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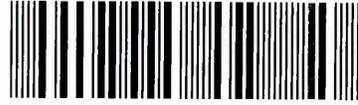
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**Immunophenotyping as a Profiling Tool in
Human Leucocyte Antigen Incompatible
Renal Transplantation**

Katherine Lillian Mounsey

**A doctoral project report submitted in partial fulfilment of the
requirements of Sheffield Hallam University for the degree of
Doctor of Professional Studies**

June 2016

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ABSTRACT

Human leucocyte antigen incompatible (HLAi) transplantation represents a key strategy for improving access for sensitised patients to the preferable treatment, of transplantation, for end-stage renal failure (ESRF). This laboratory based observational research study aimed to investigate key populations of recipient lymphocytes present at various stages of the HLAi transplant procedure, and to assess the data obtained in combination with other pertinent laboratory and clinical information, in order to build up an immunological profile.

Data was collected from blood samples provided by seven prospective and thirteen retrospective HLAi kidney transplant recipients, and nine normal control individuals. Flow cytometry was utilised to examine lymphocyte subsets, and to perform T and B cell immunophenotyping. HLA-specific antibody definition was carried out using a single antigen bead-based assay, employing Luminex technology. Longitudinal data for the prospective participants, and collective data on the retrospective cohort, was scrutinised for any trends or significant associations.

The effects of preconditioning and immunosuppression received were evident in the lymphocyte subset results of both groups of patients. Pre-treatment results in the prospective cohort also confirmed a chronic renal failure effect of reduced cell counts. Both groups of participants showed increased populations of memory T cells following transplantation, suggesting reconstitution from within this compartment. A peak in HLA-DR expression on T cells, at six months post-transplantation, was noted in the prospective group. Conversely, memory B cells remained depressed in both cohorts, with the repopulating B cells demonstrating a transitional or mature B cell phenotype in the prospective participants. A possible link between increased populations of plasmablasts post-transplantation, and detected levels of HLA-specific antibodies was indicated.

The results demonstrated some intriguing trends and patterns that are worthy of further investigation, and it is recommended that elements of this pilot study are extended into larger prospective studies. As this study highlights, there is still much knowledge to be gained and potential laboratory support that could be given, that may improve the delivery of HLAi transplant programmes and thereby access to the superior treatment of transplantation for sensitised patients.

ABBREVIATIONS

ABOi	ABO blood group incompatible
AHG	Anti-human globulin
AiT	Antibody incompatible transplantation
AMR	Antibody mediated rejection
APC	Allophycocyanin
APC	Antigen presenting cell
ATG	Anti-thymocyte globulin
ATN	Acute tubular necrosis
BD	Becton Dickinson
BHLY	Bradford Hull Leeds York
BP	Band pass
BSHI	British Society for Histocompatibility and Immunogenetics
BSI	British Society for Immunology
BTS	British Transplantation Society
CD	Cluster of differentiation
CDC	Complement dependent cytotoxicity
CMV	Cytomegalovirus
CPA	Clinical Pathology Accreditation
CREG	Cross-reactive group
cRF	Calculated reaction frequency
CVID	Common variable immunodeficiency
DBD	Donation after brain death
DCD	Donation after circulatory death
DD	Deceased donor
DGF	Delayed graft function
DNA	Deoxyribonucleic acid
DoH	Department of Health
DSA	Donor specific antibody
EDTA	Ethylenediaminetetraacetic acid
EFI	European Federation for Immunogenetics
ESRF	End-stage renal failure
FACS	Fluorescence activated cell sorter
FBS	Foetal bovine serum

FC	Flow cytometry
FFP	Fresh frozen plasma
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box P3
FSC	Forward scatter
FSGS	Focal segmental glomerulosclerosis
HAR	Hyper acute rejection
HD	Haemodialysis
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HLAi	Human leucocyte antigen incompatible
HSC	Haematopoietic stem cell
ID	Identification
IFN	Interferon
Ig	Immunoglobulin
IHW	International Histocompatibility Workshop
IL	Interleukin
IMGT	Immunogenetics (International Immunogenetics information system)
IP-10	Interferon-gamma-inducible protein 10
IPD	Immuno polymorphism database
IPEX	Immunodysregulation, polyendocrinopathy, enteropathy X-linked
IRAS	Integrated Research Application System
LD	Live donor
LRD	Live related donor
LTHT	Leeds Teaching Hospitals Trust
Mab	Monoclonal antibody
MCF	Mean channel fluorescence
MDT	Multi-disciplinary team
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MMF	Mycophenolate mofetil
NDAD	Non-directed altruistic donor
NGAL	Neutrophil gelatinase-associated lipocalin
NHS	National Health Service

NHSBT	National Health Service Blood and Transplant
NIMA	Non-inherited maternal antigens
NK	Natural killer
ODT	Organ Donation and Transplantation
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Peritoneal dialysis
PE	Phycoerythrin
PE	Plasma exchange
PP	Plasmapheresis
PRA	Panel reactive antibody
QC	Quality control
R & D	Research and Development
RCT	Randomised Control Trial
REC	Research Ethics Committee
RSSO	Reverse sequence specific oligonucleotide
RTE	Recent thymic emigrant
SAB	Single antigen beads
SLE	Systemic lupus erythematosus
SOP	Standard operating procedure
SSC	Side scatter
SSI	Site specific information
SSP	Sequence specific primers
TCR	T cell receptor
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TREC	T-cell receptor excision circle
Treg	Regulatory T cell
UK	United Kingdom
USA	United States of America
UTI	Urinary tract infection
v/v	Volume for volume

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1.1 Background to the study

Antibody incompatible transplantation (AiT) describes the crossing of the previously 'taboo' antibody barrier, to either human leucocyte antigen (HLA) or ABO blood group antigens. Since the turn of the century, developments in AiT, as a treatment for end stage renal failure (ESRF), have led to the successful introduction of programmes in most UK renal transplant centres, and worldwide, facilitating access to transplantation for recipient-donor pairs previously deemed unsuitable. This current laboratory based observational research study aimed to investigate aspects of the immune response of renal transplant recipients undergoing the specialised therapy of HLA incompatible (HLAi) transplantation. Advances in all areas of renal transplantation including surgical, laboratory, immunosuppression and post-transplant care, means that this approach to transplanting a patient, who may be unlikely to receive a donor kidney in the standard way, is successful in carefully selected, and thoroughly investigated, cases.

However, there is much that is not understood about the biological process of HLAi transplantation, both in terms of what is happening to the patient's immune system and their responses during the pre-transplant protocol, and post-transplant, when the donor kidney is *in-situ*. The main focus of this study was to examine key populations of lymphocytes present at various stages of the procedure, and to view this new data in combination with other pertinent laboratory and clinical data, in order to build up an immunological profile of HLAi transplant recipients. The development of additional ways of monitoring HLAi transplantation patients has the potential to further enhance the delivery of this treatment in a tailored, individualised manner, as information provided to clinicians may allow them to target immunosuppression and immune modulating therapies at the patients who require them, and decrease, or avoid their use, in those patients who do not. Importantly, this approach may contribute to opening up this treatment option to an increasing number of patients who are difficult to manage clinically and do not currently have access to standard transplantation. Finally, studying patients who have been through the HLAi transplantation

programme could allow insights into the long-term effects of this procedure, and potential enduring changes to these individuals' immune systems.

1.2 Justification of transplantation as a treatment choice for end-stage renal failure (ESRF)

Transplantation is the treatment of choice for patients with ESRF in terms of mortality (Newstead 2008, BSHI/BTS 2014) compared to the alternative renal replacement therapy of dialysis. Developments in the field mean that graft survival, following deceased donor transplantation, continues to improve (see table 1.1).

Table 1.1 Long-term graft survival after first adult kidney only transplant from a DBD in the UK

Year of transplant	No. at risk on day 0	% Graft survival (95% confidence interval)			
		One year	Two year	Five year	Ten year
2001-2003	2771	91 (90-92)	89 (88-90)	83 (81-84)	73 (71-75)
2004-2006	2468	93 (92-94)	91 (90-92)	85 (84-87)	
2007-2009	2114	93 (92-94)	91 (90-93)	85 (84-87)	
2010-2013	3071	94 (93-95)			

DBD – Donation following brain death; UK – United Kingdom

Adapted from NHS Blood and Transplant (NHSBT) (2015, p98)

Transplantation is also the favoured option for patients in relation to quality of life (Orr *et al.* 2007). When compared with dialysis, kidney transplantation provides a financially viable service for the National Health Service (NHS). It has been calculated that the UK transplantation programme as a whole saves the NHS £316 million per annum (Department of Health (DoH) 2013). One would think that such an all-round successful treatment should be offered to all patients in need of a transplant; however, there are important reasons why this is not currently the case.

1.3 The donor organ shortage

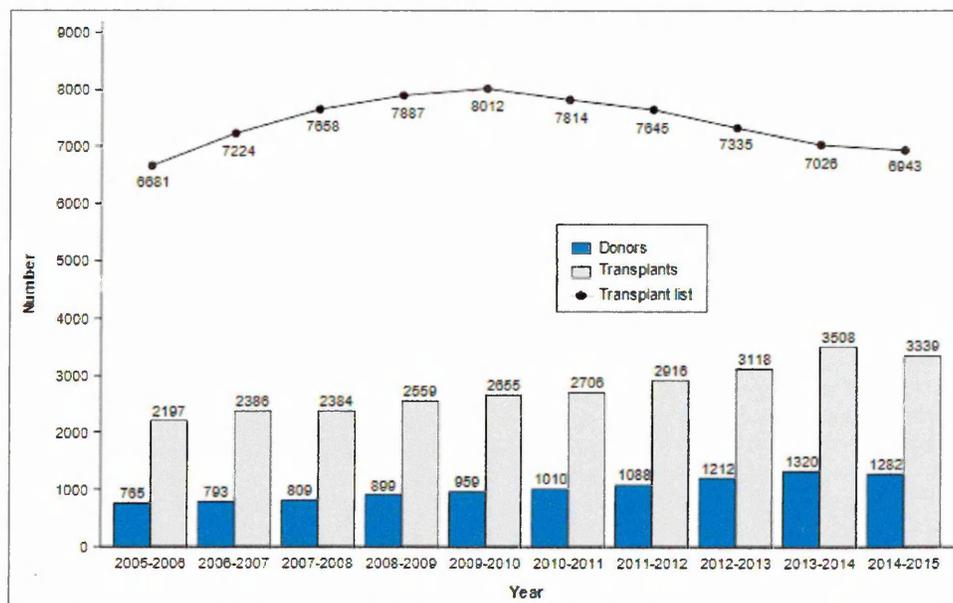
Historically, within the UK, the majority of kidneys for transplantation are obtained from deceased donors. In 2008, a report (DoH 2008) was published by the Organ

Donation Taskforce to address the issue of a plateau being reached in the number of organ donors. By 2013, the recommended actions implemented following the report had led to an increase in deceased organ donation of 50%, and an increase in transplantation of 30.5% (DoH 2013). Despite this, as indicated in figure 1.1, there remains a substantial shortfall between the total number of individuals on the organ transplant waiting list and the number of both registered donors and transplants performed. More recent recommendations (DoH 2013) aim to build further on the progress made in the UK in terms of organ donation rates from deceased donors, by firmly integrating the donation process into end of life care, and attempting to transform the attitude of the general public towards donation. A recent move to a new 'soft opt-out' system by the Welsh government represents a step towards these important changes. Effective from 1st December 2015, the Human Transplantation (Wales) Act (Wales 2013), applies 'deemed' consent to individuals who have not registered a preference as to whether or not they wish to be considered as an organ donor upon their death.

It remains clear that donor kidneys are an incredibly valuable and limited resource that are not available to all patients in ESRF. For this reason, it is essential that this resource is utilised in the most beneficial way, and in the UK this is achieved by a complex deceased donor national kidney allocation scheme, managed by NHS Blood and Transplant - Organ Donation and Transplantation (NHSBT-ODT). In previous years, the allocation scheme existed for donors following brain death (DBDs) only; however, since 2014, a national sharing scheme is also managed for donors following circulatory death (DCDs).

The shortage of deceased organ donors has resulted in initiatives to increase donation from alternative donor sources. Transplantation is regulated under the Human Tissue Act 2004 (Great Britain 2004), which allows the donation of kidneys from live donors who typically (but not necessarily) are genetically or emotionally related to the recipient. In the year 2014-15, 1,052 living donor kidney transplants were carried out, in comparison with a total of 1,880 deceased donor (DBD and DCD) transplants (NHSBT 2015). This is a key factor in relation to this research project, as not only do live donors provide an important strategy for increasing the donor pool, but in addition, in the majority of cases, the specialist treatment of HLAi transplantation is carried out with a live donor.

Figure 1.1 Number of deceased donors and transplants in the UK, April 1st 2005 – March 31st 2015, and the number of patients on the active transplant waiting list at March 31st

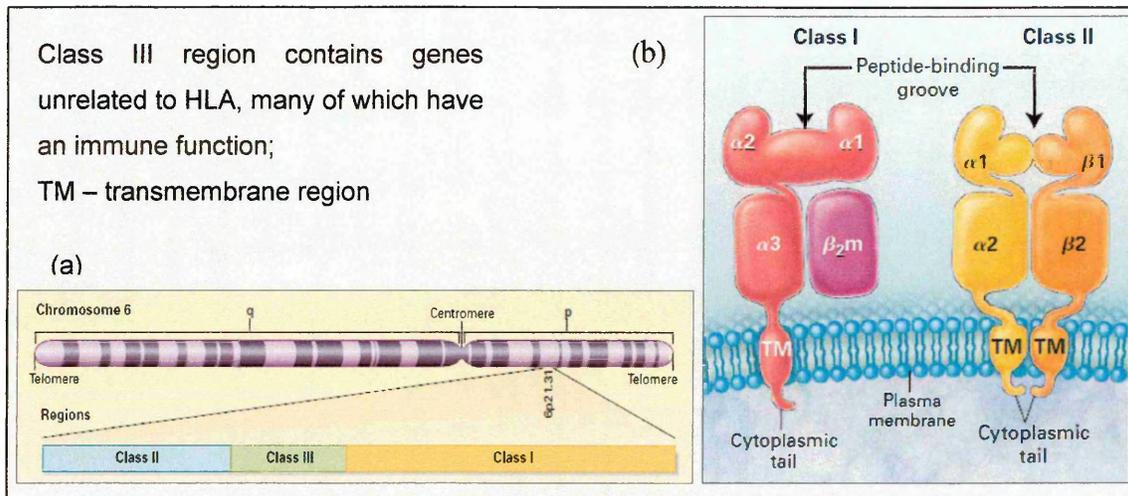


NHSBT-ODT (2015, p4)

1.4 Standard assessment of kidney transplant compatibility

In order for a kidney transplant to be successful, the compatibility of the potential donor and recipient must first be assessed. In standard transplantation, the pair must first be blood group compatible, but following on from this the immunological compatibility is examined. This work is carried out in Histocompatibility and Immunogenetics (H&I) laboratories, and two key areas relating to a potential recipient's immunological status are analysed in order to achieve the best possible outcome for the kidney transplant and ensure that the donor kidney is not immediately rejected by the recipient's immune system. Immune recognition of 'self' and 'non-self' is directed by the products of the HLA genes, which are encoded within the major histocompatibility complex (MHC), an area of the genome comprising over two hundred genes (Robinson *et al.* 2015), many of which encode proteins with immune function. Figure 1.2 (a) depicts an overview of the HLA region, which is located at position 6p21.3 on the short arm of chromosome 6. The classical HLA genes, whose products are shown in figure 1.2 (b), are organised as Class I and Class II. Class I A, B and C genes encode molecules which are expressed on all nucleated cells; Class II DR, DQ and DP molecules have more limited expression, found on antigen presenting cells (APCs) and activated T cells (Parham 2009).

Figure 1.2 The genes within the HLA region of the MHC encode HLA Class I and Class II cell surface molecules



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HLA Class I A, B and C genes each encode a heavy chain (α -chain), which combines with β -2 microglobulin (β -2m) for expression on the cell surface. β -2 microglobulin is encoded outside of the MHC, on chromosome 15. The genes encoding the Class II molecules are located centromeric to the Class I region. The classical genes consist of an A gene, e.g. DQA, which encodes the α -chain, and a B gene, e.g. DQB, which encodes the β -chain of the Class II molecule.

Both sets of proteins play a key role in immune recognition by presenting antigen to T cells, with Class I molecules presenting endogenous antigen to CD8⁺ T cells and Class II presenting exogenous antigen to CD4⁺ T cells (Parham 2009). A key feature of the HLA genes is the extensive polymorphism exhibited, with a total of over 14,000 HLA alleles currently documented (Robinson *et al.* 2015).

It has long been established that ‘tissue matching’, or attempting to achieve as close a match as possible between the potential recipient and donor HLA types results in a better transplant outcome (Opelz and Dohler 2007). As well as analysing recipient and donor ‘tissue types’, the other key focus for scientists is the analysis of a potential recipient’s sensitisation status. When individuals are exposed to ‘foreign’ HLA they are able to form antibodies directed towards them, which are capable of causing immediate irreversible rejection of a transplanted

organ. This process of forming HLA-specific antibodies is known as sensitisation, and can occur by three main routes, which all involve exposure to another individual's ('foreign') HLA; namely blood transfusion, previous transplantation or pregnancy (Magee 2006, BSHI/BTS 2014).

1.5 The problem of HLA sensitisation

The sensitisation status of a potential kidney transplant recipient has a direct impact on potential deceased donor offers, and it is acknowledged that possession of HLA specific antibodies is one of the main obstacles affecting patients' access to transplantation (BTS 2015). In standard transplantation, if a patient is known to possess antibodies against the HLA of a donor (donor specific antibody (DSA)) then the transplant is incompatible due to the potential for immediate rejection of the donor organ.

H&I scientists closely monitor a renal patient's HLA-specific antibodies in terms of detecting their presence and levels using extremely sensitive techniques, and also by determining the antibody isotype and precisely which HLA they are directed towards. These 'unacceptable antigens' are listed on the patient's record with NHSBT-ODT, and donors possessing these HLA are excluded for these individuals. In the UK in 2009, 41% of adult patients and 58% of paediatric patients on the transplant waiting list possessed HLA specific antibodies (BSHI/BTS 2014), and it has been demonstrated that these individuals' sensitisation status has a direct impact on their likelihood of being offered a deceased donor kidney (Susal and Opelz 2007, BSHI/BTS 2014). Exposure to many different HLA types due to previous transplantation with mismatched grafts or multiple blood transfusions can lead to patients awaiting transplantation with high levels of a broad range of HLA-specific antibodies, directed against common HLA types. These patients may also be incompatible with a potential live donor. The increased use of live donors and the question of how to transplant patients who are sensitised, formed the impetus for the development of strategies aimed towards improving access to transplantation. One such important strategy was the development of protocols for transplanting across the DSA barrier.

HLAi transplantation represents a key development towards 'opening the door' to transplantation to as many renal patients as possible, including those for whom it may be difficult to identify a compatible donor. This is achieved by the process of 'desensitisation', which aims to remove, or reduce, donor specific HLA antibodies from the recipient's circulation (Doxiadis, Duquesnoy and Claas 2005). The reduction of antibodies pre-transplant allows the creation of a 'window of opportunity' for the transplant, which can now be deemed compatible, to proceed.

HLAi transplantation is now established within the UK and internationally (Glotz *et al.* 2004, Montgomery *et al.* 2000, Gloor *et al.* 2003). Although the precise procedure for antibody reduction varies from centre to centre, it generally involves the same techniques, usually a combination of directly filtering or absorbing antibody from the patient's circulation, and the administration of an intensive drug regime to modulate or suppress the immune response. The success of desensitisation is affected by a number of factors including the levels of antibody present, and which particular HLA it is directed towards. The aim of the procedure is to reduce the antibody to a predefined level that is deemed will not be damaging to the kidney graft, and can be managed post-transplant. In the majority of cases, HLA antibody reduction is only temporary, and an antibody 'rebound' occurs, which may be rapid and at high levels (BTS 2006). Although there are a relatively small number of cases, and follow-up is still limited, figures from the Registry of AiT (held by NHSBT), show three year graft survival and three year patient survival, for HLAi live donor transplants, to be identical to those for deceased donor transplantation in highly sensitised patients (86% and 92% respectively) (BTS 2015).

1.6 Antibody mediated graft rejection

The role that recipient antibodies directed against donor HLA play in rejection of kidney allografts has been recognised since 1966, when Kissmeyer-Nielsen *et al.* (1966) described the phenomenon of hyperacute rejection (HAR). HAR is orchestrated by recipient pre-formed HLA-specific antibodies of the immunoglobulin (Ig)-G class, directed against the donor kidney, and can cause immediate irreversible destruction of the graft. Antibodies bind to their target antigen on the vascular endothelial cells of the kidney, activating the complement

cascade (Pescovitz 2005). This in turn initiates platelet aggregation and clotting, leading to occlusion of the blood vessels, and thrombosis of the graft. Graft necrosis is rapid, in some instances whilst the recipient is still in the operating theatre (Higgins *et al.* 2008). There is no treatment for HAR, which inevitably leads to graft loss, and can endanger the life of the recipient. The occurrence of HAR is dependent on various recipient factors including whether the DSA are circulating at the time of the transplant, antibody titre, whether the antibodies are directed towards HLA Class I or Class II antigens, and which class of antibody is present.

Whilst it is accepted that circulating IgG DSA directed towards HLA Class I A and B antigens can cause HAR, the risk of IgG DSA against Class II antigens (Pollinger *et al.* 2007), HLA-specific antibodies of the IgM class (Fuggle and Martin 2004), HLA-specific antibodies in an historic serum sample (BSHI/BTS 2014) and antibodies directed against donor antigens other than HLA (Susal and Opelz 2007), in causing early rejection are still controversial. Accelerated acute rejection within the first week post-transplant can result from memory B cells being stimulated to produce DSA, as a part of an amnestic response. Antibodies are also implicated in chronic damage to the transplanted kidney (Higgins *et al.* 2008). There has been a resurgence of interest in the role of the humoral immune response in rejection over the last few years as observations by Terasaki (2003) and others implicated the importance of antibodies, as well as cellular processes, in allorecognition and the initiation of rejection responses against the donor graft. Deposition of the complement component C4d, detectable on biopsy, and demonstration of circulating DSA are the key diagnostic indicators of antibody mediated rejection (AMR) (Pescovitz 2005). Clinical parameters that can give early warning of AMR include a rise in creatinine levels and reduction in urine production. Creatinine is a chemical product generated during the course of muscle metabolism, and processed by the kidneys to be finally excreted in the urine (The Association for Clinical Biochemistry and Laboratory Medicine 2016). A high or rising creatinine level in blood is an indicator of poor renal function.

The observations of HAR led to the development of the pre-transplant crossmatch, originally performed using a complement dependent cytotoxicity (CDC) assay (Patel and Terasaki 1969), which analyses recipient serum taken

pre-transplant for antibodies directed towards donor cells. Flow cytometric (FC) based assays are now also commonly used, and provide increased sensitivity. Historically, this final test of compatibility has been used to veto a transplant in the face of a positive result, as pre-formed IgG antibodies directed towards HLA have been seen as an absolute contraindication to transplantation (Gebel and Bray 2008). HLAi transplantation provides a way of crossing this antibody barrier, by seeking to transform a crossmatch result from positive to negative, thus allowing transplantation to proceed. As pointed out by Gebel and Bray (2008), HLAi also describes transplantation which is performed in the presence of a weak antibody, which may only be detectable using sensitive antibody screening assays such as Luminex® technology, and which does not result in a positive crossmatch. It is crucial that the antibody titre (generally reflected by the mean fluorescence intensity (MFI) value obtained from bead-based assays), class and specificity is clearly defined in all potential HLAi cases so that a judgement can be made about whether to instigate procedures to reduce antibody levels, or whether to simply adjust the post-transplant management of the patient.

1.7 Desensitisation protocols as a method of facilitating HLAi transplantation

‘Desensitisation’ describes the application of a regimen of drugs and/or treatments in an attempt to remove or reduce DSA from a potential recipient’s circulation pre-transplant. Each case is assessed individually in terms of precisely what course of treatment to implement and the HLA antibody reduction target to achieve in order for the transplant to be deemed compatible and allowed to proceed; for example a CDC crossmatch conversion from positive to negative. The clinical protocol for desensitisation varies from centre to centre, and the full procedure often incorporates newly developed and potent immunosuppressive drugs, that aim to suppress lymphocyte activation and proliferation. Treatment generally involves a regimen of immunosuppression (see table 1.2) combined with one or more of the modalities summarised in table 1.3.

Table 1.2 Immunosuppressive drugs utilised in renal transplantation in Leeds

Drug	Mode of action
Alemtuzumab	Mab targeting CD52 cell surface antigen, present on mature lymphocytes (Wiseman 2016)
Basiliximab (Simulect)	Mab targeting the α -chain of the IL-2 receptor (CD25) (Wiseman 2016). Blocks IL-2 binding and subsequent T cell proliferation
Cyclophosphamide, active metabolite phosphoramidate mustard	DNA alkylating agent (Wiseman 2016). Inhibits DNA replication and cell division
Cyclosporin	Calcineurin inhibitor, prevents production of IL-2, and subsequent T cell activation (Janeway <i>et al.</i> 2005)
Mycophenolate, active metabolite mycophenolic acid (mycophenolate mofetil (MMF))	Blocks purine biosynthesis, affecting T and B lymphocyte proliferation (Parham 2009). Inhibition of antibody production by B cells
Prednisone/ methylprednisolone	Anti-inflammatory, down regulation of gene expression of 'inflammatory mediators' (Janeway <i>et al.</i> 2005)
Tacrolimus (FK506)	Calcineurin inhibitor, prevents production of IL-2 (Janeway <i>et al.</i> 2005)

Mab – monoclonal antibody; IL – interleukin; DNA - deoxyribonucleic acid

Table 1.3 Summary of desensitisation methods employed in AIT programmes

Desensitisation method	Mechanism	Dose	Side-effects
Pooled human intravenous immunoglobulin (Ivlg)	Uncertain but thought to involve the modulation of antibody production by several different mechanisms (BTS 2006, Jordan and Pescovitz 2006)	Typically, either implemented alone at high dose (usually 2g/kg) or at lower dose in conjunction with plasmapheresis (100-500mg/kg) (BTS 2006, Jordan and Pescovitz 2006)	High dose Ivlg can cause fever/chills, severe headache, thrombosis, and nephrotoxicity. Rarely anaphylaxis (Magee 2006)
Plasmapheresis (PP)/plasma exchange (PE)	Separation of whole blood by centrifugation, and subsequent removal of antibody-containing plasma. Non-specific, so vital plasma proteins must be replaced with fresh frozen plasma (FFP), albumin and clotting factors. Double-filtration plasmapheresis , removes plasma from the circulation using a filter, and then additionally passes it through a second filter which traps only large molecules e.g. immunoglobulin, so that smaller molecules such as albumin and some clotting factors can be returned to the patient (Higgins <i>et al.</i> 2008)	Generally carried out on alternate days immediately pre- and post-transplant (Jordan and Pescovitz 2006)	Related to the removal of important components of plasma, e.g. depletion of clotting factors and hypocalcaemia and to the associated administration of FFP, which can cause fever and chills (BTS 2006, Magee 2006)
Immunoabsorption	The process of passing the patient's blood through a column which binds antibodies. For the removal of HLA directed antibodies, the process is non-specific using protein A columns, which binds all IgG subclasses, except IgG3 (BTS 2006)	Similar to plasmapheresis – see above	Depletes antibodies, but other plasma components are returned to the patient, so less adverse effects (BTS 2006)

Table 1.3 continued

Rituximab (CD20 antibody)	A chimeric humanised monoclonal antibody, which has been used in the treatment of autoimmune disorders (Magee 2006). CD20 is expressed throughout the B cell lineage, with the exception of plasma cells, which are able to persist in 'survival niches' in the lymphoid organs. Rituximab is capable of eliminating B cells (Jordan and Pescovitz 2006) and has been demonstrated to reduce HLA directed antibodies detected in serum (Vieira <i>et al.</i> 2004)	Usually given as single dose, e.g. 375mg/m ²	Severe, prolonged immunosuppression which can potentially lead to bacterial and fungal infections. Occasionally fever during administration
Splenectomy	Featured in ABOi protocols in the United States. Also used as rescue therapy in cases of severe AMR (Locke <i>et al.</i> 2007). Spleen is a secondary lymphoid organ and important site of activation of immune cells	N/A	Increased risk of infection. Surgical risk. Not recommended by BTS (BTS 2015)
Simultaneous kidney-liver transplantation	A transplanted liver is able to withstand moderate levels of HLA-specific antibodies, and can act as a 'sponge' to absorb antibodies and thereby potentially protect the kidney, allowing AiT to take place (Mosconi <i>et al.</i> 2006)	N/A	This is a risky procedure which could lead to the loss of both organs, so is not commonly used. In Sweden, liver lobes have been used in deceased donor kidney transplantation (BTS 2006)

BTS – British Transplantation Society; HLA – human leucocyte antigen; IgG – immunoglobulin-G; ABOi – ABO blood group incompatible; AMR – antibody mediated rejection; AiT – antibody incompatible transplantation

1.8 Laboratory based HLAi transplantation research reported in the literature

Despite the experience gained, questions remain around the immunological process of desensitisation and subsequent HLAi transplantation. It is recognised that it is not always realistic to aim to completely remove HLA-specific IgG, and that this is not always necessary for a successful outcome. Moreover, a reduction in antibody titre to a level that will not cause HAR and is manageable post-transplant with immunosuppressive drugs and additional sessions of plasmapheresis, will still allow for transplantation. The question of whether it is important to prevent resynthesis of antibody post-transplant has been investigated by Higgins *et al.* (2007). The study concluded that DSA levels increased within the first month post-transplant in most patients, but a correlation with clinical features of rejection was not always observed. Where rejection and rises in DSA occurred together, in some cases it was possible to achieve resolution of the rejection in the continued presence of DSA. The link between circulating DSA post-transplant and rejection, in standard transplantation, has formed the basis of other studies (Terasaki 2003) but is not clear cut, as levels of DSA in serum samples may not be truly indicative of damage to the graft. No detectable antibody, or low levels, may simply mean that antibody is being bound and retained in the kidney (BTS 2006, Higgins *et al.* 2008). The observations by Higgins *et al.* (2007) provide evidence for the theory of kidney transplant accommodation, which describes the maintenance of the graft in the presence of circulating potentially harmful antibodies. Mechanisms for graft accommodation have been suggested, but investigations around this phenomenon are ongoing.

Modulation of the antibody response, with antibody reduction treatments resulting in a long-term depletion of DSA, is another feature often observed with successful AiT (BTS 2006). Zachary, Montgomery and Leffell (2005) suggest that elimination of DSA is by an active down-regulation process, which is mediated by T lymphocytes. This is important as often T cell depleting agents are administered as part of the immunosuppression regime, and raises the question that this may be removing key regulatory T cells (Tregs) as well as those involved in initiating allorecognition.

How individual patients respond to a desensitisation protocol in terms of antibody levels is often unpredictable, and, as observed by Higgins *et al.* (2007), HLA-specific antibodies can often remain at low titre. Zachary, Montgomery and Leffell (2005) investigated antibody depletion by specific desensitisation methods in an attempt to identify factors associated with antibody persistence. Further elucidation of this issue would be valuable in predicting which patients will respond well in desensitisation programmes, resulting in a way of targeting treatment for the patients in whom resistance to desensitisation is expected. The study analysed the frequency of antibody elimination and the factors that could influence this. DSA persistence was shown to have a significant association with DSA titre at the initiation of treatment, i.e. if the antibody level was high before desensitisation treatment commenced, then it was more likely to persist following treatment and post-transplant. Zachary, Montgomery and Leffell (2005) comment that this could be a reflection of the number of B lymphocyte clones, specific for a particular donor antigen.

Both the Higgins *et al.* (2007) and Zachary, Montgomery and Leffell (2005) studies raise important questions around the topics of graft accommodation and antibody modulation, in terms of what is happening to the patient's immune system whilst they are undergoing desensitisation, and what are the key cellular mediators involved in the modulation of DSA. Zachary, Montgomery and Leffell (2005) state that transplantation, and concomitant exposure to donor antigens is essential for targeted DSA elimination to occur, and that third party HLA-specific antibodies (not directed against donor antigens) can persist.

Other studies investigating the underlying processes occurring during desensitisation include a study examining 'third party' HLA and non-HLA antibody levels, and their relationship to DSA levels (Krishnan *et al.* 2008). Another potential area of interest is complement activation in AiT patients (Hamer *et al.* 2008). A more recent report (Field *et al.* 2014) described the potential use of serum biomarkers in predicting early acute rejection in a cohort of HLAi kidney transplant recipients. Out of a series of biomarkers analysed, interferon-gamma-inducible protein 10 (IP-10) and neutrophil gelatinase-associated lipocalin (NGAL) exhibited significantly increased levels in patients who went on to develop acute rejection.

Previous work from our centre (Cherukuri *et al.* 2012) has involved immunophenotyping of renal patients undergoing 'standard' transplantation. Populations of lymphocytes studied included naïve and memory T and B cells, transitional B cells and regulatory B cells. Kamburova *et al.* (2014) also applied the laboratory tool of immunophenotyping to renal transplant recipients, to compare the results of rituximab and non-rituximab treated patients. Reconstitution, post-rituximab treatment, has been the topic of several other previous studies. Kopchaliiska *et al.* (2009) used immunophenotyping to investigate the reconstitution of B cell subsets in a group of eleven highly sensitised renal transplant recipients who had been treated with rituximab. Due to its therapeutic use in autoimmune disorders, there is a considerable amount of data concerning B cell reconstitution post-rituximab treatment in rheumatoid arthritis patients (Roll, Dorner and Tony 2008, Leandro *et al.* 2006). Splenic B cell populations have also been studied, and compared to peripheral blood cell populations, following certain desensitisation treatments (Ramos *et al.* 2007). Importantly, this study demonstrated that memory (CD27⁺) and plasma cell (CD138⁺) subsets survived in the spleen following treatment with both plasmapheresis/IVIg and rituximab. The main focus of these latter studies was in relation to reconstitution post-rituximab treatment. Although this remains relevant in terms of lymphocyte repopulation post ablative therapy, rituximab is no longer the primary desensitisation tool utilised in the Leeds centre.

The studies described above highlight the fact that questions remain around the immunological processes occurring both during desensitisation and post-transplantation, and increased understanding of this area would be a step towards predicting how patients will respond to this treatment. Immunosuppressive drugs and the desensitisation process deplete cells involved in the immune response directed towards the donor organ, but uncertainties remain about the associated immunological long-term effects, including cellular reconstitution. Also, of key importance is the effect of desensitisation on cells identified as being important in regulation of the immune response.

1.9 Data collection for this study

This research study sought to investigate HLAi transplant recipients, in order to gain an insight into the underlying immune processes underpinning individuals'

responses to desensitisation, chiefly using the laboratory based method of flow cytometry. The rationale for the choice of lymphocyte populations to study was two-fold; firstly based around some of the literature described above, and secondly around the T cell and B cell populations that are known to be important in the allo-immune response to the transplanted kidney. The defining feature of HLAi transplantation patients is that they possess DSA. However, the detection of donor relevant HLA-specific antibodies in the circulation may be viewed as a symptom, with the source of the problem being that these potential recipients all have immunological memory of certain HLA. Therefore, immune memory was a key element that was considered when designing the study.

Three sets of data were collected on each participant peripheral blood sample; a commercial kit was utilised to study lymphocyte subsets present, commercial fluorophore labelled antibodies were grouped into 'panels' for immunophenotyping of key T and B lymphocyte populations and the specificity of HLA antibodies present was analysed. The rationale behind the lymphocyte populations investigated, by immunophenotyping, follows.

1.9.1 Recent thymic emigrants (RTE) immunophenotyping

This T cell panel was included to analyse the populations of naïve and memory T cells present, and to identify recent thymic emigrants (RTEs). The cell surface markers utilised were CD4, CD45^{RO} and CD31. CD4 'helper' T cells were defined as naïve or 'resting' T cells, negative for CD45^{RO} (therefore presumed to be positive for CD45^{RA}) or activated effector or memory T cells (CD45^{RO} positive). Different isoforms of the transmembrane tyrosine phosphatase CD45 marker are created by the alternative splicing of mRNA from the CD45 gene (Parham 2009). The larger of the CD45 isoforms is CD45^{RA}, present on naïve T cells, facilitating relatively weak signals in response to specific antigen. In contrast, the smaller extracellular domain of CD45^{RO} allows stronger signals in response to antigen by its more effective association with the T cell receptor (TCR) and other co-receptors (Parham 2009). In the peripheral blood circulation of a healthy adult, approximately half of the $\alpha\beta$ T cells are naïve and half are memory (Parham 2009).

Furthermore, naïve T cells that have recently undergone their development and exited the thymus into the peripheral circulation, termed RTEs, were identified by this panel as being CD4⁺CD45^{RO}-CD31⁺. During T cell development in the thymus, the TCR gene undergoes rearrangements in order to generate the huge diversity seen in the receptor molecule. During this process, gene fragments are deleted as circular excision products, or T cell receptor excision circles (TRECs). Although TRECs can be used as a marker of recent thymic development, they are not replicated, so dilute out with each cellular division (Miltenyi Biotec 2016, Kohler and Thiel 2009). Expression of the CD31 marker has been identified as correlating with the presence of TRECs, and hence has become a marker for RTEs (Kohler and Thiel, 2009). The number of RTEs leaving the thymus decreases with age due to thymic involution, the replacement of the T cell producing tissue of the thymus with fatty tissue, which begins one year following birth in humans (Parham 2009, Ribeiro and Perelson 2007).

1.9.2 Activated T cell immunophenotyping

This immunophenotyping panel was included in order to investigate HLA-DR expression levels on CD4 and CD8 T cells. Monoclonal antibodies (Mabs) directed against CD3, CD4 and HLA-DR were included, and data was gathered on the CD3⁺CD4⁺HLA-DR⁺ and CD3⁺CD8⁺HLA-DR⁺ populations. Whilst HLA Class I molecules are expressed by all nucleated human cells, Class II expression is limited to specialised cells of the immune system, particularly antigen presenting cells. Resting T cells do not express Class II molecules, but expression is induced on activated T cells (Parham 2009). An increase in the percentage of T cells expressing HLA-DR may imply an increase in the activation status of T cells present, ultimately leading to an increase in effector cells. However, it could also correspond with already activated T cells undergoing proliferation, and thereby increasing in percentage terms. Both of these scenarios are of interest to assess in a transplantation setting.

1.9.3 Regulatory T cell (Treg) immunophenotyping

The cell markers used for this panel consisted of CD4, CD25 and the intracellular protein Forkhead Box P3 (FoxP3). Effector T cells (CD4⁺CD25⁺) were identified by this panel, alongside regulatory T cells (Tregs). The latter represent a distinct population of CD4 T cells, which are characterised as expressing high levels of

CD25 in conjunction with the transcription factor FoxP3 (CD4⁺CD25^{hi}FoxP3⁺), sometimes referred to as 'scurfin'. The gene for FoxP3 is located on the X-chromosome and is uniquely expressed in Treg cells (Dijke, Weimar and Baan 2008, Kang, Tang and Bluestone 2007). Tregs are so called due to their 'suppressor' qualities in regulating other effector T cells, of particular importance in preventing 'self-reactivity' to auto antigens. Initial investigations leading to the discovery of FoxP3 and greater understanding of the role of Tregs were instigated by research into certain autoimmune conditions (U.S. National Library of Medicine 2016). Immunodysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome is caused by mutations in the FoxP3 gene, resulting in a reduction or absence of Tregs, and dysregulation of the immune response (van der Vliet and Nieuwenhuis 2007). The severe and systemic nature of IPEX clearly demonstrates the essential role that Tregs play in controlling the immune response.

In normal function, the suppressive effect of Tregs, initiated when an autoreactive CD4 T cell and Treg cell interact with the same APC, is achieved by the production and release of cytokines, such as interleukin-4 (IL-4), interleukin-10 (IL-10) and transforming growth factor-beta (TGF- β). These immune modulating cells are of interest to monitor in an HLAi transplantation setting, due to their potential role in the dampening of the allo-response and ultimately the achievement of transplant tolerance.

In this study, Tregs were analysed according to the method of Arumugakani, Wood and Carter (2008) (detailed in section 2.8 of this report), which is carried out using peripheral blood mononuclear cells (PBMCs) isolated from whole blood.

1.9.4 Memory B cell immunophenotyping

For B lymphocytes, it was deemed to be important to include cell markers characteristic for both naïve and mature B cells, in order to attempt to ascertain the status of the reconstituting immune system, post-transplantation. Memory B cells can reside in the secondary lymphoid organs, in a quiescent state, for the life-time of an individual. These long-lived memory clones are different to the effector B cells that participate in a primary immune response, in that they are isotype switched and have undergone somatic hypermutation (Parham 2009).

Thus, the secondary response initiated by memory B cells is both stronger and faster than when the antigen was originally encountered. This is clearly of key relevance in the setting of HLAi transplantation where an antibody barrier is being crossed, and information gained around the presence of memory B cells (albeit in the periphery) may be useful to tie in with an emergent alloresponse.

The cell surface marker CD27 was employed to distinguish between naïve (negative for CD27), and memory B cells (positive for CD27). The memory B cells were then further defined as either isotype class switched (IgD negative) or IgM memory B cells (IgD dull/positive). The stage of development of the circulating memory B cells could be important, as switched (IgD negative) memory B cells secrete high affinity IgG antibody.

1.9.5 Transitional B cell immunophenotyping

The cell surface markers CD19, CD24, CD38 and CD27 were utilised to investigate different populations of B cells present. Three sets of data were recorded. Firstly, cells positive for CD19, CD38 and CD24 were noted as being mature B cells. B lymphocytes possessing the immature phenotype CD19⁺CD38^{hi}CD24^{hi} were designated as transitional B cells (Cherukuri *et al* 2012), and CD19⁺CD38^{hi}CD27⁺ as plasmablasts, on the developmental pathway to becoming plasma cells.

1.9.6 Plasma B cell immunophenotyping

The characteristic marker, CD138, was used to identify any circulating plasmablasts, using two phenotyping strategies. Plasmablasts were defined as being CD20⁻ (CD19⁺CD138⁺CD20⁻). In addition, information was gathered on the population of CD19⁺ B cells that expressed CD38 and CD138 (CD19⁺CD38⁺CD138⁺). CD20 is expressed throughout the B cell lineage, but importantly not on plasma cells, which is relevant in the context of the use of rituximab. Consequently, CD20⁻ plasma cells are able to persist long-term in 'survival niches' located in the secondary lymphoid organs (Ramos *et al.* 2007). It was planned to investigate the relationship between HLA-specific antibody levels and cell populations present.

1.10 Risk assessment for HLAi transplantation

HLAi transplantation is generally defined as transplantation proceeding where the recipient possesses HLA-specific antibodies, which are relevant in the context of the donor HLA type, in a current sample. Often, the definition infers crossmatch positivity due to current DSA. However, for the purposes of this study, potential recipients were considered as participants if they had any level of DSA present, directed against a live donor, which necessitated some degree of transplant pre-conditioning.

The assessment of recipient-donor compatibility is carried out as an analysis of risk versus benefit. This is in terms of the risk of the likelihood of unmanageable immediate or early rejection of the graft balanced against the benefit to the patient of receiving a kidney transplant. This analysis must be viewed in the context of the chance of the patient receiving a kidney graft from another source (either a deceased donor offer, or a living donor), and the clinical urgency of the individual case. An evaluation of recipient versus donor alloreactivity, informed by a detailed antibody profile of the recipient and usually a pre-transplant recipient versus donor crossmatch, is a crucial part of this decision making process.

1.10.1 The Leeds approach

The British Transplantation Society guidelines for AiT (2015) state that potential HLAi transplant recipients should be risk assessed according to parameters known to affect transplant outcome, including positive CDC crossmatch, high FC crossmatch, cumulative levels of DSA in excess of 10,000 MFI, presence of multiple DSAs, presence of HLA Class I directed DSA, potential use of a deceased donor and presence of repeat HLA mismatches.

This guidance is followed in Leeds, where each potential HLAi transplantation has always been managed in a 'case by case' approach, and the precise pre-transplant work-up is individualised, according to the clinical and laboratory profile of the recipient-donor pair. Currently, in order to gather laboratory data from the same time point, a 'baseline' crossmatch is recommended by the laboratory, to service users, which comprises a CDC crossmatch, FC crossmatch and HLA-specific antibody definition using a single antigen bead (SAB) based

method. If multiple DSAs are present, the MFIs generated by the antibody screening assay are added together as a total for HLA Class I and Class II directed antibodies. The total cumulative MFI and Class I directed cumulative MFI are then used in combination with the CDC and FC crossmatch results to generate a 'challenge score' which is intended to provide some guidance on how much clinical intervention would be required to remove the DSA and allow the transplant to proceed. For example, negative results for both crossmatches, and cumulative Class I directed DSA of less than 3,000 MFI corresponds with a challenge score of 0. At the other end of the risk spectrum, if both crossmatches are positive, and the associated cumulative Class I directed antibodies are greater than 5,000 MFI, with overall DSA greater than 18,000 MFI, the challenge score generated by this algorithm is 4.

Alongside the challenge score, a current case is often likened to a previous historic case, in order to build experience into the process of putting together the desensitisation protocol. The risk assessment is also incorporated into the decision of the laboratory results profile which will be interpreted as 'compatible' for each HLAi recipient-donor pair. However, as a general rule, the pre-transplant CDC crossmatch should be T and B cell negative, and a corresponding reduction in the MFI of the DSA present should be evident.

1.11 Objectives of the study

The objectives of the study were:

- To monitor and gain knowledge of any fluctuations occurring in key populations of lymphocytes during the process of HLAi transplantation, and to correlate this with other laboratory and clinical factors, with particular reference to DSA MFI levels.
- To examine the long-term effects of HLAi transplantation on recipients' immune systems, and investigate the immune reconstitution process, post-transplantation.

It was anticipated that completion of both of these objectives would lead to an increased scientific understanding of what is happening to an individual's immune

system during the process of HLAi transplantation. This would then feed into the overall objectives below:

- To support the development of an additional laboratory 'tool', some components of which may feed into the local risk assessment strategy described above, to allow clinicians to target immunosuppressive drugs and adjust the treatment for HLAi transplantation as a part of a personalised management plan. This would allow more informed decisions for patient care, with the aim of improving transplant outcome.
- To aid in creating an expanded evidence base in order to support the effective management of resources involved in the field of AiT.

2.1 Project overview

The project consisted of two approaches to investigating changes in the phenotypes and phenotype profiles of peripheral blood lymphocytes pre and post-HLAI kidney transplantation. The study involved analysis of blood samples obtained from –

- (1) a prospective participant cohort of patients undergoing HLAI transplantation, recruited between November 2011 and November 2012, and
- (2) a retrospective participant group, of HLAI kidney transplant recipients, transplanted at St James's University Hospital, Leeds, between 2001 and 2011. This group was recruited between June 2012 and June 2013.

Each of the study samples collected was analysed for the following:

- Lymphocyte subsets (absolute counts and population percentages)
- T and B cell immunophenotypes
- Specificity of HLA antibodies present

2.2 Research Ethics Review

An application was submitted to the NHS National Research Ethics Service (NHS Research Ethics Committee (REC)) and the NHS Research and Development office (NHS R&D) through the Integrated Research Application System (IRAS) (Health Research Authority 2016). Supplementary information submitted included: the study protocol, evidence of scientific review and statistician comments (the latter two both from Sheffield Hallam University), copies of the letter for participants' GPs, participant information sheets and consent forms. The latter documents are included in this report as appendices 1-3.

The NHS REC application was reviewed at the Leeds Central REC meeting on May 20th 2011, with full approval finally granted in July 2011 (REC reference number 11/YH/0102). As part of the NHS R&D approval sought, and as participant samples were to be collected at multiple sites, Site Specific Information (SSI) forms were completed and submitted to:

Leeds Teaching Hospitals NHS Trust (R&D reference number IM11/9743) - approval granted August 2011.

Bradford Teaching Hospitals NHS Foundation Trust (R&D reference number 1341) - approval granted October 2011.

Hull & East Yorkshire Hospitals NHS Trust (R&D reference number R1217) - approval granted January 2012.

York Hospitals NHS Foundation Trust (R&D reference number YOR-A02069) - approval granted April 2012.

A project site file was collated and stored in the Transplant Immunology Laboratory, St. James's Hospital, Leeds (Chief Investigator's office). This site file was subject to an audit by Sheffield Hallam University Research Ethics Committee in July 2012.

2.2.1 Obtaining informed written consent

The initial approach to all patient participants was via a nursing or medical member of the renal transplantation care team at each NHS site. A project information leaflet, and a consent form were provided to all potential participants, and time to consider participation in the project was given. There was time to ask questions or clarify points if this was required by potential participants. It was made clear to each participant that their involvement (or non-involvement) in the study was voluntary, and would not affect their clinical care in any way. A member of the renal clinical care team recorded the informed consent of participants who were willing to proceed with the study; this was in the format of a written consent form, which was signed and dated. A copy of the consent form was then given to the participant and the original was stored in the project site file by the Chief Investigator. Participants were able to withdraw their consent at any time during the study. Contact details were provided on the information leaflet, for gaining any further information about the study, at any point during its progression, or for learning about the study outcome.

2.3 Anonymisation of study participants and samples

In order to comply with information governance regulations and also to allow certain analyses to be performed 'blind', all patient participants were allocated an identification number, by the Chief Investigator, upon recruitment. The participants were numbered sequentially in the order of their recruitment to the study, irrespective of which cohort they were recruited to, meaning that within the prospective and retrospective groups, the identification numbers were not sequential. The normal controls recruited were also numbered in a similar way; however sequential numbers were used for this cohort. In order that the results of the study could be interpreted in a meaningful way, the Chief Investigator kept a record of the identity of all participants.

For the prospective cohort, from whom multiple blood samples were collected, in addition to the participant number, each sample was allocated a letter, in order to allow identification of longitudinal samples. For example, sample 1A is the first study sample collected from participant 1, sample 1B is the second study sample collected from the same participant, and so on.

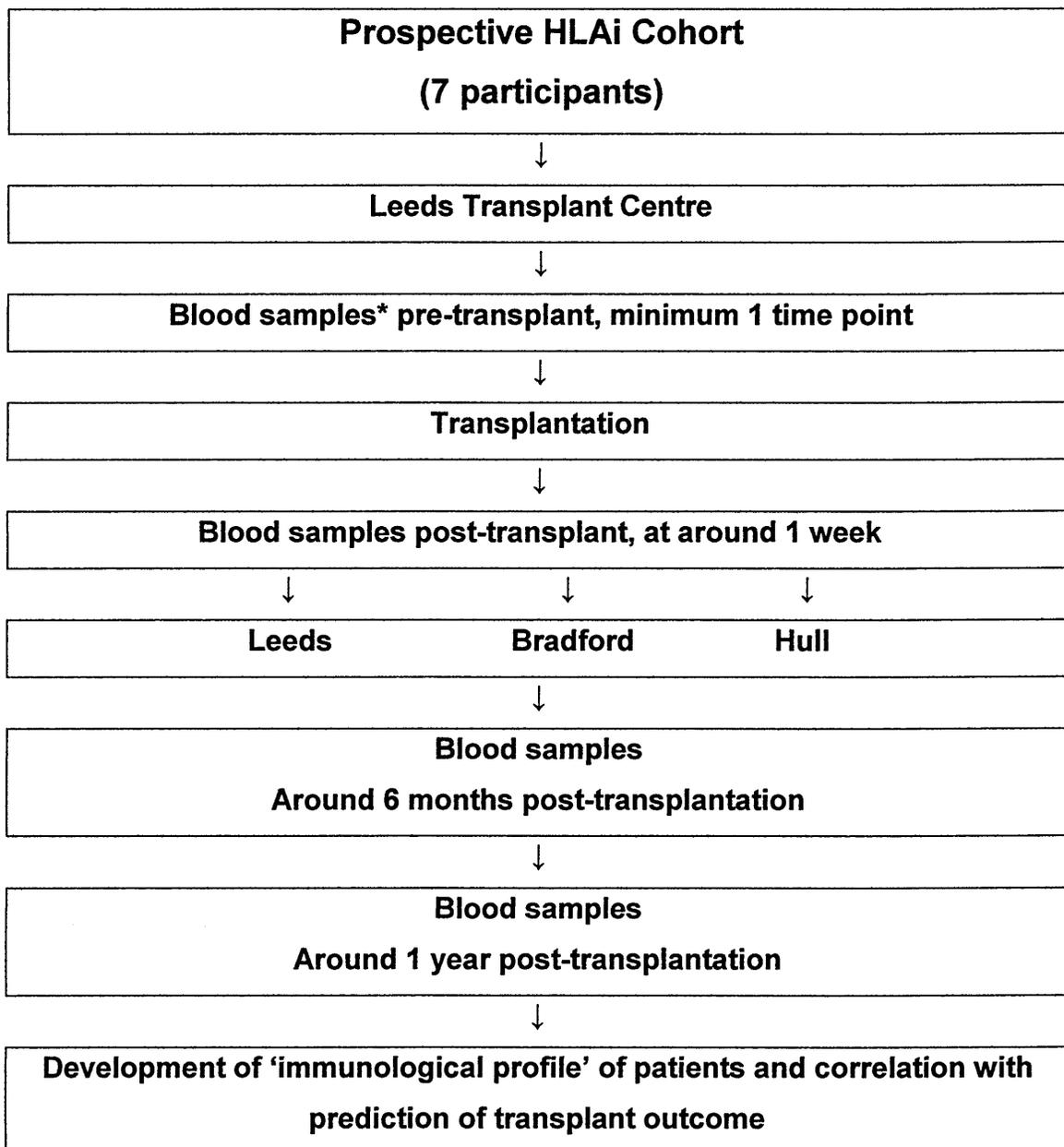
2.4 Description of the study cohort

2.4.1 Prospective study cohort

Within the year's timeframe allotted for sample collection and analysis of the prospective cohort, eight patients were progressed down this route for HLAi kidney transplantation. Seven consented to be included in the study, and longitudinal blood samples were collected by specialist and research nurse colleagues (according to the schedule detailed in figure 2.1). One patient lacked the capacity to consent to the study, so was not approached for participation. Of the seven consenting participants, three were based in Leeds, two were from Bradford and two from Hull.

The Chief Investigator was alerted to potential participants by regular communication with specialist nurse colleagues, and also by remaining vigilant to patient-donor pairs being booked into the laboratory work diary, who were under consideration for transplantation by this specialised route.

Figure 2.1 Planned schedule for sample collection from the cohort of prospective HLAi transplantation participants recruited to the study



HLAi – HLA incompatible

* Blood samples: EDTA blood sample to examine different populations of lymphocytes present, by flow cytometry immunophenotyping. Plain clotted (serum) sample to examine HLA-specific antibodies (may have been taken as part of routine blood samples at some time points).

Although R&D approval was granted by four recruitment sites, no prospective participants were subsequently recruited from York.

For reasons that will be expanded upon and explored in the discussion of this report, the sample collection for the prospective cohort did not always proceed straightforwardly according to the schedule in figure 2.1. Table 2.1 details actual samples received on the seven consenting participants.

Table 2.1 Summary of sample collection time points of the prospective HLAi transplantation participants recruited to the study

Participant ID	Gender	Pre-transplant sample collection time points			Post-transplant sample collection time points			Location
		A	B	C	C	D	D	
1	M	A	B	C	Transplant cancelled			Bradford
2	F	A	B		Transplant cancelled			Leeds
3	M	A	B		C	Patient re-located from Leeds		Leeds
7	M	A			B	C	D	Leeds
9	F	A	B		C	D		Bradford
11	F	A			B	C		Hull
14	F	A	B		C	D		Hull

ID – identification; M – male; F – female

Allocation of participant ID and sample collection time points are described in section 2.3.

Shaded rows indicate participants for whom it was not possible to obtain scheduled post-transplantation samples.

Further explanation of the timing of the study samples collected from each prospective participant follows below. The days prior (-) or post (+) the scheduled transplant date are indicated, where the day of transplant is regarded as Day 0.

Participant 1:

1A - Day -16 (pre-antibody removal treatment)

1B - Day -9

1C – Day -1

For this participant, data is also included from two routine clinical samples denoted 1 and 1D:

Sample 1 - -4.5 months (HLAi 'baseline' crossmatch)

Sample 1D – Day +11 (cancelled)

Participant 2:

2A – Day -11 (pre-antibody removal treatment)

2B – Day -1

Participant 3:

3A – Day -8 (HLAi 'baseline' crossmatch, participant commenced on MMF)

3B – Day -1

3C – Day +8

Participant 7:

7A – Day -8 (pre-transplant crossmatch, participant commenced on MMF)

7B – Day +5

7C - +8 months

7D - +13 months

Participant 9:

9A – Day -12 (pre-transplant crossmatch, participant commenced on MMF)

9B – Day -5

9C - +6 months (participant on haemodialysis following transplant nephrectomy)

9D - +1 year

Participant 11:

11A – Day -9

11B - +6 months

11C - +1 year

Participant 14:

14A – Day -14 (pre-antibody removal treatment)

14B – Day 0

14C - +6 months

14D - +1 year

2.4.2 Retrospective study cohort

Due to the specialised nature of the HLAi transplantation programme, the laboratory files for patients previously transplanted by this route are stored in a separate filing system to the standard transplanted patients. In Leeds, a laboratory file is created for each potential transplant recipient, and contains a hard copy of all their laboratory reports, some laboratory data and all pertinent correspondence. In order to identify the retrospective cohort, these files were located and cross referenced against a list of HLAi patients, provided by the Local Collaborator (Leeds).

Of the resultant group of thirty-two potential participants, five were immediately excluded; four patients had died since their transplant of causes unrelated to their treatment and one patient had relocated and transferred to a distant centre. This left a final figure of twenty-seven potential retrospective participants to consider, which were split across the four sites as follows:

Leeds – 15, Bradford – 2, York – 3, Hull – 7

Specialist nurse colleagues in Leeds were consulted to advise whether any of the potential participants should be excluded for any reason, for example if they did not regularly attend clinic appointments. It was decided that the Leeds cohort would be prioritised for participation, due to the practicalities of sample collection

and transport. Renal research nurse colleagues provided the chief investigator with the appropriate 'post-transplant' or 'dialysis' clinic lists each week, which were then scrutinised for the identified patients. Clinic appointments of potential participants would then be communicated back to the renal research nurses, who would approach, and collect samples from consenting patients. Of the fifteen patients in Leeds, one had subsequently transferred to Harrogate, and another to Hull. Of the remaining thirteen patients, eleven were recruited and included in the study.

Specialist nurse colleagues in Bradford also helped with providing clinic appointment dates for the two potential participants attending at this site. One of these patients was recruited into the study, the second patient did not consent within a time period to allow inclusion.

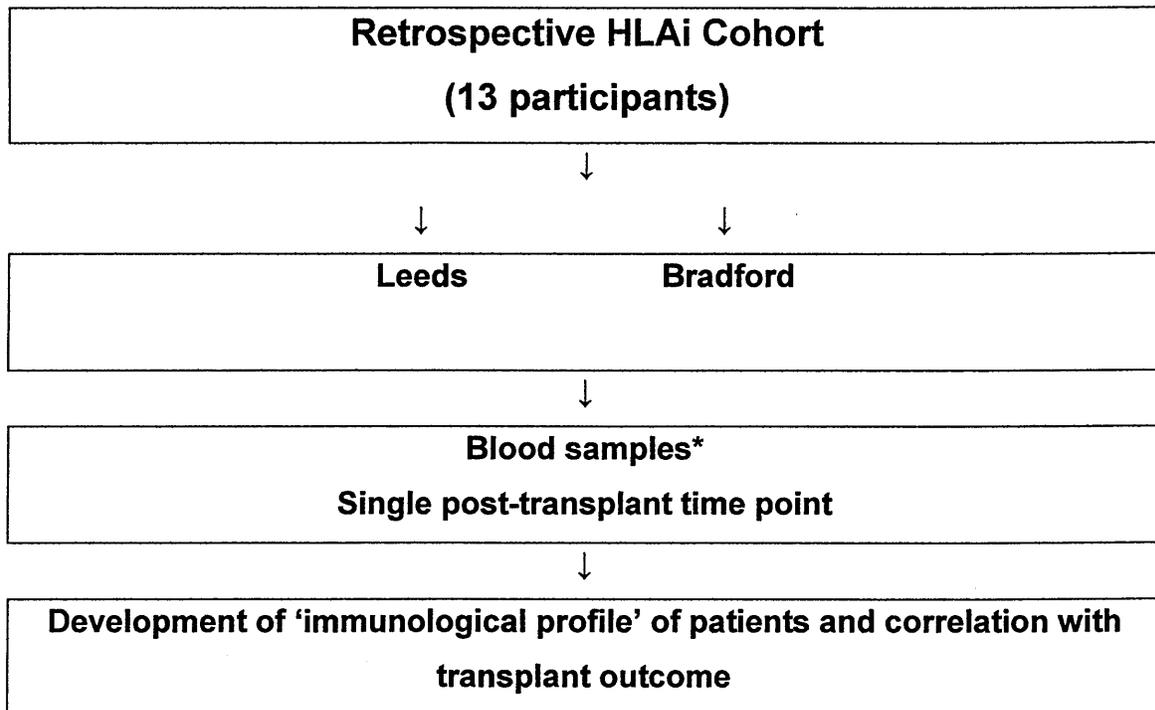
Of the three potential participants usually attending clinics in York, one was recruited at a clinic in Leeds. The remaining two York patients and the seven patients from Hull were not approached for recruitment, as the time period set for sample collection was exceeded before this was possible.

In this manner, a total of thirteen retrospective patient participants were recruited to take part in the study. The pathway of scheduled sample collection is detailed in figure 2.2. Further detail on the samples received from the retrospective participants, including time period post-transplantation, is shown in table 2.2.

2.4.3 Normal controls

During the course of the study, a total of nine normal controls were recruited from NHS colleagues, all from within the renal transplantation care team. They were all age (within 5 years) and gender matched with a retrospective and/or a prospective participant. All normal controls signed a consent form for participation in the study, and samples were collected by a nurse or medical colleague, as detailed in figure 2.3. Further details on the normal control group are shown in table 2.3.

Figure 2.2 Planned schedule for sample collection from the cohort of retrospective HLAi transplantation participants recruited to the study



HLAi – HLA incompatible

* Blood samples: EDTA blood sample to examine different populations of lymphocytes present, by flow cytometry immunophenotyping. Plain clotted (serum) sample to examine HLA-specific antibodies.

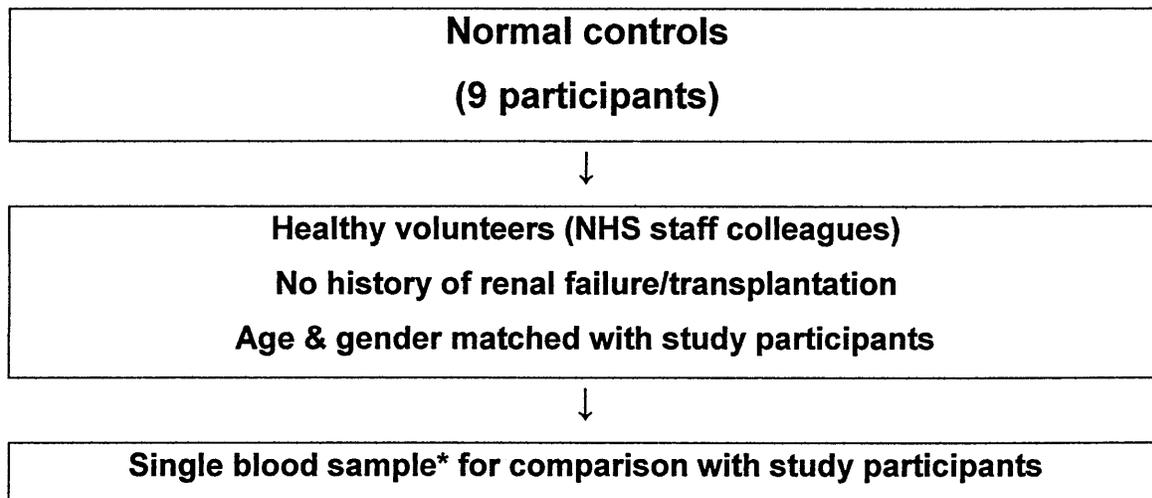
Although R&D approval was granted by four recruitment sites, no retrospective participants were subsequently recruited from York or Hull.

Table 2.2 Sample collection time points of the retrospective HLAi transplantation participants recruited to the study

Participant ID	Gender	Time post-transplantation	Location
4	F	8 months	York
5	F	9 months	Leeds
6	M	11 months	Leeds
8	F	11 years	Leeds
10	F	2 years	Leeds
12	F	7.5 years	Leeds
13	F	5 years	Leeds
15	F	4 years	Bradford
16	M	8 years	Leeds
17	F	8 years	Leeds
18	M	4 years	Leeds
19	F	4 years	Leeds
20	M	6 years	Leeds

ID – identification; F – female; M - male

Figure 2.3 Planned schedule for sample collection from the cohort of normal control participants recruited to the study



* Blood sample: EDTA blood sample to examine different populations of lymphocytes present, by flow cytometry immunophenotyping.

Table 2.3 Details of the normal control participants recruited to the study

Normal control participant ID	Gender	Age (years)	Matched patient participant ID (matched with normal control for gender & age +/- 5 years)	
			Prospective cohort	Retrospective cohort
1	M	38	7	
2	M	50		6
3	F	40	14	12
4	F	51		17
5	F	27		13
6	F	40	11	5
7	F	54		15
8	F	30		10
9	M	28	1	16

ID – identification; M – male; F – female

Each normal control was matched with a study participant, from either, or both of, the prospective cohort and/or the retrospective cohort, for gender and age (\pm 5 years).

2.5 Key technology utilised in this research: flow cytometry

The main laboratory technology used for this research study was flow cytometry. Flow cytometry literally describes the measurement of cells as they pass in single file past a specific point of 'interrogation'. The strength of flow cytometry lies in that it allows very rapid analysis of many thousands of single human cells, or 'events'. Essentially, as each cell passes through a laser beam, information is collected about its morphology and, when used in conjunction with fluorophore tagged antibodies, its surface antigen profile.

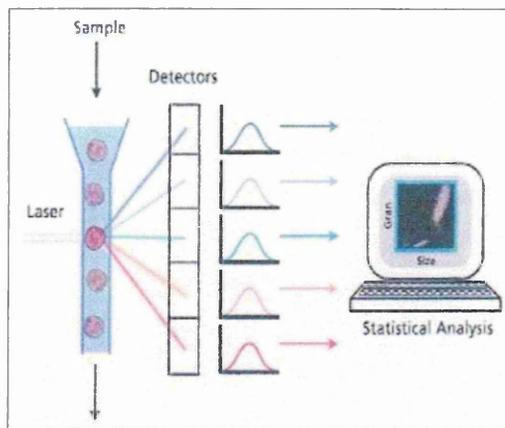
A typical bench-top flow cytometer consists of the following (a simplified diagrammatic representation of which is shown in figure 2.4):

- A laser light source
- A flow cell, where the cells are forced into single file to a point where the light source is focused. The flow cytometer utilised in this study contained a cuvette flow cell, which facilitates the injection of the cell sample through the centre of a stream of liquid (sheath fluid)
- A lens system which focuses the light into the flow cell
- Optical components which collect and focus light of different colours (i.e. wavelengths) onto the detectors
- Electronics to amplify and process the resulting signals
- A computer in order to analyse the complex data generated by the flow cytometer, and display it in a useful and informative manner.

(Chapel *et al.* 1999, Ormerod 1999)

The flow cytometer can measure both light scattered by individual cells, and fluorescence. In the first instance, the data from the analysis can be displayed as a 'dot-plot' with each dot representing a single event, or cell (Chapel *et al.* 1999).

Figure 2.4 The basic principle of a flow cytometer



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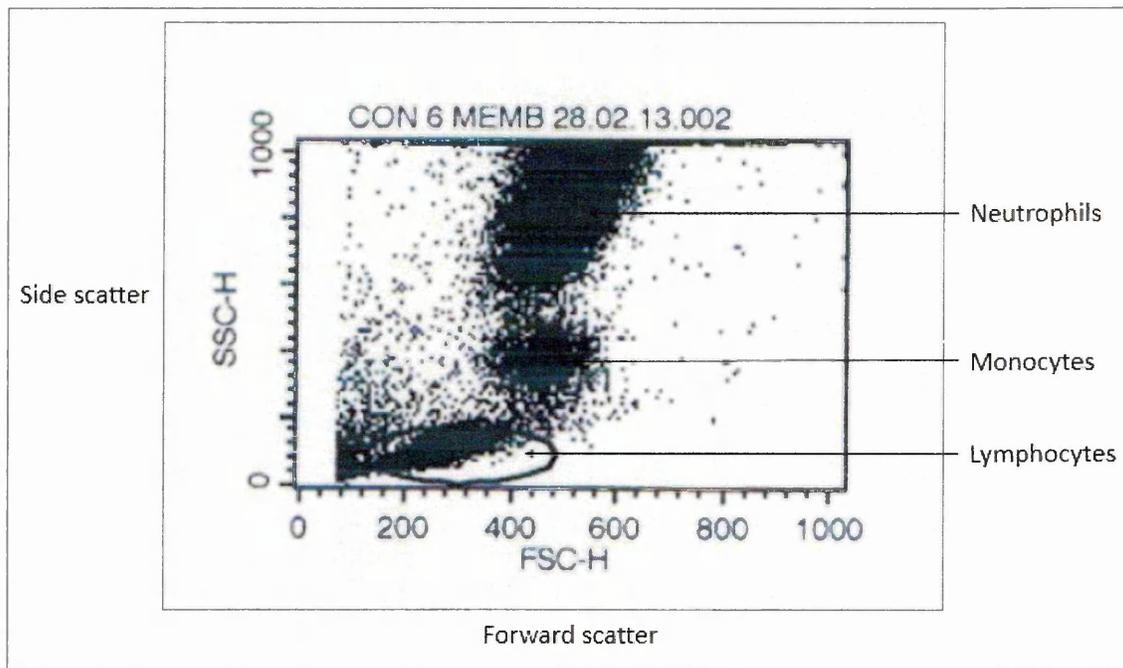
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A simplified diagrammatic overview showing the key features of a flow cytometer, including the laser light source focussed on the flow cell where the cells contained within the sample are forced into single file for analysis. Emitted light of different wavelengths is collected by the detectors and analysed by the electronics and computer software.

When a sample of cells from human whole blood is run through a flow cytometer, a characteristic dot-plot can be viewed as a two-dimensional graph of side scatter versus forward scatter, where side scatter represents light scattered by cellular granularity, and forward scatter represents light scattered by cell size (figure 2.5). This initial analysis then allows the selection, by 'gating', of a particular cell type, e.g. lymphocytes, for further analysis.

If the cells to be analysed are first stained with a fluorescence-labelled monoclonal antibody, then more detailed information can be collected, from the flow cytometer, concerning cellular surface antigen expression. Fluorescence describes the phenomenon of a molecule being excited by light of a specific wavelength, and then losing this excitation energy by emitting light of a longer wavelength (Omerod 1999). Emitted light can then be measured by the flow cytometer, and if multiple fluorochromes are used, which emit light at different wavelengths, then multiple analyses of the same cell can be collected. The difference between the absorption and emission spectra is known as the 'Stokes shift' (Wulff 2006).

Figure 2.5 An example of a whole blood dot-plot obtained during the study



SSC – side scatter (cellular granularity); FSC – forward scatter (cell size)

The characteristic dot-plot for human whole blood cells, where side scatter represents light scattered by cellular granularity, and forward scatter represents light scattered by cell size. Different cell populations may be selected by 'gating' for further analysis. The main populations identifiable from a sample of whole blood are indicated, and an example of the lymphocyte selection is presented.

Due to the fact that the emission spectra of two, or more, fluorochromes can overlap, compensation must often be applied to allow for any emitted light from one fluorochrome spilling into the detection area of another fluorochrome to be corrected. The excitation and emission wavelengths of the fluorochromes employed for use in this study are shown in table 2.4.

The flow cytometer used for this study, a FACSCalibur (Becton Dickinson) is a two-laser instrument, with one 488nm argon-ion laser and a second 630nm helium-neon laser. The 630nm laser can excite allophycocyanin (APC), with all the other fluorochromes in table 2.4 being excited by the 488nm laser. This instrument is routinely able to detect four-colour fluorescence. The instrument is in regular use for a routine NHS diagnostic service, and, as such, is calibrated and maintained according to relevant Clinical Pathology Accreditation (CPA) and European Federation for Immunogenetics (EFI) accreditation standards.

Immunofluorescence in flow cytometry describes how monoclonal antibodies, labelled with a fluorochrome, can be used to identify cells of the immune system. During haematopoiesis, different populations of blood cells arise from stem cells via a process of development along various lineages. At each stage of this process, cells at a particular stage of development along a certain lineage, will express characteristic cell surface protein markers, which can be categorised according to the cluster of differentiation (CD) nomenclature (Ormerod 1999). These CD markers can be visualised using flow cytometry in combination with immunofluorescence, a technique known as immunophenotyping. The cells of interest are first selected from the dot-plot of forward scatter versus side scatter, and then analysed further using the fluorescent signal from the monoclonal antibody-fluorochrome conjugate, which will directly bind its target surface antigen wherever present, in a process of direct staining.

Table 2.4 The excitation and emission properties of the fluorochromes used in this study

Fluorochrome	Excitation wavelength (nm)	Emission wavelength (nm)
Allophycocyanin (APC)	650	660
Fluorescein isothiocyanate (FITC)	494	520
Phycoerythrin (PE)	496	578
Peridinin chlorophyll-A protein (PerCP)	482	678
Alexa Fluor	495	519

nm – nanometre

A selection of conjugated monoclonal antibodies used together in a panel can be used to identify and define a population of a distinct set of immune cells. This approach was taken in this study in the methods described below. Isotype controls were used as a negative control in all immunophenotyping tests. This constituted a control immunoglobulin, of the same mouse immunoglobulin isotype as the test antibody, with the appropriate bound fluorochrome, which was not directed against human cells. Any background staining can then be observed and accounted for. In addition, original antibody titration experiments included unstained cells which were uniformly negative.

2.6 Staining whole blood for lymphocyte subset analysis, using Multitest™ antibody panels and Trucount™ tubes (Becton Dickinson (BD))

2.6.1 Summary of the assay

The BD Multitest™ kit (product code 340504) was used to allow identification and enumeration of the three main populations of lymphocytes: T cells, B cells and NK cells. The kit further differentiates T cells into 'helper' CD4⁺ and 'cytotoxic' CD8⁺ subsets. A full list of reagent and kit manufacturers and suppliers used in this study is included as appendix 4.

The kit consists of two different 'cocktails' of monoclonal antibody reagents (described more fully in table 2.5), in 1ml of buffered saline with 0.1% sodium azide (BD Biosciences 2011). Each monoclonal antibody is labelled with a fluorochrome. When whole blood is added directly to the monoclonal antibody 'cocktails', the antibodies bind specifically to their surface antigen targets on the cells in the blood. This then allows fluorescence detection and identification using a flow cytometer.

Within the Trucount™ tubes a mesh grid contains a lyophilised pellet, which dissolves upon addition of the kit reagents and blood sample, releasing a known quantity of fluorescent beads. This allows the analysis software to perform a calculation comparing the test cellular events with the known bead events resulting in absolute counts (cells/μl) for the numbers of each lymphocyte subset. The correct lot number of Trucount™ tubes was entered into the software for each use, in order that the correct absolute count of beads was used for the analysis.

Table 2.5 The composition of the two cocktails of monoclonal antibody reagents in the Trucount™ tubes

Conjugated monoclonal antibody	Targeted cell population	CD markers used to define population
'Cocktail' 1 (tube 1):		
CD3-FITC	T lymphocytes	CD3 ⁺
CD8-PE	'Cytotoxic' T lymphocytes	CD3 ⁺ CD8 ⁺
CD45-PerCP	Total lymphocytes	CD45 ⁺
CD4-APC	'Helper' T lymphocytes	CD3 ⁺ CD4 ⁺
'Cocktail' 2 (tube 2):		
CD3-FITC	T lymphocytes	CD3 ⁺
CD16-PE & CD56-PE	NK cells	CD16 ⁺ CD56 ⁺
CD45-PerCP	Total lymphocytes	CD45 ⁺
CD19-APC	B lymphocytes	CD19 ⁺

CD – cluster of differentiation

The MultiSET software generates absolute counts (cells/ μ l) and % ratios for each of the targeted cell populations shown

2.6.2 Method

Study participant whole blood was collected into an ethylenediaminetetraacetic acid (EDTA) anticoagulant. Two 7ml tubes of blood were taken from each participant. In the majority of cases the blood samples were analysed on the same day that they were taken, and all were analysed within 24 hours. Blood samples from Bradford and Hull were transported at ambient temperature.

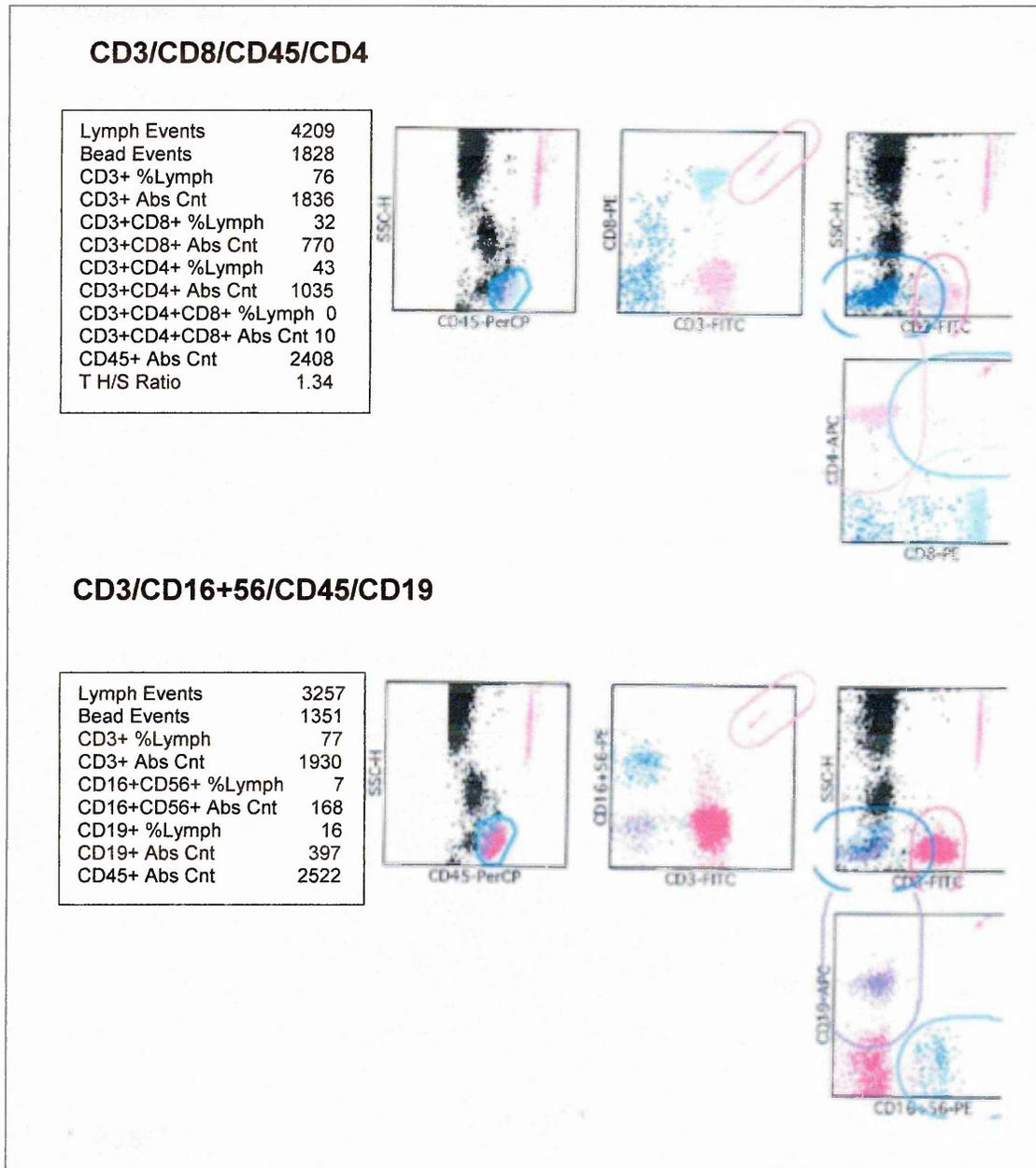
Two Trucount™ tubes were used for each test. These were labelled with the participant identification, and tube number (1 and 2). 15µl of each of two Multitest™ monoclonal antibody ‘cocktails’ was added, just above the mesh grid, to the appropriate tube. A tube of EDTA anti-coagulated blood was mixed by careful inversion, and 50µl was added to each of the Trucount™ tubes, taking care that the blood was pipetted into the bottom of the tube, adjacent to the monoclonal antibodies. The contents of the tubes were mixed gently by manual agitation, and incubated for 15 minutes, at room temperature. 450µl of BD FACS™ lysing solution (diluted 1:10 with distilled water) was then added to each tube, and the contents were incubated for 15 minutes at room temperature. The samples were then analysed on a FACSCalibur™ flow cytometer (Becton Dickinson), using the MultiSET software (BD). All study participant tests were analysed within one hour of being set up. An example of the data generated by the MultiSET software is shown in figure 2.6.

Following the protocol for routine laboratory analysis (Cellular Immunology Standard Operating Procedure (SOP)) the following acceptance criteria were applied. The information required was obtained from the quality control (QC) section present on the test report and all tests fulfilled these criteria:

- The lymphocyte total count was 100% +/- 10%. This figure is provided as a ‘lymphosum’ and consists of the sum of the percentage of T cells, B cells and NK cells. A figure which was markedly different to 100% would have indicated that the lymphocyte gate was not accurately placed.
- The difference in the percentage of CD3 cells between tube 1 and tube 2 was <5%. This comparison of the CD3 percentage obtained from the two tubes provides a technical check, for instance for pipetting errors.
- The CD3 absolute count range was similar (+/-5%) between tube 1 and tube 2. Similarly to above, this also provides a technical check.

In addition, a further check consisted of examining the sum of the results of the T helper and T suppressor cells (% ratio and absolute counts) and assessing if this was approximately equal to the total T lymphocyte count result.

Figure 2.6 A representative example of the raw data, generated at the point of acquisition, by the Multitest™ kit for the enumeration of T lymphocytes, B lymphocytes and NK cells



The gating strategy for the Multitest™ kit is shown, with tube 1 analysis in the top panel, and tube 2 analysis in the lower panel. Tube 1 analysis first identifies the lymphocyte population (CD45⁺), then from this identifies the CD3⁺CD8⁺ cells and the CD3⁺CD4⁺ cells. The analysis of tube 2 also first gates the CD45⁺ population, then from this, the CD19⁺ cells and the CD3⁺CD16⁺CD56⁺ cells. The MultiSET software (BD) generates a report from this data detailing absolute counts (cells/ μ l) and % ratios for each of the targeted cell populations.

2.7 Staining whole blood for immunophenotyping analysis

Participant blood samples were collected into EDTA anticoagulant blood tubes for this assay. All commercial monoclonal antibodies had been previously titrated from the manufacturer's recommendations to ensure optimal staining. The same procedure was followed for each immunophenotyping panel.

Sufficient 5ml polystyrene round bottom tubes (Falcon) were labelled for the immunophenotyping panels to be set up, including one tube for an isotype control. A volume of 7.5µl of each commercial monoclonal antibody was added to the appropriate tube, except all APC labelled antibodies, of which 2µl was added. The participant EDTA blood sample was gently mixed by inversion, and 75µl added to each immunophenotyping panel tube. The contents of the tubes were carefully mixed by manual agitation, and incubated in the dark, at room temperature, for 30 minutes.

3 ml of BD FACS™ lysing solution (diluted 1:10 with distilled water) was then added to each tube, with a further incubation in the dark, at room temperature, for 15 minutes. Following this incubation, the tubes and contents were centrifuged at 250g for 5 minutes. The supernatant was decanted, and the cells resuspended in 3ml of 1% (volume for volume (v/v)) foetal bovine serum (FBS) (Invitrogen) in phosphate buffered saline (PBS). The tubes were again centrifuged at 250g for 5 minutes. The supernatant was decanted, and the washing procedure repeated once more. The supernatant was decanted, and the cells were resuspended in approximately 500µl 0.5% (v/v) formaldehyde (BDH) in PBS. All study samples were then stored at 4°C and analysed on the flow cytometer within 24 hours. The flow cytometer was set to collect a total count of 20,000 events for the analysis.

Specifically, the immunophenotyping panels, which were all set up following the general method described above, are described in more detail below. A checklist, using a tick box format, was designed, and used during assay set up for every sample, to ensure all commercial antibodies were added correctly. In all cases, the lymphocytes were first selected by gating as shown in figure 2.5.

2.7.1 T cell immunophenotyping panels

Three immunophenotyping panels that identified specific populations of T cells were used for this study. The rationale for their inclusion is discussed in the introduction of this report. Two of the panels examined cell surface markers, whilst the third (regulatory T cell (Treg) panel) involved the analysis of an intracellular marker, and is described separately in section 2.8.

One T cell panel analysed naïve ($CD4^+CD45^{RO-}$) and memory ($CD4^+CD45^{RO+}$) T cells present, and identified the population of recent thymic emigrants (RTEs) as possessing the phenotype $CD4^+CD45^{RO-}CD31^+$. Table 2.6 details the composition of this immunophenotyping panel, and the gating strategy employed is described in figure 2.7.

A second T cell panel, the 'activated' T cell panel, assessed HLA-DR positivity on CD4 and CD8 T cells. Table 2.7 details this panel, and the gating strategy employed is described in figure 2.8.

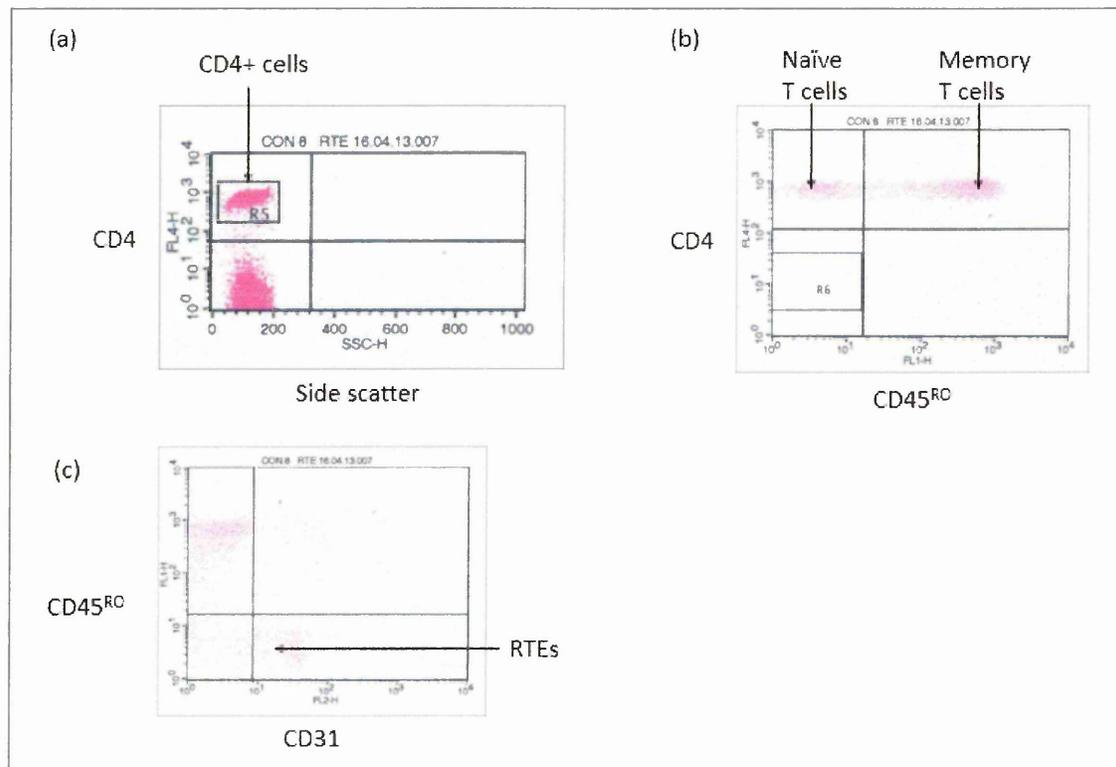
Table 2.6 'Recent thymic emigrants' (RTE) immunophenotyping panel

Antibody specificity	Fluorescent conjugate	Supplier & product code
CD4	APC	BD Biosciences 345771
CD31	PE	BD Biosciences 340297
CD45 ^{RO}	FITC	BD Pharmingen 555492

Table 2.7 'Activated' T cell immunophenotyping panel

Antibody specificity	Fluorescent conjugate	Supplier & product code
CD3	PerCP	BD Biosciences 345766
CD4	APC	BD Biosciences 345771
HLA-DR	PE	BD Biosciences 347401

Figure 2.7 The gating strategy for the RTE immunophenotyping panel



(a) FL4 – CD4, SSC – side scatter

Following the initial selection of the lymphocyte population, the CD4⁺ cells were selected by gating (R5)

(b) FL4 – CD4, FL1 – CD45^{RO}

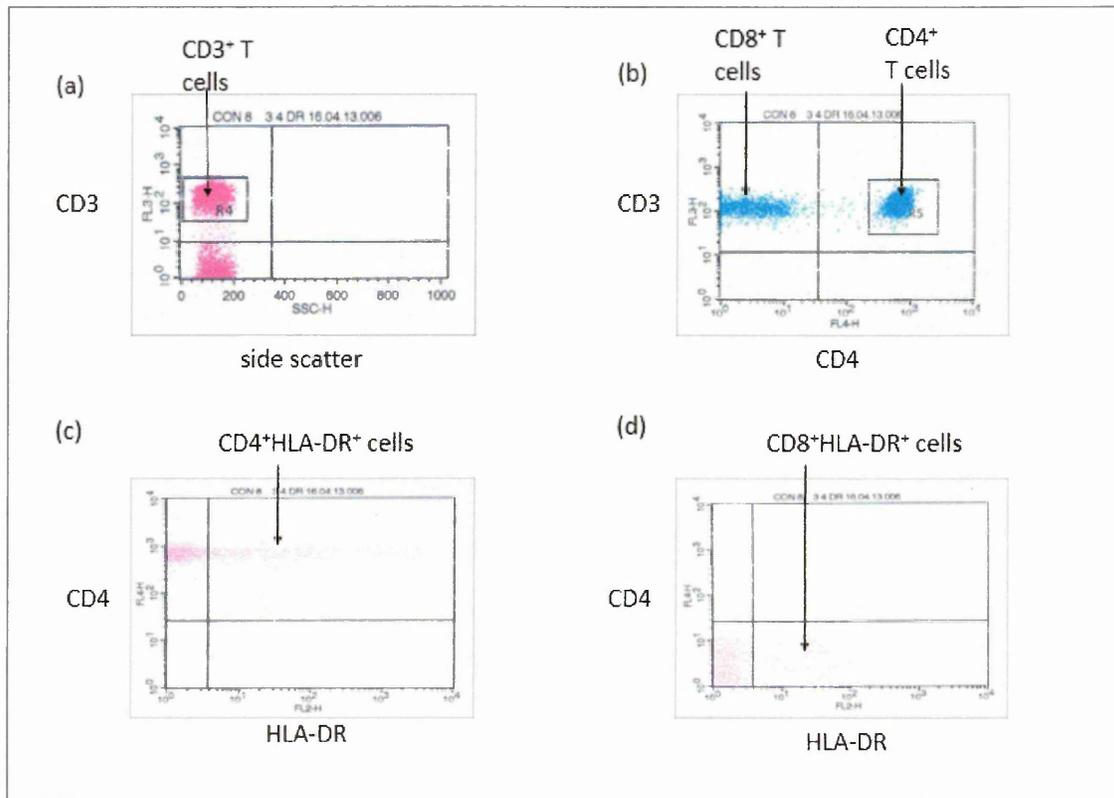
The naïve T cells CD45^{RO-} (CD45^{RA+}) (upper left quadrant) and memory T cells CD45^{RO+} (upper right quadrant) cell populations were identified

(c) FL1 – CD45^{RO}, FL2 – CD31

The RTEs can be identified as a percentage of CD4⁺ cells (from gate R5), shown in the lower right quadrant, with the phenotype CD4⁺CD45^{RO-}CD31⁺

(Gate R6 was not used in this analysis)

Figure 2.8 The gating strategy for the activated T cell immunophenotyping panel



(a) FL3 – CD3, SSC – side scatter

Following the initial selection of the lymphocyte population, the CD3⁺ T cells were gated (R4)

(b) FL3 – CD3, FL4 – CD4

From gate R4, the CD4⁺ (upper right quadrant) or CD4⁻ (CD8⁺) (upper left) cells could then be selected

(c) FL4 – CD4, FL2 – HLA-DR

Gating on the CD4⁺ cells in (b), the **CD4⁺HLA-DR⁺** population were identified in the upper right quadrant

(d) FL4 – CD4, FL2 – HLA-DR

Gating on the CD4⁻ cells in (b), the **CD8⁺HLA-DR⁺** population were identified in the lower right quadrant

2.7.2 B cell immunophenotyping panels

The study also employed three immunophenotyping panels to analyse specific populations of B cells. As with the T cell panels described above, the rationale for studying these cell markers was described in the introduction. The B cell panels all examined cell surface antigens.

The first B cell panel was used to assess 'memory' B cells by employing the CD27 cell marker. In this manner, the B cell populations could be analysed for naïve (CD27⁻) and memory (CD27⁺) B cells, and the memory B cells could be further divided into 'switched' and 'non-switched', according to IgD expression. The target markers used for this panel are shown in table 2.8, and the gating strategy is shown in figure 2.9.

A second B cell panel, for 'transitional' B cells aimed to identify CD38⁺CD24⁺ cells, to gain information about the maturity of B cells present within peripheral blood. This panel is described more fully in table 2.9, and figure 2.10.

Finally, an immunophenotyping panel to identify any plasmablasts or plasma cells present was used. Two different strategies were followed, employing the cell markers CD19, CD38, CD138 and CD20 on the study samples. This 'plasma cell' panel is described in table 2.10 and figure 2.11.

The following isotype control commercial antibodies were included in each assay:

Mouse IgG1-APC – BD Biosciences (345818)

Mouse IgG1-FITC – BD Biosciences (345815)

Mouse IgG2-PE - BD Pharmingen (555574)

Mouse IgG1-PerCP - BD Biosciences (345817)

Table 2.8 'Memory' B cell immunophenotyping panel

Antibody specificity	Fluorescent conjugate	Supplier & product code
CD19	PerCP	BD Biosciences 345790
CD27	PE	BD Pharmingen 555441
IgD	FITC	BD Pharmingen 555778

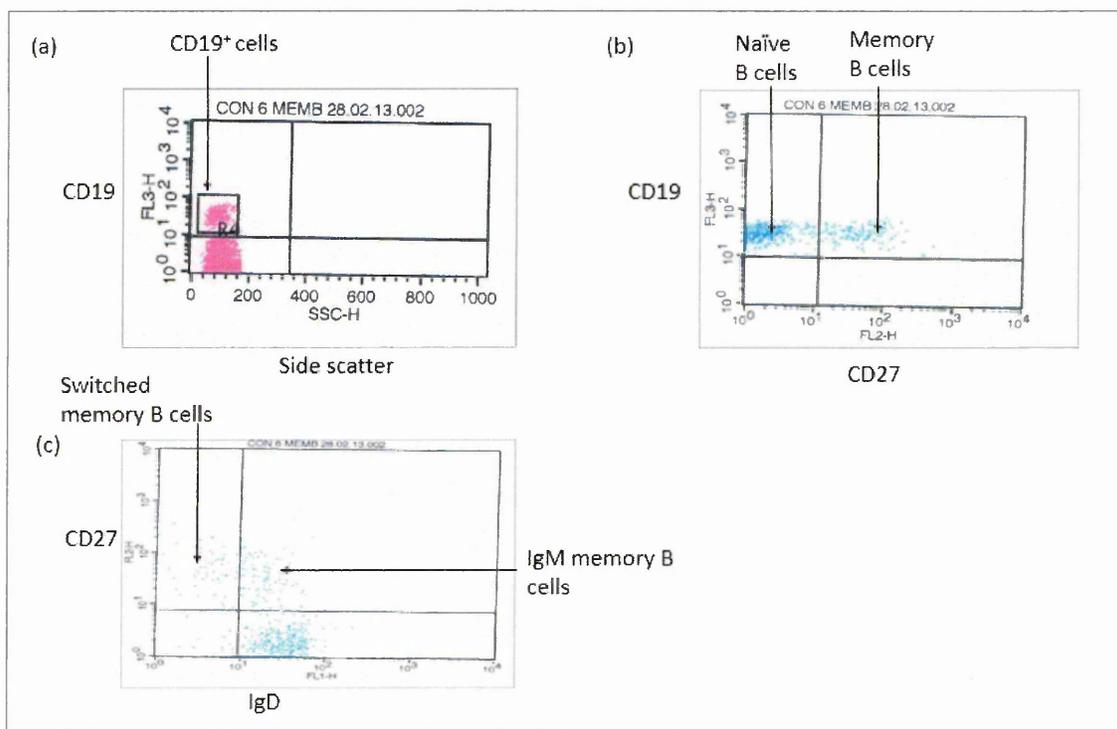
Table 2.9 'Transitional' B cell immunophenotyping panel

Antibody specificity	Fluorescent conjugate	Supplier & product code
CD19	PerCP	BD Biosciences 345790
CD24	PE	BD Pharmingen 555428
CD38	APC	BD Biosciences 345807
CD27	FITC	BD Biosciences 555440

Table 2.10 'Plasma' B cell immunophenotyping panel

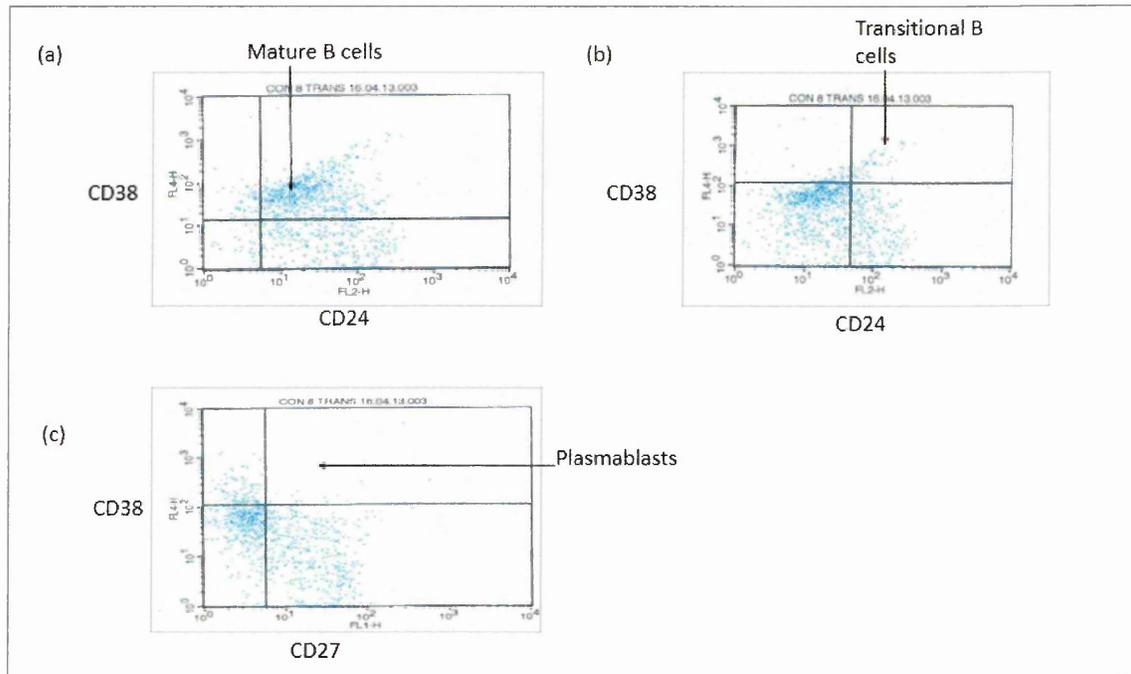
Antibody specificity	Fluorescent conjugate	Supplier & product code
CD19	PerCP	BD Biosciences 345790
CD38	APC	BD Biosciences 345807
CD138	FITC	BD Pharmingen 552723
CD20	PE	BD Biosciences 345793

Figure 2.9 The gating strategy for the memory B cell immunophenotyping panel



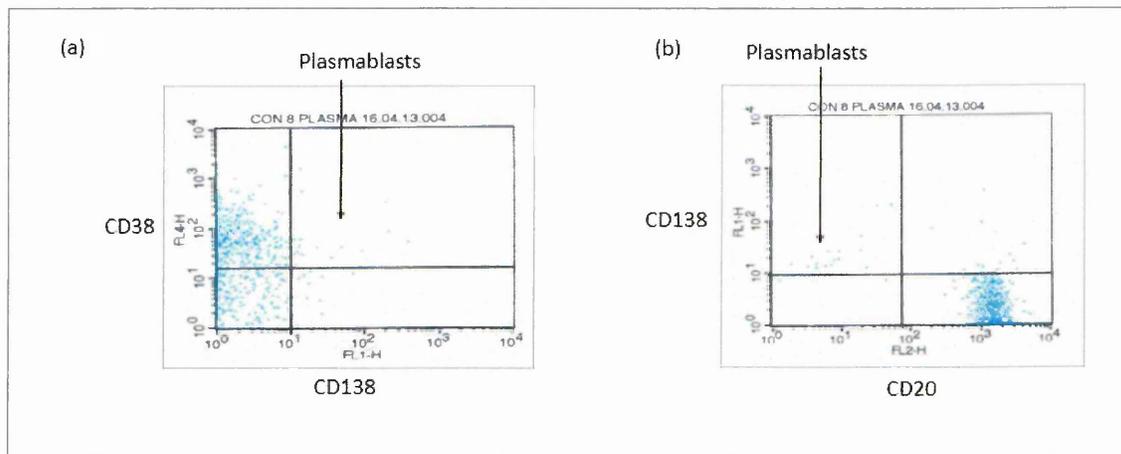
- (a) FL3 – CD19, SSC – side scatter. Following the initial selection of the lymphocyte population, the CD19⁺ B cells were gated (R4)
- (b) FL3 – CD19, FL2 – CD27. The CD19 B cells were separated into **CD27⁻ naïve** (upper left quadrant) and **CD27⁺ memory** (upper right quadrant) populations
- (c) FL2 – CD27, FL1 – IgD. The memory B cells were then further analysed according to IgD expression, which is not identified on 'switched' memory B cells. **IgG 'switched' memory cells (IgD⁻)** fall within the upper left quadrant; **IgM memory cells (IgD⁺)** in the upper right quadrant, and naïve B cells are lower right

Figure 2.10 The gating strategy for the transitional B cell immunophenotyping panel



- (a) FL4 – CD38, FL2 – CD24. Following the selection of the CD19⁺ population, as shown for the memory B cell strategy shown in figure 2.9, the CD38 and CD24 expression was analysed, identifying the **mature B cells (CD19⁺CD38⁺CD24⁺)** in the upper right quadrant
- (b) FL4 – CD38, FL2 – CD24. The quadrants were re-drawn on this plot in order to obtain data on the **CD19⁺CD38^{hi}CD24^{hi} transitional B cells** (upper right)
- (c) FL4 – CD38, FL1 – CD27. In addition, this panel included CD27 which is a marker for memory B cells (upper and lower right quadrants). The upper right quadrant was noted as containing the **CD19⁺CD38^{hi}CD27⁺ plasmablast** population

Figure 2.11 The gating strategy for the plasma B cell immunophenotyping panel



(a) FL4 – CD38, FL1 – CD138. The CD19⁺ population was first selected, as shown for the memory B cell strategy shown in figure 2.9. The CD19⁺ population was then analysed further according to CD38 and CD138 expression, to identify the **plasmablast** population (upper right quadrant) as being **CD19⁺CD38⁺CD138⁺**

(b) FL1 – CD138, FL2 – CD20. A plot of CD138 versus CD20 was then used to analyse the CD19⁺ cells further. Using this strategy, **plasmablasts** were defined as the population of cells that were **CD19⁺CD138⁺CD20⁻** (upper left quadrant)

2.8 Intracellular staining of human FoxP3 in peripheral blood mononuclear cells (PBMCs)

The immunophenotyping panel employed to study regulatory T cells (Tregs) contained a monoclonal antibody directed against human FoxP3, alongside cell surface markers (table 2.11). Unlike the immunophenotyping panels already described, whose target CD markers were all expressed on the cell surface, FoxP3 is contained within the cell nucleus, and required a method for intracellular staining. The method was performed using PBMCs, so these first required separation from whole blood. Participant blood samples were collected into EDTA anticoagulant blood tubes for this assay.

Table 2.11 Tregs immunophenotyping panel

Antibody specificity	Fluorescent conjugate	Supplier & product code
CD4	APC	BD Biosciences 345771
CD25	PE	BD Biosciences 341011
FoxP3	Alexa Fluor	BD Pharmingen 560047

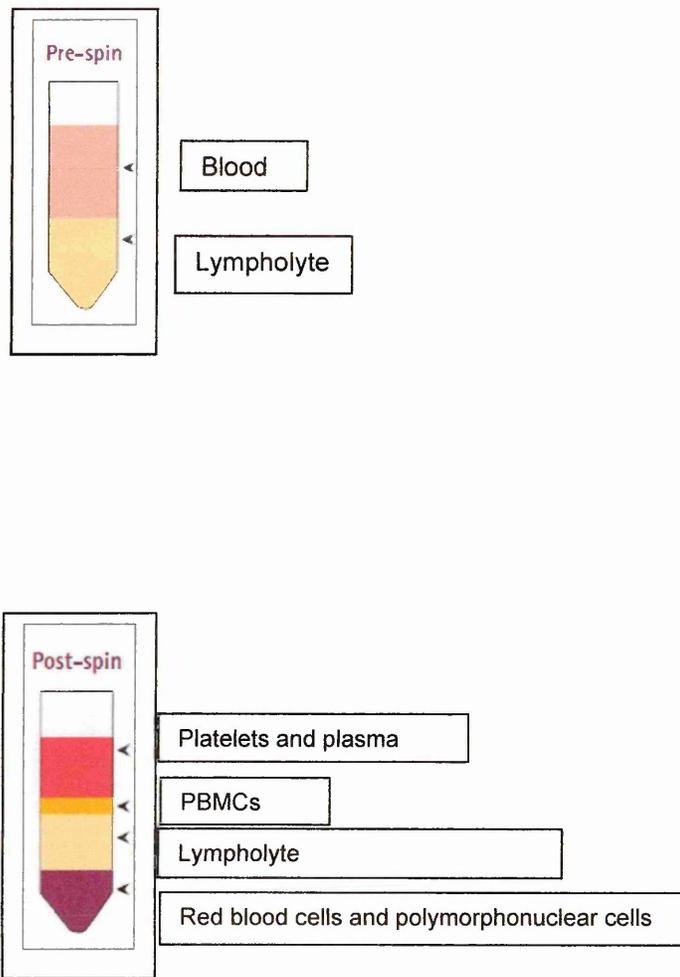
2.8.1 Preparation of PBMCs from whole blood

Lympholyte®-H cell separation media (Cedarlane CL5015) was taken from storage at 4°C and allowed to reach room temperature. Lympholyte® ('lymphoprep') density gradient medium (density of 1.077g/ml) facilitates the isolation of PBMCs. During centrifugation, granulocytes and erythrocytes are sedimented through the separation medium due to their increased density compared to PBMCs. A 'buffy coat' layer containing PBMCs is suspended on top of the lympholyte, whilst the plasma, and platelet layer is above (figure 2.12).

Two 15ml 'V-bottomed' tubes were labelled for each participant. 3ml of lympholyte was added to each of the two 15ml tubes, using a plastic pastette. The blood sample was gently mixed, and 3ml of whole blood was carefully 'layered' onto the lympholyte. The tubes were then centrifuged, at room temperature, at 500g for 20 minutes, to separate the blood into layers of plasma/platelets, PBMCs and polymorphonuclear cells/erythrocytes.

The PBMC buffy coat layer was then carefully removed from each tube using a plastic pastette, and transferred to a fresh, labelled 15ml tube, containing approximately 2 ml of PBS/FBS (1%). The tubes were topped up with PBS/FBS, and centrifuged at 150g, for 10 minutes, to wash the cells and remove the lympholyte.

Figure 2.12 Isolation of PBMCs using Lympholyte®-H cell separation media



Adapted from Cedarlane (2014)

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Lympholyte® density gradient medium facilitates the isolation of PBMCs. Whole blood is first 'layered' onto the lympholyte. During centrifugation, granulocytes and erythrocytes are deposited through the medium due to their increased density compared to PBMCs. A buffy coat layer containing PBMCs becomes suspended on top of the lympholyte, whilst the plasma, and platelet layer is above.

Following centrifugation, the supernatant was decanted, and the cells resuspended in PBS/FBS. The tubes were again topped up with PBS/FBS for a second wash, centrifuging as above. The supernatant was again removed, and the cells resuspended in 1ml PBS/FBS.

2.8.2 Counting of PBMCs

A mix of 450 μ l of 0.4% (v/v) trypan blue (Sigma T8154) and 50 μ l of cells was made up in a 5 ml Falcon tube. Trypan blue assists in the visualisation of viable cells by dye exclusion methodology with live cells not taking up the stain and dead cells staining blue (Strober 2001). A haemocytometer and microscope were used to calculate the number of cells present in this mix, and therefore in the isolated PBMC sample, and the cells resuspended in PBS/FBS to a concentration of 1×10^6 per ml in the 15 ml tube.

1 ml (1×10^6 cells) of PBMCs was then aliquoted into each of two labelled 5 ml Falcon tubes. Each tube was topped up with PBS/FBS, and the cells washed by centrifugation at 250g for 5 minutes.

2.8.3 Cell surface staining of PBMCs

The supernatant was decanted from each tube, and the resultant cell pellet containing 1×10^6 cells, was resuspended by tapping the tube. The following antibodies were then added to the tubes:

Tube 1: Test 2 μ l CD4-APC
 5 μ l CD25-PE

Tube 2: Control 2 μ l APC isotype control
 5 μ l PE isotype control

The contents of the tubes were gently mixed by agitation, and then incubated in the dark, at room temperature, for 20 minutes. Following incubation, each tube was topped up with PBS/FBS, and the cells washed as before. The cells were then re-suspended, and a second wash carried out.

2.8.4 PBMCs fixation and permeabilisation

The human FoxP3 buffer set (BD Pharmingen 560098) was prepared, as follows:

FoxP3 Buffer A, containing diethylene glycol and formaldehyde, (10x concentration supplied) was diluted 1:10 in distilled water.

FoxP3 Buffer B, containing $\leq 0.09\%$ sodium azide, (50x concentration supplied) was diluted 1:50 in buffer A. The resulting solution is then known as buffer C.

Following the second wash, the supernatant was decanted, and the cell pellet was gently vortexed in order to prevent the cells 'clumping' together. 2 mls of diluted buffer A was then added to each of the two tubes of cells, in order to 'fix' the cells. The cells were incubated with buffer A for 10 minutes, in the dark at room temperature. Following incubation, the tubes and contents were centrifuged at 250g for 5 minutes, and the resultant supernatant removed. The tubes were then briefly vortexed, before the cells were resuspended in 500 μ l of buffer C, in order to permeabilise the cells, for 30 minutes, in the dark, at room temperature.

2.8.5 Intracellular staining of PBMCs

Following incubation, the cells were washed twice in PBS/FBS as previously described. After the second wash, the following antibodies were added to the cell pellets in the tubes:

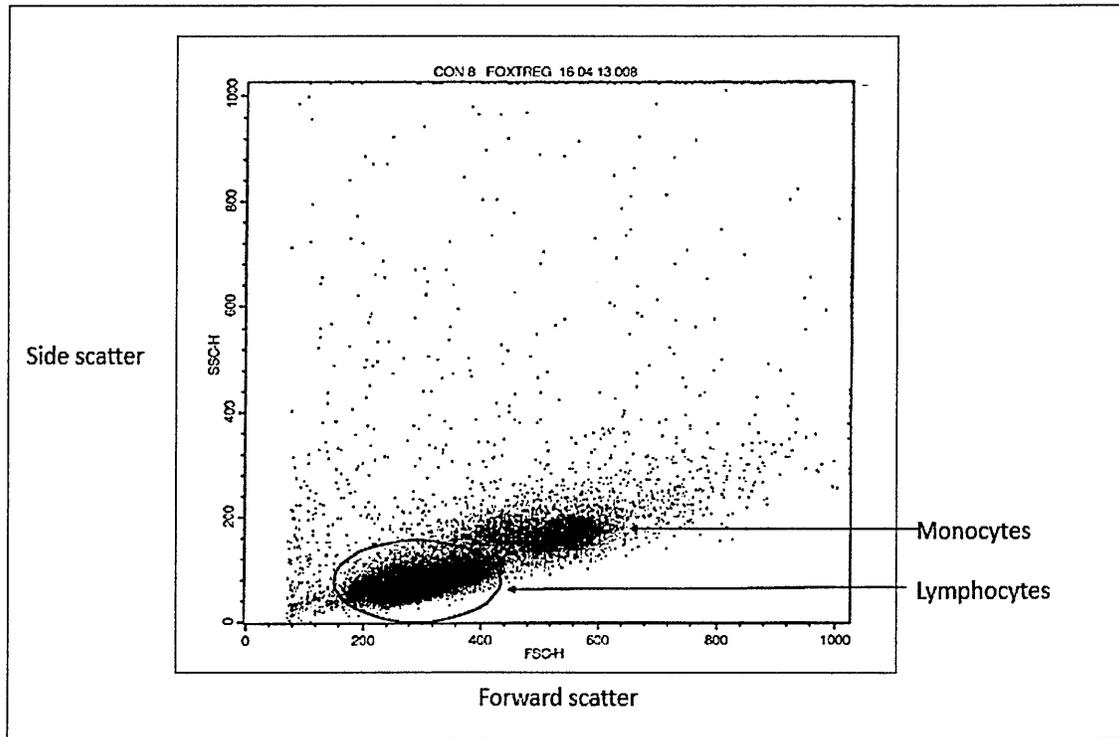
Tube 1: Test 15 μ l FoxP3-Alexa fluor

Tube 2: Control 15 μ l FITC isotype control (BD Biosciences 345815).
(FITC and Alexa fluor share the same properties in terms of fluorescence wavelength, hence the use of the FITC isotype control).

The contents of the tubes were gently mixed, and then incubated in the dark, at room temperature, for 30 minutes. Following incubation, the cells were washed twice, as before and then, finally, the cells were resuspended in approximately 500 μ l PBS/0.5% formaldehyde. All study samples were stored at 4°C and analysed on the flow cytometer within 24 hours.

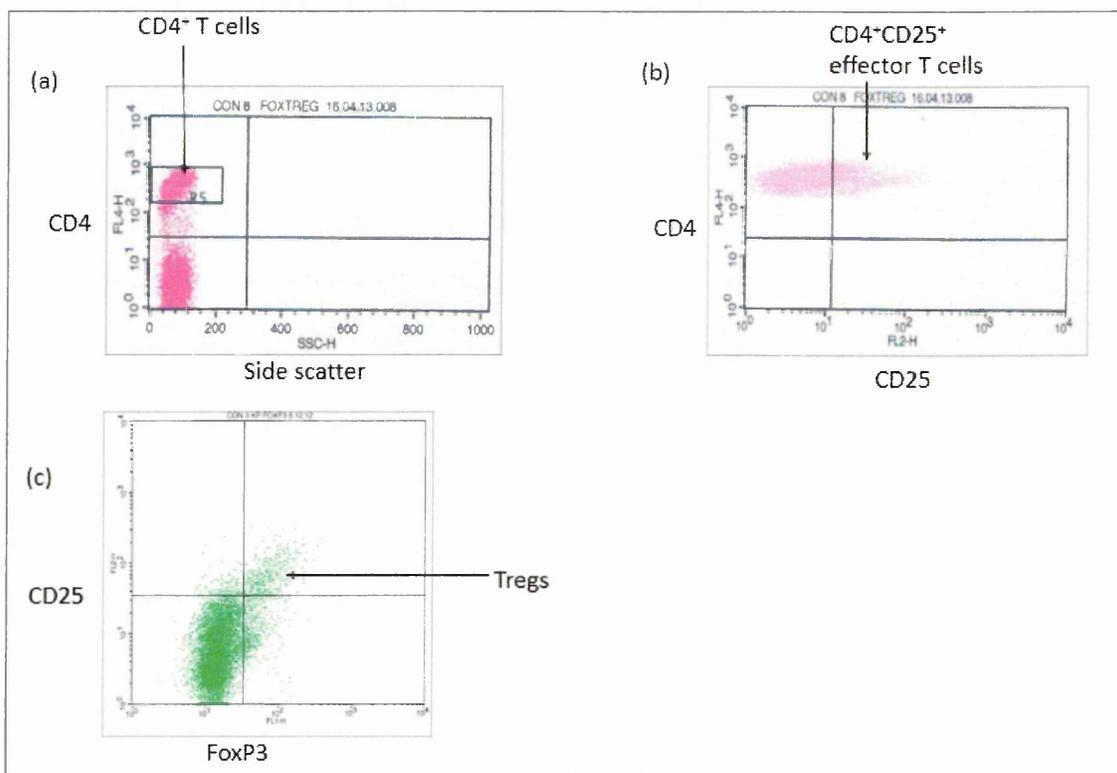
An example of the data obtained from this panel is shown in figures 2.13 and 2.14.

Figure 2.13 A dot plot of side scatter versus forward scatter for separated PBMCs



The initial dot-plot of side scatter versus forward scatter for the Tregs panel demonstrated a different appearance to those obtained in the analyses using whole blood, as the polymorphonuclear cells were removed during the lympholyte PBMC isolation. Thus, only populations of monocytes and lymphocytes remained visible (lymphocytes gated; monocytes can be discerned as a separate population of slightly larger cells shifted along the FSC axis). A gate was drawn around the lymphocytes which were then used for subsequent analyses.

Figure 2.14 The gating strategy for the Treg immunophenotyping panel



(a) FL4 – CD4, SSC – side scatter. Following the selection of the lymphocyte population, as described in figure 2.13, the CD4⁺ cells were identified (R5) and gated.

(b) FL4 – CD4, FL2 – CD25. The CD4⁺ cells were further analysed according to CD25 expression, with both **CD4⁺CD25⁺ cells** (upper right quadrant) and CD4⁺CD25⁻ cells (upper left quadrant) present

(c) FL2 – CD25, FL1 – FoxP3. Data on the Tregs was then collected, by plotting CD25 expression versus FoxP3. The population of **Tregs** was identified in the upper right quadrant as **CD4⁺CD25^{hi} FoxP3⁺**

All the data from the immunophenotyping panels were stored on the computer linked to the flow cytometer, so that the final analysis and recording of positive events in gated areas could be carried out simultaneously for all samples, for each panel. In this manner, the results from each panel were scrutinised for all participants and normal controls together, in an attempt to maintain consistency in gating and selecting cell populations, thereby minimising bias. This reanalysed 'panel-by-panel' data was additionally briefly reviewed by an expert adviser for flow cytometry (Dr Clive Carter), who was 'blinded' to the sample identity, again to ensure scientific rigour and minimise bias.

2.9 Key technology utilised in this research: Luminex xMap®

Luminex xMAP is similar to a flow cytometry based system, in that it is a bench top instrument which utilises fluorescence detection for the analysis of biological samples. In the case of Luminex, assays are designed around colour-coded polystyrene beads, or microspheres (Luminex Corporation 2014). The instrument consists of a LABScan™ 100 flow analyser, combined with a Luminex® XY platform (allowing automated sample analysis) and a sheath delivery system (One Lambda 2013). The majority of H&I service laboratories employ Luminex technology for routine HLA-specific antibody screening and/or HLA typing.

2.10 HLA-specific antibody detection using Luminex technology

The LABScreen® Single Antigen assay (One Lambda), designed for use on the Luminex instrument, is capable of detecting and specifically identifying HLA Class I and Class II antibodies, of the IgG class. Earlier cell-based methods were often not able to uniquely define the HLA target of the antibody, due to cellular expression of multiple HLAs, further complicated by the phenomenon of linkage disequilibrium. Single antigen solid-phase assays utilising purified HLA have since revolutionised HLA-specific antibody detection (Pei *et al.* 2003, Stastny *et al.* 2009). The purified HLAs are coated onto Luminex microspheres, which are uniquely identified by internal fluorescent tags. The assay is analysed using Luminex xMAP technology, which works by using two lasers; a classification laser which excites and analyses the internal fluorescent dye mixture, which contains a red-infrared ratio specific for each bead, and a reporter laser which excites and analyses any fluorescence on the outside of the bead.

During the assay, a diagrammatic representation of which is shown in figure 2.15, serum from the individual being investigated is incubated with the HLA-coated Luminex microspheres. Any HLA-specific antibodies present in the serum bind to their target HLA coated on the beads. Bound IgG is then labelled with a secondary anti-human IgG antibody, which is conjugated with phycoerythrin (PE). The Luminex classification laser identifies each bead, and the reporter laser detects and analyses the PE signal resulting from bound, 'PE-tagged' IgG. Negative and positive control beads are included in the assay. The negative control bead is a 'blank' bead which is not coated with any HLA, whilst the positive control bead is coated with purified human IgG (One Lambda 2013).

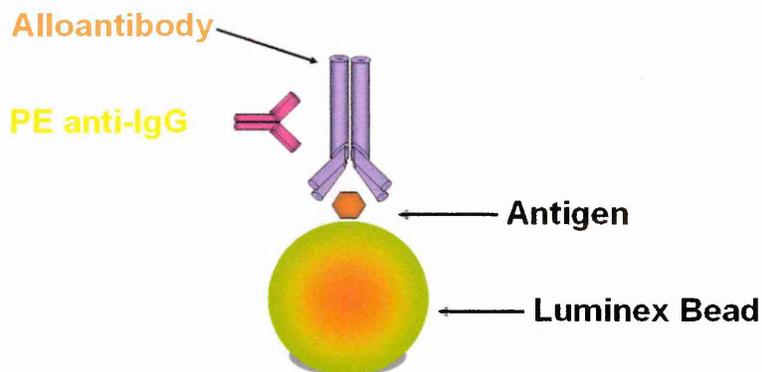
The LABScreen® Single Antigen assay Class I combi beads (LS1A04 - One Lambda) and the LABScreen® Single Antigen assay Class II combi beads (LS2A01) were used in this study, which, respectively, detect and define antibodies directed against HLA-A, B and Cw and antibodies directed against HLA-DR, DQ and DP. Local worksheets were used to document and analyse the results, alongside the supplied kit worksheets, for each lot number, which detailed the specificity of each bead.

This assay was performed on participant separated serum samples, which were prepared from a whole blood sample collected in a plain clotted tube (containing no anticoagulant). Serum samples were then frozen and stored (-20°C) until towards the end of the study, when all samples had been received, and then run together as a batch. The serum was retrieved from the freezer, and allowed to defrost at room temperature, before being used in this assay.

2.10.1 Separation of serum from whole blood samples, for HLA-specific antibody analysis

All retrospective cohort and prospective cohort study participants had their blood analysed for the presence of HLA-specific antibodies. HLA-specific antibodies are present in the serum component of whole blood, which was separated from a plain clotted blood collection tube for each participant, and stored frozen (-20°C) until analysis. The serum was separated in a manner that ensured that it was free from contaminating lipids and red blood cells.

Figure 2.15 The basic principle of the LABScreen® assay (One Lambda)



Wortley (VHBio)

Reproduced with permission from A. Wortley

A single purified HLA is coated onto each set of uniquely identifiable microspheres. Any alloantibody present in the patient sample being analysed will bind to its specific HLA target. Bound IgG is then detected using a phycoerythrin (PE)-labelled anti-human IgG antibody.

A plain clotted (without gel separator) blood tube was collected for each participant. Upon receipt, each sample was checked to ascertain that the blood had clotted, and placed in a refrigerator (4°C) for at least 1 hour, to allow the clot to retract. The tube was then centrifuged at 800g, for 5 minutes. The liquid serum component of the blood was then taken off with a plastic pastette, and aliquoted into a labelled V-bottomed centrifuge tube (12mls). This tube was centrifuged at 800g for 5 minutes.

Upon completion of centrifugation, each serum sample was removed from the V-bottomed tube, using a plastic pastette, and aliquoted into at least two 1.5 ml microfuge tubes. Care was taken to leave behind any red cell pellet, so that the serum was free from red cell contamination. The serum was then stored in the 1.5ml microcentrifuge tubes at -20°C.

2.10.2 Preparation for the LABScreen® single antigen assay

The samples to be tested were recorded on a 'batch plate layout', which included positive ('in-house' for Class I or Class II) and negative (LS-NC – One Lambda) control sera, in order to be certain of each serum sample's location on the test

plate. The Class I and Class II beads, and the assay buffer were removed from the refrigerator and allowed to reach room temperature. Both Class I and Class II sets of beads were vortexed thoroughly.

The wash buffer (x10 concentration supplied with kit, <1% sodium azide) was diluted 1:10 in distilled water. The assay was performed in a filter plate which was 'pre-wetted' before adding any test sera or reagents, by adding 300µl of wash buffer to each of the wells. The filter plate was then incubated on a plate shaker for 10 minutes. After 10 minutes, the buffer was removed from the filter plate wells, by the use of a vacuum manifold, attached to a laboratory sink tap.

2.10.3 Incubation of beads and sera

The method for routine laboratory analysis was followed (Transplant Immunology SOP). This is a locally validated method, which deviates from the manufacturer's method, mainly in terms of the volumes of test sera and reagents used.

10µl of each serum sample was carefully aliquoted to a well on the filter plate, according to the 'batch plate layout'. 2.5 µl of Class I beads were added to the Class I test samples, and 2.5 µl of Class II beads to the Class II tests. Beads and serum were mixed with the pipette tip during the addition of the beads. The contents of the filter plate were carefully mixed on the vortexer, and then incubated in the dark, at room temperature, for 30 minutes. Following incubation, non-specifically bound antibody was removed by washing the contents of each well with 150 µl of wash buffer. The wash buffer was mixed with the contents of each well upon addition, and then removed from the wells by using the vacuum manifold. A further 3 wash steps were then carried out, each using 200 µl of wash buffer.

2.10.4 Addition of secondary conjugated detection antibody

The supplied PE conjugated anti-human IgG was diluted with wash buffer 1:100, and then 50µl was added to each test well. The contents of each well were mixed with the pipette tip, upon each addition. The plate was then covered, and gently vortexed, before incubation in the dark, at room temperature, for 30 minutes.

Following incubation, the contents of each well was washed using 150µl wash buffer as above. A further 4 washes were then carried out using 200µl of wash buffer. The beads contained in each well were finally resuspended in 80µl of PBS and mixed using the pipette tip. The plate was carefully vortexed, and then analysed immediately on the Luminex platform.

2.10.5 Set-up of the Luminex instrument

Luminex set-up and analysis of the single antigen beads (SAB) assay was facilitated by the expert Antibody Section Lead (Mrs Kat Cullen). The standard routine daily start-up and shut-down procedures were followed, including laser warm-up, washing and calibration. A 'new multi-batch' was started, the appropriate bead lot numbers entered, and the batch information was entered into the Luminex software before starting the analysis. In all aspects, the samples were run in a manner similar to routine serum samples analysed in the H&I laboratory, so that the results could be compared to those previously obtained for participants. Assay set-up and analysis is validated locally to a standard acceptable to both CPA and the EFI. The results from the single antigen assays were analysed using HLA Fusion™ analysis software (One Lambda, Inc.).

In order for the results to be valid:

The mean channel fluorescence (MCF) of the positive control bead was > 500.

The mean channel fluorescence (MCF) of the negative control bead was < 1500.

The ratio of the positive control/negative control must be >2.

A minimum bead count of 50 for each bead was used for analysis, based upon local validation data.

The antibody screening data was analysed in a 'batch' to minimise any bias and ensure that data from all the tests was interpreted in a consistent manner. In line with local validation data, and in order to allow comparison with previous routine antibody screening results, beads with a mean fluorescence intensity (MFI) of 1000 or higher were considered to be positive. A 'first read' was initially performed noting all positive beads with an MFI of 1000 or greater. Beads with reactivity of 500-1000 MFI were also recorded on the worksheet as being 'weak'. The data was then scrutinised further, paying particular attention to whether or not the

positive beads for an antigen were 'clustered' together on the results read-out, and to check that all beads for a given antigen were positive in order for the antibody to be confidently assigned. Strong, genuine antibodies tend to appear consecutively in the raw data, with similar MFI results, and with all beads bearing the particular antigen being positive. There are often several beads detecting antibodies directed against a single antigen present within the kit, and if not all appear to be clearly positive, then the antibody may be weak, non-genuine, or against a single allelic variant of the antigen. A percentage panel reactive antibody (% PRA) was calculated for each test by dividing the number of positive beads by the total number of beads for each panel, and multiplying by 100. Where necessary, individual antibody profiles were converted to a percentage calculated reaction frequency (% cRF) in order to reflect the frequency of the detected specificities in the UK organ donor pool. An online tool provided by NHSBT (2016a), used for this conversion, compares the unacceptable antigens of an individual against the HLA types of 10,000 blood group identical organ donors, in order to generate a percentage which is representative of the incompatible potential donors.

The analysis until this point was performed 'blind', in that the donor HLA mismatches and the patients' previous antibody profiles were not examined until later in the process. It was hoped that this, too, may reduce bias and ensure scientific rigour of the study. Subsequently, the antibody screening data was also studied within the context of the participant's own HLA type. Since it is not possible for individuals to form antibody against self-antigen (excepting allelic antibodies), knowledge of the participant's HLA type is important when analysing antibody data, in order to avoid assigning 'self'-directed antibodies. Verification of analysis and advice on interpretation was sought from the expert antibody screening section lead, in the case of complex antibody profiles.

2.11 HLA typing to assist in the interpretation of antibody screening results

In order to allow a full exploration of the antibody screening results, following the initial data analysis, they were subsequently required to be reviewed in the context of the participant's HLA type (for the reasons explained above). In addition, it was necessary to have access to the full HLA type of the transplant

donor, in order to assess if DSA was present in a participant's antibody screening profile. In several cases this meant that additional HLA typing was required.

Due to the nature of the HLA molecule bound to the beads, the LABScreen® single antigen assay is capable of detecting antibody directed against the DQ α -chain (encoded by the DQA1 gene), the DP α -chain (encoded by the DPA1 gene) and the DP β -chain (encoded by the DPB1 gene). These genes are not routinely typed, for the purposes of live donor transplantation, so this prior information was lacking in places. Therefore, in the instance of a participant appearing to possess an antibody directed against DQ α , DP α or DP β , some additional HLA typing was undertaken, in order to allow the results to be interpreted fully.

Five recipient-donor pairs had additional HLA typing work carried out in order to allow full interpretation of the participant's HLA antibody screening data. Routine DNA samples from the five recipients and their respective donors were located in the laboratory DNA archive. DNA samples are routinely stored at -35°C for a minimum of 10 years, according to laboratory protocol. The 10 DNA samples located were originally extracted from whole blood collected into EDTA, by one of two routinely employed DNA extraction methods; rapid salting-out (an 'in-house' method), or using the Maxwell® 16 DNA extraction instrument (Promega) with the Maxwell® 16 Blood DNA Purification kit (AS1010).

Two different HLA typing methods were employed to obtain the information required; a commercial polymerase chain reaction (PCR)-sequence specific primers (SSP) assay, which is routinely used in the H&I laboratory in Leeds for HLA-DPB1 typing, and a Luminex based PCR-reverse sequence specific oligonucleotide probes (RSSO) assay. The latter is routinely used within the H&I laboratory for HLA-A, B, C, DRB1, DRB3/4/5 and DQB1 typing.

2.11.1 HLA-DPB1 typing using AllSet™ Gold PCR-SSP kit (Life Technologies)

Two participant-donor pairs were lacking the HLA-DPB1 typing data, which was required to fully interpret the participant's antibody screening profile. The four DNA samples were HLA-DPB1 typed using AllSet+™ Gold PCR-SSP (54070D – Life Technologies) trays according to standard routine laboratory protocol. The

DNA was quantitated prior to testing using a Nanodrop 1000 spectrophotometer (Thermo Scientific), and the concentration was adjusted to 50ng/μl. The PCR results were analysed both manually and by using the UniMatch® Plus software, which is supplied with the kits.

2.11.2 HLA typing using LABType® PCR-RSSO typing kits (One Lambda)

The ten DNA samples were HLA typed for HLA-DPA1/DPB1 and/or DQA1/DQB1 using LABType® PCR-RSSO typing kits, according to standard routine laboratory protocol. This local protocol follows a fully validated method which deviates from the manufacturer's method, mainly in terms of the volumes of test DNA/PCR product and reagents used.

The details of the kits were as follows:

LABType SSO Class II DPA1/DPB1 (03RSSO2PZ – One Lambda)

LABType SSO Class II DQA1/DQB1 (03RSSO2QZ – One Lambda)

The DNA was quantitated prior to testing using a Nanodrop 1000 spectrophotometer (Thermo Scientific), and the concentration was adjusted to 20ng/μl.

As the use of the above kits is fully validated for clinical use for HLA-DQB1 typing only, wherever it was possible the HLA-DQB1 type or HLA-DPB1 type obtained was cross-referenced to that already held on the laboratory database for the individual, in order to verify the HLA-DQA1/DPA1 result respectively. Where no HLA-DPB1 result was already available for the individual, the LABType® result was verified against the Allset+™ Gold DPB1 result.

2.12 Data analysis

2.12.1 General strategy for analysis of lymphocyte subsets and immunophenotyping laboratory data

In order to examine the data generated during this study with no bias, the lymphocyte subset results and the data from the immunophenotyping panels were initially analysed independently without segregating participants according

to clinical outcome. Any trends or patterns were noted, and outliers investigated. Following this initial approach, the data was also interpreted in the light of clinical criteria, e.g. whether the patient experienced graft rejection or not. By using this strategy, the scientific rigour of the study was maintained, whilst still yielding the fullest interpretation of the data.

Each participant's lymphocyte subset results were initially viewed as both absolute counts and percentage of total lymphocytes, side by side, as it was noted that the two graphs could look very different, and it was deemed possible that a false perception of what was happening to each lymphocyte subset could be gained if the results were presented in one format only. The absolute counts of cells were more informative as these gave a true indication of any change to an individual's immune cell profile during the HLAi transplant protocol. For example, it was by examining the absolute cell counts that the effect of the conditioning for the HLAi transplantation, in terms of eliminating an individual's key immune cells, could be seen. The length of time that an individual remained immunocompromised post HLAi transplantation was also evident. During this time the percentages of cells present could remain fairly high for some populations; however they may be a percentage of a very small number. Although, at first glance, they could be misleading, percentages were also interesting as they indicated how the cell populations changed relative to each other and also how the different cell populations recovered post conditioning and transplantation.

2.12.2 Clinical and additional laboratory data collection

Clinical data, such as that related to the conditioning protocol followed by each patient, immunosuppressive drugs received and transplant outcome, was obtained by a combination of means. Where possible, information was gathered from the Leeds Teaching Hospitals (LTH) Pathology electronic results system (Telepath) or the LTH renal medicine electronic results system (Bradford/Hull/Leeds/York - BHLY). However, the majority of clinical data was obtained with the help of clinical and research colleagues via access to the main LTH results server, and directly from the patients' medical case notes.

Additional laboratory data, such as crossmatch and HLA-specific antibody screening results, was obtained from the Transplant Immunology results database or from the patients' laboratory files. In certain instances, in particular to collect data related to antibody MFI levels, it was necessary to locate and review the raw laboratory data.

The serum creatinine levels and, where required, the CD45⁺ cell counts, for each participant were accessed from the LTH Pathology electronic results system (Telepath) or the LTH renal medicine electronic results system (BHLY).

2.12.3 Presentation and analysis of the study results

The study raw data was first collated into Microsoft Excel spreadsheets, and subsequently transferred to GraphPad Prism version 6.07 for Windows software. All following graphing and analysis of the data was performed using GraphPad. The data for the collective cohorts was analysed by one-way analysis of variance (ANOVA), using the Kruskal-Wallis test, followed by Dunn's multiple comparisons test (with 95% confidence interval). The Kruskal-Wallis probability (p)-value is provided for each analysis, and significant p-values indicated for the multiple comparisons. Dunn's multiple comparisons test reported the p-value in the following manner: >0.05 (not significant), ≤0.05 (*), ≤0.01 (**), ≤0.001 (***), ≤0.0001 (****). For the analyses performed on the stratified retrospective cohort, assessing the differences between two sets of data, the Mann-Whitney t-test was performed. Advice on scrutinising the data was taken from an expert advisor, statistician Dr Karen Kilner (Sheffield Hallam University).

CHAPTER 3 LYMPHOCYTE SUBSET ANALYSIS OF PROSPECTIVE AND RETROSPECTIVE HLAi TRANSPLANT RECIPIENTS

3.1 Summary of the study participants

3.1.1 The prospective participant cohort

Seven prospective HLAi transplant participants were recruited to the study and longitudinal samples were collected as detailed in section 2.4.1. An overview of this cohort is presented in tables 3.1 and 3.2, showing key laboratory and clinical data for the group. In order to provide an indication of renal function post-transplantation, the serum creatinine levels were accessed for each participant; these are displayed in table 3.3. Most recent creatinine levels, and levels obtained at times closest to both six months and one year post-transplantation, were recorded.

Table 3.1(a) indicates that, of the seven prospective participants, two were ABO blood group incompatible, in addition to being HLAi cases. Four of the participants had been previously transplanted, and it is of interest that, in two of these cases, preparation for transplantation took place against a background of repeat donor HLA mismatches. For participant 2, the repeat mismatches of HLA-A2, DR4, and DQ8 were present, which had also been represented in the previous related donor (mother). These HLA specificities were present as DSA against the current potential donor (half-sister). Participant 7 also demonstrated a repeat HLA mismatch with a previous deceased donor, for HLA-DR4. HLA-DR4 was not, however, present as a DSA in this participant, pre-transplantation.

As indicated in table 3.2, participants 1 and 2 did not progress to transplantation. In both cases this was due to the crossmatch and HLA-specific antibody results (shown in table 3.1(b)) indicating that the recipient versus donor incompatibility remained evident on the day prior to the scheduled transplantation, resulting in the procedure being cancelled. These two cases showed similarities, in that they both involved patients with multiple HLA Class I and Class II directed antibodies, which did not respond sufficiently to the antibody removal treatment.

Table 3.1(a) Key laboratory data for the prospective participant cohort

Participant ID	Gender	Relationship of donor	Recipient blood group	Donor blood group	HLA mismatch grade	Sensitising event (where known)
1	M	Step-father	A	A	1-2-0	Previous DCD transplant (graft nephrectomy 6 days post-transplant), multiple blood transfusions
2	F	Half-sister	O	O	1-1-1	Previous LRD transplant, blood transfusion
3	M	Father	O	O	0-0-1	Previous DD transplant, blood transfusion
7	M	Mother	A	A	0-0-1	Previous DD transplant, blood transfusion
9	F	Husband	O	A1	1-0-1	Pregnancy (multiple)
11	F	Partner	A	A	1-2-2	Pregnancy (multiple)
14	F	Friend	O	A1	1-1-0	Pregnancy

Bold italics - denotes ABO blood group incompatible (ABOi) transplant pair

ID – identification; M – male; F – female; HL A - human leucocyte antigen; DCD - deceased donor after circulatory death
LRD - live related donor; DD - deceased donor

HLA mismatch grade - calculated according to NHS Blood and Transplant (NHSBT) policy. The mismatches (at a 'broad' specificity level) between the recipient and donor at HLA-A, B and DR were taken into account, in the recipient versus donor direction. Therefore, 2-2-2 represents the worst scenario for mismatching, and is a poor HLA match. Conversely, 0-0-0 indicates no mismatches present, and the best possible HLA match.

Table 3.1(b) Crossmatch and DSA data at initial and pre-transplant assessment time points for the prospective participant cohort

Participant ID	Initial assessment			Pre-transplant assessment		
	DSA present (and MFI)	CDC crossmatch result	Flow cytometric crossmatch result	DSA present (and MFI)	CDC crossmatch result	Flow cytometric crossmatch result
1	A2 (6472) B44 (17,478) DR11 (13,413) DP4 (DPB1*04:02) (14,058)	T cell neg B cell pos	T cell pos B cell pos	A2 (14,862) B44 (3662) DR11 (3560) DP4 (DPB1*04:02) (4670)	T cell neg B cell pos	T cell pos B cell pos
2	A2 (800) B7 (1340) DR4 (250) DQ8 (5882)	T cell pos B cell pos	T cell pos B cell pos	A2 (5139) B7 (500) DR4 (207) DQ8 (11,438)	T cell pos B cell pos	T cell pos B cell pos
3	DQ2 (14,986)	T cell neg B cell neg	T cell neg B cell pos	DQ2 (9229)	T cell neg B cell neg	T cell neg B cell pos
7	DQ7 (3588)	T cell neg B cell neg	T cell neg B cell neg*	DQ7 (2143)	T cell neg B cell neg	T cell neg B cell neg
9	DP1 (2478) DP3 (2677) DQ8 (665)	T cell neg B cell neg	T cell neg B cell neg	DP1 (1300) DP3 (1561) DQ8 (41)	T cell neg B cell neg	T cell neg B cell pos

Table 3.1(b) continued

11	DR13 (2318)	T cell neg B cell neg	T cell neg B cell neg*	DR13 (2576)	T cell neg B cell neg	T cell neg B cell neg
14	A24 (724**)	T cell neg B cell neg	T cell neg B cell neg	A24 (41)	N/A Flow cytometric crossmatch only performed	T cell neg B cell pos

*had previously been T cell negative, B cell positive

**previously >1000

ID - identification; DSA - donor specific antibody; MFI - mean fluorescence intensity; CDC - complement dependent cytotoxicity;
neg - negative; pos - positive

All crossmatches were performed according to laboratory standard operating procedures (SOPs). CDC crossmatch positivity was scored using the International Histocompatibility Workshop (IHW) scoring system, and assessed against the negative (background) control. Flow cytometric crossmatch results were interpreted in relation to the results obtained with the negative controls. The threshold for positivity was calculated according to the internally validated policy at the time of analysis. All crossmatch protocols complied with standards for Clinical Pathology Accreditation (CPA) and the European Federation for Immunogenetics (EFI) accreditation.

Crossmatch results and MFI values are from the same serum sample, wherever this data was available. These results represent a 'snapshot' of the patient's laboratory work-up. Previous historic MFI values for DSAs may have been higher, with different crossmatch results obtained.

Table 3.2 Key clinical data for the prospective participant cohort

Participant ID	Pre-conditioning protocol	Perioperative immuno-suppression	Immuno-suppression	Post-transplant plasma exchange	Rejection episodes	Complications	Current clinical status
1	Cyclophosphamide PE (x7) Low dose Ivlg	N/A	N/A	N/A	N/A	Transplant did not proceed	On HD. Active on DD waiting list. Ongoing investigations for potential LD transplantation
2	MMF PE (x5) Low dose Ivlg	N/A	N/A	N/A	N/A	Transplant did not proceed	On PD. Active on DD waiting list and entered into NHSBT paired-pooled scheme
3	MMF PE (x3) Low dose Ivlg	Alemtuzumab	Tacrolimus MMF Prednisolone	Days +4 and +9	Post-transplant biopsy showed no rejection	Poor urine output. Biopsy indicated acute tubular injury with one artery showing mural thrombus	Relocated post-transplantation. Returned to Leeds 16 months later with failing transplant (BK virus and rejection). On PD and active on DD waiting list
7	MMF (started 6 weeks previously)	Alemtuzumab	Tacrolimus MMF Prednisolone	Days +4, +7-10, +15	Post-transplant biopsy showed no rejection	Deteriorating graft function noted one month post-transplant. Biopsy showed no rejection.	Stable function

Table 3.2 continued

9	MMF PE (x5) Low dose IvIg	Alemtuzumab Methyl- prednisolone	Tacrolimus MMF Prednisolone	Days +5, +10, +20	Post- transplant biopsy showed no rejection	DGF	Transplant failure (renal stenosis). Biopsy showed coagulative necrosis consistent with infarction. Graft nephrectomy 1 month later. Subsequent DBD donor transplant (post study samples) Stable function
11	None*	Alemtuzumab Methyl- prednisolone	Tacrolimus MMF Prednisolone	Days +7, +12, +20	None. Primary graft function	Post-transplant low dose IvIg alongside PE	Stable function
14	PE (x6) IvIg	Alemtuzumab Methyl- prednisolone Alemtuzumab	Tacrolimus	Days +5, +10, +20	Post- transplant biopsy showed rejection, treated with methyl- prednisolone	DGF (x3 dialysis sessions)	Stable function

*elective post-transplant PE (x3) and IvIg

ID – identification

MMF – mycophenolate mofetil

DD - deceased donor

DGF - delayed graft function

NHSBT – National Health Service Blood and Transplant

Mural thrombus - a blood clot that has adhered to the wall of a blood vessel; BK virus - member of polyomavirus family, and named using initials of the renal transplant patient it was first isolated from; renal stenosis - narrowing of the artery supplying blood to kidneys.

PE - plasma exchange

N/A - not applicable

LD - live donor

DBD - donation after brain death

IvIg - intravenous immunoglobulin

HD - haemodialysis

PD - peritoneal dialysis

Table 3.3 Serum creatinine levels for the prospective participant cohort

Participant ID	Creatinine ($\mu\text{mol/L}$)		
	Approximately 6 months post-transplantation	Approximately 1 year post-transplantation	Latest (time post-transplant date)
1	N/A	N/A	1193 (2 years and 6 months)
2	N/A	N/A	1060 (2 years and 4 months)
3	Out of region (199 at 3 months post-transplant)	Out of region (745 at 16 months post-transplant)	686 (2 years and 4 months)
7	137	120	135 (1 year and 9 months)
9	415	287	595 (1 year and 9 months)
11	96	73	80 (1 year and 8 months)
14	198	185	185 (1 year and 6 months)

ID - identification

$\mu\text{mol/L}$ – micromoles per litre

N/A - not applicable

Adult reference range for serum creatinine (Leeds & Bradford Pathology Service 2011):

female 49-90 $\mu\text{mol/L}$, male 64-104 $\mu\text{mol/L}$

As shown in table 3.1(b), for participant 1, the MFI values obtained for DSAs directed against HLA-B44, DR11 and DP4 reduced over the course of the pre-conditioning treatment, but the HLA-A2 antibody MFI level increased from 6,472 to 14,862. This situation was reproduced with participant 2, who experienced an increase in the MFI value of HLA-A2 antibody over the treatment period, from a pre-assessment figure of 800 to 5,139 pre-transplantation. This was accompanied by an increase in HLA-DQ8 directed DSA from an MFI of 5,882 to 11,438. A possible explanation for this antibody 'escape' from desensitisation, put forward at the time, was that a DSA blocking factor was being removed during the plasma exchange cycles. It had previously been reported (Kosmoliaptsis *et al.* 2009) that IgM HLA-specific antibodies could block IgG HLA-specific antibodies from binding to the HLA molecules immobilised on the single antigen beads in the Luminex assay, leading to reduced readings for IgG DSAs. An alternative explanation is that complement fixation by HLA specific antibodies can interfere with the binding of the secondary IgG detection antibody, thereby affecting the fluorescence levels detected and leading to an inaccurate measurement of allo-antibody present, or false negative results (Schnaidt *et al.* 2011). These phenomena illustrate the technical shortcomings of the single antigen bead assay, and are now known as the prozone effect (Schnaidt *et al.* 2011). Several strategies of pretreating test sera are now employed by H&I laboratories to counteract these difficulties, including heat inactivation, and the use of dithiothreitol (DTT) and EDTA.

The remaining five prospective participants progressed to transplantation as planned, with the final report reiterating the proviso that there was a risk of accelerated acute rejection due to historic or current low level DSAs. Table 3.1(b) indicates that for participant 9, the pre-transplant assessment flow cytometric crossmatch had converted to B cell positive. However, it was noted that the positivity observed was weak, and the HLA-DQ and DP antibodies responsible had reduced in terms of their MFI value following pre-treatment. Similarly, a B cell positive flow cytometric crossmatch was observed for participant 14, at pre-transplant assessment. However, in the setting of a reduced DSA, and no further incompatibilities, this was not deemed to be a contraindication.

As indicated in table 3.2, participant 3 relocated to a different transplant centre, and was lost to follow-up until their return 16 months later with a failing graft. A creatinine level of 745 (table 3.3) reflects this graft failure. Of the four transplanted participants from whom follow-up samples were obtained, 3 had stable graft function (7, 11 and 14), and a fourth (9) suffered graft failure due to renal stenosis. The results in table 3.3 are consistent with these outcomes, showing improving or relatively stable creatinine results for participants 7, 11 and 14, whilst those for participant 9 remained high.

3.1.2 The retrospective participant cohort

Thirteen retrospective HLAi transplant participants were recruited, as described in section 2.4.2. An overview of this cohort is provided in tables 3.4 and 3.5, showing key laboratory and clinical data for the group. As with the prospective cohort, selected serum creatinine results were recorded (appendix 5).

As indicated in table 3.4(a), of the 13 retrospective participants, two were ABO blood group incompatible, in addition to being HLAi cases. In this context, participant 5 is of interest, as a transplant using the same donor had been previously attempted, using rituximab and plasma exchange for the pre-conditioning protocol. However, the anti-A blood group titre was not sufficiently reduced using this conditioning protocol in order for the transplant to proceed. It was therefore decided, by the clinical team, that a specific anti-A absorption column (protein-A column) would be used, in a second attempt at transplantation, alongside plasma exchange.

The DSAs present pre-transplantation are listed in table 3.4(a). Three cases (participants 6, 8, and 17) had historic DSA, which were no longer detectable in most recent serum samples. Two cases (13 and 17) involved HLA-specific antibodies, which were cross-reactive with the donors' HLA, causing a positive crossmatch. Where it is known, the sensitising event is shown. This can often be difficult to ascertain, especially in the case of blood transfusion or pregnancy. Sometimes this is due to incomplete medical records, or poor communication between the clinical team and the laboratory. Often, the patient may be unsure, or unwilling to disclose, if they have ever received a blood transfusion, or, in the case of female patients, if they have ever conceived.

Table 3.4(a) Key laboratory data for the retrospective participant cohort

Participant ID	Gender	Relationship of donor	Recipient blood group	Donor blood group	HLA mismatch grade	DSA present	Sensitising event (where known)
4	F	Husband	A	O	0-2-2	B8, B17, DR3, DR7, DR52	Pregnancy (x3)
5	F	Husband	B	A1	0-2-2	B62, DR1, DR53, DQ5	No previous transplants
6	M	Wife	B	O	1-2-2	A28 (historic)	No previous transplants
8	F	Husband	O	O	2-2-2	B12 (historic)	Blood transfusion
10	F	Father	A	A	0-1-1	DR4	
12	F	Sister	A	A	1-1-1	B7	
13	F	Father	O	O	1-1-0	A11 (cross-reactive), B27 (cross-reactive)	Previous DD transplant
15	F	Son	O	O	1-1-1	DR7	No previous transplants, 1 known pregnancy
16	M	Father	B	O	2-1-2	A3, A11, B35, DR4, DR11, DQ3	Previous transplant and blood transfusions
17	F	Sister	O	O	0-1-1	B52 (cross-reactive), B35 (cross-reactive), DR7 (historic)	Pregnancy (x1), blood transfusions
18	M	Wife	O	O	0-2-1	B12	Blood transfusion
19	F	Husband	B	A2	2-2-1	A9, DR1, DQ5	Blood transfusion, pregnancy
20	M	Partner	A	O	2-1-1	A1	

HLA mismatch grade - calculated according to NHS Blood and Transplant (NHSBT) policy. The mismatches (at a 'broad' specificity level) between the recipient and donor at HLA-A, B and DR were taken into account, in the recipient versus donor direction. Therefore, 2-2-2 represents the worst scenario for mismatching, and is a poor HLA match. Conversely, 0-0-0 indicates no mismatches present, and the best possible HLA match.

Donor specific antibody

DD - deceased donor

F - female

M - male

HLA - human leucocyte antigen

transplant pair

incompatible (ABOi)

transplant pair

Table 3.4(b) Crossmatch data at initial and pre-transplant assessment time points for the retrospective participant cohort

Participant ID	Initial assessment		Pre-transplant assessment	
	CDC crossmatch result	Flow cytometric crossmatch result	CDC crossmatch result	Flow cytometric crossmatch result
4	T cell neg, B cell neg	T cell pos, B cell pos	T cell neg, B cell neg	T cell pos, B cell pos (Autologous result: T cell pos, B cell pos)
5	T cell neg, B cell neg	T cell neg, B cell neg (3 months prior to transplant)	T cell neg, B cell neg	T cell pos, B cell pos
6	T cell neg, B cell neg	T cell neg, B cell neg	T cell neg, B cell neg	T cell neg, B cell neg
8	T cell pos, B cell pos (AHG-CDC)	T cell pos, B cell pos	T cell neg, B cell neg	T cell neg, B cell neg
10	T cell neg, B cell neg	T cell neg, B cell pos	T cell neg, B cell neg	T cell neg, B cell pos
12	T cell neg, B cell pos	T cell pos, B cell pos	T cell neg, B cell neg	T cell pos, B cell pos
13	T cell neg, B cell neg	T cell neg, B cell pos	T cell neg, B cell neg	T cell neg, B cell neg
15	T cell neg, B cell neg	T cell neg, B cell pos	T cell neg, B cell pos	T cell neg, B cell neg
16	T cell pos, B cell pos	T cell pos, B cell pos	T cell neg, B cell pos (Autologous result: B cell pos)	T cell pos, B cell pos
17	T cell neg, B cell neg	T cell pos, B cell pos	T cell neg, B cell neg	T cell pos, B cell pos
18	T cell neg, B cell neg	T cell pos, B cell pos	T cell neg, B cell neg	T cell pos, B cell pos
19	T cell neg, B cell neg	T cell neg, B cell pos	'LIFECODES DSA' results: HLA Class I and Class II negative	N/A
20	T cell neg, B cell neg	T cell neg, B cell pos	T cell neg, B cell neg	T cell neg, B cell neg

ID - identification; CDC - complement dependent cytotoxicity; neg - negative; pos - positive; AHG-CDC - anti-human globulin enhanced CDC assay; LIFECODES Donor Specific Antibody (DSA) (supplied by Immucor) - a Luminex screening assay for the presence of recipient antibodies directed against HLA isolated from donor cells.

Unless stated otherwise, crossmatch results are from allo-crossmatches (recipient versus potential donor). All crossmatches were performed according to laboratory standard operating procedures (SOPs). CDC crossmatch positivity was scored using the International Histocompatibility Workshop (IHW) scoring system, and assessed against the negative (background) control. Flow cytometric crossmatch results were interpreted in relation to the results obtained with the negative controls. The threshold for positivity was calculated according to the internally validated policy at the time of analysis. All crossmatch protocols complied with standards for Clinical Pathology Accreditation (CPA) and, latterly (since 2006), the European Federation for Immunogenetics (EFI) accreditation.

Crossmatch results and MFI values are from the same serum sample wherever this data was available. These results represent a 'snapshot' of the patient's laboratory work-up, and previous historic MFI values for DSAs may have been higher, with different crossmatch results obtained. The protocol for laboratory work-up for HLAi transplantation was not yet firmly established for some of the retrospective participants, and thus the initial assessment sample may be an 'historic' result rather than a 'baseline' result.

Table 3.5 Key clinical data for the retrospective participant cohort

Participant ID	Pre-conditioning protocol	Perioperative immuno-suppression	Immuno-suppression	Post-transplant plasma exchange	Rejection episodes	Other complications	Current clinical status
4	PE (x6) Low dose Ivlg	Alemtuzumab	Tacrolimus MMF Prednisolone	Yes x2	None	Transfused immediately prior to transplant surgery	Good function
5	MMF Ivlg Protein A column PE	Alemtuzumab	Tacrolimus MMF Prednisolone	Yes and protein A column	Post-transplant biopsy showed acute tubular necrosis (no evidence of rejection)	UTI	Graft dysfunction and failure (rejection) 21 months post-transplant (post study samples). Subsequent DCD donor transplant
6	MMF (started 2 weeks previously)	Methyl-prednisolone Simulect	Tacrolimus MMF Simulect	Days +3-5, +7-10, +15-21 (x3)	Post-transplant biopsy showed tubulitis treated with methyl-prednisolone	Hepatitis B positive	Stable function
8	PE (x6?) Ivlg (3 weeks)	Methyl-prednisolone Simulect	Tacrolimus MMF Prednisolone Simulect	Day +14	None	Diabetes mellitus	Stable function
10	MMF (2 weeks)	Methyl-prednisolone Simulect	Tacrolimus MMF	None known	None	None known	Stable function

Table 3.5 continued

12	MMF (2 weeks), PE (x3)	Methyl- prednisolone Simulect	Tacrolimus MMF Simulect	Days +4 and +9	None	Herpes labialis	Graft failure approximately 8 years post- transplant. Subsequent live donor transplant 9 years post- transplant
13	None (previous DSA had declined)	Methyl- prednisolone Simulect	Tacrolimus MMF	None	Day 19, treated with ATG	None known	Stable function
15	MMF (2 weeks)	Methyl- prednisolone Simulect	Tacrolimus MMF Simulect Prednisolone	Days +3, +7, +10	Post-transplant biopsy - ATN ?rejection, treated with methyl- prednisolone (x3)	None known	Stable function
16	PE (x10) Rituximab Ivlg	Methyl- prednisolone Simulect	Tacrolimus MMF Prednisolone Simulect	Yes x7	? vascular rejection, treated with methyl- prednisolone	Recurrent FSGS	Graft failed 4 months post- transplant. Patient deceased 10 years post- transplant
17	MMF (2 weeks)	Methyl- prednisolone Simulect	Tacrolimus MMF Simulect Cyclosporin Prednisolone	Yes x9	Early acute vascular rejection, treated with PE	Chronic rejection 8 years post- transplant	On DD waiting list. Under investigation for potential live donor transplant

Table 3.5 continued

18	MMF (2 weeks) PE (x5)	Methyl- prednisolone Simulect	Tacrolimus MMF Prednisolone Simulect	Days +5, +10, +21	Humoral rejection day 5 post-transplant. Treated with PE (x5), methyl- prednisolone (x4) and Ivlg	acute pyelonephritis	Patient deceased 6 years post- transplant (cardiac event)
19	MMF (2 weeks) Rituximab PE (x5) Ivlg	Methyl- prednisolone Simulect	Tacrolimus MMF Prednisolone Simulect	Day +3	None known	DGF Diabetes	Graft failing 6 years post- transplant. Renal obstruction, staghorn calculi. On PD
20	MMF (2 weeks)	Methyl- prednisolone Alemtuzumab	Tacrolimus MMF	Days +5-7, +7- 10, +21	None	None known	Stable function

ID – identification

Ivlg - intravenous immunoglobulin

DD - deceased donor

DGF - delayed graft function

ATN - acute tubular necrosis

ATG - anti-thymocyte globulin

staghorn calculi - upper urinary tract stones caused by recurrent infection

PE - plasma exchange

MMF – mycophenolate mofetil

PD - peritoneal dialysis

DCD - deceased donor after circulatory death

UTI - urinary tract infection

FSGS - focal segmental glomerulosclerosis

Table 3.4(b) displays initial assessment and pre-transplantation assessment crossmatch results for the cohort. Participant 4 was noted for persistent positivity in the flow cytometric crossmatch. Results from an autologous crossmatch and antibody screening allowed the allo-crossmatch results to be explained by the presence of non-HLA antibodies, and the transplantation proceeded as scheduled.

The crossmatch results for participant 5 appear to denote a worsening situation; however, the initial assessment samples were assessed 3 months prior to the pre-transplant samples, so reflect a historic result. The crossmatch results for participant 15 (table 3.4(b)) also appear somewhat incongruous, as the pre-transplant samples indicated a B cell positive result for the CDC assay, whilst the flow cytometric assay was negative. However, the positivity observed was noted as being weak, and attributed to an IgM antibody, and therefore not considered a contraindication for the transplantation to proceed.

As indicated in table 3.5, the conditioning treatment for participant 19 included the administration of rituximab, a monoclonal antibody directed towards the B cell surface marker CD20 (Beimler, Susal and Zeier 2006). The presence of this drug in the recipient peripheral blood samples can result in a false B cell positive crossmatch, or can mean that there are insufficient circulating B cells remaining to perform the assay. For this reason, a different pre-transplantation assay, Lifecodes DSA, was performed to assist in result interpretation for this participant.

3.2 Prospective participant results

The results from the prospective cohort were analysed separately from the retrospective cohort results, as the former all have multiple sample time points, and were viewed against the background of the individualised transplant schedule for each participant. Full data sets were obtained for all the prospective participants. For each participant, a 'procedural timeline' is first presented, followed by graphs of the longitudinal lymphocyte subset results, both as absolute counts (cells/ μ l) and as a percentage of total lymphocytes (CD45⁺).

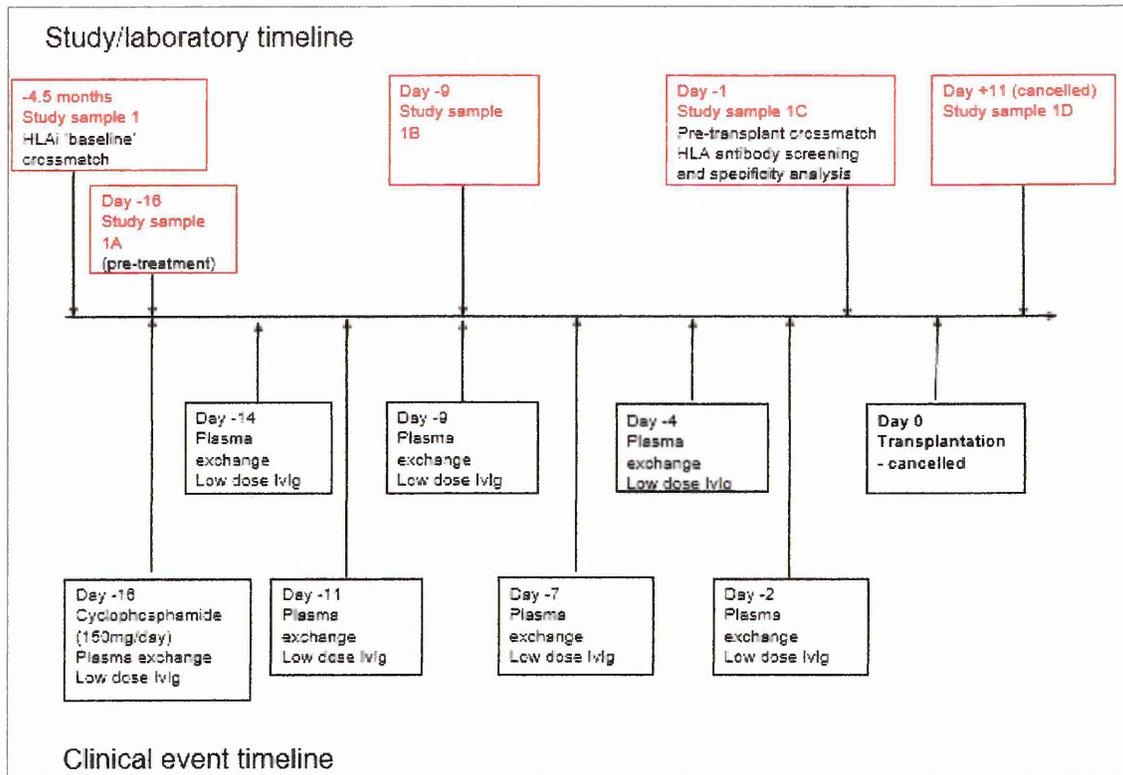
3.2.1 Participant 1

A procedural timeline is shown for participant 1 in figure 3.1. Results from two clinical samples from this patient are included. Sample 1 (-4.5 months) was taken at the point of the HLAi 'baseline assessment of challenge' crossmatch, in order to help interpret crossmatch results for the complex case of this individual. A further sample (1D – Day +11) was taken for clinical reasons, to elucidate why the required antibody reduction had not been achieved for this patient.

Figure 3.2 shows the results of lymphocyte subset analysis for participant 1. The absolute cell counts showed a steady decrease in cell numbers up until the day prior to transplant. The fact that this decrease in cell numbers was proportional across cell types is indicated in that the percentages of the cell markers represented does not alter between the point of baseline crossmatch (-4.5 months) and Day -9 (figure 3.2 (b)). However, 1 day prior to transplant (Day -1) the percentage of CD3⁺ and CD4⁺ cell populations appeared to increase, most likely due to the sudden dip in the absolute count of CD45⁺ cells observed between the week prior and the day prior to transplantation.

A clinical sample, taken 11 days post the scheduled transplantation (Day +11), indicated that the cell populations were starting to recover in terms of both numbers and percentages, as expected once the conditioning regimen was halted. In this manner, participant 1 represents a good example of why it is important to view the absolute counts of cell populations alongside the percentages, as, at first glance, figures 3.2 (a) and 3.2 (b) appear to contradict each other, and it is only when the absolute count of CD45⁺ cells is considered that the plots have meaning.

Figure 3.1 Timeline showing key study, laboratory and clinical interventions for participant 1



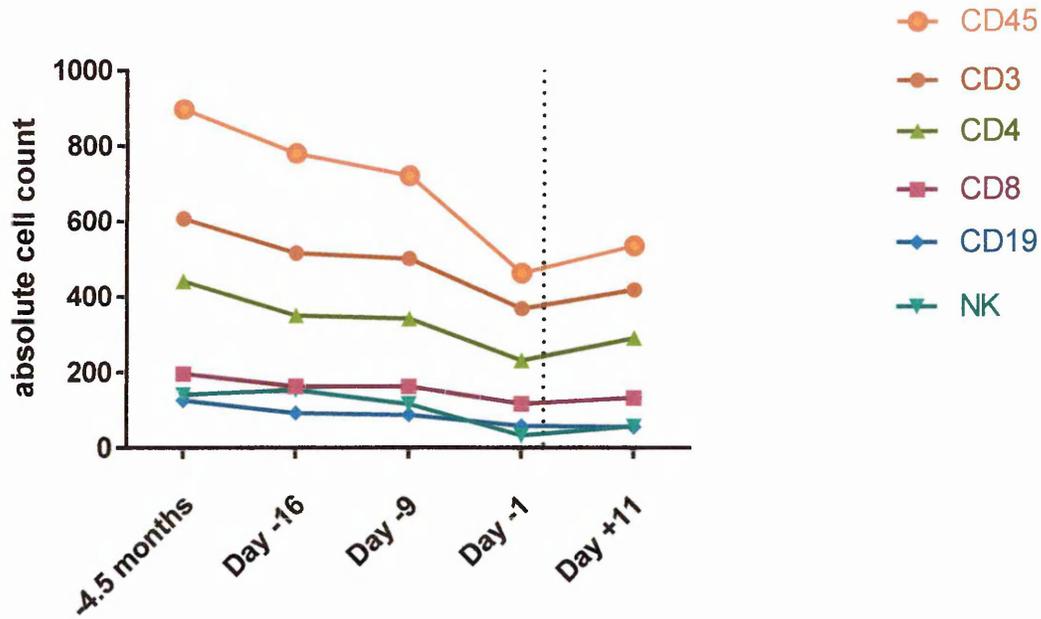
HLA - human leucocyte antigen
 mg/day - milligrams per day

HLAi - HLA incompatible
 Ivig - intravenous immunoglobulin

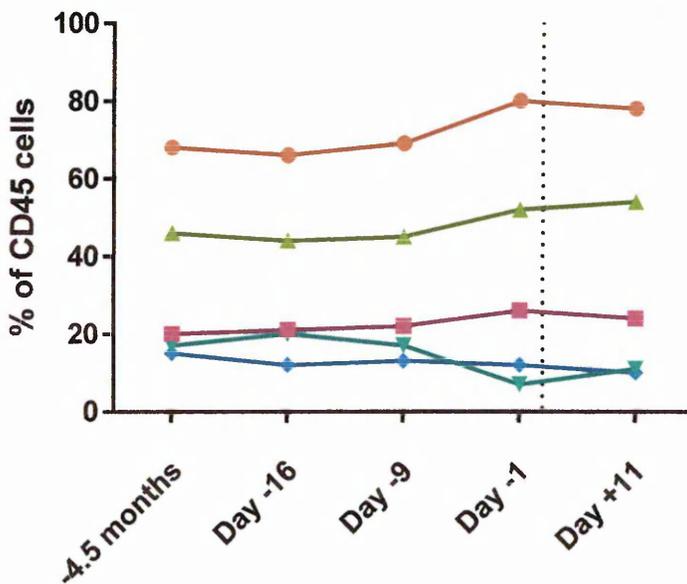
The timeline is not drawn to scale. Study samples are indicated in red text/textbox. Study/laboratory interventions are shown on the top of the timeline. Clinical interventions are indicated on the bottom of the timeline.

Figure 3.2 Longitudinal results of lymphocyte subset analysis for participant 1

(a)



(b)

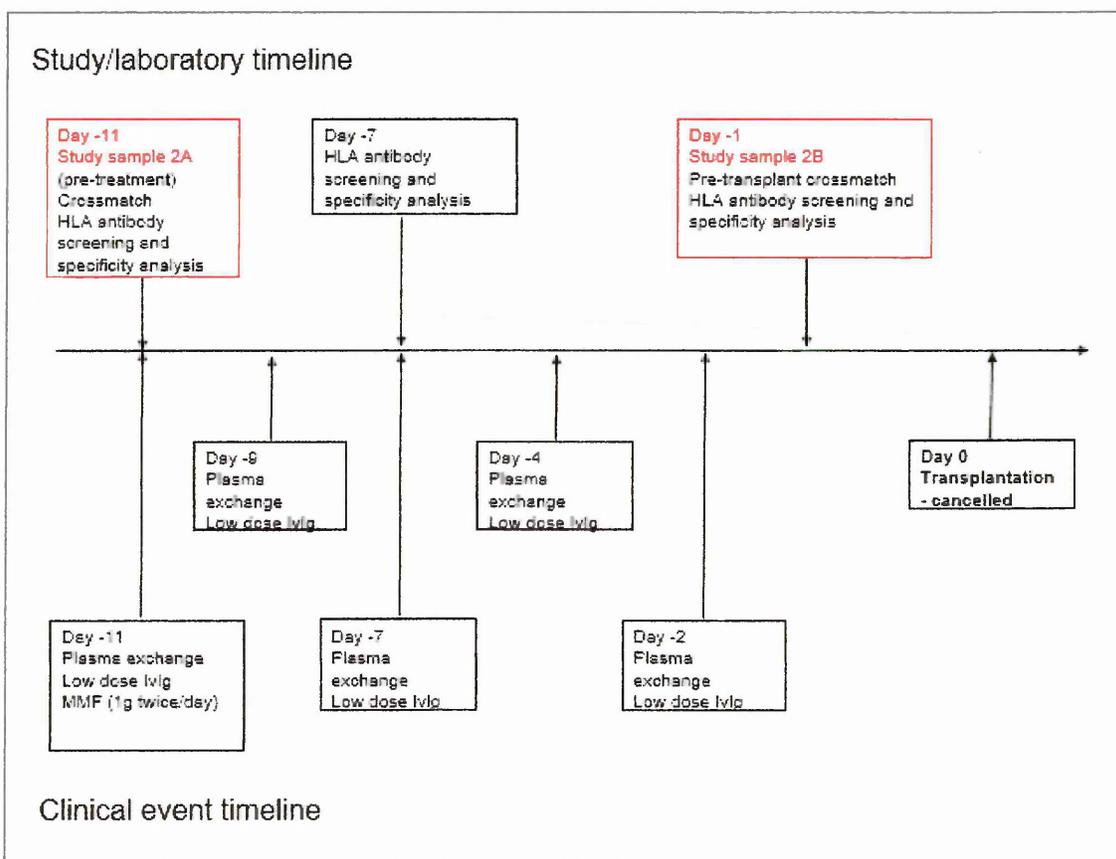


(a) Absolute cell counts (cells/μl) plotted at the sample collection time points indicated on the x-axis; (b) Lymphocyte subset percentages plotted as a percentage of total lymphocytes (CD45⁺). A dotted line indicates Day 0 (scheduled day of transplant – cancelled). Sample time points are described fully in figure 3.1.

3.2.2 Participant 2

A procedural timeline is shown for participant 2 in figure 3.3. Figure 3.4 shows the results of lymphocyte subset analysis for participant 2, who was monitored over two time points, and differs quite markedly from participant 1, in that between the samples taken pre-treatment and 1 day prior to the scheduled transplant, there was an increase in the total numbers of CD45⁺ cells, CD3⁺ cells and CD4⁺ cells (figure 3.4 (a)). This was also demonstrated for the CD3⁺ and CD4⁺ populations in terms of their overall percentages (figure 3.4 (b)).

Figure 3.3 Timeline showing key study, laboratory and clinical interventions for participant 2



HLA - human leucocyte antigen

Ivlg – intravenous immunoglobulin

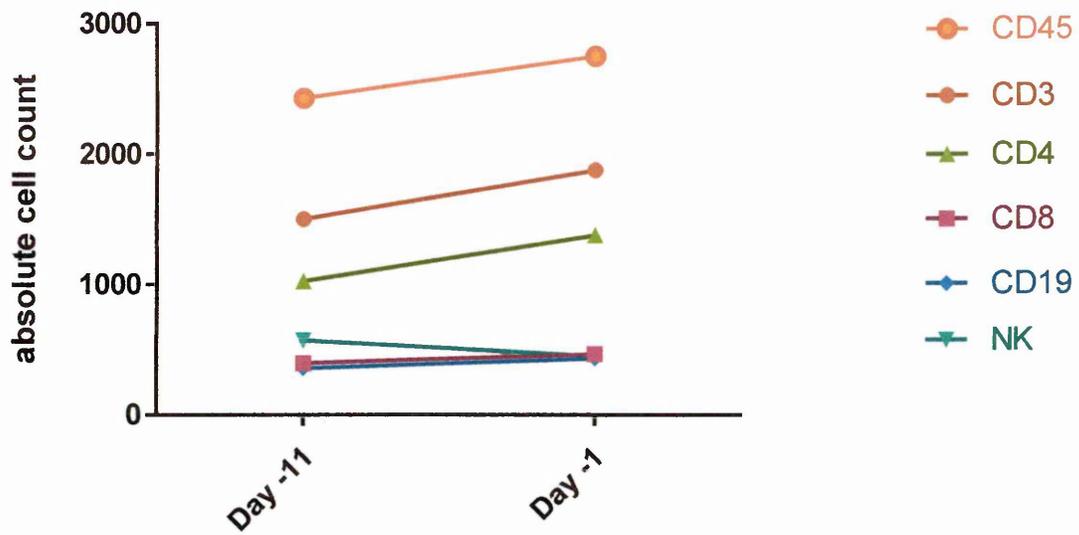
MMF - mycophenolate mofetil

g twice/day - gram twice per day

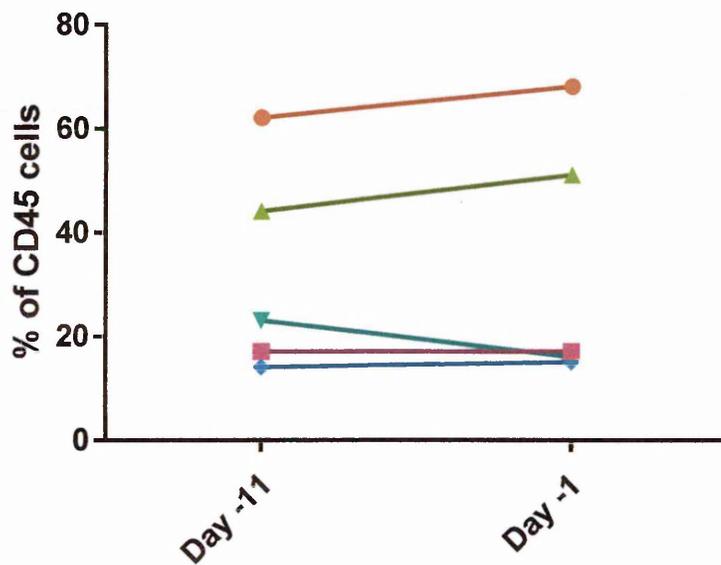
The timeline is not drawn to scale. Study samples are indicated in red text/textbox. Study/laboratory interventions are shown on the top of the timeline. Clinical interventions are indicated on the bottom of the timeline.

Figure 3.4 Longitudinal results of lymphocyte subset analysis for participant 2

(a)



(b)



(a) Absolute cell counts (cells/ μ l) plotted at the sample collection time points indicated on the x-axis; (b) Lymphocyte subset percentages plotted as a percentage of total lymphocytes (CD45⁺). Sample time points are described fully in figure 3.3.

In addition, it was noted that participant 2 possessed an absolute cell count in excess of double that of participant 1 across all cell types. Appendix 6 demonstrates that within the 'normal' population there is variation in cell counts of the lymphocyte populations. Therefore, as the differences in cell counts were apparent pre-treatment, it is likely that this simply represents the natural variation observed between individuals.

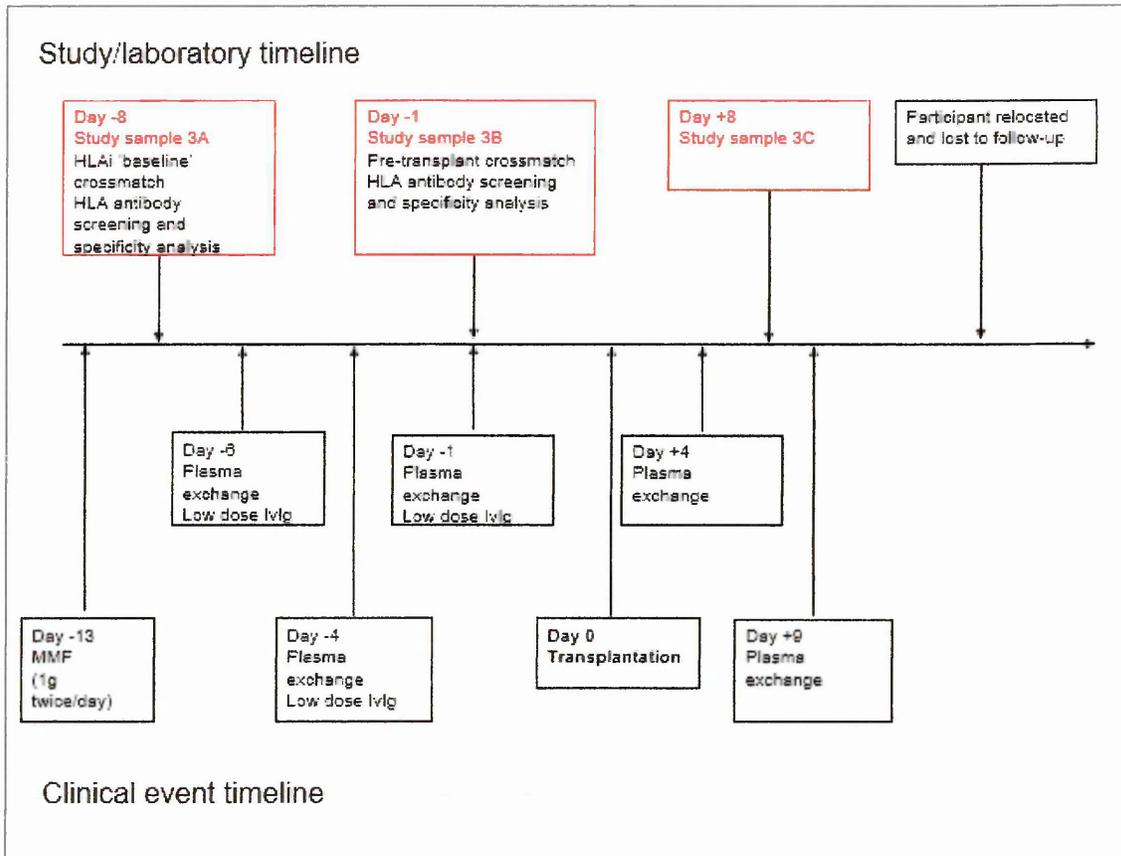
3.2.3 Participant 3

A procedural timeline is shown for participant 3 in figure 3.5. Figure 3.6 shows the results of lymphocyte subset analysis for participant 3, who was successfully transplanted following his antibody removal conditioning. However, this participant subsequently relocated to a different centre and, therefore, the collection of further study samples was not possible. Interestingly, participant 3 showed an increase in cell counts between their baseline crossmatch sample (Day -8) and the day prior to transplant (Day -1), most markedly in the CD45⁺, CD3⁺, CD4⁺ and CD8⁺ populations (figure 3.6 (a)). All lymphocyte subsets were subsequently depleted by eight days post transplantation (Day +8). Participant 3 returned to Leeds sixteen months post-transplantation, with a failing graft, with both rejection and BK virus infection identified as the cause.

3.2.4 Participant 7

A procedural timeline is shown for participant 7 in figure 3.7. Figure 3.8 shows the results of lymphocyte subset analysis for participant 7, indicating the reduction in lymphocyte numbers immediately post-transplantation, and recovery at 8 months and 13 months post-transplantation. Although the profile for the percentage of NK cells present (figure 3.8 (b)) shows a striking increase at five days post-transplantation (Day +5), all lymphocyte subsets were depleted at this point (figure 3.8 (a)). Therefore, this increase in percentage is not as significant as it may first appear.

Figure 3.5 Timeline showing key study, laboratory and clinical interventions for participant 3



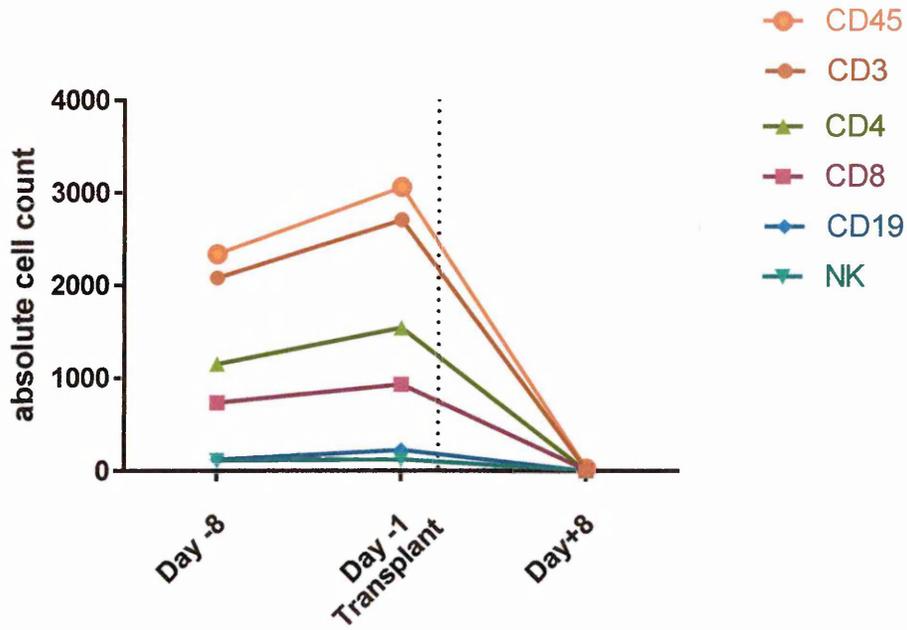
HLA - human leucocyte antigen
 MMF - mycophenolate mofetil
 Ivlg - intravenous immunoglobulin

HLAi - HLA incompatible
 g twice/day - gram twice per day

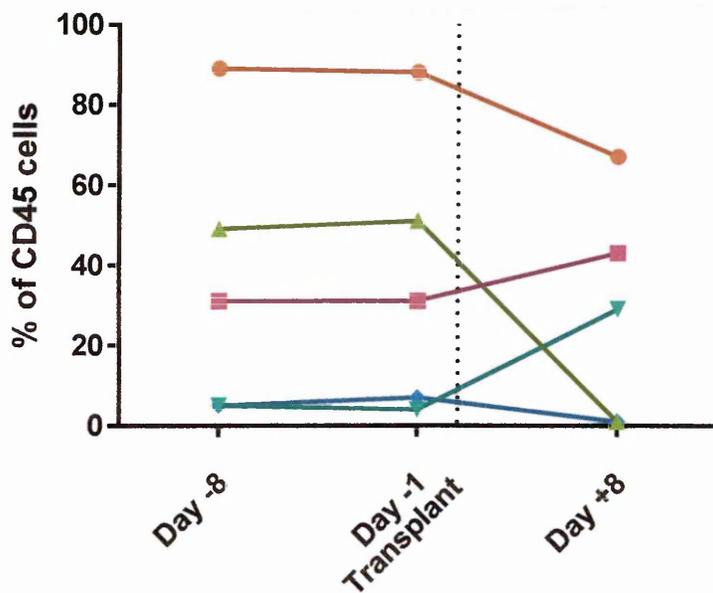
The timeline is not drawn to scale. Study samples are indicated in red text/textbox. Study/laboratory interventions are shown on the top of the timeline. Clinical interventions are indicated on the bottom of the timeline.

Figure 3.6 Longitudinal results of lymphocyte subset analysis for participant 3

(a)

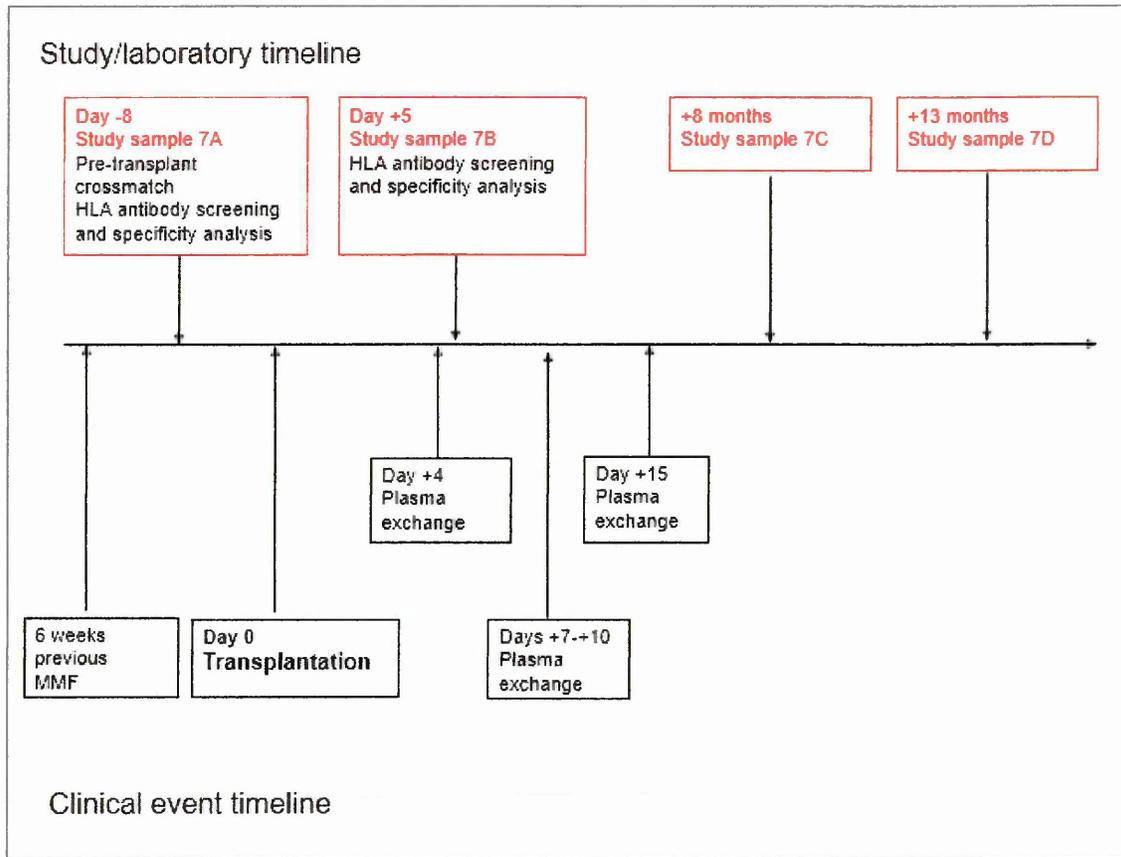


(b)



(a) Absolute cell counts (cells/ μ l) plotted at the sample collection time points indicated on the x-axis; (b) Lymphocyte subset percentages plotted as a percentage of total lymphocytes (CD45⁺). A dotted line indicates Day 0 (day of transplant). Sample time points are described fully in figure 3.5.

Figure 3.7 Timeline showing key study, laboratory and clinical interventions for participant 7



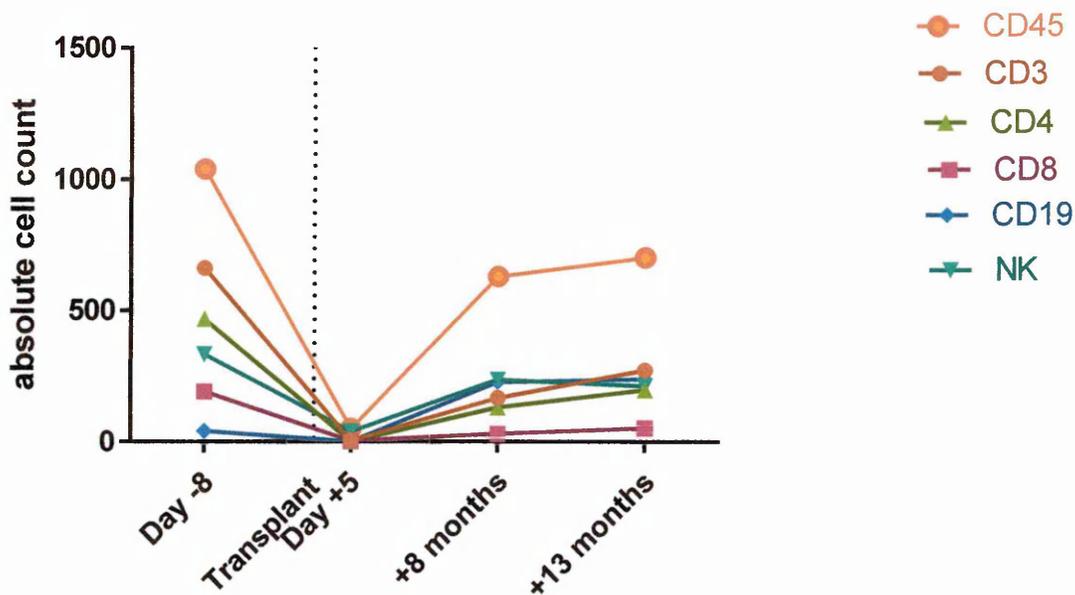
HLA - human leucocyte antigen

MMF - mycophenolate mofetil

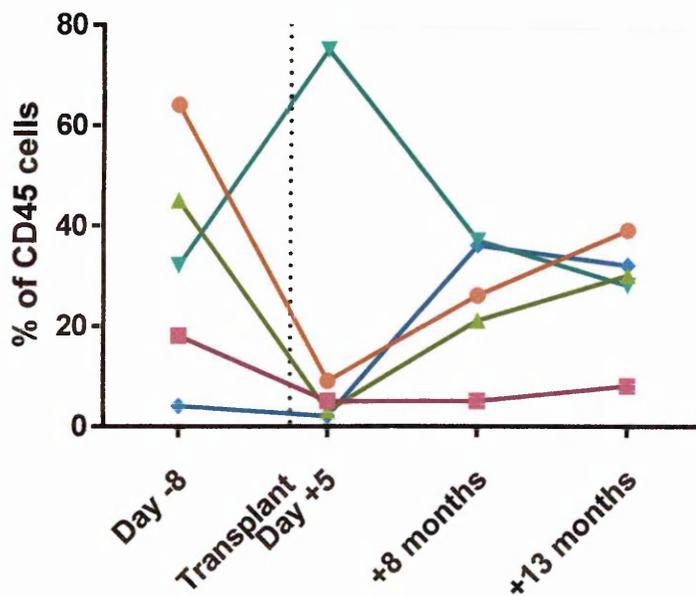
The timeline is not drawn to scale. Study samples are indicated in red text/textbox. Study/laboratory interventions are shown on the top of the timeline. Clinical interventions are indicated on the bottom of the timeline.

Figure 3.8 Longitudinal results of lymphocyte subset analysis for participant 7

(a)



(b)



(a) Absolute cell counts (cells/μl) plotted at the sample collection time points indicated on the x-axis; (b) Lymphocyte subset percentages plotted as a percentage of total lymphocytes (CD45⁺). A dotted line indicates Day 0 (day of transplant). Sample time points are described fully in figure 3.7.

3.2.5 Participant 9

A procedural timeline is shown for participant 9 in figure 3.9. Figure 3.10 shows the results of lymphocyte subset analysis, indicating the fall in total lymphocyte count at 1 week post-transplantation, and recovery at six months and one year post-transplantation. No immediate post-transplantation blood samples were collected from this recipient, due to there being insufficient cells present to allow full immunophenotyping. The CD45⁺ cell count for the Day +7 time point was obtained from routine blood count results available on the Leeds Teaching Hospitals Trust (LTHT) electronic results system (Telepath).

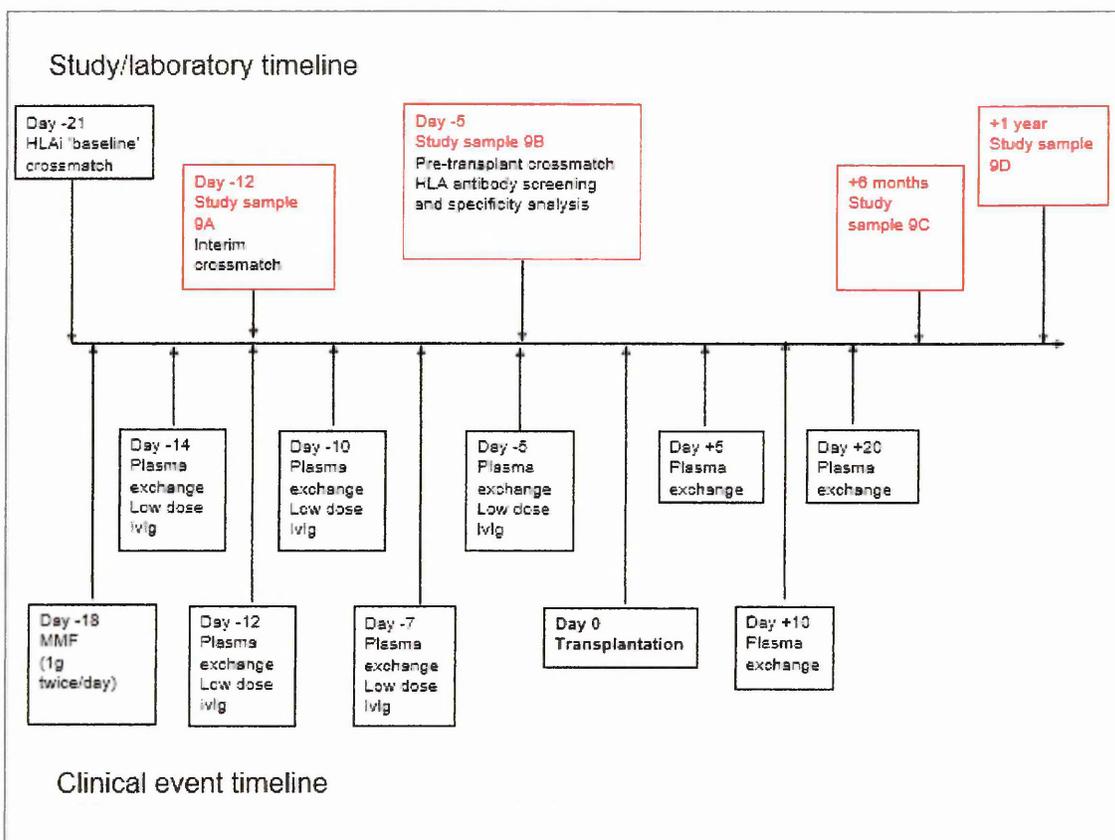
3.2.6 Participant 11

A procedural timeline is shown for participant 11 in figure 3.11, and figure 3.12 shows the results of lymphocyte subset analysis. A reduction in total lymphocyte count at one week post-transplantation, and recovery at six months and one year post-transplantation are evident (figure 3.12 (a)). No immediate post-transplantation blood samples were collected from this recipient, due to there being insufficient cells present to allow full immunophenotyping. The CD45⁺ cell count for the Day +7 time point was obtained from routine blood count results as for participant 9, above.

3.2.7 Participant 14

A procedural timeline and results of lymphocyte subset analysis are shown for participant 14 in figures 3.13 and 3.14 respectively. The fall in total lymphocyte count at one week post-transplantation, and recovery at six months and one year post-transplantation are indicated in figure 3.14 (a). No immediate post-transplantation blood samples were collected from this recipient, due to there being insufficient cells present to allow full immunophenotyping. The CD45⁺ cell count for the Day +7 time point was obtained from routine blood count results.

Figure 3.9 Timeline showing key study, laboratory and clinical interventions for participant 9



HLA - human leucocyte antigen

HLAi - HLA incompatible

MMF - mycophenolate mofetil

g twice/day - gram twice per day

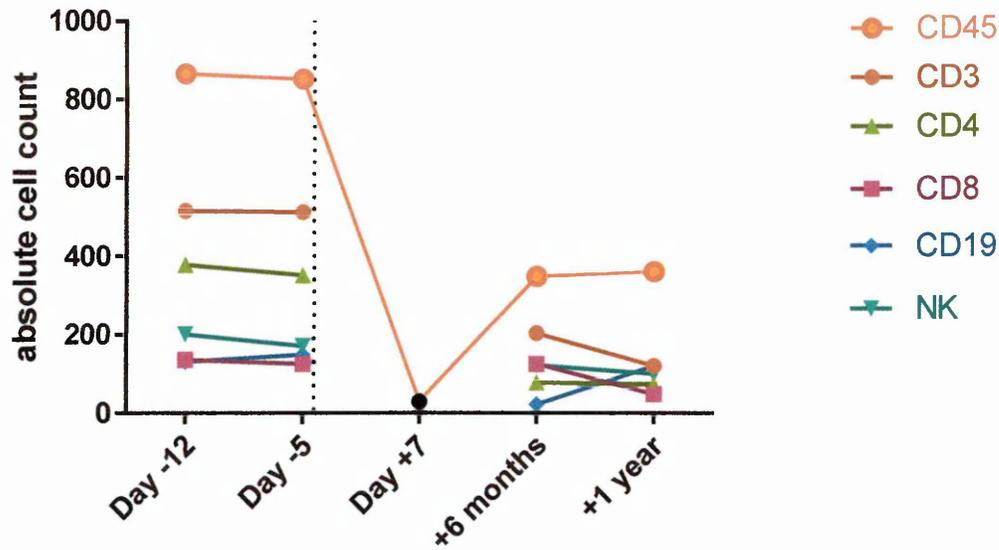
Ivlg - intravenous immunoglobulin

The timeline is not drawn to scale. Study samples are indicated in red text/textbox. Study/laboratory interventions are shown on the top of the timeline. Clinical interventions are indicated on the bottom of the timeline.

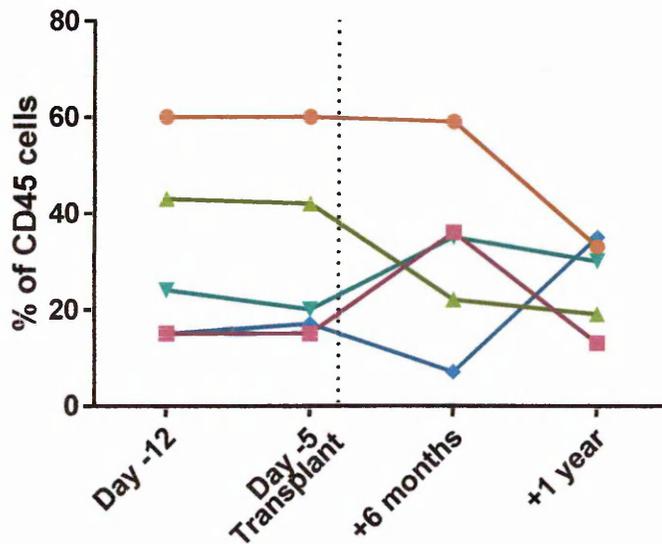
Day -5 sample was originally intended to be day -1, but transplantation was postponed for four days, at short notice, for logistical reasons.

Figure 3.10 Longitudinal results of lymphocyte subset analysis for participant 9

(a)

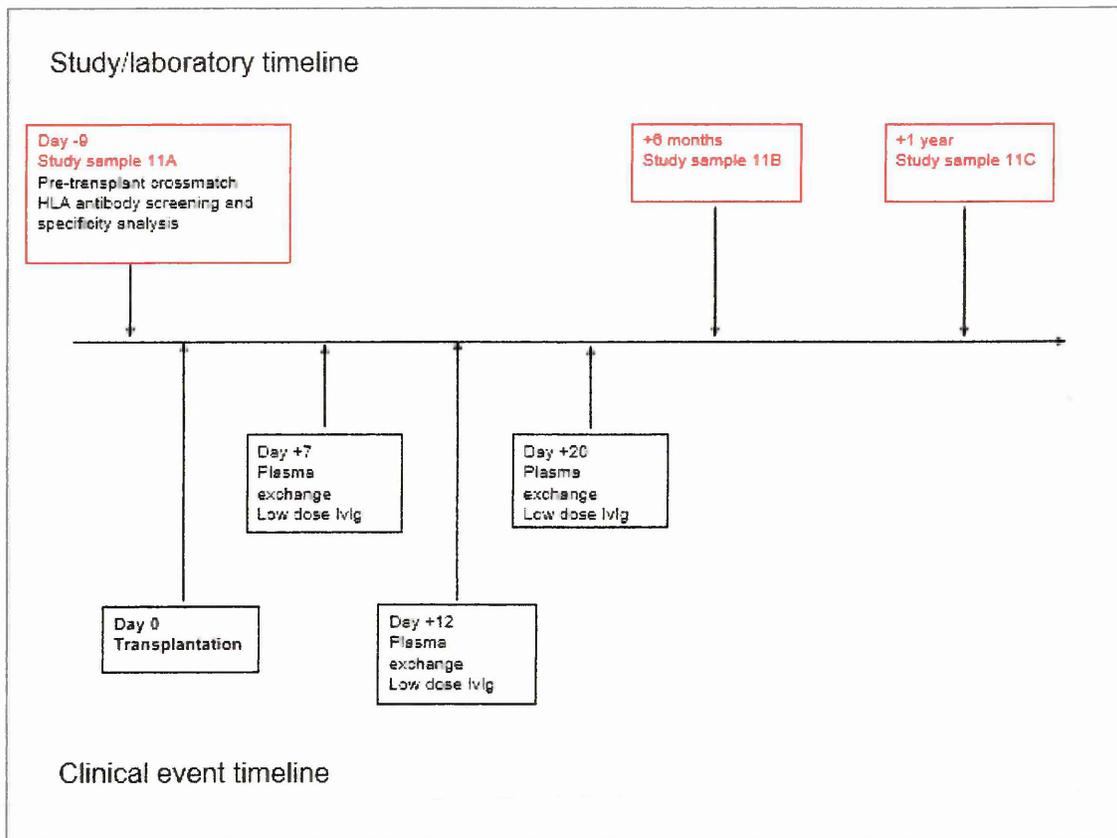


(b)



(a) Absolute cell counts (cells/μl) plotted at the sample collection time points indicated on the x-axis. The CD45⁺ cell count at Day +7 was obtained from routine blood count results available on the Leeds Teaching Hospitals Trust (LTHT) electronic results system (Telepath), indicated by black-filled data point; (b) Lymphocyte subset percentages plotted as a percentage of total lymphocytes (CD45⁺). A dotted line indicates Day 0 (day of transplant). Sample time points are described fully in figure 3.9.

Figure 3.11 Timeline showing key study, laboratory and clinical interventions for participant 11



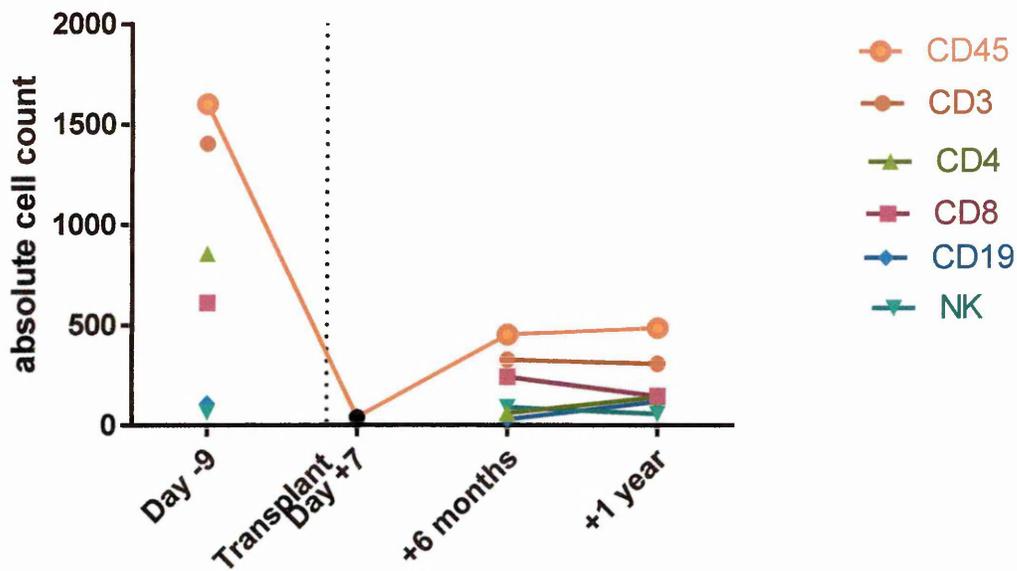
HLA - human leucocyte antigen

Ivlg - intravenous immunoglobulin

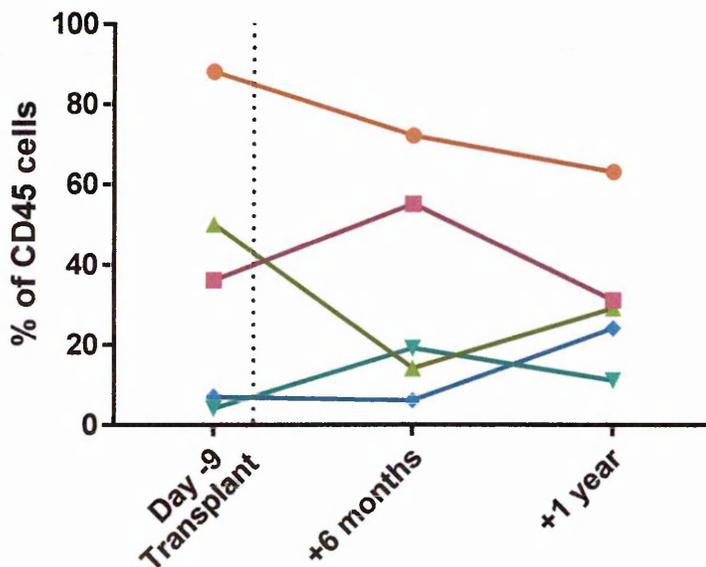
The timeline is not drawn to scale. Study samples are indicated in red text/textbox. Study/laboratory interventions are shown on the top of the timeline. Clinical interventions are indicated on the bottom of the timeline.

Figure 3.12 Longitudinal results of lymphocyte subset analysis for participant 11

(a)

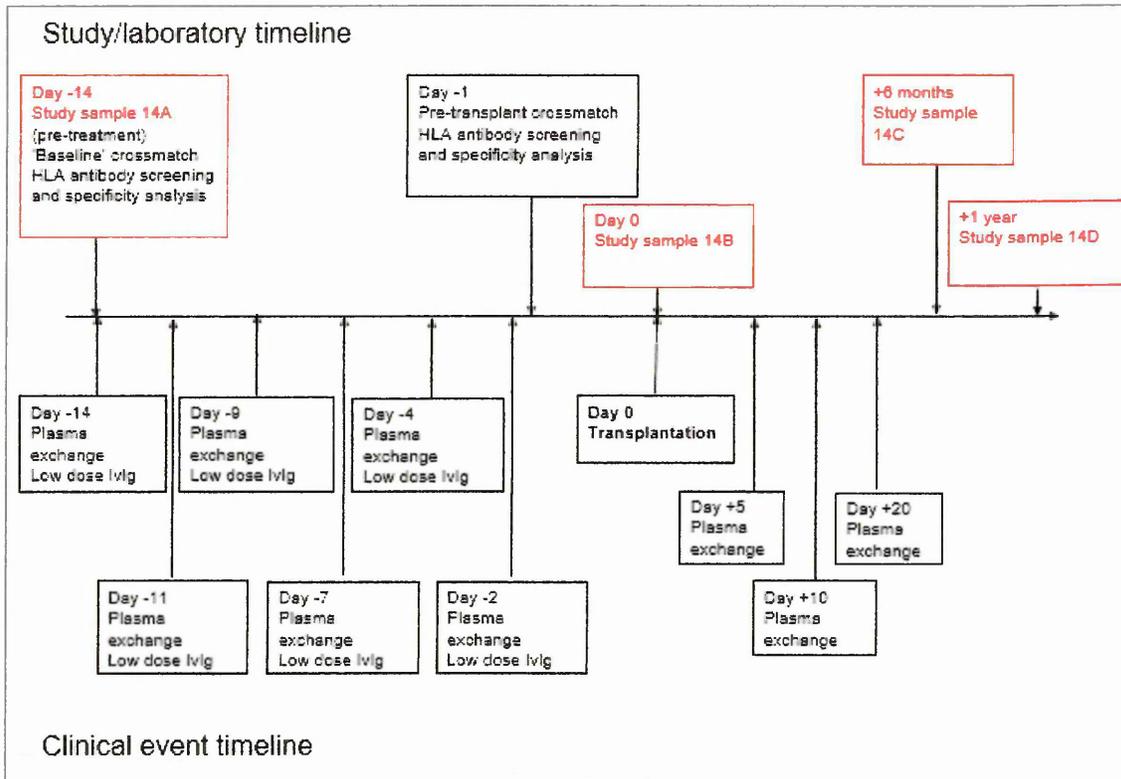


(b)



(a) Absolute cell counts (cells/ μ l) plotted at the sample collection time points indicated on the x-axis. The CD45⁺ cell count at Day +7 was obtained from routine blood count results available on the LTH electronic results system (Telepath), indicated by black-filled data point. (b) Lymphocyte subset percentages plotted as a percentage of total lymphocytes (CD45⁺). A dotted line indicates Day 0 (day of transplant). Sample time points are described fully in figure 3.11.

Figure 3.13 Timeline showing key study, laboratory and clinical interventions for participant 14



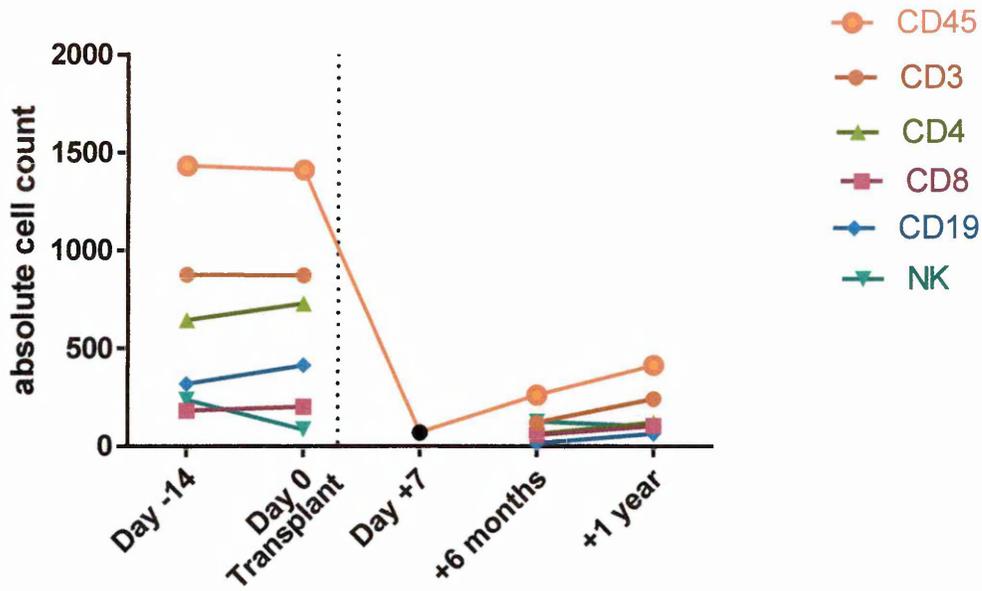
HLA - human leucocyte antigen

Ivlg - intravenous immunoglobulin

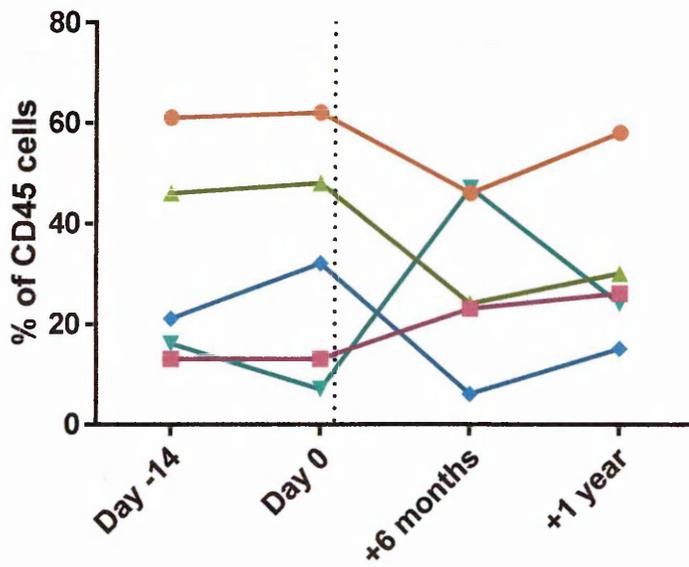
The timeline is not drawn to scale. Study samples are indicated in red text/textbox. Study/laboratory interventions are shown on the top of the timeline. Clinical interventions are indicated on the bottom of the timeline.

Figure 3.14 Longitudinal results of lymphocyte subset analysis for participant 14

(a)



(b)



(a) Absolute cell counts (cells/μl) plotted at the sample collection time points indicated on the x-axis. The CD45⁺ cell count at Day +7 was obtained from routine blood count results available on the LTHT electronic results system (Telepath), indicated by black-filled data point; (b) Lymphocyte subset percentages plotted as a percentage of total lymphocytes (CD45⁺). A dotted line indicates Day 0 (day of transplant). Sample time points are described fully in figure 3.13.

Both participants 2 and 3 showed an increase in absolute cell counts between samples taken at baseline and the day prior to transplant, particularly in the CD45⁺, CD3⁺ and CD4⁺ populations (figures 3.4 (a) and 3.6 (a)). This pattern is in contrast to transplanted participants 9 and 14. Sample time points for participants 7 and 11 did not allow this comparison. The conditioning protocols for participants 2 and 3 were similar in that they both received MMF at the start of treatment, followed by plasma exchange and low dose Ivlg. However, this is unlikely to provide the explanation for the increase in cell counts observed, as participants 9 and 14 received similar conditioning.

All five transplanted patients showed a fall in lymphocyte counts immediately post-transplantation. For participants 3 and 7 this was demonstrated in the samples collected according to the original schedule (Day +8 for participant 3 and Day +5 for participant 7). However, neither of these samples contained sufficient cells to allow full immunophenotyping analysis to be undertaken. This was found to be caused by a recent alteration to the general immunosuppression protocol, which included the addition of the drug alemtuzumab, which had rendered this sample time point uninformative due to the total reduction of cells, and therefore subsequent participants (9, 11 and 14) were not sampled at this point.

In order to check that all participants did indeed have insufficient cells for full immunophenotyping analysis at this point, and also to indicate the fall in total lymphocytes, immediately post-transplant, on the participant graphs for absolute cell counts, a CD45⁺ cell count was taken from the routine haematology test results on the LTHT results server (Telepath). The use of the CD45⁺ cell count figure from Telepath, for participants 9, 11 and 14, allowed the graphs showing absolute cell counts to indicate the extent of the depletion of total lymphocytes.

All transplanted participants for whom follow-up was possible (participants 7, 9, 11 and 14) showed some recovery of their total lymphocyte counts over the first year post-transplantation. However, consistent for all cases, the cell counts did not recover to pre-treatment levels, and in most cases (9, 11 and 14) the one year post-transplant sample indicated a CD45⁺ cell count of less than half of that of the pre-transplant sample, and a value much reduced compared to the mean for

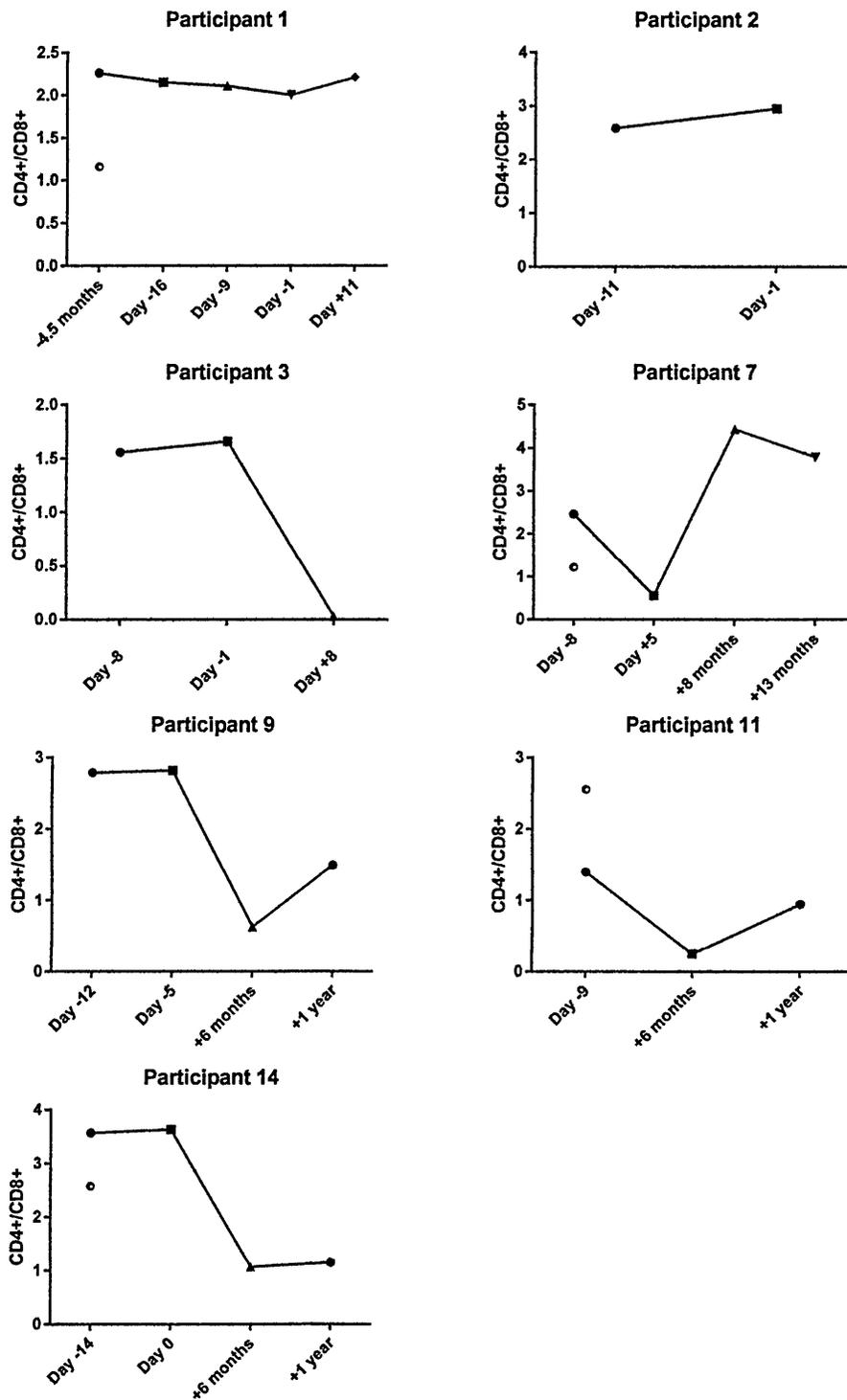
the normal controls of 2,130 cells/ μ l. However, with the exception of participant 14, the transplanted participants all showed a recovery of CD19⁺ cells to near pre-transplantation cell counts. The delayed B cell recovery, observed in participant 14, may be due to treatment with methyl-prednisolone for a rejection episode post-transplantation.

For participant 7, the cell recovery for the individual lymphocyte populations was not in proportion to the pre-transplant levels, with CD8⁺ cells in particular making a slower recovery. This is indicated in figure 3.8 (b), in that the proportions of cell types present were not consistent with the pre-transplantation sampling point. With the exception of the CD19⁺ subset, participants 9, 11 and 14 appeared to make only a small recovery of their lymphocyte subsets by one year post-transplantation (figures 3.10, 3.12 and 3.14), and indeed participants 9 and 11 showed a decrease in cell counts of both CD3⁺ and CD8⁺ cell populations between six months and one year post-transplantation (figures 3.10 (a) and 3.12 (a)). For all four transplanted participants, the proportions of cell types did not reconstitute consistently in relation to the pre-transplant sample (indicated on the lymphocyte subsets percentages graphs shown in figures 3.8 (b), 3.10 (b), 3.12 (b) and 3.14 (b)). This demonstrates that the four participants remained immunocompromised at one year post-transplantation, both in terms of absolute cell counts and in terms of relative proportions of cellular populations as a percentage of overall lymphocytes.

3.2.8 CD4:CD8 T cell ratios for the prospective transplant participants

The Multitest™ kit provides a ratio for CD4:CD8 T lymphocytes, calculated by dividing the absolute count of the CD4⁺ cells by the absolute count of the CD8⁺ cells. This was plotted longitudinally for each prospective participant for their sample collection time points (figure 3.15). For participants for whom there was an age and gender matched normal control recruited to the study, this CD4:CD8 ratio is also indicated on the graph.

Figure 3.15 Prospective participants CD4:CD8 T cell ratios



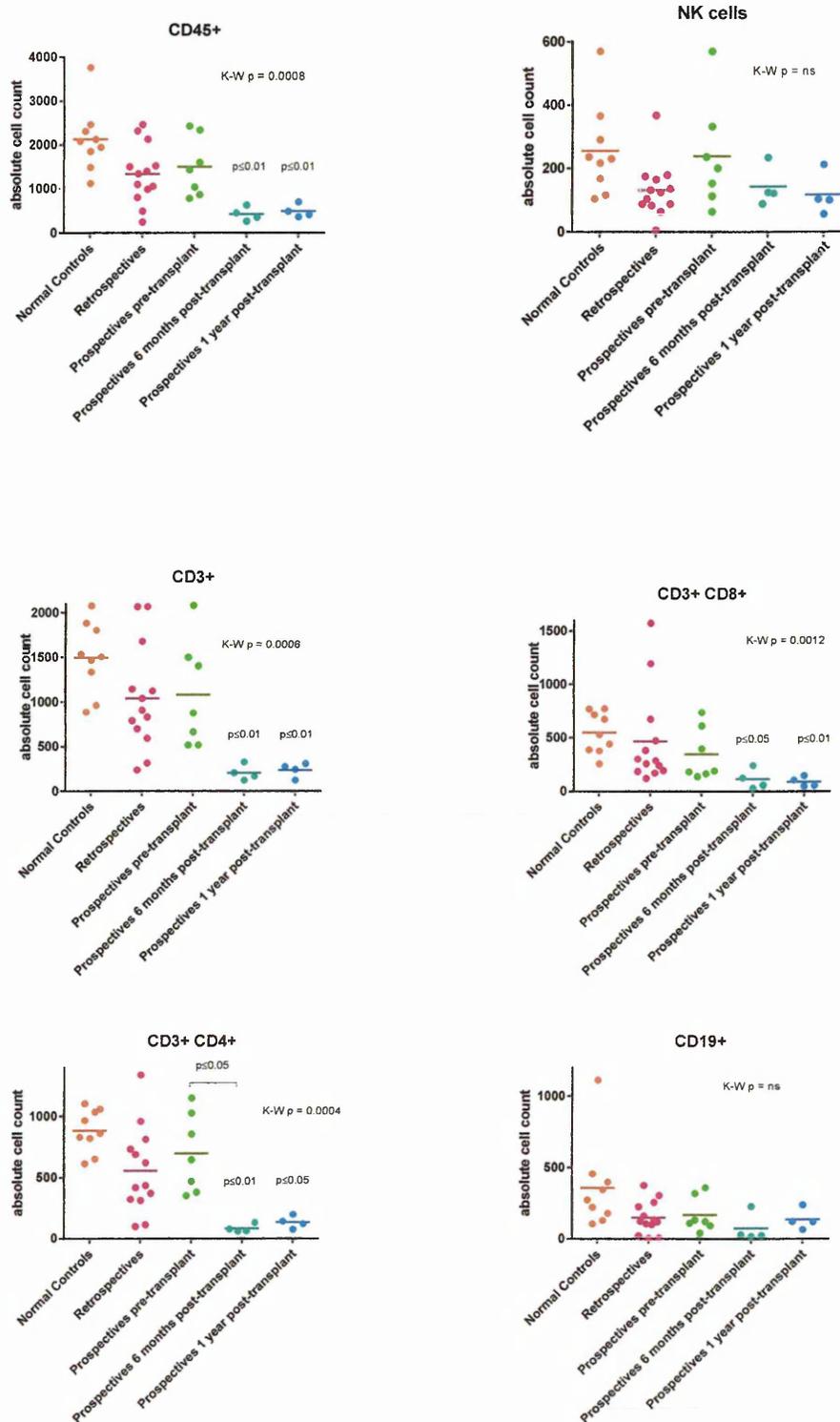
The CD4:CD8 T cell ratios are shown longitudinally for each prospective participant. x-axis - sample time points as described in individual participant timelines, in section 3.2. Where an age and gender matched normal control has been recruited to the study, the CD4:CD8 ratio for the control is indicated in red (for participants 1, 7, 11 and 14).

As indicated in figure 3.15, the absolute count of CD4⁺ cells is greater than that of CD8⁺ cells in healthy individuals, denoted by a CD4:CD8 ratio of >1. In the participants who were not transplanted, the CD4:CD8 ratio remained steady; whereas the transplanted participants all displayed dramatic changes in their ratios. Where an immediate post-transplant sample was analysed (participants 3 and 7) the ratio was close to zero, a reflection of the fall in both cell populations. Participant 7 demonstrated the highest increase in their CD4:CD8 ratio, peaking at a value of 4.4 at eight months post-transplantation. This reiterates that the recovery of the CD8⁺ T lymphocyte population was particularly slow in this participant. For the remainder of the participants (9, 11 and 14), the CD4:CD8 ratio was lowest in their six month post-transplantation sample (range 0.3 to 1.0), and then showed some recovery in their one year post-transplantation sample (range 1.0 to 1.5); although in all cases the ratio did not reach the pre-transplantation value. It is also noteworthy that for participant 14, the CD4:CD8 ratio (3.6) was relatively high in the pre-treatment sample.

3.3 Retrospective participant results

A total of thirteen retrospective HLAi transplant patients were analysed at a single post-transplant time point and full datasets for lymphocyte subset analysis were obtained. For the purpose of initial analysis, the results from the single time points for the retrospective cohort were plotted alongside the results from the normal controls. The prospective cohort results from a pre-transplant sample, and also six months and one year post-transplant were also included for comparison (figure 3.16).

Figure 3.16 Lymphocyte subset analysis for the retrospective cohort, alongside the normal controls, and prospective cohort



Absolute cell counts (cells/ μ l); K-W – Kruskal-Wallis test; significant p values are versus the normal control cohort, unless indicated otherwise; ns - not significant; horizontal line indicates mean. Normal controls, n= 9; retrospective cohort, n= 13; prospective cohort pre-transplant, n= 7; prospective cohort six months post-transplant, n= 4; prospective cohort one year post-transplant, n= 4.

The absolute count of each population of lymphocytes was studied, for the five study groups; normal controls, retrospective participants and prospective participants pre-transplantation, six months post-transplantation and one year post-transplantation (figure 3.16). The graph for total lymphocytes showed that there was much variation observed within the groups of normal controls, retrospective participants and the prospective participants pre-transplantation. A similar amount of variation between individuals within each of the aforementioned three cohorts was evident for the absolute counts of CD3⁺ and CD4⁺ cells. Conversely, the graphs for CD8⁺, CD19⁺ and NK cells display less variation within these cohorts.

The prospective pre-transplantation cohort had a mean absolute count of total lymphocytes that was reduced compared to the normal controls (not significant). With the exception of NK cells, this was the case for the cell counts of each of the lymphocyte subsets studied, for the prospective cohort prior to any interventions. The six month and one year post-transplantation groups demonstrated a drastic reduction in total lymphocytes ($p \leq 0.01$ compared to the normal controls) due to the lymphoablative effect of the transplantation conditioning protocol and the continued use of immunosuppression. A significant reduction in absolute cell counts, for these groups, was also observed for the CD3⁺, CD8⁺ and CD4⁺ subsets.

The mean CD45⁺ cell count for the retrospective cohort was also reduced compared to the normal controls (not significant) indicating that some transplant patients never totally recover their lymphocyte cell counts to a 'normal' level. These findings were mirrored in the analyses for CD3⁺, CD8⁺, CD4⁺, CD19⁺ and NK cells, in terms of the mean values being consistently reduced compared to the normal control group (not significant).

Within each graph, certain data points were striking. The graph of the absolute count of CD8⁺ cells showed two retrospective participants who had higher counts than all other participants. These outlying data belong to participants 8 and 18, with absolute cell counts of 1,573 and 1,194 cells/ μ l respectively. Retrospective participant 17 was somewhat of an outlier for CD4⁺ cells, with an absolute cell

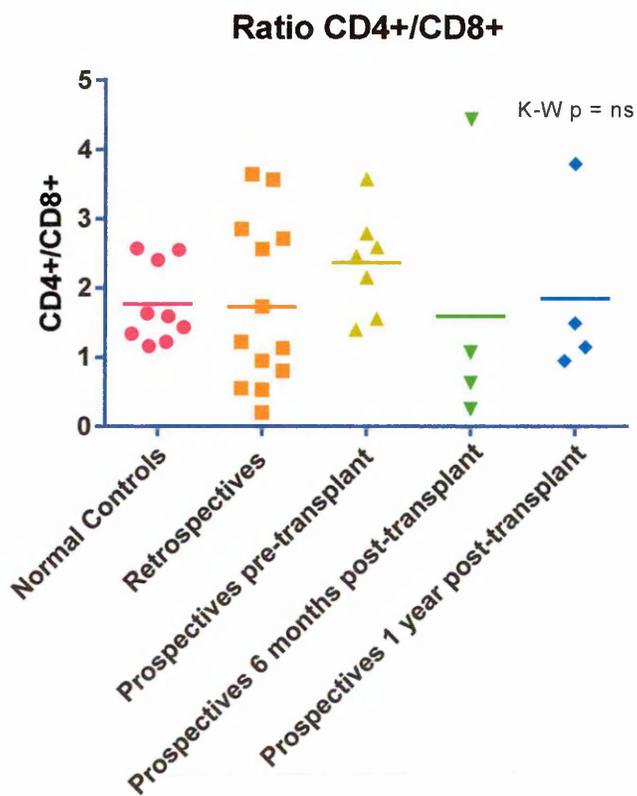
count of 1,339 cells/ μ l. Retrospective participant 8 also had a much higher than the mean number of NK cells, with a count of 368 cells/ μ l.

Participant 7 differed markedly to the rest of the prospective cohort on the graphs for NK and CD19⁺ cells in that over the course of the year post transplantation, they made a stronger recovery for these cell types compared to the other three transplanted participants. In both cases the data points for participant 7 are above the mean. However, as previously mentioned, in relation to figure 3.8, this participant made a comparatively slow recovery of their CD8⁺ cells.

The graph for CD19⁺ B cells demonstrated the serious depletion of these cells not only in most of the prospective group at the 6 month post-transplantation time point, but interestingly also in many of the retrospective group (not significant). Here participants 4, 8 and 19 had very low counts.

CD4:CD8 ratios were also plotted for the retrospective cohort, alongside the normal control cohort and the three time points for the prospective cohort (figure 3.17). From this, it was clear that there was a greater range of values for the transplant recipient cohorts compared with the normal controls. The normal controls all showed a CD4:CD8 ratio of greater than 1, and less than 3, with a mean of 1.8. The retrospective cohort, in particular, showed a large spread in CD4:CD8 ratio results, from a minimum value of 0.2 to a maximum of 3.6. Despite this a similar mean value of 1.7 was observed. Several participants, most notably 4, 6 and 8, had an inverted CD4:CD8 ratio (0.53, 0.55, 0.2 respectively) due to their CD4⁺ cell count being lower than their CD8⁺ cell count. As previously noted, prospective participant 7 was striking in showing a high CD4:CD8 ratio at the sample time points equivalent to six months and one year post-transplantation.

Figure 3.17 CD4:CD8 T cell ratios for the retrospective cohort, alongside the normal controls, and prospective cohort



K-W – Kruskal-Wallis test; ns - not significant; horizontal line indicates mean. Normal controls, n= 9; retrospective cohort, n= 13; prospective cohort pre-transplant, n= 7; prospective cohort six months post-transplant, n= 4; prospective cohort one year post-transplant, n= 4

3.4 Normal control participant results

Nine normal controls were recruited to the study and assessed at a single time point, described in section 2.4.3. The results for the normal controls are shown in appendix 6. Appendix 7 shows a comparison of the median results for the nine normal controls with published data for healthy adults (n = 51) (Bonilla and Oettgen 1997). The study data for normal controls shows similar values to the published data although, except in the case of NK cells, slightly higher absolute counts were observed across all subset populations.

3.5 Further stratified analysis of the results of lymphocyte subsets

3.5.1 HLA-DR mismatching analysis of the data from the retrospective participant cohort

Due to the fact that the retrospective cohort had a larger number of individual participants than the prospective cohort, an analysis of the impact of HLA-DR mismatching on the study data, was first attempted using this study group. The retrospective cohort was stratified according to HLA-DR mismatches present between each individual donor and recipient pair, in the recipient versus donor direction. One group consisted of zero or 1 HLA-DR mismatches ($n = 8$) whilst the second group was of recipient and donor pairs with 2 HLA-DR mismatches ($n = 5$). The results from the lymphocyte subset analysis were plotted for the two groups, for each cell type (data not shown). No differences between the two cohorts were observed from these analyses.

3.5.2 The effect of alemtuzumab on lymphocyte subsets in the retrospective participant cohort

The results from lymphocyte subset analysis were re-examined in light of which participants had received alemtuzumab as a part of their transplantation schedule. Three of the retrospective participants (4, 5, and 20) received alemtuzumab, but no correlation was observed between this and persistent low cell counts (data not shown).

3.5.3 The effect of time since transplantation on CD45⁺ cell absolute counts, in the retrospective participant cohort

The time since transplantation, in months, was examined to ascertain any effect on CD45⁺ cell counts, but no correlation was observed (data not shown).

3.5.4 The effect of the use of MMF in the prospective participant cohort, pre-transplantation, on lymphocyte subsets

Three prospective participants (3, 7 and 9) received pre-conditioning with MMF prior to the first study sample being taken. Further analysis showed no correlation

between this and the reduced cell counts observed, in comparison to the normal controls (data not shown).

3.5.5 Reanalysis of the data from the retrospective cohort, to examine a possible association between lymphocyte subset results and a post-transplantation rejection episode

As indicated in table 3.5, five retrospective participants (6, 13, 15, 17 and 18) experienced a rejection episode post-transplantation. The CD45⁺ cell counts differed significantly ($p = 0.0451$) between the group of participants who experienced a post-transplant rejection episode and those who did not, using the Mann-Whitney test (table 3.6). No association between rejection episodes and the other lymphocyte subsets data was observed (data not shown).

Table 3.6 The CD45⁺ absolute cell count for retrospective participants who experienced a post-transplant rejection episode versus participants with no post-transplant rejection episode

	Participants who experienced post-transplant rejection (n = 5)	Participants with no post-transplant rejection (n = 8)	P value (Mann Whitney test)
Median CD45 ⁺ absolute cell count (cells/ μ l)	1524	1025	0.0451

3.6 Discussion of the results of lymphocyte subset analysis

The lymphocyte subset results presented for participant 1 were predictable following treatment with cyclophosphamide, a powerful alkylating agent, which thereby inhibits DNA replication and cell division (Parham 2009). Due to its cytotoxic action, cell apoptosis occurs in all rapidly dividing cells, leading to bone marrow suppression (Parham 2009). As stated previously, participant 1 presented a particularly difficult desensitisation case as they possessed high levels of both HLA Class I and Class II antibodies. Therefore, in this case, the

objective of using cyclophosphamide in conditioning was to eradicate the recipient's immune system, reducing the numbers of activated B cells producing donor-directed antibodies, and subsequently reducing the capacity of the patient to respond to the incompatible transplant.

The five transplanted prospective participants also demonstrated the lymphoablative effect of the immunosuppressive drugs administered, with the reduction in cell counts of all lymphocyte subsets immediately post-transplantation. Despite some degree of recovery, particularly in the B cell subset, all four participants, with follow-up, remained immunocompromised at one year post-transplantation. The drug alemtuzumab was used to target recipient lymphocytes peri-operatively, and the effect of this was evident in all of the transplanted prospective participants. Alemtuzumab, a drug originally approved for the treatment of cancer, is a monoclonal antibody targeted against the cell marker CD52 (Parham 2009), which is present on all mature lymphocytes, but absent on bone marrow stem cells. This observed scenario fits in with the literature regarding alemtuzumab, which is known to cause lymphopenia, with a reported recovery of CD19 cells within 3-12 months, whilst CD4 and CD8 T cells may remain lowered for three years (Morales *et al.* 2008).

Interestingly, the retrospective cohort also displayed mean absolute cell counts that were reduced compared to the mean of the normal controls. This may be an indication of the long-term effect of the preconditioning and the continued use of immunosuppressive drugs. In an attempt to ascertain whether the use of alemtuzumab, in particular, had also affected the lymphocyte recovery of the retrospective cohort, the data was reanalysed according to whether or not the patient received this drug, but no correlation was observed. In addition, no correlation was observed between the time since transplantation and total lymphocyte cell counts, indicating that it is not the case that the most recently transplanted patients were responsible for the reduced mean value.

The observations described above suggest that some transplanted patients never recover their lymphocyte subset counts to 'normal' levels. However, the results from the prospective (pre-transplant) cohort, compared to the normal

controls, point towards a 'chronic renal failure' effect, so it may be the case that the recovery of lymphocyte counts could be to pre-transplant levels rather than to 'normal' levels. Although three prospective participants received pre-conditioning with MMF prior to the first study sample being taken, this does not account for the low cell counts observed pre-transplantation. It seems likely that the immune systems of patients awaiting transplantation have already been compromised, either from the illness itself or the impact of dialysis. This phenomenon has been previously documented, with reports of reduced absolute cell counts for T cells (CD3⁺), 'helper' T cells (CD4⁺) and 'cytotoxic' T cells (CD8⁺) in patients undergoing long-term haemodialysis (Raska *et al.* 1983, Chida, Sakurai and Yoshiyama 1986). Furthermore, it was also reported that changes in lymphocyte subset cell counts are specifically linked with multiple blood transfusions in dialysis patients (Nanishi *et al.* 1986), although this could not be confirmed in the current study.

A chronic reduction in lymphocyte subsets, demonstrated in some of the retrospective participants in this study, can have serious repercussions, in terms of the risk of infection and malignancy. For example, alongside other risk factors, the long-term reduction of CD4⁺ T cells and CD19⁺ B cells, in renal transplant recipients, has been shown to correspond with solid cancer and lymphoma development (Ducloux *et al.* 2002).

The observation that several retrospective participants had elevated cell counts for CD8⁺, CD4⁺ or NK populations may be explained by an infection. In particular, participant 8 appeared as an 'outlier' with an increased cell count for both CD8⁺ and NK cells, compared to the mean, so may have had a viral infection. However, a review of the patient's laboratory file provided no evidence for this and further investigation was not possible due to information governance issues. Conversely, participant 8 was one of three retrospective participants (4, 8 and 19) who demonstrated particularly low cell counts for the CD19⁺ subset. These participants were all at different time points post-transplantation, at eight months, eleven years and four years respectively. However, a factor that they did have in common was the continued use of prednisolone, which is the most likely explanation of this observation.

An altered CD4:CD8 ratio was a characteristic observed in many of the transplant recipients, and an inverted CD4:CD8 ratio persisted in several of the retrospective participants, i.e. CD8⁺ cells present in greater numbers than CD4⁺ cells. This is often associated with chronic or opportunistic viral infections, such as herpes virus and cytomegalovirus (CMV) (Schooley *et al.* 1983) and this is most likely the causative factor in participant 6 who had Hepatitis B. An inverted CD4:CD8 ratio, of less than 1, has also been linked with ageing and 'immunosenescence', and carries an increased morbidity and mortality risk irrespective of age (Luz Correa *et al.* 2014). Excluding participant 6, four further retrospective participants had an inverted CD4:CD8 ratio. Participants 4, 8 and 18 were the three eldest participants in the cohort of retrospective transplant patients, so age may have been a factor which affected their T cell reconstitution. Participant 4 was sampled at just eight months post-transplantation, which was another factor that most likely influenced their value for the CD4:CD8 ratio. The remaining participant who exhibited an inverted CD4:CD8 ratio was just over the median age of the cohort. These observations suggest that reconstitution of the T cell population, to within normal ranges, is less efficient in older patients.

CHAPTER 4 T AND B CELL IMMUNOPHENOTYPING ANALYSIS OF PROSPECTIVE AND RETROSPECTIVE HLAi TRANSPLANT RECIPIENTS

4.1 Results of T cell immunophenotyping

Three T cell immunophenotyping antibody panels were used for the study; a 'recent thymic emigrants' (RTE) panel, an 'activated' T cell panel and a 'regulatory T cell' (Treg) panel. The results are presented for the prospective and retrospective study participants in figures 4.1-4.14. The target cell populations for each panel, and the gating strategy employed, are described in sections 2.7 and 2.8.

Incomplete data sets were collected for 3 prospective participants, due to sample and/or time constraints, or because there were insufficient lymphocytes present for immunophenotyping.

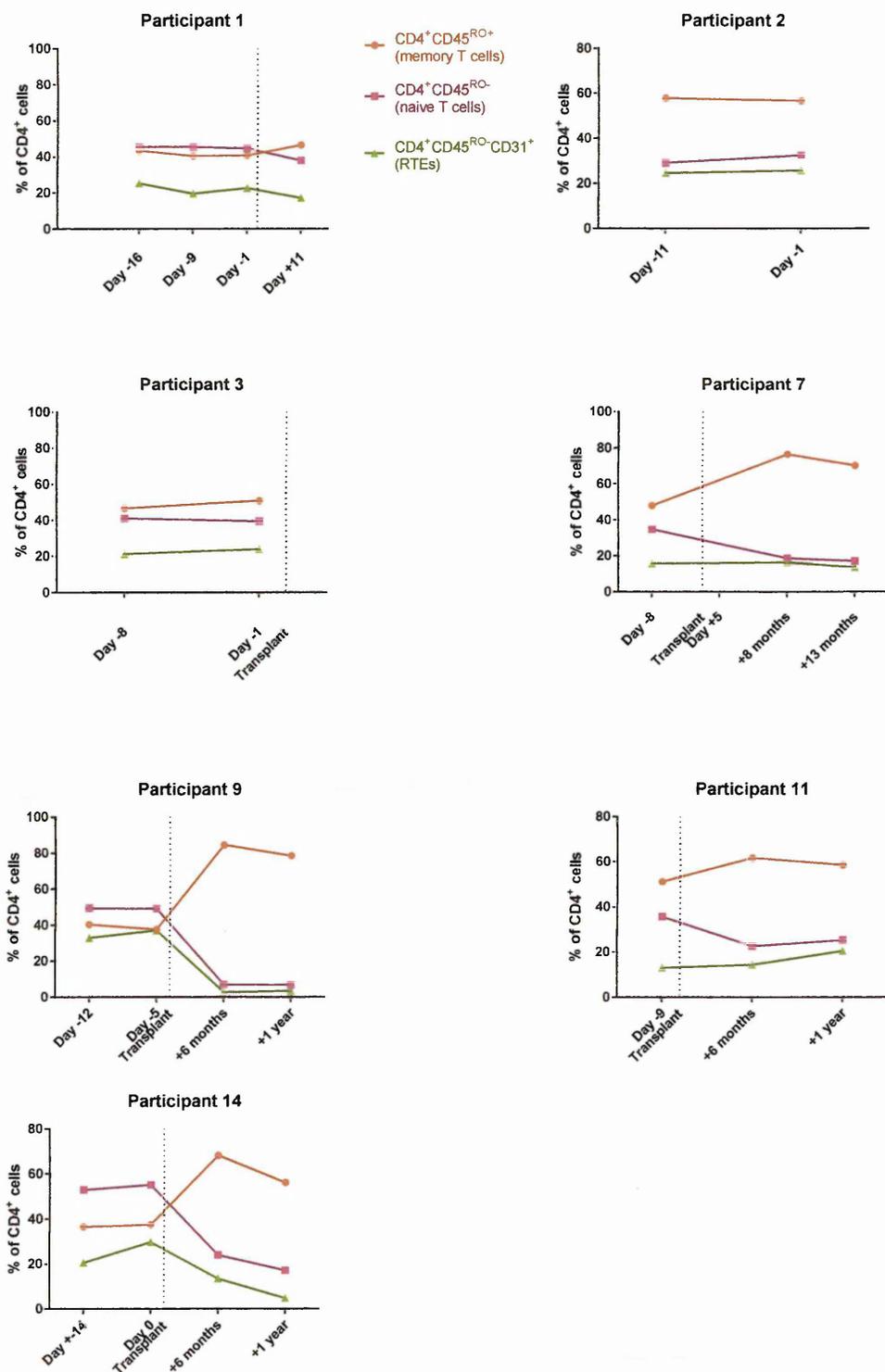
- Participant 1, Day -9 – no regulatory T cell data was collected.
- Participant 3, Day -8 and Day -1 samples, and participant 7, Day -8 – the intracellular FoxP3 marker was not performed for the Treg panel. Instead, CD127 phenotyping was performed for regulatory T cells (data not shown).
- Participant 3, Day +8, and participant 7, Day +5.

An incomplete data set was collected for one retrospective participant. For participant 6, the intracellular FoxP3 staining was not carried out due to this participant being Hepatitis B positive. Instead, data for Tregs was collected using the CD127 marker (data not shown), which, unlike intracellular FoxP3 staining, did not require isolation of PBMCs. The CD127 marker was carried out on whole blood, thereby necessitating less intervention with the sample.

4.1.1 Recent thymic emigrants (RTE) immunophenotyping

The results for the prospective participants from the RTE panel are shown in figure 4.1. The percentage of cells gated, for each population identified by this panel, is indicated for each participant longitudinally for their individual sampling time points.

Figure 4.1 Recent thymic emigrants (RTE) immunophenotyping results for the prospective participant cohort



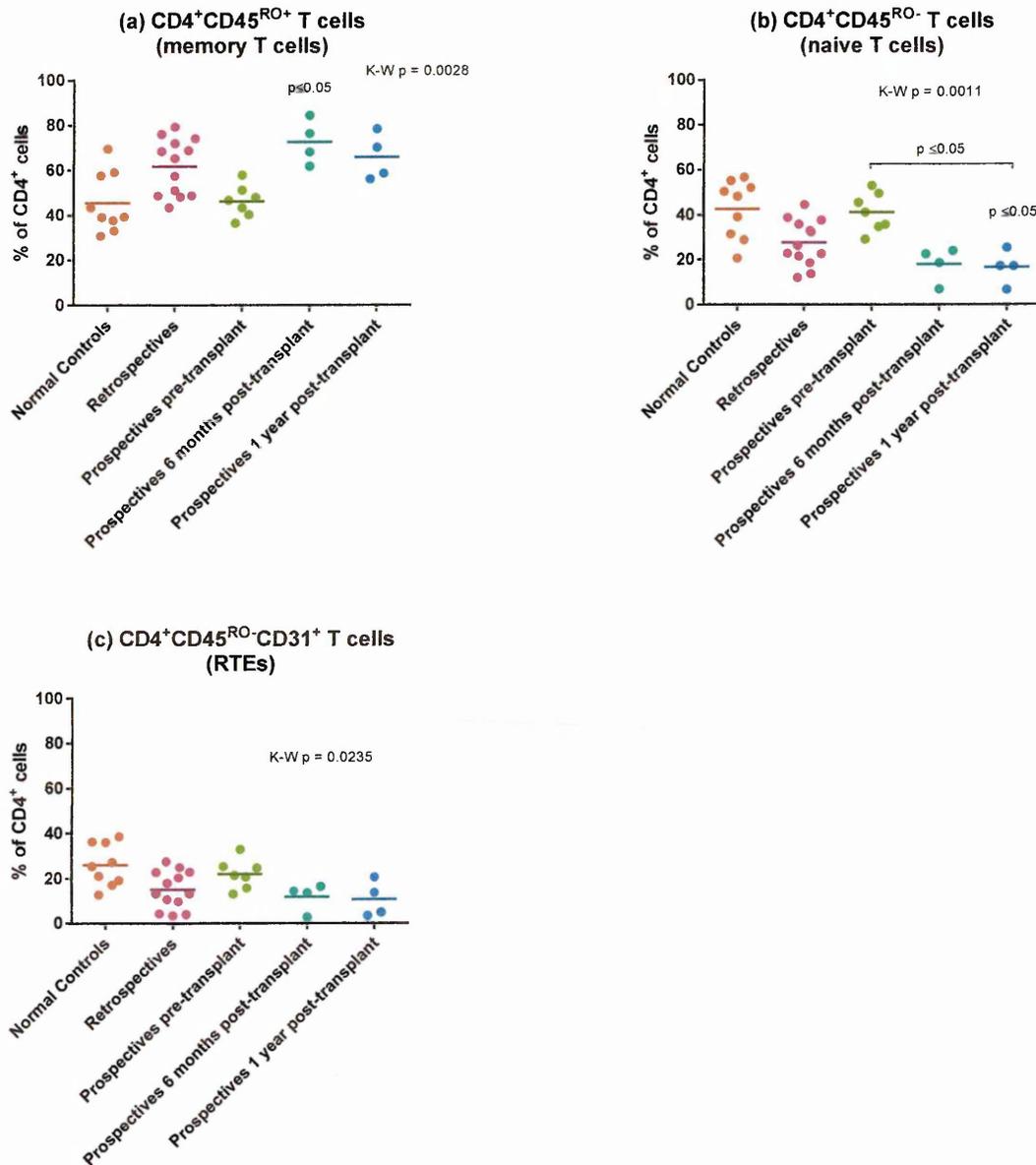
y axis– percentage of each cell population identified; x axis - sample time points as described in individual participant timelines, in section 3.2. A dotted line indicates Day 0 (day of transplant/scheduled transplant).

Figure 4.2 shows the results from the single time points for the retrospective cohort, plotted alongside the results for the normal controls, and the prospective cohort for comparison (from pre-transplant, six months and one year post-transplant time points).

Figure 4.1 shows that four of the seven prospective participants had a larger percentage of memory T cells ($CD4^+CD45^{RO+}$) compared to naïve T cells ($CD4^+CD45^{RO-}$) present at the pre-transplantation time points. Participants 1, 9 and 14 display the reverse, with larger populations of naïve T cells. There was little variation in population percentages seen in participants 1, 2 and 3, for whom there are no post-transplant analysis time points. The only exception to this was participant 1, who showed an increase in memory T cells between the sample taken on the day prior to the scheduled transplant (Day -1) (41%), and 11 days post the scheduled transplant (Day +11) (47%). It is plausible that this increase in activated memory T cells indicates an ongoing immune stimulus, which was unchecked by the desensitisation treatment. This corresponds with the increase in lymphocyte subsets, in this patient, discussed in section 3.2.1.

In contrast to participants 1, 2 and 3, who had no post-transplant follow-up, transplanted participants 7, 9, 11 and 14 showed dramatic changes in the T cell populations detected, as would be expected from the results of lymphocyte subset analysis, discussed in chapter 3. When considering figure 4.1, for the transplanted participants 7, 9, 11 and 14, it should be borne in mind that the post-transplantation time points represent a reconstitution of T cell populations from an absolute cell count of almost zero (see figures 3.8, 3.10, 3.12 and 3.14). However, due to there being insufficient cells to analyse in the immediate days following transplantation, this is not represented on the plots. This is the case for all similar graphs in this chapter.

Figure 4.2 Recent thymic emigrants (RTE) immunophenotyping results for the retrospective, normal control and prospective participant cohorts



y axis– the percentage of each cell population identified. K-W – Kruskal-Wallis test; significant p values are versus the normal control cohort, unless indicated otherwise; horizontal line indicates mean. Normal controls, n= 9; retrospective cohort, n= 13; prospective cohort pre-transplant, n= 7; prospective cohort 6 months post-transplant, n= 4; prospective cohort 1 year post-transplant, n= 4.

The graphs for participants 7, 9, 11 and 14 are similar in that there was a trend for an increase in memory T cells and a decrease in naïve T cells post-transplantation, when compared to the pre-transplantation time point(s). This suggests that the reconstitution of CD4⁺ T cells is predominantly from the memory compartment rather than with recent thymic emigrants. This observation is also evidenced by the decrease in RTE cells post transplantation in participants 9 (+6 months) and 14 (+6 months). It is also of note that, in all cases, there remained differences in the percentages of these T cell populations at one year post-transplantation, compared to pre-transplantation, suggesting that the effects of the HLAi transplant protocol are long-lived.

Whilst participant 11 fits with the overall trend described above, the increase in the memory T cell population, compared to pre-transplantation, was not as marked when compared with transplanted participants 7, 9 and 14, increasing from 51% (Day -9) to 62% (+6 months). For comparison, participant 9 had pre and post-transplantation values of 37% (Day -5) and 84% (+6 months) respectively. Also in contrast with the other aforementioned transplanted participants, figure 4.1 shows that participant 11 did not display such disparate cell percentages at the one year post-transplantation time point (compared to pre-transplantation). This participant is also the only one of the four to show an increase in RTEs at one year post-transplantation (from 13% pre transplantation (Day -9) to 20% at one year post-transplantation), suggesting thymic dependent re-population. This may be a reflection of the fact that although this participant received alemtuzumab, they did not have any pre-conditioning prior to transplantation.

The plot for participant 14 (figure 4.1) is of note for indicating an increase in RTEs between the pre-treatment sample (Day -14) and the day of transplantation (Day 0). This is in contrast to the other participants where there were sufficient samples to allow for this comparison. It has previously been shown that this participant demonstrated a slight increase in the absolute count and percentage of CD4⁺ cells between these time points (figure 3.14), so the patient may have experienced an increase in thymic activity due to the pre-conditioning protocol followed.

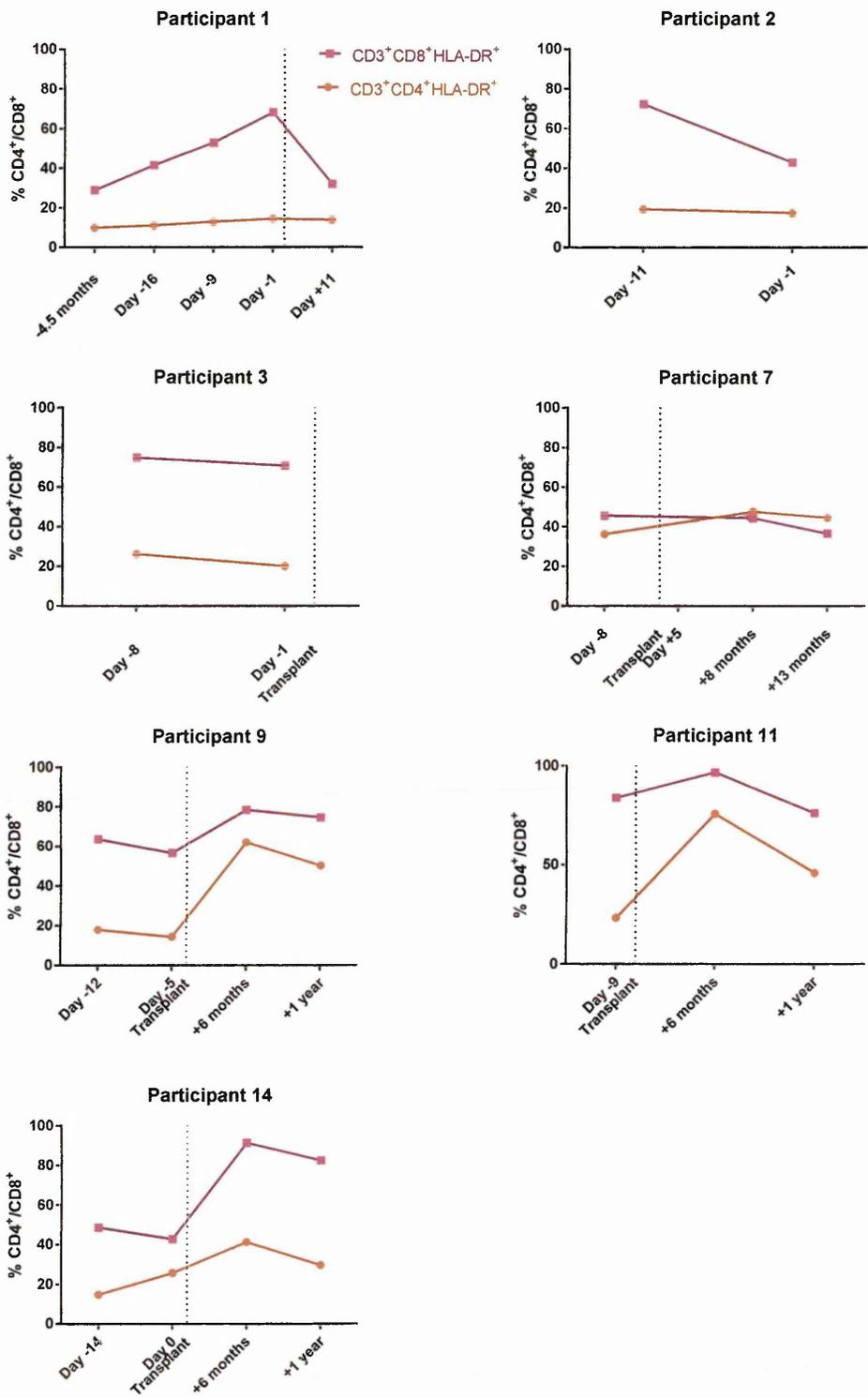
The trend for the population of memory T cells being increased, in percentage terms, in HLAi transplant recipients is repeated in figure 4.2, which shows the retrospective participant cohort alongside the normal controls and specific time points for the prospective participants. Figure 4.2 (a) displays a mean value of 46% for the prospective group pre-transplantation, indicating no difference with the normal controls (45%). In contrast to these figures, the mean for the retrospective cohort is 62%, whilst the prospective cohort has percentages for this population of cells of 73% ($p \leq 0.05$) and 66% at 6 months and 1 year post-transplantation respectively. As would be predicted, the converse of this picture is evident in figure 4.2 (b) which shows the population percentages for naïve T cells. These observations suggest that the prospective cohort would continue to slightly decrease their memory T cell populations, but only to within the range of the retrospective figures, which remained increased when compared with normal controls, again indicating a long-term effect of HLAi transplant conditioning and immunosuppression.

In contrast, figure 4.2 (c) indicates that the populations of RTEs remained reduced in percentage terms in the retrospective cohort, with a mean value of 15% compared to 26% for the normal controls. This provides further evidence that the reconstitution of the T cell compartment, post-transplantation, is largely thymic independent.

4.1.2 Activated T cell immunophenotyping

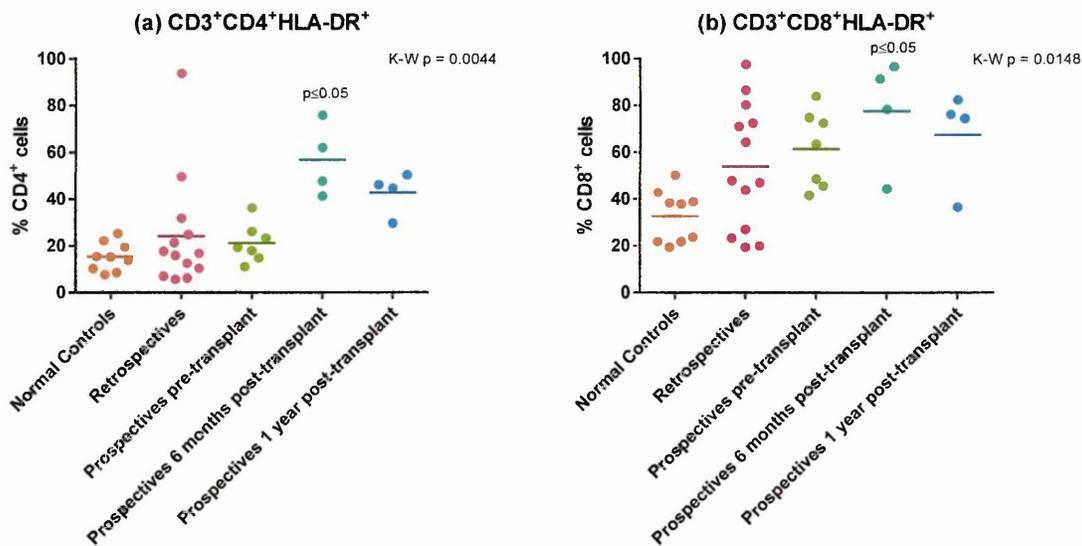
The expression of HLA-DR on CD4⁺ and CD8⁺ T cells is shown in figures 4.3 and 4.4, for the prospective and retrospective cohorts respectively. All participants possessed a greater percentage of CD3⁺CD8⁺HLA-DR⁺ cells than CD3⁺CD4⁺HLA-DR⁺ cells pre-transplantation (figure 4.3), and this relationship continued post-transplantation in all cases, except for participant 7 (discussed in further detail below). Participants 1 and 2, neither of whom were transplanted due to persistent DSAs at too high a level to proceed, were both interesting in terms of these cell populations. For participant 1, the percentage of CD3⁺CD4⁺HLA-DR⁺ cells did not alter over the HLAi conditioning protocol.

Figure 4.3 Activated T cell immunophenotyping results for the prospective participant cohort



y axis– percentage of each cell population identified; x axis - sample time points as described in individual participant timelines, in section 3.2. A dotted line indicates Day 0 (day of transplant/scheduled transplant).

Figure 4.4 Activated T cell immunophenotyping results for the retrospective, normal control and prospective participant cohorts



y axis– the percentage of each cell population identified. K-W – Kruskal-Wallis test; significant p values are versus the normal control cohort; horizontal line indicates mean. Normal controls, n= 9; retrospective cohort, n= 13; prospective cohort pre-transplant, n= 7; prospective cohort 6 months post-transplant, n= 4; prospective cohort 1 year post-transplant, n= 4.

However, the percentage of CD3⁺CD8⁺HLA-DR⁺ cells steadily increased over the course of the treatment, for participant 1, from 29% at initial assessment (-4.5 months), peaking at 68% on the day prior to transplantation (Day -1). The percentage of this cell population reduced by 11 days post-transplantation to a level similar to -4.5 months, at 32%. As a comparison, the mean figure for this population of cells from the normal control cohort was 33% (indicated in figure 4.4). This observation is in contrast to all other participants for whom multiple pre-transplantation samples were analysed, in that the percentage of CD3⁺CD8⁺HLA-DR⁺ cells decreased over the course of the desensitisation treatment. This is very clear in the case of participant 2, also not transplanted, who had a relatively high percentage of CD3⁺CD8⁺HLA-DR⁺ cells at pre-treatment (Day -11) of 72%, which declined to 43% by the day prior to the scheduled transplant (Day -1).

Of the transplanted participants with follow-up, the plots for 9, 11 and 14 (figure 4.3) show similarities, in that the six month post transplantation samples indicate an increase in HLA-DR positivity on both CD4⁺ and CD8⁺ T cells when compared to the pre-transplantation levels. These percentages were then seen to decrease at the one year post-transplantation time points. For these participants, the lines on the plot for the two populations of cells appear to parallel each other. Figure 4.4 also demonstrates this trend for the prospective cohort. However, in all three cases, HLA-DR expression on both populations of cells had not returned to pre-transplantation levels by one year post-transplantation.

Participant 7 differed to transplanted patients 9, 11 and 14, in that the increase in the population percentage of their CD3⁺CD4⁺HLA-DR⁺ cells exceeded the percentage of the CD3⁺CD8⁺HLA-DR⁺ population, at eight months post-transplantation, the only participant to show this pattern. This participant already possessed a relatively high proportion of CD4⁺HLA-DR⁺ T cells, prior to transplantation, as shown in figure 4.4(a), where the data point for participant 7 lies relatively high above the mean for the group (21%), at the pre-transplantation time point, at 36%. The percentage of CD8⁺HLA-DR⁺ T cells remained stable, for this participant, over this time period with a value of 45% pre-transplantation (Day -8) and 44% at 8 months post-transplantation. In common with the 3 other participants, both cell population percentages had reduced by 13 months post-transplantation. Figure 4.4(b) clearly shows participant 7 as an 'outlier' for the samples equivalent to six months (44%) and one year (37%) data points. For the CD3⁺CD8⁺HLA-DR⁺ cells, at 37%, this population was at a level below that obtained pre-transplantation. Participant 11 was the only other participant to have a lower percentage of CD3⁺CD8⁺HLA-DR⁺ cells present at 1 year post-transplantation, compared to pre-transplantation. However, as participant 7 was sampled at eight months and thirteen months post-transplantation, it is not possible to assess fluctuations that occurred at precisely six months and one year post-transplantation.

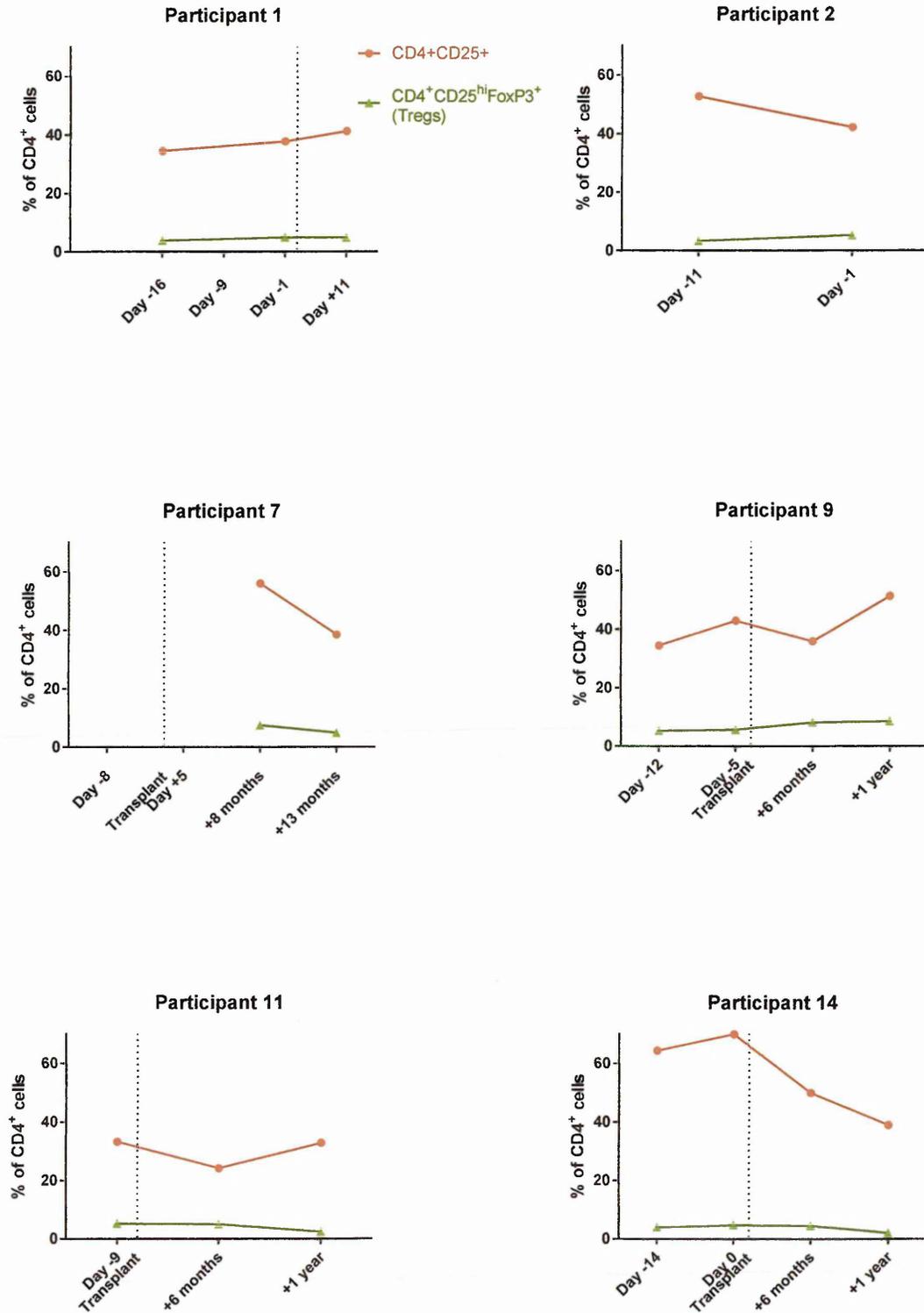
Figure 4.4 (a) compares five different cohorts of participants for their percentage of CD4⁺HLA-DR⁺ T cells and indicates that the retrospective cohort and the prospective cohort pre-transplantation do not differ to the normal controls for this

population. However, there are two clear exceptions to this within the retrospective cohort; participant 4 with 94%, and, to a lesser degree, participant 8 with 49% CD4⁺HLA-DR⁺ T cells. The mean value for this group was 24%. Participant 4 was sampled at only eight months post-transplantation, and the percentages obtained exceeded the values for the prospective cohort at a similar time point of six months. This participant also displayed a high percentage of CD3⁺CD8⁺HLA-DR⁺ cells present, indicated in figure 4.4 (b), of 97%, compared to a mean value for the group of 54%. It is possible that this could be an effect of alemtuzumab administered at the point of transplantation; however, two further participants (5 and 20) who were also given this drug, did not show this effect. The higher than the mean value for participant 8, in figure 4.4 (a), is also of interest, as this patient was sampled at eleven years post-transplantation, and was noteworthy for possessing no B cells during the lymphocyte subset analysis, most likely due to the continued use of prednisolone as a part of their immunosuppression. Figure 4.4 (b) shows the retrospective cohort appears to separate into two groups around the mean value of 54%. It is also of interest that, in contrast to the results shown in figure 4.4 (a), the prospective cohort, pre-transplantation, already have increased values for this population of cells compared with the normal controls (not significant).

4.1.3 Regulatory T cell (Treg) immunophenotyping

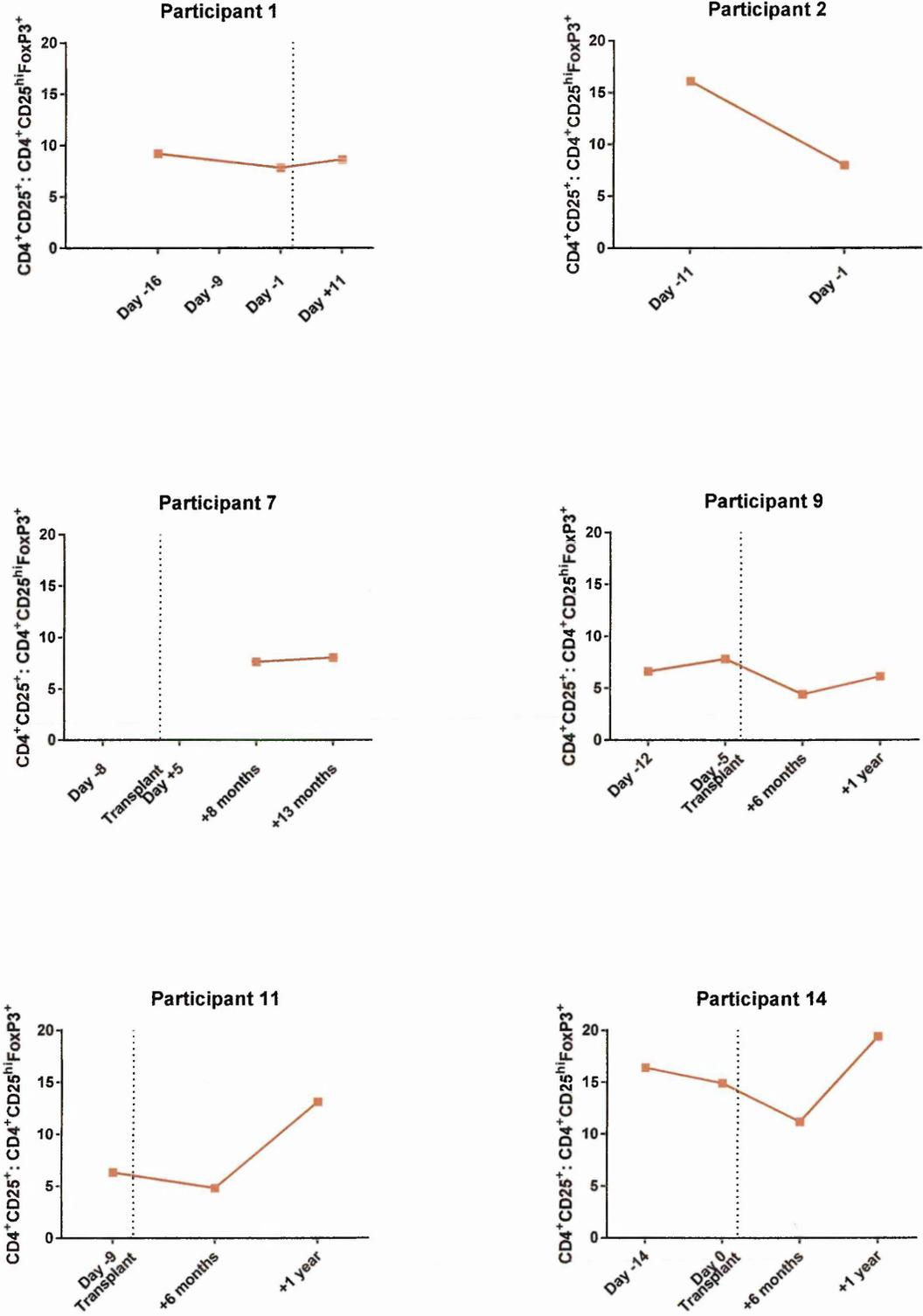
Data is presented for the prospective cohort for the populations of CD4⁺CD25⁺ (effector T cells) and CD4⁺CD25^{hi}FoxP3⁺ (Tregs), in figures 4.5 and 4.6, with the latter depicting a calculated ratio of CD4⁺CD25⁺ T cells:Tregs.

Figure 4.5 Regulatory T cell (Treg) immunophenotyping results for the prospective participant cohort



y axis– percentage of each cell population identified; x axis - sample time points as described in individual participant timelines, in section 3.2. A dotted line indicates Day 0 (day of transplant/scheduled transplant).

Figure 4.6 Ratio of CD4⁺CD25⁺ T cell: Treg cell populations for the prospective participant cohort



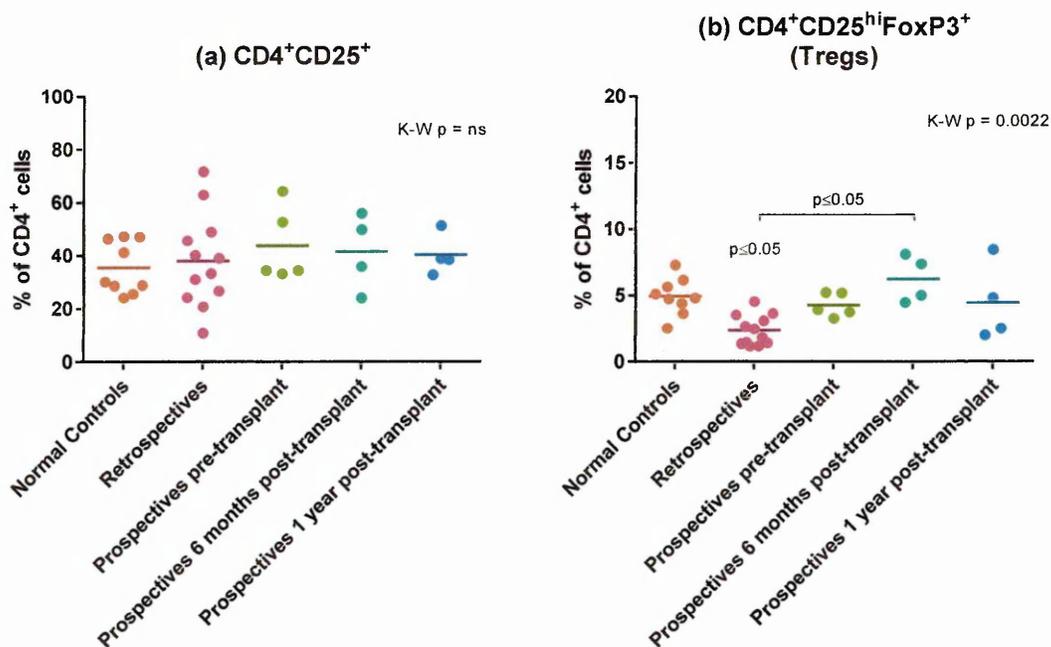
y axis— calculated ratio of CD4⁺CD25⁺:CD4⁺CD25^{hi}FoxP3⁺ T cells; x axis - sample time points as described in individual participant timelines, in section 3.2. A dotted line indicates Day 0 (day of transplant/scheduled transplant).

The collective Treg immunophenotyping results are shown in figure 4.7, displaying the single time points for the retrospective cohort, plotted alongside the results from the normal controls and the prospective cohort for comparison (from pre-transplant, six months and one year post-transplant time points). The analyses performed using the CD127 cell surface marker, in place of intracellular FoxP3 staining, did not provide any noteworthy data, and were difficult to compare with the Tregs immunophenotyping analyses carried out on the majority of the participant samples. Hence, these data are not presented in this report.

Prospective participants 7, 11 and 14 showed a reduction in the Treg cell population post-transplantation, between six months and one year time points (figure 4.5). In participants 7 and 14 there was a concurrent decrease in the CD4+CD25+ T cells; however this population of cells shows an increase in participant 11 (figure 4.5). For participant 11, this has resulted in an increase of the CD4+CD25+:Treg ratio (figure 4.6), between six months and one year post-transplantation. Participant 9 is the exception amongst the transplanted prospective participants, in that the percentage of Tregs present for this participant showed a sample by sample increase from pre-transplantation to one year post transplantation (figure 4.5). For this participant, the percentage of Tregs present at one year post-transplantation was therefore greater than pre-transplantation, at 13.8% compared to 6.7%. However, the CD4+CD25+:Treg ratio remained steady over this time, due to a concurrent increase in effector T cells. At one year post-transplantation, the two other transplanted participants for whom this comparison is possible (11 and 14) both had reduced percentages of Tregs present compared to pre-transplantation.

A further observation from these data is that an increase in percentages of Tregs was evident over the course of the HLAi conditioning in participants 1, 2, 9 and 14 (figure 4.5). For participant 2, this was reflected by a decrease in the CD4+CD25+:Treg ratio (figure 4.6). In participant 1, who was not transplanted this had started to reduce by 11 days post the scheduled transplant.

Figure 4.7 Regulatory T cell (Treg) immunophenotyping results for the retrospective, normal control and prospective participant cohorts



y axis – percentage of each cell population identified. K-W – Kruskal-Wallis test; significant p values are versus the normal control cohort, unless indicated otherwise; ns - not significant; horizontal line indicates mean. Normal controls, n= 9; retrospective cohort, n= 12; prospective cohort pre-transplant, n= 5; prospective cohort 6 months post-transplant, n= 4; prospective cohort 1 year post-transplant, n= 4.

Figure 4.7 shows that there is no difference between the different cohorts of participants for the population of CD4⁺CD25⁺ cells (4.7 (a)). The retrospective cohort had reduced percentages of Tregs compared to the normal controls ($p < 0.05$), with a mean of 5.4% and 8.6% respectively (figure 4.7 (b)). Within this cohort, participants 10, 12, 13 and 18 showed particularly low populations of Tregs.

4.2 Results of B cell immunophenotyping

The results from the three B cell immunophenotyping panels used for the study, 'memory' B cell, 'transitional' B cell and 'plasma' B cell panels, are shown in graphical format for both the prospective and retrospective participants. The target cell populations and the gating strategy employed for each panel are described in section 2.7.

Incomplete data was obtained on prospective participants 3, Day +8, and 7, Day +5, as it was identified by the Multitest™ kit that there were insufficient cells present for full immunophenotyping panels to be performed. Also, for retrospective participants 4, 8 and 19 an insufficient absolute count of B cells was identified by the Multitest™ kit for the B cell immunophenotyping panels to be performed, so T cell data only was collected for these samples.

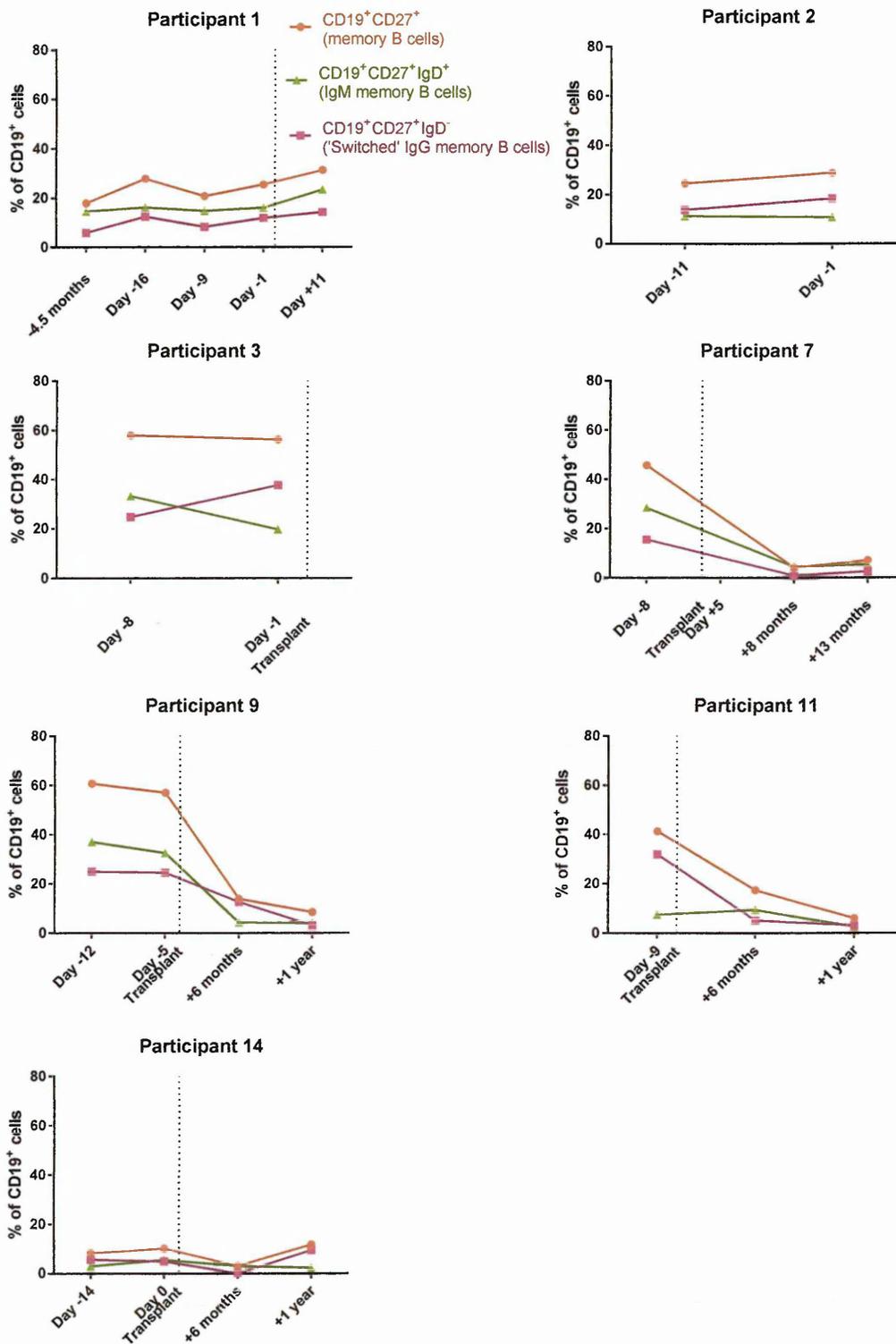
4.2.1 Memory B cell immunophenotyping

The results for the prospective participants from the memory B cell immunophenotyping panel are shown in figure 4.8. Figure 4.9 shows the results from the single time points for the retrospective cohort, plotted alongside the results from the normal controls, and the prospective cohort for comparison (from pre-transplant, six months and one year post-transplant time points).

Participants 1 and 2, who did not progress to transplantation, both showed an increase in memory B cells over the course of their conditioning treatment. Participant 1 had a greater percentage of IgM memory B cells, whilst for participant 2 it was the 'switched' IgG memory B cells which predominated. In addition, participant 1 showed a sharp increase in CD27⁺ memory B cells at the 11 day post scheduled transplant time point.

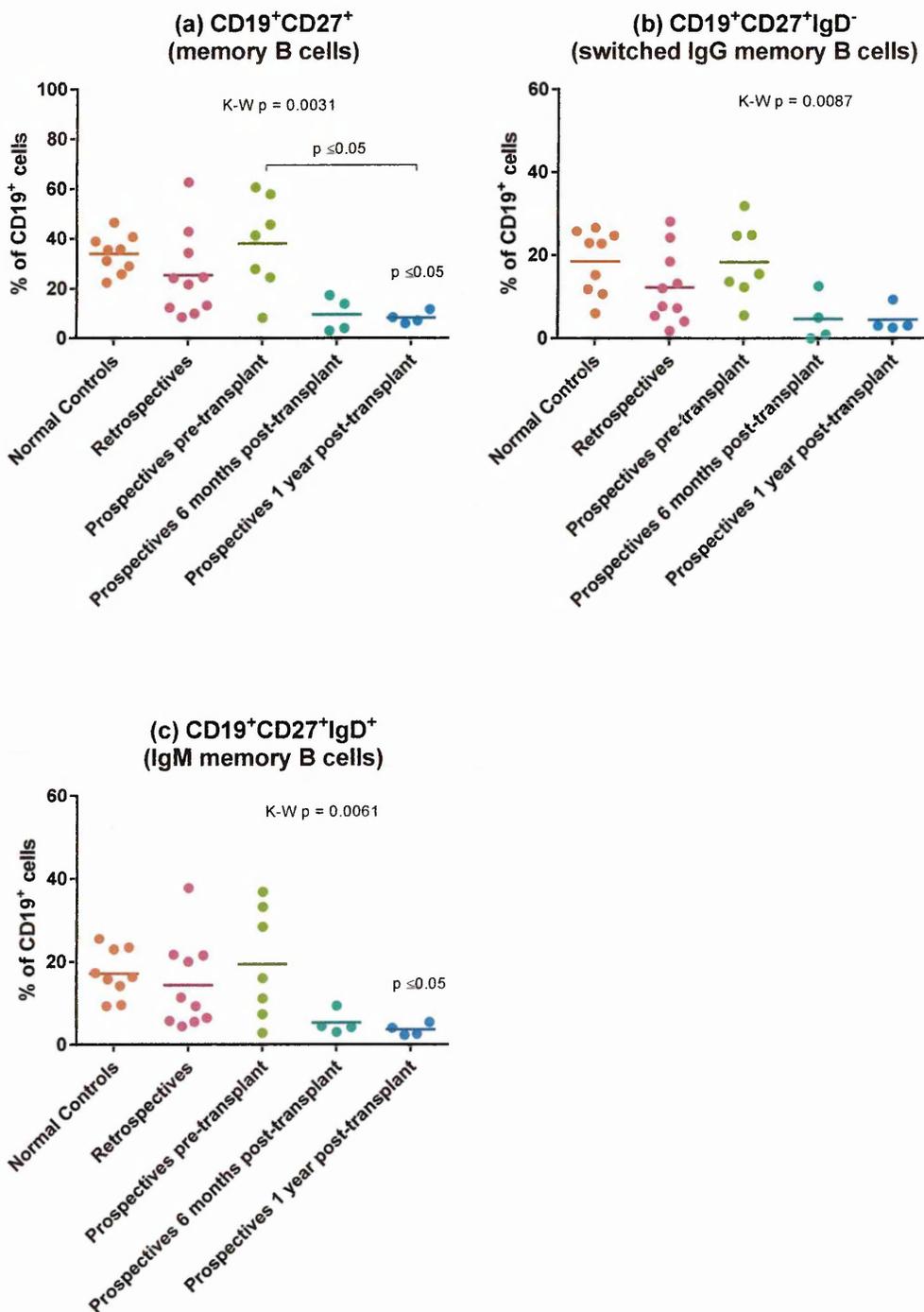
The only other participant who exhibited an increase in memory B cells prior to transplantation was participant 14 (although this comparative time point was only analysed for participants 3, 9 and 14). Participant 3 had a comparatively high percentage of memory B cells present, but the overall percentage of CD27⁺ cells did not alter over the course of the conditioning treatment, although the predominant cells appeared to change from IgM memory to 'switched' IgG memory cells.

Figure 4.8 Memory B cell immunophenotyping results for the prospective participant cohort



y axis– percentage of each cell population identified; x axis - sample time points as described in individual participant timelines, in section 3.2. A dotted line indicates Day 0 (day of transplant/scheduled transplant).

Figure 4.9 Memory B cell immunophenotyping results for the retrospective, normal control and prospective participant cohorts



y axis – percentage of each cell population identified. K-W – Kruskal-Wallis test; significant p values are versus the normal control cohort, unless indicated otherwise; horizontal line indicates mean. Normal controls, n= 9; retrospective cohort, n= 10; prospective cohort pre-transplant, n= 7; prospective cohort six months post-transplant, n= 4; prospective cohort one year post-transplant, n= 4.

Of the transplanted participants, 7, 9 and 11 showed a similar pattern of a dramatic decrease in the percentage of memory B cells observed at six months post-transplantation, compared to pre-transplantation. For participant 7, these levels had not recovered by thirteen months post-transplantation, increasing from 4% at eight months post-transplantation to 7% for CD27⁺ B cells, compared to a pre-transplantation level of 46%. A similar picture was evident for participant 9, whose population of CD27⁺ B cells measured 57% pre-transplantation, and 8% at one year post-transplantation. Interestingly, this participant showed a further decrease in memory B cells between the six months and one year time points, indicating that this population of cells had not yet started to return. This was also the case for participant 11, whose populations of memory B cells also displayed further decreases between the six months and one year time points. Figure 4.9 also clearly shows the reduction in the mean percentage of memory B cells for the prospective cohort between the pre- and the post-transplantation samples ($p \leq 0.05$). This overall picture suggests that the population of returning B cells post-transplantation is not derived from memory cells, unlike the observation previously described for T cells in section 4.1.1.

Participant 14 had a different pattern of memory B cell populations, in that the percentage of CD27⁺ cells present was comparatively low, at 8% pre HLAi transplantation conditioning treatment (Day -14). At six months post-transplantation, the memory population of B cells remained low, but by one year showed an increase to a level which exceeded pre-treatment levels at 12%. This was largely due to an increase in isotype class 'switched' B cells. This participant was the only one of the four transplanted patients whose levels of memory B cells at one year exceeded their pre-treatment level. However, the overall low levels observed in this participant, could be a reflection of suppressed B cell counts (noted in chapter 3), possibly post treatment with methyl-prednisolone.

Figure 4.9 shows the collective data for the different study cohorts for the memory B cell panel. It is striking that there was more variation in the percentage of memory B cells present in the transplant patient cohorts compared to the normal controls, particularly for total CD27⁺ B cells (figure 4.9 (a)). However, whilst the mean value of the prospective cohort pre-transplantation did not differ to the normal controls (38% compared with 34% for CD27⁺ B cells), the retrospective

cohort showed a trend towards a lower mean value of memory B cells across all populations (not significant), with a mean of 25% for CD27⁺ B cells (figure 4.9 (a)). These observations suggest that the elimination of memory B cells by the conditioning regime and post-transplantation immunosuppression is a long-term phenomenon. No correlation was observed between memory B cell population and the time elapsed since transplantation, indicating that the outlying participant results with particularly low percentages present, did not represent those participants who were most recently transplanted (data not shown).

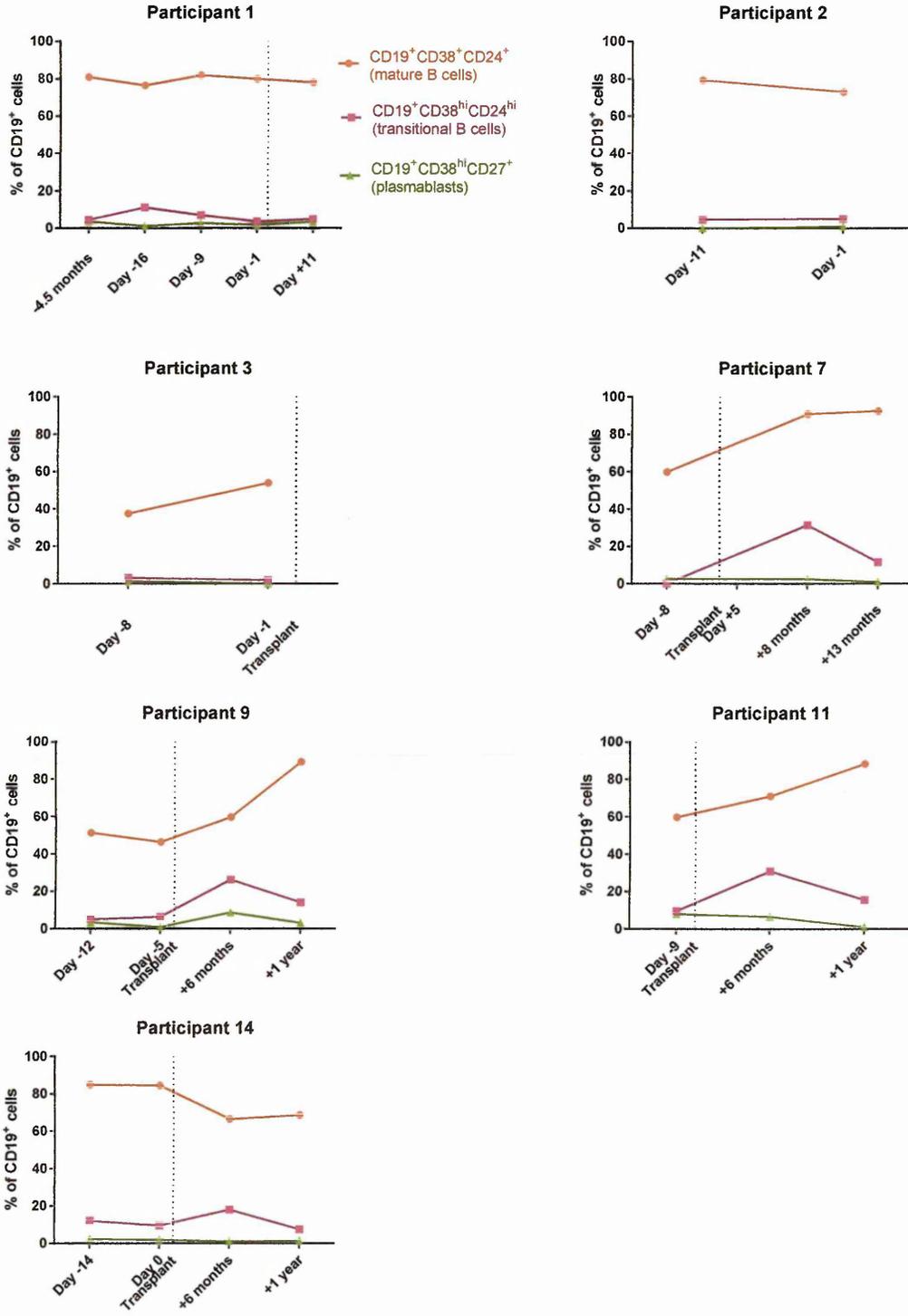
Within the retrospective cohort, participant 17 was of note for being an exception to the pattern described above, having the highest percentage of memory B cells, with a population of 63% CD27⁺ cells. The matched normal control for participant 17 had a value of 36% for this population of cells, whilst the mean for the normal controls was 34%. In particular, this participant had an expanded population of IgM memory (IgD⁺) cells present at 38%. Participant 17 was 8 years post-transplantation and experiencing chronic graft rejection, which may have been a factor influencing this phenomenon.

4.2.2 Transitional B cell immunophenotyping

The results for the prospective participants from the transitional B cell panel are shown in figure 4.10, whilst figure 4.11 shows the same data, but without the mature B cell population (CD19⁺CD38⁺CD24⁺), so that variations in the transitional B cell (CD19⁺CD38^{hi}CD24^{hi}) and plasmablast (CD19⁺CD38^{hi}CD27⁺) populations can be seen more clearly. The results from the collective cohorts are displayed in figure 4.12.

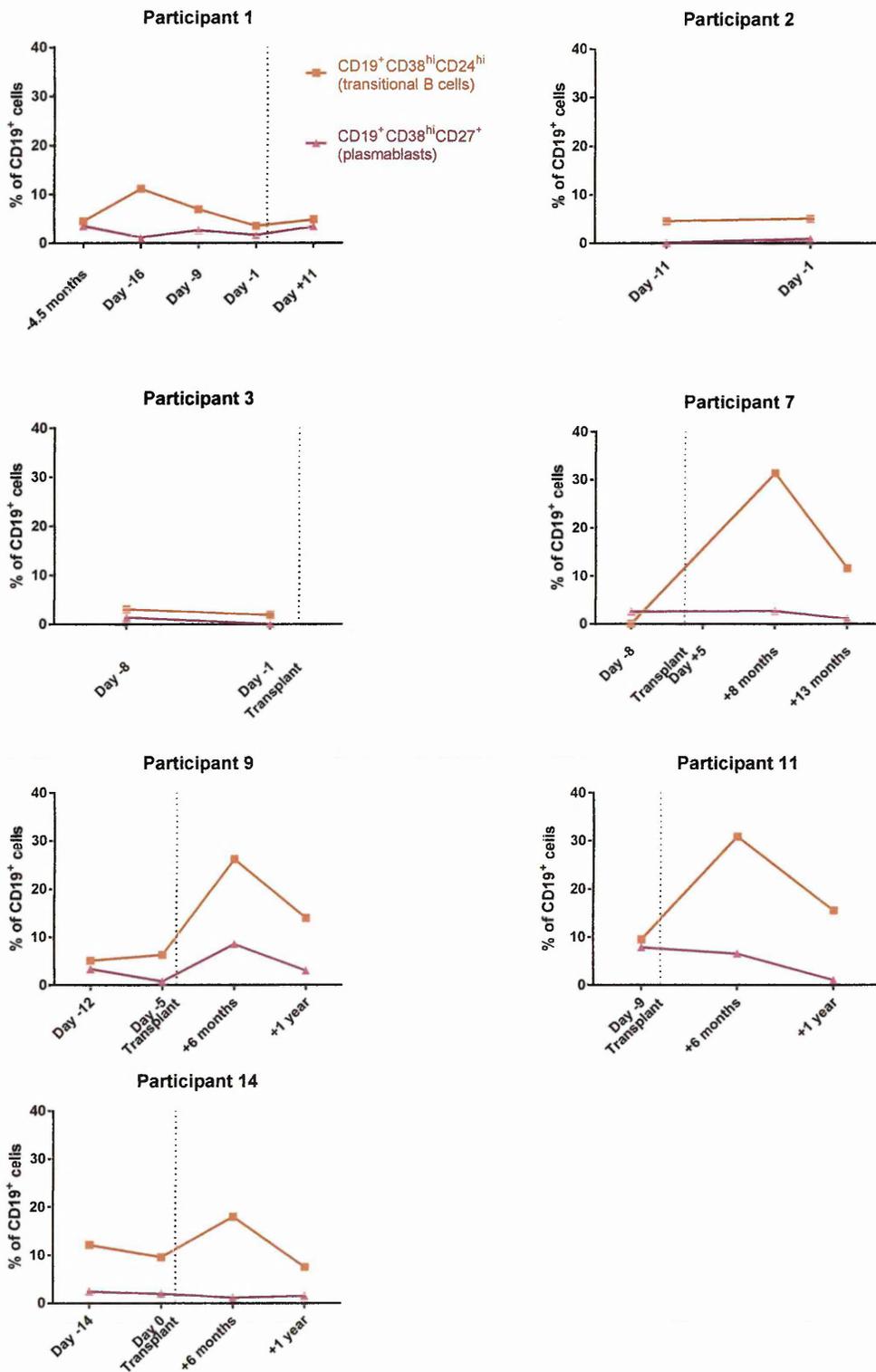
Little change in transitional B cell populations for participants 1 and 2, who did not progress to transplantation, was seen (figure 4.10). Participant 3 exhibited approximately half of the percentage of mature B cell population, as participants 1 and 2. This participant also differed by showing an increase in this cell population during the conditioning treatment (figure 4.10). Unfortunately, as the participant was lost to follow up, it was not possible to track further changes.

Figure 4.10 Transitional B cell immunophenotyping results for the prospective participant cohort



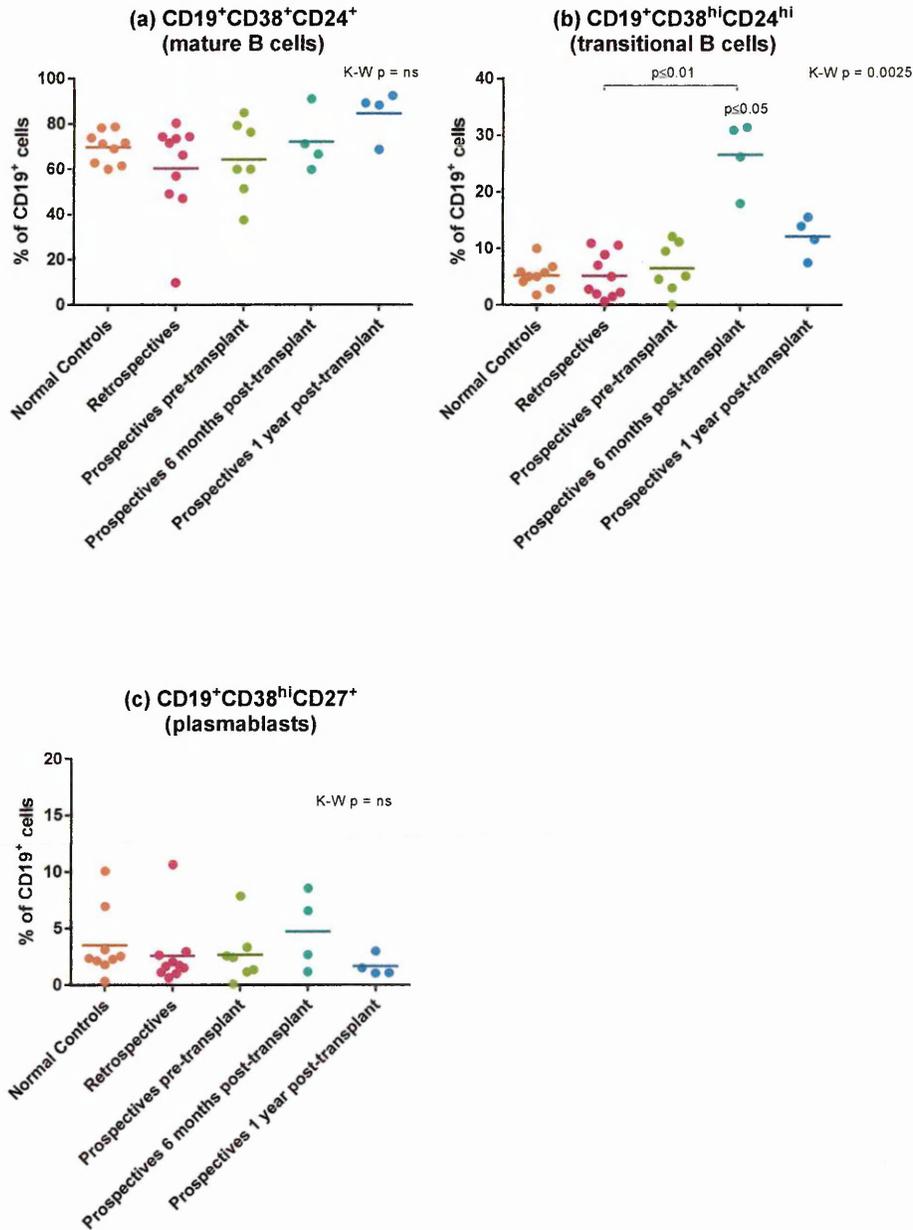
y axis– percentage of each cell population identified; x axis - sample time points as described in individual participant timelines, in section 3.2. A dotted line indicates Day 0 (day of transplant/scheduled transplant).

Figure 4.11 Results of analysis of CD38^{hi} cell populations from the transitional B cell panel, for the prospective participant cohort



y axis– percentage of each cell population identified; x axis - sample time points as described in individual participant timelines, in section 3.2. A dotted line indicates Day 0 (day of transplant/scheduled transplant).

Figure 4.12 Transitional B cell immunophenotyping results for the retrospective, normal control and prospective participant cohorts



y axis – percentage of each cell population identified. K-W – Kruskal-Wallis test; significant p values are versus the normal control cohort, unless indicated otherwise; ns - not significant; horizontal line indicates mean. Normal controls, n= 9; retrospective cohort, n= 10; prospective cohort pre-transplant, n= 7; prospective cohort six months post-transplant, n= 4; prospective cohort one year post-transplant, n= 4.

The results from the remainder of the transplanted participants suggest that both mature and transitional B cell populations reconstituted efficiently post-transplantation. In contrast to the memory B cell results, all four of the transplanted patients with follow-up (participants 7, 9, 11 and 14) showed a recovery of mature B cells by six months post-transplantation. For participants 7, 9 and 11, this population represented a greater percentage than in the pre-transplantation sample, and continued to increase up to one year post-transplantation. This observation can also be seen in figure 4.12 (a). Participant 14 differed in their response in that at six months post-transplantation, the mature B cell population had reconstituted to a lower percentage than the pre-transplantation sample (66% compared to 85%). This increased slightly to 69% at one year post-transplantation.

Mirroring the observation described above, and more clearly seen in figures 4.11 and 4.12 (b), participants 7, 11 and 14 also showed an increase in transitional B cell populations at six months ($p \leq 0.05$), compared to pre-transplantation, although this had decreased by one year post-transplantation. A similar pattern was observed for participant 9; however here the increase in population size at six months was also evident for plasmablasts.

No difference was seen between percentages of cell populations studied using the transitional B cell panel for the normal controls, the retrospective cohort and the prospective cohort pre-transplantation (figure 4.12). Regarding the prospective cohort pre-transplantation, this would suggest that chronic renal failure and its treatment does not affect these cell populations. The fact that the mean values for the retrospective cohort are not dissimilar to the normal controls implies that either these cell populations did not increase post-transplantation, as observed in the prospective cohort, or that they had declined back to pre-transplantation 'normal' levels.

Retrospective participant 13 is a clear outlier in figure 4.12 (a). This patient was transplanted 5 years ago, and was striking for possessing a mature B cell population of just 10%, compared to 79% for their matched normal control, and a mean value of 60% for the retrospective cohort.

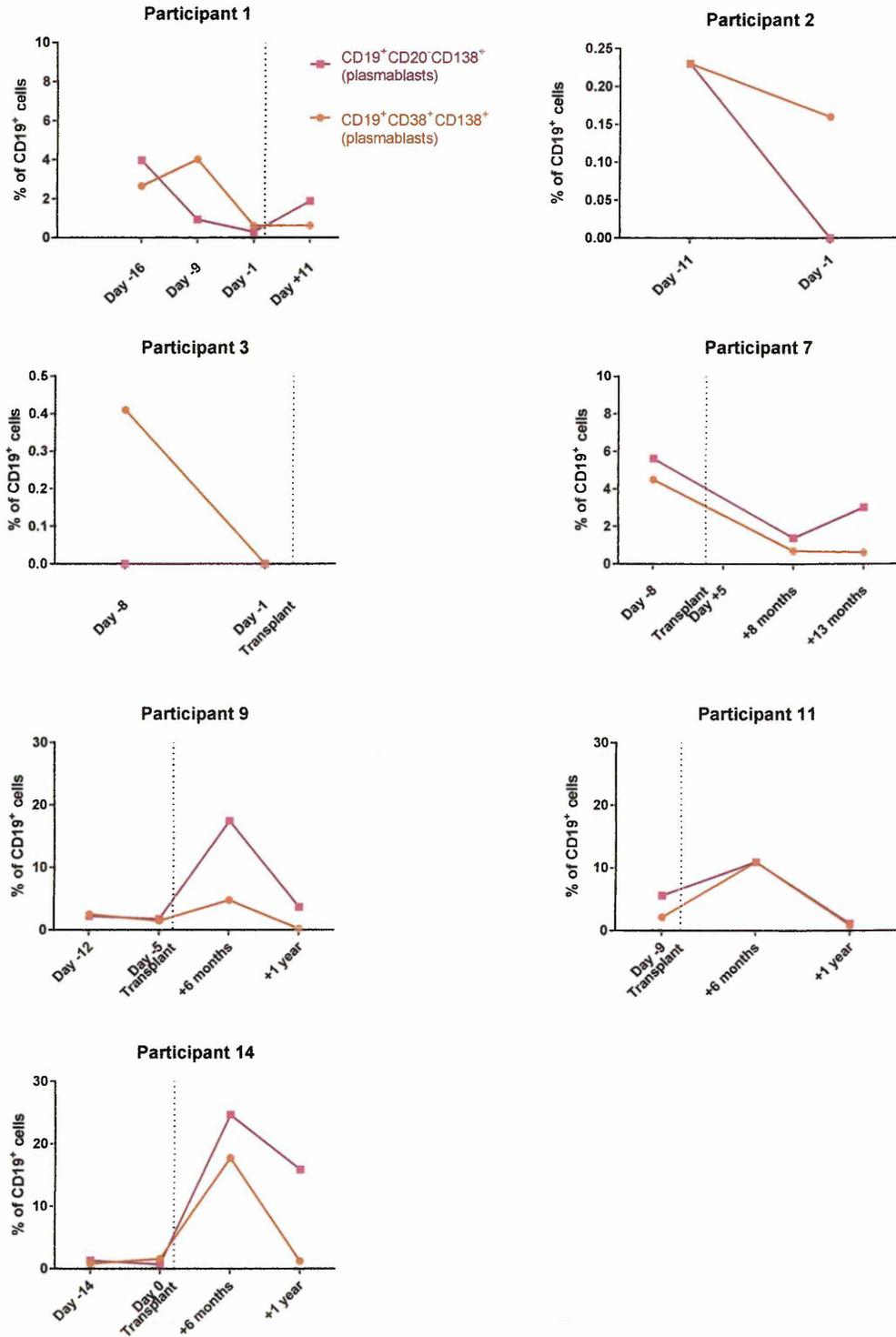
Several participants noteworthy for their plasmablast population percentages can be seen in figure 4.12 (c). The overall picture for this population of cells in the normal controls, the retrospective cohort and the prospective cohort pre-transplantation was of little variation around the mean. However, there are exceptions to this, particularly retrospective participant 5 who, with a value of 11% differed markedly to the rest of the cohort which has a mean of 3%. Prospective participant 11 also had a high value for this population of cells, pre-transplantation, at 8%. Two normal controls also differed to the trend for the rest of this group (mean of 4%); controls 1 (10%) and 9 (7%). It is possible that these individuals were undergoing some sort of immune stimulus, although overall the percentages are low.

4.2.3 Plasma B cell immunophenotyping

The results for the prospective participants from the plasma B cell panel are shown in figure 4.13. The percentages of cell populations recorded for this panel were small, but demonstrated some large changes for some participants. Hence, in figure 4.13 the scale on the y-axis is not consistent between participants, in order that the individual changes in each participant can be more easily visualised. The collective cohort results are shown in figure 4.14.

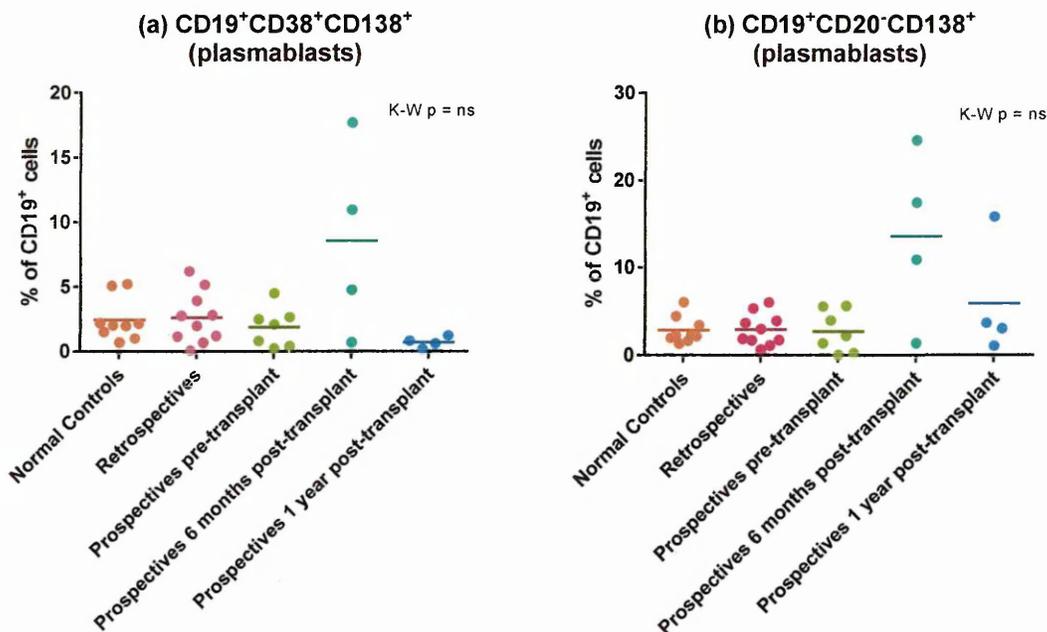
In figure 4.13 it is apparent that the plasmablast cells (CD19⁺CD20⁻CD138⁺) underwent an overall decrease during the course of pre-treatment, in both participants who did not progress to transplantation (1 and 2). For participant 1, this cell population then appeared to rebound, at 11 days post-scheduled transplant date, when the treatment was halted and the patient was not transplanted.

Figure 4.13 Plasma B cell immunophenotyping results for the prospective participant cohort



y axis– percentage of each cell population identified; x axis - sample time points as described in individual participant timelines, in section 3.2. A dotted line indicates Day 0 (day of transplant/scheduled transplant).

Figure 4.14 Results of immunophenotyping analysis from the plasma B cell panel for the retrospective, normal control and prospective participant cohorts



y axis – percentage of each cell population identified. K-W – Kruskal-Wallis test; ns - not significant; horizontal line indicates mean. Normal controls, n= 9; retrospective cohort, n= 10; prospective cohort pre-transplant, n= 7; prospective cohort 6 months post-transplant, n= 4; prospective cohort 1 year post-transplant, n= 4.

Of the transplanted participants, 9, 11 and 14 showed a similar pattern for both plasmablast populations. All participants started with relatively low levels of these cell populations, and then all experienced a ‘spike’ in the 6 month post-transplantation sample. By one year, the percentage of these cell populations had declined, although only participants 9 and 11 showed values which had returned to close to the pre-transplantation values. These observations are also evident in figures 4.14 (a) and (b) (not significant). Participant 7 differed, as these populations of cells did not appear to repopulate to the same level at eight months post-transplantation, compared to pre-transplantation, although the values for this participant were small. In addition, the plasmablasts (CD19⁺CD20⁻CD138⁺) for this participant showed a slight increase between eight and thirteen months (figure 4.13).

No difference was seen, in the plasma cell populations studied, between the normal controls, the retrospective cohort and the prospective cohort pre-transplantation (figures 4.14 (a) and (b)). This observation suggests that the process described above for the prospective participants is short-lived, either caused by the conditioning or the transplant itself. However, several retrospective participants were of note; in figure 4.14 (a) participant 13 differs by being practically CD38⁻. Several participants (5, 10, 16 and 18) appeared to have populations of plasmablasts present (figure 4.14 (b)).

4.3 Further stratified analysis of the results of immunophenotyping, in the retrospective participant cohort

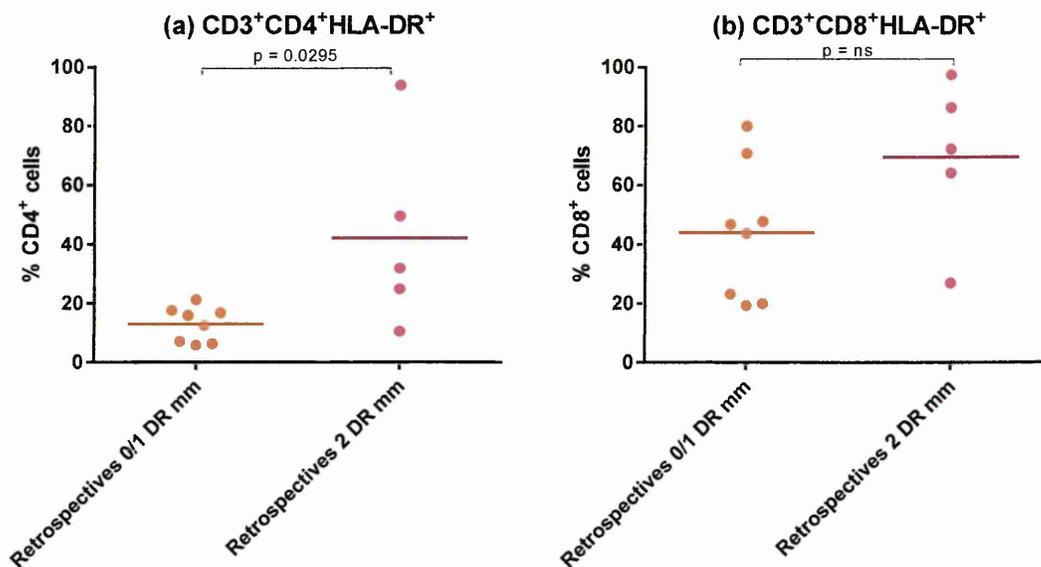
4.3.1 HLA-DR mismatching analysis

As described in section 3.5.1, the retrospective participant cohort was stratified according to the recipient-donor HLA-DR mismatch grade. The results from this analysis for the activated T cell panel (0/1 HLA-DR mismatches n = 8, 2 HLA-DR mismatches n = 5) are shown in figure 4.15. The 2 HLA-DR mismatched group had a higher percentage of HLA-DR⁺ T cells recorded; this was particularly striking in the CD3⁺CD4⁺HLA-DR⁺ cell population (p = 0.0295). The remainder of the immunophenotyping panels analysed in this manner, stratified according to HLA-DR mismatch, showed no differences between the groups (data not shown).

4.3.2 The effect of alemtuzumab on immunophenotyping results

The results from the immunophenotyping analysis were re-examined in light of which participants had received alemtuzumab, in place of basiliximab, as part of their transplantation schedule. Three of the retrospective participants (4, 5, and 20) received alemtuzumab, but no correlation was observed between this and the immunophenotyping data collected (data not shown).

Figure 4.15 Results from the retrospective cohort, for the activated T cell immunophenotyping panel, stratified according to HLA-DR mismatches



y axis – percentage of cell population identified; ns - not significant according to Mann Whitney test; horizontal line indicates mean. Retrospective cohort, 0/1 HLA-DR mismatches, n= 8; retrospective cohort, 2 HLA-DR mismatches, n = 5.

4.3.3 Reanalysis of the data, to examine a possible association between the immunophenotyping results and a post-transplantation rejection episode

As indicated in table 3.5, five retrospective participants (6, 13, 15, 17 and 18) experienced a rejection episode post-transplantation. No association between this and the immunophenotyping data was observed (data not shown).

4.4 Discussion of T and B cell immunophenotyping results

4.4.1 Lymphocyte recovery post-HLAI transplantation

The immunophenotyping results from the transplanted prospective participants highlighted similarities in how the different populations of lymphocytes reconstituted post-transplantation. CD4⁺ T cell reconstitution largely took place from within the memory T cell (CD4⁺CD45^{RO+}) compartment. In contrast, RTEs (CD4⁺CD45^{RO-}CD31⁺) accounted for little of the post-transplant reconstitution, evidenced by this population of cells either remaining at a relatively steady percentage, or showing a decrease by one year post-transplantation, compared to pre-transplantation levels. A different phenomenon was observed for B cells, with memory B cell populations remaining notably reduced at one year post-transplantation. This was associated with an efficient reconstitution of the CD19⁺CD38^{hi}CD24^{hi} transitional B cell population, which peaked at 6 months post-transplantation. These cells reflect repopulation of the B cell compartment from immature B cells.

Despite differences in the induction (peri-operative) immunosuppression received (alemtuzumab in the prospective cohort and mostly basiliximab in the retrospective cohort), a similar pattern of changes in the lymphocyte populations was observed in the retrospective cohort of patients. In the retrospective cohort, increased memory T cell populations were noted when compared to the normal control cohort. This phenomenon is not explained by examining the time since transplantation, i.e. the participants who were more recently transplanted do not correlate with the higher data points for memory T cells (data not shown). Alongside this, there was a trend for the population of RTEs to remain reduced compared to the normal controls. Neither of these observations were noted in the cohort of prospective participants pre-transplantation, indicating that this effect was due to an aspect of the transplantation process, and not caused by renal failure and/or its treatment.

In terms of B cell populations, a lower mean value of memory B cells in the retrospective cohort was seen compared to the normal controls indicating that the elimination of peripheral memory B cells was long-lived. However, the results for

the transitional B cells do not differ greatly for the retrospective cohort compared to the normal controls. This suggests that either the percentage of this B cell population has declined back to within 'normal' levels in the retrospective group, or, more likely, that the use of basiliximab in the majority (ten of thirteen) of this participant cohort has resulted in a different pattern of B cell populations observed post-transplantation, compared with the alemtuzumab treated prospective participants.

The observations around lymphocyte recovery post-transplantation, noted above, should be viewed in terms of knowledge of the process of haematopoiesis. However, the additional consideration of the mode of suppression (alemtuzumab or basiliximab) of the immune response, must also be taken into account. In relation to the former, thymic involution, which initiates during infancy, results in a reduction of naïve T cells exiting into the periphery via this route over time. Hence, the expression of the CD45^{RO} marker is affected by an individual's age, with the CD45^{RO+} variant being prevalent in adults, whilst the CD45^{RO-} population (CD45^{RA+}) decreases with age (Lazuardi *et al.* 2005). This is 'compensated' for by the fact that mature peripheral T cells are more long-lived than mature B cells, and are self-renewing (Parham 2009). In adults, then, it is unlikely that recovery of T cell populations would be as a result of increased thymic activity. Instead, the T cell populations observed post-transplantation correspond with an expansion of mature memory T cells in the secondary lymphoid tissues. It has been suggested that in adults, naïve T cells may also expand in the periphery (Kohler and Thiel 2009), but these cells would most likely have been eliminated during the pre-transplant induction process.

Conversely, the mature B cell repertoire consists of short-lived cells, which are continually renewed by the bone marrow (Parham 2009). Thus, the space created in the periphery by the use of the induction drugs could promote replenishment of the B cell populations from this route. Simultaneously, the peripheral memory B cell population would take longer to recover, although these cells could survive in the secondary lymphoid organs.

It has been suggested that due to its lympho-depleting properties, alemtuzumab may promote a state of transplant tolerance (Cherukuri *et al.* 2012). In a study previously carried out at this centre (Cherukuri *et al.* 2012), patients undergoing 'standard' renal transplantation and enrolled in a randomised control trial (RCT), were investigated for various T and B cell phenotypes approximately two years post transplantation. Alemtuzumab treated patients exhibited increased populations of naïve, transitional and regulatory B cells, whilst there was a shift towards a memory phenotype in the T cell subsets. The observations detailed in the current study agree with those described by Cherukuri *et al.* (2012), in alemtuzumab treated patients. The depletion of the peripheral memory B cell compartment, and a shift towards a more naïve B cell phenotype can be deemed to be a benefit of this mode of immunosuppression, potentially limiting the initiation of the secondary immune response.

It is noteworthy that one of the participants identified as having a peripheral population of plasmablasts present, participant 16, received the therapeutic monoclonal antibody rituximab as part of their transplant conditioning. As previously mentioned, rituximab targets the CD20 cell marker present on the majority of B cells, but absent on plasma cells. Participant 16 was tested at eight years post transplantation, which had unfortunately failed at four months due to recurrence of original disease. It was interesting to note that 89% of this participant's B cells were now expressing the CD20 marker. A further retrospective participant (19) also received rituximab pre-transplantation, but at four years post-transplantation this individual possessed insufficient B cells to phenotype, most likely due to the continued use of prednisolone.

Kamburova *et al.* (2014) reported the long-term depletion of peripheral B cells by rituximab remaining evident in low counts noted at 24 months post-treatment, suggesting that both of the aforementioned retrospective participants had most likely recovered from the rituximab effect by the time of testing. In addition, it has been reported (Anolik *et al.* 2007, Kamburova *et al.* 2014) that B cell depletion observed in rituximab treated patients, corresponds with an increased transitional B cell phenotype in the repopulating cells. The results for participant 16 concur with this observation, as they exhibited the joint highest value for the transitional

B cell population within the retrospective cohort, indicating the possibility that HLAi transplant conditioning may continue to affect lymphocyte phenotype many years post treatment.

4.4.2 Changes in T cell expression of HLA-DR

Participant 1 was notable in terms of the activated T cell panel for demonstrating a steady increase in the CD3⁺CD8⁺HLA-DR⁺ cell population over the course of the desensitisation treatment. This increase was noted between the samples for -4.5 months and Day -1, which represents a time course of in excess of two weeks, during which time this participant underwent seven sessions of plasma exchange treatment and received Ivlg. HLA-DR is designated as a late activation marker on T lymphocytes, and is upregulated in response to activation of the T cell receptor (TCR) (Reddy *et al.* 2004). The kinetics of HLA-DR upregulation has been demonstrated to be rapid; a study examining the maturation of monocytes to dendritic cells induced by IL-15 reported an increase and a peak in HLA-DR expression over the course of eight days (Saikh *et al.* 2001). Reddy *et al.* (2004) studied the expression of cell surface activation markers and cytokine secretion of stimulated PBMCs, and noted that a rise in HLA-DR expression was initiated from 24 hours following stimulation. Further studies have reported detecting inducible expression of HLA-DR between 48-72 hours (Ferenczi *et al.* 2000) and between 48-60 hours (Rea, McNerlan and Alexander 1999) following T cell activation. This reported literature indicates a feasible timeframe for participant 1 to experience such an increase in HLA-DR expression, and it is possible that this was in response to the desensitisation treatment that this participant underwent, although this was not observed in any of the other participants.

Plasma exchange has been reported to cause changes in lymphocyte subsets and antigen expression, including upregulating HLA-DR (Reeves and Winters 2014). However, elsewhere it has been stated that plasmapheresis (PP) modulates T lymphocyte activation, and is associated with a decrease in HLA-DR expression (Sadeghi *et al.* 2013). This seeming discord may represent the fact that these two modes of plasma removal differ in that plasma exchange involves a replacement fluid being transferred to the patient, while plasmapheresis does not. The use of Ivlg introduces yet another complicating

factor into the analysis, as this too has been shown to have an effect on T cell activation, decreasing the proliferation and cytokine secretion of CD8⁺ T cells (Trepanier, Chabot and Bazin 2013). It is therefore possible that the repeated plasma exchange treatments received by participant 1 contributed to the increase observed in activated CD8⁺ T cells, and this upregulation of an activation marker could be deemed as being undesirable in a patient undergoing desensitisation treatment in preparation for transplantation.

The rise in percentages of both CD4⁺HLA-DR⁺ and CD8⁺HLA-DR⁺ T cells in the transplanted prospective participants 9, 11 and 14, at the six months post-transplantation time point, is also worthy of discussion. In addition to being a late marker of T cell activation, HLA-DR expression on CD8⁺ T cells is also known to be associated with T cell proliferation (Speiser *et al.* 2001), and in this instance could be representative of returning T cell populations post-alemtuzumab induction. However, it is interesting to note that participant 7 did not exhibit the same marked response.

A large degree of variation between individuals is also evident in the retrospective cohort for the CD3⁺CD8⁺HLA-DR⁺ population of cells. Possible explanations for this again include T cell activation or proliferation. In addition, it is interesting to note that when the retrospective cohort was segregated according to HLA-DR mismatches present between the donor and recipient, the population of CD4⁺HLA-DR⁺ T cells was significantly increased in the group with 2 DR mismatches ($p = 0.0295$). This group also had a higher mean value for the CD8⁺HLA-DR⁺ T cell population (not significant). This evidence points towards recipients possessing a larger percentage of activated T cells (both CD4⁺ and CD8⁺) when their grafts have been poorly HLA matched. However, no link with rejection episodes was seen.

4.4.3 Changes in the frequency of Tregs over the course of HLAi transplantation

All of the prospective participants for whom the data points were available (1, 2, 9 and 14) showed an increase to some degree in the percentage of Tregs present over the conditioning period for HLAi transplantation. This trend has been

reported in the literature to be associated with repeated plasma exchange treatments in patients with severe systemic lupus erythematosus (SLE) (Barath *et al.* 2007). Barath *et al.* (2007) suggested that this observation in their patient cohort could be linked with the removal of interferon-alpha (IFN- α) and autoreactive lymphocytotoxic antibodies by the plasma exchange.

It has been reported (Morales *et al.* 2008) that alemtuzumab induction correlates with an increase in the population size of Tregs (defined as CD4⁺CD25⁺FoxP3⁺) for up to six months post-transplantation, compared to normal controls and a non-alemtuzumab treated cohort. This agrees with the combined results shown for the prospective cohort (figure 4.7 (b)). An earlier study (Ciancio *et al.* 2005) also suggested an increase in Tregs post-transplantation in alemtuzumab treated patients, whilst noting that this population was not increased in a daclizumab treated cohort. In the current study, the majority (ten) of the retrospective participants received the immunosuppressive drug basiliximab (Simulect) at the point of transplant. Basiliximab is a monoclonal antibody directed against the α -chain of the interleukin (IL) -2 receptor, or CD25. The mode of action of this drug is by binding the IL-2 receptor present on activated T cells, thereby blocking IL-2 binding and subsequent T cell proliferation (Vondran *et al.* 2010). Similarly to basiliximab, daclizumab also targets the CD25 molecule. As previously noted, this study suggests that the effect of basiliximab on the CD25 molecule is short-lived as there was no difference in the percentage of CD25⁺ cells between the retrospective cohort and the normal controls. Previous studies have demonstrated a decrease in CD4⁺CD25⁺ cells following basiliximab induction; however post-transplantation follow up was limited to two weeks (Zhao *et al.* 2012) and six weeks (Vondran *et al.* 2010). Vondran *et al.* (2010) concluded that the reduction in CD25 expressing T cells was due to down regulation, but that the suppressive properties of Tregs was maintained, measured by using *in vitro* assays.

The results obtained from the retrospective cohort in this study clearly show a reduction in the percentage of CD4⁺CD25⁺FoxP3⁺ cells present compared to the normal controls. This was not observed by Ciancio *et al.* (2005) as an effect of daclizumab; however their study reported on a post-transplantation follow up of

only one year. Another study with a longer follow-up time (van de Berg *et al.* 2012) reported that Tregs were decreased at both six months and two years post-transplantation, in patients with stable graft function, but had returned to levels which were similar to normal controls within five years. The retrospective cohort in this study consists of participants who are between eight months and eleven years post-transplantation, at a single sampling time point. The results for these participants differ somewhat from those reported elsewhere for patients following anti-CD25 induction therapy, so this may indicate other factors at play alongside the immunosuppressive drugs used. As Tregs have been determined as being key in inducing and maintaining tolerance in the transplantation setting (Villard 2006), a reduction in this population of cells is a non-desirable trend.

4.4.4 Plasmablast populations in the prospective and retrospective participant cohorts post-HLAI transplantation

There were changes noted in the plasmablast populations of the transplanted prospective participants and, in addition, several of the retrospective cohort were noted as demonstrating populations of these cells. These observations are investigated and discussed further in chapter 5.

CHAPTER 5 HLA ANTIBODY DETECTION AND SPECIFICITY ANALYSIS IN PROSPECTIVE AND RETROSPECTIVE HLA_i TRANSPLANT RECIPIENTS

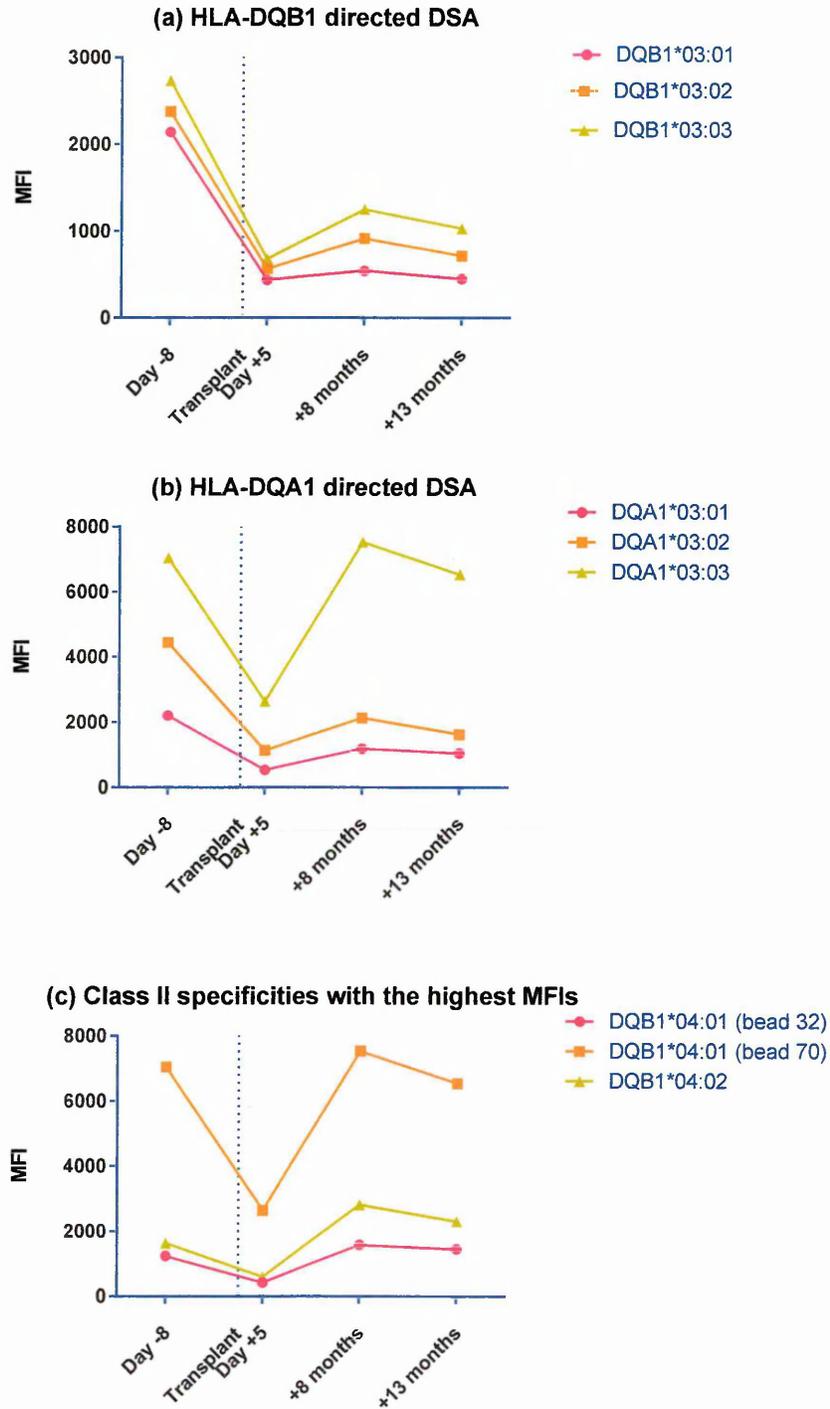
5.1 HLA-specific antibodies in the transplanted prospective participants

The objective of the antibody screening analyses was to assess HLA-specific antibodies, including donor specific antibodies (DSA), present in the recipients' serum, pre and post-transplantation, and to examine this data in the light of the results from the lymphocyte subset and immunophenotyping data, described in chapters 3 and 4. The results presented from the prospective participants are focussed around the four recipients who were transplanted and for whom follow-up samples were available (participants 7, 9, 11 and 14). The complete serum screening results, from the LABScreen® Single Antigen (One Lambda) assay, for each participant, are shown in appendices 8-11. The results were further examined, in order to determine any association between participant's antibody profiles and changes in lymphocyte populations. Where necessary, antibody serum screening results performed as part of the routine laboratory work-up for transplantation were obtained from the antibody raw data files, and combined with the antibody screening results obtained during this study, in order that an antibody profile was available for each sample time point.

5.1.1 HLA-specific antibody profile of prospective participant 7

One pre-transplant sample and three post-transplant samples were analysed, for this participant. Figure 5.1 shows the changes in the levels of DSA present (5.1 (a) and (b)). Due to the potential for cross-reactivity the levels for all HLA-DQ3 specific antibodies are shown. Similarly, all DQA1*03 directed antibodies were plotted in figure 5.1 (b). Also presented are the results of other selected HLA Class II specific antibodies, which represented those detected with the highest MFI levels (5.1(c)).

Figure 5.1 HLA antibody profiles for selected HLA specificities in prospective participant 7



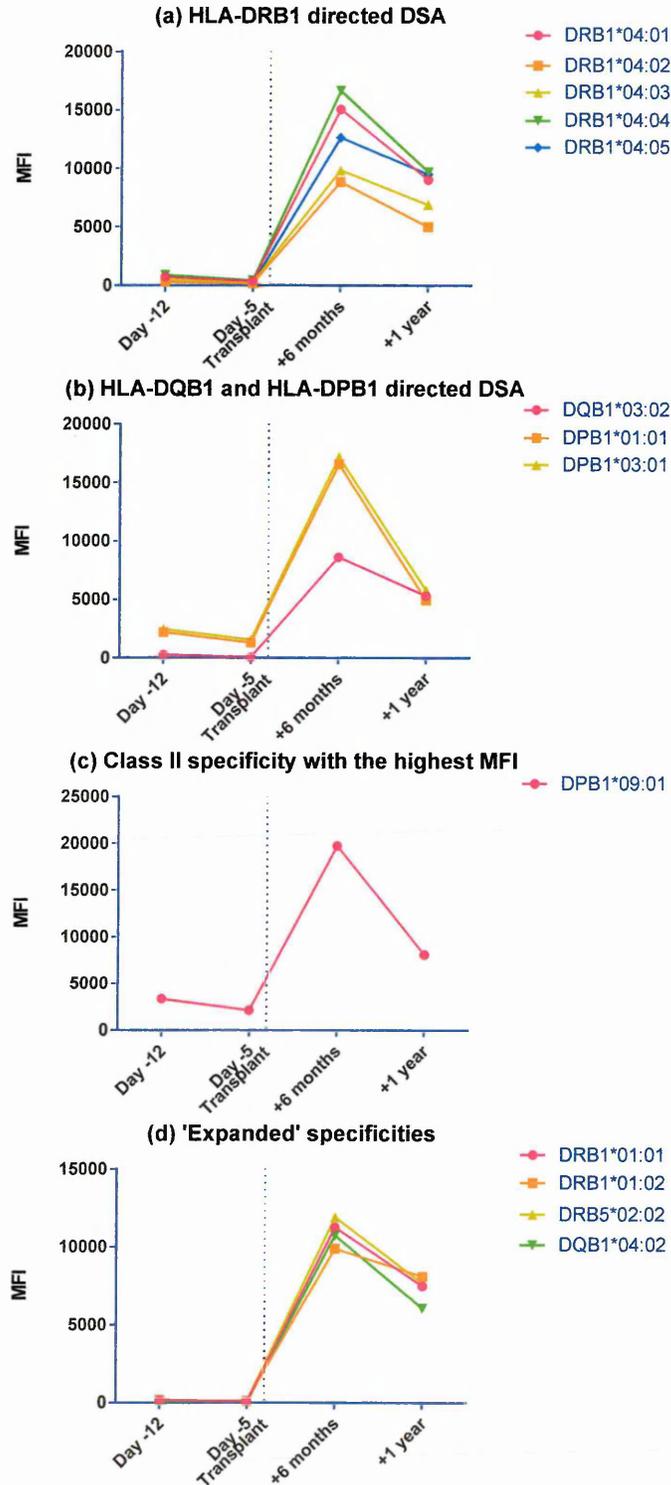
HLA - human leucocyte antigen; MFI – mean fluorescence intensity; DSA – donor specific antibody; (bead 32/bead 70) – indicates bead identity from LABScreen® assay. Sample time points are described fully in the individual participant timeline in section 3.2.

Participant 7 showed a general trend of a decrease in antibody levels at five days post-transplantation, as would be predicted with a patient going through an antibody removal protocol. With the exception of DQA1*03:03, the eight month sample showed a small resurgence of antibody, which had started to decline again by thirteen months post-transplantation. In contrast, the 'spike' in antibody directed against the epitopes encoded by HLA-DQA1*03:03, in the +8 months sample, was particularly striking (figure 5.1 (b)), exceeding pre-transplantation level, and remaining high at one year post-transplantation. This was in contrast to the other DSA levels, which remained below pre-transplant levels at one year post-transplantation. The observation regarding DQA1*03:03 is also mirrored in figure 5.1 (c), as the data for DQA1*03:03 is identical to that for DQB1*04:01 (bead 70), due to the epitopes encoded by these two genes being immobilised together, as the complete Class II molecule, on the same bead (bead 70). The profile for DQB1*04:01 identified on a separate bead (bead 32, possessing a different DQ α chain), confirms that the reactivity observed with bead 70 was chiefly due to the DQA encoded molecule present. Extended HLA typing of the patient and donor, for the DQA1 locus, during the course of this study, revealed that the DQA1*03:03 antibody was potentially donor relevant, as the donor was mismatched with the recipient for DQA1*03 (see appendix 14).

5.1.2 HLA-specific antibody profile of prospective participant 9

For participant 9, two post-transplant samples were analysed (+6 months and +1 year), (see appendix 9). The results from Day -12 and Day -5 samples were available as part of routine laboratory transplant work-up (data not shown). The results from all four samples were then used to extract the data presented in figure 5.2, which shows the selected antibody profiles for this participant, in terms of DSA, and the changes in the level of the Class II directed antibody with the highest MFI post-transplantation (HLA-DPB1*09:01). Finally, due to the complexity of this recipient's full antibody profile, the HLA Class II specific antibodies were examined in order to assess if they were broadening out over time, particularly between the pre and post-transplant time points, but also between six months and one year post-transplant. An 'expansion' of the HLA Class II directed antibodies was evident in both post-transplantation samples (+6 months and +1 year), with antibodies against HLA-DR in particular, which were previously undetected in this patient.

Figure 5.2 HLA antibody profiles for selected HLA specificities in prospective participant 9



HLA - human leucocyte antigen; MFI – mean fluorescence intensity; DSA – donor specific antibody; sample time points are described fully in the individual participant timeline in section 3.2.

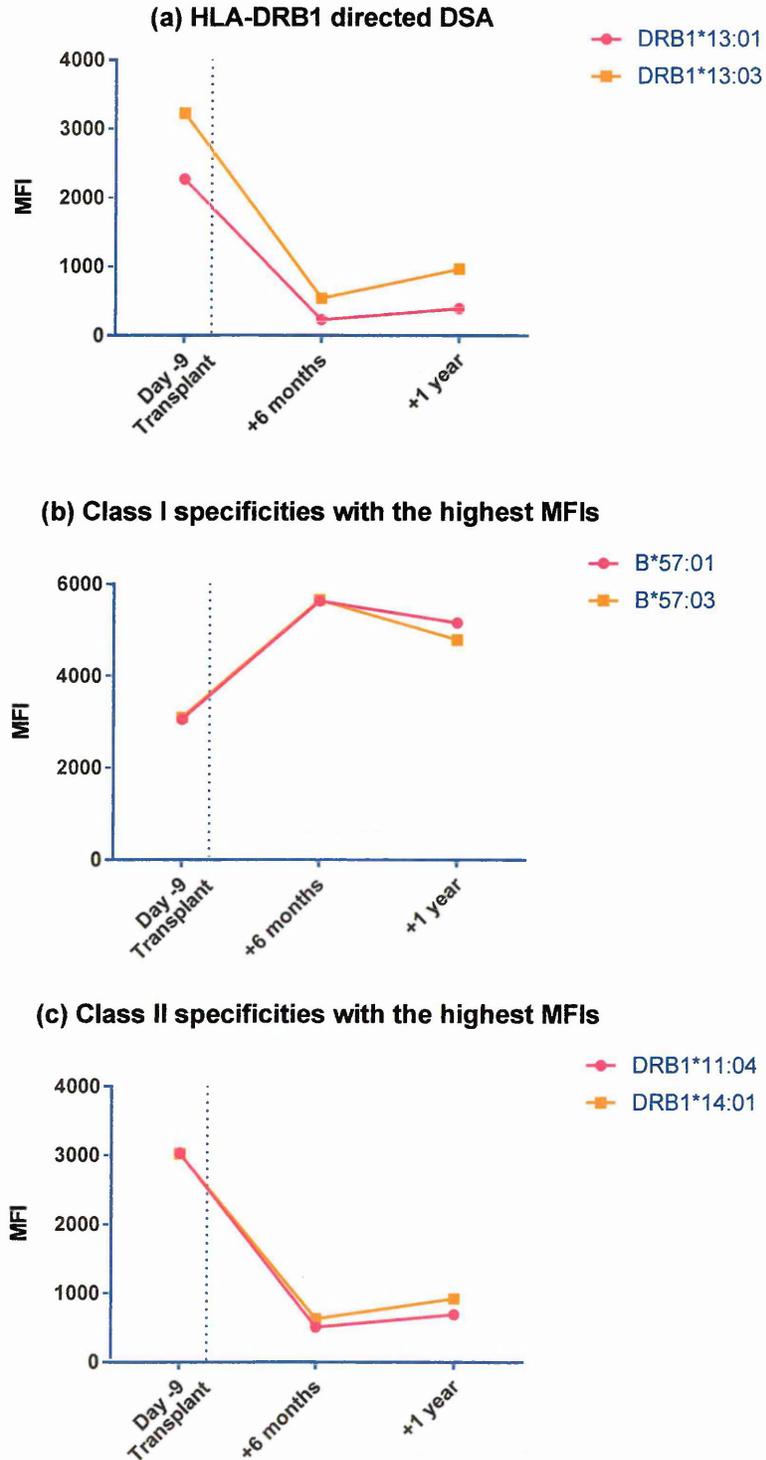
A total of sixteen 'de-novo' antibodies were detected in the post-transplantation samples; of these, antibody directed against HLA-DPB1*15:01 and DPB1*23:01 were present in the +6 months sample only, whilst HLA-DRB1*10:01 antibody was in +1 year only. The 'expanded' HLA directed antibodies detected with the highest MFI levels are included in figure 5.2(d).

Participant 9 showed a 'spike' in both DSA and other selected HLA-specific antibodies at six months post-transplantation (figure 5.2), which was much more prominent than in participant 7. The one year post-transplantation time point sample indicated that antibody levels had started to decrease, in most cases, although this was to a level which still exceeded pre-transplantation levels. There were some exceptions to this pattern within the 'expanded' specificities, with several of the 'de novo' antibodies continuing to increase in MFI level between 6 months and 1 year post-transplantation (data not shown). One such newly emergent antibody was directed against DRB1*10:01, which had an MFI level of 925 recorded at 6 months, increasing to 2615 at 1 year post-transplantation. As previously indicated in table 3.2, this participant unfortunately suffered a failed transplant, and underwent a graft nephrectomy one month after the transplantation. In addition, they also received six units of blood around this time. Both of these events will have had an impact on their subsequent HLA-directed antibody repertoire.

5.1.3 HLA-specific antibody profile of prospective participant 11

Two post-transplant samples were analysed for participant 11 (appendix 10). The results from the Day -9 sample were available as part of routine laboratory transplant work-up (data not shown). The data from all three samples was then presented in figure 5.3. The MFI levels of the DSA present were plotted, alongside plots showing the changes in the levels of the Class I and Class II specificities with the highest MFI.

Figure 5.3 HLA antibody profiles for selected HLA specificities in prospective participant 11



HLA - human leucocyte antigen; MFI – mean fluorescence intensity; DSA – donor specific antibody; Sample time points are described fully in the individual participant timeline in section 3.2.

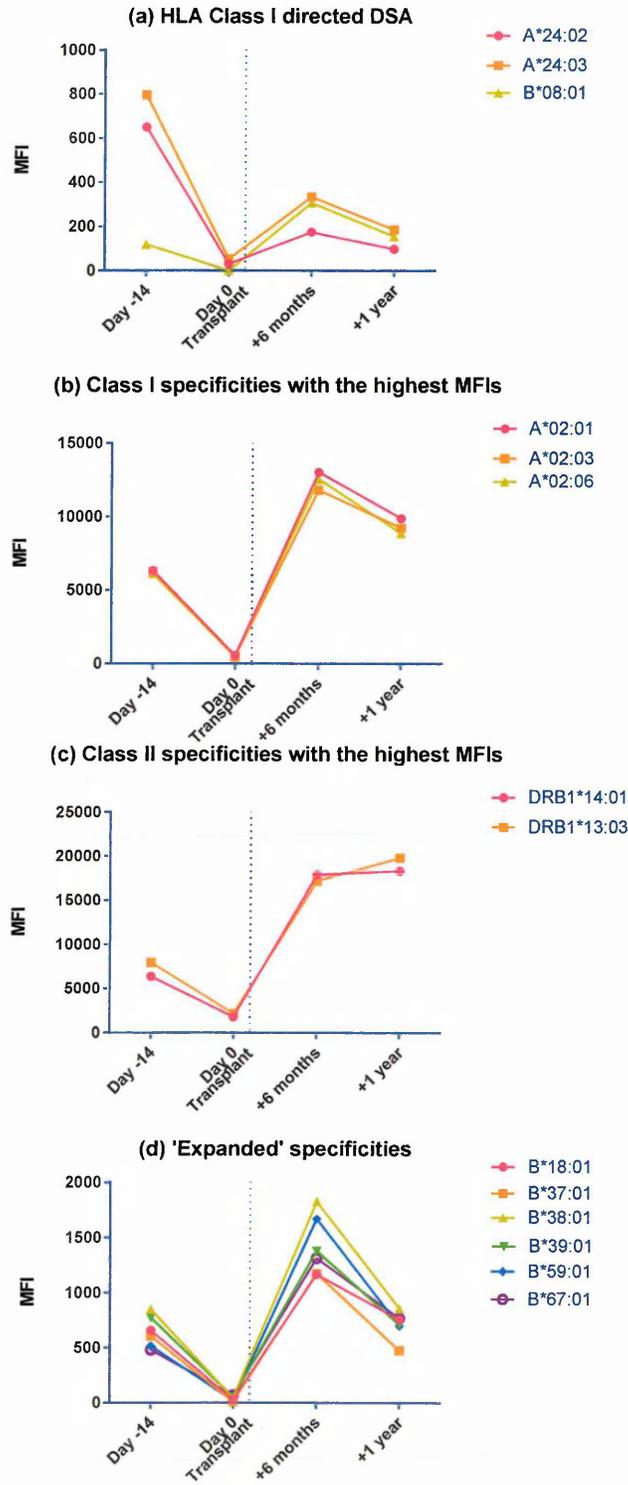
Following the decline in HLA Class II directed antibodies between the pre-transplantation and 6 month post-transplantation samples, a slight increase in HLA Class II antibodies, both DSA and the specificities with the highest MFI, between the six month and one year post-transplantation sampling time points was observed. However, as figures 5.3 (a) and (c) indicate, this increase was still considerably lower than the pre-transplantation levels recorded at Day -9. In contrast to this, figure 5.3 (b) shows a steep increase in levels of selected HLA Class I antibodies between the point of transplantation and 6 months post-transplantation, which had declined little by one year post-transplantation. The MFI reading for these antibodies remained higher than the pre-transplantation level.

5.1.4 HLA-specific antibody profile of prospective participant 14

For participant 14, two post-transplant samples were analysed (+6 months and +1 year, appendix 11). The results from Day -14 and Day 0 were available as part of routine laboratory transplant work-up (data not shown). Figure 5.4 shows data gathered from all four samples, indicating the changes in the levels of DSA (5.4(a)), but also the HLA-specific antibodies with the highest MFI, (5.4(b) and (c)) and the 'expanded' HLA specificities (5.4(d)). To assess the 'expanded' HLA directed antibodies, the profiles for the six month post-transplant and the one year post-transplant samples were examined, and any HLA directed antibodies which were not present in the pre-transplantation study samples were noted.

As would be expected, a fall in HLA-specific antibodies between the pre-treatment sample (Day -14) and the day of transplant was seen (figure 5.4). Consistent across the four graphs, a 'spike' in antibody levels was then observed at the six month post-transplantation time point. In figures 5.4 (b), (c) and (d), this rise was in excess of the pre-transplantation levels. The increase in DSA levels observed in figure 5.4 (a) (+6 months) was also in excess of pre-transplantation levels for HLA-B*08:01; however for A*24 antibody, the MFI level observed at +6 months was less than pre-transplantation levels (an MFI of 175 compared to 650 for A*24:02, and 335 compared to 797 for A*24:03).

Figure 5.4 HLA antibody profiles for selected HLA specificities in prospective participant 14



HLA - human leucocyte antigen; MFI – mean fluorescence intensity; DSA – donor specific antibody; sample time points are described fully in the individual participant timeline in section 3.2.

In all cases, with the exception of the highest Class II specificities (figure 5.4 (c)), the antibody levels were declining by the one year time point. In contrast, antibodies directed towards the beads for HLA-DRB1*14:01 and DRB1*13:03 had increased slightly further by 1 year. Figure 5.4 (d) indicates a general upregulation of the antibody response in this participant, particularly around the six month post-transplantation time point. However, in contrast to participant 9, the 'broadening' of the antibody response in the six month post-transplantation sample did not represent any 'de novo' reactivity; rather the antibodies noted had all been detected at some point over the previous eight years of records held for this patient.

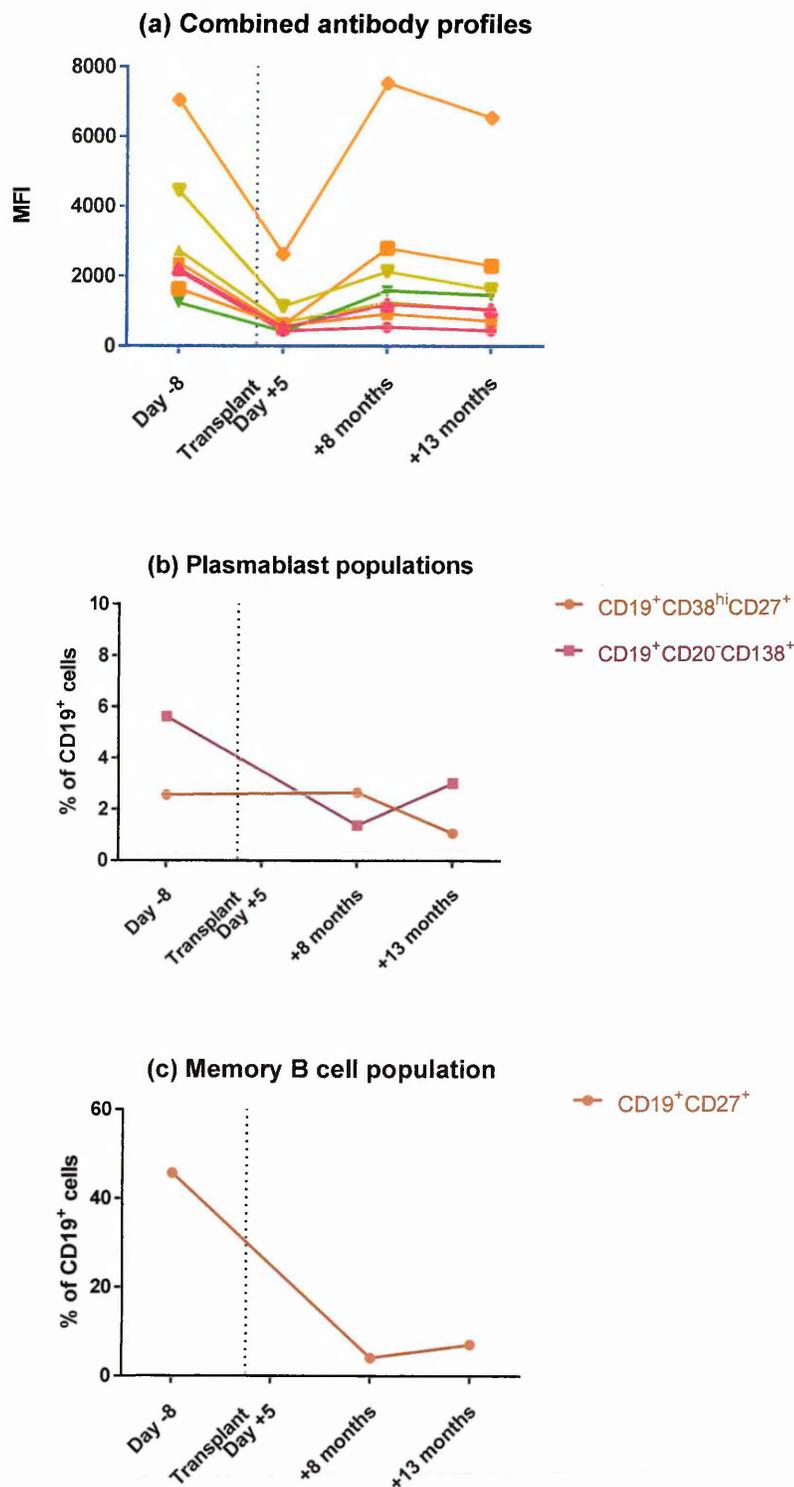
5.2 Re-examination of the B cell immunophenotyping analyses, alongside the HLA-specific antibody profiles, in the prospective participant cohort

The HLA-specific antibody profiles presented in section 5.1 were aligned with the results from the B cell immunophenotyping panels (chapter 4), with particular regard to the cells involved in antibody generation, in order to shed light on any link between these two sets of data. Again, the data presented is for the four transplanted participants (7, 9, 11 and 14). For each participant, the HLA-specific antibody screening data, shown in figures 5.1-5.4, was combined on a single graph, and is presented with the graphs showing the percentage of cell populations recorded, at each sample time point, for three B cell populations; plasmablasts bearing the phenotype CD19⁺CD38^{hi}CD27⁺ and CD19⁺CD20⁺CD138⁺, and the memory B cell population (CD19⁺CD27⁺).

5.2.1 Participant 7 - combined longitudinal antibody profiles, with selected B cell populations

Figure 5.5 presents the combined HLA-specific antibody screening profiles for participant 7, together with selected B cell immunophenotyping data. With the exception of the DSA directed against HLA-DQA1*03:03, the levels of the HLA-specific antibodies represented in figure 5.5 (a) remained relatively stable post-transplantation, with a small resurgence at six months, which did not reach pre-transplantation levels. These mainly small changes are reflected in the B cell populations in (b) and (c), which show little fluctuation between +8 months and +13 months samples.

Figure 5.5 Combined graphs of longitudinal HLA-specific antibody levels, with plasmablast and memory B cell populations, for participant 7



MFI – mean fluorescence intensity. Sample time points are described fully in the individual participant timeline in section 3.2. The antibody data sets are intended to provide an overview of antibody responses; for full specificities refer to figure 5.1.

5.2.2 Participant 9 - combined longitudinal antibody profiles, with selected B cell populations

The combined HLA-specific antibody screening profiles for participant 9, together with selected B cell immunophenotyping data, is shown in figure 5.6. Here it can be seen that the obvious increase in overall HLA antibody levels at +6 months (5.6 (a)) is reflected in a similar peak in plasmablast cell populations (5.6 (b)). Simultaneously, the memory B cell population remains reduced post-transplantation.

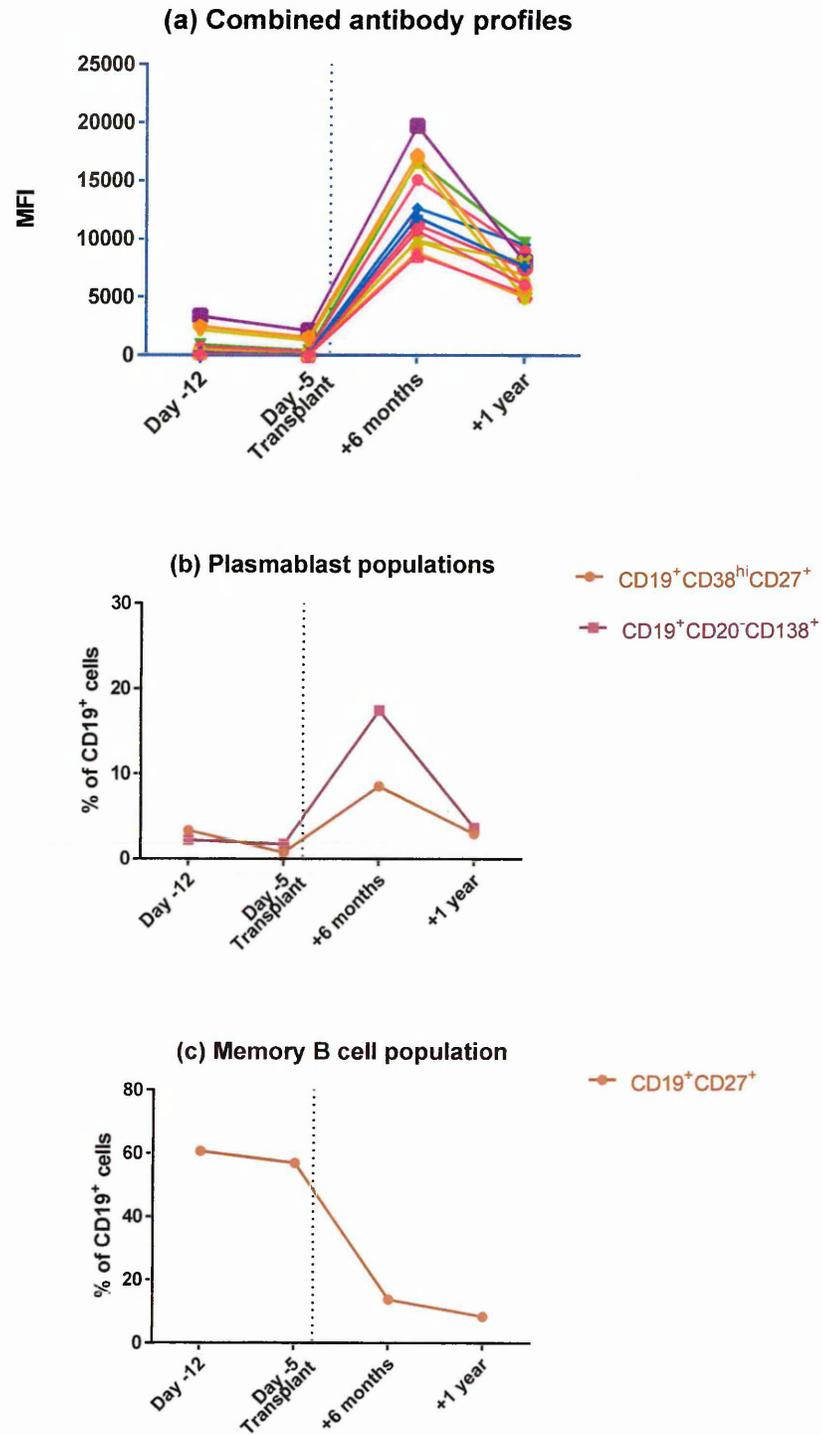
5.2.3 Participant 11 - combined longitudinal antibody profiles, with selected B cell populations

Figure 5.7 shows the combined HLA-specific antibody screening profiles for participant 11, together with the selected B cell immunophenotyping data. Small changes in plasmablast populations appear to correspond with mainly low levels of HLA Class II directed antibodies post-transplantation. The MFI levels of the highest Class I antibody specificities (detailed in figure 5.3 (b)) appear to be in contrast to this overall picture; however there is a peak in the CD19⁺CD20⁻CD138⁺ plasmablast population at six months post-transplantation which coincides with this.

5.2.4 Participant 14 - combined longitudinal antibody profiles, with selected B cell populations

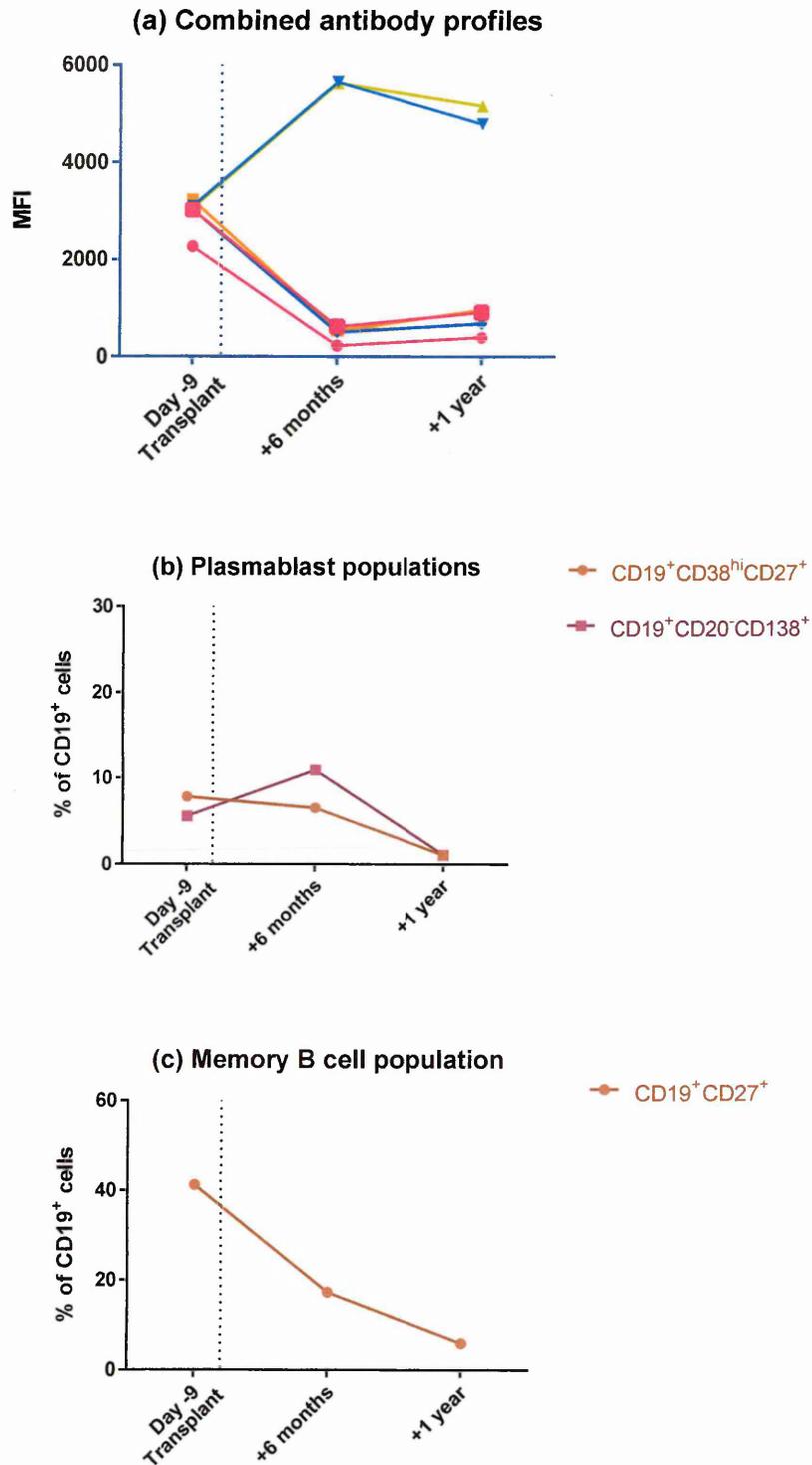
The combined HLA-specific antibody screening profiles for participant 14, together with the selected B cell immunophenotyping data, are shown in figure 5.8. The resurgence of HLA directed antibodies displayed at the six months post-transplantation time point (5.8 (a)), appears at the same time point as a peak in the plasmablast population (CD19⁺CD20⁻CD138⁺).

Figure 5.6 Combined graphs of longitudinal HLA-specific antibody levels, with plasmablast and memory B cell populations, for participant 9



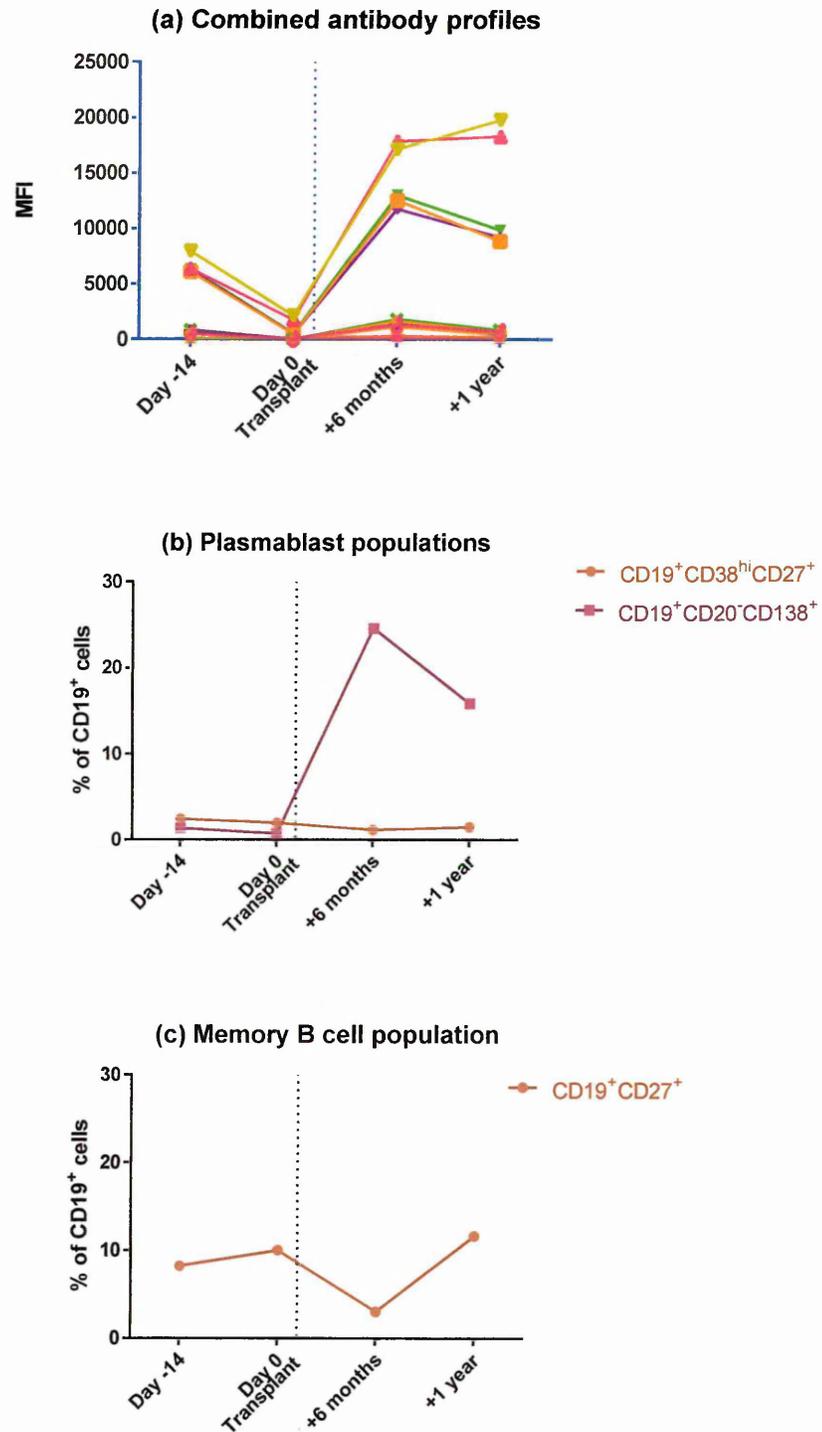
MFI – mean fluorescence intensity. Sample time points are described fully in the individual participant timeline in section 3.2. The antibody data sets are intended to provide an overview of antibody responses; for full specificities refer to figure 5.2.

Figure 5.7 Combined graphs of longitudinal HLA-specific antibody levels, with plasmablast and memory B cell populations, for participant 11



MFI – mean fluorescence intensity. Sample time points are described fully in the individual participant timeline in section 3.2. The antibody data sets are intended to provide an overview of antibody responses; for full specificities refer to figure 5.3.

Figure 5.8 Combined graphs of longitudinal HLA-specific antibody levels, with plasmablast and memory B cell populations, for participant 14



MFI – mean fluorescence intensity. Sample time points are described fully in the individual participant timeline in section 3.2. The antibody data sets are intended to provide an overview of antibody responses; for full specificities refer to figure 5.4.

5.3 Post-transplantation HLA-specific antibodies in the retrospective participants

Serum antibody screening of the retrospective participants indicated an extremely diverse group of patients, with several of the individual participants' results (appendices 12 and 13) worthy of comment. The following were of particular interest.

Participant 4 was a female patient, who possessed DSA against HLA-B8, B17 and DR3, pre-transplantation. Her post-transplantation study sample showed only DSA against HLA-B8 to be present, although further DSA had been identified in routine screening samples post-transplantation. This participant has since tested negative for DSA, demonstrating antibody fluctuations.

Participant 5 provided an example of an individual possessing clear and strong antibodies directed towards various HLA Class I specificities, although none of these were DSA. Interpretation of the screening results were aided by the fact that the antibodies were of a high MFI level and showed clear 'clustering' around related HLA, for instance those belonging to the same 'broad' group or cross-reactive group (CREG). Conversely, the same participant displayed complex results for HLA Class II antibody screening, including DSA (directed towards HLA-DR1, DQ8 and DR53). Interestingly, the HLA-DP type expressed by the donor for this participant, was not represented on the screening test panel (DPB1*16:01), so it was not possible to assess if this DSA was present.

Participant 6 also demonstrated a complex reactivity pattern for their Class II results, illustrating the difficulty of separating alloreactivity against the HLA-DQ α chain from that against the DQ β chain. In this participant it appeared that positive beads originally attributable to DQ3 were in fact most likely due to reactivity against the product of HLA-DQA1*03:02. This is relevant, in this case, as the donor was subsequently typed within this study as DQA1*03.

Participant 10 was an example of an individual whose results were not clear-cut for antibodies to HLA Class I, with lots of weak and background reactivity present. This confused the results, making it difficult to decipher genuine HLA-directed antibodies. It is interesting to note that amongst the antibodies detected in the study sample for this participant were those against non-inherited maternal

antigens (NIMA), HLA-A29 and Cw16. The Class II results for this participant were also complex with weak background reactivity present. However, clear DSA were observed against HLA-DR4 (also a NIMA) and DP3.

Participant 13 was an example of a patient who had clear sensitisation resulting from a previous transplant. HLA-A1, A11, B57, Cw6, DR13 and DR52 directed antibodies were detected, amongst the profile of Class I and Class II specificities, representing the HLA mismatches present with a previous deceased donor. This case illustrates the benefit of having full details of previous donor types and sensitising events as an aid to the interpretation of antibody screening results.

Participant 16 was an extremely complex case, with an extensive sensitisation history and antibody profile. Clear high MFI levels of DSAs (HLA-A3, A11, B35, DR4, DR11, DR52, DR53 and DQ7) were present in the antibody profile. This individual was so highly sensitised to HLA that negative tests were mostly attributable to the patient's own HLA type. However, it was noteworthy that despite having a history of previous transplantation, this patient did not possess HLA-DP specific antibodies.

Participant 17 supplied a later routine screening sample which confirmed the presence of DSA against HLA-B5 and cross reactive antibodies against B35.

Participant 19 possessed HLA-B46 specific antibody. Although this does not represent a DSA, it was of interest due to the low frequency that this antigen is observed in the general population. Class II DSA had been previously detected in routine post-transplantation screening samples, despite the current study sample being negative for these antibodies, again indicating how antibody levels can fluctuate.

5.4 Stratified analysis of the retrospective participant cohort according to the HLA-specific antibody screening results

5.4.1 Analysis of the retrospective participant cohort according to the presence or absence of DSA

The retrospective study cohort was stratified according to whether DSA (Class I or Class II) was present or absent. DSAs were present in eight of the retrospective participants and five participants had no DSA. The results from the

lymphocyte subset analysis were plotted for the two groups, for each cell type. The normal controls were used for comparison (data not shown). No differences between the segregated cohorts were observed from these analyses.

In a similar manner, the results from the B cell immunophenotyping panels were then reanalysed according to DSA presence (n = 7) or absence (n = 3) (data not shown). The results showed no striking differences between the 3 groups (DSA positive, DSA negative, normal controls) for any of the immunophenotyping panels. The cohort numbers were deemed to be too small for any further stratification to be undertaken, e.g. dividing into HLA Class I or Class II DSA, or overall HLA antibody positive or negative.

5.4.2 Analysis of the retrospective participant cohort according to 'high' or 'low' MFI value

A further stratification was carried out on the retrospective participants, according to whether they had 'high' levels of circulating HLA-specific antibody (with an MFI value of >5000) or 'low' levels of HLA-specific antibody present (MFI <5000). The data from the B cell immunophenotyping panels were plotted accordingly (retrospectives with high MFI, n = 6, retrospectives with low MFI, n = 4). Again, the results showed no clear differences between the 3 groups (high MFI, low MFI, normal controls) for each of the immunophenotyping panels.

5.5 Re-examination of the B cell immunophenotyping analyses, alongside the HLA-specific antibody screening results, for four of the retrospective participants

In section 4.2.3., it was noted that four of the retrospective participants had a population of plasmablast cells (CD19⁺CD20⁺CD138⁺) present. The percentage of plasmablasts, for these four participants all appeared above the mean for the cohort. This data has been reproduced, alongside similar plasmablast data (CD19⁺CD38^{hi}CD27⁺) from the transitional B cell panel, and the memory B cell data (CD19⁺CD27⁺), in figure 5.9. Each of the four participants noted have been assigned a colour, in order to compare their individual results for these cell populations. The HLA-specific antibody screening results for the four participants are shown in table 5.1, in order to examine any associations between these sets of data.

As would be expected, figure 5.9 (a) indicates that the four participants also showed a relatively high percentage of CD19⁺CD38^{hi}CD27⁺ cells, which are likely to represent the same plasmablast population, as the cells detected possessing the phenotype CD19⁺CD20⁻CD138⁺. Figure 5.9 (b) indicates that for three of the four participants highlighted (5, 10 and 16), the plasmablast populations corresponded with a depleted memory B cell pool. Participant 18, however, possessed a percentage of memory B cells which was above the mean for the cohort. The plasmablast populations identified in participants 5, 10 and 16 correspond with the extensive sensitisation (including DSA) and high MFI levels, shown in table 5.1. In contrast, participant 18 did not possess any DSA and had a narrow sensitisation of 2% PRA (albeit with a corresponding cRF of 26%), representing two antibody specificities (HLA-B8 and HLA-B82), at a fairly high MFI level of 12,996.

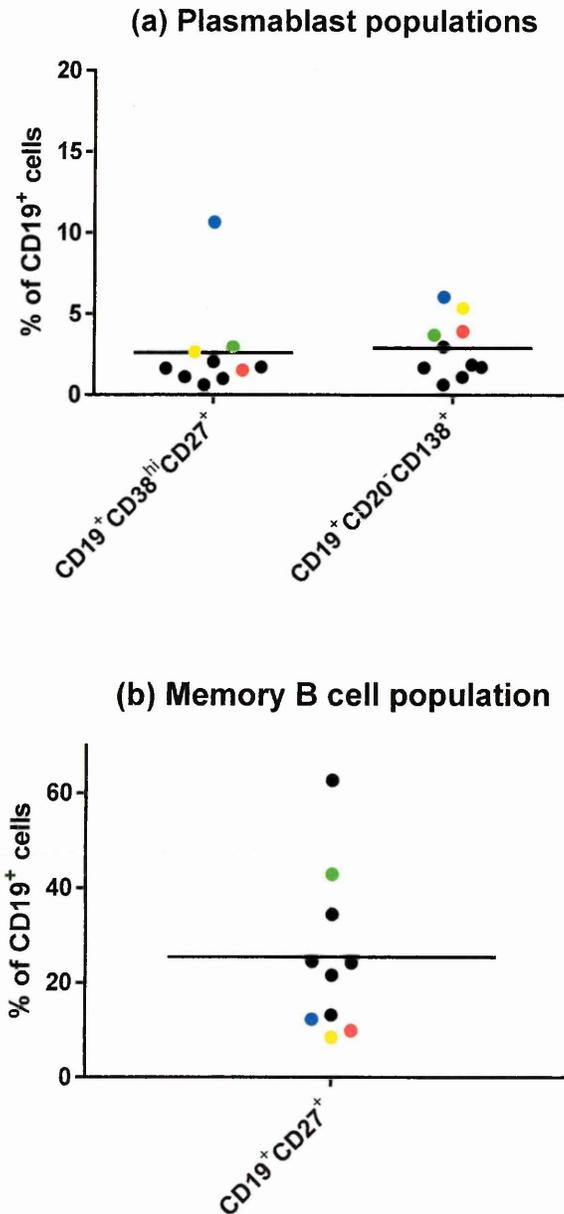
Table 5.1 HLA-specific antibody screening results for the four retrospective participants highlighted in figure 5.9

Retrospective participant	% PRA		Highest MFI		% cRF	DSA
	Class I	Class II	Class I	Class II		
	5	36	23	19,819		
10	4	41	1474	4836	95	Class II present
16	81	51	18,387	20,271	100	Class I and Class II present
18	2	0	12,996	N/A	26	None

% PRA – percentage panel reactive antibody; MFI – mean fluorescence intensity; % cRF – percentage calculated reaction frequency; DSA – donor specific antibody.

This data is a summary of that shown more fully in appendices 12 and 13.

Figure 5.9 Plasmablast and memory B cell populations for the retrospective cohort, highlighting the four participants noted for populations of plasmablasts



Y axis – percentage of each cell population identified. Horizontal line indicates mean. Retrospective cohort, n= 10.

The coloured data points indicate the four participants noted for plasmablast populations; blue – participant 5, red – participant 10, yellow – participant 16, green – participant 18. The remainder of the cohort are indicated by black data points.

5.6 Results from HLA typing performed to assist in the interpretation of HLA-specific antibody screening results

As described in section 2.11, supplementary HLA typing was carried out on five recipient and donor pairs, for HLA loci which were not typed as a part of the standard routine laboratory work-up for transplantation. This genetic typing information was required in order to allow full interpretation of the antibody data generated by this study. The HLA typing data obtained on the five recipients and their respective donors is presented in appendix 14.

5.7 Discussion of HLA-specific antibody detection in the prospective and retrospective HLAi transplant recipients

5.7.1 General observations of the problems encountered in interpreting results from HLA-specific antibody screening, as demonstrated in the retrospective participant cohort

The HLA-specific antibody screening results from the retrospective participant cohort can be used to highlight some of the difficulties often encountered with interpreting this type of data. The importance of viewing this information in the context of HLA typing results and with a knowledge of the frequencies of HLA types in different populations is also emphasised. Due to the nature of the cohort, being HLAi transplant patients, the screening results were complex, with many HLA specificities to decipher, often with much weak background reactivity, cross-reactivity or allelic reactivity present.

The choice of test methodology and commercial kit used for the detection of HLA-specific antibodies has an obvious influence on the results obtained. Solid-phase single antigen systems now provide the 'gold standard' within H&I laboratories (Roberts *et al.* 2014) and microbead analysis is recommended for monitoring patients undergoing HLAi transplantation (BTS 2015). Single-antigen tests can excel in providing clear, definitive interpretation of HLA-directed antibodies, without the confusing factor of picking apart reactivity against the numerous antigens expressed on a single cell, yet they too are not without their intrinsic drawbacks (Middleton, Jones and Lowe 2014, Roberts *et al.* 2014). The

phenomenon of HLA linkage disequilibrium, where certain genes are characteristically inherited together as a haplotype, has historically made cellular based antibody screening assays difficult to interpret. However, the representation of antigens in a commercial kit still affects the results obtained. For instance, in the case of participant 5, it was not possible to identify reactivity towards the HLA-DP antigen encoded by DPB1*16:01, despite this being donor relevant. In this kind of scenario HLA epitope analysis can be informative, by identifying shared epitopes between the HLA targets of antibodies detected by the assay, and donor mismatches which are not represented. Participant 5 showed extensive HLA-DP sensitisation (appendix 13), with antibodies directed against a range of HLA sharing the 84DEAV epitope (University of Piau 2013). DPB1*16:01 also shares this epitope, so it may be postulated that DSA was likely to be present against this donor relevant mismatch.

Single antigen kits can help facilitate the definition of antibodies directed against the α -chain of the HLA Class II molecule, but this can often be unclear and, in some cases, the antibody may be misassigned as a β -chain directed antibody. Due to the HLA Class I molecule consisting of a single heavy chain (associated with β -2 microglobulin), corresponding to a single Class I gene, interpretation is usually more straightforward. Although Class II HLA-DQA and DPA are not as polymorphic as their HLA-DQB and DPB counterparts (Robinson *et al.* 2015), antibodies directed against them are now routinely identified. This is relevant to participant 6, where it was difficult to separate reactivity against the β -chain encoded by HLA-DQB1*03 and the α -chain product of HLA-DQA1*03:02, due to these two chains often being paired in the same HLA molecule. It is important to define HLA-specific antibodies as clearly as possible in order to identify 'windows' of opportunity for transplantation, particularly in highly sensitised individuals, and in order to ensure that donor kidneys are not unnecessarily denied to patients waiting for a transplant.

Since memory B cells are able to generate antibodies upon re-exposure to antigen for the lifetime of an individual, it is important to ascertain the sensitisation history of potential transplant patients wherever possible. It is also good practice to ensure that a patient's sensitisation profile is viewed in the context of their own HLA type, in order to exclude reactivity against 'self' antigens. An understanding

of HLA expression and linkage disequilibrium is required in order to avoid unintentionally re-exposing patients to previously encountered HLA. As demonstrated with participant 13, it is often possible to identify the complete HLA mismatches of a previous donor within the antibody profile of a sensitised patient. Due to linkage disequilibrium, HLA-B57 and Cw6, and DR13 and DR52, are usually found together on the same haplotype, and this was most likely the case in the previous donor for participant 13.

HLA types are represented at different frequencies within certain ethnic groups; a phenomenon associated with the diverse microbiological pressures experienced by different populations. This can have serious repercussions for the chances of finding someone a matched donor or for excluding donors in sensitised patients. The case of participant 18 demonstrates this, in that HLA-B8 is a common antigen, present in 25% of the UK organ donor pool (NHSBT 2016b), so possession of this antibody significantly reduces this patient's chances of being offered a deceased donor kidney. Conversely, participant 19 was shown to have formed antibody against HLA-B46, which has a reported frequency of <1% in the UK organ donor pool (NHSBT 2016b). This antigen is expressed almost exclusively in individuals from south-east Asia, with reported frequencies of up to 14% in these populations (Akesaka *et al.* 2000). Initial preconceptions may indicate that it is unlikely that this participant would have been exposed to this antigen via the standard routes of sensitisation. However, the patient's nationality is documented as being Filipino, so it is possible that they received a blood transfusion in their country of origin. However, the formation of a single HLA Class I antibody, directed towards a low frequency antigen, often suggests an alternative route of sensitisation, such as molecular mimicry. This describes a potential cross reactivity between the immune response towards pathogens and HLA, due to amino acid sequence similarities. Although it is considered safe practice in our centre to list these antibodies, if they are consistently present, this does highlight the importance of ascertaining that detected antibodies are indeed clinically relevant (Middleton, Jones and Lowe 2014).

In the course of attempting to identify the most suitable live donor for participant 10, both parents had been HLA typed, and it was of interest to note that this

participant had formed antibodies directed against the non-inherited maternal HLAs. It is thought that a tolerogenic effect is generated to these HLA, due to the exposure by an individual to NIMA *in utero*. Claas *et al.* (1988) reported some time ago that, in a cohort of multi blood transfused, highly sensitised patients, 50% did not form antibodies against NIMA, creating a possible 'window' for transplantation. Although not borne out in this case, such strategies can sometimes aid in identifying the most compatible donors for patients awaiting transplantation.

5.7.2 Post-transplantation detection of HLA-specific antibodies in four prospective participants

Participants 7, 9 and 14 all demonstrated an increase in HLA-specific antibodies in general terms, by the six month post transplantation time point, which was mainly declining by one year post-transplantation. Obtaining a genuine 'picture' of DSA levels post-transplantation presents difficulties, as only levels in serum are detected, and it is not possible to ascertain if DSA is being produced but bound to target antigens present on the donated graft. However, this process of sequestration of the antibodies may only provide a partial explanation for their absence or reduction post-transplantation (Stastny *et al.* 2009). For participant 7, the pattern observed for the DSA targeting DQA1*03:03 encoded epitopes was striking as being different to the other antibodies present, by peaking to a high MFI level post-transplantation. Unlike the DSA directed towards the HLA-DQB1*03 encoded antigens in this participant, it may be that the plasma cells producing the HLA-DQA1*03:03 antibody could have 'escaped' destruction in survival niches within the secondary lymphoid organs.

Participants 9 and 14, in particular, showed similar antibody dynamics, with increased levels at six months post-transplantation. These participants both underwent transplantation which was incompatible for the ABO blood group antigens, as well as being HLAi. Therefore, they received similar transplant conditioning, which may explain the pattern of antibody resurgence post-transplantation. In addition, ABO antibody rebound is common, but often associated with a clinical picture of graft accommodation when in excess of three weeks post-transplantation. The features of this phenomenon include the

presence of both increased ABO antibody titres, alongside C4d deposition, with no antibody mediated rejection (Koo and Yang 2015). It is possible that a rebound in ABO antibody titres could have been associated with an upregulation of other antibodies, including those directed towards HLA, around this time period. Both patients suffered delayed graft function post transplantation, with participant 14 also having biopsy proven early rejection, which required treatment with methyl-prednisolone.

Participant 9 showed a broadening out of their HLA directed antibody specificities by six months post-transplantation, including the production of new specificities, which was most likely a direct response to losing their graft (Nair and Heeger 2012) and receiving multiple blood transfusions. The immunological challenge of receiving and then losing their transplanted kidney most likely stimulated this antibody production, which may have developed and 'expanded' to include new specificities, a process known as 'epitope spreading' (BSHI/BTS 2014), facilitated by shared epitopes within HLA molecules. In addition, the blood transfusions would have been from different donors, and despite leukodepletion of red cell transfusions it has been evidenced that HLA sensitisation still occurs by this route, either due to remaining leucocytes or cell debris present, or because of low level HLA expression on erythrocytes (Rees and Kim 2015). Finally, although the transplanted donor kidney was removed, it is likely that transplanted donor tissue would have remained *in situ* causing ongoing immunological stimulation (Nair and Heeger 2012). Participant 14, who showed a similar pattern of antibody 'spiking' at six months post-transplantation, also demonstrated an associated broadening out of their antibody response at this time point. However, in this case the antibodies detected had all been previously documented for this participant.

Participant 11 showed different patterns of detection for antibodies directed against HLA Class I and Class II. Class II directed antibodies, including DSA, remained at a fairly low level post-transplantation. Simultaneously, Class I directed antibodies with the highest MFI, showed a sharp increase post-transplantation. Antibodies targeting two different alleles encoding the HLA Class I antigen B57 showed a peak in MFI at six months post-transplantation, which had started to decline by the one year post-transplantation time point.

5.7.3 Possible links between plasmablast populations and HLA-specific antibodies present, in the prospective and retrospective participant cohorts, post-HLAI transplantation

Figures 5.5-5.8 suggest a link between the plasmablast populations detected by the CD19⁺CD20⁻CD138⁺ and CD19⁺CD38^{hi}CD27⁺ cell markers, and the patterns of post-transplantation antibody production in the four transplanted prospective participants. This is key in illuminating the question of where the site of antibody production lies for each individual, which could have implications for clinical intervention. The 'spike' in antibody production shown by participants 9 and 14, at six months post-transplantation, seems to correlate with an increase in the plasmablast population and a drop in the percentage of memory B cells. Participant 11 also demonstrated an increase in plasmablasts of the phenotype CD19⁺CD20⁻CD138⁺ associated with an increase in HLA Class I directed antibodies at six months post-transplantation.

This possible connection between peripheral plasmablast populations and antibody production is interesting, as it suggests a potential new approach to monitoring HLAI transplant patients post-transplantation. In the main, a resurgence of HLA-specific antibodies in the four transplanted prospective participants was accompanied by an increase in peripheral plasmablast populations, and both cells and antibodies then showed a decline. It could be hypothesised that a situation where both parameters continued to increase would indicate a poor prognosis for transplant outcome. In this scenario, plasmablasts present in the periphery represent a target for clinical intervention, perhaps with increased immunosuppression, accompanied with plasma exchange to clear the antibody burden. A worse clinical picture may be represented by a sustained increase in HLA directed antibodies, which is not accompanied by the detection of peripheral plasmablast populations. This may indicate that the antibody producing cells are 'hidden' in the survival niches of the secondary lymphoid organs, where it is impossible to target them with standard therapies. The combined results for participant 7 hints at this situation, with the DQA1*03:03 antibody, which appears to be inconsistent with the pattern obtained for their other antibodies. However, this antibody started to decline by the one year post-transplantation time point. It is also interesting to note that the two prospective

participants (1 and 2) who underwent desensitisation, but did not get transplanted, demonstrated an increase in HLA-specific antibodies (HLA-A2 directed, and HLA-A2 and DQ8 directed respectively) combined with a decrease in plasmablasts, over the course of their treatment (figure 4.13).

The observations concerning the plasmablast populations are thought provoking, yet it should be borne in mind that these cell populations are present in low percentages, so that seemingly substantial fluctuations represent a very small number of cells in absolute terms. Plasma cells (CD138⁺) are reported to be present in bone marrow as 0.2-2% of leucocytes (Stemcell Technologies 2015), yet a reference for peripheral blood is not provided. It is possible that the severe leucodepletion caused by alemtuzumab promotes the migration of plasmablasts from the secondary lymphoid tissues to the periphery, but investigation of this would require further work.

If the association between antibody levels and plasmablast populations, indicated by the four prospective participants, is a consistent finding (or can be reproduced in further transplant patients), then observations concerning this trend should also be apparent within the retrospective cohort. The results collated in figure 5.9 indicate that increased peripheral plasmablast populations associated with a relatively high % PRA (with a corresponding high % cRF) and MFI level (table 5.1), in participants 5, 10 and 16, are also linked with a reduced percentage population of memory B cells. Participant 18 appears to differ, possessing a relatively high percentage of both plasmablasts and memory B cells, yet restricted sensitisation to just two antigens, with a high level of antibody. It may be postulated that a pattern of increased numbers of plasmablasts, producing large quantities of antibodies targeting different epitopes, could lead to a concurrent reduction in the memory B cell pool. Conversely, participant 18 may represent a different 'picture' of a single clone of plasma cells producing antibody targeting a shared epitope of HLA-B8 and B82, which is not reflected in a reduction of the memory B cell population.

6.1 Summary of the study findings

This study gathered data from blood samples provided by seven prospective and thirteen retrospective HLAi kidney transplant recipients, and nine normal control individuals. Three sets of data were collected on each of the transplant participants; lymphocyte subsets, T and B cell immunophenotyping and HLA-specific antibody screening. The results obtained from this study are firstly summarised, and presented as a set of characteristics making up a profile for each group of participants, where possible. Although there were some generalisations that could be drawn from the study participants, there were also many exceptions. The evidence for any recommendations for changes in practice, which can be supported by this work, will then be described, and suggestions for further work to progress this study area will be made.

Several difficulties and limitations were encountered whilst performing this study, and these will be described as a part of this discussion. Two limitations were of particular importance when considering the results obtained; firstly the heterogeneous nature and complexity of the study group, and secondly the small number of prospective participants recruited. Further strengths and weaknesses of the study, and possible ways that the latter could have been addressed, are also considered. Finally, the future direction for HLAi transplantation will be reflected upon.

The key study findings are summarised in table 6.1 for the transplanted prospective and retrospective participants. The prospective participants who were not transplanted have not been included in this profile, as it was not possible to draw generalisations from just two individuals.

Table 6.1 Profiles of the prospective and retrospective participant groups generated from the key findings of the study

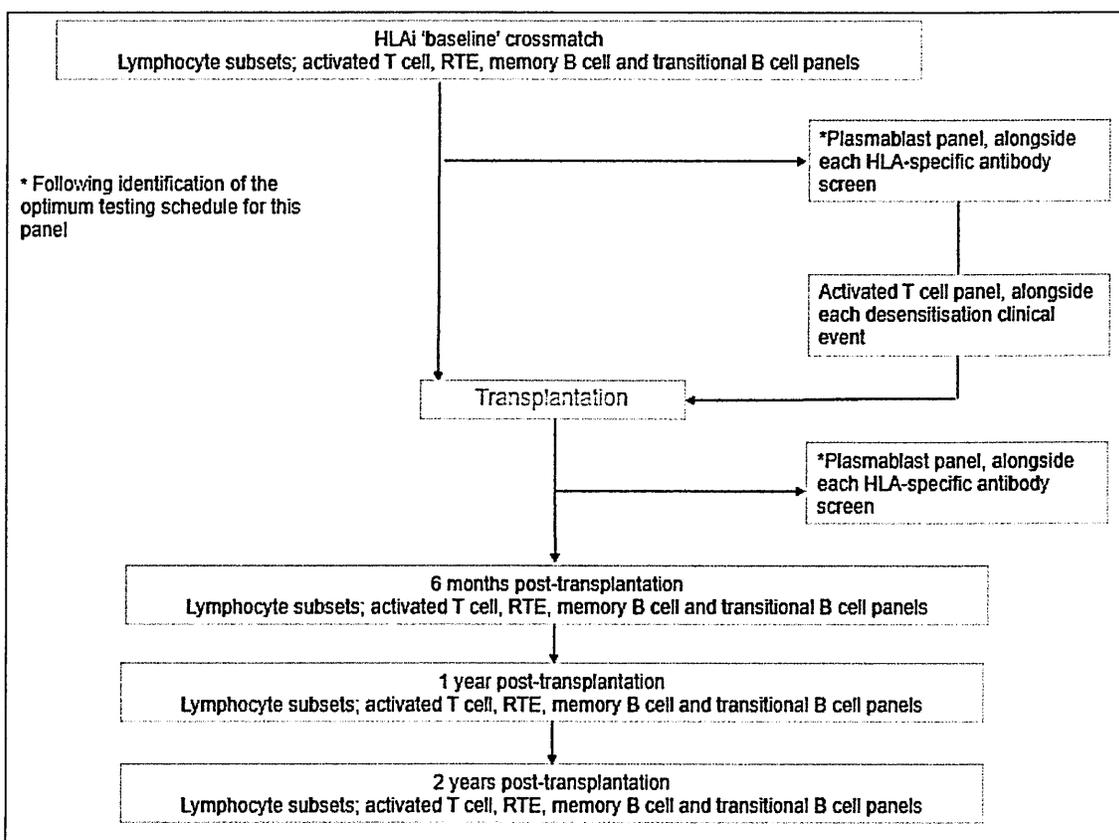
Laboratory characteristic	Prospective participant cohort (transplanted)	Retrospective participant cohort
Lymphocyte subsets	<ul style="list-style-type: none"> • Pan-lymphocyte drop in cell counts immediately post-transplant (alemtuzumab induced) • Some recovery in CD19⁺ cell counts, but remained immunocompromised one year post-transplant • Pre-treatment results suggest reduced cell counts associated with chronic renal failure 	<ul style="list-style-type: none"> • Mean absolute cell counts consistently reduced compared to normal controls • Demonstrates long-term effect of pre-conditioning and immunosuppression. Also likely that cell counts were depressed pre-transplant
CD4:CD8 ratio	<ul style="list-style-type: none"> • Altered CD4:CD8 ratio 	<ul style="list-style-type: none"> • Altered CD4:CD8 ratio persists post-transplant • Several participants had inverted ratio
RTEs	<ul style="list-style-type: none"> • T cell reconstitution is from within memory compartment 	<ul style="list-style-type: none"> • Increased memory T cells and reduced naive T cells compared to normal controls. • RTEs remain decreased compared to normal controls
Activated T cells	<ul style="list-style-type: none"> • Peak in HLA-DR expression on both CD4⁺ and CD8⁺ subsets observed at 6 months post-transplant 	<ul style="list-style-type: none"> • CD3⁺CD4⁺HLA-DR⁺ population no different to normal controls, with two exceptions • Large spread around mean for CD3⁺CD8⁺HLA-DR⁺
Tregs	<ul style="list-style-type: none"> • Decreased between 6 months and 1 year post-transplant • Mostly steady effector T cell:Treg ratios 	<ul style="list-style-type: none"> • Depressed Treg populations compared to normal controls
Memory B cells	<ul style="list-style-type: none"> • Sustained reduction in memory subsets, indicating repopulation not from within this compartment 	<ul style="list-style-type: none"> • Elimination of memory B cells in periphery is long-lived
Transitional B cells	<ul style="list-style-type: none"> • Efficient reconstitution of transitional and mature B cells by 6 months post-transplant 	<ul style="list-style-type: none"> • No different to normal controls, with a few exceptions
Plasma B cells	<ul style="list-style-type: none"> • Increase in plasmablast population at 6 months post-transplant, reducing at 1 year. Participant 7 increased between 6 months and 1 year 	<ul style="list-style-type: none"> • No overall difference in plasmablast populations, with several notable exceptions
HLA-specific antibodies	<ul style="list-style-type: none"> • Decreased at time of transplant • Resurgence at 6 months, declining by 1 year, participant 11 is exception • Possible link with plasmablast population 	<ul style="list-style-type: none"> • Potential association between HLA-specific antibodies and plasmablast populations noted in four participants

6.2 Recommendations for change in practice and further work in relation to cellular immunophenotyping HLAi transplant patients

This research project represents a pilot study into the merit of immunophenotyping HLAi transplant patients. Although it was not possible, in all cases, to extract a common thread from each panel, the results presented by these participants demonstrated some intriguing trends and patterns that are worthy of further investigation. It is therefore recommended that elements of this pilot study are extended into a larger prospective study, and that this should be continually monitored by rolling audit. In a sense, a version of the prospective arm of the current study should continue, and run alongside the schedule for routine laboratory work-up for HLAi transplantation. Figure 6.1 presents a potential schedule for best utilisation of these tests. The lymphocyte subset analysis provides an informative 'snapshot' of a potential transplant participant's immune system, for a relatively simple test, and forms a basis for any subsequent immunophenotyping undertaken. The panels which analysed populations of RTEs, and memory and transitional B cells, were most informative regarding the reconstitution of participants' lymphocyte populations post-transplantation. If the lympho-ablative drug alemtuzumab is continued in use then these panels would inform upon repopulation of the immune system, and could potentially alert the renal care team of any patients not reconstituting in the expected pattern, or with persistently low cell population percentages.

While the activated T cell panel was also informative post-transplantation, the results from participant 1, who was not transplanted, demonstrate a potential for utilising these immunophenotyping markers during the pre-transplant treatment protocol. Along with other test results, changes in these cell populations may correspond with an immune profile which is non-responsive to the desensitisation treatment. The possible association between rising MFI levels of HLA-specific antibodies and changes in the percentage of the population of peripheral plasmablasts warrants further investigation, which would need to be carried out alongside HLA-specific antibody screening, according to the current schedule.

Figure 6.1 Possible schedule for T and B cell immunophenotyping for future prospective patients undergoing HLAi transplantation



More regular identification of this cell population, may provide an answer to the question of whether it is possible to detect an increase in plasmablasts, prior to an increase in antibody levels. This would then potentially provide an opportunity for clinical intervention. Once the optimum testing schedule for this immunophenotyping panel was determined, this could then be employed alongside the panels already mentioned. HLAi transplantation often presents complex immunological cases and these additional tests would contribute additional laboratory data, which may potentially aid the clinical management of these patients. However, it is acknowledged that it would take time for this test repertoire to become embedded into the laboratory work-up, and to ascertain precisely how this new knowledge contributes to informing clinicians of the overall clinical 'picture' of the patient.

These tests may also provide additional valuable data, on an 'ad-hoc' basis, for patients undergoing investigations post-transplantation. For instance, the plasmablast panel could potentially be employed alongside post-transplantation testing for DSA, in patients who are experiencing a decline in graft function, and are possibly starting to reject their transplant. If the association between peripheral plasmablast populations and HLA-specific antibodies is proven, this could provide key information in the problematic scenario of diagnosing AMR in the presence of C4d staining but absence of circulating DSA.

In terms of the data already generated from the immunophenotyping panels employed during this study, it may be interesting to return to the raw data and review it for different cell populations. Kohler and Thiel (2009) suggest that, following the decrease in thymic activity correlated with ageing, the naïve T cell pool may be repopulated by peripheral expansion of CD31-CD45^{RA+} T cells. It may be of interest to track this population of cells (CD31-CD45^{RO-}) to ascertain any recovery made in the central naïve CD4⁺ T cell pool. It would also be worth reviewing the activated T cells panel in order to identify if there is evidence of a HLA-DR^{hi} cell population present in any of the participants. Due to the role of HLA-DR as a late marker of T cell activation, it has been suggested that these cells may be chronically activated, and could be indicative of immunosenescence (Barathan *et al.* 2015).

6.3 Limitations of the study

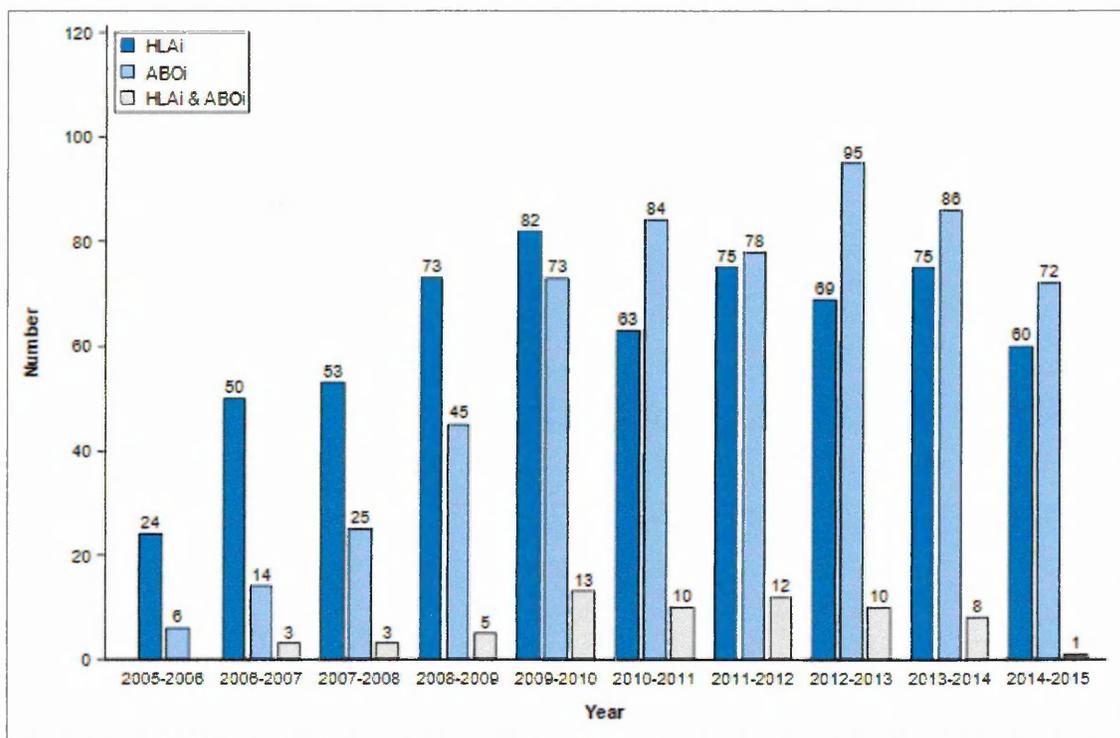
As already described, transplant patients who are being progressed down the HLAi route are assessed in an individualised manner, on a case by case basis. This inevitably meant that the cohort of patients upon which the study was based was extremely diverse, not only in terms of sensitisation history, crossmatch status, DSA present, degree of HLA mismatching between recipient and donor, relationship of donor, and cause of renal failure, but also in the precise desensitisation protocol followed and immunosuppressive drugs administered. One of the main limitations of the study has been the difficulties this has subsequently presented in comparing participants and drawing generalisations between participants. In many ways each of the study participants was unique,

and although commonalities have been identified between patient immunophenotyping results, there have been many exceptions and 'outliers' noted to the overall trend.

During the planning phase of the study, it was anticipated that the prospective cohort would form the main focus, as, at the time, it was envisaged that the HLAi route to transplantation would be a growth area in our centre. However, during the prolonged time spent preparing for, and acquiring, research ethics approval, there was a shift in our approach to the transplantation of patients with an incompatible live donor. The introduction of the paired/pooled scheme by NHSBT-ODT, meant that this was becoming the first choice method towards transplantation for these patients, and consideration for a desensitisation programme was usually only after several unsuccessful rounds in the scheme. This was combined with a move away from managing extremely complex cases, for example multiple antibodies present at high MFI levels, directed against both HLA Class I and Class II antigens, with desensitisation. The outcome of these changes was that, despite an initial prediction of approximately twelve patients progressing down this route, just seven were recruited over the specified time frame, and unfortunately only five of these were transplanted. This local experience is reflected in a similar national trend of a plateau in the number of HLAi transplantations being performed over the timeframe of this study, depicted in figure 6.2 (BTS 2015). Similarly, the reasons suggested for this concur with those described above, indicating that the circumstances in Leeds are part of a larger national development.

The project plan had allowed for this eventuality by describing a dual approach, with a retrospective participant group to also be recruited. Indeed, much interesting data was gathered from the retrospective cohort, but again the group was extremely heterogeneous. As these participants had been previously transplanted, a single sample collection time point was deemed appropriate. This, combined with the fact that the recipients were inevitably at varying time points post-transplantation, also affected the utility of the data, and the conclusions which could be drawn.

Figure 6.2 Number of HLAi and ABOi kidney only transplantations performed each year in the UK, from April 1st 2005 – March 31st 2015



NHSBT (2015, p44)

However, the inclusion of this cohort allowed some of the long-term effects of HLAi transplantation to be perceived. It is anticipated that if this pilot study was extended into future use, with prospective participants, this would allow the collection of more longitudinal data over an extended time frame, with prolonged follow-up. Regular audit would continue to assess the potential impact of these supplementary tests.

Sample collection was further hindered at times by a lack of communication, something which is often the case with laboratory-based studies where the chief investigator is 'one-removed' from the participants. More recently, and since sample collection for this study ceased, a multi-disciplinary team (MDT) meeting to discuss complex cases, which are largely focussed around HLAi and ABOi potential recipient-donor pairs, has been initiated in Leeds, which would be ideal

to facilitate putting this work into routine use, or a similar study in the future. This type of setting is recommended for facilitating excellent clinical and laboratory communication around possible AiT cases (BTS 2015). This is crucial in building up local experience and knowledge, based upon the continuous assessment of previous cases, in order to provide evidence for the risk assessment required to proceed with future cases.

The specialised nature of this approach to transplantation, coupled with the situation described above, meant that the numbers recruited to the study cohorts were small. This made the application of statistical methods somewhat challenging, and hence, some of the discussion and conclusions drawn from the study data have been anecdotal in nature. In addition, the small numbers involved made it difficult to tie any observations in with clinical outcomes, or to stratify the group for analysis.

In an observational study of a small, yet diverse patient group, it is perhaps predictable that it has been difficult to draw conclusions that can be attributed to the process of transplantation, and specifically HLAi transplantation. The effects of the conditioning drugs on the immunophenotyping results are evident, but it is not possible to extricate this from the study to allow the effects of transplantation itself to be clear. The collection of further data, with prolonged follow-up, would help with this and allow the group to be stratified for analysis. A future possibility could also involve a multi-centre approach to recruit more participants.

Another problem encountered due to unforeseen changes during the time of planning and seeking approvals for the study protocol, was due to an alteration in the general immunosuppressive drug regimen for renal transplant patients in Leeds. The introduction of alemtuzumab, a drug resulting in severe lymphopenia, for all HLAi transplant patients caused problems for the proposed schedule of collection of study samples. It was originally planned to collect a study sample approximately one week post-transplantation for the prospective participants, but when the first two were sampled, it was confirmed that insufficient lymphocytes were present for full analysis. This will remain a problem for immunophenotyping

these types of patients post-transplantation, for instance if attempting to monitor plasmablast populations as described above. As previously alluded to in the discussion of chapter five, the monitoring of small percentages of a small absolute number of cells must be acknowledged as a limitation of this study, and examining cell populations post-alemtuzumab treatment exacerbates this problem.

Finally, an unavoidable limitation of the study involved the technology employed. Each peripheral blood sample analysed merely provided a 'snapshot' of the populations of lymphocytes present at a particular time point. Furthermore, no information may be gleaned of what was occurring in the lymphoid organs. Memory B cells and plasma cells are known to remain in survival niches, such as the spleen, where desensitisation treatments cannot target them (Ramos *et al.* 2007). In order to gain a more thorough 'picture' it would, therefore, be necessary to examine lymphoid tissue samples and bone marrow extracts, which would clearly require additional research ethics approval. Although this limitation is acknowledged, peripheral blood represents a 'window' into the immune system and was an appropriate sample choice for this pilot study.

An inherent limitation of the technology of flow cytometry employed in this study is that it provides information about cell phenotype but not necessarily function. In order to fully understand the role of the cells present, cytokine expression studies would be required. Finally, some of the advantages and disadvantages of bead-based HLA-specific antibody screening methods have already been mentioned.

A research project of this nature, which is carried out in the 'working world' will inevitably have shortcomings related to the technology utilised, the study design and the interpretation of the data. Therefore, an attempt has been made to acknowledge these weaknesses, and to take them into account when drawing conclusions.

6.4 Comments on the future direction of HLAi transplantation

The success of kidney transplantation as a treatment means that it is essential that access to donor organs is an equitable and transparent process. However, this must be balanced against the best utility of this valuable resource. Various strategies to improve the access to transplantation for sensitised patients have been investigated (Fuggle and Martin 2004; Doxiadis, Duquesnoy and Claas 2005). However, despite prioritisation in the deceased donor national kidney allocation scheme (NHSBT 2016c) for highly sensitised patients, defined as $\geq 85\%$ cRF, these potential recipients continue to experience increased waiting times. Notably, most recent analyses (Bradbury 2016) have indicated that prioritisation of long waiting patients, for a deceased donor kidney offer, has failed to address the problem for the most highly sensitised individuals.

Within the UK, there are currently two main strategies for approaching transplantation for recipients who have a potential live donor who is HLA incompatible. Alongside HLAi transplantation, the national living donor kidney sharing schemes, organised by NHSBT-ODT, represent the second part to this dual tactic. The paired/pooled scheme facilitates national sharing of live donor kidneys within an algorithm that allows paired exchanges of kidneys, or more complicated chains of exchanges. These chains can now be initiated by a non-directed altruistic donation (NDAD), from a live donor who wishes to donate to the pool.

As standard compatible live donor transplants have superior outcomes to HLAi transplants, it is recommended that potential HLAi recipient-donor pairs enter the exchange scheme before proceeding down the HLAi transplantation route (BTS 2015). However, it is acknowledged that clinical urgency may prompt a more direct progression to HLAi transplantation. In addition, individual choice may lead some potential recipients towards this route sooner, with their preferred live donor of choice.

In any case, due to the nature of sensitisation to HLA, not all patients will be matched with a live donor in the paired-pooled scheme. For this reason, a place for HLAi transplantation as an option should remain. Indeed, in certain cases

these two options may be combined, with the removal of unacceptable antigens when entering the paired-pooled scheme, if it is deemed that these antibodies can be dealt with clinically peri- and post-transplantation by some degree of antibody removal therapy (BTS 2015). Using the same principle, some centres are using this approach of removing low level HLA-specific antibodies listed with NHSBT, for sensitised patients waiting for deceased donor transplantation.

Pre-conditioning for HLAi transplantation in the deceased donor setting also provides another area where this type of transplant may be utilised for a sensitised patient. However, this is difficult to manage logistically due to the inability to predict an offer of a deceased kidney, and is usually reserved for clinically urgent cases. In addition, current graft and patient survival rates for HLAi deceased donor transplantation are considerably below those for all other types of transplantation. Yet, it must be reiterated that these types of decisions are based around a risk assessment; the long-term mortality rates of a deceased donor HLAi transplantation still compare well with the mortality associated with continued dialysis (BTS 2015).

Increasingly acceptable outcomes and continued clinical need ensures that HLAi transplantation, in its various guises, will remain firmly embedded in a 'multi-faceted' strategy of transplanting sensitised patients. However, these transplants will most likely remain as individualised cases, progressed on a risk assessed basis. As this study highlights, there is still much knowledge to be gained and potential H&I laboratory support that could be given, that may improve the delivery of these programmes and thereby access to the superior treatment of transplantation.

Total word count = 42,902

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Appendix 1 A copy of the letter for participants' GPs, submitted with the NHS
REC application and granted final approval in July 2011

Version 1.0 22/03/11

Immunological Profiling in AiT Recipients Version 1

The Leeds Teaching Hospitals 
NHS Trust

Pathology Directorate
Department of Transplant Immunology
CPA Accredited Laboratory
EFI Accredited Laboratory

St James's University Hospital
Level 09 Gledhow Wing
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LS9 7TF UK

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<http://www.leedsth.nhs.uk/>

Address

Date

Dear Dr

**Re: Patient:
Date of birth:**

The above named patient has consented for recruitment into the 'Immunological Profiling in Renal Transplant Recipients Receiving an Antibody Incompatible Kidney' study. As part of the study protocol, blood samples will be collected, for laboratory analysis, pre- and/or post-kidney transplantation at set time points, dependent on whether the patient has been recruited as part of the study group or as a control. No changes will be made to their routine clinical care or medication.

This letter is purely for information purposes. There will be no requirement for any additional clinical input from the patient's normal primary care practitioners.

Please do not hesitate to contact me if you have any further queries.

Yours sincerely,

**Katherine Mounsey DipRCPath
Principal Clinical Scientist**

Chairman Mike Collier Chief Executive Maggie Boyle

The Leeds Teaching Hospitals incorporating: Chapel Allerton Hospital Leeds Chest Clinic Leeds Dental Institute
Seacroft Hospital St James's University Hospital The General Infirmary at Leeds Wharfedale Hospital

Version 2.0 15/06/11

The Leeds Teaching Hospitals 
NHS Trust

PARTICIPANT INFORMATION SHEET

Immunological Profiling in Renal Transplant Recipients

Receiving an Antibody Incompatible Kidney

Introduction

You are being invited to take part in a research study. Before you decide whether to take part, it is important for you to understand why the research is being done, and what it will involve. Please take time to read the following information carefully, and discuss it with others if you wish.

Please ask us if there is anything that is not clear, or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this information sheet and for considering taking part in this study.

Background to the study

As you know, transplantation provides the best treatment for patients with advanced kidney failure. However, sometimes it can be very difficult identifying a compatible donor for patients who have anti-donor antibodies. Antibody incompatible transplantation (AiT) is a specialised procedure that helps patients with anti-donor antibodies to be transplanted, usually with a living donor. However, there is much that it is not understood about the biological process of this procedure.

What is the purpose of the study?

The purpose of the study is to examine the different populations of immune cells, present in the blood, at different stages of AiT. This information will be combined with other laboratory and clinical results, with the aim of building up an 'immunological profile' of AiT patients. In the future, this may allow us to carry out additional monitoring of patients undergoing AiT, with the overall long-term aim of improving transplant outcome.

Why have I been chosen?

You have been chosen because you are either preparing to be transplanted with a living donor, or you have been transplanted in the past with a living donor kidney, at St James's Hospital, and you are attending follow-up sessions in the transplant clinic. If you have previously had, or are preparing for, an antibody incompatible kidney transplant, then you are being invited to be part of the study group. If you have had, or are preparing for, a standard live donor kidney transplant, then you are being invited to be part of the comparison group for the study.

What will I have to do?

If you are preparing for transplantation with an antibody incompatible living donor, you will be asked to donate approximately 15 mls of blood (about 3 teaspoons) 3 times during the fortnight before your transplant, and 3 times during the fortnight following your transplant, then again at approximately 6 months and 1 year following your transplant. You may also be asked to donate an additional 10mls of blood (about 2 teaspoons) at the 6 months and 1 year time points. These blood samples would be taken when we see you in the clinic (either at St James's or at your referral centre).

If you have been transplanted with an antibody incompatible living donor in the past, you will be asked to donate between 15-25 mls of blood (about 3-5 teaspoons) once, when we see you in the clinic for follow up. In addition, we may wish to test samples that you have previously given as part of your routine care, and which are stored in the laboratory.

If you are preparing for transplantation with a standard living donor, you will be asked to donate 15 mls of blood (about 3 teaspoons) once before your transplant, and 15-25 mls of blood once during the week following your transplant, and between 6 months to a year after your transplant. These blood samples would be taken when we see you in the clinic (either at St James's or at your referral centre).

If you have been transplanted with a standard living donor in the past, you will be asked to donate between 15-25 mls of blood (about 3-5 teaspoons) once, when we see you in the clinic for follow up.

We will study your blood samples in the laboratory (at St James's) to analyse the different populations of immune cells present before and after transplantation. Any leftover cells will be disposed of. We may also test for the presence of anti-donor antibodies, and examine these antibodies in greater detail. This may include testing samples that you have previously given as part of your routine care, and which are stored in the laboratory. Any new samples given for this purpose will be stored in the laboratory, according to laboratory protocol.

- **You will not be asked to give blood if you are anaemic**

Your interest in participation will be established by a member of your clinical care team, who will then discuss the study further with you. If you agree, they will obtain your written consent, and plan the day on which the blood sample may be taken. You will receive a copy of this information sheet to keep.

What are the benefits?

There are no immediate benefits to you, and participation in the study will have no influence on your treatment. However, the long term aim of this study is to increase our understanding of antibody incompatible kidney transplantation. In the future we hope that this will help doctors to be able to modify treatment, with the aim of improving transplant outcome.

What are the risks?

The risks are very small. The amount of blood taken (up to 25mls, about 5 teaspoons) is small, and is not harmful (for example the volume is very much less than the loss of a clotted haemodialysis circuit).

What if I do not want to take part?

Involvement in this study is entirely voluntary. It is up to you to decide whether or not you wish to take part. If you decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. If you give your consent to take part, you are still free to withdraw at any time without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the care that you receive.

Will my taking part in this study be kept confidential?

All information collected from the study will be kept entirely confidential.

What will happen to the results of the research study?

The results of the study will be published in medical or scientific journals, and presented at scientific meetings. In addition, the results will be written up (in fully anonymised form), by Ms Mounsey, as a thesis, and submitted as part-fulfilment of a Doctorate in Professional Studies (Biomedical Science) to Sheffield Hallam University. A summary sheet of research findings will be available from Ms Mounsey.

What if there is a problem?

Any complaint about the way you have been dealt with during the study, or any possible harm you might suffer, will be addressed. If you have a concern about any aspect of this study, you should ask to speak to the member of the clinical care team who took your consent, or Ms Mounsey who will do their best to answer your questions. If you remain unhappy and wish to complain formally, you can do this by contacting the Patient Advice and Liaison Service (PALS), contact telephone number: 0113 206 7168.

Who is organising and funding the research?

The Chief Investigator of this study is Ms Katherine Mounsey, Principal Clinical Scientist, based within the Transplant Immunology Laboratory at St James's Hospital. Ms Mounsey is carrying out this study as part-fulfilment of a Doctorate in Professional Studies (Biomedical Science) at Sheffield Hallam University. Ms Mounsey is supervised by Dr Brendan Clark (Consultant in Histocompatibility and Immunogenetics - H&I, at St James's) in collaboration with Dr Chas Newstead (Consultant Renal Physician).

Further contact information

If you would like further information about the study, you can contact any of the following:

Ms Katherine Mounsey
Transplant Immunology
Gledhow Wing, Level 9
St James's University Hospital
Beckett Street
Leeds
LS9 7TF
Tel: 0113 206 4579
Email: katherine.mounsey@leedsth.nhs.uk

Dr Clive Carter
Cellular Immunology
Gledhow Wing, Level 9
St James's University Hospital
Beckett Street
Leeds
LS9 7TF
Tel: 0113 206 4579
Email: clive.carter@leedsth.nhs.uk

For Leeds clinic:

Rosalyn Wheatley (Research Nurse) or Kay Tobin (Senior Research Nurse), Renal Research, St James's University Hospital, Leeds
Transplant Co-ordinators Joanne Barwick, Georgina Speak or Kathryn Brady, Renal and Transplant Medicine, St James's University Hospital, Leeds

For Bradford clinic:

Transplant Co-ordinators Claire Burton, Michael Speight or Helen Phelan, Renal Medicine, St Luke's Hospital, Bradford

For York clinic:

Consultant Renal Physicians Dr David Border, or Dr Colin Jones, Renal Medicine, York Hospital

For Hull clinic:

Consultant Renal Physician Dr David Eadington or Transplant Co-ordinator Julie Hill, Renal Medicine, Hull Royal Infirmary

If you would rather initially discuss your participation with a knowledgeable individual not directly involved in the study:

Patient Advice and Liaison Service
Patient Relations Department
Trust Headquarters
St James's Hospital
Leeds
LS9 7TF
Tel: 0113 206 7168
Email: patient.relations@leedsth.nhs.uk

Tufail Anwar (Research Nurse)
Renal Medicine,
St Luke's Hospital,
Bradford
BD5 0NA
Tel: 01274 365536

The Leeds Teaching Hospitals

NHS Trust PARTICIPANT CONSENT FORM

Immunological Profiling in Renal Transplant Recipients

Receiving an Antibody Incompatible Kidney

(Please initial box)

1. I confirm that I have read and understand the information sheet for the above study, and have had the opportunity to ask questions.
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without my medical care or legal rights being affected.
3. I understand that even if I withdraw from the above study, the data and samples collected from me will be used in analysing the results, unless I specifically withdraw consent for this. I understand that my identity will remain anonymous.
4. I consent to my GP being notified of my participation in this study.
5. I consent to storage, including electronic, of personal information for the purposes of this study. I understand that any information that could identify me will be kept strictly confidential, and no personal information will be included in the study report or other publication.
6. I agree to allow the use of any information or results arising from this study for presentation or publication. I understand that my identity will remain anonymous.
7. I understand that relevant sections of my medical notes and data, collected during the study, may be looked at by individuals from the Renal Care Team, from regulatory authorities or from the NHS Trust, where it is relevant to me taking part in this research. I give permission for these individuals to have access to my records.
8. I agree to take part in the study. I understand this includes giving fresh blood samples and, if necessary, the testing of samples that I have previously given as part of my routine care, and which are stored in the laboratory.

Signatures:

NAME OF THE PATIENT	DATE	SIGNATURE
---------------------	------	-----------

NAME OF THE PERSON TAKING THE CONSENT	DATE	SIGNATURE
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Appendix 3 Copy of the participant information sheet and consent form for the normal controls, submitted with the NHS REC application and granted final approval in July 2011

Version 1.0 15/06/11

The Leeds Teaching Hospitals 
NHS Trust

PARTICIPANT INFORMATION SHEET (NORMAL CONTROLS)

Immunological Profiling in Renal Transplant Recipients

Receiving an Antibody Incompatible Kidney

Introduction

You are being invited to take part in a research study. Before you decide whether to take part, it is important for you to understand why the research is being done, and what it will involve. Please take time to read the following information carefully, and discuss it with others if you wish.

Please ask us if there is anything that is not clear, or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this information sheet and for considering taking part in this study.

Background to the study

Transplantation provides the best treatment for patients with advanced kidney failure. However, sometimes it can be very difficult identifying a compatible donor for patients who have anti-donor antibodies. Antibody incompatible transplantation (AiT) is a specialised procedure that helps patients with anti-donor antibodies to be transplanted, usually with a living donor. However, there is much that it is not understood about the biological process of this procedure.

What is the purpose of the study?

The purpose of the study is to examine the different populations of immune cells, present in the blood, at different stages of AiT. This information will be combined with other laboratory and clinical results, with the aim of building up an 'immunological profile' of AiT patients. In the future, this may allow us to carry out additional monitoring of patients undergoing AiT, with the overall long-term aim of improving transplant outcome. In order for the research study to have scientific rigour, it is necessary to obtain laboratory data from 'normal' individuals, against which to compare the patient results.

Why have I been chosen?

As an NHS staff colleague, you are being invited to be part of the 'normal' comparison group for the study. In order to draw scientifically valid conclusions from the study data, it is necessary to compare the results from kidney transplant patients with 'normal' controls. You will be able to participate in the study as a 'healthy volunteer' if you are able to confirm that you have no history of renal failure or transplantation.

What will I have to do?

As a **healthy volunteer**, you will be asked to donate approximately 15 mls of blood (about 3 teaspoons) once.

We will study your blood sample in the laboratory (at St James's) to analyse the different populations of immune cells present. Any leftover cells will be disposed of.

Your interest in participation will be established by the Chief Investigator, who will then discuss the study further with you. If you agree, they will obtain your written consent, and plan the day on which the blood sample may be taken, by a member of the renal clinical care team. You will receive a copy of this information sheet to keep. Your blood sample results will be anonymised for the purpose of analysis, with only data on your gender and age being stored. You will receive no feedback on your individual sample results.

What are the benefits?

There are no benefits to you. However, the long term aim of this study is to increase our understanding of antibody incompatible kidney transplantation. In the future we hope that this will help doctors to be able to modify treatment, with the aim of improving transplant outcome.

What are the risks?

The risks are very small. The amount of blood taken (up to 15mls, about 3 teaspoons) is small, and is not harmful.

What if I do not want to take part?

Involvement in this study is entirely voluntary. It is up to you to decide whether or not you wish to take part. If you decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. If you give your consent to take part, you are still free to withdraw at any time without giving a reason.

Will my taking part in this study be kept confidential?

All information collected from the study will be kept entirely confidential.

What will happen to the results of the research study?

The results of the study will be published in medical or scientific journals, and presented at scientific meetings. In addition, the results will be written up (in fully anonymised form), by Ms Mounsey, as a thesis, and submitted as part-fulfilment of a Doctorate in Professional Studies (Biomedical Science) to Sheffield Hallam University. A summary sheet of research findings will be available from Ms Mounsey.

What if there is a problem?

Any complaint about the way you have been dealt with during the study, or any possible harm you might suffer, will be addressed. If you have a concern about any aspect of this study, you should ask to speak to Ms Mounsey, who will do her best to answer your questions.

Who is organising and funding the research?

The Chief Investigator of this study is Ms Katherine Mounsey, Principal Clinical Scientist, based within the Transplant Immunology Laboratory at St James's Hospital. Ms Mounsey is carrying out this study as part-fulfilment of a Doctorate in Professional Studies (Biomedical Science) at Sheffield Hallam University. Ms Mounsey is supervised by Dr Brendan Clark (Consultant in Histocompatibility and Immunogenetics - H&I, at St James's) in collaboration with Dr Chas Newstead (Consultant Renal Physician).

Further contact information

If you would like further information about the study, you can contact either of the following:

Ms Katherine Mounsey
Transplant Immunology
Gledhow Wing, Level 9
St James's University Hospital
Beckett Street
Leeds
LS9 7TF
Tel: 0113 206 4579
Email: katherine.mounsey@leedsth.nhs.uk

Dr Clive Carter
Cellular Immunology
Gledhow Wing, Level 9
St James's University Hospital
Beckett Street
Leeds
LS9 7TF
Tel: 0113 206 4579
Email: clive.carter@leedsth.nhs.uk

If you would rather initially discuss your participation with a knowledgeable individual not directly involved in the study:

Patient Advice and Liaison Service
Patient Relations Department
Trust Headquarters
St James's Hospital
Leeds
LS9 7TF
Tel: 0113 206 7168
Email: patient.relations@leedsth.nhs.uk

Tufail Anwar (Research Nurse)
Renal Medicine,
St Luke's Hospital,
Bradford
BD5 0NA
Tel: 01274 365536

The Leeds Teaching Hospitals

NHS Trust

PARTICIPANT CONSENT FORM (NORMAL CONTROLS)

Immunological Profiling in Renal Transplant Recipients

Receiving an Antibody Incompatible Kidney

(Please initial box)

1. I confirm that I have read and understand the information sheet for the above study, and have had the opportunity to ask questions.
2. I understand that my participation is voluntary and that I am free to withdraw at any time. I understand that my status and position in the workplace will not be affected by the decision that I make.
3. I understand that even if I withdraw from the above study, the data and samples collected from me will be used in analysing the results, unless I specifically withdraw consent for this. I understand that my identity will remain anonymous.
4. I consent to storage, including electronic, of personal information for the purposes of this study. I understand that any information that could identify me will be kept strictly confidential, and no personal information will be included in the study report or other publication.
5. I agree to allow the use of any information or results arising from this study for presentation or publication. I understand that my identity will remain anonymous.
6. I confirm that:
 - I am fit and healthy
 - I have never suffered from renal failure
 - I have never had a transplant.
7. I confirm that I do not have, or suspect I have, an infection, and have not been exposed to an infection, which may endanger my colleague in taking my blood sample.
8. I agree to take part in the study. I understand this includes giving a fresh blood sample.

Signatures:

NAME OF THE PATIENT	DATE	SIGNATURE

NAME OF THE PERSON TAKING THE CONSENT	DATE	SIGNATURE

Appendix 4 A summary of manufacturers and suppliers of reagents used in the study

General laboratory chemicals were manufactured by BDH and supplied by VWR.

Becton, Dickinson and Company, BD Biosciences

The Danby Building
Edmund Halley Road
Oxford Science Park, Oxford OX4 4DQ
England
<http://www.bd.com/uk/>
Products supplied by BD Biosciences.

Cedarlane

1210 Turrentine Street
Burlington, NC, 27215
<https://www.cedaranelabs.com/>
Products supplied by VH Bio Limited, Unit 11B Station Approach, Team Valley
Trading Estate, Gateshead, NE11 0ZF
<http://www.vhbio.com/>

Invitrogen (Thermo Fisher Scientific Incorporated)

3 Fountain Drive
Inchinnan Business Park
Paisley
UK
Supplied by Life Technologies Limited (Thermo Fisher Scientific Incorporated).
<http://www.lifetechnologies.com/uk/en/home.html>

One Lambda Incorporated (Thermo Fisher Scientific Incorporated)

21001 Kittridge,
Canoga Park,
California,
USA.

<http://www.onelambda.com/en/home.html>

Supplied by VHBio Limited, Unit 11B Station Approach, Team Valley Trading Estate, Gateshead, NE11 0ZF

<http://www.vhbio.com/>

Promega Corporation

2800 Woods Hollow Road,

Madison, WI 53711

USA

<http://www.promega.co.uk/>

Supplied by Promega UK, Southampton, UK

Sigma-Aldrich Limited

The Old Brickyard,

New Road,

Gillingham,

Dorset

SP8 4XT

<https://www.sigmaaldrich.com/united-kingdom.html>

Supplied by Sigma-Aldrich Company Limited.

Appendix 5 Serum creatinine levels for the retrospective participant cohort

Participant ID	Creatinine ($\mu\text{mol/L}$)		
	Approximately 6 months post-transplantation	Approximately 1 year post-transplantation	Latest (time post-transplant date)
4	166	163	167 (2 years and 7 months)
5	117	131	491 (3 years)
6	119	130	159 (3 years)
8	123	132	115 (12 years and 9 months)
10	118	118	105 (4 years)
12	105	104	111 (9 years and 7 months)
13	129	131	145 (7 years)
15	118	115	81 (5 years and 3 months)
16	695	1058	913 (9 years and 5 months)
17	206	198	607 (9 years and 4 months)
18	283	257	282 (5 years and 5 months)
19	148	243	201 (5 years and 3 months)
20	140	117	144 (7 years)

ID – identification

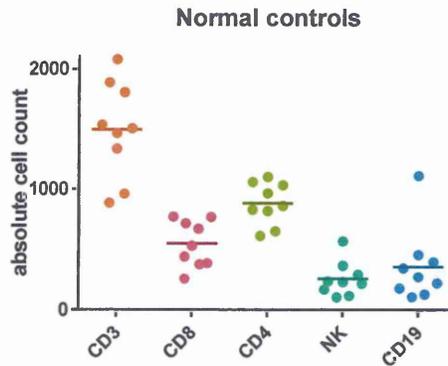
$\mu\text{mol/L}$ – micromoles per litre

Adult reference range for serum creatinine (Leeds & Bradford Pathology Service 2011):

Male 64-104 $\mu\text{mol/L}$; Female 49-90 $\mu\text{mol/L}$

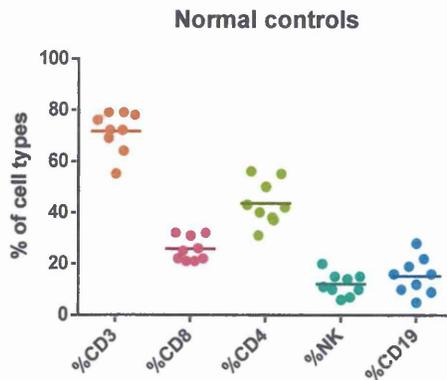
Appendix 6 Results of lymphocyte subset analysis for the normal controls

Results of lymphocyte subset analysis (absolute cell counts) for the normal controls



Absolute cell counts (cells/ μ l). Horizontal line indicates mean.
Normal controls, n= 9.

The lymphocyte subset percentages of total lymphocytes for the normal controls.



Lymphocyte subsets plotted as a percentage of total lymphocytes (CD45⁺) cells.
Horizontal line indicates mean. Normal controls, n= 9.

Appendix 7 A comparison of the median results of lymphocyte subsets for the study normal controls data with published data for healthy adults

Lymphocyte subset	CD45⁺	CD3⁺	CD8⁺	CD4⁺	CD19⁺	NK
Study data	2.1	1.5	0.5	0.9	0.3	0.2
Published data	1.8	1.2	0.4	0.7	0.2	0.3

Absolute counts ($\times 10^9/l$)

Published data is for healthy adults (n = 51) (Bonilla and Oettgen 1997)

Study normal controls (n = 9)

Appendix 8 Results from the LABScreen® Single Antigen assay for prospective participant 7

Sample ID	% PRA	HLA specificities (MFI >1000)	Highest MFI	DSA present in sample (MFI)
HLA Class I results:				
Day -8	0	None detected	N/A	N/A
Day +5	0	None detected	N/A	N/A
+8 months	0	None detected	N/A	N/A
+13 months	0	None detected	N/A	N/A
HLA Class II results:				
Day -8	18	DQ4, DQ7, DQ8, DQ9	7049	DQ7 (2143)
Day +5	3	DQA1*03?	2642	DQA1*03 (DQA1*03:01=537, DQA1*03:02=1135, DQA1*03:03=2642)
+8 months	10	DQ4, DQ9, DQA1*03:01?	7537	DQ3? (DQB1*03:03 = 2167[bead 069]+418[bead 045]+1174[bead 044]) DQA1*03? (DQA1*03:01=1193, DQA1*03:02=2137, DQA1*03:03=7537)
+13 months	10	DQ4, DQ9, DQA1*03:01?	6541	DQ3? (DQB1*03:03 = 1678[bead 069]+337[bead 045]+1077[bead 044]) DQA1*03? (DQA1*03:01=1050, DQA1*03:02=1629, DQA1*03:03=6541)

ID - identification; % PRA – percentage panel reactive antibody, calculated for each test by dividing the number of positive beads by the total number of beads for each panel, and multiplying by 100; HLA - human leucocyte antigen; MFI – mean fluorescence intensity; DSA – donor specific antibody; N/A – not applicable.

Appendix 9 Results from the LABScreen® Single Antigen assay for prospective participant 9

Sample ID	% PRA	HLA specificities (MFI >1000)	Highest MFI	DSA present in sample (MFI)
HLA Class I results:				
+6 months	0	None detected	N/A	N/A
+1 year	0	None detected	N/A	N/A
HLA Class II results:				
+6 months	74	DR1, DR4, DR9, DR11, DR12, DR13, DR14, DR15, DR16, DR17 (self) , DR18 , DR51, DQ4, DQ6, DQ7, DQ8, DQ9, DP1, DP3, DP5, DP6, DP9, DP10, DP11, DP13, DP14, DP15, DP17, DP19, DP23	19,742	DR4 (DRB1*04:05 = 12,679, 04:04 = 16,687, 04:01 = 15,099, 04:02 = 8864, 04:03 = 9851) DQ8 (03:02 = 10,341[bead 068], 8431[bead 042], 7863[bead 043], 7891[bead 082], DP1 (15,778[bead 083], 17,408[bead 046], DP3 (17,750[bead 091], 16,707[bead 084], 17,117[bead 048]
+1 year	64	DR1, DR4, DR9, DR10, DR11, DR12, DR13, DR14, DR15, DR16, DR18 , DR51, DQ4, DQ6, DQ7, DQ8, DQ9, DP1, DP3, DP5, DP6, DP9, DP10, DP11, DP13, DP14, DP17, DP19	9719	DR4 (DRB1*04:01 = 9045, 04:04 = 9719, 04:05 = 9529, 04:02 = 5026, 04:03 = 6904) DQ8 (03:02 = 6867[bead 068], 5120[bead 042], 4606[bead 043], 4661[bead 082], DP1 (5478[bead 046], 4428[bead 083], DP3 (5997[bead 048], 5871[bead 091], 5485[bead 084]

ID - identification; % PRA – percentage panel reactive antibody, calculated for each test by dividing the number of positive beads by the total number of beads for each panel, and multiplying by 100; HLA - human leucocyte antigen; MFI – mean fluorescence intensity; DSA – donor specific antibody; N/A – not applicable.

Samples Day -12 and Day -5, data not shown, but available as part of routine laboratory transplant work-up.

Appendix 10 Results from the LABScreen® Single Antigen assay for prospective participant 11

Sample ID	% PRA	HLA specificities (MFI >1000)	Highest MFI	DSA present in sample (MFI)
HLA Class I results:				
+6 months	4	B57, B58, B63	5658	None
+1 year	4	B57, B58, B63	5164	None
HLA Class II results:				
+6 months	0	None detected	N/A	N/A (weak DRB1*13:03 = 542)
+1 year	0	None detected	N/A	N/A (weak DRB1*13:03 = 967)

ID - identification; % PRA – percentage panel reactive antibody, calculated for each test by dividing the number of positive beads by the total number of beads for each panel, and multiplying by 100; HLA - human leucocyte antigen; MFI – mean fluorescence intensity; DSA – donor specific antibody; N/A – not applicable.

Day -9, data not shown, but available as part of routine laboratory transplant work-up.

Appendix 11 Results from the LABScreen® Single Antigen assay for prospective participant 14

Sample ID	% PRA	HLA specificities (MFI >1000)	Highest MFI	DSA present in sample (MFI)
HLA Class I results:				
+6 months	44	A2, A28, B5, B12, B13, B15, B16, B17, B18, B21, B35, B37, B40, B41, B46, B53, B55, B56, B59, B67, B70, B78	13,032	None
+1 year	39	A2, A68, A69, B13, B35, B41, B44, B45, B46, B47, B49, B50, B51, B52, B53, B55, B56, B57, B58, B60, B61, B71, B72, B62, B63, B75, B76, B77, B78	9889	None
HLA Class II results:				
+6 months	25	DR3, DR5, DR6, DR7, DR8, DR9, DR52, DQ2	17,886	None
+1 year	25	DR7, DR8, DR9, DR11, DR12, DR13, DR14, DR17, DR18, DR52, DQ2	19,806	None

ID - identification; % PRA – percentage panel reactive antibody, calculated for each test by dividing the number of positive beads by the total number of beads for each panel, and multiplying by 100; HLA - human leucocyte antigen; MFI – mean fluorescence intensity; DSA – donor specific antibody; N/A – not applicable.

Day -14 and Day 0, data not shown, but available as part of routine laboratory transplant work-up.

+6 months was analysed as a part of the routine antibody screening laboratory work.

Appendix 12 HLA Class I results, from the LABScreen® Single Antigen assay, for the retrospective participants

Participant ID	% PRA	HLA specificities (MFI >1000)	Highest MFI	DSA present in sample (MFI)
4	1	B8	1025	B8 (1025)
5	36	A1, A23, A24, A25, A32, A36, A80, B8, B13, B27, B37, B38, B44, B45, B46, B47, B49, B50, B51, B52, B53, B57, B58, B59, B63, B75, B76, B77, B82, Cw15	19,819	None
6	3	Cw9, Cw10	11,696	None
8	0	None detected	N/A	N/A
10	4	A29, A68, B67, Cw16	1474	None
12	6	A23, A24, B57, B58	2491	None (Weak B7 = 911)
13	25	A1, A11, A23, A24 (self) , A29, A36, A80, B49, B50, B54, B55, B56, B57, B58, B63, B76, Cw2, Cw4, Cw5, Cw6, Cw15, Cw17, Cw18	14,582	A11 (cross-reactive) (A*11:02 = 1862, A*11:01 = 761)
15	0	None detected	N/A	None
16	81	A2, A3, A11, A23, A24, A25, A26, A34, A66, A29, A30, A31, A32, A33, A74, A36, A43, A68, A69, B8, B13, B18, B35, B37, B38, B39, B41, B42, B44, B45, B46, B47, B48, B49, B50, B51, B52, B53, B54, B55, B56, B57, B58, B59, B60, B61, B64, B65, B67, B71, B72, B62, B63, B75, B76, B77, B78, B82, Cw9, Cw10, Cw15, Cw17	18,387	A3 (6981), A11 (A*11:01 = 15,425, A*11:02 = 17,816), B35 (15,680)

Appendix 12 continued

17	64	A1, A3, A11, A23, A24, A26, A34, A66, A29, A30, A31, A33, A74, A36, A43, A80, B7, B13, B27, B35, B42, B46, B47, B48, B49, B50, B51, B52, B53, B54, B55, B56, B57, B58, B60, B61, B67, B71, B72, B73, B62, B63, B75, B76, B77, B78, B81, B82, Cw9, Cw10	14,557	B51 (B*51:01 = 2922, B*51:02 = 6900) [B35 (6735) B52 (1925) cross-reactive]
18	2	B8, B82	12,996	None (weak B*44:02 = 913, B*45 = 575)
19	1	B46	3454	None
20	4	A1, A26, A36, A43	4549	A1 (4549)

ID - identification; % PRA – percentage panel reactive antibody, calculated for each test by dividing the number of positive beads by the total number of beads for each panel, and multiplying by 100; HLA - human leucocyte antigen; MFI – mean fluorescence intensity; DSA – donor specific antibody; N/A – not applicable

Appendix 13 HLA Class II results, from the LABScreen® Single Antigen assay, for the retrospective participants

Participant ID	% PRA	HLA specificities (MFI >1000)	Highest MFI	DSA present in sample (MFI)
4	0	None detected	N/A	N/A
5	23	DR1, DR103, DR9, DR10, DR53, DQ7 (self) , DQ8, DP1, DP3, DP5, DP6, DP9, DP10, DP13, DP14, DP15, DP17, DP18, DP28 DPA*02:01?	3979	DR1 (01:01 = 1213, 01:02 = 861), DQ8 (03:02 = 1181+758+53+92), DR53 (DRB4*01:03 = 1162, DRB4*01:01 = 756) (DQ5 is 'weak')
6	8	DQ7, (DQ8, DQ9?) DQA1*03:02	8840	?DQ8 (03:02 = 1325+311+547+460) DQA1*03:02?? Donor is DQA1*03
8	0	None detected	N/A	N/A
10	41	DR1, DR4, DR12, DR15, DR16, DR51, DR52 (self) , DQ2, DQ4, DQ7 (self) , DP1, DP2, DP3, DP4, DP6, DP11, DP15, DP18, DP19, DP28	4836	DR4 (DRB1*04:02 = 2324, 04:04 = 3693, 04:05 = 576, 04:03 = 2854, 04:01 = 2181) (DQ8 03:02 = 815+534+484+292 - weak DSA) DR53 = weak DSA DRB4*01:03 = 674, DRB4*01:01 = 515) DP3 (03:01 = 2571, 1215, 1061)
12	0	None detected	N/A	N/A
13	22	DR8, DR11, DR12, DR13, DR14, DR15, DR16, DR17, DR18, DR52	4338	None
15	0	None detected	N/A	N/A

Appendix 13 continued

16	51	DR4, DR7, DR8, DR9, DR10, DR11, DR12, DR13, DR14, DR15, DR16, DR17, DR18, DR52, DR53, DQ2, DQ6, DQ7, DQ8, DQ9	20,271	DR4 (DRB1*04:04=19,158, 04:05=17,795, 04:03=17,493, 04:02=18,208, 04:01=17,624) DR11 (11:01=14,891, 11:04=18,225), DQ7 (03:01=11,740+10,563+11,914+11,300+8024), DR52 (DRB3*01:01=13,148, 02:02=13,572, 03:01=3982) DR53 (DRB4*01:01=9287, 01:03=13,047)
17	11	DQ7, DQ8, DQ9	3904	None
18	0	None detected	N/A	N/A
19	24	DR7, DR9, DR53, DQ2, ?DQ4, DP3, DP6, DP9, DP14, DP17 DQA1*02:01?	19,799	None (weak DR1 = 01:01 = 876, 01:02 = 616)
20	0	None detected	N/A	N/A

ID - identification; % PRA – percentage panel reactive antibody, calculated for each test by dividing the number of positive beads by the total number of beads for each panel, and multiplying by 100; HLA - human leucocyte antigen; MFI – mean fluorescence intensity; DSA – donor specific antibody; N/A – not applicable

Appendix 14 Supplementary HLA typing results to assist in the interpretation of the LABScreen® assay results

Participant ID	HLA type (LABType® SSO)	HLA type (AllSet™ Gold SSP)	HLA mismatches	Comments
Participant 5	DPA1*01:03 DPB1*04:01, 04:02		DPB1*16:01	DPB1 types agree with those already held on record
Donor	DPA1*01:03 DPB1*04:01, 16:01			
Participant 6	DQA1*01:01+, 02:01 DQB1*02:02+, 05:03+		DQA1*03 DQB1*03	DQB1 types agree with those already held on record
Donor	DQA1*01:01+, 03:01+ DQB1*03:05, 05:01+			
Participant 19	DPA1*02:02+ DPB1*02:01, 05:01 DQA1*04:01, 06:01 DQB1*03:01+	DPB1*02:01, 05:01	DPA1*01:03 DPB1*04:01 DQA1*01:01+, 05:05+ DQB1*05	Concordance between AllSet™ and LABType® DPB1 data DQB1 types agree with those already held on record
Donor	DPA1*01:03 DPB1*02:01, 04:01 DQA1*01:01+, 05:05+ DQB1*03:01+, 05:01+	DPB1*02:01, 04:01		

Appendix 14 continued

Participant 10	DPA1*01:03	DPB1*04:01, 04:02	DPB1*03:01	Concordance between AllSet™ and LABType® DPB1 data
	DPB1*04:01, 04:02			
Donor	DPA1*01:03	DPB1*03:01, 04:01	DQA1*03:01+ DQB1*03:01+	DQB1 types agrees with those already held on record
	DPB1*03:01, 04:01			
Participant 7	DQA1*01:01+	DQA1*01:01+, 03:01+ DQB1*03:01+, 05:01+	DQA1*03:01+ DQB1*03:01+	DQB1 types agrees with those already held on record
	DQB1*05:01+			
Donor	DQA1*01:01+, 03:01+	DQA1*01:01+, 03:01+ DQB1*03:01+, 05:01+	DQA1*03:01+ DQB1*03:01+	DQB1 types agrees with those already held on record
	DQB1*03:01+, 05:01+			

HLA mismatches – these are assessed in the recipient versus donor direction, i.e. which HLA the recipient's immune system will 'see' as being foreign in the donor graft. These HLA represent the potential targets for a recipient antibody response.

+ indicates a 'string' of possible alleles is present