Prognostic Immune Markers for Chronic Allograft Injury in Renal Transplant Recipients

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Abstract

Introduction:

Alloimmunity is a major contributor to chronic allograft injury. There are currently no routine clinical cell-based assays that allow quantification of the recipients' alloimmune response towards a graft. Previous work from our group identified indirect alloimmune responses to non-polymorphic regions of HLA Class 1. The aim of this thesis was to assess the alloimmune response in renal transplant recipients (RTRs) by using synthetic peptides to non-polymorphic regions of HLA Class 2.

Methods:

Responses to newly synthesized HLA Class 2 peptides were tested in RTRs via an γ-interferon ELISPOT assay. Cell surface staining techniques and Luminex technology were used to identify the T-cell subsets driving the immune responses and subsequent cytokine production respectively.

Results:

Increased responses to HLA Class 2 derived peptides were detected in renal transplant recipients compared to healthy controls. The activated effector memory subset of T-cells was expanded in RTRs compared to healthy controls and generated these responses. T effector memory cell dependent TNF-α and IL-2 and T regulatory dependent IL-10 synthesis in the presence of specific peptide antigen was detected.

Conclusion:

A potential reproducible assay of T cell alloreactivity has been identified to help stratify RTRs at risk of an ongoing alloimmune response but needs further testing in a larger multicentre study.

Acknowledgments & Dedication

My time in research has been accompanied by a lot of changes in my personal life- I lost a mother, became a wife, a daughter-in-law, and a mother. The work presented in this thesis would not have been possible without the support of many people.

First and foremost, my supervisor Dr Simon Ball who has dedicated a lot of his own time in his role as supervisor. He has been extremely supportive through a rocky few years and I will always be grateful for his guidance, wisdom and support. I thank Prof Lorraine Harper for all her encouragement and support. Helen Smith deserves a special mention- she taught and guided me through a lot of the lab techniques I required for the completion of this thesis and assisted me in collecting and processing a lot of samples when I could not. A special thank you to my colleagues, Andy and Shazia- for always lending a helping hand and a listening ear and encouraging me.

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To my son Rayan, thank you always bringing a smile to my face. I promise to spend more of my weekends with you now! Finally, and most importantly, my husband, Abhishek- you are my pillar of strength and support. Your unnerving faith in my ability to achieve my goals is invaluable to me. Thank you for all your love and support, I could not have done this without you.

This thesis is dedicated to my mother, who isn't here to see this day but whom I believe is always watching over me- and to my father, who has taken to the role of being my mother and father more than I could have ever imagined.

List of Abbreviations

6-MP	6- mercaptopurine
6-TG	6-thioguanine
ABMR	Antibody Mediated Rejection
ACR	Albumin Creatinine Ratio
ADCC	Antibody-dependent Cellular Cytotoxicity
APC	Antigen Presenting Cell
CAI	Chronic Allograft injury
CCR7	Chemokine Receptor 7
CMV	Cytomegalovirus
CNI	Calcineurin Inhibitor
CsA	Ciclosporin A
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T Lymphocyte Associated Protein
DAMP	Danger/Damage Associated Molecular Pattern
DC	Dendritic Cell
DGF	Delayed Graft Function
DMSO	Di-methyl Sulphoxide
DSA	Donor Specific Antibody
eGFR	estimated Glomerular Filtration Rate
FDA	Food and Drug Administration
GBM	Glomerular Basement Membrane
GVHD	Graft Versus Host Disease
HC	Healthy Control
HEV	High Endothelial Venule
HLA	Human Leukocyte Antigen
ICAM-1	Intracellular Adhesion Molecule-1
IFTA	Interstitial Fibrosis and Tubular Atrophy
IL	Interleukin
LDA	Limiting Dilution Assay
LFA-1	Lymphocyte Function Associated Antigen-1

MHC	Major Histocompatibility Complex
miH	Minor Histocompatibility Complex
MLR	Mixed Lymphocyte Reaction
MMF	Mycophenolate Mofetil
MPA	Mycophenolic Acid
mTOR	mammalian target of rapamycin
NDSA	Non-donor Specific Antibody
NFAT	Nuclear Factor of Activated T-cells
NK	Natural Killer
PAMP	Pathogen-associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor
PRA	Panel Reactive Antibodies
PRR	Pattern Recognition Receptor
PRT	Panel of Reactive T-cells
PTC	Peritubular capillaries
RTR	Renal Transplant Recipient
STAT	Signal Transducer and Activator of Transcription
Tcm	T central memory cell
TCR	T-cell Receptor
Tem	T effector memory cell
T_{fh}	T follicular cell
TG	Transplant Glomerulopathy
TGF-β	Tissue Growth Factor- beta
Th	T helper cell
TNF-α	Tumour Necrosis Factor-alpha
Treg	T regulatory cell
USRDS	United States Renal Data System
VCAM-1	Vascular Cellular Adhesion Molecule-1

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CHAPTER 1
Background and Literature Review

1 Background and Literature Review

1.1 Introduction

Transplantation is the optimal form of renal replacement therapy in many patients with end stage renal failure bringing improved survival and quality of life (Fiebiger et al., 2004). In a landmark study (Wolfe et al., 1999) comparing mortality between patients on dialysis awaiting a transplant, patients receiving a transplant and patients deemed not fit for transplant and continuing on dialysis, mortality was 68% lower in transplanted patients. These data have been replicated in a more recent smaller study from Scotland (Oniscu et al., 2005). In addition, transplantation is more cost effective than long-term dialysis (Sharif and Baboolal, 2011). Deceased donor one year graft survival in the UK has increased from 83% for the period of 2000-2002 to 93% for the period 2009-2013 (NHSBT, September 2014) which may be attributed to antimicrobial prophylaxis, better diagnosis and treatment of post-operative complications and increasing potency of immunosuppressive drugs preventing acute rejection. There have been significant effects on 1-year graft survival but, long term kidney allograft attrition rates have remained relatively constant and 10-year survival is approximately 75% (Lodhi and Meier-Kriesche, 2011).

Life expectancy beyond 10 years post renal transplantation is still considerably less than in the general population (Briggs, 2001). An analysis of the United States Renal Data System (USRDS) of 7040 deaths in renal transplant recipients from 1988-97 revealed the 3 main categories of causes of death were from cardiovascular disease (42%), malignancy (9%) and infection (18%) (Ojo et al., 2000). 31% of deaths were from unknown causes (Ojo et al., 2000). Unpublished data from 6 European Registries revealed similar statistics (Briggs, 2001). Sellares et al studied 315 allograft recipients who underwent indication biopsies ranging from 6 days to 32 years post-transplant. The findings of the study showed that 60 kidneys progressed to failure in the follow-up period (median 31.4 months). The researchers concluded that biopsies of many failures manifest features of antibody-mediated or mixed rejection (Sellares et al., 2012). Several other large retrospective studies have concluded that alloimmunity remains the most common mechanism leading to graft failure in renal transplant recipients (Gomez et al., 2013, El-Zoghby et al., 2009). El-Zoghby & colleagues histologically studied graft loss in 1317 allografts over 50.3 +/- 32.6 months of follow up.

Transplant glomerulopathy was the single most specific histological diagnosis in grafts that failed (El-Zoghby et al., 2009). Interstitial fibrosis & Tubular Atrophy (IFTA) was attributed to immunological processes in 8.5% of all graft losses. Gomez & colleagues studied 487 grafts over 114 months follow up and concluded that in 37% of the grafts that survived over 15 years, chronic allograft injury (CAI) was the main cause of failure in 66% of those cases however no differentiation between the causes of CAI was presented (Gomez et al., 2013).

1.2 Chronic allograft injury

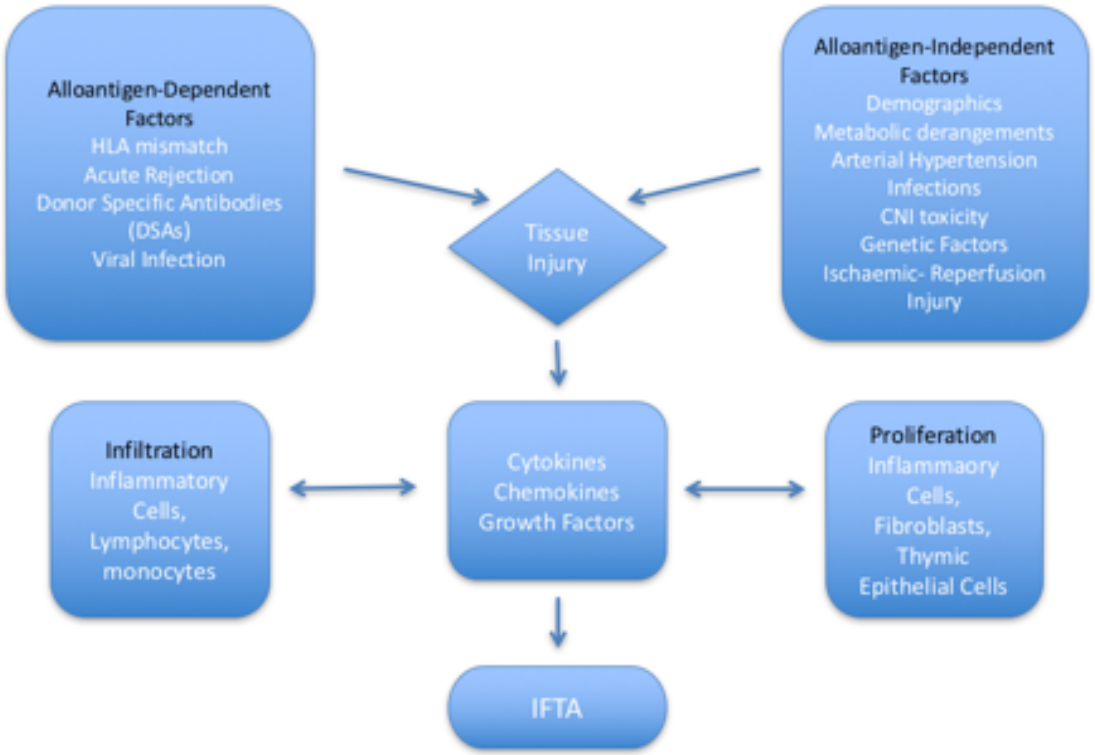
Despite improvements in immunosuppressive protocols CAI remains a major barrier for long-term graft survival. Its pathogenesis is multifactorial and clinically characterized by a slow, often discontinuous loss of graft function associated with proteinuria and hypertension (Paul, 1999). Histological changes in CAI usually precede functional deterioration and include interstitial fibrosis/tubular atrophy accompanied by vascular changes and glomerulosclerosis. In 2003 Nankivell et al published data on the natural history of CAI by studying 961 kidney transplant biopsy specimens from 120 renal transplant recipients (Nankivell et al., 2003). They observed two distinctive phases of injury. An initial phase, within 1 year of transplantation, with early tubulointerstitial damage associated with and attributed to ischaemic injury, prior severe rejection and subclinical rejection and a later phase, beyond one year, characterized by microvascular and glomerular injury. Use of Calcineurin inhibitor (CNI) exacerbated arteriolar hyalinosis, glomerulosclerosis and tubulointerstitial damage. Stegall and colleagues showed a similar progression of CAI but encouragingly fewer, less severe and less progressive chronic histologic changes in the first 5 years after transplantation (Stegall et al., 2011)

The non-specific histological changes and the association of CAI with immunologically relevant and non-immunological variables limit its clinical utility. The role of the immune system and immune mediated risk factors are discussed further in section 1.4. There are several non-immune mediated risk factors associated with the development of CAI. Increasing donor age has been linked with an increased risk of CAI. This was previously thought to be due to reduced renal mass leading to secondary hyperperfusion and glomerulosclerosis. However, pathological lesions observed in CAI correlate with changes

seen in ageing kidneys (Melk et al., 2000) leading to suggestions that accelerated senescence of renal cells might lead to deterioration of graft function (Melk and Halloran, 2001). Human Cytomegalovirus (CMV) infection is also an established risk factor for developing CAI. Animal studies found that CMV infection intensified the inflammatory response and expression of Intracellular adhesion molecule-1 (ICAM-1), vascular cellular adhesion molecule-1 (VCAM-1), tissue growth factor (TGF)-beta, platelet derived growth factor (PDGF) and connective tissue growth factor resulting in rapid progression of collagen production and subsequently accelerated chronic rejection compared to uninfected animals (Lautenschlager et al., 1999). Delayed graft function (DGF) confers an increased risk of acute rejection and subsequently a higher prevalence of earlier onset CAI (Troppmann et al., 1995). The results observed with living-unrelated donors are better than with cadaveric Human Leukocyte Antigen (HLA) matched donors (Gjertson and Cecka, 2000). Donor brain death is an independent risk factor for graft failure associated with increased levels of for example endothelial E-selectin, ICAM-1 and VCAM-1(Pratschke et al., 2001). The contribution of Calcineurin inhibitors (CNIs) to the development of CAI is also well established. Large studies show that the pathological features of CNI nephrotoxicity are almost universal by 10 years post transplant (Solez et al., 1998). The role of CNI toxicity is thought to be mediated via endothelial dysfunction (Oflaz et al., 2003). Additionally, CNIs induce hyperlipidaemia and hypertension in recipients- other potential CAI contributors (Satterthwaite et al., 1998). Figure 1.1 below shows alloantigen dependent/independent factors in relation to CAI.

Figure 1.1.Alloantigen Independent/Dependent Factors leading to CAI

(Heemann and Lutz, 2013)



The presence of CAI is inferred from chronic allograft dysfunction on the evaluation of clinical parameters such as renal function (changes in serum creatinine or glomerular filtration rate (GFR) and proteinuria), plasma levels of immunosuppressive drugs, and non-specific histological findings in the graft. The primary barrier to successful transplantation at all stages is alloimmunity. This requires lifelong maintenance of immunosuppression, which paradoxically may contribute to allograft loss through various other mechanisms. The role of alloantigen dependent factors in the development of CAI is suggested by various risk factors which include (Yates and Nicholson, 2006)

1 A history of acute rejection.

2 The detection of anti-HLA antibody.

3 The effect of prior sensitization through transplantation on long-term graft outcome.

4 Increased levels of HLA incompatibility associated with worse graft outcome.

The assessment of pre-transplant immunological risk is a critical part of improving renal transplant outcome and currently involves HLA matching between donor and recipient and routine screening for Panel Reactive Antibodies (PRA) which predict worse outcome. However, these measures are focused on humoral immunity. T-cell mediated immunity as described in section 1.4 is a critical mediator of CAI and is currently not routinely assessed. Prognostic immune makers sensitive and specific enough to allow us to know the functional state of the alloimmune response at different stages during renal transplantation may allow reprogramming of the immune system to achieve a state of donor hyporesponsiveness or tolerance in order to reduced or even eliminate immunosuppression safely- eventually leading to improved graft outcome.

1.3 Histopathology of CAI

CAI is not a specific histological appearance. It may include arteriosclerosis, glomerular lesions and sclerosis, multilayering of the peritubular capillaries (PTC) and IFTA. In 1964, the term "transplant glomerulopathy" (TG) was used to describe the glomerular changes in CAI involving enlargement of the glomeruli, swelling of the endothelial and mesangial cells, mesangial matrix expansion and widening of the subendothelial zone with interposition of mesangial cells and matrix (Hamburger et al., 1964). Immunofluorescent studies of grafts with CAI show in most cases a non-diagnostic pattern of immunoglobulin deposition, although some show linear IgG deposits along the glomerular basement membrane or granular deposits of IgG and IgA in peripheral capillary loops (Habib et al., 1987). On electron microscopy examination circumferential multilamellation was found on the majority of CAI specimens and more than 7 layers of the basement membrane was found to be specific for a large number of CAI specimens (Paul, 1999). This is now recognized as a feature of chronic antibody mediated rejection.

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1.3.1 Banff Classification

In attempts to improve the reproducibility of renal transplant histology, particularly when reporting clinical studies, in 1991 a group of pathologists, nephrologists and transplant surgeons developed a scheme for the standardization of nomenclature and criteria reporting. This was finally published in 1993 after a series of revision meetings (Solez et al., 1993).

Since then, the Banff group meet every 2 years and the Banff criteria has undergone considerable revision over the years the latest being in 2013 (Haas et al., 2014). The principal diagnostic categories were: normal, antibody mediated rejection (immediate or delayed), borderline changes (suspicious for acute rejection with no intimal arteritis), acute rejection- T-cell mediated (Grades IA, IB, IIA, IIB, III), chronic allograft nephropathy (CAN-Grade I-III) and other (Racusen et al., 1999).

In 2005, the term CAN was eliminated from the diagnostic categories as it was felt the term did not encompass other potential causes of late scarring such as chronic hypertension, CNI toxicity, chronic obstruction, bacterial pyelonephritis and viral infection (Solez et al., 2007). Banff classification was further updated recognizing separate pathology of antibody mediated rejection and T-cell mediated rejection and incorporated C4d staining- a marker of antibody mediated rejection (Solez et al., 2008). Antibody mediated rejection (Category 2) was classified into acute antibody mediated rejection with C4d positivity, presence of circulating anti-donor antibodies and evidence of acute tissue injury and chronic antibody mediated rejection was defined similarly with evidence of chronic tissue injury such as double contours of the glomerular basement membrane (GBM), multi-layering of the PTC, IFTA or intimal thickening of the arteries. T-cell mediated rejection was classified into Acute T-cell mediated (similar to grades above) and chronic T-cell mediated rejection (or chronic allograft arteriopathy) with arterial intimal fibrosis with mononuclear cell infiltration and formation of neo-intima.

In 2007 PTC grading was introduced and C4d staining of the peritubular capillaries was graded based on the percentage of stained tissue on immunofluorescence and immunohistochemistry staining that has linear, circumferential staining pattern in PTC (Solez et al., 2008). C4d deposition without morphological evidence of rejection was added to the antibody mediated category (Solez et al., 2008). This was based on findings that C4d deposition occurs in 2-26% of histologically normal ABO-compatible grafts with the higher frequency in HLA- sensitized patients (Mengel et al., 2005, Haas et al., 2006). In addition these studies along with others showed C4d staining along the PTC in 25-80% of ABO-incompatible transplants but with evidence of antibody-mediated rejection (ABMR) in only 4-12% (Haas et al., 2006, Dickenmann et al., 2006).

The most recent revision in 2013 has included the C4d negative ABMR in its criteria as multiple subsequent studies supported the existence of ABMR with negative or minimal C4d deposition (Haas, 2011, Haas, 2013).

1.4 Role of the immune system in CAI

1.4.1 Innate Immunity

The innate immune system provides first line of defense against tissue damage and invading pathogens (Matzinger, 2014). Activation of vascular endothelium and the induction of various pro-inflammatory cytokines such as interleukin (IL)-1, IL-6 and Tumour Necrosis Factor (TNF)-α can be demonstrated early post-transplantation. The up-regulation of pro-inflammatory cytokines combined with increased expression of adhesion molecules (Koo et al., 1998) results in an early infiltrate of inflammatory cells, including macrophages (McLean et al., 1997). This early inflammatory response also triggers the egress of graft tissue-resident, bone marrow–derived dendritic cells (DCs) (Larsen et al., 1990a, Larsen et al., 1990b). Cells of the innate immune system including dendritic cells bear receptors (pattern recognition receptors-PRRs) that recognize and respond to molecules expressed by both pathogens (pathogen-associated molecular patterns (PAMP)) and also damaged tissue- damage/danger associated molecular patterns (DAMPS). All forms of graft injury can result in the expression of DAMPs, engagement of these patterns by PRRs and thus innate immune system activation (Cucchiari et al., 2016). A number of factors influence innate immune system activation in kidney transplantation. In donation before cardiac death; upregulation of the complement system, sympathetic overactivity and intracranial hypertension induced cytokine surges all contribute to activation of cell-adhesion molecules and leukocyte infiltration to the grafted kidney (Cucchiari et al., 2016). The most potent and best-studied activator of the innate immune system in the context of renal allotransplantation is ischaemia-reperfusion injury. Clinically, ischaemia-reperfusion injury is associated with delayed graft function, graft rejection, chronic rejection and chronic allograft dysfunction (Schroppel and Legendre, 2014, Yarlagadda et al., 2009). Ischaemia-reperfusion affects many regulatory systems that drive an inflammatory reaction within the kidney graft (Kalogeris et al., 2012). Chemokines and cytokines together with other factors promote the inflammatory response leading to activation

of the innate immune system as well as the adaptive immune system (Salvadori et al., 2015, Satpute et al., 2009). Amelioration of ischaemia-reperfusion injury may be one way in which extracorporeal machine perfusion improves outcomes such as delayed graft function (Nicholson and Hosgood, 2013)

Antigen presenting cells (APCs) bridge the gap between innate and adaptive immunity. DCs are key cells in this regard. In the absence of noxious stimuli DCs express low levels of proteins of the B7 family, CD80 and CD86. The T-cell receptor CD28 engages proteins of the B7 family, which provide a T-cell co-stimulatory signal. Co-stimulation is essential for T-cell activation (Cucchiari et al., 2016). It follows that absence of B7 protein expression results in reduced T-cell activation and proliferation. Thus, in an inflamed or acutely damaged kidney DAMPs such as toll like receptors engage PRRs on DCs resulting in enhanced B7 protein expression on the surface of DCs. Engagement of CD28 by B7 proteins provides the necessary co-stimulation that T-cells need to proliferate upon antigen recognition (Cucchiari et al., 2016). Cytotoxic T lymphocyte antigen 4 (CTLA-4) is structurally similar to CD28 and is an important negative regulator of T-cell activation (Ville et al., 2015). In contrast to CD28, which is constitutively present on T-cells, CTLA-4 is only expressed transiently following T-cell activation (except on $FoxP3^{+}$ cells where it is present constitutively) (Wing et al., 2008). CTLA-4 is thought to raise the threshold for T-Cell Receptor (TCR) signaling thereby preventing response to self-antigens. Although the precise mechanism is unclear, Qureshi and colleagues propose that CTLA-4 mediated trans-endocytosis and hence removal of B7 proteins reduces availability of these ligands to the stimulatory CD28 molecule (Qureshi et al., 2011). CD28 and CTLA-4 work together and the balance between them is critical in determining the outcome of T-cell responses; agents to modulate both pathways are in use. Blocking CD28 activation favours immunosuppression as would be desirable after organ transplantation (Lenschow et al., 1996) whilst on the other hand abrogating CTLA-4 mediated signaling enhances T-cell responsiveness (Leach et al., 1996).

Further regulation of DC activation is provided for by natural Killer (NK) cells (Raulet, 2004). NK cells interact with DCs through various cell surface receptors that are activating or inhibitory thus “quantifying danger” (Martin-Fontecha et al., 2004). Lastly the complement system (another component of the innate immune system) produces C3a and C5a which

directly activate intragraft T-cells and APCs (Zhou et al., 2007, Strainic et al., 2008). Early graft inflammation, especially that due to ischaemia-reperfusion thus sets in play a sequence of events including innate immune system activation that eventually contribute to the development of CAI (Hauet et al., 2001, Land, 2013).

1.4.2 Adaptive Immunity

Recipient adaptive immune response to an allograft can be divided up into humoral (antibody mediated) and cellular (lymphocyte mediated) mechanisms. Histocompatibility antigens play a central role in both pathways.

1.4.2.1 Major histocompatibility complex (MHC)

Histocompatibility antigens differ between members of the same species and are therefore targets of the immune response in allogeneic transplantation. In all vertebrate species, histocompatibility antigens (termed Human Leukocyte antigens (HLA) in humans) can be divided into a single albeit multigenic, major histocompatibility complex (MHC) and numerous minor histocompatibility (miH) systems. Incompatibility between donor and recipient for either MHC or miH leads to an immune response against the graft, more vigorous for MHC than miH mismatches. HLA matching significantly reduces the risk of graft rejection and failure in solid organ transplantation (Dunn et al., 2011, Rizzari et al., 2011).

The highly polymorphic HLA system can be subdivided into two major classes: MHC Class 1 and MHC Class 2. In general, MHC Class 1 molecules (HLA-A, -B, and -C) present endogenous peptides of 8–11 amino acids in length that can be recognized by $CD8^+$ T-cells, while MHC Class 2 molecules (HLA-DR, -DQ, and -DP) present exogenous peptides of 13–18 amino acids in length that can be recognized by $CD4^+$ T-cells. MHC Class 1 molecules consist of a polymorphic α chain and a non-polymorphic β2-microglobulin and have a closed peptide-binding groove. On the other hand, MHC Class 2 molecules are rather more open but similar to their Class 2 counterparts have polymorphic and non-polymorphic regions. Crystallography reveals MHC Class 2 molecules consist of 2 chains (α and β), each with distal and proximal domains. The highly polymorphic α-1 and β-1 regions are distal, whilst

the proximal α-2 and β-2 regions and well conserved and relatively non-polymorphic (Fairchild and SpringerLink (Online service), 2013).

A combination of MHC and peptide forms a compound epitope that is engaged by the antigen specific TCR. The peptide-binding groove is usually occupied by many different peptides, often derived from MHC proteins, or other self-proteins which are replaced by those derived from pathogens during infection (Chicz et al., 1992). It is the specific role of MHC Class 1 and Class 2 molecules in presenting antigenic peptide to the TCR that underlies their significance as barriers to transplantation. The highly polymorphic nature of MHC antigens is an important driver to humoral sensitisation stimulated by pregnancy, blood transfusion and prior transplantation but the immune mechanisms involved in these responses are not fundamentally different to any other antigen. The cellular immune response to alloantigen is however fundamentally different at least in magnitude, because MHC molecules bind a diverse range of endogenous peptides, which are therefore normally presented at the cell surface. Allogeneic MHC generates a correspondingly wide range of compound epitopes distinct from the repertoire generated by syngeneic MHC. These are therefore recognised as foreign and engaged by the TCR in the so-called 'direct alloimmune response'. The cellular immune response to MHC alloantigens is consequently unique in its diversity and therefore the number of T-cells that can be recruited to an immune response (Felix et al., 2007, Ely et al., 2008).

1.4.2.2 Humoral Immunity

The humoral (or antibody mediated) response involves B-Cells that recognize target antigen. In the case of renal transplantation target antigens are primarily MHC Class 1, MHC Class 2, MICA and the ABO blood group antigens. Antibody dependent cellular cytotoxicity (ADCC) is where antibody acts as a bridge between target tissue and effector cells resulting in tissue damage (Miltenburg et al., 1989). Alternatively, antibody can activate the classical complement pathway and deposit complement traces to cause endothelial damage via macrophage and neutrophil recruitment (Flowers et al., 2011). B cells and plasma cells differentiated from naïve B cell proliferation has also recently been shown to occur via the indirect pathway of allorecognition (Lanzavecchia et al., 2006). Grafts have been shown to be

infiltrated by B cells and present graft-derived antigens to alloreactive T cells (Alegre et al., 2007).

Several investigators have detected an association of circulating HLA-antibodies with an increased risk of long-term graft loss (Terasaki et al., 2007, Worthington et al., 2003). These antibodies may be donor specific (DSA) or non-donor specific (NDSA) (Hourmant et al., 2005). Although, the relative roles DSAs and NDSAs play in graft rejection is yet to be comprehensively established, it is clear that the presence of either significantly correlates with lower graft survival, poor transplant function, and proteinuria (Hourmant et al., 2005). Using HLA Class 2 single antigen beads Cai and colleagues determined that the majority of non-donor HLA antibody specificities detected in transplant recipients were due to sharing of the same epitope with donor antigen (Cai et al., 2006).

A major discovery has been the detection of complement fragment C4d in PTCs of patients with acute rejection and is linked to DSAs (Mauiyyedi et al., 2002). C4d deposition reflects complement activation via the classical pathway and represents traces of remaining alloantibodies (Regele et al., 2002). A positive correlation has been found between acute rejection episodes and the presence of CD20+ B cells and plasma cells infiltrating allografts (Hippen et al., 2005). Depletion of CD20+ Bells using a chimeric anti-CD20 monoclonal antibody (Rituximab) has been effective in treating some but not all cases of acute humoral rejection (Becker et al., 2006).

1.4.2.3 Cellular Adaptive Immunity

The antigen-specific or adaptive cellular immune response to a graft occurs in two main stages. In the afferent arm donor antigens stimulate recipient lymphocytes, which become activated, proliferate, and differentiate while sending signals for growth and differentiation to a variety of other cell types. In the efferent arm effector leukocytes migrate into the organ and donor specific alloantibodies synthesised causing tissue damage. The principle targets of immune response to allogeneic tissue are the MHC molecules, which are present on donor cells.

1.4.2.3.1 Cellular Allorecognition

Cellular allorecognition refers to T-cell recognition of genetically encoded polymorphisms between members of the same species. The principal targets of the immune response to allogeneic tissues are the MHC molecules, which are present on donor cells. This recognition of allograft MHC antigen is the primary event that ultimately leads to graft rejection. T-cells involved in allorecognition can be sensitized against alloantigens via one of 2 distinct (but not mutually exclusive) pathways first described by Lechler and Batchelor in the 1980s (Lechler and Batchelor, 1982),

- Direct pathway where the recipient T-cells recognize intact donor MHC molecules complexed with peptide on donor stimulator cells.
- Indirect pathway where recipient APC processes the donor-MHC antigen prior to presentation to recipient T-cells in a self- restricted manner.

In the last ten years, a number of publications have shown that intact donor cell-surface molecules, including MHC, can be transferred to recipient APCs leading to activation of T-cells which can directly recognize allograft cells- termed semi-direct allorecognition (Jiang et al., 2004, Afzali et al., 2008).

Direct Pathway

In order for recipient T-cells to directly bind to intact allogeneic peptide-MHC complexes, donor APC must migrate out of the graft to make direct contact with recipient T-cells within secondary lymphoid tissue. The importance of donor APC in causing graft rejection was demonstrated by prolonged survival of donor-derived APC- depleted allogeneic thyroid grafts in the absence of immunosuppression in causing graft rejection (Lafferty et al., 1976). Furthermore, MHC Class 2 deficient mice also rejected grafts when reconstituted with CD4+ T-cells (Pietra et al., 2000). It is believed that the direct pathway is responsible for early rejection because of the high frequency of T-cells capable of recognizing allogeneic MHC on donor dendritic cells (Caballero et al., 2006) Also, donor DCs are depleted rapidly after engraftment due to apoptosis and eliminated by the recipient immune system (Caballero et al., 2006). Allogeneic MHC on DCs derived from the graft will be occupied by a wide range of different endogenous peptides derived from donor tissue, both from non-polymorphic proteins

but also from MHC proteins themselves (Daniel et al., 1998). These combinations of peptide and MHC generate a range of compound epitopes that can be engaged by recipient TCR

Indirect Pathway

The indirect pathway describes the presentation of foreign MHC molecules on recipient APCs to recipient T lymphocytes. All proteins in a donor graft different to the recipient can potentially induce an antigraft response via this pathway. Antigen delivery may occur via 3 mechanisms:

a. Recipient DCs resident in secondary lymphoid tissue engulf antigens from the graft that are present in the circulation.
b. Recipient DCs resident in secondary lymphoid tissue engulf donor cells that migrate there.
c. Recipient APCs can migrate into grafts where they pick up antigen and then migrate to secondary lymphoid tissue.

Benichou et al showed that donor derived peptides were dissolved from the groove of recipient MHC molecules supporting this pathway (Benichou et al., 1992). In addition, skin graft mouse models where APC from the graft were incapable of activating CD4 T-cells, rejection still occurred involving recipient CD4 T-cells (Auchincloss et al., 1993).

Lechler & colleagues made a direct comparison of both the direct and indirect pathways in the same group of longstanding renal transplant recipients, some with good graft function and some with a diagnosis of CAI (Baker et al., 2001). Frequencies of alloreactive $CD4^+$ T-cells stimulated through the direct pathway were conducted by performing limiting dilution assays that permitted the enumeration of donor and third party specific T-cells. $CD4^+$ T-cells from all patients (irrespective of CAI or not) demonstrated donor- specific hyporesponsiveness by the direct pathway. However, patients with CAI had significantly higher responses of $CD4^+$ T-cells indirectly activated by allogeneic peptides implicating its importance in the pathogenesis of CAI. Subsequent studies however suggest that both pathways may persist and be of relevance for the pathological changes of CAI. Mouse studies demonstrated priming of T-cells by both pathways when recipient dendritic cells were co-cultured with allogeneic dendritic cells or endothelial cells (Suciu-Foca et al., 1999). Bestard et al tested alloresponses

by both pathways in 34 long standing renal transplant recipients (>2 years post transplant) via γ-interferon (Bestard et al., 2008). Directly primed alloreactive T-cell frequencies positively correlated with serum creatinine but no correlation was found with indirect alloresponses. The longer the patient had a transplant, the higher the donor-specific alloresponse by the indirect pathway possibly as with time donor-derived APC are replaced by recipient APC. This study concluded that both pathways were detectable and may have a role in affecting long-standing transplant function.

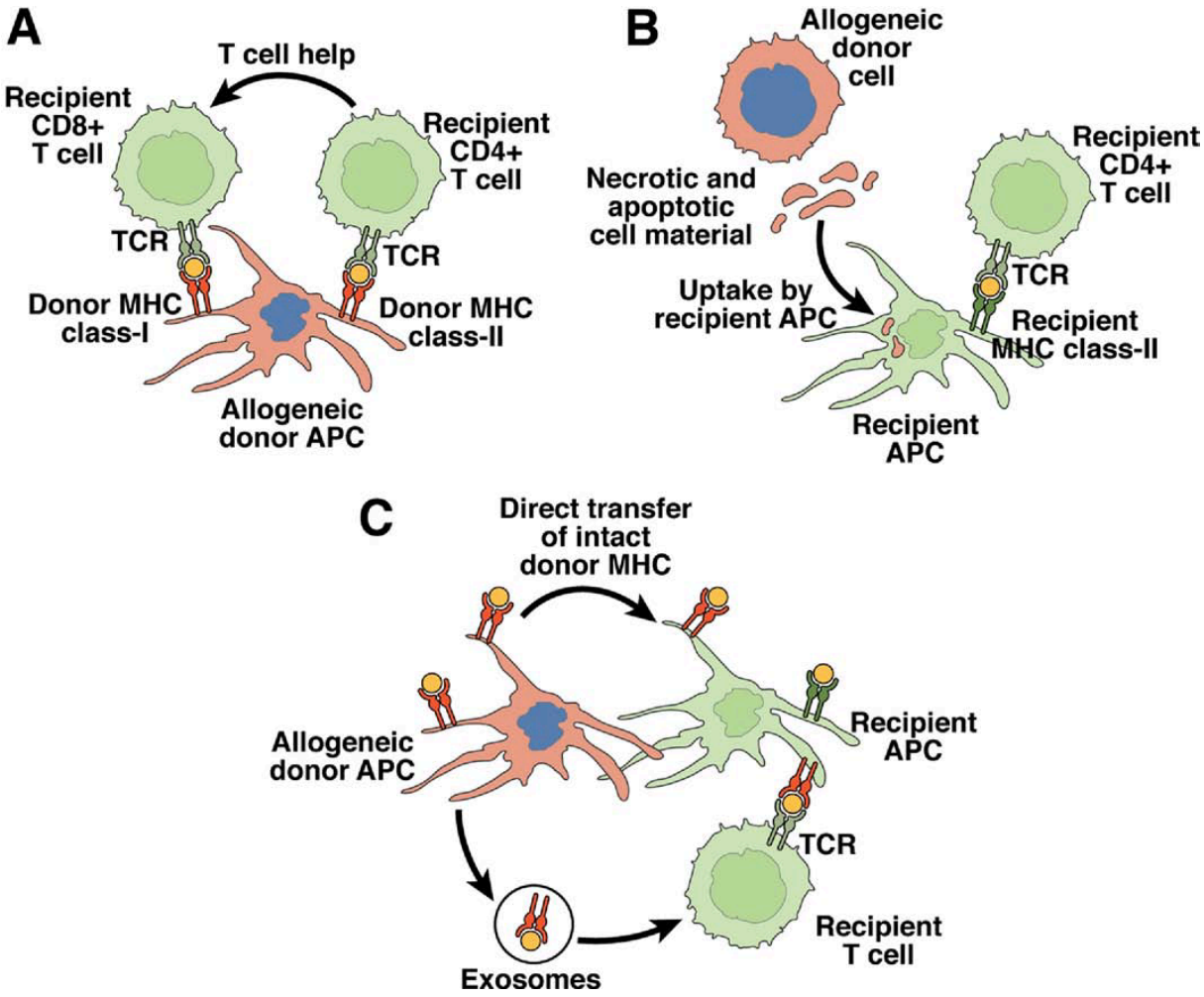
Semidirect pathway

The semidirect pathway allows the activation of both CD4+ and CD8+ cells by the same APC. There is evidence that this occurs via the direct transfer of MHC-peptide complexes from donor to recipient APC as shown in Figure 1.2C. This mode of transfer can be via the release and uptake of exosomes, or via direct cell-to-cell contact (Afzali et al., 2008).

Figure 1.2. Pathways of Allorecognition.

A.Direct pathway, B. Inirect Pathway, C.Semi-direct Pathway

(Licona-Limon et al., 2013)



1.4.3 Epitope spreading/mapping

In the indirect pathway of T-cell activation, since a continuous supply of donor allopeptides is processed and presented by host APCs, self- MHC restricted T helper cells may perpetuate the alloimmune response causing chronic rejection (Sayegh et al., 1994, Benichou and Fedoseyeva, 1996, Bradley, 1996). There is evidence in T-cell responses that intramolecular and intermolecular epitope spreading occurs during the course of autoimmune disease (Goebels et al., 2000, McRae et al., 1995, Chan et al., 1998), and indeed chronic infections. It is therefore possible that epitope spreading could be a feature of chronicity of T-cell reactivity and inflammation in (chronic) allograft rejection. In which case rejection is initiated by T cell recognition of immunodominant epitopes. Further enhancement of this acute rejection pathway is through a larger more diversified repertoire of T cells that are able to recognize and thus react to subdominant or cryptic epitopes. Peripheral lymph node APCs including antigen specific B cells and macrophages are critical to processing tissue debris and then presenting such epitopes to T cells.

1.4.4 Transplantation Tolerance

Transplant tolerance is defined as the maintenance of stable graft function without the clinical evidence of immunosuppression. This can include true tolerance defined as “the absence of any detectable detrimental immune response and no immune-suppression”, and operational tolerance defined as “the gross phenotype of tolerance with an immune response or deficit that has no significant clinical impact” (Girlanda and Kirk, 2007). An essential part of T-cell mediated immunity is to develop non-responsiveness to naturally occurring self-antigens whilst mounting an effective immune response to “foreign” antigens (Nashan et al., 1997). Initial tolerance is through a process of negative selection in the thymus during development of T-cells (Alessiani et al., 1996). High-affinity interactions of the TCR on immature thymocytes with self-antigen on thymic stromal cells results in apoptosis and elimination of such T-cells. Only T-cells with TCR of low affinity to self-antigens are released to the periphery. In the periphery, thymus derived CD25+ nTregs suppress other types of cell activation by complex mechanisms. Adaptive (antigen-induced) Tregs are generated in the periphery and act through various mechanisms including the secretion of cytokines such as

IL-10 and TGF-β. Given the presence of donor-reactive (non-self reactive) cytopathic T-cells, alloimmunity may in some circumstances be subject to the action of $CD4^{+}FOXP3^{+}$Tregs to effector T-cells that inhibit the action of donor reactive effector T-cells. This is one potential mechanism of peripheral tolerance. In these circumstances rejection or tolerance depends on the balance between donor-reactive cytopathic effector and cytoprotective Tregs.

The immune tolerance network (www.immunetolerance.org) has been set up to establish collaboration amongst research groups conducting clinical research trials looking at immune tolerance in areas of autoimmune disease, allergies and more. It also collects data on rare cases of RTRs who have become tolerant of their grafts (Matthews et al., 2003). Their focus in transplantation medicine is thus on biomarkers of tolerance and interventional trials to achieve complete and planned withdrawal of all immunosuppression whilst maintaining graft function.

1.5 T-cells in transplantation

T-cell mediated immunity is the central element of the adaptive immune system and includes a primary response by naïve T-cells, effector functions by activated T-cells and persistence of antigen-specific memory T-cells. The process is more complex however and includes other coordinated responses with other effector cells such as macrophages, natural killer cells, mast cells, basophils, eosinophils and neutrophils (Winearls, 1995). T Lymphocytes develop and mature in the thymus and express a unique TCR as part of this process (Alessiani et al., 1996). Mature T Lymphocytes (naïve T-cells) circulate though the blood and lymphatic system and reside in secondary lymphoid organs. Naïve T-cells have not been activated until they encounter foreign antigen presented to them on DCs also known as APCs in this context. DCs acquire antigen in non-lymphoid tissues and migrate into secondary lymphoid organs guided by inflammatory stimuli and cytokines. APCs generate antigenic peptides from a pathogenic agent of self-antigen by antigen processing and display then on the cell surface in the context of MHC molecules via TCR engagement, which triggers a cascade of intracellular signaling events resulting in activation of the naïve T-cell.

1.6 T-cell subsets

Thymic selection results in the appearance of T-cells with two types of TCR.

1. The majority of T-cells, which are disulfide-linked heterodimers of Ig superfamily proteins, express αβ chains in the TCR. αβTCR T-cells represent mature T-cells that circulate through secondary lymphoid organs and have a wide range of antigen recognition.
2. γδ chains are expressed by a small fraction of T-cells and are less heterogenic than αβTCR T-cells. They are not fully understood and reside in skin and other mucosal surfaces.

αβTCR T-cells are subdivided into groups based on lineage markers or functional activities. Based on lineage markers 2 groups exist:

1. $CD4^+$ T-cells- these express the co-receptor molecule CD4 and recognize antigen in the context of MHC Class 2 molecules expressed on B cells, macrophages and DCs. They produce cytokines as effector T helper cells.
2. CD8+ T-cells- express the co-receptor CD8 and activated by antigenic peptides presented by MHC Class 1 and form effector cytotoxic T lymphocytes (CTL).

There are various ways to further characterize and group T-cells. Cell surface markers can be used to identify T-cells subsets, which correlate with function. This includes the identification of naïve, effector memory and regulatory cells. Activated T-cells may be differentiated on the basis of cytokine production, for example CD4 derived Th cells into IFN-γ (Th1) vs. IL4/IL5 (Th2) or CD8 T-cells into Tc1 or Tc2 subsets, yields another method of classifying these lymphocytes. These are also associated with particular patterns of transcription factor expression. Advancing knowledge about T-cell biology reveals that groups of T-cells that were initially thought to exclusively derive from one lineage may have contributions from other lineage derived cells. CD8 T regulatory cells were first described in 1970 but subsequently we have realized that a substantial contribution to T-cell mediated suppression comes from CD4 cells specifically the CD4+CD25+ compartment (Wang et al., 2009). Allowing for this complexity, it is still useful to divide T-cells by cell surface markers or cytokine profile. These are presented below.

1.6.1 Effector T-cells

1.6.1.1 $CD4^+$ Helper cells

$CD4^+$ T-cells can differentiate from pluripotent naïve cells to various different committed T helper (Th) cells:

Th1 cells

The defining features of Th1 cells are the expression of IFN-γ and differentiation mediated through signal transducers and activators of transcription STAT1 and STAT4 proteins (Weaver et al., 2007, Safinia et al., 2010). Th1 cells enhance pro-cell-mediated immunity, are of critical importance to delayed type hypersensitivity reactions and mediate the response to intracellular pathogens such as mycobacteria(Safinia et al., 2010).

Th2 cells

IL-4 and IL-2 are critical for Th2 differentiation. IL-4 induced STAT6 upregulates GATA3, the master regulator which through various mechanisms drives Th2 differentiation (Luckheeram et al., 2012). Key effector cytokines that Th2 cells produce include IL-4, IL-5, IL-6, IL-10 and IL-13. Th2 cells significantly contribute to the immune response to extracellular parasites such as helminthes and play a major role in the pathogenesis of allergic diseases such as asthma. Th2 produced IL-4 is involved in allergic inflammation, mediating IgE class switching and secretion by B cells (Steinke and Borish, 2001).

Th17 cells

IL-6, IL-21, IL-23, and TGF-β are the major signaling cytokines involved in Th17 cell differentiation, and retinoic acid receptor-related orphan receptor gamma-T (ROR γ t) is the their regulator (Luckheeram et al., 2012). Th17 cells produce the highly pro-inflammatory IL-17 and are central in the response to invading pathogens. Other cytokines expressed by Th17 cells include IL-21 and IL-22. IL-22 is known to both mediate inflammation and exhibit tissue protective properties. It works synergistically with IL-17 to enhance expression of antimicrobial peptides thus amplifying innate responses to infections (Liang et al., 2006). These cells are significant contributors to human autoimmune diseases (Afzali et al., 2007).

T regulatory cells (Tregs)

Tregs play a critical role in the maintenance of tolerance, down-regulation in the amplitude of productive immunity and prevention of autoimmune disease. A number of early studies have shown a positive correlation between Treg function and allograft survival in solid organ transplants (Demirkiran et al., 2006, Salama et al., 2003) The mechanisms by which they exert their function are still not fully known but immunosuppressive cytokines such as IL-10, IL-35 and TGF-β play a role (Roth et al., 2011). There is enough evidence to say Tregs participate in all cell-mediated responses affecting Th1, Th2, Th17, CTL and B cell reactions against self and non-self antigens (Corthay, 2009). The majority of Tregs appear within the $CD4^+$ T-cell subset and are characterized by the expression of the α-chain of the IL-2 receptor (CD25) and transcription factor Foxp3 that is essential for the development of naïve Tregs (nTregs) (Guerra et al., 2011). Activation of Tregs via stimulation of the TCR mediates function via direct cell contact through inhibitory molecules such as CTLA-4 or via secretion of IL-10 and TGF- β. IL-10 can reduce T-cell activation by suppressing differentiation of Th1 and Th2 cells therefore reducing cytokine production of IL-2, IL-5 and TNF-α (Roth et al., 2011). They can also indirectly inhibit T-cell activation by down-regulating MHC and co-stimulatory molecules on APC (Roth et al., 2011). Foxp3+ Tregs may arise from the thymus (nTregs) of may be induced in the periphery (iTregs). One important question is the extent to which these phenotypes are stable, a question that has particularly arisen for iTregs.

Th9 cells

This recently described Th cell subset preferentially produces IL-9. Th9 cell development is IL-2 dependent and strongly enhanced by TGF-β and IL-4. These cells have been shown to drive autoimmunity in experimental models of encephalitis and allergic asthma but also have beneficial effects by inhibiting melanoma cell growth (Schmitt et al., 2014) and enhancing host immunity during parasitic infections (Licona-Limon et al., 2013).

T_{fh}

T_{fh} or T follicular helper cells specialize in providing cognate help to B cells and are thus of fundamental importance in the generation of T-cell dependent B cell responses (Ma et al., 2012). These cells depend on the expression of the master regulator transcription factor Bcl6.

Th22 cells

IL-22 production was initially thought to be restricted to Th17 cells (Fujita, 2013). Recently however a unique subset of T helper cells which produce IL-22 but unlike Th17 cells lack the ability to produce IL-17 and IFN-γ have been described (Eyerich et al., 2009). Th22 cells, which express the skin homing chemokine receptors CCR4 and CCR10, reside in the normal skin but are enriched in and thought to drive inflammatory skin diseases (Fujita, 2013, Akdis et al., 2012).

1.6.1.2 CD8 T-cells

Until relatively recently, effector $CD8^+$ T-cells were thought to exist only as cytotoxic cells that secrete γ-interferon and TNF. Given the broad range of Th cells that derive from $CD4^+$ cells researchers have sought to establish whether CD8 T-cells could differentiate in a similar manner in response to environmental cues. Initially in vitro experiments revealed that CD8+ T-cells could be induced to produce other cytokines. Thus after activation in the presence of IL-4, CD8+ T-cells acquire secretion of IL-4 and IL-5, while production of γ-interferon and cytotoxic ability are decreased. Two $CD8^+$ T-cell subpopulations were defined on the basis of their Type 1 or Type 2 cytokine secretion profiles, and were termed Tc1 and Tc2, respectively (Kemp et al., 2005, Sad et al., 1995). Subsequently Tc9, Tc17 and CD8 T memory cell subsets have been described (Mittrucker et al., 2014). An overview of the different CD8 T-cell subsets is provided in Table 1.1.

Table 1.1. Overview of effector $CD8^+$ T-cell subpopulations

(Mittrucker et al., 2014)

Type	Polarizing cytokines in vitro	Important transcription factors	Effector molecules	Function
Tc1	IL-2, IL-12	T-bet, Blimp-1, Id2, IRF4	IFN-γ, TNF-α, granzymes, perforin	Immunity against intracellular pathogens and tumors
Tc2	IL-4	GATA3	IL-5, IL-13, IL-4, granzymes, perforin	Propagation of Th2-mediated allergy, contribution to arthritis
Tc9	TGF-β, IL-4	?/IRF4	IL-9, IL-10	Inhibition of $CD4^+$ T-cell-mediated colitis, propagation of Th2-mediated allergy, anti-tumor response
Tc17	TGF-β, IL-6, IL-21	RORγt, RORα, IRF4	IL-17, IL-21	Propagation of autoimmunity, immunity to viral infections, contribution to anti-tumor response
$CD8^+$ Treg	TGF-β	?/Foxp3	TGF-β, IL-10, granzymes, perforin	Regulation of T-cell-mediated responses

1.6.2 Memory T-cells

Compared to naïve T-cells, memory cells are highly sensitive to antigen and function rapidly producing effector cytokines (Rogers et al., 2000) and thus allograft damage. In clinical transplantation, and in experimental settings in which animals are maintained outside of a pathogen-free environment T-cells specific for directly presented alloantigen are predominantly memory cells. This is assumed to be consequent upon heterologous immunity that is to say cross reactivity between allogeneic MHC and self- restricted responses to pathogen associated antigen. This situation differs from the generation of memory cells specific for extrinsic antigen including indirectly presented alloantigen. In this situation the generation of T-cell memory reflects exposure to alloantigen following blood transfusion, pregnancy and previous transplantation.

As we age, the proportion of memory phenotype T-cells increases, and it is established that memory T-cells pose a significant barrier to inducing tolerance to allografts (Adams et al., 2003). When a previous antigen is encountered, memory T lymphocytes are rapidly mobilized to deliver a recall response. This enhanced memory response is often beneficial and provides immunity against recurrent pathogens. However, in transplant recipients this poses a formidable hurdle to tolerance induction. Alloantigenic stimulation of naïve T-cells provides the most direct source of alloreactive memory T-cells (Lin et al., 2015).

Memory cells ($CD3CD45RA^{neg}$) can be divided on the basis of cell surface markers into central memory (Tcm; $CD45RA^{neg}CCR7^{pos}CD62L^{pos}$) or effector memory (Tem; $CD45RA^{neg}CCR7^{neg}/CD62L^{neg}$) subsets. These subsets exhibit differing pathways of trafficking and mobilization on antigen re-exposure. The latter population becomes the dominant population after T-cell depletion (Surh and Sprent, 2008). Although T-cell depletion, a component of induction therapy prior to renal allotransplantation is considered to favor the induction of immunological tolerance (Le Moine et al., 2009), the emergence of effector memory cells in this context can have deleterious effects on the graft inducing putative ‘lymphopaenic rejection’ (Le Moine et al., 2009, Monti et al., 2008). T memory cell proliferation under conditions of lymphopaenia is termed homeostatic proliferation and yields a population of T memory cells that exhibit resistance to the suppressive effects of Tregs

(Yang et al., 2007), costimulatory blockade (Le Moine et al., 2009, Wu et al., 2004) and a variety of immunosuppressive agents (Le Moine et al., 2009). This may be relevant to autoimmune phenomena following lymphocyte depletion with alemtuzumab and to the pathogenesis of chronic graft versus host disease.

T-cells with features of memory cells can also be generated in the absence of antigen exposure and homeostatic proliferation, via heterologous immunity. Memory T-cells are generated during heterologous immunity due to infectious or environmental stimuli and have the potential to cross react with allogeneic MHCs on donor organ cells (Lin et al., 2015). Rat models demonstrate that viruses can induce the heterologous generation of alloreactive memory T-cells, which markedly hinder the response to tolerance induction regimes (Williams et al., 2002, Adams et al., 2003). Thus heterologous immunity or T-cell cross-reactivity between viral epitopes and "alloantigen" ensures a continued stimulus for memory T-cells and highlights another mechanism via which memory T-cells can contribute to chronic graft rejection (Selin and Brehm, 2007).

1.7 T-cell subset markers

1.7.1 Naïve T-cell markers

Naïve T-cells circulating in the blood express L-selectin (CD62L), CC chemokine receptor 7 (CCR7) and leucocyte function antigen-1 (LFA-1). These mediate the rolling, adhesion and extravasation of cells through the high endothelial venules (HEVs) in peripheral lymph nodes and mucosal lymphoid organs.

1.7.2 Effector T-cell markers

Once T-cells are activated phenotypic changes occur-CD69 and CD25 (IL-2Ra) are expressed very early upon activation. Other important surface receptors are CD40 and CD28 the latter being a co-stimulatory signal binding to CD80 and CD86 enhancing IL-2 production and increasing T-cell activation. TNF receptor family molecules OX-40, CD27 and 4-1BB are also found on primary activated T-cells.

1.7.3 Memory T-cell markers

CD45RO is an activation marker exhibited on memory T-cells. Additionally, adhesion molecules such as CD44 and CD11a promote efficient interactions with APCs. Sallusto et al (Sallusto et al., 1999a) showed that the expression of CCR7 a chemokine receptor that controls homing to secondary lymphoid organs divides human memory T-cells in 2 subsets:

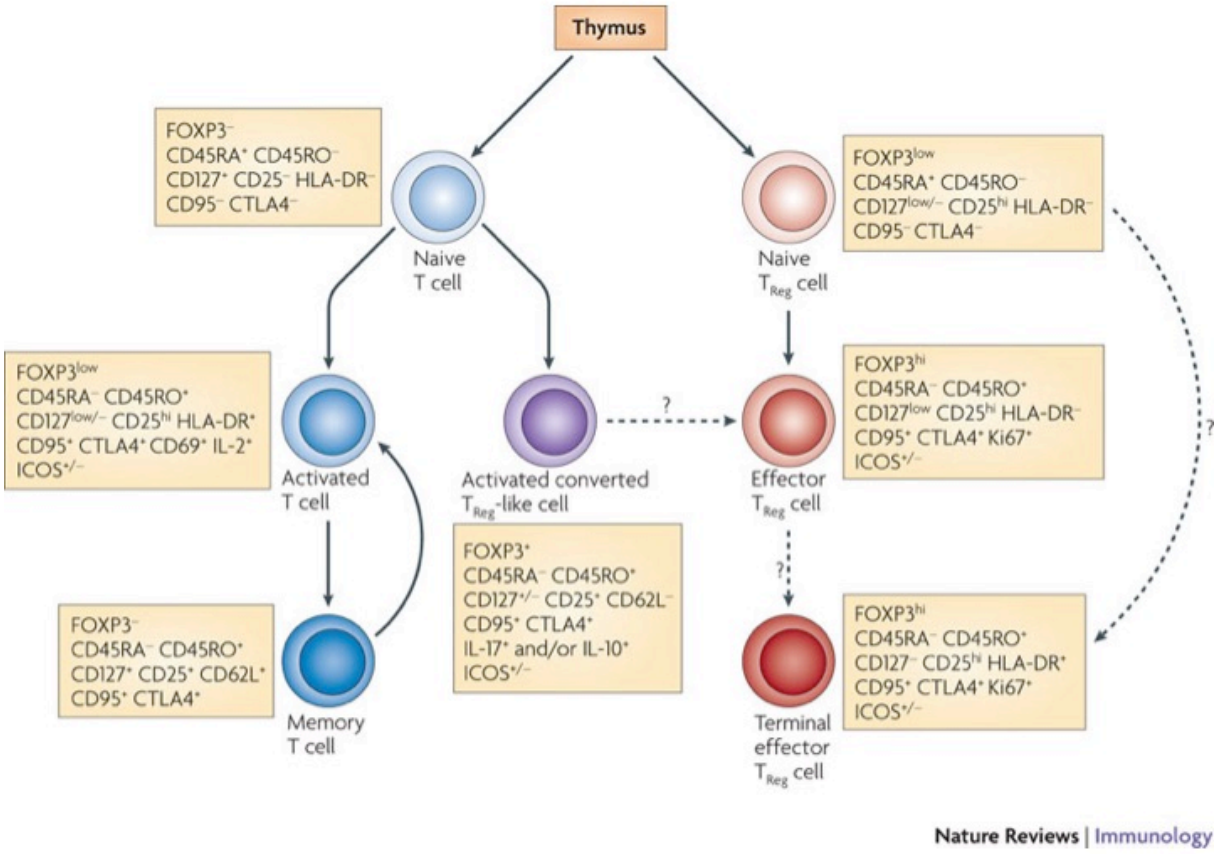
1. $CCR7^-$ memory cells express receptors for migration to inflamed tissue and have immediate effector function and are termed effector memory T-cells (Tems).
2. $CCR7^+$ memory cells express lymph node homing receptors and lack immediate effector function, these are termed T central memory cells (Tcms). $CCR7^+$ cells however efficiently stimulate dendritic cells and differentiate into $CCR7^-$ effector cells upon secondary stimulation (Sallusto et al., 1999b).

1.7.4 Regulatory T-cell markers

Many studies indicate CD25 is a crucial cell surface marker for the T regulatory subset (Kasiske et al., 2000b, Szenohradszky et al., 1999). In 2003, several elegant studies discovered a marker and functional regulator of Treg cell development and function, transcription factor Foxp3 (Hollander et al., 1997, Kasiske et al., 2000a). However, as Foxp3 is an intracellular protein, it cannot be used to separate human Treg cells for functional studies or in vivo expansion during cellular therapy, thereby limiting its use in the human setting. Liu et al demonstrated that CD127 is an excellent cell surface biomarker of human Tregs especially when used in combination with CD25 (Jiang et al., 2004). CD127 expression inversely correlated with Foxp3 expression and is expressed at low levels on a majority of Treg cells (Jiang et al., 2004).

Figure 1.3. Overview of Naïve, Memory and Effector T-cell markers

(Sakaguchi et al., 2010)



1.8 Immunosuppression in renal transplant recipients (RTRs)

Maintenance immunosuppression in RTRs generally consists of steroids, a Calcineurin inhibitor (CNI) agent (cyclosporin or tacrolimus) and an antiproliferative agent (azathioprine or mycophenolate mofetil). The introduction of CNIs in the 1980s vastly improved short-term allograft outcome. Experts in the field of renal transplantation have varying views on the use of induction agents at the time of transplantation and to this date no consensus on this topic has been reached. The long-term side effects of steroids and CNIs are well known and currently the aim of most research on immunosuppression on RTRs is the effect of steroid and/or CNI avoidance or withdrawal.

1.8.1 Immunosuppressive Agents

Available Immunosuppressive agents can be divided into 5 categories

a. corticosteroids
b. immunophilin-binding agests: ciclosporin, tacrolimus
c. mammalian target of rapamycin (mTOR) inhibitors: sirolimus, everolimus
d. antiproliferative agents: azathioprine, mycophenolate mofetil (MMF)
e. antibodies : basiliximab, alemtuzamab, daclizumab (antilymphocyte and antithymocyte globulins)

These agents and their use in significant clinical trials in renal transplantation are discussed in further detail below.

1.8.1.1 Induction agents

Currently, 2 induction agents, both IL-2 receptor antagonists (basiliximab and daclizumab) have been FDA approved for use in kidney transplantation. The underlying idea behind using these agents is to decrease the risk of acute allograft rejection and to allow the use of a lower intensity maintenance regime. However, the benefit of this is still debated within the transplantation community and the use of induction agents is center specific. Both antibodies block the IL2 receptor a-chain (CD25) thus preventing the formation of high affinity IL2 (Martin, 2012). A pooled analysis of two trials that compared daclizumab with placebo

showed significantly fewer acute rejection episodes in patients treated with daclizumab (Ekberg et al., 2000) however no difference in graft survival, patient survival or serum creatinine concentration at 3 years (Bumgardner et al., 2001). Three similar trials using basiliximab as the induction agent showed similar results versus placebo. (Nashan et al., 1997, Kahan et al., 1999, Ponticelli et al., 2001).

A 3rd induction agent, alemtuzamab- an anti-CD52 T-cell and B-cell depleting antibody has been tested clinically in renal transplantation in a number of randomized trials. The "3C" multicenter study, compared induction with alemtuzamab followed by low dose tacrolimus and MMF versus basiliximab induction followed by tacrolimus and MMF and steroids maintenance in over 800 RTRs (Group et al., 2014). Early results of this study have shown a reduced risk of biopsy proven acute rejection in the alemtuzamab group (Group et al., 2014). A further study compared alemtuzamab induction followed by tacrolimus monotherapy versus basiliximab induction followed by tactrolimus and MMF maintenance in over 100 RTRs and found no major difference in 1-year transplant outcome between the 2 groups (Welberry Smith et al., 2013).

1.8.1.2 Corticosteroids

Early data from the Collaborative Transplant Study database showed better 5-year graft survival in patients who had been switched to a steroid free regime suggesting their detrimental effects exceeded the benefits over long term graft outcome (Opelz, 1994). Contradicting this, a Canadian multicenter steroid withdrawal study found better 5-year graft survival in patients who continued on "standard" dose prednisolone versus "ultra-low" dose (Sinclair, 1992). A study from Leiden showed increased acute rejection episodes in patients where steroids were withdrawn one-year post transplantation (Hollander et al., 1997). In a meta-analysis of nine corticosteroid withdrawal trials, the risk of acute rejection and risk of graft failure was increased (Kasiske et al., 2000a). Since this was published several other trials have also demonstrated this. Long term data to determine the appropriate timing of corticosteroid withdrawal, and the patient population for whom this is acceptable practice is lacking. The potential for earlier graft loss to acute rejection may negate any benefit of avoiding adverse effects with withdrawal or avoidance.

1.8.1.3 Azathioprine

Azathioprine is a thiopurine; it is metabolized into 6-mercaptopurine (6-MP) through reduction by glutathione. It is next enzymatically converted to 6 thioguanine (6-TG) ultimately ending up being incorporated into replicating DNA and blocking de novo purine synthesis (Nielsen et al., 2001). Data from the Collaborative Transplant Study Registry suggested that azathioprine does not improve long term outcome when used in conjunction with Ciclosporin A (CsA) and steroids (Opelz, 1994). Similar results were found in a meta-analysis of five trials comparing the same regimes as above (Kunz and Neumayer, 1997). A UK based trial found no difference in graft survival between patients on triple therapy, dual therapy with CsA and azathioprine, or monotherapy with CsA only (Griffin and Salaman, 1991).

1.8.1.4 Mycophenoloic Acid (MPA)

Mycophenolic acid works by depleting guanosine nucleotides preferentially in T and B lymphocytes thus inhibiting their proliferation (Allison, 2005). MPA when used in conjunction with cyclosporin A (CsA) decrease the incidence of acute rejection in the first 6 months after transplantation by approximately 50% compared with placebo or azathioprine (Group, 1995, Group, 1996).

1.8.1.5 Calcineurin inhibitors (CNIs)

CsA and tacrolimus (FK506) are both calcineurin inhibitors. Both drugs block T-cell proliferation by inhibiting calcineurin phosphatase thus blocking activation of the transcription factor NFAT (Nuclear factor of activated T-cells) (Reynolds and Al-Daraji, 2002). The subtle difference in mechanism of action between the two drugs involves the cytoplasmic immunophilin they each bind to prior to inhibiting calcineurin phosphatase. CsA binds to cyclophilin, a cytoplasmic propyl peptidyl isomerase whilst tacrolimus binds to the cytoplasmic immunophilin, FK binding protein 12 (FKBP12) (Hoorn et al., 2012). Concerns over CsA's nephrotoxicity and other side effects have instigated several trials of CsA withdrawal studies over the years. One report found that virtually all recipients of kidney-pancreas transplants who had received ciclosporin based immunosuppression had nephrotoxicity 10 years after transplantation (Nankivell et al., 2004). Analysis of over 12,000

transplant recipients from the Collaborative Transplant Study showed better outcome at 5 years in those who remained on CsAs versus those that remained on azathioprine and corticosteroids only after 1 year post transplantation (Opelz, 1994).

Comparing tacrolimus to CsA, several early studies showed a significant decrease in the rate of acute rejection in the tacrolimus group in the first year post transplantation in a meta-analysis comparing tacrolimus with ciclosporin (in 3 studies both patient groups received azathioprine and steroids) (Knoll and Bell, 1999, Solez et al., 1993, Vincenti et al., 2002, Johnson et al., 2000, Margreiter and European Tacrolimus vs Ciclosporin Microemulsion Renal Transplantation Study, 2002). Margreiter's European study showed no difference in patient or graft survival at 6 months (Margreiter and European Tacrolimus vs Ciclosporin Microemulsion Renal Transplantation Study, 2002). Similarly Johnson's trial when analyzed at 3 years showed no significant difference in renal function, patient or graft survival between the 2 tacrolimus groups (tacrolimus with azathioprine and tacrolimus with MMF) and ciclosporin (+MMF) group (Irish et al., 2003). A study published in 2007 collaborating with the ELITE-Symphony trial compared standard dose ciclosporin, MMF and steroids versus daclizumab induction, MMF and steroids in combination with low dose ciclosporin, or low-dose tacrolimus or low-dose sirolimus. They found that at 12 months post transplantation, there was better allograft survival, better mean eGFR and less episodes of acute rejection in those patients who had the low-dose ciclosporin regime (Ekberg et al., 2007).

Studies looking at the long-term outcomes of tacrolimus versus ciclosporin have been less straightforward. A Study from Miami Florida looked at the 8-year survival of 150 kidney transplant recipients randomized to receive tacrolimus/sirolimus, tacrolimus/MMF or ciclosporin/sirolimus. Overall patients who had tac/MMF maintenance therapy had significantly lowers rates of acute rejection, renal dysfunction, and dyslipidaemia and protocol violation. However graft survival did not significantly differ in the 3 groups at 8 years post transplantation (Guerra et al., 2011). To date, clinical trials of ciclosporin versus tacrolimus have not determined which regime is better for chronic immunosuppression in renal transplant recipients.

1.8.1.6 Sirolimus

Sirolimus and everolimus are both mammalian target of rapamycin (mTOR) inhibitors. mTOR is a protein kinase that controls cell growth, proliferation, and survival. mTOR inhibitors bind to this kinase thus inhibiting its function (Ballou and Lin, 2008). Initial trials with sirolimus included its use in combination with ciclosporin: one included sirolimus or azathioprine and the other sirolimus or placebo in combination with steroids and ciclosporin. Both trials showed significantly reduced acute rejection rates within the first 6-12 months post transplantation, similar patient and graft survival at 12 months, but worse creatinine clearance in the sirolimus groups at 12 months (Kahan, 2003, MacDonald and Group, 2001). Further trials with sirolimus then compared its use versus ciclosporin. Both studies and a further analysis pool showed no difference in acute rejection rates, no difference in patient or graft survival at 12 months, but a better creatinine clearance at 12 months in the sirolimus group (Groth et al., 1999, Kreis et al., 2000, Morales et al., 2002). Early post transplantation complications of sirolimus (poor wound healing, lymphoceles and delayed graft function) seen in these studies have limited the use of sirolimus de novo. Instead an important multicenter study evaluated the use of Sirolimus in an early CNI withdrawal regimen where patients were randomized to either CNI withdrawal or conversion to Sirolimus or CNI continuation 3 months post transplantation (Johnson et al., 2001, Oberbauer et al., 2003). There was a significantly increase rate of acute rejection in the CNI withdrawal group (9.8% Vs. 5.1%). There was no difference in patient or graft survival at 24 months, but the CNI withdrawal group had improved eGFR, lower serum creatinine level at 12 months, which persisted at 24 months. This data suggests that sirolimus may have a favourable role in CNI free maintenance therapy however the potential long term effects are unknown. Current studies are also evaluating the role of sirolimus conversion for CNI-induced nephrotoxicity.

1.8.1.7 Other immunosuppressive agents in kidney transplantation

Everolimus similar to sirolimus is a mammalian target of rapamycin (mTOR) inhibitor. Its benefit over CNI based treatment was assessed in a 2-year multicentre study (Holdaas et al.) where patients with a kidney transplant of over 6 months was converted to everolimus with CNI withdrawal or minimization or remained on CNI. As with sirolimus, there was no overall renal benefit with conversion to everolimus but more adverse events and discontinuation (Holdaas et al., 2011).

Belatacept is a second-generation variant of abatacept. It differs from its parent molecule by two amino acids within the region that binds CD28-B7 (CD80, CD86), the co-stimulatory molecules required for full T-cell activation. The US FDA approved Belatacept in 2011 based on 3-year results of 2 open labeled multicenter trials (BENEFIT and BENEFIT-EXT) that demonstrated comparable survival between belatacept and CsA (Vincenti et al., 2012, Durrbach et al., 2010). At 5 years follow up patients on belatacept had a significantly better eGFR but there was no difference in graft loss or patient death between the 2 groups (Rostaing et al., 2013).

1.8.2 Individualized immunosuppression

The use of immunosuppression to prevent allograft rejection is problematic, because these agents not only have a narrow therapeutic index, they have predictable side effects even within a therapeutic window. In the case of CNIs in particular, the narrow therapeutic index is exacerbated by significant pharmacokinetic variability, which can in part be addressed using therapeutic drug monitoring. These side effects may be a consequence of non-specific immunosuppression or other "off-target" effects by potent inhibitors of pleiotropic signaling pathways. There is good evidence that reduced exposure to immunosuppression is associated with acute rejection and in all probability chronic rejection, although it may be difficult to separate the latter from the former. Conversely, immunosuppression may lead directly to infections, including those such as BK virus that impacts directly on allograft function or contribute to malignancy. A range of "off-target" effects include diabetes mellitus, hyperlipidaemia and nephrotoxicity. There are therefore potential benefits and costs associated with different exposure to immunosuppression. The evidence for risk of acute rejection in patients who undergo immunosuppression tapering post transplantation is conflicting, positive results are often reported over short periods of follow-up but may be subject to publication bias. Johnson and colleagues (Johnson et al., 2001) found a minimal risk of acute rejection upon withdrawal of ciclosporin and maintenance on a sirolimus/steroid regime 3 months post transplantation. There was no difference in patient and graft survival between the groups and instead renal function and blood pressure improved when ciclosporin was withdrawn. In contrast, a more recent ciclosporin withdrawal trial was aborted due to

high rates of acute rejection albeit maintenance regime included a different treatment regime (MMF/prednisolone rather than sirolimus) (Asberg et al., 2013). Recently published data on 15 year follow up after CNI withdrawal showed a significantly increased number of acute rejection episodes within 18 months of CNI withdrawal and no difference in patient or graft survival (Roodnat et al., 2014).

Improved outcomes in renal transplantation over the course of the last two decades have arguably been due to increased exposure to immunosuppression in the general transplant population, this suggests caution when interpreting the literature on the withdrawal of immunosuppression. This caution is also supported by growing literature on outcomes in the setting of reduced concordance or simply increasing variability of exposure to CNIs. In many studies of immunosuppression withdrawal in renal transplantation, the selection of subjects is based on clinical criteria along with the contemporaneous histology, the role of which is primarily to rule out acute rejection. Indeed, in such populations the predicted rate of many "hard outcome measures" is low making any extrapolation into higher risk populations difficult. A reasonable question may be to ask whether it would be possible to stratify transplant recipients not only on the basis of prognosis but also likely or at least hypothesized response to treatment. A theoretical ideal would be to quantify not only the concentration of particular immune-suppressive drugs as currently done, but their effects on the responses of specific mediators of cellular and humoral immunity primarily responsible for rejection. This ideal would be further benefited if a distinction were possible between alloimmunity and immunity of infectious organisms. If a measure of donor reactive immune response could predict which patients could tolerate immunosuppressive drug reduction with minimal risk of allograft injury and which could not, it could be possible to tailor therapy for an individual patient in a truly scientific manner. This would be true stratified medicine applied to transplantation.

1.9 Immune Monitoring to predict CAI

A critical feature in many cases of CAI is believed to be an ongoing alloimmune response either via the cellular or antibody mediated pathways as described extensively above. Currently in clinical practice, immune reactivity can be estimated by measuring anti-HLA

antibodies present in the circulation, monitoring immunosuppression drug levels, or by histological evaluation of the graft. More recently, attempts have been made to evaluate renal allograft tissue injury using transcriptomic or proteomic technologies however the principle of measuring tissue injury and its mechanism at a given time-point is highly analogous to the way in which histology is currently used.

Cellular immune assays can either be donor antigen specific or antigen nonspecific. A combination of assays may allow us to identify a cohort of RTRs at risk of immune mediated CAI. Table 1.2 summarizes immune monitoring assays currently available or under investigation and a few are reviewed in greater detail below.

Table 1.2. A summary of peripheral blood based immune monitoring assays to predict CAI

MLR: Mixed Lymphocyte Reaction, PBMC: Peripheral Blood Mononuclear cells, LDA: Limiting Dilution Assays, ELISPOT; Enzyme Linked Immunosorbent Assay. Modified from (Cravedi and Mannon, 2009)

Assays	**Material Needed**	**Technique**	**Advantages**	**Disadvantages**
Cell based assays				
MLRs	PBMCs	Recipient PBMCs are cultured with irradiated donor cells. Proliferation of recipient PBMCs is assessed by titrated thymidine uptake	Relatively easy to perform Inexpensive	Time consuming Poor reproducibility Radioactive material
LDAs	PBMCs	Frequency of alloreactive T-cell precursors is estimated through mixing serial dilutions of recipient cells with donor cells and measuring cytokine secretion, proliferation or cytotoxicity	Sensitive	Complex technique Time consuming
ELISPOT	PBMCs	Recipient PBMCs are cultured with donor cells in culture wells coated with a capture antibody specific for the cytokine of interest. Each cell primed to the stimulating antigen(s) can be detected by the "spots", resulting from the binding of secreted cytokine with a labelled secondary antibody	Reproducible Relatively easy to perform	Relatively expensive on a large scale

Assays	**Material Needed**	**Technique**	**Advantages**	**Disadvantages**
Direct cytotoxicity assays	PBMCs	The lytic activity of recipient cytotoxic lymphocytes toward donor cells that have been loaded with ^{51}Cr is measured		Radioactive material Complex assay Time consuming
Immune phenotyping	PBMCs	Surface and intracellular staining with antibodies	Easy to perform Sensitive	Difficult to standardize
Cylex ™ ImmunKnow® assay	PBMCs	T-cell activation after mitogen Phytohemagglutinin-L stimulation is measured. This activity is quantified by measuring the ATP produced by the activated cells	Low cost Reproducible	Not effective post CD4 T-cell depletion therapy
Genotyping	PBMCs Graft biopsy	Analysis of gene expression		Expensive
Serum based assays				
Alloantibody	Serum	Lymphocyte crossmatch (via CDC or flow cytometry) Solid phase assays with recombinant HLA proteins (ELISA, flow cytometry, Luminex®)	Sensitive assay	Potentially expensive
CD30	Serum	ELISPOT		

1.9.1 Measuring humoral responses

1.9.1.1 Donor specific antibody formation

The measurement of anti-HLA antibodies pre and post renal transplantation is routinely used in clinical practice. The presence of pre-transplant antibodies against HLA encoded antigens especially those that are expressed by the donor has been shown to be associated with acute rejection. The presence of anti-HLA antibodies is associated with poor graft outcome, delayed graft function (DGF) at 3 months and acute rejection at 3months and increased graft loss at 3 years (Susal et al., 2009). Hence, the monitoring of post transplant development of antibodies directed against donor-specific HLA Class 1 and HLA Class 2 is of paramount interest. This production of de novo donor specific antibody formation post transplantation often indicates the onset of acute antibody- medicated (humoral) rejection.

1.9.1.2 CD30

CD30 (also known as TNFRSF8) is a cell membrane protein of the TNF Receptor family. It is expressed on activated B and/or T lymphocytes and is thought to be a co-stimulatory molecule balancing regulation between Th1 and Th2 responses (Cravedi and Mannon, 2009). The soluble form- sCD30, can be measured in the serum of most individuals. Studies have shown an increase in sCD30 in patients with haematological malignancies, viral infections and inflammatory conditions such as SLE and Rheumatoid arthritis (Oflazoglu et al., 2009, Gerli et al., 1995) A study in 206 RTRs, showed increased levels of pre-transplant sCD30 was predictive of poor graft outcome (Heinemann et al., 2007). A Chinese study in 231 RTRs showed that patients who suffered an acute rejection episode had significantly higher serum levels of sCD30 at Day 5 post transplantation but the levels diminished to baseline thereafter (Dong et al., 2006). It is thus postulated that monitoring of sCD30 pre and post transplantation may identify a subgroup of patients at increased risk or acute rejection in the early post transplant period.

1.9.2 Measuring cellular immune responses

1.9.2.1 Mixed lymphocyte reactions (MLR) and Limiting Dilution Assays (LDAs)

A 2-way MLR assay involves mixing peripheral blood lymphocytes from 2 individuals and measuring lymphocyte proliferation by thymidine uptake. A 1-way MLR involves inactivating donor lymphocytes and only allowing recipient lymphocytes to proliferate. In LDAs recipient and donor peripheral blood lymphocyte are mixed in serial dilutions and cytokine secretion/proliferation measured as the estimated frequency of alloreactive T cells. Although these methods are useful research tools and relatively inexpensive, they are labour intensive and of limited reproducibility, limiting their use in the clinical setting.

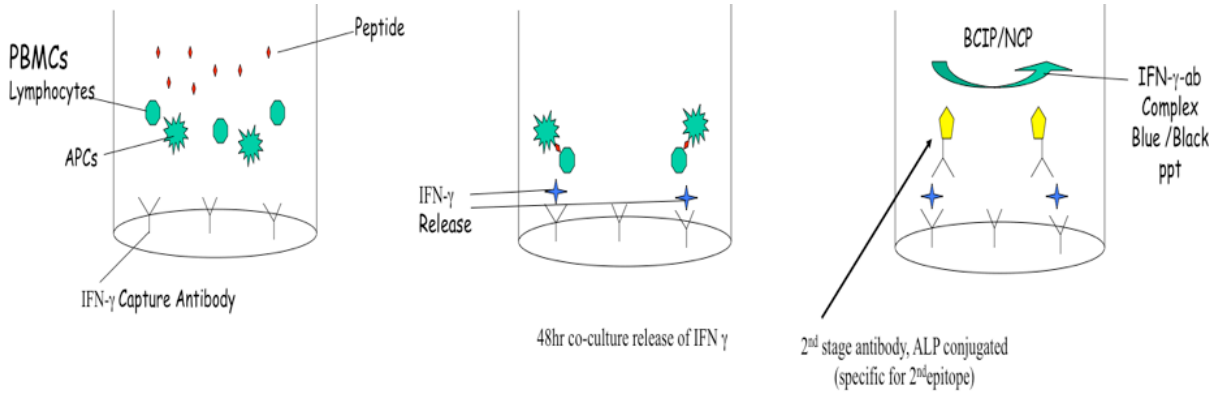
1.9.2.2 Enzyme-linked Immunosorbent Assay (ELISPOT)

Figure 1.4 shows a diagrammatic representation of a direct ELISPOT assay, which essentially combines an MLR assay with the concept of an ELISA assay. ELISPOT assays consist of tissue wells coated with antibody specific to the cytokine of interest e.g γ- interferon where T cells are cultured with donor cells or 3rd party cells. Whilst in culture, cytokines secreted remain bound using a secondary labeled antibody. Bound antibody is detected at the bottom of the wells as a "spot" which signifies a single T cell that has been primed to the stimulating antigens in vivo. Cameras on computerized plate readers then enumerate the spots digitally.

It has been hypothesized that measuring direct alloreactivity of T cells primed to donor antigens using the ELISPOT technique may identify a cohort of patients at a higher immunological risk allowing for tailored immunosuppression. Several studies have investigated this hypothesis. A study in 55 RTRs showed that ELISPOT measured alloreactivity to donor-derived antigens post transplantation significantly correlated with worse graft function (measured as eGFR) 6 to 12 months post transplantation (Hricik et al., 2003). Nather et al. showed similar results in a cohort of patients in addition to showing that pre-transplant donor-reactive ELISPOT frequency was expanded in patients with an acute rejection episode post transplantation (Nather et al., 2006). Another group used synthetic peptides corresponding to donor HLA-DR molecules and found an increased frequency of ELISPOT responses in patients with acute rejection in HLA-DR mismatched recipients (Najafian et al., 2002).

Poggio et al. were the first group to describe a Panel of T-cell reactivity (PRT) similar to Panel reactive antibodies (PRA) routinely used to measure anti-HLA antibodies in proposed renal transplant recipients (Poggio et al., 2006). They assessed reactivity of activated antidonor T cells in a haemodialysis population to a panel of allogeneic stimulator cells from healthy controls and deceased donors via γ-interferon ELISPOT assays. A significant finding was that assessment of T cell reactivity based on PRT did not predict humoral sensitization based on PRA and vice versa (Poggio et al., 2006). 12% of patients were PRA^{+}/PRT^{+}, 20% PRA^{+}/PRT^{-} and 34% PRA^{-}/PRT^{+} (Poggio et al., 2006). Initial data in 7 of the haemodialysis patients who received a renal transplant showed that a low frequency PRT positivity correlated with better transplant outcome at 1 year (Poggio et al., 2007). Subsequently, other studies have shown a significant correlation with increased PRT mean frequency with episodes of acute rejection within 6 to 12 months post transplantation (Kim et al., 2007, Koscielska-Kasprzak et al., 2009). Again, these studies suggest that a PRT may identify a subgroup of patients at a higher immunological risk (independent of PRA) or cellular-immune mediated rejection post transplantation and allow for individualized immunosuppression to minimize this risk.

Figure 1.4. A diagrammatic representation of the ELISPOT assay



1.9.2.3 Phenotyping peripheral lymphocytes

Monitoring and properly balancing the inflammatory and regulatory sides of the immune system are important in transplantation. Effector/memory T-cells associated with acute rejection (Pearl et al., 2005) can be detected and differentiated from naïve T-cells by staining for CD25, CD45RA, CD45RO, and CD62L (Seddiki et al., 2006). Regulatory T-cells, which are thought to be crucial for immunoregulation and transplant tolerance, stain positive for CD4, CD25, and FoxP3 (Sakaguchi et al., 2010, Wood and Sakaguchi, 2003). Detection of FoxP3 requires intracellular staining, fixation, and permeabilization, which impair cell viability. Staining of CD127 (IL-7 receptor) provides an alternative to intracellular FoxP3 staining. In $CD4^{+}CD25^{+}$ cells, the CD127 expression was correlated inversely with FoxP3 expression, and indeed CD4+CD25+CD127lo cells showed suppressive activity (Pelzl et al., 2003). Furthermore, an increased number of $CD4^{+}$ $CD25^{+}CD127^{hi}$-activated T-cells and a decreased number of $FoxP3^{+}$ regulatory T-cells have been associated with chronic humoral rejection in renal transplant recipients (Vallotton et al., 2011a, Braudeau et al., 2007) whereas tolerant recipients have been found to have normal numbers of regulatory T-cells similar to healthy controls. A huge advantage of flow cytometry is the capacity to quantify many immune cell subsets with great flexibility however, because flow cytometer configuration and setup, and data acquisition and analysis, will be different among centers, standardization is a challenge.

1.10 Summary of preliminary work

In this chapter I have explained the cellular responses involved in T lymphocyte activation and the justifications for the importance of the indirect allorecognition pathway in long-term renal allograft injury. The precise definition of indirect allo-epitopes would benefit the assessment of donor-specific responses pre and post transplantation to guide immunosuppressive therapy. In transplantation, this application is limited by the diversity of donor and recipient HLA combinations. Previous work in our research group and others has identified the use of the ELISPOT technique to allow the detection of antigen-specific T lymphocytes at relatively low frequencies. By studying peptides derived from the full length of HLA-A2 (which is highly polymorphic and present in 50% of the donor population) and testing responses in allosensitised haemodialysis patients via γ-interferon ELISPOT assay we were able to identify indirect allo-epitopes from conserved sequences shared by range of different alloantigens i.e. “public T cell epitopes”. In a further study in RTRs our group demonstrated responses to peptide sequences derived from the non-polymorphic α3 domain of HLA Class 1. Moreover, responses were generally to autologous sequences, i.e. “cryptic self epitopes” and were associated with markers of CAI such as eGFR.

It is based on this work that I investigated responses to non-polymorphic HLA Class 1 and Class 2 peptides as markers of an ongoing alloimmune response in RTRs in a cross sectional and longitudinal study.

1.11 Hypothesis & Aims of this thesis

T-cell mediated immunity is a critical mediator of acute rejection. It is likely that T-cells also contribute to CAI and to chronic antibody mediated rejection. Prognostic immune makers sensitive and specific enough to allow us to know the functional state of the alloimmune response may allow us to reprogramme the immune system to achieve a state of tolerance in order to reduce or even eliminate immunosuppression safely- eventually leading to improved graft outcome.

As part of this MD I wish to test two main hypotheses:

1. Responses to synthetically derived HLA peptides can be used as an additional prognostic immune marker to identify a group of renal transplant recipients' at risk of CAI.
2. Phenotypic expression of $CD4^+$ T-cell markers can be used as an additional tool to identify a group of renal transplant recipients' at risk of CAI.

My aims are to:

- Define peptides derived from the non-polymorphic region of HLA Class 2 that are predicted to bind to HLA DR.
- Measure responses to these peptides in renal transplant recipients and healthy controls.
- Optimize mixes of different HLA peptides for screening in a large number of renal transplant recipients.
- Optimize ELISPOT assay to improve reproducibility.
- Correlate responses to peptides with other markers of chronic graft injury.
- Define the subset of lymphocytes responding to HLA derived peptides and examine their phenotypic expression in the peripheral blood of renal transplant recipients.
- Examine other cytokines produced by responses to HLA derived peptides.

CHAPTER 2
MATERIALS AND METHODS

2 Materials and Methods

2.1 Materials

2.1.1 Clinical Material

All materials used were from patients who had undergone a kidney transplant and were under routine follow up at the Queen Elizabeth Hospital Birmingham or their base referring hospital if it had a dedicated transplant patient follow up service. Control samples were from normal donors at the Centre for Inflammation and Translational Research at Birmingham University. Ethical approval was obtained prior to the study from the Warwickshire Research Ethics Committee (RRK 3341) and the NRES Committee West Midlands- Solihull (RRK 4148). *Good practice* was adhered to, consisting of informed consent being obtained, the use of patient information leaflets and study specific consent forms, anonimisation of specimens with a unique identifying number, and, as stated on the application to the Regional Ethics Committee, specimens not used immediately were stored anonymously using the unique identifying number. Records of the stored specimens were maintained in a secure way by the principal investigator. Clinical Details of the participants were stored on a secure password protected server where all clinical data of patients are accessible in a clinical research file only accessible to the principal investigator and myself.

2.1.2 Buffers, Media, Solutions and Reagents

Dimethyl Sulfoxide (DMSO) (Sigma- Aldrich, Dorset, UK)

Tissue Culture Medium (Complete Medium)

Roswell Park Memorial institute (RPMI) 1640 (Sigma, Poole, UK)	50ml
L-glutamine (Sigma, Poole, UK)	2mM
Penicillin (Sigma, Poole, UK)	100IU/ml
Streptomycin (Sigma, Poole, UK)	100IU/ml
Foetal calf serum (PAA labs, Somerset, UK)	5% v/v

Ficoll Plaque (Sigma- Aldrich, Dorset, UK)

RPMI 1640 (Sigma, Poole, UK)

Phosphate Buffered Saline (PBS)

PBS tablet (Oxoid)	1 tablet

Sterile distilled water	100ml
Magnetic Activated Cell Sorting (MACs) Buffer	
Sterile PBS (pH 7.2)	500ml
Bovine Serum Albumin	0.5%
EDTA	2mM
Lipopolysaccharide (LPS) (Sigma- Aldrich, Dorset, UK)	100ng/ml
Brefeldin A (Ebioscience, ID 00-4506-51)	1:1000

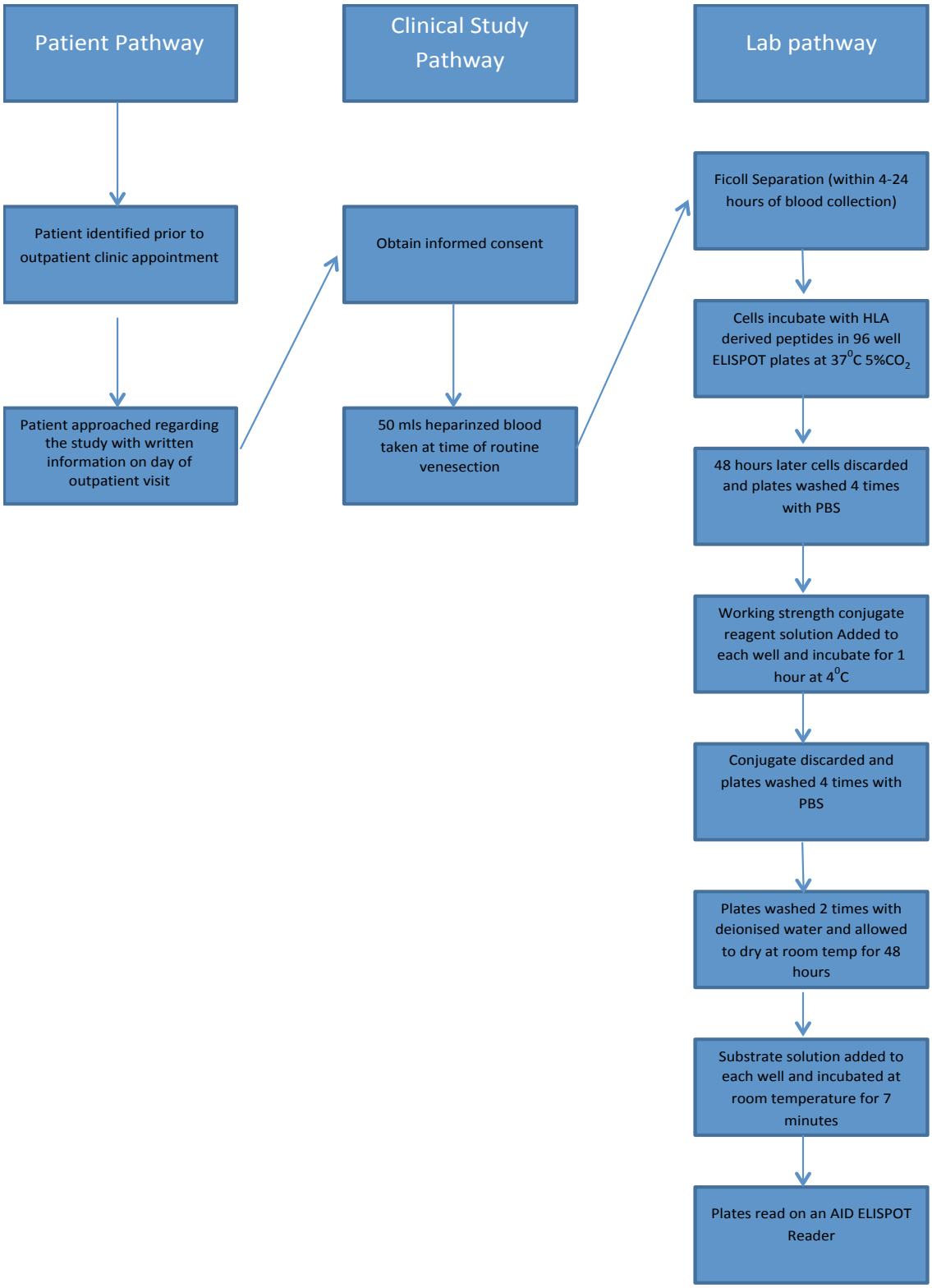
2.2 Methods

2.2.1 Patient Recruitment

2.2.1.1 Cross-Sectional Study

All renal transplant recipients attend a routine follow up clinic held twice weekly at the Queen Elizabeth Hospital Birmingham NHS trust. Potential recruits were screened a week prior to them attending the clinic. Inclusion criteria were any patient who had undergone a single organ kidney transplant (including ABO incompatible) at least 1 year ago. Patients with previous failed transplants were also included if they had a current functioning transplant. Exclusion criteria included a last-measured haemoglobin of less that 100g/dl or presence of a blood borne virus. Patients were approached at the beginning of clinic and given time to read the information leaflet (Appendix 1.1) and ask any questions. After written valid consent, samples (50mls of heparinized blood) were taken at the time of routine bleeding. If further samples were required these were taken only 3 months after the previous set with written valid consent again. Figure 2.1 shows an overview of the clinical and laboratory pathway for this study.

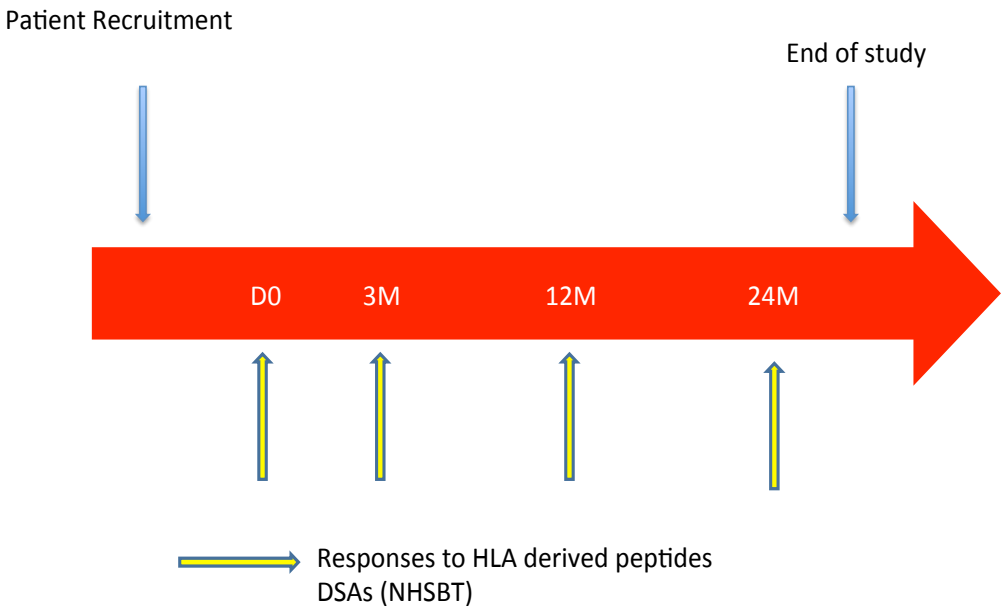
Figure 2.1. Overview of the study design for measuring responses to HLA derived peptides in renal transplant recipients.



2.2.1.2 Longitudinal Study

Patients for the longitudinal study were approached at a pre-transplant appointment (for live transplant recipients only) or at the time of admission for a transplant (live and cadaveric recipients). Patients were introduced to the study and given a written information leaflet detailing the study (Appendix A1.2, page 158). If patients were approached at a pre-transplant appointment they were then approached again at the time of admission for transplantation to obtain written valid consent. If patients were approached for the first time at time of admission they were given at least 1 hour with the information leaflet to formulate any questions before written valid consent was obtained. Inclusion criteria were any patient receiving a kidney transplant (including ABO incompatible transplants) of consenting age and exclusion criteria was last measured haemoglobin of less than 100g/dl. Figure 2.2 shows an overview of the study design.

Figure 2.2. Overview of the study design measuring responses to HLA derived peptides in a longitudinal study



2.2.2 Peptide Design and Synthesis

15 Peptides to the non-polymorphic α3 domain of HLA Class 1 were synthesized using Fmoc Chemistry (GL Biochem, Shanghai, China) as previously described and published by our laboratory (Hanvesakul et al., 2007, Smith et al., 2011). Putative HLA Class 2 binding peptides derived from sequences centered on the non-polymorphic β-2 domain of HLA Class 2 were assessed using the EPIMATRIX algorithm (www.epivax.com). In brief the EPIMATRIX programme analyses overlapping 9-mer frames offset by one amino acid. Each frame is then scored for predicted binding to common HLA Class 2 types. The EPIMATRIX raw binding score predicted for each 9-mer sequence is normalised to a distribution of scores derived from a set of randomly generated 9-mer sequences resulting in a z-score for each 9-mer. A z-score of 1.64 defines the upper 5% and 2.32 the upper 1% of the distribution. Peptides in these regions are increasingly likely to bind to the relevant MHC molecule (De Groot et al., 2008) and the majority of published T-cell epitopes fall within this range of binding scores. The physicochemical properties of peptides were predicted using the program at www.expasy.org and adjustment of their precise length undertaken to optimise solubility, stability and chemical synthesis. 9 non-polymorphic HLA Class 2 peptides were also synthesized using Fmoc chemistry (GL Biochem, Shanghai, China).

Peptide Preparation

Peptides are stored at -80°C at 20mg/mL stock solutions dissolved in Dimethyl Sulfoxide (DMSO). When required, aliquots were thawed at room temperature for 60 minutes. 20ul of peptide was diluted with 20ul of tissue culture medium (to make a concentration of 10mg/ml). To make the desired final concentration of 40ug/ml, 4ul of the 10mg/ml diluted peptide was added to 996ul of complete medium. 50ul of the final dilution was added to each well. Once thawed and diluted peptides were stored at 4^0C for 1 week only.

2.2.3 Isolation of Peripheral Blood Mononuclear Cells

PBMCs were isolated from whole blood using standard methodology of density gradient centrifuge. 50mls of heparinized blood was collected from patients after valid informed consent at the time of their routine follow up blood sampling. Samples were kept at room

temperature and processed within 4 hours of collection. 15mls Ficoll Plaque (Sigma-Aldrich, Dorset, UK) was placed in 2 separate 50mls capacity Accuspin™ tubes (Sigma- Aldrich, Dorset, UK) and centrifuged for 2 minutes at 2500rpm to allow the Ficoll to settle in the lower chamber. 25mls of collected whole blood was added to each Accuspin™ tube and made up to 50mls with 10mls of RPMI 1640 (Sigma, Poole, UK). The Accuspin™ tubes are then subjected to a 15-minute spin in the centrifuge at 2400rpm. After this time blood is separated and a fine disc of PBMCs are identifiable. This layer was carefully isolated and washed 2 more times with RPMI 1640 before pursuing further experiments. Accuspin™ tubes are fitted with a high-density polyethylene barrier, which allows a better yield of PBMCs during whole blood separation. Analysis by previous colleagues within our laboratory demonstrated a better yield than conventional methods of cell separation in renal transplant recipients. PBMCs were used for further study straight away and not frozen or store overnight.

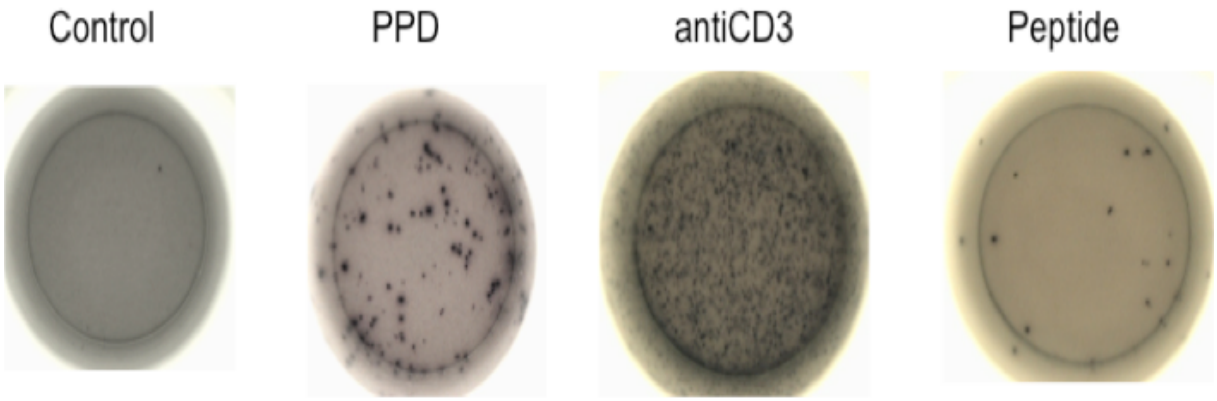
2.2.4 Enzyme-Linked Immunosorbent SPOT assays

A γ- interferon Enzyme-lined immunosorbent (ELISPOT) assay was used according to manufacturer's instructions (Oxford Immunotec, UK). PBMCs were isolated from peripheral blood by Ficoll-density gradient centrifugation as described in 2.2.3. A total of $4x10^5$ PBMCs were added to each well in a final volume of 50µl complete medium along with 50µl of peptide to give a final concentration of 20µg/ml. Peptides were used either singly or in mixes.

Negative control wells contained responder PBMCs plus medium alone. Positive control wells included Purified Protein Derivatives (PPD) (SSI, Copenhagen, Denmark) at a final concentration of 10µg/ml, and an anti-CD3 based positive control supplied by Oxford Immunotec, the manufacturer of the ELISPOT assay. PBMCs were cultured at 37^0C, 5% CO_2 for 48 hours, then discarded and washed 4 times with Phosphate Buffered Saline (PBS). Plates were then incubated at room temperature for 2 hours with 50µl per well of diluted secondary conjugate antibody (Oxford Immunotec). This was then discarded and washed a further 4 times with PBS. 50µl per well of substrate (equilibrated to room temperature) was then added the plates allowed to develop for 7 minutes. Plates were then washed twice with distilled water and allowed to air-dry at room temperature for a further 24 hours. Figure 1.4, page 48 shows a diagrammatic representation of the ELISPOT assay. Spot counts were then

counted on an AID ELISPOT reader and frequency of positive cells registered. Typical examples are shown in Figure 2.3. By previously examining the responses to a wide range of HLA derived peptides in healthy controls we are able to define an upper limit of normal of 7 spots per well (Smith et al., 2010).

Figure 2.3. Examples of typical appearance of γ-interferon spots as visualized on a developed γ-interferon ELISPOT assay with a positive response.



2.2.5 Optimization studies

To further develop and evaluate the utility and reproducibility of an ELISPOT assay for the assessment of allograft specific cellular immunity, a series of optimization tests were carried out to assess optimal temperature and incubation time for plate development and the reproducibility of reading plates with the AID ELISPOT reader. These studies were carried out at two sites- our laboratory and at Oxford Immunotec to further test reproducibility of the assay.

γ-interferon responses to HLA Class 1 derived peptide mixes were tested in RTRs. In the first experiment, assays were duplicated on two 96-well bottom plates and cultured at 37^0C and 5% CO_2 as described in section 2.2.4 (page 42). One plate was developed after 24 hours of incubation time and the 2nd plate developed after 48 hours of incubation under the same conditions. In a second set of experiments, assays were also duplicated and incubated for 48

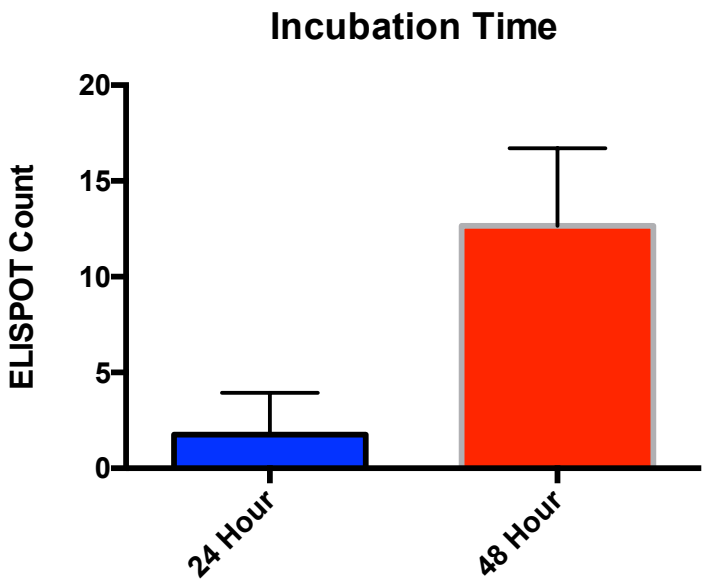
hours at 37^0C and 5% CO_2. After the addition of conjugate antibody and substrate, plates were allowed to develop for 7 minutes and then washed twice with distilled water. One plate was then allowed to air-dry at room temperature for a further 24 hours and another plate in a dry oven (37^0C) for 4 hours before spots were enumerated on an AIDS ELISPOT reader.

Incubation Time

Comparing responses between 24 hours and 48 hours of incubation showed a significant difference in ELISPOT counts with a more positive response after 48 hours of incubation (Figure 3.5). Responses were compared using a paired t test (Wilcoxon test; $p<0.0001$). This suggests that it was not possible to reduce incubation time below 48 hours without compromising sensitivity.

Figure 2.4. Comparing optimal incubation times for plate development in a γ-interferon ELISPOT assay.

ELISPOT plates incubated with $4x10^5$ PBMCs per well (duplicate plates from the same patient) and 50µl of HLA Class 1 derived peptide mixes were incubated for 24 AND 48 hours and developed as per manufactures instructions. Spots were enumarated on an AID ELISPOT reader. 48 hours of incubation showed a significant increase in responses to HLA derived peptide mixes compared to 24 hours of incubation (mean response 12.65/well Vs. 1.753/well; $p<0.0001$).

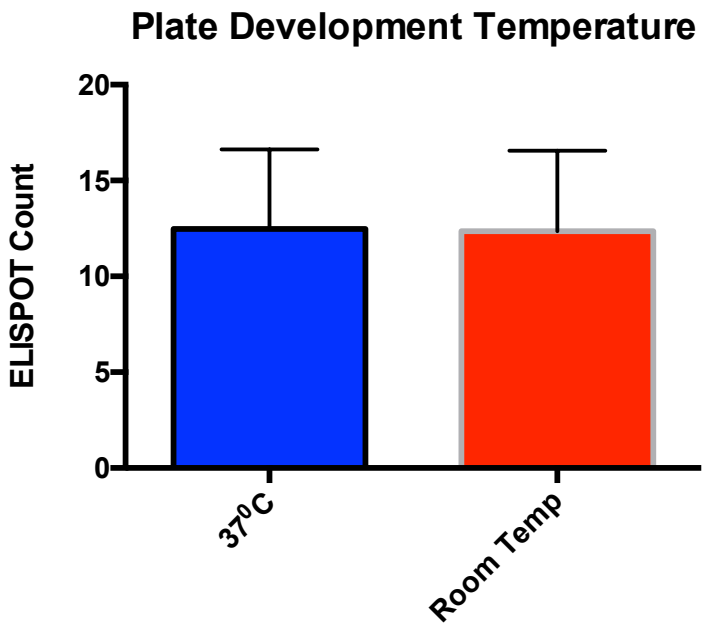


Temperature

Comparing responses between temperatures for spot formation, there was no significant difference in allowing plates to develop either for 4 hours in a dry (37^0C) oven or at room temperature (18-24^0C) for 24 hours (Figure 3.6).

Figure 2.5. Comparing optimal temperature for plate development in a γ-interferon ELSIPOT assay.

ELISPOT plates incubated with $4x10^5$ PBMCs per well (duplicate plates from the same patient) and 50µl of HLA Class 1 derived peptide mixes were incubated for 48 hours and developed as per manufactures instructions. For the final drying step, after the addition of conjugate and substrate, plates were either developed for 4 hours in a dry oven at 37^0C or for 24 hours at room temperature before being read on an AID ELISPOT reader. There was no significant difference in responses between the final step for plate development (mean response 12.48/well Vs 12.36/ well; p=0.08).



2.2.6 Cell staining and Flow Cytometry

Monoclonal antibodies used for surface staining included CD4 eFluor450, CD25 PE-Cy7, CD45RO APC, CD127-PE and CCR7 FITC

Table 2.1. List of primary antibodies used for flow cytometry.

Abbreviation used Phycoerythrin (PE), Cyanine 7 (Cy7), Allophycocyanin (APC), Fluorescein isothiocyanate (FITC). Dilution is expressed as volume of antibody used per $1x10^5$ PBMCs.

Target	Conjugate	Species	Isotype	Clone	Company	Catalogue Number	Dilution
CD4	eFluor450	Mouse	IgG_{2b}	OKT4	eBiosciences	48-0048-42	1µl
CD25	Pe-Cy7	Mouse	IgG1	BC96	eBiosciences	25-0259-42	3µl
CD45RO	APC	Mouse	IgG_{2a}	UCHL-1	BD	304210	1.6µl
CD127	PE	Mouse	IgG1	eBioRDR5	eBiosciences	12-1278-42	2µl
CCR7	FITC	Mouse	IgG_{2a}	150503	R&D	FAB197F	5µl

$1x10^6$ fresh PBMCs were incubated with monoclonal antibodies for 30 minutes at 4^0C then washed in MACS buffer twice after centrifugation at 1800 rpm. Cells were suspended in 500ul of MACS buffer and flow cytometry was performed immediately on a Beckman Coulter (CyanTMADP, High Wycombe, UK) gating on a minimum of $2x10^5$ events on the mononuclear cell gate based on forward and side scatter. Data was collected and analysed using Summit v4.3 software.

Negative Controls

Unstained cells and isotype controls (Table 2.2) were used for every antibody in every batch of experiments to confirm that primary antibody staining was specific and not a result of non-specific binding to antibody or any other proteins.

Table 2.2. List of Isotype Control Antibodies used fo flow cytometry

Isotype	Conjugate	Clone	Company	Catalogue Number
Mouse IgG2b, kappa	eFluor450	eBMG2b	eBiosciences	48-4732
Mouse IgG1, kappa	Pe-Cy7	P3.6.2.8.1	eBiosciences	25-4714
Mouse IgG2a, kappa	APC	MOPC-173	BD	400219
Mouse IgG1, kappa	PE	P3.6.2.8.1	eBiosciences	12-4714
Mouse IgG2a	FITC	20102	R&D	IC003F

Steps taken to ensure robustness of all flow data included

- Preparation of all cell suspensions in a standardized manner
- Used of fresh cells and no fixation of cells for analysis later
- Use of negative control and isotype stains in all batch experiments
- Use of the same antibody clones
- Compensation of the cytometer prior to each analysis to avoid spectral overlap and measurement artefact
- Analysis on a large number of events (10,000 events for single antibody stains, and 200, 000 events for test samples)

When using flow cytometry to analyse the expression of cell surface markers that is present in a small percentage of cells there is a risk that errors in calibration or compensation could give erroneous results. Dead or dying cells could also cause an apparent difference, however the use of viability stains and isotype controls to set compensation correctly avoided this.

2.2.7 Cell Sorting and peptide stimulation assays with different $CD4^{+}CD25^{Hi}$ Subsets

Patients previously responsive to HLA Class 1 and Class 2 peptides were used for these experiments. PBMCs were isolated as previously described in Section 2.2.3, page 56. $4.8x10^{4}$ PBMCs were saved in complete medium at room temperature for use later and $1x10^{7}$ PBMCs were surface stained with antibody using the methods described in Section 2.2.5. The stained cells were finally suspended in 500ul MACS and sorted at The Centre for Translational Inflammation Research (CTIR) using a MoFLo Legacy cell sorter. Cells were counted and sorted into 3 categories

1. Tems- T effector memory cells ($CD4^{+}CD25^{Hi}CD45RO^{+}CD127^{Hi}CCR7^{-}$)
2. Tcms- T central memory cells ($CD4^{+}CD25^{Hi}CD45RO^{+}CD127^{Hi}CCR7^{+}$)
3. Tregs- T Regulatory cells ($CD4^{+}CD25^{Hi}CD45RO^{+}CD127^{Lo}$)

A 96-well bottom plate (Oxford Immunotec, UK) was prepared containing peptides to which the patients were known to be reactive (active) or unreactive (inactive) to (Appendix 3.4). Sorted cells were then collected in sterile FACs tubes and $2x10^{3}$ cells were added into the corresponding well (Appendix 3.4) including the saved PBMCs. The cells were cultured at $37^{0}C$, 5% CO_2 for 48 hours and developed using the techniques mentioned above (Section 2.2.4).

2.2.8 Cytokine Assays

Supernatants from transplant recipients and healthy controls used in the peptide stimulation assays were saved in 96-well bottom plates and stored at $-80^{0}C$ for these experiments. The assay was performed according to manufacturer's instructions using the following human Bioplex Custom Premixed Multiplex (Bio-Rad Hertfordshire, UK): IL-10, TNF-α, IL-2, IL-1Ra and IL-1β. Plates were washed using the Bio-Rad Bioplex Pro plate washer and the assay was read using the Bio-plexTM200 system (Bio-Rad, Hertfordshire, UK). One standard was used for all 5 cytokines. 500µl of Standard Diluent was resuspended by vortex for 30 seconds and incubated on ice for 30 minutes. 60µl of thawed samples was added to the wells on a u-bottomed ELISA plate as shown in plate plan Appendix 3.5 and incubated on ice.
A standard dilution series was then carried out to produce an eight-point standard dilution curve with a fourfold dilution between each point. 8 polypropylene 1.5ml tubes were labeled

S2-S8 and Blank. 150µl of the appropriate diluent was added to tubes S2-S8. Standard left on ice was resuspended and 50µl added to tube S2. This was resuspended by vortex and a further 50µl added to tube S3 and process was continued until tube S8. Standards were then transferred to the appropriate wells on the ELISA plate. 7475µl of assay buffer was added to a falcon tube. Beads were resuspended by vortex at medium speeds for 30 seconds and 575µl of each bead was added to the buffer. This was covered with aluminum foil to be protected from light. 50ul of the coupled beads were added to each well of an assay plate supplied with the kit. This was then washed twice using the magnetic plate washer and wash buffer prepared earlier. 50ul of the standards and samples were transferred from the ELISA plate to the appropriate well of the assay plate. This was then incubated on a horizontal microplate shaker at room temperature for 30 seconds at 1000rpm and 30mins at 350rpm.

Detection antibody was prepared 10 minutes before use by adding 2700µl of the detection antibody diluent to a falcon tube, vortex for 30 seconds and then adding 300µl of antibody to the diluent. After incubation the assay plate was washed 3 times and 25µl of antibody was added to each well. The incubation process was then repeated as above. Streptavidin-RPE was prepared 10 minutes before use by adding 5950µl of assay buffer in a falcon tube along with 50ul of Streptavidin-RPE after vortex for 20 seconds.

After incubation with antibody, the assay plate was washed 3 times and 50ul of the diluted Streptavidin-RPE was added to each well. This was incubated as above for 10 minutes. The plate was washed for a final 3 times and 120µl of assay buffer added to each well. This was sealed and protected from light and placed on a microplate shaker for 30 seconds at 1000rpm. The plates were read immediately on a Bio-plexTM200 system (Bio-Rad, Hertfordshire, UK).

2.2.9 Inhibition assays of PBMC responses to HLA derived peptides

15 patients (10 responsive to HLA Class 1 derived peptides and 5 responsive to HLA class 2 peptides) were used for these experiments. PBMCs were isolated as described (2.2.3) and $1x10^7$ were surface stained and sorted using the methods as described (0). A 96-well plate was

prepared containing peptides to which the patient was known to be reactive and unreactive to as shown in Appendix 3.6.

Inhibitors of T-cell activation used for this experiment included:

1. Ciclosporin A (Sigma- Aldrich, Dorset, UK) at concentrations of 100ng/ml and 200ng/ml
2. Sirolimus (Pfizer) at concentrations of 250ng/ml and 500ng/ml
3. Abatacept (Bristol-Myers Squibb) at concentrations of 500ng/ml and 1ug/ml
4. ShK (Stichodactyla helianthus Neurotoxin) (Bachem, Germany) at concentrations of 10nM and 50nm

All inhibitors were diluted according to manufacturer's instructions. ShK is a Kv1.3 channel inhibitor, which targets T effector memory cells. In animal studies Kv1.3 blockers have treated and prevented disease in rat models of multiple sclerosis, type 1 diabetes and rheumatoid arthritis (Beeton et al., 2001, Beeton et al., 2006). The inhibitors were added to the wells as shown in Appendix 3.6 followed by $2x10^3$ sorted cells and cultured at 37^0C, 5% CO_2 for 48 hours. After incubation, cell culture supernatant cytokine concentrations were analysed as described in 2.2.8 and γ-interferon spot enumerated via the ELISPOT assay (2.2.4).

2.2.10 IL-10 Staining

50 mls of heparinized blood was taken from two patients used previously for the cytokine experiments (2.2.8). PBMCs were isolated and counted using techniques previously described (2.2.3). $5x10^6$ cells were diluted in 5mls of PBS and 50ul was added to each well of a 96-well plate (Appendix 3.7). This was done for both patients. The remaining cells were incubated with monoclonal antibodies at pre-determined concentrations (Table 2.3) for 30 minutes at 4^0C and then washed in MACs buffer twice (Centrifuged 1800 RPM). Cells were counted and suspended at a final concentration of $1x10^7$ cells per 500ul MACs buffer prior to cell sorting (MoFLo High Speed Cell Sorter-Beckman Coulter). T regulatory cells ($CD4^+CD25^{Hi}CD45RO^+CD127^{lo}$) were isolated and collected in a sterile FACs tube

containing 200ul of MACS buffer. Cells were counted, washed again with MACS buffer and resuspended in RPMI 1640 with 5% Human A/B serum at $1x10^4$ cells per ml.

Table 2.3. List of primary antibodies used for IL-10 staining experiments

Abbreviation used Allophycocyanin (APC), Cyanine 7 (Cy7), Phycoerythrin (PE), Peridinin chlorophyll (PerCP). Dilution is expressed as volume of antibody used per $1x10^5$ PBMCs

Target	Conjugate	Species	Isotype	Clone	Company	Catalogue Number	Dilution
CD4	APC-Cy7	Mouse	IgG_{2b}	OKT4	BioLegend	317417	1µl
CD25	Pe-Cy7	Mouse	IgG1	BC96	eBiosciences	25-0259-42	3µl
CD45RO	APC	Mouse	IgG_{2a}	UCHL-1	BD	340438	1.6µl
CD127	PerCP 5.5	Mouse	IgG1	A019D5	BioLegend	351321	2µl
IL-10	PE	Rat	IgG1	JES3-9D7	eBiosciences	12-7108-41	5ul
CD4	V500	Mouse	IgG1	RPA-T4	BD	560768	5ul

Cell Stimulation

The aim of the experiment was to stain for intracellular IL-10. We have already demonstrated the production of extracellular soluble IL-10 (in the supernatant from Tregs stimulated with active Peptide). For this experiment the same active peptides (Tra 33 and Tra 34) were added to the testing wells (Appendix 3,7 Rows A, B, C & E) for a 48-hour stimulation.

Controls

Our Positive control included staining for monocytes and intracellular IL-10 PE after 48 hours of stimulation with LPS.

Our negative control included incubating PBMCs alone with Tregs and no active or inactive peptide.

Plate Plan

Figure A3.7, page 156 shows the plate plan used for this experiments. As mentioned earlier, $5x10^5$ PBMCs in 50ul were added to each well used. $2x10^3$ sorted Tregs in 200ul was added to each well in the testing rows (Row A), negative control row (Row D- this also contained a HLA derived peptide the patient had previously known to be unresponsive to), supernatant row (Row E- we pooled and collected the supernatant from these wells as in the initial experiments to measure IL-10 production if required), and the unstimulated row (Row F). 100ng/ml LPS (Sigma-Aldrich) was added to each well of the positive control row (Row G). Brefeldin (Ebioscience) at 1:1000 was added to all the rows except the FMO and supernatant rows (Rows B, C & E) 6 hours before the end of the 48-hour stimulation.

After a 48-hour stimulation, cells were pooled from the wells of each row and collected in labeled standard sterile FACs tubes as shown in Table 2.4. The cells were washed twice in PBS. Viability stain eFluor506 (Ebioscience ID 65-0866-14) at a concentration of 2ul per test (1:10 diluted with PBS) was added to cells from all rows except the positive control row (row G) and incubated for 30 minutes at 4^0C. Cells were then washed once again in PBS and resuspended in 200ul of MACS buffer. Surface stains were then added to the tubes as shown in Table 2.4 and again incubated for 30 minutes at 4^0C. Compensation tubes were simultaneously stained using control PBMCs. Cells were then washed once in MACS buffer. Rows B & C were used as the FMO (fluorescence minus one) controls for CD45RO and CD127. In these experimental tubes all surface stains were added except for the

corresponding marker instead of which their isotype was added. 6.2ul of a PerCP Cy5.5 k IgG1 isotype (BD) was added and 10ul of APC k IgG2a (BD) was added.
Cells were then permeabilized and fixed using a Cytofix kit (BD, Catalogue No 554714) as per manufacturer recommendations.
PE IL-10 intracellular stain was then added to all experimental tubes apart from Tube E from which the supernatant was collected into a 2ml Accuspin^{TM} tube and frozen at -80^{0}C to be analysed later. Cells were incubated with the intracellular stain for 30 minutes at 4^{0}C, washed once with MACs buffer and finally re-suspended in 200ul MACs buffer for FACS analysis (Becton Dickinson LSRII).

Table 2.4. List of Experimental tubes and staining used for the IL-10 experiment

Tube	Description	Viability stain eFLuor506	CD4 APC- Cy7	CD25 Pe Cy7	CD45RO APC	CD127 Per CP Cy5.5	CD14 V500	IL-10 PE
A	AP Test sample	✔	✔	✔	✔	✔	✖	✔
B	CD127 FMO	✔	✔	✔	✔	✖	✖	✔
C	CD45RO FMO	✔	✔	✔	✖	✔	✖	✔
D	IP test sample	✔	✔	✔	✔	✔	✖	✔
E	Supernatent	✔	✔	✔	✔	✔	✖	✔
F	Negative control	✔	✔	✔	✔	✔	✖	✔
G	Positive Control	✖	✔	✖	✖	✖	✔	✔

2.2.11 Statistical Analysis

Continuous variables are expressed as median and interquartile range or mean and standard deviation for those normally distributed. Normality of distribution was established using a Kolmogorov Simonov test. Categorical variables were compared using the χ^2 test. Mean ELISPOT counts were compared with negative control using the t test for independent samples. The significance of any differences were assessed using a Mann-Whitney U non-parametric method and of any correlation with Pearson's rank correlation coefficient for nominal terms or Cohen's kappa statistic for categorical terms. Statistical analyses were undertaken using Minitab v16 (Minitab Inc., PA, USA) and IBM SPSS v21.0 and p values of 0.05 or less reported as significant.

CHAPTER 3
RESULTS

The use of HLA derived peptides as tools to predict Chronic Allograft Injury

3 Results: The use of HLA derived peptides as tools to predict CAI

In a previous study, indirect allo-epitopes from HLA-A2 were shown to arise not only from sequences of high polymorphism but also from relatively conserved sequences shared by a range of different alloantigens (Hanvesakul et al., 2007) The existence of such shared or 'public' indirect epitopes had been predicted by others to account for the fact that non-donor specific alloantibody is a risk factor for allograft loss (Tambur et al., 2000). Responses to peptides identical in sequence to self were also identified, similar to those 'cryptic self antigens' described in animal models of transplantation(Boisgerault et al., 2000, Lovegrove et al., 2001). If the response to these shared epitopes is representative of indirect alloimmunity in general, then they could act as surrogates from which to infer a wider relationship between clinical outcomes and this route of allorecognition. Furthermore, the small number of alleles for each epitope and the relative promiscuity of their binding to different HLA-DR suggested that an assay based on responses to such HLA derived peptides might be applied across a population using a practical number of different synthetic antigens.

Using such an assay we tested the responses to synthetic HLA derived peptides in a cross sectional and longitudinal study of renal transplant recipients and correlated these responses with clinical parameters indicative/suggestive of CAI.

3.1 Cross sectional study of T-cells responses to non-polymorphic (autologous) HLA derived peptides

Previous work undertaken in the laboratory had investigated immune responses to a range of peptides derived from HLA Class 1. A novel finding was the identification of γ-interferon responses to peptides derived from the regions of Class 1 HLA that exhibit little polymorphism in particular from the α3 domain. Furthermore, long term renal transplant recipients were significantly more likely to respond to these peptides than healthy controls (51/110 Vs. 1/18, 46.4% Vs. 5.5%, $p<0.001$) (Smith et al., 2011). The presence of responses to these non-polymorphic HLA derived peptides were also associated with CAI, defined by a reduced and falling estimated glomerular filtration rate (responders vs. non-responders; eGFR at recruitment: 39.5 Vs. 48.8ml/min, $p=0.015$; annual rate of deterioration of eGFR -4.1 vs. -1.3ml/min/year, $p=0.008$) (Smith et al., 2011). This

suggests that irrespective of the precise mechanism of their acquisition, these responses reflected a relevant change in the immune repertoire of renal transplant recipients.

Summary of Aims

1 Define peptides derived from the non-polymorphic region of HLA Class 2 predicted to bind to HLA-DR.
2 Measure immune responses to these peptides in renal transplant recipients and healthy controls.
3 Optimise mixes of different HLA derived peptides for screening of large numbers of renal transplant recipients using small volumes of blood.
4 Optimise the ELISPOT assay technique to improve reproducibility.

3.2 In silico definition of HLA Class 2 derived peptides

Putative HLA Class 2 binding peptides derived from sequences centred on the non-polymorphic β-2 domain of HLA Class 2 were assessed using the EPIMATRIX algorithm (www.epivax.com). The physicochemical properties of peptides were predicted using the program at www.expasy.org and adjustment of their precise length undertaken to optimise solubility, stability and chemical synthesis. Table 3.1 (page 52, 53) shows the peptides that were synthesized using Fmoc chemistry (GL Biochem, Shanghai, China).

Table 3.1. Sequences of non-polymorphic HLA Class 2 derived Single Peptides

These sequences were derived from the non-polymorphic β2 domain of HLA Class 2. GRAVY (grand average hydropathy) denotes the solubility of the peptides. EpiMatrix raw scores are normalized with respect to a score distribution derived from a very large set of randomly generated peptide sequences. Any peptide scoring above 1.64 on the EpiMatrix "Z" scale (approximately the top 5% of any given peptide set) has a significant chance of binding to the MHC molecule for which it was predicted.www.expasy.org. 20 peptides were synthesized and 18 (in bold) were used for further study.

Peptide	Sequence	residues in parent	MW	Length	Isoelectric point	GRAVY	Instability Index	Hits (top 10%)	Hits (top 5%)	High (Top 1%)	Epi score
DRE1	HFFNGTERVRLLERCIYNQ	45-63	2394.2	19	8.23	-0.642	S	8	7	4	10.6
DRE1A	HFFNGTERVRLLERSIYNQ	45-63	2378.22	19	8.75	-0.816	S	8	8	3	18.1
DRE2	GEYRAVTELGRPD	74-86	1461.72	13	4.87	-1.085	S	8	6	0	12.5
DRE2A	EYRAVTELGRP	75-85	1289.67	11	6.24	-0.927	S	8	7	0	14.27
DRE3	ERVRLLERSIY	51-61	1432.82	11	8.84	-0.573	U	7	5	2	7.54
DRE3A	ESFTVQRRVHPKVTV	116-130	1781.99	15	10.84	-0.487	U	7	6	0	13.26
DRE3B	ESFTVQRRVQPKVTV	116-130	1772.99	15	10.84	-0.507	U	8	6	0	13.79
DRE3C	ESFTVQRRVYPKVTV	116-130	1772.99	15	9.99	-0.36	U	7	6	0	11.21
DRE4	KVTVYPSKTQPLQHH	127-141	1761.95	15	9.7	-1.047	U	7	5	2	3.96
DRE4A	KVTVYPSKTQPLQ	127-139	1487.43	13	9.7	-0.715	U	7	5	2	5.74
DRE5	SIEVRWFRNGQEEK	155-169	1776.89	14	5.95	-1.5	U	6	5	1	6.96
DRE6	GDWTFQTLVMLETVPRSGE	180-198	2165.05	19	4.43	-0.211	S	8	8	2	16.99
DRP6A	GDWTFQTLVMLETVPRS	180-196	1978.98	17	4.37	-0.006	U	8	8	2	22.37
DRE6B	WTFQTLVMLETVPRS	182-196	1806.93	15	6	0.253	U	8	8	2	24.15
DRE6C	WTFQTLVMLETVPRSGE	182-198	1994.2	17	4.86	-0.006	S	8	8	2	18.77
DRE6D	QTLVMLETVPRSG	185-197	1429.76	13	6	0.169	U	8	7	2	16
DRE6E	HFFNGTERVRLLE	45-57	1616.84	13	6.77	-0.492	S	7	6	1	5.82
DRE7	SPLTVEWRARSESAQSK	211-227	1930.99	17	8.46	-1.071	U	8	5	2	5.92
DRE8	GLFIYFRNQKGHSGLQ	245-260	1863.97	16	9.99	-0.481	S	8	8	5	25.11

3.3 Interferon-γ responses to HLA Class 2 derived single peptides

A total of 18 peptides (13-19 amino acids long) derived from the non-polymorphic β2 domain of HLA DR were selected for further study (Table 3.1, page 52 & 53). This selection was on the basis of EpiMatrix Z score, solubility and ease of synthesis. On the basis of previous studies using HLA Class 1 peptides, single Class 2 peptides were incubated with $4x10^5$ PBMC's in γ-interferon ELISPOT plates (Oxford Immunotec) for 48 hours at 37^0C 5% CO2. At this time point plates were developed according to the manufacturers instructions and enumerated on an AID ELISPOT reader. The methodology is described in detail in section 2.2.4 page 57.

An irrelevant peptide was used as negative control. Responses to anti-CD3 and to PPD were used as positive controls. The definition of a response in this assay was previously defined as > 7 spots per well, based upon previous analysis (Smith et al., 2010).

The frequency of γ-interferon producing cells in PBMCs was assessed in 80 renal transplant recipients more than 1-year post-transplantation. At recruitment they were on stable immunosuppression with no treatment for acute rejection within the previous 3 months. The demographics and clinical details of this group are shown in Table 3.2. In addition, the response in 19 healthy volunteers (mean age 32.2 ± 10.8 years) was determined.
A response to at least one HLA Class 2 derived peptide was observed in 55 of 80 renal transplant recipients compared with 4 of 19 healthy controls (p=0.0002; Fisher's Exact). All 4 healthy volunteers had a history of previous antigen exposure via pregnancies.

Table 3.2. Patient Demographics for the 80 patients recruited into the cross sectional study to test γ-interferon responses to HLA Class 2 derived single peptides.

Characteristic	All patients (n=80)
Male (%)	51 (63.8)
Recipient age, mean (SD)	49.7 (13.31)
Time since transplant, median/yr. (interquartile range)	5.4 (3.8-11.4)
eGFR on day of study (ml/min/1.73 m^2), mean (SD)	36.8 (14.5)
Urinary albumin:creatinine ratio (mg/mmol), median (interquartile range)	3.95 (1.25-18.5)
Number of recruits with previous acute rejection (%)	32
Immunosuppression on day of recruitment, n (%)	
Corticosteroids	70
MMF	45
Azathioprine	48
Calcineurin inhibitors	88
HLA mismatch, median (interquartile range)	
HLA A	1.0 (0.0-1.0)
HLA B	1.0 (0.0-1.0)
HLA DR	0.0 (0.0-1.0)
Ethnicity (%)	
White	80
Indo-Asian	6
Afro-Caribbean	10
Mixed race	0
Other	4
Type of transplant (%)	
ABO compatible living donation	31
ABO incompatible living donation	15
Cadaveric	54
CMV status (%)	
D-/R-	27
D+/R-	23
D-/R+	17
D+/R+	33
Underlying Cause of ESRF (%)	
Glomerular	26
Hereditary Structural/Cystic Disease	25
Diabetes Mellitus	10

Characteristic	All patients (n=80)
Vascular	19
Interstitial	6
Others	14

Figure 3.1. γ-interferon responses to HLA Class 2 derived peptide in 19 healthy volunteers

ELISPOT plates incubated with $4x10^5$ PBMCs per well and 50µl of HLA Class 2 peptide were incubated for 48 hours and developed as per manufactures instructions. The frequency of cells synthesizing γ-interferon was enumerated on an AID ELISPOT reader. Only 4 of the 19 healthy volunteers responded to Class 2 HLA derived peptides (DRE2, DRE6B, DRE8, DRP6A but all had previous antigen exposure via previous pregnancies.

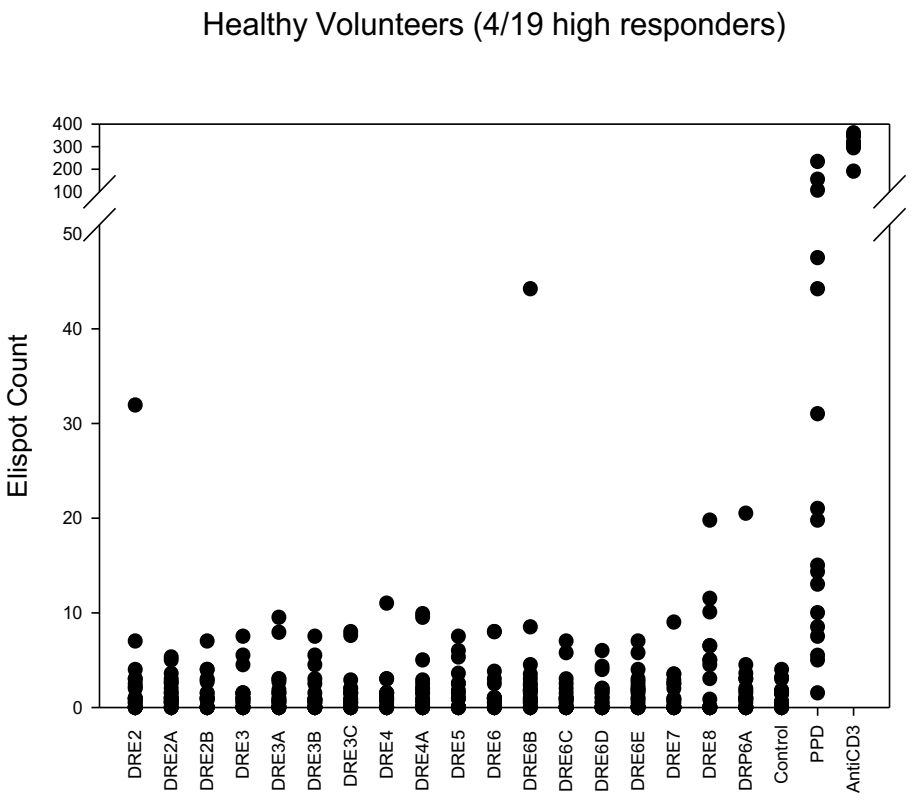
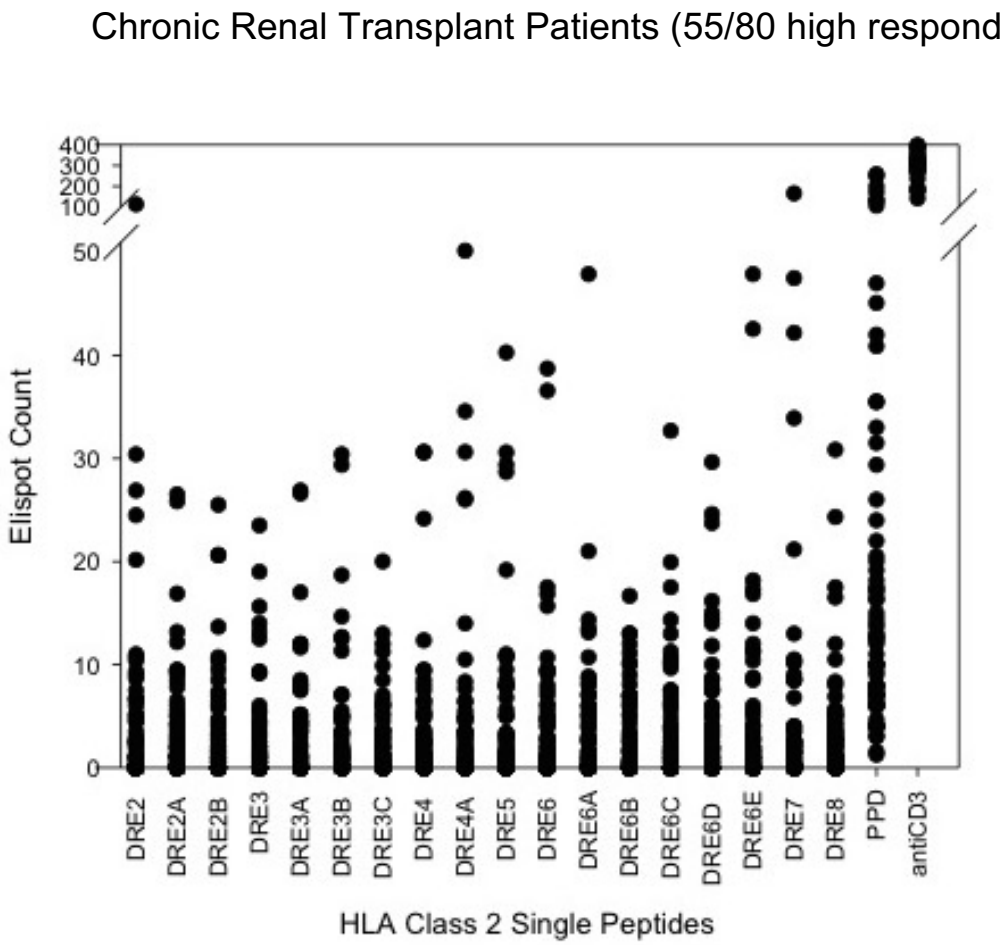


Figure 3.2. γ-interferon responses to HLA Class 2 derived peptide in 80 Renal Transplant Recipients

ELISPOT plates incubated with $4x10^5$ PBMCs per well and 50µl of HLA Class 2 peptide were incubated for 48 hours and developed as per manufactures instructions. The frequency of cells synthesizing γ-interferon was enumerated on an AID ELISPOT reader. 55 of the 80 renal transplant recipients responded to HLA Class 2 derived peptides. Responses were seen in at least 1 patient with all Class 2 peptides with the most number of responses seen in response to DRE4A, DRE5, DRE6, DRE6E and DRE7.



3.4 Correlation between responses to HLA Class 1 and Class 2 derived peptides

54 of the renal transplant recipients recruited had been previously assessed for their response to HLA Class 1 derived peptide (Smith et al., 2011). We compared these previous responses to HLA Class 1 peptides to the subsequently measured responses to HLA Class 2 peptides in the 54 patients who were in both groups. 25 patients responded to both sets of peptides whilst 20 patients did not respond to either (Chi-squared test; $p<10^{-4}$). Although tested at different time points the 45 patients whose responses correlated had stable graft function over the few years between testing. Of the 9 patients who did not correlate in responses 7 had stable graft function between the two time points tested. Interestingly, 2 of 7 patients who previously did not respond to HLA Class 1 derived peptides but subsequently responded to HLA Class 2 derived peptides, had deteriorating allograft function with positive DSAs implying a link between T-cell alloreactivity and a humoral response with de-novo DSA production. Any such speculation must however be moderated by the fact that patients had to survive with a functioning graft to be included in the later study.

Table 3.3. Correlation between responses to HLA Class 1 and Class 2 derived peptides in 54 Renal Transplant Recipients

We compared the correlation between responses to Class 1 and Class 2 HLA derived peptides. 54 patients had been previously assessed for their responses to HLA Class 1 derived peptide (Smith et al., 2011). All patients had stable graft function between testing points and statistically, responses between Class 1 and Class 2 HLA peptides correlated (Chi-squared test; $p<10^{-4}$).

	Response to Class 1 Peptide	**No Response to Class 1 Peptide**
Response to Class 2 Peptide	25	7
No Response to Class 2 peptide	2	20

3.5 γ-interferon responses to peptide mixes

The screening for responses to HLA derived peptides in these experiments required 45 mls of blood on each occasion. This was because responses to large numbers of different peptides were measured. Also the frequency of peptide specific response was likely to be low so that high numbers of PBMCs per well were used (400,000 PBMC's per well). On that basis the response to mixtures of different peptides derived from the α3 domain of Class 1 and β2 domain of Class 2 HLA were defined. Some peptides to which specific γ-interferon production was found, overlapped and/or were homologues of one another. On that basis the inclusion of overlapping or homologous sequences within the same mix was avoided. The combination of different peptides was otherwise non-purposeful. Table 3.4 shows the 7 Class 1 derived peptide mixes and 3 Class 2 derived peptide mixes that were defined.

Table 3.4. HLA Class 1 and Class 2 peptide mixes composition

Peptide mixes were defined to reduce the volume of patient blood required on each test. Frozen peptides were stored in aliquots at 1mg/ml. On the day of experiments they were mixed with complete media at the required volumes and stored at 4^{o}C for a further 1 week.

Peptide Mix						
Class 1						
Mix 2	Tra30	Tra39	Tra51	Tra02	Tra14	
Mix 3	Tra31	Tra40	Tra46	Tra49	Tra06	Tra19
Mix 4	Tra31	Tra40	Tra52	Tra06	Tra19	
Mix 6	Tra33	Tra41	Tra53	Tra08aTra24		
Mix 7	Tra34	Tra42	Tra10	Tra29		
Mix 8	P1	P20	P45/46			
Mix 9	P2	P21	P45/46a			
Class 2						
DR Mix 1	DRE6c	DRE2	DRE7			
DR Mix 2	DRE6d	DRE3c				
DR Mix 3	DRE6e	DRE4a				

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3.5.1 γ-interferon responses to HLA Class1 derived peptide mixes

The frequency of γ-interferon producing cells to Class 1 mixes were tested in 70 transplant recipients and 17 healthy controls and showed a significantly increased number of responses in transplant recipients (35/70 Vs. 4/17,p=0.04). The mean response across all tests was significantly higher in transplant recipients than in healthy controls (5.5 ± 0.3 Vs. 2.9 ± 0.35, $p<0.001$).

Figure 3.3. γ-interferon responses to HLA Class 1 derived peptide mixes in 17 healthy controls

ELISPOT plates incubated with $4x10^5$ PBMCs per well and 50µl of HLA Class 1 derived peptide mixes were incubated for 48 hours and developed as per manufactures instructions. The frequency of cells synthesizing γ-interferon was enumerated on an AID ELISPOT reader. Only 4 of the 17 healthy controls responded to these peptides. Reponses were tested in triplicate to 2 different concentrations. Responses were seen to Mix 2 and Mix 9.

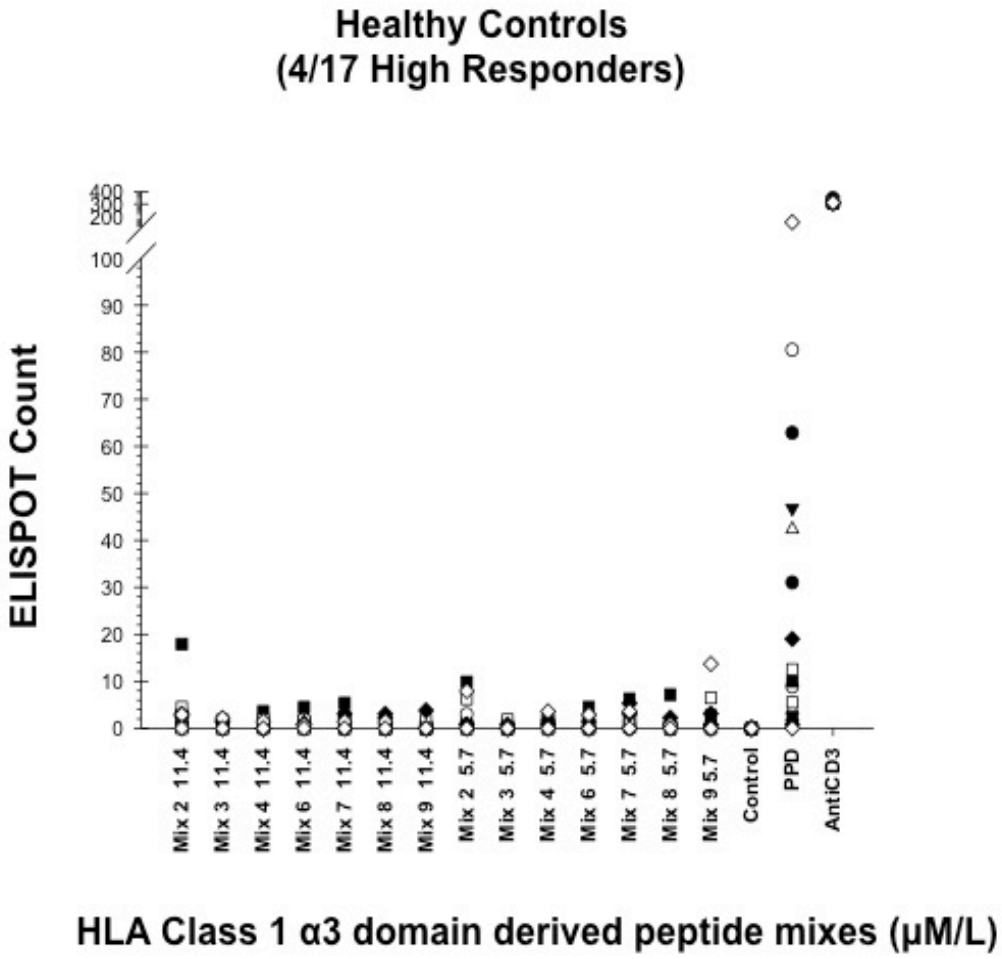
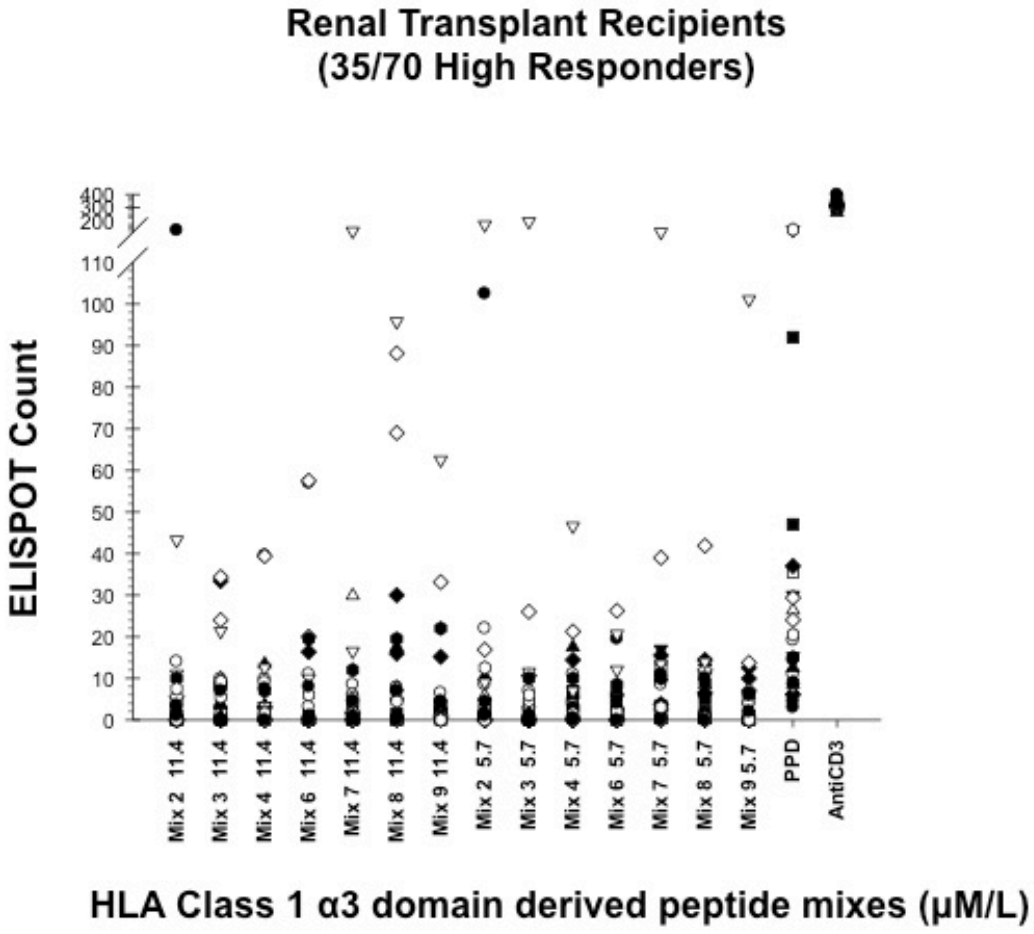


Figure 3.4. γ-interferon responses to HLA Class 1 derived peptide mixes in 70 Renal Transplant Recipients

ELISPOT plates incubated with $4x10^5$ PBMCs per well and 50µl of HLA Class 1 derived peptide mixes were incubated for 48 hours and developed as per manufactures instructions. The frequency of cells synthesizing γ-interferon was enumerated on an AID ELISPOT reader. 35 of 70 Renal Transplant responded to HLA Class 1 derived peptide mixes. Mixes were tested in triplicate at 2 concentrations as per healthy controls. Responses to all peptides by at least 1 patient were seen.



3.6 Correlation between responses to single peptides and peptide mixes

A total of 35 patients were tested to both the single and mixed peptides derived from HLA Class 1. 21 patients responded to both single and mixed peptides whilst 8 patients did not correlate in their responses. 7 of 8 of patients responded to at least one of the single peptides screened but not to peptide mixes. Table 3.2 shows the significant correlation between responses to Class 1 single peptides and peptide mixes (Chi squared test; p=0.01).

Table 3.5. Correlation between responses to HLA Class 1 single peptides and peptide mixes in 35 Renal Transplant Recipients

We directly compared the correlation between γ-interferon responses to HLA derived single peptides and peptide mixes in an ELISPOT assay in 35 renal transplant recipients and found a significant correlation (Chi squared test; p=0.01). Only 8 of the 26 patients did not correlate in their responses.

	Response to Class 1 Single Peptide	**No Response to Class 1 Single Peptide**
Response to Class 1 Peptide Mix	21	1
No Response to Class 1 Peptide Mix	7	6

3.7 Correlation between γ-interferon responses and circulating Donor specific antibodies (DSAs)

By September 2012, 130 patients had been tested to HLA derived Class 1 or Class 2 peptide mixes. These patients were recruited in a number of studies however all were at least 1-year post transplantation at the time of testing. For 95 patients we had corresponding circulating DSA data at the time of testing. Figure 3.5 shows the correlation between γ-interferon responses and the presence of DSAs. Although this did not show a significant correlation (Chi-squared test; p=0.09), as this was a cross-sectional study we cannot say how responses vary over time.

Table 3.6. Correlation between γ-interferon responses to HLA derived peptide mixes and presence of circulating DSAs.

	Response to HLA derived peptide mixes	No Response to HLA derived peptide mixes
Presence of circulating DSAs	18	23
Absence of circulating DSAs	14	41

3.8 2-year follow up data: Correlation with graft loss, graft function and patient death

We collected 2-year follow up data for the 130 RTRs tested to HLA Class 1 or HLA Class 2 derived peptide mixes. 5 patients were transferred back to their base hospitals so no follow up data was available and hence excluded from the analysis. Table 3.7 shows the 2-year follow up data for 125 RTRs; 43 RTRs responded to the peptide mixes and 82 RTRs did not. Overall, there was no significant difference in graft function (eGFR) between the two groups 2 years after testing. A small number of patients lost their graft function or died 2 years after testing but there was no significant difference between the two groups.

Table 3.7. 2-year follow up data for 125 RTRs tested to HLA Class 1 or Class 2 derived peptide mixes.

Clinical data 2 years post testing was collected for 125 RTRs whose responses were tested via γ-interferon ELISPOT assays to HLA Class 1 or Class 2 derived peptide mixes. There was no significant difference in ΔeGFR (ml/min/year) between RTRs who tested positive or negative to the peptide mixes. There was no significant difference in the number of patients who lost their graft function or died between the 2 groups. RTRs who tested positive to the mixes had an overall higher ACR 2 years post testing which was significantly different to the group with negative responses.

*ACR: albumin:creatinine ratio.

	Positive Responses n=43	**Negative Responses n=82**	**p value**
ΔeGFR (ml/min/year), mean (SD)	-0.81(3.31)	0.04(0.82)	0.09 (Student's t test)
*ACR (mg/mmol), median (interquartile range)	11.4 (55.7)	3.7 (13.5)	0.02 (Mann Whitney U test)
Graft loss (%)	6 (13.9)	13 (15.9)	0.68 (Chi-squared test)
Patient death (%)	2 (4.6)	5 (6.1)	0.73 (Chi-squared test)

3.9 Longitudinal study of T-cells responses to non-polymorphic (autologous) HLA derived peptides

As part of a study of CMV responses in 100 consecutive unselected renal transplant recipients, the response to HLA derived peptides was also studied using the methodology described above (Section 2.2.4, page 57). This group of patients was separate to the ones in the cross-sectional study. γ interferon secretion to HLA Class 1 and 2 derived peptides mixes were measured pre-transplant, and at 3,12 and 24-months post transplantation. Donor-specific antibody samples were collected at these time points as well along with clinical data including serum creatinine, urine albumin: creatinine ration (ACR), biopsy results (performed if clinically indicated), treatment for acute rejection (presumed or biopsy proven), infection episodes including CMV, graft failure, and patient death.

3.9.1 Patient Demographics

The demographics of a 100 patients recruited from February 2009 and June 2011 are shown in Table 3.8. The transplant population was an average 44.9 yrs. with 57% males and the majority of Caucasian origin (73%). There was a roughly even split between live and cadaveric donation and an overall low levels of HLA mismatch.

Table 3.8. Patient Demographics for the 100 patients recruited into the longitudinal study

Characteristic	All patients (n=100)
Male (%)	57
Recipient age, mean (SD)	44.9 (14.3)
HLA mismatch, median (interquartile range)	
HLA A	1.0 (1.0-2.0)
HLA B	1.0 (1.0-1.0)
HLA DR	1.0 (0.0-1.0)
Ethnicity (%)	
White	73
Indo-Asian	16
Afro-Caribbean	9
Mixed race	1
Other	1
Type of transplant (%)	
ABO compatible living donation	31
ABO incompatible living donation	21
Cadaveric	48
CMV status (%)	
D-/R-	26
D+/R-	22
D-/R+	17
D+/R+	35
Underlying Cause of ESRF (%)	
Glomerular	37
Hereditary Structural/Cystic Disease	29
Diabetes Mellitus	8
Vascular	11
Interstitial	4
Others	11
Patients withdrawn from study by 24M (%)	
Withdrew consent	10
Failed transplants	7
Patient death	4

A total of 10 patients withdrew from the trial at various time points, 7 patients lost their graft function and 4 others died by the end of the 24M period. All samples collected before patient withdrawals were included in the analysis. Samples at 12M and particularly 24M were hard to obtain as a lot of patients were referred back to their parent hospital trust after transplantation.

Table 3.9. A summary of the number of patient samples successfully processed at the chosen time points

10 patients withdrew consent, 7 patients lost their graft function, and 4 patients died during the study. A significant number of patients were referred back to their parent trust 3 months post transplantation.

Time point	Total number of samples
D0	92
3M	78
12M	66
24M	43

3.9.2 Overall ELISPOT Responses

Table 3.10 summarizes the total number of positive and negative ELISPOT responses in all 100 patients. Table 3.11 shows the consecutive responses in 36 transplant recipients. There is a consistent overall trend of more negative than positive responses at all time points with an increasing number of positive responses at 12 months. By 24 months however, 5% to 9% of patients lose their positive responses to the HLA derived peptides. No patients with consecutive data lost their responses and gained them back again. All patients in this cohort had induction therapy with basiliximab and maintenance therapy with steroids, tacrolimus and mycophenolate. 1 patient was converted to sirolimus at18-months post transplant and 2 others were converted to azathioprine at 12-months post treatment. None of these patients had a change in ELISPOT response after a change in immunosuppression.

Overall, 21% of patients had positive ELISPOT responses at 24-months post transplantation. This is comparable to the cross sectional study where 33% of all RTRs tested responded, albeit at various times post transplantation.

Table 3.10. Overall ELISPOT Responses to HLA derived peptides obtained during the 2-year study

Table summarizing the overall ELISPOT responses to HLA derived peptide mixes pretransplant (n=92), 3M post transplant (n=78), 12M post transplant (n=66) and 24M post transplant (n=43). The definition of a response in this assay was previously defined as > 7 spots per well, based upon analysis.

	Positive Responses, n (%)	Negative Responses, n (%)
Pre-transplantation n=92	15 (16%)	77 (84%)
3M n=78	14 (18%)	64 (82%)
12M n=66	17 (26%)	49 (74%)
24M n=43	9 (21%)	34 (79%)

Table 3.11. ELISPOT Responses in 36 patients for whom we had consecutive data at each time point

Table summarizing consecutive γ-interferon responses to HLA derived peptide mixes in 36 renal transplant recipients. The definition of a response in this assay was previously defined as > 7 spots per well, based upon analysis.

	Positive Responses, n (%)	Negative Responses, n (%)
Pre-transplantation	8 (22%)	28 (78%)
3M	6 (17%)	30 (83%)
12M	11 (31%)	25 (69%)
24M	8 (22%)	28 (78%)

3.9.3 ELISPOT Responses and Acute Rejection Episodes

All acute rejection episodes were confirmed by histology according to the 2007-revised Banff classification (Solez et al). Of the 100 patients recruited, 22 patients had 1 or more episodes of acute rejection within the first year post transplantation. A total of 4 patients had an acute rejection episode between 12 to 24 months post transplantation, 2 of them having had an episode of rejection previously.

Statistical analysis using Chi-squared or Fisher's exact (based on the minimum expected count) were used to test if there was a relationship between patients who had 1 more acute rejection episodes and their ELISPOT responses. There was no link to say that patients with positive ELISPOT responses had subsequently more episodes of acute rejection, or that acute rejection episodes lead to positive ELISPOT responses.

3.9.4 ELISPOT Responses and Biopsy proven chronic rejection

In our cohort of 100 patients there were only a small number of patients with histology proven CAI within 2 years of transplantation, hence any association was not possible to conclude. None of the 6 patients with biopsy proven chronic rejection had preceding positive ELISPOT response within 2 years of transplantation. There was only 1 patient with biopsy proven chronic rejection at 8-months post transplantation whom at 3-month was ELISPOT negative but at 12 months was ELISPOT positive and remained positive on subsequent testing.

3.9.5 ELISPOT Responses and Episodes of Infection

A high proportion of patients (57%) had infective episodes within the first 2 years of transplantation. A further 2% had culture negative episodes that were treated as infection. There were a total of 92 culture positive and 8 culture negative episodes of infection in a 24-month period post transplantation. For the purpose of statistical analysis, an infective episode was defined as a culture positive episode- bacterial, fungal or viral. Episodes of infection were grouped into occurring within 3-months post transplantation, between 3 and 12-months post transplantation and between 12 and 24-months post transplantation and compared with ELISPOT responses before and after these periods. Multiple episodes of infection were

counted as a single episode if it occurred within each time period for a single patient. If an episode of infection had missing ELSPOT responses either pre of post infective episode, they were not included in the analysis.

Table 3.12. Comparisons between episodes of infection and ELISPOT responses before and after the episodes

As expected the number of infective episodes reduced with time away from transplantation. Statistical analyses did not prove that ELISPOT responses were either predictive of or changed due to infective episodes

Infective Episodes	Pre Episode		Post Episode	
	ELISPOT +	ELISPOT -	ELISPOT +	ELISPOT -
Pretransplantation-3M	9	30	8	24
3M-12M	5	25	8	21
12M-24M	5	14	5	7

CMV viraemia episodes were analysed separately and considered positive if >200copies/ml were isolated in a peripheral blood sample. Only a small number of episodes of CMV viraemia occurred in our cohort of 100 patients over a 2-year period post transplantation. Statistical analyses using Fisher's exact did not show any significance between CMV viraemia episodes and ELISPOT responses either before or after the viraemia episode.

Table 3.13 Comparisons between episodes of CMV viraemia and ELISPOT responses before and after the episodes

A CMV viraemia episode was defined as >200 copies/ml on serum PCR. There was no significant correlation between γ-interferon ELISPOT responses to HLA peptides and a CMV viraemia episode albeit the very low numbers.

CMV Viraemia Episodes	Pre Episode		Post Episode	
	ELISPOT +	ELISPOT -	ELISPOT +	ELISPOT -
Pretransplantation-3M	1	4	1	4
3M-12M	1	10	4	4
12M-24M	2	1	1	1

3.9.6 ELISPOT responses and presence of circulating DSAs

Of the 100 patients recruited onto the longitudinal study, we had pre-transplant circulating DSA data for 79 patients. 15 patients had the presence of circulating DSAs pre-transplant but all were ELISPOT negative at the time. Of these 15 patients only 3 patients had an episode of acute rejection within 3-months post transplantation. 12 other patients were ELISPOT positive pre-transplantation with no evidence of circulating DSAs. Of these, 3/12 patients had at least one episode of acute rejection within 12-months post transplantation. 2/12 patients subsequently developed circulating DSA. We were able to analyse circulating DSA data at 0,12 or 24 months for only 25 patients. It is interesting to note that the 8 patients we pre-transplantation DSAs but where ELISPOT, became ELISPOT positive 12-months later.

CHAPTER 4
RESULTS

Responses to non-polymorphic (autologous) HLA derived peptides are mediated through $CD4^+$ T effector memory cells

4 Results: Responses to non-polymorphic (autologous) HLA derived peptides are mediated through $CD4^+$ T effector memory cells

The phenotype of cells responding to non-polymorphic HLA derived peptides has previously been demonstrated to lie in the $CD4^+CD25^{Hi}$ lymphocyte compartment by intracellular staining (Smith et al., 2011). This section aims to define with greater acuity the $CD4^+$ T lymphocyte subset responsible for the production of γ-interferon and other cytokines.

4.1 Gating Strategy

In human lymphocytes, CD25 expression is continuously distributed so that there is not a clearly defined CD25 positive population, containing Treg cells. For the purpose of functional studies this is not necessarily a problem, since in this setting it is often only necessary to demonstrate differential effects generated by phenotypically distinct populations, (although even this may be complicated by the fact that the $CD25^{Hi}$ population includes activated T lymphocytes). On the other hand, for the purposes of correlating the frequency of expression of a particular cell surface phenotype in peripheral blood, this imprecision in gate definition is problematic, still more so if any such approach is extended beyond a single operator. A number of different approaches have been taken in order to indirectly derive such a '$CD25^{Hi}$ gate' however they are potentially confounded by circular arguments or dependence upon other variables that are also difficult to standardise.

In this chapter I will show that the lymphocyte population responsible for RTRs' γ-interferon production in response to non-polymorphic HLA derived peptides has the cell surface phenotype: $CD4^+CD25^{Hi}CD45RO^+CD127^+CCR7^-$. The expression of this phenotype in peripheral blood as a proportion of $CD4^+CD25^{Hi}$ lymphocytes in RTRs (n=35) and HCs (n=17) is shown in Fig 4.1 for populations based upon gating strategies for CD25 expression that included progressively greater proportions of $CD4^+$ T lymphocytes. There was a highly statistically significant difference in the expression of $CD25^{Hi}CD45RO^+CD127^+CCR7^-$ lymphocytes when the CD25 gate was set to include 5% or less of $CD4^+$ lymphocytes. The significance of this difference was greatest between 1.25% and 5%. A CD25 gate of 2.5% of $CD4^+$ T lymphocytes was therefore chosen for all subsequent studies.

Figure 4.1. The proportion of cells CD45RO^{+}CD127^{+}CCR7^{-} (as a function of CD4^{+}CD25^{Hi} lymphocytes) in RTR Vs. HC incorporating different levels of CD25^{Hi} expression

The proportion of cells CD45RO^{+}CD127^{+}CCR7^{-} (as a function of CD4^{+}CD25^{Hi} lymphocytes) in 35 RTRs immunosuppressed with a calcineurin inhibitor and 17 HCs. This proportion was significantly higher in RTRs than in HCs up to a gate incorporating the highest 5% of CD25 expression and was most significant when the gate was set to the highest 2.5% of CD4^{+} T lymphocytes: RTR median = 8.9 % (IQR 4.2-13.4) healthy control median = 2.8% (IQR 1.7-5.2) (p<0.001).

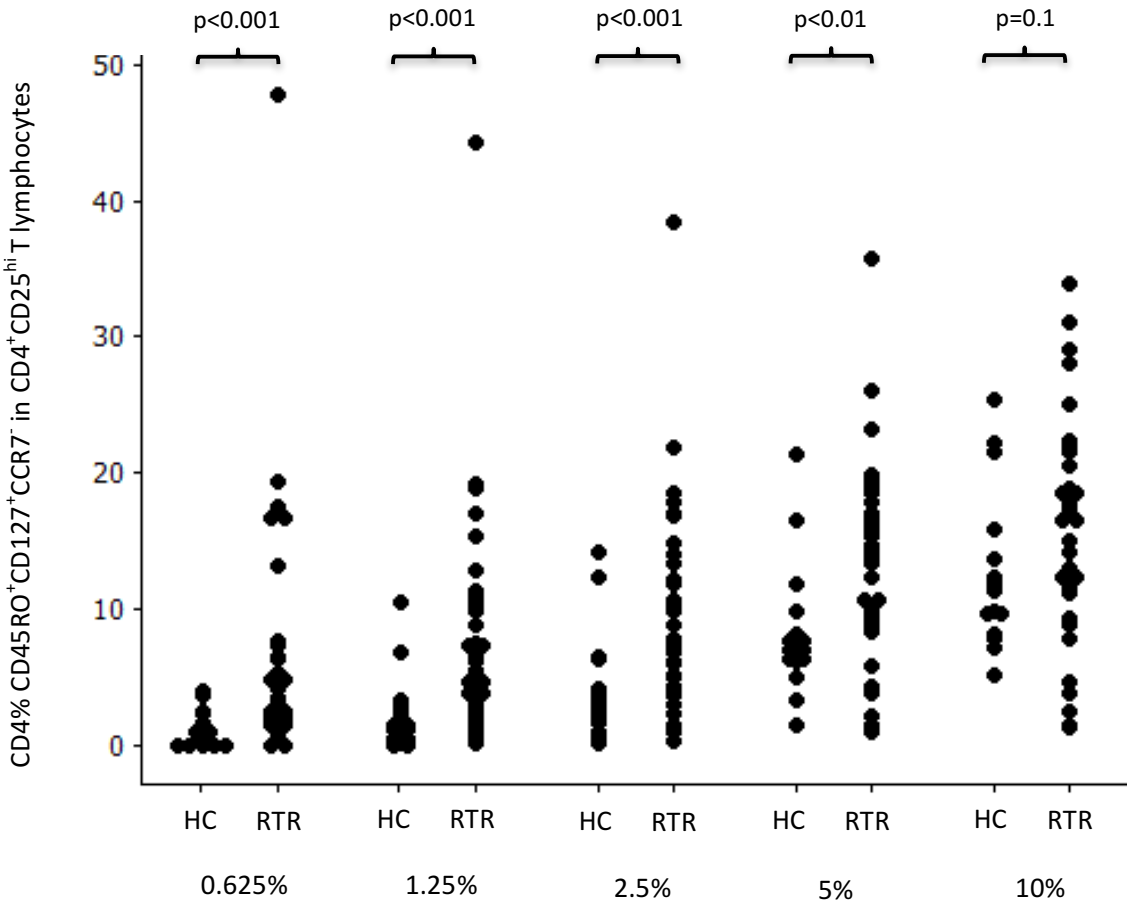
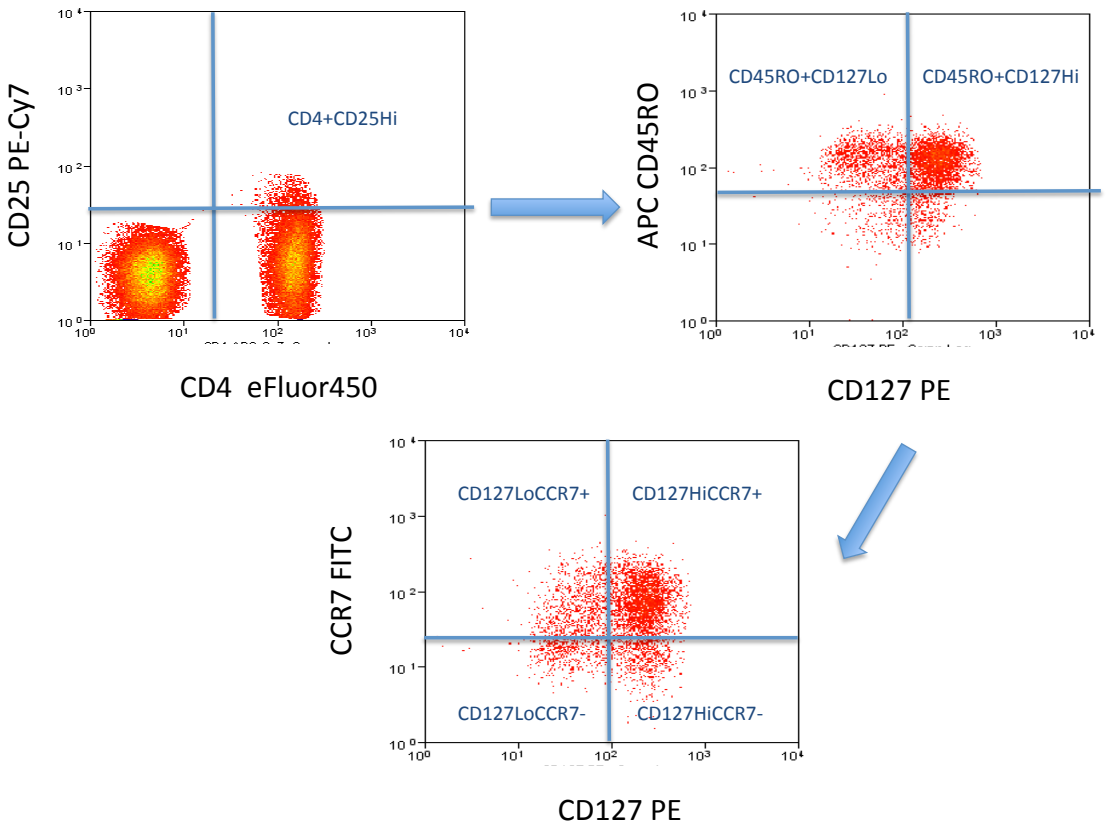


Figure 4.2. Gating strategy to sort $CD4^+$ subset

$1x10^7$ PBMCs were surface stained with antibody using the methods above (Section 2.2.5, page 40). The stained cells were finally suspended in 500ul MACS and sorted at The Centre for Translational Inflammation Research (CTIR) using a MoFLo Legacy cell sorter. Cells were counted and sorted into 3 categories

1. Tems- T effector memory cells ($CD4^+CD25^{Hi}CD45RO^+CD127^{Hi}CCR7^-$)
2. Tcms- T central memory cells ($CD4^+CD25^{Hi}CD45RO^+CD127^{Hi}CCR7^+$)
3. Tregs- T Regulatory cells ($CD4^+CD25^{Hi}CD45RO^+CD127^{Lo}$). Liu et al demonstrated that CD127 is an excellent cell surface biomarker of human Tregs especially when used in combination with CD25 (Jiang et al., 2004). CD127 expression inversely correlated with Foxp3 expression and is expressed at low levels on a majority of Treg cells (Jiang et al., 2004). Hence Foxp3 staining wasnot performed to identify Treg cells.

$CD25^{Hi}$ were defined as the top 2.5% of $CD4^+CD25^+$ cells for reasons described in Section 4.1.



4.2 γ-interferon responses to HLA derived peptides by different $CD4^+CD25^{Hi}CD45RO^+$ subsets

The strategy used to deduce the $CD4^+$ T lymphocyte subset responsible for cytokine production was to purify T-cell subsets by flow cytometry as shown in the section above. These subsets were then added to peptide-stimulated PBMC cultures quantified by γ-interferon ELISPOT. This strategy was used to combine sensitivity with the potential to undertake functional studies.

19 patients were studied on the basis of a consistent response to one or more HLA derived peptides in earlier studies. This included, 12 patients responsive to Class 1 derived HLA peptides (tra30, tra34, tra33, tra02, tra14, tra31) and 7 patients responsive to Class 2 derived HLA peptides (DRE3c, DRE6b, DQ1, DRE2, DRE3b, DRE2a, DRE6c, DRE4a) were chosen.

A significant increment in the response to active Class 1 and Class 2 derived peptides was only seen when the $CD4^+CD25^{Hi}CD45RO^+CD127^{Hi}CCR7^-$ subset was added back to the cell culture. Mean Response to Class 1 peptides was 136 spots/well and to class 2 peptides was 121 spots/well as shown in Figures 4.3 and 4.4. The mean frequency when any other subset of cells was titrated back to active peptide was under 10 spots/well.

Figure 4.3. Interferon-γ responses in the presence of Class 1 HLA derived peptides and different $CD4^{+}CD25^{Hi}CD45RO^{+}$ subsets.

Four flow sorted lymphocyte subsets: $CD4^{+}CD25^{Hi}CD45RO^{+}CD127^{+/-}CCR7^{+/-}$ (Figure 4.2, page 76) were titrated back into γ-interferon ELISPOT wells containing $4x10^{5}$ PBMC's with active (AP) (tra30, tra34, tra33, tra02,tra14, tra31) or control (IP) HLA derived peptide (11.4μmol/L) and cultured for 48 hours at $37^{0}C$ and 5%CO_2. A γ-interferon ELISPOT response was observed only in the presence of active peptide with the $CD4^{+}CD25^{Hi}CD45RO^{+}CD127^{+}CCR7^{-}$ (average 136spots/well) population but not any of the other three subsets The control peptide wells all returned 4 spots or less. Results from all 12 patients are shown.

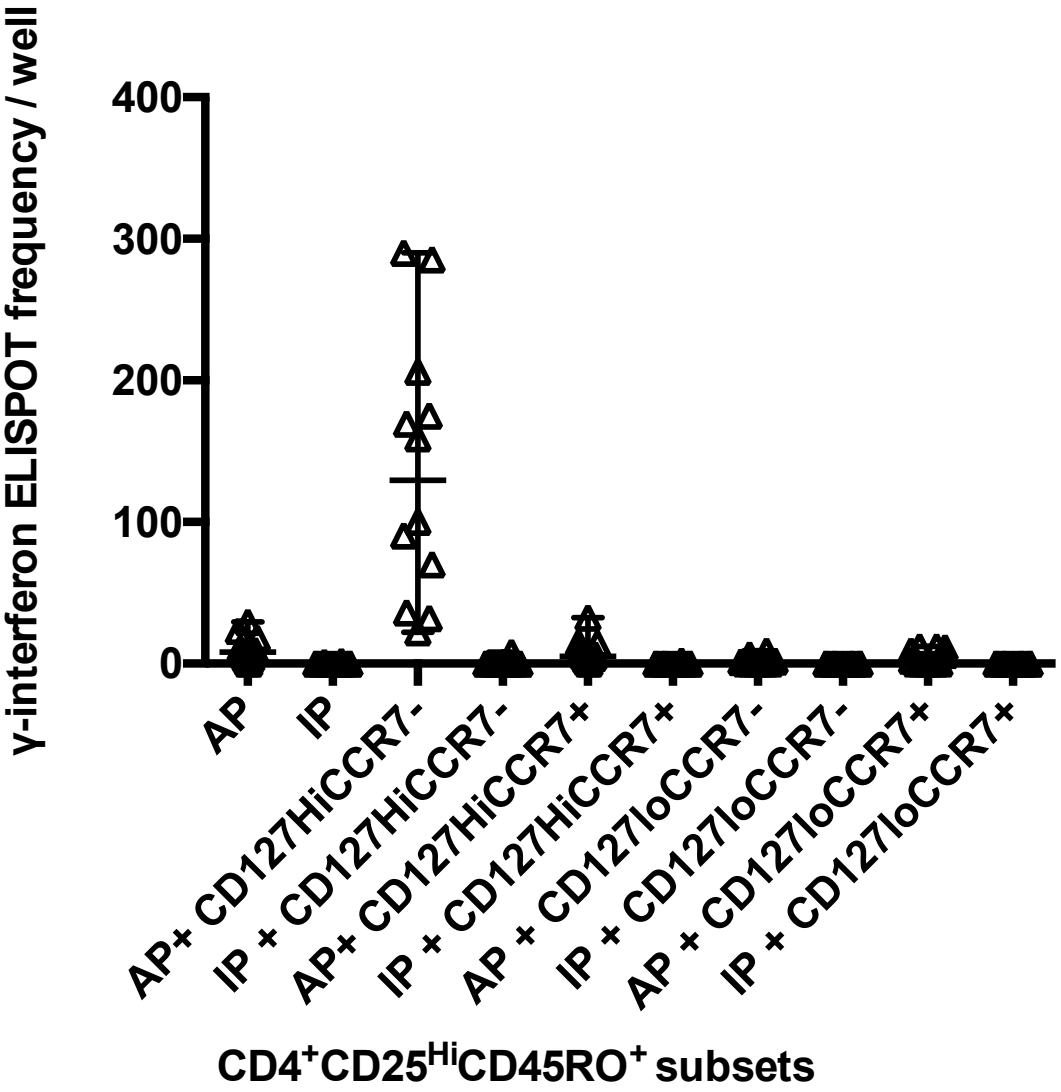
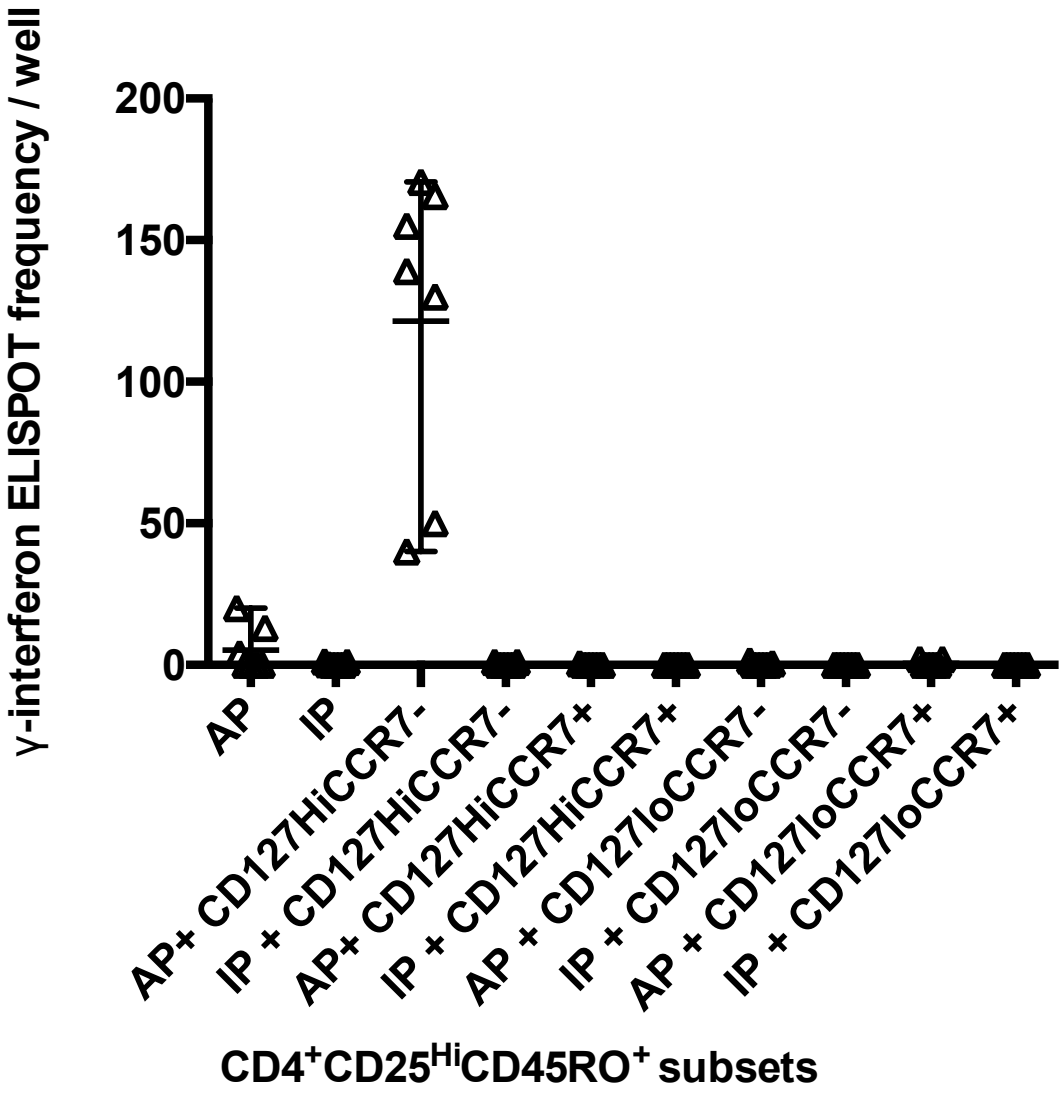


Figure 4.4. Interferon-γ responses in the presence of Class 2 HLA derived active peptide and different $CD4^{+}CD25^{Hi}CD45RO^{+}$ subset

Four flow sorted lymphocyte subsets: $CD4^{+}CD25^{Hi}CD45RO^{+}CD127^{+/-}CCR7^{+/-}$ (Figure 4.2, page 76) were titrated back into γ-interferon ELISPOT wells containing 4x105 PBMC's with active (AP) (DRE3c, DRE6b, DQ1, DRE2, DRE3b, DRE2a, DRE6c, DRE4a) or control (IP) HLA derived peptide (11.4μmol/L) and cultured for 48 hours at 37^{0}C and 5%CO2. A γ-interferon ELISPOT response was observed only in the presence of active peptide with the $CD4^{+}CD25^{Hi}CD45RO^{+}CD127^{+}CCR7^{-}$ (average 121spots/well) population but not any of the other three subsets The control peptide wells all returned 4 spots or less. Results from all 7 patients are shown.

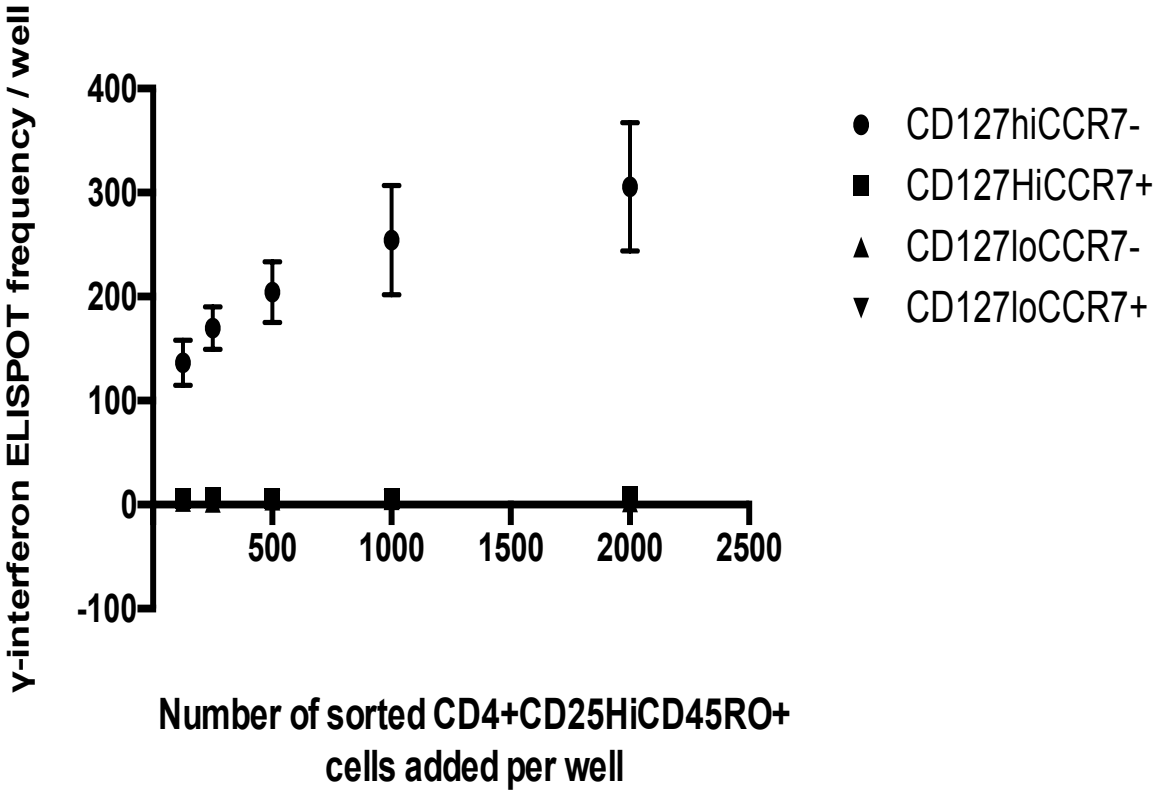


4.3 Interferon-γ responses to HLA derived peptides by different $CD4^{+}CD25^{Hi}CD45RO^{+}$ subsets at different doses

Dose titration responses in 13 patients to Class 2 peptides showed a progressive increase in γ-interferon ELISPOT frequency to active peptide when increased numbers of $CD4^{+}CD25^{Hi}CD45RO^{+}CD127^{Hi}CCR7^{-}$ sorted cells were titrated back. Mean responses are shown in Figure 4.3. Responses in all other subsets in any amount showed an average frequency of less than 10 spots/well.

Figure 4.5. Interferon-γ responses in the presence of Class 2 HLA derived active peptide and different $CD4^{+}CD25^{Hi}CD45RO^{+}$ subsets in increasing number of cell numbers.

Four flow sorted lymphocyte subsets: $CD4^{+}CD25^{Hi}CD45RO^{+}CD127^{+/-}CCR7^{+/-}$ (Figure 4.2, page 76) were titrated back into γ-interferon ELISPOT wells containing $4x10^{5}$ PBMC's with active (DRE2, DRE3b, DRE3c and DRE6c) or control HLA derived peptide (11.4μmol/L) and cultured for 48 hours at $37^{0}C$ and 5%CO_2. A progressive increase in γ-interferon ELISPOT frequency was observed only in the presence of active peptide with the $CD4^{+}CD25^{Hi}CD45RO^{+}CD127^{+}CCR7^{-}$ population but not any of the other three subsets .The control peptide wells all returned 4 spots or less. Results from all 13 patients are shown below.


y-interferon ELISPOT frequency / well
400
300
200
100
0
-100
500
1000
1500
2000
2500
Number of sorted CD4+CD25HiCD45RO+ cells added per well
CD127hiCCR7-
CD127HiCCR7+
CD127loCCR7-
CD127loCCR7+

4.4 Effect of lymphocyte activation inhibitors on γ-interferon responses to HLA derived peptides

15 patients (10 previously responsive to HLA Class 1 derived peptides and 5 previously responsive to HLA class 2 peptides) were studied for γ- Interferon responses by T-effector memory cells in the presence of active peptide alone and in the presence of different inhibitors of lymphocyte activation.

Table 4.1 shows the median number of γ-interferon responses with and without inhibitors and their comparisons. All inhibitors reduced the effect of T effector memory cell responses to active peptide, however the effect was less marked with Sirolimus and Abatacept. Figure 4.6 shows median responses in all 15 patients. 3 of 15 patients had less than 50% inhibition with ciclosporin at 100mg/ml. Overall Ciclosporin at 100mg/ml and 200mg/ml and ShK at 10nM and 50nM have the most profound effect (less than 40% production compared to that in the absence of inhibitors) whilst the effect is less progressively marked with Abatacept and Sirolimus. There was no significant difference in inhibition between the doses of inhibitors used when compared with each other

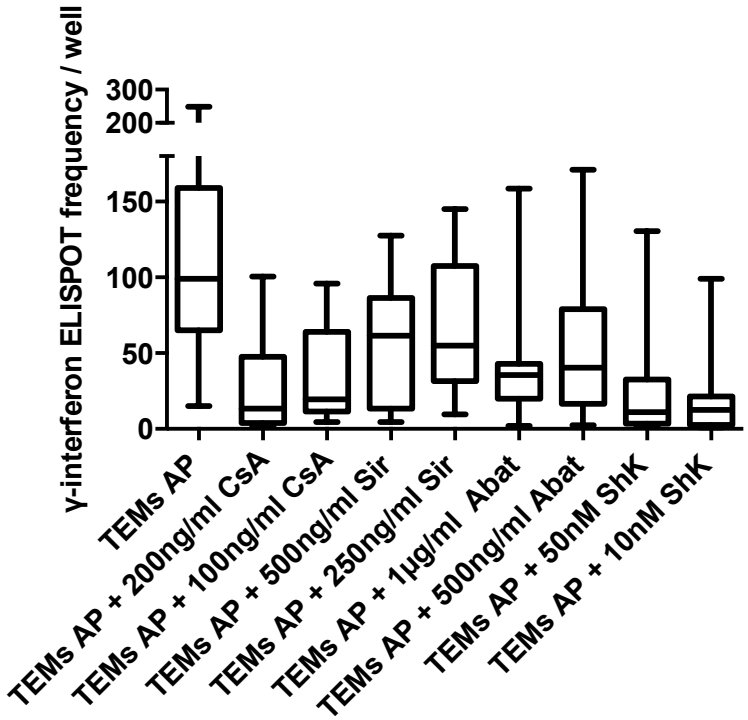
Table 4.1. Table showing the mean γ-interferon responses of 15 patients to active peptide alone and in the presence of lymphocyte activation inhibitors at different doses.

$4x10^5$PBMC's from 15 RTRs previously identified to respond to HLA derived peptides, were enriched with 10^3 sorted autologous $CD4^+CD25^{Hi}CD45RO^+CD127^{Hi}CCR7^-$ T lymphocytes and cultured in the presence or absence of 'active' peptide and of Ciclosporin (200ng/ml and 100ng/ml), Sirolimus (50ng/ml and 25ng/ml), Abatacept (1µg/ml), Shk peptide (10nM and 50nM- a Kv1.3 channel inhibitor which targets T effector memory cells).Cell culture supernatant cytokine concentrations were analysed at 24 hours using a multiplex assay and γ-Interferon ELISPOTs enumerated at 48 hours of culture. Results are shown as the median frequency of γ-Interferon responses per well. The p values show comparisons of responses between responses with and without each inhibitor (significant values in red). Responses were tested in triplicate.

	Median γ-Interferon responses/well (IQR)	% inhibition median (IQR)	P value
TEMS + AP	108		
TEMs AP + 100ng/ml CsA	11.5 (15)	70 (33.8)	0.0002
TEMs AP + 200ng/ml CsA	13.5 (43.5)	82.7 (26.1)	0.0001
TEMs AP + 250ng/ml Sir	55 (76)	36.7 (41)	0.0953
TEMs AP + 500ng/ml Sir	61.5 (73)	46 (38)	0.0323
TEMs AP + 500ng/ml Abat	40.5 (62.5)	40.3 (47.4)	0.0256
TEMs AP + 1µg/ml Abat	35.5 (23)	73.9 (42.6)	0.0140
TEMs AP + 10nM ShK	12.5 (19)	85.6 (18.3)	<0.0001
TEMs AP + 50nM ShK	32.5 (29)	87.5 (31.6)	0.0001

Figure 4.6. γ-interferon responses to HLA derived peptides in the presence of lymphocyte activation inhibitors in 15 RTRs.

$4x10^5$ PBMC's from each RTR previously identified to respond to HLA class 1 and Clss 2 derived peptides, were enriched with 10^3 sorted autologous $CD4^+CD25^{Hi}CD45RO^+CD127^{Hi}CCR7^-$ T lymphocytes and cultured in the presence or absence of 'active' peptide and of Ciclosporin (200ng/ml and 100ng/ml), Sirolimus (50ng/ml and 25ng/ml), Abatacept (1µg/ml), Shk peptide (10nM and 50nM - a Kv1.3 channel inhibitor which targets T effector memory cells). Cell culture supernatant cytokine concentrations were analysed at 24 hours using a multiplex assay and γ-interferon ELISPOTs enumerated at 48 hours culture. Results are shown as the median (triplicatewells) frequency of γ-Interferon responses per well.



4.5 Peripheral expansion of activated T effector memory cells in renal transplant recipients

4.5.1 Patient Demographics

Cell surface markers from the peripheral blood of 37 RTRs and 17 HCs were studied. Table 4.2 shows the demographics of the 37 renal transplant recipients. 26 patients were male and 11 patients female with a median age of 49.2 years (range 19-70 years). At the time of the study, median time from transplantation was 10.5 years (range 2 to 40.1 years).

All patients received our department's standardized immunosuppression with basiliximab at induction followed by steroids, CNI and MMF/azathioprine maintenance. All but 2 patients (n=35) were on CNI based immunotherapy the time of the study with prednisolone (n=31) and an antiproliferative agent (n=21). 2 patients had their CNI therapy withdrawn and were on dual therapy with prednisolone and an antiproliferative agent.

Patients were divided into 2 categories for analysis. Those with stable kidney function (n=20) and those with CAI (n=17) defined based upon KDIGO guidelines as eGFR <40ml/min or proteinuria >500mg/day (Kidney Disease: Improving Global Outcomes Transplant Work, 2009) or those with biopsy proven chronic allograft rejection (interstitial fibrosis, glomerular sclerosis, tubular atrophy). Patients in the CAI group did not have evidence of CNI toxicity (defined as either biopsy evidence of CNI damage or 2 consecutive levels out of target range in the previous 3 months) or any other cause of CAI such as viral infections or circulating DSAs. The control group included 17 healthy subjects with 12 women and 5 men aged 26 to 59 years, mean 37.2 years.

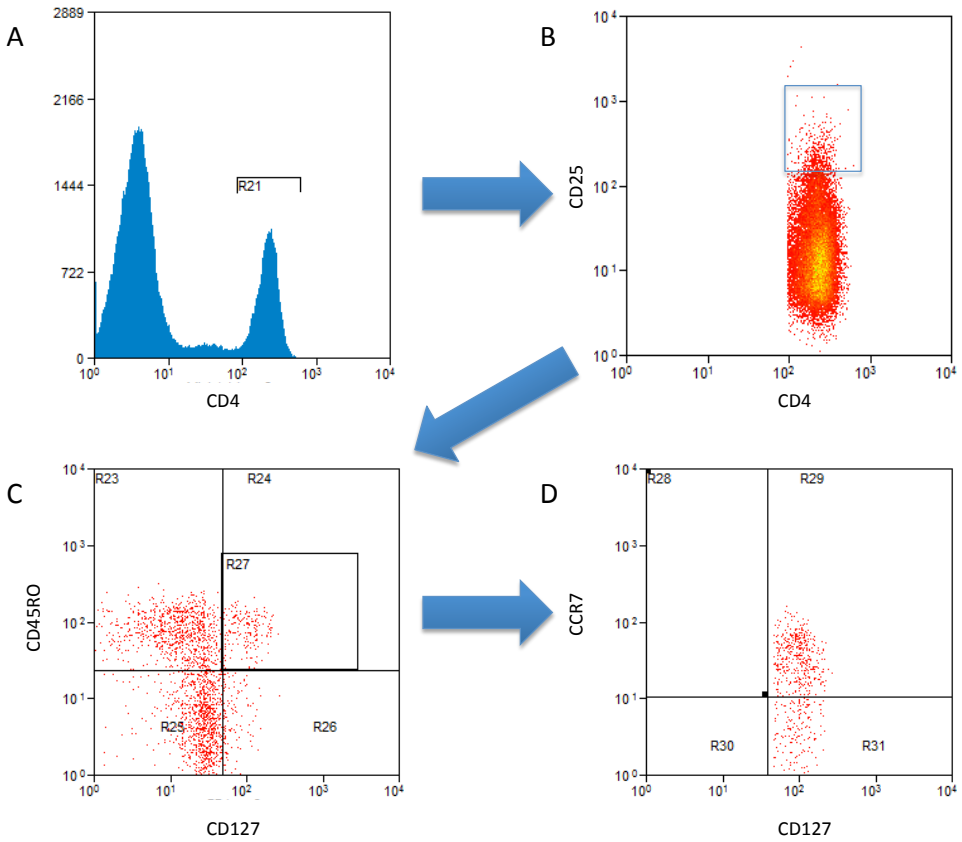
Table 4.2. Patient demographics for the 37 patients used to study cell surface markers in Renal Transplant Recipients

[a] indicates significance by student's t test, [b] indicates significance by Mann-Whitney U test, [c] indicates significance by Chi-squared test. N/A not applicable, NS not significant.

	Stable graft function	Chronic Allograft Injury	P-value
Number of patients	20	17	N/A
Recipient age: mean ± SD (range)	47.4 ± 12.7 (19-67)	51.4 ± 10.9 (31-70)	NS
Recipient sex M: F (% male)	13:7 (65%)	13:4 (76.5%)	NS
CMV status (%)			
D-/R-	40	29.4	NS
D+/R-	15	29.4	NS
D-/R+	15	17.6	NS
D+/R+	30	23.6	NS
Time (yrs.) since transplantation: mean ± SD (range)	8.5 ± 7.2 (2-25.3)	12.9 ± 9.9 (3.5-40.1)	NS
Recipient Creatinine: mean ± SD (range)	128 ± 43.7 (65-198)	232.4 ± 79.8 (10.2- 375)	0.02 [a]
Recipient ACR: mean ± SD (range)	5.6 ± 8.5 (0-29.3)	83.4 ± 139.7 (0.3-51.8)	0.017 [b]
Recipient eGFR: mean ± SD (range)	53.6 ± 20.9 (27-90)	25.6 ± 12 (11-63)	0.005 [a]
Immunosuppression regimen			
Steroids	17/20	15/17	NS [c]
CNI (tacrolimus/ciclosporin)	19/20	16/17	NS [c]
Antiproliferative (MMF/azathioprine)	18/20	10/17	NS [c]
CAI diagnosis			
Creatinine >150µmol/L	N/A	16/17	N/A
ACR >30	N/A	8/17	N/A
Biopsy proven	N/A	10/17	N/A
Previous episodes of acute rejection	7/20	7/17	NS [c]

Figure 4.7. Gating strategy used for phenotyping cell surface markers in 37 renal transplant recipients and 17 healthy controls.

Lymphocytes were gated on $CD4^+$ cells (A) and the top 2.5% $CD25^{Hi}$ of $CD4^+$ cells (B). T effector memory cells were identified as being $CD45RO^+$ and $CD127^{Hi}$ (C) and then identified as being either peripheral ($CCR7^-$) or central ($CCR7^+$) (D).

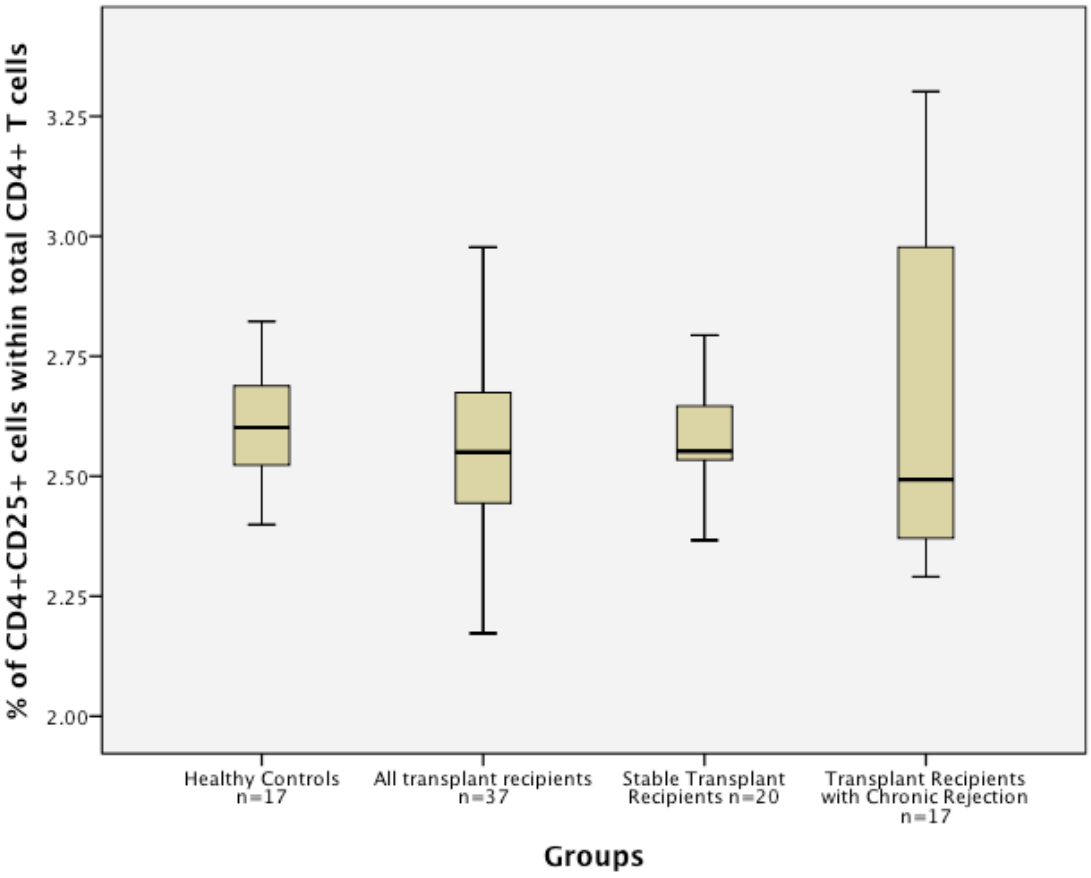


4.5.2 $CD4^{+}CD25^{Hi}$ cells in Renal Transplant Recipients

The percentage of $CD4^{+}$ T-cells after transplantation did not differ from that in healthy individuals (41.3% (IQR 13.8) Vs. 32.9% (IQR 16.2); p=0.06). This was in keeping with van De berg et al (van de Berg et al., 2012b) who showed no difference in percentages but lower absolute numbers at 6 month, 2 years and 5 years post transplantation with our average age of transplant being 10.5 years. Figure 4.8 shows the percentage of $CD4^{+}CD25^{Hi}$ cells in all 4 groups with no significant differences.

Figure 4.8. Box plot showing the number of $CD4^{+}CD25^{Hi}$ cells within total $CD4^{+}$ T-cells.

Lymphocytes from the PBMCs of 37 RTRs and 17 HCs were stained with cell surface antibodies and analysed for various phenotyping cell surface markers of T lymphoctyes (Figure 4.7, page 112) There was no significant difference in the number of $CD4^{+}CD25^{Hi}$ cells within the $CD4^{+}$ T-cell population as analysed by a non-parametric Kruskal-Wallis test (p=0.65) between RTRs and HCs or within RTRs with stable function or features or CAI.



4.5.3 $CD4^{+}CD25^{Hi}CD127^{Lo}$ & $CD4^{+}CD25^{Hi}CD127^{Hi}$ cells in Renal Transplant Recipients

As shown in several studies (Codarri et al., 2007, Seddiki et al., 2006) the majority of $CD4^{+}CD25^{+}$ T-cells were $CD45RO^{+}CD127^{Lo}$ in a representative healthy subject as shown from one example in Figure 4.9 (A). Only a small percentage (4.99% in this example) of $CD4^{+}CD25^{+}$ expressed $CD127^{Hi.}$ The same analysis in a stable renal transplant recipient showed that the $CD4^{+}CD25^{+}CD45RO^{+}CD127^{Lo}$ population was markedly reduced (34.5% in this example Figure 4.9 B) compared with a healthy control. Interestingly there was also a major increase (19.6%) in the proportion of $CD4^{+}CD25^{+}CD45RO^{+}CD127^{Hi}$ population. The differences in these populations remained similar overall in patients with stable transplant function and those with CAI as shown by one example in figure 4.9 (C).

The percentage of Tregs ($CD4^{+}CD25^{Hi}CD45RO^{+}CD127^{Lo}$) within $CD4^{+}CD25^{Hi}$ T-cells in healthy controls was significantly higher than in transplant recipients (84.8% (IQR 32.3) Vs. 48.4% (IQR 23.4); $p<0.05$). There was no significant difference in the proportion of these cells between transplant recipients with stable function and CAI (48.1% (IQR 28.2) Vs. 53.1% (IQR 25.6); p=0.63). Figure 4.10 shows the number of Tregs in all 4 groups.

The percentage of Tems ($CD4^{+}CD25^{Hi}CD45RO^{+}CD127^{Hi}$) within the $CD4^{+}CD25^{Hi}$ T-cells in transplant recipients was significantly higher than in healthy controls (21.8% (IQR 24.4) Vs. 7% (IQR 24.8); p=0.02), even when confounded for age (Pearson's correlation). However there was no significant difference in the proportion of these cells between transplant recipients with stable function and CAI (21.2% (IQR 24.8) Vs. 24.7% (IQR 23.1); p=0.62) unlike what Coddari and colleagues described (Codarri et al., 2007). Figure 4.11 (page 118) shows the number of Tems in all 4 groups.

Figure 4.9. $CD4^+CD25^+$ flow cytometry profiles on one healthy donor (A), one stable renal transplant recipient (B) and one recipient with CAI (C).

Lymphocytes from the PBMCs of 2 RTRs and 1 HC were stained with cell surface antibodies and analysed for various phenotyping cell surface markers of T lymphoctyes (Figure 4.7, page 112). The vast majority of $CD4^+CD25^+$ T-cells are $CD45RO^+CD127^{Lo}$ in healthy controls (A) while this proportion reduced in renal transplant recipients (B & C). $CD45RO^+CD127^{Hi}$ population was expanded in RTRs (B&C).

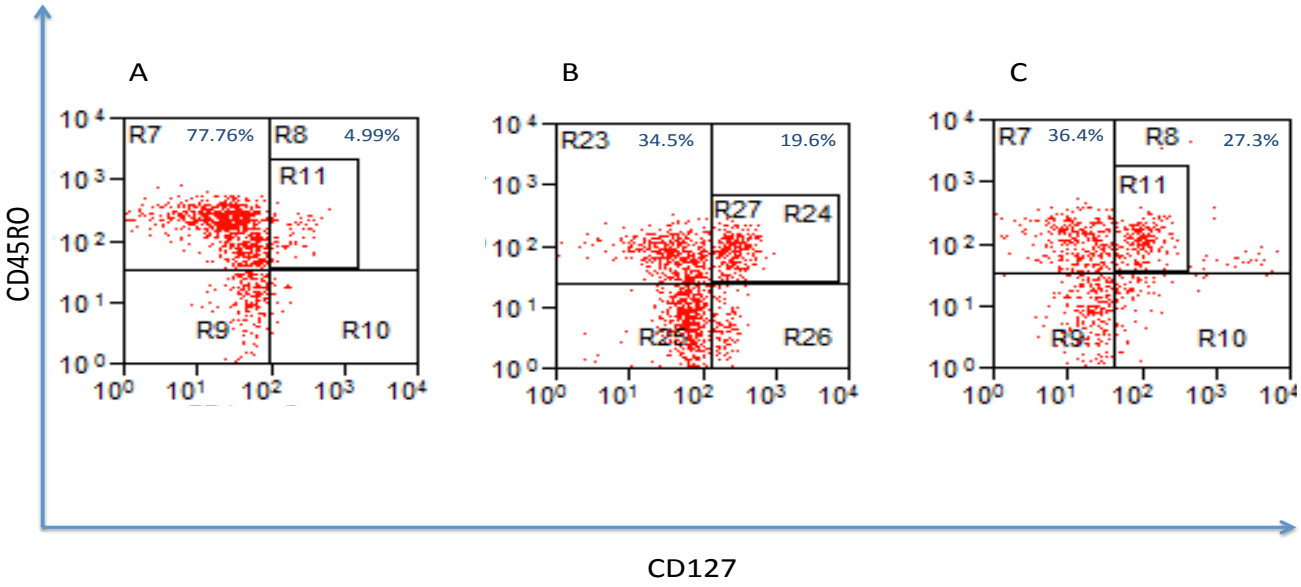


Figure 4.10. Box plot showing the proportion of Tregs within $CD4^{+}CD25^{Hi}$ cells.

Lymphocytes from the PBMCs of 37 RTRs and 17 HCs were stained with cell surface antibodies and analysed for various phenotyping cell surface markers of T lymphoctyes (Figure 4.7, page 112) There was a significant difference in the proportion of $CD4^{+}CD25^{+}CD45RO^{+}CD127^{Lo}$ population between HCs and RTRs but not between recipients with stable function or CAI. Statistical analyses were done using the non-parametric independent t test (MannWhitney U test) between groups.

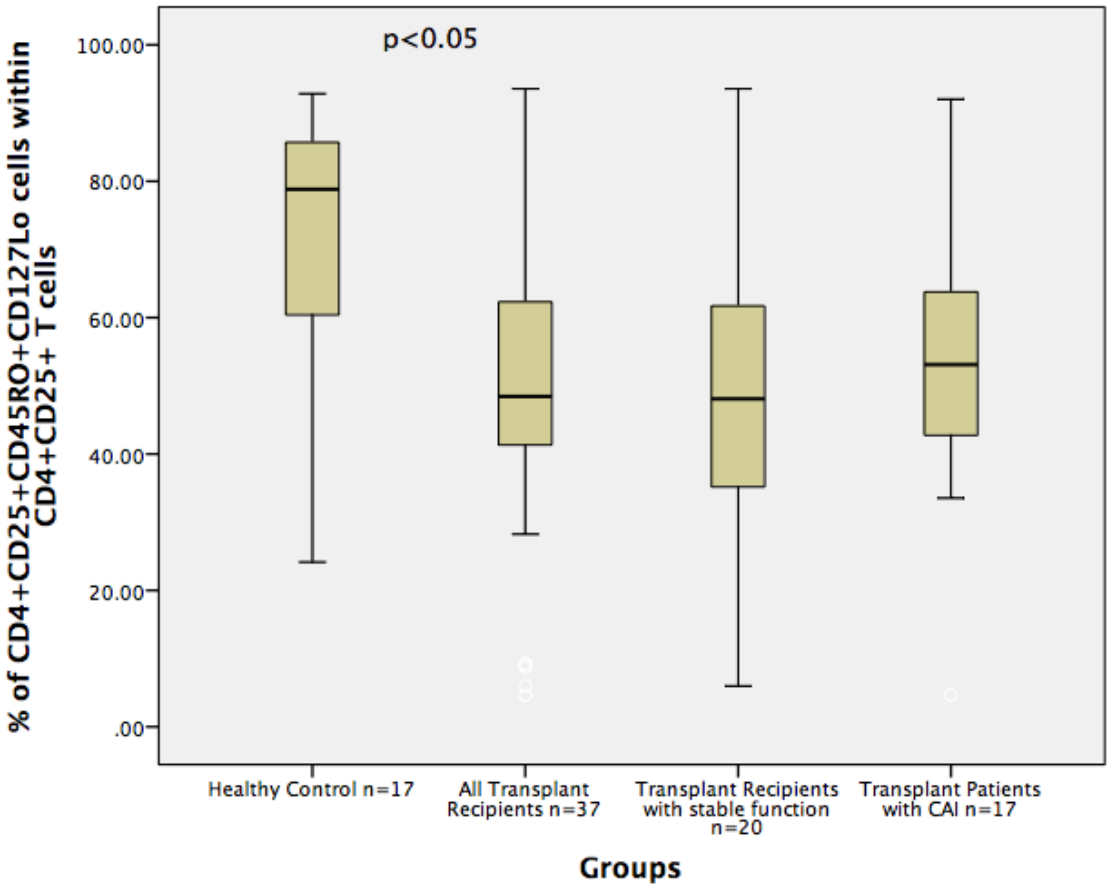
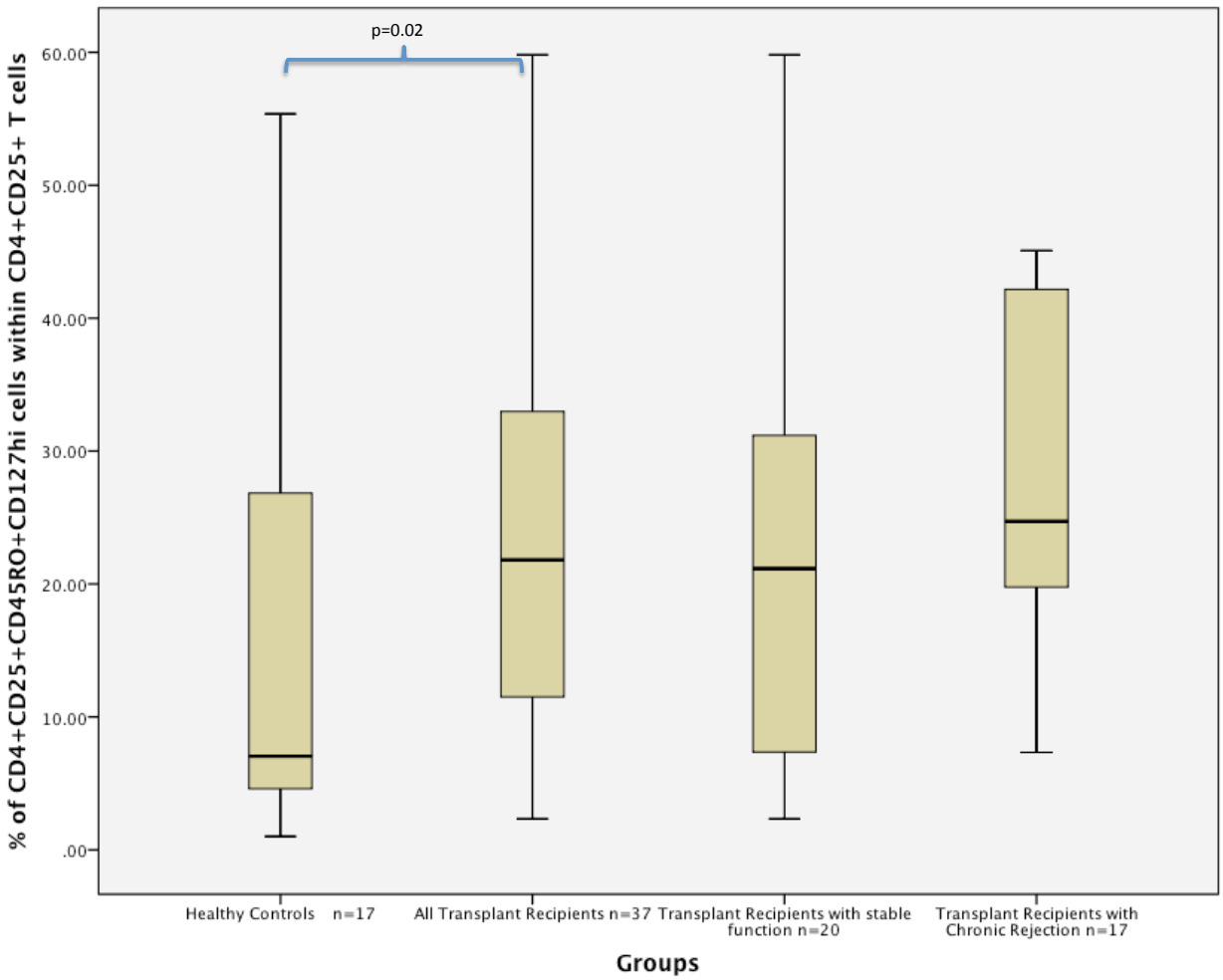


Figure 4.11. Box plot showing the proportion of Tems within $CD4^{+}CD25^{Hi}$ cells.

Lymphocytes from the PBMCs of 37 RTRs and 17 HCs were stained with cell surface antibodies and analysed for various phenotyping cell surface markers of T lymphoctyes (Figure 4.7, page 112). There was a significant difference in $CD4^{+}CD25^{+}CD45RO^{+}CD127^{Hi}$ cells between RTRs and HCs but not between recipients with stable function or CAI. Statistical analyses were done using the non-parametric Mann-Whitney U test between groups.



4.5.4 CCR7 Chemokine Receptor Expression in Renal Transplant Recipients

Sallusto and colleagues described the expression of CCR7 (a chemokine receptor that controls homing to secondary lymphoid organs) divides human memory T-cells into two subsets:

1. $CD4^{+}CD25^{+}CD45RO^{+}CD127^{Hi}CCR7^{-}$ ($CCR7^{-}$Tems)
2. $CD4^{+}CD25^{+}CD45RO^{+}CD127^{Hi}CCR7^{+}$(T central memory cells (Tcms))

As described earlier in this chapter, I have shown that cells making γ-interferon to non-polymorphic HLA derived peptides are $CD4^{+}CD25^{+}$ $CD127^{Hi}$ $CD45RO^{+}CCR7^{-}$ (Tem)-a phenotype that is over-expressed in renal transplant recipients compared to healthy controls. In the previous section I find that although $CD4^{+}CD25^{+}$ $CD127^{Hi}$ $CD45RO^{+}$ 'activated T cells' are relatively over-expressed in renal transplant recipients, I could not find a correlation with the presence of chronic allograft injury.

I subsequently analysed the proportion of $CD4^{+}CD25^{+}$ $CD45RO^{+}CD127^{Hi}$ $CCR7^{-}$ (Tem) and $CD4^{+}CD25^{+}$ $CD45RO^{+}CD127^{Hi}$ $CCR7^{+}$ (Tcm) shown in Figures 4.12 and 4.13 respectively. In particular, the proportion of Tems is significantly increased in renal transplant recipients with chronic allograft injury (19.8% (IQR 21.5) vs. 9.2% (IQR 21.5); p=0.03).

Figure 4.12. Box plot showing an increased proportion CCR7-Tems in transplant recipients vs. healthy controls.

Lymphocytes from the PBMCs of 37 RTRs and 17 HCs were stained with cell surface antibodies and analysed for various phenotyping cell surface markers of T lymphoctyes (Figure 4.7, page 112). There was an increased in this population in RTRs Vs. HC 10.8% (IQR 16.9) Vs. 4.5% (IQR 17.5); p=0.23) although not statistically significant. More interestingly, this population was further expanded in transplant recipients with chronic rejections compared to those with stable function (19.8% (IQR 21.5) Vs. 9.2% (IQR 13.7);p=0.03).

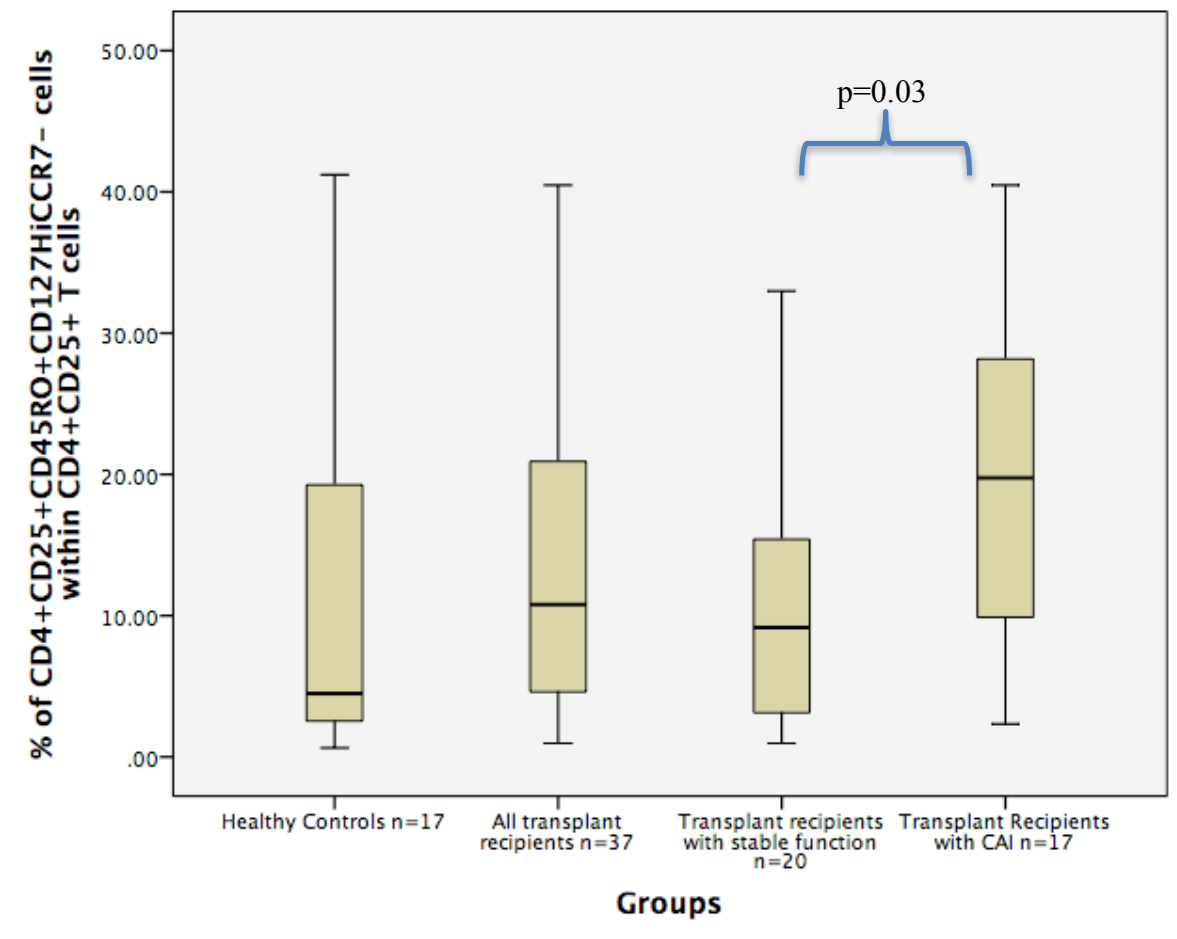
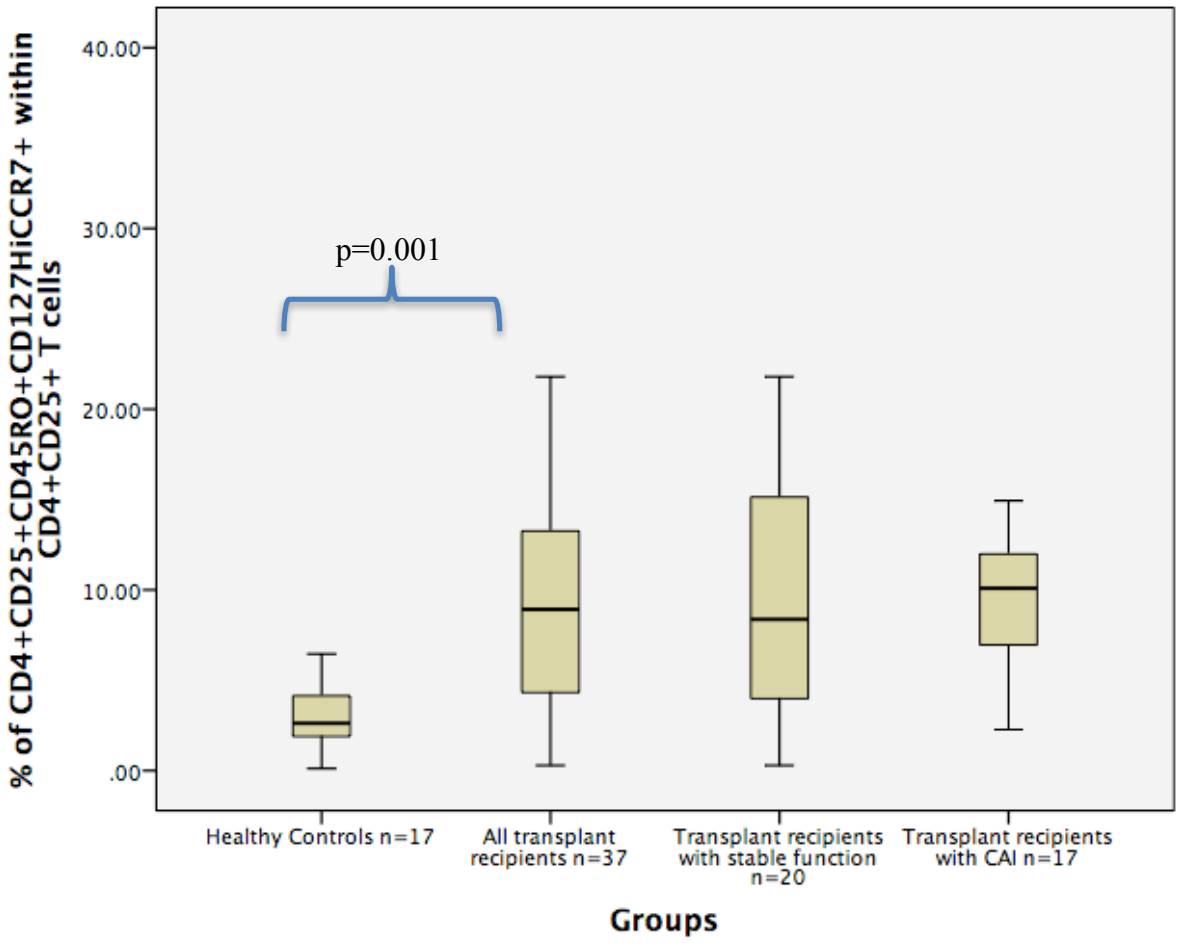


Figure 4.13. Box plot showing an increased population of Tcms in transplant recipients vs. healthy controls.

Lymphocytes from the PBMCs of 37 RTRs and 17 HCs were stained with cell surface antibodies and analysed for various phenotyping cell surface markers of T lymphoctyes (Figure 4.7, page 112). There was a significant increase in $CCR7^{+}$ Tcms in the peripheral blood of transplant recipients Vs. healthy controls (8.9% (IQR 9.2) Vs. 2.6% (IQR 3.5); p=0.001) as reflected by the higher number of total $CD4^{+}CD45RO^{+}CD127^{Hi}$ cells in RTRs. There was no significant difference in this population as calculated by Mann Whitney Test between transplants with stable function and those with CAI (8.4% (IQR 12) Vs. 9.9% (IQR 13.9); p=1.0).

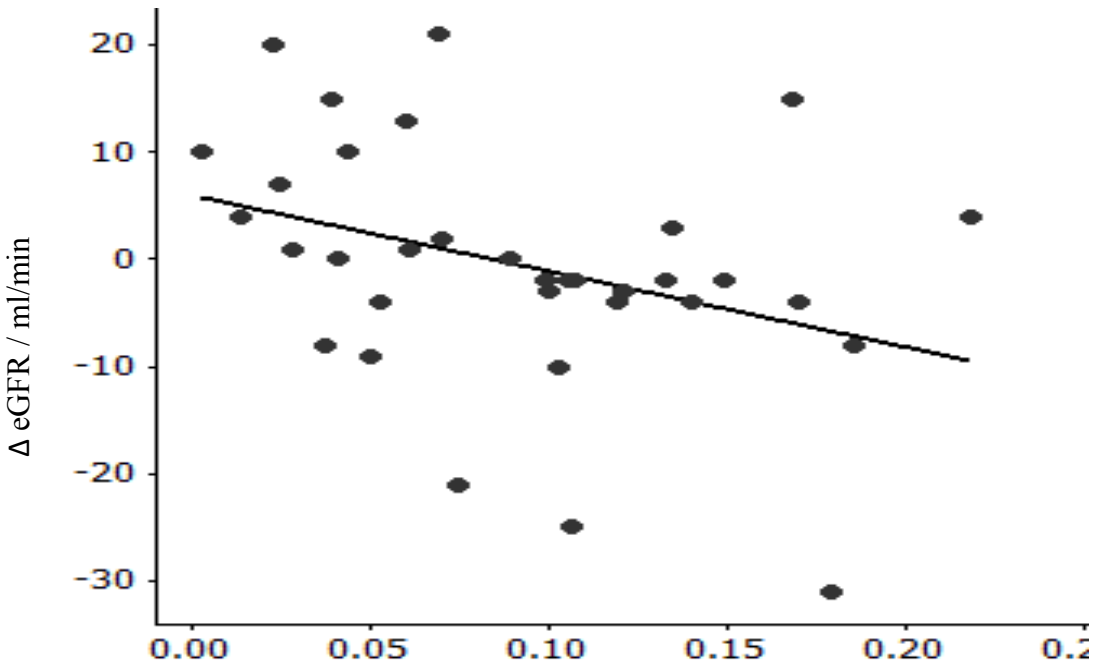


4.6 Correlations between surface markers and clinical markers of renal transplant dysfunction

This finding was supported by a second analysis in which the relationship between the frequency of T effector memory cells and change in estimated GFR (ΔeGFR) from recruitment (that is to say the time of lymphocyte enumeration) to most recent follow-up (801 ± 80 days post recruitment) was investigated using Spearman rank order correlation. Figure 4.15 shows in RTRs there was a weak but significant correlation between decline in renal function over the period following lymphocyte subset quantification (801 ± 80 days) and representation of this subset in peripheral blood $CD4^+$ T lymphocytes (r=-0.35, p=0.03). This test was limited by the small number of samples. There was no correlation between the number of T effector memory cells and urine albumin creatinine ratio (data not shown).

Figure 4.14. Correlation between number of Tems and change in estimated GFR

Relationship between change in (CKD-Epi) estimated GFR (ΔeGFR) from recruitment to most recent follow-up (801 ± 80 days post recruitment) in 37 RTRs and peripheral blood representation of $CD4^+CD25^{Hi}CD45RO^+CD127^+CCR7^-$ T lymphocyte within the $CD4^+CD25^{Hi}$ population Correlation calculated using Spearman's rank correlation coefficient (r=-0.35, p=0.03)..

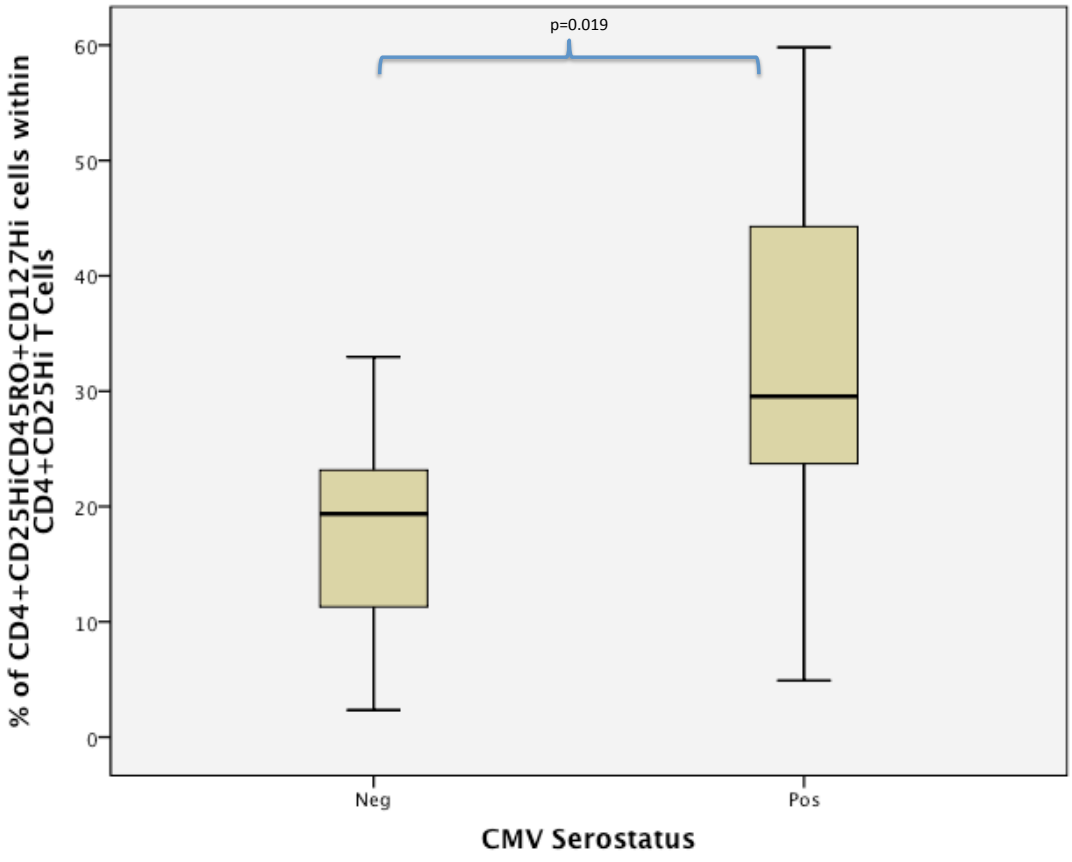


4.7 Association between CMV serostatus and circulating T lymphocyte subsets

Highly differentiated T-cells contribute to graft rejection (Reinke et al., 1994a, Reinke et al., 1994b). In healthy individuals these cells are specifically observed in CMV seropositive individuals with the pool further expanded in transplant patients. In our cohort of 37 transplant recipients 15 (40.5%) were CMV seropositive. Correlations between CMV serostatus and percentage of Tems showed a higher number of Tems in seropositive vs. seronegative status (31.8% ± 17.5 Vs. 19.8% ± 12.3; p= 0.019) as shown in Figure 4.15. As we did not have the CMV serostatus of our healthy control population, we were unable to conclude if our increase in Tems in RTRs was purely due to a high number of them (40.5%) being CMV seropositive.

Figure 4.15. Box plot showing an increase in percentage of $CD4^{+}CD25^{Hi}CD45RO^{+}CD127^{Hi}$ cells in CMV seropositive recipients vs. seronegative recipients.

Lymphocytes from the PBMCs of 37 RTRs were stained with cell surface antibodies and analysed for various phenotyping cell surface markers of T lymphocytes (Figure 4.7, page 112). Clinical data collected including the patient's CMV serostatus. The number of Tems ($CD4^{+}CD25^{Hi}CD45RO^{+}CD127^{Hi}$) was compared in CMV seropositive and seronegative individuals and showed a significant difference, being higher in CMV seropositive RTRs (29% (IQR 20) Vs. 19.5% (IQR 11.5);p=0.019).



4.8 Cytokine release in response to non-polymorphic (autologous) HLA derived peptides in renal transplant recipients

In a further set of experiments, autologous lymphocyte subsets were supplemented back to PBMC cultures in the presence of active or control peptide- n=6 for Class 1 peptides and n=5 for Class 2 peptides. PBMC's were cultured alone or following the addition of sorted autologous $CD25^{Hi}CD45RO^{+}CD127^{+}CCR7^{Hi}$ activated effector cells or $CD4^{+}CD25^{Hi}CD45RO^{+}CD127^{Lo}$ Tregs in duplicate. At 24 hours, supernatants were harvested and frozen and subsequently thawed for an assay of cytokine concentration. A panel of 5 cytokines (IL-10, TNF-α, IL-2, IL-Ra and IL-1b) was measured using the human Bioplex Cytokine Kit and analysed on a Bio-plexTM200 System (Bio-Rad, Hertfordshire, UK). A parallel set of experiments was performed on healthy controls (n=6 for Class 1 and n=6 for Class 2). For each cytokine eight standards ranging from 2 to 32,000pg/ml was used. The manufacturer quoted typical lower limit of assay sensitivity for IL-10 corresponds to those achieved in these experiments. Outliers from the standard curve were automatically removed using the Bioplex software manager and excluded from analysis.

4.8.1 IL-10

Interleukin-10 is a Th2 type cytokine shown to inhibit the development and function of Th1 cells, suppress inflammation, and enhance humoral pathways of the immune response (Gaffen and Liu, 2004). The role of IL-10 in transplant rejection is uncertain; evidence from animal models has suggested a role for the immunoregulatory cytokine IL-10 in promoting graft survival (Lowry et al., 1995) whilst others have provided evidence supporting the role of IL-10 in rejection (Merville et al., 1995, Weimer et al., 1996).

Negligible IL-10 release was seen following addition of any T-cell subset using the peripheral blood of healthy controls (median 2.6 pg/ml (IQR 2.3)) either in presence of active or control Class 1 and Class 2 peptide. In transplant patients however, the addition of Tregs in the presence of active peptide as opposed to control peptide showed an exponential significant release in IL-10 (Class 1: 26.1pg/ml (IQR 20.4) Vs. 2.6pg/ml (IQR 9.4); p=0.0087, Class 2: 170.6 pg/ml (IQR 122.4) Vs. 28.9 pg/ml (IQR 25.1); p=0.0079). This was not seen with the addition of $CD25^{Hi}CD127^{Hi}$ cells in the presence of active peptide. The response was consistent among all 6 patients however higher levels of IL-10 concentration was seen in the presence of active Class 2 peptides compared to Class 1 peptides (median 170.6pg/ml (IQR 122.4) Vs. 26.05pg/ml (IQR 20.44).

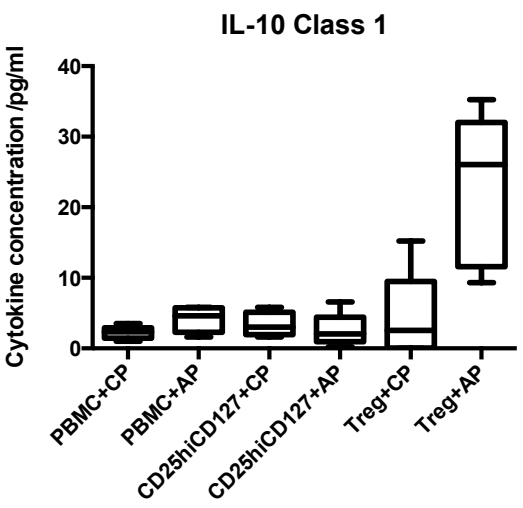
Figure 4.16. Graphs showing the average release of IL-10 when different $CD4^{+}CD25^{Hi}CD45RO^{+}$ subsets are added to wells in the presence of HLA Class 1 (A) and HLA Class 2 (B) derived peptide the patients were previously known to be responsive to.

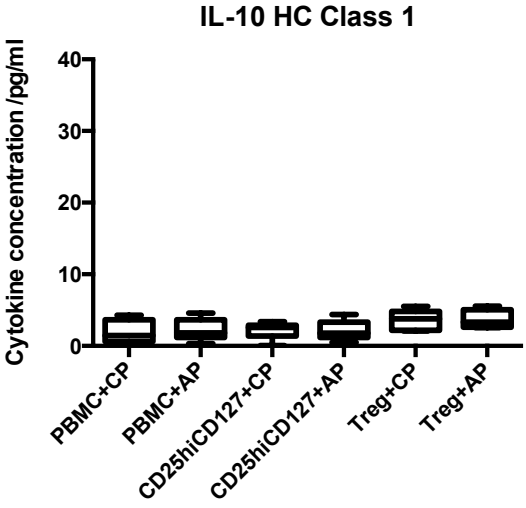
RTRs' PBMCs were cultured at $4x10^5$ per well in 96-well flat bottomed plates in duplicate in the presence of 'active' peptide (AP) (Class 2 derived: DRE6C, DRE3B or Class 1 derived: Tra 33) or control peptide (CP) at 11.4 µmol/L, at 37^0C and 5%CO_2. To these cultures were added $2x10^3$ autologous T lymphocyte subsets $CD4^{+}CD25^{Hi}CD45RO^{+}CD127^{+}CCR7^{-}$ ($CD25^{Hi}CD127^{+}$) or $CD4^{+}CD25^{Hi}CD45RO^{+}CD127^{-}CCR7^{+/-}$ (Tregs). Following 24 hours of culture supernatants were harvested and frozen. The cell culture supernatants from 6 individual experiments were subsequently thawed and the concentrations of IL-10 assayed with a human Bioplex Cytokine Kit and analysed on a Bio-plexTM200 System (Bio-Rad, Hertfordshire, UK). The median concentration and IQR is shown for each cell mixing experiment. There was an increased response of IL-10 in RTRs in response to active Class 1 and 2 peptides in the presence of Tregs:

Class 1: Treg +CP Vs Treg +AP (2.56pg/ml (9.4) Vs. 26.1pg/ml (20.4); p=0.0087)

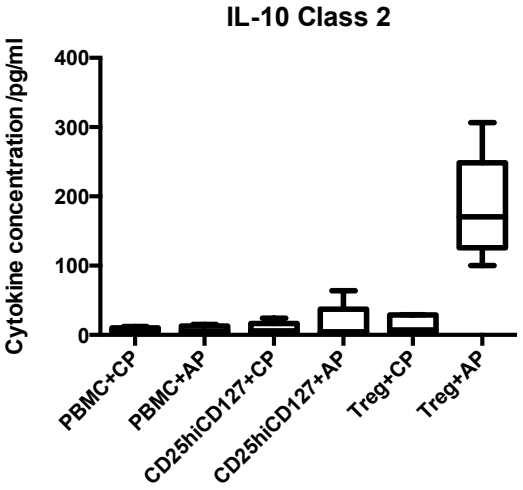
Class 2: Treg +CP Vs Treg +AP (7.1pg/ml (25.1) Vs. 170.6pg/ml (122.4); p=0.079)

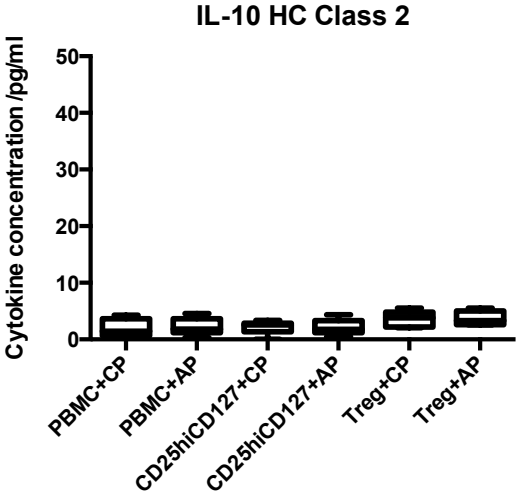
A


IL-10 Class 1
Cytokine concentration /pg/ml
40
30
20
10
0
PBMC+CP
PBMC+AP
CD25hiCD127+CP
CD25hiCD127+AP
Treg+CP
Treg+AP


IL-10 HC Class 1
Cytokine concentration /pg/ml
40
30
20
10
0
PBMC+CP
PBMC+AP
CD25hiCD127+CP
CD25hiCD127+AP
Treg+CP
Treg+AP

B


IL-10 Class 2
Cytokine concentration /pg/ml
400
300
200
100
0
PBMC+CP
PBMC+AP
CD25hiCD127+CP
CD25hiCD127+AP
Treg+CP
Treg+AP


IL-10 HC Class 2
Cytokine concentration /pg/ml
50
40
30
20
10
0
PBMC+CP
PBMC+AP
CD25hiCD127+CP
CD25hiCD127+AP
Treg+CP
Treg+AP

4.8.2 TNF-α

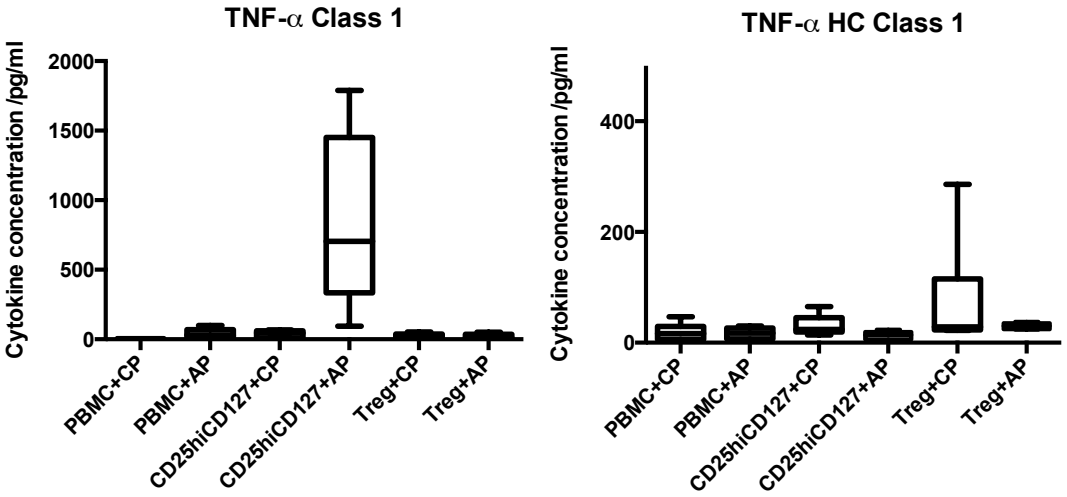
Tumour Necrosis Factor-alpha is a lymphocyte and macrophage derived cytokine that is pleiotropic in its actions. It plays an inflammatory and immunoregulatory role orchestrated by its two receptors TNFR1 and TNFR2. As allograft rejection is mediated by both innate and adaptive immune responses, it is not surprising that TNF-α is largely implicated in kidney allograft rejection. In rat experimental models, TNF-α mRNA expression is upregulated in tissue samples during acute and chronic rejection (Gasser et al., 2004, Herrero-Fresneda et al., 2005), as it is in human renal tissue during acute rejection (Noronha et al., 1993, Oliveira et al., 2002). In Chronic allograft nephropathy, TNF-α is also upregulated and may be specifically implicated in the intimal hyperplasia of arterial walls observed in late graft failure (Zegarska et al., 2002).

TNF-α was found in small quantities in the experiments using the peripheral blood of healthy controls. There was no significant variation with the addition of specific T-cell subsets or in the presence of active or control peptide in healthy controls. TNF-α concentrations were increased in the presence of active peptide (Class 1 and 2) and PBMCS compared with control peptide (Class 1: 29.7 pg/ml (IQR 56.8) Vs. 2.33 pg/ml (IQR 2.9); p=0.002, Class 2: 29.8 pg/ml (IQR 20.3) Vs. 3.8 pg/mol (IQR 0.5); p=0.0079). This concentration was further significantly increased with the addition of $CD25^{Hi}CD127^{Hi}$ cells in the presence of active peptide (Class 1; 703.3 pg/ml (IQR 116.8); Class 2: 779.3 pg/ml (IQR 935.6)) but reduced in the presence of Tregs (Class 1: 11pg/ml (IQR 29.3); Class 2: 4.6 pg/ml (IQR 2.8)). This suggests that Tregs inhibit peptide specific responses in cell culture but does not define the specificity of the Treg response. The response to $CD25^{Hi}CD127^{Hi}$ cells was only seen with active peptide and not control peptide (Class 1: 703.3pg/ml (IQR 116.8) Vs. 43.8pg/ml (IQR 56.2), p=0.002; Class 2: 779.3 pg/ml (IQR 935.6) Vs. 2.7pg/ml (IQR 1.9), p=0.0079). Cytokine concentrations did not differ in the presence of Class 1 or Class 2 peptides overall.

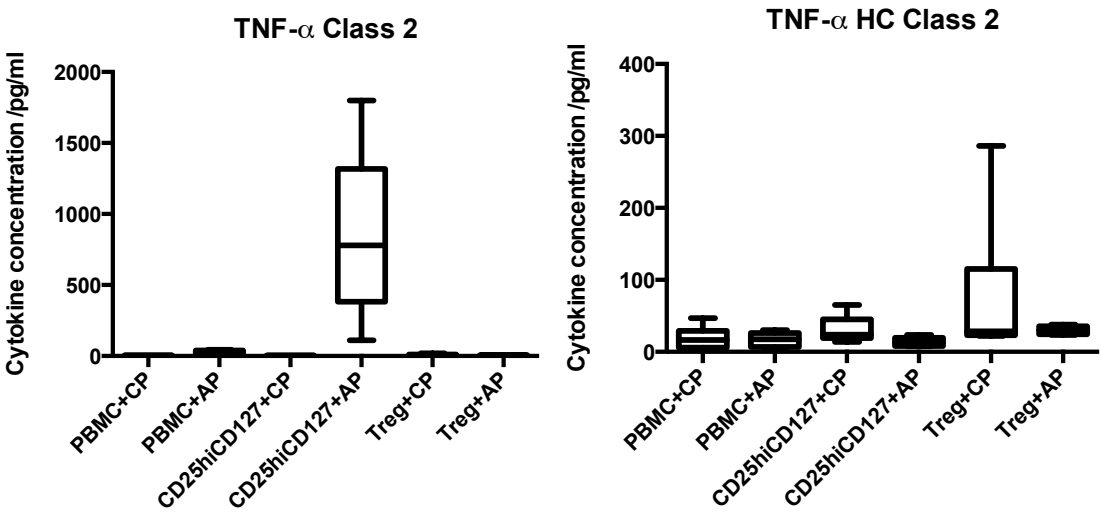
Figure 4.17. Graphs showing the average release of TNF-α when different $CD^{4+}CD25^{Hi}CD45RO^{+}$ subsets are added to wells in the presence of HLA Class 1 (A) and HLA Class 2 (B) derived peptide the patients were previously known to be responsive to.

RTR's PBMCs were cultured at $4x10^5$ per well in 96-well flat-bottomed plates in duplicate in the presence of 'active' peptide (AP) (class 2 derived: DRE6C, DRE3B or class 1 derived: Tra 33) or control peptide (CP) at 11.4 µmol/L, at 37^0C and 5% CO2. To these cultures were added $2x10^3$ autologous T lymphocyte subsets $CD4^+CD25^{Hi}CD45RO^+CD127^+CCR7^-$ ($CD25^{hi}CD127^+$) or $CD4^+CD25^{Hi}CD45RO^+CD127^-CCR7^{+/-}$ (Tregs). Following 24 hours of culture supernatants were harvested and frozen. The cell culture supernatants from 6 individual experiments were subsequently thawed and the concentrations of TNF-α assayed with a human Bioplex Cytokine Kit and analysed on a Bio-plexTM200 System (Bio-Rad, Hertfordshire, UK). The median concentration and IQR is shown for each cell mixing experiment. There was an overall increased response of TNF-α only in the presence of $CD4^+CD25^{Hi}CD127^+CCR7^-$ Tems in response to active peptides compared to control peptides (Class 1: 703.3pg/ml (IQR 116.8) Vs. 43.8pg/ml (IQR 56.2), p=0.002; Class 2: 779.3 pg/ml (IQR 935.6) Vs. 2.7pg/ml (IQR 1.9), p=0.0079). Note the difference in y-axis ranges between renal transplant recipients and healthy controls

A


TNF-α Class 1
Cytokine concentration /pg/ml
2000
1500
1000
500
0
PBMC+CP
PBMC+AP
CD25hiCD127+CP
CD25hiCD127+AP
Treg+CP
Treg+AP
TNF-α HC Class 1
Cytokine concentration /pg/ml
400
200
0
PBMC+CP
PBMC+AP
CD25hiCD127+CP
CD25hiCD127+AP
Treg+CP
Treg+AP

B


TNF-α Class 2
Cytokine concentration /pg/ml
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1500
1000
500
0
PBMC+CP
PBMC+AP
CD25hiCD127+CP
CD25hiCD127+AP
Treg+CP
Treg+AP
TNF-α HC Class 2
Cytokine concentration /pg/ml
400
300
200
100
0
PBMC+CP
PBMC+AP
CD25hiCD127+CP
CD25hiCD127+AP
Treg+CP
Treg+AP

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4.8.3 IL-2

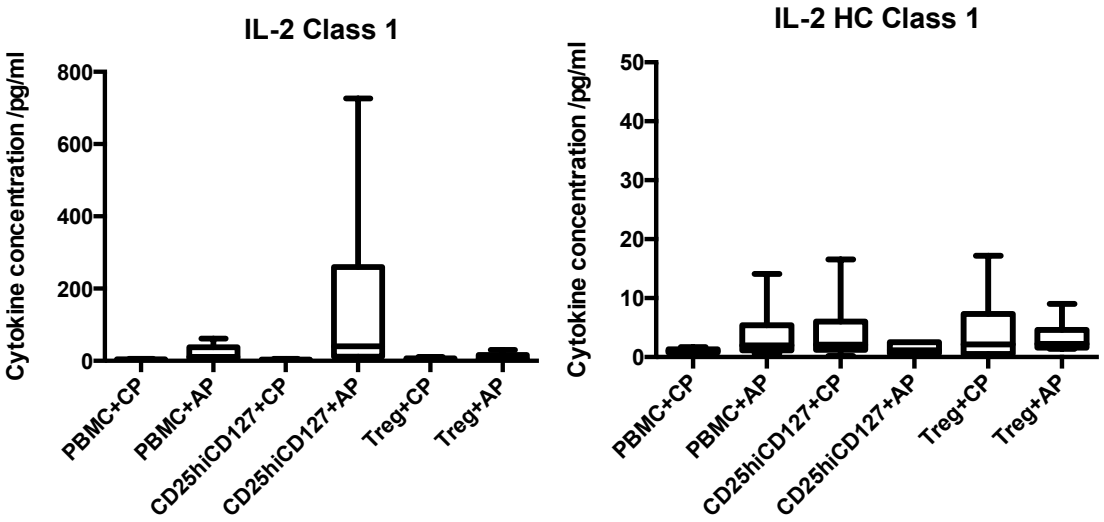
The complexity of the cytokine system and the pleiotropic effects of each cytokine explain why therapeutic strategies to modulate cytokines have not been fruitful. To date, only blockade of IL-2R has translated into clinical utility, with the development of basiliximab and anti-CD25 monoclonal antibodies that target the IL-2/IL-2R pathway.

IL-2 release was increased in the presence of active peptide and PBMCs compared to control peptide and PBMCs (Class 1: 10.8pg/ml (IQR 35.4) Vs. 2.1pg/ml (IQR 2.8), p=0.04; Class 2: 18pg/ml (IQR 13.5) Vs. 1.9pg/ml (IQR 1.3), p=0.0079). Responses were further increased with the addition of $CD25^{Hi}CD127^{Hi}$ Tems and active peptide (Class 1: 4.4pg/ml (IQR; Class 2: 45.5pg/ml (IQR 1938.9)). Interestingly, the concentrations of IL-2 were also significantly reduced by the addition of Tregs in the presence of active peptide (Class 1: 30pg/ml; Class 2: 13pg/ml) but not as marked as compared to TNF-α. This again was consistent with both classes of peptides.

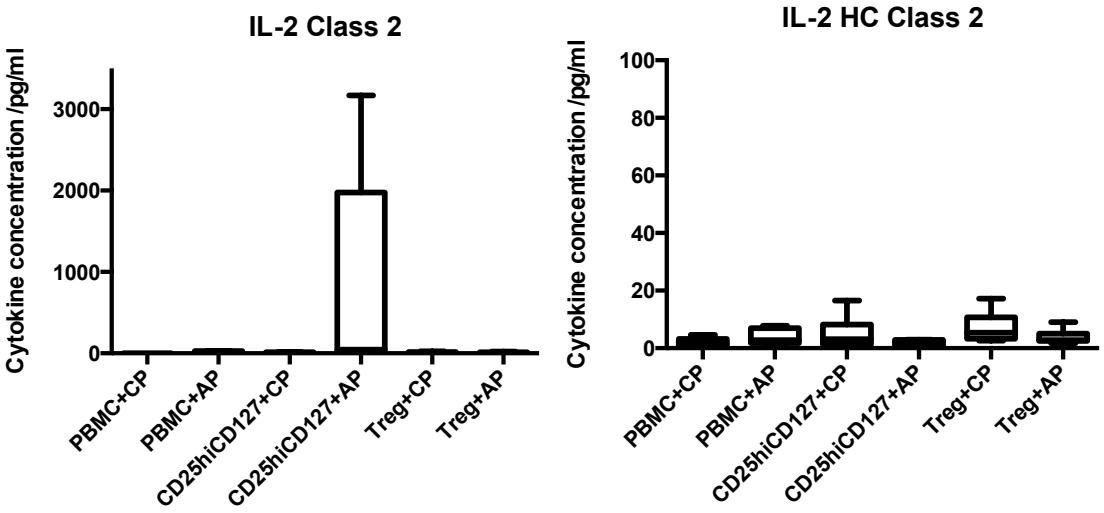
Figure 4.18. Graphs showing the average release of IL-2 when different $CD4^{+}CD25^{Hi}CD45RO^{+}$ subsets are added to wells in the presence of HLA Class 1 (A) and HLA Class 2 (B) derived peptide the patients were previously known to be responsive to.

RTR's PBMCs were cultured at $4x10^{5}$ per well in 96-well flat-bottomed plates in duplicate in the presence of 'active' peptide (AP) (class 2 derived: DRE6C, DRE3B or class 1 derived: Tra 33) or control peptide (CP) at 11.4 μmol/L, at 37^{0}C and 5% CO2. To these cultures were added $2x10^{3}$ autologous T lymphocyte subsets $CD4^{+}CD25^{Hi}CD45RO^{+}CD127^{+}CCR7^{-}$ ($CD25^{hi}CD127^{+}$) or $CD4^{+}CD25^{Hi}CD45RO^{+}CD127^{-}CCR7^{+/-}$ (Tregs). Following 24 hours of culture supernatants were harvested and frozen. The cell culture supernatants from 6 individual experiments were subsequently thawed and the concentrations of IL-2 assayed with a human Bioplex Cytokine Kit and analysed on a Bio-plexTM200 System (Bio-Rad, Hertfordshire, UK). The median concentration and IQR is shown for each cell mixing experiment. There was an overall increased response of IL-2 only in the presence of $CD4^{+}CD25^{Hi}CD127^{+}CCR7^{-}$ Tems in response to active peptides compared to control peptides (Class 1: 40.44 pg/ml (IQR 247.7) Vs. 2.48 pg/ml (IQR 2), p=0.002; Class 2: 45.7 pg/ml (IQR 1938.9) Vs. 6 pg/ml (IQR 10.5), p=0.0079).

A


IL-2 Class 1
Cytokine concentration /pg/ml
800
600
400
200
0
PBMC+CP
PBMC+AP
CD25hiCD127+CP
CD25hiCD127+AP
Treg+CP
Treg+AP
IL-2 HC Class 1
Cytokine concentration /pg/ml
50
40
30
20
10
0
PBMC+CP
PBMC+AP
CD25hiCD127+CP
CD25hiCD127+AP
Treg+CP
Treg+AP

B


IL-2 Class 2
Cytokine concentration /pg/ml
3000
2000
1000
0
PBMC+CP
PBMC+AP
CD25hiCD127+CP
CD25hiCD127+AP
Treg+CP
Treg+AP
IL-2 HC Class 2
Cytokine concentration /pg/ml
100
80
60
40
20
0
PBMC+CP
PBMC+AP
CD25hiCD127+CP
CD25hiCD127+AP
Treg+CP
Treg+AP

4.8.4 IL-1Ra & IL-1β

IL-1 is an important mediator of inflammation and tissue damage in multiple organs, both in experimental models of disease and in human diseases. The IL-1 family consists of two agonists, IL-1Ra and IL-1b, two receptors- IL-1R1 and IL-1RII and a specific receptor antagonist IL-1Ra. A human recombinant form or IL-1Ra is used in the treatment of rheumatoid arthritis.

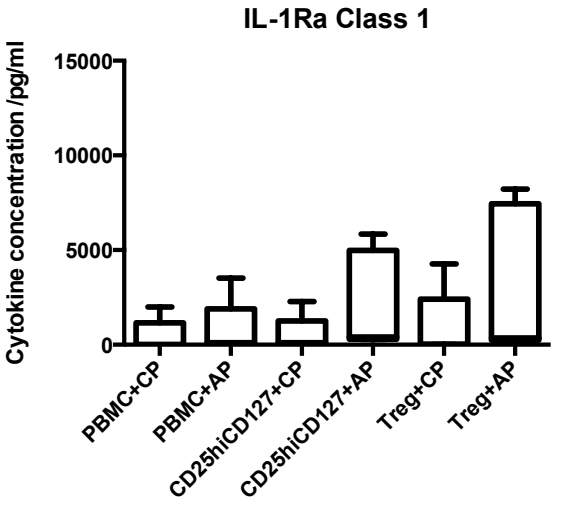
Results for IL-1Ra production were harder to decipher any significance from. Using Class 1 peptides there was overall a high background of IL-1Ra concentration (Median 593.4pg/ml; range 169.4- 2989) in all the wells using PBMCs from healthy controls. This was consistent amongst all 6 healthy controls and also using Class 2 peptides in healthy controls. In contrast to the contrary changes in peptide specific TNF-α and IL-10 production with sorted autologous activated effector cells or Tregs, addition of either subset was associated with elevated IL1Ra concentrations in response to the active peptide.

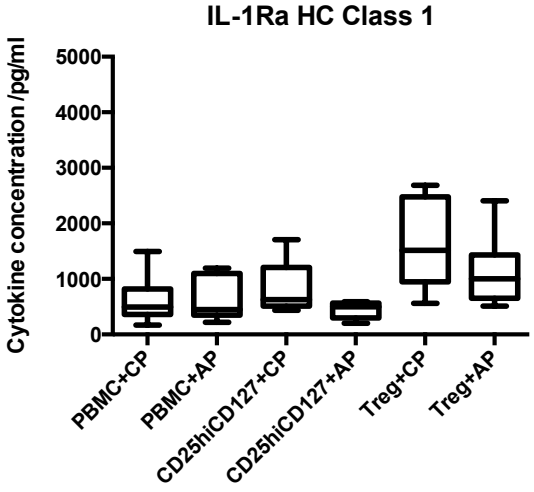
IL-1β concentrations reflected that of TNF-α and IL-2. Minimal levels were found in with PBMCs of healthy controls with either Class 1 or Class 2 peptides. In the presence of active peptide (Class 1 or Class 2) compared to control peptide there was an increase in IL-1β cytokine concentration in the presence of $CD25^{hi}CD127^{+}$ lymphocyte subset (Class 1: 96.2 pg/ml (IQR 234) Vs. 1 pg/ml (IQR 2.9), p=0.002; Class 2: 374.9 pg/ml (IQR 468.1) Vs. 0.7 pg/ml (IQR 3.6), p=0.0079). This was not seen with the addition of the Tregs subset.

Figure 4.19. Graphs showing the average release of IL-1Ra when different $CD4^{+}CD25^{Hi}CD45RO^{+}$ subsets are added to wells in the presence of HLA Class 1 (A) and HLA Class 2 (B) derived peptide the patients were previously known to be responsive to.

RTR's PBMCs were cultured at $4x10^{5}$ per well in 96-well flat-bottomed plates in duplicate in the presence of 'active' peptide (AP) (Class 2 derived: DRE6C, DRE3B or Class 1 derived: Tra 33) or control peptide (CP) at 11.4 μmol/L, at 37^{0}C and 5% CO2. To these cultures were added $2x10^{3}$ autologous T lymphocyte subsets $CD4^{+}CD25^{Hi}CD45RO^{+}CD127^{+}CCR7^{-}$ ($CD25^{hi}CD127^{+}$) or $CD4^{+}CD25^{Hi}CD45RO^{+}CD127^{-}CCR7^{+/-}$ (Tregs). Following 24 hours of culture supernatants were harvested and frozen. The cell culture supernatants from 6 individual experiments were subsequently thawed and the concentrations of IL-1Ra assayed with a human Bioplex Cytokine Kit and analysed on a Bio-plexTM200 System (Bio-Rad, Hertfordshire, UK). The median concentration and IQR is shown for each cell mixing experiment. Note the difference in Y-axis ranges between the graphs. The background release of IL-1Ra was high overall but more so with the Class 1 peptides experiments. There was no significant variation in levels between each cell mixing experiment.

A


IL-1Ra Class 1
Cytokine concentration /pg/ml
15000
10000
5000
0
PBMC+CP
PBMC+AP
CD25hiCD127+CP
CD25hiCD127+AP
Treg+CP
Treg+AP


IL-1Ra HC Class 1
Cytokine concentration /pg/ml
5000
4000
3000
2000
1000
0
PBMC+CP
PBMC+AP
CD25hiCD127+CP
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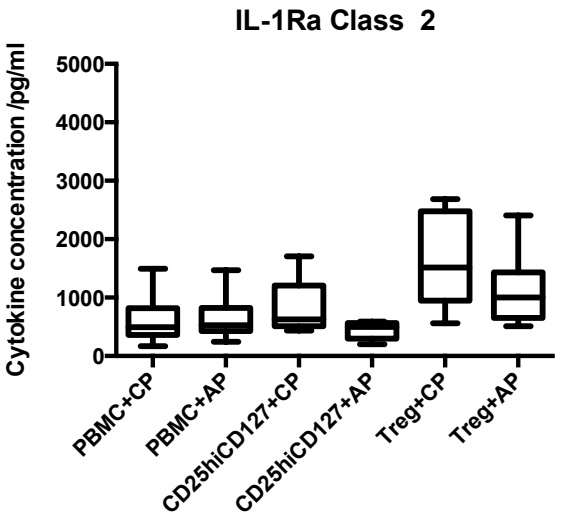
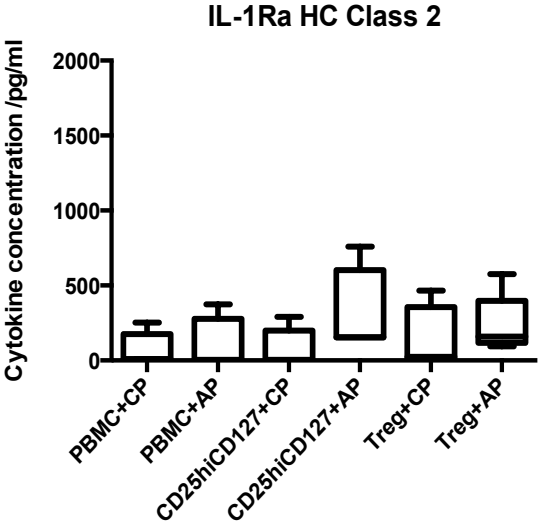
Treg+CP
Treg+AP

B


IL-1Ra Class 2
Cytokine concentration /pg/ml
5000
4000
3000
2000
1000
0
PBMC+CP
PBMC+AP
CD25hiCD127+CP
CD25hiCD127+AP
Treg+CP
Treg+AP

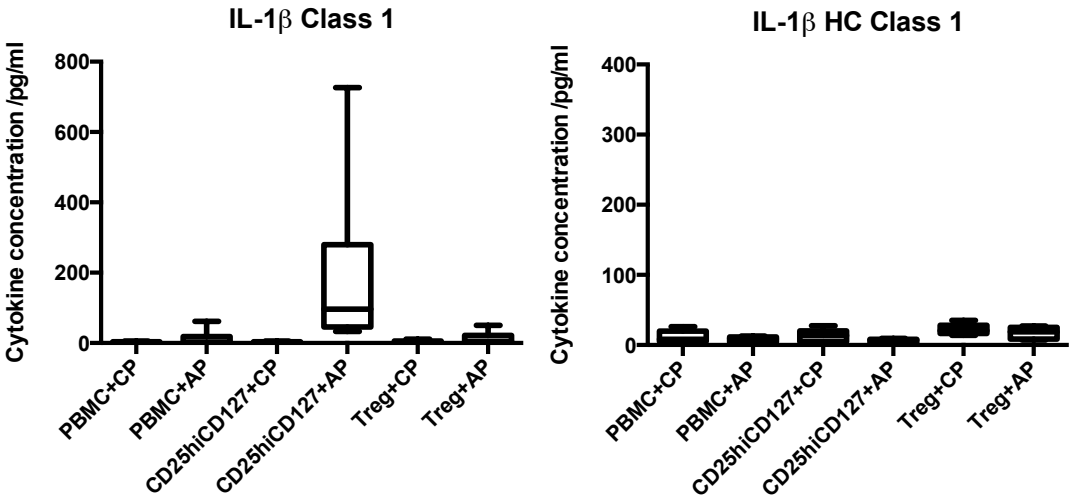

IL-1Ra HC Class 2
Cytokine concentration /pg/ml
2000
1500
1000
500
0
PBMC+CP
PBMC+AP
CD25hiCD127+CP
CD25hiCD127+AP
Treg+CP
Treg+AP

.

Figure 4.20. Graphs showing the average release of IL-1β when different $CD4^{+}CD25^{Hi}CD45RO^{+}$ subsets are added to wells in the presence of HLA Class 1 (A) and HLA Class 2 (B) derived peptide the patients were previously known to be responsive to.

RTR's PBMCs were cultured at $4x10^{5}$ per well in 96-well flat-bottomed plates in duplicate in the presence of 'active' peptide (AP) (class 2 derived: DRE6C, DRE3B or class 1 derived: Tra 33) or control peptide (CP) at 11.4 µmol/L, at 37^{0}C and 5% CO2. To these cultures were added $2x10^{3}$ autologous T lymphocyte subsets $CD4^{+}CD25^{Hi}CD45RO^{+}CD127^{+}CCR7^{-}$ ($CD25^{hi}CD127^{+}$) or $CD4^{+}CD25^{Hi}CD45RO^{+}CD127^{-}CCR7^{+/-}$ (Tregs). Following 24 hours of culture supernatants were harvested and frozen. The cell culture supernatants from 6 individual experiments were subsequently thawed and the concentrations of IL-1β assayed with a human Bioplex Cytokine Kit and analysed on a Bio-plexTM200 System (Bio-Rad, Hertfordshire, UK). The median concentration and IQR is shown for each cell mixing experiment. There was an increased response of IL-1β in response to Class 1 and 2 peptides in RTRs only in the presence of $CD4^{+}CD25^{Hi}CD127^{+}CCR7^{-}$ (Class 1: 96.2 pg/ml (IQR 234) Vs. 1 pg/ml (IQR 2.9), p=0.002; Class 2: 374.9 pg/ml (IQR 468.1) Vs. 0.7 pg/ml (IQR 3.6), p=0.0079).

A


IL-1β Class 1
Cytokine concentration /pg/ml
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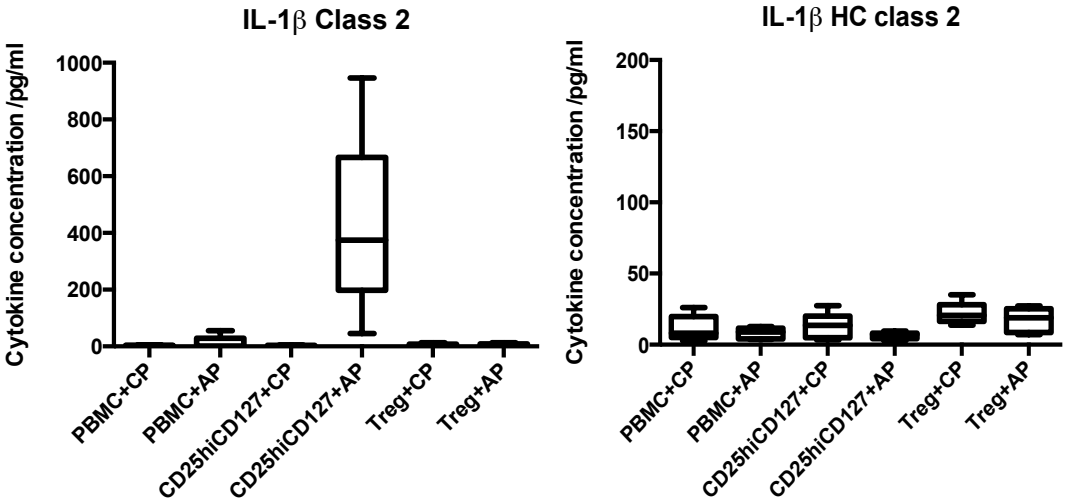
600
400
200
0
PBMC+CP
PBMC+AP
CD25hiCD127+CP
CD25hiCD127+AP
Treg+CP
Treg+AP
IL-1β HC Class 1
Cytokine concentration /pg/ml
400
300
200
100
0
PBMC+CP
PBMC+AP
CD25hiCD127+CP
CD25hiCD127+AP
Treg+CP
Treg+AP

B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Cytokine concentration /pg/ml
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400
200
0
PBMC+CP
PBMC+AP
CD25hiCD127+CP
CD25hiCD127+AP
Treg+CP
Treg+AP
IL-1β HC class 2
Cytokine concentration /pg/ml
200
150
100
50
0
PBMC+CP
PBMC+AP
CD25hiCD127+CP
CD25hiCD127+AP
Treg+CP
Treg+AP

4.8.5 Summary of responses

These data using PBMC's from renal transplant recipients suggests that autologous HLA derived peptides stimulate γ-interferon, TNF-α, IL-2 and IL-1β production from activated effector memory cells and IL-10 production in the presence of Tregs however the specificity of these responses is not defined.

Table 4.3. Summary of median cytokine production (pg/ml) when T lymphocyte subsets are stimulated in culture with HLA- Class 1 derived peptides transplant patients have been known to be responsive to (active peptide-AP) Vs unresponsive to (control peptide-CP) in the presence of PBMCS alone, addition of T effector memory cells ($CD25^{Hi}CD127^{+}$) or addtion of Tregs.

	PBMC +CP	PBMC +AP	$CD25^{Hi}CD127^{+}$ +AP	Treg + AP
IL-10	2.3 (IQR 1.5)	4.6 (IQR 3.5)	2.1 (IQR 9.4)	26.1 (IQR 20.4)
TNF-α	2.3 (IQR 2.9)	29.7 (IQR 56.8)	703.3 (IQR 116.8)	11 (IQR 29.3)
IL-2	2.1 (IQR 2.8)	10.8 (IQR 35.4)	40.4 (IQR 247.7)	4.9 (IQR 13.9)
IL-1Ra	9.3 (IQR 1150)	102.7 (IQR 1873)	408.3 (IQR 4688)	356.8 (IQR 7206)
IL-1β	1.5 (IQR 2.9)	2.7 (IQR 16.9)	96.2 (IQR 234)	4.3 (IQR 18.1)

Table 4.4. Summary of median cytokine production (pg/ml) when T lymphocyte subsets are stimulated in culture with HLA- Class 2 derived peptides transplant patients have been known to be responsive to (active peptide-AP) Vs unresponsive to (control peptide-CP) in the presence of PBMCS alone, addition of T effector memory cells ($CD25^{Hi}CD127^{+}$) or addtion of Tregs.

	PBMC +CP	PBMC +AP	$CD25^{Hi}CD127^{+}$ +AP	Treg + AP
IL-10	6.4 (IQR 6.1)	5.3 (IQR 12.7)	4.6 (IQR 36.1)	170.6 (IQR 122.4)
TNF-α	3.8 (IQR 0.5)	29.8 (IQR 20.3)	779.3 (IQR 935.6)	4.6 (IQR 2.8)
IL-2	1.9 (IQR 1.3)	18 (IQR 13.4)	45.7 (IQR 1938.9)	5 (IQR 9.6)
IL-1Ra	8.7 (IQR 169.7)	4.1 (IQR 275.8)	154.9 (IQR 447.6)	159.9 (IQR 279.5)
IL-1β	2.6 (IQR 2.4)	0.5 (IQR 28.9)	374.9 (IQR 468.1)	1.6 (IQR 8.1)

4.9 IL-10 production is specific to T regulatory cells when stimulated by HLA derived peptides

IL-10 production was seen in the supernatant of Tregs co-cultured with HLA derived peptides. To determine if IL-10 production was specific to T regulatory cells we performed the same cytokine release experiments on 2 previously responsive patients but instead blocked cytokine release from the cells (using Brefeldin) and stained for intracellular IL-10 in Tregs as described in Section 2.2.8, page 44.

A significant number of total lymphocytes were lost due to the intracellular staining method. 500,000 PBMCs were isolated per patient but after the experimental process only 5% of lymphocytes were gated compared to 60% when we used surface staining methods only. The majority of $CD4^{+}CD25^{Hi}$ cells were $CD45RO^{+}CD127^{Hi}$ however the number of $CD45RO^{+}CD127^{Lo}$ (Tregs) cells was unusually low. Within this small number of cells any intracellular IL-10 production was undetectable.

CHAPTER 5
DISCUSSION

5 Discussion

Chronic allograft injury remains a barrier to improved long-term renal graft outcome. Although alloantigen-independent factors contribute to CAI sufficient data exists that emphasize the importance of ongoing immunological damage as a major mechanism for this process as is described in this thesis. The contribution of indirect allorecognition to the ongoing immune process is well established in several experimental models of transplantation (Benichou et al., 1992, Vella et al., 1999, Watschinger et al., 1994, Pettigrew et al., 1998) and in it has been associated with chronic rejection in clinical studies of kidney (Najafian et al., 2002), heart (Ciubotariu et al., 1998) and lung transplantation (Reznik et al., 2001). Given the role of cellular immunity in transplant rejection, its quantification might be expected to provide valuable diagnostic and prognostic value – however development of a clinically relevant and widely applicable assay has proved difficult (Waanders et al., 2008, Iacomini and Sayegh, 2006, Sawitzki et al., 2009)- one important factor being the diversity of donor and recipient HLA. Additionally, the low precursor frequency of indirectly primed T cells makes this pathway difficult to quantify. Preliminary work in our research group on the identification of responses to non-polymorphic HLA derived peptides in RTRs via a γ-interferon ELISPOT assay has allowed us to detect low frequency indirect alloimmune responses.

5.1 The use of HLA derived peptides as tools to predict CAI

Colin Watts and colleagues have over many years studied antigen processing and amongst other findings have shown that antibody binding can determine the pattern of peptides generated during antigen processing and indeed this has varying effects on the presentation of specific peptide epitopes following processing of whole antigen (Watts, 2012).

It is therefore possible that alloantibody binding of antigen enhances the presentation of otherwise cryptic self epitopes as described in animal models of transplantation ((Boisgerault et al., 2000, Lovegrove et al., 2001) and thus possible that the T-cell responses that we observe are testament to a prior alloantibody response – which of course does not have to be donor specific. γ-interferon production by lymphocytes incubated with autologous HLA derived peptides is associated with chronic renal allograft dysfunction (Smith et al., 2011) and chronic graft versus host disease (GVHD) following allogeneic bone marrow transplantation

(Smith et al., 2010). It is in this context that we further investigated responses to autologous HLA derived peptides in a clinical setting in a single population of RTRs recruited with minimal exclusion criteria suggesting our findings may be broadly relevant.

The ELISPOT technique provides the most sensitive way for detection and quantification of antigen-specific responses of in vivo activated T-cells (Heeger et al., 1999). Unlike limiting dilution assays, culture times in ELIPSOT are shorter: consequently, the frequency measured corresponds to the true number of T-cells that have been encountered in vivo. A study by Zhang and colleagues in 11 different laboratories across Europe and the United States used the ELISPOT assay to measure antigen- specific T-cell responses to a CMV peptide (Zhang et al., 2009). Results produced were reproducible using identical reagents and a standardized protocol even in the hands of ELIPSOT inexperienced investigators (Zhang et al., 2009). Our own results with the optimization studies performed at two different sites yielded comparable results confirming the reproducibility of this assay.

Our studies looked at the detection of T-cell responses to synthetic HLA Class 1 and Class 2 derived peptides. With synthetic peptides the exact amino acid length and concentration is known making the assay reproducible. One disadvantage is the creation of neoepitopes by dissecting proteins into peptides. Peptides may be synthesized that do not occur in vivo because of the absence of natural splicing sites at relevant positions on the full protein (Waanders et al 2008). This is however unlikely in short culture periods as in an ELISPOT assay.

In the first instance we demonstrated, as with HLA Class 1, RTR's lymphocytes produce γ-interferon to a number of non-polymorphic Class 2 derived peptides that were predicted to bind HLA-DR promiscuously. These responses were less frequent in healthy controls suggesting but not proving that the response is a feature of exposure to alloantigen.
The fact that responses to HLA class 2 derived peptides correlated closely with those to HLA Class 1, suggests that saturation was approached with respect to identifying RTRs with the potential to generate such a response. This is consistent with the finding that responses to HLA class 1 derived peptide was associated with a clinical phenotype, since had significant reclassification occurred with a wider range of epitopes such an association would seem

unlikely, assuming that responses to HLA Class 2 derived peptides are mechanistically equivalent to those to Class 1.

Correlation of responses between single peptides and mixes of different peptides derived from the α3 domain of HLA A2 (Class 1) and β2 domain of HLA-DR (Class 2) allowed us to significantly reduce the volume of whole blood required per test per patient.

2 year follow up data on patients tested to HLA derived mixes showed low rates of graft failure and loss, hence any statistical survival analysis was not powered sufficiently. My data did not appear to show an association with single time point ELISPOT responses and 2-year graft function as measured by eFGR. There was a positive correlation with worse proteinuria at 2-years however confounding factors such as infection or recurrent disease were not corrected for. Serial time point analysis of ELISPOT responses with clinical outcome may give a better understanding of recipient risk, which was then attempted in the longitudinal study.

In the longitudinal study responses were tested to Class 1 and 2 mixes pretransplantation and at 3,12, and 24 months post transplantation. An increasing number of responses were seen at 12 months' post transplantation however 5-9% of patients lost their positive responses at 24 months' post transplantation. One potential explanation for this could be a reduction in the number of effector memory cells with time away from transplantation as we found this was the subset of $CD4^+$ T lymphocytes, which contributed to the peptide response. No significant correlation was found between post-transplant ELISPOT responses and acute transplant rejection before or after testing. This result although disappointing may reflect previous studies where responses to donor derived antigens were increase during an episode of acute rejection in cardiac allografts but decreased after successful treatment (van Besouw et al., 2005). Additionally, all our transplant recipients received induction therapy with basiliximab, which selectively depletes a subset of T-cells. Other studies showing a negative correlation of pre-transplant ELISPOT results and acute rejection had used similar induction therapy protocols to ours (Reinsmoen et al., 2008). Our small numbers of biopsy proven CAI (6% in 2 years follow up) made it impossible to decipher any correlation to ELISPOT responses.

Infective episodes including CMV did not interfere with testing ELISPOT response although the number of CMV viraemia episodes was low in this study.

5.2 The use of $CD4^+$ T Lymphocyte surface makers as tools to predict CAI

In previous studies we identified the phenotype of responding cells as $CD4^+CD25^{hi}$ by intracellular γ-interferon staining. We have extended this characterisation by titrating increasingly defined subsets of $CD4^+CD25^{hi}$ T-cells, back into autologous HLA peptide stimulated PBMC cultures. There was titration of γ-interferon producing cells only with the $CD4^+CD25^{Hi}CD45RO^+CD127^+CCR7^-$ phenotype: 'activated effector memory' T-cells. This titration was also reflected in the supernatant concentrations of TNF-α. The need to add exogenous autologous peptide to identify these in vitro phenomena is serendipitous in that it allowed their original definition. The rapid expression of γ-interferon by memory cells following stimulation (within 6 hours in previously described flow cytometry studies), their consistent observation with a range of different class 1 and 2 derived peptide sequences and their association with a clinical phenotype, suggest that these observations are however unlikely to represent a purely in vitro phenomenon but reflect a biologically relevant alteration in transplant recipients' immune repertoire.

All lymphocyte activation inhibitors reduced the responses to peptides in the presence of T effector memory cells only- suggesting a pathway of action. This response was less marked however with sirolimus and abatacept. A small number of patients also showed reduced inhibition to ciclosporin at a dose of 100ng/ml, which corresponds to the lower quartile trough concentration in the BENEFIT study (at 1year). These findings illustrate how relatively subtle intra- and inter-individual variations in calcineurin inhibitor exposure could influence the expression of HLA peptide specific T-cell memory. In this assay, the relative resistance of peptide specific activated effector memory cell responses to CD28 blockade may relate mechanistically to the observation that, referred to CNI based therapy, such treatment is less effective with respect to the prevention of acute rejection whilst inhibiting pathogen specific primary responses to a greater degree. These findings echo those in a recent report by Xu & colleagues where mTOR inhibitors also have limited efficacy against this subset (Xu et al.,

2014). My findings suggest mechanisms that may in part account for differing effects of maintenance immunosuppressive regime.

Our findings of the phenotypic expression of $CD4^+$ T lymphocytes reflect the literature. The majority of $CD4^+CD25^+$ T-cells lack CD127 expression in healthy controls but this number is reduced in RTRs. The increased representation of the $CD4^+CD25^{Hi}CD45RO^+CD127^+CCR7^-$ subset in RTRs is consistent with the findings of others on CCR7 undifferentiated $CD4+CD25^{Hi}CD45RO^+CD127^+$ populations. Two groups have reported increased representation of the latter population in long-term RTRs in association with CAI (Alonso-Arias et al., 2009) or chronic humoral rejection (Vallotton et al., 2011b). Another group did not confirm these findings, albeit with a different approach to case definition and lymphocyte isolation (van de Berg et al., 2012a). Our finding of a relationship between progressive CAI and peripheral representation of the $CD4^+CD25^{Hi}CD45RO^+CD127^+CCR7^-$ subset is interesting but this analysis was not part of the original design and needs repetition in a study adequately powered to undertake multivariable analysis.

Highly differentiated T cells contribute to graft rejection (Reinke et al., 1994a) and these cells are observed in CMV seropositive healthy controls and further expanded in RTRs (Reinke et al., 1994b). Previous work from our own group has identified expansion of CD4+CD28- cells predominantly of the effector-memory phenotype post transplantation in CMV seropositive individuals (Shabir et al., 2016). Work presented here has shown an expansion of $CD4^+CD25^{Hi}CD45RO^+CD127^{Hi}$ (Tems) in the peripheral blood of CMV seropositive RTRs when compared to healthy controls. I was unable to prove a difference in the peripheral population of Tems in CMV seropositive patients with stable function compared to CMV seropositive patients with CAI (data now shown). I am unable to conclude from this work if the expansion of Tems in RTRs was purely an effect of CMV serostatus, as we did not investigate the CMV serostatus of healthy controls.

The predominant contribution of HLA derived peptides to the MHC Class 2 ligandome was reported more than 20 years ago (Chicz et al., 1992). These autologous peptides are therefore candidates for self-restricted engagement by T-cell receptors, resulting in self-tolerance through thymic deletion and agonist selection of natural Tregs (Hsieh et al., 2004). We

considered whether γ-interferon production to autologous HLA derived peptides might reflect alteration of a normal regulatory response to such peptides. Production of IL-10 in PBMC cultures following addition of both autologous HLA derived peptide and Tregs identified these as likely target epitopes for Tregs. This occurred with 3 different peptide sequences selected on the basis of predicted or demonstrated high affinity binding to a range of HLA-DR, suggesting that these observations are likely to reflect a mechanism that involves conventional self restricted T-cell receptor engagement. The finding of novel Treg epitopes derived from HLA is reminiscent of reports by de Groot and colleagues of so called 'Tregitopes' derived from the non-polymorphic regions of IgG (Cousens et al., 2013). Other ubiquitous cellular proteins are also candidate Treg epitopes (van Herwijnen et al., 2012, Cousens et al., 2013).

Importantly in these experiments Treg dependent IL-10 synthesis required the addition of specific peptide antigen. Although it is possible that IL-10 production arose from a distinct PBMC population, this does not detract from the peptide specific link between the detection of IL-10 and the presence of Tregs. This requires further investigation beyond the current study however the parsimonious explanation is that Tregs synthesize IL-10 in response to these peptides. In an attempt to determine if IL-10 production was specific to Tregs I attempted intracellular staining for IL-10 in repeat cytokine release experiments. The intracellular staining method resulted in a low yield of lymphocytes within which the number of Tregs was extremely low. A number of points to consider were raised from the failure of this experiment. Firstly, isolating and staining a small population of $CD4^{+}CD25^{hi}CD45RO^{+}CD127^{lo}$ cells proved to be ambitious in the first place. Secondly, Brefeldin was added 6 hours before the end of a 48-hour stimulation as suggested by previous studies in the literature and manufacturer recommendations. However, we did not perform a time comparison for the addition of Brefeldin. Early addition to the stimulation but have limited the release of IL-10 from cells into the supernatant. Due to time restraints we were unable to perform any further experiments to clarify this.

In light of recent experimental evidence of phenotypic instability in $CD25^{hi}Foxp3^{+}$ Treg's (Bailey-Bucktrout et al., 2013, Sharma et al., 2013, Lal et al., 2011), one possible explanation of these findings is that autologous HLA peptide specific, natural Tregs acquire an effector

phenotype in the presence of an inflammatory response. In which case observations of an association between γ-interferon production to autologous HLA derived peptides and a disease phenotype (CAI (Smith et al., 2011) or chronic GVHD (Smith et al., 2010)) could reflect loss of regulatory as much as acquisition of effector function. An alternative but related explanation is that T effector or induced Treg responses to cryptic self-epitopes arise from naïve T lymphocytes during the course of an inflammatory response (Drakesmith et al., 1998). Since productive immunity may be associated with epitope spreading, then responses to autologous sequences could emerge from allogeneic responses, possibly influenced by other effector mechanisms. For example antibody binding to whole antigen has been reported to enhance presentation of certain peptide epitopes whilst diminishing others (Simitsek et al., 1995, Watts, 2012); an additional perspective on the potential role of alloantibody in determining T-cell responses to HLA (Tambur et al., 2000). These findings may also be relevant to other settings characterised by acute and chronic inflammation in the presence of immunosuppression, such as autoimmune disease. Indeed the role of autoimmunity in cGVHD is well described and in this setting we have documented γ-interferon production to autologous HLA Class 1 derived peptides (Smith et al., 2010).

Treg therapy attempts to address this balance between effector and tolerogenic phenotypes. 2 broad possibilities for this therapy exist in humans- 1) induction of Tregs from naïve precursors in vivo or 2) infusion of autologous Tregs expanded from peripheral blood ex vivo (Issa et al., 2010). Recent evidence has demonstrated that Tregs can be expanded in vitro from healthy human blood and retain their suppressive function (Putnam et al., 2009). Joffre and colleagues showed that the administration of in vitro allo-stimulated and expanded host Tregs induces donor-specific tolerance to allogeneic bone marrow grafts (Joffre et al., 2008). The safety of using expanded Tregs in human patients is not certain. As previously mentioned there are some suggestions in the literature that Tregs retain the capacity to differentiate into effector populations (Bailey-Bucktrout et al., 2013). Our own findings of IL-10 production in the presence of autologous HLA peptides and Tregs may support this although a direct causative link cannot be confirmed. Reassuringly, the first trial of ex vivo expanded Treg therapy in humans for the treatment of GVHD did not report any unexpected side effects (Trzonkowski et al., 2013). The administration of Tregs to transplanted patients in order to induce tolerance and allow dose reduction in their immunosuppressive treatment is a realistic

target and our preliminary findings are potentially relevant to the development and safe application of Treg therapy. In conclusion these data identify autologous derived peptides that stimulate IL-10 production in the presence of Tregs. We hypothesize that this reflects a normal functional relationship, which in the setting of antigen driven immune activation and inflammation is redirected to be pro-inflammatory (Sharma et al., 2013, Bailey-Bucktrout et al., 2013).

5.3 Limitations of this study

Perhaps the biggest limitation of this study are the low numbers of recruits overall especially for the longitudinal study. Accounting for patient withdrawal, we lost several samples as patients were sent back to their base hospital for follow up. With low numbers the rates of CAI were also low to make any significant correlation difficult. Lack of protocol transplant biopsies was also a confounding factor to this. Due to the low numbers and high dropout rates in the longitudinal study we were unable to decipher any definitive correlations between ELISPOT responses and the course of a transplant in terms of alloimmune risk.

Working with freshly- isolated cells we could not reproduce assay results using the same cell material. Circadian rhythms, nutrition, infection and stress will all change the cellular composition of peripheral blood mononuclear cells. Hence a major drawback of our study is that using freshly isolated material our T-cell assays were technically not fully standardized. Although ELISPOT technique provides the most sensitive way for detection of antigen-specific responses in vivo, we do not know how many patients had subclinical rejection leading to allosensitization.

Renal failure necessitating transplantation has diverse aetiology and furthermore most RTRs have concomitant comorbidities giving rise to a fairly heterogeneous group of patient. Such variance undoubtedly impedes the development of a unified biomarker for predicting allograft survival. Studies across much groups of patients are thus necessary to define the contribution of underlying disease processes to the transplant outcome.

5.4 Future work

Several experimental and clinical trials are underway evaluating the therapeutic role of peptides in human diseases such as coeliac, diabetes, asthma and multiple sclerosis.
The ultimate goal for renal transplant recipients would be a desensitization program where patients with HLA specific antibodies on the transplant waiting list would be vaccinated with peptides and antibody levels monitored pre and post operative to essentially induce peripheral tolerance by allowing substation for more specific tolerance and reduction of the current quadruple load immunosuppression.

OUTSMART Trial

Work from this thesis has contributed to the grant application for the OUTSMART trial. The general aim of this multicentre trial is to determine whether combinations of analyses of immune responsiveness against the transplant are better than anti-HLA antibody and conventional histology at identifying patients: a) with active immune responses against their transplant and b) at high risk of transplant dysfunction and graft loss. Blood and biopsy-based assays measuring donor-specific immune responsiveness in kidney transplant recipients using flow cytometry, ELISPOT, RT-PCR and microarrays will be used to assess whether a combination of these have better diagnostic, prognostic and mechanistic value than anti-HLA antibody and conventional histology. The data generated will inform the development of tailored therapeutic interventions to prevent premature graft failure.

5.5 Final Conclusion

In summary, this body of work has aimed to identify a set of immunological biomarkers that may facilitate the stratification of the risk of progressive CAI. We have identified a set of synthetic HLA derived peptide mixes that transplant recipients respond to irrespective of both donor and recipient HLA type. Furthermore, we have identified the subgroup of responding cells and have shown peripheral expansion of these cells in renal transplant recipients.
Further work has identified γ-interferon and TNF-α production in the presence of T effector cells and active peptide and IL-10 in the presence of Tregs and active peptide- in short the balance as it were between effector cells and Tregs as determinants of chronic rejection or

transplant tolerance. The findings from this small study do not of course establish a cause and effect relationship between indirect T cell alloreactivity and the risk of progressive CAI. I conclude that further work in a larger multicenter study and involvement of protocol biopsies may allow the development of an alloimmune assay to be used pre transplantation and during the course of a transplant to help stratify a renal transplant recipient's risk of developing CAI.

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Appendix 1
Patient Information Leaflets and Consent Forms

APPENDIX 2
SEQUENCES OF PEPTIDES

Appendix 2 Sequences of Peptides

Table A2.1. Sequences of non-polymorphic HLA Class 1 derived Single Peptides.

Their sequences were derived from 4 different 15 to 17 amino acid sequences of the non-polymorphic domain of the α3 domain of HLA Class 1. Polymorphic residues are shown in red italic and serine substitutions for cysteine in blue. Pegylation with KR wraps were added to some peptides as shown to increase their solubility.

Name	Peptide	Sequence		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
Tra30		192-206		H	*A*	*V*	S	D	H	E	A	T	L	R	C	W	A	L	
Tra31		192-206		H	*P*	*I*	S	D	H	E	A	T	L	R	C	W	A	L	
Tra33		192-206		H	*A*	*V*	S	D	H	E	A	T	L	R	**S**	W	A	L	
Tra34		192-206		H	*P*	*I*	S	D	H	E	A	T	L	R	**S**	W	A	L	
Tra39		202-216		R	C	W	A	L	*S*	F	Y	P	A	E	I	T	L	T	
Tra40		202-216		R	C	W	A	L	*G*	F	Y	P	A	E	I	T	L	T	
Tra41		202-216		R	**S**	W	A	L	*S*	F	Y	P	A	E	I	T	L	T	
Tra42		202-216		R	**S**	W	A	L	*G*	F	Y	P	A	E	I	T	L	T	
Tra46		268-282		K	P	L	T	L	R	W	E	*L*	S	S	Q	P	T	I	
Tra49				L	T	L	R	W	E	*L*	S	S	Q	P	T	I	P	I	
Tra02		280-294	KR	P	T	I	P	I	V	G	I	I	A	G	L	V	L	*F*	RK
Tra06		280-294	KR	P	T	I	P	I	V	G	I	I	A	G	L	V	L	*L*	RK
Tra08		280-294	KR	P	T	I	P	I	V	G	I	I	A	G	L	*A*	*V*	*L*	RK
Tra10		280-294	KR	P	T	*V*	P	I	V	G	I	I	A	G	L	*A*	*V*	*L*	RK
Tra14		282-296	KR	I	P	I	V	G	I	I	A	G	L	V	L	*F*	G	A	RK
Tra19		282-296	KR	I	P	I	V	G	I	I	A	G	L	V	L	*L*	G	A	RK
Tra24		282-296	KR	I	P	I	V	G	I	I	A	G	L	*A*	*V*	*L*	*A*	*V*	RK
Tra29		282-296	KR	*V*	P	I	V	G	I	I	A	G	L	*A*	*V*	*L*	*A*	*V*	RK
	P1			H	S	M	R	Y	F	F	*T*	S	V	S	R	P	G	R	
	P2			M	R	Y	F	F	T	S	V	S	R	P	G	R	G	E	
	P20			S	D	W	R	F	L	R	G	Y	H	Q	Y	A	Y	D		
	P21			*W*	R	F	L	R	G	Y	H	Q	Y	A	Y	D	G	K		

Table A2 1 Sequences of non-polymorphic HLA Class 2 derived Single Peptides

These sequences were derived from the non-polymorphic β2 domain of HLA Class 2. GRAVY (grand average hydropathy) denotes the solubility of the peptides. EpiMatrix raw scores are normalized with respect to a score distribution derived from a very large set of randomly generated peptide sequences. Any peptide scoring above 1.64 on the EpiMatrix "Z" scale (approximately the top 5% of any given peptide set) has a significant chance of binding to the MHC molecule for which it was predicted.www.expasy.org

Peptide	Sequence	residues in parent	MW	Length	Isoelectric point	GRAVY	Instability Index	Hits (top 10%)	Hits (top 5%)	High (Top 1%)	Epi score
DRE1	HFFNGTERVRLLERCIYNQ	45-63	2394.2	19	8.23	-0.642	S	8	7	4	10.6
DRE1A	HFFNGTERVRLLERSIYNQ	45-63	2378.22	19	8.75	-0.816	S	8	8	3	18.1
DRE1B	HFFNGTERVRLLE	45-57	1616.84	13	6.77	-0.492	S	7	6	1	5.82
DRE1C	ERVRLLERSIY	51-61	1432.82	11	8.84	-0.573	U	7	5	2	7.54
DRE1D	HFFNGTERVRLLERSIY	45-61	2136.12	17	8.75	-0.5	S	8	7	3	14.25
DRE2	GEYRAVTELGRPD	74-86	1461.72	13	4.87	-1.085	S	8	6	0	12.5
DRE2A	EYRAVTELGRP	75-85	1289.67	11	6.24	-0.927	S	8	7	0	14.27
DRE3	ESFTVQRRVEPKVTV	116-130	1773.97	15	8.85	-0.507	U	7	6	0	7.12
DRE3A	ESFTVQRRVHPKVTV	116-130	1781.99	15	10.84	-0.487	U	7	6	0	13.26
DRE3B	ESFTVQRRVQPKVTV	116-130	1772.99	15	10.84	-0.507	U	8	6	0	13.79
DRE3C	ESFTVQRRVYPKVTV	116-130	1772.99	15	9.99	-0.36	U	7	6	0	11.21
DRE4	KVTVYPSKTQPLQHH	127-141	1761.95	15	9.7	-1.047	U	7	5	2	3.96
DRE4A	KVTVYPSKTQPLQ	127-139	1487.43	13	9.7	-0.715	U	7	5	2	5.74
DRE5	SIEVRWFRNGQEEK	155-169	1776.89	14	5.95	-1.5	U	6	5	1	6.96
DRE6	GDWTFQTLVMLETVPRSGE	180-198	2165.05	19	4.43	-0.211	S	8	8	2	16.99
DRP6A	GDWTFQTLVMLETVPRS	180-196	1978.98	17	4.37	-0.006	U	8	8	2	22.37
DRE6B	WTFQTLVMLETVPRS	182-196	1806.93	15	6	0.253	u	8	8	2	24.15
DRE6C	WTFQTLVMLETVPRSGE	182-198	1994.2	17	4.86	-0.006	S	8	8	2	18.77
DRE6D	QTLVMLETVPRSG	185-197	1429.76	13	6	0.169	U	8	7	2	16
DRE7	SPLTVEWRARSESAQSK	211-227	1930.99	17	8.46	-1.071	U	8	5	2	5.92
DRE8	GLFIYFRNQKGHSGLQ	245-260	1863.97	16	9.99	-0.481	S	8	8	5	25.11

Table A2.3. HLA Class 1 and Class 2 peptide mixes composition

Frozen peptides were stored in aliquots at 1mg/ml. On the day of experiments they were mixed with complete media at the required volumes and stored at 4^{o}C for a further 1 week.

Peptide Mix						
Class 1						
Mix 2	Tra30	Tra39	Tra51	Tra02	Tra14	
Mix 3	Tra31	Tra40	Tra46	Tra49	Tra06	Tra19
Mix 4	Tra31	Tra40	Tra52	Tra06	Tra19	
Mix 6	Tra33	Tra41	Tra53	Tra08aTra24		
Mix 7	Tra34	Tra42	Tra10	Tra29		
Mix 8	P1	P20	P45/46			
Mix 9	P2	P21	P45/46a			
Class 2						
DR Mix 1	DRE6c	DRE2	DRE7			
DR Mix 2	DRE6d	DRE3c				
DR Mix 3	DRE6e	DRE4a				

APPENDIX 3
PLATE PLANS

Appendix 3 Plate Plans

Figure A3.1. Plate Plan for non-polymorphic HLA Class 1 derived peptides

Tra 30	Tra 46 + 49	complete			PPD	Anti CD3
Tra 31	Tra 02 + 14					
Tra 33	Tra 06 + 19					
Tra 34	Tra 08 + 24					
Tra 39	Tra 10 + 29					
Tra 40	p1 + p2					
Tra 41	p20 + p21					
Tra 42						

Figure A3.2. Plate plan for non-polymorphic HLA Class 2 derived peptides

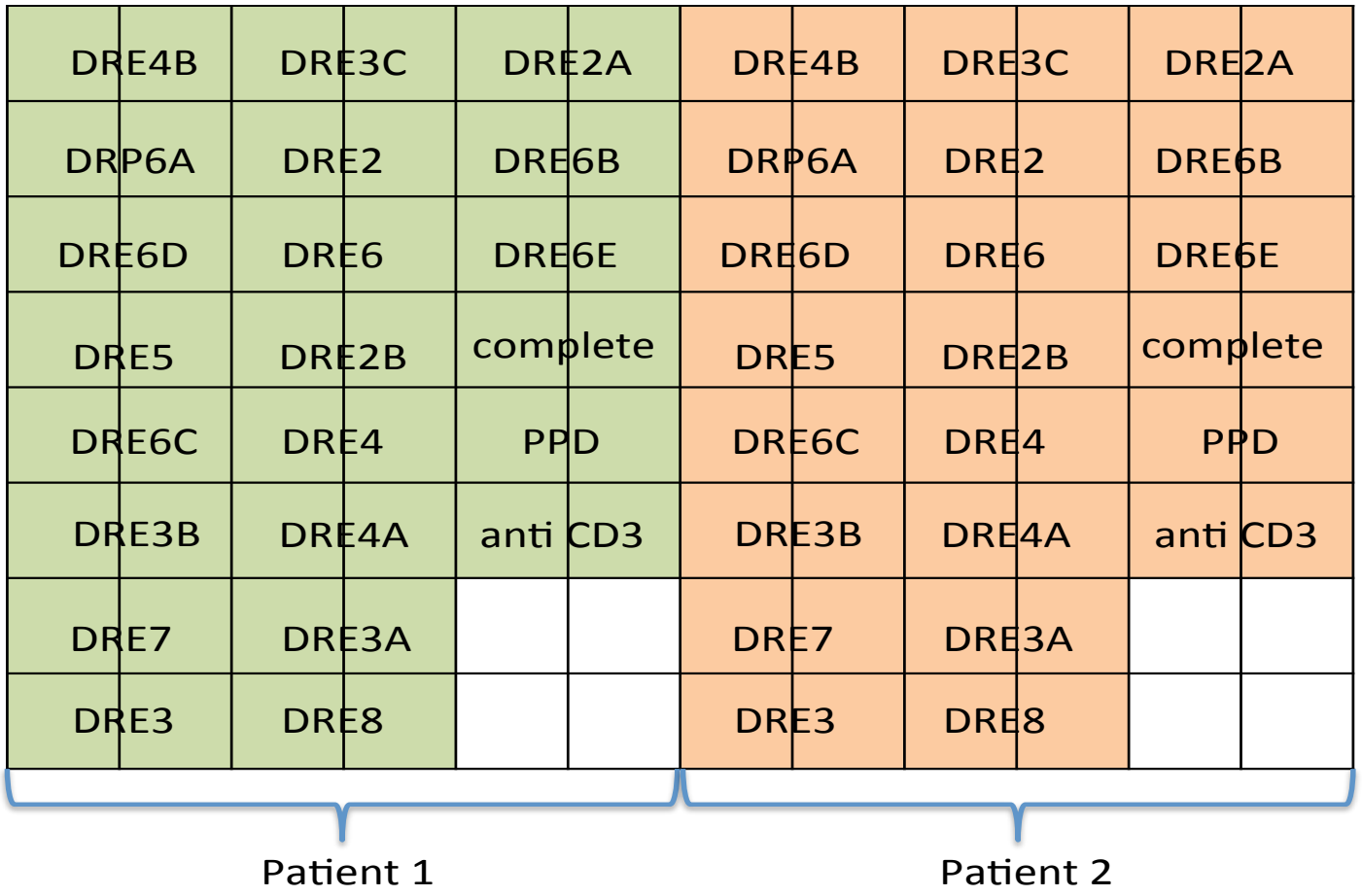

Figure A3.3. Plate Plan for HLA Class 1 and 2 peptide mixes

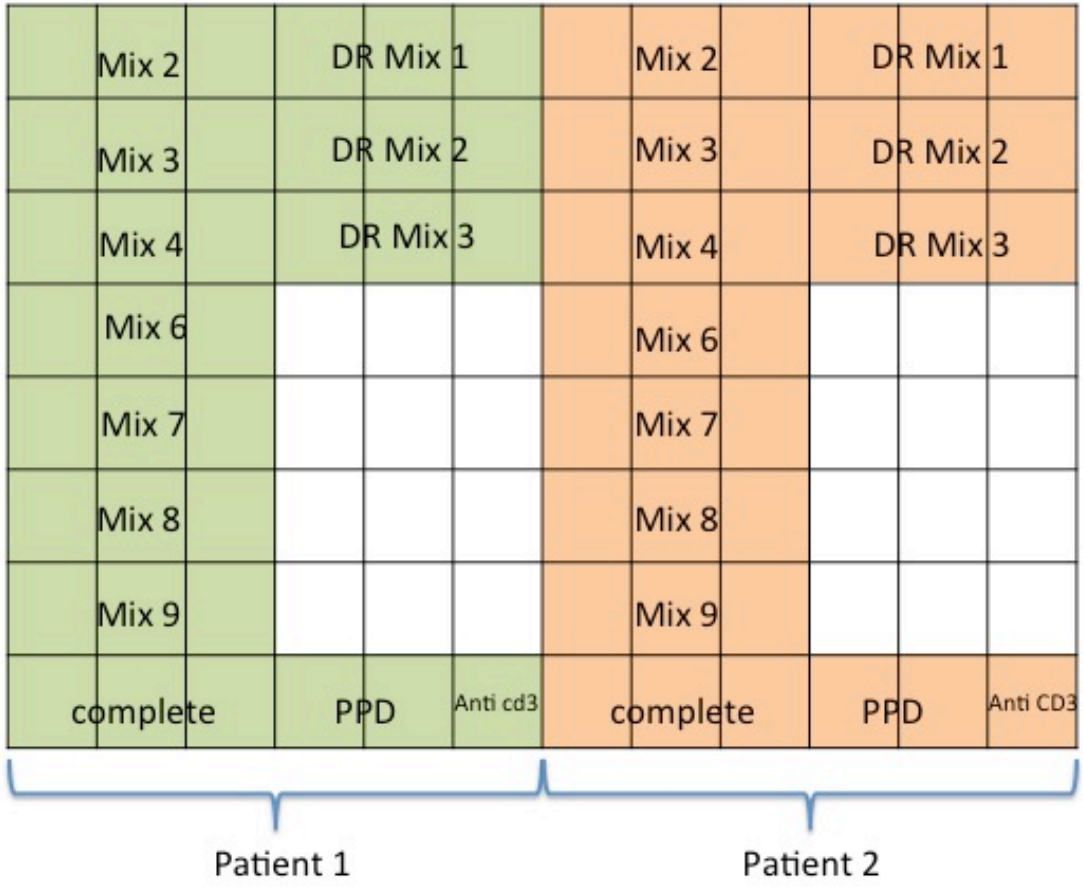

Figure A3.4. Plate plan for Peptide Stimulation assays with different subsets of $CD4^{+}CD25^{Hi}$ subsets

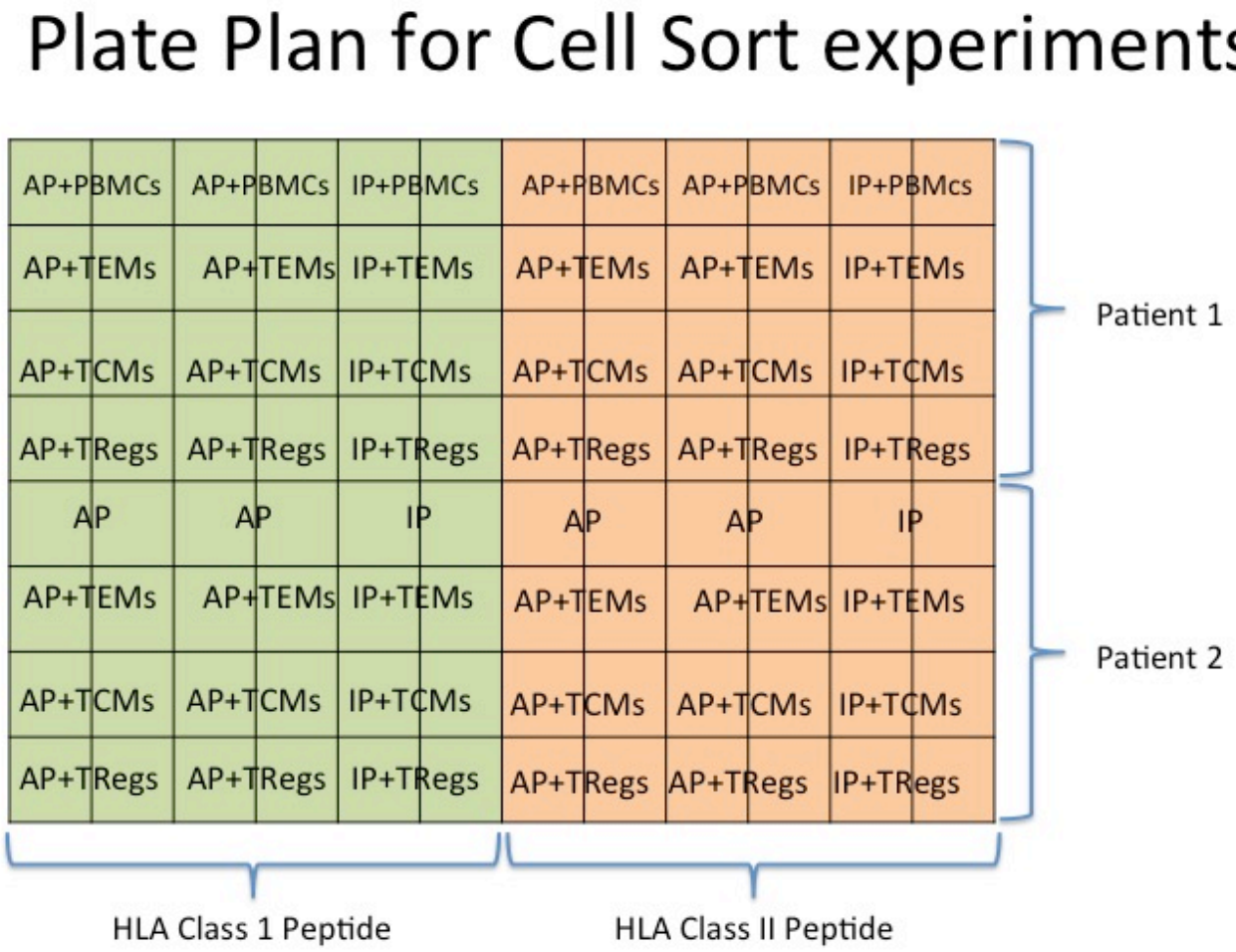

Figure A3.5. Plate plan for Cytokine Assay (Luminex) Studies

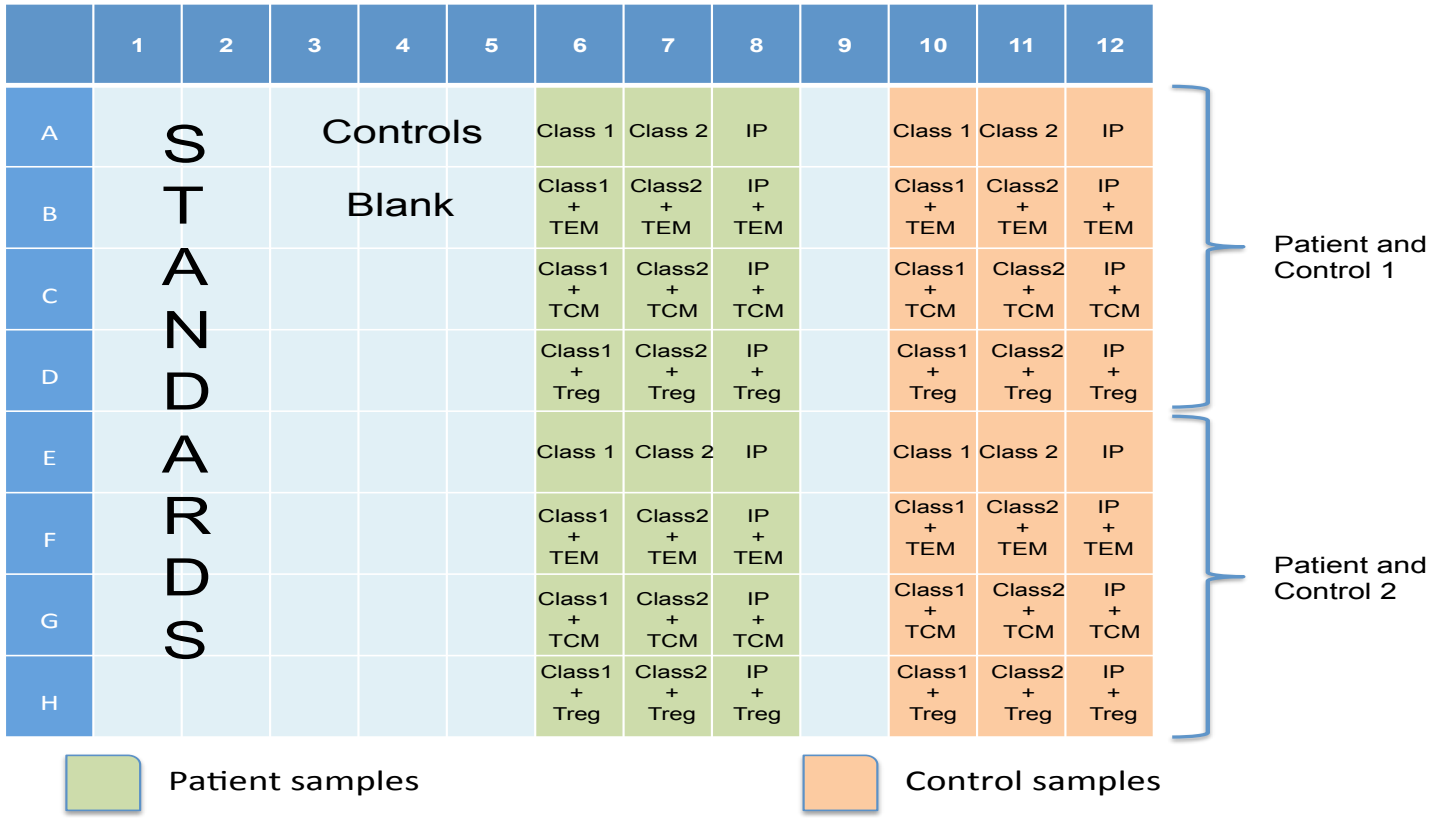

Figure A3.6. Plate Plan used for Inhibition Assays

TEMS			AP+TEMs+50nM ShK			IP+TEMs+500ng/ml Abat			AP+TRegs+50nM ShK		
AP+TEMs			AP+TEMs+10nM ShK			IP+TEMs+50nM ShK			IP+TRegs		
AP+TEMS+200ng/ml CsA			IP+TEMs			IP+TEMs+10nM ShK			IP+Tregs+200ng/ml CsA		
AP+TEMs+100ng/ml CsA			IP+TEMS+200ng/ml CsA			TRegs			IP+TRegs+500ng/ml Sir		
AP+TEMs+500ng/ml Sir			IP+TEMs+100ng/ml CsA			AP+TRegs			IP+TRegss+1ug/ml Abat		
AP+TEMs +250ng/ml Sir			IP+TEMs+500ng/ml Sir			AP+Tregs+200ng/ml CsA			IP+TRegs+50nM ShK		
AP+TEMs+1ug/ml Abat			IP+TEMs +250ng/ml Sir			AP+TRegs+500ng/ml Sir					
AP+TEMs+500ng/ml Abat			IP+TEMs+1ug/ml Abat			AP+TRegss+1ug/ml Abat					

Figure A3.7. Plate Plan used for intracellular IL-10 staining experiment