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**Investigations into the Outcomes of HLA Antibody
Incompatible Renal Transplantation and the Influence of
Immune Memory.**

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A thesis submitted to the University of London
for the degree of Doctor of Philosophy

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Abstract

Antibodies specific for donor HLA molecules are seen as a contraindication to renal transplantation. The presence of these antibodies suggests a patient has previously been exposed to non-self HLA, with resulting immune response and memory generation. Immune memory allows for a rapid response upon re-exposure, manifesting as rapid acute rejection. Antibody removal pre-transplant facilitates transplantation across HLA antibody incompatibilities, however there is no rationale as to which patients will benefit from this treatment. I have developed a testing procedure to identify which patients are suitable for antibody removal and the amount required to allow transplantation from a specified donor.

I hypothesised that patients producing HLA specific antibody to previous mismatches will have both cellular and humoral memory to those antigens, and therefore be at greater risk of rejection and graft failure, compared to patients with HLA specific antibody but no memory of the mismatches with which they are presented. By observing outcomes I have investigated the effect that a repeat HLA mismatch with antibody, and therefore presumed memory, has on outcome, with patients in this category having reduced graft survival at 5 years and a shorter time to rejection.

Whilst HLA specific antibody is relatively simple to define in the laboratory, donor specific memory T cells are not. I have also developed a novel assay to identify donor specific memory T cells pre-transplant, through their production of IL-17 on stimulation. This predicted T cell mediated rejection with 100% accuracy in the patient group tested. However the presence of memory T cells did not correlate with repeat mismatches or the presence of HLA specific antibody.

The conclusions are that repeat mismatches influence humoral memory but are not obviously indicative of T cell memory, which cannot in this assay system be predicted based on the presence of a repeat mismatch or HLA specific antibody.

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Also, to my partner Tony, and the whole of my family, for their never ending support and constant cheering on, even when I didn't seem to be getting very far.

This is for my daughter, Rose, who I hope will one day be proud, and my Dad, I'm sorry you didn't get to see me finish this, but I hope you knew I would.

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Abbreviations

AAMR	Acute antibody mediated rejection
Ab	Antibody
Ag	Antigen
ALG	Anti-lymphocyte globulin
AlloAb	Alloantibody
AlloAg	Alloantigen
AMR	Antibody mediated rejection
APC	Antigen presenting cell
ATG	Anti-thymocyte globulin
AutoAb	Auto reactive antibody
AZA	Azathioprine
BCR	B cell receptor
BPPE	Biopsy proven rejection episode
CDC	Complement dependant cytotoxicity
CDR	complementarity determining region
CIT	cold ischaemic time
CNI	calcineurin inhibitor
cRF	calculated reaction frequency
CTL	cytotoxic T lymphocyte

DFPP	double filtration plasmapheresis
DNA	deoxyribonucleic acid
DSA	donor specific antibody
DTT	dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
ELISPOT	Enzyme-linked immunospot assay
ESRD	End stage renal disease
FITC	Fluorescein isothiocyanate
FXM	Flowcytometric crossmatch
HLA	Human Leucocyte antigen
HLAi	HLA antibody incompatible
IA	Immunoadsorption
IFN γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IVIg	Intravenous immunoglobulin
mAb	monoclonal antibody
MdX	median channel value
MFI	median fluorescence intensity
MHC	major histocompatibility complex

MLR	Mixed lymphocyte reaction
MMF	Mycophenolate mofetil
ODT	Organ donation and transplant
PBL	Peripheral blood lymphocyte
PBMC	peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PEX	Plasma exchange
PRA	Panel reactive antibody
PTC	Peritubular capillary
RMF	Relative median fluorescence
SPA	Solid phase assay
TAC	tacrolimus
TCMR	T cell mediated rejection
TCR	T cell receptor
Th	Helper T cell
TNF α	Tumour necrosis factor alpha
XM	crossmatch

1 Introduction

1.1 Renal Transplantation

End stage renal disease (ESRD) is the failure of the kidneys to effectively filter waste from the blood and produce biochemically appropriate urine. It is defined by the Kidney Disease Quality and Outcomes Initiative guidelines as a reduction in the estimated glomerular filtration rate (GFR) to $\leq 15\text{ml per minute per } 1.73\text{m}^2$. ESRD develops due to damage to the kidneys caused by various conditions and diseases which may be acquired or congenital, including diabetes mellitus, chronic hypertension, renal tumours, polycystic kidney disease, various autoimmune diseases and infections. If untreated renal failure will ultimately prove fatal. Treatment in the form of dialysis can be effective in the medium to long term. However, it is time consuming for the patients, expensive for the health care system and does not provide a cure. Additionally there is a significant level of morbidity and mortality associated with long term dialysis, with the average life expectancy in the UK on dialysis being 4 – 20 years depending on the age at which dialysis commenced and the underlying disease causing renal failure [1].

Transplantation of a functioning kidney from either a living or deceased donor is currently the only “cure” for ESRD, allowing the patient a relatively normal quality of life. Over the past century renal transplantation has become a common, accepted and viable treatment option, with the half-life of a kidney transplanted from a living donor being up to 15.3 years, and 11.9 years when the organ is from a deceased donor [2].

1.2 History of Renal Transplantation

In the modern era initial interest in kidney transplantation began at the beginning of the 20th century driven by advances in surgical skills, particularly in vascular surgical techniques [3].

The first successful experimental organ transplant was reported in 1902 by Ullmann, in which he performed an autograft in a dog, moving the kidney from its native position to the neck.

The organ remained somewhat functional with demonstrated urine output. Ullmann followed

this up by performing dog to goat and then pig to man, all showed short term function but ultimate failure [3]. In 1907 Jaboulay attempted xenotransplantation into two patients suffering renal failure, one from a goat and another from a pig, both failed by day 3 [3].

Continued developments in surgical technique, particularly suturing, allowed further work on organ transplants, with a number of auto- and then allo-grafts being reported by Jaboulay and then Carrel [4]. Whilst no allografts functioned for long the physical ability to perform the transplants had been demonstrated. Interest in organ transplantation then waned as further attempts in both animals and then humans all resulted in failure. A lack of knowledge and understanding of the process of rejection made success unlikely.

The first human kidney allograft was carried out in the Ukraine by Voronoy in 1936 [4]. This was blood group incompatible (B into O) and the kidney never functioned, leading to the death of the patient 2 days later. Voronoy continued to perform human kidney allografts, carrying out a total of 6 between 1936 and 1949, without success.

By the early 1950s interest in renal transplantation had been revived and it had become apparent to investigators that immunological mechanisms were involved. Simonsen in Denmark and Dempster in London both studied graft rejection and suggested that humoral and cellular mechanisms were likely [5-8]. In 1954 Joseph Murray and J. Hartwell Harrison performed the 1st successful kidney transplant in humans between identical twins [4]. The kidney functioned well despite a lack of immunosuppression. Current knowledge tells us this is due to the donor and recipient being genetically identical [9]. Further attempts between genetically disparate individuals however resulted in failure due to rejection [9].

In the 1950s the knowledge that rejection was an immune mediated mechanism led to various efforts at immunosuppression with varied success, initially total body irradiation and bone marrow transplantation, which had high rates of associated mortality [10, 11]. In the late 1950s the immunosuppressive effect of 6-mercaptopurine was discovered and its ability to prolong the survival of renal transplants in dogs reported [12]. This was closely followed in

1960 by the development of the immunosuppressive agent azathioprine, and the use of this, together with prednisolone, became a standard regimen following reports of success from Starzl et. al. [13].

In parallel with the improvements in surgical technique and drug treatment the understanding of the immune system involvement had been growing with the identification of first the mouse, and then the human, major histocompatibility complex (MHC), in humans the Human Leucocyte Antigen (HLA) system. The MHC was first identified in mice following a series of experiments by George Snell in the 1930s using inbreeding to achieve pure strains in which each individual mouse is genetically identical except at one locus. Using transplantation of tumours between strains the genetic system involved in the acceptance or rejection of a tumour graft was identified [14]. Initially called the H locus, this became the H-2 locus following the identification of Antigen II, by Peter Gorer. Initially believed to be involved in the blood group antigen system, Antigen II was finally found to be part of the H system identified by Snell. The H-2 system is now known to be the mouse MHC, found on chromosome 17.

Identification of the human MHC followed, aided both by the knowledge gained from the early attempts at kidney allograft transplants plus that from skin grafting of burn victims during the second world war. Peter Medawar led the Medical Research Council war wounds committee investigation into the use of skin transplantation and concluded that rejection is a systemic process, at the time believed to be 'governed by at least 7 antigens that are freely combined' [14]. He also found that transplant immunity could be adoptively transferred from one sensitised patient to another by lymphocytes but not serum.

The 1st human leucocyte antigen, "mac", was described by Dausset in 1958. Identification of further antigens followed and in 1965 the first international Histocompatibility and Immunogenetics workshop was held, where a standardised nomenclature for these antigens was described. The first full report was published in 1968 for the 1st 8 antigens identified. At

this time it was thought that there was only 1 locus for HLA (HL-A), however by 1975 it was realised that HL-A was the product of multiple loci and these became HLA-A and HLA-B.

Techniques in identifying an individual's HLA antigens, known as tissue typing, became routine from 1962 allowing some form of matching between donor and recipient. Additionally crossmatching between donor cells and recipient serum to identify those pairs in which hyperacute rejection may occur was introduced routinely from 1966 [15, 16].

From this point on kidney transplantation for patients in end stage renal failure became a more viable option. Refinements in HLA typing and crossmatching, plus in organ retrieval, continued and in 1976 the immunosuppressive drug cyclosporine was introduced, accompanied by a great improvement in graft survival rates [9].

In more recent years our knowledge of the immune system involvement has grown exponentially. Typing to a high resolution at multiple HLA loci to allow a greater level of matching has become routine, along with the use of highly sensitive methods for detection and identification of HLA specific antibodies.

1.3 The Immune System

The primary role of the immune system as a whole is to protect an individual from infection.

Immunity and immune responses can generally be divided into two groups, the innate response and the acquired or adaptive response. Innate immunity is the first line of defence.

It is comprised of physical barriers such as the skin and mucosal surfaces along with some immune cells such as macrophages, neutrophils and NK cells [17]. Innate immunity provides rapid, non-specific protection against bacteria, fungi and parasites, relying on a combination of secreted anti-microbial proteins and invariant receptors to recognise invading microorganisms [18].

The acquired immune response is far more specific than the innate, and its activation results in the generation of memory to a particular pathogen, allowing for a rapid and specific response

upon re-exposure [19]. The central effector cells to the adaptive immune response are the T and B cells.

T cells recognise foreign antigen in the form of peptides derived from pathogens presented in the context of a self MHC molecule [20], whereas B cells recognise small antigenic areas on larger intact molecules via an immunoglobulin cell surface receptor [21].

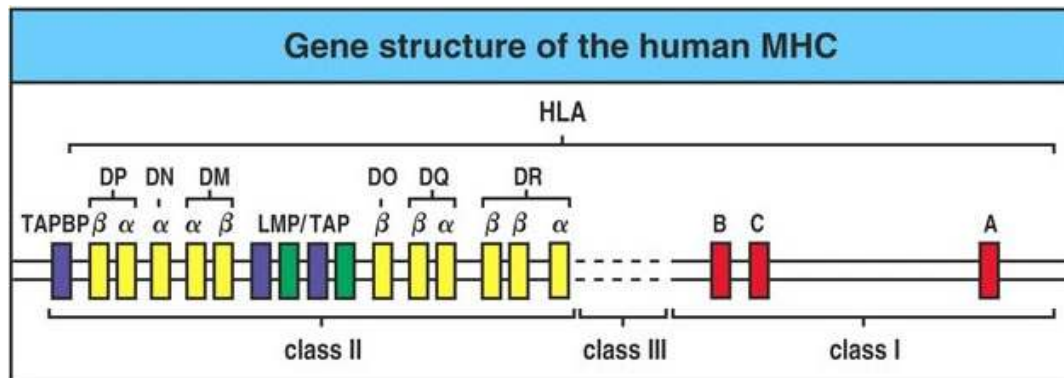
Whilst they are seen as separate systems there is in fact much cross talk between the innate and adaptive arms, for example the production of cytokines is required for T and B cell activation but can also be used to recruit cells such as the macrophages to sites of infection [22].

In order to avoid the development of auto immune disease, an important aspect of a functional immune system is to be able to discriminate between self and non-self [19]. In organ transplantation however the transplanted tissues are seen as non-self due to differences in the cell surface molecules, principally the MHC but also the ABO blood group antigens, minor histocompatibility antigens and the endothelial cell antigens, all of which have been reported to elicit an immune response directed at the transplanted tissue [17].

1.4 Structure and Function of the MHC

The major histocompatibility complex (MHC) is a highly polymorphic group of over 200 genes, which in humans is located on the short arm of chromosome 6. Many of the functions attributed to the genes in this complex are associated with the immune system and an individuals' ability to combat disease. The MHC is divided into three regions, or classes, Class I, Class II and Class III, represented in figure 1 below, the gene products of which differ in their structure, function and tissue distribution [23].

Figure 1 - Representation of the Genetic Structure of the Human MHC region on Chromosome 6 [18].

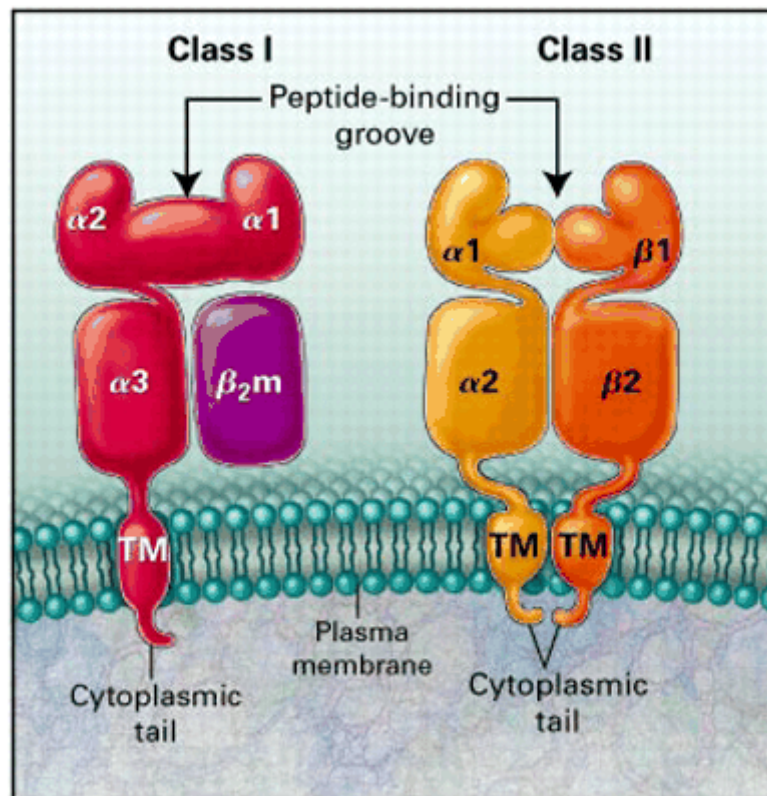


In humans the MHC class I and II genes encode the cell surface expressed glycoproteins, HLA. HLA molecules are central to the adaptive immune system as they enable the discrimination between healthy and diseased cells, which is achieved through the presentation of peptides by the HLA molecules to T cells [25]. The Class I and Class II molecules differ in their structure, patterns of expression and function. The classical Class I products, HLA A, B and C, are expressed at varying densities on the surface of most nucleated cells in the body and present cytoplasmic and virally derived peptides to CD8+ cytotoxic T cells. The Class II products, HLA DR, DQ and DP, are generally expressed only on professional antigen presenting cells (APC), such as dendritic cells, macrophages, B cells, monocytes, Langerhans cells, dendritic cells, in addition to activated T cells, endothelial and epithelial cells [26] and present externally derived peptide which has been internalised and processed by the cell prior to presentation to CD4+ helper T cells [19]. Interaction between a T cell receptor (TCR) and a complementary HLA molecule can, when accompanied by the appropriate co-stimulatory signals, initiate an immune response against the presenting cell or against the source of the peptide being presented. Of note, in regards to renal transplantation, expression of HLA Class II molecules can be induced on the surface of renal tubular and glomerular epithelial cells, particularly at times of stress to the organ, as seen during transplantation and in renal disease [27, 28].

The MHC Class I complex is comprised of genes encoding the classical HLA-A, -B and -C which are most commonly described, in addition to the non-classical HLA E – F which have more limited variability and tissue distribution [17]. Each gene encodes a 45-kd transmembrane

Class I α chain, or heavy chain, which is associated with a 12-kd invariant β 2 microglobulin or light chain, encoded on chromosome 15, plus peptide, prior to expression on the cell surface [23]. The Class I α chain is folded into 3 domains, α 1, α 2 and α 3 and then associated with the β 2 microglobulin chain prior to expression, as represented in figure 2 below. The majority of the variation between Class I molecules is found in the α 1 and α 2 regions encoded by exons 2 and 3, whereas the α 3 region, encoded by exon 4, is more conserved [25]. The α 1 and α 2 regions fold to form the peptide binding groove, and it is this area which dictates the range of peptides that can be bound and presented to the cells of the immune system by the HLA molecule. The variation in amino acids in the α 1 and α 2 regions are at positions that can alter the peptide binding site, meaning that different HLA molecules are able to present a different range of peptides to the immune system. The α 3 region is the part of the molecule involved in both β 2 microglobulin association and interaction with the CD8 molecule on the cytotoxic T cells and is believed to be more conserved to maintain these functions [29].

Figure 2 - Structure of the HLA Class I and II molecules [30].



The MHC Class II complex is comprised of 3 main regions, HLA-DR, -DQ and -DP. Each Class II molecule is made up of 2 polypeptide chains, α and β , both of which are encoded within the Class II region. Other than the DR α chain, which has only 2 alleles, each of these loci are polymorphic, giving rise to even greater variation in the Class II gene products. Each chain has 2 domains and here the $\alpha 1$ and $\beta 1$ regions, encoded by exon 2, create the peptide binding groove, seen in figure 2 above. Again it is the variation in each of these that dictates the range of peptides that can be bound and presented.

An individual will inherit a single set of MHC genes, known as a haplotype, from each parent, meaning a complete MHC region will include 2 of each HLA gene. These are then co-dominantly expressed so an individual will have the ability to express 2 each of the HLA-A, -B, -C, -DR, -DQ and -DP molecules, known as heterozygote [31]. In some cases an individual will inherit the same gene from each parent at one or more loci, so for example, only 1 type of HLA-A molecule will be expressed, known as homozygote. Throughout evolution it is believed

that heterozygotes have a survival advantage as the possession of multiple HLA alleles increased the chances of expressing an HLA molecule capable of presenting a suitable pathogen derived peptide to the T cells to fight the infection [25].

The genes encoding the HLA molecules are highly polymorphic, by April 2013 there were 7089 Class I and 2065 Class II HLA alleles registered on the IMGT/HLA database [32]. It is believed that such polymorphism has developed within the population to ensure that there will be at least some individuals whose HLA molecules are able to present suitable pathogen derived peptides to the appropriate antigen specific T cells to survive the disease and maintain continuation of the species [31]. However it is this polymorphism which creates some of the major difficulties presented in organ transplantation.

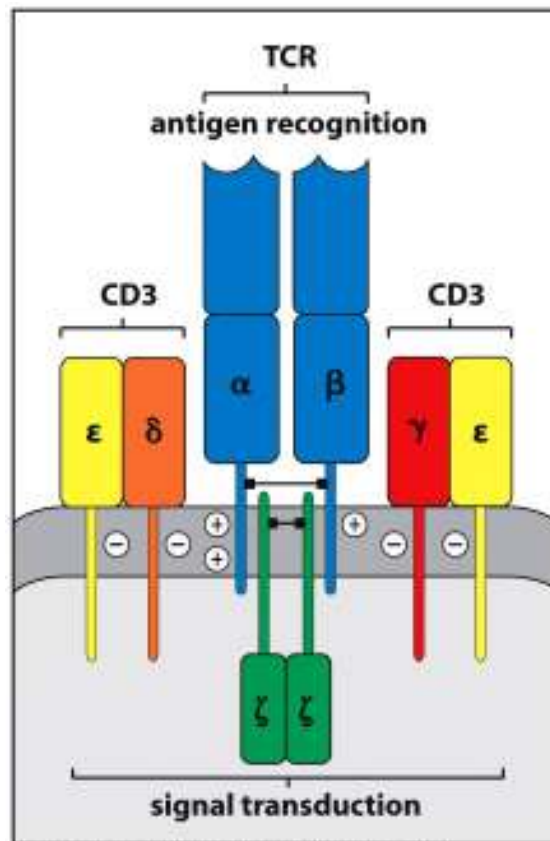
1.5 Development and Education of Lymphocytes

Lymphocytes are cells central to the adaptive immune system, and can be broadly split into T cells and B cells. They both develop from undifferentiated precursors from the bone marrow and follow ordered and controlled stages of development marked by various gene rearrangement and proliferation phases. They require specific signals from specialised microenvironments, namely the bone marrow for B cells and the thymus for T cells. In these microenvironments they encounter specialised cells which provide signals and growth factors to encourage or cease development.

1.5.1 T cells.

Mature T cells express a complex known as the T cell receptor (TCR) on their surface. It is this which recognises and interacts with a specific antigen in the context of an MHC molecule. Each T cell in circulation has a subtly different version of the TCR allowing interactions with different antigens, giving them a different specificity. This great diversity allows the immune system to recognise many different potential pathogens.

The TCR is made up of a collection of polypeptides, the central pair of which are most commonly the α and β chain, and more rarely the γ and δ chains, combined with accessory chains to form a heterodimer with one antigen binding site, represented in Figure 3 below.

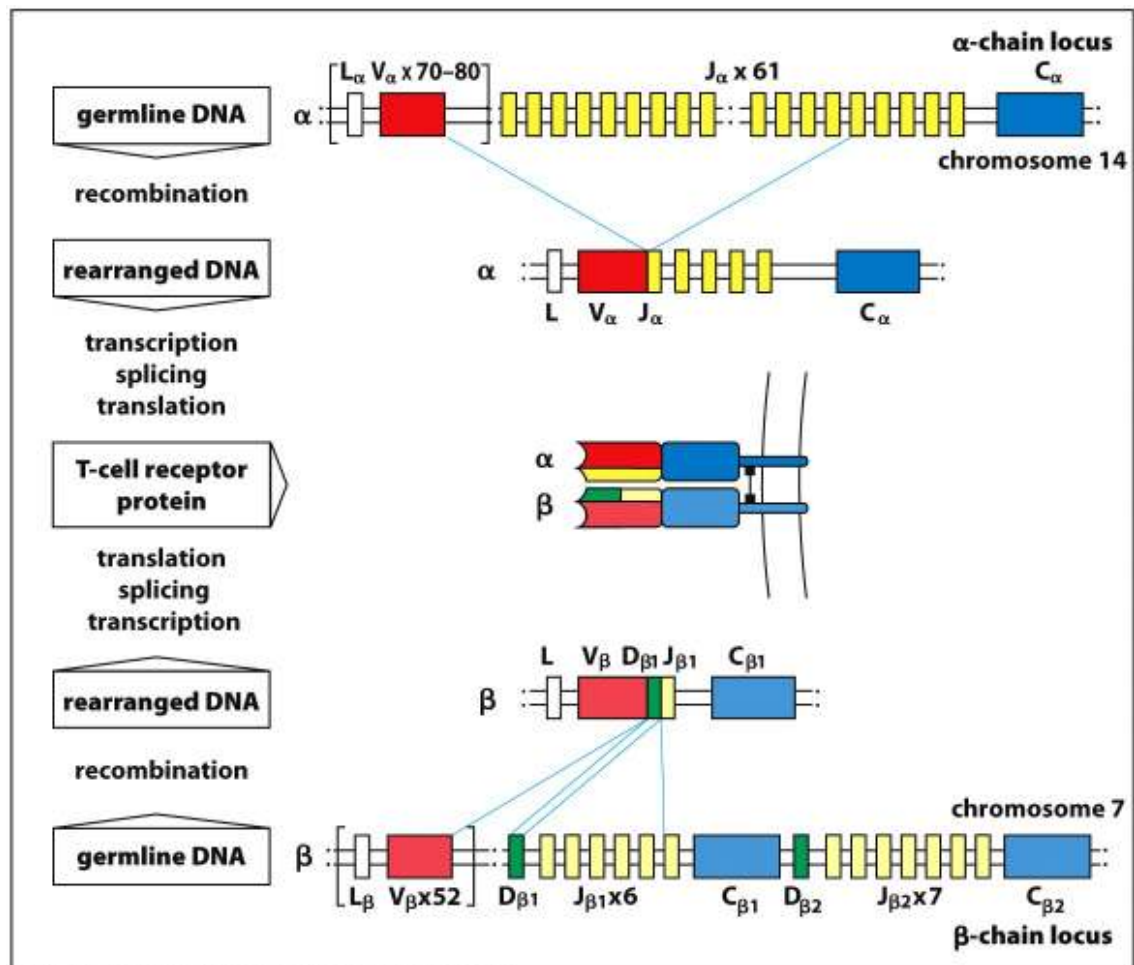


All T cell precursors start with the same collection of gene segments, which, during maturation in the thymus, randomly rearrange and combine to build a complete gene to encode the variable regions of each chain, represented in Figure 4 below. The gene segments for the β chain are from three groups – V, D and J, and one segment from each group is taken to make up a complete and unique VDJ gene to encode the variable region of the β chain, which is then combined with one of two constant (C) β chain genes to produce a complete β chain gene.

The multiple gene segments available allow for hundreds of different VDJ β chain combinations.

The α chain variable region genes contain just V and J gene segments and one α chain constant gene, however the variation it is possible to generate is immense.

Figure 4 – The Sequence of Gene rearrangement in the T cell Receptor [33].



The rearrangement of TCR germline DNA sequences and the pairing of the α and β TCR products create a theoretical repertoire diversity of approximately 10^{15} different T cells in humans [34].

Within the variable domains of these chains there are three hypervariable regions, known as the complementarity determining regions (CDR) 1 – 3, the main diversity being concentrated in CDR3, which is at the centre of the antigen binding site and interacts with the peptide being presented in the groove of the corresponding MHC molecule. The CDR1 and 2 regions are less

variable and make contact with the two α helices forming the peptide binding groove on the MHC molecule.

In order to prevent reactivity to self, or autoimmunity, the development and 'education' of T cells must ensure that they are able to recognise the bodies many different tissues as self and not attack unless instructed to do so, for example in malignancy, but still maintain the ability to recognise and eliminate foreign pathogens [35].

In order to prevent potentially deleterious self-reactive T cells being released into the periphery, those which have created T cell receptors that recognise self peptides with high affinity, must be recognised and removed. There are two selection processes in the thymus, positive and negative. Positive selection selects for TCR gene rearrangements which can recognise self MHC:self peptide complexes, and therefore are capable of functioning in self restricted responses. If the TCR has no affinity for self MHC then they will receive signals to die by apoptosis. However T cells that respond with high affinity to complexes of self peptide and self MHC class I or II molecules are also eliminated in a process called negative selection. Approximately 98% of the T cells that develop will die in the thymus by apoptosis through positive and negative selection [34].

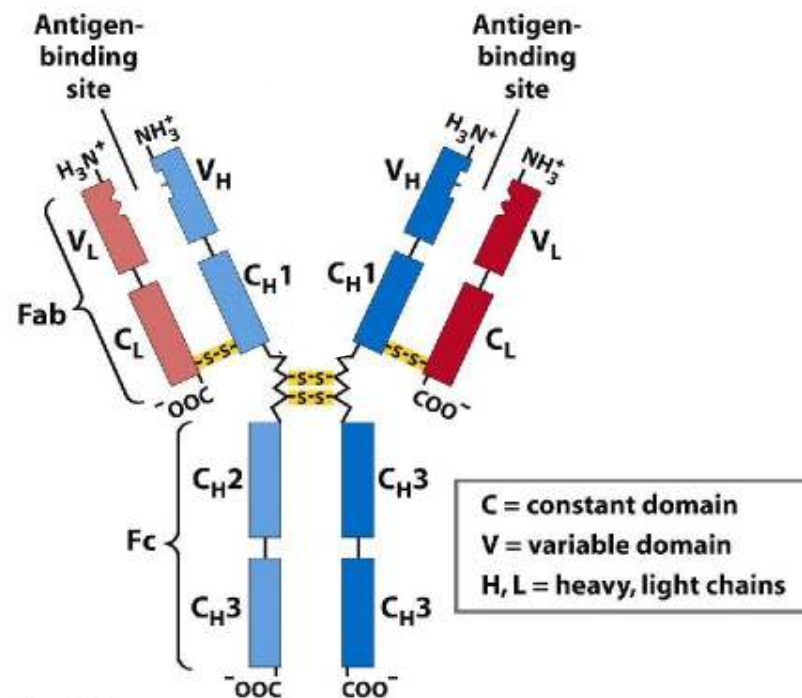
During their development in the thymus the T cells will also receive signals to differentiate into different effector cells, namely the CD4⁺ T helper cells and the CD8⁺ Cytotoxic T cells, which will recognise peptide presented in MHC class II or class I molecules respectively. The CD4/CD8 molecules along with a CD3 complex form the complete functional TCR complex required to be ligated for signal transduction and subsequent cellular activation.

1.5.2 B Cells

B cells also develop from precursors produced in the bone marrow, which is where their initial maturation continues. These precursors require marrow stromal cells to develop, which retain the cells in the bone marrow and provide developmental signals.

B cells express an antigen receptor analogous to the TCR, known as the B cell receptor complex (BCR). It is composed of a surface immunoglobulin (sIg) as the antigen receptor, coupled with a signal transduction complex. The sIg molecule is composed of four chains, 2 heavy and 2 light, each with variable and constant regions, which form 2 identical antigen binding sites, shown in Figure 5 below.

Figure 5 - Diagram of the Structure of an Immunoglobulin molecule [36].

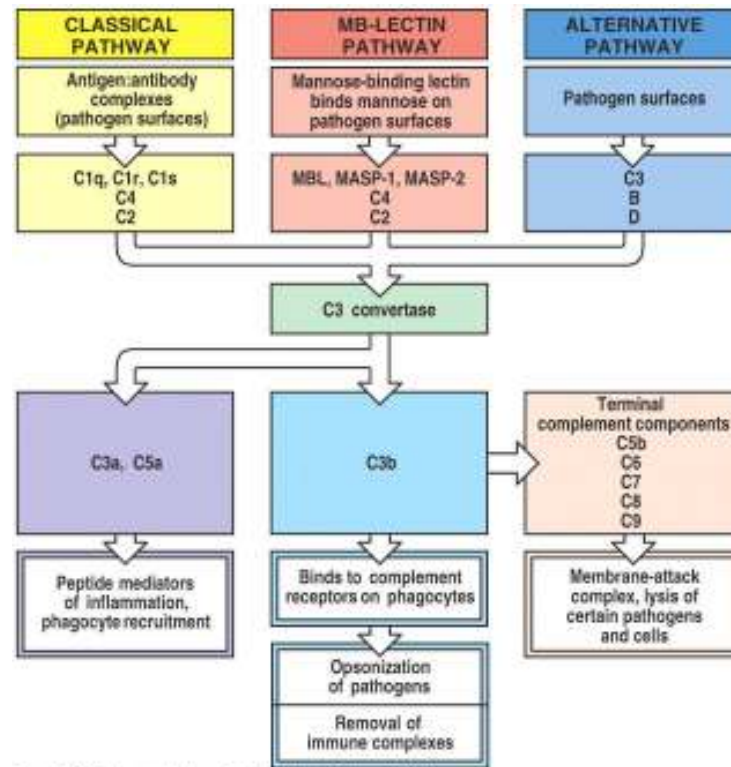


Like the TCR, the BCR is highly variable and generated through successive gene rearrangements. The heavy chains again develop from groups of gene segments, V, D and J, which combine to form a functional variable region gene, which is combined with a constant region gene to allow production of a complete heavy chain. The light chain variable regions are encoded by gene segments from two groups, v and J, which are also rearranged and combine with a constant light chain gene. Once a complete and functional sIg molecule is expressed self-reactive clones are again removed by positive and negative selection before the cells are released into the periphery to undergo further maturation. Naïve B cells, those that have yet to meet their cognate antigen, recirculate through lymphoid tissue.

1.6 Antibody

One of the main roles of a B cell in the acquired immune response is to produce antibody to a given pathogen in order to fight an infection, humoral immunity particularly protects against extracellular pathogens [37]. The antibody is a secreted form of the sIg presented in the BCR. Depending on the activation signals received, and subsequent gene rearrangements that occur, the secreted antibody can have one of a number of structures and functions. They may bind to the pathogen, preventing it from entering the target cell, these are known as neutralising antibodies. They may bind to the pathogen and facilitate its uptake and destruction by phagocytic cells, known as opsonisation. The antibody may bind to the pathogen and activate the classical complement cascade, leading to the destruction of the target cell. The complement cascade is a step wise series of proteolytic cleavage reactions whereby inactive complement components in the circulation are cleaved and become active, which then go on to cleave the next factor in the cascade. For the classical complement pathway, triggered by antibody, there are nine active components, C1 – C9, with the final C5-C9 forming the membrane attack complex, which is inserted into the membrane of the target cell causing it to lyse. The cleavage of each component leads to the production of various fragments, some of which play other roles in the immune response. For example, C5a has inflammatory and chemotactic properties, recruiting further phagocytes to the site of activation. C3b can bind to the target and act as an opsonin, promoting the uptake and removal of the antigen target by phagocytes. See figure 6 below.

Figure 6 – Diagram presenting The complement cascade [18].



Initially the antibody produced in a humoral response will be of the IgM class, which has a pentameric structure, with 5 IgM molecules joined by 5 J chains. This is the first antibody type seen in an antibody response and, whilst it's a potent activator of the complement cascade, it is often of low affinity for the target antigen.

The most abundant antibody class found in the serum is IgG. Unlike IgM, IgG is a monomer, binding as a single IgG molecule. Within the IgG class there are 4 subclasses, IgG1 – 4, defined by the functional (Fc) portion of the heavy chain and with varying effector functions. IgG1 and 3 are potent activators of complement, whereas IgG2 and 4 have limited to no ability to do this. IgG can bind to, and activate other immune cells, such as the monocytes, neutrophils and NK cells via the heavy chain γ receptors Fc γ RI, II and III.

IgA is the main Ig molecule secreted on mucosal surfaces, for example in the gut. It can be found as a monomer when in circulation, but requires dimeric formation to be secreted. As with IgM, an IgA dimer is formed by the joining of two monomers with a single J-chain.

Binding of the J-chain to the polymeric immunoglobulin receptor (pIgR) expressed on epithelial

cells allows the antibody to be transported to the luminal surface of the epithelium. The antibody is then released by cleavage of the pIgR, although part of the receptor remains bound to the IgA dimer to become the 'secretory component', with the final molecule comprising the complete secretory IgA [18]. Again IgA can fix complement and interact with immune cells such as the neutrophils.

IgE is the main antibody class used to fight parasitic infections and seen in allergy, where it binds to and activates mast cells.

A fifth class of antibody is IgD. IgD is coexpressed with IgM on the surface of all naïve, and most mature, B cells. Its expression ceases once the B cell has been activated and very little, if any, is secreted at any time. The function of IgD is as yet unclear [18].

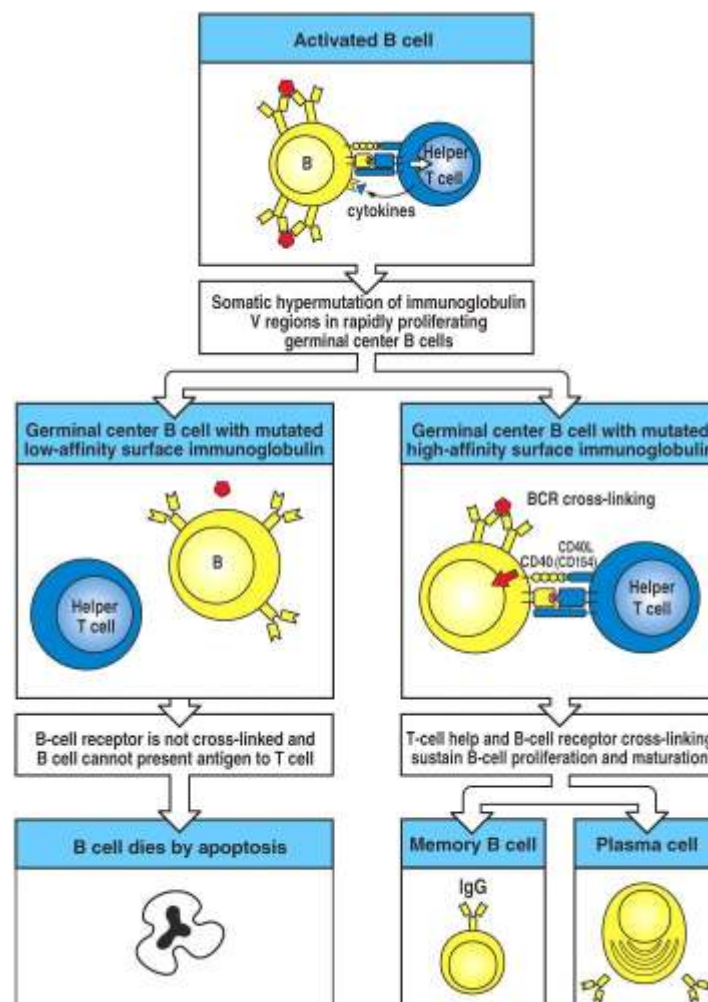
1.6.1 Production of Antibody

Antibodies are a major effector in the adaptive immune response. When a B cell encounters and binds to its cognate antigen via the BCR in the secondary lymphoid tissue, lymph node or spleen, the entire Ag/BCR complex is internalised and the antigen broken down, processed, into multiple short peptides. These peptides are then loaded into the MCH class II molecules and presented on the B cell surface. If the B cell then encounters an activated CD4⁺ T helper (Th) cell which is expressing a TCR cognate to the peptide:MHC on its surface, the T cell will provide signals for the B cell to become activated, in a process represented in figure 7 below.

Some of these B cells will develop into short lived plasma cells, secreting low affinity IgM antibody. Others undergo rapid division and, when interacting with the follicular dendritic cells and Th cells, begin to form a germinal centre. During this rapid phase of division the cells begin the process of somatic hypermutation and affinity maturation, during which further gene alterations ensue, generally involving subtly changing the variable regions by point mutations, deletions or insertions of single nucleotides, and thus also changing the specificity of the antibody. Often these changes will lead to an antibody of lower, or no, affinity for the target antigen, and these cells will receive apoptosis signals. However if the new antibody

product is of higher affinity the cells will receive survival signals from the follicular dendritic cells presenting the antigen. These surviving cells also receive signals from the appropriate Th cells to differentiate and undergo isotype switching, whereby the functional heavy chain gene is changed from IgM class to either IgG, IgA or IgE, dependant on the signals received and the type of pathogen encountered. These surviving B cells then follow one of a number of possible routes. They may re-enter the process of proliferation, mutation and selection or develop into an antibody secreting plasma cell or a memory B cell [38].

Figure 7 - Diagram demonstrating the requirement for T cell help in the activation of B cells and generation of an antibody response [18].



The plasma cells migrate to specialised survival niches in the bone marrow where they continue to produce circulating antibody. These plasma cells are terminally differentiated, unable to divide and secrete antibody continuously even in the absence of antigen.

Controversy exists as to the life span of these cells. The fact that antibody can persist and be detected in the plasma long after an infection has been resolved, and in the case of vaccination for example, for the life time of the individual, indicates that long term production of antibody by plasma cells is somehow facilitated. There are currently three competing concepts. The first is that the plasma cells may be short lived, generated from memory B cells in a process driven by persistent antigen. The second is there is continuous generation of long lived plasma cells with a defined half-life, again derived from memory B cells in the circulation. The third is that the plasma cells generated have unconditional survival due to the environment provided in the bone marrow niches and are independent of the memory B cell pool [38]. The number of survival niches is finite which limits the number of long lived plasma cells [39]. Plasma cells are rare and make up only 0.5% of all bone marrow cells and 0.05% of the peripheral blood cells [38].

The memory B cells re-enter circulation where they can provide a rapid response if re-challenged with the same antigen. These memory B cells are not terminally differentiated and do not continuously secrete antibody. If these cells re-encounter their cognate antigen they can become rapidly reactivated, some of which will then become plasma cells secreting antibody and others of which will form another germinal centre and restart the affinity maturation process.

The response seen when first encountering an antigen is known as the primary response, and tends to peak at 5 – 10 days following exposure, however when re challenged with the same pathogen, even years later, a secondary, or second set, response will ensue via the memory cells, allowing a rapid response peaking at 3 – 5 days.

Once an antibody response has been stimulated and plasma cells generated it is often possible to continue to be able to detect a specific antibody for the lifetime of the individual. However this is not always the case, particularly when the antigenic stimulus has been removed and years have passed. In these cases whilst no antibody can be detected in circulation there will

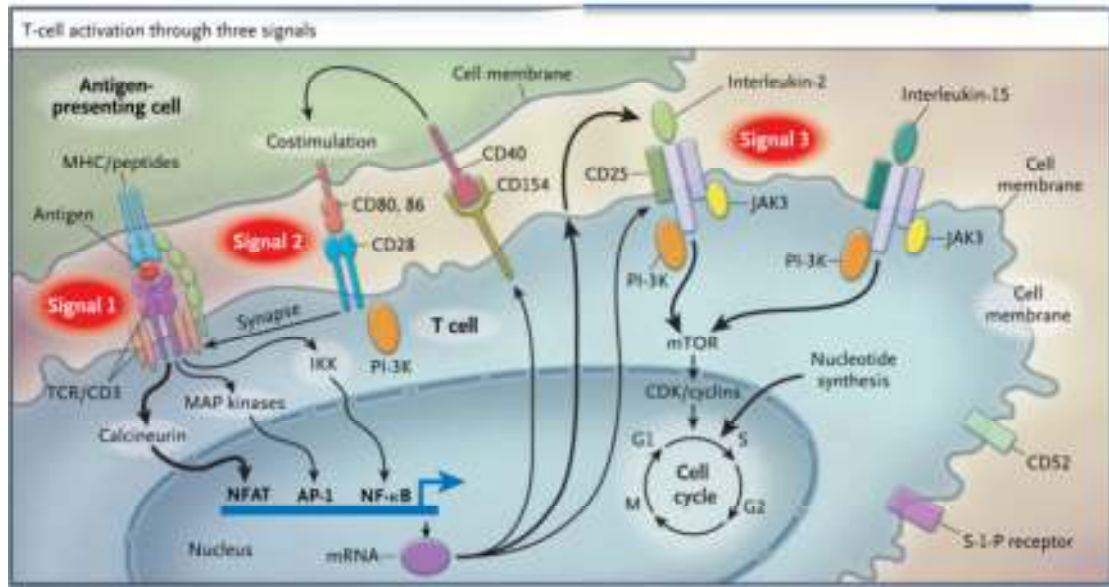
still be a pool of memory cells, B and T, primed to respond if the individual is re-challenged with the same antigen. Subsequent challenges with the same antigen can result in an anamnestic response, where antibody production increases very rapidly to eliminate the antigen. Memory B cells can become activated within hours of re-exposure to an antigen and differentiate into plasma blasts and then plasma cells secreting antibody, it is not yet clear if this activation requires some or any T cell help in the same way as it is required in the initial response [38].

1.7 Generation of an acquired immune response.

Generation of a response requires the interaction of multiple cell types under the correct conditions and results in various effector mechanisms to fight infection.

The first step in generating a response is the uptake of the invading pathogen e.g. bacteria or viral particle, by specialised antigen presenting cells (APC), macrophage, dendritic cell or B cell, which transport it to the lymph node via the lymphatic system. During transport the antigen is processed and broken down into shorted peptides which are then presented in an MHC class II molecule on the surface of the cell, in addition to this the cell also starts to express high levels of costimulatory and adhesion molecules. Once in the lymph node the APC moves into the T cell rich areas where it becomes surrounded by CD4⁺ Th cells. If a Th cell has a TCR complementary to the peptide:MHC CII complex being presented by the APC and receives adequate costimulatory signals, such as those received via ligation of CD28/CD80/86 and CD40/CD40L, it becomes activated, represented in figure 8 below. The T cells then begin a process of maturation, differentiation and proliferation driven by various signals and cytokines. Activated Th cells may then encounter their cognate B cell in the lymphoid tissue providing signals to start an antibody response. T cell help via an activated Th cell is also required for activation of CD8⁺ cytotoxic T cells. If a T cell encounters its cognate antigen in the absence of costimulation it will be rendered anergic and unable to respond. Activation of a naïve T cell will result in the formation of both effector and memory populations.

Figure 8 - The three signal pathway to T cell activation [40].



1.8 Allorecognition and the alloresponse.

As discussed earlier the main problem encountered in the early years of transplantation was that of general immediate rejection of the foreign organ by the recipient. The first long term success was achieved by transplanting a kidney between genetically identical twins. We now know that this was successful due to the fact that the recipient's immune system did not recognise the new kidney as foreign as they shared the same genes [9].

It is vitally important within an organism that the immune system can recognise self in order to prevent attack against self tissues and autoimmune disease. In order to maintain this integrity self-compatible tissues are recognised as such by the expression of the histocompatibility antigens [17]. Transplantation involves the introduction of non-self tissues to an individual which express the major and minor histocompatibility antigens encoded by the donor genes. If the graft comes from a genetically identical donor, e.g. an identical twin, it is called syngeneic, and will not elicit a significant immune response as it expresses the same antigens as self. However, if the transplant is between individuals who are genetically disparate, allogeneic, then under normal circumstances in an immunocompetent individual without immunosuppression, an adaptive immune response to the foreign tissue will ensue, leading to the destruction and rejection of the transplanted tissues [41]. This reaction against non-self is

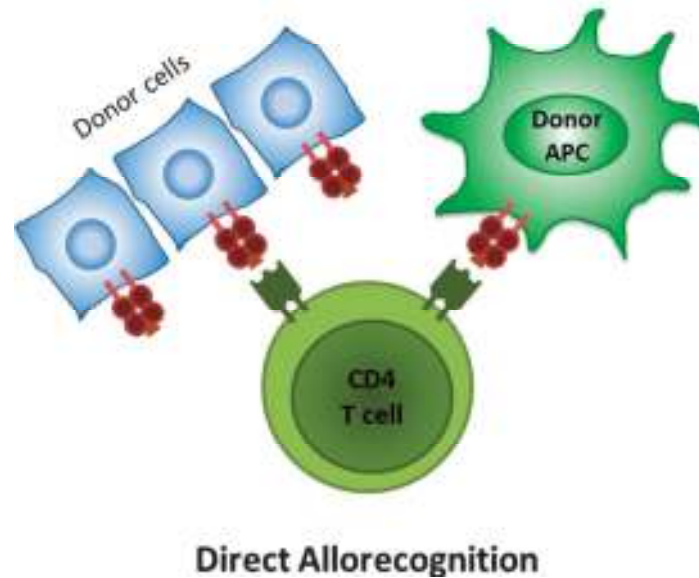
known as the alloresponse and the targets to which the response is raised are called the alloantigens (alloAg), recognised in the process of allorecognition. In man there are many potential alloAg, however the ones which elicit the strongest immune response are the HLA Class I and II molecules, encoded by the MHC [17].

T cells play a pivotal role in allorecognition [41]. There are three pathways of allorecognition described – direct, indirect and semi-direct [41].

In the direct pathway allospecific T cells are able to recognise the donor MHC molecules as foreign directly. It is hypothesised that the allograft, when transplanted, brings with it passenger donor APCs [42] which traffic to the secondary lymphoid tissue of the recipient and initiate direct responses [41]. The donor APCs provide the costimulatory signals required to activate the T cells. Lafferty et al. [43] were able to demonstrate that cultured thyroid tissue has a prolonged survival after transplantation due to the loss of passenger APCs.

Unlike a normal response, where foreign antigen must be processed and the presented in the groove of a self HLA on a self APC before a T cell can be activated, the donor APCs are directly presenting intact foreign HLA molecules to the recipient T cells, represented in figure 9 below, which explains the immediate nature of the direct pathway activation [35]. It is believed that the direct pathway dominates the immediate post-transplant period reducing over time as the donor derived APC are depleted [44].

Figure 9 - Model of Direct Allorecognition [45].



Direct Allospecific T cells are found in uniquely high numbers in the circulation of most individuals, with an estimated 10% of the recipient T cells being able to react directly with donor alloAg [34]. Compared to a more normal level of 1 in 10^5 - 10^6 of T cells being able to react to any other given antigen [46]. In an immediate post-transplant draining lymph node analysis it was demonstrated that >90% of the allospecific T cells were of the direct pathway. The high number of cells capable of reacting to foreign HLA molecules directly accounts for the strength of reaction seen [44]. The reason for this high level of alloreactivity is due to the high level of inherent cross reactivity of the TCR [44].

Two models have been proposed to account for the high frequency of direct alloreactive T cells –The high determinant density and the multiple binary complex models.

High determinant density suggests that alloreactive TCR can directly recognise amino acid differences on the allo HLA molecule regardless of the peptide it is presenting. Therefore every HLA molecule on the surface of a foreign cell could act as a ligand for the allospecific T cells. This leads to a high frequency of ligands for potential allospecific T cells, meaning that T

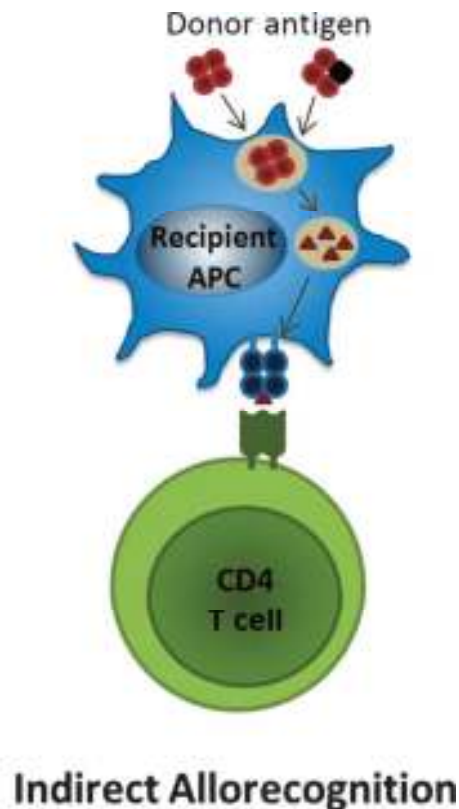
cells expressing TCR of lower affinity are able to respond due to a high density of targets expressed on the cell [44].

The multiple binary complex model suggests that it is the peptide bound in the groove of the alloHLA molecule that is of greatest importance. Differences in the HLA molecules leads to a different set of peptides being presented compared to those presented by self, so each peptide-allo HLA complex is recognised by a different alloreactive T cell and only one mismatch can stimulate a large number of diverse T cells [44].

It is thought that both mechanisms probably contribute to direct allorecognition [44].

The second pathway of allorecognition follows a more conventional route of antigen uptake, processing and presentation in a self MHC class II restricted manner. This is the indirect pathway. In this pathway the alloAg, in the form of foreign HLA molecules shed from the graft, are processed as any exogenous antigen would be by host APCs and then the resulting peptides presented to the host T cells in the context of self HLA molecules, see figure 10 below. The indirect pathway takes longer to become apparent due to the time taken to process and present the peptide. However, unlike in the direct pathway, where donor APCs and therefore the response, are thought to diminish with time, the alloAg will continue to be shed for the lifetime of the graft and therefore continually stimulate an immune response. The indirect response is dominated by the CD4⁺ Th cells [35]. There are fewer allospecific Th cells with indirect specificity compared to those with direct [41]. As the T cell help for B cells to become activated requires interaction of a B cell presenting peptide:self MHC Class II to an appropriate activated Th cell, alloAb can only be produced via the indirect pathway [47]. So the presence of class switched alloantibody (AlloAb) is indicative of help provided by indirect pathway T cells [41].

Figure 10 - Model of Indirect Allorecognition [45].



The third pathway of allorecognition described is that of semi-direct allorecognition. It is suggested that intact surface donor MHC:peptide complexes are acquired by recipient APC via cell-to-cell contact or in an exosomal manner. Recipient APC can then present intact donor HLA CI and II molecules to both CD4+ and CD8+ T cells in addition to the processed peptides in self HLA molecules. This could lead to crosslinking of a CD4+ Th cell from the indirect pathway with a CD8+ cytotoxic T cell recognising the intact donor HLA CI molecule in a direct manner. However there is no direct evidence of an *in vivo* role for this pathway in allorecognition and rejection [44].

1.9 Alloantibody and its Production.

In an immune response to a typical pathogen, production of antibody specific for that pathogen by activated B cells would be expected. This is also the case in the allo immune response. In much the same way as other antibody production, the B cells specific for the allo Ag are activated by helper T cells specific for the same antigen, in a T cell dependent process which begins when an individual is exposed to non-self HLA molecules [37].

The area of recognition by an immune cell on an antigen is called the epitope, and whilst a T cell and a B cell may recognise and react to the same antigen, the epitope that they recognise is thought to be different. A T cell epitope is in the form of a linear peptide created from the intracellular processing of the pathogenic protein presented in the context of a Class I or Class II HLA molecule on the surface of the cell, for CD8+ and CD4+ T cells respectively. The determinant recognised by the antibody is a conformational or structural epitope, which is found on the 3D structure of the antigen, often involving amino acids from different points on the linear protein which have been brought together by peptide folding to produce the epitope. The epitope is generally a small area on the surface of a larger molecule.

HLA molecules are large proteins which often possess many antigenic determinants, or epitopes, on their surface. Some of these epitopes will be limited to a single HLA molecule, private epitopes, whereas others will be found on the surface of multiple HLA molecules, public epitopes [48]. If an antibody response is raised to a mismatched epitope on the surface of a foreign HLA molecule and the epitope is shared by a number of HLA molecules, antibody raised to this HLA molecule will also be found to react with all the other HLA molecules which express the same epitope. These are known as cross reactive groups or CREGs [49], therefore a single HLA mismatch may lead to the production of antibody specific for multiple HLA antigens. In order to generate HLA specific antibody the activated and responding B cell must receive signals from corresponding helper T cells which recognise and respond to peptide presented in the context of self MHC Class II. In the context of the alloresponse the peptide is generated through internalisation and processing of the non-self HLA molecule and will therefore activate the CD4+ helper T cells via the indirect allorecognition pathway. As such, the presence of HLA specific antibodies is indicative of a response via the indirect allorecognition pathway [44], however depending on the epitope to which the antibody was raised and the cross reactive nature of HLA specific antibodies, presence of an antibody to a particular HLA antigen does not necessarily indicate that the T cell help and therefore the T cell memory was raised to that particular HLA antigen.

1.10 Rejection of Solid Organs following Transplantation.

In a typical immunocompetent healthy individual transplantation of a solid organ from a non-genetically identical donor will result in rejection [46]. Only a few amino acid differences between the donor and recipient HLA molecules can be sufficient to cause rejection [46]. The speed and pathology of the rejection varies depending on the sensitisation status of the patient pre transplant and the effector mechanisms involved in the response. The types of rejection can broadly be divided into humoral, brought about by antibody responses, and cellular, where predominantly T cells will bring about the damage and destruction to the graft. In many cases however a mixed picture of both cellular and humoral mechanisms will be observed. They are also divided on the basis of the speed of the rejection response, with four main categories – Hyperacute, accelerated acute, acute and chronic.

1.10.1 Antibody Mediated Rejection

The presence of preformed antibody directed towards donor HLA molecules have long been known to be a definite contraindication to transplantation. Kissmeyer-Nielsen et al.[50] in 1966 reported hyperacute rejection of kidney allografts in association with pre-existing humoral antibodies against donor cells. Patel and Terasaki [16] reported in 1969 that in a group of 30 patients transplanted in the presence of antibodies cytotoxic to donor lymphocytes, 24, or 80%, lost their kidneys to hyperacute rejection. In addition to hyperacute rejection, HLA specific antibodies have also been associated with both acute and chronic rejection [51].

Hyperacute rejection of a graft is rejection or loss which occurs within 24 hours of transplantation, although often it will be within minutes of reanastomosis of the organ [52]. It is virtually always irreversible and occurs when an organ is transplanted in the presence of high levels of donor specific antibody, specific for either the HLA mismatches presented by the donor or due to blood group incompatibilities between donor and recipient to which the recipient has antibody e.g. a blood group A kidney being transplanted into a blood group O

recipient. At the time of reperfusion the kidney fails to “pink up” and becomes flaccid, mottled and anuric [46]. Antibody becomes deposited on the vascular endothelium where it activates the classical complement cascade, resulting in endothelial necrosis, platelet deposition and initiation of the coagulation cascade [53]. There is not usually a cellular element to this type of rejection, with biopsy often showing no mononuclear cell infiltrate [54]. Hyperacute rejection is now a very rare occurrence due to improvements in HLA specific antibody detection and pre transplant crossmatching [51] in addition to blood group matching.

Acute antibody mediated rejection (AAMR) usually occurs within days to weeks post-transplant [46], although may occur years post-transplant, often coinciding with a decrease in immunosuppression [53]. 20-30% of all acute rejection episodes will include an element of antibody mediated damage [52]. AAMR can be divided into accelerated acute rejection, generally occurring within the first two to four days post-transplant, or acute rejection, characterised by a sudden onset of graft dysfunction within the first few weeks post-transplant [52]. Accelerated AAMR is predominantly due to ABO or HLA specific antibody produced rapidly post-transplant in an anamnestic response due to the presence of immune memory to the HLA mismatches presented by the graft, in a classic ‘second set’ response [46]. In these cases the antibody prior to transplant is of low level and may not be detected by the antibody screening methods used, or in the case of HLA antibody incompatible transplantation, the antibody has been removed to a level deemed safe to transplant. Grafts rejected in this way appear similar to those rejected hyperacutely, being enlarged and soft with numerous foci of haemorrhage. There may be evidence of both humoral and cellular elements, although the cellular component does not generally predominate.

Acute AMR is characterised by a sudden loss of function, due to inflammation [46], with patients presenting with a sudden rise in serum creatinine, fluid retention and possible fever and graft tenderness [55]. Patients are often sensitised pre-transplant, although the risk of AAMR is also increased due to non-compliance with immunosuppressive medication in

previously unsensitised patients, where de novo antibody production can be responsible [55]. Common histological features include fibrinoid necrosis, microthrombi, acute tubular injury, neutrophil and macrophage infiltration [53], in addition C4d deposition on the peritubular capillaries, indicating complement activation, can often be seen on immunofluorescence staining [56]. In patients where de novo antibody production is responsible, especially in patients who are non-compliant, the histopathology is reported to be different to that in acute AMR in sensitised recipients, with a greater mix of humoral and cellular mechanisms [57, 58].

This form of rejection can be reversed if treated early following initiation of the response. However, it remains undesirable as damage sustained by the graft during the rejection episode can affect the long term function of the graft, and the comorbidity that is associated with the increased immunosuppression required to treat the rejection episode [59].

Chronic AMR presents as slow, progressive loss of graft function over months to years [53]. In recent years evidence has been presented that donor specific antibody plays an important role in chronic rejection, with circulating HLA specific antibody to Class I and/or Class II mismatches being found in a large proportion of renal allograft recipients and associated with late graft loss [60, 61]. Both de novo donor specific and non-donor specific antibody are commonly detected in patients diagnosed with chronic AMR [52]. Einecke et al.[62] attributed 63% of late graft losses seen in their study of 27 patients to chronic AMR. El-Zoghby et al.[63] found 20% of the 153 renal transplants studied were lost to acute or chronic AMR. In a study of patients transplanted with a positive crossmatch, Bentall et al. [64] found over 90% of the biopsies taken at 5 years showed histological evidence of chronic AMR.

There has been a concerted effort over the past 30 years to standardise the diagnosis of renal allograft rejection on biopsy through the generation of the Banff classification system [65] which aims to provide histopathologists with a guided framework around which various forms of allograft pathology can be diagnosed.

Diagnostic criteria for chronic antibody mediated rejection include histological evidence of chronic injury with at least two of the following – arterial intimal fibrosis without elastosis, duplication of glomerular basement membrane, multilaminated peritubular capillary (PTC) basement membrane and interstitial fibrosis with tubular atrophy. In addition there must be evidence for antibody action or deposition in the tissue, such as C4d in the PTC, plus detection of circulating donor specific antibody [53].

1.10.2 T cell Mediated Rejection.

Even in the presence of immunosuppression T cell mediated rejection (TCMR) remains a significant problem and is the dominant phenotype seen in early rejection [66]. Acute TCMR is diagnosed in 5-10% of unsensitised patients within the first year of transplantation [55]. T cells may be activated via the direct, indirect or semi direct pathways when donor alloAg is presented via the donor or recipients own APCs. However the lesions produced by the activated T cells are the same, independent of the route of activation [66]. The lesions produced are also similar regardless of the mismatch provoking the response – Class I, II or non-HLA [66].

The initial response in a previously unsensitised recipient will require donor antigen from the graft to be presented in secondary lymphoid tissue by antigen presenting cells, originating either from the donor, in direct allorecognition, or the recipient's own APCs in indirect allorecognition. Once activated in a lymph node the effector T cells return to the graft where they initiate rejection [46].

In a previously sensitised recipient circulating memory T cells previously primed by exposure to the same non-self HLA, or those which recognise the mismatched antigens due to crossreactivity with antigens encountered during a previous infection, heterologous immunity [67], are able to enter and respond to the graft immediately without the requirement for initial priming in the secondary lymphoid organs.

The effector T cells responding to the mismatched antigens in a primary response will be composed of naïve T cells which have been primed in the secondary lymphoid organs and some memory T cells which may have been previously primed by infection and possess crossreactive TCR [68]. Effector T cells can home to the graft via two routes, firstly through recognition of antigen on the endothelium and secondly through their general role of patrolling the tissues [66]. Both memory and effector CD4+ helper and CD8+ cytotoxic T cells enter the graft [66]. Recognition of their cognate antigen by CD8+ T cells will lead to activation of their cytolytic activity, including release of perforin and granzymes A and B, resulting in destruction of the target cells. In addition they release chemokines and cytokines which act to recruit more cells into the graft including further T cells and macrophages [46]. CD4+ effector T cells activate both the cytotoxic T cells and the B cells, to produce antibody. They also release cytokines recruiting further cellular infiltration, along with inducing the upregulation of expression of HLA on the surface of the endothelial and epithelial cells of the graft, encouraging further antigen recognition by T cells.

1.11 Immunosuppression

The development of immunosuppressive drugs was a key feature in the ability to offer organ transplantation as a viable treatment option. The first attempts at immunosuppression for organ transplantation involved total body irradiation in the late 1950s. However the side effects encountered with this radical treatment were generally fatal to the patient [10, 11, 69]. Since this time much work has been focussed on the development of immunosuppressive agents to facilitate successful transplantation. The majority of these agents focus mainly on the T cell arm of the immune response as, along with inhibiting T cell mediated rejection, inhibition of the T cells should in turn inhibit the development of alloantibody due the T cell requirement in this process, and the activation of other cellular effectors of rejection such as macrophages. The actions of T cells can be inhibited in three main ways – depletion of the cells, restricting their ability to traffic and by blocking their response pathways. The small

molecule drugs, such as azathioprine (AZA), ciclosporine, tacrolimus (TAC), mycophenolate mofetil (MMF) and Sirolimus all have mechanisms of action which interfere with cellular response pathways during activation. The first immunosuppressive agent to gain widespread use in renal transplantation was AZA [70] which, on metabolism, releases 6-mercaptopurine which is further converted to a number of compounds which act to halt DNA synthesis, thereby preventing cell proliferation. In addition 6-mercaptopurine is believed to convert co-stimulatory signals via CD28 from activatory to apoptotic [71], leading to depletion of activated T cells. Whilst AZA may still be included in current IS regimens, its widespread use was reduced with the discovery of the calcineurin inhibitors (CNI), ciclosporine and then tacrolimus. Both these agents act by engaging with the immunophilins, cyclophilin and FK506 binding protein 12 (FKBP12) respectively, which in turn inhibit calcineurin activity, required for the de-phosphorylation and translocation of nuclear factor of activated T cells (NF-AT) into the cell nucleus, where it enhances the transcription of the genes encoding pro-inflammatory cytokines such as IL-2 [9]. IL-2 is a key cytokine in the signals required to promote activation and proliferation of T cells. The agents Sirolimus and everolimus also engage with the immunophilin FKBP12. However the complex that is created does not affect calcineurin, but instead inhibits the action of the mammalian target of rapamycin (mTOR). The mTOR pathway, activated by cytokines such as IL-2, provides the 3rd signal in T cell activation, see figure 8 above, and its inhibition leads to a blockade on cell proliferation and cell-cycle progression. MMF is another small molecule drug which, on metabolism, releases mycophenolic acid. Mycophenolic acid inhibits the enzyme inosine monophosphate dehydrogenase, which is required for purine synthesis and therefore DNA replication, again required for cell proliferation following activation [40]. Immunosuppressive regimens which use a combination of MMF and CNI are now commonplace and have been shown to improve both graft and patient survival, and reduce the incidence of rejection episodes [72, 73]. Therapeutic antibodies are another group of immunosuppressive agents and can lead to either cellular depletion or inhibition of action depending on their target. Depleting antibodies are

those which activate pathways leading to the destruction of cellular targets and can be specific to T cells, B cells or both. Anti-thymocyte globulin (ATG) and anti-lymphocyte globulin (ALG) are polyclonal antibodies raised in animals through inoculation with human derived thymocytes or lymphocytes, and then subsequent isolation of the cellular specific IgG fraction [40]. A typical course of ATG lasts 3 – 10 days and leads to a profound and long lasting lymphopenia [74] through complement mediated lysis and opsonisation and removal of target cells [75]. Alemtuzumab (Campath) is a humanised monoclonal antibody (mAb) raised against cell marker CD52 and leads to destruction of CD52+ lymphocytes via complement mediated lysis, causing long term depletion of these cells from circulation. OKT-3 (muromonab-CD3) is a mouse derived monoclonal antibody specific for the T cell receptor complex associated marker CD3. Infusion of OKT3 initially leads to rapid lysis of circulating T cells followed by a lack of expression of CD3 in the returning T cell compartment [76] leading to an inability of these cells to respond to stimulation. Non-depleting antibodies include basiliximab which is a mAb specific for the IL-2 receptor α chain, CD25. CD25 is expressed on activated T cells and increases responsiveness of the cell to IL-2. Blocking CD25 through mAb binding reduces the activatory effect of IL-2 on T cells. Belatacept is a cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) Ig fusion protein. CTLA-4 is a transiently expressed cell surface molecule seen after T cell activation. Like CD28 it can bind to CD80 and CD86 expressed on APCs, but unlike CD28, engagement of CTLA-4 leads to a negative signal being transduced. Binding of CTLA-4Ig fusion protein to CD80 and CD86 on the APC blocks interaction with CD28 on the T cell and prevents co-stimulation from occurring leading to anergy of the responding T cells [77].

Corticosteroids have long been a mainstay of immunosuppressive regimens and the two most commonly used in renal transplantation are prednisolone and prednisone, which is metabolised into active prednisolone [9]. The mechanisms of action are wide including acting as agonists for the glucocorticoid receptors leading to interference with gene transcription and production of pro-inflammatory cytokines along with other anti-inflammatory and immunomodulatory effects [78].

Whilst the majority of immunosuppressive agents target T cells, a number of agents are now being investigated for use in reduction of alloantibody production or modulation of the damaging effects of alloantibody on a graft. These include the use of intravenous immunoglobulin (IVIg), CD20 specific mAb Rituximab, proteasome inhibitor bortezomib and complement component C5a specific mAb eculizumab. The use and mechanisms of action of all these agents are discussed in 'transplanting the sensitised patient' below.

No single immunosuppressive agent is able to provide complete prevention of rejection at tolerated doses and therefore the majority of centres worldwide utilise combinations of agents to provide the best protection from rejection and minimise the potential side effects. Some immunosuppressive are used as induction agents, given in the weeks, days or hours pre transplant, ATG, basiliximab and Rituximab are common examples of this, often in patients who are deemed to be at higher risk of early acute rejection episodes. These agents may be required again for treatment of active rejection episodes.

One major problem with all immunosuppressive agents are the associated side effects, which can be the general increase in infection and cancers, such as skin cancer and post-transplant lymphoproliferative disorder, found with most agents, to more agent specific effects, such as nephrotoxicity and an increase in diabetes and hypertension with CNI, and diarrhoea and anaemia with MMF. In general a patient, once transplanted, will remain on immunosuppression for the lifetime of the graft, so the potential side effects of immunosuppression must be weighed up against the benefits achieved through transplantation. This is particularly important in the context of higher risk antibody incompatible transplantation where stronger immunosuppressive agents and regimens may be required both to achieve transplantation and to treat any subsequent rejection episodes.

1.12 HLA matching and outcome.

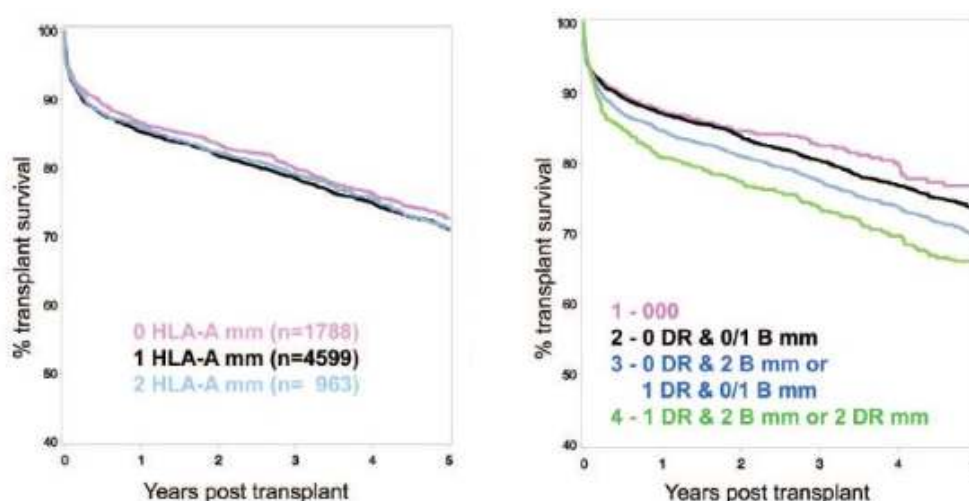
Due to the immunogenicity of HLA molecules, and their central role in allograft rejection, HLA matching at a minimum of the HLA A, B and DR loci has long been considered a benefit to long term transplant outcomes, with many studies providing evidence of increasing numbers of mismatches at these loci being associated with decreasing graft survival [79-81]. HLA matching remains an important factor in allocation algorithms used in many countries for the national allocation of deceased donor organs [82]. However with the introduction of ever more potent immunosuppressive regimens in recent years the importance of HLA matching on the long term graft survival has been called into question. Su et al. examined the effects of HLA mismatching on graft survival for transplants performed between 1995 and 1998 in the United States, and found that in the 1995 cohort 3-6 antigen mismatches were associated with a higher risk of graft failure, but the effect reduced each year until in the patients transplanted in 1998 only a 6 antigen mismatch was significantly associated with a risk of graft failure [83], however these patients were only followed up for a median of 2.2 years so the effects on long term survival were not investigated. Martins et al. analysed the longer term (8 – 20 year) outcomes from 1314 deceased donor renal transplants split into four eras based on immunosuppressive protocols in use at their centre from pre 1990 to post 2000. They found that HLA matching was significantly associated with graft loss in the early eras from pre 1990 to 1996 but that since the introduction of mycophenolate mofetil, anti thymocyte globulin, tacrolimus and sirolimus to their immunosuppressive repertoire HLA matching was no longer a significant factor in graft loss [84]. Of note, whilst the differences in graft survival may not be significant, in all published studies HLA matched organs continue to show the best overall graft survival compared to mismatched organs.

The apparent reduction in the influence of HLA matching and the increasing numbers of patients on transplant waiting lists worldwide has led some national registries to reduce the importance of HLA matching on allocation, such that the United Network of Organ Sharing

(UNOS) only considers matching at HLA DR and the California Transplant Donor Network no longer includes HLA matching in its' allocation algorithm [85].

In the UK however HLA matching at HLA A, B and DR is still factored into the matching algorithm with '000' matches being favoured over other match grades and a stepwise importance of increasing mismatches. Analysis of the factors effecting outcome of renal transplants performed in the UK from deceased donors between 1995 – 2001 by Johnson et al., on behalf of NHS Blood and Transplant (NHSBT), found that whilst HLA A matching bore no significant influence on graft outcome, matching at both HLA B and DR did still exert a significant influence on longer term graft survival, with a cumulative effect of increasing mismatches at these loci [86]. The survival curves for these loci presented by this group can be seen in figure 11 below. Following this analysis the UK has retained its policy on favouring well matched grafts where possible at all three previously mentioned loci [87]. It should however be noted that the authors did observe improvements in outcomes in patients transplanted in the latter time period of the study regardless of other influencing factors, which was suggested could reflect changes in immunosuppressive regimens.

Figure 11 - The effect of HLA matching on UK Renal Transplant 5 year graft survival rates for deceased donor transplants carried out between 1995 and 2001. [86]



Other non-immune factors have also been found to exert a significant influence on long term graft survival including recipient and donor age, donor cause of death, initial disease of the recipient, length of cold ischaemic time of the organ, the length of time the recipient was waiting for an organ, donor and recipient body weight ratio, and patient ethnicity [86, 88, 89].

One longer term impact of reducing HLA matching between donor and recipient is the increase in sensitisation of these patients. In the US the third most common reason for being on the transplant waiting list is due to failure of a previous graft [85] and it has been found that greater numbers of mismatches presented by the first graft leads to a higher level of sensitisation to HLA in patients following failure of the graft and return to the waiting list [90]. By reducing the importance of HLA matching there is the risk that patients will become more sensitised following failure of the primary graft reducing their chances of a re-graft in future, a concern borne out in a study by Meier-Kiresche et al.[85]. In the UK paediatric patients are given priority for well-matched kidneys partly in an effort to reduce the risk of sensitisation, as their age at the time of first transplant means that they are likely to require more than one graft over their lifetime. If matching is reduced and patients do in general become more sensitised it is likely that the demand for HLA antibody incompatible transplantation could rise in the future.

1.13 HLA sensitisation.

Patients who produce Abs specific to HLA are known as sensitised. The production of HLA specific antibodies generally requires exposure to non-self HLA antigens. There are three main routes of sensitisation – previous transplantation, pregnancy and blood transfusion.

Transplantation, and subsequent rejection or loss of the organ, often produces the strongest response with the longest lived Ab production. In pregnancy the foetus acts in many ways as a graft, harbouring HLA Ags of the father, to which a response may be made, particularly in women who have had multiple pregnancies and blood transfusions [91]. As a consequence women are more likely to be sensitised, which is evidenced by the fact that a 2006 audit of the

UK renal transplant waiting list found 33% of the female patients were sensitised in comparison to only 17% of the males [92]. Transfusion of blood products also leads to exposure to non-self HLA and, despite the current practice of leucodepletion, is a common route to sensitisation [93]. Approximately 50% of patients receiving multiple blood transfusions will develop some level of HLA sensitisation [94]. However, the antibodies and response following a transfusion are often more transient in nature. In addition, HLA specific antibodies are found in 1% of the population through no obvious cause [95], although more recently Morales-Buenrostro et al. found up to 63% of a group of 424 healthy male blood donors produced detectable HLA specific antibody [96], possibly due to the antibodies detected actually being specific for pathogens, but binding to shared epitopes [97], or due to antibody binding to epitopes exposed through the kit manufacturing process but not naturally revealed [98]. The specificity and strength of HLA specific antibody produced by a patient can vary and diminish over time [99] and in sensitised patients the strength and breadth of the HLA specific antibody can change following further sensitising events and also in response to alternative pro-inflammatory events such as infection or trauma [100]. Therefore it is recommended that patients awaiting a renal transplant are regularly monitored [99].

1.14 Methods for Detection and Identification of HLA specific Antibody.

A large proportion of the work carried out by the transplantation laboratory involves the detection and identification of HLA specific antibody both in patients awaiting renal transplantation and in monitoring those who have been transplanted.

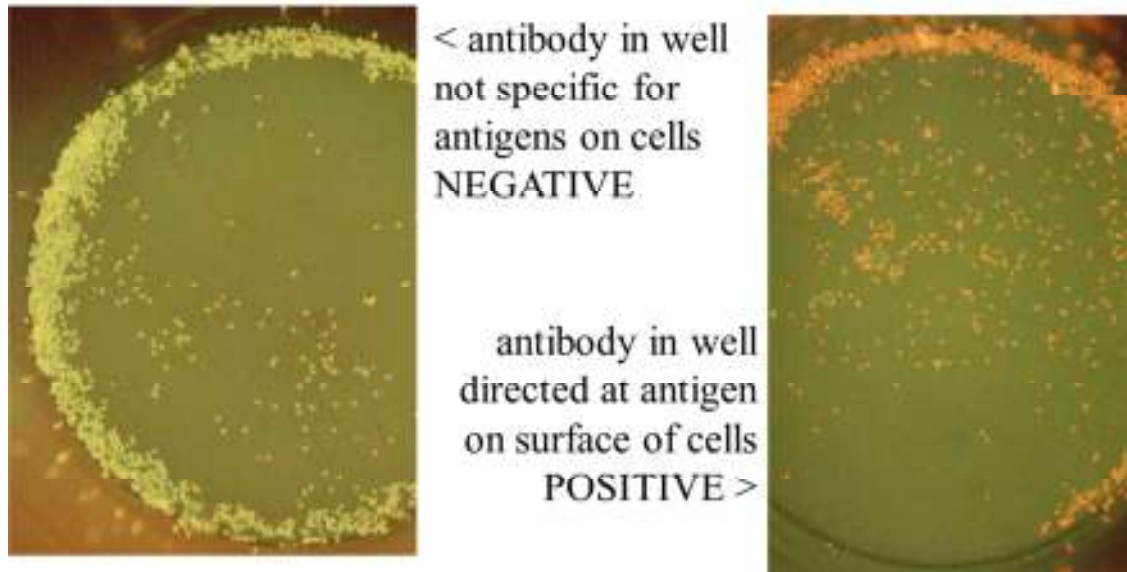
There are two main routes by which potential donor specific antibody may be identified. The first is through screening patient serum samples to detect the presence of, and identify the specificity of, antibody specific for HLA. The second is through crossmatching a patient against a potential donor to detect actual donor specific reactivity. Broadly these assays can be defined as being cell-based or solid phase.

1.14.1 Cell Based Assays

1.14.1.1 *Complement Dependent Microlymphocytotoxicity (CDC).*

The CDC assay was first described in 1964 by Terasaki and McClelland [15]. The basic premise of the test is that cells expressing a variety of HLA antigens are incubated with patient sera. Antibody present in the sera specific for antigens on the surface of the cells will bind. Rabbit derived complement is then added and any bound antibody that is of the correct class and/or subclass, of sufficient titre and binds with high enough affinity will activate the classical complement cascade leading to the generation of the final membrane attack complex (MAC) and perforation the cell membrane. Cell death, indicating the presence of a complement fixing antibody specific for an antigen on the cell, can then be visualised by the use of vital dye staining. Dye is able to enter cells whose membranes have been perforated by MAC generation, and is unable to enter cells which have not been damaged by complement fixation. Staining can be observed using a fluorescence microscope. When using a stain composed of ethidium bromide, acridine orange and haemoglobin living cells appear green and lysed or perforated cells appear red. Strong, or high titre, antibody may lead to death of all the cells present, whereas weaker antibody may lead to only some of the cells being observed to have died. The proportion of living to dead cells can be estimated and a score attached. International scoring generally follows '0' for no cell death, through 2, 4, 6 and 8, representing up to 20%, 40%, 60% and up to and over 80% of the cells in the test well being seen to have died.

Figure 12 - Example of Cells Observed in a CDC Assay (in-house photography).



This method can be used for both the detection and identification of HLA specific antibody, and for donor crossmatching. For identifying HLA specific antibody a patients' serum sample is tested against a panel of cells of known HLA type, and analysis of the reaction patterns can indicate which HLA antigens the antibody in the sample is specific for. The cell panels can be generated locally through use of donor or volunteer cells, or be purchased as pre-plated frozen cell panels. The cell panels need to be designed to ensure that as many common HLA antigens as possible are represented. A significant problem with the design of cell panels can be caused by linkage disequilibrium, where combinations of HLA alleles in a haplotype are found at a higher frequency than would be expected based on the frequencies of each individual allele. So if two antigens, for example HLA A1 and B8, are often presented together it can complicate HLA antibody specificity analysis in designating which antigen the antibody is directed at. Therefore it is necessary to design the panel to ensure that these antigens are represented on some cells separately. When cell panels are generated locally the composition of the panel can vary between batches meaning that, when monitoring a patient over the longer term, variations in panel reactive antibody (PRA) value can occur not through changes to the patients' antibody status but due to changes in the panel composition. The differences in

panel composition between laboratories also means that PRA values cannot be compared with great accuracy between centres [101].

The method described above is often referred to as the National Institutes of Health (NIH) direct CDC. Since its introduction a number of variations have been described in an effort to increase the sensitivity of the method. One such variation is the indirect antihuman globulin (AHG) CDC described by Johnson et al. in 1972 [102]. Here, following incubation of cells and serum, rabbit anti-human IgG antibodies are added prior to complement addition to amplify the cytotoxic response. This is believed to allow antibodies of titre or classes which are unable to efficiently fix complement to also be detected. Antibodies of the IgM class are able to efficiently fix complement and can cause positive reactions in the CDC assay. However they may not be HLA specific, and, even if they are, the significance of IgM HLA specific antibody in solid organ transplantation has not been clarified [103-105]. Pre-treatment of serum with Dithiothreitol (DTT), which reduces disulphide bonds, causes the breakdown of the IgM pentameric structure, required for IgM to fix complement, and reduces its interference with the CDC assay, meaning any positive reactions observed can be generally assumed to be due to IgG class antibodies. Even with the variations on the technique available the CDC assay remains relatively insensitive, generally only detecting high titre complement fixing antibodies [106] and whilst this is important in avoiding hyperacute rejection [16] both low level complement fixing and non-complement fixing antibodies have been associated with higher incidence of acute rejection and worse outcomes post-transplant [107] neither of which can be detected using the conventional CDC assay. In addition it requires a relatively large number of viable cells, analysis is subjective and the assay requires a high degree of manual dexterity. Often the cells used in a simple CDC assay are unseparated, meaning that, when derived from peripheral blood, the majority will be T lymphocytes, mainly expressing HLA Class I only; up to 20% of the cells will be B cells expressing HLA Class I and II. To accurately assess the presence of antibody to both classes it is necessary to physically separate the T and B cells into separate

populations to be used in separate assays adding further to the technical challenges of this assay.

1.14.1.2 *Flow Cytometric Screening and Crossmatching.*

Cell based flow cytometric assays for HLA specific antibody detection use target lymphocytes, donor derived for the crossmatch and cultured cell lines for screening, which are incubated with patient serum to allow any antibody present to bind to targets on the cells. Bound antibody is then detected through the addition of a fluorescence labelled secondary antibody which is detected and quantified using a flow cytometer. The class and subclass of antibody can be defined by the addition of different secondary antibodies, most commonly IgG specific but also IgM, IgA and IgG 1 – 4 subclasses can be identified [92, 108, 109]. Flow cytometric based techniques have greater sensitivity compared to CDC [110]. Screening of patient sera on a large scale using flow cytometry with cellular targets is generally limited to the detection, rather than identification, of HLA specific antibody. Various cell lines are pooled in an effort to ensure that all the major HLA specificities are covered within the pool. Cell targets can be derived from pools of chronic lymphocytic leukaemia patients [111], PBLs [112, 113] and Epstein Barr Virus transformed lymphoblastoid cell lines [114, 115]. Any samples testing positive can then be further analysed using either individual cell lines or other antibody identification techniques. In 1983 Garovoy et al. first described the use of flow cytometry for crossmatching in transplantation [116]. Initially they used a single colour technique to detect IgG binding to lymphocytes and found that it was up to 50 times more sensitive than the standard CDC XM test. Further work led to the introduction of the two colour [117], and then three colour [118], crossmatching methods where addition of various alternative fluorescence labelled antibodies to target antigens on different cell populations allows for the easy differentiation of cell types in one test sample, so T cells and B cells can be separated on analysis through addition of differently coloured CD3 and CD19 specific monoclonal antibodies respectively. Analysis of the FXM is generally based on comparison of the patient/donor reactions to a negative control, where, in addition to reactions with recipient sera, the donor

cells are incubated with a validated negative control serum, generally acquired from a pool of unsensitised AB blood group males. Reactivity of the patient serum above that seen with the negative control indicates that there is antibody present in the patient sera that is binding to the donor cells. The level of reactivity above the negative control required to constitute a positive FXM result is defined locally by each laboratory. A positive T cell crossmatch is indicative of the presence of donor HLA class I specific antibodies, as T cells, unless activated, generally express only HLA class I. A positive B cell crossmatch can be indicative of either low level HLA class I specific antibody, as B cells express higher levels of HLA class I than T cells [119, 120], or donor HLA class II specific antibody. A summary of the causes of positive FXM can be seen in table 1 below.

Table 1 - Influence of HLA class specificity of Antibody on Crossmatch Result.

Antibody	T cell FXM result	B cell FXM result
No HLA specific antibody	Negative	Negative
Class I and II HLA specific Ab	Positive	Positive
High titre Class I HLA specific Ab	Positive	Positive
Low titre Class I HLA specific Ab	Negative	Positive
Class II HLA specific Ab	Negative	Positive
Autoantibody	?Negative	Positive

Whilst a positive CDC XM has been shown to predict hyperacute rejection post-transplant [16], the flow cytometric crossmatch (FXM), being more sensitive and detecting antibodies of both lower titre and non-complement fixing types, has been shown to detect weaker donor specific antibody and identify patients who are at increased risk of antibody mediated rejection which would have otherwise been missed using CDC XM alone [121]. The T cell FXM in particular has been shown to have a greater value in predicting future antibody mediated rejection in comparison to the CDC XM [122-125]. The B cell crossmatch remains more controversial however with studies both supporting [126-129] and failing to support [130-133]

a correlation between a positive B cell FXM and poor graft outcome. There is still much controversy surrounding positive cut off levels for the FXM, particularly for B cell crossmatches where higher background binding can be observed [134, 135]. Each centre must validate how much reactivity above negative control is significant and could be classed as being potentially detrimental to the outcome of the transplant when performed under the clinical conditions in their unit. Whilst the basic FXM method is similar between centres standardisation of the technique is difficult to achieve due to variations in the method, differences between reagents and cytometers used and differences in the calculations used to generate the reaction value. These variations again highlight the requirement for centres to assess and set their own cut-offs based on the clinical situation in their unit. Despite the difficulty in setting cut off levels for the FXM, the greater sensitivity of the FXM in detection of donor specific antibody compared to CDC XM means that in many centres it has largely replaced the use of the CDC XM in recent years.

1.14.1.3 *Autoantibodies.*

All cell based assays have the disadvantage of detecting antibodies to non-HLA targets, so called autoantibodies, along with alloantibody. Autoantibodies are not HLA specific and will bind to targets on both donor and self-derived cells, and have been shown to have no effect on transplant outcome [136, 137]. The influence of these antibodies can be determined by performing auto-crossmatches whereby a patients' own cells are incubated with their own serum samples in the same assay set up as a conventional crossmatch. Any reactivity observed can be subtracted from the donor crossmatch to provide a more indicative crossmatch result. However this is easier to apply to FXM where definitive numerical values are achieved, and must always be carried out in conjunction with in depth antibody specificity analysis, particularly in highly sensitised patients, to ensure that all or most of the reactivity observed can be accounted for by non-HLA specific antibody. Early studies investigating the cause of B cell positive FXM have reported up to 94% of B cell positive FXM could be due to non-HLA specific autoantibodies based on detection of potential DSA using screening techniques [138].

However, with the development of more sensitive screening and antibody identification methods this figure was reduced to 50% in more recent reports [139]. By including positive B cell FXM due to autoantibodies in analysis of the influence of a positive B cell FXM in renal transplant outcome then it is unlikely that an association would be found, highlighting the importance of investigation into the causes of a positive FXM result and if necessary testing for autoantibodies in conjunction to HLA specific antibodies by running an auto FXM test in parallel with the donor FXM.

1.14.2 Solid Phase Assays.

The alternative to cell based methodologies are the so called 'solid-phase assays' (SPA). SPAs were first introduced in the 1990s and rapidly gained favour due to their increased sensitivity and specificity when compared to the cell based methods [140]. SPA are generally commercial kits which utilise intact HLA molecules that have been either solubilized and purified from cell membranes or recombinant HLA antigens from transfected cell lines [141] and then immobilised onto a solid matrix, most commonly a microtitre plate or polystyrene microparticles. The benefit of using purified HLA molecules, when compared to whole cells, is that generally only HLA specific antibody is detected, with far less interference from non-HLA- and auto- antibodies. The SPAs also allow for easy differentiation of HLA class I and II antibodies through separating these into either different testing wells or bead populations. With all the SPAs available there are three levels of testing –

1. Detection – provides a yes/no answer to the presence of HLA specific antibody.
2. Panel Reactivity – allows medium resolution of antibody specificity.
3. Single Antigen – allows high resolution of antibody specificity.

Detection methods use pools of HLA glycoproteins, generally separated into class I and II, and cover at least the majority of the HLA antigens found commonly in the population. They provide a rapid and more cost effective method of high throughput screening, allowing the

identification of positive samples requiring more detailed analysis using higher resolution kits. Panel reactive, or phenotype panel, kits again separate HLA class I and II, and limit the HLA antigens present in each test well, or on each bead population, to that found on one donor cell. So, for class I the maximum number of different antigens present would be 2 each of HLA A, B and Cw, and for class II it would be limited to a maximum of 2 each of the HLA DRB1, 3, 4 or 5, DQ and, depending on the kit, DP. Analysis of reactivity patterns allows identification of antibody specificity, although in highly reactive sera some specificities may be masked by the presence of others, particularly in the case of antibodies to Cw, DQ and DP [141]. It is thought that the phenotype panel kits may best reflect the in vivo situation as the panel may include phenotypes to which the patient has more than one donor specific antibody, allowing the assessment of the combined reactivity of these antibodies [109]. The most sensitive of the tests available are the single antigen panels, where each test well or bead population is coated in a single HLA specificity allowing for high resolution antibody analysis. The single antigen methods are particularly useful in analysis of highly reactive sera giving the most accurate information available as to the exact HLA antigens to which a patient produces antibody [142].

In general the SPA routinely detect IgG class antibodies only, however they can be adapted to detect IgM [143, 144], IgG1-4 [145, 146], IgA [147] and more recently to directly assess complement fixation through the detection of the deposition of complement cascade components C4d [148] or C1q [149].

All SPAs provide an analyser derived numerical read out allowing analysis of the results to be more objective than CDC and also they are believed to be semi-quantitative giving an indication as to the titre of the antibody present [92, 150].

1.14.2.1 *Enzyme-Linked Immunosorbant Assay (ELISA).*

The first commercially available SPA for HLA antibody detection and identification was in the form of an ELISA kit called PRA-STAT, launched in 1994 by SangStat. The initial aim of the PRA-STAT kit was to provide a method for HLA antibody identification that was more robust than

the CDC test, without the requirement for viable cells, validated complement , subjective analysis, detection of non-HLA specific antibodies and autoantibodies, and which gave a standard panel against which all patients could be tested [151]. Whilst the PRA-STAT kit is no longer available, other commercial ELISA kits are still routinely used in many laboratories, and have been found to be more sensitive than CDC in detecting HLA specific antibody [152]. For ELISA based methods the HLA antigens are immobilised in the well of a microtitre plate and patient serum is added. Any antibody specific for the antigens in the well will bind during incubation and, following a wash step, a secondary antibody, specific for human IgG, is added, which will in turn bind to any patient antibody present in the well. This secondary antibody is conjugated to a reporter molecule, such as alkaline phosphatase, which on addition of a substrate, such as p-nitrophenyl phosphate, will result in a colour change in the well. This colour change is then measured using spectroscopy to assess the optical density and, in general, the greater the colour change the higher the level of antibody in the well.

Disadvantages of ELISA based methods include the requirement for large serum volumes, relatively long incubation times and low throughput capacity in comparison to other SPAs available.

1.14.2.2 *Flow Cytometry*

Flow cytometric based SPAs are similar to more traditional cell based flow screening, but instead of cellular targets the HLA antigens are immobilised onto microparticles. For detection tests these are separated into two pools of class I and class II coated beads. On incubation with patient sera any HLA specific antibody will bind to the beads and is then detected using a FITC-conjugated secondary human IgG specific antibody. The level of fluorescence is then assessed using a flow cytometer and the fluorescence signal achieved with the patient sample is compared to that achieved with a negative control serum, with the level of fluorescence above the negative control being indicative of the level of antibody binding. Low titre antibodies, perhaps to only single specificities, may however be missed as the level of

fluorescence seen may not reach the positive threshold. In these circumstances the architecture of the peak must be examined by an experienced technician to detect small changes to the curve shape, or the presence of small secondary peaks, which may indicate the presence of a low titre antibody.

Antibody identification methods utilise pools of beads with different fluorescence properties due to variations in red fluorescence staining. Each bead is coated with either a single phenotype or single HLA antigen and can be in pools of 8 – 12 different bead populations. On analysis the different bead populations in the pool are separated due to the variations in red fluorescence staining and bound antibody is identified through the green fluorescent staining of the FITC conjugated secondary antibody. Read out is based on median channel shift and comparison to a negative control [153]. The antibody identification panels available for flow cytometric analysis are limited in size by the ability to resolve different bead populations, meaning that, particularly in the single antigen setting, a panel size of 28 antigens requires 4 bead pools and therefore 4 sample preparations. Whilst this can be representative of a population many antigens are not included and therefore antibody specificities may go undetected.

1.14.2.3 *Luminex*

Luminex based methods are the most recently introduced SPA for HLA specific antibody testing. Similar to the flow based methods, the Luminex assays use pools of microparticles coated in purified or recombinant HLA molecules. The microparticles are specifically designed to be analysed using a Luminex flow analyser, and up to 100 different microparticles can be distinguished in one test based on the ratio of red to infra-red internal dye in each bead. Equipped with two lasers, the Luminex analyser simultaneously uses a red laser to identify the microparticle and a green laser to measure the R-phycoerythrin (PE)-conjugated secondary antibody bound to the analyte coating the bead, in the case of HLA antibody definition this will be a patient derived HLA specific antibody bound to HLA antigen coating the bead. Analysis of

the combination of these two signals allows either the presence of antibody, in detection tests, or the specificity of antibody, in identification tests, to be defined. The amount of PE conjugated antibody bound to a bead is given as a mean fluorescence intensity (MFI) value and can give an indication as to the titre of antibody present in the patient serum [150]. There are currently two manufacturers that produce Luminex kits for HLA specific antibody detection and identification, One Lambda and Gen-Probe, and whilst both aim to provide the same information, there are differences in manufacturing processes, assay method, reaction calculations and panel composition.

Luminex based methods have been found to be the most sensitive of all the SPA currently available [154], however the clinical relevance of antibody detected by Luminex only, or by solid phase but not CDC, is a matter of great controversy. A number of studies have been reported where the clinical effect of SPA only detected antibodies on transplant outcome have been investigated. The general trend for the majority of reports is a detrimental effect of these antibodies, either by a reported reduction in graft survival or through an increase in observed rejection episodes [155-158]. However this is not always the case with further reports finding no association between SPA only antibodies and outcome, particularly in the case of Luminex single antigen only detected antibodies [159, 160]. With regard to low level donor specific antibody in renal transplantation consensus does now appear to be in favour of relevance, at least in the long term outcomes [161-163]. Roelen et al. [164] in a recent review concluded that low level DSA are not a contraindication to transplant, but are a risk factor for acute rejection.

1.15 Accommodation

One possible explanation for a lack of detrimental effect being seen with all donor HLA specific antibodies could be the development of accommodation. Accommodation was first described in patients receiving ABO blood group incompatible transplants, where despite the initial reduction in ABO antibody titres following antibody removal and transplantation, the antibody

returned to circulation yet antibody mediated damage to the organs was not observed [165]. The antibody does appear to be able to bind to the graft and fix complement, a finding borne out by the fact that many biopsies from these patients with functioning grafts show positive staining for C4d but no other histology indicating AMR [166]. Accommodation is defined as the acquired resistance of the transplanted organ to antibody mediated injury and is believed to involve Ab induced endothelial cell expression of pro-survival and cryoprotective proteins, along with regulation of the terminal complement components [167]. Jindra et al. [168] found that crosslinking HLA class I molecules on the surface of endothelial cells with low dose antibody induced cell survival through stimulation of the mTOR-2 pathway which led to an upregulation in the expression of anti-apoptotic protein Bcl-2. Salama et.al. [169] reported that expression of another pro-survival protein, Bcl-xL, was also upregulated in the endothelial cells of patients who had circulating DSA but no demonstrable AMR on biopsy, following HLA antibody incompatible renal transplantation. In a mouse xenograft model, in addition to Bcl-2 and Bcl-xL, expression of other pro-survival genes such as A20 and cryoprotective proteins hemoxygenase and nitric oxide have been reportedly upregulated in the endothelial cells of accommodated cardiac xenografts but not in rejected hearts [170]. Ding et al. [171] using another mouse xenograft model found that accommodation could be induced by regular injection of low doses of donor specific antibody, and that this effect was likely to be due to the increase in expression of complement regulatory protein, decay accelerating factor (DAF), which has been found to inhibit hyperacute rejection. This evidence suggests that particularly in patients who undergo antibody removal transplantation, the phenomenon of accommodation could at least in part explain why not all DSA are detrimental in the outcome data achieved.

1.16 Transplantation of Sensitised Patients.

Patients who produce any amount of HLA specific antibody are said to be sensitised. In the UK a patient who produces HLA specific antibody reactive to >85% of a cell panel representative

of the general population are defined as being 'Highly Sensitised' by Organ Donation and Transplant (ODT). Traditionally this level was defined by the patients' treatment centre laboratory, using locally derived or kit based cell panels, to generate a panel reactive antibody (PRA) value, which represented the percentage of positive reactions against the panel. Whilst this gave some indication as to the breadth of reactivity of a patient against a population of donors the value achieved was highly dependent on the composition of the testing panel. In recent years ODT have published a computer based algorithm whereby the specificities of the antibody produced by a patient can be input, and a virtual % calculated reaction frequency (cRF) can be generated, by comparing the HLA types of the past 10000 blood group compatible donors and calculating the number of these donors with which a positive crossmatch may be expected based on the antibody specificity. The values achieved range from 0% in unsensitised patients to 100% for the most highly sensitised patients, and give an indication to both the clinicians and patients as to their chances of receiving an organ offer through the national allocation system.

The first option open to sensitised and highly sensitised patients is to wait for an offer of an organ from a compatible deceased donor through the national allocation system. However this group of patients generally have much longer waiting times compared to unsensitised patients. A recent report from the American Organ Procurement and Transplant Network (OPTN) suggested that the numbers of offers decreased with increasing sensitisation, such that patients with cRF of 80-84 % had an offer rate of only 40% that of unsensitised patients, and this decreased dramatically to 1% in those patients classified as having 100% cRF [172]. Vo et al. reported that in the US only 6.5% of the highly sensitised patients of the national waiting list are transplanted annually compared to an overall transplantation rate in all groups of 20% [173]. A UK analysis of the waiting times for patients registered between 1998-2005 showed that unsensitised patients had a median waiting time of 788 days, patients with a cRF of 61-84% waited 1696 days and the highly sensitised patients, cRF >85% waited 2232 days [92].

An audit of UK kidney waiting list data by ODT indicates that in 2006 23% of patients awaiting a first transplant and 52% of patients awaiting a second, or subsequent graft, were sensitised [92]. An audit of the patients awaiting transplant at our centre in January 2013 found 56% of the waiting list were sensitised and 54% of these were classified as highly sensitised due to having a cRF of >85%. With the advent of new technology allowing for more sensitive and sophisticated detection and identification of HLA specific antibodies it is likely that the number of patients on the waiting list classified as sensitised will continue to increase, this has been demonstrated on analysis of the numbers of patients awaiting transplant on the UNOS database who are highly sensitised increasing by 25% in the year 2002-2003 corresponding with the widespread introduction of Luminex based solid phase antibody detection systems across the US [174].

Phelan et al. have reported that in the Republic of Ireland waiting times for patients with a PRA of greater than 50% can be more than double that of patients who produce no HLA specific antibody, with a combination of being blood group O and sensitised having the longest waiting times [175].

Longer waiting times are associated with an increase in morbidity and mortality, with approximately 2-4% of patients on the renal transplant waiting list dying annually without receiving a graft [176]. In addition a longer waiting time, and therefore longer time on dialysis, is also associated with poorer long term outcomes post-transplant, in terms of both graft and patient survival [177].

Transplantation of an organ from a living donor offers good outcomes and can often be preferable to a deceased donor transplant. However in our centre approximately 25% of the patients assessed for transplantation from a potential living donor will have antibody to this donor preventing a straight forward, low risk, direct transplant.

Another option open to these patients who have a willing but incompatible donor is to enter into the national paired scheme. Here a patient, and any associated potential living donors,

are registered in a pool with ODT, and four times a year an allocation 'matching run' is performed to identify compatible combinations of pairs, whereby the donor of one pair will donate to the recipient of another pair and vice versa. This scheme is useful to patient and donor combinations who are ABO blood group incompatible and/or HLA antibody incompatible, although highly sensitised patients are still less likely to achieve a match than those that are unsensitised [178, 179]. The numbers of potential transplants from one run are increased by the inclusion of both two-way and three-way swaps, four-way swaps are also possible, however de Klerk et al. in a recent analysis of the Dutch waiting list, found that the numbers of possible transplants from a single run were not significantly increased by allowing both four-way or unlimited chains [180]. At our centre, since 2007 a total of 73 patients have been included in the paired matching run and over the past six years 18 of these have received a transplant through the scheme. Having a high level of sensitisation reduces the possibility of being matched and of those who have not been transplanted through the scheme at our centre the majority are highly sensitised, highlighting the difficulties faced in transplanting these patients.

In recent years a third option has become more routinely available to these patients, antibody removal. Antibody removal, or de-sensitisation, is a term that covers a variety of therapies aimed at reducing or removing the donor specific antibody produced by a patient to allow successful transplantation from either a given living donor or through the deceased donor scheme. Strategies have been developed to allow transplantation across both HLA and ABO blood group antibody incompatibilities [181]. However in this thesis I shall be concentrating on those applied to HLA specific antibody removal only.

One of the earliest reports of successful renal transplantation following HLA specific antibody removal in the more recent era came from Taube et al. in 1984 [182]. They reported transplantation in a group of 5 patients all of whom were sensitised through previous failed transplants and were in urgent need of a subsequent graft. The antibody removal method

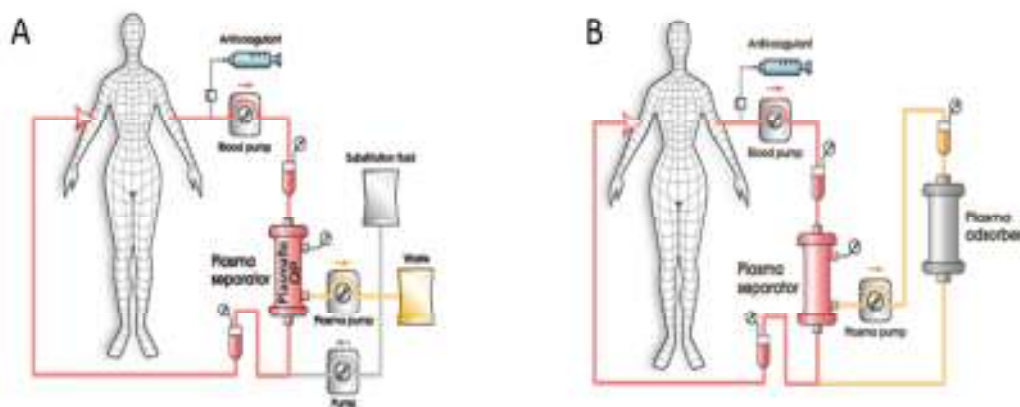
adopted in this group of patients followed the regimen designed for removal of glomerular basement membrane specific antibodies in Goodpasture's syndrome and consisted of thrice weekly plasma exchange (PEX) continued until the HLA specific antibody titres, assessed using CDC, fell below 1 in 10. In total the patients received 6 – 10 sessions of PEX. All 5 patients had a significant reduction in PRA and were transplanted with a deceased donor organ, having a positive CDC crossmatch pre-treatment, which was reduced to negative immediately prior to the transplant. All 5 patients suffered multiple rejection episodes, and one patient died 6 weeks post-transplant due to septicaemia. Following this Palmer et al. in 1989 reported a series of 10 highly sensitised patients who underwent HLA specific antibody removal using extracorporeal immunoadsorption (IA), of which 7 went on to receive a transplant [183]. This method required the patient to be treated with plasma exchange. The plasma volume removed was then passed down a staphylococcal protein A column to remove the IgG antibody, prior to being returned to the patient. Patients received 3-18 treatment sessions, with PRA assessment following each session. All patients showed a reduction in PRA and of those transplanted both the CDC and flow cytometric crossmatches (when available) were reduced to negative pre transplant. Overall there were only 6 reported episodes of rejection in the seven patients, one of the seven transplanted grafts failed to function and one had failed by one year. However in 1996 the same group published a follow-up report stating that from this series all the grafts that were functioning at one year, remained so by 5 years [184]. Whilst these two series involved multiple antibody removal sessions for a significant length of time prior to transplantation, in 1996 Higgins et al. reported the use of IA immediately prior to transplantation in a series of 13 patients [185]. All 13 patients were sensitised with PRA ranging from 63-100% and had been waiting for a deceased donor transplant for at least 2 years. In this series antibody removal was not initiated until a deceased donor organ had been identified, the patient then underwent one or two sessions of IA, totalling up to 22 hours of IA pre transplant. All 13 patients had the flow cytometric crossmatch reduced to negative. 6 grafts failed within the first 6 months and 7 functioned, with a median graft survival at 26

months of 53.8%. Of note the cold ischaemic time (CIT) for the donor organs ranged from 28-62 hours, a period of time that would no longer be deemed acceptable, and may be in part responsible for the poor initial outcomes. Following these early reports there have been a number of published single and multi-centre studies relating to HLA specific antibody removal prior to renal transplantation with a variety of success [186-191]. To date the majority of the protocols designed for desensitisation involve some combination of PEX or IA, intravenous immunoglobulin and/or Rituximab.

Plasma exchange and immunoadsorption are two methods of physical antibody removal. When treated with PEX a patient's blood is removed via a cannula and subject to centrifugation or double membrane filtration to separate the plasma from the cellular components [192, 193]. The cellular fraction is then returned in addition to plasma replacement, either albumin or fresh frozen plasma from a donor. It is not specific for antibody and removes all plasma proteins including clotting and complement components. Exchange of a single blood volume will remove up to 63% of the plasma proteins, including antibodies [194]. IA is more specific for antibody and involves separating the plasma from the cellular components and then passing this down a sepharose-bound staphylococcal protein A or protein G column to which IgG type antibodies will bind with high affinity, prior to reintroduction of the filtered blood to the patient [195]. A diagram representing these two methods can be seen in figure 13 below. IA has the advantage over PEX of being antibody specific and does not require the replacement of large plasma volumes. Additionally it does not affect coagulation [196]. Three to six courses of IA can lead to a reduction in IgG of up to 90% [195]. However, IA is significantly more expensive than PEX and in addition, those columns utilising protein A do not remove antibodies of the IgG3 subclass as efficiently as IgG1,2 + 4 [197] and since IgG3 subclass antibodies are able to fix complement very efficiently [198] this may be of relevance in antibody removal prior to transplantation. Antibody removal through PEX or IA alone does not have a long term effect, with a rebound in antibody levels following an antibody removal session being seen within 3 hours due to diffusion of antibody

in the extravascular areas back into circulation [199, 200]. This can in part be compensated for by carrying out multiple sessions in a relatively short period of time. Antibody removal does not stop antibody production and removal of large levels of antibody may in fact activate homeostatic feedback mechanisms, causing an increase in the production levels of antibody by plasma cells [201]. In an attempt to prevent resynthesis and rebound of antibody most protocols for desensitisation using PEX or IA involve the addition of immunosuppressant drugs (mycophenolate mofetil (MMF) and tacrolimus (tac)), intravenous immunoglobulin (IVIg) and/or anti-CD20 monoclonal antibody, Rituximab, or splenectomy [192].

Figure 13 - Diagram representing the mechanical process of antibody removal by PEX (a) and IA (b) [202].



IVIg is the term given to a number of commercially available preparations primarily composed of IgG monomers isolated from the blood of up to 1000 donors, often using ethanol precipitation, and then pooled [193]. IVIg has been used as an antibody replacement therapy in a number of immunodeficiency conditions such as hypogammaglobulinemia and severe combined immunodeficiency [203] and since 1981 it has been recognised as a treatment option for autoimmune diseases such as chronic idiopathic thrombocytopenic purpura [204]. There have been many theories proposed as to the mechanism of action of IVIg in combating antibody production, or its effects, and it is likely that many actions are involved [205]. IVIg is believed to effect the actions of the complement cascade by binding C3b and C4b and preventing them binding to the cell membrane so inhibiting the generation of the terminal

membrane attack complex of the complement cascade [206]. It has also been proposed that IVIg neutralises circulating antibodies through anti-idiotypic actions [203], although this has been questioned by subsequent studies [206]. IVIg is also believed to have effects on the cellular components of the immune system through binding to targets such as the Fcγ receptors on neutrophils, macrophages, NK cells and mast cells leading to non-specific effects such as inhibition of cytokine production and adhesion molecule activity [195]. It has also been shown to bind to the inhibitory receptor FcγIIb, inhibit expression of CD19 on activated B cells, induce apoptosis of B cells and inhibit alloreactive T cells [207, 208]. The use of IVIg in autoimmune diseases highlighted the apparent immunosuppressive effects of IVIg and in 1994 Tyan et al. reported the use of IVIg to suppress production of HLA specific antibody in highly sensitised renal and cardiac transplant candidates to allow transplantation with an HLA antibody incompatible organ [209]. Since this time the use of IVIg both in desensitisation regimens and to treat antibody mediated rejection episodes has been widely reported. In terms of its use in desensitisation two main protocols have emerged, the use of high dose IVIg, usually 2.0g/kg, alone, or low dose IVIg, from 100mg/kg, in conjunction with PEX or IA [195]. The group at John Hopkins University in Baltimore first described the use of PEX with low dose IVIg for antibody removal, both for desensitisation prior to renal transplantation and as a rescue therapy for transplanted patients with acute humoral rejection, reported by Montgomery et al. in 2000 [210]. They reported a series of 7 patients treated with PEX, IVIg at 100mg/kg, tac and MMF, 3 of which were post-transplant and suffering acute humoral rejection and 4 pre transplant who had positive CDC (n=1) or flow cytometric (n=3) crossmatches against their potential living donor. The patients received 2 – 10 sessions of PEX and were transplanted once the crossmatch became negative. All 4 patients had one or more episodes of antibody mediated rejection but they reported a 100% graft survival at 1 year. Jordan et al in 2003 reported a series of 45 highly sensitised patients treated with high dose IVIg, at 2g/kg, to reduce anti-donor activity [211]. 28 of these patients had potential living donors with whom they had a positive crossmatch. In these patients IVIg was added to the

CDC XM and if a reduction in reactivity was seen the patients were given a single dose of IVIg, a further XM, and, if negative, rapidly transplanted. A similar strategy was employed with the patients awaiting a deceased donor. 42 patients were transplanted, all with a negative CDC XM but 7 remained flow XM positive. Antibody mediated rejection was seen in 31% of the patients, leading to graft loss in 7%. However patient and graft survival at 2 years was 98% and 89% respectively. Over the past decade many groups have reported similar series of patients who have been treated with a variety of desensitisation regimens which include PEX and IVIg but additionally adapted to include other agents such as rituximab and anti thymocyte globulin (ATG). Rituximab is a humanised chimeric monoclonal antibody directed against CD20 expressed on developing and mature B cells from late-pro B stage to memory B cells, but not on plasma cells. Infusion of Rituximab causes a rapid and prolonged depletion of circulating B cells and is currently licensed for treatment of non-Hodgkin's lymphoma [212] and post-transplant lymphoproliferative disease [195]. It is also under investigation for use in various autoimmune diseases as it is believed to reduce antibody production in certain situations, however since CD20 is not expressed on the main IgG producing plasma cell it is unlikely to cause immediate or potentially even long term effects on alloantibody production. It has been proposed that eliminating the early B cells would ensure that no new alloantibody producing plasma cells could be generated, and in addition there would be no B cells available to present antigen to alloreactive T cells so preventing the generation of new alloantibody producing clones [212]. Vieira et al. [213] in 2004 reported a series of 9 patients who all had a PRA of >50% and had been treated with a single dose of Rituximab. 7 of the 9 patients showed a potential reduction in PRA within 6 months of dose, although this was not confirmed using antibody specificity analysis in all cases, and one patient went on to be transplanted having converted to a negative XM following treatment. Ramos et al. investigated the splenic B cell populations in spleen samples taken from 25 patients who had been subject to various desensitisation regimens [214]. They noted that the addition of rituximab to the therapy did reduce the numbers of naïve B cells present in the spleen, but had no effect on the numbers of

memory B cells and plasma cells. Of the regimens involved the only one where a reduction in the numbers of memory B cells was seen involved the addition of anti-thymocyte globulin (ATG) to the treatment plan, however this again did not cause a reduction in the plasma cell population. ATG is a polyclonal antibody preparation generated by inoculation of animals, often rabbits, goats or horses, with human lymphocyte or thymocyte preparations. The resulting purified IgG fraction contains antibody specific to many cell surface antigens found on T and B cells, NK cells and macrophages. Its administration leads to rapid and prolonged lymphopenia. It is used both as an induction agent prior to transplantation and also as a method of treating acute rejection episodes. Stegall et al. carried out a comparison of the efficacy of three different desensitisation regimens [188]. Group 1 received high dose IVIg alone (n=13), group 2 received PEX, low dose IVIg and Rituximab (n=32) and group 3 received PEX, low dose IVIg, Rituximab and ATG (n=16). In group 1 only 38% achieved a negative XM compared to 84% and 88% in groups 2 and 3. Of those transplanted, 80% of group 1 and only 37% and 29% if groups 2 and 3 had one or more antibody mediated rejection (AMR) episodes. They concluded that the regimen used for group 3 was the most effective, however the rate of AMR post-transplant was still significant. Marfo et al. carried out an analysis encompassing the results from 21 published reports of renal transplantation following desensitisation between 2000 and 2010 [195]. There were a total of 725 patients included who were transplanted following desensitisation to remove donor specific antibody using various protocols. Overall the patient survival rate was 95% and the graft survival 86% with a median follow up time of 2 years. However, what was striking was the rate of acute T cell mediated rejection being 36% and acute antibody mediated rejection at 28%, which they reported to be significantly higher than the typical rate of <10% seen in unsensitised patients.

A new immunosuppressive agent, bortezomib, has recently been reported to be effective in treatment of acute antibody mediated rejection of renal allografts which was refractory to other forms of treatment [215]. Bortezomib is a proteasome inhibitor which can cause depletion of antibody producing plasma cells. Inhibition of the proteasome causes an

accumulation of unfolded proteins leading to cell cycle arrest and apoptosis. Plasma cells are particularly susceptible to apoptosis through proteasome inhibition due to their high protein production. In addition to its effects on plasma cells, proteasome inhibition can also prevent antigen presentation to the immune system by stopping protein degradation and therefore the production of peptides required for incorporation into the groove of HLA molecules and their subsequent presentation on the cell surface [215]. Whilst the effect of bortezomib on PRA levels of highly sensitised patients awaiting transplant has so far been reported to be minimal [216], at least in the absence of other immunosuppression, with a demonstrable ability to reduce alloantibody production in rejecting patients, it is likely that bortezomib may become incorporated into antibody removal protocols pre transplant.

Currently pre transplant HLA specific antibody testing is most useful in predicting AMR, which constitutes 5-30% of all rejection episodes [217]. However 70 – 95% of rejection episodes seen following solid organ transplantation are cellular in nature, and little can be done to accurately predict these [217]. In a recent study, Poggio et al., found no correlation between pre transplant PRA values and incidence of acute cellular rejection post-transplant [218].

Historically labs have used HLA antibody information as a marker for immune memory. However since antibodies to HLA can cross react widely between specificities due to shared epitopes, the original sensitising antigen is not always obvious. Since T cell help is required for the production of HLA specific antibody, there will be T cell memory for the initial sensitising antigen or antigens, but not necessarily for all the HLA antigens to which antibody has been produced. It would therefore be helpful to be able to identify if any of the HLA antigens presented by a potential donor are able to invoke a memory T cell response and therefore acute rejection.

1.17 T cell Memory and Transplantation

The human immune system is constantly being challenged by environmental antigens which results in the generation of multiple memory T cells. These T cells are primed to respond

rapidly upon re-exposure to the initial stimulus. Memory T cells possess a number of properties that allow for a rapid response compared to naïve T cells. Naïve T cells are limited in their migration from circulation to mainly the secondary lymphoid tissues [219]. Once stimulated, a naïve T cell, within the confines of a secondary lymphoid organ, will differentiate into either an effector T cell or a memory T cell, dependent on the signals it receives [220, 221]. These effector and memory T cells can then leave the secondary lymphoid organ and enter circulation, from which they can migrate into peripheral tissues. Memory T cells are able to migrate through both lymphoid and non-lymphoid tissues [222, 223]. In addition to a wider ability to migrate, memory T cells have fewer requirements for activation, being able to interact with their cognate antigen in the periphery potentially without the need for co-stimulation from APCs [224] and having far lower activation thresholds, allowing for responses to lower doses of antigen [225]. Expression of cytokines and their receptors, chemokines and adhesion molecules are all altered on becoming a memory T cell, which is thought to allow for more rapid migration to sites of infection and inflammation compared to naïve T cells [226]. Following an infection a population of memory T cells generated to the stimulus are maintained in circulation until re-exposure [226]. This maintenance is most likely to be due to a low level of homeostatic proliferation of the memory T cells rather than being long lived cells [226].

Immune memory to non-self HLA antigens can be generated through exposure to non self HLA via previous transplants, blood transfusions and, in women, pregnancy. Additionally, alloreactive memory T cells have been found in apparently unsensitised individuals [226]. There are two suggested routes of generation of alloreactive memory T cells without exposure to non-self HLA – cross reactivity with infectious agents, or heterologous immunity, [227, 228] and homeostatic proliferation [229]. A number of published studies have demonstrated the ability of virally induced memory CD4+ and CD8+ T cells to cross-react with alloantigen in both mice and humans [230-233]. One example, from Burrows et al. [232] demonstrated that HLA B8 restricted CD8+ T cells generated in response to an EBV infection were able to crossreact

with various allo-HLA molecules including HLA B44. The ability of these memory T cells generated through heterologous immunity to mount a rejection response has been demonstrated by Pantenburg et al. who reported that there was no difference between the rejection of skin allografts in mice when the memory T cells had been generated in response to *Leishmania* and were crossreactive, compared to those generated through previous allo exposure [234]. Homeostatic proliferation is another potential method to generate alloreactive memory T cells in the absence of exposure to non-self HLA. Here it is believed that naïve T cells are capable of proliferating and differentiating into memory like T cells, which have acquired properties associated with the memory phenotype, such as the ability to infiltrate tissues [235]. This proliferation is initiated by the individual becoming lymphopenic such as during an infection or following lymphoablative treatment for disease or transplant rejection [236]. The memory T cells generated by homeostatic proliferation appear to be less efficient in their response compared to those generated through exposure to infection or non-self HLA as Chalasani et al. demonstrated that memory T cells generated this way were less able to reject cardiac allografts compared to memory T cells generated by allo antigen exposure [235]. It is likely that memory T cells generated by alloantigen recognition via both the direct and indirect pathways will exist.

1.18 Methods to Identify Alloreactive Memory T cells.

Identification of allospecific T cells is more technically challenging than detecting HLA specific antibody. Binding of antibody to its target antigen is a relatively simple process which can be detected in the various methods described earlier. The binding and consequent activation of a T cell is a more complex process and as such more difficult to assay.

1.18.1 Mixed Lymphocyte Reaction (MLR)

Historically the mixed lymphocyte reaction, or MLR, has been used in an attempt to identify recipient T cell reactivity to a potential donor. The MLR measures recipient T cell proliferation after culture with donor cells *in vitro* using ³H-thymidine incorporation. It primarily measures

CD4 T cell reactivity via the direct pathway in cases of HLA class II mismatch [237]. It requires approximately 5 days to complete and the use of radioactivity, which would be best avoided [217]. The results achieved are also highly variable and no conclusive data have been published to indicate that the results correlate with transplant outcome [237, 238].

Additionally this method only detects the proliferation capacity of the recipient cells. However many effector cells can only proliferate poorly on stimulation despite having very powerful effector functions such as the ability to secrete cytokines [239, 240] and therefore such reactivity may be missed. Over 20 years ago variations on the MLR were introduced to assess T cell alloreactivity by analysing the ability of T cells to kill allogeneic target cells in the cytotoxic T lymphocyte assay (CTL) [239]. The CTL assay assesses CD8 T cell mediated killing of donor target cells via the direct pathway [237]. Additional variations include testing in limiting dilution in order to determine the frequency of the alloantigen reactive T cells. Here serial dilutions of responder and stimulator cells are mixed in culture and reactivity assessed by measuring proliferation, cytokine secretion or cytotoxicity [241]. Again the information gained from these assays is not always indicative of the post-transplant outcome, in addition the assays are labour intensive and the results not often reproducible [238, 239, 241].

1.18.2 Flow Cytometric Detection Methods.

A novel variation on the MLR is to use flow cytometric detection of the intracellular stain carboxyfluorescein succinimidyl ester (CFSE) which binds to intracellular proteins. The fluorescence detected is halved with each cell division and can therefore give an indication of the extent of proliferation. Flow cytometric based methods have the advantage of being highly sensitive, allowing phenotypic analysis of different cell populations, for example splitting CD4+ and CD8+ T cells, and the ability to analyse other markers simultaneously [242]. These tests however again require days of culture and are, at a basic level, only detecting cell proliferation [239].

Another flow cytometric based method for assessing T cell reactivity uses intracellular cytokine detection. Waldrop et al. [243] reported a method whereby T cells are co-cultured with antigen presenting cells, which themselves had previously been incubated with specific viral peptides. A secretion inhibitor is added, ensuring that any cytokines produced in response to this stimulation accumulate within the cells and can then be detected using fluorescent anti-cytokine antibodies by flow cytometry. This assay is relatively quick, requiring only a few hours of incubation, but it is expensive and works only when there are high frequencies of responding cells ($>1:10000$) [239]. Suchin et al. adapted this assay to assess T cell alloreactivity post-transplant in mice [244].

A more direct method to assess which specific antigens a T cell can bind to is to use fluorescence labelled multimeric complexes of peptide plus either a class I or class II MHC molecule, called tetramers. Binding of a T cell to one of these tetramers indicates that the cell possesses a TCR complementary to the peptide/MHC complex being presented. Creation of these tetramer complexes is technically challenging, so only a limited number of peptide/MHC complexes are available. In addition it has been found that binding of the T cell to a complex does not necessarily correspond with a functional effect, limiting the use of this method in analysis of the potential consequence of a transplant. [239]

1.18.3 Enzyme-Linked Immunospot (ELISPOT) Assay.

In recent years another promising method has been reported utilising the enzyme-linked immunospot assay (ELISPOT). This was first described in 1990 as a method of detecting cytokine secretion from single lymphocytes following stimulation [241]. A number of groups have since used this method to probe for alloreactivity in T cells. Briefly, ELISPOT plates with synthetic membrane bases are coated with cytokine specific capture antibodies. Responder PBLs are added along with donor stimulator cells and incubated for 24-48 hours. Activated T cells produce cytokines which are captured directly onto the well membrane. Following washing steps the cytokine production is visualised using biotinylated enzyme linked detection

antibodies. Spots develop on the membranes at the sites of the cytokine secreting cells and are then counted using a computer-assisted ELISPOT image analyser [239, 245]. The advantages of this method are that it provides functional information following a short culture period and it will detect low frequencies of responder cells. However it is expensive and only one cytokine can be measured in each well [239]. Heeger et al. reported that IFN γ production detected by ELISPOT within the first 24 hours of culture correlated with the presence of activated memory T cells and that detection of these in increasing numbers correlated with the risk of post-transplant rejection episodes [245]. Interestingly they also found that the strength of alloreactivity appeared to be independent of the level of antigen mismatching [245]. Hricik et al. used the same method to assess IFN γ production by PBLs in response to donor and 3rd party cells in serial samples taken post-transplant [246]. They reported a correlation between IFN γ production in samples taken in the early post-transplant period and subsequent renal function, independent of other factors associated with reduced graft function, namely the quality of the donor organ, effects of immunosuppressive drugs, recurrent disease and hypertension and presensitisation. They did however suggest that the level of IFN γ production may be a surrogate marker for general immune reactivity, as they also found a correlation between IFN γ production in response to 3rd party cells and poor graft outcome [246]. Danielle van den Boogaardt et al.[247] again used the ELISPOT technique to measure the production of IFN γ and IL-10 by PBLs stimulated by donor cells post-transplant in 8 patients with early biopsy proven acute rejection and 8 with stable renal function. They found the patients with rejection had significantly higher numbers of IFN γ producing cells compared to the stable renal patients, and conversely a higher number of IL-10 producing cells in the stable patients compared to the rejectors. Suggesting that IFN γ , a classical Th1 cytokine [248], indicates immune reactivity, whereas IL-10, thought by some to be a Th2 cytokine [249], may indicate a more 'tolerant' environment. This highlights the potential benefits of testing for more than one cytokine. A number of groups, including Poggio et al. [218], have adapted the ELISPOT method to create a test to define pretransplant T cell alloreactivity analogous to

the panel reactive antibody (PRA) analysis used to assess humoral sensitisation. Here they stimulate patient PBLs with a panel of donor cells covering the major HLA antigens found in the population and assess reactivity based on numbers of IFN γ producing cells to generate a so called panel reactive T cell (PRT) value. They found a correlation between PRT value and post-transplant incidence of acute rejection, however they found no correlation between the PRA and PRT values, suggesting that PRA may not be indicative of cellular immunity. A similar study published by Andree et al. confirmed both of these observations [250]. Both groups also found a correlation between the PRT level and length of time on dialysis prior to transplant [251], indicating that long term dialysis patients may have a generally heightened immune status prior to transplantation. Of note, two retrospective studies found the predictive value of measuring IFN γ producing cells pre transplant was negated by the use of ATG induction therapy, which suggests that ATG may be able to inhibit memory T cell responses [252, 253].

All of the above reported ELISPOT assays are thought to be testing only the direct allorecognition pathway, the dominant pathway in the early alloimmune response. Since memory T cells may have been primed via either the direct or indirect pathway, and the production of alloantibody requires T cell help via the indirect pathway, assessment of indirect memory T cells prior to transplant would be useful. The ELISPOT assay has also been adapted to do this by Najafian et al. [254] and Poggio et al. [255]. Here they measured T cell reactivity to cytoplasmic membrane protein preparations from donor cells, which had been processed and presented by recipient antigen presenting cells. Both groups reported higher numbers of IFN γ producing T cells in patients with poor graft function indicating chronic rejection, compared to those with stable function.

1.19 T cell Cytokines and Rejection.

When naïve T cells are first activated they are commonly thought to only produce IL-2 for at least the first 24 hours and require 3 – 6 days before complete differentiation into effector cells, which are then capable of secreting other cytokines such as IFN γ , IL-4, IL-5, IL-10.

However, it has been reported that prior to production of IL-2, naïve T cells initially produce TNF α before differentiation and development of other effector functions [256]. Unlike naïve cells, memory T cells are able to produce all these cytokines within hours upon reactivation. This difference in cytokine production allows the differentiation between naïve and memory T cell responses in culture [245]. The cytokines produced also give an indication as to the cell type responding. There are three widely recognised CD4⁺ T cell subgroups – Th1, Th2 and Th17 – each with distinct functions and cytokine production profiles [257]. Commitment of a naïve T cell to one of these three lineages is dependent on the cell interaction with the APC, the cytokines to which they are exposed, the costimulatory molecules involved and the type of antigen [258]. Th1 cells produce IL-2, IL-3, IFN γ and TNF α and act against intracellular pathogens. Th2 produce IL-4, IL-5, IL-6, IL-9 and IL-13 and are involved in humoral and cell mediated immunity [259]. Th-17 cells produce IL-17, IL-21 and IL-22 and are thought to be mainly primed to fight bacterial, fungal and protozoal infections [258]. All three of these cell types have been implicated in rejection of renal allografts [257, 259, 260]. Historically it has been believed that alloreactive Th1 cells induce rejection and alloreactive Th2 cells induce tolerance, and therefore the classical Th1 cytokines are associated with rejection and Th2 cytokines with tolerance. However evidence is mounting that both Th1 and Th2 cells are capable of inducing allograft rejection [261].

There is much data in the literature to implicate Th1 cells with allograft rejection [262]. Upon activation Th1 cells produce IL-2, which promotes proliferation of alloreactive cytotoxic T cells. They activate other cell types to manifest their effector functions, such as delayed type hypersensitivity response by macrophages. They are also able to directly damage the allograft through Fas/Fas ligand mediated cellular cytotoxicity [260].

IFN γ , along with IL-2 and TNF α , are classically seen as Th1 cytokines. However it is also produced by NK cells, Tregs and CD8⁺ T cells [263, 264], so the presence of IFN γ cannot be directly linked to Th1 activity [257]. As outlined in the ELISPOT review above, several studies

have associated the production of IFN γ with acute and chronic rejection. However this is not always the case. Sadeghi et al. found that levels of IFN γ in the serum of renal allograft recipients at 24 months post-transplant was significantly higher in patients who had not experienced rejection episodes compared to those with chronic rejection and healthy controls [265]. Using animal subjects, Wang et al. found that IFN γ knockout mice rejected xenografts faster than the wild type mice [266]. This leaves the role of IFN γ as slightly confused with potential effects in both rejection and induction of tolerance.

Although classically Th2 cells, and the cytokines they produce, have often been associated with tolerance or acceptance of a graft [261], the ability of Th2 cells to induce allograft rejection has been demonstrated by a number of studies which have shown that adoptively transferred Th2 cell lines are able to induce rejection in animal models [260]. IL-5, produced by Th2 cells, recruits and activates eosinophils in transplanted tissue [267]. There have been a number of studies indicating that both IL-5 and eosinophils may be present during rejection episodes [268]. IL-4 is a prototypic cytokine produced by Th2 cells and promotes B cell proliferation, Ig class switching and survival of T cells [269, 270], in addition it can induce macrophage activation, promote Th1 responses [259] and induce increased expression of adhesion molecules on endothelial cells [267]. It is also produced by NK cells, mast cells, eosinophils and basophils [259]. In clinical transplantation studies IL-4 has been associated with chronic renal allograft rejection. Uboldi de Capei et al. reported that in renal transplant recipients, development of chronic allograft rejection was associated with a high IL-4 producing genotype [271]. Whereas D'Elios et al. reported that CD4⁺T cell clones isolated from human kidney allografts during acute rejection produce high levels of IFN γ upon restimulation *in vitro*, but do not produce IL-4 or IL-5, indicating that IL-4 produced by T cells may not be involved in acute rejection episodes [272].

Th17 cells are thought mainly to be involved in directing other effector cells to act in infections caused by extracellular pathogens [257, 273]. Th17 cells, as a separate lineage from Th1 and

Th2 cells, were first described by two groups in 2005 [274, 275]. They are defined by their ability to produce IL-17, although it can also be produced by other T cell subsets, including CD8+ T cells, and have been identified to play major roles in the pathogenesis of inflammation and various autoimmune diseases, where IFN γ and Th1 cells had been previously thought to dominate [260, 273]. The cytokine, IL-17, had been described 10 years earlier by Yao et al. [276] and investigations into its involvement in transplant rejection had been started long before the exact producing cell had been identified. Van Cooten et al. [277] reported that renal allograft biopsies taken during active rejection episodes from 6 patients could all be stained positive for the presence of IL-17 compared to none of the biopsies taken pre transplant or from a stable control group of patients. Loong et al. [278] reported the presence of both IL-17mRNA in renal biopsies and IL-17 protein in the urine of patients with borderline rejection when compared to healthy controls. A number of other studies have linked the presence of IL-17 and IL-21, another classical Th17 cytokine, to both acute and chronic rejection [260, 279]. Additionally 2 groups working with murine cardiac allograft models have described the finding that IL-17 producing cells are able to mediate rejection in mice unable to mount Th1 responses [280, 281]. IL-17 is thought to mediate inflammation through recruitment and activation of neutrophils [257, 282], so the involvement of IL-17, and the Th17 cells, in allograft rejection is likely to be through the recruitment of neutrophils into the graft [283]. After transplantation of an organ neutrophils are one of the first effector cells to infiltrate the allograft and act as effector cells in rejection [260].

The various cytokines produced by these three T cell subsets, as well as having effector functions in graft rejection also act to regulate the responses by the other subsets. So, for example IFN γ and IL-4 inhibit Th17 cells [257] and the Th2 cytokines, in particular IL-4 and IL-10, are able to inhibit Th1 responses [260]. Each cell type and cytokine has at various stages in research been linked to both acute and chronic rejection, as well as a number of reports of links with tolerant profiles [260].

1.20 Impact of Immune Memory on Transplantation.

Immunological memory to non-self HLA antigens presents a number of challenges in solid organ transplantation. Terasaki et al. reported in a study of graft survival rates from living donor transplants that the 3 year survival of husband to wife transplants was 87% if there had been no pregnancies, however this dropped to 76% if there had been previous pregnancies in the couple [284]. This suggests that previous exposure, and potentially generation of immune memory, can impact on long term survival of the graft. Historically graft survival rates in patients receiving their second or subsequent cadaveric donor renal allografts have been lower than those receiving primary grafts [285-287]. However with the advent of better HLA matching, more sensitive crossmatching and HLA specific antibody screening methods the long term graft survival rates are now similar between first and second transplants [288]. In a single centre study Coupel et al. reported that, overall, recipients of second transplants had a graft survival rate similar to that of the primary graft recipients at 10 years. However all patients included in this study received cadaveric grafts, which compared to living donors, allowed for greater matching and avoidance of HLA specificities to which the patients had antibody, with 64% of the re-graft group receiving organs with ≤ 2 HLA mismatches, out of a possible 6 recorded, compared to only 24% of the primary graft group [289]. A review of the outcome data from UNOS 1997-2002 indicates that patients receiving a second or subsequent graft from a living donor have poorer outcomes compared to primary allograft recipients unless the donor and recipient are well HLA matched [290]. This would suggest that regardless of the donor source outcomes of second and subsequent renal allografts have poorer long term outcomes when HLA mismatching is greater. Many patients awaiting a second renal allograft are sensitised due to either rejection of the first graft, or due to withdrawal of immunosuppression following non-immune failure of the first graft, which gives the immune system full ability to mount a cellular and humoral response against the residing non-functioning graft. Patients who return to the transplant waiting list for a second graft will, if offered, receive a transplant which is generally well matched and lacking those mismatches to

which antibody has been produced, however the wait could be long and an offer may never be made. These patients may well have a potential living donor available. However living donors tend to be less well matched and may well harbour HLA antigens to which the potential recipient is sensitised. It is these pairs to whom antibody removal may be offered as an alternative to waiting for transplantation through the deceased donor scheme or paired exchange scheme.

One problem facing laboratories supporting transplantation is that it is not always possible to know exactly which non-self HLA a patient has been previously exposed to. Patients receiving multiple blood transfusions could have been exposed to countless non-self or mismatched HLA antigens, and responded to some, or all, of these, however the HLA types of these donor blood units would not commonly be known. Additionally, in patients who have been sensitised by pregnancies in earlier life, it is not always possible to gain the HLA types of previous partners, and/or children, and therefore the exact HLA antigens to which the patient has been exposed are not available. This is also true for patients receiving transplants many years previously, as HLA typing techniques were not as advanced and minimal information may be available. In practice highly sensitised patients will often not have one single sensitising event and may in fact have received previous transplants, had multiple blood transfusions and pregnancies, making identification of all the non-self HLA antigens to which they have been exposed virtually impossible and therefore predicting which HLA antigens will invoke a strong memory response equally impossible. In addition, as discussed earlier, not all memory T cells are generated through exposure to non-self HLA, so knowledge of previous HLA exposure may still not accurately predict the presence of donor specific memory T cells in a recipient.

It is widely reported [157, 291-294] that not all HLA specific antibodies are deleterious to a transplanted organ. Even looking back to the seminal work by Terasaki and Patel, only 80% of the patients with a positive CDC XM suffered hyperacute rejection. Whilst this is a high number, why didn't the other 20% of the transplants suffer the same fate? As has been

proven with time the CDC XM test used in this first report is not very sensitive and detects only high titre complement fixing antibodies, generally believed to be detrimental to graft outcome, yet only 80% of these transplants failed immediately. The present day Luminex single antigen methods give the highest and most sensitive resolution of donor specific antibody available, yet there is still no clear conclusion as to which DSA will be detrimental and which will not. With an increasing number of patients requesting assessment for antibody removal and incompatible transplantation it is becoming ever more important to be able to identify which antibodies are the most detrimental and provide an accurate risk assessment, both to the clinicians and the patients, as to the likelihood of both successful antibody removal to facilitate transplantation and of the potential outcome post-transplant. Undoubtedly the titre of HLA specific antibody, particularly at the time of transplant, will play a part. However as the conflicting reports in the literature suggest both low and high titre antibodies have been implicated, or not, in rejection of grafts. Additionally with the introduction of the Luminex based solid phase assays what constitutes high titre is not yet clearly defined. The characteristics of the antibody in terms of its ability to fix complement may also play a part. However, with the introduction of routine C4d staining on biopsy it is clear that not all episodes of AMR appear to involve complement fixation [295]. Smith et al. on reporting an adaptation of the Luminex single antigen bead assay to detect complement fixing antibodies found that in cardiac recipients the 1 year graft survival of patients with complement fixing DSA was 29% compared to 90% for those with no DSA, however for those patients with non-complement fixing DSA it was 54%, still significantly lower than those without any DSA [148]. Wahrmann et al. using the same method carried out a similar analysis in renal recipients and found no difference in graft survival between those with complement fixing and non-complement fixing DSA, both had significantly increased rates of rejection in comparison to those patients without any DSA [296]. We suggest that it may not just be the presence of the antibody but the fact that the HLA antigen is 'known' to both the humoral and cellular arms of the immune response and can lead to activation of primed memory T cells. The antibody

produced will only be specific for a small epitope on the HLA molecule and, as discussed earlier, these epitopes may be shared by multiple HLA antigens, meaning that sensitisation through exposure to one or two foreign HLA antigens can lead to the generation of antibody to multiple HLA molecules. However, are all these antibodies equal? We will investigate if in fact only patients challenged with the same mismatched HLA antigen to which the antibody was raised are at higher risk of failure following an antibody incompatible transplant due to the presence of B and T cell immune memory, compared to those in whom the antibody is reacting due to epitopes shared with previously mismatched antigens, and therefore lacking at least the T cell memory.

Traditionally when assessing patients for renal transplantation we aim to choose the most compatible donor available. This is achieved through HLA typing and matching, and avoiding all mismatches to which a patient has antibody, through extensive antibody screening and crossmatching, and, we suggest, incidentally avoiding HLA specificities to which T cell memory may have been generated. However in the context of antibody removal transplantation, the fact that there is donor specific antibody present pre transplant automatically increases the risk that the graft will be rejected. From the HLA antibody removal studies published to date it is clear that whilst HLA antibody incompatible transplantation provides a viable treatment option for some highly sensitised patients, the rates of success vary considerably and there is no distinct rationale as to which transplants are likely to be successful and which are not.

We believe it is very important to ensure that the immune risk between the potential donor and recipient pair is extensively assessed in order to minimise the risk and provide a realistic prediction as to the success of the potential transplant to the patients, allowing an informed choice prior to proceeding to transplant. In some cases there may be more than one incompatible potential donor, extensive immune risk assessment of each would provide a better basis for choosing the donor with whom the best outcome may be achieved. A number of non-immune factors could be taken into account including age, sex and health of the donor.

However from an immunological point of view the 'immune history' of the patient is a good place to start. This should include HLA specific antibody analysis and detection of memory T cell reactivity, along with knowledge of previous exposure to non-self HLA. What the memory T cell assays discussed earlier have established is that donor reactive memory T cells, often as defined by rapid IFN γ production on stimulation, are detrimental to the long term outcome of renal transplants. However researchers have often focussed the results on the ability to predict patients in whom reduced immunosuppressive protocols may be permissible and potentially the prediction of tolerance. In this study the patients we are focussing on are those known to be highly sensitised and reactive to their donor based on HLA specific antibody detection. Often these patients have multiple potential donors to whom they are sensitised and we would like to be able to probe for memory T cell reactivity to these specific donors in order to make an informed choice as to which donor would present the least immunological barriers to transplant and potentially provide the best outcome. The very nature of HLA antibody incompatible transplantation means that all these patients will require significant immunosuppression and we need to be able to choose the 'best' donor from a 'bad bunch'.

The ELISPOT method described previously produces interesting results, however it only allows for the detection of one cytokine per well, it requires specialised equipment not found in every transplantation laboratory and it is expensive. In this project we set out to develop a method to probe for T cell memory to specific donor HLA antigens using techniques and equipment familiar to the transplantation laboratory. In addition we wanted to test for multiple cytokines produced upon stimulation in order to assess the profile of the reacting cells. The results from both the T cell and antibody assays could then be combined to provide a detailed risk assessment of the potential transplant.

Using these assays and observing the outcomes of HLA antibody incompatible renal transplants in relation to the results generated we aim to investigate the effect of immune memory on the response to HLA antibody incompatible renal transplants.

1.21 - Aims and Objectives

The aims of this project are to:

1. Develop a laboratory testing repertoire to effectively assess the potential for antibody removal in sensitised patients.
2. To assess the outcomes of these transplants for the effect of immune memory to HLA antigens presented by the donor.
3. To develop an assay to effectively detect the presence of donor specific memory T cells in a recipient pre-transplant.
4. To assess the relationship between presence of HLA specific antibody and donor specific T cell memory.
5. Generate a rationale using these results to provide a detailed risk assessment pre transplant as to the likelihood of success, and highlighting which patients will be at highest risk of post-transplant rejection episodes and graft failure.

2 Materials and Methods

Complete methods used for the individual tests are described in full in Chapter 3 for antibodies and Chapter 4 for T cells.

All work was carried out using clinical samples taken for routine testing. Where tests were performed over and above that required routinely only cells or serum surplus to requirements were used, therefore no samples were taken specifically for this project. Ethical approval for this project was obtained from the Outer South East London Research Ethics Committee under project number 07/H0805/42. Where cells from deceased donors were used all donor families had consented to the use of samples in research.

The majority of the laboratory work for the antibody assays and all the laboratory work for the T cell assays was performed by myself. In the latter stages of the project, where the antibody assessment methods were introduced into the routine work, the staff in the serology section of the laboratory, under my supervision, performed the practical aspects whilst I performed the data analysis.

2.1 Patient Data Collection.

In order to assess the effect of immune memory and the presence of HLA specific antibody on renal transplant outcomes, data from a large group of patients needed to be collected. The main emphasis of the analysis is centred on the patients in whom there is demonstrable donor specific antibody, based on single antigen bead analysis and/or flow crossmatching and the requirement for antibody removal. However in order to analyse these in the context of outcomes seen in all groups of patients at our centre, data for control groups was also collected.

The HLA antibody incompatible transplants included in this analysis were performed at Guys Hospital between May 2005 and October 2012. Due to the wide geographical area and number of referring renal units that are served by our laboratory to ensure sufficient data

could be collected the various control groups collected were limited to patients who received both their transplant and subsequent follow up at the Guys Hospital based renal unit only. Data for all transplants performed between January 2005 and December 2010 were collected. During this time a number of ABO blood group incompatible and combined HLA antibody and ABO blood group incompatible transplants were performed, to ensure that records of adverse events and the outcome data were not skewed by the presence of ABO incompatible transplants all these were excluded from the analysis.

For the investigations into the effect of immune memory in relation to antibody the patients were categorised into 6 groups based on their sensitisation status and the presence of repeat HLA mismatches presented by the transplanted organ. Repeat mismatches were defined as HLA antigens to which the patient had previously been exposed either via a previous transplant or pregnancy. Clearly some patients in the sensitised group who had neither recorded transplants or pregnancy may have been sensitised via blood transfusion, however routine HLA typing of blood units is not performed and could not be accounted for, these patients were therefore allocated to the no repeat mismatch group. Additionally it was only possible to include patients as having pregnancy repeat mismatches when a pregnancy was recorded, there may well be female patients in the no repeat mismatch group who have had unrecorded pregnancies. A breakdown of the six groups and the numbers of patients involved are presented in table 2 below.

Table 2 - Description of Patient Groups Studied.

Group Number	Status	Number of Patients
Group 1	HLA antibody negative, repeat mismatch negative	169
Group2	HLA antibody negative, repeat mismatch positive	5
Group 3	3 rd Party HLA specific antibody positive, repeat mismatch negative	53
Group 4	3 rd Party HLA specific antibody positive, repeat mismatch positive	11
Group 5	Donor HLA specific antibody positive, repeat mismatch negative	31
Group 6	Donor HLA specific antibody positive, repeat mismatch positive	23

Various data were collected for each of these patients. Clinical data were obtained from the hospital provided Electronic Patient Record (EPR) system. HLA related data were obtained from our local database system, initially ORD and subsequently Manzen. Data collected included transplant date, donor and recipient HLA type, gender and age, type of donor (living or deceased), relationship of donor and recipient in the case of living donors, recipient sensitisation status, details of previous sensitising events - including previous transplants and pregnancies with the HLA mismatches involved, induction therapy used, dates and outcomes of any 'for cause' biopsies performed, date and cause of graft loss if it occurred, and graft and patient survival up to 5 years post-transplant. Additionally in the HLA antibody incompatible groups the crossmatch relative median fluorescence (RMF) and LABScreen single antigen bead median fluorescence intensity (MFI) values pre-treatment, for those requiring antibody removal, and pre transplant for all these patients were collected.

For the investigations into immune memory and T cells similar data were collected, where patients were identified as being sensitised through their HLA antibody status, the HLA types

of the recipient and donor were recorded and the presence of any potential repeat HLA mismatches identified through comparison with the HLA types of previous donors, partners or children. For the patients assessed using the T cell assay developed, who were subsequently transplanted, the occurrence of T cell mediated rejection episodes were also recorded in addition to the induction therapy received.

The biopsy results for both Chapter 3 for antibody investigations and Chapter 4 for T cell investigations were taken from analysis and diagnosis by our local histopathologists. When relevant the cause and time of graft loss was also confirmed. The presence of rejection on biopsy was diagnosed by the histopathologists using the most recent Banff criteria, available at the time of the biopsy, and for the purposes of this analysis I have included all biopsies regarded as 'suspicious of' either antibody or T cell mediated rejection as representing a biopsy proven rejection episode in addition to those where the diagnosis was absolute. All rejection episodes were broadly divided into those with antibody mediated rejection, those with T cell mediated rejection and those where both antibody and T cell mediated mechanisms were observed. The date of each biopsy proven rejection episode (BPRES) was recorded and the time between transplant and the 1st BPRES was calculated. The initial biopsy results were provided for clinical reasons and were therefore read unblinded. However the biopsies from patients who received HLA antibody incompatible transplants were subsequently re-analysed by a different histopathologist in a blinded fashion to confirm diagnosis.

HLA typing of both donors and recipients was undertaken by the DNA section of our laboratory using LIFECODES SSO HLA typing kits produced by Gen-Probe for the Luminex. These kits provide low to medium resolution HLA typing.

2.2 Statistical Analysis.

All statistical analysis carried out for this project was performed using the GraphPad Prism 6 statistical software package.

The data used for all comparative tests was analysed for Gaussian distribution using the D'Agostino-Pearson normality test. Due to the small and diverse populations the majority were not found to be of Gaussian distribution and were therefore subject to non-parametric analysis. The caution attached to these test types are that they are of lower power and are likely to infer less significance than the reality, particularly in small sample sizes. Where appropriate, two-tailed analysis was used with a 95% confidence setting.

Comparisons of antibody readout values between two groups of patients were analysed using the non-parametric Mann-Whitney U test, which analyses the significance between median values of two groups. Where more than two groups were analysed together the non-parametric one way ANOVA test, Kruskal-Wallis, was employed. The p values generated through these calculations were recorded.

The relationship between predicted and actual values, along with the relationship between values achieved with two methodologies, were assessed using linear regression analysis and the R^2 value recorded. R^2 is a value between 0 and 1, the closer the value is to 1 the greater the relationship. If R^2 is 0.0 there is no relationship between the two variables.

Survival analysis was performed using Kaplan-Meier survival curves where patients were censored at the end of their follow up period, if less than five years, or if an adverse event, such as death or graft loss, occurred that was not related to immunological loss of the renal allograft. The survival curves were compared using the Mantel-Cox logrank method where two curves were compared or the logrank for trend when three or more curves were compared. The logrank test and logrank for trend test both produce p values, which were recorded.

For analysis of cytokine production in the T cell assay described in Chapter 4 the data were arranged in 2x2 contingency tables and subject to Fisher's exact 2 tailed analysis, where the null hypothesis was that there is no difference between the two outcomes in the two groups that would not be expected to be seen in random population sampling. This method is ideal

for small sample sizes, as tested in this study. P values were generated, where a small P value indicates that the correlation observed would rarely happen through random population sampling, that the null hypothesis is incorrect and that there is a difference in results between groups that is large enough to be significant. A large P value, up to 1.0, indicates that there is no significant difference between the two outcomes in the two groups other than that which would be expected through random population sampling.

3 Antibodies in HLA Antibody Incompatible Renal Transplantation and their Relationship to Immune Memory.

3.1 Introduction and Aims

In 2005 the clinicians at our centre became interested in antibody removal as a route to transplant highly sensitised patients, initially those who were in urgent need of a transplant due to a failure of access for dialysis, but subsequently those who were highly sensitised and were unlikely to be offered a transplant via any other route. Central to the success of any antibody removal programme is the ability to monitor donor specific antibody levels to assess the success of the desensitisation treatment and to provide advice on when the antibody is of a suitable level to perform the transplant. In addition to this it was felt that it was essential to develop the ability to predict which patients would benefit from antibody removal and what the likelihood of a successful post-transplant outcome would be. Therefore the aims for this part of the project were -

- Introduction and validation of new Luminex technology for antibody monitoring in highly sensitised patients.
- Development of methods to assess patient and donor pairs for potential antibody removal.
- Assessment of post-transplant outcomes following antibody incompatible transplantation in relation to antibody titre and the effect of immunological memory.
- Generation of a risk assessment model based on the results collected above.

3.2 Methods.

As outlined in the introduction there are various methods available to detect and identify HLA specific antibodies. At this unit we use flow cytometry for donor crossmatching and bead based solid phase assays for HLA antibody detection and identification. As outlined in the introduction these methods are sensitive, timely and provide objective results on which clinical decisions can be based. Three main methods have been employed when gathering data for this project –

- FlowPRA single antigen bead screening
- LABScreen single antigen bead screening
- 3-Colour Flow Cytometric crossmatching

Initially I shall describe each method and then refer back to these when outlining the exact testing carried out.

3.2.1 FlowPRA Single Antigen Antibody Identification Method.

Prior to starting this project FlowPRA single antigen beads were the local antibody identification method of choice. FlowPRA Single Antigen beads are produced by OneLambda (Canoga Park, CA) and allow for rapid flow cytometric detection of HLA specific antibodies. The bead mix consists of a number of different bead groups with varying fluorescent properties which can be separated on the FL2 channel based on their channel shifts. 4 vials, each containing 8 bead groups, are provided to cover the panel, with each having 7 different HLA specific beads plus a control bead group, giving a total of 28 different HLA antibody specificities to be detected. Following incubation with patient sera, binding of any HLA specific antibody is detected through the addition of a FITC-conjugated anti-human IgG antibody, which is then detected through shifts on the FL1 channel. FL1 shifts for each bead group are then compared to the equivalent bead group tested with the negative control sera. The median channel value (Mdx) for the negative control is subtracted from that achieved with the

test sera to give a final Mdx value, and in general the greater this value is the greater the titre of antibody present in the serum. The method used in this laboratory is a locally validated variation on the manufacturers' guidelines, allowing the use of fewer beads and increasing the number of tests achieved from one kit. The results gained using this variation have been validated by comparison with results achieved using the kit manufacturers' instructions, with previously well characterised positive and negative patient sera. The method is the same for both Class I and Class II single antigen beads. Briefly –

A 96 well 'v' bottomed titre plate is labelled numerically for each serum sample plus a negative and positive control, with each sample being allocated 4 wells, one for each bead mix.

Each vial of beads is mixed well and 2µl is added to the bottom to the appropriate well.

10µl of patient sera is then added to each of the four designated test wells and mixed well by repeat pipetting.

The plate is then sealed, wrapped in aluminium foil and incubated at room temperature for 30 minutes on an orbital mixer set at 200rpm.

Following incubation the beads are washed by adding 150µl of kit provided wash buffer, diluted 1:10 with distilled water to provide a working concentration. The plate is sealed and centrifuged at 3450g for 3 minutes. The waste is then gently decanted and, remaining inverted, blotted on absorbent paper.

The wash step is repeated a further 2 times to give a total of 3 washes.

A working dilution of FITC-conjugated anti-human IgG is produced by diluting the conjugate 1:100 with wash buffer. 50µl is then added to each well.

The plate is re-sealed and wrapped in aluminium foil before a further 30 minute incubation at room temperature on an orbital mixer set at 200rpm.

After incubation the plate is centrifuged at 3450g for 3 minutes and the diluted conjugate decanted as previously described.

The beads are then washed twice with 150µl of wash buffer as previously described.

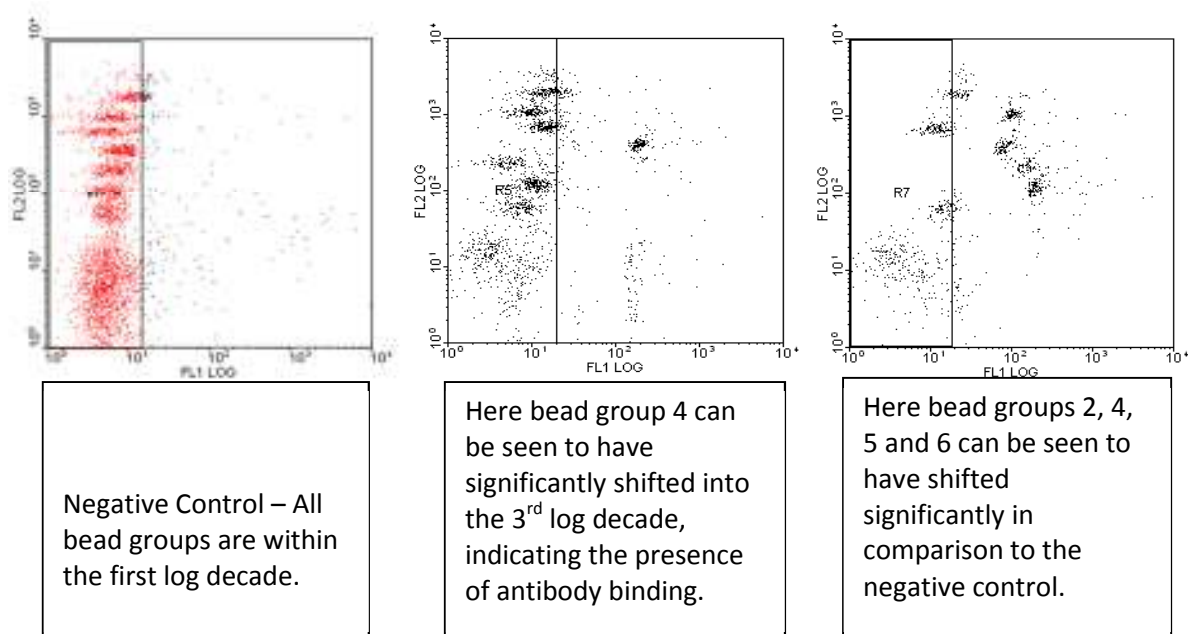
Following the decanting of the final wash buffer the plate is transferred to a fume cabinet and 200µl of 1% Formaldehyde is added to all wells and mixed well.

The plate is then sampled using a Beckman Coulter FC500 flow cytometer.

Median Channel Fluorescence (Mdx) values for each bead group are automatically exported to an Excel file for data analysis.

The sample Mdx minus the negative control Mdx values are calculated along with visual comparison of the bead plots. Observation of any shift in the control bead group can give an indication as to non-specific antibody binding to the beads.

Figure 14 - Example of dot plots gained from flow cytometric analysis of FlowPRA single antigen beads.



3.2.2 LABScreen Single Antigen Screening Method.

The start of this project coincided with the introduction of Luminex based antibody analysis methods to our local testing repertoire. LABScreen single antigen beads are again produced by

OneLambda (Canoga Park, CA) and are designed to be analysed using a Luminex fluoroanalyser as described in the introduction. All samples included in this analysis were tested using our in-house validated variation on the manufacturers recommended method. The method used is the same for both the Class I and Class II specific beads.

Briefly –

Wells on a 96-well filter plate are vertically labelled for the number of samples to be tested, including wells for both a negative and positive control.

Any unassigned wells must be covered using plastic plate sealers (alpha laboratories).

In the test wells the filter is dampened by addition of 250µl of sterile water and incubated at room temperature for 5 minutes. After this time water is completely removed using a vacuum manifold system.

The vial of test beads is then well mixed and 3µl are added to each test well.

12µl of patient or control serum is then added to the bead suspension and mixed by repeat pipetting.

The plate is then covered and wrapped in aluminium foil before being placed on an orbital plate mixer set at 500rpm to incubate for 30 minutes at room temperature.

Following this incubation the beads are washed by the addition of 250µl of kit provided wash buffer that has previously been diluted to a concentration of 1:20 with distilled water. The wash buffer is removed using the vacuum manifold. This wash procedure is repeated a further two times to give a total of 3 washes.

After the final removal of wash buffer 100µl of manufacturer provided PE conjugated anti human IgG is added, having been previously diluted 1:100 with wash buffer.

The plate is then re-covered and wrapped again in aluminium foil prior to a further 30 minute incubation on the orbital plate mixer set at 500rpm at room temperature.

After the incubation the beads are again washed. Initially 150µl of wash buffer is added to each well and the wells drained using the vacuum manifold. A further two washes each using 250µl of wash buffer are then performed.

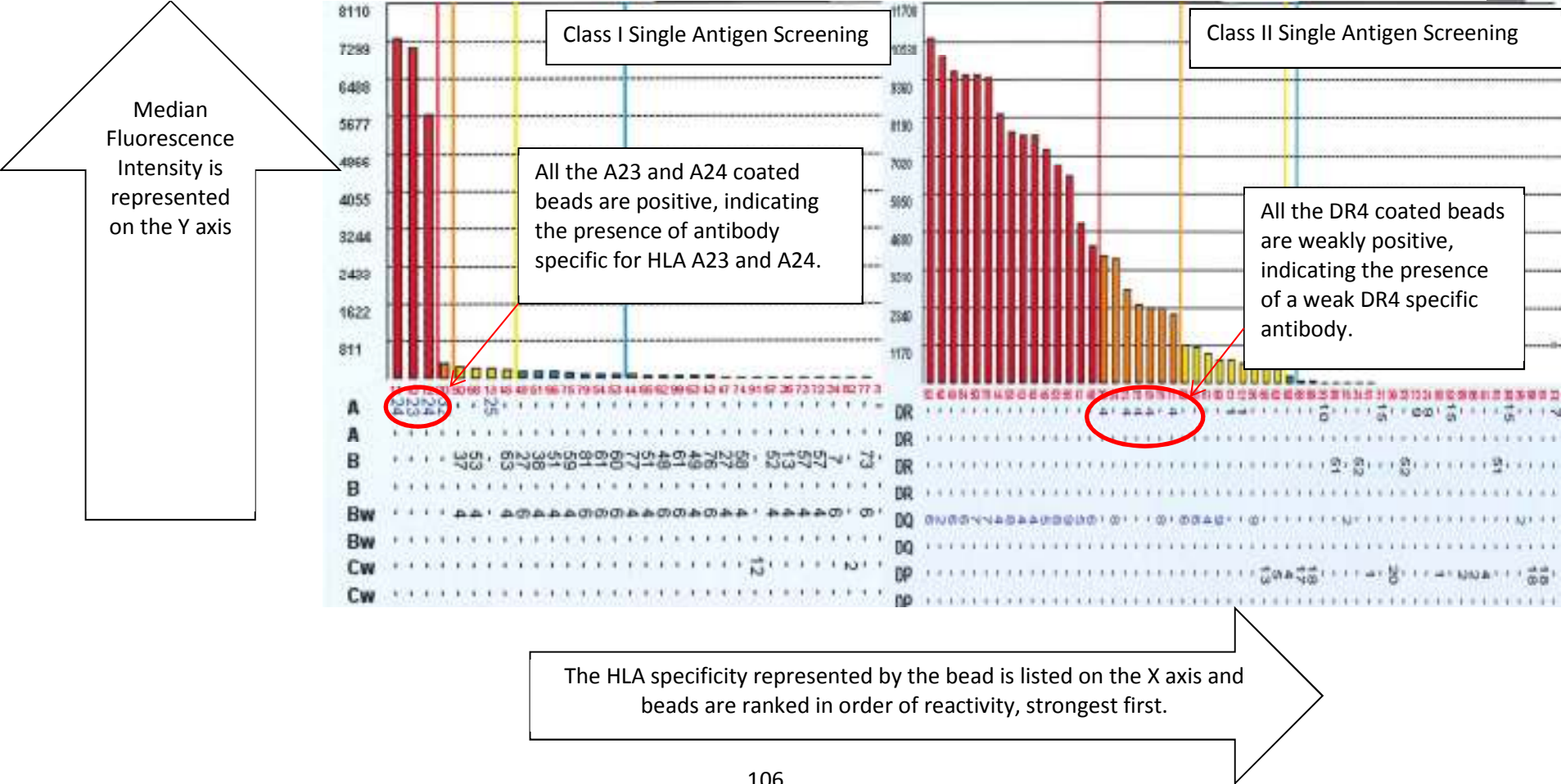
Following the final wash the beads are re-suspended in 80µl of phosphate buffered saline before being read using a Luminex analyser.

As discussed in the introduction the Luminex analyser collects a range of data from the bead sets in use, the most commonly referenced of which is the median fluorescence intensity (MFI). The Luminex output from each run is published as a .csv file which can then be imported for analysis using an appropriate software package. For the initial group of patients analysed this was performed using HLA Visual which was later replaced with HLA Fusion, both produced by OneLambda for analysis of all their Luminex kits. Both software packages can use the same formulas to calculate results. Therefore the results achieved with both programmes are comparable. Both programmes are fully interactive for the user, and whilst they have pre-programmed values and specificity suggestions they both allow manual setting of cut-off ranges and assignment of specificities. The reactivity of each test bead is 'corrected' for non-specific binding using the value gained with the internal negative control bead, specific for each test well, and also for background binding or fluorescence of each bead by accounting for binding seen with the negative control serum run with each batch of tests. This calculation is carried out by the analysis software which then produces bar graphs ranking the beads in order of the highest to lowest MFI values. Initially the kit manufacturer recommended that beads with an MFI value of greater than 1000 be considered as positive. Analysis should however consider results previously achieved, the pattern of antibody binding in terms of conforming to known crossreactive or shared epitopes, the presence of apparent 'self' reactivity and the HLA antigens involved in known sensitising events before specificities are assigned. The profile of the bar charts can also be useful in assigning cut-off levels for a patient, where obvious demarcation between groups of beads lends itself to act as a cut-off

point. At our centre we look to place the cut-off between 1-2000 MFI but depending on the profile presented and the patient history this can be increased or decreased.

An example of the results produced can be seen in figure 15 below.

Figure 15 - Example of results produced by HLA Visual and HLA Fusion software when analysing LABScreen single antigen Beads.



3.2.3 3-Colour Flow Crossmatching.

At Guys Hospital the flow crossmatch (FXM) has been our front line crossmatching method for 20 years and as such we have robust cut off criteria based on long term clinical outcomes.

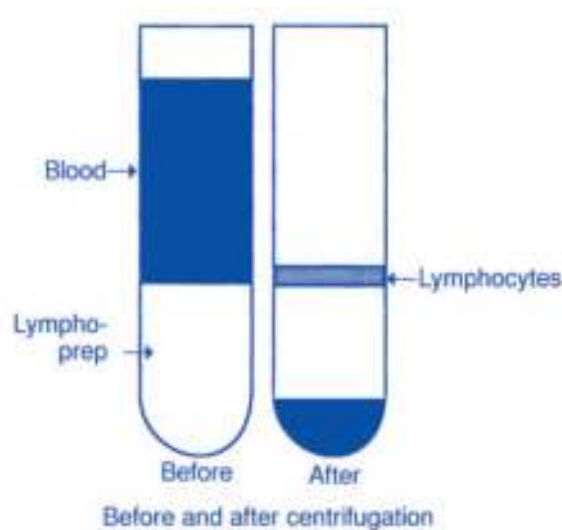
3.2.3.1 Isolation of Peripheral Blood Lymphocytes.

Lymphocytes are isolated using density centrifugation on a ficoll gradient from 20mls EDTA anticoagulated fresh donor peripheral blood samples. Briefly,

Peripheral blood was diluted at a ratio of 1:1 using phosphate buffered saline (PBS) (Sigma-Aldrich), layered onto equal volumes of density centrifugation medium lymphoprep (Axis-Shield), and centrifuged at 1690g for 15 minutes, see figure 16 below.

The isolated lymphocyte layer was removed by pipette and the cells washed twice using PBS, centrifuging at 700g for 3 minutes to recover the cells each time.

Figure 16 - Diagram depicting lymphocyte isolation using Lymphoprep (Axis-Shield)



The cells were then washed for a third time using locally produced Flow Diluent comprised of PBS, 0.1% Foetal Bovine Albumin and 0.1% Sodium Azide.

Following removal of the wash buffer the cells are re-suspended in Flow Diluent to a concentration of 5×10^6 – 5×10^7 cells/ml.

3.2.3.2 Flow Crossmatch Method.

All samples are tested in duplicate, using a 3-colour flow crossmatch method.

5ml Falcon tubes, two per serum sample to be tested, including a negative and positive control, are labelled appropriately.

The negative control is commercially prepared human serum provided by the National Institute of Biological Standards and Controls (NIBSC) for flow cytometric crossmatching, using serum prepared from donations provided by blood group AB unsensitised males.

The positive control is an in-house serum preparation from a pool of at least eight highly sensitised renal patients and is rigorously tested to ensure all the major specificities are included and that positivity can be expected with all donor cells encountered.

To the bottom of each tube 20µl of serum is added, using a fresh pipette tip for each sample and ensuring the serum is well mixed prior to pipetting.

30µl of PBLs are then added to the side of each tube and the tubes briefly centrifuged to 400g to bring the cells to the bottom of the tube.

The cells and serum are then mixed well using a vortex and incubated at room temperature (20-25°C) for 30 minutes.

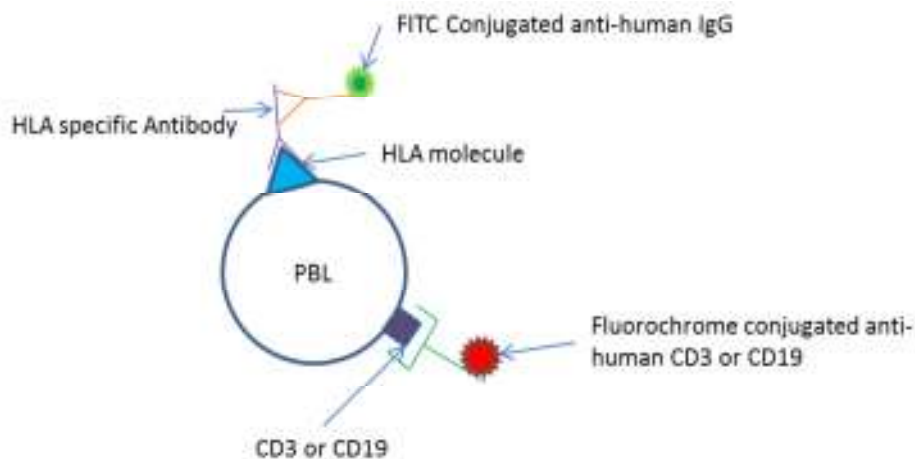
Following incubation 2mls of Flow Diluent is introduced into each tube and the tubes centrifuged at 400g for 3 minutes to recover the cells. The wash buffer is then vigorously decanted and the cells washed a further two times in the same manner.

After the third wash and removal of the wash buffer 4µl of FITC conjugated polyclonal rabbit anti-human IgG F(ab')₂ antibody (Dako) is added to the bottom of each tube, using a fresh pipette tip for each addition. Following this 8µl of a 1:1 'megamix' of PE conjugated monoclonal mouse anti-human CD3 antibody and PE-Cy5 conjugated monoclonal mouse anti-human CD19 antibody (both Dako) is also added to the bottom of each tube, again ensuring a fresh pipette tip is used for each addition.

The tubes are then mixed well using a vortex and then incubated at 4°C for 30 minutes.

Following incubation the cells are given a final wash using 4ml Flow Diluent and centrifuging at 400g for 3 minutes. The wash buffer is decanted and the cells are resuspended in 500µl of Flow Diluent ready for analysis using a Beckton Dickinson FACSCalibur flow cytometer.

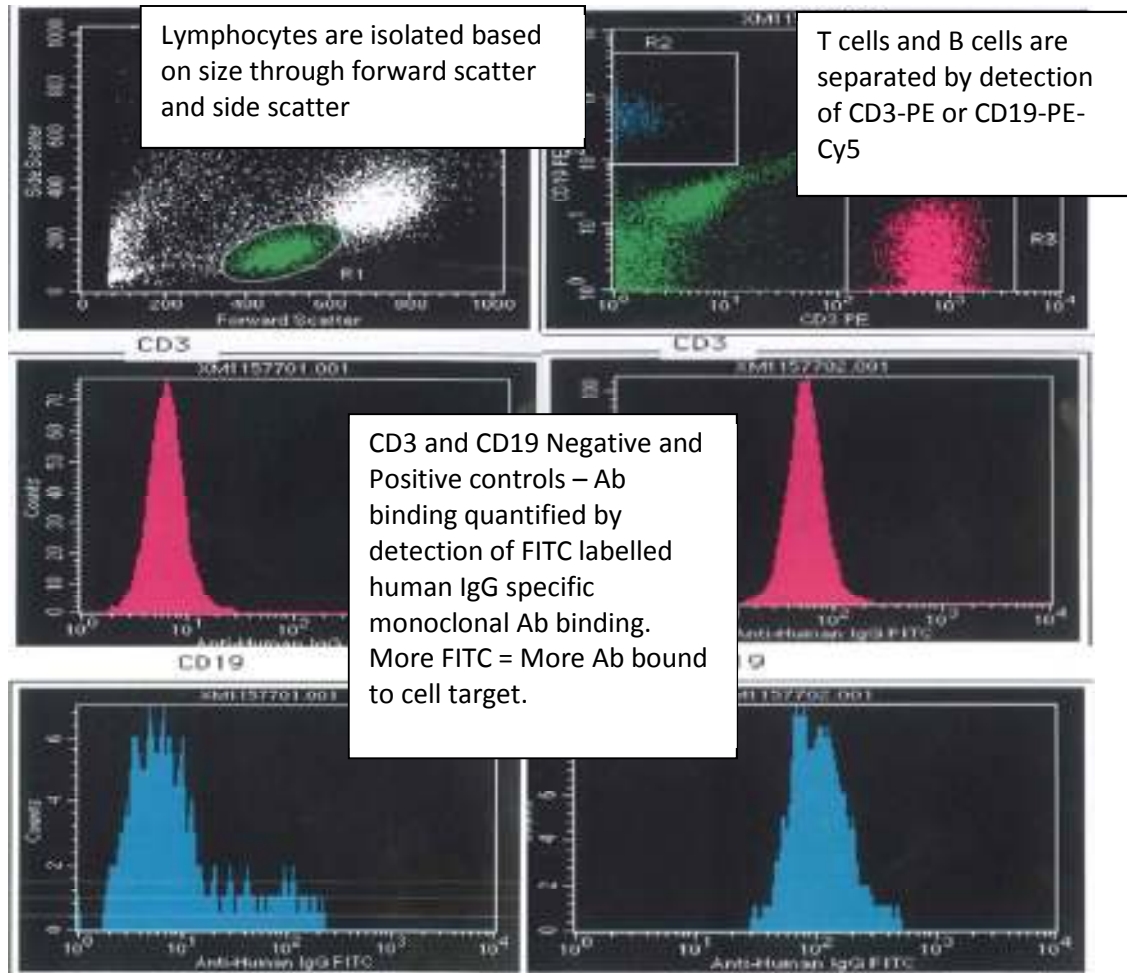
Figure 17 - Diagram Depicting Staining Used in the 3-Colour Flow Crossmatch Technique.



On flow cytometric analysis the lymphocytes are initially identified based on size through forward scatter and side scatter, all further analysis is based on the cells in this region (R1). The cell type can be identified as either a CD3 expressing T cell (R3) or CD19 expressing B cell (R2) through fluorescence emitted from either PE for the CD3 or PE-Cy5 for the B cell. For FXM using PBL, where B cells are in lower proportion, cell counting continues until a minimum of 1500 B cell events in R2 have been collected. Any recipient derived IgG type antibody in the serum specific for an HLA molecule expressed by the donor will have bound to the donor PBL, and this will be detectable by the fluorescence produced by the FITC conjugated to the anti-human IgG polyclonal Ab when passed through a laser. An example of the readout achieved with a negative and positive control can be seen in figure 18 below. The fluorescence emitted by the FITC is collected on a log scale and the median channel (MDX) at which the fluorescence is detected is collected as the readout for that sample, the higher this channel the more FITC

conjugated anti-human IgG is bound to the cell and therefore the greater the amount of recipient derived donor specific antibody is bound to the cell.

Figure 18 - Example Readout for Negative and Positive Controls seen with 3-Colour FXM technique.



The Mdx for both the T cells and B cells for each tube is collected and the duplicates compared to ensure they are within the limits of reproducibility, to be within 80% comparability, and the average of the two values is taken.

The relative median fluorescence (RMF) for each cell type, for each serum sample tested, is then calculated by dividing the Mdx from the sample with the Mdx achieved with the appropriate cell type in the negative control.

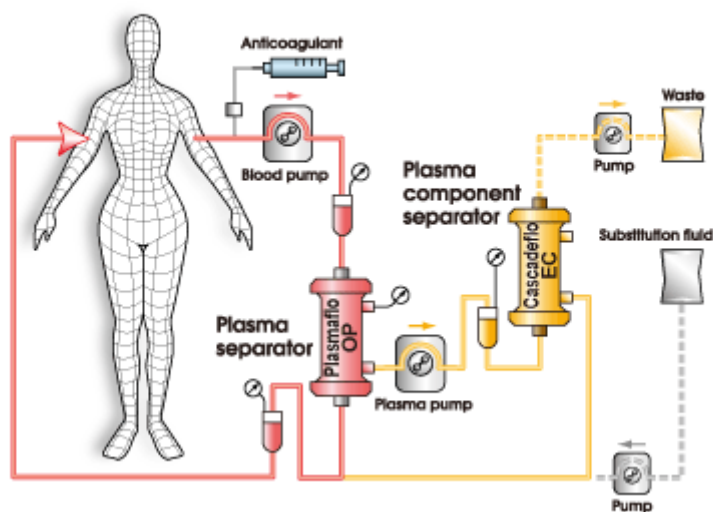
$$\text{Relative Median Fluorescence (RMF)} = \frac{\text{MDX WITH PATIENT SAMPLE}}{\text{MDX WITH NEGATIVE CONTROL}}$$

In our centre for crossmatches against living donors any RMF ≥ 2.3 is deemed positive and potentially a contraindication to transplantation. To ensure test validity the positive control must achieve an RMF of ≥ 4.0 .

3.3 Development of the Titre Crossmatch

The initial patients in whom antibody removal was attempted at our centre were all approached in a manner similar to that reported by many centres. Patients, identified as a candidate for Ab removal by the clinical team, often due to clinical urgency, underwent antibody removal indefinitely until a negative crossmatch was achieved, with either their designated living donor or a deceased donor when no living donor was available. This approach led to very long and costly treatment periods and in a number of cases was not rewarded with a transplant, which was disappointing for both the patient and the clinical team. The general regimen adopted included PEX, low dose IVIg and in some cases rituximab. Whilst PEX is generally well tolerated side effects can occur including reaction to the replacement albumin or fresh frozen plasma, hypotension, thrombosis, cardiac arrhythmias and myocardial infarctions [297], some of which were experienced by our initial patient group and became more common with increasing sessions of PEX. The first change made was to replace traditional PEX with double filtration plasmapheresis (DFPP). DFPP is similar to PEX in that the plasma is initially separated from the blood cells. However instead of then being discarded and the volume replaced, the plasma is filtered for a second time, through a smaller filter size which allows the passage of plasma components with smaller molecular weights, <70000 molecular weight, but traps and removes those with larger molecular weights, such as antibodies [298, 299]. The filtered plasma, minus the immunoglobulins, can then be returned with the blood cells to the patient. DFPP was initially developed as an alternative to PEX for the specific removal of immunoglobulins without the requirement for replacement of large volumes of fluid with Albumin [300].

Figure 19 - Diagram Representing the Mechanical Process of DFPP [202]



The second area for change was highlighted following discussion with our renal unit, where it was decided that a more stratified approach was required whereby patients with living donors were assessed for the feasibility for antibody removal during a work-up period prior to commencing the treatment. Central to assessment process was the ability to predict which patients would benefit from antibody removal and, if so, how much treatment would be needed prior to transplant. From our experiences with the initial patients it was apparent that not all antibodies appeared to be equal in terms of ease of removal, a finding that has been confirmed by other groups [301]. So it was decided that the method by which the most information regarding the likely success of antibody removal would be achieved would be to perform a single 3L volume antibody removal procedure and then assess the level of antibody that had been removed and from this predict if sufficient antibody could be removed and if so the number of sessions that would be required pre transplant in order to achieve a negative crossmatch.

At Guys Hospital we feel that the flow crossmatch gives the most accurate reflection of donor and recipient antibody compatibility of all the antibody analysis tests available. This is both in terms of detecting antibody that will bind to the actual HLA molecules presented by the donor and the level of reactivity, which may well reflect donor expression levels of HLA, which cannot

be accurately assessed by SPA since in vivo these levels can vary both over time, between individuals and in response to common drug treatments such as statins [302]. Whilst HLA expression levels on PBLs may not be a true reflection of expression in the kidney it is likely to be more comparable than antigen representation on the SPA formats, particularly the single antigen assays, where beads are coated in very high concentrations of single HLA specificities.

In order to assess the possibility of achieving successful antibody removal to facilitate an antibody incompatible transplant we aimed to develop and assess the use of a titre FXM using recipient serum samples taken immediately prior to, and after, a single 3L DFPP and assaying against the potential living donor. We hoped to gain an indication as to whether the reactivity was reduced, and, if so, predict the number of DFPP sessions required to achieve a negative crossmatch prior to transplant. Discussion with the clinicians involved centred around what would be an acceptable crossmatch result required before a transplant could proceed. It was decided that, for at least the early part of the programme, a negative crossmatch, so an RMF of <2.3 for both T and B cells, would be required, this was later expanded to an RMF of <4.0 , the local cut off for deceased donor FXM, in patients with harder to remove antibodies. Therefore the aim of the titre crossmatch assessment would be to predict the potential number of DFPP sessions required to achieve this.

3.3.1 Titre FXM Method.

The Titre FXM method follows the same technique used for the standard 3-colour FXM at Guys. The variation is in the serum samples used.

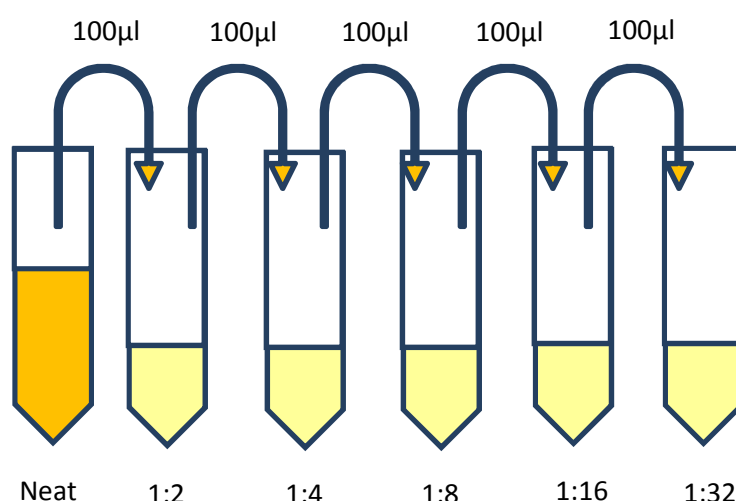
The pre and post serum samples are titred using serial dilutions of the patient serum in negative control serum (NIBSC). Samples were titred to 1:32 pre PEX/DFPP and 1:16 post PEX/DFPP with the rationale that antibody levels with significant reactivity beyond these levels could not be reduced to negative with a realistic number of DFPP sessions pre transplant.

In addition to a donor based crossmatch an auto FXM was also performed using the samples at neat only to ensure any reactivity observed was donor specific and not non-specific reactivity.

3.3.1.1 Serial Dilution of Serum Samples.

Nine 250µl eppendorff tubes were labelled pre – 1:2, 1:4, 1:8, 1:16 and 1:32, Post – 1:2, 1:4, 1:8 and 1:16. The initial serum storage tube for each sample was taken as the neat. To each of the eppendorff tubes 100µl of negative control serum (NIBSC) was added. 100µl of the appropriate patient serum was then added to the first, 1:2, tube and mixed well. From this a further 100µl was taken and added to the 1:4 tube and mixed well. This process was continued until all the dilutions were prepared, represented in figure 20 below.

Figure 20 - Diagram representing serial dilution of serum samples.



From this point each dilution was treated as an individual serum sample and the titre FXM set up following the standard 3-colour FXM protocol above, with a total of 26 assay tubes, two for each serum sample plus two for the negative control and two for the positive control. The results were collected and the RMF values calculated for each serum dilution. The reduction in antibody strength, based on a reduction in the RMF value at neat and/or in the dilution at which a negative result was achieved, was assessed and the potential number of DFPP sessions required to achieve a negative flow crossmatch pre transplant calculated. Where either the T or B cell crossmatch was stronger, as demonstrated by positivity to a higher titre against one of the cell populations, the number of DFPP sessions predicted was based on the strongest

crossmatch result. When the RMF value at 1:16 dilution of the post removal sample was not negative, i.e. the RMF was greater than 2.3 for this sample, it was reported that it would not be possible to remove the antibody to a level that would allow for a negative crossmatch pre-transplant. Where a reduction was seen and the crossmatch became negative at titres of 1:16 or below on the post removal sample the predicted number of antibody removal sessions was calculated based on a similar reduction of titre being expected with each antibody removal session. As had been observed with our original antibody removal patients, and previously discussed in the introduction, there is often a 'bounce back' in antibody levels seen, starting a few hours after the end of the antibody removal session. This is believed to be due to synthesis of new antibody and movement of antibody in the extravascular spaces into the circulation. To account for this an additional antibody removal session was added to the total number required pre transplant. For example, a reduction from 1:16 to 1:8 would indicate a requirement for 4 DFPP sessions pre-transplant, based on each session reducing the strength by one titre, with an additional session to cover possible antibody 'bounce back', giving a total predicted number of pre transplant antibody removal sessions of 5. A reduction of 1:8 to 1:2 would indicate a requirement of two sessions to achieve a negative crossmatch and then an addition treatment to cover potential bounce back, giving a total of 3 sessions. If however the titre decreased but the RMF values at neat remained unchanged then the number of antibody removal treatments recommended would follow the calculation above, with the additional caveat that the requirement for further antibody removal treatments could not be excluded.

3.4 Results

3.4.1 Introduction and Validation of Luminex Technology for Monitoring Donor Specific Antibody in Highly Sensitised Renal Transplant Recipients.

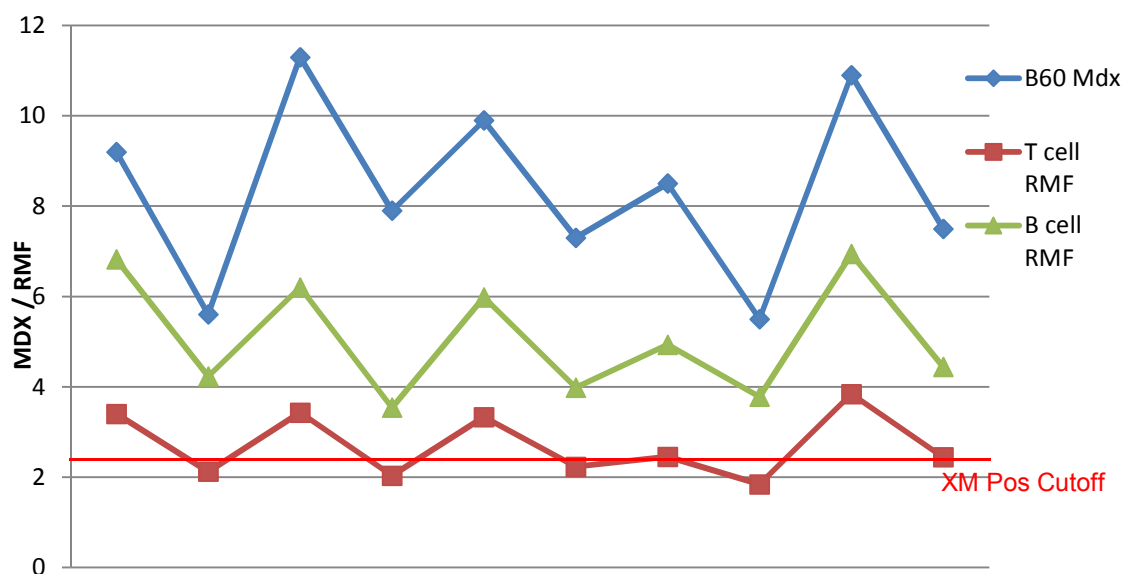
3.4.1.1 Scene setting background work prior to project

At the time of commencement of this project the Renal Unit at Guys Hospital had transplanted 3 highly sensitised patients following antibody removal. As part of the process our laboratory had been monitoring the levels of donor specific antibodies using our routine method of choice at the time, FlowPRA single antigen detection beads, in conjunction with numerous donor flow crossmatches both pre and post-transplant. However with our commitment to embracing new technology and service improvement the decision to introduce Luminex technology was taken. As part of the enormous validation process required, the assessment of the use of LABScreen single antigen beads to monitor HLA DSA in antibody removal patients was required.

Samples were selected from these three patients, who had previously been screened using the current FlowPRA single antigen method and both T and B cell flow crossmatches. Previous monitoring of these patients using the FlowPRA bead method in combination with flow crossmatches had highlighted the fact that changes in fluorescence detected on FlowPRA beads generally followed those of the flow crossmatch, giving similar indications as to the increasing and decreasing trends of the donor specific antibody levels. This therefore allowed post-transplant monitoring of DSA to be carried out using FlowPRA beads, rather than repeated crossmatches, and using the values achieved to correlate back to pre transplant crossmatches and predict if the antibody had reached levels at which a positive crossmatch would be expected were it to be performed. A representative example of such monitoring can be seen in figure 21 below which shows both T and B cell flow crossmatch RMF and corresponding FlowPRA single antigen bead fluorescence values from a patient undergoing

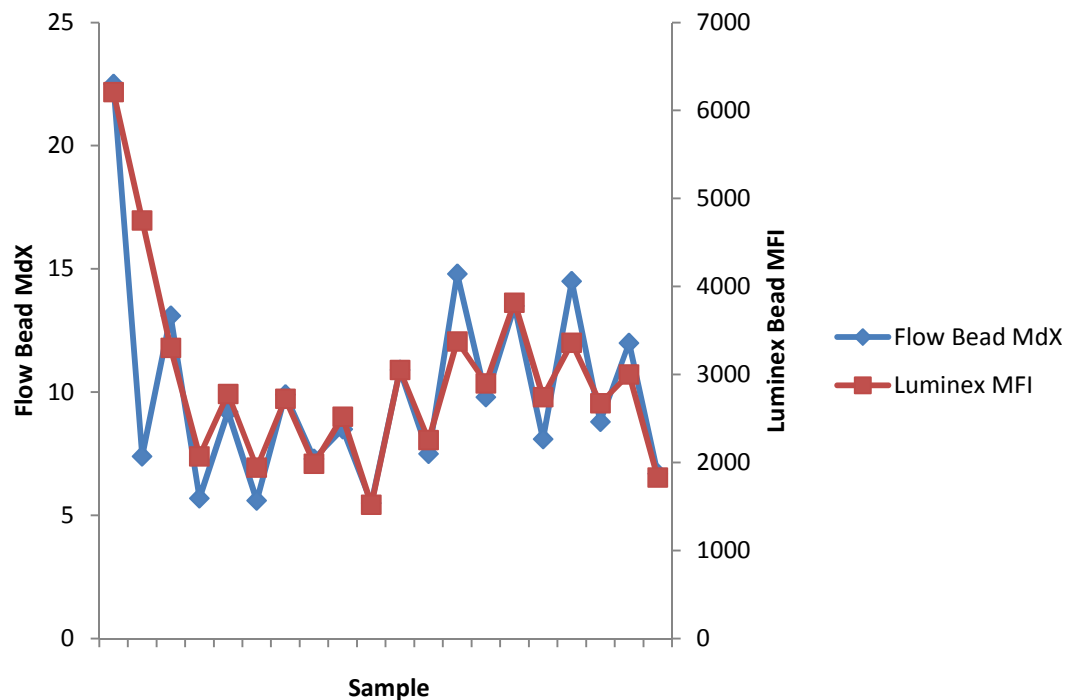
removal of an antibody specific for the mismatch HLA B60. Since the antibody involved is to a Class I mismatch both the T and B cell crossmatch results are relevant. The trendlines for all three data sets can be seen to follow similar patterns of increasing and decreasing values.

Figure 21 - Serial samples from a patient undergoing antibody removal measured using FlowPRA single antigen beads (MDX) and T and B cell flow crossmatch (RMF).



During the validation of the LABScreen single antigen beads these samples were analysed using the new method and the results compared to those achieved previously to assess the ability of the new single antigen beads to be used in the same manner. A representative example of the results achieved can be seen in figure 22 below, where serial samples from the same patient featured in figure 21 above who was undergoing HLA specific antibody removal, focused on removal of HLA B60 specificity, were tested using both FlowPRA and LABScreen single antigen methods.

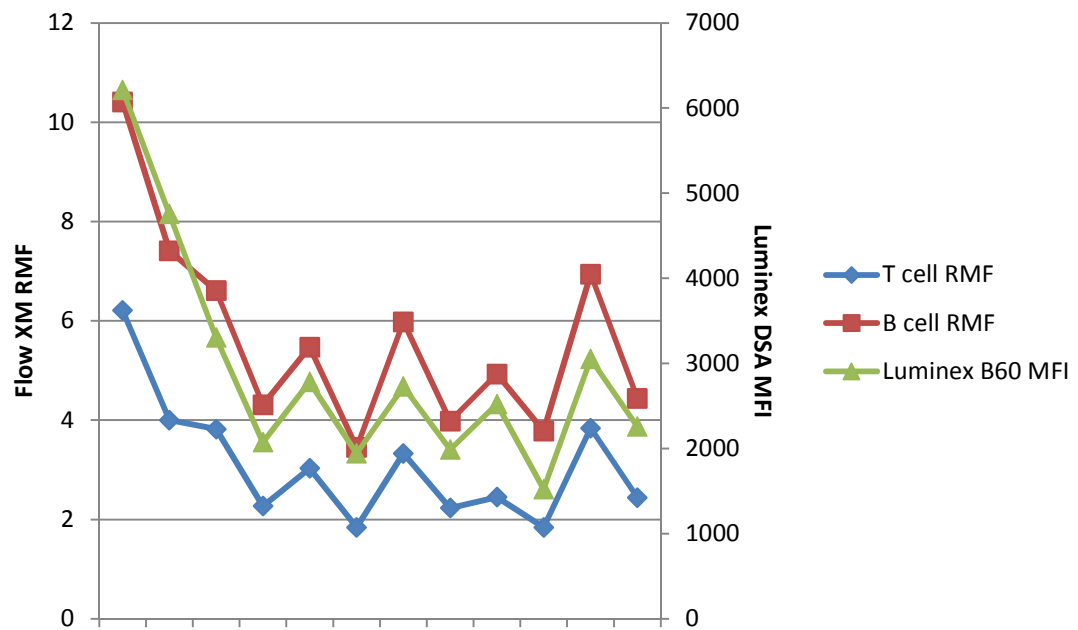
Figure 22 - Comparison of FlowPRA (Flow bead) MdX values and LABScreen (Luminex) MFI values gained from testing of serial samples for the presence of HLA B60 specific Antibody.



As can be seen in figure 22 above the results achieved with both kits showed very similar changes in fluorescence between each sample, as indicated by changing MdX or MFI values. This indicated that LABScreen single antigen beads could be used in the same manner as the FlowPRA beads to monitor changes in donor specific antibody levels.

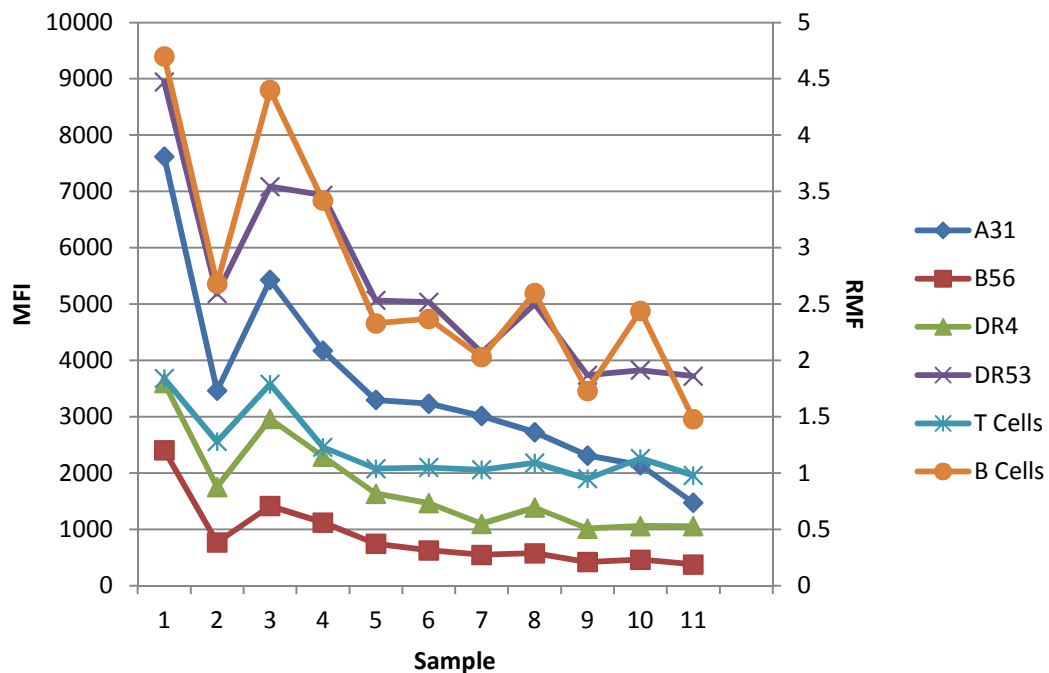
This was further confirmed by comparing the trend in LABScreen MFI values with that seen for the flow crossmatch RMF values. An example of this can be seen in figure 23 below, which, using samples from the same patient described in figures 21 and 22, plots the T and B cell RMF values achieved on crossmatching on the same chart as the MFI values for HLA B60 from the LABScreen single antigen beads, the antibody of interest, and allows comparison of the trendlines from the two tests.

Figure 23 - Trends in donor specific antibody levels as monitored by LABScreen (Luminex) single antigen beads and T and B cell flow crossmatching (RMF).



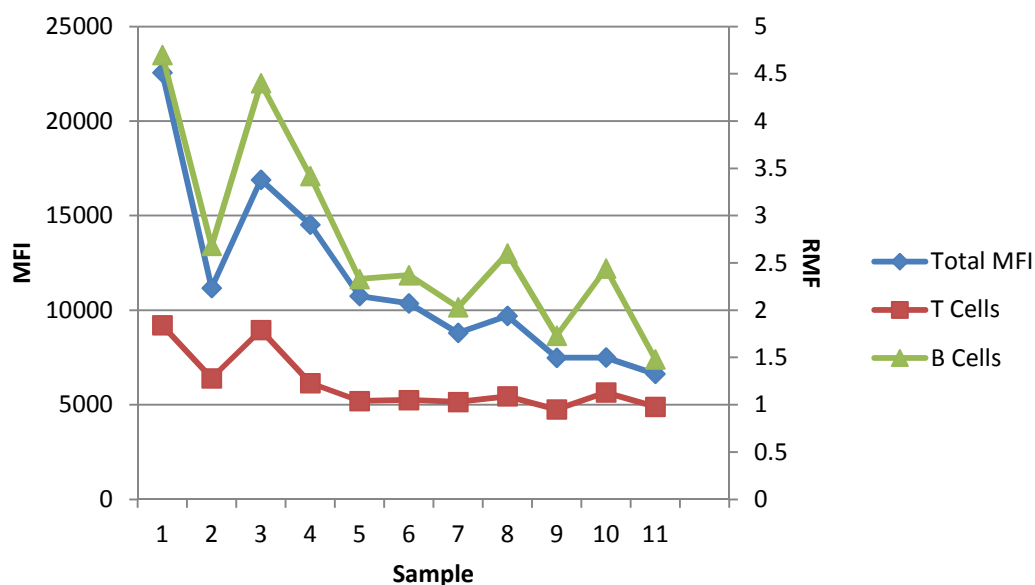
The examples above were from a patient undergoing antibody removal for a single donor specific antibody directed at HLA B60. To confirm that LABScreen single antigen MFI values could be equally useful in monitoring a number of donor specific antibodies in one patient similar analysis was carried out using serial samples from a patient with potential donor specific antibody to HLA A31, B56, DR4 and DR53. The trend comparison of the individual MFI values with the T and B cell flow crossmatch RMF values can be seen in figure 24 below.

Figure 24 - Comparison of MFI values for multiple donor specific antibodies (A1, B56, DR4 and DR53) with RMF values from T and B cell flow crossmatches from testing serial samples from one patient.



Whilst the trends are clearly comparable, visually the changes in the different specificities are complicated. As the T and B cells express multiple HLA antigens on their surface, compared to just the single specificity as seen on the beads, I decided to investigate if the cumulative MFI value, achieved by addition of all the individual MFI values, would also correlate with the antibody level trends indicated by flow crossmatching. Figure 25 below represents the same data seen in figure 24 above, but the individual MFI values for each donor specific antibody have been combined to give a total DSA MFI value, plotted against the RMF values achieved with the T and B cell flow crossmatches. In this example it can be seen that to ensure the antibody is at a level that would be negative on flow crossmatch, $RMF < 2.3$, the combined MFI of the beads would need to be less than 9000 for this patient donor pair.

Figure 25 – Data presented in Figure 24 but simplified by calculating the cumulative MFI values for multiple donor specific antibodies (A1, B56, DR4 and DR53) compared to the RMF values achieved with flow crossmatching on T and B cells.



These initial results indicated that the LABScreen screening methods yielded comparable results to those achieved with FlowPRA screening and that both could reflect the patterns and changes in fluorescence seen on crossmatching.

3.4.2 Titre FXM Assessment.

During this study titre crossmatches between 117 recipient and donor pairs have been performed using serum samples taken before and after a test antibody removal treatment. In addition a further 34 titre crossmatches have been carried out using a single serially diluted serum sample from patients who did not receive a test antibody removal treatment. As an ideal all the patients assessed using a test DFPP and titre FXM would first have had an initial FXM to ensure that the DSA present would be sufficient to give a positive result. However, due to time constraints, clinical urgency, and restricted donor availability this was not always possible. In these cases crossmatch results were predicted to be positive, and the need for antibody removal assumed, based on the results achieved from the antibody screening methods employed, initially FlowPRA and then LABScreen Single Antigen bead testing. In some of these cases a positive crossmatch was not found, even with the pre DFPP/PEX sample,

and these are the patients in whom a negative crossmatch both pre and post was reported. A comparison of the screening and crossmatch results will be performed in the next section.

The results for all the titre crossmatches performed can be seen in table 3 below. Each row represents a single crossmatch pair. The donor HLA mismatches to which the recipient produces HLA specific antibody, as detected by LABScreen single antigen beads, are listed, split into HLA Class I and II specificities. The flow crossmatch results are divided into those achieved with the pre treatment sample (column marked pre PEX/DFPP) and those with the post treatment sample (column marked post PEX/DFPP). The patients who did not receive a test antibody removal treatment and were crossmatched using a single pre DFPP/PEX sample are marked as 'nt' (not tested) in the post DFPP/PEX column. Listed for each recipient and donor pair are the T and B cell flow crossmatch RMF values achieved with the neat serum sample, and for each of these the titre at which the last positive crossmatch value was achieved. Those where the result was positive at neat only are marked as 'neat'. Those where the result was negative at neat are marked as 'neg'.

Table 3 - Final Titre and flow crossmatch T and B cell RMF results from 117 titre flow crossmatches between potential HLA antibody incompatible donor and recipient pairs, using serially diluted samples taken prior to and following a single antibody removal treatment using DFPP or PEX, and 34 titre flow crossmatches using single serum samples from patients not treated with antibody removal.

Crossmatch Pair	Class I specificities	Class II specificities	Pre PEX/DFPP				Post PEX/DFPP			
			T cell titre	RMF at neat	B cell Titre	RMF at neat	T cell titre	RMF at neat	B cell Titre	RMF at neat
1	A3, Cw5	DR17, DQ2	neg	1.35	neg	2.26	neg	1.28	neg	2.12
2	A3, Cw5	DR17, DQ2, DQ7	neg	1.41	neat	3.21	neg	1.16	neg	1.54
3	A32, B61	DQ7	neg	2.25	1: 2	3.92	neg	1.74	neat	2.71
4	A33, B58	DR15, 51 DQ6	neat	2.43	1: 8	8.61	neg	1.3	1:2	4.91
5	A33, B58, Cw10	DR15, DR51, DQ6	neg	1.65	1: 2	5.11	neg	1.23	neat	2.68
6	none	DR13, DQ6	neg	1.05	1: 2	3.34	neg	0.94	neg	1.23
7	B57	DR13, DQ6	neg	1.18	>1:32	11.98	nt	nt	nt	nt
8	none	DR13, DQ6	neg	1.02	1: 8	5.91	nt	nt	nt	nt
9	A31	none	neg	1.02	neg	1.25	neg	1.02	neg	1.59
10	none	DQ3, DQ6	neg	1.04	>1:32	8.43	nt	nt	nt	nt
11	none	DQ3, DQ6	neg	1.12	>1:32	11.12	neg	1.09	>1:16	9.29
12	A1, B35, Cw4	D11, DR52, DQ7	neg	1.41	>1:32	34.22	neg	1.06	>1:16	12.12
13	A30, Cw15	DR11, DP0201	neg	1.66	1: 8	9.6	neg	1.25	1: 2	5.5

Crossmatch Pair	Class I specificities	Class II specificities	Pre PEX/DFPP				Post PEX/DFPP			
			T cell titre	RMF at neat	B cell Titre	RMF at neat	T cell titre	RMF at neat	B cell Titre	RMF at neat
14	Cw15	DR11, DP0201	neg	1.46	1: 8	9.04	nt	nt	nt	nt
15	none	DQ5	neg	0.96	neg	1.5	neg	1.03	neg	1.6
16	A3	none	neat	2.52	neg	2.13	neg	2	neg	2
17	B35	none	neg	1.13	neg	1.22	neg	0.97	neg	0.93
18	A1, A3, B8, B44	DR13, DR17, DR52, DQ2	neat	2.97	1:8	8.68	neat	2.81	1: 4	6.84
19	A1	DQ2 DR53	neg	1.15	>1:32	9.33	neg	1	>1:16	8
20	A66	none	neg	2.29	1:2	3.18	neg	1.48	neg	1.69
21	A2, A3, B57	DR4, 53 DQ5	1:8	21.89	1:32	27.21	1:4	8.5	1: 8	14.62
22	A2, A3, B57	DR4, DR53, DQ5	1:2	6.19	1:8	7.14	neat	2.57	1: 2	3.46
23	B7	DP0401	neg	1.15	neg	2.21	nt	nt	nt	nt
24	A1, B52	DR13, 15 DR51,52 DQ6	neg	1.16	>1:32	6.77	neg	1.15	>1:16	6.27
25	A29, B44 Bw4 Cw5	DR7 ?DQ2	>1:32	34.42	>1:32	50.88	>1:16	21.69	>1:16	35.2
26	B7	DR15, DR51	neg	1.01	>1:32	4.05	neg	1.06	1: 8	6.38
27	A1	DQ2	neg	1.51	1:16	3.84	neg	1.35	1: 8	4.77
28	Cw5	none	neg	1.14	neg	0.86	nt	nt	nt	nt

Crossmatch Pair	Class I specificities	Class II specificities	Pre PEX/DFPP				Post PEX/DFPP			
			T cell titre	RMF at neat	B cell Titre	RMF at neat	T cell titre	RMF at neat	B cell Titre	RMF at neat
29	A2	DR11, DQ7	neg	2.21	>1:64	13.39	nt	nt	nt	nt
30	A2, B18	none	>1:32	45.73	>1:32	23.13	nt	nt	nt	nt
31	A2, B44, Cw5	DR4, DQ7	>1:32	53.06	>1:32	35.31	nt	nt	nt	nt
32	A23, B44, B64	DR7 DQ2	neg	1.41	neat	3.28	nt	nt	nt	nt
33	A3, A29, B44	DQ5	neg	1.68	>1:32	21.47	neg	1.26	>1:16	16.84
34	B18	none	neg	2.02	neg	2.03	neg	1.09	neat	10.3
35	A3, B7, B57, Cw7	DR15, DR51, DQ9	1:16	9.86	>1:32	12.61	1:2	6.26	>1:16	13.92
36	B58	none	neg	1.22	neg	1.37	neg	1.11	neg	1.33
37	B57, Cw6	DR7, DP13, DP18	neg	1.11	neg	1.63	neg	0.96	neg	1.25
38	A11	DR103, 4, 53 DQ5	neg	2.22	>1:32	23.51	neg	1.34	>1:32	17.08
39	B44	none	neg	0.96	neg	1.06	nt	nt	nt	nt
40	B35, Cw4	DQ2 DR53	neg	2.17	>1:32	8.39	neg	1.6	>1:16	8.02
41	A2	none	>1:32	24.78	>1:32	17.96	1:8	11.84	1: 8	8.09
42	A2	none	>1:32	26.9	>1:32	22.67	>1:16	16.4	>1:16	11.45
43	A32, B60,	DR53, DQ7, DQ8	1:32	23.22	>1:64	32.64	1:8	8.25	1:32	17.91
44	B63	none	neg	1.05	neg	1.84	nt	nt	nt	nt

Crossmatch Pair	Class I specificities	Class II specificities	Pre PEX/DFPP				Post PEX/DFPP			
			T cell titre	RMF at neat	B cell Titre	RMF at neat	T cell titre	RMF at neat	B cell Titre	RMF at neat
45	B8, B45	DR17, DR52, DQ2	neg	1.69	1:16	18.05	neg	1.38	1: 8	12.8
46	B45	DR17, DR52, DQ2	>1:32	8.09	1:16	9.25	nt	nt	nt	nt
47	A26, B8, B60, Cw7, Cw10	none	1:8	6.85	1:16	9.03	1:2	4.26	1:4	6.56
48	A2, B7, B8, Cw7	none	1:16	5.63	1: 2	2.26	1:4	4.17	neg	1.79
49	B18, Cw7	none	1:2	3.23	neg	2.04	neat	2.69	neg	1.98
50	A24, B44, Cw7	DR4, DR53	1:8	17.47	1: 8	8.94	nt	nt	nt	nt
51	A23, A26, B44, Cw4	DR15, DR51	1:8	21.12	1:16	20.35	nt	nt	nt	nt
52	A23, A26, B44, Cw4	DR15, DR51, DP5	1:16	24.31	1:16	14.41	1:8	14.43	1:8	10.5
53	A1, Cw7	DR15, DP51, DP10	neat	3.58	neat	6.44	neat	2.35	1:2	4.5
54	A1, B8	none	neg	2.26	neat	3.5	neg	1.42	neg	1.48
55	A29, B57	none	1:8	6.6	1: 8	6.26	1:2	2.96	1:2	3.1
56	A3	none	neat	3.01	1: 2	2.49	neg	1.61	neg	1.68
57	A36, A74, B44, B72, Cw2	DR11, DR15, DQ6	>1:32	21.21	>1:32	20	nt	nt	nt	nt
58	A1, A2, Cw4, Cw7	DR1, DR15, DR51	>1:32	25.73	>1:16	12.56	>1:16	15.72	1:8	6.92
59	A3, A32, B63, Cw7	DR15, DR51	neg	1.68	neat	2.72	neat	2.72	neg	1.5
60	A2, Cw12	DQ7	neg	1.39	>1:32	8.9	neg	1.12	>1:16	8.48

Crossmatch Pair	Class I specificities	Class II specificities	Pre PEX/DFPP				Post PEX/DFPP			
			T cell titre	RMF at neat	B cell Titre	RMF at neat	T cell titre	RMF at neat	B cell Titre	RMF at neat
61	B44, Cw5	none	neg	1.39	neg	1.27	neg	1.16	neg	0.87
62	none	DQ7	neg	1.03	>1:32	8.74	neg	0.94	>1:16	7.85
63	B8	DQ2, DQ7, DQA5	neg	1.59	>1:32	15.61	neg	1.3	>1:16	13.7
64	B8	DQ2, DQ7, DQA5	neg	2.23	>1:32	6.73	neg	1.79	>1:16	6.19
65	B8	none	neg	1.6	neat	3.16	neg	1.1	neg	1.52
66	none	DQ7	neg	1.3	1:16	3.8	neg	1.22	1:4	3.25
67	none	DQ6, DP20	neg	1.18	1:2	3.49	nt	nt	nt	nt
68	Cw7	DR11, DQ7	neg	1.27	1:2	3.31	neg	0.99	neat	2.31
69	A1	none	1:8	5.76	1:8	5.31	1:4	5.08	1:4	4.18
70	B44, Cw5	DR52	neat	3.25	1:2	4.17	neg	1.75	neg	2.13
71	A2, B13, Cw6	DR7, DQ2	neg	1.9	1:4	3.61	neg	1.17	neg	2.25
72	none	DR17, DR52	neg	0.97	neg	0.88	neg	0.89	neg	0.66
73	A2, A24, B57, B62	none	neat	3.39	1:8	6.31	neat	3.36	1:4	5.78
74	A3, A29, B45, Cw6	DQ2 DR53	1:2	4.05	1:2	4.33	nt	nt	nt	nt
75	A3, A29, B45, Cw6	none	1:2	3.92	1:4	4.7	neg	2.15	neat	2.75
76	none	DQB4, DQA4	neg	0.93	>1:32	7.1	neg	0.95	>1:16	7.01

Crossmatch Pair	Class I specificities	Class II specificities	Pre PEX/DFPP				Post PEX/DFPP			
			T cell titre	RMF at neat	B cell Titre	RMF at neat	T cell titre	RMF at neat	B cell Titre	RMF at neat
77	B7	DQ6	1:2	4.02	>1:64	16.37	nt	nt	nt	nt
78	Cw2,9	DQ5	>1:16	11.98	>1:16	19.07	>1:16	8.54	>1:16	17.81
79	A68	DR7, DQ2	1:2	6.26	1:4	8.56	neat	2.34	1:2	3.67
80	A1, A3, B60, Cw10	none	1:2	3.55	1:4	6.85	neg	2.24	1:2	3.88
81	A1	none	neg	2.17	1:2	5.08	neg	1.85	1:2	4.15
82	A3, A30, Cw9	none	neat	2.92	1:2	5.35	neg	2.11	1:2	3.35
83	none	DR11, DR13, DQ6	neg	1.43	>1:32	35.34	neg	1.43	>1:16	41.67
84	A2, B55	none	neg	1	neg	0.94	neg	1.04	neg	0.76
85	A2, B27	DR4	1:8	5.95	neat	4.94	1:2	3.35	neat	3.2
86	A24, B44, B51, Cw2, Cw16	none	1:4	3.93	1:8	4.04	neat	3.27	1:2	4.07
87	B7, B44, Cw5	none	1:8	8.25	1:8	5.93	1:2	5.1	1:4	4.73
88	none	DR8, DQ4	neg	1.36	1:16	13.38	nt	nt	nt	nt
89	A2	none	neg	1.13	neg	0.92	nt	nt	nt	nt
90	Cw10	none	1:4	5.34	1:2	2.82	neat	3	neg	1.81
91	Cw15, Cw16	none	1:2	3.48	neg	1.96	neat	2.38	neg	1.67

Crossmatch Pair	Class I specificities	Class II specificities	Pre PEX/DFPP				Post PEX/DFPP			
			T cell titre	RMF at neat	B cell Titre	RMF at neat	T cell titre	RMF at neat	B cell Titre	RMF at neat
92	none	DQ5	neg	1.11	neg	2.15	neg	1.03	neg	1.52
93	B13	DR11, DQ7	1:16	25.02	>1:32	22.02	1:4	8.54	1:8	8.96
94	B57	none	neg	1.42	neg	1.18	neg	1.29	neg	1.03
95	A1, A33, B57	none	>1:32	49.22	>1:16	52.59	>1:32	49.86	>1:16	44.51
96	A2, B57, Cw7	none	neg	1.71	neg	2.17	neg	1.07	neg	1.03
97	A68	none	1:16	24.01	1:16	16.27	1:8	10.77	1:8	9.69
98	A31, B56	DR4, DR53	neg	1.84	1:4	4.7	neg	1.28	1:2	2.68
99	B35	none	neat	2.31	neg	1.9	neg	1.41	neg	1.03
100	A2, B57	DR14	neg	2.02	neat	2.55	neg	1.23	neg	1.07
101	B44, Cw5	DP3	neat	2.42	1:2	4.2	neg	1.91	neat	3.78
102	none	DR17, DR52	neg	0.97	1:4	8.41	neg	0.93	1:4	8.43
103	A1	DQ6, DQ8	neg	1.18	1:32	2.94	neg	1.08	1:16	2.83
104	none	DR13, DQ6	neg	1	>1:32	14.09	neg	1.03	>1:16	13.02
105	B62, Cw9	DR13, DQ6	1:2	4.45	>1:32	7.83	neat	2.88	>1:16	6.35
106	B13, Cw6	DR11	1:8	5.72	>1:32	18.01	1:4	3.74	>1:16	13.8
107	A2, B44	none	neg	1.53	neg	1.55	neg	1.12	neg	1.01

Crossmatch Pair	Class I specificities	Class II specificities	Pre PEX/DFPP				Post PEX/DFPP			
			T cell titre	RMF at neat	B cell Titre	RMF at neat	T cell titre	RMF at neat	B cell Titre	RMF at neat
108	A1, A36, B18	?DR51, DQ6	1:4	5.61	>1:32	9.33	1:2	3.65	>1:16	8.58
109	A3, B72	DQ5	neat	2.9	1:4	4.35	neg	1.91	1:4	2.86
110	A2, B62, Cw10	DR4, DQ7	1:8	11.56	>1:32	13.54	1:4	6.56	>1:16	9.94
111	B44	DR15, DR51	1:4	9.34	1:16	20.03	1:2	4.9	18	10.44
112	A1	DR17, DR52	neg	1.13	>1:32	9.37	neg	1.03	1:8	10.86
113	A3, Cw7	DR4, DQ7, DQ8	1:2	4.39	1:2	5.11	neg	1.71	neg	2.02
114	A2, Cw17	DR18, DR52	1:16	9.78	1:4	4.19	1:4	5.99	1:2	3.18
115	A33, Cw14	none	neg	1.54	neg	1.38	neg	1.24	neg	1.15
116	B8	DR17, DR52	1:8	6.58	>1:8	10.31	1:2	2.67	1:4	4.17
117	B57	DR7	1:2	5.37	1:4	6.31	nt	nt	nt	nt
118	B62, Cw9	DR13, DR52	1:4	6.39	1:8	10.4	neat	2.36	1:2	4.47
119	A2	none	>1:32	8.1	>1:32	8.55	1:8	5.26	>1:16	6.51
120	A2	none	1:16	5.57	>1:32	9.81	nt	nt	nt	nt
121	B53	DP1, DP9	neg	1.2	1:8	4.92	nt	nt	nt	nt
122	A3, B60	DR8	1:4	8.64	>1:32	27.97	neat	3.62	>1:16	16.03
123	none	DR11	neg	0.98	1:4	2.95	neg	0.97	neat	2.51

Crossmatch Pair	Class I specificities	Class II specificities	Pre PEX/DFPP				Post PEX/DFPP			
			T cell titre	RMF at neat	B cell Titre	RMF at neat	T cell titre	RMF at neat	B cell Titre	RMF at neat
124	A1	none	neg	1.07	1:2	3.43	nt	nt	nt	nt
125	none	DQ2	neg	1.21	1:8	5.28	neg	1.1	1:4	4.17
126	A2	DR4, DQ7	neg	1.28	1:32	13.89	nt	nt	nt	nt
127	none	DR4, DQ7	neg	1.41	1:32	14.15	neg	1.08	1:8	9.06
128	none	DR15, DR51, DQ6	neg	1.2	1:16	14.14	neg	0.98	1:4	8.2
129	B57, B58	none	neat	2.34	1:4	5.73	nt	nt	nt	nt
130	B57	none	neg	2.22	1:4	4.94	neg	1.77	1:4	3.98
131	B44	none	1:2	4.66	1:4	6.58	neat	2.8	1:2	4.26
132	A2, B44, Cw5	DR4, DR53	>1:32	30.99	>1:32	60.77	>1:16	19.82	>1:16	36.59
133	Cw5	none	1:2	3.57	1:2	4.05	nt	nt	nt	nt
134	A1, A3, B7, B8	none	1:32	12.81	1:64	6.28	1:16	8.09	1:32	6.18
135	A31, B44, B60, Cw5	DR1, DR8, DQ4, DQ5	>1:32	20.73	>1:32	29.95	>1:16	21.3	>1:16	29.96
136	B7	DR15, DQ6	1:4	5.23	1:32	18.94	nt	nt	nt	nt
137	B7	DR15, DQ6	neat	3.23	1:16	16.5	neg	1.91	1:8	10.62
138	A1, B8, Cw2	DR16, DR17, DR51, DR52	neg	1.68	>1:32	22.27	neg	1.2	>1:16	21.84

Crossmatch Pair	Class I specificities	Class II specificities	Pre PEX/DFPP				Post PEX/DFPP			
			T cell titre	RMF at neat	B cell Titre	RMF at neat	T cell titre	RMF at neat	B cell Titre	RMF at neat
139	none	DR13, DP3	neg	1.48	>1:32	18.02	neg	1.31	>1:16	15.75
140	Cw6	none	1:8	4.05	1:8	3.7	nt	nt	nt	nt
141	Cw6	DR12, DR52	1:4	4.55	1:2	3.61	1:2	3.73	neat	2.82
142	A23, A80, B57, Cw18	none	>1:32	13.89	>1:32	5.23	>1:16	12.95	>1:16	4.33
143	A3, B35, B51, Cw15	DR1	1:16	14.51	>1:32	21.95	1:4	8.79	>1:16	19.33
144	A3	none	1:8	11.11	1:8	6.73	nt	nt	nt	nt
145	B35	none	neg	1.27	neg	1.49	neg	1.12	neg	1.26
146	B57	none	neg	1.35	1:2	2.66	nt	nt	nt	nt
147	A24, B44	none	neg	1.26	neg	1.26	nt	nt	nt	nt
148	A26, A32, B35, B60, Cw10	DQ7	1:4	5.15	1:16	11.39	1:2	3.4	1:8	7.84
149	A11	DQ6	1:2	4.06	1:32	11.88	neat	2.73	1:16	9.72
150	none	DR53	neg	0.96	>1:32	4.37	nt	nt	nt	nt
151	A11, Cw5	DR11, DR52	neg	1.84	1:8	9.22	neg	1.76	1:4	5.43

3.4.3 Analysis of the Predictive Value of the Titre Crossmatch assessment.

Of the patients assessed using this method, 31 went on to be transplanted with an organ from the donor with which they were assessed. Table 4 below summarises the titre crossmatch results using pre and post test PEX/DFPP samples. Listed for each donor and recipient pair are: the pre and post test antibody removal T and B cell RMF values at neat, and titre to which positivity reached using these samples from the original test antibody removal titre flow crossmatch assessment, number of antibody removal sessions predicted to be required pre transplant using the original titre flow crossmatch assessment results ,as previously discussed, and the actual number of antibody removal sessions that were required prior to transplant to achieve a negative crossmatch. The values in the final two columns are the numbers of antibody removal sessions, predicted or required respectively. Where no antibody removal was either predicted or required '0' is listed. None of the patients, who following assessment using the test antibody removal and titre XM method, and being found suitable for an antibody removal transplant, have subsequently failed to receive a graft following antibody removal. Three patients were treated with induction therapy of the anti-CD20 mAb Rituximab, which is known to generate false positive B cell flow crossmatches due to binding to CD20 expressed on the B cell surface and, due to its chimeric nature, being detected as a bound antibody by the anti-human IgG FITC conjugate. As, at our centre, Rituximab is given one month prior to transplant these patients could not be assessed by flow crossmatch prior to transplant and therefore their results will not be included in the following analysis.

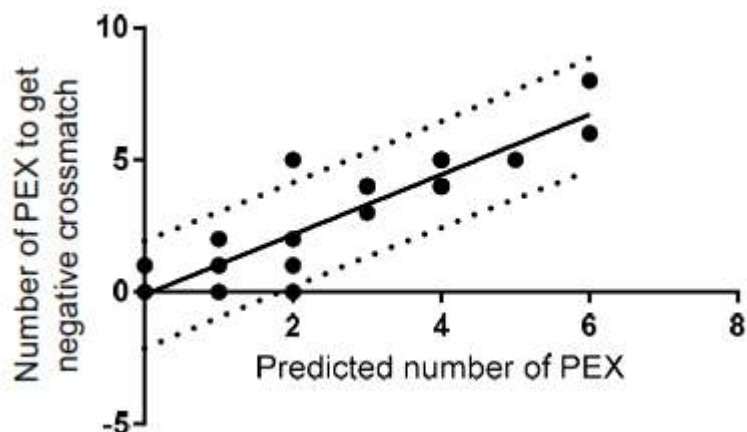
Table 4 - Summary of the antibody removal requirements in the transplanted group of patients, including the predicted number of antibody removal sessions based on the original test antibody removal and titre flow crossmatch assessment, and the actual number of antibody removal treatments given prior to transplant.

	Pre DFPP				Post DFPP					
Patient No.	T cell RMF at neat	T cell titre	B cell RMF at neat	B cell titre	T cell RMF at neat	T cell titre	B cell RMF at neat	B cell titre	Predicted number of Ab removal	Actual number of Ab removal prior to transplant
HLAi 5	2.32	1:2	3.08	1:8	1.47	Neat	1.66	Neat	1	1
HLAi 6	2.5	1:2	5.44	1:8	1.45	Neat	2.32	1:2	2	NT - Rituximab
HLAi 7	3.47	1:2	3.3	1:4	2.5	Neat	2.48	Neat	2	1
HLAi 8	6.39	1:4	10.4	1:8	2.36	neat	4.47	1:2	3	4
HLAi 9	3.03	neat	3.5	1:2	2.31	Neat	2.57	Neat	2	NT - Rituximab
HLAi 10	1.37	neat	3.08	1:2	1.24	Neat	2.62	Neat	2	5
HLAi 11	1.84	neat	4.7	1:4	1.28	neat	2.68	1:2	2	2
HLAi 12	3.01	neat	2.49	1:2	1.61	Neat	1.68	Neat	1	1
HLAi 13	3.92	1:2	4.7	1:4	2.15	Neat	2.75	Neat	2	2
HLAi 14	14.01	1:8	6.37	1:4	8.51	1:4	4.6	1:2	4	4
HLAi 15	9.78	1:16	4.19	1:4	5.99	1:4	3.18	1:2	4	5
HLAi 16	1.27	neat	3.31	1:2	0.99	Neat	2.31	Neat	2	2
HLAi 17	1.84	neat	9.22	1:8	1.76	Neat	5.43	1:4	4	NT - Rituximab
HLAi 18	1.69	neat	18.1	1:16	1.38	Neat	12.8	1:8	6	6
HLAi 19	2.26	neat	3.5	neat	1.42	Neat	1.48	Neat	1	1
HLAi 20	5.35	1:2	12.5	1:8	2.61	Neat	5.5	1:2	4	4
HLAi 21	6.19	1:2	7.14	1:8	2.57	Neat	3.46	1:2	3	3
HLAi 22	2.52	neat	2.13	neat	2	Neat	2	Neat	1	0
HLAi 23	1.01	neat	3.89	1:4	0.94	Neat	5.49	1:2	3	4
HLAi 24	1.65	neat	5.11	1:2	1.23	Neat	2.68	Neat	2	2

	Pre DFPP				Post DFPP					
Patient No.	T cell RMF at neat	T cell titre	B cell RMF at neat	B cell titre	T cell RMF at neat	T cell titre	B cell RMF at neat	B cell titre	Predicted number of Ab removal	Actual number of Ab removal prior to transplant
HLAi 26	1.71	neat	3.05	1:2	1.07	Neat	1.03	Neat	1	0
HLAi 27	9.56	1:4	18.9	>1:32	3.79	Neat	10.2	1:8	6	8
HLAi 28	1.11	neat	2.15	neat	1.03	Neat	1.52	Neat	0	1
HLAi 32	1.39	neat	1.27	neat	1.16	Neat	0.87	Neat	0	0
HLAi 38	1.18	neat	3.23	neat	1.03	Neat	1.4	Neat	1	2
HLAi 39	9.34	1:4	20.0	1:16	4.9	1:4	10.4	1:8	5	5
HLAi 40	1.85	neat	2.56	1:2	1.65	Neat	1.95	Neat	0	0
HLAi 41	2.31	neat	1.9	neat	1.41	Neat	1.03	Neat	0	0
HLAi 42	3.58	neat	6.44	neat	2.35	Neat	4.5	1:2	4	5
HLAi 43	3.48	1:2	1.96	neat	2.38	Neat	1.67	Neat	2	0
HLAi 44	3.23	1:2	2.04	neat	2.69	Neat	1.98	Neat	3	3

Linear regression assessment of the association between the predicted number of antibody removal sessions and the actual number required prior to transplant to achieve a negative crossmatch gave an R^2 value of 0.87 ($p < 0.0001$), indicating good correlation, shown in figure 26 below. In addition a two tailed paired t-test analysis found there was no significant difference between the predicted number and the required number $p=0.23$.

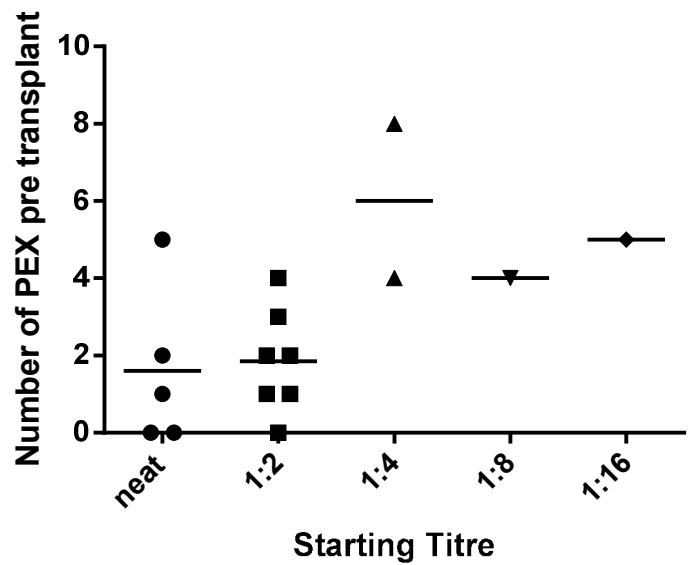
Figure 26 - Linear regression analysis of the association between the predicted and actual number of antibody removal treatments required pre transplant.



3.4.4 Influence of starting titre on number of antibody removal sessions needed pre transplant.

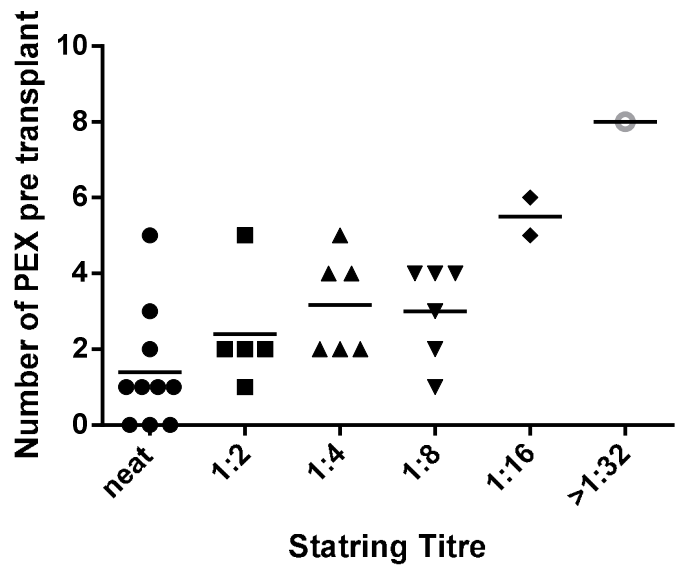
To assess if the starting titre as assessed by FXM related to the number of antibody removal sessions required prior to transplant, the number of sessions of PEX was plotted against the starting titre for all positive T and B cell crossmatches. The data is presented in figure 27 and figure 28 below.

Figure 27 - T cell FXM starting titre vs number of Antibody removal treatments required pre transplant.



Mean	1.6	1.9	6	4	5
Median	1	2	6	4	5

Figure 28 - B cell FXM starting titre vs number of antibody removal sessions required pre transplant.



Mean	1.4	2.4	3.17	3	5.5	8
Median	1	2	3	3.5	5.5	8

For both the T and B cell starting titre there appears to be a general increase in number of antibody removal sessions required prior to transplant with increasing starting titre. The T cell values may be affected by the influence of antibody removal for Class II specific antibody not reflected in the T cell titre value. It can however be seen that there is a wide variation in the number of antibody removal treatments required in the lower titre antibodies, with for example a B cell FXM positive only at 1:2 requiring between 1 and 5 antibody removal sessions pre transplant.

3.4.5 Effect of Antibody Specificity on the ability to successfully remove the antibody.

Observation of the reduction in titre seen with a single antibody removal treatment over all the patients highlighted the fact that not all patients responded in the same way, with some showing a reduction of multiple titres and others showing no reduction in either titre or strength. We assessed if the difference in response was due to specificity of the antibody, being directed at either HLA class I or class II or both. The values are shown in table 5 below. Reduction in titre was classed as the difference in the number of dilutions where positivity was seen between the pre and post samples, for example a reduction from 1:8 to 1:2 is a reduction of 2 titre strengths and classed as '2'. '-' indicates negative at neat both pre and post, '0' indicates no reduction in titre with a single DFPP, '1' indicates a reduction of 1 dilution and '2' indicates a reduction of 2 dilutions.

Table 5- Summary of the reduction in titre on crossmatch and the donor HLA specific antibody specificities present.

Titre Crossmatch Pair	Class I Specific Antibody	Class II specific Antibody	Reduction in Titre	
			T cells	B cells
1	A3, Cw5	DR17, DQ2	-	1
2	A3, Cw5	DR17, DQ2, DQ7	-	1
3	A32, B61	DQ7	-	1
4	A33, B58	DR15, 51 DQ6	1	2
5	A33, B58, Cw10	DR15, DR51, DQ6	-	1
6	none	DR13, DQ6	-	1
7	A31	none	-	-
8	none	DQ3, DQ6	-	0
9	A1, B35, Cw4	D11, DR52, DQ7	-	0
10	A30, Cw15	DR11, DP0201	-	2
11	none	DQ5	-	-
12	A3	none	1	-
13	B35	none	-	-
14	A1, A3, B8, B44	DR13, DR17, DR52, DQ2	0	1
15	A1	DQ2 DR53	-	0
16	A66	none	-	1
17	A2, A3, B57	DR4, 53 DQ5	1	2
18	A2, A3, B57	DR4, DR53, DQ5	1	2
19	A1, B52	DR13, 15 DR51,52 DQ6	-	0
20	A29, B44 Bw4 Cw5	DR7 DQ2	0	0
21	B7	DR15, DR51	-	2
22	A1	DQ2	-	1
23	A3, A29, B44	DQ5	-	0
24	B18	none	-	-
25	A3, B7, B57, Cw7	DR15, DR51, DQ9	3	0
26	B58	none	-	-
27	B57, Cw6	DR7, DP13, DP18	-	-
28	A11	DR103, 4, 53 DQ5	-	0

Titre Crossmatch Pair	Class I Specific Antibody	Class II specific Antibody	Reduction in Titre	
			T cells	B cells
29	B35, Cw4	DQ2 DR53	-	0
30	A2	none	2	2
31	A2	none	0	0
32	A32, B60,	DR53, DQ7, DQ8	2	1
33	B8, B45	DR17, DR52, DQ2	-	1
34	A26, B8, B60, Cw7, Cw10	none	2	2
35	A2, B7, B8, Cw7	none	2	1
36	B18, Cw7	none	1	1
37	A23, A26, B44, Cw4	DR15, DR51, DP5	1	1
38	A1, Cw7	DR15, DP51, DP10	0	0
39	A1, B8	none	-	1
40	A29, B57	none	2	2
41	A3	none	1	1
42	A1, A2, Cw4, Cw7	DR1, DR15, DR51	1	1
43	A3, A32, B63, Cw7	DR15, DR51	-	1
44	A2, Cw12	DQ7	-	0
45	B44, Cw5	none	-	-
46	none	DQ7	-	0
47	B8	DQ2, DQ7, DQA5	-	0
48	B8	DQ2, DQ7, DQA5	-	0
49	B8	none	-	1
50	none	DQ7	-	2
51	Cw7	DR11, DQ7	-	1
52	A1	none	1	1
53	B44, Cw5	DR52	1	1
54	A2, B13, Cw6	DR7, DQ2	-	2
55	none	DR17, DR52	-	-
56	A2, A24, B57, B62	none	0	1
57	A3, A29, B45, Cw6	none	1	1
58	none	DQB4, DQA4	-	0

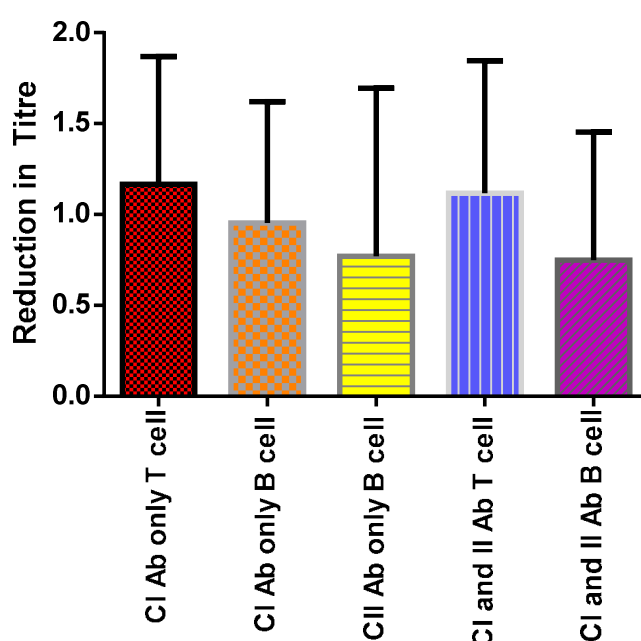
Titre Crossmatch Pair	Class I Specific Antibody	Class II specific Antibody	Reduction in Titre	
			T cells	B cells
59	Cw2,9	DQ5	0	0
60	A68	DR7, DQ2	1	1
61	A1, A3, B60, Cw10	none	1	1
62	A1	none	-	0
63	A3, A30, Cw9	none	1	0
64	none	DR11, DR13, DQ6	-	0
65	A2, B55	none	-	-
66	A2, B27	DR4	2	0
67	A24, B44, B51, Cw2, Cw16	none	2	2
68	B7, B44, Cw5	none	2	1
69	Cw10	none	2	1
70	Cw15, Cw16	none	1	-
71	none	DQ5	-	-
72	B13	DR11, DQ7	2	2
73	B57	none	-	-
74	A1, A33, B57	none	0	0
75	A2, B57, Cw7	none	-	-
76	A68	none	1	1
77	A31, B56	DR4, DR53	-	1
78	B35	none	1	-
79	A2, B57	DR14	-	1
80	B44, Cw5	DP3	1	1
81	none	DR17, DR52	-	0
82	A1	DQ6, DQ8	-	1
83	none	DR13, DQ6	-	0
84	B62, Cw9	DR13, DQ6	1	0
85	B13, Cw6	DR11	1	0
86	A2, B44	none	-	-
87	A1, A36, B18	DR51, DQ6	1	0

Titre Crossmatch Pair	Class I Specific Antibody	Class II specific Antibody	Reduction in Titre	
			T cells	B cells
88	A3, B72	DQ5	1	0
89	A2, B62, Cw10	DR4, DQ7	1	0
90	B44	DR15, DR51	1	1
91	A1	DR17, DR52	-	2
92	A3, Cw7	DR4, DQ7, DQ8	1	1
93	A2, Cw17	DR18, DR52	2	1
94	A33, Cw14	none	-	-
95	B8	DR17, DR52	2	1
96	B62, Cw9	DR13, DR52	2	2
97	A2	none	2	0
98	A3, B60	DR8	2	0
99	none	DR11	-	2
100	none	DQ2	-	1
101	none	DR4, DQ7	-	2
102	none	DR15, DR51, DQ6	-	2
103	B57	none	-	0
104	B44	none	1	1
105	A2, B44, Cw5	DR4, DR53	0	0
106	A1, A3, B7, B8	none	1	1
107	A31, B44, B60, Cw5	DR1, DR8, DQ4, DQ5	0	0
108	B7	DR15, DQ6	1	1
109	A1, B8, Cw2	DR16, DR17, DR51, DR52	-	0
110	none	DR13, DP3	-	0
111	Cw6	DR12, DR52	1	1
112	A23, A80, B57, Cw18	none	0	0
113	A3, B35, B51, Cw15	DR1	2	0
114	B35	none	-	-
115	A26, A32, B35, B60, Cw10	DQ7	1	1
116	A11	DQ6	1	1

Titre Crossmatch Pair	Class I Specific Antibody	Class II specific Antibody	Reduction in Titre	
			T cells	B cells
117	A11, Cw5	DR11, DR52	-	1

Analysis of the titre reduction seen on crossmatch in relation to the relevant antibody specificities found no significant difference between the groups – Class I only, Class II only and Class I and II in relation to T and B cell crossmatches respectively. Kruskal-Wallis 1 way non-parametric ANOVA analysis yielded a p value of 0.063, which whilst not significant does indicate a trend to there being a difference between the mean values. Graphical representation of the data in figure 29 indicates that patients with class II antibody, detected on B cell crossmatches, appear to show the smallest overall reduction in titre with one antibody removal session, suggesting that HLA Class II specific antibodies may be more challenging to remove than HLA Class I specific antibodies. However, the 95% confidence error bars presented on the graph do show the wide variability observed within each group and indicate that any trends observed should be approached with caution.

Figure 29 - Reduction in titre by cell and antibody specificity following a single antibody removal treatment.



3.4.6 Comparison of FXM results with LABScreen Single antigen

Screening Results.

MFI values generated from LABScreen single antigen analysis are believed to be a semi-quantitative value representing the strength of an antibody [150] . Whilst FXM is our method of choice at Guys, it requires fresh donor derived PBLs to be collected for each FXM, which is not always easy to arrange due to the availability and location of the donor. Therefore we wanted to assess if MFI values could be used in highly sensitised patients to accurately predict crossmatch results and act as a surrogate marker for the assessment of the requirement for, and likely success of, HLA specific antibody removal.

By observing reaction patterns in our early antibody removal patients similar to those shown in figure 25 above, it was noted that the combined DSA MFI in different patient and donor pairs that equated to a predicted positive crossmatch showed great variation, indicating that it may not be possible to predict a positive crossmatch based on MFI values. In addition 22 of the titre crossmatches predicted to be positive based on historical screening results were found to be negative on both the T and B cell crossmatch prior to any antibody removal. To investigate the relationship between LABScreen single antigen bead MFI values and crossmatch results, samples from 58 patients who had received a test antibody removal treatment and had a titre flow crossmatch, along with samples from a further 10 patients who had been assessed by a single sample titre crossmatch, were tested at neat using LABScreen single antigen screening beads. The MFI values for each donor specificity represented on the bead panel were collected. Where more than one bead was present for a single specificity, such as for HLA A24 and HLA DR4 seen in the example results provided in table 4, the average MFI of all the specific beads was recorded, unless the high resolution donor type was known and represented by a bead on the panel. If more than one specificity was involved then the total sum of the MFI of all the beads was calculated, both separately for Class I and Class II plus a total of both Class I and II, as the total DSA MFI value had been found to mirror trends in crossmatch results over time in the patients who had previously undergone antibody removal. This gave a total of 126

corresponding crossmatch and single antigen bead results. The MFI and RMF values along with the specificities involved are summarised in table 6 below, where a sample was not tested either due to no HLA specific antibody to that class having been previously detected, or due to the patient providing only a pre DFPP sample, then 'nt' is marked in the appropriate column.

Table 6 – Table providing a summary of the T and B cell Titre Crossmatch RMF values and corresponding donor specific antibody MFI values in patient samples provided pre and post a test antibody removal treatment, with the relevant antibody specificities (Class I or II) listed.

Crossmatch Pair	Class I specificities	Class II specificities	Pre PEX/DFPP				Post PEX/DFPP			
			T cell RMF at neat	B cell RMF at neat	Total Class I MFI	Total Class II MFI	T cell RMF at neat	B cell RMF at neat	Total Class I MFI	Total Class II MFI
1	A3, Cw5	DR17, DQ2	1.35	2.26	1996	3595	1.28	2.12	4946	3498
2	A3, Cw5	DR17, DQ2, DQ7	1.41	3.21	1996	6252	1.16	1.54	4946	5774
3	A32, B61	DQ7	2.25	3.92	7144	2657	1.74	2.71	13345	2270
5	A33, B58, Cw10	DR15, DR51, DQ6	1.65	5.11	8446	12580	1.23	2.68	2819	20667
6	none	DR13, DQ6	1.05	3.34	nt	10052	0.94	1.23	nt	8697
7	B57	DR13, DQ6	1.18	11.98	1706	16616	nt	nt	nt	nt
8	none	DR13, DQ6	1.02	5.91	nt	16616	nt	nt	nt	nt
14	Cw15	DR11, DP0201	1.46	9.04	9353	20914	nt	nt	nt	nt
15	none	DQ5	0.96	1.5	nt	8279	1.03	1.6	nt	9782
16	A3	none	2.52	2.13	14056	nt	2	2	11553	nt
18	A1, A3, B8, B44	DR13, DR17, DR52, DQ2	2.97	8.68	19834	20752	2.81	6.84	21788	13502
22	A2, A3, B57	DR4, DR53, DQ5	6.19	7.14	32734	22376	2.57	3.46	31374	15691
23	B7	DP0401	1.15	2.21	2298	668	nt	nt	nt	nt

Crossmatch Pair	Class I specificities	Class II specificities	Pre PEX/DFPP				Post PEX/DFPP			
			T cell RMF at neat	B cell RMF at neat	Total Class I MFI	Total Class II MFI	T cell RMF at neat	B cell RMF at neat	Total Class I MFI	Total Class II MFI
24	A1, B52	DR13, 15 DR51,52DQ6	1.16	6.77	6714	41168	1.15	6.27	3141	44074
26	B7	DR15, DR51	1.01	4.05	nt	16412	1.06	6.38	nt	17318
27	A1	DQ2	1.51	3.84	6829	12411	1.35	4.77	7434	13230
28	Cw5	none	1.14	0.86	4852	nt	nt	nt	nt	nt
34	B18	none	2.02	2.03	5061	nt	1.09	10.3	2520	nt
35	A3, B7, B57, Cw7	DR15, DR51, DQ9	9.86	12.61	46588	46319	6.26	13.92	43187	30936
36	B58	none	1.22	1.37	12369	nt	1.11	1.33	14421	nt
44	B63	none	1.05	1.84	9587	nt	nt	nt	nt	nt
46	B45	DR17, DR52, DQ2	8.09	9.25	1185	11759	nt	nt	750	11672
47	A26, B8, B60, Cw7, Cw10	none	6.85	9.03	46770	nt	4.26	6.56	45195	nt
48	A2, B7, B8, Cw7	none	5.63	2.26	34128	nt	4.17	1.79	39132	nt
49	B18, Cw7	none	3.23	2.04	19017	nt	2.69	1.98	20788	nt
52	A23, A26, B44, Cw4	DR15, DR51, DP5	24.31	14.41	30843	24781	14.43	10.5	34785	25966
53	A1, Cw7	DR15, DP51, DP10	3.58	6.44	14013	22123	2.35	4.5	10851	23321

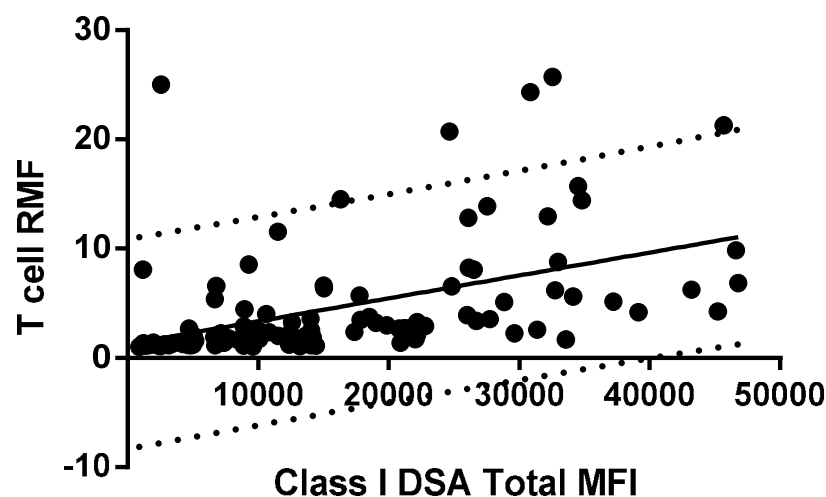
Crossmatch Pair	Class I specificities	Class II specificities	Pre PEX/DFPP				Post PEX/DFPP			
			T cell RMF at neat	B cell RMF at neat	Total Class I MFI	Total Class II MFI	T cell RMF at neat	B cell RMF at neat	Total Class I MFI	Total Class II MFI
55	A29, B57	none	6.6	6.26	15016	nt	2.96	3.1	nt	nt
58	A1, A2, Cw4, Cw7	DR1, DR15, DR51	25.73	12.56	32555	34697	15.72	6.92	34522	42298
59	A3, A32, B63, Cw7	DR15, DR51	1.68	2.72	33562	24134	2.72	1.5	21206	25304
60	A2, Cw12	DQ7	1.39	8.9	7395	3614	1.12	8.48	8908	8855
61	B44, Cw5	none	1.39	1.27	20895	nt	1.16	0.87	14053	nt
63	B8	DQ2, DQ7, DQA5	1.59	15.61	5193	7694	1.3	13.7	3457	13652
64	B8	DQ2, DQ7, DQA5	2.23	6.73	9133	7622	1.79	6.19	7741	8799
70	B44, Cw5	DR52	3.25	4.17	12592	4586	1.75	2.13	10094	2206
71	A2, B13, Cw6	DR7, DQ2	1.9	3.61	14192	24718	1.17	2.25	4637	13642
77	B7	DQ6	4.02	16.37	10629	7500	nt	nt	nt	nt
80	A1, A3, B60, Cw10	none	3.55	6.85	27723	nt	2.24	3.88	29618	nt
81	A1	none	2.17	5.08	12378	nt	1.85	4.15	12812	nt
82	A3, A30, Cw9	none	2.92	5.35	22801	nt	2.11	3.35	22168	nt
83	none	DR11, DR13, DQ6	1.43	35.34	nt	21205	1.43	41.67	nt	30300
86	A24, B44, B51, Cw2, Cw16	none	3.93	4.04	26013	nt	3.27	4.07	22183	nt

			Pre PEX/DFPP				Post PEX/DFPP			
Crossmatch Pair	Class I specificities	Class II specificities	T cell RMF at neat	B cell RMF at neat	Total Class I MFI	Total Class II MFI	T cell RMF at neat	B cell RMF at neat	Total Class I MFI	Total Class II MFI
87	B7, B44, Cw5	none	8.25	5.93	26114	nt	5.1	4.73	28857	nt
91	Cw15, Cw16	none	3.48	1.96	17838	nt	2.38	1.67	17377	nt
92	none	DQ5	1.11	2.15	nt	11206	1.03	1.52	nt	9288
93	B13	DR11, DQ7	25.02	22.02	2574	23062	8.54	8.96	9284	14014
96	A2, B57, Cw7	none	1.71	2.17	22036	nt	1.07	1.03	13198	nt
98	A31, B56	DR4, DR53	1.84	4.7	10026	12598	1.28	2.68	4234	6854
99	B35	none	2.31	1.9	7003	nt	1.41	1.03	nt	nt
100	A2, B57	DR14	2.02	2.55	13640	1706	1.23	1.07	5017	nt
101	B44, Cw5	DP3	2.42	4.2	10156	10751	1.91	3.78	6641	10490
103	A1	DQ6, DQ8	1.18	2.94	2721	17943	1.08	2.83	2177	14915
105	B62, Cw9	DR13, DQ6	4.45	7.83	8964	7165	2.88	6.35	8902	10230
106	B13, Cw6	DR11	5.72	18.01	17777	3204	3.74	13.8	18520	3863
110	A2, B62, Cw10	DR4, DQ7	11.56	13.54	11507	8977	6.56	9.94	24833	33381
112	A1	DR17, DR52	1.13	9.37	1427	28010	1.03	10.86	900	28114
116	B8	DR17, DR52	6.58	10.31	6800	6711	2.67	4.17	4700	3711

Crossmatch Pair	Class I specificities	Class II specificities	Pre PEX/DFPP				Post PEX/DFPP			
			T cell RMF at neat	B cell RMF at neat	Total Class I MFI	Total Class II MFI	T cell RMF at neat	B cell RMF at neat	Total Class I MFI	Total Class II MFI
117	B57	DR7	5.37	6.31	6685	3400	nt	nt	nt	nt
118	B62, Cw9	DR13, DR52	6.39	10.4	15042	5412	2.36	4.47	9930	3054
125	none	DQ2	1.21	5.28	nt	18356	1.1	4.17	nt	14130
134	A1, A3, B7, B8	none	12.81	6.28	26100	nt	8.09	6.18	26520	nt
135	A31, B44, B60, Cw5	DR1, DR8, DQ4, DQ5	20.73	29.95	24651	46745	21.3	29.96	45645	37184
139	none	DR13, DP3	1.48	18.02	nt	7145	1.31	15.75	nt	12602
142	A23, A80, B57, Cw18	none	13.89	5.23	27547	nt	12.95	4.33	32191	nt
143	A3, B35, B51, Cw15	DR1	14.51	21.95	16324	2617	8.79	19.33	32951	11850
146	B57	none	1.35	2.66	1200	nt	nt	nt	nt	nt
148	A26, A32, B35, B60, Cw10	DQ7	5.15	11.39	37200	10381	3.4	7.84	26700	9734

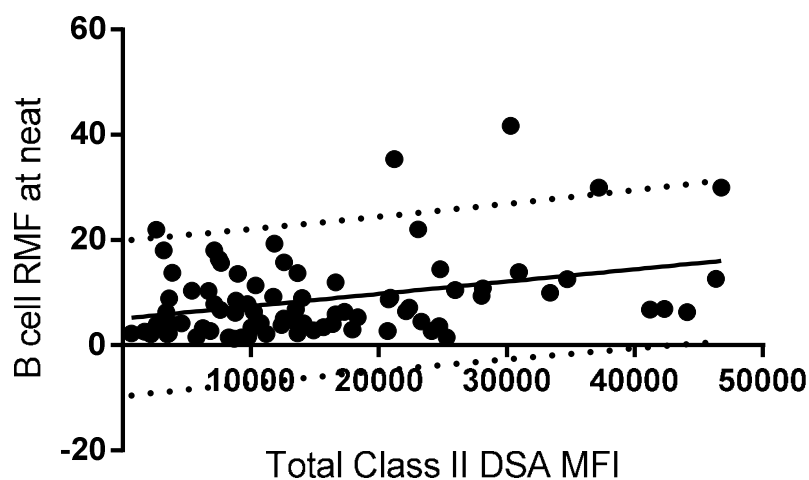
The bead MFI values were compared to the crossmatch RMF values to assess if the cumulative donor specific bead MFI values could predict a crossmatch result in these highly sensitised patients. Due to the differences in expression of HLA class I and class II molecules between T and B cells, the analysis split the results into three groups – the association between Class I MFI and T cell RMF, the association between Class II MFI and B cell RMF and the association between combined Class I and II MFI and B cell RMF. Initial linear regression analysis of all three groups indicated that whilst there was some association between MFI and RMF it was not possible to confidently predict an RMF value for either T or B cells from the MFI values within the 95% confidence window. The analysis is shown in figures 30, 31 and 32 respectively below. In these figures the dots represent corresponding RMF and cumulative DSA MFI values from a single sample, the solid line is the line of regression and the two dotted lines represent the 95% confidence interval.

Figure 30 – Linear regression analysis of total Class I DSA MFI against T cell RMF



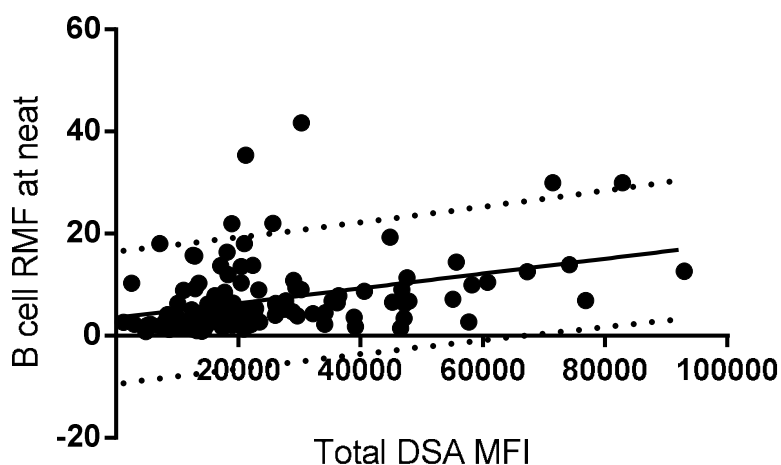
$$R^2 = 0.22$$

Figure 31 – Linear regression analysis of total Class II DSA MFI against B cell RMF.



$$R^2 = 0.12$$

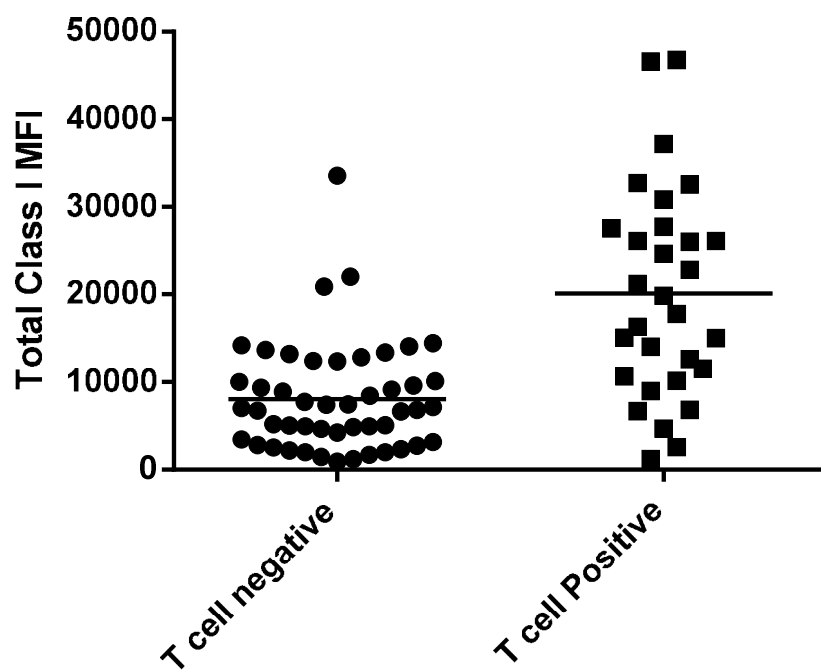
Figure 32 – Linear regression analysis of total DSA MFI against B cell RMF.



$$R^2 = 0.14$$

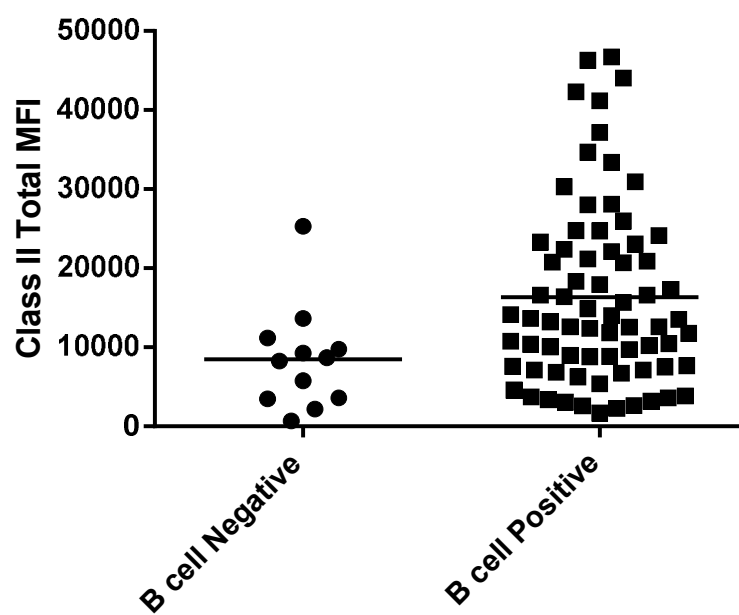
To assess if there was a difference in total DSA MFI values between those crossmatches that were positive at neat and those that were negative the three data sets were also analysed using the unpaired non-parametric Mann-Whitney test. The results are shown in figures 33, 34 and 35 respectively below.

Figure 33 - Total Class I DSA MFI against negative and positive T cell crossmatches at neat.



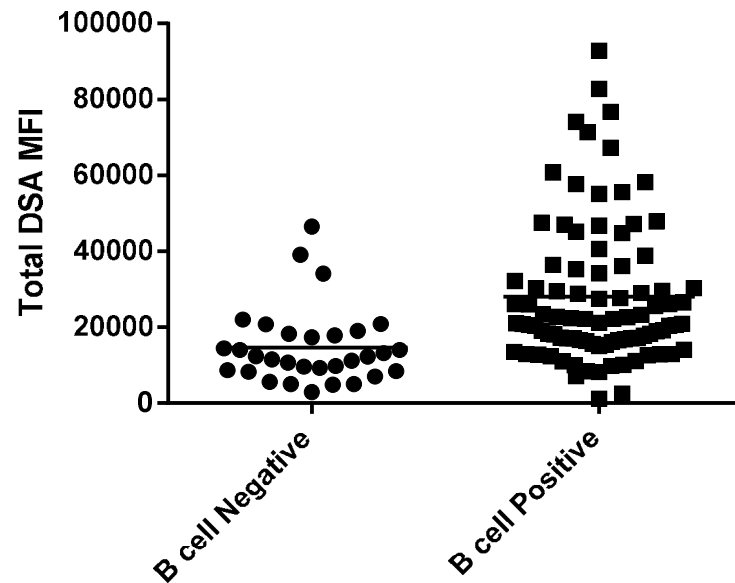
$p = <0.0001$

Figure 34 – Total Class II DSA MFI against negative and positive B cell crossmatches at neat.



$p = 0.0125$

Figure 35 - Total Class I and II DSA MFI against negative and positive B cell crossmatches at neat.

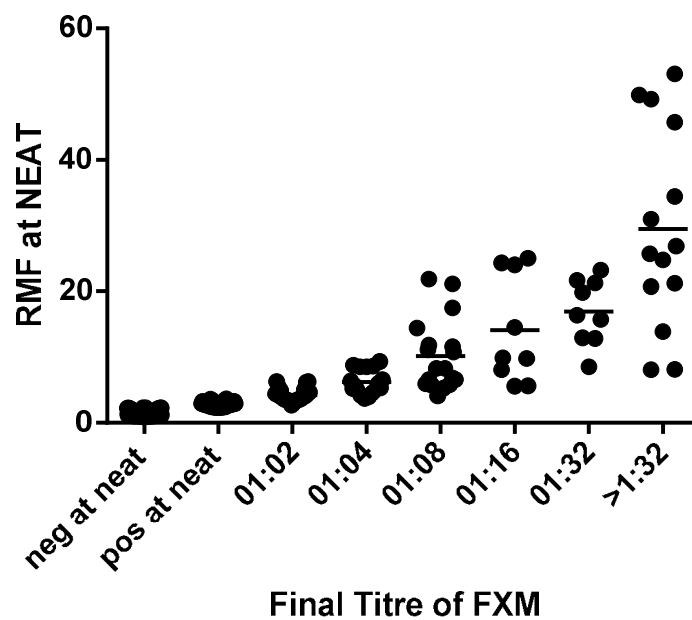


$p = <0.0001$

The p values achieved for all three groups indicate a significant difference between the median MFI value achieved for the crossmatches which were negative and those which were positive. However the range of MFI values in all groups show considerable overlap, particularly in the lower values, indicating again that total DSA MFI may not be accurately predictive of a FXM result. The B cell crossmatches however do indicate that a total Class II DSA MFI of greater than 30000 and a total combined Class I and II DSA MFI of greater than 50000 are always associated with a positive B cell crossmatch. Up to these values however there seems to be equal likelihood of a positive or negative crossmatch. The T cell crossmatches indicate that a total Class I DSA MFI of greater than 20000 is commonly associated with a positive crossmatch, but again at lower values there is no association.

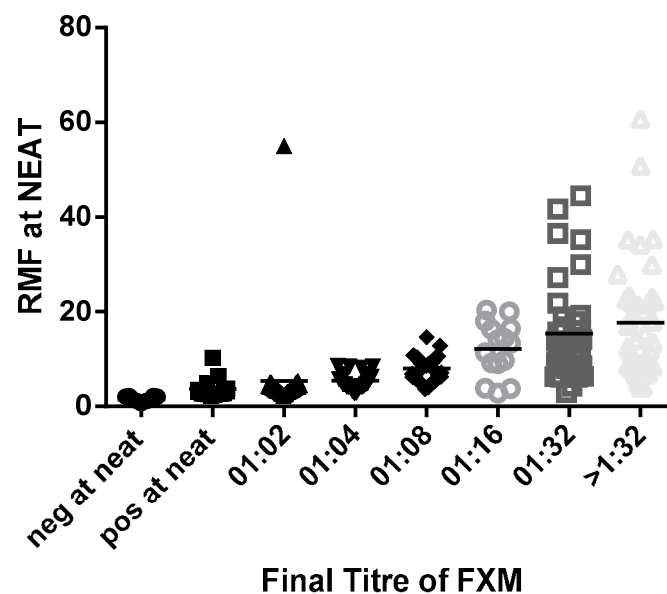
The titre crossmatch involves multiple dilutions and sample tubes in comparison to a routine crossmatch, making it more time consuming, costly and demands a higher volume of donor cells. To assess if it was possible to predict the final titre achieved from the results gained against the neat sample only the results were separated into final titre and plotted against the RMF value at neat. Kruskal-Wallis non-parametric ANOVA analysis was employed to assess if there was significant variation between any of the median values for the respective titre groups.

Figure 36 - T cell RMF values at neat against final titre of crossmatch.



$p = <0.0001$

Figure 37 – B cell RMF values at neat against final titre of crossmatch.

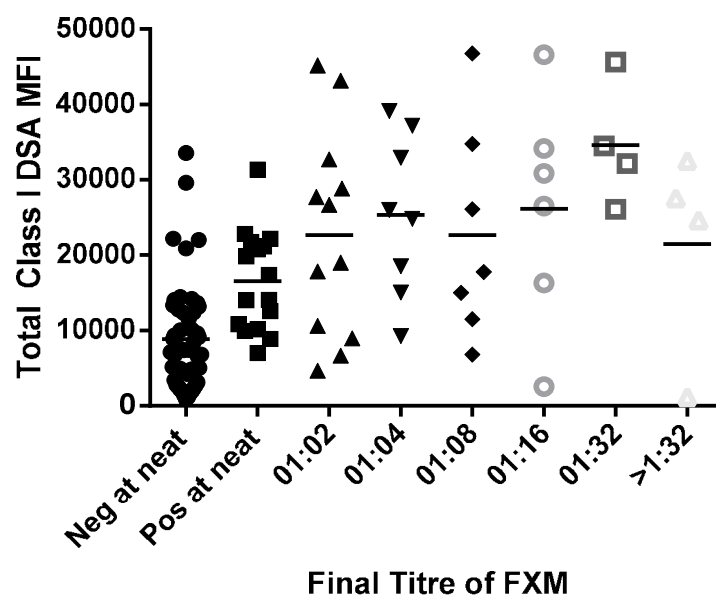


$p = <0.0001$

As can be seen in figure 36 and 37 above there is an obvious trend between the median RMF values achieved at neat and the final titre of the crossmatch, both for the T cell and the B cell, with increasing RMF values at neat being associated with a higher final titre. It can also be seen however that the higher the titre the wider the range of starting RMF value, with overlap being seen between groups.

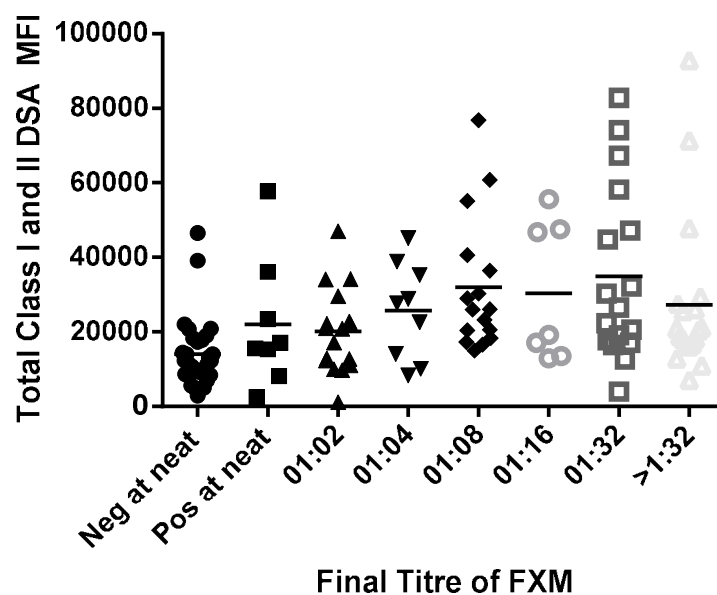
To assess if the single antigen bead MFI values also followed a similar trend, potentially allowing prediction of a final titre prior to performing a crossmatch, the same analysis was carried out replacing the RMF values with the total DSA MFI values. The results were split into two groups – the T cell titre against total class I DSA MFI and the B cell titre against combined class I and II total DSA MFI.

Figure 38 - Total Class I DSA MFI against final titre of the T cell crossmatch.



$p = <0.0001$

Figure 39 – Total Class I and II DSA MFI against final titre of the B cell crossmatch.



$p = 0.0002$

As can be seen, whilst the ANOVA analysis indicates that there is a significant difference between at least one pair of the median MFI values of the groups, there is clearly no trend similar to that seen with the RMF values relating to increasing MFI values at neat

corresponding with increasing titre values. Again indicating that the MFI values achieved with the single antigen beads may not be indicative of RMF values on crossmatch.

3.4.7 Association with Post-transplant Outcome.

Whilst it is apparent that antibody levels vary between patients based on the results achieved with both the flow crossmatches and with the single antigen beads the significance of these levels in relation to post-transplant outcome needed to be investigated. By comparing pre transplant antibody results with post-transplant outcomes in our HLA antibody incompatible groups we hoped to be able to define a level, either pre-treatment or pre transplant, at which there was a higher risk of post-transplant complications and failure.

Data was collected for all the HLA antibody incompatible transplants – where potential donor specific antibody had been detected using single antigen screening beads with or without a positive T or B cell flow crossmatch. In those patients where antibody removal pre transplant was required due to a positive crossmatch, both the pre-treatment and pre transplant crossmatch RMF values and total DSA MFI values were collected. In the patients who were single antigen bead positive only, not requiring antibody removal, the pre transplant values only were collected. In addition in patients where the graft was lost during the time period studied the time in days to graft loss and the cause of graft loss – rejection or other, non-immunological causes, such as death of the patient with a functioning graft – was also recorded. The incidence of biopsy proven rejection episodes (BPPE) in the first year that included a diagnosis of antibody mediated rejection was also recorded. It can be seen that a number of patients in the HLA antibody incompatible group who did not require antibody removal have been included despite having low levels of HLA specific antibody, in their immediate pre transplant sample. However in all patients historic levels of antibody were all greater than 2000 MFI, so on this basis have been included in the analysis. The data is summarised in table 7 below.

It must be noted that with regard to immunosuppressive regimens this group of patients in particular are mixed. Due to the evolving nature of HLA antibody incompatible transplantation patients have not all been treated the same. As discussed earlier a few patients have received Rituximab as part of their induction therapy. In addition, as will be discussed fully later, more recently transplanted patients have received Campath or ATG induction therapy. All other patients have received Basiliximab induction. Our standard triple therapy immunosuppression regimen, consisting of MMF, tacrolimus and prednisolone, has been followed in at least the immediate post-transplant period for all patients. Due to the already small numbers involved, for the following analysis all the patients have been included regardless of the induction therapy received. A separate analysis comparing outcomes in patients who did and did not receive Campath or ATG induction therapy is included later.

Table 7 - Summary of the antibody specificities, MFI and RMF results pre-treatment and pre-transplant in the HLA antibody incompatible patients.

Patient	Antibody Specificity	Pre Treatment			Pre Transplant			Antibody removal required	Time to graft loss	Cause of Graft Loss	AMR in first year post-transplant	Any BPPE in first year
		T cell RMF	B cell RMF	Total DSA MFI	T Cell RMF	B cell RMF	Total DSA MFI					
HLAi 1	A2, B18	3.81	5.03	5721	Rituximab - NT	Rituximab - NT	1518	y	-	-	No	No
HLAi 2	B62, Cw9, DR13, DR52	7.11	11.54	20454	1.25	1.76	3790	y	9	other	No	No
HLAi 3	Bw4 (B49)	2.58	3.2	5860	1.57	Rituximab - NT	2735	y	614	other	No	Yes
HLAi 4	A3, DP2, DP4	1.47	8.18	28182	0.97	2.79	12342	y	-	-	Yes	Yes
HLAi 5	A31, B56, DR4, DR53	1.45	4.38	16895	1.07	2.02	6830	y	-	-	No	No
HLAi 6	A2, B42, DR18	14.73	14.16	18319	2.12	2.9	12723	y	478	Rejection	Yes	Yes
HLAi 7	Cw7, DR11, DQ7	1.08	3.45	14020	1.05	2.89	20305	y	-	-	Yes	Yes
HLAi 8	A11, B44, Cw5, DR11, DR52	1.84	9.22	22321	Rituximab - NT	Rituximab - NT	10190	y	-	-	No	No
HLAi 9	A31, B8, B45, Cw6, DR17, DR52	13.89	9.54	38669	3.45	4.21	25008	y	48	Rejection	Yes	Yes
HLAi	A11, B55, C9, DR14, DR52	2	11.6	26779	1	0.85	6665	y	-	-	Yes	Yes

		Pre Treatment			Pre Transplant							
Patient	Antibody Specificity	T cell RMF	B cell RMF	Total DSA MFI	T Cell RMF	B cell RMF	Total DSA MFI	Antibody removal required	Time to graft loss	Cause of Graft Loss	AMR in first year post-transplant	Any BPPE in first year
10												
HLAi 11	A2, A3, B57, DR53 DQ5	2.26	5.55	35223	1.47	3.93	30617	y	-	-	Yes	Yes
HLAi 12	A3, B35, DR1, DQ5	1.76	2.49	11046	1.29	2.01	8431	y	-	-	Yes	Yes
HLAi 13	DQ7	0.94	5.49	9999	1.09	6.07	15089	y	-	-	No	No
HLAi 14	B58, DR15, DR51, DQ6	1.47	5.42	47453	1.14	2.86	19328	y	200	Rejection	Yes	Yes
HLAi 15	A23, B8, B44	3.73	9.64	27103	2.13	3.96	11686	y	-	-	Yes	Yes
HLAi 16	DR4, DQ7	nt	nt	-	1.01	0.75	1669	n	-	-	No	No
HLAi 17	B44, C5, DR4	nt	nt	32902	1.19	2.09	8600	n	-	-	No	No
HLAi 18	Bw4 B63	nt	nt	-	1.2	1.45	3300	n	-	-	Yes	Yes

		Pre Treatment			Pre Transplant							
Patient	Antibody Specificity	T cell RMF	B cell RMF	Total DSA MFI	T Cell RMF	B cell RMF	Total DSA MFI	Antibody removal required	Time to graft loss	Cause of Graft Loss	AMR in first year post-transplant	Any BPRES in first year
HLAi 19	B44	nt	nt	-	1.24	1.22	780	n	-	-	Yes	Yes
HLAi 20	DR11	nt	nt	-	1.19	1.6	600	n	-	-	No	No
HLAi 21	B7	nt	nt	-	1.34	1.74	1200	n	-	-	Yes	Yes
HLAi 22	B35	nt	nt	-	0.9	1.05	500	n	1324	other	No	No
HLAi 23	B44	nt	nt	-	0.88	1.14	3800	n	-	-	No	No
HLAi 24	B62, Cw9, DR13, DR52	2.5	5.31	7260	1.26	1.83	4500	y	-	-	No	No
HLAi 25	DQ1	0.9	2.79	8577	0.92	1.47	3454	y	970	other	No	No
HLAi 26	A24	2.8	3.79	8475	1.45	1.92	3656	y	-	-	No	Yes
HLAi 27	DP0301	2.18	4.76	10320	1.36	2.98	6310	y	316	Rejection	No	Yes

		Pre Treatment			Pre Transplant							
Patient	Antibody Specificity	T cell RMF	B cell RMF	Total DSA MFI	T Cell RMF	B cell RMF	Total DSA MFI	Antibody removal required	Time to graft loss	Cause of Graft Loss	AMR in first year post-transplant	Any BPPE in first year
HLAi 28	Bw6	2.32	3.08	4028	1.09	1.46	692	y	208	other	No	Yes
HLAi 29	B35	3.19	3.75	7500	1.05	Rituximab - NT	2860	y	-	-	No	No
HLAi 30	A3	3	3.89	10098	1.29	2.37	5478	y	-	-	No	No
HLAi 31	A3, A29, B45, Cw6	2.99	4.27	8484	1.53	1.9	6920	y	-	-	Yes	Yes
HLAi 32	A3	11.14	6.18	6350	1.98	2.53	5181	y	237	Rejection	Yes	Yes
HLAi 33	A1, B8	2.41	3.66	18442	1.17	1.12	6626	y	-	-	No	No
HLAi 34	A2, B57, DQ9	1.71	3.05	15300	1.31	1.13	6959	y	-	-	No	No
HLAi 35	DQ5	0.99	2.71	9763	nt	nt	10905	y	-	-	No	No
HLAi 36	Cw7	nt	nt	-	0.88	1.68	1498	n	-	-	No	No

		Pre Treatment			Pre Transplant							
Patient	Antibody Specificity	T cell RMF	B cell RMF	Total DSA MFI	T Cell RMF	B cell RMF	Total DSA MFI	Antibody removal required	Time to graft loss	Cause of Graft Loss	AMR in first year post-transplant	Any BPPE in first year
HLAi 37	DQA5	nt	nt	-	0.94	2.77	6529	n	-	-	No	No
HLAi 38	Cw12, DR15	nt	nt	-	1.01	1.6	10700	n	-	-	No	No
HLAi 39	A30	nt	nt	-	1.39	2.21	3448	n	-	-	No	No
HLAi 40	A24	nt	nt	-	1.01	1.06	2830	n	231	Rejection	No	Yes
HLAi 41	A11	nt	nt	-	2.19	1.37	6145	n	2049	Rejection	Yes	Yes
HLAi 42	Cw10	nt	nt	-	1.14	2.76	3000	n	-	-	No	No
HLAi 43	B75	nt	nt	-	1.08	1.64	1400	n	-	-	No	No
HLAi 44	A2	nt	nt	-	0.97	0.71	1400	n	-	-	No	Yes
HLAi 45	A30	nt	nt	-	0.93	1.02	800	n	-	-	No	Yes

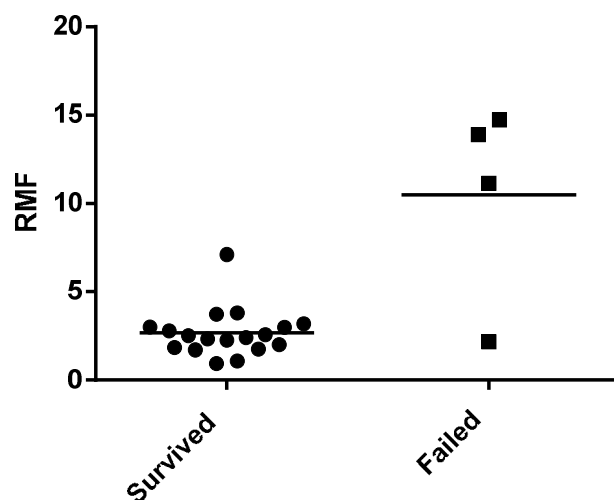
		Pre Treatment			Pre Transplant							
Patient	Antibody Specificity	T cell RMF	B cell RMF	Total DSA MFI	T Cell RMF	B cell RMF	Total DSA MFI	Antibody removal required	Time to graft loss	Cause of Graft Loss	AMR in first year post-transplant	Any BPPE in first year
HLAi 46	A2	nt	nt	-	0.99	1.89	780	n	-	-	No	No
HLAi 47	B35	nt	nt	-	nt	nt	8000	n	-	-	No	No
HLAi 48	B14	nt	nt	-	1.01	2.05	2000	n	-	-	No	No
HLAi 49	DR1	nt	nt	-	0.99	1.04	800	n	-	-	No	No
HLAi 50	B8	nt	nt	-	0.97	1.26	4500	n	-	-	No	No
HLAi 51	DQ7	nt	nt	-	1.07	1.04	917	n	-	-	No	No
HLAi 52	Cw8, DQ9	nt	nt	-	1	1.76	5500	n	-	-	No	No
HLAi 53	A32	nt	nt	-	0.91	0.86	2000	n	-	-	No	Yes
HLAi 54	Cw7	nt	nt	-	0.94	0.99	1300	n	-	-	No	No

3.4.8 Association with Graft Survival.

For the purposes of the following analysis outcome was defined as graft survival or failure, due to rejection, over the course of the study period, with a median follow up time of 1351 days (range - 249 – 3096). Failures due to other non-immunological causes were censored from the analysis. There were 5 such failures recorded in this patient group, 2 patients died with a functioning graft, 1 graft was lost due to renal vein thrombosis secondary to prolonged hypotension, 1 was lost due to recurrent disease and 1 was lost following patient death due to infection of the graft.

The initial analysis of data centred round comparing the pre-treatment RMF, T or B cell, and total DSA MFI values with occurrences of graft loss in the patients who required antibody removal prior to transplant. Secondly, the pre-transplant values were also analysed including all the HLA antibody incompatible transplants, regardless of the need for antibody removal. As previously discussed T cells, unless activated, express only HLA Class I, whereas B cells express both HLA Class I and II. This means in general terms that T cell positive FXM are due to Class I specific antibody whereas B cell positive FXM can be due to Class I and/or II specific antibody. To account for the different expression of HLA on the T and B cells the groups analysed were - T cell RMF pre-treatment in patients with HLA Class I or Class I and Class II specific antibody, B cell RMF pre-treatment in all patients, T cell RMF pre transplant in patients with HLA Class I or Class I and Class II specific antibody, B cell RMF pre-transplant in all patients, Total DSA MFI pre-treatment and Total DSA MFI pre-transplant. Due the small patient numbers involved, particularly in the failure due to rejection group, the data for each group was analysed using the non-parametric t-test, Mann-Whitney U, and the results can be seen in figures 40 – 45 below. The p values achieved are indicative of significant differences between groups. However, due to the small numbers involved, they must be treated with caution. The dot plots presented below provide a visual representation of the differences and trends seen.

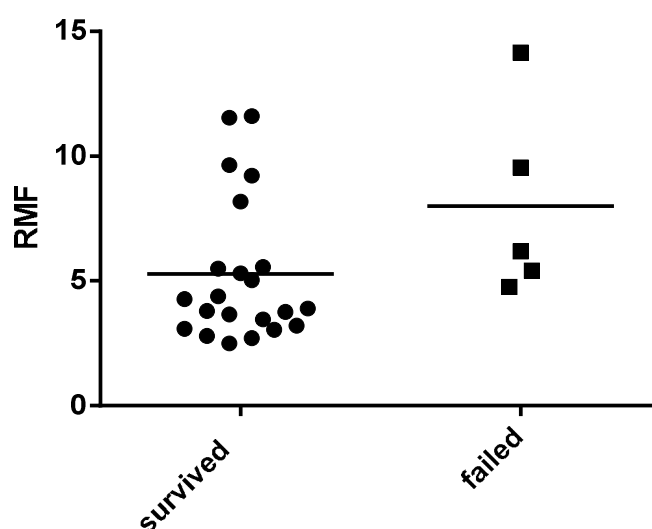
Figure 40 - Graft survival or failure due to rejection vs Pre-treatment T cell RMF in patients with HLA class I or Class I and II specific antibody.



$p = 0.0424$

Figure 40 above indicates that there is a significant difference in pre-treatment T cell RMF values between the group who had functioning grafts throughout the study period and those whose grafts failed. It would indicate that a starting T cell RMF of greater than 10 indicates a strong risk of graft failure due to rejection.

Figure 41 – Graft survival or failure due to rejection vs pre-treatment B cell RMF in all HLA antibody incompatible transplant patients.

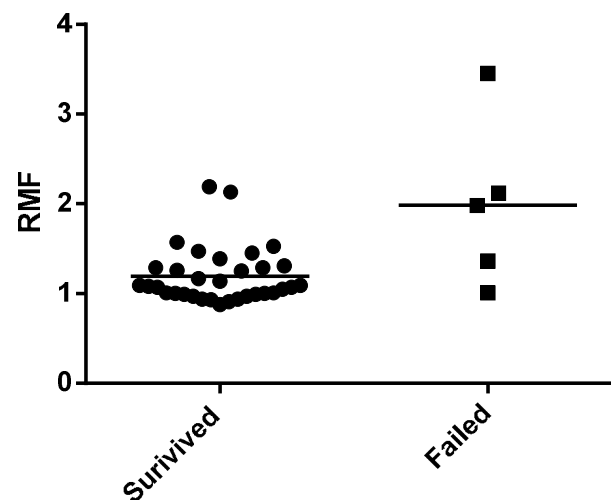


$p = 0.0550$

The B cell RMF values pre-treatment are not as clearly associated with graft survival or failure as the T cell values above, although there is a difference in the mean RMF values in each

group. There is obvious crossover of values, however the group of patients whose grafts failed due to rejection all had an RMF of >5, compared to less than a third of those where the graft survived.

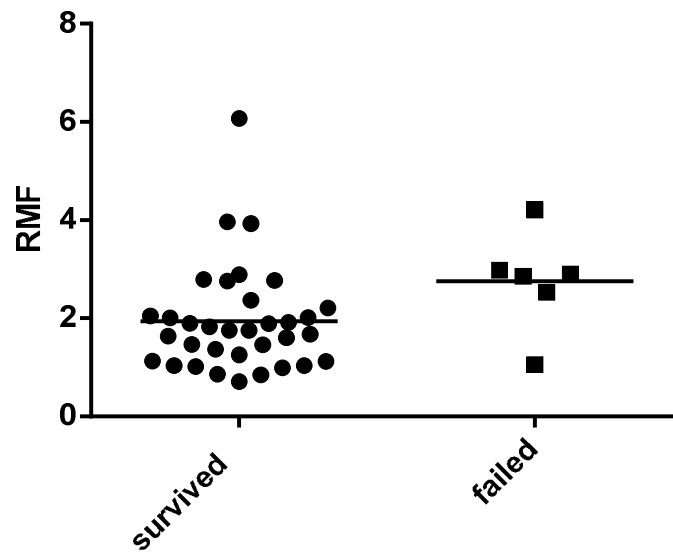
Figure 42 – Graft survival or failure due to rejection vs pre-transplant T cell RMF in patient with HLA Class I or HLA Class I and Class II specific antibody.



$p = 0.0265$

Again the pre transplant T cell RMF values are significantly different between the two groups. However there is less of a clear difference than that seen with the pre-treatment values. It can be seen however that the graft survival group all have negative T cell flow crossmatches pre transplant, with RMF values below 2.3, however in the failure group, 1 of the five has a positive T cell crossmatch, although less than the cut off of 4 used in the latter part of the study, with two of the others nearing the positive threshold.

Figure 43 - Graft survival or failure due to rejection vs pre-transplant B cell RMF in patients with HLA Class II or HLA Class I and Class II specific antibody



p = 0.0291

The difference in the B cell pre transplant RMF values is statistically significant. However the dot plot shows that the range of values seen in both groups is similar. In the majority of cases where the graft was lost the RMF was greater than 2.3. There is one clear outlier in the survival group with a B cell RMF of 6 pre-transplant. This reactivity was explained by the infusion of IVIg prior to the FXM as the FXM had been negative prior to this infusion, therefore the transplant proceeded on this basis.

A scatter plot comparing Total DSA MFI between two groups: 'survived' and 'failed'. The y-axis is labeled 'Total DSA MFI' and ranges from 0 to 50,000 in increments of 10,000. The 'survived' group is represented by black circles, with a horizontal line indicating a mean MFI of approximately 14,000. The 'failed' group is represented by black squares, with a horizontal line indicating a mean MFI of approximately 24,000. The 'failed' group shows significantly higher MFI values, with several points above 30,000.

Group	Total DSA MFI (approximate values)
survived	10000, 10000, 11000, 12000, 13000, 14000, 15000, 16000, 17000, 18000, 20000, 22000, 25000, 27000, 28000, 35000
failed	6000, 10000, 18000, 38000, 47000

The pre-treatment MFI values are found to not be significantly different between the two groups. In this case it may be possible to conclude that a combined DSA MFI of greater than 38000 is associated with future graft failure to rejection, however with the small numbers and a graft failure also seen in patients with an MFI similar to, or below, the mean of the survival group this should be treated with caution.

A scatter plot comparing Total DSA MFI between two groups: 'survived' and 'failed'. The y-axis is labeled 'Total DSA MFI' and ranges from 0 to 40,000. The 'survived' group is represented by black circles, with a mean MFI of approximately 5,500. The 'failed' group is represented by black squares, with a mean MFI of approximately 11,500. The plot shows that the 'failed' group has a significantly higher mean MFI compared to the 'survived' group.

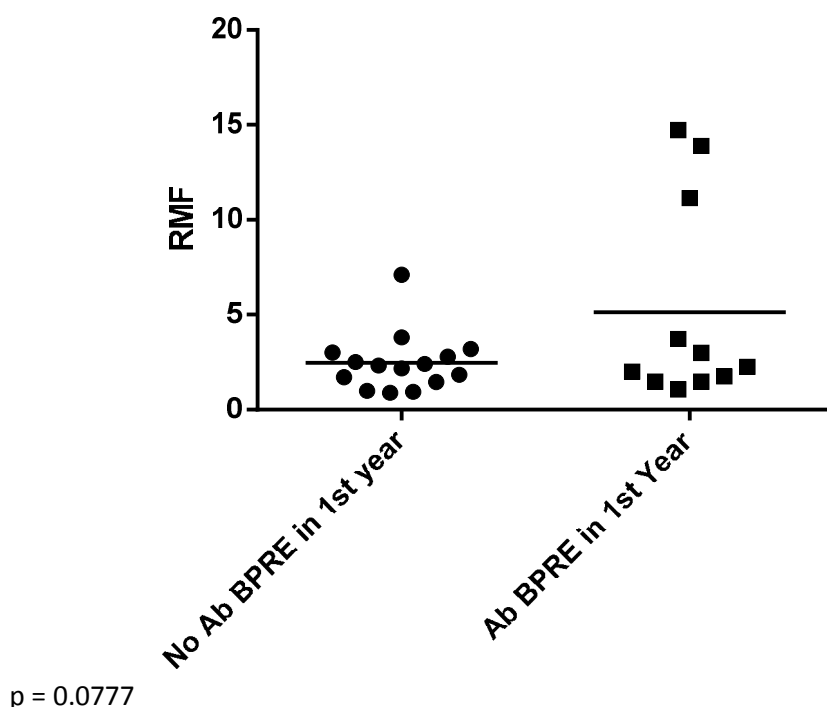
170

The pre transplant total DSA MFI values are also not significantly different between the two groups, and it is clear that the values achieved in both groups are within the same range and therefore it is not possible to associate a pre transplant DSA MFI value with future loss due to rejection.

3.4.9 Association with Antibody Mediated Rejection in the First Year Post Transplant.

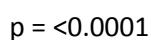
A similar analysis was performed comparing T and B cell RMF and total DSA MFI values in both the pre-treatment and pre transplant samples with the incidence of patients diagnosed with one or more episodes of rejection involving antibody during the first year post-transplant.

Figure 46 - Pre-treatment T cell RMF values compared to incidence of antibody mediated rejection in the first year post-transplant.



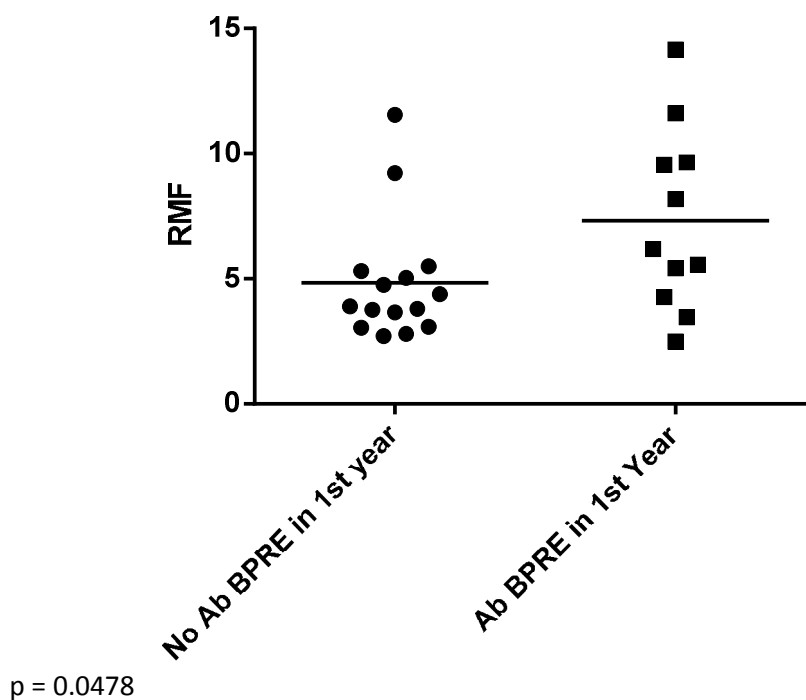
The T cell RMF values pre-treatment, shown in figure 46 above, whilst having a significant difference in the mean values between the two groups, show considerable crossover. It may be once again possible to suggest that patients with an RMF of greater than 10 are likely to suffer an episode of AMR in the first year, although these were also the patients who lost their

Figure 47 - Pre transplant T cell RMF values compared to incidence of antibody mediated rejection in the first year post-transplant.



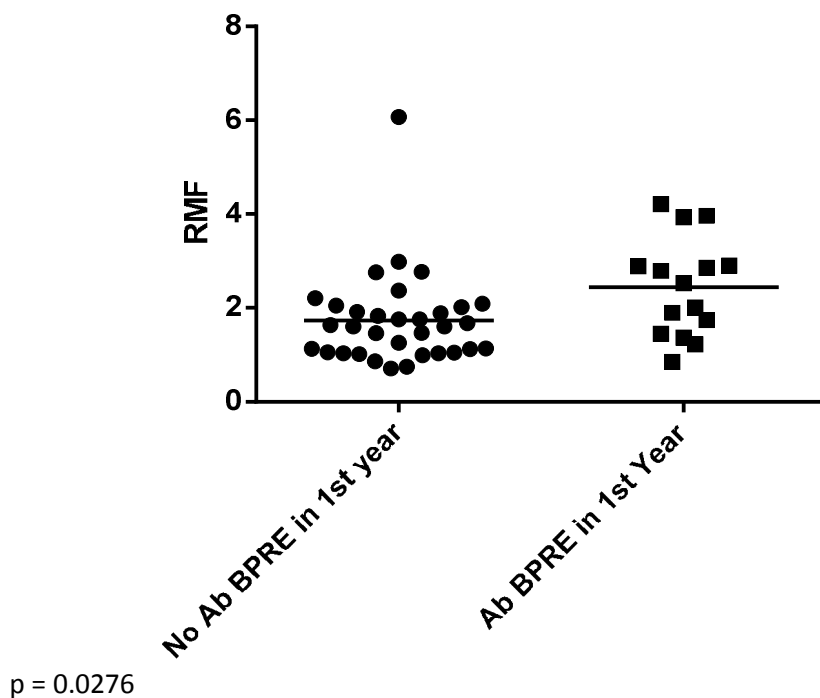
172

Figure 48 - Pre-treatment B cell RMF values compared to incidence of antibody mediated rejection in the first year post-transplant.



Whilst the mean B cell pre-treatment RMF values are just significantly different, as figure 48 above indicates the ranges of values in both groups are very similar and, based on this data, it is not possible to suggest a starting B cell RMF above which a patient is at risk of one or more episodes of AMR in the first year. Increasing patient numbers may allow for more significant conclusions to be drawn in the future.

Figure 49 - Pre transplant B cell RMF values compared to incidence of antibody mediated rejection in the first year post-transplant.



Again, whilst statistically significant, the data in figure 49 above, clearly show that there is no obvious association between the pre transplant B cell RMF values and the incidence of AMR in the first year.

$p = 0.0128$

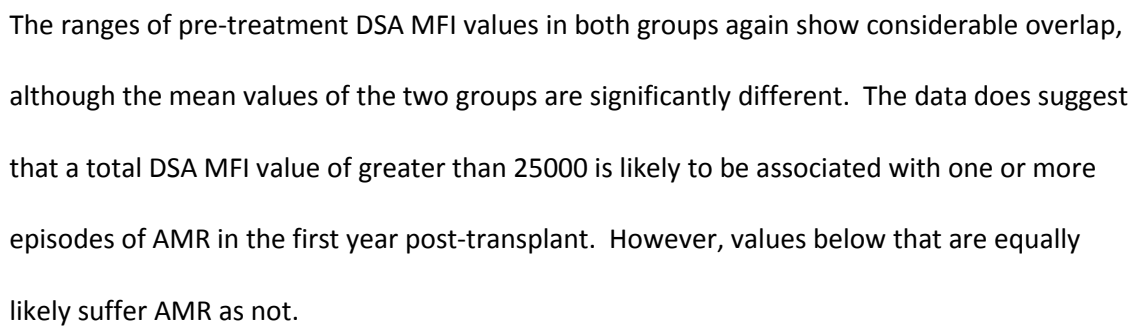
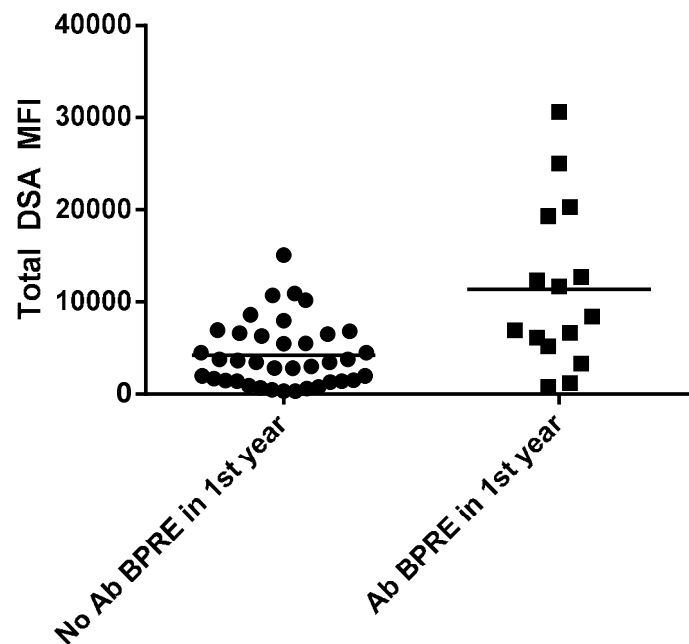


Figure 51 - Pre transplant total DSA MFI values compared to incidence of antibody mediated rejection in the first year post-transplant.



$p = <0.0001$

The difference in the mean values of the total DSA MFI pre transplant are highly significantly different, but once again there is considerable overlap in the lower values. The data indicates that a total DSA MFI value of greater than 10000 pre transplant is highly indicative of AMR post-transplant, but at values lower than that it is not possible to predict occurrence of AMR based on the MFI values.

A similar analysis was carried out for overall incidence of BPRES in the first year – combining all the episodes of T cell and antibody mediated rejection, graphs not shown. As can be seen from the information in table 7 eight patients suffered episodes of TCMR only in the first year. When including these additional patients the only values to yield a significant difference were the T cell RMF values pre-treatment, giving a p value of 0.0023. The other groups had p values ranging from 0.2454 – 0.3147, which are not statistically significant. Therefore there appears to be no association between antibody titres, measured by RMF or MFI, and incidence of T cell mediated rejection.

Overall 6 of the 54 HLA antibody incompatible grafts have been lost to rejection. 5 of these came from the group requiring antibody removal due to a positive crossmatch and all were due to either AMR or combined AMR and TCMR. The group of patients who were DSA positive but crossmatch negative lost only 1 graft, this was due entirely to TCMR.

3.4.10 Assessment of transplant outcome and the effect of immune memory.

At the start of this study it was hypothesised that patients who had preformed donor specific antibody to an HLA antigen to which they had previously been exposed were likely to have both T and B cell immune memory to this antigen, and therefore show a more rapid and vigorous response when re-challenged with the same antigen compared to patients who were DSA positive, but not challenged with a repeat mismatch. In order to assess the effect of pre formed donor specific antibody and the presence of repeat mismatches on the outcome of renal transplants data for both study and control groups was collected as described in the methods section.

To assess the effect of pre formed donor specific antibody on renal transplant outcomes, patients were categorised as being HLA specific antibody negative, 3rd Party antibody positive and donor specific antibody positive.

To assess the effect of the presence of repeat mismatches presented by the donor organ, patients were categorised as being repeat mismatch negative or repeat mismatch positive.

To assess the effect of the presence of repeat mismatches to which preformed donor specific antibody was also present or absent, indicating the presence of immune memory directly to some or all of the mismatches being presented, each of these groups were then subdivided into those who received a graft presenting a repeat HLA mismatch from either a previous graft or pregnancy and those who were not challenged with a repeat mismatch.

For purposes of the assessment of immune memory on outcomes for patients whose grafts failed during the study period, those who failed due to reasons other than rejection were censored, in addition to those who died with a functioning graft. Loss due to rejection was defined by reference to the histological reports of biopsies taken between transplant and return to dialysis, or patient death.

Overall 20 grafts were lost due to non-immunological causes. 13 patients died with a functioning graft, 4 grafts were lost to recurrent disease, 1 deceased donor organ failed to function and was removed, 1 was lost following major post biopsy bleed and 1 was lost due to renal vein thrombosis secondary to prolonged hypotension in the immediate post-transplant period.

The demographics of the patient groups can be seen in table 8 below.

For data analysis follow up to 5 years, or 1825 days, has been included.

Table 8 - Demographics of all six groups of patients included in the Chapter 3 antibody analysis.

		No HLA Specific Antibody, Repeat Mismatch Negative	No HLA Specific Antibody, Repeat Mismatch Positive	3 rd Party Antibody Positive, Repeat Mismatch Negative	3 rd Party Antibody Positive, Repeat Mismatch Positive	Donor Specific Antibody Positive, Repeat Mismatch Negative	Donor Specific Antibody Positive, Repeat Mismatch Positive
Number of Recipients		169	5	48	11	31	23
Median (range) Age at time of Transplant	Donor	44 (3 - 75)	42 (24-55)	49.5 (12-67)	50 (23-66)	44 (20-65)	41 (20-68)
	Recipient	44 (17-77)	48 (39-62)	43 (18-73)	49 (26-64)	46 (25-72)	41 (20-68)
Sex (M:F)	Donor	82:87	2:3	29:19	10:1	15:16	16:7
	Recipient	116:53	2:3	23:25	5:6	14:17	10:13
Donor Type - Deceased:living		76:93	3:2	27:21	3:8	9:22	1:22
Median Cumulative Number of Mismatches (A,B,DR)		3	2	2	3	3	3
Median Number of Previous Transplants		0	1	0.5	1	0	1
Median time of follow up		2040 (974-3075)	1828 (1354-2784)	1634 (1060-2989)	1780 (1136-2294)	1383 (249-3096)	1320 (319-2200)
Mean %cRF at time of transplant		0	0	51	56	58	62

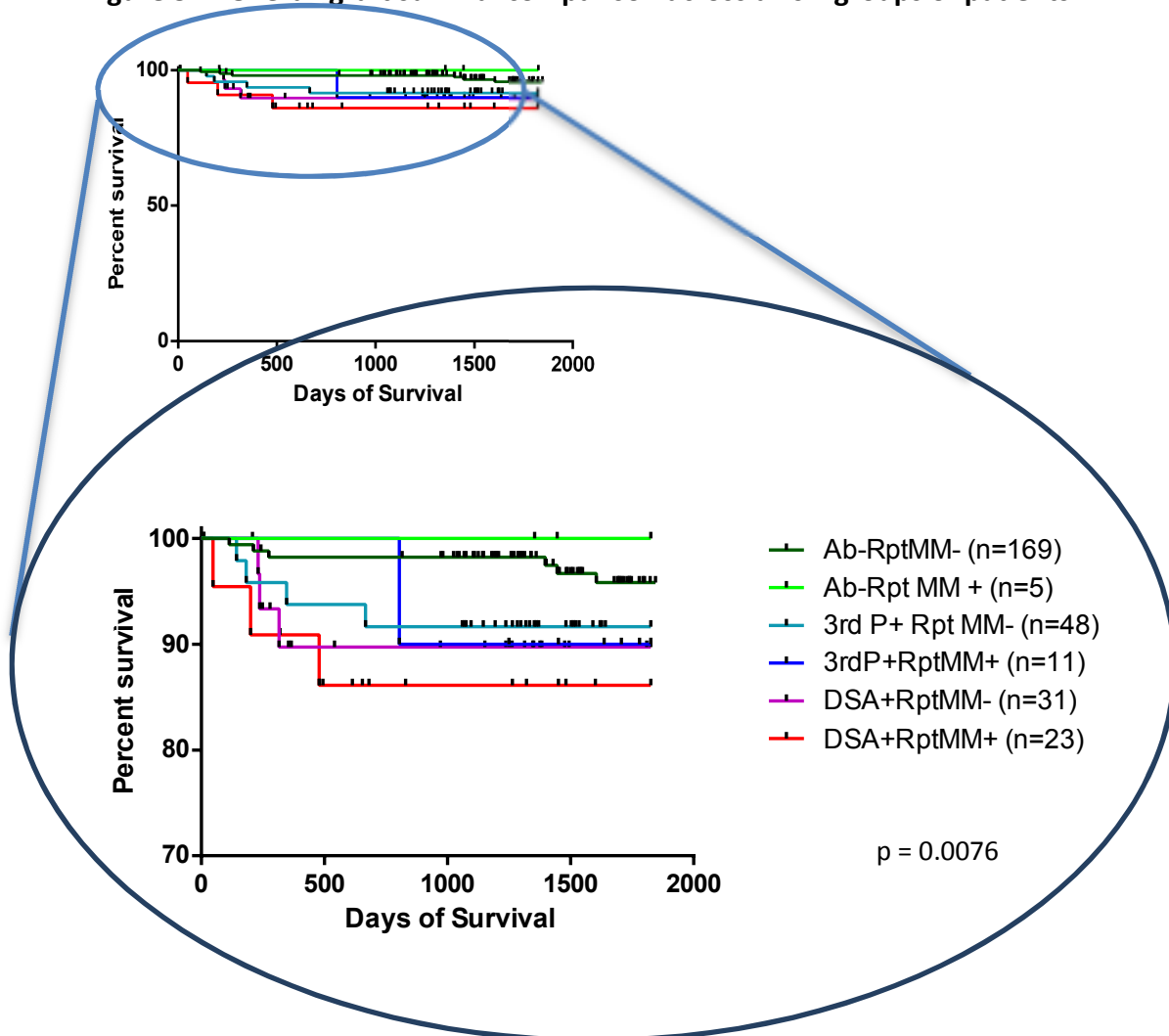
		No HLA Specific Antibody, Repeat Mismatch Negative	No HLA Specific Antibody, Repeat Mismatch Positive	3rd Party Antibody Positive, Repeat Mismatch Negative	3rd Party Antibody Positive, Repeat Mismatch Positive	Donor Specific Antibody Positive, Repeat Mismatch Negative	Donor Specific Antibody Positive, Repeat Mismatch Positive
Mean DSA MFI Pre treatment		-	-	-	-	9549	21869
Mean DSA MFI Pre Transplant		-	-	-	-	4067	9030
Mean T cell RMF Pre treatment		-	-	-	-	3.01	4.00
Mean T cell RMF pre Transplant		-	-	-	-	1.16	1.36
Mean B cell RMF pre treatment		-	-	-	-	3.94	7.26
Mean B cell RMF pre transplant		-	-	-	-	1.66	2.36

Graft survival analysis was performed by generating Kaplan-Meier curves for each data set.

The p values quoted are generated by the logrank test or the logrank test for trend.

Initially the overall graft survival over five years was compared between all six groups of patients. Since no single group fell below a survival of 85% the 'y' axis representing percentage of patients surviving had been edited to show only 70% and above to allow for easier observation of trends, as represented in figure 52 below. This is the case for all the following Kaplan-Meier curves presented.

Figure 52 - Overall graft survival comparison across all six groups of patients.

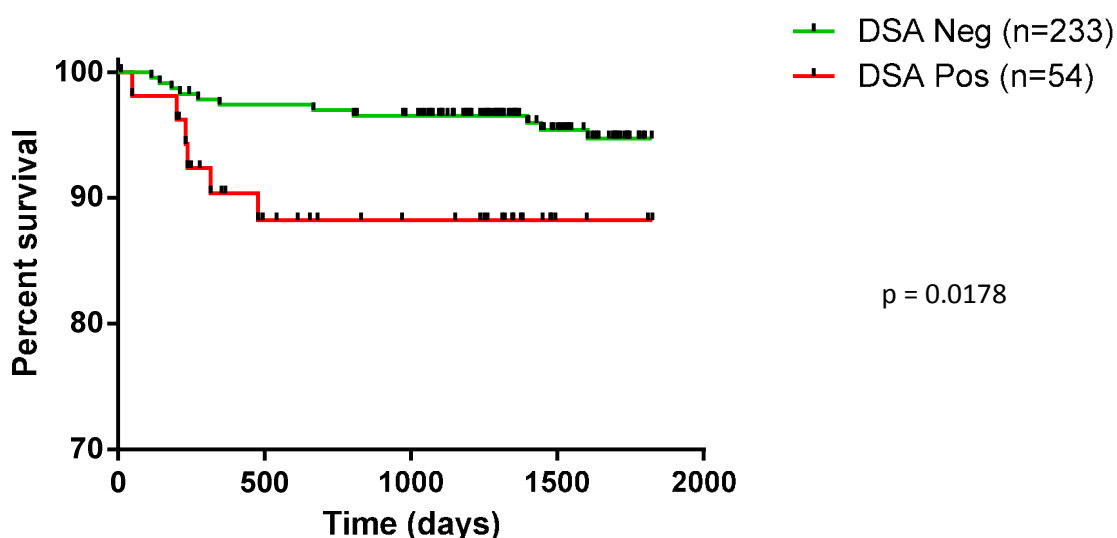


The overall graft survival analysis presented in figure 52 above indicates that patients who produce no HLA specific antibody have the best 5 year graft survival rates, with survival decreasing based on the presence of 3rd party then donor specific antibody, with the group showing the lowest 5 year graft survival rates being those who produce donor specific antibody and are presented with a repeat mismatch. A more in depth analysis of these differences follows.

3.4.11 The effect of HLA specific antibody on renal transplant outcomes.

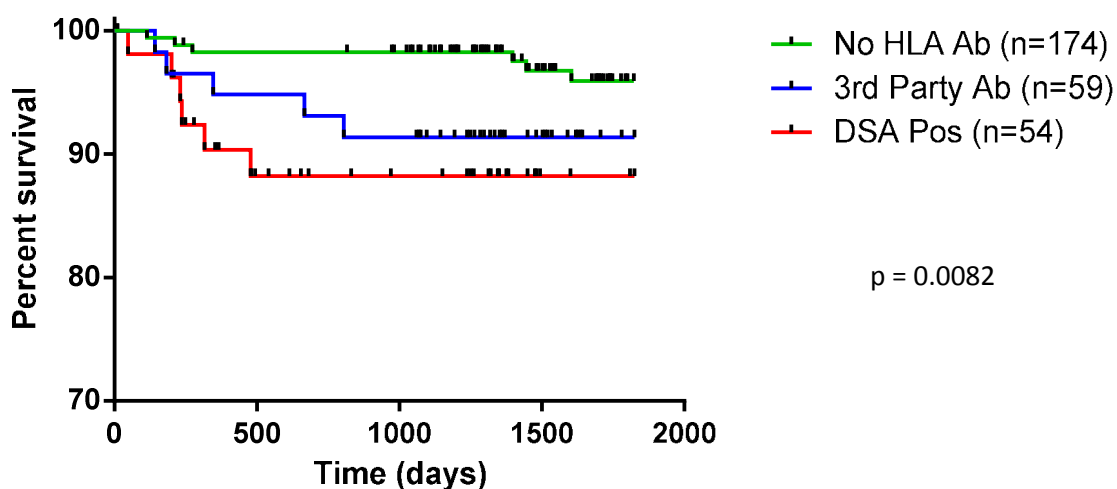
Initially the effect of the presence or absence of donor HLA specific antibody on renal transplant outcome was compared. The graft survival in these two groups can be seen in figure 53 below.

Figure 53 - Renal graft survival based on the presence or absence of donor HLA specific antibody.



Whilst there is a clear difference in outcome between the DSA negative and positive groups to assess if the presence of any HLA specific antibody is associated with an increase in graft loss due to rejection the absence of any HLA specific antibody, the presence of 3rd party HLA specific antibody only or donor HLA specific antibody on renal transplant outcomes was compared. The graft survival in these 3 groups can be seen in figure 54 below.

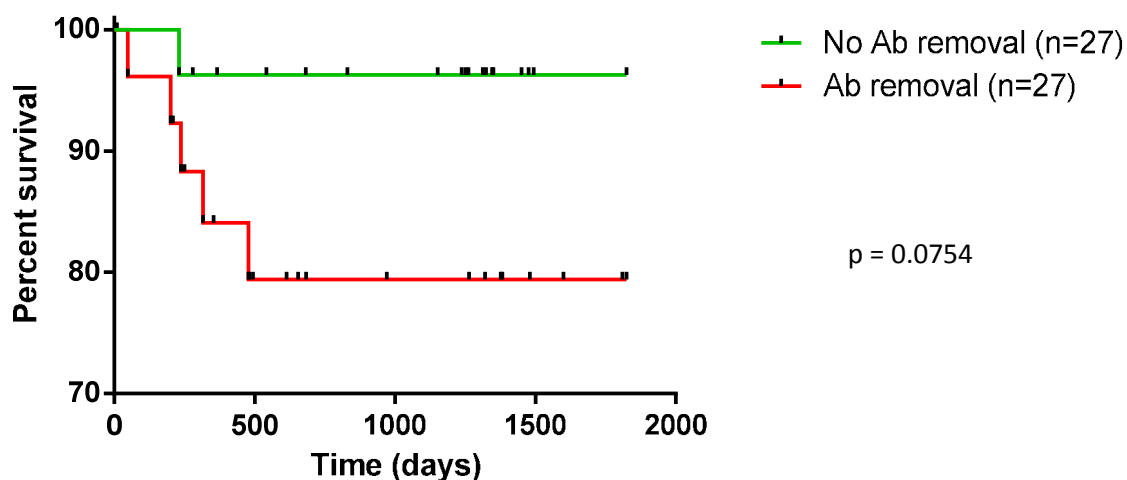
Figure 54 - Renal graft survival based on the presence or absence of HLA specific antibody.



The group of patients with preformed donor specific antibody clearly have lower graft survival compared to the other two groups, however the group producing 3rd party HLA specific antibody also have lower graft survival than those who are HLA specific antibody negative.

To assess the effect of the titre of DSA on outcomes, graft survival rates in the DSA positive group of patients who required antibody removal due to positive crossmatch were compared with the DSA positive patients who were crossmatch negative and did not receive antibody removal pre transplant. The survival curve generated can be seen in figure 55 below.

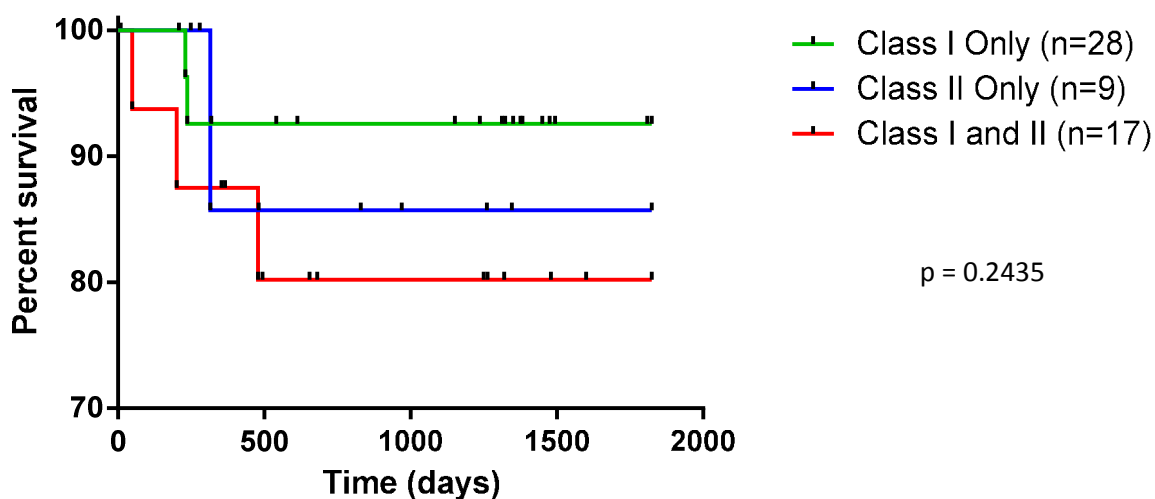
Figure 55 - Renal graft survival in DSA positive patients based on the requirement for antibody removal.



The curve presented in figure 55 above clearly shows that titre of antibody may well be important in graft survival and confirms the earlier finding that antibody detected by crossmatching, rather than just on single antigen screening beads, may be more significant in longer term outcomes.

The final analysis of the DSA positive patients was to assess if the HLA class to which the antibody was directed had an impact on graft survival rates in this group. Patients were divided into those with HLA Class I only DSA, HLA Class II only DSA and those with both HLA Class I and Class II DSA. The curve generated can be seen in figure 56 below.

Figure 56 - Renal graft survival in DSA positive patients based on the HLA Class to which the DSA was directed.

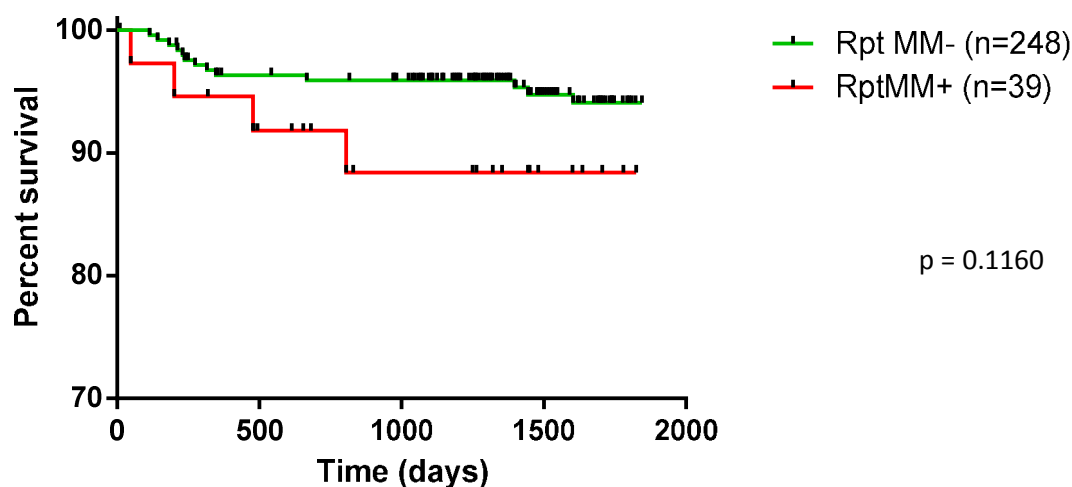


Although not statistically significant, the survival curves in this analysis suggest that antibody to HLA Class I only are the least detrimental to outcome, with a potential cumulative effect where patients transplanted across both HLA Class I and Class II DSA have the lowest five year graft survival rates.

3.4.12 The effect of the presence of repeat HLA mismatches on renal transplant outcomes.

To investigate the effect of the presence of one or more repeat HLA mismatches on renal transplant outcomes the patients were initially divided into those presented with a repeat mismatch and those which were not, regardless of the presence of HLA specific antibody. The survival curve generated can be seen in figure 57 below.

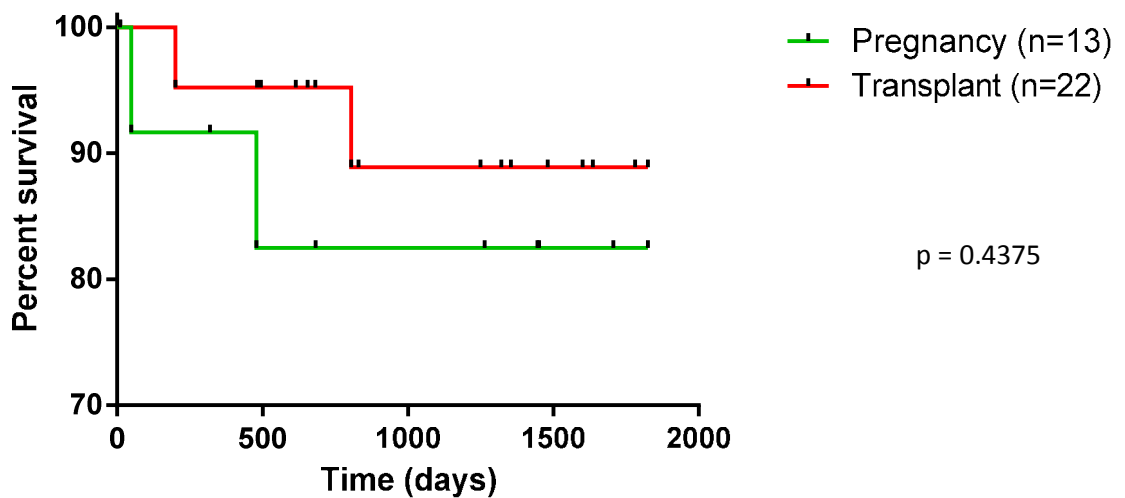
Figure 57 - Renal graft survival comparison between patients presented with a repeat HLA mismatch and those which were not.



Although not statistically significant, this analysis indicates that the presence of a repeat HLA mismatch may impact on the 5 year graft survival rates. However it must be noted that half the small number of repeat mismatch positive patients also had DSA, which could be influencing the results.

The patients presented with a repeat HLA mismatch had all previously been exposed to non-self HLA through either a previous transplant or pregnancy. To assess if the route of previous exposure had an impact on graft survival in patients with a repeat mismatch, regardless of HLA antibody status, the repeat mismatch positive group were divided into those exposed via transplant and those via pregnancy. The survival curve generated can be seen in figure 58 below.

Figure 58 - Renal graft survival comparison in patients presented with a repeat HLA mismatch based on previous exposure through either pregnancy or transplant.

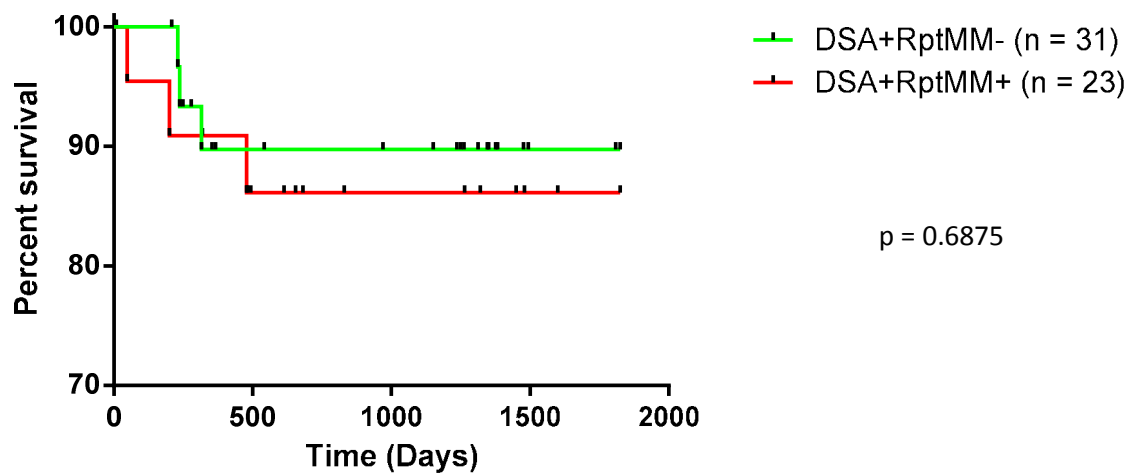


Again the small numbers involved hamper any meaningful statistical comparison, however the trend of the data suggests that previous HLA exposure via pregnancy may be more detrimental than via transplant to graft survival, although this is not statistically significant and would require greater patient numbers for confirmation.

3.4.13 The effect of immune memory on renal transplant outcomes.

The hypothesis when starting this investigation was that patients transplanted with a kidney which presented an HLA mismatch to which they had previously been exposed, and produced HLA specific antibody, demonstrating the presence of immune memory, would have lower overall graft survival compared to patients who were presented with an HLA mismatch to which they produced demonstrable antibody but to which they had not previously been directly exposed, the antibody believed to be a consequence of epitope crossreactivity. To assess this the DSA positive patients were divided into those presented with repeat mismatches to which they had produced antibody and those who were not. The survival curve generated can be seen in figure 59 below.

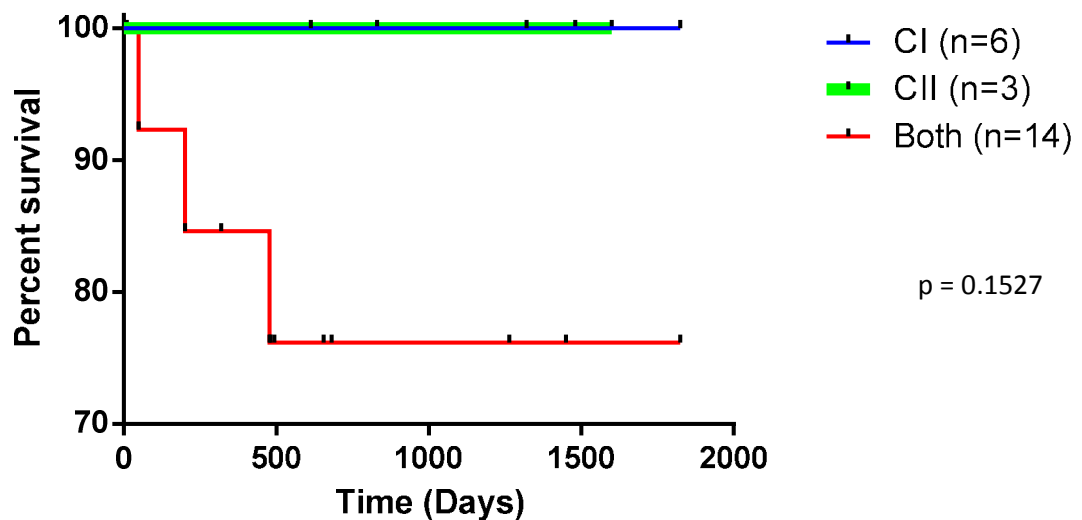
Figure 59 - Renal graft survival comparison in patients presented with a repeat HLA mismatch to which they produce antibody and those that were not.



This data would suggest that there is no difference in 5 year outcomes between those patients who produce DSA and are presented with a repeat mismatch and those who are not.

To assess if the HLA Class of the repeat mismatch with DSA has an effect the DSA positive, repeat mismatch positive patients were further subdivided into those who were presented with just a Class I, those with just a Class II and those with both Class I and Class II repeat mismatches. The survival curve generated can be seen in figure 60 below.

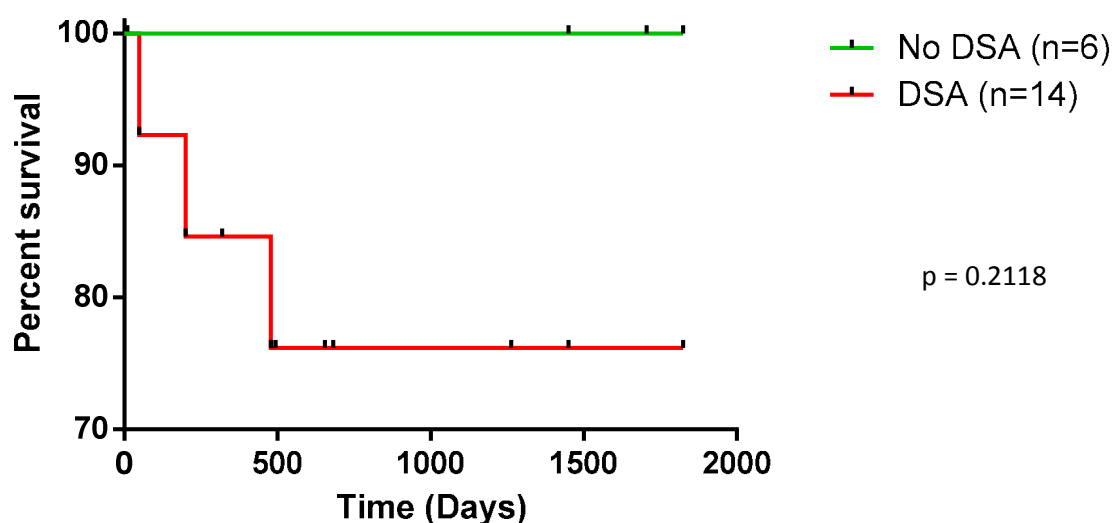
Figure 60 - Renal graft survival comparison in patients presented with a repeat HLA mismatch to which they produce antibody based on HLA Class.



Again, despite the small numbers involved, there does appear to be a trend suggesting that those patients who are presented with both HLA class I and class II repeat mismatches, to which they also produce antibody, have lower graft survival rates. This again does not reach statistical significance and would require a larger group of patients to study before the trend observed could be confirmed.

To assess if the presence of HLA Class I and II repeat mismatches is detrimental in all grafts, regardless of antibody status, all patients who received a graft presenting both HLA class I and II repeat mismatches during the study period were divided into those with DSA and those without, and the groups compared. The survival curves generated can be seen in figure 61 below.

Figure 61 - Renal graft survival comparison in patients presented with repeat HLA Class I and II mismatches with and without donor specific antibody.



Again, despite the small numbers, a trend, whilst not statistically significant, does appear to be present where patients who have previously been exposed to a combined HLA Class I and II mismatch, to which they have formed an immune response culminating in IgG class antibody generation, have worse outcomes compared to those who have previously been exposed but do not appear to have formed an antibody response.

3.4.14 The Effect of Immune Memory on Biopsy Proven Rejection

Episodes.

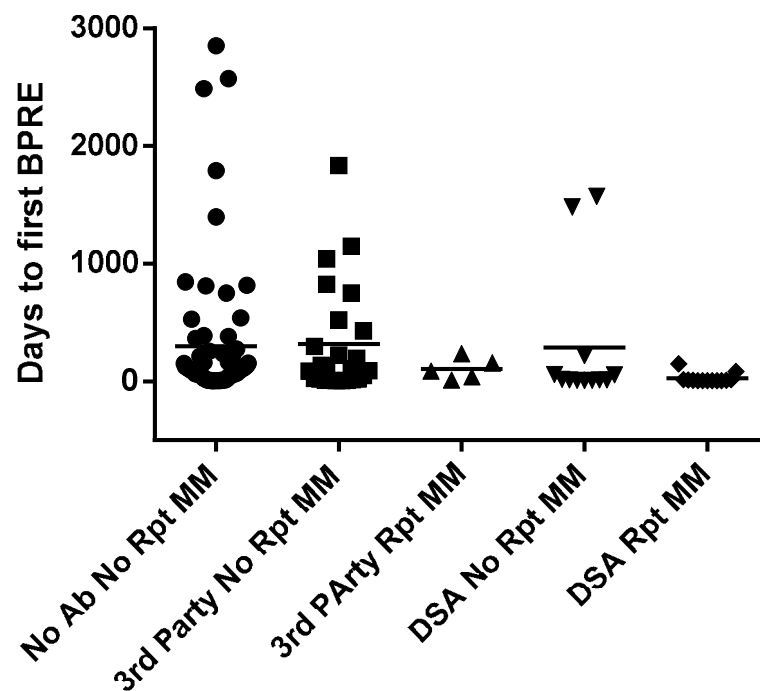
Whilst overall graft survival, or loss to rejection, is a good indicator of the effects of the various parameters on outcome, patients may well suffer one or more episodes of immunological rejection which do not, at least in the immediate study period, result in graft loss. Data was collected for all the patients investigated in this study relating to the number and timing of biopsy proven rejection episodes (BPRE). The most common renal graft biopsies performed at our centre are 'for cause' biopsies, where a change in renal function, as indicated by a rise in serum creatinine or reduction in estimated glomerular filtration rate, suggests the graft is not functioning as well as previously seen. Biopsies are analysed by the histopathology department and reported on with reference to the current Banff criteria for diagnosis of

causes of renal allograft dysfunction. For the purposes of this analysis 'for cause' biopsies that indicated definite, or were 'suspicious of', T cell mediated rejection (TCMR) or antibody mediated rejection (AMR) were classed as a BPRES. The date of the first BPRES for each patient, the total number of BPRES over the study period and the aetiology of the rejection, being TCMR, AMR or both, were all recorded. The time in days to the first BPRES was calculated by subtraction of the transplant date from the biopsy date, to give the difference in days. For the purpose of the initial investigation all BPRES, whether TCMR, AMR or both, were combined, separate analysis of the different types of rejection is detailed later. The time to first BPRES was chosen as a distinct end point as immune memory responses are known to occur more rapidly in response to a stimulus to which the individual has previously been exposed, a second set response, when compared to a primary response in an individual naïve to a particular non-self antigen. Therefore when investigating the influence of immune memory on transplant outcomes it was hypothesised that patients who received a graft bearing mismatches to which their immune system had previously been exposed would generate a response and have earlier rejection episodes than those who were not presented with a mismatch of which they had memory. The time to first BPRES was calculated for all patients who has a BPRES over the study period in five of the six groups of patients. In the HLA specific antibody negative, repeat mismatch positive group of patients only one suffered a BPRES, at day 13 post-transplant. However, they could not be included in the analysis as a single value for a group cannot be subject to statistical analysis. The mean time in days for each group was then compared using a non-parametric one way ANOVA, Kruskal-Wallis test. A summary of the results can be seen in table 9 and figure 62 below.

Table 9 – Median and range of time to 1st BPPE in each of the six patient groups.

Patient Group	Number of patients with BPPE	Mean time to 1st BPPE (days)	Range of time to 1st BPPE (days)
HLA Specific Antibody Negative, Repeat mismatch Negative. (n=169)	73	296	5 – 2853
HLA Specific Antibody Negative, Repeat mismatch Positive. (n=5)	1	13	-
3 rd Party antibody positive, repeat mismatch negative. (n=53)	25	318	7 – 1835
3 rd Party antibody positive, repeat mismatch positive. (n=11)	4	106	12 – 236
Donor specific antibody positive, repeat mismatch negative. (n=31)	12	289	6 – 1573
Donor specific antibody positive, repeat mismatch positive. (n=23)	13	25	5 – 149

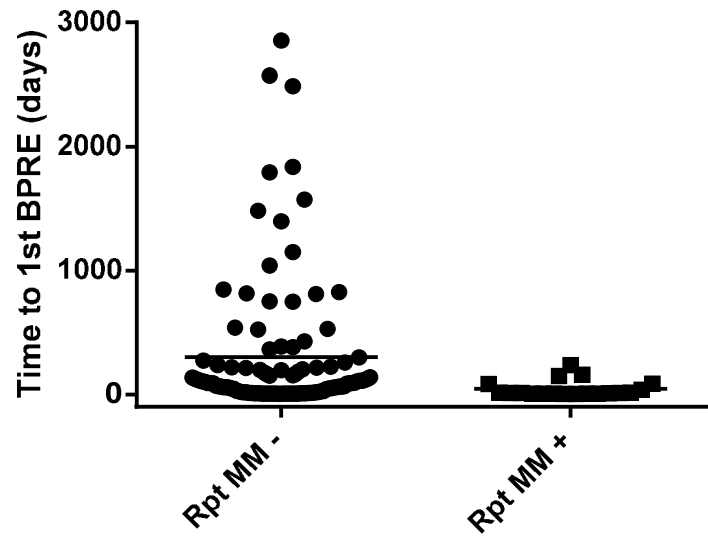
Figure 62 - Time to first biopsy proven rejection episode in all groups of patients.



$p = 0.0014$

The mean time to 1st BPPE is different between the various patient groups. Within each antibody status group the patients who are presented with a repeat mismatch all have a shorter time to 1st BPPE compared to the repeat mismatch negative patients. There were no obvious differences between the patients who made up the majority of each group and those that could be classed as outliers. One potential reason for late rejection could be related to patient non-compliance with the immunosuppressive medication, or rejection following immunosuppression reduction or withdrawal, although this was not noted in the patient records. To further assess the effect of a repeat mismatch, regardless of the presence of HLA specific antibody, the time to 1st BPPE was compared between the patients presented with a repeat mismatch and those who were not. The data was analysed using an un-paired t-test. The comparison graph can be seen in figure 63 below.

Figure 63 - Comparison of time to 1st BPPE between patients with and without a repeat mismatch.

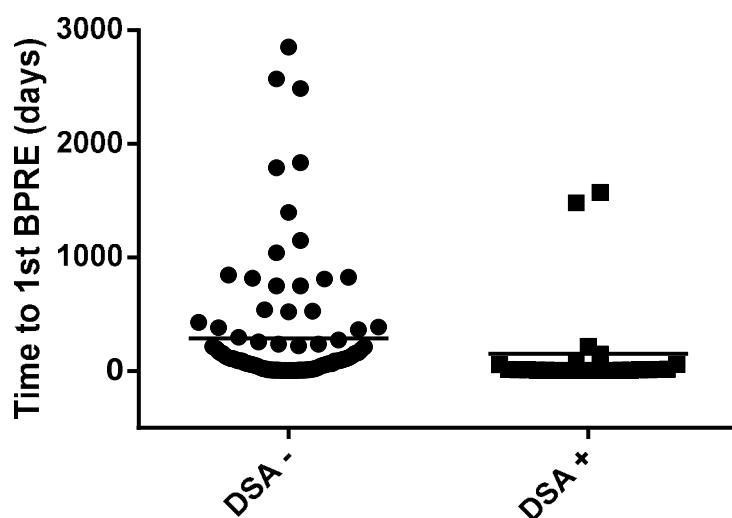


$p = 0.0467$

There is a clear difference between these two groups, not just in the mean time to 1st BPPE, but also in the range of time points that this is seen, with patients presented with a repeat mismatch having a shorter time to first BPPE and all being earlier than the mean time in the no repeat mismatch group.

A similar analysis was carried out to compare those patients who were DSA positive with those who were not. The comparison graph can be seen in figure 64 below.

Figure 64 - Time to 1st BPARE in patients with and without DSA.

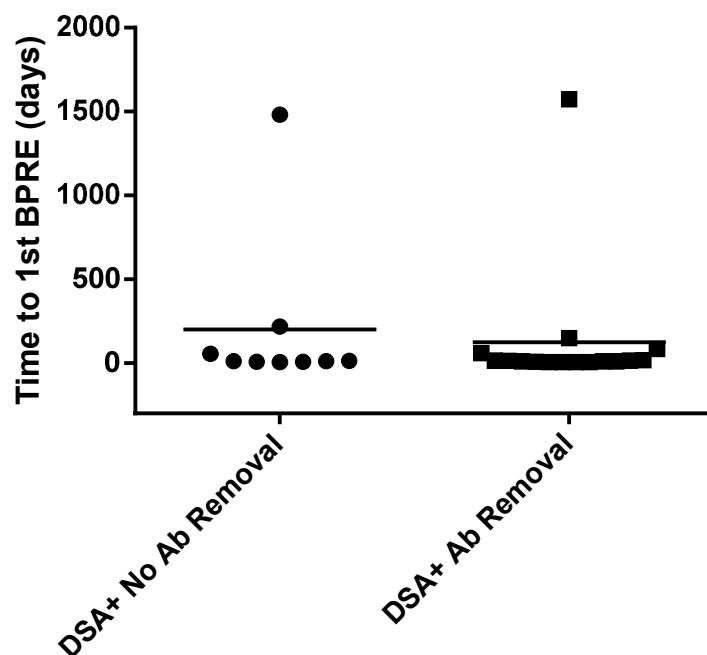


$p = 0.2316$

Whilst there is a difference in mean time to 1st BPARE between groups, it is not statistically significant. There are two clear outliers in the DSA positive group where there was a longer time to first BPARE than seen with the other rejecting patients. These patients demonstrate no obvious differences from the other patients in terms of characteristics. Other than these patients the time to first BPARE is generally earlier than many of the DSA negative group.

To assess if there is an effect of DSA titre on time to first BPARE a similar analysis was carried out limited to the DSA positive patients, regardless of repeat mismatch status, comparing time to 1st BPARE in those who required antibody removal due to a positive flow crossmatch and those who were single antigen bead positive only and did not receive antibody removal pre transplant. The comparison graph can be seen in figure 65 below.

Figure 65 - Time to 1st BPPE in DSA positive patients with and without the requirement for antibody removal.

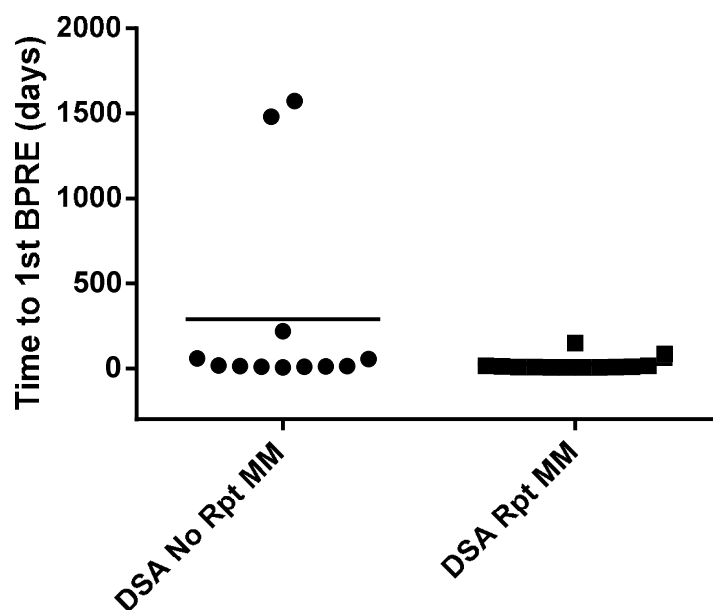


p = 0.6656

This indicates that there is little difference in the mean time to 1st BPPE between the patients with DSA at levels requiring antibody removal compared to those who did not require antibody removal pre-transplant.

The final analysis was to investigate the effect of immune memory on time to 1st BPPE by comparing the patients who were DSA positive with and without the presence of a repeat mismatch. Analysis was carried out as previously described and the comparison graph can be seen in figure 66 below.

Figure 66 - Time to 1st BPPE in DSA positive patients with and without a repeat mismatch.



p = 0.0355

This analysis indicates that patients who have been previously exposed to an HLA mismatch and generated an immune response, and therefore immune memory, as demonstrated by the production of IgG class HLA specific antibody, have shorter times to rejection episodes diagnosed on biopsy compared to those patients who produce DSA but not in direct response to the same mismatch.

The data presented above regarding time to 1st BPPE has combined instances of both TCMR and AMR as total BPPE. Data was however collected for the aetiology of the rejection episode recorded. Table 10 below summarises the total number and percentage of patients in each group to suffer a BPPE and of these how many included a T cell or antibody element.

Table 10 - Summary of the number of patients suffering BPRE and its aetiology in all groups.

	Antibody negative, Repeat mismatch negative	Antibody negative, repeat mismatch positive	3rd party antibody positive, repeat mismatch negative	3rd party antibody positive, repeat mismatch positive	Donor specific antibody positive, repeat mismatch negative	Donor specific antibody positive, repeat mismatch positive.
Total number of Patients	169	5	53	11	31	23
Number of patients with BPRE	73	1	25	4	12	13
% of patients with BPRE	43	20	47	36	39	56
% of BPRE with T cell involvement	100	100	100	100	83	54
% of BPRE with antibody involvement	18	0	20	75	33	92

The proportion of patients diagnosed with BPRE across all groups are similar, although the DSA positive with repeat mismatch group do clearly have a higher rate of 56% compared to a range of 20 - 47% across the other groups. The type of rejection diagnosed clearly varies with a T cell element being found in all BPRE in all groups except the DSA positive patients. The % of BPRE with an antibody element also varies widely, ranging from 0 – 75% in the non-DSA groups up to 92% in the DSA positive repeat mismatch positive group. Perhaps most striking is the difference between the two DSA positive groups where only 33% of the BPRE diagnosed had an antibody element in the repeat mismatch negative group compared to 92% in the repeat mismatch positive group. Fishers 2 tailed analysis of the effect of DSA in the presence of a repeat mismatch compared to DSA with no repeat mismatch on occurrence of BPRE with an

antibody element gave a significant difference with $p=0.0026$. The data can be seen in table 11 below.

Table 11 – 2x2 comparison of repeat mismatch status in patients who produce pre transplant donor specific antibody and were subsequently diagnosed with antibody mediated rejection on biopsy.

	No Ab mediated rejection	Ab mediated rejection
DSA positive, Repeat MM negative	27	4
DSA positive, Repeat MM positive	11	12

As discussed earlier a local audit of rejection rates in 2010 found higher than expected rates of T cell mediated rejection across all patients. This led to a change in the immunosuppressive regimen used at the end of 2010. Whilst this change will not have influenced the data collected for patients in the control groups some of the patients in the HLA antibody incompatible transplant group did benefit from the review. One major change that was made was to give patients with donor specific antibody induction therapy of Campath or anti-thymocyte globulin (ATG) prior to transplant. By depleting recipient T cells from the circulation it was believed that rates of T cell mediated rejection would be reduced. To assess if this change in immunosuppressive regimen has had an effect on the data presented above a smaller comparison in the DSA positive groups was performed comparing rates in patients who did and did not receive T cell depleting induction therapy. The results are presented in table 12 below.

Table 12 - Summary of BPRE in DSA positive patients before and after the use of T cell depleting induction therapy.

	No pre transplant T cell Depletion	Pre transplant T cell depletion
Total number of Patients	39	15
Number of patients with BPRE	18	7
% of patients with BPRE	46	47
% of BPRE with T cell involvement	78	43
% of BPRE with antibody involvement	56	86

The percentage of patients with one or more BPRE in each group is the same. However, it does appear that the proportion of these which had T cell involvement has indeed decreased, although has certainly not been eliminated. Of interest, the proportion of BPRE with antibody involvement is greater in the group treated with T cell depletion pre transplant compared to those without T cell depletion, although this is not statistically significant with $p=0.35$. To investigate if this could be due to differences in the patients included in the two groups in terms of immunological complexity a breakdown of the type of transplant and antibody status of the patients is shown in table 13 below.

Table 13 - Summary of patients with and without pre transplant T cell depletion.

	No pre transplant T cell Depletion	Pre transplant T cell depletion
Number of DSA positive, Repeat mismatch negative	25	6
Number of DSA positive, Repeat mismatch positive	14	9
Number of patients needing Ab removal.	16	11
Mean MFI pre treatment	14458	20729
Mean MFI pre transplant	6549	10425
Number of grafts lost to rejection	4 (10%)	2 (13%)

As can be seen from the data in Table 13 above, the group of patients receiving T cell depletion therapy pre transplant include a higher proportion of DSA positive repeat mismatch positive patients, a higher proportion of patients requiring antibody removal with higher average DSA MFI values both pre-treatment and pre transplant. However the percentage of patients losing their grafts to rejection in each group is similar, perhaps indicating that pre transplant T cell depletion may reduce the incidence of graft loss to rejection in the patients with greater immune complexity.

3.5 Discussion

Prior to starting this project our unit had successfully transplanted three patients following removal of donor HLA specific antibody, either alone or in addition to removal of ABO blood group antibody. However, antibody removal had also been unsuccessfully attempted in a further three patients. For all these patients, both the transplanted and the untransplanted, the treatment periods were long, often involving months of twice weekly antibody removal therapy in addition to their usual dialysis treatments. These treatment sessions are time consuming for the patients and clinicians in addition to being costly to the healthcare system. There are also logistical problems associated with this approach, especially for a busy transplant unit without dedicated operating theatres, in that once the window of opportunity for transplant is reached the operation must happen rapidly. This requires availability of the transplant team, the facilities and most importantly the donor, when a living donor is being used, or the offer of a deceased donor where no living donor is suitable. Following the unsuccessful attempts at antibody removal, in addition to the logistical problems encountered with the successful transplants, it was decided that a more stratified approach was required to identify which patients would benefit from antibody removal therapy, how much antibody removal would be required prior to the transplant, so that a date could be booked, and if possible to provide an indication as to the risk of failure and adverse events post-transplant. As a unit we devised a strategy whereby patients would be treated with a single antibody removal session and samples taken prior to and immediately after would be assessed for changes in donor specific reactivity. It was hoped that the information generated would allow assessment as to the likely success of antibody removal in achieving a transplant, in addition to an estimate as to the number of sessions required pre transplant to achieve a negative flow crossmatch. This information would then be used to allow the transplant to be scheduled in an organised fashion with adequate notice for the donor. At Guys Clinical Transplantation Laboratory we have over 20 years' experience of the use of flow cytometric crossmatching in assessing the suitability of a patient and donor pair to proceed to transplant, it was therefore

logical to use this method in assessing the effectiveness of antibody removal in these patients. The FXM has the benefit over the CDC XM of being more sensitive and detecting antibodies of lower titre and non-complement fixing isotypes, both of which have been associated with poorer long term graft survival [303]. Analysis of both the strength of reactivity at neat in the pre and post samples, along with the reduction in the titre at which the crossmatch becomes negative, was used to predict how many antibody removal sessions would be required to achieve a negative flow crossmatch pre transplant. During the course of this study 117 recipient and donor pairs have been assessed using this method in addition to 34 pairs assessed without a test PEX or DFPP. The results achieved showed great variation in reactivity levels and in the ability for antibody to be removed, with some pairs showing little or no reduction both on dilution and following antibody removal and others becoming negative following just one session. It has previously been reported that some antibody specificities are more resistant to removal than others. Zachary et al. [301] reported that in their experience Class II specific antibodies showed greater resistance to removal than those specific for Class I antigens. To assess if this was also the case in our group of patients we analysed the difference in the number of titres reduced by a single antibody removal treatment in these patients, dividing them into those with just Class I, Class II or Class I and II DSA. We found that the greatest reduction in titre was seen on the T cell FXM, detecting Class I antibodies only, in patients with Class I or Class I and II DSA. The patients showing the least reduction in titre were those with Class II or Class I and II DSA detected using the B cell FXM. Whilst our results did not reach statistical significance they did indicate a similar finding to that reported by Zachary et.al.. This would suggest that patients with Class II DSA will require higher treatment volumes prior to transplantation compared to those with just a Class I specific DSA.

31 patients assessed using the titre crossmatch method went on to be transplanted from the donor with which they had been assessed. There have been no instances of patients being assessed as suitable for antibody removal, using the titre XM method, who we have subsequently tried and failed to transplant. However, we cannot comment on the number of

patients who we have ruled as being unsuitable to transplant with an organ from the prospective donor, but in whom the transplant could have proceeded successfully following antibody removal. The predicted number of antibody removal treatments and actual number needed to achieve a negative crossmatch pre transplant in this group were compared. The results indicated that this pre-treatment assessment did accurately predict the number of treatments required in most cases. The starting titre, particularly with the B cell positive FXM, also showed a requirement for an increasing number of antibody removal sessions pre transplant with an increasing starting titre, however in the lower titres there was considerable variation in the requirement, with, for example, some patients positive only at neat requiring no antibody removal whereas others required up to 5 sessions. In the patients where a different number were needed to that predicted, the majority appeared to require a greater number of removal sessions than first predicted, indicating an underestimate. There are a number of possible reasons for this. The first is that patients referred to us for potential HLA antibody incompatible transplantation are often assessed early in process to ensure we have accurate antibody information. They are then entered into at least two matching runs of the paired exchange scheme, with the aim of receiving a compatible graft. If they are not successful in the paired exchange matching runs they are then worked up for a direct antibody incompatible graft. This means there can be at least 6 months between the initial assessment and the commencement of the final antibody removal treatments, during this time we often find, for as yet unexplained reasons, there is an apparent increase in the antibody levels. This may be due to the longer time on dialysis, infections, vaccinations or blood transfusions during the waiting period. Another possible explanation could be that many of our HLA antibody incompatible transplants are pairs that have been referred from other units, often in distant parts of the country, and whilst the patient attends our unit for the test antibody removal treatment, the donor often provides blood locally which is then transported to our laboratory for testing. Indeed some of our donors have been from other countries including America, Africa and Thailand, meaning the donor blood is in transit for a considerable period of time

before being processed in our laboratory. It is possible therefore that the cells used for the assessment crossmatch are not in the optimum condition for testing, with potentially lower expression levels of the target HLA antigens. This would lead to lower reactivity on crossmatch and therefore a reduced estimate of the number of antibody removal sessions required. For the final crossmatch prior to transplant the donor blood is taken and processed within hours, meaning the quality of the test cells and expression levels of HLA on their surface will be greater. One solution to avoid the need for fresh cells for a crossmatch would be to assess the antibody levels using solid phase assays, such as the LABScreen single antigen beads. The single antigen beads have the disadvantage of being very costly, indeed a single one off screen with these beads for HLA Class I and II is over twice as expensive as a titre FXM, but if the results were indicative of the potential crossmatch result and remove the need to courier donor blood then the cost could perhaps be justified. The cost of carrying out serial dilution analysis using single antigen beads in the same way as the titre FXM would however be prohibitive. Therefore it is the values achieved at neat that would need to be indicative of a final crossmatch. Background work at the beginning of the project using serial samples from our early HLA antibody incompatible transplants validated the use of LABScreen single antigen beads for post-transplant antibody monitoring in these patients, as the relative variations in fluorescence seen with these beads correlated well with that seen with the FlowPRA single antigen beads. In addition the relative variations in MFI values also reflected the changes in RMF on FXM, meaning for a particular patient and donor pair an MFI value could be estimated to indicate if the level of DSA post-transplant had exceeded that which would give a positive FXM, alleviating the need for the post-transplant FXM used in the early patients. It was also shown that in patients with multiple DSA the MFI values for each specificity could be combined to give a total DSA MFI value that also well reflected the changes in reactivity seen on FXM, a technique which has also been adopted by other units [304]. A number of patients who underwent test antibody removal and titre FXM were found to have a negative FXM with a sample taken pre-treatment, not requiring antibody removal. These patients had all been

identified as being at risk of a positive XM due to the antibody specificities detected on screening, either by our laboratory or the laboratory serving the referring unit. So another area of interest was to find if there is a DSA MFI level above which a positive FXM is expected in these highly sensitised patients. To assess this, samples from 58 of the patients who had received a test DFPP and 10 patients who had not, which had all been tested against their prospective donor by titre FXM were also tested at neat only using LABScreen single antigen beads. The total Class I DSA MFI or total Class I and II DSA MFI values were then compared to the RMF values at neat from the T and B cell FXM respectively. In addition the MFI values achieved at neat were also compared to the titre at which the FXM became negative. Both these analyses found no clear correlation between the single antigen bead MFIs and the FXM results. There did not appear to be an MFI threshold above which a positive FXM would be expected for either the T or B cells. There were instances of strongly positive FXM with very low MFI values and weakly positive or negative FXM with very high MFI values. In addition the DSA MFI values at neat did not give any indication as to the titre to which the crossmatch would reach. Therefore our data would suggest that, at least in the highly sensitised patients, it is not possible to confidently predict a crossmatch result based on single antigen bead MFI values. By relying on these values alone there would be the risk of encountering an unexpectedly strongly positive crossmatch during final work up, along with the risk of subjecting a patient to unnecessary antibody removal in the face of a negative FXM or indeed excluding a patient from further work up for an antibody incompatible transplant. In 2010 Gloor et al. [304] reported a strong association between XM results and single antigen bead MFI levels. Their analysis was stratified on crossmatch strength and type, negative XM had the lowest MFI values, FXM with a channel shift of less than 300 had the next highest MFI values, followed by those positive FXM with channel shifts greater than 300 and finally those where the CDC XM was positive had the strongest MFI values. However the data presented by the group does show considerable overlap of MFI values in each group, with no obvious MFI level at which the XM result could be confidently predicted. The authors did note that some

strongly positive XM had weak or negative DSA on screening. In addition the study did not include any DSA to HLA-Cw, -DQA or -DPB as the HLA types at these loci were not known, which may well influence the results seen. There are a number of possible explanations as to why the bead and XM results do not always equate. For a number of years it has been known that, as with most antibody detection methods, the Luminex beads are susceptible to the prozone effect [305]. Prozone is the phenomenon whereby high titre antibodies are apparently either not detected or detected only at very low levels, leading to false negative results. Testing of the sera at dilution often reveals the true nature of the strength of the antibody present. There have been a number of theories suggested to explain this phenomenon, including steric hindrance, where in theory so much antibody is competing for the binding sites available that only relatively few manage to bind the target. Dilution reduces the number of antibodies competing per antigen available, allowing more to bind and therefore be detected on assay [306]. This is likely to be a greater problem in high titre sera with the single antigen screening beads where the density of antigen present on the beads is far higher than would be seen in vivo. More recently other suggestions have been published. The presence of HLA specific pentameric IgM antibodies in the sera have been suggested to block the binding sites available to the IgG antibodies [307], Kosmoliaptsis et al. reported that the prozone effect can be avoided through treatment of the sera with DTT, analogous to that used to eradicate the influence of IgM antibodies in the CDC tests [308]. Further to this work Schnaidt et al. [309] suggested that the effect of DTT on reducing prozone was not necessarily due to the removal of the IgM antibodies, but actually due to the additional effect DTT has on complement proteins in the serum. Classical complement component C1, made of C1q, which interacts with the pathogen surface or the antibody bound to the target, and proteases C1r and C1s, is also sensitive to cleavage by DTT. Schnaidt et al. hypothesised that the prozone phenomenon was due to the binding of C1 in the test serum to the antibody bound to the target antigens on the beads. This then blocks the binding sites for the secondary detection antibody, commonly targeted at the Fc region, so giving a false negative result.

Ethylenediaminetetraacetic acid (EDTA) is a common anticoagulant used for blood collection. One property of EDTA is to chelate metal ions, including Ca^{2+} , a property to which C1 is sensitive. C1 is also sensitive to heat inactivation, where incubation of sera at 56°C for 30 minutes, leads to inactivation of C1. Schnaidt et al. went on to test sera treated with DTT, C1 inhibitor C1INH or heat inactivated, and plasma taken from EDTA anticoagulated samples. They found that prozone seen with the untreated sera was virtually eliminated with all the treatment methods and that it was reinstated by the addition of an external source of C1. This does indicate that blocking by C1 is a major factor in the prozone phenomenon. With this knowledge it is therefore possible to suggest that samples where a low or negative MFI value was recorded, yet a strongly positive FXM result found, were actually suffering a prozone effect and treatment of the sera to remove C1 may reveal a different result. With this information our laboratory recently performed its own evaluation of sera treatment for routine testing and all sera are now heat inactivated by incubation at 56°C as part of the sample processing on arrival in the laboratory. Of note, in the evaluation performed the effect on crossmatching was also assessed and it appears that heat inactivation of the sera does not alter the FXM results, indicating that prozone is less commonly seen using this method. Another possible explanation for the low MFI – high FXM results could be the detection of non-specific, or non-HLA antibodies, on crossmatch. It is unlikely that non-specific auto reactive antibodies can explain the difference seen in the majority of these cases, as all patients also had an auto FXM performed to rule out the presence of auto antibody. However other, as yet undefined, targets could be responsible for the increased crossmatch results. The alternative difference seen between the bead MFI and the FXM RMF values, was that high DSA MFI values were found in some patients where the crossmatch was weakly positive or negative. One cause could be the prozone effect on the FXM. However this would have been seen as an increase in reactivity on dilution, which was not observed. Another more likely explanation is that the antibodies being detected using the single antigen bead method are not in fact specific for epitopes that are accessible on the native HLA molecule, but directed at

epitopes exposed during the manufacturing process used to create the beads. These epitopes are most likely due to incorrect folding of the HLA protein and, for Class I molecules, a lack of association with the $\beta 2$ microglobulin. These alterations in conformation lead to the detection of apparent HLA specific antibodies by the beads, which are most likely clinically irrelevant [98, 310, 311]. The detection of such antibodies has been given as a reason to explain the high number of individuals, up to 63% in one study [96], who are apparently sensitised despite receiving no alloimmunising events. Another possible explanation for weak and negative FXM results in the presence of high titre antibody could be that the donor expression levels of HLA antigens are low either due to changes in the promoter regions of the HLA gene or due to the immunomodulatory influence of drugs such as statins. Guidelines published by the European Federation of Immunogenetics, to which we comply, suggest that when genotyping an individual at high resolution the method used should allow the detection of genes more commonly associated with changes in the promoter region leading to reduced or no expression of the given antigen. In cases where an obvious and unexpected discrepancy was found between bead MFI and FXM RMF the donors were typed by sequencing and therefore this should not be a cause. To absolutely confirm the HLA expression by a given donor the cells could be HLA typed using the more traditional cytotoxic typing method which provide the phenotype rather than genotype. This was not performed as our laboratory does not include this test in our repertoire. However in the future it may be of relevance to investigate in some cases. Statins are commonly prescribed drugs aimed at lowering blood cholesterol levels, which have also been found to have immunomodulatory effects, some of which are through the reduction of expression of HLA class I molecules on some cell types [302], and effect HLA class II expression on other cell types [312]. If potential donors have been prescribed these then it is possible that the FXM result could be falsely negative or weak due to lower target expression.

It should be noted that all the antibody analysis included in this study has been performed using LABScreen single antigen beads. There are however other single antigen beads available

from a different manufacturer, Gen-probe. These beads are also for use with the Luminex analyser and comprise a similar panel and methodology. Due to the prohibitive cost the samples were not tested using both kits available. It is possible that testing with kits from the alternative manufacturer could help identify true HLA specific antibodies and provide different MFI values, which could be of better correlation with the FXM results. Another kit related concern is that lot numbers of the kits used changed on a number of occasions during the study period. There is the theoretical possibility that antigen density on the beads can vary significantly between lots [110, 313], meaning it is possible that the MFI values derived could be different between lots. However this should not be significantly common problem as in theory it would only affect samples in which maximum binding to all the antigens on the bead is achieved. At our laboratory all new lot numbers undergo rigorous validation before being put into routine use, which includes testing multiple samples to check for consistency of results, in terms of specificity and MFI value, with previous lots, and testing with Class specific mAbs, which gives an indication of changes in Ag density. Variations between lots in recent years have been found to be small.

Patients testing positive by both FXM and beads, could still show less correlation than expected due to maximum binding, or saturation, being achieved on either the beads or the FXM [307]. Here all the targets available are bound meaning that the maximum possible MFI or RMF that can be achieved, has been. Due to differences in the antigen expression levels between cells and the single antigen beads it is likely that this would not occur at the same level for both.

Groups have had varying success in associating bead MFI values with both CDC and flow XM results [142, 150, 314, 315], with the greater certainty surrounding the prediction of those which will be negative based on negative screening results. The crossmatch, either CDC or FXM, has long been seen as a requirement prior to transplant, with a negative crossmatch indicating low risk of early rejection. Therefore it has been seen as important for screening

results to predict XM results. However, in deciding how to assess patients who were eligible for antibody removal and the risk of post-transplant complications and graft loss, I decided to investigate if either the FXM RMF values or the single antigen bead MFI values were associated with post-transplant outcomes in our HLA antibody incompatible group. There were 54 HLA antibody incompatible transplants included in the analysis based on the detection of DSA on the single antigen beads, of which 27 required pre transplant antibody removal due to the presence of a positive FXM on either the B cells alone or both the T and B cells. Initially I analysed the association between the various antibody values, pre-treatment and pre transplant, and whether a graft had been lost due to rejection only during the study period. When drawing conclusions from these results it must be remembered that there is a wide variation in the follow up time in this group of patients, from 249 to over 3000 days. Firstly the T and B cell RMF values and DSA MFI values were compared to survival in the group of patients requiring antibody removal pre transplant. For the crossmatch results in this group the T cell RMF seemed to show the greater association with future graft loss, particularly in the pre-treatment sample, where an RMF of >10 was found in all of the patients who lost their graft and none of the patients who did not. Whilst there was a significant difference in the mean values of the two groups, the B cell RMF showed less association and whilst all the patients who lost their graft had an RMF of >5 an equal number of patients in the survival group also had similar starting B cell RMFs. The pre-treatment DSA MFI value also showed a difference between the two groups and here the majority of the failed transplants had a total DSA MFI of greater than 38000 where none of the surviving grafts were above this level. The analysis of the pre-transplant values included all patients classified as HLA antibody incompatible. The results with these samples were less conclusive with again the only possible association being found with the T cell RMF being >2.0. The range of B cell RMF values and total DSA MFI values in both groups were similar, despite both having different mean values, meaning that these data would suggest it is not possible to predict future outcome using these values. A number of the graft losses due to rejection recorded were in fact lost due to TCMR rather than AMR,

therefore an association between antibody values and loss may not be entirely expected.

Whilst graft survival or failure is a conclusive outcome, other post-transplant complications can occur. HLA antibody incompatible transplants are associated with higher rates of antibody mediated rejection episodes post-transplant compared to antibody compatible grafts [195] and whilst the majority of these rejection episodes can be resolved if treated promptly, the occurrence of, and damage caused by, early AMR is associated with a reduction in long term graft survival [303, 316, 317]. The ability to predict which HLA antibody incompatible grafts are at most risk of AMR in the first year post-transplant would assist in both identifying patients who require even more vigilant follow up, and allow the laboratory to convey to clinicians and patients an indication of risk for AMR, and therefore risk of reduced long term survival. The incidence of AMR in the first year post-transplant, as diagnosed on biopsy, was recorded for all the HLA antibody incompatible transplants. The T and B cell RMF and total MFI values both pre-treatment, in the patients requiring Ab removal, and pre transplant in all the HLA antibody incompatible grafts were compared between the patients who did and did not suffer at least one episode of AMR in the first year post-transplant. The T cell RMF and total DSA MFI values both pre-treatment and pre transplant did indicate a level above which AMR would be likely. For the T cells patients with a pre-treatment RMF >10 or a pre transplant RMF >2 all suffered AMR, and were generally the same patients who went on to lose their graft. However below these values there were no differences between the groups. For the total DSA MFI all patients with a pre-treatment value of >25000 or pre transplant value of >10000 were diagnosed with AMR in the first year, although at lower values there were no differences. Neither the pre-treatment or pre transplant B cell RMF values were associated with an increased risk of AMR, despite the mean for each group being significantly different. The same analysis with total BPPE, regardless of the aetiology, found no association of incidence with antibody values. This would indicate that the level of DSA detected has no association with T cell mediated rejection. It must be noted however that a number of patients were transplanted after a change in immunosuppressive protocol in 2010 and

received T cell depletion induction therapy, which could possibly skew the results observed with regard to TCMR episodes. This will be investigated in more depth later in the discussion.

From our data, despite the small patient numbers, it is possible to conclude that the T cell RMF value appears to be the most predictive of both future graft loss and occurrence of AMR in the 1st year. Patients presenting with a T cell RMF of >10 prior to treatment and those with a total DSA MFI value of >38000 appear to be at the greatest risk of graft failure, although patients with lower values also failed. Those with a pre-treatment T cell RMF of >10 or a total DSA MFI of >25000 appear to be at most risk of AMR within the first year, although, again, patients with lower values also suffered AMR. It is of interest to note that of the 27 patients who were classified as DSA positive on bead analysis but were FXM negative, and did not receive antibody removal prior to transplant, only one patient lost their graft due to rejection and on all biopsies this was classified as only being of T cell in nature. In the group who required antibody removal there were five graft losses due to rejection and all five were either solely AMR or combined AMR and TCMR. This would suggest that, in this group at least, it is the detection of antibody on the FXM which is most relevant and, that the DSA detected on single antigen screening is only clinically relevant when associated with a positive FXM, regardless of the MFI value. This directly contradicts a study published by Hirai et al. [318] who reported that in a retrospective analysis of their patients DSA MFI values of 800 – 2500 were highly associated with both acute and chronic rejection. These patients all had negative CDC and T cell XM. In a similar analysis Thielke et al. failed to find any association between pre transplant sensitisation as detected by FXM and the incidence of BPRE post-transplant in their group of antibody incompatible transplants [190]. Data published by Gloor et al. [304] also showed that whilst high baseline DSA levels did correlate with an increased risk of AMR, AMR was also frequently found in patients with low DSA titres. They concluded that baseline DSA as measured by both XM and single antigen beads was not sufficient to predict subsequent AMR. They also reported a similar finding, that baseline T cell crossmatching results correlated with graft loss, where patients with strongly positive pre-treatment crossmatches were at greatly

increased risk of graft loss compared to patients with lower antibody levels. Amico et al. [163] published a retrospective study where 334 consecutive renal transplants were investigated for the presence of DSA detected by single antigen bead screening at the time of transplant and its association with the development of AMR post-transplant. The patients all had negative CDC XM but had not been assessed by FXM. 67 of the patients were found to have DSA on the day of transplant following single antigen screening and 55% of these went on to suffer an AMR episode within 200 days compared to just 6% of those that were DSA negative. When comparing the patients with DSA who did and did not present AMR on biopsy they found no difference between the cumulative MFI values for each group. In addition the patients who were diagnosed with AMR had a 20% lower five year graft survival rate compared to both the DSA negative and DSA positive but no AMR groups, which were both the same. Unfortunately since no FXM were performed by this group it is not possible to assess if the FXM results would have shown greater association. In general these published studies agree with the findings presented here in terms of the lack of association of the bead MFI values with outcome, and a lack of consensus between positive screening beads and a positive crossmatch. This does call into question the MFI cut off values that should be employed when registering unacceptable antigens for potential renal transplant recipients with the national allocation systems. There is clearly the risk that specificities may be registered that would not be associated with a positive crossmatch and others may not be registered that would.

Since only a limited association was found between antibody strength prior to transplant and graft outcome, it is clear that this is not the only factor associated with the success or failure of an antibody incompatible graft. The hypothesis being tested by analysing our outcome results is that immune memory may play a major role in graft survival or failure, and in the incidence of rejection post-transplant in the antibody incompatible group. The presence of true IgG class alloantibody indicates that an immune response, through exposure to non-self HLA, and presentation of allopeptide by an allospecific B cell to a helper T cell via the indirect pathway of allorecognition in a germinal centre has occurred [47]. As previously discussed, HLA specific

antibodies recognise small epitopes on the accessible areas of HLA molecules. An epitope may be specific for a single HLA antigen or be found on multiple HLA antigens, leading to a single antibody clone being reactive against numerous HLA antigens. The helper T cell providing the activatory signals to the presenting B cell will however be specific for an entirely different epitope. The indirect Th cell recognises linear epitopes on peptides, usually 13 – 17 amino acids in length, generated through the processing of the HLA molecule by the B cell and then presented in the context of a self HLA class II molecule. In recent years there has been considerable interest in identifying the epitopes to which antibodies react [319], both in order to explain antibody reactivity patterns seen and also to identify HLA antigens which may present fewer immunogenic epitopes to an individual [320]. However the technical challenges involved has meant there has been less work investigating the epitopes to which indirect Th cells are reactive. The studies published to date are limited and have drawn mixed conclusions, with some suggesting that, whilst there may be multiple peptides generated, there is usually a single immunodominant peptide responsible for generating the response [321] and others suggesting that there may be multiple immunogenic epitopes, including some generated from the more conserved regions of the HLA molecules [322] indicating an ability for wide crossreactivity similar to that seen with antibody. Either way, it is possible that whilst exposure to a limited number of non-self HLA molecules may generate a wide ranging alloantibody response and apparent B cell memory, the T helper cell memory may be limited to just the original alloantigen. My starting hypothesis was that when transplanting across HLA antibody barriers the presence of a repeat HLA mismatch with donor specific antibody presents a greater likelihood of activating a full memory immune response, and likely rejection episode, than the presence of an antibody which is just epitope crossreactive and to which there may not be full immune memory. To examine this I collected outcome data from not just our HLA antibody incompatible transplant group, but also various control groups, based on HLA antibody status, namely those patients who did not produce any detectable HLA antibody pre transplant and those in whom HLA specific antibody was present, but donor specificities

had never been detected. These groups were then further subdivided into those patients who received a graft during the study period that presented a repeat HLA mismatch to which they had previously been exposed, via a previous transplant or pregnancy, and those receiving a graft which did not present a repeat mismatch. Analysis was based on graft losses due to rejection only up to five years post-transplant. Patients were censored if the graft failure was due to death of the patient, graft loss through causes other than rejection and at the end of their follow-up period if less than five years. Initial analysis of the effect of antibody indicated, as expected, that the patients with pre formed donor specific antibody showed the lowest graft survival rates up to five years of 89% and HLA antibody negative at 96%. When the analysis was further subdivided to include those patients with 3rd party, non- DSA, as a separate group, they were found to have an intermediate survival rate of 92%. The presence of 3rd party antibodies pre transplant has previously been associated with lower graft survival rates than those patients in whom no HLA specific antibody has been detected [323], although in earlier studies this could often be potentially explained by incomplete antibody analysis, excluding loci such as HLA Cw, DQ or DP, all of which have been included in this analysis. One potential explanation could be that these patients had previously produced DSA, the levels of which had been modulated over time to become undetectable, but which returned following transplant to cause rejection. However, only two anamnestic antibody responses were seen in this group of patients and neither resulted in graft loss. Another possible explanation could be the presence of donor specific antibody to antigens other than HLA, such as the major histocompatibility complex class I chain-related molecule A (MICA) [324] which have been associated with rejection and graft loss, these were not tested for in this study. The cause of graft loss in this group was found to be mainly due to T cell mediated rejection, with only one patient losing their graft to AMR. Interestingly the patient who lost their graft to AMR was the only graft loss to rejection in the 3rd party antibody positive with repeat mismatch group, and this was over two years post-transplant, so is probably due to non-compliance and de novo antibody production rather than a memory response. The presence of 3rd party antibody

indicates that an immune response to non-self HLA has occurred in the past and therefore raises the possibility that alloreactive memory T cells may be present, accounting for an increased immune reactivity to further mismatches. Assessment of the effect of the strength of DSA, as determined by the requirement for antibody removal pre transplant, in the DSA positive group found that those requiring antibody removal had a significantly lower graft survival rate of 79% compared to 97% in the patients who did not require antibody removal. To assess if the class of HLA to which the antibody was directed was a factor, the DSA positive patients were split into those with just HLA Class I, just HLA Class II and those who had DSA to both HLA class I and II mismatches. I found that those with just HLA Class I had the highest survival rates of 93% compared to 86% for the patients with Class II alone and 80% for those with both. One explanation could be that those patients with both HLA class I and II specific antibody had antibody to more donor antigens than either group alone, and we were seeing a cumulative effect. However this was not the case with the mean number of mismatches to which there was antibody in the surviving group being 1.8 and 2.6 in the failed group, which whilst different is not significantly so ($p=0.3244$). Bentall et al. [64] recently published similar findings, reporting that patients with DSA to HLA Class I only had superior 5 year graft survival rates of 85% compared to just 63% in those with Class II alone or both Class I and II. They also concluded that single antigen bead MFI levels were not associated with graft survival rates in these patients. However Amico et al. [163] found no association of DSA Class and outcome, however all patients in this study were XM negative so single antigen bead MFI values alone were used in DSA assessment. We then investigated the effect of the presence of at least one repeat mismatch, regardless of antibody status, on outcome. Here, those without a repeat mismatch had a 95% graft survival compared to 88% in the repeat mismatch group. When the source of the original sensitising event was investigated it was found that those presented with pregnancy repeat mismatches had a lower graft survival of 82% compared to the transplant repeat mismatches of 88%. Since a number of the patients transplanted in the pregnancy repeat mismatch group received an organ from either their partner, who may be fully

mismatched, or their child, who would present a haplotype mismatch, we investigated if this was associated with a greater number of repeat mismatches compared to the transplant repeat mismatch group. This was the case with the mean number of pregnancy repeat mismatches being 3.9 compared to 1.9 in the transplant repeat mismatch group ($p=0.0009$). This could therefore be a cumulative effect, with a greater number of repeat mismatches being associated with worse outcome.

To assess the effect of combined DSA and repeat mismatches graft survival in the DSA positive group was calculated based on the additional presence of a repeat mismatch. Here the patients who were both DSA positive and were presented with repeat mismatches had a slightly worse outcome of 87% compared to 90% for the DSA positive repeat mismatch negative group. Further, in the DSA positive repeat mismatch positive group all graft failures were in patients presented with both HLA class I and II repeat mismatches, from prior pregnancy, to which they had DSA. Two of the three failures in the DSA positive repeat mismatch negative group were female, so previous pregnancy exposure cannot be ruled out, although it was not recorded. In addition, whilst previous pregnancy and transplant exposure was assessed as a repeat mismatch, it is not possible to include or exclude repeat mismatches from previous blood transfusions, immune memory from which could not be accounted for. The presence of both class I and II repeat mismatches was however only found to be detrimental to graft survival when DSA was also present, with no failures being seen in patients without DSA, indicating repeat mismatches alone are not sufficient to lead to rejection.

Whilst graft survival or loss is a solid end point, the variability in follow-up times for these patients could skew the data presented and re-analysis in a further five years could present greater, or smaller, differences between these groups. The speed at which an immune response is seen is also an indication of the presence of immune memory. Both primed memory T and memory B cells can be seen to respond within hours of re-exposure to an

antigen [38, 325]. I hypothesised that patients who were exposed to repeat mismatches and therefore potentially possessed immune memory would have shorter times to the first episode of biopsy proven rejection. Since the follow up time for all patients was long enough to have allowed acute rejection episodes to occur, the time to first BPPE was compared between all six groups, based on antibody and repeat mismatch status, and showed that overall the patients presented with repeat mismatches had shorter mean times to first BPPE regardless of the presence of DSA. Indeed further analysis based solely on the presence or absence of a repeat mismatch, regardless of DSA status, found that the time to first BPPE was significantly lower in the repeat mismatch positive group compared to the repeat mismatch negative patients ($p=0.0467$), indicating that the presence of DSA does not represent the full extent of immune memory or reactivity present. In contrast the presence of DSA, regardless of repeat mismatch status, does not significantly affect the time to first BPPE ($p=0.2316$). Additionally the strength of DSA, as defined by the need for antibody removal, was also not associated with a difference in time to first BPPE ($p=0.6656$), indicating that the strength of antibody is not associated with a more rapid immune response. The combination of DSA and repeat mismatch was significantly associated with a shorter time to 1st BPPE compared to DSA positive repeat mismatch negative ($p=0.0355$).

I also investigated the effect of DSA and/or repeat mismatches on the overall proportion of patients to suffer one or more BPPE. The values were similar across all groups except in those patients with DSA and a repeat mismatch, where 56% of patient had a BPPE, compared to a range of 20-43% in the other groups. A breakdown of the aetiology of BPPE showed that TCMR was more common in all groups except the DSA positive, repeat mismatch positive group where only 54% of BPPE included a T cell component compared to 83% in the DSA positive repeat mismatch negative group. AMR was significantly more common in the DSA positive repeat mismatch positive group with 92% of BPPE including an antibody component compared to 33% in the DSA positive repeat mismatch negative group. The 3rd party positive repeat mismatch positive group also had a higher proportion of patients with antibody

involvement, where 75% of BPRES included AMR compared to just 20% of the 3rd party positive repeat mismatch negative.

It must be noted that the patients in the DSA positive repeat mismatch negative group had lower overall antibody levels on all tests used and only 39% required antibody removal, whereas 65% of the DSA positive repeat mismatch positive group required antibody removal. It could be suggested that this is the reason behind the differences in survival and time to BPRES in these groups. However the reduced survival rates in the third party antibody groups, particularly those with repeat mismatches, and the lower time to 1st BPRES in the 3rd party positive, repeat mismatch positive group, indicates that previous exposure, even in the absence of DSA generation, can result in immune memory generation and a more rapid response upon re-exposure potentially due to direct T cell memory responses. Only two patients in the 3rd party antibody positive group showed an anamnestic antibody response post-transplant, indicating the presence of low level undetected DSA pre-transplant, the remainder of patients with AMR produced de novo DSA sometime post-transplant.

It may perhaps be expected that patients who are DSA positive and presented with a repeat mismatch have higher DSA levels than those not presented with a repeat mismatch. Whilst often DSA to an antigen is assumed to be a single specificity, it is in fact most likely that multiple B and T cell clones are involved in generating antibody specific for a single non-self HLA antigen to which they are exposed. Therefore antibodies generated will be specific for more than one epitope presented by the antigen, whereas they may only bind to single epitopes on crossreactive antigens. Multiple clones of antibody binding to beads or cells would lead to higher reactivity readings than seen with lower levels of binding of single antibody clones to crossreactive epitopes.

One limitation of the data presented is the eclectic mix of induction therapy included. It can be noted that the rate of TCMR seen in our control group of antibody negative patients is high at 43%, and an internal audit aimed at tackling this rate led to a change in immunosuppression

protocol in 2010. Firstly cyclosporine A was replaced with tacrolimus in all patients, both those previously transplanted and for all further transplants. Additionally whilst basiliximab was a commonly used induction agent, patients identified as higher risk due to the presence of DSA pre transplant were given either the anti-CD52 mAb, Campath, or, in those patients with contraindication to this, anti-thymocyte globulin (ATG). Campath causes profound and long term depletion of T cells, in addition to variable depletion of B cells, NK cells, dendritic cells and monocytes [326] and its use as an induction agent pre transplant has been reported to be associated with lower rates of acute rejection compared to other forms of immunosuppression [327]. The use of T cell depleting induction agents in the final 15 DSA positive patients could well skew the data in terms of TCMR. Comparison of BPRE between those patients transplanted before the introduction of Campath and those after showed that overall there were a similar proportion of patients diagnosed with BPRE (46% vs 47%) but that, unsurprisingly, the Campath treated group had almost half the incidence of TCMR (43% vs 78%). However there was significantly more AMR in the Campath treated group (86% vs 56%). One explanation for this could be that when observing the immune status of the patients, those transplanted with Campath included a greater proportion of patients with repeat mismatches and more patients requiring antibody removal, indicating a higher level of antibody pre transplant. Various groups reporting on the use of Campath induction in renal transplantation have also encountered higher than expected AMR [326, 327] although this is not uniformly the case [328, 329]. Zachary et.al. [301] have reported that the use of T cell depleting agents during the removal of antibody prior to transplant results in an increase or recurrence of DSA in these patients. They suggest that T cells may be required for the active down regulation of DSA production and that elimination of these cells removes this regulatory mechanism allowing unfettered antibody production. Of note it has been reported that memory T cells are somewhat resistant to depletion by Campath. Therefore these could be responsible for the observed rates of rejection. Overall the actual proportion of grafts lost in the study period is however similar between the two groups (10% vs 13%), indicating the use

of T cell depleting induction agents may improve graft survival rates. However this may also be reflective of the shorter follow up times available for the Campath treated group of patients. Despite the use of T cell depleting agents, 43% of the patients with Campath induction still had TCMR on biopsy. Published observations of the T cell populations following Campath induction have revealed that >90% of the T cells are depleted, however those remaining are of the effector memory T cell phenotype [330], believed to be somewhat resistant to depletion. Profound depletion of T cells may then activate homeostatic proliferation of both the newly emerging naïve T cells into memory T cells and also expansion of the undepleted effector memory T cells [236, 331, 332]. Effector memory T cells initiate their effector function in the periphery so those remaining would still be capable of involvement in graft rejection episodes [330].

The profile of donor specific antibody production post-transplant could also have a significant effect on graft survival and incidence of rejection episodes [333]. However, whilst this would be an area of interest to pursue in these patients, the aim of this project was to develop a method of risk assessment pre transplant, based on antibody and sensitisation status, in order to categorise potential antibody incompatible patients on risk.

3.6 Conclusions

The data presented here suggests that antibody detected by single antigen beads alone should be treated with caution as it may not be a contraindication to transplant. Assessment of the need for antibody removal, and the number of treatments required by performing a test antibody removal and titre crossmatch, provides an efficient and cost effective method of identifying those patients in whom antibody removal may be possible, and allows efficient transplant planning to be put in place. HLA antibody incompatible patients presented with a repeat mismatch are at higher risk of both graft failure and rejection episodes indicating that immune memory does play a part in the post-transplant outcome. Whilst the data may not change our future approach to HLA antibody incompatible transplantation, it will certainly

allow us to provide a risk assessment for each recipient and donor pair and guide the clinicians in the risk and timing of potential rejection episodes.

4 Memory T cells in HLA Antibody Incompatible Transplantation.

4.1 Introduction and Aims

As discussed in the introduction whilst there are a number of assays described to test for recipient T cell memory to donor HLA, none described to date is particularly accurate at defining donor specific T cell reactivity pre-transplant and they often focus on the production of a single cytokine as a marker of T cell activation. However, since there is no clear cytokine to choose that is absolutely linked with rejection or tolerance I decided that it is important to select a range of cytokines produced across the three previously described Th cell subtypes that could be assessed to identify potential increased immune risk to the allograft and possibly give an indication as to the mediators of any potential future rejection episodes. The activation of Th cells was particularly of interest as they are required for activation of both cytotoxic T cells and the B cells to produce antibody, of particular relevance in HLA antibody incompatible transplants. For this reason I decided initially to test for the production of IFN γ , TNF α , IL-2, IL-5, IL-8 and IL-17 by responder cells. In designing an assay to probe for memory T cells I also decided that for ease of integration into the routine testing repertoire of a clinical transplantation laboratory the methods and equipment used should be familiar to staff and commonly found.

4.1.1 Aims for this study.

The aim for our T cell assay was as follows –

- To develop a method to assess the response of T cells found in the peripheral blood of highly sensitised transplant candidates to the HLA antigens expressed by a potential donor.
- By assessing the cytokines produced at various time points we hoped to be able to identify the presence of memory T cells of all the subsets described above.

- To use this information to stratify the risk of acute cellular rejection post-transplant.

4.2 Methods

T cells may respond to proteins other than HLA expressed on the surface of cells derived from other individuals. In order to just identify recipient cells which would respond to the mismatched HLA antigens I decided not to use whole donor or stimulator cells. Initially I investigated the use of HLA coated beads designed for HLA antibody specific detection on the Luminex platform as the stimulator particles. The patient cells were to be cultured with different HLA-luminex beads, culture medium sampled at various time points and tested for the presence of the cytokines listed above.

Basic Techniques used for all the following tests.

4.2.1 Peripheral Blood Mononuclear Cell (PBMC) Isolation.

All work was carried out under sterile conditions.

Peripheral blood was diluted at a ratio of 1:1 using phosphate buffered saline (PBS) (Sigma-Aldrich), layered onto equal volumes of density centrifugation medium lymphoprep (Axis-Shield), and centrifuged at 600g for 20 minutes.

The isolated lymphocyte layer was removed by pipette and the cells washed twice using 10ml PBS, as described in the previous chapter.

Culture medium was made, comprised of:

500ml RPMI 1640 without L-glutamine (Gibco),

5ml 100x L-Glutamine (Gibco),

5ml 100 µg/ml Penicillin streptavidin (Gibco),

5ml sodium pyruvate (Gibco),

5ml 100x non-essential amino acids (Gibco),

5ml 1M HEPES buffer (Gibco),

45ml sterile filtered human AB serum (Sigma –Aldrich),

Cells were then re-suspended in the culture medium to give a concentration of 250000 cells per 200µl.

Cell counting was performed using a haemocytometer and viability assessed using trypan blue staining.

4.2.2 Cytokine Analysis.

Detection of cytokines in the supernatant was performed using the Fluorokine® MAP multianalyte profiling kit from R&D Systems, for use with the Luminex® 100™. All the cytokines we wished to detect required only the use of base kit A. This was chosen as it had the capability of detecting multiple cytokines simultaneously in a limited sample volume.

4.2.2.1 Principle of the Assay.

Luminex specific colour-coded microparticles are provided pre-coated with analyte specific antibodies. Combinations of these beads can be created by the user to set up an panel specific to the analytes being tested. The microparticles are mixed with the sample, or standard, and, the bead bound antibody binds to the analyte of interest if present. Following washing of the beads a biotinylated antibody cocktail specific to the analyte(s) of interest is added to the test wells. Following further incubation and wash steps, a streptavidin-phycoerythrin conjugate is added. This will bind to the biotinylated detection antibodies. Following another period of incubation and washing the microparticles are re-suspended in buffer and read using the Luminex analyser which, using two lasers, will determine which bead is being detected, and therefore which analyte, and how much phycoerythrin is bound and therefore how much analyte is present. In order to quantify the amount of analyte being detected, pre-defined standards can be run to create a standard curve for each analyte from which the analyte concentration in the test sample can be calculated.

4.2.2.2 Cytokine Assay Method.

All supernatant samples had been stored at -20°C, as suggested by the manufacturers, and thawed immediately prior to testing.

All buffers and diluents were prepared as instructed in the kit guidelines. Briefly -

Wash Buffer Concentrate provided was diluted 20ml wash buffer concentrate with 480ml deionised water.

Microparticle concentrates for each analyte were prepared in the mixing bottle provided within 30 minutes of use. For a 96 well testing plate 50 µl of each microparticle concentrate was added to the bottle and this total volume was then diluted with 5ml of the microparticle diluent provided.

The biotin antibody cocktail was prepared by adding 50 µl of each of the analyte relevant biotin antibody concentrates to the 5.25ml vial of Biotin Antibody Diluent provided. This could then be stored at 2-8°C for 1 month.

The streptavidin-PE detection conjugate was diluted by adding 55 µl to 5.5ml working wash buffer. This was exposed to minimum light at all times and, once made, was stored in a foil wrapped tube. Once prepared this could be stored at 2-8°C for 1 month.

The kit standards, Standard cocktail 1 and Standard cocktail 2, were reconstituted by addition of 1ml of 1x Calibrator diluent RD5K provided. They were then left for 15 minutes with intermittent gentle agitation. 7 dilutions were then made using 7 labelled polypropylene tubes where 300 µl of Calibrator diluent was added to tube 1 and 200 µl each to tubes 2 – 7. 100 µl of each reconstituted standard was then added to tube 1 and mixed well. 100 µl of the contents of tube one was then added to tube 2, which was again well mixed before further transfer of 100 µl from tube 2 to tube 3. This was continued until all 7 dilutions had then been prepared. The initial calibrator diluent provided the 8th standard and served as a blank. Kit Lot appropriate standard values for each analyte contained within the standard cocktails were

detailed with the concentration at neat provided as pg/ml. All eight standards were tested on each cytokine analysis run.

A template for the Luminex 100 analyser was created using the template wizard. A pre-defined bead region, as defined by the lot specific kit insert, was assigned for each analyte being tested. A minimum count of 50 events per bead region was set with a flow rate of 60µl/Min and a sample size of 50 µl. The doublet discriminator gates were set to 7500 and 15500.

4.2.2.3 Test Procedure

1. All reagents were prepared as described above.
2. A 96 well filter plate was labelled and pre-wet by adding 100 µl of wash buffer, which was then removed using a vacuum manifold.
3. 50 µl of the microparticle mix was added to each test well.
4. 50 µl of standard or sample was added to the appropriate test well and mixed by repeat pipetting.
5. The plate was covered and wrapped in foil to prevent exposure to light and then incubated for 3 hours at room temperature on a horizontal orbital microplate shaker set at 500 rpm.
6. Following incubation the liquid was removed from the plate again using the vacuum manifold and the beads washed by addition of 100 µl of wash buffer which was again removed using the vacuum system. This was repeated three times.
7. 50 µl of diluted biotin antibody cocktail was then added to each test well and the plate re-covered and wrapped in foil to be incubated for 1 hour at room temperature on the orbital shaker.
8. The wash procedure in step 6 was repeated.

9. 50 µl of diluted Steptavidin-PE was added to each well and the plate recovered and wrapped in foil and incubated at room temperature for 30 minutes on the orbital shaker.
10. The wash procedure in step 6 was repeated.
11. The microparticles were resuspended in 100 µl of was buffer and the plate mixed for 2 minutes on the orbital shaker.
12. The plate was then read within 90 minutes using the Luminex analyser.

4.2.2.4 Data Analysis

Data was collected by the Luminex as MFI units for each bead region. To ensure that no cytokines were present in the culture medium used, this was tested following each culture assay performed. In addition each assay included wells containing just test cells with no stimulator particles to ensure that the cells were not producing significant amounts of cytokines without specific stimulation. The analyte specific MFI values for these negative control wells were subtracted from the positive control wells and each stimulator test well to give overall cytokine production values for each test well. These resulting MFI values were then plotted using Microsoft Excel to provide a visual interpretation of the each cytokine produced in each test well at each time point.

The raw MFI results gained for all the experiments outlined in this chapter can be found in Appendix 1 – 4.

4.3 Initial Experiments

The first two experiments were carried out to ascertain if we could set up a system in which cellular cytokine responses to HLA antigen presented on a solid phase could be detected.

For these first tests the stimulator particles used were Luminex specific LIFECODES Lifescreen Class I and Class II ID beads produced by Gen-Probe for HLA specific antibody analysis. These kits utilise 5.6 micron microspheres designed to be analysed using the Luminex platform. The

beads in these kits are designed to detect panel reactive HLA Class I or Class II specific IgG antibodies in serum. The beads are coated with affinity purified Class I or Class II HLA glycoproteins isolated from different individuals. The class I kit comprises of 50 and the class II of 30 test beads, each coated in one individuals HLA class I or class II antigens. The respective panels of each can be seen in tables 14 and 15 below.

Table 14 - Gen-probe Class I PRA bead composition

BEAD	DONOR	HLA-A*		HLA-B*		HLA-C*	
103	634	0101	6602	5201	5801	0718	1202
104	458	2402	2601	1513	4001	0702	0801
111	636	0201	3001	0702	5601	0102	0702
112	116	2301	8001	4501	8101	1601	1801G
113	247	0101	6901	0801	3508	0701	1203
114	205	1101	2901	3505	4601	0102	0401
115	941	3002	6802	1516	4403	0701G	1402
117	209	2402	3001	1302	4001	0602	0702
118	364	2501	3303	5501	5801	0303	0602
119	374	0205	2301	4501	5001	0602	-
120	544	2402	3303	1501	3701	0304	0602
121	498	3301	3402	3501	5301	0401	-
124	117	2402	3201	3801	4801	0801	1203
125	106	1101	3101	3901	4002	0304	0702
126	531	3402	8001	1801	5301	0202	0602
127	017	3001	8001	1801	7801	0202	1601
128	739	0102	0301	3501	4901	0401	0701
129	012	2402	3101	3501	5201	0401	1202
130	720	3601	8001	0702	1801	0202	1505
132	308	2301	6601	0801	3910	0702	1203
133	215	0201	0301	5001	5702	0602	1801G
134	765	2402	6801	3901	5501	0303	0702
135	181	4301	-	1503	-	1802	-
136	342	0101	6901	0801	7301	0701	1505
137	088	3001	-	4201	5001	0602	1701
138	397	0308	3402	1510	5703	0304	0701G
139	144	3101	3201	4001	4002	0202	0304
140	373	0201	2902	1302	4403	0602	1601
141	477	0201	2501	0801	2705	0102	0701
142	590	0101	3101	0801	1401	0701	0802
144	316	2601	3301	1402	4201	0802	1701G
145	412	0201	2902	1302	1501	0304	0602
146	325	0201	2601	2705	3801	0102	1203
147	490	2301	6602	0702	4410	0401	0702
148	337	0101	0301	1501	3906	0304	0702
149	311	0301	2902	5101	5601	0102	1502
150	804	0301G	-	0702G	5101G	0102	0702
151	402	1101	2402	5901	6701	0102	0702
152	467	2902	6802	1510	4901	0304	0701G
154	730	0101	0201	0702	3701	0602	0702
155	645	3004	7401	3501	4102	0401	1701G
156	517	1101	-	1502	5401	0102	0801
157	127	3002	-	1402	7801	0802	1601
161	479	2501	3303	4001	5301	0304	0401
162	770	0101	3303	0702	5703	0701G	0702
163	432	0201	3601	0702	1503	0202	0702
164	230	0301	2402	4701	5101	0602	1402
165	633	0201	2301	1402	1516	0802	1601
166	908	2501	6801	2702	2705	0102	0202
167	812	2402	6801	4901	8201	0302	0701

Table 15 - Gen-Probe Class II PRA Kit Composition.

BEAD	DONOR	DRB1*		DRB3*		DRB4*		DRB5*		DQB1*	
115	372	04:03	14:04	02:02		01:03				03:02	05:03
117	770	10:01	14:04	02:02						05:01	05:03
118	013	01:03								05:01	
119	316	03:02		01:01						04:02	
120	517	04:05	12:02	03:01		01:03				03:01	04:01
121	325	01:01G	01:03							03:01	05:01
122	814	11:02	13:03	01:01	02:02					03:01	
125	397	13:02	15:03	03:01				01:01		06:02	06:09
126	126	03:01		02:02						02:01	
127	730	10:01	12:01	02:02						03:01	05:01
128	088	03:02	08:06	01:01						04:02	06:02
129	981	04:05	15:02			01:03		01:02G		04:01	05:01
130	373	07:01				01:01	01:03			02:02	
132	876	01:02	13:02	03:01						05:01	06:09
134	484	03:01	11:01	02:02						02:01	03:19
135	590	03:01	07:01	01:01		01:01				02:01	02:02
136	812	09:01				01:01	01:03			02:02	
137	328	01:01	04:01			01:01				03:01	05:01
138	307	03:01	08:01	01:01						02:01	04:02
139	016	04:03	04:06			01:03				03:02	
141	432	10:01	15:03					01:01		05:01	06:02
142	064	01:03	08:04							04:02	05:01
143	077	16:02						02:02	02:03	05:02	
144	490	03:02	09:01	01:01		01:01				02:02	04:02
145	671	12:01	14:01	01:01	02:02					05:01	05:03
146	830	11:06	15:01	02:02				01:01		03:01	06:02
147	022	09:01	15:01			01:03		01:01		05:02	03:03
148	467	08:04	15:03					01:01		03:19	06:02
149	531	07:01	13:03	01:01		01:01				02:02	03:01
150	114	07:01	16:01			01:01		02:02		02:02	05:02

The cells used were PBMCs extracted from peripheral blood of two healthy unsensitised volunteers, from this point named 'Cell 1' and 'Cell 2'.

In these experiments 250000 volunteer PBMCs were incubated in a 'U' bottomed 96-well plate with 3µl of class I, class II or class I+II beads at neat, 1:10 and 1:100 dilutions, with and without the addition of CD28 (molecular probes) for costimulatory signals, of which 2µl of neat or a 1:100 dilution of stock 1µg/ml was added.

Two negative control wells were included, one containing culture medium only and one containing just cells but no HLA-luminex beads.

A positive control well was included containing PBMCs plus human T cell activatory CD3+CD28+ dynabead stimulator beads (Gibco), to provide evidence that the cells were capable of responding in a detectable manner.

Each combination was tested in triplicate.

A summary of the well contents can be seen in table 16 below.

Table 16 - Plate Layout for Experiment One.

Well Number	Contents	
1	Negative Control - Medium Only	
2	Negative control - Cells only	
3	Positive control - CD3/28 0.2µl	
4	Positive control - CD3/28 0.1µl	
5	Positive control - CD3/28 0.05µl	
6	Class I Beads - Neat	Without CD28
7	Class I Beads - 1:10	
8	Class I Beads - 1:100	
9	Class I Beads - Neat	With CD28
10	Class I Beads - 1:10	
11	Class I Beads - 1:100	
12	Class II Beads - Neat	Without CD28
13	Class II Beads - 1:10	
14	Class II Beads - 1:100	
15	Class II Beads - Neat	With CD28
16	Class II Beads - 1:10	
17	Class II Beads - 1:100	
18	Cells + CD28 (neat)	
19	Cells + CD28 (1:100)	
20	Class I + II beads + CD28 neat	
21	Class I + II beads + CD28 1:100	

Test plates were then incubated at 37°C for up to 72 hours with the first cell and 7 days with the second cell.

During this time 50 µl of supernatant was sampled from each well at various time points. For the first cell, Cell 1, this was at 16, 24, 48 and 72 hours. This was then adapted to 24, 72 hours

and 7 days for the second cell, Cell 2. After each sample was taken, 50 µl of fresh culture medium was replaced.

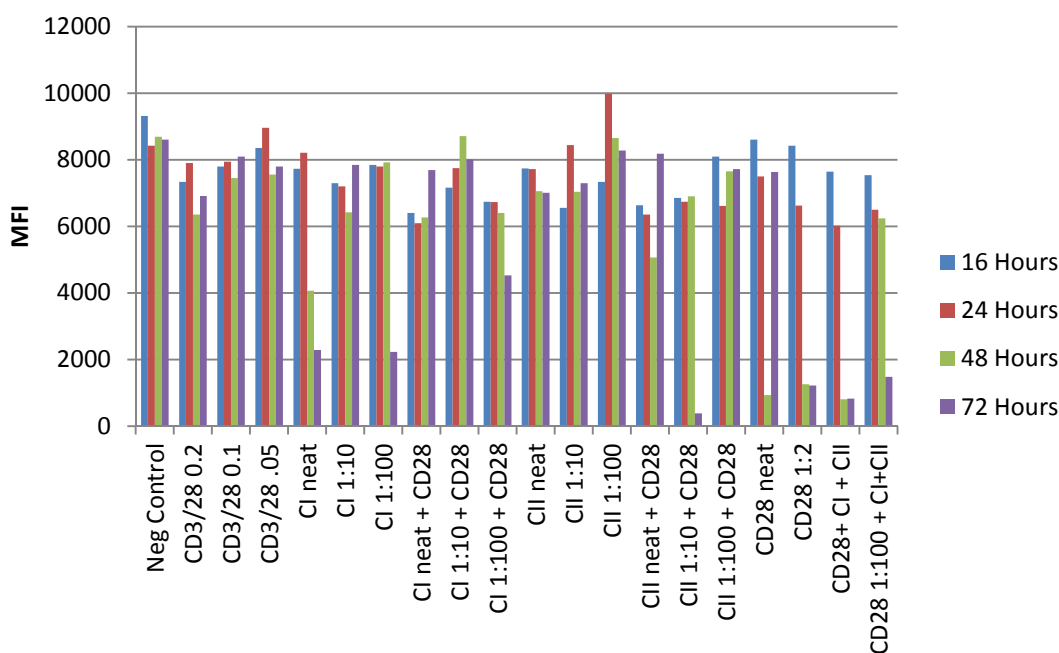
The supernatant samples were frozen immediately and stored at -20°C prior to testing for the presence of cytokines.

Analysis of the cytokines was carried out as previously described and the MFI values gained from the negative control, culture medium only well, were subtracted from those values gained with the test wells to give a calculated total MFI value for each cytokine in each well. The cytokines initially tested for were IFN-γ, TNF-α, IL-5, IL-8 and IL-17.

4.3.1 Initial Results.

As may have been expected, due to the fact that the PBMCs were from unsensitised healthy individuals, no significant cytokine production was observed in any test well at any time point other than the positive control, with the exception of the IL-8 cytokine, which was found to be produced in all test wells including the cells only negative control well, as shown in figure 67.

Figure 67- Example of IL-8 detection in all test wells with cell 1



'Off-record' discussion with other users of the R&D Fluorokine MAP test system indicated that positive detection of IL-8 in all test wells was a common occurrence and may be due to non-specific binding in the test system. Another potential explanation could be that there was unspecific macrophage activation or oxidative stress of the cells, although it may be expected that other cytokines in the test system would also have been produced if this was the case.

In view of these results it was decided that a similar experiment should be carried out using cells from a sensitised renal patient who had undergone previous organ transplantation, had produced detectable HLA specific antibodies and may well have T cell memory to specific HLA antigens.

4.4 Experiment 2.

Cells remaining after extraction for routine flow crossmatching tests from a sensitised renal patient were used for the next experiment in line with our ethical approval.

Patient one was a female, long term renal patient. Her HLA type was HLA A1,2 B8,44 Cw5,7 DR4,7 DR53 DQ2,7. She had received two previous renal transplants in 1979 and 1982, both of which included HLA class I mismatches, but had not been typed for HLA class II as it was not routine at the time of transplant. Serum screening revealed she had produced multiple HLA class I and II specific antibodies. She was being assessed for a potential antibody removal transplant from a donor presenting mismatches to which she had antibody including DR11, and against whom she had a positive FXM. As she had received previous transplants and produced HLA specific antibody I hoped that her T cells may respond to stimulation by HLA antigens.

It was decided that in addition to using the Gen-probe Class I and II ID beads as detailed previously, beads coated in single specificities would also be used. These single antigen beads, purchased from OneLambda (Canoga Park, CA), and can be purchased as vials of 96 mixed specificities or as vials containing a single group of beads coated in a single specificity. Since the patient had antibody to HLA DR11 and possibly DR52, proven by flow crossmatch, single

antigen beads coated in HLA DR11 and DR52 were selected along with the self-specificity HLA DR7 to assess potential background reactivity against beads rather than the HLA molecules on their surface. The plate was set up following the plate layout in table 17 below.

Each well was tested in duplicate. 250000 patient cells were added to each well along with the bead volumes as detailed in table 17.

Each reaction combination was tested with and without plate bound CD28, where 200µl of a 1:100 dilution of CD28 was introduced to the appropriate test wells, incubated for 30 minutes to allow binding to the well surface and then washed three times using 250µl PBS per wash.

50µl of supernatant was sampled from each well and stored at -20oC prior to cytokine analysis. The time points sampled were 24 and 48 hours and day 7. After each sample was taken, 50µl of fresh culture medium was replaced.

Cytokines analysed were IFN-γ, TNF-α, IL-2, IL-5, IL-8 and IL-17.

Table 17 - Plate layout for T cell experiment 2.

Well number	Contents
1	Neg Medium Only, No CD28
2	Neg Medium Only, with CD28
3	Positive Control, no CD28
4	Positive Control, with CD28
5	Negative, cells only, no CD28
6	Negative, Cells only, with CD28
7	CI mixed beads 5 µl, no CD28
8	CI mixed beads 5µl, with CD28
9	CII mixed beads 5µl, no CD28
10	CII mixed beads 5µl, with CD28
11	CI+II mixed beads 5µl, no CD28
12	CI+II mixed beads 5µl, with CD28
13	DR11 2µl, no CD28
14	DR11 2µl, with CD28
15	DR52 2µl, no CD28
16	DR52 2µl, with CD28
17	DR7 2µl, no CD28
18	DR7 2µl, with CD28
19	DR11 10µl, no CD28
20	DR11 10µl, with CD28
21	DR52 10µl, no CD28
22	DR52 10µl, with CD28
23	DR7 10µl, no CD28
24	DR7 10µl, with CD28
25	DR11 + 52, no CD28
26	DR11 + 52, with CD28

4.4.1.1 Results from Experiment 2.

MFI values for each cytokine tested at each time point in each test well were collected. These were plotted as bar charts for each cytokine using excel. These results are presented below in figure 68 – figure 73 below.

Figure 68 – Figure indicating detection of IFN-g production, as an MFI value, in each test well at each test time point in experiment 2.

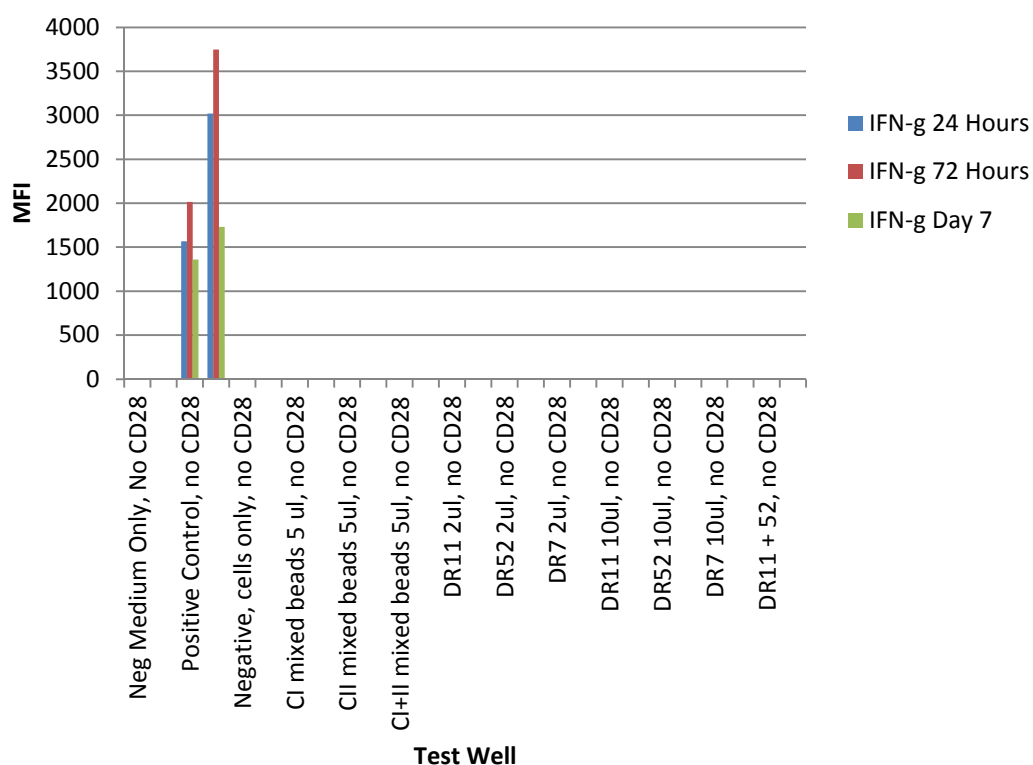


Figure 69 - Figure indicating detection of TNF-a production, as an MFI value, in each test well at each test time point in experiment 2

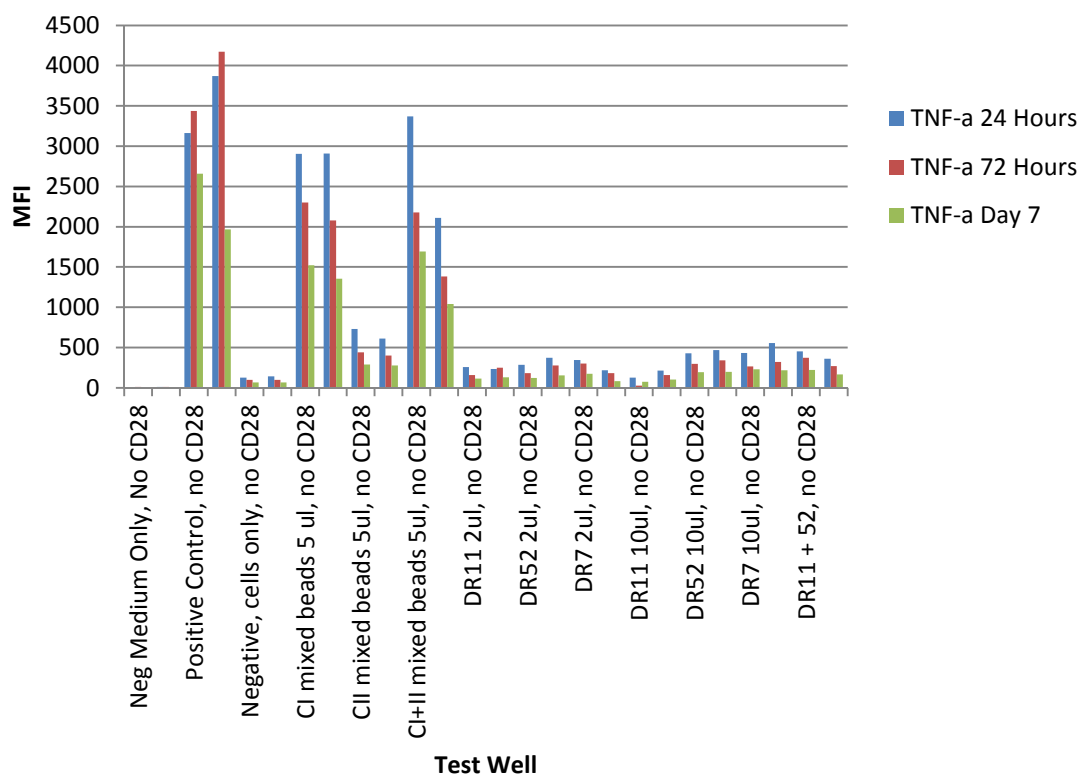


Figure 70 - Figure indicating detection of IL-2 production, as an MFI value, in each test well at each test time point in experiment 2

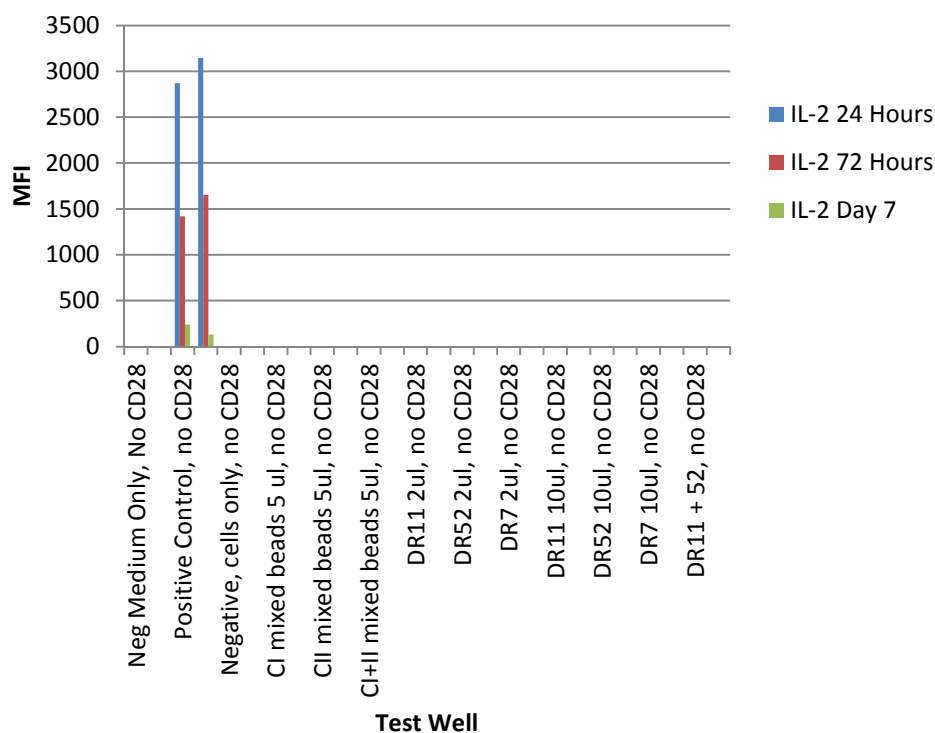


Figure 71 - Figure indicating detection of IL-5 production, as an MFI value, in each test well at each test time point in experiment 2.

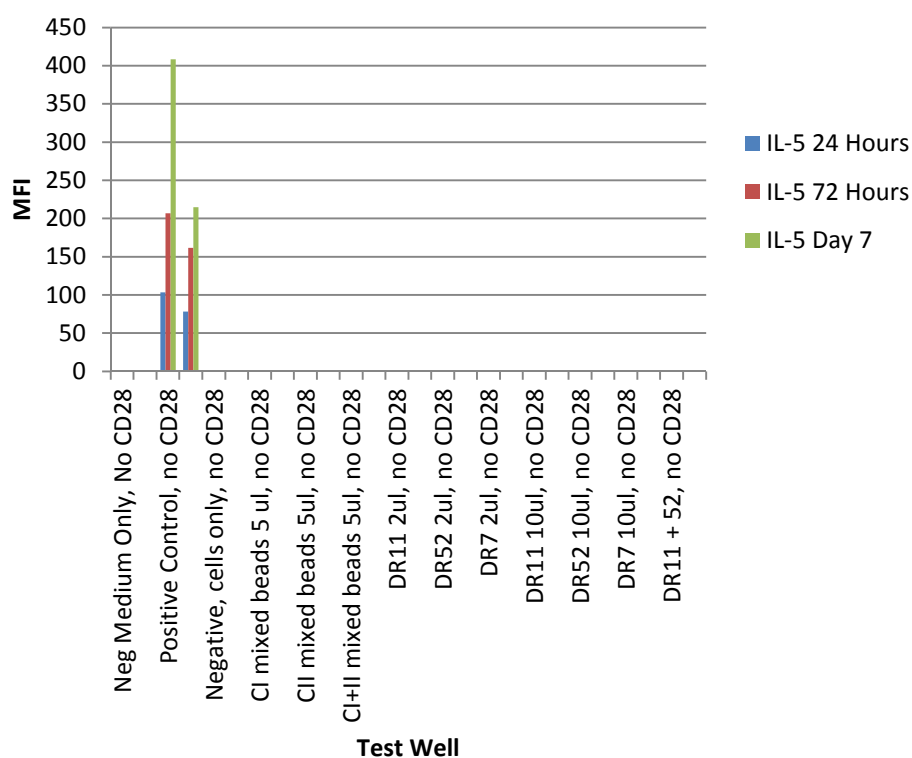


Figure 72 - Figure indicating detection of IL-8 production, as an MFI value, in each test well at each test time point in experiment 2.

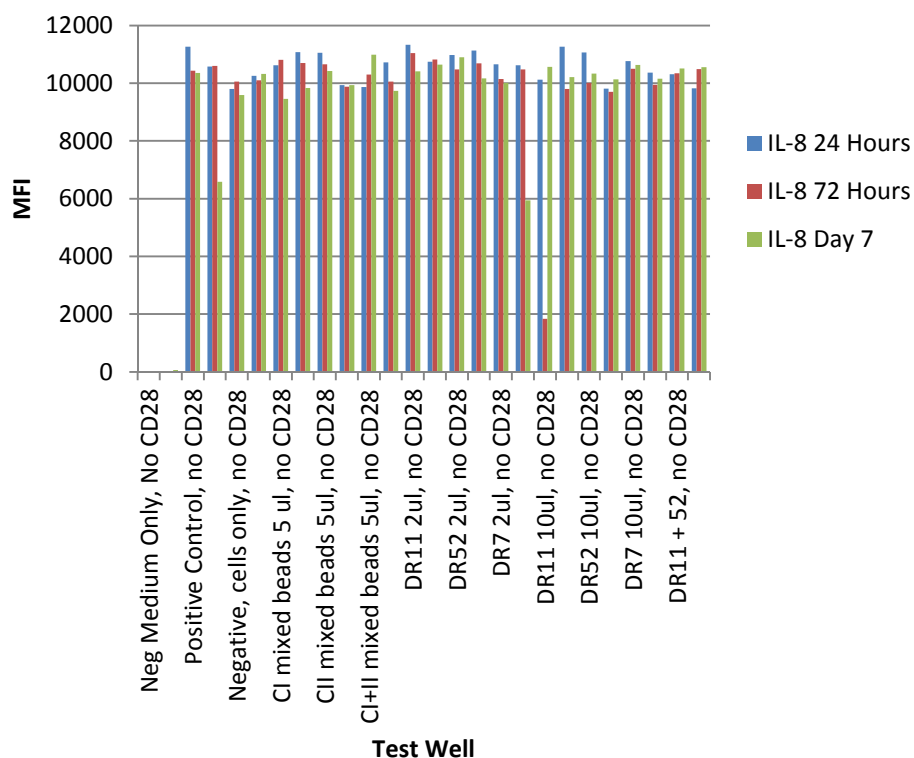
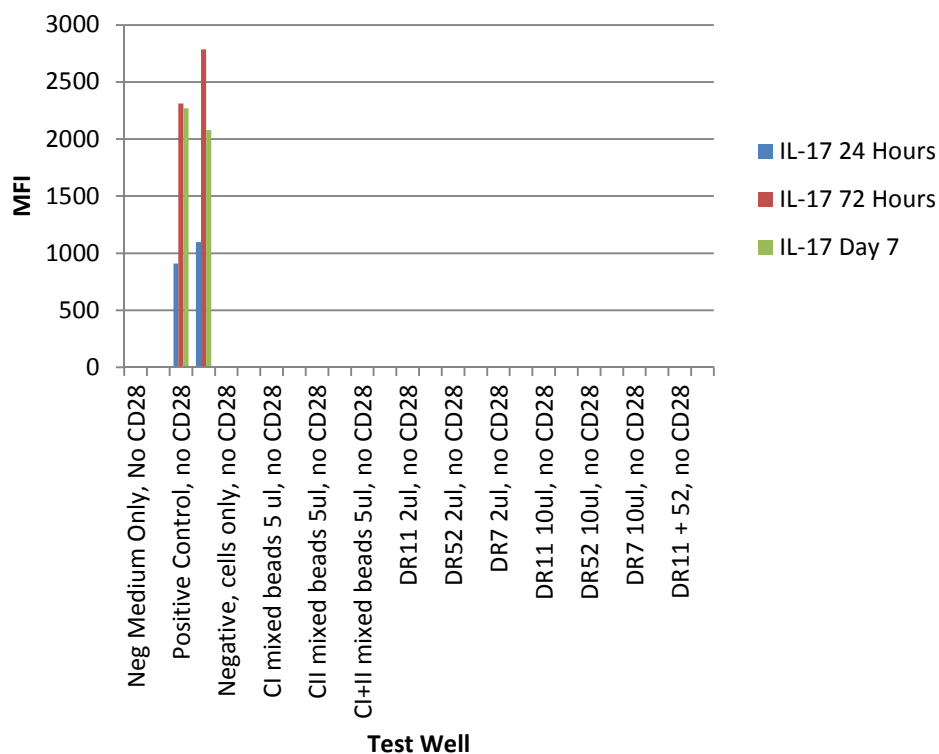


Figure 73 - Figure indicating detection of IL-17 production, as an MFI value, in each test well at each test time point in experiment 2.



As can be seen from the graphs above, other than IL-8 as previously discussed, the only cytokine produced at detectable amounts was TNF α , the majority of which was found in wells containing the Class I mixed beads, although lower levels were also found in the wells containing class II mixed beads and a small amount in the wells containing the single antigen beads or peptide, at all time points.

The presence of CD28 did not appear to influence the cytokine production.

4.4.2 Experiment 2 - Discussion

TNF α is a pro-inflammatory cytokine produced by T cells, macrophages and NK cells and its detection in the test wells containing the class I or II mixed beads, but not in the cell only negative control well, indicated that some cellular activation was occurring in response to the presence of HLA coated beads. Lower levels were detected in the single antigen bead wells, including the well containing self HLA molecule DR7. Production above the level seen in the cell only negative control well was not found in the test wells containing HLA derived peptides.

Since the patient had received two previous grafts, both with known HLA class I mismatches, and produced antibody specific to those mismatches, it would be quite safe to assume that there should be some direct T cell memory to the Class I HLA antigens. The production of TNF α in the Class I bead containing wells could reflect this.

The HLA antigen coating the single antigen beads is derived from recombinant sources and is potentially not of the exact conformation found *in vivo*. In addition, it is likely that the peptide being presented in the groove of each of the HLA molecules on the single antigen bead surface is the same. Since in the normal immune response the TCR recognises and responds to both the HLA molecule and the peptide it presents, it is possible only a few or none of the T cells in the culture recognised the peptide being presented. *In vivo* a cell would present many different cell or pathogen derived peptides in the same HLA molecule over its' surface, allowing T cells of many different specificities to respond. The peptide being presented is thought to be important in the TCR recognition process [42]. The class I and II mixed ID beads

are coated with HLA antigens purified from different donor cell sources and as such are thought to contain a wide variety of peptides in their presentation grooves. It could be this difference in peptide variety which explains why the Class I and II mixed beads showed the greatest reaction.

Both the class II mixed beads and the class II single antigen beads show lower levels of stimulation compared to the class I mixed beads. The patient produces a number of HLA class II specific antibodies. However since the class II HLA types of her two previous donors are unknown, the exact class II specificities to which she has previously been exposed are not known, therefore it is possible that the T cell memory is limited to one or two specificities, whilst the antibody produced is to epitopes found on a number of HLA antigens and the DR11 antibody is just a consequence of shared epitopes and cross reactivity.

The mild reaction above baseline seen in the wells containing the DR7 coated beads could potentially be explained by either the fact that the DR7 on the bead surface is not in the 'natural' conformation and is being recognised as 'non-self', or that the peptide being presented is in fact causing the response. As discussed in the introduction there are two widely recognised, non-mutually exclusive, theories used to explain the high numbers of direct alloreactive T cells found in the peripheral blood of most individuals, and these centre around whether it is the HLA molecule, or the peptide which is being presented, that causes the stimulation. The high determinant density theory suggests that it is the allogeneic HLA molecule itself that is directly recognised by the alloreactive T cell and the peptide bound to the HLA molecule is of secondary importance. The multiple binary complex theory suggests that it is the peptide being presented by the non-self HLA molecules that is the primary point of recognition and the non-self HLA molecules themselves are of secondary importance. It is possible that the DR7 HLA molecules on the single antigen Luminex beads are presenting a peptide to which some of the patient alloreactive T cells can respond and reactivity is seen following the multiple binary complex theory.

The strongest reaction was seen with the class I mixed beads. However using the mixed beads would not allow us to be able to identify which HLA antigens are causing the reaction and therefore whether the reaction would be donor specific. The best way to assess specific donor reactivity would be to incubate the cells with donor derived HLA antigens, which would also contain donor derived peptides in the grooves of the HLA molecules, and therefore represent a better *in vitro* simulation of the potential stimulatory incompatibilities that would be encountered *in vivo*.

Two kits aimed at testing for donor specific HLA antibodies are available, which allow the user to strip intact HLA molecules from the surface of donor cells and immobilise them on either a Luminex compatible bead or the surface of a micro-ELISA plate. Previous work in our laboratory had validated that the donor HLA molecules bound to the micro-ELISA plate was of sufficiently natural conformation to detect HLA specific antibody present in patient serum. I decided to investigate the use of these kits to repeat the previous T cell assays using donor derived HLA molecules.

4.5 Experiment 3.

These experiments utilised both the LIFECODES donor specific antibody detection kit for Luminex and the LIFECODES MicroAMS ELISA based kit, both produced by Gen-Probe. Both kits use the same principle, Class I and Class II HLA glycoproteins are solubilized, via cell lysis, from donor lymphocytes, and captured using class-specific monoclonal antibodies which have been immobilized in the microwells of the micro AMS plate or on the surface of Luminex beads. The lysate containing the glycoproteins can be stored at -80°C for up to two years, allowing for future and post-transplant testing.

4.5.1 Lysate Preparation

Both methods follow the same protocol for lysate preparation.

Lymphocytes are isolated from donor peripheral blood or spleen samples following the density gradient centrifugation cell separation method outlined earlier in the chapter.

The packed cell volume of the final cell pellet produced must be estimated. This can be done by adding an equal volume of water to an identical tube and measuring this volume of water.

It is estimated that a packed cell volume of 10 μ l would equate to approximately 30×10^6 lymphocytes.

To every 10 μ l of packed cells, 100 μ l of diluted lysis buffer is added. The lysis buffer is diluted by addition of 10 μ l lysis buffer provided to 90 μ l of sterile deionised water.

Cells and diluted lysis buffer are mixed well and vortexed to encourage cell lysis.

The lysate is then centrifuged at 1000-1500 rcf for 5 minutes to pellet the cell membranes and debris.

The supernatant is transferred to a clean tube and can either be frozen at -80°C for up to two years or kept on ice and used immediately.

4.5.2 Donor Specific Beads.

The donor HLA specific beads were prepared following the kit instruction, briefly:

The donor cell lysate was defrosted and centrifuged at 8000-12000 rcf for 4-5 minutes.

The vial of test beads provided in the kit was centrifuged at 600-800 rcf for 30 seconds and then vortexed to thoroughly resuspend the beads.

The number of test wells required for each donor lysate was calculated and enough beads and lysate were mixed in a small tube to cover all wells plus extra to allow for pipetting loss. Each test well required 8 μ l lysate plus 5 μ l beads. After thorough mixing the beads and lysate were incubated for 30 minutes at room temperature.

In a deviation from the kit instructions the beads were then washed prior to incubation with the recipient cells to remove any residual lysate buffer. This was achieved by addition of 50 μ l

of sterile PBS, gentle vortex mixing, then centrifuging at 600-800 rcf for 30 seconds after which the supernatant was gently decanted. The beads were washed a total of three times and finally resuspended in enough cell culture medium to allow the addition of 10 µl of bead suspension to each test well.

4.5.3 ELISA plate preparation.

The donor HLA specific microELISA plate was prepared following the manufacturer's instructions, briefly:

Enough Class I and II specific ELISA wells to prepare all the test wells needed were removed from the appropriate pouches, and fitted to the 96 well plate frame provided in the kit.

The number of Class I and Class II test wells required was calculated and the lysate diluted as follows:

For each Class I well, 2 µl of lysate was diluted in 14 µl of lysate and conjugate diluent (LCD) provided in the kit.

For each Class II well, 4 µl of lysate was diluted in 12 µl of LCD.

15 µl of the appropriately diluted lysate was added to each test well, the plate covered and then incubated at 37°C for 40 minutes.

Following incubation the wells were washed 3 times, each time adding 140µl of PBS and then vigorously decanting the well contents and blotting with absorbent paper.

These first three experiments using the donor specific kits were aimed at assessing the ability to detect donor specific T cell responses using these platforms and to compare the results achieved using the bead and ELISA plate methods to ascertain which, if either, would be the preferable format for further testing.

Three patients undergoing investigations for HLA antibody incompatible renal transplantation were identified and cells from both the patients and donors, excess to routine testing requirements, were used to perform the assays.

Donor cell lysate was prepared as outlined above and, using these, both Luminex beads and microELISA plates were created.

The assays using the microELISA format were performed in the ELISA wells provided in the kit, those performed using the beads as stimulators were carried out in a standard 'U' bottomed 96 well plate.

Testing included wells with and without the addition of soluble CD28 where 2µl of neat stock 1µg/ml was added to each test well as appropriate. 250000 PBMCs were added to each test well as appropriate and the plates sealed and incubated at 37°C. The testing combinations are outlined in table 18 below.

Table 18 - Test combinations used in Experiment 3.

Negative control – Medium only
Negative control – Cells + medium
Positive Control – Cells + CD3/28 stimulator beads
Class I ELISA without CD28
Class I ELISA with CD28
Class II ELISA without CD28
Class II ELISA with CD28
Luminex Beads with CD28
Luminex Beads without CD28

50µl of culture supernatant was collected at 24, 72 and 120 hours from each well and tested for the presence of the cytokines IFN γ , TNF α , IL-2, IL-5, IL-8 and IL-17 using the method previously outlined.

The MFI values gained with the negative control, cells only well were subtracted from the test wells to give an indication of cytokine production above baseline.

4.5.3.1 Pair One.

The potential recipient was a female patient who had received two previous renal transplants. She was highly sensitised, producing multiple Class I and II HLA specific antibodies. The HLA types, previous mismatches and HLA specific antibodies involved are outlined in table 19 below.

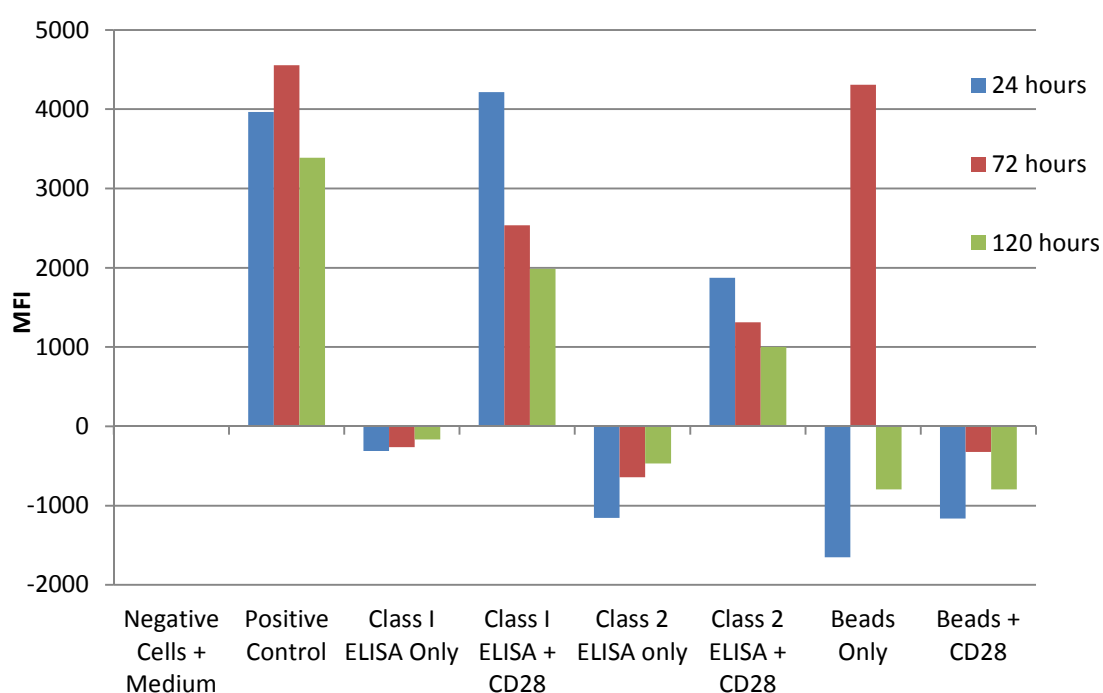
Table 19 - Pair One HLA information.

Patient 1 HLA type	A2,68 B42,52 Bw4/6 Cw16,17 DR8,17 DR52 DQB4,7
Donor 1 HLA type	A1,36 B18 Bw6 Cw4,5 DR15,17 DR51,52 DQB2,6
Previous Mismatches	A1, B18. Class II HLA type of previous donors not complete, may be repeat mismatches.
Donor Specific Ab	A1, A36, B18, DQ2
Crossmatch Result	T & B cell positive.

4.5.3.2 Pair One Results.

No production of IFN γ , IL-2, IL-5, IL-8 and IL-17 was seen above baseline, other than in the positive control, however production of TNF α was detected in both the Class I and II ELISA wells tested with additional CD28 at all time points. TNF α was additionally detected at 72 hours in the 'beads without CD28' well. The results for TNF α can be seen in figure 74 below.

Figure 74 - TNF α production in Pair One.



4.5.4 Pair Two.

The potential recipient was a male renal patient who had received one previous transplant. He was highly sensitised, producing multiple Class I and II HLA specific antibodies. The HLA types, previous mismatches and HLA specific antibodies involved are outlined in table 20 below.

Table 20 - Pair Two HLA information.

Patient 2 HLA Type	A3,26 B37,61 Bw4,6 Cw2,6 DR13 DR53 DQB6
Donor 2 HLA Type	A26,68 B37,71 Bw4,6 Cw6,7 DR11,13 DR52 DQB6,7
Previous Mismatches	Cw7
Donor Specific Ab	Cw7, DR11, DQ7
Crossmatch Results	B cell positive

4.5.4.1 Pair Two Results.

Due to a technical problem involving evaporation in some wells, only data for 24 and 72 hours are available for this pair.

No production of IFN γ , IL-2, IL-5 and IL-8 was seen above baseline in any of the test wells at either time point, other than in the positive control. IL-17 production was detected in the class II ELISA well with CD28 at 72 hours. TNF α production was detected in all test wells and most time points tested. The IL-17 results can be seen in figure 75 and the TNF α in figure 76 below.

Figure 75 - IL-17 Production in Pair Two

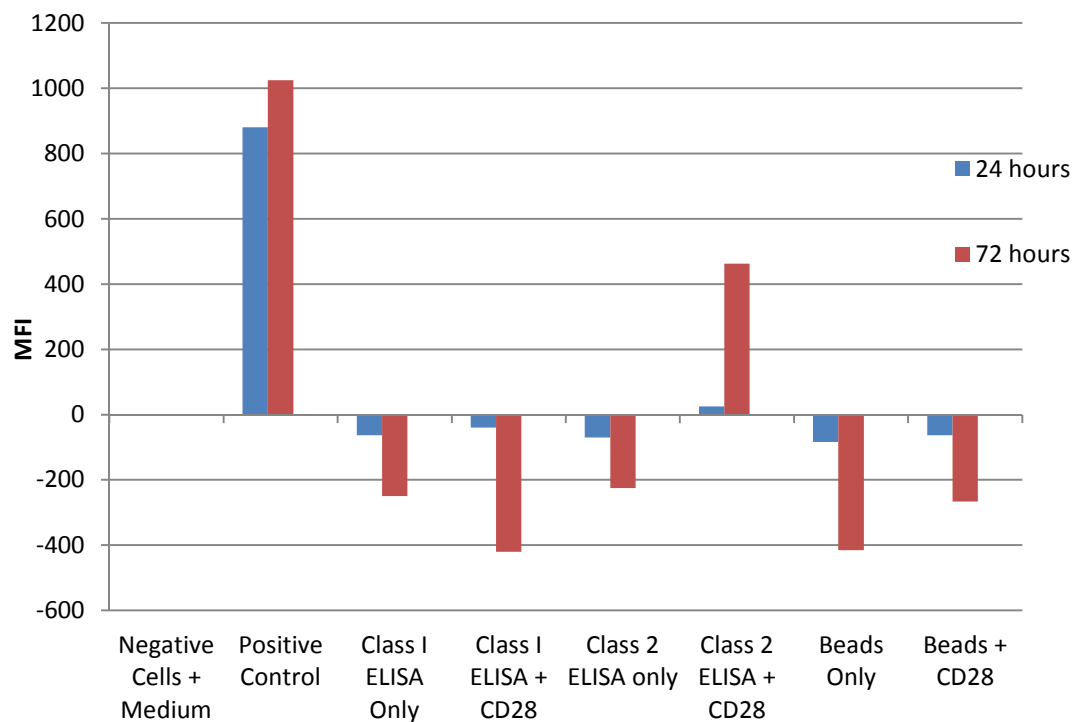
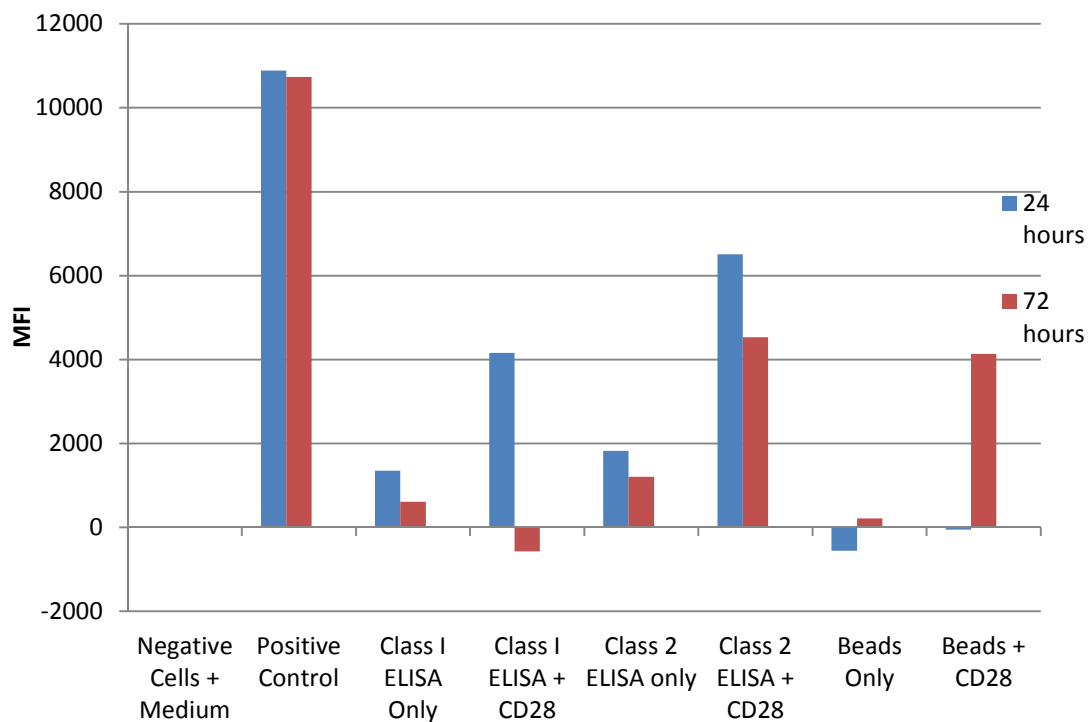


Figure 76 - TNF α production in Pair Two



4.5.5 Pair Three.

The third patient was a male renal patient who had received two previous grafts. He was highly sensitised producing multiple Class I and II HLA specific antibodies. The HLA types, previous mismatches and HLA specific antibodies involved are outlined in table 21 below.

Table 21 - Pair Three HLA Information.

Patient 3 HLA Type	A2,68 B53,65 Bw4,6 Cw4,5 DR1,13 DR52 DQ5,6
Donor 3 HLA Type	A3,68 B53,65 Bw4,6 Cw4,8 DR13 DR52 DQ6
Previous Mismatches	No repeat mismatches presented by donor 3.
Donor Specific Antibodies	A3
Crossmatch Results	T & B cell positive

4.5.5.1 Pair Three Results.

No production of any of the cytokines tested was detected in any of the test wells other than the positive controls.

4.5.6 Experiment Three Discussion.

This set of three experiments gave some interesting results.

The mismatches between pair one included two Class I repeat mismatches from two previous transplants, to which HLA specific antibody had been produced. One of her two previous donors had not been fully HLA class II typed so we were unable to assess the presence of repeat class II mismatches from this donor, however there were no repeat mismatches from the second donor at DR and DQ. We expected to see a response to the donor Class I antigens and potentially a response to the Class II. The main responding cytokine detected was TNF α which was found predominantly in the Class I and II ELISA test wells where soluble CD28 had been added, and additionally at 72 hours only in the beads only test well.

The mismatches between pair two included a repeat Cw7 mismatch from a previous graft, to which HLA specific antibody had been produced. There were no repeat HLA class II mismatches from the previous donor graft, although the patient has produced multiple HLA class II specific antibodies. We expected to see a response to the donor Class I antigens, but

potentially not to the Class II. The main responding cytokine detected was again TNF α in both the Class I and Class II ELISA test wells containing additional CD28, plus in the beads + CD28 well at 72 hours only. In addition some IL-17 was detected in the Class II ELISA well with CD28 at 72 hours only. Unfortunately due to evaporation of the culture medium no results are available for 120 hours for this pair.

The mismatches between pair three did not include any repeats at any loci from his two previous donor grafts. There was a single antibody specificity incompatibility involved to the mismatched HLA A3. Detection of this antibody is believed to be due to epitope crossreactivity with the previously mismatched HLA A1 and A11 from the two previous grafts, as antibodies to all the specificities known to share the epitope in the cross reactive epitope group (CREG) can be detected by single antigen bead screening. Since there were no Class I repeat mismatches and there were no Class II mismatches between the donor and recipient we did not expect to detect any reactivity with this pair. This was the case, and other than the positive control, no cytokine production was detected in any test well above the negative control baseline.

TNF α appears to have been the major cytokine detected using this assay so far. TNF α is a pro-inflammatory cytokine produced by a variety of cell types, including monocytes/macrophages, lymphocytes, endothelial cells and fibroblasts. It elicits many effects during the immune response including immune cell activation and trafficking, cell survival and differentiation [334, 335]. Excessive production and dysregulation in the TNF signal pathways have been linked to a number of autoimmune diseases including rheumatoid arthritis, multiple sclerosis and Crohn's disease. Due to the nature of the cells separated from the peripheral blood of the patients for use in this assay, TNF α production detected here can be limited to T cells, Th1, Th2, Th17 and CTLs have all been found to produce TNF α on stimulation, monocytes and NK cells. Monocytes are phagocytic mononuclear cells which make up 10-15% of the circulating white blood cells. They are bone marrow derived and following a brief period of circulation (24 – 72 hours) enter the tissues where they undergo further differentiation into either macrophages or dendritic

cells, dependent on the signals received and the local tissue environment [336-338]. They are central to both the innate and adaptive immune systems, microbial products such as lipopolysaccharide can directly activate monocytes to produce pro-inflammatory cytokines such as TNF α and IL-1 β , and anti-inflammatory cytokines such as IL-10.

Monocytes/macrophages may also be stimulated by Th1 cells in an antigen dependent fashion, whereby the antigen specific T cell recognises processed peptide presented in an HLA class II molecule on the surface of the macrophage. In addition to the TCR ligation, CD40L on the T cell binds to CD40 on the macrophage surface, providing a co-stimulatory signal for the T cell and the macrophage. This CD40L-CD40 interaction plus production of IFN γ by the Th cell, activates the macrophage to produce cytokines including TNF α and also activates the T cell. In addition activated CD8 $^{+}$ CTLs can also activate macrophages to produce TNF α .

The TNF α production seen in our assay could potentially be explained as being due to non-allospecific reactivity of monocytes. However this does not explain the differences seen in TNF α production between the cell only negative control wells and the stimulator wells, where the only difference is the presence of donor derived HLA molecules, suggesting some form of allospecific recognition. Also, the addition of CD28, for costimulation, appears to lead to an increased level of cytokine production compared to the wells where no CD28 is added, again this would indicate a more specific response is occurring. Finally, the responses seen were generally as expected, whereby the patient cells exposed to repeat mismatched HLA antigens responded and the patient who had not previously been exposed to the HLA antigens present did not respond. These factors all suggest that some form of T cell recognition and consequent activation is occurring. Another possible explanation could be that we are not detecting memory responses but naïve responses. Naïve T cells have a unique ability to produce TNF α rapidly after activation and prior to acquiring effector functions [256]. Recent work has suggested that both naïve CD4 $^{+}$ and CD8 $^{+}$ T cells found in the periphery are able to rapidly produce TNF α following TCR ligation and interaction with an APC, many hours before cell division or production of other classical cytokines such as IL-2 or IFN γ [339, 340]. It is possible

that direct recognition of the donor derived HLA molecules in addition to CD28 binding could lead to activation of allospecific naïve T cells in the culture.

The detection of TNF α in our assay could be of interest when comparing it to the post-transplant outcome, as TNF α has been linked to rejection of renal transplants in multiple ways. A number of groups have studied the effect of polymorphisms in a number of cytokine genes and their relationship to cytokine production and transplant outcome. TNF α is one such cytokine, where polymorphism in the encoding gene has a functional effect on the production of TNF α . A single nucleotide polymorphism (SNP) in the promoter region of the gene at position -308 is associated with a 6-7 fold increase in production of TNF α in vitro [341-343]. A number of studies have looked at the effect of either donor or recipient TNF α high production genotype and post-transplant outcome. The general consensus is that high TNF α production is associated with an increased risk for acute rejection, late rejection and vascular rejection, although this risk decreases with good matching at HLA DR [344, 345]. TNF α production by monocytes has also been associated with the development of transplant glomerulopathy in renal allograft recipients, with high levels of TNF α secretion from the transplant recipient cultured PBMCs correlating with the development of transplant glomerulopathy but not with HLA specific antibody production or C4d staining on biopsy [346]. Culture experiments identified the main TNF α producing cells as monocytes [346]. Further work on monocytes in renal transplantation have identified CD14+CD16+ monocytes as being associated with subclinical atherosclerosis in renal graft recipients [347]. CD14+CD16+ monocytes are considered to be highly proinflammatory due to their ability to produce high levels of proinflammatory cytokines such as TNF α [348]. In addition higher numbers of circulating CD14+CD16+ monocytes have been identified in the blood of patients suffering from end stage renal failure when compared to healthy controls [349] indicating a higher immune activation status. High levels of TNF α in the serum and urine of renal allograft patients have also been found during episodes of acute rejection [350, 351].

The fact that only TNF α was found to be produced at detectable levels is slightly confusing as other cytokines might have also been expected. It was decided for the next experiments to change the array of cytokines being detected to IFN γ , TNF α , IL-2, IL-4, IL-5, IL-10 and IL-17, to reflect the potential cell types being activated.

The results from experiment three indicated that as a concept the assay showed some promise, with reactivity, or lack thereof, being detected when expected in previously sensitised patients. Comparison of the results achieved using the beads vs the ELISA based format indicated that the ELISA format gave the best results, especially when an external source of CD28 was added. It was decided that all further work would be based on the ELISA format with the addition of CD28. The benefit of the ELISA method was that it allowed for separation of the Class I and Class II HLA antigens, giving a greater amount of information about the sensitisation status of a patient. A reduction in the number of test wells also meant that we could add an additional random 3rd party donor stimulator cell into the assay for each patient, to observe further potential reactivity.

4.6 Experiment 4

Over a number of months a further 34 donor and recipient pairs were identified with cells excess to routine testing requirements. For each pair recipient cells were separated and frozen in in-house sterile freezing mix composed of RPMI 1640 with Glutamine (Gibco), Heat inactivated Foetal Bovine Serum (Gibco) and Dimethyl Sulphoxide (Sigma) in a 45:45:20 ratio, and stored at -80°C, lysate was generated from the donor cells and stored at -20°C until used. Each experiment was set up in batches of 6 pairs, to fill one plate. The test wells were as described in table 22 below.

Table 22 - Experiment 4 plate set up.

Negative Control – Cells only
Positive Control – Cells plus CD3/28 stimulator beads
Patient cells vs donor lysate
Patient cells vs donor lysate
Patient cells vs 3 rd party lysate

- The ELISA wells were prepared as described previously.
- 250000 recipient cells were added to each well.
- 2µl of 1ng/ml CD28 was added to each well.
- Plates were incubated at 37°C.
- Supernatant was sampled at 18, 72 and 120 hours and stored at -20°C for testing later.
- Cytokine production was analysed as previously described.

Full details of patient and donor/3rd party types and sensitisation can be found in appendix 4.

4.6.1.1 Experiment 4 Results.

Overall the results from tests with 30 patient cells were included in analysis, all were tested both against HLA molecules isolated from their potential donors and with those isolated from random 3rd party cells. Results from 4 Patient/Donor pairs were excluded due to lack of cells or poor cell viability. In total this gave results from 60 patient/donor test pairs to analyse.

Patients were divided into two groups, sensitised and unsensitised, based on HLA antibody production and/or previous transplants and recorded pregnancies.

One cytokine, IL-4, was not detected in any assay in either the donor specific test wells or in the positive controls. The standards provided with the test kit did produce detectable results on the Luminex test, indicating that the kit was able to detect IL-4. Possible explanations for this could be that there was either no IL-4 produced by any test cell at any point under any stimulation, or that there was some form of inhibition occurring during the Luminex assay detecting IL-4. Therefore the IL-4 results are not included in analysis.

A full summary of all the data generated can be found in appendix 4.

4.6.1.2 Unsensitised Patients.

There were 6 patients recorded as being unsensitised. Of these 2 were female patients and 4 male. All 6 had been screened on multiple occasions and produced no detectable HLA specific antibody. In addition all 6 had no recorded sensitising events, however previous unrecorded transfusions, and pregnancies in the female patients, could not be excluded. An overview of the results achieved in these patients can be seen in table 23 below. The patient and donor pair number assigned at the start of testing is listed under 'No.' and the gender of the patient is listed as 'F' for female and 'M' for male. If a response was observed the HLA class (CI or CII) to which the response was seen is indicated either to the donor or to the 3rd party stimulator, under the column labelled 'Response'. If no response was seen this is indicated by 'none'. Where a response was recorded the time point at which it was first observed is recorded in the column 'time to first response', again divided into responses to the donor or 3rd party stimulator. The cytokines detected in the response are listed in the 'cytokines produced' column. The HLA mismatches presented by either the donor or the 3rd party stimulators are listed in the column 'mismatches present'.

Table 23 - Overview of results observed in unsensitised patients.

No	Sex	Response		Time to 1st Response		Cytokines Produced		Mismatches Present	
		To Donor	To 3rd Party	To Donor	To 3rd Party	To Donor	To 3rd Party	Donor	3rd Party
1	F	CI	CI	24 hrs	24 hrs	IFN γ	TNF α , IL-5	A2, A68, B60, Cw10	A2, B61, Cw2
		CII	None	24 hrs	-	IFN γ	-	DR11, DR53, DQ7	DR4, DQ8
2	M	None	None	-	-	-	-	B35, Cw4	A68, B60, Cw10
		None	None	-	-	-	-	DR15, DR51, DQ5	DR7, DR11, DQ7
3	M	CI	CI	24 hrs	24 hrs	IL-10	IFN γ , TNF α , IL-10	A1, B35, Cw4	A33, B44, B65, Cw5, Cw8
		CII	CII	24 hrs	24 hrs	IFN γ , TNF α , IL-10, IL-17	IFN γ , TNF α , IL-2, IL-10, IL-17	DR1, DQ5	DR1, DQ5
4	M	CI	CI	24 hrs	24 hrs	TNF α , IL-10	TNF α , IL-10	A1, A24, B44, Cw5	A1, A24, B8, B44, Cw5, Cw7
		CII	CII	24 hrs	24 hrs	TNF α , IL-10	TNF α	DR13, DQ6	DR13, DQ6
5	F	None	None	-	-	-	-	A3, B52, Cw1	A2, B35, Cw4
		CII only	CII only	24 hrs	24 hrs	IFN γ	IFN γ	DR1, DQ5	DR15, DR51, DQ6
6	M	None	None	-	-	-	-	A1, A24, B44, Cw5	A3, A32, B7, B35, Cw4
		None	None	-	-	-	-	DR13, DQ6	DR4, DR53, DQ7

Following my hypothesis that early responses would be seen in the patients who were sensitised and also presented with repeat mismatches it was expected that none of these 6 patients would show a response. However, this was the case in only 2 of the patients, with the other 4 showing detectable cytokine production of various profiles. It is noticeable that the cytokines produced, and the timing of their production, is comparable in both the donor specific and 3rd party wells for each patient.

Looking at each patient individually, patient 1 shows an early IFN γ response to both class I and II of the donor but TNF α and IL-5 in response to the class I of the 3rd party only. This indicates

that there may be a certain specificity to the response and that it is specific to the different mismatches presented by each donor cell. This patient is a 51 year old female, therefore previous unrecorded pregnancy or blood transfusion cannot be excluded. The patient went on to be transplanted from the test donor with an ABO incompatible kidney, requiring Rituximab induction, which is still functioning 3 years post-transplant and has had no recorded rejection episodes, observed both through functional testing and biopsy.

Patient 2 showed no response to either the donor or third party, as expected. This patient has been transplanted with a kidney from the test donor, which is still functioning 3 years post-transplant with no recorded rejection episodes.

Patient 3 showed a similar response to both the donor and 3rd party class I and II, with the greatest range of cytokines and similarity in profiles being seen with the two class II tests. Interestingly both the donor and 3rd party cells shared the same Class II mismatches of DR1 and DQ5, which could again indicate some level of specificity in the reaction. This patient is a 39 yr old male and has had no recorded sensitising events, although previous unrecorded blood transfusions cannot be excluded. He was transplanted with a kidney from this donor which is still functioning 3 years post-transplant with no recorded rejection episodes.

Patient 4 showed similar responses to both the class I and II donor and 3rd party. In this case both the donor and 3rd party were HLA identical so similar responses would be expected. This is a 49 year old male who has no recorded sensitising events, although, once again, unrecorded blood transfusions cannot be excluded. The patient went on to receive a kidney from this donor which is still functioning 3 years post-transplant with no recorded rejection episodes.

Patient 5 is a 23 year old female. Her cells showed no response when stimulated with the class I HLA molecules, however IFN γ production was detected in response to both the donor and 3rd party Class II HLA molecules. In this case however no HLA mismatches are shared by the donor and 3rd party. She went on to receive an ABO incompatible transplant from this donor,

requiring rituximab induction, which is functioning well at 3 years post-transplant with no episodes of biopsy proven rejection.

Patient 6 showed no response to either the donor or third party, as expected. This patient has been transplanted with a kidney from the test donor, which is still functioning 3 years post-transplant with no recorded rejection episodes.

4.6.2 Sensitised Patients.

There were 24 patients tested who were classified as sensitised, either due to previously recorded transplants or pregnancies, or due to the fact that they produced HLA specific antibody detected in multiple samples on screening. 19 of these were female and 5 male. 10 patients were sensitised through pregnancy, 12 through a previous, failed, renal transplant and 2 had unidentified sensitising events, presumed to be blood transfusion. Overall, when both donor specific and 3rd party responses were included there were 48 T cell response combinations to be analysed. Of these a response was seen in 39/48 cases, 3/39 to Class I only, 7/39 to Class II only, 29/39 to both Class I and Class II. 9/48 showed no response. A summary of patient details and responses can be seen in table 24 below.

Table 24 - Overview of results observed in the sensitised patients.

No	Sex	Observed Response		Repeat mismatch		Antibody Present		Time to 1st Cytokine		Cytokines Produced		Mismatches	
		Donor	3rd Party	Donor	3rd Party	Donor	3rd Party	Donor	3rd Party	Donor	3rd Party	Donor	3rd Party
7	F	CI	CI	CI	CI	CI	-	24 hrs	24 hrs	TNFα, IL-10	TNFα, IL-10	A24,31 B7,62 Cw9	A3, B60, Cw10
		CII	CII	CII	CII	-	-	24 hrs	24 hrs	IL-10	IL-10	DR12,15 DR51 DQ6	DR15, DR51, DQ6
8	F	CI	-	CI	-	CI	-	Day 3	24 hrs	IL-5, IL-17	-	A66, B41, Cw17	A24, 31, B7,62 Cw9
		CII	CII	CII	CII	-	-	24 hrs	24 hrs	TNFα, IL-10	TNFα, IL-10, IL-17	DR13, DR52, DQ7	DR12, DR52, DQ7
9	F	CI	CI	CI	CI	CI	CI	24 hrs	Day 5	IFNγ, IL-2	IL-5	A3,32 B7 Cw7	A3 B57 Cw4
		CII	CII	CII	CII	CII	CII	24 hrs	24 hrs	TNFα, IL-10	TNFα, IL-5, IL-10	DR4, 53 DQ7,8	DR4, 7 53 DQ8,9
10	F	CI	CI	-	CI	CI	CI	24 hrs	Day 5	IFNγ	IL-5, IL-17	Cw2,7	A1,32 B35,55 Cw4
		CII	-	CII	-	CII	CII	24 hrs	-	IFNγ, IL-2, IL-5, IL-17	-	DR4,15 51,53	DR11,14 DQ5,7
11	F	CI	CI	CI	-	CI	CI	24 hrs	24 hrs	IL-10	IL-10	A2 B37 Cw6	A26 B37 Cw6
		CII	CII	-	-	-	-	24 hrs	24 hrs	TNFα, IL-10	IL-10	-	DR13,15 51 DQ6
12	M	-	-	-	CI	-	CI	-	-	-	-	-	A2,11 B44, Cw5
		CII	CII	-	-	CII	CII	24 hrs	24 hrs	TNFα	TNFα	DQ7	DR4,11 53 DQ7,8
13	F	CI	CI	CI	CI	CI	CI	24 hrs	24 hrs	TNFα, IL-2, IL-17	IFNγ, TNFα, IL-2, IL-5	A3,29 B45 Cw6	A3 B57,65 Cw6,8
		CII	CII	CII	-	-	-	24 hrs	24 hrs	TNFα	TNFα, IL-2	DR17, DQ2	DR1,13
14	F	-	-	-	-	CI	CI	-	-	-	-	A33,68 B71 Cw10	A1,3 B35,39 Cw7
		-	-	-	-	-	-	-	-	-	-	DR10,13 52	DR1,11 52

No	Sex	Observed Response		Repeat mismatch		Antibody Present		Time to 1st Cytokine		Cytokines Produced		Mismatches	
		Donor	3rd Party	Donor	3rd Party	Donor	3rd Party	Donor	3rd Party	Donor	3rd Party	Donor	3rd Party
												DQ2	
15	F	CI	CI	CI	-	CI	CI	24 hrs	24 hrs	TNFα, IL-2, IL-17	IFNγ, TNFα	A2 B49,51 Cw6	A3,29 B8,45 Cw6
		CII	CII	-	-	-	CII	Day 3	Day 3	IL-17	IL-2, IL-5, IL-17	DR9,13 DQ5	DR17
16	F	-	-	-	-	-	-	-	-	-	-	A1,3 B7 Cw7	A1,24 B8,44 Cw5,7
		-	-	-	-	-	-	-	-	-	-	DR8 DQ4	DR13,17 DQ2
17	F	CI	-	CI	-	CI	-	24 hrs	-	TNFα	-	A1,3 B57,65 Cw6,8	A2,33 B49,51 Cw7,16
		-	-	CII	CII	-	-	-	-	-	-	DR13, DQ6	DR9,13 53 DQ6
18	F	CI	CI	CI	-	CI	CI	24 hrs	24 hrs	TNFα, IL-5,IL-10, IL-17	TNFα, IL-5	A2, B60, Cw10	A2, B41, B44, Cw5,17
		CII	CII	-	-	-	-	Day 3	Day 3	IL-5	IL-5	-	DR4, 13 DR53
19	F	CI	-	CI	-	-	CI	24 hrs	-	TNFα	-	B27,44 Cw2,5	A23,80 B52,57 Cw16,18
		CII	CII	CII	-	CII	CII	24 hrs	24 hrs	TNFα, IL-2	IFNγ, TNFα	DQ7	DR7
20	F	CI	CI	CI	-	CI	-	Day 5	Day 3	IFNγ, IL-17	IFNγ, IL-5, IL-10	B57, Cw6	A11 B55 Cw9
		CI	CII	-	-	-	-	Day 3	Day 3	IFNγ	IFNγ, TNFα, IL-5	-	DR10, 14 DQ5
21	F	CI	CI	-	-	-	CI	24 hrs	24 hrs	IFNγ, TNFα,IL-2, IL-10, IL-17	TNFα, IL-10	-	A2, B18
		CII	CII	-	-	-	-	24 hrs	24 hrs	TNFα, IL-10	TNFα, IL-10	-	DR7, DR53
22	M	CI	CI	CI	-	CI	CI	24 hrs	24 hrs	IFNγ, TNFα,IL-2	IFNγ, TNFα,IL-2	A23,80 B57 Cw16,18	A3 B7,57 Cw6,7
		CII	CII	-	-	-	-	24 hrs	24 hrs	IFNγ, TNFα	IFNγ, TNFα	-	-
23	M	-	-	-	CI	CI	CI	-	-	-	-	A1,3 B35 Cw4	A2 B44 Cw2,5
		CII	CII	-	-	-	-	24 hrs	24 hrs	IL-10	IFNγ, TNFα, IL-2, IL-5, IL-10, IL-17	DR4 DR53 DQ8	DR4 DR53 DQ8

No	Sex	Observed Response		Repeat mismatch		Antibody Present		Time to 1st Cytokine		Cytokines Produced		Mismatches	
		Donor	3rd Party	Donor	3rd Party	Donor	3rd Party	Donor	3rd Party	Donor	3rd Party	Donor	3rd Party
24	M	CI	CI	CI	CI	CI	CI	24 hrs	24 hrs	IL-10	TNF α , IL-10	A2 B62 Cw9	A2 B18,62 Cw9
		CII	CII	-	-	-	-	24 hrs	24 hrs	TNF α , IL-10	TNF α , IL-10	DR13, DR52	DR4,11 52,53 DQ7,8
25	F	-	-	-	CI	-	CI	-	-	-	-	B18 Cw7	A1,32 B8,44 Cw5,7
		CII	CII	-	CII	CII	CII	Day 5	24 hrs	IL-17	IFN γ , TNF α , IL-10	DR11 DR52 DQ7	DR12,17 DR52 DQ2,7
26	M	CI	CI	-	-	CI	CI	24 hrs	24 hrs	TNF α	TNF α , IL-10	A1 B35 Cw4	A1 B7 Cw4,12
		CII	-	CII	CII	CII	CII	24 hrs	-	IL-10	-	DR11 DR52 DQ7	DR14,15 DR51,52 DQ6
27	F	-	-	CI	-	CI	CI	-	-	-	-	A2 B44 Cw5	A23,66 B49
		-	-	CII	-	CII	-	-	-	-	-	DR4 DR53 DQ8	DR15 DR51 DQ6
28	F	CI	CI	CI	-	-	-	24 hrs	24 hrs	TNF α , IL-10, IL-17	TNF α , IL-10	A1,26 B37	A3 B35,62 Cw4,9
		CII	CII	CII	CII	-	-	24 hrs	24 hrs	TNF α , IL-10	TNF α , IL-10	DR13	DR1,13 DQ2
29	F	CI	CI	CI	CI	CI	CI	24 hrs	24 hrs	TNF α	TNF α	A11 B55 Cw9	A33 B53,71 Cw10
		CII	CII	CII	CII	CII	CII	24 hrs	Day 3	TNF α	TNF α	DR14 DR52	DR13 DR52 DQ2
30	F	-	-	CI	CI	CI	CI	-	-	-	-	A11 B44 Cw5	A31 B8,45 Cw6
		-	-	CII	CII	CII	CII	-	-	-	-	DR11 DR52 DQ7	DR17 DR52 DQ2,7

The group in whom I expected to see the greatest responses were those where there was demonstrable donor specific antibody and this antibody was directed at a mismatch to which the recipient had previously been exposed, classified as a repeat mismatch (Ab+RptMM+). The group in whom we expected to see the fewest responses were those where there was no donor specific antibody and no previous exposure to the mismatches presented by the potential donor or 3rd party (Ab-RptMM-). A response was defined as production of any cytokine, or combination of cytokines, at any of the time points tested.

Analysis of correlation was performed using Fisher's exact 2 tailed test and the p value recorded.

Table 25 - 2x2 table - Class I DSA positive Repeat Mismatch Positive vs DSA negative Repeat mismatch negative.

Class I	Response	No Response
Ab+RptMM+	16	6
Ab-RptMM-	9	12

Analysis of the Class I information gives a p value of 0.0666, indicating that the association between the antibody and repeat mismatch status with the response outcome is approaching statistical significance.

Table 26 - 2x2 table - Class II DSA positive Repeat Mismatch Positive vs DSA negative Repeat mismatch negative.

Class II	Response	No Response
Ab+RptMM+	8	4
Ab-RptMM-	22	10

Similar analysis of the Class II information gives a p value of 1.000 indicating there is no association between the antibody and repeat mismatch status with the response outcome.

Additionally combination of the Class I and II results indicates no statistically significant association of the antibody and repeat mismatch status with an observed response (p=0.3623).

Cytokine specific analysis, comparing production of each individual cytokine against antibody and repeat mismatch status, also failed to produce any statistically significant results ($p=0.3548 - p=1.00$), with the exception of IL-10, where production of IL-10 in the Class I group was associated with a lack of antibody production and repeat mismatches ($p=0.0414$).

There was also no significant difference in the timing of cytokine production between the two groups with 19/24, or 80%, of the Ab+RptMM+ group producing a cytokine response within 24 hours, compared to 25/31, or 81%, of the Ab-RptMM- group.

Comparison of general sensitisation status and response again yielded no significant results, with sensitisation being associated with any response giving a p value of 1.000, a Class I response giving a p value of 0.508, a Class II response $p=0.293$.

Production of donor specific antibody, regardless of the presence of a repeat mismatch, was also not associated with generation, or absence, of a cytokine response, $p=0.8476$.

Presence of a repeat mismatch, regardless of the production of a donor specific antibody, was again not associated with generation or absence of a cytokine response, $p=0.4273$.

Association between gender of the patient and cytokine production was also analysed and there was found to be no statistical significance ($p=1.000$).

In conclusion the original hypothesis behind the development of the test, that greater numbers of T cell responses would be observed in patients presented with repeat HLA mismatches, particularly where donor specific antibody is present, compared to patients who were unsensitised or who were not presented with repeat mismatches, was not confirmed.

4.6.3 Transplanted Patients.

Previously published results from the groups investigating the IFN γ ELISPOT method, described in the introduction, indicated an association of T cell responsiveness, as measured by IFN γ production, and rejection episodes post-transplant [241]. Of the 30 patients tested in this group of experiments, 14 went on to be transplanted with an organ from the donor used as a

stimulator in the donor specific test wells. A summary of these patients can be seen in table 27 below. This table includes the relevant cytokine data taken from tables 23 and 24 presented previously, and presents the specific responses seen following stimulation of patient cells by HLA isolated from samples provided by the subsequent donor. In this table the donor/recipient pair is indicated in the 'No.' column and corresponds with the identification number assigned during the initial experiments represented previously in tables 23 and 24. The 'type of transplant' column indicates if the patients received an antibody compatible graft, an ABO blood group incompatible graft, an HLA antibody incompatible graft or a combined ABO and HLA antibody incompatible graft. The 'mismatch' column shows the mismatches presented by the donor cells. The 'induction therapy' column indicates the induction therapy administered to the recipient prior to transplant – none, IVIg alone, Campath, Rituximab or combined rituximab and campath. The 'T cell mediated rejection on biopsy' indicates with 'yes' or 'no' if T cell mediated rejection was diagnosed on post transplant biopsys at any point during the first 2 years post transplant. The 'class I' and 'class II' response columns list the cytokines detected at any sampling time point in response to donor class I or class II specific stimulation during the culture experiments described previously.

Table 27 - Overview of results observed in transplanted patients.

No	Type of Transplant	Mismatches	Induction Therapy	T cell mediated rejection on Biopsy	Class I Response	CII Response
1	ABOi	A2,68 B60 Cw10 DR11 DR53 DQ7	Rituximab	No	IFN γ (weak)	None
2	Compatible	B35 Cw4 DR15 DR51 DQ5	None	No	None	None
3	Compatible	A1 B35 Cw4 DR1 DQ5	None	No	IL-10	IFN γ , TNF α , IL-10, IL-17 (weak)
4	Compatible	A1,24 B44 Cw5 DR13 DQ6	None	No	TNF α , IL-10	TNF α , IL-10
5	ABOi	A3 B52 Cw1 DR1 DQ5	Rituximab	No	None	IFN γ
6	Compatible	A1 A24 B44 Cw5 DR13	None	No	None	None
11	HLA/ABOi	A2, B37, Cw6	Campath	No	TNF α , IL-10	TNF α , IL-10
13	HLAi	A3, 29 B45 Cw6 DR17 DQ2	IVIg	Yes	TNF α , IL-2, IL-17	TNF α (weak)
16	Compatible	A1,3 B7 Cw7 DR8 DQ4	None	No	None	None
21	Compatible	No mismatches	None	Yes	IFN γ , TNF α , IL-2, IL-10, IL-17	TNF α , IL-10
23	HLA/ABOi	A1,3 B35 Bw6 Cw4 DR4 DR53 DQ8	Campath	No	None	IL-10
28	ABOi	A1 A26 B37 DR13	Rituximab	Yes	TNF α , IL- 10, IL-17	TNF α , IL-10
29	HLAi	A11 B55 Cw9 DR14 DR52	Campath	No	TNF α	TNF α
30	HLAi	A11 B44 Cw5 DR11 DR52 DQ7	Rituximab + IVIg	No	None	None

The transplanted group is made up of a mixture of patients who received a compatible graft, a graft which was ABO blood group incompatible, a graft which was HLA antibody incompatible or one which presented both ABO and HLA antibody incompatibilities. The variation in transplant type also led to a variation in induction therapy used, which included some

occasions of Campath for lymphocyte depletion, Rituximab for B cell depletion and no cell depleting induction therapy. The post-transplant outcomes in terms of the occurrence of T cell mediated rejection episodes in patients treated with Campath need to be treated with caution as T cell depletion would inhibit the ability for a T cell mediated response to be mounted. In our centre the patients who do not receive Campath or Rituximab are treated with anti-CD25 mAb Basiliximab for induction. All patients then receive triple therapy of mycophenolate mofetil (MMF), Tacrolimus (Tac) and steroids in the immediate post-transplant period.

All patients have been followed up of a minimum of 2 years. All recorded episodes of biopsy proven T cell mediated rejection were from 'for cause' biopsies taken at times of clinically implied suspected rejection. As previously outlined biopsy analysis was performed by histopathologists and reported in the context of the internationally agreed Banff criteria [352].

3 of the 14 transplanted patients suffered from one or more episodes of biopsy proven T cell mediated rejection. 1/3 received an HLA antibody incompatible transplant, 1/3 received an ABO blood group incompatible transplant and one received an antibody compatible transplant, which also presented no HLA mismatches. None of these patients received Campath induction. All three patients showed T cell responsiveness to their donor as indicated by cytokine production during the assay.

11 of the 14 transplanted patients suffered no episodes of T cell mediated rejection. 4/11 produced no cytokines during the assay, 6/11 produced a very limited response, often with only one cytokine being detected to either class I or class II, 1/11 showed a wider ranging response to the donor HLA class II molecules. Three of these 11 patients did receive Campath induction therapy, therefore observation of TCMR would be less likely in the short term post-transplant period. The cytokine production profiles of these patients were limited, showing production of TNF α and/or IL-10.

The three patients in whom TCMR was diagnosed all showed a wide range of cytokine production on stimulation in the Class I test wells. The major difference in cytokine production

between the rejector and non-rejector group is that all those who had an episode of TCMR produced the cytokine IL-17 on stimulation. Of the transplanted group, only 1 patient produced IL-17 who did not go on to have a biopsy proven episode of rejection. Comparison of the timings of the IL-17 production showed that 3/3 patients who suffered TCMR produced IL-17 in response to the Class I molecules at days 3 – 5. IL-17 production in the patient who did not suffer TCMR was in the class II stimulator well and detected in the first 24 hours only. In addition, as discussed earlier, the cytokine production profiles with the donor specific wells and the 3rd party test wells are generally similar, possibly suggesting that this is an indication as to general responsiveness rather than anything donor specific. Comparison of the profiles seen in these 4 patients against their potential donor and the 3rd party showed that in the 3 rejectors the profiles were indeed similar with the exception of IL-17 production, which was only seen in the donor specific well, however in the patient who didn't reject, IL-17 was seen in both the donor and 3rd party wells. Of note, one patient in the TCMR group received an apparent HLA identical transplant, yet still suffered an episode of TCMR and still responded on stimulation in vitro. Since the only apparent addition to the test wells was donor derived HLA molecules, differences, potentially in the peptide being presented may be responsible for stimulating the patient cells.

The mean time to 1st episode of biopsy proven TCMR in this group was 11.3 days, with a range of 6 – 15 days. Patient 28 had a donor organ nephrectomy at day 7 due to uncontrolled T cell mediated rejection. Patient 13 and Patient 21 have retained there grafts which remain functional at 3.5 and 2.8 years post-transplant respectively.

Analysis of the IL-17 data using Fisher's exact test gives a p value of 0.0027 indicating strong statistical significance, the data used for analysis is presented in table 28 below. Whilst the numbers involved are too small to draw genuinely significant conclusions these results would indicate that IL-17 production on stimulation in vitro could be indicative of future TCMR episodes and warrants further investigation.

Table 28 - 2x2 table presenting analysis of IL-17 production at day 3 of culture following stimulation with potential donor cells and subsequent diagnosis of T cell mediated rejection on post transplant biopsy.

	IL-17 at day 3 of culture	No IL-17 at day 3 of culture
T cell mediated rejection	3	0
No T cell mediated rejection	0	11

IFN γ production did not appear to be associated with future rejection episodes, with only one of the TCMR group producing IFN γ on stimulation compared to 3 in the non-TCMR group.

Only 2/14 transplanted patients showed a detectable IL-2 response, interestingly both these two patients were again in the TCMR group. None of the non-TCMR showed an IL-2 response. Production of IL-2 was again detected later on in the assay period, at days 3-5.

None of the transplanted patients showed an IL-5 response.

The production of TNF α or IL-10 also does not appear to be linked with future episodes of TCMR, $p=0.1923$ and $p=0.5227$ respectively. All of the TCMR group produced both TNF α and IL-10 on stimulation, however 4/11 of the non-TCMR also produced TNF α and/or IL-10.

Further investigation, with a larger sample size, may be able to give a better indication as to their association with future TCMR episodes.

4.7 Discussion.

The rationale behind the development of this test system was to assess if memory T cells could be stimulated *in vivo* to produce detectable cytokines in response to donor derived HLA molecules. It was hoped that this system could help to identify patients who had memory T cell responses to their potential donors, particularly in the HLA antibody incompatible group, in order to help identify the most suitable donor, when multiple donors were available, and those patients who were at high risk of developing T cell mediated rejection. Sindhi et.al. recently reported that 70-95% of rejection episodes in renal allografts include a cellular component [217], however most solid organ transplantation laboratories do not routinely assess a patients' cellular reactivity to a potential donor, relying on HLA specific antibody status to act as a marker for overall immune reactivity against a given donor and thus avoiding hyperacute and acute antibody mediated rejection. In part this is due to the ease and reliability of HLA specific antibody testing methods compared to those assays currently available for assessment of T cell reactivity.

Prior to testing it was assumed that patients in my study who were sensitised to their potential donor, in terms of HLA specific antibody production, and had had previous sensitising events involving one or more of the HLA antigens presented by the potential donor, would be more likely to produce a rapid detectable cytokine response on stimulation due to the presence of donor specific memory T cells. Whereas unsensitised patients, or those whose previous sensitising events had not included any of the HLA antigens presented by the potential donor, would have milder responses, potentially later in the testing period, if any response at all. This made the assumption that patients would only possess memory T cells to HLA antigens to which they had previously been exposed. However, analysis of the data generated found no correlation between detected cytokine responses and previous exposure to HLA. Indeed wide ranging cytokine production was detected in 4/6 of the unsensitised patient group.

Comparison of patients in whom we had previously detected potential donor specific antibody and who had previously been exposed to one or more of the HLA mismatches presented by

the donor (Ab+Rptmm+) with patients who produced no potential donor specific antibody and had not been previously exposed to any of the HLA mismatches showed no statistical difference in cytokine production ($p=0.362$). However when divided into class I and class II results the association of a class I repeat mismatch with antibody and cytokine production was nearing significance ($p=0.0666$), indicating that there was potential correlation between an observed response and previous sensitisation. Presence of a repeat mismatch alone, regardless of antibody status, and presence of donor specific antibody alone, regardless of repeat mismatch status, were also not significantly associated with cytokine production, $p=0.4273$ and $p=0.8476$ respectively. Additionally production of no single cytokine could be associated with previous HLA sensitisation. This overall result should perhaps not have been unexpected as they generally agree with previous observations made by groups developing the IFN γ ELISPOT method discussed in the introduction. Heeger et.al. and Nickel et.al. have both reported that donor specific T cell reactivity as demonstrated by IFN γ production in the ELISPOT system, was independent of a patients previous sensitisation events, HLA specific antibody production, age and donor HLA mismatches [245, 353]. Andree et.al. and Poggio et.al. also demonstrated that T cell reactivity against a panel of stimulator cells, which provides a 'Panel reactive T cell' (PRT) score is also independent of a patients sensitisation status [250, 354]. However these two groups did report a correlation between PRT and HLA mismatches, previous transplants and being female, which was not found in this current study. One of the strongest correlations reported was between IFN γ production and length of time on dialysis[251]. Due to difficulty in obtaining complete historical dialysis records for all our patients we were unable to assess this in our group of patients. The detection of IFN γ responses, or indeed other cytokines, following stimulation by antigens to which a patient has not previously been exposed is not unexpected as it has been estimated that 10% of mature naïve T cells in circulation exhibit cross-reactivity against HLA molecules to which they have not previously been exposed [240, 355, 356].

It is perhaps not surprising that sensitisation as measured by HLA antibody production is not associated with T cell reactivity, as both the original IFN γ ELISPOT and our T cell assay are observing early cytokine production and therefore are likely to be detecting direct and possibly indirect alloresponses rather than just the indirect T cell responses required for allo antibody production. Najafian et.al. and Poggio et.al. have both reported an alternative IFN γ ELISPOT assay which detects indirect T cell reactivity via stimulation with donor HLA derived peptides [254, 255], however whilst both associate post-transplant T cell reactivity with poor renal function and chronic allograft nephropathy, neither reported an observed relationship with HLA specific antibody production.

The cytokines tested for in this assay included IFN γ , TNF α , IL-2, IL-4, IL-5, IL-10 and IL-17, although due to lack of detection in any test, including all the positive controls, meant that IL-4 could not be included in the analysis. Two of the most commonly detected cytokines were TNF α and IL-10, often together but also individually. As discussed previously the presence of TNF α could be due to activation of naïve T cells or memory Th1 cells in response to TCR ligation, or due to non allospecific activation of monocytes. In the initial results from experiment 3 it appeared that maximal TNF α production was being detected in the wells presenting HLA mismatches to which the recipient was previously sensitised and therefore suggested that it may be being produced in an allospecific response by T cells. However continuation of the assay with a further 30 pairs showed that TNF α production was a little more non-specific, appearing even in the test wells of unsensitised patients. No correlation between TNF α production and either presence of repeat mismatches or HLA antibody sensitisation could be found.

Comparison of the responses seen with donors and the respective third parties shows that the profile of cytokines produced in response to stimulation is very similar. This is in agreement with reports from Hricik et.al. [246] that numbers of IFN γ producing T cells were similar in response to both donor and 3rd party, indeed in their study it was the number of IFN γ

producing cells in response to the 3rd party stimulators, not the donor stimulators, that showed statistical significance with acute rejection post-transplant. It is possible therefore that detection of cytokine responses after allo stimulation is more a reflection on immune reactivity status than donor specific reactivity. Whilst, as previously discussed, a number of studies have correlated the number of IFN γ producing cells with both acute rejection episodes post-transplant and renal function, as measured by creatinine or glomerular filtration rates, at 3 and 6 months, none to this authors knowledge has provided a definition of 'acute rejection episode' or described the nature of the rejection, defining it as cellular or antibody mediated or both. In the report by Hricik et.al. [246] 29% of the transplanted patients included were sensitised, producing HLA specific antibody, although it is not reported if these antibodies were donor specific, nor were the crossmatch results reported, therefore it is possible that AMR could be the cause of the reported acute rejection episodes. A number of the other studies included patients in whom antibody removal was required to facilitate transplantation, however the nature of acute rejection episodes reported post-transplant was not documented. So whilst numbers of IFN γ producing T cells in response to stimulation may be indicative of future acute rejection episodes there is not enough reported data to indicate that these patients would benefit from immunosuppressive therapy aimed at the T cell or humoral components of the immune response.

Whilst the results from all 30 pairs were indicative of the ability to detect cytokine responses on stimulation, it is the results from the 14 pairs who went on to be transplanted that yield the most direct information regarding cytokine production and clinical outcome. Cytokine analysis in our group of 14 patients who went on to be transplanted with an organ from the donor providing the stimulator cells in the assay showed some interesting results. 3 of these patients went on to suffer TCMR episodes, whilst 11 showed no TCMR episodes, however 2/11 did suffer antibody mediated rejection (AMR) episodes, probably related to the fact they were HLA antibody incompatible transplants. Overall the TCMR group produced a wider range of cytokines compared to the non-TCMR group. 4/11 non-TCMR group produced no cytokines on

stimulation, of the remaining seven, 6 produced very limited cytokine responses with TNF α and IL-10 being the most commonly detected cytokines, with only 1 producing a wider response. Of note, only 1/3 TCMR group produced IFN γ on stimulation compared to 2/11 non-TCMR patients, neither of which received Campath or other lymphoablative induction therapy, which would have questioned the validity of this observation. Comparison of the characteristics of the 14 patients who were tested and subsequently transplanted shows a very immunologically diverse group. The three patients with TCMR were very varied, all were sensitised as defined by the detection of HLA specific antibody on serum screening, one received an HLA identical cadaveric graft, one being apparently unsensitised to the donor and receiving an ABO incompatible graft and one sensitised patient who received an HLA incompatible graft following antibody removal with a donor to whose mismatches they had previously been exposed. In the 11 non-TCMR group, 3 patients were sensitised and received HLA incompatible grafts, following antibody removal, from donors to whose HLA mismatches they had previously been exposed, one also required HLA antibody removal but the mismatches presented by the donor were not repeats from previous sensitising events and seven were unsensitised patients receiving HLA and ABO antibody compatible grafts. Therefore it could be concluded that previous HLA mismatches and HLA antibody production are not indicators of future TCMR episodes alone.

Whilst TNF α was universally detected in the TCMR group, it was also detected in 4/11 non-TCMR group, so could not be significantly correlated to post-transplant outcome in relation to TCMR episodes. The finding of TNF α and IL-10 being produced in the same test wells is somewhat confusing as TNF α is a classical pro-inflammatory cytokine and IL-10 an anti-inflammatory cytokine, the production of which is reported to inhibit TNF α production [357]. Both are produced by activated monocytes but also by different T cell populations, with TNF α being associated with Th1 responses and IL-10 with Th2. The cytokine profiles observed don't appear to fit with any typical Th1 or Th2 pattern, where the detection of IFN γ might be expected to be accompanied by IL-2 and TNF α , signifying a Th1 type response, whereas IL-4, IL-

5 and IL-10 might signify a Th2 response. This highlights the complexity of the responses generated and the fact that multiple cell types might be involved. It is virtually impossible to dissect out which cells types are being activated using cytokine analysis alone, more detailed flow cytometric analysis of the cultured cells could possibly elucidate the different cell types involved and may be of interest in future work.

IL-10 has previously been associated with graft acceptance and stable function. Higher numbers of CD4+ IL-10 producing cells have been associated with stable graft function in lung transplantation [358], renal transplantation [247] and in the absence of graft versus host disease in bone marrow transplant recipients [359]. It has been suggested that a higher ratio of IL-10 producing cells to those producing IFN γ as measured on ELISPOT in response to donor specific stimulation, is an indicator of stable graft function and low IL-10 producing cell numbers associated with acute rejection [247]. Individuals can be divided into high and low producers of certain cytokines, including IL-10, by analysis of single nucleotide polymorphisms (SNPs) in the promoter regions of the cytokine genes. There is a SNP at -1082 in the IL-10 gene, with the -1082G being associated with higher IL-10 production and -1082A with lower production [271]. It has been reported that patients with the higher producing genotype are protected from chronic rejection in comparison with the lower producing group [271].

However other studies have demonstrated that high levels of IL-10 in the urine and plasma of transplant recipients is associated with development of renal allograft rejection [360]. In addition analysis of intrarenal mRNA expression of the gene encoding IL-10 found a correlation between high levels of mRNA and development of acute rejection [361]. Several cell types produce IL-10 including activated monocytes, regulatory T cells and Th2 cells [362, 363]. IL-10 is the main cytokine produced by both Th2 and regulatory T type 1 (Tr1). In addition activated Tr1 cells will produce transforming growth factor – beta (TGF- β) and activated Th2 cells would produce IL-4 and IL-5 [363, 364]. In our assay IL-10 production was only accompanied by IL-5 in 5 of the 39 IL-10 positive tests, so these could be due to Th2 responses. Unfortunately TGF- β was not included in our cytokine testing panel so we cannot draw conclusions as to the origin

of the IL-10 with regard to Tr1 cells, but can assume that not all the incidences of IL-10 are due to Th2 production. IL-10 production within the transplanted group did not appear to correlate with TCMR, with 2/3 TCRM group and 4/11 non-TCRM group producing IL-10 on stimulation. None of the transplanted patients produced an IL-5 response in conjunction with IL-10, so it is unlikely to indicate Th2 activation.

Our finding that IFN γ production did not appear to be associated with TCMR episodes post-transplant is in agreement with other reported findings that IFN γ production is not a positive indicator of rejection in all cohorts. Two retrospective studies using the IFN γ ELISPOT failed to find an association with acute rejection episodes, however this is thought to have been due to the treatment of patients with ATG pre transplant, which would inhibit a T cell response [252, 253]. Analysis of cytokines in the serum of patients post-transplant by Sadeghi et.al. [265] found serum levels of IFN γ were significantly elevated at 24 months post renal transplant in patients without any apparent rejection episodes compared with both healthy controls and chronically rejecting patients. Ghafari et.al. [365] also found that serum levels of IFN γ did not correlate with episodes of acute rejection within the first month when measured pre transplant and at 7 and 14 days post-transplant.

Of all the cytokines analysed in the transplanted patients in this study the striking difference between the two groups was the production of IL-17, where all 3 TCRM patients produced IL-17 on stimulation compared to only 1 of the non-TCMR group. The correlation of the detection of IL-17 with an episode of biopsy proven TCMR was statistically significant, with a p value of 0.0027. The numbers on which this analysis is based are small, however the strength of this finding cannot be dismissed on this basis. Whilst, as expected, the responses to both the donor and 3rd party stimulators were similar for the majority of the cytokines detected in these patients, IL-17 was only produced in the donor specific wells and not the third party. In addition the profile of production was the same for all three patients, where IL-17 did not

become detectable until day 3 and then remained so until day 5. In two of the three patients this was also accompanied by IL-2 production.

Of note, results found in the original development experiment 3 also detected a patient who produced IL-17 at day three, this time in response to class II stimulation. This data has not been included in the analysis for the transplanted patients due to the differences in set up and cytokines analysed. However this patient was also transplanted with a kidney from the donor used as a stimulator and he too went on to suffer an episode of TCMR in the early post-transplant period. Of the other two patients tested in that set of experiments, who did not produce IL-17 at any time point, one went on to also receive a transplant from their respective stimulator donor and whilst they suffered multiple episodes of AMR, no TCMR could be detected on biopsy. If the results from these two patients are also included in the analysis the p value, from Fisher's exact test, becomes even smaller at $p = 0.0021$.

As discussed in the introduction IL-17 is a relatively newly identified cytokine and its main producing cell, Th17, even more recently defined. Initially it was reported that T cell production of IL-17 was limited to just CD4+ helper T cells, however mounting evidence has been published to suggest that CD8+ cytotoxic T cells are equally capable of producing IL-17 on stimulation [366]. In addition, Shin et.al. reported that only T cells of the memory CD45RO+ phenotype are capable of producing IL-17 as they found no expression of IL-17 mRNA by naïve CD45RA+ cells on stimulation [366]. When separated CD4+CD45RO+ and CD8+CD45RO+ T cell populations were cultured and stimulated by ionomycin/PMA the memory CD4+ T cells were able to respond without further signals, however the memory CD8+ T cells appeared to require further costimulatory signals, possibly from CD4+ T cells, APCs or NK cells, although the exact nature of these signals could not be specified [366]. In addition to memory T cells, NK cells, $\gamma\delta$ T cells and neutrophils have also been associated with IL-17 production.

Initial work investigating the conditions required for induction of a naïve T helper precursor cell to commit to becoming a Th17 cell was carried out using mouse derived cells. It has been

commonly reported that the naïve CD4⁺ T cells require exposure to TGF- β , IL-21 and dendritic cell-derived IL-6 in order to differentiate into an IL-17 secreting Th17 cell [367-369]. Some groups later confirmed these requirements in human naïve T cells, showing that TGF- β , IL-23 and/or IL-21 can lead to differentiation into IL-17 producing cells [370-372]. However more recently published work reported by Evans et.al. [373] suggests that human naïve CD4⁺ T cells require different conditions before commitment to the Th17 line. They reported that stimulation of naïve T cells in the presence of TGF- β and IL-6 does not cause differentiation of these cells to Th17, and the presence of TGF- β may in fact inhibit their generation. They instead demonstrated that generation of these cells requires stimulation through the T cell receptor as well as interaction with Toll-like receptor activated monocytes. They also report that methods used to generate Th17 cells from naïve T cells failed to stimulate the production of substantial numbers, <2%, and that the greatest numbers of Th17 cells, up to 28% of the population, were generated following stimulation of memory T cells via their TCR and interaction with activated monocytes. Since prior to stimulation <0.5% of the population were IL-17⁺ it is suggested that the Th17 cells came not from expansion of an existing population but from directed differentiation into IL-17 producing Th17 cells from memory T cells. Of note the culture period used where peak responses were seen following interaction with monocytes and stimulation of the TCR by anti-CD3 mAb was 3 days.

All three of the patients who suffered TCMR produced IL-17 in response to donor HLA class I stimulation which was first detected at day 3 of the assay. From this one possible scenario could be drawn. As discussed previously TNF- α production was also noted, this can be produced in abundance by activated monocytes and it's detection early in this assay could indicate that the recipient monocytes present in the culture are being activated by some aspect of the donor lysate being introduced into the well, they may well be taking up and processing the donor HLA class I molecules and presenting them in the context of self HLA class II on their surface. Memory T cells present in the culture which possess a TCR complementary to donor HLA derived peptide in self HLA class II molecules on the surface of

the monocyte, may become activated and ushered down the route of Th17 differentiation by both ligation of their TCR and further interaction with activated monocytes. The data reported by Evans et.al. [373] would suggest that this could indeed lead to IL-17 production being detectable at day 3. This would also suggest the presence of donor specific memory T cells capable of an indirect response in the patient. The mean time to biopsy proven TCMR episode in this group of three patients is 11.3 days, with a range of 6 – 15 days, it is likely that as all these were 'for-cause' biopsies that the initiation of the rejection episode was earlier than these timings would suggest, indicating that all three suffered early acute rejection potentially due to activation of memory T cells.

Another possible explanation is that the IL-17 is being produced by activated memory CD8+ cytotoxic T cells, potentially recognising the donor HLA Class I molecules present in the test well directly, and receiving co-stimulatory signals either directly from interaction with other cells in the culture or from cytokines being produced by other cells present. If this were the case however it may be expected that a response would be seen prior to day 3.

Only 1 patient, patient 21, who had TCMR on biopsy produced detectable IFN γ in addition to IL-17 and other cytokines. This is of interest as Harrington et.al. have reported that IFN γ , when added to in vitro culture, inhibits the generation of Th17 cells [274] and therefore it might be expected that both may not be found in the same culture. This again highlights the complexity of defining the exact cell types involved in this assay.

One patient in the transplanted group who did not suffer an episode of TCMR also produced low but detectable levels of IL-17 on stimulation. The timescale of this response was however completely different from that seen in the TCMR group, with the IL-17 response being detected at 24 hours only and not being detectable at later time points. It is possible that this detection was due to non-specific cytokine production, an artefact of the cytokine Luminex assay or that the patient did indeed suffer a sub-clinical episode of TCMR for which a biopsy was not taken.

Since the identification of the cytokine IL-17 a number of groups have investigated its' role in rejection of solid organ transplants. Strehlau et.al. investigated the intragraft expression of a number of immune activation genes in 60 renal allograft biopsies, among the cytokines investigated IL-17 was shown to be expressed solely in samples taken from rejecting organs, but it was not a reliable indicator of all rejection episodes as it was not found to be expressed in all rejecting samples [374]. However it is not clear from the report if diagnoses of rejection on all the biopsies were T cell mediated. Following this study, Van Kooten et.al. demonstrated that the cytokine IL-17 could be detected by immunofluorescent staining in all of the renal biopsies studied compared to none of the biopsies taken from healthy control or pre transplantation [277], in addition they demonstrated that the expression of IL-17 protein was significantly increased in the infiltrating cells in acute TCMR. More recently Loverre et.al. [375] demonstrated that in 50 renal allograft biopsies, the presence of IL-17 secreting cells was significantly found in those where acute TCMR had been diagnosed compared to those where antibody mediated rejection, BK nephropathy and calcineurin inhibitor nephrotoxicity had been diagnosed. Additionally, identification of the cell source of IL-17 indicated that 80% of the IL-17 producing cells present in the biopsy sample were CD4+ Th17. This confirms numerous previous reports of the involvement of IL-17 producing CD4+ Th17 cells in the pathogenesis of acute TCMR in renal transplants [376-378]. Th17 cells have also been implicated to have a pathogenic role in lung [379, 380], liver [381], small bowel [382] and heart [281] allograft rejection. The majority of studies implicating IL-17 and Th17 cells in allograft rejection have concentrated on the detection of IL-17 protein or mRNA on biopsy. It would be of interest on expansion of this assay to a wider test group to assess IL-17 production on allograft biopsy in comparison to production in the pre-transplant assay.

IL-17 is an overarching term used to describe a family of structurally homologous cytokines IL-17A – IL-17F [383]. Human Th17 cells have been shown to produce two isoforms of IL-17, IL-17A and IL-17F [260]. In a number of studies using animal models it is IL-17A, not IL-17F, that has been found to be implicated in transplant rejection [260] and inflammation [384]. The

cytokine detection kit used in this assay has been confirmed to detect IL-17A only, with negligible cross-reactivity with other isoforms of IL-17 (personal communication with R&D systems).

One patient in the transplanted TCMR group received an HLA identical kidney. She had received one previous renal transplant and was sensitised, producing a wide range of HLA specific antibodies but due to the lack of HLA mismatches, based on 2 digit HLA typing at all loci, between her and the donor it was assumed that we would not see a T cell response on assay, and that TCMR post-transplant was unlikely. However production of a number of cytokines was observed after donor stimulation, including IL-17 at day 3, and TCMR was reported on biopsy post-transplant. There are two possible explanations for this, the first is that there were differences in the minor histocompatibility antigens being presented by the donor, or that at a higher resolution there were differences in the HLA antigens between donor and recipient. Minor histocompatibility antigens (mHA) are peptides derived from cell proteins encoded by polymorphic genes, the presentation of which is restricted to specific HLA molecules [385]. The Collaborative Transplant Study reported that the 10 year graft survival rate for renal transplants between HLA identical siblings stands at 82.5%, and whilst some losses will be due to non-immune factors, some are believed to be due to immunologically driven loss, with Terasaki reporting that up to 56% of the losses could be due to immune mediated causes [386, 387]. It has been suggested that responses to the mHA may be the cause of at least some of these graft losses [388, 389]. Mismatches in the mHA between donor and recipient in combination with the correct HLA antigen required for presentation of the mHA could trigger T cell responses [390]. There are now a total of 10 Y-chromosome encoded and 14 autosomal encoded recognised mHA [391]. Of note, 3 of the HLA antigens involved in our pair include HLA A1, B8 and B44, all of which are able to present various mHA. Two of the potential mHA are A1/HY and B8/HY which could be discounted as the donor and recipient were both female and therefore Y chromosome encoded mHA are not relevant. There are 5 HLA B44 restricted mHA, all of which have tissue distribution limited to hematopoietic cells

and B cells, so whilst they may be relevant to the T cell culture assay as the donor HLA molecules are derived from some of these cells, it is unlikely that they would be relevant in a solid organ transplant. This leaves one potential mHA, HA-3, which is restricted to presentation by HLA A1 and has broad tissue distribution. Typing for mHA has not been performed in this study so differences between donor and recipient cannot be confirmed, but it remains a possible target for T cell activity.

Another theory behind the observed reactivity could be due to mismatches at a higher resolution. The most obvious target when observing the HLA types involved would be HLA B44. HLA B*44:02 and *44:03 are the two most commonly represented of the B44 group of antigens in the population. They differ by only one amino acid at position 156, with B*44:02 having an asparagine and B*44:03 having a leucine at this position. This difference is located on the $\alpha 2$ helix, and whilst it doesn't much alter the repertoire of peptides that can be presented by the B44 molecules, it does render them targets for CD8+ T cells which are able to discriminate between the two types [392]. However higher resolution typing at HLA B44 revealed both donor and recipient to be HLA B*44:02. Complete high resolution typing for the pair was not performed, so it cannot be discounted that there were other unrevealed mismatches at the 4 digit level that could be responsible for the reactivity observed.

The discovery that in our population IL-17 production in response to donor stimulation pre-transplant correlated well with episodes of TCMR post-transplant indicates that this assay could be used as a tool to aid decisions regarding pre transplant induction therapy. In 2010 our renal unit introduced a three tier risk based immunosuppressive protocol. Patients were deemed to be at higher risk of rejection when they were being transplanted in the presence of donor specific antibodies detected on Luminex single antigen bead screening, regardless of the crossmatch result, with those presented with repeat mismatches as being at greatest risk. These patients are now given Campath as part of their induction routine. Introduction of this T cell assay could help identify which patients are at need of Campath induction therapy and

which would potentially gain no benefit from this treatment. Whilst this could initially be for the patients involved in HLA antibody incompatible transplants, as the data showed, 2/3 patients with TCMR did not fall into this category, therefore expansion of the test to a wider group of patients may also prove clinically useful. Unfortunately due to the culture time required before an IL-17 response could be detected it would not be suitable for pre transplant assessment for deceased donor organs where rapid implantation is required. Additionally, since the IL-17 production in these cases was donor specific a panel reactive T cell test, akin to that reported by Heeger et.al. for IFN γ , would also potentially not be of benefit.

If further work did indeed confirm that IL-17 detection in this assay correlated with TCMR episodes in a larger study it could be possible to transfer this knowledge to a different platform, such as ELISPOT. However if the theory that activated monocytes are required to initiate Th17 responses is true then it would be necessary to include monocytes in the ELISPOT assay, which has been reported to be avoided by some groups by removal of adherent cells prior to final ELISPOT testing [389].

Overall the results indicate that the presence of memory T cells as detected by IL-17 production on stimulation correlates with the development of TCMR post-transplant. However the results achieved across all patients do not appear to correlate with previous HLA exposure or the presence of DSA. This would suggest that the prediction of either direct or indirect T cell memory based on either repeat mismatching or the presence of DSA is inadequate and the immune memory may be more complex.

5 Final Conclusions and Future Aims.

The hypothesis put forward at the start of this study was a simple one, suggesting that the outcomes of renal transplants across HLA antibody incompatibilities would be negatively influenced by immune memory. Patients presented with repeat mismatches to which they had made antibody being more likely to demonstrate both cellular and humoral memory responses, indicated by earlier and more vigorous rejection episodes, than patients in whom donor specific antibody could be detected but who were not presented with a repeat mismatch. It was assumed that the presence of donor specific antibody prior to transplant indicates previous activation of both the cellular and humoral immunity via the indirect pathway, and that by re-challenging a recipient with the same mismatched antigen both these arms of the adaptive immune response would be rapidly re-activated, leading to early rejection. The presence of DSA without a repeat mismatch was assumed to be due to antibody epitope cross reactivity from previous sensitising events that would not necessarily indicate the presence of T cell memory. This however did not account for the presence of direct alloreactive memory T cells, which would not be expected to be involved in the alloantibody response, but that could still initiate early rejection. When analysing the time to 1st BPRE the mean time in all patients presented with a repeat mismatch was considerably shorter regardless of the antibody status, in part this could be accounted for by the presence of direct memory T cells not indicative of antibody presence. There may also be a cumulative effect of both the number of repeat mismatches and the number of specificities to which DSA is generated. When graft survival in the DSA positive repeat mismatch positive group were compared dividing them into class I only, class II only and both class I and II, the single classes showed 100% graft survival over the study period and it was those patients presented with both class I and II that showed poor 5 year graft survival rates. This could be an effect of both more mismatches and antibody clones, in addition to the likelihood of direct CD4+ Th cell

memory to the class II mismatched antigens activating both the humoral arm and the direct cytotoxic T cells specific for the class I mismatches.

I had hoped that by combining results from the antibody tests and the T cell assay it would be possible to link both arms of the immune response, however using the assay methods described in this thesis it is clear that there is only a very limited relationship between antibody production and T cell memory. Whilst I theorise that the T cell assay developed could be detecting a memory response from the indirect Th17 cells, direct alloresponses could also be occurring accounting for some of the other cytokine production. In a wider group of patients it may be possible to link the production of other cytokines with both direct and indirect T cell activation.

The production of IL-17 on stimulation in vitro appears to correlate closely with TCMR post-transplant, if this cytokine is being produced by Th17 cells then it is unlikely that it would correlate with humoral immunity. Th2 type T cells are most closely associated with a humoral immune response and none of the cytokines tested for, that were typical of a Th2 response, appeared to correlate with humoral immunity in terms of recipient antibody production. IL-4 production by Th2 cells is closely linked to B cell proliferation and Ig class switching, it is therefore unfortunate that the IL-4 detection in our assay system appeared to fail, either due to kit failure or potentially due to a lack of Th2 cytokine producing cells in the isolated PBMCs. Since IFN γ is also not a classical Th2 cytokine it is possible that other reported assays of T cell memory, which also found no association with humoral immunity are being skewed by the cytokine profiles being selected. The cytokines produced by the different T cell subsets are believed to influence each other's action, it may only be truly possible to observe and link T cell memory with antibody status by isolating and assaying Th2 cells alone without the interference of other cytokine producing T cell groups.

Part of the rationale behind the initial hypothesis was that both T cell and antibody mediated rejection would be influential on outcomes of HLA antibody incompatible transplants.

However as the breakdown of aetiology of BPRES in the different patient groups showed, in the antibody positive (DSA or non-DSA) repeat mismatch positive groups the proportion of antibody mediated BPRES was high, and in the DSA positive group exceeded the TCMR quite considerably. One possible explanation could be that the Th2 memory T cells required for naïve B cell activation, along with the upregulation and honing of the antibody response, are not in fact in the graft but isolated in the secondary lymphoid organs interacting with both naïve and memory B cells in newly formed germinal centres and therefore not appearing on biopsy as a classical T cell mediated rejection episode, but still being highly influential in the antibody response and rejection episode.

The presence of donor specific Th2 cells have been linked to chronic rejection and since the current thinking is that the majority of chronic graft damage is due to the action of DSA this could be due to the link between Th2 and DSA production.

In terms of T cell memory it has been noted that CD4+memory T cells can have a Th2 bias following re-stimulation so increasing antibody responses [226], which could be highly influential on antibody production post-transplant. D'Elia et.al. used cytokine staining on renal allograft biopsies from patients with acute and borderline rejection to characterise the subsets of Th cells and noted that the majority of graft infiltrating Th cells are Th1, with Th2 cells being extremely rare [272].

With regards to the presence of DSA and repeat mismatches, overall it is difficult to separate the effect of antibody strength and the presence of repeat mismatches on either overall graft survival or BPRES post-transplant. The DSA positive repeat mismatch positive group had significantly higher DSA levels in terms of strength on testing and the need for antibody removal pre transplant. When observing the effect of the strength of antibody in terms of

FXM RMF or MFI on beads on graft failure due to rejection or occurrence of AMR, only the T cell RMF values pre-treatment and pre transplant showed some correlation, although not in all cases. Both B cell RMF and bead MFI levels showed only very limited association, certainly not enough to rule a transplant as being at too high risk of failure to be attempted. This suggests that the strength of antibody prior to transplant may not be a useful sole prognostic indicator for post-transplant outcomes. However, when graft survival was analysed on the basis of the requirement for antibody removal pre transplant it was found that those requiring antibody removal did have decreased graft survival in comparison to those who did not, indicating that having any strength of positive FXM pre-treatment was an automatic risk factor in terms of failure. However, the group of DSA positive patients requiring antibody removal included a far higher proportion of patients who were also presented with a repeat mismatch. When all groups were combined based on the presence of a repeat mismatch, those that were presented with a repeat mismatch had worse overall survivals, however once again the proportion of patients with additional DSA was greater in the repeat mismatch group. Therefore potentially a combination of repeat mismatch with DSA provides the worst outcome and should be treated as those at the greatest risk of graft failure due to rejection, with additional caution given to those where there is a combination of both HLA Class I and II mismatches. In terms of the speed of a rejection event following transplant all patients presented with a repeat mismatch had the fastest time to 1st BPPE, regardless of the antibody status, with the exception of one patient in the antibody negative repeat mismatch positive group, the fastest time to BPPE was seen in the DSA positive repeat mismatch positive group. Neither the presence of DSA nor the requirement for antibody removal influenced the time to first BPPE, so it can therefore be concluded that the main factor, under assessment, influencing the speed of a rejection episode is the presence of a repeat mismatch, possibly indicating the involvement of memory T cells from the direct allorecognition pathway.

It is clear from both the antibody related and the T cell assay results that immune memory to HLA antigens is not as simple as the original hypothesis suggests and that potentially the T cell memory response and the antibody response do not appear to be as closely linked as we first hypothesised, with responses in the T cell assay system not correlating with the production of HLA specific antibody or the presence of repeat mismatches. Additionally, whilst the donor specific antibody positive with repeat mismatch group had the worst post-transplant outcomes, this could not categorically be separated from the amount of antibody present pre-transplant. Since the requirement for T cell help for memory B cell responses on stimulation is an unknown quantity and may in fact not be required [38], in addition to the fact that the antibody secreting plasma cells are long lived and continuously producing antibody at some level this is perhaps not a surprising conclusion.

Longer term follow up of these patients may provide some more conclusive results as to the effect of repeat mismatches on graft survival.

The time to rejection is shorter in those patients showing sensitisation to HLA through antibody production and being presented with a repeat mismatch, indicating that at some level immune memory in either arm of the adaptive response can be predicted through the presence of repeat mismatches.

I have developed a process and accompanying repertoire of tests to be employed to assess the ability to remove donor specific antibody to allow antibody incompatible transplantation to proceed, some of which detected can provide a limited ability to predict post-transplant outcome and identify patients at risk of early rejection episodes and graft failure.

I have also developed an assay which appears, in the small numbers presented, to detect the presence of donor specific memory T cells pre-transplant capable of eliciting TCMR post-transplant with 100% accuracy, interestingly in patients who may not necessarily have been thought to possess them. Further development of this assay and assessment in larger patient

cohorts, potentially in all living donor renal transplants, will be an important continuation of this project.

Investigation into the isolation of Th2 cells and a separate assay system to detect Th2 memory cells capable of driving the antibody response may allow closer linking of T cell and humoral memory. With expansion of the starting hypothesis to suggest that the presence of Th2 memory T cells, potentially primed by repeat mismatches, may be more indicative of poorer post-transplant outcomes in patients receiving HLA antibody incompatible transplants.

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7 Appendix 1.

The following tables present the raw MFI values achieved with the beads for each test cytokine, in each testing combination at each time point sampled for the two cells from healthy volunteers tested in the initial experiment 1, described in T cell Chapter 4.

Experiment 1 – Volunteer Test Cell 1 – Raw Cytokine MFI Results.

Test Day	Sample	IFN-g	IL-17	IL-5	IL-8	TNF-a
16 Hours	Neg Control	7.5	5	1	9317	3637.5
	CD3/28 0.2	129	153	29	7336.5	5004.5
	CD3/28 0.1	12	14	3	7793	4033
	CD3/28 .05	13.5	26.5	4	8350.5	3194
	CI neat	7	8	1	7726.5	8454
	CI 1:10	6	5	1	7291	2956
	CI 1:100	7	6	1	7846	3323
	CI neat + CD28	6	6	1	6395	4076
	CI 1:10 + CD28	6	7	1	7162	4004
	CI 1:100 + CD28	7	6	1	6736	5032
	CII neat	6.5	9	5	7740	9965
	CII 1:10	6	6	1	6557	2981.5
	CII 1:100	5.5	5	1	7334	2504
	CII neat + CD28	7.5	7	1	6626	6628.5
	CII 1:10 + CD28	7	6	0	6852.5	3538
	CII 1:100 + CD28	5	5	1	8090	3120
	CD28 neat	8	6	1	8603	5822.5
	CD28 1:2	6	5	1	8421.5	3193.5
	CD28+ CI + CII	10	8	1	7639	10754.5
	CD28 1:100 + CI+CII	13	8	1	7533.5	12410
24 Hours	Neg Control	9	6	1	8414	3160.5
	CD3/28 0.2	528	184.5	73	7902	5575
	CD3/28 0.1	19	27.5	4	7933.5	4279
	CD3/28 .05	29	33.5	9	8960	3233
	CI neat	10	7	1	8205	9428
	CI 1:10	6	5.5	1	7200	3384.5
	CI 1:100	7	5	1	7790	3299
	CI neat + CD28	6.5	6	1	6096	4306
	CI 1:10 + CD28	8	7	1	7745	4516
	CI 1:100 + CD28	7.5	6	1	6724	6096
	CII neat	8	7	4	7720	9784
	CII 1:10	7	5	1	8442	3000
	CII 1:100	6	6	1	9972.5	2834.5
	CII neat + CD28	8	7	1	6347	6557.5
	CII 1:10 + CD28	7	5.5	1	6732.5	3757
	CII 1:100 + CD28	6	5	1	6611	3355
	CD28 neat	6.5	5	1	7492	5587
	CD28 1:2	6	6	1	6618	4527.5
	CD28+ CI + CII	11.5	8	1	6011	10691
	CD28 1:100 + CI+CII	14	8	1	6496	10879
NO II	Neg Control	7	6	1	8690	2633.5

Test Day	Sample	IFN-g	IL-17	IL-5	IL-8	TNF-a
	CD3/28 0.2	6183	495	1133	6352.5	5788
	CD3/28 0.1	363.5	216.5	39	7450	3281.5
	CD3/28 .05	166.5	164.5	72	7558	2619
	CI neat	8	19	1	4064	6992
	CI 1:10	6.5	5	1	6420.5	2764.5
	CI 1:100	6	5	0	7914	2994
	CI neat + CD28	6	7.5	1	6263.5	3323
	CI 1:10 + CD28	6	6	1	8702.5	3797
	CI 1:100 + CD28	6	6	1	6401	5007
	CII neat	6	7	4	7049.5	7206.5
	CII 1:10	5	5	1	7033	2387
	CII 1:100	6	6	1	8646	2243.5
	CII neat + CD28	6	6	1	5059	5384
	CII 1:10 + CD28	5.5	5	0	6897	3142
	CII 1:100 + CD28	6	5	1	7645	2901
	CD28 neat	5	4	0	927	735
	CD28 1:2	5	5	0.5	1256.5	814.5
	CD28+ CI + CII	6	6	0	806.5	1581
	CD28 1:100 + CI+CII	11	7	1	6231.5	9569
72 Hours	Neg Control	6	6	1	8597	2197
	CD3/28 0.2	8798	921.5	1729.5	6906	4600
	CD3/28 0.1	442	299	46	8088	2179
	CD3/28 .05	165	301.5	110	7796	2297
	CI neat	6	37	1	2280.5	1789
	CI 1:10	6	7	1	7843	1854
	CI 1:100	6	5	1	2230.5	2046.5
	CI neat + CD28	6	8	1	7692	2742
	CI 1:10 + CD28	5	5	1	8009	2820
	CI 1:100 + CD28	7	6	0	4522	3768
	CII neat	8	14	4	7001	6267
	CII 1:10	6.5	6	1	7293	2275.5
	CII 1:100	9	9	1	8271	1855
	CII neat + CD28	7	14	1	8182.5	4985
	CII 1:10 + CD28	5	4.5	1	379.5	607
	CII 1:100 + CD28	6	5	1	7716	1809
	CD28 neat	6.5	11	1	7629.5	3977
	CD28 1:2	5	4	0	1219.5	551.5
	CD28+ CI + CII	7	6	0	827	1155
	CD28 1:100 + CI+CII	7.5	5	1	1479	1781

Experiment 1 – Volunteer Test Cell 2 – Raw Cytokine MFI Results.

Test Day	Sample	IFN-g	IL-17	IL-5	IL-8	TNF-a
Day 3	no CD28 Neg	7	7	2	22	7
	no CD28 Neg	6	6	1	18	7
	Pos 1	6	9	1	15937	1147.5
	Pos 2	6	11	3	14771	337
	no CD28 CI	6	7	1	15327	482.5
	No CD28 CI	5	13	1	14623	903
	No CD28 CII	6	6	1	14300.5	137
	No CD28 CII	6	8	1	15297.5	755
	No CD28 CI+II	6	8	1	15219	148.5
	No CD28 CI+II	6	7	1	16746	798.5
	Sol CD28 Neg	6	7	1.5	16	7
	Sol CD28 Neg	6	7	1	20.5	8
	Sol CD28 CI	6	7	1	13869	540.5
	Sol CD28 CI	9	11	1	14975	5573.5
	Sol CD28 CII	6	10	0	15608.5	221.5
	Sol CD28 CII	6	8	1	15719	278
	Sol CD28 CI+II	6	7	1	15282	514
	Sol CD28 CI+II	6	11	3.5	14765	274
	PB CD28 Neg	7	7	1	17	8
	PB CD28 Neg	6	7	1	18.5	7
	PB CD28 CI	7	18	1	13941	4655
	PB CD28 CI	7	8	1	14429	2357
	PB CD28 CII	7	11	6	15610	4740
	PB CD28 CII	5	8.5	4	14040	3066
	PB CD28 CI+II	7	7	2	15141	4429.5
	PB CD28 CI+II	7	11	11	13360	4738.5
Day 5	no CD28 Neg	7	7	1	23	8.5
	no CD28 Neg	6	7	1	25	8
	Pos 1	821	1451.5	14370	15367.5	3703.5
	Pos 2	930.5	903.5	8395	16756.5	3189
	no CD28 CI	5	7	1	14475.5	430
	No CD28 CI	5	12	1	15385	749.5
	No CD28 CII	6	9	1	16146	880.5
	No CD28 CII	6	7	1	15972	621.5
	No CD28 CI+II	6	7	1	15630.5	568.5
	No CD28 CI+II	6	9.5	4	14183	264.5
	Sol CD28 Neg	6	7	1	20	8
	Sol CD28 Neg	6	6	1	16	9
	Sol CD28 CI	5.5	7	1	13198	459.5
	Sol CD28 CI	7	22	1	14001	4761
	Sol CD28 CII	7	17	1	15163	1665
	Sol CD28 CII	6	7	1	15481	233.5
	Sol CD28 CI+II	7	7	1	17208	323
	Sol CD28 CI+II	5	8	2	13217	1302
	PB CD28 Neg	6	7	1	20.5	8
	PB CD28 Neg	6	7	1	21	7
	PB CD28 CI	7	16	1	14857.5	3158
	PB CD28 CI	6	11	2	5647	2449.5
	PB CD28 CII	8	14	8	14572	4266.5
	PB CD28 CII	8	14	7.5	14183	3688

Test Day	Sample	IFN-g	IL-17	IL-5	IL-8	TNF-a
	PB CD28 CI+II	6	8	1	15100	1720.5
	PB CD28 CI+II	6	23	1	14038	1644
Day 7	no CD28 Neg	6	6	0	12	8
	no CD28 Neg	6	7	1	11	7
	Pos 1	571	1045	10611.5	16688	2803
	Pos 2	612.5	1229	6735	16231	2528
	no CD28 CI	6	11	1	15724.5	655
	No CD28 CI	6	7	1	15349	239.5
	No CD28 CII	6	8	1	16055.5	410
	No CD28 CII	6	10	1	14768.5	498
	No CD28 CI+II	6	11	3	16899	460
	No CD28 CI+II	6	9	2	15423	239
	Sol CD28 Neg	5	6	1	18	7.5
	Sol CD28 Neg	7	7	1	18	8
	Sol CD28 CI	7	14	2	14496	2537.5
	Sol CD28 CI	5	10	1	14860	1058.5
	Sol CD28 CII	6	8	1	15159.5	212.5
	Sol CD28 CII	7	26	1	13727.5	1273
	Sol CD28 CI+II	5.5	7	2	9563	995.5
	Sol CD28 CI+II	6	6.5	1	11.5	8.5
	PB CD28 Neg	7	7	0	9	7
	PB CD28 Neg	6	6	1	11.5	8
	PB CD28 CI	6	34	1.5	14476.5	3173.5
	PB CD28 CI	7	10	1	13971	1729
	PB CD28 CII	7	16.5	13	15179.5	3121.5
	PB CD28 CII	6	19	8	15172	2920.5
	PB CD28 CI+II	6	8	2	16042	1424
	PB CD28 CI+II	5	60	1	16381	1291

8 Appendix 2

The following tables present the raw MFI values achieved with the beads for each test cytokine, in each testing combination at each time point sampled for the two cells from patients tested in the experiment 2, described in T cell Chapter 4.

Experiment 2 – Test Patients Cells 1 – Raw Cytokine MFI Data.

Test Day	Sample	IFN-g	IL-17	IL-2	IL-5	IL-8	TNF-a
24 hours	Neg Medium Only, No CD28	6.5	5.0	5.0	1.0	23.0	8.8
	Neg Medium Only, with CD28	6.0	5.5	5.5	1.0	10.5	9.5
	Positive Control, no CD28	1566.3	909.5	2871.5	103.3	11259.5	3162.5
	Positive Control, with CD28	3019.5	1097.8	3146.5	78.3	10576.0	3871.0
	Negative, cells only, no CD28	6.0	5.0	4.5	1.0	9802.5	125.0
	Negative, Cells only, with CD28	6.5	5.5	4.8	0.5	10253.0	141.5
	CI mixed beads 5 ul, no CD28	7.0	5.0	5.0	1.0	10622.3	2904.8
	CI mixed beads 5ul, with CD28	7.0	5.5	5.5	1.0	11072.3	2908.5
	CII mixed beads 5ul, no CD28	6.8	5.0	5.0	1.0	11048.5	728.8
	CII mixed beads 5ul, with CD28	5.8	5.0	4.0	1.0	9936.5	611.3
	CI+II mixed beads 5ul, no CD28	6.0	6.3	5.5	1.0	9866.8	3368.3
	CI+II mixed beads 5ul, with CD28	7.0	5.0	4.5	1.0	10720.5	2108.3
	DR11 2ul, no CD28	7.0	4.8	6.0	0.8	11325.3	257.5
	DR11 2ul, with CD28	6.0	5.0	4.5	1.0	10743.0	233.8
	DR52 2ul, no CD28	6.0	5.5	5.0	1.0	10969.8	286.0
	DR52 2ul, with CD28	6.0	4.5	4.8	0.5	11130.8	371.5
	DR7 2ul, no CD28	5.3	4.5	5.0	1.0	10657.0	343.8
	DR7 2ul, with CD28	6.0	4.0	5.5	1.0	10624.5	218.3
	DR11 10ul, no CD28	6.0	5.0	5.0	1.0	10119.0	128.5
	DR11 10ul, with CD28	6.0	5.0	5.0	1.0	11267.0	212.5
	DR52 10ul, no CD28	6.0	5.5	5.0	1.0	11058.3	427.5
	DR52 10ul, with CD28	6.5	5.0	4.8	1.0	9811.0	466.8
	DR7 10ul, no CD28	6.3	4.5	4.5	1.0	10763.5	431.3
	DR7 10ul, with CD28	6.5	4.5	4.5	1.0	10367.0	554.0
	DR11 + 52, no CD28	6.0	4.0	6.0	0.0	10305.	451.0

Test Day	Sample	IFN-g	IL-17	IL-2	IL-5	IL-8	TNF-a
						0	
	DR11 + 52, with CD28	6.0	4.0	7.0	1.0	9826.5	361.5
	DR7 peptide, no CD28	6.0	5.0	5.0	1.0	9513.0	121.0
	DR7 peptide, with CD28	6.0	5.0	5.0	1.0	9833.5	189.0
	DR17 peptide, no CD28	6.0	5.0	5.0	0.0	10062.0	199.0
	DR17 peptide, with CD28	5.5	4.5	5.0	0.5	10224.	190.0
72 hours	Neg Medium Only, No CD28	6.5	4.3	4.5	1.0	10.0	10.0
	Neg Medium Only, with CD28	6.0	5.0	4.5	1.0	10.0	10.0
	Positive Control, no CD28	2013.0	2312.3	1420.5	206.8	10430.0	3437.0
	Positive Control, with CD28	3745.8	2786.0	1654.0	161.5	10602.3	4173.8
	Negative, cells only, no CD28	6.5	4.8	4.8	1.0	10053.5	97.8
	Negative, Cells only, with CD28	6.5	4.5	5.5	1.0	10100.5	98.8
	CI mixed beads 5 ul, no CD28	6.0	6.5	6.0	1.0	10806.3	2299.0
	CI mixed beads 5ul, with CD28	6.5	5.5	5.3	0.5	10692.0	2079.3
	CII mixed beads 5ul, no CD28	6.0	4.5	5.0	1.0	10648.0	442.0
	CII mixed beads 5ul, with CD28	6.5	5.5	4.5	1.0	9879.0	402.3
	CI+II mixed beads 5ul, no CD28	6.0	5.5	5.5	1.0	10297.0	2178.5
	CI+II mixed beads 5ul, with CD28	6.0	6.0	4.5	1.0	10054.8	1381.5
	DR11 2ul, no CD28	6.0	4.0	5.5	0.5	11044.0	156.8
	DR11 2ul, with CD28	6.5	5.3	4.5	1.0	10817.8	251.0
	DR52 2ul, no CD28	5.5	4.0	5.0	1.0	10477.0	181.3
	DR52 2ul, with CD28	6.5	5.0	5.0	0.5	10688.5	278.8
	DR7 2ul, no CD28	6.0	5.5	5.3	1.0	10141.3	300.8
	DR7 2ul, with CD28	5.5	5.0	4.0	0.5	10474.5	182.8
	DR11 10ul, no CD28	5.0	4.0	1.0	1.0	1836.5	25.5
	DR11 10ul, with CD28	6.0	5.0	4.0	1.0	9803.0	159.5
	DR52 10ul, no CD28	6.0	4.0	5.0	1.0	10007.5	297.5
	DR52 10ul, with CD28	6.0	5.5	5.0	1.0	9699.5	342.5
	DR7 10ul, no CD28	5.5	6.0	5.5	1.0	10500.0	266.3
	DR7 10ul, with CD28	5.8	4.5	5.0	0.8	9945.3	320.0
	DR11 + 52, no CD28	5.5	9.5	6.0	1.0	10339.0	372.0
	DR11 + 52, with CD28	5.0	5.0	7.0	1.0	10491.0	270.5
	DR7 peptide, no CD28	6.0	5.0	4.0	1.0	10201.5	86.0
	DR7 peptide, with CD28	5.8	4.8	4.0	1.0	10645.5	139.5

Test Day	Sample	IFN-g	IL-17	IL-2	IL-5	IL-8	TNF-a
7 days	DR17 peptide, no CD28	6.0	4.0	5.0	1.0	9907.0	160.0
	DR17 peptide, with CD28	6.3	4.5	6.0	1.0	9162.3	145.8
	Neg Medium Only, No CD28	5.0	4.5	4.0	0.5	11.5	8.8
	Neg Medium Only, with CD28	5.0	4.5	4.5	1.0	69.0	9.3
	Positive Control, no CD28	1360.0	2269.5	238.0	408.5	10355.5	2658.0
	Positive Control, with CD28	1729.5	2079.3	131.0	214.8	6588.8	1966.0
	Negative, cells only, no CD28	5.5	4.5	5.0	1.0	9593.3	67.5
	Negative, Cells only, with CD28	5.5	4.5	4.5	1.0	10320.3	66.0
	CI mixed beads 5 ul, no CD28	6.3	7.0	5.0	1.0	9457.5	1521.5
	CI mixed beads 5ul, with CD28	6.0	6.5	5.5	1.0	9837.0	1353.5
	CII mixed beads 5ul, no CD28	6.5	5.0	4.5	1.0	10424.5	290.3
	CII mixed beads 5ul, with CD28	6.0	5.0	4.5	1.0	9933.3	279.5
	CI+II mixed beads 5ul, no CD28	6.0	6.0	5.0	1.5	10987.8	1693.3
	CI+II mixed beads 5ul, with CD28	5.5	6.0	5.0	1.0	9735.8	1041.5
	DR11 2ul, no CD28	5.3	4.0	5.0	1.0	10412.8	113.0
	DR11 2ul, with CD28	5.5	4.5	5.0	1.0	10646.0	128.8
	DR52 2ul, no CD28	5.0	4.0	4.5	1.0	10895.0	122.8
	DR52 2ul, with CD28	6.0	4.0	5.0	1.0	10165.8	153.0
	DR7 2ul, no CD28	6.0	4.5	4.5	1.0	10009.0	174.5
	DR7 2ul, with CD28	5.3	4.0	2.5	0.5	5945.5	82.3
	DR11 10ul, no CD28	5.0	4.0	5.0	1.0	10559.5	74.5
	DR11 10ul, with CD28	5.0	4.0	5.0	1.0	10209.5	103.0
	DR52 10ul, no CD28	5.8	4.0	5.3	1.0	10335.3	195.3
	DR52 10ul, with CD28	6.0	4.8	5.5	1.0	10131.8	199.8
	DR7 10ul, no CD28	5.8	6.0	5.0	1.0	10636.5	231.5
	DR7 10ul, with CD28	5.5	4.5	4.0	1.0	10151.3	219.5
	DR11 + 52, no CD28	5.5	8.0	4.5	0.5	10513.0	221.0
	DR11 + 52, with CD28	6.0	4.0	5.0	1.0	10554.5	164.5
	DR7 peptide, no CD28	6.0	4.0	4.0	1.0	10415.0	72.0
	DR7 peptide, with CD28	6.0	4.0	5.0	1.0	9836.0	78.0
	DR17 peptide, no CD28	6.0	4.0	6.0	1.0	10214.0	117.5
	DR17 peptide, with CD28	5.5	5.0	4.5	1.0	10436.0	110.0

9 Appendix 3 – Experiment 3

The following tables list the raw MFI values achieved for each test cytokine bead group, at each time point and in each test combination, for the three patient/stimulator pairs tested in T cell experiment 3 outlined in Chapter 4.

Test Pair 1 – Raw Cytokine MFI Results

Class I

		IFN-g	IL-17	IL-2	IL-5	IL-8	TNF-a
24 Hours	Neg Control - Medium Only	7	3	3	1	11	8.5
	Neg Control - Cells Only	166.5	200	161	14	11400	2438.5
	Positive Control	6149	276	5060	525	10190	11816.5
	CI	7	6.5	48	1	12627	256.5
	CI + CD28	8	5	35	1	12576	440
72 Hours	Neg Control - Medium Only	7	4	2	0	783	8
	Neg Control - Cells Only	146	120.5	11	14	9572	2323
	Positive Control	8529	410	206	2049	6841	10370
	CI	7	20	28	1	12070	258.5
	CI + CD28	7	8	41.5	1	12215	361
120 Hours	Neg Control - Medium Only	7	3	4	1	20	8
	Neg Control - Cells Only	102.5	44	4	12	10847	1309.5
	Positive Control	9434	315.5	24.5	2467	7808	8556.5
	CI	6	9	11	1	12095	84
	CI + CD28	7	4.5	10	1	11029	109.5

Class II

		IFN-g	IL-17	IL-2	IL-5	IL-8	TNF-a
24 Hours	Neg Control - Medium Only	6	3	4	1	12	8
	Neg Control - Cells Only	7	8	10	1	12495	1019.5
	Positive Control	2697	245	7402	1042	11882	11725
	CII	8	4	17	1	11932	149.5
	CII + CD28	7	5	14	1	12200	187
72 Hours	Neg Control - Medium Only	6	3	4	1	18	9
	Neg Control - Cells Only	6	13	15	1	12995	1147
	Positive Control	3977	344.5	23.5	5674.5	6795	12664.5
	CII	7	6	17	1	13507.5	204
	CII + CD28	6	6	24	1	12534	257
120 Hours	Neg Control - Medium Only	6	3	4	1	8	7.5
	Neg Control - Cells Only	6	3	4	1	11	8
	Positive Control	9	3	9	2	138	9.5
	CII	8	5	9	1	12184	70
	CII + CD28	6	5	8	1	12674	64

Beads

		IFN-g	IL-17	IL-2	IL-5	IL-8	TNF-a
24 Hours	Neg Control - Medium Only	6	3	3	1	12	8
	Neg Control - Cells Only	7	6	13	1	12108	119
	Positive Control	2269	199	6656.5	373.5	10984.5	6489

72 Hours	Beads	6	4	4	1	957	9
	Beads + CD28	7	3	3	1	986	10
	Neg Control - Medium Only	6	3	3	1	29	8
	Neg Control - Cells Only	6.5	8	27	2	3532	501
	Positive Control	3687.5	427.5	3883	1978.5	7514.5	12744.5
	Beads	6	1	1	1	2	5
120 Hours	Beads + CD28	6	2	1	0	1	7
		IFN-g	IL-17	IL-2	IL-5	IL-8	TNF-a
	Neg Control - Medium Only	x	x	x	x	x	x
	Neg Control - Cells Only	x	x	x	x	x	x
	Positive Control	x	x	x	x	x	x
	Beads	x	x	x	x	x	x
	Beads + CD28	x	x	x	x	x	x

Test Pair 2 – Raw Cytokine MFI Results.

Class I

		IFN-g	IL-17	IL-2	IL-5	IL-8	TNF-a
24 Hours	Neg Control - Medium Only	7	4	3	1	11	8
	Neg Control - Cells Only	8	88.5	27	2	11568	743
	Positive Control	5118	969	3759	2285	10935	11631
	CI	8	25	12	1	11490	2096
	CI + CD28	6	49	19	1	10613	4903.5
72 Hours	Neg Control - Medium Only	6	4	3	1	13	7.5
	Neg Control - Cells Only	9	420	22	3	11617.5	568
	Positive Control	8796.5	1444.5	316	5519.5	8536.5	11302
	CI	6	170.5	89	1	11195	1179
	CI + CD28	x	x	x	x	x	x
120 Hours	Neg Control - Medium Only	x	x	x	x	x	x
	Neg Control - Cells Only	x	x	x	x	x	x
	Positive Control	x	x	x	x	x	x
	CI	x	x	x	x	x	x
	CI + CD28	x	x	x	x	x	x

Class II

		IFN-g	IL-17	IL-2	IL-5	IL-8	TNF-a
24 Hours	Neg Control - Medium Only	6.5	3	4	1	12	8
	Neg Control - Cells Only	7	19	18	1	11024	4195.5
	Positive Control	3885	1474.5	3666	2529	10260	13339
	CII	7.5	18	14	1	12122.5	2564.5
	CII + CD28	27	114	29	2	10236	7255
72 Hours	Neg Control - Medium Only	6	170.5	89	1	11195	1179
	Neg Control - Cells Only	6	3	4	1	11	8
	Positive Control	5008	1270	11	4707	8257.5	10653.5
	CII	6	195	53	1	10256	1773
	CII + CD28	57	883	34	2	8916.5	5104
120 Hours	Neg Control - Medium Only	x	x	x	x	x	x
	Neg Control - Cells Only	x	x	x	x	x	x
	Positive Control	x	x	x	x	x	x
	CII	x	x	x	x	x	x
	CII + CD28	x	x	x	x	x	x

Beads

		IFN-g	IL-17	IL-2	IL-5	IL-8	TNF-a
24 Hours	Neg Control - Medium Only	6	4	3	1	13	8
	Neg Control - Cells Only	7	5	9	1	10792	208.5
		IFN-g	IL-17	IL-2	IL-5	IL-8	TNF-a
	Positive Control	1180	1213.5	9300	2452	10881	9692
	Beads	6.5	5	12	2	11363	189.5
	Beads + CD28	8	25	26	1	11240	694
72 Hours	Neg Control - Medium Only	6	4	4	1	27	7
	Neg Control - Cells Only	4	3	4	1	334	65
	Positive Control	2918	2361	46	6698	4532.5	6854
	Beads	3	5	21	1	1836	786
	Beads + CD28	7	154	65	2	2670.5	4707
120 Hours	Neg Control - Medium Only	x	x	x	x	x	x
	Neg Control - Cells Only	x	x	x	x	x	x
	Positive Control	x	x	x	x	x	x
	Beads	x	x	x	x	x	x
	Beads + CD28	x	x	x	x	x	x

Test Pair 3 – Raw Cytokine MFI Results.

Class I

		IFN-g	IL-17	IL-2	IL-5	IL-8	TNF-a
24 Hours	Neg Control - Medium Only	6	3	4	1	10	8
	Neg Control - Cells Only	8	28	17	1	12167	1986.5
	Positive Control	343.5	174	1614	59.5	11124	5953
	CI	7	4	5	1	12987	1674
	CI + CD28	9	19	33	1	12123	6200.5
72 Hours	Neg Control - Medium Only	6	4	4	1	11	8
	Neg Control - Cells Only	7	78	12	1	11399	1085
	Positive Control	2307	488	269.5	997	9634	5639
	CI	6	4	6	1	11917	821
	CI + CD28	7	25	14	1	10808	3619.5
120 Hours	Neg Control - Medium Only	6	3	4	1	10	8
	Neg Control - Cells Only	7	93	27.5	1	12854	797.5
	Positive Control	2564.5	420	48	1133	10125	4184
	CI	6	3	5	1	12237.5	630
	CI + CD28	6	19.5	14	1	11577	2786

Class II

		IFN-g	IL-17	IL-2	IL-5	IL-8	TNF-a
24 Hours	Neg Control - Medium Only	6	3	3	1	11	8
	Neg Control - Cells Only	8	7	7	1	11756	1973.5
	Positive Control	126	111	1591.5	68.5	11841	6524
	CII	6	5	4	1	12472	829.5
	CII + CD28	8	16	21	2	12065	3862
72 Hours	Neg Control - Medium Only	6.5	3	4	1	11	8
	Neg Control - Cells Only	7	42	23	1	11949.5	1506
	Positive Control	607	167	11.5	806	9625.5	4875.5
	CII	7	4	22	1	12705.5	442
	CII + CD28	6	15	7	2	11062	2398
120 Hours	Neg Control - Medium Only	6	3	3	1	9	8
	Neg Control - Cells Only	6	23.5	36	1	12351	1189
	Positive Control	553	99	7	631	9927	3160
	CII	6	4	16	1	12706	328.5
	CII + CD28	6	13	8	3	11654	1800

Beads

		IFN-g	IL-17	IL-2	IL-5	IL-8	TNF-a
24 Hours 72 Hours	Neg Control - Medium Only	6	3.5	4	1	14	8
	Neg Control - Cells Only	5.5	3	4	1	11257	431
	Positive Control	10	41	809.5	13	12594	1359
	Beads	7	3	3	1	12288	333.5
	Beads + CD28	7	7	11	1	12322.5	822
	Neg Control - Medium Only	6	4	4	1	20	8
72 Hours 120 Hours	Neg Control - Cells Only	8	5	9	1	11751.5	1252.5
	Positive Control	70.5	401	97	360	11234.5	6433.5
	Beads	6	5	5	1	11030	5394
	Beads + CD28	5	4	6	2	582	763.5
	Neg Control - Medium Only	x	x	x	x	x	x
120 Hours	Neg Control - Cells Only	x	x	x	x	x	x
	Positive Control	x	x	x	x	x	x
	Beads	x	x	x	x	x	x
	Beads + CD28	x	x	x	x	x	x

10 Appendix 4 – Experiment 4 Information

Patient Sensitisation and Donor/3rd Party Mismatch information – the table below summarises the patient sensitisation information and the HLA mismatches presented by the donor or 3rd party stimulator cells. ‘Patient’ represents the patient number assigned during testing. ‘Gender’ indicates the sex of the patient’ ‘Sensitised’ indicates the patient has received a previous transplant and/or produces demonstrable HLA specific antibody detected using LABScreen mixed bead testing. ‘Donor MM’ and ‘3rd Party MM’ lists the HLA class I and II mismatches presented by the donor or 3rd party stimulator cells. ‘HLA Ab’ lists any HLA specific antibody identified on serum screening which is specific for one or more mismatches presented by either the donor or 3rd party stimulator.

Patient	Gender	Sensitised	Donor MM	HLA Ab	3rd party MM	HLA Ab
1	F	No	A2,68 B60 Cw10 DR11 DR53 DQ7	No	A2, B61, Cw2, DR4, DQ8	no
2	M	No	B35 Cw4 DR15 DR51 DQ5	No	A68, B60, Cw10, DR7, DR11, DQ7	no
3	M	No	A1 B35 Cw4 DR1 DQ5	No	A33, B44, B65, Cw5, Cw8, DR1, DQ5	no
4	M	No	A1,24 B44 Cw5 DR13 DQ6	No	A1, A24, B8, B44, Cw5, Cw7, DR13, DQ6	no
5	F	No	A3 B52 Cw1 DR1 DQ5	No	A2, B35, Cw4, DR15, DR51, DQ6	no
6	M	No	A1 A24 B44 Cw5 DR13	No	A3, A32, B7, B35, Cw4, DR4, DR53, DQ7, DQ8	no
7	F	Yes	A24, 31 B7, 62 Cw9 DR12,15 DR51, DQ6	Yes - Ab = A31	A3, B60, Cw10, DR15, DR51, DQ6	no

Patient	Gender	Sensitised	Donor MM	HLA Ab	3rd party MM	HLA Ab
8	F	Yes	A66, B41, Cw17, DR13, DR52, DQ7	Yes - Ab = A66	A24, A31, B7, B62, Cw9, DR12, DR52, DQ7	no
9	F	Yes	A3, A32 B7 Cw7 DR4 DR53 DQ7,8	Yes - Ab = A3, cw7, DR4, DQ7,8	A3, B57, Cw4, DR4, DR7, DR53, DQ8, DQ9	Yes - Ab = A3, DR4, DR7, DR53, DQ8, DQ9
10	F	Yes	Cw2,7 DR4,15 DR51,53	Yes - Ab = Cw2,7 DR4,15 DR51,53	A1, A32, B35, B55, Cw4, DR11, DR14, DQ5, DQ7	Yes - Ab = A21, A32, Cw4, DR11, DQ5
11	F	Yes	A2, B37, Cw6	Yes - Ab = A2, B37	A26, B37, Cw6, DR13, DR15, DR51, DQ6	Yes - B37
12	M	Yes	DQ7	Yes - Ab = DQ7	A2, A11, B44, Bw4, Cw5, DR4, DR11, DR53, DQ7, DQ8	Yes - Ab = A2, B44, DQ7, DQ8
13	F	Yes	A3, 29 B45 Cw6 DR17 DQ2	Yes - Ab = A3, A29, B45	A3, B57, B65, Cw6, Cw8, DR1, DR13	Yes - Ab = A3, B57, B65
14	F	Yes	A33, 68 B71 Cw10 DR10, 13 DR52 DQ2	Yes - Ab = Cw10	A1, A3, B35, B39, Cw7, DR1, DR11, DR52	Yes - Ab = Cw7
15	F	Yes	A2 B49,51 Cw16 DR9,13 DQ5	Yes - Ab = A2, B49, B51	A3, A29, B8, B45, Cw6, DR17	Yes - Ab = A3, DR17
16	F	Yes	A1,3 B7 Cw7 DR8 DQ4	No	A1, A24, B8, B44, Bw4, Cw5, Cw7, DR13, DR17, DQ2	no
17	F	Yes	A1,3 B57,65 Cw6,8 DR13 DQ6	Yes - Ab = A1, A3, B57	A2, A33, B49, B51, Cw7, Cw16, DR9, DR13, DR53, DQ2	no
18	F	Yes	A2 B60 Cw10	Yes - Ab = A2	A2, B41, B44, Bw4, Cw5, Cw17, DR4, DR13, DR53	Yes - Ab = A2, B41, B44
19	F	Yes	B27,44 Bw4 Cw2,5 DQ7	Yes - Ab = DQ2	A23, A80, B52, B57, Bw4, Cw16, Cw18, DR7	Yes - Ab = A80, DR7
20	F	Yes	B57 Cw6	Yes - Ab = B57	A11, B55, Cw9, DR10, DR14, DQ5	no
21	F	Yes	No mm	No	A2, B18, DR7, DR53	Yes - Ab = A2
22	M	Yes	A23,80 B57 Cw16,18	Yes - Ab = A23, A80, B57, Cw18	A3, B7, B57, Cw6, Cw7	Yes - Ab = B57, Cw6
23	M	Yes	A1,3 B35 Bw6 Cw4 DR4 DR53 DQ8	Yes - Ab = B35	A2, B44, Cw2, Cw5, DR4, DR53, DQ8	Yes - Ab = A2, Cw5

Patient	Gender	Sensitised	Donor MM	HLA Ab	3rd party MM	HLA Ab
24	M	Yes	A2 B62 Cw9 DR13 DR52	Yes - Ab = A2	A2, B18, B62, Cw9, DR4, DR11, DR52, DR53, DQ7, DQ8	Yes - Ab = A2
25	F	Yes	B18 Cw7 DR11 DR52 DQ7	Yes - Ab = DR11	A1, A32, B8, B44, Cw5, Cw7, DR12, DR17, DR52, DQ2, DQ7	Yes - Ab = B8, DR17, DQ2
26	M	Yes	A1 B35 Cw4 DR11 DR52 DQ7	Yes - Ab = A1, B35, Cw4, DR11, DR52, DQ7	A1, B7, Cw4, Cw12, DR14, DR15, DR51, DR52, DQ6	Yes - Ab = A1, Cw4, Cw12, DR14, DR15, DR51
27	F	Yes	A2 B44 Cw5 DR4 DR53	Yes - Ab = B44, Cw5, DR4, DR53	A23, A66, B49, DR15, DR51, DQ6	Yes - Ab = A23, A66, B49
28	F	Yes	A1 A26 B37 DR13	No	A3, B35, B62, Cw4, Cw9, DR1, DR13, DQ2	no
29	F	Yes	A11 B55 Cw9 DR14 DR52	Yes - Ab = A11, B55, Cw9, DR14, DR52	A33, B53, B71, Cw10, DR13, DR52, DQ2	Yes - Ab = A33, B53, B71, Cw10, DR13, DR52
30	F	Yes	A11 B44 Cw5 DR11 DR52 DQ7	Yes - Ab = A11, B44, Cw5, DR11, DR52	A31, B8, B45, Cw6, DR17, DR52, DQ2, DQ7	Yes - Ab = B8, B45, DR17, DR52

The following pages list, on an individual patient basis, the raw median fluorescence intensity (MFI) values achieved for each cytokine bead group, in each test well, at each time point for experiment 4 described in section 4.6. The cytokine detected is listed in the first column, and the results are divided into day 1, 3 and 5 sampling time points and further divided into those achieved in the negative control control well, the positive control well and test wells 1, 2 and 3 at each time point. Where test wells 1 and 2 are replicates derived from donor stimulators and test 3 is the response seen to the third party stimulator. There are two tables per patient, the first presenting the raw results in the class I stimulator wells and the second from the class II stimulator wells.

Patient 1 – Cytokine Raw MFI Results.

Class I

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	179	8753	431	546	312	97	10989	246.5	306	175	79	8159	267.5	305	284
TNF α	5612.5	10263.5	5298	5469	6288	3967	7166	3391.5	3724	4211	2785	5035	2251	2484	2879
IL-2	31.5	18798	32	13	7	35	251	150	29	76.5	121	40	127.5	127	452
IL-5	4	389.5	4	4	3	3	1150.5	6	3.5	2	6	761	5	49	400
IL-17	53	2616.5	53	26	16	128	6310.5	111.5	84	74.5	288.5	3987	476	325	359

Class II

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	365.5	10031	478	402.5	380.5	230	12287	242	203	148	440.5	9428	85.5	65	46
TNF α	8053	13078.5	5530.5	6096.5	7101	5262.5	10227	3529	4094	4328	3287.5	5913	1933	2221	2696
IL-2	10	24283	13	8	11	172.5	201	47	62	33	87	30	126	107.5	143.5
IL-5	7	922.5	4	5	5	7	3753.5	4	5	5	8	2158.5	8	4	4
IL-17	51	4623	26	58	20	179	5476.5	96.5	260	92.5	550.5	3011	163	438.5	120.5

Patient 2 – Raw Cytokine MFI Results.

Class I

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	171.5	7469	456.5	280.5	304.5	97.5	8487.5	214	213	150	31.5	4658	67	56.5	40
TNF α	8738	8090	1934.5	2988	2966	6006	7499.5	1260.5	2252	1788.5	3520	5253	801	1187	1011.5
IL-2	14	6953	25.5	26	21	171.5	90.5	40	72	61	310	43	46	100.5	67
IL-5	1	232	1	1	1	3	533	1	1.5	1	2	295	1	1	1
IL-17	18	459	19	10.5	14	67	1714	28	90	40	82	612.5	21	48	72.5

Class II

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	394.5	7991	400.5	394.5	406.5	192	10329.5	261.5	155	308	52	7026.5	60.5	43	87
TNF α	7224	6944	2554	3297	2299	5191.5	5262	1507	2144	1392	3677	3173.5	912.5	1175	796
IL-2	24	11428	43	14	24	39.5	378.5	60	62	24	92.5	16	91	69	35
IL-5	2	349	1	1	1	2	1158	1	1	1	1	718	1	1	1
IL-17	42	598	22.5	24.5	23.5	59	2036	92.5	51.5	114.5	23	1000.5	47	22	61

Patient 3 – Raw Cytokine MFI Results.

Class I

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	65.5	501	35	94	96	34	435	29	78	57	18	153.5	12	26.5	27
TNF α	3185	5142	3215.5	3249	6537	2103	3556.5	2065.5	2279	3950	1298	1765	1219	1250.5	2190.5
IL-2	15	283	7	20	9	14	41	8	16	7	12	15.5	5.5	13	7
IL-4	10	11	10	12	11	7	7	7	8	7	7	7.5	8	6	9
IL-5	1	4	1	1	2	1	4	1	1	1	1	3	1	1	2
IL-10	2307.5	3317.5	3310.5	2841	5430	29	67.5	44	45.5	90	32.5	102.5	91	49	93.5
IL-17	85	213.5	18	61	21	84	162.5	16	63	22	62	88	18	33	12

Class II

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	10	217	186.5	96.5	84	8	230.5	107.5	51	39	13	98	36	21	19
TNF α	1956	2975	4635.5	3670	2727.5	1989.5	2367.5	2805	2447	1880	1185.5	1414.5	1638	1166	1180
IL-2	4	221	15	14	24	7	26.5	12	14	38	23	21	22	8	31
IL-4	11	11	11	11	11.5	7	8	8	6.5	9	7	8	7.5	7.5	8
IL-5	1	3	1	1	1	1	2	2	1	1	1	2	1.5	1	1
IL-10	2102.5	3122.5	3800	2888.5	2469	99	90	49.5	37	30	50	75	46	28.5	36
IL-17	15	132.5	84	53.5	86	35	105.5	64	51.5	81	83	179.5	35	20	83.5

Patient 4 – Raw Cytokine MFI Results.

Class I

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	147	5755	62.5	63	58	504	9953	52.5	47	132.5	154.5	7292.5	18	24	101.5
TNF α	2006	7015	2337	2288.5	2123	964.5	10305.5	992	1140	1211.5	487	6399	469	1011	1050
IL-2	1073.5	22032.5	5.5	6	9	251	15288	7	9	55	46.5	336	3	13	52
IL-4	4	335.5	3	4	3	3	109	3.5	4	2	2.5	7	2	2	3
IL-5	43	3901	1.5	2	2	59	8662	2	2	2	34	5877	2	1	4
IL-10	1734	7197	1984	1716	1514.5	506	1747	538.5	634	572	114	51	110	124	88.5
IL-17	133	2457	6	5	6	443	15039	16	9	50	296	14312	8	26	48

Class II

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	92	4541	109	56.5	95	126	8111.5	1056	77	382	127	6589	691.5	36.5	502
TNF α	1889.5	7119	2536	2502	2497	939.5	9451.5	1366	1398	1410	756	6120.5	1267	992	1197
IL-2	265	20705	689	7.5	13.5	82	20975	404	14	52	50	899	91	16	27
IL-4	4	389	3	3	4	3	246.5	4	3	3	3	12	2	3	3
IL-5	12	3569	18	1	2	10	6491	107	1.5	5	8	4778	92	1	5.5
IL-10	1450	6907	1843.5	1653	1458	356	1308.5	574.5	660	622.5	98	52.5	103	114.5	91.5
IL-17	15	2078	27	6	13	44	14998	207	24	226	38	15520	682.5	27	405.5

Patient 5 – Raw Cytokine MFI Results.

Class I

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	2600.5	10597	2384.5	2351	1652.5	1331	10905.5	1487	1321	944	444.5	8783	467	516	255.5
TNF α	6481.5	10559	4052	2508	3828	4298.5	7296.5	2914	1791	2648.5	2405	4429.5	1217	1340.5	1595
IL-2	32.5	1635	13	18	19	14.5	53	11	16	11	19	27	14	13.5	70
IL-5	3	191	3	1	2	3	170	3	1	1.5	1	84.5	2	2	1
IL-17	689	2113.5	554	583.5	545.5	288	1061	230	263.5	271	141	473	136	160.5	145

Class II

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	774.5	8422.5	3344	1508.5	1996	385.5	8153.5	1676	727	1050	94.5	5151	830.5	237.5	314
TNF α	4880	7609	4107	2195.5	2397.5	3289	4899	2248	1425.5	1586.5	2040.5	3221	1745	861.5	808.5
IL-2	17	4140.5	15	14	13	17	62.5	12	12	15	44.5	34	13	15	28.5
IL-5	2	226	1	1	1	2	215.5	1	1	1	2	112	1	1	1
IL-17	356	1907.5	391	339	480	183	897	140	152	213	212	380	85.5	111	117

Patient 6 – Raw Cytokine MFI Results.

Class I

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	87	4896	22	15	16	352	8785	14	13	14	95	9024	9	8	8
TNF α	1231	7312	1691	1607	1672.5	539	7625	643	729	630	221	7081.5	247.5	241	222
IL-2	113	21675	28	3	3	59	19082	17	4	2	18.5	2402	10	2	2
IL-4	3	68	3	4	3	3	86	3	2	3	3	6	3	3	2
IL-5	21	1167	29	25	46	23	2912	15	18	28	14	5127	9	9	15
IL-10	623	4427.5	710	526	634	216	2044	148	191	214	64	172.5	43	47.5	63
IL-17	36	1509	3	3	4	305	5113	3	2	3	170	7198	2.5	2	3

Class II

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	553	3093	275	23	39	1121	10831.5	394	12	417	416	10057	132	8	117
TNF α	1715.5	5265	1882	2165.5	2319	879	10090.5	680	654	1083	354	6490	256.5	230	420
IL-2	4111.5	18744	3365	10	10	2158	21424.5	1474.5	12	61	1148	4452	687.5	7	15
IL-4	4	53	5	3	3.5	3	369.5	3	3	3	3	29	3	3	3
IL-5	76	989	112	34.5	48.5	120	6685	66	16	75	127	5394	43	10	44
IL-10	841	3842	1015	732.5	844.5	144.5	6059.5	154	82	285	34	272.5	34	17	66
IL-17	106	1087	90	4	3.5	176	15138	93	4	58	185	15033.5	45	3	37

Patient 7 – Cytokine Raw MFI Results.

Class I

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	54	7040	125	73	137	163.5	7180.5	87.5	47	89	52	4348.5	25	17	29
TNF α	2818	6789	4175	4321	4509	1891	4274.5	2398.5	2536	2419.5	1149	2155	1162	1222	1184.5
IL-2	212	8873.5	133.5	76	161	129	816	76.5	57	74	41	46	21	31.5	24.5
IL-4	11	12	11	12	11	10	8	11	11	10	7	8	7	8	8
IL-5	1	58.5	1	1	2	2	52.5	1	1.5	1	1	31.5	0.5	2	1
IL-10	2011	3798	3563	3874.5	3547.5	527.5	640.5	834	918	611	13	16	14	14	12.5
IL-17	58.5	1075	20	22	10	140.5	936	30	20	14	193	536	31	25	30.5

Class II

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	108.5	10705	127	111	121	207	8869	102	105	x	65	4753	27	39	42
TNF α	4145.5	8377.5	4086	3839	4304	2839.5	5487	2181	2289.5	x	1618.5	2015	1244	1197	1355
IL-2	123	5726.5	121.5	219.5	187.5	163	226	187	113	x	71	15	75	63	51.5
IL-4	10	13	11	11	10	11	8	8	8	x	8	5.5	7	7	7
IL-5	1	37	1	1	2	1.5	28.5	4	1.5	x	1	12	1	3	1
IL-10	2547.5	4449	3742	2616	2712.5	898.5	938	663.5	693.5	x	24	12	21	16	15
IL-17	24	1152	21	13	15	541	1071.5	176.5	115	x	773	414.5	142.5	434	239

Patient 8 – Cytokine Raw MFI Results.

Class I

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	49	5977.5	95	103	49.5	45	6865	74	81.5	88	34	4759	98	211.5	100
TNF α	2132	3132	1566.5	2181	1378	1199	2589	922	1283	625.5	650	1644	523	757	338.5
IL-2	25	1927	13.5	10	16.5	19	222	27	32.5	54	126.5	21	42	41	48.5
IL-4	12	11	12.5	11	11	8	8	9	5	9	7	7	7	7	7
IL-5	1	34	1	2	1	1	37	1	1	1	1	29	21	3	1
IL-10	2383	2251	1017	1619	758.5	941	823.5	252.5	527	132	47.5	20	22	28	22
IL-17	62	559	26	22	12	71.5	1142	68.5	51.5	57	200.5	611.5	320	247	126

Class II

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	17	7633	64	75	64	23	7794	46	77	111	13.5	5503	35	56	89
TNF α	1114	3875.5	1383	1472	1416	640	2854.5	471.5	721	674.5	436.5	1951	413	371	420.5
IL-2	14.5	1853	11	22	22	134	103	54.5	27	81	75	15	37	52.5	37.5
IL-4	11	10	10	10	10	8	9	8	8	8	7	7	5	6	6.5
IL-5	1	71	1	1	2	4	79.5	3	1	3	9	59.5	4	1	9
IL-10	472	1633	720	802.5	676	100	509	115	180	116.5	8	14	13	14	7
IL-17	17	443.5	15	40.5	20	52	1014	57	75	70	137	492.5	147.5	151.5	215

Patient 9 – Raw Cytokine MFI Results.

Class I

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	2098	9767	6000	380.5	156	4298	9748	7015	213	285.5	2415	9769.5	4010.5	349.5	311.5
TNF α	1732	7251	1612.5	574	599	900	5449.5	1078	202	230	516	3058	611	164	221
IL-2	5417	20107	5096.5	16.5	18	1050	3623	640	54	45	79	45	43	139	120
IL-4	4	6	4	4	3	3	4	3	3.5	3	3	3	3	3	3
IL-5	107.5	1470	76	2	1	88	2769	144	3	4	93	1791.5	95	246	298.5
IL-10	1660.5	3654	1072	530	540	466.5	745.5	330	117.5	92	153	70.5	28	79	40
IL-17	225	2401	212.5	6	4	950.5	12426.5	1582	12	31	1843	9635	953	127	246

Class II

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	84	11497	144.5	138.5	133	3833	10789	207	195	167	3714.5	9372	823	541	339
TNF α	643	6592	463	655.5	1236	615	4518	199.5	292	492.5	1156	2094.5	397	249	605
IL-2	41	20845	5	8	15	387.5	2917.5	72	108.5	71.5	146.5	32	142	203	112
IL-4	4	6	3	4	3	3	4	3	2.5	3	3	3	3	4	3
IL-5	2	1455.5	1	1	2	40	1593.5	21	4	11	106	900.5	405	198.5	793
IL-10	623.5	4052	419	824	753.5	156	469	94	170	123	95	36	62.5	97.5	52.5
IL-17	10	3466	2	4	4	676	9352	30	28	26	672	6313	165	428	194

Patient 10 – Raw Cytokine MFI Results.

Class I

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	169	4509.5	246	214	186.5	95	8165	79.5	88.5	121	334	4746	35	29	207
TNF α	6208.5	8069	4921	4968.5	4652	4397	7698.5	3425	3327	3055.5	2463	3918	1682.5	1735	1808
IL-2	26	15558	22.5	9	9	94	114	32.5	35	212	78	151.5	55	63	117
IL-5	2	334	1	1	1	2	1736	1	1	5	15	1201.5	1	1	267.5
IL-17	45.5	1385	20	25	17	79	6355.5	58.5	24	246	484	4112.5	236	74	1348

Class II

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	75	4473	86	159.5	202	80	4869.5	113.5	84	118	151.5	2399.5	93	42	168
TNF α	5684	10281	4158.5	7326	5149	3878	6537	3445	3655	3269	2359	4166	2187.5	2057	1936
IL-2	10	22893	10	15	12	72	111	280	72	76	133	27	61.5	150	138.5
IL-5	0	796	1	1	2	1	1506	100	7	16	683	927.5	957	320	215.5
IL-17	29	1907.5	31	55	53	71	3089	201	210	139	709	1584	810	476.5	769.5

Patient 11 – Raw Cytokine MFI Results.

Class I

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	30.5	3898	84	96	68.5	22	8736	50.5	45.5	39	13	5767	25	24	20
TNF α	1753.5	3832.5	1926	2680.5	2278	1434.5	4029	1387.5	1339	1480.5	1013	2303	783	1249	1131
IL-2	5	10333	4	4	4	4.5	4012.5	14	4.5	4	33	481	43	8	9
IL-4	12	12	11	12	12	8	10	8	10.5	10	8	9	8	9	9
IL-5	1	41	1	1	2	2	75	2	1	1	1	47	1	1	1
IL-10	848	1592	1218.5	2078	1649	395	401.5	469	360.5	384	62	73	32	132.5	141.5
IL-17	9.5	593.5	8	7	4.5	10	882	14	8	6	25	818.5	50	23	18

Class II

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	50.5	2999	67	107.5	82	35	6464.5	39	45	52.5	17	3839.5	17	23	24
TNF α	1813.5	4081	1877.5	2223.5	2395	1620	3368.5	1178.5	1383.5	1537.5	1032	1916.5	605	987	994
IL-2	4	7112	3	3	3	4	3109	6	7	9	43	449	18.5	15	52
IL-4	9	12.5	12	11	12.5	8	10	8	9	8	10	9	8	7.5	10
IL-5	1	25	1	1	1	1	45	1	2	1	1	29.5	1	1	2
IL-10	734.5	1276.5	1321	1698	1748.5	446	234	451.5	399	469	48	50	24.5	36	49
IL-17	6	490	6	5.5	6	15	907	7	15.5	22	63.5	962	26.5	42	74.5

Patient 12 – Raw Cytokine MFI Results.

Class I

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	142	6315.5	10	6	6	1662	14104.5	10	7	7	425	11917.5	8	7	6
TNF α	1825	10010.5	3477.5	720	606	3011	17205	2109	390.5	334	1415	15208	820.5	145.5	139
IL-2	1703	24233	52	12	6	351	26268.5	107.5	18	12	17	26760.5	63	16	10
IL-5	1	77	1	1	1	11	4406	1	1	1	7	3088.5	1	1	1
IL-17	390.5	2386	10	9	9	4151.5	19241.5	12	10	10	2747	18099	10	8	9.5

Class II

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	7	5905	7	8	8	8	13131	8	7	7	7	12263.5	7	6	7
TNF α	663.5	9243	1207	2055	2642	506	15402.5	792	1138	1737.5	171	12614	292	546	658.5
IL-2	61.5	24705	24.5	8	5	96.5	26483	30	13	12	58	26138	56	15	15
IL-5	1	36.5	1	1	0	2	8364	1	1	1	2	7833	1	1	1
IL-17	8	1105	9	9	9	10	15812.5	11	10.5	10	12	19347	14	10	11

Patient 13 – Raw Cytokine MFI Results.

Class I

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	7	30	6	6	6	8	17	8	6	7	8	11	8	8	7
TNF α	351	499.5	560.5	272	431.5	218.5	369	340.5	146.5	285	132	201	207	144	189
IL-2	114.5	587.5	62	61	56	49	28	165	75	114	35	42	119.5	78	68.5
IL-5	1	43	1	2	2	1	74.5	9	2	7	1	38	9.5	3	32
IL-17	48	550.5	14.5	20	29	216	586	111	197	79	135	479	183	357	147

Class II

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	7	13	7	7	7	7.5	10	7	8	11	7	8	7	7	11
TNF α	549	646.5	658	432	521	734	497	391.5	243	319	523	318	226	197.5	241
IL-2	87	91	19	53	85	113	104	139	131	132	91	51	67	47	102
IL-5	2	8	0	1	1	4	14	1	12	4	3.5	9	1	10	7
IL-17	19	106.5	11	10.5	20	473	433	65.5	102.5	135	337	449	116	68	146

Patient 14 – Raw Cytokine MFI Results.

Class I

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	308.5	8823	109.5	263	209.5	135	8640	54	122	140	125	5531	63	79	71
TNF α	2657	3666	673.5	987	2591.5	1506	3164	363	552.5	1284	1089	1621	355.5	466.5	961.5
IL-2	4	11927.5	11	4	5	42	60.5	30	23	68.5	118	6	97.5	51.5	93
IL-4	3	4	3	3	4	3	3	2	3	2.5	3	3	3	3	2
IL-5	1	189.5	1	1	1	1	328	1	1	2	6	192.5	1	5	38
IL-10	1933	3406.5	388	584	771.5	177	261.5	74	63	95	32	15	33	77	27.5
IL-17	21	1322.5	13	14	9	135	4343	69	20	38	245	1938	165.5	94	141

Class II

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	84	9835.5	146	120	70.5	51	9456.5	173	84	35.5	55	7516	59.5	45	63
TNF α	2213	4600	1079	1148	1849.5	1513	3486	488.5	692	960	960.5	2147.5	330	538	858
IL-2	3	11884.5	21	4	5	52	51	23	20	43	243	5	41	66	91
IL-4	3	4	3	3	3	3	4	3	4	3	3.5	3	3	2	3
IL-5	1	75.5	1	1	1	0	104.5	0.5	1	0.5	16	62	1	6	3
IL-10	1163	2156.5	441	439	436	133.5	128.5	89	82	46	83	12.5	107	23.5	13
IL-17	10	1263	17	4	8	36	2816.5	49	61.5	36	132	1634.5	130	89	71

Patient 15 – Raw Cytokine MFI Results.

Class I

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	6	23	7	8	13	18	37	14	11	9.5	20.5	40	42	13.5	11
TNF α	576	603	612.5	793.5	1428	333.5	342	273	306.5	576.5	198.5	242.5	230	235	464
IL-2	7	133	10	8.5	8	206	185	250	260	68	174	211	523.5	392.5	112
IL-5	1	7	1	1	2	2	5	14	3	2	2	5	14	3	2
IL-17	8	74	17	7.5	6	736.5	1261	735	750.5	204	866.5	2729	1956.5	1258	442.5

Class II

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	8	48	8	6	8	8	20	12	10	9	9	14	13	15	12
TNF α	1167	1314	592	636	699	555	426	271	259	289.5	357.5	371	184.5	168	201
IL-2	10	111	10	9	15	136.5	21	207	119	114	208	32	147	182.5	296
IL-5	1	5	1	1	1	9	4	3	9.5	7.5	38	3	15	55	276
IL-17	16	43	18.5	7	10	351	37	829	509	525	963	87	1653.5	1102.5	1211

Patient 16 – Raw Cytokine MFI Results.

Class I

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	190.5	6794	63	36	45.5	481	10125	111	39	42	160	9928	49.5	17	17
TNF α	1175	7990	936	923	967.5	528.5	11152	515	563	488	304	7334.5	305	323	291.5
IL-2	18.5	22151.5	3	2	2	14	16913	9	2	2	6	651.5	9	8	3
IL-4	4	265	3	3	3	3	242	3	3	3	3	15	2.5	3	4
IL-5	2	6005.5	1	1	1	7	13738.5	2	1	1	5	10599	1	1	1
IL-10	463	9428.5	364	375	277.5	116	1683	126	114.5	79	30	99.5	27	26	18
IL-17	7	6222	3	3	3	99	21077	5	2	3	77	20713.5	12	3	2

Class II

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	46	5155	154	91.5	56.5	38	9634	130	147	54	133	8403	70	151	28
TNF α	1073.5	5830	1608	985	1386.5	566.5	10181	851	532.5	652.5	383.5	6292	532	389	349
IL-2	2	21038	4	3	4	3	8254	3	23	7	22	136	9	12	10
IL-4	4	143	4	3	3	3	108	2	2	2	3	9	3	2	3
IL-5	2	3769	2	1	2	1.5	8468	2	1	2	24	6211	2	1	2
IL-10	375	6583	671	325	481	83	1584.5	227.5	86.5	97	18.5	71.5	58	20	15.5
IL-17	3	4971	4	3	3	4	19969	3	3	2	397.5	18645	6	4.5	2

Patient 17 – Raw Cytokine MFI Results.

Class I

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	7	9	7.5	11.5	11.5	8.5	10	8	7	7	8	7	8	8	8
TNF α	656	525	589	1205	527	454	282	351.5	661	238	251.5	158	182.5	364	132
IL-2	6	31	5	7	6	15	10	6	5	6	13	8	7	6	6
IL-5	1	1	1	1	1	0	1	1	1	1	0	1	1	1	1
IL-17	18	28	19	21	17.5	18	23.5	14	16	14	18	20	17	14	14

Class II

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	9	8	13	9.5	14	10	11	9	11	10	8	8	8	8	8
TNF α	619.5	717	505	441	540.5	364.5	384	243	220	253	199	221.5	128.5	121	140
IL-2	7	21	5	7	5	12	22	7	6	5	17	14	13	6	4.5
IL-5	1	1	1	1	1	0	1	1	1	1	0	1	0	1	1
IL-17	17.5	17	16	17	17	24	20	15	16	16	18	17	12	14	13

Patient 18 – Raw Cytokine MFI Results

Class I

Cytokine	Day 1					Day 3					Day 5				
	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	27.5	3523	185	94	109	70	3688	158.5	110	170	43	1720	415	238	234
TNF α	1271	4006	4392.5	4004	3274	1042	3183	2445	2909	1920.5	953	2493.5	1806	1714	1245.5
IL-2	15	837.5	15.5	9.5	14	81	257	40.5	22	61	130	15	201	297	192
IL-4	12.5	11	12	10	11	9	10	9	9	10	8	7	8	7	8
IL-5	1	5	1	2	1	2	7	6	1	1	24.5	4	518	2	359.5
IL-10	527	1168	907	1643	583	158	1065.5	404	984	135.5	5	28	40	15.5	5.5
IL-17	16.5	100.5	22	28.5	25	72	240	66	96	55.5	322.5	233.5	429	414	292.5

Class II

Cytokine	Day 1					Day 3					Day 5				
	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	146	7476.5	111	98	106	343	8281	90.5	115.5	129	144.5	4907.5	153	119	51.5
TNF α	3864	9131	3195	4524	3078	2574	6788.5	1706	2439	2163.5	1627.5	4797	1316.5	1596	1283
IL-2	15	2011	21	20	20	35	417.5	54.5	58	45	98	29	100.5	92	70.5
IL-4	10	12	12	10	12	11	10	12	10	11	8	8	8	8	11
IL-5	1	11	1	1	2	2	101.5	3	1	26	2	74.5	79.5	41.5	72
IL-10	1010.5	868	560	737	512	308	422	181	293	165	5	17	5	9.5	6
IL-17	17.5	145.5	24	24	21	117	568.5	162.5	103	48	105	469	615	379	104

Patient 19 – Raw Cytokine MFI Results

Class I

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	114	6025	352.5	679	221	379.5	12209	1325	1911	2208	233.5	10704.5	443	1164	1477
TNF α	6845	7573.5	6489	6760.5	6626	5903	11544	5850.5	5353	5078	3422	8411.5	2972	3508	3519
IL-2	6	16447	10	4	4	10	19823	12.5	8	53.5	14	275	13	18	18
IL-4	4	113	4	3	4	3	549	4	3	3	3	17	3	3	2
IL-5	28	6291.5	8	10.5	6	28	20426.5	7	7	4	15.5	17972	4	4	4
IL-10	3289	1423	755.5	851	609	2209.5	2924	276	284	240	533	40	42	44.5	52
IL-17	12	363	5	4	4	50.5	4274	25	11	8	75.5	4033.5	13	13	15

Class II

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	115.5	5089	211.5	501	242	998	10589	534	2637	2809	562.5	9860	261	1103.5	1368
TNF α	5553	8070	7681	7158	7989	4272	9485	5760	5022.5	5717	3188	6901	3482	3110	3210
IL-2	8	15900	4	7	4	21	9466.5	21	16	100	12.5	139	46	18	26
IL-4	5	131	3	3	3	4	311	2	4	3	2.5	12	3	2.5	3
IL-5	9	5909.5	7	10	13	16	17663.5	16	7	8.5	10	15149.5	14	6	8
IL-10	518	1474.5	671.5	587.5	586.5	182	2819.5	257	206.5	209	45	45	43	25.5	26
IL-17	5	413.5	4.5	5	5	14	5029	11	15	7.5	15	4095	11	12	9

Patient 20 – Raw Cytokine MFI Results.

Class I

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	45	1959	94.5	39	23	1000	3332	859	1403	1976	3956.5	3538	5021	3487.5	3701.5
TNF α	3326	3501	1676.5	1510	1742	1681.5	2522.5	919	1000.5	1251	2705.5	2150	2156.5	1805.5	1712
IL-2	45.5	378	41	40	54	42	43.5	54	100	72	26	31	14	17.5	21
IL-4	4	4	3	3	3.5	3	3	3.5	3	3	3	3	2	3	3
IL-5	7	56.5	3	3	3	6	303.5	3	4	25.5	6.5	243	10	10	180
IL-10	127	179	89.5	80	64	48.5	71	71.5	124	153	17	18	106	64	63
IL-17	29	81	12.5	10	5.5	167	300.5	102.5	212	61	154.5	242	227	341	61.5

Class II

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	14	2757	14	13	21	593.5	4515.5	2278	514	1334	3618.5	4243	4631	3764.5	4978
TNF α	2265	3617	1491	784.5	1710	1257	2721.5	916.5	613	1492	2764	2515	2786	2086.5	3154
IL-2	20.5	1175	22	37	65	59.5	226	68	85	80.5	17	25	27	18	22
IL-4	3	2	3	2	3	3	3	3	2	2	4	3	2	2	2
IL-5	2	62	1	2	1	18	221	2.5	2	68	56	195	4	9	130
IL-10	124	100.5	109	62.5	100	70	70.5	151	55	67	57	60	90.5	54.5	19
IL-17	8	15	4	5	7.5	141	153	102.5	129.5	97	230.5	317	95	160	114

Patient 21 – Raw Cytokine MFI Results.

Class I

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	10	155	19	23	13	10	92	19.5	58	13	10	34	32	112	16.5
TNF α	891.5	1349	1857	2851	1123	669	642	1434	1551	754	744	620	968	1489	688
IL-2	10	1236	21.5	34	47	40	531	207	111.5	33	199.5	216	139	134	145
IL-4	11	11	12	12	12	8	7	8	8	7	8	8.5	8	7	6
IL-5	1	48	1	1	1	1	42	2	1	1	3	26	3	3	1
IL-10	1126	2014	2082	2057	1376	51	30	90	95.5	75	18	33	8	12	18.5
IL-17	8	50	24.5	24	53	17	52	194	307	33	45	57	261.5	754	88

Class II

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	9	565	12	16	14	23	488	15	12	12	59	323.5	12	10	14
TNF α	1346	1236	3916	2065.5	1982	966	1036	742	1190	1136	808	960	505	862.5	936
IL-2	6	1520.5	20	17	27	346	248	141.5	14	69	404.5	64	393	16	139
IL-4	11	11	11	10.5	11	9	8	7	7	8	7	8	6	7	7
IL-5	1	52.5	1	1	1	5	46	2	1	2	12	31.5	7	1	1
IL-10	1086	1417	2209.5	2790.5	2411	67	80	39	103	103	11	32	5	25	13
IL-17	6.5	117.5	6	16	11	214	186	61	17	23.5	339.5	950	247.5	16	29.5

Patient 22 – Raw Cytokine MFI Results.

Class I

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	16	435	66.5	103	104.5	168.5	915	115	344	698	121	547	150	234	328
TNF α	5108.5	6679	4773	4376.5	7718.5	2520	4772	2712.5	2338.5	5090	1781.5	2458.5	1574	1483	2943
IL-2	18	104	3	11	25	11	23.5	21.5	34	18.5	29	14	47	45.5	6
IL-4	2	3	3	3	2.5	2	3	3	2	2.5	2	2	3	2	2
IL-5	1.5	2	1	1.5	1	1	2	0	0	1	1	1	1	1	1
IL-10	853.5	504	385.5	282.5	356.5	173.5	120	65.5	54.5	91	73	34	35	33	21
IL-17	4	30	8	3	6	55	251	32	24	23	60	291.5	154	36	14

Class II

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	11	324	162.5	222.5	284	44	721	141	241	677.5	35.5	306	47	92	296
TNF α	3957	6408.5	4639	5835.5	7639	2122	3903	2599	3280	5080	1183.5	2092	1412	1955	3285.5
IL-2	5.5	93	7	16	8.5	11	42	16	24.5	17	43.5	69.5	18.5	25	11.5
IL-4	2	3	3	3	3	2	2.5	3	2	2	3	2	3	2	3
IL-5	1	5	1	1	0	0	4	1	1	1	1	3	1	1	1
IL-10	352.5	330	130	250	316.5	72	81.5	48	65	118.5	19	19	12.5	14	29
IL-17	3	7	4	3	6	15	64	7	11	40.5	47	102	7	10	40.5

Patient 23 – Raw Cytokine MFI Results.

Class I

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	17	4121.5	17.5	31	11	16.5	7726	135.5	27	12	13	5582	61	26	9
TNF α	808	5056.5	521.5	526	503	443	4424	347	317	243.5	303	2303.5	202	175	111
IL-2	10	6142.5	5	3	4	112	193	221.5	47	17	38	7	251	52	16.5
IL-4	4	8	4	4.5	3	3	5	3	3	3	3	2.5	2.5	2.5	2
IL-5	1	677	1	1	1	41	2701	54	5	7.5	464	1579.5	98	107	8
IL-10	529	878	520	458	300.5	91	358	89	99.5	50.5	45	19	35.5	31.5	11
IL-17	5	318.5	4	3	3	72	3419	37	7	5	195	1984	98	9	6

Class II

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	78	4478	33	61	159	345	7788	16	217.5	2630	356.5	4877.5	11	430.5	5033
TNF α	1558	4443	825.5	2088	968	957.5	3545	348	1566	1032	614	2051	159	1347	2490
IL-2	329.5	5066	122	7	131	240	152	36	235.5	3038.5	54	10	90	270.5	110.5
IL-4	5	7	3	4	4	2	4	3	3	4	2	4	2.5	3	5
IL-5	21	605	8	3	4	217	2017.5	6	10	383.5	484	1287.5	7	490	3192.5
IL-10	352	588.5	515	2601.5	441.5	50	276.5	99	930.5	290	15.5	24	41	86.5	277.5
IL-17	16	247	8	3	6.5	73	2698.5	8	9	204	333	1532.5	8	211.5	464

Patient 24 – Raw Cytokine MFI Results.

Class I

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	386	5487.5	192.5	231	271.5	179	3692.5	106	143.5	159	48	1538	32	41	45
TNF α	3951	6867	3650	3549	3618	2269	3567	2307.5	2383	2349.5	1232.5	1981	1357	1246	1564.5
IL-2	28	2102.5	8	18	13	23.5	444	7	9	11	16	124.5	5	13	6
IL-4	13	14	14	12.5	14	9	8	9	10	9	9	6.5	5	9	9
IL-5	2	8	1	2	1	2	8	1	3	1	1.5	6	1	2	1
IL-10	6233.5	10319	6871	6491	6601	102	130	131.5	123	176	21	90	79.5	63	51
IL-17	264	640.5	95	117	99.5	171	288.5	58	83	88.5	138	149	33	52	57.5

Class II

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	177	9168	213.5	219	233.5	78.5	7304	111	133	136	28	3681	39	36	33.5
TNF α	2838.5	5271	3532.5	3396	3407	1775	3770	1957	2120	2045.5	1051	1844	1188	1280	1272
IL-2	19	2119	9	11	9.5	14.5	94	9	10	10	12	22	8	7	9
IL-4	13	13	15	12.5	15	8	9	9	6	9	9	10	8.5	8.5	9
IL-5	9	38	1	2	2	9.5	33	1	1	3	10	26	1	2	1
IL-10	3768	6385	7196	5874	5578	52	354	114	64.5	68.5	16	47.5	69	55.5	63.5
IL-17	156.5	1572	109	93	78	145.5	898.5	69.5	81	63.5	113.5	673.5	34	46	34

Patient 25 – Raw Cytokine MFI results

Class I

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	9	5287	19.5	11	20	25	5732	13	10.5	20.5	33.5	3971	8	9.5	18
TNF α	2034	2012	1381	644	696	717	1667	716.5	239	464	417	1398	522	100.5	244.5
IL-2	6	13656	6	6	17	47.5	3202	18	35	27	97.5	72.5	141	50	148
IL-4	13	11	11	10	10	7	8	8	8	8	8	5	5	5	5
IL-5	0	104	1	0	1	1	100	2	1	1	1	65	2	1	3
IL-10	2127.5	1902	2059	1047.5	1045	553	245.5	378	109.5	173	37.5	60	9	8	24
IL-17	10	971	8.5	18	19	37	852.5	32	47.5	55	174.5	624	40	49.5	161

Class II

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	9	7000.5	15	13	392	43	7259	18	9	101	42	4702.5	97	24	30
TNF α	328	1462	371	321	3937	263	2131	219	168	1148	130	976	142	155	382
IL-2	10	14808	19	9	34	30	3562.5	32	14	23	218	198	209	155	203
IL-4	13	10	11	10	11	8	7	7	7	6	5.5	4	5	5	4
IL-5	1	91.5	0	1	1	1	104	0.5	2	1	1	59	1	2	0
IL-10	428	1611	720	731	7516	68	235	87	79	823	32	43	67	39	21
IL-17	19	1720	22	24	41	60	1355	53	40	46	344	844	714.5	736	65

Patient 26 – Raw Cytokine MFI Results.

Class I

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	155.5	1140	48	60	24	497	6814	41	394	16	161	4160	20	113.5	9
TNF α	4195	6402	4875	4247	4085	2567	8825	2450	2200	2780	1231.5	5033.5	1503.5	1155	1512
IL-2	45	11048	5	22	3	42.5	4480	9	113	3	7.5	28	41	36	11
IL-4	3	49	3	2	3	3	70	3	3	3.5	2	5	3	3	3
IL-5	1	1601	1	2	2	4	10048.5	2	3.5	1	2	7339	1	6	1
IL-10	1582	1518	1417	869	906	319	909	375.5	250	240	53	25	47	46	34
IL-17	31	658	4	3.5	8	292	7045.5	6	8	17	150	5132	14	8	19

Class II

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	95	3549	32	244	107	579	7635	216	257	317.5	291	5450.5	111	153	275
TNF α	5899.5	7011	4632	3873	4552.5	3342	6594.5	2728.5	2185	2792	2195	3792	1644	1356	1686
IL-2	112.5	9976	3.5	2	5	46	386	86	48	34.5	19	14	121	53	76
IL-4	4	27.5	4	3	3	2	11	3	2	2	3	3	3	4	3
IL-5	5	827	1	1	1	4	4973.5	3	2	2	3	3602	4	1	7
IL-10	1416	1053.5	1182	1133.5	1367.5	277	946.5	248	230.5	324.5	41	22	31	55.5	46
IL-17	8	401	4	4	4	10	4811.5	22	4	4	11	2763	47	4	21

Patient 27 – Raw Cytokine MFI Results.

Class I

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	446	7608	47.5	70	31	7076	11477.5	46	957	29	4829	9849	17.5	243	9
TNF α	3217	8380	2070	2358	2391	2351	12550	1190	1306.5	1222	1356	10852	530	607.5	495.5
IL-2	226	24653.5	9	4	7	200	23444	27	6	4	11	23034	52	8	6
IL-4	10	82.5	11	10	13	10	452.5	8	7	7	6	37	6	5	7
IL-5	1	210	1	1	2	101.5	1748	1	1	1	102	2917.5	1	1	1
IL-10	139	1866	25.5	39	31.5	389.5	4949.5	9.5	13	11	29	54	3	3	3
IL-17	39	1271.5	6	7	7	918	9121.5	6	5.5	6	621	11414.5	7	6.5	6

Class II

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	51.5	6035	51	46.5	40.5	85	11714.5	220	30.5	27	17	10925.5	55	9	10
TNF α	3601	8446.5	1912	1770	1507.5	2491	12884.5	1283	1066	842	1045	10151	542	494	388
IL-2	6	23864	6	8	6	6	23330	250	6	5	5.5	22895.5	36	6	5
IL-4	11	60	11.5	10	11	8	339.5	7	8	8	6	57	7	7	7
IL-5	1	127.5	2	1	1	1	2302.5	2	1	1	1	3591.5	1	1	1
IL-10	99.5	2171	33	20.5	31.5	51	5594	21	8	13	9	67.5	3	2.5	3
IL-17	8	1251.5	7	6.5	6	6	10864	35	5	7	6	13101	10	6	4

Patient 28 – Raw Cytokine MFI Results.

Class I

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	44	1276	276.5	185	123	21	686.5	97	57	52	13	380.5	53	33.5	23
TNF α	2090.5	3441	3956	4083	4264	1474	1475	2284	2429	2563	1036.5	941	1599.5	1630	1740
IL-2	10	4908	13	9	12	36	3200	45	8	14	13	1487.5	107	61.5	15
IL-4	12	16	13	13	13	9	9	9	8	9	8	10	8	8	7
IL-5	1	4.5	2	1	2	1	5	1	1	1	1	5	1	1	1
IL-10	1886	2029	2386	3108	3650	59	48	144	182.5	238	29	127	13.5	28.5	101.5
IL-17	22.5	466	59	29	17	33.5	325	188	49	17	34	228	683	184	26

Class II

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	139.5	12294	111	135	421	61	11317.5	47	60	168	37.5	8524	26	25	92
TNF α	2779	3886	3712	4069	4598	1853	3331	2069.5	2324	2821	1182.5	2313	1351	1488	1802.5
IL-2	13	16683	7	12	42	38	649	11	10	70.5	92	28	7	6	44
IL-4	12	13	13	12	12	8	8	8	8	8	7	8	8	9	8
IL-5	2	45	1	1	1	2	53.5	1	1.5	1	2	31	1	1	2
IL-10	888	3955.5	3048.5	3512	2155	64	99	124	192	195	7	30	47.5	41	21.5
IL-17	125	3392.5	17.5	29.5	152	647	2461.5	18	44	452.5	1436.5	1632	15	52	941

Patient 29 – Raw Cytokine MFI Results.

Class I

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	822	10523	513	210	128.5	4294	10353	2234.5	131	68	2457.5	9909.5	870.5	34	29.5
TNF α	4372	12203	5672	5755.5	5987	4025	9994	3853	3565	3224.5	2720	9047	2325	1995	1974
IL-2	2585.5	23504.5	847.5	12	3	427	6369	247	4.5	2	15	82	8.5	6	9
IL-4	3	378	3	3	3	4	116	3	3	2	2	6	3	3.5	3
IL-5	105	8983	49	2	3	402.5	18381.5	40	2	2	334.5	16648.5	27	1	2
IL-10	680.5	4766.5	786	555.5	610	221	782	194.5	152	120	34.5	47	28	21	29
IL-17	130	3693	42.5	5	5	1266	10039	205.5	32.5	7	949	7806	213.5	12	6

Class II

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	352	10131	152	77	136.5	949	10731	402	51	78.5	949	8215	93.5	17	22.5
TNF α	5391	10590	6588	6756	6607.5	3272	9170	4004	3583.5	3879.5	3272	6657	2010	1689.5	2234
IL-2	2532.5	23037	4	3	3	536	659	7	2	2	536	36	4	2	5
IL-4	4	246	3	3	3	3	44	4	3	3	3	4	2	3	2
IL-5	126	7841	3	5	5	89.5	17020.5	2.5	4	4	89.5	11882	2	3.5	2
IL-10	2426	5018	478	446	388.5	927	544	121	109	107.5	927	109	19	21	17
IL-17	128	3655	6	4	5	590.5	11082	13	6	45	590.5	7553	12	4	16.5

Patient 30 – Raw Cytokine MFI Results.

Class I

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	29	4402	129	98.5	65.5	19	5361	173	122	71	93.5	2480.5	322	526	332
TNF α	2939	6195.5	2772	2812	2618.5	1630	4086	1779	1638	1746	861	2088	1038	1008.5	1028.5
IL-2	14	12742	21	8	8	64	587	71	73	55	189	26	325	238	145
IL-5	1	350.5	2	1	2	1	643	2	16	2	107	391	61	95	4
IL-17	15	661	15	30	23	121.5	2545	97	79	99.5	631	1492	360	556.5	811.5

Class II

	Day 1					Day 3					Day 5				
Cytokine	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3	Neg	Pos	Test 1	Test 2	Test 3
IFN γ	44	5647	109	97	74	29	7738	91	99.5	97	69	4549	104	150	228
TNF α	3910	7720.5	2921	3959	2576	2538	5747	1882	2716	1394	1815	3231	971	1924	839.5
IL-2	5	17829	16	12	8	52	540	87	30	65	131	27	150	158	138
IL-5	2	752	2	2	1	3	1614	3	3	1	3	1117.5	3	2	2
IL-17	15	1294.5	18	17	22	170	4402	233	139	74	833.5	2672	868.5	322.5	564