B LYMPHOCYTE BIOMARKER ANALYSIS TO RISK-STRATIFY CLINICAL OUTCOMES IN RENAL ALLOGRAFT RECIPIENTS

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I confirm that the work submitted is my own, except where work which has formed part of jointly authored publications has been included. My contribution and the other authors to this work has been explicitly indicated below. I confirm that appropriate credit has been given within the thesis where reference has been made to the work of others.

PUBLICATIONS:

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3. Alemtuzumab induction is safe and permits the avoidance of steroids in the majority of renal patients. Seitz A, Robb M, Ahmad N, McLean A, Taube D, Johnson R, Baker R. 'Six of The Best' Platform presentation, British Transplant Society Congress, Harrogate March 2017
4. Alemtuzumab Induction Has Been Safe in the United Kingdom and Achieves Long-Term Steroid Avoidance in More than 80% of Low Risk Renal Transplant Recipients. Seitz A, Robb M, Balasubramanian S, McLean A, Taube D, Johnson R, Baker R. British Transplant Society Congress, Bournemouth March 2015 and American Transplant Congress, Philadelphia May 2015

Abstracts

1. DP Incompatible Transplants – A Single Centre Review. Seitz Adrienne, Mounsey Katherine, Foster Ruth, Hughes Pamela, Baker Richard, Clark Brendan. British Transplant Society Congress, Brighton March 2018 and 32nd European Immunogenetics and Histocompatibility Conference, Venice Lido Italy May 2018
2. Alemtuzumab induction is safe, and permits the avoidance of steroids in the majority of renal patients. Seitz A, Robb M, Ahmad N, McLean A, Taube D, Johnson R, Baker R. American Transplant Congress, Chicago May 2017

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ABBREVIATIONS

ABMR	Antibody-mediated rejection
ALBERT	Assessment in Leeds of Biomarkers Early after Renal Transplantation
APC	Antigen Presenting Cell
APC	Allophycocyanin
APCeFluor780	Allophycocyanin-eFluor780
ASC	Antibody Secreting Cell
ATG	Antithymocyte Globulin
AUC	Area Under the Curve
BBR	Bead-B-cell Rosette
BCR	B-Cell Receptor
BV421	Brilliant violet 421
BV510	Brilliant violet 510
CAMR	Chronic Antibody Mediated Rejection
CD	Cluster of Differentiation
CDCC	Complement-Dependent Cytotoxicity Crossmatch
CDR	Complementarity Determining Regions
CD40L	CD40 Ligand
CEN	Composite Endpoint Negative
CEP	Composite Endpoint Positive
CIT	Cold Ischaemia Time
CLP	Common Lymphoid Progenitor
CMV	Cytomegalovirus
CREG	Cross Reactive Group
CRF	Calculated Reaction Frequency
DBD	Donation after Brain Death
DCD	Donation after Cardiac Death
DGF	Delayed Graft Function
DNA	Deoxyribonucleic Acid
DSA	Donor Specific Antibody
DTT	Dithiothreitol

eGFR	Estimated Glomerular Filtration Rate
ELISA	Enzyme linked Immunosorbent Assay
FAB	Fragment Antigen Binding region
FBS	Foetal Bovine Serum
FC	Fragment Crystallizable region
FITC	Fluorescein Isothiocyanate
FXM	Flow cytometric Crossmatch
HLA	Human Leukocyte Antigen
HVR	Hypervariable Region
HSCT	Haematopoeitic Stem Cell Transplant
HSP	Highly Sensitised Patient, cRF>85%
IFN	Interferon
IL	Interleukin
IMDPH	Inosine-5'-Monophosphate Dehydrogenase
ITAM	Immune Receptor Tyrosine based Activation Motif
JAK	Janus Kinase
KIR	Killer Ig-like Receptor
LD	Live Donor
LSA	Lifecodes Single Antigen
MCF	Median Channel Fluorescence
MFI	Median Fluorescent Intensity
MHC	Major Histocompatibility Complex
MyD88	Myeloid Differentiation Factor 8
NHS-BT	National Health Service – Blood and Transplant
NGS	Next Generation Sequencing
NK	Natural Killer Cell
PAMPs	Pathogen Associated Molecular Patterns
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin

PerCpCy5.5	Peridinin Chlorophyll protein-Cyanine 5.5
PeCy7	Phycoerythrin-Cyanine 7
PMA	Phorbol Myristate Acetate
PMT	Photomultiplier tube
RNA	Ribonucleic Acid
ROC	Receiver Operating Characteristic
rSSO	Reverse sequence specific oligonucleotide
SAB	Single Antigen Bead
SAPE	Streptavidin PE
SD	Standard Deviation
SNP	Single Nucleotide Polymorphism
SSP	Sequence Specific Primer
STAT	Signal Transducer and Activator of Transcription
TCMR	T-cell Mediated Rejection
TI	T-cell Independent
TLR	Toll Like Receptor
TRIF	Toll/IL-1 Receptor-domain-containing-adaptor protein Inducing IFN β
Tyk	Tyrosine Kinase
UPCR	Urine Protein Creatinine Ratio
3D	3-Dimensional

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ABSTRACT

There is increasing interest in understanding how the immune phenotype may be associated with allograft outcomes, and efforts are being made to identify biomarkers that can predict outcome and guide clinical management. This report describes the evaluation of potential biomarkers associated with the B-cell phenotype. Firstly, an observational study using UK registry data was undertaken to compare the medium-term outcomes of adult recipients of their first renal transplant receiving either alemtuzumab or basiliximab. This study concluded that alemtuzumab induction permits steroid avoidance in significantly higher numbers of patients without any obvious penalty. The effect of both induction agents on the B-cell phenotype was studied prospectively in a cohort of adult renal transplant recipients, with time-dependent alterations assessed against clinical outcomes. In particular, the immunomodulatory function of B-cells was explored within the transitional B-cell (TrB) population as this has been demonstrated to contain high concentrations of regulatory B-cells. Changes within TrB subsets were found to be associated with rejection and reduced graft survival. CD9 expression was also investigated to determine its value in highlighting regulatory B-cells (Bregs). Increased expression was demonstrated within TrBs including those producing IL-10, however it was not an exclusive marker for Bregs.

Secondly, B-cells as antibody producers were assessed. Two assays to determine the presence of alloreactive memory B-cells were considered. The first assay involved the non-specific stimulation of peripheral blood mononuclear cells (PBMCs) into antibody secreting cells, with the collection of cell supernatant for the assessment of HLA antibodies. The second method aimed to quantify alloreactive B-cells through the co-incubation of PBMCs with single antigen beads. Finally, a retrospective study of HLA-DP antibody incompatible renal transplants was carried out; findings demonstrate that these transplants should be considered high risk, and laboratory tests cannot further risk stratify these patients.

1 INTRODUCTION

Renal transplantation remains the treatment of choice for patients with end stage renal disease. Despite an improvement in short-term allograft survival, the median lifespan of a renal transplant from a deceased donor remains at 15 years, and it is increasingly common for patients to receive more than one allograft in their lifetime. A significant proportion of late graft loss is due to immune-mediated graft damage, of which antibody-mediated rejection (AMR) remains a significant problem.[1,2] There is increasing evidence surrounding the importance of B-cells in allograft survival, not only through the production of HLA-donor specific antibodies which are implicated in antibody mediated rejection, but also through other effector functions including antigen presentation and cytokine secretion. The long-term survival of an allograft depends on the sustained use of immunosuppression, which itself is associated with morbidity, including cardiovascular disease, infection, and malignancy. Additionally, immunosuppressive agents, for example calcineurin inhibitors, can contribute to toxicity within the graft. Therefore, there is a fine balance between over immunosuppressing a patient which results in unwanted outcomes, and reducing the immunosuppressive burden, increasing the risk of immune-mediated allograft damage. The current practice for monitoring allograft function includes measuring serum creatinine, urine protein excretion and blood pressure. The gold standard remains a needle biopsy, however, this is invasive, and sampled areas may not be representative of the whole graft. There is therefore an unmet need to develop non-invasive biomarkers that can predict the clinical course of an allograft at an early stage, allowing the clinician to tailor treatment to the individual patient.

1.1 IMMUNE RESPONSES TO FOREIGN TISSUE

The human immune system has evolved as a highly sophisticated defence system that functions to distinguish between self and non-self antigens. From an evolutionary perspective this was essential to survive attacks by pathogens, but inconvenient consequences are occasional reactivity to self, manifest by autoimmune disease and reactivity to other members of the species (alloreactivity). This latter phenomenon only came to light when early pioneers attempted to transplant organs between individuals. Alloreactivity occurs due to the wide genetic diversity that is present

within the outbred human population. Only identical twins share the same genetic material and are thus syngeneic. It is for this reason that the first successful human kidney transplant was carried out between identical twins in 1954 with minimal immunosuppression.[3]

1.1.1 HUMAN LEUKOCYTE ANTIGENS (HLA)

The area of the human genome with the greatest genetic variation between individuals is located on the short arm of chromosome 6 (6p21.3) in the Major Histocompatibility Complex (MHC, Figure 1.1). This is a densely packed region with over 200 genes that encode glycoprotein products called Human Leukocyte Antigens (HLA). These molecules are widely expressed on the surface of human cells and are intimately involved in antigen presentation and activation of the adaptive immune system. HLA heterogeneity both within a population and the individual ensures a broad repertoire of response to pathogens, ensuring survival. An example of this can be seen in the West African population, where individuals possessing either HLA-B*53, or the HLA-DRB1*13:02-DQB1*05:01 haplotype are protected from severe forms of malaria.[4]

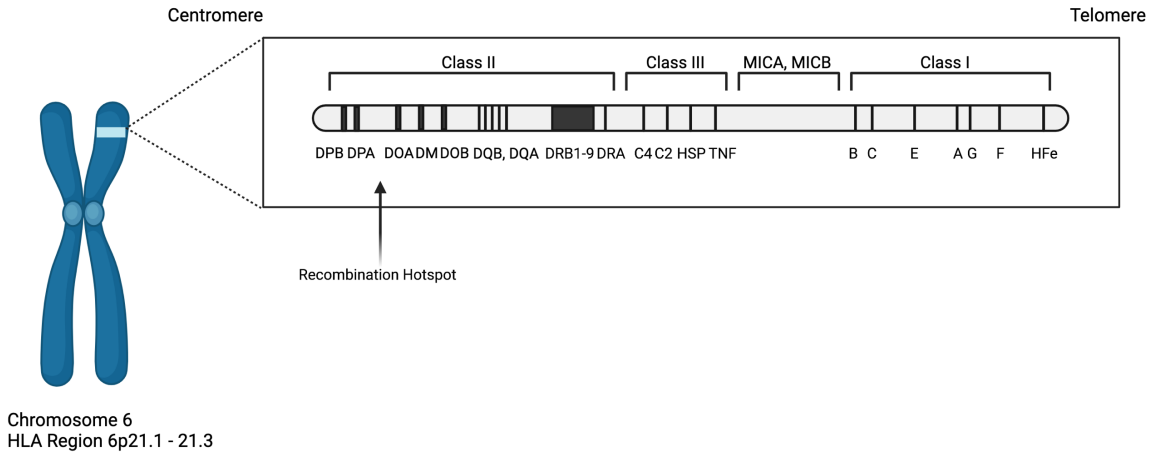


Figure 1.1. Schematic of HLA region.

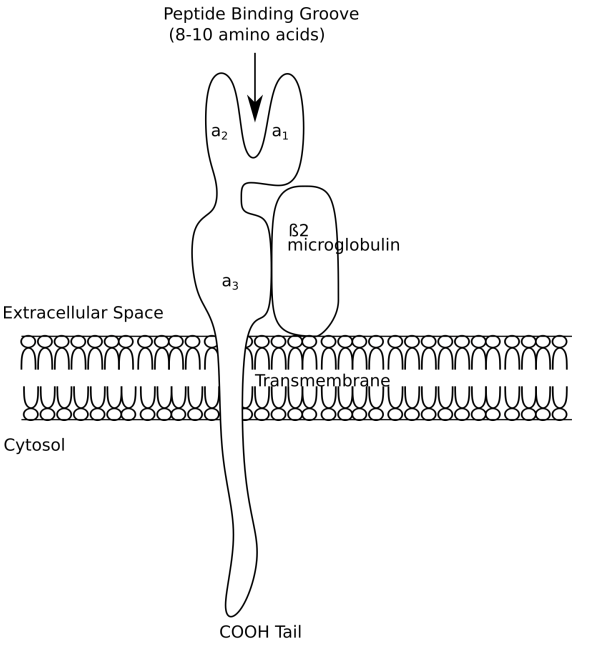
The Human MHC is found on the short arm of chromosome 6 and encodes distinct classes of cell surface molecules. Created with BioRender.com

1.1.1.1 STRUCTURE AND FUNCTION OF HLA MOLECULES

HLA are grouped into classes dependent on their structural similarities, function, and distribution patterns. Classical class I HLA (HLA -A, -B, and C) are expressed on the cell surface of all nucleated cells. The Class I genes produce a 43kda molecule (α chain) that has three extracellular domains α_1, α_2 and α_3, a transmembrane region, and a short cytoplasmic tail. The domains are each coded for by separate exons, with exons 2 and 3 exhibiting the most polymorphism. These two exons encode the α_1 and α_2 domains which interact to form the peptide binding groove allowing the presentation of short 8-10 amino acid peptides to its cognate T-cell receptor.[5] It is the variation in α_1 and α_2 that dictates which peptides can be presented. The α_3 domain is non-covalently associated with the invariant light chain of $ß_2$ microglobulin (encoded on chromosome 15) and interacts with the CD8 co-receptor of cytotoxic T cells during antigen presentation (Figure 1.2a). Class I proteins present peptides derived from defective intracellular proteins, or viruses from within the cell to the T-cell receptor on $CD8^+$ T cells, which activate mechanisms that ultimately destroy the cell. Class I HLA are also important ligands for killer inhibitory receptors (KIRs) that are expressed on the surfaces of NK cells, which is important for tumour surveillance.[6]

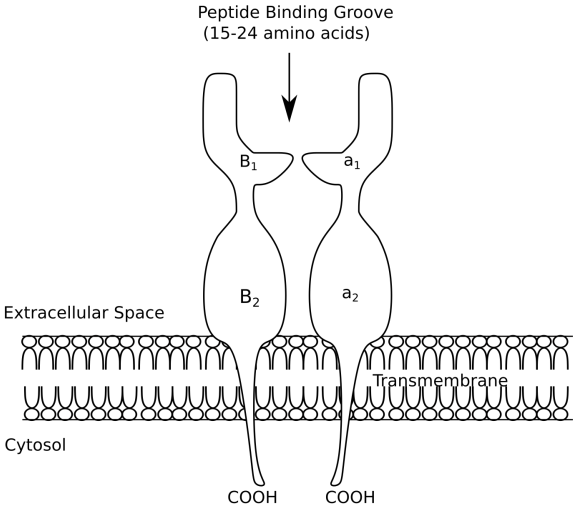
Class II HLA (DR, DQ and DP) are made up of two non-covalently linked chains, an alpha (33-35kda) and a beta (26-28kda) chain.[5] In the case of HLA-DR, the alpha chain is relatively conserved, with polymorphism exhibited in the beta chain. However, both the α and β chains in HLA-DP and HLA-DQ are highly polymorphic, and biodiversity is increased further in class II HLA due to the different combinations that can be achieved between the α and β chains. The peptide binding groove is created by the interaction of the α1 and β1 domains. It presents peptides of 15-24 amino acids in length to its cognate T cell receptor on $CD4^+$ T cells, Figure 1.2b. These peptides are usually derived from extracellular pathogens.

A) HLA CLASS I



EXON	1	2	3	4	5
PROTEIN DOMAIN	Leader Sequence	a1	a2	a3	Transmembrane Region

B) HLA CLASS II



a chain EXON	1	2	3	4	5
PROTEIN DOMAIN	Leader Sequence	a1	a2	Tm/Cyt	3'UT

ß chain EXON	1	2	3	4	5
PROTEIN DOMAIN	Leader Sequence	ß1	ß2	Tm/Cyt	3'UT

Figure 1.2. Structure of A) HLA Class I and B) HLA Class II.[7]

The exons responsible for coding each corresponding protein domain has been indicated. Tm – transmembrane region, Cyt – cytosolic region, 3'UT – 3' untranslated region.

The MHC is inherited from each parent as a haplotype, and gene expression is co-dominant. Therefore, in heterozygotes, up to 12 different classical HLA alleles can be expressed on a cell surface, 2 from each locus, and each allele will have the ability to bind to a different repertoire of peptides. This forms the molecular basis of human immunogenetic diversity. The number of different alleles at each locus is shown in Table 1.1 and underpins human heterogeneity. It is this diversity, together with ubiquitous expression that makes HLA the main immunological barrier to transplantation.[8]

HLA Type	A	B	C	DR	DQA1	DQB1	DPA1	DPB1
No. of alleles (DNA)	6291	7562	6223	3536	264	1930	216	1654
No. of proteins	3896	4803	3681	2476	114	1273	80	1064

Table 1.1. Assigned number of alleles and proteins for each HLA locus as of September 2020 [9]

1.1.1.2 TISSUE DISTRIBUTION OF HLA

Class I HLA are constitutively expressed on the cell surface of all nucleated cells. Class II HLA are primarily found on the cell surface of professional antigen presenting cells. However, in an inflammatory environment, this expression can be upregulated in other cells, including vascular endothelial and activated T cells.[10] As demonstrated in Figure 1.3, the tissue distribution of HLA is widely variable across the human body and reflects the function of HLA in antigen presentation. There is increased HLA expression on lymphocytes and within the primary lymphoid structures, whereas minimal expression is found in the immune-privileged sites (central nervous system, testes, retina).

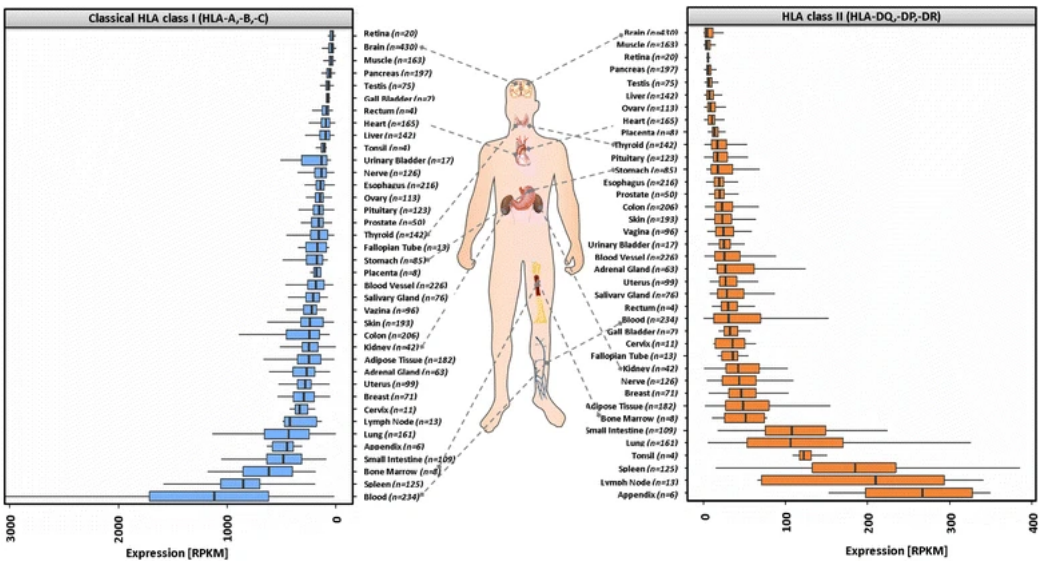


Figure 1.3. Tissue distribution of HLA.

Classical HLA class I (blue) and class II (red) expression levels have been determined using NGS RNA-Seq data from human non-cancer tissues. This figure has been reproduced without any changes from the open access article: Boegel, S., Loewer, M., Bukur, T. *et al*. HLA and proteasome expression body map. *BMC Med Genomics* 11, 36 (2018). https://doi.org/10.1186/s12920-018-0354-x [11] using the Creative Commons license: https://creativecommons.org/licenses/by/4.0/

1.1.1.3 HLA EPITOPES

Epitopes refer to parts of an antigenic molecule that interact with the host immune system (usually an antibody or antigen receptor). In proteins, these epitopes can be continuous (linear strands of amino acids) or discontinuous (non-contiguous amino acids that are brought together in the tertiary and quaternary structure of the molecule). In transplantation, the improved molecular typing techniques, and ability to determine the 3-dimensional (3D) structure of HLA has led to increasing interest in assessing mismatches in HLA epitopes. Epitope mismatches can be evaluated in terms of immunogenicity and antigenicity. Immunogenicity refers to the ability of an antigen to induce an immune response and is dependent on the structural configuration of the immunizing epitopes in reference to the host's epitopes; the mismatched epitopes having the potential to induce the immune response. Conversely, antigenicity is the

interaction between the epitope and the final products of the immune response, for example, the T-cell receptor or antibodies.

1.1.1.3.1 HLAMATCHMAKER

The most widely used HLA epitope tool is HLAMatchmaker, an algorithm that systematically defines possible HLA epitopes.[12] Developed by Duquesnoy *et al*, the original HLAMatchmaker was developed by using linear amino acid sequences together with the known 3D structure of Class I HLA to define polymorphic amino acid sequences that could be accessed by HLA antibodies. It was postulated that polymorphisms in linear 3 amino-acid sequences were essential for reactivity, and these were called 'triplets'. Triplets however did not take the folding of the HLA protein into account. Subsequent iterations of HLAMatchmaker took this into consideration, leading to the definition of 'eplets'. These are small clusters of amino acid residues, linear or discontinuous, found on the surface of HLA molecules in a 3.0-3.5 angstrom radius. They are equivalent to the CDR-H3 or 'functional epitope' of an antigen and are essential to determining antibody specificity (Figure 1.4).[13] Although this eplet registry is comprehensive, not all eplets have been found to induce antibody production and are classified as theoretical eplets: their clinical relevance is not yet known. Commonly used in conjunction with HLAMatchmaker, is the HLA epitope registry also developed by Duquesnoy and colleagues (epregistry.com.br)[14] which contains an evolving list of antibody-verified eplets for each HLA locus. These antibodies have been verified using multiple different methods including human monoclonal antibodies, sera from multiparous women, mouse monoclonal antibodies and elution and absorption techniques.

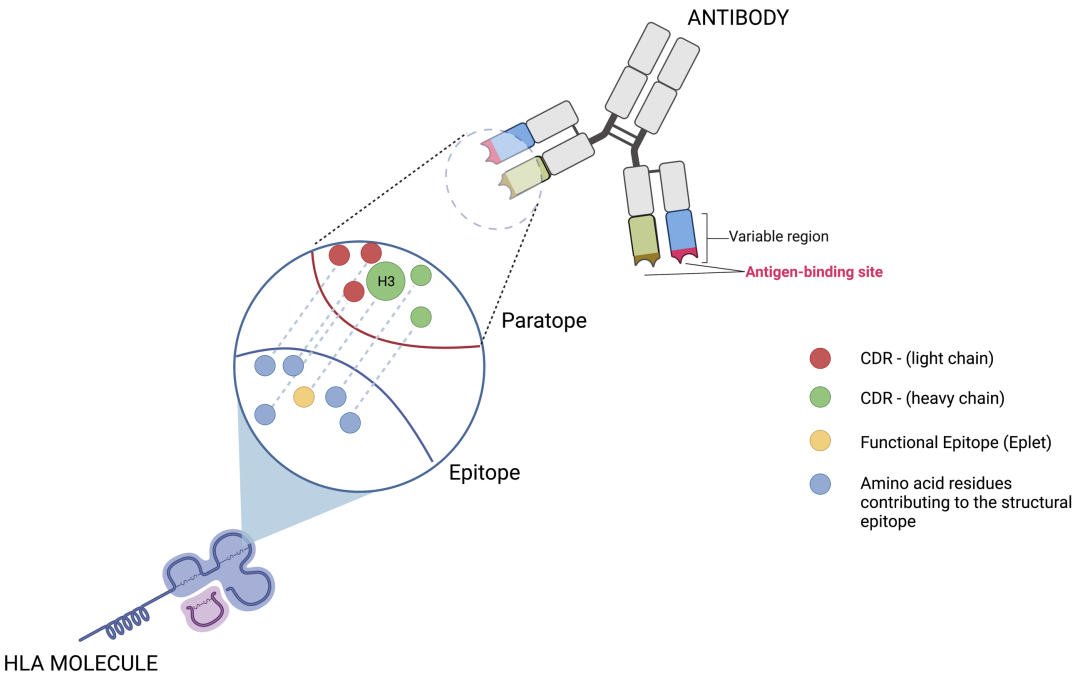


Figure 1.4. Schematic of the HLA antigen-antibody interface.

The binding surface on the antibody (the paratope) is made up of six complementarity determining regions (CDR). CDRL1-L3 refer to the regions found on the antibody light chain, CDRH1-H3 are found on the heavy chain. The specificity of the antibody is usually determined by CDR-H3, a central loop which binds to a specific short amino acid sequence, also known as the functional epitope or eplet.[15] The remaining CDRs contribute to the stability of antigen binding and affect the affinity of the antibody. The binding surface of HLA (antigenic determinant or epitope) comprises of a central functional epitope (eplet) which binds to CDR-H3, surrounded by amino acid residues that are in contact with the with rest of the antibody CDRs, contributing to the structural epitope.

Image created using Biorender.com

HLAMatchmaker has been used to assess the immunogenicity of epitope mismatches in renal transplantation. A retrospective study demonstrated that patients who received a renal transplant with a high HLA class II eplet mismatch load were more likely to develop *de novo* donor-specific HLA antibodies (DSAs).[16] Furthermore, compliance with immunosuppressive medication was shown to minimise any detrimental effects associated with this high mismatch load.[17] However, single epitope mismatches have been associated with the development of de novo DSAs.[18,19] This suggests that it is not just the mismatch load, but also the biological properties associated with each epitope mismatch that can affect immunogenicity.

HLAMatchmaker is currently used in the Eurotransplant acceptable mismatch programme for highly sensitised patients (HSPs).[20] In this programme, HLAMatchmaker compares the epitopes that the recipient possesses with the epitopes found in their antibody profile. A permissive epitope profile is constructed based on epitopes to which they have not developed antibodies; only donors with HLA antigens made up of these epitopes are accepted. A recent study has reported a superior 10-year graft survival in highly sensitised patients (HSPs) who received renal transplants through the Eurotransplant acceptable mismatch programme compared with HSPs who were transplanted through the routine unacceptable mismatch allocation.[16]

Other epitope tools are available. Kosmoliaptsis *et al* considers how polymorphisms affect the electrostatic charge of the HLA molecule, which is important in peptide binding and the HLA – T cell receptor interaction.[21,22] Spierings developed the PIRCHE (**P**redicted **I**ndirectly **R**e**C**ognizable **H**LA **E**pitopes) -II, a tool that focuses on epitope mismatches that are targets for T cells during indirect allorecognition.[23] Initially used for stem cell transplantation, it has been applied retrospectively to renal transplantation where high PIRCHE scores were associated with reduced graft survival.[24,25] Similarly, the T-cell Epitope model developed by Fleischhauer is routinely used to determine permissive and non-permissive HLA-DP mismatches when searching for unrelated donors in stem cell transplantation.[26] The newest freely-available mismatch tool is the HLA Epitope MisMatch Algorithm (HLA-EMMA) which attempts to determine the immunogenicity of the mismatches by measuring 'solvent accessibility' of the amino acid mismatches that could be accessible to B-cell receptors.[27]

Recently, the utility of the class II eplet, amino acid and electrostatic mismatch methods were compared in 596 renal recipients. The authors determined that the 3 methods were highly correlated with each other. Furthermore, high mismatch scores obtained from using each method were associated with the development of *de novo* class II donor specific antibodies.[28]

1.2 ALLORECOGNITION

Allorecognition refers to the immunological recognition by T cells of non-self antigens between genetically disparate individuals within the same species. Recipient T cells may encounter alloantigens by via the following 3 pathways: the direct, indirect and semi-direct pathways.

Direct allorecognition is thought to be an important mechanism associated with acute cellular rejection. As the graft is re-perfused, passenger donor leukocytes,[29,30] expressing donor derived HLA molecules, enter the recipient circulation and encounter naive alloreactive recipient T cells within the secondary lymphoid tissue.[31,32] Direct recognition initiates a polyclonal T cell response that can involve a large proportion of the T-cell repertoire.[33,34] Once primed these cells mature, acquire the trafficking molecules necessary to access the transplanted graft, enter the circulation and travel to the transplanted organ (Figure 1.5). This is thought to be the main mechanism underlying $CD8^+$ mediated cytotoxic tubular damage (tubulitis).[35]

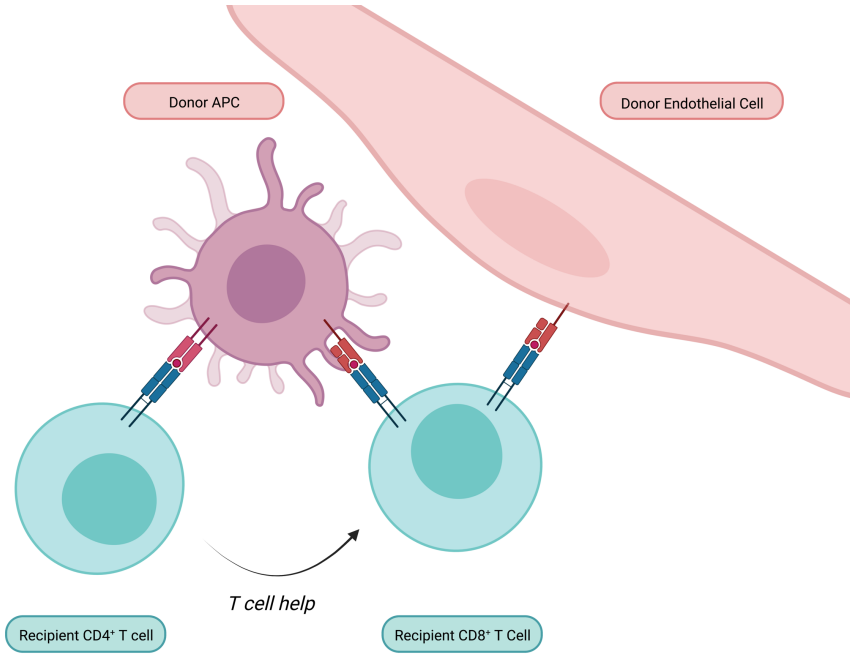


Figure 1.5. Direct Allorecognition.
On revascularisation of the graft, donor antigen presenting cells (APCs) bearing donor derived HLA molecules and peptides interact with recipient T cells. Donor HLA class II molecules interact with the T cell receptor (TCR) expressed on recipient $CD4^+$ T cells. Donor HLA Class I molecules interact with TCR on $CD8^+$ T cells. Activated recipient $CD4^+$ T cells provide T cell help in the form of cytokines to activate $CD8^+$ T cells. $CD8^+$ T cells migrate to the graft and target donor cells for cytotoxicity. Donor cells are depicted in red, recipient cells in green.
(Image created using Biorender.com)

In indirect allorecognition, which is the normal physiological pathway of foreign antigen processing, recipient antigen presenting cells (APCs) take up and process donor proteins that have been shed from the graft for presentation to T cells in peptide fragments (Figure 1.6). This mechanism emerges as dominant after the disappearance of donor derived passenger leukocytes and is thought to be instrumental in the development of chronic antibody mediated rejection.[36] Recently, Charmetant *et al* proposed a new model of allorecognition – 'inverted indirect allorecognition'. They demonstrated in a murine model that donor $CD4^+$ T cells could

recognise intact recipient class II MHC molecules expressed on BCR-activated allospecific B-cells and provide the second signal for further activation.[37]

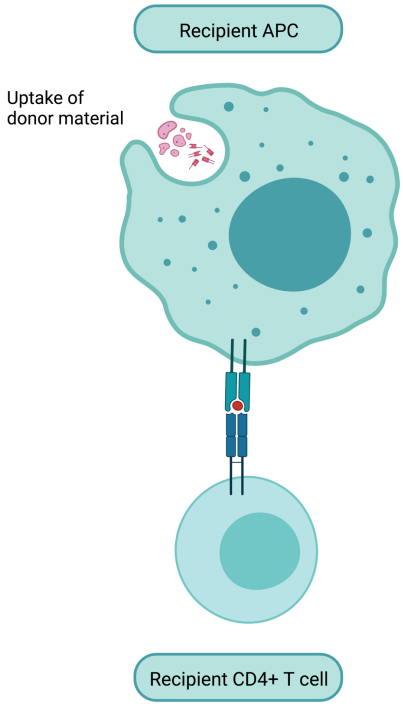


Figure 1.6. Indirect Allorecognition.
Recipient antigen presenting cells (green) take up donor material (red) for processing and loading onto to recipient HLA Class II molecules. These are presented to recipient $CD4^+$ T cells.
(Image created using Biorender.com)

Finally, with semidirect allorecognition, recipient dendritic cells acquire intact donor HLA (recently demonstrated through the transfer of exosomes) and present this as an intact protein to recipient T cells for activation (Figure 1.7).[38] The physiological importance of this pathway remains to be determined.

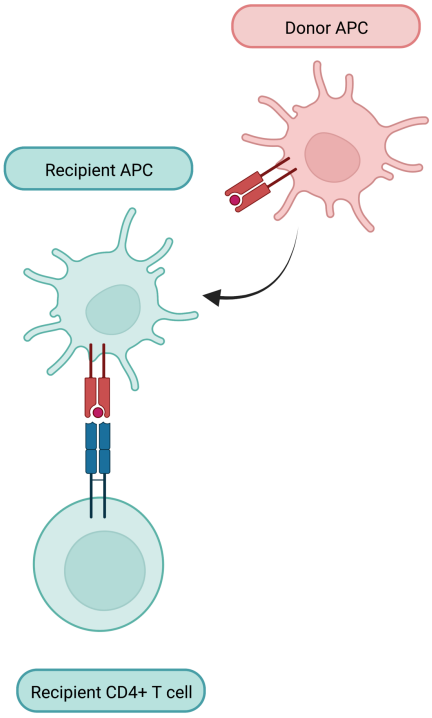


Figure 1.7. Semi Direct Allorecognition.

Intact donor HLA-antigen complexes are transferred (via direct contact or exosomes) from donor to the surface of recipient antigen presenting cells. Recipient cells are depicted in green, donor cells in red.

(Image created using Biorender.com)

A distinctive feature of the T cell response to alloantigen presented via the direct pathway is that it can involve a large proportion (between 1-10%) of the T cell repertoire, whereas only approximately 0.01% of the T cell repertoire will respond to a specific foreign peptide presented by a self MHC molecule.[39–41] Two theories attempt to explain this widespread activation in allorecognition: the multiple binary complex, and high determinant density models.

Multiple Binary Complex Model [42]

Described by Matzinger, the presented peptide is central to activation. There is homology between the structural components of self and non-self MHC molecules which allows recipient self-restricted T cells to bind to the foreign MHC as they would

to self MHC. However, there is polymorphism within the peptide binding groove therefore the peptides presented by non-self MHC will be significantly different to those presented by self MHC. The non-self MHC can therefore present a pool of novel peptides, thus activating T cells with a wide range of specificities.

High Determinant Density Model [43]

Proposed by Bevan, it is the non-self MHC molecule that is central to activation. Recipient T cells can recognise the structural differences in the exposed residues of non-self MHC molecules, irrespective of the peptide presented in the peptide binding groove. Donor derived antigen presenting cells possess a high density of these non-self residues, allowing for activation of multiple recipient T-cell clones, which may recognise these antigens with low, medium, or high affinity.

1.2.1 THE CONSEQUENCES OF ALLORECOGNITION

1.2.1.1 T-CELL-MEDIATED REJECTION (TCMR)

This is associated with an abrupt rise in serum creatinine, fluid retention and sometimes fever and graft tenderness. The incidence of acute TCMR is approximately 5-10% during the first year following renal transplantation.[44,45] Pathologically, an accumulation of mononuclear cells (usually activated T-cells and macrophages) is seen in the interstitium, together with inflammation of the tubules and sometimes the arteries.[46] Activated recipient $CD4^+$ and $CD8^+$ T-cells migrate from the circulation to the transplanted organ and enter the interstitial space. $CD8^+$ T-cells release cytotoxic granules containing perforin and granzyme A+B. Proinflammatory cytokines and chemokines are released by macrophages and $CD4^+$ T-cells, which contribute to tissue inflammation by priming cytotoxic T-cells, stimulating the humoral response, activating NK cells and recruiting further neutrophils and macrophages to the graft. Infiltrating T-cells and macrophages invade the tubules, resulting in tubulitis. In this inflammatory environment, tubular epithelium cells upregulate ICAM-1 and co-stimulatory molecules for T-cells, driving inflammation. In extreme cases, this can lead to rupture of the basement membrane and leakage of tubular proteins into the interstitium,

furthering graft dysfunction and proteinuria. Infiltration of the vascular endothelium of small and medium sized arteries can lead to endothelialitis which can be associated with haemorrhage.

1.2.1.2 ANTIBODY MEDIATED REJECTION (ABMR)

Donor specific antibodies bind to foreign HLA molecules on the graft endothelium, initiating antibody mediated injury through multiple mechanisms. The amount of circulating antibody, antibody isotype as well as the individual antibody specificity and expression level of its target will play a role in determining the pathogenesis of subsequent injury and severity of clinical outcome.

First described in 1966 by Kissmeyer-Nielson *et al*, hyperacute rejection is a catastrophic event that usually occurs soon after the reperfusion of the transplanted organ, and frequently leads to graft loss.[47] In the 1960s, this occurred in up to 28% of regrafts, and was recognised to be caused by preformed complement fixing antibodies in the recipient.[47,48] Donor specific antibodies enter the donor vasculature, bind to the vascular endothelium and activate the complement and clotting cascades. This leads to the formation of thrombi which occlude the vasculature resulting in ischaemia and necrosis of the graft. For hyperacute rejection to occur, preformed complement fixing antibodies must be present in the recipient circulation (see Figure 1.9). These can be IgM antibodies against ABO blood group antigens, or high affinity IgG antibodies usually directed against Class I HLA. Hyperacute rejection is now avoided in most cases through the transplantation of ABO compatible organs, and by implementing crossmatch techniques between donor and recipient to inform on the presence of donor specific HLA antibodies prior to transplantation. Non-complement fixing DSAs play a more indolent role in the pathogenesis of antibody mediated rejection. The Fc portion of the endothelial cell-bound DSA can initiate antibody-dependent cytotoxicity via the ligation of Fc receptors on natural killer (NK) cells and macrophages. Direct interaction of HLA antibodies with class I HLA on endothelial cells can result in multiple functional and phenotypic changes within the endothelial cell, including upregulation

of adhesion molecules, class II HLA, and the secretion of chemokines and cytokines, thus promoting recruitment and activation of leukocytes, and facilitating ongoing allograft damage.[49–52] Low level crosslinking of HLA class I by HLA antibody can activate anti-apoptotic pathways within the endothelial cell leading to increased endothelial cell turnover and proliferation, and the duplication of the glomerular basement membrane seen with transplant glomerulopathy.

Currently, active and chronic ABMR is diagnosed using the Banff criteria,[53,54] and requires:

- morphologic evidence of antibody interaction with the endothelium,
- immunohistologic evidence of antibody interaction (linear C4d staining in peritubular capillaries) and
- serologic evidence of donor specific antibodies.

It has been recognised that C4d deposition can be present without morphologic evidence of active rejection, and conversely, C4d deposition can be absent in ABMR.[55,56] Therefore, in the 2017 iteration of the Banff criteria increased expression of gene transcripts associated with ABMR was accepted as an alternative to C4d staining.[57]

1.3 DEVELOPMENT AND DIFFERENTIATION OF B LYMPHOCYTES

The development of B-cells is tightly regulated and takes place within the primary lymphoid tissue (foetal liver, and foetal/adult bone marrow). The first development stages do not require contact with a foreign antigen but depend on the interaction with bone marrow stromal cells. The first B-lineage cell arises from common lymphoid progenitors (CLP) as a pro-B-cell, which expresses the earliest B-lineage markers involved in signal transduction (CD19, CD45R), in addition to growth factor receptors (e.g. IL-7, CD25) and CD38. The cell proceeds through several stages involving the random rearrangement of its immunoglobulin gene segments, generating antibody diversity. The V, D and J gene segments in the heavy chain locus are rearranged first, and if successful, results in the expression of an intact μ heavy chain. The μ chain pairs with two 'surrogate' light chains to form a pre-B receptor. The presence of the pre-B

receptor is important to allow the cell to pass through the first checkpoint as a pre-B-cell. Additional markers displayed by the pre-B-cell include CD24 (unknown function). Following the successful rearrangement of V and J in the light chain locus, the pre-B-cell becomes an immature B-cell that expresses a complete IgM that associates with the membrane-bound proteins Igα and Igβ to form a functional B-cell receptor (BCR). At this point, the immature B-cell is tested for autoreactivity. The self-tolerant immature B-cell enters the periphery as the transitional B-cell (TrB) which expresses high levels of surface IgM and IgD (Figure 1.8). Transitional B-cells represent approximately 4% of the peripheral B-cells in healthy adults.[58]

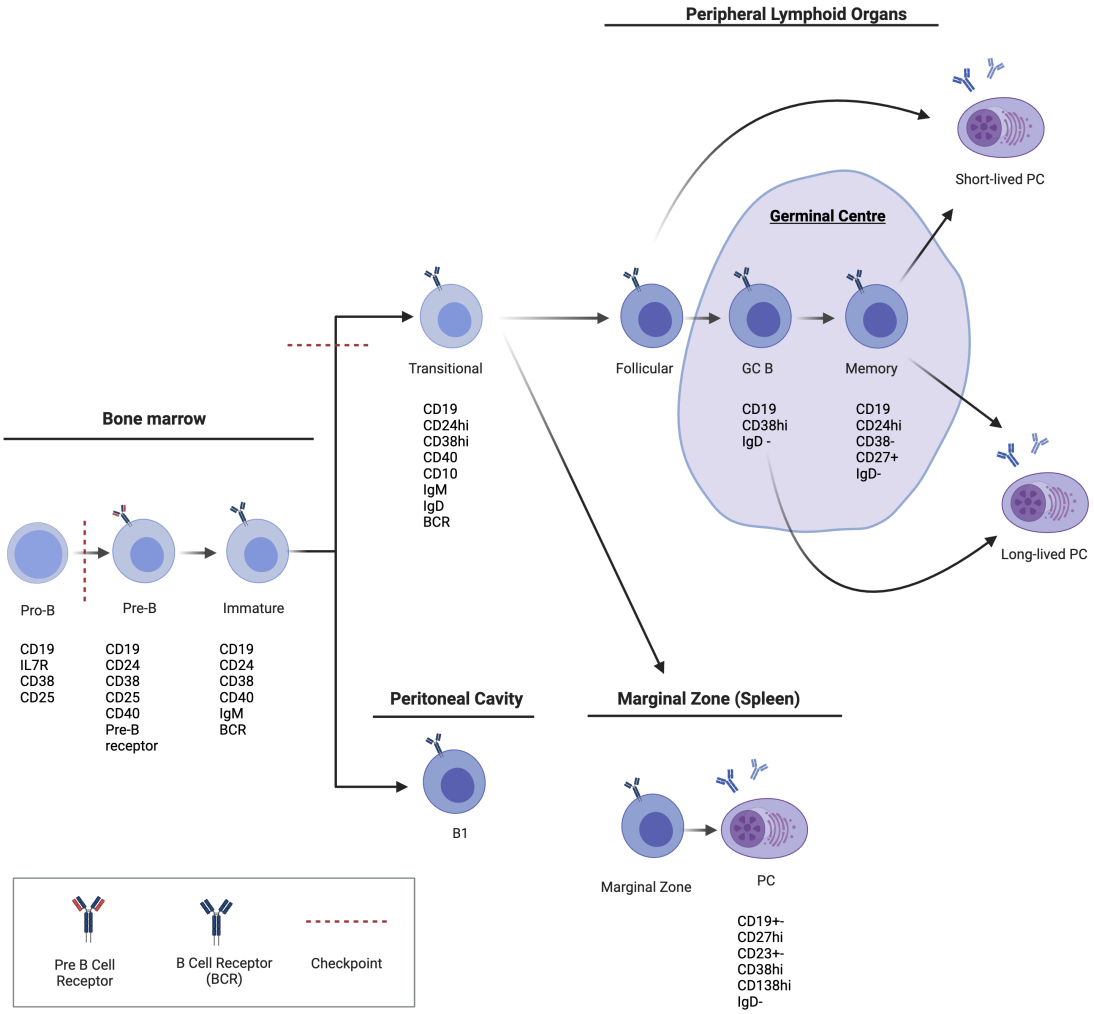


Figure 1.8. Development and Differentiation of human B-cells, with common surface markers

The antigen-independent stage begins within the primary lymphoid organs and relies on the interaction with stromal cells. The immature B-cell enters the peripheral circulation as a TrB. There are 3 types of mature B-cells: B1, marginal zone, and follicular. Further development is antigen dependent, occurs in the periphery, and is characterised by clonal expansion, class switch recombination and somatic hypermutation. This results in the generation of primed memory cells and long-lived plasma cells. Image created with BioRender.com

1.3.1 B-CELL ACTIVATION

Further activation and maturation of the B-cell is antigen dependent, requiring two signals. The first signal is delivered through antigen binding to the B-cell receptor (BCR). The molecules Igα and Igβ associated with the BCR contain a single immune-receptor tyrosine-based activation motif (ITAM) in their cytosolic tails, allowing for signal transduction when the BCR binds to an antigen. The second signal is delivered either by T helper cells (T-cell dependent activation) or by certain microbial antigens (T-cell independent activation).

1.3.1.1 T-CELL DEPENDENT ACTIVATION

B-cell activation in response to protein antigens require antigen specific T-cell help. The antigen binds to the BCR, is internalised, processed, and presented on the cell surface within the MHC class II-peptide complex where it may be recognised by the cognate TCR on a helper cell. In turn, the T-cell becomes activated and provides the second B-cell activation signal (which involves T-cell dependent costimulatory ligands and cytokines including CD28 and CD40 ligand [CD40L], IL-21, IL-4). This interaction generates short lived plasma cells and memory cells with low binding affinity to the antigen, together with the development of microanatomical structures within the secondary lymphoid tissue called germinal centres.[59] Germinal centres contain a specialised subset of $CD4^+$ T-cells, T follicular helper cells, which support B-cells as they undergo clonal expansion, somatic hypermutation and class switch recombination resulting in the generation of primed memory cells, and long-lived plasma cells. These generated B-cell clones express the BCR with the highest affinity to the activating antigen. A proportion of the plasma cells will persist in survival niches (the bone marrow or secondary lymphoid organs), where they continuously produce low level antibodies independent of further T-cell help.[60]

The memory cells recirculate, and upon re-encounter with the priming antigen, will rapidly proliferate and differentiate into short and long lived plasmablasts, augmenting the antibody levels (Figure 1.8).

1.3.1.2 T-CELL INDEPENDENT (TI) ACTIVATION

B-cells can be activated and induced to produce antibodies without T-cell help as signal 2 can also be delivered by the binding antigen. This usually occurs with antigens that are commonly found on the surface of pathogens. TI activation is advantageous as this results in a rapid B-cell response to the invading pathogen. However, the B-cell clones do not undergo affinity maturation or develop into memory cells, both of which require T-cell help. The TI antigens are broadly split into two groups, TI-1 and TI-2.

1.3.1.2.1 TI-1 ANTIGENS

TI-1 antigens (also described as mitogens), bind to toll like receptors (TLRs), inducing polyclonal B-cell activation and a non-specific antibody response.

1.3.1.2.1.1TOLL LIKE RECEPTORS

Toll like receptors (TLR) are a family of receptors that recognise molecular patterns characteristic of pathogenic microorganisms, often called pathogen-associated molecular patterns (PAMPS). TLRs are instrumental in initiating inflammatory responses and priming adaptive responses. There are 10 TLR genes in humans which encode different receptors, each able to recognise distinct molecular patterns produced by pathogens (for example lipopolysaccharides and CpG oligonucleotides). TLR ligation provides one mechanism by which B-cells can be activated independent of T-cell interaction. Whereas most of the TLRs are expressed on the cell surface, three TLRs (TLR 3, TLR 7, and TLR 9) are found in endosomes, allowing the recognition of the breakdown products of pathogens. TLRs are widely expressed by many cells of the immune system as well as other non-immune cells for example fibroblasts and epithelial cells. Most TLRs signal via the adapter proteins called the myeloid differentiation factor 8 (MyD88), leading to the production of proinflammatory cytokines.[61] TLR2 and TLR4 agonists in addition are able to induce IL-10 expression through the MyD88 or Toll/IL-1 receptor-domain-containing adaptor protein inducing IFNβ (TRIF, also known as TICAM1) by macrophages and dendritic cells.[62] TLRs are also expressed on human B-cells at most stages of development, including the CLP, however different B-cell subsets will express different TLR patterns. Peripheral naïve B-cells for example express low levels of TLRs 1, 7, and 9, whereas memory B-cells

express high levels of TLR 1, 6, 7, 9 and 10, and plasma cells express further TLRs including TLR 3 and 4.[63–65] TLR ligation therefore plays a role in B-cell development, differentiation and activation, together with antibody and cytokine secretion.[61]

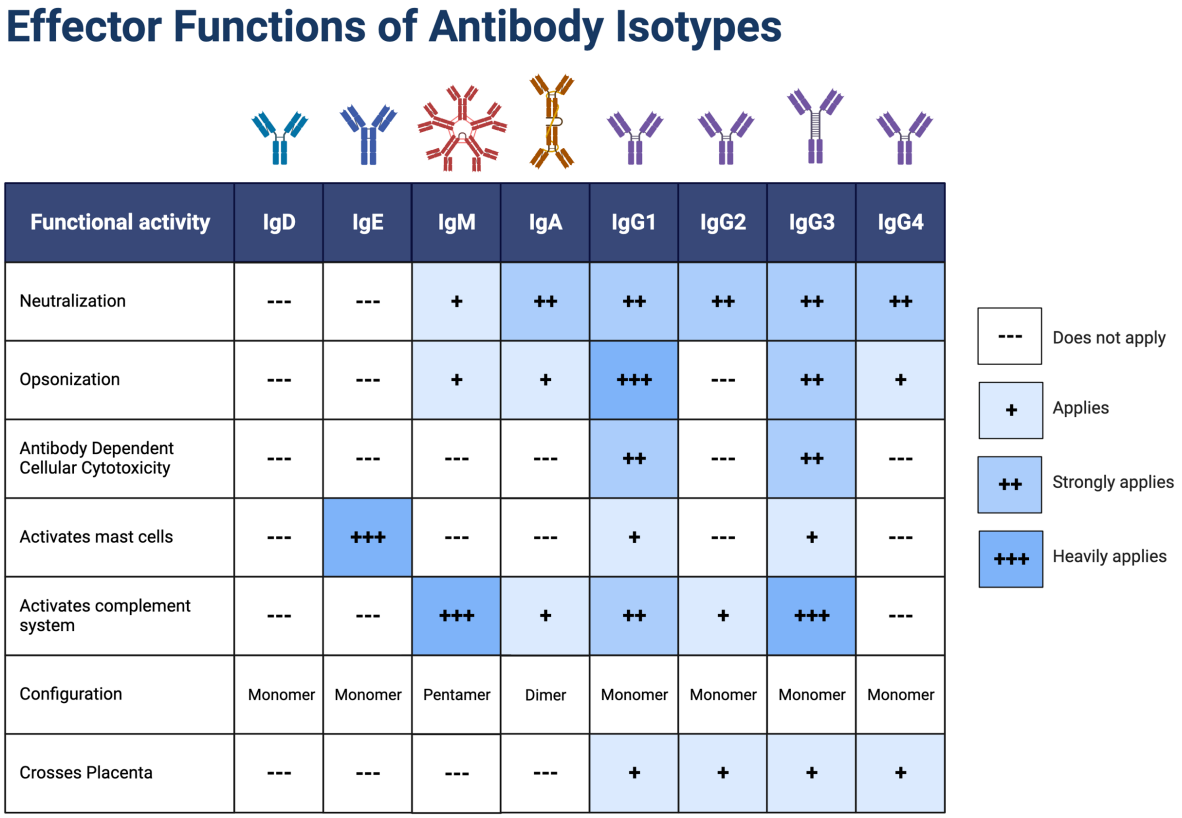
1.3.1.2.2 TI-2 ANTIGENS

TI-2 antigens, for example bacterial capsular polysaccharides, glycolipids or glycoproteins, are characterised by a highly repetitive structure. This repetitive structure allows several epitopes of TI-2 antigens to crosslink multiple BCRs at the same time, resulting B-cell activation and a rapid IgM response.[66,67] Whereas TI-1 antigens can activate immature and mature B-cells, TI-2 antigens can only activate mature B-cells.[68]

1.4 EFFECTOR FUNCTIONS OF B-CELLS

1.4.1 ANTIBODY PRODUCTION

B-cells were first considered as antibody producers in 1890 following the discovery of circulating antitoxins to diphtheria and tetanus.[69] Subsequently, it was proposed that cells displaying preformed antibody receptors produced antitoxins, and these cells could be induced to produce more antibody receptors that were specific to the activating antigen.[70] Electrophoresis of sera by Tiselius *et al* in the 1930s demonstrated that antibodies were gamma globulins, and in 1948 Fagraeus *et al* correlated antibody production with plasma cell development following immunisation.[71,72] Structurally, antibodies consist of paired heavy and light chains which are comprised of variable and constant regions. Light chains have a single variable and constant region, whereas heavy chains have one variable and 3 constant regions. The variable regions determine antigen specificity whereas the constant regions interact with other elements of the immune system. There are 5 different classes of antibodies that provide differing effector functions. Figure 1.9 provides an overview of the different antibody classes.



Functional activity	IgD	IgE	IgM	IgA	IgG1	IgG2	IgG3	IgG4
Neutralization	---	---	+	++	++	++	++	++
Opsonization	---	---	+	+	+++	---	++	+
Antibody Dependent Cellular Cytotoxicity	---	---	---	---	++	---	++	---
Activates mast cells	---	+++	---	---	+	---	+	---
Activates complement system	---	---	+++	+	++	+	+++	---
Configuration	Monomer	Monomer	Pentamer	Dimer	Monomer	Monomer	Monomer	Monomer
Crosses Placenta	---	---	---	---	+	+	+	+

Figure 1.9. Effector Functions of Antibody Isotypes

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1.4.1.1 ALLOSENSITISATION AND HISTOCOMPATIBILITY

In the context of transplantation, a patient is considered to be sensitised when they develop reactivity to alloantigens. The molecular correlate of sensitisation is the formation of highly specific IgG by an iterative process of genetic recombination following T-cell dependent activation of B-cells. The exposure to mismatched alloantigens occurs through three principle routes: paternal antigens *in utero* during pregnancy, by blood transfusion (contaminated by leukocytes) and previous transplants. A minority of cases may be attributable to other events including vaccination, infections, or sexual exposure. The risk of developing antibodies has been shown to increase with the amount of exposure to foreign HLA,[73] and certain sensitising events, for example pregnancy, can result in a stronger and more durable immune response.[74] The presence of IgG donor specific HLA antibodies has been associated with poor graft outcomes. [47,75–77] Preformed IgG DSAs have been implicated in hyperacute rejection and ABMR within the first year post transplant.[78,79] Additionally, the development of *de novo* DSAs, especially class II DSAs are associated with increased microcirculation inflammation, glomerulopathy, capillary basement membrane multilayering and C4d staining on biopsy, with reduced graft survival after biopsy.[80–82] Once a DSA develops, up to 40% of patients will subsequently lose their graft.[82] On the other hand, not all DSAs have been associated with allograft damage and loss. Recent studies show that 30% of non-sensitised patients can develop *de novo* donor specific antibodies following transplantation without demonstrating evidence of rejection on biopsy.[82,83] This can possibly be explained by the characteristics of the antibody itself, for example, titres, IgG subclasses, the antibody's ability to fix complement, and affinity. Alternatively, graft characteristics such as target antigen expression levels and the endothelial cell signalling response to antibody binding may play a role. Low level DSAs for example, have been found to be protective by preventing cell lysis when subsequently exposed to higher levels of DSA. This is thought to be through the activation of the phosphoinositide-3-kinase protein kinase B (PI3/Akt) signalling pathway which upregulates anti apoptotic proteins and promotes proliferation, thus abrogating the cytotoxic effects of complement.[84–86]

H&I laboratories aim to assess risk by measuring HLA antibodies prior to transplantation.[47,75–77] There are 3 commonly used methods for determining the presence of donor specific antibodies:

Cell Dependent Cytotoxicity Crossmatch

First described by Paul Terasaki, the cell dependent cytotoxicity (CDC) crossmatch is used to determine the presence of complement fixing IgM and IgG antibodies by incubating donor cells with recipient serum in the presence of complement and a cell viability stain.[87] The crossmatch is reported based on the relative proportion of viable and dead cells visualised at the end of the incubation period. A positive crossmatch was associated with hyperacute rejection, and was therefore at the time, considered a veto to transplantation.[87] The initial crossmatch method has since been modified to improve sensitivity, and to separate donor T and B-cells, allowing the assessment of potential class I (T-cell reactivity) and class II (B-cell reactivity) HLA DSAs. Some protocols include the pre-treatment of serum using dithiothreitol (DTT) to remove IgM antibodies. The CDC assay continues to be used for antibody screening and histocompatibility testing however there are inherent problems with the assay. CDC assays can only test for complement fixing donor specific antibodies. Non-complement fixing antibodies, or low titre antibodies cannot be ruled out with a negative CDC crossmatch. Furthermore, as the test output measures cell viability, the test sensitivity and specificity are heavily influenced by the quality of donor cells.

Flow Cytometric Crossmatch

The flow cytometric crossmatch (FXM) is considered up to 250 times more sensitive than the standard CDC crossmatch technique and measures total IgG DSAs (complement fixing and non-complement-fixing).[88] The fluorescence obtained from each cell is proportional to the bound antibodies and allows a more quantitative assessment of DSAs compared with the CDC crossmatch. Similar to the CDC crossmatch, it does not determine the presence of HLA donor specific antibodies, simply antibodies that bind to donor lymphocytes.

Virtual Crossmatch

The final method for determining histocompatibility is to perform a virtual crossmatch. Recipient sera are screened for IgG HLA antibodies, and specificities are compared with the HLA type of the donor. Screening for antibodies previously involved performing multiple crossmatches on a locally maintained or a commercially sourced cell panel. These have been superseded by solid phase testing including enzyme linked immunosorbent assay (ELISA) and the current gold standard test utilises Luminex technology where HLA are purified from cell lines and coated onto polystyrene microbeads. Antibodies bind to the HLA and are visualised via a fluorochrome-conjugated reporter antibody using flow cytometry. The differing ratio of internal dyes within each bead allow hundreds of antigen specificities to be tested in a single assay, resulting in a rapid and a highly sensitive screening tool (see Figure 2.2a). Demonstrating HLA antibodies with this method has been shown to be more sensitive than the flow crossmatch, however, relies on the assumption that these antibodies will also bind to donor cells. Local laboratory experience and validation studies will provide a level of DSA (class I, II and even specific loci) that typically is associated with flow or CDC crossmatch positivity. The virtual crossmatch has been increasingly used for selected patients who have stable HLA antibody profiles and well documented sensitisation histories as it minimises the laboratory contribution to the organ cold ischaemic time. The Luminex HLA antibody assay however has its own limitations. Laboratories are reliant on a comprehensive bead panel that is representative of antibodies found in their patient and donor population. There is increasing evidence that false positive profiles can occur, possibly as the result bead manufacturing process.[89] Usually, a combination of crossmatch techniques will be performed at the time of offer to inform on compatibility and risk.

HLA-specific antibodies have been referred to in the literature using the following commonly used terms: anti-HLA-antibodies, and HLA-antibodies. 'Anti-HLA-antibodies' has also been used to describe idiotypic antibodies. Therefore, for the purposes of this thesis, I will be referring to HLA-specific antibodies as 'HLA antibodies'.

1.4.2 ANTIGEN PRESENTATION

B-cells express HLA class I and II, and can present both endogenous and exogenous peptides to T-cells. Whereas other APCs present exogenous peptides following the non-specific uptake of antigen, B-cells bind to specific antigens via the B-cell receptor (BCR).[90,91] The BCR and antigen will internalise and enter the endosomal compartment, allowing for antigen processing. The peptides presented to $CD4^+$ T-cells via HLA class II therefore are those to which the B-cells are highly specific, therefore establishing a cognate link between the T and B-cell. This augments both the T-cell response to the activating B-cell, and B-cell activation following T-cell help.[90,91] Following activation and clonal expansion, the B-cell becomes the predominant antigen presenting cell. Expression of co-stimulatory molecules including CD80, CD86 and OX40L allow the B-cells to prime and activate T-cells almost as effectively as dendritic cells, driving a highly specific and robust immune response.

1.4.3 CYTOKINE SECRETION

B-cells can modulate the immune system through the secretion of pro-inflammatory and regulatory cytokines.[92–97] The type and the amount of cytokine produced varies across the B-cell subsets, influenced by the activating signal and microenvironment. Similar to Th1 and Th2 responses, activated B-cells can be distinguished depending on their distinct patterns of cytokine secretion. Harris *et al* identified two populations of effector B-cells (Be-1 and Be-2) that produced different cytokine profiles depending on their stimulating environment. Be-1 cells, primed by antigens and Th1 cells, secreted cytokines that were associated with type 1 responses (IFN-γ and IL-12). Be-2 cells on the other hand, primed by Th2 cells and antigens, produced cytokines associated with type 2 responses (IL-2, IL-4 and IL-13).[97] Through the production of different cytokines, these B-effector cells were able to drive the differentiation of naïve $CD4^+$ T-cells to either Th1 or Th2 cells.[97]

1.4.3.1 INTERLEUKIN-10 (IL-10)

Interleukin-10 (IL-10), a key cytokine involved in immune regulation, was first described as a Th2-type cytokine when it was initially found to be produced by murine Th2 cells, inhibiting Th1 cell activation and cytokine production.[98,99] It is now known that IL-10 is widely expressed by macrophages, dendritic cells, various T-cell subsets and B-cells.[100–102] Additionally, endothelial, epithelial, and some tumour cells have also been shown to express IL-10.[103–105] It affects multiple cellular processes which include reducing the production of inflammatory cytokines, suppressing the proliferation of T-cells, inducing FoxP3 regulatory T-cells, and inhibiting antigen presentation.[106,107] IL-10 mediates its action by binding to the IL-10 receptor. The IL-10 receptor consists of 2 subunits (IL-10R1 and IL-10R2). IL-10 binds to IL-10R1 with high affinity which results in a conformational change, allowing IL-10R1 to oligomerise with IL-10R2.[108] Closely associated with the intracellular domains of IL-10R1 and IL-10R2 are Janus Kinase (JAK) 1 and tyrosine kinase (Tyk) 2 which are brought together allowing for phosphorylation.[109,110] Jak1/Tyk2 phosphorylate two further residues on IL-10R1 which are required for the recruitment and activation of signal transducer and activator of transcription (STAT) 3.[110,111] Activated STAT3 homodimers translocate from the cytoplasm to the nucleus allowing the transcription of several regulatory factors that inhibit the transcription of pro-inflammatory cytokines and chemokines.[112,113] The IL-10 receptors are expressed mainly by leukocytes, and expression is upregulated following activation of various cells suggesting its importance in regulatory activity.[102]

IL-10 has direct effects on T-cell responses, by inhibiting chemokine and cytokine production. It is important in maintaining FoxP3 expression in regulatory T-cells together with promoting their survival.[107] It can act directly on $CD4^+$ cells, inducing non-responsiveness.[114] Indirectly, IL-10 can affect T-cell activation by decreasing the production of pro-inflammatory chemokines and cytokines by macrophages and limiting antigen presentation via the downregulation of cell surface MHC class II expression.[115]

IL-10 plays a complex role in regulating B-cell function, and possibly the timing of IL-10 ligation in relation to B-cell activation determines whether it has an inhibitory or stimulatory effect. For example, when purified human B-cells were cultured with *Staphylococcus aureus* Cowan I, if IL-10 was added during the initial activation, it facilitated apoptosis of the activated B-cells. However, if IL-10 was added after 72 hours of culture, it prevented apoptosis, and supported B-cell differentiation.[116] It can inhibit B-cell activation by reducing the expression of costimulatory molecules including CD80 and CD86.[117] IL-10 itself can directly suppress immunoglobulin production, and in a model of allergy, can skew specific isotype formation to an IgG4 dominant phenotype.[118] Finally, an autocrine effect on the expansion of IL-10 competent B-cells has been described.[119] IL-10 has also been demonstrated to prevent the apoptosis of germinal B-cells and induce differentiation of activated B-cells, and enhance immunoglobin production.[120,121]

Human B-cells can produce IL-10 following CD40 stimulation alone, yet when the activating stimulus includes the B-cell receptor together with CD40, the proinflammatory cytokines TNF-α, lymphotoxin and IL-6 are produced.[122] Further work showed that different cytokines were produced by different B-cell subsets, where memory cells were found to be responsible for producing the majority of lymphotoxin and TNF-α following BCR and CD40 ligation, yet naïve B-cells mainly produced IL-10 following CD40 activation alone.[123] The plasticity of transitional B-cell cytokine production has been investigated in the context of renal transplantation, where the ratio of IL-10 to TNF-α production was measured following TLR and CD40 stimulation.[124]

1.5 REGULATORY B LYMPHOCYTES (BREGS)

B-cells with immunosuppressive capacity were first described in guinea pigs in the 1970, however, Mizoguchi first presented the term 'regulatory B-cell' in a murine model of colitis, where $CD1d^{+}$ B-cells were found to produce IL-10 and inhibit disease progression through the downregulation of inflammatory cascades.[125,126]

Subsequently, IL-10 producing B-cells were shown to suppress inflammation in murine models of experimental autoimmune encephalitis, lupus, and arthritis.[127–129]

It was not entirely clear whether or how these findings would translate to humans until the observation that patients receiving the anti-CD20 monoclonal antibody rituximab were at increased risk of developing psoriasis, or exacerbations of ulcerative colitis, leading to the hypothesis that rituximab was depleting B-cells with immunoregulatory function.[130,131] This has led to an increase in studies focussing on the phenotypic and functional characteristics of immunosuppressive B-cells through the secretion of IL-10, or other immunosuppressive actions. In humans, Bregs have been described in different B subsets in models of alloimmunity and autoimmunity, and have been summarised in Table 1.2. Although there is currently no specific marker for Bregs (either transcriptional or cell surface), it is accepted that Bregs typically produce IL-10.[94,95,132,133]

In addition to inhibitory cytokine secretion, Bregs can also exert their regulatory activity through direct cell-to-cell interactions. The cell surface molecules that have been implicated in this regulatory activity include CD80, CD86, CD40, CD1d which are able to inhibit effector T-cell function, induce regulatory T-cells (observed via increase of FoxP3 and CTLA-4 expression) and induce apoptosis of the target cell.[134–136] It is thought that $CD25^{+}$ B-cells can induce expression of FoxP3 and CTLA-4 on Treg cells through this direct cell contact.[96]

The differing cell-surface phenotypes of B-cells with IL-10 capacity have led to a lack of consensus surrounding the ontogeny of Bregs. The first model suggests that Bregs, like regulatory T-cells, develop from a single B-cell lineage.[137,138] In the second model, B-cells can acquire a regulatory phenotype in response to the local microenvironment, and consequently, Bregs can arise from different B-cell populations.[139] Table 1.2 lists the different human regulatory B-cell phenotypes currently described in the literature.

	Phenotype	Mechanism of suppression	In vitro model	Reference
Immature	$CD19^{+}CD24^{hi}CD38^{hi}$	IL-10, PD-L1	CD40L activation in SLE patients	95
TrB	$CD19^{+}CD24^{hi}CD38^{hi}$	IL-10:TNF-α	CPG and CD40L activation in Renal transplant recipients	124,140,141
Naive	$CD19^{+}CD27^{-}$	IL-10	CD40L activation in multiple sclerosis patients	123
B10 cells	$CD19^{+}CD24^{hi}CD27^{+}$	IL-10	LPS and CPG activation in healthy donors, and patients with RA, SLE, SS, and MS	94
Memory	$CD19^{+}CD24^{hi}CD27^{+}$	IL-10, HLA-G expression	CPG and CD40L activation in renal transplant recipients	142
Plasmablasts	$CD27^{int}CD38^{+}$	IL-10	Healthy donors, activation with CPG, IL-2, IL-6, IFN-γ	143
$CD25^{+}$	$CD19^{+}CD25^{high}$ $CD27^{high}$ $CD86^{high}CD1d^{high}$	IL-10, TGF-β Direct cell contact	Healthy donors, ANCA-vasculitis. CPG, CD40L, IL-4	96,144

Table 1.2. Phenotype of Bregs in humans

(SLE: systemic lupus erythematosus, RA: rheumatoid arthritis, SS: Sjogren syndrome, MS: multiple sclerosis, LPS: lipopolysaccharide)

1.5.1 ACTIVATION OF REGULATORY B-CELLS

Bregs can be activated to produce IL-10 by a combination of signalling pathways including TLR and BCR ligation, and co-stimulation mediated by CD40, CD80/86, and cytokines.[145] There are two main models describing Breg activation, and in both models, TLR and CD40 ligation are considered essential.[146,147] CD40 is a membrane associated protein and member of the TNF superfamily. It is expressed on B-cells and other cells including monocytes, macrophages, dendritic cells, platelets, and endothelial cells. Its ligand, CD40L (also known as CD154) is expressed primarily on activated T-cells.[148] The CD40-CD40L interaction provides the co-stimulation necessary for the maturation of the B-cell, isotype switching and somatic hypermutation.[149] However, prolonged signalling with high density of CD40L can have inhibitory effects, for example it can reduce antibody secretion by the B-cell. [150] In a murine model of autoimmune encephalitis when B-cells were incubated with antigen providing BCR stimulation only, IL-10 was not produced. However, when CD40 was added to the culture, IL-10 was produced and this correlated with clinical recovery.[151] Similarly in a murine model of collagen induced arthritis, therapy with an agonistic CD40 monoclonal antibody was associated with IL-10 production by B-cells, and clinically, disease progression was controlled.[152]

In the first model of described by Fillatreau *et al*, Breg activation is a multi-step process. The first step includes TLR ligation to initiate IL-10 production. As the immune response develops, the B-cells gain further immunosuppressive function, where BCR and CD40 binding is required to promote Breg survival and augmented IL-10 secretion.[139,153] This model has been developed following investigations of murine experimental autoimmune encephalomyelitis (EAE). After naïve B-cells were activated with TLR4 for 72 hours, they were able to produce enough IL-10 to inhibit the proliferation and differentiation of $CD4^+$ T-cells.[139] Mice lacking IL-10 competent B-cells developed early chronic EAE, due to an uncontrolled pathogenic Th1 response. However, if the B-cells lacked CD40L only, the mice went on to develop chronic EAE, which occurred after 10 days.[151] This suggests that CD40 is required for sustained IL-10 production.[151]

In the second model, favoured by Mizoguchi *et al* in the context of autoimmune diseases, Breg activation occurs via different pathways. Mesenteric lymph node B-cells, or alternatively B1 cells are activated by TLR signalling (either lipopolysaccharide or CPG) representing an 'innate-type' Breg subset, whereas follicular B-cells receive stimulation by CD40 with or without BCR ligation resulting in adaptive-type B regs.[154]

In humans, *in vitro* studies have demonstrated that TLR9 ligation is crucial for the development of Bregs and is the strongest stimulus for IL-10 production; this can be enhanced with CD40 stimulation.[155,156] Bouaziz *et al* demonstrated that the duration of TLR9 ligation was important in the development of IL-10 capacity across the different B-cell subsets. Short term activation (5 hours) of purified B-cells using CPG-B, phorbol myristate acetate (PMA) and ionomycin resulted in IL-10 production by $CD19^{+}CD27^{+}$ memory and $CD19^{+}CD24^{+}CD38^{+}$ transitional B-cells.[155] The authors found that TLR and BCR stimulation (using CPG-B and anti-Ig) for 48 hours resulted in maximal IL-10 production by purified B-cells. A longer stimulation period also revealed IL-10 producing naïve cells which upregulated TLR9 expression following BCR ligation.[155] When purified tonsillar B-cells were stimulated with CPG alone, an upregulation of activation markers including CD40 was noted, however the proliferation of B-cells and their production of IL-10 was not apparent until the cells were co-cultured with CD40L-expressing cell line.[157]

These experiments support the concept that not only the B-cell subset but other factors including the B-cell activation status and the specific microenvironment can contribute to Breg formation. [158]

1.5.2 REGULATORY B-CELLS IN RENAL TRANSPLANTATION

Previous studies conducted in Leeds focussed on the immune phenotype of allograft recipients in a randomised controlled trial, and demonstrated that following alemtuzumab induction, patients had higher numbers of B-cells including naïve, TrB, and regulatory subsets compared with those receiving basiliximab induction.[140] Bregs were characterised not only by IL-10 secretion, but additionally by TNF-α expression,

using a ratio of IL-10:TNF-α to describe cytokine polarisation from different B subsets.[124] In healthy volunteers, TrBs were found to have the most anti-inflammatory profile based on the IL-10:TNF-α ratio and were capable of suppressing pro-inflammatory Th1 polarisation in autologous T-cells *in vitro*. However, TrBs from patients with rejection, were unable to suppress the expression of Th1 cytokines by T-cells. In allograft recipients, higher numbers of TrBs were associated with better graft function, and less HLA antbodies.[140] A low TrB IL-10:TNF-α ratio was associated with poor graft outcomes in patients with graft dysfunction. In stable patients, a reduced ratio was predictive of worse outcomes.

Human TrBs can be further subdivided into T1 and T2 subsets. T1 represents a more immature cell with increased expression of CD24, CD38, IgM and CD10 compared with T2.[140,159] Additionally, T1 cells exhibit a higher IL-10:TNF-α ratio through conserved levels of IL-10 but decreased TNF-α expression.[124] In healthy subjects, the ratio of T1:T2 cells is approximately 25:75.[141] Patients with chronic antibody mediated rejection (CAMR) have been shown to have an altered distribution of T1 and T2 cells, with a decreased T1:T2 ratio compared with other patients who had graft dysfunction but no evidence of CAMR on biopsy.[141]

1.6 THE DIFFERING EFFECTS OF INDUCTION AND MAINTENANCE IMMUNOSUPPRESSIVE AGENTS ON THE B-CELL PHENOTYPE

Most immunosuppressive agents routinely used in renal transplantation can affect B-cells and B-cell subsets, both directly and indirectly. Routine steroid-sparing immunosuppression in Leeds includes Alemtuzumab induction followed by tacrolimus monotherapy. The second line regime, used in approximately 30% of the transplant population, includes Basiliximab followed by tacrolimus and mycophenolate mofetil (MMF). Alemtuzumab is a humanised monoclonal antibody against CD52 that is highly expressed on lymphocytes, monocytes and NK cells. The binding of alemtuzumab to CD52 targets the cell for antibody dependent cellular cytolysis, and complement mediated lysis, resulting in the profound depletion of the cells. Following depletion,

monocytes rapidly repopulate the periphery, followed by NK cells.[160] Lymphocyte repopulation can take up to a year, and often B-cells will repopulate exceeding pre-induction levels.[140,161] Basiliximab is a chimeric monoclonal antibody that binds with high affinity to the α chain (CD25) of the IL-2 receptor, preventing IL-2 ligation that is required for the activation of T-cells. In healthy individuals, up to 65% of the peripheral B-cell population can express CD25, and therefore basiliximab can potentially have direct effects on B-cell function.[162] Brisslert *et al* initially investigated the phenotypic and functional differences of $CD25^+$ and $CD25^-$ B-cells. They demonstrated that $CD25^+$ B-cells are larger and more granular, and expressed more surface immunoglobulin and co-stimulating molecules (CD27 and CD80) than $CD25^-$ B-cells. They noted that $CD25^+$ B-cells failed to secrete immunoglobulins on stimulation with IL-2, but during mixed lymphocytic reactions, $CD25^+$ B-cells more efficiently activated allogenic T-cells compared with $CD25^-$ B-cells. IL-2 blockade reduced this T-cell proliferation. The authors concluded that $CD25^+$ B-cells played a significant role in antigen presentation and activation of T-cells.[162] Subsequently, $CD25^+$ cells have been shown to secrete higher levels of IL-10 compared with $CD25^-$ cells, and these cells were present in higher numbers in ANCA positive vasculitis patients during remission than those experiencing active disease, suggesting a potential regulatory role.[144] $CD25^+$ B-cells have also been shown to enhance FoxP3 and CTLA-4 expression in regulatory T-cells.[96] Basiliximab induction may therefore affect this regulatory potential. *In vivo* studies have also demonstrated that basiliximab induction can lead to an increased memory B-cell population.[140,163]

The differing mechanisms of action (MOA) of the induction agents, as well as the potential effects of maintenance immunosuppression on B-cells will need to be considered when determining these biomarkers of graft outcome. Table 1.3 summarises the common immunosuppressive agents, together with the potential effects on B-cells.

Immunosuppressive Agent	Mechanism of Action	Peripheral B-cell effects	References
Alemtuzumab	Monoclonal CD52 antibody, depletes lymphocytes	Elevated naïve and TrBs on repopulation. Reduced memory cells	140,161,164,165
Basiliximab	Anti CD25 antibody	Increased total B-cells, and memory cells. No effect on TrBs. ?affect regulatory potential through CD25 blockade. Indirect: reduced plasma cell generation (reduced IL-2 production by T-cells)	140,144,163,166
Tacrolimus	Calcineurin inhibitor	No direct effect on B-cells or B-cell subsets. Indirect: Reduced T-cell dependent activation, differentiation and immunoglobulin secretion	167–170
Ciclosporin	Calcineurin inhibitor	Reduction of TrBs, reduced IL-10 capacity. Indirect: Reduced T-cell dependent activation, differentiation and immunoglobulin secretion	170
Sirolimus	Mammalian target of rapamycin inhibitor	Increased TrBs, reduced B-cell proliferation/activation	167,171
Prednisolone	Widespread anti-inflammatory actions	Induces apoptosis, reduces B-cell reconstitution after HSCT, reduces naïve and TrBs.	168,169
Mycophenolate	IMPDH inhibitor, inhibits purine synthesis	Inhibits B-cell proliferation + plasma cell differentiation. Indirect: Reduced T-cell dependent activation	168,169,172
Azathioprine	Inhibits purine synthesis	Reduces TrBs and naïve B-cells	168,169,172
Rituximab	Chimeric anti CD20 monoclonal antibody, depletes B-cells	Transient increase in TrBs	173,174

Table 1.3. Common Immunosuppressive agents and their effects on B-cells in the periphery.

IMPDH – inosine-5'-monophosphate dehydrogenase, TrB – transitional B-cell, HSCT – haemopoietic stem cell transplantation

1.6.1 B-CELLS IN ALLOTRANSPLANTATION

B-cells were initially thought to be involved in with allograft rejection as an adjunct to T-cell mediated rejection. However, it is increasingly recognised that B-cells can contribute directly to graft damage not only through antibody production but also through efficient antigen presentation. They can shape the T-cell response through cytokine production and co-stimulation. The differentiation into memory B-cells and plasma cells provides immune memory which must be considered in the case of highly sensitised patients or patients requiring regrafts. Current immunosuppressive regimes have successfully targeted T-cell activation and proliferation, improving the rates and severity of TCMR. However, despite this, there has not been an improvement in long term graft survival, and chronic antibody mediated rejection has become the predominant cause of immune-mediated allograft injury. Unfortunately, current therapeutics are not as effective at treating ABMR and are not able to successfully target individual B-cell subsets. The mechanistic understanding of the B-cell response to allotransplantation is required to develop therapies that can target individual B-cell subsets improve graft survival.

1.7 AIMS AND OBJECTIVES

1.7.1 TO ASSESS THE DIFFERENCES IN MEDIUM – TERM CLINICAL OUTCOMES FOR ALLOGRAFT RECIPIENTS RECEIVING EITHER ALEMTUZUMAB OR BASILIXIMAB AS AN INDUCTION AGENT.

Despite satisfactory short-term outcomes associated with alemtuzumab based immunotherapy, the widespread adoption of alemtuzumab as an induction agent in renal transplantation has not occurred, possibly due to concerns regarding the long-term safety profile. The longer-term outcomes in standard risk adult patients receiving their first kidney alone transplant will therefore be assessed by interrogating the UK NHS Blood and Transplant (NHSBT) renal registry. Clinical outcomes (including renal function, patient, graft and rejection free survival) will be compared between alemtuzumab and other induction agents. Data returns on cause of death and cause of graft loss will be assessed for any signals suggestive of increased malignancy or infection associated with alemtuzumab use.

1.7.2 TO PROSPECTIVELY STUDY THE UTILITY OF TRBS AS AN EARLY BIOMARKER OF ALLOGRAFT OUTCOME

Kidney transplant recipients have well-defined quantitative and qualitative TrB phenotypes, and changes in these phenotypes correlate with clinical outcomes. The next step is to test the utility of these biomarkers prospectively and investigate whether they can be utilised to guide clinical management.

A composite endpoint will be used to assess the utility of the biomarkers, and will comprise of surrogate markers that have been previously associated with adverse graft outcomes:

- The development of *de novo* donor specific antibodies [76,77,175,176]
- A 30% reduction in estimated glomerular filtration rate (eGFR) between 3 - 18 months [177]

- Histological diagnosis showing immune-mediated changes, including antibody mediated, T-cell mediated or borderline rejection according to the Banff 2013 criteria, evidence of transplant glomerulopathy, or recurrent disease.[178,179]
- Proteinuria (urine protein-creatinine ratio, UPCR>50) [180]

1.7.2.1 HYPOTHESES

1) A low 3-month TrB, T1:T2 ratio is predictive of later adverse graft outcomes.
2) In patients with a troubled graft, TrBs, and T1:T2 ratio can risk stratify patients at risk of poor graft outcomes.

1.7.3 TO DEVELOP AN *IN VITRO* MODEL OF THE IMMUNE MEMORY RESPONSE

1.7.3.1 HYPOTHESIS:

In patients who have experienced a previous sensitising event, the memory assay will uncover additional HLA antibody specificities to those identified in routine serum screening. These additional specificities may potentially reflect an existing capacity to form a memory response.

1.7.4 TO ASSESS THE ROLE OF PREFORMED HLA-DP DONOR SPECIFIC ANTIBODIES ON CLINICIAL OUTCOMES IN RENAL TRANSPLANT RECIPIENTS

There is limited data surrounding the clinical outcomes following renal transplants in the presence of HLA-DP donor specific antibodies (DSAs). Until recently, HLA-DP sensitisation was not considered in the UK allocation algorithm. A retrospective analysis of transplants in the presence of HLA-DP DSAs will be performed, and clinical outcomes assessed to determine any factors that may help with risk stratifying these types of transplants.

2 GENERAL MATERIAL AND METHODS

2.1 DNA EXTRACTION AND QUANTITATION

Three different methods of DNA extraction were used.

2.1.1 DNA EXTRACTION USING MAGNETIC BEADS (FIGURE 2.1)

The Maxwell® 16 DNA extraction system (Promega) permits the automated extraction of DNA from batches of 16 different samples. Whole blood samples were centrifuged for 10 minutes at 1450 x *g* to obtain a buffy coat. Using a pastette, 500µl of buffy coat was transferred to well 1 of the corresponding reagent cartridge. A magnetic plunger was added to well 7 and the reagent cartridge was inserted into the Maxwell instrument. Molecular grade water (400µl) was added to an elution tube which was placed in front of each cartridge. During the DNA extraction programme, cells were lysed, and paramagnetic MagneSil® particles were added to the lysed cells in order to capture the DNA. The paramagnetic particles with bound DNA were moved stepwise through a series of wash steps. The final heat-elution step delivered extracted DNA into the elution tube. The elution tube was placed onto a magnet to separate the paramagnetic particles from the DNA which was transferred into an Eppendorf tube for quantitation and storage.

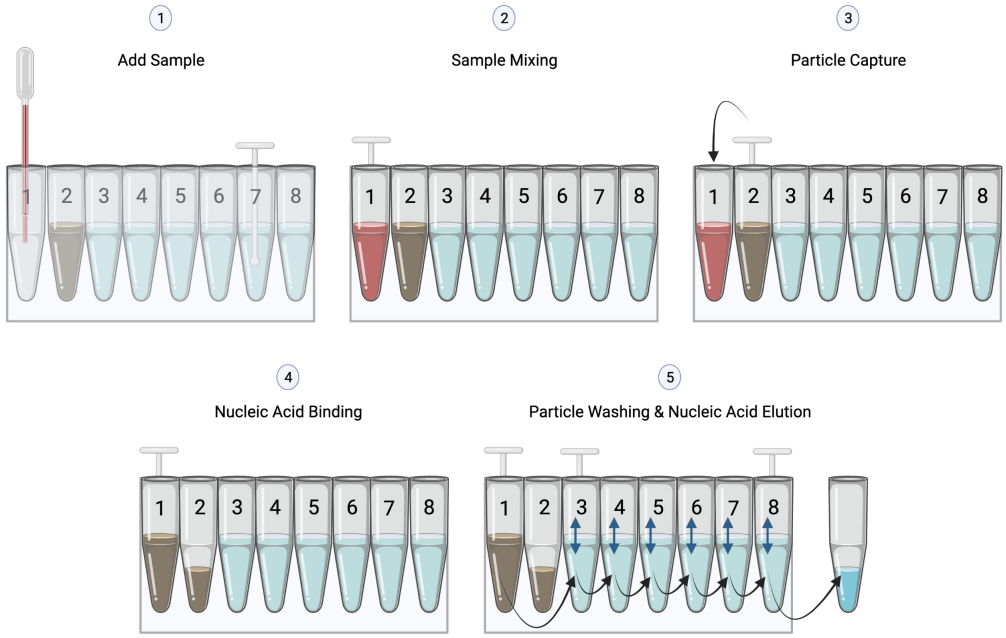


Figure 2.1. DNA Extraction using the Maxwell® DNA extraction system. Created with Biorender.com

2.1.2 DNA EXTRACTION USING SPIN COLUMNS

The QIAmp® DSP DNA Blood Mini Kit (QIAGEN) was used to extract DNA from high-risk or clinically urgent patient samples. Whole blood samples were centrifuged at 1450g for 10 minutes to obtain a buffy coat. QIAGEN protease (20µl) and 200µl of buffy coat were added to a 1.5ml Eppendorf tube and vortexed briefly. The lysis buffer AL (200µl) was then added to the tube and mixed thoroughly. The Eppendorf tube was placed in a heat block at 55-57°C for 10 minutes to allow lysis of the cells. Following incubation, the tube was centrifuged briefly to remove any droplets from the inside of the lid. The DNA was precipitated by adding 200µl of 96% ethanol to the sample and vortexing. The sample was then transferred to a spin column placed in a 2ml collection tube. This was centrifuged for 1 minute at 16162 x *g*. The spin column was placed into a clean collection tube and the filtrate was discarded. The column was washed once by adding 500µl of buffer AW2 to the spin column prior to centrifuging for 3 minutes at 16162 x *g*. The spin column was transferred to a clean 1.5ml Eppendorf tube. Between 100-

150µl of molecular grade water was added to the spin column depending on the quality of the initial buffy coat obtained. The spin column was incubated at room temperature for 1 minute to allow the precipitated DNA to dissolve in the water. Following this, the sample was centrifuged at 16162 x *g* for 1 minute to collect the DNA in the clean Eppendorf tube for quantitation.

2.1.3 DNA EXTRACTION USING ETHANOL PRECIPITATION

Whole blood was centrifuged at 1450 x *g* for 10 minutes to obtain a buffy coat. Concentrated red cell lysis buffer (5x RCL, 2ml) was added to a 15ml v-bottomed tube and diluted with 8ml of molecular grade water and 1-2ml of buffy coat was added. This was incubated for 5 minutes at room temperature on a Spiramix roller (Denley) to ensure mixing. The tube was centrifuged at 1450 x *g* for 10 minutes. The supernatant was carefully discarded, and the white cell pellet was re-suspended by in 1ml of 5x RCL buffer prior to transfer to a 1.5ml Eppendorf tube. This was vortexed and then centrifuged at 16162 x *g* for 2 minutes. The cell pellet was washed by resuspending in 1ml of molecular water and centrifuging at 16162 x *g* for 2 minutes. The supernatant was discarded. The cell pellet was then resuspended in the following: molecular grade water (200µl), 5x proteinase K buffer (80µl), 10% SDS (40µl) and Proteinase K 10% solution (30µl). The cells and mixture were incubated at 54-56°C for 10 minutes using a heat block. Cold 6M sodium chloride solution (200µl) was added to the tube. The sample was vortexed for at least 15 seconds to precipitate protein whilst keeping the DNA in solution. The sample was centrifuged at 16162 x *g* for 5 minutes. The supernatant was poured into a clean 1.5ml Eppendorf and the pellet of cellular debris was discarded. Cold 96% ethanol was added to the Eppendorf tube to precipitate the DNA. The tube was vortexed and then centrifuged at 16162 x *g* for 2 minutes. The supernatant was discarded. A final wash step was performed by resuspending the pellet was in 200µl of cold 70% ethanol prior to centrifuging at 16162 x *g* for 2 minutes. The supernatant was discarded and the excess 70% ethanol was removed by carefully wiping the inside of the tube. Depending on the size of the DNA pellet, between 100-600µl of molecular grade water was added to dissolve the DNA. The

DNA was placed in an incubator at 35-39°C for a minimum of 10 minutes prior to quantitation.

2.1.4 DNA QUANTITATION

DNA samples were obtained and allowed to come to room temperature. The NanoDrop One Spectrophotometer (ThermoFisher Scientific) was initialised, and the loading pedestal cleaned with a lens tissue. As DNA samples were dissolved in water, 2µl of molecular grade water was loaded onto the pedestal to test its UV absorbance and provide a 'blank' or background value against which the absorbance of DNA could be measured. The DNA was vortexed and 2µl was loaded onto the pedestal for testing. The DNA concentration was noted. The ratios of sample absorbance at 260/280 nm and 260/230 nm were also recorded, allowing the assessment of DNA purity and contaminants.

2.2 HLA TYPING

HLA typing was performed by the Transplant Immunology Laboratory in St James's University Hospital. Three complementary methods were routinely used:

2.2.1 POLYMERASE CHAIN REACTION – REVERSE SEQUENCE SPECIFIC OLIGONUCLEOTIDE (PCR-RSSO)

Overview

This method was used to determine the initial HLA type of renal live donors, recipients and for verification typing of imported renal donors. The ONELAMBDA LABType® kit was used, which allowed the batch typing of HLA – A, B, C, DRB1, DRB3/4/5, DQA1, DQB1, DPA1 and DPB1 loci using a 96 well plate format. The gene of interest was amplified by PCR using locus specific biotinylated primers. The biotinylated PCR product was then hybridised with a mix of up to 100 fluorescent Luminex microspheres, containing differing allele group discriminatory oligonucleotide probes on the bead surface. This allowed the biotinylated amplicons to bind to their complementary oligonucleotide probes. A secondary conjugate of streptavidin and phycoerythrin (SAPE) was then added to bind to the biotinylated PCR product allowing detection (Figure 2.2).

Brief Method

DNA was extracted, adjusted to a concentration of 20ng/ml, and 0.8 microlitres of the DNA was added to a 96 well PCR plate for each locus tested. The LABType® D-mix and appropriate primers were allowed to thaw at room temperature. A master mix containing the appropriate volumes of primer, D-mix and DNA polymerase was made according to the number of PCR reactions, adding 4 excess reactions per HLA locus (Table 2.1).

Reagent	Volume per Reaction (µl)
D-mix	5.6µl
Primer	1.6µl
Taq	0.06µl
DNA	0.8µl

Table 2.1. LabType® Reaction volumes. Volumes of D-mix, Primer, DNA and DNA polymerase required per reaction

The master mix (7.5µl) was added to each reaction well. A negative and positive control reaction was included for each locus tested. The PCR plate was tightly sealed and centrifuged to ensure that all the contents were collected at the bottom of each reaction well. The plate was placed on a thermocycler (Applied Biosystems Geneamp PCR System 9700) programmed for the following amplification reaction (Table 2.2):

Number of Cycles	Step	Temperature (°C)	Time (seconds)
1	1	96	180
5	1	96	20
	2	60	20
	3	72	20
30	1	96	10
	2	60	15
	3	72	20
1	1	72	600
End	1	4	--

Table 2.2. LABType PCR programme

Following amplification, the biotinylated PCR products were transferred to a 96-well Luminex tray and placed on the LABXpress desktop bench top robot for hybridisation. The precalculated volumes of denaturation buffer, neutralisation buffer, wash buffer, SAPE solution and microbeads suspended in hybridisation buffer were added to the correct buckets and loaded onto the robotic platform.

The following hybridisation steps were performed by the LABXpress bench top robot: Denaturation solution (1.25µl) was added to each PCR product in the hybridisation tray and incubated at room temperature for 10 minutes. Following this, 3µl of neutralisation solution was added to each PCR product and mixed several times. Hybridisation buffer containing Luminex microspheres (19µl) was added to each well and mixed. The PCR tray was then placed into a pre-heated thermal cycler and held at 60°C for 15 minutes. Following this, each well was washed twice using 100µl of wash buffer. Twenty-five microlitres of 1x SAPE solution was then added to each well, and the hybridisation tray was incubated at 60°C for 5 minutes. The plate was then washed, and the contents of each well was resuspended in 50µl of wash buffer for acquisition by the LabScan 3D analyser.

2.2.2 POLYMERASE CHAIN REACTION – SEQUENCE SPECIFIC PRIMER (PCR-SSP)

Overview

This method was used to perform verification HLA typing on renal wait list recipients, their potential live donors, and as a secondary method for urgent HLA typing of deceased organ donors in an 'on call' setting. A commercial PCR kit (Ready Gene, Inno-train Diagnostik) was used, allowing for the analysis of HLA-A, -B, -C, -DRB1, DQB1, -DRB3-5 and DPB1 loci.

Brief Method

DNA was extracted and adjusted to a concentration of 50ng/µl using molecular grade water. The PCR trays containing pre-aliquoted primers for each locus of interest and the ReadyGene PCR master mix were defrosted. DNA polymerase (Thermoprime Plus) and molecular grade water were added to the master mix according to the specified volumes (Table 2.3).

Locus	Number of Reactions	Volume of mH_2O to add (µl) (6µl/well)	Volume of PCR mix (µl) (3µl/well)	Volume of Taq polymerase (µl) (0.08µl/well)	Volume of DNA (µl) (1µl/well)
A	24	168	84	2.2	26
B	48	324	162	4.3	52
C	24	168	84	2.2	26
DRB1, DRB3-5	24	168	84	2.2	26
DQ	8	60	30	0.8	10
DP	48	324	162	4.3	52

Table 2.3. ReadyGene PCR Volumes.

Volumes of molecular grade water, PCR master mix, Taq polymerase and DNA required for each HLA locus.

Each HLA locus under test (excluding HLA-DQB1) had a negative control well. Aliquots of the DNA polymerase/master mix (10µl) were added to each negative control well prior to the addition of the appropriate volume of DNA. Following this, 10µl of the PCR mixture was added to each well (apart from the negative control wells). The PCR tray was firmly sealed, vortexed and spun down to ensure that the samples collected in the bottom of each tube. The PCR tray was placed in a thermocycler that had been programmed according to Table 2.4.

Number of Cycles	Step	Temperature (°C)	Time (seconds)
1	1	96	120
10	1	96	15
	2	65	60
20	1	96	15
	2	61	50
	3	72	30
End	1	4	--

Table 2.4. ReadyGene PCR Programme

Following PCR amplification, the amplicons were loaded onto a 2.5% agarose gel containing ethidium bromide. A 100mbp electrophoresis ladder (2µl, Promega) was added to lane number 9 for each sample. The samples were run out on the gel for 20-25 minutes using 150 volts. The agarose gel was transilluminated with UV light and a photograph taken. The lane positions of the positive and negative reactions were compared with the ReadyGene interpretation worksheets to obtain an HLA type.

2.2.3 'REAL TIME' POLYMERASE CHAIN REACTION (RTPCR)

Overview

The LinkSeq™ (Linkage Biosciences) typing kit utilises a real-time PCR/quantitative PCR platform (QuantStudio™, ThermoFisher) to rapidly determine the HLA type (A, B, C, DRB1, DRB3/4/5, DQB1, DQA1, DPB1, DPA1) of deceased organ donors. The QuantStudio™ instrument performs a PCR programme and then generates a melt curve of the PCR products. During PCR-SSP, SYBR® Green, a dye which fluoresces when bound to double stranded DNA, is incorporated into the PCR products. Following the PCR programme, the products are heated, resulting in the dissociation of DNA into single strands. With this, there is an abrupt loss of fluorescence; a product specific melt curve is generated and analysed by the QuantStudio.

Brief Method

The LinkSeq master mix (pre-aliquoted in kit) and typing tray (384-well plate) were brought to room temperature. The plate was centrifuged at 400g for 1 minute to ensure that the reagents were located at the bottom of each well. DNA polymerase (92µl) was added to the master mix and carefully mixed by inversion. The mixture (10µl) was then added to the water control well. Molecular grade water and DNA was added to the buffer to achieve a total added volume of 2308µl DNA at a concentration of 1.3ng/µl. The DNA/master mix/DNA polymerase mix was then decanted into a reagent reservoir to allow loading onto a 12-channel pipette. Aliquots of 10µl were added to each well (apart from the water control well). The plate was sealed and centrifuged at 400 x *g* for 1 minute. The plate was then loaded onto the QuantStudio

real-time PCR instrument to run the PCR programme and construct the DNA dissociation curves.

2.3 THE PRINCIPLE OF LUMINEX TECHNOLOGY

The detection of HLA antibodies and HLA typing via rSSO utilise Luminex technology. The Luminex analyser is a type of flow cytometer that employs two lasers to interrogate microbead arrays. The red classification laser excites the internal fluorochromes within each bead, allowing the software to differentiate individual bead populations within the bead mix. The green reporter laser excites the phycoerythrin (PE) molecules that are attached to the reporter antibody (in the case of HLA antibody detection) or the SAPE molecules that have attached to the hybridised biotinylated PCR products. HLAFusion is a software that translates the pattern of PE fluorescence obtained into either HLA antibody specificities, or HLA type (Figure 2.2).

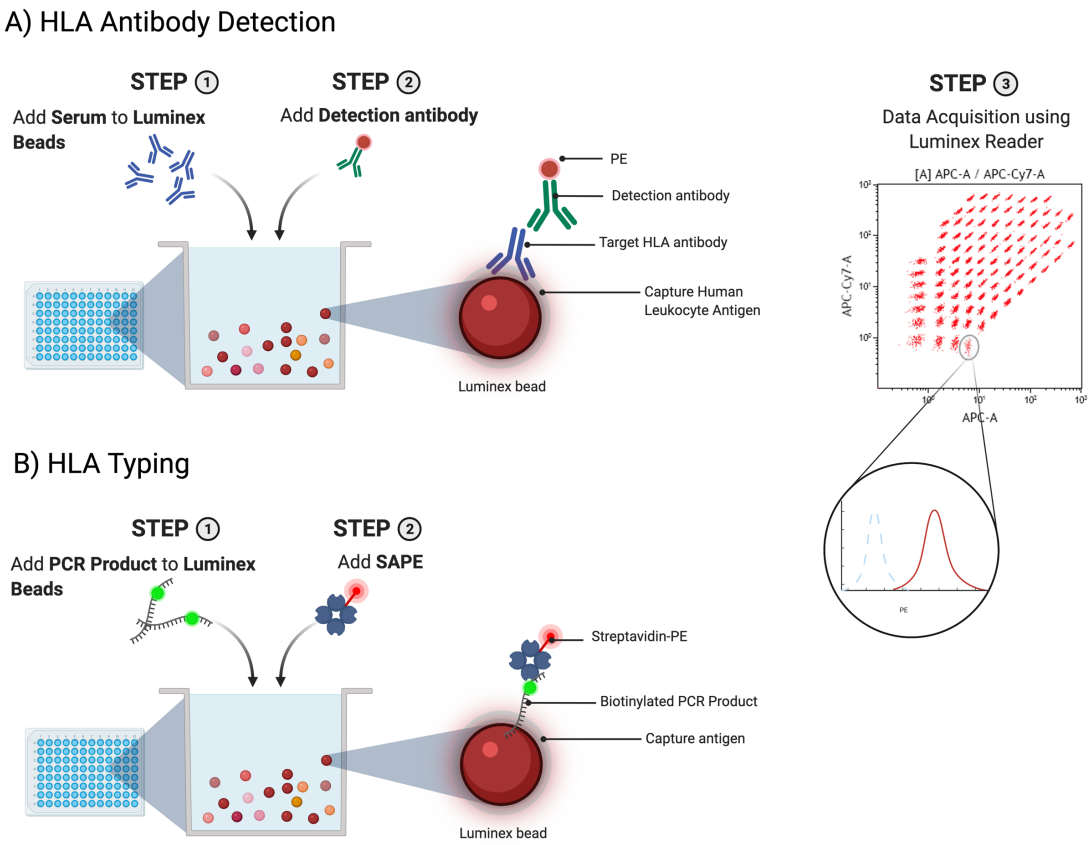


Figure 2.2. The principle of Luminex Technology for A) HLA Antibody detection and B) HLA typing.

Luminex beads contain two internal fluorescent dyes at different concentrations which can be configured into an array. The surface of each bead is coated with differing capture HLA. Step 1: The target analyte is incubated with the Luminex microspheres, and specific analytes are captured by the surface antigens on each bead. Step 2: In the case of HLA antibody detection, a reporter antibody that is conjugated with PE is added. For HLA typing, the biotinylated PCR product binds to the beads. Streptavidin-PE (SAPE) is added which binds to the biotinylated products. Step 3: The sample is acquired using a Luminex flow cytometer, and the PE fluorescence is calculated for each bead. Created using Biorender.com

2.4 ASSESSMENT OF HISTOCOMPATIBILITY

Histocompatibility assessment was performed by the Transplant Immunology Laboratory in St James's University Hospital according to the local policies. This included a combination of one or more of the following tests: Complement Dependent cytotoxicity crossmatch (CDCC), Flow crossmatch (FXM), assessment of recipient HLA antibodies in the current or time of offer serum using single antigen beads (SABs).

2.4.1 COMPLEMENT DEPENDENT CYTOTOXICITY CROSSMATCH (CDCC)

Donor and recipient PBMCs were isolated using Ficoll centrifugation as described in section 2.11. The T and B lymphocytes were then separated using magnetic beads (Dynabeads) using CD8 for T-cells and CD19 for B-cells. Aliquots of current and historic recipient serum were placed in a water bath at 56°C for 30 minutes to inactivate complement and then allowed to cool to room temperature for 10 minutes. Two oiled Terasaki trays were obtained. Sera were added to the crossmatch trays according to Figure 2.3. AB serum was used as the negative control. Anti-B-cell and Anti-lymphocyte sera were used for the positive control. The 'Allo' crossmatch (donor lymphocytes added to recipient serum) was performed in the first tray. The 'Auto' crossmatch (recipient lymphocytes added to recipient serum) was performed in the second tray. T and B lymphocytes (1µl) from donor or recipient were added to the relevant wells. The trays were then incubated at room temperature for 40 minutes. Freeze dried rabbit complement (Cedar Lane) was allowed to thaw on the bench for 5 minutes prior to reconstitution in 1ml of molecular grade water. Five microlitres of reconstituted complement was then added to each well of the Terasaki trays. The trays were then incubated at 18-22°C for 40 minutes. Acridine orange/ethidium bromide dye (2µl) was then added to each well and the trays were incubated in the dark at room temperature for 10 minutes prior to reading using an inverted fluorescent microscope. The relative proportion of live (green) and dead (red) cells were assessed and compared with the negative control and positive control wells to provide a positive/negative crossmatch result with a score indicating the 'strength' of positivity (see Table 2.5).

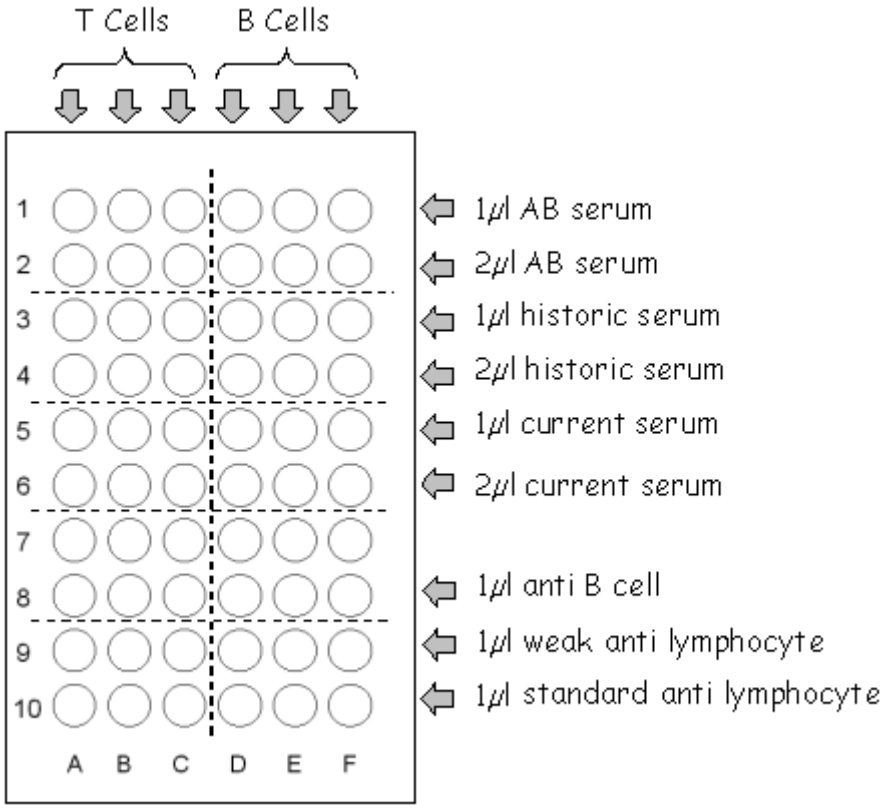


Figure 2.3. Layout of the CDC crossmatch tray.

Figure obtained from the SJUH transplant immunology laboratory SOP SLF2IMTS020 version 7.2

0	Unreadable / Invalid / No cells	
1	0 - 10 %	Negative
2	11 - 20 %	Probably negative
4	21 - 50%	Weak positive
6	51 - 80%	Positive
8	81 - 100%	Strong positive

Table 2.5. International Histocompatibility Workshop CDC crossmatch scoring system.

Figure obtained from the SJUH transplant immunology laboratory SOP SLF2IMTS020 version 7.2

2.4.2 FLOW CROSSMATCH (FXM)

This was performed by the Transplant Immunology Laboratory at St James's University Hospital. A suspension of donor lymphocytes was obtained using Ficoll density centrifugation as described in (Section 2.11), or by infiltrating donor lymph node and/or spleen with phosphate buffered saline (PBS). The cells were counted using a Neubauer chamber and adjusted to a concentration of $5x10^6$ cells/ml in 1% foetal bovine serum supplemented PBS (FBS-PBS).

Sera (recipient, negative control, positive control, 50µl) were added to numbered falcon tubes to allow testing in triplicate for each type of serum. Fifty microlitres of donor cell suspension was added to each tube. The contents of the tube were mixed gently and then incubated at 37°C in a water bath. Following incubation, the tubes were placed in a DiaCent automatic cell washer (BioRad laboratories) and washed with 1% FBS in PBS. FITC anti-human IgG was diluted 1:40 with PBS and 50µl was added to each tube. The tubes were mixed well and 2µl of neat anti-CD3-PE or 2µl of neat anti-CD19-PE were added to the appropriate tubes. The tubes were incubated at 2-8°C in the dark for 30 minutes. The cells were washed with cold 1% FBS-PBS using the DiaCent cell washer and the cell pellets were resuspended with 300µl of 1% formaldehyde for flow cytometric analysis.

2.4.3 ASSESSMENT OF HLA ANTIBODIES IN SERUM OR CELL SUPERNATANT USING ONELAMBDA SINGLE ANTIGEN BEADS (SABS)

Routine HLA antibody screening and interpretation were performed by the Transplant Immunology Laboratory in St James's University Hospital using the methods described in section 2.4.3.1. The positivity threshold for HLA antibody reporting was a normalised median fluorescent intensity (MFI) > 2000. Patients were screened on a quarterly basis as guided by the European Federation for Immunogenetics (EFI) Standards.[181]

2.4.3.1 POST TRANSPLANT MONITORING AS PART OF THE ALBERT STUDY

Serum and cell supernatant were stored in aliquots at -80°C as they were collected. To reduce inter-test variability, samples from the same patient were batched and tested at the same time, using the LABXpress benchtop robotic system. All sera were diluted 1:50 using 5% EDTA to minimise the prozone effect.[182] 10µl of either serum or concentrated cell-supernatant was added to a 96-well plate. A positive and negative control was added to the start of each batch, and the plate was loaded onto the LABXpress tray deck. The required volumes of reagents were calculated by the LabFusion software, based on the number of samples tested. Wash buffer, PBS, Class I and Class II SABs, and IgG PE conjugates were added to the reagent racks. The following steps were carried out by the LABXpress:

Class I or Class II beads (5µl of 1:2 dilution with bead diluent) were added to each sample and mixed well. The plate was then incubated for 30 minutes in the dark at room temperature. Non-specific binding was removed by 3 wash steps using wash buffer. PE-conjugated IgG (100µl of 1:100 dilution) was added to each well, and the plate was incubated for 30 minutes in the dark at room temperature. Finally, the plates were washed 5 times and the sample/beads were suspended in 80µl of PBS for acquisition on the Luminex-200 or Labscan-3D platforms.

Serum reactivity was calculated using HLAFusion software by measuring the fluorescent signal for each HLA-coated bead, after correcting for the non-specific binding to the negative control bead within the sample. The following equations were used:

$$a)\ Normalised\ MFI = S\#N - (BG\#N + (SNC\ bead - BGNC\ bead))$$

$$b)\ NBG\ ratio = \frac{S\#N\ /\ bead}{BG\#\ /\ BGNC\ bead}$$

Equation 1. The Formulae for determining the threshold of positivity for HLA antibody detection using ONELAMBDA single antigen beads.
Formula for calculating the normalised median fluorescence intensity value for each HLA-coated bead. B) Formula for calculating the normalised background ratio to indicate the strength of the reaction above the negative control bead. S#N – sample specific fluorescent value for bead #N, SNC bead – sample-specific fluorescent value for the negative control bead, BG#N – background negative control (NC) serum fluorescent value for #N, BGNC bead – background NC serum fluorescent value for negative control bead

For serum, a normalised (MFI) >1000 was considered positive. For the preliminary analyses of cell supernatant, all specificities with MFI >0 were considered positive.

2.4.3.2 SERUM TESTING FOR HLA ANTIBODIES USING LIFECODES SINGLE ANTIGEN (LSA) (IMMUCOR) BEADS

Assigned wells of the filter plate were pre-wet by adding 300µl of distilled water. After 5 minutes the water was aspirated using a vacuum manifold. The LSA beads were thawed and vortexed and 20µl were added to each assigned well. 5µl of patient sera were then added to each assigned well and mixed with gentle pipetting. The plate was covered and incubated in the dark at room temperature for 30 minutes on a plate shaker.

After 30 minutes of incubation, a wash step was performed. Wash buffer (100µl) was added to each well and the beads were resuspended using gentle pipetting. The plate was then gently aspirated using the vacuum manifold. Two further wash steps were performed using 250µl of wash buffer.

The pre-diluted secondary conjugate (25µl of 1:10 dilution) was added to each well. The plate was covered and incubated for 30 minutes in the dark on a plate shaker.

The beads were resuspended in 80µl of wash buffer and the data was collected using the Luminex 200 instrument. Serum reactivity was calculated using the Match IT! Antibody software (v 1.3, Immucor).

2.5 PATIENT RECRUITMENT TO THE ALBERT STUDY

Recruitment to the ALBERT (**A**ssessment in **L**eeds of a **B**iomarker **E**arly after **R**enal **T**ransplantation) study commenced on 30/03/2016 after ethical approval was obtained (16/YH/0025). Patients were recruited into 3 groups (Figure 2.4). Group 1 compromised of recently transplanted patients. Group 2 were transplanted patients who were offered a 'for cause' biopsy (FCB) to investigate a deterioration in eGFR from baseline, or new onset proteinuria. Group 3 were patients on the renal transplant waiting list, who had experienced a previous sensitising event.

All newly transplanted adult patients, who had not yet achieved the composite endpoint, were approached. This included standard and high immunological risk patients, and patients who had received repeat transplants. Additionally, samples were obtained from consenting healthy volunteers for use in preliminary work.

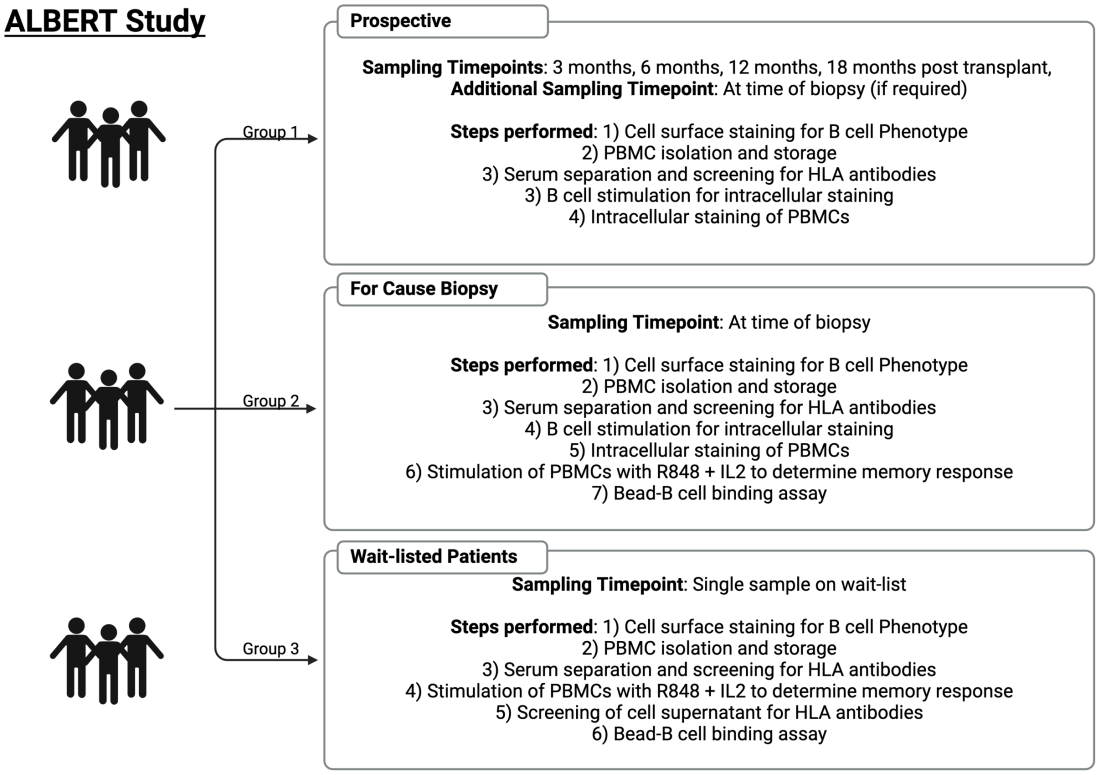


Figure 2.4. ALBERT Study Protocol Design.

Three different groups of patients were recruited, and consent obtained. Group 1 patients were recent post-transplant recipients who were prospectively followed and had blood samples collected at set time points (3, 6, 12, 18 months post-transplant with additional sample collection if they were offered a biopsy). Group 2 patients were post-transplant recipients who were offered a biopsy after 1 year post transplant. Blood samples were collected at the time of biopsy. Group 3 pre-transplant patients who were active on the renal transplant wait list. Blood samples were collected at one timepoint only. Clinical parameters were obtained from all patients at the time of blood sampling.

Image created with BioRender.com

2.6 CYTOMEGALOVIRUS (CMV) PROPHYLAXIS

Patients were assigned high, intermediate, and low risk categories for CMV reactivation depending on donor and recipient CMV status, and whether alemtuzumab was used as the induction agent (Table 2.6). High CMV risk patients received 200 days of valganciclovir prophylaxis. Intermediate risk patients received 100 days of prophylaxis, and low risk patients did not receive any prophylaxis.

Donor CMV Status	Recipient CMV Status	Alemtuzumab	Risk Status	Prophylaxis (days)
+	-	✓/×	High	200
+	+	✓	Intermediate	100
+	+	×	Low	Nil
-	+	✓	Intermediate	100
-	+	×	Low	Nil
-	-	✓/×	Low	Nil

Table 2.6. Definition of CMV Risk Status, with the duration of CMV prophylaxis required.

+ positive, - negative. ✓ denotes if alemtuzumab was used as an induction agent. × - alemtuzumab was not used.

2.7 ALLOGRAFT BIOPSIES

Only 'for cause' renal allograft biopsies were performed. Indications included delayed graft function (DGF), a sustained elevated urinary protein/creatinine ratio >50mg/mmol, or a sustained rise in creatinine. C4d deposition was assessed using immunohistochemistry staining. Biopsies were scored using the Banff 2017 criteria.[54]

2.8 CLASSIFICATION OF HLA MISMATCH LEVELS

The mismatch levels between donor and recipient have been summarised in Table 2.7, using the following NHS-BT mismatch categories: Level 1 000 A,B,DR, Level 2 0DR and 0/1B, Level 3 0DR and 2B or 1DR and 0/1B, Level 4 1DR and 2B or 2DR, Table 2.7 [183].

Level	Summary of Mismatches at A,B and DR loci:	A, B, DR Mismatches Included:
1	000	000
2	0DR and 0/1 B	100, 010, 110, 200, 210
3	0DR and 2B or 1DR and 0/1B	001, 011, 101, 111, 201, 211, 120, 020, 220
4	1DR and 2B or 2DR	021, 121, 221, 002, 102, 202, 012, 112, 212, 022, 122, 222

Table 2.7 NHSBT-ODT Mismatch Levels [183]

2.9 IMMUNOSUPPRESSION

The standard steroid-sparing immunosuppression regime consisted of 30mg of subcutaneous Alemtuzumab and 1g of intravenous methylprednisolone at induction, followed by tacrolimus monotherapy aiming for a trough level of 9-14 ng/ml within the first three months, 5-9ng/ml thereafter. In the presence of a 2-DR HLA mismatch, mycophenolate mofetil (MMF) was added. If Alemtuzumab was contraindicated, the second line regime, used in approximately 30% of the transplant population, included methylprednisolone induction followed by two doses of Basiliximab 20mg at day 0 and 4. Maintenance immunosuppression for these patients included tacrolimus and MMF. Table 2.8 lists the absolute contraindications for Alemtuzumab induction.

Transplants associated with a higher immunological risk, for example, HLA or ABO incompatible transplants, received augmented immunosuppression with MMF, prednisolone or plasma exchange. The decision to augment immunosuppression was made on a case-by-case basis by the consultant clinician at the time of transplantation.

Contraindications for Alemtuzumab Use
Recipient does not provide consent for use of Alemtuzumab
Known hypersensitivity to Alemtuzumab
Recent use of Rituximab (within 6 months)
Total white cell count less than 2.0 x10^9/L at the time of transplantation
Hereditary nephritis with deafness in recipient, e.g. Alports syndrome (due to risk of alveolar haemorrhage)
Recipient past medical history includes high risk malignancy: • Haematological • Colorectal • Thyroid • Breast • Anal/cervical/vulval/penile
Virology • Recipients with Human Immunodeficiency Virus (HIV) • BK virus resulting in previous graft loss

Table 2.8. List of absolute contraindications for the use of Alemtuzumab induction

2.10 CELL SURFACE STAINING - SURFACE B-CELL PHENOTYPE

The B-cell phenotype was determined by flow cytometry using a panel of monoclonal antibodies that was designed and optimised by the candidate (Table 2.9). Whole blood (300µl) was washed twice in ice-cold phosphate buffered saline supplemented with 1% foetal bovine serum (PBS-1%FBS). Cells were incubated using the optimal concentration of monoclonal antibodies (Table 2.9) in the dark at room temperature for 20 minutes. Red cells were lysed using 3ml of BD-FACSlyse buffer (BD Biosciences). Cells were washed twice with 3mls of ice cold PBS-1%FBS and strained to remove clumped cells prior to fixing with 200µl of PBS-3%Formaldehyde.

B Surface Phenotype Panel				
Monoclonal Antibody	**Clone**	**Manufacturer**	**Catalogue #**	**Volume (µl)**
Fc Block		BD Pharmingen	564219	2
Anti-Human CD24-FITC	ML5	BD Pharmingen	555427	10
Anti-Human CD27-PE	M-T271	BD Pharmingen	555441	10
Anti-Human IgM-PerCPCy5.5	G20-127	BD Pharmingen	561285	2
Anti-Human CD38-PECy7	HIT2	BD Pharmingen	560677	2
Anti-Human CD10-APC	HI10a	BD Pharmingen	332777	4
Anti-Human CD19-APCeFluor780	HIB19	eBioscience	47-0199	1
Anti-Human IgD-BV421	IA6-2	BD Horizon	562518	2
Anti-Human CD9-BV510	M-L13	BD Horizon	563640	3

Table 2.9. Monoclonal antibodies used in the B Surface Phenotype panel. FITC- fluorescein isothiocyanate, PE- phycoerythrin, PerCpCy5.5 – peridinin chlorophyll protein-cyanine 5.5, PeCy7 – phycoerythrin-cyanine 7, APC – allophycocyanin, APCeFlour780 – allophycocyanin-eFlour780, BV421 – Brilliant violet 421, BV510 – brilliant violet 510.

2.11 PBMC ISOLATION AND STORAGE

Whole blood was layered over lympholyte-H (CedarLane) in a 1:1 ratio and centrifuged at room temperature for 20 minutes without brake at 644 x *g*. The mononuclear layer was aspirated from the plasma-lympholyte interface, and cells were washed twice

using RPMI-1640 (Sigma Aldrich). PBMCs were counted using a Neubauer chamber, and the cell density was adjusted as required:

For storage, and subsequent batch processing to determine B-cell intracellular cytokines, PBMCs were adjusted to a density of $2.5x10^6$ cells/ml in FBS-10% dimethyl sulfoxide (DMSO) and were frozen at using a 'Mr Frosty' container at -80°C in 1ml aliquots.

For stimulation with R848/IL-2, PBMCs were adjusted to a density of $1x10^6$cells/ml in RPMI.

In preliminary experiments, B lymphocytes were isolated from PBMCs using positive selection with CD19 coated magnetic beads (Miltenyi Biotec) following manufacturer's instructions.

2.12 PBMC STIMULATION FOR INTRACELLULAR STAINING

PBMCs were quickly thawed using a water bath set at 37°C. DMSO was washed from the cells using warmed PBS-10% FBS, followed by two washes with B-cell media (Table 2.11). Cells were suspended in 1ml of B-cell media and placed in culture in a 12 well flat bottom plate with 1ml of pre-mixed RPMI, CpG ODN-2006-1 (final concentration 10µg/ml, Alpha Diagnostic International) and soluble CD40L (sCD40L, final concentration 1µg/ml, Gibco). Cells were cultured for 24 hours at 37°C, 5%CO_2. Phorbol-12-myristate-13-Acetate (PMA, 0.05µg/ml, Sigma Aldrich), Ionomycin (0.5µg/ml, Sigma Aldrich), Brefeldin-A (4µl of 1000x solution, eBioscience) and monensin (2µl of 1000x solution, eBioscience) were added for the last 5 hours of incubation. During preliminary experiments, either isolated B-cells or PBMCs were cultured for <24, 24 or 48 hours as described above, with PMA, ionomycin, brefeldin-A and monensin added for the last 5 hours.

2.13 INTRACELLULAR STAINING OF PBMCS

Following the incubation, cells were washed twice with ice cold PBS-1%FBS prior to staining. Cells were incubated with LIVE/DEAD Fixable violet dead cell stain kit (Invitrogen) in the dark at 4°C for 30 minutes, following manufacturer's instructions.

Following this, an antibody panel that had been designed and optimised by the candidate was used to determine B-cell subsets and their intracellular cytokines (Table 2.10). Cells were washed twice with ice-cold PBS-1%FBS, and were then incubated with required monoclonal antibodies using the method described above (Table 2.10). Cells were fixed and permeabilised using the BD cytofix/cytoperm kit (BD Biosciences) to allow staining for intracellular cytokines. Anti-Human IL-10-PE and Anti-Human TNF-α-FITC were added, and cells were incubated at 4°C in darkness for 30 minutes. They were washed twice and strained to remove clumped cells prior to fixing with PBS-3%Formaldehyde.

B Functional Intracellular Panel				
Monoclonal Antibody	**Clone**	**Manufacturer**	**Catalogue #**	**Volume (µl)**
Fc Block		BD Pharmingen	564219	2
Anti-Human TNFα-FITC	Mab11	BD Pharmingen	552889	20
Anti-Human and Viral IL-10-PE	JES3-9D7	BD Pharmingen	559337	20
Anti-Human CD9-PerCPCy5.5	M-L13	BD Pharmingen	561329	5
Anti-Human CD24-PECy7	ML5	BD Pharmingen	561646	3
Anti-Human CD3-APC	SK7	eBioscience	17-0036	2
Anti-Human CD19-APCeFluor780	HIB19	eBioscience	47-0199	1
Anti-Human CD38-BV421	HIT2	BD Horizon	562444	5

Table 2.10. Monoclonal antibodies used in the B Functional Intracellular panel FITC- fluorescein isothiocyanate, PE- phycoerythrin, PerCpCy5.5 – peridinin chlorophyll protein-cyanine 5.5, PeCy7 – phycoerythrin-cyanine 7, APC – allophycocyanin, APCeFlour780 – allophycocyanin-eFlour780, BV421 – Brilliant violet 421, BV510 – brilliant violet 510

	Volume	Manufacturer
B-Cell Media (for 50ml)		
IMDM	44ml	Gibco
Human Insulin	250µl (final concentration 0.24iU/ml)	Sigma
FBS	5ml (10%)	Gibco
Gentamicin	82µl	Gibco
200mM L-Glutamine	500µl	Sigma
Wash Buffer (for 1000ml)		
PBS	990ml	
FBS	10ml	Gibco
EDTA 0.5M	2ml	

Table 2.11. Reagents and Culture Media

2.14 ACQUISITION OF DATA USING FLOW CYTOMETRY + GATING STRATEGY

Samples were acquired on the day of sample processing using a BDFACSCanto (BD Biosciences) flow cytometer. Daily cytometer setup and tracking checks were performed, allowing consistent fluorescence intensity target values to be obtained for each experiment over the duration of the study period. Acquisition settings included a stopping gate of 30,000 $CD19^+$ events. Spectral compensation was adjusted for using either BD FACSDiva (BD Biosciences) or Kaluza Analysis Software (Beckman Coulter). Raw flow data were analysed using Kaluza. Initial gating procedures for TrBs were established on healthy controls based on CD24 and CD38 expression.[184,185] It has been previously demonstrated that an approximate T1:T2 ratio in healthy controls is 25:75 - this was applied to the patient sample. Additionally, in renal transplant recipients, the T1 population can be distinguished from T2 and naïve populations based on the surface expression of IgM and CD10.[141] The gating strategies for B-cell surface and intracellular phenotyping are shown in Figure 2.5 and Figure 2.6 respectively.

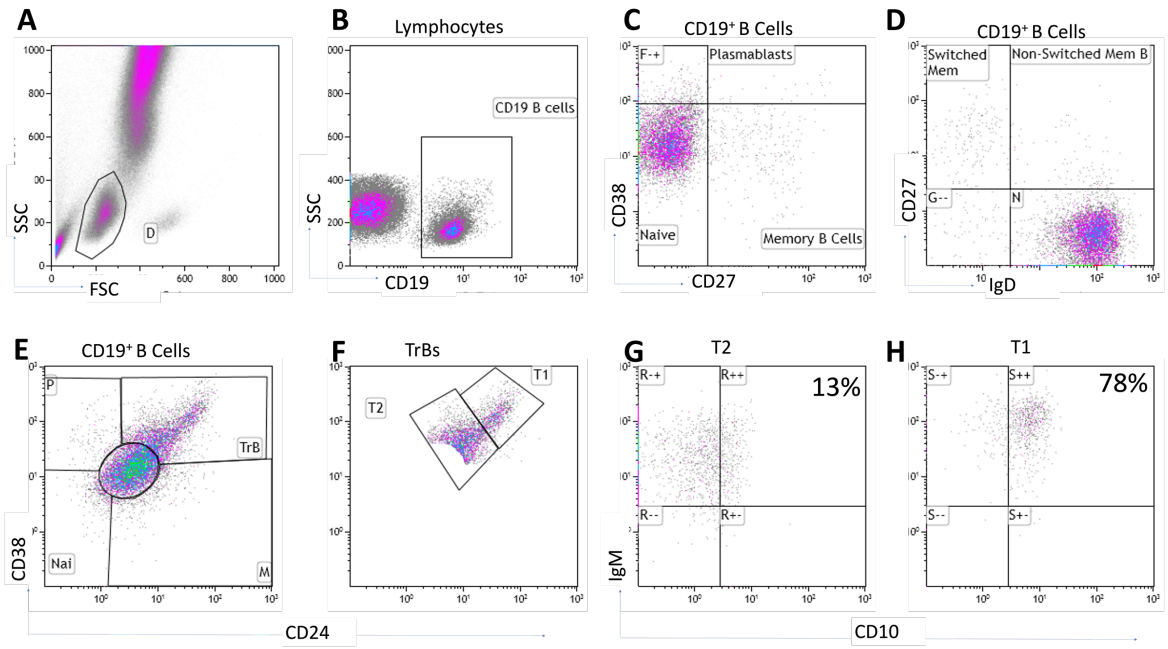


Figure 2.5. Representative Scatter Plots for Resting B-cell Panel. **Lymphocytes are identified by their forward and side scatter profile. B) B-cells are gated based on $CD19^{+}$ expression. C-E) Different B subsets are identified using the expression of different cell surface markers. Plasmablasts: $CD19^{+}CD27^{hi}CD38^{hi}CD24^{-}$, Memory: $CD19^{+}CD24^{+}CD27^{+}CD38^{-}$, Naïve: $CD19^{+}CD24^{+}CD38^{+}CD27^{-}IgD^{+}$, TrBs: $CD19^{+}CD24^{++}CD38^{++}$ F-H) TrBs can be further subdivided into T1 $CD24^{+++}CD38^{+++}CD10^{+}IgM^{+}$, and T2 $CD24^{++}CD38^{++}$. Sample obtained from a 44year old recipient, 6 months post-transplant/alemtuzumab induction**

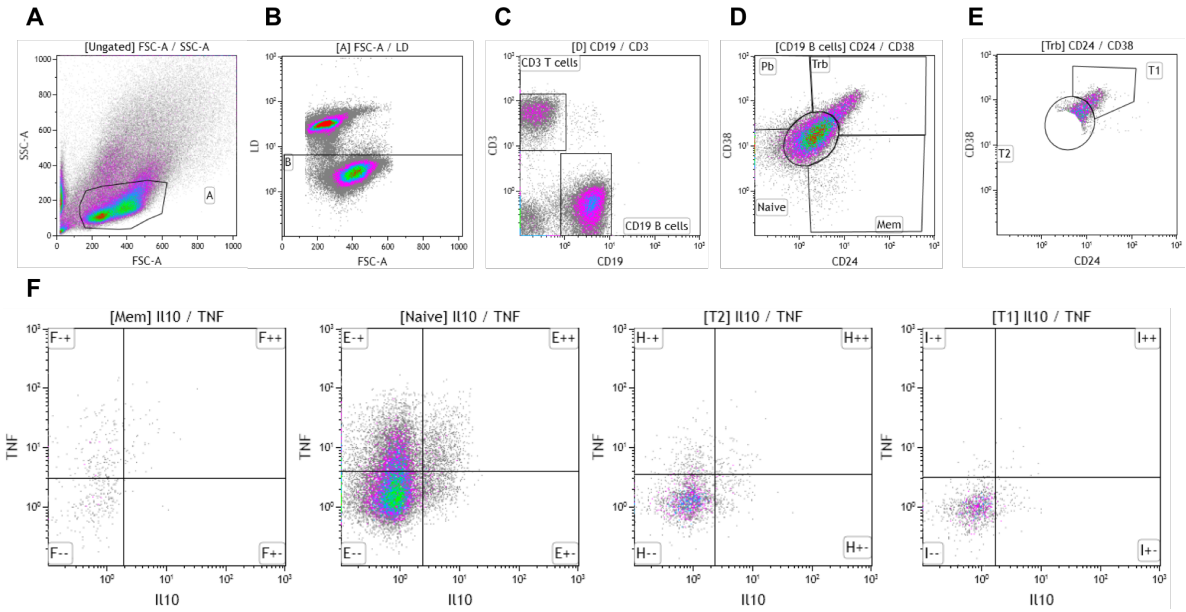


Figure 2.6. Representative Scatter Plots for Intracellular Phenotype Panel. PBMCs were cultured for 24 hours with CPG and CD40L. Brefeldin A, monensin, PMA and ionomycin were added for the last 5 hours of culture. Cells were surface stained, fixed and permeabilised prior to the addition of Anti Human IL-10 and Anti Human TNF-α. A) Lymphocytes were identified by their forward and side scatter profile. B) Dead cells that stained brightly with the viability dye were excluded from analysis. C+D) Different B subsets were identified by the surface expression of CD24 and CD38. TrBs are defined as $CD19^{+}CD24^{hi}CD38^{hi}$, Naïve $CD19^{+}CD24^{+}CD38^{+}$, Memory $CD19^{+}CD24^{+}CD38^{-}$. E) TrBs can be further subdivided: T1 $CD24^{+++}CD38^{+++}$, T2 $CD24^{++}CD38^{++}$. F) The IL-10:TNF-α ratio was calculated from each subset, dividing the singly positive IL-10 cells by singly positive TNF-α cells. This sample was obtained from a 35-year-old recipient 3 months post-transplant/alemtuzumab induction.

2.15 ASSESSING THE SINGLE NUCLEOPTIDE POLYMORPHISMS (SNPS) ASSOCIATED WITH CYTOKINE GENE EXPRESSION USING PCR-SSP (POLYMERASE CHAIN REACTION – SEQUENCE SPECIFIC PRIMER)

There are several single nucleotide polymorphisms (SNPs) in specific genes that have been associated with the expression levels of cytokines. The gene expression of various cytokines (TNF-α, TGF-β, IFN-γ, IL-10 and IL-6) were inferred using a commercial polymerase chain reaction – sequence specific oligonucleotide primers (PCR-SSP) kit (ONELAMBDA, Canoga Park), according to the manufacturer's instructions. Briefly, DNA was purified from leukocytes, and suspended in molecular-grade water to achieve a concentration of 100ng/μl, with the A260/A280 ratio of 1.65-1.80.

Deoxynucleoside triphosphate-buffer mix (D-mix, 180μl per sample) and the PCR microtube tray containing pre-aliquoted primers (Table 2.12) were thawed at room temperature.

	H	G	F	E	D	C	B	A
1	23-43 P1477.21 Neg Control	70-14 P1551.11 TNFA-A	70-27 P1552.11 TNFA-G	70-27 P1860.10 TGFB10-TX	70-04 P1859.9 TGFB10-CX	70-27 P1861.8 TGFB25-CY	70-1 P1862.11 TGFB26-GY	70-04 P1561.10 IL10-1
2	70-22 P1562.10 IL10-2	70-41 P1563.11 IL10-3	327-29 P1564.10 IL10-4	327-38 P1565.10 IL10-5	327-38 P1633.10 IL-6(-174C	327-38 P1634.11 IL-6(-174G	327-29 P1668.10 IFNG-T	327-50 P1665.10 IFNG-A

Table 2.12. DNA Primer Tray Map

Molecular grade water (1μl) was added to each negative control well. Following this, 1μl of Taq polymerase (5u/μl) was added to each D-mix tube, and 9μl of this mixture was added to each negative control well. Purified DNA (19μl) was then added to the D-mix/Taq polymerase tube, and 10μl of this final mixture was added to each test well. The PCR plate was then sealed and centrifuged to ensure that the D-mix/DNA/Taq

polymerase was thoroughly mixed with the pre-aliquoted primers. The tray was then placed on a thermocycler. Table 2.13 lists the cycles and temperatures used for the polymerase chain reaction.

Number of Cycles	Step	Temperature (°C)	Time (seconds)
1	1	96	130
	2	63	60
9	1	96	10
	2	63	60
20	1	96	10
	2	59	50
	3	72	30
End	1	4	--

Table 2.13. OneLambda Polymerase Chain Reaction Program for Cytokine Expression

After completing the PCR reaction, the contents of each well in the PCR tray were carefully transferred to a 2.5% agarose gel containing ethidium bromide. An electrophoresis ladder (2μl) was added to lane number 9 for each sample. The samples were then run out on a gel for 20-25 minutes using 150 volts. The gel was transferred to a UV transilluminator and photographed. The lane positions of positive and negative reactions were noted and compared with the accompanying worksheet (Figure 2.7). This SNP analysis allowed the results 'High', 'Intermediate' and 'Low' to be assigned to the gene expression of the relevant cytokine.

2.16 CALCULATIONS AND STATISTICAL ANALYSES

Statistical calculations were performed using either SPSS v 26 or Prism v9 (GraphPad), with a significance value considered at $p<0.05$, unless otherwise stated.
Normally distributed continuous variables were reported with mean and standard deviation (SD). Variables with non-normal distribution were reported with median and interquartile range (IQR). Continuous variables were assessed using either Mann-Whitney tests or Kruskal-Wallis tests, unless otherwise stated. Categorical variables were assessed using Chi-squared tests unless otherwise stated. Survival analyses were performed by constructing Kaplan Meier curves with log-rank comparisons. Allograft loss was defined when the patient returned to dialysis or received a further transplant. Multivariate analyses were performed using cox regression.

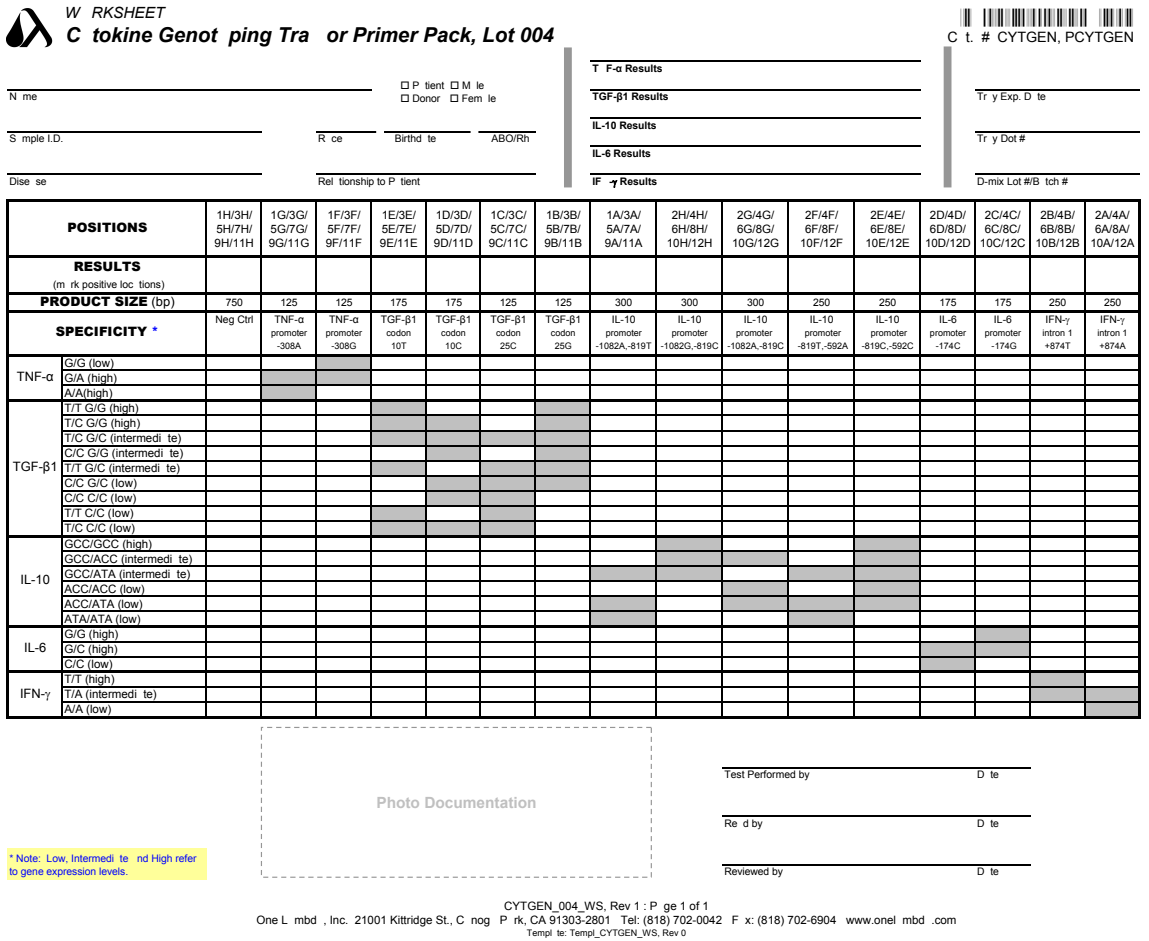
Calcineurin inhibitor (CNI) variability, or mean absolute deviation, was calculated using the following equation:

$$CNI\ Variability = \frac{1}{n}\sum_{i=1}^{n}\frac{|x_i - \overline{x}|}{\overline{x}} \times 100$$

Equation 2. Calcineurin Inhibitor Variability.

Also known as the mean absolute deviation [186,187]. x = trough CNI value, $\overline{x}$ = mean trough CNI level, n = number of CNI values

The CNI variability was calculated for two periods of follow up: 0-3 months post-transplant, and >3 months post-transplant, to allow for the intentional reduction in trough tacrolimus levels after 3 months.



W RKSHEET
C tokine Genot ping Tra or Primer Pack, Lot 004

C t. # CYTGEN, PCYTGEN

N me

☐ P tient ☐ M le
☐ Donor ☐ Fem le

S mple I.D.

R ce

Birthd te

ABO/Rh

Dise se

Rel tionship to P tient

T F-α Results

TGF-β1 Results

IL-10 Results

IL-6 Results

IF -γ Results

Tr y Exp. D te

Tr y Dot #

D-mix Lot #/B tch #

POSITIONS		1H/3H/ 5H/7H/ 9H/11H	1G/3G/ 5G/7G/ 9G/11G	1F/3F/ 5F/7F/ 9F/11F	1E/3E/ 5E/7E/ 9E/11E	1D/3D/ 5D/7D/ 9D/11D	1C/3C/ 5C/7C/ 9C/11C	1B/3B/ 5B/7B/ 9B/11B	1A/3A/ 5A/7A/ 9A/11A	2H/4H/ 6H/8H/ 10H/12H	2G/4G/ 6G/8G/ 10G/12G	2F/4F/ 6F/8F/ 10F/12F	2E/4E/ 6E/8E/ 10E/12E	2D/4D/ 6D/8D/ 10D/12D	2C/4C/ 6C/8C/ 10C/12C	2B/4B/ 6B/8B/ 10B/12B	2A/4A/ 6A/8A/ 10A/12A
RESULTS (m rk positive loc tions)																	
PRODUCT SIZE (bp)		750	125	125	175	175	125	125	300	300	300	250	250	175	175	250	250
SPECIFICITY *		Neg Ctrl	TNF-α promoter -308A	TNF-α promoter -308G	TGF-β1 codon 10T	TGF-β1 codon 10C	TGF-β1 codon 25C	TGF-β1 codon 25G	IL-10 promoter -1082A,-819T	IL-10 promoter -1082G,-819C	IL-10 promoter -1082A,-819C	IL-10 promoter -819T,-592A	IL-10 promoter -819C,-592C	IL-6 promoter -174C	IL-6 promoter -174G	IFN-γ intron 1 +874T	IFN-γ intron 1 +874A
TNF-α	G/G (low)																
	G/A (high)																
	A/A(high)																
TGF-β1	T/T G/G (high)																
	T/C G/G (high)																
	T/C G/C (intermedi te)																
	C/C G/G (intermedi te)																
	T/T G/C (intermedi te)																
	C/C G/C (low)																
	C/C C/C (low)																
	T/T C/C (low)																
	T/C C/C (low)																
IL-10	GCC/GCC (high)																
	GCC/ACC (intermedi te)																
	GCC/ATA (intermedi te)																
	ACC/ACC (low)																
	ACC/ATA (low)																
	ATA/ATA (low)																
IL-6	G/G (high)																
	G/C (high)																
	C/C (low)																
IFN-γ	T/T (high)																
	T/A (intermedi te)																
	A/A (low)																

* Note: Low, Intermedi te nd High refer to gene expression levels.

Photo Documentation

Test Performed by D te

Re d by D te

Reviewed by D te

CYTGEN_004_WS, Rev 1 : P ge 1 of 1

One L mbd , Inc. 21001 Kittridge St., C nog P rk, CA 91303-2801 Tel: (818) 702-0042 F x: (818) 702-6904 www.onel mbd .com

Templ te: Templ_CYTGEN_WS, Rev 0

Figure 2.7. ONELAMBDA Cytokine PCR Worksheet

3 THE OUTCOME OF ALEMTUZUMAB AS AN INDUCTION AGENT FOR RENAL TRANSPLANTATION IN THE UNITED KINGDOM

3.1 INTRODUCTION

The forerunner of the humanised monoclonal antibody alemtuzumab, Campath-1, was originally distinguished by its ability to deplete human lymphocytes which led to speculation regarding its potential role in solid organ transplantation.[188,189] Initial observations demonstrated that this new antibody was able to reverse episodes of acute cellular rejection and to reduce rejection rates when used as an induction agent.[190,191]

The first renal transplant recipients treated with alemtuzumab had satisfactory short and medium term outcomes when compared to conventional immunosuppressive regimes and interest developed in more widespread adoption.[192,193] Experience in the USA was also positive in a few small series when compared to historical controls over short term follow up.[194–198] However, the initial enthusiasm for alemtuzumab was tempered by several concerning observations. Firstly, alemtuzumab was employed in potentially "tolerogenic" studies involving the minimisation of conventional immunosuppression with poor results [199–201] possibly due to homeostatic repopulation by mature T cells in the absence of regulatory cells. [199,200] Secondly it became apparent that the use of alemtuzumab was associated with a small but significant incidence of *de novo* autoimmune disease including immune thrombocytopenic purpura and thyroid disease.[201–203] Finally, there were numerous reports of increased infective complications although largely in small series.[204–207] Thus widespread usage was deferred pending the results of prospective randomised controlled trials (RCTS). Two small RCTs in the United Kingdom demonstrated reduced rates of acute rejection when alemtuzumab and tacrolimus monotherapy was compared to a conventional steroid avoidance regime consisting of tacrolimus, mycophenolate mofetil and an anti-CD25 monoclonal antibody.[208,209] Two larger multicentre RCTs also demonstrated that alemtuzumab was associated with lower early rejection rates following low immunological risk renal transplantation.[210,211] Despite these studies widespread

adoption of alemtuzumab based immunotherapy has not occurred, possibly due to persistent concerns regarding its long-term safety. There are conflicting reports regarding an increased incidence of neoplasia following the usage of alemtuzumab and there is also evidence of long-lasting alteration in the immune profile of recipients.[212–215] Data on long-term infection risk is poor making it difficult to draw firm conclusions.[216] However there is some evidence that outcomes are improving as experience with the agent grows.[217] Consequently, it is important to closely monitor outcomes from large registries for renal transplant recipients undergoing induction with alemtuzumab. The UK NHS Blood and Transplant (NHSBT) renal transplant data registry was interrogated to look at the longer-term outcomes in standard risk adult patients who underwent kidney-alone transplantation. The results are presented here.

The study was conceived by the candidate with support from their supervisory team. Statisticians from NHS-BT accessed the NHS-BT registry data and performed statistical analyses. Data interpretation and manuscript preparation was performed by the candidate.

3.2 MATERIALS AND METHODS

A retrospective analysis was performed on data collected and held by UK NHSBT. Transplanting centres are asked as a matter of routine to complete and return a transplant record for each recipient. Inclusion criteria comprised of standard risk adult (age ≥18 years) patients receiving their first renal transplant in the United Kingdom between 2005 and 2013. High immunological risk patients who had received an ABO or HLA-incompatible transplant, or highly sensitised recipients with a calculated reaction frequency (cRF) greater than or equal to 85% were excluded from analysis. Due to the way data was collected for the UK registry, there is a possibility that the alemtuzumab group may have included transplants that were donor specific antibody (DSA) positive, yet flow crossmatch negative. However many of these transplants will have been excluded on the basis of a cRF greater than or equal to 85%. HLA mismatch was recorded using the NHSBT mismatch level as described in Table 2.7.

Patients were stratified into two groups according to induction immunosuppression- alemtuzumab (Group A) and control (Group B). The majority of patients in group A received 30mg of alemtuzumab, either intravenously or subcutaneously. In some transplanting centres, younger recipients (Age <60) received a second 30mg dose [211]. Patients in group B received any other induction agent, which was most commonly the IL-2 receptor antagonist basiliximab (95% of Group B). Patients who did not receive an induction agent (0.66% of Group B) were also included in this group.

Analysis of follow-up data was performed on all transplants divided into living and deceased donor groups which were analysed separately. All patients identified in the analysis had complete data for the variables of interest, unless otherwise specified. Patient survival was defined as the time from transplant to patient death, with censoring for patients still alive at the last follow-up or at 5 years. Death censored graft survival was defined as time from transplant to graft failure, censoring for death with a functioning graft and grafts still functioning at last follow-up or at 5 years. Rejection-free survival was defined as the time from transplant to first recorded rejection episode, censoring for graft failure, patient death, at 5 years or last follow-up if earlier.

Recorded rejection episodes included biopsy proven rejection, and suspected rejection that received treatment. Estimated glomerular filtration rate (eGFR) was used to compare graft function between the groups, with patients excluded from this analysis if they had returned to dialysis.

Kaplan-Meier estimates were used to analyse rates of patient survival, death-censored graft survival, and rejection-free survival. Associated p-values were calculated using the log-rank test. Cox proportional hazards regression models were fitted to analyse the effect of factors on death censored graft survival. The models were developed using a stepwise variable selection method, and the importance of each factor was re-assessed at each stage of the model development. Table 3.3 lists the factors included in the final model. Univariate comparisons were performed using Fishers exact test for categorical variables. The significance level was defined as $p<0.05$. All statistical analyses were performed using Statistical Analysis Software (version 9.3).

3.3 RESULTS

A retrospective analysis was performed on data collected and held by NHSBT between 2005 and 2013. Within this period, 13,816 patients were included in the study cohort. Of these, 1661 (12.0%) patients were reported as receiving alemtuzumab induction (group A). They were compared with 12,155 control patients (group B) who received any other induction agent, most commonly the anti-IL-2 receptor antagonist, basiliximab (95% of Group B). Twenty seven percent of all recipients received a graft from a live donor.

Table 1. summarises the study cohort demographics. If a specific patient demographic was not reported, this was coded within the demographic table as 'not reported'. Of note, within the deceased donor cohort, 49.4% of Group A patients received a DBD graft, compared with 63.6% in the control group. These recipients were also older (mean age 52.4, SD 13.4, compared with 50.6, SD 13.4 in Group B), and had received grafts with a higher mismatch level as described in Table 2.7 (67.0% level 3 or 4, compared with 55.2% level 3 or 4 in Group B) from older donors (mean age 50.5, SD 16.3 vs 48.7, SD 15.8, $p<0.0001$). Primary renal disease differed between the two groups; however, variables were missing for a high proportion of patients. In the deceased donor cohort, the primary renal disease was not reported in 34.7% (n=3072) of the control group, and 31.1% (n=386) of the alemtuzumab group. In the live-donor cohort, the primary renal disease was not reported in 38.5% (n=1265) of the control group, and 43.6% (n=184) of the alemtuzumab group.

In the live-donor cohort, alemtuzumab recipients were older (mean age 47.7, SD 13.2, compared with mean age 45.0, SD 13.8 in Group B, $p=0.0001$), however they received grafts from younger donors (mean age 46 SD 13.0 vs 47.3 SD 12.2, $p=0.04$).

Patients who received alemtuzumab were more likely to be maintained on calcineurin inhibitor monotherapy (68% calcineurin inhibitor monotherapy, 31% calcineurin inhibitor plus an antiproliferative), whereas patients in the control group were more likely to be maintained on a combination of calcineurin inhibitor and an

antiproliferative (88% calcineurin inhibitor plus antiproliferative, 9% calcineurin inhibitor monotherapy)

Factor	Deceased donor			Living donor		
	Control	**Alemtuzumab**	**p-value**	**Control**	**Alemtuzumab**	**p-value**
Donor Type			**<0.0001**	3289 (88.6%)	422 (11.4%)	
DBD	5639 (63.6%)	612 (49.4%)				
DCD	3227 (36.4%)	627 (50.6%)				
Recipient Age (years, SD)	50.6 (13.4)	52.4 (13.4)	**<0.0001**	45.0 (13.8)	47.7(13.2)	**0.0001**
Recipient Gender			0.34			0.36
Male	5735 (64.7%)	825 (66.6%)		2092 (63.6%)	280 (66.4%)	
Female	3126 (35.2%)	413 (33.3%)		1196 (36.4%)	142 (33.6%)	
Not reported	5 (0.1%)	1 (0.1%)		1 (0%)	0 (0%)	
Recipient Ethnic Group			**<0.0001**			**<0.0001**
White	7006 (79.0%)	815 (65.8%)		2823 (85.8%)	262 (62.1%)	
Asian	1019 (11.5%)	269 (21.7%)		270 (8.2%)	85 (20.1%)	
Black	624 (7.0%)	100 (8.1%)		139 (4.2%)	35 (8.3%)	
Other	217 (2.5%)	55 (4.4%)		57 (1.7%)	40 (9.5%)	
Mismatch Level			**<0.0001**			**0.046**
1	1066 (12.0%)	95 (7.7%)		318 (9.7%)	38 (9.0%)	
2	2908 (32.8%)	313 (25.3%)		478 (14.5%)	64 (15.2%)	
3	4220 (47.6%)	688 (55.5%)		1533 (46.6%)	171 (40.5%)	
4	672 (7.6%)	143 (11.5%)		960 (29.2%)	149 (35.3%)	
cRF			0.18			0.11
0-19	7579 (85.5%)	1077 (86.9%)		2601 (79.1%)	355 (84.1%)	
20-39	483 (5.5%)	49 (4.0%)		196 (6.0%)	18 (4.3%)	

40-59	393 (4.4%)	56 (4.5%)		207 (6.3%)	20 (4.7%)	
60-84	411 (4.6%)	57 (4.6%)		285 (8.7%)	29 (6.9%)	
Dialysis Modality			**0.02**			**0.05**
Dialysis (PD/HD)	7979 (90%)	1084 (87.5%)		2371 (72.1%)	280 (66.4%)	
Pre-emptive	882 (9.9%)	154 (12.4%)		915 (27.8%)	142 (33.6%)	
Not reported	5 (0.1%)	1 (0.1%)		3 (0.1%)	0 (0%)	
Primary Renal Disease			**<0.0001**			**<0.0001**
Hypertension	556 (6.3%)	108 (8.7%)		151 (4.6%)	22 (5.2%)	
Diabetes	662 (7.5%)	163 (13.2%)		181 (5.5%)	45 (10.7%)	
GN	1537 (17.3%)	195 (15.7%)		662 (20.1%)	73 (17.3%)	
Polycystic kidneys	1211 (13.7%)	177 (14.3%)		353 (10.7%)	46 (10.9%)	
Pyelonephritis/ Interstitial Nephritis	647 (7.3%)	70 (5.7%)		237 (7.2%)	25 (5.9%)	
Other	1181 (13.3%)	140 (11.3%)		440 (13.4%)	27 (6.4%)	
Not reported	3072 (34.7%)	386 (31.1%)		1265 (38.5%)	184 (43.6%)	
Donor Age (years, SD)	48.7 (15.8)	50.5 (16.3)	**<0.0001**	47.3(12.2)	46.0 (13.0)	**0.07**
CIT			0.43			0.99
<12 hours	2065 (23.3%)	276 (22.3%)		3276 (99.6%)	421 (99.8%)	
>12 hours	6801 (76.7%)	963 (77.7%)		13 (0.4%)	1 (0.2%)	
CMV mismatch			**<0.0001**			**<0.0001**
High Risk (D+/R-)	1871 (21.1%)	252 (20.4%)		570 (17.3%)	72 (17.1%)	
Intermediate Risk (R+)	4110 (46.3%)	484 (39%)		1291 (39.3%)	138 (32.7%)	

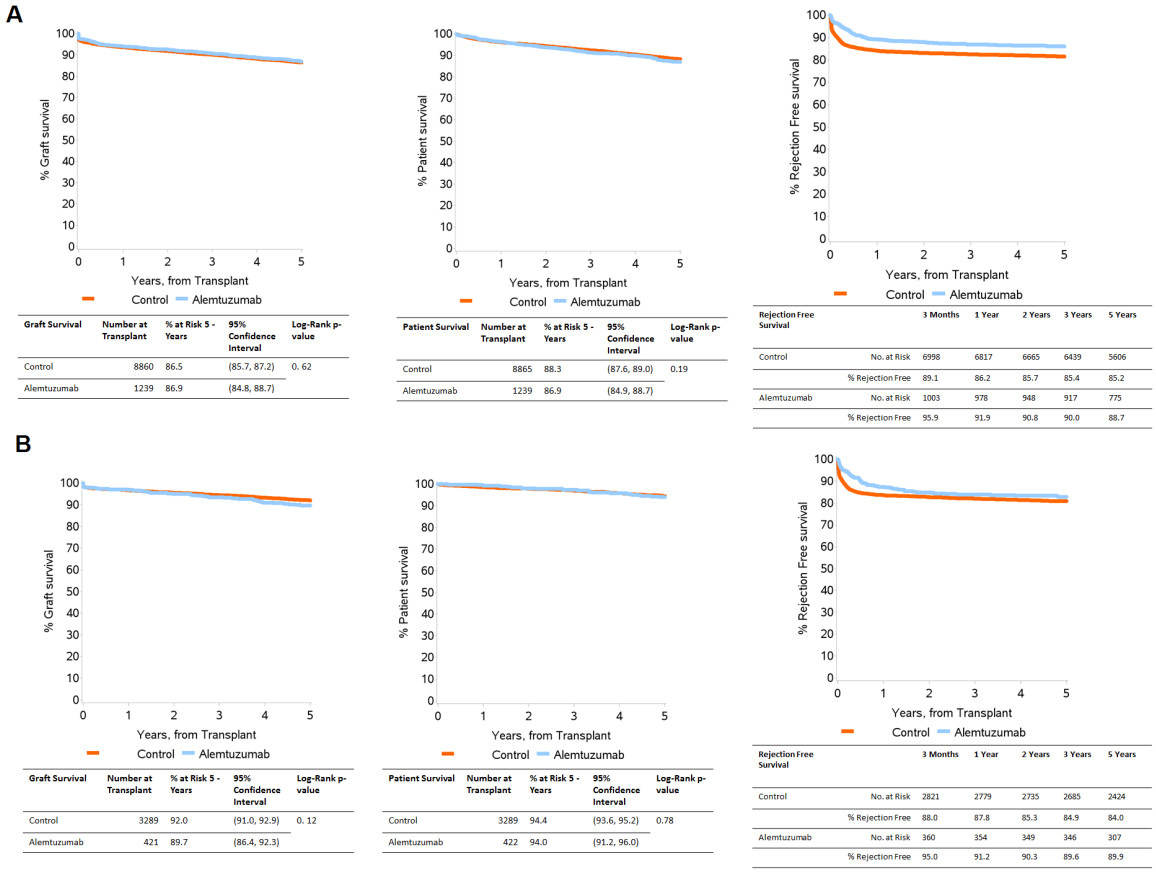
Low Risk (D-/R-)	2153 (24.3%)	260 (21.0%)	1024 (31.1%)	111 (26.3%)
D or R unknown	732 (8.3%)	243 (19.6%)	404 (12.3%)	101 (23.9%)

Table 3.1. Patient Demographics.

cRF– calculated reactive frequency, GN- glomerulonephritis, PD – peritoneal dialysis, HD – haemodialysis, D – Donor, R – Recipient, SD – standard deviation

3.3.1 ALEMTUZUMAB INDUCTION DOES NOT AFFECT 5-YEAR PATIENT SURVIVAL

During the 5-year follow up, 13815 transplants were performed, and there were 1337 recorded deaths. Figure 3.1 demonstrates the 5-year patient survival estimates (graft survival, patient survival and rejection free survival) for this cohort, divided into the two treatment groups. The alemtuzumab group is depicted in blue, and the control group in red. Panel A demonstrates the 5-year survival estimates following deceased donor transplants, and panel B following living donor transplants. The use of alemtuzumab did not affect the 5-year patient survival, which was 86.9%, compared with 88.3% in the group B (p-value 0.19, 95% Confidence intervals 84.9-88.7, and 87.6-89.0 respectively).



Graft Survival	Number at Transplant	% at Risk 5 - Years	95% Confidence Interval	Log-Rank p-value
Control	8860	86.5	(85.7, 87.2)	0. 62
Alemtuzumab	1239	86.9	(84.8, 88.7)	

Patient Survival	Number at Transplant	% at Risk 5 - Years	95% Confidence Interval	Log-Rank p-value
Control	8865	88.3	(87.6, 89.0)	0.19
Alemtuzumab	1239	86.9	(84.9, 88.7)	

Rejection Free Survival		3 Months	1 Year	2 Years	3 Years	5 Years
Control	No. at Risk	6998	6817	6665	6439	5606
	% Rejection Free	89.1	86.2	85.7	85.4	85.2
Alemtuzumab	No. at Risk	1003	978	948	917	775
	% Rejection Free	95.9	91.9	90.8	90.0	88.7

Graft Survival	Number at Transplant	% at Risk 5 - Years	95% Confidence Interval	Log-Rank p-value
Control	3289	92.0	(91.0, 92.9)	0. 12
Alemtuzumab	421	89.7	(86.4, 92.3)	

Patient Survival	Number at Transplant	% at Risk 5 - Years	95% Confidence Interval	Log-Rank p-value
Control	3289	94.4	(93.6, 95.2)	0.78
Alemtuzumab	422	94.0	(91.2, 96.0)	

Rejection Free Survival		3 Months	1 Year	2 Years	3 Years	5 Years
Control	No. at Risk	2821	2779	2735	2685	2424
	% Rejection Free	88.0	87.8	85.3	84.9	84.0
Alemtuzumab	No. at Risk	360	354	349	346	307
	% Rejection Free	95.0	91.2	90.3	89.6	89.9

Figure 3.1. 5-Year Survival in Alemtuzumab and Control Groups.
Kaplan Meier curves depicting graft survival (first column), patient survival (second column), and rejection-free survival (third column) over a 5-year period in A) deceased donor recipients, and B) live-donor recipients. Patients in the Alemtuzumab cohort are depicted in red, Control cohort in blue.

The reported causes of death (818 records returned out of 1337 events) were compared between the Alemtuzumab and Control groups. The commonest reported cause of death across both groups was infection, accounting for 30% of the total deaths. This was followed by death due to cardiovascular causes (24%), and death due to malignancy (22%), see Table 3.2. Despite a significant difference in the age and mismatch level of recipients in group A, no differences were noted in the causes of death between the two groups, and importantly, alemtuzumab was not associated with an increased number of deaths due to infection or malignancy. However, in 519 cases, the cause of death was not reported back to NHSBT.

	Alemtuzumab		Control	
Cause of death uncertain/ not determined	0	(0%)	11	(1.5%)
Myocardial ischaemia and infarction	11	(10.4%)	65	(9.1%)
Hyperkalaemia	0	(0%)	5	(0.7%)
Cardiac - miscellaneous	9	(8.5%)	62	(8.7%)
Elevated PVR	0	(0%)	1	(0.1%)
Pulmonary embolus	0	(0%)	10	(1.5%)
Cerebro-vascular accident	5	(4.7%)	30	(4.2%)
Gastro-intestinal haemorrhage	0	(0%)	8	(1.1%)
Haemorrhage - miscellaneous	4	(3.8%)	17	(2.4%)
Mesenteric infarction	2	(1.9%)	9	(1.3%)
Pulmonary infection	17	(16.0%)	111	(15.6%)
Infection - miscellaneous	2	(1.9%)	25	(3.5%)
Septicaemia	13	(12.3%)	73	(10.3%)
Liver disease	0	(0%)	9	(1.3%)
Renal Failure	0	(0%)	4	(0.6%)
Recurrent primary disease - malignant	1	(1.1%)	1	(0.1%)
Patient refused further treatment	0	(0%)	3	(0.4%)
Suicide	0	(0%)	2	(0.3%)
Therapy ceased for any other reason	0	(0%)	4	(0.6%)
ESRF treatment withdrawn for medical reasons	1	(0.9%)	5	(0.7%)
Uraemia caused by graft failure	0	(0%)	1	(0.1%)
Pancreatitis	2	(1.9%)	1	(0.1%)
Bone marrow depression	0	(0%)	1	(0.1%)
Lymphoma	12	(11.3%)	68	(9.6%)
Malignant disease: Lymphoproliferative disorders (except lymphoid malignant disease possibly induced by immunosuppressive therapy)	0	(0%)	2	(0.3%)
Dementia	0	(0%)	2	(0.3%)

	Alemtuzumab		Control	
Sclerosing (or adhesive) peritoneal disease	2	(1.9%)	2	(0.3%)
Perforation of peptic ulcer	0	(0%)	1	(0.1%)
Perforation of colon	0	(0%)	4	(0.6%)
Non-lymphoid malignant disease	11	(10.4%)	89	(12.5%)
Cardiac tamponade	1	(0.9%)	1	(0.1%)
ARDS	0	(0%)	2	(0.3%)
Respiratory failure	0	(0%)	15	(2.1%)
Multi-system failure	3	(2.8%)	25	(3.5%)
Accident unrelated to treatment	1	(0.9%)	3	(0.4%)
Donor organ failure	1	(0.9%)	4	(0.6%)
Other identified cause of death	8	(7.6%)	35	(4.9%)
Unknown	0	(0%)	1	(0.1%)

Table 3.2. Reported Cause of Death

During the follow up period, 13815 transplant were performed and 1337 deaths were recorded. 818 records containing causes of death were returned to NHSBT; 519 records missing

3.3.2 GRAFT SURVIVAL AND FUNCTION

During the period of study, a total of 13,816 transplants were performed and were included in the following analyses. The 5-year Kaplan-Meier death censored estimate for graft survival (Figure. 1) was 86.5% in Group B-DD compared with 86.9% in Group A-DD (p-value 0.62). In live-donor recipients, the 5-year Kaplan-Meier death-censored estimate for graft survival was 92.0% in the Group B, and 89.7% in Group A (p-value 0.12). Further multivariable analyses were performed using a Cox proportional hazards model to adjust for significant factors (donor age, recipient age, waiting time, recipient ethnic group, primary renal disease, sensitisation, level of mismatch, cold-ischaemia time, and transplant centre as a random effect) that had been found to affect the estimation of 5-year death-censored graft survival. Despite adjusting for these factors, alemtuzumab was not an independent risk factor for graft survival (alemtuzumab hazard ratio for 5-year graft survival was 1.35, CI 0.91-2.00 in the living donor cohort, and 0.95, CI 0.78-1.15 in the deceased donor cohort). A further analysis was performed after selecting for recipients aged 60 and above, and alemtuzumab induction was not associated with reduced transplant survival (Figure 3.2, p=0.29).

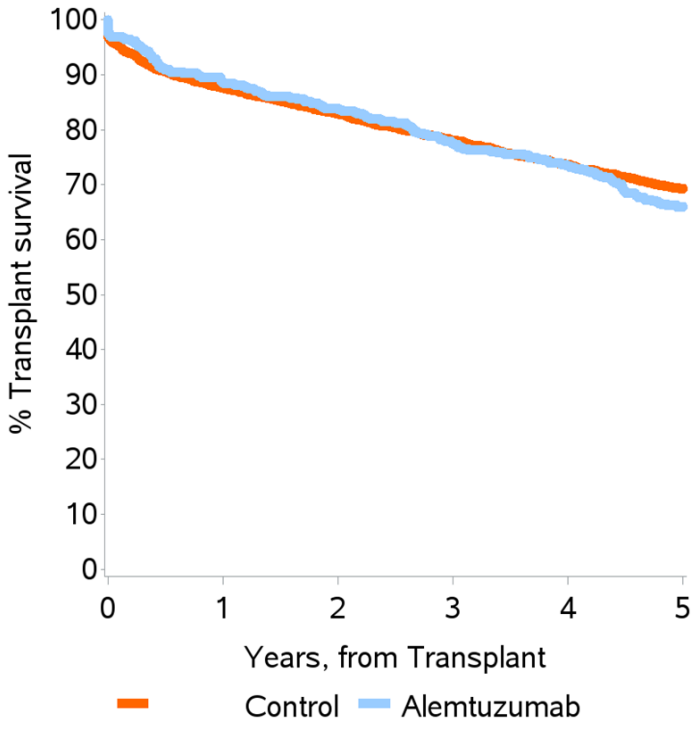


Figure 3.2. 5-year transplant survival in Recipients aged over 60yrs.

Kaplan Meier curves comparing the 5-year transplant survival in recipients aged 60 or over, who received a deceased donor kidney.

Factors Included in the 5-year Death Censored Survival Model

Deceased Donors	Living Donors
Donor Age	Donor Age
Recipient Age	Recipient Age
Waiting Time	Recipient Ethnicity
Recipient Ethnicity	
Sensitisation (cRF)	
Primary Renal Disease	
NHSBT Mismatch Level	
Cold Ischaemia Time	
Transplant Centre	

Table 3.3. Factors Included in the 5-Year Death Censored Survival Model.
cRF – calculated reaction frequency

The median eGFR for recipients in the alemtuzumab and control groups were similar at all timepoints. At 5 years, the median eGFR was 50 (Q1-Q3 38-67.5) in Group A-DD, and 49 (Q1-Q3 36-64) in Group B-DD. In the living donor cohort, the median eGFR at 5 years was 57.5 (Q1-Q3 4-70) in the Group A, compared with 54 (Q1-Q3 42-65) in Group B.

3.3.3 CAUSE OF GRAFT LOSS

During the study period, 13,816 transplants were performed (from living donors and deceased donors), and were included in the Kaplan Meier analysis of graft survival. After 5 years of follow up, 1587 grafts had failed but only 1288 records detailing the causes of graft loss were returned to NHS-BT. An analysis was performed on these 1288 returns for the cause of graft loss. Table 3.4 summarises the reported causes of graft loss in each treatment group. The commonest cause of graft loss was rejection whilst taking immunosuppressive medication (3.97% Group A, 4.17% Group B), followed by graft loss due to non-viable kidney. Importantly, alemtuzumab induction was not associated with a significant difference in graft loss due to neoplasia, or infection of the graft. Similarly, there was no difference between the frequency of grafts lost due to a major systemic illness. This category encompassed a variety of systemic insults including pancreatitis, myocardial infarction, multi-organ failure, and importantly, systemic sepsis.

	Group A (Alemtuzumab) Number of Grafts lost, N(%)	Group B (Control) Number of Grafts lost, N(%)	P value
Hyperacute Rejection	0 (0)	5 (0.44)	>0.999
Rejection while taking immunosuppressive drugs	66 (40.5)	507 (45.0)	0.31
Rejection after stopping all immunosuppressive drugs	3 (1.84)	33 (2.93)	0.611
Recurrent primary renal disease	13 (7.98)	75 (6.65)	0.508
Vascular or ureteric operative problems (excluding vascular thrombosis)	8 (4.91)	103 (9.14)	0.074
Vascular (arterial or venous thrombosis)	9 (5.52)	99 (8.78)	0.176
Infection of Graft	6 (3.68)	35 (3.11)	0.635
Removal of functioning graft	1 (0.613)	5 (0.444)	0.556
Non- viable kidney	23 (14.1)	127 (11.3)	0.296
Major systemic Illness	4 (2.45)	31 (2.75)	>0.999
De novo primary renal disease	2 (1.23)	12 (1.06)	0.694
Neoplasia	2 (1.23)	7 (0.621)	0.318
Other	26 (16.0)	88 (7.81)	0.002
TOTAL	163	1127	

Table 3.4. Reported Causes of Graft Loss within 5 years of transplantation. During the 5-year follow up, 1587 grafts had failed, however only 1288 records detailing the causes of graft loss were returned. Therefore 299 records are missing from this analysis

3.3.4 REJECTION AND STEROID-FREE SURVIVAL

Figure 1 shows the Kaplan-Meier estimates for rejection free survival over a 5-year period. Patients were further subdivided according to whether they had received a graft from a live donor or a deceased donor. In the deceased donor groups, the use of alemtuzumab had a protective effect on rejection free survival at all time points ($p<0.01$, log-rank test, 1df). In patients with a reported rejection episode, the median time to rejection was 46 days in group B. This was increased to 150 days in the group A. At the end of the 5-year follow up period, 88.7% of patients who had received alemtuzumab were rejection-free, compared with 85.2% in the control group. In the live-donor cohort, for cases with a reported rejection episode, the median time to rejection was increased from 40 days to 170 days in those who had received alemtuzumab. Although a larger percentage of live-donor recipients in the group A were rejection free at all time points, this did not achieve statistical significance. The 5-year rejection free survival was 89.9% in group A compared with 84.0% in group B.

Importantly, at every time-point, a larger proportion of Group A patients were maintained on a steroid-free immunosuppressive regimen compared with Group B (Table 3.5).

		3 Month	**1 Year**	**2 Year**	**3 Year**	**4 Year**	**5 Year**
Deceased Donor	Alemtuzumab	1071 (90.4%)	923 (83.3%)	831 (82.3%)	764 (81.1%)	724 (80.0%)	708 (81.6%)
	Control	1621 (19.4%)	1987 (24.9%)	2210 (30.6%)	2246 (32.8%)	2225 (34.8%)	2124 (35.3%)
		3 Month	**1 Year**	**2 Year**	**3 Year**	**4 Year**	**5 Year**
Living Donor	Alemtuzumab	361 (87.6%)	319 (79.6%)	299 (81.0%)	283 (83.0%)	277 (83.2%)	260 (81.0%)
	Control	633 (20.2%)	860 (27.9%)	952 (33.2%)	973 (35.3%)	984 (37.5%)	959 (38.3%)

Table 3.5. Steroid Free Survival

3.4 DISCUSSION

Alemtuzumab has been used in the United Kingdom as an induction agent for renal transplantation since the 1980s but widespread adoption has not occurred.[190] Two small single centre randomised controlled trials yielded promising short term results which suggested that its use was associated with lower rates of acute rejection and minimal side effects [208,209]. These results were replicated in two large multicentre RCTs although with more significant side effects, possibly due to the routine addition of mycophenolate mofetil to the maintenance regime.[210,211] However, the maximum period of follow up in any of these studies was only three years.

Despite these promising results there has been some reluctance from the renal transplant community to adopt alemtuzumab as a standard induction agent. Some of this reluctance is probably caused by concerns over long term safety especially the long-lasting effects on the lymphoid compartment.[213] This has led to concerns over significant infections and the development of neoplasia. There have been many selected reports of increased infection rates but many of these have been in high-risk patients treated with a high total burden of immunosuppression (e.g. simultaneous pancreas-kidney transplants and HLA incompatible patients). Reports on the rates of malignancy are conflicting but there is no clear evidence that alemtuzumab is associated with greater risk of neoplasia than basiliximab. Another concern has been the development of autoimmune phenomena, in particular autoimmune cytopenias of the myeloid series, e.g. idiopathic thrombocytopenic purpura (ITP).[202,203] There is anecdotal evidence for this phenomenon in renal transplantation but little formal evidence. There is some evidence that this toxicity might be improved by dosing according to recipient weight.[218]

The apparent incongruity between good outcome and lack of widespread usage of alemtuzumab in the UK was explored. Since individual studies are limited by relatively small numbers and short periods of follow-up the UK database for renal transplantation held by NHSBT was analysed to see whether there was any longer-term danger signal regarding the use of alemtuzumab as an induction agent. This is

the first such registry analysis assessing the medium-term outcomes following alemtuzumab induction in renal recipients. This study demonstrates that in patients with standard immunological risk, alemtuzumab induction is associated with a similar 5-year patient and graft survival compared with conventional immunosuppression in both the deceased donor (DCD and DBD) and live donor populations.

Analysis of the database between 2005 and 2013 yielded 13,816 recipients of an adult first kidney-only transplant with standard immunological risk. The patients treated with alemtuzumab were generally older, more likely to have received a DCD graft, more poorly matched and had received older donor kidneys. They were also more likely to have been maintained on tacrolimus monotherapy as maintenance immunosuppression. During this period the bulk of the patients treated with alemtuzumab in the UK were either part of a large multicentre trial (c. 400) [211] or were derived from centres who were pursuing steroid avoidance in standard risk patients. As a result, recipients were less likely to be on corticosteroids. With the increased morbidity and costs associated with the long term use of corticosteroids, there has been a drive to minimise its use in maintenance immunosuppression protocols.[219] Whilst achieving a 5-year mortality and graft survival that is similar to contemporary immunosuppression, this study has shown that alemtuzumab induction is associated with an improved rejection-free survival in the deceased donor cohort at all time points. This is notable as most of these patients were maintained on a steroid-free immunosuppressive regime. Although this has not translated to an improvement in cardiovascular mortality at 5 years, the benefits of a steroid-sparing regime may become apparent in future years. In the live donor cohort, alemtuzumab was associated with an improved rejection-free survival, however this did not achieve statistical significance.

Other groups report similar 5-year mortality data when alemtuzumab was compared with either basiliximab or anti-thymocyte globulin (ATG) in deceased donor cohorts.[220,221] One meta-analysis concluded that mortality following alemtuzumab was similar to IL-2 receptor antagonists after 12 months of follow up.[222] Tan *et al* confirm

the 1-year safety of alemtuzumab in a living donor cohort.[223] Additionally, a RCT showed similar one and two year survival when comparing alemtuzumab and tacrolimus monotherapy with daclizumab, tacrolimus and MMF.[208] There have been some recent studies emanating from the United States, but these have compared induction with alemtuzumab against ATG and looked at short term outcomes only. A systematic review suggested that outcomes including mortality and graft survival were similar between alemtuzumab and ATG in the short term.[224] However a paired analysis suggested a short term benefit in favour of ATG in terms of patient survival despite less resource usage in patients on alemtuzumab.[225] A Cochrane analysis suggested that both ATG and alemtuzumab reduce rejection rates but at the expense of increased cytomegalovirus (CMV) rates without any major effects on other important outcome measures.[226]

The association between alemtuzumab induction and improved graft survival is less clear. In live donors, alemtuzumab induction was associated with improved 1-year graft survival although this was not statistically significant.[223] Hanaway *et al* report equivalent 3-year graft survival in a RCT when alemtuzumab induction was compared to basiliximab in low risk patients, and ATG in high risk patients [210], and Chan *et al* show that alemtuzumab with tacrolimus monotherapy had similar 2-year graft survival when compared with daclizumab.[208] La Mattina *et al.* found reduced graft survival when alemtuzumab was compared with contemporary induction, however on multivariate analysis, alemtuzumab was not an independent risk factor for graft loss. They concluded that the inferior graft survival was due to the increased risk of antibody-mediated rejection and incidence of CMV disease in a higher risk cohort. Additionally, patients who received alemtuzumab were less likely to be maintained on tacrolimus and underwent rapid steroid tapering compared with the controls. Furthermore, alemtuzumab patients were typically maintained on calcineurin inhibitors at levels lower than the control.[220] Serrano *et al.* attempted to address the poor early outcomes and performed a registry analysis to study the primary outcome of overall death-censored graft survival during different transplanting eras. They found that alemtuzumab was only associated with poor graft survival in the earliest

era (2003-2005) when compared with ATG. They concluded that poor early outcomes with alemtuzumab use were related to under immunosuppression rather than the ineffectiveness of alemtuzumab, and clinical outcomes were improving with experience.[217] Maintaining patients on a calcineurin inhibitor (CNI) based regime compared with a CNI-free regime, was associated with improved outcomes.[62] There has been some concern regarding the use of alemtuzumab induction amongst older recipients. Hurst *et al* investigated the safety profile of alemtuzumab in the elderly population, and concluded that alemtuzumab induction was associated with increased death and reduced graft survival, possibly related to over immunosuppression in that cohort, however they did not report differences in the causes of death (infection, malignancy, cardiac) in the alemtuzumab cohort compared with other induction agents.[227]

In this study, the finding that the death and graft survival rates are not significantly different for either agent is very reassuring and suggests that at least in the medium term there is no significant difference in outcomes between either induction agent. This is confirmed by the data on graft function which demonstrates parity between the two agents. Furthermore, there was no difference in transplant survival for recipients aged >60 years who received Alemtuzumab compared with other induction agents. In the UK, alemtuzumab induction consisted of a single 30mg dose, with some patients aged <60 years receiving a second 30mg dose, perhaps accounting for the different observed outcomes.

Concern over the increased potential for the profound lymphocyte depletion by alemtuzumab to cause increased death rates or graft failure by either infection or neoplasia is partly allayed by the data showing no significant differences in causes of graft loss or patient death. There is conflicting data surrounding the risk of malignancy following alemtuzumab use, and this is likely due to differences in the period of follow-up. Two RCTs that utilised a steroid sparing regime found similar rates of malignancy when comparing alemtuzumab to control after 1-year,[209] and 3 years [210] of follow up. Kirk *et al*. interrogated the OPTN/UNOS database, and found that when records were

censored at 730 days, the incidence of posttransplant lymphoproliferative disease (PTLD) was not increased with alemtuzumab use.[212] However, one US registry study that utilised mandatory local and regional cancer reporting found that with a median follow up period of 3.5 years, alemtuzumab induction was associated with an increased risk of non-Hodgkin Lymphoma, virus related cancers, thyroid cancer, and colorectal cancer. Although other induction agents, including basiliximab were investigated in this study, the reference population did not receive an induction agent.[215] Clatworthy *et al* report no increase in the rate of malignancy (skin, solid organ and PTLD) after 10 years of follow up, in a small cohort of patients who received alemtuzumab for the treatment of biopsy proven rejection. Many of these patients had received a cumulative dose of alemtuzumab exceeding the 30mg used for induction in renal transplantation.[228]

Similarly, there are differing reports on the incidence of infections following alemtuzumab induction. One retrospective study reported no difference in rates of infections.[197] Another retrospective study compared alemtuzumab with basiliximab and ATG and found an increase in infectious complications (overall, opportunistic and CMV related), with alemtuzumab. Although overall survival was similar to the control, they also noted an increase in death related to sepsis.[220] When alemtuzumab was compared with basiliximab, one RCT reported more infective episodes requiring hospitalisation in the same number of patients.[209] Another RCT found in low-risk patients, alemtuzumab induction was associated with similar overall infection rates when compared to basiliximab induction, however the rate of serious infections was increased. In high-risk patients, there were similar rates of serious infections, but the overall infection rate was decreased when compared with ATG.[210]

In addition to noticing an improved rejection free survival within the Alemtuzumab group, a difference in the timing of rejection episodes was also observed, with a higher proportion of Group B patients experiencing rejection at an earlier time point compared with Group A patients. In patients who developed a rejection episode, the median time to rejection in Group A was 150 (deceased donor cohort), and 170 days

(live donor cohort). Similar patterns have been noted elsewhere,[197,209,210] and is important clinically when determining the timing and frequency of post-transplant follow up. Alemtuzumab results in the depletion of the lymphocyte compartment, and subsequent repopulation can take up to 1 year to achieve baseline levels,[140,229] and an increased median time to rejection may be related to this repopulation.

The use of registry data has its limitations, and missing records for cause of graft loss (20%), and cause of death (40%), make it difficult to draw firm conclusions. The regime for administering alemtuzumab is variable within group A with some units preferring subcutaneous administration of alemtuzumab to intravenous. In addition, some units give a second dose to patients under 60 years old at 24 hours after transplantation. Furthermore, the control group are heterogeneous, and some may not have received induction therapy.

This study has included data obtained from multiple UK transplanting centres, and each centre will have their own protocol for maintenance immunosuppression and, the decision to use alemtuzumab may have been affected by both donor and recipient characteristics which will introduce bias to the clinical outcomes. There are several important variables (e.g. tacrolimus levels or incidence of non-fatal neoplasia) that are not collected by the registry and these cannot be taken into account in the analysis. Clearly the findings are limited by all the usual inherent problems in analysing large retrospective databases and ultimately, only associations can be demonstrated.

To conclude, despite the concerns over long term safety regarding the use of alemtuzumab in renal transplant recipients, UK registry data is reassuring and suggests similar overall performance to alternative induction agents. However, alemtuzumab induction does permit the avoidance of steroids as part of maintenance immunosuppression in a significantly higher proportion of patients without any obvious penalty.

4 THE ALBERT (ASSESSMENT IN LEEDS OF A BIOMARKER EARLY AFTER RENAL TRANSPLANTATION) STUDY RESULTS

4.1 THE DESCRIPTION OF THE B-CELL SURFACE PHENOTYPE FROM 3-18 MONTHS POST TRANSPLANT

Ninety-five patients were consented to the prospective arm of the ALBERT study. The demographics for these patients are described in Table 4.1. Although alemtuzumab is the first-line induction agent at Leeds, approximately 30% of the population received basiliximab due to patient-specific contradictions.

The B-cell surface phenotype was compared between the two induction groups, and the composition of B-cell subsets were described over the follow-up period. In a healthy population, the absolute B-cell count and frequencies of B-cell subsets will change with increasing age.[230] Acknowledging that alemtuzumab depletes B-cells, and the phenotypes described in this cohort represent B-cell reconstitution as opposed to the age - related developmental changes, the prospective phenotypes were also reviewed alongside an age-dependent reference range described by Morbach *et al*.[230] The median age of the study cohort was 48 years.

	Total	Alemtuzumab	Basiliximab	p-value
Number	95 (100%)	68	27	
Recipient Age (yr.)	48	49	48	0.818
Recipient Gender (m)	59 (62%)	38 (56%)	21 (78%)	0.038
Recipient Gender (f)	36 (38%)	30 (44%)	6 (22%)	
Recipient Ethnicity				0.709[1]
Caucasian	85 (90%)	59 (87%)	26 (96%)	
Asian	8 (8%)	7 (10%)	1 (4%)	
Afro Caribbean	1 (1%)	1 (2%)	0	
Other	1 (1%)	1 (2%)	0	
Cause of ESRD				0.415
Diabetes and HTN	13 (14%)	8 (12%)	5 (19%)	
Glomerulonephritis	36 (38%)	24 (13%)	12 (44%)	
Inherited	14 (15%)	9 (13%)	5 (19%)	
Other	19 (20%)	16 (24%)	3 (11%)	
Unknown	13 (14%)	11 (16%)	2 (8%)	
Donor Type				0.957
LD	26 (27%)	18 (27%)	8 (30%)	
DBD	45 (47%)	33 (49%)	12 (44%)	
DCD	24 (25%)	17 (25%)	7 (26%)	
Pre-emptive	26 (27%)	20 (29%)	6 (22%)	0.612
Graft Number				0.044
1	71 (75%)	50 (74%)	21 (78%)	
2	20 (21%)	17 (25%)	3 (11%)	
3	4 (4%)	1 (2%)	3 (11%)	
NHSBT HLA Mismatch Level				0.209
1	15 (16%)	9 (13%)	6 (22%)	
2	24 (25%)	21 (31%)	3 (44%)	

	Total	Alemtuzumab	Basiliximab	p-value
3	35 (22%)	23 (22%)	12 (44%)	
4	21 (22%)	15 (22%)	6 (22%)	
Maintenance Immunosuppression*				
Tacrolimus	93 (98%)	67 (99%)	26 (96%)	0.49[1]
Sirolimus	2 (2%)	1 (2%)	1 (4%)	
MMF	48 (43%)	20 (25%)	28 (88%)	**<0.001**
Azathioprine	6 (5%)	3 (4%)	3 (10%)	0.225
Prednisolone	25 (22%)	17 (21%)	8 (25%)	0.421

Table 4.1. Demographic characteristics of the ALBERT study prospective cohort (stratified according to induction agent)

***Maintenance immunosuppression at initial discharge from hospital**

4.1.1 THE DIFFERING EFFECTS OF INDUCTION AGENTS ON THE B-CELL PHENOTYPE

4.1.1.1 %B-CELLS AND CALCULATED B LYMPHOCYTE COUNT

The groups were initially analysed separately according to induction agent (Alemtuzumab and Basiliximab). Within each induction agent group, there was no significant difference in the %B-cells obtained at each time point (Alemtuzumab mean 3m 14.84, 6m 17.16, 12m 15.52, 18m 15.7 p=0.6853, Basiliximab median 3m 6.1, 6m 5.1, 12m 4.47, 18m 7.010, p=0.1588). The median %B-cells obtained from a healthy cohort aged 26-50 years is 9.2% (range 7.2-11.2).[230] Patients who received alemtuzumab induction demonstrated higher %B-cells at all time points compared with the reference population. Conversely, patients who received basiliximab had lower median %B-cells compared with the reference population. For patients who received alemtuzumab, the calculated B-cell count increased at each time point (mean rank difference 3 vs 6m -46.44 p=0.0012, 3 vs 12m -51.50 p<0.0001, 3 vs 18m -61.92,

p<0.0001). A significant increase in the B-cell count was also noted in the Basiliximab between 3 and 18m (mean rank difference -25.43, p=0.0073).

The two induction groups were then compared with each other. At each time point, Alemtuzumab patients were found to have higher %B-cells compared with Basiliximab patients (Figure 4.1 and

%B-cells					
Timepoint	**Alemtuzumab %B**		**Basiliximab %B**		**p-value**
	Median	**IQR**	**Median**	**IQR**	
3 months	12.42	18.71	6.100	4.75	**0.0009**
6 months	15.69	19.50	5.160	5.93	**<0.0001**
12 months	14.74	15.63	4.490	4.57	**<0.0001**
18 months	15.73	15.48	7.010	4.50	**0.0023**

Table 4.2), however there was no statistically significant difference in the median calculated B lymphocyte count between these groups (Figure 4.1 and Table 4.3). These results reflect the total depletion of the lymphocyte compartment following alemtuzumab induction, and the subsequent repopulation of B-cells prior to T-cells.[160,164]

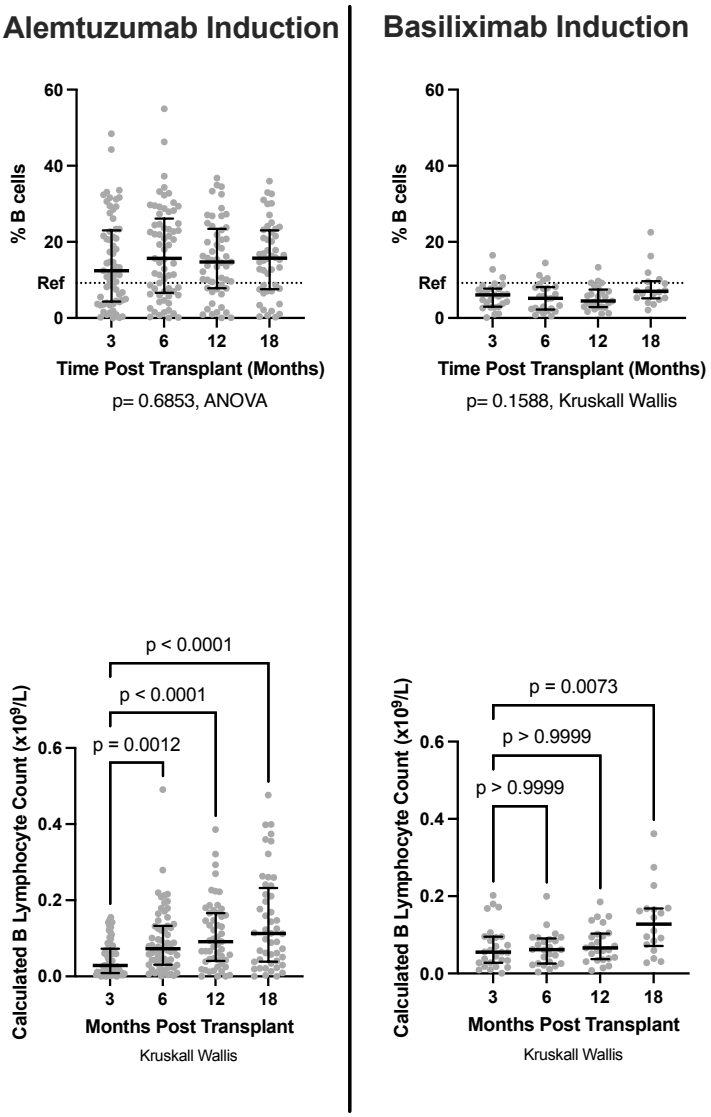


Figure 4.1. Comparison of B-cell phenotype by Induction Agent.

Group 1 patients had venous blood drawn at 3 months, 6 months, 12 months and 18 months post-transplant. The resting B-cell phenotype was assessed by flow cytometry. The left side of the figure demonstrates data obtained from the Alemtuzumab group, and the right side Basiliximab group. The top panel shows %B-cells and the bottom panel shows the calculated B lymphocyte count. Individual values, median and interquartile range are shown. Unless otherwise stated, comparisons were made using the Kruskall Wallis test. The Dunn's multiple comparisons test was performed if a difference was noted (using 3 months as the control). The dotted line denotes the median reference value for a healthy adult population.[230]

%B-cells					
Timepoint	**Alemtuzumab %B**		**Basiliximab %B**		**p-value**
	Median	**IQR**	**Median**	**IQR**	
3 months	12.42	18.71	6.100	4.75	**0.0009**
6 months	15.69	19.50	5.160	5.93	**<0.0001**
12 months	14.74	15.63	4.490	4.57	**<0.0001**
18 months	15.73	15.48	7.010	4.50	**0.0023**

Table 4.2. Mann-Whitney Test comparing the %B-cells obtained from Alemtuzumab and Basiliximab patients over the follow up period.

IQR – interquartile range

Calculated B Lymphocytes ($x10^9$/L)					
Timepoint	**Alemtuzumab Calc B**		**Basiliximab Calc B**		**p-value**
	Median	**IQR**	**Median**	**IQR**	
3 months	0.02870	18.71	0.05526	0.06388	**0.0566**
6 months	0.07289	19.50	0.06173	0.10187	0.2176
12 months	0.09115	15.63	0.06642	0.12546	0.2280
18 months	0.1127	15.48	0.1276	0.19358	0.7053

Table 4.3. Mann-Whitney Test comparing the calculated B lymphocytes obtained from the Alemtuzumab and Basiliximab groups over the follow up period.

IQR – interquartile range

4.1.1.2 B-CELL SUBSETS DEFINED USING CD27/IGD EXPRESSION

The B-cell subsets were then assessed using CD27 and IgD expression (see Figure 4.2 for the gating strategy). Patients who received Alemtuzumab were found to have more %naive ($CD27^-IgD^+$) cells, reduced %class-switched memory ($CD27^+IgD^-$) cells and %non-switched memory ($CD27^+IgD^+$) cells compared with a normal adult population (median %naive 65.1%, range 58-72%, median %class-switched memory 13.2, range 9.2-18.9, median %non-switched memory 15.2, range 13.4-21.4).[230] Over the period of follow up, the alemtuzumab %naive cells decreased (median value 3m 97.10, 6m 96.52, 12m 94.91, 18m 92.54, $p<0.0001$ Kruskal-Wallis test), and class-switched memory cells increased (median value 3m 0.7050, 6m 1.330, 12m 2.140, 18m 3.570, $p<0.0001$ Kruskal-Wallis test). However at the end of the follow up period, the %naive cells remained higher, and %class-switched memory cells were lower, than the corresponding adult reference range.[230] An increase in non-switched memory cells was noted at 12m and 18m compared with the earlier timepoints (median value 3m 1.230, 6m 1.220, 12m 1.285, 18m 1.840, $p=0.0466$ Kruskal-Wallis test), although this remained below the adult reference range. Finally, $CD27^-IgD^-$ cells were noted to increase over the follow up period (median value 3m 0.5850, 6m 0.6700, 12m 1.290, 18m 1.780 $p<0.0001$), however these values remained less than the adult reference range (median 3.3, range 2.1-5.3).[230] Figure 4.3 demonstrates the change in CD27/IgD subsets together with comparisons using the Kruskal-Wallis test and Dunn's multiple comparisons test. A dotted line represents the median normal adult value.[230]

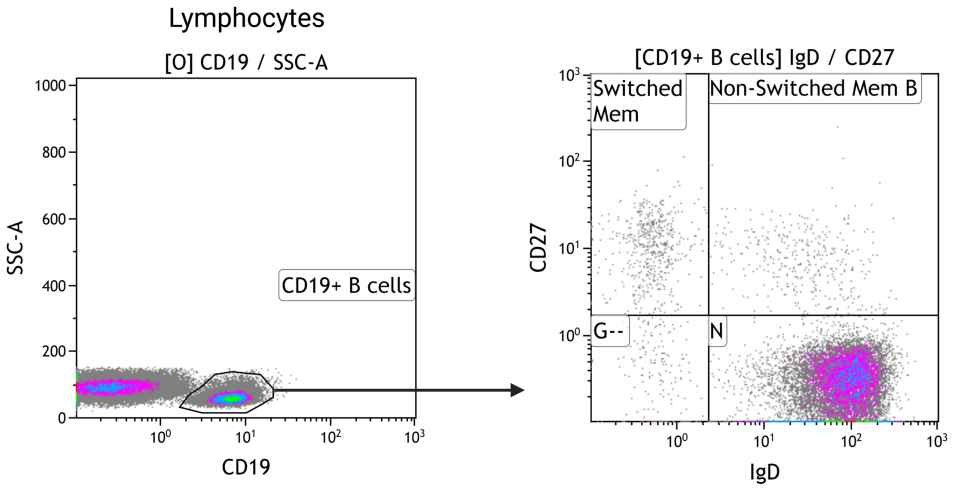


Figure 4.2. Definition of B-cell subsets using CD19, CD27, IgD expression.
Naïve - $CD19^{+}CD27^{-}IgD^{+}$, Non-Switched Memory – $CD19^{+}CD27^{+}IgD^{+}$, Class-switched memory – $CD19^{+}CD27^{+}IgD^{-}$, Exhausted memory – $CD19^{+}CD27^{-}IgD^{-}$
Sample obtained from a 60-year-old recipient 6 months after transplantation/alemtuzumab induction

The basiliximab induction patients were noted to have slightly increased %naive cells, and reduced non-switched, class-switched and $CD27^{-}IgD^{-}$ cells compared with the reference adult population. No statistically significant changes were seen within each subset during the follow up period in the basiliximab group (Figure 4.3).

On direct comparison between the two induction agents, alemtuzumab patients demonstrated higher %naive, and lower %non-switched memory cells at all time points compared with basiliximab patients. Alemtuzumab patients also had reduced %class switched and $CD27^{-}IgD^{-}$ cells at all time points, however this only reached statistical significance for 3, 6, and 12 months (class switched), and 3, 6 months ($CD27^{-}IgD^{-}$ [Table 4.4, Table 4.5, Table 4.6, Table 4.7]).

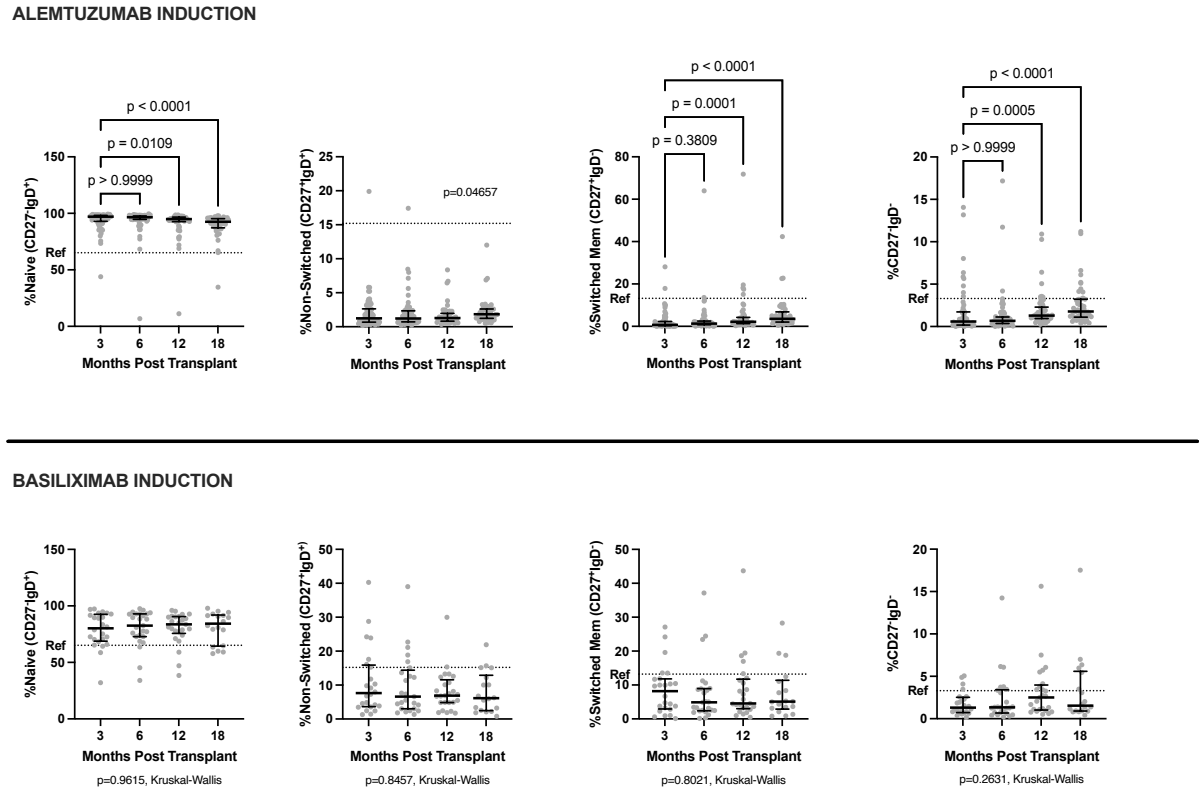


Figure 4.3. Comparison of B-cell subsets using CD27 and IgD expression.

Group 1 patients had venous blood drawn at 3 months, 6 months, 12 months, and 18 months post-transplant. The resting B-cell phenotype was assessed by flow cytometry. The upper panel includes data obtained from the Alemtuzumab group, and the bottom panel, Basiliximab group. From left to right, graphs depict %Naive, %Non-switched memory, %Switched memory and %CD27-IgD-. Individual values, median and interquartile range are shown. Comparisons were made using the Kruskal-Wallis test. The Dunn's multiple comparisons test was performed if a difference was noted (using 3 months as the control). A dotted line represents the median adult reference value described by Morbach et al.[230]

%Naive ($CD27^-IgD^+$)					
Timepoint	**Alemtuzumab**		**Basiliximab**		**p-value**
	Median	**IQR**	**Median**	**IQR**	
3 months	97.10	5.26	80.15	23.73	**0.0009**
6 months	96.52	3.01	82.58	20.15	**<0.0001**
12 months	94.91	3.95	83.73	14.83	**<0.0001**
18 months	92.54	8.05	84.36	27.50	**0.0023**

Table 4.4. Mann-Whitney test comparing the %Naive ($CD27^-IgD^+$) subsets by induction agent.

IQR – interquartile range

%Non-Switched Memory ($CD27^+IgD^+$)					
Timepoint	**Alemtuzumab**		**Basiliximab**		**p-value**
	Median	**IQR**	**Median**	**IQR**	
3 months	1.230	1.97	7.625	12.25	**<0.0001**
6 months	1.220	1.62	6.600	11.37	**<0.0001**
12 months	1.285	1.13	6.880	6.71	**<0.0001**
18 months	1.840	1.39	6.135	10.42	**<0.0001**

Table 4.5. Mann-Whitney test comparing the %Non-Switched Memory ($CD27^+IgD^+$) subsets by induction agent.

IQR – interquartile range

Switched Memory ($CD27^+IgD^-$)					
Timepoint	**Alemtuzumab**		**Basiliximab**		**p-value**
	Median	**IQR**	**Median**	**IQR**	
3 months	0.7050	1.98	8.200	8.89	**<0.0001**
6 months	1.330	1.79	4.880	6.52	**<0.0001**
12 months	2.140	2.88	4.560	8.70	**0.0018**
18 months	3.570	4.81	5.085	8.53	0.2638

Table 4.6. Mann-Whitney test comparing the %Switched memory subsets by induction agent.

IQR – interquartile range

$CD27^-IgD^-$					
Timepoint	**Alemtuzumab**		**Basiliximab**		**p-value**
	Median	**IQR**	**Median**	**IQR**	
3 months	0.5850	1.55	1.305	1.793	**0.0063**
6 months	0.6700	0.77	1.340	2.73	**0.0036**
12 months	1.290	1.35	2.500	2.96	**0.0780**
18 months	1.780	2.12	1.545	4.68	0.8673

Table 4.7. Mann-Whitney test comparing the $CD27^-IgD^-$ subsets by induction agent.

IQR – interquartile range

4.1.1.3 B-SUBSETS DEFINED BY CD24/CD38 EXPRESSION

Finally, B-cell subsets were assessed by CD24 and CD38 expression (see Figure 4.4 for gating strategy and Figure 4.5 for representative examples of B subsets following alemtuzumab and basiliximab induction) to investigate transitional B-cells and plasmablasts. In the healthy adult population aged 26-50 years, the median value of %TrBs was 2.0 (range 1.0 - 3.6) and the median value of %plasmablasts was 1.0 (0.6-1.6).[230]

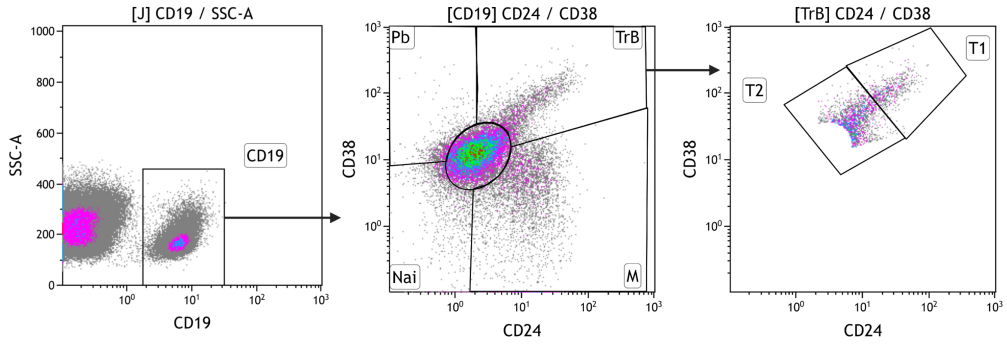


Figure 4.4. Definition of B-cell subsets using CD19, CD24 and CD38 expression. Sample obtained from a healthy volunteer (age 31 years). Naïve - $CD19^{+}CD24^{+}CD38^{+}$, Plasmablasts – $CD19^{+}CD24^{-}CD38^{hi}$, Memory - $CD19^{+}CD24^{hi}CD38^{-}$, Transitional – $CD19^{+}CD24^{hi}CD38^{hi}$

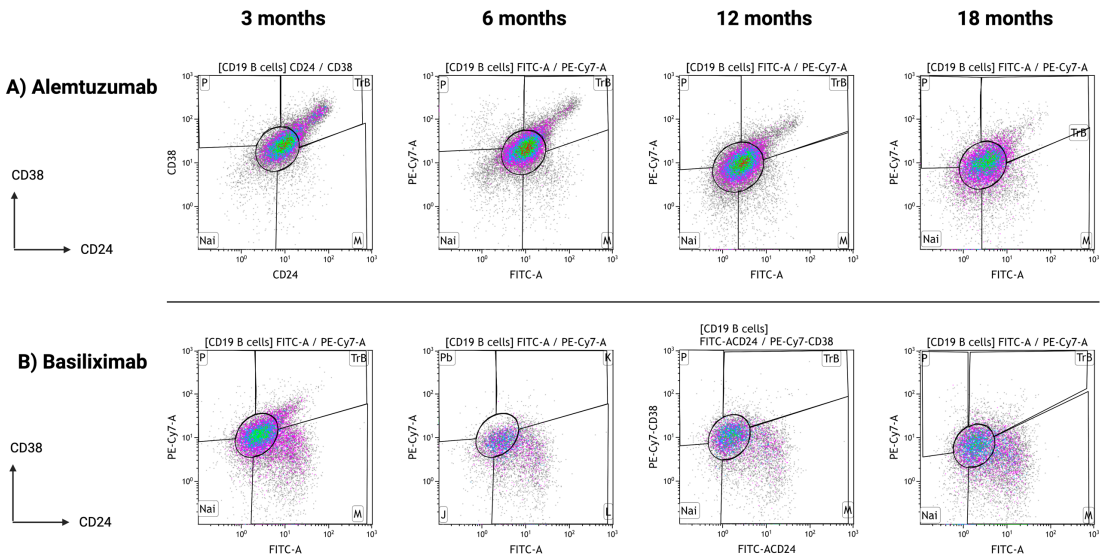
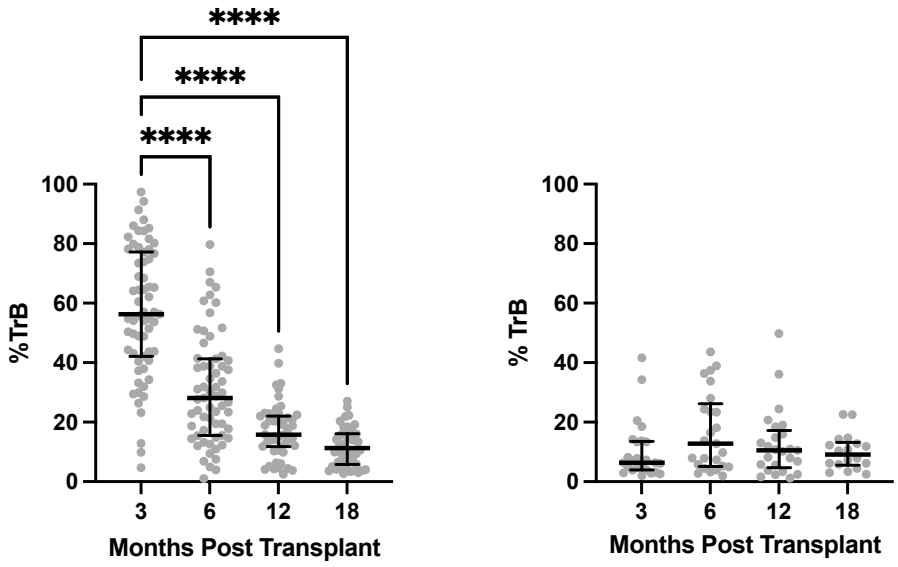


Figure 4.5. Representative example of transitional B-cell subsets at different time points post-transplant.

Samples obtained from: Panel A) 58-year-old recipient following alemtuzumab induction, Panel B) 20-year-old recipient following basiliximab induction.

4.1.1.3.1 TRANSITIONAL B-CELLS ($CD24^{HI}CD38^{HI}$)

When compared against the normal reference range described above, both alemtuzumab and basiliximab patients were shown to have higher %TrBs at all time points following transplantation (reference TrBs = 2%). Whereas patients who received basiliximab had a similar proportion of TrBs over the follow up period (median 3m 6.77%, 6m 12.77%, 12m 10.55%, 18m 9.125%, p=0.3265, Kruskall-Wallis test), patients who received alemtuzumab had high proportions of TrBs at 3 months post-transplant that decreased over the follow up period (mean %TrB at 3m 57.35, 6m 31.40, 12m 17.19, 18m 11.77, p<0.0001, Figure 4.6). The two groups were directly compared at each time point, and patients who received alemtuzumab had more TrBs than those who received basiliximab for the first post-transplant year (Table 4.8). At 18 months, Alemtuzumab patients had more TrBs than Basiliximab patients, however this difference was not statistically significant.



A) Alemtuzumab Induction B) Basiliximab Induction

Figure 4.6. Transitional B-cells during follow up period.

Prospective study patients were divided into two groups according to induction agent A) Alemtuzumab, and B) Basiliximab. The proportion of transitional B (TrBs) cells were defined using $CD19^{+}CD24^{hi}CD38^{hi}$ expression. Individual values, median and interquartile range shown. Comparisons were made using ordinary one-way analysis of variance with Dunnetts's multiple comparisons test. ** denotes $p<0.0001$**

Transitional B-cells ($CD24^{+}CD38^{+}$)					
Timepoint	**Alemtuzumab**		**Basiliximab**		**p-value**
	Median	**IQR**	**Median**	**IQR**	
3 months	55.74	36.32	6.77	9.74	**<0.0001**
6 months	28.09	24.44	12.77	21.07	**0.0004**
12 months	15.76	10.25	10.55	12.49	**0.0084**
18 months	11.33	9.70	9.125	7.66	0.3309

Table 4.8. Mann-Whitney test comparing the proportion of transitional B-cells obtained from different induction groups during the follow up period.
IQR – interquartile range

4.1.1.3.2 T1 AND T2 SUBSETS, AND T1:T2 RATIO

The transitional B-cells were examined further and TrBs were divided into T1 and T2 cells using the relative expression of CD24 and CD38 as described previously (Figure 4.4). Patients who received Alemtuzumab induction were found to have a high proportion of T1 cells at 3 months that decreased over time, and a low proportion of T2 cells that increased over time. This resulted in a high T1:T2 ratio that reduced over the follow up period. The top panel of Figure 4.7 demonstrates the %T1, %T2 and T1:T2 ratios obtained from the Alemtuzumab group during the follow up period. On the other hand, no significant differences were noted in the %T1, %T2, and T1:T2 ratios in patients who received Basiliximab induction (Figure 4.7, bottom panel).

The two induction groups were directly compared at each time point, and the results are demonstrated in Table 4.9,Table 4.10 and Table 4.11. Patients who received alemtuzumab induction had significantly higher %T1, lower %T2 and a higher T1:T2 ratio compared with basiliximab patients at 3 months. There was however no statistically significant difference in the %T1, %T2 and T1:T2 ratios between the two induction groups for the rest of the follow up period, although there was a trend towards a higher T1:T2 ratio in the Alemtuzumab patient group at 12 months and 18 months (Table 4.11).

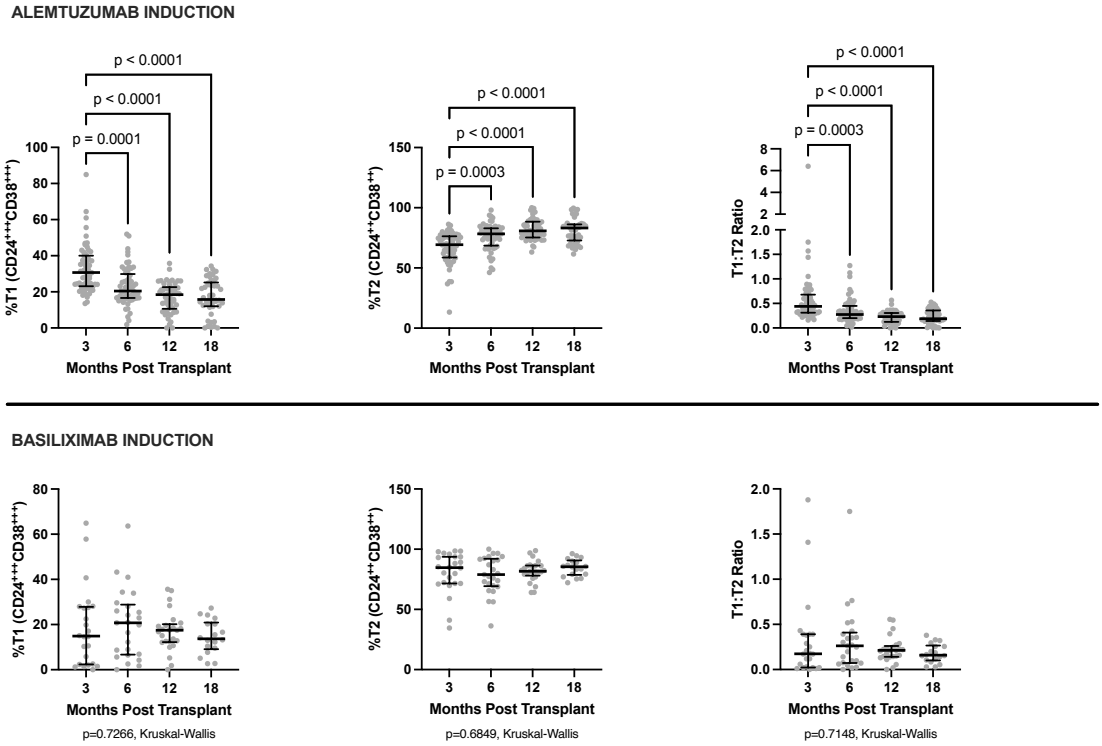


Figure 4.7. Transitional B ($CD24^{+}CD38^{+}$) subsets during follow-up.

Group 1 patients had venous blood drawn at 3 months, 6 months, 12 months, and 18 months post-transplant. The resting B-cell phenotype was assessed by flow cytometry. The upper panel demonstrates data obtained from the Alemtuzumab group, and the bottom panel, Basiliximab group. From left to right, graphs depict %T1, %T2, and T1:T2 ratios^{-}. Individual values, median and interquartile range are shown. Comparisons were made using the Kruskal-Wallis test. The Dunn's multiple comparisons test was performed if a difference was noted (using 3 months as the control).

%T1					
Timepoint	**Alemtuzumab**		**Basiliximab**		**p-value**
	Median	**IQR**	**Median**	**IQR**	
3 months	30.67	16.93	14.88	25.46	**<0.0001**
6 months	20.45	13.27	20.76	22.12	0.3379
12 months	18.50	12.05	17.49	7.93	0.6836
18 months	15.78	13.13	13.67	11.82	0.2421

Table 4.9. Mann-Whitney Test comparing %T1 cells between alemtuzumab and basiliximab groups during follow up.

IQR – interquartile range

%T2					
Timepoint	**Alemtuzumab**		**Basiliximab**		**p-value**
	Median	**IQR**	**Median**	**IQR**	
3 months	69.31	17.66	84.70	22.17	**<0.0001**
6 months	78.34	14.30	78.95	22.72	0.3647
12 months	80.78	13.18	81.70	8.36	0.7996
18 months	83.22	13.31	85.44	12.02	0.1230

Table 4.10. Mann-Whitney test comparing %T2 cells between alemtuzumab and basiliximab groups during follow up.

IQR – interquartile range

T1:T2 Ratio					
Timepoint	**Alemtuzumab**		**Basiliximab**		**p-value**
	Median	**IQR**	**Median**	**IQR**	
3 months	0.4400	0.39	0.1750	0.37	**<0.0001**
6 months	0.2747	0.25	0.2630	0.34	0.3065
12 months	0.2308	0.18	0.2123	0.12	0.6497
18 months	0.1885	0.22	0.1599	0.17	0.2247

Table 4.11. Mann-Whitney test comparing T1:T2 ratios obtained from Alemtuzumab and Basiliximab induction groups at all time points.

IQR – interquartile range

4.1.1.3.3 PLASMABLASTS ($CD24^{-}CD38^{+}$)

Morbach *et al* defined their plasmablast populations using a smaller gate, therefore the values obtained from the ALBERT prospective study patients could not be directly compared with this reference population. Within the alemtuzumab induction group, the proportion of %plasmablasts increased with time, yet no statistically significant differences were noted in patients who received basiliximab induction (Figure 4.8).

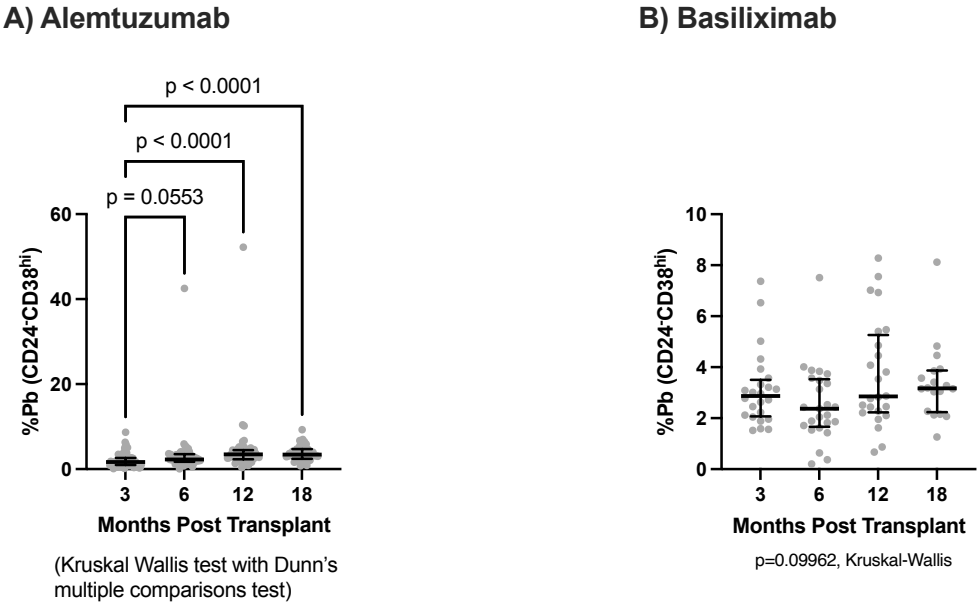


Figure 4.8. Plasmablasts ($CD24^{-}CD38^{+}$) obtained during follow up. Patients who received alemtuzumab induction, and B) Basiliximab induction. Dot plots show individual values, median and interquartile range.

The two induction groups were then compared at each time point and although alemtuzumab patients had fewer plasmablasts at 3 months (1.68% alemtuzumab vs 2.87% basiliximab, p=0.0005), there were no significant differences at 6, 12 and 18 months (Table 4.12).

Plasmablasts ($CD24^{-}CD38^{+}$)					
Timepoint	**Alemtuzumab**		**Basiliximab**		**p-value**
	Median	**IQR**	**Median**	**IQR**	
3 months	1.680	1.65	2.870	1.44	**0.0005**
6 months	2.270	1.80	2.370	1.86	0.8188
12 months	3.480	2.16	2.850	3.04	0.9475
18 months	3.430	2.27	3.170	1.63	0.4946

Table 4.12. Mann-Whitney test comparing $CD24^{-}CD38^{+}$ Plasmablasts (Pbs) between induction agents.

IQR – interquartile range

To summarise, following renal transplantation, recipients were found to have key differences in their B-cell subsets when compared with an age-matched reference population.[230] Allograft recipients were found to have a more phenotypically immature B-cell population with increased naïve and transitional B-cells, and fewer non-switched and switched memory B-cells. This difference was more marked in the alemtuzumab cohort which had the highest proportion of naïve and TrBs at 3 months post-transplant. These values decreased with time but remained higher than the reference population at 18 months.

4.1.2 DISCUSSION

Alemtuzumab is a humanised monoclonal antibody against CD52 which is found on lymphocytes. The binding of alemtuzumab to CD52 targets the cell for lysis through the complement pathway, and antibody dependent cellular cytolysis, resulting in the depletion of lymphocytes. Basiliximab on the other hand is a non-depleting monoclonal antibody that binds with high affinity to CD25, therefore blocking IL-2 binding and inhibiting T-cell proliferation. CD25 is also expressed on activated B-cells, therefore CD25 blockade on B-cells can interfere with B – T-cell interactions and affect the differentiation of activated B-cells towards plasma cells. *In vivo* studies have demonstrated increased total B-cells and memory cells following basiliximab induction.[140,163,166]

Patients had their first study blood sample drawn at 3 months post-transplant. After alemtuzumab induction, patients initially demonstrated reduced B-cell counts which gradually increased over the follow up period to levels comparable with patients who had received basiliximab. Additionally, a difference in peripheral B-cell phenotype was noted between the two induction groups with alemtuzumab patients demonstrating a shift towards more naïve and TrBs, and fewer memory cells. Within the memory cell compartment, alemtuzumab patients were found to have a higher proportion of non-switched memory and fewer class-switched and exhausted memory cells compared with basiliximab patients. The observed differences in B-cell phenotype from the two induction groups reduced as time progressed except for the naïve B-cells which remained significantly elevated, and class-switched memory cells which were significantly reduced at 18 months follow up in the alemtuzumab group. These findings are consistent with previous studies.[140,231] Heidt *et al* demonstrated that naïve B-cells were increased in the repopulating B-cell compartment following alemtuzumab induction, and these cells remained dominant for at least 12 months after induction, even in patients who were not on maintenance immunosuppression.[164] Additionally, they noted a reduction in memory cells.[231] This higher naïve:memory cell ratio may be due to a block in the differentiation from naïve to memory cells, a phenomenon which has previously been described in multiple

sclerosis patients.[161] It could also be the normal recapitulation of B-cell ontogeny or due to the effect of calcineurin inhibitors on B-cell activation. The long-term dominance of naïve cells has been described in a cohort of kidney transplant recipients who were maintained on triple immunosuppression, and this was independent of a subsequent switch from tacrolimus to sirolimus.[231] The noted differences in B-cell phenotype could also be attributed to the different maintenance immunosuppression regimes associated with each induction agent. The Leeds immunosuppression protocol includes an antiproliferative agent in addition to a calcineurin inhibitor when patients receive basiliximab induction. Therefore, in this study cohort, 88% of basiliximab patients received MMF for maintenance immunosuppression compared with 20% of alemtuzumab patients. MMF has been demonstrated to affect B-cells through the reduced T-cell dependent activation of B-cells, as well as directly inhibiting B-cell proliferation and plasma cell differentiation. A limitation affecting the analysis was that whilst the use of MMF post transplantation was documented, the dose for each patient was not recorded. Similarly, mycophenolic acid levels were not tested, therefore it is possible that some patients were maintained on subtherapeutic or supratherapeutic levels, affecting B-cell subsets and recapitulation.

Azathioprine can reduce B-cells overall, as well as reduce naïve and TrB populations.[168,169] Although a higher proportion of basiliximab patients received azathioprine compared with the alemtuzumab group (10% vs 4%), this difference was not statistically significant. The observed reduction in naïve and TrBs in the basiliximab patients therefore cannot be solely attributed to azathioprine use. Finally, steroids have been shown to affect B-cell repopulation after stem cell transplantation,[232] and are associated with a dose-dependent reduction in TrBs.[168] All patients in this study population received a single 500mg dose of methylprednisolone at the time of induction, and a similar proportion of patients in each group received steroids as part of their initial maintenance immunosuppression regime. Steroids alone, are therefore less likely to be the cause of the differing phenotype.

In conclusion, the data presented here shows a differing B-cell phenotype between the alemtuzumab and basiliximab induction groups, with increased naïve and transitional B-cells seen in the alemtuzumab group. Although the differences in maintenance immunosuppression could contribute to this differing phenotype, it is likely due to the depleting effect of alemtuzumab. The difference between the induction agents was most marked early post-transplant, and when searching for B-cell phenotypic markers associated with clinical outcomes, patients will need to be analysed separately according to induction agents until at least 1 year following transplantation. It will be important to identify any deviations from 'normal' repopulation, and to determine whether this deviation is associated with different clinical outcomes. If maintained, the observed differences in the TrB and naïve populations between induction agents may become clinically relevant as TrBs have previously been shown to be enriched with Bregs.[95,124]

4.2 B-CELL SURFACE PHENOTYPE AND CLINICAL OUTCOMES FOR ALBERT STUDY PATIENTS

4.2.1 PROSPECTIVE STUDY

The aim of this prospective study was to investigate the differences in B-cell phenotypes following renal transplantation, and to prospectively determine any associations between B-cell phenotype and subsequent clinical outcome. A composite endpoint was initially assessed. This included graft loss and the following surrogate markers that have previously been associated with adverse graft outcomes:

- The development of de novo donor specific antibodies [76,77,175,176]
- A 30% reduction in eGFR between 3 - 18 months [177]
- Histological findings showing immune-mediated changes, including antibody mediated, T-cell mediated or borderline rejection according to the Banff 2013 criteria, evidence of transplant glomerulopathy, or recurrent disease.[178,179]
- Recurrent proteinuria.[180] This was defined as the urine protein creatinine ratio (uPCR)>50 (units) on two or more consecutive occasions after 3 months post-transplant. At this point, it was expected that the contribution of the native kidneys towards proteinuria was minimised, and together with the frequency of urinary tract infections.

Patient recruitment to the ALBERT study occurred between 30/03/2016 and 25/10/2018. All newly transplanted (kidney alone) adult patients who had not yet achieved the composite endpoint and received their post-transplant care at Leeds were eligible to participate in the ALBERT study (Group 1 – prospective study). Patients were first approached in the outpatient setting and were provided with verbal and written information about the study. Further contact was made with the patient to obtain their consent at their 3-month appointment. Fifty-three percent of eligible patients were recruited to the ALBERT prospective study. A second cohort (Group 2 - For Cause biopsy) included patients who were more than 1-year post-transplant and were offered a 'for cause' biopsy for either the deterioration of renal function, or recurrent proteinuria with uPCR>50. These patients were approached at their pre-

biopsy assessment appointment, and formal consent to enrol in the study obtained on the day of biopsy (see Figure 2.4 for study protocol design).

Patients who were recruited to the ALBERT Group 1 study donated blood samples at set time points (3 months, 6 months, 12 months, and 18 months) following their transplant. Alemtuzumab induction results in profound lymphocyte depletion, with gradual repopulation occurring throughout the subsequent year.[140] It can take up to 6 weeks for B-cells to be observed in peripheral blood, and over 8 weeks for peripheral B-cells to return to pre-induction levels.[231] For this reason the first time-point for sampling was 3 months post-transplant. However, there were still cases where the B-cell events were insufficient to confidently gate on individual B-cell subsets. These cases were removed from analysis. Additional samples were collected if patients were offered a 'for cause' biopsy. Clinical information was collected during the study period, with a median follow up of 1701 days (range 132-2129 days). Over the period of follow up, 12% of alemtuzumab, and 4% of basiliximab patients lost their graft. Table 4.1 lists the ALBERT study patient demographics stratified according to induction agent.

Six patients who had received alemtuzumab, and 4 patients who had received basiliximab underwent a for cause biopsy and subsequent treatment prior to the 3-month timepoint. These patients were excluded from assessment of B-cell subsets as

prospective biomarkers of outcome (Figure 4.9).

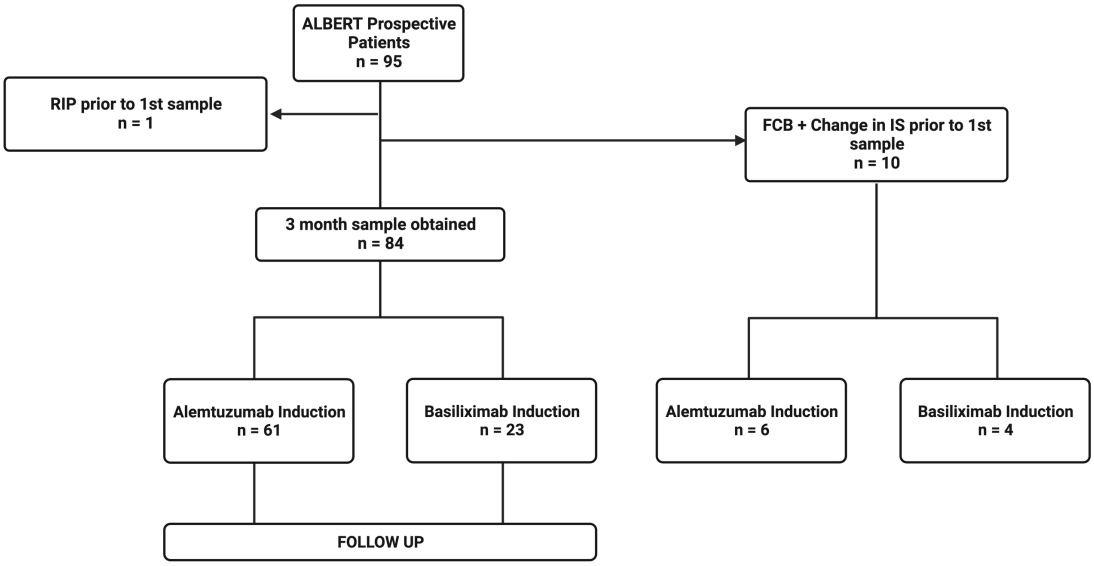


Figure 4.9. Flow chart demonstrating the ALBERT prospective study groups. FCB – for cause biopsy, IS – immunosuppression

4.2.1.1 ALEMTUZUMAB INDUCTION – 3 MONTHS POST TRANSPLANT

As demonstrated in the previous chapter (4.1), alemtuzumab induction is associated with a distinct change in B-cell population, with a skew towards an immature phenotype (with increased transitional B-cells, naïve cells, and increased T1 cells) that was most evident at 3 months post-transplant. Although the first sampling point was delayed to 3 months to allow for peripheral B-cell repopulation, this may not have occurred in all cases. For this reason, subsets were evaluated using values obtained as a percentage of the $CD19^{+}$ gate (%cells) as well as the calculated cell count ($x10^{9}$/L).

4.2.1.1.1 COMPOSITE ENDPOINT

Over the period of follow up, 25 of 61 alemtuzumab patients achieved the composite endpoint. The patient demographics can be viewed in Table 4.13, and a summary of endpoints in Table 4.14. When composite endpoints were reviewed, recurrent proteinuria alone was most frequently seen, affecting 7/25 patients. This was followed by *de novo* DSA alone, which was found in 6/25 patients. The 3-month phenotypes were compared between the 25 alemtuzumab composite endpoint positive (CEP) patients and the 33 composite endpoint negative (CEN) patients. The results of this comparison can be seen in Table 4.15 and Table 4.16.

Variable	TOTAL	Composite Endpoint Negative (CEN)	Composite Endpoint Positive (CEP)	p-value
Number	61	36	25	
Age ($\bar{x}$ years, SD)	48.3 (13.9)	49.7 (12)	47.1 (15.9)	0.470
Gender				0.116
M	33 (54%)	16 (44%)	17 (68%)	
F	28 (46%)	20 (56%)	8 (32%)	
Primary Renal Disease				0.569
DM/HTN	8 (13%)	3 (8%)	5 (20%)	
GN	19 (31%)	10 (28%)	9 (36%)	
Inherited	9 (15%)	6 (17%)	3 (12%)	
Infection/Obstruction	15 (25%)	10 (28%)	5 (20%)	
Other	10 (16%)	7 (19%)	3 (12%)	
Preemptive				0.238
Yes	17 (28%)	8 (22%)	9 (36%)	
No	44 (72%)	28 (78%)	16 (64%)	
Donor Type				0.227
DBD	31	16 (44%)	15 (60%)	
DCD	16	9 (25%)	7 (28%)	
LD	14	11 (31%)	3 (12%)	
HLA Mismatch Level				0.708
1	8	6 (17%)	2 (8%)	
2	21	13 (36%)	8 (32%)	
3	21	11 (31%)	10 (40%)	
4	11	6 (17%)	5 (20%)	

Variable	TOTAL	Composite Endpoint Negative (CEN)	Composite Endpoint Positive (CEP)	p-value
Graft number				0.615
1	46	26 (72%)	20 (80%)	
2	14	9 (25%)	5 (20%)	
3	1	1 (3%)	0	
Median CIT (hrs, IQR)	12.42 (6)	12 (10)	13.7 (7)	**0.05**
DGF				0.183
Yes	13 (21%)	7 (19%)	6 (24%)	
No	48 (79%)	29 (81%)	19 (76%)	
CNI variability 3-18 months ($\bar{x}$, SD)	24.4 (7.51)	24.4 (8.71)	24.4 (5.37)	0.997

Table 4.13. Clinical demographics stratified according to whether composite endpoints were met during the follow up period.

Number of patients	Proteinuria	De novo DSA	Disease recurrence	Biopsy Proven Rejection	>30% reduction in eGFR from 3-18 months	Graft Loss
7	✓	×	×	×	×	×
6	×	✓	×	×	×	×
2	✓	✓	×	×	×	×
2	✓	×	×	✓	✓	✓
1	✓	×	×	✓	×	✓
1	✓	×	✓	×	✓	✓
1	✓	×	✓	×	×	✓
1	✓	×	✓	×	×	×
1	✓	✓	×	✓	✓	✓
1	×	×	×	×	✓	×
1	✓	✓	×	✓	×	×
1	✓	×	×	×	✓	✓
25	18	10	3	5	6	7

Table 4.14. Table demonstrating the endpoints met during the follow up period.

Sixty-one patients who had received alemtuzumab induction had their bloods drawn at the 3-month time point. Three samples were excluded due to insufficient B-cell events. Therefore 58 samples were included in the 3-month prospective analyses. In one of these samples, there were no T1 events. A valid T1:T2 ratio could not be obtained for this sample, and this was excluded for any analyses that compared T1:T2 ratios. The number of valid samples included in each B cell subset analysis has been documented in Table 4.15 and Table 4.16.

Of note, CEP patients had fewer calculated B-cells (median $0.0215x10^9$/l CEP vs $0.0594x10^9$/l CEN, p=0.150) and reduced B-cells as a proportion of the lymphocyte gate at 3 months (median 19% CEP vs 10% CEN, p=0.05) compared with CEN patients, although this did not meet clinical significance. CEP patients were noted to have a higher %gated $CD19^+CD27^+CD38^-$ memory cells (2.28% CEP vs 0.82% CEN, p=0.0021), and $CD19^+CD27^+IgD^+$Non-Switched Memory cells at 3 months compared with CEN patients (2.38% CEP vs 1.08% CEN, p=0.03), however the overall switched:non-switched memory cell ratio was not statistically significant (see Table 4.15 and Table 4.16).

Calculated lymphocyte subset (x10^9/L)	CEN Median Value	IQR	CEP Median Value	IQR	p value
$CD19^+$ B-cells (CEN n=33, CEP n=25)	0.0594	0.0830	0.0215	0.0497	0.150
$CD19^+CD27^+CD38^-$ Memory Cells (CEN n=33, CEP n=25)	0.000524	0.000709	0.000539	0.00110	0.583
$CD19^+CD27^-IgD^+$ Naïve (CEN n=33, CEP n=25)	0.0572	0.0767	0.0209	0.0498	0.121
$CD19^+CD27^+IgD^+$ Non-Switched Memory (CEN n=33, CEP n=25)	0.000458	0.00126	0.000449	0.000673	0.821
$CD19^+CD27^+IgD^-$ Class switched Memory (CEN n=33, CEP n=25)	0.000338	0.000494	0.000375	0.000829	0.572
$CD19^+CD24^{hi}CD38^{hi}$ Transitional B-cells (CEN n=33, CEP n=25)	0.0278	0.0391	0.0165	0.0374	0.357
$CD19^+CD24^{+++}CD38^{+++}$ T1 (CEN n=33, CEP n=25)	0.00686	0.00942	0.00564	0.00918	0.550
$CD19^+CD24^{++}CD38^{++}$ T2 (CEN n=33, CEP n=25)	0.0187	0.0265	0.0102	0.0243	0.293

Table 4.15. Mann Whitney test comparing the 3-month B-cell phenotype (calculated) between CEN and CEP patients.

Median values and interquartile range (IQR) are included in the table. CEN – composite endpoint negative, CEP – composite endpoint positive

Lymphocyte Subset (%gated)	CEN Median Value	IQR	CEP Median Value	IQR	p value
$CD19^+$ B-cells (CEN n=33, CEP n=25)	18.9	22.8	10.7	13.81	0.050
$CD19^+CD27^+CD38^-$ Memory Cells (CEN n=33, CEP n=25)	0.820	1.30	2.28	5.83	**0.0021**
$CD19^+CD27^-IgD^+$ Naïve (CEN n=33, CEP n=25)	97.6	2.44	96.2	9.60	0.072
$CD19^+CD27^+IgD^+$ Non-Switched Memory (CEN n=33, CEP n=25)	1.08	1.37	2.38	3.09	**0.0304**
$CD19^+CD27^+IgD^-$ Class switched Memory (CEN n=33, CEP n=25)	0.52	0.78	1.16	4.39	0.081
$CD19^+CD24^{hi}CD38^{hi}$ Transitional B-cells (CEN n=33, CEP n=25)	54.8	37.67	62.13	30.42	0.296
$CD19^+CD24^{+++}CD38^{+++}$ T1 (CEN n=33, CEP n=25)	27.6	14.8	31.0	19.0	0.268
$CD19^+CD24^{++}CD38^{++}$ T2 (CEN n=33, CEP n=25)	72.4	14.97	68.5	20.28	0.245
T1:T2 Ratio (CEN n=32, CEP n=25)	0.380	0.303	0.450	0.460	0.296
S:NS Memory Ratio (CEN n=33, CEP n=25)	0.541	1.40	1.06	1.68	0.550

Table 4.16. Mann Whitney test comparing the 3-month B-cell phenotype (%gated) between CEN and CEP patients.

T1:T2 and S:NS ratios were calculated for each individual patient. The median values and interquartile range (IQR) for each group are included in the table.

CEN – composite endpoint negative, CEP – composite endpoint positive.

Receiver operating characteristic curves were constructed which defined the optimum cut-off value of $CD27^{+}CD38^{-}$ Memory cells = 1.185%. This classified patients at risk of meeting the composite endpoint with a sensitivity of 80% and specificity of 67%, AUC 0.733. Using Kaplan Meier estimates, patients with >1.185% memory cells had a reduced composite survival, compared with patients who had $\leq$1.185% memory cells, with a log-rank hazard ratio of 4.39, p=0.001 (Figure 4.10).

Similar assessments were performed focussing on % gated $CD19^{+}CD27^{+}IgD^{+}$ non-switched memory cells (Figure 4.11). The optimum cut-off defined by the AUC was 1.51% NS memory cells (AUC 0.6667, p=0.0309, sensitivity 64%, specificity 64%), however represented decreased sensitivity and specificity when compared with that obtained when assessing the %gated memory cells described above. Patients with >1.51% NS memory cells at 3 months were more likely to reach the composite endpoint compared with patients who had <= 1.51% NS (Hazard ratio 3.512, p=0.0015 log-rank).

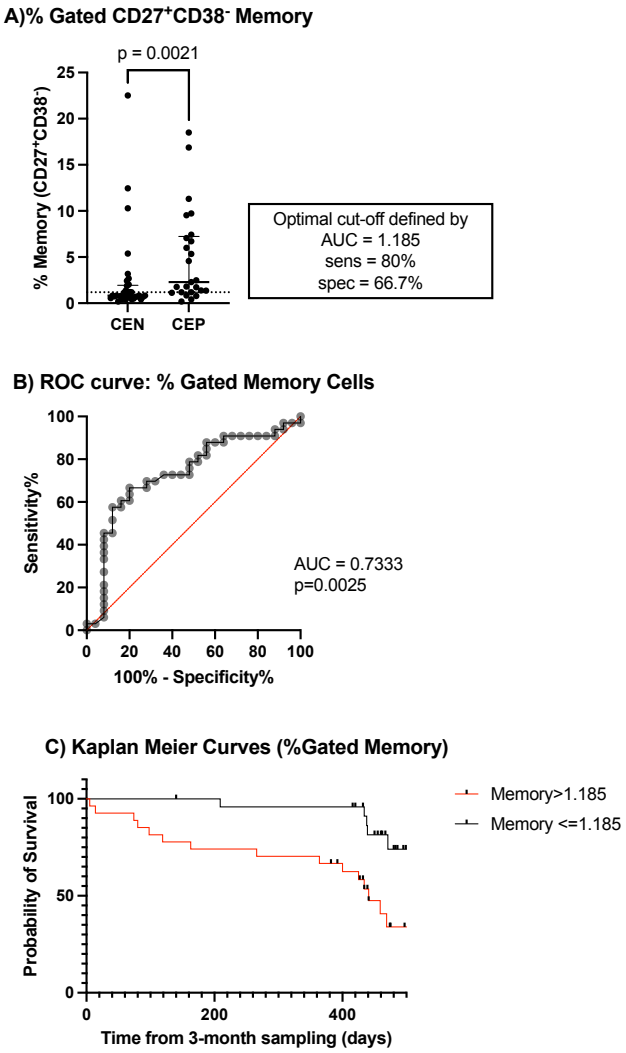


Figure 4.10. Assessment of 3-month CD27⁺CD38- Memory B-cells as a biomarker of outcome.

Patients were split into two groups according to whether they met or did not meet the composite endpoint. This included graft loss, recurrent proteinuria, >30% reduction in eGFR from 3-18 months, de novo DSA, biopsy proven rejection, recurrent disease). A) Dot plots comparing the %gated memory cells between the two groups. Individual values, median and interquartile ranges are shown. B) ROC constructed from the 3-month % gated memory cells C) Kaplan Meier curves comparing composite-endpoint-free survival in patients stratified according to %gated memory cells. CEN – composite endpoint negative, CEP – composite endpoint positive

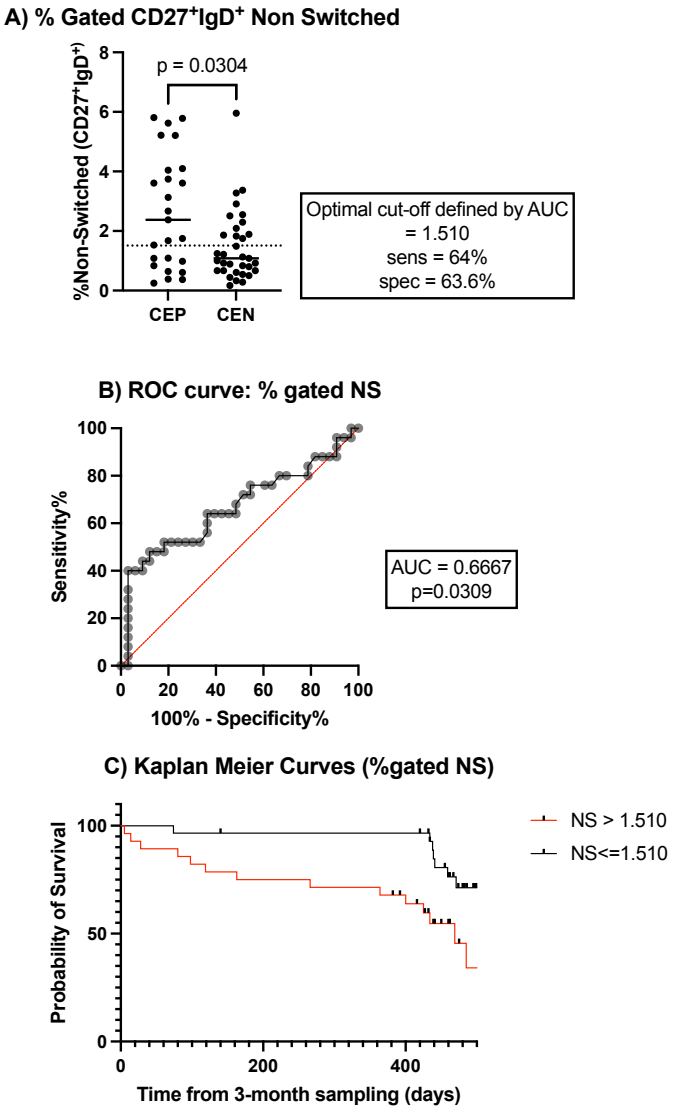


Figure 4.11. Assessment of 3-month CD27⁺IgD⁺ Non-Switched Memory B-cells as a biomarker of outcome.

Patients were split into two groups according to whether they met or did not meet the composite endpoint. This included graft loss, recurrent proteinuria, >30% reduction in eGFR from 3-18 months, de novo DSA, biopsy proven rejection, recurrent disease). A) Dot plots comparing the %gated non-switched memory cells between the two groups. Individual values, median and interquartile ranges are shown. B) ROC constructed from the 3-month % gated NS memory cells C) Kaplan Meier curves comparing composite-endpoint-free survival in patients stratified according to %gated NS memory cells. CEN – composite endpoint negative, CEP – compositive endpoint positive

4.2.1.1.2 GRAFT SURVIVAL

The 3-month B-cell phenotype was then assessed to see whether there was an association with subsequent allograft survival. Patients were divided into two groups – graft survival (GS) and graft loss (GL) and their 3-month B-cell phenotypes were compared. The clinical characteristics for both graft survival and graft loss groups have been listed in Table 4.17. During the follow up period, 7 patients subsequently lost their graft. All patients had received one or more biopsies prior to allograft loss. Two of the seven patients had evidence of disease recurrence on their biopsy (both recurrent IgA nephropathy). Four patients had evidence of rejection prior to graft loss (their biopsy results have been included in Table 4.20). The final patient had 2 biopsies. The first biopsy at 6 weeks post-transplant was reported as patchy acute tubular necrosis with moderate arteriosclerosis and severe arteriolar hyalinosis. The second biopsy (8 months post-transplant) was reported as interstitial fibrosis and tubular atrophy (IFTA) grade 1.

Variable	TOTAL	Graft Survival	Graft Loss	p-value
Number	61	54	7	
Age (years, SD)	48 (13.8)	49 (13.6)	42 (15.2)	0.177
Gender				0.693
M	33 (54%)	30 (56%)	3 (43%)	
F	28 (46%)	24 (44%)	4 (57%)	
Primary Renal Disease				0.283[1]
DM/HTN	8 (13%)	6 (11%)	2 (29%)	
GN	19 (31%)	16 (30%)	3 (43%)	
Hereditary	9 (15%)	8 (15%)	1 (14%)	
Other	15 (25%)	15 (28%)	0	
Unknown	10 (16%)	9 (17%)	1 (14%)	
Preemptive				0.074[1]
Yes	18 (29%)	18 (33%)	0	
No	43 (71%)	36 (67%)	7 (100%	
Donor Type				0.740
DBD	31 (51%)	27 (50%)	4 (57%)	
DCD	16 (26%)	15 (28%)	1 (14%)	
LD	14 (23%)	12 (22%)	2 (29%)	
HLA Mismatch Level				0.700
1	8 (13%)	8 (15%)	0	
2	21 (34%)	18 (33%)	3 (43%)	
3	21 (34%)	18 (33%)	3 (43%)	
4	11 18%)	10 (19%)	1 (14%)	
Graft number				0.398
1	46 (75%)	42 (78%)	4 (57%)	

Variable	TOTAL	Graft Survival	Graft Loss	p-value
2	14 (23%)	11 (20%)	3 (43%)	
3	1 (2%)	1 (2%)	0	
Median CIT (hrs, Q1-Q3)	12 (6)	12.4 (6)	13.7 (12)	0.497
DGF				0.637
Yes	13 (22%)	11 (21%)	2 (29%)	
No	47 (78%)	42 (79%)	7 (71%)	
Immunosuppression				
Calcineurin Inhibitor (Tacrolimus or Ciclosporin)	59 (97%)	52 (96%)	7 (100%)	0.605
Antiproliferative (MMF or Azathioprine)	10 (16%)	7 (13%)	3 (30%)	0.08
Steroids	16 (26%)	14 (26%)	2 (29%)	0.653[1]
CNI variability 3-18 months	24.4 (7.5)	24 (7.54)	27 (7.23)	0.329

Table 4.17. The clinical characteristics of Alemtuzumab prospective study patients stratified according to graft survival.
Maintenance immunosuppression refers to the immunosuppression regime at the time of the 3-month blood sample. Continuous data were analysed using the Mann-Whitney test. Categorical data were analysed using the chi-squared test, unless otherwise indicated. [1]Fishers exact test.

Three out of the 61 patient samples (Graft survival, GS n=3) were excluded from the subsequent analysis due to insufficient B-cell events. Therefore 58 samples (GS n=51, Graft Loss, GL n=7) were included in the 3-month prospective analyses. In one of these samples, there were no T1 events. A valid T1:T2 ratio could not be obtained for this sample, and this was excluded for any analyses that compared T1:T2 ratios (GS n=1). The number of valid samples included in each B cell subset analysis has been documented in Table 4.18 and Table 4.19.

Patients who lost their graft during the follow up period were noted to have a fewer %gated B-cells, a lower T1:T2 ratio, and a higher class-switched:non-switched memory ratio, however these differences were not statistically significant. The comparison of lymphocyte subsets (% gated) between graft survival (GS) and graft loss (GL) patients can be seen in Table 4.19.

Calculated lymphocyte subset (x10^9/L)	GS Median Value	IQR	GL Median Value	IQR	p value
$CD19^+$ B-cells (GS n=51, GL n=7)	0.0511	0.0680	0.0112	0.0188	**0.040**
$CD19^+CD27^+CD38^-$ Memory Cells (GS n=51, GL n=7)	0.000534	0.000742	0.000292	0.000930	0.467
$CD19^+CD27^-IgD^+$ Naïve (GS n=51, GL n=7)	0.0505	0.0680	0.0107	0.0182	**0.031**
$CD19^+CD27^+IgD^+$ Non-Switched Memory (GS n=51, GL n=7)	0.000537	0.00123	0.000290	0.000257	0.225
$CD19^+CD27^+IgD^-$ Class switched Memory (GS n=51, GL n=7)	0.000339	0.000339	0.000409	0.000868	0.972
$CD19^+CD24^{hi}CD38^{hi}$ Transitional B-cells (GS n=51, GL n=7)	0.0254	0.0403	0.00929	0.019	0.082
$CD19^+CD24^{+++}CD38^{+++}$ T1 (GS n=51, GL n=7)	0.00721	0.00886	0.00146	0.00429	**0.024**
$CD19^+CD24^{++}CD38^{++}$ T2 (GS n=51, GL n=7)	0.0163	0.0163	0.00449	0.0145	0.144

Table 4.18. Comparison of 3 month calculated lymphocyte subsets (x10^9/l) obtained from Graft Survival (GS) and Graft Loss (GL) patients.

Calculations were made using the Mann-Whitney test. IQR – interquartile range

Lymphocyte Subset (% Gated)	Median Value	GS IQR	Median Value	GL IQR	p value
$CD19^+$ B-cells (GS n=51, GL n=7)	17.16	20.57	6.81	8.73	0.065
$CD27^+CD38^-$ Memory (GS n=51, GL n=7)	1.15	3.57	1.76	5.52	0.194
$CD19^+CD27^-IgD^+$ Naïve (GS n=51, GL n=7)	97.36	6.33	96.19	6.18	0.347
$CD19^+CD27^+IgD^+$ Non-Switched Memory (GS n=51, GL n=7)	1.49	2.06	2.67	2.52	0.301
$CD19^+CD27^+IgD^-$ Class switched Memory (GS n=51, GL n=7)	0.65	1.72	1.16	3.52	0.335
$CD19^+CD24^{hi}CD38^{hi}$ Transitional B-cells (GS n=51, GL n=7)	56.3	32.42	79.43	35.48	0.279
$CD19^+CD24^{+++}CD38^{+++}$ T1 (GS n=51, GL n=7)	30.70	16.63	24.22	17.77	0.123
$CD19^+CD24^{++}CD38^{++}$ T2 (GS n=51, GL n=7)	69.19	17.48	75.6	17.75	0.103
T1:T2 Ratio (GS n=50, GL n=7)	0.440	0.375	0.320	0.300	0.102
S:NS Ratio (GS n=51, GL n=7)	0.565	1.46	1.048	2.54	0.559

Table 4.19. Comparison of 3 month %gated lymphocyte subsets obtained from Graft Survival (GS) and Graft Loss (GL) patients.

Calculations were made using the Mann-Whitney test. T1:T2 and S:NS ratios were calculated for each individual patient. The median values and interquartile range (IQR) for each group are included in the table.

There were however statistically significant differences when calculated values for each lymphocyte subset were considered. Patients who lost their graft during the follow up period were found to have fewer $CD19^+$ B (median $0.0112x10^9$/l GL vs $0.0511x10^9$/l GS, p= 0.04), $CD27^-IgD^+$ naïve (median $0.0505x10^9$/l GL vs $0.0107x10^9$/l GS, p=0.031), and $CD24^{+++}CD38^{+++}$ T1 cells (median $0.00146x10^9$/l GL vs $0.00721x10^9$/l GS, p=0.024) at 3 months than those whose grafts remained functioning, (Table 4.18).

Receiver operating characteristic curves were constructed to determine the cell counts that would separate the two groups with optimum sensitivity and specificity (Figure 4.12). Calculated $CD27^-IgD^+$ Naïve cells $<0.0135x10^9$/L was the best marker of subsequent graft loss (AUC = 0.7514, sensitivity 71%, specificity 78%, p=0.0324). Calculated $CD19^+$ B-cells $<0.01553x10^9$/L was also a good classifier with (AUC = 0.7405, sensitivity 71%, specificity 78%, p=0.0410.

Kaplan Meier curves were then constructed using the cut-off values determined by the ROC analyses (Figure 4.13). Patients with a low calculated $CD19^+$ count ($<0.01553x10^9$/L) were associated with reduced allograft survival (Hazard ratio 7.406, 95% CI of ratio 1.346 – 40.76, p=0.0048), with 29% of patients losing their graft over the subsequent 5-year follow up period. Alternatively, only 4.8% of patients with a high 3-month $CD19^+$ count lost their graft during the follow up period. Similarly, a low $CD27^-IgD^+$ Naïve count was associated with reduced graft allograft survival (Hazard ratio 8.594, 95% CI of ratio 1.766 – 41.82, p=0.0015). Kaplan meier curves demonstrated that patients with a reduced T1 cell count at 3 months also had an increased risk of subsequent allograft failure, but this did not reach statistical significance (Hazard ratio 3.004, 95%CI of ratio 0.6059-14.89, p=0.1016).

Having demonstrated that graft loss was associated with a reduced $CD19^+$ count, the 7 graft loss patients were investigated separately to see whether there was a 3- month phenotype that could differentiate graft loss due to disease recurrence or allograft rejection. The calculated B-cells were similar between the 2 groups (median calculated $CD19^+$ $0.0123x10^9$/L Rejection vs $0.0112x10^9$/L Disease recurrence p=0.857), and

although the differences were not statistically significant, there was a trend towards increased TrBs (median %gated TrB 82 rejection, 50 disease recurrence, p=0.629), with an overall reduced T1:T2 ratio (median T1:T2 ratio 0.182 rejection, 0.325 disease recurrence, p=0.4) in those who had evidence of rejection on biopsy.

As previous studies have highlighted the T1:T2 ratio as a potential biomarker of reduced graft survival, and as demonstrated in the previous chapter, the T1:T2 ratio decreases with time post transplant.

Finally, for the purposes of tracking potentially clinically significant values of the T1:T2 ratio with time, a ROC curve analysis was performed. This is because previous studies have highlighted the T1:T2 ratio as a potential biomarker of reduced graft survival,[141] and the T1:T2 ratio decreases with time post-transplant as described in Section 4.1). Although the two groups were not significantly different, a cut off value of 0.3250 classified the two groups with a sensitivity of 71%, specificity 65%, AUC = 0.6891, p=0.1071 (Figure 4.14).

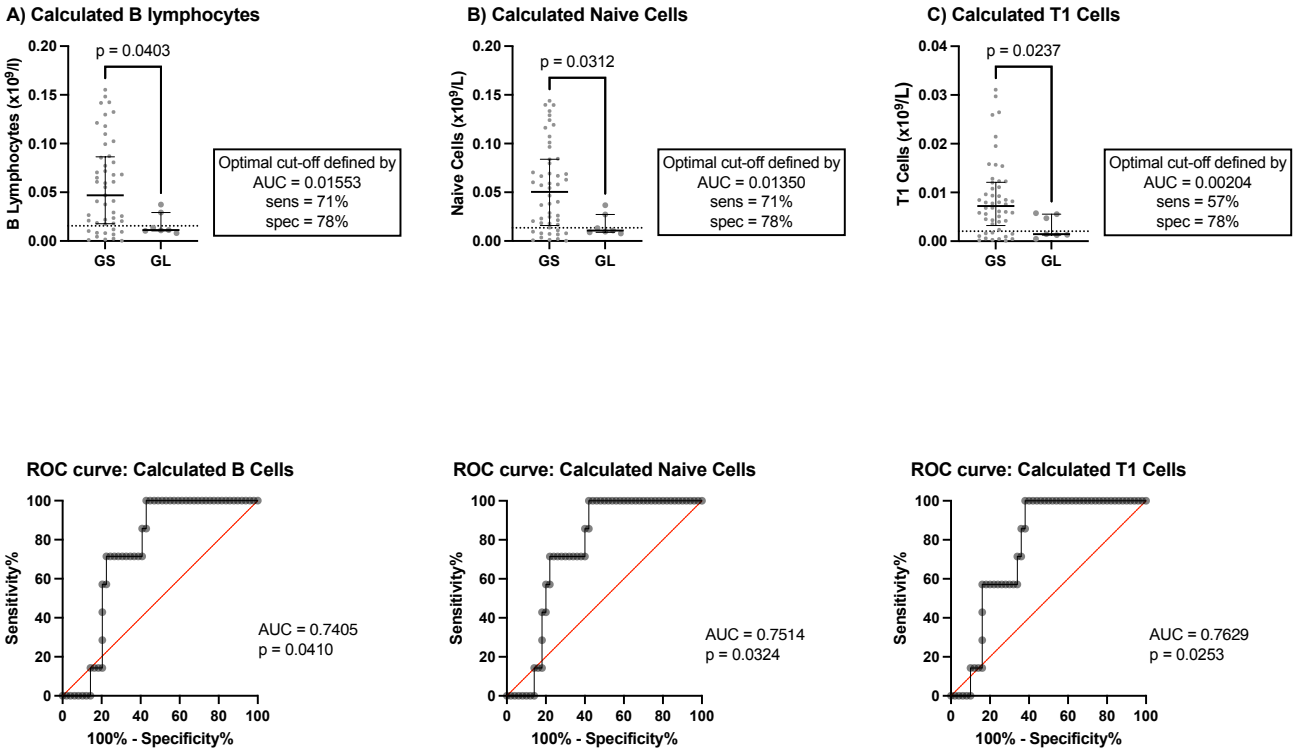
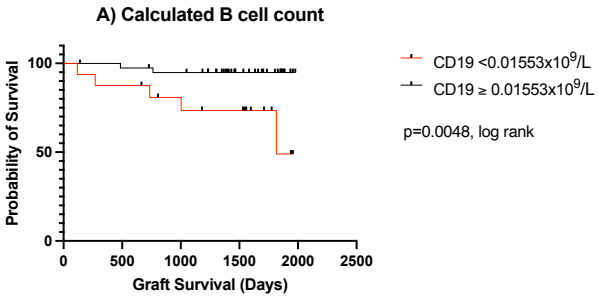
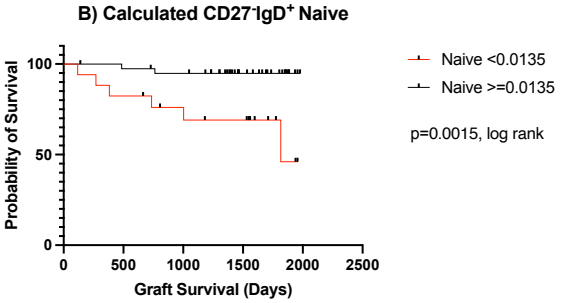


Figure 4.12. 3-month B-cell surface phenotype in Alemtuzumab patients.

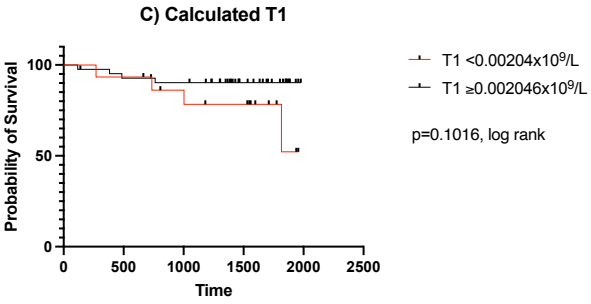
Patients were split into two groups according to graft survival (GS) or graft loss (GL) during the follow up period. The top panel contains comparisons between the two groups using the Mann-Whitney test. Individual values, median and interquartile ranges are shown. The bottom panel contains receiver operating characteristic curves constructed from the 3-month A) calculated B lymphocytes, B) Calculated $CD27^{-}IgD^{+}$ Naïve C) Calculated $CD24^{+++}CD38^{+++}$ T1 cells.



No. at risk	3-month blood draw (T=0)	300 days	1000 days	1500 days
CD19≥0.01553x 10^9/L	42	41	37	21
CD19<0.01553x 10^9/L	16	15	12	10



No. at risk	3-month blood draw (T=0)	300 days	1000 days	1500 days
Naïve≥0.0135	41	40	37	21
Naïve<0.0135	17	16	12	10



No. at risk	3-month blood draw (T=0)	300 days	1000 days	1500 days
T1≥0.00204	43	42	38	21
T1<0.00204	15	15	12	10

Figure 4.13. Kaplan Meier survival estimates of graft loss in the 5 years following the 3-month blood sampling in Alemtuzumab patients.

Patients were split into two groups based on optimal thresholds obtained from ROC curve analyses. A) calculated B lymphocytes, B) Calculated $CD27^-IgD^+$ Naïve C) Calculated $CD24^{+++}CD38^{+++}$ T1 cells

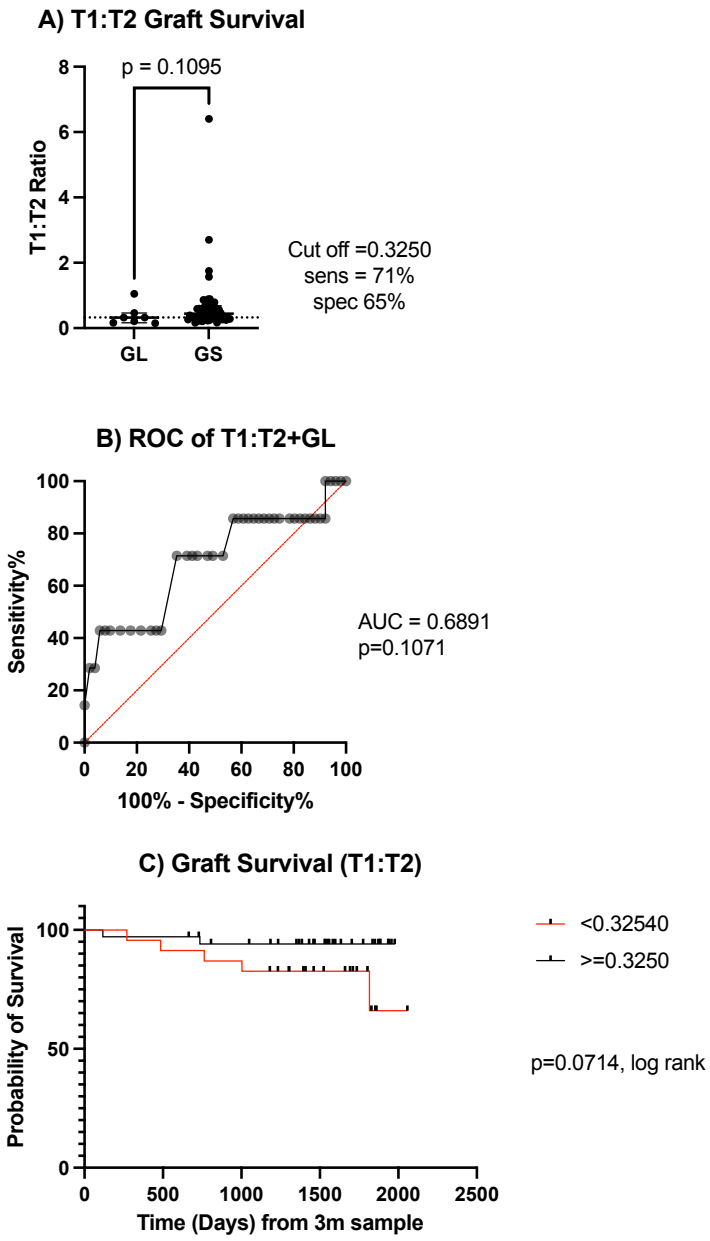


Figure 4.14. Assessment of 3-month T1:T2 ratio as a biomarker of graft loss. Patients were split in 2 groups (GS and GL). A) Mann Whitney test comparing the two groups with individual values, median and IQR shown, B) ROC curve analysis, C) Kaplan Meier survival curves using optimal thresholds defined by ROC curve analysis.

4.2.1.1.3 REJECTION

During this follow up period, 5 out of 61 patients were found to have an episode of biopsy proven rejection (2x mixed TCMR and ABMR, 2x ABMR 1x borderline), and 4/5 of rejection patients subsequently lost their graft. The biopsy results for these patients can be viewed in Table 4.20.

Three out of the 61 patient blood samples were excluded from the subsequent analysis due to insufficient B-cell events. Therefore 58 samples were included in the 3-month prospective analyses. In one of these samples, there were no T1 events. A valid T1:T2 ratio could not be obtained for this sample, and this was excluded for any analyses that compared T1:T2 ratios (NR n=1). The number of valid samples included in each B cell subset analysis has been documented in Table 4.21 and Table 4.22. The calculated B-cell phenotype was first assessed between the 'Rejection' (R) and 'No rejection' (NR) groups; no statistically significant differences in the calculated lymphocyte subsets (Table 4.21). The %gated values were then assessed: There were no statistically significant differences in the proportion of $CD19^{+}$ B-cells or memory cells between the two groups (Table 4.22), however, patients who subsequently went on to develop rejection episodes had a higher proportion of transitional B-cells (median 84.4% Rejection vs 54.8% No Rejection p=0.044). Interestingly despite having a higher proportion of TrBs, rejection patients had a reduced T1 cells (median 17.1% Rejection vs 30.7% No-Rejection, p=0.0256), and increased T2 cells (median 83% Rejection vs 69.3% No-Rejection, p=0.0255), resulting in a lower overall T1:T2 ratio when compared with non-rejection patients (Median Ratio 0.210 Rejection vs 0.440 No-Rejection, p=0.0259).

ID	Time post Tx	G	cg	mm	t	ct	i	ti	ci	v	cv	aah	ptc	c4d	Polyoma	Banff Class	Diagnosis
60	61				1.0	1.0	1.0	2.0	1.0	0.0	1.0	1.0	1.0			3,5,6	Borderline changes - suspicious for acute T-cell-mediated rejection, IFTA Gr 1, Suboptimal/inadequate biopsy
67	6	0.0	0.0	0.0	0.0	1.0	2.0	3.0	2.0	0.0	0.0	0.0	0.0	0.0	3.0	5,6	IFTA gr1-2, BK virus nephropathy
67	17	1.0	0.0	1.0	1.0	1.5	1.0	3.0	1.0	0.0	1.0	1.0	0.0	0.0	0.0	3,5	Borderline changes suspicious for acute TCMR, IFTA gr 1
67	24	1.0	0.0	1.0	2.0	1.0	1.5	2.0	1.0	0.0	1.0	0.0	1.5	2.0	0	2,4,5	Acute antibody mediated rejection, acute TCMR grade 1A, IFTA grade 1
67	30	1.0	1.0	1.0	3.0	2.5	3.0	3.0	?	1.0	2.0	0.0	1.0	3.0	0.0	2,4,5	Acute AMR, Acute TCMR gr 2A, IFTA grade 3
77	0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	3.0	0.0	0.0	0.0	6	Patchy acute tubular injury
77	3																Insuffiecient ?RAS
77	7	1.0	1.0	2.5	1.0	1.0	1.0	1.0	1.0	2.0	2.0	0.0	1.0	0.0	0.0	2,4,5	acute antibody mediated rejection, acute TCMR grade IIB, IFTA gr 1
77	7	1.5	0.0	1.0	1.0	1.0	0.0	0.0	0.0	0.0	1.5	1.0	1.0	0.0	0	3,6,4	Borderline changes- suspicious for acute T-cell mediated rejection, Acute tubular injury, morphological features suspicious for TMA ? ABMR
98	0	1.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	3.0	0.0	0.0	0.0	2,6	suspicious of antibody mediated changes ? Acute antibody mediated rejection, arterolar hyalinosis
98	3	0.0	1.0	2.0	0.0	1.5	0.0	1.0	2.0	0.0	0.0	2.0	0.0	0.0	0	5	IFTA grade II
98	3	0.0	0.0	1.0	0.0	1.0	0.0	0.0	1.0	0.0	1.0	3.0	0.0	0.0	0.0	5,6	IFTA grade 1, vascular hyalinosis
98	11	1.0	0.0	1.0	1.0	1.0	1.0	1.5	1.0	0.0	1.0	1.0	1.0	1.0	0.0	3,5,	borderline changes suspicious for acute TCMR, IFTA grade 1. possible ABMR
108	4	1.0	1.0	1.0	0.0	1.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0	0.0	2,5	Antibody mediated changes - chronic active antibody mediated rejection, IFTA gr 1
108	5	2.5	1.0	1.0	0.0	1.0	0.0	1.0	1.0	0.0	2.0	1.0	0.0	0.0	0	2,5	antibody mediated changes - chronic active antibody mediated rejection, IFTA gr 1

Table 4.20. Histology Results for patients with evidence of rejection during follow up.

Time post-transplant has been reported in months. If the histopathologist reported a result that was in-between 2 Banff scores, for example Glomerulitis: between G1 - G2, the result was coded as G = 1.5. AMR, ABMR – antibody mediated rejection, RAS – renal artery stenosis, TCMR – T-cell mediated rejection

Calculated lymphocyte subset (x10^9/L)	Median Value No Rejection	NR IQR	Median Value Rejection	R IQR	p value
$CD19^+$ B-cells (NR n=53, R=5)	0.0408	0.0736	0.0136	0.0379	0.142
$CD19^+CD27^+CD38^-$ Memory Cells (NR n=53, R=5)	0.000551	0.000811	0.000194	0.00344	0.068
$CD19^+CD27^-IgD^+$ Naïve (NR n=53, R=5)	0.0392	0.0712	0.0133	0.0372	0.274
$CD19^+CD27^+IgD^+$ Non-Switched Memory (NR n=53, R=5)	0.000508	0.000878	0.000185	0.000783	0.341
$CD19^+CD27^+IgD^-$ Class switched Memory (NR n=53, R=5)	0.000368	0.000570	0.000101	0.000370	0.300
$CD19^+CD24^{hi}CD38^{hi}$ Transitional B-cells (NR n=53, R=5)	0.0238	0.0400	0.0108	0.0378	0.691
$CD19^+CD24^{+++}CD38^{+++}$ T1 (NR n=53, R=5)	0.00646	0.00990	0.00472	0.00660	0.287
$CD19^+CD24^{++}CD38^{++}$ T2 (NR n=53, R=5)	0.0152	0.0259	0.00933	0.0328	0.838

Table 4.21. Mann Whitney comparison of calculated lymphocyte subsets in rejection (R) and no rejection (NR) groups.

Median values and interquartile range (IQR) for each group are included in the table.

Lymphocyte Subset (% Gated)	Median Value No Rejection	No Rejection IQR	Median Value Rejection	Rejection IQR	p value
$CD19^+$ B-cells (NR n=53, R=5)	14.74	20.47	6.81	7.99	0.142
$CD19^+CD27^+CD38^-$ Memory (NR n=53, R=5)	1.19	4.66	1.19	3.73	0.747
$CD19^+CD27^-IgD^+$ Naïve (NR n=53, R=5)	97.1	7.05	97.5	3.41	0.851
$CD19^+CD27^+IgD^+$ Non-Switched Memory (NR n=53, R=5)	1.53	2.46	1.68	2.37	0.936
$CD19^+CD27^+IgD^-$ Class switched Memory (NR n=53, R=5)	0.66	2.83	0.74	2.29	0.914
Switched:NS Mem Ratio (NR n=53, R=5)	0.619	1.50	0.679	1.95	0.816
$CD19^+CD24^{hi}CD38^{hi}$ Transitional B-cells (NR n=53, R=5)	54.8	30.29	84.4	32.98	**0.0436**
$CD19^+CD24^{+++}CD38^{+++}$ T1 (NR n=53, R=5)	30.7	15.70	17.1	20.61	**0.0256**
$CD19^+CD24^{++}CD38^{++}$ T2 (NR n=53, R=5)	69.2	16.82	83.0	20.93	**0.0255**
T1:T2 Ratio (NR n=52, R=5)	0.440	0.366	0.210	0.47	**0.0259**

Table 4.22. Mann Whitney-U comparison of 3-month B-cell phenotype (%gated) in rejection (R) and no rejection (NR) patient groups.

T1:T2 and S:NS ratios were calculated for each individual patient. The median values and interquartile range (IQR) for each group are included in the table.

Receiver operating characteristic curves (ROCs) were constructed using the %TrB, %T1, %T2 and %T1:T2 ratios to determine the optimum cut off values that would separate the R and NR groups. As demonstrated in Figure 4.15, the best predictor of subsequent allograft rejection was the %T1 with an area under the curve (AUC) of 0.8 on ROC analysis. A T1 cut-off of <17.66% had a sensitivity of 80% and a specificity of 96%. %T2 (AUC 0.7981) and T1:T2 ratios (AUC 0.7981) were also very good predictors of subsequent allograft rejection. The T2 cut off >82.04% was associated with a sensitivity of 80% and specificity of 96%, and the T1:T2 ratio cut off <0.215 had a similar sensitivity of 80% and specificity of 96%. These cut-off values were used to construct Kaplan Meier rejection free survival estimates which can be viewed in Figure 4.16. Over the subsequent 5 years, 67% of patients with a low %T1 (<17.66), high %T2 (>82.05) and low T1:T2 ratio (<0.215) developed biopsy proven allograft rejection, whereas only 2% of patients with high %T1, low %T2 and high T1:T2 ratios had allograft rejection. As the T1:T2 ratio includes the %T1 and %T2 values, the transitional cell T1:T2 ratio was used for further statistical analyses. Table 4.24 compares the clinical characteristics between patients who had a low T1:T2 ratio and a high T1:T2 ratio at 3 months post-transplant. There were no statistically significant differences between the two groups, however patients with a low T1:T2 ratio tended be younger in age (median age 39, IQR 17) than those with a high ratio (median age 52, IQR 19, p=0.054). Patients with a low T1:T2 ratio also had a higher variability in calcineurin inhibitor levels between 3-18 months post-transplant, but this was not statistically significant.

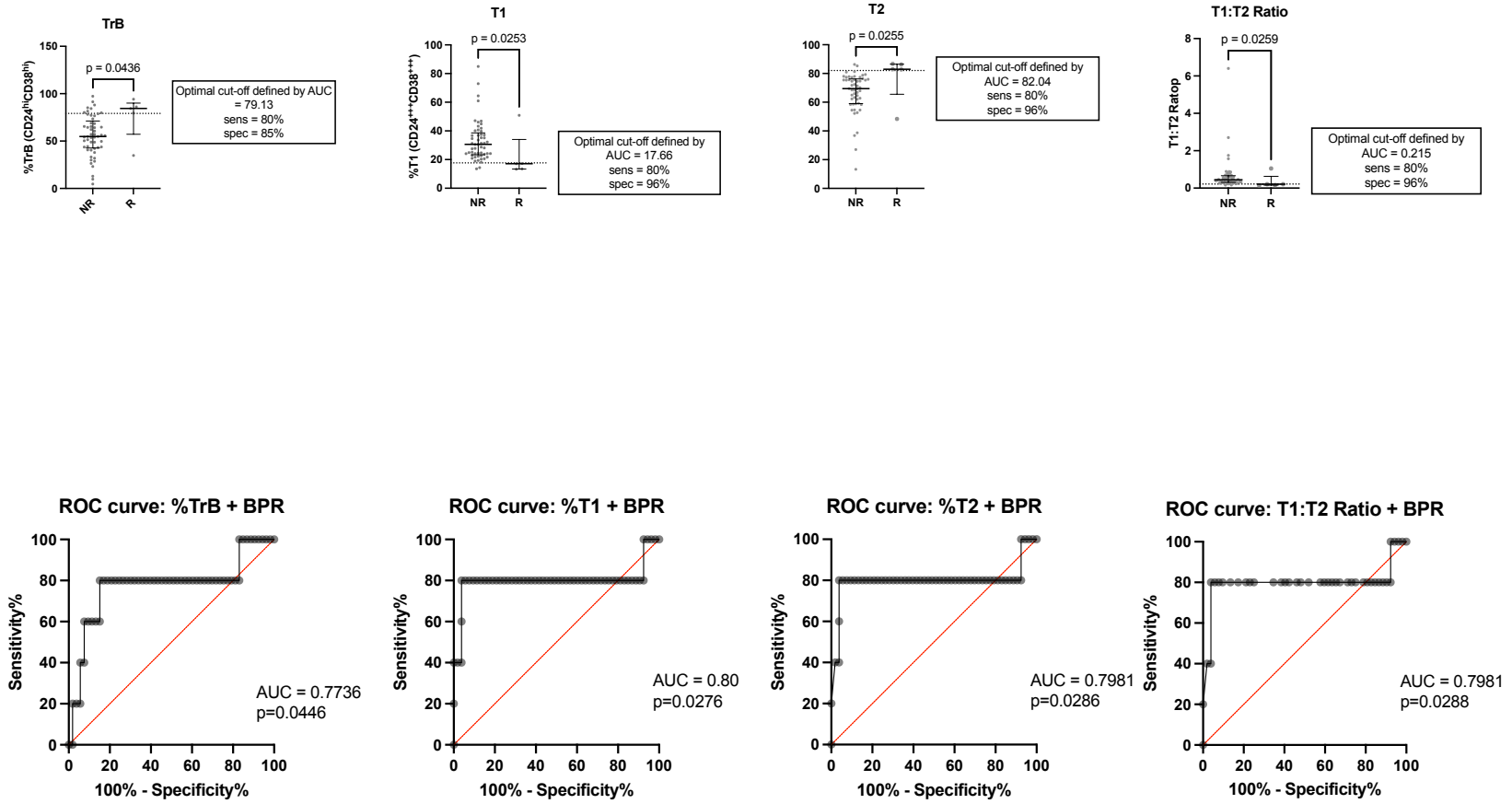
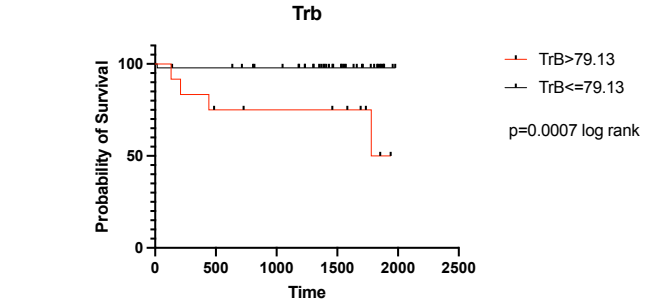
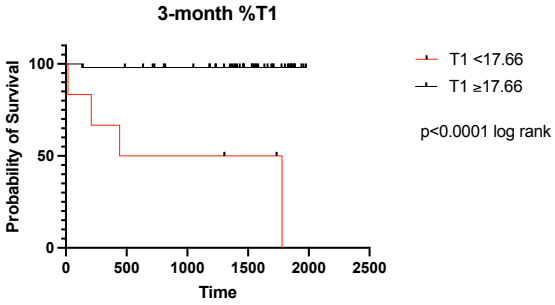


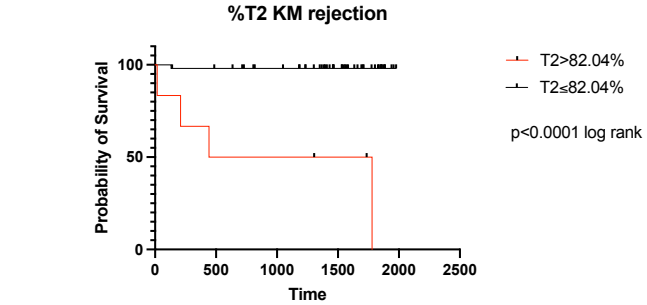
Figure 4.15. Receiver operating characteristic curve analysis for transitional B-cells (TrBs), T1, T2 cells and T1:T2 ratio as markers for subsequent allograft rejection.



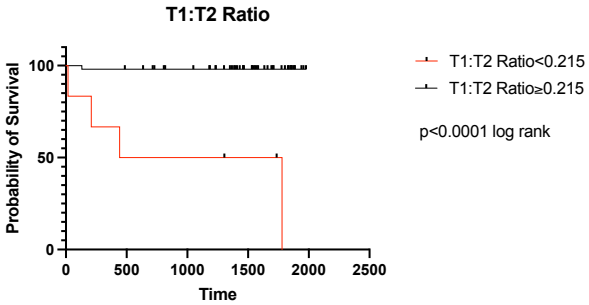
No. at risk	3-month blood draw (T=0)	300 days	1000 days	1500 days
TrB≤79.13	46	45	41	24
TrB>79.13	12	5	8	7



No. at risk	3-month blood draw (T=0)	300 days	1000 days	1500 days
T1≥17.66	52	51	45	28
T1<17.66	6	5	4	3



No. at risk	3-month blood draw (T=0)	300 days	1000 days	1500 days
T2 >82.04	52	52	45	28
T2 ≤82.04	6	5	4	3



No. at risk	3-month blood draw (T=0)	300 days	1000 days	1500 days
T1:T2 >= 0.215	51	51	45	28
T1:T2 <0.215	6	5	4	3

Figure 4.16. Kaplan Meier curves estimating 5-year rejection free survival over 5 years from blood sampling (3 months post-transplant).

To see whether the transitional cell T1:T2 ratio, was associated with subsequent rejection independent of other variables affecting rejection free survival (high calcineurin inhibitor variability, NHS-BT mismatch levels, regrafts), a multivariate cox proportional hazards analysis was performed. This showed that a low T1:T2 ratio was independently associated with subsequent allograft rejection (adjusted hazards ratio 62; 95% CI 3.31-1161, p=0.006), Table 4.23.

	Hazard Ratio (95% CI)	**P value**
T1:T2 ratio <0.215	62.0 (3.31-1161)	**0.006**
Regraft vs 1st graft	1.89 (0.093-38.4)	0.679
FK variability 3-18 months	1.214 (0.888-1.66)	0.225
NHSBT mismatch (levels 3 or 4 vs levels 1 or 2)	1.32 (0.162-10.663)	0.797

Table 4.23. Rejection - Multivariate analysis

Variable	Study Population	T1:T2 Ratio <0.215	T1:T2 Ratio ≥0.215	p-value
Number	57	6	51	
Recipient Age (yr, med + IQR)	49 (22)	39 (17)	52 (19)	0.054
Recipient Gender				0.102
Male	29 (51%)	5 (83%)	28 (55%)	
Female	28 (49%)	1 (17%)	23 (45%)	
Recipient Ethnicity				0.852
Caucasian	51 (90%)	6 (100%)	45 (88%)	
Asian	4 (7%)	0	4 (100%)	
Afro-Caribbean	1 (2%)	0	1(100%)	
Other	1 (2%)	0	1 (100%)	
Cause of ESRD				0.939
Diabetes and Hypertension	7 (12%)	1 (17%)	6 (12%)	
Glomerulonephritis	18 (32%)	1 (17%)	15 (33%)	
Inherited	9 (16%)	1 (17%)	8 (16%)	
Other	14 (25%)	2 (33%)	12 (24%)	
Unknown	9 (16%)	1 (17%)	8 (16%)	
Pre-emptive Transplant				0.570
Yes	15 (26%)	1 (17%)	14 (28%)	
No	42 (74%)	5 (83%)	37 (73%)	
Donor Type				0.937
LD	12 (23%)	1 (17%)	11 (22%)	
DBD	29 (50%)	3 (50%)	26 (51%)	
DCD	16 (27%)	2 (33%)	14 (28%)	
Graft Number				0.900
1	44 (77%)	5 (83%)	39 (77%)	
2	12 (21%)	1 (17%)	11 (22%)	
3	1 (2%)	0	1 (2%)	
NHSBT HLA Mismatch Level				0.400
1	8 (14%)	0	8 (14%)	

Variable	Study Population	T1:T2 Ratio <0.215	T1:T2 Ratio ≥0.215	p-value
2	18 (32%)	3 (50%)	15 (29%)	
3	22 (39%)	3 (50%)	19 (37%)	
4	9 (16%)	0	9 (18%)	
Delayed Graft Function?				0.683
Yes	14 (25%)	2 (33%)	12 (24%)	
No	42 (75%)	4 (67%)	38 (76%)	
Maintenance Immunosuppression*				0.105
Tacrolimus	56 (98%)	5 (83%%)	51 (100%)	
Sirolimus	1 (2%)	1 (17%)	0	
Antiproliferative (Azathioprine/MMF)?				0.237
Yes	9 (16%)	2 (33%)	7 (14%)	
No	48 (84%)	4 (67%)	44 (86%)	
Prednisolone?				0.125
Yes	13 (23%)	3 (50%)	10 (20%)	
No	44 (77%)	3 (50%)	41 (80%)	
3-month eGFR (med, IQR)	42 (22)	30 (31)	42 (19)	0.254
3-month UPCR (med, IQR)	15 (24)	31 (57)	14 (20)	0.138
CNI variability 3-18 months	24.0 (7.22)	29 (8.9)	23.3 (6.4)	0.073

Table 4.24. The comparison of clinical characteristics for patients with a high T1:T2 ratio (>=0.215) and a low T1:T2 ratio (<0.125) at 3 months post-transplant. Although 61 patients were initially included in the analysis, only 57 valid T1:T2 ratios could be obtained. Categorical data were analysed using Chi-square tests. Continuous data were analysed using the Mann Whitney test. Med – median, IQR – interquartile range, MMF – mycophenolate mofetil

4.2.1.1.4 DISEASE RECURRENCE

Three patients developed disease recurrence during the follow up period (1 x membranous, 2 x IgA). Patients were divided into two groups depending on the recurrence of disease on biopsy. There were no differences in the B-cell phenotype (%gated and calculated) when the two groups were compared (Mann Whitney Test).

4.2.1.1.5 RECURRENT PROTEINURIA

Three out of the 61 patient blood samples were excluded from the subsequent analysis due to insufficient B-cell events (No Proteinuria n=3). Fifty-eight samples were included in the 3-month analyses for the development of recurrent proteinuria. In one of these samples, there were no T1 events. A valid T1:T2 ratio could not be obtained for this sample, and this was excluded for any analyses that compared T1:T2 ratios (Recurrent Proteinuira, RP n=1). The number of valid samples included in each B cell subset analysis has been documented in Table 4.25 and Table 4.26.

Eighteen patients developed recurrent proteinuria during the follow up period. The calculated B-cell subsets (Table 4.25) and % gated B-cell subsets (Table 4.26) were compared using the Mann Whitney test (Proteinuria = 18, no proteinuria = 40). There were no statistically significant differences in the calculated B-cells and calculated B-cell subsets obtained from the two groups. However, patients who developed recurrent proteinuria had a higher 3-month frequency of $CD19^{+}CD27^{+}CD38^{-}$ memory cells compared with patients who did not develop recurrent proteinuria (Memory cells 3.14% recurrent proteinuria vs 0.85% no proteinuria, p=0.022). Similarly, patients who developed recurrent proteinuria had a higher 3-month frequency of $CD19^{+}CD27^{+}IgD^{+}$ non-switched memory cells (non-switched memory cells 2.9% recurrent proteinuria vs 1.08% no proteinuria, p=0.041). A higher frequency of $CD19^{+}CD27^{+}IgD^{-}$ switched memory cells were also noted in the proteinuria group, with an increased switched:non-switched ratio, however these differences did not reach statistical significance.

ROC curve analysis classified the development of recurrent proteinuria using a cut off value of %gated Memory cells > 1.185%, with a sensitivity of 79%, specificity of 61% and AUC 0.07051, p=0.0239 (Figure 4.17b). Kaplan Meier curves were constructed using this cut-off value (Figure 4.17c) and patients with high 3-month frequencies of memory cells >1.185% were more likely to develop proteinuria with a hazard ratio of 4.544, CI 1.584-13.03, p=0.0105.

Calculated lymphocyte subset (x10^9/L)	Median Value NP	IQR	Median Value RP	IQR	p value
$CD19^+$ B-cells (NP n=40, RP n=18)	0.0594	0.0820	0.0251	0.0311	0.137
$CD19^+CD27^+CD38^-$ Memory Cells (NP n=40, RP n=18)	0.000545	0.000723	0.000404	0.00108	0.894
$CD19^+CD27^-IgD^+$ Naïve (NP n=40, RP n=18)	0.0572	0.0829	0.0238	0.0298	0.105
$CD19^+CD27^+IgD^+$ Non-Switched Memory (NP n=40, RP n=18)	0.000552	0.00120	0.00348	0.000601	0.641
$CD19^+CD27^+IgD^-$ Class switched Memory (NP n=40, RP n=18)	0.000340	0.000532	0.000367	0.000721	0.929
$CD19^+CD24^{hi}CD38^{hi}$ Transitional B-cells (NP n=40, RP n=18)	0.03302	0.0419	0.0188	0.214	0.183
$CD19^+CD24^{+++}CD38^{+++}$ T1 (NP n=40, RP n=18)	0.00775	0.00997	0.00564	0.00650	0.239
$CD19^+CD24^{++}CD38^{++}$ T2 (NP n=40, RP n=18)	0.0187	0.0309	0.0116	0.014	0.214

Table 4.25. Mann-Whitney test comparing calculated 3-month B-cell subsets in patients with no recurrent proteinuria (NP) and recurrent proteinuria (RP) in the subsequent follow up period.

Recurrent proteinuria was defined as urine protein creatinine ratio (UPCR) >50 on two or more occasions. Median values and interquartile range (IQR) for each group are shown.

Lymphocyte Subset (%gated)	Median Value UPCR<50	IQR	Median Value Recurrent UPCR>50	IQR	p value
$CD19^+$ B-cells (NP n=40, RP n=18)	19.0	21.76	10.45	9.42	0.059
$CD19^+CD27^+CD38^-$ Memory (NP n=40, RP n=18)	0.85	1.33	3.14	6.14	**0.022**
$CD19^+CD27^-IgD^+$ Naïve (NP n=40, RP n=18)	97.5	2.86	94.75	10.98	0.089
$CD19^+CD27^+IgD^+$ Non-Switched Memory (NP n=40, RP n=18)	1.08	1.45	2.90	1.45	**0.041**
$CD19^+CD27^+IgD^-$ Class switched Memory (NP n=40, RP n=18)	0.620	1.15	1.32	5.22	0.085
$CD19^+CD24^{hi}CD38^{hi}$ Transitional B-cells (NP n=40, RP n=18)	56.3	36.25	55.85	31.15	0.743
$CD19^+CD24^{+++}CD38^{+++}$ T1 (NP n=40, RP n=18)	28.1	15.10	30.0	16.6	0.841
$CD19^+CD24^{++}CD38^{++}$ T2 (NP n=40, RP n=18)	71.4	15.33	69.30	18.1	0.800
T1:T2 Ratio (NP n=40, RP n=17)	0.390	0.31	0.435	0.400	0.816
Switched:NS Ratio (NP n=40, RP n=18)	0.541	1.71	1.16	1.57	0.564
Plasmablasts (NP n=40, RP n=18)	0.700	0.94	0.750	0.59	0.743

Table 4.26. Association between % gated 3-month B-cell subsets and recurrent proteinuria in the subsequent follow up period.

Comparisons were made using the Mann-Whitney Test. UPCR – urine protein creatinine ratio. T1:T2 and S:NS ratios were calculated for each individual patient. The median values and interquartile range (IQR) for each group are included in the table.

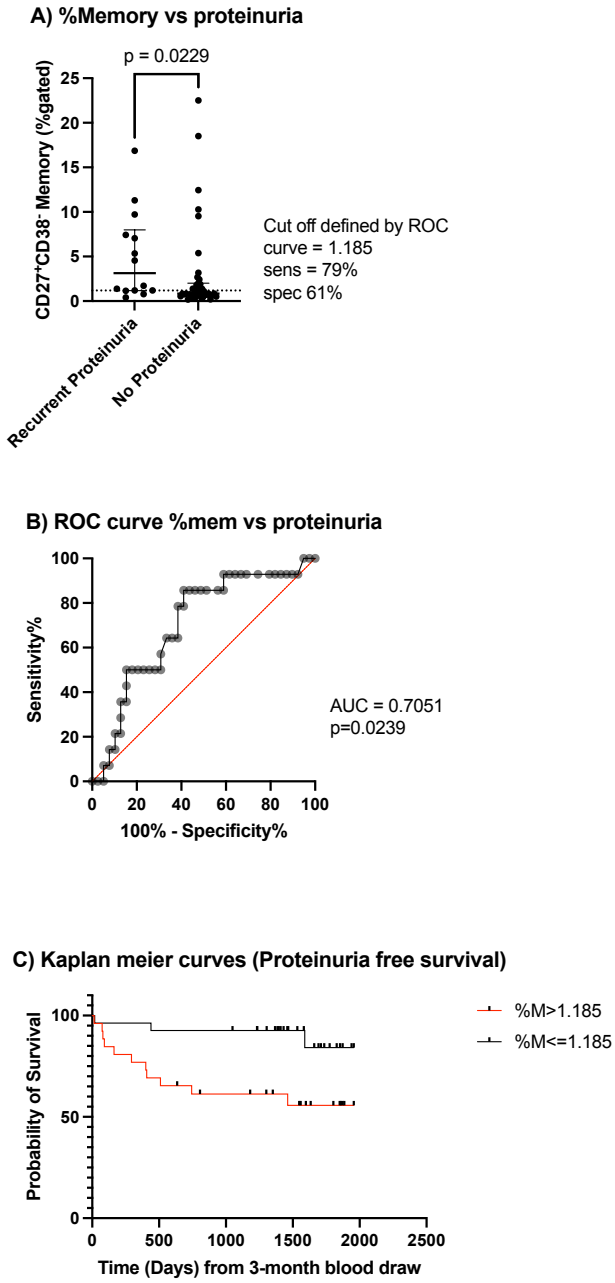


Figure 4.17. Assessment of 3-month $CD19^{+}CD27^{+}CD38^{-}$ memory cells as a biomarker of recurrent proteinuria.

Patients were divided into two groups (Recurrent Proteinuria and No Proteinuria). A) Dot plot comparing %gated Memory cells obtained from the two groups using the Mann Whitney Test. Individual values, median and IQR shown B) ROC curve analysis C) Kaplan Meier survival curves demonstrating recurrent proteinuria-free survival in patients with high and low %Memory cells as defined by ROC curve.

As the pathogenesis of proteinuria can be varied, the demographics and some clinical characteristics of patients with recurrent proteinuria were reviewed in Table 4.27). Biopsies were performed in 80% of these patients. For those who were not offered a biopsy, one patient had poorly controlled diabetes, and 2 patients were under management for recurrent urinary tract infections, and the proteinuria was thought to be related to these issues. Reviewing the patients who received a biopsy, 5 patients demonstrated evidence of rejection (TCMR, ABMR, mixed or borderline features), and 3 had evidence of recurrent disease. One patient was switched to sirolimus as there were features in keeping with calcineurin inhibitor toxicity, and two patients were found to have features associated with infection (1x BK nephropathy, 1x bacterial infection). The patient with BK nephropathy on the first biopsy was found to have features associated with rejection on subsequent biopsies.

ID	Primary Disease	Biopsy	Results	UTIs	Sirolimus	Comment
7	MCGN	Yes x1	Borderline TCMR		No	
11	FSGS	No	NA	Yes	No	
15	Membranous	Yes x 1	Recurrent membranous	No	No	
16	GN	Yes x 1	IFTA grade I/segmental sclerosis	No	No	
60	Hypertension	Yes x 2	Borderline TCMR IFTA grade III	No	No	
61	T2DM	No		No	No	Poorly controlled DM
63	T2DM	Yes x 1	CNI toxicity	No	Yes	
67	SLE	Yes x 3	IFTA Gr 1, BKN IFTA, TCMR Mixed ABMR + TCMR, IFTA Gr 1	No	No	
72	IGAN	Yes x 2	Recurrent IGA Recurrent IGA	No	No	

ID	Primary Disease	Biopsy	Results	UTIs	Sirolimus	Comment
77	T2DM	Yes x 3	ATN RAS Mixed TCMR, ABMR, IFTA Gr1	No	No	
82	ADPKD	Yes x 2	ATN with moderate arteriosclerosis IFTA Gr 1	No	No	
85	Chronic pyelonephritis	Yes x 1	IFTA Gr 1	No	No	
95	ADPKD	Yes x 3	IFTA ?rejection ?infection IFTA Gr 1 IFTA ? infection ? rejection	No	No	
98	Interstitial Nephritis	Yes x 3	?ABMR IFTA Gr 2 IFTA + vascular hyalinosis	No	No	
100	Congenital renal dysplasia	No	NA	Yes	No	
108	Unknown	Yes x 2	Active ABMR Active ABMR	No	No	
115	IgA Nephropathy	Yes x 1	Recurrent IgA	No	No	
118	Renovascular disease	Yes x1	Infection	Yes	No	

Table 4.27. Clinical features of patients with recurrent proteinuria

The patients who were thought to have recurrent proteinuria associated with bacterial infection, poorly controlled diabetes and sirolimus use (highlighted in grey in Table 4.27) were removed from the patient set, and calculations were repeated (Table 4.28 and Table 4.29). Upon recalculation, no significant differences were noted in %gated or calculated B cell subsets. This suggests that the findings noted above (increased memory cells associated with increased risk of proteinuria) may have been associated with other causes of non-immune mediated proteinuria (e.g. recurrent UTIs), acknowledging as well that with such small patient numbers, patient selection can significantly affect results.

Calculated lymphocyte subset (x10^9/L)	Median Value UPCR<50	IQR	Median Value Recurrent UPCR>50	IQR	p value
$CD19^+$ B-cells (NP n=40, RP n=13)	0.0593	0.0820	0.0209	0.0279	0.064
$CD19^+CD27^+CD38^-$ Memory Cells (NP n=40, RP n=13)	0.000545	0.000723	0.000292	0.000601	0.209
$CD19^+CD27^-IgD^+$ Naïve (NP n=40, RP n=13)	0.0572	0.0829	0.0204	0.028	0.060
$CD19^+CD27^+IgD^+$ Non-Switched Memory (NP n=40, RP n=13)	0.000552	0.00120	0.000176	0.000389	0.141
$CD19^+CD27^+IgD^-$ Class switched Memory (NP n=40, RP n=13)	0.000340	0.000533	0.000176	0.000615	0.482
$CD19^+CD24^{hi}CD38^{hi}$ Transitional B-cells (NP n=40, RP n=13)	0.0330	0.0419	0.0184	0.0203	0.114
$CD19^+CD24^{+++}CD38^{+++}$ T1 (NP n=40, RP n=13)	0.00775	0.00997	0.00554	0.00569	0.134
$CD19^+CD24^{++}CD38^{++}$ T2 (NP n=40, RP n=13)	0.0185	0.309	0.0100	0.012	0.141

Table 4.28. Redo Calculation: Association between calculated 3-month B-cell subsets and recurrent proteinuria in the subsequent follow up period.
Comparisons were made using the Mann-Whitney Test. UPCR – urine protein creatinine ratio

Lymphocyte Subset (%gated)	Median Value UPCR<50	IQR	Median Value Recurrent UPCR>50	IQR	p value
$CD19^+$ B-cells (NP n=40, RP n=13)	18.97	21.76	8.35	8.17	0.068
$CD19^+CD27^+CD38^-$ Memory (NP n=40, RP n=13)	0.85	1.33	1.38	5.01	0.229
$CD19^+CD27^-IgD^+$ Naïve (NP n=40, RP n=13)	97.53	2.86	97.3	6.46	0.134
$CD19^+CD27^+IgD^+$ Non-Switched Memory (NP n=40, RP n=13)	1.08	1.45	1.75	2.77	0.499
$CD19^+CD27^+IgD^-$ Class switched Memory (NP n=40, RP n=13)	0.62	1.15	1.16	4.34	0.482
$CD19^+CD24^{hi}CD38^{hi}$ Transitional B-cells (NP n=40, RP n=13)	56.29	36.25	64.7	34.22	0.272
$CD19^+CD24^{+++}CD38^{+++}$ T1 (NP n=40, RP n=13)	28.1	15.1	28.7	16.63	0.755
$CD19^+CD24^{++}CD38^{++}$ T2 (NP n=40, RP n=13)	71.41	15.33	70.1	17.65	0.755
T1:T2 Ratio (NP n=40, RP n=13)	0.39	0.31	0.410	0.36	0.736
Switched:NS Ratio (NP n=40, RP n=13)	0.541	1.71	1.169	1.99	0.499
Plasmablasts (NP n=40, RP n=13)	0.70	0.94	0.72	1.07	0.678

Table 4.29. Redo Calculation: Association between % gated 3-month B-cell subsets and recurrent proteinuria in the subsequent follow up period.
Comparisons were made using the Mann-Whitney Test. UPCR – urine protein creatinine ratio. T1:T2 and S:NS ratios were calculated for each individual patient. The median values and interquartile range (IQR) for each group are included in the table.

4.2.1.1.6 DEVELOPMENT OF *DE NOVO* HLA ANTIBODIES AND DONOR SPECIFIC ANTIBODIES

During the follow up period, 30 patients developed *de novo* HLA antibodies, and 10 patients developed *de novo* donor specific antibodies above the predefined cut-off (MFI>1000).

Three out of the 61 patient blood samples were excluded from the subsequent analysis due to insufficient B-cell events (no DSA n=2, DSA n=1, no HLA antibodies n=2, HLA antibodies n=1). Therefore 58 samples were included in the 3-month prospective analyses (no DSA n=49, DSA n=9 and no HLA antibodies n=29, HLA antibodies n=29). In one of these samples, there were no T1 events. A valid T1:T2 ratio could not be obtained for this sample, and this was excluded for any analyses that compared T1:T2 ratios (no DSA n=1, no HLA antibody n=1). The number of valid samples included in each B cell subset analysis has been documented in Table 4.30 through to Table 4.33.

The 3-month calculated B-cell phenotype (calculated and % of parent gate) were not associated with the development of either *de novo* HLA antibodies or DSAs over the follow up period (Table 4.30,
Table 4.32). It was noted however that patients who developed DSAs had almost twice the 3-month switched memory:non-switched memory cell ratio than patients who did not develop DSA however this was not statistically significant (1.12 vs 0.565, p=0.360).

Lymphocyte Subset (Calculated, x10^9/L)	Median Value No DSA	No DSA IQR	Median Value DSA	DSA IQR	p value
$CD19^+$ B-cells (No DSA n=49, DSA n=9)	0.0335	0.0733	0.0374	0.0889	0.991
$CD19^+CD27^-IgD^+$ Naïve (No DSA n=49, DSA n=9)	0.0328	0.0729	0.0474	0.0718	0.911
$CD19^+CD27^+IgD^+$ Non-Switched Memory (No DSA n=49, DSA n=9)	0.000432	0.000878	0.000472	0.00180	0.859
$CD19^+CD27^+IgD^-$ Switched Memory (No DSA n=49, DSA n=9)	0.000324	0.000528	0.000387	0.00927	0.423
$CD19^+CD24^{hi}CD38^{hi}$ TrBs (No DSA n=49, DSA n=9)	0.0208	0.0332	0.0394	0.0496	0.449
$CD19^+CD24^{+++}CD38^{+++}$ T1 (No DSA n=49, DSA n=9)	0.00574	0.00916	0.00773	0.0112	0.533
$CD19^+CD24^{++}CD38^{++}$ T2 (No DSA n=49, DSA n=9)	0.0131	0.0248	0.0273	0.0405	0.563
$CD27^+CD38^+$Plasmablasts (No DSA n=49, DSA n=9)	0.000204	0.000555	0.000252	0.00133	0.533

Table 4.30. Calculated 3-month B-cell phenotype and the development of DSAs over the subsequent follow up period.

Comparisons were made using the Mann-Whitney Test. Median values and interquartile range (IQR) are included for each group. DSA – donor specific antibody

Lymphocyte Subset (% Gated)	Median Value No DSA	No DSA IQR	Median Value DSA	DSA IQR	p value
$CD19^+$ B-cells (No DSA n=49, DSA n=9)	14.10	21.3	16.93	16.54	0.991
$CD19^+CD27^-IgD^+$ Naïve (No DSA n=49, DSA n=9)	97.40	4.23	94.20	15.03	0.456
$CD19^+CD27^+IgD^+$ Non-Switched Memory (No DSA n=49, DSA n=9)	1.59	2.06	1.99	3.50	0.664
$CD19^+CD27^+IgD^-$ Switched Memory (No DSA n=49, DSA n=9)	0.635	1.29	0.935	6.36	0.290
$CD19^+CD24^{hi}CD38^{hi}$ TrBs (No DSA n=49, DSA n=9)	54.40	34.16	65.00	26.76	0.136
$CD19^+CD24^{+++}CD38^{+++}$ T1 (No DSA n=49, DSA n=9)	29.40	15.33	26.50	24.25	0.947
$CD19^+CD24^{++}CD38^{++}$ T2 (No DSA n=49, DSA n=9)	70.40	16.17	72.70	24.57	0.894
T1:T2 Ratio (No DSA n=48, DSA n=9)	0.415	0.323	0.365	0.640	0.991
$CD27^+CD38^+$Plasmablasts (No DSA n=49, DSA n=9)	0.705	0.86	0.82	2.06	0.221
Switched Mem:NS Mem Ratio (No DSA n=49, DSA n=9)	0.565	1.39	1.12	2.77	0.306

Table 4.31. % Gated 3-month B-cell phenotype and the development of DSAs over the subsequent follow up period.

Comparisons were made using the Mann-Whitney Test. T1:T2 and S:NS ratios were calculated for each individual patient. The median values and interquartile range (IQR) for each group are included in the table. DSA – donor specific antibody

Lymphocyte Subset (Calculated, x10^9/L)	Median Value No HLA A	No HLA A IQR	Median Value De novo HLA A	De novo HLA A IQR	p value
$CD19^+$ B-cells (No HLA A n=29, HLA A n=29)	0.0238	0.0598	0.0374	0.0994	0.700
$CD19^+CD27^-IgD^+$ Naïve (No HLA A n=29, HLA A n=29)	0.0231	0.0588	0.0572	0.0819	0.422
$CD19^+CD27^+IgD^+$ Non-Switched Memory (No HLA A n=29, HLA A n=29)	0.000432	0.000706	0.000395	0.00132	0.883
$CD19^+CD27^+IgD^-$ Switched Memory (No HLA A n=29, HLA A n=29)	0.000324	0.000521	0.000412	0.000773	0.566
$CD19^+CD24^{hi}CD38^{hi}$ TrBs (No HLA A n=29, HLA A n=29)	0.0164	0.0263	0.0322	0.0429	0.201
$CD19^+CD24^{+++}CD38^{+++}$ T1 (No HLA A n=29, HLA A n=29)	0.00472	0.00651	0.00686	0.00972	0.213
$CD19^+CD24^{++}CD38^{++}$ T2 (No HLA A n=29, HLA A n=29)	0.0100	0.0167	0.0176	0.0330	0.195
$CD27^+CD38^+$Plasmablasts (No HLA A n=29, HLA A n=29)	0.000512	0.000688	0.00545	0.00114	0.441

Table 4.32. Comparisons between calculated 3-month B phenotype and the development of de novo HLA antibodies.
Comparisons were made using the Mann-Whitney Test. Median values and interquartile range (IQR) are included for each group. HLA A – HLA antibody

Lymphocyte Subset (%gated)	Median Value No HLA A	No HLA A IQR	Median Value De novo HLA A	HLA A IQR	p value
$CD19^+$ B-cells (No HLA A n=29, HLA A n=29)	12.1	21.3	15.8	18.94	0.786
$CD19^+CD27^-IgD^+$ Naïve (No HLA A n=29, HLA A n=29)	97.1	5.53	97.53	5.98	0.692
$CD19^+CD27^+IgD^+$ Non-Switched Memory (No HLA A n=29, HLA A n=29)	1.72	2.78	1.49	2.01	0.840
$CD19^+CD27^+IgD^-$ Switched Memory (No HLA A n=29, HLA A n=29)	0.720	2.42	0.660	2.07	0.680
$CD19^+CD24^{hi}CD38^{hi}$ TrBs (No HLA A n=29, HLA A n=29)	50.9	41.31	62.1	27.74	0.316
$CD19^+CD24^{+++}CD38^{+++}$ T1 (No HLA A n=29, HLA A n=29)	31.0	20.62	28.1	15.26	0.907
$CD19^+CD24^{++}CD38^{++}$ T2 (No HLA A n=29, HLA A n=29)	69.0	20.01	71.4	15.66	0.919
T1:T2 Ratio (No HLA A n=28, HLA A n=29)	0.45	0.458	0.39	0.335	0.968
$CD27^+CD38^+$Plasmablasts (No HLA A n=29, HLA A n=29)	0.705	1.27	0.74	0.88	0.652
Switched Mem:NS Mem Ratio (No HLA A n=29, HLA A n=29)	0.563	1.46	0.771	1.88	0.566

Table 4.33. Comparisons between %gated 3- month B phenotype and the development of de novo HLA antibodies.

Comparisons were made using the Mann-Whitney test. T1:T2 and S:NS ratios were calculated for each individual patient. The median values and interquartile range (IQR) for each group are included in the table. HLA A – HLA antibody

4.2.1.1.7 RENAL FUNCTION

The mean eGFR for the alemtuzumab cohort at 3 months was 44.13ml/min/1.73m^2 (standard deviation 16.9). To determine if the 3-month B-cell phenotype was associated with the 3-month renal function, a comparison was made between each lymphocyte subset (calculated and % gated) and the 3-month eGFR for 58 patients. The 3-month eGFR was weakly correlated with the calculated $CD19^+CD27^+IgD^-$ class switched memory cells (Spearman R 0.330, p=0.012). There was a negative correlation between %gated transitional B-cells and 3-month eGFR (Spearman R -0.449, p<0.001), however, %T1, %T2 and T1:T2 ratios were not seen to be correlated with 3-month function. As other factors could affect the 3-month eGFR, hierarchical multiple regression models were constructed to control for the 3-month tacrolimus levels, cold ischaemia time and delayed graft function. The 3-month %transitional B-cells and 3-month calculated class switched memory cells were entered into separate models as new predictors. When controlled for tacrolimus levels, CIT and DGF, the 3-month calculated class switched memory cells remained positively associated with eGFR (standardized β=0.404, p=0.003, Table 4.37), however there was no significant association between eGFR and 3-month transitional B-cells (Table 4.36).

Calculated Lymphocyte Subset	Spearman R (eGFR @ 3 months)	p value
$CD19^+$ B-cells (n=58)	0.245	0.066
$CD19^+CD27^-IgD^+$ Naïve (n=58)	0.236	0.077
$CD19^+CD27^+IgD^+$ Non-Switched Memory (n=58)	0.150	0.265
$CD19^+CD27^+IgD^-$ Class switched Memory (n=58)	0.330	**0.012**
$CD19^+CD24^{hi}CD38^{hi}$ Transitional B-cells (n=58)	0.077	0.571
$CD19^+CD24^{+++}CD38^{+++}$ T1 (n=58)	0.115	0.396
$CD19^+CD24^{++}CD38^{++}$ T2 (n=58)	0.087	0.521

Table 4.34. Correlation analyses between B-cell phenotype (calculated) and renal function

Lymphocyte Subset (% gated)	Spearman R (eGFR @ 3 months)	p value
$CD19^+$ B-cells (n=58)	0.146	0.268
$CD19^+CD27^-IgD^+$ Naïve (n=58)	-0.132	0.759
$CD19^+CD27^+IgD^+$ Non-Switched Memory (n=58)	-0.050	0.709
$CD19^+CD27^+IgD^-$ Class switched Memory (n=58)	0.216	0.103
$CD19^+CD24^{hi}CD38^{hi}$ Transitional B-cells (n=58)	-0.449	**<0.001**
$CD19^+CD24^{+++}CD38^{+++}$ T1 (n=58)	0.013	0.924
$CD19^+CD24^{++}CD38^{++}$ T2 (n=58)	-0.032	0.811
T1:T2 Ratio (n=57)	0.044	0.745
S:NS Ratio (n=58)	0.186	0.165

Table 4.35. Correlation analyses between 3-month % gated lymphocyte subset and renal function.

T1:T2 and S:NS ratios were calculated for each individual patient. The median values and interquartile range (IQR) for each group are included in the table.

	Unstandardised B (95% CI)	Coefficients Std Error	Standardised Coefficients Beta	P value
Step 1				
(Constant)	50.058 (31.997-68.119)	9.001		<0.001
DGF	-11.381 (-22.332- -0.431	5.457	-0.279	0.042
CIT (hrs)	0.441 (-0.444-1.327)	0.441	0.135	0.322
Tacrolimus	-0.876 (-2.316-0.563)	0.717	-0.161	0.227
Step 2				
Constant	58.086 (38.525-77.647)	9.744		<0.001
DGF	-9.730 (-20.560-1.101)	5.395	-0.239	0.077
CIT (hrs)	0.337 (-0.534-1.208)	0.751	0.103	0.441
Tacrolimus	-0.356 (-1.864-1.153)	0.751	-0.065	0.638
%TrB	-0.210 (-0.431-0.012)	0.110	-0.267	0.063

Table 4.36 Linear model assessing 3-month %TrB as a predictor of 3-month eGFR.

Note R^2=0.119 for Step 1; ΔR^2=0.058

	Unstandardised B (95% CI)	Coefficients Std Error	Standardised Coefficients Beta	P value
Step 1				
(Constant)	50.954 (32.391-69.3518)	9.247		<0.001
DGF	-11.625 (-22.706 - -0.545)	5.519	-0.285	0.040
CIT (hrs)	0.489 (-0.424 - 1.402)	0.455	0.145	0.287
Tacrolimus	-1.017 (-2.576 - 0.542)	0.777	-0.173	0.196
Step 2				
Constant	45.303 (27.826 - 62.779)	8.701		<0.001
DGF	-14.126 (-24.459 - -3.793)	5.145	-0.347	0.008
CIT (hrs)	0.299 (-0.551-1.149)	0.423	0.089	0.483
Tacrolimus	-0.532 (-2.001-0.937)	0.731	-0.090	0.470
Calculated $CD19^+CD27^+IgD^-$	7050.433 (2595-11504)	2217	0.404	0.003

Table 4.37. Linear model assessing 3-month calculated switched memory cells as a predictor of 3-month eGFR.

Note R^2=0.121 for Step 1; ΔR^2=0.148

4.2.1.1.8 ALEMTUZUMAB 3-MONTH SUMMARY

To summarise, as early as 3 months post transplantation, there are signals within the reconstituting B lymphocyte subsets that are associated with subsequent adverse markers of clinical outcome. The composite outcome was first assessed. Although this was not statistically significant, patients meeting the composite endpoint were more likely to have fewer B-cells (both calculated B lymphocytes and %gated). These patients also tended to have more $CD27^{+}CD38^{-}$ memory B-cells, of which non-switched memory B-cells were significantly higher than in those who did not meet the composite endpoint.

Individual endpoints were then considered separately. As recurrent proteinuria alone was the most common composite endpoint achieved, it was not surprising to see that a high %gated $CD27^{+}CD38^{-}$ memory cells (>1.185%) was also associated with the subsequent development of recurrent proteinuria (HR 4.544, CI 1.584-13.03, p=0.0105), however on excluding patients with possible non-immune related causes of proteinuria, this difference was no longer seen. Renal function (eGFR) was correlated with calculated class switched memory cells. Patients who subsequently lost their allograft during the follow up period were found to have significantly reduced calculated B-cells, $CD19^{+}CD27^{-}IgD^{+}$ naïve and $CD19^{+}CD24^{+++}CD38^{+++}$ T1 transitional B-cells compared with those that did not lose their allograft. Receiver operating characteristic curves demonstrated that a low calculated $CD27^{-}IgD^{+}$ Naïve cells $<0.0135x10^{9}/L$ was the best marker of subsequent graft loss.

When rejection events were assessed, the B-cell count/frequency was not associated with adverse outcomes. However, patients who had a rejection episode during the follow up period were found to have significantly elevated transitional B-cell frequencies compared with those that did not have rejection. When the transitional subset was further analysed, patients who had rejection episodes demonstrated lower frequencies of T1 transitional cells and higher frequencies of T2 transitional cells, resulting in a lower T1:T2 ratio. ROC analyses showed that a low T1:T2 ratio less than

0.215 was associated with increased risk of rejection. Multivariate cox proportional hazards analysis showed that this low 3-month T1:T2<0.215 ratio was independently associated with subsequent allograft rejection (HR 62, 95% CI 3.31-1161, p=0.006).

4.2.1.2 ALEMTUZUMAB INDUCTION – 6 MONTH PHENOTYPE AS A MARKER OF CLINICAL OUTCOMES

Similar analyses were performed using the 6-month samples. When assessing each surrogate marker of adverse outcome individually, patients were removed from analysis if they had already reached that end point. (For example, if a patient had evidence of allograft rejection at 4 months post-transplant, they were removed from the composite endpoint analysis as well as the analysis of allograft rejection at further time-points. If they had not met the endpoint for other surrogate markers, they were still included for analysis of that marker).

4.2.1.2.1 COMPOSITE ENDPOINT

Four patients achieved the composite endpoint prior to their 6-month clinic visit and were therefore removed from this analysis. Of the remaining 57 patients, 2 had insufficient B-cell events and were therefore excluded. A total of 55 patients had their 6-month phenotype assessed against the composite outcome, however a further two patients (1 CEP and CEN) had insufficient transitional B cell events for gating. These were excluded from the assessment of transitional B cells, subsets and T1:T2 ratio. Table 4.38 and Table 4.39 compare calculated lymphocyte subsets and % gated subsets with whether patients met the composite endpoint (CEP – composite endpoint positive, CEN – composite endpoint negative). There were no statistically significant differences in calculated subsets when comparing the two groups, however CEP patients had fewer B-cells than CEN patients. CEP patients also had more $CD19^{+}CD27^{+}CD38^{-}$ memory cells, of which, there were more $CD19^{+}CD27^{-}IgD^{-}$ class switched memory cells (Table 4.38).

When the %gated subsets were considered, CEP patients were found to have a lower frequency of $CD24^{+++}CD38^{+++}$ T1 cells (19.2% CEP vs 23.9% CEN, p=0.0295) and a higher frequency of $CD24^{++}CD38^{++}$ T2 cells (80% CEP vs 75% CEN, 0=0.018), resulting in a lower T1:T2 ratio which was statistically significant (0.24 vs 0.32, p=0.027). Also of note, CEP patients had a higher switched:non-switched memory cell ratio at 6 months compared with CEN patients (1.18 CEP vs 0.746 CEN, p=0.0167), see Table 4.39.

Calculated lymphocyte subset (x10^9/L)	CEN Median Value	IQR	CEP Median Value	IQR	p value
$CD19^+$ B-cells (CEN n=35, CEP n=20)	0.0915	0.0869	0.0684	0.157	0.588
$CD19^+CD27^+CD38^-$ Memory Cells (CEN n=35, CEP n=20)	0.00157	0.00180	0.00236	0.00325	0.670
$CD19^+CD27^-IgD^+$ Naïve (CEN n=35, CEP n=20)	0.0894	0.0819	0.0646	0.155	0.565
$CD19^+CD27^+IgD^+$ Non-Switched Memory (CEN n=35, CEP n=20)	0.00125	0.00171	0.000859	0.00173	0.282
$CD19^+CD27^+IgD^-$ Class switched Memory (CEN n=35, CEP n=20)	0.000881	0.00149	0.00172	0.00223	0.207
$CD19^+CD24^{hi}CD38^{hi}$ Transitional B-cells (CEN n=34, CEP n=19)	0.0238	0.0242	0.0208	0.0607	0.911
$CD19^+CD24^{+++}CD38^{+++}$ T1 (CEN n=34, CEP n=19)	0.00572	0.00518	0.00581	0.00655	0.528
$CD19^+CD24^{++}CD38^{++}$ T2 (CEN n=34, CEP n=19)	0.0191	0.0146	0.0159	0.0482	0.926

Table 4.38. Comparison between 6-month calculated lymphocyte subsets and whether patients subsequently met the composite endpoint during follow up. Patients had their blood drawn at their 6-month post-transplant follow up. B-cells subsets were determined using flow cytometry. Comparisons have been made using the Mann Whitney test. CEN – composite endpoint negative, CEP – compositive endpoint positive.

Lymphocyte Subset (% Gated)	CEN Median Value	IQR	CEP Median Value	IQR	p value
$CD19^+$ B-cells (CEN n=35, CEP n=20)	22.6	18.76	14.4	14.95	0.146
$CD19^+CD27^+CD38^-$ Memory Cells (CEN n=35, CEP n=20)	1.62	1.75	1.71	2.26	0.262
$CD19^+CD27^-IgD^+$ Naïve	96.9	2.63	97.0	3.55	0.853
$CD19^+CD27^+IgD^+$ Non-Switched Memory (CEN n=35, CEP n=20)	1.32	1.20	0.94	1.78	0.246
$CD19^+CD27^+IgD^-$ Class switched Memory (CEN n=35, CEP n=20)	1.04	1.58	1.33	1.88	0.164
$CD19^+CD24^{hi}CD38^{hi}$ Transitional B-cells (CEN n=34, CEP n=19)	27.2	17.83	34.6	15.92	0.278
$CD19^+CD24^{+++}CD38^{+++}$ T1 (CEN n=34, CEP n=19)	23.9	13.08	19.2	6.43	**0.0295**
$CD19^+CD24^{++}CD38^{++}$ T2 (CEN n=34, CEP n=19)	75.8	12.64	80.0	6.68	**0.018**
T1:T2 Ratio (CEN n=34, CEP n=19)	0.315	0.245	0.240	0.102	**0.027**
Switched Mem:NS Mem Ratio (CEN n=35, CEP n=20)	0.746	1.03	1.18	1.42	**0.0167**

Table 4.39. Comparison between 6-month %gated lymphocyte subsets and whether patients subsequently met the composite endpoint during follow up. Comparisons were made using the Mann Whitney test. T1:T2 and S:NS ratios were calculated for each individual patient. The median values and interquartile range (IQR) for each group are included in the table. CEN – composite endpoint negative, CEP – composite endpoint positive

The T1, T2 cells, T1:T2 ratio and the switched:non-switched memory cell ratios were investigated further. ROC curves were constructed to define an optimal cut-off for each variable. A %T1 cut off <20.86 was associated with meeting the composite endpoint (sensitivity 63.2%, specificity 67.7%) with an area under the curve (AUC) of 0.681, p=0.03. A %T2>80% was associated with meeting the composite endpoint (sensitivity 52.7%, specificity 77%) with an AUC of 0.6966, p=0.0185. A T1:T2 ratio <0.2488 was associated with meeting the composite endpoint with a sensitivity 57.9% and specificity 65.7, AUC=0.6842, p=0.0273 (Figure 4.18). Finally, a high switched:non-switched memory ratio >0.958 was associated with meeting the composite endpoint (AUC = 0.6977, sensitivity 63%, specificity 65%, p=0.0172, Figure 4.20). The AUC values obtained from these ROC curves were less than 0.7, therefore these subsets were unlikely to effectively discriminate between the two groups. Kaplan Meier curves were constructed for the TrB subsets (Figure 4.19). There was initial crossover between the 2 survival curves for each TrB subset, which subsequently separated as time progressed. These differences (using the log-rank test) did not achieve statistical significance. Similarly, a low S:NS ratio was associated with a reduced composite-endpoint-free survival, however this was not statistically significant (log rank p=0.1359, Figure 4.20c).

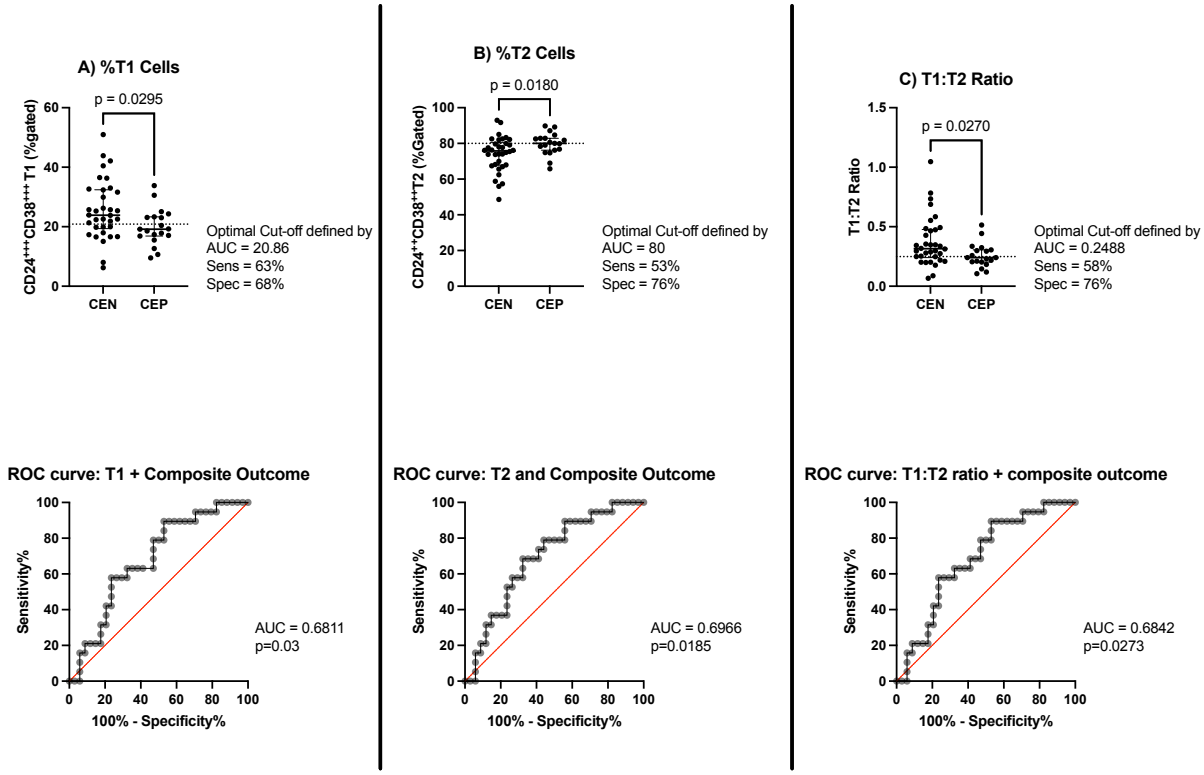


Figure 4.18. Assessment of 6-month phenotype as a marker of outcome. **Patients were split into two groups according to whether they met the composite endpoint (which included graft loss, recurrent proteinuria, >30% reduction in eGFR from 3-18 months, de novo DSA, biopsy proven rejection, recurrent disease). 6-month post-transplant blood samples were obtained, and the B-cell phenotype was assessed by flow cytometry. The top panel shows the differences in each subset between the two groups. The bottom panel contains the receiver operating characteristic curves comparing phenotype with outcome. A) %gated $CD24^{+++}CD38^{+++}$ T1 cells, B) %$CD24^{++}CD38^{++}$ T2 cells, C) T1:T2 Ratio. CEN – composite endpoint negative, CEP – composite endpoint positive**

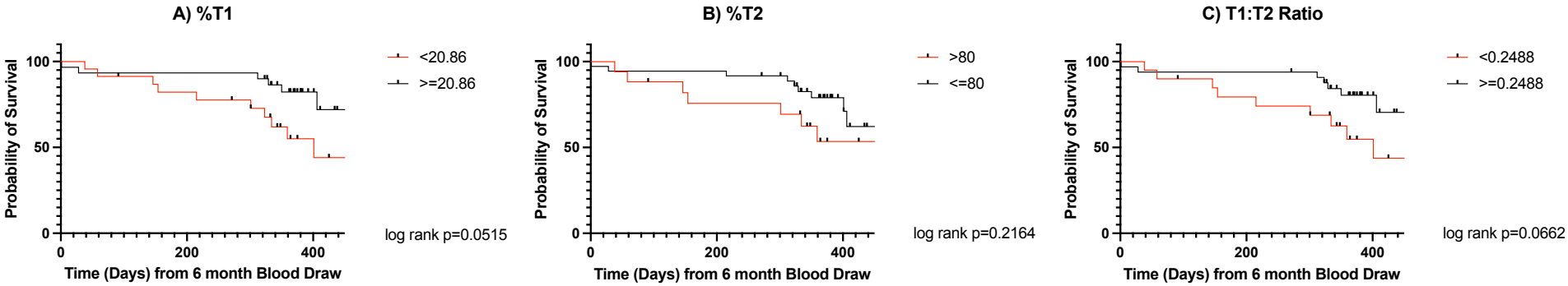


Figure 4.19. Kaplan Meier curves comparing composite-endpoint-free survival from 6-month post-transplant in patients stratified according to transitional B-cell subsets.

A) %gated $CD24^{+++}CD38^{+++}$ T1 cells, B) %gated $CD24^{++}CD38^{++}$ T2 cells, C) T1:T2 Ratio

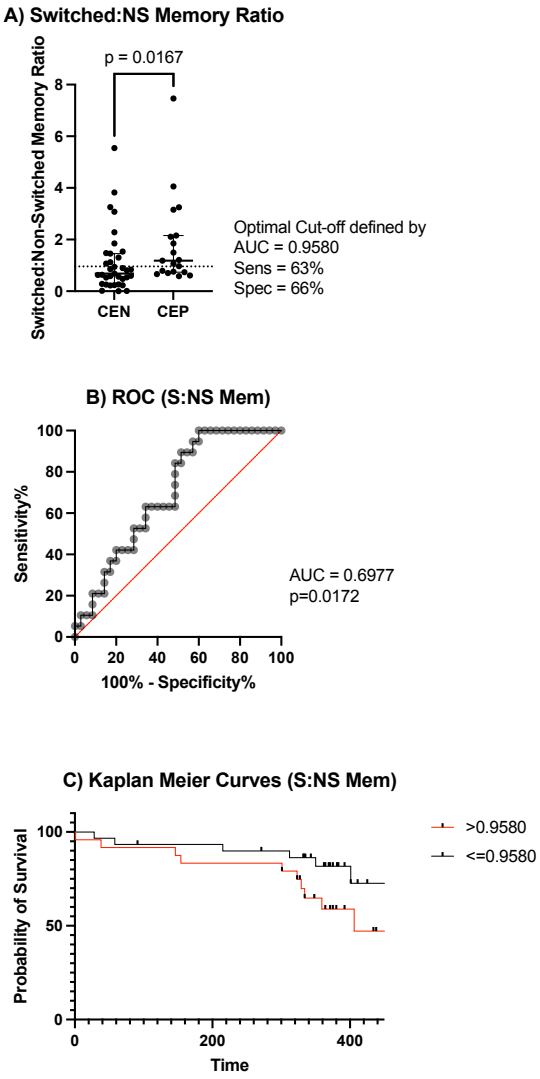


Figure 4.20. Assessment of the 6-month Class Switched:Non-Switched Memory ratio (S:NS ratio) as a biomarker of subsequent outcome.

Patients were divided into two groups according to whether they went on to meet the composite endpoint. A) Dot plots comparing the S:NS ratio between the two groups, with individual values, median and interquartile range shown. B) Receiver operating characteristic curve C) Kaplan Meier survival estimates of composite endpoint free survival from the 6-month blood draw stratified according to high or low S:NS ratio. CEN – composite endpoint negative, CEP – composite endpoint positive.

4.2.1.2.2 GRAFT SURVIVAL

Graft survival was then considered as a separate endpoint. One patient lost their graft prior to the 6-month timepoint. Sixty-one patients had their blood drawn at 6 months post-transplant. A further 3 patients were excluded due to insufficient B cell events. The remaining 57 patients were separated into two groups according to whether they subsequently lost their graft (GS = graft survival, n=51 GL = graft loss, n=6) over the follow up period. One further patient in the GL group had insufficient transitional B cells for analysis, and was excluded for the purposes of TrB and TrB subset analyses, see
Table 4.40 and
Table 4.41.

When considering calculated subsets, there were no statistically significant differences between the two groups. However, patients who lost their graft had fewer 6-month T1 cells compared with those who did not lose their graft (T1 = 0.00875x10^9/l GL vs 0.0190x10^9/l GS, p=0.058,
Table 4.40). There were also no statistically significant differences in %gated subsets between the GS and GL groups (
Table 4.41), however patients who subsequently lost their grafts had a lower proportion of T1 (18% GL vs 23% GS, p=0.07), higher T2 (81% GL vs 76% GS, p=0.054), lower T1:T2 ratio (0.22 GL vs 0.29 GS, p=0.074), and higher S:NS memory ratio (1.83 GL vs 0.856 GS, p=0.051) that approached statistical significance (
Table 4.41).

Although the differences in the T1:T2 ratio were not statistically significant, further investigations were performed to determine a possible cut off value that may be associated with subsequent graft loss (Figure 4.21). This was to see if the T1:T2 ratios associated with graft loss reduced with time post-transplant, in keeping with the trends noted in the previous section (The Description of The B-cell Surface phenotype from 3-18 months post transplant). Using a cut off value of <0.2640, ROC curve analysis classified the two groups reasonably well with an AUC=0.7255, sensitivity 83%

and specificity 61%, p=0.0728. When Kaplan Meier curves were constructed using the cut off defined by the ROC curve, patients with a low T1:T2 ratio had an increased but not statistically significant risk of graft loss (HR 6.362, CI 1.269-31.89, p=0.0508).

Lymphocyte Subset (calculated, x10^9/L)	Median Value GS	IQR	Median Value GL	IQR	p value
$CD19^+$ B-cells (GS n=51, GL n=6)	0.0877	0.101	0.0429	0.0786	0.212
$CD19^+CD27^-IgD^+$ Naïve (GS n=51, GL n=6)	0.0842	0.101	0.0394	0.0753	0.194
$CD19^+CD27^+IgD^+$ Non-Switched Memory (GS n=51, GL n=6)	0.00107	0.00162	0.000558	0.00130	0.153
$CD19^+CD27^+IgD^-$ Class switched Memory (GS n=51, GL n=6)	0.00116	0.00132	0.00121	0.00205	0.95
$CD19^+CD24^{hi}CD38^{hi}$ Transitional B-cells (GS n=51, GL n=5)	0.0233	0.0241	0.0108	0.0242	0.111
$CD19^+CD24^{+++}CD38^{+++}$ T1 (GS n=51, GL n=5)	0.00590	0.00485	0.00161	0.00452	0.058
$CD19^+CD24^{++}CD38^{++}$ T2 (GS n=51, GL n=5)	0.0190	0.0154	0.00875	0.0202	0.145

Table 4.40. 6 month B-cell phenotype (Calculated) comparisons according to subsequent graft survival.

Comparisons were made using the Mann Whitney test. IQR – interquartile range

Lymphocyte Subset (% Gated)	Median Value GS	IQR	Median Value GL	IQR	p value
$CD19^+$ B-cells (GS n=51, GL n=6)	20.9	18.97	10.7	3.18	0.115
$CD19^+CD27^-IgD^+$ Naïve (GS n=51, GL n=6)	96.7	2.75	96.8	5.59	1.000
$CD19^+CD27^+IgD^+$ Non-Switched Memory (GS n=51, GL n=6)	1.22	1.43	0.96	0.97	0.322
$CD19^+CD27^+IgD^-$ Class switched Memory (GS n=51, GL n=6)	1.19	1.52	1.39	3.10	0.524
$CD19^+CD24^{hi}CD38^{hi}$ Transitional B-cells (GS n=51, GL n=5)	28.1	19.41	27.9	15.37	0.675
$CD19^+CD24^{+++}CD38^{+++}$ T1 (GS n=51, GL n=5)	22.6	12.10	18.01	6.27	0.07
$CD19^+CD24^{++}CD38^{++}$ T2 (GS n=51, GL n=5)	76.3	12.10	81.4	6.82	0.054
T1:T2 Ratio (GS n=51, GL n=5)	0.30	0.210	0.22	0.095	0.074
Switched Mem:NS Mem Ratio (GS n=51, GL n=6)	0.856	0.955	1.83	2.11	0.051

Table 4.41. 6-month B-cell phenotype (% gated) comparisons according to subsequent graft survival.

T1:T2 and S:NS ratios were calculated for each individual patient. The median values and interquartile range (IQR) for each group are included in the table. Comparisons were made using the Mann Whitney Test.

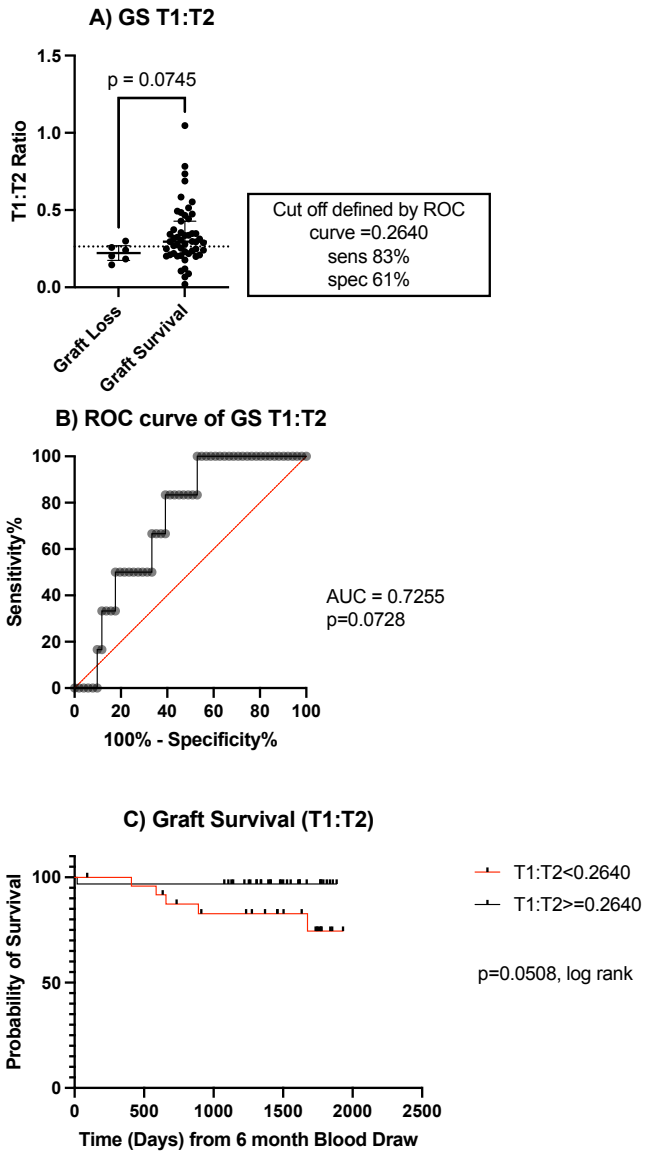


Figure 4.21. Assessment of 6-month T1:T2 ratio as a marker of subsequent graft loss. Patients were placed into two groups (Graft loss and Graft survival) and their T1:T2 ratio was assessed. A) Comparison (Mann Whitney test) of T1:T2 ratios between the two groups with individual values, median and IQR shown. B) ROC curve analysis C) Kaplan Meier curves comparing graft survival in patient with a high (>0.2640) and low (<0.2640) T1:T2 ratio

4.2.1.2.3 REJECTION

Sixty-one patients had their blood drawn at 6-months post-transplant. One patient had biopsy proven rejection between 3- and 6-months post-transplant and was excluded from this analysis. A further 2 patients (R n=1 and NR n=1) were excluded from analysis due to insufficient B cell events. The remaining 58 patients were divided into two groups (rejection, R, n=5 and no rejection, NR, n=53) depending on whether they had a rejection episode in the subsequent follow up period, and their 6-month B-cell phenotypes were analysed. A further patient within the rejection group had insufficient transitional B cells and was excluded from the analysis of transitional B cells and their subsets (see Table 4.42 and Table 4.43).

Although patients with rejection had fewer calculated B-cells compared with patient who did not have rejection (median $0.0496x10^9$/L R vs $0.0846x10^9$/L NR), this difference was not statistically different (p=0.297). Patients with rejection however were found to have fewer calculated non-switched memory cells, and transitional cells (overall, T1 and T2) compared with those who did not have rejection (Table 4.42). Similar differences were noted in the % gated TrB and memory populations (Table 4.43). Firstly, looking at the transitional cells, similar %TrBs were obtained (median %TrB 27.73 NR, 23.2 R. p=0.294), however, as with the 3-month values, patients in the rejection group had lower %T1, higher %T2 and therefore a reduced T1:T2 ratio compared with the no-rejection group. Within the memory compartment, the %non-switched ($CD27^+IgD^+$) memory cells were higher in the no-rejection group compared with the rejection group (median %NS 1.22 NR vs 0.62 R, p=0.045), and this resulted in a higher switched:non-switched memory cell ratio (median 0.875 NR vs 2.66 R p=0.022), Table 4.43.

ROCs were constructed to determine the optimum cut-off values for T1, T2, T1:T2 and S:NS memory cell ratios (Figure 4.22 and Figure 4.24). The %T1, %T2 and therefore T1:T2 ratios were strong predictors of allograft rejection (Figure 4.22). ROC analysis using a threshold T1:T2 ratio of 0.1911 was associated with a sensitivity of 75% and specificity of 91%, area under the curve 0.8066, p=0.0423. When patients were

divided into two groups according to T1:T2 ratio, a low T1:T2 ratio <0.1911 was associated with a reduced 5-year rejection free survival and 38% of patients with a low ratio developed rejection compared with only 2% of patients with a high ratio.

In addition to the T1:T2 ratio, the 6-month S:NS memory cell ratio was also a good predictor of allograft rejection with an area under the curve of 0.8380 on ROC analysis with a p=0.0251. Using a S:NS ratio threshold of >2.134 was associated with a sensitivity of 75% and specificity of 82%. Patients were then divided into those with a high S:NS ratio >2.134 and a low S:NS ratio $\leq$ 2.134 and over the following 5 years, 23% of those with a high S:NS ratio experienced allograft rejection as opposed to 2% of those with a low S:NS ratio (Figure 4.24).

Lymphocyte Subset (Calculated, x10^9/L)	Median Value NR	IQR	Median Value R	IQR	p value
$CD19^+$ B-cells (NR n=53, R n=5)	0.0846	0.106	0.0496	0.0750	0.297
$CD19^+CD27^-IgD^+$ Naïve (NR n=53, R n=5)	0.0827	0.106	0.0473	0.070	0.236
$CD19^+CD27^+IgD^+$ Non-Switched Memory (NR n=53, R n=5)	0.00107	0.00165	0.000307	0.000642	**0.024**
$CD19^+CD27^+IgD^-$ Class switched Memory (NR n=53, R n=5)	0.00106	0.00149	0.00121	0.00267	0.965
$CD19^+CD24^{hi}CD38^{hi}$ Transitional B-cells (NR n=53, R n=4)	0.0233	0.0248	0.00574	0.0101	**0.015**
$CD19^+CD24^{+++}CD38^{+++}$ T1 (NR n=53, R n=4)	0.00590	0.00531	0.00107	0.00133	**0.005**
$CD19^+CD24^{++}CD38^{++}$ T2 (NR n=53, R n=4)	0.0190	0.157	0.00467	0.00883	**0.023**
$CD27^+CD38^+$Plasmablasts (NR n=53, R n=5)	0.000353	0.000677	0.00020	0.000193	0.189

Table 4.42. Comparison of 6-month calculated lymphocyte subsets in patients with rejection (R) and no rejection (NR).

Groups were compared using the Mann-Whitney U test. Median values and interquartile range (IQR) are included.

Lymphocyte Subset (%gated)	Median Value No Rejection	NR IQR	Median Value Rejection	R IQR	p value
$CD19^+$ B-cells (NR n=53, R n=5)	20.9	19.55	9.34	12.36	0.187
$CD19^+CD27^-IgD^+$ Naïve (NR n=53, R n=5)	97.0	2.76	95.8	3.23	0.756
$CD19^+CD27^+IgD^+$ Non-Switched Memory (NR n=53, R n=5)	1.22	1.45	0.62	0.64	**0.045**
$CD19^+CD27^+IgD^-$ Class switched Memory (NR n=53, R n=5)	1.14	1.48	2.28	2.05	0.248
$CD19^+CD24^{hi}CD38^{hi}$ Transitional B-cells (NR n=53, R n=4)	28.73	19.38	23.2	24.5	0.294
$CD19^+CD24^{+++}CD38^{+++}$ T1 (NR n=53, R n=4)	22.56	10.77	14.1	10.83	**0.037**
$CD19^+CD24^{++}CD38^{++}$ T2 (NR n=53, R n=4)	77.55	10.24	85.9	10.32	**0.034**
T1:T2 Ratio (NR n=53, R n=4)	0.295	0.190	0.176	0.119	**0.041**
$CD27^+CD38^+$Plasmablasts (NR n=53, R n=5)	0.360	0.58	0.300	0.44	0.504
Switched Mem:NS Mem Ratio (NR n=53, R n=5)	0.875	1.03	2.66	4.96	**0.022**

Table 4.43. Comparison of 6-month %gated subsets in patients with rejection and no rejection.

Groups were compared using the Mann-Whitney U test. T1:T2 and S:NS ratios were calculated for each individual patient. The median values and interquartile range (IQR) for each group are included in the table.

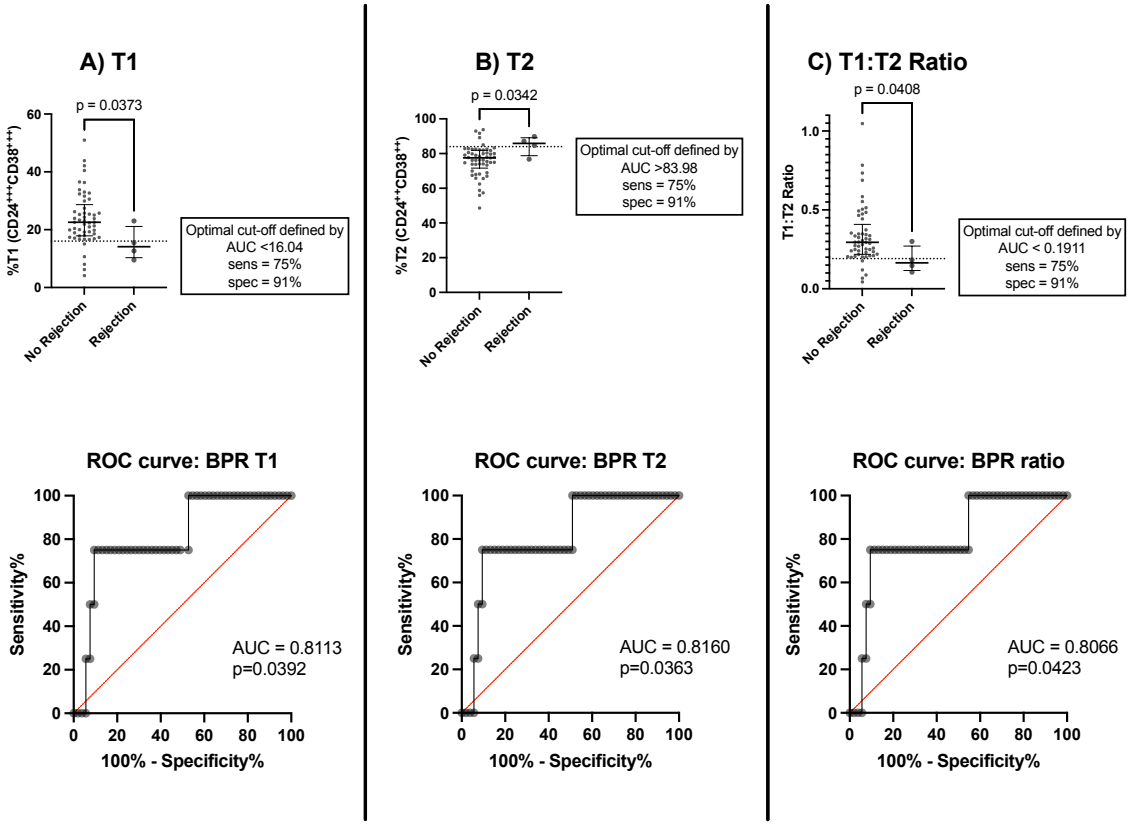
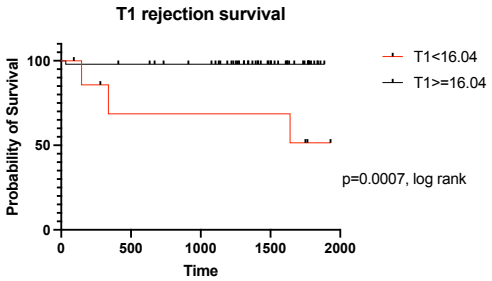
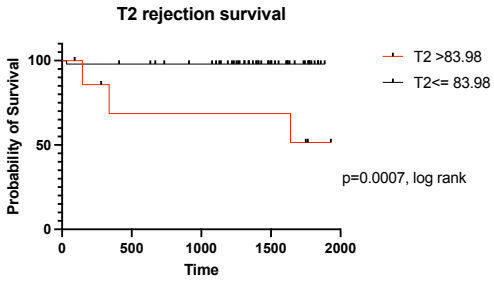


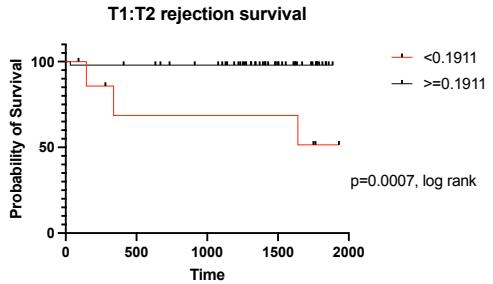
Figure 4.22. Receiver operating characteristic curve analysis using the 6-month A) %T1, B) %T2 and C) T1:T2 Ratio as markers of subsequent allograft rejection.
The top panel demonstrates comparisons between the two groups (rejection and no rejection) using the Mann Whitney test. Individual results, median and interquartile ranges are shown. The optimal cut-off values defined by the ROC analysis are also shown with associated sensitivities and specificities. The bottom panel shows the ROC analysis including area under the ROC for each TrB subset and ratio.



No. at risk	6-month blood draw (T=0)	300 days	1000 days	1500 days	2000 days
T1≥16.04	49	49	43	21	1
T1<16.04	8	5	5	5	1

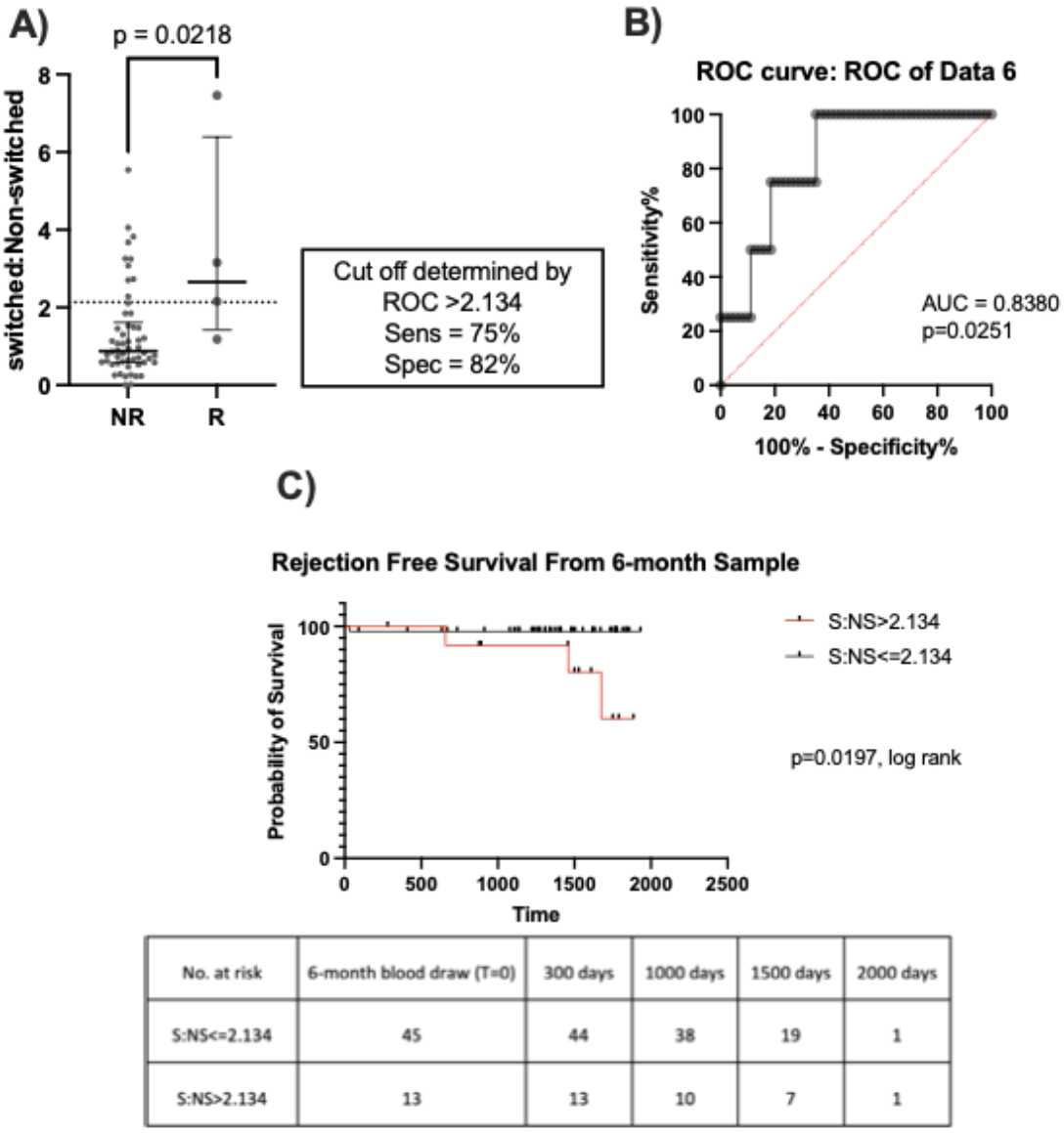


No. at risk	6-month blood draw (T=0)	300 days	1000 days	1500 days	2000 days
T2≤83.98	49	49	43	21	1
T2>83.98	8	6	5	5	1



No. at risk	6-month blood draw (T=0)	300 days	1000 days	1500 days	2000 days
T1:T2≥0.1911	49	49	43	21	1
T1:T2<0.1911	8	5	5	5	1

Figure 4.23. Kaplan Meier Curves comparing the 5 year rejection free survival according to 6-month %T1, %T2 and T1:T2 ratio.



No. at risk	6-month blood draw (T=0)	300 days	1000 days	1500 days	2000 days
S:NS<=2.134	45	44	38	19	1
S:NS>2.134	13	13	10	7	1

Figure 4.24. A high S:NS ratio at 6months is associated with a reduced 5-year rejection free survival in patients receiving alemtuzumab induction.
Patients were divided into two groups: Rejection, R and No Rejection, NR and their B-cell phenotype was assessed using flow cytometry. A) Patients with rejection had a higher Switched Memory ($CD19^+CD27^+IgD^-$):Non-switched Memory ($CD19^+CD27^+IgD^+$) ratio (Mann Whitney test with individual values, median and IQR shown). B) Receiver operating characteristic curve classifying the two groups according to the S:NS ratio. C) Kaplan Meier estimates of rejection free survival in patients with a high 6-month S:NS ratio and low S:NS ratio. AUC = area under the curve

4.2.1.2.4 RECURRENT PROTEINURIA

61 patients had their blood drawn at 6 months. Between 3 and 6 months, 4 patients developed proteinuria, and were excluded from the following analysis. Additionally., Four patients had insufficient B cells for analysis and were excluded. Patients were divided into two groups according to the development of proteinuria during the follow up period (recurrent proteinuria n=13, no recurrent proteinuria n=40). Comparisons using the Mann Whitney test did not yield statistically significant differences in the calculated B-cells and B-cell subsets. However, when %gated frequencies were reviewed, patients who developed proteinuria had fewer transitional B-cells compared with those who did not develop proteinuria (not statistically significant). The make-up of the transitional subsets was however significantly different, and patients who developed recurrent proteinuria were found to have a reduced T1:T2 ratio compared with those that did not develop recurrent proteinuria (T1:T2 ratio 0.231 recurrent proteinuria vs 0.315 no proteinuria, p=0.011, Table 4.44).

Lymphocyte Subset (%gated)	Median Value No Proteinuria	NP IQR	Median Value Recurrent Proteinuria	RP IQR	p value
$CD19^+$ B-cells (NP n=40, RP n=13)	21.73	20.0	20.65	18.81	0.865
$CD19^+CD27^+CD38^-$ Memory (NP n=40, RP n=13)	1.72	1.91	1.71	2.07	0.535
$CD19^+CD27^-IgD^+$ Naïve (NP n=40, RP n=13)	96.44	2.76	97.07	3.17	0.852
$CD19^+CD27^+IgD^+$ Non-Switched Memory (NP n=40, RP n=13)	1.24	1.34	0.72	1.25	0.160
$CD19^+CD27^+IgD^-$ Class switched Memory (NP n=40, RP n=13)	1.12	1.40	1.33	1.98	0.642
$CD19^+CD24^{hi}CD38^{hi}$ Transitional B-cells (NP n=40, RP n=13)	28.4	20.48	24.7	29.68	0.861
$CD19^+CD24^{+++}CD38^{+++}$ T1 (NP n=40, RP n=12)	23.9	13.74	18.6	5.15	**0.010**
$CD19^+CD24^{++}CD38^{++}$ T2 (NP n=40, RP n=12)	75.8	13.29	80.6	5.63	**0.009**
T1:T2 Ratio (NP n=40, RP n=12)	0.315	0.255	0.231	0.078	**0.011**
$CD27^+CD38^+$Plasmablasts (NP n=40, RP n=13)	0.390	0.61	0.27	0.36	0.204
Switched Mem:NS Mem Ratio (NP n=40, RP n=13)	0.823	0.93	1.50	2.55	0.193

Table 4.44. Comparison of 6-month %gated subsets in patients with recurrent proteinuria (RP) with no proteinuria (NP).

Groups were compared using the Mann-Whitney test. T1:T2 and S:NS ratios were calculated for each individual patient. The median values and interquartile range (IQR) for each group are included in the table.

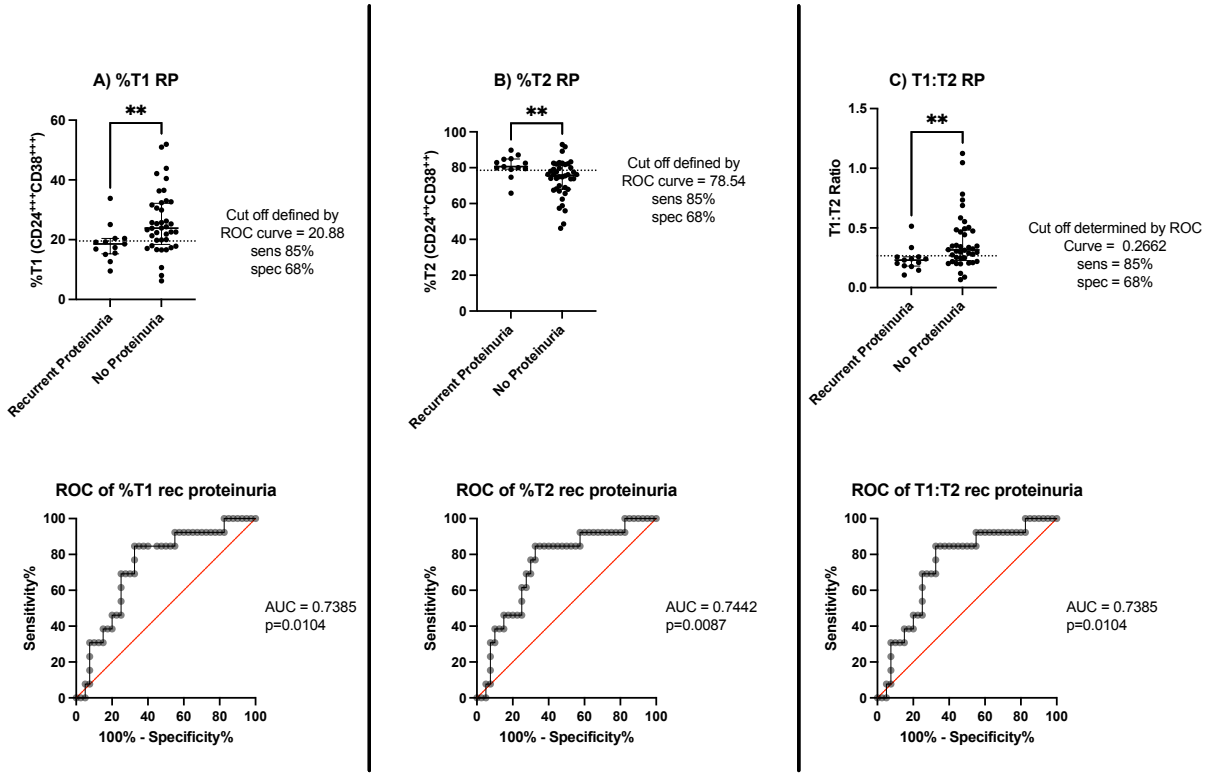


Figure 4.25. Receiver operating characteristic curve analysis using the 6-month A) %T1, B) %T2 and C) T1:T2 Ratio as markers of developing recurrent proteinuria (RP).

The top panel demonstrates comparisons between the two groups (RP and no proteinuria) using the Mann Whitney test. Individual results, median and interquartile ranges are shown. The optimal cut-off values defined by the ROC analysis are also shown with associated sensitivities and specificities. The bottom panel shows the ROC analysis including area under the ROC for each TrB subset and T1:T2 ratio.

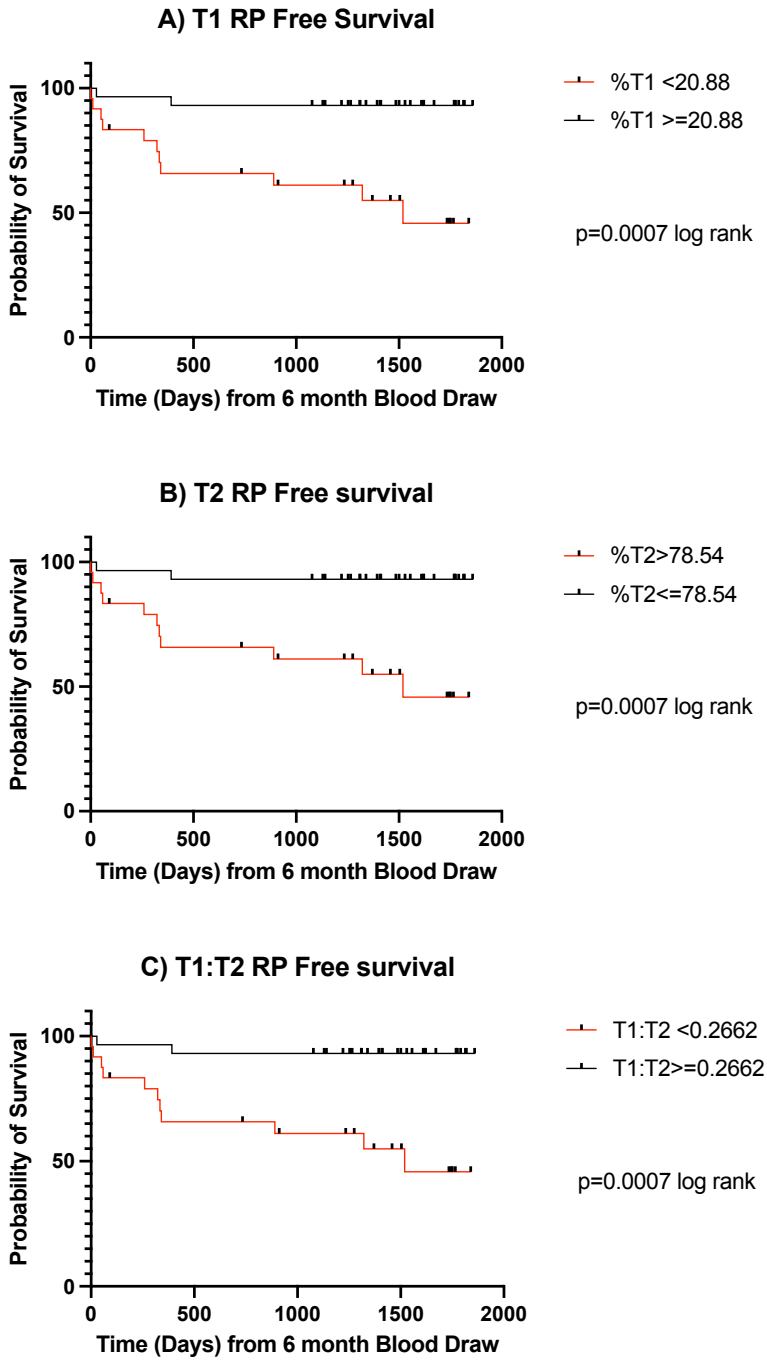


Figure 4.26. Kaplan Meier curves estimating the recurrent proteinuria (RP) – free survival after the 6-month blood draw

%T1 cut-off 20.88%, B) %T2 cut-off 78.54% and C) T1:T2 ratio 0.2602

ROC curves were constructed to determine the optimum cut off values for %T1 (%T1 <20.88, sensitivity 85%, specificity 68%, AUC=0.7385, p=0.0104), %T2 (%T2>78.54, sensitivity 85%, specificity 68%, AUC 0.7442, p=0.008) and the T1:T2 ratio (Ratio<0.2662 sensitivity 85%, specificity 68%, AUC=0.7385, p=0.0104), see Figure 4.25. When Kaplan Meier curves were constructed, a low 6-month T1:T2 ratio<0.2662 was associated with a reduced proteinuria-free survival with a log rank hazard ratio of 8.567, p=0.0007, CI 2.245-21.02 (Figure 4.26). The analysis was repeated with patients excluded if they were thought to have developed proteinuria secondary to a non-immune mediated cause (Table 4.45). As with previous calculations, patients with a lower T1:T2 ratio were at risk of developing recurrent proteinuria (Ratio 0.231 RP vs 0.309 No Proteinuria, p=0.032). Additionally, although it was not statistically significant, there was a trend towards an increased switched:non-switched memory ratio (1.85 recurrent proteinuria, 0.823 no proteinuria, p=0.056).

Lymphocyte Subset (%gated)	Median Value No Proteinuria	IQR	Median Value Recurrent Proteinuria	IQR	p value
$CD19^+$ B-cells (NP n=40, RP n=9)	22.3	18.49	11.7	13.89	0.193
$CD19^+CD27^+CD38^-$ Memory (NP n=40, RP n=9)	1.62	1.73	1.71	2.47	0.781
$CD19^+CD27^-IgD^+$ Naïve (NP n=40, RP n=9)	96.9	2.77	97.1	3.51	0.103
$CD19^+CD27^+IgD^+$ Non-Switched Memory (NP n=40, RP n=9)	1.24	1.19	0.700	0.77	0.030
$CD19^+CD27^+IgD^-$ Class switched Memory (NP n=40, RP n=9)	1.07	1.41	1.33	2.31	0.511
$CD19^+CD24^{hi}CD38^{hi}$ Transitional B-cells (NP n=40, RP n=8)	28.4	20.50	24.7	24.54	0.606
$CD19^+CD24^{+++}CD38^{+++}$ T1 (NP n=40, RP n=8)	23.6	13.65	18.6	5.75	**0.032**
$CD19^+CD24^{++}CD38^{++}$ T2 (NP n=40, RP n=8)	76.1	13.59	80.6	6.45	**0.030**
T1:T2 Ratio (NP n=40, RP n=8)	0.309	0.255	0.231	0.0738	**0.032**
$CD27^+CD38^+$Plasmablasts (NP n=40, RP n=9)	0.390	0.57	0.270	0.40	0.185
Switched Mem:NS Mem Ratio (NP n=40, RP n=9)	0.823	0.94	1.85	2.53	0.056

Table 4.45. Redo Calculation: Comparison of 6-month %gated subsets in patients with recurrent proteinuria and without recurrent proteinuria.
(Patients with non-immune mediated causes of proteinuria removed from analysis). T1:T2 and S:NS ratios were calculated for each individual patient. The median values and interquartile range (IQR) for each group are included in the table. Groups were compared using the Mann-Whitney test.

4.2.1.2.5 DE NOVO DSA

Sixty one patients had their blood drawn at 6 months post transplant. Four patients developed DSAs between 3 and 6 months post transplant therefore were excluded form subsequent analysis. Furthermore, there were insufficient B cell events in 4 patients who were also excluded. The remaining 53 patients were divided into two groups according to the development of *de novo* DSAs. Six out of 53 patients developed *de novo* DSA after their 6-month blood draw. No statistically significant differences were noted in the B-cell phenotypes (calculated and %gated) between the two patient groups.

4.2.1.2.6 DISEASE RECURRENCE

Four of 61 patients were excluded from analysis due to insufficient B cell events. Patients were divided into two groups according to disease recurrence (recurrence n=3, no recurrence n=57) after 6 months. No statistically significant differences were noted when B-cell phenotypes (calculated and %gated) were compared between the two groups.

4.2.1.2.7 RENAL FUNCTION

To assess renal function, only patients who had not met the composite endpoint were considered (n=55). Correlation analyses were performed between 6-month eGFR and B-cell subsets (see Table 4.46 and Table 4.47) and statistically significant correlations were noted between eGFR and calculated $CD19^{+}$ B-cells, non-switched memory, switched memory and naïve cells. A negative correlation was also noted between eGFR and %transitional B-cells (spearman R = -0.292, p=0.032).

Calculated Lymphocyte Subset	Spearman R (eGFR @ 6 months)	p value
$CD19^+$ B-cells	0.296	**0.030**
$CD19^+CD27^-IgD^+$ Naïve	0.323	**0.017**
$CD19^+CD27^+IgD^+$ Non-Switched Memory	0.294	**0.031**
$CD19^+CD27^+IgD^-$ Class switched Memory	0.324	**0.017**
$CD19^+CD24^{hi}CD38^{hi}$ Transitional B-cells	0.150	0.278
$CD19^+CD24^{+++}CD38^{+++}$ T1	0.085	0.542
$CD19^+CD24^{++}CD38^{++}$ T2	0.190	0.169

Table 4.46. Correlation analyses between B-cell phenotype (calculated) and renal function (6-month eGFR)

Lymphocyte Subset (% gated)	Spearman R (eGFR @ 6 months)	p value
$CD19^+$ B-cells	0.119	0.392
$CD19^+CD27^-IgD^+$ Naïve	0.150	0.279
$CD19^+CD27^+IgD^+$ Non-Switched Memory	0.097	0.457
$CD19^+CD27^+IgD^-$ Class switched Memory	0.103	0.457
$CD19^+CD24^{hi}CD38^{hi}$ Transitional B-cells	-0.292	**0.032**
$CD19^+CD24^{+++}CD38^{+++}$ T1	-0.032	0.816
$CD19^+CD24^{++}CD38^{++}$ T2	0.036	0.794
T1:T2 Ratio	-0.034	0.806
S:NS Ratio	0.112	0.420

Table 4.47. Correlation analyses between 6-month % gated lymphocyte subset and renal function (6- month eGFR). T1:T2 and S:NS ratios were calculated for each individual patient. The median values and interquartile range (IQR) for each group are included in the table.

Hierarchical multiple regression controlling for previous (3-month eGFR) and 6-month tacrolimus levels was then performed. As collinearity existed between calculated $CD19^+$ B-cells and the calculated subsets, the final model included $CD19^+$ B-cells alone and there was no statistically significant association between calculated $CD19^+$ B-cells and 6-month eGFR (see Table 4.48).

	Unstandardised B (95% CI)	Coefficients Std Error	Standardised Coefficients Beta	P value
Step 1				
(Constant)	13.327 (4.716-21.938)	4.287		0.003
Previous eGFR	0.837 (0.695-0.978)	0.070	0.878	<0.001
Tacrolimus	-0.460 (-1.406-0.486)	0.471	-0.072	0.334
Step 2				
Constant	11.000 (2.275-19.724)	4.341		0.015
Previous eGFR	0.786 (0.639-0.933)	0.073	0.825	<0.001
Tacrolimus	-0.238 (-1.187-0.712)	0.472	-0.037	0.617
Calculated $CD19^+$B-cells	26.65 (-0.919-54.2)	13.7	0.146	0.058

Table 4.48. Linear model assessing 6-month calculated $CD19^+$ B-cells as a predictor of 6-month eGFR. Note R^2=0.744 for Step 1; ΔR^2=0.018

Similarly, a hierarchical multiple regression model controlling for previous eGFR and 6-month tacrolimus levels was constructed with %TrBs as the new predictor variable, and %TrBs were not found to be predictors of 6-month renal function (Table 4.49)

	Unstandardised B (95% CI)	Coefficients Std Error	Standardised Coefficients Beta	P value
Step 1				
Constant	13.327 (4.716-21.938)	4.287		0.003
Previous eGFR	0.837 (0.695-0.978)	0.070	0.878	<0.001
Tacrolimus	-0.460 (-1.406-0.486)	0.471	-0.072	0.334
Step 2				
Constant	11.728 (1.640-21.815)	5.020		0.024
Previous eGFR	0.853 (0.701-1.0006)	0.076	0.895	<0.001
Tacrolimus	-.0521 (-1.494-0.451)	0.484	-0.082	0.287
%TrB	0.043 (-0.095-1.81)	0.069	0.048	0.536

Table 4.49. Linear model assessing 6-month %TrB as a predictors of 6-month eGFR.

Note $R^2 = 0.744$ for Step 1; $\Delta R^2 = 0.002$

4.2.1.2.8 ALEMTUZUMAB – 6 MONTH SUMMARY

As with the 3-month B-cell phenotype, differences were noted in the 6-month phenotype that were associated with inferior graft outcomes.

Composite Endpoint:

A high 3-month %memory cell population was associated with an increased risk of meeting the composite endpoint. The 6-month %memory cell population was also raised in those who met the composite endpoint, but this was not statistically significant. It was interesting to note that a high switched:non-switched memory cell ratio indicating a more differentiated B-cell population at 6 months was associated with meeting the composite endpoint (Mann Whitney test). However, the ROC curve analysis demonstrated a marginal discrimination between the two groups, and differences in survival curves did not achieve statistical significance. Similarly, when the individual values were compared between the two groups using the Mann Whitney test, a low 6-month %T1, high T2, and a low calculated T1:T2 ratio (<0.2488) was significantly associated with patients subsequently meeting the composite endpoint, however the discrimination between the two groups using ROC curve analyses was marginal.

Graft Survival:

A 3-month phenotype which included low calculated B-cells, naïve cells and T1 cells was associated with graft loss. There were no statistically significant differences in the corresponding 6- month values between the GS and GL groups. However, a trend towards a higher 6-month switched:non-switched memory cell ratio and lower T1:T2 ratio was noted in patients (<0.2640) who subsequently lost their graft.

Rejection Free Survival:

There were statistically significant differences between the rejection and no rejection groups when the TrB populations were assessed. Patients in the rejection group had fewer overall TrBs, as well as fewer T1 and T2 cells. When the %gated frequencies were assessed, patients in the rejection group had lower %T1 and higher %T2 cells, and therefore a lower T1:T2 ratio, similar to the results noted at 3 months. ROC curve analyses showed excellent discrimination the rejection and no-rejection groups, with AUC values >0.8 for %T1, %T2 and the T1:T2 ratio. Interestingly, reflective of the gradual decrease in transitional B-cells and the T1:T2 ratio over time following alemtuzumab induction (as noted in 4.1.1.3.2 page 109), the threshold determined by the ROC curve to classify patients at risk of rejection was lower (<0.19) at 6 months than that determined by the 3-month ROC curve (<0.215). Kaplan Meier survival estimates demonstrated that patients with a T1:T2 ratio <0.19 had a reduced 5-year rejection free survival, with a hazard ratio of 17.09 (CI 1.093-267.3, p=0.0007 log rank). Finally, although this was not noted at 3 months, a high 6-month switched to non-switched memory cell ratio >2.134 was significantly associated with a reduced 5-year rejection free survival (Hazard ratio 9.114, CI 0.942-88.25, p=0.0197).

Recurrent Proteinuria:

Patients who developed recurrent proteinuria were found to have fewer (although non-significant) transitional B-cells compared with patients who did not develop proteinuria. However significant differences were noted in the transitional subsets. Patients with a low 6-month %T1, high %T2 and therefore a low T1:T2 ratio (<0.2662)

were at risk of reduced proteinuria-free survival (hazard ratio 8.567, CI 2.245-21.02, p=0.007). These differences remained statistically significant even when patients with non-immune mediated causes of proteinuria were removed from analysis.

Renal function:

Although there was correlation between 3-month calculated class switched memory cells and renal function, when 6-month samples were assessed, and previous renal function and current tacrolimus levels were corrected for, there was no statistically significant association between the subsets and eGFR.

4.2.1.3 ALEMTUZUMAB 12-MONTH PHENOTYPE

4.2.1.3.1 COMPOSITE ENDPOINT

Five patients met the composite endpoint prior to their 12-month visit. One patient transferred away from Leeds. The 12-month sampling was missed in a further 10 patients, therefore 41 patients had their 12-month phenotypes assessed. There were no significant differences in the calculated or %gated B-cell phenotypes between the 2 groups (CEP n = 13, CEN n = 28).

4.2.1.3.2 GRAFT SURVIVAL

One patient lost their graft between the 6 and 12 month timepoints (mixed TCMR and ABMR on final biopsy). As with previous timepoints, the remaining patients were separated into two groups (GL – Graft Loss, and GS – Graft Survival) and had their 12-month B-cell phenotypes assessed using flow cytometry. The different B-cell subsets were then compared. The calculated $CD19^{+}$ B-cells were similar between the two groups (median B-cells $0.0892x10^{9}$/l GS vs $0.0848x10^{9}$/l GL, p=0.859), and no statistically significant differences were demonstrated between the two groups when calculated subsets were analysed.

Lymphocyte Subset (%gated)	Median Value GS	IQR	Median Value GL	IQR	p value
$CD19^+$ B-cells (GS n=40, GL n=6)	15.6	14.5	16.87	17.6	0.917
$CD19^+CD27^-IgD^+$ Naïve (GS n=40, GL n=6)	95.1	3.17	95.0	10.3	0.988
$CD19^+CD27^+IgD^+$ Non-Switched Memory (GS n=40, GL n=6)	1.27	1.13	1.01	0.94	0.351
$CD19^+CD27^+IgD^-$ Class switched Memory (GS n=40, GL n=6)	2.10	2.05	2.52	4.71	0.796
$CD19^+CD24^{hi}CD38^{hi}$ Transitional B-cells (GS n=40, GL n=6)	16.8	10.1	15.7	8.29	0.773
$CD19^+CD24^{+++}CD38^{+++}$ T1 (GS n=40, GL n=6)	19.0	11.0	11.5	2.18	**0.021**
$CD19^+CD24^{++}CD38^{++}$ T2 (GS n=40, GL n=6)	79.5	11.1	88.3	2.45	**0.011**
T1:T2 Ratio (GS n=40, GL n=6)	0.237	0.168	0.130	0.028	**0.012**
$CD27^+CD38^+$Plasmablasts (GS n=40, GL n=6)	0.360	0.28	0.505	0.80	0.939
Switched Mem:NS Mem Ratio (GS n=40, GL n=6)	1.81	1.59	2.03	2.61	0.573

Table 4.50. Mann Whitney Test comparing 12-month B-cell phenotype (%gated) with allograft loss.

T1:T2 and S:NS ratios were calculated for each individual patient. The median values and interquartile range (IQR) for each group are included in the table. GS – graft survival, GL – graft loss

The TrB subsets were further investigated using ROC curve analysis (Figure 4.27). The 12-month T1:T2 ratio offered the best discrimination using a cut-off value of 0.1424 (AUC 0.8130, sensitivity 100%, specificity 78%, p=0.0141). Kaplan Meier curves were constructed, and patients with a low 12-month T1:T2 ratio <0.1424 had a reduced graft survival over the subsequent 5 years (p=0.0003, log rank), Figure 4.28. Patients with graft loss were then divided into two groups according to evidence of rejection on their biopsies. Although the differences were not statistically significant, patients with rejection had a lower median T1:T2 ratio compared with those who had no rejection (median T1:T2 = 0.1093 rejection, 0.1310 no rejection, p=0.7000).

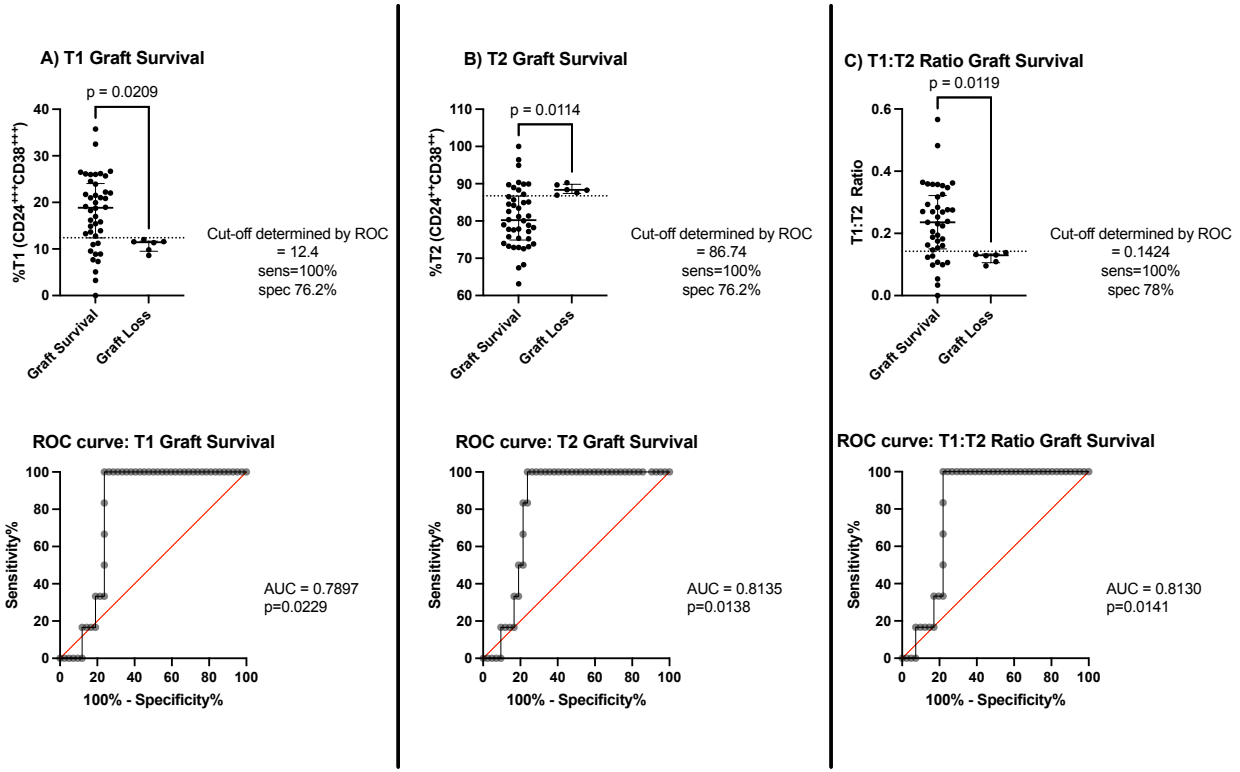


Figure 4.27. Receiver operating characteristic curve analysis using the 12-month A) %T1, B) %T2 and C) T1:T2 Ratio as biomarkers of allograft loss. The top panel demonstrates comparisons between the two groups (Graft Survival and Graft Loss) using the Mann Whitney test. Individual results, median and interquartile ranges are shown. The optimal cut-off values defined by the ROC analysis are also shown with associated sensitivities and specificities. The bottom panel shows the ROC analysis including area under the ROC for each TrB subset and ratio.

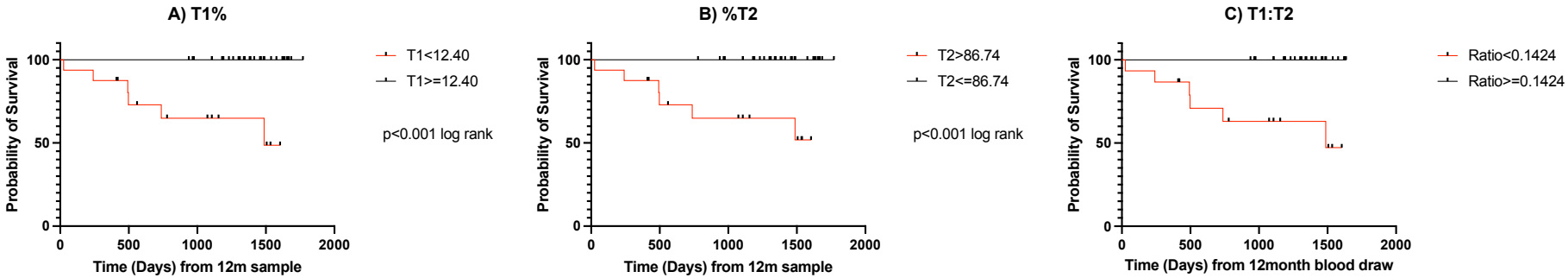


Figure 4.28. Kaplan Meier Curves comparing the 5-year graft survival according to the 12-month A) %T1 cells, B) %T2 cells, and C) T1:T2 ratio

4.2.1.3.3 REJECTION

Two patients had evidence of rejection on a for cause biopsy that was performed between 6- and 12- months post-transplant. These patients were removed from analysis. The remaining patients were divided into two groups (Rejection, and No Rejection, NR) and their 12-month B-cell phenotypes were assessed using flow cytometry. The calculated $CD19^{+}$ B-cells were similar between the two groups (median value $0.0749x10^{9}$/L NR vs $0.1109x10^{9}$/L R, p=0.754). Similarly, there were no statistically significant differences between the two groups when calculated values were assessed. When the %gated subsets were assessed, there was a trend towards increased %T2 cells and decreased T1:T2 ratio in R compared with NR groups (Table 4.51).

Lymphocyte Subset (%gated)	Median Value NR	IQR	Median Value R	IQR	p value
$CD19^+$ B-cells (NR n=42, R n=2)	15.3	14.3	23.0	-	0.301
$CD19^+CD27^-IgD^+$ Naïve (NR n=42, R n=2)	94.9	3.63	96.7	-	0.222
$CD19^+CD27^+IgD^+$ Non-Switched Memory (NR n=42, R n=2)	1.20	1.12	0.76	-	0.164
$CD19^+CD27^+IgD^-$ Class switched Memory (NR n=42, R n=2)	2.24	2.23	1.37	-	0.315
$CD19^+CD24^{hi}CD38^{hi}$ Transitional B-cells (NR n=42, R n=2)	15.8	10.40	20.3	-	0.485
$CD19^+CD24^{+++}CD38^{+++}$ T1 (NR n=42, R n=2)	18.5	11.13	9.21	-	0.113
$CD19^+CD24^{++}CD38^{++}$ T2 (NR n=42, R n=2)	80.8	12.0	90.0	-	0.085
T1:T2 Ratio (NR n=42, R n=2)	0.228	0.168	0.102	-	0.076
$CD27^+CD38^+$Plasmablasts (NR n=42, R n=2)	0.370	0.43	0.190	-	0.202
Switched Mem:NS Mem Ratio (NR n=42, R n=2)	2.03	2.02	1.78	-	0.808

Table 4.51. Mann Whitney test comparing 12-month %gated B subsets with rejection.

T1:T2 and S:NS ratios were calculated for each individual patient. The median values and interquartile range (IQR) for each group are included in the table.

Although the differences in the T1:T2 ratio were not statistically significant, further investigations were performed to determine a possible cut off value that may be associated with subsequent rejection. This was to see if the T1:T2 ratios associated with rejection reduced with time post-transplant, in keeping with the trends noted in

the previous section (The Description of The B-cell Surface phenotype from 3-18 months post transplant).

Further analysis was performed on the T1:T2 ratio (Figure 4.29). Although the differences were not statistically significant, ROC curve analysis classified the two groups using a cut-off value of T1:T2<0.1162 with an AUC=0.8810 (sensitivity 100%, specificity 83%, p=0.0714). When the Kaplan Meier curves were constructed using this cut off value, patients with a low T1:T2<0.1162 displayed a statistically higher incidence of rejection in the subsequent 5 years (p=0.0024, log rank, Figure 4.29c)

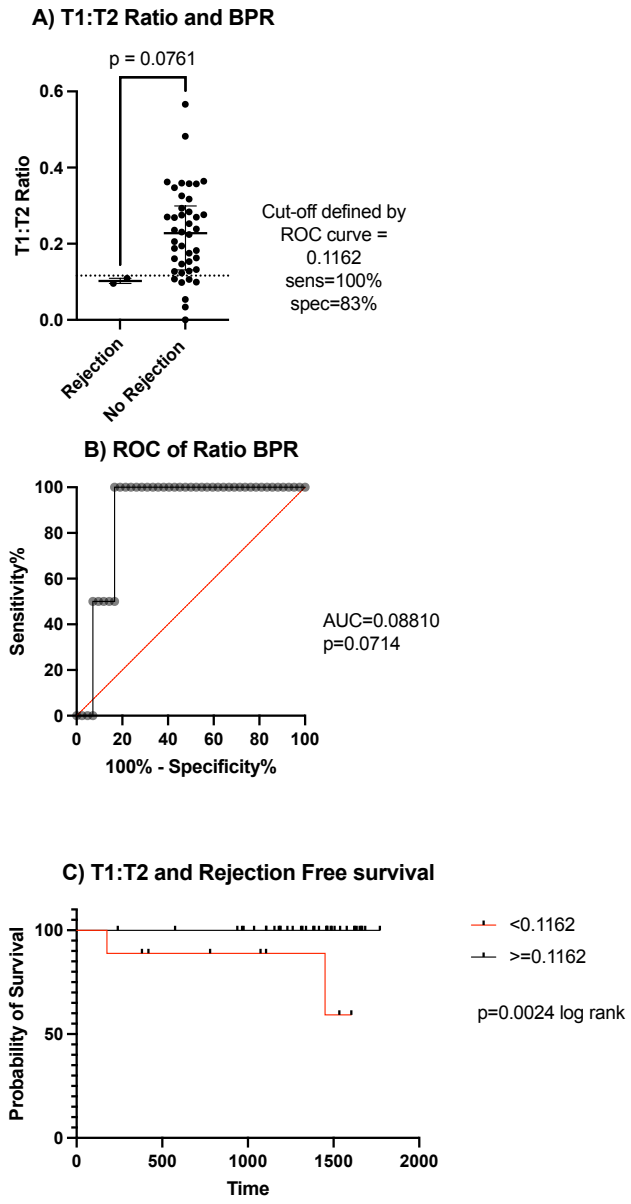


Figure 4.29. Assessing the 12-month T1:T2 ratio as a biomarker for rejection. Patients were divided into two groups according to rejection (R) and no rejection (NR) and their 12-month T1:T2 ratios were assessed. A) Dot plot showing the T1:T2 ratios obtained from the two groups (individual values, median and IQR shown), B) ROC curve analysis, C) Kaplan Meier curves estimating rejection free survival from the 12-month blood draw based on high and low T1:T2 ratios defined by the ROC curve.

4.2.1.3.4 DISEASE RECURRENCE

Patients were divided into two groups depending on the development of recurrent disease in the subsequent follow up period (no disease recurrence n=46, disease recurrence n=1). There were no differences in the B-cell phenotype (%gated and calculated) when the two groups were compared.

4.2.1.3.5 DEVELOPMENT OF DE NOVO DSAS

Patients were divided into two groups depending on the development of DSAs during subsequent follow up (DSA n=3, no DSA n=29). There were no differences in the B-cell phenotype (%gated and calculated) when the two groups were compared.

4.2.1.3.6 RECURRENT PROTEINURIA

Patients were divided into two groups depending on the development of recurrent proteinuria which was defined as UPCR>50 on two or more occasions (recurrent proteinuria n=9, no proteinuria n=33). There were no differences in the B-cell phenotype (%gated and calculated) when the two groups were compared. Similarly, when patients with non-immune mediated proteinuria were excluded, there were no differences in the B-cell phenotype (%gated and calculated) when the two groups were compared.

4.2.1.3.7 RENAL FUNCTION

Correlation analyses were performed between each individual B-cell subset and 12-month eGFR. No statistically significant correlations were noted.

4.2.1.3.8 ALEMTUZUMAB 12- MONTH SUMMARY

Acknowledging that overall, fewer samples were included at the 12-month assessment compared with other time points, some differences were still noted in the B-cell phenotype with respect to graft survival and rejection free survival.

Graft survival:

A low T1:T2 ratio <0.1424 was an excellent marker of subsequent graft loss (AUC 0.8130, sensitivity 100%, specificity 78%, p=0.0141) on univariate analysis. When the T1:T2 ratio was compared at all timepoints, patients who lost their graft during the follow up period had a reduced median T1:T2 ratio compared with those who did not lose their graft (Figure 4.31a). When the patients with graft loss were scrutinised further, considering evidence of rejection in for cause biopsies prior to graft loss, it was interesting to note that the median T1:T2 ratio was reduced in patients with rejection compared to patients with no rejection (Figure 4.30).

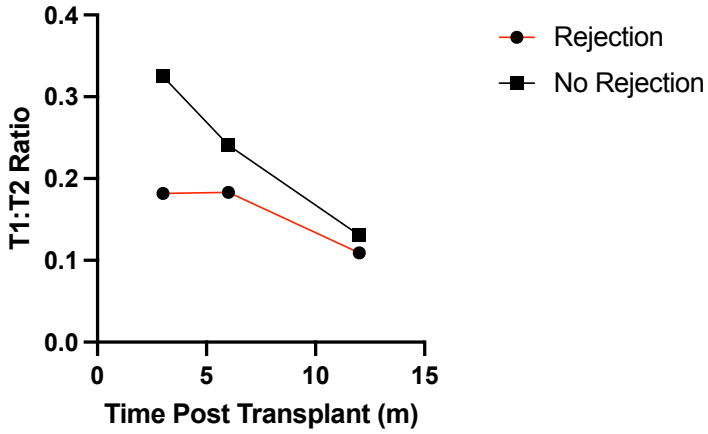


Figure 4.30. T1:T2 ratio obtained from Graft Loss patients stratified according to evidence of rejection on biopsy

Rejection free survival:

As with the phenotype observed at 3 and 6 months, patients with a low T1:T2 ratio displayed a statistically higher incidence of rejection when compared with patients who had a high T1:T2 ratio (Figure 4.29c). When compared at all timepoints, the median T1:T2 ratio was reduced in patients who developed rejection (Figure 4.31b).

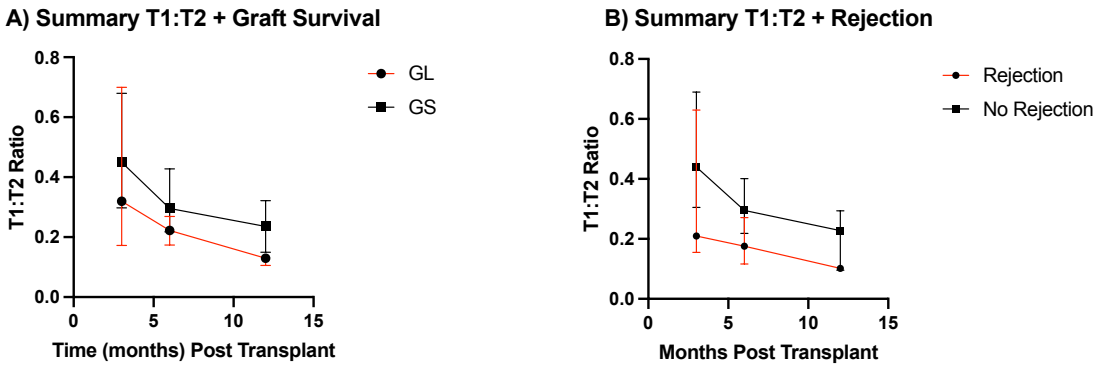


Figure 4.31. Summary of T1:T2 ratios in A) patients stratified according to graft survival and B) Patients stratified according to rejection.

Plots show the median and interquartile range for each time point.

4.2.1.4 BASILIXIMAB INDUCTION – 3MONTHS POST TRANSPLANT

4.2.1.4.1 COMPOSITE ENDPOINT

Twenty-one patients received Basiliximab induction and were followed up for a median period of 1744 days. During this follow up period, 7 patients met the composite endpoint. A summary of the composite endpoints can be seen in Table 4.52. The patient demographics are visible in Table 4.53. As seen with the alemtuzumab induction patients, proteinuria alone and *de novo* DSA alone were the two commonest endpoints seen in this cohort.

Number of patients	Proteinuria	De novo DSA	Disease recurrence	Biopsy Proven Rejection	>30% reduction in eGFR from 3-18 months	Graft Loss
2	✓	×	×	×	×	×
3	×	✓	×	×	×	×
1	✓	✓	×	✓	×	×
1	✓	×	×	×	✓	✓
7	4	4	0	1	1	1

Table 4.52. Table demonstrating the different endpoints noted in the Basiliximab cohort

It was interesting to note that in contrast to patients in the alemtuzumab group, a high 3 month %B-cell was associated with meeting the composite endpoint ($CD19^+$ 7.64% CEP vs 4.29% CEN, p=0.01, Figure 4.32a and
Table 4.55). This was also reflected in the calculated B-cell count, although this did not reach statistical significance (calculated B-cell $0.0853x10^9$/l CEP vs $0.0547x10^9$/l CEN, 0=0.172, Table 4.54). When individual calculated B-cell subsets were reviewed, it was noted that patients with a high calculated 3-month TrB count were more likely to achieve the composite endpoint compared with patients who had a low 3-month TrB count (calculated TrB $0.00572x10^9$/l CEP vs $0.00374x10^9$/l CEN, p=0.012). There were no statistically significant differences in the calculated T1 or T2 subsets, or T1:T2 ratio. ROC curve analysis of 3-month % B-cells defined the optimal cut off between the two groups as %B>6.395, which was associated with a sensitivity of 71% and specificity

79%, and AUC 0.8469, p=0.0112 (Figure 4.32b). When Kaplan Meier curves were constructed using this cut off point, there was early separation between the curves, which then crossed around 500 days after the 3-month blood draw (Figure 4.32c). This was reflected in the results of the curve comparisons; there was a trend towards statistical significance when the Gehan-Breslow-Wilcoxon test, which adds weight to early events, was applied (p=0.0551).

Variable	TOTAL	CEN	CEP	p-value
Number	21	14	7	
Age ($\bar{x}$ years, SD)	46 (16)	48 (14)	42 (19)	0.474
Gender				1.000[1]
M	16 (76%)	11 (79%)	5 (71%)	
F	5 (24%)	3 (21%)	2 (29%)	
Primary Renal Disease				0.833
DM/HTN	5 (24%)	4 (29%)	1 (14%)	
GN	8 (38%)	5 (36%)	3 (43%)	
Inherited	4 (19%)	2 (14%)	2 (29%)	
Infection/Obstruction	3 (14%)	2 (14%)	1 (14%)	
Other	1 (5%)	1 (7%)	0	
Preemptive Transplant?				0.574[1]
Yes	4 (19%)	2 (14%)	2 (29%)	
No	17 (81%)	12 (86%)	5 (71%)	
Donor Type				0.523
DBD	11 (52%)	7 (50%)	4 (57%)	
DCD	6 (29%)	5 (36%)	1 (14%)	
LD	4 (19%)	2 (14%)	2 (29%)	
NHSBT HLA Mismatch Level				0.522
1	6 (29%)	5 (36%)	1 (14%)	
2	3 (14%)	1 (7%)	2 (29%)	
3	9 (43%)	6 (43%)	3 (43%)	
4	3 (14%)	2 (14%)	1 (14%)	
Graft number				0.392
1	16 (76%)	10 (71%)	6 (86%)	

Variable	TOTAL	CEN	CEP	p-value
2	2 (10%)	1 (7%)	1 (14%)	
3	3 (14%)	3 (21%)	0	
Median CIT (hrs, IQR)	13 (4)	13.4 (6)	13 (7)	0.898
DGF				1.000[1]
Yes	1 (5%)	1 (7%)	0	
No	20 (96%)	13 (93%)	7 (100%)	
CNI variability 0-3 months ($\bar{x}$, SD)	30 (15)	29 (18)	31 (10)	0.711
CNI variability 3-18 months ($\bar{x}$, SD)	24 (8)	23 (8)	25 (7)	0.686

Table 4.53. Basiliximab patient demographics stratified according to whether the composite endpoints were met during follow up.

[1]Fishers exact test. IQR – interquartile range, SD – standard deviation, $\bar{x}$ – mean

Lymphocyte Subset (Calculated, x10^9/L)	Median Value CEN	IQR	Median Value CEP	IQR	p value
$CD19^+$ B-cells (CEN n=14, CEP n=7)	0.0547	0.0467	0.0853	0.137	0.172
$CD19^+CD27^-IgD^+$ Naïve (CEN n=14, CEP n=7)	0.0424	0.0255	0.0599	0.113	0.197
$CD19^+CD27^+IgD^+$ Non-Switched Memory (CEN n=14, CEP n=7)	0.00321	0.00840	0.0130	0.0178	0.535
$CD19^+CD27^+IgD^-$ Class switched Memory (CEN n=14, CEP n=7)	0.00467	0.00464	0.00928	0.00933	0.322
$CD19^+CD24^{hi}CD38^{hi}$ Transitional B-cells (CEN n=14, CEP n=7)	0.00374	0.00417	0.00525	0.00589	**0.012**
$CD19^+CD24^{+++}CD38^{+++}$ T1 (CEN n=14, CEP n=7)	0.0005	0.0012	0.0012	0.0049	0.094
$CD19^+CD24^{++}CD38^{++}$ T2 (CEN n=14, CEP n=7)	0.0033	0.0029	0.0046	0.0011	0.056

Table 4.54. Comparison of 3-month calculated B-lymphocyte subsets between CEN and CEP patients.

Comparisons were made using the Mann Whitney Test. CEN – composite endpoint negative, CEP – composite endpoint positive, IQR – interquartile range

Lymphocyte Subset (%gated)	Median Value CEN	IQR	Median Value CEP	IQR	p value
$CD19^+$ B-cells (CEN n=14, CEP n=7)	4.29	3.61	7.64	2.64	**0.01**
$CD19^+CD27^-IgD^+$ Naïve (CEN n=14, CEP n=7)	73.0	27.83	81.4	22.51	0.743
$CD19^+CD27^+IgD^+$ Non-Switched Memory (CEN n=14, CEP n=7)	9.13	15.06	10.3	12.82	0.971
$CD19^+CD27^+IgD^-$ Class switched Memory (CEN n=14, CEP n=7)	9.70	11.95	6.7	9.59	0.743
$CD19^+CD24^{hi}CD38^{hi}$ Transitional B-cells (CEN n=14, CEP n=7)	7.3	9.78	6.35	28.91	0.799
$CD19^+CD24^{+++}CD38^{+++}$ T1 (CEN n=14, CEP n=7)	14.7	14.6	22.5	47.23	0.255
$CD19^+CD24^{++}CD38^{++}$ T2 (CEN n=14, CEP n=7)	85.0	22.8	76.6	76.6	0.287
T1:T2 Ratio (CEN n=14, CEP n=7)	0.173	0.361	0.293	1.29	0.233
$CD27^+CD38^+$Plasmablasts (CEN n=14, CEP n=7)	0.14	0.21	0.15	0.09	0.535
Switched Mem:NS Mem Ratio (CEN n=14, CEP n=7)	0.600	1.95	0.774	0.740	0.913

Table 4.55. Comparison of 3-month %gated B-lymphocyte subsets between CEN and CEP patients.

T1:T2 and S:NS ratios were calculated for each individual patient. The median values and interquartile range (IQR) for each group are included in the table. Comparisons were made using the Mann Whitney Test. CEN – composite endpoint negative, CEP – compositive endpoint positive

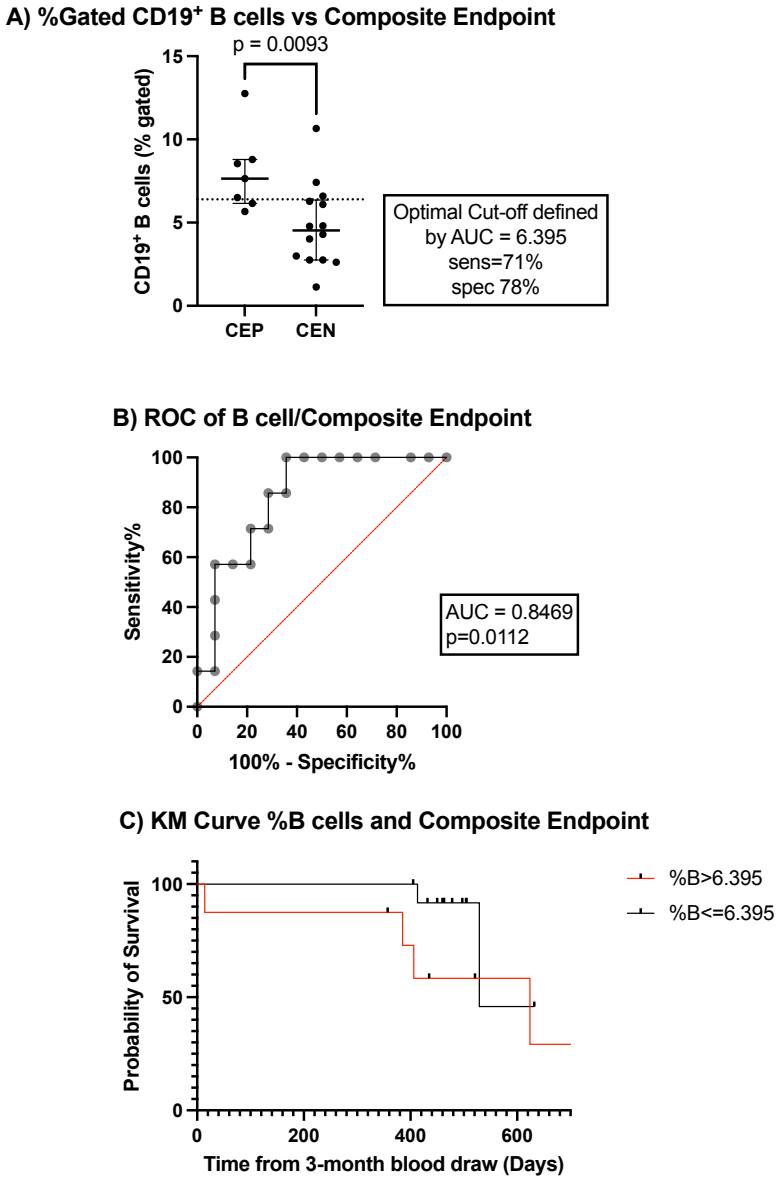


Figure 4.32. Assessment of 3-month %Gated CD19⁺ B-cells as a biomarker of clinical outcome.

Patients were divided into 2 groups according to whether they met or did not meet the composite endpoint. This included graft loss, recurrent proteinuria, >30% reduction in eGFR from 3-18 months, de novo DSA, biopsy proven rejection, recurrent disease. A) Dot plots comparing the %gated B-cells between the two groups. Individual values, median and interquartile ranges are shown. B) ROC curve constructed from the 3-month %gated CD19⁺ B-cells. C) Kaplan Meier estimates of composite-endpoint-free survival in patients stratified according to high and low %B-cells. CEN – composite endpoint negative, CEP – composite endpoint positive

The differences in calculated 3-month TrBs were investigated further. ROC curve analyses defined the optimum cut off value to differentiate the two groups as $0.004573x10^9$/l, with a sensitivity of 100%, specificity 71% and AUC 0.8367, p=0.0138 (Figure 4.33a and Figure 4.33b). When Kaplan Meier estimates were constructed (Figure 4.33c), patients with high calculated TrBs>$0.004573x10^9$/l were associated with a reduced composite-endpoint-free survival (log rank 0.0348).

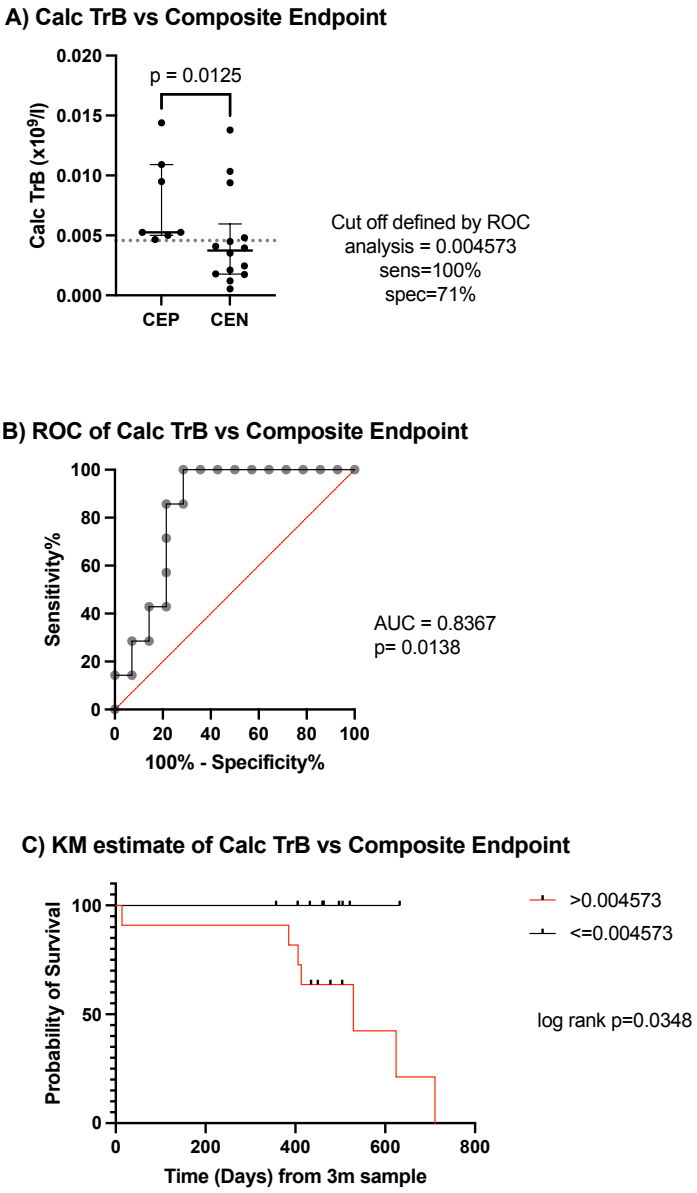


Figure 4.33 Assessment of 3-month calculated $CD19^{+}CD24^{+}CD38^{+}$ transitional B-cells (TrBs) as a biomarker of clinical outcome.

Patients were divided into 2 groups according to whether they met or did not meet the composite endpoint (including graft loss, recurrent proteinuria, >30% reduction in eGFR from 3-18 months, de novo DSA, biopsy proven rejection, recurrent disease). A) Dot plots comparing the calculated TrBs between the two groups. Individual values, median and interquartile ranges are shown. B) ROC curve constructed from the 3-month calculated TrBs C) Kaplan Meier estimates of composite-endpoint-free survival in patients stratified according to high and low calculated TrBs.

4.2.1.4.2 GRAFT SURVIVAL

One out of 21 patients lost their graft (at approximately 22 months). This patient had 2 biopsies during their follow up, and each was reported as BK virus nephropathy and interstitial fibrosis/tubular atrophy (IFTA) grade 1. No differences were noted in this patient's 3-month B-cell phenotype (calculated and % gated) compared with the rest of the cohort.

4.2.1.4.3 REJECTION

One patent was found to have evidence of chronic active antibody mediated rejection on biopsy approximately 14 months post-transplant. They were offered a biopsy because of new onset recurrent proteinuria. The biopsy was reported as g2-3, cg1, mm1, t0, ct0, i0, ti1, ci1, v0, cv1-2, aah1, ptc1 c4d0, polyoma virus 0. There was additionally a *de novo* DQA DSA, therefore the patient was treated with pulsed steroids and 7 sessions of plasma exchange. Mycophenolate mofetil and prednisolone were added to their maintenance immunosuppression. When their 3-month B-cell phenotype was analysed, no statistically significant differences were noted (calculated and %gated) between this patient, and the rest of the basiliximab cohort.

4.2.1.4.4 RECURRENT PROTEINURIA

Five out of 21 patients developed recurrent proteinuria during the follow up period. A trend towards increased 3-month %B-cells was noted in patients who subsequently developed recurrent proteinuria compared with those without recurrent proteinuria (%Gated B-cells 7.5% recurrent proteinuria vs 4.8% no proteinuria, p=0.06, Table 4.56).

Lymphocyte Subset (%gated)	Median Value No Recurrent Proteinuria	IQR	Median Value Recurrent Proteinuria	IQR	p value
$CD19^+$ B-cells (NP n=16, RP n=5)	4.81	4.14	7.53	5.46	0.06
$CD19^+CD27^-IgD^+$ Naïve (NP n=16, RP n=5)	72.7	26.5	85.2	23.2	0.858
$CD19^+CD27^+IgD^+$ Non-Switched Memory (NP n=16, RP n=5)	9.13	12.8	9.15	16.9	0.858
$CD19^+CD27^+IgD^-$ Class switched Memory (NP n=16, RP n=5)	9.93	11.1	4.77	6.73	0.370
$CD19^+CD24^{hi}CD38^{hi}$ Transitional B-cells (NP n=16, RP n=5)	7.16	9.62	5.86	24.0	0.654
$CD19^+CD24^{+++}CD38^{+++}$ T1 (NP n=16, RP n=5)	14.6	23.6	23.6	44.1	0.591
$CD19^+CD24^{++}CD38^{++}$ T2 (NP n=16, RP n=5)	85.0	22.1	76.1	45.0	0.654
T1:T2 Ratio (NP n=16, RP n=5)	0.173	0.345	0.313	1.08	0.591
$CD27^+CD38^+$Plasmablasts (NP n=16, RP n=5)	0.150	0.20	0.125	1.16	0.720
Switched Mem:NS Mem Ratio (NP n=16, RP n=5)	0.774	1.63	0.531	0.72	0.698

Table 4.56. Comparison of 3-month B-cell phenotype and the development of recurrent proteinuria in Basiliximab induction patients.

Recurrent proteinuria is defined as a urine protein:creatinine ratio (UPCR) >50 on two or more occasions. T1:T2 and S:NS ratios were calculated for each individual patient. The median values and interquartile range (IQR) for each group are included in the table.

As proteinuria can be caused by multiple different processes, patient files and biopsy data were reviewed to determine the possible cause of proteinuria. As described in Table 4.57, the causes were heterogenous and could be considered a mixture of immune-mediated and other causes.

Patient ID	Primary Disease	Biopsy?	Results	Converted to sirolimus?
25	IgAN	Yes x3	1) No abnormality 2) Minimal patchy ATN 3) Recurrent IgA	No
33	Unknown	Yes x1	FSGS	No
34	Pyelonephritis	Yes x2	1) BK nephropathy 2) BK nephropathy	No
51	Polycystic kidney disease	Yes x1	Antibody mediated rejection	No
80	Primary membranous	No	(En bloc transplant. Not biopsied due to kidney size. Proteinuria presumed to be secondary to hyperfiltration). Proteinuria resolved 3yrs post-transplant	No

Table 4.57. Possible explanations for recurrent proteinuria in Basiliximab cohort

4.2.1.4.5 DEVELOPMENT OF *DE NOVO* HLA ANTIBODIES AND DSAS

Thirteen out of 21 patients developed *de novo* HLA antibodies, and 4 patients developed *de novo* donor specific antibodies. These included both HLA class I and class II DSAs. No statistically significant associations were noted (either calculated or %gated) with the B-cell phenotype and the development of *de novo* HLA antibodies or DSAs.

4.2.1.4.6 RENAL FUNCTION

No statistically significant correlations were noted between eGFR and B-cell subsets.

4.2.1.4.7 BASILIXIMAB- 3 MONTH SUMMARY

Compared with the alemtuzumab cohort, fewer patients received basiliximab as an induction agent, therefore fewer events were documented during the follow up period. Despite this, some differences were noted when considering outcomes.

Composite Endpoint

Basiliximab patients who had high 3-month %gated B-cells were more likely to meet the composite endpoint during follow up. Similarly, these patients had a higher calculated B-cells than those who did not meet the composite endpoint; however this difference was not statistically significant. This observation was in contrast to that observed for the alemtuzumab patients – where those who met the composite endpoint were found to have fewer B-cells at 3 months than those who did not meet the composite endpoint.

High calculated transitional B-cells at 3months was also found to be associated with meeting the composite endpoint in the basiliximab cohort, although no differences were noted in the T1 or T2 subsets. When ROC curve analysis was performed, a high 3-month calculated TrB > 0.004573×10^9/l was the better marker of outcome, with a sensitivity 100%, specificity 71%, AUC=0.8367, p=0.0138. When the Kaplan Meier estimates were constructed, this cut-off resulted in curve separation, and patients with a high 3-month TrB count were found to have a reduced composite-endpoint-free survival (p=0.00348 log rank).

Recurrent proteinuria

A trend towards increased %B-cells was also noted in patients who developed proteinuria, however this difference was not statistically significant.

When other individual endpoints (graft survival, rejection free survival, the development of de novo HLA antibodies and DSAs) were assessed, no statistically significant differences were noted in the B-cell phenotype.

4.2.1.5 BASILIXIMAB INDUCTION – 6 MONTH OUTCOMES

Due to smaller patient numbers at this point onwards, when statistical analyses were performed, a p value <0.1 was considered statistically significant.

4.2.1.5.1 RECURRENT PROTEINURIA

4 patients removed due to recurrent proteinuria occurring between 3-6 months. Of the remaining 18 patients, 3 subsequently developed recurrent proteinuria. No statistically significant differences were noted in the %gated phenotype between the two groups. Patients who developed recurrent proteinuria had more calculated $CD19^+$ B-cells (RP $0.102x10^9$/l B-cells vs no RP $0.0502x10^9$/l, p=0.039) of which there were more naïve cells at 6 months ($CD19^+CD27^+IgD^+$ Naïve: $0.0955x10^9$/l vs $0.0452x10^9$/l, p=0.039, Table 4.58).

Lymphocyte Subset (Calculated, x10^9/L)	Median Value No RP	No RP IQR	Median Value RP	RP IQR	p value
$CD19^+$ B-cells (No RP n=15, RP n=3)	0.0502	0.0589	0.102	-	**0.039**
$CD19^+CD27^-IgD^+$ Naïve (No RP n=15, RP n=3)	0.0452	0.048	0.0955	-	**0.039**
$CD19^+CD27^+IgD^+$ Non-Switched Memory (No RP n=15, RP n=3)	0.00331	0.00816	0.00939	-	0.130
$CD19^+CD27^+IgD^-$ Class switched Memory (No RP n=15, RP n=3)	0.00309	0.00349	0.00660	-	0.164
$CD19^+CD24^{hi}CD38^{hi}$ Transitional B-cells (No RP n=15, RP n=3)	0.00839	0.0138	0.00510	-	1.000
$CD19^+CD24^{+++}CD38^{+++}$ T1 (No RP n=15, RP n=3)	0.00239	0.00395	0.00130	-	1.000
$CD19^+CD24^{++}CD38^{++}$ T2 (No RP n=15, RP n=3)	0.00156	0.00311	0.000958	-	1.000
$CD19^+CD27^+CD38^+$ Pbs (No RP n=15, RP n=3)	0.0012	0.000235	0.0025	-	0.738

Table 4.58. Comparison of 6-month calculated B-cell phenotype and the development of recurrent proteinuria in Basiliximab induction patients. Recurrent proteinuria (RP) is defined as a urine protein:creatinine ratio, UPCR >50 on two or more occasions. IQR – interquartile range

The calculated B lymphocytes, and calculated naïve cells were investigated further as shown in Figure 4.34 and Figure 4.35.

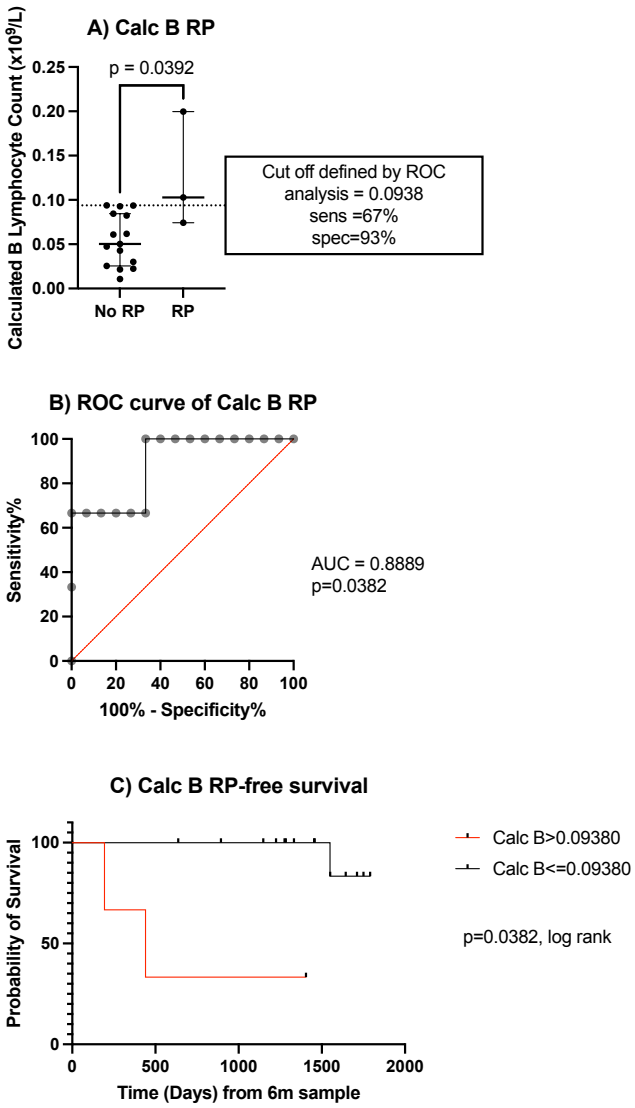


Figure 4.34 6-month calculated CD19⁺ B lymphocytes as a biomarker of recurrent proteinuria.

Patients were divided into two groups according to the development of recurrent proteinuria (RP) defined by 2 x UPCR readings >50). A) Dot plots comparing the calculated B lymphocyte count between the two groups with individual values, median and interquartile range shown B) ROC curve C) Kaplan Meier estimates of recurrent proteinuria-free survival using cut offs defined by ROC curve analysis

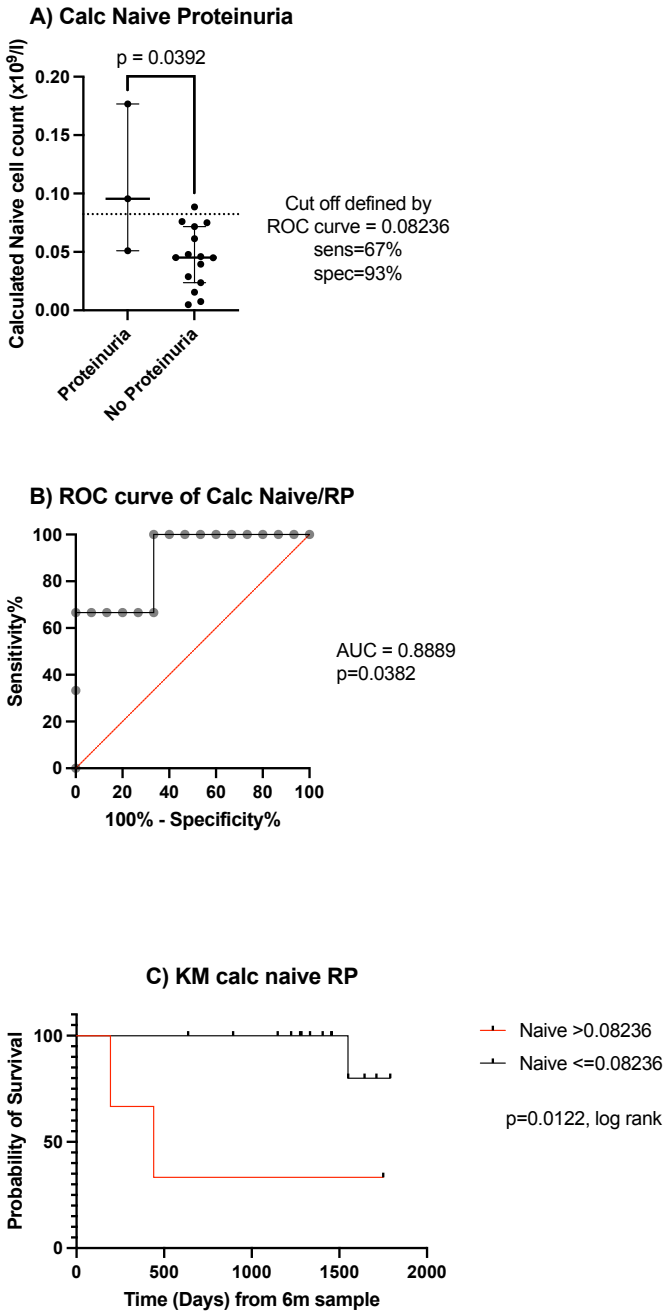


Figure 4.35. Assessment of the 6-month calculated $CD19^{+}CD27^{-}CD38^{+}$ Naïve Cells as a biomarker of Recurrent Proteinuria.

Patients were divided into two groups according to the development of recurrent proteinuria (2x UPCR >50). A) Dot plots with Mann Whitney test comparing the two groups with individual values, median and IQR shown B) ROC curve analysis C) Kaplan Meier Estimates using the cut off values defined by ROC curve analysis

A high calculated 6-month B-cell count > 0.09380x10^9/l was associated with recurrent proteinuria, with a sensitivity of 67%, specificity 93% and AUC =0.8889, p=0.0382. When Kaplan Meier estimates were constructed, a high B-cell count was associated with a reduced recurrent proteinuria-free survival (hazard ratio 19, CI 0.4011-909.9, p=0.0005 log rank, Figure 4.34). Similarly, a high calculated naïve count >0.08236x10^9/l was associated with recurrent proteinuria with a sensitivity of 67%, specificity 93%, AUC =0.8889, p=0.0382. When Kaplan Meier estimates were performed a high naïve count was associated with reduced proteinuria-free survival with a hazard ratio of 11.3, CI 0.4766-268.1, p=0.0122, log rank (Figure 4.35).

4.2.1.5.2 GRAFT SURVIVAL

No significant differences were noted in the B-cell phenotype (%gated and calculated) when graft survival and graft loss patients were compared. The graft loss (GL) patient's %TrB and T1:T2 ratio were tracked alongside the median %TrB and T1:T2 ratio values obtained from the basiliximab cohort (Figure 4.36). Between 3-8 months post-transplant, the GL patient had fewer %TrBs, but a higher T1:T2 ratio than the median for the basiliximab cohort, however beyond 12 months post-transplant, this trend reversed, and the GL patient had more %TrBs with a lower T1:T2 ratio than the cohort average. This occurred after biopsies confirming BK virus nephropathy. It is possible that this change in phenotype occurred following a change to immunosuppression, or as a result of the viral infection.

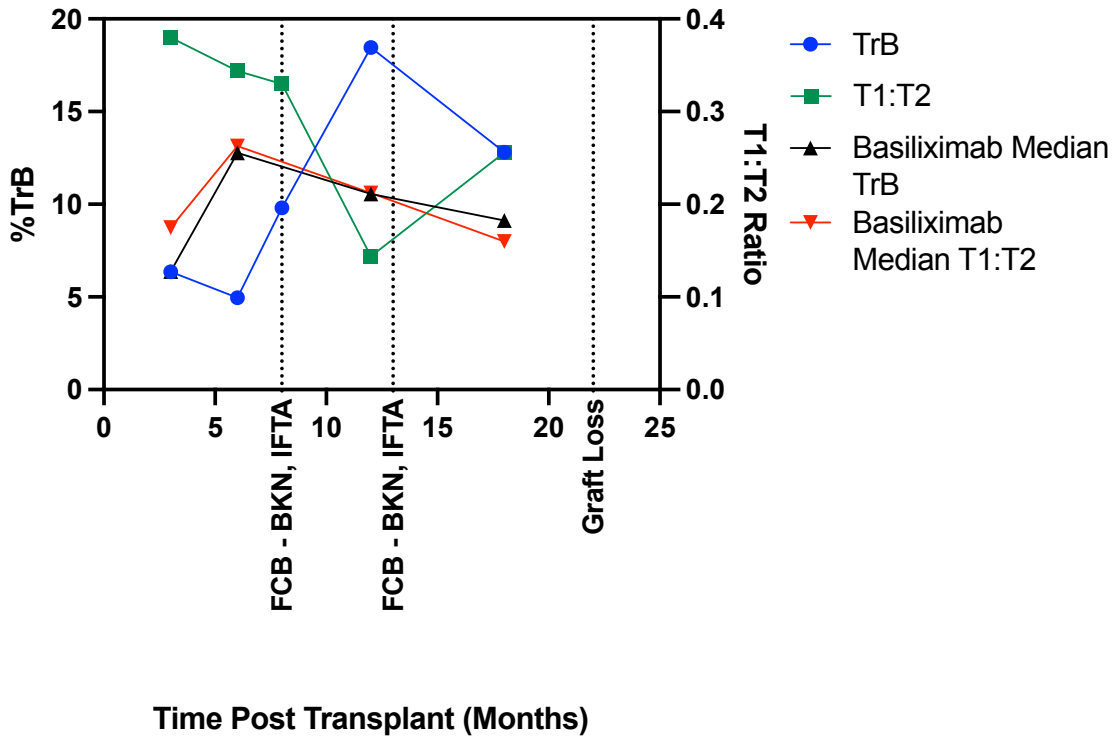


Figure 4.36. The post-transplant transitional B-cell population in a patient with graft loss.

The patient's TrB phenotype is tracked with the median values for %TrB and T1:T2 ratio obtained from the basiliximab cohort at similar time points. FCB – for cause biopsy, BKN – BK nephropathy, IFTA – interstitial fibrosis and tubular atrophy

4.2.1.5.3 REJECTION

No significant differences were noted in the B-cell phenotype (%gated and calculated) when the rejection (n=1) and no rejection groups (n=19) were compared. Although no statistically significant differences were noted between the rejection patient and no rejection cohort at 3 and 6 months, it was interesting to see how the individual values obtained from the rejection patient compared to the median values obtained from the basiliximab cohort (Figure 4.37). Although 3-month %TrB and T1:T2 ratio were higher than the median values obtained from the basiliximab cohort, the T1:T2 ratio reduced and remained below the median cohort value from 6 months. The %TrB in the rejection patient reduced and remained below the median %TrB obtained from the basiliximab cohort at 12-months. This occurred 2 months before the patients was

offered a biopsy which demonstrated features associated with acute antibody mediated rejection.

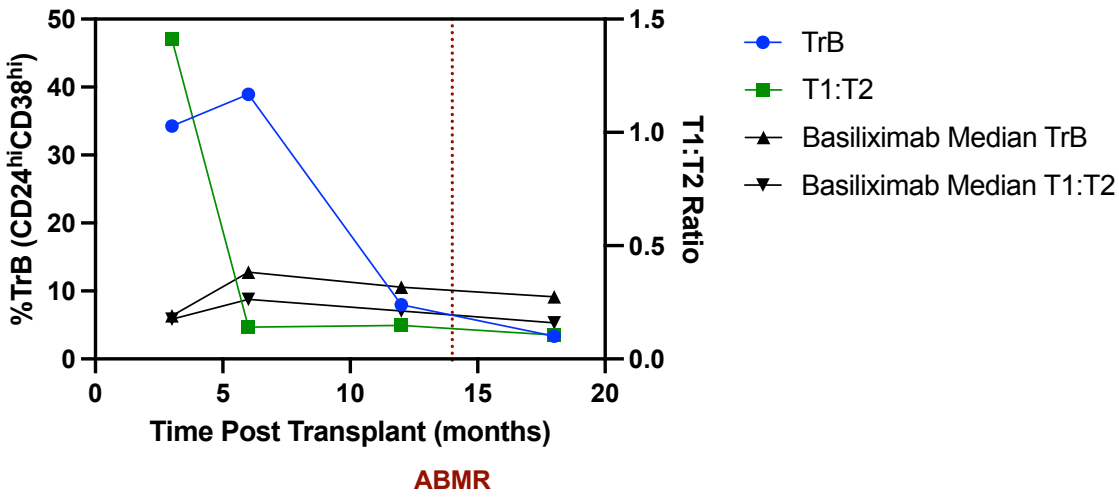


Figure 4.37. The post-transplant transitional B-cell population in a basiliximab patient with rejection.

No other statistically significant associations were noted when other outcomes were investigated.

No further comparisons were made using 12-month time points due to low sample and event numbers.

4.2.1.6 SUMMARY OF PROSPECTIVE FINDINGS

Table 4.59 summarises the statistically significant findings from the prospective ALBERT study.

	Endpoints	3 months	6 months	12 months
Alemtuzumab	CEP	↓ % $CD19^+$ B cells ↑ % $CD27^+CD38^-$ Mem ↑ % $CD27^+IgD^+$ NS mem	↓ T1:T2 ratio ↑ S:NS mem ratio	No significant differences
	Graft Loss	↓ calc $CD19^+$ B cells ↓ calc $CD27^-IgD^+$ Naïve ↓ calc $CD24^{+++}CD38^{+++}$ T1	No differences	↓ T1:T2 ratio
	Rejection	↑ % $CD24^{hi}CD38^{hi}$ TrBs ↓ T1:T2 ratio	↓ calc and % $CD27^+IgD^+$ NS ↓ calc $CD24^{hi}CD38^{hi}$ TrBs ↓ calc $CD24^{+++}CD38^{+++}$ T1 ↓ calc $CD24^{++}CD38^{++}$ T2 ↓T1:T2 ratio ↑ S:NS ratio	No significant differences
	Disease recurrence	No significant differences	No significant differences	No significant differences
	Recurrent Proteinuria	No significant differences	↓T1:T2 ratio	No significant differences
	De novo DSA	No significant differences	No significant differences	No significant differences
	Renal function	Correlated with calc $CD27^+IgD^-$ switched mem	No significant correlation	No correlation
Basiliximab	CEP	↑ %CD19 ↑ calc $CD24^{hi}CD38^{hi}$ TrB	No significant differences	
	Graft Loss	No significant differences	No significant differences	
	Rejection	No significant differences	No significant differences	
	Disease recurrence			
	Recurrent Proteinuria	No significant differences	↑calc $CD19^+$ ↑calc $CD27^-IgD^+$Naïve	
	De novo DSA	No significant differences	No significant differences	
	Renal function	No significant correlation	No significant correlation	

Table 4.59. ALBERT Prospective Study Results: Summary of Statistically Significant Findings.

CEP – composite endpoint positive, DSA – donor specific antibodies, NS – non switched memory, TrB – transitional B cell, S:NS – switched:non-switched memory B-cell, calc- calculated

4.2.2 FOR CAUSE BIOPSIES – ALBERT STUDY GROUP 2

Twenty-seven patients with a troubled graft were offered a 'for cause' biopsy at a median of 3 years (IQR 6 years) after transplantation. Indications for a biopsy included deterioration in renal function, and new onset proteinuria. The median eGFR was 19ml/min/1.73m^2 (interquartile range [IQR] 16.5), median urea 19mmol/l (IQR 13) and median UPCR was 68.6mg/mmol, (IQR 16.5). Blood samples were collected on the day of biopsy to determine the surface B-cell phenotypes, and the presence of donor specific antibodies (DSAs). Four patients were excluded from this study as biopsies were performed within the first transplant year. This was to avoid any inherent differences associated with the repopulation of B-cells following alemtuzumab induction (Figure 4.6). Two further samples were excluded as the proportion of B-cells obtained were <1%. Table 4.60 describes the demographics for patients who received a late (>1yr) for cause biopsy and were included in the subsequent analysis.

Variable	Study Population
Number	21
Recipient Age (yr, med + IQR)	41 (27)
Recipient Gender	
Male	12 (57.1%)
Female	9 (43%)
Recipient Ethnicity	
Caucasian	19 (90.5%)
Asian	1 (4.8%)
Chinese	1 (4.8%)
Cause of ESRD	
Diabetes and Hypertension	1 (4.8%)
Glomerulonephritis	8 (38.1%)

Variable	Study Population
Inherited	4 (19%)
Other	4 (19%)
Unknown	4 (19%)
Donor Type	
LD	4 (19%)
DBD	10 (47.6%)
DCD	7 (33.3%)
Graft Number	
1	18 (85.7%)
2	2 (9.5%)
3	1 (4.8%)
NHSBT HLA Mismatch Level	
1	1 (4.8%)
2	5 (23.8%)
3	10 (47.6%)
4	5 (23.8%)
Induction Agent	
Alemtuzumab	11 (52.4%)
Basiliximab	3 (14.3%)
Unknown	7 (33.3%)
Maintenance Immunosuppression*	
Tacrolimus	18 (85.7%)

Variable	Study Population
Ciclosporin	1 (4.8%)
Sirolimus	1 (4.8%)
MMF	6 (29%)
Azathioprine	4 (19%)
Prednisolone	6 (28.6%)
Baseline eGFR (med, IQR)	19 (16.5)
Baseline UPCR (med, IQR)	68.6 (16.5)
Median Interval: transplant to biopsy (yrs, IQR)	3 (6)
DSA Present	8 (38%)
Allograft rejection on biopsy	10 (48%)

Table 4.60. Demographic characteristics of the final cohort of patients receiving a late for cause biopsy.

***Maintenance immunosuppression at the time of biopsy. UPCR- urine protein-creatinine ratio, IQR – interquartile range, MMF – mycophenolate mofetil**

To investigate whether there was a distinct phenotype associated with rejection, patients were initially divided into two groups according to the histology results R (rejection, Banff classes 2,4 n=10), NR (no rejection, Banff classes 1, 5 and 6, n=9), and B (borderline changes suspicious for T-cell mediated rejection, Banff class 3, n=1)[54]. The B-cell phenotypes were compared using the Mann Whitney test. Figure 4.38 demonstrates the B-cell subsets across the rejection and non-rejection groups. No statistically significant differences were appreciable between the two groups with respect to the proportion of naïve, memory (including class switched and non-switched, class switched:non-switched memory ratio), transitional cells or plasmablasts.

Further analysis focussed on the transitional B-cell subsets as previous studies had highlighted these populations as possible biomarkers of allograft rejection.[141,233] T1 and T2 cells were differentiated using the gating strategy described in Figure 2.5. Patients in the Rejection group were noted to have less T1 cells compared with the No-Rejection group (median %T1 9.465 Rejection, 19.37 No-Rejection, p=0.0355 Mann-Whitney test). Although the rejection group demonstrated more %T2 cells than those with no-rejection, this difference was not statistically significant (median %T2 87.68, median %T1 80.44, p=0.0630, Mann-Whitney test). Overall, patients with rejection had a reduced T1:T2 ratio compared with those without rejection (median T1:T2 ratio 0.11 Rejection, 0.27 no-rejection, p=0.0408, Figure 4.39). Receiver operating characteristic curves were then constructed to determine the optimal cut-off values to differentiate rejection from no-rejection using the different TrB parameters (second panel of Figure 4.39). A T1:T2 ratio of 0.2 was a good marker of differentiating the two groups, with a sensitivity of 80% and specificity of 90%, AUC 0.7700, p=0.0215. The best overall marker of rejection was low %T1 cells <16.56 (sensitivity 90%, specificity 80%, AUC 0.78, p=0.0343). One patient (ID: AL93) was assigned Banff category III (borderline, suspicious for acute T-cell mediated rejection). It was interesting to see that the T1:T2 ratio for this patient was reduced and similar in value to those seen with patients who were in the rejection group.

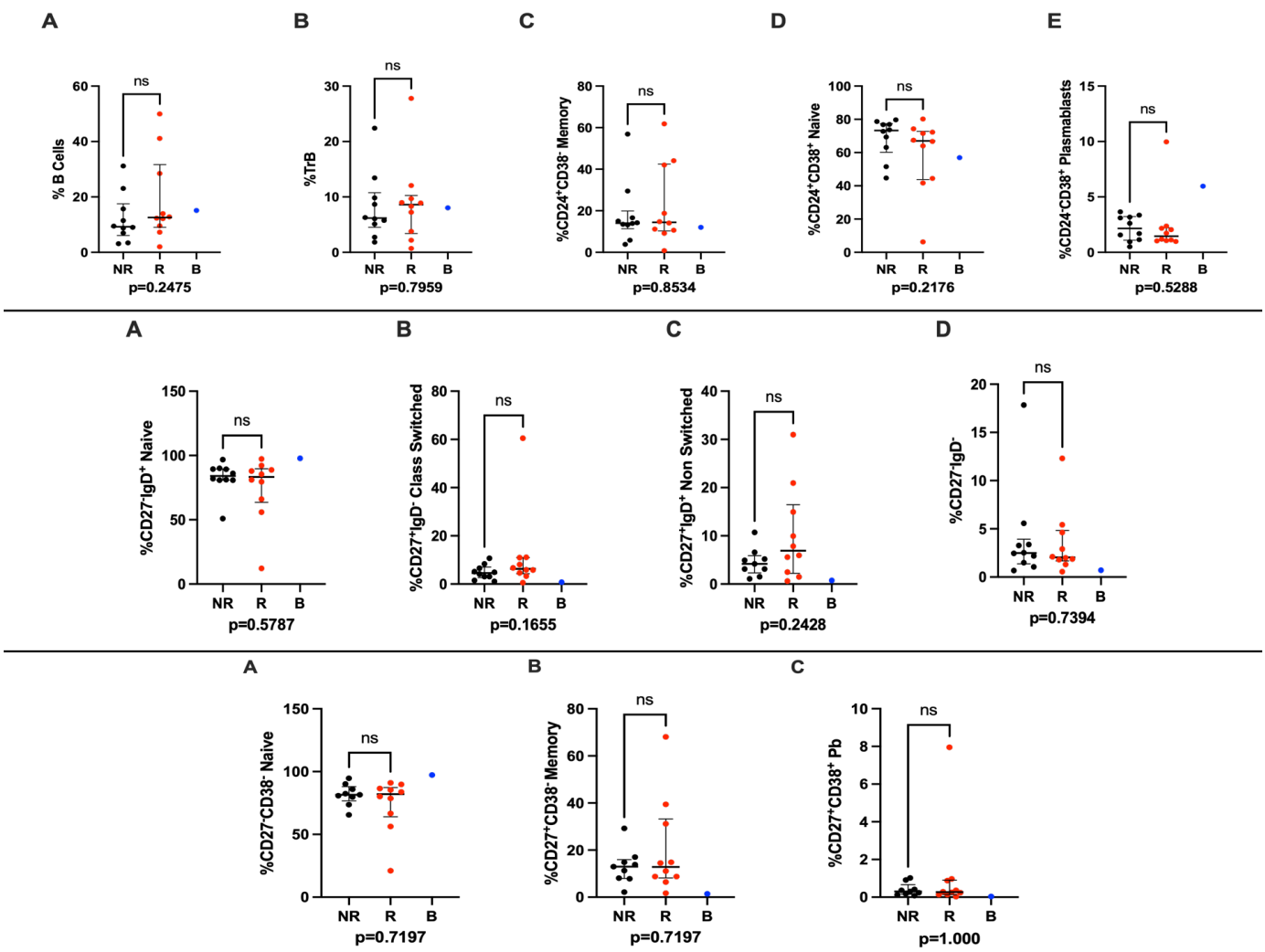


Figure 4.38. B-cell phenotype in patients with a troubled graft.

Whole blood was collected from patients at the time of a 'for cause' biopsy. Patients were separated into groups according to histology results (Rejection -R, No rejection – NR, Borderline -B), and the different B-cell phenotypes were compared. The first panel demonstrates A) the % gated B-cells from the lymphocyte population, and B-D) the different B subsets using CD24 and CD38 expression (B – transitional B-cells, C- memory, D – Naïve, E- plasmablasts). The second panel demonstrates the different B subsets using CD27 and IgD expression: A) Naïve B) Class switched memory C) Non-switched memory D) $CD27^{-}IgD^{-}$. The final panel demonstrates the B subsets using CD27 and CD38 expression: A) Naïve B) Memory C) Pb. Individual values, median and interquartile ranges are shown. Comparisons were made using the Kruskal Wallis test.

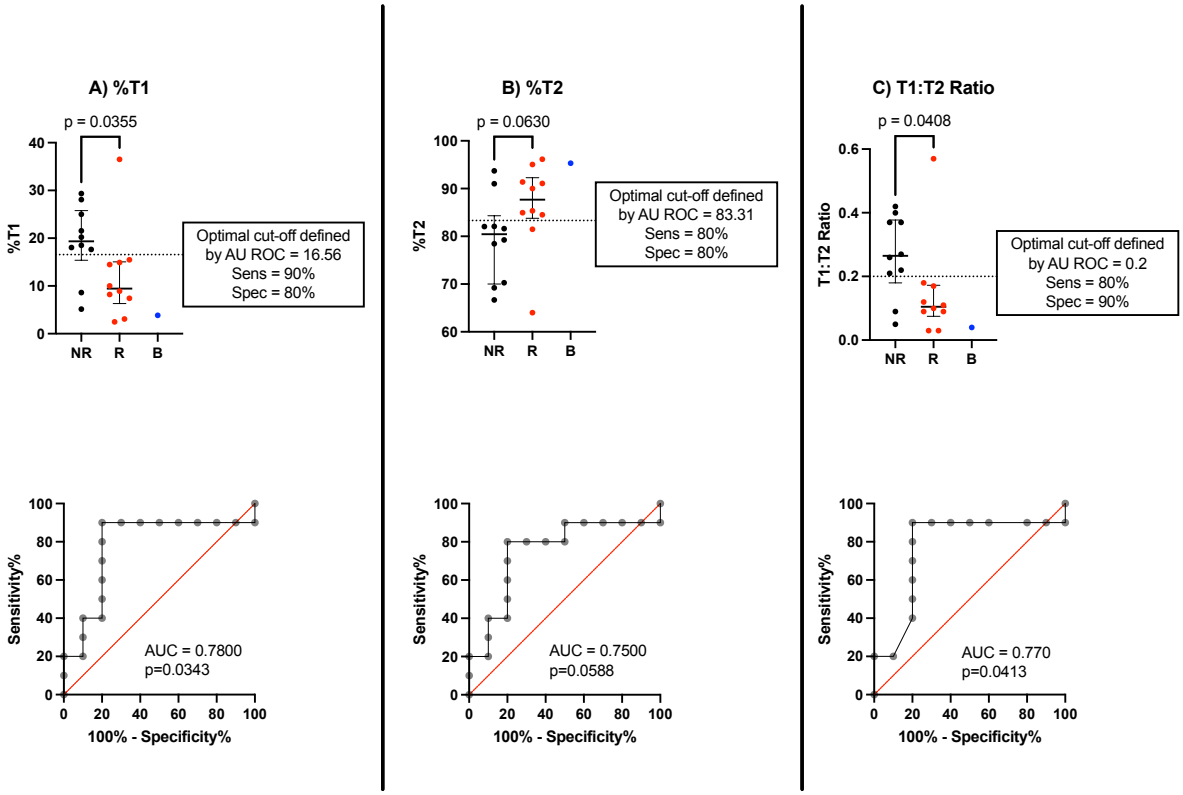


Figure 4.39. Receiver operating characteristic curve analysis of markers of rejection. A) %T1 cells, B) %T2 cells, C) T1:T2.

Patients were divided into groups according to histology results (R – rejection, NR – no rejection, B – borderline). The transitional B-cell populations were assessed from whole blood using flow cytometry. The top panel contains scatter plots with comparisons using the Mann Whitney test between the R and NR groups. Individual results, median and interquartile ranges are shown. The optimal cut-off values defined by the ROC are also demonstrated. The bottom panel shows the ROC analyses including area under the ROC for each population.

4.2.2.1 IN PATIENTS WITH A TROUBLED GRAFT, THERE IS A DISTINCT PHENOTYPE THAT IS ASSOCIATED WITH REDUCED GRAFT SURVIVAL.

Having demonstrated that in patients with a troubled graft, there is a distinct phenotype that is associated with biopsy proven rejection, the next step was to determine whether this phenotype was associated with subsequent allograft loss. Clinical outcomes were obtained over a median follow up of 781 days (range 68 – 1920 days) from biopsy.

Over this period, 10 patients subsequently lost their graft. Patients were divided into two groups (graft survival, GS and graft loss, GL) and their B-cell phenotypes at the time of biopsy (prior to any intervention) were compared. There was no significant difference in the % B-cell obtained when comparing the two groups (Figure 4.40A). Similarly, there was no difference in the proportion of transitional, memory cells, naïve cells or plasmablasts defined by CD24 and CD38 expression (Figure 4.40B-E).

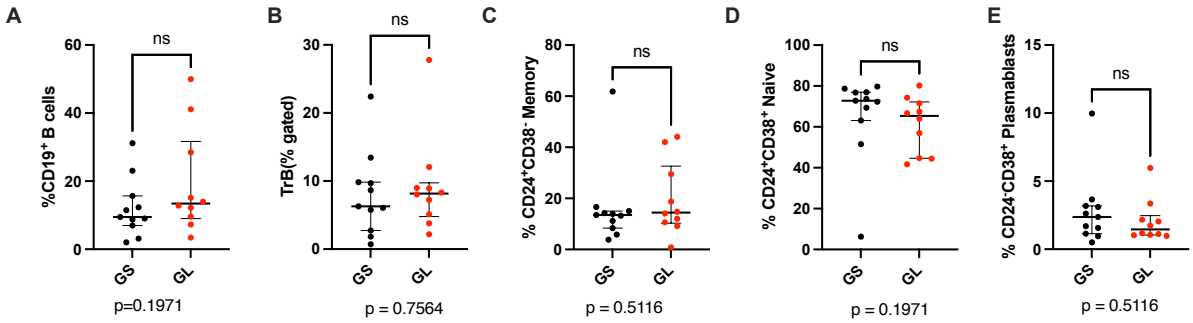


Figure 4.40. Comparison of A) $CD19^{+}$ B-cells, B) $CD24^{++}CD38^{++}$ Transitional B-cells, C) $CD24^{+}CD38^{-}$ Memory, D) $CD24^{+}CD38^{+}$ Naïve, E) $CD24^{-}CD38^{+}$ Plasmablasts in patients offered a late 'for cause' biopsy.

Patients were divided into 2 groups (GS – graft survival, GL – graft loss) according to graft survival during the follow up period. The B-cell phenotype was assessed from whole blood using flow cytometry. Groups were compared using the Mann Whitney test with individual results, median and interquartile range shown.

Analysis within the transitional population however demonstrated significant differences in the T1 and T2 populations. GS patients had a significantly higher proportion of T1 cells (median %T1 18.51 GS, 7.82 GL, p=0.0089), a lower proportion of T2 cells (median %T2 81.63 GS, 91.23 GL, p=0.0288), and subsequently, a higher T1:T2

ratio (median ratio 0.23 GS, 0.09 GL, p=0.0093) compared with the GL patients. Receiver operating characteristic curves (ROC) were constructed to determine the proportion of T1 and T2 cells, and the T1:T2 ratio that would separate the two groups with optimum sensitivity and specificity (Figure 4.41). The best predictors of subsequent allograft loss were the T1:T2 ratio<0.175 which classified cases with a sensitivity 80%, specificity 82% and area under the curve (AUC) 0.8409, p=0.0063 and % gated T1 cells <14.69 (sensitivity of 80% and specificity 82%, AUC = 0.8455, p=0.0075). Figure 4.42 demonstrates the Kaplan-Meier survival curves comparing graft survival in patients with high (>0.175) and low (<0.175) T1:T2 ratios.

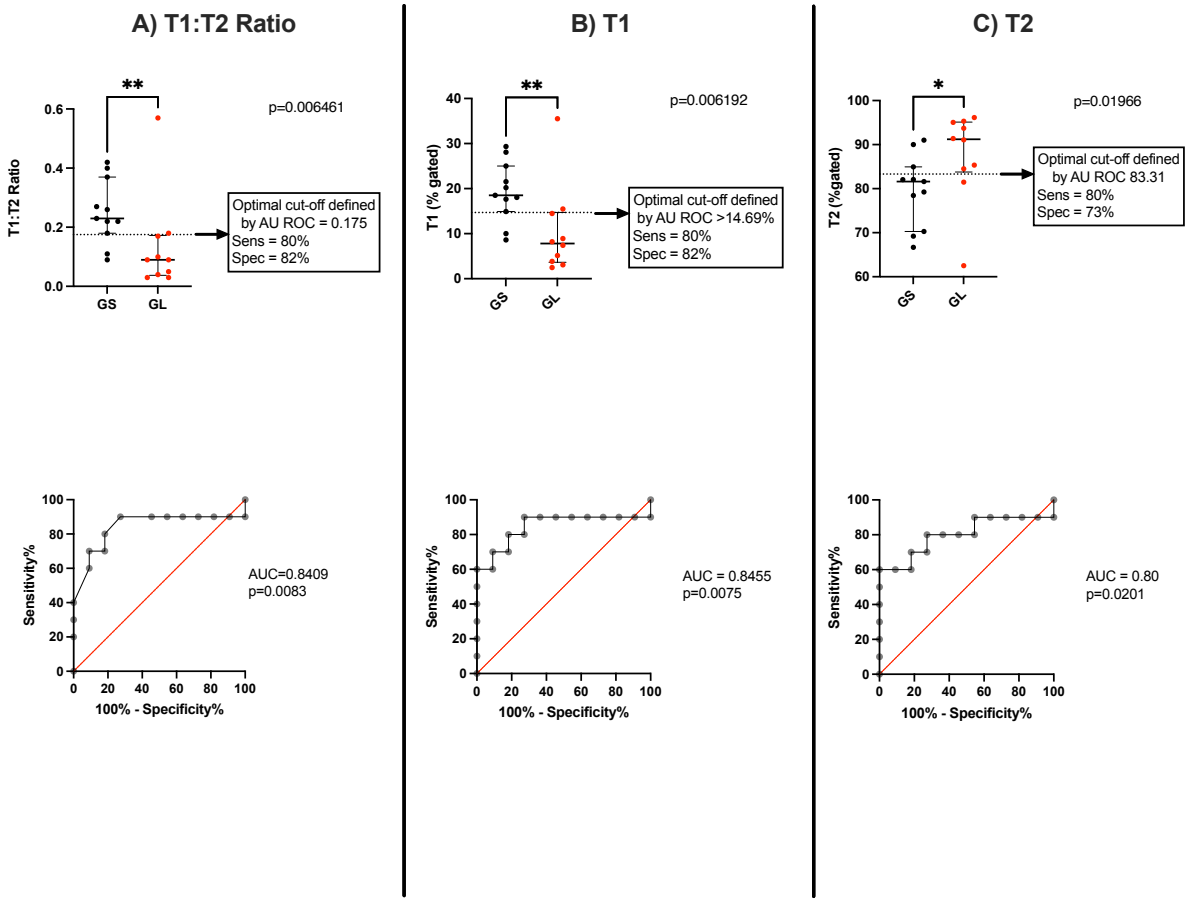
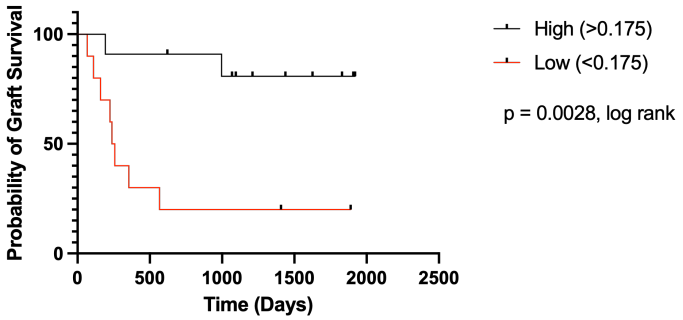


Figure 4.41. Receiver operating characteristic curve analysis as a marker of allograft loss using A) T1:T2 Ratios, B) %T1 and C) %T2 cells.

Patients were divided into 2 groups (GS – graft survival, GL – graft loss) according to graft survival during the follow up period. The transitional B-cell populations were assessed from whole blood using flow cytometry. The top panel demonstrates the comparisons between the two groups using the Mann Whitney test. Individual results, median and interquartile ranges are shown. The optimal cut-off values defined by the ROC are also demonstrated. The bottom panel shows the ROC analyses including area under the ROC for each population.



No. At Risk	At Biopsy (0 days)	500 days	1000 days	1500 days
High (>0.175)	11	11	9	5
Low (<0.175)	10	4	3	2

Figure 4.42. Kaplan Meier Curves estimating graft survival from time of biopsy according to measured T1:T2 Ratios.

Further ROC analyses were performed to compare the utility of the T1:T2 ratio against other currently available biomarkers of graft loss including eGFR at time of biopsy, the presence of DSAs, and proteinuria (Figure 4.43). The only other good biomarker of allograft loss was a low eGFR <21 at the time of biopsy (sensitivity 90%, specificity 81.82%). The calculated area under the curve for eGFR however was reduced compared with the curve generated by the T1:T2 ratio (AUC 0.8091, p=0.0167 for eGFR compared with AUC 0.8409, p=0.0083 for T1:T2 ratio).

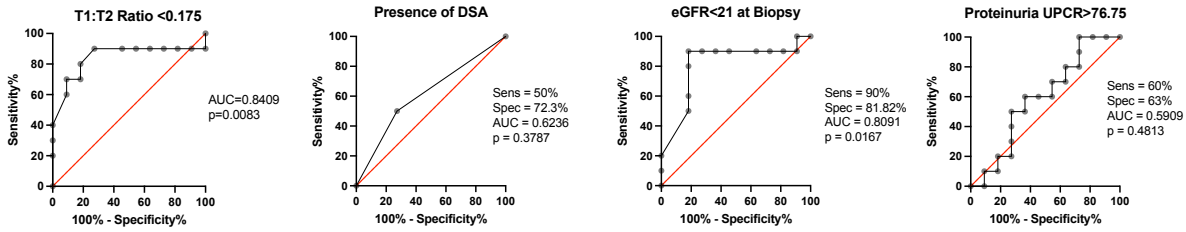


Figure 4.43. ROC analyses comparing the utility of the T1:T2 ratio as a biomarker of subsequent allograft loss with current available biomarkers.

The histology results for these patients were compared according to the T1:T2 ratio at the time of biopsy. Although the differences were not statistically significant, a higher proportion of patients with a low T1:T2 ratio exhibited higher Banff g, ptc, c4d, and t scores compared with those who had a high T1:T2 ratio (Figure 4.46).

Univariate and multivariate analyses were performed to determine other variables that may affect subsequent graft survival. The demographic characteristics of the cohort stratified according to T1:T2 ratio can be viewed in Table 4.61. Variables were entered into a Cox proportional hazards model (see Table 4.62). Although graft type, evidence of rejection on histology, and T1:T2 ratios were significantly associated with reduced graft survival on univariate analysis, an eGFR<20 at the time of biopsy was the only significant variable associated with subsequent graft loss in the final multivariable model (hazard ratio 24.5, p=9.48, p=0.03). When the patients were stratified according to eGFR at the time of biopsy (eGFR<20 and eGFR>20), a low T1:T2 ratio was associated with reduced graft survival when eGFR<20 (but not when eGFR>20), however this was not statistically significant (p=0.081, log rank), see Figure 4.44.

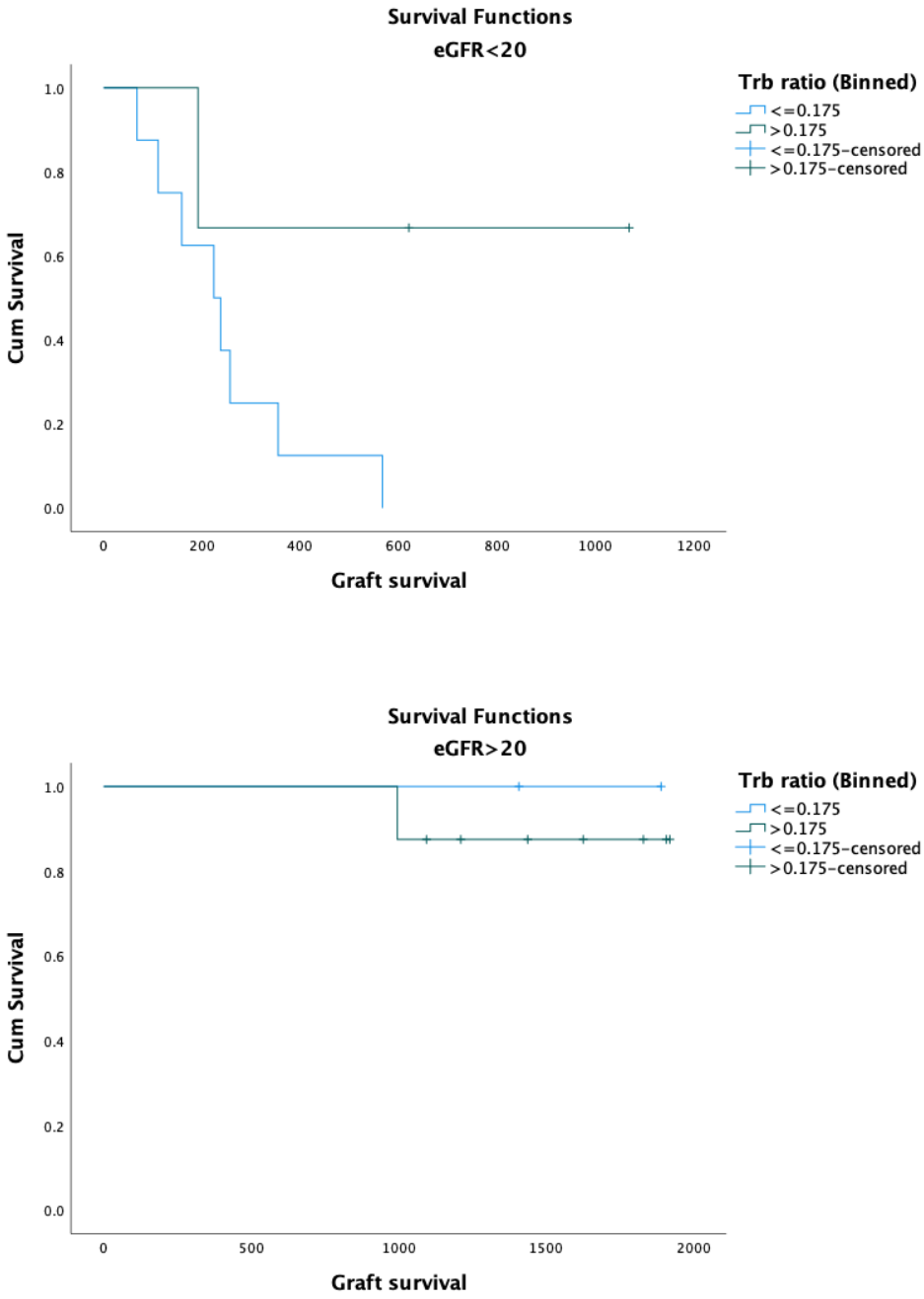


Figure 4.44. Kaplan Meier Curves estimating allograft survival according to T1:T2 ratios in patients who are stratified by renal function.

The top panel shows patients with eGFR<20, the bottom panel eGFR>20.

Further analyses were performed to determine whether there was a correlation between serum urea and different B-cell phenotypes as uraemia has previously been demonstrated to influence B-cell function and maturation.[234] There was no significant relationship between %gated B-cells and serum urea (r= -0.6978, p=0.7638). Similarly, there was no significant relationships between TrBs (r=-0.2773, p=0.2236), T1 (r=-0.2680, p=0.2402), T2 (r=0.1263, p=0.5853) and T1:T2 ratios (r=-0.2089, p=0.3635) when compared with serum urea at the time of biopsy (Figure 4.45).

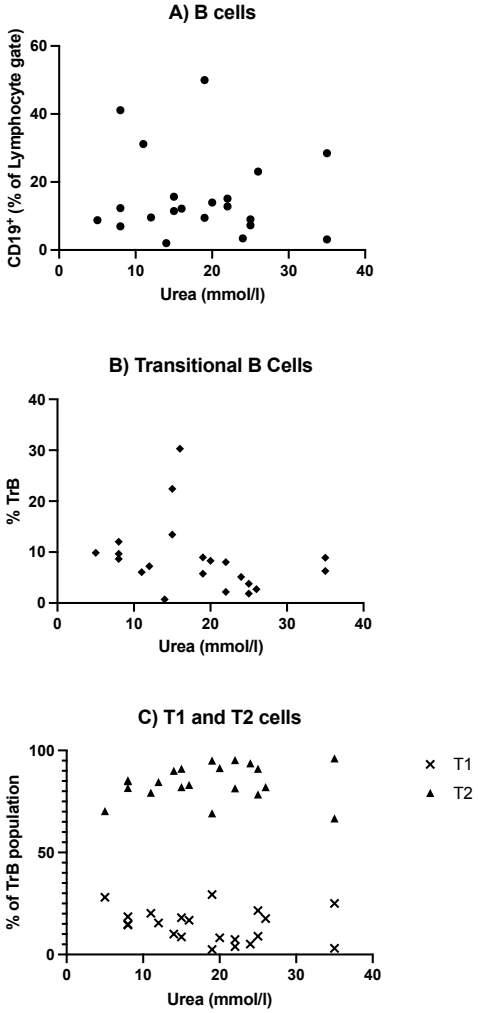


Figure 4.45. Assessment of relationship between serum urea (mmol) and A) B-cells, B) Transitional B-cells, C) T1 and T2 cells in patients who received a 'for cause' biopsy

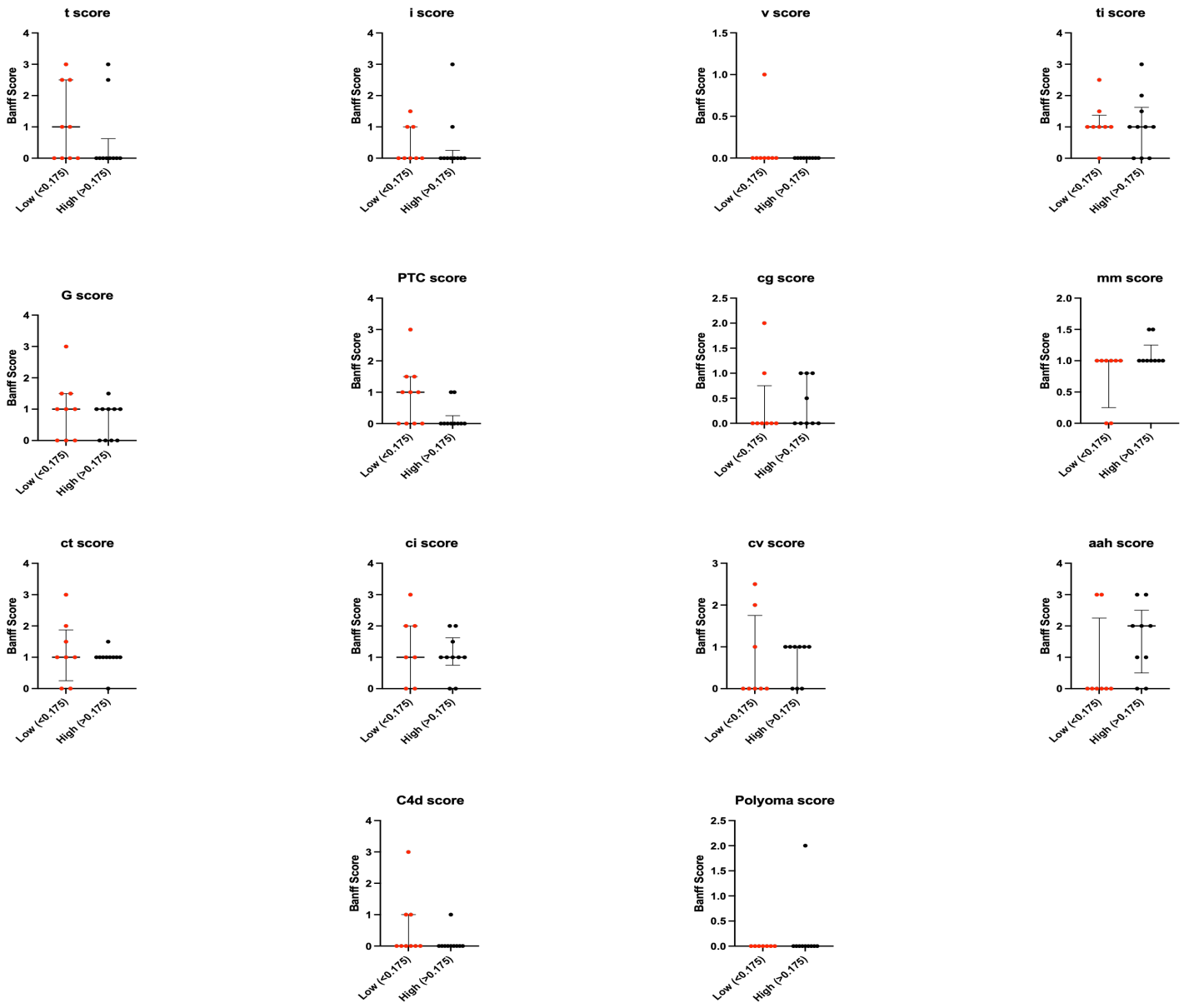


Figure 4.46. Comparison of Banff Histological scores in patients who received a late for cause biopsy. Patients had their B-cell phenotype analysed at the time of biopsy and were grouped according to T1:T2 ratios.

Similar analyses were performed to see if different B-cell subsets were associated with subsequent graft loss. CD27/CD38 (Figure 4.47) and CD27/IgD (Figure 4.48) cell surface expression was considered, however no statistically significant differences were present between the two groups.

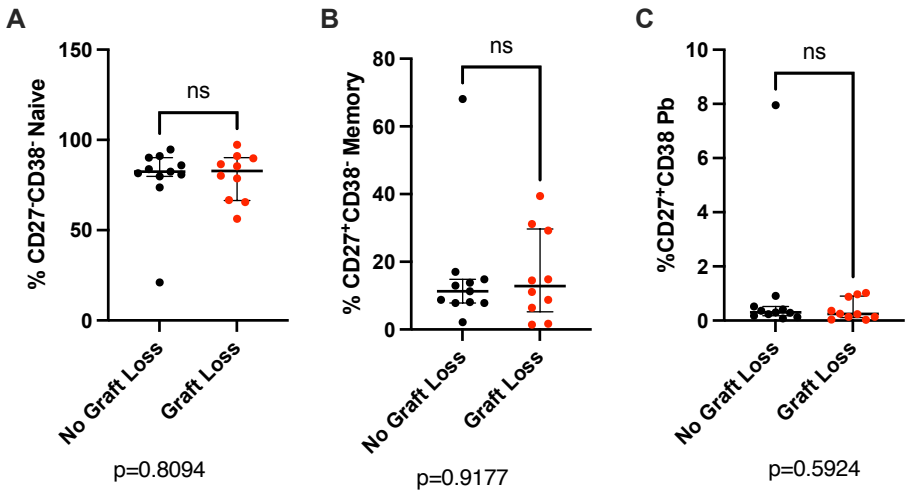


Figure 4.47. Comparison of A) % $CD27^{-}CD38^{-}$ Naïve, B) % $CD27^{+}CD38^{-}$ Memory, C) %$CD27^{+}CD38^{+}$ Plasmablasts in patients undergoing late for cause biopsies. Patients were divided into 2 groups according to whether they subsequently lost their graft in the follow up period. The B-cell phenotype was assessed from whole blood using flow cytometry. Groups were compared using the Mann Whitney test with individual results, median and interquartile range shown.

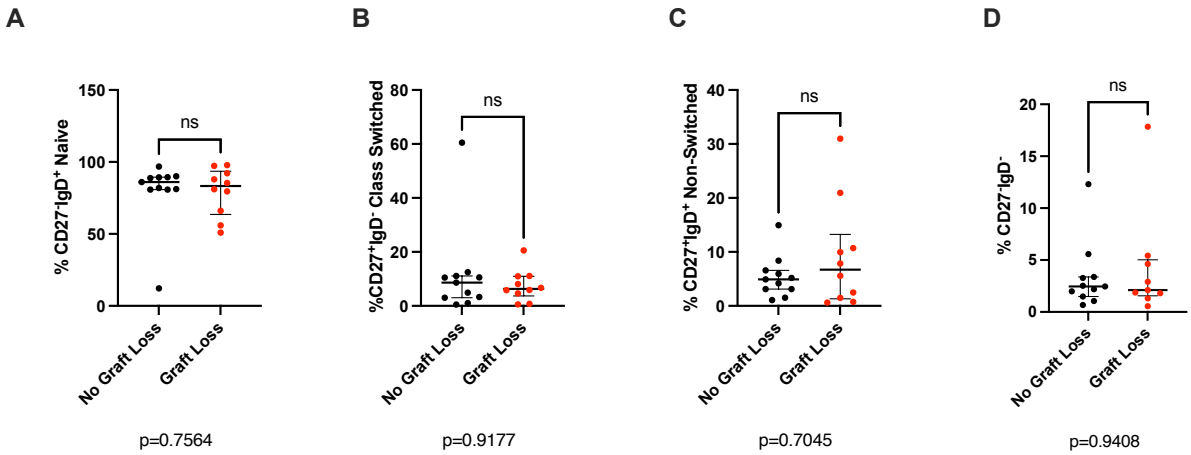


Figure 4.48. Comparison of A) % $CD27^{-}IgD^{-}$ I, B) % $CD27^{+}IgD^{-}$ Class Switched Memory, C) % $CD27^{+}IgD^{+}$ Non-Switched Memory, and D) $CD27^{-}IgD^{-}$ cells in late for cause biopsy patients.

Patients were divided into 2 groups according to graft loss during the follow up period. The B-cell phenotype was assessed from whole blood using flow cytometry. Groups were compared using the Mann Whitney test with individual results, median and interquartile range shown.

Variable	Study Population	T1:T2 Ratio >0.175	T1:T2 Ratio <0.175	p-value
Number	21	11 (52%)	10 (48%)	
Recipient Age (yr, med + IQR)	41 (27)	47 (12)	39 (18)	0.423
Recipient Gender				**0.030**
Male	12 (57.1%)	9 (82%)	3 (30%)	
Female	9 (43%)	2 (18%)	7 (70%)	
Recipient Ethnicity				0.214
Caucasian	19 (90.5%)	11 (100%)	8 (80%)	
Asian	1 (4.8%)	0	1 (10%)	
Chinese	1 (4.8%)	0	1 (10%)	
Cause of ESRD				0.917
Diabetes and Hypertension	1 (4.8%)	1 (5%)	0	
Glomerulonephritis	8 (38.1%)	4 (36%)	4 (40%)	
Inherited	4 (19%)	2 (18%)	2 (20%)	
Other	4 (19%)	2 (18%)	2 (20%)	
Unknown	4 (19%)	2 (18%)	2(20%)	
Donor Type				0.383
LD	4 (19%)	1 (9%)	3 (30%)	
DBD	10 (47.6%)	5 (46%)	5 (50%)	
DCD	7 (33.3%)	5 (46%)	2 (20%)	
Graft Number				0.476
1	18 (85.7%)	9 (82%)	9 (90%)	
2	2 (9.5%)	2 (18%)	0	

Variable	Study Population	T1:T2 Ratio >0.175	T1:T2 Ratio <0.175	p-value
3	1 (4.8%)	0	1 (10%)	
NHSBT HLA Mismatch Level				0.786
1	1 (4.8%)	1 (9%)	0	
2	5 (23.8%)	3 (27%)	2 (20%)	
3	10 (47.6%)	4(36%)	6 (29%)	
4	5 (23.8%)	3 (27%)	2 (20%)	
Induction Agent				1.000
Alemtuzumab	11 (52.4%)	6 (55%)	5 (50%)	
Basiliximab	3 (14.3%)	2 (18%)	1 (10%)	
Unknown	7 (33.3%)	3 (27%)	4 (40%)	
Maintenance Immunosuppression*				
Tacrolimus	18 (85.7%)	10 (91%)	8 (80%)	0.586[1]
Ciclosporin	1 (4.8%)	0	1 (10%)	0.476
Sirolimus	1 (4.8%)	1 (9%)	0	1.000
MMF	6 (29%)	3 (27%)	3 (30%)	1.00
Azathioprine	4 (19%)	0	4 (40%)	**0.035**
Prednisolone	6 (28.6%)	1 (9%)	5 (50%)	0.063
Baseline eGFR (med, IQR)	19 (16.5)	30 (24)	15 (7)	**0.014**
Baseline UPCR (med, IQR)	68.6 (16.5)	61.5 (241.7)	76.8 (116.9)	0.863
Median Interval: transplant to biopsy (yrs, IQR)	3 (6)	2 (2)	3 (12)	0.138
DSA Present	8 (38%)	3 (27%)	5 (50%)	0.387

Variable	Study Population	T1:T2 Ratio >0.175	T1:T2 Ratio <0.175	p-value
Allograft rejection on biopsy	10 (48%)	3 (27%)	8 (80%)	**0.035**

Table 4.61. Demographic characteristics of the final cohort of patients receiving a late for cause biopsy, stratified according to T1:T2 ratio.

***Maintenance immunosuppression at the time of biopsy. UPCR- urine protein-creatinine ratio, IQR – interquartile range, MMF – mycophenolate mofetil**

Variable	HR (95% CI)	p-value	HR (95% CI)	p-value
	Univariate		Multivariate	
T1:T2 Ratio <0.175	7.26 (1.492-35.186)	**0.014**	1.714 (0.131 – 22.411)	0.681
DSA Present at time of biopsy	2.040 (0.588-7.074)	0.261		
Proteinuria (UPCR>50 vs <50)	0896 (0.253-3.180)	0.896		
eGFR at time of biopsy (<20 vs >20)	24.416 (2.90-205.92)	**0.003**	24.504 (1.297-463.112)	**0.033**
Years since transplant (>3 vs<3)	1.513 (0.390-5.872)	0.550		
Donor Type (LD Ref)				
DBD	0.230 (0.046-1.158)	0.203		
DCD	0.444 (0.098-2.003)	0.291		
Regraft vs 1st graft	0.408 (0.052-3.234)	0.408		
Induction (alem ref)				
Basiliximab	0.626 (0.075-5.218)	0.665		
Unknown	0.783 (0.195-3.141)	0.731		
Evidence of rejection on biopsy				
Yes	19.001 (2.32-155.38)	**0.006**	11.046 (0.511 – 238.871)	0.126
Borderline	5.709 (0.356-91.44)	0.356	1.655 (0.042-65.654)	0.789

Table 4.62. Univariate and Multivariate analyses of factors associated with graft survival in ALBERT patients receiving a late for cause biopsy.

To summarise, in patients with a troubled graft who received a late for cause biopsy, there was a distinct B-cell phenotype that was associated with evidence of rejection on biopsy and subsequent reduced 5-year graft survival. Patients with evidence of rejection on biopsy had fewer %T1 $CD24^{+++}CD38^{+++}$ cells, more %T2 $CD24^{++}CD38^{++}$ cells, and a lower T1:T2 ratio than those patients who did not have rejection. ROC curve analyses using a T1:T2 ratio of 0.2 successfully differentiated the rejection from no-rejection groups with sensitivity of 90.91% and specificity of 81.82%, AUC 0.7893, p=0.0215. A low T1:T2 ratio was also associated with higher Banff microvascular inflammation scores, however this was not statistically significant. Additionally, on univariate analysis a low T1:T2 <0.175 was associated with subsequent graft loss (hazard ratio 7.26, p=0.014 cox proportional hazards model). Multivariate analysis showed that a low eGFR<20 at the time of biopsy however was the single independent risk factor of subsequent graft loss.

4.2.3 DISCUSSION

In addition to antigen presentation and antibody secretion, B-cells can modulate the immune system through the secretion of pro-inflammatory and anti-inflammatory cytokines. The regulatory B-cell phenotype has yet to be fully defined however it is accepted that regulatory B-cells typically produce IL-10. In humans, the $CD24^{+}CD38^{+}$ transitional B-cell population has been extensively studied in both autoimmunity and alloimmunity. Acknowledging that B-cells have the capacity to produce both proinflammatory and anti-inflammatory cytokines, Cherukuri *et al* demonstrated that $CD24^{+}CD38^{+}$ transitional B-cells (TrBs) were reduced in renal transplant patients with a troubled graft, and these cells were also unable to suppress Th1 immune response.[124] Furthermore, TrBs could be defined into the very immature T1 and more mature T2 cells based on CD10, IgM, CD24 and CD38 expression.[124,141] The T1 cells were observed to have the most regulatory phenotype, through the relative increased production of IL-10 compared with TNF-α.[124]

A difficulty with using cytokine secretion to define B regulatory capacity Is the requirement for an often prolonged cell culture which has the potential to alter the cell phenotype. Additionally, the *in vitro* activation of regulatory B-cells may not necessarily be representative of the environment *in vivo.* The aim of this study was to assess if the surface B-cell phenotype could be used as a marker of clinical outcome.

4.2.3.1 PROSPECTIVE STUDY

The prospective B-cell surface phenotype following transplantation was first assessed. In this cohort, it was important to review the subsets using frequencies as a percentage of the parent gate as well as calculated values. This was because as described in the previous chapter, alemtuzumab induction causes B-cell depletion, and any subsequent clinical outcomes may be a function of not only the subtype of reconstituting B-cells, but also the number of cells in the peripheral circulation.

4.2.3.1.1 COMPOSITE OUTCOMES

Due to the relatively short follow up period for this cohort, a decision was made to assess the B-cell phenotype against a composite endpoint which comprised of surrogate markers for poor outcome (the development of de novo donor specific antibodies, 30% reduction in eGFR between 3-18 months, immune-mediated changes or recurrent disease on biopsy and recurrent proteinuria). Although no consistent trends were noted at the various time points, it was interesting to see that the 3-month % B-cell was a marker of meeting the composite endpoint for both alemtuzumab and basiliximab patients, however in the alemtuzumab cohort, low %B-cells were associated with an increased risk of meeting the endpoint, yet in the basiliximab cohort, low %B-cells were associated with a reduced risk of meeting the endpoint. Assuming that each individual endpoint occurred as a result of an alloimmune reaction, a possible explanation could be that the B phenotype following alemtuzumab is more immature with increased transitional and regulatory B-cells. Patients with low B-cell frequencies would have fewer regulatory cells at a time where $CD4^+$ and $CD8^+$ lymphocytes frequencies would have expected to return to pre

alemtuzumab levels. This could result in a relatively unchecked immune system and increasing the risk of poorer outcomes. Supporting this theory is the case series where Clatworthy *et al* reported early T-cell mediated rejection following rituximab and attributed this to the depletion of immunoregulatory B-cells.[235] In the Basiliximab group of the ALBERT prospective study, it was noted that high 3-month calculated transitional B-cells were associated meeting the composite endpoint. No differences were noted in the T1:T2 ratio. Alfaro *et al* found that patients (who received either ATG or basiliximab induction) with a low frequency of B-cells at six months post-transplant were more likely to develop acute rejection in the first transplant year.[236] In a single centre matched cohort study, Todeschini *et al* were able to predict the development of DSA by early changes in the B-cell phenotype, where low 1-month post-transplant B-cell counts were significantly associated with the development of DSA at 1 year.[165] This correlated with worse long-term outcomes. There may also be other patient factors that can affect the rate and type of B-cell repopulation. For example, age related changes in the bone marrow, which can lead to the impaired development and function of B-cells, have been documented.[237,238] In multiple sclerosis patients, B-cell repopulation after rituximab has been found to be influenced by the presence of $NKG2C^+$ NK cells with enhanced ADCC function – high levels of $NKG2C^+$ NK cells were associated with reduced B-cell numbers.[239] Alternatively, low peripheral B-cell could be a reflection of the accumulation of B-cells elsewhere, for example in lymph nodes and spleen, or within the graft itself.

A finding was that alemtuzumab patients were at increased risk of meeting the composite endpoint if they had high 3-month % $CD27^+CD38^-$ memory cells >1.185% including high %non-switched memory cells. Following alemtuzumab induction, it would be expected that the majority of repopulating B-cells will derive from the bone marrow and this will be reflected as increased transitional or naïve cells with few memory cells. An increased frequency of peripheral memory cells therefore could suggest repopulation in the periphery from previously sequestered memory cells. Additionally, at 6-months, a high switched:non-switched ratio was also associated with an increased risk of meeting the composite endpoint. Although documented at a

different timepoint, elevated switched memory $IgD^{-}CD27^{+}$ cells at 1 year post transplant have been associated with DSA development.[165]

The individual endpoints were then then assessed:

4.2.3.1.2 REJECTION

A high 3-month frequency of transitional B-cells post alemtuzumab was found to be associated with subsequent rejection episodes during the follow up period. In the basiliximab cohort, only one patient was found to have a rejection episode. Although not statistically significantly different, this rejection patient also had a 3-month %TrB that was greater than the 3-month median of the basiliximab cohort (Figure 4.37). These findings contradict current literature however which describes an increased risk of rejection with fewer transitional B-cells. Svachova *et al*, for example showed that lower transitional B-cells in the 3rd post-transplant month were associated with a higher risk of subsequent allograft rejection.[233] Shabir *et al*, also concluded that TrB frequencies were associated with protection from any type of rejection at all time points up to 360 days post-transplant.[240] However, in both case series listed above, patients received either basiliximab or ATG induction. Alfaro *et al* on the other hand did not notice any differences in either the frequency (% of B lymphocyte gate) or absolute count (cell/µl) of transitional B-cells at 3 or 6months post-transplant in rejection and no-rejection patients.[236] Although not statistically significant, when the 3-month calculated transitional B-cell counts were reviewed, patients who had rejection had fewer calculated transitional B-cells at 3 months (median value $0.0108x10^{9}$/l rejection vs $0.238x10^{9}$/l no rejection, p=0.691).

At 6 months however, a low calculated TrB population was associated with subsequent rejection episodes in alemtuzumab patients (calculated TrBs $0.00590x10^{9}$/l no rejection vs $0.00107x10^{9}$/l rejection, p=0.005). Further work will need to be performed to evaluate the IL-10 capability in these cells. In this cohort, the relative frequencies of $CD19^{+}CD24^{+++}CD38^{+++}$ T1 and $CD19^{+}CD24^{++}CD38^{++}$ T2 cells were key in establishing the

risk of subsequent rejection, and the T1:T2 ratios at 3 and 6 months were good biomarkers of allograft rejection over the 5-year follow up period. Furthermore, multivariate analysis of 3-month data showed a low T1:T2 ratio to be independently associated with an increased risk of allograft rejection with a hazard ratio of 62, and when the T1:T2 ratio was compared at all timepoints, the median T1:T2 ratio was reduced in patients who developed rejection (Figure 4.31b), supporting its use as a biomarker of allograft rejection in alemtuzumab patients. Interestingly, in keeping with the evident reduction in T1:T2 ratio with time following alemtuzumab, when ROC curve analyses were performed, the cut-off values offering the optimal sensitivity and specificity of subsequent allograft rejection decreased at each time point (3 months: <0.215, 6 months <0.1911, 12 months <0.1162).

The basiliximab cohort was appreciably smaller than the alemtuzumab cohort and contained only 24 patients. One patient developed an episode of rejection during the follow up period. No statistically significant associations were seen between the 3-month frequency of TrBs and rejection. This could either be due to small patient numbers, or the overall low frequency of TrBs in basiliximab patients. However, when this patient's B-cells were tracked over time, a switch in phenotype was noted between 6 and 12 months post-transplant, where there was a reduction in T1 cells, resulting in a lower T1:T2 ratio. This switch in phenotype occurred 2 months before the patient was biopsied for a 50% rise in creatinine and new onset proteinuria (Figure 4.37). This supports the conclusion that although in some cases, a 3-month sample may be helpful in identifying at risk patients, the B-cell phenotype is dynamic and therefore the close and frequent monitoring of subsets will be required to track changes in B-cell subsets with time.

In addition to differences seen in the T1:T2 ratio, the 6-month S:NS memory cell ratio was also a good predictor of allograft rejection in the alemtuzumab cohort. Class switched memory cells are generated following interaction with their cognate T-cells, therefore a high S:NS ratio could indicate a more primed alloimmune system. In this cohort, a S:NS ratio at 6 months >2.134 (driven by reduced non-switched memory

cells), was associated with a reduced rejection free survival during the subsequent 5 years of follow up. The memory phenotype, including the relative frequencies of switched and non-switched memory B-cells have been extensively studied in autoimmune diseases. Simon *et al*, for example demonstrate that in systemic sclerosis, patients with diffuse cutaneous systemic sclerosis (dcSSc) were found to have significantly elevated frequencies of $CD19^{+}CD27^{+}IgD^{-}$ class switched memory cells (within the $CD19^{+}CD27^{+}$ fraction) compared with patients with localised cutaneous systemic sclerosis (lcSSc). Furthermore, patients with dcSSc and higher class switched memory cells were more likely to have more severe form of the disease, exhibiting pulmonary fibrosis compared with those who had lower frequencies of class switched memory cells. They concluded that the reduced non-switched memory B-cell frequencies could be a potential biomarker of disease severity in systemic sclerosis.[241] Similar findings have been noted in primary Sjogren syndrome and SLE.[242,243] The increased frequency of class switched memory cells has also been suggested as a potential biomarker of IgA nephropathy,[244] and implicated in the pathogenesis of focal segmental glomerulosclerosis.[245] This leads to the question as to whether patients in this cohort who experienced rejection episodes also had an autoimmune condition in their medical history. One of five patients had lupus nephritis as their primary disease, and one patient had a history of ulcerative colitis, however their autoimmune conditions were clinically quiescent during the follow up period. Other factors, for example persistent viral infections, may also explain differences in the S:NS ratio, and would need further exploration.

4.2.3.1.3 GRAFT SURVIVAL

Seven patients in the alemtuzumab cohort, and 1 patient in the basiliximab cohort lost their graft during the follow up period. Although no significant differences were noted in the basiliximab cohort, some differences were noted in the alemtuzumab cohort. Looking first at the transitional subsets, at 3 months, patients who lost their graft had significantly fewer calculated T1 cells than those who did not lose their graft. There was a trend towards fewer calculated T1 cells and a lower %T1 and T1:T2 ratio at 6

months in graft loss patients, however this was not statistically significant. Finally at 12 months, a low T1:T2 ratio <0.1424 was a good marker of graft loss over the subsequent 5 years, with a sensitivity 100%, and specificity 78%. When the T1:T2 ratio was reviewed at all timepoints, patients who lost their graft during the follow up period had a reduced median T1:T2 ratio compared with those who did not lose their graft. This suggests that in addition to highlighting patients at risk of rejection, the T1:T2 ratio may also be a marker of reduced allograft survival. As 4 of 7 graft loss patients were found to have rejection prior to graft loss, the T1:T2 ratios were interrogated further within the graft loss group. Although the differences were not statistically significant, the T1:T2 ratios obtained from patients who had rejection were lower than the T1:T2 ratios of patients who lost their graft due to other reasons. The current literature is limited with respect to assessing T1:T2 ratios and renal allograft survival. However, Cherukuri *et al* demonstrated that low T1:T2 ratios <0.17 obtained from patients with a troubled graft were associated with either return to dialysis or a 2-fold reduction in eGFR in the 5 years following the biopsy.[141] The important distinction to make from Cherukuri's study is that in the ALBERT prospective cohort, patients were clinically stable, yet a low T1:T2 ratio was also found to be a marker of reduced allograft survival. Further work will need to be carried out to determine whether these observations in the ALBERT prospective cohort can be replicated in other transplanting centres, and in larger numbers to determine clinically relevant cut-off values for each time point.

Other potential markers for reduced allograft survival were noted at the 3-month timepoint in the alemtuzumab cohort. These included low calculated B-cells < 0.01553×10^9/l and naïve B-cells <0.01350×10^9/l. As discussed above, this could reflect a more immature and immunomodulatory B-cell phenotype following alemtuzumab induction, decreasing the risk of allograft rejection. The peripheral phenotype of tolerant allograft recipients (defined as having stable graft function greater than one year without immunosuppression) has previously been investigated and these patients have been found to have higher circulating $CD19^+$ B-cells compared with patients taking immunosuppression. It is however difficult to discern whether this difference is

due to the presence or absence of immunosuppression, which can affect peripheral B-cells, or whether it is a function of a 'tolerant phenotype'.[246] Low circulating peripheral B-cells have been documented in cases of active allograft rejection and could represent B-cell infiltration into the graft.

4.2.3.1.4 DEVELOPMENT OF DSAS

In alemtuzumab patients, although a low 3-month T1:T2 ratio was associated with subsequent rejection episodes during the follow up period, it was surprising that the B-cell phenotype was not associated with the development of *de novo* HLA antibodies or *de novo* donor specific antibodies. A possible explanation for this is the nature of how DSAs were reported. The standard laboratory practice reports pre-transplant HLA antibodies using a combination median fluorescent intensity (MFI) > 2000 and relative strength defined by the reaction score. However, post-transplant, DSAs are only tested for if there is clinical concern, and a more cautious threshold is applied with all DSAs >1000 MFI considered positive. This strategy was followed for the analysis of ALBERT study patients, which had the possibility of over calling the presence of DSAs post-transplant, especially as serum samples were tested at set timepoints according to the study protocol between 3-18 months, irrespective of the clinical picture. The incidence of *de novo* DSAs reported in the current literature varies, but ranges between 2-10% for the first year post renal transplantation.[247–251] In this study, *de novo* DSAs were found in 16% of alemtuzumab patients, and 21% of basiliximab patients, supporting the hypothesis that the threshold for positivity was set too low. The DSA data would need reanalysing using different thresholds, together with a review of all the antibody data sets in each patient's screening history to determine an optimum threshold. This was not feasible for the purposes of this thesis.

4.2.3.1.5 RECURRENT PROTEINURIA

Recurrent proteinuria alone was the most frequently occurring endpoint in both cohorts. This was defined as the urine protein-creatinine ratio > 50mg/mmol on two

or more occasions. As consistent trends in the B-cell phenotype were not observed across the different timepoints, and as there were several possible causes for the development of proteinuria, it was difficult draw firm conclusions with respect to the at-risk B-cell phenotype. In the Alemtuzumab cohort, high 3-month %memory cells (>1.185%) and a low 6-month T1:T2 ratio were associated with an increased risk of developing recurrent proteinuria. However, when patients who were thought to have developed proteinuria due to non immune-mediated processes were removed from this analysis, there was no statistically significant differences between the two groups (calculated and % gated) at 3 months. At 6 months, a low T1:T2 ratio remained a risk factor for developing recurrent proteinuria after patients with possible non-immune mediated causes of proteinuria were removed from analysis. As proteinuria has been associated with reduced graft survival and with allograft rejection, it was unsurprising to see that a low T1:T2 ratio was associated with all 3 outcomes in the alemtuzumab cohort.

In the Basiliximab cohort, recurrent proteinuria was associated with increased calculated B-cells, including elevated $CD19^{+}CD27^{-}IgD^{+}$ naïve cells at the 6-month timepoint. Although these results were statistically significant, only 4 events occurred in this cohort, making it difficult to draw firm conclusions.

A limitation with this study was that c-reactive protein was not routinely measured at each clinic appointment. As urinary tract infections are common early post-transplant, there was a possibility that the definition of UPCR >50 on two or more occasions would capture patients with recurrent urinary tract infections. It is possible that active UTIs may change the peripheral B-cell phenotype; one patient with recurrent proteinuria in the alemtuzumab cohort had features associated with a bacterial urinary tract infection on an indication biopsy. Serial blood pressure readings, c-reactive protein and HbA1c levels would be helpful to collect in any further analysis of proteinuria.

Renal Function

No associations were noted with the B-cell subsets and renal function in the basiliximab group. However, at 3-months, on univariate analysis, calculated class switched memory B-cells were correlated with eGFR yet %transitional B-cells were inversely correlated with eGFR. When corrected for delayed graft function, cold ischaemia time and tacrolimus levels, no association was noted between transitional B-cells and eGFR, however calculated class switched memory B-cells remained correlated with eGFR. Additionally at 6 months, correcting again for tacrolimus levels and previous (3-month eGFR), overall calculated B-cells, including naïve and memory (non-switched and class switched) were correlated 6-month eGFR. No statistically significant associations were seen with %gated subsets. This finding is surprising and not in keeping with current literature. It could be explained the inaccuracies associated with using calculated values, which will be discussed further in the limitations section. Alfaro *et al* tracked B-cell subsets post transplantation and performed standard statistical analyses as well as cluster analyses to determine markers associated with outcome. They found on standard statistical analyses that from 3 months post transplantation, transitional B-cells were correlated with eGFR, and class-switched memory cells were inversely correlated with eGFR.[236] Although not directly built into their analysis, they investigated the incidence of delayed graft function (DGF), and patients with the highest tertile of class switched memory B-cells also had the highest incidence of DGF. In the ALBERT cohort, DGF, length of cold ischaemia time and tacrolimus levels were considered when assessing 3-month data. For 6-month data, tacrolimus levels and previous eGFR were considered. When Alfaro *et al* performed a cluster analysis of B-cell phenotype on the same patients, they determined that low transitional B-cells and high plasma cells were associated with reduced renal function.[236]

4.2.3.2 FOR CAUSE BIOPSY

The B-cell surface phenotype in patients who were offered a late 'for cause' biopsy were then assessed. These patients were included in the analysis if they were at least

1 year post-transplant to avoid any differences that may occur with B-cell repopulation after alemtuzumab induction. The median time to biopsy in this cohort was 3 years post-transplant. Fifty percent of this cohort received alemtuzumab as an induction agent, and 14% basiliximab. The remaining 33% were recorded as 'unknown induction agent'. Based on the date of transplantation, it is likely that they did not receive any induction. Although there were no statistically significant differences in % transitional B-cells obtained between the R and NR groups, low levels of $CD24^{+++}CD38^{+++}$ T1 cells, which have previously been demonstrated to have the most regulatory capacity [124,141] at the time of biopsy was associated with an increased risk of rejection. This low % gated T1 resulted in a lower T1:T2 ratio, and receiver operator curves successfully classified patients into rejection and no-rejection groups using a T1:T2 ratio cut-off of 0.2, with a sensitivity of 90%, specificity 82%, area under the curve 0.7893, p= 0.0215. Furthermore, a low T1:T2 ratio <0.175 was associated with a reduced 5-year survival, with a hazard ratio of 7.26, p=0.014 on univariate analysis. When patients with high T1:T2 ratios >0.175 and low T1:T2 ratios < 0.175 were compared, there were no statistically significant differences in the type of induction agent. Cherukuri *et al* noted a similar finding in a cohort of consecutive patients who were offered a biopsy in the setting of a troubled graft. Using a T1:T2 ratio value <0.17, they were able to identify patients who were at risk for allograft deterioration, which they defined as a 2-fold reduction in eGFR or dialysis dependency in the subsequent 5 years.[141] Although the median time to biopsy in their cohort was 99 months (standard deviation 64 months), their cohort included patients who received both alemtuzumab and basiliximab induction, in similar frequencies. Therefore, they were able to conclude that a low T1:T2 ratio in a troubled graft was predictive of clinical outcomes irrespective of induction agent.[141] Other authors have demonstrated that lower absolute numbers and frequencies of transitional B-cells are associated with a reduced rejection free survival, however these patients were assessed early post-transplant.[233,240]

One biopsy patient was diagnosed with Banff Category III (borderline/suspicious for TCMR). This patient had a transitional B-cell phenotype that was similar to patients in the rejection group. Furthermore, he lost his graft during subsequent follow up.

Although this is an example of one case, and firm conclusions cannot be drawn from this data, it would be interesting to study the transitional B-cell phenotype in a group of borderline cases to see whether it could help risk stratify patients for graft loss.

4.2.3.3 LIMITATIONS

This study has several limitations which will influence the conclusions drawn:

Patient numbers, enrolment, and timing of first sample

Patients enrolled in the prospective ALBERT study had their first review 3 months after transplantation. This was to allow the peripheral repopulation of B-cells following alemtuzumab induction to a level that enabled assessment using flow cytometry.[161] This set timing had its limitations in that early rejection episodes were missed in both groups. In some cases, depending on patient factors, the first time point had to be excluded as the frequency of B-cells was too low for assessment. As discussed above, low peripheral B-cells have been associated with poorer outcomes following transplantation, possibly due to the reduced frequency of immunoregulatory cells; in the ALBERT alemtuzumab cohort, low % B-cells at 3 months was associated with an increased risk of meeting the composite endpoint. Similarly, patients with very low transitional B-cell populations would be excluded from the assessment of T1 and T2 subsets due to the inherent inaccuracies with gating. This study has shown that 3-month B-cell frequency (defined as a percentage of the lymphocyte gate) was associated with reaching the composite endpoint (increased in Basiliximab, and decreased in alemtuzumab), therefore excluding patients with low B-cells will introduce bias to the data.

To reflect the type of patients that would be managed in a transplanting centre, consecutive patients were approached to participate in the study. This included patients who had previously received multiple transplants, and those who may have received immunomodulatory therapies prior to transplant for treatment of their native kidney disease. These previous treatments may have a long-term effect on their

peripheral B-cell phenotype and could partially explain why some findings in this cohort are different to those documented elsewhere. A time of offer or pre-transplant sample would be useful to have as a baseline comparator.

The use of 'for cause' biopsies

Patients were offered a biopsy if there was unexplained deterioration in allograft function, or the development of persistent proteinuria. As up to 30% of surveillance biopsies performed within the first post-transplant year can demonstrate findings consistent with subclinical rejection, it is possible that several patients in the prospective study cohort were misclassified, thus affecting the utility of the different surface B-cell phenotypes as biomarkers of outcome in prospective patients, especially as both subclinical TCMR and ABMR have been associated with reduced graft survival.[252–255] Similarly, the group 2 study patients had their B-cell phenotype assessed only at the time of a for cause biopsy, thus introducing a selection bias.

Duration of follow-up

The median graft survival for a renal allograft from a deceased donor is approximately 15 years, whereas the median follow-up for the prospective study was 1701 days. It is acknowledged that not all events will have occurred during this assessment period. An attempt was made to address this by using a pre-defined composite endpoint which incorporated other clinical features that have been associated with decreased allograft survival. It will be useful to repeat an assessment of clinical outcomes in the future to see if the conclusions made here are applicable to long term outcomes. It is important to note that during the period of follow up, in some cases, reflecting patient specific circumstances, the maintenance immunosuppression regimes were amended. At the 18-months follow up, only 69% of the alemtuzumab and 86% of the basiliximab cohort remained on the same immunosuppressive agents that they were prescribed on discharge following their transplant potentially influencing the B-cell phenotype.

Assessment of individual B-cell subsets

Although some signals of poor allograft outcome associated with individual B-cell subsets were noted, it is important not to review these results in isolation. Alfaro *et al* for example performed cluster analyses on normalised frequencies of different B-cell subsets obtained at varying time points post-transplant. They identified different B-cell signatures that were associated with reduced eGFR at the time of sampling, together with an increased risk of allograft rejection within the first post-transplant year.[236] Larger patient population with complete datasets will be required to perform this type of analysis. It will also be important to assess B-cells within the context of other cells within the immune system. Whilst multiple serial measurements of the B-cell phenotype in the prospective patients were performed, replicate analyses were not performed at each timepoint. This will need to be performed in the future to assess repeatability, inter-user and inter-assay variability, and confirm at risk thresholds should this be introduced as a routine assay. Assessment of peripheral blood may not necessarily reflect the B-cell dynamics within the graft. Staining an extra core of tissue for B-cells and subsets following allograft biopsy may provide additional information.

Flow cytometric analysis of B-cells

Analysing flow cytometry plots can be subjective. Especially in cases where there are few events (for example B-cells early post-transplant, or analysis of transitional subsets), precise gating is important, otherwise this can lead to inaccuracies. An attempt was made to minimise these inaccuracies by ensuring that at least 30000 $CD19^+$ events were acquired. Flow plots were excluded from analysis if there were less than 2% B-cells of the lymphocyte gate as it was felt that there would be too few events to confidently gate individual subsets. Samples that had <5% B-cells of the lymphocyte gate underwent further scrutiny to ensure enough events were available within each subset prior to inclusion in the analysis. Analysis of flow plots was performed by a single user in an unbiased manner using templates obtained from healthy volunteers, and in the case of transitional cells, using a ratio of 25% T1 to 75% T2 as previously described.[141] Additionally, CD10 and IgM expression was used to

confirm placement of the T1/T2 gates. A consistent finding in this study was that transitional B-cells were elevated in patients who had evidence of rejection (either at the time of, or subsequent to sampling. This is not in keeping with the current published literature. Whilst there could be other mechanistic or biological explanations for this, there are other potential explanations related to the technical aspects of flow cytometry:

- As described above, patients were excluded from analysis if there were insufficient B-cells events. This could lead to a sampling bias where patients exhibiting a phenotype that may be associated with less favourable outcomes were systematically excluded.
- The gating strategy used – some groups will exclude $CD27^{+}$ cells prior to the analysis of CD24 and CD38 expression to determine the transitional B-cell population. A decision was made not to follow this strategy for the surface phenotype as CD27 could not be included in the intracellular panel. The reasoning behind this was to ensure consistency between the two gating strategies (surface and intracellular) to allow for direct comparisons.

Although gating and analysis was performed by a single user in an unbiased manner using additional markers, not all regulatory B-cells may have been captured. Introducing other surface markers that are associated with IL-10 capacity, for example CD9, (which will be discussed in chapter 5: Investigating CD9 As a potential marker of Regulatory B-cells), may be helpful adjuncts to the gating strategy.

<u>The assessment of subsets as a percentage of parent gate and calculated number</u>

Due to the reconstitution of the B lymphocyte population following depletion with alemtuzumab induction, B-cell subsets were assessed both as a percentage of the parent gate and calculated numbers. Calculated numbers were derived by measuring the subsets against the lymphocyte count obtained from the matched full blood count. It is acknowledged that this method of calculating numbers does have inbuilt inaccuracies. The optimal method for determining absolute counts would have been to use for example the Trucount tube (BD biosciences) which allows for accurate measurement of different lymphocyte subsets by including a set number of test beads

per tube. However, this test would have added a further £144 per sample tested, which was cost prohibitive.

In conclusion, the analysis of the B-cell phenotype early post-transplant following alemtuzumab induction and in recipients with a troubled graft have highlighted that changes in transitional B-cells, and a reduced T1:T2 ratio, signalling the reduction of $CD24^{+++}CD38^{+++}$ T1 cells which are thought to have the highest regulatory potential, may be important markers in determining the increased risk of allograft rejection and subsequent graft loss. Furthermore, the changes within the memory B-cell subset may also provide signals suggestive of less favourable allograft outcomes which will warrant further investigation. Univariate analyses were performed in the majority of cases, however there will be other factors apart from the B-cell phenotype that will affect outcomes. Multivariate analyses and therefore a larger test population will be required to further assess these clinical factors.

5 INVESTIGATING CD9 AS A POTENTIAL MARKER OF REGULATORY B-CELLS

5.1.1 INTRODUCTION

In humans, a regulatory phenotype has been described in different B-cell subsets in models of both alloimmunity and autoimmunity (Table 1.2). Although there is currently no specific marker (transcriptional or cell surface) for B Regulatory cells (Bregs), it is accepted that Bregs typically produce IL-10, and it is through the effects of IL-10 on other cells that Bregs exert their regulatory effects.[94,95,132] In addition to IL-10 secretion, B regulatory capacity can also be determined by assessing the relative production of regulatory to pro-inflammatory cytokines (IL-10:TNF-α ratio).[124]

A potential novel marker of regulatory B-cells Is CD9, a member of the tetraspanin family. It is expressed on hematopoietic stem cells and most subsets of leucocytes as well as at high levels on endothelial cells. Through interaction with other tetraspanin molecules and tetraspanin-enriched microdomains (TEMS), CD9 has been shown to influence multiple cellular mechanisms including cell differentiation, signalling, migration, adhesion, and motility.[256–258] CD9 expression has also been associated with IL-10 secretion in antigen presenting cells in both murine and human models.[259]

CD9 was first highlighted as a potential marker of Bregs by Sun *et al* following the transcriptomic analysis of IL-10^{+} and IL-10^{-} murine B-cells. CD9^{+} expression was associated with IL-10 secretory capacity, and these CD9^{+} B-cells were more efficient at suppressing T-cell proliferation compared with CD9^{-} B-cells *in vitro*.[260] In a murine model of asthma, reduced CD9^{+} IL-10 producing B-cells were associated with increased airway hyperresponsiveness, and these features of asthma resolved following the adoptive transfer of CD9^{+} B-cells.[261]

In humans, patients with severe asthma were found to have reduced CD9^{+}CD19^{+} cells.[259] In the field of transplantation, higher CD9 expression is prominent in the transitional CD24^{+}CD38^{+} B-cell population.[262] Following lung transplantation, patients

with increased numbers of $CD19^+CD9^+CD24^+CD38^+$ cells were less likely to develop bronchiolitis obliterans, the most common feature of chronic immune-mediated lung allograft dysfunction.[263] Finally, $CD24^{hi}CD38^{hi}$ transitional B-cells in renal transplant patients with stable graft function were shown to express CD9.[262]

Therefore, CD9 was investigated as a potential marker for Bregs in the ALBERT study. In renal allograft recipients, the relative surface expression of CD9 across the various B subsets was compared. Subsequently, the IL-10:TNF-α ratio was determined for $CD9^+$ and $CD9^-$ B-cells following stimulation with CD40L, CPG, PMA and Ionomycin as described in section 2.12: PBMC stimulation for intracellular staining.

5.1.2 RESULTS

5.1.2.1 SURFACE CD9 EXPRESSION VARIES ACROSS THE B SUBSETS

The surface expression of CD9 was tested in 97 ALBERT study samples. Thirty-one samples were excluded from the analysis due to insufficient numbers of B-cells (<1% B-cells). One sample was excluded as it was not possible to confidently gate around each B-cell population despite obtaining sufficient B-cell events. Sixty-five samples were therefore included in the final analysis, with a median time to sampling of 112 days from transplantation (range 7-381 days).

Whole blood (300µl) was stained with monoclonal antibodies as described in section 2.10 to detect the surface expression of CD19, CD9, CD24, CD38, CD27, IgD, IgM and CD10. The following gating strategy (Figure 5.1) was used to assess CD9 expression on $CD19^+$ B-cells and B-cell subsets. The $CD9^+$ gate was placed using an isotype control.

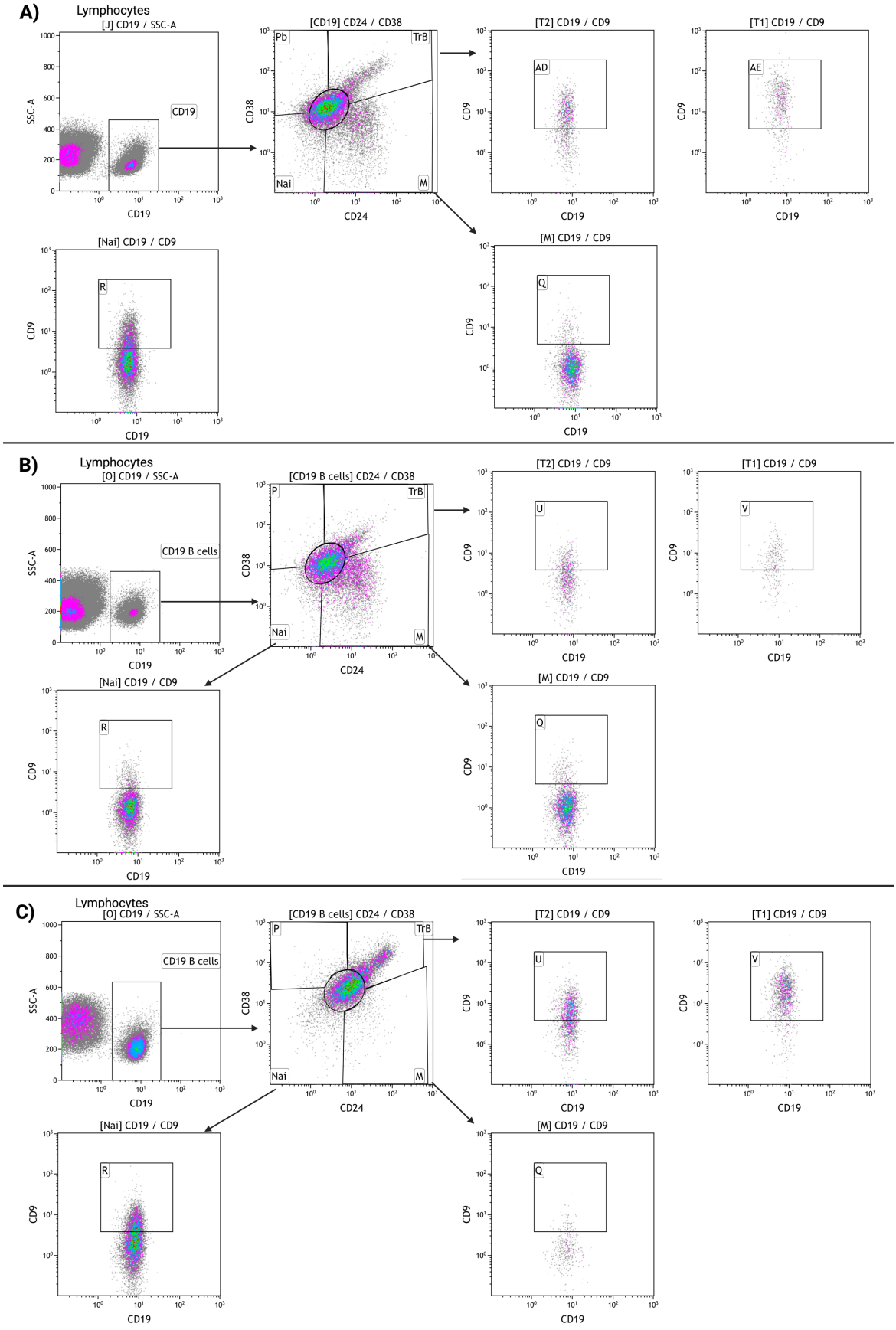


Figure 5.1. Gating Strategy to determine surface CD9 expression

Figure 5.1 Gating Strategy to determine surface CD9 expression – continued: Whole blood was stained with monoclonal antibodies as described in section 3.10. B-cells were identified from the lymphocyte gate using CD19 expression. CD24 and CD38 expression were used to differentiate the B-cell subsets: Plasmablasts (P): $CD19^{+}CD27^{hi}CD38^{hi}CD24^{-}$, Memory: $CD19^{+}CD24^{+}CD27^{+}CD38^{-}$, Naïve: $CD19^{+}CD24^{+}CD38^{+}CD27^{-}IgD^{+}$, T1 $CD19^{+}CD24^{+++}CD38^{+++}$, T2: $CD19^{+}CD24^{++}CD38^{++}$. CD9 expression was determined within each subset. Representative plots are included from A) Healthy Volunteer (age 31), B) Basiliximab induction (3m post-transplant, recipient age 28 years), C) Alemtuzumab induction (3m post-transplant, recipient age 59). Although not shown in this figure, the CD9 positive gate was placed using an isotype control.

The proportion of cells expressing CD9 was significantly different across the B-cell subsets, ($p<0.0001$, Figure 5.2c). Multiple comparisons with adjusted p values showed that there were no significant differences between CD9 expression in the memory and naïve subsets, however the proportion of cells expressing CD9 were significantly higher in the $CD24^{hi}CD38^{hi}$ transitional populations, with the highest proportion of cells expressing CD9 in the $CD24^{+++}CD38^{+++}$ T1 population. Similarly, the median fluorescent intensity (MFI) obtained from $CD9^{+}$ cells was different across the B-cell subsets, with increased fluorescent intensity seen in the more immature B-cell subsets (naïve and transitional). The $CD9^{+}$ cells demonstrating the highest fluorescent intensity were the $CD24^{+++}CD38^{+++}$ T1 cells ($p<0.0001$, Kruskall Wallis with Dunns multiple comparisons, Figure 5.2d). The percentage of CD9 positive cells and the fluorescent strength of CD9 expression obtained from the different B-cell subsets can be seen in Table 5.1 and Table 5.2. Here it is observed that CD9 expression is highest within the $CD24^{hi}CD38^{hi}$ transitional B-cell population, which have previously been demonstrated to have significant regulatory activity.[124,140,141]

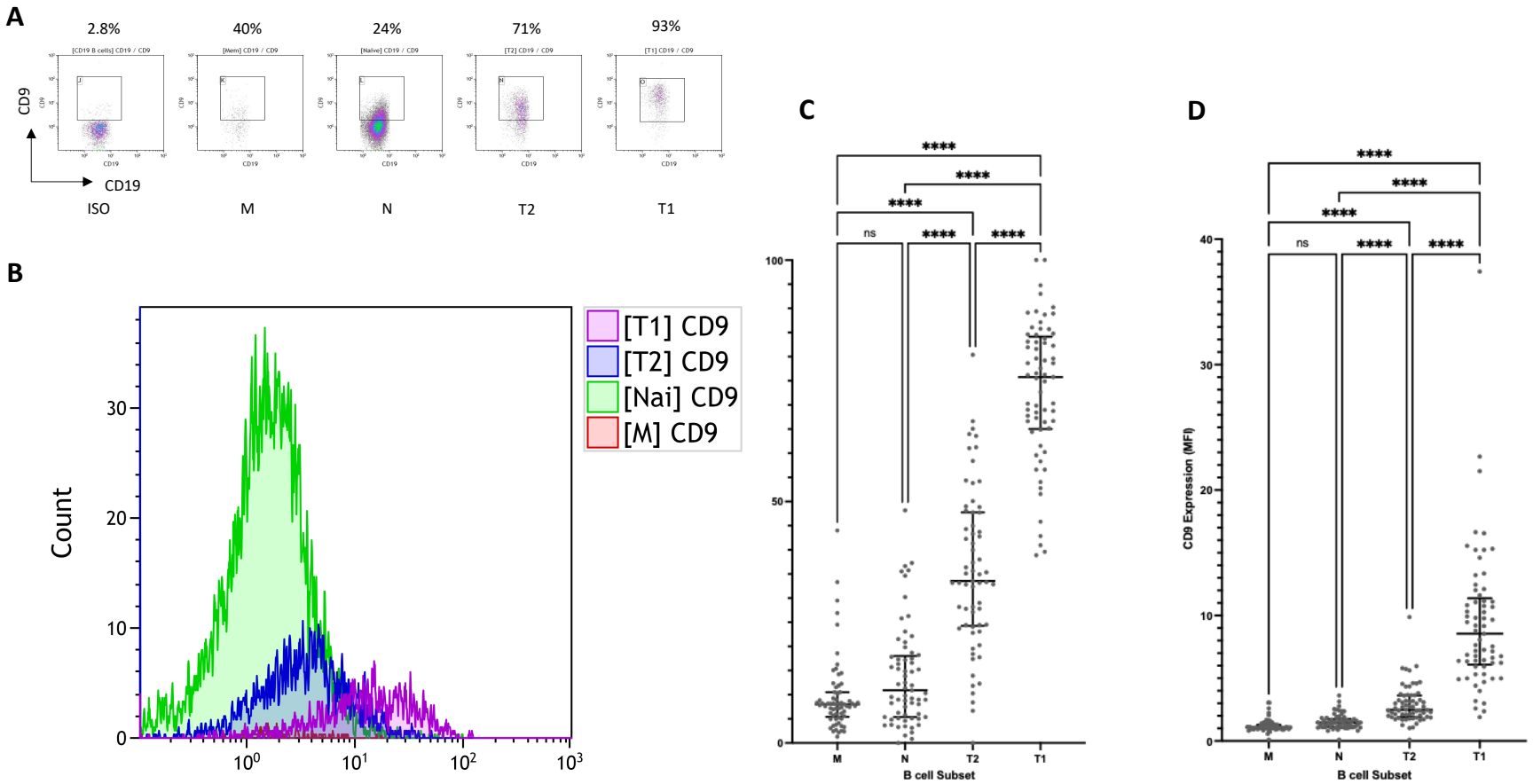


Figure 5.2. Surface Expression of CD9. Whole blood samples were stained using the panel described in Table 2.9 to assess CD9 expression across different B subsets.

Figure 5.2. Surface Expression of CD9 continued:

A) Representative scatter plots demonstrating CD9 expression across Memory (M), Naïve (N), T2, and T1 subsets compared with the isotype control. B) Representative overlay graph demonstrating the relative difference in CD9 expression using median fluorescent intensity (MFI) across M, N, T2 and T1 cells. The representative patient for A) and B) is a 47-year-old ALBERT group 2 patient who received a for cause biopsy 3 years post transplantation/Alemtuzumab induction. C) Cumulative scatter plots of 65 ALBERT prospective study samples (median time to sampling 112 days from transplantation, 20% Basiliximab induction, 80% alemtuzumab induction) comparing the proportion of $CD9^{+}$ cells demonstrated within each B subset. D) Cumulative scatter plots comparing the CD9 expression (MFI) across the different B subsets in the same 65 ALBERT prospective study samples. Subsets were compared using the Kruskal-Wallis test followed by the Dunns multiple comparisons test to compare the mean rank difference of each group. ** denotes a p value <0.0001.**

B-cell subset	% expressing CD9	IQR
Memory ($CD24^{+}CD38^{-}$)	8	5.105
Naïve ($CD24^{+}CD38^{+}$)	10.9	12.64
T2 ($CD24^{++}CD38^{++}$)	33.5	23.5
T1 ($CD24^{+++}CD38^{+++}$)	75.8	19.13

Table 5.1. CD9 expression (%gated) in different B-cell subsets.

B-cell subset	MFI (median)	IQR
Memory ($CD24^{+}CD38^{-}$)	1.09	0.295
Naïve ($CD24^{+}CD38^{+}$)	1.47	0.645
T2 ($CD24^{++}CD38^{++}$)	2.48	1.71
T1 ($CD24^{+++}CD38^{+++}$)	8.55	5.3

Table 5.2. CD9 Expression (fluorescent strength measured by MFI) on different B-cell subsets.

MFI – median fluorescent intensity

5.1.2.2 CD9 EXPRESSION DECREASED FOLLOWING STIMULATION WITH CD40L/CPG IN B-CELLS.

Having demonstrated increased CD9 expression in transitional B-cells, the next step was to investigate whether CD9 could be a useful marker of B-cells with regulatory capacity. PBMCs isolated from 178 ALBERT study samples (median time from transplantation 197 days, range 76-449 days, 32% basiliximab induction, 68% alemtuzumab induction) were stimulated with CPG, CD40L, PMA and ionomycin as described in section 2.12 *PBMC stimulation for intracellular staining.* A separate 'control' well was set up alongside each 'test' well where PBMCs were incubated unstimulated in plain B-cell culture media. It was noted that the percentage of B-cells expressing CD9 decreased following stimulation with CD40L/CPG/PMA/ionomycin compared with the unstimulated controls (mean of differences 11.47, $p<0.0001$, paired t test, Figure 5.3). This suggests that CD9 expression can be influenced by culture conditions. A similar phenomenon was noted when cells from a healthy volunteer was stimulated. CD9 was expressed by 7.71% of resting B-cells, however this reduced to 5.01% following stimulation. In a second healthy volunteer, 30% of B-cells expressed CD9 following stimulation with PMA/Ionomycin only, however, this reduced to 19.24% after stimulation with CPG/CD40L/PMA/ionomycin.

Despite this overall reduction in CD9 expression, the distinct B-cell subsets following stimulation could still be differentiated by the relative expression of CD9 (Figure 5.4A). PBMCs isolated from 265 ALBERT study samples (median time from transplantation 327 days, range 16-4561 days, 31% basiliximab induction, 69% alemtuzumab induction) were stimulated with CPG, CD40L, PMA and ionomycin as described in section 2.12 *PBMC stimulation for intracellular staining.* T1 cells expressed the highest level of CD9 (mean MFI 8.028), followed by T2 cells (mean MFI 2.059, $p<0.0001$). Furthermore, gating on the $CD9^+$ transitional B-cells increased the T1:T2 ratio when compared with all TrBs (median ratio in $CD9^+$ cells 0.4254 vs median ratio of all TrBs 0.2431, $p<0.001$, Figure 5.4B).

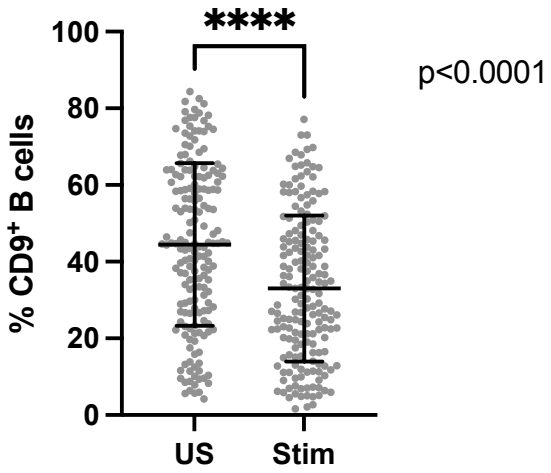


Figure 5.3. Comparison of cell surface CD9 expression on B-cells (unstimulated and stimulated) following 24-hour incubation.

Samples were obtained from 178 ALBERT study samples (median time from transplantation 197 days, range 76-449 days, 32% basiliximab induction, 68% alemtuzumab induction) PBMCs were cultured for 24 hours. Unstimulated (US) cells were incubated in plain B-cell media. Stimulated cells (Stim) were cultured with CPG and CD40L. Brefeldin A, monensin, PMA and ionomycin were added for the last 5 hours of culture. The percentage of $CD9^+$ B-cells are shown, together with the mean and standard deviation.

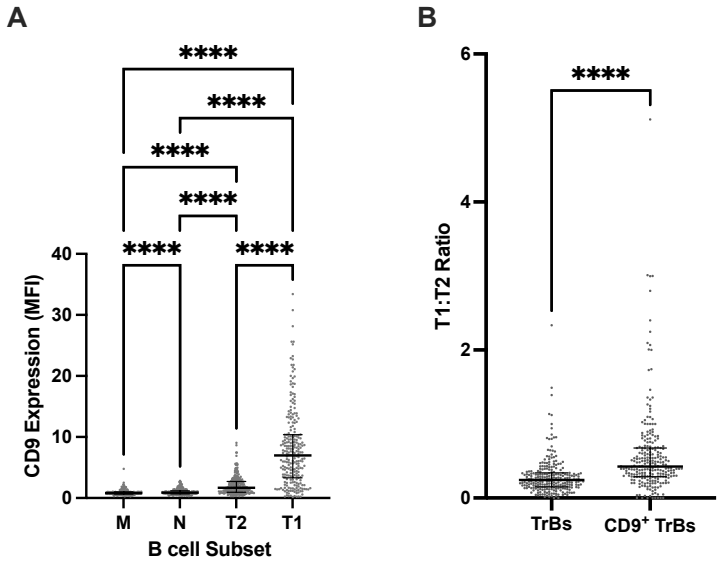


Figure 5.4. Surface expression of CD9 following stimulation with CPG/CD40L. Samples were obtained from 265 ALBERT study patients (median time from transplantation – 327 days, range 16-4561 days, 31% basiliximab induction, 69% alemtuzumab induction). Comparison of A) CD9 expression (median fluorescent Intensity) across different B-cell subsets and B) the T1:T2 ratios obtained from all transitional B-cells and CD9+ transitional B-cells. Isolated PBMCs were cultured in CPG and CD40L for 24 hours. Brefeldin A, monensin, PMA and ionomycin were added for the last 5 hours of culture. Individual values are shown, together with the median and interquartile range. Groups were compared using the Wilcoxon matched-pairs signed rank test. ** denotes a p-value <0.0001.**

5.1.2.3 CD9 EXPRESSION IS ASSOCIATED WITH AN INCREASED REGULATORY POTENTIAL

Following stimulation with CPG, CD40L, PMA and ionomycin, the regulatory potential was determined by assessing the percentage of IL-10 producing cells, and by calculating the IL-10:TNF-α ratios obtained from each B-cell subset (Figure 5.5). The more immature B-cell subsets displayed increased regulatory potential, and the T1 subset had the highest proportion of $IL10^+$ cells (T1 vs T2 mean rank difference 93.86, p=0.0004, T1 vs Naïve mean rank difference 144.7, p<0.0001, T1 vs memory mean rank difference 237.3, p<0.0001). Similarly when the regulatory potential was assessed

using the IL-10:TNF-α ratio, T1 cells were found to have the highest ratio (Mean rank difference: T1 vs T2 140.9, $p<0.0001$, T1 vs Naïve 281.6, $p<0.0001$, T1 vs memory 399.5, $p<0.0001$, Figure 5.5). This is in keeping with previous findings.[124]

The expression of CD9 was then compared with regulatory capacity (Figure 5.6). Gating on $CD9^+$ cells within each subset increased the % of IL-10 producing cells when compared with the overall subset. Gating on $CD9^+$ cells also increased the IL-10:TNF-α ratio compared to the ratio obtained from the overall subset (Median of differences in the IL-10:TNF-α ratio: $CD24^+CD38^-$ Memory – 0.04759, $p<0.0001$, $CD24^+CD38^+$ Naïve – 0.52, $p<0.0001$, $CD24^{++}CD38^{++}$T2 – 0.1118, $p<0.0001$, $CD24^{+++}CD38^{+++}$T1 – 0.3003, $p<0.0001$). These experiments show that cells expressing CD9 have an increased regulatory potential demonstrated by increased IL-10 production, and a skew towards a more regulatory cytokine profile measured by the IL-10:TNF-α ratio.

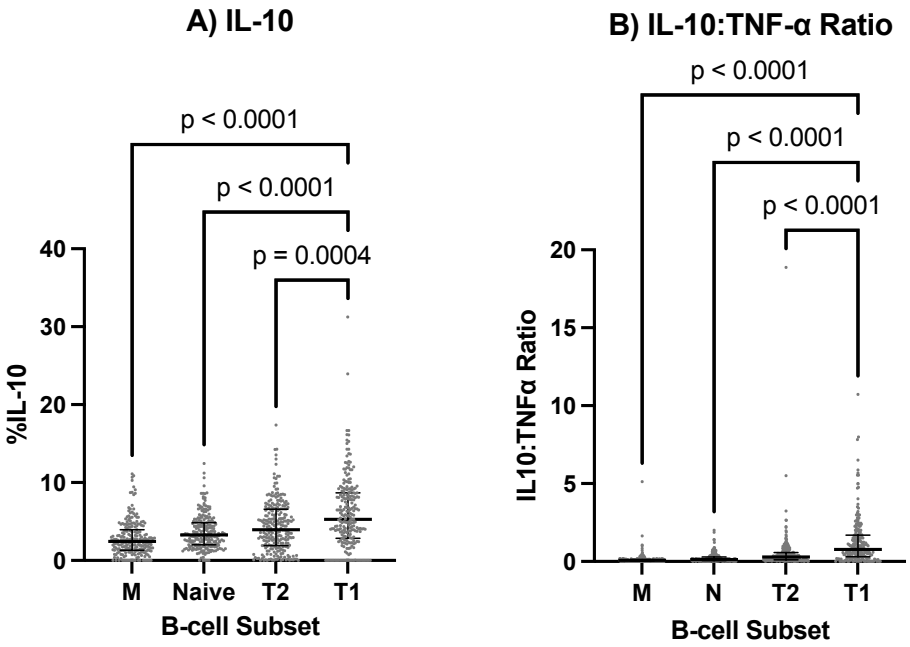


Figure 5.5 Transitional Cells have increased regulatory capacity.

Samples were obtained from 265 ALBERT study patients (median time from transplantation – 327 days, range 16-4561 days, 31% basiliximab induction, 69% alemtuzumab induction). PBMCs were isolated and stimulated with CPG and CD40L for 24 hours. PMA, ionomycin, brefeldin-A and monensin were added for the last 5 hours of incubation. Cells were then stained using the antibody panel described in Table 2.10 to determine the regulatory capacity defined by A) % cells expressing IL-10 and by the B) IL-10:TNF-α ratio in each subset. Individual values, median and interquartile ranges are depicted. Comparisons were made using the Kruskal Wallis test with Dunns multiple comparisons test, using T1 as the control group.

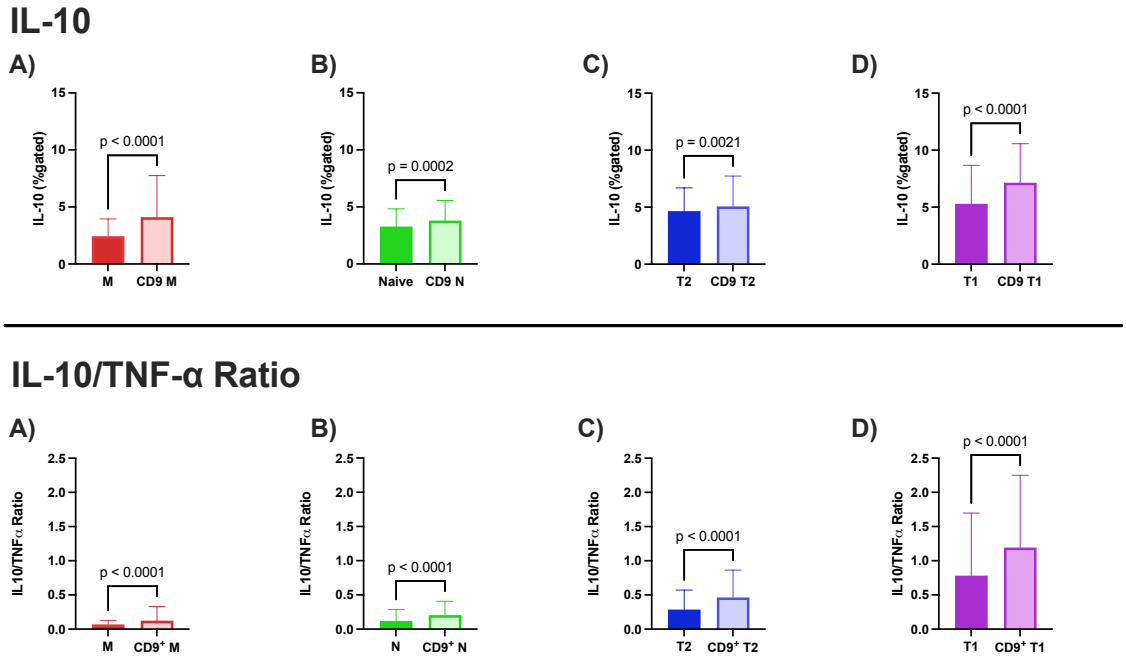


Figure 5.6. CD9 expression is associated with increased regulatory capacity. Samples were obtained from 265 ALBERT study patients (median time from transplantation – 327 days, range 16-4561 days, 31% basiliximab induction, 69% alemtuzumab induction). PBMCs were isolated and stimulated with CPG and CD40L for 24 hours. PMA, ionomycin, brefeldin-A and monensin were added for the last 5 hours of incubation. Cells were then stained using the antibody panel described in Table 2.10 to determine the regulatory capacity defined by IL-10 and by the IL-10:TNF-α ratio in each subset. The top panel compares the the % of IL-10 positive cells obtained from all cells in each B-cell subset with the % of IL-10 positive cells obtained from the $CD9^+$ cells within that subset. The bottom panel shows the IL-10:TNF-α ratios obtained from all cells in each B-cell subset compared with the ratios obtained from the $CD9^+$ cells within each subset. A) $CD24^+CD38^-$ Memory B) $CD24^+CD38^+$ Naïve C) T2 D) T1. The bar charts demonstrate the median values and interquartile range for each group. Comparisons were made using the Wilcoxon matched-pairs signed rank test.

Although $CD9^+$ cells displayed a more regulatory phenotype (increased IL-10 production and increased IL-10:TNF-α ratio) CD9 cannot be considered an exclusive

marker for Bregs because CD9 expression did not capture all IL-10 producing cells (see Figure 5.7).

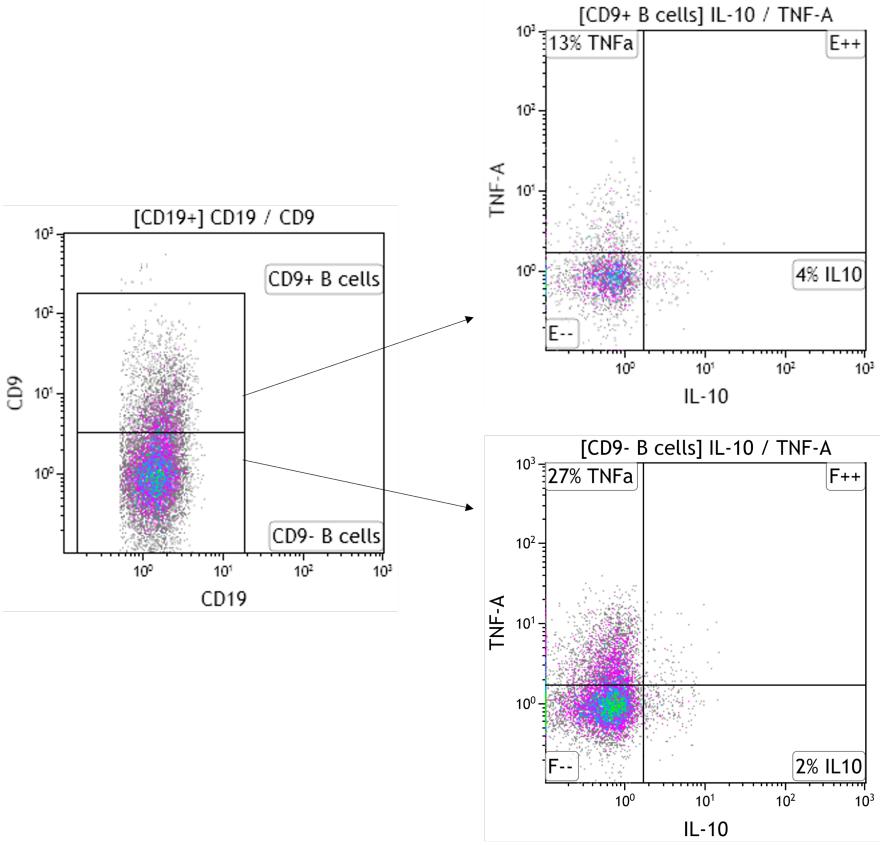


Figure 5.7. CD9$^+$ expression does not capture all IL-10 producing cells.
PBMCs were isolated and stimulated with CPG and CD40L for 24 hours. PMA, ionomycin, brefeldin-A and monensin were added for the last 5 hours of incubation. Cells were then stained using the antibody panel described in Table 2.10 to determine the regulatory capacity defined by IL-10 and by the IL-10:TNF-α ratio in each subset. These representative plots (obtained from a 47 year old 'For Cause' Biopsy patient, who had blood sampling 3 years post transplantation/alemtuzumab induction) show the % cells expressing IL-10 and TNF-α. In this patient sample, 4% of CD9$^+$ cells express IL-10, yet 2% of CD9$^-$ also express IL-10.

5.1.2.4 UTILITY OF CD9 EXPRESSION AS A BIOMARKER OF CLINICAL OUTCOME

The utility of measuring CD9 expression as a biomarker of graft survival in the 5 years following a late 'for cause biopsy' was then tested. Eleven 'Group 2' patients (described in section 4.2.1.3) had their B-cell subsets characterised together with CD9 expression using flow cytometry on the day of biopsy. Patients who lost their graft over the follow up period were found to have a lower $CD9^{+}$ T1:T2 ratio than patients who did not lose their graft (median ratio 2.078 graft survival vs 1.812 graft loss, p=0.0815), however this difference was not statistically significant. No other differences were noted between the two groups with respect to CD9 expression, however overall patient numbers were small and firm conclusions could not be made.

5.1.2.5 SUMMARY OF FINDINGS

CD9 expression was increased in the transitional B-cell population, with the highest expression observed within the $CD24^{+++}CD38^{+++}$ T1 transitional subset. The cells demonstrating high CD9 expression overlaps with the distribution of previously described Bregs. Following the stimulation protocol for regulatory B-cells, a reduction in CD9 expression was seen compared with the unstimulated controls, bringing into question the stability of CD9 as a marker for B regs. Nevertheless, following stimulation, $CD9^{+}$ cells were associated with higher IL-10 production, and a higher IL-10:TNF-α ratio compared with the overall subset suggesting a marker for B-cells with regulatory capacity. CD9 however, did not capture all IL-10 producing cells.

5.1.3 DISCUSSION

A specific marker for human B regulatory (Bregs) cells remains elusive. The hallmark feature of Bregs is their ability to produce IL-10, and these cells have been demonstrated in several human B-cell subsets. In particular, the $CD19^{+}CD24^{hi}CD38^{hi}$ transitional B-cell population have gained increasing interest as they have been found to contain higher frequencies of IL-10 producing cells and have been demonstrated to be increased in operationally tolerant renal allograft recipients.[264]

This study of renal allograft recipients has found that cells expressing CD9 have a significant overlap with this $CD19^{+}CD24^{hi}CD38^{hi}$ population: $CD19^{+}CD24^{+++}CD38^{+++}$ T1

cells and $CD19^{+}CD24^{++}CD38^{++}$ T2 cells had the highest frequency of CD9 expressing cells, and these cells demonstrated the highest CD9 expression measured by median fluorescent intensity. This finding was also observed by others: Bigot *et al* found a significantly higher frequency of $CD9^{+}$ cells in the $CD19^{+}CD24^{hi}CD38^{hi}$ B-cells compared with $CD24^{int}CD38^{int}$ and $CD24^{+}CD38^{-}$ cells in the peripheral blood of healthy volunteers.[262] Brosseau *et al* observed an association between $CD9^{+}$ B-cells and the $CD19^{+}CD24^{hi}CD38^{hi}$ transitional B-cell phenotype, and these cells were reduced in the peripheral blood of patients with severe asthma compared with healthy volunteers.[261] Mohd Jaya *et al* tested human tonsillar cells and found the majority of $CD24^{hi}CD38^{hi}$ transitional B-cells within $CD9^{+}$ B-cells compared with $CD9^{-}$ B-cells.[265] The transitional cell T1:T2 ratio has recently been suggested as a biomarker of renal allograft outcome, with a higher ratio >0.17 associated with subsequent graft stability in patients who received a late for cause biopsy at an average of 99 months following transplantation.[141] This study demonstrates that gating on $CD9^{+}$ transitional B-cells almost doubles the T1:T2 ratio compared with the T1:T2 ratio obtained from the overall transitional B-cell population, suggesting CD9 expression captures the transitional cells with the highest regulatory potential.

As IL-10 production is the hallmark feature of regulatory B-cells, PBMCs were stimulated with CPG/CD40L/PMA/ionomycin to activate regulatory cells. Following stimulation, $CD9^{+}$ cells were found to be associated with increased regulatory capacity defined by increased IL-10 production. B-cells can produce both pro-inflammatory and regulatory cytokines, therefore Bregs can also be defined using the IL-10:TNF-α ratio, with a high ratio correlating with a skew towards increased regulatory capacity.[124] Here, it has been demonstrated for the first time that CD9 expression in B-cell subsets is associated with an increased IL-10:TNF-α ratio. However, it was noted that CD9 was not a fully inclusive marker of Bregs as gating on $CD9^{+}$ did not capture all IL-10 producing cells. Similar results have recently been published by Bigot *et al* who found that although $CD24^{hi}CD38^{hi}$ TrBs expressed CD9, CD1b and ICOS-L, $IL\text{-}10^{+}$ cells were only enriched in $CD24^{hi}CD38^{hi}$ cells expressing CD1b and ICOS-L.[262] In a study of

operationally tolerant liver recipients, only 38-85% of IL-10 producing cells were CD9 positive.[266]

Interestingly, *in vitro* stimulation of B-cells using CPG/CD40L/PMA/ionomycin was associated with a reduction in CD9 expression compared with unstimulated B-cells (Figure 5.3). This was not noted in murine studies, although LPS was frequently used to activate Bregs instead of CD40L, and the duration of the assay was significantly shorter (5 hrs compared with this study's 24-hour protocol).[260] A similar phenomenon was noted by Mohd Jaya *et al* who purified and sorted $CD19^+CD9^+$ and $CD19^+CD9^-$ cells from human tonsil cells. Following incubation with a CD40-agonist, anti-IgM and IL-2 in the presence of mesenchymal cells for up to a week, they demonstrated that purified $CD9^-$ cells regained CD9 expression (up to 40%) whereas CD9 was downregulated in the purified $CD9^+$ cells (by up to 70%).[265] Brosseau *et al* however have reported an association between CD9 expression and IL-10 production, and have validated $CD9^+$ B-cell frequency as a predictive marker of lung allograft stability.[263] Their protocol only included stimulation of cells with PMA and ionomycin for 5 hours. These findings show that CD9 expression can be influenced by culture conditions, and therefore may not be a stable marker for Bregs. Further investigation will be required to characterise the effect of different Breg activation protocols on CD9 expression. Mechanistically, it would also be interesting to investigate whether blocking CD9 with a neutralising antibody would affect IL-10 production.

After demonstrating an overlap between CD9 expression and transitional B-cells, together with increased regulatory capacity using the IL-10:TNF-α ratio, CD9 expression was investigated as a marker of outcomes in patients who had a for cause biopsy. Although, as described in the previous chapter, a low overall T1:T2 ratio was associated with evidence of rejection on biopsy, and an increased risk of graft loss in the subsequent 5 years, there was no association between CD9 expression and clinical outcomes. This was likely due to the small numbers in each group, limiting any firm conclusions. Further prospective studies will need to be performed to answer this question.

To summarise, although CD9 is not an exclusive marker for Bregs, its expression is increased in the $CD24^{hi}CD38^{hi}$ transitional B-cell population, a population that has been shown to have increased regulatory capacity. An association between CD9 and increased regulatory capacity was demonstrated through increased IL-10 production and an increased IL-10:TNF-α ratio. Further work will need to be performed to determine the prospective utility of CD9 expression as a biomarker of clinical outcomes.

6 DETERMINING THE PRESENCE OF ALLOREACTIVE B-CELLS IN THE PERIPHERY

Circulating HLA antibodies are produced from two sources – long lived plasma cells that survive in bone marrow niches and circulating memory B-cells. However, memory B-cells can be present in the circulation without declaring themselves through the production of antibodies.[267] On re-exposure to their cognate antigen, they can rapidly proliferate and generate antibodies without the need for further T-cell help, potentially leading to early ABMR. Obtaining a thorough sensitisation history is important to identify previous exposure to HLA that are not evident through routine screening, and some transplanting centres may wish to avoid repeat exposure with a new graft. To truly understand the kinetics, and the full potential of the immune response to an allograft, peripheral memory B-cells will need to be interrogated in addition to routine serum screening.

In transplant candidates, efforts are now being made to understand the phenotype and trafficking of alloreactive B-cells,[268] and research groups are employing two broadly different methods to achieve this. Firstly, membrane bound B-cell receptors on intact cells are identified using HLA tetramers (which are streptavidin-biotin complexes of four HLA molecules conjugated to a fluorochrome). The tetramer-bound cognate B-cells can be visualised using flow cytometry, and additional surface markers can be used to differentiate subsets.[269] Secondly, memory cells can be cultured *in vitro*, and differentiated into antibody secreting cells (ASCs). The allospecific immunoglobulins (Igs) produced in the culture supernatant can then be quantified,[270] or the number of Ig secreting cells can be enumerated using an ELISpot assay.[267,271]

These methods have their limitations, and it is increasingly accepted that a proportion of B-cells can recognise the non-HLA portions of the tetramer (including streptavidin-biotin, and the fluorochrome itself),[272–274] resulting in non-specific binding. Collecting cell supernatant is sensitive and reflects the production of antibodies over the whole culture period. However, it is difficult to determine the origin of each antibody specificity and assumes that the amount of antibody obtained is proportional to the

size of a single B-cell clone rather than the presence of multiple clones. The ELISpot assay will only account for the number of HLA specific immunoglobulin secreting cells that are viable at the end of the culture period.[274] One novel technique to assess alloreactive B-cells is to co-culture cells with single antigen beads (SABs), and quantitate the bead-bound cells using flow cytometry as described by Degauque *et al.*[275] SABs are polystyrene beads that contain differing ratios of 2 fluorophores. Each bead is coated with a different HLA molecule and configured in an array. When excited by the red laser, this allows the reactivity to multiple HLA specificities to be assessed in one reaction and is currently used to screen for HLA antibodies in serum (Figure 2.2a). This has the benefit over tetramers in that only the HLA antigens coating the bead surface are available for binding to the alloreactive cells. Surface staining with antibody-conjugated fluorochromes prior to culturing with the SABs will allow the cell type, in addition to the HLA specificity to be identified and quantitated using flow cytometry. Finally, a direct comparison can be made to the specificities obtained from the patient's serum, as the same SABs are used.

This chapter describes the preliminary experiments surrounding the development of two assays that could be implemented to determine identify B-cell memory for potential patients who have experienced a previous sensitising event. A combination of two different techniques were used:

- the non-specific stimulation of peripheral memory B-cells to ASCs as described by Lanzavecchia,[270] followed by assessing the culture supernatant for the presence of HLA antibodies.
- Binding of alloreactive B-cells to ONELAMBDA single antigen beads (SABs) to detect potential reactivity using a flow cytometer.

6.1 METHODS

See Chapter 2: General Material and Methods

2.4.3 – Assessment of HLA antibodies in serum or cell supernatant using ONELAMBDA Single Antigen Beads (SABs)

2.5 – Patient recruitment to the ALBERT study

2.10 – Cell Surface Staining - Surface B-cell Phenotype

2.11 – PBMC Isolation and Storage

2.16 – Calculations and Statistical Analyses

6.1.1 METHOD 1 – THE NON-SPECIFIC STIMULATION OF PBMCS WITH R848/IL-2

Freshly isolated PBMCs ($1x10^6$) were placed into 75ml culture flasks with 25ml RPMI-10%FBS. R848 (1µg/ml, Invivogen) and IL-2 (10ng/ml, Sigma Aldrich) were added, and cells were cultured for up to 12 days at 37°C, 5%CO_2. Control conditions were set up in parallel, where PBMCs were cultured in RPMI alone.

After the required incubation time, cells and supernatant were harvested and centrifuged for 5 minutes at 400 x *g* to separate the supernatant from the cell pellet. The supernatant was decanted into an Amicon Ultra-15 Centrifugal filter device fitted with a 100Kda filter (Merck Millipore) and concentrated up to 100 times by centrifuging at 500 x *g* for 30 minutes. Following this, the protein concentration of the supernatant was quantified using a Nanodrop spectrophotometer by measuring absorbance at 280nm. The supernatant was stored at -80°C. The cell pellet was washed with ice-cold PBS-1%FBS, and then stained as described above using the fixable viability kit, followed by surface staining with monoclonal antibodies to assess the development of antibody secreting cells (Table 6.1. Monoclonal Antibodies used for cell surface staining following R848/IL-2 stimulation.).

Surface Staining Following R848/IL-2 Stimulation				
Monoclonal Antibody	**Clone**	**Manufacturer**	**Catalogue #**	**Volume (µl)**
Fc Block		BD Pharmingen	564219	2
Anti-Human CD24-FITC	ML5	BD Pharmingen	555427	10
Anti-Human CD25-PE	M-A251	BD Pharmingen	555432	15
Anti-CD27-PerCPeFluor710	LG.7F9	eBioscience	46-0271	1
Anti-Human CD38-PECy7	HIT2	BD Pharmingen	560677	2
Anti-Human CD138-APC	DL101	BioLegend	352308	5
Anti-Human CD19-APCeFluor780	HIB19	eBioscience	47-0199	1
Anti-Human IgD-BV421	IA6-2	BD Horizon	562518	2

Table 6.1. Monoclonal Antibodies used for cell surface staining following R848/IL-2 stimulation.

6.1.2 METHOD 2 – DEMONSTRATING THE PRESENCE OF ALLOSPECIFIC B-CELLS USING SINGLE ANTIGEN BEADS (SABS)

Freshly isolated PBMCs ($1x10^6$) were stained with the viability stain (LIVE/DEAD fixable violet dead cell stain kit, Invitrogen), and then incubated with Anti-Human IgD-BV421, Anti-Human CD27-PE, Anti-Human CD19-FITC and 2µl of Fc block (BD biosciences) for 20 minutes in the dark at 4°C. Cells were washed twice with ice-cold PBS-1%FBS and were incubated with 5µl of Class I or Class II SABs for 20 minutes in the dark at room temperature. They were washed 3 times and fixed with PBS-3%formaldehyde prior to acquisition.

6.2 RESULTS

6.2.1 METHOD 1: THE NON-SPECIFIC STIMULATION OF PBMCS WITH R848/IL-2

6.2.1.1 IN VITRO DIFFERENTIATION OF B-CELLS INTO ANTIBODY SECRETING CELLS (ASCS)

PBMCs were cultured with R848 and IL-2, using methods modified from previous authors.[270,276] To determine the optimum culture duration, cell viability was determined at different time points. The cell viability was higher with stimulated cells compared with unstimulated cells at all time points (data not shown). With stimulated cells, viability decreased as the incubation time increased, and after 9 days of culture, it was not possible to identify different cell populations. In addition to cell viability, the B phenotype was assessed. Antibody secreting cells (ASCs, $CD19^{+}CD24^{-}CD38^{hi}$), class switched memory cells ($CD27^{+}IgD^{-}$) and plasma cells, ($CD19^{lo}CD27^{hi}CD38^{hi}$ and $CD19^{lo}CD138^{+}CD38^{+}$) were compared. ASCs and plasma cells increased over the 9-day stimulation period, and the proportion of class-switched memory cells peaked at day 6 (Figure 6.1A). The results from 10 experiments were collated, and the proportion of ASCs peaked following 9 days of stimulation (Figure 6.1B) suggesting that phenotypically, the optimal culture duration was 9 days.

Cell culture supernatant was collected and concentrated to test for the presence of HLA antibodies using SABs. The concentration was assessed by measuring A280 protein absorption using a nanodrop spectrophotometer. This parameter was chosen as the culture media contained foetal bovine serum (FBS), and similar values would suggest a uniform concentration factor. This allowed the antibody specificities to be standardised against the concentration of the supernatant. The concentration of cell supernatant was not statistically significant between unstimulated and stimulated cells (difference between means: 0.7944 ± 1.725, p=0.6487, Figure 6.2).

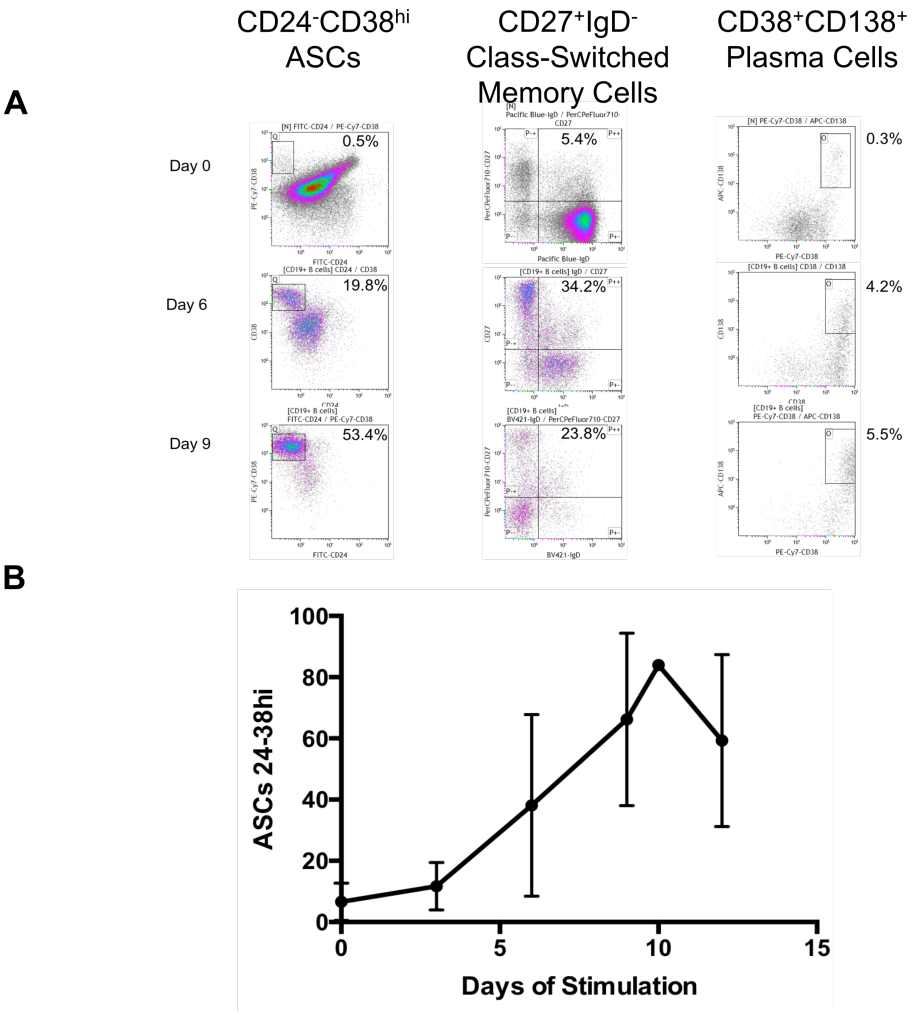


Figure 6.1. Non-specific Stimulation of PBMCs with R848/IL-2 – Analysis of Surface Phenotype

$1x10^6$ PBMCs were cultured with R848 and IL-2 for up to 12 days. Following culture, the cells were surface-stained. A) Representative scatter plots depicting the change in cell phenotype from Day 0 to Day 9. Antibody secreting cells (ASCs, $CD24^-CD38^{hi}$), and Plasma cells ($CD38^+CD138^+$) increase in number over 9 days of stimulation. Class Switched memory cells ($CD27^+IgD^-$) peak at day 6. B) Cumulative data from 10 samples (2 healthy volunteers, 8 patients) which show the relationship between %-gated antibody secreting cells and number of days of stimulation. The %-gated antibody secreting cells peak at day 9.

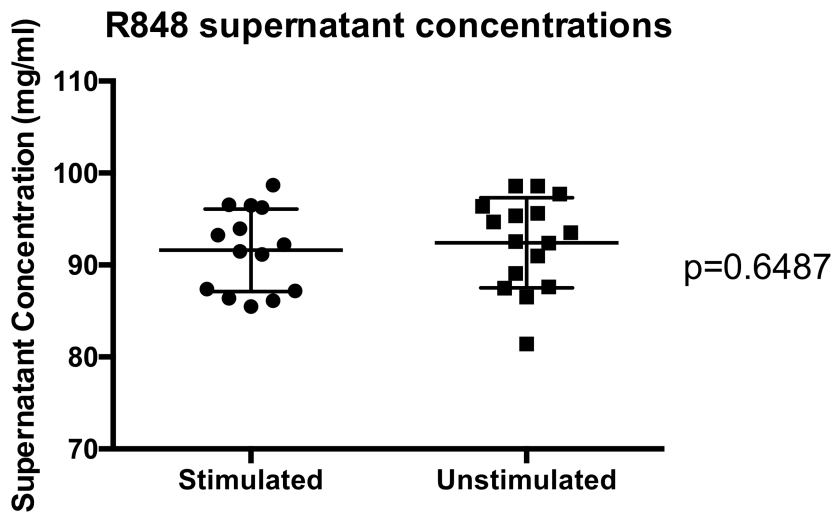


Figure 6.2. Comparison of Cell supernatant concentrations

PBMCs were incubated for up to 12 days in the presence of R848 and IL-2. Unstimulated PBMCs were cultured in RPMI alone. The cell supernatant was harvested and concentrated up to 100 times using an Amicon centrifuge filter device. The concentration of the supernatant was tested by measuring the A280 protein absorption using a spectrophotometer. The two culture conditions were compared using an unpaired t-test, with the individual values, mean and standard deviation shown.

When the cell supernatant was concentrated to approximately 90mg/ml, antibody specificities could be identified after 3 days of culture; however, more antibody specificities were visible after 9 days of stimulation, and these were present at higher intensities (Figure 6.3b). When compared against unstimulated cells, more HLA antibodies (measured by trimmed mean fluorescence) were obtained from the supernatant of stimulated cells compared with unstimulated cells at all time points (Figure 6.3b).

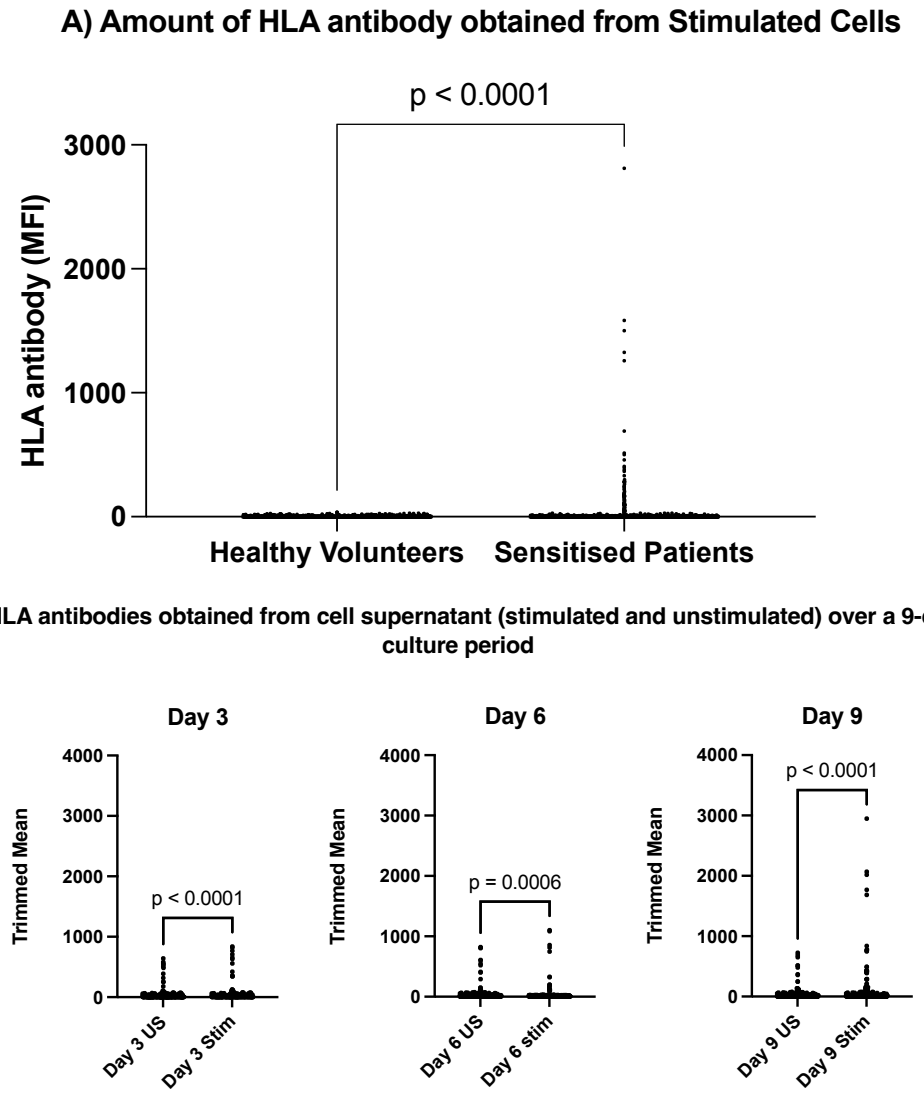


Figure 6.3. HLA antibody detection in cell supernatant following stimulation with R848 and IL-2.

Amount of HLA antibody (median fluorescent intensity) detected in the supernatant of stimulated PBMCs obtained from 3 unsensitised healthy volunteers and 3 sensitised patients. B) Amount of HLA antibody (Trimmed Mean) detected in the supernatant of unstimulated and stimulated PBMCs.

The cell supernatant obtained from 3 healthy unsensitised volunteers (HV) and 3 sensitised wait list patients were then compared. After 9 days of stimulation with R848/IL-2, sensitised patients were found to have more HLA antibodies in their cell

supernatant compared with healthy volunteers (HV: median MFI 0, IQR 0.4522, range 0 – 35, SP: median MFI 0.3075, IQR 1.555, range 0 -2812 p<0.0001 [Figure 6.3a]).

Some reactivity was noted in the unstimulated supernatants, albeit at lower intensities, which could represent either non-specific binding, or the production of antibodies by any ASCs that were present at the start of the culture period. To correct for this, the MFI values obtained from the stimulated cell supernatant were normalised using the values obtained from the unstimulated cells as the negative control.

6.2.1.2 DETERMINING THE POSITIVE CUT-OFF THRESHOLD FOR HLA ANTIBODIES IN CELL SUPERNATANT

Initially, all normalised MFI values <0 were considered. However, as the normalised MFIs obtained from healthy unsensitised volunteers ranged from 0 – 35, the cut off was adjusted to twice the upper limit of normal, or 70 MFI. The local measurement uncertainty calculations determined for each single antigen bead lot tested using the LABXpress robotic platform were also reviewed (see Table 6.2). Taking the uncertainty of measurement into consideration (acknowledging that this calculation would need to be repeated in the future using cell supernatant), this created a 'grey area' between nMFI 35 (upper limit of healthy volunteer result) and 70 (2x the upper limit) that would require increased scrutiny.

Class I, Lot 011	MU Range	Av MU	%CV
MFI <1000	5.5-41.2	13.87	10.4
MFI 1000-2000	25.3-45.6	35.41	8.49
MFI 2000-5000	53.0-601.6	109.33	11.2
Class II, Lot 012	**MU Range**	**Av MU**	**%CV**
MFI <1000	20.5-26.2	23.88	24.65
MFI 1000-2000	78.9-108.4	93.63	17.11
MFI 2000-5000	92.3-310.22	195.49	17.22

Table 6.2. Measurement Uncertainty calculations for each bead using the LABXpress platform.

The positive control serum was tested on 10 runs. The measurement uncertainty (MU, $SD/\sqrt{N}$) and coefficient of variance was calculated for each bead at different MFI levels. The normalised MFI range 0-5000 was included in this table as cell supernatant values did not exceed 3000 MFI. SD- standard deviation, N- number of values

The normalised MFIs (nMFI) obtained from each patient cell supernatant were ranked from highest to lowest values, and any 'steps' in the data were noted. This was in accordance with the local policy for determining the presence of HLA antibodies. The specificities were then analysed, looking for clustering of antibodies from the same broad antigen group or CREGs (cross reactive groups), and reactivity patterns were compared with the matched serum sample. For example, in Figure 6.4, the specificities with the highest values (B*57:01, B*57:03, B*58:01) were clustered

around 100 MFI. These represent specificities from the broad B17 group. As these are the only B17 beads within the panel that have clustered together >2x the upper normal limit, these could be considered positive. A second step is visible around 50 MFI (B*15:16) in the grey area, and a third step at around 15 MFI. Based on values obtained from healthy controls, specificities with nMFI <15 could be considered negative.

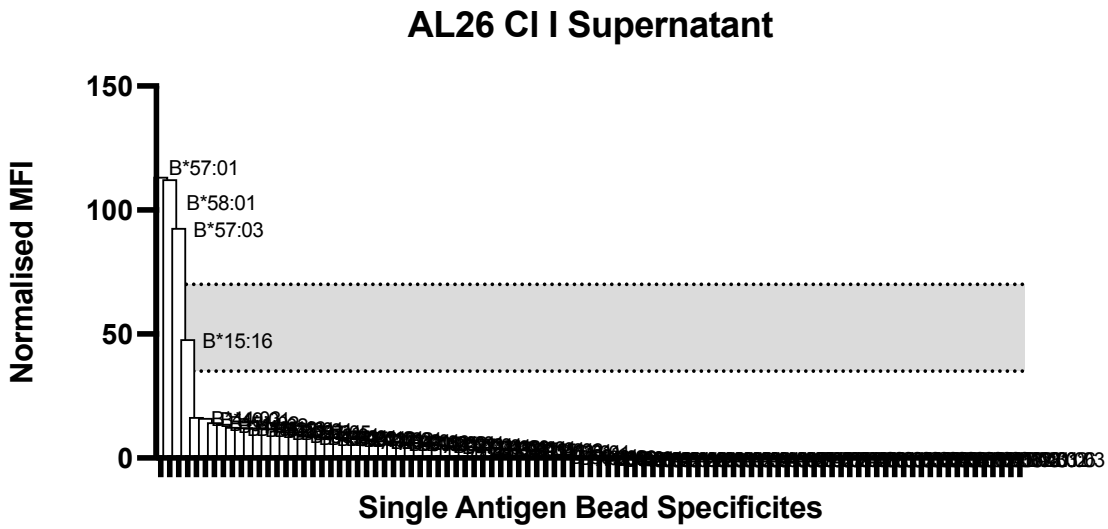


Figure 6.4. HLA antibody profile obtained from the cell supernatant of patient AL26. PBMCs were cultured for 9 days. Stimulated cells were cultured with R848 and RPMI, unstimulated cells were cultured in RPMI alone. Following the period of incubation, the supernatant was concentrated using an Amicon centrifugal filter. The supernatant was tested using ONELAMBDA single antigen beads. The stimulated supernatant was normalised using values obtained from the unstimulated supernatant. A 'grey area' has been marked between MFI =35 (the highest value obtained from healthy volunteers) and MFI = 70 (2x the upper limit of normal) has been marked with a dashed line.

6.2.1.3 INDIVIDUAL PATIENT RESULTS

	Total
Number	6
Recipient Age (yr)	36
Recipient Gender (m)	2
Recipient Gender (f)	4
Recipient Ethnicity	
Caucasian	5
Asian	1
Cause of ESRD	
Glomerulonephritis	3
PKD	1
Reflux	2
Sensitisation history	
Transfusion	0
Transplant	5
Pregnancy	2
On Immunosuppression?	
Yes	2
No	4
ALBERT study group	
Prospective	2*
For Cause Biopsy	1
Wait List	5

Table 6.3. R848 Stimulation – Patient Demographics.

***2 patients were recruited to the ALBERT study whilst on the wait list, however received a transplant and re-consented to follow up as part of the prospective group.**

Table 6.3 contains the patient demographics, and Table 6.4 describes the serum and cell supernatant results of 6 sensitised patients, together with their reported sensitising events. When assessing transplantation as a sensitising event, some donor HLA types (for example HLA-C, HLA-DQA1) were inferred using linkage association (see Supplementary Information: SLFOIMTM095, Common haplotypes and HLA association). This was because donor HLA typing was performed according to the minimum requirements at the time (for example in 1995, this was low resolution typing for HLA-A, -B and -DR only) and donor DNA was not available for re typing.

Two of the 6 patients did not have any HLA antibodies in their cell supernatant. In the other 4 patients, both class I and class II specificities were found in the cell supernatant. Overall, 26 HLA antibody specificities were found in the cell supernatant. Sixty-five percent of these specificities were also demonstrated in the matched serum sample. HLA antibodies were identified in the cell supernatant, but not in the matched serum sample in 3 patients (AL26, AL29 and AL39). These patients are described in further detail below.

6.2.1.3.1 AL26

AL26 has a history of 1 transplant from his father, but no previous transfusions. The graft failed and he returned to dialysis in 2009, with subsequent removal of the graft. Prior to his transplant no HLA antibodies were demonstrated in his serum. Following the graft nephrectomy, he was found to have multiple class I and II HLA antibodies in his serum. His cell supernatant demonstrated B57 and B58 antibodies in the positive region (however these specificities were not apparent in the current serum or historical profile), and a B*15:16 antibody in the grey area. B57 and B58 could be explained by the B58 mismatch from the failed transplant. B15 antibodies had been present in the past but were not present in the current serum. Figure 6.5 shows the results obtained from the cell supernatant.

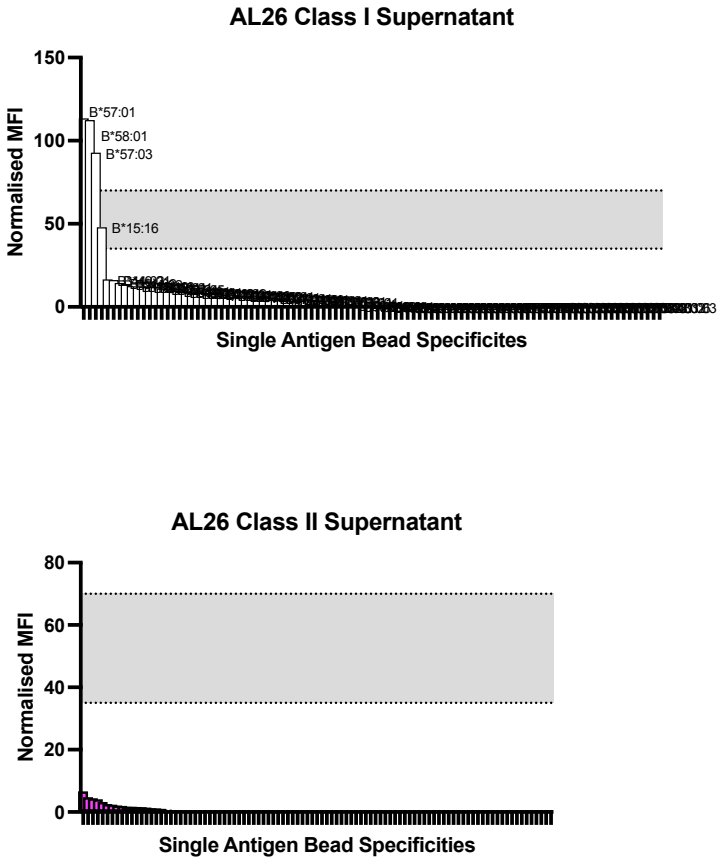


Figure 6.5. Cell supernatant results obtained from AL26.

A grey area has been delineated between MFI 35-70.

6.2.1.3.2 AL29

AL29 developed end stage renal disease secondary to MCGN and received a renal transplant in 1995. Sensitisation history also included prior pregnancies. She was offered a for cause biopsy due to increasing proteinuria and graft dysfunction. Immunosuppression at the time of biopsy included azathioprine and prednisolone. The biopsy sample was suboptimal, however there was evidence of borderline TCMR, and ABMR (g3, ptc3, c4d1). The matched serum sample demonstrated multiple DSAs (A1, B18, Cw7, DR11, DQ7). Similarly in the supernatant, DSAs were also observed (B18, Cw7, DQ7). B8 was demonstrated in both serum and supernatant, and Cw8 was noted in the grey area of the supernatant but not the current serum, although it had been identified in the cumulative profile. On review of the class II supernatant (Figure 6.7), the DQA1 specificities DQA1*05:01, 05:03, 05:05 and 06:01 were reported as

positive. This is because, although they were associated with DQB1*03:01 specificities within the bead kit, there was clustering of 4 DQB1*03:01 beads around nMFI 1000-1500, and a cluster of the final DQB1*03:01 beads around 200 nMFI. If all reactivity was caused by the same DQB1*03:01 antigen, all 6 beads would be expected to cluster at the same nMFI. This split in strengths could be explained by differing reactivity to the DQA1 chain. Furthermore, the positive reactivity to DQA1*05:01, DQA1*05:03, DQA1*05:05 and DQA1*06:01 can be explained through the shared epitope 40GR.[14] The DQA reaction pattern described above was not seen in the matched serum (Figure 6.6). The DQA reaction seen in the supernatant could possibly be explained by the prior transplant, however the donor DNA was not available for further testing.

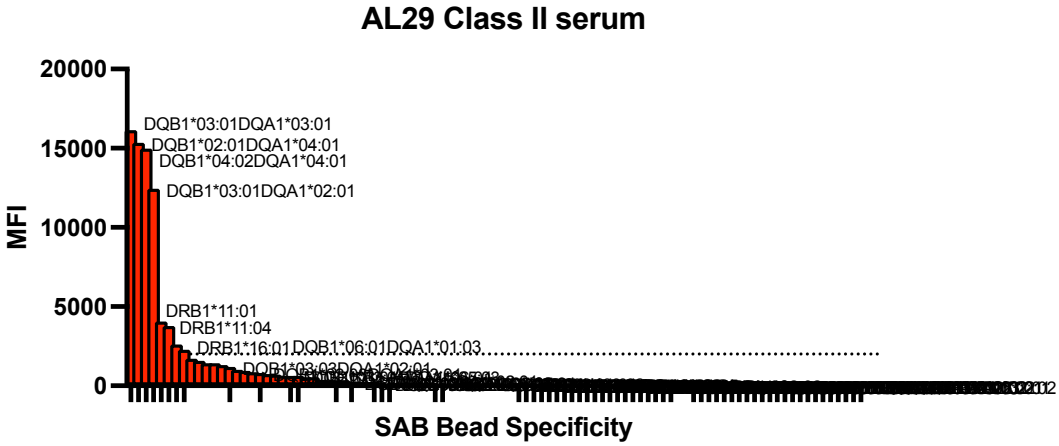


Figure 6.6. AL29 Class II Serum results

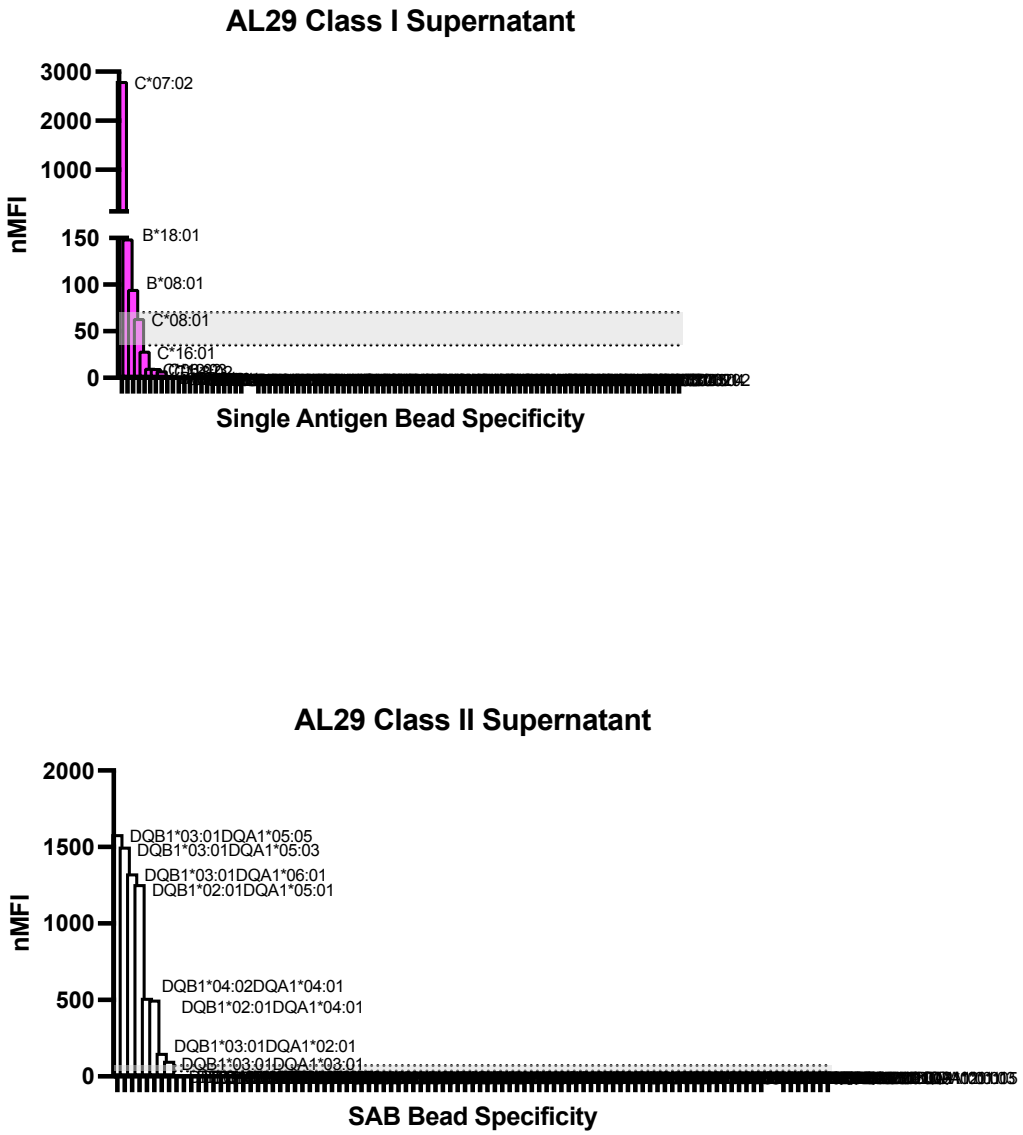


Figure 6.7 AL29 Cell Supernatant Results.

6.2.1.3.3 AL39

AL39 has a history of two previous transplants, but no transfusions or pregnancies. The class I antibodies found in the cell supernatant were also present in the serum. Some of the class I specificities in the cell supernatant could be explained by mismatches from the first transplant (B44, B45, Cw5), but no specificities directed towards the second transplant were present. DPA1*02:01 and DPA1*02:02 antibodies were demonstrated at moderate levels (MFI 100-200) in the cell

supernatant, but were not present in the matched serum, or in the patient's cumulative profile. All beads coated with these specificities within the microbead panel were positive, and they were clustered at similar levels, adding strength to the result obtained. On review of the patient's sensitising history, DPA1*02:01 or DPA1*02:02 could not be explained by the most recent transplant (donor 2 HLA type DPA1*01:03). A possible explanation is that these specificities could be directed against donor 1, although the HLA-DPA type was not available to confirm this hypothesis (Figure 6.8).

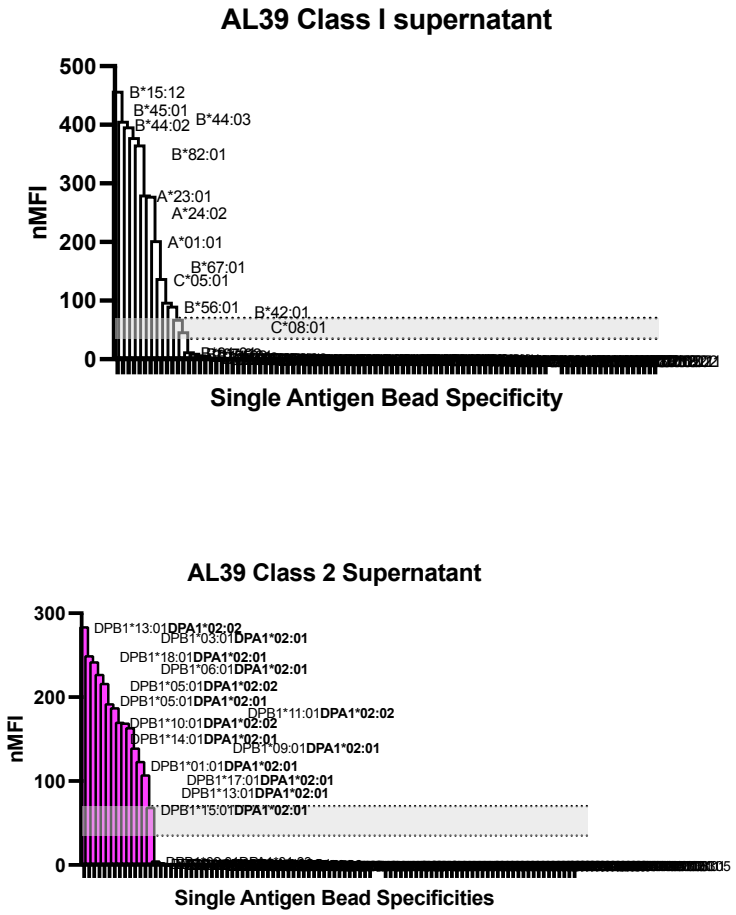


Figure 6.8. AL39 Cell Supernatant Results

ID	Recipient HLA Type		Sensitising Events/Donor HLA type		Serum HLA Antibody Profile	Supernatant HLA Antibody profile
AL39 (F)	A*02:01 B*50:01 C*04:01 DQB1*02:02 DQA1*02:01 DRB1*07:01 DRB3*02:02 DPB1*03:01 DPA1*01:03	A*68:01 B*53:01 C*06:02 DQB1*03:01 DQA1*05:05 DRB1*11:01 DRB4*01:01 DPB1*04:01 -	Two transplants (2002, 2014). 1st graft lost – recurrent FSGS, 2nd graft lost – cellular rejection No transfusions, No pregnancies		Current sample (29/09/16): A1,3,23,24,29,43 B7,13,18,42,44,45,46,51,52,54,55,56,57,63,67,76,81,82 Cw1,5,7,8,9,10,12,15,16 DQ5,6 Cumulative Profile: A1 B13,44(12),45(12),76,82 DQ5,6,8,9, DQA1*03:02	Sample date (29/09/16) A1, A23, A24, B*44:02. B*44:03, B*45:01, B76, B*67:01, B*82:01 Cw5, DPA1*02:01, DPA1*02:02
			Donor 1: A2 **B44(12**) **Cw5** DR7 DQ2	 - - - - DQ3		
			Donor 2: A*02:01 **B*51:01** **C*01:02** DRB1*07:01 DRB3*02:02 DQA1*02:01 DQB1*02:02 DPA1*01:03 **DPB1*02:01**	 **A*03:01** **B*56:01** **C*15:02** DRB1*11:01 DRB4*01:01 DQA1*05:05 DQB1*03:01 - DPB1*04:01	Current sample (MFI>10 000) A1,3,23,24 B42,44,45,56,63,67,76,81,82 Cw5,8	

ID	Recipient HLA Type		Sensitising Events/Donor HLA type		Serum HLA Antibody Profile	Supernatant HLA Antibody profile
AL26 (M)	A1 B7 Cw7 DR3 DQ2	A30(19) B8 - DR15(2) DQ6	One transplant (2007). Graft nephrectomy 2009. No transfusions, no pregnancies		Current sample (15/07/16): A11,26,28,29,33,34,43,66,74 B40,41 Cw2,5,8,10,12,16 DR4,7,9 DQ3,6, DQA1*01:02. Cumulative Profile: A2,3,9,10,11,28,29,31,32,33,36,43,74 B5,12,13,14,15,16,22,27,35,40,41,47,48,53,70 Cw2,3,6,8,12,16 DR4,7,9 DQ3, DQA1*01:02 Current Sample MFI>10000: DR7	Sample Date:15/07/16 <u>B57(17), B58(17), B63(15)</u>
			Donor: **A3** **B58(17)** Cw7 **DR7** **DQ9**	 A30(19) B7 - DR15(2) DQ2		

ID	Recipient HLA Type		Sensitising Events/Donor HLA type		Serum HLA Antibody Profile	Supernatant HLA Antibody profile
A29 (F)	A2 B62(15) DR4	A11 B55(22) DR14(6)	One transplant (functioning) 1995. No transfusions. Previous pregnancies		Current sample: 14/09/2016 A1,23,29 B8,18,27,37,38,39,47,51,54,57,59,73,78,B*13:02 Cw1,2,4,5,6,7,8,12,14,15,16 DR11,16 DQ7, DQA1*04:01 Cumulative Profile: B8,16,18,54,56,59,73 Cw1,4,5,6,7,8,12,15,16,18 DQ7, DQA1*04:01,05:01 Current sample>10000 MFI: B8,18 Cw1,7,12,16 DQ2,7,DQA1*04:01	Sample date: 14/09/2016 B8,18 C*07:02 DQB1*03:01 DQA1*05:01,*05:03,*05:05,*06:01 DQA1*04:01
			Donor: **A1** **B18** DR4 23 **C*07**	A2 B55(22) **DR11(5)** **DQA1*05:01** **DQB1*03:01**		
AL05 (F)	A*24:02 B*13:01 C*06:02 DRB1*07:01 DRB4*01:01 DQB1*02:02 DQA1*02:01	A*30:01 B*44:03 C*07:01 - - - -	24 3 previous pregnancies. No transplants. No transfusions		Current sample: 24/04/16 A2 B17 DR1,103,2,3,4,5,6,8,9,10,51,52 Cumulative Profile: A2 B17 DR1,103,2,3,4,5,6,8,9,10,51,52	Sample date: 24/04/16 Nil (All nMFIs < 5)

ID	Recipient HLA Type		Sensitising Events/Donor HLA type	Serum HLA Antibody Profile	Supernatant HLA Antibody profile
AL37 (F)	A*01:01 B*07:02 C*07:02 DRB1*12:01 DRB3*02:02 DQB1*03:01 DQA1*04:02	A*24:02 B*51:01 C*16:02 DRB1*13:02 DRB3*03:01 DQB1*06:04 DQA1*05:05	2x transplants. Transfusions Donor 1: (1989) A24(9) **B35** B51 DR6 **DR10** Donor 2: (1991) A1 **A2** B7 **B44** **DR4** DR6	Current sample: A2 DR4,16, DRB4*01:01, DRB4*01:03 Cumulative Profile: A2,28 B17 DR1,2,4,9,10,16,47,53 DP1,3,4,18,19,28	Sample date: Nil (All nMFIs <5)
AL72 (M)	A*11:01 B*55:01 C*03:03 DRB1*04:07 DQB1*03:01 DRB4*01:01 DQA1*03:01 DPB1*03:01 DPA1*01:03	A*26:01 B*45:01 C*06:02 DRB1*07:01 DQB1*02:02 - DQA1*02:01 DPB1*04:02	1x Transplant. No transfusions, No pregnancies <u>Donor 1:</u> A26(10) B45(12) Cw6 DR7 DQ2 DR53 DQA1*02:01	Current Sample: 24/02/17 DQ4,5,6,8,9 Cumulative Profile: DQ4,5,6,8,9 DP14 Current sample>10000 MFI Nil	Sample Date: 24/02/17 DQ5,6

Table 6.4. Stimulated cell supernatant results

Table 6.4. Stimulated cell supernatant results continued:

Donor/Recipient mismatches are marked in bold. Antibody specificities that appear in cell supernatant but not the matched serum are underlined. Donor alleles highlighted in red are inferred using linkage association (see Supplementary Material, page 382).[277–279] nMFI = median fluorescent intensity normalised to the supernatant obtained from unstimulated cells. Serum specificities highlighted in green represent specificities with high MFI values obtained from serum that were also present in the supernatant

6.2.2 SUMMARY OF FINDINGS

These preliminary studies have determined that the optimum duration for the non-specific stimulation of PBMCs with R848/IL-2 was 9 days. This resulted in a change in B-cell phenotype towards antibody secreting cells and at the end of the culture period, and IgG HLA antibodies could be detected in the cell supernatant. The cell supernatant obtained from sensitised patients had more HLA antibody specificities at higher MFI values when compared with unsensitised volunteers. When PBMCs obtained from sensitised patients were stimulated for 9 days, 65% of HLA specificities found in the supernatant were also present in the matched serum sample. In cases where the supernatant demonstrated additional HLA antibodies, these either could be attributed to a previous transplant, or had been present in the patient's historic serum profile.

6.2.3 METHOD 2: DEMONSTRATING THE PRESENCE OF ALLOSPECIFIC B-CELLS USING SINGLE ANTIGEN BEADS (SABS)

6.2.3.1 IDENTIFICATION OF SABS USING FACSCANTO

SABs were identified by acquiring a suspension of Class I or Class II beads in PBS using the FACSCanto flow cytometer. Photomultiplier Tube (PMT) voltages were adjusted so that each discrete bead group could be visualised and gated separately based on the differing ratio of internal dyes (Figure 6.9A). ONELAMBDA 'calibrate' beads were used to compensate for bead fluorescence. PMT voltages were adjusted further to ensure that most of the beads remained within the first log scale when plotted against other fluorophores (Figure 6.9B).

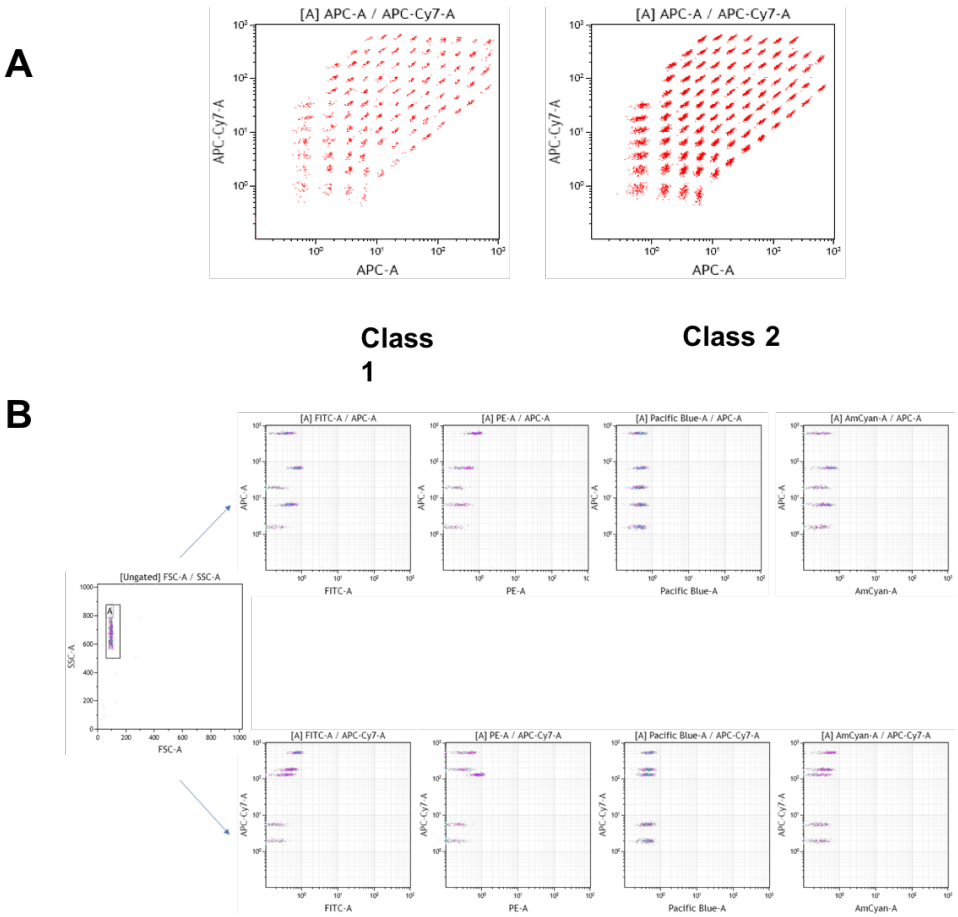


Figure 6.9. Visualisation of ONELAMBDA Single Antigen Beads using a FACSCanto flow cytometer.

5µl of either Class I or Class II SABs were added to 100µl of PBS for acquisition. A) Forward scatter (FSC), Side Scatter (SSC), APC and APC-Cy7 Photomultiplier Tube (PMT) voltages were decreased to allow separation of bead groups based on the differing ratio of internal dyes. Each discrete group of beads is coated with a different Human Leukocyte Antigen. B) ONELAMBDA 'calibrate' and 'control' beads were used to compensate for bead fluorescence. PMT voltages were adjusted to ensure that most of the beads fell within the first log scale for each fluorophore.

6.2.3.2 DEMONSTRATION OF ALLOREACTIVE B-CELLS USING SINGLE ANTIGEN BEADS (SABS)

Pre-stained PBMCs were incubated with single antigen beads in an attempt to quantify alloreactive B-cells. PBMCs ($1x10^6$) were surface stained (Table 2.9) prior to incubating with 5µl of washed SABs. Cells were gated following the strategy described by Degauque to allow identification of HLA specific B-cells (Figure 6.10).[275] First, non-viable cells were excluded. B-cells and bead-B-cell rosettes (BBR) were selected based on $CD19^+$ expression. The specificities of the $CD19^+$ BBR could then be determined by plotting APC against APC-Cy7, and by gating around each group. Different BBR subsets could be identified based on expression of CD27 and IgD. Non-specific binding to the SABs was accounted for by disregarding bead populations that had binding values that were equal to or less than the negative control bead (bottom left bead cluster in Figure 6.10). Additionally, the lymphocyte gate could be interrogated, providing information on the relative frequencies of the different B-cell subsets that did not form BBRs. Figure 6.11 is a photograph demonstrating B-cell binding to single antigen beads.

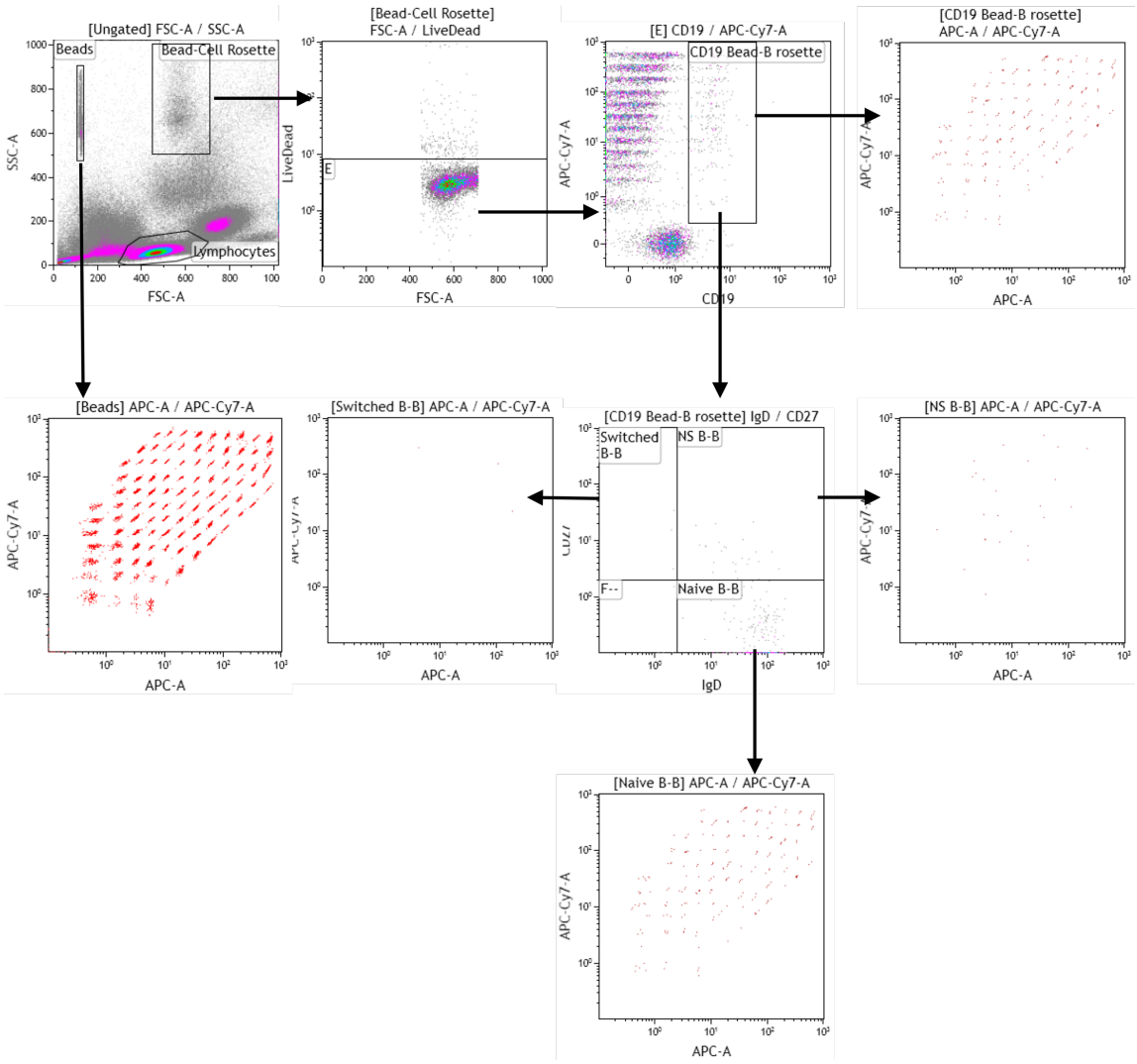


Figure 6.10. Identification of Allospecific B-cells

After co-incubation, lymphocytes and SABs are gated based on their forward and side scatter. After exclusion of non-viable cells, B-cells and Bead-B-cell rosettes (BBRs) are identified based on CD19 expression. Individual HLA specificities can be identified based on the internal fluorochromes within each bead. Furthermore, different B subsets (Naïve, Non-switched memory, and switched memory) can be identified based on CD27 and IgD expression, and HLA specificities for each subset can be determined. The gating strategy has been modified from Degauque *et al*[280]

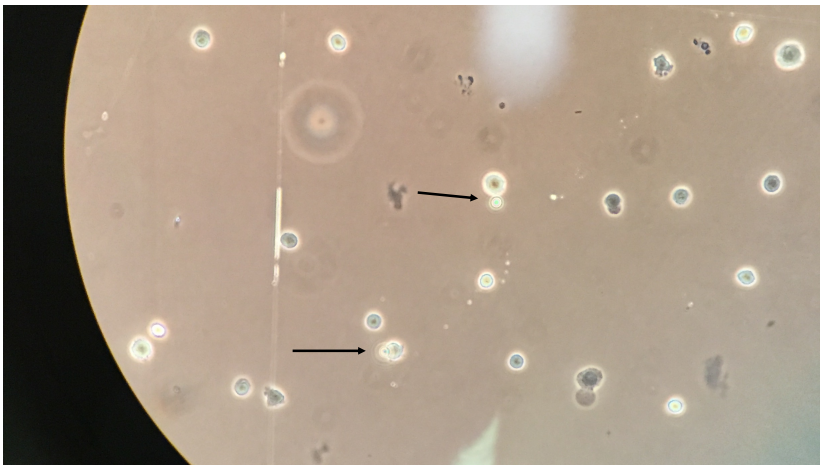


Figure 6.11. Photograph demonstrating B-cell binding to single antigen beads

6.2.3.3 SENSITISED PATIENTS DEMONSTRATED A HIGHER FREQUENCY OF ALLOREACTIVE B-CELLS COMPARED WITH HEALTHY UNSENSITISED VOLUNTEERS

The frequency of $CD19^+$ cells obtained from the HLA class I and class II bead-cell rosette gates were compared between 3 healthy unsensitised volunteers and 2 sensitised wait list patients.

Table 6.5 contains information about their sensitisation status. A higher proportion of $CD19^+$ bead-B-cell rosettes (BBR) were present in the sensitised patients compared with the healthy volunteers (median BBR: 2.410 unsensitised volunteers, 38.09 sensitised patients, p= 0.0095, Mann-Whitney test, Figure 6.12). The panel reactive - BBR was calculated for these patients. This was calculated in a similar way that the panel reactive antibody (PRA) is usually calculated for a microbead kit. The number of bead groups that formed bead-B-cell rosettes was counted and expressed as a proportion of the number of class I and class II bead groups in the microbead kit. For example, if BBRs formed with 69 bead groups out of the 198 class I and class II bead groups, the panel reactive- BBR was 35%. The serum panel reactive antibody (PRA) and panel reactive-BBR were compared (Table 6.5) however there was no association between the two values.

Patient	Route of sensitisation	Serum PRA	cRF	Panel Reactive -BBR
AL01	Transplant, transfusion	63%	100%	35%
AL05	Pregnancy	34%	100%	45%

Table 6.5. Sensitisation status, PRA, cRF and Panel Reactive-BBR for 2 sensitised wait-list patients.

PRA – panel reactive antibody, cRF – calculated reaction frequency, BBR – Bead B-cell rosette. Panel Reactive -BBR was determined by calculating the percentage of the single antigen bead kit panel that were bound to B-cells.

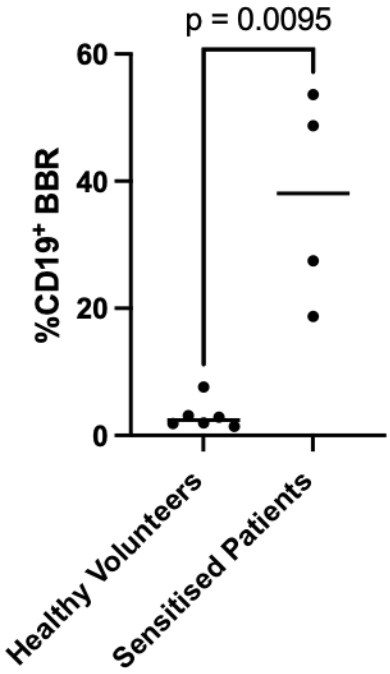


Figure 6.12. Proportion of $CD19^+$ Bead-B-cell rosettes obtained from healthy unsensitised volunteers and sensitised patients.

HLA Class I and Class II BBRs were included from 3 healthy volunteers and 2 sensitised patients on the renal transplant wait list. Class I and Class II BBRs have been reported separately, therefore each subject will be represented by 2 data points.

6.2.3.4 ASSESSMENT OF ALLOSPECIFIC B-CELLS IN SENSITISED PATIENTS UNDERGOING A FOR CAUSE BIOPSY

Seven renal transplant recipients who were undergoing a 'for cause' biopsy had their bloods drawn. PBMCs were isolated and the B-cells were surface-stained using monoclonal antibodies to characterise different subsets as described above. Following the antibody staining protocol, the PBMCs were then co-cultured with washed single antigen beads (SABs). Both HLA class I and class II Bead-B-cell rosettes (BBRs) were found. The frequencies of BBRs visible in different subsets ($CD19^+CD27^-IgD^+$ naïve, $CD19^+CD27^+IgD^+$ non-switched, and $CD19^+CD27^+IgD^-$ switched memory cells) of sensitised patients were then investigated. Resembling the expected frequencies within peripheral blood, Naïve BBRs were most frequently observed compared with non-switched and switched memory cells ($p<0.0001$, Friedman test).

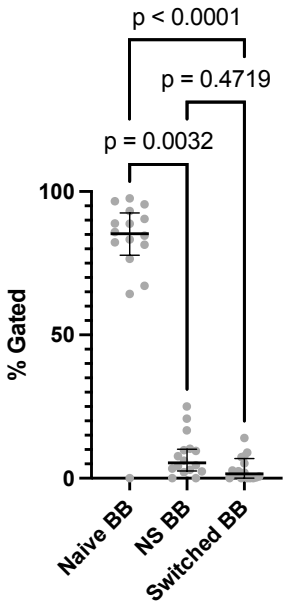


Figure 6.13. Frequency of Bead-B-Cell Rosettes (BB) within different B-cell subsets. Resting PBMCs were isolated using Ficoll centrifugation and stained using monoclonal antibodies prior to incubation with Class I and Class II single antigen beads as described in section 2.10. $CD19^+CD27^-IgD^+$ Naïve, $CD19^+CD27^+IgD^+$ Non-switched memory, $CD19^+CD27^+IgD^-$ switched memory BBRs were measured as a percentage of the parent gate ($CD19^+$ BB). Comparisons were made using the Friedman's test with Dunn's multiple comparisons test. Individual values, median and interquartile range are shown.

The patients were then separated into two groups depending on the presence of features associated with rejection on the biopsy (No Rejection =3, Rejection = 4). See Table 6.6 for biopsy results. Although not statistically significant, patients with histological features of rejection had higher frequencies of BBRs, which could reflect increased alloreactive B-cells in the peripheral circulation, or non-specific binding, which will need further investigation (Figure 6.14).

ID	Banff Class	Diagnosis/Comment
AL16	5,6	IFTA grade 1, mesangiolysis and capillary dilatation in 1 glomerulus
AL35	5,6	IFTA gr 1, early recurrent membranous on electron microscopy. BK nephropathy
AL75	5,6	IFTA gr I/II, severe arteriolar hyalinosis
AL53	2,4	Suboptimal sample. Features of ongoing active TCMR + ABMR. t1, ptc 1, c4d3
AL73	2,4,5,6	?Antibody mediated changes - ?acute/chronic active antibody mediated rejection, g2, acute t-cell mediated rejection gr 1a, IFTA gr 2, severe arteriolar hyalinosis
AL108	2,5	2 -?Antibody mediated changes - chronic active antibody mediated rejection, 5 - IFTA gr 1 (g1, PTC 1)
AL55	2,4	Antibody mediated changes? Acute antibody mediated rejection, Acute T-cell rejection Grade IIA (g1, ptc 0, c4d 3, v1)

Table 6.6. Biopsy results from sensitised patients undergoing assessment of allospecific B-cells

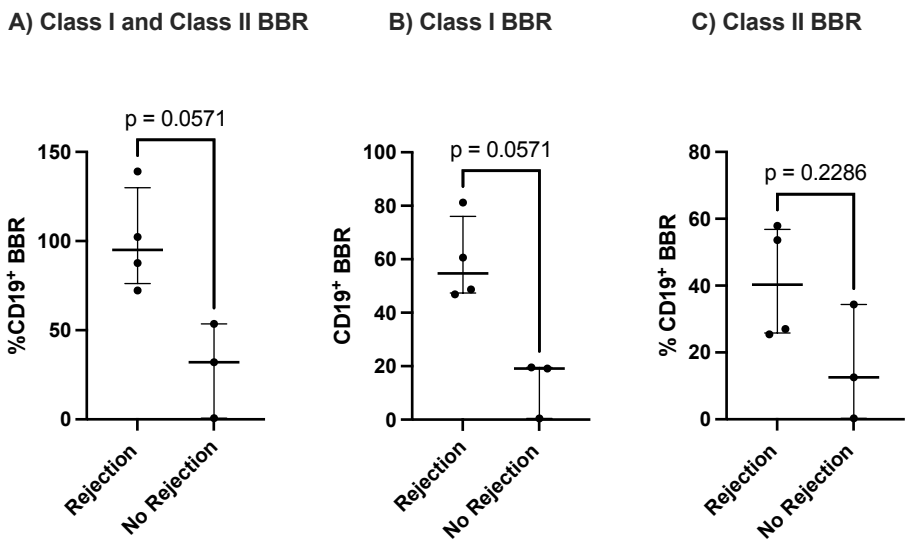


Figure 6.14. Frequency of Bead—B-cell rosettes in renal allograft recipients who were offered a 'for cause' biopsy.

Patients were separated into two groups (Rejection and no rejection) based on histological features present in the biopsies. Resting peripheral blood mononuclear cells were obtained and stained using a live/dead discriminator. Cells were washed and then stained with CD19, CD27 and IgD fluorochrome- conjugated monoclonal antibodies. These pre-stained PBMCs were then incubated with washed class I and class II single antigen beads. A) Total (Class I and Class II) B-cell- bead rosette, B) Class I BBR, C) Class II BBR.

When the BBR subsets were compared, there were no statistically significant differences between the rejection and no-rejection groups (Figure 6.15).

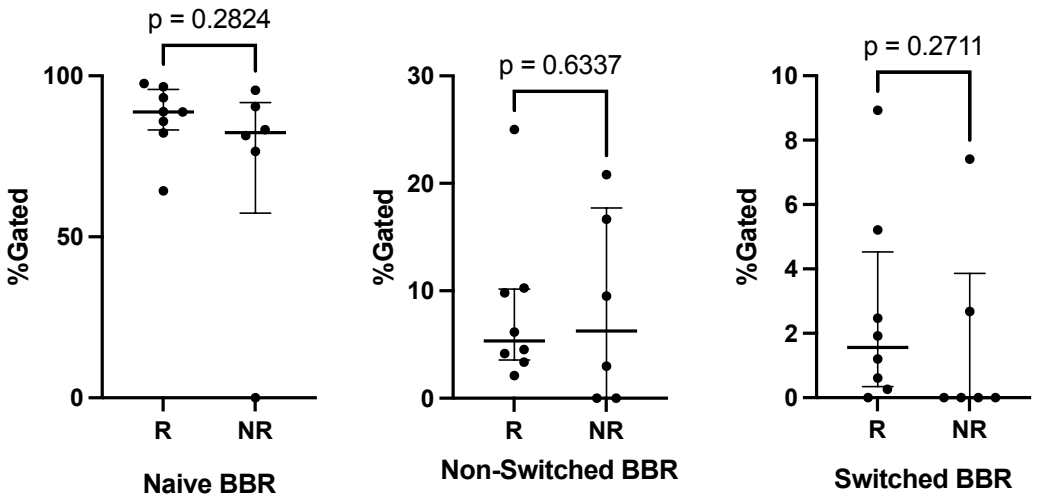


Figure 6.15. Frequency of different B subset – bead rosettes in patients with R- rejection and NR – no rejection on biopsy.

To summarise, these experiments represent the early stages of assay development where pre-stained PBMCs were incubated with single antigen beads to determine the frequency of alloreactive B-cells. This demonstrates proof of concept, and bead-B-cell binding was visualised using flow cytometry. Sensitised patients had an increased frequency of alloreactive B-cells as measured by Bead-B-cell rosettes compared with non-sensitised volunteers. When patients received a for-cause biopsy, there was a trend towards increased BBRs patients with histological features of rejection compared with those who did not have features of rejection. This was not statistically significant.

6.3 DISCUSSION

Despite the improvement in short term renal allograft survival, the median survival remains unchanged at approximately 15 years,[281] and a significant proportion of late graft loss is secondary to immune-mediated damage, including chronic antibody mediated rejection.[1] Renal allograft survival is reduced in the presence of pre-transplant HLA antibodies and DSAs,[77,282,283] and the development of *de novo* DSAs have been associated with worse outcomes.[80] The level of immune risk therefore is currently assessed through the measurement of serum HLA antibodies, however the potential contribution of memory cells to the antibody pool may not fully be appreciated. Assessing B-cell memory is difficult for two reasons: alloreactive B-cells are relatively rare and are present in less than 5% of the peripheral B-cell population, and whereas long lived plasma cells produce low level antibodies, memory cells are quiescent.[284] This gap in the understanding of patient sensitisation is increasingly important as renal recipients are likely to receive more than one graft in their lifetime. In cases where serum HLA antibodies are not detected, laboratories must decide whether to list mismatches from a previous graft as unacceptable antigens thus limiting transplanting opportunities or permit repeat mismatches with the risk of uncovering an anamnestic alloresponse. New pharmacological agents, for example imlifidase, have been introduced into routine clinical use, permitting transplants that would otherwise have been vetoed. Careful consideration needs to be made as to whether any delisted antibody specificities have arisen from previous pregnancies or transplants, as repeated alloantigen exposure can reactivate existing memory cells resulting in augmented DSA production.

6.3.1 METHOD 1: NON-SPECIFIC STIMULATION OF PBMCS TO UNCOVER IMMUNE MEMORY

Lanzaveccia *et al* previously described a method for uncovering the memory B-cell potential using non-specific stimulation of B-cells through toll-like receptor ligation. Through this assay, they were able to demonstrate the presence of immune memory to previous vaccinations 17 years after the immunising event.[270] This assay has been

modified and applied to renal patients who have experienced defined sensitising events. Surface staining of cultured cells by flow cytometry confirmed the switch in cell phenotype to antibody secreting cells ($CD24^{-}CD38^{hi}$), class-switched memory cells ($CD27^{+}IgD^{-}$) and plasma cells ($CD38^{+}CD138^{+}$), with a peak in memory and plasma cells visible between 6 and 9 days of cell culture. After 9 days of incubation, the most HLA antibodies at higher MFIs were found in the cell supernatant. The cell supernatant was tested from unsensitised volunteers and sensitised patients, and sensitised patients were found to have more HLA antibody specificities with higher MFIs compared with the cell supernatant obtained from unsensitised volunteers. Both Class I and Class II HLA antibodies were found in the cell supernatant and 65% of HLA specificities found in cell supernatant were also present in the matched serum sample. Importantly, no 'self' HLA specificities were found in the cell supernatant. Interestingly, when HLA specificities were found in both serum and supernatant, the levels of antibodies found in the sera were high, and often reached bead-saturating levels (MFI approximately 15 - 20,000). This phenomenon was also noted by Wehmeier *et al* when they compared serum and cell supernatant DSA in renal transplant recipients.[285] Similarly, using ELISpot testing, Lucia *et al* demonstrated a weak but significant correlation between the frequency of memory B-cells displaying HLA antibodies and the strength of the same HLA specific antibody circulating in the serum.[271] Mechanistically, this could be explained by the presence of additional HLA-specific memory cell clones replenishing the circulating HLA antibody levels.

In this study, the highest amount of HLA antibody observed in the cell supernatant was a Class I DSA (Cw7, MFI 3000), which was 10 times higher than levels obtained from the cell supernatant in other sensitised patients. Other DSAs were found in the supernatant and accompanying serum. This patient received a biopsy on the same day as blood collection, and this had histological features of mixed antibody and cellular rejection. Although this level of antibody production was only noted in the supernatant of one patient, it is tempting to speculate that this is a result of either a large memory B-cell clone, or a highly metabolically active clone. A recent study investigating HLA antibodies in cell supernatant found individuals who had

pretransplant DSAs in serum and matched supernatant more frequently developed ABMR in the first year post transplant compared with serum DSA positive, supernatant DSA negative patients.[285] Lucia *et al* assessed the frequency of memory B-cell using ELISpot and found that patients undergoing ABMR had higher frequencies of donor-specific memory B-cells in peripheral blood, and these patients had more severe histological lesions.[271] Similarly, when PBMCs obtained from patients undergoing a 'for cause' biopsy were incubated with ONELAMBDA single antigen beads, a trend towards increased bead-B-cell rosettes in patients who had histological features of acute rejection on their biopsy was demonstrated (See section 6.3.2, page 309).

In three patient samples, 9 HLA antibodies that were not present in the matched serum were identified. These antibody specificities could either be attributed to a previous transplant or had been present in the patient's cumulative profile, and therefore were consistent with the patient's sensitisation history. In one patient (AL39), the antibody specificities obtained from the cell supernatant were directed against their first renal transplant that had occurred 15 years prior to blood sampling, demonstrating the longevity of memory B-cells.[267,271,286]

Conversely, in 2 patients, no HLA antibodies were detected in the supernatant despite a broad HLA antibody profile in the matched serum. This could be explained either by the absence of peripheral memory B-cells, or by the low frequency of memory B-cells in the peripheral blood that were not sampled during the blood draw. An alternative explanation could be that the amount of HLA antibodies produced in the cell supernatant were below the positive threshold set for this assay. Further work titrating B-cell frequencies with supernatant antibody production could be performed to determine the minimum frequency of B-cells required to produce a positive result. In this study these were not thought to be false negative results from ineffective stimulation as the B-cell phenotypes were compared between day – 0 and day – 9 and cells were demonstrated to have successfully differentiated into antibody secreting cells (ASCs). A potential method for increasing this confidence would be to test the

cell supernatant for IgG antibodies that may be present following common childhood vaccinations.

Other groups have described discrepancies in the HLA specificities obtained from serum and the memory compartment (either through collection of cell supernatant or via ELISpot testing), and this suggests that memory cells and long-lived plasma cells contribute different antibody repertoires to the immune response.[267,271,285,287]

Two out of the 5 sensitised patients were taking immunosuppressive medication at the time of blood sampling. HLA antibodies were found in the cell supernatant of both patients, demonstrating the feasibility of performing this assay on immunosuppressed patients.

There are limitations to this study. In some patients, due to the timing of previous transplants, the full HLA type of the donor was not available. HLA-C and HLA-DQA1 types had to be inferred using common associations and haplotypes therefore the IgG HLA antibodies found in the cell supernatant could not always be directly attributed with certainty to the previous transplant.

A disadvantage to this method is the inability to directly measure the size of the B-cell clone responsible for the production of HLA antibody. Quantifying memory B-cells is possible using ELISpot testing, however this is more labour intensive. An attempt was made to standardise this assay to allow comparisons between samples – 1 million PBMCs were placed into culture, the cell supernatant was concentrated by the same factor, and confirmed using A280 protein absorption using spectrophotometry. A possible way to further standardise this assay would be to first isolate B-cells from PBMCs. The B-cells could be counted, and a set fraction returned to the remaining PBMCs. This extra step however would be associated with added costs, and as cells are lost during each wash step, further manipulation and isolation could affect the overall count and viability of the B-cells. Collecting cell supernatant does have an advantage over ELISpot testing as all IgG HLA antibodies that have accumulated over

the culture period can be measured, potentially increasing the assay sensitivity. ELISpot on the other hand only measures the IgG secreting cells that have survived to the end of the culture period, potentially missing some B-cell clones. Furthermore, the same single antigen bead kits that are used for HLA antibody screening in serum can be used to test the cell supernatant, permitting direct comparisons. Memory cells appear in low frequencies in the peripheral circulation, and although quantitation of these cells would be interesting and was attempted by co-incubating pre-stained cells with single antigen beads (see section 6.2.3), it is the ultimately the presence or absence of the cells that will aid clinical decision making.

The immune system remains dynamic process, and a single blood sample will only provide a snapshot in time of a dynamic process. Serial measurements over time, similar to the serum screening schedule performed when a patient is on the renal transplant waiting list, may be more useful. Further testing will need to be performed in a larger cohort of patients.

A method that can uncover the peripheral memory response has been demonstrated. This uses technology that is accessible to most H&I laboratories and may provide additional information of pre-transplant risk. This assay will be useful when assessing live donor pairs where the donor may repeat mismatches associated with pregnancy, and in regrafts, prior to the removal of 'other unacceptable antigens'. Finally, this method may be applicable when considering delisting strategies in the context of novel peri-transplant agents.

6.3.2 METHOD 2: DEMONSTRATING THE PRESENCE OF ALLOSPECIFIC B-CELLS USING SINGLE ANTIGEN BEADS (SABS)

This method was developed to supplement the information obtained from the non-specific stimulation of memory B-cells as described above. As cells from sensitised patients were used to test the parameter of both this assay and the R848/IL-2 assay, frequently there were insufficient PBMCs to perform both assays, resulting in incomplete data sets. Using a gating strategy modified from Degaque and Akl, $CD19^{+}$

B-cells bound to single antigen beads could be visualised using a flow cytometer (Bead-B-cell rosettes, BBR).[280,288] PBMCs could be surface stained prior to co-incubation and frequency of alloreactive B-cells could be determined within different subsets. Although this was demonstrated in very small numbers, sensitised patients had a higher frequency of BBRs compared with unsensitised volunteers. These results are similar to findings reported by Akl *et al* who demonstrated a higher frequency of HLA specific B-cells (both donor specific and non-donor-specific) in transplanted renal recipients who had circulating serum HLA antibodies compared to healthy volunteers.[288]

Patients who received an allograft biopsy were then assessed, and there was a trend towards increased BBRs in those who had evidence of rejection. Whilst this could be non-specific binding to the beads, this is in keeping with observations from other groups.[271,288] The BBR phenotype was then investigated to see if there was a difference between rejectors and non-rejectors. Although the median frequency of switched memory BBRs was increased in patients with histological features of rejection, this was not statistically significant, and would warrant further investigation.

A disadvantage with enumerating allospecific B-cells using tetramers is the non-specific binding of B-cells to the non-HLA portion of the tetramers.[273,274] In this assay, B-cell binding to the negative control bead was noted, and this was controlled for by disregarding bead binding that was equal to or less than the binding seen in the negative control bead. This has a potential to decrease the overall sensitivity of the assay which may limit its utility. An attempt to limit this non-specific binding could include testing the effects of reduced temperatures and increased agitation during the incubation with beads, and the addition of extra wash steps.

Further work should include repeating this test using larger patient numbers. The next step would include a comparison of the HLA specificity of BBRs together with the BBR phenotype. This can be compared with the HLA antibody screen results from the matched serum and the patient's sensitisation history.

7 ISOLATED PRE-EXISTING HLA-DP DONOR SPECIFIC ANTIBODIES ARE ASSOCIATED WITH POORER OUTCOMES IN RENAL TRANSPLANTATION

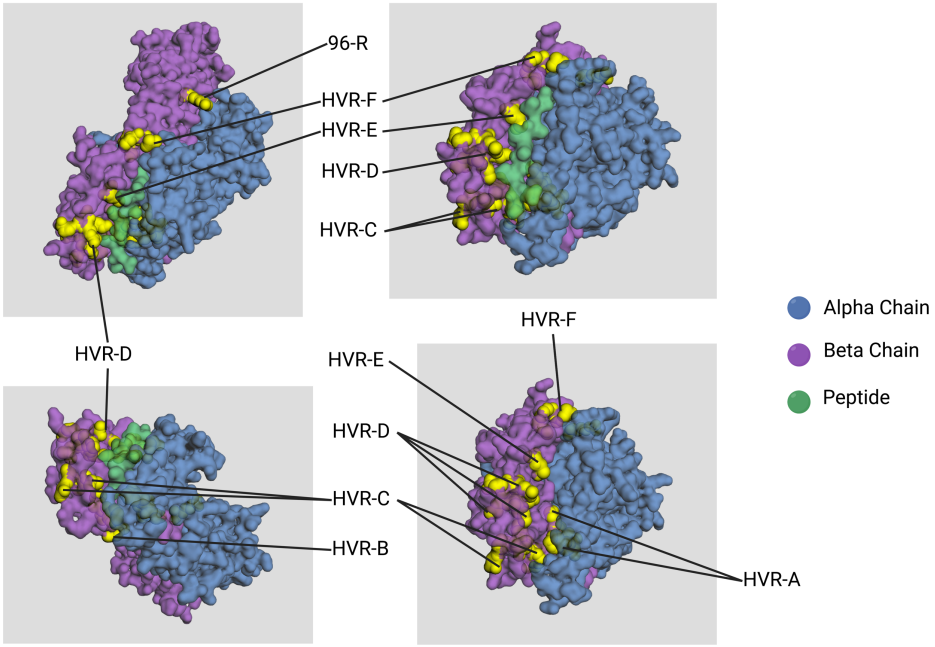
7.1 INTRODUCTION

The importance of donor-specific antibodies (DSAs) in renal transplantation has long been recognised and led to the establishment of the pre-transplantation crossmatch.[47] Understanding of the pathogenesis of antibody-mediated damage to renal allografts has increased over the last three decades as a result of technological advances in both the detection of antibodies and their associated injury pathways.[289,290] As increased sensitivity and improved definition of antibody analysis has become available, scrutiny has turned to the relative importance of different antibodies contributing to graft injury.

Antibody-mediated damage of renal allografts is recognised as a major cause of graft loss and the role of HLA Class II antibodies has been increasingly acknowledged.[52] While renal endothelial cells may not express Class II HLA constitutively, they have been demonstrated to express these molecules following inflammatory stimuli.[291,292] The subsequent development of class II antibodies may lead to acute antibody mediated rejection (ABMR) or more insidious chronic antibody mediated rejection (CAMR) associated with the development of transplant glomerulopathy. Although attention was initially focused on antibodies against HLA-DR it has been increasingly recognised that HLA-DQ and HLA-DP antibodies are also important.[52]

HLA-DP is a class II human leukocyte antigen formed by a heterodimer of two peptide chains, DPα and DPβ which are encoded by the DPA1 and DPB1 genes respectively. The DPA1 and DPB1 genes are located at the centromeric end of the major histocompatibility complex. There is at least 1 recombination hotspot between DPB1 and DQB1, which means that a donor and recipient pair can be fully matched at HLA A, B, C, DR and DQ, yet mismatched at HLA-DP.[293] The covalent association between the α_1 and β_1 domains create the peptide binding groove, and much of the polymorphism

associated with HLA-DP is derived from 6 hypervariable regions which are illustrated in Figure 7.1.



Hypervariable Region	A	B	C	D	E	F
Amino Acid Position	8-11	33-36	55-57	65-69	76	84-87

Figure 7.1. Crystal Structure of HLA DPB1*04:01/DPA1*01:03.
The hypervariable regions (HVR) A, B, C, D, E and F are highlighted on the beta chain, together with amino acid position 96. The peptide chain has been removed from the bottom-right panel to better visualise the position of HVR-A, HVR-C and HVR-D at the base of the peptide binding groove. Underneath the images is a table that demonstrates the different amino acid positions that correspond to each HVR. The 3D structures were obtained from phla3d.com.br. [294,295] **Figure created using Biorender.com**

Early data suggested that performing transplants in the presence of HLA-DP antibodies was not detrimental to graft outcomes, although no distinction was made between DSA and non-DSA.[296] A study of 4900 cadaveric renal transplants showed that while HLA-DP mismatch was not associated with a deleterious effect in first transplant

recipients, each HLA-DP mismatch was associated with a step-wise reduction in 1-year graft survival rates for re-transplants. This was particularly significant in sensitised recipients with >50% reactivity of preformed lymphocytotoxic antibodies.[291] HLA-DPB mismatches at the epitope level were also associated with reduced graft survival in re-transplants only, suggesting that HLA-DP antibodies may be a contributing factor.[292] More recently, several case reports have demonstrated the pathogenicity of preformed HLA-DP DSAs identified by flow crossmatch, [297,298] or solid phase assays alone.[299] 'Third party' HLA-DP antibodies with cross-reactive epitopes have also been implicated in CAMR.[300]

This is a retrospective study of a single centre experience with HLA-DP antibody incompatible renal transplants defined by single antigen beads (SABs). In this group, there was no T-cell positivity in the flow crossmatch, and B-cell positivity occurred in 32% of patients. In addition to assessing clinical outcomes, clinical and laboratory parameters that could potentially risk stratify these transplants were examined.

7.2 METHODS

7.2.1 PATIENT SELECTION

In this retrospective study, the time of offer (TOO) or current sera were assessed in all adult renal transplant recipients who were transplanted between January 2013 and February 2020. The group of patients with pre-existing HLA-DP DSAs in the absence of other HLA DSAs formed the primary study group (DPDSA). The DPDSA cohort was compared with 3 other groups. The first group included patients who had HLA-DP antibodies that were not donor specific (DPnDSA group). The second group included highly sensitised patients (HSP) with a calculated reaction frequency (cRF) greater than 85% but who had no HLA-DP antibodies in the TOO sera (HSP group). The final group (control group) were standard immunological risk recipients (with cRF <85%) who received contemporaneous transplants that were matched in a 2:1 ratio with the DPDSA cohort according to donor type. This was to account for changes in both the local crossmatching (removal of the CDC crossmatch) and national UK allocation policies that occurred during the study period. Patients were excluded from the analysis if there was an historical HLA-DSA which was not present in the TOO sample. This was to limit adverse outcomes that could be attributed to anamnestic B-cell responses. Similarly, regrafts with HLA mismatches that repeated mismatches of previous transplants were excluded. ABO-incompatible and all other HLA antibody incompatible transplants were excluded. Figure 7.2 demonstrates the study design.

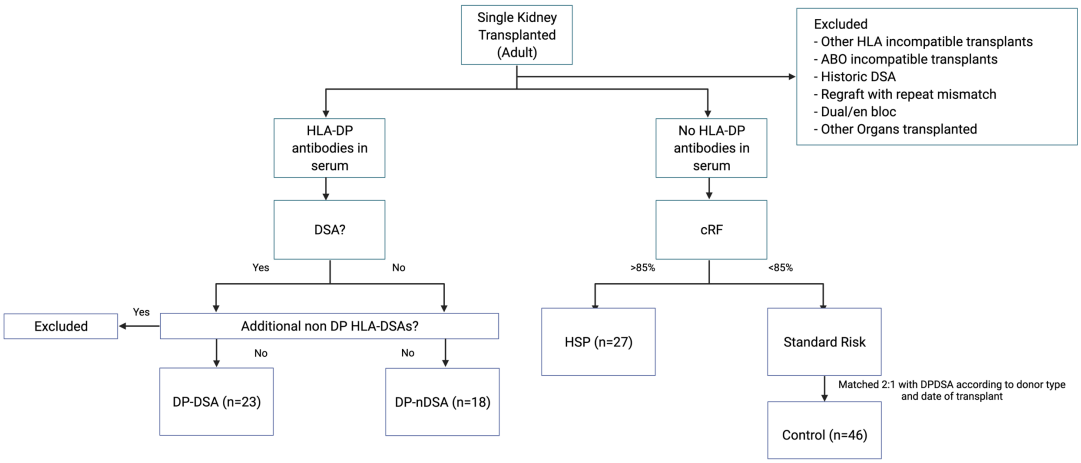


Figure 7.2. HLA-DP incompatible transplant study Design.

Created using Biorender.com

Patients had HLA typing in line with requirements for the UK allocation scheme (the minimum requirement included HLA-A, B, C, DRB1, DRB3/4/5, DQB1 until September 2019. After September 2019, the requirement expanded to include HLA-DQA1, DPB1 and DPA1).[301,302] For DPDSA patients, the HLA-DPB1 and HLA-DPA1 types were confirmed for both donors and recipients using reverse sequence specific oligonucleotides (LABType, ONE LAMBDA, Canoga Park CA) to allow differentiation between DPA1 and DPB1 DSAs (Donor DPA1 – 15/23, donor DPB1 – 23/23, recipient DPA1 19/23, recipient DPB1 22/23).

7.2.2 HLA ANTIBODY SCREENING AND HISTOCOMPATIBILITY TESTING

In accordance with standard practice, wait-list patients were screened quarterly using LABScreen Mixed beads (ONE LAMBDA). If positive, the specificities were characterised using LABScreen Single Antigen beads (ONE LAMBDA, SAB) according to manufacturer instructions. To overcome the prozone effect, sera were pre-treated with 6% EDTA to achieve a 1:50 EDTA:serum dilution. The protocol for reporting HLA antibodies included a positivity threshold of normalised median fluorescent intensity (MFI)>2000 combined with a ratio score of 6 or more after correcting for non-specific binding to the negative control bead (Equation 1). If the antibody specificity was

represented by more than one bead, and all beads were positive, an average MFI was calculated for the antibody. If only some of the beads were positive, the represented allelic antibodies were reported with the average MFI obtained for each positive allelic antibody. If more than one HLA-DP DSA was detected, the MFIs obtained from each DSA were added together, and this cumulative MFI was included in the analysis. The TOO sera from the DPDSA group (21 out of 23) were retested using the locally validated LifeCodes Single Antigen microbead assay (Immucor, Norcross GA) to confirm the presence of DPDSAs.[303]

A combination of complement dependent cytotoxicity crossmatch (CDC), flow crossmatch (FXM) and SAB analysis were used to determine histocompatibility and risk of proceeding with the transplant. Transplants were progressed in the case of 'technical' positive wet crossmatches if the reactivity could not be attributed to the presence of a donor-relevant HLA antibody. If DSAs were identified pre-transplant, the decision to progress was made based on individual patient history and risk appetite. As donor HLA-DP typing was not mandated for organ allocation, it was not always apparent that an HLA-DP incompatible offer had been received until after the transplant had occurred. This was usually known by the next working day following TOO serum testing or after the donor HLA-DP typing was performed locally.

7.2.2.1 HLA MATCHMAKER

HLAMatchmaker and https://epregistry.com.br were used to determine any 'exposed antibody-verified' DPB1 epitope mismatches that corresponded with the recipient DSA profile.[15]

7.2.2.2 T-CELL EPITOPE (TCE) ALGORITHM

The T-cell epitope (TCE) algorithm has been used to predict the effect of HLA-DPB1 mismatches following unrelated haematopoietic stem cell transplantation.[304] HLA-DP alleles are placed into different TCE groups based on epitope groups that are associated with high, medium and low reactivity. The calculator determines whether a DP mismatch might be tolerated (permissive) and those that would increase risk (non-permissive) following stem cell transplantation. Ideally epitope mismatches would be

in the same group (permissive). Depending on level of reactivity between the epitope groups, non-permissive mismatches can increase the risk of graft versus host disease, or host versus graft activity which can result in rejection or non-engraftment.

7.2.3 DONOR HLA-DPB1 EXPRESSION LEVELS

Two single nucleotide polymorphism (SNP) variants (rs9277534G and rs2281389A/G) that are present in the 3' untranslated region of HLA-DPB1 have been shown to be associated with differing HLA-DPB1 transcript levels. [305,306] These SNPs have been described to be in linkage disequilibrium with certain HLA-DPB1 alleles in Caucasians.[305] DPDSA patients were categorised into low and high expression groups inferred from the donor HLA-DPB1 alleles.

7.2.4 ROUTINE IMMUNOSUPPRESSION

Standard immunosuppression consisted of alemtuzumab induction with tacrolimus monotherapy, or basiliximab induction with tacrolimus and mycophenolate mofetil (MMF) as previously described.[209] MMF was routinely added if there were two HLA-DR mismatches between donor and recipient. If an HLA-DP incompatible transplant occurred, the decision to augment immunosuppression was made by a clinician at the time of transplantation or soon afterwards informed by further donor characterisation and recipient antibody testing.

7.2.5 ALLOGRAFT BIOPSIES

Only 'for cause' renal allograft biopsies were performed. Indications included delayed graft function (DGF), a sustained elevated urinary protein/creatinine ratio >50mg/mmol, or a sustained rise in creatinine. C4d deposition was assessed using immunohistochemistry staining. Biopsies were scored using the Banff 2017 criteria.[54] Biopsies receiving more than one Banff diagnosis (categories 2+3 or 2+4) were categorised as mixed rejection.

7.3 RESULTS

Between January 2013 and February 2020, 1355 adult kidney transplants were performed. The study included 114 patients (23 DPDSA, 18 DPnDSA, 27 HSP, 46 Control) with a median follow up of 1197 (range 1-2517) days. Throughout this period 33 recipients had biopsy-proven rejection which encompassed ABMR, TCMR, borderline, and mixed rejection (15 DPDSA, 6 DPnDSA, 5 HSP, 7 Control). Twenty-four patients had biopsy proven ABMR (15 DPDSA, 4 DPnDSA, 3 HSP, 2 control). Twenty grafts failed (7 DPDSA, 6 DPnDSA, 4 HSP, 3 Control).

7.3.1 PATIENT CHARACTERISTICS

The patient characteristics are shown in Table 7.1. HLA-DP antibodies were associated with increased sensitisation as defined by cRF. Seventy percent of the DPDSA cohort had a cRF≥85%. A higher proportion of DPDSA and DPnDSA patients had a sensitisation history including blood transfusions or prior renal transplants. Thirty-nine percent of the total cohort were regrafts.

Variable	TOTAL	DPDSA	DPnDSA	HSP	Control	p-value
Number	114 (100%)	23 (20%)	18 (16%)	27 (24%)	46 (40%)	
Age (years, SD)	46 (14)	43 (12)	46 (13)	45(14)	47 (15)	0.717[3]
Gender						**0.002[1]**
M	60 (53%)	10 (44%)	8 (44%)	8 (30%)	34 (74%)	
F	54 (48%)	13 (57%)	10 (56%)	19 (70%)	12 (26%)	
Primary Renal Disease						
DM/HTN	15 (13%)	3 (13%)	1 (6%)	3 (11%)	8 (17%)	
GN	25 (22%)	6 (26%)	7 (38%)	7 (26%)	5 (11%)	
Infection/Obstruction	19 (17%)	5 (22%)	2 (11%)	8 (30%)	4 (9%)	

Variable	TOTAL	DPDSA	DPnDSA	HSP	Control	p-value
Other	55 (48%)	9 (39%)	8 (44%)	9 (33%)	29 (63%)	
Median cRF (Q1-Q3)	66 (0-96)	94 (69-98)	84 (21-95)	95 (87-97)	0 (0)	**<0.001**[4]
cRF ≥ 85%	52 (46%)	16 (70%)	9 (50%)	27 (100%)	0	**<0.001**[1]
Sensitisation History						
Blood transfusion	59 (52%)	19 (83%)	12 (67%)	18 (67%)	10 (22%)	**<0.001**[1]
Pregnancy	37 (32%)	9 (39%)	5 (28%)	14 (52%)	9 (20%)	0.067[1]
Previous Transplant	47 (41%)	15 (65%)	10 (56%)	16 (59%)	6 (13%)	**<0.001**[1]
Preemptive	14 (12%)	3 (13%)	2 (11%)	0	9 (20%)	0.068[2]
Donor Type						**0.023**[2]
DBD	68 (60%)	15(65%)	5 (28%)	20 (74%)	28 (61%)	
DCD	37 (33%)	7 (30%)	8 (44%)	6 (22%)	16 (35%)	
LD	9 (8%)	1 (4%)	5 (28%)	1 (4%)	2 (4%)	
Donor Age (years, SD)	48 (16)	45 (16)	49 (22)	43 (12)	51 (17)	0.209[3]
HLA Mismatch Level						**0.001**[2]
1	19 (17%)	5 (22%)	3 (17%)	8 (30%)	3 (7%)	
2	28 (25%)	7 (30%)	1 (6%)	5 (19%)	15 (33%)	
3	57 (50%)	10 (44%)	7 (39%)	14 (52%)	26 (57%)	
4	10 (9%)	1 (4.3%)	7 (39%)	0	2 (4%)	
Graft number						**<0.001**[2]
1	70 (61%)	8 (35%)	10 (56%)	10 (37%)	42 (91%)	
2	36 (32%)	11 (48%)	6 (33%)	16 (59%)	3 (7%)	
3	7 (6%)	3 (13%)	2 (11%)	1 (4%)	1 (2%)	

Variable	TOTAL	DPDSA	DPnDSA	HSP	Control	p-value
4	1 (1%)	1 (4%)	0	0	0	
Median CIT (hrs, Q1-Q3)	14 (11-17)	17 (13-18)	13 (9-17)	15 (12-17)	13 (10-14)	**0.002**[4]
DGF	32 (28%)	10 (44%)	6 (33%)	11 (41%)	5 (11%)	**0.007**[1]
Induction Agent						0.115[1]
Alemtuzumab	80 (70%)	15 (66%)	16 (89%)	21 (78%)	28 (61%)	
Basiliximab	34 (30%)	8 (35%)	2 (11%)	6 (22%)	18 (39%)	
Maintenance Immunosuppression						
Tacrolimus	100%	100%	100%	100%	100%	
MMF	51 (45%)	20 (87%)	5 (28%)	8 (30%)	18 (39%)	**<0.001**[1]
Prednisolone	17 (15%)	10 (44%)	2 (11%)	3 (11%)	2 (4%)	**<0.001**[2]
Augmented Immunosuppression:	24 (21%)	17 (74%)	1 (6%)	4 (15%)	2 (4%)	**<0.001**[2]

Table 7.1. Patient Demographics.

DBD – donation after brain death, DCD – donation after cardiac death, LD – live donor, SD- standard deviation, DM – diabetes mellitus, HTN – hypertension, GN- glomerulonephritis, cRF – calculated reaction frequency, CIT – cold ischaemic time, Q1 – 25th percentile, Q3 – 75th percentile,[1]Chi Squared Test, [2]Fisher's Exact Test, [3]One way Analysis of Variance, [4]Kruskall Wallis Test

HLA mismatches were recorded using the United Kingdom NHS-BT mismatch categories (Table 2.7).[183] Three DPDSA patients received a kidney that was fully matched at the HLA-A,-B,-C,-DR and -DQ loci. DPnDSA and Control patients were more likely to receive a Level 3 (A/B/DR 001, 011, 101, 111, 201, 211, 120, 020, 220) or 4 (A/B/DR 021, 121, 221, 002, 102, 202, 012, 112, 212, 022, 122, 222) mismatch kidney compared with the DPDSA and HSP groups. Highly sensitised patients including DPDSA patients received grafts with a median cold ischaemic time (CIT) that was significantly greater than the remainder of the study cohort. These differences reflect the national

allocation policy of the relevant era which prioritised HSPs to receive 000 mismatched DBD kidney grafts after paediatric recipients.

As donor HLA-DP typing was not required for organ allocation, the presence or absence of HLA-DP DSAs was not always known at the time of offer which may have influenced decision making. However, DPDSA patients were more likely to be maintained on augmented immunosuppression at the time of transplant compared with the control groups. Four patients received prophylactic perioperative plasma exchange and intravenous immunoglobulin.

Forty-one patients had at least one HLA-DP antibody in their TOO sera, and 70% had antibodies against several HLA-DPB1 antigens (median number of specificities 10, IQR 11). Twenty-three patients had one or more HLA DPB1-DSA, with a median cumulative MFI 11009 (range 2141-47349). Additional HLA-DPA1 DSAs could not be excluded in 5 DPDSA recipients due to the configuration of the HLA-DPA1 and HLA-DPB1 antigens within the microbead kits.

Twenty-one of the 23 DPDSA TOO sera were retested using Immucor kits and this confirmed HLA-DP DSAs in 16/21 samples. Two samples contained DP20 antibodies, which were not represented in the Immucor kit. The other 3 samples which tested negative using Immucor had a mean MFI of 2570. These results are demonstrated in Table 7.7.

HLA-DPB1*04:01 was the most frequently occurring allele within both donor and recipient populations, in keeping with representation in the UK population.[307] Eight recipients were homozygous for HLA-DPB1*04:01.

7.3.2 ROUTINE LABORATORY TESTS ARE UNABLE TO RISK STRATIFY TRANSPLANTS WITH PREFORMED HLA-DP ANTIBODIES

7.3.2.1 COMPLEMENT DEPENDENT CYTOTOXICITY/FLOW CROSSMATCH TESTING

An attempt was made to risk stratify DPDSA patients using routine laboratory methods. The patient groups were compared with FXM results. Out of 95 'wet' crossmatches performed, 19 generated a positive B-cell flow crossmatch (BFXM) result. A higher proportion of BFXM positivity occurred in patients with HLA-DP antibodies (DPDSA and DPnDSA groups) compared with patients who did not have HLA-DP antibodies (p=0.0776, Chi Squared test, Table 7.2. In the 5 patients where HLA-DPA DSAs could not be excluded, a FXM was performed in 4 patients (BFXM positive in 2 of 4 and negative in 2 of 4 patients). One transplant proceeded following a virtual crossmatch (DPA1 DSA noted. Either DPA1*02:01 average MFI 7126 or DPA1*02:02 average MFI 6323). This multidisciplinary decision was made to minimise cold ischaemic time with the local experience at the time that DPA1 antibodies were unlikely to cause FXM positivity.

Group (N)	FXM B Negative	FXM B Positive
DP-DSA (22)	15 (68%)	7 (32%)
DP-non-DSA (18)	12 (67%)	6 (33%)
HSP (24)	21 (88%)	3 (12%)
Control (31)	28 (90%)	3 (10%)
Total (95)	**76 (80%)**	**19 (20%)**

Table 7.2. Comparison of B-cell flow crossmatch reactivity.

The crossmatch results for 19 cases have been excluded (15 virtual crossmatches in the control group, 1 virtual crossmatch in the DP-DSA group, 1 inconclusive result in the DP-non-DSA group, 2 missing crossmatch records in the DPnDSA group).

Two DPnDSA samples generated a T-cell positive FXM but T-cell negative CDC crossmatch result. These transplants were progressed because the concurrent serum samples did not contain HLA-DSAs that could be attributed to the reactivity. All other T-cell crossmatches (CDC and FXM) were negative. There was 1 CDC B-cell positive crossmatch in the DPDSA group which was attributed to the HLA-DP DSA. Fifteen transplants in the control group proceeded following a virtual crossmatch and were coded as 'BFXM negative' for subsequent analyses.

A correlation analysis was performed to see whether the TOO cumulative MFI was associated with an increased median channel fluorescence (MCF) shift obtained from the BFXM. The values for cumulative MFI and MCF were not correlated (R^2 value = 0.28) and a high cumulative MFI was not associated with BFXM positivity (BFXM negative median DP-DSA MFI 9931.5, range 2141-22252, BFXM positive median DP-DSA MFI 11277, range 2788-47349, p=0.2666, Figure 7.3).

The inferred donor HLA-DP antigen expression levels were also considered.[308] Seventeen donors had high expression levels; 1 donor/recipient pair was removed from this analysis because only a virtual crossmatch had been performed. A positive crossmatch was obtained in 31% of cases where donors were inferred to have high DP expression levels. The cumulative DSA associated with the positive crossmatch ranged from 2788-28997 MFI. The negative crossmatches were associated with DSA MFIs ranging from 2204 - 22252. For cases with low expression (n= 6), a positive crossmatch was obtained in 33% of cases, with an associated DSA MFI of 10350-47439, Table 7.3. Negative crossmatches were associated with DSAs ranging from 2141-19136 MFI. This can be interpreted as no correlation *in vitro* between measured HLA-DP MFI and donor-specific reactivity measured by BFXM.

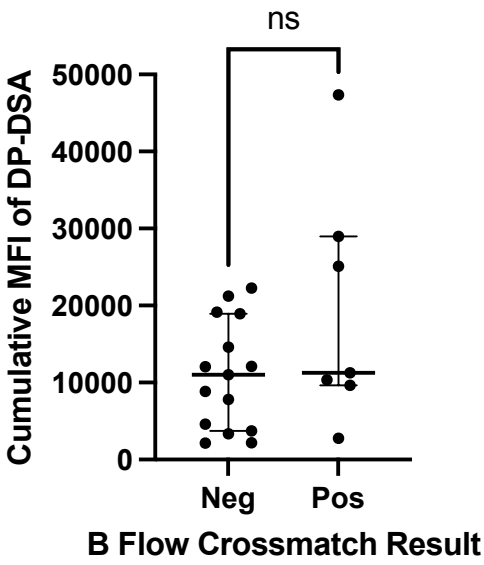


Figure 7.3. Scatter plots comparing the cumulative DP-DSA (MFI) with B flow crossmatch results.

Individual results, median and interquartile range are shown.

	High Expression	Low Expression
FXM positive	5 (31%)	2 (33%)
FXM negative	11 (69%)	4 (67%)
Total	16	6

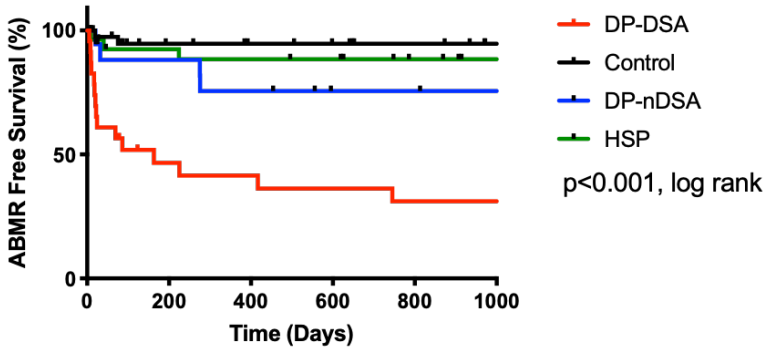
Table 7.3. Comparison of B Flow Crossmatch Results with inferred donor HLA-DP expression in the DPDSA group.

p>0.999 (Fisher's exact test).

7.3.3 HLA-DP ANTIBODIES AND ABMR FREE SURVIVAL

The relationship between the antibody profile and clinical episodes of ABMR was then studied. Throughout the follow up period, 109 'for cause' biopsies were performed (33 DPDSA, 16 DPnDSA, 30 HSP, 30 Control) and 24 patients had biopsy proven ABMR (15 DPDSA, 4 DPnDSA, 3 HSP, 2 control). The Kaplan-Meier survival estimates for ABMR-free survival for the 4 cohorts is demonstrated in Figure 7.4. DPDSA patients were observed to have significantly reduced ABMR-free survival compared with the control group (Hazard ratio, HR 19.026, $p<0.001$), with a median time to ABMR of 22 days. Univariate analyses using the Cox proportional hazards model found that patient group (DPDSA HR 19 $p<0.001$, DPnDSA HR 4.54 $p=0.081$), cRF>85% (HR 3.37, $p=0.01$), regrafts (HR 2.76, $p=0.016$) and BFXM positivity (HR 3.655, $p=0.03$) were associated with a reduced ABMR free survival. These were entered into a multivariable model (Table 7.4), and DPDSA remained the single variable that was associated with reduced ABMR-free survival (HR 9.578, $p=0.012$).

BFXM positivity was investigated further by constructing Kaplan-Meier curves for each cohort under study, comparing ABMR-free survival with BFXM positivity (Figure 7.5 a-d). In the DPDSA and control groups, BFXM positivity was not associated with a significant difference in ABMR-free survival. There was a trend towards a reduced ABMR-free survival in BFXM positive HSP recipients, however this was not statistically significant ($p=0.192$). Interestingly, a positive BFXM was associated with a reduced ABMR-free survival in the DPnDSA group (Figure 7.5B, median survival 276 days, HR 8.483, $p=0.0253$).



Time (Days)		0	30	100	200	300	500	1000
Number at risk	DP DSA	23	15	12	10	9	8	7
	DP nDSA	18	16	15	15	13	12	9
	HSP	27	26	24	24	23	22	15
	Control	46	38	33	31	30	28	19

Figure 7.4. Kaplan Meier curve demonstrating the ABMR-free survival up to 1000 days for patients within the DP-DSA, DP-nDSA, HSP and Control Groups. ABMR- antibody mediated rejection

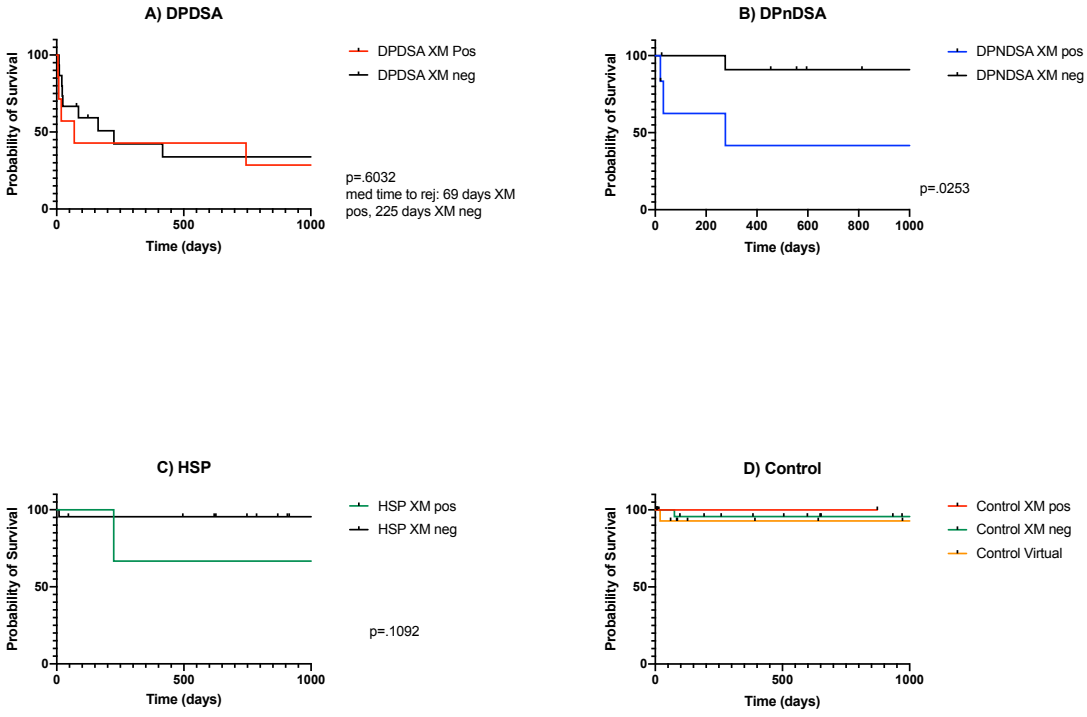


Figure 7.5. Kaplan Meier Curves demonstrating ABMR-free survival for the first 1000 days, stratified according to BFXM results in each of the 4 cohorts. DPDSA B) DPnDSA C) HSP D) Control

Variable	HR (95% CI)	P-value	HR(95% CI)	p-value
	Univariate		Multivariate	
Group				
DP-DSA vs Control	19.026 (4.340-83.412)	**<0.001**	9.578 (1.653-55.497)	**0.012**
DP-nDSA vs Control	4.540 (0.831-24.794)	0.081	2.677 (0.413-17.358)	0.302
HSP vs Control	2.121 (0.354-12.697)	0.410	0.715 (0.073-6.971)	0.773
CIT (long vs short, hrs)	1.347 (0. 598-3.034)	0.472		
cRF>=85% vs <=85%	3.374 (1.339 - 8.499)	**0.010**	1.726 (0. 530-5.618)	0.365
DGF (Yes vs No)	1.649 (0.721-3.769)	0. 236		
Donor Type (LD ref)				
DBD	0.588 (0.171-2.019)	0.399		
DCD	0.333 (0.079-1.393)	0.132		
Mismatch Level (1-Ref)				
2	0.963 (0.279-3.331)	0.953		
3	0.848 (0. 299-2.409)	0.757		
4	0.974 (0. 189-5.025)	0.975		
Regraft vs 1st graft	2.760 (1.206-6.314)	**0.016**	1.362 (0.541-3.433)	0.512
Induction (alemtuzumab ref)	0.594 (0.222-1.591)	0.300		
CNI Variability (hi vs low)				
<3month	1.780 (0.769-4.120)	0.178		
>3month	1.994 (0.758-5.247)	0.162		
BFXM Positivity	3.655 (1.547-8.635)	**0.003**	1.483 (0.584-3.762)	0.407

Table 7.4. Cox Regression Analysis to assess variables associated with Antibody Mediated Rejection free survival.

CIT – cold ischaemia time, median CIT 13.52 hours, LD – living donor, cRF – calculated reaction frequency, DGF – delayed graft function, HR – hazard ratio. Median CNI variability <3mo 26.88 . Median CNI variability >3mo 24.96.

7.3.4 HLA-DP ANTIBODIES AND REJECTION FREE SURVIVAL

Similar models were constructed to investigate the variables associated with reduced overall rejection free survival (encompassing ABMR, TCMR and mixed rejection). DPDSA was associated with an increased risk of rejection on univariate analysis (HR 6.129, $p<0.001$), however this was not statistically significant in the multivariable analysis (HR 2.855, $p=0.093$, Table 7.5).

Variable	HR (95% CI)	P-value	HR(95% CI)	P-value
	Univariate		Multivariate	
Group				
DP-DSA vs Control	6.129 (2.488-15.101)	**<0.001**	2.855 (0.841-9.700)	0.093
DP-nDSA vs Control	1.974 (0.663-5.876)	0.222	1.040 (0.281-3.850)	0.953
HSP vs Control	1.029 (0.326-3.243)	0.962	0.346 (0.068-1.745)	0.198
CIT (long vs short, hrs)	1.068 (0.540-2.115)	0.850		
cRF>=85% vs <=85%	2.141 (1.053 – 4.354)	**0.035**	1.958 (0.679-5.649)	0.214
DGF (Yes vs No)	1.412 (0.684-2.913)	0.351		
Donor Type (LD ref)				
DBD	0.429 (0.146-1.256)	0.125		
DCD	0.400 (0.125-1.278)	0.122		
Mismatch Level (1-Ref)				
2	0.892 (0.258-3.082)	0.856		
3	1.430 (0.536-3.814)	0.475		
4	1.484 (0.354-6.220)	0.589		
Regraft vs 1st graft	2.131 (1.073-4.231)	**0.031**	1.505 (0.687-3.299)	0.307
Induction (alem ref)	0.743 (0.335-1.649)	0.466		
CNI Variability (hi vs low)				
<3mo	2.040 (0.994-4.188)	0.052		
>3mo	1.252 (0.592-2.646)	0.557		
BFXM Positivity	2.823 (1.304-6.113)	**0.008**	1.564 (0.676-3.618)	0.296

Table 7.5. Cox Regression Analysis to assess variables associated with Rejection Free Survival.

CIT – cold ischaemia time, median CIT 13.52 hours. Median CNI variability <3mo 26.88 . Median CNI variability >3mo 24.96.

7.3.5 BIOPSY RESULTS

Eighty-one percent of the DPDSA biopsies had rejection, of which ABMR and mixed rejection were the most common diagnoses. In the DPnDSA group, 68.75% of the biopsies were reported as rejection. Nevertheless, there was a higher proportion of mixed rejection (37.5%). Conversely, HSP and Control patients were more likely to receive an alternative diagnosis (Figure 7.6a). The indication biopsies were assessed for histological lesions that are associated with inferior clinical outcomes.[178,179] The DPDSA biopsies were associated with higher microvascular inflammation (p=0.0346), higher C4d scores (p<0.0001), and higher transplant glomerulopathy scores (p=0.015) compared with the control patients (Figure 7.6b). There were higher cg scores in the DPDSA biopsies compared with the DPnDSA biopsies (mean rank difference 16.58, p=0.0384), however the difference in MVI scores were not statistically significant. Interestingly, less tubular atrophy was found in the DPDSA (mean rank difference 18.02, p=0.0331) and DPnDSA (mean rank difference 21.57, p=0.0439) patients and less fibrosis in the DPDSA patients (mean rank difference 21.61, p=0.0174) when compared with controls. This could not be explained by donor age. Although the median time to biopsy was shorter in the DPDSA group compared with the other groups, this difference was not statistically significant (DPDSA: 69 days, IQR 207.5, DPnDSA: 157 days, IQR 394.55, Control: 143 days, IQR 433.25, p=0.3615). The DSA profiles obtained from the DPDSA group over the period of follow-up, together with initial post-transplant management and subsequent clinical outcomes are depicted in Table 7.7.

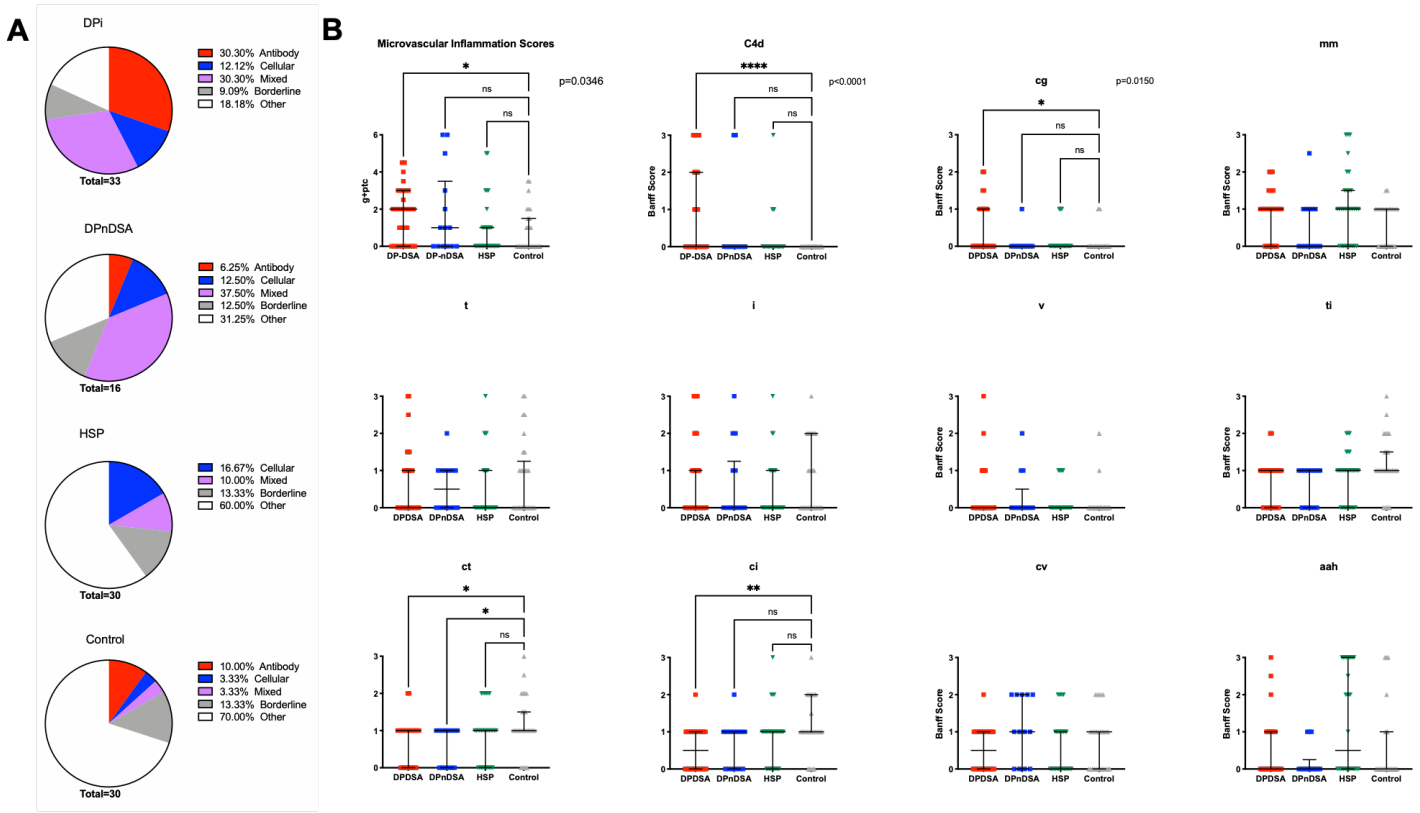
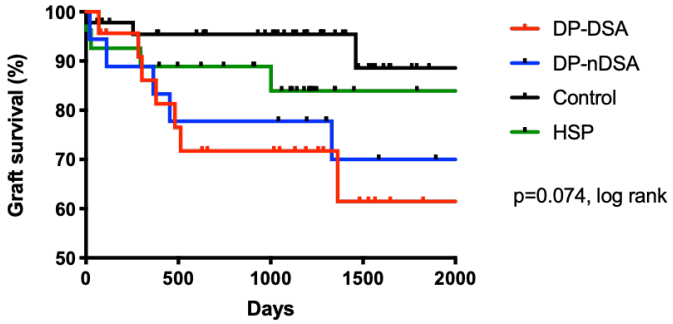


Figure 7.6. Analysis of Banff histological lesions from indication biopsies performed in each cohort.

A) Proportion of biopsy diagnoses (ABMR, TCMR, mixed rejection, borderline rejection or other). B) Scatter plots demonstrating the individual scores, median and interquartile range for each Banff lesion. The microvascular inflammation scores are the sum total of g + ptc scores. Comparisons were made using the Kruskal-Wallis test

7.3.6 GRAFT SURVIVAL

Having established that HLA-DP antibodies were associated with reduced ABMR-free survival, the presence of HLA-DP antibodies was then compared with graft survival. The Kaplan-Meier curves comparing death-censored graft survival across the 4 groups are depicted in Figure 7.7. Univariate analyses using the Cox proportional hazards model identified 4 variables associated with reduced graft survival which included DPDSA (HR 5.218, p=0.048), delayed graft function (HR 3.376, p=0.016), Regraft (HR 7.461, p=0.002), and high CNI variability[186,187] more than 3 months post-transplant (HR 9.505, p<0.001). In the multivariable analysis, DP-DSA was not associated with graft loss. Regrafts remained the single independent variable for reduced graft survival (HR 5.135, p=0.028 [Table 7.6]). The documented causes of graft loss in the DPDSA group (n=7) included ABMR with ischaemia-reperfusion injury, chronic ABMR, recurrent FSGS, BK nephropathy, CMV disease with evidence of chronic ABMR and chronic allograft nephropathy (n=2).



Time (Days)		0	30	100	200	300	500	1000	1500
Number at risk	DP DSA	23	23	22	21	20	17	14	6
	DP nDSA	18	18	18	17	17	15	15	10
	HSP	27	26	26	26	25	23	19	4
	Control	46	46	43	42	41	39	33	13

Figure 7.7. Kaplan-Meier survival curves depicting the estimated death censored Graft Survival in the first 2000 days for the patients within the DP-DSA, DP-nDSA, HSP and Control Groups.

Variable	HR (95% CI)	P-value	HR(95% CI)	P-value
	Univariate		Multivariate	
Group				
DP-DSA vs Control	5.014 (1.296-19.398)	**0.020**	1.577 (0.223-11.147)	0.648
DP-nDSA vs Control	3.911 (0.932-16.407)	0.062	1.029 (0.141-7.525)	0.977
HSP vs Control	2.872 (0.549-11.041)	0.239	0.595 (0.072-4.955)	0.631
CIT (long vs short)	1.040 (0.422-2.564)	0.933		
cRF≥85% vs ≤85%	2.624 (1.084-7.606)	**0.034**		
DGF (Yes vs No)	3.380 (1.398-8.169)	**0.007**	1.769 (0.536-5.835)	0.349
Donor Type (LD Ref)				
DBD	0.673 (0.148-3.051)	0.608		
DCD	0.666 (0.135-3.290)	0.618		
Mismatch Level (1-Ref)				
2	0.376 (.105-1.350)	0.134		
3	0.394 (.136-1.145)	0.087		
4	0.522 (0.099-2.743)	0.443		
Regraft vs 1st graft	5.445 (1.942-15.266)	**0.001**	5.135 (1.190-22.161)	**0.028**
Induction (alem ref)	0.591 (0.197-1.767)	0.346		
CNI Variability (hi vs low)				
<3mo	2.068 (0.810-5.284)	0.129		
>3mo	2.820 (0.876-9.081)	0.082	2.618 (2.396-19.748)	0.145
BFXM Positivity	2.981 (1.101-8.071)	**0.032**	1.767 (0. 443-7.044)	0.420

Table 7.6. Cox Regression Analysis to assess variables associated with Death Censored Graft survival.

CIT – cold ischaemia time, median CIT 13.52 hours. Median CNI variability <3mo 26.88 . Median CNI variability >3mo 24.96.

7.3.7 FUNCTION AND PROTEINURIA

The median eGFR of the whole cohort was 40, 45 and 42ml/min/1.73m^2 for 3 months, 1 year and 3 years post-transplant, with no statistically significant difference across the groups. For the purposes of this assessment, if a graft failed, the eGFR was coded as 5ml/min/1.73m^2.

DPDSA patients had more proteinuria compared with controls at 3 to 9 months post-transplant, but this was not statistically significant.

7.3.8 EPITOPE ANALYSIS

An attempt was made to further risk-stratify DPDSA transplants using models that assess HLA at an epitope level.

7.3.8.1 T-CELL EPITOPE (TCE) ALGORITHM

Donor and recipient pairs were classified using the TCE algorithm into permissive and non-permissive groups,[304] and survival curves were calculated using ABMR-free and graft survival as endpoints. Classification of permissive and non-permissive donor and recipient pairs did not risk stratify DPDSA transplants (Figure 7.8).

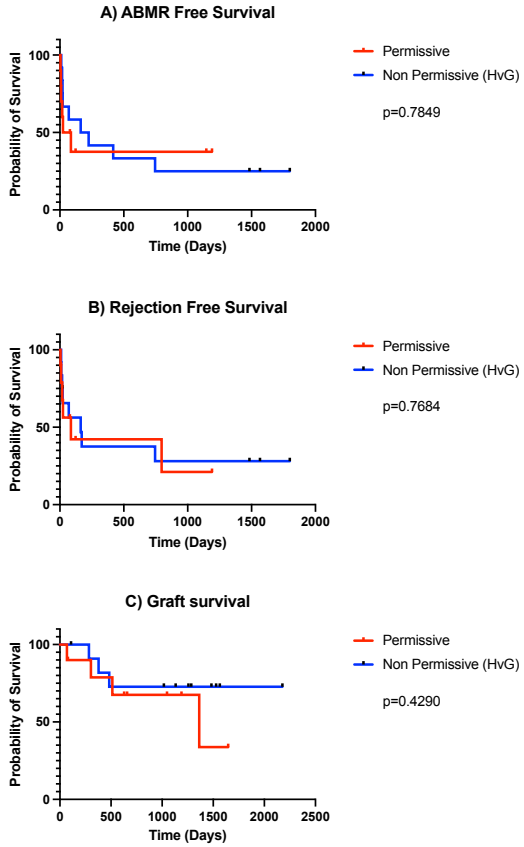


Figure 7.8. Kaplan Meier estimates comparing A) ABMR free survival, B) Rejection Free (TCMR, ABMR, borderline rejection) survival, and C) Graft survival. Donor/recipient pairs were classified into permissive and non-permissive (in the host versus graft direction) DP mismatches according to the T-cell Epitope Algorithm.[304] Curve comparisons were made using the log-rank test.

7.3.8.2 HLA MATCHMAKER

HLAMatchmaker and https://epregistry.com.br were used to determine any 'exposed antibody-verified' DPB1 epitope mismatches that corresponded with the recipient DSA profile.[15] Two recipients were excluded due to the presence of HLA-DPA1 DSAs in the TOO sample. ABMR-free survival and death-censored graft survival were studied by comparing the presence or absence of each antibody derived epitope. There was a trend towards reduced but non-statistically significant ABMR-free survival (HR 1.867, p=0.3308) and graft survival (HR 2.979, p=0.2880) in donor/recipient pairs with an 84DEAV mismatch. Additionally, a 96R mismatch was associated with reduced ABMR-

free survival (HR 10.47, p=0.0040) but a trend towards improved graft survival (HR 0.33, p=0.4435). It was difficult to draw firm conclusions due to the small patient numbers and multiple epitope mismatches within each donor/recipient pair (see Figure 7.9, Figure 7.10, Figure 7.11, Figure 7.12, Figure 7.13).

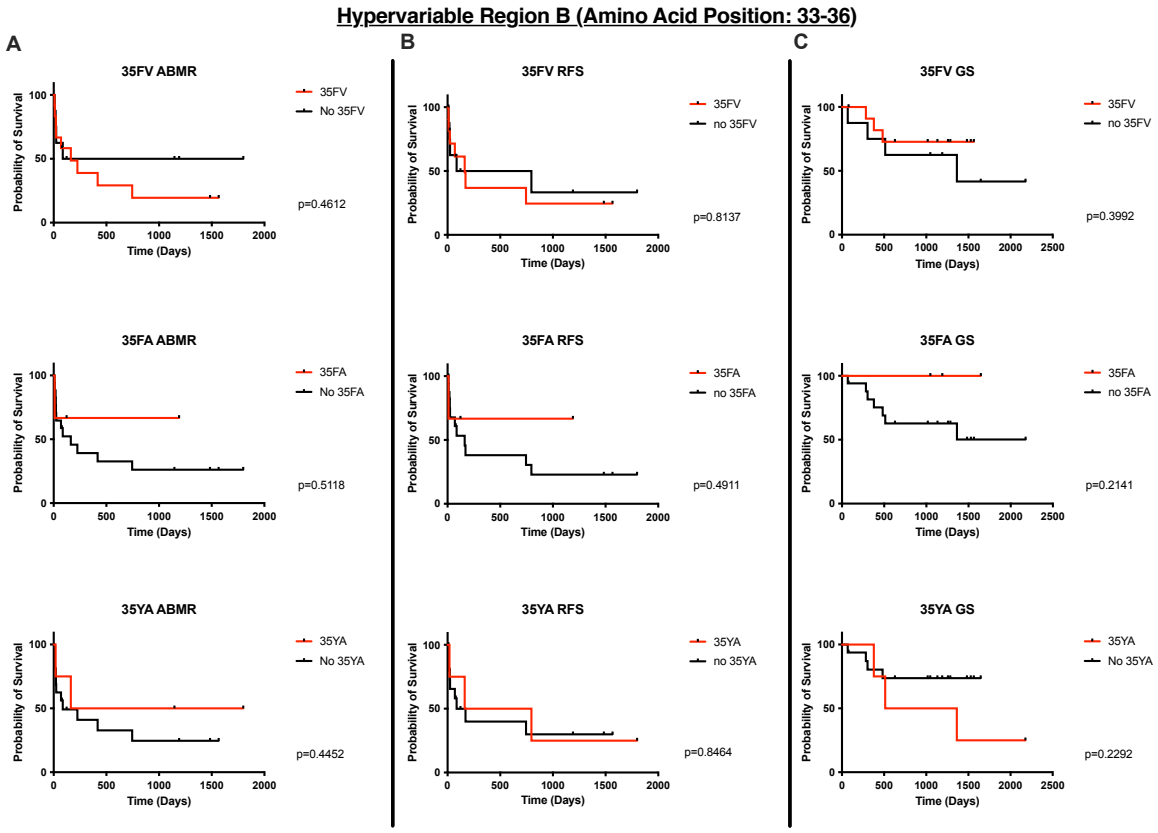


Figure 7.9. Clinical Outcomes associated with DPDSAs directed against HVR B. Kaplan Meier estimates of A) ABMR-free survival, B) Rejection Free survival and C) Graft survival comparing the presence/absence of DSAs directed against eplets present in HVR B. Outcomes against 35FV are visible in the top panel, 35FA in the middle panel, and 35YA in the bottom panel. Curves were compared using the log-rank test, and p values are demonstrated on each graph.

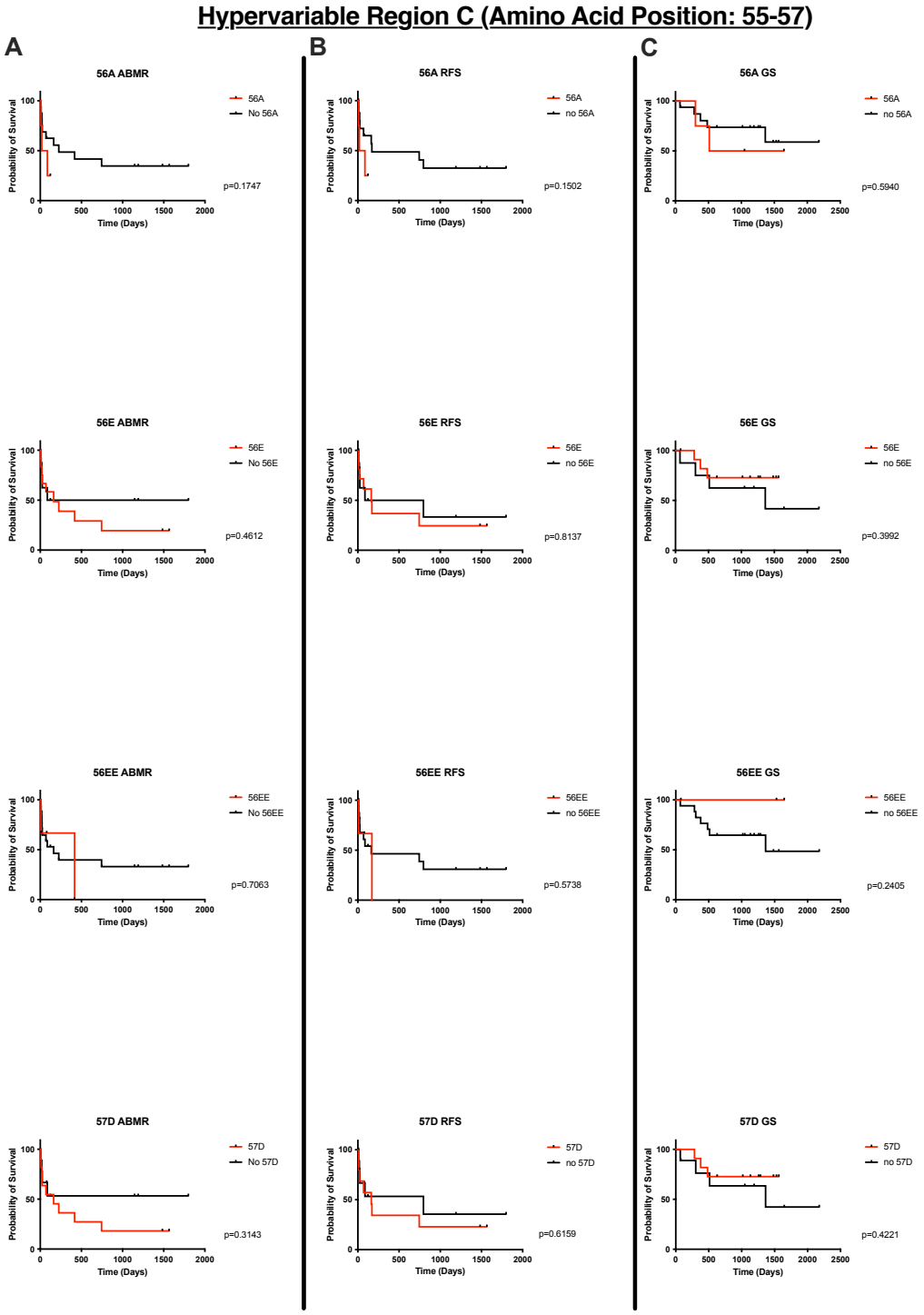


Figure 7.10. Clinical outcomes associated with DPDSAs directed against HVR C. Kaplan Meier estimates of A) ABMR-free survival, B) Rejection Free survival and C) Graft survival comparing the presence/absence of DSAs directed against eplets present in HVR C. The first row demonstrates outcomes against 56A, row 2 56E, row 3 56EE, row 4 57D. Curves were compared using the log-rank test, and p values are demonstrated on each graph.

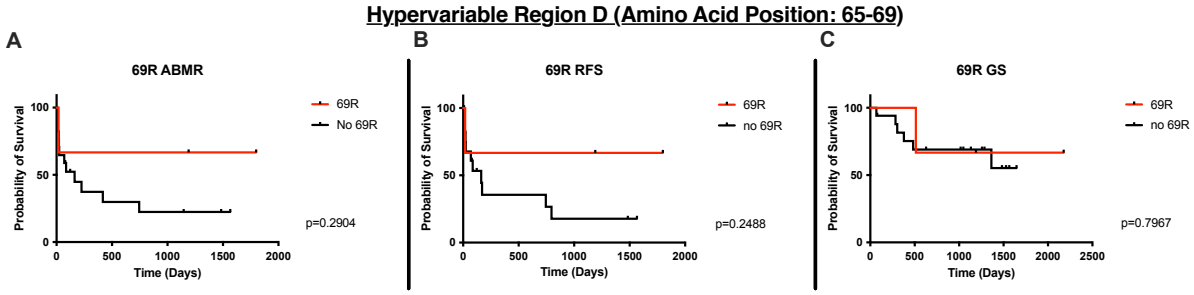


Figure 7.11. Clinical outcomes associated with DPDSAs directed against HVR D. Kaplan Meier estimates of A) ABMR-free survival, B) Rejection Free survival and C) Graft survival comparing the presence/absence of DSAs directed against 69R. Curves were compared using the log-rank test, and p values are demonstrated on each graph.

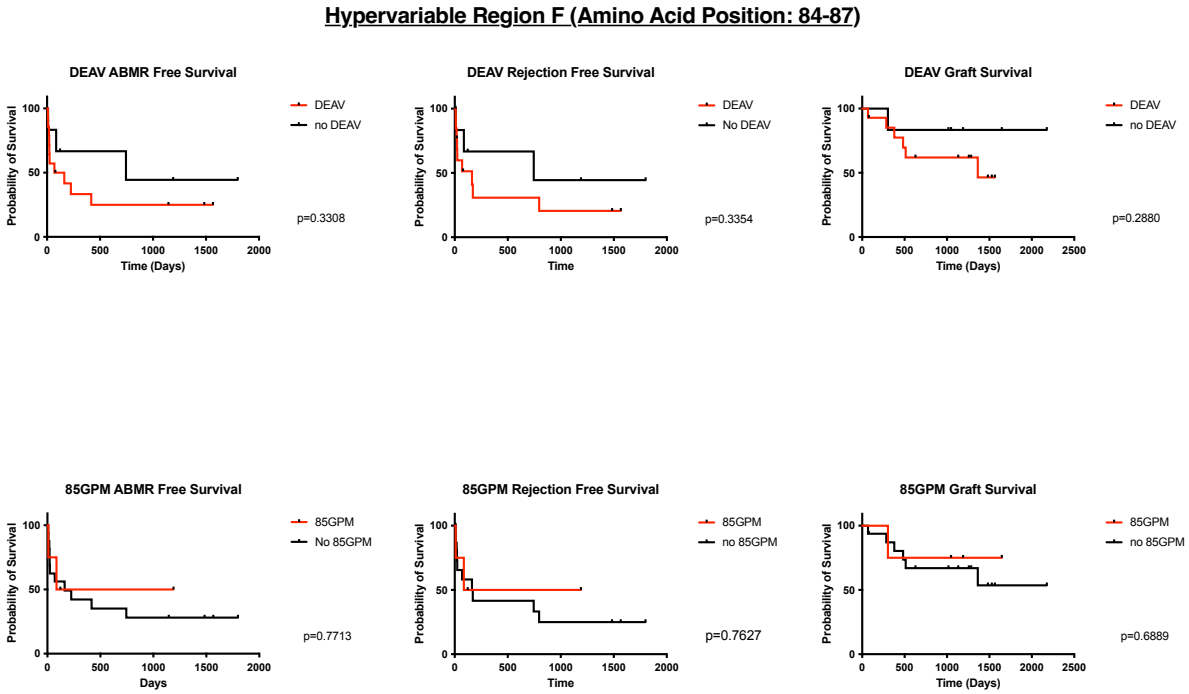


Figure 7.12. Clinical outcomes associated with DPDSAs directed against HVR F. Kaplan Meier estimates of A) ABMR-free survival, B) Rejection Free survival and C) Graft survival comparing the presence/absence of DSAs directed against 84DEAV (top row) and 85GPM (bottom row). Curves were compared using the log-rank test, and p values are demonstrated on each graph.

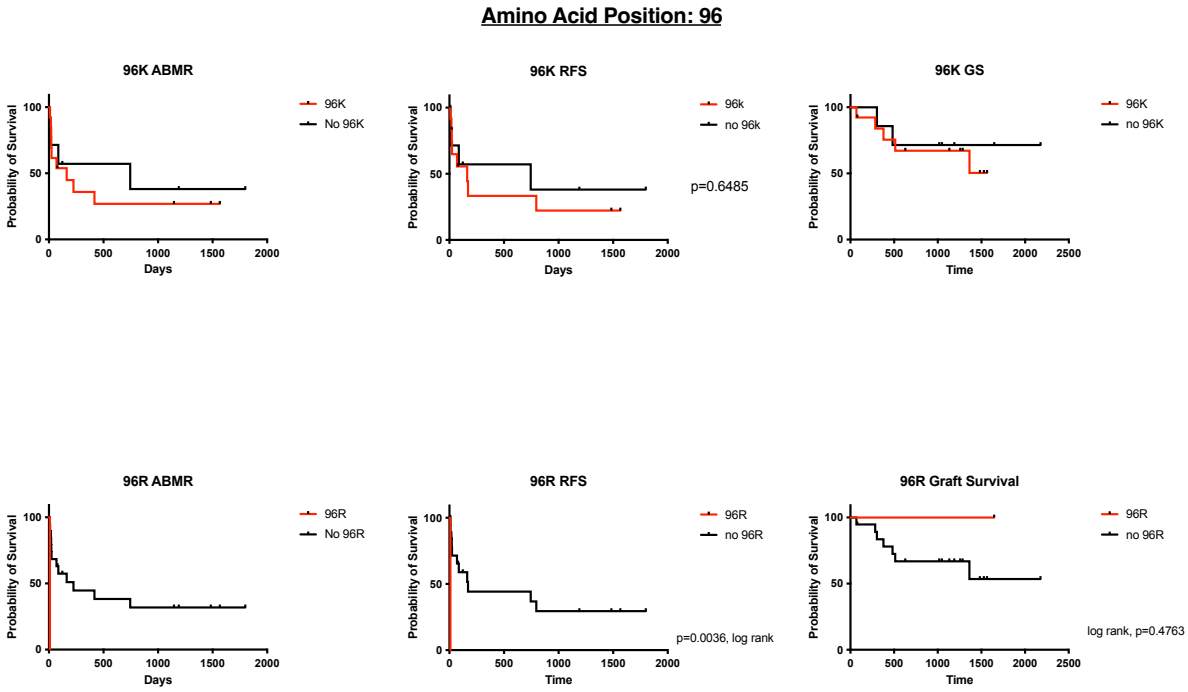


Figure 7.13. Clinical outcomes associated with DPDSAs against amino acid position 96.

Kaplan Meier estimates of A) ABMR-free survival, B) Rejection Free survival and C) Graft survival comparing the presence/absence of DSAs directed against 96K (top row) and 96R (bottom row). Curves were compared using the log-rank test, and p values are demonstrated on each graph.

Mismatches in the hypervariable regions (HVRs) C and F have been associated with the development of *de novo* DSAs following transplantation.[309,310] Both regions are easily accessible to antibodies and T-cell receptors due to their exposed positioning on the peptide binding groove (Figure 7.1). In a recent study, Daniëls *et al* noticed ABMR in cases where the HLA-DP DSA was directed against certain 'high risk' eplets which included 84DEAV, 85GPM + 56A, and 56E.[310] These eplets are positioned within hypervariable regions F (84DEAV and 85GPM), and C (56A, 56E). A further analysis was therefore performed to see whether DP DSAs directed against these two higher risk HVRs were associated with reduced clinical outcomes. DPDSA patients were separated into two groups – those that had DSAs against HVR C and HVR F, and those with DSAs against other regions (Figure 7.14). Patients who had DP-DSAs directed towards epitopes in HVR C or F had a reduced ABMR-free survival compared to those who had

DP-DSAs directed towards epitopes in other regions (p=0.0397, log rank). DP-DSAs against eplets in HVR C and/or F were associated with a trend towards reduced graft survival, however this was not statistically significant (p=0.2, log rank).

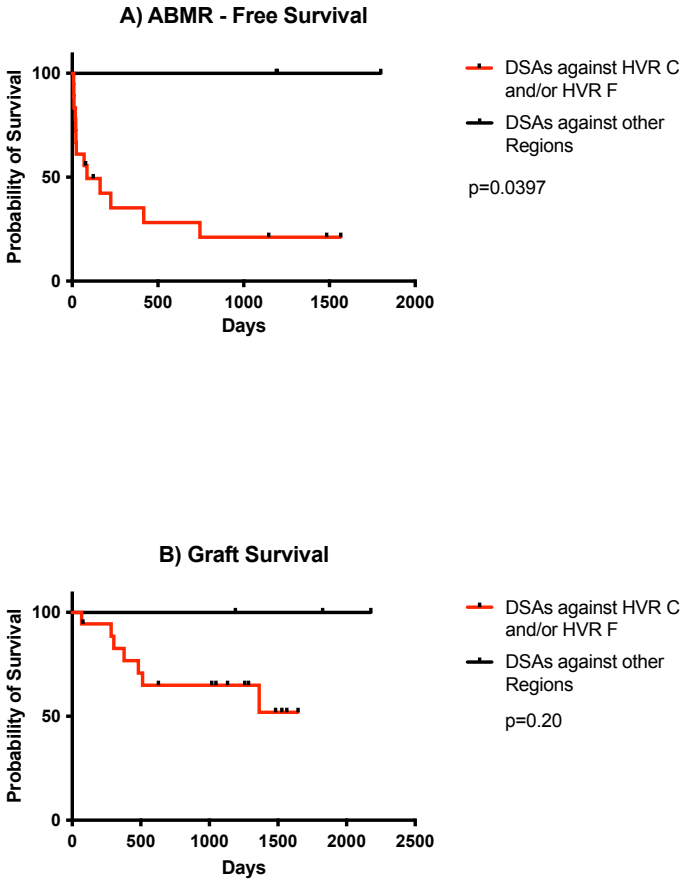


Figure 7.14. Clinical outcomes in patients with DPDSA.

Patients were divided into two groups depending on whether the DPDSAs were directed against eplets present in the HVR C or F regions (red, n=18) and DSAs directed at other regions (n=3). A) ABMR free survival, B) Graft survival. Comparisons were made using the log rank test.

7.3.9 DONOR HLA-DPB1 EXPRESSION LEVELS

Finally, DPDSA patients were categorised into low and high expression groups inferred from the donor HLA-DPB1 alleles (Figure 7.15). [305,306] Donor HLA-DPB1 expression levels were not associated with rejection free survival, however there was a trend towards reduced graft survival in high donor HLA-DPB1 expressors, (HR 2.505, p=0.3578).

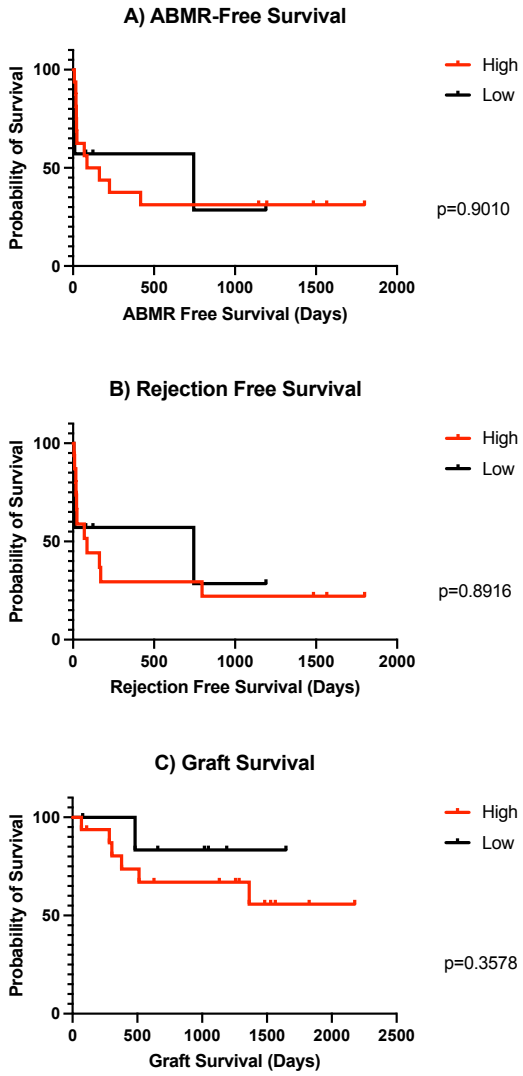
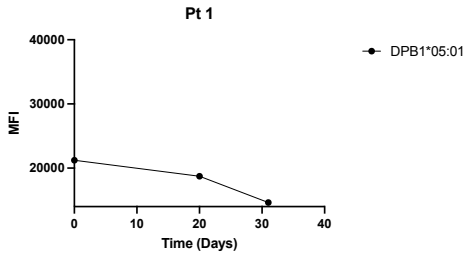
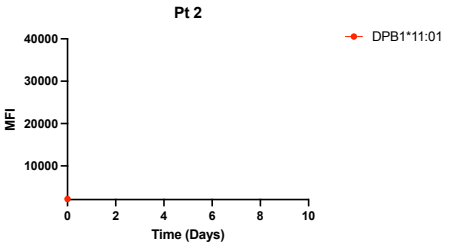
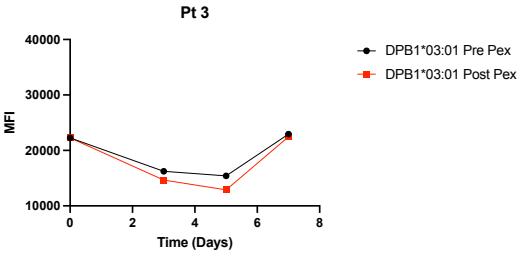
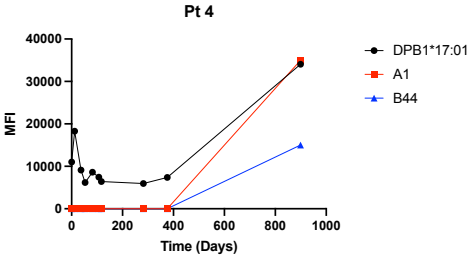
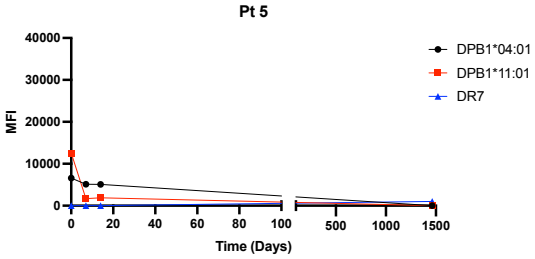
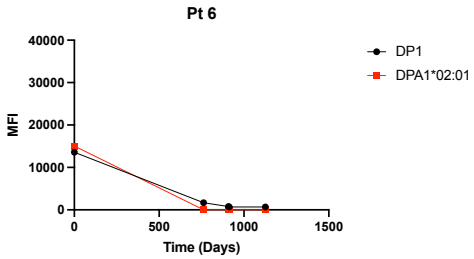
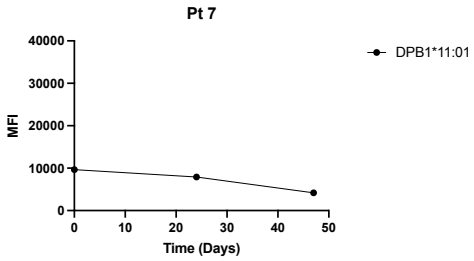
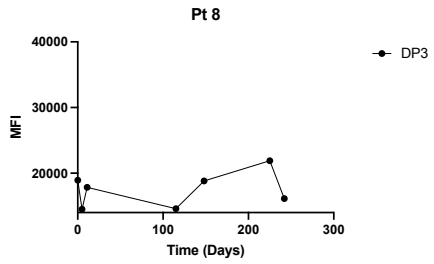
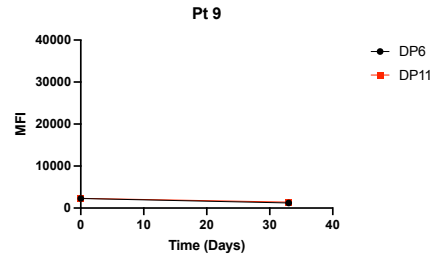
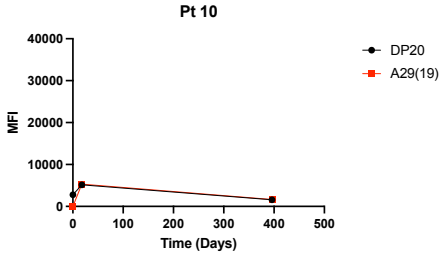
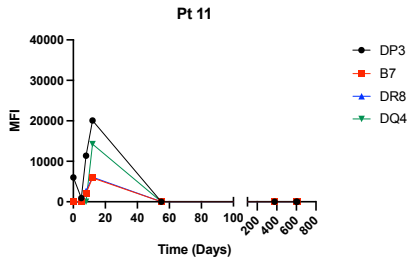
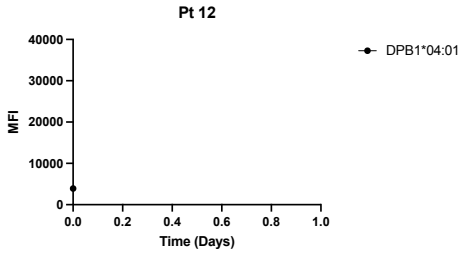
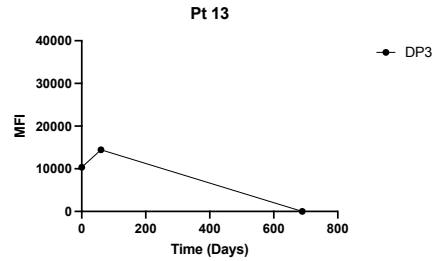
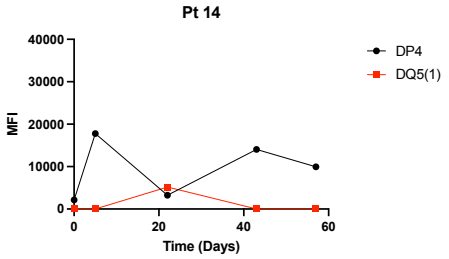
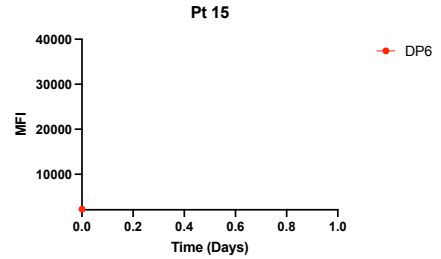
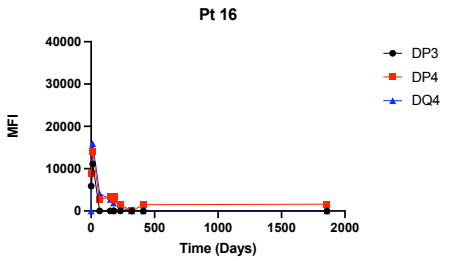


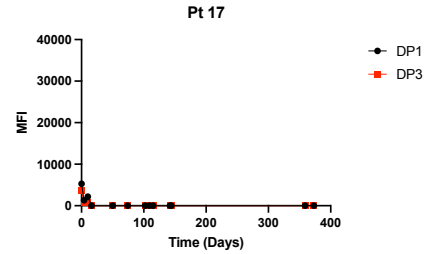
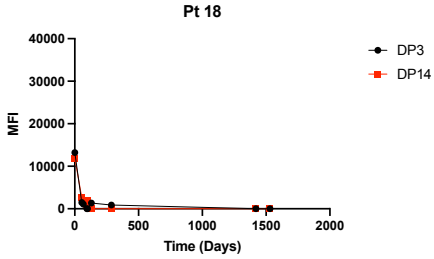
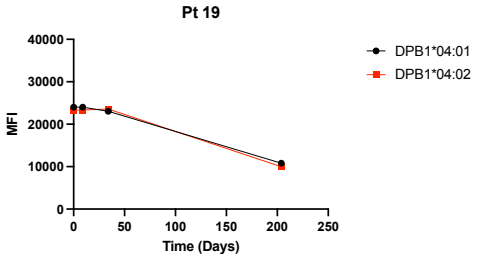
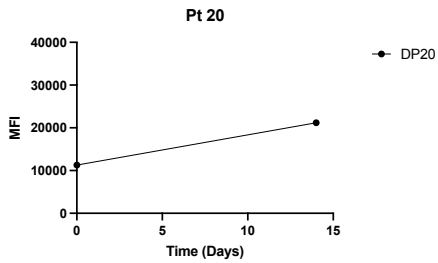
Figure 7.15. Kaplan Meier curves estimating A) ABMR free survival, B) overall rejection free survival, C) Graft survival according to inferred donor HLA-DPB1 expression levels.[305]

Patient	HLA-DP DSA Present using ONELAMBDA and Immucor microbead kits?	IS at time of transplant	ABMR (Days)	Graft Loss (Days)
	Unable to retest	Basiliximab Tacrolimus, MMF, Prednisolone	Yes (24)	Yes (69)
	No	Alemtuzumab Tacrolimus, MMF	No	No
	Yes	Pre-emptive plasma exchange Alemtuzumab Tacrolimus, MMF, Prednisolone	Yes (20)	No
	Yes	Basiliximab Tacrolimus, MMF	Yes (10)	Yes (482)

Patient	HLA-DP DSA Present using ONELAMBDA and Immucor microbead kits?	IS at time of transplant	ABMR (Days)	Graft Loss (Days)
	Yes	Basiliximab Tacrolimus, MMF, Prednisolone	No	No
	Yes	Alemtuzumab Tacrolimus, MMF	No	Yes (1363)
	Yes	Basiliximab Plasma Exchange Tacrolimus, MMF, Prednisolone	Yes (18)	Yes (513)
	Yes	Alemtuzumab Tacrolimus	Yes (9)	Yes (284)

Patient	HLA-DP DSA Present using ONELAMBDA and Immucor microbead kits?	IS at time of transplant	ABMR (Days)	Graft Loss (Days)
	Unable to retest	Basiliximab Tacrolimus, MMF	No	No
	No – DP20 not present in LifeCodes kit	Basiliximab Tacrolimus, MMF	No	No
	Yes	Alemtuzumab Tacrolimus, MMF	Yes (22)	No
	Yes	Basiliximab Tacrolimus, MMF, Prednisolone	No	No

Patient	HLA-DP DSA Present using ONELAMBDA and Immucor microbead kits?	IS at time of transplant	ABMR (Days)	Graft Loss (Days)
	Yes	Alemtuzumab Tacrolimus, MMF	Yes (745)	No
	No	Alemtuzumab Tacrolimus	Yes (9)	No
	No	Alemtuzumab Tacrolimus	No	No
	Yes	Basiliximab Plasma Exchange Tacrolimus, MMF, Prednisolone	Yes (170)	No

Patient	HLA-DP DSA Present using ONELAMBDA and Immucor microbead kits?	IS at time of transplant	ABMR (Days)	Graft Loss (Days)
	Yes	Alemtuzumab Tacrolimus, MMF, Prednisolone	Yes (163)	Yes (379)
	Yes	Alemtuzumab Plasma Exchange Tacrolimus, MMF, Prednisolone	Yes (69)	No
	Yes	Alemtuzumab Tacrolimus, MMF, Prednisolone	Yes (5)	No
	No - DP20 not present in LifeCodes Kit	Alemtuzumab Tacrolimus, MMF	Yes (8)	No

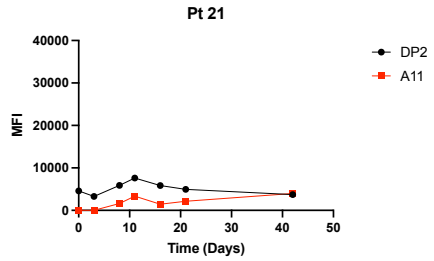
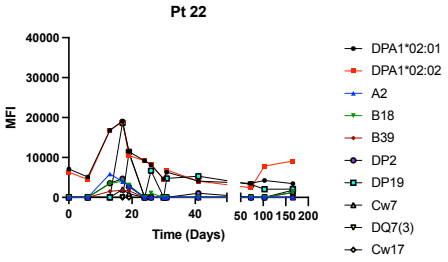
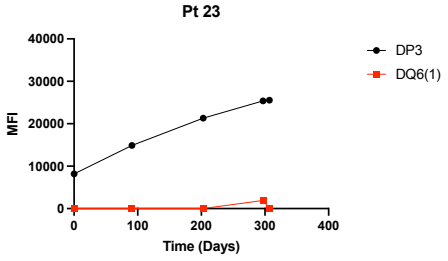
Patient	HLA-DP DSA Present using ONELAMBDA and Immucor microbead kits?	IS at time of transplant	ABMR (Days)	Graft Loss (Days)
	Yes	Alemtuzumab Tacrolimus, MMF, Prednisolone	No	No
	Yes	Alemtuzumab Tacrolimus, MMF	Yes (17)	No
	Yes	Alemtuzumab Tacrolimus, MMF	Yes (86)	Yes (303)

Table 7.7. Graphs demonstrating the kinetics of the donor specific antibodies for each DPDSA patient over the period of follow up.

Clinical outcomes (including ABMR and Graft loss) are included. MFI = median fluorescent intensity, IS = immunosuppression. Samples were tested in real-time therefore the uncertainty of measurement associated with determining serial MFI values could not be controlled for. Routine/real-time sera were testing using ONELAMBDA SAB kits. The time of offer (TOO) samples were retested using Immucor Kits

7.4 DISCUSSION

Chronic antibody mediated rejection is a major cause of renal allograft loss and it is strongly linked to the development of donor-specific HLA Class II antibodies.[52,75] Class II DSAs are associated with the process of transplant glomerulopathy leading to interstitial fibrosis, tubular atrophy and eventual graft failure.[311] A primary site of allorecognition is the donor endothelium and while HLA-DR expression is usually higher in the resting state the expression of HLA-DQ and DP antigens are induced by inflammatory stimuli such as rejection or ischaemia-reperfusion injury, possibly mediated by γ-interferon.[312]

The HLA-DP antigen consists of a heterodimer of two peptide chains DPα and DPβ which are derived from the polymorphic DPA1 and DPB1 genes respectively. Population genetic studies have revealed strong linkage disequilibrium between DPA1 and DPB1 but only weak linkage with HLA-DR and DQ.[313] Consequently, there is an 80% chance of a DP mismatch even if an unrelated donor and recipient are fully matched at the A, B, C, DR and DQ loci. Initial experiments in mixed lymphocyte reactions revealed inconsistent results between different DP types, limiting its utility in clinical practice.[314] With the advent of molecular typing, it became clear that substantial polymorphism exists within the DPB1 gene.[315] Furthermore, associations have been discovered relating to allelic variation and expression levels in both autoimmunity and the development of GvHD in stem cell transplantation.[305,316]

In the United Kingdom HLA-DP antibodies have not been used historically to define unacceptable antigens in the national deceased donor kidney allocation scheme. The introduction of solid phase assays for HLA antibody detection and readily available molecular typing methods has led to a reappraisal of the role of HLA-DP in renal transplantation.[317] This study was undertaken to address this situation and to try to guide the clinician when faced with DP-DSAs particularly as these may become apparent after the renal transplant has occurred.

An early registry-based study found no relationship between DP mismatch and outcomes in first transplants.[291] Nevertheless there was a deleterious effect on graft survival in subsequent grafts especially in recipients with cRF>50%. A European study of 291 patients showed that HLA-DP antibodies were common, present in nearly half of recipients with DSAs.[318] Whereas the presence of class II DSAs were associated with poorer graft survival, there was no additive effect of HLA-DP antibodies. Other studies have reported deleterious effects, but the DP-DSAs were usually present with other HLA-DSAs making it difficult to disentangle specific effects of the HLA-DP antibodies.[319] There have also been case reports suggesting that isolated DP antibodies can mediate significant graft damage with ABMR and early graft loss implying that such antibodies may be directly pathogenic.[297,299,320] A French study reported 26 patients with HLA-DP DSAs and demonstrated an association with a significantly increased risk of a positive FXM, ABMR and graft loss compared to unsensitised controls. This risk was similar to recipients with DSAs against HLA-A, HLA-B, HLA-DR and HLA-DQ.[321] A recent retrospective study identified 13 patients with pre-existing isolated HLA-DP DSAs, six of whom experienced ABMR and three lost their grafts.[310]

There is good evidence that there is a phenotypic difference between pre-existing HLA-DSAs and *de novo* DSAs. *De novo* antibodies tend to be HLA class II antibodies and are associated with more chronic damage at the time of biopsy with worse clinical outcome.[81] Against this background the impact of isolated pre-existing HLA-DP DSAs have been assessed over a seven-year period. During this time, donor HLA-DP typing was not routinely performed therefore transplants would proceed in the setting of a negative crossmatch, and knowledge of pre-existing HLA-DP DSAs often only became apparent following transplantation. This was not surprising as recent evidence suggests that HLA-DP antibody levels with MFIs < 10,000 are associated with a negative CDC crossmatch and even above 10,000, only 70% will register as positive.[310] This may be due to the lower expression levels of HLA-DP antigens compared with other human leukocyte antigens on resting cells. [50]

In this study kidney transplant recipients with isolated pre-existing HLA-DP DSAs were compared with two other sensitised groups (DPnDSA and HSP) and a third control group. Unsurprisingly, the three sensitised groups included more females and sensitising events especially blood transfusion and previous transplantation. There were differences in donor type with only one living donor in the DPDSA group. The UK kidney allocation system prioritises implantation of sensitised and long-waiting patients over geographical proximity. Transplants therefore tended to be better matched in the sensitised patients where there was a high proportion of regrafts (65% of the DPDSA group and 63% of the HSP group). Cold ischaemia times were also longer in the sensitised patients, and this may partially explain the associated increased rates of delayed graft function, although alloimmune mechanisms may also be operating. For example, the longer CIT may have led to increased ischaemia-reperfusion injury with upregulation of HLA-DP expression.

The presence of DP-DSAs often were reported following the transplant and the clinicians usually commenced MMF, prednisolone, or both. As a result, augmented immunosuppression was used in 74% of patients with DP-DSAs. This was not seen in the HSP population as patients who received transplants following delisting or desensitising strategies were excluded from this study. It is acknowledged that the presence or absence of DP-DSAs was not always available to the clinician on the day of transplant which may have influenced decision making.

In this study 32% of DPDSA patients had a positive B-cell crossmatch which was increased compared to other groups but not significantly so. There was no correlation between the measured HLA-DP antibodies in the DPDSA group and the total donor-specific reactivity as measured by the BFXM. This is consistent with previous data describing a negative CDC crossmatch in patients with DP-DSA levels less than 10,000 MFI, and approximately 30% of those with MFI greater than 10,000.[310] However DP-DSAs were associated with significant episodes of ABMR with more than half (15 of 23) suffering from ABMR (median time to ABMR 22 days, twenty-fifth centile 14, seventy-fifth centile 125 days). After multivariate analysis, HLA-DP-DSA was the single factor

that was associated with the development of clinical ABMR (HR=9.6). Though it does not prove causality this supports the observations of others that TOO HLA-DP DSAs are associated with significant clinical events.[298,322] Moreover, in this study cohort, the BFXM did not add any further information. Mechanistically this raises the question as to whether HLA-DP DSAs are directly pathogenic or simply a marker of an increased immunoreactive phenotype, a hypothesis that warrants further study. Recent observations that HLA-DQ DSAs can bind to the donor endothelium and modulate the generation of T-regulatory cells support possible indirect mechanisms.[323]

DPDSA renal transplant biopsies did show evidence of increased microvascular inflammation, C4d deposition and transplant glomerulopathy although it is acknowledged that in the absence of protocol biopsies there may have been a lower threshold to perform biopsies in this group. This did translate into a trend towards lower graft survival in the DPDSA group although this did not reach statistical significance, possibly due to low numbers overall. There was also no association with graft function or proteinuria and a larger series will be required to address this.

An attempt was made to evaluate certain high-risk antibody-verified eplets, such as 84DEAV mismatch, but there were insufficient numbers to draw valid conclusions about individual eplet mismatches.[310] However, reduced ABMR-free survival and a trend towards reduced graft survival if DP-DSAs were directed against HVR C and/or HVR F regions were noted. These findings are in concordance with a recent study which observed ABMR in cases of pre-transplant DP-DSAs which were directed against 84DEAV, 85GPM (HVR-F), 56A and 56E (HVR-C).[310] These observations highlight HVR C and HVR F as potential areas of increased antigenicity and warrant further investigation. In this cohort, there was no significant association between inferred donor HLA-DPB1 expression levels and clinical outcomes. This is not in keeping with the findings described following stem cell transplantation.[305]

There is increasing evidence of processing artefacts associated with the production of Luminex microbead arrays, which may result in false positivity, especially among the

class II HLA.[89] There was an attempt to address this using assays from two different manufacturers and a consensus was obtained in 16 of 21 TOO samples tested. Two samples were not in agreement due to differing antibody specificities included in the assay kits. In the remaining 3 samples, DPDSAs were detected using the ONELAMBDA, but not the Immucor assay. The overall 'strength' of these DSAs were relatively low, and inconsistencies could be explained by differing assay sensitivities as a result of varying antigen densities in the presence of low-level antibodies, or by the conformational changes of antigens found on the different bead kits. Although these 3 patients did not lose their grafts during the follow up period, 1 did exhibit early ABMR, therefore further investigation is required to test the clinical utility of using a combination of bead kits for risk stratification in the presence of HLA-DP antibodies.

A 10% rate of antibody mediated rejection was noted in the control (standard immunological risk) group, however there was no AMR in the HSP group. Whereas the HSPs would have undergone a detailed longitudinal characterisation of their HLA antibody profile over a prolonged wait time, the control group would not have been as closely scrutinised. In addition, all patients are routinely screened for HLA antibodies using the LABScreen mixed bead test, with a reflex for further characterisation using ONELAMBDA SABs if positive. It is possible that samples test negative using the mixed screen, yet are positive on testing with SAB. Unfortunately, testing all samples from every wait listed patient with SAB is cost prohibitive, and it is acknowledged that there may be the rare case where a patient who screened negative in the control group may have an uncharacterised DSA.

There is currently no consensus method for calculating the antibody 'strength' when a panel includes more than one bead per antigen or allele specificity. The local practice is to calculate the average MFI over all beads, unless there is a clear allelic antibody, at which point the MFI for the specific allelic antibody is reported, which can underestimate the amount of antibody present. Alternatively, adding the MFI obtained from each bead can lead to the overestimation of the antibody amount.

Non-HLA antibodies, which may have contributed to BFXM positivity in the DPnDSA cohort in the absence of measurable HLA-DSAs, were not investigated, and their role could not be excluded in the transplant outcomes.[324]

In summary, the clinical progress of a cohort of patients who received a kidney transplant with pre-existing HLA-DP DSAs is described. Despite augmented immunosuppression approximately half these cases suffered from biopsy proven ABMR within the first 6 months that was not further informed by the FXM. This rejection was associated with increased histological damage and a trend towards worse graft survival. Kidney transplant recipients with pre-existing DP-DSA should be considered a high-risk immunological group and are subjected to close monitoring in the first six months after transplantation.

8 FINAL CONCLUSION

In transplantation, B-cells play a significant role in allograft outcomes through antigen processing and presentation, cytokine and antibody production, and contribution to immune memory, a corollary of which is antibody mediated rejection (ABMR). Current therapeutic agents have not been as effective at treating ABMR as they have been with treating TCMR, and ABMR has become the predominant cause of immune-mediated allograft injury. This study sought to evaluate potential B-cell biomarkers of allograft outcome. In chapter 3, a retrospective study using UK registry data was performed to compare the 5-year outcomes of adult renal transplant recipients receiving either alemtuzumab or alternate agents (usually basiliximab). This demonstrated that alemtuzumab induction was not inferior to basiliximab induction in terms of 5-year patient and graft survival. However, for deceased donors, alemtuzumab induction was associated with a protective effect on rejection free survival despite fewer patients receiving long-term steroids as part of their immunosuppressive regime. There were no differences in reported cause of death or cause of graft loss, however, it is acknowledged that several records were missing, which is an inherent problem with interrogating registry data. Having demonstrated non-inferiority at 5 years, it would be useful to repeat this analysis to determine if these differences are maintained in the long-term. The effect of both induction agents on the B-cell phenotype was then studied in a cohort of adult renal transplant recipients. Early differences were noticed in the transitional cell, naïve and memory B-cell populations, which were maintained until at least 1 year post transplant. These findings confirm those reported by others.[140,163,166,231] The surface B-cell phenotypes were then studied, and changes in transitional B-cells (TrBs) including reduced $CD24^{+++}CD38^{+++}$ T1 cells which are thought to have the highest regulatory potential, may be markers of poor clinical outcomes, both in terms of clinically stable transplant recipients early post-transplant, and those with a troubled graft.

These findings are encouraging and will need confirming in larger multi-centre studies. The current gold standard for demonstrating the presence of Bregs is through IL-10

production. Although this study has confirmed that transitional cells, especially $CD24^{+++}CD38^{+++}$ T1 cells contain the highest numbers of Bregs (defined both by IL-10 production and the IL-10:TNF-α ratio),[124] a prospective assessment of IL-10 capacity by these cells post-transplant will be useful, acknowledging that stimulation of cells *in vitro* will alter the cellular phenotype, and may not necessarily correspond with the *in vivo* environment. The data obtained from this study highlights the importance of using caution with newer pharmaceutical agents that may alter the TrB population or the T1:T2 ratio. Daratumumab, for example, is a human monoclonal IgGκ antibody that targets CD38 which is highly expressed on plasma cells, inducing apoptosis via the ADCC, antibody and complement dependent cytotoxicity mechanisms.[325] Although daratumumab has been developed primarily to target malignant plasma cells in multiple myeloma, as the key role of plasma cells is antibody production, it is unsurprising that daratumumab has been explored in transplantation, including the reduction of HLA antibodies pre-transplant and treatment of refractory ABMR.[326,327] Daratumumab was used to treat sensitised macaques who received sequential skin grafts prior to a renal transplant. These macaques had significantly reduced DSA levels compared with controls, however, subsequently demonstrated a rapid rebound in DSA levels and developed severe T-cell mediated rejection (TCMR).[326] A case report has recently been published, documenting the development of severe TCMR within 72 hours of transplantation in what would otherwise have been considered a standard immunological risk transplant. This recipient had received a final infusion of daratumumab only a few weeks prior to transplantation.[327] This suggests that not only plasma cells have been targeted by anti CD38, but other immunomodulatory cells, including regulatory B-cells that have been demonstrated in the $CD24^{hi}CD38^{hi}$ population. Krejcik *et al* found that in multiple myeloma patients, in addition to plasma cells, daratumumab depleted $CD19^{+}CD24^{+}CD38^{+}$ regulatory B-cells which produced IL-10.[328]

CD9 expression was then demonstrated to be increased in the $CD24^{hi}CD38^{hi}$ transitional B-cell population, and expression was associated with increased IL-10 production and a higher IL-10:TNF-α ratio. Whilst CD9 expression may add confidence

to gating strategies for TrBs, following the stimulation protocol, it was not a stable marker for Bregs and it did not account for all IL-10 producing cells. Further work investigating the mechanistic association between CD9 expression and IL-10 production will be interesting, together with a prospective analysis of $CD9^+$ B-cells and clinical outcome.

In the final chapters, HLA antibody production by B-cells was explored. In Chapter 6, two novel methods were described that evaluated the contribution of peripheral alloreactive memory B-cells to the sensitisation of renal patients. The first method determined the specificities of HLA antibodies produced by circulating memory B-cells after non-specific stimulation. The second method sought to determine the frequency of alloreactive B-cells within different B-cell subsets. It involved co-incubating pre-stained peripheral B-cells with single antigen beads and visualising them using flow cytometry. Although further work will be required before either assay can be introduced into routine use, an attractive feature of both methods is that they utilise reagents and equipment frequently found in most H&I labs, and therefore could eventually be added to the H&I routine test repertoire. Assessing the memory B-cell contribution to sensitisation will be of utmost value during the implementation of delisting strategies in highly sensitised patients to facilitate transplantation; it is becoming increasingly common to list patients for their 3rd and 4th renal transplant. The National Institute for Health and Care Excellence (NICE) approval of Imlifidase (Idefirix) to enable desensitisation has allowed H&I laboratories to expand HLA antibody delisting thresholds beyond what has previously been considered safe.[329] However, due to the nature of Imlifidase, induction agents cannot be administered for several days, potentially resulting in an unchecked anamnestic response, and a rebound of donor specific antibodies with early antibody mediated rejection is expected.[330–332] Knowledge of the memory B-cell contribution to HLA sensitisation may be helpful as specificities produced by memory cells could be avoided during the first, more cautious attempts at antibody delisting. In Chapter 7, the clinical outcomes following transplantation in the presence of pre-existing HLA-DP donor specific antibodies were assessed. This study represents the largest cohort of patients in a

single centre receiving this type of transplant. Until recently, due to the conflicting reports surrounding the risk of transplanting across this barrier, the national allocation system did not take HLA-DP sensitisation into account. This study shows that patients receiving this type of transplant, despite augmented immunosuppression, are at increased risk of antibody mediated rejection within the first 6 months post-transplant, with increased histological damage, and a trend towards reduced graft survival. Furthermore, the data confirm findings obtained elsewhere that performing a wet crossmatch does not risk stratify these patients.[310] Whilst not necessarily a veto to transplantation, crossing HLA-DP-DSAs should be considered high immunological risk, and these patients should be monitored closely post-transplant and augmented immunosuppression considered. The interaction of HLA-DP antibodies with different hypervariable regions was assessed, and whilst the data did not yield statistically significant results in terms of graft survival, HLA-DP antibodies directed at hypervariable regions C and F were associated with reduced ABMR-free survival. This suggests differing antigenicity associated with the antibody interactions within distinct hypervariable regions, requiring further investigation. As technology improves, allowing for the rapid HLA typing of donors to higher resolution, information gained from this study may help to finesse the assessment of risk when performing HLA-DP incompatible transplants, allowing for 'permissive' vs 'non-permissive' transplants.

Taken together the findings of this thesis identify the necessity of understanding in detail the individuals' immune parameters towards ensuring the best outcomes of transplantation.

9 APPENDIX

9.1 ETHICS APPROVAL

The Leeds Teaching Hospitals NHS
NHS Trust

Date: 07/03/2016

Our Ref: Anne Gowing

Research & Innovation Department
34 Hyde Terrace
Leeds
LS2 9LN

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Dr Richard J Baker
Consultant Renal Physician
Leeds Teaching Hospitals NHS Trust
Renal Unit, Lincoln Wing, St James's
University Hospital, Beckett Street, Leeds
Leeds
LS9 7TF

Dear Dr Baker

Re: **NHS Permission at LTHT for: Assessment in Leeds of Biomarkers Early after Renal Transplantation (ALBERT) study**
LTHT R&I Number: RL15/399(154894)
REC: 16/YH/0025

I confirm that *NHS Permission for research* has been granted for this project at The Leeds Teaching Hospitals NHS Trust (LTHT). NHS Permission is granted based on the information provided in the documents listed below. All amendments (including changes to the research team) must be submitted in accordance with guidance in IRAS. Any change to the status of the project must be notified to the R&I Department.

The study must be conducted in accordance with the *Research Governance Framework for Health and Social Care*, ICH GCP (if applicable), the terms of the Research Ethics Committee favourable opinion (if applicable) and NHS Trust policies and procedures (see http://www.leedsth.nhs.uk/research/) including the requirements for research governance and clinical trials performance management listed in appendix 1 and 2 . NHS permission may be withdrawn if the above criteria are not met including the requirements for clinical trials performance

The Leeds Teaching Hospitals NHS Trust participates in the NHS risk pooling scheme administered by the NHS Litigation Authority ""Clinical Negligence Scheme for NHS Trusts"" for: (i) medical professional and/or medical malpractice liability; and (ii) general liability. NHS Indemnity for negligent harm is extended to researchers with an employment contract (substantive or honorary) with the Trust. The Trust only accepts liability for research activity with NHS Permission

The Trust therefore accepts liability for the above research project and extends indemnity for negligent harm. Should there be any changes to the research team please ensure that you inform the R&I Department and that s/he obtains an appropriate contract, or letter of access, with the Trust if required.

Yours sincerely

Anne Gowing
Research Governance Manager

Approved documents
The documents reviewed and approved are listed as follows:-

Document	*Version*	*Date of document*
NHS R&D Form		29 Jan 2016
Protocol	1.0	17 Feb 2016
CSU Approval		14 Dec 2015
REC Letter confirming favourable opinion		25 Feb 2016
Participant information sheet	1.1	22 Feb 2016
Participant information sheet Healthy controls	1.0	17 Feb 2016
Consent form	1.0	17 Feb 2016

Conditions of NHS Permission for Research:

Appendix 1

Governance requirements:

Managerial approval within the Clinical Support Unit must be obtained before starting the study and healthcare staff should be suitably informed about the research their patients are taking part in and information specifically relevant to their care arising from the study should be communicated promptly.

Agreements must be in place with appropriate support departments.

Arrangements must be in place for the management of financial and other resources provided for the study, including intellectual property arising from the research.

All data and documentation associated with the study must be available for audit/monitoring by authorised Trust or external agencies.

All members of the research team, where applicable, have appropriate employment contracts or letter of agreement to carry out their work in the Trust.

Each member of the research team must be qualified by education, training and experience to discharge his/her role in the study. Students and new researchers must have adequate supervision, support and training.

The research must follow the protocol approved by the relevant research ethics committee. Any proposed amendments to or deviations from the protocol must be submitted for review (as necessary) by the Research Ethics Committee, the Research Sponsor, regulatory authority and any other appropriate body. Where the amendment has resource implications within the CSU, the Directorate research lead/clinical director and R&I should be notified.

Adverse Events in clinical trials of investigational medicinal products must be reported in accordance with the Medicines for Human Use (Clinical Trials) Regulations 2004.

Procedures should be in place to ensure collection of high quality, accurate data and the integrity and confidentiality of data during processing and storage in line with Trust Information Governance Policies and arrangements must be made for the appropriate archiving of data when the research has finished. Records must normally be kept for 15 years.

In compliance with the Health Research Authority (HRA) regulations, clinical trials (and other studies falling within the HRA definition) must be registered on a publically accessible database (such as https://clinicaltrials.gov/) prior to commencement. Studies sponsored by LTHT will be registered by the R&I Department.

Findings from the study should be exposed to critical review through accepted scientific and professional channels.

All members of the research team involved in seeking informed consent adheres to GCP standards. Investigators are directed to the R&I website for further information about training in consent for clinical trials.

Studies involving the use of human tissues must be performed in compliance with the code of practice of the Human Tissue Authority.

If you are not able to comply with these requirements, NHS permission to conduct the research in LTHT will be suspended.

Appendix 2

Commercially Sponsored and funded studies.

In line with Trust Standing Financial Instructions there must be a research agreement with the commercial funder signed by the R&I Department (on behalf of the Leeds Teaching Hospitals NHS Trust). Investigators do not have the authority to sign research agreements on behalf of the Trust.

NHS permission for this project to be carried out in the Trust is granted on the understanding that you:

Provide recruitment information when requested by R&I on the Clinical Trial Tracker (available on the CSU Research Hub)

Work with R&I to resolve blocks and delays on trials to ensure that LTHT meets the NIHR benchmarks.

NIHR Benchmarks for Performance in Initiating & Delivering Clinical Research
LTHT clinical trial performance is measured against 2 national benchmarks to improve the initiation and delivery of clinical trials approved by the Trust. NIHR funding to the Trust is conditional on meeting these benchmarks.

Initiation – it should take no more than 70 days from receipt of a valid research application (signed SSI form) by the R&I Department to the recruitment of (ie consenting) the 1st patient to the trial

Delivery – for all trials hosted by the Trust the agreed number of patients must be recruited within the agreed recruitment period

The Trust submits quarterly performance reports to the Department of Health setting out our performance.

For more information about the benchmarks and the work we are doing to support clinical trial management please see the R&I website.
http://www.leedsth.nhs.uk/research/

9.2 PATIENT INFORMATION LEAFLET

The Leeds Teaching Hospitals NHS
NHS Trust

Renal Unit

Lincoln Wing

St. James's University Hospital

Beckett Street

Leeds

West Yorkshire

LS9 7TF

Tel: 0113 243 3144

Assessment in Leeds of Biomarkers Early after Renal Transplantation (ALBERT) Study

Participant information sheet

Introduction

You are being invited to take part in a research study. Before you decide, 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. 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.

Background to the study

As you know, transplantation provides the best treatment for patients with advanced kidney failure. On average, successful kidney transplants last around 15 years. Approximately half of these transplants are lost due to transplant failure.

On examination under a microscope, these failing transplanted kidneys display scarring. Our own body defence mechanisms play an important role in causing this scarring. On the other hand some patients have very good long term kidney function without scarring.

What is the purpose of the study?

We aim to study the various mechanisms that control this phenomenon and their relative importance. By doing this it may be possible to identify patients who are at a risk of developing scarring or those who may enjoy stable long term function. This will benefit patients by possibly enabling doctors to modify the treatment given after transplantation.

Why have I been chosen?

You have been chosen because you had kidney transplantation and being followed up in the Leeds transplant clinic. The process described above may or may not be active in your case, but we intend to study these mechanisms in patients who are:

1. Waiting on the transplant waiting list
2. Newly transplanted

3. Patients who already have a transplant and who need to undergo a transplant kidney biopsy for the usual clinical reasons

What will I have to do?

You will be requested to **donate 30mls of blood** and **provide 100 mls of urine** in a container which will be provided to you when we see you in the clinic.

We will then study your blood and urine in the laboratory to analyse and understand the various mechanisms contributing to the scarring of the transplant.

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

Your interest in participation will be established by Dr. Seitz, Research Registrar or the Chief Investigator, Dr Richard Baker, who will then discuss the study further with you. If you agree, he or one of the medical team will obtain your written consent and plan the day on which blood sample will be taken. You will receive a copy of this information sheet and a copy of the consent to keep.

What are the benefits?

Even though no immediate benefits are seen, the long term aim would be to study the various mechanisms that cause scarring of the transplant. In the future we hope that doctors will be able to modify treatment after transplantation aiming to achieve better long term transplantation function.

What are the risks?

The risks are very small. The amount of blood taken 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 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 decide 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 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 journals and presented at scientific meetings.

Who is organising and funding the research?

The study is funded by the Yorkshire Kidney Research Fund and the Chief investigator is Dr. Richard Baker, Consultant Nephrologist at St. James's University Hospital. Dr. Adrienne Seitz, Research Registrar in Nephrology and Transplantation is a co-investigator.

Further contact Information

If you want further information about the study, you can contact the principal investigators of the project.

Chief Investigator:

Dr. Richard Baker,

Renal Unit,
St. James's University Hospital,
Beckett Street,
Leeds- LS97TF
Tel: 0113 2066875

Dr Adrienne Seitz
Renal Unit,
St. James's University Hospital,
Beckett Street,
Leeds- LS97TF.

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

Sister Kay Tobin,
Senior Renal Transplant Research Nurse,
Renal Research Office,
Lincoln Wing,
St. James's University Hospital,
Beckett Street,
Leeds- LS97TF.
Tel: 0113 2064119

10 SUPPLEMENTARY MATERIAL

SOP Index code: SLFOIMTM095 Version 2.0

COMMON DR/DQ ASSOCIATIONS

DQ2	DR7, DR9, DR17
DQ4	DR4, DR8, DR18
DQ5 (1)	DR1, DR10, DR14, DR16, DR103
DQ6 (1)	DR13, DR15
DQ7 (3)	DR4, DR11, DR12
DQ8 (3)	DR4, DR8
DQ9 (3)	DR7, DR9

DR51	DRB5*	DR2, DR1*
DR52	DRB3*	DR3, DR5, DR6
DR53	DRB4*	DR4, DR7, DR9
* RARELY OBSERVED HAPLOTYPE		

Bw4/Bw6 ASSOCIATIONS

Bw4		
A23	B27	B*51:03
A24	B37	B52
A*24:03	B38	B53
A25	B44	B57
A32	B47	B58
B13	B49	B59

Bw6			
B7	B*39:05	B54	B67
B*07:03	B*40:05	B55	B70
B8	B*40:08	B56	B71
B*15:08	B41	B*56:03	B72
B*15:11	B42	B60	B73
B18	B45	B61	B75
B*27:08	B46	B62	B76
B35	B48	B64	B78
B39	B50	B65	B81
			B*82:01

B C ASSOCIATION

Bw4	Bw6	Common Association	Rare Association
	B*07	C*07:02 C*07:04	C*03:02/04 C*05 C*07:01 C*08 C*14 C*15 C*17
B*08:02/08:03	B*08:01	C*07:01 C*07:01	C*03:02/04 C*04 C*05 C*07:02 C*15
B*13		C*06	C*03:02/04 C*05 C*07 C*16:01
	B*18	C*04 C*05 C*07:01 C*12:03, 07:01	C*01 C*02 C*03:02/04 C*06 C*07:04 C*12:04/05
B*27	B*27:08	C*01 C*02	C*03 C*04 C*05 C*06 C*07 C*12 C*15
	B*35	C*04	C*02 C*03 C*06 C*07:02 C*12:03 C*15 C*16:01
B*37		C*06	C*01 C*16:01
B*38		C*12:03	C*04 C*06 C*07:01

	B*39	C*07 C*12:03	C*02 C*06
	B*41	C*07 C*17:01	C*04 C*07:01 C*16:02
	B*42	C*17:01	
B*44	B*44:09	C*04 C*05 C*07:04 C*16:01	C*02 C*03 C*07 C*14 C*15
	B*45	C*06	C*04 C*16:01
	B*46	C*01 C*02	
B*47		C*06	C*15
	B*48	C*08:01/08:03	C*04 C*08:02 C*15
B*49		C*07:01	C*06
	B*50	C*06	C*05
B*51		C*01 C*02 C*14 C*15 C*16:02	C*03 C*04 C*05 C*07 C*12:03 C*16:01

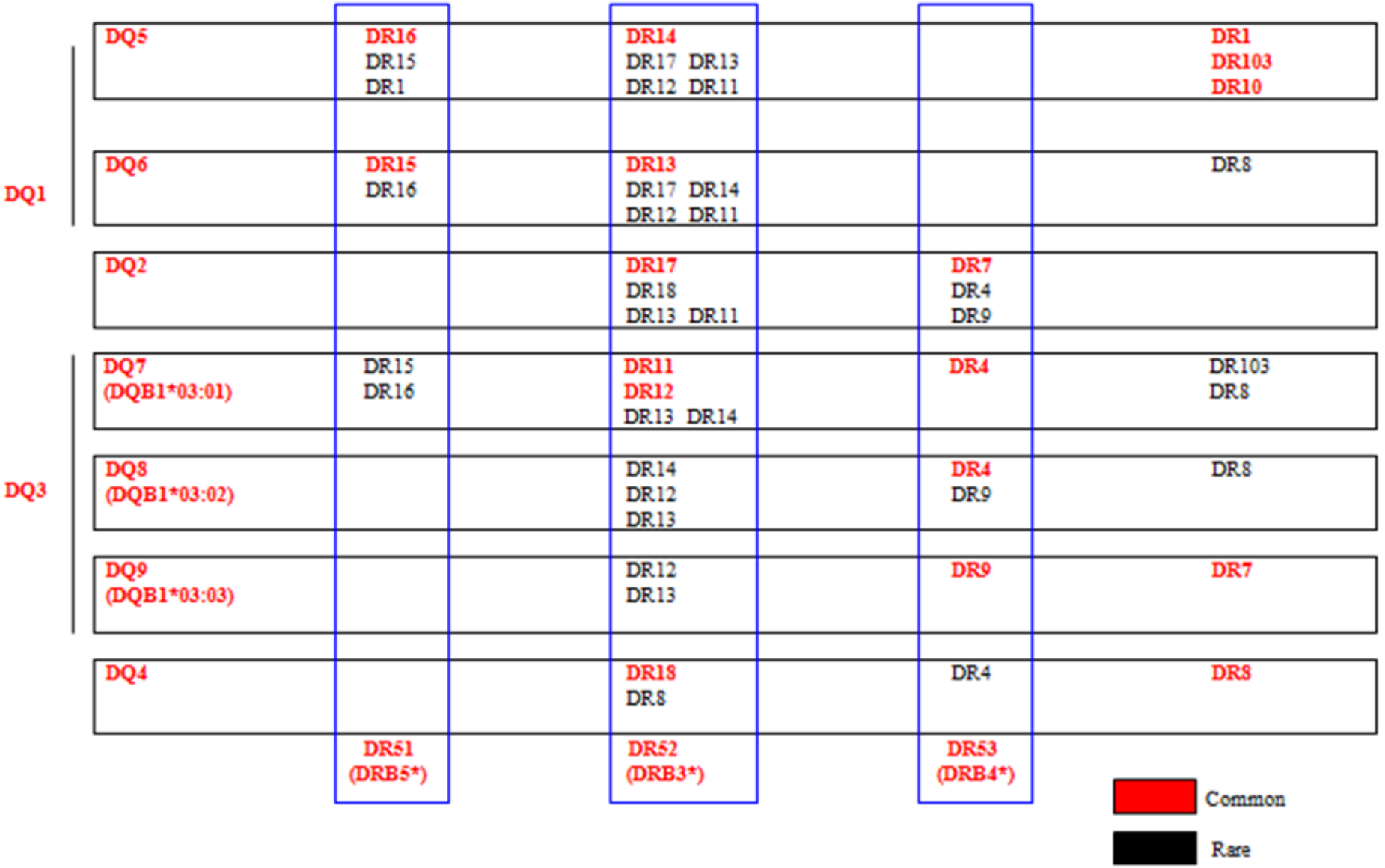
B*52		C*12	C*03:02/04 C*07:01 C*16
B*53		C*04 C*14	C*06 C*07:01

Bw4	**Bw6**	**Common Association**	**Rare Association**
	B*54	C*01	
	B*55	C*03:03	C*01 C*12:03/06
	B*56	C*01	C*15 C*17:01
B*57		C*06	C*02 C*03 C*04 C*07 C*12:03 C*16:01 C*18
B*58		C*02 C*03:02 C*07	C*08:02 C*14 C*16:01
B*59		C*01	
	B*40:01+	C*03:04	C*02 C*03:03 C*04 C*07 C*12:03 C*15

	B*40:02+	C*02 C*08:01/08:03 C*15	C*01 C*12:02
B*15:24	B*15:01	C*03:03 C*03:04	C*01 C*04 C*05 C*07 C*08:01/08:03 C*12:03
B*15:16/15:17		C*07	C*14
	B*14:01	C*08	C*04
	B*14:02	C*08	C*02
	B*67	C*12:03	
	B*15:09	C*02 C*03 C*07:04	C*07:04
	B*15:10/15:18	C*03:02/04 C*07	C*02
	B*15:03/15:46	C*02	C*08 C*12:03
	B*73	C*07 C*15	C*15:05
	B*15:02/08/11	C*01 C*08:01/08:03	C*03:03 C*08:01/08:03

	B*15:12/14/19	C*07:02	
B*15:13		C*08:01/08:03	
	B*78	C*16:01	
	B*81	C*08:04 C*18:01	
	B*82	C*03:02/04	

DR DQ ASSOCIATION


DQ1
DQ3
DQ5
DR16
DR15
DR1
DR14
DR17 DR13
DR12 DR11
DR1
DR103
DR10
DQ6
DR15
DR16
DR13
DR17 DR14
DR12 DR11
DR8
DQ2
DR17
DR18
DR13 DR11
DR7
DR4
DR9
DQ7
(DQB1*03:01)
DR15
DR16
DR11
DR12
DR13 DR14
DR4
DR103
DR8
DQ8
(DQB1*03:02)
DR14
DR12
DR13
DR4
DR9
DR8
DQ9
(DQB1*03:03)
DR12
DR13
DR9
DR7
DQ4
DR18
DR8
DR4
DR8
DR51
(DRB5*)
DR52
(DRB3*)
DR53
(DRB4*)
Common
Rare

DQB1 DQA1 ASSOCIATION

DQB1	DQA1		
	VERY COMMON	INTERMEDIATE	RARE
02:01	05:01 05:03 05:05		
02:02	02:01 03:02 03:03		
03:01	03:02 03:03 04:01 05:01 05:03 05:05 06:01		
03:02	03:01	03:02 03:03	
03:03	03:02 03:03	02:01	
03:04		03:01	

03:05			03
04:01	03:02 03:03		
04:02	04:01	03:01	
05:01	01:01	01:02 01:04	
05:02		01:02 01:04	
05:03	01:04	01:03	
05:04			01:02
06:01	01:03		
06:02	01:02		

06:03	01:03		01:02
06:04	01:02		
06:09	01:02		

DRB1 DQB1 DQA1 HAPLOTYPES

Common							Rare			
DRB1*01:01 DQB1*05:01 DQA1*01:01	DRB1*04:03 DQB1*03:02 DQA1*03:01	DRB1*08:01 DQB1*04:02 DQA1*04:01	DRB1*10:01 DQB1*05:01 DQA1*01:04	DRB1*12:01 DQB1*03:01 DQA1*05:01	DRB1*13:04 DQB1*03:01 DQA1*05:05	DRB1*15:01 DQB1*06:02 DQA1*01:02	DRB1*01:01 DQB1*05:04 DQA1*01:02	DRB1*04:05 DQB1*02:02 DQA1*03:02	DRB1*08:06 DQB1*03:01 DQA1*05:05	DRB1*13:03 DQB1*03:02 DQA1*03
DRB1*01:02 DQB1*05:01 DQA1*01:01	DRB1*04:04 DQB1*03:02 DQA1*03:01	DRB1*08:02 DQB1*04:02 DQA1*04:01	DRB1*11:01 DQB1*03:01 DQA1*05:01	DRB1*12:01 DQB1*03:01 DQA1*05:03	DRB1*14:01 DQB1*05:03 DQA1*01:04	DRB1*15:02 DQB1*06:01 DQA1*01:03	DRB1*03:01 DQB1*06:02 DQA1*01:02	DRB1*04:05 DQB1*02:02 DQA1*03:03	DRB1*08:09 DQB1*03:03 DQA1*03:02	DRB1*13:05 DQB1*03:01 DQA1*05:01
DRB1*01:03 DQB1*03:01 DQA1*05:01	DRB1*04:05 DQB1*04:01 DQA1*03:02	DRB1*08:03 DQB1*06:01 DQA1*01:03	DRB1*11:01 DQB1*03:01 DQA1*05:03	DRB1*12:01 DQB1*03:01 DQA1*05:05	DRB1*14:01 DQB1*05:02 DQA1*01:04	DRB1*15:03 DQB1*06:02 DQA1*01:02	DRB1*03:02 DQB1*02:01 DQA1*05:01	DRB1*04:05 DQB1*05:03 DQA1*01:03	DRB1*08:09 DQB1*03:03 DQA1*03:03	DRB1*13:05 DQB1*03:01 DQA1*05:03
DRB1*01:03 DQB1*03:01 DQA1*05:03	DRB1*04:05 DQB1*04:01 DQA1*03:03	DRB1*08:03 DQB1*03:01 DQA1*06:01	DRB1*11:01 DQB1*03:01 DQA1*05:05	DRB1*12:01 DQB1*05:01 DQA1*01:04	DRB1*14:02 DQB1*03:01 DQA1*05:01	DRB1*16:01 DQB1*05:02 DQA1*01:02	DRB1*03:02 DQB1*02:01 DQA1*05:03	DRB1*04:06 DQB1*04:02 DQA1*03:01	DRB1*11:01 DQB1*02:02 DQA1*03:02	DRB1*13:05 DQB1*03:01 DQA1*05:05

DRB1*01:03 DQB1*03:01 DQA1*05:05	DRB1*04:05 DQB1*03:02 DQA1*03:02	DRB1*08:04 DQB1*03:01 DQA1*04:01	DRB1*11:01 DQB1*06:02 DQA1*01:02	DRB1*12:02 DQB1*03:01 DQA1*06:01	DRB1*14:02 DQB1*03:01 DQA1*05:03	DRB1*16:02 DQB1*03:01 DQA1*05:01	DRB1*03:02 DQB1*02:01 DQA1*05:05	DRB1*04:09 DQB1*03:01 DQA1*03:02	DRB1*11:01 DQB1*02:02 DQA1*03:03	DRB1*14:01 DQB1*05:01 DQA1*01:04
DRB1*01:03 DQB1*05:01 DQA1*01:01	DRB1*04:05 DQB1*03:02 DQA1*03:03	DRB1*08:04 DQB1*03:01 DQA1*05:01	DRB1*11:02 DQB1*03:01 DQA1*05:01	DRB1*13:01 DQB1*06:03 DQA1*01:03	DRB1*14:02 DQB1*03:01 DQA1*05:05	DRB1*16:02 DQB1*03:01 DQA1*05:03	DRB1*04:01 DQB1*03:02 DQA1*03:02	DRB1*04:09 DQB1*03:01 DQA1*03:03	DRB1*11:01 DQB1*05:02 DQA1*01:02	DRB1*14:01 DQB1*06:02 DQA1*01:02
DRB1*03:01 DQB1*02:01 DQA1*05:01	DRB1*04:06 DQB1*03:02 DQA1*03:01	DRB1*08:04 DQB1*03:01 DQA1*05:03	DRB1*11:02 DQB1*03:01 DQA1*05:03	DRB1*13:02 DQB1*06:04 DQA1*01:02	DRB1*14:03 DQB1*03:01 DQA1*05:01	DRB1*16:02 DQB1*03:01 DQA1*05:05	DRB1*04:01 DQB1*03:02 DQA1*03:03	DRB1*04:11 DQB1*04:02 DQA1*03:01	DRB1*11:02 DQB1*02:02 DQA1*03:02	DRB1*14:02 DQB1*03:02 DQA1*03:01
DRB1*03:01 DQB1*02:01 DQA1*05:03	DRB1*04:07 DQB1*03:02 DQA1*03:01	DRB1*08:04 DQB1*03:01 DQA1*05:05	DRB1*11:02 DQB1*03:01 DQA1*05:05	DRB1*13:02 DQB1*06:09 DQA1*01:02	DRB1*14:03 DQB1*03:01 DQA1*05:03	DRB1*16:02 DQB1*05:02 DQA1*01:02	DRB1*04:03 DQB1*03:04 DQA1*03:01	DRB1*07:01 DQB1*02:02 DQA1*03:02	DRB1*11:02 DQB1*02:02 DQA1*03:03	DRB1*14:04 DQB1*05:02 DQA1*01:04
DRB1*03:01 DQB1*02:01 DQA1*05:05	DRB1*04:07 DQB1*03:01 DQA1*03:02	DRB1*08:04 DQB1*04:02 DQA1*04:01	DRB1*11:03 DQB1*03:01 DQA1*05:01	DRB1*13:03 DQB1*03:01 DQA1*05:01	DRB1*14:03 DQB1*03:01 DQA1*05:05		DRB1*04:03 DQB1*03:05 DQA1*03	DRB1*07:01 DQB1*02:02 DQA1*03:03	DRB1*11:04 DQB1*06:03 DQA1*01:03	DRB1*15:01 DQB1*06:03 DQA1*01:02

DRB1*03:02 DQB1*04:02 DQA1*04:01	DRB1*04:07 DQB1*03:01 DQA1*03:03	DRB1*08:06 DQB1*06:02 DQA1*01:02	DRB1*11:03 DQB1*03:01 DQA1*05:03	DRB1*13:03 DQB1*03:01 DQA1*05:03	DRB1*14:04 DQB1*05:03 DQA1*01:04		DRB1*04:03 DQB1*03:04 DQA1*03:01	DRB1*08:01 DQB1*03:02 DQA1*03:01	DRB1*12:02 DQB1*03:03 DQA1*03:02	DRB1*15:03 DQB1*02:02 DQA1*03:02
DRB1*04:01 DQB1*03:01 DQA1*03:02	DRB1*04:08 DQB1*03:01 DQA1*03:02	DRB1*09:01 DQB1*02:02 DQA1*03:02	DRB1*11:03 DQB1*03:01 DQA1*05:05	DRB1*13:03 DQB1*03:01 DQA1*05:05	DRB1*14:05 DQB1*05:03 DQA1*01:04		DRB1*04:04 DQB1*04:02 DQA1*03:01	DRB1*08:02 DQB1*03:02 DQA1*03:01	DRB1*12:02 DQB1*03:03 DQA1*03:03	DRB1*15:03 DQB1*02:02 DQA1*03:03
DRB1*04:01 DQB1*03:01 DQA1*03:03	DRB1*04:08 DQB1*03:01 DQA1*03:03	DRB1*09:01 DQB1*02:02 DQA1*03:03	DRB1*11:04 DQB1*03:01 DQA1*05:01	DRB1*13:03 DQB1*02:02 DQA1*02:01	DRB1*14:06 DQB1*03:01 DQA1*05:01		DRB1*04:05 DQB1*03:01 DQA1*05:01	DRB1*08:04 DQB1*06:02 DQA1*01:02	DRB1*13:01 DQB1*03:03 DQA1*03:02	DRB1*15:03 DQB1*05:01 DQA1*01:02
DRB1*04:01 DQB1*03:02 DQA1*03:01	DRB1*07:01 DQB1*02:02 DQA1*02:01	DRB1*09:01 DQB1*03:03 DQA1*03:02	DRB1*11:04 DQB1*03:01 DQA1*05:03	DRB1*13:04 DQB1*03:01 DQA1*05:01	DRB1*14:06 DQB1*03:01 DQA1*05:03		DRB1*04:05 DQB1*03:01 DQA1*05:03	DRB1*08:06 DQB1*03:01 DQA1*05:01	DRB1*13:01 DQB1*03:03 DQA1*03:03	
DRB1*04:02 DQB1*03:02 DQA1*03:01	DRB1*07:01 DQB1*03:03 DQA1*02:01	DRB1*09:01 DQB1*03:03 DQA1*03:03	DRB1*11:04 DQB1*03:01 DQA1*05:05	DRB1*13:04 DQB1*03:01 DQA1*05:03	DRB1*14:06 DQB1*03:01 DQA1*05:05		DRB1*04:05 DQB1*03:01 DQA1*05:05	DRB1*08:06 DQB1*03:01 DQA1*05:03	DRB1*13:02 DQB1*05:01 DQA1*01:02	

HLA A ALLELES AND ANTIGENS		
DNA	**Split**	**Broad**
A*01	A1	-
A*02	A2	-
A*03	A3	-
A*23	A23	A9
A*24	A24	
A*25	A25	A10
A*26	A26	
A*34	A34	
A*66	A66	

HLA B ALLELES AND ANTIGENS					
DNA	**Split**	**Broad**	**DNA**	**Split**	**Broad**
B*07	B7	-	B*41	B41	-
B*08	B8	-	B*42	B42	-
B*13	B13	-	B*44	B44	B12
B*14:01	B64	B14	B*45	B45	
B*14:02	B65		B*46	B46	-
B*15:01	B62	B15	B*47	B47	-
B*15:02	B75		B*48	B48	-
B*15:03	B72	B70	B*49	B49	B21
B*15:09	B70	-	B*50	B50	

HLA C ALLELES AND ANTIGENS		
DNA	**Split**	**Broad**
C*01	Cw1	-
C*02	Cw2	-
C*03:02	Cw10	Cw3
C*03:04		
C*03:03	Cw9	
C*04	Cw4	-
C*05	Cw5	-
C*06	Cw6	-
C*07	Cw7	-

A*11	A11	-
A*29	A29	A19
A*30	A30	
A*31	A31	
A*32	A32	
A*33	A33	
A*74	A74	
A*68	A68	A28
A*69	A69	
A*36	A36	-
A*43	A43	-

B*15:10	B71	B70	B*50:02	B45	B12
B*15:12	B76	B15	B*51	B51	B5
B*15:13	B77		B*52	B52	
B*15:14	B76		B*53	B53	-
B*15:16	B63		B*54	B54	B22
B*15:17	B63		B*55	B55	
B*15:18	B71	B70	B*56	B56	
B*15:19	B76	B15	B*57	B57	B17
B*18	B18	-	B*58	B58	
B*27	B27	-	B*59	B59	-
B*35	B35	-	B*67	B67	-

C*08	Cw8	-
C*12	-	-
C*14	-	-
C*15	-	-
C*16	-	-
C*17	-	-
C*18	-	-

A*80	A80	-

B*37	B37	-	B*73	B73	-
B*38	B38	B16	B*78	B78	-
B*39	B39		B*81	B81	-
B*40:01	B60	B40	B*82	-	-
B*40:02	B61		B*83	-	-

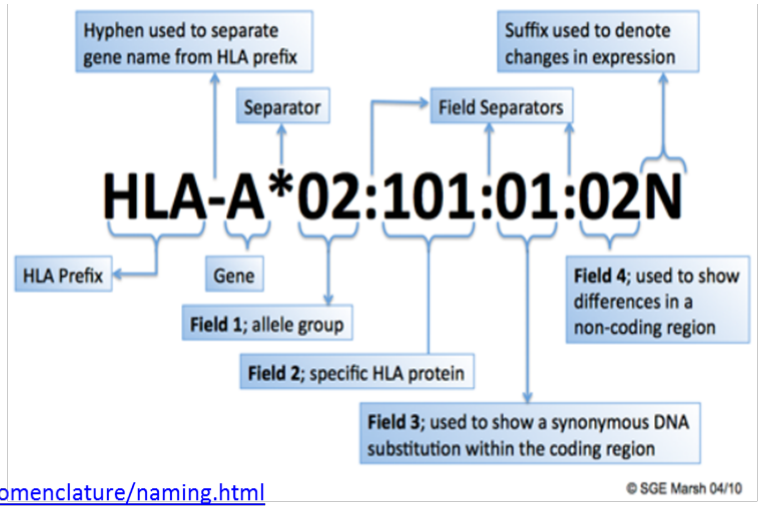
HLA DR ALLELES AND ANTIGENS		
DNA	**Split**	**Broad**
DRB1*01	DR1	-
DRB1*01:03	DR103	-
DRB1*03:01	DR17	DR3
DRB1*03:02	DR18	
DRB1*03:03	DR18	
DRB1*03:04	DR17	
DRB1*04	DR4	-
DRB1*07	DR7	-
DRB1*08	DR8	-

HLA DQ ALLELES AND ANTIGENS		
DNA	**Split**	**Broad**
DQB1*02	DQ2	-
DQB1*03:01	DQ7	DQ3
DQB1*03:02	DQ8	
DQB1*03:03	DQ9	
DQB1*03:04	DQ7	
DQB1*03:05	DQ8	
DQB*04	DQ4	-
DQB1*05	DQ5	DQ1
DQB1*06	DQ6	

DRB1*09	DR9	-
DRB1*10	DR10	-
DRB1*11	DR11	DR5
DRB1*12	DR12	
DRB1*13	DR13	DR6
DRB1*14	DR14	
DRB1*14:15	DR8	-
DRB1*15	DR15	DR2
DRB1*16	DR16	

DRB3*	-	DR52

DRB4*	-	DR53
DRB5*	-	DR51



From: http://hla.alleles.org/nomenclature/naming.html

Nomenclature	Indicates
HLA	the HLA region and prefix for an HLA gene
HLA-DRB1	a particular HLA locus i.e. DRB1
*HLA-DRB1*13*	a group of alleles that encode the DR13 antigen or sequence homology to other *DRB1*13* alleles
*HLA-DRB1*13:01*	a specific HLA allele
*HLA-DRB1*13:01:02*	an allele that differs by a synonymous mutation from *DRB1*13:01:01*
*HLA-DRB1*13:01:01:02*	an allele which contains a mutation outside the coding region from *DRB1*13:01:01:01*
*HLA-A*24:09N*	a 'Null' allele - an allele that is not expressed
*HLA-A*30:14L*	an allele encoding a protein with significantly reduced or 'Low' cell surface expression
*HLA-A*24:02:01:02L*	an allele encoding a protein with significantly reduced or 'Low' cell surface expression, where the mutation is found outside the coding region
*HLA-B*44:02:01:02S*	an allele encoding a protein which is expressed as a 'Secreted' molecule only
*HLA-A*32:11Q*	an allele which has a mutation that has previously been shown to have a significant effect on cell surface expression, but where this has not been confirmed and its expression remains 'Questionable'

HLA NOMENCLATURE

RISK HAPLOTYPES FOR "COMMON" NULL ALLELES

HLA-Ready Gene reaction (all multiplex)	Possible null allele	Haplotype	Deceased donor report to NHSBT	Frequency of null allele in local population Based on local audit results: R. McCurtin 2011
A lane 22 small (100bp)	**A*24:09N**	A*24; B*40:01+ OR A*24; B*27	Complete in HLA allele field In 'Comments' section: "Null allele A*24:09N present"	0% (out of 20 people with risk haplotype)
B lane 14 small (185bp)	**B*51:11N**	A*02; B*51; C*15; DRB1*04	Complete in HLA allele field. In 'Comments' section: "Null allele B*51:11N present"	5% (out of 20 people with risk haplotype)

C lane 17 Large (190bp)	**C*04:09N**	B*44:03+; C*04	Complete in HLA allele field. In 'Comments' section: "Null allele C*04:09N present"	15% (out of 20 people with risk haplotype)
DR lane 23 small & large (130bp & 215bp)	**DRB4*01:03:01:02N**	DRB1*07; DQB1*0303+	Leave DR53 field blank. Complete DRB3/4/5 allele fields#	N/A Common
DR lane 24 small & large (180bp & 265bp)	**DRB5*01:08N**	DRB1*15:02+	Leave DR51 field blank. Complete DRB3/4/5 allele fields#	0% (out of 20 people with risk haplotype)

 # Allele fields for expressed DRB3/4/5 (if present) must also be completed to prevent NHSBT validation error

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