Immunological monitoring of the B-cell compartment in renal transplant recipients.

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Immunological monitoring of the B-cell compartment in renal transplant recipients

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Abstract

B cells contribute to chronic allograft deterioration, negatively impacting graft survival, and curtailing the lifespan of a resource already in short supply. Given this, identifying alloreactive B cells could generate an important target in the battle against rejection. This study described an IgG-detecting ELISPOT used to determine if the risk of developing antibody-mediated rejection (AMR) could be predicted pre-transplantation by *in vitro* analysis of allospecific B cells. This method failed to discriminate accurately B-cell responses to donor antigen. An alternative approach used was to detect peripheral HLA-specific B cells. Circulating HLA–A*0201 and –DQB1*0301 B cells were identified at higher frequency in sensitised patients, and this correlated with the level of serum alloantibody. Expression of HLA-DQB1*0301 B cells were at a higher frequency than HLA-A*0201 B cells in those with serum *de novo* donor-specific antibody (dnDSA). Next, levels of B-cell activating factor (BAFF) were investigated. Excess BAFF has been related to rejection and the development of DSA. Here elevated serum BAFF, low BAFF-receptor and DSA were all associated with deteriorating graft function. In addition intrarenal $CD19^{+}$ cells, BAFF and BAFF-receptor identified with acute AMR.

In contrast to a pathogenic role of B cells, a small population may be protective. The presence of regulatory B cells, defined by IL-10 production were higher in those with stable graft function, and identified with naïve B cells rather than memory B cells when compared to those with deteriorating grafts. The $CD19^{+}CD24^{high}CD38^{high}$ subset was also elevated in stable patients, and the ability to supress T-cell activation and secretion of the Th1 cell pro-inflammatory cytokine, IFN-γ was altered as a function of allograft stability.

These data demonstrated characteristics within the B-cell compartment associated with stable graft function. The ability to monitor these cells may have clinical implications for predicating the risk of rejection, to dictate immunosuppressive therapy and promote allograft survival.

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LIST OF ABBREVIATIONS

AAMR	Acute antibody-mediated rejection
AMR	Antibody-mediated rejection
ANCA	Anti-neutrophil cytoplasmic antibodies
APCs	Antigen-presenting cells
APC	Allophycocyanin
APC-Cy7	Allophycocyanin-Cy7
APRIL	A proliferation-inducing ligand
ASC	Antibody-secreting cell
B1/2	B-cell type 1/2
BAFF	B-cell activating factor
BAFF-R	B-cell activating factor receptor
BANK1	B-cell scaffold protein with ankyrin repeats 1
Bcl	B-cell lymphoma
BCMA	B-cell maturation antigen
BCR	B-cell receptor
Be1/2	B effector 1 or 2 cell
BLyS	B lymphocyte stimulator
Bregs	Regulatory B cells
BSA	Bovine serum albumin
CAML	Calcium-signal modulating cyclophilin ligand
CAMR	Chronic antibody-mediated rejection
CD	Cluster of differentiation
CDC	Complement-dependent cytotoxicity
CIA	Collagen-induced arthritis
CKD	Chronic kidney disease
CpG	C-phosphate-G
CSFE	Carboxyfluorescein succinimidyl ester
CSR	Class-switch recombination
CV	Coefficient of variance

CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor
DC	Dendritic cell
DBD	Donation after brainstem death
DCD	Donation after circulatory death
DNA	Deoxyribonucleic acid
DnDSA	*De novo* donor-specific HLA antibody
DSA	Donor-specific HLA antibody
DS-B cell	Donor HLA-specific B-cell
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein Barr virus
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot
ESRF	End stage renal failure
FACS	Fluorescently activated cell sorting
FCM	Flow-cytometric crossmatch
FCS	Foetal calf serum
FDA	Food and Drug Administration
FDC	Follicular dendritic cell
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box P3
FSGS	Focal segmental glomerulosclerosis
GC	Germinal centre
G-CSF	Granulocyte-colony stimulating factor
GFR	Glomerular filtration rate
GN	Glomerulonephritis
GvHD	Graft-versus host disease
HBV	Hepatitis B virus
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HSCT	Hematopoietic stem cell transplantation

IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ITAM	Immuno-receptor tyrosine-based activation motif
ITIM	Immuno-receptor tyrosine-based inhibition motif
LN	Lupus nephritis
LPS	Lipopolysaccharide
LSA	Luminex single antigen
LT	Lymphotoxin
MFI	Mean-fluorescent intensity
MHC	Major histocompatibility complex
MICA	MHC class I polypeptide-related sequence A
MS	Multiple sclerosis
mTORC	Mammalian target of rapamycin
MZ	Marginal zone
MZP	Marginal-zone precursor
NDSA	Non-donor-specific HLA antibody
NF-kB	Nuclear factor-kappa B
NICE	The National Institute for Health and Care Excellence
NK	Natural killer
ns	Not significant
OD	Optical density
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PE	Phycoerythrin
PE-Cy7	Phycoerythrin-Cy-7
PI3K	Phosphatidylinositide 3-kinase
PIP3	Phosphatidylinositol (3, 4, 5)-triphosphate
PKD	Polycystic kidney disease
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptor

RA	Rheumatoid arthritis
RAG	Recombinase-activating gene
RD	Rapidly deteriorating
RPM	Revolutions-per-minute
RPMI	Roswell Park Memorial Institute
sd	Standard deviation
SD	Slowly deteriorating
SFU	Spot-forming unit
SHM	Somatic-hypermutation recombination
SLE	Systemic lupus erythematosus
SPA	Solid phase assay
ST	Stable
T1/2	Transitional-type 1/2
TACI	Transmembrane activator
TALL-1	TNF- and APOL-related leukocyte expressed ligand
TCR	T-cell receptor
Tfh	Follicular helper T-cell
TGF	Tumour growth factor
Th	T-helper
THANK	TNF homologue that activates apoptosis, nuclear factor-kappaB
TI	T-cell independent
TIM	T-cell Immunoglobulin mucin
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAF	TNF receptor associated factor
Tregs	Regulatory T cells
UDA	Unacceptable defined antigen
US	Unsensitised

Chapter 1

Introduction

1.0 Introduction

Renal transplantation is the preferred therapeutic modality to treat end stage renal failure (ESRF), improving quality of life, increasing life expectancy and being cost-effective (Wolfe et al., 1999, Howard et al., 2009). To receive these benefits, every transplant must be optimised to maximise long-term allograft survival, not least because the demand for organs cannot be satisfied by the supply donated. However, despite advances in organ preservation and immunosuppression, rejection is a major obstacle governing the success of transplantation. Allograft rejection occurs following the transplantation of an organ into a genetically disparate recipient, evoking an immune response directed against recognition of alloantigens leading to destruction.

Vast improvements over the past two decades in our understanding of the molecular mechanisms of rejection, together with immunosuppression management and immunological assessment of a recipient–donor pairing, have contributed towards attaining excellent short-term allograft survival; surpassing 90% during the first-year. However, in the long-term this impact is not conveyed as demonstrated by data reporting allograft half-life survival. In the United States, a deceased-donor transplant survived 6.6 years in 1989; in 1995, 8 years and by 2005, 8.8 years. Following a living-donor transplant, the change was minimal from 11.4 years in 1989 to only 11.9 years in 2005 (Lamb et al., 2011). These data reflect relative stagnation in attrition rate beyond the first-year of transplant.

In the early period post-transplant, T cells are the principal mediators of acute rejection. This immune response follows recognition of donor alloantigens presented to recipient T cells most commonly by donor antigen-presenting cells (APCs). Donor APCs can travel from the graft to lymphoid organs rich in T cells, presenting alloantigen and allowing differentiation of T cells into various subsets. These return to invade and destroy the graft, producing inflammatory cytokines to attract further T cells, ultimately leading to necrosis, basement-membrane rupture and tubular atrophy (Robertson and Kirby, 2003, Bonsib et al., 2000). Current therapies target the initial

stages of T-cell activation, and these strategies successfully control acute T-cell-mediated rejection.

In the long-term, allografts fail due to a combination of immunological and non-immunological factors. The latter include delayed graft function, prolonged cold ischaemia time, inadequate immunosuppression, recurrence of disease, infection and complications due to pre-existing donor-related factors. Thus there is complexity beyond preventing initial rejection and the potential for effective intervention can be limited. Therefore targeting the immunological response, specifically HLA antibody directed against the allograft, may hold the most effective strategy towards improving allograft longevity.

B cells produce antibody and their contribution as important mediators in humoral allograft rejection is well established. Specifically, the presence of B cells specific for donor HLA expressed on the allograft can cause immediate or hyperacute antibody-mediated rejection (AMR). The deposition of anti-HLA antibodies on allograft endothelium activates the complement pathway within the graft, causing endothelial necrosis, platelet deposition, local coagulation and ultimate graft loss (Colvin, 2007). Accordingly, technology affording their detection has greatly improved and has led to avoidance of high-risk situations. Post-transplantation, the detection of HLA antibodies formed against the allograft are incontrovertibly associated with poorer long-term graft survival (Hidalgo et al., 2009, Loupy et al., 2012, Einecke et al., 2009, Cornell et al., 2008).

A limitation in defining sensitisation status through analysis of patient serum is that this reflects plasma cells actively secreting antibody and not the B-cell compartment as a whole. This is particularly relevant in the case of acute antibody-mediated rejection (AAMR). Although not as immediate as hyperacute rejection, AAMR can cause major graft dysfunction by the rapid generation of complement-fixing antibodies (Terasaki, 2003). This response is most commonly engendered by previous exposure to HLA expressed by the allograft. Since, the presence of donor-specific HLA antibodies (DSA) pre-transplantation are, in general, a contraindication to proceeding with the

transplant, AAMR may be attributed to activation of a secondary immune response as a result of memory B cells. Equally, in chronic AMR (CAMR), undetected pre-existing DSA or those developed in the post-transplantation period are implicated in antibody-mediated allograft damage as demonstrated by histological evidence (Colvin, 2007). Thus more detailed characterisation of B cells beyond their secreted products could provide valuable information to anticipate graft dysfunction, aid prediction of both acute and chronic rejection, and allow treatment prior to the development of irreversible graft lesions.

The questions central to this thesis relate to the ability to detect and characterise B cells in renal transplant recipients with differing levels of allograft stability. This introduction provides an overview of the origin, development and functions of B cells, with emphasis on the role of B-cell activating factor (BAFF) and regulatory B cells in the context of renal transplantation. The introduction concludes by describing the principle aim and objectives of this thesis.

1.1 The origin and development of B cells

B cells provide both specific and long-term immunity and are a critical component of the adaptive immune response. First, in response to antigen, B cells undergo a series of genetic rearrangements generating high-affinity antibody-producing plasma cells. Second, immunological memory which imparts long-lived immune protection against a vast range of foreign antigens is generated. To achieve this requires co-operation with T cells. Following capture of antigen, B cells process and present antigenic peptides to $CD4^+$ T cells. This interaction not only provides B cells with the help they require for activation into plasma or memory B cells but also drives $CD4^+$ T-cell activation. This third role as antigen-presenting cells (APCs) is important during the development of an immune response.

In detail, during embryonic life B cells originate in the bone marrow from pluripotent haematopoietic stem cells seeded by the developing foetal liver (Muller et al., 1994). Early B-cell development occurs with differentiation of a pluripotent haematopoietic precursor to a multipotent progenitor cell expressing a tyrosine kinase receptor, FLT3 (Hardy et al., 2007). This binds to its ligand on stromal cells allowing the next differentiation stage giving rise to the common lymphocyte progenitor; this includes T- and B-cell progenitors. With regard to B cells, the pro-B-cell is formed first and is characterised by the cell surface marker CD22. The generation of antibody diversity begins at this pro-B-cell stage with functional rearrangement of gene segments comprising variable (V), diversity (D), joining (J) of the heavy (H) chain (V_H, D_H, J_H) together with V & J of the light (L) chain (V_L, J_L). The H chain is part of the core antibody structure. Five types of H chain exist: γ, μ, δ, α and ε which confer the class of antibody as IgG, IgM, IgD, IgA and IgE. Antibodies have two identical H chains containing several constant domains and a single variable domain and two L chains containing one variable domain and one constant domain. The antigen-binding site of an antibody contains the variable region of one H chain and one L chain. There are three stages in the H and L chain gene rearrangements that depend on two enzymes; recombinase-activating gene (RAG) 1 and RAG2. First, pro-B cells rearrange the D & J segments of the H chain. Second, joining the rearranged DJ segment with an upstream V region. This requires the stromal cell-derived cytokine, IL-7 (Milne and Paige, 2006). The third is the functional rearrangement of VDJ leading to μ-H chain at the cell surface to form part of the pre-B-cell receptor (BCR). The pre-BCR must be functional for B cells to develop further and enter the pre-B-cell stage defined by expression of CD19 and CD20 at the pre-B-cell stage.

The immature B-cell stage is completed by expression of IgM on the cell surface, defined by the μ-H chain, and is followed by antigen-specific negative selection. B cells reacting with self-antigens present in the bone marrow are eliminated by central tolerance mechanisms which include anergy (Hartley et al., 1991), clonal deletion (Nemazee and Burki, 1989) and receptor-editing (Gay et al., 1993). Only a small percentage, approximately 5%, of the B-cell pool will survive past the first selection stage and enter the periphery (Anolik, 2013). These immature B cells, now

independent of stromal factors migrate from the bone marrow, via the blood stream to the secondary lymphoid organs and begin to express IgD, defined by δ-H chain along with IgM and CD21.

Transitional B cells are the intermediate stage between immature B cells derived from the bone marrow to mature B cells in the periphery. In mice, immature B cells differentiate to transitional type 1 (T1), type 2 (T2) (Loder et al., 1999) and/or type 3 (T3) (Allman et al., 2004). The classification is dependent on expression of IgD and CD21 (Carsetti et al., 1995). T1 cells are found in the bone marrow, spleen and blood. T2 are thought to be derived from T1 and are present in the spleen. T3 are proposed as an anergic splenic population failing to reach maturity (Merrell et al., 2006, Srivastava et al., 2005). T1 and T2 cells progress to become a mature follicular or marginal zone (MZ) B-cell (Allman et al., 2004). The fate of this occurs at the T1 or T2 stage and is dependent on the nature of the BCR (Pillai et al., 2005, Meyer-Bahlburg et al., 2008).

The BCR consists of a surface antigen-binding immunoglobulin plus Igα (CD79a) and Igβ (CD79b) chains; B cells deficient in the Igα cytoplasmic tail are unable to develop beyond the T1 stage (Torres et al., 1996). In addition, signalling via B-cell activating factor (BAFF) to BAFF receptor (BAFF-R) regulates B-cell expansion and survival and is proposed to control development of T1 to T2 to mature B cells. Their critical role is demonstrated by their deficiency in mice that are unable to develop beyond T1 cells and lack both T2 and mature B cells (Schiemann et al., 2001, Mackay et al., 2003).

Both follicular and MZ B cells represent mature naïve populations with differing functions. Follicular B cells generate a conventional T-cell-dependent response and MZ B cells react to T-cell independent antigens, contributing to an innate response; these routes of B-cell response are described in detail in the next section, ‘The B-cell response to antigen’.

In humans, B-cell maturation upon exit from the bone marrow is less understood. Transitional B cells have been identified as early emigrant cells previously selected for self-tolerance (Sims et al., 2005). Evidence from studies of autoimmunity indicates a

check point is lost at this stage in patients with systemic lupus erythematosus (SLE) where elevated levels of immature B cells, both polyspecific and autoreactive cells are present within the circulating B-cell compartment having failed deletion (Yurasov et al., 2005, Meffre and Wardemann, 2008). Expansion of transitional cells, identified through high expression of CD24 and CD38, are also reported in infection and more recently in transplantation (Blair et al., 2010, Sims et al., 2005, Das et al., 2012, Newell et al., 2010). Moreover, *in vitro* these cells are capable of producing IL-10, mediating immune suppression of T cells (Blair et al., 2010). The regulatory capacity identified in the transitional B-cell subset has important implications for immune competence and tolerance and accordingly there is active interest in further characterising these cells in humans. This is discussed in more detail under the section 'Regulatory B cells'.

Transitional B cells that survive selection to avoid auto-reactivity develop into naïve B cells (Palanichamy et al., 2009, Suryani et al., 2010). It is not clear in humans whether, as with mice, MZ B cells directly develop from transitional cells. Evidence suggests murine MZ B cells develop as a separate lineage of naïve B cells from those that occupy the follicles (Vossenkamper et al., 2012). However, in humans, MZ B cells have features of memory B cells and somatic mutations indicative of involvement in a germinal centre (GC) response (Weill et al., 2009). The GC reaction is described in more detail in the next section.

1.1.1 Mature B cells – The B-cell response to antigen

The further maturity of B cells occurs following encounter with antigen. Unlike T cells, they can recognise antigen in a native or unprocessed form as it circulates through B-cell-rich areas of the secondary lymphoid organs (Tarlinton and Lew, 2007). Antigen can also activate B cells by travelling on the cell surface of dendritic cells into follicular areas of the lymph nodes or spleen (Qi et al., 2006). The B-cell response is initiated following engagement of the BCR by antigen; however the BCR itself has no intrinsic signalling capacity and, to transduce signal, must be associated with CD79a and CD79b which do. Both have immune-receptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic domains (Clark et al., 1992). Phosphorylation of the ITAM tyrosines

by src kinases initiates a signalling cascade generating intermediates such as phosphatidylinositol-(3-4-5)-triphosphate (PIP3) and phosphatidylinositol-3 kinase (PI3K) (Gold et al., 1990). The expression of cell surface receptors, for example CD19, can lower the threshold for B-cell activation by augmenting signals through the BCR. Moreover, CD19 expresses tyrosine residues on its cytoplasmic domain and following phosphorylation can allow binding of other proteins such as PI3K (Carter and Fearon, 1992). Other pro-activation signals are provided by BAFF (Scholz et al., 2008) and are discussed in detail later in this chapter. BCR signalling is also negatively regulated by inhibitory cell surface receptors, CD22, CD72 and FcγRIIB, which express immune-receptor tyrosine-based inhibitory motifs (ITIMs). Ligation of these receptors results in phosphorylation of ITIMs and employment of phosphatases such as SHIP, SHP-1 and SHP-2 to degrade PIP3 thus terminating activation signalling (Pritchard and Smith, 2003). The progression of B-cell activation following successful aggregation of the BCR can be either T-cell-dependent or T-cell-independent.

1.1.1.1 T-cell-independent B-cell response to antigen

B cells can be induced to produce antibody in the absence of T-cell help, as the second activating signal usually delivered by the T-cell can be provided by the antigen itself. These antigens are derived from microbial constituents and activate B cells by cross-linking the BCR, inducing polyclonal activation of B cells regardless of antigen specificity.

Two main classes of T-cell-independent (TI) antigens exist and are broadly classified as either TI-1 or TI-2. TI-1 antigens are intrinsically mitogenic, inducing polyclonal B-cell activation through toll-like receptors (TLRs). TLRs are a class of pattern recognition receptors (PRR), which recognise conserved microbial structures such as the bacterial polysaccharide, lipopolysaccharide (LPS) or unmethylated single-stranded DNA motifs for example, CpG oligonucleotides (Manicassamy and Pulendran, 2009). Many cells of the immune system express TLRs and respond to stimulation via TLR agonists, such as LPS or CpG. These include dendritic cells, macrophages, mast cells, T cells and B cells and non-immune cells, including fibroblasts and epithelial cells. B cells, in particular,

express many TLRs, for example, TLR2, TLR3, TLR5, TLR7, TLR9, and can respond to a variety of ligands (Barr et al., 2007). The response is to proliferate, secrete antibody and cytokines or act as APCs.

TI-2 antigens are not intrinsically mitogenic and are characterised by their repetitive polysaccharide structure in the form of glycoproteins, glycolipids or capsules expressed on the surface of pathogens such as *Streptococcus pneumoniae* and *Nisseria meningitidis* (Satterthwaite et al., 1998). With an organised and repetitive form, epitopes of TI-2 antigens simultaneously engage and cross-link multiple BCRs to induce rapid IgM responses (Dintzis et al., 1983).

1.1.1.2 T-cell-dependent B-cell response to antigen

In response to T-cell-dependent antigen, naïve follicular B cells are activated in the T-cell-rich extra-follicular areas of secondary lymphoid tissue, including spleen, lymph nodes and Peyer's patches in the gut or tonsil. B-cell activation occurs via two signals, the first is delivered through the BCR, and the second, required for proliferation and differentiation, is delivered by helper T (Th) cells that recognise the processed antigenic peptides, presented in the context of HLA class II molecules, on the B-cell surface. In addition, the interaction between CD40 ligand (CD40L) expressed on the Th cell and CD40 receptor expressed on the B-cell are essential for efficient activation of naïve B cells (Gray et al., 1996, Tarlinton, 2006).

Activated B cells have two destinations; either they migrate to the T-zone: red pulp border, marginal sinus-bridging channel and into the red pulp of the spleen to form extra-follicular plasmablasts, (MacLennan et al., 2003) or they continue to interact with T cells at the T–B border of the spleen or interfollicular region of the lymph node, migrate into the follicle and proliferate to form a germinal centre (GC) (Kerfoot et al., 2011). Evidence suggests the initial response to antigen is dictated by B cells with the greatest specificity for antigen; these quickly differentiate into short-lived antibody-secreting plasma cells whereas those with low affinity encounter the GC reaction (Paus et al., 2006). Here B cells undergo rapid proliferation and form B-cell concentrated

follicles, the GC. This also contains specialised subsets of cells, follicular helper T (Tfh) cells and follicular dendritic cells (FDC). Both are important in maintaining the GC, Tfh cells through the secretion of IL-21 and high-level expression of ICOS and CXCR5 (Vinuesa et al., 2005) and FDC by localising the antigen on their surface within the follicles (Tarlinton et al., 2008b). The GC reaction results in clonal expansion, class-switch recombination (CSR) of the heavy chain, somatic hypermutation (SHM) of V_H genes and affinity maturation where there is selection of cells carrying a BCR with the highest affinity for the antigen. This modification of the BCR achieves greater affinity for antigen. The final progeny of B cells can migrate as antibody-secreting plasma cells, usually to the bone marrow or leave the GC as a long-lived population of memory B cells. These events are summarised in Figure 1.1.

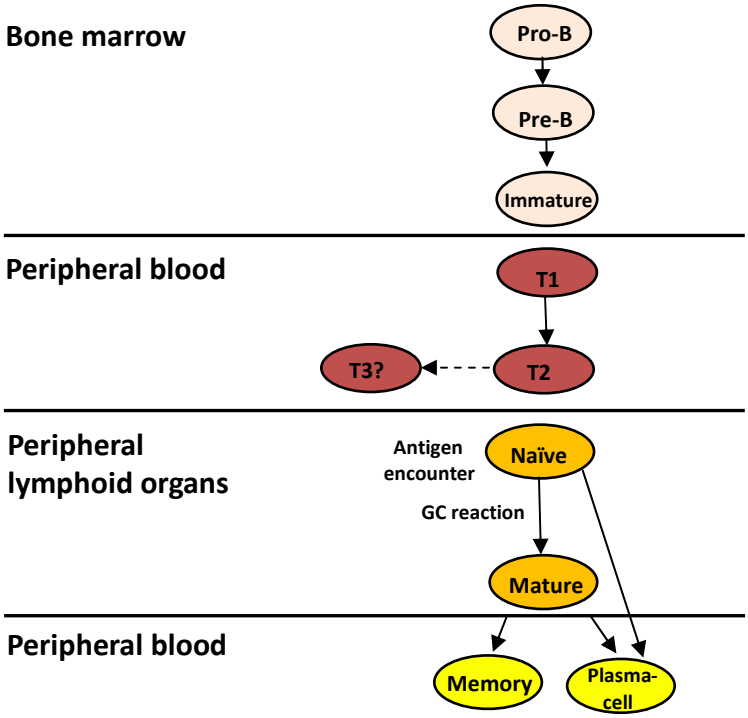


Figure 1.1 Human B-cell development
B cells originate in the bone marrow, as they progress to maturity, a number of differentiation stages occur. First formed is the pro-B cell where generation of antigen diversity begins. The pre-B cell stage is defined by possession of a functional BCR and expression of CD19 & CD20. Further selection processes occur at the immature B-cell stage by central tolerance mechanisms; those which survive enter the periphery as transitional B cells. Transitional B cells (T1 & T2) are an intermediate of immature and mature B cells. T3 cells may represent an anergic splenic population that fail to mature. Transition to maturity occurs following engagement with antigen by the BCR and associated signalling pathways. B cells proliferate to form a B-cell rich follicle, the germinal centre (GC). The final progeny of B cells can migrate as antibody-secreting plasma cells or a long-lived population of memory B cells.

1.1.1 The development of humoral immunity

1.1.2.1 Memory B cells

B-cell memory is responsible for providing long-term humoral immunity through both long-lived antibody-secreting plasma cells in the bone marrow and memory B cells circulating through the lymphatic system, driving the rapid secondary immune response. These latter cells do not themselves secrete antibody rather, following re-exposure to antigen, they can elicit an immune response capable of generating and replenishing the plasma cell compartment. In addition, antibody production is of both greater magnitude and higher affinity for the antigen than the primary response (Slifka and Ahmed, 1998). Their ability to respond rapidly to antigenic re-stimulation can be attributed to the precursor frequency of antigen-specific B cells being greater than that of antigen-binding naïve cells (McHeyzer-Williams and McHeyzer-Williams, 2005). In addition, memory B cells are more optimally placed to encounter antigen at sites of antigen drainage (McHeyzer-Williams and McHeyzer-Williams, 2005). They have also been located in the splenic marginal zone, mucosal epithelium and next to GC within secondary lymphoid organs (Aiba et al., 2010, Ehrhardt et al., 2005). Recently, murine memory B cells were located in the lung and draining lymph nodes following influenza infection (Onodera et al., 2012). In addition to differential localisation, other subsets of memory B cells exist together with the classical IgG-type, identified by expression of IgA and IgM, commonly involved in intestinal immune responses (Benckert et al., 2011, Pape et al., 2011).

Why an individual B-cell becomes a memory or plasma cell following a GC reaction (or vice versa) is not clear. A temporal switch model has been proposed whereby in early GC reactions memory cells are produced and conversely in late reactions plasma cells are produced (Shlomchik and Weisel, 2012). Evidence in animal models demonstrates early failure of the GC has minimal impact on memory but not plasma cell constitution (Wang and Carter, 2005, Inamine et al., 2005). Taken together with the uncertainty of function roles, differential localisation and subsets of memory B cells, these factors may have important implications in disease progression or resistance, and for

designing potential therapy to target memory B cells during infection or to diminish the alloresponse following transplantation.

1.1.2.2 Plasma cells

Plasma cells predominantly reside in the bone marrow. These cells are terminally differentiated and responsible for antibody secretion, although generally short-lived; are capable of persisting for many years (DiLillo et al., 2008, Slifka et al., 1998). The signals that enable long-term plasma cell survival may be mediated by up-regulation of anti-apoptotic molecules such as Bcl2 and Mc11 (Spets et al., 2002, Peperzak et al., 2013). Mc11, in particular, has been shown to be critical for plasma cell survival. There is also dependence on access to specialised niches formed by stromal elements in normal and inflamed tissue (Tarlinton et al., 2008a). This migration towards niches is reliant on expression of survival factors such as APRIL, IL-6 and CXCL12 (Tarlinton et al., 2008a, Chu and Berek, 2013). CXCL12 was the first of these to demonstrate function during migration: under its influence immature plasma cells migrate into the blood stream and enter the bone marrow (Radbruch et al., 2006). Furthermore, in synovial tissue from patients with rheumatoid arthritis (RA), infiltrating plasma cells consistently express CXCR3, while synovial fibroblasts produce its ligand, CXCL9, possibly providing a mechanism for retaining these cells in the inflammatory environment (Tsubaki et al., 2005). However, it remains to be determined how plasma cell homeostasis is maintained with the continual addition of new specificities without any loss of the existing population.

1.1.2.3 Longevity of B-cell memory

The memory response to antigen can be elicited many years after immunisation or infection and a number of mechanisms for this are proposed. First, that a population of long-lived plasma cells exist or that antigen persists providing constant antigenic stimulation (Lanzavecchia and Sallusto, 2009). However, it is unclear how these memory cells are maintained in the absence of persistent antigenic stimulus. For example, serum antibodies to the vaccinia virus have been detected more than half a

century post-vaccination with no further exposure to antigen. In humans, evidence suggests memory B cells are maintained through homeostatic proliferation (Bernasconi et al., 2002). By measuring memory B-cell responsiveness to polyclonal stimuli in the absence of specific antigen, memory cells will generate plasma cells maintaining constant serum antibody levels (Lanzavecchia et al., 2006). Alternatively a 'bystander effect' may exist where memory B cells respond to non-specific T cell signals as a result of an immune response to antigen of unrelated specificity (Banchereau et al., 1994). However, this hypothesis is undermined by data demonstrating memory B-cell proliferation is dependent on antigen specificity (Benson et al., 2009). In summary, the mechanism underlying the maintenance of B-cell memory is unclear.

Memory B cells can up-regulate survival molecules such as Bcl2 (Good et al., 2009). In addition, BAFF is implicated in maintaining memory B-cell survival as mutational studies in a BAFF receptor, TAC1, result in reduced frequency of memory B cells (Salzer et al., 2009). The direct contribution of these factors to the overall persistence of memory B-cell populations remains to be established.

1.1.3 The B-cell lineage

The process of B-cell development described does not apply to all B cells. In mice, it has long been established that two populations exist, B1 and B2 (Kantor and Herzenberg, 1993, Montecino-Rodriguez and Dorshkind, 2012). Unlike standard B cells, referred to as B2 cells, B1 cells develop during foetal and perinatal life and reside in the peritoneal and plural cavities; they are self-renewing and important in the innate immune system. Independent of T-cell help, they produce low-affinity, polyreactive, IgM antibodies and can be the first cells to respond to antigen (Martin and Kearney, 2001, Griffin et al., 2011). B1 cells can be identified by differential expression of C5. $C5^+$ cells are classed as B1a and produce broadly reactive IgM. B1b cells lack C5 expression and generate long-lasting memory-type IgM response independent of T cells (Baumgarth, 2011).

Given the location of B1 cells in foetal/neonatal peritoneal and pleural cavities, human B1 cells are difficult to isolate and characterise and an equivalent population has yet to be identified. C5-expressing human B cells in cord blood are assumed to be the murine equivalent (Montecino-Rodriguez and Dorshkind, 2012). Recently, an alternative B1 cell phenotype, $CD20^{+}CD27^{+}CD43^{+}$ was postulated to share common features with murine B1 cells (Verbinnen et al., 2012). However, more detailed analysis indicated this phenotype included pre-plasmablasts since secretion of IgG can be induced, a feature which would preclude them from being pure innate B1 type cells (Covens et al., 2013).

In contrast to B1 cells, B2 cells develop in the post-natal period in the conventional way described in mice and humans. They are continuously replaced throughout life from bone marrow precursors. Circulating in the blood and secondary lymphoid organs, they can respond to T-dependent and T-independent antigen. A number of subsets exist corresponding to the level of activation, maturation and differentiation.

1.1.3.1 Defining human B-cell subsets in peripheral blood

Until the advent of monoclonal antibody technology, descriptions of cellular characteristics were very primitive. The ability to identity cell surface receptors, known by cluster of differentiation (CD) molecules allowed for detailed examination of B cells, indeed all cells and their associated subsets. In 1980, five years after monoclonal antibodies were first described (Kohler and Milstein, 1975), Nadler and colleagues reported B1, now known as CD20, as the first B-cell-specific surface molecule (Stashenko et al., 1980). The introduction of immunophenotyping cells by flow cytometry has allowed classification of a number of B-cell subsets based on expression of cell surface markers; these include a variety of CD molecules and immunoglobulin isotypes. Universal to all B-cell subsets is expression of CD19 and in the peripheral blood there are three major populations of circulating B cells; transitional, mature naïve and memory B cells, each identifiable through cell surface markers.

1.1.3.2 Transitional B cells

Human transitional B cells are found in bone marrow, spleen and peripheral blood and were first identified by high expression of CD24 and CD38 (Sims et al., 2005, Cuss et al., 2006, Carsetti et al., 2004). In the periphery, three populations of $CD19^{+}$ cells can be discriminated using CD24 and CD38. The smallest population expresses high levels of both CD24 and CD38 and in healthy individuals represents from 2-4% of peripheral B cells (Sims et al., 2005, Carsetti, 2004, Marie-Cardine et al., 2008). (Incidentally, in the bone marrow this population can comprise up to 50% (Marie-Cardine et al., 2008).) Additional analysis of CD27 expression differentiates mature from memory cells: $CD24^{high}CD38^{-}$ are memory cells and $CD24^{low}CD38^{+}$ are mature naive B cells. CD27 is absent on $CD24^{high}CD38^{high}$ cells (Carsetti, 2004, Cuss et al., 2006, Tangye et al., 1998).

In contrast to mice, T1 and T2 populations are not as clear and the distinction between subsets is rather subjective. Some studies have reserved the brightest expression of both CD24 and CD38 for T1 cells (Palanichamy et al., 2009, Vossenkamper et al., 2013).

Recently, CD21 and IgD have also been employed, with high expression of both identifying with T2 cells (Suryani et al., 2010). Furthermore, *in vitro* studies showed $CD21^{high}$ T2 cells to be more mature by exhibiting greater proliferation and IgG secretion. In addition, analysis of these subsets following hematopoietic stem-cell transplantation (HSCT) demonstrated T1 as the primary emigrants from the bone marrow, repopulating peripheral blood prior to T2. Significantly higher expression of CD21 on T2 cells compared to T1 cells has also been reported in human bone marrow samples suggesting differentiation may also occur here (Agrawal et al., 2013).

1.1.3.3 Mature naïve B cells

Naïve B cells reside in lymphoid follicles and represent approximately 60-70% of total B cells. They are characterised by co-expressing both IgM and IgD, while having no CD27 (Weill et al., 2009). As noted above, the $CD19^{+}$ $CD24^{low}CD38^{+}$ phenotype is also used to define mature naïve B cells.

1.1.3.4 Memory B cells

Early investigations determined that memory B cells expressed CD27 defined their expression using CD27; this population represents approximately 25% of circulating B cells (Maurer et al., 1990, Agematsu et al., 1997). In addition to CD27, the identity of IgG^+ switched memory cells was confirmed by their loss of IgD expression. IgD^- cells signify the accumulation of somatic mutations in Ig V region genes, a defining feature of isotype class-switched memory cells (Tangye and Tarlinton, 2009, Klein et al., 1998). Memory B cells expressing IgM and/or IgD phenotypically resemble the classical Ig class-switched memory cells but without having undergone isotype switching. The introduction of IgD allowed identification of three populations, $CD19^+CD27^-IgD^+$, naïve B cells; $CD19^+CD27^+IgD^+$, un-switched memory B cells and $CD19^+CD27^+IgD^-$, memory B cells (Agematsu et al., 1997, Maurer et al., 1992).

CD27 and IgD were regarded as unique markers of all memory B cells (Klein et al., 1998) until minor populations of $CD27^-$ memory B cells were identified (Sanz et al., 2008, Fecteau et al., 2006, Ma et al., 2006, Wei et al., 2007). Further analysis of this population revealed IgM expression rather than IgD (Wu et al., 2011). While both $CD27^+$ and $CD27^-$ populations could elicit antibody secretion in response to viral antigen (Wirths and Lanzavecchia, 2005), fewer $CD27^-$ cells have somatic mutations suggesting an heterogeneous memory population (Wu et al., 2011).

Advances in flow cytometry have allowed multiple cell surface and intracellular markers to be explored simultaneously, allowing detailed characterisation of cellular subsets and importantly enables identification of changes within disease settings.

1.1.4 Effector functions of B cells

B cells are classically associated with antibody production. However, accumulating evidence demonstrates their functional versatility with important antibody-independent functions exerted via secretion of cytokines and antigen presentation to either promote or suppress the immune response.

1.1.4.1 Cytokine production

B cells are highly plastic in their capacity to produce cytokines, modulating both the humoral and cellular immune response. Activated B cells produce a wide range of cytokines and are classified as B-effector 1 (Be1) or B-effector 2 (Be2). Analogous to Th1 and Th2 cells, Be1 cytokines include pro-inflammatory cytokines targeting effector T-cell subsets, while Be2 cells produce cytokines which favour regulatory T-cell (Treg) expansion (Harris et al., 2000, Zhong et al., 2007). The production of cytokines can be elicited by stimuli such as TLR agonists or Th cells. TLR agonists are particularly potent mediators of cytokine secretion and this function is augmented by ligation of CD40 (Barr et al., 2007). Moreover, the stimulant can dictate both type and amount of cytokine produced. For example, engagement with different TLRs gives rise to different cytokines: IL-13 is produced in response TLR1 and TLR2 stimulation, while TLR7 and TLR9 engagement induced production of IL-10 (Agrawal and Gupta, 2011). Similarly, stimulation with CD40L can drive B cells to produce IL-10 and not lymphotoxin (LT); however a combination of CD40L and antigen (anti-immunoglobulin) and B cells secrete elevated levels of LT and less IL-10 (Duddy et al., 2007). The level of B-cell maturation can also determine cytokine production. For example, both naïve and memory B cells produce IL-13 in response to TLR1 and TLR2 ligation; however only memory B cells produce IL-6 and TNF-α (Agrawal and Gupta, 2011). Moreover, the production of pro-inflammatory cytokines such TNF-α and LT is most prominent from memory B cells (Duddy et al., 2007).

In the disease setting, altered capacity of B cells to produce cytokine can be measured as an indicator of dysregulation within the immune system. In autoimmunity, B cells isolated from patients with muscular sclerosis (MS) and activated with CD40L and TLR9 showed reduced secretion of IL-10 and elevated levels of LT compared to healthy controls (Bar-Or et al., 2010). Similarly, in patients infected with *Porphyromonas gingivalis,* B cells produced large quantities of IL-8 and TNF-α in response to stimulation with TLR4, unlike healthy controls who were essentially unresponsive (Jagannathan et al., 2009). These studies highlight how the microenvironment during disease can alter expression of cytokines – which could have both positive and

negative effects. For example, the secretion IL-10 by B cells has a protective or suppressive role in autoimmune disease as demonstrated in animal models where depletion directly impacted the severity of disease (Mauri et al., 2003, Yanaba et al., 2009, Mizoguchi et al., 2002, Fillatreau et al., 2002).

IL-10-producing B cells can also induce Treg expansion or suppress Th1 or Th17 responses (Fillatreau et al., 2008, Zhong et al., 2007). These cells are referred to as regulatory B cells (Bregs) and are described in more detail later in this chapter. Conversely, cytokine production by B cells could be pathogenic, enhancing disease progression. This is evident in $CD27^+$ Be1 cells which are elevated in the salivary glands of patients with primary Sjögren's syndrome (Youinou et al., 2007). Similarly, B cells stimulating the production of TNF-α and IFN-γ by $CD4^+$ T cells have been associated with development of MS (Duddy et al., 2007, Harp et al., 2010). Furthermore, B cells secreting TNF-α and LT could exacerbate inflammatory T-cell responses in MS. Importantly, depletion of B cells decreased CD4 and CD8 pro-inflammatory responses and also attenuated disease (Bar-Or et al., 2010).

Cytokine-producing B cells are implicated in the formation and maintenance of lymphoid tissue. Within the ectopic lymphoid tissues are B cells producing IFN-γ, IL-6, TNF-α and LT (Daridon et al., 2007). Ectopic lymphoid tissues develop at areas of inflammation or infection in peripheral, non-lymphoid organs. It is not understood if these tissues lead or are produced in response to disease, but B cells contribute to their formation as shown by B-cell-deficient mice failing to develop successful tertiary lymphoid tissue (Weyand and Goronzy, 2003). Moreover LT derived from B cells was important in creating successful lymphoid tissue architecture (Fu et al., 1998). In the setting of autoimmunity, B-cell ectopic lymphoid structures have been identified in patients with lupus nephritis (Chang et al., 2011). Furthermore, depleting B cells altered the lymphoid architecture through elimination of TNF-α- and LT-secreting cells (Duddy et al., 2004, Ansel et al., 2000). Similarly, depleting B cells in synovial biopsies from patients with RA failed to develop ectopic follicles and reduced autoreactive T cells (Takemura et al., 2001). Together these studies suggest cytokine-producing B cells contribute to exacerbated disease through maintaining ectopic lymphoid tissues. For

therapeutic purposes, a potentially effective strategy would target cytokines involved in a pathogenic response while sparing suppressive or regulatory cytokines having a positive impact on disease progression.

1.1.4.2 Antigen presentation

In vivo, activated B cells represent the largest population of antigen-presenting cells and are particularly efficient due to their specificity and high affinity of the BCR for antigen (Balin et al., 2008, Lund and Randall, 2010). In addition, their capacity to clonally expand allows them to become the principal APCs during an immune response (Tarlinton et al., 2008b). The capability of B cells to act as APCs was first demonstrated using EBV-transformed B cells that effectively presented and processed an exogenous antigen, tetanus toxoid, resulting in antigen-specific T-cell proliferation (Lombardi et al., 1987). Similarly, B cells isolated from healthy donors stimulated with CD40L acted as APC to generate antigen-specific $CD4^+$ T-cell lines (Lanzavecchia, 1985, Lapointe et al., 2003).

The mechanisms for effective antigen presentation are believed to be reliant on formation of the immunological synapse following encounter with specific antigen and reorganisation of the B-cell membrane (Grakoui et al., 1999). In the immunological synapse, BCR and antigen become aggregated and subsequently internalised allowing antigen degradation and presentation on the B cell's surface in association with HLA class II molecule to $CD4^+$ T cells (Batista et al., 2001). This establishes a cognate link between B and T cells due to the specificity of the antigen presented to the T-cell receptor (TCR) (Lanzavecchia, 1985). Activation of T cells by B cells is distinct from other APC that bind antigen non-specifically as B cells process and present antigen that binds specifically to the BCR therefore the peptides presented will be enriched for the specific antigen and facilitate engagement with the T-cell receptor (Clark et al., 2004). This cognate interaction provides the T-cell help required for B-cell activation while allowing the B-cell to drive T-cell activation. Evidence suggest this can both amplify and co-regulate, promoting B- and T-cell clonal expansion and memory (Ron and Sprent, 1987, Ahmed and Gray, 1996, Shimoda and Koni, 2007, Linton et al., 2000). The

importance of this interaction is highlighted by a study of allograft rejection where MHC class II-deficient B cells prevented T-cell activation (Crawford et al., 2006). Similarly, chimeric mice with defective MHC-class II had prolonged cardiac survival (Noorchashm et al., 2006). In patients with MS, memory and naïve B cells had the ability to induce $CD4^+$ T-cell proliferation to neuro-antigens which was almost absent in healthy controls (Harp et al., 2010). In addition, B cells induced IFN-γ secretion by autologous $CD4^+$ T cells suggesting a contribution in the Th1 response.

These studies show B cells can effectively process and present antigen to T cells, priming and initiating effector responses allowing T-cell mediated responses independent of antibody.

1.1.5 Summarising B-cell development

The process of B-cell development described begins in primary lymphoid tissue and progresses to maturation in secondary lymphoid tissue resulting in a population of cells expressing a hugely diverse range of BCRs capable of recognising an enormous variety of antigenic specificities. The differentiation of activated B cells into antibody-secreting cells forms the cellular basis of humoral immunity, providing both specific and long-term immunity. In addition, B cells have the inherent apparatus to effectively present antigen to T cells, and this coupled with secretion of cytokines can potentially exacerbate disease progression. In contrast, a small population of IL-10-producing B cells can exert immune suppression. A greater understanding of the B-cell compartment as a whole is emerging and will aid in designing strategies of how best to abolish or exploit these cells in disease.

1.2 The role of BAFF in the immune system

The B-cell activating factor (BAFF), a member of the tumour necrosis factor (TNF) superfamily is essential for B-cell selection, differentiation and survival (Batten et al., 2000, Mackay et al., 2010, Mackay and Schneider, 2009). Also known as TALL-1 (Shu et al., 1999), THANK (Mukhopadhyay et al., 1999), BLyS (Moore et al., 1999)and zTNF4 (Gross et al., 2000), it exists as a homotrimeric type II transmembrane protein until proteolytic cleavage by a furin protease releases the soluble cytokine (Moore et al., 1999, Mackay et al., 1999). Soluble BAFF can remain as a homotrimer or a capsid-like assembly of 20 trimers and can bind to one of three receptors: BCMA (B-cell maturation antigen), TACI (transmembrane activator) and CAML (calcium-signal modulating cyclophilin ligand) and BAFF receptor (BAFF-R), all are predominantly expressed on B cells (Mackay and Browning, 2002).

1.2.1 BAFF and its receptors

BAFF is principally expressed by cells of the innate immune system including monocytes, macrophages, neutrophils, dendritic cells & follicular dendritic cells (Mackay et al., 2003) and to a lesser extent T cells and activated B cells (Mackay and Leung, 2006, Chu et al., 2007). Expression is also described by non-haematopoietic cells. Some examples are fibroblast-like synoviocytes in the synovium in patients with RA (Alsaleh et al., 2007), astrocytes in patients with MS (Krumbholz et al., 2005) and osteoclasts in patients with multiple myeloma (Abe et al., 2006). This suggests possible niches exist, promoting the survival and function of B cells and influencing disease progression.

The production of BAFF can be stimulated by cytokines such as IFN-γ, IL-10, granulocyte colony-stimulating factor (G-CSF) (Mackay et al., 2003) and by engagement of TLR4 and TLR9 (Mackay et al., 2003, Boule et al., 2004).

Although BAFF binds all three receptors; there is higher affinity for BAFF-R and TACI (Mackay et al., 2003). The expression of these receptors on B cells varies according to

the stage of maturation. TACI is expressed on mature B cells such as marginal zone (MZ) and B1 cells (Ozcan et al., 2009) and BCMA is restricted to plasmablasts and plasma cells (O'Connor et al., 2004).

BAFF-R is the dominant receptor for BAFF and is expressed at low levels by immature B cells, increasing as B cells reach maturity such that expression is found on all mature B cells with the exception of plasmablasts and plasma cells (Ng et al., 2004, Darce et al., 2007). In addition, constitutive expression is found on regulatory T cells and is upregulated on activated T cells (Mackay and Leung, 2006). BAFF-R has a key role for inducing B-cell survival as demonstrated using BAFF-R-, BCMA- and TACI-deficient mice to show that BAFF differentiation and survival signal in transitional and mature B cells is critically dependent on BAFF-R only (Mackay et al., 2003).

The ligation of BAFF to its receptors activates specific TNF-associated factors (TRAFs) to regulate signal transduction in B cells (Figure 1.2). These TRAFs induce the (nuclear factor) NF-κB-signalling pathways which have pivotal roles in a range of immune responses, (Miller et al., 2006, Morrison et al., 2005) and can also promote regulation of Bcl-2 proteins important in cell proliferation and survival (Do et al., 2000). Ligation of BAFF with BAFF-R primarily results in activating the non-classical NF-κB2 pathway whereas binding BCMA or TACI leads to activation of the classical NF-κB1 pathway. This signalling initiates a cascade of events to promote B-cell survival and growth (Mackay and Schneider, 2009, Rickert et al., 2011).

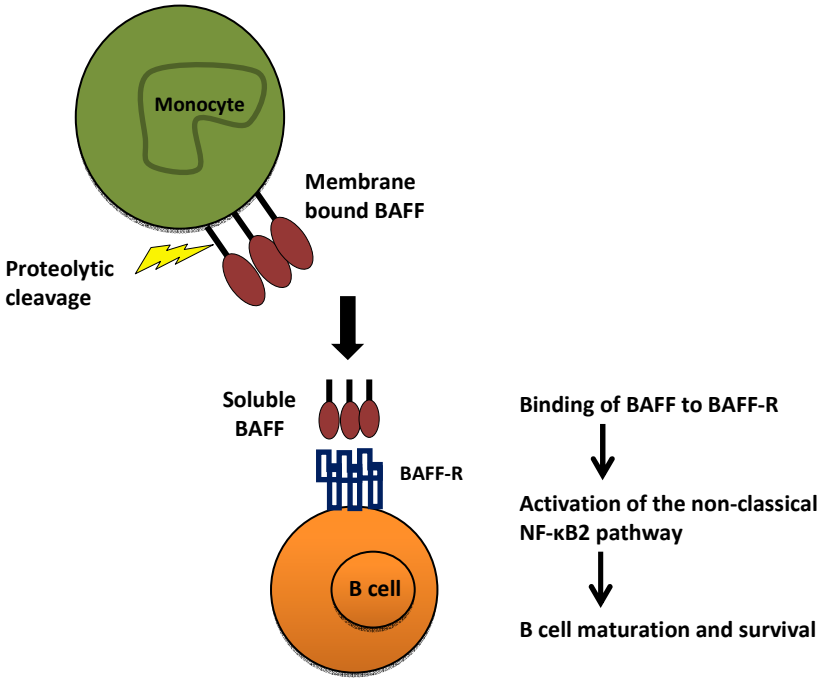


Figure 1.2 BAFF signalling through BAFF-receptor mediates B-cell growth and survival
B-cell activating factor (BAFF) is predominantly produced by innate immune cells such as macrophages, monocytes, neutrophils, dendritic cells, and follicular dendritic cells. BAFF is proteolytically cleaved at the cell surface to produce soluble cytokines which exist in the common trimeric form of TNF-family ligands. Upon binding to BAFF-receptor (BAFF-R), TNF receptor-associated factors (TRAFs) are recruited resulting in the activation of the non-classical NF-κB2 pathway promoting B-cell maturation and survival.

1.2.2 Biological functions of BAFF and BAFF receptor

B-cell survival is dependent on both the nature of BCR engagement and the availability of cytokines, namely BAFF (Anderson et al., 2007, Goodnow et al., 2005). Firstly, differentiation of B cells from immature to mature or MZ B cells requires a functional BCR consisting of a surface immunoglobulin molecule plus Igα and Igβ chains. In a murine model, B cells are unable to develop beyond the immature stage following deletion of the Igβ cytoplasmic tail (Reichlin et al., 2001). Secondly, signalling via BAFF is required, as its absence is associated with almost complete failure to proceed to maturity (Schiemann et al., 2001, Gross et al., 2001). It is now well established that maturation of transitional B cells into mature B cells is critically dependent on BAFF

(Waldschmidt and Noelle, 2001) (Kalled, 2006, Woodland et al., 2006). In a murine model, deficiency of BAFF and B cells fail to mature beyond the T1 stage to B2 or MZ B cells (Mackay et al., 2003). However, B1 and memory cells remain unaffected (Benson et al., 2008). In addition, BAFF-R is necessary to support survival signals as deletion results in the loss of mature B cells (Shulga-Morskaya et al., 2004).

There is direct interplay between BAFF/BAFF-R signalling and the BCR directly influencing B-cell maturation and survival. When BAFF is limited, ligation of the BCR by antigen results in anergy and reduced survival of immature transitional B cells (Mackay et al., 2010). Equally, during excessive production of BAFF, improper B cell maturation of splenic B cells occurs, affecting transitional T2 B cells, MZ B cells and a vastly expanded B-cell compartment (Gross et al., 2000). Furthermore, recombinant BAFF can induce survival and maturation of immature B cells (Batten et al., 2000, Rolink et al., 2002).

In addition to promoting B-cell survival, BAFF is also implicated in inducing protein synthesis, cell growth and glycolysis through activating a protein kinase pathway, mTORC1 (mammalian target of rapamycin complex 1). Cellular growth and survival effects of BAFF on B cells are mediated by the kinases, mTOR and PIM2, as studies which inactivated either kinase rendered B cells unable to respond by either survival or growth (Woodland et al., 2008).

BAFF also has a speculated role in regulating T cell immunity. *In vitro* studies show BAFF can deliver co-stimulation to T cells (Huard et al., 2001) and, in association with BAFF-R, induce proliferation of suboptimally stimulated T cells (Ng et al., 2004). Furthermore, blocking secretion of BAFF by dendritic cells can inhibit T-cell activation. (Huard et al., 2004).

Collectively there is clear demonstration in animal models that signalling via BAFF has an essential role in survival of immature B cells and complete B-cell maturation; these observations are yet to be fully extrapolated to humans.

1.2.3 The role of BAFF and disease

Given that central role of BAFF in controlling B-cell survival, it has been implicated in both autoimmunity and tolerance. In murine models of autoimmunity, transgenic-BAFF mice have increased frequency of B cells, high levels of serum BAFF and develop SLE-type features (Mackay et al., 1999, Khare et al., 2000, Gross et al., 2000). The development of this lupus-like disease was T-cell-independent resulting in chronic activation and promotion of low-affinity self-reactive B cells (Groom et al., 2007). In addition, the progression of arthritis was supressed in mice deficient in BAFF (Gross et al., 2001). In humans, excessive BAFF production measured in sera is associated with disease activity in patients with SLE, RA and Sjörgen's syndrome (Groom et al., 2002, Mackay et al., 2003, Sellam et al., 2011).

During infection, elevated serum BAFF levels are described in the context of bacterial, viral and parasitic infections (Vincent et al., 2013). Moreover, levels of BAFF can correlate with disease progression in HIV & hepatitis C infection and acute malaria (Rodriguez et al., 2003, Sene et al., 2007, Nduati et al., 2011).

During viral infection, increased BAFF is dependent on IFN and monocytes can release BAFF in response to IFN treatment (Nardelli et al., 2001). HIV-infected patients treated with IFN therapy in conjunction with anti-retroviral agents demonstrated both higher titres and broader specificity of anti-HIV antibodies and increased serum BAFF (Adalid-Peralta et al., 2008). Similarly in bacterial infection, patients with active pulmonary tuberculosis had both elevated BAFF and IFN levels (Liu et al., 2012). It remains to be established if heightened BAFF expression is a direct effect of infection by BAFF-producing cells or merely a bystander effect of inflammation.

In cancer, BAFF may contribute to oncogenesis in both solid and haematological tumours (Vincent et al., 2013). Some examples of increased serum BAFF levels are seen in non-Hodgkin's lymphoma (Briones et al., 2002) oral cavity cancers (Jablonska et al., 2011) and multiple myeloma (Fragioudaki et al., 2012). Elevated serum BAFF was also noted in non-lymphoid epithelial cells of breast cancer, suggesting a wider

role of activity (Pelekanou et al., 2008). In contrast, epithelial cell-derived BAFF in prostate cancer has shown to be protective; limiting tumour survival (Di Carlo et al., 2009). This suggests further understanding of BAFF in disease dynamics is required, in particular assessing BAFF-R phenotype, as this could have important implications toward the rationale of targeting BAFF as a therapeutic strategy.

1.2.4 The presence of BAFF following transplantation

B cells are recognised to contribute towards the process of acute and chronic allograft rejection, primarily through the production of alloantibody. The recent identification of BAFF in solid organ transplantation has been met with some interest. In murine models, BAFF-deficient mice demonstrated prolonged cardiac allograft survival, dependent on BAFF-R rather than BCMA or TACI (Ye et al., 2004). In addition, blocking BAFF with monoclonal antibodies increased survival in MHC-disparate islet allografts (Parsons et al., 2012).

To date, human studies have predominantly assessed BAFF in patient sera and have associated heightened expression with negative outcome in both stem cell (Sarantopoulos et al., 2009) and renal transplantation (Xu et al., 2009a, Zarkhin et al., 2009, Pahl et al., 2010). Firstly, in allogeneic hematopoietic stem cell transplantation (HSCT) elevated BAFF levels are associated with chronic graft versus host disease (GvHD). Focusing on renal transplant recipients, higher levels of surface BAFF on CD4 and CD8 T cells has been identified with abnormal function five years post-transplant (Xu et al., 2009a). Similarly, rejecting patients had elevated serum BAFF which correlated with increased incidence of HLA class I donor-specific antibodies (DSA) and decreased clearance of creatinine (Zarkhin et al., 2009). Furthermore patients with ESRF have elevated serum BAFF levels. Here, expression of BAFF-R was also assessed and found to be significantly reduced on transitional B cells. (Pahl et al., 2010).

Elevated BAFF levels are linked with antibody-mediated rejection (AMR), in particular following induction therapy with alemtuzumab, an anti-CD52 monoclonal antibody targeting T and B cells but more profoundly T cells. B cells can recover beyond baseline

levels within one year whereas T cells can take three years to achieve even half the baseline level (Bloom et al., 2006). Patients treated with alemtuzumab in the absence of calcineurin-inhibitors have increased frequency of AMR and DSA (Pascual et al., 2008, Cai et al., 2004). Moreover, patients have heightened expression of BAFF (Bloom et al., 2009). Together, these studies suggest excessive production of BAFF may create a dysregulated environment for B cells, leading to the activation and expansion of alloreactive B cells.

Recently, a cohort of patients with stable graft function at enrolment was assessed for the expression of BAFF-R transcripts and it was found that those who developed graft dysfunction had elevated levels. Furthermore, high level of serum BAFF could predict development of DSA, translating to a 38.3% risk compared to 10.7% in patients with low serum BAFF (Thibault-Espitia et al., 2012). A similar study measured serum BAFF levels in patients undergoing HLA-incompatible renal transplantation and associated elevated serum BAFF with increased risk of developing AMR (Banham et al., 2013b). Together these studies indicate the potential to measure serum levels of BAFF during the post-transplant period to provide an indication, perhaps earlier than a rise in serum creatinine, of graft dysfunction.

A more detailed approach to identify BAFF and BAFF-R involvement in renal injury is direct localisation by immunohistochemistry within the kidney. This has previously been shown in graft loss biopsies from those with acute rejection and interstitial fibrosis/tubular atrophy where BAFF stained strongly in the perinephric tubular epithelial cell cytoplasm and cell membrane but was absent or weak in protocol biopsy sections (Xu et al., 2009a). Furthermore there was correlation between intensity of expression for BAFF, C4d and IgG, although IgG was with weaker intensity (Xu et al., 2009b). A study of chronically rejecting patients found BAFF was overexpressed in serum and by gene analysis and the source within the tissue was inflammatory cells infiltrating the graft (Thaunat et al., 2008). To date, these are the only two published data sets concerning renal transplantation; however intrarenal inflammation is a common feature of lupus nephritis and BAFF has been identified within renal tissue in this disease setting. Here both BAFF and BAFF-R accumulated in interstitial

inflammatory cells and BAFF-R was restricted to a small population of cells, consistent with B cells (Neusser et al., 2011). Similarly, BAFF was abundantly expressed in the renal interstitium and associated with intrarenal B cells. Furthermore there was significant correlation between intrarenal B cells and the level of renal function (Sun et al., 2013). Together these studies demonstrate that, in rejecting allografts and lupus nephritis, BAFF can be localised within renal tissue to areas of infiltrating inflammatory cells and correlate with clinical parameters, implicating BAFF in the pathogenesis of disease and as a potential target for therapeutic intervention.

1.2.5 BAFF as a therapeutic target in renal transplantation

The importance of BAFF in B-cell biology suggests it as an interesting therapeutic target. Accordingly, an anti-BAFF human monoclonal antibody, belimumab, has been developed and approved for treatment in SLE by the USA Food and Drug Administration (FDA) (Vincent et al., 2013). However, due to cost-per-quality-adjusted life year, it has not been approved for use in the UK by The National Institute for Health and Care Excellence (NICE). In the USA, belimumab has been used in a Phase II trial studying desensitisation in renal transplant patients (ClinicalTrials.gov identifier: NCT01025193). However, the trial was terminated for failure to reach the primary outcome measures in effectively decreasing antibody levels and permitting transplantation. In the UK, a Phase II trial is currently approved but not yet recruiting to study the effect of belimumab in preventing rejection post-transplant (ClinicalTrials.gov identifier: NCT01536379).

In renal transplantation, the optimal time to target B cells may be at the time of transplant, as ‘induction therapy’. Early targeting of the B-cell compartment is proposed to be beneficial, coinciding with the period of intense B-cell selection, as during this time three quarters of developing B cells alter their specificity through receptor editing or deletion (Cornall et al., 1995, Pelanda and Torres, 2006). In a murine model, humoral transplant tolerance was acquired following ablative therapy whereby B cells with specificity for self-antigen were supressed from migrating to the follicle and developing into mature B cells resulting in clonal deletion of alloreactive

specificities (Parsons et al., 2011). Therefore early shaping of the repertoire could prevent production and development of alloantibody in the months or years post-transplant (Chhabra et al., 2013). However, employing rituximab, an anti-CD20 therapy, at induction did not produce a favourable outcome and was implicated in an increased rate of acute rejection which included both cellular and humoral responses (Knechtle et al., 2009, Knechtle et al., 2003, Clatworthy et al., 2009). This may be attributed to the deletion of regulatory B cells; this is described in the next section.

An alternative is to target BAFF in conjunction with anti-CD20 monoclonal antibody. In a murine model, blocking BAFF signalling increased the efficiency of anti-CD20 monoclonal antibody by decreasing MZ and follicular B cells (Gong et al., 2005). Combining anti-BAFF antibody and B cell depletion was employed in NOD mice. This strategy showed delayed repopulation of autoreactive B cells and anti-islet autoantibody levels were reduced (Zekavat et al., 2008).

This is yet to be applied to human studies. However, targeting BAFF in transplantation has a number of issues: first it is unlikely to be useful as an induction therapy due to the time taken to be effective; second, it is unlikely to be useful for sensitised patients as memory B-cell activation is independent of BAFF signalling. Therefore, the most likely scenario for the interruption of the BAFF pathway is in conjunction with B cell-depleting therapy to deplete the recovering B-cell population of precursor B cells which contain alloreactive cells, thus preventing an alloresponse against the graft in the future.

1.2.6 Summarising the role of BAFF in the immune system

BAFF has a pivotal role in B-cell development and survival. Increasing evidence, in a range of pathologies, identifies an increased level of BAFF with disease activity. However it remains to be determined if there is a direct role in generating the manifestations of diseases or it is merely a marker of chronic inflammation. In the post-transplant setting, elevated BAFF levels are associated with rejecting allografts and its co-existence with alloantibody is suggestive of its making a contribution to

humoral rejection. Accordingly, it is a potential target for therapeutic intervention to enhance efficacy of B-cell immunotherapy by affecting the composition of the B cell compartment, impairing the ability to mount an alloresponse.

1.3 Regulatory B cells

The activities of B cells described place them as pathological mediators during an immune response; in contrast, a distinct population exists to promote immune suppression. The notion that B cells could exert regulation was proposed over forty years ago by Morris and Moller (Morris and Moller, 1968) demonstrating murine B cells from immunised donors could produce antibodies which, upon antigenic stimulation, suppressed primary immune responses. At a similar time a specific T cell population was identified to negatively regulate, as opposed to augmenting an immune response, (Gershon and Kondo, 1970) prompting others to investigate if a population comparable to the negative feedback system seen in T cells exists in B cells. The first indication emerged through adoptive transfer studies where splenocytes depleted of B cells were unable to inhibit delayed-type hypersensitivity reactions (Katz et al., 1974). Evidence followed that suppression was independent of antibody. Here mice immunised with sheep erythrocytes induced the development of antigen-specific suppressive T cells (Shimamura et al., 1982). Interest in these cells did not remerge for some time, until a demonstration using the murine model of MS deficient of B cells, experimental autoimmune encephalomyelitis (EAE), was exacerbated and these mice failed to undergo spontaneous remission unlike their wild-type counterparts (Wolf et al., 1996). Similar observations followed in animal models of inflammatory bowel disease (Mizoguchi et al., 2002) and RA (Mauri et al., 2003).

These studies provided evidence of B-cell-mediated immune suppression independent of antibody. This protection is now attributed to B cells producing the anti-inflammatory cytokine, IL-10. The central role of this cytokine has been confirmed in animal models where manipulation directly impacted severity of disease (Mauri et al., 2003, Yanaba et al., 2009, Mizoguchi et al., 2002, Fillatreau et al., 2002).

1.3.1 Regulatory B cell phenotypes in mouse and man

Regulatory B cells do not have a unique set of cell surface markers or a defined transcription factor related to their activation and function in mouse or man. In murine models, a number of distinct subsets are described to exert protective capacity including: B10 cells, $CD19^{high}CD5^{+}CD1d^{high}$ (Yanaba et al., 2008); transitional 2 marginal zone precursor (T2-MZP) cells, which are $CD21^{high}CD23^{high}CD1d^{high}$ (Evans et al., 2007); $CD1d^{high}$ B1b cells, which are $CD5^{-}B220^{low}CD11b^{+}IgM^{+}CD1d^{high}$ (Mizoguchi et al., 2002); and $CD1d^{high}$ Tim-1^{+} $CD5^{+}$ (Ding et al., 2011). Prominent among them are B10 cells and T2-MZP cells. B10 cells are a rare splenic population that produce, and are the predominant B-cell source of IL-10 following stimulation with LPS. *In vivo* adoptive transfer reduced inflammation during contact-hypersensitivity and EAE (Matsushita et al., 2010, Yanaba et al., 2008).

$CD21^{high}CD23^{high}CD1d^{high}$ T2-MZP cells identified in the collagen-induced arthritis (CIA) model produce IL-10 in response to CD40 stimulation. Similar to B10 cells, adoptive transfer could prevent development of arthritis in syngeneic recipient mice (Evans et al., 2007). This phenotype has also been identified in a murine model of lupus-like disease: these MRL/lpr mice expand in response to agonistic CD40 monoclonal antibody and produce IL-10 (Blair et al., 2009). Importantly their suppressive capacity was investigated and provides evidence that both *in vitro* and *in vivo* it is mediated by both IL-10 and cell-cell contact with $CD4^{+}$ T cells (Mauri et al., 2003, Mizoguchi et al., 2002).

In human peripheral blood, only relatively recently was their existence demonstrated in helminth-infected MS patients as a distinct population of $CD1d^{+}$ cells producing high levels of IL-10 (Correale et al., 2008). Since then others have sought to identify and correlate expression in disease settings. However, direct comparisons between studies are difficult due to the lack of a unique phenotype, and the additional challenge of their low frequency, further impacted by immunosuppression. In peripheral blood of normal healthy individuals, IL-10-producing B cells are found to represent <1% (Iwata et al., 2011) with approximately 60% expressing high levels of CD24 and CD38

(Kalampokis et al., 2013). An emerging pattern is found of human IL-10-producing B cells being enriched within this immature transitional B-cell subset with growing evidence to support their suppressive role (Blair et al., Flores-Borja et al., 2013, Newell et al., 2010, Bouaziz et al., 2010). This includes the ability to inhibit production of Th1 cytokines, IFN-γ and TNF-α (Blair et al., 2010) and maintain Tregs (Flores-Borja et al., 2013).

Of note are two further phenotypes exhibiting regulatory capacity. First, a population of IL-10-producing B cells to parallel murine B10 cells. In contrast to mice, $C5^{+}$ cells are not the main source of IL-10-producing cells in humans. Although C5 is expressed at high levels in the foetal spleen (Antin et al., 1986), it is significantly reduced in cord blood with less than 2% secreting IL-10. In peripheral blood they have been identified within the $CD24^{high}CD27^{+}$ subset (Iwata et al., 2011). However, functional studies revealed that suppressive capacity was not confined to this subset, as both $CD24^{high}CD27^{+}$ and $CD24^{low}CD27^{-}$ populations inhibited production of IFN-γ by Th1 cells, independently of IL-10. In contrast, the IL-10-producing $CD24^{high}CD27^{+}$ subset inhibited TNF-α production by monocytes.

Second is a B-cell subset expressing high levels of CD25 and secreting high levels of IL-10 (Amu et al., 2007, Brisslert et al., 2006). This phenotype was depressed in patients with active ANCA-related vasculitis compared to those in remission (Eriksson et al., 2010). A more detailed $CD25^{high}$ Breg subset, $CD25^{high}CD27^{high}CD86^{high}$ $CD1d^{high}IL\text{-}10^{high}TGF\text{-}\beta^{high}$ cells has recently been described in healthy controls to suppress $CD4^{+}$ T cell proliferation and stimulate an increase in Tregs (Kessel et al., 2012).

Together these studies show a population of IL-10-producing B cells exists in humans, but there remains no uniformity in the assignment of a unique phenotype to fully characterise or define functionality.

1.3.2 Activation of regulatory B cells

Activation of B cells with regulatory function was first described following induction of EAE and CIA, where production of IL-10 was antigen-specific, dependent on both ligation of the BCR by antigen and CD40 by CD40L (Fillatreau et al., 2002, Mauri et al., 2003). CD40L belongs to the TNF-family and is expressed on T cells, crosslinking CD40 on B cells, inducing their maturation and development (Banchereau et al., 1994). A number of studies detail CD40 binding with its ligand as essential for activation of Bregs. For example, CD40-deficent mice suffer more severe EAE coupled with elevated Th1 and Th17 responses (Mizoguchi et al., 2000). In the murine model of lupus, administering anti-CD40 antibody could correct the deficit in number of Bregs and delay progression of disease (Blair et al., 2009). Similarly, anti-CD40 treatment prevented development of arthritis by promoting IL-10 production rather than a Th1 cell response (Mauri et al., 2000).

To activate IL-10 production by effector and memory B cells stimulation via the BCR and CD40 are sufficient, but in naïve B cells they depend on additional signalling through TLRs (Fillatreau et al., 2002). TLR agonists are the strongest stimuli for inducing IL-10 secretion by B cells (Barr et al., 2007). Those most commonly employed are lipopolysaccharide (LPS) from Gram-positive bacteria or CpG-containing oligonucleotides that mimic bacterial DNA. The requirement for TLR signalling has been explored in animal models of disease. In mice, TLR2 and TLR4 were critical for development of Bregs and, in their absence, developed a chronic form of EAE similar to that observed in IL-10-deficient mice (Lampropoulou et al., 2008). These mice also exhibited heightened activation of autoreactive Th1 and Th17 cells (Park et al., 2005). Furthermore, the adoptive transfer of B cells activated with LPS protected non-obese diabetic mice from insulitis (Tian et al., 2001).

In contrast, humans B cells express TLR9 and the agonist CpG, a ligand of TLR9 is a potent inducer of IL-10 (Bouaziz et al., 2010, Barr et al., 2007). *In vitro* studies show this can be enhanced when coupled with CD40L (Blair et al., 2010, Gantner et al., 2003). Furthermore, the addition of agents which induce BCR signalling can augment

IL-10 secretion. This is based on disease progression in the absence of CD19, the receptor pivotal in BCR signalling as mice deficient of CD19 suffer severe EAE (Yanaba et al., 2008). In peripheral blood from healthy volunteers, inclusion of an antibody against the BCR enhanced production of IL-10 by B cells in conjunction with CpG which interestingly was reduced in the presence of CD40L (Bouaziz et al., 2010).

The signals to drive activation of Bregs have not yet been established unequivocally and different models describe their ontogeny. Fillatreau and colleagues support a model for induction of IL-10 by B cells encompassing TLR signalling, BCR engagement and CD40 ligation. This multistep process begins with an initial TLR-dependent phase to commence IL-10 secretion and is followed by BCR recognition and CD40 interaction to sustain IL-10 production and maintain B-cell survival (Lampropoulou et al., 2010). Alternatively, Mizoguchi & Bhan propose Breg populations are generated from existing subsets via distinct activation pathways. Here 'innate type' Bregs develop from MZ B cells primed by inflammatory signals from TLR agonists, LPS or CpG. In contrast, 'acquired type' Bregs are generated via CD40 engagement with or without BCR ligation (Mizoguchi and Bhan, 2006). The exact signals inducing Breg maturation and activation remain to be determined; it is possible in the context of disease that a range of different stimuli may exist.

1.3.3 Mechanisms of immune suppression by B cells

Following the first demonstration by Janeway and colleagues that B-cell deficient mice are unable to control EAE (Wolf et al., 1996), it was proposed B cells fail to act as APCs which would normally activate T cells and induce Th2 cell differentiation. Consequently, the Th1 cell population is allowed to expand and migrate into the central nervous system allowing EAE to manifest (Adorini et al., 1997). This was accepted until evidence directed the cause of exacerbated disease to a deficiency in IL-10-producing B cells (Fillatreau et al., 2002). Similar adoptive-transfer studies followed implementing IL-10 as the primary mediator of suppressive activity exerted by B cells through failure to ameliorate inflammation (Matsushita et al., 2008, Mizoguchi et al.,

2002) Reinforcing this was the demonstration that transfer of IL-10-producing cells could actually improve the disease state in CIA (Mauri et al., 2003).

These studies place IL-10 as the hallmark suppressive feature of Bregs and evidence suggests a similar reliance in humans. Analysis of B cells from patients with SLE and MS show reduced frequency of IL-10-producing cells compared to healthy controls (Duddy et al., 2007, Blair et al., 2010). Conversely, in tolerant renal transplant recipients these cells are elevated (Newell et al., 2010), suggesting their presence offers a level of immune protection. In conjunction with secretion of IL-10, the mechanisms of suppression by Bregs include suppression of T-cell responses, inhibiting antigen presentation and promoting expansion of Tregs (Figure 1.3).

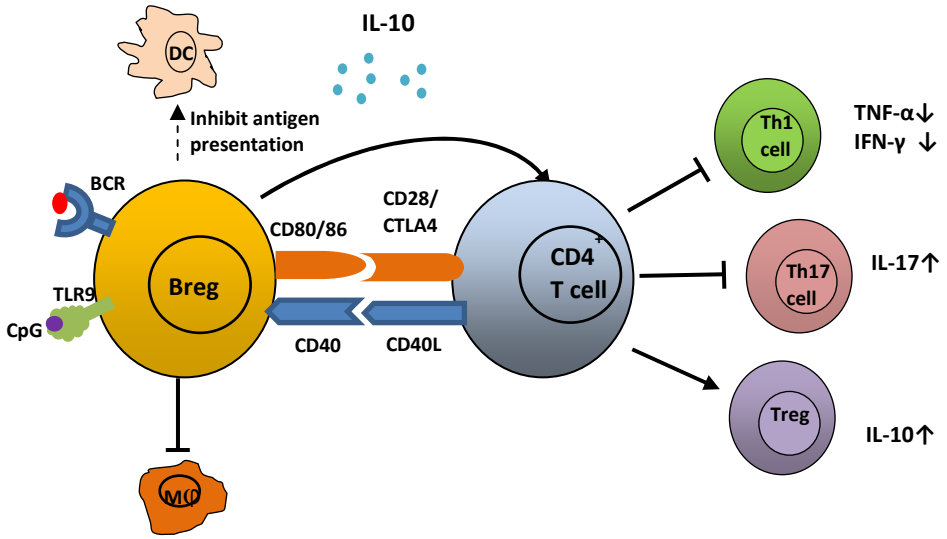


Figure 1.3 Activation and effector mechanisms of regulatory B cells
The release of IL-10 by regulatory B cells (Bregs) occurs following ligation of antigen with the B-cell receptor (BCR) and CD40 with CD40L on $CD4^+$ T cells together with co-stimulatory molecules, CD80/86 and CD28/CTLA4. Secretion of IL-10 is enhanced by Toll-like receptor 9 (TLR9) binding a ligand such as CpG. Activated Bregs inhibit activation of macrophages (Mφ) and differentiation of T helper (Th) Th17 cells & Th1 cells, the latter resulting in reduced secretion of pro-inflammatory cytokines, TNF-α & IFN-γ while maintaining regulatory T cells (Tregs). Antigen processing and presentation by dendritic cells (DC) may also be an indirect consequence of IL-10 production by B cells.

1.3.3.1 Suppression of T-cell responses

The capacity of Bregs to suppress $CD4^+$ T-cell responses are described in numerous murine models of autoimmunity (Fillatreau et al., 2008, Mauri and Ehrenstein, 2008, Bouaziz et al., 2008). This ability to dictate the differentiation of T cells has important implications on disease progression and IL-10-producing B cells are involved with supressing pathological T-cell responses during arthritis (Evans et al., 2007), MS (Fillatreau et al., 2002, Matsushita et al., 2010) & lupus (Blair et al., 2009). This is reliant on production of IL-10, suppressing polarisation of Th1 and inhibiting Th2 responses, thus restoring the Th1/Th2 balance (Pestka et al., 2004). Recently Th17 cells were demonstrated to increase during the acute-stage of CIA, correlating with a reduced number of B10 cells (Yang et al., 2012). Moreover adoptive transfer of B10 cells delayed the onset of arthritis and corresponded with a reduced frequency of Th17 cells. Similar findings have also been reported using $CD21^{high}CD23^{high}CD1d^{high}$ T2-MZP cells lacking IL-10: here CIA was exacerbated concurrently with a marked increase in both Th1 and Th17 cells (Carter et al., 2012).

Studies using B cells stimulated with CpG and anti-Ig from healthy volunteers have also demonstrated suppression of $CD4^+$ T cell proliferation (Bouaziz et al., 2010). Similarly the $CD19^+CD24^{high}CD38^{high}$ subset could inhibit the differentiation of Th1 cell production of pro-inflammatory cytokines (Blair et al., 2010), partially dependent on IL-10. Furthermore this regulatory capacity relied on engagement of co-stimulatory molecules, CD80 and CD86, as addition of monoclonal antibodies against either could reverse suppression.

1.3.3.2 Inhibition of antigen presentation

As an indirect consequence, secretion of IL-10 by B cells affects the innate immune system inhibiting antigen-processing and presentation by dendritic cells (Moore et al., 2001). This has been demonstrated in IL-10 producing $CD1d^{high}CD5^+$ cells during EAE (Matsushita et al., 2010). Furthermore dendritic cells from B-cell-deficient mice produced higher amounts of IL-12 resulting in an enhanced Th1 cell response (Moulin

et al., 2000). Conversely, IL-10 from B cells suppressed production of IL-6 and IL-12 by dendritic cells inhibiting the development of Th1 and Th17 cells (Lampropoulou et al., 2008).

Macrophages are also a target of IL-10 produced by B cells and can negatively impact the immune response by inhibiting their activation. In a murine tumour model this favoured the development of an 'M2' phenotype associated with promoting tumour angiogenesis and metastasis (Wong et al., 2010).

1.3.3.3 Supporting the differentiation of regulatory T cells

Increasing evidence suggests an important role of IL-10-producing B cells in generating and maintaining Tregs. An early study in a murine corneal transplant model showed generation of both suppressive $CD4^+$ and $CD8^+$ T cells following culture with purified antigen-specific B cells (Ashour and Niederkorn, 2006). During EAE, the expansion of Tregs depended on direct MHC-mediated contact (Sun et al., 2008a) and interactions with B7 co-stimulatory molecules (Mann et al., 2007). Subsequently IL-10 producing B cells were identified as inducing Treg development and protection against autoimmune inflammation (Gray et al., 2007). In a model of arthritis, IL-10-deficient B cells demonstrated not only to reduce expression of Tregs but also promote an increase of Th1 and Th17 cells (Carter et al., 2011). This dual regulatory role – inhibiting Th1 and Th17 cell differentiation, hence production of pro-inflammatory cytokines and promoting Tregs rather than effector T cells is also reported of $CD19^+CD24^{high}CD38^{high}$ cells isolated from healthy individuals (Flores-Borja et al., 2013). Moreover, when applied to patients with RA, failure to convert naïve T cells to functional Tregs or prevent Th17 cell development was observed. Similarly, $CD25^{high}CD27^{high}CD86^{high}CD1d^{high}IL\text{-}10^{high}TGF\text{-}\beta^{high}$ cells from healthy individuals could suppress $CD4^+$ T-cell proliferation and stimulate an increase in Tregs (Kessel et al., 2012). However rather than IL-10, this was dependent on TGF-β. In humans the role of TGF-β is unclear: suppression of Th1 cell differentiation by $CD19^+CD24^{high}CD38^{high}$ cells is partially dependent on IL-10 but not TGF-β (Blair et al., 2010). Certainly in mice, TGF-β has an important role by enhancing the function of some Bregs subsets (Noh and

Lee, 2011, Tian et al., 2001) and inducing conversion of effector to Treg cells (Singh et al., 2008).

The engagement of co-stimulatory molecules, CD80 and CD86, has proposed importance for generating Tregs. In EAE, CD86 on B cells can trigger activation of Tregs via IL-10 (Mann et al., 2007). In healthy individuals, CD86 is found over-expressed on Bregs in parallel with its ligand, CTLA-4, on Tregs during co-culture, suggesting CD86 is important in their activation (Kessel et al., 2012). Similarly, CD40-activated naïve B cells, following engagement of CD80 and CD86 promoted effective conversion from effector T cells to Tregs. In addition, these allospecific Tregs had potent suppressive capacity, more so than Tregs generated by immature dendritic cells (Tu et al., 2008, Zheng et al., 2010).

These studies indicate B cells exert their regulatory function via secretion of IL-10 and, in a contact-dependent manner, perhaps acting as a catalyst for Treg differentiation which raises interest of their use in a therapeutic setting. It remains to be established if Bregs mediate suppression *in situ* with T cells or migrate to sites of inflammation.

1.3.4 Regulatory B cells in disease

Regulatory B cells were first described in murine models of autoimmune disease as discussed. Accordingly much effort has focused to identify equivalence in human conditions. An association was first described by reduced frequencies of IL-10-producing B cells in relapsing MS compared to healthy controls (Duddy et al., 2007). Following this, MS patients with helminth infection had ameliorated symptoms in the presence of IL-10-producing B cells. Here IL-10 production was restored in response to antigenic challenge and CD40 stimulation inhibiting $CD4^+$ T-cell proliferation and secretion of IFN-γ (Correale et al., 2008). Impairment in the function of Bregs is also reported in patients with lupus (Lemoine et al., 2011, Blair et al., 2010). IL-10-producing cells were enriched in the $CD19^+CD24^{high}CD38^{high}$ cells in SLE patients. However there is dysfunction in their regulatory properties by failure to respond to CD40 stimulation and inability to suppress production of Th1 cytokines as

demonstrated in healthy controls (Blair et al., 2010). The suppressive capacity of these cells was dependent on CD40:CD40L and CD80:CD86 interactions and on the production of IL-10.

A pattern is emerging associating the transitional B-cell $CD24^{high}CD38^{high}$ subset and favourable outcome of disease. This is based on studies monitoring B-cell profiles in patients in long-term clinical remission from SLE, (Anolik et al., 2007, Palanichamy et al., 2009) and tolerant renal transplant recipients (Newell et al., 2010) who revealed a high ratio of transitional B cells compared to memory B cells.

During infectious disease, IL-10-producing B cells are predominantly reported for their pathogenic rather than protective role. During infection by parasites such as *Schistosoma mansoni*, B cells are stimulated to express Fas ligand, leading to the apoptosis of $CD4^+$ T cells, offering protection from a Th1 response (Lundy and Boros, 2002). In bacterial infection, IL-10-producing B cells inhibit protective immunity against *Salmonella* by suppressing NK cells, neutrophils and pro-inflammatory T cells (Neves et al., 2010). In *Listeria* infection, IL-10-producing B cells were expanded, inhibiting macrophage activation and increasing the bacterial load, thus promoting T-cell expansion (Horikawa et al., 2013).

In viral infection, HIV-infected individuals had increased frequency of $CD19^+CD24^{high}CD38^{high}CD10$ cells that expanded in advancing disease and were unresponsive to activation through the BCR (Malaspina et al., 2006). In this setting IL-10-producing cells indicate a role in maintaining viral load. Patients with chronic hepatitis B infection (HBV) had increased IL-10-producing B cells and IL-10 in serum which correlated with disease activity. *In vitro* studies showed predominantly $CD19^+CD24^{high}CD38^{high}$ cells were able to supress HBV-specific $CD8^+$ T-cell responses dependent on IL-10 (Das et al., 2012). This indicates a pathogenic role of IL-10 acting to suppress liver inflammation and the bystander effect of this is limiting HBV-specific $CD8^+$ T-cell response.

In cancer, fewer details exist but akin to infectious disease Bregs may disrupt immune suppression, consequently promoting tumour cell growth. The mechanisms include suppression of $CD8^+$ T cells and conversion of Tregs (Fremd et al., 2013). In tumour immunology, the protective effect offered during autoimmune disease, converting naïve T cells to Tregs (Wei et al., 2005) may be reversed. In a murine model of breast cancer, Bregs producing TGF-β convert $CD4^+$ T cells to Tregs supporting metastasis by allowing escape from the immune system (Olkhanud et al., 2011). Unlike previous observations, a role for IL-10 was not reported here (Inoue et al., 2006).

Together these data suggest Bregs can manipulate the immune response during infection by pathogens and promote tumour development by influencing both innate and adaptive immune responses. Given this, targeting Bregs may be of interest to release the suppressive restraints posed by these cells.

1.3.5 Regulatory B cells in transplantation

The suppressive capacity of B cells can be described in experimental graft-versus-host disease (GvHD) after allogeneic bone marrow transplantation by their ability to supress proliferation of $CD4^+$ T cells and inhibit the Th1 cell response (Rowe et al., 2006). Interestingly, the repopulating APCs preceding the pre-transplant irradiation regimen were predominantly IL-10-producing B cells. Moreover these cells may account for preventing GVHD by attenuating the alloresponse since B cells defective in IL-10 production suffered more severe disease.

In clinical renal transplantation, B cells are regarded primarily as detrimental to allograft survival by virtue of their capacity to present antigen to T cells and produce highly damaging donor-specific antibody. However, this negative stance is shifting as evidence emerges of certain B-cell subsets being beneficial and potentially promoting transplant tolerance (Newell et al., 2010, Pallier et al., 2010, Sagoo et al., 2010). This stems from studying recipients experiencing stable graft function in the absence of immunosuppression. By addressing B cells' phenotypes in these patients, the frequency of IL-10-producing transitional and naïve B cells is increased (Newell et al.,

2010). Furthermore when comparing patterns of gene expression; these studies showed 30 were increased by 2-fold in tolerant recipients and of those 30, 22 were B cell-specific with roles in activation and differentiation. Interestingly, three genes were identified solely in tolerant patients and all expressed by transitional B cells. A separate cohort of tolerant patients had increased B-cell numbers expressing CD1d and CD5. Here genes relating to the B-cell cycle and inhibitory molecules were over expressed, in particular BANK1 (B-cell scaffold protein with ankyrin repeats 1), an important protein involved in preventing hyperactive B-cell responses mediated by CD40 (Pallier et al., 2010). Interestingly, a similar inhibitory phenotype is observed in B cells of tolerant rats in a long-term cardiac allograft model (Le Texier et al., 2011). A further study found no significant increase in IL-10-producing B cells in tolerant recipients but a distinct gene expression profile identified with transitional B-cell biomarkers (Sagoo et al., 2010).

The functional significance of Breg expression and transplant tolerance has not been established but there are suggestions that B-cell depletion at the time of transplant could be a contributing factor by favouring the repopulation of naïve and transitional B cells (Salinas et al., 2013), akin to B cell reconstitution observed following induction with Alemtuzumab (Heidt et al., 2012a, Knechtle et al., 2009, Cherukuri et al., 2012). A commonly used therapy to deplete B cells in transplantation is rituximab, a monoclonal antibody targeting CD20 expressed on the cell surface. This can successfully decrease DSA titre and effectively supress post-transplant T cell-mediated rejection in sensitised patients (Becker et al., 2006).

In the post-transplantation setting, patients who received induction therapy with rituximab suffered a higher prevalence of acute T-cell-mediated rejection (Clatworthy et al., 2009). However, in a similar study, lower rates of rejection were reported (Tyden et al., 2009). The key difference between these studies was the timing of B cell depletion. The study reported by Clatworthy and colleagues administered two doses of rituximab at day zero and a second dose at day seven as opposed to a single dose at day zero, suggesting there may be a critical window of time during which is it possible

not to abolish a protective population of B cells and thus promote T-cell-mediated rejection.

Early investigations in autoimmune disease provided evidence that B-cell depletion exacerbated disease features; in the transplant setting this may shift the balance of allograft stability to promote rejection rather than tolerance. Currently B-cell-depleting therapy cannot distinguish between protective and pathogenic B cells and it would be beneficial to develop strategies to enhance Bregs *in vivo.* Investigations in animal models identify mechanisms of suppressive capacity to induce tolerance. For example, adoptive transfer of B cells expressing Fas-ligand can promote acceptance of male-to-female skin graft (Minagawa et al., 2004). In solid organ transplantation there are many more antigenic targets and the demonstration that monoclonal anti-TIM-1 antibodies stimulate production of IL-10, preventing rejection of pancreatic allografts (Ding et al., 2011), raises encouraging prospects to investigate selective therapy augmenting the generation of Bregs and thus promote allograft survival.

1.3.6 Summarising the role and functions of Bregs

Understanding the role of Bregs in humans is in its infancy but evidence clearly demonstrates a population of B cells, distinct from T cells, capable of modulating effector responses in a contact-dependent and independent manner, predominantly driven through secretion of IL-10. The current lack of a consensual identity hinders characterising their function or extracting conclusions from across the literature and the impetus remains to discover a unique identifier, perhaps a 'master gene regulator', analogous to Foxp3 in Tregs. The depletion of B cells in autoimmunity and pre-transplantation has augmented disease and rejection, highlighting both the importance of these cells and the requirement for targeted therapeutic strategies to eradicate harmful effector B cells while promoting the beneficial regulatory subsets which induce immune suppression and tolerance.

1.4 The role of B cells in renal transplantation

B cells have diverse functions in renal transplantation (Figure 1.4). Aside from the recent identification of regulatory B cells, in the clinical setting, B cells are predominantly considered pathogenic mediators of allograft rejection due to two key activities. First, their capacity to present antigen to T cells evoking effector responses and, second, their ability to produce donor-specific HLA antibody against the allograft.

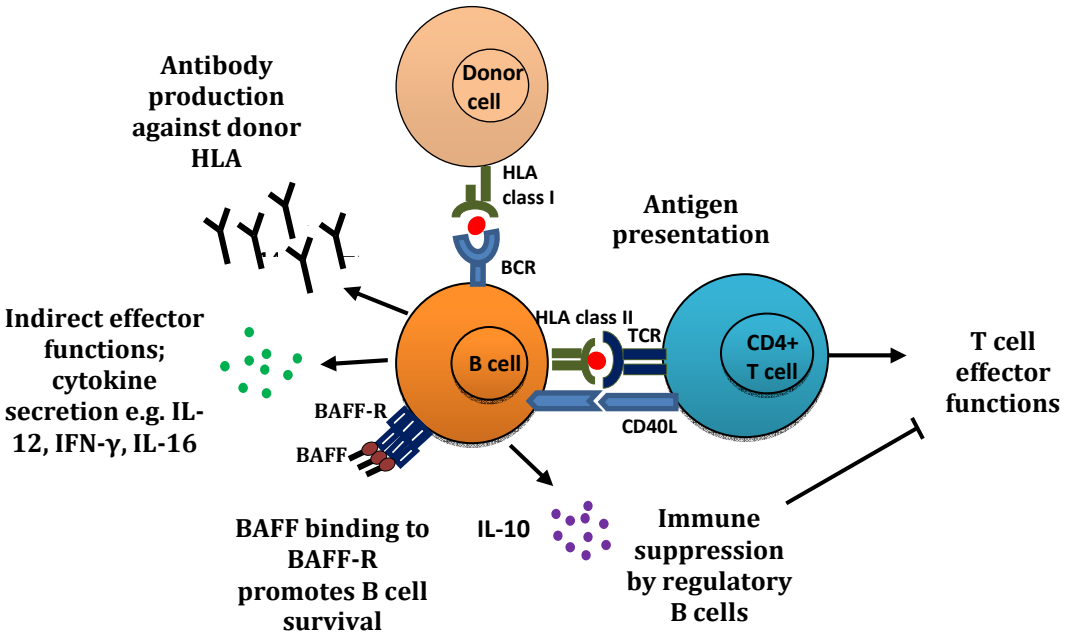


Figure 1.4 The roles of B cells in transplantation
B cells have multiple immunological functions. In addition to their primary role as antibody-producing cells they are efficient antigen-presenting cells (APCs) due to both specificity and high affinity of their B-cell receptor (BCR) for antigen which can drive activation of $CD4^+$ T cells. They also have capacity to clonally expand becoming numerically dominant during an immune response. Indirect effector functions are predominantly exerted through cytokines, in co-operation with T-cell stimulation, pro-inflammatory cytokines; IL-12 and IFN-γ are secreted. Production of IL-16 can mediate dendritic cell maturation. The B-cell activating factor (BAFF) binding to its receptor (BAFF-R) promotes B-cell maturation and survival. Excessive levels of serum BAFF are reported during allograft rejection. In contrast to the pathological role of B cells, a small population are described to down-regulate the immune response. Regulatory B cells (Bregs) are proposed to mediate suppression of damaging T-cell responses via secretion of IL-10.

1.4.1 Antigenic targets in allograft rejection

The major histocompatibility complex (MHC) in humans is the HLA system (this originally stood for 'human leukocyte antigen' but now is just 'HLA' since it is known that these molecules have a much wider distribution that just leucocytes). The HLA system has a critical role in regulating immunity. These molecules present peptides, derived from protein antigen, in a form that is recognised by T cells and in doing so initiate and propagate the immune response.

HLA molecules are classified as HLA class I or class II. Classical HLA class I molecules are HLA-A, -B and –C and are expressed on all nucleated cells and platelets. Classical HLA class II molecules are HLA-DR, -DQ and -DP and are predominantly expressed on APCs, although during an inflammatory response their expression can be induced on other cell types (Jackson et al., 2009). Clustered on the short arm of chromosome 6, the genes coding HLA molecules are the most polymorphic in the human genome with more than 8000 known alleles (www.ebi.ac.uk/imgt/hla/). Therefore finding an identical donor expressing the same HLA type for each patient is virtually impossible. As a result, transplantation proceeds in the presence of 'non-self' or mismatched HLA which is principally responsible for the immune response to alloantigen.

In addition to these major classes of HLA, there are minor histocompatibility antigens, such as MHC class I polypeptide-related sequences A (MICA) and B (MICB). The clinical significance is not clear. However both have been implicated in allograft loss in recipients well matched for HLA (Stastny et al., 2009, Dragun et al., 2008). Other non-HLA antigenic targets of the immune response in transplantation include vimentin (Carter et al., 2005), angiotensin II type I receptor (Dragun et al., 2005) and endothelial antigens (Sun et al., 2008b). Immune response to all of these has been implicated in poorer outcome. However, of recent years the primary focus in allograft rejection has been the effect of antibody against HLA, and specifically donor-specific HLA antibody (DSA).

1.4.2 The role of B cells in presenting alloantigen

B cells can present antigen to T cells. As with other professional APCs, they bind and process antigen into peptides which are presented on the cell surface as HLA/peptide complex. The fate of the processed antigen being presented by HLA class I or class II is dependent upon its entry into the cell. Endogenous protein antigens in the cytoplasm are processed and presented in the context of HLA class I to cytotoxic $CD8^+$ T cells. Conversely, exogenously generated peptides are presented on HLA class II to $CD4^+$ T cells.

In response to allogeneic transplantation, APC mediate the immune response by a process of allorecognition where donor HLA expressed on the allograft disparate to the recipient are recognised by T cells. This occurs via the direct or indirect pathway (Lechler and Batchelor, 1982). The direct pathway describes the recognition of intact donor HLA on the surface of donor APCs by TCRs on recipient T cells. The indirect pathway involves donor HLA antigen being taken up by recipient APCs and processed into peptides which are presented in the context of HLA class II to recipient T cells. Following transplantation, the direct pathway is prominent as donor APCs migrate from the graft to secondary lymphoid organs and in doing so stimulate recipient T cells which migrate to the allograft and initiate an inflammatory response. This is a major factor in acute rejection (Benichou, 1999). The indirect pathway emerges when donor APC are exhausted and recipient APC maintain the immune response by processing antigen shed by the graft and presenting to recipient T cells.

In the post-transplant setting, B cells have an important role in allograft rejection by presentation of allogeneic peptides to T cells via the indirect pathway. Both animal models and clinical studies suggest this pathway is dominant in chronic rejection (Noorchashm et al., 2006, Taylor et al., 2007). B cells are very effective APCs and represent the largest population *in vivo* (Lund and Randall, 2010). This efficiency is attributable to the BCR; allowing B cells to extract antigen at very low quantity or tethered to the plasma membrane of target cells, such as those undergoing apoptosis (Tarlinton et al., 2008b, Ciechomska et al., 2011). In addition, their capacity to clonally

expand allows them to become the numerically dominant APC (Thaunat et al., 2010). Furthermore, the cognate interaction between T and B cells; previously described in ‘‘B cell development – Effector functions of B cells – Antigen presentation”, may serve to amplify and co-regulate the alloresponse. The direct extent is not known as it is difficult to ascertain since B cells have multiple functions therefore their absence would impact other aspects of immunity.

$CD4^+$ helper T cells are key regulators in allorecognition, helping and controlling $CD8^+$ T cells but also providing essential help to activate B cells leading to antibody formation and plasma cells (details were outlined in “B-cell development - Mature B cells - The B-cell response to antigen”). With respect to transplantation, alloantibodies directed against HLA molecules expressed on the transplanted graft are important mediators of allograft rejection. The binding of antibody to donor HLA antigens, predominantly expressed on vascular endothelium, triggers a series of events leading to the activation of the complement, clotting and kinin cascades, resulting in vascular thrombosis, ischaemia and ultimately necrosis (Afzali et al., 2007).

1.4.3 Alloantibody production by B cells

B-cell activation is a multistep process, initiated by binding of antigen and ultimately production of antibody-secreting plasma cells to clear the invading antigen. Following transplantation, B cells are well placed for activation by antigens derived from the allograft transported via the lymphatics to draining lymph nodes by dendritic cells. There is also evidence of a humoral response occurring locally within the graft. Cytokines secreted by B cells are important in the formation and maintenance of lymphoid tissue. Lymphoid neogenesis is detected in the rat model of chronic allograft rejection (Thaunat et al., 2005). Furthermore, the presence anti-MHC class I antibodies implicates the role of B cells in these structures.

A heightened presence of B cells have also been found within the renal allograft biopsies of failing grafts, (Sarwal et al., 2003) and B-cell infiltrates associated with

adverse outcome (Muorah et al., 2009, Zarkhin et al., 2008). These studies place B cells as important mediators of allograft rejection.

An important co-stimulator of B cells is BAFF, as previously described. Suffice it to say, elevated levels of BAFF are associated with declining renal graft function (Bloom et al., 2009, Banham et al., 2013a, Thibault-Espitia et al., 2012). The direct involvement of BAFF in sustaining B-cell activation and driving alloantibody production is not known but its central role in both B-cell survival and function is suggestive of a contributory role in dysregulated immunity.

The presence of pre-existing memory B cells, generated as a result of pregnancy, blood transfusion or previous transplantation, results in the production of high affinity antibody-secreting cells upon re-exposure to antigen. Interestingly, increased alloantibody production occurs in less than half of sensitised recipients indicating that alloantigen does not always evoke a memory B-cell response (Stegall et al., 2009). In the early post-transplantation setting, acute antibody-mediated rejection (AAMR) occurs when high levels of alloantibody are present and is likely due to a secondary immune response mediated by memory B cells. Evidence for this follows the identification of circulating HLA-specific B cells using tetramers which upon stimulation produce DSA (Zachary et al., 2007, Mulder et al., 2003). Similarly, memory cells from peripheral blood of sensitised patients were isolated and induced to secrete DSA (Han et al., 2009). The production of DSA by pre-existing memory B cells is a major obstacle in achieving long-term allograft survival.

1.4.4 Role of B cells in transplant tolerance

In contrast to the pathogenic function of B cells in allograft survival, there is evidence to suggest a role in tolerance. This is highlighted by the success of blood group or ABO-incompatible paediatric heart transplantation (West et al., 2001, Henderson et al., 2012). The reduced risk of hyperacute rejection was attributed to lack of ABO antibodies which do not occur until five to six months after birth, resulting in spontaneous tolerance to blood group antigens (Fan et al., 2004). This demonstrates a

case of acquired tolerance in humans and is proposed to result from clonal deletion. Since donor-reactive cells are dependent on alloantigen for their development, B-cell-depleting therapy eliminates the probability of an alloantigen-specific germinal centre response (Parsons et al., 2009, Parsons et al., 2011). However, depleting B cells at the time of transplant may not be favourable as previously described in the context of regulatory B cells. An additional explanation is the nature of the antigen presented by B cells to T cells and their cognate interaction. This is proposed to promote T-cell immunity as endogenous or pinocytosed antigens presented by B cells can lead to T-cell tolerance (Chen and Jensen, 2008). In particular, antigen presented by naïve B cells stimulates naïve T cells towards Tregs (Reichardt et al., 2007, Chen and Jensen, 2007). Interestingly, the recent identification of a higher proportion of naïve and transitional B cells in stable transplant patients in the absence of immunosuppression suggests repopulation with immature and naïve B cells may facilitate B-cell transplant tolerance (Newell et al., 2010, Sagoo et al., 2010). Moreover, this may explain why the transfer of naïve B cells promotes murine skin allograft acceptance (Fuchs and Matzinger, 1992). Collectively, evidence suggests certain populations of B cells could be exploited by therapeutic strategies to harness clinical transplant tolerance.

1.4.5 HLA antibody-mediated allograft rejection

Mismatched ‘non-self’ HLA molecules are targets for an immune response and antibodies against these can be raised following sensitising events. If a transplanted organ expresses HLA against any of these pre-existing HLA antibodies, there is an increased risk of both rejection and reduced allograft survival (Kerman et al., 1996, Susal and Opelz, 2002, Wahrmann et al., 2006).

It is well established that the presence of pre-formed HLA antibodies is a contraindication to transplantation potentially resulting in hyperacute rejection (Patel and Terasaki, 1969). Hyperacute rejection rapidly follows transplantation, usually within 24 hours, as a direct result of DSA or blood-group antibody produced in response to antigen on allograft endothelial cells. This type of rejection has fortunately become a very rare occurrence, largely due to advances in pre-transplant antibody

detection and assessment of immunological compatibility between the donor and recipient. However, antibodies are still implicated in acute and chronic humoral rejection, and major barriers to successful transplantation.

1.4.6 Defining and categorising antibody-mediated rejection

Renal allograft rejection is classified by histopathological diagnosis of biopsy tissue. To standardise definition, the Banff classification scheme was introduced in 1993 (Solez et al., 1993). Six categories were defined, but only one of those included an antibody-mediated component and this was hyperacute rejection. The 1997 revision of the Banff classification recognised the participation of antibody and hyperacute rejection was renamed antibody-mediated rejection (AMR) which included hyperacute rejection and accelerated acute rejection (Racusen et al., 1999). The 1997 update published in 2003 further placed a contributory role of HLA antibody with allograft rejection (Racusen et al., 2003). This was aided by the description of staining for C4d, an inactive cleaved component of the classical complement pathway. (Feucht et al., 1993). Deposition of C4d on peritubular capillaries provided evidence of antibody involvement, given the relationship between antibody binding and complement activation in allografts, and became accepted as a marker of AMR (Racusen et al., 2003). However, evidence showed AMR could be C4d-negative, indeed in up to two-thirds of cases (Sis and Halloran, 2010). Equally, positive staining for C4d can occur in the absence of anti-donor activity. Accordingly, Banff criteria reflect the uncertainty surrounding the role of C4d as a marker of allograft rejection has led to the inclusion of 'C4d deposition without the morphological evidence of active rejection' (Solez et al., 2008). In addition, AMR was further defined as acute or chronic.

1.4.6.1 Acute antibody-mediated rejection

The criteria for diagnosing AAMR are morphological evidence of tissue injury, C4d positivity and detection of DSA (Sis et al., 2010). As noted above there is uncertainty of C4d expression and AAMR in the absence of C4d is classed as 'suspicious of' or 'consistent with' AAMR (Racusen et al., 2004). Between 30-40% of sensitised patients

experience AAMR and it is a major cause of morbidity and early graft loss (Stegall et al., 2010). Moreover, AAMR can contribute towards the development of chronic rejection and transplant glomerulopathy (TG) (El-Zoghby et al., 2009, Issa et al., 2008). Furthermore there is correlation between the incidence and levels of DSA post-transplant as patients with low level DSA do not present with AAMR on biopsy, whereas those who develop high levels very often do (Burns et al., 2008).

1.4.6.2 Chronic antibody-mediated rejection

Diagnosing chronic antibody-mediated rejection (CAMR) is complicated by the number of non-immune factors involved such as age of the kidney, chronic ischaemia, drug toxicity, hypertension, diabetes and atherosclerosis (Kreis and Ponticelli, 2001, Fellstrom, 2001).

According to Banff criteria, diagnosing CAMR include demonstration of C4d positivity, DSA and at least one feature of histological findings such as peritubular capillary basement membrane thickening, glomerular double contours or interstitial fibrosis/tubular atrophy, and/or intimal thickening of arteries (Colvin, 2009). TG which results in glomerular capillary endothelium injury is diagnostic of CAMR and associated with reduced allograft survival, although CAMR can exist in its absence (Gloor et al., 2007). In AAMR, the role of DSA in pathogenesis is well defined (Solez et al., 2008). However in chronic rejection this is not fully understood, as DSA can be absent in the presence of histological evidence of AMR and C4d positivity, but DSA may be an important developmental element. Evidence shows that recipients who develop CAMR have higher HLA antibody titres in the first year post-transplant (Kwun and Knechtle, 2009).

1.4.7 The impact of HLA antibody on allograft survival

It has long been established that HLA antibody has a negative impact on allograft survival. In 1971, Terasaki and colleagues reported the presence of HLA antibodies prior to transplantation significantly decreased allograft survival (Terasaki et al., 1971).

Now clear evidence exists that pre-formed DSA pose a risk of immediate rejection, while post-transplant DSA significantly impairs allograft function and survival (Lefaucheur et al., 2010, Terasaki, 2003). With regard to HLA class, both HLA class I and/or class II correlate with a poorer prognosis of graft survival (Rifle et al., 2005, Susal et al., 2009). Furthermore, patients with DSA compared to non-DSA have significantly lower allograft survival (Lefaucheur et al., 2010). Pre-existing DSA are particularly detrimental, as even in the absence of clinical AMR results in worse long-term allograft survival (Lefaucheur et al., 2010, Haas et al., 2007).

De novo DSA (dnDSA) have a particular association with late allograft loss (Cooper et al., 2011, Ginevri et al., 2012, Everly et al., 2013), with the presence of antibodies to both HLA class I and II being associated with increased risk of graft failure than the presence of antibodies to either class alone (Wiebe et al., 2012). Moreover, anti-HLA-DQ antibodies are implicated with a significantly greater risk of rejection and reduced allograft survival than antibodies to other class II isotypes (Willicombe et al., 2012, DeVos et al., 2012).

These data suggest immunogenic relevance in HLA antibody type and development of rejection. In general, HLA class I are associated with acute humoral rejection (Crespo et al., 2001), while HLA class II have a particularly important negative impact on long term graft survival (Langan et al., 2007, Campos et al., 2006), possibly due the expression of HLA class II on the endothelial cells of peritubular and glomerular capillaries (Muczynski et al., 2001, Gloor et al., 2007). These differences in immunogenicity are not an inherent property of the HLA molecule itself but reflect how the HLA epitopes, comprising triplets of amino acids, are presented to recipient HLA (Doxiadis et al., 1996). During an alloresponse, the development of HLA antibody is directly related to the number of mismatched or non-self-triplets presented on the HLA molecule (Dankers et al., 2004, Kosmoliaptsis et al., 2008). Moreover, the physiochemical characteristics between mismatched HLA class I molecules influences the production of specific alloantibody post-transplant (Kosmoliaptsis et al., 2009).

Evidence demonstrates a strong association between HLA antibody and AMR, and subsequent allograft failure. The development of sensitive methods of detecting these antibodies has afforded more accurate definition and detailed analysis of their impact on transplant outcome.

1.4.8 Clinical immunology: HLA antibody detection

Immunological assessment of a recipient–donor pairing can help predict the potential risk of post-transplant AMR. The most clinically relevant HLA antibodies are DSA. However not all DSA cause deteriorating graft function or rejection. It is not clear why differences in pathogenicity exist, but the ability to activate complement could be an important factor (Wahrmann et al., 2006, Wahrmann et al., 2009).

A number of methods are employed to allow detection of HLA antibody. Initially, this was achieved using cell-based assays, most commonly by complement-dependent cytotoxicity (CDC) assay. Prior to transplantation, a CDC assay can detect DSA in recipient serum, specifically against HLA molecules expressed on donor cells. Anti-donor activity is visualised by the addition of a source of complement and a cell viability stain. A positive CDC assay is indicative of hyperacute rejection and considered an absolute contraindication to transplantation. However, a negative CDC assay cannot rule out presence of low titre DSA and a more sensitive flow cytometric test is also included. This is 10- to 250-fold more sensitive than the CDC assay and can be used to detect HLA antibody directed against HLA class I using T cells and HLA Class II using B cells (Mehra et al., 2013). Together the CDC and flow cytometric assays provide information of anti-donor activity.

For specific identification and characterisation of HLA antibody, more sensitive solid phase assays (SPA) have been introduced. SPA utilise purified HLA derived from transfected cell lines or solubilisation from cell membranes. These purified HLA are coupled to a solid phase such as a plastic plate or latex beads to measure serum HLA antibody, revealing previous antigenic exposure and estimate the degree of circulating antibody. The clinical relevance of pre-transplant DSA detected by SPA at levels below

those detected by CDC is demonstrated by their association with reduced renal function (Lefaucheur et al., 2009). The most widely used SPA in transplantation laboratories are multiplex quantitation assays, namely Luminex technology. Here patient sera are exposed to a panel of purified HLA class I and class II molecules coupled to microbeads which determines relative antibody strength, specificity and identifies unacceptable antigens, if necessary to the single-antigen level. This information, combined with the CDC assay and flow cytometric cross-match gives the most informative evaluation of a recipient's immune status.

A strategy to increase the predictive value of SPA to include functional complement activation has been developed to better identify clinically relevant DSA. Using a modified form of the SPA used to detect HLA antibodies, the addition of a source of human complement and an antibody reactive against the complement fragment, C4d allows detection of DSA-induced complement activation (Smith et al., 2007, Bartel et al., 2008). In a clinical context, C4d fixing-DSA could predict allograft survival in cardiac allograft recipients (Smith et al., 2007), and in renal allograft recipients with low-levels of DSA in the context of negative CDC assay and flow cytometric cross-match, was predictive for AMR (Lawrence et al., 2013).

Based on the same principle, an alternative SPA encompassing the detection of C1q, the first component in the complement cascade has been developed. The detection of C1q-fixing DSA are shown to be predictive of allograft rejection and graft loss (Yabu et al., 2011, Sutherland et al., 2012). This assay is reported to have higher sensitivity compared to detection of C4d by SPA (Tyan, 2012). However both are relatively new assays and data is currently limited.

Defining immunological risk using improved SPA could better identify unacceptable donor-antigens and patients at risk of AMR to potentially improve outcome following renal transplantation.

1.4.9 Summarising the role of B cells in transplantation

Multiple factors participate towards graft dysfunction and ultimate loss. The contribution of B cells and specifically the production of antibodies against the graft are a significant component in the process of allograft injury.

The increased sensitivity of tests for detecting HLA antibodies has shifted the attention from simply identifying patients at risk of rejection to providing a relative level of risk. This has increased access to transplant by defining DSA and allowing monitoring in antibody-removal programmes. Moreover, has assisted clinically in the diagnosis of humoral rejection thus allowing appropriate therapy to target the B-cell compartment.

1.5 Aims and Objectives

Aim

To develop and implement methods of detailing the B-cell compartment in renal transplant recipients, and to assess if there were characteristics of these cells which could be harnessed to indicate renal allograft stability.

Objectives

1. To determine if the risk of a renal transplant recipient developing antibody-mediated rejection post-transplantation could be predicted pre-transplantation using *in vitro* analysis of allospecific B cells.

2. To identify and characterise donor HLA-specific B cells within the circulation, and determine whether HLA-DQ-specific B cells had any defining characteristics to account for the observed prevalence of *de novo* HLA-DQ antibodies post-transplantation.

3. To determine if there was a relationship between differential expression of BAFF and level of allograft function; to promote its use as an effective biomarker post-transplantation.

4. To investigate phenotypic and functional properties of regulatory B cells, and determine if their presence or suppressive capability differed depending on level of graft function.

Chapter 2

General materials and methods

2.0 Materials and methods

2.1 GM 1 Recruitment of study patients and blood sample collection

Renal transplant recipients were recruited during attendance at routine clinic appointments at the Royal London Hospital, Barts and The London NHS Trust, London, UK.

The study was ethically approved by a local ethics committee (Central London, REC1; Research Ethics Reference Number: 07/H0707/10; Appendix A) and written informed consent was obtained to participate. Venous blood samples were collected in ethylene diamine tetra-acetic acid (EDTA) vacutainers (Becton Dickinson (BD), UK) and processed within four hours for analysis of peripheral blood mononuclear cells (PBMC; 40 ml). A clotted sample (6 ml) was also collected for serum retrieval. Blood tubes were centrifuged for 10 minutes at 2000 revolutions per minute (RPM), serum collected from the top layer and stored at -20 ^{o}C until further use.

Staff working within Barts and The London NHS Trust or the Centre for Immunology & Infectious Disease, Queen Mary University of London served as blood donors for use as healthy controls as detailed in the ethics application (Appendix A).

2.2 GM 2 Immunosuppressive regimes

Immunosuppression protocols variably included anti-CD25 induction therapy with Basiliximab (Simulect; Novartis Pharmaceuticals) or Daclizumab (Zenapax; Roche) or, anti-thymocyte globulin (ATG). A number of patients were transplanted prior to the introduction of induction therapy.

Maintenance immunosuppression included a calcineurin-inhibitor, anti-metabolite and rapidly tapering steroids.

2.3 GM 3 Defining level of allograft function in patient cohorts

Patients were separated into three cohorts by level of allograft function based on eGFR (estimated glomerular filtration rate) measured over a 36-month period or 12 months if six or more samples had been collected. This could more accurately reflect current graft function; particularly if there was deterioration.

GFR (glomerular filtration rate) is used for diagnosis, staging and determining rate of deteriorating renal impairment by measuring serum creatinine. Creatinine is a waste product of muscle metabolism which enters the blood stream and is maintained at a steady level depending on kidney function. Elevated levels of creatinine can indicate renal function is impaired. The rate at which creatinine is cleared is proportional to the GFR. Previously, this required a urine sample taken over a 24-hour period. A more practical approach is to estimate GFR using serum extracted from whole blood and apply the Modification of Diet in Renal Disease (MDRD) equation (Levey et al., 1999). This formula (186 x (Creatinine/88.4)$^{-1.154}$ x (Age)$^{-0.203}$ x (0.742 if female) x (1.210 if black) = eGFR (ml/min/1.73m^2) also accounts for patient age, sex and race providing a more accurate measure than would serum creatinine measurements alone. eGFR measurements are less accurate at near-normal levels of renal function (Levey et al., 2014) (>60 ml/min). In this study, all patients had an eGFR of <60 ml/min.

The criteria for patient group allocation was based on the presence or absence of a progressive process toward declining allograft function, and determined by linear regression analysis. This method was used by Dudley and colleagues in the "creeping creatinine study" to define patients with chronic deterioration in allograft function (Dudley et al., 2005). Based on this, eGFR measurements were plotted against time and a negative gradient with a significance value of P less than 0.05 and an R^2 of more than 0.35 was considered to demonstrate rapidly deteriorating (RD) graft function. A negative gradient with an R^2 of between 0.1 and 0.35 were defined to have slowly deteriorating (SD) graft function, and the stable (ST) group was defined as having no significant change in function. While it was recognised that using the gradient of the

slope would have been more informative of how eGFR changes as a function of time rather than how well a line fits the data, as demonstrated by the R^2 value; the method detailed by Dudley and colleagues was used for a well-regarded clinical trial, and was therefore adopted here.

2.4 GM 4 Peripheral blood mononuclear cells (PBMC) separation

Peripheral whole blood (40 ml) was diluted 1:2 with sterile phosphate buffered saline (PBS; PAA laboratories, UK), layered over 15 ml of Ficoll-Hypaque solution (GA Healthcare, UK) in 50 ml Falcon tubes (BD) and centrifuged for 30 minutes at 2000 RPM. Lymphocytes were collected from the interface, washed in RPMI-1640 (Invitrogen, UK) and centrifuged at 1700 RPM for 10 minutes. If necessary, red blood cells were lysed (ACK buffer; Invitrogen) for five minutes at room temperature, then washed with RPMI for 5 minutes at 1400 RPM, this was the standard centrifuge speed and time unless otherwise stated. Supernatant was discarded and cell pellets resuspended in RPMI-1640 supplemented with 5% L-glutamine, 5% penicillin, 5% streptomycin (Sigma-Aldrich, UK) and 10% heat inactivated sterile-filtered (0.2 µm; VWR, UK) foetal calf serum (FCS; PAA laboratories); this medium was used throughout and shall be referred to as complete medium. Cells were counted using a haemocytometer under a light microscope and viability evaluated by Trypan blue (0.4%, Invitrogen) exclusion.

2.5 GM 5 $CD19^+$ cell isolation by positive selection

Immediately following PBMC isolation, CD19 cells were selected using magnetic-bead separation (Miltenyi Biotec) as per manufacturer's instructions. Briefly 1 x 10^7 PBMC were resuspended in 80 µl MACS buffer (PBS/0.5% FCS/0.05% EDTA) with 20 µl CD19 microbeads in a 15 ml Falcon tube (BD) and incubated for 20 minutes at 4 °C. Cells were washed with 2 ml MACS buffer, resuspended in 500 µl MACS buffer and applied to a column (MS column; Miltenyi Biotec) attached to a magnetic plate. The labelled PBMC suspension was allowed to run completely through the column before being

washed three times with 500 µl MACS buffer. The column was removed from the magnet and the positively selected cells attached to the column were released by depressing the plunger. Cells were washed and resuspended in complete medium at a concentration of 1 x 10^6/ml.

2.6 GM 6 Cellular phenotyping by flow cytometry

Cells were stained with fluorchrome-conjugated monoclonal antibodies analysed using a flow cytometer (LSRII analyser; BD Biosciences, UK). The optimal antibody concentration was determined by titration experiments. Unless otherwise stated, 0.5 µg/million cells provided adequate staining. Cells were gated for lymphocytes on the basis of forward and side scatter profiles. The relevant labelled isotype control antibodies were included in all experiments and used to set voltage and compensation parameters. All cells with a staining intensity higher than the upper limit obtained using the isotype control (<0.5%) were considered positive. Data was analysed using Flowjo software (Treestar Inc.).

2.6.1 GM 6.1 Extracellular staining for CD19$^+$ cell subsets

Cells were transferred to FACS tubes (BD), centrifuged and the cell pellet resuspended in 50 µl FACS buffer (PBS/2% FCS) and stained with a cocktail of mouse anti-human monoclonal antibodies or relevant isotype controls (Table 2.1). Cells were stained in the dark at room temperature for 20 minutes, washed in FACS buffer and analysed by flow cytometry.

Antibody Specificity	Fluorochrome	Clone	Source species and isotype	Company	Reference
CD19	APC-Vio 770	LT19	Mouse IgG1, κ	Miltenyi Biotec	(Tedder et al., 2002)
CD27	PerCP-Cy 5.5	M-T271	Mouse IgG1, κ	Biolegend	(Tangye and Tarlinton, 2009)
IgD	FITC	IA6-2	Mouse IgG2a, κ	BD Biosciences	(Wei et al., 2007)
CD24	PE-Cy7	ML5	Mouse IgG2a, κ	BD Biosciences	(Sims et al., 2005)
CD38	V450	HIT2	Mouse IgG1, κ	BD Biosciences	(Sims et al., 2005)
BAFF receptor	PE	11C1	Mouse IgG1, κ	BD Biosciences	(Mackay and Mackay, 2002)

Table 2.1 Extracellular antibodies used to identify B-cell subsets by flow cytometry

2.7 GM 7 Polyclonal agonists applied during CD19+ cell culture

Purified CD19+ cells were cultured with polyclonal agonists to induce proliferation and differentiation. Details of concentration and combination with additional supplements are detailed in Table 2.2.

Stimulants	Working Concentration	Company	Reference
R848 (Resiquimod), TLR7/8 ligand Recombinant human IL-2	2.5 µg/ml 1000 U/ml	Autogen Bioclear Peprotech	(Tomai et al., 2000)
Type B CpG oligonucleotide CpG-B ODN 2006, TLR9 ligand (CpG)	10 µg/ml	Invivogen	(Bernasconi et al., 2002)
CpG Recombinant human CD40 ligand (CD40L)	10 µg/ml 1 µg/ml	Invivogen R&D Systems	(Gantner et al., 2003)
CpG CD40L Goat anti-human IgA+IgG+IgM (anti-Ig)	10 µg/ml 1 µg/ml 20 µg/ml	Invivogen R&D Systems Jackson ImmunoResearch Laboratories, Inc.	(Bouaziz et al., 2010)

Table 2.2 Polyclonal agonists used to simulate B-cell proliferation and differentiation

2.8 GM 8 HLA antibody detection in serum samples

LABScreen single-antigen (LSA) microbeads (One Lambda, USA) were used to detect IgG antibodies to HLA class I and II antigens as prescribed by the manufacturer. In brief, 300 µl of wash buffer was added to the required wells of a 96-well filter plate and incubated at room temperature. After 10 minutes, the wash buffer was aspirated using a vacuum manifold. 20 µl of each serum was added per well with 2.5 µl of

microbeads and the plate was incubated in the dark for 30 minutes with shaking at room temperature. Following incubation, 275 µl wash buffer was added to each well and aspirated using the vacuum manifold. This step was repeated four times. Finally, 100 µl of PE-conjugated anti-human IgG was added to each well and incubated for a further 30 minutes in the dark at room temperature while shaking. After incubation, the plate was washed as stated above and 80 µl PBS added to each well. The plate was read using a Luminex 100 IS analyser (One Lambda), and data exported to HLA fusion software (One Lambda) for analysis. To normalise data, the Baseline formula was used where the LSA microbead value is subtracted from the negative control microbead value for both the patient and negative control serum. These two values are then subtracted to give a normalised value.

Baseline formula

[(Raw Value of N) – (Raw Value of NC)] - [(Raw Value of N) – (Raw Value of NC)]

Patient Sample **Negative control serum**

N = LSA test beads, NC = negative control bead

To assign the strength of each anti-HLA reaction, results were expressed as mean fluorescence intensity (MFI); an MFI greater than 500 were defined as very weak positive, above 1000 MFI were weak positive and above 2000 MFI as positive. These tests were performed by the Clinical Transplantation Laboratory, Barts and The London NHS Trust, London, UK.

2.9 GM 9 HLA-typing by PCR-SSP

HLA Class I and Class II type was derived by DNA typing using the polymerase-chain reaction (PCR) with sequence-specific primers (SSP). Patients and donors were typed at the HLA-A, -B, -C, HLA-DRB1/B3/B4/B5 and HLA-DQB1 loci. These tests were performed by the Clinical Transplantation Laboratory, Barts and The London NHS Trust, London, UK.

2.10 GM 10 Statistical Analysis

Experimental data was analysed using Prism software (GraphPad Software Inc., USA; Version 6.02). A *p* value of less than 0.05 (<0.05) was considered statistically significant. Data analysis is summarised by mean, median and standard deviation (sd). For categorical variables, differences were assessed by Chi squared test. The non-parametric Kruskal-Wallis test with correction for multiple comparisons was used for analysis of differences between patient groups. If the *p* value was significant, where the difference lied was explored by investigating the largest median difference between groups and the Mann-Whitney test was performed. The Mann-Whitney test was also used to investigate differences between unpaired data within groups.

Chapter 3

Pre-transplantation assessment of the alloresponse to donor antigen

3.1 Introduction

Achieving long-term allograft survival can be compromised by detrimental effects of memory B cells; this is increasingly relevant as the number of sensitised patients awaiting transplantation expands. The existence of a pre-existing humoral response to alloantigen is often identifiable by the presence of donor-specific HLA antibody (DSA) in serum, resulting in a positive crossmatch upon incubation with donor cells. However, alloreactive memory B cells generated as a result of pregnancy, blood transfusion or previous transplant can exist in the absence of detectable serum antibody. Upon transplantation, memory B cells become activated, resulting in a highly damaging secondary immune response. These cells can have a dual role in promoting allograft injury; primarily due to the effect of alloantibodies, leading to antibody-mediated rejection (AMR) and reducing graft survival (Rifle et al., 2005, Campbell et al., 2007), but also acting as antigen-presenting cells (APCs), contributing to graft rejection by directly activating allospecific T cells. In the clinical setting, pre-transplantation assessment does not include methods to address the potential for alloreactive memory B cells; their presence is unfortunately discovered in light of acute antibody-mediated rejection (AAMR).

Within the live-donor transplantation programme, certain donor–recipient pairs are at heightened risk of developing AAMR. Of those are women sensitised during pregnancy as a result of maternal immunisation to paternal antigens inherited by the foetus. In general, pre-formed alloantibodies are detected during pre-transplantation assessment and, in the absence of any contraindication, transplantation generally proceeds to a favourable outcome. However, AAMR can occur and is reported following both offspring–mother and husband–wife transplantation (Rosenberg et al., 2004, Bohmig et al., 2000, Matsuo et al., 2009). Given this, the policy of some transplant centres is not to transplant women with the donor whom they have been pregnant with (van Kampen et al., 2002). However with cadaveric donors in short-supply and increased allograft survival offered by live-donation, for many recipients their husband or offspring is the best available option. Accordingly, a method to

determine the presence of memory B cells reactive against donor-antigens; indicative of AAMR, would benefit patients with undetectable serum HLA antibody.

Detection and enumeration of antibody-secreting B cells was first described using an enzyme-linked immunosorbent spot assay (ELISPOT) (Czerkinsky et al., 1983). Following this, demonstration that memory B cells could be stimulated to produce antibody using polyclonal activators resulted in its application to monitor antigen-specific memory B cells (Bernasconi et al., 2002). Frequently employed mitogens are CpG (TLR 9 agonist), pokeweed mitogen in combination with *Staphylococcus aureus* Cowen, CD40-ligand (CD40L), IL-2 & IL-10 and R848 (TLR7 and 8 agonist) together with IL-2 (Pinna et al., 2009, Crotty et al., 2004, Cao et al., 2010). The introduction of effective agents to successfully induce polyclonal activation of memory B cells has allowed application of ELISPOT in a number of disease settings, including memory B cells specific for viral antigen, for example to the anthrax vaccine (Crotty et al., 2004) and HIV antigens (Titanji et al., 2006). In addition, is utilised to detect antigen-specific B cells of the malaria-causing parasite, *Plasmodium falciparum,* (Dorfman et al., 2005) and blood-group antibodies following ABO-incompatible heart transplantation (Fan et al., 2004). Recently designed is an ELISPOT to detect and enumerate HLA-specific memory B cells. Synthetic HLA molecules are applied as the detection matrix and this assay has been employed in a small cohort of patients as a tool for monitoring post-transplant alloreactivity (Heidt et al., 2012b).

ELISPOT is a well-established method for detecting memory B cells. However until the publication by Heidt and colleagues, (Heidt et al., 2012b) its application has predominantly been restricted to the memory recall response to viral antigens. This study sought to develop and apply an IgG-detecting ELISPOT to measure and quantify the humoral response to alloantigen. By studying a cohort of women, as pre-emptive renal allograft recipients from either their husband or offspring, the study aimed to establish if this method can provide clinical information of allosensitistion not currently detected by conventional pre-transplant assessment and, thus, reduce the risk of AMMR.

3.2 Materials and methods

Materials and methods detailed in Chapter 2, General Methods (GM) are indicated.

GM1 **Recruitment of study patients**

GM4 **Peripheral blood mononuclear cells (PBMC) separation**

GM5 **$CD19^+$ cell isolation from PBMC**

GM6 **Phenotyping $CD19^+$ cells by flow cytometry**

GM7 **Polyclonal agonists applied during $CD19^+$ cell culture**

GM8 **Detection of HLA antibody in serum by Luminex technology**

GM 9 **HLA-typing by PCR-SSP**

GM10 **Statistical analysis**

Responder-stimulator cell preparation

Responder and stimulator cells (donor-type and third-party) were HLA-typed by PCR-SSP (GM 9).

Stimulator cells – Third-party and donor-type

PBMC were isolated from fresh peripheral whole blood as previously described (GM 4). PBMC (1 x 10^6/ml) were resuspended in RPMI-1640 supplemented with 5% L-glutamine, 5% penicillin, 5% streptomycin and 10% FCS (complete medium) and transferred to a 12-well tissue culture plate (Becton Dickinson; BD) before exposure to gamma irradiation at 30 Gy. Following this, cell viability was assessed by Trypan blue (0.4%; Invitrogen) exclusion. Cells were washed and resuspended in complete medium at 1 x 10^6/ml.

Responder cells

$CD19^+$ cells were isolated from PBMC as described in GM 4 & 5 and resuspended in complete medium prior to cell culture. Cells were phenotyped by flow cytometry to provide pre-culture B-cell subset frequency (GM 6, Table 2.1).

Allogeneic and polyclonal cell culture conditions

For polyclonal cell culture (GM 7), 100 µl 10^6/ml $CD19^+$ were cultured together with either R848 (2.5 µg/ml) and IL-2 (1000 U/ml) or a CpG-mixture (CpG-B 2006 (10 µg/ml); CD40L (1 µg/ml) and anti-IgG-IgM-IgA (anti-Ig; 20 µg/ml)) to give a final volume of 200 µl in complete medium.

For allogeneic cell culture, 100 µl 10^6/ml $CD19^+$ responder B cells and irradiated (30 Gy) 10^5/ml donor-type or third-party donor PBMC were resuspended in complete medium.

200 µl of each cell culture were added in triplicate to a 96-well U-bottom plate (BD) and incubated at 37 ^oC in 5% CO_2 for five days prior to transfer to the ELISPOT assay or retained in the plate for phenotype analysis by flow cytometry at day seven.

IgG-detecting ELISPOT assay

At day five post-culture, 96-well filter plates (Millipore, MAHA N4510) were pre-wet with 70% ethanol (50 µl for 1 minute), washed five times with sterile water and coated with 100 µl goat anti-human IgG (1.25 µg/ml, Sigma-Aldrich) and incubated overnight at 4 ^{o}C. Following incubation, the plate was washed five times with PBS and blocked with 200 µl complete medium for two hours at room temperature.

At day six post-culture, 200 µl of cell cultures were plated in duplicate into the pre-coated wells and incubated for 24 hours at 37 ^{o}C, 5% CO_2. For development, the plate was washed five times with PBS and 100 µl mouse anti-human IgG Fc biotin-conjugated antibody (1 µg/ml; Sigma-Aldrich) in PBS/0.5% FCS added per well and incubated for two hours at room temperature in a humid chamber. The plate was washed and 100 µl of streptavidin–alkaline phosphate (1 µg/ml; Sigma-Aldrich) applied per well for 45 minutes at room temperature in the dark. After a further wash, 100 µl of the development reagent, BCIP/NBT (Mabtech) was added for 10 minutes at room temperature. This reaction was stopped by extensive washing under running water. The plate was allowed to dry overnight and analysed using an automated ELISPOT plate reader (AID).

Post-culture phenotype analysis by flow cytometry

Post-culture, responder $CD19^{+}$ cells were assessed for expression of B-cell subsets by flow cytometry (GM 6, Table 2.1). Stimulator cells (donor-type and third-party) were also assessed for expression of CD4 using a mouse anti-human eFlour 450-labeled monoclonal antibody (Clone SK3; eBioscience). To confirm the efficacy of irradiation, both irradiated and non-irradiated cells were examined.

3.3 Results

This study aimed to determine if the alloresponse to donor-antigen could be measured by the frequency of antibody-secreting cells (ASC), utilising an IgG-detecting ELISPOT. Purified $CD19^+$ cells (responder cells) cultured with donor-type PBMC (stimulator cells) were assessed for spot-forming units (SFU), each equating to a single IgG-secreting B cell.

$CD19^+$ cells represent a small percentage of lymphocytes even in healthy individuals (approximately 5-10%) and are reduced in patients with ESRF (Descamps-Latscha and Chatenoud, 1996, Pahl et al., 2010). Therefore to maximise cell number available for analysis in the pre-transplant study cohort, methods were initially optimised in healthy women, sensitised through pregnancy. Sensitisation was defined by serum analysis for HLA antibody against paternal antigen by Luminex technology.

Optimising polyclonal activation of IgG-producing B cells detected by ELISPOT

$CD19^+$ cells purified from PBMC of sensitised women (n = 4) were polyclonally activated with TLR agonists previously described to induce expansion and differentiation of human memory B cells: R848 and IL-2 (Pinna et al., 2009) and CpG-B 2006 together with CD40L and anti-human IgG-IgM-IgA (CpG-mixture) (Bouaziz et al., 2010). The optimal concentration of each TLR agonist was assessed (Figure 3.1C). In both cases, the higher concentration of R848 (1000 U/ml) and CpG (10 μg/ml) promoted the greater expansion of ASC as detected by the ELISPOT assay ($p = 0.43$). In addition, the number of ASC was marginally higher at a seven-day interval compared to day-five or day 10 ($p = 0.7$; Figure 3.1D). Due to the limited number of $CD19^+$ cells available, it was only possible to assess this during culture with the 'CpG-mixture'.

Comparing effectiveness of the TLR agonists, cell culture with the CpG-mixture induced greater expansion of ASC (96 ± 92 SFU vs. 58 ± 72 SFU by ELISPOT for CpG vs. R848 respectively, Figure 3.1C). Phenotypic analysis demonstrated the majority of cells were non-switched memory B cells, particularly following culture with R848 and IL-2 (Figure

3.1E). The distinct pre-culture memory B-cell population was no longer present, however, overall a larger proportion of memory B cells existed following cell culture with the CpG mixture (31.26 ± 12.41% vs. 16.32 ± 9.25% for CpG vs. R848 respectively; Figure 3.1E). This, together with a greater proportion of ASC by ELISPOT, resulted in its use hereafter to polyclonally stimulate $CD19^+$ cells and served as the positive control.

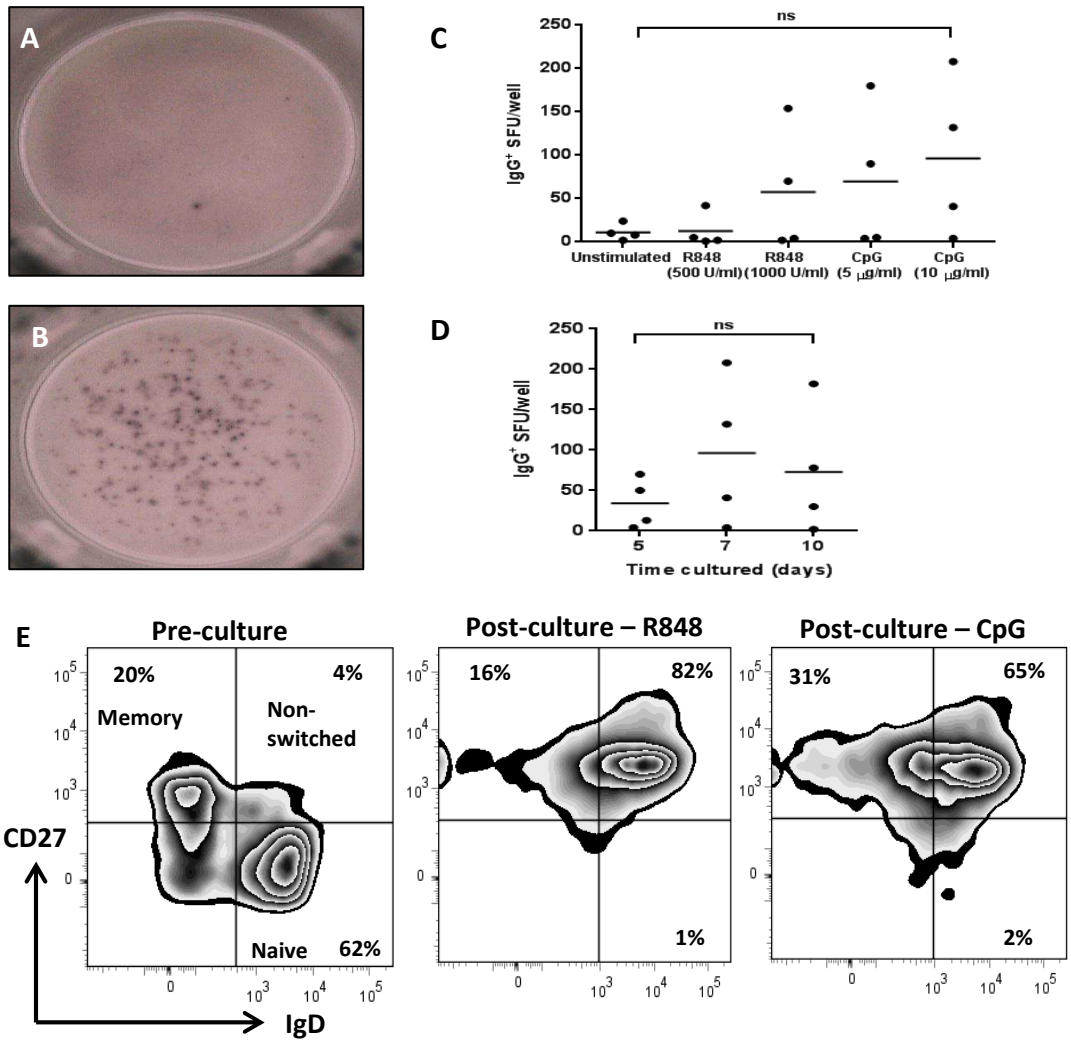


Figure 3.1 Optimising polyclonal expansion and differentiation of antibody-secreting and memory B cells

$CD19^+$ cells from women sensitised by pregnancy (n = 4) as defined by serum analysis for HLA antibody were isolated from PBMC and cultured for 7 days with TLR agonist cocktails; R848 & IL-2 or 'CpG mixture'. Post-culture, cells were assessed for IgG-secretion by ELISPOT. Representative images of (A) unstimulated and (B) CpG-activated B cells are shown. (C) The dot-plot represents mean number of spot-forming units (SFU) per duplicate well following culture at two concentrations with the TRL agonists. (D) At day 7 post-culture, the number of SFU detected following culture with the CpG mixture was marginally higher. ns is not-significant derived from Kruskal-Wallis test. (E) Representative flow-cytometric plots show $CD19^+$ cell co-expression of CD27 and IgD at day zero and day 7 with R848 & IL-2 or CpG mixture. Percentages denote proportion in each quadrant with phenotype typical of naïve, non-switched memory and memory B cells.

Measuring the alloresponse of recipient CD19⁺ cells to donor-antigen by an IgG detecting ELISPOT in sensitised healthy controls

An IgG-detecting ELISPOT was applied to measure the alloresponse to donor-antigen using $CD19^+$ cells isolated from the same cohort of women described, cultured with PBMC from their husband (donor-type stimulator cells). To control for specificity, cells were also stimulated with third-party PBMC, derived from a donor not expressing HLA matching the donor or which the recipient had pre-formed antibodies against.

The frequency of IgG-secreting B cells following culture with a range of concentrations of donor-type or third-party cells was investigated (Figure 3.2). To prevent excessive proliferation of stimulator cells; donor-type and third-party PBMC were irradiated at 30 Gy; a standard dose applied in numerous responder: stimulator assays (Game et al., 2003, Tapirdamaz et al., 2010, Tanaka et al., 2012). The ELISPOT failed to discriminate between the responses to donor-type or third-party stimulation. Since the most evident separation was achieved at the lowest concentration of stimulator cells (1 x 10^5/ml donor-type PBMC: 1 x 10^6/ml $CD19^+$ cells) when compared with non-specific stimulation by third-party donor cells, this concentration was applied in the pre-transplant study cohort. Due to the limited number of $CD19^+$ cells, it was not possible to show the phenotype of these populations by flow cytometry.

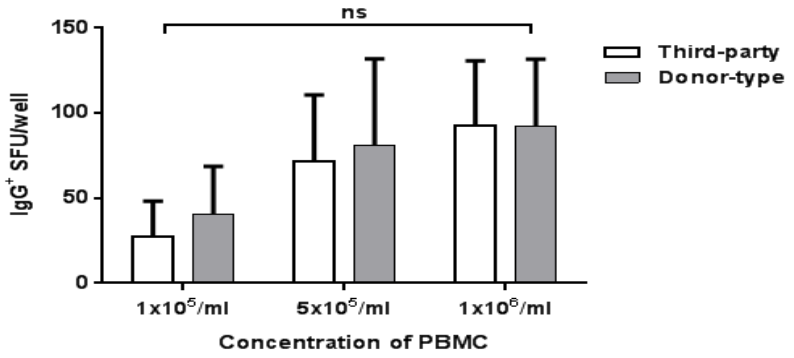


Figure 3.2 The IgG-detecting ELISPOT failed to discriminate between donor-type or third-party stimulation

Responder $CD19^+$cells (n = 4; 1 x 10^6/ml) were assessed for spot-forming units (SFU) 7 days after culture with irradiated (30 Gy) third-party or donor-type PBMC at a range of concentrations.

The bar-chart shows the mean SFU for duplicate wells (error bars represent standard deviation) at each concentration of stimulator cells. ns is not-significant derived from Kruskal-Wallis test by comparing the median SFU following third-party to donor-type cell culture at each concentration (p = 0.07).

Measuring the alloresponse in the pre-transplantation study cohort

To examine capability for measuring the humoral immune response before transplantation, a cohort of potential recipients (n = 12) were identified with defined pre-sensitisation to their donor or where a memory response was suspected as a result of pregnancy. In the majority of cases the donor was the father of a woman's offspring (83%). Patients were separated by level of sensitisation as detected in serum by Luminex single-antigen (LSA) analysis and demographics are shown in Table 3.1.

	Unsensitised UnSens (n = 4)	**Sensitised NDSA (n = 3)**	**Sensitised DSA (n = 5)**
Age, median (range)			
Recipient	57 (52-63)	33 (28-46)	50 (30-60)
Donor	36 (31-52)	36 (32-45)	46 (34-55)
Relationship			
Husband: offspring, n	2: 2	3: 0	5: 0
Previous transplant, n (%)	0	1 (33)	3 (60)
***HLA mismatches,* n ± sd**			
-A, mean ± sd	1 ± 0	1.7 ± 0.6	1.4 ± 0.6
-B, mean ± sd	1.5 ± 0.6	2 ± 0	1.4 ± 0.6
-C, mean ± sd	1.3 ± 0.5	1.3 ± 0.6	1.5 ± 0.6
-DR, mean ± sd	1 ± 0.8	1.3 ± 1.2	1.8 ± 0.5
-DQ, mean ± sd	1 ± 0	0.7 ± 0.6	1.6 ± 0.6
Pre-transplant assessment,			
Positive, n (%)			
T-cell FCM	0	0	3 (60)
B-cell FCM	0	0	4 (80)
CDC assay	0	0	0

Table 3.1 Patient demographics for the pre-transplantation cohort

FCM, flow-cytometric crossmatch; CDC, complement-dependent cytotoxicity; DSA, donor-specific HLA antigen; NDSA, non-donor-specific HLA antigen; sd, standard deviation; UnSens, unsensitised.

Measuring the frequency of antibody-secreting cells to donor-antigen by ELISPOT

The IgG-detecting ELISPOT was applied to donor-recipient pairs, prior to transplantation. Clinically, identifying a memory B-cell response in recipients where sensitisation to a potential donor is not identified by serum analysis for HLA antibody would benefit these patients at risk of AAMR; therefore of particular interest was the unsensitised (UnSens) group. Figure 3.3 shows the number of ASC detected by ELISPOT when $CD19^+$ cells from each of the three groups remained unstimulated (negative control) or co-cultured with the polyclonal agonist (positive control) or with donor-type or third-party PBMC.

The number of ASC observed following each culture condition was compared between groups, overall no significant difference was found ($p = 0.06$). Examining the B-cell response to donor-type cell stimulation showed while ASC expanded when compared to unstimulated cells in all three groups; this failed to reach a level of statistical significance, and although the number of ASC were greater in the DSA group, the difference between groups was minimal (48 ± 38, 32 ± 17 and 40 ± 17 IgG^+ SFU/well for DSA, NDSA and UnSens groups respectively). Moreover, the response to third-party stimulation was of similar magnitude in each group, questioning the specificity of the donor-type response.

Next each culture condition was compared to unstimulated cells within patient groups. Following polyclonal stimulation, when compared with unstimulated cultures, there was expansion of ASC in sensitised patients whether their antibodies were NDSA (20 ± 15 vs. 165 ± 32 IgG^+ SFU/well; unstimulated vs. polyclonal stimulation, $p = 0.06$) or significantly so, DSA (18 ± 17 vs. 144 ± 115 IgG^+ SFU/well; unstimulated vs. polyclonal stimulation, $p = 0.03$), which was absent in the UnSens group (16 ± 11 vs. 20 ± 36 IgG^+ SFU/well; unstimulated vs. polyclonal stimulation, $p = 0.82$).

If the number of ASC detected by ELISPOT following allogeneic stimulation was compared to unstimulated cells within groups, the response to did not alter significantly with either third-party or donor-type cells.

Overall these data demonstrate this ELISPOT cannot discriminate between the response to third-party and donor-type cell stimulation nor does it suggest patients with known sensitisation to their potential donor are more responsive to *in vitro* stimulation by donor cells, as measured by capacity to produce ASC.

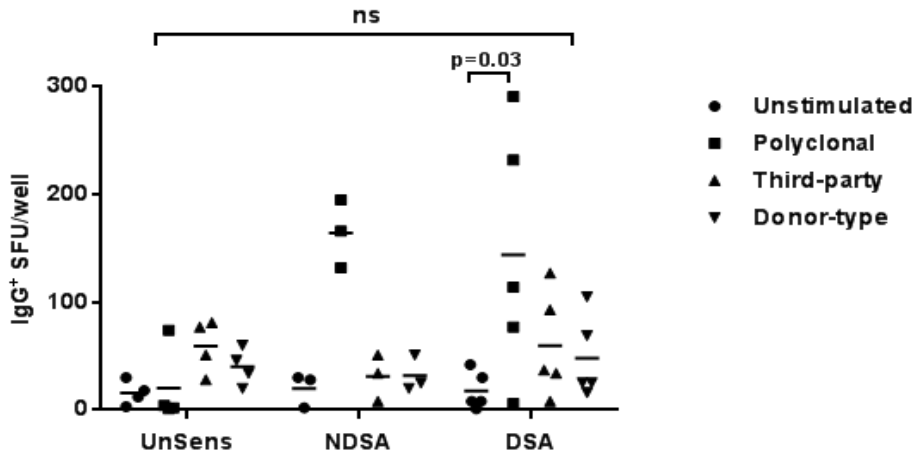


Figure 3.3 Assessing alloreactivity between donor-recipient pairs prior to transplantation by IgG-detecting ELISPOT
$CD19^+$ cells were assessed for ASC by an IgG-detecting ELISPOT at day seven post-culture following stimulation with a polyclonal agonist (positive control), third-party or donor-type allogeneic PBMC. Unstimulated cells served as the negative control.
The dot-plot shows mean values of spot-forming units (SFU) of duplicate wells for each patient group: unsensitised (UnSens; n = 4), with donor-specific HLA antibodies (DSA; n = 5) or non-DSA (NDSA; n = 3) as defined by serum analysis for HLA antibodies by Luminex technology. There were no statistical significances between groups (p = 0.06) derived from the Kruskal-Wallis test. Comparing within groups, there was a significant difference in the number of ASC in patients with DSA following polyclonal stimulation compared to unstimulated cells (p = 0.03 by Mann-Whitney test.

To characterise the cellular representation post-culture; the frequency of naïve and memory B cells were assessed. First, the pre-culture frequency of memory B cells were not significantly altered between groups (20.39% ± 7.5%, 14.19% ± 5.74 and 25.88% ± 14.45, p = 0.46; UnSens, NDSA and DSA groups respectively). Post-culture, expression (percentage or absolute number) of both naïve (p = 0.76) and memory (p = 0.72) B cells also did not alter significantly between groups (Figure 3.4A and B). However, as the figures demonstrate, there are differences in the distribution of naïve and memory B cells following cell-culture conditions between the UnSens group and both the DSA and NDSA groups, but the small number of patients investigated results in a lack of statistical power. To confirm if these observed differences reflect pre-existing sensitisation would require a larger sample size. In particular, there may be statistical significance post-culture with donor-type cells, where memory B-cell frequency was seen to expand in sensitised patients when compared to the UnSens group (6.43% ± 2.98%, 21.14% ± 4.12 and 25.38% ± 7.76; UnSens, NDSA and DSA groups respectively, Figure 3.4B). Moreover, patients with DSA appeared to be more responsive to memory B-cell activation following polyclonal stimulation when compared to the UnSens group (4.23% ± 2.12%, 21.43% ± 16.75 and 34.94% ± 25.26; UnSens, NDSA & DSA groups respectively).

Similarly, while lacking statistical significance, naïve B-cell distribution post-culture was reduced in both sensitised groups compared to the UnSens group (47.02% ± 11.05%, 18.79% ± 6.64 and 15.00% ± 8.79; UnSens compared to NDSA and DSA groups, Figure 3.4A).

Next distribution of both B-cell subsets within each patient group were compared by the frequency of unstimulated cells against culture conditions (polyclonal agonists or third-party and donor-type stimulator cells). Again no statistical differences were found. Particularly the UnSens group were relatively unaffected by cell-culture conditions, overall representing the lowest frequency of memory B cells (Figure 3.4B), and predominance of naïve B cells compared to sensitised patients (Figure 3.4A). For sensitised groups, the observed frequency of naive and memory B cells supported the

ELISPOT data; where B cells are responsive to the CpG mixture, and there was no notable difference between third-party and donor-type cell stimulation.

Overall, the small number of patients studied limits the application of statistical tests and future studies would benefit from a larger cohort. However, the preliminary results obtained by the Ig-G detecting ELISPOT were not encouraging to pursue this study further.

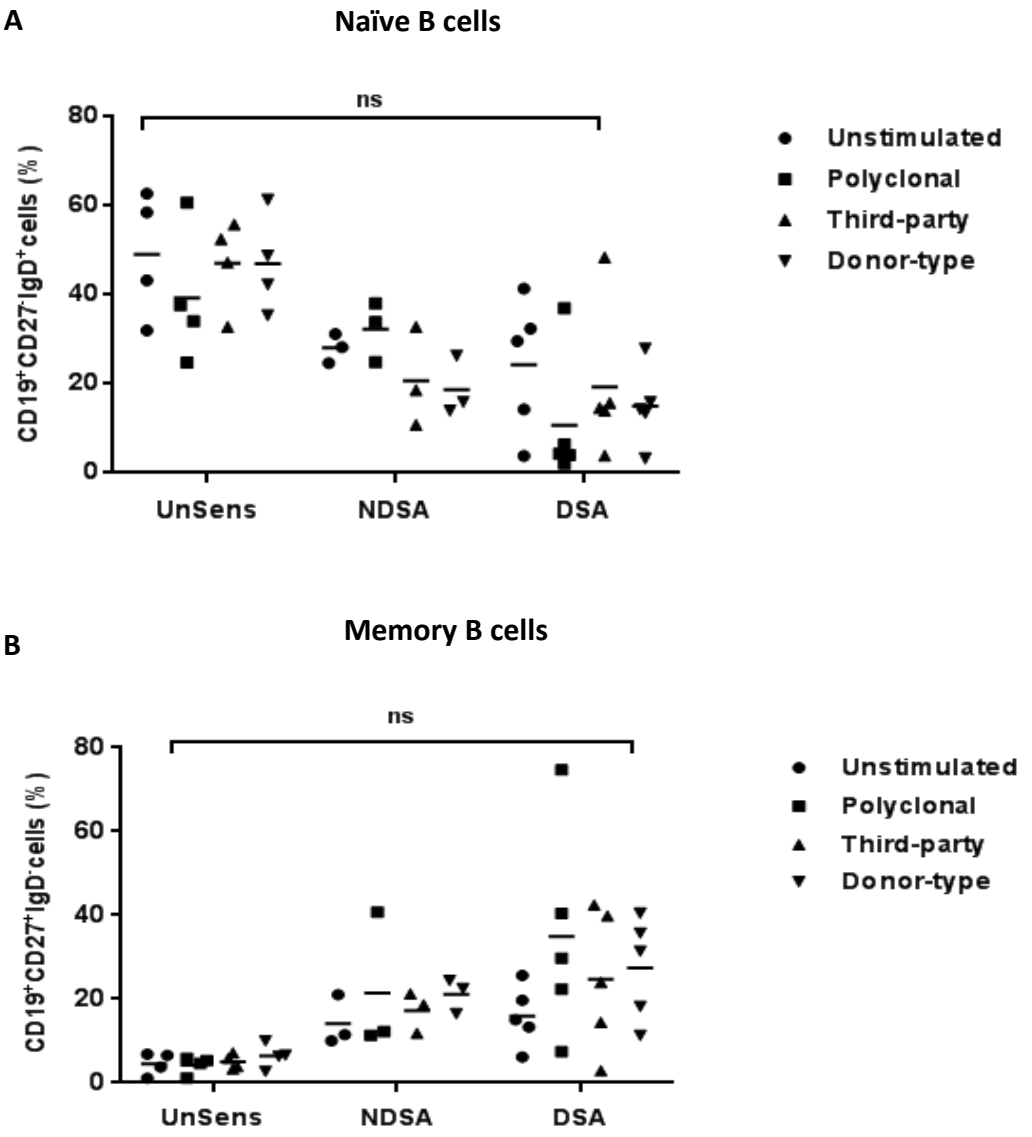


Figure 3.4 Post-culture phenotyping of recipient CD19⁺ cells for expression of naïve and memory B cells

$CD19^+$ cells isolated from PBMC were cultured for 7 days with a polyclonal agonist; third-party or donor-type allogeneic cells, stained for CD19, CD27 & IgD and analysed by flow cytometry.

The dot-plots show the frequency of naïve ($CD19^+CD27^-IgD^+$; A) and memory ($CD19^+CD27^+IgD^-$; B) B cells for each patient group defined as unsensitised (UnSens; n = 4) or with serum defined HLA antibody as donor-specific antigen (DSA; n = 5, or non-DSA (NDSA; n = 3). Statistical analysis was performed using the Kruskal-Wallis test comparing the frequency of naïve and memory B cells between groups ($p = 0.76$ and $p = 0.72$ for naïve and memory B-cell frequency respectively). Comparing unstimulated cells with culture conditions within each group was performed by Mann-Whitney test. This did not show any statistical difference (all $p > 0.05$).

3.4 Discussion

The ability to measure memory B-cell activity, as a potential route to producing alloantibody, would benefit certain patients entering the transplantation programme. In particular are those at heightened risk of AAMR; this generally describes a person having a second transplant sensitised by the first, and also includes women sensitised toward paternal antigen through pregnancy who are pre-emptive recipients of an allograft from that donor. Current methods employed during pre-transplant assessment assume compatibility between donor and recipient in the absence of DSA detectable in serum.

This study attempted to measure the frequency of allospecific B cells in pre-transplant recipients by an IgG-detecting ELISPOT assay where allogeneic donor cells served to stimulate responder B cells, analogous to the transplant environment. To control for specificity, unstimulated $CD19^+$ cells served as a negative control and demonstrated a low-level of IgG-secretion by B cells. As a positive control or a measure of total memory B-cell responsiveness, regardless of antigen specificity, $CD19^+$ cells were activated with polyclonal activators, the TLR9 agonist; CpG or R848, a TLR 7 and 8 agonist. Both induce BCR stimulation in the absence of T-cell help, (Poeck et al., 2004) and CpG in particular has been cited as a potent activator of memory B cells (Bernasconi et al., 2003, Hornung et al., 2002). In this study, $CD19^+$ cells from women sensitised through pregnancy, upon TLR ligation, demonstrated a greater response to polyclonal stimulation by CpG than R848, as detected by the IgG-detecting ELISPOT assay, and CpG was chosen for use in subsequent investigations with the pre-transplantation cohort.

Refining for specificity, reactivity toward allogeneic antigen was measured and minimal difference was found in the number of ASC between unsensitised and sensitised patients in response to donor-antigen. Moreover, the response to third-party antigen was comparable. Third-party donor cells were selected based on expression of HLA molecules distinct from the donor or pre-formed antibodies defined by serum analysis. Due to the limited number of HLA-typed third-party donors, it was not possible to

avoid all HLA antibody specificities in highly sensitised patients; while DSA were avoided, NDSA could not. However, given that unsensitised patients demonstrated comparable frequency of ASC in response to third-party antigen, this was probably not the contributory cause. An alternative possible explanation was the concentration of T cells in stimulator populations was too high, potentially activating B cells in the absence of BCR triggering. T cells activated by third-party antigen are capable of stimulating B cells via non-cognate interaction between CD40 on B cells and CD40L on T cells and cytokine production (Lanzavecchia et al., 1983). More recently it was demonstrated that while naïve B cells require BCR stimulation to proliferate in response to CpG, in the presence of T-cell help, memory B cells can respond with T-cell help alone to result in proliferation of memory B cells and differentiation to plasma cells (Bernasconi et al., 2002).

Overall, in these experiments, while recognising this was a very small cohort, I have to conclude that the application of this ELISPOT could not distinguish between the third-party and antigen-specific allogeneic response, limiting its value as a tool for interrogating any donor-specific response.

In parallel to the ELISPOT, the phenotype of recipient cells post-culture was addressed to determine the distribution of naïve and memory B cells. On appearance, memory B cells were elevated in sensitised patients following polyclonal, third-party and donor-type stimulation, and as demonstrated by ELISPOT and ASC number, memory B-cell frequency was comparable in response to either third-party or donor-type cells. However, potentially due to the small number of patients investigated together with the variance in B-cell subset frequency between patients no significant differences in both naïve and memory B cell distribution were found between groups.

Supporting their activation status as cells which have not previously encountered antigen, unsensitised patients demonstrated a low frequency of memory B cells coupled with greater frequency of naïve B cells, unaltered in response to polyclonal or allogeneic stimulation. This was supported by the absence of detectable serum HLA antibody and suggests these patients may not be at risk of AAMR. As only a small

proportion of patients experience AAMR following transplantation, and given the cohort size of this study, it is quite probable to have not captured any patients who would actually suffer AAMR. Further study in a larger cohort and retrospective analysis post-transplant to establish if this method can identify those at higher risk is necessary. However the preliminary data generated by ELISPOT were not encouraging and did not warrant further effort.

The key feature of ELISPOT is the inherent sensitivity: it is capable of detecting antibody secretion at the single-cell level. However, in addition to the issue of antigen-specificity, there are numerous factors limiting its clinical application. First, access to donor and third-party cells, together with a source of irradiation may be impractical. In addition, ELISPOT gives information only of that population of responder cells, for additional phenotypic information it must be used in conjunction with flow cytometry requiring a relatively large number of B cells, while generally achievable pre-transplant, would probably not be feasible following transplant when B-cell numbers are affected by immunosuppression. Moreover, a limiting factor of this assay, and equally any functional assay accessing B cells from the periphery, is the unknown degree of representation in lymphoid organs as these cells may only represent a fraction of those with alloantibody-producing capability. Finally, a specific limitation of this IgG-detecting ELISPOT is the lack of specificity for HLA. Recently, synthetic HLA molecules have been constructed and applied to the ELISPOT detection matrix to enumerate HLA-specific memory B cells. (Heidt et al., 2012b). While technically feasible, use of HLA monomers is very expensive and together with high polymorphism of the HLA system would require patient-specific design, limiting general clinical application.

Concluding remarks

Examining the presence of DSA in serum provides an individual's antibody repertoire, but is not informative about the patient's latent memory B cells. This study described a method for detecting allospecific B cells in the periphery. In this small cohort, observations were not encouraging to suggest this method be applied in the pre-transplant setting as an indication of alloreactivity. An alternative approach might be

to directly identify circulating HLA-specific B cells by flow-cytometry to predict the B-cell response to donor antigen. This could potentially be a valuable tool in both the pre- and post-transplant setting. Such an approach is described in the next chapter.

Chapter 4

Identifying donor HLA-specific B cells in the periphery

4.1 Introduction

The presence of HLA antibody is an identifying feature of alloreactivity and is associated with both early and late graft loss (Everly et al., 2009, Worthington et al., 2003, Zhang et al., 2005). Retrospective analysis of patients with failed grafts shows a higher prevalence of HLA antibody (Mizutani et al., 2005, Lee et al., 2009). Moreover, their absence is predictive of superior graft survival (Lefaucheur et al., 2010, van Timmeren et al., 2009). Accordingly, much effort is centred on characterising HLA antibodies and the advent of solid-phase assays coupled with Luminex technology has increased the sensitivity of our ability to detect them.

In the pre-transplant setting, finer definition of HLA antibodies permits a wider selection of permissible donors for sensitised patients and provides a useful tool during desensitisation protocols to assess the efficacy of treatment. During the post-transplant period, early detection and diagnosis of antibody-mediated rejection (AMR) could prevent development of chronic rejection and transplant glomerulopathy (TG) (El-Zoghby et al., 2009, Issa et al., 2008). In either setting, a patient's profile of HLA antibody specificity is defined by analysing serum. These specificities reflect the presence of B cells actively secreting antibody; yet little is known of the cells themselves as to their prevalence, relationship with secreted levels of alloantibody or phenotypic characteristics.

Donor HLA-specific B cells (DS-B cells) are very rare in the circulation; however identification is feasible and has been demonstrated through the use of HLA tetramers. Tetramers are conventionally used to detect HLA-specific T cells by acting as surrogate APC binding with high avidity to a T-cell receptor specific for the given HLA molecule (Altman et al., 1996, Burrows et al., 2000). These complexes consist of four-identical HLA-molecules embedded with biotin-labelled peptide bound to a fluorescently labelled streptavidin molecule, allowing detection and enumeration by flow-cytometry (Altman, 2004). This approach has been transferred to enable identification of antigen-specific B cells by exploiting the nature of the B-cell receptor (BCR) to bind native,

unprocessed antigen. This was first applied in mice to detect B cells specific for a nominal antigen (R4A peptide) (Newman et al., 2003). In humans, DS-B cells were detected in women sensitised to HLA-A2 via pregnancy, demonstrating tetrameric HLA-A2 was capable of recognising HLA-A2-specific BCR (Mulder et al., 2003). This method was later applied to renal transplant recipients (Zachary et al., 2007). Here, a higher frequency of B cells bound to HLA-A*0201, HLA-A*2402 & HLA-B*0702 in sensitised compared to non-sensitised patients. Moreover, cell-culture with polyclonal agonists induced secretion of HLA antibody sharing specificity with the HLA antigen of the tetramer. Collectively, these studies showed DS-B cells can be detected in the periphery by utilising the capability of a BCR to bind synthetic HLA molecules; importantly, this proved sufficiently sensitive in immunosuppressed patients. In addition to peripheral identification of DS-B cells, HLA-specific pentamers (a development on the use of tetramers) have been successfully applied *in situ* to examine a murine model of the alloresponse (Panoskaltsis-Mortari et al., 2008). Using H2-pentamers, allospecific $CD19^{+}$ cells were identified within spleen sections by immunohistochemistry.

The study by Zachary and colleagues demonstrated differential expression of DS-B cells post-transplant as an index of sensitisation. The ability to achieve this through analysing peripheral blood is an attractive concept to explore further. Of particular interest is the development of *de novo* donor-specific HLA-antibodies (dnDSA) formed against the allograft in patients who had no evidence of sensitisation prior to transplant. dnDSA are increasingly recognised as contributing toward antibody-mediated injury and reduced allograft survival (Terasaki et al., 2007, Willicombe et al., 2011, Yabu et al., 2011, Wiebe et al., 2012). Indeed, dnDSA are attributed with a 10-fold increase in the risk of allograft failure, resulting in 40% lower graft survival at 10 years (Wiebe et al., 2012). Specifically, HLA-class II antibodies are more prominent than class I (Everly et al., 2013, Gloor et al., 2007). Recently DQ antibodies are reported as the most prevalent dnDSA and associated with inferior outcome (Everly et al., 2013, DeVos et al., 2012, Willicombe et al., 2012, Freitas et al., 2013). This could indicate discordance between matching at the HLA-DR and HLA-DQ loci. Currently, HLA-DQ is not included in HLA-matching algorithms used for organ allocation programmes due to

the supposed strong linkage disequilibrium between HLA-DR and HLA-DQ (Navarrete et al., 1985). However, the frequency of HLA-DQ incompatibility in HLA-DR-matched transplant recipients is not known; mismatching for HLA-DQ may me a major risk factor for DSA development. These data suggest characterising the B cells responsible for their secretion could promote understanding for this apparent enhanced immunogenicity.

In addition to characterising DS-B cells in patients with dnDSA, there are further clinical applications. First, detection of HLA antibody in serum can be transient and identifying peripheral DS-B cells could benefit patients with historic unacceptable-defined HLA antibodies (UDA) who are awaiting transplantation or where historic serum is not available and the presence of donor-specific memory B cells are suspected. This poses a particular risk for recipients previously sensitised through pregnancy, affecting women receiving allografts from their offspring or the father of their children. Second, detecting DS-B cells could minimise the impact of dnDSA arising in the immediate post-transplant period. This suggests DSA are potentially missed during analysis of serum which could be attributed to a high MFI cut-off or lack of historic sera, resulting in a misleading negative-crossmatch.

While the detrimental effect on allograft survival of DSA directed against HLA-A, -B and -DR proteins is well established; with an emerging view of similar involvement by HLA-DQ, little is known of the B cells responsible for their production. This study aimed to detect, characterise and quantify DS-B cells in transplanted patients with varying degrees of sensitisation to their allograft, as determined by serum analysis. Detailing their magnitude may allow for monitoring the effectiveness of treatment, and describing phenotypic characteristics may provide insight for development of targeted therapy to aid their removal.

4.2 Materials and methods

Materials and methods detailed in Chapter 2, General Methods (GM) are indicated.

GM1 Recruitment of study patients

GM2 Immunosuppressive regimen

GM3 Defining level of allograft function in patient cohorts

GM4 Peripheral blood mononuclear cells (PBMC) separation

GM5 $CD19^+$ cell isolation from PBMC

GM6 Phenotyping $CD19^+$ cells by flow cytometry

GM8 Detection of HLA antibody in serum by Luminex technology

GM10 Statistical analysis

Detection of DS-B cells using HLA-coated microbeads by flow-cytometry

LABScreen single-antigen (LSA) microbeads (One Lambda) in conjunction with Luminex technology are conventionally used to detect HLA antibody in serum samples. Measuring 5.6 µm, these polystyrene beads are coated with HLA-antigen of a single specificity derived from a transfected human cell line. In this study, these properties were exploited to detect B cells with a BCR specific for the same HLA molecule as was coupled to the microbead surface. The microbeads contain a fluorochrome allowing detection by flow cytometry. Using the 635 nm red-diode laser, this excites the fluorochrome-labelled microbeads to emit light at 658-712 nm and the flow cytometer can be programmed to analyse cells in this channel. Additional B-cell markers can be

examined simultaneously by selecting fluorochromes excited by different lasers and emitting light in different channels.

$CD19^+$ cells were isolated from PBMC (GM 4 & 5) and stained with the extracellular antibody B-cell panel or isotype controls (GM 6, Table 2.1). 3 x 10^5 $CD19^+$ cells were resuspended in 50 µl FACS buffer (PBS/2% FCS) and 5 µl LABScreen single-antigen microbeads (One Lambda) bearing HLA-A*0201, HLA-A*0203 or HLA-DQB1*0301 and incubated in the dark for 45 minutes with mixing every 15 minutes. Without washing, $CD19^+$ cells were analysed immediately by flow cytometry. A minimum of 250,000 events were collected and data were exported to Flowjo (Tree star Inc.) for analysis. Double-positive $CD19^+HLA^+$ cells were selected above a background threshold of 0.05% based on cells stained with only the CD19 antibody.

Statistical analysis of rare events by flow cytometry

For analysing a population of cells representing <1% of the total, Poisson distribution statistics were applied. This describes the probablity of a random event occuring in a fixed volume. Based on the lowest frequency of positive events expected for $CD19^+HLA^+$ cells (0.01% or 30 events), a minimum of 2.5 x 10^5 total events were aquired (Hedley and Keeney, 2013). This was achieved from a starting population of 3 x 10^5 $CD19^+$ cells and equates to a 20% Poisson coefficent of variation (CV) of the counting error. This is defined as 100/√positive events counted. In this case, the actual CV is 18.25% (100/√30).

Ethical approval for the observation study of *de novo* donor specific antibody development in a single renal transplant centre

HLA antibody data and renal allograft outcome details were taken from information collated and stored by the Clinical Transplantation Laboratory, Barts and the London NHS Trust. This research study audited current practice to assess *de novo* HLA antibody production and graft outcome. Since the data analysed was anonymised and from established procedures it did not require specific ethical approval.

4.3 Results

Establishing the principle of identifying DS-B cells in the periphery

To establish the feasibility of identifying DS-B cells in peripheral whole blood using a flow-cytometric based method, two groups of transplanted patients were selected based on sensitisation to HLA-A*0201, as this antigen is expressed at high frequency within the donor population. Both groups had received an allograft mismatched for this antigen and during the post-transplant period tested positive or negative for anti-HLA-A*0201 (HLA-A2) in serum samples by Luminex single-antigen (LSA) analysis. The anti-HLA-A2 group (n = 5) had an MFI of >1000 (range; 1230-8675 MFI) and the unsensitised (anti-HLA$^-$) group (n = 5) all had an MFI <150. The ability of CD19$^+$ cells isolated from these patients to bind microbeads coated with recombinant HLA-A*0201 was assessed by flow cytometry (Figure 4.1). A small population of CD19$^+$anti-HLA-A*0201 cells is evident in the 'test' (Figure 4.1B) but not the negative control (Figure 4.1A).

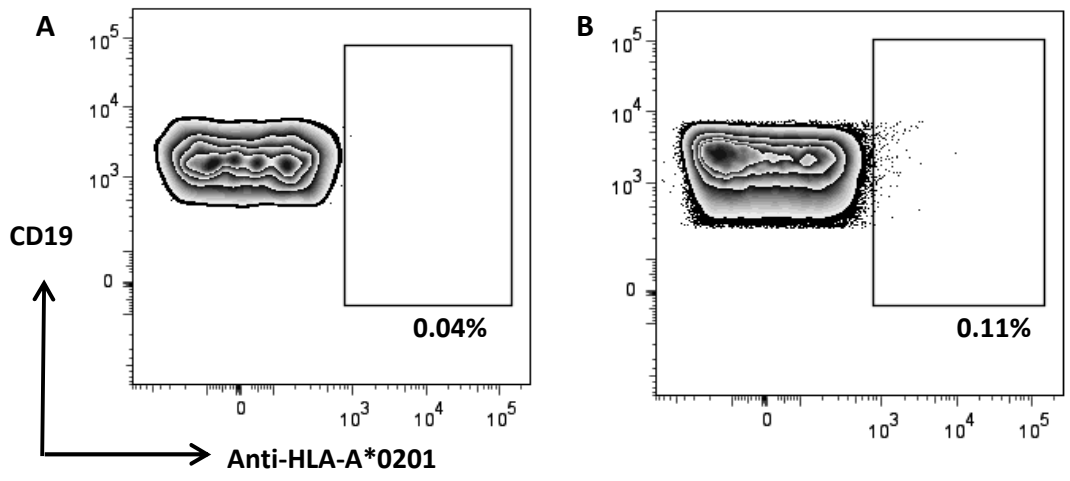


Figure 4.1 Post-transplantation detection of CD19$^+$ anti-HLA-A*0201 cells by flow cytometry
CD19$^+$ cells (3 x 10^5) from HLA-A2 mismatched patients (n = 5) were isolated from PBMC and binding to microbeads coated with HLA-A*0201 assessed by flow cytometry.
Representative flow-cytometric plots show the negative control, CD19 cells stained for CD19 only (A) used to determine the percentage of anti-HLA-A*0201 positive CD19$^+$ cells (B).

In an effort to increase the detection frequency of $CD19^+$ anti-HLA-A*0201 cells, $CD19^+$ cells were cultured with a polyclonal agonist against TLR7 (R848) and IL-2, known to induce B-cell expansion (Pinna et al., 2009). However, this resulted in high-level non-specific staining which could not be eliminated by stringent gating of lymphocytes (data not shown); therefore using cultured $CD19^+$ cells was rejected. In addition, the efficacy of pentamers of HLA-A*0201 was investigated in a small number of sensitised patients (n = 4). Compared to HLA-coated microbeads, this method suffered from considerable non-specific staining (data not shown). As a consequence, and due to their high-cost, they were not utilised further and all subsequent investigations employed HLA-coated microbeads with freshly isolated $CD19^+$ cells.

The volume of microbeads (suspended at a constant concentration) used per test was assessed in relation to the detected frequency of $CD19^+$ anti-HLA-A*0201 specific cells in sensitised patients (n = 3) and 5 µl proved to have an adequate concentration to produce saturation at a standardised concentration of $CD19^+$ cells (Figure 4.2).

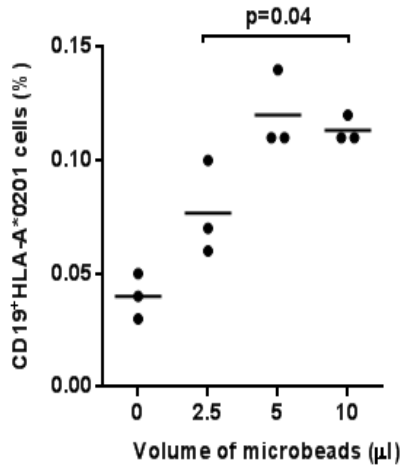


Figure 4.2 Optimising HLA-A*0201 microbead concentration used per test
$CD19^+$ cells (2×10^5) from HLA-A2 mismatched patients (n = 3) were isolated from PBMC and binding to 2.5 µl, 5 µl or 10 µl of microbeads was assessed by flow cytometry for frequency of $CD19^+$ anti-HLA-A*0201 specific cells.
The dot-plot shows the mean percentage of $CD19^+$ anti-HLA-A*0201 specific cells and increasing volumes of microbeads at constant concentration. 5 µl was an adequate volume per test as determined by comparing the frequency of $CD19^+$ anti-HLA-A*0201 specific cells to other volumes by the Kruskal-Wallis test (p = 0.04), and was used throughout.

Selecting a negative control

Selecting a negative control was problematic due to the difficulty identifying an irrelevant HLA-specificity with a clear absence of potential memory B cells. Initially, HLA-A*4301 microbeads were used as antibodies against this antigen are relatively rare, but this did not give uniform negativity in all patients. Next, $CD19^+$ cells from unsensitised male volunteers were investigated as employed by Zachary and colleagues (Zachary et al., 2007). However, using $CD19^+$ cells from each patient labelled only with CD19 antibody allowed for more stringent selection of antigen-specific B cells. While not an ideal negative control, this gave the best consistency, and was used across the study cohort.

Investigating level of specificity of donor-specific B cells toward HLA-coated microbeads

Most often antibody-specificity is defined at the level of the broad HLA-A2 serotype. The serotype encompasses the genotypes of many HLA-A2 alleles, usually against epitopes expressed by HLA-A*0201,*0202,*0203,*0206 and *0207. To address the level of allelic specificity $CD19^+$ cells isolated from sensitised patients had toward HLA-coated microbeads, HLA-A*0203 microbeads were applied in conjunction with HLA-A*0201 microbeads to those with serum antibody specificity against only HLA-A*0203. However, after extensive review, I could find only two patients who met these criteria, and the results were inconclusive. Therefore this study was unable to determine the application of this approach to the allelic level of antibody specificity.

Elevated frequency of peripheral CD19+ anti-HLA-A*0201 cells in sensitised patients

Transplanted patients with donor-specific anti-HLA-A*0201 antibody defined by serum analysis demonstrated a higher frequency of $CD19^+$ cells capable of binding HLA-A*0201 microbeads compared to unsensitised patients (0.12 ± 0.05% and 0.04 ± 0.01%, anti-HLA-$A2^+$ and anti-HLA^- respectively; p=0.008, Figure 4.3A). Assessing if a correlation exists between heightened level of serum antibody and frequency of cells bearing that specificity showed a positive trend, however the relationship was not significant (R^2=0.52; p=0.17, Figure 4.3B).

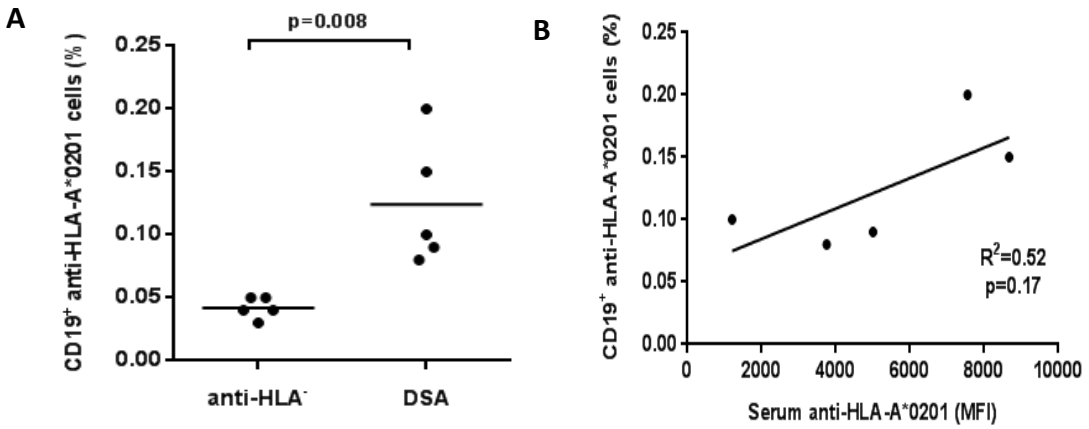


Figure 4.3 Increased frequency of CD19+ anti-HLA-A*0201 cells in patients with anti-HLA-A*0201 DSA detected in serum

CD19+ cells from patients mismatched for HLA-A2; without HLA antibodies (anti-HLA-; n = 5) or with donor-specific HLA antibody (DSA; n = 5) were isolated from PBMC and assessed for ability to bind microbeads coated with HLA-A*0201 by flow cytometry.

A. The dot-plot shows frequency of CD19+ cells bound to HLA-A*0201 microbeads was significantly higher in sensitised patients (p=0.008 by the Mann-Whitney test comparing anti-HLA- group against DSA group).

B. The dot-plot shows there is not a statistically significant relationship between CD19+ anti-HLA-A*0201 cell frequency and the level of circulating anti-HLA-A*0201 shown as mean fluorescence intensity (MFI) in serum. P value derived from linear regression analysis.

These data confirmed the principle and methodology of the assay – that is was capable of detecting B-cell reactivity to donor-specific antigen presented on microbeads – and it was therefore applied to further patient groups.

Characterising DS-B cells in patients transplanted with allografts expressing HLA-A*0201 and HLA-DQB1*0301

Study group demographics

The frequency of DS-B cells was examined to determine if this parameter represented a useful index of sensitisation to HLA expressed by the allograft. Two antigens, HLA-A2 (HLA-A*0201) and HLA-DQ7 (HLA-DQB1*0301) were selected: HLA-A2, a HLA Class I antigen with high frequency within the donor population, and HLA-DQ7, a common HLA Class II antigen found in our transplanted patients.

Patients transplanted with an HLA-A2-expressing allograft were separated into three groups based on sensitisation prior to transplantation (Table 4.1). Unsensitised (US) patients were all negative prior to transplantation by CDC crossmatch, T and B-cell flow-crossmatch and for DSA by Luminex single-antigen (LSA) analysis. The US group was separated by post-transplant sensitisation into two groups, those that did not have any detected HLA antibodies (anti-HLA^{-}), and those whom developed *de novo* DSA (dnDSA). The third group, pre-sensitised (PS) patients had varying levels of sensitisation: all tested positive for HLA-A*0201 in serum by LSA analysis (MFI: range 1000-10035, median 1050); 43% tested positive by T and B cell flow-crossmatch and had historically defined HLA-A2 by CDC assay. All patients were negative by CDC assay at the time of transplantation. None of the patients transplanted with an HLA-DQB1*0301-expressing allograft were sensitised to this antigen prior to transplantation. The demographics of these patients are shown in Table 4.2.

Patients transplanted with a graft expressing HLA-A*0201			
Pre-transplant	**Unsensitised (US)**		**Sensitised (PS)**
Post-transplant	**Anti-HLA$^-$**	***De novo* DSA (dnDSA)**	**DSA**
Total number of patients, n (%)	5 (29.4)	5 (29.4)	7 (41.2)
Recipient			
Gender (F,M)	2,3	2,3	2,5
Age, median (range) (years)	44 (40-70)	33 (28-58)	52 (43-54)
Time post-transplant, median (range) (months)	60 (12-120)	12 (12-60)	24 (12-180)
***Type of allograft*, n (%)**			
Live donation	2 (40)	2 (40)	1 (14)
DBD	1 (20)	3 (60)	5 (72)
DCD	2 (40)	0	1 (14)
***HLA mismatches,* n ± sd**			
A, mean ± sd	1.6 ± 0.6	1.6 ± 0.6	1.7 ± 0.5
B, mean ± sd	1.6 ± 0.6	1.4 ± 0.6	1.3 ± 0.5
DR, mean ± sd	0.8 ± 0.5	1 ± 0.7	0.7 ± 0.8
Cause of ESRF, n (%)			
IgA nephropathy	0	1 (20)	0
Diabetes	1 (20)	0	1 (14)
FSGS	1 (20)	0	0
GN	1 (20)	0	1 (14)
PKD	0	0	1 (14)
Other/Unknown aetiology	2 (40)	4 (80)	4 (58)

Table 4.1 Demographics for patients transplanted with a graft expressing HLA-A*0201

Abbreviations: DBD, donation after brainstem death; DCD, donation after circulatory death; DSA, donor-specific HLA antibody; ESRF, end-stage renal failure; FSGS, focal and segmental glomerulosclerosis; GN, glomerulonephritis; PKD, polycystic kidney disease; sd, standard deviation.

Patients transplanted with a graft expressing HLA-DQB1*0301		
	Anti-HLA$^-$	***De novo* DSA (dnDSA)**
Total number of patients, n (%)	7 (50)	7 (50)
Recipient		
Gender (F,M)	2,5	4,3
Age, median (range) (years)	59 (40-70)	39 (27-61)
Time post-transplant, median (range) (months)	60 (12-120)	24 (12-60)
***Type of allograft*, n (%)**		
Live donation	2 (40)	2 (40)
DBD	1 (20)	3 (60)
DCD	2 (40)	0
***HLA mismatches,* n ± sd**		
A, mean ± sd	1.1 ± 0.6	1.8 ± 0.4
B, mean ± sd	1.6 ± 0.5	1.0 ± 0.7
DR, mean ± sd	0.7 ± 0.5	1.0 ± 1
Cause of ESRF, n (%)		
IgA nephropathy	1 (14)	0
Diabetes	2 (28)	0
GN	0	2 (28)
Hypertension	2 (28)	2 (28)
PKD	1 (14)	0
Other/Unknown aetiology	1 (14)	3 (42)

Table 4.2 Demographics for patients transplanted with a graft expressing HLA-DQB1*0301

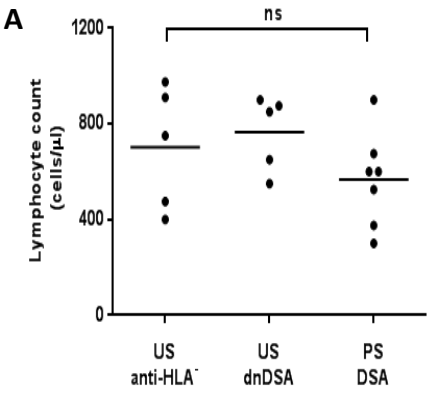
Abbreviations: DBD, donation after brainstem death; DCD, donation after circulatory death; DSA, donor-specific HLA antibody; ESRF, end-stage renal failure; FSGS, focal and segmental glomerulosclerosis; GN, glomerulonephritis; PKD, polycystic kidney disease; sd, standard deviation.

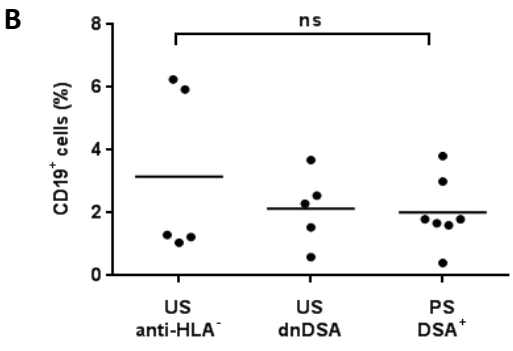
Analysing peripheral blood from HLA-A*0201 transplanted patients

Total lymphocyte and CD19⁺ cell populations

To identify any difference between groups, PBMC and $CD19^+$ cell constitution were assessed. The total PBMC count was determined following lymphocyte isolation by density gradient centrifugation of peripheral whole blood within four hours of venesection (Figure 4.4). The difference between the absolute number of PBMC was minimal (702 ± 229 cells/µl; 765 ± 156 cells/µl and 568 ± 198 cells/µl, for anti-HLA^-, dnDSA & DSA groups respectively; p = 0.33 comparing anti-HLA^- against other groups).

Following PMBC isolation, $CD19^+$ cells were positively selected by magnetic bead separation. There were no significant differences between groups in frequency, (3.15 ± 2.4%; 2.13 ± 1.15% and 2.01 ± 1.09% for anti-HLA^-, dnDSA and DSA groups, respectively; p<0.99, Figure 4.4B) or absolute numbers (21 ± 17 cells/µl; 16 ± 11 cells/µl; 11 ± 7% for anti-HLA^-, dnDSA & DSA groups, respectively; p = 0.72, Figure 4.4C).





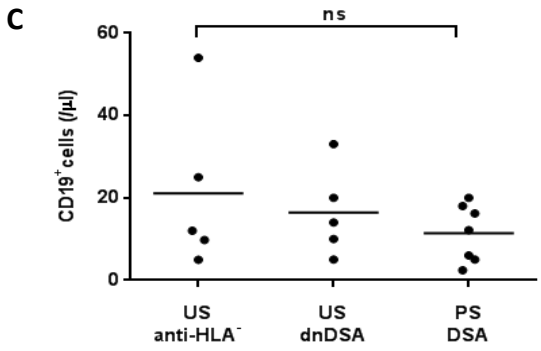


Figure 4.4 Comparable total lymphocyte and $CD19^+$ cell counts in HLA-A2 transplanted study groups
Dot-plots show the PBMC counts (A) for each group based on lymphocyte isolation by density centrifugation of fresh peripheral whole blood (40 ml) with both frequency (B) & number (C) of positively selected $CD19^+$ cells. p values (all p > 0.05; ns is not significant) derived from comparing anti-HLA^- group (n = 5), *de novo* donor-specific HLA antibody (dnDSA; n = 5) and DSA (n = 7) groups by Kruskal-Wallis test.
US, unsensitised; PS, pre-sensitised.

*Detecting CD19⁺ anti-HLA-A*0201 cells by flow cytometry*

The frequency of donor-specific anti-HLA-A*0201 B cells (DS-A2 B cells) were assessed post-transplant. Patients identified with preformed alloantibody prior to transplant demonstrated the highest frequency of $CD19^+$ cells capable of binding HLA-A*0201 microbeads compared to those with either dnDSA or unsensitised (0.23 ± 0.2%; p = 0.005, 0.09 ± 0.03%; p = 0.02 and 0.04 ± 0.03%; DSA, dnDSA and anti-HLA^- groups respectively).

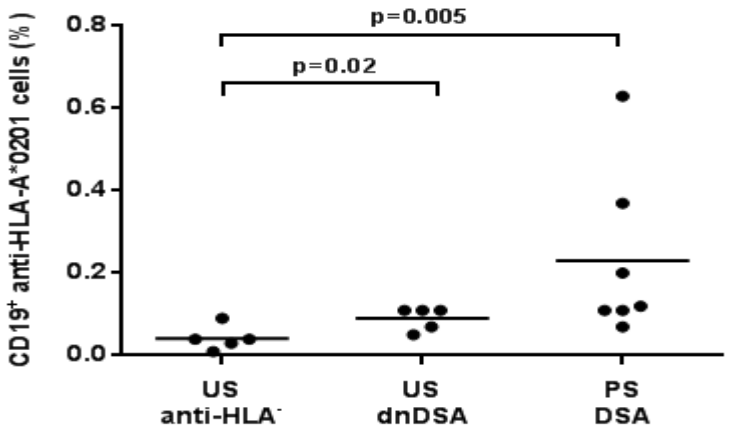


Figure 4.5 Elevated frequency of DS-A2 B cells in HLA-A*0201 transplanted patients with dnDSA and DSA

$CD19^+$ cells isolated from PBMC were assessed for binding microbeads coated with HLA-A*0201 by flow cytometry.

Dot-plot shows percentage of $CD19^+$ anti-HLA-A*0201 cells for the three groups. Pre-sensitised (PS) patients with donor-specific antibody (DSA; n = 7) had the highest frequency compared to those without detectable HLA antibody (anti-HLA^-; n = 5) patients and those with *de novo* DSA (dnDSA; n = 5). A significant difference existed between patients without HLA antibody (anti-HLA^-) and both dnDSA and DSA groups. p values derived from comparing HLA- group with other groups by Mann-Whitney test.

To probe the relationship between DS-A2 B cells and elevated levels of serum alloantibody, linear regression analysis was performed and a significant, if small correlation observed (R^2= 0.27, p = 0.04; Figure 4.6A). To investigate if elevated frequency of DS-A2 B cells correlated with level of graft function, the same analysis was applied to rate of deterioration, extracted from measuring GFR over a period of up to 36 months. No significant association was found (R^2 = 0.07, p = 0.29). When groups were separated based on graft function (as defined in GM 3), there was comparable frequency with stable function (40%, 40%, and 43% for anti-HLA-, dnDSA and DSA groups, respectively, Figure 4.6B). Rapidly deteriorating graft function was only observed in patients with dnDSA (20%; p = 0.36), or DSA (43%; p = 0.2).

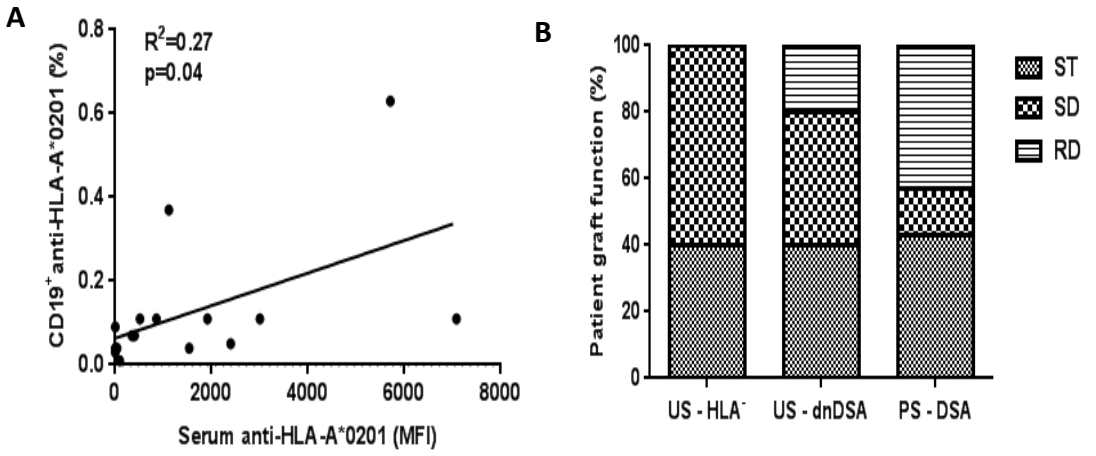


Figure 4.6 Correlation between the frequency of DS-A2 B cells and serum HLA-A*0201 antibody level

A. Linear-regression analysis assessing the frequency of $CD19^+$ anti-HLA-A*0201 cells compared to the level of serum anti-HLA-A*0201 expressed as mean fluorescence intensity (MFI) as determined by Luminex technology for all patients (n = 17) showed a significant correlation.

B. Assessing the level of graft function in patient groups. The frequency of patients with stable (ST), slowly deteriorating (SD) and rapidly deteriorating (RD) are shown as a percentage (%) of the total for each group. Only patients with donor specific HLA antibody (DSA; n = 7) or *de novo* DSA (dnDSA; n = 5) had RD graft function.

Detailing the phenotype of DS-A2 B cells

The phenotype of DS-A2 B cells were analysed to distinguish frequency of memory ($CD19^+CD27^+IgD^-$), naïve ($CD19^+CD27^-IgD^+$) and transitional B cells ($CD19^+CD38^{high}CD24^{high}$). The last group is proposed to have regulatory capacity (Blair et al., 2010, Flores-Borja et al., 2013). Moreover, elevated expression of both naïve and transitional B cells is associated with tolerance in renal transplant recipients (Newell et al., 2010). Figure 4.7 compared the distribution of these phenotypes between groups for the total B-cell population and DS-B cells.

Overall no significant differences were found in the distribution of B-cell subsets for total $CD19^+$ cells and DS-A2 B cells between groups (naïve B cells; $p = 0.33$, memory B cells; $p = 0.67$ and transitional B cells; $p = 0.67$). As the figures illustrate, there was often wide variance in the frequency of the B-cell subsets measured between individual patients. On appearance, PS patients with DSA had a higher frequency of naïve B cells for both total and DS-A2 B cells (52.13 ± 16.13% and 43.07 ± 18.94%; total and DS-A2 B cells) compared to those with dnDSA (25.35 ± 18.87% and 27.08 ± 19.83%) or US patients (32.42 ± 18.49% and 24.43 ± 19.48%; Figure 4.7A), and increasing the sample size number may confirm a significant difference.

Comparing the frequency of memory B cells within each group, the most notable difference was between total $CD19^+$ cells and $CD19^+$ DS-A2 cells in the anti-HLA^- group (26.89 ± 10.61% vs. 11.78 ± 6.79%; $p = 0.09$, Figure 4.7B).

Similar to memory B-cell frequency, the dnDSA group had an elevated frequency of DS-A2 transitional B cells, although neither was to a level of statistical significance. However, when the frequency of total $CD19^+$ cells and $CD19^+$ DS-A2 transitional cells were compared within each group, in each case transitional B cells were enriched in the DS-A2 B-cell subset (5.27% vs. 28.86%; $p = 0.02$, 6.07% vs. 25.46%; $p = 0.02$ and 4.02% vs. 15.19%; $p = 0.04$, for anti-HLA^-, dnDSA and DSA groups respectively, Figure 4.7C).

The frequency of B cells expressing BAFF-R was also measured and the difference between groups was minimal, for both total and DS-A2 B cells ($p = 0.33$; Figure 4.7D). Similarly, serum BAFF levels were not statistically different (1032.1 ± 460.9 pg/ml; 797.8 ± 242.7 pg/ml and 804.5 ± 345.6 pg/ml; $p = 0.33$ for DSA, dnDSA and anti-HLA$^-$ groups respectively, Figure 4.7E).

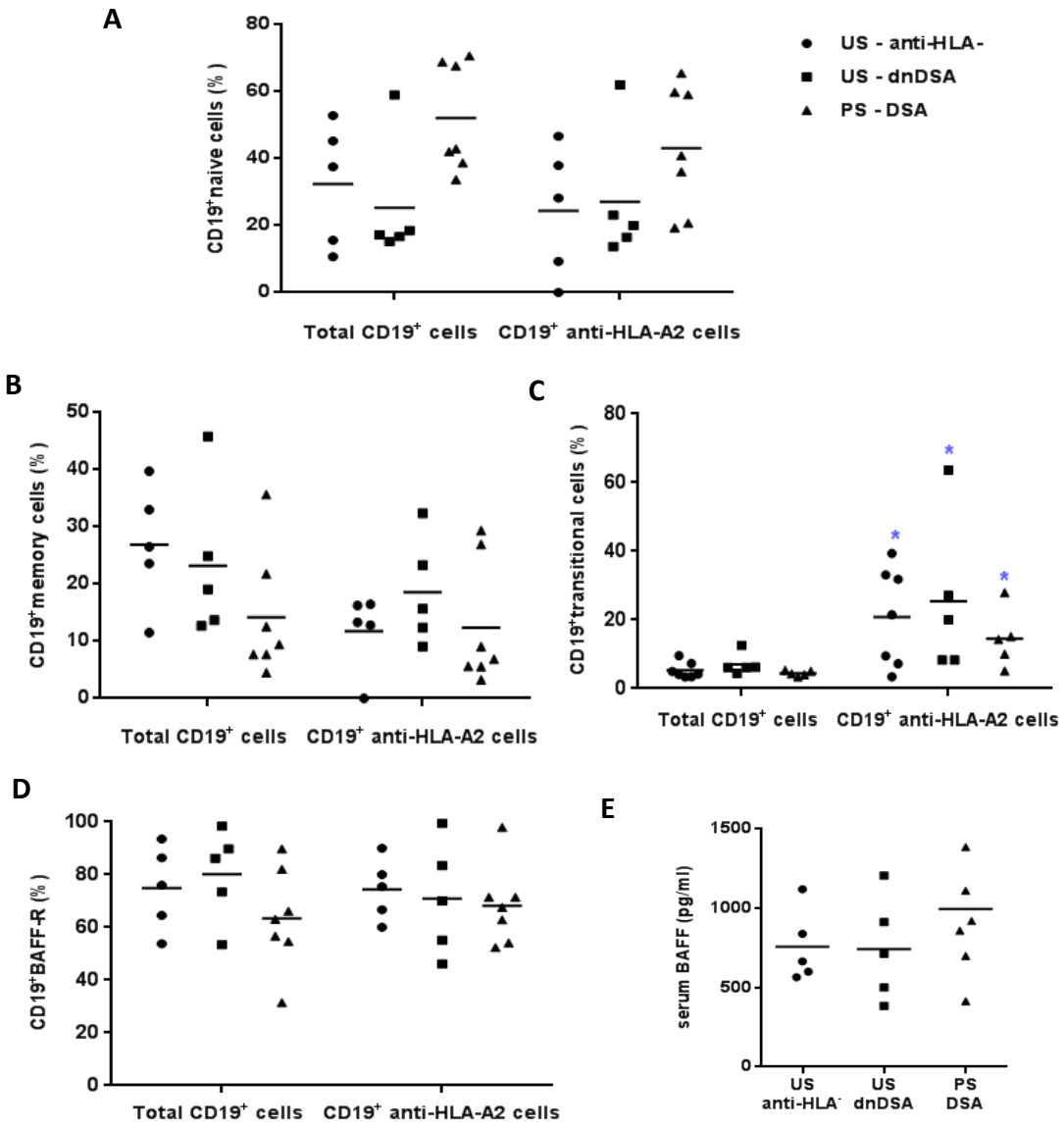


Figure 4.7 Comparing the frequency of naïve, memory and transitional B cells and expression of BAFF in patient groups for total and DS-A2 B cells

The dot-plots show frequency of naïve (A), memory (B), transitional (C) B cells for total CD19+ cells and CD19+ anti-HLA-A*0201 cell populations in each patient group.

A. Unselected naïve B cells were present at a higher frequency in patients with donor-specific HLA antibody (DSA; n =7), when compared to the *de novo* DSA (dnDSA; n =5) and without HLA antibody (anti-HLA-) groups. The difference was not significant (p = 0.33 by Kruskal-Wallis test).

B. Comparing total memory B-cell frequency and DS-A2 memory B cells between groups did not show a significant difference (p = 0.67 by Kruskal-Wallis test). DS-A2 memory B cells were reduced compared to total CD19+ cells in the anti-HLA- group, this did not reach a level of statistical significance (p = 0.09 by Mann-Whitney test).

C. The frequency of total CD19+ cells compared to DS-A2 transitional B cells were not significantly different between groups (p=0.67 by Kruskal-Wallis test). All groups showed transitional B cells were enriched in the DS-A2 B-cell subset compared to total CD19+ cells (anti-HLA- group; p=0.02, dnDSA group; p = 0.02 and the DSA group; p = 0.04 by Mann-Whitney test). D. CD19+BAFF-R+ cells and serum BAFF levels (E) did not alter significantly between groups.

Summarising the presence of DS-A2 B cells in the study cohort

The percentage of DS-A2 B cells was significantly elevated in patients with preformed DSA and dnDSA compared to unsensitised patients. Furthermore, there was significant correlation between serum level of alloantibody and frequency of DS-A2 B cells. More detailed phenotypic analysis revealed patients with DSA had a higher frequency of naive B cells and a reduced memory B cell population compared to other groups, albeit not to a statistically significant level. When DS-A2 B-cell phenotypes were examined compared to the total B cell population, the most notable difference was the heightened presence of DS-A2 transitional B cells; this was apparent for all groups.

Next, the same analysis was applied to patients transplanted with an HLA-DQB1*0301 expressing allograft and these findings compared to the prevalence and phenotypic characteristics observed for DS-A2 B cells.

Analysing peripheral blood from HLA-DQB1*0301 transplanted patients

Total lymphocyte and CD19$^+$ cell populations

The difference in the absolute number of PBMC between the two groups (there are no pre-sensitised patients in this cohort) was minimal (768 ± 210 and 636 ± 335 cells/µl, for anti-HLA$^-$ and dnDSA groups, respectively; p = 0.78, Figure 4.8A). Both the frequency of CD19$^+$ cells, (1.98 ± 1.38 and 2.54 ± 0.89% for anti-HLA$^-$ and dnDSA groups, respectively; p = 0.26, Figure 4.8B) and number of CD19$^+$ cells were not significantly altered between groups (15 ± 13 and 19 ± 6 cells/µl for anti-HLA$^-$ and dnDSA groups, respectively; p = 0.05, Figure 4.8C).

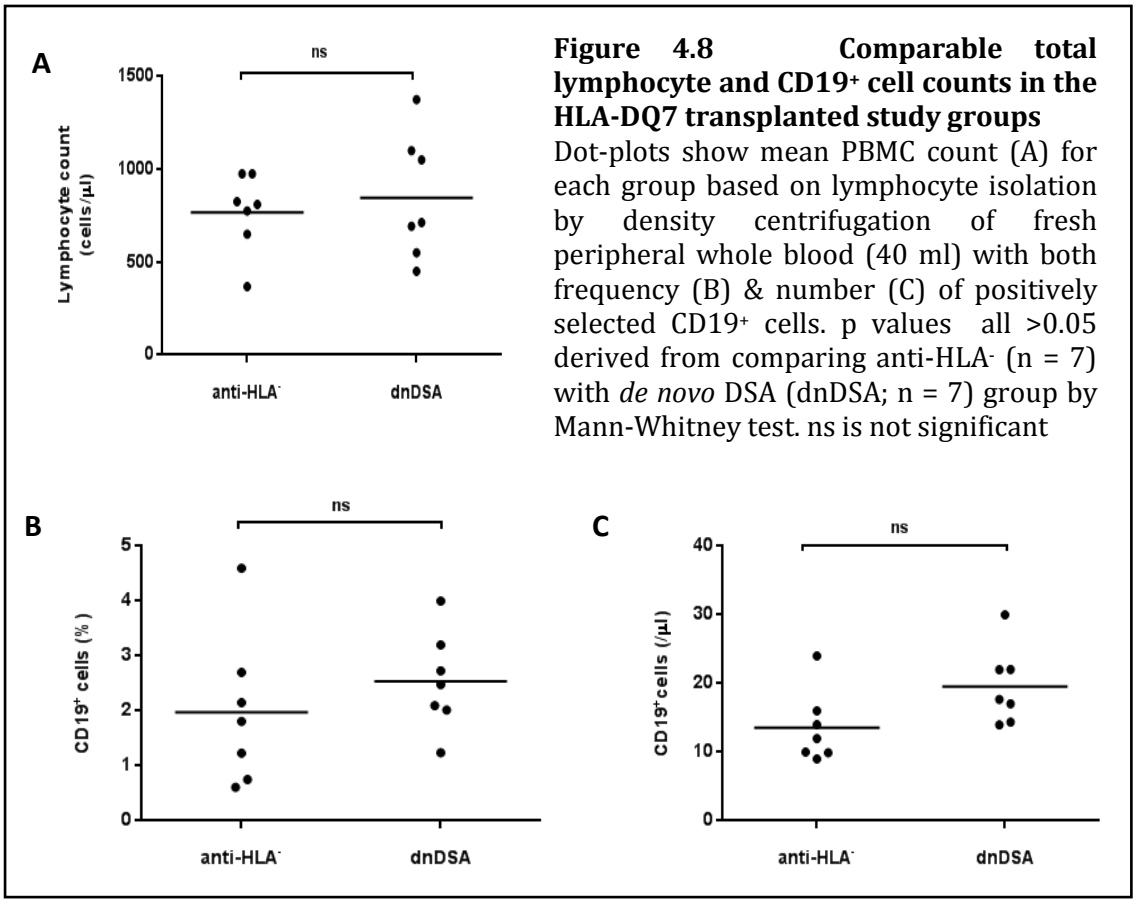


Figure 4.8 Comparable total lymphocyte and CD19⁺ cell counts in the HLA-DQ7 transplanted study groups
Dot-plots show mean PBMC count (A) for each group based on lymphocyte isolation by density centrifugation of fresh peripheral whole blood (40 ml) with both frequency (B) & number (C) of positively selected CD19⁺ cells. p values all >0.05 derived from comparing anti-HLA⁻ (n = 7) with *de novo* DSA (dnDSA; n = 7) group by Mann-Whitney test. ns is not significant

*Detecting CD19⁺ anti-HLA-DQB1*0301 cells by flow cytometry*

The frequency of donor-specific anti-HLA-DQB1*0301 B cells (DS-DQ7 B cells) post-transplant were significantly higher in patients with dnDSA (0.21 ± 0.17% and 0.07 ± 0.03%; dnDSA and anti-HLA⁻ groups, respectively, p = 0.03; Figure 4.9A). Linear regression analysis demonstrated a small, but significant correlation between the frequency of DS-DQ7 B cells and level of serum alloantibody (R^2= 0.37, p = 0.03; Figure 4.9B). Comparing for level of graft function, a greater proportion of patients without HLA antibody was stable (57% vs. 14%, p = 0.09 for anti-HLA⁻ vs. dnDSA groups; Figure 4.9C). Moreover, an increased number of patients with dnDSA had rapidly deteriorating function (29% vs. 43%, p = 0.19 for anti-HLA⁻ vs. dnDSA groups).

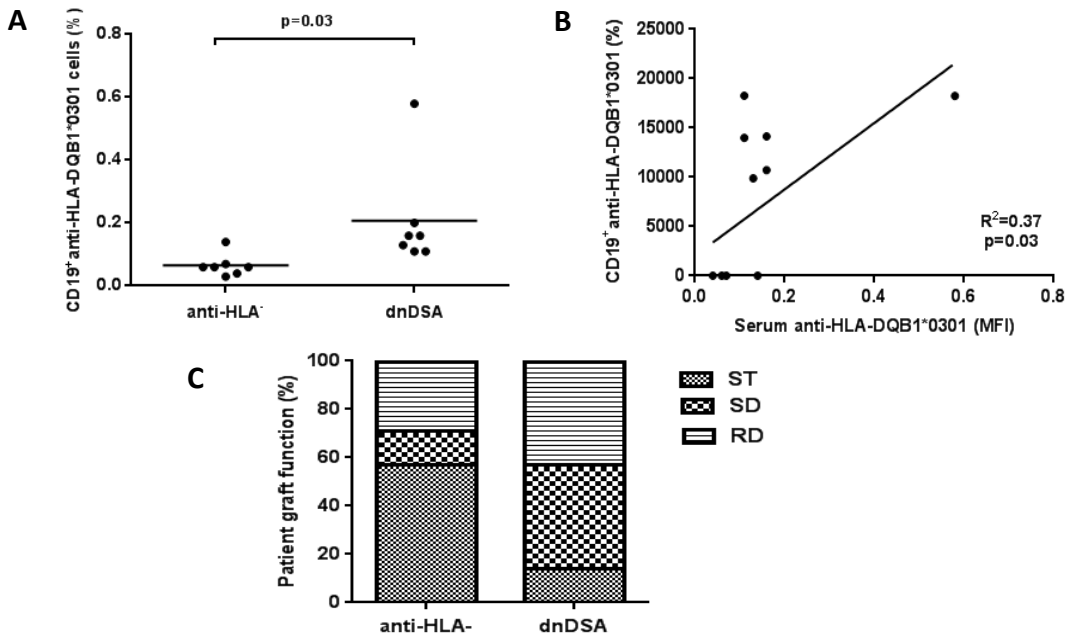


Figure 4.9 Elevated frequency of DS-DQ7 B cells in patients with serum HLA-DQ7 alloantibody

$CD19^+$ cells from patients mismatched with a HLA-DQB1*0301 expressing allograft (n = 7) were isolated from PBMC and assessed for binding to microbeads coated with HLA-DQB1*0301 by flow cytometry compared to those without HLA antibodies (anti-HLA-; n = 7).

A. Dot-plot showing the frequency of $CD19^+$ cells bound to HLA-DQB1*0301 microbeads was significantly higher in patients with *de novo* DSA (dnDSA; p = 0.03 by Mann-Whitney test). B. Linear regression analysis showed a significant correlation between $CD19^+$ anti-HLA-DQB1*0301 cell frequency and level of serum anti-HLA-DQB1*0301 (MFI) as determined by Luminex technology. C. The frequency (%) of patients with stable (ST), slowly deteriorating (SD) and rapidly deteriorating (RD) graft function. A higher proportion of patients without post-transplant HLA antibody had stable function.

Detailing the phenotype of DS-DQ7 B cells

The frequency of both total and donor-specific naïve and memory B cells did not differ significantly on the basis of presence or absence of serum alloantibody (Figure 4.10A and B). Similar to observations in HLA-A2 transplanted patients, DS transitional B cells were elevated compared to total $CD19^+$ cells; however, this was not statistically significant, possibly in part because of a large standard deviation (Figure 4.10C). Also, expression of BAFF-R did not differ between groups (Figure 4.10D). Serum BAFF was elevated in patients with dnDSA, although due to wide variation was not statically significant (1001.9 ± 272.6 and 1831.1 ± 1843.7 pg/ml; anti-HLA^- and dnDSA groups respectively; p = 0.45, Figure 4.10E).

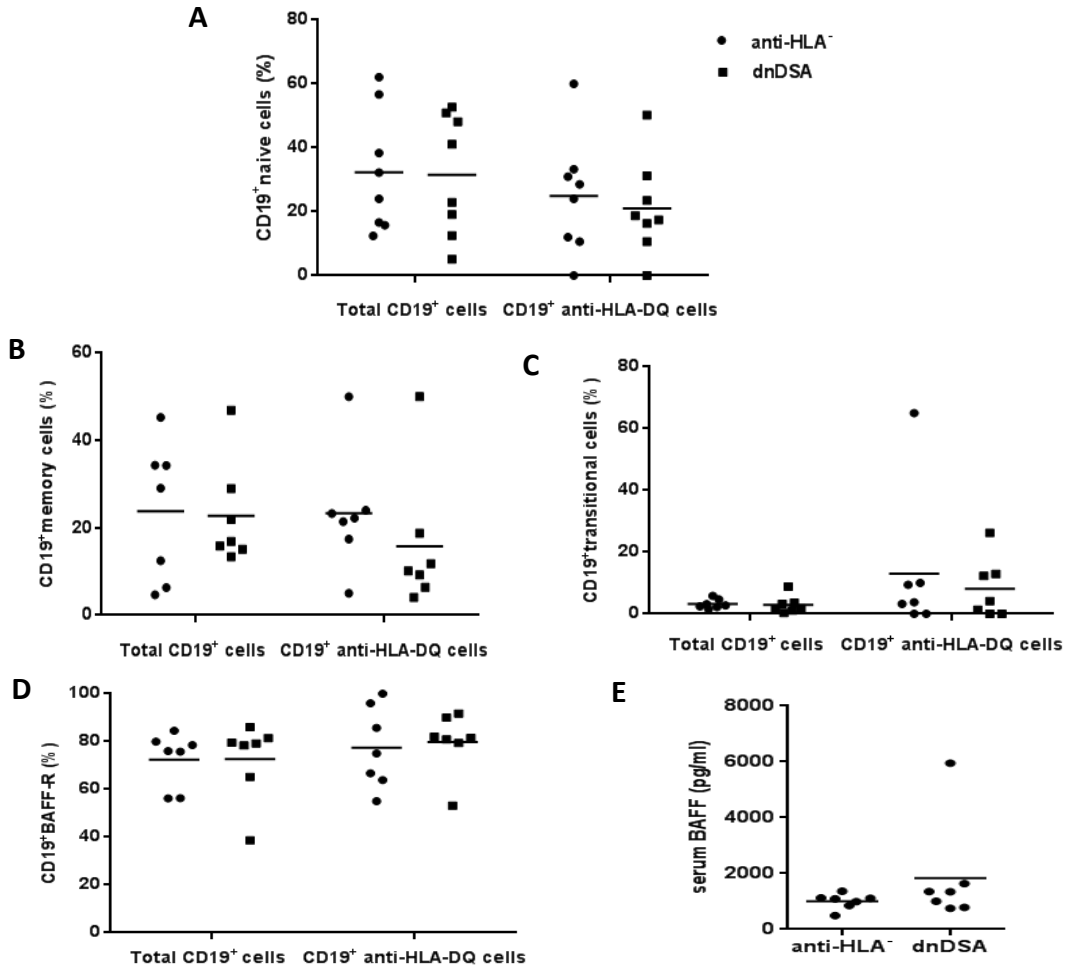


Figure 4.10 Comparing the frequency of naïve, memory and transitional B cells and expression of BAFF in patient groups for total and DS-DQ7 B cells
The dot-plots show frequency (%) of naïve (A), memory (B), transitional (C) B cells and BAFF-R (D) by total CD19+ cells and DS-DQ7 B cells, and serum BAFF levels (E). No statistically significant differences existed between those without HLA antibodies (anti-HLA-; n = 7) and *de novo* donor-specific antibodies (dnDSA; n = 7) when comparing frequencies by Mann-Whitney test.

Comparing phenotypic expression of DS-DQ7 and DS-A2 B cells in patients with dnDSA

De novo anti-HLA-DQ antibodies are reported as the most prominent HLA-type and may impact allograft survival as much as antibodies against HLA-A, -B and –DR (DeVos et al., 2012, Everly et al., 2013). Here, DS-DQ7 B cells in patients with dnDSA were present at greater frequency than patients with dn-HLA A2 (0.2 ± 0.17% and 0.09 ± 0.03%, DS-DQ7 and DS-A2 B cells respectively, p = 0.02 Figure 4.11A). The phenotype of these cells was compared and, interestingly, while there was minimal difference in percentages of naïve and memory B cells (Figure 4.11B and C), transitional B cells were

significantly reduced in those with dn-DQ7 DSA (7.1 ± 3.6% and 2.9 ± 2.9%; p = 0.03 Figure 4.11D). In addition, the level of serum BAFF was elevated in this group, although again, not significantly so (1843.7 vs. 812.9 pg/ml; dn-HLA DQ7 and dn-HLA A2 groups respectively; p = 0.26) (data not shown).

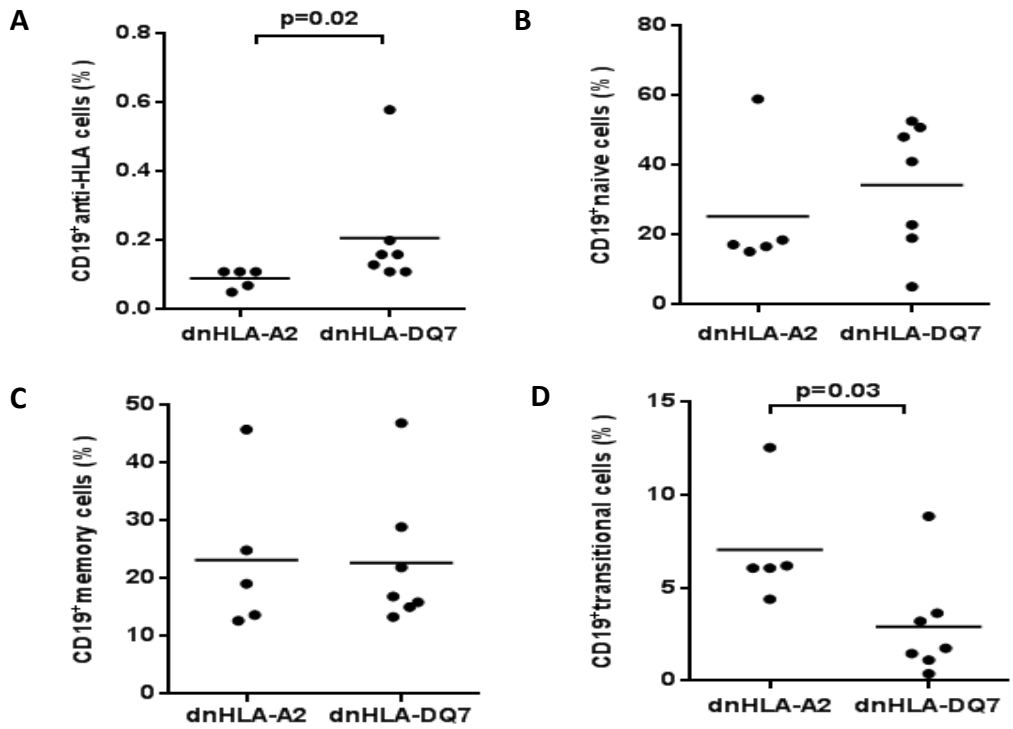


Figure 4.11 Increased frequency of donor-specific transitional B cells in patients with *de novo* serum HLA-A2 compared to and dnHLA-DQ7 antibodies
The dot-plots show elevated frequency of DS-DQ7 (n = 7) compared to DS-A2 B cells (n = 5) in patients with *de novo* donor-specific antibodies (dnDSA; A) with phenotypic representation for naïve (B), memory (C) and transitional B cells (D). The frequency of DS-A2 transitional B cells was significantly higher in patients with dnDSA (p = 0.03). P values derived comparing the two groups by Mann-Whitney test.

Relating level of graft function with type of dnDSA (Figure 4.12), a greater proportion of patients with dn-A2 DSA (40%) had stable function compared to dn-DQ7 DSA (14%). Furthermore, rapidly deteriorating graft function associated with dn-DQ7 DSA (43% vs. 20%, p=0.27; dn-DQ7 and dn-A2 DSA respectively). To date, biopsy-proven AMR has occurred in 29% (n = 2/7) of these patients compared to 40% (n = 2/5) with dn-A2 DSA. Allograft loss has not been experienced by individuals in either group.

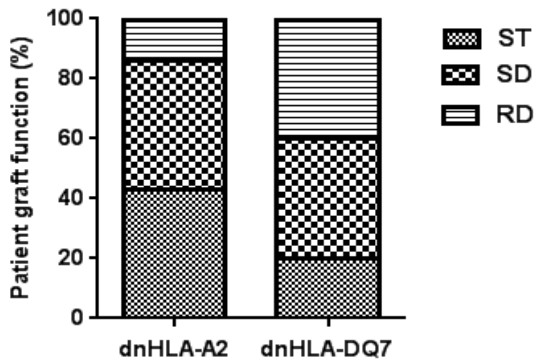


Figure 4.12 Deteriorating graft function associated with presence of serum *de novo* HLA-DQ7 antibodies
The bar chart shows the percentage of patients with dn-A2 & dn-DQ7 DSA with stable (ST), slowly deteriorating (SD) and rapidly deteriorating (RD) graft function as determined by regression analysis of GFR over time. A greater proportion of patients with dn-DQ7 DSA had deteriorating graft function (43% vs. 20%).

This study cohort was too small to make any general assumptions relating the incidence of *de novo* DQ antibodies on allograft survival; therefore an observation investigation was conducted with a wider context of patients transplanted in a single centre over a four-year period.

Investigating the incidence of de novo DQ antibodies post-transplant within a single renal transplantation centre

The incidence of *de novo* DQ antibody was compared to anti-HLA-A, -B, -C & -DR in 434 patients who received a renal transplant from either a living or deceased donor (excluding HLA antibody-incompatible, blood-group-incompatible or simultaneous kidney-pancreas transplant) between 4th November 2007 and 9th October 2012 at the Royal London Hospital, London, UK. Patients included only those assessed for HLA antibody by LSA analysis prior to transplantation to maximise detection at low expression levels. The remaining patients were assessed for dnDSA and results are summarised in Figure 4.13.

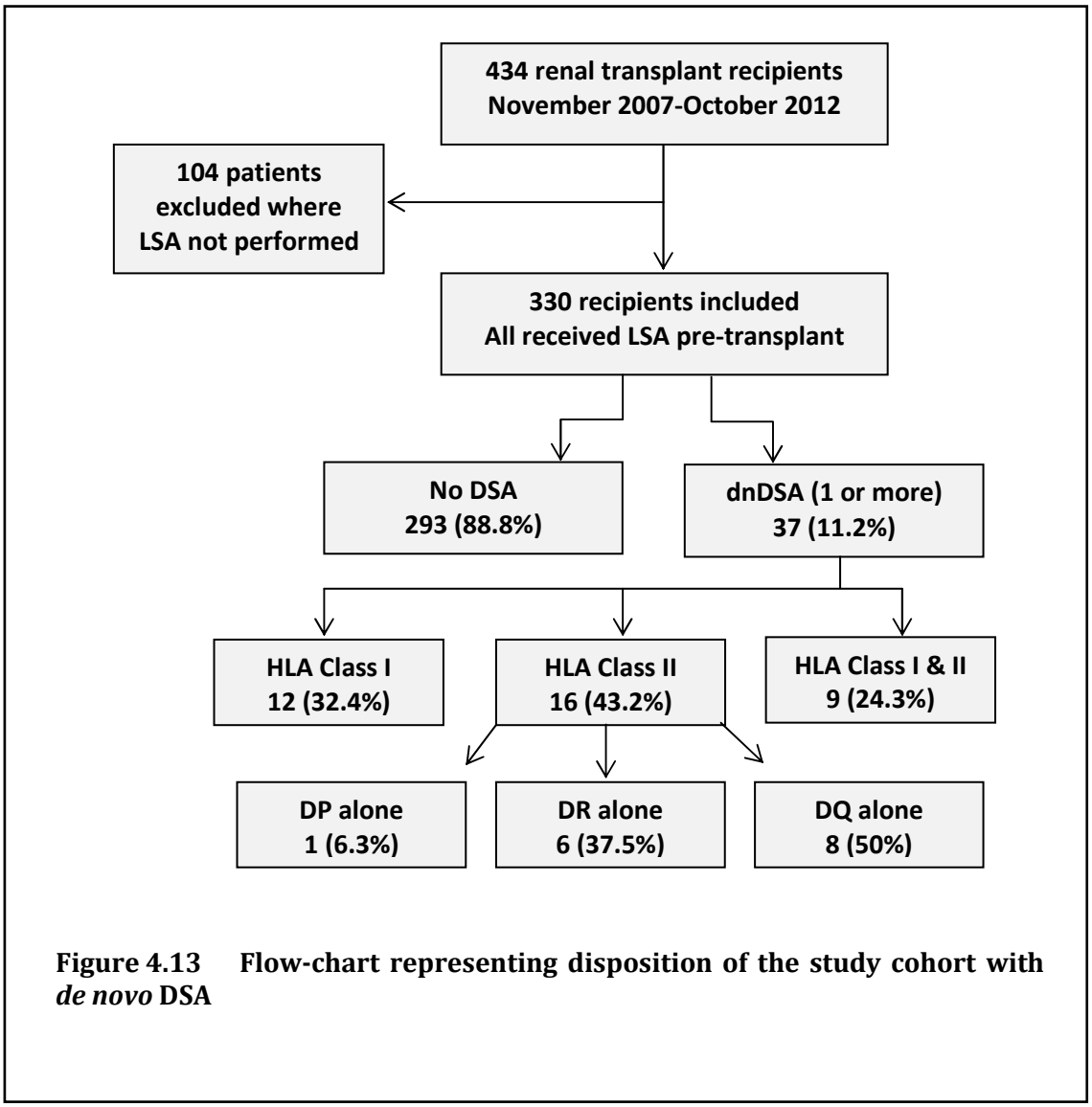


Figure 4.13 Flow-chart representing disposition of the study cohort with *de novo* DSA

Of the 330 patients included, 37 (11.2%) developed dnDSA and, of those, 13 had DQ antibodies (35.1%). There was similar frequency of HLA-A (n = 14, 37.8%) and HLA-DR (n = 14, 37.8%) and a lesser percentage of antibodies to HLA-B (n = 10, 27%), HLA-C (n = 4, 10.8%) and HLA-DP antibodies (n = 1, 2.7%). dnDQ antibodies were detected at a slightly higher frequency in isolation (21.6%) compared to dn-DR (16.2%) and dn-A antibodies (16.2%).

Examining the development of dnDSA in relation to HLA-incompatibility expressed by the allograft (Figure 4.14A), patients transplanted with a mismatch at the HLA-DQ locus had the highest prevalence of dnDSA (55.6% compared to 44.4%, 30.6%, 13.3% and 35.3% for HLA-A, -B, -C and -DR dnDSA, respectively). This was significantly so when compared to patients mismatched with an allograft expressing HLA-C (p=0.02). The most common of the DQ antibodies detected were DQ7, and all patients mismatched for this HLA type developed dnDSA (Figure 4.14B).

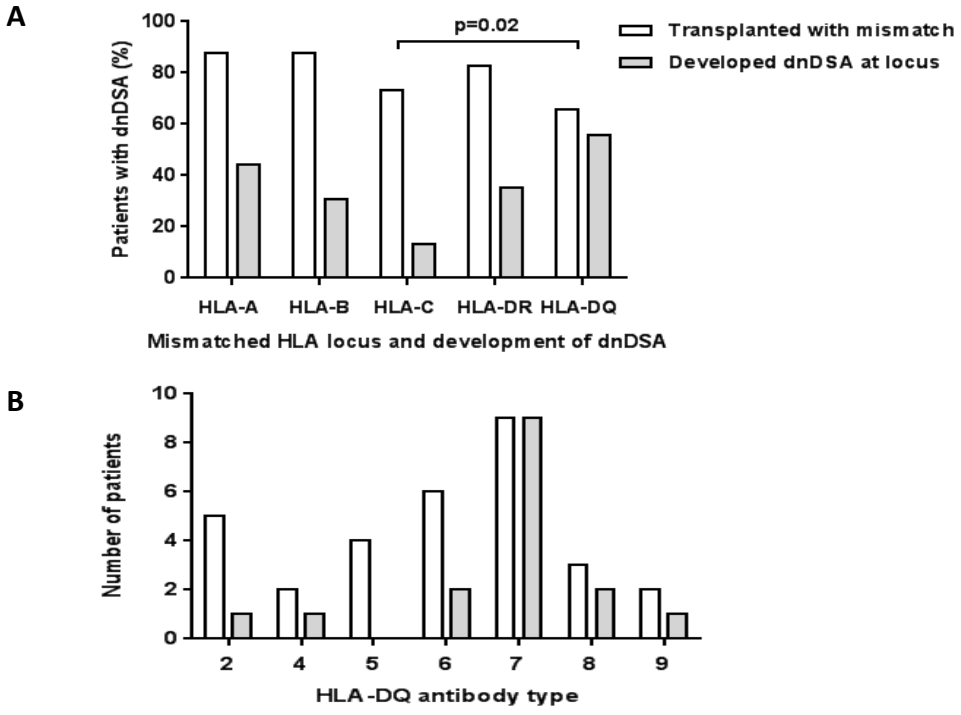


Figure 4.14 Analysing HLA mismatching and corresponding dnDSA production
A. The bar-chart relates percentage of patients (n = 37) transplanted with HLA-A, -B, -C, -DR & -DQ mismatched allograft with the frequency of developing dnDSA. There was a significant difference in those transplanted with an allograft mismatched at HLA-DQ compared to HLA-C (p = 0.02 by Chi squared test).
B. Post-transplant, patients mismatched for DQ7 all developed dnDSA (n = 9).

Time of appearance and strength of dnDSA

The exact time of development of antibodies was not known; an indication was given by the first time dnDSA were recorded following LSA analysis. This was at a median time of 2.6 months (range, 0.03-59.4) and a mean of 8.7 ± 14 months. Further examination showed HLA-class I antibodies had the shorter time to detection (median, 2.3 months (range, 0.03-15.2) and mean 4.4 ± 5.4 months) compared to HLA-class II antibodies (median, 3.6 months (range, 0.3-59.4) and mean 15.2 ± 19.1 months). Assessing dnDQ antibody; these antibodies appeared at a significantly later time than HLA-class I antibodies (median, 8.7 months (range, 0.3-42.6) and mean 15.8 ± 16.9 months; $p = 0.04$).

The level of dnDSA was assessed for all HLA loci. Comparing those directed against HLA-class I and -class II, MFI values were very similar (mean, 5932 (482-12826) & 5492 (454-13662). Investigating for individual HLA antibody, the highest mean MFI was observed for HLA-B antibodies and HLA-DQ antibodies (Figure 4.15A). The lowest levels of dnDSA were HLA-C and HLA-DP and were also the least frequent.

Assessing level of graft function, as determined by regression analysis of eGFR over the last 12 months, we saw that half of the patients had stable function, and a small fraction had deteriorating function (Figure 4.15B). Graft failure had occurred in 30% of patients; of those, AMR was confirmed by biopsy in 27% (Figure 4.15B). Of those, the rapidly deteriorating group represented the highest frequency of biopsy-proven AMR (16% vs. 57%, $p = 0.07$ for ST vs. RD groups, respectively). Addressing the detrimental impact of dnDQ antibodies and allograft function, none of the categories examined (level of graft function, rate of failure and biopsy proven AMR) were significantly over-represented compared to other dnDSA ($p > 0.05$; Figure 4.15C).

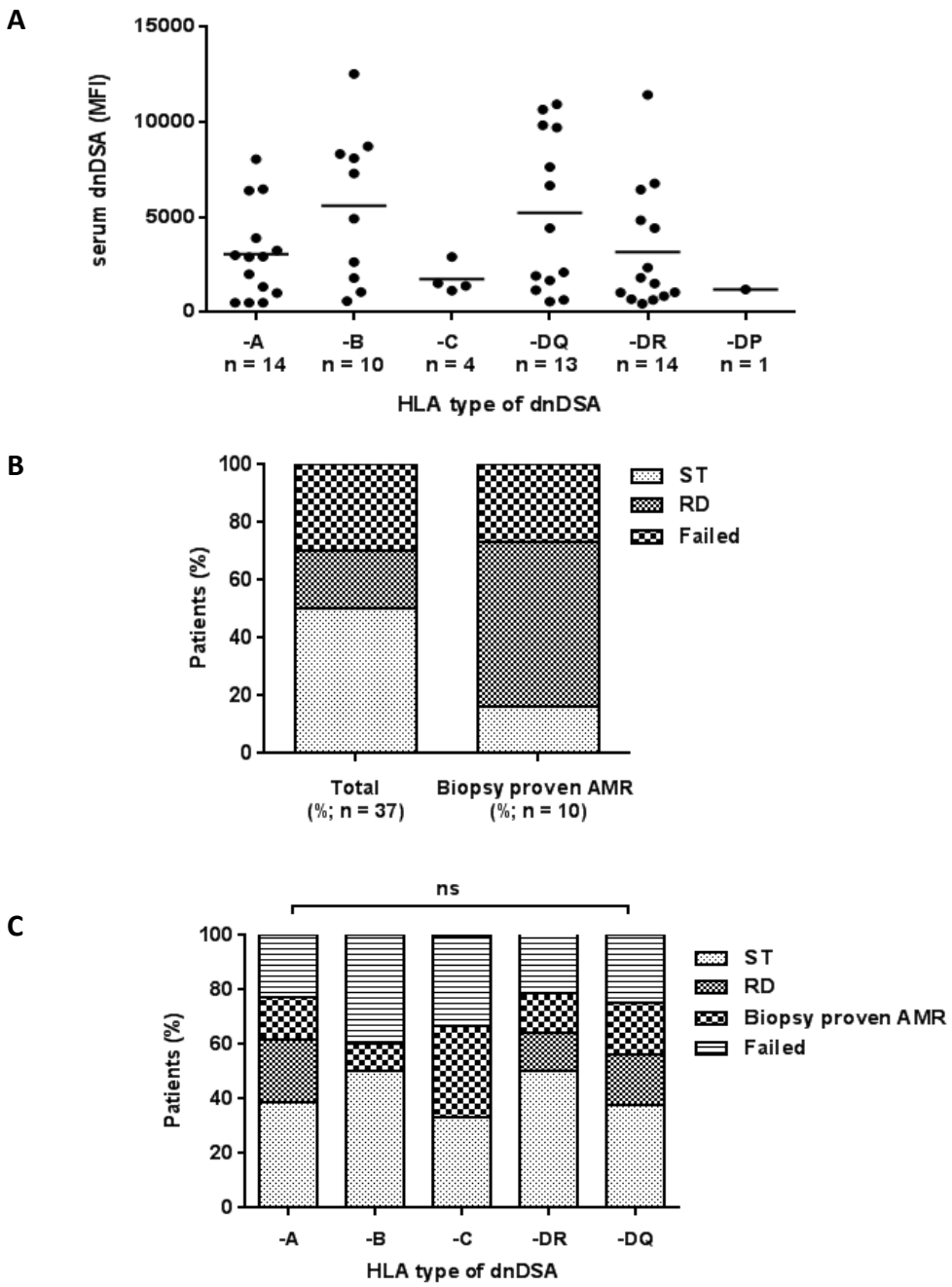


Figure 4.15 Investigating the level and frequency of dnDSA and associated level of graft function

A. Dot-plot shows the frequency of patients with serum HLA-A, -B, -C, -DR, -DQ & -DP *de novo* donor-specific HLA antibodies (dnDSA) against relative strength (mean fluorescent intensity; MFI) as determined by Luminex technology. The mean MFI value is shown. Comparing MFI values between those with dnDSA-DQ antibodies and other HLA types of dnDSA did not indicate any significant differences (p = 0.23 by Kruskal-Wallis test).

B. Bar-graph shows the frequency of patients (%) with level of graft function. Those with rapidly deteriorating (RD) graft function had a higher incidence of biopsy proven AMR (4 of 7 patients) than the stable (ST) group (3 of 19 patients; p = 0.07 by Chi squared test).

C. Comparing HLA-A, -B, -C, -DR, -DQ & -DP dnDSA with graft function & failure, and biopsy proven AMR did not show an over presentation of categories in those with dn-DQ antibodies (p>0.05 by Chi squared test).

4.4 Discussion

Allograft rejection is multifaceted. However, a prevailing view is held of a causative role for DSA (Terasaki and Cai, 2008, Einecke et al., 2009). In a primate model, following progression of chronic antibody-mediated rejection (CAMR), four stages are identified: the presence of DSA, leading to deposition of C4d, development of transplant glomerulopathy (TG), followed by raised creatinine and ultimate loss of graft function (Smith et al., 2008). In transplanted renal patients, allograft failure occurs more frequently in those with HLA antibody (Terasaki et al., 2007, Lefaucheur et al., 2010). In particular, the presence of DSA is predictive of inferior graft outcome (Worthington et al., 2003, Mao et al., 2007, Lachmann et al., 2009). Thus, the detrimental effect of DSA on allograft function and survival is well established. More detailed examination during the post-transplant period has disclosed that pathogenicity of DSA is not equal. Recently, a number of studies observe a prevalence of dnDQ DSA and an association with allograft dysfunction (Hidalgo et al., 2009, Willicombe et al., 2012, DeVos et al., 2012). Given evidence supporting a negative impact of DSA on allograft loss, identification in serum is relied upon as a prognostic marker of dysfunction. However, this may not capture the complete profile of alloreactivity and details of the cells responsible for producing antibody are largely unknown.

The method described to identify DS-B cells directly from peripheral blood provides a novel approach to monitor their presence and prevalence and could be easily adapted to clinical practice. Moreover it offers a lower cost alternative to the application of HLA-specific tetramers as described by Zachary and colleagues (Zachary et al., 2007). In this study at least, the high degree of non-specific staining experienced with HLA-A*0201 pentamers was reduced using HLA-coated microbeads.

The theoretical basis of identifying HLA-specific B cells by flow-cytometry relies upon the capability of a BCR, unlike a TCR, to recognise soluble, unprocessed antigen, in this instance coated to the surface of microbeads. The ability to utilise microbeads in this way may be possible because of their size, together with the high density of BCR

expressed per B-cell (approximately 10^5/cell; (Unanue et al., 1971)). With a diameter of 5.6 µm, they are slightly smaller than a B-cell (average diameter of 7 µm) and potentially capable of cross-linking and engaging with a number of BCRs expressing antigen-specificity toward the HLA presented on the bead. This interaction was sufficiently stable for detection by flow-cytometry. Through coupling fluorescently labelled antibodies targeted at B cells and utilising the inherent fluorescent property of the HLA-coated microbeads, it was possible to identify a small population of DS-B cells. To increase the stringency of this method, future studies would benefit from the inclusion of an additional fluorochrome such as 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) to allow for selection of only viable cells.

The ability to detect DS-B cells in peripheral blood is considerably preferable to isolating cells from bone-marrow aspirates, as described by Perry and colleagues (Perry et al., 2008). While they reported aspirate removal as being well-tolerated without post-operative complications, this method is logistically far more involved and expensive compared to the relative ease of taking blood during routine clinic appointments. They cite adopting this method following unsuccessful detection of DS-B cells in peripheral blood; however a low volume was analysed (20 ml). Moreover, their method of isolating antibody-secreting B cells relied on intracellular expression of immunoglobulin κ and λ chains. Intracellular staining requires a process of permeablisation and fixation, resulting in cell loss, potentially reducing the very small population of interest. In this study, a minimum of 40 ml was drawn, generating approximately one-million $CD19^+$cells or 2.3%; from this a median of 0.11% or 330 $CD19^+$ anti-HLA^+ cells were detected per million $CD19^+$ cells (range, 0.01-0.63% or 28-1750). This is a higher frequency than cited for women sensitised via preganancy using teteramer recognition of HLA-A2-specific B cells (179/10^6 $CD19^+$ cells) (Mulder et al., 2003). However given the permanent antigenic stimulation offered by the allograft, a higher frequency should probably be expected.

This study decribed the identification of cells at low-frequency and the validity of these data is governed by Poisson statistics. Accuracy increases with cell-number aquired by the flow-cytometer. Using a method previously decribed to determine cell-number

requirements and level of precision (Hedley and Keeney, 2013), the Poisson coefficient of variation (CV) of the counting error was calculated at 18.25%. To increase the stringency or lower the CV would require more cells or events. Obviously the volume of blood requested from patients is limited. However, during this study, cells were required to optimise and test parameters; should this method be applied in clinical practice, the CV could be improved by increasing the number of cells acquired with minimal allocation for controls.

This study established that a higher frequency of peripheral DS-A2 B cells was present in sensitised (0.16%) compared to non-sensitised patients (0.04%), and to a level comparable with that previously described in women sensitised to HLA-A2 via pregnancy (0.11%) (Mulder et al., 2003). Both of these values are considerably lower than those reported by Zachary et al., (4.1%) which may be explained by differences in B-cell selection methods (Zachary et al., 2007). In this, and that of Mulder and colleagues, positive $CD19^{+}$ cell selection was employed rather than T cell-depletion, the latter having potential to allow inclusion of cells expressing lower levels of CD19.

With reference to the frequency of DS-DQ7 B cells, I am not aware of any publications on this to date. The rationale for examining the binding of this antigen to B cells was based on the reported elevated frequency of dnDQ antibodies following transplantation and their impact on reducing allograft survival (Willicombe et al., 2012, Everly et al., 2013, DeVos et al., 2012). Accordingly, it was of interest to identify differences in prevalence or phenotypic characteristics compared to DS-B cells from patients with dn-A2 DSA. Firstly, DS-B cells were present at significantly higher levels in patients with serum dnDSA against HLA-DQ7 and HLA-A2 compared to unsensitised patients. Moreover, there was significant correlation between the increased presence of DS-B cells and level of serum antibody (as determined by MFI). Comparing the presence of DS-B cells, DS-DQ7 B cells were at a significantly higher overall frequency than DS-A2 B cells.

Further examination of memory and naïve B cell distribution by DS-B cells compared to the total B-cell population did not reveal an associated phenotype or a distinction in

those patients with dn-DQ7 DSA. However DS-A2 memory B cells were reduced in patients without HLA antibody and could relate with the absence of DSA development. Obviously these data represented a very small cohort and wider investigations may reveal differences. This could include assessing HLA-specificity on plasmablasts. In mice, over-expression of autoantibody is associated with prolonged survival of extrafollicular plasmablasts (William et al., 2005). Extrafollicular plasmablasts are formed from activated B cells. These are short-lived and responsible for production of early antibody with specificities encoded by the primary repertoire (Jacob and Kelsoe, 1992). It is possible these cells are responsible for early *de novo* alloantibody production. Recently described was a subset of phenotype, $CD20^{+}CD27^{+}CD43^{+}CD70^{-}CD69^{-}$. This has been proposed as the human equivalent of murine B1 cells and demonstrated the capacity to differentiate into pre-plasmablasts and plasma cells *in vitro* (Covens et al., 2013). In addition, circulating plasmablasts spontaneously producing anti-citrullinated protein antibody (ACPA) have been identified in patients with rheumatoid arthritis. Moreover the ACPA-specific B cells were not confined to $CD20^{+}$ memory B cells (Kerkman et al., 2013). These studies support more detailed phenotypic analysis of DS-B cells at the level of B cell differentiation into antibody-secreting cells and could aid towards developing strategies beyond those currently targeted by CD20 depletion.

DS-B cells were examined for phenotypic expression of transitional B cells. Exhibiting high levels of CD24 & CD38, they are ascribed with regulatory capacity via their ability to suppress Th1 cytokines and promote development of regulatory T cells (Flores-Borja et al., 2013, Blair et al., 2010). Moreover, a reduced presence was observed during ESRF (Kim et al., 2012) and following renal transplantation correlated with poor graft function (Cherukuri et al., 2012). Chapter 6 of this thesis is dedicated to considering their role. Suffice it to say at this stage that transitional B cells were enriched in DS-B cells compared to total B-cell populations for all patient groups. In addition, DS-transitional B cells from patients with dn-A2 DSA were present at a significantly higher frequency compared to those with dn-DQ7 DSA. This coupled with superior graft function in patients with dn-A2 DSA is an interesting observation, compatible with a

potentially protective role of these cells in limiting a pro-inflammatory response and damage to the allograft.

In terms of graft function and serum alloantibody, patients with stable graft function were equally represented in the HLA-A2 transplanted group (US, dnDSA and DSA). Conversely, a lower frequency of patients with dn-DQ7 DSA had stable renal function (14%) compared to those without DSA (57%). This, together with the correlation between DS-DQ7 B cells and level of serum alloantibody, suggests an association with allograft dysfunction. In both cohorts, patients were tested at a single time-point and the natural history of emerging DS-B cells prior to detection of DSA in serum is not known. Equally their prevalence during progression from normal to acute allograft dysfunction was not determined. Establishing if these cells appear prior to presentation of changing clinical features, such as rising creatinine or proteinuria, requires further investigation.

In summary, DS-B cells can be identified in peripheral blood, their frequency was elevated in patients with dnDSA and a positive association existed between their frequency and level of serum alloantibody. Moreover, deteriorating graft function prevailed in patients with dn-DQ7 DSA. To place these findings in a wider context, an observational study was performed on an entire cohort transplanted over a four-year period to determine the prevalence of dnDQ DSA and their impact on allograft survival. dnDSA occurred at a rate of 11%; this is slightly lower than recent reports of approximately 18%, albeit for larger cohorts (DeVos et al., 2012, Willicombe et al., 2012). The appearance of dnDSA at a median of three-months is consistent with others, where the majority are detected within six-months post-transplantation (Cooper et al., 2011, Gill et al., 2010, Piazza et al., 2001). Collectively, these data indicate monitoring DS-B cells or serum dnDSA should be concentrated in the early months post-transplant. Several risk factors can influence the development of dnDSA, including non-compliance (Sellares et al., 2012), immunosuppression minimisation, or use of less potent immunosuppression (Ginevri et al., 2012) and for these patients identification could allow potential intervention prior to irreversible chronic damage.

The levels of dnDQ DSA were comparable to those of HLA-B DSA, and together were the only antibodies detected at greater than 5000 MFI. Compared to HLA-A, B, C or DR antibodies, DQ antibodies were not over represented and, at 35%, was below the lower range reported by others (52-91%) (DeVos et al., 2012, Everly et al., 2013, Willicombe et al., 2012). This variation may be reflective of differing MFI cut-off values. Moreover, retrospective analysis of samples excluded from this study due to absence of pre-transplant LSA analysis could increase the dnDSA cohort.

Two points of particular interest transpire from this analysis: first, dnDQ DSA were the most prevalent detected alone. Second, the incidence of DQ antibodies; of the 37 patients who developed dnDSA, 66% were mismatched at HLA-DQ and, of those, 56% developed dnDQ-DSA, representing the highest proportion. Further analysis showed DQ7 antibodies were the most common DQ allele to provoke an immune response, in line with a recent study (DeVos et al., 2012). Indeed every patient mismatched with an allograft expressing HLA-DQ7 subsequently developed dn-DQ7 antibodies, an observation not seen with other HLA alleles, indicating that immunogenicity may not be equal between alleles. To address this, the level of graft function and incidence of failure, together with frequency of biopsy-proven AMR was examined. In this cohort, dnDQ DSA was not found to be particularly pathogenic by these categories assessed. However, at present, the mean time post-transplantation is 3.6 years. If equal assessment is performed in five or ten years, it may be shown to have a contributing role in chronic allograft damage.

Concluding remarks

This study described the application of flow-cytometry-based methods for detecting and enumerating DS-B cells in peripheral blood of patients identified with serum DSA. Applied in conjunction with cell-surface markers used to define B-cell subsets, it was possible to further detail phenotypic characteristics. Moreover, positive correlation between DS-B cell frequency and level of serum alloantibody indicated a level of both sensitivity and specificity of the method.

Presently, post-transplant assessment of an ongoing alloresponse is limited to analysing serum, usually together with biopsy. The ability to detect DS-B cells could enrich the clinical information available, and may also hold potential following HLA-incompatible transplantation for monitoring the effectiveness of agents targeting B cells. Recent evidence implementing dnDSA and, in particular, DQ antibodies with graft damage suggests an application to aid identification and assess magnitude of an emerging alloresponse, potentially prior to manifestation detectable in serum, allowing more timely therapeutic intervention and limiting irreversible graft damage.

Chapter 5

Analysing expression of BAFF as a biomarker of allograft function

5.1 Introduction

B cells are increasingly recognised as important mediators of transplant survival, promoting antibody-mediated rejection (AMR) but also potentially supporting allograft longevity. However their activity post-transplant is currently not addressed until a patient presents with symptoms suggestive of allograft dysfunction. If appropriate, B-cell-mediated pathology is examined by serum analysis for HLA antibodies and ideally allograft biopsy; together they can identify and diagnose AMR. Pursuing these clinical investigations initially relies upon measuring changes in levels of creatinine and proteinuria to gauge renal function. Yet at this time injury to the allograft may have already occurred and alternative modes to immunologically monitor functional changes have not been widely explored. An attractive candidate is the B-cell activating factor, BAFF, a cytokine critical for B-cell survival and maturation, as demonstrated in BAFF-deficient mice where B cells fail to reach maturity (Mackay et al., 2003). Equally, over-expression resulted in increased B-cell survival, development of pathogenic autoantibodies and manifestation of a lupus-like disease (Groom et al., 2007).

Given the central role of BAFF in B-cell survival, serum levels are reported in autoimmune diseases, including systemic lupus erythematosus (SLE), rheumatoid arthritis and Sjörgen's syndrome (Pers et al., 2005, Gottenberg et al., 2009, Hegazy et al., 2010). In each disease modality, BAFF is identified in excess and proposed to contribute toward development and survival of autoantibodies. Moreover, studies of SLE have correlated elevated serum BAFF (sBAFF) with disease activity (Petri et al., 2008, Carter et al., 2013) and in multiple myeloma, reduced sBAFF correlated with clinical responsiveness and favourable prognosis (Fragioudaki et al., 2012). In the context of transplantation, elevated sBAFF could predict the development of graft versus host disease (GvHD) (Sarantopoulos et al., 2007, Sarantopoulos et al., 2009). Collectively these studies demonstrate BAFF as a biomarker of disease activity and this may equally apply to monitoring allograft function. Recent reports link increased sBAFF levels with ability to predict the development of DSA, (Thibault-Espitia et al., 2012) and increased risk of experiencing AMR when undergoing HLA-incompatible renal

transplantation (Banham et al., 2013b). However, these studies have not addressed involvement of BAFF in the post-transplantation period as an indication of deteriorating function resulting from immunological activity or association with renal injury by direct localisation within the kidney. Indeed published data of intrarenal expression of BAFF is limited: to date only two studies have identified BAFF in allograft biopsies; first, following loss as a result of acute antibody-mediated rejection (AAMR) (Xu et al., 2009a) and second, in two patients with chronic antibody-mediated rejection (CAMR) (Thaunat et al., 2008). Neither explored differential expression depending on the level of renal graft function or examined the presence of BAFF-R. The localisation of BAFF and BAFF-R within renal tissue to areas of infiltrating inflammatory cells in conjunction with presence of DSA and elevated levels of sBAFF could provide evidence for measuring sBAFF as an effective marker of AMR, offering a low risk and relatively inexpensive alternative to the invasive biopsy procedure. Moreover this could obviate issues with sample quality and retrieval. It is reported that 0.02% of the kidney is presented by the biopsy section and it is known that the sampled core may not always be entirely representative (Rush, 2006).

Understanding the biology that governs the function of B cells may allow for advances in immunological monitoring to detect changes prior to irreversible damage to the allograft and influence the fate of transplant survival. Defining BAFF expression in an evolving B-cell compartment could potentially identify dysfunction and enable timely intervention to treat AMR or monitor the response to treatment. In the small study by Thaunat and colleagues, they demonstrated that, despite B-cell depletion with rituximab, alloantibody production persisted in patients with graft loss as a result of CAMR (Thaunat et al., 2008). Tertiary lymphoid organs were evident in the explanted grafts and proposed to support the humoral response. In addition to detecting intrarenal BAFF by immunohistochemistry, expression at the gene and protein level was elevated. This raises the possibility of utilising sBAFF measurements as an indicator of B-cell depletion effectiveness and also following alterations or reductions in maintenance immunosuppression.

To summarise, excessive BAFF may be indicative of dysregulation in the B-cell compartment, suggesting a role in disease pathogenesis. The development of a non-invasive test to monitor allograft function could allow early intervention to prevent rejection and ensuing injury to improve long-term transplant outcome. This study explores if there is correlation between BAFF localisation within the allograft, together with elevated sBAFF and the presence of DSA, as this could provide evidence of humoral rejection, perhaps limiting the requirement for biopsy.

5.2 Materials and methods

Materials and methods detailed in Chapter 2, General Methods (GM) are indicated.

GM1 Recruitment of study patients

GM2 Immunosuppressive regime

GM3 Defining level of allograft function in patient cohorts

GM4 Peripheral blood mononuclear cells (PBMC) separation

GM5 $CD19^+$ cell isolation from PBMC

GM6 Phenotyping $CD19^+$ cells by flow cytometry

GM8 Detection of HLA antibody in serum by Luminex technology

GM10 Statistical analysis

Detection of BAFF by ELISA

Human BAFF detecting ELISA (R&D Systems) was used to measure serum levels as per manufacturer's instructions. In brief, serum samples were thawed and diluted 1:2 in calibrator diluent before applying to a microplate pre-coated with mouse monoclonal antibody against human BAFF (as provided in the kit; therefore at unknown concentration) and incubated with shaking for three hours at room temperature. The plate was washed four times with wash buffer and residual liquid removed after each wash with blotting. A polyclonal antibody against BAFF conjugated to horseradish peroxidase provided in the kit was applied to each well and incubated with shaking for an hour at room temperature. Following this, the plate was washed again four times

and developed for 30 minutes using equal volumes of neat tetramethylbenzidine (TMB) and neat hydrogen peroxide. This reaction was stopped by addition of sulphuric acid (2N) and the plate analysed using an automated microplate reader (Labtech) set at an absorbance wavelength of 450 nm with wavelength correction at 540 nm. Data were exported to Excel and, using the concentration of serially diluted positive controls against absorbance, generated a linear regression curve used to estimate the concentration of BAFF in serum samples.

Renal biopsies and Immunohistochemistry

Immunohistochemistry was carried out on 3 µm sections of renal biopsies. The sections were cut on a rotary microtome (ThermoFisher Scientific), floated on a warm water bath, to remove creases and dried in a 60 °C oven over night before staining.

Immunohistochemistry was performed to detect expression of CD19 BAFF and BAFF-R. Mouse anti-human monoclonal antibodies were used against CD19 (clone LE-CD19; Dako) and BAFF (clone T7-241; LifeSpan BioSciences). A polyclonal rabbit anti-human antibody was used to detect BAFF-R (C-terminus, LifeSpan BioSciences). Staining was performed using the Dako Envision FLEX detection system with an Autostainer (Dako Link 48). Initial optimisation was carried out on tonsil tissue and antibodies were tested at two pH (pH 9.0 and 6.0), at a range of concentrations and also with and without the pre-treatment linker system (Dako). The slides were examined by a consultant histopathologist and the optimised conditions gave the strongest and most accurate staining with the least background.

In brief, the cut paraffin-embedded tissue were mounted onto slides before being de-waxed, rehydrated and treated with FLEX peroxidase-blocking reagent (Dako) to block endogenous peroxidase. To reduce non-specific background staining, neat horse serum and an avidin-biotin blocking kit (Invitrogen) were also used. Deparaffinised tissue sections were treated with heat-induced epitope retrieval using Target retrieval solution (Dako) at high pH (pH 9.0, CD19) or low pH (pH 6.0, BAFF & BAFF-R) and the PT linker system (Dako); this was a series of water baths. The antigen retrieval process

allowed access of the primary antibody to the antigen which can become masked during the tissue fixation process. The slides were cooled to 65 ^{o}C transferred to wash buffer at room temperature and then moved to the Autostainer. Slides were stained with optimised concentrations of CD19 (1:50), BAFF (1:200) or BAFF-R (1:200) antibodies for 30 minutes. FLEX/HRP detection reagent and FLEX DAB+ chromagen reagent (Dako) were used to visualise the target antigen. Here the secondary antibody labelled with horseradish peroxidase (HRP) converts 3, 3’ diaminobenzidine (DAB) to brown; this was the visualising chromagen. Counter-staining of nuclei used FLEX Hematoxylin (Dako). Following staining, slides were mounted and examined by a consultant histopathologist. Slides were graded by percentage expression in the core. Tonsil tissue was used for negative and positive controls.

5.3 Results

To assess differential expression of sBAFF and BAFF-R as a factor of allograft function, three cohorts were selected (details of patient group allocation were outlined in Chapter 2, GM 3) and defined as stable (ST; n = 31), slowly deteriorating (SD; n = 29) and rapidly deteriorating (RD; n = 33). Patient demographics are shown in Table 5.1.

Allograft function	**Stable (ST)**	**Slowly deteriorating (SD)**	**Rapidly deteriorating (RD)**
Total number of patients, n (%)	31 (33.3)	29 (31.2)	33 (35.5)
Recipient			
Gender (F,M)	11,20	9,20	14,19
Age, mean (range) (years)	45 (22-68)	48 (26-70)	46 (22-67)
Time post-transplantation, mean (range) (years)	6 (1-21)	9 (1-25)	8 (1-23)
***Type of allograft*, n (%)**			
Live donation	19 (61)	11 (38)	8 (24)
DBD	11 (36)	14 (48)	22 (67)
DCD	1 (3)	4 (14)	3 (9)
***HLA mismatches,* n ± sd**			
A, mean ± sd	1.1 ± 0.6	1.3 ± 0.7	1.4 ± 0.5
B, mean ± sd	1.0 ± 0.6	1.3 ± 0.6	1.2 ± 0.4
DR, mean ± sd	0.9 ± 0.6	0.9 ± 0.7	0.7 ± 0.7
HLA antibody - post-transplantation			
Negative	19 (61)	11 (38)	10 (30)
NDSA	5 (16)	4 (14)	5 (15)
DSA	7 (23)	14 (48)	18 (55)
Cause of ESRF			
IgA nephropathy	3 (10)	2 (7)	2 (6)
FSGS	2 (6)	3 (10)	1 (3)
GN	7 (23)	4 (14)	4 (12)
Hypertension	2 (6)	0	4 (12)
PKD	1 (3)	2 (7)	3 (9)
Diabetes	2 (6)	0	0
Other/Unknown	14 (45)	18 (62)	19 (58)

Table 5.1 Patient demographics for the study cohort
Abbreviations: DBD, donation after brainstem death; DCD, donation after circulatory death; DSA, donor-specific HLA antibody; ESRF, end-stage renal failure; FSGS, focal and segmental glomerulosclerosis; GN, glomerulonephritis; PKD, polycystic kidney disease; sd, standard deviation.

Investigating serum BAFF levels in patients with different allograft function

Elevated serum BAFF levels correlate with reduced graft function

Serum BAFF levels were measured at a single time-point post-transplantation. Patients with ST graft function had significantly lower levels compared to those with SD or RD graft function (662.3 ± 326.9 pg/ml, 877.9 ± 388.8 pg/ml; p = 0.03 & 1096.1 ± 999.2 pg/ml; p = 0.009 for ST, SD & RD groups respectively; Figure 5.1A). When separated by low (n = 60; 64.5%) or high (n = 33; 35.5%) sBAFF level relative to the mean value (878.8 pg/ml), there was a significant, if small correlation between the level (for both low (p = 0.03) and high (p = 0.04) and rate of allograft deterioration, extracted from regression analysis (Figure 5.1B).

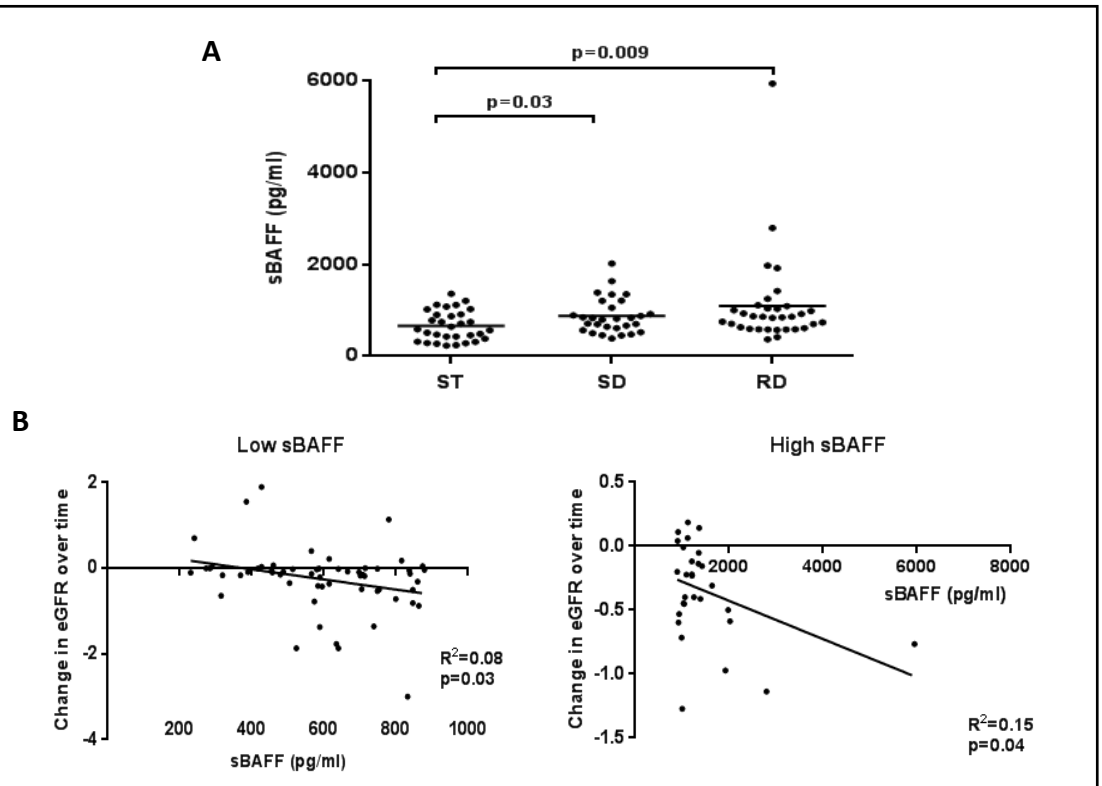


Figure 5.1 Elevated serum BAFF in patients with deteriorating allograft function

A. Dot-plot representing serum BAFF (sBAFF) levels measured by ELISA for patients with stable (ST; n = 31), slowly deteriorating (SD; n = 29) and rapidly deteriorating (RD; n = 33) graft function. sBAFF was significantly elevated in both SD & RD groups compared to the ST group (p values by Mann-Whitney test).

B. Linear regression analysis showed significant correlation with level of sBAFF (low and high) and rate of deteriorating graft function (gradient of eGFR over time) in patients with either low or high sBAFF (< or > mean 878.8 pg/ml).

Correlating level of serum BAFF with donor-specific HLA antibody

Expression of sBAFF was examined in relation to patients' HLA antibody status (Figure 5.2A). Overall, DSA was more prevalent in patients with high levels of sBAFF ($sBAFF^{high}$) compared to low sBAFF ($sBAFF^{low}$). Equally, $sBAFF^{low}$ was associated with the absence of DSA ($sBAFF^{high}$ - 60.6% vs. 28.3% and $sBAFF^{low}$ - 24.2% vs. 58.4% for DSA vs. HLA negative groups, respectively; $p = 0.0009$).

Examining sBAFF level and DSA together with allograft function, there was no statistically significant difference between the prevalence of DSA, high levels of sBAFF and poor-graft function. Equally, patients without HLA antibody did not have statistically significant lower levels of sBAFF.

A relationship between elevated sBAFF and the presence of DSA was investigated further to support the hypothesis that excess sBAFF may maintain a dysregulated B-cell compartment and the production of alloantibody. There was a weak positive trend toward rate of declining graft-function and sBAFF level in patients with DSA (R^2=0.11, $p = 0.19$, Figure 5.2B), which was absent in patients with NDSA ($R^2 = 0$, $p = 0.99$). Furthermore, of those where a biopsy was performed, AMR was entirely restricted to patients with DSA and $sBAFF^{high}$ (33.3%).

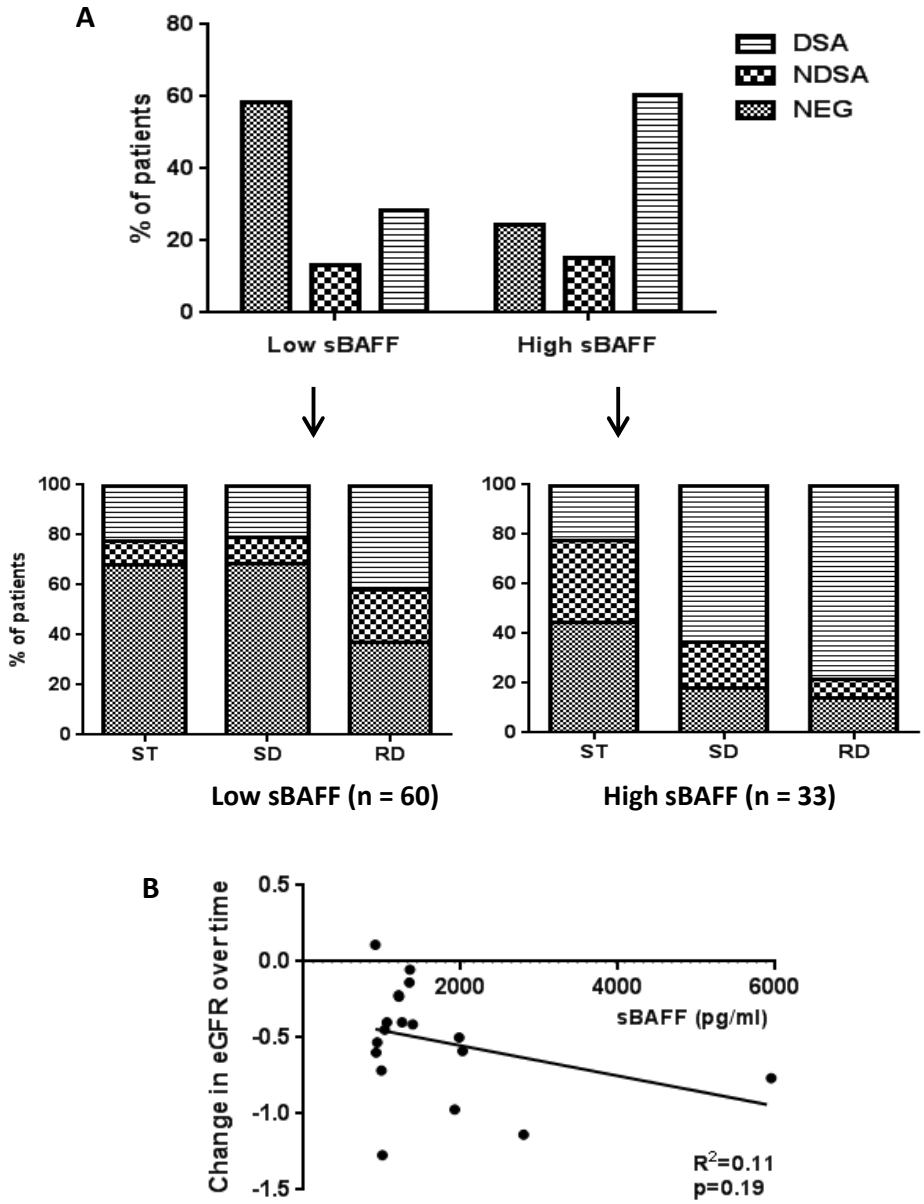


Figure 5.2 Level of serum BAFF and presence of donor-specific HLA antibody
A. Bar-chart representing the frequency of patients with low (n = 60) or high levels of sBAFF (n = 33; < or > mean value 878.8 pg/ml) and presence of donor-specific HLA antibody (DSA), non-DSA (NDSA) or negative for HLA antibody. Relating this to graft function, the majority of stable (ST), slowly deteriorating (SD) groups with low sBAFF did not have HLA antibody. In contrast, SD and rapidly deteriorating (RD) groups with high sBAFF had a higher prevalence of DSA.
B. Linear-regression analysis assessing the level of sBAFF in relation to rate of graft deterioration (extracted from the gradient of eGFR over time) showed the absence of a statistically significance difference.

Measuring serum BAFF as a marker of early renal dysfunction

To evaluate BAFF levels during developing renal dysfunction and its potential application as a biomarker, sBAFF levels were analysed where stored serum was available for the duration of a patient's transplant (n = 3; Figure 5.3). For patients 1 & 3, a rise in serum creatinine (sCr) appeared in conjunction with increasing sBAFF. For patient 2, sBAFF increased prior to sCr. Overall, measurements of sBAFF were more prone to fluctuation over time than sCr, reflected by lower R^2 and p values. With limited samples available it was not possible to determine if sBAFF was detected prior to a rise in sCr. Both sCr and sBAFF reached their peak value at the point of allograft failure.

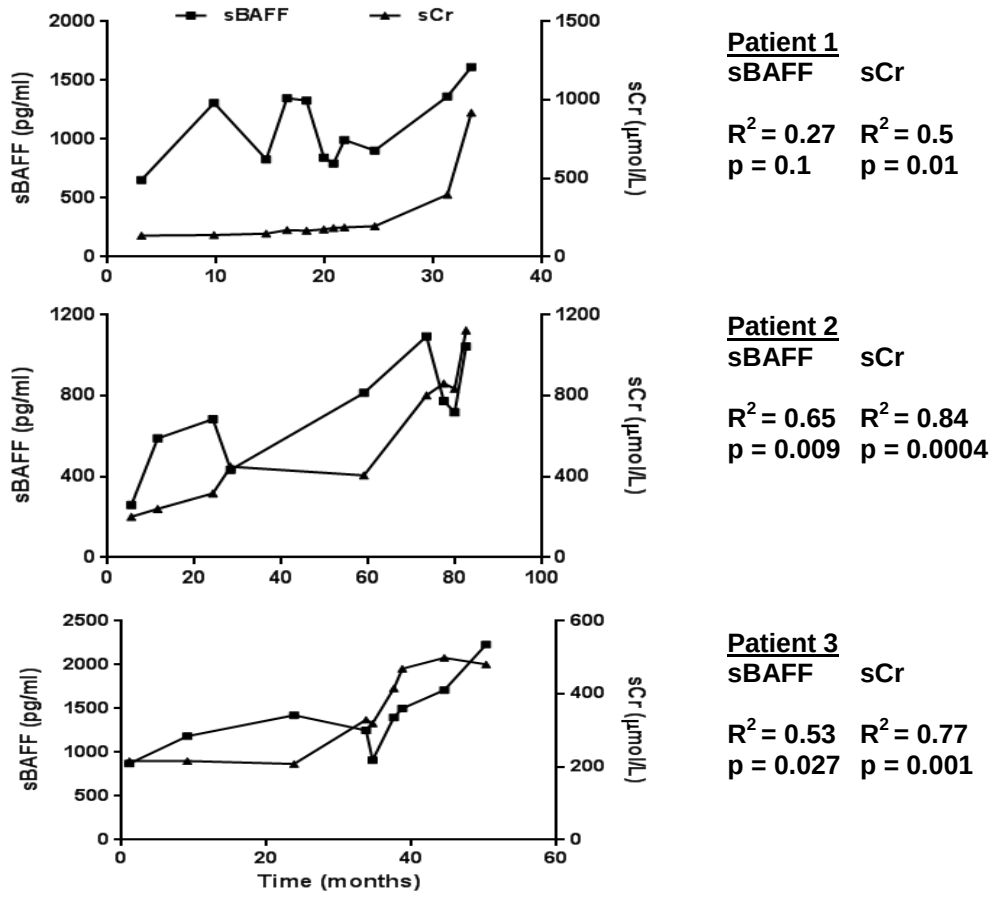


Figure 5.3 Monitoring serum creatinine together with BAFF levels over the duration of allograft survival for three patients was inconclusive
Assessing level of sBAFF in relation to serum creatinine (sCr) from time of transplant through to allograft failure (n = 3). Linear-regression analysis was performed for both sBAFF and sCr over time; R^2 and p values are stated for each patient.

Summarising serum BAFF levels in renal transplant recipients

Serum BAFF levels were elevated in patients with slowly and rapidly deteriorating graft function. When separated based on serum level, there was significant correlation between declining renal function and high sBAFF. The potential contribution towards deregulating homeostasis within the B-cell compartment is highlighted by the prevalence of DSA in these patients.

Phenotyping CD19⁺ cells for expression of BAFF receptor

BAFF-R is primarily expressed by B cells and is the principal receptor for BAFF (Bossen and Schneider, 2006). To investigate if elevated sBAFF is due to lack of available receptor, BAFF-R was measured on the cell surface of $CD19^+$ cells isolated from PBMC in the three groups. The initial constitution of lymphocytes (512 ± 210, 628 ± 273 and 645 ± 262 cells/µl for ST, SD and RD, respectively; p = 0.29) and $CD19^+$ cells (19 ± 15, 17 ± 14 and 20 ± 14 cells/µl for ST, SD and RD, respectively; p = 0.68) was not significantly different between groups (Figure 5.4A). However, the frequency of BAFF-R was reduced in patients with RD graft function (84.3 ± 16.6, 75.6 ± 18.3 and 67.5 ± 17.5 % for ST, SD and RD respectively, p = 0.002; Figure 5.4B).

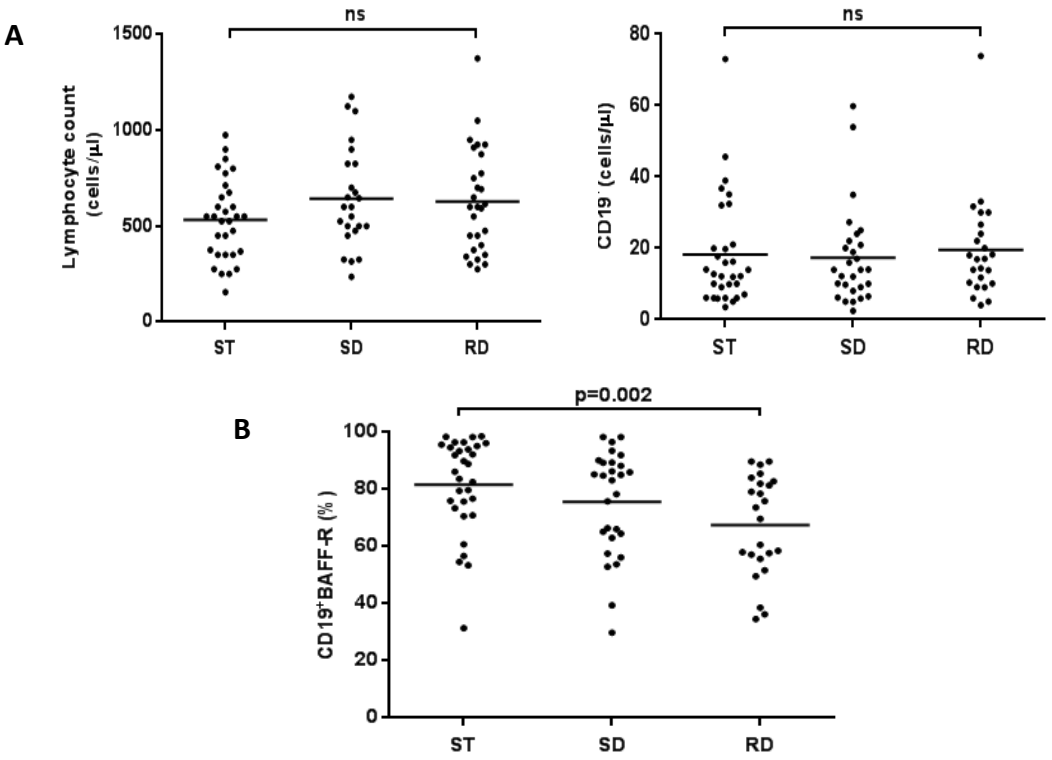


Figure 5.4 Expression of BAFF-R on $CD19^+$ cells is reduced in patients with deteriorating allograft function

$CD19^+$ cells isolated from PBMC were stained with anti-human monoclonal antibodies against CD19 and BAFF-R and analysed by flow cytometry.

A. Initial evaluation of lymphocyte and $CD19^+$ cell number showed minimal difference between patient groups; stable (ST; n = 31), slowly deteriorating (SD; n = 28) and rapidly deteriorating (RD; n = 24) graft function. There was no significant (ns) difference between groups by Kruskal-Wallis test.

B. Expression of BAFF-R was significantly reduced on $CD19^+$ cells from patients with RD graft function (p = 0.002) and not SD graft function (p= 0.15). P values derived by comparing ST to other groups by Mann-Whitney test.

The frequency of $CD19^+$ cells and expression of BAFF-R were examined to determine if increased sBAFF was related to a reduced numbers of B cells. Patients with $sBAFF^{high}$ had a significantly lower number and percentage of $CD19^+$ cells ($sBAFF^{high}$ cohort: n = 31; 2.43 ± 1.68%, 13 ± 7 cells/µl compared to $sBAFF^{low}$ cohort: n = 53; 3.65 ± 2.12%, 22 ± 16 cells/µl, p = 0.01 for both percentage and number; Figure 5.5A). Next, reduced expression of BAFF-R on $CD19^+$ cells was investigated to determine if there was a correlation with increased sBAFF. Patients were separated based on low or high percentage of $CD19^+BAFF\text{-}R^+$ cells; defined by the mean value (75.3%; n = 30). Serum BAFF levels in the $CD19^+BAFF\text{-}R^{low}$ group were not significantly higher ($CD19^+BAFF\text{-}R^{low}$ group, mean 1014 pg/ml (median 847, range 369-5947 pg/ml) vs. $CD19^+BAFF\text{-}R^{high}$ group, mean 816 pg/ml (median 749, range 231-2799 pg/ml); p = 0.17). Moreover, examination by linear-regression analysis did not reveal a significant correlation (R^2 = 0.09, p = 0.12; Figure 5.5B).

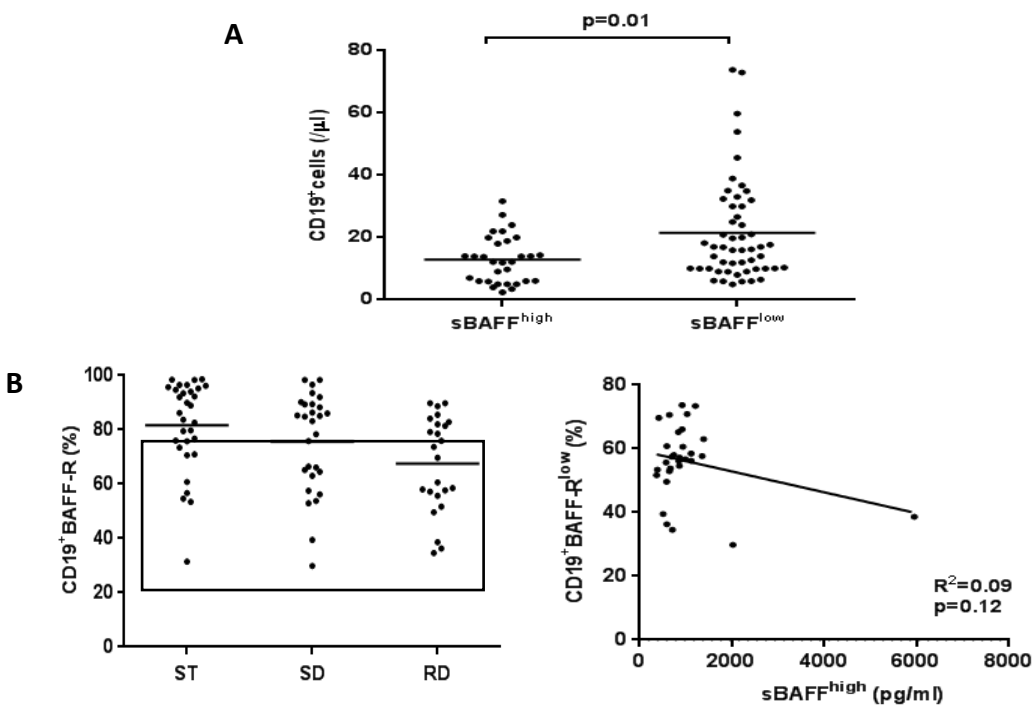


Figure 5.5 $CD19^+$ cells were reduced in patients with elevated sBAFF but there was no significant correlation with low expression of BAFF-R

A. Patients with elevated sBAFF (> mean value of 879 pg/ml; n = 31) had reduced numbers of $CD19^+$ cells compared to those with low sBAFF (n = 53; p = 0.01). P value derived by Mann-Whitney test.

B. Reduced frequency of BAFF-R on $CD19^+$ cells (< mean value of 75.3% shown in boxed area; n = 30) were assessed in conjunction with sBAFF levels. Analysis by linear regression did not support a statistically significant relationship.

More detailed phenotypic analysis of B-cell subsets and elevated sBAFF demonstrated a significantly lower distribution of naïve B cells ($CD19^+CD27^-IgD^+$) when compared to the $sBAFF^{low}$ cohort (30.9 ± 15.4% vs. 43.6 ± 18.2%, p=0.002, Figure 5.6). Memory B cells ($CD19^+CD27^+IgD^-$) were not significantly differently expressed between groups.

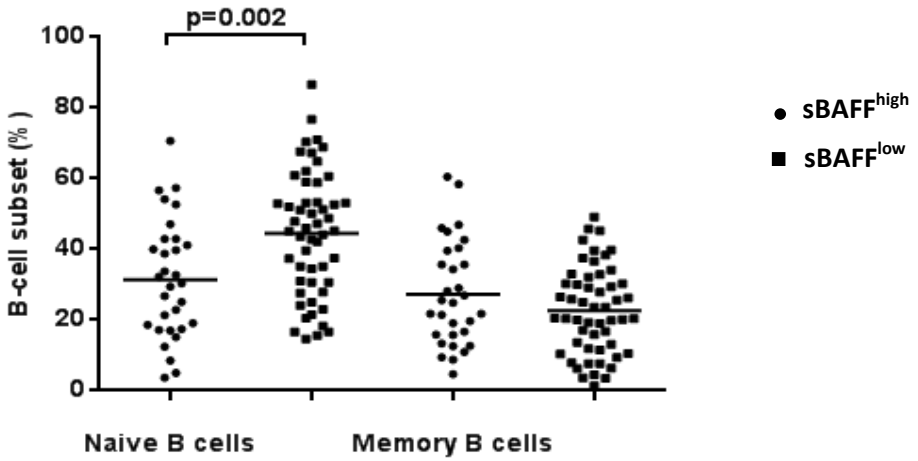


Figure 5.6 Phenotypic distribution of naïve and memory B cells altered with expression level of sBAFF

$CD19^+$ cells stained with anti-human monoclonal antibodies against CD19, CD27 and IgD were assessed by flow cytometry. Naïve B cells ($CD19^+CD27^-IgD^+$) were elevated in patients with low levels of sBAFF ($sBAFF^{low}$; n = 53) compared to patients with high levels of sBAFF ($sBAFF^{high}$; n = 31). There was no statistical difference in the frequency of memory B cells ($CD19^+CD27^+IgD^-$) between groups. P values derived by Mann-Whitney test.

Investigating if elevated serum BAFF is a bystander effect of raised serum creatinine

The correlation between elevated sBAFF and impaired renal function was investigated by analysing expression in different disease settings. Patients were selected on the basis of the presence or absence of a condition believed to be linked aetiologically to B-cell involvement: they were diagnosed with polycystic kidney disease (PKD; n = 21), SLE (without lupus nephritis, LN; n = 21) and with LN (n = 21). PKD is a cystic genetic disorder often resulting in chronic kidney disease (CKD). Without a direct immunological component, patients with already impaired renal function were to serve as the negative control group. Conversely, B cells contribute to the pathogenesis of SLE by the production of autoantibodies and acting as APC to present self-antigens to autoreactive T cells (Lipsky, 2001, Renaudineau et al., 2004). Patients with SLE were divided into two cohorts based on renal involvement (+/- LN) and served as positive-control groups. For the purpose of this comparative study, clinical manifestation of renal impairment for PKD and LN groups were based on sCr measurements.

Serum BAFF was significantly elevated in patients with SLE, irrespective of the presence or absence of nephritis (mean values, 377.4 ± 117.2, 812.1 ± 385.5 & 758.7 ± 344.2 pg/ml for PKD, SLE & LN groups, respectively; $p<0.0001$; Figure 5.7A). Indeed, renal function was worse in the PKD cohort, compared to the LN cohort, but sBAFF levels were still higher in the latter group (Figure 5.7A). The severity of PKD is classified in stages by level of renal function. Patients in this cohort were not separated by disease stage as reflected by the wide range of sCr measurements (mean, 231.4 µmol/L (range, 117-475); Figure 5.7B). Equally, the LN group had variable sCr due to an individual's disease state or effectiveness/compliance with treatment (mean, 122 µmol/L (range, 85-493); Figure 5.7B). However, when separated by renal function, the same correlation as seen with transplanted patients existed between elevated sBAFF and poor renal function (Figure 5.7C). This may represent correlation with either or both of degree of aggressiveness of immune response and renal dysfunction. Overall, both LN and PKD groups have renal impairment. However, unlike LN, PKD lacks

immune-related aetiology and the low sBAFF expression in these patients suggests elevated sBAFF is not merely a bystander effect of raised sCr, although it may make a contribution.

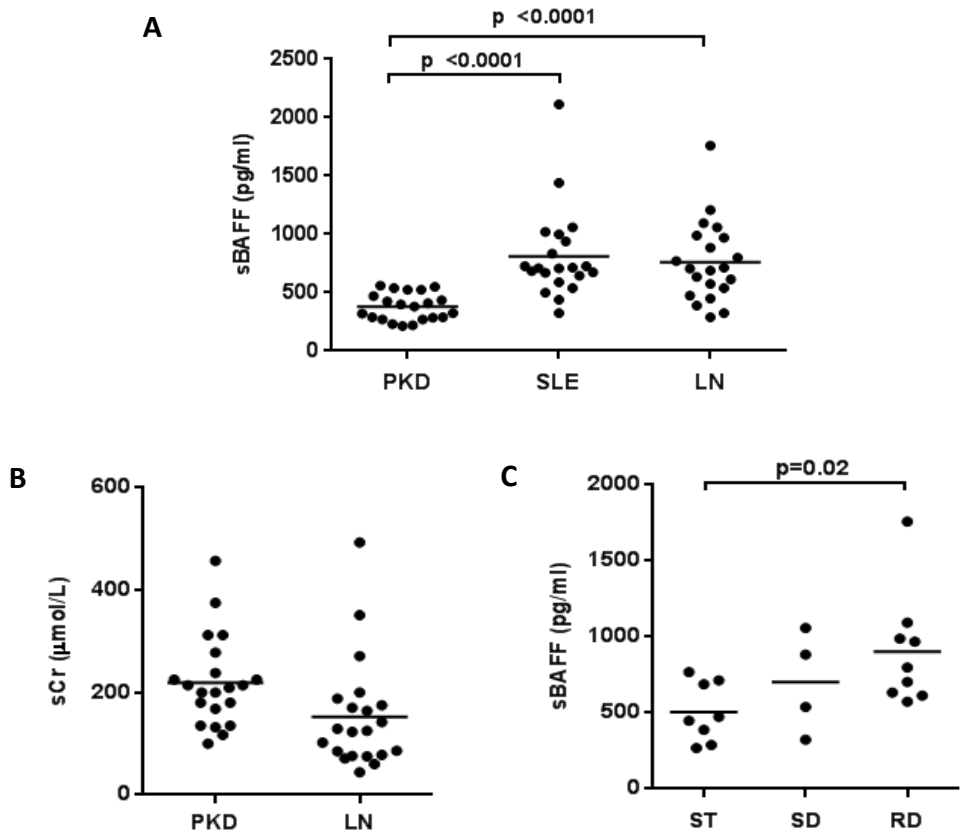


Figure 5.7 Elevated expression of sBAFF was not a bystander effect of raised serum creatinine

Three cohorts of patients were assessed for expression of sBAFF based on B-cell involvement in disease aetiology.

A. Dot-plot representing sBAFF levels measured by ELISA. Serum levels were significantly lower in patients with polycystic kidney disease (PKD; n = 21) compared to SLE (n = 21) and lupus nephritis (LN; n = 21). There was minimal difference in sBAFF levels for SLE patients with or without LN.

B. Dot-plot shows both PKD and LN patients had renal impairment based on raised serum creatinine (sCr) compared to the normal range (60-100 µmol/l), however only LN patients had elevated sBAFF levels (A).

C. Separating LN patients by renal function based on measuring eGFR over time, showed those with rapidly deteriorating (RD; n =9) renal function had elevated sBAFF compared to stable (ST; n =8) and slowly deteriorating (SD; n = 4) patients, analogous to what was seen in transplanted patients.

P values derived by Mann-Whitney test.

Immunohistochemistry of renal allograft biopsies

Cohorts of patients with and without AAMR were assessed for presence of B-cell infiltrates and associated expression of BAFF-R and soluble BAFF by immunohistochemistry using the same renal allograft tissue sections used previously to diagnose AAMR. Antibodies reactive to CD19, BAFF-R and BAFF were applied and initially optimised in tonsil tissue (Figure 5.8). A reliable staining pattern was established and, using an isotype-matched immunoglobulin, demonstrated a clear negative control for all antibodies. Transferring these methods to renal tissue (in which it can be difficult to clearly define antigen) required the use of multiple blocking methods, as detailed in the materials and methods, and the optimised method employed hydrogen peroxide, horse serum and an avidin-biotin block.

Intrarenal expression of CD19, BAFF-R and BAFF was examined in tissue sections from patients with (n = 16; Figure 5.9A) and without AAMR (n = 11; Figure 5.9B). Staining intensity was graded as a percentage of the core sections (Table 5.2). The density of $CD19^+$ cell infiltrates were variable among AAMR patients, associating with small populations of sub-capsular interstitial lymphoid aggregates and ranging from <1-10%. BAFF-R expression showed a similar distribution to $CD19^+$ cells, consistent with inflammatory cell accumulation. There was occasional expression on podocytes, mesangial cells and apoptotic bodies. In general, expression was lower than CD19. The distribution of BAFF was more diffuse, ranging from <1–20%. BAFF was noted to stain strongly with $CD19^+$ cell infiltrate and was also associated with podocytes, fibroblasts and mesangial cells with some tubular staining. Patients without rejection had a lower percentage of CD19 infiltrates (<1-2%), similarly BAFF-R (<1-3%) and soluble BAFF (<1-5%) which did not reach the level seen in some patients with AAMR. Overall those with AAMR appeared to show a higher staining intensity for CD19 (2.58 ± 2.61% vs. 1.67 ± 0.52%; p = 0.57), BAFF-R (2.3 ± 2.75% vs. 1.31 ± 0.8%; p = 0.99) and BAFF (4.36 ± 5.79% vs. 2.44 ± 1.4%; p = 0.69) compared to those without rejection (Figure 5.10A). However, there was wide variation in these data within groups and no statistical difference was demonstrated (Table 5.2).

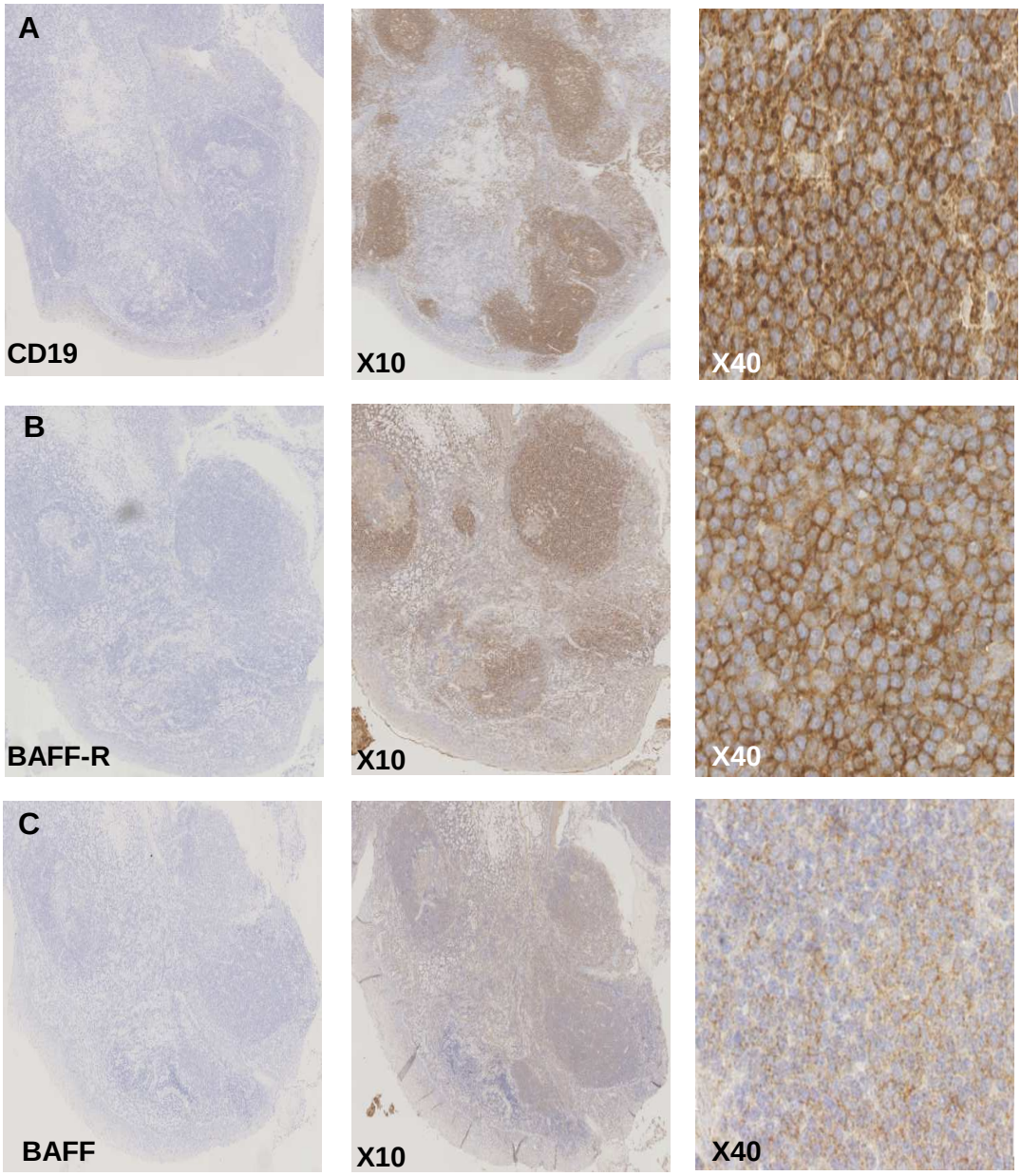


Figure 5.8 Optimising methods in tonsil tissue stained with CD19, BAFF-R and BAFF

Immunohistochemistry was performed with monoclonal antibodies directed against CD19 and BAFF-R, and a polyclonal antibody against soluble BAFF in tonsil tissue to optimise staining prior to application in renal biopsy material. The negative control is shown on the left of each set of images, followed by the stained sections at x 10 and x 40 magnification.

A. Positive-staining for CD19 was associated with dense regions of inflammatory infiltrates

B. Staining for BAFF-R showed a similar pattern to CD19, although less diffuse.

C. Soluble BAFF was not as prominent as the receptor; at higher magnification (x40) staining was not only confined to the cell membrane.

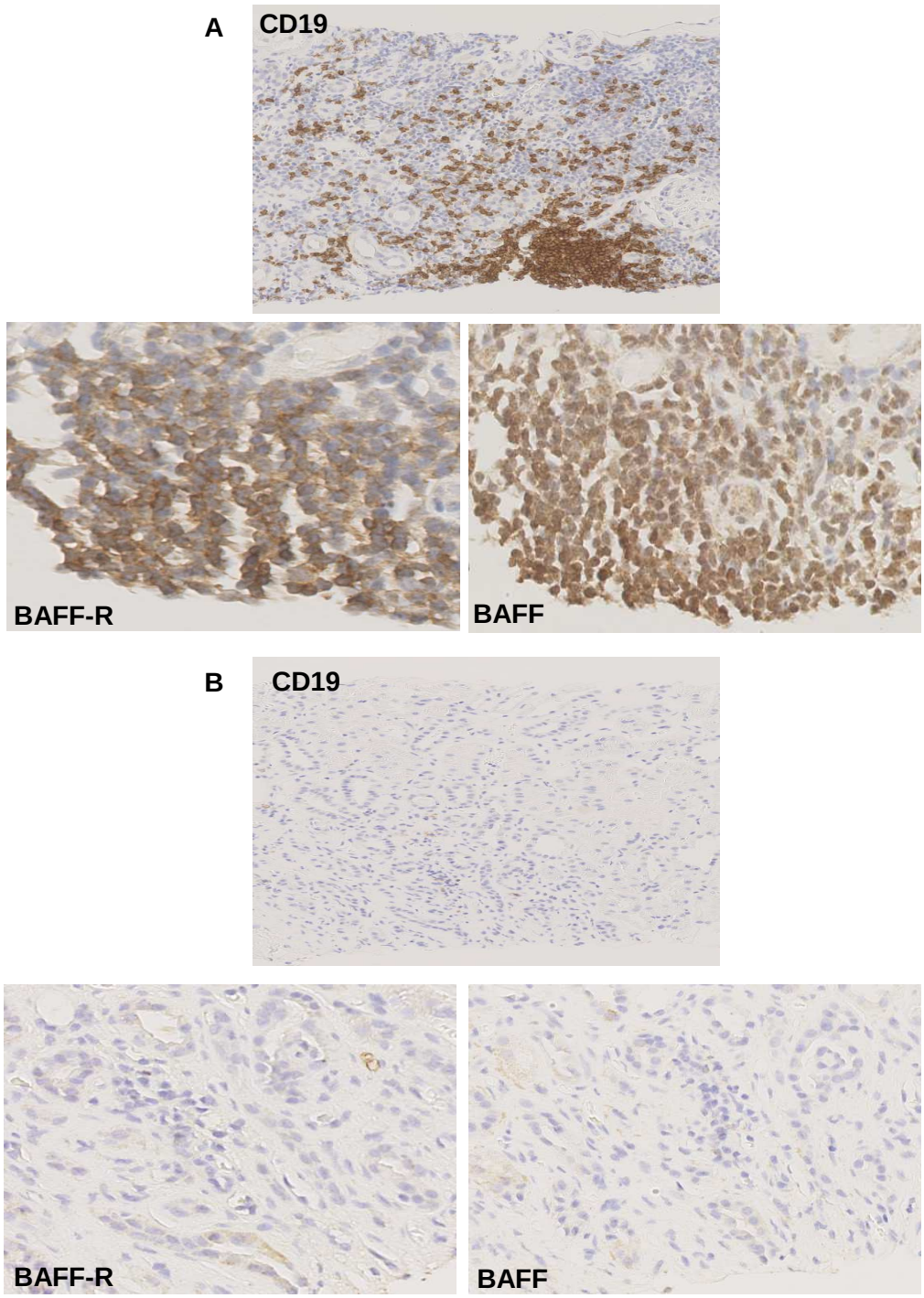


Figure 5.9 Localisation of lymphocyte aggregates within renal allograft tissue stained with CD19, BAFF-R and BAFF

Immunohistochemistry was performed with monoclonal antibodies directed against CD19 and BAFF-R and a polyclonal antibody against soluble BAFF in renal allograft tissue. Representative figures show a patient with AAMR (A) and a patient without evidence of rejection (B).

A. Application of a CD19 antibody showed positive staining in association with inflammatory infiltrates within the core. BAFF-R showed a similar pattern to CD19 with more diffuse distribution of BAFF.

B. A patient without rejection had no positively for CD19, BAFF-R or BAFF by immunohistochemistry.

	CD19 (%)	BAFF-R (%)	BAFF (%)	sBAFF (pg/ml)	DSA (%)
AAMR (n=16), mean **Range**	2.58 <1-10	2.3 <1-10	4.36 <1-20	758.71 142-2677	100
AAMR with BAFF^{low} (n=10 of 16) **BAFF^{low} = ≤2% intrarenal BAFF**	1.35	0.9	1.2	652.03	
AAMR with BAFF^{high} (n=6 of 16) **BAFF^{high} = >2% intrarenal BAFF** **P value compares AAMR with BAFF^{low} and BAFF^{high}**	3.25 **p =0.41**	2.83 **p=0.26**	8.33 **p=0.0001**	936.5 **p=0.77**	
Without AAMR (n = 11), mean **Range** **P value compares AAMR with patients without AAMR**	1.67 <1-2 **p=0.57**	1.31 <1-3 **p=0.9**	2.44 <1-5 **p=0.68**	622 142-1027 **p=0.9**	27

Table 5.2 Summarising intrarenal expression of CD19, BAFF-R and BAFF in patients with or without AAMR

Renal biopsy sections were stained with antibodies against CD19, BAFF-R and BAFF and examined by immunohistochemistry. The table shows mean values for staining intensity (%) for each antibody assessed. Serum BAFF (sBAFF) values are shown, together with presence of donor-specific HLA antibodies (DSA).

P values derived by Mann-Whitney test comparing AAMR group against those without AAMR and those with AAMR and low intrarenal BAFF (BAFF^{low}; ≤2%) against AAMR with high intrarenal BAFF (BAFF^{high}; >2%).

The AAMR group was investigated further by separation based on expression of BAFF; determined as greater or less than 2% staining intensity (Table 5.2). The staining intensity of CD19, BAFF-R and BAFF was compared in those classified as 'BAFF^{high}' or 'BAFF^{low}'. In each case, the percentage of staining was higher in the BAFF^{high} group (CD19: 3.25% vs. 1.35%, p=0.41; BAFF-R: 2.83% vs. 0.9%, p = 0.26 and BAFF: 8.33% vs. 1.2%, p = 0.0001 for BAFF^{high} vs. BAFF^{low} respectively). This was significantly so for intrarenal BAFF.

The relationship between serum and intrarenal BAFF was next examined. First, comparing groups, those with AAMR did not have especially higher levels of sBAFF (758.7 ± 656.1 pg/ml vs. 605.1 ± 275.3 pg/ml; p = 0.47). Comparing levels of intrarenal BAFF, patients with AAMR had elevated levels (3.9 ± 5.55% vs. 2.05 ± 1.4%; p = 0.29). As these data show, there was wide variation in expression of both serum and intrarenal BAFF.

By linear-regression analysis, there was a positive, albeit not quite statistically significant trend between intrarenal, sBAFF levels and AAMR (R^2 = 0.22, p = 0.067; Figure 5.10B). When separated by expression of high intrarenal BAFF (>2%), the level of sBAFF increased but again not significantly (937.5 pg/ml vs. 622 pg/ml; p= 0 .42).

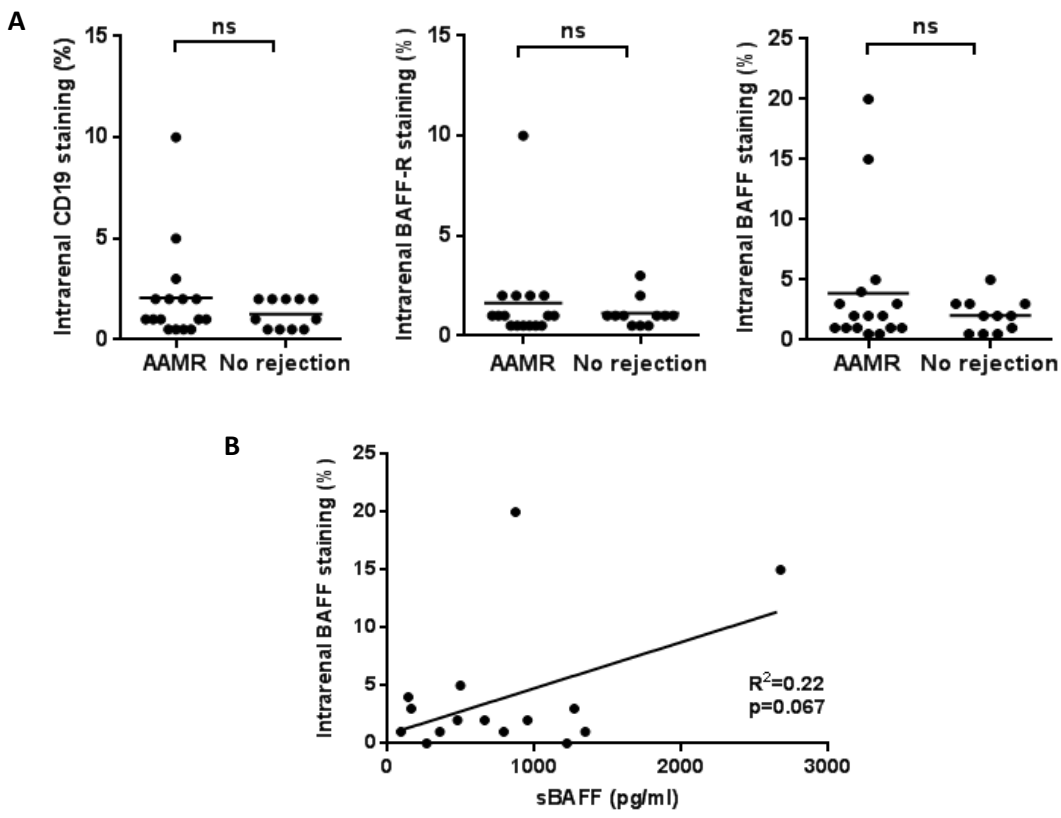


Figure 5.10 Increased intensity of staining for intrarenal CD19, BAFF-R and BAFF in patients with AAMR

A. Intensity of staining for CD19, BAFF-R and BAFF was graded as a percentage of the core biopsy. In each case, AAMR patients (n = 16) had higher expression levels than those without rejection (n = 11). ns is not significant by Mann-Whitney test.

B. Linear-regression analysis of serum BAFF (sBAFF) levels (pg/ml) and intrarenal BAFF expression (%) in patients with AAMR showed a positive trend, however a statistically significant correlation did not exist.

Clinical outcome and intrarenal expression of BAFF

Patients with AAMR exhibited variable intrarenal expression of CD19, BAFF-R and BAFF. In particular, BAFF expression ranged from 1-20%. Investigating the clinical outcome of those expressing more than 2% (median value) of the core section (n = 6), two patients had dnDSA compared to none of those with low intrarenal BAFF. Graft function was variable and one patient died with a functioning graft. Of those with low intrarenal BAFF, two patients suffered graft loss as a result of CAMR. As expected, all patients with AAMR had DSA, of those without AAMR, 27% had DSA and evidence of CAMR.

5.4 Discussion

BAFF is an important effector molecule of the B-cell compartment, responsible for regulating cell maturation and survival. Given this, it has been widely investigated in autoimmune disease where aberrant expression is proposed to enhance B-cell activity, increase auto-antibody production and exacerbate pathology (Vincent et al., 2012, Scholz et al., 2013). Similar investigations have been applied to transplant recipients and elevated BAFF levels reported during allograft rejection, (Xu et al., 2009b, Banham et al., 2013a) and the development of DSA (Thibault-Espitia et al., 2012). These studies begin to provide a rationale for measuring expression levels of BAFF to give information of graft function and potentially allow for more timely treatment of rejection, minimising irreversible graft damage and promoting allograft longevity.

This study measured sBAFF during the post-transplant period and associated elevated levels with deteriorating (slow and rapid) graft function, together with a prevalence of DSA and AMR. Moreover, a significant correlation existed between the level of sBAFF and degree of functional allograft deterioration. The comparable B-cell numbers between groups suggests BAFF production is independent of B-cell frequency and supported by Bloom and colleagues who report increased levels in alemtuzumab-treated patients were not altered by frequency of $CD20^{+}$ cells (Bloom et al., 2009). These data are in contrast to the close relationship reported by others where B-cell numbers decrease in response to BAFF neutralisation (Batten et al., 2000, Gross et al., 2000). However, these murine studies do not reflect the altered B-cell compartment in immunosuppressed transplant recipients. Moreover, DSA was more prevalent in those with high sBAFF where patients are most likely received intravenous immunoglobulin treatment; further altering B-cell numbers.

Binding of BAFF to BAFF-R has a critical role in B-cell homeostasis by preventing apoptosis (Schneider, 2005). Patients with rapidly deteriorating graft function showed BAFF-R was down-regulated and this may account for an excess of unbound BAFF. While elevated BAFF was associated with low expression of BAFF-R in some patients,

this was not a uniform observation. Elevated BAFF may promote survival of alloantibody-producing B cells. In autoimmunity, the generation of B cells in a micro-environment rich in BAFF leads to increased autoreactive B cells by failing to respond to cell-death-promoting factors (Lesley et al., 2004). Similarly, in mice, over-expression of BAFF made B cells less susceptible to anti-CD20-mediated depletion (Gong et al., 2005). It is possible that elevated BAFF levels promote alloreactive B-cell development and decrease susceptibility to elimination and may explain predominance of DSA in those with high sBAFF and absence in those with reduced BAFF levels.

The role of BAFF in supporting the survival of B-cell subsets was investigated by phenotypic analysis of naïve, memory and transitional B cells. In conjunction with signalling through the BCR, BAFF is demonstrated to mediate survival of transitional B cells (Batten et al., 2000, Goodnow et al., 2005). The expression of BAFF-R on IL-$10^{+}CD24^{high}CD38^{high}$ cells were investigated (described in Chapter 6). In brief, this small cohort demonstrated very high expression of BAFF-R on transitional B cells in those with stable and slowly deteriorating graft function and to a lesser degree in those with rapidly deteriorating graft function. Assuming BAFF is bound with BAFF-R on these transitional B cells, this interaction may favour their survival as observed in a murine model of autoimmunity where over-expression of BAFF alters the process of B-cell maturation, promoting survival of transitional B cells (Mackay et al., 1999).

Memory B cell frequency increased in patients with elevated BAFF. In mice, BAFF has been demonstrated to be essential for formation of splenic germinal centres (Yan et al., 2000) and in human $CD27^{+}$ memory B cells, BAFF enhances their survival and promotes generation of plasma cells *in vitro* (Avery et al., 2003). This supporting role in survival could explain why memory B cells increase in patients with elevated BAFF. Increased BAFF could potentially break immune tolerance during B-cell maturation, providing a critical role in promoting the humoral immune response.

Measurement of sBAFF as a reliable marker of B-cell mediated allograft dysfunction is reliant on both accuracy and specificity and a number of parameters were assessed to

address this. Firstly, in transplanted patients factors can affect allograft function such as infection, drug nephrotoxicity and malignancy, potentially altering expression of BAFF. Here, those with elevated sBAFF or low BAFF-R did not have any clinically remarkable events at the time of sample procurement as measured by C-reactive protein (CRP) to contribute toward raised sBAFF. However excluding interfering factors in sBAFF measurements requires further investigation. Secondly, the possible bystander effect consequential of raised sCr was examined using patient samples with PKD; a disease modality absent of B-cell involvement in aetiology in parallel to SLE; a disease where elevated sBAFF is established (Cheema et al., 2001, Morimoto et al., 2007). Raised sBAFF was not seen in PKD patients compared to those with SLE both with and without nephritis. It may have been expected that sBAFF be more pronounced in conjunction with nephritis. However, a similar study investigating sBAFF levels in SLE patients with/without nephritis did not find a significant association (Eilertsen et al., 2011). Examining renal function in LN patients supported the correlation found in the transplanted cohort of elevated sBAFF and worsening allograft function. Future studies would benefit measuring the impact renal function has on sBAFF levels using eGFR data in patients with a non-immunological disease, in this case the PKD population. Finally, this study attempted to address if sBAFF increased prior to the elevation of sCr, which would offer its application as an early marker of deteriorating function. Unfortunately too few samples were available over the duration of allograft survival to draw any conclusions and this requires further investigation.

To address the reliability of BAFF as a marker of B-cell activity in renal injury, histological analysis of biopsy material was performed. In this cohort, a number of patients with AAMR had significant infiltrate of $CD19^{+}$ cell aggregates. Expression of BAFF-R and BAFF accumulated at these areas, suggesting the predominant source of BAFF to be inflammatory cells infiltrating the graft. Comparing intensity of staining for CD19, BAFF-R and BAFF to those without AAMR, although greater did not reveal any statistically significant difference, which is in part due to extremes of staining intensity. When the AAMR cohort were separated based on higher expression of intra-renal

BAFF, an interesting observation was the presence of dnDSA noted in a third of these patients; absent in those with low intrarenal BAFF. Although in terms of clinical outcome there was nothing remarkable in this, dnDSA are attributed with increased risk of allograft failure, (Wiebe et al., 2012) and heighten B-cell activity may contribute toward this. This study described a very small number of patients and long-term follow-up of those with prominent $CD19^{+}$ cell infiltrate, associated with BAFF-R and BAFF in a larger cohort is required. Of final note, a correlation was found between increasing intensity of intrarenal BAFF and sBAFF. This did not quite reach a level of statistical significance but the principle of association is important as it provides evidence that elevated sBAFF levels are transferable to patients with active humoral rejection identified in the allograft. This is further supported by the correlation of DSA and elevated sBAFF. Collectively these data provided evidence of BAFF during AAMR and in the presence of alloantibody supporting the notion of a non-invasive alternative to performing biopsy, carrying no risk to the patient or the inherent issue of variability of sample size, and quality or lack of injured tissue. Clinically the application of a BAFF-detecting ELISA would be easy as it is already a well-established method and measuring sBAFF levels could indicate potential for developing AMR. This may be particularly beneficial in CAMR, most frequently diagnosed when a graft is failing such that when biopsy is performed damage to the allograft is already apparent and intervention with treatment may be too late to reverse lesions. Equally useful could be monitoring sensitised patients to assess effectiveness of antibody-removal treatment.

Concluding remarks

B cells are implicated in the pathogenesis of transplant rejection through production of alloantibody. Elevated BAFF may support this by promoting survival of B cells, potentially allowing alloreactive cells to manifest, leading to B-cell-mediated pathology and graft dysfunction. The activity of B cells post-transplant is not measured. Here elevated sBAFF, low expression of BAFF-R and DSA correlated with deteriorating graft function. Performing immunohistochemistry provided direct evidence of BAFF associated with infiltrating B cells within allografts of patients with AAMR. Together,

these data demonstrated BAFF as a potential biomarker of dysregulation within the B-cell compartment and of humoral mediated allograft rejection.

Chapter 6

Characterising and investigating functional capabilities of regulatory B cells in renal transplant recipients

6.1 Introduction

The presence and role of B cells with suppressive capacity have not been widely explored following transplantation. Research primarily focuses on their position in autoimmunity where they are believed to regulate disease progression, since depletion, in some cases, leads to exacerbation of symptoms. This regulatory function is partially mediated through secretion of IL-10 and a number of methods are described to activate production by peripheral B cells. In general, this is achieved via TLR stimulation with CpG and CD40 ligation, both with (Bouaziz et al., 2010) or without BCR engagement (Iwata et al., 2011). Following activation, intracellular expression of IL-10 can be identified at the single cell level by flow cytometry following a short stimulation with phorbol myristate acetate (PMA), ionomycin and monensin. PMA activates a protein kinase pathway, triggering calcium release and a cascade of cellular responses to mediate cytokine production (Truneh et al., 1985). Used in conjunction with PMA is ionomycin, an ionophore produced by *Streptomyces conglobatus* to transport ions across the lipid bilayer. In this context, it is utilised to stimulate production of intracellular cytokines. During the last stages of stimulation monensin is added to prevent cytokines exiting the cell by inhibiting their transport across the endoplasmic reticulum and preventing export to the Golgi complex resulting in enhanced detection. Employing these methods has allowed IL-10-producing B cells to be identified at relatively low frequency. In peripheral blood of healthy controls the reported range is between 1 and 7% (Bouaziz et al., 2010, Iwata et al., 2011, Blair et al., 2010, Das et al., 2012).

An obstacle in studying regulatory B cells (Bregs) is the lack of a unique surface phenotype, and a consensual view is yet to be achieved. A number are ascribed to human B cells with regulatory function which include $CD19^{+}CD24^{high}CD38^{high}$ (Blair et al.), $CD19^{+}CD24^{high}CD27^{+}$ (Iwata et al., 2011) and $CD19^{high}CD1d^{high}CD5^{+}$ 'B10' (Yanaba et al., 2008). There does appear to be agreement that IL-10-producing cells are enriched in the $CD24^{high}CD38^{high}$ B-cell compartment (Blair et al., 2010, Flores-Borja et al., 2013, Bouaziz et al., 2010). This was initially described as a suppressive B-cell phenotype lacking in patients with SLE, (Blair et al., 2010) and has since been included in a B-cell

signature of tolerance in renal transplant recipients, those with stable graft function in the absence of immunosuppression (Newell et al., 2010). Here tolerance was linked with increased frequency of $CD24^{high}CD38^{high}$ transitional B cells expressing IL-10, and also naïve B cells. In a separate study, tolerant allograft recipients displayed a distinct phenotype with increased expression of inhibitory molecules and expanded activated and memory B cells (Pallier et al., 2010). These data suggest regulatory B cells reside in committed subsets and importantly their frequency is positively associated with good graft function.

IL-10-producing B cells can be characterised by expression of cell-surface markers creating a detailed, if not unique phenotype of these cells. This can aid identification and allow potential numerical deficits in disease settings to be revealed. The functional role of these cells to control effector immune response, in addition to secretion of IL-10, is dependent on direct contact with $CD4^+$ T cells (Mauri et al., 2003, Blair et al., 2010, Mizoguchi et al., 2002, Kessel et al., 2012). The anti-inflammatory effects exerted on the suppression of $CD4^+$ T-cell differentiation, specifically T helper 1 (Th1) cells responsible for producing pro-inflammatory cytokines can be measured *in vitro*. Activated $CD4^+$ T cells can be defined as Th1 or Th2 cells based on their pattern of cytokine expression (Mosmann et al., 1986). Th1 cells predominately secrete pro-inflammatory cytokines, IFN-γ, TNF-α and lymphotoxin, but also IL-2 involved in cell-mediated immunity. Th2 cell cytokines include IL-4, IL-5 and IL-13 associated with the humoral response, and IL-10 with anti-inflammatory effects (Zhu et al., 2010). *In vivo* $CD4^+$ T cells are activated from naïve T cells following binding by specific antigen. This requires two signals: first engagement of the T cell receptor (TCR) with an antigen presented in association with the MHC receptor complex; the second follows ligation of co-stimulatory receptors – CD28 on T cells and CD86 on antigen-presenting cells (June et al., 1994) – resulting in cytokine production, cytokine receptor expression and proliferation of activated T cells. *In vitro*, naïve T cells can be non-specifically activated in the absence of antigen by a number of agents. This is usually achieved by direct cross-linking of the TCR on a large proportion of responder cells with anti-CD3 antibodies bound to a tissue culture plate. Although this method does not require the

presence of accessory molecules, the response may be suboptimal, (Jenkins et al., 1990) and combining with CD28 can enhance T-cell activation and proliferation (Lenschow et al., 1996). This method has been used to evaluate regulatory activity of $CD19^{+}CD24^{high}CD38^{high}$ cells (Blair et al., 2010). Co-culture of these cells with naïve T cells reduced the frequency of pro-inflammatory cytokines, TNF-α and IFN-γ produced by Th1 cells. This analysis was then applied to patients with SLE who lacked the suppressive capacity seen in healthy controls. It is possible that this type of suppression is impaired in those with poor graft function post-transplantation.

In response to contact with T cells, B cells produce cytokines. Secretion can also occur constitutively, in response to antigen presentation or TLR stimulation, all of which can indirectly modulate T-cell behaviour (Lund, 2008, Lund et al., 2005). The commitment of naïve T cells to become 1) effector Th1 and Th2 cells, 2) protective regulatory T cells (Tregs), or 3) cytopathic Th17 cells is influenced by the cytokine environment (Strom and Koulmanda, 2009). Th1 cell cytokines, TNF-α, IL-1β and IL-6 can promote differentiation to the damaging Th17 phenotype (Acosta-Rodriguez et al., 2007) preventing commitment to Tregs. (Bettelli et al., 2006). $CD19^{+}CD25^{high}CD27^{high}CD86^{high}CD1d^{high}CD10^{high}TGF\text{-}\beta^{high}$ cells, defined as Bregs can enhance expression of Foxp3 and CTLA-4 on Tregs and supress proliferation of $CD4^{+}$ T cells which interestingly was dependent not on IL-10 but on TGF-β. (Kessel et al., 2012). This suggests although IL-10 is regarded as pivotal in regulatory cells inhibiting pro-inflammatory responses, their cytokine production may extend beyond this. Indeed a range of other cytokines produced by B cells demonstrates immune regulation. For example, IL-12 can promote Th1 differentiation, IL-4 induces Th2 maturation, and IL-6 and TGF-β are important in Th17 cell development. (Hamze et al., 2013). Furthermore IL-6, TNF-α and IL-10 can indirectly modulate T-cell responses through interactions with macrophages (Wong et al., 2010). Evidence of suppressed Th1 cell cytokines by $CD19^{+}CD24^{high}CD38^{high}$ cells (Blair et al., 2010) may have implications following transplantation due to their association with allograft rejection. Prominent are IL-6, implicated in cardiac allograft rejection, (Diaz et al., 2009) and TNF-α and IL-1β in chronic renal allograft rejection (De Serres et al., 2011). An additional

cytokine important in regulating B-cell survival and maturation is BAFF (B-cell activating factor). Studies in animal models suggest this may also be essential in development or promotion of Bregs. In BAFF-deficient mice there is impaired maturation of transitional B cells, (Schiemann et al., 2001) and BAFF-transgenic mice promote development of Tregs (Walters et al., 2009). Moreover, BAFF induced the differentiation of IL-10-producing B cells capable of suppressing T-cell proliferation and Th1 cytokine production, and their *in vivo* transfer inhibited arthritis (Yang et al., 2010). The significance of BAFF and other cytokines in elevation of Bregs, and during their interaction with $CD4^+$ T cells following renal transplantation to advocate a suppressive or regulatory response has yet to be addressed.

In summary, research into human regulatory B cells has yielded diverse results and in the absence of a definitive phenotype it is difficult to unify a coherent function. However it is clear that, in the context of autoimmunity and observations in tolerant renal transplant recipients, B cells play an important role in maintaining and controlling the immune response.

The purpose of this study was to identify IL-10-producing B cells, further define association with naïve, memory and transitional B cells and, progressing beyond the phenotypic level, to determine if regulation resides in $CD19^+CD24^{high}CD38^{high}$ cells by assessing suppression of Th1 cell pro-inflammatory cytokines and explore secreted products during contact with $CD4^+$ T cells. Collectively findings are related to the level of renal allograft function.

6.2 Materials and methods

Materials and methods detailed in Chapter 2, General Methods (GM) are indicated.

GM1 Recruitment of study patients

GM2 Immunosuppressive regime

GM3 Defining level of allograft function in patient cohorts

GM4 Peripheral blood mononuclear cells (PBMC) separation

GM5 $CD19^+$ cell isolation from PBMC

GM6 Phenotyping $CD19^+$ cells by flow cytometry

GM7 Polyclonal agonists applied during $CD19^+$ cell culture

GM8 Detection of HLA antibody in serum by Luminex technology

GM10 Statistical analysis

$CD19^+$ cell culture

$CD19^+$ cells (1 x 10^6/ml) in RPMI-1640/10% FCS with 5% L-glutamine, 5% penicillin and 5% streptomycin (complete medium) were cultured with TLR agonists to induce proliferation and differentiation (GM 7, Table 2.2). This included R848 and IL-2, CpG alone or in combination with CD40L and anti-human IgG-IgM-IgA (anti-Ig). Cells were added at 100 µl/well to a 96-well U-bottom plate (Becton Dickinson; BD) and incubated at 37 ^oC in 5% CO_2 for five days.

Stimulating CD19$^+$ cells for intracellular cytokine detection

Post-culture cells were transferred to FACS tubes (BD), washed, resuspended in 800 µL complete medium and stimulated for five hours with phorbol 12-myristate 13-acetate (PMA, 50 ng/ml; Sigma-Aldrich, UK) and ionomycin (500 ng/ml; Sigma-Aldrich). Brefeldin A (3 µg/ml; ebioscience, USA) and monensin (2 µM; ebioscience) were added for the last two hours. Tubes were centrifuged to collect the cells, the medium discarded, cells resuspended and washed in 1 ml FACS buffer (PBS/2% FCS).

Post-culture extracellular staining of CD19$^+$ cells for analysis by flow cytometry

Following stimulation for intracellular cytokine analysis, cells were resuspended in 50 µl FACS buffer and stained with the B-cell panel of antibodies as described in GM 6, Table 2.1.

Post-culture intracellular staining of CD19$^+$ cells for analysis by flow cytometry

Following extracellular staining, cells were washed in 1 ml FACS buffer, resuspended in 100 µl fixation buffer (4% formaldehyde; ebioscience) and incubated in the dark with shaking for 20 minutes at room temperature. Cells were washed and resuspended in 50 µl permeabilisation buffer (0.1% saponin; ebioscience) with rat anti-human IL-10-APC (0.5 µg/ml; Clone JES3-19F1, BD Biosciences) or for the negative control, a blocking antibody, rat anti-human IL-10 (0.5 µg/ml; Clone JES3-19F1, BD Biosciences), for 20 minutes in the dark at room temperature. The negative control was re-stained with anti-human IL-10-APC (0.5 µg/ml) to confirm efficacy of the blocking antibody. Cells were washed with 1ml permeabilisation buffer, resuspended in 500 µl fixation buffer and analysed on the flow cytometer.

CSFE staining of CD19+ cells

100 µL of $CD19^+$ cells (1 x 10^6/ml) were incubated with 5 µM CFSE (Molecular Probes, Life Technologies) in 1 ml FCS at 37 ^oC for six minutes with mixing every two minutes. Cells were quenched with 5 ml of ice-cold FCS for five minutes, washed and quenched again to remove any remaining CFSE. Cells for culture were resuspended in complete medium. 100 µl of stained cells were analysed at day zero and day five by flow cytometry.

Functional assays – CD4+T: B-cell co-cultures

PBMC and CD19+ cell isolation

PBMC were isolated from peripheral whole blood (40 ml) within four hours of venesection as detailed in GM 4. For studies using the whole $CD19^+$ cell fraction, CD19 cells were isolated by magnetic bead separation as detailed in GM 5. The positively selected $CD19^+$ cells were retained and the negative fraction was utilised to isolate $CD4^+CD25^-$ naïve T cells. For cell-sorting experiments, PBMC (5 x 10^6 cells) were reserved for naïve T-cell isolation.

Cell-sorting by Fluorescently Activated Cell Sorting (FACS)

For cell-sorting experiments by FACS, PBMC were resuspended in 1 ml FACS buffer. 20 µl was removed for isotype & single antibody-stained controls and the remaining stained with CD19-APC-H7, CD24-PerCP 5.5 and CD38-V450 for 30 minutes in the dark at room temperature. Cells were washed and resuspended in FACS buffer before analysis using a FACS Aria (BD Biosciences). Voltages and compensation parameters were set using the controls and $CD19^+$ cells selected from the total lymphocyte population. From $CD19^+$ cells, CD24 & CD38 cells were gated to distinguish three populations: transitional ($CD19^+CD24^{high}CD38^{high}$), memory ($CD19^+CD24^{high}CD38^-$) and mature naïve cells ($CD19^+$ $CD24^+CD38^+$). FACS cells were collected under sterile

conditions into FACS tubes containing complete medium with FCS at 20%. At the end of the sort, cells were washed and resuspended in complete medium prior to cell culture.

Naïve T-cell isolation

Naïve T cells ($CD4^+CD25^-$) were isolated by magnetic-bead purification using naïve $CD4^+$ T cell microbeads (Miltenyi Biotec) as per manufacturer instructions. Briefly, PBMC (5 x 10^6 cells) were resuspended in 80 µl MACS buffer (PBS/0.5% FCS/0.05% EDTA), 10 µl microbeads and incubated for 20 minutes at 4 °C in a 15 ml Falcon tube. Cells were washed with 2 ml MACS buffer, resuspended in 80 µl MACS buffer and 20 µl biotin microbeads and incubated again for 20 minutes at 4 °C. Cells were washed in 2 ml MACS buffer and applied to a pre-prepared column (MS column; Miltenyi Biotec) on a magnetic plate to retain cells bound to the microbeads. The labeled PBMC suspension was allowed to run completely through the column before being washed three times with 500 µl MACS buffer. The negative fraction contained naïve T cells. After the flow through was complete, cells were washed and resuspended in complete medium at a concentration of 1 x 10^6/ml.

$CD4^+$ T-cell suppression assay

Pre-culture preparation for naive T-cell activation by CD3 antibody

100 µl of anti-CD3 (10 µg/ml; clone HIT3a; BD Biosciences) in PBS was applied to a 96-well round bottom microplate (BD) and incubated at 37 ^oC for a minimum of two hours. Immediately prior to naïve/B-cell subset transfer, the antibody solution was expelled from the plate and wells washed twice with PBS. After each wash the plate was inverted and blotted against clean paper towels to remove residual liquid.

Naïve T-cell co-culture with CD19, CD24, CD38 subsets

$CD19^+$ cells or $CD19^+CD24^{high}CD38^{high}$; $CD19^+CD24^{high}CD38^-$; $CD19^+$ $CD24^+CD38^+$ FACS populations (1 x 10^6/ml) were cultured 1:1 with $CD4^+CD25^-$ cells (1 x 10^6/ml). $CD4^+CD25^-$ cells without $CD19^+$ cells or $CD19^+$ subsets served as positive controls. Cells were cultured for 96 hours at 37 ^oC in 5% CO_2.

Post-culture analysis

Cytokine detection in culture supernatant

Supernatant was removed from culture wells without disturbing the cell pellet and stored at -20 ^oC until required.

Stimulating cells $CD4^+$ T cells for intracellular cytokine detection

Post-culture cells were stimulated for intracellular cytokine analysis previously described.

Extracellular staining of $CD4^+$ T cells for analysis by flow cytometry

Following stimulation for intracellular cytokine analysis, cells were resuspended in 50 µl FACS buffer and stained with mouse anti-human CD4-eFluor 450 (0.2 µg/ml, Clone SK3; eBioscience) and CD69-FITC (0.2 µg/ml, Clone FN50; BD Biosciences) or isotype controls (eFluor 450 & FITC Mouse IgG1, κ) as previously described.

Intracellular staining of $CD4^+$ T cells for analysis by flow cytometry

Following extracellular staining, cells were prepared for intracellular cytokine analysis as previously described. Expression of TNF-α-PE (0.5 µg/ml, Clone MAb11; BD Biosciences) and IFN-γ- APC (0.5 µg/ml, Clone B27; BD Biosciences) were investigated.

Cytokine detection in serum and culture supernatant

IL-10 detecting ELISA

IL-10 in serum and culture supernatant was investigated by ELISA following the manufacturer's instructions (DuoSet ELISA System; R&D Systems). In brief, a 96-well flat-bottom microplate (BD Biosciences) was coated with 100 µl per well of mouse anti-human IL-10 antibody (2 µg/ml), sealed with paraffin film and incubated at room temperature overnight. Next day, the plate was washed three times with wash buffer (PBS/0.05% Tween 20). After each wash the plate was inverted and blotted against clean paper towels to remove any remaining wash buffer. A blocking solution (PBS/1% BSA) was applied and incubated at room temperature for one-hour. The plate was washed as outlined above and positive controls (recombinant human IL-10) prepared by serial dilution (decreasing from 2000 pg/ml) or samples (1:1 with PBS/1% BSA) applied to the plate, covered with paraffin film and incubated in the dark for two hours at room temperature. The plate was washed and biotinylated goat anti-human IL-10 (150 ng/ml) added to each well before being sealed and incubated for two hours. The plate was decanted again, washed and 100 µl streptavidin conjugated to horseradish-peroxidase (HRP) reagent applied to each well for 20 minutes in the dark at room temperature. After washing, the substrate solution (1:1; H_2O_2: Tetramethylbenzidine) was applied for 20 minutes in the dark at room temperature. This reaction was stopped with sulphuric acid (2N H_2SO_4) and the optical density (OD) of the developed colour analysed using a microplate reader (Labtech) set to 450 nm with a correction value of 540 nm. Data were exported to Excel and using the concentration of serially diluted positive controls against absorbance generated a linear regression curve used to estimate the concentration of IL-10 in the samples.

Human cytokine array assay

Identification of cytokines in the B-T cell co-culture supernatant employed a Proteome Profiler, Human Cytokine Array assay (Panel A; R&D systems). This enabled detection of 36 cytokines and chemokines (anti-C5a, CD40 Ligand, G-CSF, GM-CSF, GRO, I-309, sICAM-1, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, p70, IL-13, IL-16, IL-17, IL-17E, IL-23, IL-27, IL-32α, IP-10, I-TAC, MCP-1, MIF, MIP-1α, MIP-1β, Serpin E1, RANTES, CXCL12, TNF-α, sTREM-1) impregnated onto a nitrocellulose membrane. The method followed the manufacturer's instructions. In brief, 500 µl of a blocking buffer was applied onto the membrane in a four-well multiplate and incubated at room temperature on a rocking platform for one-hour. Culture supernatant was centrifuged to collect cell debris and the upper fraction, together with the antibody detection cocktail (supernatant-antibody mixture) was incubated at room temperature for one-hour. After incubation, the blocking buffer was removed from each membrane, the supernatant-antibody mixture applied, and the membranes incubated overnight at 4 ^{o}C on a rocking platform. Next day, membranes were removed and washed on a rocking platform; this was repeated for a total of three washes. The membranes were removed; excess buffer allowed to drain and placed back into the cleaned multiplate with streptavidin-HRP reagent (1:200) and incubated for 30 minutes at room temperature on a rocking platform. The membranes were washed again as described. After the third wash, each membrane was removed, allowed to drain and placed on a plastic sheet held in an autoradiography cassette. Chemi Reagent Mix was applied onto the membrane and covered with a plastic sheet. After one minute, excess Chemi Reagent Mix and any air bubbles between the plastic sheets were removed with smoothing. The membranes were exposed to X-ray film in a dark room and level of spot development assessed. Generally, one to three minutes was sufficient time. The X-ray film was scanned and the images analysed using Image J software (National Institutes of Health).

6.3 Results

Identification of IL-10-producing B cells

To evaluate if numbers of IL-10-producing B cells are associated positively with stable graft function, three groups of patients were investigated: those with stable (ST; n=5); slowly deteriorating (SD; n=5), and rapidly deteriorating (RD; n=6). The demographic and clinical characteristics of patients are shown in Table 6.1.

	Stable (ST)	**Slowly deteriorating (SD)**	**Rapidly deteriorating (RD)**
Total number of patients, n (%)	5 (31.3)	5 (31.3)	6 (37.5)
Recipient			
Gender (F,M)	1,4	1,4	1,5
Age, mean (range; years)	45 (31-57)	45 (33-52)	43 (32-55)
Time post-transplant, mean (range; years)	6 (1-10)	13 (1-24)	11 (3-23)
***Type of allograft*, n (%)**			
Live donation	3 (60)	2 (40)	2 (30)
DBD	2 (40)	3 (60)	4 (70)
***HLA mismatches, n* ± sd**			
A, mean ± sd	1.2 ± 0.45	0.75 ± 0.96*	1.5 ± 0.55
B, mean ± sd	1.4 ± 0.55	1 ± 0.82*	1.2 ± 0.41
DR, mean ± sd	0.8 ± 0.84	0.5 ± 0.58*	0.33 ± 0.52
HLA antibody - post-transplant, n (%)			
Negative	3 (60)	4 (80)	3 (50)
NDSA	2 (40)	1 (20)	1 (20)
DSA	0	0	2 (30)
Cause of ESRF, n (%)			
IgA nephropathy	0	0	1 (16.7)
FSGS	0	0	1 (16.7)
GN	4 (80)	1 (20)	1 (16.7)
Hypertension	0	0	1 (16.7)
Other/Unknown aetiology	1 (20)	4 (80)	2 (33.3)

Table 6.1 Demographics of patient groups separated by allograft function
Abbreviations: DBD, donation after brainstem death; DSA, donor-specific HLA antibody; NDSA, non-donor-specific HLA antibody; ESRF, end-stage renal failure; FSGS, focal segmental glomerulosclerosis; GN, glomerulonephritis; sd, standard deviation. *Donor type missing for one patient.

Phenotypic analysis of peripheral blood in study groups

Total lymphocyte and CD19⁺ cell populations

The total PBMC count was assessed following lymphocyte isolation by density-gradient centrifugation of peripheral whole blood within four hours of venesection (Figure 6.1). Minimal differences existed between groups (450 ± 169 cells/µl; 570 ± 84 cells/µl; and 558 ± 198 cells/µl; p = 0.44, for ST, SD and RD groups respectively). Following PMBC isolation, $CD19^+$ cells were positively selected by magnetic-bead separation. Patients with stable function seemed to have a higher percentage (5.14 ± 2.45%; 2.76 ± 1.89% and 3.55 ± 0.8%; p = 0.43 for ST, SD and RD groups respectively) and number (23.01 ± 0.28 cells/µl; 15.13 ± 9.1 cells/µl and 20.09 ± 9.02%; p = 0.26 for ST, SD and RD groups respectively) of $CD19^+$ cells, but in neither case was the difference statistically significant.

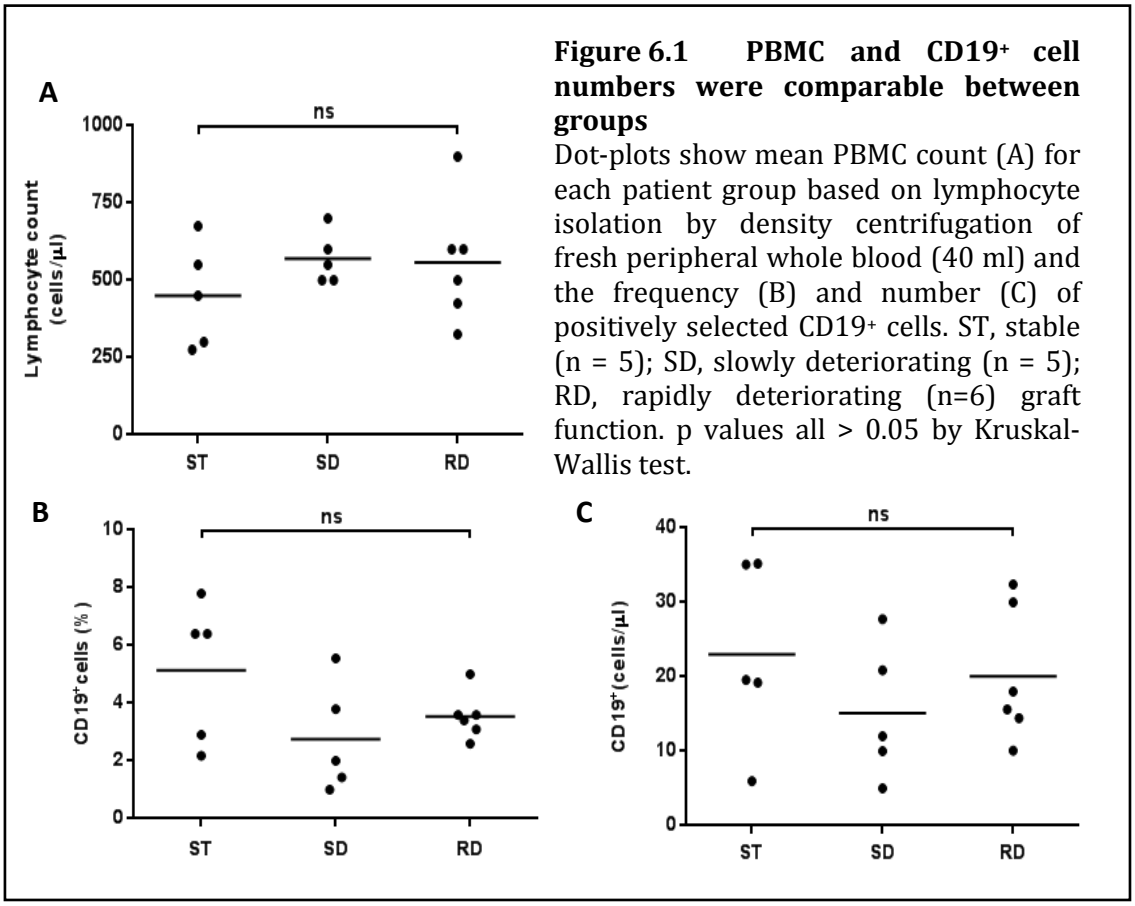


Figure 6.1 PBMC and $CD19^+$ cell numbers were comparable between groups
Dot-plots show mean PBMC count (A) for each patient group based on lymphocyte isolation by density centrifugation of fresh peripheral whole blood (40 ml) and the frequency (B) and number (C) of positively selected $CD19^+$ cells. ST, stable (n = 5); SD, slowly deteriorating (n = 5); RD, rapidly deteriorating (n=6) graft function. p values all > 0.05 by Kruskal-Wallis test.

Optimising cell-culture conditions to promote CD19+ cell expansion in peripheral blood of healthy controls

CD19$^+$ cells were investigated for IL-10 production post-culture with TLR agonists known to promote B-cell expansion and differentiation. This included the TLR7/8 agonist, R848 (Pinna et al., 2009), and the TLR9 agonist, CpG-B (Bouaziz et al., 2010). The ability of these agonists, with combinations of additional stimulants, to promote IL-10 production was assessed. First, expansion of CD19$^+$ cells was determined in healthy controls (n = 3). CD19$^+$ cells were labelled with CSFE to identity proliferation and cultured for five days in the presence of the following: R848 and IL-2; CpG-B; CpG-B and CD40L; or CpG-B 2006, CD40L and anti-human IgG-IgM-IgA (anti-Ig). Although small, the greatest proliferation of CD19$^+$ cells was observed with a combination of CpG, CD40L and anti-Ig (Figure 6.2A & B).

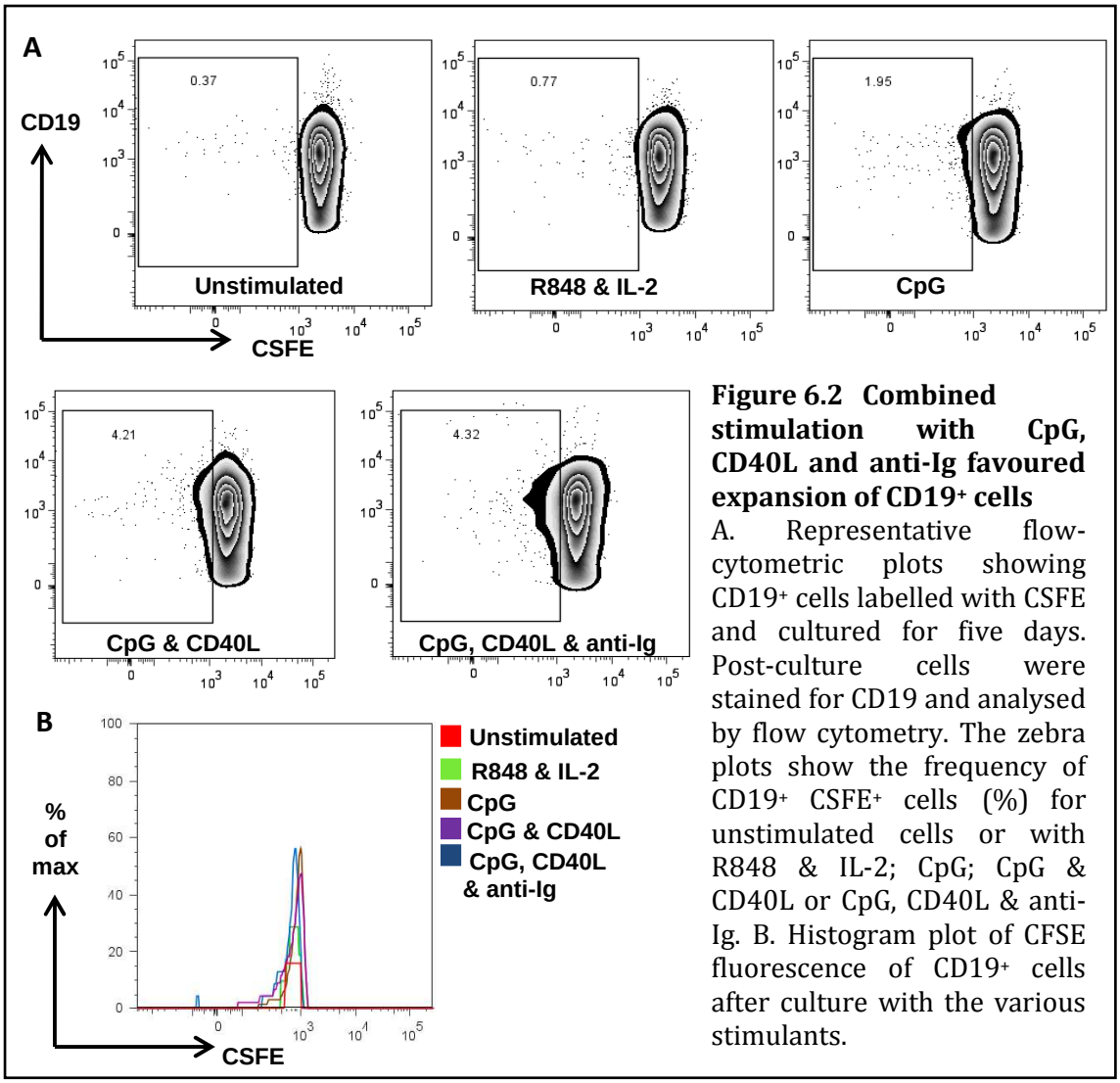


Figure 6.2 Combined stimulation with CpG, CD40L and anti-Ig favoured expansion of CD19+ cells
A. Representative flow-cytometric plots showing CD19+ cells labelled with CSFE and cultured for five days. Post-culture cells were stained for CD19 and analysed by flow cytometry. The zebra plots show the frequency of CD19+ CSFE+ cells (%) for unstimulated cells or with R848 & IL-2; CpG; CpG & CD40L or CpG, CD40L & anti-Ig. B. Histogram plot of CFSE fluorescence of CD19+ cells after culture with the various stimulants.

Next, each TLR agonist was tested to establish optimal IL-10 production. Following culture and a short stimulation with PMA, ionomycin & monensin, expression of $CD19^{+}IL\text{-}10^{+}$ cells was assessed by flow cytometry. The gating strategy to determine $CD19^{+}IL\text{-}10^{+}$ cells employed a blocking antibody matched to the test antibody as the matched isotype control lacked specificity and could not be used for accurate discrimination. In agreement with $CD19^{+}$ cell proliferation; the combination of CpG, CD40L and anti-Ig gave the highest percentage of $CD19^{+}IL\text{-}10^{+}$ cells and was used thereon in (Figure 6.3).

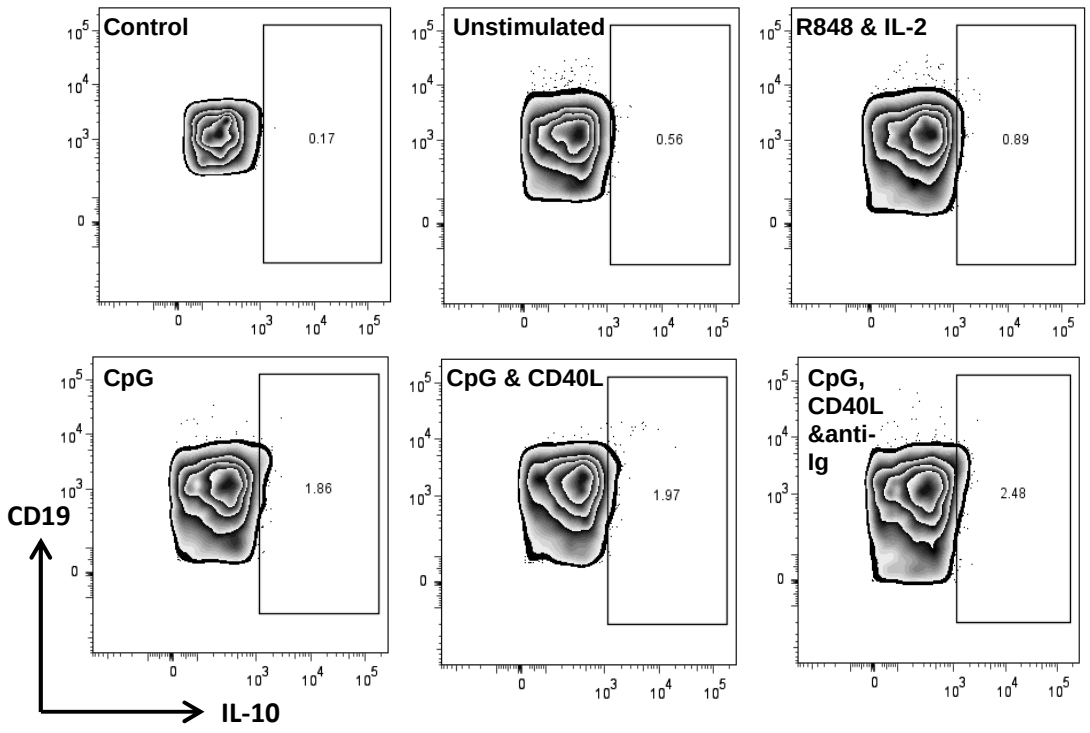


Figure 6.3 Stimulating $CD19^{+}$ cells with CpG, CD40L and anti-Ig promoted the highest frequency of IL-10-producing cells

$CD19^{+}$ cells were cultured for five days either alone; with R848 & IL-2; CpG; CpG & CD40L or CpG, CD40L & anti-Ig and with PMA, ionomycin & monensin for the last five hour. Post–culture, cells were assessed by flow cytometry for cell surface CD19 and intracellular expression of IL-10.

Representative flow cytometry zebra plots show the percentage of IL-10 producing $CD19^{+}$ cells for each condition. Culture with CpG, CD40L & anti-Ig promoted the greatest, albeit low, frequency of $CD19^{+}$ $IL\text{-}10^{+}$ cells.

Identifying IL-10-producing CD19+ cells in peripheral blood from patient groups

The conditions optimised in healthy controls were applied to patient groups. Those with stable graft function had a higher percentage and number of $CD19^+IL\text{-}10^+$ cells (1.4% and 0.32 cells/µl; Figure 6.4A and B) than the SD group (0.37%; p = 0.06 and 0.07 cells/µl; p = 0.06) and significantly more than the RD group (0.20%; p = 0.004 and 0.04 cells/µl; p = 0.009). Investigating if a relationship existed between the percentage of $CD19^+IL\text{-}10^+$ cells and level of graft function based on eGFR measurements taken over time found there was significant correlation between elevated frequency and superior graft function ($R^2 = 0.36$, p = 0.02; Figure 6.4C).

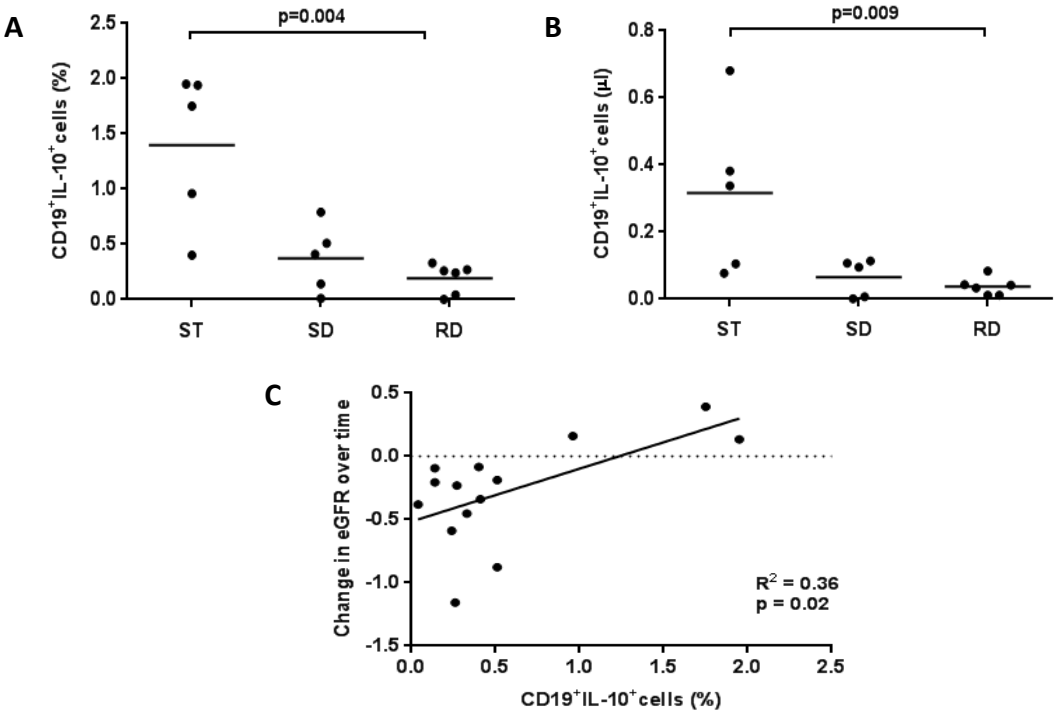


Figure 6.4 Patients with stable graft function had significantly higher frequency and number of $CD19^+IL\text{-}10^+$ cells

$CD19^+$ cells were cultured for five days in the presence of CpG, CD40L & anti-Ig and assessed for cell surface CD19 and intracellular expression of IL-10 by flow cytometry. The dot plots show the percentage (A) and number (B) of $CD19^+IL\text{-}10^+$ cells for each group. The stable (ST; n = 5) group have a higher number and percentage of $CD19^+IL\text{-}10^+$ cells compared to those with slowly deteriorating (SD; n = 5) graft function, and this is significantly so when compared to the rapidly deteriorating (RD; n = 6) group. P values shown are derived from Mann-Whitney tests.

(C) Linear regression analysis demonstrated significant correlation between $CD19^+IL\text{-}10^+$ cell frequency (all patients) and the rate of allograft deterioration extracted from measuring eGFR over time.

Phenotypic characteristics of CD19$^+$IL-10$^+$ cells

IL-10-producing CD19$^+$ cells were examined for expression of cell surface markers associated with naïve, memory and transitional B cells.

CD19$^+$IL-10$^+$ transitional B cells

Transitional B cells are defined as expressing high levels of CD24 and CD38 (Carsetti et al., 2004). Previous studies in autoimmunity and during infection show CD19$^+$IL-10$^+$ cells are enriched within the CD24^{high}CD38^{high} subset (Blair et al., 2010, Flores-Borja et al., 2013, Bouaziz et al., 2010, Das et al., 2012). This was also demonstrated in renal transplant recipients. Post-culture analysis by flow cytometry showed CD19$^+$IL-10$^+$ cells expressed CD24 and CD38 at high levels compared to CD19$^+$IL-10$^-$ cells (Figure 6.5).

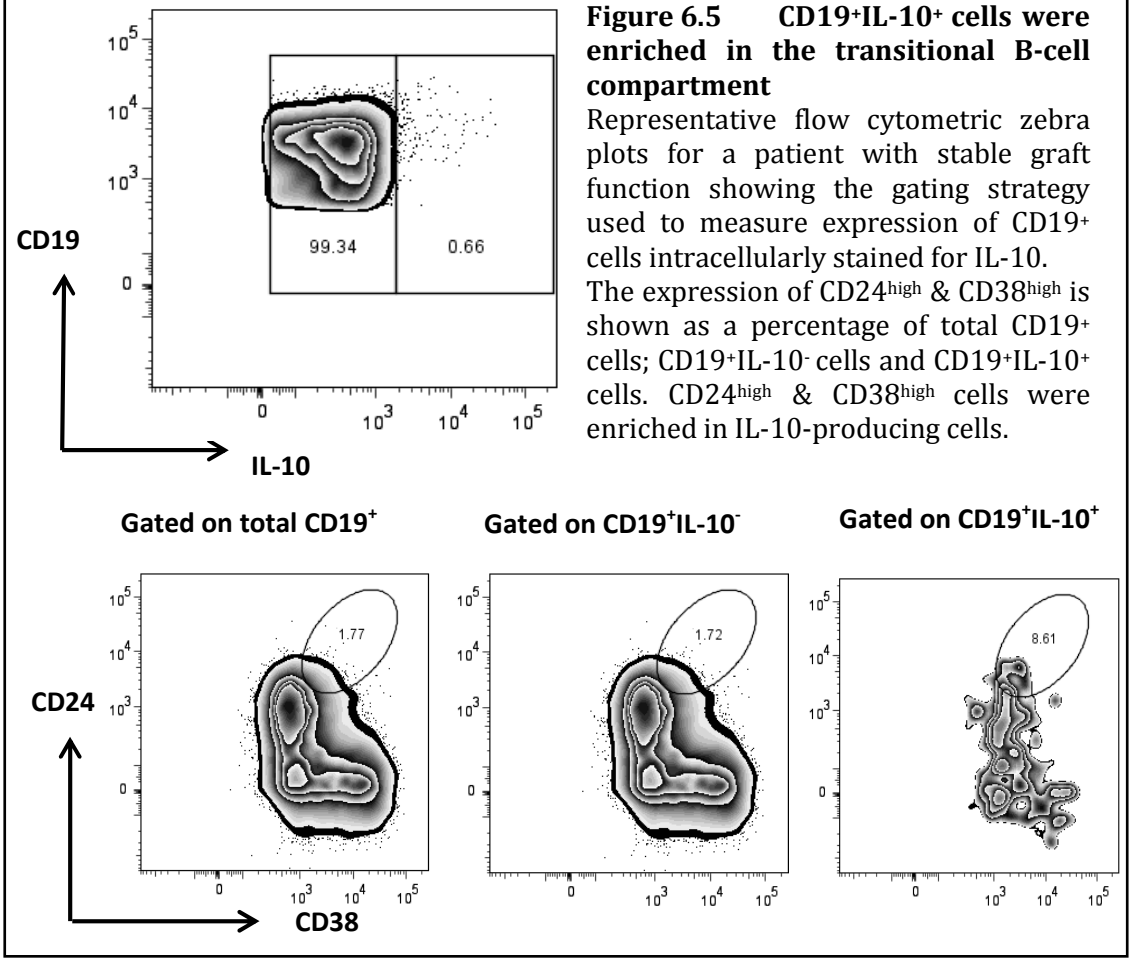


Figure 6.5 CD19$^+$IL-10$^+$ cells were enriched in the transitional B-cell compartment
Representative flow cytometric zebra plots for a patient with stable graft function showing the gating strategy used to measure expression of CD19$^+$ cells intracellularly stained for IL-10. The expression of CD24^{high} & CD38^{high} is shown as a percentage of total CD19$^+$ cells; CD19$^+$IL-10$^-$ cells and CD19$^+$IL-10$^+$ cells. CD24^{high} & CD38^{high} cells were enriched in IL-10-producing cells.

In addition, regardless of graft function, $CD19^{+}IL\text{-}10^{+}$ cells were enriched within the $CD24^{high}$ $CD38^{high}$ subset (18.57% vs. 8.42% and 9.64% for ST, SD and RD groups respectively; Figure 6.6). While there was no significant differences found between groups (p = 0.8); both the ST group (18.57% vs. 5.73%; p = 0.03) and the RD group (9.64% vs. 2.62%; p = 0.009) had a significantly greater representation of this subset by IL-10 positive compared to IL-10 negative cells

To determine if a lower percentage of IL-10 producing $CD24^{high}$ $CD38^{high}$ cells correlated with rate of deterioration, eGFR was measured over time and linear regression analysis performed. No significant correlation was found (R^2 = 0.04, p = 0.46; data not shown), this was likely due to variance in the percentage of IL-10-producing $CD24^{high}$ $CD38^{high}$ cells expression between patients.

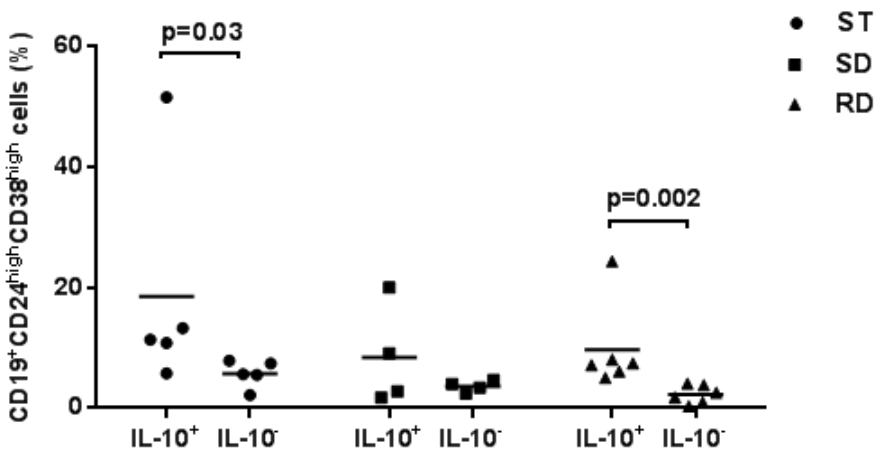


Figure 6.6 $CD19^{+}IL\text{-}10^{+}$ cells were enriched within the $CD24^{high}$ $CD38^{high}$ subset in all patient groups

$CD19^{+}$ cells were cultured with CpG, CD40L & anti-Ig for five days with PMA, ionomycin and monensin added for the last five hours. Cells were surface stained for CD24 and CD38 prior to intracellular staining for IL-10 and analysed by flow cytometry.

The dot-plot shows the frequency (%) of $CD19^{+}IL\text{-}10^{+}CD24^{high}CD38^{high}$ cells post-culture in patients with stable (ST; n = 5), slowly deteriorating (SD; n = 5) and rapidly deteriorating (RD; n = 7) graft function. No significant difference was found between groups (p=0.8). $CD24^{high}CD38^{high}$ cells were expanded in IL-10-producing B cells in all patient groups, and to a significant level in the ST and RD groups. Comparison between $CD24^{high}$ $CD38^{high}$ cell expression and 1) $CD19^{+}IL\text{-}10^{+}$ or $CD19^{+}IL\text{-}10^{-}$ populations was performed using Mann-Whitney test, and 2) $CD19^{+}IL\text{-}10^{+}CD24^{high}CD38^{high}$ cells between patient groups by Kruskal-Wallis test.

CD19⁺IL-10⁺ naïve and memory B cells

Expression of naïve and memory cell phenotypes within IL-10$^+$ and IL-10$^-$ B-cell populations was investigated by flow cytometry using CD27 and IgD as extracellular markers to distinguish naïve, CD19$^+$CD27$^-$IgD$^+$, from memory, CD19$^+$CD27$^+$IgD$^-$, cells (Sanz et al., 2008). The gating strategy employed is shown in Figure 6.7. The composition of these phenotypes was found to be differentially expressed depending on level of allograft function.

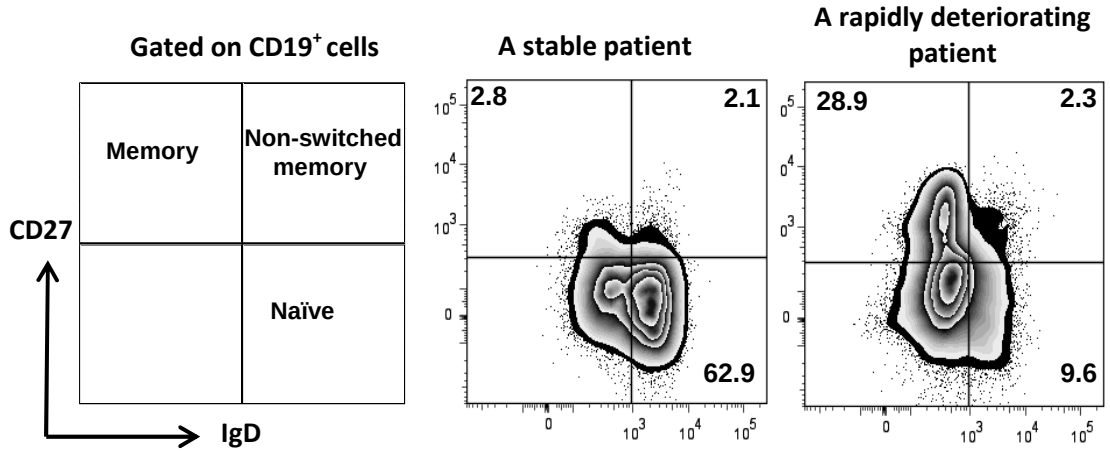


Figure 6.7 The frequency of naïve and memory B cells differed depending on the level of graft function
Naïve (CD19$^+$CD27$^-$IgD$^+$) and memory (CD19$^+$CD27$^+$IgD$^-$) B cells were evaluated by analysing cell surface expression of CD27 and IgD.
Representative flow cytometric zebra plots show the percentages of naïve and memory B cells for a patient with stable graft function and a patient with rapidly deteriorating graft function. The frequency of memory B cells was lower, while naïve B cells were at a greater frequency in this patient with stable function compared to a patient with deteriorating function. This pattern of expression was seen within the groups and are shown in Figure 6.8

Naïve and memory B cell expression on CD19⁺IL-10⁺ cells

The expression of IL-10$^+$ and IL-10$^-$ naïve B cells between patient groups was compared and did not show any statistically significant difference ($p = 0.3$). In stable patients, naïve B cells were present in equal proportions in both IL-10$^+$ and IL-10$^-$ B-cell populations (51.05 ± 25.41%; vs. 48.92 ± 20.42%; $p = 0.89$). The IL-10$^+$ B-cell population

representation was lower in the RD groups, albeit not to the level of statistical significance (17.91 ± 21.41% vs. 40.60 ± 25.11%; p = 0.09, Figure 6.8A).

The frequency of IL-10$^+$ memory cells was reduced in the ST group (6.09 ± 4.76%; 15.54 ± 9.16% and 26.44 ± 21.60% for ST, SD and RD groups respectively, Figure 6.8B), however expression was variable within groups and statistically significance differences were not found (p = 0.2). Comparing for differences in memory B-cell populations that produced IL-10 within groups, found there was little difference in the proportions of IL-10$^+$ and IL-10$^-$ cells in all three groups.

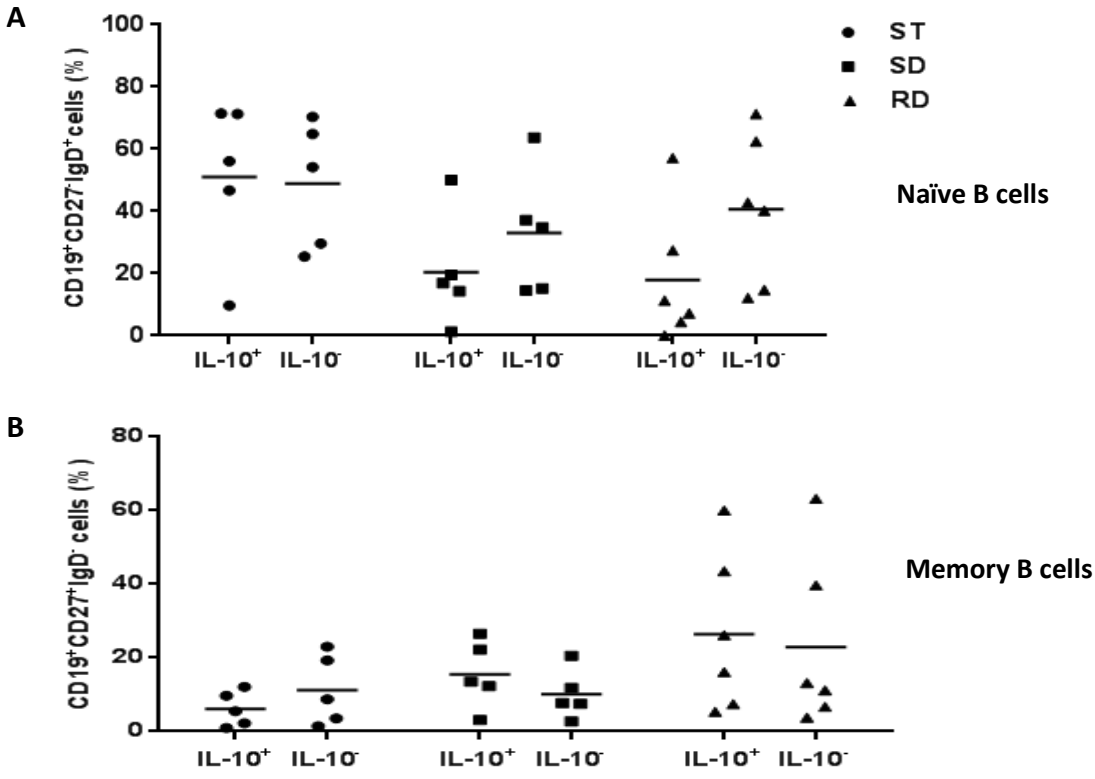


Figure 6.8 The frequency of CD19$^+$IL-10$^+$ naïve and memory B cells were not significantly altered with level of allograft function

CD19$^+$ cells were cultured with CpG, CD40L and anti-Ig for five days with PMA, ionomycin and monensin added for the last five hours. Cells were surface stained for CD27 & IgD to distinguish naïve (CD27$^-$IgD$^+$) from memory (CD27$^+$IgD$^-$) prior to intracellular staining for IL-10 and analysed by flow cytometry.

The dot-plots show the frequency of naïve (A) and memory (B) B cells separated by their capacity to produce IL-10 in patients with stable (ST; n = 5), slowly deteriorating (SD; n = 5) and rapidly deteriorating (RD; n = 6) graft function. Comparing CD19$^+$IL-10 positive and negative naïve and memory B cell populations in each group by Kruskal-Wallis test did not show any significant differences. Similarly, comparing CD19$^+$IL-10 positive and negative cells within each group by Mann-Whitney test did not show any significant differences.

Expression of BAFF receptor on IL-10 producing cells

Chapter 5 reported that stable patients had a higher percentage of $CD19^+BAFF\text{-}R^+$ cells. Here, the ST group also had a significantly higher percentage of B cells expressing BAFF-R than the RD group (92.53 ± 3.11%, 62.25 ± 30.78%; p = 0.09 and 58.08%; p = 0.004, ST, SD and RD, respectively, Figure 6.9). The percentage of $CD19^+BAFF\text{-}R^+$ cells was also significantly reduced for IL-10 producing cells in the SD compared to the ST group (95.39 ± 4.5% vs. 66.55 ± 18.78%; for ST and SD groups respectively; p = 0.009).

The level of BAFF-R positivity was evaluated in IL-10-producing transitional B cells. Both the ST and SD group demonstrated that BAFF-R was expressed on virtually all cells (98.71 ± 1.98% and 99.04 ± 1.93% for ST and SD groups respectively). In the RD group, expression was significantly less frequent than the ST group (79.15 ± 21.79%; p = 0.04). Moreover, there was a significant difference when comparing the frequency of total $CD19^+BAFF\text{-}R^+$ cells against the IL-10-producing transitional B-cell population in the SD group (p = 0.02).

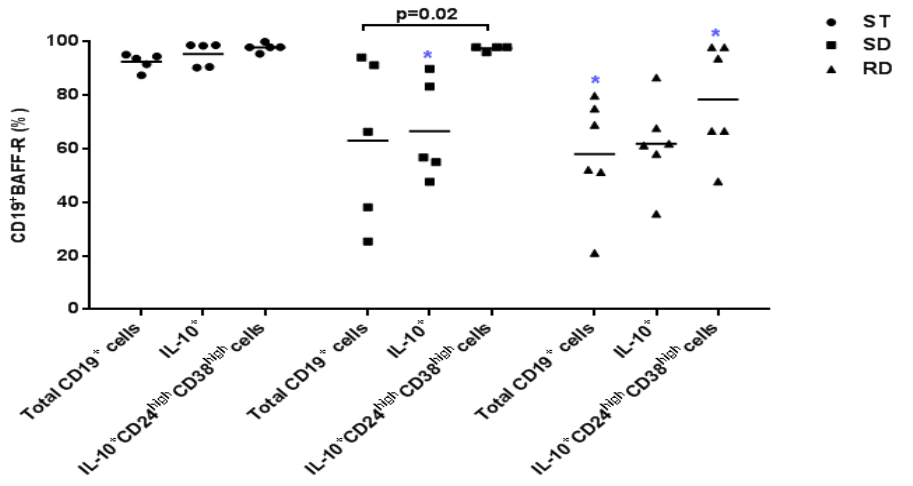


Figure 6.9 The level of BAFF-R positivity by $CD19^+IL\text{-}10^+$ cells was associated with stable graft function

$CD19^+$ cells cultured with CpG, CD40L and anti-Ig for five days were surface-stained for BAFF-R, CD24 & CD38 prior to intracellular IL-10 and analysed by flow cytometry. The dot-plot shows the percentage of $CD19^+$ $BAFF\text{-}R^+$ cells for total $CD19^+$, $CD19^+IL\text{-}10^+$ & $CD19^+$ $IL\text{-}10^+$ $CD24^{high}$ $CD38^{high}$ cells in each patient group. $BAFF\text{-}R^+$ cells were expanded in the unselected B-cell population in patients with stable (ST; n = 5) graft function. p values (*) derived from Mann-Whitney test by comparing percentage of total $CD19^+$, $CD19^+IL\text{-}10^+$ & $CD19^+$ $IL\text{-}10^+$ $CD24^{high}$ $CD38^{high}$ cells in the ST group against the slowly deteriorating (SD; n = 5) group and the rapidly deteriorating group (RD; n =6). $CD19^+$ $BAFF\text{-}R^+$ $IL\text{-}10^+$ $CD24^{high}$ $CD38^{high}$ cells were elevated in the SD group when compared to total CD19+ cells (p=0.02 by Mann-Whitney test).

The frequency of IL-10-producing B cells and presence of HLA antibody

A relationship between an increased frequency of IL-10-producing B cells and absence of HLA antibody was investigated. Patients from all study groups were examined for presence of HLA antibody (Figure 6.10A). Those without HLA antibodies (negative) did not have a significantly higher percentage of IL-10-producing B cells than those with DSA (0.61%, 0.87%; and 0.39%; p = 0.4, negative, NDSA and DSA respectively). When patients with DSA were examined further, all had rapidly deteriorating graft function (100%; p = 0.07) compared to those with NDSA (20%), and those without antibody (33%; Figure 6.10B).

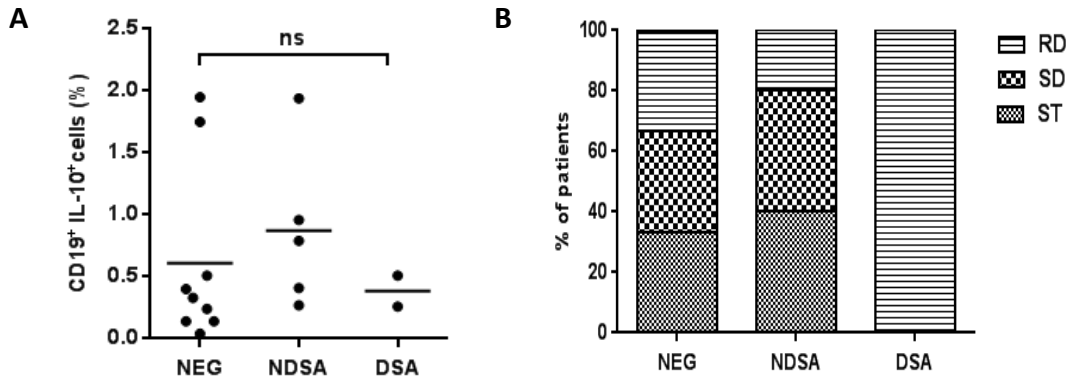


Figure 6.10 Comparing the frequency of IL-10 producing B cells and presence of HLA antibodies
Serum samples were analysed for HLA Class I & II antibodies by Luminex technology. Patients were divided into three groups; those with donor-specific HLA antibodies (DSA; n = 2) non-DSA (NDSA; n = 5) and no detected HLA antibodies (Neg; n = 10).
A. The dot-plot shows percentage of $CD19^{+}IL\text{-}10^{+}$ cells and HLA antibody status for all study patients. There was no significant difference between groups when compared by Kruskal-Wallis test (p=0.4).
B. The study groups were separated based on presence or absence of serum HLA antibodies. Those with DSA were all categorised as having rapidly deteriorating (RD) graft function (p=0.07 by Chi squared test) unlike those with slowly deteriorating (SD) or stable (ST) graft function.

Investigating the suppressive capacity of $CD19^{+}CD24^{high}CD38^{high}$ cells

The relevance of $CD19^{+}CD24^{high}CD38^{high}$ cells as important regulators during the immune response has emerged to represent regulatory B cells, which in SLE (Blair et al., 2010) and rheumatoid arthritis (Flores-Borja et al., 2013) lack suppressive capacity. Their functional role was investigated in patients grouped by allograft function. The demographics and clinical characteristics are shown in Table 6.2.

	Stable (ST)	**Slowly deteriorating (SD)**	**Rapidly deteriorating (RD)**
Total number of patients, n (%)	5 (33.3)	5 (33.3)	5 (33.3)
Recipient			
Gender (F,M)	2,3	2,3	1,4
Age, mean (range) (years)	48 (46-52)	47 (35-53)	47 (33-56)
Time post-transplant, mean (range) (years)	7 (1-20)	9 (2-24)	14 (6-23)
***Type of allograft*, n (%)**			
Live donation	2 (40)	4 (80)	2 (40)
DBD	2 (40)	1 (20)	3 (60)
DCD	1 (20)	0	0
***HLA mismatches, n* ± sd**			
A, mean ± sd	1 ± 0.71	1.25 ± 0.5*	1.2 ± 0.84
B, mean ± sd	1 ± 0.71	1.25 ± 0.5*	1.2 ± 0.45
DR, mean ± sd	1 ± 0.71	1.25 ± 0.96*	0.8 ± 0.45
HLA antibody - post-transplant, n (%)			
Negative	4 (80)	3 (60)	3 (60)
NDSA	0	0	2 (40)
DSA	1 (20)	2 (40)	0
Cause of ESRF, n (%)			
Diabetes	1 (20)	1 (20)	0
IgA nephropathy	1 (20)	0	1 (20)
FSGS	2 (40)	0	0
GN	0	0	1 (20)
Hypertension	0	0	1 (20)
PKD	1 (20)	1 (20)	0
Other/Unknown aetiology	0	3 (30)	2 (40)

Table 6.2 Demographics of patient groups separated by allograft function
Abbreviations: DBD, donation after brainstem death; DCD, donation after circulatory death; DSA, donor-specific HLA antibody; NDSA, non-donor-specific HLA antibody; ESRF, end-stage renal failure; FSGS, focal segmental glomerulosclerosis; GN, glomerulonephritis; PKD, polycystic kidney disease; sd, standard deviation. *Donor type missing for one patient

Establishing methods to investigate suppression of pro-inflammatory T-cell cytokines by $CD19^+$ cells in healthy controls

The effect of $CD19^+$ cells on the production of the pro-inflammatory T-cell cytokines, TNF-α and IFN-γ was investigated initially using peripheral blood from healthy controls (n = 4) to establish methods. To maximise available cell number, the whole B-cell fraction was used as opposed to CD19, CD24 & CD38 subsets.

Optimising T-cell activation from naïve to effector cells

Anti-CD3 antibodies, immobilised on a tissue culture plate were used to induce activation of naïve T cells ($CD4^+CD25^-$) via the TCR. Optimal proliferation required a higher concentration of antibody (10 µg/ml) than the range (0.5-2 µg/ml) suggested in previous publications (Flores-Borja et al., 2013, Abraham et al., 2008, Paulsen et al., 2011). In addition the co-stimulatory molecule, CD28, was included as this enhanced proliferation of effector T cells (Figure 6.11).

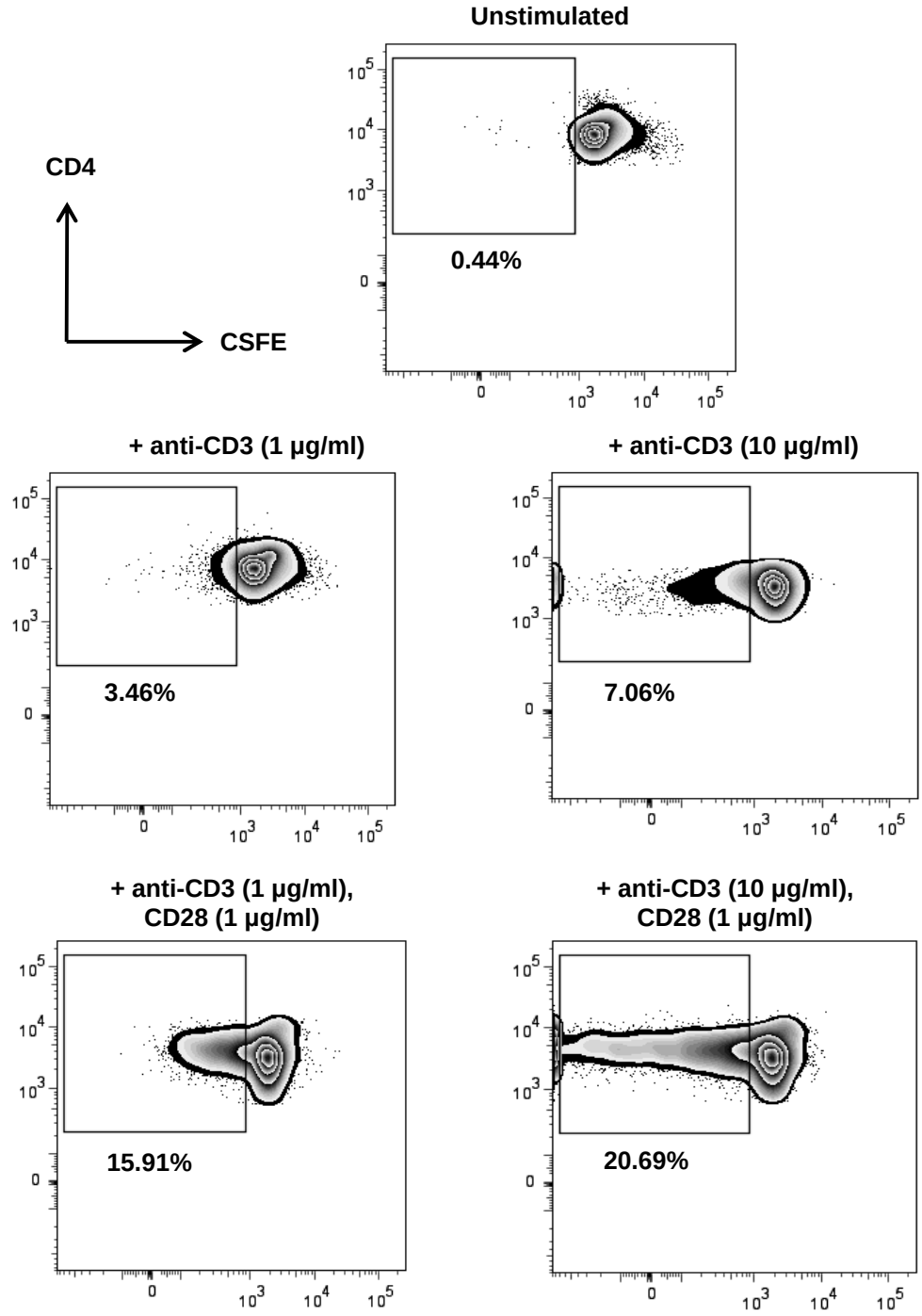


Figure 6.11 Combining anti-CD3 antibody and CD28 gave optimal T-cell activation

Autologous naïve T cells ($CD4^+CD25^-$) labelled with CSFE were cultured with plate-bound anti-CD3 and CD28 for 96 hours. Post-culture, cells were stained with CD4 and analysed by flow cytometry.

Representative CSFE profiles of $CD4^+$ T cell proliferation are shown with percentage of $CD4^+CSFE^+$ cells for unstimulated $CD4^+$ T cells; with anti-CD3 (1 µg/ml); anti-CD3 (10 µg/ml); anti-CD3 (1 µg/ml) & CD28 (1 µg/ml) or anti-CD3 (10 µg/ml) & CD28 (1 µg/ml). The last gave optimal naïve T-cell activation and was used thereon in.

Suppression of CD4+ T-cell proliferation during co-culture with CD19+ cells

Purified $CD19^+$ cells were co-cultured with autologous naïve T cells activated with anti-CD3 and CD28 to investigate any inhibitory effects. The frequency of effector T cells post-culture with $CD19^+$ cells together with CpG, CD40L and anti-Ig was reduced when compared to naive T cells cultured alone (Figure 6.12). Although the reduction was small, these data demonstrated a level of $CD4^+$ T-cell reduction in the presence of $CD19^+$ cells and justified further investigation to determine if $CD24^{high}$ $CD38^{high}$ B cells were responsible.

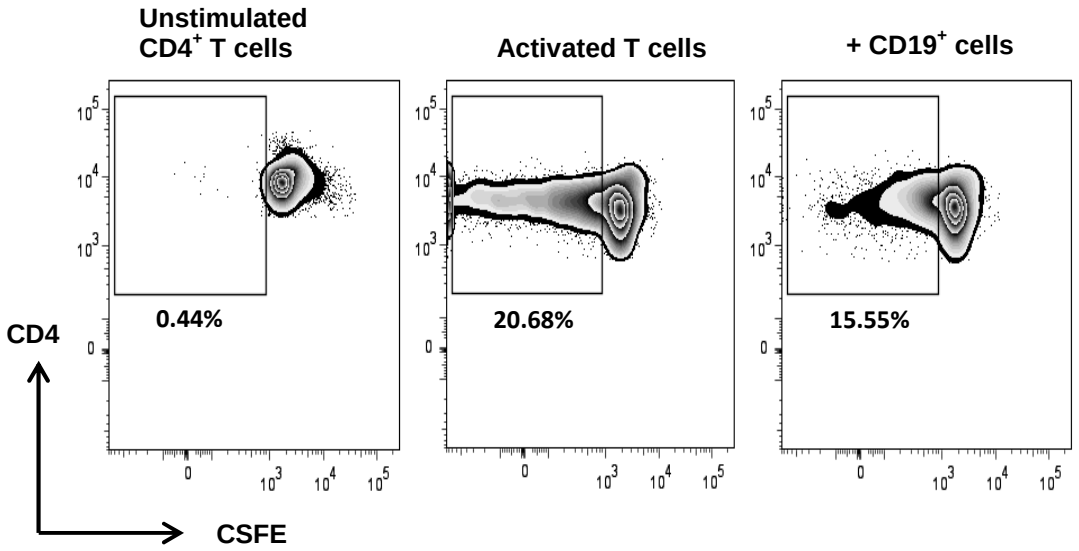


Figure 6.12 Proliferation of $CD4^+$ T cells was marginally reduced in the presence of $CD19^+$ cells in healthy controls
Autologous naïve T cells ($CD4^+CD25^-$) from healthy controls (n = 4) were activated with anti-CD3 and CD28 for and cultured in the presence or absence of $CD19^+$ cells for 96 hours. Post-culture $CD4^+CSFE^+$ cells were assessed by flow cytometry.
Representative CSFE profiles of effector T-cell proliferation ± $CD19^+$ cells are shown with percentage of proliferating T cells. Co-culture with $CD19^+$ cells demonstrated a small reduction in the frequency of $CD4^+$ T cells.

Rational for including polyclonal B-cell agonists in T: B co-culture assay

As described, the frequency of IL-10-producing CD19 cells was maximised in the presence of CpG, CD40L and anti-Ig. To promote expansion of these cells, CpG, CD40L & anti-Ig were included within the T: B cell co-culture environment. Investigations found in their absence, $CD4^+$ T cell frequency went up (Figure 6.13). In addition, the ratio of $CD4^+$: $CD19^+$ cells were more effective at 1:1 (1 x 10^6/ml) compared to 1:2 (13.95% & 4.93%, 1:1 & 1:2 respectively). These were not significant findings (p = 0.8), however the B-cell agonists were included in all co-culture conditions at a 1:1 ratio with naïve $CD4^+$ T cells.

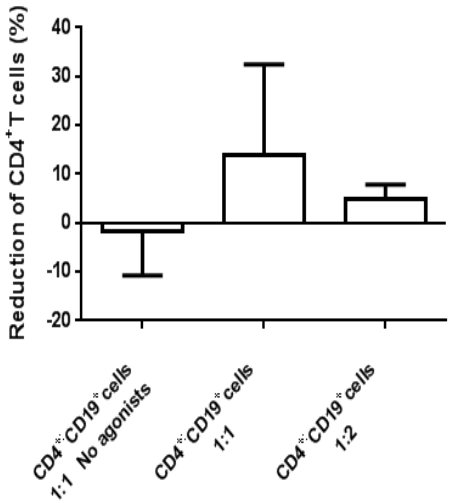


Figure 6.13 Reduced frequency of $CD4^+$ T cells with inclusion of B-cell agonists in co-culture assays
Autologous naïve T cells ($CD4^+CD25^-$) from healthy controls (n = 4) were activated by anti-CD3 and CD28 in the presence of $CD19^+$ cells +/- CpG, CD40L and anti-Ig for 96 hours.
The bar-chart shows post-culture staining with CD4 antibody and analysis by flow cytometry. The percentage reduction subtracts frequency of $CD4^+$ T cells cultured alone from other conditions which included $CD19^+$ cells. Co-culture of $CD4^+$ T cells with equal number of $CD19^+$ cells and inclusion of CpG, CD40L and anti-Ig was most effective. However there was no significant difference found (p = 0.8 by Kruskal-Wallis test).

Suppression of TNF-α-secreting CD4⁺ T cells in the presence of CD19⁺ cells

Next, suppression of TNF-α and IFN-γ by $CD19^+$ cells was investigated (Figure 6.14). The frequency of $CD4^+TNF\text{-}\alpha^+$ cells was not significantly reduced (41.09 ± 12.28% to 29.46 ± 10.92%; p = 0.1). $CD4^+IFN\text{-}\gamma^+$ cells were unaffected (34.97 ± 2.96% increased to 37.16 ± 9.35%).

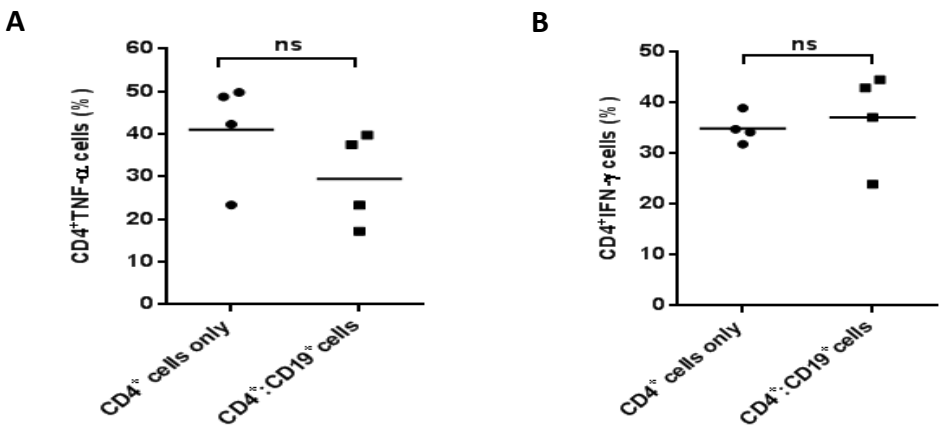


Figure 6.14 Reduced expression of TNF-α but not IFN-γ by CD4⁺ T cells following culture with CD19+ cells from healthy controls
Autologous naïve T cells ($CD4+CD25^-$) from healthy controls (n = 4) were activated by anti-CD3 and CD28 and co-cultured ± $CD19^+$ cells for 96 hours. Post culture cells were surface stained for CD4 and intracellularly for TNF-α and IFN-γ for analysis by flow-cytometry.
The dot-plots show the percentage of $CD4^+TNF\text{-}\alpha^+$ cells (A) and $CD4^+IFN\text{-}\gamma^+$ cells (B) post-culture. $CD4^+TNF\text{-}\alpha^+$ cells were marginally reduced in the presence of $CD19^+$ cells compared to $CD4^+$ T cells cultured alone (p = 0.1 by Mann-Whitney test). There was no reduction in the frequency of $CD4^+IFN\text{-}\gamma^+$ cells post-culture with $CD19^+$ cells.

In summary, these data demonstrated the principle of the impact of $CD19^+$ cells on naïve $CD4^+$ cells activation, and the detection of pro-inflammatory Th1 cytokines by flow cytometry; these methods were then applied to patient groups.

Evaluating suppression of pro-inflammatory T-cell cytokines by subsets defined by CD19, CD24 and CD38 phenotypes in patient groups

To determine if functional regulatory properties resided in $CD19^{+}CD24^{high}CD38^{high}$ cells, these populations were isolated by flow cytometry using fluorescently activated cell-sorting (FACS) based on cell surface marker expression of CD19, CD24 and CD38.

Phenotypic analysis of peripheral blood in study groups

Total lymphocyte and $CD19^{+}$ cell populations

Total PBMC were isolated from peripheral whole blood and a total lymphocyte count calculated. No significant difference existed between groups (770 ± 276 cells/µl, 647 ± 223 cells/µl and 965 ± 263 cells/µl; p = 0.22 for ST, SD and RD groups, respectively, Figure 6.15A). Likewise, the percentage (4.82 ± 3.53%, 2.36 ± 1.77% and 5.43 ± 3.99%; p = 0.11 for ST, SD and RD groups respectively, Figure 6.15B) and number of $CD19^{+}$ cells was not significantly different between groups (38 ± 35 cells/µl, 8 ± 17 cells/µl and 40 ± 34 cells/µl; p = 0.24 for ST, SD and RD groups respectively, Figure 6.15C). Therefore any differences in the suppressive capacities of B cells were not attributed to the initial lymphocyte or $CD19^{+}$ cell constitution.

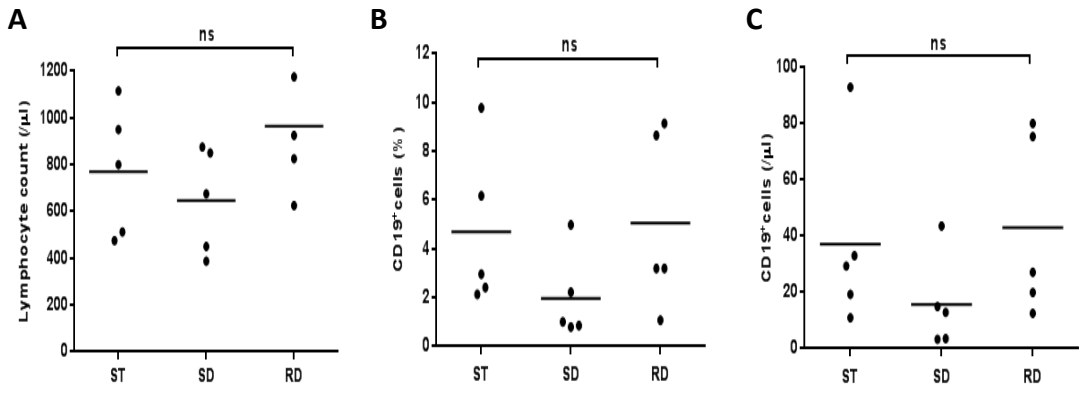


Figure 6.15 PBMC and $CD19^{+}$ cell numbers were comparable between groups
Dot-plots show mean PBMC count (A) for each patient group based on lymphocyte isolation by density centrifugation of fresh peripheral whole blood (40 ml) and the frequency (B) and number (C) of positively selected $CD19^{+}$ cells. ST, stable (n = 5); SD, slowly deteriorating (n = 5); RD, rapidly deteriorating (n = 5) graft function. p values all > 0.05 by Kruskal-Wallis test.

Characterisation of transitional, memory & mature naïve B cells in healthy controls

PBMC were separated into three populations based on expression of CD19, CD24 and CD38 by FACS (Figure 6.16). This was first optimised in healthy controls and analysis revealed three distinct subsets consistent with that shown by others (Carsetti et al., 2004, Blair et al., 2010, Flores-Borja et al., 2013). These populations have been defined as: transitional, $CD19^{+}$ $CD24^{high}CD38^{high}$; memory, $CD19^{+}$ $CD24^{high}CD38^{-}$; and mature naïve cells; $CD19^{+}$ $CD24^{+}CD38^{+}$.

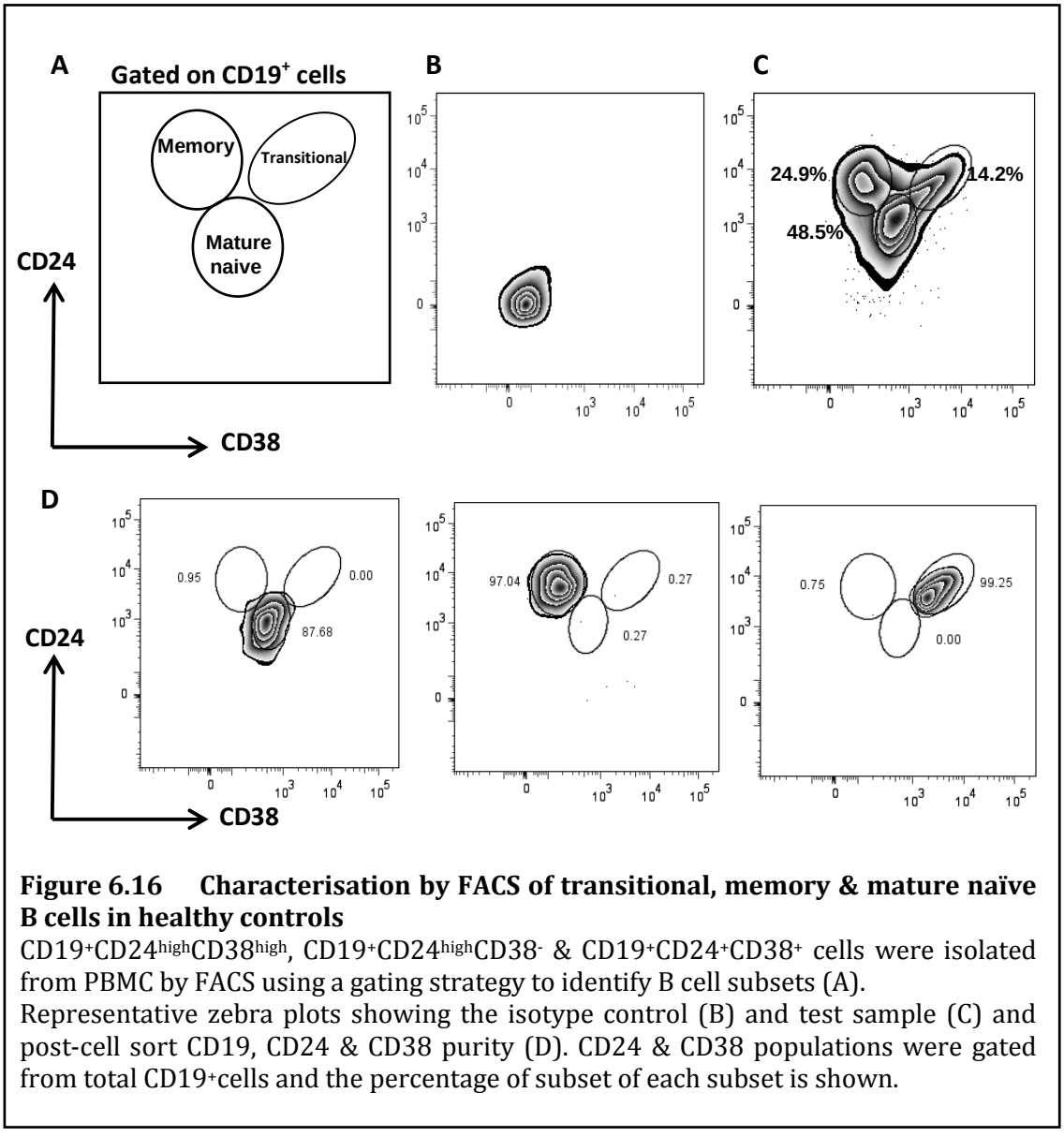


Figure 6.16 Characterisation by FACS of transitional, memory & mature naïve B cells in healthy controls
$CD19^{+}CD24^{high}CD38^{high}$, $CD19^{+}CD24^{high}CD38^{-}$ & $CD19^{+}CD24^{+}CD38^{+}$ cells were isolated from PBMC by FACS using a gating strategy to identify B cell subsets (A).
Representative zebra plots showing the isotype control (B) and test sample (C) and post-cell sort CD19, CD24 & CD38 purity (D). CD24 & CD38 populations were gated from total $CD19^{+}$cells and the percentage of subset of each subset is shown.

Identifying transitional, memory & mature naïve B cells in study groups

FACS analysis of CD19, CD24 & CD38 subsets in healthy controls identified three distinct populations (Figure 6.16C). However, in transplanted patients, these populations were not always evident; in particular, the mature naïve subset was often unclear (Figure 6.17).

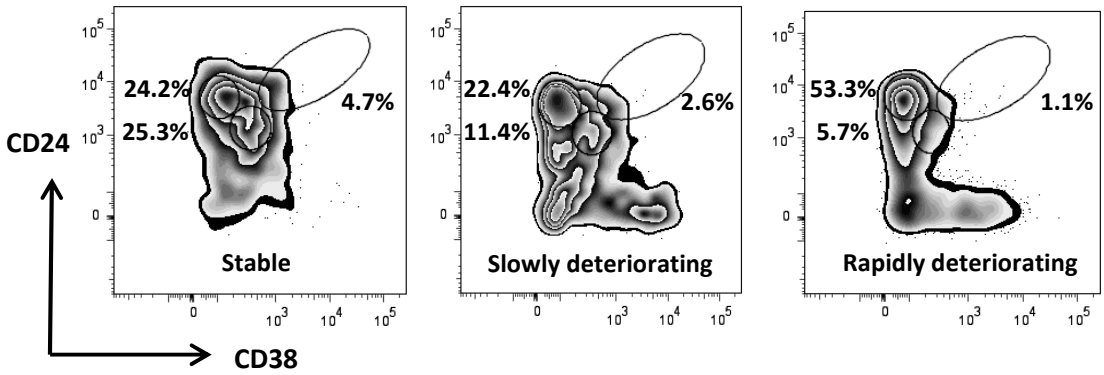


Figure 6.17 Variation in the constitution of transitional, memory & mature naïve B cells between study groups
Representative zebra plots of $CD19^{+}$, CD24 & CD38 populations following FACS for a patient from each study group.
$CD19^{+}CD24^{high}CD38^{high}$, $CD19^{+}CD24^{high}CD38^{-}$ & $CD19^{+}CD24^{+}CD38^{+}$ populations varied depending on graft function. The frequency (%) of each subset is shown. Overall the percentage of $CD19^{+}CD24^{high}CD38^{high}$ cells was lower in patients with deteriorating graft function (slow and rapid).

Transitional B-cell populations

The frequency of transitional B cells was significantly lower in patients with deteriorating graft function (4.2 ± 0.64%; 2.54 ± 0.7%; p = 0.02 and 2.13 ± 0.45%; p = 0.008, for ST, SD and RD groups respectively, Figure 6.18A). This reduction appears a feature of graft dysfunction as both SD and RD groups have significantly lower expression. However even the ST group had a numerical deficit compared to healthy controls (13.95 ± 0.25%). Although only reflective of two individuals, these data are consistent with others reporting expression of approximately 10% (Blair et al., 2010, Flores-Borja et al., 2013).

The expression $CD24^{high}$ $CD38^{high}$ cells was examined in relation to level of graft function. There was significant correlation between elevated frequency and superior graft function by linear regression analysis of eGFR over time (R^2 = 0.67, p = 0.0002; Figure 6.18B).

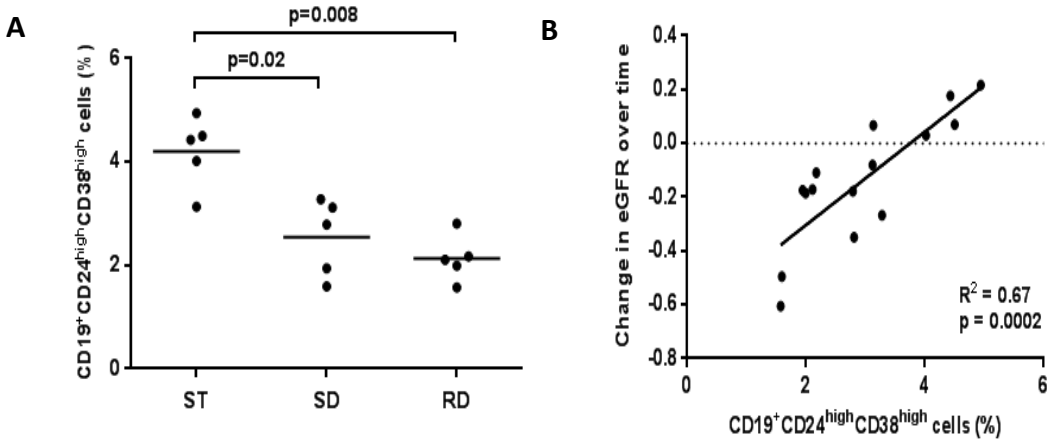


Figure 6.18 Patients with stable function had significantly higher frequency of $CD19^{+}CD24^{high}CD38^{high}$ cells than those with deteriorating function
PBMC stained with CD19, CD24 and CD38 were analysed by FACS.
A. Dot-plot shows mean percentage of $CD19^{+}CD24^{high}CD38^{high}$ cells for each group. The stable (ST; n = 5) group had significantly higher frequency compared to both slowly deteriorating (SD; n = 5) and rapidly deteriorating (RD; n = 5) patients (p values by Mann-Whitney test). B. Linear regression analysis demonstrated a significant correlation between $CD19^{+}CD24^{high}CD38^{high}$ cell frequency (all patients) and the rate of allograft deterioration extracted from measuring eGFR over time.

Mature naïve and memory B cell populations

Memory and mature naïve B cell frequencies were not significantly different in groups with different allograft function. The memory B-cell population was elevated in patients with rapidly deteriorating function (28.05 ± 7.72%; 27.38 ± 11.37% and 48.63 ± 8.2%; p = 0.07 for ST, SD and RD groups respectively, Figure 6.19A). Accordingly, naïve B cells were reduced (30.66 ± 20.24%; 18.71 ± 8.95% and 8.69 ± 2.3%; p=0.08 for ST, SD and RD groups respectively, Figure 6.19B).

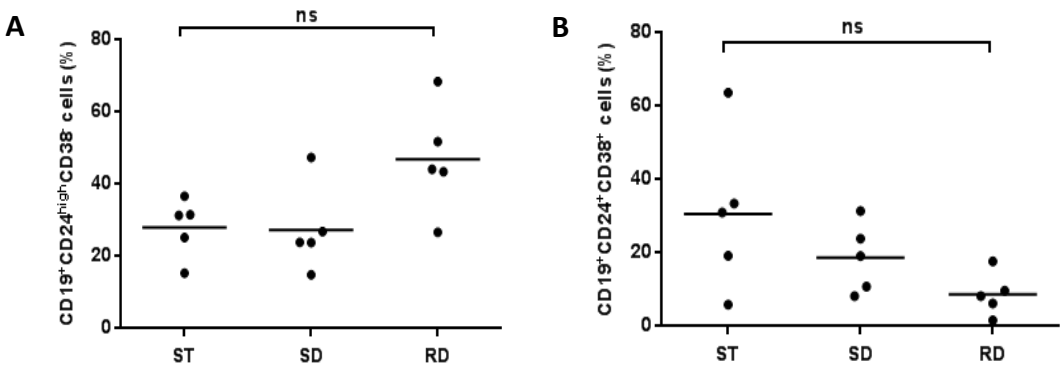


Figure 6.19 Memory and naive B cells were not significantly altered as a factor of allograft function
PBMC were stained for CD19, CD24 and CD38 were analysed by FACS. Dot-plots show percentage of memory B cells; $CD19^{+}CD24^{high}CD38^{-}$ (A) and naïve B cells; $CD19^{+}CD24^{+}CD38^{+}$cells (B). The frequency of naïve (p = 0.08) and memory (p = 0.07) did not significantly alter when comparing the stable (ST; n = 5), slowly deteriorating (SD; n = 5) and the rapidly deteriorating (RD; n = 5) function groups by Kruskal-Wallis test.

$CD19^{+}CD24^{-}CD38^{high}$ cell population in deteriorating function groups

A number of patients had a fourth distinct population of B cells, with no CD24 but highly expressed CD38 (seen in the SD patient shown in Figure 6.17). Interestingly 80% of the RD group and 60% of the SD group presented this phenotype compared to only 20% of the ST group. Although more of the RD group had this population, as a percentage of total cells, when it was present, it was found at higher frequency in SD patients (10.49 ± 6.29% vs. 3.22 ± 2.3%, SD vs. RD; p = 0.105). This population was not included in the subsequent functional analysis as it was identified too late in the study, but it may be of interest in further investigations.

Investigating suppression of activated T cells by $CD19^{+}CD24^{high}CD38^{high}$ cells

To investigate potential suppressive capacity of $CD19^{+}CD24^{high}CD38^{high}$ cells, FACS-separated subsets expressing CD19, CD24 & CD38 were co-cultured with naïve autologous T cells activated with anti-CD3 & CD28 using methods optimised in healthy controls as previously described. Activated $CD4^{+}$ T-cell frequency was assessed in conjunction with the activation marker, CD69. CD69 is one of the earliest cell surface antigens expressed by T cells following activation and is detectable within an hour of ligation of the TCR/CD3 complex. (Ziegler et al., 1994). The extent of T-cell suppression by each B-cell subset was investigated for each group by identifying the difference in the frequency of $CD4^{+}CD69^{+}$ T cells cultured in the absence and presence of B-cell subsets (Figure 6.20). Overall there was a significant difference between groups ($p = 0.01$). Examining this further, $CD4^{+}CD69^{+}$ T cells were reduced to the greatest extent in the ST group when co-cultured with $CD19^{+}CD24^{high}CD38^{high}$ cells (10.00 ± 4.69%, 1.07 ± 11.13%; $p = 0.3$ and 1.99 ± 5.27%; $p = 0.09$ for ST, SD and RD groups respectively). However due to the small sample size, the number of variables and wide variance in the frequency $CD4^{+}CD69^{+}$ T cells between patients, no statistically significant differences are found.

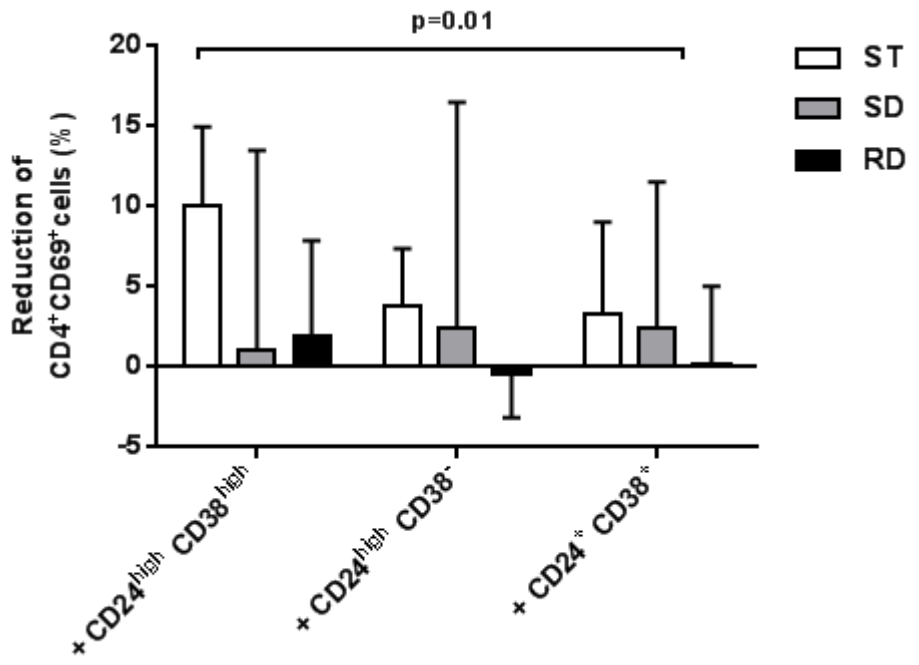


Figure 6.20 Reduced frequency of activated T cells in the presence of $CD19^{+}CD24^{high}CD38^{high}$ cells from patients with stable graft function

FACS-separated subsets (defined by various CD19, CD24 and CD38 phenotypes) were cultured 1:1 with autologous naïve T cells activated with anti-CD3 antibody & CD28 for 96 hours. Post-culture cells were stained with CD4 & CD69 and analysed by flow cytometry.

The bar-graph shows the mean percentage of $CD4^{+}CD69^{+}$ T cells (error bars represent standard deviation) for each patient group; stable (ST; n = 5); slowly deteriorating (SD; n = 5) and rapidly deteriorating (RD; n = 5) graft function following culture with the various B-cell subsets. Comparing culture of $CD4^{+}$ cells alone against culture in the presence of the B-cell subsets showed a significant difference by Kruskal-Wallis test (p = 0.01). In the ST group, there was the greatest reduction in the frequency of $CD4^{+}CD69^{+}$ T cells during co-culture with $CD19^{+}CD24^{high}CD38^{high}$ cells, however examining this further by Mann-Whitney test did not show a significant differences when compared to either the SD and RD groups.

Investigating suppression of pro-inflammatory cytokines, IFN-γ and TNF-α by $CD19^{+}CD24^{high}CD38^{high}$ cells

Suppression of IFN-γ-secreting $CD4^{+}$ T cells was pronounced in patients with stable graft function but not restricted to $CD19^{+}CD24^{high}CD38^{high}$ cells

The isolated subsets defined by pattern of expression of CD19, CD24 & CD38 were next investigated to determine if a particular population was responsible for increased capacity to supress the production of the Th1 pro-inflammatory cytokine, IFN-γ (Figure 6.21A). By examining the level of reduction of $CD4^{+}$IFN-γ^{+} T cells when $CD4^{+}$ T cells were cultured alone or with B-cell subsets, there was a significant difference between groups (p = 0.03). The ST group demonstrated the greatest reduction with all subsets ($CD24^{high}CD38^{high}$: 12.32 ± 2.5%, $CD24^{high}CD38^{-}$: 11.76 ± 4.13% and $CD24^{+}CD38^{+}$: 10.86 ± 4.43%). Further examination did not reveal any statistically significant differences between groups. As with $CD4^{+}CD69^{+}$ T-cell reduction, this study lacked the statistical power to find statistically significant difference at this level. However, since the largest median difference between groups existed when comparing the ST group against the RD group following culture with either $CD24^{high}CD38^{high}$ (p = 0.09) or $CD24^{high}CD38^{-}$ (p = 0.06) B cells; an increase of statistical power in this analysis through a greater sample size may have demonstrated enhanced suppressive capability of IFN- γ and stable graft function.

Suppression of TNF-α-secreting $CD4^{+}$ T cells by $CD19^{+}CD24^{high}CD38^{high}$ cells was greatest in patients with stable allograft function

Next the capacity of $CD19^{+}$ cells of various subsets defined by their expression of CD24 and CD38 cells to suppress TNF-α secretion by $CD4^{+}$ T cells was investigated. Again for each group the frequency of $CD4^{+}$TNF-α^{+} T cells following co-culture with the B-cell subsets was compared (Figure 6.21B). As the bar graph shows, there was considerable variation between patients in the reduction of $CD4^{+}$TNF-α^{+} T cells and no statistically significant difference was found (p = 0.13). However, in contrast to the suppression of IFN-γ-secreting $CD4^{+}$ T cells, the ST group demonstrated maximal suppressive capacity

in the presence of the $CD24^{high}CD38^{high}$ subset ($CD24^{high}CD38^{high}$: 10.24 ± 2.71%, $CD24^{high}CD38^{-}$: 7.42 ± 4.87% and $CD24^{+}CD38^{+}$: 7.2 ± 4.23%). Again, this data may be enriched by the inclusion of additional patients to the study to increase the statistical power.

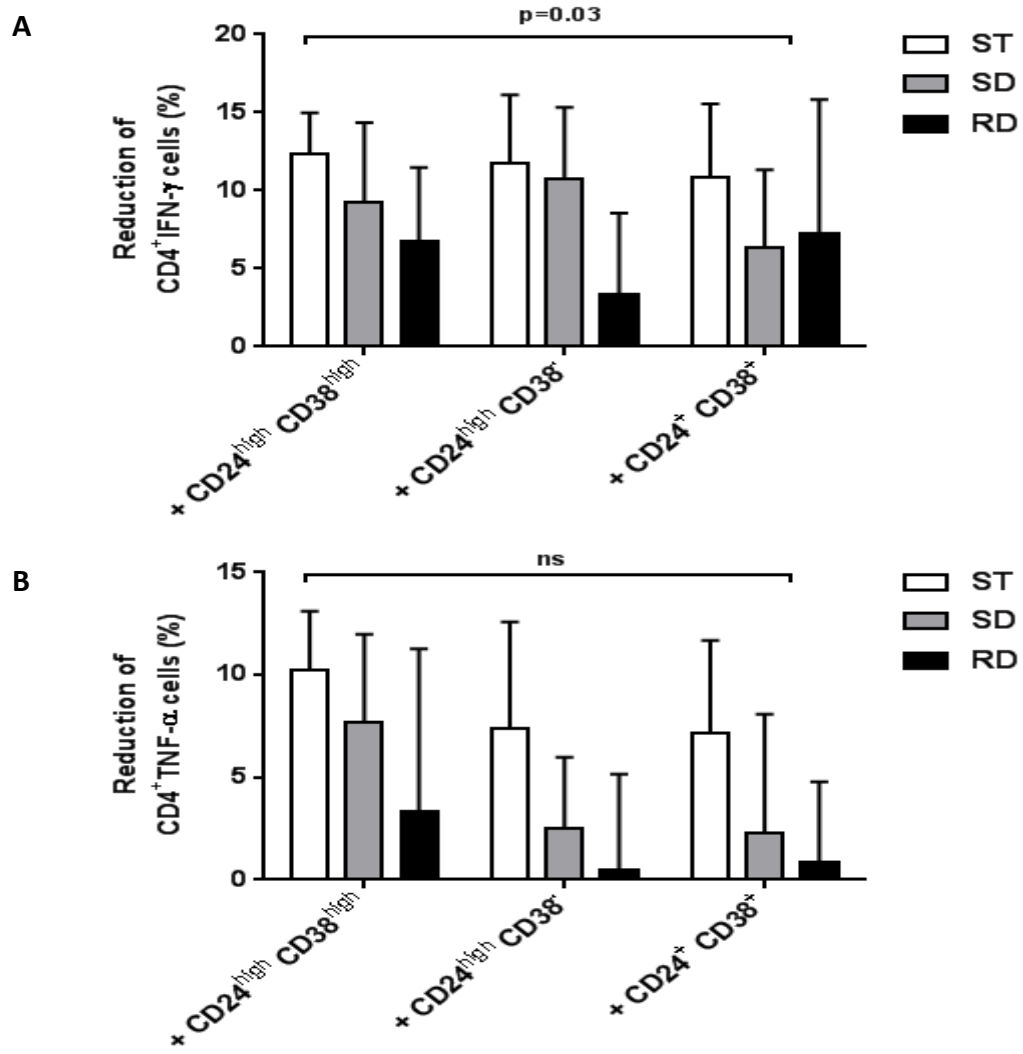


Figure 6.21 Suppression of IFN-γ and TNF-α-producing $CD4^{+}$ T cells by subsets of $CD19^{+}$ CD24 and CD38 expressing cells

CD19, CD24 & CD38 subsets were separated by FACS and cultured 1:1 with autologous naïve T cells activated with anti-CD3 antibody & CD28 for 96 hours. Post-culture cells were stimulated with PMA, ionomycin & monensin for five hours and analysed by flow cytometry for expression of CD4 and intracellular IFN-γ and TNF-α.

The bar-graphs show the mean level of $CD4^{+}IFN\text{-}\gamma^{+}$ (A) and $CD4^{+}$ $TNF\text{-}\alpha^{+}$ (B) cell reduction (error bars represent standard deviation) during co-culture with CD19, CD24 & CD38 subsets compared to $CD4^{+}$ T cells cultured alone.

There was a significant difference in the level of $CD4^{+}IFN\text{-}\gamma^{+}$ reduction between groups ($p = 0.03$) but not $CD4^{+}$ $TNF\text{-}\alpha^{+}$ ($p = 0.13$) by Kruskal-Wallis test. Further examination of $CD4^{+}IFN\text{-}\gamma^{+}$ reduction by Mann-Whitney test could not find any statistically significant differences between groups due to the lack of statistical power; potentially attributable to the small sample size and number of variables.

Suppression of activated CD4⁺ T cells and pro-inflammatory cytokines by CD19⁺CD24^{high}CD38^{high} cells was maximal in patients with stable graft function

To summarise the suppressive capacity of CD19^{+}CD24^{high}CD38^{high} cells on T-cell activation and pro-inflammatory cytokine secretion by each group (Figure 6.22), patients with stable graft function demonstrated the highest reduction in the frequency of activated T cells and both TNF-α and IFN-γ producing T cells. The difference between groups did not quite reach a level of statistical significance (p = 0.05).

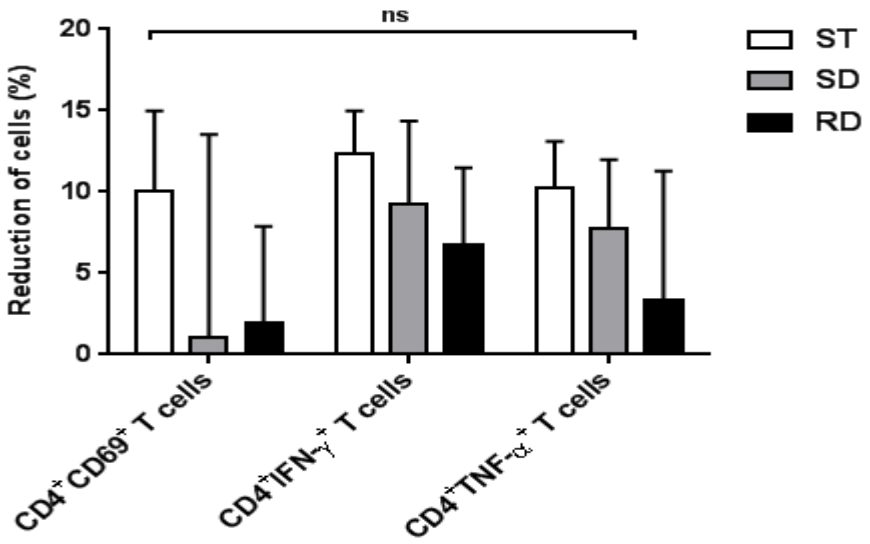


Figure 6.22 Greater reduction of activated T cells, TNF-α and IFN-γ producing T cells following CD4⁺: CD19⁺CD24^{high}CD38^{high} cell co-culture in stable patients
Investigating the reduction of CD4⁺ T cells expressing CD69, IFN-γ and TNF-α in patients with stable (ST) compared to slowly deteriorating (SD) and rapidly deteriorating (RD) graft function following co-culture with CD19⁺CD24^{high}CD38^{high} cells.
The bar-chart shows the mean percentage (error bars represent standard deviation) of CD4⁺ T-cell reduction post-culture. Reduction is based on the difference of CD4⁺ cells cultured alone compared with CD19⁺CD24^{high}CD38^{high} cells. The ST group demonstrated higher reduction of all CD4⁺ T cell subsets. There was no significant difference in the frequency of CD4⁺ T-cell subsets between groups (p=0.05 by Kruskal-Wallis test).

Profiling cytokine expression following $CD4^+$: B cell subset co-culture

Analysing serum and culture supernatant for IL-10

Secretion of IL-10 is a primary mechanism of suppression by B cells. To assess if IL-10 was elevated during $CD4^+$ T cell-culture with $CD19^+CD24^{high}CD38^{high}$ cells, supernatant was collected and analysed by ELISA (Figure 6.23). A uniform observation in all patient groups was that activated T cells produced high levels of IL-10. This was reduced in all the B-cell subset cultures but there was no significant difference between the different subsets or patient groups. Serum samples were also analysed and interestingly IL-10 was only detected in patients with rapidly deteriorating function

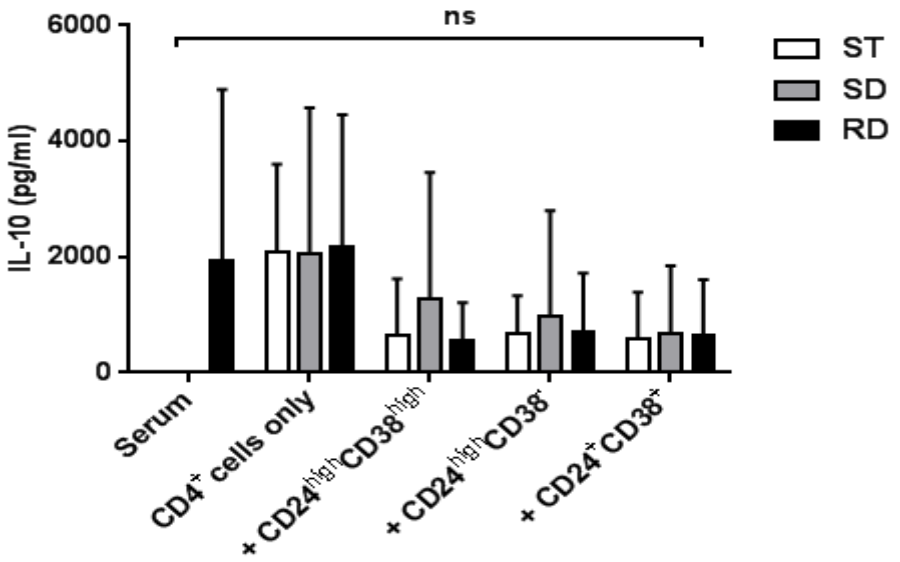
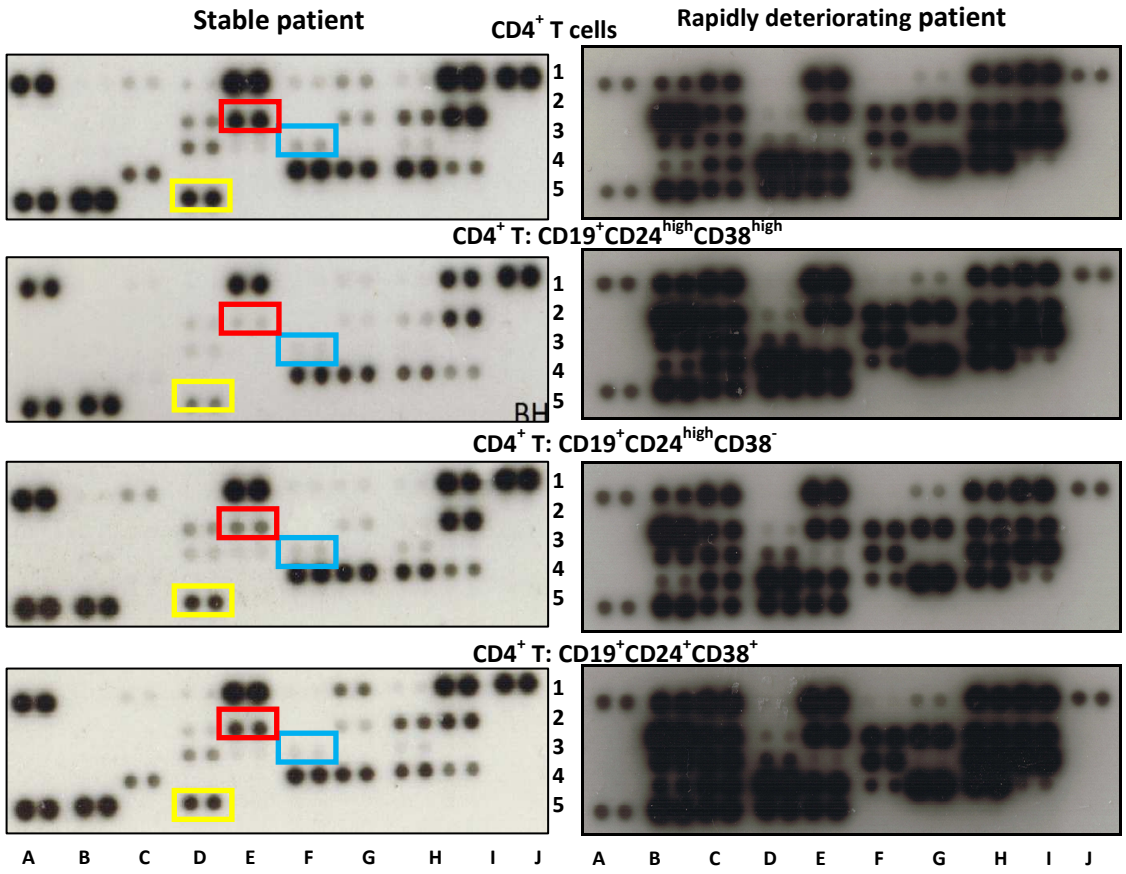


Figure 6.23 Levels of IL-10 in post-culture supernatant were not elevated in the presence of $CD19^+CD24^{high}CD38^{high}$ cells for any group
Serum and post-culture supernatant were analysed for IL-10 by ELISA for patients with stable (ST), slowly deteriorating (SD) or rapidly deteriorating (RD) graft function.
The bar-chart shows the mean level of IL-10 (error bars represent standard deviation) in serum or following cell culture for $CD4^+$ T cells alone or in the presence of subsets isolated from $CD19^+$ cells with different patterns of CD24 and CD38 expression. Activated $CD4^+$ T cells cultured alone produced the highest levels and no significant difference existed between patient groups by Kruskal-Wallis test ($p = 0.6$). Serum IL-10 was only detected in the RD group.

Analysing culture supernatant by cytokine array

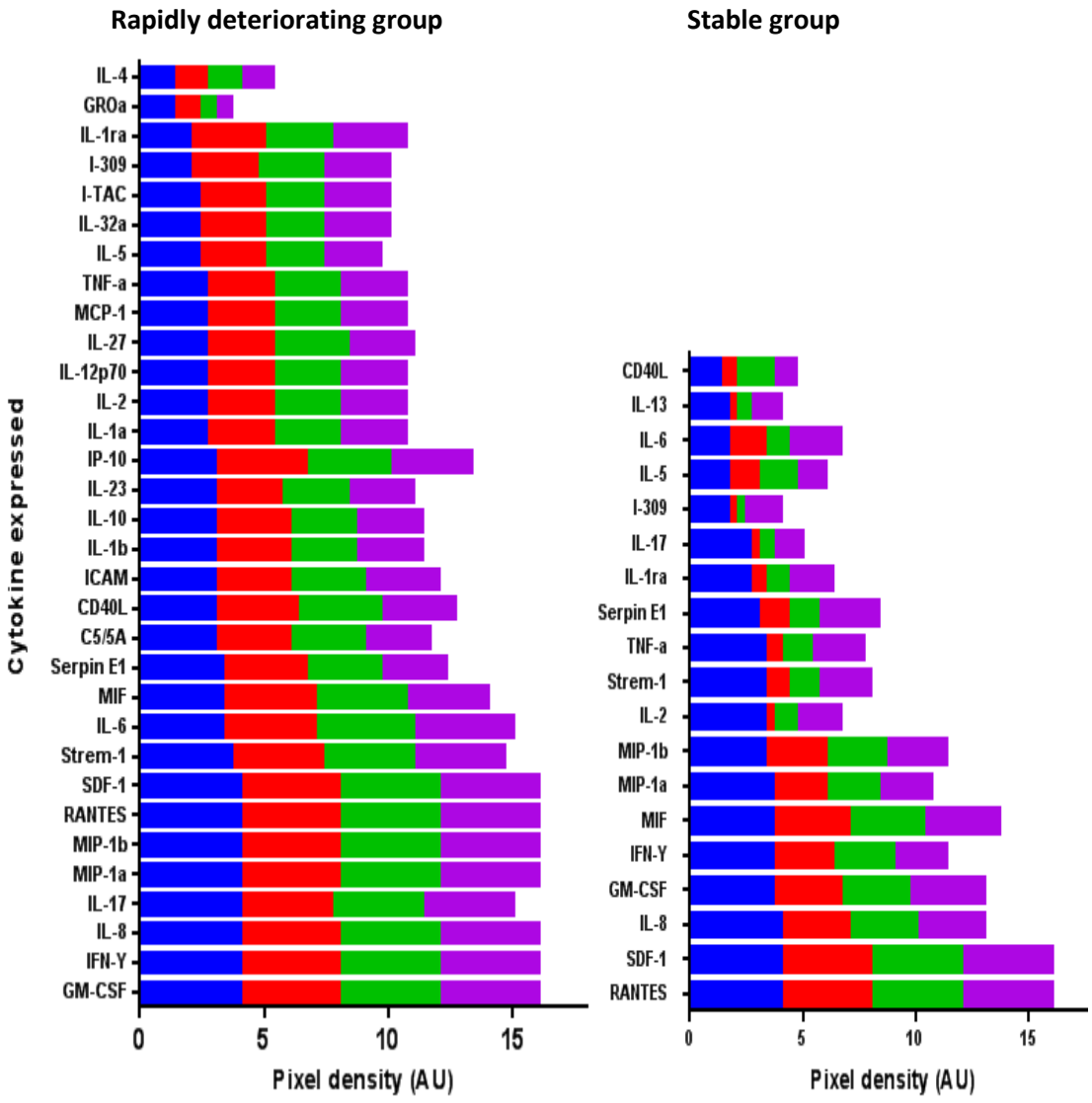
Analysis of IL-10 in serum or post-culture supernatant did not reveal any clear distinction between the cell subsets. Therefore, a wider approach was taken and supernatant was assessed against a panel of cytokines and chemokines associated with T-cell activation. A marked difference was observed in the overall presence of cytokines in supernatant from patients in the ST group (n = 3) compared to the RD group (n = 3, Figure 6.24). The SD group were not included in this analysis due to financial constraints on the project.



	A	B	C	D	E	F	G	H	I	J
1	+ve con.	C5/5a	CD40L	G-CSF	GM-CSF	GROa	I-309	sICAM-1	IFN-γ	+ve con.
2		IL-1α	IL-1β	IL-1ra	IL-2	IL-4	IL-5	IL-6	IL-8	
3		IL-10	IL-12	IL-13	IL-16	IL-17	IL-17E	IL-23	IL-27	
4		IL-32	IP-10	I-TAC	MCP-1	MIF	MIP-1α	MIP-1β	Serpin E1	
5	+ve con.	RANTES	SDF-1	TNF-α	sTREM-1					-ve con.

Figure 6.24 Analysis of culture supernatant by cytokine array assay showed heightened reactivity in those with rapidly deteriorating graft function

Post-culture supernatant from CD4+ T cells cultured alone or with CD19, CD24, CD38 subsets were analysed for the presence of cytokines and chemokines by cytokine array in patients with stable (n = 3) and rapidly deteriorating graft function (n = 3). Supernatant was applied to membranes containing anti-cytokine antibodies. After detection by secondary antibody, a chemiluminiscence signal of the membranes was captured and the intensity of each spot was estimated by densitometric analysis. Representative data are shown from a patient with stable and deteriorating graft function. The table shows the panel of cytokines included in array analysis and the coloured boxes show those significantly reduced (red, IL-2; yellow, TNF-α and blue; IL-17).



Cytokines not detected

G-CSF
IL-13
IL-16
IL-17E

G-CSF	IP-10	GROα
IL-10	IL-12	IL-16
ICAM	IL-23	IL-1β
IL-17E	IL-27	IL-32a
C5/5a	IL-1	IL-4
I-TAC	MCP-1	

Figure 6.25 Increased range and heightened production of cytokines and chemokines during CD4+ T cell: CD19, CD24, CD38 subset co-culture in patients with rapidly deteriorating graft function

The bar graphs shows mean pixel density (arbitrary units; AU) for each cytokine ranging from high (pixel density = 4) to low (pixel density = 1) for stable and rapidly deteriorating patients as derived from the cytokine array assay. Cytokines not detected are listed in the tables.

The assay is semi-quantitative and relative expression levels were determined by analysing pixel density of each spot (Figure 6.25). Cytokines detected in supernatant from the RD group demonstrated reactivity against the majority of the panel and there were no significant differences found when comparing cultures with B-cell subsets to $CD4^+$ T cells alone.

In contrast, the ST group demonstrated less reactivity to the panel. This included IL-10, supporting the previous findings using ELISA. However there was marked reduction of IL-2, TNF-α and IL-17, particularly by $CD19^+CD24^{high}CD38^{high}$ cells when compared with the level of pixel density of $CD4^+$ T cells cultured alone (p=0.003; Figure 6.26). It was not possible to apply any further statistical analysis due to the lack of statistical power governed by the small sample size and number of variables. Figure 6.26 clearly demonstrates that IL-2, TNF-α and IL-17 expression are reduced in the presence of $CD19^+CD24^{high}CD38^{high}$ cells, to a greater extent than co-culture with $CD19^+CD24^{high}CD38^-$ and $CD24^+CD38^+$ cells.

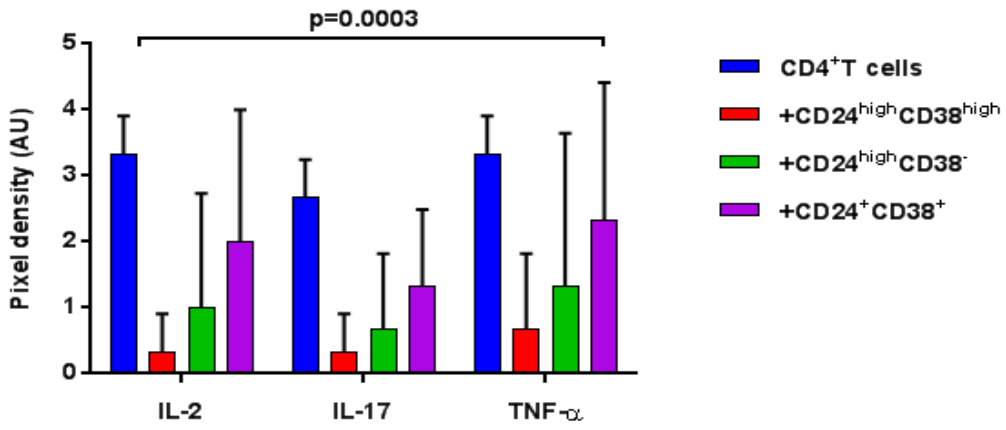


Figure 6.26 IL-2, IL-17 and TNF-α were significantly reduced during $CD4^+$ T cell: $CD19^+CD24^{high}CD38^{high}$ co-culture in patients with stable graft function
The bar–chart shows the mean pixel density (error bars represent standard deviation) for each cytokine analysed by human cytokine array assay for stable patients (n = 3).
Co-culture with $CD19^+CD24^{high}CD38^{high}$ cells caused the greatest reduction in IL-2, TNF-α and IL-17. Comparing $CD4^+$ T cells alone with co-culture with CD19, CD24, CD38 cell subsets was statistically significant by Kruskal-Wallis test (p = 0.0003), however the small sample size lacked the statistical power to perform any further statistical analysis. AU= arbitrary units.

6.4 Discussion

Promoting the longevity of renal allograft survival will prevent patients returning to dialysis and potentially awaiting re-transplantation. The composition of the B-cell compartment may influence a favourable outcome post-transplantation through the suppressive function of IL-10-producing and transitional B cells. This study sought to establish if their prevalence increased in those with stable graft function, and if there was capacity in these cells to attenuate an inflammatory immune response by $CD4^+$ T cells.

IL-10 secretion by B cells is a primary mechanism to suppress other immune cells. This study showed that by stimulating $CD19^+$ cells via TLR-9 it was possible to detect IL-10-producing B cells at low frequency, and the most effective expansion included CD40 and anti-Ig. The mechanism for this would follow the two-step model proposed by Lampropoulou et al., that first TLR stimulation primes B cells towards IL-10 production and second, this is potentiated towards a regulatory phenotype upon BCR and CD40 ligation (Lampropoulou et al., 2010). There are conflicting reports as to the requirement for CD40-CD40L interactions. In animal models of autoimmune disease, CD40 stimulation was critical for suppressive capacity, (Mauri et al., 2003) and mice with CD40-deficent B cells did not recover from experimental autoimmune encephalomyelitis (EAE; (Fillatreau et al., 2002)). In contrast, investigating human IL-10-producing cells found only CpG and anti-Ig were necessary (Bouaziz et al., 2010). In this study, $CD19^+$ cells were stimulated for five days rather than two days as shown by Bouaziz and colleagues. It is possible that cells respond via the innate pathway in the short-term and later by the CD40-dependent adaptive pathway.

The frequency of IL-10-producing B cells was lower in renal transplant recipients than figures reported in the peripheral blood of healthy controls (~5%) (Bouaziz et al., 2010, Iwata et al., 2011, Blair et al., 2010, Das et al., 2012), and is what would be expected given the overall relative lymphopenic state of transplant recipients. When lymphocyte constitution was evaluated, this did not identify any differences between the three

groups of patients. It did however highlight the level of lymphopenia still present at a considerable period of time post-transplantation. With a median of seven years, and a number of patients over twenty years, the average lymphocyte count (~600 cells/µl) remains below the normal range (1000-4800 cells/µl). This is interesting since induction therapy with basiliximab is non-depletional with its target being the IL-2 receptor on activated T cells. It is possible lymphopenia is a consequence of maintenance immunosuppression with MMF, which is known to affect lymphocyte constitution (Heidt et al., 2008). Similarly reconstitution of B cells does not return to the normal range and patients with stable graft function did not display any significant differences in percentage or number of cells. This agrees with data following a larger cohort of renal transplant recipients where B-cell counts were low regardless of allograft stability, even in patients experiencing either subclinical or acute rejection (van de Berg et al., 2012).

For frequency of IL-10-producing B cells, it is difficult to draw a perfect comparison with other studies since the methods used to stimulate cells, culture time and details of analysis by flow cytometry differ. There may also be bias towards certain phenotypes as a consequence of the nature of the stimulation employed. For example in this study and that of Bouaziz and colleagues, anti-Ig stimulation of B cells was included, (Bouaziz et al., 2010) where in others it was not (Das et al., 2012, Iwata et al., 2011). Also as previously mentioned, CD40 is not always included, so there is variation in the methods employed to promote and identify $CD19^{+}IL\text{-}10^{+}$ cells.

Comparing the frequency of IL-10-producing B cells between groups presented clear associations with stable graft function which diminished with deteriorating graft function. A correlation between their presence and a positive impact on disease has also been reported in autoimmunity where patients with SLE (Blair et al., 2010) and multiple sclerosis (Duddy et al., 2007) exhibited reduced frequency. In the temporal setting of viral infection, IL-10-producing B cells were enriched during active flares of hepatitis B (Das et al., 2012). Furthermore in ‘tolerant’ renal transplant recipients, those with stable function in the absence of immunosuppression, there was increased

frequency of IL-10-producing B cells (Newell et al., 2010). In addition they reported IL-10-producing B cells as enriched in the $CD24^{high}CD38^{high}$ cell subset. We also found this association, as have others (Blair et al., 2010, Duddy et al., 2007). As to why IL-10-producing cells reside within this phenotype is not clear; possibly this subset expresses receptors to efficiently stimulate IL-10 secretion. Interestingly in this study, expression of BAFF-R was significantly higher on $IL\text{-}10^{+}$ B cells in stable patients, suggesting transitional cells may be at a higher state of activation.

Assignment of a distinct phenotype demonstrated IL-10-producing B cells were predominantly naïve in stable patients but in other patient groups were at a lower frequency. Naïve B cells constitute a major type in peripheral B cells and may be important in promoting allograft survival. Although ineffective APC for naïve T cells, they are not quiescent as shown in animal models: antigen presented by these cells can induce a tolerant state (Fuchs and Matzinger, 1992) or promote development of Tregs (Reichardt et al., 2007). In renal transplant recipients, an increased number of naïve cells has been associated with tolerance (Newell et al., 2010). Furthermore, following treatment for acute rejection with rituximab, a higher number of naïve B cells correlated with graft survival (Zarkhin et al., 2011). IL-10-producing memory B cells were also investigated and increased frequency was associated with patients with deteriorating function – in both the $IL\text{-}10^{+}$ and total B-cell population. This pattern was a reverse to the observed increased frequency of naïve B cells in the stable group. This shift from naïve to memory B-cell constitution marked declining graft function and could be applied as a negative prognostic marker post-transplantation. Indeed, increased circulating memory B cells have been found during ESRF, (Kim et al., 2012) and associated with the incidence of acute rejection (Zarkhin et al., 2011). However, interpretation may be difficult in immunosuppressed patients who suffer persistent infections.

IL-10 secretion by B cells is a hallmark feature of their regulatory properties and is utilised to identify them in peripheral blood. This requires intracellular analysis, limiting functional investigations due to the fixation and permeabilisation process. To

circumnavigate this, $CD19^{+}CD24^{high}CD38^{high}$ cells are reported to have regulatory capacity (Blair et al., 2010, Flores-Borja et al., 2013) and were selected in this study to investigate functional suppressive properties of B cells. A reduced frequency of $CD19^{+}CD24^{high}CD38^{high}$ cells was a feature of graft dysfunction as patients with deteriorating graft function (slow or rapid) had significantly lower expression compared to stable patients. However even stable patients had a numerical deficit relative to healthy controls. Although only reflective of two individuals, this is consistent with others citing re-constitution at approximately 10% (Blair et al., 2010, Flores-Borja et al., 2013). A reduction in transitional B cells was also observed during ESRF (Kim, Chung et al. 2012), and post-renal transplantation correlated with significantly worse graft function (Cherukuri, Salama et al. 2012). The latter study also measured reconstitution of B-cell subsets following induction with either alemtuzumab or basiliximab and reported an association between higher expression of transitional and naïve B cells with stability of graft function following alemtuzumab. Similarly Heidt et al., report dominance of these B-cell subsets with alemtuzumab and not basiliximab induction (Heidt et al., 2012a). Together these studies suggest alemtuzumab rather than non-depletional induction therapy favours transitional and naïve B cell reconstitution and presumably due to the more profound lymphocyte depletion. In contrast, this study demonstrated correlation of these B-cell subsets and stable graft function following basiliximab induction. A possible explanation is the relatively short post-transplant follow-up time monitoring B-cell repopulation (Cherukuri et al., at 2 years and Heidt et al., up to 12 months post-transplantation). In this study, the median time was seven years and, perhaps given time and favourable conditions, the B-cell compartment will reach a level of homeostasis independent of induction therapy.

As a consequence of identifying $CD19^{+}CD24^{high}CD38^{high}$ cells in the patient groups, an additional subset of cells expressing low levels of CD24 and high levels of CD38 were noted; predominantly in those with deteriorating (slow and rapid) graft function. These $CD19^{+}CD24^{-}CD38^{high}$ cells could be plasmablasts or circulating plasma cells. The inclusion of additional B-cell markers such as CD27 would confirm this as most plasmablasts express high levels of both CD27 and CD38 (Wirths and Lanzavecchia,

2005). Plasma cells can then be distinguished from plasmablasts through expression of CD138 (Medina et al., 2002, Oracki et al., 2010). Further refinement for transitional B-cell selection may include CD10 and IgD as both are associated with T1 and T2 transitional B cells (Blair et al., 2010, Wirths and Lanzavecchia, 2005). Together these markers could provide information as to the possible origin of $CD19^{+}CD24^{-}CD38^{high}$ cells; this may warrant further analysis as to their function and apparent association with deteriorating allograft function.

Evaluating the functional suppressive capacity of transitional B cells demonstrated the $CD19^{+}CD24^{high}CD38^{high}$ cell subset as the most effective inhibitors of T-cell activation in patients with stable graft function and in agreement with observations in healthy individuals (Blair et al., 2010). However while pro-inflammatory cytokines, in particular, IFN-γ, were reduced to the greatest extent in stable patients, this was not entirely restricted to the $CD19^{+}CD24^{high}CD38^{high}$ subset for either IFN-γ or TNF-α production by Th1 cells. These data suggested all of the B-cell subsets had the functional capacity to control excessive production of IFN-γ by inhibiting activation of naïve to effector Th1 cells, and this declined with the rate of deteriorating graft function (slow to rapid). The small sample size number in this study together with the number of variables within the analysis, restricted gaining more detailed statistical information, and the inclusion of more patients in this study may have enriched the data.

While lacking any statistical significance, it was noted that the reduction in $CD4^{+}TNF\text{-}\alpha^{+}$ cells was at its greatest during co-culture with the $CD19^{+}CD24^{high}CD38^{high}$ subset in patients with stable function. It is possible that within the transitional B-cell phenotype there are distinct populations: those which develop into mature B cells and those which assume a regulatory capacity. Perhaps the environment experienced by those with declining graft function favours the promotion of mature cells but without a definitive marker uniquely expressed by Bregs it is difficult to dissect this further. It would also be interesting to explore if effector T cells are converted into Tregs at an increased rate in stable patients, promoting a balance between effector responses and

immune tolerance. In patients with rheumatoid arthritis, $CD19^{+}CD24^{high}CD38^{high}$ cells failed to convert naïve T cells to Tregs or suppress Th17 development as is seen in healthy controls (Flores-Borja et al., 2013). It is therefore reasonable to suppose this mechanism is disrupted following transplantation.

The suppressive activity of $CD19^{+}CD24^{high}CD38^{high}$ cells to inhibit Th1 cell differentiation *in vitro* is mediated by the release of IL-10 (Blair et al., 2010). In this study the concentration of IL-10 following co-culture with naïve T cells was not restricted to this subset. Activated T cells were the highest producers, presumably as a result of the intense method used to promote naïve to effector cells, driving expression of this classically Th2 type cytokine. Interestingly, IL-10 was only detected in serum from patients with rapidly deteriorating graft function which does not correlate with the low frequency of IL-10-producing cells seen in this group, but this may reflect the general immune environment in these patients. As shown by the cytokine array assay, these patients displayed high reactivity to almost the entire panel, suggesting dysregulation of the cytokine network implying an environment which may be hostile for Bregs to reside, affecting stability, rendering the capacity of cells to act in a suppressive manner or promoting differentiation to a mature B-cell phenotype rather than a regulatory one. In contrast, cytokine expression in stable patients demonstrated a much less reactive pattern and importantly indicated $CD19^{+}CD24^{high}CD38^{high}$ cells as mediators in the reduction of TNF-α, IL-2 and IL-17. The reduction of TNF-α supports our previous finding that inhibition of TNF-α–producing $CD4^{+}$ T cells occurs during co-culture with this B-cell subset. In a wider context, the identification of these three cytokines could provide information of the potential mechanisms involved in immune suppression. For example, IL-2 is a key cytokine necessary for proliferation and differentiation of T cells. The involvement of IL-17 has been associated with renal allograft rejection where increased $CD4^{+}IL\text{-}17^{+}$ T cells are described in renal biopsies from patients undergoing acute T-cell-mediated rejection (Loverre et al., 2011). Moreover, serum IL-17 expression is increased in acutely rejecting liver and renal allografts (Crispim et al., 2009, Fabrega et al., 2009).

An important mediator in transplant rejection is the development of HLA antibodies, especially those that are donor-specific. In this small cohort, patients with DSA had a lower frequency of IL-10-producing B cells. This analysis could not be applied to transitional B cells as only two of the fifteen patients studied had DSA. In a larger cohort of renal transplant patients, those with DSA had a significantly lower number of transitional B cells (Cherukuri et al., 2012). It is an attractive possibility that regulatory B cells suppress development of HLA antibodies directed against the allograft.

Concluding remarks

In summary, this study defined an association between increased frequency of IL-10-producing cells and transitional B cells with stable graft function, where in both cases elevated cell frequency correlated with superior graft function. Furthermore transitional B cells from those with stable graft function had superior ability to suppress T-cell activation and secretion of the Th1 cell pro-inflammatory cytokine, TNF-α. These data raise the possibility of analysing and monitoring B-cell phenotypes to predict renal allograft function, and potentially guide immunosuppressive therapy. In addition, heightened activity in the cytokine milieu was characteristic of declining allograft function, suggesting dysregulation of B cells in their ability to restrain inflammation by promoting differentiation of pro-inflammatory over immunoregulatory T cells.

Chapter 7

Conclusion

7.1 Concluding remarks

B cells are increasingly recognised as important mediators in renal transplantation, not only in facilitating AMR, but also in their role in potentially promoting allograft survival. This study aimed to gain a better understanding of the biology that governs the function of these cells by investigating their phenotypic and functional characteristics.

Given the well-defined detrimental effect of HLA antibodies on allograft survival, over the past decade there has been considerable refinement in the methodology to aid their definition. This has benefited patients pre-transplantation through avoidance of DSA, or where DSA cannot be avoided, has allowed monitoring of DSA levels during desensitisation protocols, such that where possible transplantation can proceed if levels are sufficiently reduced. In the post-transplantation period, their identification has allowed for appropriate treatment. These scenarios demonstrate how AMR is effectively avoided and managed. However there are limitations to the approach of assessing B-cell responses by serological measurement of HLA antibody, and this study looked beyond B cells actively secreting antibodies against the allograft.

Limiting analysis of B-cell activity to serum measurement for DSA could be misleading, as it excludes detection of memory B cells which can exist in the absence of detectable levels in serum, as demonstrated against hepatitis B antigen (West and Calandra, 1996, Bauer and Jilg, 2006). In Chapter 3, a method was described to detect antibody-secreting cells directed against HLA molecules expressed on donor cells using an IgG-detecting ELISPOT. This was an attractive approach allowing memory B cells to be detected prior to transplantation, and identify those patients potentially at risk of developing AAMR. However as the results demonstrated, this method failed to effectively discriminate the B-cell response to donor cells from that of third-party cells. Although these data were reflective of a very small cohort, results were not encouraging to pursue further. Since this project progressed to investigate other aspects of the B-cell compartment, Heidt and colleagues published their method of detecting HLA-specific B cells by ELISPOT (Heidt et al., 2012b), demonstrating a

superior approach to the one we described by refining the characterisation of antibody-secreting cells to include details of HLA specificity. However, neither method would be especially adaptable to clinical laboratory practice. Accessibility of donor cells, coupled with a means of irradiation limit the feasibility of these ELISPOT applications, and the approach described in Chapter 4, where HLA-specific B cells were identified from peripheral blood, would be more amenable to routine clinical practice.

Measuring the presence of circulating B cells with donor-antigen specificity is an attractive concept to assess the risk of rejection, and may influence decision-making in the type and amount of immunosuppression. Using the methods described in Chapter 4, donor HLA-specific B cells (DS-B) cells could be effectively identified in sensitised recipients. The further demonstration that their frequency correlated with level of serum HLA antibody suggested a level of both sensitivity and specificity. Once methods to detect DS-B cells had been established and optimised in patients with serum HLA antibodies against HLA-A*0201, it was of interest to investigate DS-B cells from patients transplanted with an allograft expressing HLA-DQB1*0301 (DQ7) who had developed *de novo* donor-specific HLA antibodies (dnDSA). In our centre, and also reported by DeVos and colleagues (DeVos et al., 2012), this was the most common DQ antibody in those with dnDSA. Again the frequency of DS-B cells correlated with the level of serum anti-HLA-DQ7. While there was no specific phenotypic signature associated with these cells, they were present at significantly higher frequency than DS-B cells from patients with dnDSA against HLA-A2. Moreover the frequency of transitional B cells, attributed with regulatory capacity (Blair et al., 2010, Flores-Borja et al., 2013) were reduced in patients with dnDSA against HLA-DQ7, and the proportion of patients with rapidly deteriorating graft function was greater compared to those with dnDSA against HLA-A2. Together these data suggest transitional B cells may serve to protect allograft survival. However investigating if the DQ7 allele was perhaps more immunogenic than others by measuring clinical outcomes in a larger cohort with dnDSA did not suggest anti-DQ7 DSA were more pathogenic than dnDSA of other specificities. However it did suggest that HLA-DQ antibodies require equal consideration as is currently given to those with HLA-A, -B and –DR. As routine

monitoring of HLA antibodies becomes more frequent, the long-term impact of HLA-DQ DSA will become clearer.

Measuring a product of B-cell activation in serum could allow for monitoring of an evolving B-cell compartment, and BAFF is an attractive candidate for this given its essential role in B-cell maturation and survival (Mackay et al., 2003, Groom et al., 2007). Excess serum BAFF has been associated with rejection and the development of DSA (Thibault-Espitia et al., 2012, Banham et al., 2013a, Xu et al., 2009b), and Chapter 5 described how levels of BAFF were investigated in transplanted patients, to identify if a relationship existed between differential expression and level of allograft function. The data demonstrated that elevated serum BAFF, low BAFF-R and DSA were all associated with deteriorating graft function. While serum BAFF was elevated in those with both slowly and rapidly deteriorating allografts, the reduced percentage of BAFF-R expressing B cells was restricted to those with rapidly deteriorating graft function. This suggests that serum BAFF levels may be governed by allograft stability rather than the quality of graft function, and for BAFF-R expressing B cells it is the quality of graft function that is important.

This study provides evidence for measuring BAFF as a biomarker of deteriorating graft function. In addition, the prevalence of DSA in patients with elevated serum BAFF is suggestive of deregulated homeostasis in the B-cell compartment. To support the clinical application of measuring serum BAFF levels, a number of investigations were performed. First, the ability to demonstrate serum BAFF levels increased prior to serum creatinine was not successful due to the limited number of stored samples available for the duration of a patient's functioning allograft. However, the increased surveillance of DSA in patients over recent years will result in more detailed collections of stored serum and this question may be addressed. Second, as to whether levels of serum BAFF increased as a potential bystander effect of raised creatinine were investigated. By measuring levels in patients with diseases lacking B-cell involvement this was eliminated as a contributing factor. Finally, expression of CD19, BAFF and BAFF-R were examined at the intrarenal level. The identification of BAFF and BAFF-R

within lymphoid aggregates of renal biopsy tissue in patients with AAMR placed BAFF at the site of renal injury. Together, these data promote the concept of serum BAFF as a marker of B-cell mediated pathology, and future studies in larger cohorts of patients could add validity to these findings.

The final chapter of this thesis investigated B cells in a protective, rather than pathological role. The recent identification of a small population of human B cells with suppressive capacity, (Blair et al., 2010, Flores-Borja et al., 2013, Iwata et al., 2011, Yanaba et al., 2008) prompted the question as to their prevalence in patients with stable compared to deteriorating graft function. Since a definitive phenotype of Bregs is yet to be demonstrated, methods for identification of IL-10-producing B cells followed those previously described (Bouaziz et al., 2010, Das et al., 2012). These cells associated with stable graft function and their predominance with transitional & naïve B cells and a paucity of memory B cells suggested a regulatory response serving to limit immunopathology to prevent rejection. However, the lack of IL-10 in both serum and supernatant from subsets of cells expressing CD19, CD24 and CD38 cultured with $CD4^+$ T cells were not supportive of a role of IL-10, and it is possible other mechanisms served by B-cell subsets contribute to immunological graft stability. This was highlighted by the identification that levels of IL-2 and IL-17 were reduced in $CD4^+$ T cells co-cultured with $CD19^+CD24^{high}CD38^{high}$ cells.

The role of B cells beyond antibody production or antigen-presentation to promoting allograft survival has only re-emerged in recent years, and whether these positive attributes of only a very small number of cells can supersede negative pro-inflammatory effects are largely unknown. Further investigations into whether Bregs can promote Tregs, which in turn prevail over Th1 or Th17 activation could provide more detailed information of the role of Bregs, and may suggest that targeting B cells for depletion may be detrimental for long-term allograft survival.

In summary, this study has demonstrated methods for: effective detection and phenotyping of HLA-specific B cells, measuring serum BAFF levels as an indication of graft function and identifying populations of B cells described to have regulatory capacity. Together these data increase our understanding of B cells in renal transplant recipients, and may have important implications in the clinical setting for predicting the risk of rejection thus dictating immunosuppressive therapy and ultimately promoting allograft function and survival. However it is recognised that graft outcomes are affected by non-immunological mechanisms. It is also recognised that the studies described in this thesis are representative of a small number of patients, at a single time-point post-transplantation. On several occasions during this study, the small sample size together with the number of variables to assess resulted in a lack of statistical power to perform analysis which may have otherwise been significant had the sample size been larger. Therefore future studies should include larger cohorts, to monitor phenotypic stability or changes in the B-cell compartment over a longer time-frame, and to explore the functional versatility of B cells. This would benefit our knowledge of how the favourable attributes of B cells could be promoted, while targeting their harmful effects.

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APPENDICES

Appendix A

A1 Approved original application (2007)

A2 Approved application upon site change to Barts and London NHS trust (2011)

A3 Approved notice of substantial amendment (2013)

Appendix B

B1 Abstracts arising from the thesis

Appendix A1

Approved original application (2007)

[redacted] APPLICANT'S CHECKLIST

All studies except clinical trials of investigational medicinal products

REC Ref:	
Short Title of Study:	Immunological monitoring of B cells in transplant recipients v1
CI Name:	Dr Anthony N Warrens
Sponsor:	Imperial College London

Please complete this checklist and send it with your application

- Send ONE copy of each document (except where stated)
- ALL accompanying documents must bear version numbers and dates (except where stated)
- When collating please do NOT staple documents as they will need to be photocopied.

Document	Enclosed?	Date	Version	Office use
Covering letter on headed paper	◉ Yes ○ No	10/07/2007	[redacted]	
NHS REC Application Form, Parts A&B	Mandatory	10/07/2007	1	
Site-Specific Information Form (for SSA)	◉ Yes ○ No	10/07/2007	1	
Research protocol or project proposal (6 copies)	Mandatory	10/07/2007	1	
Summary C.V. for Chief Investigator (CI)	Mandatory	10/07/2007	[redacted]	
Summary C.V. for supervisor (student research)	○ Yes ◉ No			
Research participant information sheet (PIS)	◉ Yes ○ No			
Research participant consent form	◉ Yes ○ No			
Letters of invitation to participants	○ Yes ◉ No			
GP/Consultant information sheets or letters	○ Yes ◉ No			
Statement of indemnity arrangements	○ Yes ◉ No		[redacted]	
Letter from sponsor	◉ Yes ○ No			
Letter from statistician	○ Yes ◉ No		[redacted]	
Letter from funder	○ Yes ◉ No		[redacted]	
Referees' or other scientific critique report	◉ Yes ○ No			
Summary, synopsis or diagram (flowchart) of protocol in non-technical language	○ Yes ◉ No			
Interview schedules or topic guides for participants	○ Yes ◉ No			
Validated questionnaire	○ Yes ◉ No	[redacted]	[redacted]	
Non-validated questionnaire	○ Yes ◉ No			
Copies of advertisement material for research participants, e.g. posters, newspaper adverts, website. For video or audio cassettes, please also provide the printed script.	○ Yes ◉ No			

[redacted]

An application form specific to your project will be created from the answers you give to the following questions.

1. Is your project an audit or service evaluation?

○ Yes ◉ No

2. Select one research category from the list below:

○ Clinical trials of investigational medicinal products
○ Clinical investigations or other studies of medical devices
○ Other clinical trial or clinical investigation
○ Research administering questionnaires/interviews for quantitative analysis, or using mixed quantitative/qualitative methodology
○ Research involving qualitative methods only
◉ Research limited to working with human tissue samples and/or data
○ Research tissue bank

If your work does not fit any of these categories, select the option below:

○ Other research

2a. Please answer the following questions:

a) Will you be taking new tissue samples primarily for research purposes (i.e. excluding surplus tissue)?	◉ Yes	○ No
b) Will you be using newly obtained surplus tissue (i.e. left over from tissue taken in the course of normal clinical care)?	○ Yes	◉ No
c) Will you be using existing stored tissue identifiable to the researcher?	○ Yes	◉ No
d) Will you be using only existing stored tissue not identifiable to the researcher?	○ Yes	◉ No
e) Will you be using identifiable data?	◉ Yes	○ No
f) Will you be using only anonymised or pseudonymised data?	○ Yes	◉ No

3. Is your research confined to one site?

◉ Yes ○ No

4. Does your research involve work with prisoners?

○ Yes ◉ No

5. Do you plan to include in this research adults unable to consent for themselves through physical or mental incapacity?

○ Yes ◉ No

6. Is the study, or any part of the study, being undertaken as an educational project?

○ Yes ◉ No

NHS Research Ethics Committee NHS
Application form for research limited to working with human tissue samples and/or data

This form should be completed by the Chief Investigator, after reading the guidance notes. See glossary for clarification of different terms in the application form.

Short title and version number: (maximum 70 characters – this will be inserted as header on all forms)
Immunological monitoring of B cells in transplant recipients v1

Name of NHS Research Ethics Committee to which application for ethical review is being made:
Hammersmith Hospital

Project reference number from above REC:
Submission date: 12/06/2007

PART A

A1. Title of the research

Full title: Immunological monitoring of B cells in transplant recipients

Key words: B cells; antibodies; kidney transplantaiton; rejection

A2. Chief Investigator

Title:	Dr
Forename/Initials:	Anthony N
Surname:	Warrens
Post:	Reader and Honorary Consultant Physician
Qualifications:	BSc BM BCh DM PhD FRCP
Organisation:	Imperial College London
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Telephone:	020 8383 2307
Fax:	020 8383 2788
Mobile:	07803 941 336

A copy of a current CV (maximum 2 pages of A4) for the Chief Investigator must be submitted with the application

A3. Proposed study dates and duration

Start date: 01/08/2007
End date: 31/07/2010
Duration: Years: 3 ; Months: 0

A4. Primary purpose of the research: *(Tick as appropriate)*

- ☐ Commercial product development and/or licensing
- ☑ Publicly funded trial or scientific investigation
- ☐ Educational qualification
- ☐ Establishing a database/data storage facility
- ☐ Other

Question(s) 5 disabled.

A6. Does this research require site-specific assessment (SSA)? (Advice can be found in the guidance notes on this topic.)

○ Yes ◉ No

If No, please justify:

It involves routine laboratory tests, currently in the use in our laboratory, on blood samples that will be drawn form patients.

If Yes, an application for SSA should be made for each research site on the Site-Specific Information Form and submitted to the relevant local Research Ethics Committee. Do not apply for SSA at sites other than the lead site until the main application has been booked for review and validated by the main Research Ethics Committee.

Management approval to proceed with the research will be required from the R&D office for each NHS care organisation in which research procedures are undertaken. This applies whether or not the research is exempt from SSA. R&D applications in England, Wales and Scotland should be made using the Site-Specific Information Form.

PART A: Section 1

A7. What is the principal research question/objective? *(Must be in language comprehensible to a lay person.)*

Is it possible to predict the risk of a patient developing rejection following transplantation by studying how strongly their cells react to donor cells in the test tube?

A8. What are the secondary research questions/objectives? *(If applicable, must be in language comprehensible to a lay person.)*

None

A9. What is the scientific justification for the research? What is the background? Why is this an area of importance? *(Must be in language comprehensible to a lay person.)*

Organ rejection is still a significant problem in clinical transplantation. We are now beginning to understand that this can often be caused by antibodies. This is of particular interest because we now have techniques avalalable by which antibodies can be removed or neutralised. In this work, we will attempt to predict the likelihood that one individual will generate antibodies to another if he receives an organ from that person. We propose to do so by developing a test for the quantification of cells of the lineage that is responsible for producing antibodies (B cells) which have receptors that will recognise donor cells. This has been done for the other main class of lymphocytes (T cells), but with our increasing knowledge of the potential damage that can be done by antibodies, we belive the time is now right for this to be done for B cells also.

A10-1. Give a full summary of the purpose, design and methodology of the planned research, including a brief explanation of the theoretical framework that informs it. It should be clear exactly what will happen to the research participant, how many times and in what order.

This section must be completed in language comprehensible to the lay person. It must also be self-standing as it will be replicated in any applications for site-specific assessment on the Site-Specific Information Form. Do not simply reproduce or refer to the protocol. Further guidance is available in the guidance notes.

In this work, we will attempt to predict the likelihood that one individual will generate antibodies to another if he receives an organ from that person and that we are able to predict the extent to which a rejection process is due to antibodies after the transplant has taken place. We propose to do so by developing a test for the quantification of cells of the lineage that is responsible for producing antibodies (B cells) which have receptors that will recognise donor cells.

Patients being considered for renal transplantation are currently screened for pre-formed antibodies specific for donor antigens. However, such tests only reflect the current or historical presence of cells that are actively secreting antibodies. The differentiation of immature B cells to plasma cells is only one outcome of B cell activation; memory B cells are also generated and, although they do not secrete antibody, their presence implies that the recipient could mount a highly damaging "second set" response on re-exposure to the same antigen. Finally, we currently have no way of assessing the potential for graft-specific B cell responses in a given donor-recipient combination where the recipient has not been sensitised.

All three of these components of the B cell compartment (plasma cells, memory cells and naïve B cells) may contribute to graft damage, albeit with different degrees of immediacy. Hence, a series of tests that detect the frequency of donor-specific cells of each of these components would potentially be clinically useful in predicting the outcome of a given donor-recipient combination. They could be applied both in advance of a proposed transplant and also as a monitoring tool following transplantation to guide the immunosuppressive management of these patients and thus allow the clinicians to strike an appropriate balance between under- and over-immunosuppression.

A) Development of an ELISPOT assay to determine antigen-specific B cell frequencies:
We have started establishing and validating an IgG-detecting ELISPOT assay. Plastic plates are coated with an anti-human IgG antibody. Cells are then added and the presence of IgG-secreting cells determined by the local capture of secreted IgG by the fixed anti-IgG, producing a "spot". Spots are expressed as spot-forming cells (SFC) per million input cells.

This IgG detection system will be used to determine the frequency of plasma cells and memory B cells. Spots will be produced from each cell type with different kinetics (see below). To determine the frequency of antigen-specific naïve B cells, a parallel ELISPOT assay will be established to detect the secretion of IgM. This will use the same principle employed above, but with a human IgM-specific capture antibody bound to the plate.

Before applying this assay to donor-specific B lymphocytes, we will first determine the sensitivity, specificity and reproducibility of the test and optimise the time courses necessary to identify each cell type using a less complex nominal antigen-specific response to hepatitis B vaccine. This is useful because it is easy to find individuals who are hepatitis-immune and other who are hepatitis-naïve.

Sixteen volunteers who have previously been immunised with hepatitis B vaccine will have 10 mL blood drawn to show that they are immune on the basis a high antigen-specific titre. On a second occasion, peripheral blood mononuclear cells will be isolated from 100 mL fresh blood drawn from these volunteers. Control samples will be taken from sixteen individuals known to have been neither infected with the virus nor immunised in whom the absence of specific antibody will be confirmed. They will be subjected to the same two rounds of venesection.

Cells will then be cultured in the presence or absence of the immunising hepatitis B antigen for various periods of time in order to induce (in the case of the immunised individuals) memory B cells to differentiate into plasma cells. It is predicted that spots will develop in assays performed on cells cultured for 1-2 days, representing secretion from plasma cells, and also on cells cultured for 3-5 days, representing secretion from memory cells that had then differentiated into plasma cells. If this is confirmed, the frequency of plasma cells will be calculated from the number of "early" spots and the frequency of memory cells from the difference between the number of "late" and "early" spots. In the case of naïve individuals, this culture will be expected to generate antigen-specific IgM-secreting cells from primary B cells over a period of 7-10 days. The types of cells will be confirmed by studying their surface markers.

B) Development of parallel limiting dilution assay (LDA) analysis to determine antigen-specific B cell

precursor frequencies
This will be performed in a subset (eight controls and eight hepatitis-immune volunteers) of the above volunteers from whom 200mL rather than 100mL of blood will be drawn. There is no "gold standard" with which to compare the data obtained from the above ELISPOT system since this represents the measuring of a hitherto unmonitored component of immunity. Accordingly, we feel it would be appropriate to compare these data in parallel with an alternative established assay of precursor frequency, the limiting dilution assay (LDA). Lymphocytes will be prepared as described above, including their isolation form peripheral blood and their culturing with antigen for various periods of time. Then, rather than be used in an ELISPOT assay, they will be used in a LDA.

Cell populations will be purified and plated out in limiting numbers so that frequencies of antibody producing cells can be determined. Antibody production will be determined by harvesting the supernatants and assaying for human IgG (memory or plasma cells) or IgM (naïve B cells) using ELISA based-methods. Optimised LDA assays incorporating ELISA-based readouts for T cell sub-populations and their effector cytokines are well established within our department.

C) Application of these tests of B cell precursor frequencies to clinical renal transplantation
Having validated the assay in this simple nominal antigen system, we shall next apply it to the determination of humoral reactivity against donor antigens. We shall identify sixteen individuals who have received living donor kidney transplants and who themselves and whose donors are prepared to make blood available for this project. We shall identify eight recipients who have never undergone antibody-mediated rejection and eight who have.

200 mL of blood will be drawn from each of the recipients and 100 mL from the donors and used in the assays outlined above.

We further predict that it will be possible using these assays on pre-transplant samples to differentiate early from late humoral rejection. We are currently expanding our live donor transplant programme in West London and are currently performing between 10 and 15 live donor transplants a month. Hence, we predict that during the course of this project it will be possible to examine eight patients prospectively and, depending on there being large enough numbers of people with rejection, differentiate (i) those who have mounted a antibody-mediated rejection episode in the first ten days following transplantation (in whom we predict we will detect pre-existing memory B cells) from (ii) those who have mounted their first antibody-mediated rejection episode after the first three weeks following transplantation (in whom we predict we will detect a high level of naïve antigen-specific B cells, implying a de novo B cell response) and (iii) those who never mount a antibody-mediated rejection episode (in whom we predict there will be low levels of naïve and memory B cells and plasma cells). We expect to request the drawing of 50 mL of blood on three occasions on each of these recipients and their donors.

A10-2. In which parts of the research have patients, members of the public or service users been involved?

- ☐ As user-researchers
- ☐ As members of a research project group
- ☐ As advisor to a project
- ☐ As members of a departmental or other wider research strategy group
- ☑ None of the above

Please provide brief details if applicable:

A10-3. Could the research lead to the development of a new product/process or the generation of intellectual property?

○ Yes ○ No ◉ Not sure

Question(s) 11 disabled.

A12. Give details of any clinical intervention(s) or procedure(s) to be received by research participants over and above those which would normally be considered a part of routine clinical care. *(These include uses of medicinal products or devices, other medical treatments or assessments, mental health interventions, imaging investigations and taking samples of human biological material.)*

Additional Intervention	Average number per participant		Average time taken (mins/hours/days)	Details of additional intervention or procedure, who will undertake it, and what training they have received.
	Routine Care	Research		
Venepuncture	0	2	seconds	Blood will be taken only by people who normally take blood as part of their clinical duties.
Venepuncture	3	0	seconds	Blood will be taken only by people who normally take blood as part of their clinical duties. In these cases additional blood will be taken during venepuncture being performed for the purposes of routine clinical care

A13. Give details of any non-clinical research-related intervention(s) or procedure(s). *(These include interviews, non-clinical observations and use of questionnaires.)*

Additional Intervention	Average number per participant	Average time taken (mins/hours/days)	Details of additional intervention or procedure, who will undertake it, and what training they have received.

Question(s) 14 disabled.

A15. What is the expected total duration of participation in the study for each participant?

Either a one-off donation of blood or a series of doantions over 12 months

Question(s) 16 disabled.

A17. What is the potential for pain, discomfort, distress, inconvenience or changes to lifestyle for research participants?

None beyond the trivial discomfort of venepuncture.

A18. What is the potential for benefit to research participants?

None until after the completion of the study.

A19. What is the potential for adverse effects, risks or hazards, pain, discomfort, distress, or inconvenience to the researchers themselves? *(if any)*

None

A20. How will potential participants in the study be (i) identified, (ii) approached and (iii) recruited?
Give details for cases and controls separately if appropriate:

1) Laboratory and clinical staff will be invited to doante as part of the inital studies.
2) All live donor–recipient paris at the West London Renal and Transplant centre will be considered. They will be approached during the course of their clincial care by one of the team looking after them. the purpose of the research will be explained and they will be given the opportunity to ask questions. It will be made clear that refusal to particiapte will in no way compromise their clinical care.

A21. Where research participants will be recruited via advertisement, give specific details.

☑ Not Applicable

If applicable, enclose a copy of the advertisement/radio script/website/video for television (with a version number and date).

A22. What are the principal inclusion criteria? *(Please justify)*

1) Hepatitis immune and non-immmune labopratory volunteers.
2) Live donor-recipient pair who are undergoing or who have unergone renal donation and transplantation at the West London Renal and Transplant Centre.

A23. What are the principal exclusion criteria? *(Please justify)*

Donor and recipient unable to give informed consent.

A24. Will the participants be from any of the following groups? *(Tick as appropriate)*

- ☐ Children under 16
- ☐ Adults with learning disabilities
- ☐ Adults who are unconscious or very severely ill
- ☐ Adults who have a terminal illness
- ☐ Adults in emergency situations
- ☐ Adults with mental illness (particularly if detained under Mental Health Legislation)
- ☐ Adults with dementia
- ☐ Prisoners
- ☐ Young Offenders
- ☐ Adults in Scotland who are unable to consent for themselves
- ☑ Healthy Volunteers
- ☑ Those who could be considered to have a particularly dependent relationship with the investigator, e.g. those in care homes, medical students
- ☐ Other vulnerable groups

Justify their inclusion.

In order to establish the assay, we plan to use hepatitis B-immune and non-immune people. This is a good system since the antigen is known and available and an individual's immune status will be known to him/herself. The amount of blood will be small for a health person.

In order to ensure that no corercion will be involved, a notice will be put up asking people to volunteer to give blood to this study.

☐ No participants from any of the above groups

Question(s) 24 1-5 disabled.

A25. Will any research participants be recruited who are involved in existing research or have recently been involved in any research prior to recruitment?

◉ Yes ○ No ○ Not Known

If Yes, give details and justify their inclusion. If Not Known, what steps will you take to find out?

A large proportion of transplant recipeints are in controlled trials in our unit in order to be continually developing improvements in treatment. Since this is not an interventioanl study, this will not be a confounding feature.

A26. Will informed consent be obtained from the research participants?

◉ Yes ○ No

If Yes, give details of who will take consent and how it will be done. Give details of any particular steps to provide information (in addition to a written information sheet) e.g. videos, interactive material.

If participants are to be recruited from any of the potentially vulnerable groups listed in A24, give details of extra steps taken to assure their protection. Describe any arrangements to be made for obtaining consent from a legal representative.

If consent is not to be obtained, please explain why not.

Consent will be taken by a study investigator or a registrar or consultant in the Renal Unit.

Copies of the written information and all other explanatory material should accompany this application.

A27. Will a signed record of consent be obtained?

◉ Yes ○ No

If Yes, attach a copy of the information sheet to be used, with a version number and date.

A28. How long will the participant have to decide whether to take part in the research?

As long as they wish.

A29. What arrangements have been made for participants who might not adequately understand verbal explanations or written information given in English, or who have special communication needs? *(e.g. translation, use of interpreters etc.)*

All patients will have arrangements in place for translation, if necessary, to obtain consent for surgery. This will be used to seek research consent also.

Question(s) 30 disabled.

A31. Does this study have or require approval of the Patient Information Advisory Group (PIAG) or other bodies with a similar remit? *(see the guidance notes)*

○ Yes ◉ No

A32a. Will the research participants' General Practitioner (and/or any other health professional responsible for their care) be informed that they are taking part in the study?

○ Yes ◉ No

If Yes, enclose a copy of the information sheet/letter for the GP/health professional with a version number and date.

A32b. Will permission be sought from the research participants to inform their GP or other health professional before this is done?

○ Yes ◉ No

If No to either question, explain why not

This study has no implicaitons for their subsequent care or for any adverse effects with which they may present to their GPs.

It should be made clear in the patient information sheet if the research participant's GP/health professional will be informed.

A33. Will individual research participants receive any payments for taking part in this research?

○ Yes ◉ No

A34. Will individual research participants receive *reimbursement of expenses* or any other *incentives or benefits* for taking part in this research?

○ Yes ◉ No

A35. Insurance/indemnity to meet potential legal liabilities

Note: References in this question to NHS indemnity schemes include equivalent schemes provided by Health and Personal Social Services (HPSS) in Northern Ireland.

A35–1. What arrangements will be made for insurance and/or indemnity to meet the potential legal liability of the *sponsor(s)* for harm to participants arising from the *management of the research*?

Note: Where a NHS organisation has agreed to act as the sponsor, indemnity is provided through NHS schemes. Indicate if this applies (there is no need to provide documentary evidence). For all other sponsors, describe the arrangements and provide evidence.

○ NHS indemnity scheme will apply
◉ Other insurance or indemnity arrangements will apply (give details below)

Imperial College London indemnification

Please enclose a copy of relevant documents.

A35–2. What arrangements will be made for insurance and/or indemnity to meet the potential legal liability of the *sponsor(s) or employer(s)* for harm to participants arising from the *design of the research*?

Note: Where researchers with substantive NHS employment contracts have designed the research, indemnity is provided through NHS schemes. Indicate if this applies (there is no need to provide documentary evidence). For other protocol authors (e.g. company employees, university members), describe the arrangements and provide evidence.

○ NHS indemnity scheme will apply to all protocol authors
◉ Other insurance or indemnity arrangements will apply (give details below)

Imperial College London indemnification

Please enclose a copy of relevant documents.

A35–3. What arrangements will be made for insurance and/or indemnity to meet the potential legal liability of *Investigators/collaborators* and, where applicable, *Site Management Organisations*, arising from harm to participants in the *conduct of the research*?

Note: Where the participants are NHS patients, indemnity is provided through NHS schemes or through professional indemnity. Indicate if this applies to the whole of the study (there is no need to provide documentary evidence). Where non–NHS sites are to be included in the research, including private practices, describe the arrangements which will be made at these sites and provide evidence.

○ All participants will be recruited at NHS sites and NHS indemnity scheme or professional indemnity will apply
◉ Research includes non–NHS sites (give details of insurance/indemnity arrangements for these sites below)

Imperial College London indemnification

Please enclose a copy of relevant documents.

Question(s) 36 disabled.

A37. How is it intended the results of the study will be reported and disseminated? *(Tick as appropriate)*

- [x] Peer reviewed scientific journals
- [x] Internal report
- [x] Conference presentation
- [ ] Other publication
- [ ] Submission to regulatory authorities
- [ ] Access to raw data and right to publish freely by all investigators in study or by Independent Steering Committee on behalf of all investigators
- [ ] Written feedback to research participants
- [ ] Presentation to participants or relevant community groups
- [ ] Other/none e.g. Cochrane Review, University Library

A38. How will the results of research be made available to research participants and communities from which they are drawn?

None beyond answers to A37

A39. Will the research involve any of the following activities at any stage (including identification of potential research participants)? *(Tick as appropriate)*

- [ ] Examination of medical records by those outside the NHS, or within the NHS by those who would not normally have access
- [ ] Electronic transfer by magnetic or optical media, e-mail or computer networks
- [ ] Sharing of data with other organisations
- [ ] Export of data outside the European Union
- [ ] Use of personal addresses, postcodes, faxes, e-mails or telephone numbers
- [ ] Publication of direct quotations from respondents
- [ ] Publication of data that might allow identification of individuals
- [ ] Use of audio/visual recording devices
- [ ] Storage of personal data on any of the following:
  - [ ] Manual files including X-rays
  - [x] NHS computers
  - [ ] Home or other personal computers
  - [ ] University computers
  - [ ] Private company computers
  - [ ] Laptop computers

Further details:

Password-protected files with master data and codes idetifying patines. In all other places, these anonymised codes will be used.

A40. What measures have been put in place to ensure confidentiality of personal data? Give details of whether any encryption or other anonymisation procedures have been used and at what stage:

Password-protection of any files from which it would be possible to identify anyone.

A41. Where will the analysis of the data from the study take place and by whom will it be undertaken?

Hammersmith Hospitals NHS Trust

A42. Who will have control of and act as the custodian for the data generated by the study?

The Principal Investigator

A43. Who will have access to research participants' or potential research participants' health records or other personal information? *Where access is by individuals outside the normal clinical team, justify and say whether consent will be sought.*

No-one ooutside the normal clinical team

A44. For how long will data from the study be stored?

5 Years **0** Months

Give details of where they will be stored, who will have access and the custodial arrangements for the data:

Password protection on NHS computer with access only to research team who also have normal clinical access to patient data.

A45–1. How has the scientific quality of the research been assessed? *(Tick as appropriate)*

- [x] Independent external review
- [ ] Review within a company
- [ ] Review within a multi–centre research group
- [x] Review within the Chief Investigator's institution or host organisation
- [ ] Review within the research team
- [ ] Review by educational supervisor
- [ ] Other

Justify and describe the review process and outcome. If the review has been undertaken but not seen by the researcher, give details of the body which has undertaken the review:

The details of the project have been reviewed within the Principal Investigator's institution by individuals knowledgeable in this area of science and medicine but who are not directly involved in this project.

It has also been reviewed externally in an application for funding from NHS Blood and Transplant, an application which we have been told has been provisionally successful.

A45–2. How have the statistical aspects of the research been reviewed? *(Tick as appropriate)*

- [ ] Review by independent statistician commissioned by funder or sponsor
- [ ] Other review by independent statistician
- [ ] Review by company statistician
- [ ] Review by a statistician within the Chief Investigator's institution
- [ ] Review by a statistician within the research team or multi–centre group
- [x] Review by educational supervisor
- [ ] Other review by individual with relevant statistical expertise

In all cases give details below of the individual responsible for reviewing the statistical aspects. If advice has been provided in confidence, give details of the department and institution concerned.

Title:	Forename/Initials:	Surname:
Dr	Anthony	warrens

Department:	Immunology
Institution:	Imperial College London
Work Address:	Department of Immunology Hammersmith Campus, Imperial College LOndon
Postcode:	W12 0HS
Telephone:	020 8383 2307
Fax:	020 8383 2788
Mobile:	07803 941 336
E–mail:	a.warrens@imperial.ac.uk

Please enclose a copy of any available comments or reports from a statistician.

Question(s) 46–47 disabled.

A48. What is the primary outcome measure for the study?

Extent of correlation between in vitro levels of B cell anti–donor activity and clinical outcomes.

A49. What are the secondary outcome measures? *(if any)*

None

A50. How many participants will be recruited?
If there is more than one group, state how many participants will be recruited in each group. For international studies, say how many participants will be recruited in the UK and in total.

16 hepatitis–immune and 16 non–hepatitis immune volunteers
24 kidney donors and 24 kidney transplant recipients.

A51. How was the number of participants decided upon?

Formal sample size calculation

If a formal sample size calculation was used, indicate how this was done, giving sufficient information to justify and reproduce the calculation.

Assuming a mean number of "spots" in the control group of 100 and of 150 in the "Rejector" group with a value of sigma of 50, the number of patients required in each group to identify a difference at a significance level of 0.05 with a power of 80% is 16. To allow for technical faiures, this is being increased to 25.

A52. Will participants be allocated to groups at random?

○ Yes ◉ No

A53. Describe the methods of analysis (statistical or other appropriate methods, e.g. for qualitative research) by which the data will be evaluated to meet the study objectives.

Parametric significance testing

A54. Where will the research take place? *(Tick as appropriate)*

- [x] UK
- [ ] Other states in European Union
- [ ] Other countries in European Economic Area
- [ ] Other

If Other, give details:

A55. Has this or a similar application been previously rejected by a Research Ethics Committee in the UK, the European Union or the European Economic Area?

- ○ Yes
- ◉ No

A56. In how many and what type of host organisations (NHS or other) in the UK is it intended the proposed study will take place?

Indicate the type of organisation by ticking the box and give approximate numbers if known:

	Number of organisations
☑ Acute teaching NHS Trusts	
☐ Acute NHS Trusts	
☐ NHS Primary Care Trusts or Local Health Boards in Wales	
☐ NHS Trusts providing mental healthcare	
☐ NHS Health Boards in Scotland	
☐ HPSS Trusts in Northern Ireland	
☐ GP Practices	
☐ NHS Care Trusts	
☐ Social care organisations	
☐ Prisons	
☐ Independent hospitals	
☐ Educational establishments	
☐ Independent research units	
☐ Other (give details)	

Other:

A57. What arrangements are in place for monitoring and auditing the conduct of the research?

Either or both of Hammersmith Hospitals NHS Trust or Imperial College may audit the study as part of the requirements under the research Governance framework.

Question(s) 57a disabled.

A58. Has external funding for the research been secured?

◉ Yes ○ No

If Yes, give details of funding organisation(s) and amount secured and duration:

Organisation:	NHS Blood and Transplant
Address:	Prof D Anstee, Research Coordinator, NHS Blood National Blood Service Southmead Road, Bristol
Post Code:	BS10 5ND
UK contact:	
Telephone:	0117 991 2100
Fax:	0117 959 1660
Mobile:	
E-mail:	
Amount (£):	TBC Duration: 24 Months

A59. Has the funder of the research agreed to act as sponsor as set out in the Research Governance Framework?

○ Yes ◉ No

Has the employer of the Chief Investigator agreed to act as sponsor of the research?

◉ Yes ○ No

Lead sponsor *(must be completed in all cases)*

Name of organisation which will act as the lead sponsor for the research:

Imperial College London

Status:

○ NHS or HPSS care organisation ◉ Academic ○ Pharmaceutical industry ○ Medical device industry ○ Other

If Other, please specify:

Address:	Clinical Research Office, G02 Sir Alexander Fleming Building Imperial College London, Exhibition Road, London
Post Code:	SW7 2AZ
Telephone:	020 7594 1188
Fax:	020 7594 1792
Mobile:	
E-mail:	gary.roper@imperial.ac.uk

Sponsor's UK contact point for correspondence with the main REC *(must be completed in all cases)*

Title: Mr	Forename/Initials: Gary	Surname: Roper
Work Address:	Hammersmith Campus Ducane Road London	
Post Code:	W12 0HS	
Telephone:		
Fax:		
Mobile:		
E-mail:		

Co-sponsors

Are there any co-sponsors for this research?

○ Yes ◉ No

A60. Has any responsibility for the research been delegated to a subcontractor?

○ Yes ◉ No

A61. Will individual *researchers* receive any personal payment over and above normal salary for undertaking this research?

○ Yes ◉ No

A62. Will individual *researchers* receive any other benefits or incentives for taking part in this research?

○ Yes ◉ No

A63. Will the host organisation or the researcher's department(s) or institution(s) receive any payment or benefits in excess of the costs of undertaking the research?

○ Yes ◉ No

A64. Does the Chief Investigator or any other investigator/collaborator have any direct personal involvement (e.g. financial, share-holding, personal relationship etc.) in the organisations sponsoring or funding the research that may give rise to a possible conflict of interest?

○ Yes ◉ No

A65. Research reference numbers: *(give any relevant references for your study):*

Applicant's/organisation's own reference number, e.g. R&D (if available):
Sponsor's/protocol number:
Funder's reference number:
Project website:

A66. Other key investigators/collaborators *(all grant co-applicants or protocol co-authors should be listed)*

Title: Dr	Forename/Initials: Paul	Surname: Brookes
Post:	Senior Sceintist	
Qualifications:	PhD	
Organisation:	Hammersmith Hospitals NHS Trust	
Work Address:	Immunology, G Block Hammersmith Hospital London	
Postcode:	W12 0HS	
Telephone:	020 8383 3226	
Fax:		
Mobile:		
E-mail:	p.a.brookes@imperial.ac.uk	

Title: Dr	Forename/Initials: Cristina	Surname: Navarrete
Post:	Reader	
Qualifications:	PhD	
Organisation:	National Blood Service	
Work Address:	National Blood Service Colindale London	
Postcode:	NW7	
Telephone:		
Fax:		
Mobile:		
E-mail:	denny.williams@nbs.nhs.uk	

Question(s) 67 disabled.

PART A: Summary of Ethical Issues

A68. What are the main ethical issues with the research?

Summarise the main issues from the participant's point of view, and say how you propose to address them.

Taking blood from an individual who might conceivably benefit from the results of teh research but to whom the application of the research will not be available immediately.

Indicate any issues on which you would welcome advice from the ethics committee.

Question(s) 69–71 disabled.

PART B: Section 5 – Use of newly obtained human biological material

1. What types of human tissue or other biological material will be included in the study?

Blood

2. Who will collect the samples?

Individuals involved inthe routine clinical care of the pateints or one of the investigators in the study.

3. Will the samples be: *(Tick as appropriate)*

☑ Obtained primarily for research purposes?
☐ Surplus (i.e. left over from tissue taken in the course of normal clinical care for diagnostic or therapeutic purposes)?

4. Will informed consent be obtained from donors for use of the samples:

In this research?

◉ Yes ○ No

In future research?

○ Yes ◉ No

5. Will the samples be stored:

In fully anonymised form? *(link to donor broken)*

○ Yes ◉ No

In linked anonymised form? *(linked to donor but donor not identifiable to researchers)*

○ Yes ◉ No

If Yes, say who will have access to the code and personal information about the donor.

In a form in which the donor could be identifiable to researchers?

◉ Yes ○ No

If Yes, please justify:

So that it will be possible to determine the clinical implicaitons of having a particular response in vitro in the study.

6. What types of test or analysis will be carried out on the samples?

Ability of cells to recognise a stimulus in vitro.

7. Will the research involve the analysis of human DNA in the samples?

○ Yes ◉ No

8. Is it possible that the research could produce findings of clinical significance for individuals? *(May include relatives as well as donors)*

◉ Yes ○ No

9. If so, will arrangements be made to notify the individuals concerned?

◉ Yes ○ No ○ Not applicable

If No, please justify. If Yes, say what arrangements will be made and give details of the support or counselling service.

If clinical applicability becomes obvious early on and there is a significant implciaiton for a study particpant, the doctors looking after him will be informed.

10. Give details of where the samples will be stored, who will have access and the custodial arrangements.

Histocompatibility and Immunogenetics Lab, Hammersmith Hospital.
They will be avialable onlto to Drs Brookes and Warens and the technician employed on this study.

11. What will happen to the samples at the end of the research?

◉ Destruction

○ Transfer to research tissue bank
(If the bank is in England, Wales or Northern Ireland a licence from the Human Tissue Authority will be required to store the tissue for possible further research.)

○ Storage by research team pending ethical approval for use in another project
(Unless the researcher holds a licence from the Human Tissue Authority, a further application for ethical review should be submitted before the end of this project.)

○ Storage by research team as part of a new research tissue bank
(The bank will require a licence from the Human Tissue Authority. A separate application for ethical review of the tissue bank may also be submitted.)

○ Not yet known

Please give further details of the proposed arrangements:

PART B: Section 7 - Declarations

Declaration by Chief Investigator

1. The information in this form is accurate to the best of my knowledge and belief and I take full responsibility for it.
2. I undertake to abide by the ethical principles underlying the Declaration of Helsinki and good practice guidelines on the proper conduct of research.
3. If the research is approved I undertake to adhere to the study protocol, the terms of the full application of which the main REC has given a favourable opinion and any conditions set out by the main REC in giving its favourable opinion.
4. I undertake to seek an ethical opinion from the main REC before implementing substantial amendments to the protocol or to the terms of the full application of which the main REC has given a favourable opinion.
5. I undertake to submit annual progress reports setting out the progress of the research.
6. I am aware of my responsibility to be up to date and comply with the requirements of the law and relevant guidelines relating to security and confidentiality of patient or other personal data, including the need to register when necessary with the appropriate Data Protection Officer.
7. I understand that research records/data may be subject to inspection for audit purposes if required in future.
8. I understand that personal data about me as a researcher in this application will be held by the relevant RECs and their operational managers and that this will be managed according to the principles established in the Data Protection Act.
9. I understand that the information contained in this application, any supporting documentation and all correspondence with NHS Research Ethics Committees or their operational managers relating to the application:

   – Will be held by the main REC until at least 3 years after the end of the study.

   – May be disclosed to the operational managers or the appointing body for the REC in order to check that the application has been processed correctly or to investigate any complaint.

   – May be seen by auditors appointed by the National Research Ethics Service to undertake accreditation of the REC.

   – Will be subject to the provisions of the Freedom of Information Acts and may be disclosed in response to requests made under the Acts except where statutory exemptions apply.

Optional – please tick as appropriate:

☑ I would be content for members of other RECs to have access to the information in the application in confidence for training purposes. All personal identifiers and references to sponsors, funders and research units would be removed.

Signature: .....

Print Name: Anthony Warrens

Date: 17/06/2007 (dd/mm/yyyy)

Declaration by the sponsor's representative

If there is more than one sponsor, this declaration should be signed on behalf of the co-sponsors by a representative of the sponsor nominated to take the lead for the REC application.

I confirm that: *(tick as appropriate)*

- ☑ This research proposal has been discussed with the Chief Investigator and agreement in principle to sponsor the research is in place.
- ☑ An appropriate process of scientific critique has demonstrated that this research proposal is worthwhile and of high scientific quality.*
- ☑ Any necessary indemnity or insurance arrangements, as described in question A35, will be in place before this research starts.
- ☑ Arrangements will be in place before the study starts for the research team to access resources and support to deliver the research as proposed.
- ☑ Arrangements to allocate responsibilities for the management, monitoring and reporting of the research will be in place before the research starts.
- ☑ The duties of sponsors set out in the NHS Research Governance Framework for Health and Social Care will be undertaken in relation to this research.**

* Not applicable to student research (except doctoral research).
** Not applicable to research outside the scope of the Research Governance Framework.

Signature: .....

Print Name: P. Henley

Post: Research Facilitator

Organisation: Imperial College

Date: (dd/mm/yyyy) 11/07/2007

Appendix A2

Approved application upon site change to Barts and London NHS trust (2011)

Welcome to the Integrated Research Application System

IRAS Project Filter

The integrated dataset required for your project will be created from the answers you give to the following questions. The system will generate only those questions and sections which (a) apply to your study type and (b) are required by the bodies reviewing your study. Please ensure you answer all the questions before proceeding with your applications.

Please enter a short title for this project (maximum 70 characters)
Immunological monitoring of B cells in renal transplant recipients

REC details:

Name of main REC:
Central London REC1

REC Reference Number:
07/H0707/104

NRES form lock code:

1. Select one category from the list below:

- ○ Clinical trial of an investigational medicinal product
- ◉ Study only involving data or tissues not identifiable to the researcher

If your work does not fit any of these categories, select the option below:

- ○ Other study

2. Does the study involve the use of any ionising radiation?

○ Yes ◉ No

3. In which countries of the UK will the research sites be located? *(Tick all that apply)*

- ☑ England
- ☐ Scotland
- ☐ Wales
- ☐ Northern Ireland

3a. In which country of the UK will the lead NHS R&D office be located:

- ◉ England
- ○ Scotland
- ○ Wales
- ○ Northern Ireland
- ○ This study does not involve the NHS

4. Do you plan to include any participants who are children?

○ Yes ◉ No

5. Do you plan to include any participants who are adults unable to consent for themselves through physical or mental incapacity?

○ Yes ◉ No

Answer Yes if you plan to recruit participants aged 16 or over who lack capacity, or to retain them in the study following loss of capacity. Intrusive research means any research requiring consent in law. This includes use of identifiable tissue samples or personal information, except where application is being made to the NIGB Ethics and Confidentiality Committee to set aside the common law duty of confidentiality in England and Wales. Please consult the guidance notes for further information on the legal frameworks for research involving adults lacking capacity in the UK.

6. Is the study, or any part of the study, being undertaken as an educational project?

◉ Yes ○ No

Site-Specific Information Form

Is the site hosting this research a NHS site or a non-NHS site? *NHS sites include Health and Social Care organisations in Northern Ireland. The sites hosting the research are the sites in which or through which research procedures are conducted. For NHS sites, this includes sites where NHS staff are participants.*

- (●) NHS site
- ( ) Non-NHS site

This question must be completed before proceeding. The filter will customise the form, disabling questions which are not relevant to this application.

One Site-Specific Information Form should be completed for each research site and submitted to the relevant R&D office with the documents in the checklist. See guidance notes.

The data in this box is populated from Part A:

Title of research:
Immunological monitoring of B cells in renal transplant recipients

Short title: Immunological monitoring of B cells in renal transplant recipients

Chief Investigator:

Title	Forename/Initials	Surname
Professor	Anthony N	Warrens

Name of NHS Research Ethics Committee to which application for ethical review is being made:
Central London REC1

Project reference number from above REC: 07/H0707/104

1-1. Give the name of the NHS organisation responsible for this research site

Barts and The London NHS Trust

1-2. In which country is the research site located?

- (●) England
- ( ) Wales
- ( ) Scotland
- ( ) Northern Ireland

1-3. Is the research site a GP practice or other Primary Care Organisation?

( ) Yes (●) No

2. Who is the Principal Investigator or Local Collaborator for this research at this site?

Select the appropriate title: ◉ Principal Investigator ○ Local Collaborator

	Title	Forename/Initials	Surname
	Professor	Anthony N	Warrens
Post	Dean for Education		
Qualifications	DM PhD FHEA FRCP		
Organisation	Queen Mary University		
Work Address	Barts and The London School of Medicine and Dentistry		
	Garrod Building, Turner Street		
	London		
PostCode	E1 2AD		
Work E-mail	a.warrens@qmul.ac.uk		
Work Telephone	02078822261		
Mobile	07803941336		
Fax	02078827187		

a) Approximately how much time will this person allocate to conducting this research? *Please provide your response in terms of Whole Time Equivalents (WTE).*
5%

b) Does this person hold a current substantive employment contract, Honorary Clinical Contract or Honorary Research Contract with the NHS organisation or accepted by the NHS organisation? ◉ Yes ○ No

A copy of a current CV for the Principal Investigator (maximum 2 pages of A4) must be submitted with this form.

3. Please give details of all locations, departments, groups or units at which or through which research procedures will be conducted at this site and describe the activity that will take place.

Please list all locations/departments etc where research procedures will be conducted within the NHS organisation, describing the involvement in a few words. Where access to specific facilities will be required these should also be listed for each location.

Name the main location/department first. Give details of any research procedures to be carried out off site, for example in participants' homes.

	Location	Activity/facilities
1	Clinical Transplantation Laboratory, The Royal London Hospital, Whitechapel, E1 1BB	All laboratory based activites. Procedures include cell isolation, cell culture and flow cytometry

5. Please give details of all other members of the research team at this site.

1

	Title	Forename/Initials	Surname
	Ms	Louise	Onions
Work E-mail	l.onions@qmul.ac.uk		
Employing organisation	Queen Mary University		
Post	Research assistant		
Qualifications	BSc MSc BSHIdip		

Role in research team: researcher

a) Approximately how much time (approximately) will this person allocate to conducting this research? *Please provide your response in terms of Whole Time Equivalents (WTE).*
FT

b) Does this person hold a current substantive employment contract, Honorary Clinical Contract or Honorary Research Contract with the NHS organisation or accepted by the NHS organisation? ◉ Yes ○ No

A copy of a current CV for the research team member (maximum 2 pages of A4) must be submitted to the R&D office.

6. Does the Principal Investigator or any other member of the site research team have any direct personal involvement (e.g. financial, share-holding, personal relationship etc) in the organisation sponsoring or funding the research that may give rise to a possible conflict of interest?

○ Yes ◉ No

7. What is the proposed local start and end date for the research at this site?

Start date:	01/04/2011
End date:	01/04/2014
Duration (Months):	36

10. How many research participants/samples is it expected will be recruited/obtained from this site?

110 participants
This includes:
10 healthy volunteers
40 post-transplant patients
30 pre-transplant donor/recipient pairs (60)

11. Give details of how potential participants will be identified locally and who will be making the first approach to them to take part in the study.

Healthy volunteers will be recruited via institution wide email to make contact with the research team.
Pre- and post-transplant patients will be indentified by the research team and approached to consent to taking part in the study while attending routine clinic appointments.
In some cases letters will be sent to patients requesting their participation.

12. Who will be responsible for obtaining informed consent at this site? What expertise and training do these persons have in obtaining consent for research purposes?

Name	Expertise/training
AN Warrens	Many years of participating in clinical research. To attend research governance training at BLT in June.
L Onions	Consented patients for this study for 18 months at Hammersmith Hospital. She has sufficent time and expertise to answer questions relating to the study. Any questions beyond this will be refered to the clinical team. To attend research governance training at BLT in 31/03/2011.

15-1. Is there an independent contact point where potential participants can seek general advice about taking part in research?

No

15-2. Is there a contact point where potential participants can seek further details about this specific research project?

The participant information sheet has contact details for the CI.

16. Are there any changes that should be made to the generic content of the information sheet to reflect site-specific issues in the conduct of the study? *A substantial amendment may need to be discussed with the Chief Investigator and submitted to the main REC.*

No

Please provide a copy on headed paper of the participant information sheet and consent form that will be used locally. Unless indicated above, this must be the same generic version submitted to/approved by the main REC for the study while including relevant local information about the site, investigator and contact points for participants (see guidance notes).

17. What local arrangements have been made for participants who might not adequately understand verbal explanations or written information given in English, or who have special communication needs? *(e.g. translation, use of interpreters etc.)*

All patients will have arrangements in place for translation services.

18. What local arrangements will be made to inform the GP or other health care professionals responsible for the care of the participants?

None, not relevant for this study

19. What arrangements (e.g. facilities, staffing, psychosocial support, emergency procedures) will be in place at the site, where appropriate, to minimise the risks to participants and staff and deal with the consequences of any harm?

None, not relevant for this study

20. What are the arrangements for the supervision of the conduct of the research at this site? *Please give the name and contact details of any supervisor not already listed in the application.*

Consultant Nephrologist
Dr Raj Thuraisingham
Renal Office
The Royal London Hospital
London, E1 2AD

21. What external funding will be provided for the research at this site?

- ○ Funded by commercial sponsor
- ◉ Other funding
- ○ No external funding

Please give details of the funding:
Funded by a grant from NHS Blood and Transplant

Type of funding	Details (including breakdown over years if appropriate)
(i) Block grant	£151,665
(ii) Per participant	
(iii) Other (give details)	

Which organisation will receive and manage this funding?
Queen Mary University

23. Authorisations required prior to R&D approval

This section deals with authorisations by managers within the NHS organisation. It should be signed in accordance with the guidance provided by the NHS organisation. This may include authorisation by clinical supervisors, line managers, service managers, support department managers, pharmacy, data protection officers or finance managers, depending on the nature of the research. Managers completing this section should confirm in the text what the authorisation means, in accordance with the guidance provided by the NHS organisation.

This section may also be used by university employers or research support staff to provide authorisation to NHS organisations, in accordance with guidance from the university.

Declaration by Principal Investigator or Local Collaborator

1. The information in this form is accurate to the best of my knowledge and I take full responsibility for it.
2. I undertake to abide by the ethical principles underpinning the World Medical Association's Declaration of Helsinki and relevant good practice guidelines in the conduct of research.
3. If the research is approved by the main REC and NHS organisation, I undertake to adhere to the study protocol, the terms of the application of which the main REC has given a favourable opinion and the conditions requested by the NHS organisation, and to inform the NHS organisation within local timelines of any subsequent amendments to the protocol.
4. If the research is approved, I undertake to abide by the principles of the Research Governance Framework for Health and Social Care.
5. I am aware of my responsibility to be up to date and comply with the requirements of the law and relevant guidelines relating to the conduct of research.
6. I undertake to disclose any conflicts of interest that may arise during the course of this research, and take responsibility for ensuring that all staff involved in the research are aware of their responsibilities to disclose conflicts of interest.
7. I understand and agree that study files, documents, research records and data may be subject to inspection by the NHS organisation, the sponsor or an independent body for monitoring, audit and inspection purposes.
8. I take responsibility for ensuring that staff involved in the research at this site hold appropriate contracts for the duration of the research, are familiar with the Research Governance Framework, the NHS organisation's Data Protection Policy and all other relevant policies and guidelines, and are appropriately trained and experienced.
9. I undertake to complete any progress and/or final reports as requested by the NHS organisation and understand that continuation of permission to conduct research within the NHS organisation is dependent on satisfactory completion of such reports.
10. I undertake to maintain a project file for this research in accordance with the NHS organisation's policy.
11. I take responsibility for ensuring that all serious adverse events are handled within the NHS organisation's policy for reporting and handling of adverse events.
12. I understand that information relating to this research, including the contact details on this application, will be held by the R&D office and may be held on national research information systems, and that this will be managed according to the principles established in the Data Protection Act 1998.
13. I understand that the information contained in this application, any supporting documentation and all correspondence with the R&D office and/or the REC system relating to the application will be subject to the provisions of the Freedom of Information Acts and may be disclosed in response to requests made under the Acts except where statutory exemptions apply.

Signature of Principal Investigator or Local Collaborator:

Print Name: A. N. Williams

Date: 29/7/11

Appendix A3

Approved notice of substantial amendment (2013)

NHS
National Patient Safety Agency

National Research Ethics Service

NOTICE OF SUBSTANTIAL AMENDMENT

For use in the case of all research other than clinical trials of investigational medicinal products (CTIMPs). For substantial amendments to CTIMPs, please use the EU-approved notice of amendment form (Annex 2 to ENTR/CT1) at http://eudract.emea.eu.int/document.html#guidance.

To be completed in typescript by the Chief Investigator in language comprehensible to a lay person and submitted to the Research Ethics Committee that gave a favourable opinion of the research ("the main REC"). In the case of multi-site studies, there is no need to send copies to other RECs unless specifically required by the main REC.

Further guidance is available at http://www.nres.npsa.nhs.uk/applicants/review/after/amendments.htm.

Details of Chief Investigator:

Name:	Professor Anthony Warrens
Address:	Barts and The London School of Medicine & Dentistry Garrod Building, Room 2.46 Turner Street, Whitechapel London, E1 2AD
Telephone:	020 7882 2261
Email:	a.warrens@qmul.ac.uk
Fax:	020 7882 7187

Full title of study:	Immunological monitoring of B cells in renal transplant recipients
Name of main REC:	Central London REC1
REC reference number:	07/H0707/104
Date study commenced:	01/01/2009
Protocol reference *(if applicable)*, current version and date:	V3 04/2013
Amendment number and date:	2, April 2013

Type of amendment (indicate all that apply in bold)

(a) Amendment to information previously given on the NRES Application Form

Yes *No*

If yes, please refer to relevant sections of the REC application in the "summary of changes" below.

(b) Amendment to the protocol

Yes *No*

If yes, please submit either the revised protocol with a new version number and date, highlighting changes in bold, or a document listing the changes and giving both the previous and revised text.

(c) Amendment to the information sheet(s) and consent form(s) for participants, or to any other supporting documentation for the study

Yes *No*

If yes, please submit all revised documents with new version numbers and dates, highlighting new text in bold.

Is this a modified version of an amendment previously notified to the REC and given an unfavourable opinion?

Yes ***No***

Summary of changes

Briefly summarise the main changes proposed in this amendment using language comprehensible to a lay person. Explain the purpose of the changes and their significance for the study. In the case of a modified amendment, highlight the modifications that have been made.

If the amendment significantly alters the research design or methodology, or could otherwise affect the scientific value of the study, supporting scientific information should be given (or enclosed separately). Indicate whether or not additional scientific critique has been obtained.

1. Changes to REC approved documents

The participant information sheets (PIS) and consent forms have been changed to include use of surplus tissue biopsy material. The old (consent form, version 2 02/2011; PIS, version 3 02/2011) and new (consent form, version 3 04/2013; PIS, version 4 04/2013) documents are included in this submission.

2. Protocol amendment

The main research design shall remain unchanged; however we would like to broaden our knowledge of the B cell compartment in renal transplant recipients to include the investigation of cellular markers of B cell survival and activation in renal biopsy material. This would only be in patients where biopsy material is stored and surplus to clinical requirements. This will not significantly affect the study other than to increase the scientific value.
The previous protocols (National Blood Authority Protocol V1 & V2 03/2011) and the new protocol (V3 04/2013) are included in this submission.

Any other relevant information

Applicants may indicate any specific ethical issues relating to the amendment, on which the opinion of the REC is sought.

List of enclosed documents

Document	*Version*	*Date*
Participant information sheet – Barts and The London	3	02/2011
Participant information sheet – Barts and The London	4	04/2013
Participant consent form – Barts and The London	2	02/2011
Participant consent form – Barts and The London	3	04/2013
National Blood Authority Protocol	1	Not listed
Immunological monitoring of B cells in renal transplant recipients – Protocol V2 03/2011	2	03/2011
Immunological monitoring of B cells in renal transplant recipients – Protocol V3 04/2013	3	04/2013

Declaration

- I confirm that the information in this form is accurate to the best of my knowledge and I take full responsibility for it.
- I consider that it would be reasonable for the proposed amendment to be implemented.

Signature of Chief Investigator: .....

Print name: 16 iv 13

Date of submission: A.N. WARREN

Appendix B1

Abstracts arising from the thesis

B1 Abstracts arising from the thesis

World Transplant Congress, San Francisco, July 2014

Oral presentation

RENAL TRANSPLANT STATUS CORRELATES WITH EXPRESSION OF BAFF AND LOCALISES WITHIN INTRA-GRAFT LYMPHOID AGGREGATES DURING ACUTE ANTIBODY-MEDIATED REJECTION

Louise Onions, MSc[1], Arun Gupta, MSc[2], Joanne E Martin, MD[3], Carmelo Puliatti, MD[4], Roberto Cacciola, MD[4], Cristina Navarrete, PhD[5] and Anthony N Warrens, MD[1,2].

[1]Translational Medicine and Therapeutics, William Harvey Research Institute, Barts and the London School of Medicine and Dentistry, London, United Kingdom; [2]Clinical Transplantation Laboratory, Barts and the London NHS Trust, London, United Kingdom; [3]Cellular Pathology, Barts and the London School of Medicine and Dentistry, London, United Kingdom; [4]Transplant Surgery, Barts and the London NHS Trust, London, United Kingdom and [5]Histocompatibility and Immunogenetics Laboratory, NHSBT, London, United Kingdom.

Purpose of Study: To measure serum and intra-renal expression of B cell-activating factor (BAFF) as a marker of B cell-mediated pathology.

Methods: CD19+ cells from patients with deteriorating (n=33), stably-impaired (n=29) and stable (n=31) grafts were examined for CD19, CD27, IgD & BAFF-receptor (BAFF-R) expression by flow cytometry. Serum BAFF (sBAFF) levels were measured by ELISA and HLA antibodies using Luminex technology. Renal biopsy sections from cohorts with (n=16) and without (n=11) acute antibody-mediated rejection (AAMR) were studied by immunohistochemistry.

Results: The stable group had significantly lower sBAFF and more CD19+BAFF-R+ cells. Those with above average sBAFF (sBAFFhigh) had a higher rate of allograft deterioration and greater prevalence of donor-specific HLA antibody compared to those with below average sBAFF levels. Moreover, naïve B cells (CD19+CD27-IgD+) were reduced and memory B cells (CD19+CD27+IgD-) increased in sBAFFhigh patients. Examination of biopsy sections demonstrated a higher frequency of CD19+ cells associated with small populations of sub-capsular interstitial lymphoid aggregates in patients with AAMR. BAFF-R expression showed a similar distribution to CD19, but BAFF more often had a diffuse distribution across the core. The presence or absence of AAMR made no difference to sBAFF levels.

Conclusion: BAFF is a central component of the B cell compartment regulating cell maturation and survival. Here elevated expression of sBAFF and low frequency of BAFF-R on CD19 cells correlate with deteriorating graft function and intra-renal expression of CD19, BAFF-R and BAFF is increased during AAMR. These data provide

direct evidence of BAFF expression on infiltrating B cells during humoral rejection, and a rationale for measuring expression levels of BAFF as a biomarker of allograft function.

European Society for Organ Transplantation, Vienna, September 2013

Poster presentation

RENAL TRANSPLANT STATUS CORRELATES WITH EXPRESSION OF BAFF AND REGULATORY B CELLS

Louise Onions,[1] Arun Gupta,[2] Cristina Navarrete[3] & Anthony N Warrens[1,2]

[1]William Harvey Research Institute, Barts and the London School of Medicine and Dentistry, Queen Mary, University of London, London; [2]Clinical Transplantation Laboratory, The Royal London Hospital, Barts Health NHS Trust, London; [3] Histocompatibility & Immunogenetics Laboratory, NHSBT Colindale Centre, London

Background: Dysfunction in the B cell compartment may contribute to chronic allograft deterioration. B cell activating factor (BAFF), if in excess, has been associated with rejection and development of donor-specific antibody (DSA). Here we examined BAFF and regulatory B cells (Breg) to identify the relationships between differential expression and level of function of the allograft.

Methods: CD19+ cells from patients with deteriorating (gp 1), stably-impaired (gp 2) and well-functioning (gp 3) grafts were examined for CD19 & BAFF-R or cultured for Breg analysis and stained for CD19, CD24, CD38, IL-10. FACS-sorted-cells were stained with CD19, CD24 & CD38. Serum BAFF (sBAFF) levels were measured by ELISA. HLA antibodies were assessed using Luminex technology.

Results: Gp 1 had higher sBAFF and lower CD19+BAFF-R+ cells. DSA with elevated sBAFF or low BAFF-R were only detected in gp 1. IL-10 producing-B cells, enriched in the CD24highCD38high compartment were lower in gp1 and cell-sorting showed the distinct CD19+CD24highCD38high subset was minimal.

Conclusion: The expression of BAFF and Bregs correlates with deteriorating graft function, suggesting important roles in regulating B cell homeostasis. We are currently examining functional regulatory properties.

British Transplant Society Annual Congress, February 2013

Poster presentation

Identifying dysregulation of the B cell compartment in patients with deteriorating renal graft function

Louise Onions,[1] Arun Gupta,[2] Cristina Navarrete[3] & Anthony Warrens[1]

[1]William Harvey Research Institute , Barts and the London School of Medicine and Dentistry, London; [2]Clinical Transplantation Laboratory, The Royal London, London; [3] Histocompatibility & Immunogenetics Laboratory, NHSBT Colindale Centre, London

Introduction: B cells have multiple immunological functions. In transplantation their defining role has been the secretion of HLA antibody against the allograft. However more detailed analysis of these cells suggests additional factors may contribute to dysfunction in the B cell compartment. Here we investigate expression of the B cell activating factor (BAFF), a member of the TNF family essential for B cell selection, differentiation and survival. Excessive BAFF production has been associated with the development of autoimmune disorders and recent studies in renal transplant recipients show elevated serum BAFF (sBAFF) to be associated with rejection and the development of donor-specific antibody (DSA). In this study we measured sBAFF and the receptor to identify any differential expression depending on the level of function of the allograft. In addition we investigated if the presence of regulatory B cells, thought to play an important role in B cell homeostasis, would also differ depending on graft function.

Method: Peripheral whole blood was collected from renal transplant recipients with deteriorating (gp 1), stably impaired (gp 2) and well-functioning (gp 3) grafts a minimum of one year post-transplant. CD19+ cells were isolated by positive selection and stained with anti-human CD19 & BAFF-R. CD19+ cells for Breg analysis were cultured for 5 days in the presence of CpG-B 2006 (3ug/ml). Post culture cells were treated with PMA (50ng/ml), ionomycin (500ng/ml) and monensin (2mM) stained with CD19, CD24, CD38, washed, fixed, permeabilised and stained with intracellular IL-10 and analysed by flow cytometry. sBAFF levels were measured by ELISA. HLA antibodies were assessed using Luminex technology and HLA Single Antigen beads.

Results: Patients in gp1 (n=20) had significantly higher sBAFF levels than those in gp 2 (n=10) and gp 3 (n=16) (mean values 1005.66, 681.74, 664.70pg/ml respectively; gp 1 vs. gp 2 $p=0.047$, gp 1 vs. gp 3 $p=0.125$). In addition, expression of BAFF-R on CD19+ cells is downregulated in gp 1 patients (62.87%) compared to gp 2 & 3 (86.71 & 87.54%; $p<0.001$). DSA with elevated sBAFF level (higher than the overall mean; >784.03pg/ml) or low BAFF-R (lower than the overall mean; 79.03%) were only detected in gp 1 patients. Furthermore the rate of graft deterioration showed a significant correlation with elevated sBAFF level ($p=0.004$) but not with decreased expression of BAFF-R. In a smaller cohort of patients (n=5) there was an increase in IL-10 producing B cells in gp 3 patients compared to gp 1 (1.09 vs. 0.27%; $p=0.04$). In

addition these cells were enriched in the CD24highCD38high compartment (gp 1; 31.84 vs. 5.18% & gp 3; 30.66 vs. 5.51%)

Conclusion: These data show that expression of sBAFF and the receptor correlate with deteriorating graft function suggesting an important role in regulating B cell homeostasis. In addition, preliminary data shows cells with a regulatory B cell phenotype are associated with stable graft function but further investigations are necessary to link these cells with down regulation of the immune response.

British Transplant Society Annual Congress, February 2012

Oral presentation

Elevated BAFF Correlates With Deteriorating Renal Graft Function

L Onions,[1] A Gupta,[2] R Thuraisingham[3] & AN Warrens[1]

1.Translational Medicine and Therapeutics, William Harvey Research Centre, London
2. Clinical Transplantation Laboratory, The Royal London Hospital, London
3. Department of Nephrology, The Royal London Hospital, London

Introduction: The B cell activating factor (BAFF) of the tumour necrosis family is essential for B cell selection, differentiation and survival[1] and therefore an important regulator of B cell immunity. In animal models, it has been shown that excess BAFF can lead to the development of autoimmune disorders[2] and that BAFF is upregulated in patients with SLE[3]. In renal transplant patients, increased levels of BAFF were detected in those treated with Campath-1H[4]. However, a key question remains as to whether the dysregulation of B cell activation could be a contributing factor in alloantibody production and if aberrant expression of BAFF correlates with allograft function. Given this and the association of B cells in renal allograft rejection, we investigated if BAFF and its receptor are differentially expressed depending on the level of function of the allograft.

Method: Peripheral whole blood was collected from renal transplant recipients with deteriorating (gp 1), stably impaired (gp 2) and well-functioning (gp 3) grafts a minimum of one year post-transplant. $CD19^+$ cells were isolated by positive selection, stained with anti-human CD19 & BAFF-R (BD Biosciences) and analysed by flow cytometry. Serum BAFF levels were measured by ELISA (R&D Biosysytems). The presence of HLA antibodies were assessed using Luminex technology and HLA Single Antigen beads (One Lambda).

Results: Patients in gp1 (n=23) had significantly higher serum BAFF levels than those in gp 2 (n=8) and gp 3 (n=14) (mean values 1706pg/ml, 675pg/ml, 774pg/ml respectively; gp 1 vs. gp 2 p=0.04, gp 1 vs. gp 3 p=0.02). In addition, expression of BAFF-R on $CD19^+$ cells is downregulated significantly in gp 1 patients (60%) compared to gp 3 patients (73.7%; p=0.02) but not gp 2 patients (69.8%; p=0.27). Stratified by HLA antibody

status of study patients, donor-specific antibodies (DSA) were only detected in gp 1 patients (DSA, 41%, non-DSA 27%, negative 32%) compared to gp 2 (non-DSA 14%; negative 86%) and gp 3 patients (non-DSA 21%; negative 79%). However, no correlation was found between the presence of HLA antibody (DSA or non-DSA) and high expression BAFF (>1223pg/ml, the overall mean) or downregulation of BAFF-R (<68.3%, the overall mean).

Conclusion: B cells are increasingly recognised as key mediators in allograft injury. These data show that expression of BAFF and its receptor significantly correlate with renal graft function, suggesting that measurement of BAFF could act as a biomarker of deteriorating graft function. However, it does not appear that BAFF is a mediator in enhanced alloantibody production. Further research into B cell regulation and their dependence on BAFF expression could provide important information as to whether B cell survival factors should be utilised as potential markers of humoral rejection.

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A Novel Method To Detect HLA-specific B cells In Renal Transplant Recipients

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Introduction:
The presence of donor-specific HLA antibodies is associated with acute and chronic renal allograft rejection. In an effort to minimise their damaging effects, highly sensitive and specific solid-phase assays have been developed to aid their detection in patient serum. However, investigating HLA antibodies in serum alone will only reflect the presence of B cells actively secreting antibody and may not reflect the overall B cell sensitisation status, such as the potential presence of memory B cells. Although such cells are very rare in the circulation; a more sensitive approach would be to identify circulating HLA-specific B cells as a method of assessing B cell alloreactivity between recipient–donor pairs.

Method:
Peripheral whole blood was collected from HLA-A*0201 sensitised renal transplant recipients a minimum of one year post-transplant. Non-sensitised healthy males served as controls. PBMCs were prepared by Ficoll-Hypaque separation and $CD19^+$ cells isolated by positive selection. A minimum of 10^6 $CD19^+$ cells were stained with anti-human CD19 followed by Luminex Single HLA Antigen HLA-A*0201 beads (One Lambda) and analysed by flow cytometry. All cells with a staining intensity higher than the upper limit obtained using the non-sensitised control were considered positive.

Results:

Sensitised renal transplant recipients known to have HLA-A*0201 antibody (MFI >1000) as previously defined by Luminex technology were tested for the presence of HLA-A2 specific B cells. Using beads coated with HLA-A*0201 it is possible to show the presence of a small population of B cells capable of binding to the beads as assessed by flow cytometry. The data shows a significantly higher proportion of $CD19^{+}$ cells which bind HLA-A2 beads in sensitised (n=5) compared to non-sensitised subjects (n=5; 0.124 ± 0.049% and 0.042 ± 0.008%; p=0.007 sensitised patients and non-sensitised controls respectively).

Conclusion:

This preliminary data shows differences in B cell reactivity in sensitised patients compared to controls which can be effectively demonstrated using a simple technique. This method can detect and quantify HLA-specific B cells which could provide useful information for predicating the B cell response to donor antigens and be a valuable tool in both the pre- and post-transplant setting.