190 kidneys transplanted.
The impact on eGFR is presented for: *every additional 10 years of donor age; **for 1 unit of log(μmol/L creatinine); †for every additional 1 h cold ischemia. Only seven kidneys were transplanted with mismatch level III; these were analyzed combined with the level II group.

transplant function is not clear, but presumably reflects organ-specific differences, possibly relating to the ability of the kidney to maintain its blood supply, through autoregulation, even during systemic hypotension. By the same reasoning, the higher incidence of PNF and poor graft function in uncontrolled, as opposed to controlled, DCD kidney transplants (25) perhaps relates to cessation of autoregulation during the prolonged periods of asystole commonly observed. Irreversible cortical necrosis from profound hypotension, thought responsible for PNF in uncontrolled DCD kidney transplants, was not a feature of our series.

The surprising nature of our results mandates careful consideration of possible sources of error that obscure a true detrimental impact of agonal phase instability on transplant outcome. First, like all retrospective studies it suffers from the potential for confounding variables and reporting bias. Against this, the data on each donor was collected prospectively and held centrally. Equally, we chose deliberately to examine only a limited number of recipient factors to avoid weakening the strength of the statistical analysis, and it is theoretically possible that a deleterious impact of the agonal phase on outcome was masked by somehow assigning kidneys from donors with poor agonal phase characteristics to better 'quality' recipients. However this was not the case, because kidneys were allocated according to the same algorithm that is used nationally for DCD and DBD kidneys, as demonstrated by the very few patients who received a level III mismatched kidney. This algorithm does not consider agonal-phase characteristics. Furthermore, for most donors, a pair of kidneys was transplanted into different recipients, making selection bias inherently difficult. Finally, donor age and CIT were included deliberately in the analysis to act as internal, positive controls; it is unlikely that recipient selection could have been manipulated to negate the impact of agonal phase on eGFR, yet maintain the impact of age and CIT.

Another potential source of error is that outcome comparisons in this study were performed by analysis of 3- and 12-month eGFR and graft and patient survival at one and three years following transplantation and it is possible that the impact of the agonal phase characteristics on transplant outcome does not become apparent until later. This will be addressed in future studies, but seems unlikely; we have recently reported that 1- and 5-year survival rates for DCD kidney transplants performed at our center between 1996 and 2006 are similar to contemporaneous DBD kidney transplants (6). It is also possible that an association between agonal phase characteristics and eGFR may have been revealed if larger numbers of DCD

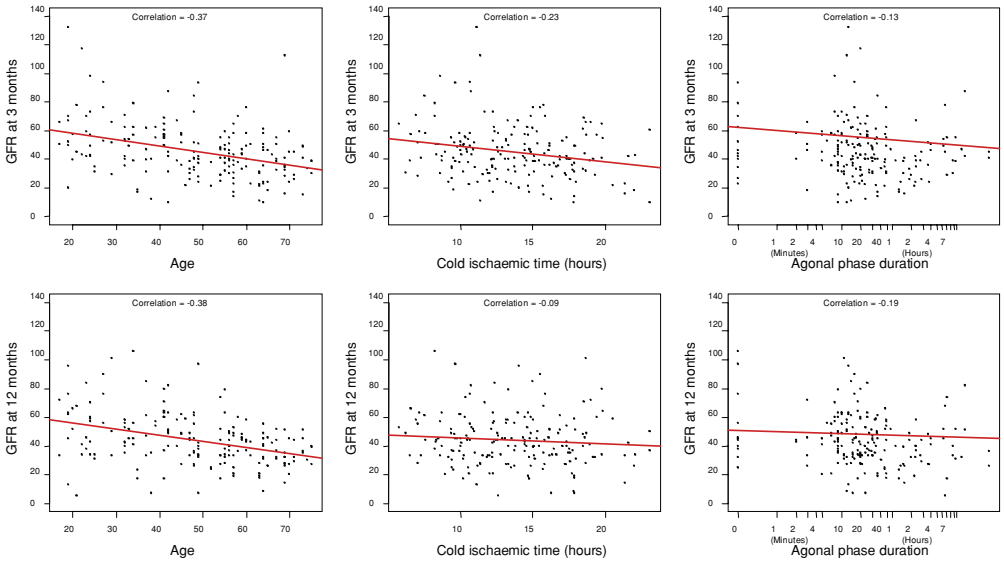

Figure 5: Relationships of donor age, cold ischemic time and agonal phase duration to kidney transplant function at 3 and 12 months, with fitted univariate linear regressions. Scatter plot diagrams depicting univariate relationships between donor age, cold ischemic time and agonal phase duration to kidney transplant function at 3 and 12 months.

transplants had been included in the study. Against this, there was no apparent trend in the impact of individual agonal-phase variables on eGFR (donor hypotension and hypoxia are associated with higher values) and likewise higher global agonal phase scores did not confer a greater risk of poor graft function (Table 2). Similarly, agonal phase instability was not confined to donors with agonal phases of greater than 1 h, reflecting our policy of pursuing DCD donors despite the development of instability during the assessment stage prior to WLST, and thus analysis of unfavorable agonal phase characteristics on kidney function is influenced by the 19.7% of all donors with agonal scores $\geq$3 (Figure 3B). In contrast, our data, representing one of the largest single center-experiences in DCD transplantation (5,26), identified donor age and CIT as independent predictors of outcome, which mirrors the findings of a recently published analysis of 2562 DCD kidney transplant records in the United Network for Organ Sharing (UNOS) database (9).

Analysis of the paired outcomes of kidneys from each donor has not been reported previously but, by essentially excluding donor-specific variables, provides important confirmation of the detrimental impact of CIT on graft outcome. Notably, the impact of CIT on eGFR that was evident at 3 months was not sustained at 12 months, either on multivariable and univariate regression analysis, or on analysis of paired outcomes. Presumably, this reflects a reversible component to the damage induced by long cold ischemic times, albeit recovery takes several months.

Although our data indicate that DCD kidney donation rates can be improved, without prejudicing transplant outcome, by lengthening waiting time from WLST, a policy of waiting a minimum of 4 h is undoubtedly labor intensive and logistical problems are created by the organ recovery team being mobilized for extended periods. Nevertheless, with the marked expansion in the DCD program, we now perform approximately twice as many DCD as DBD kidney transplants and estimate that our policy of waiting a minimum of 4 h following WLST is responsible for generating 20% of the deceased kidney transplants performed annually in our center. The expansion in the DCD program was unexpected and indicates that numbers of potential DCD donors, at least in our region, are several-fold greater than those of DBD donors (Figure 1B). The UK Organ Donation Taskforce, on behalf of the Department of Health, has targeted a 50% increase in organ donation within 5 years (3). Our experience suggests that this could be readily achieved by expanding DCD donation. DCD kidney recovery rates in the United Kingdom [currently 9.0 per million population (pmp) annually] are gradually increasing (2); matching to those in our center (currently 35.2 pmp, Figure 1D) would approximately double the number of deceased-donor

kidney transplants currently performed in the United Kingdom each year (~1500), with a quarter of these provided by pursuing donors for more than 1 h after WLST.

In summary, a minority of DCD referrals proceed to kidney recovery. Our results demonstrate that DCD kidney numbers are increased substantially, without compromising transplant outcome, by extending the waiting time from WLST to beyond 1 h. The presence of unfavorable agonal phase characteristics should not be regarded as a contraindication to kidney recovery, but CIT should be minimized to optimize transplant outcome.

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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