The Diverse Roles of Human B Lymphocytes in Renal Transplantation

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Thesis submitted in accordance with the requirements for the degree of PhD

University of Leeds

School of Medicine

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Declaration

I confirm that the work submitted is a presentation of my original research work, except where work which has formed part of jointly-authored publication has been included. Contribution of the co-authors and myself for the publication has been explicitly indicated in the ‘Publications’ section. I confirm that appropriate credit has been given within the thesis where reference has been made to the work of others.

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2013

University of Leeds

Dr Aravind Cherukuri
Publications

In Print:


*WS MP collected and analysed the data and written the manuscript, AC collected the data and contributed towards data analysis and manuscript preparation. The remaining co-authors participated in the routine clinical care of the patients. RJB conceived the clinical trial and contributed towards data analysis and manuscript preparation

**Oral communications:**


2. Regression of Proteinuria during the First Three Years after Renal Transplantation Is Associated with Improved Graft Outcomes. A Cherukuri, James Tattersall, Matthew Welberry Smith, Chas Newstead, Andrew Lewington, Richard Baker. {American Transplant Congress, San Diego- 2010}

3. Immune monitoring in renal transplantation-HLA antibody and beyond {Joint national symposium of British Transplant Society and British Society for Histocompatibility and Immunogenetics-2010}


5. For Cause Renal Biopsies Are Heterogeneous Irrespective of Clinical Presentation. A. Cherukuri, S. Balasubramanian, P. Prasad, C. Angel, A. Lewington, C. Newstead, B. Clark, R. Baker {American Transplant Congress, Seattle, 2013}

6. Human Regulatory B Cells (Bregs) Are Characterized by Both IL-10 and TNF-α expression and Are Reduced in Numbers with Altered Function in Renal Transplant Recipients with Immunological Graft Injury, A. Cherukuri A. Salama,
C. Carter, B. Clark, D. Rothstein, R. Baker\{ British Transplant Society Congress, Bournemouth-2013\}

7. Transitional B cell (TrB) T1/T2 ratio is a marker for graft dysfunction in human kidney transplant recipients (KTRs) \{World Transplant Congress, San Francisco; Young Investigator Award; British Transplant Society Congress, Glasgow, 2014\}
To my mom, dad and teachers
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Renal transplantation is the treatment of choice for patients with end stage renal disease. Despite the significant advances made over the last fifty years, one predicament that still perplexes the transplant community is late allograft loss. The two major contributing factors include the limitations with the clinical utility of various markers for early diagnosis and lack of appropriate therapy. This thesis deals with the issue of early diagnosis and tries to establish a link between the clinical, histological and immunological phenotype with a view to identify prognostic markers. Firstly, low-grade proteinuria is clinically analysed for its utility to predict graft outcomes. Secondly, a disjunction between the clinical and histological phenotype and more importantly the limited utility of the clinical phenotype to determine the prognosis for a troubled allograft in light of clinical dysfunction is considered. Thirdly, a novel definition of human B regulatory cells is proposed with a view to address the discrepancy in the current literature with regards to their identification. Fourthly, a link between the histological phenotype of late allograft dysfunction is correlated with the frequency and function of regulatory B cells. Here, the functional diversity of the B cells, specifically within a small sub-group of B regulatory cells in relation to histological abnormalities is considered. Finally, the phenotype and functionality of the Bregs are explored for their use as potential markers for allograft outcomes and the utility of a simple phenotype tested in a prospective sample of patients from a randomized controlled trial.
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<tr>
<td>AAMR</td>
<td>Acute Antibody Mediated Rejection</td>
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<td>ACR</td>
<td>Acute Cellular Rejection</td>
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<td>APC</td>
<td>Antigen Presenting Cell</td>
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<td>CNI</td>
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<tr>
<td>DSA</td>
<td>Donor Specific Antibody</td>
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<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunoabsorbant Assay</td>
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<td>ESRD</td>
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<td>FITC</td>
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<td>IFTA</td>
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Preface

I have always dreamt of becoming a physician. My interest in renal transplantation developed initially whilst working at a renal transplant centre in India before my arrival in the UK in 2001. My interest and enthusiasm to learn more in the field of renal transplantation grew further since my introduction to Richard Baker, a great inspiring mentor. At one of the earlier interactions, we deliberated to look into clinical markers that predict long-term graft outcomes in renal transplantation. In Leeds, at St. James’s University Hospital renal unit we had the privilege of being able to analyse proteinuria in depth as a clinical marker as every patient at every clinic visit gets their urine checked for random protein excretion by urinary protein creatinine ratio. We planned to use two very basic and cheap clinical markers, namely proteinuria and serum creatinine to identify troubled grafts and understand any potential immunological alterations that account for their plight.

My interest to look into B lymphocytes and their function developed out of a mere coincidence when I stumbled on two patients who were given alemtuzumab for induction treatment and who had given me blood samples for assessment of their overall lymphocyte reconstitution. These patients had a significantly higher proportion of B lymphocytes in their blood when compared to healthy volunteers. This has indeed kick started my work on the B cell phenotype and its significance in renal transplantation. In this thesis, I have tried to link the clinical phenotype of renal transplant recipients to a histological phenotype and finally correlate this to the immunological phenotype mainly centred on the functionality of B lymphocyte subsets.

The scope of a thesis entitled ‘the diverse roles of human B lymphocytes in renal transplantation’ is very broad. Renal transplantation provides the best form of renal replacement therapy by not only improving the quality of life of patients with end stage
renal disease but also increasing their overall survival (Port, Wolfe et al. 1993, Wolfe, Ashby et al. 1999). However renal transplant do not last for ever and one of the major problems we face at present is lack of improvement of long-term outcomes despite significant advancements made in the management of early complications after transplantation.

In the introduction, I have attempted to provide an overview and also insight into the need to understand the spectrum of immunopathology that besieges chronic allograft loss, with particular emphasis on the role of B lymphocytes. B lymphocytes have traditionally been studied in relation to the production of antibodies. Doubt over the role of antibodies in the rejection of renal allografts emerged from early rodent models of transplantation. Moreover microscopic examination of failing renal allografts failed to demonstrate the signature of antibody mediated damage, namely the deposition of immunoglobulins. However two major advances in the last decade have changed this situation. The discovery by Feucht that the human complement component C4d is covalently bonded at sites of antibody-mediated complement activation has revealed hitherto unseen evidence of antibody mediated damage in renal allografts (Feucht, Felber et al. 1991, Feucht and Mihatsch 2005). Secondly new technologies have been developed to detect human anti-HLA antibodies in patient’s serum with previously unimaginable sensitivities. I have provided a detailed overview of the emergence and significance of antibody detection methodology and the role of post-transplant HLA specific antibody detection in the current day transplant clinical practice.

More diverse roles of B lymphocytes have emerged over the last decade and now it is established that certain B cell subsets are capable of regulating immune response. A brief history of the discovery of the B regulatory cells follows, as the study of these cells has become pivotal to our better understanding of immunopathology. An overview of the role of B cells as regulators of immune response and their significance in the context of human renal transplantation follows.
The introduction concludes with concise statements of the objectives and aims of the clinical studies and experimental work to be described in this thesis.

Chapter-3 describes the clinical studies performed to understand the utility of low-grade proteinuria as a marker of premature graft loss. Previous studies from other groups have suggested that proteinuria, as early as three months after transplantation can predict long term graft loss. Here, the impact of changes in this dynamic variable over time on clinical outcomes is presented. More importantly, a link between the clinical and histological phenotype of troubled grafts is explored which sets a background for the experimental work on human B cell subsets that continues in the following chapters.

Chapter-4 attempts to identify regulatory B cells by pro or anti-inflammatory cytokine polarization. The inherent heterogeneity even within a small subset of the putative B regulatory cells in humans is also described. Next, these cells are studied in the context of renal allograft dysfunction with a particular emphasis on chronic antibody mediated rejection. The utility of these cells as potential markers of clinical outcomes in troubled renal allografts is then explored. The following chapter is a descriptive summary of a randomized controlled trial performed in Leeds, comparing two steroid free immunosuppression regimes using either alemtuzumab or basiliximab. Here I come back to the point that kindled my interest in the role of B cells in transplantation- What is the nature and role of the repopulated B cells in maintaining allograft function when patients receive induction therapy with alemtuzumab?

The findings of this thesis and their implications are discussed in the final chapter.
Chapter-1: Introduction

The incidence of End Stage Renal Disease (ESRD) requiring renal replacement therapy in the UK is estimated at 107 per million population in 2010 and this represents a considerable increase over the last decade. Patients with ESRD requiring dialysis have a significantly higher mortality rate when compared to general population. It is estimated that the 5 year survival of men and women starting dialysis is worse than those suffering from colon and breast cancer respectively (Parfrey and Foley 1999). This high rate of mortality emanates from a substantially higher incidence of cardiovascular disease in these patients. As shown in Figure 1, it is alarming to note that the effect of ESRD on cardiovascular mortality is notably worse in relatively younger patients. In the UK, it is reckoned that the mortality of young patients on dialysis is 25 times higher than general population.

![Graph showing cardiovascular disease mortality by age, ethnicity and gender in general population and in dialysis patients.](image)

*Figure 1: Cardiovascular disease mortality by age, ethnicity and gender in general population and in dialysis patients*  
*(Parfrey and Foley 1999)*
1.1 Renal Transplantation-clinical outcomes

Renal transplantation offers a long-term survival benefit for patients with end stage renal disease when compared to dialysis (Wolfe, Ashby et al. 1999), as well as a significant cost saving. Moreover, renal transplantation has been shown to attenuate the progression of cardiovascular disease in patients with ESRD (Meier-Kriesche, Schold et al. 2004) making it the best form of renal replacement therapy available.

J.E. Murray’s first account on the usage of immunosuppressive drug therapy in human kidney transplantation reported a 70% graft loss within the first year (Murray, Merrill et al. 1963). However renal allograft survival has increased enormously over the past few decades mainly due to improvements in first year survival and a significant improvement in the level of graft function achieved at one year post-transplantation (Hariharan, McBride et al. 2002, Lamb, Lodhi et al. 2011) (Figure 2). There is however a discordance between acute rejection rates and long term graft survival after 1996 since graft outcomes have remained relatively unchanged despite further improvements in the rates of acute rejection (Hariharan, Johnson et al. 2000). This phenomenon is partly attributed to the lack of improvement in the long-term outcomes of low-risk renal transplants including living donor transplants (Lamb, Lodhi et al. 2011) and to a trend towards fewer late acute rejections showing a complete response to therapy. (Meier-Kriesche, Schold et al. 2004). Further major improvements in the rate of acute rejection and its treatment are unlikely.
Figure 2: (A) Cumulative half-lives of the grafts between 1989 and 2005. (B) Significant improvement in the 0-1 year graft attrition rate during the period 1989-2009.

(Lamb, Lodhi et al. 2011)
1.2 Allograft loss

1.2.1 Chronic allograft dysfunction

It is essential to understand the pathology of allograft loss in order to address the problems underlying the goal of long-term graft maintenance. Approximately half of all the allografts are lost when patients die with a functioning graft. The other half are lost due to graft failure. Several investigators have attempted to clarify the causes of graft failure but the process is complicated by the finding that late failing grafts display similar histological findings characterized by interstitial fibrosis and tubular atrophy, also called chronic allograft nephropathy (CAN) (Solez, Axelsen et al. 1993). The term CAN has been rejected since it encourages diagnostic nihilism and obscures the probable heterogeneous nature of progressive graft damage. Hereafter the process will be called chronic allograft dysfunction (CAD). It has become increasingly clear that the underlying pathophysiology of CAD is multifactorial. There are immunological contributions to this process leading to graft rejection and non-immunological factors including viral infections and the nephrotoxicity of immunosuppressive drugs.

Over the past decade there was a significant shift in the understanding of the pathology of CAD. There was an initial emphasis on the importance of calcineurin inhibitor (CNI) drug toxicity in the pathogenesis of CAD. Two distinctive phases of allograft injury were proposed- an initial phase of early tubulo-interstitial damage from ischaemic injury and rejection and a later phase of progressive histological changes (arteriolar hyalinosis with luminal narrowing, increasing glomerular sclerosis and additional tubulo-interstitial damage) accompanied by the use of CNIs. This latter phase was shown to be universal at 10 years post-transplantation (Nankivell, Borrows et al. 2003). Interestingly Nankivell et al. have reported that rejection was uncommon in the latter phase. Based on this paradigm, several authors have reported some success with minimization of CNI in patients with CAD (Stoves, Newstead et al. 2004, Garcia, Pinheiro-Machado et al. 2006). However it should be noted that patients had excellent graft function with a
mean isotopic glomerular filtration rate (GFR) of 50.2ml/min even at 6 to 10 years of follow-up in the study reported by Nankivell et al despite the histological findings. Also, arteriolar hyalinosis and fibrointimal thickening were not necessarily specific to the diagnosis of CNI toxicity.

In contrast to the diagnostic scepticism provided by the usage of interstitial fibrosis and tubular atrophy (IFTA) or CAN as an aetiology for chronic allograft loss, and challenging the existing dogma that most chronic allograft damage is relate to CNI induced damage, F.G Cosio’s group from Mayo Clinic have shown that most cases of kidney allograft failure can be attributed to a specific cause if sufficient histological and clinical information is available. Interestingly glomerular pathology which includes transplant glomerulopathy, recurrent disease and de novo glomerular disease has been shown to be the single most common cause of allograft loss and more importantly CNI toxicity as a cause of IFTA has only been reported in 2.1% of patients (El-Zoghby, Stegall et al. 2009). In a prospective histological and clinical analysis of “for cause biopsies” Halloran’s group from Canada have shown that the commonest cause of late allograft failure is antibody mediated graft damage and in keeping with the observations from Cosio’s group, they have also shown that drug toxicity and non-specific scarring are relatively uncommon (Sis, Campbell et al. 2007, Einecke, Sis et al. 2009). The significance of antibody mediated rejection as a cause of long-term graft loss has thus been underscored.

1.2.2 Allograft rejection

As it is now established that chronic antibody mediated rejection (CAMR) plays a significant role in the demise of an allograft, I shall elucidate the evolution of our understanding of the pathology of allograft rejection and the role of various immunological processes underpinning the pathological outcomes with a particular emphasis on CAMR.
Rejection of skin allografts has been one of the most potent and convenient experimental models for the study of immunologically mediated tissue destruction. Impelled by the exigencies of war, skin allograft rejection was first studied by Medawar in humans and then in animals (Gibson and Medawar 1943, Medawar 1944, Medawar 1945). In 1943, Gibson and Medawar working at the Glasgow Royal Infirmary provided a detailed description of the outcome of treating a young woman with a combination of both skin pinch autografts and homografts from her brother. This patient with thermal burns was treated with 52 pinch autografts from her thigh and 50 homografts from her brother. A second set of 28 pinch homografts from the same donor were transplanted to a different part of the raw area 15 days after the application of the first. Biopsy specimens from these grafts were collected at each change of dressings and examined histologically. Whilst the autografts grew and coalesced to form a continuous sheet of skin tissue, the growth and development of homografts was rather different. The first sets of homografts were similar to the autografts initially but started to degenerate by day 15 and were lost by day 23. The second set homografts showed a far rapid dissolution by day-8. Based on the histological observations, a process of active immunization has been postulated that would account for the destruction of foreign epidermis. This was clearly one of the earliest demonstrations of acute and secondary accelerated acute rejection in the context of human transplantation. Even prior to Medawar, it was Peter Gorer working at Guy’s Hospital in London at the time, who showed that rejection of murine tumours was due to the antigens present on the donor tissue and that these grafts induced alloantibodies that could agglutinate donor erythrocytes (Amos, Gorer et al. 1954).

1.2.2.1 Kidney transplant as an immunogenic stimulus

The central problem in transplantation is the immune response of the host against the transplanted antigens of the donor. The successful outcome of any transplant depends on the modulation of this response through medical intervention. Both innate and adaptive immune responses are activated by transplantation and play a crucial role in
the process of allograft rejection. Unlike the adaptive system, the innate immune system comprises inflammatory cells (dendritic cells, monocytes, macrophages, neutrophils and other cells) that do not express rearranging receptors, have limited clonal expansion, and, in the most part, do not generate memory. Cells of the innate immune system instead express germ-line encoded pattern recognition receptors (PRR) that detect conserved pathogen associated molecular patterns (PAMP) present in microbes but not shared by mammalian cells (Palm and Medzhitov 2009, Oberbarnscheidt, Zecher et al. 2011). The innate immune system also encompasses non-cellular mediators capable of microbial recognition—for example, complement proteins. Activation of the innate immune system by microbial ligands causes inflammation, the first line of defence against infection, but more importantly induces the maturation and migration of antigen presenting cells (APC) to secondary lymphoid tissues where they trigger primary T cell and B cell responses. Recognition of PAMP by PRR forms the cornerstone of the Janeway theory and provides a robust explanation for how the adaptive immune response to a large variety of pathogens is initiated by the innate immune system. These cells are being increasingly appreciated as significant players in the process of allograft rejection (LaRosa, Rahman et al. 2007, Liu and Li 2010). In part this is based on clinical findings that depletion of T cells with alemtuzumab is not effective in preventing rejection and that rejection occurs in association with monocytic and eosinophilic inflammation (Kirk, Hale et al. 2003, Wu, Bond et al. 2006). There is also an emerging understanding that the innate immune system has a role in the induction of transplant tolerance (Morelli and Thomson 2007). The adaptive immune response begins with recognition of self from non-self by both T and B lymphocytes through their respective receptors.

1.2.2.2 Major Histocompatibility Complex (MHC)

Immune system is a remarkable defence mechanism against myriad potentially pathogenic microorganisms that inhabit the world we live in and is often compared to the Red Queen in Alice’s Adventures in Wonderland, (Danilova 2006) who must keep
moving just to avoid falling behind. Because of the perpetual conflict with pathogens, the immune system is in constant flux. This is exemplified by differences in the immune systems of animals within the same phylogenetic group.

The question remained as to the nature of the inciting antigens on the foreign tissue that were spurring the immune response. George Snell, working in Maine, had described the genes responsible for the genetic behaviour of tumour transplants in mice and named them the histocompatibility genes. The prefix ‘histo’ was used because he felt that the same genes which determine the susceptibility or resistance to tumour transplants probably also determine susceptibility or resistance to tissue transplants and these genes were symbolized as group by letter-‘H’ (Snell 1948). Jean Dausset and his group in Paris discovered antibodies to a leukocyte antigen called MAC in the sera of French patients who received multiple blood transfusions. This antigen is now known as HLA-A*0201 (Dausset 1958). There were numerous other groups interested in these leukocyte antigens which were designated as Human Leukocyte Antigens (HLA). The genes of the MHC in humans (HLA) are located on the short arm of chromosome six and they encode distinct classes of cell surface molecules- HLA-class-I and HLA-class-II. Humans have three main highly polymorphic MHC class I genes, known as HLA-A, HLA-B and HLA-C. These genes transcribe proteins which, along with β2-microglobulin form the class-I molecules. There are six MHC class II genes in humans, namely HLA-DPA1, HLA-DPB1, HLA-DQA1, HLA-DQB1, HLA-DRA and HLA-DRB1. These genes encode the class II molecules which are dimers of an alpha and beta chain (Halloran, Wadgymar et al. 1986).

The structure of MHC molecules has been elucidated at the molecular level. Initially the structure of HLA-A*0201 was determined and it revealed the presence of a nonapeptide in the groove formed by the β-pleated sheet in between the two α-helices that form the recognition site for the T cell receptor. The identification of this peptide has
finally shed light on our understanding of MHC restriction and antigen processing. The description of the structure of HLA class-2 molecule, HLA-DR1 followed next (Brown, Jardetzky et al. 1993, Stern, Brown et al. 1994). Figure 3 compares the molecular structures of HLA-A2 and DR-1 as described by Brown et al. According to the data available from the IMGT/HLA database (http://www.ebi.ac.uk/imgt/hla/stats.html), a total of 3296 HLA-A, B and C a chain alleles have been identified. For renal transplant recipients, tissue type is routinely reported for HLA-A, B and DR for the purpose of the identification of the best possible match.

Figure 3: HLA DR1 (class-2) superimposed on HLA-A2 (class-1)

(a). One DR-1 αβ heterodimer and one HLA-A2 molecule. (b). Top view showing the overall similarity of the peptide binding grooves. (c). Side view of the α1 domain helical regions showing differences at both ends. (d). Side view of the helical regions of the DR1-β1 and HLA-A2-α2 domains (Brown, Jardetzky et al. 1993).
Given both their polymorphism and ability to elicit strong immune responses, the presence of allogeneic HLA antigens on the allograft is the most important determinant of the alloresponse and therefore the most influential factor in determining the intensity of graft rejection. This was shown for the first time in the mouse models of allogeneic skin grafting where grafts that differed at MHC loci were promptly rejected (Billingham, Brent et al. 1954). In fact the collaborative transplant study in 1985 has highlighted the importance of appropriate HLA matching to promote better graft survival as shown in Figure 4 (Opelz 1985, Cecka 1997).

![Figure 4: HLA-A, B and DR mismatches and graft survival (1982-1984)](Cecka 1997)

1.2.2.3 Minor histocompatibility antigens

However it should also be noted that there are numerous genetic loci, outside of the MHC region, coding for minor histocompatibility antigens that were capable of inducing allograft rejection (Eichwald and Silmser 1955, Graff and Bailey 1973). In 1990 Rammensee and his colleagues separated distinct peptides from MHC molecules thereby providing evidence for minor histocompatibility antigens to be peptides...
(Rotzschke, Falk et al. 1990, Wallny and Rammensee 1990). These antigens are encoded by polymorphic genes that belong to non-HLA related genes (Dzierzak-Mietla, Markiewicz et al. 2012). In the context of transplantation, the fact that genetic polymorphisms are present in endogenous proteins expressed by the donor and not the recipient suggests that these proteins are recognised as non-self and therefore they can elicit a donor specific response (Robertson, Chai et al. 2007, Bleakley, Otterud et al. 2010).

1.2.2.4 *Allorecognition-direct, indirect and semi direct pathways*

One of the most striking features of the T cell response provoked by the MHC incompatible cells is its vigour as reflected by the rejection of the solid organ transplants and graft vs. host disease in bone marrow transplant recipients. The strength of this response is accounted for by the uniquely high precursor frequency of T cells with allospecificity. This feature of the alloresponse was first detected by for class-I reactive T cells (Skinner and Marbrook 1976, Lindahl and Wilson 1977).

![Figure 5: Pathways of Allorecognition](image-url)
Direct allorecognition (Figure 5a) is defined as the recognition by the recipient T cells of the intact MHC alloantigens displayed on the surface of the donor antigen presenting cells (APC) carried within the allograft. Two facts are central to the characterization of the direct pathway.

1. The high precursor frequency of the alloreactive T cells that recognize allogeneic MHC molecules.

2. The rules of self MHC restriction are apparently disregarded in the direct binding of the TCR to the allogeneic MHC molecules.

The recognition of the nominal antigen in the context of self MHC molecules and recognition of the alloantigen is brought about by overlapping populations of T cells (Lombardi, Sidhu et al. 1989). Approximately half of the cells involved in the alloresponse have been shown to be primed previously to a nominal antigen (Lombardi, Sidhu et al. 1990). The precise nature of the ligand recognised by the alloreactive T cells still remains unclear. There are two different hypotheses that make very different assumptions about the nature of the ligand bound by the alloreactive T cells and provide explanations for the wide repertoire of the alloreactive T cells.

**The multiple binary complex hypothesis** (Figure 6), proposed by Matzinger and Bevan in 1977 proposes that the antigen binding grooves of MHC molecules expressed on normal cells are occupied by an extremely diverse repertoire of peptides derived from the processing of proteins presented with class I or class II MHC molecules. Alloreactive T cells are specific for individual complexes of MHC and the peptide. As a consequence, a single allogeneic MHC molecule is able to stimulate a large number of distinct T cell clones (for distinct peptides) (Matzinger and Bevan 1977).

**The high determinant-density hypothesis** (Figure 6) proposed by Bevan explains that the attention of the TCR is focussed on the exposed residues of the non-self MHC
molecule irrespective of the occupancy of its antigen binding site. Therefore, at least in theory, all non-self MHC molecules of any given isotype (for example HLA-DR), presented by the APC could act as the ligand for the alloreactive T cell. Harding and Unanue have shown that only a small fraction of class II molecules presented by the APC will be occupied by a particular allospecific peptide. There may be 100 fold higher available number of ligands, or determinant density, per cell for the alloreactive T cells than is available for a conventional self-restricted antigen specific T cell response. Therefore, cells of lower affinity for an antigen specific response may be called into alloreactive repertoire; such that cells of low, intermediate and high affinity for the allo-MHC molecule could lead to the generation of a high precursor frequency (Harding and Unanue 1990).

Figure 6: Ligand bound by alloreactive T cells

The interaction of the donor APC and the recipient T cells has been shown to occur predominantly in secondary lymphoid organs (Larsen, Morris et al. 1990). Once the donor APC induces the activation of the alloreactive T cells, these effector cells migrate
back to the graft and destroy the allograft. This pathway is primarily responsible for process of acute cellular rejection of the allograft (Warrens, Lombardi et al. 1994).

Evidence for the existence of an alternative route for allorecognition was provided by Lechler and Batchelor through experiments in a rat model of transplantation (Lechler and Batchelor 1982). They observed that MHC incompatible kidney allografts depleted of indigenous passenger leucocytes were permanently accepted without immunosuppression in certain donor/recipient combinations but were lost to rejection in others. This second route for allorecognition is called the indirect pathway (Figure 5B). Here allogeneic MHC and other donor antigens are processed and presented by the recipient APC. In contrast to the direct pathway, this is the normal mechanism of T cell stimulation by nominal antigens (i.e. processed peptides restricted by self MHC molecules). The first direct demonstration of the indirect pathway was by Sherwood, Brent and Rayfield, who showed that host APCs could present both class I and class II antigens to syngeneic lymphocytes and bring about skin allograft rejection (Sherwood, Brent et al. 1986). Alloantigens shed from the graft are naturally processed as exogenous antigens by self-APCs, promoting alloreactive helper T cells that recognise allopeptides bound to autologous MHC class II molecules. This suggests that the indirect pathway might contribute to long term allograft damage as the graft has lost its dendritic cells and the direct pathway becomes hyporesponsive.

B lymphocytes require the presence of CD4+ T cells to induce class switching and to differentiate into antibody secreting plasma cells. These T cells must have been activated by MHC class II molecules either on dendritic cells or alternatively autologous class II positive B cells. B cells may represent important APCs in the context of the alloreponse since although numerically inferior they do possess B cell receptors with high affinity for alloantigens and they retain the ability to proliferate in response to antigen (c.f. dendritic cells). The presence of class switched IgG alloantibodies is therefore indirect proof for T cell help provided through indirect pathway (Lanzavecchia
In experimental animals, it has also been shown that cardiac allograft survival was significantly prolonged in mice with defective B cell antigen presentation (Nozaki, Rosenblum et al. 2008).

In the murine system, Benichou et al. showed that T cells collected from mice that had been sensitized by allogeneic splenocyte infusion or skin grafts proliferate to synthetic peptides derived from the polymorphic regions of the $\alpha$ and $\beta$ chains of the allogeneic class II MHC molecule presented by self APCs (Benichou, Takizawa et al. 1992). A number of other investigators have demonstrated T cell responses to synthetic peptides derived from allogeneic donor MHC molecules. In these experiments it has been established that the priming of CD4+ T cells by the indirect route can bring about a full range of effector responses including DTH (Waaga, Chandraker et al. 1998), priming of CD8+ CTLs (Lee, Grusby et al. 1994, Popov, Fedoseyeva et al. 1995), production of alloantibodies (Steele, Laufer et al. 1996) and regulatory cytokine secretion (Vella, Magee et al. 1999). Taken together, these experiments show that T cell activation by the indirect pathway occurs and can orchestrate the whole gamut of alloreactive effector responses. One of the more impressive examples is the work by Fabre and colleagues who initially showed that peptides from allogeneic MHC molecules could stimulate both the production of alloantibodies and alloreactive CD4+ T cells (Parker, Dalchau et al. 1992). They then went on to show that immunization with isolated denatured chains of class I and class II rat MHC molecules could accelerate the rejection of allogeneic skin grafts (Dalchau, Fangmann et al. 1992). This feat was then repeated by the immunization of graft recipients with peptide fragments of donor MHC antigens, bringing about accelerated graft rejection even when the allografts had been depleted of APCs (Fangmann, Dalchau et al. 1992, Benham, Sawyer et al. 1996). By using peptide fragments, only indirect pathway presentation is possible in this system.
In humans, the frequency of T cells engaged in indirect recognition of synthetic DR1 peptides in an *in vitro* culture system was found to be about 100 fold lower than that of T cells participating in direct recognition of native HLA DR antigen (Liu, Sun et al. 1993).

In humans both direct and indirect pathways of allorecognition were studied simultaneously in long standing renal transplant recipients for the first time by Baker and colleagues. Using limiting dilution analysis (LDA), they have demonstrated donor specific direct pathway hyporesponsiveness defined either by cell proliferation or cytokine secretion. This phenomenon was not dependent on the diagnosis of IFTA with allograft dysfunction. Using a mixture of donor membrane proteins, they went on to demonstrate that indirect pathway activated alloreactive T cells are significantly higher in number in patients with deteriorating graft function, thereby establishing the significance of indirect pathway activation in the context of late allograft dysfunction (Baker, Hernandez-Fuentes et al. 2001). Following this, several groups have confirmed the significance of indirect allorecognition in the context of chronic allograft dysfunction using less cumbersome techniques such as ELISPOT by showing that patients with CAD have a higher frequency of circulating indirect pathway activated alloreactive CD4+ T cells (Najafian, Salama et al. 2002, Poggio, Clemente et al. 2004); patients with CAD demonstrate direct pathway hyporesponsiveness whilst those with proteinuria show indirect anti-donor alloreactivity (Bestard, Nickel et al. 2008).

The third mechanism of allorecognition is through the semi direct pathway or “linked allorecognition” (Figure 5C). Here, the recipient T cells interact with self-APC that have acquired intact donor MHC-peptide complexes from the donor cells through membrane transfer (Herrera, Golshayan et al. 2004). In the immune system, cells are capable of exchanging surface molecules and this is especially true of dendritic cells which can acquire MHC-peptide complexes from other dendritic cells and endothelial cells (Bedford, Garner et al. 1999). Therefore self APC not only have acquired intact donor
MHC but also processed these molecules and able to present these peptides thereby possessing the ability to simultaneously stimulate alloreactive CD4+ T cells by the indirect pathway and CD8+ T cells by the direct pathway (Herrera, Golshayan et al. 2004).

### 1.2.2.5 Clinical consequences of the allorecognition

#### 1.2.2.5a Hyperacute rejection

Hyperacute rejection refers to rapid rejection of either allografts or xenografts which is due to preformed donor specific antibodies. These antibodies are of two principal lineages.

1. Low affinity IgM and IgG antibodies that are specific for ABO blood group antigens and mandate ABO blood group matching in solid organ transplantation. Similar antibodies exist in humans against galactose-α-1-3-galactose epitope present in all other mammals and this constitutes one of the major impediments to successful xenotransplantation (Parker, Bruno et al. 1994).

2. High affinity IgG antibodies directed against HLA class I antigens expressed on vascular endothelium. These antibodies commonly develop as a consequence of prior immunization either by blood transfusions, pregnancies or previous allografts. In 1966, Kissmeyer Neilson described the pathology of hyperacute rejection in two cases of multiparous women who received renal transplants (Kissmeyer-Nielsen, Olsen et al. 1966). These patients had demonstrable complement fixing antibodies in their serum. It was the binding of these antibodies that triggered activation of clotting and complement cascades leading to intravascular thrombosis, ischemia and subsequent necrosis (Figure 7).
1.2.2.5b. Acute cellular rejection

Renal allografts can be rejected quite rapidly even in the absence of preformed alloantibodies. In routine clinical practice with the use of immunosuppression, this usually occurs between 5 days and 3 months after transplantation. With current therapy directed largely against T cells, the incidence of acute cellular rejection is approximately 5-10% in the first post-transplant year. Pathologically, acute cellular
rejection is manifested by the accumulation of mononuclear cells in the interstitium, accompanied by the inflammation of tubules (tubulitis) and sometimes of small and medium sized arteries (intimal arteritis) (Colvin 2006). Glomerulitis is rarely a conspicuous feature of acute cellular rejection with a predominance of CD3+ T and CD68+ macrophages. However, why the glomerulus becomes a target in a minority of cases still remains a mystery (Tinckam, Djurdjev et al. 2005).

**Tubulitis:** Tubulitis is an important lesion in the diagnosis of ACR and is believed to contribute to the abrupt rise in serum creatinine. This lesion is T cell dependent and occurs even in the absence of B cells and alloantibody (Jabs, Sedlmeyer et al. 2003). T cells are attracted to the cortical tubules probably via chemokines (CCL2, CCL5, CX3CL1) from tubular cells, made in response to cytokines from inflammatory cells. T cells, especially cytotoxic T cells enter between the tubular cells and may cause apoptosis by releasing cytolytic granules containing granzyme B and perforin or by exposure to FasL on the T cell surface. The integrin component (CD103) is postulated to help retain T cells in the epithelial layer by binding to E-cadherin expressed strongly in the distal nephron. FOXP3+ Tregs also accumulate but their function is unknown. In response tubules secrete IL15 and protease inhibitor-9 which in turn inhibit granzyme-B and perforin respectively. Infiltrating macrophages secrete TGF-β and upon chronic exposure, tubules undergo epithelial-mesenchymal transformation with the loss of E-cadherin. These cells may infiltrate the interstitium and promote fibrosis (Figure 8) (Cornell, Smith et al. 2008). Although multiple cytokines and chemokines are selectively expressed in ACR, the genes that are differentially expressed in clinical vs. subclinical ACR in protocol biopsies are T-bet, FasL and CD152 (Hoffmann, Hale et al. 2005).
**Figure 8: Postulated events in tubulitis (acute cellular rejection)**

*(Cornell, Smith et al. 2008)*

**Intimal arteritis:** Endarteritis affects large and small arteries focally both within and outside the parenchyma and can occur even without the participation of alloantibody as shown in murine models (Russell, Chase et al. 1997, Wieczorek, Bigaud et al. 2006). Apoptosis of the vascular endothelial cells is present in the sites of arteritis but this lesion is not always associated with an interstitial inflammation, thereby suggesting that a T cell pathway distinct from that of tubulitis is involved (Cailhier, Laplante et al. 2006). In this lesion, the inflammatory cells infiltrating the endothelial cell are mostly T cells and macrophages with an effector phenotype. Inflammatory cellular adhesion and trans-endothelial migration begins when abluminal chemokines (CCL4, CCL5, CXCL8) are transcytosed to the luminal surface and bound by luminal glycosaminoglycans thus creating a chemotactic gradient for inflammatory cell migration (Middleton, Patterson et
Endarteritis is detected in approximately 25-40% of ACR but rarely seen in stable grafts (Mengel, Gwinner et al. 2007). This lesion responds poorly to steroids but is reversible with anti T cell therapy (OKT3/ATG) again stressing the importance of T cells (Colvin 2006).

In summary, ACR is a complex process involving many effector cells including T cells and macrophages. Despite the well documented pre-eminence of the cellular components there is some evidence for the involvement of alloantibody. In renal transplantation, about 10% of the biopsies for ACR show evidence of intravascular fibrinoid necrosis with occasional evidence of antibody and complement deposition by immunofluorescence.

1.2.2.5c Antibody mediated rejection

Seminal work by Gorer and others has shown that mouse skin allografts can induce antibody that can agglutinate donor erythrocytes (Amos, Gorer et al. 1954, Gorer 1955). However passive transfer of the antibody at the time of engraftment failed to cause accelerated rejection in contrast to the adoptive transfer of the sensitised lymphocytes which promptly caused rejection. Moreover, T cells were considered to be the central regulators and effectors of the process of allograft rejection as lack of T cells in animal models led to the non-rejection phenotype (Manning, Reed et al. 1973). The apparent lack of response to the early transfer of the antibody was later shown to be secondary to the lack of perfusion of the skin grafts during the first few days (Gerlag, Koene et al. 1975). Evidence accumulated for the implication of antibody in chronic rejection and in 1970, Russell and colleagues identified a strong link between the presence of HLA specific antibody and chronic transplant arteriopathy (stenotic arterial lesions of the graft) (Jeannet, Pinn et al. 1970). Furthermore experimental studies in murine models have confirmed the ability of the passively transferred antibody to cause transplant arteriopathy even in immunologically deficient mice but
not in the absence of B cells (Russell, Chase et al. 1997). A further breakthrough occurred with the demonstration of the connection between the antibody and graft damage by the identification of C4d deposition as a marker of complement fixation in the tissues (Feucht, Felber et al. 1991, Collins, Schneeberger et al. 1999). Chronic antibody mediated rejection as a significant cause of chronic allograft loss is now widely recognised.

**Acute antibody mediated rejection (AAMR):** At present AAMR is diagnosed based on four criteria (Colvin and Smith 2005).

1. Clinical evidence of graft dysfunction
2. Histological evidence of tissue injury (e.g. Acute tubular injury, inflammatory infiltrate, capillary microthrombi, fibrinoid necrosis)
3. Immunopathological evidence for the action of antibodies (e.g. C4d deposition)
4. Serologic evidence for the presence of donor specific antibody at the time of the biopsy

On an average this occurs in about 7% of allografts and is seen in around 30% of allografts with acute rejection (Herzenberg, Gill et al. 2002, Mauiyedi, Crespo et al. 2002, Racusen, Colvin et al. 2003). Demonstration of C4d deposition in the capillaries, a technique pioneered by Feucht has become crucial in the diagnosis of AAMR with varying specificity (50-95%) and sensitivity (78-96%) (Bohmig, Exner et al. 2002, Mauiyedi, Crespo et al. 2002, Feucht and Mihatsch 2005). C4d is an inactive fragment derived from C4b in the classical complement pathway. C4d per se does not have a functional role but remains bound to the tissue for several days after the immunoglobulin and C1 have been released. C4d is deposited in the majority of the peritubular capillaries as an intense ring pattern, detected by immunofluorescence microscopy (Figure 9) (Regele, Bohmig et al. 2002). Electron microscopy demonstrates C4d on the surface and within the intracytoplasmic vesicles of the endothelial cells (Regele, Bohmig et al. 2002). Although anti-HLA DSA are detected in about 90% of
patients who have C4d deposition, AAMR can occur even in the absence of demonstrable circulating DSA probably due to the adsorption of the antibody onto the graft (Martin, Guignier et al. 2005).

![Immunofluorescence microscopy for C4d](image)

**Figure 9: Immunofluorescence microscopy for C4d**

*(A). Control case with CNI nephrotoxicity (B) AMR*

*(Mauityedi, Crespo et al. 2002)*

**Complement and AAMR**: In animal studies and in humans, complement fixation is essential for the pathogenesis of AAMR. Passive transfer of complement fixing
isotypes of alloantibodies can mediate AAMR in animal models of kidney and heart transplants independent of Fc receptors or NK cells (Wasowska, Qian et al. 2001, Rahimi, Qian et al. 2004). In humans, activation of C1 (composed of C1q, C1r, and C1s) is initiated by the interaction of globular domains of C1q with IgG or IgM bound to the antigen epitopes of the graft endothelium. The C1q binding potential of human IgG subclasses is as follows- IgG3>IgG1>IgG2>IgG4. Conformational changes within C1q then allow the cleavage and activation of C1r which in turn activates C1s. C1s is the enzyme that activates C2 and C4. C4 is cleaved by C1s into C4a and C4b, exposing the sulfhydryl group. The reactive sulfhydryl group forms a rapid ester or amide bond with the nearby molecules that contain hydroxyl or amino groups. C4b is inactivated by factor 1 into C4d which remains covalently bound to the surface of the capillary endothelium. Eventually C4d is cleared from the tissue after the antibody response has ended (can take place within as early as 8 days) (Collins, Schneeberger et al. 1999). Further steps leading to the formation of the membrane attack complex are demonstrated in Figure 10 (Colvin and Smith 2005).
Figure 10: Classical pathway of complement activation

(Colvin and Smith 2005)
The acute effects of complement activation are well described-

1. Chemoattraction of neutrophils and macrophages (C3a and C5a)
2. Vasospasm (PGE2 from macrophages)
3. Edema (histamine from the activated mast cells)
4. Lysis of endothelial cells (MAC)
5. Vascular microthrombi (Von Willibrand factor released by the activated endothelial cells and platelet aggregation)

**Complement independent pathway and chronic antibody mediated rejection (CAMR):** Antibody can also damage target cells through complement independent pathways which also contribute to the pathogenesis of antibody mediated rejection. Current evidence suggests that complement independent pathways contribute to the pathogenesis of CAMR (Colvin and Smith 2005). In the absence of complement or inflammatory cells, alloantibody (for example HLA class I DSA) can induce endothelial cell activation (to synthesize cytokines and chemokines), and also promote their proliferation through NF-κB (Smith, Lawson et al. 2000, Zhang and Reed 2009). The activation of the endothelial cells may be relevant to the arterial intimal proliferation, a lesion characteristic of CAMR. Antibody can also lyse target cells through low affinity Fcγ-III receptors on the surface of NK cells and macrophages (antibody dependent cell mediated cytotoxicity). Figure 11 demonstrates both complement independent and dependent pathways for endothelial cell damage (Colvin and Smith 2005).
In humans, the role of complement independent endothelial injury in the pathogenesis of CAMR has been studied indirectly through gene expression studies. These studies are pioneered by Halloran’s group who have demonstrated that endothelial transcripts (ENDATS) are preferentially expressed in patients with CAMR, and that they correlate with histological lesions and predict graft outcomes irrespective of peritubular C4d deposition thereby raising an important question on the utility of C4d staining in the diagnosis of CAMR (Sis, Jhangri et al. 2009). Furthermore, in an analysis of patients with DSA, they have shown that DSA selective transcripts demonstrated a selective high expression in the NK cells and correlated with a diagnosis of CAMR. Interestingly immunostaining of the cells in the peritubular capillaries of these patients revealed that...
most of these cells are CD16+ CD56+ NK cells and not T cells. This study highlights the importance of NK cells in the context of CAMR (Hidalgo, Sis et al. 2010).

The classical histological lesions of CAMR are (Colvin 2011) (Figure 12)

1. Transplant glomerulopathy
   a. Duplication of glomerular basement membrane (GBM)
   b. Absence of evidence of immune complex glomerulonephritis/chronic thrombotic microangiopathy
   c. Glomerulitis (majority of the cellular infiltrate-CD68+, minority CD3+)

2. Transplant arteriopathy
   a. Fibrointimal thickening of the arteries
   b. Inflammatory cells present within the thickened intima

3. Peritubular capillaropathy
   a. Peritubular capillary basement membrane multilayering
   b. Loss of peritubular capillaries over time
   c. Mononuclear cells in the peritubular capillaries
   d. C4d deposition in some cases

4. Interstitial fibrosis and Tubular atrophy
   a. Non-specific feature
   b. If present, C4d deposition may offer a diagnostic clue
**Figure 12: Histological lesions of CAMR**


When the damaged endothelium repairs itself, it also forms a new basement membrane layer. The pathologic finding of basement membrane multilamination is thought to occur by repeated episodes of injury to the endothelium by the antibody. Ivanyi and colleagues have proposed that peritubular capillaropathy can be defined as one peritubular capillary with seven or more circumferential layers or three or more peritubular capillaries with 5-6 layers (Ivanyi, Kemeny et al. 2001). What leads to the episodic antibody mediated injury is not clearly known but one potential explanation is summarized in Figure 13 (Cornell, Smith et al. 2008).
According to this scheme circulating DSA may be below the level of detection at a given time point but typical lesions persist in the graft.

Four theoretical stages for the evolution of CAMR have been described at a NIH consensus conference (Takemoto, Zeevi et al. 2004). As shown in Figure 14, this staging process proposes that appearance of DSA in the circulation marks the first stage which progresses eventually to allograft loss. Although this provides a clear view of the progression of the disease process, it is in fact an over-simplification of a rather complex immuno-pathological process. For example the time frame from the origin of DSA to the development of histological lesions can vary and same is true of the lapse between the onset of histological lesions to that of allograft dysfunction. Moreover, C4d
detection which heralds the start of stage-2 may not occur in some cases of histologically proven CAMR.

Figure 14: Proposed sequence of stages of CAMR

(Takemoto, Zeevi et al. 2004)

1.2.2.5d Accommodation

Accommodation is defined as the resistance of a graft to the acute pathological effects of allospecific antibodies and complement fixation (Koch, Khalpey et al. 2004). Alexandre and colleagues initially observed this phenomenon in the recipients of ABO incompatible renal transplants (Alexandre, Squifflet et al. 1987). They described how transient depletion of blood group specific antibodies allowed immediate graft survival. However there was rebound of the antibody concentration within the first 10 days that led to graft rejection in about 90% of the patients. Importantly, for the remaining grafts after 21 days, there was no correlation between the titre of the antibody and the occurrence of rejection.
In animal experiments, the absence of T cell help and the emergence of B cell tolerance through exposure to incompatible carbohydrate antigens leading to the development of non-complement fixing antibodies have been cited as possible mechanisms for accommodation (Ogawa, Mohiuddin et al. 2004). Also the emergence of anti-apoptotic proteins, such as B cell lymphoma-2 (Bcl-2), Bcl-XL and haem-oxygenase-1 (HO-1), has been demonstrated in rodent studies in the context of allograft accommodation (Tabata, de Perrot et al. 2003). In humans, antibody induced increase in the endothelial expression of Bcl-XL has been demonstrated as a molecular mechanism for the phenomenon of accommodation in HLA mismatched renal allografts by Salama et al. (Salama, Delikouras et al. 2001). Finally complement regulation has also been shown to be involved in the development of accommodation. Four cell surface inhibitors of complement activation have thus far been identified (Collins, Schneeberger et al. 1999).

1. CR1
2. Decay Accelerating Factor (DAF, CD55)
3. Membrane Cofactor Protein (MCP, CD46)
4. Protectin (CD59)- inhibits MAC

Inhibit both classical and alternate pathways at the level of C3 and C5 convertase

It is easy to assume that accommodation is an ‘all or none’ process. But some authors propose that there are gradations and indeed that CAMR is partially accommodated AAMR (Colvin and Smith 2005).

1.2.2.5e Tolerance

The opposite phenomenon to CAD is characterized by excellent graft function with no evidence of morphological graft damage which, if it occurs in the absence of immunosuppression, is termed tolerance. Nearly seventy years ago, Owen documented the coexistence of two types of erythrocytes in the blood of dizygotic cattle twins (freemartins). He concluded that the twins must have exchanged the erythrocytes
or their precursors in utero and that the cells survived as the calves grew into adults. This naturally acquired non-reactivity to foreign red cells with their antigens was a clear example of immunological tolerance in action (Owen 1945). The phenomenon of actively acquired tolerance was further described by Medawar and colleagues in experiments demonstrating the inability of individuals to react immunologically against foreign tissue, to which they had been previously exposed in foetal life (Billingham, Brent et al. 1953).

Instead of a strict definition of tolerance, an operationally tolerant state has been defined as long-term graft survival in a patient who is no longer taking any maintenance immunosuppression (Calne 2004). Such a state of operational tolerance is very rare in routine clinical practice and is exemplified either by non-adherence or by patients with malignancy or severe infections who discontinue immunosuppression but maintain stable graft function. There has been much interest in the identification of immune signature for operational tolerance since the identification of a similar phenotype in other transplant recipients may facilitate the safe withdrawal of immunosuppression.

**1.2.3 Markers of allograft loss or tolerance**

There is an unmet need for specific markers that can identify patients across the spectrum of immunological risk of renal transplantation (i.e. CAD vs. operational tolerance). Such risk stratification would inform the choice of immunosuppressive strategy with potentially progressive minimization of immunosuppression in patients with low-immunological risk or intensification if there is evidence of on-going immune activation. Such individualized immunosuppressive strategy may eventually help realize the ultimate goal of prolonging long term graft life.
1.2.3.1 Chronic allograft loss—Clinical markers

Late renal allograft loss is one of the biggest challenges in the field of transplantation and early diagnosis is thwarted by the inability to detect early graft injury. Two of the commonest clinical variables measured routinely in practice are serum creatinine and urine protein excretion.

Serum creatinine measured at one year was identified as an important determinant of long term allograft survival in a large registry analysis by Hariharan (Hariharan, McBride et al. 2002). In this study they demonstrated that subsets of patients with markedly reduced graft life can be identified by using renal function measured by serum creatinine at one year or the change in creatinine between 6 months and one year after transplantation (Δ creatinine). For this they have used a cut-off for serum creatinine of 1.5mg/dl and 0.3 for Δ creatinine. Patients who had renal function worse than these cut-off values demonstrated a step wise reduction in the overall graft survival and the graft half-life even after adjusting for potential confounding factors, thereby suggesting that serum creatinine can be used as a surrogate end point for eventual graft loss. However there is one significant shortcoming of this analysis which is the measurement itself. Serum creatinine is not a reliable method for estimating renal function; as it dependent on age, gender, ethnicity and body weight.

While there is no disputing the strong correlation of renal function and renal transplant outcomes, caution is advised in extrapolating this. In establishing highly associated diagnostics as predictive entities, further steps of validation are required (Armitage 2002). In another study Kaplan and colleagues further analysed the utility of serum creatinine as a predictive marker (Kaplan, Schold et al. 2003). In their analysis, serum creatinine was found to generate an odds ratio of around 2 for graft loss which suggests doubling of the risk for allograft loss. But when the utility of the variable was assessed by Receiver Operational Characteristic (ROC) curve, the area under curve value was a mere 0.67. In other words, the ability of serum creatinine to discriminate observations between the actual failure cases and the cases that do not fail is a
minimal gain from random allocation alone (analogous to an area of 0.500). More importantly, no particular range of the creatinine values had any predictive character or a discrete cut-off that could be utilized. Overall, this study highlighted the limited value of serum creatinine as a predictive marker for allograft loss although it concurred with previous finding that creatinine is a strong risk factor.

Ever since the seminal description of Bright’s disease, proteinuria has been used as a marker of renal parenchymal damage, and in most renal diseases, proteinuria is an important early prognostic marker of poor outcome (Cattran, Pei et al. 1992, Burton and Harris 1996, Harris, Burton et al. 1996, Iseki, Ikemiya et al. 2003, Reich, Troyanov et al. 2007). Furthermore, in patients without overt renal disease, low-grade proteinuria is a marker of poor cardiovascular outcome (Grimm, Svendsen et al. 1997). The origin of protein in the urine may simply be a reflection of established renal damage, but there is some evidence that it may be indicative of earlier processes in the chain of injurious events that ultimately lead to renal fibrosis and glomerulosclerosis. For example, protein may be acting as a mediator of renal injury, initiating multiple inflammatory pathways that lead to renal fibrosis and scarring (Abbate, Zoja et al. 2006). Recent evidence also suggests that low-grade proteinuria may be indicative of on-going endothelial injury (Stam, van Guldener et al. 2006). Perhaps, more importantly, there is some evidence that proteinuria is a modifiable risk factor and that reduction of protein excretion may be a worthwhile therapeutic goal leading to improved clinical outcomes (Bakris 2008).

Allograft glomerular diseases that lead to high grade proteinuria are shown to be associated with reduced kidney allograft survival (First, Vaidya et al. 1984). More recent studies show this relationship extends to lower levels of proteinuria that may or may not be associated with glomerular pathology (Halimi, Laouad et al. 2005, Amer, Fidler et al. 2007, Halimi 2013). Investigating the cause of early post-transplant proteinuria is complex because after transplantation protein may originate from the
allograft or from native kidneys, may be due to various allograft pathologies, and/or may be a side effect of immunosuppressive medications. Thus, stating that proteinuria relates to graft survival is an oversimplification because among other variables, the cause of proteinuria likely has an effect on that relationship. For example low levels of proteinuria have been attributed to several mechanisms including glomerular hyperfiltration, endothelial activation, tubular defects in the absorption of albumin, and proteinuria originating from the native kidneys, all of which may be modulated by the hemodynamic effects of calcineurin inhibitors and all of which may have a significant effect on graft outcomes (First, Vaidya et al. 1984, D'Cunha, Parasuraman et al. 2005, Myslak, Amer et al. 2006, Straathof-Galema, Wetzels et al. 2006, Amer, Fidler et al. 2007, Russo, Sandoval et al. 2007). Still, these observations emphasize the importance of monitoring urine protein excretion post-transplant, investigating the cause of even low levels of proteinuria, and considering therapy.

1.2.3.2 **Chronic allograft loss-Immunological markers- T cells**

Lymphocytes, despite their monotonous morphological appearance, have kindled the curiosity and challenged the experimental skills of investigators across the breadth of medicine. We have come to know them as either T (Thymus derived) or B (bursa of fabricius or bone marrow derived) lymphocytes and the diverse roles played by the latter in the context of human renal transplantation will be the focus of this thesis.

T cells have been regarded to have central regulatory and effector roles in graft rejection since experimental animals without T cells permanently accept foreign skin grafts from other animals with increasing phylogenic disparity (Manning, Reed et al. 1973). In the context of chronic antibody mediated rejection (CAMR), T cells also provide help for the alloresponsive B cell by both non-cognate and cognate interactions (Steele, Laufer et al. 1996). Given the potential role of the T cells in the pathogenesis
of CAMR, the measurement and characterization of T cell function may provide a marker for clinical use (see below).

During the past decade, the Th1/Th2 paradigm of the helper T cell activation has evolved to include two distinct subsets called regulatory T cells and Th17 cells. Regulatory T cells are identified by the expression of FoxP3 (Forkhead box P3), which is a member of the Forkhead/winged-helix family of transcription factors. These cells have been shown to suppress immune responses either through their interactions with dendritic cells or via cytokine mediated inactivation of responder T cells (Sakaguchi, Yamaguchi et al. 2008). Th17 cells were identified by the production of the IL-17 family of cytokines (IL17 A-F) along with IL-21 and IL-22 upon stimulation (Bettelli, Korn et al. 2007). Even though these cells have been shown to play an important role in autoimmunity and infection, their specific role in the context of CAD/tolerance is less well understood.

ELISPOT is one of the most sensitive assays to detect and quantify antigen specific responses of activated T cells and the technique has paved the way for a more objective and reproducible functional analysis of T cells (Czerkinsky, Nilsson et al. 1983). Analysis of interferon-γ (IFN-γ) production by T cells using the ELISPOT technique in response to stimulation by peptides corresponding to the polymorphic hypervariable regions of the β chains of HLA-DR molecules has been shown to predict rejection and correlate with graft function in patients undergoing renal transplantation (Najafian, Salama et al. 2002, Bestard, Nickel et al. 2008). Interestingly, patients with stable function and low IFN-γ CD4+ T cell responses demonstrated regulation of the indirect pathway alloresponse by regulatory T cells (Salama, Najafian et al. 2003). Lower frequencies of direct pathway anti-donor IFN-γ secreting CD4+ T cell responses were also seen in operationally tolerant patients (Sagoo, Perucha et al. 2010).
1.2.3.3 B lymphocytes

A simple definition of B lymphocytes is a population of cells that express clonally diverse cell surface immunoglobulin (Ig) receptors recognizing specific antigenic epitopes. Their origin can be traced to the evolution of adaptive immunity in jawed vertebrates beginning more than 500 million years ago (Cooper and Alder 2006). Mammalian B-cell development encompasses a continuum of stages that begin in primary lymphoid tissue (e.g., human foetal liver and foetal/adult marrow), with subsequent functional maturation in secondary lymphoid tissue (e.g., human lymph nodes and spleen). The functional/protective end point is antibody production by terminally differentiated plasma cells. However, these B-cell development stages and their functional identities were essentially discovered in reverse chronologic order. Before presenting various B cell markers in the context of transplantation, I shall briefly discuss the development and maturation of B cells.

1.2.3.3a Development of B cells

B cells along with the other cells of lymphoid lineage are all derived from common lymphoid progenitors which in turn arise from the pluripotent stem cells (Figure 15) (LeBien and Tedder 2008). The bone marrow stromal cells provide the external signals for this maturation process. Pluripotent stem cells next differentiate into multipotent progenitor cells which express a surface tyrosine kinase called FLT3 which is needed for differentiation into the next stage, i.e. common lymphoid progenitor. Lymphocyte development is accompanied by the expression of the receptor for IL7. IL7 which is dominantly produced by the stromal cells is essential for the growth and the survival of the developing B cells. The common lymphoid progenitor gives rise to the earliest B lineage cell, i.e. the pro-B cell in which immunoglobulin gene re-arrangement begins. A definitive B cell fate is determined by the induction of B lineage specific transcription factor, E2A. From here, further development of B cell ensues, with the re-arrangement of the heavy chain locus followed by the L chain loci. The consecutive stages of B cell
development include-early pro B cell, late pro B cell, large pre B cell, small pre B cell, immature B cell and mature B cell. The rearrangement of Ig gene occurs via an error-prone process involving the combinatorial rearrangement of the V, D, and J gene segments in the H chain locus and the V and J gene segments in the L chain loci. Pre-B cells arise from progenitor (pro-B) cells that express neither the pre-BCR nor surface Ig. Subsequently, BCR expression is a pre-requisite for the development and survival of these cells in the periphery (Lam, Kuhn et al. 1997). Once the re-arranged light chain pairs with a \( \mu \) chain, IgM can be expressed on the cell surface and the pre-B cell becomes an immature B cell. At this stage, the antigen receptor is first tested for tolerance to self-antigens (central tolerance). The fate of the immature cell depends on interactions of surface IgM (IgM). IgM interacts with Ig\( \alpha \) and Ig\( \beta \) to form a functional B cell Receptor (BCR). Ig\( \alpha \) signalling is key to the emigration of the immature B cell. Immature B cells that have no strong reactivity to self-antigens undergo maturation and carried by venous blood to the lymphoid organs (Spleen). A self-reactive immature B cell has 4 potential fates (Murphy 2008):

1. Clonal deletion: Removal of cells of particular antigen specificity
2. Receptor editing: Further gene re-arrangements that replace the autoreactive BCR with a non-self-reactive BCR
3. Anergy: a state of permanent unresponsiveness upon weak cross-linking of the antigen
4. Immunological ignorance: to self-antigen such that these cells behave as if they are not self-reactive at all.

The immature B cell that leaves the marrow on its way to the spleen is called a transitional B cell. There are three types of the mature B cell as shown in Figure 16. Antigen-induced B-cell activation and differentiation in secondary lymphoid tissues is mediated by dynamic changes in gene expression that give rise to the germinal centre
(GC) reaction. The GC reaction is characterized by clonal expansion, class switching recombination (CSR) at the Ig locus, somatic hyper mutation (SHM) of VH genes, and selection for increased affinity of a BCR for its unique antigenic epitope through affinity maturation. CSR, also known as isotype-switching, was first demonstrated in the chicken (Kincade, Lawton et al. 1970). CSR and SHM are mediated by an enzyme designated activation-induced cytidine deaminase (AID) which is induced in B cells in GCs where CSR and SHM occur (Muramatsu, Kinoshita et al. 2000, LeBien and Tedder 2008).
Figure 15: Maturation of human B cells

(LeBien and Tedder 2008)

Figure 16: Mature naïve B cells
1.2.3.3b B cells as antibody producing cells

The discovery of B cells did not originate in the identification of a cell, but rather the identification of an effector protein (i.e., Ig or antibody). Identification of serum gammaglobulin as the source of antibodies was a launching point for the eventual discovery of antibody producing cells (Tiselius and Kabat 1938). Plasma cells were suggested as a source of antibody production as early as 1948 (Fagraeus 1948). All antibodies are constructed in the same way from paired heavy and light chains that are composed of constant (C) and variable regions (V). Each light chain has a variable and constant region while the heavy chain is composed of one variable and three constant regions (CH1, CH2, and CH3). Five different classes of the antibody can be distinguished by their C region whilst differences within the V regions confer antigen specificity. Proteolytic enzymes like papain cleave the antibody molecule at its hinge yielding two identical fragments that contain antigen binding activity. These are called Fab fragments. Each Fab fragment is composed of a light chain paired with the variable region and CH1 domain of the heavy chain. The other fragment contains no antigen binding activity but was originally observed to crystallize readily and therefore called the Fc fragment. Antibodies of various classes operate in distinct places and have distinct effector functions. They are briefly summarized in Figure 17. IgG antibody further consists of 4 subclasses-IgG1, IgG2, IgG3, IgG4. Of these IgG3 has the strongest ability to fix complement, whilst IgG4 does not fix complement.
### Figure 17: Brief summary of Immunoglobulin classes

<table>
<thead>
<tr>
<th></th>
<th>Monomer</th>
<th>Dimer/monomer</th>
<th>Pentamer</th>
<th>Monomer</th>
<th>Monomer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antigen binding sites</strong></td>
<td>2</td>
<td>4/2</td>
<td>10</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>Molecular weight</strong></td>
<td>150,000</td>
<td>170,000-385,000</td>
<td>900,000</td>
<td>180,000</td>
<td>200,000</td>
</tr>
<tr>
<td><strong>% of total Ig in the serum</strong></td>
<td>80%</td>
<td>13%</td>
<td>6%</td>
<td>1%</td>
<td>0.002%</td>
</tr>
<tr>
<td><strong>Half life</strong></td>
<td>23 days</td>
<td>6 days</td>
<td>5 days</td>
<td>3 days</td>
<td>2.5 days</td>
</tr>
<tr>
<td><strong>Fixes complement?</strong></td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>no</td>
</tr>
<tr>
<td><strong>Crosses placenta?</strong></td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td><strong>Fc binds to...</strong></td>
<td>Phagocytes</td>
<td>Mast cells/basophils</td>
<td>Mast cells/basophils</td>
<td>Mast cells/basophils</td>
<td>Mast cells/basophils</td>
</tr>
<tr>
<td><strong>Biological function</strong></td>
<td>Neutralization, opsonisation, memory antibodies</td>
<td>Secretory antibody Neutralization</td>
<td>Complement activation, first response</td>
<td>Receptor on B cells</td>
<td>Antibody of allergy</td>
</tr>
</tbody>
</table>

#### Notes:
- **IgG**
  - Monomer
  - 2 antigen binding sites
  - Molecular weight: 150,000
  - 80% of total Ig in the serum
  - 23 days half life
  - Yes: Fixes complement
  - Yes: Crosses placenta
  - Phagocytes
  - Neutralization, opsonisation, memory antibodies

- **IgA (dimer only)**
  - Dimer/monomer
  - 4/2 antigen binding sites
  - Molecular weight: 170,000-385,000
  - 13% of total Ig in the serum
  - 6 days half life
  - No: Fixes complement
  - No: Crosses placenta
  - No

- **IgM**
  - Pentamer
  - 10 antigen binding sites
  - Molecular weight: 900,000
  - 6% of total Ig in the serum
  - 5 days half life
  - Yes: Fixes complement
  - No: Crosses placenta
  - No
  - Secretory antibody Neutralization

- **IgD**
  - Monomer
  - 2 antigen binding sites
  - Molecular weight: 180,000
  - 1% of total Ig in the serum
  - 3 days half life
  - No: Fixes complement
  - No: Crosses placenta
  - No
  - Complement activation, first response

- **IgE**
  - Monomer
  - 2 antigen binding sites
  - Molecular weight: 200,000
  - 0.002% of total Ig in the serum
  - 2.5 days half life
  - No: Fixes complement
  - No: Crosses placenta
  - No
  - Antibody of allergy
**Antibody and Transplantation:** The production of alloantibody (Non-IgM) usually requires T-cell help. MHC molecules are presented to recipient T cells by either the direct or indirect pathways of allore cognition. The activated T cells can then provide help for B-cell activation, antibody class switching and affinity maturation, through various T-cell-derived cytokines and co-stimulatory factors (such as ICOS (inducible T-cell co-stimulator), CD40 ligand, and CD80 and CD86). Experiments in mice indicate that, for B-cell help, the antigen that is recognized by the T cell needs to be presented by self-APCs (indirect pathway), perhaps because the T cell needs to react with and signal through self-MHC class II molecules on the surface of the B cell (i.e. B cell is acting as APC) (Mitchison 2004). By contrast, the production of IgM alloantibodies that are specific for MHC molecules and carbohydrate antigens (ABO) might not require T-cell help (Steele, Laufer et al. 1996). The B-cell response leads to the production of long-lived plasma cells, which migrate to the bone marrow and continue to produce antibody indefinitely, without requiring T-cell help (Shapiro-Shelef and Calame 2005).

The landmark report that hyperacute rejection was related to the presence of preformed donor-specific antibodies provided unequivocal evidence for the role of B cells and antibody in graft rejection (Kissmeyer-Nielsen, Olsen et al. 1966). The detection and the association of such antibodies with fibrointimal proliferation in the renal allografts (chronic allograft arteriopathy) (Jeannot, Pinn et al. 1970) emphasized the role played by the antibody in the pathogenesis of CAD.

The advent of Luminex xMAP technology (Figure 18) has provided solid phase antibody assays that can detect the presence of HLA specific antibodies with high sensitivity.
Luminex technology for antibody detection

Luminex is based on flow cytometry. 5.6 micron polystyrene microspheres are internally dyed with varying amounts of red and infrared fluorophores to obtain 100 distinct sets. The surface chemistry on these microspheres allows simple chemical coupling of capture reagents such as HLA molecules. High-tech fluidics based on flow cytometry allows profiling and analysis of these microspheres by detection lasers. Because each microsphere carries a unique signature, the Luminex system can identify which set it belongs to.
Several studies have reported an association between the development of donor specific (DSA) and non-donor specific (NDSA) HLA specific antibodies and poor graft survival (Worthington, Martin et al. 2003, Terasaki and Ozawa 2004, Hourmant, Cesbron-Gautier et al. 2005, Lachmann, Terasaki et al. 2009). In these studies, the overall prevalence of these antibodies after transplantation ranged from 12 to 60%. Such a wide variation in the prevalence rates could be the result of different monitoring techniques. In almost all these studies HLA specific antibodies were analysed at either one or two time points, with no serial evaluation to determine when they develop and if they persist at the time of graft failure. Also, clinical status at the time of DSA development may vary from normal function to acute dysfunction with rapid graft loss.

A humoral theory of transplantation has been proposed implicating these antibodies in the causation of graft rejection and loss (Terasaki P I. 2003). However some unanswered questions remain. The presence of post-transplant HLA specific antibodies in patients with no apparent graft dysfunction in part contradicts the humoral theory (Bartel, Regele et al. 2008). Also in sequential screening protocols these antibodies do not always predict acute rejection since their appearance does not precede rejection episodes (Gill, Landsberg et al. 2010). The relevance of DSA in clinical practice was examined by Nickerson’s group in Canada in 2012. In this longitudinal study, they have shown that DSAs develop in 15% of low-risk renal transplant recipients and that graft survival is significantly reduced in such patients. Interestingly, patients with DSA were more likely to have had preceding clinical and subclinical cellular rejections in the 0–6 month post-transplant period. Moreover, in those who develop graft dysfunction, DSAs typically emerge before the onset of proteinuria or a rise in creatinine. In non-adherent patients who present with DSA and dysfunction, the histology was often a mixed picture of cellular and antibody-mediated rejection. Finally, they have shown that histological antibody-mediated injury could occur in patients with DSAs in the absence of graft dysfunction and that the degree of injury could progress in these patients despite augmented immunosuppression (Wiebe,
Gibson et al. 2012). This is the most comprehensive assessment of the clinical relevance of HLA specific DSAs to date.

Complement activation by antibodies has been shown to be an important mediator of graft injury. Hence, assessment of the ability of antibodies to fix complement has gained increasing interest. C4d binding by antibodies has been shown to correlate with graft survival in small cohorts of transplant recipients (Smith, Hamour et al. 2007). Since the “signal” for C4d binding by antibodies is relatively low, the ability of antibodies to bind C1q is a current focus (Tyan 2012). Recent small series have shown that the presence of C1q-binding antibodies correlates with worse graft survival of kidney and heart transplants (Sutherland, Chen et al. 2012, Freitas, Rebellato et al. 2013, Zeevi, Lunz et al. 2013). In a recent study designed to determine the ability of C1q binding by post-transplant donor-specific antibodies to risk stratify renal transplant recipients, Loupy and colleagues have demonstrated that C1q binding by antibodies was strongly correlated with antibody-mediated rejection in the first year after transplantation, with more microvascular inflammation and injury, more C4d deposition in the peritubular capillaries of the graft, and a lower estimated glomerular filtration rate (GFR) at 1 year (Loupy, Lefaucheur et al. 2013). C1q-binding antibodies also correlated with worse 5-year graft survival, whether they were detected at the time of rejection during the first year or at 1 year, and with a higher risk of graft loss in each of three categories of estimated GFR at 1 year. However, there remains one significant caveat to this analysis. In this study, C1q binding was strongly correlated with the strength of the DSA with C1q binding being recorded predominantly in a cohort of patients with a median fluorescence intensity of >6000. This will be a potential argument against the use of such an expensive assay when the outcome is simply related to the strength of the DSA. Even though this study shows that C1q binding risk stratifies renal transplant patients, it indirectly suggests that the strength of the DSA is as strong a clinical marker.
Apart from the anti-HLA DSA, non HLA antibodies have also been studied as markers of graft outcomes in solid organ transplantation in small cohorts. Antibodies of interest include, anti-angiotensin-1 receptor antibody (AT1R-Ab), anti-endothelin type A receptor antibody (ETAR-Ab), anti-K-α1 tubulin antibody and anti-collagen-V antibody. Their clinical applicability needs testing in larger cohorts (Dragun, Catar et al. 2013).

1.2.3.3c Cytokine producing B cells

Studies of B lymphocytes and their roles in the normal immune response have traditionally focused on their potential to differentiate into Ab-producing cells, thereby contributing to cognate humoral immunity. However, in recent years, animal studies have generated a growing interest in the contribution of Ab-independent functions of B cells to immune responses (Lund, Garvy et al. 2005, Lund and Randall 2010). Multiple reports from animal models of infection and autoimmune disease have implicated activated B cells as effective APCs (Serreze, Fleming et al. 1998, Shlomchik, Craft et al. 2001, O'Neill, Shlomchik et al. 2005). B cells may also contribute to immune responses through the secretion of effector cytokines (Harris, Haynes et al. 2000, Lund and Randall 2010), and emerging animal and human studies have shown that the selective manipulation of B cell cytokines can impact on the expression of autoimmune disease models (Fillatreau, Sweenie et al. 2002, Mizoguchi, Mizoguchi et al. 2002, Duddy, Niino et al. 2007, Bar-Or, Fawaz et al. 2010).
1.2.3.3d B regulatory cells

The existence of B cells with suppressive capacity was first reported in the mid-1970s by Katz and colleagues in guinea pigs (Katz, Parker et al. 1974). In this seminal piece of work, they demonstrated that B cells suppress DTH type reactions in a series of immunization experiments. They postulated that these suppressor cells in DTH form a separate sub-population of easily identifiable Ig-coated cells that are distinct from those which become antibody producing cells. This was the first report of the ability of B cells to regulate T cell function. Following this original observation, suppressive B cells generated in mice were shown to be associated with the induction of antigen specific suppressor T cells (Shimamura, Hashimoto et al. 1982). Briefly, various milestones in the history of B regulatory cells are summarized in Table 1. As shown in this table, different approaches led to the identification of IL-10 as a key player in this suppressive function. It was in 2002, that Mizoguchi and colleagues demonstrated that IL-10 producing B cells are enriched in the CD1d\textsuperscript{hi} B cell subset and that they control autoimmune colitis and for the first time named these cells regulatory B cells (Mizoguchi, Mizoguchi et al. 2002). In humans, CD19\textsuperscript{+}CD24\textsuperscript{hi}CD38\textsuperscript{hi} transitional B cells have been shown to correlate with the CD1d\textsuperscript{hi} population in mice and they express IL-10 when studied in vitro. Interestingly, these cells, which exhibit regulatory properties in healthy volunteers, lose their ability to regulate inflammatory Th1 immune responses in patients with SLE (Blair, Norena et al. 2010). In contrast, Tedder's group from Durham, NC, have shown that IL-10 positive B cells are enriched within the memory sub-population, thereby suggesting a degree of plasticity within the regulatory B populations (Iwata, Matsushita et al. 2011).
### Table 1: History of Breg research

<table>
<thead>
<tr>
<th>Year</th>
<th>Mouse</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>1974</td>
<td>B cells suppress DTH (Neta and Salvin 1974)</td>
<td></td>
</tr>
<tr>
<td>1996</td>
<td>B cell–deficient mice develop chronic EAE (Wolf, Dittel et al. 1996)</td>
<td></td>
</tr>
<tr>
<td>2002</td>
<td>IL-10-producing B cells regulate autoimmunity (Filatreau, Sweenie et al. 2002). CD19&lt;sup&gt;+&lt;/sup&gt;CD1d&lt;sup&gt;hi&lt;/sup&gt; B cells producing IL-10 control Th2-driven colitis (Mizoguchi, Mizoguchi et al. 2002)</td>
<td></td>
</tr>
<tr>
<td>2003</td>
<td>CD40 and antigen-activated B cells prevent CIA (Mauri, Gray et al. 2003)</td>
<td></td>
</tr>
<tr>
<td>2004</td>
<td>Helminth-induced IL-10-producing B cells protect mice from anaphylaxis (Mangan, Fallon et al. 2004)</td>
<td></td>
</tr>
<tr>
<td>2007</td>
<td>CD19&lt;sup&gt;+&lt;/sup&gt;CD21&lt;sup&gt;+&lt;/sup&gt;CD23&lt;sup&gt;+&lt;/sup&gt;IL-10&lt;sup&gt;+&lt;/sup&gt;B cells protect mice from developing CIA (Evans, Chavez-Rueda et al. 2007). Apoptotic cells induce regulatory B cells (Gray, Miles et al. 2007). B cell–mediated suppression of EAE requires B7 costimulatory molecules to induce Tregs (Mann, Maresz et al. 2007).</td>
<td>Impaired IL-10-production by B cells in MS patients (Duddy, Niino et al. 2007). B cell depletion therapy may give rise to functionally competent Breg pool upon B cell repopulation in SLE patients (Anolik, Barnard et al. 2007).</td>
</tr>
<tr>
<td>2008</td>
<td>TLR-activated B cells suppress EAE by inhibiting Th1/Th17 responses (Lampropoulou, Hoehlig et al. 2008)</td>
<td>Helminth-induced CD1d&lt;sup&gt;+&lt;/sup&gt;hi IL-10-producing B cells partially ameliorate MS (Correale, Farez et al. 2008).</td>
</tr>
<tr>
<td>2009</td>
<td>GIFT15-induced Bregs suppress EAE (Rafei, Hsieh et al. 2009)</td>
<td></td>
</tr>
<tr>
<td>2010</td>
<td>Bregs prevent AIA via induction of Tregs (Amu, Saunders et al. 2010)</td>
<td>CD19&lt;sup&gt;+&lt;/sup&gt;CD24&lt;sup&gt;+&lt;/sup&gt;CD38&lt;sup&gt;+&lt;/sup&gt;IL-10-producing Bregs suppress Th1 differentiation (Blair, Norena et al. 2010). B10 cells suppress monocyte cytokine production (Iwata, Matsushita et al. 2011). Increased CD19&lt;sup&gt;+&lt;/sup&gt;CD24&lt;sub&gt;+&lt;/sub&gt;CD38&lt;sub&gt;+&lt;/sub&gt;IL-10&lt;sup&gt;+&lt;/sup&gt; B cells associated with positive outcome in renal transplant recipients (Newell, Asare et al. 2010).</td>
</tr>
<tr>
<td>2011</td>
<td>Breg IL-10 production is regulated by STIM1 and STIM2 calcium sensors (Matsumoto, Fujii et al. 2011). IL-10-producing B cells induce Tregs and inhibit Th1/Th17 cells during acute inflammation (Carter, Vasconcellos et al. 2011). B cells inhibit antitumor responses via the release of TNF-a (Schioppa, Moore et al. 2011). TIM1 is a new inclusive marker for Bregs (Ding, Yeung et al. 2011)</td>
<td></td>
</tr>
<tr>
<td>2013</td>
<td></td>
<td>CD19&lt;sup&gt;+&lt;/sup&gt;CD24&lt;sup&gt;+&lt;/sup&gt;CD38&lt;sup&gt;+&lt;/sup&gt;IL-10-producing Bregs maintain Treg while limiting Th1 and Th17 differentiation (Flores-Borja, Bosma et al. 2013).</td>
</tr>
</tbody>
</table>
**Phenotypic and functional markers of Bregs:** Various phenotypic markers that have been used to define Bregs in mice and human beings and their respective functions are summarized in Table 2. Since the discovery of the Bregs, IL-10 secretion has been considered a key trait of this population.

**IL-10:** Interleukin-10 (IL-10) was first described as cytokine synthesis inhibitory factor (CSIF), produced by mouse Th2 cells that inhibited the activation of and cytokine production by Th1 cells (Fiorentino, Bond et al. 1989). Biochemical and X-ray crystallographic analyses of human IL-10 (Walter and Nagabhushan 1995) demonstrated that it is an acid-sensitive, non-covalent homodimer of two interpenetrating polypeptide chains, similar to interferon. The functions of IL-10 are mediated through its receptor which is composed of at least two subunits that are members of the interferon receptor family - a high affinity IL-10R1 and low affinity IL-10R2. IL-10 R1 is expressed by most hemopoietic cells, although generally at measured levels of only a few hundred per cell. IL-10 R1 expression on T cells is downregulated by activation and in contrast, activation of monocytes is associated with upregulation, consistent with IL-10’s role as an inhibitory factor for these cells (Tan, Indelicato et al. 1993, Liu, Wei et al. 1994). IL-10R2 is an accessory subunit for signalling and contributes little to IL-10-binding affinity. Its principal function appears to be recruitment of a Jak kinase (Tyk2) into the signalling complex (Kotenko, Krause et al. 1997). The best characterized IL-10 signalling pathway is the Jak/stat (Janus kinase/Signal Transducer and Activator of Transcription) system. The IL-10/IL-10R interaction engages the Jak family tyrosine kinases Jak1 and Tyk2 (Finbloom and Winestock 1995), which are constitutively associated with IL-10R1 and IL-10R2 respectively. IL-10 induces tyrosine phosphorylation and activation of the latent transcription factors stat3, stat1, and in non-macrophage cells, stat5.

IL-10 potently inhibits production of IL-1, TNF and various other inflammatory cytokines and the CC/CX group of chemokines that are implicated in the recruitment of inflammatory cells by activated monocytes/macrophages (de Waal Malefyt, Abrams et
al. 1991, Moore, de Waal Malefyt et al. 2001). The inhibitory effects of IL-10 on IL-1 and TNF production are crucial to its anti-inflammatory activities, because these cytokines often have synergistic actions on inflammatory pathways and processes, and amplify these responses by inducing secondary mediators such as chemokines, prostaglandins, and PAF. Moreover, IL-10 thereby has the ability to affect both Th1 and Th2 responses. Both transcriptional and posttranscriptional mechanisms have been implicated in the inhibitory effects of IL-10 on cytokine and chemokine production. IL-10 down regulates the expression of MHC on APCs which significantly affects the T cell–activating capacity of a monocytic APC. Thus, IL-10 inhibits cytokine, chemokine, PGE2 production, and antigen presentation (Niiro, Otsuka et al. 1995, Koppelman, Neefjes et al. 1997). IL-10 also has a significant effect on the maturation and function of B cells wherein it enhances B cell survival, proliferation, Ig production and class switch (Rousset, Garcia et al. 1992). Apart from the indirect effect of this cytokine of T cells through the APCs, IL-10 is also capable of directly inhibiting pro-inflammatory cytokines by T cells (de Waal Malefyt, Yssel et al. 1993). Interestingly, activation of T cells in the presence of IL-10 can induce non-responsiveness/ anergy, which cannot be reversed by IL-2 or stimulation by anti-CD3 and anti-CD28 (Groux, Bigler et al. 1996). In the context of transplantation, IL-10 has been extensively studied in animal models of both solid organ and bone marrow transplantation with variable results. The effects of IL-10 on allograft outcomes in these models represent a complex product of multiple factors including timing, kinetics, and amounts of cytokine (Moore, de Waal Malefyt et al. 2001).
Table 2: Phenotypic markers for Bregs

<table>
<thead>
<tr>
<th>Marker</th>
<th>Expression level</th>
<th>Function</th>
<th>Limitations for their use</th>
<th>Mouse</th>
<th>Humans</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1d</td>
<td>High</td>
<td>Expressed on APC, presents lipid antigen</td>
<td>Constitutively expressed on all B subsets</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>CD5</td>
<td>Positive</td>
<td>Major ligand of CD72, found on thymocytes, T cells, thymic NKT cells and a subset of B cells</td>
<td>Upregulated upon activation</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>CD10</td>
<td>Variable</td>
<td>Zinc metalloprotease that degrades small secreted peptides. Marker for follicular lymphoma</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD19</td>
<td>Positive</td>
<td>BCR activation</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>CD21</td>
<td>Positive</td>
<td>BCR activation</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>CD23</td>
<td>Variable</td>
<td>Low affinity IgE receptor, negative regulator of IgE production</td>
<td>Down regulated upon activation</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>CD24</td>
<td>High</td>
<td>Heat stable antigen, Expressed on B cell progenitors. Provides T cell costimulation</td>
<td>Upregulated upon activation with LPS</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>CD27</td>
<td>Variable</td>
<td>Marker for memory B cells, binds to CD70. Induces T cell costimulation and B activation</td>
<td>Upregulated upon activation</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>CD38</td>
<td>Variable</td>
<td>Marker for activation, monitors intracellular Ca</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD62L</td>
<td>Negative</td>
<td>Cell adhesion molecule involved in homing of lymphocytes to lymph nodes</td>
<td>Downregulated upon migration</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>CD138</td>
<td>Variable</td>
<td>Plasma cell marker, mediates B cell-matrix interactions</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD93</td>
<td>Variable</td>
<td>B cells at early stage of maturation. Intracellular adhesion and clear apoptotic cells</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgD</td>
<td>Variable</td>
<td>Provides activation signal for B cells</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgM</td>
<td>High</td>
<td>Role in complement activation</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>Positive</td>
<td>Anti-inflammatory cytokine</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>FasL</td>
<td>variable</td>
<td>Induces apoptosis</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIM-1</td>
<td>positive</td>
<td></td>
<td>Yes</td>
<td>?</td>
<td></td>
</tr>
</tbody>
</table>
Origin and function of Bregs: The diversity in the phenotype of reported Bregs has led to controversy regarding their origin and development. Lampropoulou and colleagues have proposed that Bregs develop from the naïve follicular B cells and gain suppressive function through integrated signals provided through TLRs, engagement of CD40 and BCR (Lampropoulou, Hoehlig et al. 2008). However, in human beings and in animal models, naïve B cells represent a population that are the least common secretors of IL-10 (Evans, Chavez-Rueda et al. 2007, Blair, Chavez-Rueda et al. 2009, Blair, Norena et al. 2010). Moreover, mice that have follicular B cells and lack marginal zone B cells indeed develop spontaneous colitis. This brings us to the second model for the origin of the Bregs. According to this model, proposed by Mauri’s group, Bregs arise from a common progenitor, namely T2-marginal zone precursor B cells. The T2-MZP stage of B cell development is immature and such cells are programmed to respond quickly to environmental triggers. This model of the development which is summarized in Figure 19 (Mauri and Bosma 2012) shows that T2MZP B cells are initially activated through a TLR in response to a pathogen leading to the secretion of IL-10. Persistent inflammation causes activation of the T cells which provide further help for the activation of the Bregs which in turn leads to feedback signals to the T cells thereby modulating their responses.

Although various mechanisms have been studied in the context of B regulatory activity, TLR and CD40 mediated pathways are considered to be crucial (Mauri and Bosma 2012). Toll like Receptors (TLR) are pattern recognition receptors that recognise conserved microbial products and alert host cells of the presence of these danger signals. Although TLRs are critical for host defence, signalling through some TLRs can elicit a protective effect from inflammation.
Figure 19: Breg differentiation and regulatory mechanisms (Mauri and Bosma 2012)

Two-step pathway for the development of Bregs. Step 1: a common progenitor, namely autoreactive T2-MZP Bregs, produces IL-10 after TLR stimulation. Step 2: Bregs are further activated and release an optimal amount of IL-10 in response to CD154+CD4+ T cells. This step also requires antigen recognition. The chief mechanism of suppression is via the release of IL-10, leading to the suppression of Th1, Th17 responses, and TNF-α from monocytes.
TLR9 has been studied extensively in this context. For example, TLR-9 deficient mice develop an exacerbated lupus-like syndrome and polymorphisms in the tlr9 gene which reduce the expression of TLR9 have been shown to increase predisposition to SLE in humans (Christensen, Shupe et al. 2006, Tao, Fujii et al. 2007). Administration of TLR9 and TLR4 agonists has been shown to suppress the course of diabetes, EAE and arthritis in mice (Quintana, Rotem et al. 2000, Buenafe and Bourdette 2007, Wu, Sawaya et al. 2007). Interestingly, stimulation of B subsets by TLRs promotes differential cytokine expression. For example, stimulation of MZP T2 cells with TLR2 and TLR4 promotes IL-10 secretion which is enhanced by the addition of CD40. In contrast under similar conditions follicular B cells predominantly secrete Interferon-γ. In a similar fashion, CpG, a TLR9 ligand, has also been shown to up-regulate the production of IL-10 in neonates (Sun, Deriaud et al. 2005, Barr, Brown et al. 2007, Gray, Gray et al. 2007, Lampropoulou, Hoehlig et al. 2008).

CD40 is a membrane associated protein and a member of TNF receptor super family. Prolonged and strong signalling via CD40 has been shown to have an inhibitory effect on the secretion of antibodies by B cells (Miyashita, McIlraith et al. 1997). CD40-CD40L interaction has been shown to be a critical pathway for the activation of Bregs (Mauri, Mars et al. 2000, Fillatreau, Sweenie et al. 2002, Mauri, Gray et al. 2003, Evans, Chavez-Rueda et al. 2007). In animal models of transplant tolerance using anti-CD45RB antibodies, CD40 has again been shown to be a requirement for the induction of tolerance thereby suggesting that tolerance induction is dependent on direct T and B cell contact. Interestingly this effect was independent of IL-10 (Deng, Moore et al. 2007).

**Bregs and Transplantation:** An increasing interest in Bregs has recently emerged within the field of clinical renal transplantation. Studies in renal transplant recipients who are not maintained on immunosuppression (operationally tolerant) have shown that there is a higher expression of genes related to B cells in their peripheral blood
and urine. More importantly, these patients have higher numbers of CD24\textsuperscript{hi}CD38\textsuperscript{hi} transitional B cells and cells with elevated expression of CD5 and CD1d. When compared to patients on maintenance immunosuppression, tolerant patients also demonstrated higher levels of IL-10 secretion by transitional B cells upon non-specific stimulation with PMA and ionomycin (Newell, Asare et al. 2010, Pallier, Hillion et al. 2010, Sagoo, Perucha et al. 2010). Despite the potential for clinical applicability one major question remains - Is this signature a mere representation of the lack of immunosuppression? Further prospective studies are required to understand the clinical significance of Bregs in human transplantation.

Finally, despite multiple candidate phenotypes presented in this section, the identity of an inclusive marker for Bregs remains a holy grail in the field of Bregs. To this end, Rothstein’s group from Pittsburgh have identified T cell Ig domain and Mucin domain-1 (TIM-1) as an inclusive marker for murine Bregs. TIM-1 was expressed in the majority of IL-10+ B cells and TIM-1 specific antibody promoted tolerance in mice which is B cell dependent (Ding, Yeung et al. 2011). This marker has not been validated in humans and a search for an inclusive marker for human B regs continues. This is especially important because there is an increasing interest in the role of B cells as immune regulators in transplantation. The development of consensus regarding the Breg phenotype will take us a step forward towards not only identifying a potential biomarker but also cell-based immunotherapy.
1.3 Objectives of the thesis

The main objectives of this thesis are set out as below:

1. To study the clinical utility of proteinuria as a marker for graft outcomes in renal transplant recipients (Chapter-3)

2. To investigate the link between the clinical phenotype (defined by deteriorating creatinine and proteinuria), histological phenotype and outcomes in patients with troubled renal allografts (Chapter-3)

3. To define human B regulatory cells based on the secretion and synthesis of both IL-10 and a prototype pro-inflammatory cytokine TNF-α (Chapter-4)

4. To establish the link between the histological phenotype and Bregs in renal transplantation (Chapter-4)

5. To validate the utility of the phenotype of human B regs in a random sample of patients from a RCT of two steroid avoidance regimes (Chapter-5)
Chapter-2: Methods

2.1 Identification of allografts at risk-Utility of proteinuria

Electronic clinical records of all consecutive renal transplants performed and followed up at St. James's University Hospital, Leeds between 1988 and 2008 were searched to identify potential subjects for this part of the analysis. For the assessment of the clinical utility of proteinuria, the epidemiological study is divided into two parts.

1. The analysis of the significance of early low-grade proteinuria after transplantation (3 months post-transplantation) and its impact on graft outcomes.

2. Longitudinal analysis of low grade proteinuria and its impact on graft outcomes.

2.1.1 Measurement of proteinuria

Proteinuria was assessed using the urine protein creatinine ratio (uPCR). All transplant clinics were held during the morning, so that PCR determination represents a midmorning sample. Urinary protein was measured by an automated turbidometric method (O'Malley and Penney 1984) using the quaternary ammonium salt benzethonium chloride, and creatinine by an in-house modification of the Jaffe method, incorporating a pre-dilutional circuit (Mallinson 1985). The protein creatinine ratio was calculated as protein (g/l) × 1000 divided by creatinine (mmol/l), with units of g/μmol. The factor of 1000 is required to produce values with the same units as those in previously reported studies, as protein concentration was originally measured in mg/dl. The median of urine PCR values obtained in the 3rd post-transplant month was used for this analysis, and patients were divided into subgroups depending on the degree of proteinuria. Proteinuria was assessed for every transplant recipient at every clinic visit using a random urine protein/creatinine ratio (uPCR). uPCR was validated for
quantifying proteinuria against 24hr protein excretion at our centre (Dyson, Will et al. 1992) and had been shown to correlate very well with 24hr urine protein excretion. For example a uPCR value of 1000 equates to 1g proteinuria/24hrs. Significant proteinuria was defined as a urinary PCR value above 150 (0.15 g/24 hrs equivalent).

2.1.2 Immunosuppression

All patients received 1g of intravenous methylprednisolone at induction followed by maintenance immunosuppression which consisted of ciclosporin, azathioprine and prednisolone between 1988 and 1998. Patients who were diabetic or who had a two DR mismatch received two doses of intravenous basiliximab 20 mg at day 0 and day 4 between 1998 and 2004. Since 2004, all patients received Basiliximab along with methylprednisolone at induction and patients were maintained on a steroid free maintenance immunosuppression comprising tacrolimus and mycophenolate. Maintenance prednisolone was used in moderate to high risk renal transplant recipients as described above.

2.1.3 Longitudinal analysis of low grade proteinuria- Impact on graft outcomes

Proteinuria was calculated for every patient based on their uPCR for years 1, 2 and 3 after transplantation. Any patient with a proteinuria estimate of more than 1g/day was excluded from this analysis as the main goal was to assess the dynamics of low-grade proteinuria. Based on the annual mean uPCR estimates, patients were divided into two groups - Proteinuria absent (A) with a mean uPCR of <150 and low-grade proteinuria (P) with a mean uPCR between 150 and 990. Between years 1 to 2, patients were divided into 4 groups namely; A/A, P/A, A/P, P/P based on the evolution of low-grade proteinuria. Patients were grouped in a similar fashion between years 2 and 3 as well. Like proteinuria, mean systolic blood pressure was recorded for years 1 to 3. In
accordance to the WHO standard and the previously published Collaborative transplant study a cut off value of 140mm of Hg was used to define systolic hypertension (Opelz, Dohler et al. 2005). The primary outcome measure was graft loss up to 10 years which was determined by return to dialysis or death. Change in renal function was estimated by eGFR calculations for the first 5 years of follow-up. Patients who were lost to follow-up and those who were transferred out to a different centre were excluded from the analysis.

2.1.4 Analysis of the significance of early low-grade proteinuria

For this part of the study all consecutive patients transplanted between 1988 and 2003 were included. Patients and grafts that survived less than 3 months were excluded from the analysis. Variables affecting long-term graft and patient outcomes after transplantation were identified for inclusion in multivariate analysis. These included recipient age, sex ethnicity, cause of end-stage renal disease (glomerular disease including diabetic nephropathy, infection/obstruction, and others), donor age, sex and cause of death (cerebrovascular accident, trauma, others), type of donation (cadaveric and living), graft number, total human leukocyte antigen and HLA-DR mismatches, systolic and diastolic blood pressures at 3 months, delayed graft function, biopsy-proven acute rejection, cytomegalovirus disease, diabetes mellitus at the time of transplantation, median serum creatinine and proteinuria measured during the 3rd month after transplantation, and the era of transplantation (1988–1992, 1993–1997, and 1998–2003).

The median of all the urine PCR values obtained in the 3rd post-transplant month was used for this analysis, and patients were divided into four subgroups depending on the degree of proteinuria. Significant proteinuria was defined as a urinary PCR value above 150 (0.15 g/24 hrs equivalent). Patients with PCR less than 150 constituted group 1, whereas group 2 had a PCR between 150 and 500 (0.15 g–0.5 g/24 hrs equivalent),

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group 3 between 500 and 1000 (0.5 g–1.0 g/24 hrs equivalent), and group 4 had a PCR more than 1000 (1.0 g/24 hrs equivalent). Patient demographics were compared across the different proteinuria groups. The primary outcome measures were death-censored graft loss, death with a functioning graft, and vascular events during the lifetime of the graft. A composite vascular endpoint consisted of fatal and nonfatal myocardial infarction, unstable angina, congestive cardiac failure, cardiac arrhythmia, cerebrovascular accident (thrombotic, embolic, and haemorrhagic), transient ischemic attack, limb revascularization, and limb amputation during the lifetime of the graft. Estimated glomerular filtration rate (eGFR) achieved at 5 years after transplantation was a secondary outcome measure. GFR was estimated using the four-variable abbreviated Modification of Diet in Renal Disease formula.

2.1.5 Histological assessment of troubled allografts

For this study, all renal transplant recipients followed up at the renal transplant unit at the St. James’s University Hospital were assessed for proteinuria by uPCR along with the review of their serum creatinine. Patients who presented with proteinuria of more than 500mg (uPCR>500) on three consecutive occasions were offered a renal transplant biopsy, irrespective of their serum creatinine. Additionally patients who presented with a gradual decline of renal function determined by deterioration of their serum creatinine over a period of 2 years were also included. Deteriorating grafts or ‘troubled grafts were defined based on the calculations made in the creeping creatinine study (Dudley, Pohanka et al. 2005). An example of a patient with creeping creatinine compared to the one with stable creatinine is shown below (Figure 20).
Based on the development of persistent proteinuria and creeping creatinine, patients were divided into three groups (creeping creatinine (CC), proteinuria only (IP), creeping creatinine+proteinuria (PC). The biopsies of all the three groups of patients were examined by an independent blinded histopathologist. A serum sample was collected from all the patients for the determination of donor specific antibody within 6 weeks of the biopsy.

2.1.5.1 Serum analysis for HLA-specific antibodies:

For serum separation, venous blood samples were collected in 6ml plain vacutainer tubes with no additive. The samples were left to stand at room temperature for about 30 minutes and centrifuged at 2000 rpm for 10 minutes at room temperature. The serum was then aliquoted and stored at -20˚ C.

Serum samples were thawed at room temperature away from direct sunlight. Lab screen wash buffer was diluted (×10) in distilled water. A 96 well PVDF filter plate was

Figure 20: 1/creatinine plots for representative patients with creeping creatinine or stable creatinine

![Graph showing 1/creatinine plots for representative patients.](image)
used for the assay and the rows of wells not to be used were covered with a plate sealer. The wells were pre-wet by adding 300µl of diluted wash buffer after which the plate was placed on a plate shaker at speed-3 for 10 minutes. The wash buffer was drained on a vacuum manifold. 10µl of serum was added to the appropriate well and this was mixed with 2.5µl of labscreen mixed antigen assay beads. The plate was incubated at room temperature away from light on the plate shaker for 30 minutes. Following the incubation, nonspecific binding was removed by three washes adding 200µl of the diluted wash buffer followed by aspiration on the vacuum manifold. After the final wash, 50µl of diluted (1:100) PE conjugated anti-human IgG was added to each respective well. The plate was again incubated for 30 min as above. After incubation, three further wash steps were carried out and after the final wash step, 80µl of PBS was added to each well and the plate was analysed on the Luminex platform.

Serum reactivity was calculated by the fluorescent signal for each HLA coated bead after correction for non-specific binding to the negative control bead. All data was normalized to the lab screen negative control serum. The strength of the HLA reactivity was assigned by the normalized background ratio which was calculated by using the formula.

\[
\text{Normalized background ratio} = \frac{S\#N / \text{SNC bead}}{BG\#N / \text{BGNC bead}}
\]

\( S\#N = \text{Sample specific fluorescent value for bead #N} \)

\( \text{SNC bead} = \text{Sample specific fluorescent value for negative control bead} \)

\( BG\#N = \text{Background negative control serum fluorescent value for bead #N} \)

\( \text{BGNC bead} = \text{Background negative control serum fluorescent value for negative control bead. (Analysis of the data from this assay was performed by using the fusion software.)} \)

An NBG ratio>1.5 was considered positive for HLA antibody screen.
Samples which were positive in the screening assay were reanalysed using the single antigen Labscreen assay in a similar fashion to the screening assay apart from addition of Class I or Class II HLA beads in the place of the mixed screen beads to the respective serum samples. Allele specificity for HLA reactivity was assessed by the beads which were positive in the single antigen assay. A normalized mean fluorescent value>1000 was considered positive.

Normalized mean fluorescent value= S#N- BG#N

2.1.5.2 Histopathology

All biopsies are examined and scored by PP as per the 2011 Banff classification (Mengel, Sis et al. 2012). Microcirculatory damage which characterizes the histological phenotype of chronic antibody mediated rejection (CAMR) is defined by the presence of glomerulitis, peritubular capillaritis and transplant glomerulopathy with or without linear diffuse deposition of C4d in the peritubular capillaries. Glomerulitis (g score 0-3) lesions describe the percentage of glomeruli with occlusion of endocapillary space by mononuclear cells and endothelial swelling. Peritubular capillaritis score (ptc score 0-3) examines the number of infiltrating monocytes or neutrophils in the most affected capillary and is considered positive with a score of 1 when more than 10% of the capillaries are affected. Transplant glomerulopathy (cg score 0-3) is defined by the presence of double contours on light microscopy; with no or minimal deposition of immune complexes on immunofluorescence in the absence of hepatitis C infection or thrombotic microangiopathy. Electron microscopy was only performed in a selected number of specimens, where the lesions were equivocal on light microscopy. Microcirculatory inflammation defined by g+ptc scores (range 0-6), is also analysed to compute graft survival (de Kort, Willicombe et al. 2013).

Broad histological classes included microcirculatory damage (MCD), glomerulonephritis (GN), T cell mediated rejection (including borderline for TCMR), interstitial fibrosis and
tubular atrophy (IFTA, scarring in the absence of obvious aetiology) and others (BK virus nephropathy, ascending infection, hypertensive arteriosclerosis and amyloidosis).

2.1.6 Statistical Analysis
Statistical analysis was performed by SPSS for Windows version 20 (SPSS, Chicago, IL) and Graph pad version-6. All continuous variables were expressed as mean ± standard deviation unless stated otherwise. All categorical variables were expressed as the percentage of the population. All continuous variables were compared across the groups using either univariate analysis of variance (ANOVA) or student t-test. All categorical variables were compared by the chi-square test. Graft failure was defined as return to dialysis. The Kaplan–Meier method was used to estimate graft survival and patient groups were compared using the Log Rank test. Univariate Cox regression analysis was used to study the impact of variables on the primary outcomes. For every primary outcome, proteinuria was analysed using a multivariate Cox-proportional hazards model, adjusting for the effect of other factors that were significant ($P$<0.05) in the univariate analysis to establish its independent effect. Model fit was analysed by omnibus tests of model coefficients. Results were indicated as the hazard ratio (HR) with 95% confidence interval (95% CI). $P$ value of less than 0.05 was considered significant.

2.2 Human B Regulatory Cells & Troubled Renal Allografts

2.2.1 Study participants
Venous blood samples for PBMCs and serum samples were collected from healthy volunteers (n=18) and renal transplant recipients (n=88). Written informed consent was obtained from all the participants according to the Declaration of Helsinki. Renal
transplant recipients were grouped into those with stable graft function (S, n=40) or graft dysfunction (GD) based on their serum creatinine and proteinuria assessment as described in section 2.1.5. All patients with graft dysfunction had an indication transplant renal biopsy and based on the histology patients were divided in those with a histological phenotype of chronic antibody mediated rejection (GD-R, n=26) and with no evidence of chronic immunological damage (GD-NI, n=22). Chronic immunological microcirculatory injury was defined as the presence of glomerulitis, peritubular capillaritis and transplant glomerulopathy with or without peritubular C4d deposition in the peritubular capillaries in the presence of interstitial fibrosis and tubular atrophy. All the biopsies were scored according to the Banff-2011 criteria. All transplant recipients irrespective of their graft function were tested for the presence of HLA specific antibodies as described in section 2.1.5.1.

2.2.2 Separation of PBMC

Venous blood was collected in two 10ml K2E (18mg of Potassium EDTA) vacutainer tubes. Each blood sample was diluted with an equal volume of RPMI1640 and was layered over ficoll in a 50ml conical bottom tube and centrifuged without brake at 2000 rpm for 20 minutes at room temperature. The opaque layer of mononuclear cells was aspirated from the plasma/ficoll interface and washed twice using 1%PBS-BSA. The number of cells was counted on a Neubauer’s cell counting chamber and for the final wash the re-suspended cell pellet was centrifuged with brake at 1800 rpm for 10min. The final cell pellet was suspended in 1%PBS-BSA to a concentration of $10^7$ cells/ml. The cells were stored on ice between the washing steps.
2.2.3 Determination of absolute numbers of lymphocytes

Two trucount tubes with a specific number of absolute count beads were labelled A and B. 20\(\mu\)l of Multitest CD3/CD8/CD45/CD4 reagent (BD Pharmingen) was added to tube A. Similarly 20\(\mu\)l of the CD3/CD16/CD56/CD45/CD19 reagent (BD Pharmingen) was added to tube B. 50\(\mu\)l of undiluted blood was added to each tube. The tubes were capped, vortexed gently for 30 seconds and incubated for 15 min in the dark at room temperature. 450\(\mu\)l of the red cell lysing solution was added to each tube followed by incubation for 15 min. Samples were analysed on the flow cytometer. Various lymphocyte populations were identified by their staining characteristics. Absolute numbers (cells/\(\mu\)l) of lymphocyte subsets were determined using BD cell quest software by comparing the cellular events to the bead events.

2.2.4 Antibodies, flow cytometry, lymphocyte isolation and cell sorting

All experiments were performed on fresh cells on the same day of blood sampling. The monoclonal antibodies used for cell staining are detailed in Table-3. Once the absolute number of lymphocytes was established by the trucount method as described in the previous section, further analysis of B subsets was performed on whole blood. PBMCs were stained in 200 \(\mu\)L of whole blood at room temperature for 20 minutes using the optimal concentration of appropriate antibodies. Red cells were lysed using BD-FACSlyse. Cells were then washed with ice cold 1% FCS-PBS and fixed in formaldehyde-PBS before the analysis of their immunofluorescence either by BD-FACS Calibur or BD-LSR-Fortessa Flow cytometers. Flow cytometric data was analysed by the Kaluza software after accounting for spectral compensation.
### Table 3: Monoclonal antibodies for cell staining and their manufacturers

<table>
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<tr>
<th>Monoclonal antibody</th>
<th>Clone</th>
<th>Manufacturer</th>
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<tr>
<td>Anti-human CD19-APC H7</td>
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<td>BD Pharmingen</td>
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<td>Anti-human CD24-BV421</td>
<td>M1/69</td>
<td>Biolegend</td>
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<td>JES3/19F1</td>
<td>R &amp; D systems</td>
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#### 2.2.4.1 B and T cell enrichment by Magnetic beads and Cell sorting

Human PBMCs isolated by ficoll-paque density gradient centrifugation as described earlier were used for the enrichment of CD19+ B cells or CD4+CD25- T conv cells. $10^7$
PBMC were suspended in 2mls of ice cold RPMI1640 supplemented with 10% FBS and 1% Penicillin-Streptomycin-L-glutamine (complete RPMI) and centrifuged at 300xg for 10 min. The cell pellet was used for the isolation of B or T cells.

B lymphocytes were isolated manually from PBMCs using CD-19 coated magnetic beads by positive selection (Miltenyi Biotec) as per manufacturer’s instructions. Miltenyi MS-columns were used for positive selection, while LD columns for negative selection/enrichment. Briefly the cell pellet was resuspended in 80μl of complete RPMI along with 20μl of CD19+ beads/10⁷ cells and then incubated at 4° for 15 min. After two more washes at 300g, the cell suspension in 500μl of complete RPMI was applied to a magnetic column placed in the magnetic field. The cells were washed twice through the column with 500μl of complete RPMI. Finally, the column was removed from the magnetic field and the labelled cells were flushed in a sterile collecting tube.

Alternatively untouched B cells were enriched using the B cell isolation kit by negative selection (Miltenyi) according to the manufacturer’s instructions. In this method the cell pellet was resuspended in 40μl of complete RPMI per 10⁷ cells and then 10μl of biotin antibody cocktail added per 10⁷ cells. After 5 min of incubation at 4° a further 30μl of RPMI and 20μl anti-biotin micro beads were added for a further incubation at 4° of 10 min. After incubation, the cells were adjusted to 1ml volume with complete RPMI and the suspension applied to the magnetic as described before. In this method the untouched B cells were collected by washing through the magnetic column in the magnetic field.

Untouched CD4+ CD25lo and CD4+ CD25hi T Cells were also enriched using MACS magnetic bead kits (Miltenyi Biotec). Here, the cell pellet was suspended in 90μl of complete RPMI per 10⁷ cells along with 10μl of CD4+ T cell biotin antibody cocktail and incubated for 5 min at 4°. After a similar step, with anti-biotin micro beads and incubation (as described for the B cells), the cells were made up to a volume of 500μl and applied to a magnetic column in the magnetic field. Untouched CD4+ cells were
collected by washing through the column twice with 500μl of ice cold complete RPMI. Now the CD4 cells were centrifuged at 300g for 10 min. The cell pellet was again resuspended in 90μl of RPMI and 10μl of CD25 micro beads. After 15 min incubation at 4° in darkness, the cells were centrifuged once at 300g for 10 min and the cell pellet finally resuspended in 500μl of complete RPMI. The cell suspension was applied to a magnetic column. CD25- CD4+ T cells were washed through the column in the magnetic field. CD25+CD4+ T cells were flushed in a separate sterile tube once the column was removed from the magnetic field.

CD19+ cells and CD4 cells were centrifuged at 300g for 10 min and the cell pellet resuspended in complete RPMI at appropriate concentrations for cell culture. Pre and post enrichment concentrations and appearances of both CD4 subsets and B cells are shown in Figure 21.

**Figure 21: Enrichment of CD19+ B and CD4+ T subsets by magnetic beads**

CD20 PerCP Cy5.5, CD4-APC & CD25-PE monoclonal antibodies are used for this analysis.
2.2.5 Cell culture- cytokine detection

Magnetic bead enriched B lymphocytes were suspended in complete RPMI at a concentration of 2X10^6 cells/ml and stimulated with CpG-ODN2006 (10µg/ml) (Invivogen) and CD40L (1µg/ml) (Sigma Aldrich). The cells were cultured in 24 well flat bottom cell culture plates (Nunc, Germany) at 37°C and 5% CO₂ for 48 hours. Phorbol 12 myristate 13 acetate (PMA 50ng/ml) (Sigma Aldrich), Ionomycin (1µg/ml) (Sigma Aldrich) and Brefeldin A (1µl/ml) (Sigma Aldrich) were added for the last 5 hours of the cell culture. Cultured cells were washed twice in ice cold 1% PBS-BSA at 300×g. The cells were incubated in 10% human normal serum at 4°C for 15 min and washed once in ice cold 1% PBS-BSA before proceeding to surface staining. Appropriate amounts of monoclonal CD20-PerCP-Cy5.5, CD24-FITC, CD38-APC, TIM-1-ALexa488 and CD27-APC antibodies were added to the resuspended cells according to manufacturer’s instructions and incubated in darkness at 4°C for another 15 min. Stained cells were fixed and permeabilized using the Cytofix/Cytoperm kit (BD Pharmingen) as per manufacturer’s instructions. Cells were then stained for intracellular cytokines with IL-10-PE, IFN-γ-PE, IL17-PE, TNF-α-PerCP-Cy5.5 and TNF-α-PE for 30min in darkness at 4°C. Samples were acquired on BD-FACS Calibur flow cytometer and the data analysed by Kaluza software (Beckman Coulter). Alternatively both IL-10 and TNF-α were analysed from the cell culture supernatants of the FACS sorted B lymphocyte subsets by bead based multiplex-ELISA (Invitrogen) as described below.

2.2.6 Bead based ELISA

Untouched B lymphocytes were counted and suspended at 50×10^6 cells in 250ml of 1%PBS-BSA. Cells were then stained by CD20-PerCP-Cy5.5, CD24-PE, CD38-APC and CD27-FITC for 15 min in darkness at 4°C. The stained cells were sorted by Moflo (Beckman Coulter). Transitional B cells were selected as CD20+CD24hiCD38hiCD27- (90% purity), memory B cells were selected as CD20+CD24hiCD27+CD38- (>95%
purity) and naïve B cells as CD20+CD24+CD38+CD27- (>95% purity). Flow sorted B cell subsets were cultured as described in section 2.2.6, but in 96 well round bottom tissue culture plates (Nunc) for 48hrs. At the end of 48hrs, the culture plates were centrifuged at 500×g for 2 min and the supernatants were collected and stored for ELISA.

Bead based ELISA was performed according to the manufacturer’s instructions (Invitrogen). Briefly, 25μl of vortexed microbeads were added to the respective wells of the ELISA plate provided with the kit. The cells were washed twice with 200μl of the wash solution with 30sec in between the washes. Then 50μl of incubation buffer was added to the respective wells. 100μl of the diluent for the blank well or the appropriate standards were added to the designated wells. 50μl of the assay diluent along with 50μl of the samples were added to the wells designated for the patient samples. The plate was then covered in tin foil and incubated at room temperature for 2hrs on an orbital plate shaker (500rpm). The plate was washed twice with ice cold wash solution on a vacuum manifold. 100μl of biotinylated antibody was added to the respective wells and the plate incubated on the plate shaker again for 1hr. Again, the plate was washed twice and 100μl of streptavidin-RPE solution was added to each well and the plate incubated for 30min on the plate shaker. The plate was finally washed thrice and then the beads were resuspended in 150μl of the wash buffer. The beads from the respective wells were acquired on the Luminex platform and the concentration of the cytokines analysed from the standard curve constructed as shown in Figure 22.
Figure 22: Bead based ELISA, standard curves for IL-10 and TNF-α
2.2.7 B functional assays

A sterile round bottom cell culture plate was coated overnight at 4°C with 0.5mg/ml of monoclonal anti-human CD3 (HIT3A-BD Pharmingen). On the day of the experiment, the plate was washed twice with RPMI and then 1×10^5 magnetic bead enriched CD4^+CD25^lo T Cells in 100ml of complete RPMI were added to the respective wells. Either 100ml of complete RPMI or magnetic bead enriched CD4^+CD25^+ T cells or the flow sorted B cell subsets (transitional, memory and naïve B cells) were added (1:1) to the representative wells. The culture plate was incubated for 72hrs at 37°C in 5% CO2. For the last 6hrs of cell culture 1ml/ml of Brefeldin A along with PMA and ionomycin were added. Dead cells were examined by Trypan Blue stain and typically there was >85% cell viability as all the experiments were conducted on fresh blood samples within a few hours of phlebotomy. The cells were then surface stained with CD4-APC and CD20-PerCp-Cy5.5 followed by fixation and permeabilization (BD Perm Fix Kit). Cells were further stained for the detection of intracellular cytokines using IFN-γ-AlexaFluor488, IL-10-PE, TNF-α-AlexaFluor488 and IL-4-PE. The cells were acquired on the BD-FACSCalibur flow cytometer and data analysed by Kaluza software. For the purpose of the analysis of CD4 related cytokines, both CD4 and CD19+ B subsets were distinguished after cell culture utilising the gating strategy shown in Figure 23.
Lymphocytes were identified by forward and side scatter properties. CD4 and CD19 cells were gated as shown above.

Figure 23: B functional assays-T and B gating strategy
2.2.8 Neutralization experiments

To understand the functional significance of either IL-10 or TNF-α, the co-cultures of CD4+CD25lo T conv cells and the B subsets were repeated in 3 healthy volunteers wherein the T and B subsets were cultured alone as discussed in section 2.2.7 or with an addition of 10μg/ml of neutralizing IL-10 antibody or TNFR1/R2 antibodies or appropriate isotype (IgG2B). Cells were cultured for a total of 72hrs as mentioned previously with the addition of PIB for the last 6hrs of cell culture and the cells were washed stained and acquired on the BD FACSCalibur flow cytometer for the analysis of TNF-α+ or IFN-γ+ CD4+ T cells.

2.2.9 Statistical analysis

Statistical analysis was performed by SPSS version 17 and Graph pad Prism version 5 for Windows. Categorical variables were compared using Fisher’s exact test and continuous variables using Mann-Whitney U test for data with skewed distribution and independent sample student t-test for normally distributed data. A p-value of <0.05 was considered significant. One way ANOVA with either Tukey or Dunnet post hoc correction was used for multiple comparisons. Graft outcomes and survival were analysed by the Kaplan Meier method and groups compared by the Log Rank test. A P value of <0.05 was considered significant.

2.3 B lymphocyte phenotype & alemtuzumab in renal transplantation

The evolution of B cell phenotype was studied in the context of a randomized controlled trial and then, in a small subgroup of 14 patients, the maturation of B cells after
peripheral depletion was studied longitudinally at 3, 6 and 12 months after transplantation.

2.3.1 Trial design

Patient blood samples were collected as a part of a single-centre, prospective, open-label, randomized controlled trial (EudraCT No. 2006-000830-11, International Standard Randomised Controlled Trial Number ISRCTN94424606) The study was conducted in accordance with the principles of the International Conference on Harmonisation Harmonized Tripartite Guidelines for Good Clinical Practice and the Declaration of Helsinki. Written informed consent was obtained from all patients after receipt of approval from the Research Ethics Committee. All adult recipients of renal transplants were considered eligible: donation after brain death (DBDs), donation after circulatory death (DCDs), and living donors. Patients considered to be at high immunologic risk were excluded (high immunologic risk was defined as two HLA-DR mismatch, preoperative positive donor-specific anti-HLA antibodies, or previous immunologically mediated graft loss after less than 6 months). Patients with pre-existing leukopenia or thrombocytopenia were excluded, and female patients were required to have a negative pregnancy test and were advised on appropriate contraceptive use. Random numbers were generated using http://www.randomizer.org/ in 12 blocks of 10, each block of 10 assigned alternately to the alemtuzumab or basiliximab arms. An independent third-party (who had nothing to do with the study) then used the random numbers to generate numbered envelopes, into which the appropriate treatment arm for that number was sealed. After randomization into the study, these envelopes were opened in the predetermined sequential order for each patient, thus assigning them to the alemtuzumab or basiliximab arms as per the contents of the sealed envelope. Once the envelope was opened, the investigators and
patient knew which treatment they were to receive, because this was an open-label trial.

2.3.2 Immunosuppression

Patients were randomized on a 1:1 basis to receive either (a) basiliximab induction (20 mg intravenously iv on days 0 and 4) and a single dose of 1 g iv methylprednisolone intraoperatively followed by MMF at a starting dose of 750 mg orally, twice daily, with tacrolimus given orally to achieve trough levels of 9 to 14 ng/mL in months 0 to 3 and of 4 to 9 ng/mL thereafter or (b) alemtuzumab induction (30 mg I.V intraoperatively) and a single dose of 1 g I.V methylprednisolone, followed by tacrolimus orally to the same target levels as the basiliximab group, but no MMF. Steroids were subsequently avoided entirely unless rejection occurred or another clinical circumstance mandated their use. Alterations in mycophenolate dose were left to the discretion of treating clinicians, as was any decision to switch to mycophenolate sodium.

2.3.3 Anti-viral and antibiotic prophylaxis

In the basiliximab group, high-risk patients (D+R-) received 100 days of CMV prophylaxis with valganciclovir (dose adjusted according to renal function). In the alemtuzumab group, all patients, except low-risk patients (D-R-), received 100 days of prophylaxis. Cotrimoxazole was given for 6 months and topical oral antifungal therapy was administered for 3 months to all patients.

2.3.4 Clinical end points

The primary endpoint of the trial was non-inferiority of isotopic GFR at 1 year measured by technetium-99m diethylenetriamine penta-acetic acid clearance using the two-blood sample method. The recruitment target was set at 120 patients based on a non-
inferiority limit of 10 mL/min of isotopic GFR, with power level of 0.80 and > of 0.05, assuming a dropout rate of 15%. It was predetermined that patients who returned to dialysis would be included in the calculations using an assumed GFR of 5 mL/min. Secondary endpoints included patient and graft survival, rates of DGF, severity and rates of rejection, time to rejection, 24-hr blood pressures at 6 and 12 months, rates of cardiovascular, malignant and infective complications, percentage of patients steroid-free at 12 months, and rates of NODAT.

2.3.5 Patient Samples

Venous blood samples for serum and PBMCs were collected from consented renal transplant recipients participating in the trial. Of the 116 patients randomized for the trial, samples were obtained from 96 patients at a mean of 25 +/-2 months after transplantation. The outline of the RCT along with the primary outcome measures and sampling for DSA and B cell phenotype is summarized in Figure 24. Serum samples were analysed for the presence of DSA as described in section 2.1.5.1. PBMCs were analysed for B subsets by cell surface staining using CD20-PerCP, CD24-FITC, CD38-APC and CD27-PE as described previously.
2.3.6 Statistical analysis

Statistical analysis was performed in Graph Pad Prism version 5.04 and SPSS version 17 for Windows. Continuous variables were analysed using t tests if normally distributed. Mann Whitney tests were used if not normally distributed and were also used for nonparametric data. Categorical variables were analysed using chi-square testing, unless expected frequencies were less than five in any group when Fisher’s exact test was used. Results were analysed on a modified intention to treat basis (modified refers to the exclusion of four patients after randomization).

2.3.7 Longitudinal analysis of B subsets

A random sample of 14 patients, all of whom received alemtuzumab at induction along with 1g of intravenous methylprednisolone and who were maintained on tacrolimus monotherapy, were consented for this part of the analysis. Venous blood samples were collected at 3, 6 and 12 months after transplantation and B subsets analysed by
CD20-PerCP-Cy5.5, CD24-PE, CD38-APC, CD27-FITC and IgD-PE at these time points by cell staining as described previously. Given the small patient set, no clinical correlates were included in the analysis.

2.4 Ethical approval

Both clinical and laboratory investigations were performed after approval from the regional ethics committees. York Regional Ethics Committee (Ref No: 8/H1311/41) granted the approval for the investigations relating to the identification of the allograft at risk and defining Bregs. The Leeds East Regional Ethics Committee (Ref No: 06/Q1206/64) granted the approval for the RCT.
3.1 Introduction

Renal transplantation offers a significant survival benefit over other forms of treatment for end stage renal disease and thereby represents the best form of renal replacement therapy available at present (Wolfe, Ashby et al. 1999). However renal transplants do not last indefinitely and the late failure of allografts remains a significant problem despite improvements in early outcomes (Lamb, Lodhi et al. 2011). Renal transplant recipients have a high early and late mortality and this is a major cause of allograft loss (Gill, Rose et al. 2007, Cosio, Hickson et al. 2008). Although the survival of the renal transplant patients is superior to patients with ESRD on dialysis there is still significant room for improvement when compared to matched controls in the general population (Foley, Parfrey et al. 1998, Wolfe, Ashby et al. 1999). Unfortunately medical management of some of the important causes of allograft loss including, death from cardiovascular disease, recurrent renal disease in the allograft and chronic antibody mediated rejection (CAMR) has not yielded improved outcomes (Briganti, Russ et al. 2002, Matas 2006, Cosio, Hickson et al. 2008, Sellares, de Freitas et al. 2012). Moreover, the debate that besieges the set diagnostic criteria for CAMR is still sizeable (Einecke, Sis et al. 2009, Mengel, Reeve et al. 2009, Reeve, Einecke et al. 2009, Mengel, Sis et al. 2012, Sis, Jhangri et al. 2012).

Several authors have stressed the importance of various clinical variables that can be utilized to identify ‘grafts at risk’ at an earlier stage after transplantation. The main argument in favour of such a strategy is the principle of ‘early risk modification’ for the ‘at risk groups’. The commonest variable that gets measured routinely for every renal transplant recipient is serum creatinine (Hariharan, McBride et al. 2002). But once elevated it signifies advanced allograft damage and often insidious irreversible nephron
loss, thereby limiting its clinical utility (Chapman, O'Connell et al. 2005). Studies have shown that proteinuria, even at low levels and as early as three months after transplantation predicts adverse allograft survival (First, Vaidya et al. 1984, Fernandez-Fresnedo, Plaza et al. 2004, Halimi, Laouad et al. 2005, Amer, Fidler et al. 2007).

In a detailed histological analysis of patients with post-transplant proteinuria of >2g/day, First et al. have shown that the cause for proteinuria in these patients is quite heterogeneous with diagnoses varying from transplant glomerulopathy to renal vein thrombosis, but the commonest cause even at this level of relatively high-grade proteinuria was transplant glomerulopathy (First, Vaidya et al. 1984). The heterogeneous nature of aetiology for post-transplant proteinuria has been confirmed by Amer et al. by examining the histology of proteinuria at the first post-transplant year (Amer, Fidler et al. 2007). Various risk factors such as hypertension, reduced renal function, and increased fasting glucose are known to be associated with proteinuria (Fernandez-Fresnedo, Plaza et al. 2004). Moreover hypertension is a modifiable determinant of renal function and improvement of renal function and mortality with hypertensive agents is recognised (Walker, Neaton et al. 1992, Peterson, Adler et al. 1995). A report from the Collaborative transplant study demonstrated an association between better patient and graft outcomes and improvement in blood pressure control (Opelz, Dohler et al. 2005).

Based on current literature it is tempting to speculate that assessment of proteinuria after transplantation provides an important opportunity to intervene in patients at risk of premature allograft loss. Moreover it is well established that proteinuria is a marker of kidney damage and adverse cardiovascular outcomes in general population (Culleton, Larson et al. 2000, Jafar, Stark et al. 2001, Chronic Kidney Disease Prognosis, Matsushita et al. 2010, Hemmelgarn, Manns et al. 2010). Furthermore albuminuria has been shown to be associated with increased mortality independent of glomerular filtration rate (Chronic Kidney Disease Prognosis, Matsushita et al. 2010). There is some evidence that proteinuria is a modifiable risk factor and that reduction of
proteinuria may be a worthwhile therapeutic intervention in patients with chronic kidney disease (Bakris 2008).

However there remains an important caveat. Patients with proteinuria are also likely to have abnormal or progressively deteriorating creatinine which represents irreversible nephron loss and intervening in these patients may not necessarily yield fruitful clinical results. Although studies have examined the causes for proteinuria histologically, it is vital to stratify these patients by their renal function as well, if proteinuria were to be identified as potential marker for ‘grafts at risk’, and to plan therapeutic intervention. A more detailed understanding of the interactions between proteinuria, blood pressure and allograft outcomes would permit risk stratification of renal transplant recipients and direct appropriate therapeutic interventions specifically towards these ‘at risk’ groups. It should be realized that proteinuria is a dynamic clinical entity that changes with time. Even though early low grade proteinuria as early as 3 months after transplantation has been shown to be associated with poor graft outcomes, the impact of changes in proteinuria especially in relation to blood pressure control remains unknown.

At St. James’s University Hospital Renal Unit (SJUH), we have established urine protein/creatinine ratio (uPCR) as a method of assessment for proteinuria and, every patient who attends the outpatient department is routinely tested for proteinuria at every clinic visit (Dyson, Will et al. 1992). The unit therefore has been able to establish a comprehensive database of patients with proteinuria. In this chapter, the analysis of the impact of changes in proteinuria when assessed longitudinally on overall graft loss will be presented. In addition, from a subset of patients transplanted between 1988 and 2003, all of whom are maintained on a similar immunosuppression regime consisting of ciclosporin, azathioprine and prednisolone, results that examine the impact of proteinuria very early after transplantation on multiple clinical outcomes will be analysed. Finally, with a cross sectional study, histological features of all renal transplant recipients who had persistent proteinuria of more than 500mg/day (uPCR>50) or deteriorating creatinine will be correlated to graft loss.
3.2 Results

3.2.1 Longitudinal analysis of low-grade proteinuria-impact on graft outcomes

In this section, results describing the clinical significance of low-grade proteinuria and its evolution after renal transplantation will be presented. As described in the methods section, patients are divided into groups with and without proteinuria and the groups are analysed over the first three years after transplantation for their impact on 10 year overall graft survival.

3.2.1.1 Study population

A total of 805 patients who have undergone renal transplantation at SJUH (mean follow-up of 113 months, SD=14.2) with a functioning graft at 1 year had their urine protein excretion (uPCR) recorded. The demographic characteristics of the study population divided into groups with and without proteinuria are described in Table-4. Patients are dominantly young Caucasians with a male preponderance. The majority of the grafts originated from deceased donors and there are no pre-emptive transplants. Patients in the low-grade proteinuria group had modestly higher systolic and diastolic blood pressures; higher donor age; more antihypertensive medications in use and a lower proportion of live transplants. It is of interest to note that there was no difference in the proportion of grafts performed across various immunosuppression eras, the distribution of the primary renal diagnosis or the serum creatinine achieved at 1 year between the two groups. No patients were on sirolimus.
Table 4: Demographics

<table>
<thead>
<tr>
<th>Variable</th>
<th>Proteinuria absent (A)*</th>
<th>Low grade proteinuria (P)*</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (n)</td>
<td>139 (17.3%)</td>
<td>666 (82.7%)</td>
<td></td>
</tr>
<tr>
<td>uPC at 1 year</td>
<td>114 (25)</td>
<td>353 (171)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>uPC at 2 years</td>
<td>94 (31)</td>
<td>292 (152)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>uPC at 3 years</td>
<td>91 (30)</td>
<td>318 (171)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SBP at 1 year</td>
<td>135 (13)</td>
<td>139 (14)</td>
<td>0.002</td>
</tr>
<tr>
<td>SBP at 2 years</td>
<td>132 (16)</td>
<td>137 (15)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SBP at 3 years</td>
<td>131 (16)</td>
<td>137 (17)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DBP at 1 year</td>
<td>80 (7)</td>
<td>82 (8)</td>
<td>0.07</td>
</tr>
<tr>
<td>DBP at 2 years</td>
<td>78 (9)</td>
<td>81 (8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DBP at 3 years</td>
<td>78 (9)</td>
<td>80 (10)</td>
<td>0.01</td>
</tr>
<tr>
<td>Age</td>
<td>38 (15)</td>
<td>41 (16)</td>
<td>0.04</td>
</tr>
<tr>
<td>Gender-male%</td>
<td>65%</td>
<td>63%</td>
<td>Ns</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian%</td>
<td>83%</td>
<td>88%</td>
<td></td>
</tr>
<tr>
<td>South Asian%</td>
<td>13%</td>
<td>11%</td>
<td></td>
</tr>
<tr>
<td>Other%</td>
<td>4%</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td>Era of transplant</td>
<td></td>
<td></td>
<td>Ns</td>
</tr>
<tr>
<td>1988-1998</td>
<td>44%</td>
<td>44%</td>
<td></td>
</tr>
<tr>
<td>1998-2004</td>
<td>29%</td>
<td>29%</td>
<td></td>
</tr>
<tr>
<td>2004-2008</td>
<td>27%</td>
<td>27%</td>
<td></td>
</tr>
<tr>
<td>Graft number</td>
<td>1.1 (0.3)</td>
<td>1.1 (0.4)</td>
<td>Ns</td>
</tr>
<tr>
<td>Type of transplant</td>
<td></td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>DBD</td>
<td>67%</td>
<td>82%</td>
<td></td>
</tr>
<tr>
<td>DCD</td>
<td>14%</td>
<td>6%</td>
<td></td>
</tr>
<tr>
<td>Live</td>
<td>19%</td>
<td>12%</td>
<td></td>
</tr>
<tr>
<td>HLA mismatches</td>
<td></td>
<td></td>
<td>Ns</td>
</tr>
<tr>
<td>0-2</td>
<td>53%</td>
<td>61%</td>
<td></td>
</tr>
<tr>
<td>2-4</td>
<td>42%</td>
<td>36%</td>
<td></td>
</tr>
<tr>
<td>4-6</td>
<td>5%</td>
<td>3%</td>
<td></td>
</tr>
<tr>
<td>Primary renal diagnosis</td>
<td></td>
<td></td>
<td>0.07</td>
</tr>
<tr>
<td>Diabetes/hypertension</td>
<td>11%</td>
<td>10%</td>
<td></td>
</tr>
<tr>
<td>GN</td>
<td>25%</td>
<td>17%</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>64%</td>
<td>73%</td>
<td></td>
</tr>
<tr>
<td>Donor age</td>
<td>35 (16)</td>
<td>39 (16)</td>
<td>0.009</td>
</tr>
<tr>
<td>Initial immunosuppression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ciclosporin</td>
<td>73%</td>
<td>73%</td>
<td>Ns</td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>27%</td>
<td>27%</td>
<td>Ns</td>
</tr>
<tr>
<td>Mycophenolate</td>
<td>27.3%</td>
<td>26.9%</td>
<td>Ns</td>
</tr>
<tr>
<td>Azathioprine</td>
<td>75%</td>
<td>74%</td>
<td>Ns</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>9%</td>
<td>13%</td>
<td>0.1</td>
</tr>
<tr>
<td>Early rejection</td>
<td>9%</td>
<td>13%</td>
<td>0.1</td>
</tr>
<tr>
<td>Creatinine at 1 year</td>
<td>149.4 (61)</td>
<td>148.7 (57)</td>
<td>Ns</td>
</tr>
<tr>
<td>BP medications</td>
<td>2 (0.9)</td>
<td>2.4 (1.2)</td>
<td>0.04</td>
</tr>
</tbody>
</table>

*Standard deviation is shown for continuous variables whilst percentage for categorical variables in the parenthesis. Continuous variables are compared by student t-test and categorical variables by Chi-square test.
3.2.1.2 Low-grade proteinuria 1-3 years after transplantation & graft survival

82.7% of patients in this study had low-grade proteinuria (mean uPCR 353 ± 171) at 1 year after transplantation. By the second year, there was a net reduction in this percentage to 49.7% (mean uPCR 292 ± 152) and further to 42.1% (mean uPCR 318 ± 171) by the third post-transplant year. As expected these changes were multidirectional with patients moving from the low-grade proteinuria group (P) to the proteinuria-absent group (A) and vice versa. Thus whilst 56.3% of patients in Group-P had persistent proteinuria, 43.7% improved to Group-A by 2 years. Likewise, 17.6% of the patients in group A went on to develop low-grade proteinuria (P) by the second year. In a similar fashion between years 2 and 3, I have noticed similar dynamics with 68.2% of patients in Group-P having persistent proteinuria and 14.9% of the patients in Group-A developing low-grade proteinuria (P). DBD donor type, serum creatinine and non-glomerular aetiology for ESRD were shown to be associated with the development of low-grade proteinuria at 1 year in a multivariate logistic regression analysis (Table 5).
Table 5: risk factors for low-grade proteinuria

<table>
<thead>
<tr>
<th>Variable</th>
<th>RR</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recipient age (&gt;60yrs vs. &lt;60yrs)</td>
<td>0.97</td>
<td>0.4-2.3</td>
<td>Ns</td>
</tr>
<tr>
<td>Donor age</td>
<td>1.01</td>
<td>0.99-1.03</td>
<td>0.1</td>
</tr>
<tr>
<td>Recipient gender (male vs. female)</td>
<td>0.8</td>
<td>0.45-1.2</td>
<td>Ns</td>
</tr>
<tr>
<td><strong>Graft type</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Live</td>
<td>Ref</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>DBD</td>
<td>2.1</td>
<td>1.1-4.1</td>
<td>0.02</td>
</tr>
<tr>
<td>DCD</td>
<td>1.02</td>
<td>0.4-2.8</td>
<td>Ns</td>
</tr>
<tr>
<td><strong>Graft number</strong></td>
<td>1.26</td>
<td>0.7-2.4</td>
<td>Ns</td>
</tr>
<tr>
<td><strong>HLA mismatches</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-2</td>
<td>Ref</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>3-4</td>
<td>0.9</td>
<td>0.6-1.4</td>
<td>Ns</td>
</tr>
<tr>
<td>5-6</td>
<td>0.4</td>
<td>0.1-1.5</td>
<td>Ns</td>
</tr>
<tr>
<td><strong>Cause of ESRD: Non-glomerular vs. glomerular disease</strong></td>
<td>2.5</td>
<td>1.5-4.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Era of transplantation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1988-1998</td>
<td>Ref</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>1998-2004</td>
<td>1.01</td>
<td>0.57-1.8</td>
<td>Ns</td>
</tr>
<tr>
<td>2004-2008</td>
<td>1.7</td>
<td>0.85-3.4</td>
<td>Ns</td>
</tr>
<tr>
<td><strong>Early rejection</strong></td>
<td>1.3</td>
<td>0.6-2.7</td>
<td>Ns</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>1.01</td>
<td>0.99-1.03</td>
<td>Ns</td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>1.01</td>
<td>0.97-1.05</td>
<td>Ns</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>1.6</td>
<td>0.9-3.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Serum creatinine</td>
<td>1.01</td>
<td>1.0-1.02</td>
<td>0.04</td>
</tr>
</tbody>
</table>

RR relative risk, 95% CI 95% confidence interval for RR, DBD Donation after brain death, DCD donation after circulatory death

Next, impact of low-grade proteinuria, assessed at the three annual time points, on long-term graft survival was analysed. The Kaplan Meier survival curves in Figure 25A show that patients in the low-grade proteinuria group (P) had significantly worse graft survival when assessed at any of the three different time points (1 year 87.1% vs. 77.6%, P=0.01; 2 years 86.9% vs. 74%, P<0.001; 3 years 86.2% vs. 72.3%, P<0.001). The graphic from Figure 25B shows that low-grade proteinuria associates with adverse graft survival irrespective of blood pressure, presence of diabetes or eGFR. However,
the results did not achieve statistical significance in patients with systolic BP ≤ 140 mm Hg and eGFR > 60. It is notable that low-grade proteinuria was selectively associated with worse graft survival in younger transplant recipients (age < 60 yrs) and that a significant effect is noted in all the three eras of our immunosuppression protocols (1988-98, 1998-04, 2004-08). Proteinuria was also significantly associated with worse graft survival in patients with both proteinuric glomerular disease and non-glomerular disease as the cause of their ESRD. When renal function was analysed, patients with low-grade proteinuria at 3 years had significantly low mean eGFR at 5 years when compared to their non-proteinuric counterparts (53.9 ± 18.7 ml/min vs. 45.5 ± 23.4 ml/min, P < 0.001, independent samples t-test)

As noted earlier, proteinuria is dynamic and changes with time. Therefore the impact of modification of proteinuria over time on graft survival was next analysed. Figure 25C shows that between years 1 to 2 (A/A 91.1% vs. P/A 86.1% vs. A/P 79.2% vs. P/P 73%, P < 0.001) and 2 to 3 (A/A 87.9% vs. P/A 81.8% vs. A/P 80% vs. P/P 70.1%, P < 0.001) patients who had persistent proteinuria (Group-P/P) had the worst graft survival while those with no proteinuria throughout the first three years (Group-A/A) had the best graft survival. It is important to note that patients who showed resolution of proteinuria (Group-P/A) demonstrated significant improvements in overall graft survival.
Figure 25: Low-grade proteinuria and overall graft survival

A. Kaplan Meier survival curves demonstrate that overall graft survival for the three annual time points is significantly superior in patients with no proteinuria. B. This is a graphic representing the hazard ratio for the overall graft loss in patients with low-grade proteinuria stratified into various subgroups based on relevant clinical parameters as shown in the figure. The hazard ratios are obtained by the Univariate Cox proportional hazards model. C. Patients are divided into groups based on the progression of proteinuria between years 1-2 and 2-3 and graft survival assessed by the Kaplan Meier method. These graphs show that patients who have persistent proteinuria (P-P) through years 1-2 and 2-3 have the worst graft survival and that there is an improvement in survival for patients where proteinuria resolved (P-A).
The independent significance of proteinuria was next assessed by the multivariate Cox proportional hazard model. Potential confounders including recipient and donor age, cause of ESRD, diabetes mellitus, eGFR, early rejection after transplantation, era of transplantation and systolic blood pressure were included in the model. Table-6 summarizes the independent effect of modification of proteinuria during the time periods, 1 to 2 and 2 to 3. From the table it is apparent that there was a step wise increase in the risk of overall graft failure with the highest risk of graft failure in the Group-P/P. Again it is important to note that even after adjusting for potential confounders, resolution of low-grade proteinuria (Group-P/A) was associated with improved outcomes.

### Table 6: Multivariate analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard ratio</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proteinuria longitudinal</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Years 1-2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/A</td>
<td>Ref</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>P/A</td>
<td>1.2</td>
<td>0.6-2.5</td>
<td>Ns</td>
</tr>
<tr>
<td>A/P</td>
<td>1.4</td>
<td>0.4-5.1</td>
<td>Ns</td>
</tr>
<tr>
<td>P/P</td>
<td>2.1</td>
<td>1.1-4.1</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Years 2-3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/A</td>
<td>Ref</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>P/A</td>
<td>1.3</td>
<td>0.7-2.3</td>
<td>Ns</td>
</tr>
<tr>
<td>A/P</td>
<td>1.5</td>
<td>0.8-3.1</td>
<td>Ns</td>
</tr>
<tr>
<td>P/P</td>
<td>2.0</td>
<td>1.3-3.2</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Analysis was by Cox proportional hazards model. Variables including systolic blood pressure, eGFR, early rejection, recipient and donor age, era of transplantation, diabetes mellitus and cause of ESRD were adjusted for in the multivariate analysis.

### 3.2.1.3 The impact of proteinuria and systolic hypertension on graft survival

The assessment of proteinuria remains incomplete without consideration of blood pressure. So, the association between systolic hypertension and graft survival was examined. The Kaplan Meier survival curves in Figure 26A show that; at the three
annual time-points, patients with a mean systolic blood pressure (SBP) ≥ 140 mmHg (year-1 83.2% vs. 75.9%, P=0.03; year-2 84.8% vs. 73.7%, P=0.003; year-3 85.7% vs. 70.3%, P<0.001) had significantly worse graft survival when compared to those with SBP<140 mmHg. It is well documented that tighter blood pressure control is associated with improvement in proteinuria. Next, the impact of this interaction on long-term outcomes was tested. As shown in Figure 26B, patients were divided into four groups based on both blood pressure and the presence of low-grade proteinuria. In year 1, 2 and 3 patients with a systolic blood pressure ≥140mm Hg and proteinuria (H/P) had the worst graft survival when compared to all the other groups (year-1 N/A 85.7% vs. H/A 87.5% vs. N/P 82.6% vs. H/P 74.3%, P=0.03; year-2 N/A 87.3% vs. H/A 83.5% vs. N/P 82.2% vs. H/P 66.9%, P<0.001; year-3 N/A 87.9% vs. H/A 81% vs. N/P 80.3% vs. H/P 60.2%, P<0.001). It is important to note that at the same time points, patients with a systolic blood pressure ≥ 140mmHg in the absence of proteinuria (H/A) had remarkably better graft survival when compared to their hypertensive counterparts with proteinuria. Figure 27 shows that those patients who were persistently hypertensive (H/H) between year-1 and year-3 (H/H 86.2% vs. H/N 86.1% vs. N/H 79.7% vs. H/H 66.4%, P<0.001) had the worst graft survival and this finding corroborates with the findings of Opelz et al in the Collaborative Transplant Study (Opelz, Dohler et al. 2005). I have then performed a sub-group analysis in patients with SBP above and below 140 mm Hg between years 1 and 3. Figure 27 shows that proteinuric patients in the group with SBP>140mm Hg had a significantly worse graft survival (82.6% vs. 53.6 %, P<0.001). The trend was not as marked in the proteinuric patients who were normotensive (87.6% vs. 79.7%, P=0.1).
Figure 26: Systolic hypertension and overall graft survival

A. Kaplan Meier survival curves comparing overall graft survival for the three annual time points in various systolic blood pressure groups. B. Patients were divided into groups based on their systolic blood pressure and the presence of proteinuria, these Kaplan Meier estimates analyse overall graft survival across the various SBP-proteinuria groups.
A. Patients were divided into groups based on their systolic blood pressure (>/>= 140 mm Hg) at years 1 and 3. The Kaplan Meier survival curves show that patients with SBP>140 mm Hg at both the annual check points had the worst graft survival and interestingly, patients whose blood pressure improved at year-3 (H/N) had remarkably better graft survival. B. In a subgroup analysis of patients who were either persistently hypertensive or normotensive, the presence of low-grade proteinuria has been shown to be significantly associated with worse graft survival in the hypertensive subgroup in this set of Kaplan Meier survival curves.

In summary, persistent low-grade proteinuria was associated with adverse graft survival after renal transplantation, which improved as proteinuria resolved, facilitating the stratification of hypertensive renal transplant recipients.
3.2.1.4 Causes of graft loss

Finally, the causes of graft loss in the study population were analysed. Overall, 20.7% (N=167) of grafts were lost during the study period. In this group of patients, the dominant cause of the graft loss was death with a functioning graft (67.1%, n=112/167). Cardiovascular events (including myocardial infarction, cerebrovascular accident and sudden cardiac death) followed by infection and malignancy were the three most common causes of death. The most common cause of functional graft loss was rejection. This information was obtained from registry returns and represented a broad category including refractory acute rejection, chronic transplant glomerulopathy and a few slowly failing grafts that were not routinely biopsied. No data was available for the histology of graft losses.

3.2.2 Clinical significance of early low-grade proteinuria

Now, results that describe the impact of early proteinuria on multiple graft outcomes shall be analysed in a subset of 477 patients, all of whom were maintained on ciclosporin, azathioprine and prednisolone. As described in the methods section, patients in this series were divided into four groups based on the degree of proteinuria, measured by uPCR (Group-1, uPCR<150; Group-2, uPCR 150-500; Group-3, uPCR, 500-1000 and Group-4, uPCR>1000) and they were followed up for a mean of 10 years (+/- SD). Clinical outcomes including patient survival, death censored graft survival and cardiovascular event free graft survival were analysed in the groups of patients with and without proteinuria. Cardiovascular event was defined as a composite of fatal or non-fatal myocardial infarction, sudden cardiac death, congestive cardiac failure, cardiac arrhythmia, stroke, transient ischaemic attack and peripheral limb revascularization or amputation.
3.2.2.1 Patient characteristics-proteinuria groups

A total of 477 patients undergoing renal transplantation at SJUH between 1988 and 2003 were included in the analysis with a mean follow-up of 122 months (SD=34.8). All 477 patients had proteinuria quantitatively assessed at each clinic visit during the 3rd month after transplantation (minimum of three readings per patient), and the median of these readings was used to divide the patients into groups stratified by increasing protein excretion. The demographic characteristics of the study population divided into groups are illustrated in Table 7. The patients were relatively young compared with our contemporary practice (mean age 43±13 years) and predominantly white (89%) with a male preponderance (64%). The majority of grafts originated from deceased donors (89%) and the most common cause of death in these donors was cerebrovascular disease (44%). The rates of delayed graft function (24.5%) and acute rejection (24.3%) were fairly typical for all-comers during this era.

Further analysis of the demographics across the different groups demonstrated that recipient age (group 1: 39±13, group 2: 42±15, group 3: 46±13, group 4: 46±16 years), the prevalence of diabetes mellitus (group 1: 3.5%, group 2: 9.4%, group 3: 19%, group 4: 20%), and systolic blood pressure at 3 months (group 1: 137±18, group 2: 140±22, group 3: 142±17, group 4: 146±18 mm Hg) correlated with increasing proteinuria. Interestingly, glomerular disease as a primary diagnosis was more common in the lower protein excretion groups, although not significantly so.
Table 7: Demographics (groups divided by the degree of proteinuria)

<table>
<thead>
<tr>
<th>Demographic</th>
<th>Group-1</th>
<th>Group-2</th>
<th>Group-3</th>
<th>Group-4</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number</td>
<td>183</td>
<td>203</td>
<td>27</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Recipient age in years± SD</td>
<td>44±13.5</td>
<td>47±15.3</td>
<td>37±12.8</td>
<td>44±14.4</td>
<td>0.007</td>
</tr>
<tr>
<td>% male gender</td>
<td>57%</td>
<td>62%</td>
<td>67%</td>
<td>56%</td>
<td>0.7</td>
</tr>
<tr>
<td>% Caucasian</td>
<td>86%</td>
<td>79%</td>
<td>89%</td>
<td>72%</td>
<td>0.08</td>
</tr>
<tr>
<td>Graft number± SD</td>
<td>1.1±0.28</td>
<td>1.05±0.22</td>
<td>1.15±0.36</td>
<td>1.3±0.61</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total HLA-mismatches± SD</td>
<td>1.5±1.1</td>
<td>1.9±1.3</td>
<td>1.9±1.0</td>
<td>2.2±1.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>% CMV R-/D+</td>
<td>26%</td>
<td>24%</td>
<td>37%</td>
<td>22%</td>
<td>0.5</td>
</tr>
<tr>
<td>Donor type- live donor</td>
<td>21%</td>
<td>19%</td>
<td>4%</td>
<td>22%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DBD- donor</td>
<td>72%</td>
<td>59%</td>
<td>92%</td>
<td>54%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DCD donor</td>
<td>7%</td>
<td>22%</td>
<td>4%</td>
<td>24%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Donor age</td>
<td>46±13.6</td>
<td>46±14.1</td>
<td>38±17.7</td>
<td>49±15.7</td>
<td>0.01</td>
</tr>
<tr>
<td>% diabetic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cold ischemia time in hrs± SD</td>
<td>14.9±8.2</td>
<td>14.9±7.6</td>
<td>17.5±4.6</td>
<td>14.7±8.2</td>
<td>0.4</td>
</tr>
<tr>
<td>% Delayed graft function</td>
<td>27%</td>
<td>30%</td>
<td>5%</td>
<td>48%</td>
<td>0.03</td>
</tr>
<tr>
<td>Mean follow-up± SD</td>
<td>48±0</td>
<td>39.5±8</td>
<td>48±0</td>
<td>38.3±7.5</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Groups are compared by univariate one way ANOVA with Dunnet's post hoc correction for multiple comparisons.
3.2.2.2 Risk factors for Clinical outcomes - Significance of early proteinuria

The relationship between proteinuria at 3 months and various primary endpoints including death-censored graft loss, death with a functioning graft, and vascular events during the graft’s life were explored using univariate Cox regression analysis as illustrated in Table 8. Recipient age, ethnicity, graft number, acute rejection in the first post-transplant year, uPCR groups at 3 months, and serum creatinine at 3 months affected death-censored graft loss. Recipient age, uPCR at 3 months, primary renal diagnosis, type of donation, graft number, and diabetes mellitus affected patient survival, whereas recipient age, uPCR at 3 months, creatinine at 3 months, primary renal diagnosis, donor age, and diabetes mellitus predicted the occurrence of the vascular end point in the univariate analysis.
**Table 8: Risk factors for various clinical outcomes (3 months post-transplant)**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Death censored graft loss (95% CI)</th>
<th>P-value</th>
<th>Death with a functioning graft (95% CI)</th>
<th>P-value</th>
<th>Cardiovascular event (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recipient age</td>
<td>0.98 (0.96-0.99)</td>
<td>0.009</td>
<td>1.09 (1.07-1.11)</td>
<td>&lt;0.001</td>
<td>1.04 (1.03-1.05)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ethnicity-South Asian vs. Caucasian</td>
<td>2.5 (1.2-5.2)</td>
<td>0.02</td>
<td>0.5 (0.1-1.9)</td>
<td>0.3</td>
<td>0.97 (0.4-2.4)</td>
<td>0.9</td>
</tr>
<tr>
<td>Cause of ESRD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glomerular disease</td>
<td>Ref</td>
<td>Ref</td>
<td>Ref</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infection/obstructive disease</td>
<td>1.1 (0.5-2.4)</td>
<td>0.8</td>
<td>2.0 (1.1-3.9)</td>
<td>0.03</td>
<td>2.5 (1.3-4.8)</td>
<td>0.004</td>
</tr>
<tr>
<td>Others</td>
<td>0.98 (0.6-1.6)</td>
<td>0.9</td>
<td>1.6 (0.97-2.6)</td>
<td>0.07</td>
<td>1.7 (1.04-2.8)</td>
<td>0.04</td>
</tr>
<tr>
<td>Donor age</td>
<td>1.004 (0.99-1.02)</td>
<td>0.6</td>
<td>1.008 (0.99-1.02)</td>
<td>0.2</td>
<td>1.02 (1.002-1.03)</td>
<td>0.02</td>
</tr>
<tr>
<td>Cadaveric donor vs. live</td>
<td>0.9 (0.4-1.9)</td>
<td>0.8</td>
<td>10.6 (1.5-7.6)</td>
<td>0.02</td>
<td>2.3 (0.9-5.7)</td>
<td>0.07</td>
</tr>
<tr>
<td>Graft number</td>
<td>1.8 (1.2-2.6)</td>
<td>0.004</td>
<td>0.45 (0.2-0.99)</td>
<td>0.05</td>
<td>1.1 (0.6-1.7)</td>
<td>0.8</td>
</tr>
<tr>
<td>Early acute rejection</td>
<td>2.6 (1.7-4.1)</td>
<td>&lt;0.001</td>
<td>0.7 (0.4-1.2)</td>
<td>0.2</td>
<td>1.4 (0.9-2.1)</td>
<td>0.2</td>
</tr>
<tr>
<td>uPCR at 3 months</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group-1</td>
<td>Ref</td>
<td>Ref</td>
<td>Ref</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group-2</td>
<td>7.8 (2.0-32.2)</td>
<td>0.005</td>
<td>1.7 (0.9-3.2)</td>
<td>0.09</td>
<td>1.5 (0.8-2.8)</td>
<td>0.2</td>
</tr>
<tr>
<td>Group</td>
<td>12.0 (2.8-51)</td>
<td>0.001</td>
<td>2.5 (1.3-5.0)</td>
<td>0.008</td>
<td>2.6 (1.3-5.0)</td>
<td>0.005</td>
</tr>
<tr>
<td>---------</td>
<td>---------------</td>
<td>-------</td>
<td>---------------</td>
<td>-------</td>
<td>---------------</td>
<td>-------</td>
</tr>
<tr>
<td>Group-4</td>
<td>19.0 (4.3-83.4)</td>
<td>&lt;0.001</td>
<td>4.5 (2.2-9.4)</td>
<td>&lt;0.001</td>
<td>3.2 (1.6-6.7)</td>
<td>0.001</td>
</tr>
<tr>
<td>Creatinine at 3 months&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.006 (1.003-1.008)</td>
<td>&lt;0.001</td>
<td>1.003 (1.0-1.006)</td>
<td>0.09</td>
<td>1.004 (1.001-1.01)</td>
<td>0.003</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>1.1 (0.6-2.2)</td>
<td>0.8</td>
<td>1.7 (1.02-2.8)</td>
<td>0.04</td>
<td>1.9 (1.1-3.0)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

<sup>a</sup> Hazard ratio calculated for every year.

<sup>b</sup> Hazard ratio calculated for every μmol/L rise in creatinine.

95% CI, 95% confidence interval; CVA, cerebrovascular accident; ESRD, end-stage renal disease; HLA, human leukocyte antigen; Ref, reference group; CMV, cytomegalovirus; DGF, delayed graft function; PCR, protein creatinine ratio; BP, blood pressure (mm Hg)

Factors including recipient gender, donor cause of death, HLA mismatches, era of transplantation, DGF, CMV disease, systolic and diastolic blood pressure were not significant in the univariate analysis.
Table-9 summarizes the independent effect of proteinuria on the three primary outcome measures (death-censored graft loss, death with a functioning graft, and occurrence of vascular events during the graft’s life). From the analysis, it can be seen that the independent effect of low-grade proteinuria (uPCR 0.15–0.5) on death-censored graft loss was significant. However, the impact of early proteinuria on death with a functioning graft and composite vascular endpoint was modest when compared with its effect on death-censored graft loss. Patients in group 1 clearly had a good outlook but it should be noted that they only represented 17.8% (85 of 477) of the total population. The largest group is group 2 representing 51.4% (245 of 477) of patients.

**Table 9: Independent effect of early proteinuria on clinical outcomes**

<table>
<thead>
<tr>
<th>uPCR group</th>
<th>Hazard ratio</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Death censored graft loss</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group-1</td>
<td>Ref</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group-2</td>
<td>7.1</td>
<td>1.7-29.3</td>
<td>0.007</td>
</tr>
<tr>
<td>Group-3</td>
<td>10.5</td>
<td>2.4-45.7</td>
<td>0.002</td>
</tr>
<tr>
<td>Group-4</td>
<td>16.0</td>
<td>3.5-72</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Death with a functioning graft</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group-1</td>
<td>Ref</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group-2</td>
<td>0.95</td>
<td>0.5-1.8</td>
<td>0.9</td>
</tr>
<tr>
<td>Group-3</td>
<td>1.2</td>
<td>0.6-2.5</td>
<td>0.6</td>
</tr>
<tr>
<td>Group-4</td>
<td>2.6</td>
<td>1.2-5.4</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>Cardiovascular event</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group-1</td>
<td>Ref</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group-2</td>
<td>1.1</td>
<td>0.6-2.1</td>
<td>0.7</td>
</tr>
<tr>
<td>Group-3</td>
<td>1.3</td>
<td>0.7-2.7</td>
<td>0.4</td>
</tr>
<tr>
<td>Group-4</td>
<td>2.2</td>
<td>1.04-4.9</td>
<td>0.04</td>
</tr>
</tbody>
</table>

*a Death-censored graft survival: adjusted for the effect of recipient age, ethnicity, graft number, acute rejection, and serum creatinine at 3 mo.

*b Death with a functioning graft: Adjusted for the effect of recipient age, primary renal diagnosis, type of donation, graft number and diabetes mellitus.

*c Cumulative vascular events within the life of the graft: Adjusted for the effect of recipient age, serum creatinine at 3 months, primary renal diagnosis, donor age and diabetes mellitus.

95% CI, 95% confidence interval; Ref, reference group; uPCR, protein creatinine ratio
Analysis of the causes of graft loss across various proteinuria groups showed that a total of 187 grafts were lost during the follow-up period, and again death with a functioning graft was the dominant cause of graft loss in all groups (109 of 187, 58.3%) but particularly group 1 in which 12 of the 14 grafts were lost due to death. After death with a functioning graft, most grafts were lost due to rejection (50 of 187, 26.7%). As mentioned before, this information was obtained from registry returns and represents a broad category that includes refractory acute rejection, chronic allograft nephropathy, and a few slowly failing grafts that were not biopsied. Recurrent disease was unusual, representing only eight lost grafts in total. Although represented in higher absolute numbers in group 4 (3 of 35, 8%), there were no significant differences among the groups and we would not have expected proteinuria after only 3 months to correlate with eventual graft loss due to recurrent disease.

In summary, with these clinical data, it could be concluded that low-grade proteinuria was associated with adverse clinical outcomes. The effect was visible as early as 3 months after transplantation and when assessed longitudinally resolution of proteinuria was associated with improved outcomes, thereby suggesting that measurement of proteinuria by uPCR was a potential biomarker for graft loss.

3.2.3 Histological assessment of troubled allografts

Now, data describing the histological characteristics of patients presenting with either proteinuria or deteriorating creatinine or a combination of the both will be presented and finally the ability of clinical or histological phenotype to predict graft outcomes will be examined.
3.2.3.1 Study population

A total of 135 patients who underwent consecutive late indication renal transplant biopsy for a clinical presentation of either ‘creeping creatinine’ or isolated persistent proteinuria were included in this analysis. Of these, 32 patients presented with creeping creatinine (CC) only and 44 patients with isolated proteinuria (IP). A total of 59 patients were biopsied for a combination of proteinuria and ‘creeping creatinine’ (CP). Figure 28A summarizes the regression of the 1/creatinine plot against time across ten different time points spread over 2 years for the three clinical groups. Similarly, Figure 28B shows mean proteinuria measured at the three consecutive time points for the three groups.

Table 10 compares the demographic characteristics of the three study groups. The key differences between the groups included a significantly older age for the recipients in the CP group and longer graft vintage for both the proteinuria groups (IP and CP) when compared to the CC group. As expected there was a significantly lower serum creatinine at the time of biopsy in the IP group when compared to the other groups. There was also a trend towards a dominant Caucasian representation in the CP group. Finally it is interesting to note that the proportion of patients with glomerulonephritis as the cause for ESRD was slightly lower in the proteinuria groups, although these results did not achieve statistical significance. This finding corroborates with the previous observations in Table 5 and Table 7 suggesting that non-glomerular aetiology for ESRD is more common in patients with proteinuria. Patients were followed for a maximum of 3 years after the biopsy (mean follow-up 31.4 months +/- SD).
Figure 28: The three clinical groups and the broad histological classification

(A) The three regression plots demonstrate the changes in renal function measured by serum creatinine over time. CC Group-$R^2=0.91$, $P<0.0001$; IP Group-$R^2=0.13$, $P=0.3$; CP Group-$R^2=0.78$, $P=0.0003$. The slopes of the three groups were significantly different. $F=21.5$, $DF_n=2$, $DF_d=1332$, $P<0.0001$. (B) The mean of the three consecutive proteinuria estimates is shown for the three groups (CC, IP, and CP). The error bars represent SEM. Based on their histology, all the three groups of patients were divided into 5 broad classes - recurrent glomerulonephritis, microcirculation damage (defined by the presence of ‘g’, ‘ptc’ or ‘cg’ lesion with or without C4d deposition), IFTA-no specific cause identified, T cell mediated rejection and others (BK virus nephropathy, amyloidosis, hypertensive arteriosclerosis and ascending infection). (C) The stacked bar charts demonstrate the distribution of the five histological classes in the three clinical groups. There were no statistically significant differences in the distribution of any specific histological class between the clinical groups ($P=Ns$, Chi square test). (D) Even when the analysis was repeated by stratifying the groups based on the degree of proteinuria (< or > 1g/day), the distribution of histological classes remained comparable.
Table 10: Clinical characteristics of patient groups (CC, IP, CP)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>CC</th>
<th>IP</th>
<th>CP</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (N)</td>
<td>32</td>
<td>44</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>Recipient age in years</td>
<td>41 (17)</td>
<td>47 (17)</td>
<td>49 (15)</td>
<td>0.04</td>
</tr>
<tr>
<td>Gender-male%</td>
<td>47%</td>
<td>66%</td>
<td>58%</td>
<td>Ns</td>
</tr>
<tr>
<td>Ethnicity-Caucasian%</td>
<td>66%</td>
<td>73%</td>
<td>81%</td>
<td>Ns</td>
</tr>
<tr>
<td>Primary renal diagnosis</td>
<td></td>
<td></td>
<td></td>
<td>Ns</td>
</tr>
<tr>
<td>Diabetes and hypertension</td>
<td>10%</td>
<td>23%</td>
<td>8%</td>
<td></td>
</tr>
<tr>
<td>Glomerulonephritis</td>
<td>52%</td>
<td>33%</td>
<td>41%</td>
<td></td>
</tr>
<tr>
<td>APKD and congenital</td>
<td>19%</td>
<td>27%</td>
<td>21%</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>29%</td>
<td>17%</td>
<td>30%</td>
<td></td>
</tr>
<tr>
<td>Graft type</td>
<td></td>
<td></td>
<td></td>
<td>Ns</td>
</tr>
<tr>
<td>Live%</td>
<td>30%</td>
<td>17%</td>
<td>25%</td>
<td></td>
</tr>
<tr>
<td>DBD%</td>
<td>63%</td>
<td>69%</td>
<td>63%</td>
<td></td>
</tr>
<tr>
<td>DCD%</td>
<td>7%</td>
<td>14%</td>
<td>12%</td>
<td></td>
</tr>
<tr>
<td>HLA-MM</td>
<td>2.2 (1.2)</td>
<td>2.7 (1.2)</td>
<td>2.3 (1.2)</td>
<td>Ns</td>
</tr>
<tr>
<td>Donor age</td>
<td>43 (17)</td>
<td>40 (20)</td>
<td>48 (21)</td>
<td>Ns</td>
</tr>
<tr>
<td>Donor gender-male%</td>
<td>50%</td>
<td>56%</td>
<td>62%</td>
<td>Ns</td>
</tr>
<tr>
<td>DSA</td>
<td>22%</td>
<td>21%</td>
<td>27%</td>
<td>Ns</td>
</tr>
<tr>
<td>Time since transplant</td>
<td>64 (44)</td>
<td>124 (85)</td>
<td>95 (66)</td>
<td>0.001</td>
</tr>
<tr>
<td>Serum creatinine at biopsy</td>
<td>252 (126)</td>
<td>154 (30)</td>
<td>262 (122)</td>
<td>0.001</td>
</tr>
<tr>
<td>Immunosuppression</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Induction-methylprednisolone alone</td>
<td>35%</td>
<td>43%</td>
<td>37%</td>
<td>Ns</td>
</tr>
<tr>
<td>Induction –Basiliximab</td>
<td>53%</td>
<td>50%</td>
<td>58%</td>
<td>Ns</td>
</tr>
<tr>
<td>Induction-Alemtuzumab</td>
<td>12%</td>
<td>7%</td>
<td>5%</td>
<td>Ns</td>
</tr>
<tr>
<td>Ciclosporin</td>
<td>19%</td>
<td>34%</td>
<td>32%</td>
<td>Ns</td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>75%</td>
<td>50%</td>
<td>53%</td>
<td>Ns</td>
</tr>
<tr>
<td>Mycophenolate</td>
<td>63%</td>
<td>48%</td>
<td>61%</td>
<td>Ns</td>
</tr>
<tr>
<td>Azathioprine</td>
<td>23%</td>
<td>29%</td>
<td>20%</td>
<td>Ns</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>50%</td>
<td>50%</td>
<td>58%</td>
<td>Ns</td>
</tr>
<tr>
<td>Sirolimus</td>
<td>3%</td>
<td>4%</td>
<td>7%</td>
<td>Ns</td>
</tr>
</tbody>
</table>

Continuous variables were compared across the groups by one way ANOVA with Tukey post hoc correction for multiple comparisons. Categorical variables were compared by Chi squared test. For continuous variables SD is shown in the parenthesis.
3.2.3.2 Allograft histology and clinical presentation

The distribution of various histological classes stratified by clinical presentation at the time of the indication biopsy is first analysed. Figure 28C shows that the distribution of histological classes including glomerulonephritis, interstitial fibrosis tubular atrophy-no specific cause identified, microcirculation damage (defined by the presence of either glomerulitius, peritubular capillaritis, transplant glomerulopathy with or without deposition of C4d in the peritubular capillaries), late T cell mediated rejection and other diagnoses was quite heterogeneous with similar distribution of both glomerulonephritis and microcirculatory damage across all the clinical groups. When the histological classes were re-analysed based on the degree of proteinuria (>1g or <1g), again no significant differences were noted in the distribution of the histological classes irrespective of the degree of proteinuria (Figure 28D).

Next, a detailed analysis of all the Banff lesions was performed in 106 patients after excluding 29 patients with either GN (n=18) or other miscellaneous diagnosis (BK viral nephritis, n=6; amyloidosis, n=2; ascending infection, n=1; hypertensive vasculopathy, n=2). In Figure 29, the top panel of graphs represent the distribution of Banff scores whilst the bottom panel analyses the mean Banff scores across the groups of patients stratified by clinical presentation. Figure 29A shows that there was a trend towards a higher proportion of patients in the CC group to have both more tubulitis (t) and interstitial inflammation (i) and it was notable that the CC group had the highest ‘t’ and ‘i’ scores, although only ‘t’ scores reached statistical significance. There were no significant differences noted in the distribution and the mean levels of either intimal arteritis (‘v’) or the total inflammation (‘t’). The distribution and mean levels of microcirculatory inflammation (‘g’, ‘ptc’) were similar across the groups, whilst there was a trend towards higher mean levels of the transplant glomerulopathy score and mesangial expansion in patients with proteinuria (Figure 29B-C). All the clinical groups had prominent features of scarring as depicted by the distribution and the mean values of ‘ci’ and ‘ct’, scores (Figure 29D). Again there was a non-significant trend towards
slightly higher mean scores for these in patients with both proteinuria and creeping creatinine (CP). It has recently been demonstrated that a ‘g+ptc’ score of more than 2 is indicative of significant microcirculatory inflammation and is associated with worse graft survival in patients with de novo donor specific antibodies (de Kort, Willicombe et al. 2013). When I analysed the distribution of the biopsies with ‘g+ptc’ scores of 0, 1 to 2 or 3; no significant differences are noticed between the clinical groups (Figure 29E). Finally analysis of the deposition of C4d in the peritubular capillaries has shown a slightly but statistically significantly stronger C4d scores in patients with CC (Figure 29F). In summary, there was overall heterogeneity in the distribution of the histological diagnoses irrespective of the clinical presentation. Whilst there was a propensity towards a slightly higher degree of tubulo-interstitial inflammation in patients with CC, patients with proteinuria had more chronic changes.
Figure 29: Banff lesions and the three clinical groups

The top panel in this figure shows the distribution of the Banff scores (0, 1, >1) and the bottom panel demonstrates the mean Banff score for each lesion. (1A). Analysis of the Banff scores for tubulointerstitial inflammation ('t' tubulitis, 'v' intimal arteritis, 'i' interstitial inflammation, 'ti' total inflammation). Patients who presented with CC had a significantly higher 't' score (* CC vs. PC, P=0.04). Although there was a trend towards a higher 't' score in the CC group in comparison to the others, this did not reach statistical significance. (1B). Microcirculation inflammation scores ('g' glomerulitis, 'ptc' peritubular capillaritis) and these scores were comparable across the clinical groups. (1C). Lesions pertaining to microcirculatory deterioration ('cg' transplant glomerulopathy, 'mm' mesangial matrix expansion). Patients in the CC group had the lowest 'cg' and 'mm' scores, even though only the 'mm' score achieved statistical significance ('mm' score CC vs. PC, P=0.02). (1D) All patients had a significant amount of scarring as evidenced by these lesions ('ct' tubular atrophy, 'ci' interstitial fibrosis, 'cv' intimal fibrosis, and 'aah' arteriolar hyalinosis). Although there was a trend towards higher scores for scarring in patients with proteinuria, these results did not reach statistical significance. (1E). Microcirculatory inflammation (g+ptc) scores across the clinical groups. Again it was evident that there were no significant differences between the groups. (1F). C4d scores. Patients within the CC group had stronger scores for C4d deposition. All the Banff lesion scores were compared across the clinical groups by ANOVA with Dunnet's post hoc correction for multiple comparisons. The error bars represent SEM.
3.2.3.3 Histological class, clinical phenotype and allograft survival

Having established the histological heterogeneity in these patient groups I have proceeded further to test the prognostic significance of either the clinical presentation or the histological phenotype. Table-11 shows that none of the clinical variables were significantly associated with worse graft survival in a univariate analysis (univariate Cox proportional hazards model). In a univariate Kaplan Meier survival analysis, patients with both proteinuria and creeping creatinine (CP) had a significantly worse survival when compared to both CC (P=0.002) and P (P=0.05) groups (Figure 30). Whilst there was a trend towards slightly worse graft survival in the presence of a DSA, this did not reach statistical significance (Figure 30). For the assessment of the histological classes, I divided the patients into three broad groups - GN, microcirculatory damage (MCD) and others. When these three groups were compared for graft survival after the biopsy, patients with both GN (P=0.03) and MCD (P=0.045) had significantly worse graft loss when compared to the rest of the patients (Figure 30). Finally I have examined the significance of scarring defined by tubular atrophy, interstitial fibrosis, and arterial fibrointimal thickening. For this analysis patients were divided into tertiles bases on their composite ‘ct’, ‘cv’ and ‘ci’ scores and from Figure 30, again it can be noted that patients in the highest tertile had significantly worse graft survival when compared to the other tertiles (P<0.001).
**Table 11: Impact of clinical variables on graft loss after an indication biopsy**

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recipient age</td>
<td>1.0</td>
<td>0.98-1.02</td>
<td>0.99</td>
</tr>
<tr>
<td>Recipient gender</td>
<td>1.1</td>
<td>0.6-2.01</td>
<td>0.8</td>
</tr>
<tr>
<td>Recipient ethnicity (Caucasian vs. Soth Asian)</td>
<td>1.2</td>
<td>0.6-2.5</td>
<td>0.6</td>
</tr>
<tr>
<td>Graft number</td>
<td>1.5</td>
<td>0.7-3.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Time since transplantation</td>
<td>1.0</td>
<td>0.99-1.005</td>
<td>0.6</td>
</tr>
<tr>
<td>HLA mismatches</td>
<td>1.09</td>
<td>0.8-1.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Donor age</td>
<td>1.01</td>
<td>0.99-1.03</td>
<td>0.07</td>
</tr>
<tr>
<td>Type of donor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DBD vs. live</td>
<td>1.1</td>
<td>0.5-2.6</td>
<td>0.8</td>
</tr>
<tr>
<td>DCD vs. live</td>
<td>1.4</td>
<td>0.5-4.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

HR, Hazard ratio; 95% CI, 95% Confidence Interval

Analysis by univariate Cox proportional hazards model
Figure 30: Graft survival after the biopsy—the influence of clinical presentation and histology

Patients were followed for a maximum of 3 years after the biopsy and death censored graft survival was examined, stratified by clinical presentation and histological features using Kaplan Meier method. Survival curves were compared using the Log Rank test. When patients were grouped based on their clinical presentation, the CP group had significantly worse survival when compared to CC alone (Chi square=3.8, P=0.05) and IP (Chi square=9.6, P=0.002). Positivity of DSA at the time of biopsy was not associated with a significant difference in graft survival. Recurrent GN (Chi square=4.8, P=0.03) and MCD (Chi square=3.9, P=0.045) on the histological analysis were associated with worse graft survival. When patients were divided into tertiles based on the cumulative ‘ct’, ‘cv’ and ‘ci’ scores; patients in the highest tertile had significantly worse graft survival (Chi square=12.98, P<0.001).
To study the independent effect of these variables on graft loss, multivariate analysis was next performed using Cox’s Proportional hazard model. In this analysis (Table-12), the presence of MCD (HR 2.5, 95% CI 1.1-5.6, P=0.03) and the diagnosis of recurrent GN (HR 2.9, 95% CI 1.2-6.9, P=0.015) were independently associated with increased risk of graft loss. Most importantly, the degree of scarring noted in the biopsy was the strongest predictor of graft loss, with patients in the highest tertile of the cumulative ‘ct’, ‘cv’ and ‘ci’ scores having the worst graft survival (HR 4.5, 95% CI 1.8-10.5, P=0.001).

**Table 12: Multivariate analysis of factors predicting graft loss**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard ratio</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Histological diagnosis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GN vs. rest</td>
<td>2.9</td>
<td>1.2-6.9</td>
<td>0.015</td>
</tr>
<tr>
<td>MCD vs. rest</td>
<td>2.5</td>
<td>1.1-5.6</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Scarring</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Middle tertile vs. lowest</td>
<td>1.2</td>
<td>0.5-3.3</td>
<td>Ns</td>
</tr>
<tr>
<td>Highest tertile vs. lowest</td>
<td>4.3</td>
<td>1.8-10.5</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>DSA</strong></td>
<td>1.02</td>
<td>0.5-2.3</td>
<td>Ns</td>
</tr>
<tr>
<td><strong>Clinical presentation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IP vs. CC</td>
<td>0.8</td>
<td>0.3-2.3</td>
<td>Ns</td>
</tr>
<tr>
<td>PC vs. CC</td>
<td>1.8</td>
<td>0.8-4.2</td>
<td>Ns</td>
</tr>
</tbody>
</table>

Analysis was by multivariate Cox Proportional Hazards model.

95% CI 95% confidence interval
Figure 31 shows that there was a decline in renal function in the functioning grafts in all the three groups as measured by 1/creatinine over the next 2 years (CC group, $R^2=0.23$, $P=0.1$; P group, $R^2=0.46$, $P=0.03$; PC, $R^2=0.75$, $P=0.001$). However, despite a significant difference in the serum creatinine at the time of the biopsy in the three clinical groups, it was interesting to note the rates of decline of renal function after the biopsy, as measured by the slope of the 1/creatinine regression plots was not significantly different between the groups.

![Graph](https://via.placeholder.com/150)

**Figure 31: 1/creatinine plots for representative patients within CC, P and PC groups after the biopsy**

Serial serum creatinine levels were assessed after the biopsy in patients from all the three clinical groups. Grafts that were lost were excluded from the analysis at the time of graft loss. This graphic shows that the overall elevation of the 1/creatinine plots was not surprisingly significantly different with evidence of inequality of intercepts ($F=67.8$, $DF=960$, $DF=2$, $P<0.0001$) given the initial differences in the serum creatinine at the time of the biopsy. However the rate of deterioration of renal function as determined by the slope of 1/creatinine plots was not significantly different between the groups ($F=0.34$, $DF=2$, $DF=958$, $p=0.7$).
Finally, a sub-group analysis of graft survival was performed in the 47 patients with the histological diagnosis of microcirculatory damage (MCD). The Kaplan Meier survival curves in Figure 32 show that neither clinical presentation nor the DSA status at the time of the biopsy was associated with worse graft survival in these patients. Although there was a trend towards worse graft survival in patients with either ‘g’ (P=0.1) or ‘ptc’ (P=0.09) lesions, patients with ‘g+ptc’ score ≥3 (score≥3 vs. score-0, P=0.04) had significantly worse graft survival, highlighting the importance of the ‘g+ptc’ score (MI lesion score). Despite the lack of prognostic significance for the presence of DSA, deposition of C4d in the peritubular capillaries has been shown to be associated with a significantly adverse graft survival after the biopsy (P=0.002). As has been noted in the main analysis, worse scarring defined by higher cumulative ‘ci’, ‘cv’and ‘ct’ scores was associated with worse graft survival (highest tertile vs. lowest tertile, P=0.02). Interestingly, when the graft survival was analysed in groups stratified by the presence of MI lesion score≥3 and the detection of C4d in the peritubular capillaries, we noticed that patients with an MI score≥3 and with detectable C4d on the biopsy had the worst graft survival (Figure 33).

Therefore, histological phenotype defined by the presence of scarring and the lesions of microcirculatory damage along with a diagnosis of GN were associated with significantly worse graft survival. The prognostic significance of the histological lesions persisted even when the analysis was repeated in a small sub-group of patients with MCD alone.
47 patients who had lesions of either 'g', 'ptc' or 'cg' with or without C4d detection were analysed for graft survival based on various Banff characteristics using the Kaplan Meier method. This figure shows that clinical presentation and DSA positivity at the time of the biopsy were not significantly associated with worse graft survival. Key histological features including, the MI lesion score≥3 (Chi square=4.2, P=0.04), C4d deposition in the peritubular capillaries (Chi square=9.6, P=0.002) and the degree of scarring based on the cumulative 'ct', 'cv' and 'ci' scores (highest vs. lowest tertile, Chi square=5.2, P=0.02) were significantly associated with worse graft survival. Tubulitis, glomerulopathy, arteriolar hyalinosis, ‘g’ or ‘ptc’ lesions alone did not have statistically significant associations with graft survival.

Figure 32: Subset analysis of graft survival in patients with MCD
Figure 33: Graft survival-risk stratification by MI score and C4d deposition

Kaplan Meier survival curve demonstrating the graft survival over three years in patients divided into three groups based on MI lesion score and C4d deposition (Group-1: MI lesion<3, C4d neg; Group-2: MI lesion score≥3 and C4d neg or MI lesion score<3 and C4d pos; Group-3: MI lesion score≥3 and C4d pos). The curves are compared using the Log Rank test. In comparison to Group-1, patients who had positivity for C4d and MI lesion score≥3 had significantly worse survival (P<0.001). Although there is a trend towards worse survival in Group-2, this did not reach statistical significance.

In summary, with the data presented in this chapter, it can be concluded that low-grade proteinuria; despite being a good clinical marker for graft outcomes in renal transplant recipients is inferior to the histological phenotype of the deteriorating graft on an indication biopsy. In fact histological phenotype is probably superior to clinical phenotype once chronic graft dysfunction ensues.
3.3 Discussion

One of the major challenges for the renal transplant community at present is the lack of improvement in long-term allograft loss despite significant advances in the management of early acute rejection and superior early graft survival (Hariharan, McBride et al. 2002, Lamb, Lodhi et al. 2011). Early diagnosis of allograft injury remains one of the greatest challenges in renal transplantation. Serum creatinine levels are measured as a surrogate marker of renal function, but this is a relatively insensitive marker of established renal allograft damage, and by the time levels become consistently elevated, significant irreversible nephron loss has usually occurred (Chapman, O'Connell et al. 2005). Despite this limitation, elevated serum creatinine at 12 months after transplantation is strongly associated with a poor outcome, although it has a poor predictive value because many grafts that eventually fail have a low creatinine level at 1 year (Hariharan, McBride et al. 2002, Kaplan, Schold et al. 2003) (21, 22). Consequently, the search continues for a marker of early allograft injury.

Protocol biopsy of renal tissue has been proposed as one such method. Although safe, this procedure is invasive and incurs significant cost. Despite some initially encouraging reports, especially in the context of management of early subclinical rejection, the benefits in normal clinical practice remain unproven (Nickerson, Jeffery et al. 1998, Rush, Nickerson et al. 1998) (2, 23). Consequently, there remains a great deal of interest in less invasive methods involving analysis of urine, circulating blood cells, or serum.

3.3.1. Low-grade proteinuria- A clinical marker for adverse graft outcomes

Protein excretion in the urine is a cardinal sign of renal damage and higher levels of protein excretion predict poor outcome in many renal diseases. Low levels of albumin excretion (microalbuminuria) have been shown to be an early marker of renal parenchymal damage and a predictor for cardiovascular events in patients with renal
disease and those with no overt renal problems (Grimm, Svendsen et al. 1997, Abbate, Zoja et al. 2006, Bakris 2008, Chronic Kidney Disease Prognosis, Matsushita et al. 2010, Turin, James et al. 2013). Urine protein creatinine ratio (uPCR) provides a simple way of investigating proteinuria in patients with renal disease and it has been shown to be an accurate reflection of 24 hour protein excretion (Shaw, Risdon et al. 1983, Dyson, Will et al. 1992). Formal quantification of proteinuria for every patient at every clinic visit at SJUH provided me an opportunity to analyse this dynamic variable in a longitudinal fashion and study its impact on long-term graft survival. The majority of patients at one year had low-grade proteinuria (82.9%), although there was an improvement by year 2 (49.7%) and 3 (42.1%). It is however important to note that a significant number of these patients (86.6% at 1 year, 95.1% at year-2 and 94.4% at year-3) had very low-grade proteinuria (150mg-500mg/24hrs) and at these low levels, there is a distinct possibility that proteinuria can regress. In this study, factors including non-glomerular disease, serum creatinine and DBD grafts were associated with the development of low-grade proteinuria. It was rather surprising to note that patients with glomerular disease had a significantly lower risk in this study population. This is potentially attributable to the fact that patients with a potential diagnosis of recurrent glomerular disease would likely present with a far more significant level of urine protein excretion (>1g/day) and all such patients were excluded from the study. Another possible explanation could be that there were no pre-emptive transplants within this group and it is very unlikely that protein excretion from the native kidney would be persistent beyond the first year (Halimi 2013). However it should be noted that even in the analysis of early proteinuria, patients with glomerulonephritis are less represented in the group with proteinuria.

Low-grade proteinuria is associated with worse graft survival in subgroups stratified by various clinical variables and its regression is associated with better graft survival when compared to those with persistent proteinuria. In the non-transplant literature, remission of proteinuria has been shown to be associated with better outcomes in
patients with various glomerular diseases (Reich, Troyanov et al. 2007). Post-hoc analysis from the RENAAL (Reduction in End-points in Non-insulin dependent diabetes mellitus with Angiotensin 2 Antagonist Losartan) study provided further evidence for cardiovascular risk modification with the reduction of proteinuria and this beneficial effect was shown to be independent of blood pressure control (de Zeeuw, Remuzzi et al. 2004, de Zeeuw, Remuzzi et al. 2004). The results from the analysis of systolic hypertension and graft failure confirm the findings of Opelz et al. (Opelz, Dohler et al. 2005) showing that persistently elevated systolic blood pressure is associated with adverse clinical outcomes after renal transplantation. In fact the data presented in this chapter also demonstrates that patients with systolic hypertension can be risk stratified based on the presence of low-grade proteinuria. Low-grade proteinuria not only identifies high risk hypertensive patients at years-1, 2 and 3 but is also found to be significantly associated with worse overall graft survival in patients who are persistently hypertensive between years 1 and 3.

The uPCR method used at SJUH to determine proteinuria will pick up both albumin and non-albumin proteinuria. Halimi et al. have shown that 76% of protein excreted in patients with low-grade proteinuria is tubular, a finding confirmed by other groups demonstrating that tubular proteinuria is associated with worse graft outcomes (Teppo, Honkanen et al. 2004, Halimi, Matthias et al. 2007, Schaub, Mayr et al. 2007). This may reflect donor derived damage, ischemia reperfusion injury or previous immunologically mediated graft damage. A report from Fotheringham et al. has shown that patients with donor specific antibodies (DSA) have significantly more proteinuria and that proteinuria develops before the detection of DSA with a higher risk of graft loss in patients with more than 0.2g/day of proteinuria (Fotheringham, Angel et al. 2011). Even in the absence of significant proteinuria with its associated recurrent or de novo glomerular disease, patients with proteinuria as low as 150mg/day are shown to have diverse pathological findings including acute rejection, IFTA, ATN and arteriolar hyalinosis and suffer adverse long-term graft survival (Amer, Fidler et al. 2007). In this
analysis, the dominant cause of allograft loss was death with a functioning graft although loss of a functioning graft was seen in 32.9%, most of which was attributed to allograft rejection.

This analysis has certain limitations. It is a single centre retrospective cohort study, which limits my ability to test a wide variety of clinical parameters due to sample size. The data on the usage of ACE inhibitors and ARBs is not presented as it is incomplete with the type of antihypertensive drugs not being recorded electronically for many patients routinely. Although the commonest cause of allograft function is noted as rejection, patients are not routinely biopsied and the diagnosis is derived from the registry returns.

In summary, low-grade proteinuria is shown to be a modifiable risk factor for overall graft loss after renal transplantation; resolution of which has led to improved overall outcomes. Proteinuria measured as early as three months after transplantation is identified as a useful marker to risk stratify patients. More importantly the presence of low-grade proteinuria risk stratifies hypertensive renal transplant recipients and provides a valuable marker to identify potential patients for therapeutic intervention.

3.3.2 Histological analysis of troubled allografts

Several recent studies which analysed the phenotype of ‘late at risk’ allografts have asserted that chronic antibody mediated rejection (CAMR) is the commonest cause of late graft dysfunction and graft loss (Einecke, Sis et al. 2009, El-Zoghby, Stegall et al. 2009, Gourishankar, Leduc et al. 2010). I have systematically analysed the late ‘indication’ biopsies performed at SJUH with a view to identify a clinical phenotype associated with a specific histological phenotype and adverse graft outcome. The data presented in this chapter shows that late histological phenotype is quite heterogeneous irrespective of clinical presentation and allografts present with significant chronic damage even in the isolated proteinuria group with a stable serum. In a multivariate analysis, the histological phenotype of microcirculatory damage, the hallmark of CAMR,
and recurrent GN has been shown to be independently associated with worse graft survival. More importantly, the degree of chronic damage was the strongest predictor of graft survival after a late biopsy. This finding was replicated again in a subset of patients with MCD, thus highlighting the fact that significant chronic damage has already been established by the time a biopsy is performed for cause and thereby limiting the utility of these biopsies for therapeutic intervention.

The principle finding from the data is the degree of heterogeneity seen within the histological phenotype of late indication biopsies, irrespective of the clinical presentation. It might have been expected to see a significant contribution of recurrent GN and transplant glomerulopathy in patients presenting with proteinuria. To my surprise, the distribution of the various histological classes was comparable across the clinical groups. The distribution was not altered even in patients that presented with proteinuria of more than a gram per day.

In an analysis of the Banff lesions based on clinical presentation, a trend towards more tubulointerstitial inflammation in patients presenting with creeping creatinine and more chronic changes in patient groups with proteinuria was demonstrated. Although several groups have reported the histological phenotype of the ‘troubled allograft’, histological phenotype based on clinical presentation has not been reported before (El-Zoghby, Stegall et al. 2009, Gourishankar, Leduc et al. 2010). First et al. in the earliest report of the histological characteristics of patients presenting with persistent proteinuria have highlighted the diagnostic heterogeneity, with the spectrum ranging from transplant glomerulopathy (TG) and recurrent GN to renal vein thrombosis (First, Vaidya et al. 1984). Recurrent GN and TG as dominant histological phenotypes in patients with proteinuria have also been established by other groups as well (Yakupoglu, Baranowska-Daca et al. 2004, Amer, Fidler et al. 2007). Indeed, my findings are in agreement with these reports. In addition it is shown that the histology in patients
presenting with proteinuria is not significantly different to those presenting with creeping creatinine alone.

Several groups have highlighted the importance of proteinuria in its ability to risk stratify patients and speculated that measuring proteinuria potentially identifies a group of at ‘risk individuals’ who may theoretically benefit from therapeutic intervention (Halimi, Laouad et al. 2005, Amer, Fidler et al. 2007). Given the fact that a significant degree of chronic damage (defined by ‘ct’, ‘ci’, ‘cv’ lesions) has already been established even in patients that present with isolated proteinuria and that clinical presentation as an independent variable is not significantly associated with adverse graft survival in the multivariate analysis, it is less likely that any potential therapeutic intervention can offer significant long term benefit once clinical changes have occurred. This assumption is strengthened by the finding that the extent of chronic damage noticed in the all the three patient groups is significant with proteinuria groups (P, PC) having slightly worse chronic scarring. In the multivariate analysis, the degree of chronic damage was found to be the strongest predictor of graft survival after the biopsy. These findings also suggest that the prognostic utility of measuring DSA is limited, once the clinical phenotype has altered with the development of proteinuria and/or decline in serum creatinine. However this study does not inform the discussion over the utility of DSAs as biomarkers at earlier time points, prior to the detection of overt clinical abnormalities. Despite the limited overall utility of the detection of DSA in this population, in a small sub-group analysis of patients who had detectable lesions of microcirculatory changes (‘g’, ‘ptc’, ‘cg’), C4d deposition was shown to be associated with adverse graft survival. More importantly, detection of C4d in patients with MI lesion score ≥3 was shown to confer the worst prognostic outcome. C4d deposition has been shown to be an indicator of humoral allograft damage within the graft and its diagnostic significance lies in its specificity (Feucht, Felber et al. 1991, Regele, Bohmig et al. 2002, Mroz, Durlik et al. 2003). It is quite possible that the C4d deposition was a result of non-HLA DSA. There are certain well documented limitations for the diagnostic utility of C4d, e.g its
limited applicability in patients transplanted across the ABO blood group barrier (Haas, Rahman et al. 2006) and its lack of sensitivity in patients presenting with late CAMR (Gloor, Sethi et al. 2007, Sis, Campbell et al. 2007). Based on the findings presented in this chapter, the prognostic utility of C4d in renal transplant recipients cannot be discounted, despite these short comings.

The main limitation to this analysis emerges from its single centre and cross sectional nature. The numbers in each clinical group were small and this was especially evident in the sub-group analysis. Furthermore, the impact of modifications in treatment has not been accounted for in the analysis of graft survival.

The disjunction between clinical presentation, histological changes and the resultant clinical outcomes in patients who underwent an ‘indication biopsy’ asks for an alternative plan of investigation in renal transplant recipients. Although protocol biopsies might be considered, owing to their limited diagnostic yield (Mengel, Bogers et al. 2005), modifications are necessary. For example, genomic analysis of these biopsies could be considered as there is accumulating evidence that genomic transcripts offer significant prognostic information (Einecke, Reeve et al. 2010, Halloran, Reeve et al. 2013). Higher risk group patients might be identified by the detection of DSAs in the serum, before any clinical abnormalities occur, since they have been shown to be powerful biomarkers for poor outcomes (Lachmann, Terasaki et al. 2009, Wiebe, Gibson et al. 2012). However adoption of this process requires consensus over the best form of treatment when the DSAs are detected. Finally, it is hypothetically possible that if patients were identified and biopsied at lower levels of proteinuria, earlier histological picture with less scarring and chronic changes may emerge and this sub-group of patients may potentially benefit from therapeutic intervention.

In summary the data presented here describes the histological heterogeneity in late ‘indication biopsies’ and the striking lack of correlation between histological and clinical
phenotypes. Importantly, the histological rather than clinical phenotype has been shown to be of significant prognostic utility.

In conclusion, the data presented in this chapter shows that proteinuria, although an early and reversible predictor for graft outcomes has limited diagnostic and prognostic significance once the damage to the graft with the resultant chronic changes ensues.
Chapter-4: Human Bregs and troubled renal allografts

4.1 Introduction

This chapter presents results that characterize immune regulation by B lymphocyte subsets in healthy volunteers and renal transplant recipients based on their allograft histology. By comparing them to a random sample of patients with stable graft function, I will illustrate the potential significance of B cell mediated immune regulation in renal transplantation.

It is important to understand the role of B lymphocytes as regulators of the immune response in the context of renal transplantation since there is emerging evidence for an immune signature in operationally tolerant patients based on the peripheral blood B lymphocytes (Sagoo, Perucha et al. 2010). Recent studies have uncovered several functions of B lymphocytes beyond the production of antibodies including antigen presentation, co-stimulation, germinal centre organisation and cytokine secretion (Janeway, Ron et al. 1987, Ron and Sprent 1987, Harris, Haynes et al. 2000, Shlomchik, Craft et al. 2001, Yanaba, Bouaziz et al. 2008). An immunoregulatory role for B cells, first described in murine colitis (Mizoguchi, Mizoguchi et al. 1997), has since been supported by studies demonstrating that B cell depletion or deficiency can worsen disease, while transfer of B cell subpopulations can suppress inflammatory responses in experimental models (Fillatreau, Sweenie et al. 2002, Mizoguchi, Mizoguchi et al. 2002, Mauri, Gray et al. 2003, Hussain and Delovitch 2007, Yanaba, Bouaziz et al. 2008, Ding, Yeung et al. 2011). Such regulatory B cell (Breg) activity is predominantly IL-10 dependent. However, as yet there is no specific marker for Bregs. In various murine models, B cells expressing phenotypes such as CD5+ (B-1a) (Silverman, Srikrishnan et al. 2008), CD1dhiCD5+ (Yanaba, Bouaziz et al. 2008), CD21hiCD23lo (marginal zone) (Gray, Miles et al. 2007), CD21hiCD23hiCD24hiCD1dlo (transitional)
(Evans, Chavez-Rueda et al. 2007) and TIM-1+ (Ding, Yeung et al. 2011), have been shown to express IL-10 and exhibit regulatory activity. It is noteworthy that even in these “enriched” subsets, IL-10+ B cells remain a minority and it is less well appreciated that these same subsets may contain cells that express other cytokines, some of which are conventionally considered pro-inflammatory (Fillatreau, Sweenie et al. 2002, Mizoguchi, Mizoguchi et al. 2002, Evans, Chavez-Rueda et al. 2007, Blair, Chavez-Rueda et al. 2009).

In humans, CD19+CD24hiCD38hi Transitional B cells (TrB) isolated from peripheral blood, inhibit inflammatory cytokine expression by anti-CD3 stimulated CD4+ T cells in vitro (Blair, Norena et al. 2010). Such Th1 inhibition was dependent on the release of IL-10 and the engagement of CD80 and CD86. TrB from patients with Systemic Lupus Erythematosus (SLE) were defective in IL-10 expression, and were unable to suppress Th1 responses in vitro (Blair, Norena et al. 2010). In contrast another study demonstrated regulatory activity in human B cells, defined by IL-10 expression, was mostly represented in the CD19+CD24hiCD27+ “memory” subpopulation (Iwata, Matsushita et al. 2011). However, in this study, IL-10 expression by B cells in patients with autoimmune disorders was increased compared to healthy controls. This raises the possibility that human Breg populations are heterogeneous similar to their T cell counterparts. However these findings cast doubt upon the definition of a functional property of a cell subset purely based on one cytokine - “the master regulator-IL-10”.

Although human Bregs were defined on the basis of IL-10 expression alone, the significance of potential pro-inflammatory cytokine release by B cells with the same phenotype has not been considered. Despite limited data that human Bregs can inhibit Th1-type cytokine production by T cells, their effect on Th2-type cytokine expression is unclear. Such insights may aid further characterization of Breg subsets and clarify discrepancies in B cell “function” in inflammatory settings such as chronic antibody mediated rejection.
In this chapter, results that help further characterize B cells in human peripheral blood, based upon both pro-inflammatory (TNF-α) and anti-inflammatory (IL-10) cytokine expression will be discussed. Next the ability of various B subsets to regulate either Th1 or Th2 responses from autologous T cells in vitro will be analysed. B subsets will also be studied in renal transplant recipients with either stable graft function or dysfunction with histological evidence of CAMR, with a view to understand the role of B cell mediated immune regulation in renal transplantation.
4.2 Results

4.2.1 Human B cell subsets

PBMCs from 15 healthy volunteers were analysed according to surface expression of CD19, CD24, CD38 and CD27 as shown in Figure 34. Three distinct B cell (CD19⁺) subsets were identified including CD19⁺CD27⁺CD24^hiCD38^hi (transitional B; TrB cells), CD19⁺CD24^hiCD27⁺ (CD24^hi memory/memory B cells; a minority of memory B cells were CD24⁻) and CD19⁺CD27⁺CD24⁺CD38⁻ (mature naïve B cells).

![Figure 34: Definition of human B cell subsets](image)

CD19⁺CD5^hiCD1d^hi B cells were initially described in murine literature as B regulatory cells by their ability to synthesize and secrete IL-10.

Next the distribution of these cells within the three B cell subsets was assessed. Interestingly, the majority of the CD5^hiCD1d^hi B cells were contained within the TrB population as shown in Figure 35. The next step was to see if the phenotypic
differences match cytokine expression by these cells. To address this, these B cell subsets were analysed for cytokine expression.

**Figure 35: CD1d\(^{hi}\)CD5\(^{+}\) B cells and the three B subsets.**

Representative dot plots of CD1d\(^{hi}\)CD5\(^{+}\) B cells (CD19 gate; left panel), analysed for their expression of CD24, CD38 and CD27 (right panels) and cumulative data (mean ± SEM, n=5 subjects; *P<0.0001, **P=0.0001, ***P=0.005 by ANOVA).

### 4.2.2 Stimulation of B lymphocytes for cytokine expression

B cells were isolated from PBMCs by MACS magnetic bead separation and were stimulated for 5hrs with PMA, ionomycin and Brefeldin-A (PIB) or for 48hrs with CPG with or without CD40L. PIB was added to these experiments for the last 5hrs of the cell culture. Figure 36 shows that PIB caused the least expression of IL-10 whilst a combination of CPG and CD40L caused maximal expression. It is noteworthy that the same stimuli that led to maximal expression of IL-10 by B cells also caused an even higher expression of TNF-\(\alpha\). Next, other potentially pro-inflammatory cytokines were
analysed using the same stimulation protocol. At 48hrs whilst there was strong expression of both IL-10 and TNF-α, the percentage positivity for both IFN-γ and IL-17 was minimal (Figure 37). Based on these initial experiments, it was decided to explore the cytokine expression of both IL-10 and TNF-α in the three B subsets as described in section 4.2.1.

**Figure 36: Stimulation of B lymphocytes**

Representative of 3 individual experiments. PBMC and CD19+ gates were selected and CD19+ cells analysed for various cytokines after stimulation by appropriate stimulus as shown for 48hrs

**Figure 37: Analysis of B cell expression of various cytokines by flow cytometry**

Representative of 3 individual experiments. Purified B cells were stimulated for 48hrs and analysed for various cytokines on the B cell gate.
4.2.3 Cytokine polarization-B cell subsets

Magnetic bead separated B cells were stimulated with CPG and CD40L as described before. The three B cell subsets including the transitional, memory and naïve cells were analysed for the expression of both IL-10 and TNF-α. Intracellular staining for IL-10 demonstrated that a significantly higher proportion of both memory and TrB cells express IL-10 compared to naïve B cells (Figure 38A-B). These same B cell subsets expressed TNF-α at a surprisingly high frequency, approximating or even surpassing that of IL-10 expression. TrB cells contained the fewest TNF-α⁺ B cells (Figure 38C-D).

IL-10/TNF-α ratio was used as a measure of cytokine polarization. Such a ratio takes into account the overall cytokine balance either in a pro or anti-inflammatory direction, combining the results of both IL-10 and TNF-α expression by any cell population. Taken together, TrB cells had the highest IL-10/TNF-α ratio whilst naïve B cells had the lowest ratio (Figure 38E). This indicates that the CD24⁺CD38⁺ TrB population exhibited increased cytokine polarization towards IL-10 relative to other B cell subpopulations.

When the B cells were analysed for the expression of both IL-10 and TNF-α at multiple time points, the nature of cytokine polarization within these B subsets remained stable when analysed at 12, 24 and 48hrs (Figure 38H).

So far the results demonstrated that as a subset, TrB cells had the most anti-inflammatory cytokine profile upon in vitro stimulation. However, individual cells can potentially secrete and synthesize more than one cytokine. To address this, individual B cells within each subpopulation were examined to see if they could express more than simply IL-10, using dual-intracellular staining after cell stimulation. This analysis revealed that a significant fraction of IL-10⁺ B cells also co-express the pro-inflammatory cytokine, TNF-α. Whilst TrBs showed the most enhanced IL-10 expression alone, memory B cells had dominant dual IL-10⁺ and TNF-α⁺ populations. Both memory and naïve B cells were enriched for pure TNF-α⁺ cells (Figure 38F-G).

These experiments distinguished both memory and transitional cells with similar levels
of positivity for IL-10. The proportion of IL-10 positive cells that were also TNF-α positive and the total number of TNF-α positive cells within the memory cell population makes them less polarized towards an anti-inflammatory phenotype in vitro when compared to the TrB cells.

Figure 38: B cell subsets and cytokine analysis.

(A) Scatter plots showing IL-10⁺ cells within the respective B subsets after 48hr stimulation with CPG CD40L and PIB. (B) Bar chart representing cumulative results of IL-10 expression in 15 healthy volunteers expressed as mean ± SEM (* TrB vs. naïve, P=0.0002; memory vs. naïve P<0.0001, ANOVA). (C) Scatter plots showing TNF-α⁺ cells within the respective B subsets. (D) Bar chart representing cumulative results of TNF-α expression in 15 healthy volunteers
expressed as mean ± SEM (**TrB vs. memory, P=0.001; TrB vs. naïve, P=0.006, ANOVA). (E) Bar chart representing cumulative mean ± SEM of IL-10/TNF-α ratio across the three B subsets in 15 healthy volunteers (**TrB vs. memory, P=0.002; ****TrB vs. naïve, P<0.0001, ANOVA). (F) Scatter plots showing the analysis by dual staining for both IL-10 and TNF-α. (G) Bar charts showing cumulative results expressed as mean± SEM in 6 healthy volunteers for IL-10, dual and TNF-α expression by B subsets (#TrB vs. naïve, P<0.0001; TrB vs. memory, P=0.01; ## memory vs. naïve, P<0.0001; memory vs. TrB, P<0.0001; ####TrB vs. naïve, P=0.0002; TrB vs. memory, P=0.0004). (H) Graphical representation of IL-10/TNF-α ratio at 12, 24 and 48 hrs in 5 independent healthy volunteers.

As the stimulation of B cells can alter their phenotype, cytokine expression by B cell subsets was re-examined with isolation prior to stimulation in vitro. FACS (Flow Assisted Cell Sort)-purified B cell subsets were stimulated with CpG and CD40L for 48h, and IL-10 and TNF-α in culture supernatants measured by bead based ELISA. The results in Figure 39 parallel those in Figure 38, showing the highest ratio of secreted IL-10 to TNF-α being produced by the TrB cell subset.

Figure 39: IL-10/TNF-α ratios obtained from purified B cell subsets

FACS sorted CD24hiCD38hi, CD24hiCD27+ and CD24+CD38+ B cells were stimulated with CPG and CD40L for 48hrs and supernatants were collected without the addition of PIB. The graphs represent the ratio of the concentration of IL-10 and TNF-α in the supernatants for the respective cell subsets (n=11 subjects for each cell type, * TrB vs. memory, P=0.049; ** TrB vs. naïve, P=0.0002, ANOVA).
In summary, these experiments showed that CD24^{hi} CD38^{hi} TrB cells are most polarized towards an anti-inflammatory cytokine profile when analysed as a subset and when re-assessed on a per cell basis.

4.2.4 TIM-1^+ B cells and their significance

T cell Ig domain and Mucin domain-1 (TIM-1) protein belongs to a family of co-stimulatory molecules that play an important role in the effector differentiation of CD4 T cells (Kuchroo, Dardalhon et al. 2008). TIM-1 has recently been shown to be expressed preferentially on murine B cells and to be an inclusive marker for IL-10^+ murine B regulatory cells (Ding, Yeung et al. 2011).

TIM-1 expression on the surface of the B cells had next been examined and both IL-10 and TNF-\(\alpha\) expression on TIM-1^+ B cells was assessed after stimulation with CpG and CD40L. As seen in Figure 40, TIM-1 expression by human B lymphocytes is minimal and although these cells stain for both IL-10 and TNF-\(\alpha\) when stimulated by CD40L and CPG, Further experiments were not possible owing to the very small numbers of TIM-1^+ cells. TIM-1 expression on the B cell surface did not alter even when cells were stained prior to in vitro stimulation.
TIM1+ B cells were analysed from the B cell gate for both IL-10 and TNF-α in comparison to a negative control.

4.2.5 In vitro assessment of regulatory properties of B cell subsets

To determine the regulatory capacity of each B cell subset, FACS-purified populations (CD19^+^CD24^hi^CD38^hi^, CD19^+^CD24^hi^CD27^+, CD19^+^CD24^hi^CD38^hi^) from healthy volunteers were examined for their ability to inhibit anti-CD3 stimulated autologous conventional (CD4^+^CD25^-^) T cell (Tconv) cytokine expression. Tconv co-cultured with an equal number of CD4^+^CD25^hi^ regulatory T cells (Tregs) served as a positive control. All of the B, Tconv and Treg cells were initially purified by negative selection using Miltenyi magnetic beads prior to cell culture. Sorting gates and purities of the B cell subsets are shown in Figure 41.
Figure 41: Representative sort gates and purities for B cell subsets.

Dot plots showing B cell subsets prior to cell sorting (CD19 gate). Post-sort purity of CD19+ cells (lymphocyte gate). Post-sort purities of B cell subsets (CD19 gate)

After 72hrs of cell culture which included a period of stimulation with PIB for the last 6hrs, cells were washed and analysed by flow cytometry for CD4 T cell expression of TNF-α, IFN-γ and IL-4 in the cell culture conditions mentioned above. TrB cells significantly suppressed TNF-α and IFN-γ expression by autologous Tconv whilst having no effect on IL-4 expression (Figure 42). Selective suppression of pro-inflammatory cytokine expression by TrB cells was comparable with that mediated by Tregs. Neither naïve nor memory B cells were nearly as potent. None of the cell populations had a significant effect on IL-4 expression by Tconv. The summary of results for B cell mediated regulation of T cell cytokines is shown in Figure 43.
The addition of neutralizing anti-IL-10 antibody to TrB co-cultured with autologous Tconv cells restored both TNF-α (Figure 44A-B) and IFN-γ expression (Figure 44C-D) by CD4 cells to the baseline levels observed when Tconv cells were cultured alone. Thus, regulatory activity of TrBs was IL-10 dependent. The influence of TNF-α on regulatory function of memory or naïve B cells was analysed by adding neutralizing antibodies to its receptors, TNFR1 and TNFR2, during co-culture with Tconv (Figure 44). Neutralizing TNFR1 increased the ability of memory B cells to suppress TNF-α and IFN-γ expression to levels comparable to those obtained with TrBs. Neutralization of TNFR2 was somewhat less effective than neutralizing TNFR1. Neutralizing TNFR1 and TNFR2 on naïve B cells also increased suppression, but the effect was much smaller than the effect on memory B cells, perhaps due to lower IL-10 expression by the naïve subset. In summary, neutralizing IL-10 inhibited the regulatory effects of TrBs less regulatory whilst blocking TNF receptors significantly improved the suppressive capacity of both memory and naïve B cells. This highlights the significance of both IL-10 and TNF-α in regulation of CD4 T cells by B cell subsets.

These results match the initial finding of an anti-inflammatory cytokine polarization within the TrB cells and indirectly confirm that B cell subset IL-10 and TNF-α expression (IL-10/TNF-α ratio) correlate with suppressive function. To summarize so far, TrB cells have been shown to have the strongest in vitro anti-inflammatory (regulatory) potential and IL-10/TNF-α ratio serves as a good marker to assess the ability of a cell subset to actively regulate immune responses in vitro.
Figure 42: Assays of B cell regulatory function

Representative dot plots showing the frequency of TNF-α, IFN-γ, IL-4 expression amongst T conv cells in the respective cell cultures (n=6). Cells were gated on CD4 and various representative culture conditions demonstrated.
Figure 43: Cumulative results of the B cell suppression assays.

Bar graphs showing the cumulative (n=6) percentage stimulation/inhibition of the frequency of TNF-α+, IFN-γ+, or IL-4+ cells after culture with Tregs or B cell subsets. Cumulative results expressed as mean± SEM. (*TrB vs. memory, p<0.05; ** TrB vs. naive, p=0.03, #TrB vs. memory, p=0.02; ## TrB vs. naive, p=0.01). Results compared by ANOVA with Dunnet test for multiple comparisons.
Figure 44: IL-10/TNF-α ratio predicts the capacity of the B subsets to suppress autologous Th1 cytokines

Magnetic bead enriched Tconv cells (CD4⁺CD25⁻) were stimulated for 72hrs with plate bound anti-human CD-3 (0.5µg/ml) in the presence of TrB (CD24⁺CD38⁺) B cells, CD24⁺CD27⁺ memory B cells, CD24⁺CD38⁻ naïve B cells or CD4⁺CD25⁺ Treg cells with either an isotype control or neutralizing antibodies to IL-10, TNFR1, TNFR2 or a combination of TNFR1 and TNFR2. PIB was added for the last 6hrs of cell culture. Data derived from 3 healthy volunteers. (A, C) Representative scatter plots showing the detection of TNF-α and IFN-γ by intracellular staining when T conv were cultured alone or in the presence of TrB, memory and naïve B cells with matched isotype controls or neutralizing antibodies to IL-10, TNFR1 or TNFR2. (B, D) Cumulative percentage stimulation/inhibition of the frequency of TNF-α⁺ ($P=0.005$, *$P=0.02$, **$P=0.1$, ###$P=0.8$) and IFN-γ⁺ ($P=0.03$, *$P=0.008$, **$P=0.04$, ###$P=0.2$, ####$P=0.1$) T cells (mean ± SEM) after culture with the respective B subsets and neutralizing antibodies. Statistical analysis was by paired t-test.
4.2.6 Analysis of the subsets of CD24\textsuperscript{hi}CD38\textsuperscript{hi} TrB cells

In experimental animals and human beings, TrB cells have been shown to contain distinct sub-populations (T1 and T2) based on the intensity of surface expression of CD24 and CD38. To pursue further phenotypic characterization of these cell subsets, phenotypic analysis by multi-color flow cytometry was performed in 5 healthy volunteers. T-1 cells were defined as CD19\textsuperscript{+}CD24\textsuperscript{+++}CD38\textsuperscript{+++}CD27\textsuperscript{−} whereas T-2 cells as CD19\textsuperscript{+}CD24\textsuperscript{++}CD38\textsuperscript{+}CD27\textsuperscript{−}. In comparison, mature naïve B cells were labeled as CD19\textsuperscript{+}CD24\textsuperscript{+}CD38\textsuperscript{+}CD27\textsuperscript{−} (Figure 45A). Further analysis of the cell surface phenotype of these sub-population revealed that T-1 cells were IgM\textsuperscript{hi} IgD\textsuperscript{+} CD5\textsuperscript{hi} CD10\textsuperscript{hi} CD20\textsuperscript{hi} CD40\textsuperscript{hi} CD86\textsuperscript{hi} CD23\textsuperscript{+} CD1d\textsuperscript{hi} CD27\textsuperscript{−}; whereas T2 cells expressed CD20\textsuperscript{int} IgD\textsuperscript{+} IgM\textsuperscript{+} CD1d\textsuperscript{+} CD5\textsuperscript{lo} CD10\textsuperscript{lo} CD23\textsuperscript{+} CD40\textsuperscript{+} CD86\textsuperscript{lo} (Figure 45B). Interestingly, a combination of CD10 and IgM were able to clearly distinguish the T-1 subpopulation from the other two subsets of naïve B cells (Figure 45C).

Cytokine expression and functional differences between these transitional B subsets remain unknown. To address this, (CD19\textsuperscript{+}) B lymphocytes were enriched with magnetic beads and the enriched cells were stimulated as described in the previous sections. Analysis of intracellular cytokine expression for IL-10 and TNF-\(\alpha\) reveals that a similar percentage of T1 and T2 cells express IL-10 (Figure 46 A, B). A much smaller percentage of mature naïve B cells are positive for IL-10 in comparison to both T1 and T2 subsets. However, T1 cells have a lower proportion of TNF-\(\alpha\)\textsuperscript{+} cells leading to a higher IL-10/TNF-\(\alpha\) ratio in comparison to both T2 and mature B cells (Figure 46 C, D).

Next, the differences between individual TrB cells were explored with regard to simultaneous expression of both IL-10 and TNF-\(\alpha\), using dual-intracellular staining (Figure 47). This analysis revealed that a significant fraction of T1, T2 and mature naïve B cells co-expressed both IL-10 and TNF-\(\alpha\). Whilst T-1 cells had the highest frequency of B cells expressing IL-10 alone, naïve B populations were enriched for cells expressing TNF-\(\alpha\) alone.
Figure 45: Phenotypic characterization of transitional B subsets

(A). Human B cells are classified into transitional T1, T2 and mature naïve based on the surface expression of CD19, CD24, CD38 and CD27. (B). When the three subsets of cells are analyzed for the expression of other markers, T1 cells are characterized as IgM$^+$ IgD$^+$ CD5$^+$ CD10$^+$ CD20$^+$ CD40$^+$ CD86$^+$ CD23$^+$ CD1d$^+$ CD27$^-$; T2 cells as CD20$^{int}$ IgD$^+$ IgM$^+$ CD1d$^+$ CD5$^{lo}$ CD10$^{lo}$ CD23$^+$ CD40$^{lo}$ CD86$^{lo}$; and mature cells as CD20$^+$ IgM$^+$ IgD$^+$ CD5$^-$ CD1d$^-$ CD10$^-$ CD23$^+$ CD40$^-$ CD86$^-$; (C). A combination of CD10 and IgM are able to clearly distinguish T-1 subpopulation from the other two subsets of naïve B cells. Figures are representative of 5 independent experiments in healthy volunteers.
Magnetic bead enriched CD19+ B cells are stimulated with CpG and CD40L for 48hrs with the addition of PIB in the last 5 hrs of culture. (A). Scatterplots representing the IL-10 and TNFα expression after intracellular staining within T1, T2 and mature subsets in a representative healthy volunteer. (B). Cumulative results of the percentage of IL-10 + cells within each subset (n=15, *P<0.0001 mature subset compared to either T1 or T2). (D). Cumulative results of the percentage of TNFα + cells within each subset (n=15, **P=0.09, T1 vs. T2; ***P<0.0001, T1 vs. Mature & P=0.02, T2 vs. mature). (E). Cumulative results of the IL-10/ TNFα ratio within each subset (n=15, # T1 vs. T2, P<0.0001; ## T1 vs. mature, P<0.0001; T2 vs. mature, P=0.0001). The bars in each graphic represent mean whilst the error bars represent SEM. Statistical analysis was by one way ANOVA with Tukey post hoc correction for multiple comparisons.

Figure 46: Functional characterization of transitional B subsets
Magnetic bead enriched CD19+ B cells are stimulated with CpG and CD40L for 48hrs with the addition of PIB in the last 5 hrs of culture. Both IL-10 and TNFα are analysed by dual cytokine staining (representative of 5 individual experiments). Scatter plots from T1, T2 and naïve subsets with either isotype control or the respective cytokines are demonstrated. The bar chart summarizes the cumulative results (mean ± SEM). *T1 vs. mature naïve, P<0.0001; ** T1 vs. mature naïve, P<0.0001; T1 vs. T2, P=0.04. Analysis was by ANOVA with Dunnet’s correction. T1 and T2, respective TrB subsets; M, mature naïve B subset

4.2.7 Analysis of B cells in renal transplant recipients

Having established the regulatory properties of TrB cells in normal subjects, I next investigated whether quantitative differences exist in renal transplant patients with stable function versus chronic antibody mediated rejection (CAMR). B cells from healthy volunteers (HV; n=15), were compared to 88 renal allograft recipients. Patients were divided into those with; stable function (S; n=40); graft dysfunction with no histological evidence of CAMR (GD-NR; n=22); and graft dysfunction with a histological phenotype of CAMR (GD-R; n=26) based on serial serum creatinine measurements, assessment of urine samples for proteinuria, anti-HLA antibody screening and graft
histology. Clinical parameters of these subjects are detailed in Table 13. Significant differences between the groups included a longer time since transplant for GD-NR and GD-R groups and a higher usage of maintenance prednisolone for immunosuppression in the GD-R group. Histological classification with Banff categories of the GD-R group is detailed in Figure 48. The GD-R group was characterized by the phenotype of chronic antibody mediated rejection (CAMR) with evidence of either glomerulitis or peritubular capillaritis or transplant glomerulopathy with or without the deposition of C4d in the peritubular capillaries. 80% of these patients had a donor specific antibody detected in their serum at the time of the biopsy. Interestingly, 25% of these patients also had a component of T cell mediated rejection detected in their biopsies (tubulitis or interstitial inflammation). All of the grafts in this group had a significant degree of scarring as demonstrated by high ‘cv’, ‘ct’ and ‘ci’ scores.
Figure 48: Summary of Banff scores of the biopsies for GD-R group.

These findings show that the biopsies had a significant degree of chronic damage as suggested by ct, cv and ci scores. g-glomerulitis; ptc-peritubular capillaritis; cg-chronic transplant glomerulopathy; C4d-C4d deposition in the peritubular capillaries; mm-mesangial expansion; t-tubulitis; v-endothelitis; i-interstitial inflammation; ti-total inflammation; ct-tubular atrophy; cv-fibrointimal thickening; ci-interstitial fibrosis; aah-arteriolar hyalinosis. (0-absent, 1-mild & >1-moderate to severe).
### Table 13: Patient Characteristics (healthy volunteers, stable group and GDNR/GDR)

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<td>64%</td>
<td>64%</td>
<td>Ns</td>
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<td>Ethnicity-Caucasian%</td>
<td>68%</td>
<td>91%</td>
<td>92%</td>
<td>95%</td>
<td>0.03^</td>
</tr>
<tr>
<td>Primary Renal Diagnosis</td>
<td>Ns</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glomerulonephritis</td>
<td>n/a</td>
<td>15%</td>
<td>45%</td>
<td>28%</td>
<td>0.01***</td>
</tr>
<tr>
<td>Hypertension/diabetes</td>
<td>n/a</td>
<td>15%</td>
<td>9%</td>
<td>4%</td>
<td></td>
</tr>
<tr>
<td>Inherited</td>
<td>n/a</td>
<td>15%</td>
<td>14%</td>
<td>16%</td>
<td></td>
</tr>
<tr>
<td>Others#</td>
<td>n/a</td>
<td>55%</td>
<td>32%</td>
<td>52%</td>
<td></td>
</tr>
<tr>
<td>Transplantation details</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Graft number</td>
<td>n/a</td>
<td>1.06 (1.15)</td>
<td>1.26 (1.04-1.48)</td>
<td>1.1 (0.95-1.26)</td>
<td>Ns</td>
</tr>
<tr>
<td>Type of allograft donor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ns</td>
</tr>
<tr>
<td>DCD%**</td>
<td>n/a</td>
<td>12%</td>
<td>14%</td>
<td>12%</td>
<td></td>
</tr>
<tr>
<td>DBD%**</td>
<td>n/a</td>
<td>68%</td>
<td>68%</td>
<td>64%</td>
<td></td>
</tr>
<tr>
<td>Live%**</td>
<td>n/a</td>
<td>20%</td>
<td>18%</td>
<td>24%</td>
<td></td>
</tr>
<tr>
<td>Donor cause of death</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ns</td>
</tr>
<tr>
<td>Trauma%</td>
<td>n/a</td>
<td>15%</td>
<td>29%</td>
<td>20%</td>
<td></td>
</tr>
<tr>
<td>Vascular%</td>
<td>n/a</td>
<td>77%</td>
<td>47%</td>
<td>53%</td>
<td></td>
</tr>
<tr>
<td>Other%</td>
<td>n/a</td>
<td>87%</td>
<td>24%</td>
<td>27%</td>
<td></td>
</tr>
<tr>
<td>HLA mismatches</td>
<td>n/a</td>
<td>2.6 (2.1-3.1)</td>
<td>1.6 (1.0-2.3)</td>
<td>2.9 (2.5-3.5)</td>
<td>0.02****</td>
</tr>
<tr>
<td>Donor age in yrs (95% CI)</td>
<td>n/a</td>
<td>39 (33-45)</td>
<td>47 (38-55)</td>
<td>42 (35-49)</td>
<td>Ns</td>
</tr>
<tr>
<td>Allograft function</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age of the graft in months (IQR)**</td>
<td>n/a</td>
<td>56 (43-78)</td>
<td>78 (61-128)</td>
<td>83 (51-153)</td>
<td>0.04^</td>
</tr>
<tr>
<td>Best eGFR achieved (95%CI)</td>
<td>n/a</td>
<td>74 (69-79)</td>
<td>59 (48-69)</td>
<td>66 (56-76)</td>
<td>0.01***</td>
</tr>
<tr>
<td>e-GFR in ml/min (95% CI)</td>
<td>n/a</td>
<td>65 (61-70)</td>
<td>31 (25-36)</td>
<td>29 (22-36)</td>
<td>&lt;0.0001**</td>
</tr>
<tr>
<td>Proteinuria g/L (95% CI)</td>
<td>n/a</td>
<td>0.17 (0.13-0.20)</td>
<td>0.84 (0.51-1.18)</td>
<td>1.47 (0.67-2.26)</td>
<td>0.009**</td>
</tr>
<tr>
<td>Maintenance Immunosuppression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% on CNI***</td>
<td>n/a</td>
<td>97%</td>
<td>92%</td>
<td>83%</td>
<td>Ns</td>
</tr>
<tr>
<td>% on MMF/Azathioprine</td>
<td>n/a</td>
<td>71%</td>
<td>76%</td>
<td>78%</td>
<td>Ns</td>
</tr>
<tr>
<td>% on Prednisolone</td>
<td>n/a</td>
<td>7%</td>
<td>18%</td>
<td>60%</td>
<td>0.01~</td>
</tr>
<tr>
<td></td>
<td>HV</td>
<td>S</td>
<td>GD-R</td>
<td>GD-NR</td>
<td></td>
</tr>
<tr>
<td>---------------------------</td>
<td>-----------</td>
<td>------------</td>
<td>------------</td>
<td>------------</td>
<td></td>
</tr>
<tr>
<td>DSA- HLA Class-1 % positive</td>
<td>n/a</td>
<td>9%</td>
<td>2.5%</td>
<td>48%</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>DSA-HLA Class-2 % positive</td>
<td>n/a</td>
<td>0%</td>
<td>2.5%</td>
<td>36%</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Late cellular rejection%</td>
<td>n/a</td>
<td>0%</td>
<td>23%</td>
<td>25%</td>
<td></td>
</tr>
<tr>
<td>Allograft Pathology</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>GN</td>
<td>n/a</td>
<td>n/a</td>
<td>27%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>n/a</td>
<td>n/a</td>
<td>32%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFTA (scarring)</td>
<td>n/a</td>
<td>n/a</td>
<td>23%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viral Infection</td>
<td></td>
<td></td>
<td>9%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic antibody mediated changes</td>
<td></td>
<td>n/a</td>
<td>n/a</td>
<td>9%</td>
<td></td>
</tr>
<tr>
<td>GN</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glomerulitis (g)</td>
<td>n/a</td>
<td>n/a</td>
<td>0</td>
<td>44%</td>
<td></td>
</tr>
<tr>
<td>Transplant glomerulopathy (cg)</td>
<td></td>
<td>n/a</td>
<td>n/a</td>
<td>0</td>
<td>64%</td>
</tr>
<tr>
<td>Peritubular capillaritis (ptc)</td>
<td></td>
<td>n/a</td>
<td>n/a</td>
<td>0</td>
<td>36%</td>
</tr>
<tr>
<td>Peritubular capillary C4d deposition</td>
<td></td>
<td>n/a</td>
<td>n/a</td>
<td>0</td>
<td>48%</td>
</tr>
</tbody>
</table>

HV- Healthy Volunteers; S-Stable allograft function; GD-NR-Graft dysfunction-no rejection, GD-R-Graft dysfunction-rejection

~significant differences between HV and the rest; ~~ significant differences between GD-R and the rest

*significant difference between group-S and GD-R; **significant difference between S and GD-NR; S and GD-R; ***significant difference between S and GD-NR; ****significant difference between GD-NR and other groups (S vs. GD-NR, p=0.02; GD-NR vs. GD-R, P=0.007). #others include obstruction, chronic pyelonephritis, unknown causes; ##DCD-Donation after Circulatory Death donor, DBD-Donation after Brain Death donor, Live-live donor; ###. As graft age (vintage) is extremely skewed, median and inter quartile ranges have been presented and data analysed by non-parametric test; ####CNI-Calcineurin inhibitors- ciclosporin or tacrolimus. @MMF-Mycophenolate mofetil/ Mycophenolate sodium; All categorical variables were compared using Chi-square test. All continuous variables are analysed using ANOVA with Tukey post-hoc test for multiple comparisons if normally distributed and Kruskal-Wallis test for variables with skewed distribution. Adjusted P values for multiple comparisons after the post-hoc tests are presented, P<0.05 is significant.
4.2.7.1 Analysis of B lymphocyte subsets-Patient groups

All three patient sub-groups along with healthy volunteers were assessed for their B lymphocyte phenotype (Figure 49). Absolute lymphocyte numbers obtained by trucount bead analysis on flow cytometry showed that there was a trend towards the patient groups being more lymphopenic, though only the GD-R group was significantly different from the healthy volunteers (Figure 50A). Further trucount bead analysis of the B cells revealed that both GD-NR and GD-R groups had significantly lower B cells either when analysed as a percentage of the lymphocytes or as an absolute number per microliter of whole blood (Figure 50B-C). It is noteworthy that the two graft dysfunction groups were not significantly different with regards to their B cell numbers as a whole. There was a trend in the healthy volunteers towards a lower B naïve/memory ratio suggesting a relatively higher B memory cell number. This was evident again when healthy volunteers were found to have higher numbers of CD24hiCD27+ memory B cells (Figure 50D-F). Although slightly lower than healthy volunteers, stable patients had no significant change in the quantity of TrB cells (P=0.4). GD-NR group showed a trend towards lower percentage of, and a significantly lower absolute number of, TrB cells compared to HV and S patients. Compared to GD-NR patients, those with CAMR (GD-R) displayed a further decrease in the percentage of TrB cells and their absolute TrB number was not different from GD-NR group (Figure 50 G-H). This was reflective of the B cell lymphopenia in the GD-NR group. Thus, transplant patients with the phenotype of CAMR showed evidence of quantitative decreases in B cells with regulatory potential compared to those with stable graft function and to a certain extent, the GD-NR group as well.
Figure 49: B lymphocyte phenotype in the patient groups

Representative scatter plots demonstrating CD24hiCD38hi TrB cells, CD24hiCD27+ memory B cells and IgD+CD27 naïve B cells in healthy volunteers (HV), stable patients (Stable), graft dysfunction- non-immunological group (GD-NR) and graft dysfunction-CAMR group (GD-R)
Figure 50: Analysis of B lymphocyte subsets in the patient groups

Comparison of absolute number of (A). lymphocytes per \( \mu l \) of whole blood (*\( P=0.003 \)). (B). % of CD19+ B cells (*HV vs. GD-NR, \( P=0.007 \); S vs. GD-NR, \( P<0.0001 \); **HV vs. GD-R, \( P=0.08 \); S vs. GD-R, \( P=0.004 \)). (C). absolute number of B cells across the groups (*HV vs. GD-NR, \( P<0.0001 \); S vs. GD-NR, \( P=0.002 \); **HV vs. GD-R, \( P<0.0001 \); S vs. GD-R, \( P=0.003 \)). (D). B naive/memory ratio (E). % CD24hiCD27+ B cells (*HV vs. S, \( P=0.05 \)) (F). Absolute number of 24hiCD27+ B cells (*HV vs. S, \( P=0.0002 \); HV vs. GD-NR, \( P<0.0001 \); HV vs. GD-R, \( P=0.0001 \). (G). % TrB cells (*HV vs. GD-NR, \( P=0.01 \); GD-NR vs. GD-R, \( P=0.05 \); **HV vs. GD-R, \( P<0.0001 \); S vs. GD-R, \( P<0.0001 \)). (H). absolute number of TrB cells (*HV vs. GD-NR, \( P=0.0001 \); S vs. GD-NR, \( P=0.001 \); **HV vs. GD-R, \( P<0.0001 \); S vs. GD-R, \( P<0.0001 \)). Statistical analysis by one way ANOVA and post hoc correction by Tukey’s test for multiple comparisons. The bars in the graphs represent mean whilst the error bars represent SEM. HV, healthy volunteers; S, stable; GD-NR, graft dysfunction-no rejection; GD-R, graft dysfunction-CAMR
4.2.7.2 Analysis of TrB subsets - patient groups

Within the transitional B cell population, the balance of T1 and T2 TrB cells was examined in healthy volunteers (HV; n=14) and renal allograft recipients (n=88). The trends for both T1 and T2 subsets followed that of the entire TrB population with the percentage of T1 and T2 subsets from S patients being slightly lower to those from HV; GD-NR patients with a trend towards lower proportion of T1 and T2 subsets compared to HV and S patients and GD-CAMR with the lowest percentage of T1 and T2 cells when compared to S patients. When the T1 and T2 balance within the TrB population is assessed in the patient groups using the ratio of T1/T2, the ratio of T1/T2 in S patients is comparable to HV and GD-NR patients have a significantly lesser ratio when compared to the group-S. Again, GD-CAMR patients have a significantly lower ratio of T1/T2 when compared to all the other patient groups (Figure 51).

Figure 51: TrB subsets in patient groups

Comparison of the proportion of T1 cells in the study groups (GD-R vs. S, P<0.0001). Whilst there is a trend towards a lower percentage of T1 cells in S when compared to HV; GD-NR compared to S and GD-NR compared to GD-R, these results did not reach statistical significance. Comparison of the proportion of T2 cells in the study groups (GD-R vs. S, P<0.0001; GD-NR vs. GD-R, P=0.03). Whilst there is a trend towards lower percentage of T2 cells in S when compared to HV and GD-NR compared to S, these results did not reach statistical significance. (E). Comparison of the ratio of T1/T2 cells in the study groups (GD-R vs. S, P<0.0001; GD-R vs. GD-NR, P=0.02; GD-NR vs. S, P=0.04).The bars in each graph represent mean whilst the error bars represent SEM. Statistical analysis is by one way ANOVA with Tukey post hoc correction for multiple comparisons.
4.2.7.3 *Analysis of cytokine polarization in the B subsets - patient groups*

As there is a significant difference in the number of TrB cells in patients with CAMR, their functional aspect was further looked into. The first step was to assess the cytokine profiles of the TrB cells in different patient groups as described before. CPG and CD40L stimulated B cells were gated for TrB cells and intra-cellular cytokine analysis for both IL-10 and TNF-α revealed that TrB cells in all groups including healthy volunteers were not significantly different with regards to the proportion of IL-10* cells (Figure 52B). Then, the difference in cytokine polarization in the patient groups by IL-10/TNF-α ratio was assessed. Patients with stable graft function were not significantly different from healthy volunteers in their cytokine polarization. When compared to stable patients there is a trend towards a lower IL-10/TNF-α ratio in the GD-NR group. Patients in the GD-R group had the lowest IL-10/TNF-α ratio when compared to all the other groups suggesting a relative dominance of TNF-α expression in the TrB cells in this group of patients (Figure 52A). Interestingly, when whole B cells were assed for IL-10/TNF-α ratio, there was no significant difference between the clinical groups (Figure 52C). These results revealed functional alterations within the same cell subset (TrB) in different clinico-pathologic settings. As a significant number of GD-R patients were on maintenance steroids when compared to the other groups, I have re-analysed the IL-10/TNF-α ratio within the GD-R group divided into sub-groups based on the use of corticosteroids. Figure 52D shows that there was a non-significant trend towards lower IL-10/TNF-α ratio in patients on maintenance steroids. Therefore, the IL-10/TNF-α ratio in the GD-R group despite being affected by the use of corticosteroids was still significantly different from the remaining groups.
**Figure 52: TrB cytokine analysis-patient groups**

Bar graphs (mean ± SEM) comparing healthy volunteers and patient groups (HV, healthy volunteers; S, stable graft function; GD-NR, Graft dysfunction - no rejection; GD-R, Graft dysfunction-rejection) for (A-C). IL-10/TNF-α ratio-TrB (♀ S vs. GD-R, P=0.0002, φGD-NR vs. GD-R, P=0.03), %IL-10 for TrB and IL-10/TNF-α ratio whole B cells. (D). IL-10/TNF-α ratio for TrB cells in patient groups - influence of maintenance corticosteroids. Pred-maintenance corticosteroids, no pred-no maintenance corticosteroids. *S vs. GD-R (no pred), P=0.02; **S vs. GD-R (pred), P=0.0002; #GD-NR vs. GD-R (no pred), P=0.1; ##GD-NR vs. GD-R (pred), P=0.008

**4.2.7.4 In vitro assessment of B cell subset-mediated regulation - patient groups**

To determine whether the observed difference in cytokine profile of TrBs between patient groups correlated with qualitative differences in regulatory function, the ability of TrB to inhibit cytokine expression by anti-CD3 stimulated autologous Tconv cells was
examined (Figure 53). Purified TrB cells from transplant patients with stable allograft function and those from healthy volunteers exhibited similar levels of inhibition of IFN-γ and TNF-α expression by Tconv cells, while sparing IL-4 expression. In contrast, in patients with CAMR (GD-R), TrB significantly suppressed IL-4 expression but exerted a minimal effect on TNF-α and IFN-γ expression by autologous Tconv. As a control, Tregs from the same patients were assessed. Unlike their B counterparts, Tregs from patients with rejection were no different in their activity in comparison to those from patients with stable function, or healthy controls. Since the same number of TrB cells were added in each assay, the principal difference in TrB cells from GD-R and S patients was a decrease in their IL-10/TNF-α ratio. These results confirmed the advantage of the IL-10/TNF-α ratio over IL-10 expression alone in predicting Breg activity.
Figure 53: Analysis of B regulatory function in vitro in S and GD-R groups

(A). Representative dot plots for 1 out of 5 patients in the GD-R group for TNF-α, IFN-γ and IL-4 expression by Tconv cultured alone or in the presence of TrB cells or Tregs. Numbers in each plot indicate frequency of Tconv cells expressing each cytokine under respective culture conditions. (B): Graphical representation (mean ± SEM) of the cumulative results for the suppressive/stimulatory effect of the TrB cells or Tregs on the Tconv cytokine expression in HV (n=6), stable (n=7) and GD-R (n=5) groups ($ S vs. GD-R: TNF-α, P=0.0003; $$ IFN-γ, P=0.002; $$$ IL-4, P<0.0001) Statistical analysis is by ANOVA using Tukey post hoc correction.
This far the regulatory properties of TrB cells were demonstrated:

1. By the virtue of their overlapping phenotype with the murine prototype of Bregs, i.e. CD5$^{hi}$ CD1d$^{hi}$ cells.

2. By their ability to express cytokines that are polarized to an anti-inflammatory phenotype.

3. By in vitro assays to demonstrate their ability to selectively suppress pro-inflammatory Th1 responses.

4. In the clinical setting of renal transplantation, where their cytokine polarization is altered to a pro-inflammatory phenotype in the context CAMR. There was also loss of in vitro suppression of the pro-inflammatory Th1 cytokine expression by autologous Tconv cells but a paradoxical suppression of Th2 response.

4.2.7.5 Clinical significance of TrB IL-10/TNF-α ratio & the distribution of T1/ T2

Finally, the clinical significance of altered cytokine polarization and distribution of T1 and T2 within the TrB subsets was analysed. When the IL-10/TNF-α ratio for patients with stable grafts and those with rejection was analysed by building a Receiver Operating Characteristic (ROC) curve, the area under the curve was reasonable at 0.823, P<0.0001. In comparison the AUC for T1/T2 ratio was 0.88, P<0.0001. This suggested the ability of both TrB IL-10/TNF-α ratio and TrB-T1/T2 to distinguish patients with stable grafts from those with CAMR, although, T1/T2 ratio seems to offer a slightly greater AUC (Figure 54)

Next, graft outcomes were analysed over a period of 3 years after the biopsy by the Kaplan Meier method. For this analysis, only 44 patients with graft dysfunction were included as patients with stable graft function would potentially induce a bias in the favour of the TrB IL-10/TNF-α ratio. The 44 patients with graft dysfunction were divided
into two groups at the mean IL-10/TNF-α ratio of this group of 0.75. There are 28 patients with a ratio<0.75 and 15 patients with a ratio>0.75. Table 14 details the characteristics of both these sub-groups. Patients within the lower IL-10/TNF-α ratio group were more likely to be on maintenance corticosteroids, had a higher incidence of anti-HLA-DSA, and had higher mean g, ptc and cg scores on their biopsy. None of these differences were statistically significant, owing to small group numbers.

**Figure 54: Clinical significance of or T1/T2 ratio or IL-10/TNF-α**

ROC curves for T1/T2 ratio (AUC=0.88) or IL-10/TNF-α ratio (AUC=0.823) to demonstrate the ability of these parameters to distinguish stable grafts from those with CAMR.
Table 14: Characteristics of patients - IL-10/TNF-α groups

<table>
<thead>
<tr>
<th>Variable</th>
<th>Low IL-10/TNF-α ratio</th>
<th>High IL-10/TNF-α ratio</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>28</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>50 (43-56)</td>
<td>52 (47-58)</td>
<td>Ns</td>
</tr>
<tr>
<td>Time since transplant</td>
<td>108 (80-136)</td>
<td>92 (64-121)</td>
<td>Ns</td>
</tr>
<tr>
<td>HLA-MM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.92 (0.7)</td>
<td>0.7 (0.7)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.9 (0.6)</td>
<td>0.8 (0.8)</td>
<td></td>
</tr>
<tr>
<td>DR</td>
<td>0.6 (0.6)</td>
<td>0.3 (0.5)</td>
<td></td>
</tr>
<tr>
<td>Clinical features</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSA</td>
<td>46.4%</td>
<td>20%</td>
<td>Ns</td>
</tr>
<tr>
<td>eGFR at biopsy</td>
<td>27.4 (22.4-32.4)</td>
<td>33 (26.9-39)</td>
<td>Ns</td>
</tr>
<tr>
<td>Proteinuria</td>
<td>116 (63-168)</td>
<td>119 (23-215)</td>
<td>Ns</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Late TCMR</td>
<td>18%</td>
<td>20%</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>0.75 (1.06)</td>
<td>0.29 (0.48)</td>
<td>Ns</td>
</tr>
<tr>
<td>Ptc</td>
<td>0.68 (1.1)</td>
<td>0.14 (0.38)</td>
<td></td>
</tr>
<tr>
<td>C4d deposition PTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cg</td>
<td>1.19 (1.1)</td>
<td>0.57 (0.78)</td>
<td></td>
</tr>
<tr>
<td>Cv</td>
<td>0.8 (0.9)</td>
<td>0.7 (0.8)</td>
<td></td>
</tr>
<tr>
<td>Ci</td>
<td>1.9 (0.8)</td>
<td>1.3 (1.0)</td>
<td></td>
</tr>
<tr>
<td>Ct</td>
<td>1.9 (0.8)</td>
<td>1.5 (0.8)</td>
<td></td>
</tr>
</tbody>
</table>

Mean ± SD or percentages of the group are shown in the table. Statistical analysis was by non-parametric Mann Whitney U test.

When these patients were assessed for the clinical end-point of graft loss by the Kaplan Meier method, there was a trend towards worse graft survival in patients with lower IL-10/TNF-α ratio. This trend persisted even when the analysis was repeated in patients with histological evidence of CAMR (Figure 55A and D). In view of the short follow-up period, the utility of TrB IL-10/TNF-α ratio was re-analysed using a composite end-point of graft loss or a doubling of serum creatinine. The results in Figure 55B
show that patients with graft dysfunction & a low IL-10/TNF-α ratio had significantly lower survival, free of the composite end-point, when compared to their counterparts with a higher ratio. A trend nearly achieving statistical significance was seen in patients with CAMR as well (Figure 55 E). When the same analysis for the composite end-point was repeated for T1/T2 ratio, as seen in Figure 55 C and F, the trends were not as significant.

These results highlight the clinical significance of the functional alteration within a small subset of B cells as their regulatory properties were altered in patients with CAMR. Although T1/T2 ratio, which is reflective of this functional alteration, at least in part provides good separation of stable patients from those with CAMR, TrB-IL-10/TNF-α ratio provided a much useful predictor for graft outcomes.
Figure 55: Clinical significance of IL-10/TNF-α or T1/T2 (graft outcomes)

(A, D). Kaplan-Meier survival analysis for high and low IL-10/TNF-α (TrB) for graft loss over 3 years in patients with graft dysfunction (B, E). Kaplan-Meier survival analysis for the high and low IL-10/TNF-α (TrB) for 100% increase in serum creatinine (doubling of serum creatinine) and or graft loss over 3 years in patients with graft dysfunction. (C, F). Kaplan Meier survival analysis for the high and low T1/T2 (TrB) for 100% increase in serum creatinine and or graft loss over 3 years in patients with graft dysfunction. Groups compared using Log Rank test.
4.3 Discussion

Bregs have been shown to play an important role in regulating immune responsiveness through IL-10 expression in a variety of autoimmune and transplant animal models (Mizoguchi, Mizoguchi et al. 1997, Mizoguchi, Mizoguchi et al. 2000, Fillatreau, Sweeney et al. 2002, Mizoguchi, Mizoguchi et al. 2002, Evans, Chavez-Rueda et al. 2007, Gray, Miles et al. 2007, Hussain and Delovitch 2007, Mann, Maresz et al. 2007, Matsushita, Yanaba et al. 2008, Yanaba, Bouaziz et al. 2008, Ding, Yeung et al. 2011). Their role in human disease is less clear with studies showing that IL-10 is enriched in either TrB (CD24hiCD38hi) or memory B cells (CD24hiCD27+) and IL-10 expression may be unchanged, elevated, or reduced in patients with various autoimmune diseases (Duddy, Niino et al. 2007, Correale, Farez et al. 2008, Yanaba, Bouaziz et al. 2008, Blair, Norena et al. 2010, Iwata, Matsushita et al. 2011).

The results of the experiments discussed in this chapter suggest that renal transplant recipients with histological evidence of rejection have both a reduced number of, and functionally defective, CD19+CD24hiCD38hi transitional B cells, in contrast to immunosuppressed patients with stable function. By directly comparing human TrB and memory B cell subsets, shown by others to be enriched for IL-10+ Bregs (Blair, Norena et al. 2010, Iwata, Matsushita et al. 2011), I have shown that the CD24hiCD38hi TrB cell subset is most potent in suppressing pro-inflammatory cytokine expression by Tconv in vitro. Importantly, this is not due to a higher frequency of IL-10 expressing cells within the TrB population, but rather diminished expression of pro-inflammatory cytokines compared to memory B cells. In fact B cells within naïve, memory, and TrB subsets are not only capable of expressing IL-10, but also TNF-α. The results from this chapter demonstrate that a high IL-10/TNF-α ratio, which suggests cytokine polarization, is a
better indicator of B cell regulatory activity than IL-10 expression alone. This consideration may explain inconsistencies between earlier studies.

Correlation of the suppressive activity of TrB cells from patients with different clinical outcomes lends support to the validity of the IL-10/TNF-α ratio as a marker of B cell mediated immunoregulation. Despite exhibiting the same frequency of IL-10 expression, TrB cells from GD-R patients displayed a lower IL-10/TNF-α ratio and had reduced ability to inhibit Th1 T cell differentiation than those from transplant recipients with stable function (S). Th1 responses are generally considered deleterious, whereas Th2 responses promote tolerance in experimental animals (Sho, Yamada et al. 2002). In GD-R patients, this was compounded by a decrease in the number of TrB cells. While causality cannot be established in this retrospective study, these Breg “abnormalities” correlated with graft damage, in particular that mediated by CAMR. The IL-10/TNF-α ratio in TrB not only identifies patients with CAMR but in those with graft dysfunction, there was an association between a low ratio and adverse graft outcomes at 3 years justifying further validation of this simple calculation.

There are several important potential consequences of this pro- and anti-inflammatory heterogeneity in B cell function. Firstly TrB cells are not only more polarized towards an anti-inflammatory function as a subset, but individual cells within that subset are more likely to express IL-10 alone (without TNF-α co-expression) compared to either memory or naïve B cells. Thus, while the total number of memory and especially naïve B cells expressing IL-10 is not significantly lower than TrB cells, a greater proportion of the former, express or co-express inflammatory cytokines and are thus phenotypically distinct resulting in less suppression of T cell responses. Moreover, in different clinical or pathological states, the degree of polarization can change. Taken together, this B cell duality makes it unlikely that any single cytokine expressing subset will adequately define human Bregs. Rather, the findings presented in this chapter indicate that cytokine polarization is the best predictor of Breg activity, at least in an in vitro setting.
Based on these findings a model for TrB cell mediated immune regulation of T cells can be drawn (Figure 56) In the future, other combinations of both pro-inflammatory and anti-inflammatory cytokines may further refine human Breg definition.

Figure 56: Putative model for TrB mediated immune regulation

Graphic in the top panel is a hypothetical representation of Breg mediated immune regulation in patients with stable function. In this model, a polarized IL-10/TNF-α ration towards IL-10
favours Th2 differentiation and promotes graft stability and as shown in the bottom panel, reversal of this polarization results in Th1 skewing and allograft rejection. However this model has only been examined in a non-cognate fashion and need a further study to examine alloresponse.

A combination of CD40 ligand (CD40L) and Cytosine Guanine-Oligodeoxynucleotide) CPG-ODN 2006 was used to stimulate B cells for cytokine detection. CPG-ODN 2006 is Toll like receptor-9 (TLR-9) agonist and has been shown to up regulate the production of IL-10 by the B cells (Barr, Brown et al. 2007). Agonistic stimulation of TLR-9 has been shown to alter the course of various autoimmune diseases in murine models (Quintana, Rotem et al. 2000, Wu, Sawaya et al. 2007). In human beings, decreased expression of TLR-9 has been shown to increase predisposition to autoimmune conditions like Systemic Lupus Erythematosis (SLE) (Tao, Fujii et al. 2007). CD40 is a membrane associated protein and belongs to the TNF receptor superfamily. Prolonged and strong signals via cross linking of CD40 by CD40L (CD154) on the surface of T cells in vivo inhibit antibody production by B cells (Miyashita, McIlraith et al. 1997). Several studies have shown that the interaction of CD40-CD40L is a key pathway in the induction of Bregs (Mizoguchi, Mizoguchi et al. 2000, Fillatreau, Sweenie et al. 2002). A recent study by Tedder’s group in Durham NC has shown that stimulation of B cells by a combination of CD40L and CPG-ODN 2006 provides optimal stimulation for the expression of IL-10 by the B cells (Iwata, Matsushita et al. 2011). It is worthwhile to note that TLR engagement on different B cells is associated with differential expression of multiple cytokines (Gray, Gray et al. 2007, Lampropoulou, Hoehlig et al. 2008). I have focussed on the expression of TNF-α (a prototype pro-inflammatory cytokine) along with IL-10 by B cell subsets after stimulation with a combination of CPG and CD40L.

In humans, Bregs have predominantly been studied in the context of autoimmune disease. A significant reduction in the number of IL-10+ B cells was reported in patients
with multiple sclerosis (MS) (Duddy, Niino et al. 2007, Correale, Farez et al. 2008). In patients with relapsing MS, B lymphocyte mediated inflammatory cytokine responses were shown to be associated with disease activity, *in vitro* T cell proliferation and cytokine release. These responses were modified with B cell depletion using rituximab (Bar-Or, Fawaz et al. 2010). CD24hiCD38hi Bregs in patients with SLE have been shown to produce less IL-10 in response to CD40L, but not after stimulation with CpG (Blair, Norena et al. 2010). However, in SLE, TLR stimulation is an important component of B cell activation (Green and Marshak-Rothstein 2011). These B cells were also unable to suppress the differentiation of pro-inflammatory cytokine producing T cells *in vitro*. In contrast, Iwata et al. have described a significantly higher number of IL-10+ B cells following *in vitro* stimulation with CpG and CD40L in patients with various autoimmune disorders, but functional studies were not performed (Iwata, Matsushita et al. 2011). The observations in this chapter indicate that the study of IL-10 expression alone by Breg populations does not correlate well with clinical outcomes at least in the setting of clinical renal transplantation.

As a refinement to the study of the cytokine expression by the B cell subsets, the potential reasons that underlie such a difference within the cytokine profiles of the same subset of cells in different clinical conditions (i.e. stable vs. GD-R) are explored. Interestingly the transitional B cells which have been identified as potential B regulatory cells in human beings (Blair, Norena et al. 2010) are indeed heterogeneous and distinct subsets (i.e. T1 and T2) can be identified both by phenotypic markers and by their ability to express pro and anti-inflammatory cytokines *in vitro*. Although both the subsets are significantly reduced in patients with CAMR, there is a relative depletion of T1 cells in comparison to the T2 subset. The distribution of T1/T2 has been identified and validated as a simple biomarker for the prediction of graft deterioration in renal transplant recipients.
CD10, CD24 and CD38 have been previously shown to be immature markers in human bone marrow (Tedder, Clement et al. 1984, Loken, Shah et al. 1987, Meffre, Casellas et al. 2000). By using a combination of CD24, CD38, IgM and CD10, this data showed that transitional B cells can be divided into subsets with T1 cells expressing CD10\textsuperscript{hi}, CD24\textsuperscript{++} CD38\textsuperscript{+++} IgM\textsuperscript{+} and T2 cells expressing CD10\textsuperscript{lo} CD24\textsuperscript{++} CD38\textsuperscript{+++} and IgM\textsuperscript{lo}. In contrast to previous studies in both humans and mice, we noticed that T1 B cells had a relatively higher expression of CD40 and CD5 (Loder, Mutschler et al. 1999). However the initial B cells to emerge from the human bone marrow following stem cell transplantation have been shown to be CD5\textsuperscript{*} in a different study (Waddick and Uckun 1993). In murine B cells, a subset that express CD5 are predominantly found in the peritoneum and have been shown to secrete and synthesize high levels of IL-10 and are labelled as regulatory B cells (Mizoguchi, Mizoguchi et al. 1997, Mizoguchi, Mizoguchi et al. 2002). In our study, T1 B cells expressed CD5 and were differentiated from other B cells by their response to CD40L and CpG. Indeed it has been previously shown that transitional B cells can be divided into subsets that differ in response to T cell signalling (Chung, Sater et al. 2002). Using the same principle and by studying the effect of TLR mediated stimulation, it was demonstrated that T1 cells differ from T2 cells by relatively limited expression of TNF-\(\alpha\). Whilst T1 and T2 cells can be differentiated based on TNF-\(\alpha\) expression, T2 cells can be differentiated from mature naïve B cells by greater expression of IL-10 along with the other phenotypic markers thereby establishing that T2 cells are distinct from mature naïve B cells.

There are recent reports that suggest transitional B cells are characterized by reduced survival, proliferation, differentiation and chemotaxis (Carsetti, Rosado et al. 2004, Sims, Ettinger et al. 2005, Cuss, Avery et al. 2006). Both in animals and human beings, isolated BCR mediated stimulation of transitional B cells induced apoptosis or cell death by neglect rather than proliferation (Carsetti, Kohler et al. 1995, Loder, Mutschler et al. 1999, Su and Rawlings 2002, Sims, Ettinger et al. 2005). This process is believed to represent a key negative selection check point for auto-reactive B cells and thus may
play a vital role in the pathogenesis of various autoimmune diseases. However T cell signals mediated via CD40-CD40L interactions can overcome BCR mediated apoptosis of immature B cells and induce proliferation (Tsubata, Wu et al. 1993, Parry, Hasbold et al. 1994). I have also shown that transitional B cells fail to suppress pro-inflammatory cytokine expression by autologous T cells \textit{in vitro}, in patients with a histological phenotype of CAMR. The finding that not only are the transitional B cells reduced in number in CAMR but also there is an altered distribution of the subsets within the TrB cells may potentially explain the differences in the cytokine polarization noted within the TrB cells in CAMR. This is important because, T1 cells have the most anti-inflammatory polarization and despite their limited number and short life-span it is possible that these cells act as regulatory cells. Interestingly these cells are relatively reduced in comparison to T2 cells in patients with CAMR. Of course there are several possibilities:

1. These cells act as a reservoir for a much smaller number of B cells that mature into antigen-specific regulatory cells in secondary lymphoid organs

2. They are not detected in the periphery or are lost in assessing the overall population given the broad markers that are used and lack of antigen specificity in these experiments

3. T1:T2 ratio is a good predictor of antigen-driven maturation of B cells. So, if changes in B cell phenotype were analysed longitudinally over time alterations could potentially be seen in most patients, especially with the onset of immunopathology.

The findings in this chapter are supported by recent studies showing that renal transplant patients who were “operationally tolerant” (no maintenance immunosuppression), exhibit higher numbers of TrB cells than their non-tolerant counterparts, although the potentially confounding effects of immunosuppression in the latter group cannot be excluded (Newell, Asare et al. 2010, Pallier, Hillion et al. 2010, Sagoo, Perucha et al. 2010).
Patients in the GD-R group exhibited microvascular inflammation and scarring with or without complement (C4d) deposition, and had serological evidence of circulating donor specific HLA antibody in the majority of patients (80%) - all features of CAMR. However, 25% of these patients had a component of acute cellular rejection superimposed. Whether Breg abnormalities precede development of antibody-mediated rejection (suggesting causality) or are also involved in acute cellular rejection, must await prospective longitudinal study. However, the findings highlighted in this chapter raise the possibility that in addition to affecting T cell effector function, Bregs may also play a role in CAMR. Notably, CAMR with features of microcirculatory inflammation with or without C4d deposition within the peritubular capillaries is proposed as the major cause of chronic renal allograft loss (Kieran, Wang et al. 2009, Gaston, Cecka et al. 2010, Brouard, Renaudin et al. 2011, Gaston 2011).

Another limitation of these studies is that patients who had an acute cellular component to their rejection on biopsies were often initiated on treatment with corticosteroids before blood samples could be obtained. Thus, more patients with rejection were on maintenance corticosteroids. However GD-R patients who were not receiving corticosteroids still had a significantly lower number of TrB cells and these cells had a lower IL-10/TNF-α ratio when compared to both healthy volunteers and patients with stable graft function. Finally, it should be noted that the GD-NR group was heterogeneous and at least some patients may have had “immune-mediated” pathology which may explain why TrB cells and the IL-10/TNF-α ratio were reduced from S patients (stable graft function) and healthy volunteers, while remaining significantly higher than the GD-R group.

The studies described in this chapter provide novel insights into the description of B cell mediated immunoregulation in humans. Since a significant number of these cells express pro-inflammatory cytokines upon in vitro stimulation, it is not unreasonable to conclude that any definition of Breg phenotype should take this finding into account.
Using such a strategy, I have shown that CD24<sup>hi</sup>CD38<sup>hi</sup> B cells outperformed other B subsets in their regulatory potential defined by cytokine polarization, and this correlated with clinical status and was associated with medium term outcomes in renal transplant recipients. Finally, a simple measurement of TrB T1/T2 ratio on blood phenotyping may provide a valuable biomarker for predicting graft outcomes in renal transplant recipients.
Chapter-5: B lymphocyte phenotype & alemtuzumab in renal transplantation

5.1 Introduction

In the previous chapter CD24<sup>hi</sup>CD38<sup>hi</sup> transitional B cells, by virtue of cytokine polarization were shown to demonstrate immuno-regulatory properties and that these cells were shown to be reduced in number with an altered phenotype when tested in vitro in renal transplant recipients with chronic antibody mediated rejection (CAMR). Now, results that will test the utility of an immune signature based on the peripheral B lymphocyte phenotype in renal transplant recipients will be presented. The study setting was a randomized controlled trial, comparing alemtuzumab and basiliximab induction in a steroid free immunosuppression regime, wherein patients were maintained on either tacrolimus or tacrolimus with mycophenolate mofetil for maintenance immunosuppression. Alemtuzumab is a mouse monoclonal IgG<sub>1</sub>-κ antibody against CD52 and causes profound depletion of lymphocytes through complement mediated cell lysis (Hale, Bright et al. 1983). This agent has been shown to cause profound T lymphocyte depletion and is used as an induction agent in solid organ transplantation (Weaver and Kirk 2007, Newell, Cendales et al. 2008). Basiliximab is a chimeric mouse human monoclonal antibody to the α chain of IL-2 receptor and has been shown to significantly reduce the rate of acute rejection episodes when used as induction immunosuppression in renal transplant recipients (Nashan, Moore et al. 1997).

The experience in renal transplantation with alemtuzumab has generally been positive with good medium term success in published studies (Knechtle, Fernandez et al. 2004, Clatworthy, Friend et al. 2009, Cai and Terasaki 2010, Chan, Taube et al. 2011, Hanaway, Woodle et al. 2011). Further interest in alemtuzumab induction was
generated by the demonstration that the induction of tolerance in animal models can be facilitated by profound T cell depletion (Wells, Li et al. 1999). Thus, clinical studies were carried out to examine its role in novel immunosuppression regimes consisting of minimal or calcineurin inhibitor free maintenance treatment (Knechtle, Pirsch et al. 2003, Kirk, Mannon et al. 2005). These trials yielded unacceptably high rates of acute rejection; however other studies in which alemtuzumab induction was followed by long-term tacrolimus or ciclosporin monotherapy as maintenance therapy yielded acceptable results with excellent graft function (Vathsala, Ona et al. 2005, Margreiter, Klempnauer et al. 2008, Tan, Donaldson et al. 2009).

The first clinical studies in renal transplantation demonstrated that alemtuzumab caused profound and prolonged depletion of T lymphocytes (Watson, Bradley et al. 2005). CD4+ T cells take the longest time to repopulate and the reconstitution may not be complete more than one year after treatment. It has also been shown in human and animal models of both autoimmune and alloimmune disease that the reconstituted CD4+ T cell pool consists of cells with a predominant memory phenotype (CD45RO+)(Brett, Baxter et al. 1996, Trzonkowski, Zilvetti et al. 2008). Such CD4+ memory cells have also been identified in the biopsies of patients undergoing T cell mediated rejection of renal allografts after induction with alemtuzumab (Gallon, Gagliardini et al. 2006). Furthermore, a relative increase in the population of CD4+ CD25+ FoxP3+ T regulatory cells has been demonstrated in recipients of renal transplants following alemtuzumab induction (Bloom, Chang et al. 2008). It has also been shown that CD8+ T cells re-establish themselves more rapidly than CD4+ cells and that some of these cells possess a regulatory phenotype (Trzonkowski, Zilvetti et al. 2008).

Human B cells also express CD52 and they are profoundly depleted following alemtuzumab induction. The reconstitution of B cell subsets has not previously been studied in human renal transplantation. In the autoimmune field it has been demonstrated that B cells reconstitute within three months of treatment with a predominance of the mature naïve phenotype (CD19+CD27-) and that this is
associated with a sustained increase in levels of B-cell activating factor (BAFF) (Thompson, Jones et al. 2010). The recent association of certain B cell phenotypes with operational tolerance in renal allografts and the demonstration of immune regulation by certain human B cell subsets, encouraged me to study the reconstitution of B cells following alemtuzumab induction and compare this with standard basiliximab induction at our institute in Leeds, in the context of a clinical trial (Lund and Randall 2010, Mauri and Blair 2010, Newell, Asare et al. 2010, Pallier, Hillion et al. 2010, Sagoo, Perucha et al. 2010).
5.2 Results:

A total of 120 patients were recruited for this study as shown in Figure 57. Following randomisation one patient withdrew and two patients did not proceed to transplantation. One patient was lost to follow up. Of those remaining, 58 were randomised to each treatment arm. Of the enrolled 120 patients, 96 were tested for peripheral blood lymphocyte phenotype and for the detection of HLA specific antibodies.

![Flow chart showing patient recruitment into the clinical trial](image)

5.2.1 Clinical outcomes

Table 15 shows key comparative parameters for the two groups. No differences were apparent between the groups in mean age, gender ratio, causes of renal disease (including percentage of diabetics pre-transplant), transplant types, mean HLA
mismatches, CMV statuses, cold and warm ischaemic times, or percentage of extended criteria donors.

**Table 15: Characteristics of the study population**

<table>
<thead>
<tr>
<th>Demographic</th>
<th>Alemtuzumab group</th>
<th>Basiliximab group</th>
<th>P Value*</th>
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</thead>
<tbody>
<tr>
<td>Total participants</td>
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<td>58</td>
<td></td>
</tr>
<tr>
<td>No with Phenotyping</td>
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<td>45</td>
<td></td>
</tr>
<tr>
<td>No with HLA specific antibody testing</td>
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<td>45</td>
<td></td>
</tr>
<tr>
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<td>Causes of ESRD (number)</td>
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<td>Extended criteria donors</td>
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<td>Mean HLA mismatches (mean)</td>
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<tr>
<td>HLA-A</td>
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<td>Total warm ischaemia time (mean±SD)</td>
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<td>Donor pre-transplant creatinine (mean±SD)</td>
<td>89.1 ± 43.5</td>
<td>93.0 ± 45.9</td>
<td></td>
</tr>
</tbody>
</table>

*Continuous variables are compared by independent samples student t test whilst categorical variables are compared by chi-square test
5.2.1.1 Assessment of Renal function

The primary outcome measure was the isotopic GFR at 1 year after transplantation. Mean uncorrected isotopic GFR at one year was 57 ± 26 ml/min in the alemtuzumab group and 53 ± 21 ml/min in the basiliximab group (p=0.42). These figures include patients returned to dialysis by one year, each with an assumed GFR of 5 ml/min. Neither excluding such patients, nor correcting isotopic GFRs for recipient body surface area (nor both) demonstrated any difference between the groups. One year mean creatinine was 147.1 ± 93.7 µmol/L (alemtuzumab group) and 151.4 ± 46.6 µmol/L (basiliximab group; p=0.769). Latest mean MDRD eGFRs for the two groups are 57 ± 20 ml/min (alemtuzumab) and 51 ± 19 ml/min (basiliximab; p=0.106); median time to latest eGFR was 41 months (range 12.4-62.2 months, at January 2012). Patient survival was 96.6% in both groups. Four deaths occurred in total – one cardiovascular death in each group, one death from sepsis in the basiliximab group, and one death from malignancy (an EBV driven B cell lymphoma) in the alemtuzumab group. Three grafts were lost in each group – graft survival 94.8% (basiliximab group) 94.9% (alemtuzumab group, where n=59 for graft survival, since the lost to follow up patient had a functioning graft at one year, but that was the only information obtainable). Two cases of primary non-function occurred in the basiliximab group. Intractable rejection leading to graft loss was seen in both groups – two cases in the alemtuzumab group (one associated with deliberate non-compliance) and one in the basiliximab group. A case of progressive interstitial fibrosis and tubular atrophy leading to graft loss was also seen in the alemtuzumab group.

5.2.1.2 Rejection episodes

By 12 months, 6 patients had biopsy proven acute rejection in the alemtuzumab group, compared to 14 in the basiliximab group (p=0.049). A 7th patient in the alemtuzumab was clinically treated for rejection without biopsy proof and responded as would be expected in a case of rejection. Including this patient in the analysis yields a P value of 0.082. Median time to rejection was longer in the alemtuzumab group: 116 days (range
18-314 days) vs. 8 days (range 4-166), p=0.015. Death censored rejection free survival is shown in Figure 58 (log rank test p=0.063). More severe rejection episodes of Banff grade II and above occurred at similar frequencies in the alemtuzumab group (4/6) and the basiliximab group (5/14). Only 3 rejection episodes failed to return to within 10% of baseline creatinine on treatment, 2 (28.5%) in the alemtuzumab group, 1 (7.1%) in the basiliximab group.

![Death censored rejection free graft survival in alemtuzumab and basiliximab groups (curves compared by Log Rank test)](image)

**Figure 58:** Death censored rejection free graft survival in alemtuzumab and basiliximab groups (curves compared by Log Rank test)

5.2.1.3 Immunosuppression

There was no difference in mean tacrolimus levels between the two groups at 3, 6 or 12 months (p=0.238, 0.461 and 0.315 respectively) (Figure 59). Percentages of patients remaining steroid free at 12 months were 81% and 74% for the alemtuzumab and basiliximab groups respectively. Commencing steroids was at the discretion of the
treating clinicians. Reasons for steroid use at one year were as follows: alemtuzumab group: 4 patients switched to sirolimus (and prednisolone), 1 patient developed tacrolimus neurotoxicity and 1 patient was treated for PTLD. A further 6 patients were on prednisolone following rejection episodes. 81% of the alemtuzumab group were on tacrolimus monotherapy at 12 months. In the basiliximab group: one patient developed calcineurin-inhibitor-pain-syndrome (CIPS) and was changed to a steroid containing regimen, one had MMF withdrawn for leucopenia with steroids substituted, and one had neurotoxicity with tacrolimus resulting in a regimen change to one incorporating steroids. A further 13 patients were on prednisolone following rejection episodes – one of these was being concurrently treated for recurrent membranous nephropathy with a steroid containing protocol. The mean mycophenolate mofetil dose in the basiliximab group at one year was 1.2 ± 0.3g / 24h (median 1.5g / 24h).

Figure 59: Tacrolimus levels in alemtuzumab and basiliximab groups (mean ± SD).

Means compared by independent sample t test.
5.2.1.4 Other clinical outcomes and adverse events

Rates of diagnosed NODAT at 12 months for the alemtuzumab and basiliximab groups were 8.9% and 12.5% respectively (with n=56 at 12 months in each group). No statistically significant differences in NODAT rates were apparent at any of the time points (4 weeks, 6 months and 12 months). Both groups had excellent blood pressure control demonstrated by the 24hr ambulatory blood pressure measurements. Urine protein creatinine ratios measured at 3, 6 and 12 months after transplantation were similar in both the groups.

Similar numbers of hospitalisations for cardiovascular events occurred in each group – 8 (of which 4 were myocardial infarctions) and 6 (of which 4 were myocardial infarctions) in the alemtuzumab and basiliximab groups respectively. De novo neoplasia was seen in 3 patients (5.1%) in the alemtuzumab group – 2 post-transplant lymphoproliferative disorders (PTLD): one leading to death, one now in complete remission with good graft function. One thyroid carcinoma (successfully treated) was also seen. Two neoplasias were seen in the basiliximab group (3.4%) – one PTLD, and one renal cell carcinoma – both successfully treated. Hospitalisations for infective episodes occurred more frequently in the alemtuzumab group (36 vs. 30), but in a similar number of patients (22 vs. 17 in the basiliximab group). Further details are given in Table 16. CMV syndrome was seen in 3 patients in the basiliximab group, all in intermediate risk patients (D+/R− or D-/R+) who did not receive prophylaxis with valganciclovir. Only 1 patient (D+/R+) in the alemtuzumab group developed CMV syndrome after the 100 day prophylaxis period. There was no invasive CMV disease. Two cases of BK virus nephropathy occurred, both in the basiliximab group.
Table 16: Details of the hospitalization episodes over the first post-transplantation year in alemtuzumab and basiliximab groups

<table>
<thead>
<tr>
<th>Type of infective episode</th>
<th>Alemtuzumab</th>
<th>Basiliximab</th>
<th>*P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No of patients</td>
<td>No of episodes</td>
<td>No of patients</td>
</tr>
<tr>
<td>Urinary tract infection</td>
<td>17</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Gastroenteritis</td>
<td>4</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Sepsis-unknown cause</td>
<td>5</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Chest infection</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Cellulitis</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>peritonitis</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>other</td>
<td>5</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

*Groups compared by Chi-square test. Frequency is shown as absolute numbers

In summary, the results so far have shown that alemtuzumab induction allows for minimization of maintenance immunosuppression with avoidance of mycophenolate and prednisolone in a significant majority of the patients and results in comparable clinical outcomes to patients who are maintained on tacrolimus and mycophenolate after basiliximab induction. In the next few sections, an analysis of the peripheral blood B lymphocyte phenotype in both the groups will be presented.

5.2.2 Analysis of reconstituted peripheral lymphocyte phenotype

5.2.2.1 Absolute lymphocyte number in Alemtuzumab and Basiliximab groups

Figure 60 shows the differences in the mean number of lymphocytes in the peripheral blood of patients in both groups at various time points after transplantation. Lymphocyte depletion was profound in the alemtuzumab group and repopulation
occurred gradually over the first year. By 9 months after transplantation, there was no longer any statistically significant difference between lymphocytes in the two groups.

![Figure 60: Mean number of lymphocytes compared between the two groups.](image)

*Time points are marked on the x-axis and the y-axis shows the scale for the number of lymphocytes (× 10⁹ /µl of whole blood). Even though there is a marked decrease in the lymphocyte number early after transplantation, no statistically significant differences exist at the time of sampling (p=0.3, independent samples t-test)*

### 5.2.2.2 Lymphocyte phenotype in Alemtuzumab and Basiliximab groups

Figure 61 summarizes the analysis of lymphocyte subsets using the trucount beads. This analysis of the absolute numbers of lymphocyte subpopulations in the peripheral blood was carried out using trucount beads at a mean of 25 months post transplantation. Figure 62 summarizes the differences in the proportion and absolute numbers of lymphocytes between the two groups and the two control groups that include dialysis patients (n=10) and healthy volunteers (n=10).
Figure 61: Identification of lymphocyte subsets by flow cytometry.

Lymphocytes are identified by the lack of granularity and positivity for CD45. This gated population is further classified as helper-T cells (CD45+CD3+CD4+), Cytotoxic T-cells (CD45+CD3+CD8+), B cells (CD45+CD19+) and NK cells (CD45+CD16+CD56+).

The proportion and the absolute number of CD3+ T lymphocytes were less in the alemtuzumab group when compared to both healthy volunteers and dialysis patients. In large part, this was due to the significantly depleted CD4+ lymphocyte population in this group. There was also an increase in the proportion of both CD19+ B cells and NK cells in the alemtuzumab group when compared to both control groups. The absolute numbers were only increased in comparison to dialysis patients due to relative lymphopenia of this population compared to healthy controls. When the same set of controls were compared to the basiliximab group, no significant differences were demonstrated in the proportions of any of the lymphocyte subsets but the absolute number of CD3+ T cells, CD4+ T cells, CD19+ B cells and NK cells were significantly
lower than in healthy controls. This is once again a reflection of the relative lymphopenia of these patients.

Figure 62: Comparison of lymphocyte subsets in healthy volunteers, dialysis patients and alemtuzumab/basiliximab groups.
This graph shows that both alemtuzumab and basiliximab groups were lymphopenic when compared to healthy controls. The alemtuzumab group had significantly lower CD3\(^+\) T cells and CD4\(^+\) T cells whilst CD8\(^+\) T cells were comparable to those of healthy controls and dialysis patients. The proportion of B cells and NK cells were significantly higher in the alemtuzumab group when compared to both controls. The absolute numbers of these cell types were comparable to healthy controls even though they were significantly higher than the dialysis patients. The proportions of the various lymphocyte subsets in the basiliximab group were comparable to those of other controls. However the absolute number of CD3\(^+\) T cells, CD4\(^+\) T cells, B cells and NK cells were significantly lower when compared to healthy controls.

Further analysis within the CD4\(^+\) T and B cell compartments is summarized in Figure 63 and Figure 64. Within the CD4\(^+\) T cell compartment of patients treated with alemtuzumab, the ratio of naïve (CD45RA\(^+\) CD45RO\(-\)) to memory cells (CD45RA\(^-\) CD45RO\(+\)) was not significantly different to either healthy controls or dialysis patients even though a trend towards a higher ratio could be seen. In contrast the basiliximab group had a significantly higher ratio of naïve to memory CD4\(^+\) cells in comparison to both healthy controls and dialysis patients (Figure 64a).
Figure 63: Representative dot plots of the various T and B subsets in healthy volunteers, dialysis patients, alemtuzumab and basiliximab groups.
Patients in the basiliximab group had a significantly higher naïve to memory CD4+ T cell ratio, whilst a similar trend was seen in the alemtuzumab group as well, the results were not statistically significant (a). Alemtuzumab group had the highest naïve to memory B ratio when compared to both control groups and even the basiliximab group had a high naïve to memory B cell ratio when compared to healthy controls but the results were not significantly different to those of the dialysis controls (b). The proportion of transitional B cells were significantly higher in the alemtuzumab group when compared to both the controls groups but the absolute numbers were only high in comparison to the dialysis patients. In contrast, the absolute number of transitional B cells were significantly lower in the basiliximab group when compared to healthy controls (c). Whilst the proportion and absolute numbers of the B regs were significantly
lower in the basiliximab group when compared to healthy controls they were comparable in the alemtuzumab group (d).

Within the reconstituted B cell compartment, the alemtuzumab group had a significantly higher number of CD19$^{+}$CD27$^{-}$IgD$^{+}$ naive cells when compared to both healthy controls and dialysis patients. This group also had a lower number of memory B cells (both CD19$^{+}$CD27$^{-}$IgD$^{+}$ non-class switched and CD19+CD27+IgD- class switched) when compared to healthy controls but these numbers were similar to those seen in dialysis patients. This was reflected in a significantly higher naïve to memory B cell ratio in the alemtuzumab group when compared to both control groups (Figure 64b). Furthermore the alemtuzumab group had a significantly higher proportion of transitional B cells when compared to both control groups with absolute numbers that were restored to levels found in healthy controls (Figure 64c). The proportion and absolute number of CD5$^{+}$CD1d$^{hi}$ B cells in this group were comparable to healthy controls but higher than dialysis patients (Figure 64D).

There was a relative increase in the numbers of naïve B cells in the basiliximab group when compared to both the controls although this difference was not as marked as that in the alemtuzumab group (Figure 64b). However, in contrast to the alemtuzumab group, the basiliximab group had significantly lower absolute numbers of both transitional B cells (Figure 64c) and CD5$^{+}$CD1d$^{hi}$ B cells (Figure 64d).

5.2.2.3 Correlation of B cell subsets with graft function

Lymphocyte phenotype was correlated with graft function assessed by eGFR at the time of the analysis and by ΔeGFR, an indicator of the stability of graft function between 6 and 25 months after transplantation. Table 17 summarizes the correlation of absolute numbers of lymphocyte subsets with both eGFR and ΔeGFR. There was a significant correlation between graft function and the absolute numbers of B cells,
naïve B cells, transitional B cells and regulatory B cell cells in the alemtuzumab group. A similar correlation was demonstrated between these subsets and stability of graft function. These correlations remained statistically significant in a multivariate analysis after adjusting for significant confounding clinical variables including recipient age, donor age and donor retrieval creatinine (Table 18).
**Table 17: Correlation of Lymphocyte phenotype to graft function**

<table>
<thead>
<tr>
<th>Lymphocyte Phenotype</th>
<th>Alemtuzumab group</th>
<th>Basiliximab group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spearman R (eGFR)</td>
<td>p-value</td>
</tr>
<tr>
<td>CD4⁺ T cells</td>
<td>1.5</td>
<td>0.3</td>
</tr>
<tr>
<td>CD8⁺ T cells</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>CD4⁻CD8⁻ T cells</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Naive CD4⁺ T cells</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>Memory CD4⁺ T cells</td>
<td>0.02</td>
<td>0.9</td>
</tr>
<tr>
<td>NK cells</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>B cells</td>
<td>0.3</td>
<td>0.03</td>
</tr>
<tr>
<td>Naive B cells</td>
<td>0.3</td>
<td>0.03</td>
</tr>
<tr>
<td>Class un-switched Memory B cells</td>
<td>0.35</td>
<td>0.01</td>
</tr>
<tr>
<td>Class switched Memory B cells</td>
<td>-0.12</td>
<td>0.4</td>
</tr>
<tr>
<td>Transitional B cells</td>
<td>0.3</td>
<td>0.02</td>
</tr>
<tr>
<td>Cd5⁺CD1dhi B cells</td>
<td>0.34</td>
<td>0.01</td>
</tr>
</tbody>
</table>

eGFR was calculated at the time of sampling (a mean of 25 months after transplantation). ∆ eGFR was the difference between the eGFR at 6 months and at the time of sampling.
In the basiliximab group there were significant correlations between absolute numbers of CD4+ T cells, and in particular CD4+ CD45RA+ cells, and eGFR but not ΔeGFR. Naïve and transitional B cells also correlated with eGFR but not ΔeGFR. Regulatory B cells correlated with both eGFR and ΔeGFR. Both the alemtuzumab and basiliximab groups were divided into tertiles (stratified by absolute numbers of transitional B cells, regulatory B cells and naïve B cells) for further functional analysis. Figure 65 demonstrates that the patients with the highest numbers of these B cell subsets were almost entirely restricted to the alemtuzumab group, although there was some overlap with the basiliximab group in lower tertiles. There was a stepwise increase in the eGFR and ΔeGFR across the three tertiles of all three B cell subsets within the alemtuzumab group. Patients in the lowest tertile of all three B subsets had the worst eGFR and the greatest deterioration in eGFR whilst the converse was true of patients in the highest tertile. Patients with the highest tertiles of transitional and regulatory B cells had no DSA and only one patient in the highest tertile for naïve B cells had a DSA (Figure 66).

### Table 18: B cell phenotype- correlation to eGFR and ΔeGFR- multivariate analysis

<table>
<thead>
<tr>
<th>B cell type</th>
<th>R (unadjusted)</th>
<th>R (adjusted-partial correlation)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve B cells</td>
<td>0.3 (p=0.03)</td>
<td>0.4 (p=0.01)</td>
</tr>
<tr>
<td>Transitional B cells</td>
<td>0.3 (p=0.02)</td>
<td>0.43 (p=0.003)</td>
</tr>
<tr>
<td>Cd5+CD1dhi B cells</td>
<td>0.34 (p=0.01)</td>
<td>0.35 (p=0.02)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Δ eGFR</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve B cells</td>
<td>0.4 (p=0.01)</td>
<td>0.4 (p=0.004)</td>
</tr>
<tr>
<td>Transitional B cells</td>
<td>0.3 (p=0.03)</td>
<td>0.44 (p=0.002)</td>
</tr>
<tr>
<td>Cd5+CD1dhi B cells</td>
<td>0.4 (p=0.008)</td>
<td>0.3 (p=0.02)</td>
</tr>
</tbody>
</table>

*Adjusted for recipient age, donor age and donor retrieval creatinine in partial correlation analysis.
Such a clear relationship could not be demonstrated across the tertiles of the basiliximab group (Figure 67).

Figure 65: Comparison of absolute numbers of transitional and CD5+CD1dhi B cells in basiliximab and alemtuzumab groups

A comparison of the absolute numbers of naive, transitional and regulatory B cells across the three tertiles in both the basiliximab and alemtuzumab groups is made with those of healthy volunteers and dialysis patients. Patients in the highest tertile alemtuzumab group had the highest number of naive (P<0.001), transitional (P<0.001) and regulatory B cells (p<0.01) respectively.
Figure 66: Comparison of eGFR, ΔeGFR and HLA specific antibodies across the three B subset tertiles (Alemtuzumab).

For all three B cell subtypes, the lowest tertile had the lowest eGFR and the highest tertile the best eGFR (Naive B cells, lowest vs. highest tertile P=0.07; Transitional B cells, lowest vs. highest tertile, P=0.05; CD5hiCD1d+ B cells, lowest vs. highest tertile, P=0.01) Similar trends were also seen with ΔGFR as well (Naive B cells, lowest vs. highest tertile P=0.03; Transitional B cells lowest vs. highest tertile, P=0.005; CD5hiCD1d+ B cells, lowest vs. highest tertile, P=0.002). No patients within the highest tertiles of the transitional and regulatory B cells had DSA whilst only one with the highest tertile of the naive B cells had DSA.
Figure 67: Comparison of eGFR, ∆eGFR and HLA specific antibodies across the three B subset tertiles (Basiliximab)

For all three B cell subtypes, the lowest tertile had the lowest eGFR and the highest tertile the best eGFR (Naive B cells: lowest vs. highest tertile, P=0.02; Transitional B cells: lowest vs. highest tertile, P=0.05; CD5hiCD1d+ B cells lowest vs. highest tertile, P =0.03). Despite trends, ∆GFR was not significantly different across the tertiles (Naive B cells: lowest vs. highest tertile, P =Ns; Transitional B cells: lowest vs. highest tertile, P =Ns; CD5hiCD1d+ B cells lowest vs. highest tertile, P =0.052). DSA/NDSA development post-transplant did not follow a specific trend.
5.2.2.4: HLA specific antibodies

Similar number of patients in each group had HLA specific antibodies with no difference in the number of patients with donor specific or non-donor specific antibodies (Figure 68).

![Diagram with HLA specific antibodies](image)

Figure 68: HLA specific antibodies in both the groups

Patients with DSA in the alemtuzumab group had a significantly lower number of naïve, transitional and regulatory B cells when compared to those without antibody (Figure 69).

![Figure 69: Comparison of the absolute numbers of B lymphocyte subsets in patients with and without HLA-specific antibodies.](image)

In comparison to those without HLA-specific antibody, patients with DSA had significantly lower numbers of transitional B cells (No HLA antibodies 33.8/μl (95% CI 25.8 to 41.8/μl) vs. DSA 14.1/μl (95% CI 6.9 to 21.3/μl), p=0.01); regulatory B cells that express CD5 and CD1d (No HLA antibodies 41.6/μl (95% CI 29.4 to 53.8/μl) vs. DSA 17.8/μl (95% CI 9.5 to 26.1/μl), p=0.03) and a lower naïve to memory B cell ratio (No HLA antibodies 6.8 (95% CI 5.1 to 8.5) vs. DSA 2.7 (95% CI 0.9 to 4.4), p=0.005).
Such a relationship could not be demonstrated within the basiliximab group (Figure 70)

![Graph showing comparison of B lymphocyte subsets](image)

**Figure 70**: Comparison of the absolute numbers of B lymphocyte subsets in patients with and without HLA specific antibodies in the basiliximab group.

*No significant differences existed between the patients with DSA, NDSA and those with no HLA antibody.*

### 5.2.3 Validation of T-1/T-2 ratio as a clinical marker

Results discussed in the previous chapter show that transitional B cells are heterogeneous containing distinct phenotypic and functional subsets (T-1, T-2). They also conclude that the ratio of T-1 and T-2 provides a simple and valuable biomarker to predict graft outcomes. Now the TrB cells are re-analysed in both alemtuzumab and basiliximab groups which has shown distinct clinical correlations to the B phenotype based on the distribution of T-1/T-2. This provided an opportunity to validate the utility of T-1/T-2 in an independent random sample of patients all of whom received same immunosuppression. ΔeGFR was measured from 6 months post-transplantation to 3 years after blood sampling. When these patients were divided into three tertiles based on their T-1/T-2 ratio; highest percentage of patients who were in the lowest tertile of the T-1/T-2 ratio had a donor specific antibody detected at the time of the analysis. Similar to the results seen in the test set, patients in the lowest tertile had a significantly
lower (negative) ΔeGFR compared to the highest tertile thus confirming the clinical applicability of T-1/T-2 ratio (Figure 71).

![Pie charts and bar charts illustrating clinical significance of T1/T2 ratio](image)

**Figure 71: Clinical significance of T1/T2 ratio**

The top panel of pie charts show that a significantly higher proportion of patients in the lowest tertile are positive for a DSA when compared to those in the highest tertile in both Alemtuzumab and Basiliximab groups. Similarly the bar charts show that Δ eGFR is significantly lower (negative) within the lowest tertile when compared to the highest tertile (## P=0.01). The proportions of patients with DSA are compared across the tertiles by Chi square test and the Δ eGFR compared by independent t-test. The bars in the bar charts represent the mean whilst the error bars represent SEM.

5.2.4 Longitudinal assessment of B subsets after alemtuzumab induction

So far, the data presented shows that alemtuzumab causes depletion of both T and B cells and now analyzed the reconstitution of the T1, T2 and mature naive B cells after
lymphocyte depletion with alemtuzumab induction in renal transplant recipients (n=14). All 14 patients received intra-operative induction therapy with 1g of intravenous methylprednisolone along with 30mg alemtuzumab and were maintained on tacrolimus monotherapy. Serial analysis of the B subsets at 3, 6 and 12 months after transplantation in this group revealed that the proportion of mature naïve B cells (CD24+CD38+) increased over time while that of the immature T1 and T2 cells decreased accordingly (Figure 72). At 1 year after transplantation the majority of B cells comprise the mature phenotype followed by T2 cells and then T1 cells in the lowest number. In all these patients at time-0, the number of lymphocytes in the peripheral blood was zero or close to zero making it impossible to assess B subsets at that time point. Given the very small numbers in this subset of patients, clinical correlation was not possible.
Renal transplant recipients who received induction with alemtuzumab along with a single dose of methylprednisolone were followed up for 1 year and B subsets analysed longitudinally. (A). Representative scatter plots showing the T1, T2 and mature B cells at 3 months, 6 months and 1 year after transplantation. (B). Graph demonstrating T1, T2 and mature B cells at different time points after transplantation. Each bar in the graph represents mean and the error bars represent SEM. At time 0 the mean number of peripheral lymphocytes was zero thereby giving a value of ‘0’ at the starting point.

5.2.5 Summary

In summary, the clinical data presented here shows that alemtuzumab offers comparable short term graft and patient outcomes to basiliximab induction and it is
encouraging to see that the rate of early acute rejection is marginally superior in the alemtuzumab group. There are notable differences in the reconstitution of T and B cell compartments at nearly 2 years after transplantation. In an independent small group of patients the development of the B phenotype within the first post-transplant year was also examined. It is noteworthy that the B cell repopulation is dominated by an immature and a naïve phenotype which contrasts that of the T cell phenotype. More importantly, those patients who had a higher number of these immature cells experienced better graft outcomes with a superior eGFR and no DSA.
5.3 Discussion

In the setting of a RCT of two steroid avoidance regimes, a conspicuous and significant difference in the phenotype of the reconstituted lymphocyte pool following alemtuzumab induction was demonstrated. Although there was slightly less rejection in the alemtuzumab group, clinical outcomes were otherwise similar. Both groups were exposed to similar tacrolimus levels but the basiliximab group was also exposed to MMF and there is limited data to suggest that this drug may affect both B and T lymphocyte subpopulations (Weigel, Griesmacher et al. 2002, Salinas-Carmona, Perez et al. 2009). Information regarding the effects of MMF on lymphocyte populations following profound depletion is lacking, though recent experiments in primates do not suggest that MMF has a profound effect on subsets other than CD4$^+$ T cells (van der Windt, Smetanka et al. 2010).

5.3.1 Clinical outcome measures

This clinical trial with its non-superiority design shows that the clinical outcomes are similar in both groups of patients. It is encouraging to note that a high percentage of patients in both the groups (81% alemtuzumab group, 74% basiliximab group) remained steroid free at one year. The study patients remain on tacrolimus monotherapy, an approach first described by the group in Pittsburgh (Shapiro, Basu et al. 2005, Thomas, Woodside et al. 2007). A relatively simple immunosuppression regime based on a single maintenance agent has potential advantages. It is economical and enables most patients to take fewer tablets. This strategy may lead to better adherence to treatment.

Although the time to rejection episode for patients who received alemtuzumab in this study is longer when compared to that of basiliximab, there remains a trend towards reduced rejection rate in the alemtuzumab group. This finding is in agreement with that reported by Woodle’s group in Cincinnati (Hanaway, Woodle et al. 2011). They
propose that the lower rejection group in their study is attributed to the absence of DCD
donor transplants. However, DCD transplants are included in this study. Groups led by
Calne in Cambridge and Singapore have described a higher rate of late acute rejection
between 6 and 12 months (Vathsala, Ona et al. 2005, Margreiter, Klempnauer et al.
2008) and this can possibly be due to the inclusion of high immunological risk
transplants and the use of ciclosporin monotherapy, as opposed to tacrolimus. It is also
important to note that the rate of severe grade rejection (histological Banff grade 2 or
above) in both groups is similar.

5.3.2 **Alemtuzumab induction and the lymphocyte phenotype**

In this study, patients in both the alemtuzumab and basiliximab groups had total
lymphocyte counts similar to those seen in dialysis patients but were lymphopenic
when compared to healthy volunteers. It is possible that lymphopenia in both groups is
caued by a combination of two factors. Firstly these patients probably came to
transplantation with reduced numbers of lymphocytes since they were suffering with
end stage renal disease. Superimposed on this background are the effects of
immunosuppressive agents including the induction agents, basiliximab or
alemtuzumab.

The relative proportion of CD3\(^+\) T cells, even 25 months after transplantation, was
significantly lower in the alemtuzumab group when compared to both control groups.
This was largely due to a reduction in the CD4\(^+\)T cell pool. The basiliximab group had a
significantly higher naïve to memory CD4\(^+\)T cell ratio when compared to both control
groups. In contrast such a distinction was not demonstrated in the alemtuzumab group,
a finding which accords with previous studies (Pearl, Parris et al. 2005, Trzonkowski,

The phenotype of reconstituted B cells after alemtuzumab induction has not previously
been studied in renal transplant recipients. In patients with multiple sclerosis treated
with alemtuzumab, rapid recovery of B cells has been described with development of
supra-normal levels (65% above baseline) by 12 months following treatment (Thompson, Jones et al. 2010). This observation was associated with increased numbers of transitional B cells in the early phase of recovery, prolonged suppression of memory B cells, and a brisk and augmented BAFF response. Two years following alemtuzumab induction, a distinct phenotype within the regenerated B cell compartment was demonstrated for patients with good clinical outcomes which was less pronounced following basiliximab induction. A B cell repertoire with prominent naïve, transitional and regulatory components is consistent with the recent description of elevated BAFF levels in patients induced with alemtuzumab (Bloom, Chang et al. 2009). In the alemtuzumab group there was a significant increase in the naïve B cell population reflected by a high naïve/memory B cell ratio compared to both sets of controls. This is similar to recent observations in patients with multiple sclerosis and suggests a block in differentiation to the memory phenotype (Thompson, Jones et al. 2010). A similar though less significant result was demonstrated in the basiliximab group. Limited data after alemtuzumab induction for islet cell transplantation in humans has also demonstrated a loss of memory B cells although further subset analysis was not available (Toso, Edgar et al. 2009). The fact that T cells were markedly depleted in the alemtuzumab group could possibly explain the prolonged suppression of the memory B cells since naïve B cells require T cell help for maturation (Steele, Laufer et al. 1996).

Following alemtuzumab, the proportion of transitional B cells (CD19⁺CD24hi CD38hi) was high when compared to both control groups, with absolute numbers restored to levels seen in healthy volunteers despite the absolute lymphopenia. In contrast the basiliximab group had both a lower proportion and absolute number of transitional B cells when compared to healthy volunteers. Transitional B cells represent an immature phenotype, intermediate between bone marrow precursors and mature naïve B cells. Studies of B cell depletion therapies with Rituximab (anti-CD20) in autoimmune diseases have identified transitional cells as immature cells that feature prominently in
homeostatic repopulation following lymphocyte depletion (Sutter, Kwan-Morley et al. 2008, Palanichamy, Barnard et al. 2009). A recently proposed subset of the transitional cells, termed T3 cells, were shown to develop from mature naïve B cells upon chronic exposure to antigen (Merrell, Benschop et al. 2006). In the clinical setting transitional B cells have been proposed as regulatory cells in patients with systemic lupus erythematosus (Blair, Norena et al. 2010, Mauri and Blair 2010, Mauri and Blair 2010).

Another B cell subgroup associated with regulatory function (CD19^+CD5^+CD1d^hi) was also distinct in patients who received alemtuzumab induction. Whilst patients who received alemtuzumab had a comparable number of CD19^+CD5^+CD1d^hi B cells to healthy volunteers, the basiliximab group had a significantly lower proportion and absolute number of these cells. This subset has been shown to produce IL-10 and is associated with antigen-specific regulatory properties in murine experimental models (Mizoguchi, Mizoguchi et al. 2002). Interestingly, cells with such a phenotype were recently shown to be associated with operational tolerance in human kidney transplant recipients (Pallier, Hillion et al. 2010).

The observations in these patients are noteworthy but may simply reflect a phenomenon contingent upon the underlying immunosuppressive regime, especially since there were similar clinical outcomes in the two treatment arms. However, two associations increase the impact of these observations. Firstly, B cell subset numbers were shown to correlate with graft function, most closely in the group of patients treated with alemtuzumab. Secondly, similar lymphocyte phenotypes have recently been demonstrated in recipients of kidney transplants with operational clinical tolerance (Newell, Asare et al. 2010, Pallier, Hillion et al. 2010, Sagoo, Perucha et al. 2010).

Moreover, when the patients in the alemtuzumab arm were divided into tertiles according to absolute numbers of B cell subsets (either naïve, transitional or regulatory B cells) there was a significant correlation with graft function, improved stability of
function and reduced humoral allo-sensitisation. This finding suggests that it may be possible to use peripheral B cell phenotype analysis by flow cytometry to monitor or even predict immunological graft function.

There are however limitations in this analysis and caution should be exercised when interpreting the findings. All of the observations were made at a fixed time point approximately two years following transplantation and it is now necessary to prospectively study this phenomenon to document the time course, evolution and stability of these phenotypic changes. Even though the role of the immature B cells in the regulation of immune response and the change of function noticed in the same cells in patients with CAMR was discussed in the previous chapter, it is still important to study the functional significance of these cells in the regulation of the alloresponse. It is crucial to note that graft outcomes are affected by other important non-immunological mechanisms and this analysis has not taken these into account.

Such a striking relationship was not seen following basiliximab induction and it could be speculated that this phenomenon is more prominent in the regenerating B cell compartment following profound lymphocyte depletion. Certainly in animal models there is evidence that following lymphocyte depletion a B cell compartment regenerating in the presence of alloantigen is dominated by transitional B cells which can mitigate a tolerant state and prevent alloantibody production (Parsons, Vivek et al. 2011).

Recent studies in patients who have stable renal allograft function in the absence of on-going immunosuppressive medications (“operational tolerance”) have demonstrated an association with distinct B cell phenotypes. One group demonstrated a relationship between operationally tolerant patients and the presence of naïve and transitional B cells in peripheral blood (Newell, Asare et al. 2010). Other similar results suggest the intriguing possibility that certain peripheral B cell phenotypes may be predictive of good outcomes and even a potentially tolerant state (Pallier, Hillion et al. 2010, Sagoo,
Perucha et al. 2010). It is possible that antigens presented by naive B cells possibly promote tolerance by favouring the development of regulatory rather than the effector memory T cells (Fuchs and Matzinger 1992, Reichardt, Dornbach et al. 2007). Certainly transitional B cells have been shown to produce IL-10 and have regulatory functions in human autoimmune disease (Blair, Norena et al. 2010).

Patients in the basiliximab group had comparable graft function to those in the alemtuzumab group in the short term and the relationship between lymphocyte subtypes and graft function was less clear-cut. However it is possible that the mechanism of immune regulation is different following non-depletional induction therapy. The lower B cell numbers in the basiliximab group might be explained in part by the anti-proliferative effects of MMF on the B cell compartment but also possibly there is less lymphoid space for B cells to expand into since basiliximab is a non-depleting antibody.

5.3.3 Summary

In summary it is demonstrated that, in the context of a randomised controlled trial, two years after renal transplantation, alemtuzumab rather than basiliximab induction was associated with a highly significant and novel peripheral lymphocyte phenotype, although clinical outcomes were broadly similar. This phenotype was notable for the prominence of naïve and regulatory B cell subtypes. Patients with a significantly higher number of these regulatory cells had better and more stable graft functions. Intriguingly, these are similar cell types to those that are prominent in the peripheral blood of operationally tolerant renal allograft recipients. Prospective studies monitoring peripheral blood lymphocyte phenotype with long term clinical follow up are now required in patients undergoing alemtuzumab induction, to explore how this may aid clinical management.
Chapter-6: Discussion

In the preface to this thesis, I have briefly discussed a plan to address certain pertinent questions to the field of renal transplantation. Here, I will reflect upon those questions and examine whether any further insight has been gained as a result of the clinical and experimental research conducted over the last four years.

6.1 Can clinical phenotype be linked to histological picture & graft outcomes?

This is clearly a complex question as the answer depends heavily on the clinical context. Early low-grade proteinuria either measured at 3 months post-transplantation or at 1 year has been shown to be associated with worse graft outcomes and poor overall survival. The results presented in chapter-3, support previously reported data. However, proteinuria is a dynamic variable that changes with time. For example, resolution of proteinuria in a subset of non-transplant patients in the RENAAL study of diabetic nephropathy demonstrated superior clinical outcomes. However, proteinuria was assessed over a short period of only 6 months. There is a dearth of data looking at the impact of modification of proteinuria over longer periods of time. This issue has been addressed here by the longitudinal assessment of proteinuria. I have analysed patients with proteinuria less than 1g/day during three years of assessment. One major finding from this analysis is that resolution of low-grade proteinuria is associated with improved overall graft outcomes. Moreover, proteinuria helps to risk stratify hypertensive renal transplant recipients. Given these findings, low-grade proteinuria seems to be a promising clinical marker. However, this should be validated in a larger prospective multi-centre study. This is to avoid the same pitfall encountered when using creatinine as a predictive marker. We should remember that although high
creatinine is strongly associated with worse long-term graft outcomes, the predictive value is not much different from the toss of a coin. The next question that these findings pose is the potential benefit of anti-proteinuric therapy for hypertensive patients with low-grade proteinuria. This should be explored in a trial setting.

As proteinuria has been shown in our experience to be associated with worse graft survival, patients with three consecutive proteinuria assessments of more than 0.5g/day were offered an indication transplant biopsy. Surprisingly, there was no clinical correlation between the histological phenotype and clinical presentation of either isolated change in creatinine, proteinuria or both. Even more important is the finding that clinical presentation does not independently predict graft loss in this highly selective group of patients defined by graft dysfunction. From these findings, we can postulate that proteinuria whilst showing a strong clinical association in a population based analysis, is of limited significance once graft dysfunction ensues. Therefore one can argue that the actual prognostic utility of proteinuria in patients with graft dysfunction is rather limited. This could be explained by two possibilities

1. Clinical intervention once graft dysfunction ensues may not be very effective
2. Possibly the interventions are not effective, irrespective of the presentation.
3. It is too late since original observations were at three months

One potential solution can be offered to address the issue of early diagnosis. This could be addressed by early biopsies at 3 months. One major issue regarding a planned early biopsy again will be the timing. To address this, patients can be screened routinely for the presence of DSA at a regular basis through the life of that graft and those with DSA could undergo a biopsy irrespective of clinical function. Alongside conventional histology, molecular diagnostics may prove useful as a predictive tool (Einecke, Reeve et al. 2010).
6.2 Can Bregs be identified by both pro and anti-inflammatory cytokines?

Bregs have traditionally been identified by their ‘signature’ cytokine IL-10 (Mauri and Bosma 2012). Various groups have suggested that either memory or immature B cells are capable of secreting and synthesising IL-10 leading to them being classified as Bregs. However these are *in vitro* experiments and B cells can be made to express IL-10 depending on the culture conditions. Lund and colleagues have argued that B cell subpopulations in mice are capable of expressing multiple cytokines. In humans, similar evidence emanated from Bar-Or’s group, working on multiple sclerosis. They have shown that B cells are capable of expressing both pro and anti-inflammatory cytokines and they used the whole B cell IL-10/lymphotoxin ratio to distinguish healthy volunteers from patients with remitting multiple sclerosis. However, they did not identify a subset of B cells capable of expressing IL-10 or lymphotoxin. My experiments in healthy volunteers with *in vitro* B cell stimulation using CPG and CD40L aimed to address this issue. Both TLR9 (CPG agonist) and CD40-CD40L interactions have been identified as crucial for the generation and function of Bregs in animal studies. In these experiments, I have been able to demonstrate that

1. B cells are able to express both IL-10 and TNF-α.
2. This duality of expression remained valid even when analysed on a per-cell basis.
3. The differential expression of these two cytokines distinguished immature transitional B, naïve and memory B cells.
4. Transitional B cells, by the virtue of relative cytokine polarization towards IL-10, possess the most anti-inflammatory profile.
5. This anti-inflammatory profile correlates well with *in vitro* suppressive capacity.

Based on these results, immature transitional B cells can be identified as a significant population with regulatory potential based on both pro and anti-inflammatory cytokine...
expression. These results raise more questions than answers. One significant query that needs addressing in the future will be to decipher the role of other cytokines expressed by these cells. Although one can argue about the plasticity of these populations, the other major limitation is the lack of an inclusive marker. For example, TrBs which have been shown to demonstrate regulatory properties only constitute 10% of B population and on an average only 10-15% of these cells are pure expressers of IL-10. A potential more inclusive marker can probably be identified by \textit{in vitro} gene expression studies with a hope to identify a transcription factor akin to the Tregs.

6.3 Is the role of Bregs diverse in renal transplantation?

My interest in B cells and in particular, Bregs in the context of renal transplantation stemmed from the studies of operational tolerance. First, in a cross sectional study I have shown that transitional B cells are reduced in number with an abnormal pro-inflammatory cytokine profile in patients with a histological phenotype of CAMR. Histological rather than clinical identification of patient groups has been preferred in light of my findings discussed in chapter-3 suggesting histological phenotype has a stronger association with clinical outcomes. Importantly, this alteration in cytokine polarization resulted in the loss of \textit{in vitro} suppressive capacity and correlated with graft outcomes in these patients with a late indication biopsy. Secondly, I have shown that alemtuzumab induced lymphodepletion results in a repopulated phenotype which is ‘B dominant’. This is not a universal finding and patients who had a dominance of B cells; in particular cells with the phenotype of Bregs had superior and more stable graft function. This validated the utility of the phenotype of Bregs as a potential clinical marker. Finally, the issue of the diverse roles of B cells in renal transplantation. More than half of the patients with CAMR have an identifiable DSA in their serum. This is a reflection of the antibody producing role of the B cells. In contrast, patients with stable
function have a significantly higher number of B cell subsets that express an anti-inflammatory cytokine profile. Interestingly the same B cell subset, in the context of CAMR demonstrates a pro-inflammatory profile and this correlated with graft function. Therefore diversity is a feature of the B populations even when assessed for particular subsets and in fact for each individual cell.

There are certain limitations to this body of work.

1. This is a cross sectional analysis and does not address the longitudinal variability of the phenotype and function of these cells over time.

2. All these assays were performed by non-cognate stimulation. The significance of the cytokines and their suppressive capacity has not been assessed in an allo-specific manner.

3. These findings need to be replicated prospectively in larger groups of patients to assess their clinical applicability.
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