Indirect Effects of Cytomegalovirus

In Kidney Transplantation

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Abstract

Cytomegalovirus (CMV) infection is the most frequent and significant opportunistic infection in kidney transplant recipients. It is associated with direct (CMV disease) and indirect (rejection, poor graft survival) effects with resultant increases in morbidity and mortality. The mechanisms responsible for the indirect effects of CMV infection remain unclear.

In this thesis, the indirect effects of cytomegalovirus infection in kidney transplantation are studied. Firstly, the mechanism of CMV infection is investigated. Secondly, the mechanism of CMV associated kidney transplant damage is explored. Thirdly, an assessment for the role of CMV in causing immunosenescence within the kidney transplantation cohort is undertaken.

This thesis provides previously undescribed and direct evidence of immune hypo-responsiveness to latent CMV. I have shown $CD4^{+}CD27^{-}CD28^{null}$ cells are pathognomonic of prior CMV exposure and have a role in glomerular endothelial cell damage, an effect which may be mediated by NKG2D. Higher $CD4^{+}CD27^{-}CD28^{null}$ cell counts at 12 months post-transplantation predict a steeper decline in kidney allograft function thereafter. I provide novel insight into the 'indirect' effect of CMV in the pathogenesis of $CD8^{+}CD28^{null}$ cells. My study is the first to demonstrate a temporal association between elevated $CD8^{+}CD28^{null}$ cell frequencies and subsequent development of clinically relevant episodes of infection.

The findings from this thesis set the scene for future interventional research and therapeutic strategies.

Acknowledgements

My time in research has been enjoyable and rewarding. I take with me new skills, collaborations, friendships and a desire to continue high quality translational research.

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Abbreviations

CMV	Cytomegalovirus
HCMV	Human Cytomegalovirus
MCMV	Murine Cytomegalovirus
ESKD	End-stage kidney disease
D+	CMV seropositive donor
D-	CMV seronegative donor
R+	CMV seropositive recipient
R-	CMV seronegative recipient
AR	Acute rejection
BPAR	Biopsy Proven Acute Rejection
CR	Chronic rejection
ABMR	Antibody Mediated Rejection
CAI	Chronic allograft injury
NODAT	New Onset Diabetes After Transplantation
TLRs	Toll-Like Receptors
TNF-alpha	Tumour Necrosis Factor alpha
NK cells	Natural Killer cells
DCs	Dendritic cells
ANOVA	Analysis of Variance
IgG	Immunoglobulin G
IE	Immediate Early
MHC	Murine Histocompatibility Complex
HLA	Human Leukocyte Antigen
DAMPs	Damage-Associated Molecular Patterns
IFNγ	Interferon gamma
IL-10	Interleukin-10
PTLD	Post-Transplant Lymphoproliferative Disease
BPAR	Biopsy-Proven Acute Rejection
PCR	Polymerase Chain Reaction
DNA	Double-stranded Nucleic Acid
ELISPOT	Enzyme-Linked ImmunoSpot assay
CMI	Cell Mediated Immunity
NKG2D	Natural Killer Group 2D receptor
HUVEC	Human Umbilical Vein Endothelial Cells
GenC	Glomerular Endothelial Cell
IRP	Immune Risk Profile
DGF	Delayed Graft Function
eGFR	Estimated Glomerular Filtration Rate
PBMCs	Peripheral Blood Mononuclear Cells
UACR	Urine Albumin Creatinine Ratio

ALT	Alanine Transaminase
AST	Aspartate Transaminase
DBD	Deceased Brain Death Donor
DCD	Deceased Cardiac Death Donor
SEB	Staphylococcus Enterotoxin B
Ag	Antigen
Ab	Antibody
ESRF	End-Stage Renal Failure
LA	Late Antigen
APCs	Antigen Presenting Cells
PD-1	Programmed cell death protein 1
EBV	Epstein-Barr virus
FACS	Fluorescence-Activated Cell Sorting
CX3CR1	C-X3-C Motif Chemokine Receptor 1 Fractalkine receptor;
vWF	von Willebrand factor
hsCRP	high sensitivity C-reactive protein
FCS	fetal calf serum
DMSO	Dimethyl Sulfoxide
CM	Central memory-like
EM	Effector memory-like
EMRA	Effector memory cells re-expressing RA
CFSE	Carboxyfluorescein Succinimidyl Ester
ELISA	Enzyme Linked Immunosorbent Assay
CMI	Cell Mediated Immunity
PD	Peritoneal Dialysis
HD	Haemodialysis
PRE	Pre-emptive transplantation
NETS	Neutrophil Extracellular Traps
dnDSA	de novo Donor Specific anti-HLA Antibody
dnNDSA	de novo Non-Donor Specific anti-HLA Antibody
IQR	Interquartile Range
MDRD	Modification of Diet in Renal Disease
MFI	Mean Fluorescence Intensity
PBS	Phosphate Buffered saline

Chapter 1: Background and Literature review

1.1 Introduction

Kidney transplantation is the gold standard treatment for end stage kidney disease (ESKD), offering improved quality of life and life expectancy compared with maintenance dialysis [1, 2]. Figure 1.1 demonstrates the large survival advantage kidney transplantation holds over maintenance dialysis.

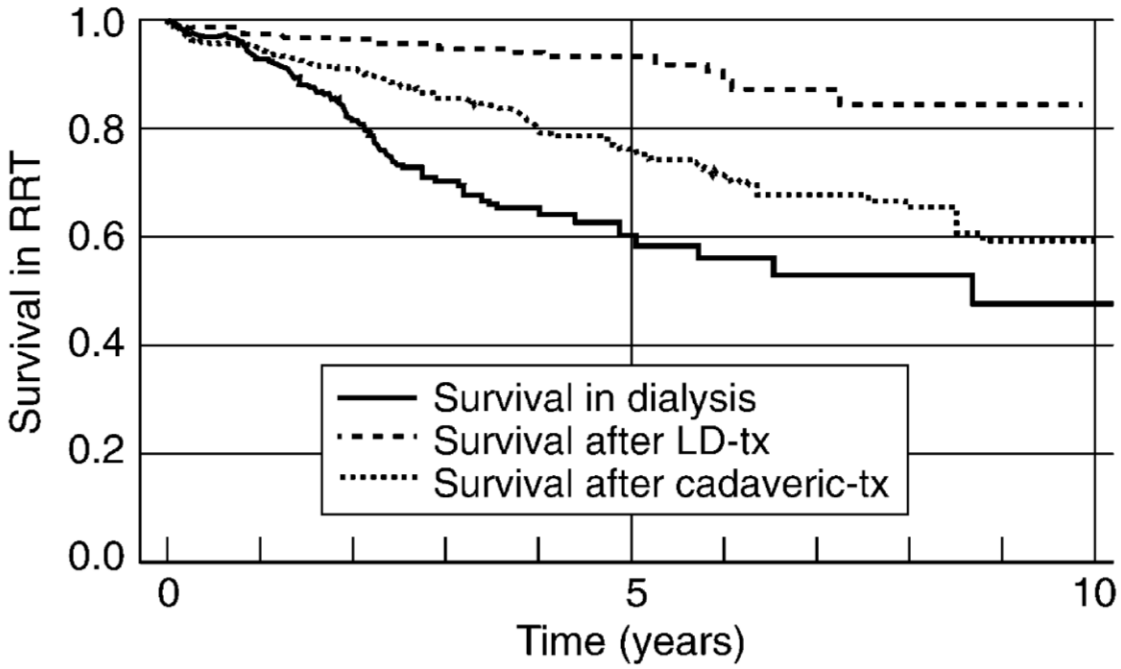

Figure 1.1: Kaplan–Meier survival curves for three groups of renal replacement therapy (RRT) patients: those transplanted with a living-related kidney donor (LD), those transplanted with a cadaveric kidney, and those remaining on dialysis. Follow-up for 10 years [184].

Potent long-term immunosuppression is used to avoid acute rejection and extend graft survival. The two main complications associated with chronic immunosuppression are opportunistic infections and cancer [3].

Cytomegalovirus (CMV) infection is the most frequent and significant opportunistic infection in kidney transplant recipients [3, 4]. Cytomegalovirus is a ubiquitous β-herpesvirus which is seemingly harmless in healthy individuals. In kidney transplantation, CMV infection is associated with significantly reduced patient and graft survival (Figure 1.2) [5]. CMV induces a strong cellular immune response and has developed strategies to evade immune surveillance [6]. The mechanism behind CMV driven damage remains unclear.

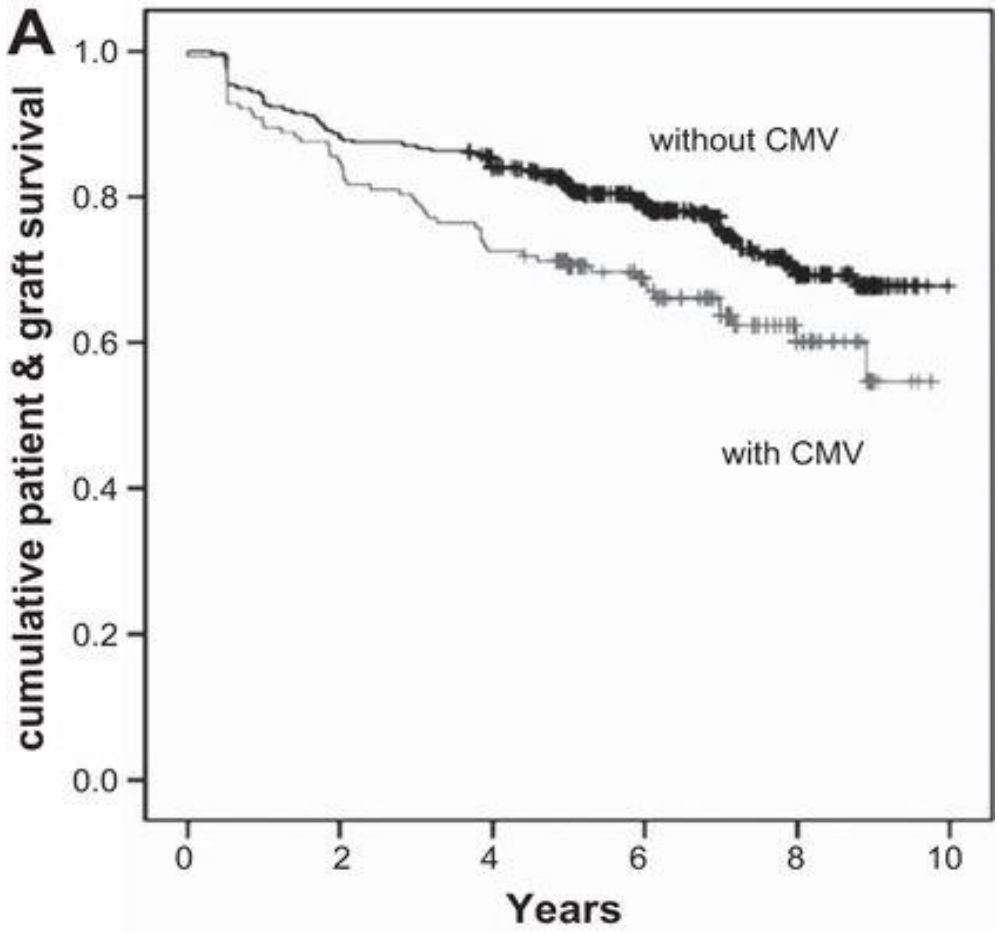

Figure 1.2: Patient and graft survival in patients with and without CMV
CMV infection following kidney transplantation significantly reduced long-term patient and graft survival (p = 0.008) [5].

CMV infection is associated with direct (CMV disease) and indirect (rejection, poor graft survival) effects [7, 8]. CMV causes direct effects by viral inclusion in the cells of various tissues with resultant cytopathic effects. CMV inclusions are seen in glomerular endothelial cells (Figure 1.3), peritubular capillaries, and tubular epithelial cells, resulting in an immunotactoid glomerulopathy and interstitial nephritis [9].

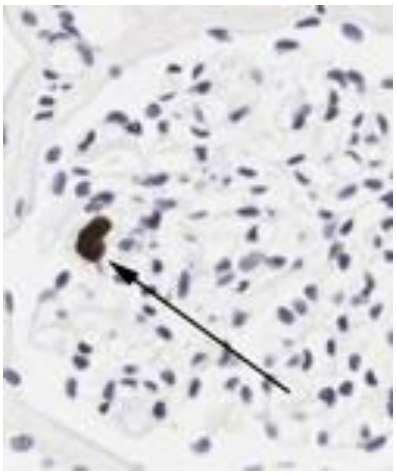

Figure 1.3 CMV in glomerular endothelial cell
Cytomegalovirus (arrow) in a glomerular endocapillary nucleus, shown by immunoperoxidase staining. Courtesy of Alexander J Howie, University College London.

The indirect effects of CMV result from an interaction between CMV and the host immune system [10]. Primary CMV infection produces a robust and specific adaptive immune response involving CMV-specific $CD4^+$ T cells, $CD8^+$ T cells, and IgG, which persist lifelong [11]. Latent CMV infection is associated with expansion of circulating, late-differentiated CMV-specific $CD8^+$ T cells [12]. As these cells play a central role in the control of CMV, it has been proposed to monitor these cells before and after transplantation to guide optimal use of anti-CMV prophylaxis and therapy [13]. Expansion of an unusual and rare subset of $CD4^+$ T cells

has been reported in CMV seropositive patients with ESKD or autoimmune conditions [14]. These cells are characterised by the absence of the co-stimulatory molecule CD28 ($CD4^+CD28null$); they are unusual in that they are cytotoxic [14]. In patients with autoimmune conditions, these $CD4^+CD28null$ cells have be shown to be CMV specific and are associated with poor patient outcomes [15, 16]. $CD4^+CD28null$ cells have been reported in a study of lung transplant recipients with associated increased risk of allograft rejection [17, 18]. Furthermore, one study demonstrated emergence of $CD4^+CD28null$ cells in four kidney transplant recipients following primary CMV infection [19], although evaluation of these cells' reactivity to alloantigen has not yet been studied.

Indirect effects of CMV are associated with poor patient and graft survival, acute rejection, chronic allograft dysfunction (interstitial fibrosis/tubular atrophy), new-onset diabetes after transplantation (NODAT) and an increased cardiovascular risk [20].

The direct cytopathic effects of cytomegalovirus on the renal allograft are now uncommon in clinical practice due to effective antiviral therapies. Unfortunately, far less is known about the efficacy of antiviral therapy in managing the indirect effects of cytomegalovirus [21]. Widespread and prolonged use of antiviral drugs has changed the natural course of CMV disease by delaying its onset and causing drug resistance.

An increasing number of immunological, clinical and epidemiological studies suggest that persistent CMV infection is associated with accelerated ageing of the immune system and with several age-related diseases. However, current evidence on whether and how CMV

infection is implicated in immunosenescence and in age-related diseases remains incomplete and many aspects of CMV involvement in immune ageing remain controversial [22].

1.2 Cytomegalovirus

1.2.1 Epidemiology

CMV affects 60% to 90% of healthy individuals worldwide and is closely linked with socioeconomic status [23]. Seroprevalence in communities has a large impact on the risk of developing CMV disease following transplantation and the methodologies used to reduce this risk [24].

CMV is present in a number of bodily secretions, such as saliva, urine and breast milk. In early childhood, transmission of CMV results from both vertical and horizontal transmission following shedding of virus in urine and respiratory secretions [6]. Sexual transmission is one of the major modes of transmission in adults. In solid-organ transplant recipients, CMV can be transmitted through the donor organ.

1.2.2 Genomics

Cytomegalovirus has a 236Kb double-stranded DNA genome, the largest of the human herpes virus species [25]. The virion is 150-200nm in diameter and icosahedral (Figure 1.4) [6]. CMV-DNA is surrounded by three layers: a tegument, a capsid with 162 protein capsomeres, and an outer envelope. Capsid encloses the tegument consisting of three phosphoproteins:

pp150, pp65, and pp71. The envelope contains lipoproteins and at least 33 structural proteins, some of which are glycosylated. These glycoproteins determine the CMV strain and consist of three distinct families of glycoprotein complexes, gcI (also known as gB), gcII, and gcIII, which are used for viral cellular entry and form the target for neutralizing antibodies [6]. The principal reservoirs of CMV are fibroblasts, myeloid cells and endothelial cells.

Six strains of human CMV have been sequenced, two laboratory (AD169 and Towne) and four clinical [26]. Our understanding of CMV pathogenesis has been aided by determination of the complete DNA sequence for AD169 by Chee and colleagues at Cambridge in 1990 [27] and subsequent further data on the additional genes carried by the clinical strains [26, 28, 29].

1.2.3 CMV cell entry

CMV infects a number of different cell types such as epithelial, smooth muscle, endothelial, macrophage and dendritic cells. Viral entry is mediated through engagement (tethering) between key surface viral glycoproteins and heparan sulphate-bearing polysaccharides (HSPGs) on the cell surface (Figure 1.5) [30]. This results in delivery of viral DNA (capsid), viral proteins and virion mRNA transcripts into the cytoplasm of the infected cell [31]. CMV can also enter cells by engaging (docking) with receptors such as the epidermal growth factor receptor (EGFR), integrins and Toll-Like receptor 2 (TLR-2) with resultant intracellular signal transduction and subsequent replication [32].

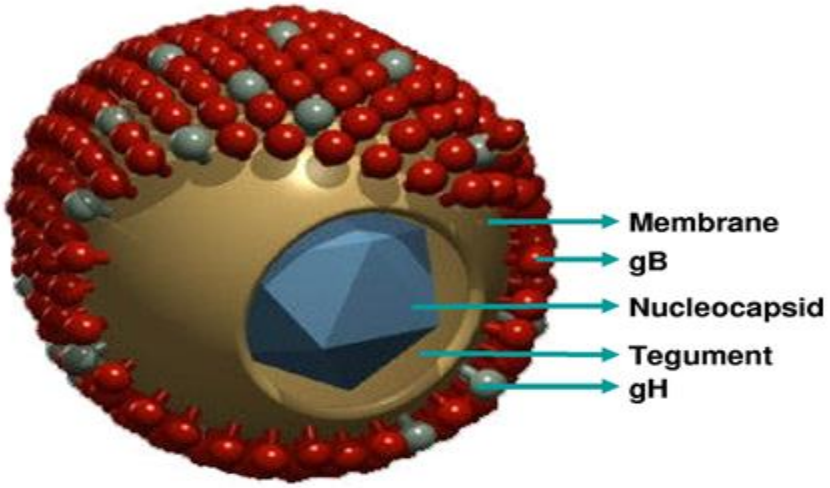

Figure 1.4 Human Cytomegalovirus [33]

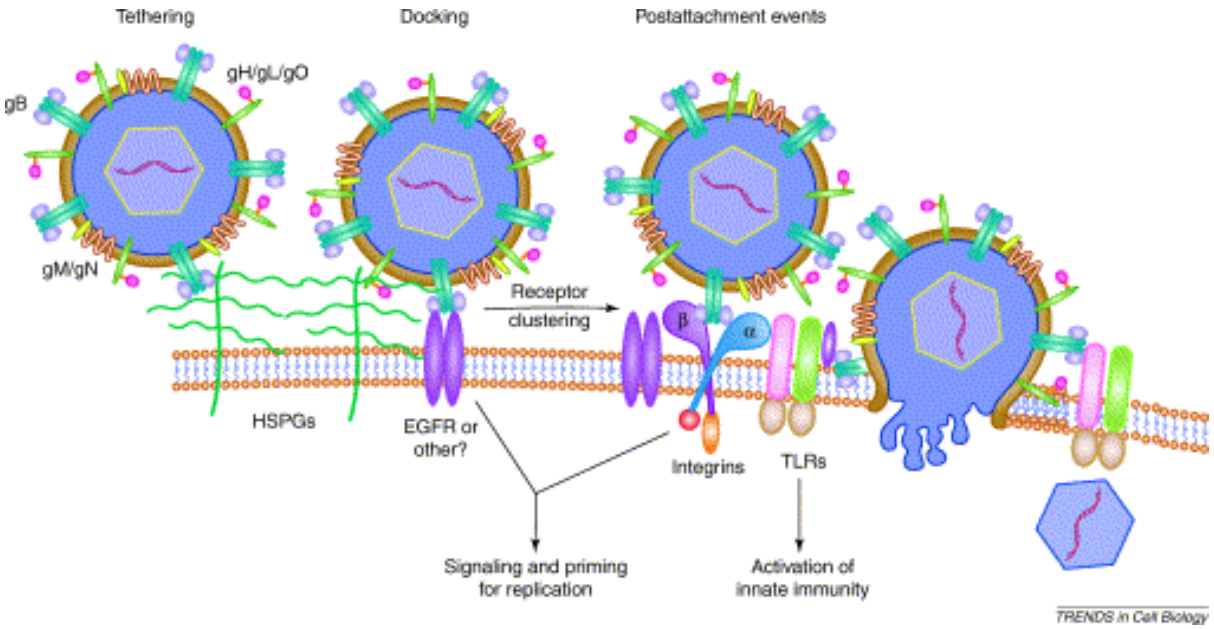

Figure 1.5 Cytomegalovirus entry into cells [30]

1.2.4 The lytic cycle

Figure 1.6 displays the CMV lytic life cycle. Following cell entry, CMV mRNA transcripts undergo translation in the cytoplasm. Viral DNA and certain viral proteins are transported to the nucleus, viral DNA is replicated through gene expression. Gene expression takes place in three steps:

- immediate-early (IE) gene expression – synthesis of proteins (IE 72, IE86) necessary for viral replication, cellular survival and escape from the host's immune surveillance. Occurs during the first 4 hours of infection.
- early (E) gene expression – synthesis of proteins key for viral replication
- late (L) gene synthesis - provides proteins that will be used to construct the new virions, within 6 to 24 hours after DNA synthesis.

This lytic cycle of replication produces at least 165 unique proteins [29]. Three key enzymes; DNA polymerase, CMV protease and Terminase are responsible for CMV replication, assembly and packaging of the capsid respectively [34, 35]. The infectious viral particle is then released from the cell.

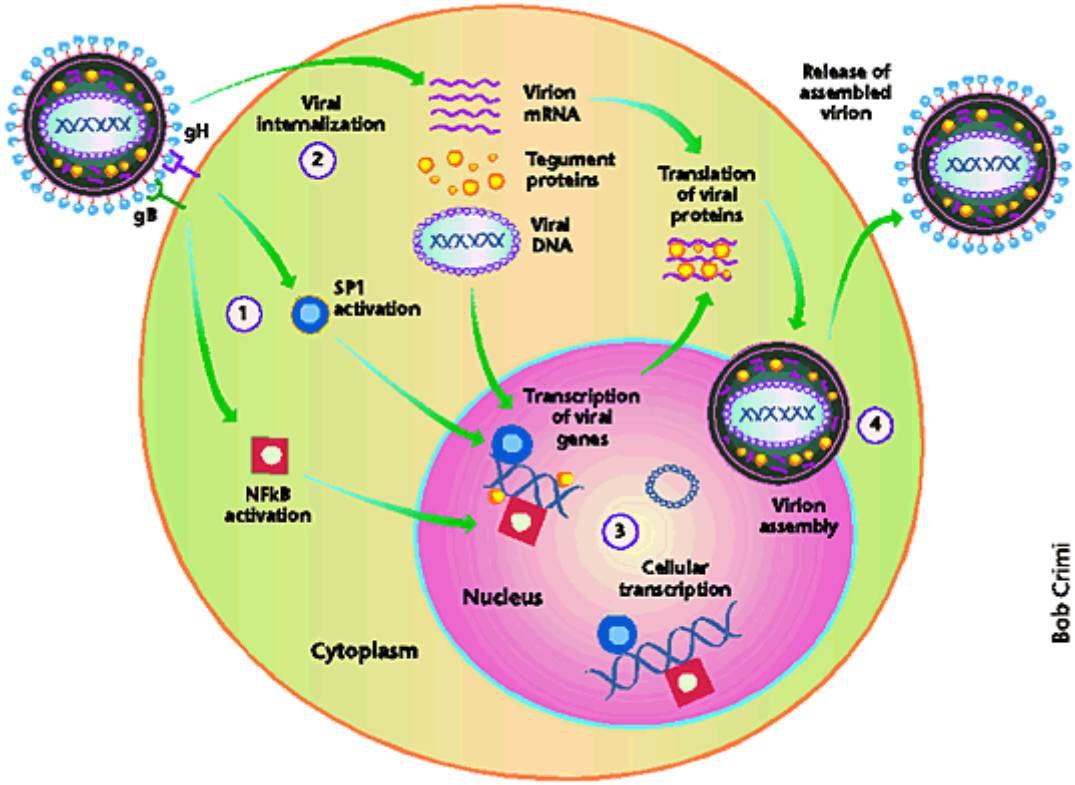

Figure 1.6 CMV lytic life cycle [31]

1.2.5 Latency

All herpesviruses, including CMV have the ability to escape immunological clearance and persist life-long in the infected host. This is known as latency. CMV latency has been described in both bone marrow progenitor cells and monocytes, although other cell types may also be important as a latent reservoir (e.g., endothelial cells) [36, 37].

The frequency of latently infected cells in blood appears to be very low. It is believed that CMV is continually reactivating and being supressed by the immune system. This suggests that even in healthy individuals the natural history of CMV replication is highly dynamic, which in turn has implications for patients when they are immunosuppressed [38]. In this context, there has been a focus on identifying the factors that may be important for triggering latent

CMV infection into an active infection. Triggers for reactivation in the healthy individual may be very different to those that are present in other settings. For example, in the case of organ transplantation, there is increasing evidence that the cytokine TNF-alpha mediates stimulation of the IE promoter in both human CMV and in murine models [39, 40]. Tissue damage at the time of organ harvesting and during transplantation surgery produces inflammation and a significant increase in pro-inflammatory cytokines, including TNF-alpha. Interestingly, CMV-specific CD8 T cells are also known to secrete large amounts of TNF-alpha, and CMV infection itself upregulates TNF-alpha production [41]. Consequently, in this scenario, a positive feedback loop may exist that leads to high-level CMV replication and a subsequent development of CMV disease.

1.3 Immune responses to Cytomegalovirus

Both the innate and adaptive immune system plays an important role in the defence against cytomegalovirus infection. In contrast to the level of response seen to other acute and persistent viruses, a substantial proportion of the immune systems efforts are devoted to the control of CMV [42].

1.3.1 Innate Immunity

The innate immune system is important in both the defence against CMV and in priming the adaptive immune response to CMV [33]. Evidence from murine [43, 44] and human models [32, 45, 46] of CMV demonstrates the important role Toll-like receptors (TLRs) play in the

innate sensing of CMV. The interaction of CMV with TLRs results in activation of signal transduction pathways, secretion of inflammatory cytokines (interferon alpha/beta) by dendritic cells and macrophages (Figure 1.6) and the subsequent activation of natural killer (NK) cells.

The role of NK cells in the immune defence against CMV has been determined from murine models of CMV infection (MCMV). Experimental studies have shown NK cells to be involved in the clearance of experimental MCMV infection [47, 48]; depletion of NK cells increased susceptibility to MCMV [49], whereas adoptive transfer of NK cells resulted in protection from MCMV [50]. There is paucity of data on the role of NK cells in the immune defence against human CMV (HCMV). In renal transplant patients, increased NK activity during primary and recurrent CMV infection has been demonstrated [51]. CMV disease and suboptimal recovery from CMV infection has been reported in patients with NK cell deficiency [52, 53].

1.3.2 Adaptive Immunity

Long-lasting immunity to CMV is vital in preventing uncontrolled viral replication and severe CMV disease. T-cell mediated immune responses are predominately responsible for controlling and restricting viral replication during primary latent infection (Figure 1.7) [33]. Although the role of B cells in the control of CMV replication has been debated, there is evidence to support their role in restricting viral dissemination and limiting severity of disease (Figure 1.6) [54, 55].

1.3.2.1 T-cell mediated immune responses

The role of T-cell immunity to CMV is supported by data from murine and human models of CMV. Figure 1.6 shows the role of T cells in the acute and latent phase of CMV infection.

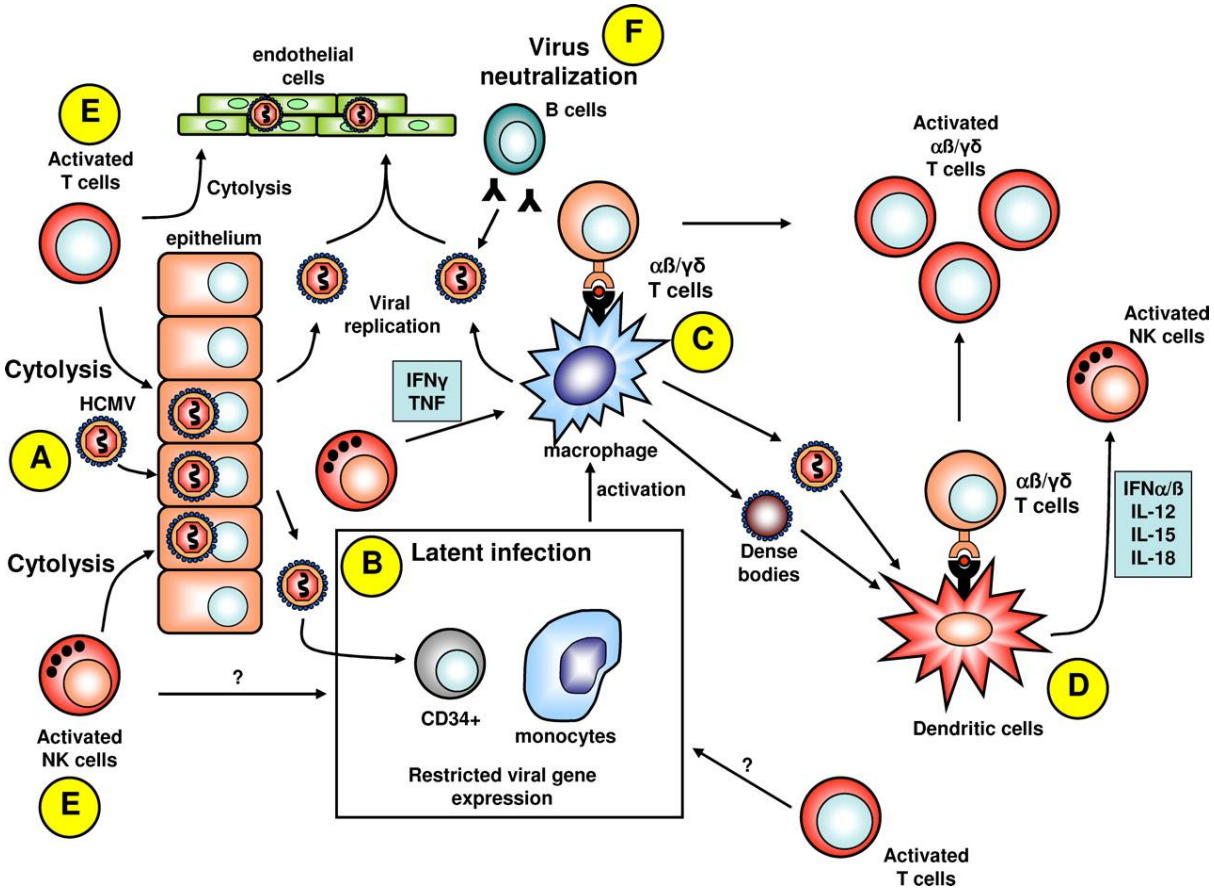

Figure 1.7 Immune control of CMV by innate and adaptive immunity [33]

1.3.2.2 Role of CD8⁺ T cells

CD8⁺ T cells play a pivotal role in the immune control of CMV [48]. In murine models, depletion of CMV-specific CD8⁺ T cells resulted in an increase in reactivation and dissemination of CMV

infection, whereas adoptive transfer of CMV-specific $CD8^+$ T cells provided protection from the infection [56, 57]. In humans, fetal $CD8^+$ T cells have been shown to expand in response to a primary CMV infection [58]. In immunocompromised patients, CMV-specific $CD8^+$ T cells are largely responsible for the control of CMV. This has been supported by data from studies of patients with AIDS [59], bone marrow transplants [60-62], and solid organ transplants [63, 64] . A dominant $CD8^+$ T-cell response to both primary and latent CMV has been shown in kidney transplant recipients [65-67].

A substantial proportion of the $CD8^+$ T-cell pool is committed to the anti-CMV response. In healthy virus carriers, 4-10% of peripheral blood $CD8^+$ T cells can be specific for CMV antigens [68-71]. In healthy elderly CMV seropositive donors, up to 40% of the $CD8^+$ T-cell pool can be CMV specific [70]. The impact this immune dominance has on the response to other pathogens/antigens remains unclear [33].

Studies in healthy CMV-seropositive donors have revealed CMV-specific $CD8^+$ T cells are directed towards a hierarchy of virus-encoded proteins, with UL123 (IE-1), UL122 (IE-2), and UL83 (pp65) as immunodominant antigens [71, 72].

Figure 1.8 demonstrates the phenotypic changes of CMV-specific T cells from acute infection to latent state [73]. During acute CMV infection, CD8 effector T cells have a $CD45RA^-$ $CD45RO^+$ $CD27^+$ $CD28^{+/-}$ $CCR7^-$ phenotype [33]. In chronic CMV infection (latent state), CMV-specific T cells are either effector memory ($CD45RA^-$ $CD45R0^+$ $CD27^-CD28^-$ $CCR7^-$) or terminally differentiated effector T cells re-expressing CD45RA (TEMRA) ($CD45RA^+$ $CD45R0^-$ $CD27^-$ $CD28^-$ $CCR7^-$) [74]. These highly differentiated effector memory T cells undergo oligoclonal

expansion (memory inflation) with resultant reduction in the naïve T-cell pool [75]. The expansion of these cells is driven by persistence of antigen, and do not require IL-7, unlike memory cells found after infection with cleared viruses.

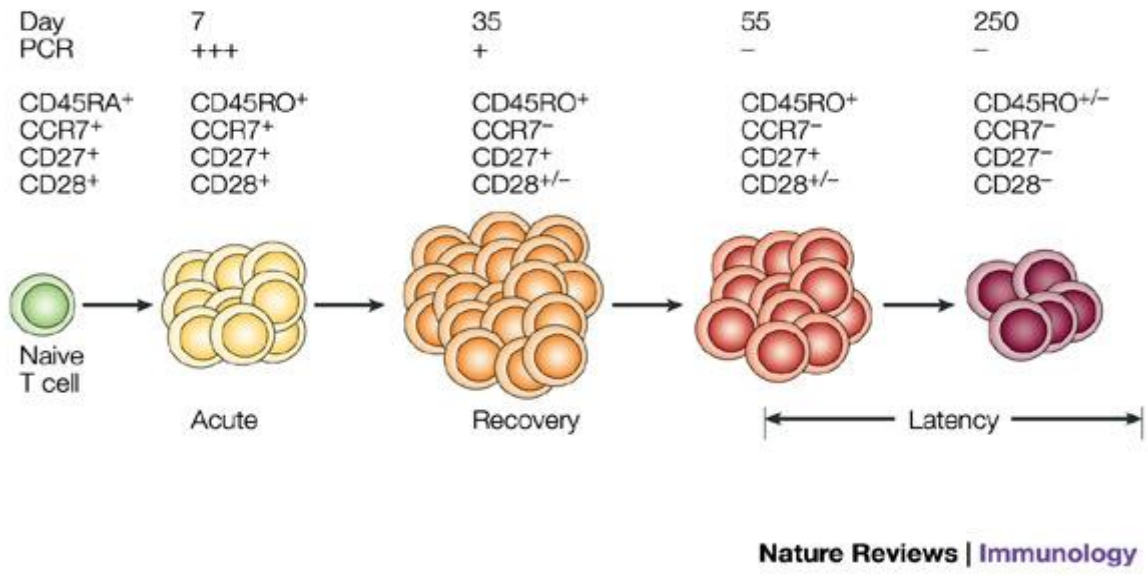

Figure 1.8 Phenotypic changes of CMV-specific T cells from acute infection to latent state [73]

1.3.2.3 Role of CD4+ T cells

The role of $CD4^+$ T cells in the control of CMV infection is becoming increasingly evident [76, 77]. In murine models of CMV infection, $CD4^+$ T cells have been shown to contribute to both the control of primary and recurrent MCMV infection [48, 78]. Deficiency of $CD4^+$ T-cell immunity to cytomegalovirus in immunocompetent children is associated with prolonged viral urinary and salivary shedding [79]. In studies of HIV-infected patients, CMV-specific $CD4^+$ T cells have been shown to be crucial for a functional CMV-specific $CD8^+$ T-cell response [80-82]. These observations have direct relevance for the control of CMV replication post-

transplantation, especially as the immunosuppressant drugs utilised to prevent organ rejection have a significant impact on CD4 T-cell function. In a study of allo-stem cell transplant patients, a single infusion of predominately CMV-specific $CD4^+$ T cells resulted in the clearing of CMV viraemia in five out of seven patients [76]. Following kidney transplantation, levels of CMV-specific $CD4^+$ T cells have been shown to correlate with cytomegalovirus control and may be useful to predict CMV disease [83]. A further study involving kidney transplant patients demonstrated the importance of CMV-specific effector-memory $CD4^+$ T cells for recovery of infection [11].

In healthy CMV seropositive individuals, a substantial proportion of circulating $CD4^+$ T cells is committed to anti-CMV immunity. A median frequency of 9.1% [71], and up to 40% in some donors occurs, with no overt compromise to the remaining protective immunity [84].

In latent CMV infections, the role of $CD4^+$ T cells in providing indirect help for maintaining virus-specific antibody responses [85] and expanding the $CD8^+$ T-cell population [61] is well established. However, CMV-specific $CD4^+$ T cells also have a direct role in controlling infection by killing virus-infected cells [86, 87]. The cytolytic activity of CMV-specific $CD4^+$ T cells is associated with a terminally differentiated phenotype (Figure 1.8, $CD4^+CD27^-CD28^{null}$) [88]. These cells are cytotoxic in that they express perforin and granzyme B in response to CMV [89].

1.3.2.4 Humoral immunity

The importance of antibodies in the effective immune response against CMV is supported by various animal and human studies [90-94]. In the guinea pig model, passive immunization with guinea pig cytomegalovirus antibodies did not prevent infection, but the antibodies increased the survival of pups [90]. In humans, the transfer of antibodies from a CMV-seropositive mother to a new-born infant was shown to be protective against CMV infection from seropositive blood transfusions [91]. It is now well established that women with preconception immunity to CMV transmit infection to the foetus at a lower frequency than women with primary infections [91].

1.4 Immune Evasion by Cytomegalovirus

Cytomegaloviruses have developed a number of strategies to subvert host immune surveillance and defence, allowing them to co-exist with their host despite an anti-CMV immune response. These include:

- Downregulation of MHC class I: results in inhibition of direct present of CMV antigens to $CD8^+$ T cells [95]
- Downregulation of MHC class II: results in inhibition of indirect presentation of CMV antigens to $CD4^+$ T cells [96, 97]
- Inhibition of NK cell activation: CMV activates NKG2A inhibitory receptor [98] and blocks NKG2D activating receptor [99]

The control of CMV replication in the immunocompromised host has been studied using class I HLA tetramers together with intracellular cytokine assays. This has revealed that patients who fail to control CMV replication have poorly functional CD4 and CD8 T-cell responses (ref). The functional ability of $CD8^+$ and $CD4^+$ T cells to produce interferon-gamma (IFN-gamma) and IL-2 in response to CMV peptides has been shown to be essential for limiting high-level replication in both renal and liver transplant recipients [100, 101]. Other markers that are associated with periods of active replication include PD-1 and perforin. However, there is paucity of data on prognostic immune markers for future CMV disease.

1.5 CMV pathogenesis after solid organ transplantation

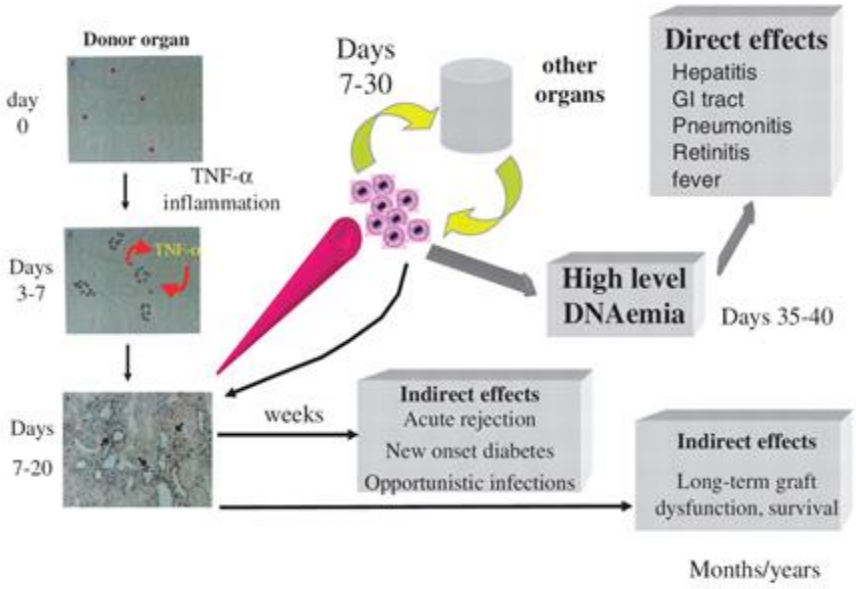

Figure 1.9 A model for CMV pathogenesis after solid organ transplantation [24]

Figure 1.9 shows a model for CMV pathogenesis after solid organ transplantation [24]. The donor organ harbours a small number of cells with latent infection (red dots). The physiological stress associated with transplantation surgery results in the release of inflammatory cytokines such tumour necrosis factor (TNF). TNF-alpha appears to be an important driver for re-activation of latent CMV in the transplanted organ and there is increasing evidence that CMV replication begins very early after the transplant surgery is complete [102]. Subsequent organ-specific replication ensues over the next 7 days, with the production of new virions. These new virions may then spread through the blood to infect other target organs, which contributes to the overall level of CMV DNAemia. In the absence of CMV prophylaxis, virus detection in the blood usually occurs between 20 and 30 days post-transplant. If left untreated, these high levels of replication will be associated with the direct effects of CMV infection. In addition, early graft infection may contribute to acute transplant dysfunction, occurrence of other opportunistic infections and affect long-term graft and patient survival (indirect effects) [24].

Figure 1.10 shows the possible mechanisms responsible for graft damage during an infection [103]. Microorganisms can have direct cytopathic effects, with resultant release of damage-associated molecular patterns (DAMPs), microbial or donor antigens (alloantigens) and possibly autoantigens (Figure 1.10a). Recipient antigen-presenting cells (APCs) or endothelial cells in the graft take up and cross-present exogenous microbial antigens to recipient T cells (Figure 1.10b). Activated APCs and/or T cells secrete cytokines that are damaging to the graft or that injure endothelial cells, thereby reducing vascular supply to the graft. Infections can induce the maturation of recipient APCs or activate endothelial cells that have taken up donor antigens (blue triangles). This results in the indirect activation of alloreactive T cells and in

graft injury mediated by pro-inflammatory cytokines and toxic soluble factors that injure blood vessels (Figure 1.10c). Similarly, infections can augment the direct activation of alloreactive T cells by enhancing the maturation of donor APCs or graft parenchymal cells, resulting in graft damage (Figure 1.10d). Cellular graft damage may release cryptic antigens (autoantigens) and DAMPs that then activate autoreactive T cells (Figure 2e). After re-encountering these antigens on cross-presenting recipient APCs or endothelial cells, these autoreactive T cells may contribute to graft damage in a phenomenon analogous to epitope spreading.

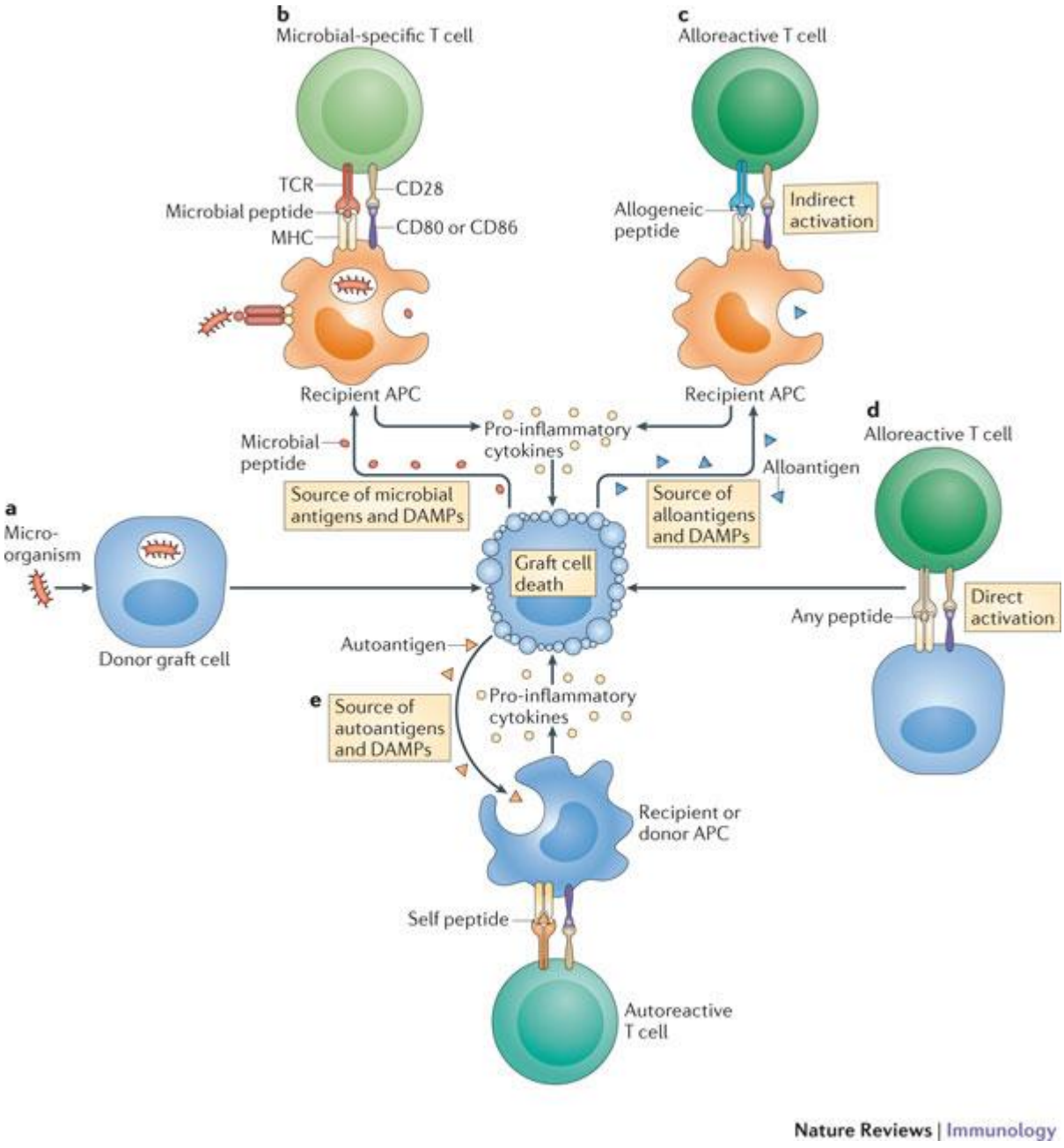

Figure 1.10 Potential causes of graft damage during microbial infections [103]

Figure 1.11 shows a possible mechanism for CMV induced endothelial damage. Latent CMV infection produces a large pool of terminally differentiated $CD8^+$, and to a lesser extent, $CD4^+$ effector T cells [104, 105]. These $CD8^+CD27^-CD28^{null}$ and $CD4^+CD27^-CD28^{null}$ cells have the potential to produce IFNγ and are armed with cytotoxic granules (perforin, granzyme B),

ready to become active as soon as viral replication occurs [106]. $CD8^{+}CD27^{-}CD28^{null}$ and $CD4^{+}CD27^{-}CD28^{null}$ cells have a potential role in vascular damage. CMV infection induces a systemic Th1 response with increased serum levels of IFNγ and TNFα [106] [107]. These cytokines have been shown to induce an upregulation of fractalkine on endothelial cells (EC) in vitro, and CMV specific effector T cells were shown to bind to these fractalkine-expressing endothelial cells. This was followed by migration of these cells to activated EC in vitro, followed by damage and apoptosis of these endothelial cells [108] and [109].

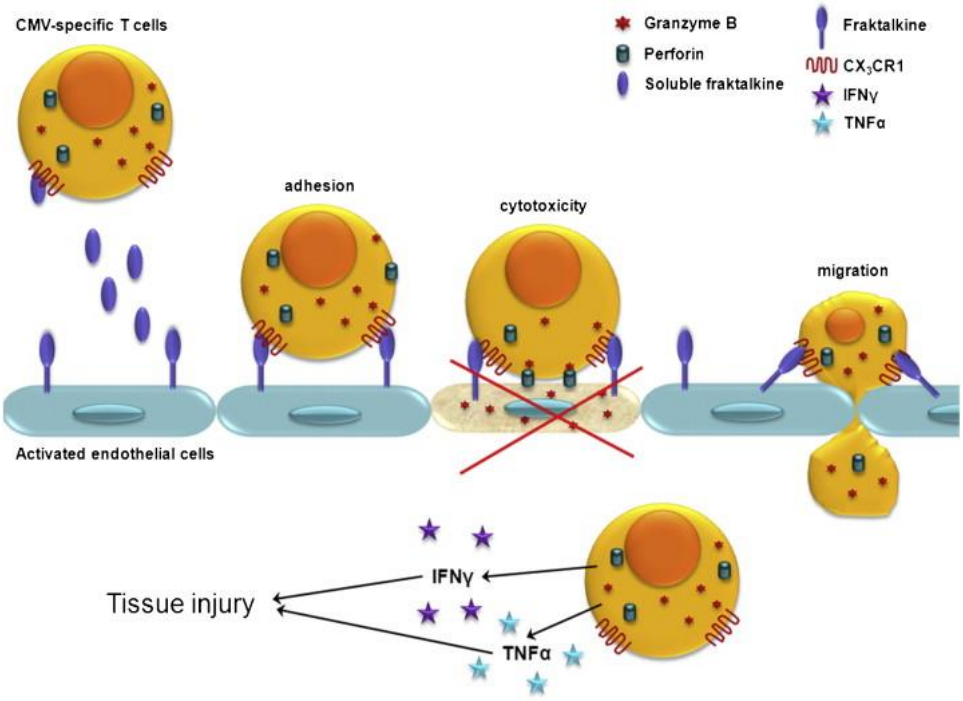

Figure 1.11 Immunopathology caused by cytomegalovirus [106]
Figure 1.11 illustrates how CMV can influence cell adhesion.

1.6 Definitions

Individuals who have no prior exposure to CMV are defined as 'CMV seronegative'. Following infection with CMV, individuals become 'CMV seropositive' lifelong due to the formation of antibodies directed against CMV and the ability of the virus to establish latency. In the context of transplantation, the organ/cell donor can be CMV seronegative (D^-) or CMV seropositive (D^+) and likewise, the recipient is either CMV seronegative (R^-) or CMV seropositive (R^+).

1.6.1 Primary CMV infection

Primary CMV infection in an immunocompetent host is defined as CMV infection in a previously seronegative individual.

In the immunocompromised host; such as a transplant recipient, primary CMV infection occurs when latently infected cells from a CMV seropositive donor are transferred to the seronegative recipient [21]. In the vast majority of cases, these latently infected cells are within the allograft; however, transfusion of viable leukocytes from seropositive blood donors can also transmit CMV [21].

1.6.2 CMV infection

CMV infection is defined as detectable viral replication in any body fluid/tissue specimen, or a significant rise in CMV-specific antibodies [6]. CMV infection can be asymptomatic, or give

rise to CMV syndrome or disease. British Transplant Society Guidelines for the diagnosis of CMV syndrome and disease in solid organ transplantation are as follows:

CMV Syndrome	**CMV disease**
1. CMV viraemia 2. One or more of: • fever • new onset severe malaise • leukopenia • atypical lymphocytosis • thrombocytopenia • hepatitis (ALT or AST >2 upper limit of normal)	In addition to criteria for CMV syndrome, evidence of organ involvement (supported by histological evidence where possible): • BM suppression • Hepatitis • Pneumonitis • Nephritis • Gastrointestinal tract • CNS • Retinitis • Myocarditis • Pancreatitis

1.6.3 Latent infection

Following primary infection, CMV establishes lifelong persistence within the host. Latency is defined as carriership of CMV genome without active replication [6]. Latent CMV has the ability to reactivate under specific stimuli.

1.6.4CMV Reactivation

CMV reactivation is new detection of CMV infection in a seropositive individual. It occurs as a result of reactivation of host's latent CMV. In the context of transplantation, this is the result of reactivation of the seropositive transplant recipient's endogenous latent CMV strain.

1.6.5 CMV Superinfection/Re-infection

CMV superinfection/re-infection occurs when both the transplant donor and recipient are seropositive (D+/R+). It occurs as a result of reactivation of exogenous latent CMV (donor origin) [21].

1.6.6 CMV disease

CMV disease is the clinical expression of active infection [6].

1.7 Clinical presentation

In transplantation, the incidence and severity of CMV disease is determined by the pre-transplant CMV serostatus of the donor (D) and recipient (R) [110, 111]. The three risk groups as defined by the donor and recipient CMV serostatus are:

1. High risk (D^+/R^-)
2. Intermediate risk (D^-/R^+, D^+/R^+)

3. Low risk (D^-/R^-)

The British Transplant Society guidelines (2011) recommend CMV prophylaxis or pre-emptive therapy for the high risk (D^+/R^-) group and for transplant recipients treated with T-cell depleting antibodies.

The effects of CMV infection in immunocompromised hosts can be divided into direct and indirect effects. In immunocompetent hosts, primary CMV infection is usually asymptomatic but can produce an infectious mononucleosis-like syndrome with fever, myalgia, pharyngitis, cervical lymphadenopathy, mild hepatitis, and splenic enlargement [6, 112]. However, primary CMV infection may take a much more aggressive course in immunocompromised individuals. Resultant tissue-invasive disease may present as pneumonitis, carditis, gastrointestinal disease, hepatitis, nephritis or retinitis [113]. Direct effects of CMV are related to viral burden, with high viral loads intimately involved in the pathogenesis of CMV infection [38].

1.7.1 Indirect effects of CMV infection

CMV associated adverse outcomes, not directly related to viral invasion of tissues are outlined below. These indirect effects of CMV infection are mediated by cellular and humoral immune responses [114].

1.7.1.1 Acute and chronic allograft rejection

Data from clinical studies [7, 8, 115], and animal models [116, 117], supports an association between CMV infection, rejection and graft loss.

The mechanism for CMV-driven rejection in not fully understood. However, an in-vitro study of a rat heart transplantation model suggests CMV infection of vascular endothelium and smooth muscle cells plays a key role in the pathogenesis of chronic rejection [116]. CMV infection up-regulates endothelial cell adhesion molecules [118-120] and enhances production of inflammatory cytokines (IL-10, TNF-alpha) and chemokines [39, 41, 43]. These cytokines and chemokines can also be produced due to allograft rejection. Therefore, CMV has the potential to both trigger and exacerbate a rejection episode.

CMV infection and disease significantly increased the risk for acute rejection in a study of 477 kidney transplant recipients not receiving CMV prophylaxis [115]. In a separate series of 259 kidney transplant patients, Sola et al identified CMV disease as an independent risk factor for chronic allograft nephropathy ($p<0.02$) [121]. Helantera and colleagues have shown persistent CMV infection in kidney allografts is associated with reduced kidney allograft function ($p=0.007$) and survival ($p=0.04$) [8]. In a separate study, CMV DNA was localised to renal tubular epithelial cells and associated with poor long-term kidney allograft function [102].

Complementing these human studies is a large body of evidence from the rat CMV model. These animal studies show CMV infection accelerates progression to poor graft function

(chronic allograft nephropathy) and graft loss [84]. In rat models, CMV can induce tubular apoptosis via the TNF-alpha/TNF-alpha receptor-1 pathway [117]. Importantly, treatment of these animals with ganciclovir reduced intimal thickness to levels seen in uninfected rats [122].

The immune response against CMV is extensive. Migration of CMV-specific lymphocytes into an organ to kill CMV-infected cells may be mistaken for acute rejection in the absence of evidence showing that CMV is present. This suggests CMV prophylaxis in the early post-transplant period may minimize the effects of CMV as a causative or exacerbating agent for acute rejection [123].

1.7.1.2 Accelerated coronary artery disease

In the general population CMV has been implicated in the pathogenesis of atherosclerosis as early as the 1970s [124]; with a number of clinical studies demonstrating it as an independent risk factor for restenosis after coronary angioplasty [125, 126]. A high incidence of CMV DNA has been demonstrated in both coronary atherectomy specimens and re-stenotic lesions [127, 128]. High antibody titres against CMV are associated with coronary artery disease and may predict restenosis risk following coronary angioplasty [129].

Although the mechanism of injury remains unclear, rat models have shown CMV infection to cause endothelial injury, increase leukocyte adhesion, and precipitate the accumulation of lipids in vascular endothelium [120]. In vitro studies indicate that CMV infects endothelial

cells, smooth muscle cells, monocytes, and macrophages, all of which have been implicated in atherosclerosis [130, 131].

In kidney transplantation, CMV infection has been shown to be an independent risk factor for atherosclerotic events; with increased incidence of arrhythmias, congestive heart failure, and vessel occlusion [131-133].

1.7.1.3 Increased incidence of opportunistic infections

CMV infections increase the susceptibility of transplant patients for other opportunistic infections (bacterial, fungal and protozoal) [134-136]. CMV interferes with the recognition of infected cells by altering the function of dendritic cells and preventing the delivery of signals required for T-cell activation [137]. CMV has been linked to an increased risk of Epstein-Barr virus (EBV) related post-transplant lymphoproliferative disease (PTLD) [138, 139]. High levels of IL-10 have been observed in the serum of patients with PTLD [140] and it has been suggested that the CMV IL-10 virokine could mediate this increased risk [141].

1.7.1.4 New-onset diabetes after transplantation (NODAT)

New-onset diabetes after transplantation (NODAT) is common, with a reported incidence ranging between 2% and 53% [142]. Data from clinical [143] and animal studies [144] suggest that CMV infection may be a predisposing factor for NODAT. A fivefold increased risk of NODAT has been reported in kidney transplant patients with asymptomatic CMV infection

compared with patients who remained free of CMV infection (figure 1.12) [143]. It has been suggested that CMV may damage the beta cells of the pancreas in a variety of ways, including direct infection and immune-mediated destruction [143].

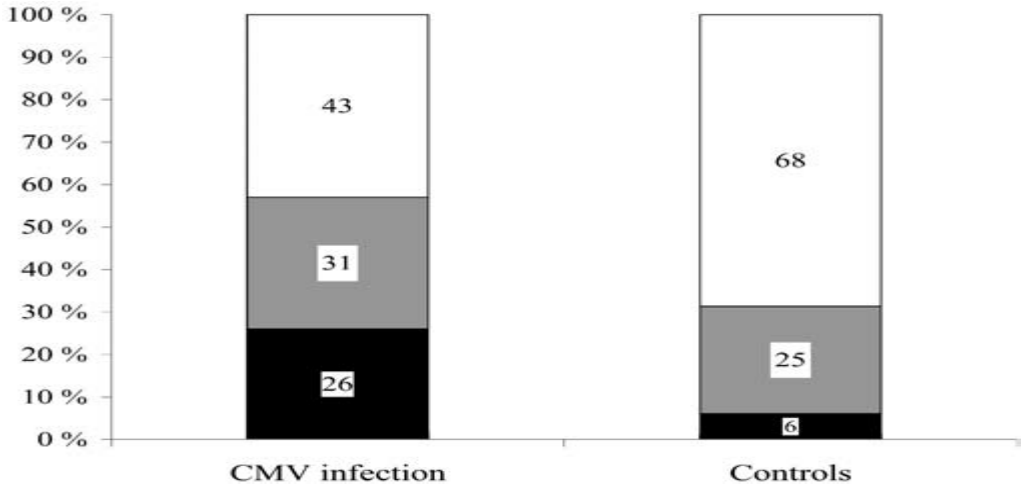

Figure 1.12 Relative proportions of patients with varying degrees of glucose tolerance according to CMV status

A significantly higher proportion of patients with CMV infection developed NODAT (p=0.003) and Impaired Glucose Tolerance (p=0.008) compared with controls.
CMV infection (*n*=61), patients without CMV infection (controls, *n*=63). White: Normal Glucose Tolerance, Grey: Impaired Glucose Tolerance, Black: Post-Transplant Diabetes Mellitus [143].

1.8 Role of CMV in immunosenescence

Aging of the immune system ('immunosenescence') is characterised by the following immune risk profile (IRP):

1. Accumulation of memory T cells, especially $CD8^{+}CD28^{null}$ cells and EMRA cells [145, 146] .
2. Inverted CD4:CD8 ratio.
3. Increased memory: naïve cell ratio within the CD8 compartment [145-147].

These markers of immunosenescence have clinical correlates in the general population, and specifically are associated with increased risk for infection and mortality, and a reduced vaccine response [147-151].

It has been suggested and increasingly accepted, that CMV infection is a major driver of the immune phenotype characteristic of immunosenescence [152-155]. The link between CMV and the immunosenescence phenotype is not clearly established in transplantation, although a recent study demonstrated expansion of $CD8^{+}CD28^{null}$ cells in 11 kidney transplant recipients experiencing primary CMV infection [156]. Increased frequencies of CD8 EMRA and $CD8^{+}CD28^{null}$ cells at the time of transplantation have been associated with freedom from early biopsy-proven acute rejection [157, 158]; CD8 EMRA frequencies were also associated with (a trend towards) increased risk of graft dysfunction over the long term in prevalent patients [159]. In a cross sectional 'extended phenotype' study, $CD8^{+}CD28^{null}$ cell frequencies were higher in patients with histological lesions of 'chronic rejection' in comparison to those with drug-free operational tolerance [160]. Surprisingly only one (very recent) study addressed infection as an endpoint, with no association found between $CD8^{+}CD28^{null}$ cell frequencies or CD4:CD8 ratio and episodes of infection [161]. These studies of transplant cohorts understandably addressed the 'transplant-specific' end measures as described; the potential role of CMV in driving the studied markers of immunosenescence was not a focus in most of these particular studies.

Finally, an increasingly recognised phenomenon is immunosenescence of the innate immune system, in particular abnormalities of neutrophils which represent the most abundant circulating immune cell and a first line of defence against pathogens [162, 163]. Yet far less

work has been undertaken in this area compared with T-cell immunosenescence, and is lacking in the field of transplantation. Overviews of this subject in transplantation to date have necessarily extrapolated data from non-transplant settings [164-166].

1.9 Monitoring of CMV-specific T-cell responses

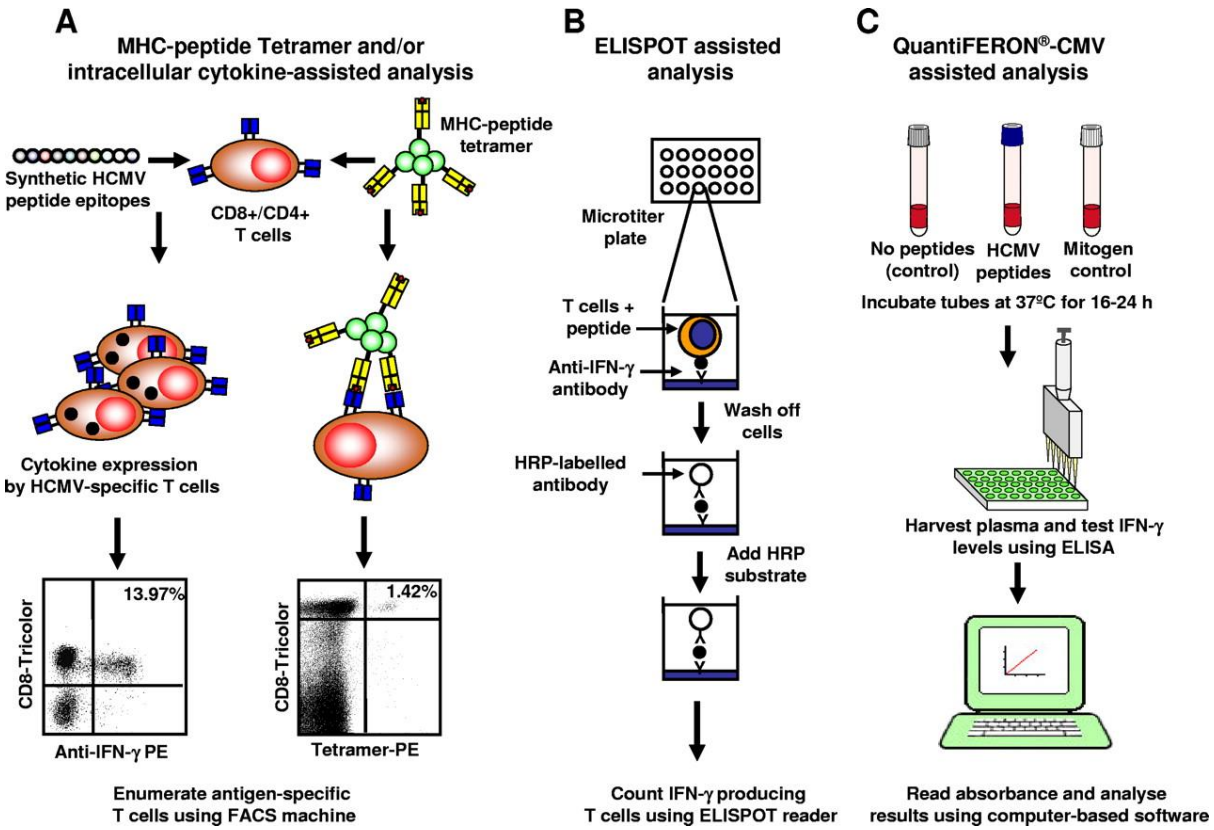

Figure 1.13 Ex vivo monitoring of CMV-specific T-cell responses [33]

The diagnosis of CMV disease is guided by clinical symptoms and signs, and supported by detection of CMV in the blood and involved tissue(s) [33, 167]. In transplantation, Quantitative PCR methods (Figure 2.3c) are routinely used for determining CMV loads and

monitoring viral DNAemia [33]. However, patients with a detectable viral load do not necessarily develop CMV disease but may receive treatment with toxic anti-viral medication regardless [33, 168].

The development of ex-vivo T-cell assays (MHC-peptide tetramers: Figure 1.13a, ELISPOT: Figure 1.13b, intra-cellular cytokine analysis by flow cytometry: Figure 1.13a) have facilitated the identification and functional analysis of CMV-specific T cells [64, 68, 169].

In transplantation, various cross-sectional studies have focused on the diagnostic and clinical utility of measuring the cell-mediated immune (CMI) response to CMV [33, 64, 83]. Although the results are somewhat conflicting, nonetheless, these studies have provided preliminary evidence that the frequency of IFN-γ-secreting CMV-specific $CD4^+$ and $CD8^+$ T cells may be clinically relevant and could be an effective tool for monitoring disease activity [64, 83]. A recent prospective study identified an association between pre-transplant CMI to CMV and post-transplant viral replication in 55 kidney or lung transplant recipients undergoing either antiviral prophylaxis or pre-emptive therapy [13]. Further prospective studies under contemporary immunosuppression are required to further evaluate the diagnostic utility of these ex-vivo T-cell assays.

1.10 Aims and Outline of the Thesis

With this background, evidently, there are significant gaps in the current literature pertaining to the indirect effects of cytomegalovirus infection following kidney transplantation. The studies presented in this thesis aim to explore both the mechanism of CMV infection following kidney transplantation and CMV-driven kidney transplant damage.

Chapter 2: Impaired Direct CD8 Priming to Donor-Derived Cytomegalovirus Following Kidney Transplantation

Chapter 2 investigates the associations between donor CMV serostatus on CMV disease risk in seropositive recipients and explores mechanism of CMV infection in D^+/R^+ transplantation. Also, utility of pre-transplant cellular immunity assays in predicting CMV infection are evaluated.

In D^+/R^- transplantation, high rates of primary CMV infection are well recognised and requirement for antiviral prophylaxis largely uncontested. CMV disease is also seen in seropositive recipients (R+), often grouped together as 'intermediate risk' irrespective of donor serostatus. However, both historical and recent evidence suggest higher infection rates in D^+R^+ transplantation compared with D^-R^+ transplantation [110, 170-172]. The latter studies focussed on CMV infection rather than symptomatic disease, and used either antiviral prophylaxis or pre-emptive therapy of CMV infection. It therefore remains unclear whether disease rates differ between D^+R^+ and D^-R^+ transplantation, particularly under contemporary immunosuppression without antiviral prophylaxis. The mechanism behind CMV infection in D^+R^+ transplantation is incompletely understood [170, 173]. Donor-derived infection and lack of protective immunity to newly infecting strains may be important (Ishibashi). An additional possible mechanism is human leucocyte antigen (HLA) mismatch between donor and recipient. However, data is conflicting with suggestion of both increased [174, 175] and reduced [176, 177] CMV disease rates with increased HLA Class II mismatch. HLA Class I mismatch has been identified as a risk factor for CMV disease in a historical study by Pouteil-Noble C [175].

The primary aims of this chapter were to evaluate the impact of donor CMV serostatus on disease risk in seropositive recipients and explore the mechanism of infection by use of HLA class I tetramers. The secondary aim was to study the utility of pre-transplant cellular immunity assays in predicting CMV infection in D^+/R^- and D^+/R^+ transplantation.

The findings will be of considerable interest in further understanding the mechanism of viral infection in solid organ transplantation and aid the clinical management of CMV infection.

Chapter 3: CMV-associated $CD4^{+}CD28^{null}$ cells in NKG2D-dependent glomerular endothelial injury and kidney allograft dysfunction

Chapter 3 focuses on the temporal association between CMV serostatus, CMV infection and $CD4^{+}CD28^{null}$ cells. It explores mechanisms of kidney allograft damage by $CD4^{+}CD28^{null}$ cells and their impact on kidney transplant outcomes.

Latent CMV infection in patients with end-stage kidney disease (ESKD) is associated with expansion of circulating, late-differentiated, cytotoxic $CD4^{+}$ T cells which have lost expression of the costimulatory molecule CD28 ("$CD4^{+}CD28^{null}$") [14]. In 2004, a study by Van Leeuwen demonstrated emergence of $CD4^{+}CD28^{null}$ cells in four kidney transplant recipients following primary CMV infection [19]. In studies involving subjects with autoimmune conditions, expansion of $CD4^{+}CD28^{null}$ cells occurs in response to CMV antigens rather than candidate autoantigens [15, 16]. In kidney transplantation, reactivity of these cells to alloantigen has not yet been studied. In lung transplantation, expansion of $CD4^{+}CD28^{null}$ cells has been associated with allograft rejection [17, 18]. $CD4^{+}CD28^{null}$ cells express the cytotoxic mediators' perforin and granzyme B [19] . They express natural killer-like receptors such as NKG2D [178]. In vitro studies suggest a pathogenic role for $CD4^{+}CD28^{null}$ cells, with endothelial cell injury via an NKG2D-dependant mechanism [179]. However, these studies involved human umbilical vein endothelial cells (HUVEC). At present, there is no data on the pathogenic role of $CD4^{+}CD28^{nulls}$ cells in kidney transplantation or their impact on kidney transplant outcomes.

The purpose of this study was to evaluate the temporal relationship of CMV serostatus, CMV infection and $CD4^{+}CD28^{null}$ cell frequencies during the first post-transplantation year. In vitro responses to CMV-derived and HLA-derived antigens were examined. The pathogenic role of $CD4^{+}CD28^{null}$ cells was evaluated using in vitro assays of glomerular endothelial cells. Finally, the relationship between $CD4^{+}CD28^{null}$ cell frequency and kidney transplant outcome was studied.

The findings will help provide potential novel strategies to interfere with $CD4^{+}CD28^{null}$ cell toxicity and may result in further improvements in allograft outcome.

Chapter 4: Immunosenescence in kidney transplantation: relationship with CMV, infection, and neutrophil dysfunction

Chapter 4 evaluates the relationship between CMV infection and immunosenescence, and explores whether immunosenescence predicts episodes of other infections. The mechanisms responsible for increased rates of infection are studied using in-vitro neutrophil assays.

Immunosenescence is defined by an immune risk profile (IRP). The immune parameters that characterise the IRP are: CMV seropositivity, clonal expansion of CMV–specific $CD8^+$ T cells, an increase in $CD8^+CD28^{null}$ cells, low $CD4^+$ T-cell, low B cells, and an inverted CD4: CD8 ratio [145]. These findings are based on studies of healthy Scandinavian octo- and nonagenarians [147, 180]. In these studies, the IRP has been associated with adverse clinical sequelae (reduced vaccine response, increased infection and mortality). As described in the earlier chapter, CMV elicits a particularly profound CD8 immune response termed 'memory inflation' and is characterised by accumulation of late-differentiated $CD8^+CD28^{null}$ and $CD8^+$ EMRA subsets [152]. It is therefore suggested that CMV represents a major driver towards immunosenescence. Studies in kidney transplantation have focused on the association between increased frequencies of $CD8^+CD28^{null}$ and $CD8^+$ EMRA cells and transplant specific outcomes (biopsy-proven acute rejection, chronic rejection and chronic allograft dysfunction) [157, 159, 160]. There are no studies to date in clinical transplantation addressing the potential role of CMV in driving immunosenescence.

In this prospective, longitudinal study of kidney transplant recipients I define the most informative single cell-phenotype marker of immunosenescence. I establish the link of CMV

latency and infection with immunosenescence, and then the relationship between immunosenescence and subsequent episodes of infection. Finally, I describe the association between immunosenescence and impaired neutrophil function *in vitro*.

The findings of this study will provide novel insights into the indirect effects of CMV infection in kidney transplantation, with resultant impact on our approach to this important virus.

Chapter 5: Transitional B lymphocytes are associated with protection from kidney allograft rejection: a prospective study.

Chapter 5 tracks the temporal relationship between CMV, B cells and clinical events in kidney transplantation.

Retrospective, cross-sectional studies report regulatory protective role for a B-cell subset in kidney transplant outcomes [181, 182]. This transitional (regulatory) B-cell subset is defined by the phenotype $CD19^{+}CD24^{hi}CD38^{hi}$. However, these studies focused on antibody-mediated rejection and were conducted as case control studies many years after transplantation. Interestingly, an association between $CD19^{+}CD24^{hi}CD38^{hi}$ cells and immunosenescence has been reported in a recent study of healthy older donors [183].

The is the first prospective study to track the evolution of transitional B cells following kidney transplantation, and evaluate the temporal relationship between cell counts, CMV, rejection, and graft function from the time of transplantation.

The findings of this study will further clarify the role of $CD19^{+}CD24^{hi}CD38^{hi}$ cells in clinical transplantation, either as biomarkers of rejection risk, or in potential cell-based therapies.

Chapter 6: General Discussion

Chapter 6 addresses the major findings from the thesis, discussing the strengths and limitations of the aforementioned studies, the clinical implications and directions for future research.

Chapter 7: Appendices

Chapter 7 includes confirmation of ethical approval, and the standard operating protocols for the laboratory work undertaken.

1.11 Study Design and Patient Selection in this Thesis

The studies presented in chapters 2 to 5 are prospective, longitudinal and single-centre. Patients admitted to the Queen Elizabeth Hospital Birmingham (United Kingdom) for a kidney transplant were approached. In total, 100 unselected kidney transplant recipients were enrolled between February 2009 and October 2010. The flow diagram in figure 1.14 summarises the patient selection and clinical outcomes for the study cohort.

All study patients received standard immunosuppression, with Basiliximab for induction followed by maintenance tacrolimus (Prograf; pre-dose level 5-8ng/ml), mycophenolate mofetil (Cellcept; 2g daily) and prednisolone (20mg daily; reducing to 5mg by 3 months post transplantation). Valganciclovir prophylaxis (100 days) was administered to CMV seronegative recipients of seropositive donors; co-trimoxazole prophylaxis was administered to all for 12 months and isoniazid prophylaxis for 12 months to those of South-Asian ethnicity or previous tuberculosis.

Serial clinical, immunological and biochemical information was collected over 48 +/- 6 months of study follow up. Regular and frequent sampling for CMV PCR, anti-HLA antibodies and detailed phenotyping of patients' peripheral blood mononuclear cells (PBMCs) by multicolour

flow cytometry add to the strengths of this prospective, longitudinal study (outlined by figure below).

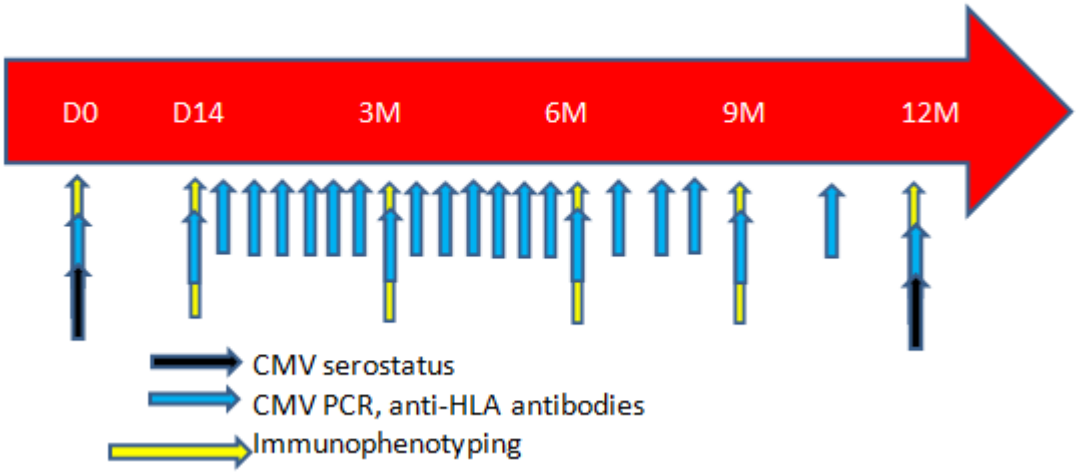

Table 1.1 provides a summary of the study plan over the first 12 months. Kidney transplant outcome data continued to be collected thereafter at three monthly intervals until end of the study.

1.12 Ethical Approval of Research Studies presented in this Thesis

The studies presented in chapters 2 to 5 in this thesis were approved by the Staffordshire Research Ethics Committee, with Research Ethics Committee reference number of (08/H1204/103), confirmation of favourable ethical opinion can be found under Appendices.

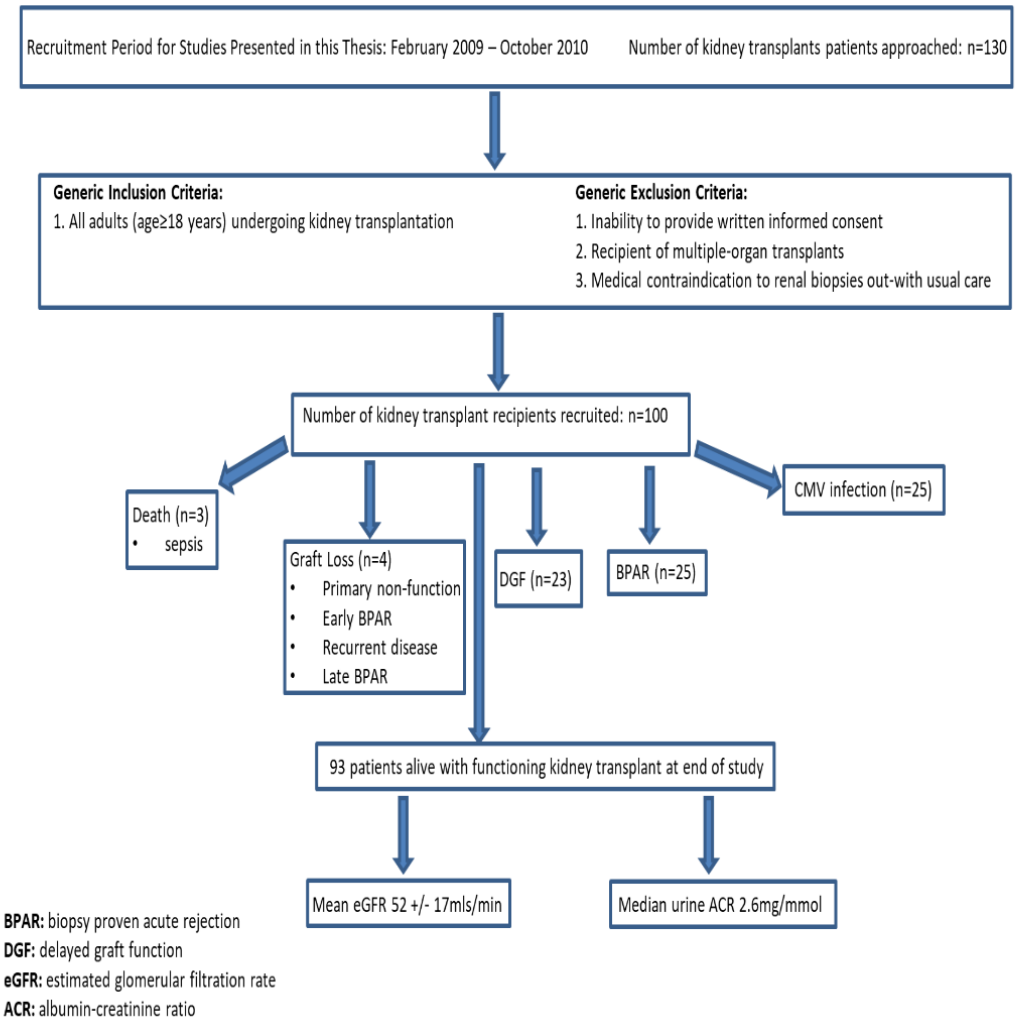

Figure 1.14 Overview of the study cohort

Table 1.1 Summary of study plan

	Pre-op	D7	D14	D21	D28	W6	W8	W10	W12	W14	W16	W20	W24	W28	W36	W40	W46	W52
Clinical Assessment																		
Donor demographics	X																	
Recipient demographics	X																	
Cause of kidney failure	X																	
Pre-transplant renal replacement therapy	X																	
Transplant source	X																	
Donor-Recipient HLA mismatch	X																	
Donor-Recipient CMV serostatus	X																	
Delayed graft function		X																
BPAR		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
CMV infection		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Other infection		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Medication list and doses	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Investigations																		
Biochemical /haematological profile	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
CNI trough level	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
eGFR	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Isotopic GFR									X									X
Urine MSU	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Urine ACR	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Oral glucose tolerance test		X							X									X
Renal biopsy	X																	
CMV PCR (save plasma)	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Anti-HLA antibodies (save serum)	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
High sensitivity c-reactive protein	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Peripheral immune function testing (cell immunophenotyping)	X		X						X				X			X		X

1.13 References

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Chapter 2: Impaired Direct CD8 Priming to Donor-Derived Cytomegalovirus Following Kidney Transplantation

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Impaired direct priming of CD8 T cells by donor-derived cytomegalovirus following kidney transplantation.

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2.1 ABSTRACT

This study aimed to clarify the risk of clinically significant CMV disease in "D+R+" transplantation under contemporary immunosuppression, and to examine recipients' cellular immune responses to CMV antigens primed directly by donor cells. In a retrospective cohort of 569 patients administered standardised basiliximab-tacrolimus-mycophenolate-corticosteroid immunosuppression, increased CMV disease rates were seen with D+R+ serostatus pairings compared with D-R+ (HR:2.61[95%CI:1.36,5.01]; p=0.004). Increased donor-recipient HLA mismatch was associated with increased disease rates in D+R+ transplantation (HR [per class 1 mismatch]:1.43[95%CI:1.12-1.82]; p=0.02). Implantation histology demonstrated CMV antigen expression in all seropositive donor organs examined (n=10). Cellular immunity to CMV presented exclusively by donor-restricted HLA within donor tissue was examined using HLA Class I-peptide tetramers. Transplant procedures whereby the donor expressed a HLA Class I allele not expressed by the recipient, and where the recipient expressed a HLA Class I allele not expressed by the donor, were prospectively followed from the time of transplantation (all donors seropositive;8 recipients seropositive;3 recipients seronegative). During the first-year post-transplantation, 4/8 seropositive recipients and 1/3 seronegative recipients displayed peripheral blood $CD8^+$ T-cell responses to CMV presented by recipient-specific HLA. Very interestingly, no recipients mounted responses to CMV presented by donor-specific HLA, suggesting the allograft of seropositive donors may represent a "reservoir" inaccessible to $CD8^+$ T-cell responses. Finally, pre-transplant assays of anti-CMV cellular immunity predicted post-transplant CMV replication less accurately in D+R+ compared to D-R+ pairings, possibly reflecting specificity of in-vitro assays for recipient (rather

than donor) HLA. These findings have important implications for clinical management and immunological understanding of donor-transmitted viral infection.

2.2 INTRODUCTION

Cytomegalovirus (CMV) infection remains an important complication of kidney transplantation, being associated with increased graft failure rates, morbidity and mortality. Although the cellular immune response to CMV, especially the $CD8^+$ T-cell response, is of primary importance in controlling infection [1-3], the CMV-specific antibody serostatus of donor (D) and recipient (R) is a useful surrogate to aid risk stratification, as it identifies individuals with latent CMV infection which may subsequently reactivate. High disease rates in primary infection (D+R- transplantation) are well recognised, and requirement for antiviral prophylaxis in this context largely uncontested. CMV disease is also seen in seropositive recipients (R+), and may occur irrespective of donor serostatus. Seropositive recipients are often grouped together as 'intermediate risk', irrespective of donor serostatus. However historical evidence suggests D+R+ transplantation is associated with increased disease rates compared to D-R+ transplantation [4]. Also, two recent trials [5,6[and a single centre study [7] showed higher infection rates in D+R+ compared with D-R+ transplantation. Because these latter studies focussed on (often asymptomatic) CMV infection, rather than symptomatic disease, and because all used either antiviral prophylaxis or pre-emptive therapy of CMV infection, it remains unclear whether disease rates differ between D+R+ and D-R+ transplantation, particularly under contemporary immunosuppression without antiviral prophylaxis.

Following D+R+ transplantation, some CMV cases may result from donor-derived infection, although the mechanism behind this is incompletely understood [4,8]. Although lack of protective immunity to newly infecting strains may be important [9] other mechanisms may

also play a role. For example, human leucocyte antigen (HLA) mismatch between donor and recipient may result in failure of "cognate" immunity to control viral infection in donor tissue. In this regard, clinical data is conflicting, with studies from the 1980s and 1990s suggesting increased CMV disease rates with increased HLA Class II mismatch [10,11], but others suggesting reduced rates in this setting [12,13]. One of these studies also identified HLA Class I mismatch as a risk factor for disease [11].

The initial purpose of this study was to evaluate the impact of donor CMV serostatus on disease risk in seropositive recipients, and whether the risk is modified by HLA Class I mismatch. We then utilised HLA-peptide tetramers to determine the specificity of cellular immunity to defined CMV peptides presented through either donor or recipient HLA alleles, and examined the development of T-cell responses (or lack of them) against CMV primed 'directly' on donor cells. These findings should be of value in the clinical management of CMV infection in this setting and are of considerable interest in further understanding the mechanism of viral infection in solid organ transplantation.

2.3 CONCISE METHODS

2.3.1 Risk of CMV disease and donor-recipient serostatus: retrospective cohort

A retrospective analysis of the prospectively maintained departmental database identified 569 adult patients (≥18 years) undergoing solitary kidney transplantation between May 2007 and June 2011 at Queen Elizabeth Hospital Birmingham. This start date was chosen because at that time a standardised immunosuppression regimen was introduced. This consisted of

Basiliximab induction followed by tacrolimus (trough level 5-8ng/ml initially, measured by liquid chromatography-tandem mass spectrometry), mycophenolate mofetil (2g daily initially) and prednisolone (20mg daily, reducing to 5mg maintenance by 3 months post transplantation). Transplantation proceeded if the cross match between donor and recipient was negative by flow cytometry and cytotoxicity.

Baseline information was collected on the pre-transplant CMV serostatus of the recipients and their donors, donor and recipient age and sex, cause of renal failure, HLA mismatch, source of transplant (live related; live unrelated; deceased donor following brain death [DBD]; deceased donor following cardiac death [DCD]). Post-operative events of delayed graft function (requirement for dialysis during the first post-operative week) and biopsy proven acute rejection (any time; any histological grade) were collected.

CMV prophylaxis with 100 days of valganciclovir was given to the D+R- group only, with dose adjustment for renal function. Testing for CMV was based on clinical suspicion of disease; no protocolised assessment of CMV infection was undertaken in this cohort. CMV disease was diagnosed according to international guidelines and was based on one or more of the following in association with the finding of CMV viraemia: fever; new onset severe malaise; leucopenia; thrombocytopaenia; hepatitis (alanine transaminase or aspartate transaminase levels greater than twice the upper limit of normal); tissue invasive disease proven by histology. For our laboratory, a copy rate of >500 copies/ml of whole blood represents significant CMV viraemia. For the purpose of the current analysis, CMV disease of any severity was evaluated (i.e. no distinction was made between mild CMV “syndrome” and severe tissue invasive disease).

2.3.2 Evaluation of the HLA restricted cellular response in CMV reinfection (seropositive donors): prospective cohort

Between February 2009 and October 2010, 36 adult recipients of kidney transplants from CMV seropositive donors were enrolled into a prospective study to evaluate the immune response to CMV (D+R-: 17; D+R+: 19). The immunosuppression and antiviral prophylaxis strategy was identical to the departmental protocol described above.

Class I CMV tetramers were used to examine HLA-specific cell mediated immunity to CMV. We first identified donor-recipient pairs for which the donor expressed an HLA-A or HLA-B antigen not expressed by the recipient, *and* where the recipient expressed an HLA-A or HLA-B antigen not expressed by the donor. We then narrowed this selection down to donor-recipient pairs for which a class I CMV tetramer specific to the mismatched HLA for both the donor and recipient was available (see below for details of available tetramers). In this way, we were able to distinguish circulating $CD8^+$ T cells which were specific for CMV epitopes presented by donor HLA (but not recipient HLA) on one hand, and recipient HLA (but not donor HLA) on the other. Using these criteria, we identified 11 recipients of kidneys from seropositive donors (8 D+/R+ pairs and 3 D+/R- donor-recipient pairs) suitable for analysis. In these patients, serial blood samples were taken for analysis post transplantation (1, 3, 6 and 12 months).

All CMV peptides used were synthesized commercially by Alta Biosciences (Birmingham, U.K.). Peptides incorporated in the tetramers (and the protein from which they are derived) were as follows: HLA-A1 restricted epitopes YSEHPTFTSQY (pp65) and VTEHDTLLY (pp50), HLA-A2

restricted epitopes NLVPMVATV (pp65) and VLEETSVML (IE-1), HLA-B7 restricted epitopes TPRVTGGGAM and RPHERNGFTVL (both pp65) and HLA-B8 restricted epitopes ELKRKMIYM and QIKVRVDMV (both IE-1). Tetramerization was carried out using Streptavidin-APC (Invitrogen, Paisley, UK).

Peripheral blood mononuclear cells (PBMC) were isolated from heparinised blood of the transplant recipient by density gradient centrifugation using RPMI-1640 medium (Sigma) and cryopreserved in fetal calf serum (FCS) containing 10% DMSO. This was performed within 6 hours following venepuncture. To identify virus-specific $CD8^+$ T cells by flow cytometry, $1x10^6$ PBMCs were stained with tetramer at 37ºC for 15 minutes, followed by staining of surface markers. Cells were then analysed on a Becton-Dickinson LSRII flow cytometer and using DIVA-software.

2.3.3 Predicting post-transplant CMV infection by pre-transplant assessment of cell mediated immune response to CMV: prospective cohort

The predictive utility of pre-transplant cell mediated immunity to CMV was evaluated in 38 adult seropositive transplant recipients receiving kidneys from either seropositive or seronegative donors (19 D+R+; 19 D-R+) between February 2009 and October 2010, using the departmental immunosuppression protocol described above. No patient received CMV prophylaxis. The frequency of circulating CMV-reactive T cells was determined prior to transplantation by antigen stimulation and subsequent detection of cytokine production. Briefly, fresh PBMCs were stimulated with CMV-derived peptides for 16 hours. Peptide pools were derived from either IE-1 (to assess the CD8 response) or pp65 (which can assess both

CD4 and CD8 responses – see below) were used at a final concentration of 1μg/ml per peptide (all Alta Biosciences, Birmingham, UK). 10μg/ml BrefeldinA (Sigma-Aldrich, Gillingham, UK) was added to block cytokine secretion after 1 hour of incubation. As a positive control cells were stimulated with staphylococcus enterotoxin B (SEB) (0.2μg/ml final concentration; Sigma-Aldrich) and unstimulated cells served as a negative control. Cells were then stained with anti-CD4 and anti-CD8 antibodies (BD Biosciences, Oxford, UK), fixed using 4% paraformaldehyde, and permeabilised with 0.5% Saponin. Intracellular Interferon (IFN)-γ was detected with anti-IFN-γ-FITC (BD Biosciences). Analysis was performed on a Beckton-Dickinson LSRII flow cytometer with FlowJo software.

The endpoint for this investigation was the development of CMV replication (irrespective of the development of symptomatic disease) within the first 12 months following transplantation. Serial whole blood samples were taken for CMV DNA PCR in these patients at day 0 (prior to transplantation), and then weeks 1, 2, 3, 4, 6, 8, 10, 12, 16, 20, 24, 28, 34, 40, 46, and 52. This cohort of patients were contained within the larger "retrospective" cohort described above. However, the clinical team remained unaware of the results of either the T-cell responses or the PCR results, and no changes in clinical management resulted from this series of experiments.

2.3.4 Immunohistochemistry analysis of implantation kidney biopsy tissue

Paraffin embedded tissue sections obtained from 5 seropositive kidney transplant donors immediately prior to transplantation ("implantation biopsies") were examined for HCMV-IE (reacts with an immediate early non-structural antigen of 68-72kDa, antibodies

IgG2a, Chemicon International), HCMV-LA (reacts with a late protein of 47-55kD, antibodies IgG2a, Chemicon International) and HCMV-pp65 (IgG1, Novacostra, CA). Antibodies against smooth muscle cells alpha actin (IgG2a, Biogenex, San Ramon, CA), vWF (IgG1, DakoCytomation, Denmark) and tissue stained by exclusion of primary antibodies were used as controls. Comparison was made with biopsies from 3 seronegative individuals.

Briefly, the sections were deparaffinized in xylene (Sigma Aldrich), rehydrate in alcohol series, post fixed with 4% neutral buffered formalin (Apoteketpharmaci, Stockholm, Sweden), treated with pepsin (Biogenex, San Ramon, CA), and then incubated in citrate buffer (Biogenex). Endogenous peroxidase was blocked by treating sections with 3% H2O2 (Sigma-Aldrich), endogenous avidin/biotin was blocked using avidin/biotin blocking kit (DakoCytomation, Glostrup, Denmark), and FC receptor blocker (Innovex Biosciences) was used to block FC-Receptors. Finally, the tissue sections were treated with background buster (Innovex Biosciences). All sections were incubated with primary antibodies as mentioned above or without primary antibodies (only diluents) overnight at 4°C. Antibodies were visualized using biotinylated secondary goat antibodies against primary mouse antibodies (Biogenex), streptavidin-conjugated horseradish peroxidase and diaminobenzidine (Innovex Biosciences).

The study was approved by the North Staffordshire Research Ethics Committee, and conducted in accordance with the guidelines of the Declaration of Helsinki.

2.3.5 Statistical analysis

Data are presented as mean ± standard deviation unless otherwise described. Continuously distributed data was compared using Student's t-test, and categorical data using Chi-square testing.

Survival analysis was conducted using Kaplan-Meier methodology, with the log rank test used to assess significance. The development of CMV disease in the retrospective analysis was considered as a time to event outcome. This analysis factored both death and graft loss as competing risks, and so the analysis was performed using a competing risks regression model using the methods described by Fine and Gray. For all analyses, initially the effect of each variable on the outcome was considered separately in a series of univariate analyses. Variables showing some evidence of effect ($p<0.15$) were included in a subsequently multivariate analysis. A stepwise backwards selection procedure was performed to retain only the statistically significant variables in the final model.

In addition, there was specific interest with regard to the influence of donor-recipient HLA mismatch on the development of CMV disease. First, a statistical interaction between donor-recipient HLA mismatch and donor-recipient CMV serostatus with regard to time to CMV disease was evaluated. When such an interaction was found, subgroup analysis (by serostatus combination) was performed using the competing risk model described above.

In the first instance, only pre-transplant demographics were considered in the model, and episodes of delayed graft function and biopsy proven acute rejection were not evaluated as

these can be considered as intermediate end points which may confound the analysis. However, in a set of secondary analyses these post-transplant events were evaluated as time-dependent covariates.

For all analyses, a type 1 error rate below 5% ($p<0.05$) was considered statistically significant.

Assessment of the cell mediated immunity assay in regard to predicting CMV replication in the first year was evaluated by the following parameters of predictive performance: sensitivity, specificity, positive and negative predictive values. These measures were calculated following the determination of the optimal cut-off value from Receiver Operating Characteristic (ROC) curve analysis. The area under the ROC curve (c-statistic) was calculated as a global measure of prognostic performance.

2.4 RESULTS

2.4.1 The incidence of CMV disease is related to donor and recipient CMV serostatus

The demographics and clinical features of the 569 patients are described in table 2.1

Table 2.1 Patient demographics

Recipient Age	47±13 years
Recipient Sex	42.4% male (241/569)
Recipient ethnicity	
White	431 (75.7%)
Indo-Asian	88 (15.5%)
African-Caribbean	42 (7.4%)
Other	8 (1.4%)
Cause of Renal Failure	
Glomerular	185 (32.5%)
Hereditary/Cystic	170 (29.9%)
Diabetes	31 (5.4%)
Vascular	29 (5.1%)
Interstitial	15 (2.6%)
Other	139 (24.4%)
Donor Age	47±15 years
Transplant Source	
Deceased donor	313 (55.0%)
-DBD[1]	-247 (43.4%)
-DCD[2]	-66 (11.6%)
Live donor	256 (45.0%)
Donor-Recipient HLA Mismatch	
HLA-A	1.0±0.7 Ag
HLA-B	1.0±0.6 Ag
HLA-DR	0.7±0.6 Ag
Donor-Recipient CMV Serostatus	
D-R-	151 (26.5%)
D-R+	122 (21.5%)
D+R+	179 (31.4%)
D+R-	117 (20.6%)

[1] Donation after Brain Death
[2] Donation after Cardiac death

In these, 77 episodes of CMV disease occurred, and were associated with donor and recipient serostatus (Figure 2.1; p=0.003). D-R- transplantation was associated with the lowest risk of

disease (3/151; 2%). CMV disease was seen in 19.8% (23/117 patients) of the D+R- group. Of these, 3 patients experienced CMV disease during the 3-month period of antiviral prophylaxis, with suboptimal valganciclovir dosing (adjusted for allograft function) in all cases. Interestingly, risk was highest in seropositive recipients of seropositive donors (D+R+) where 39/179 patients (21.9%) experienced CMV disease. In contrast 12/122 patients (9.9%) in the D-R+ group developed CMV disease.

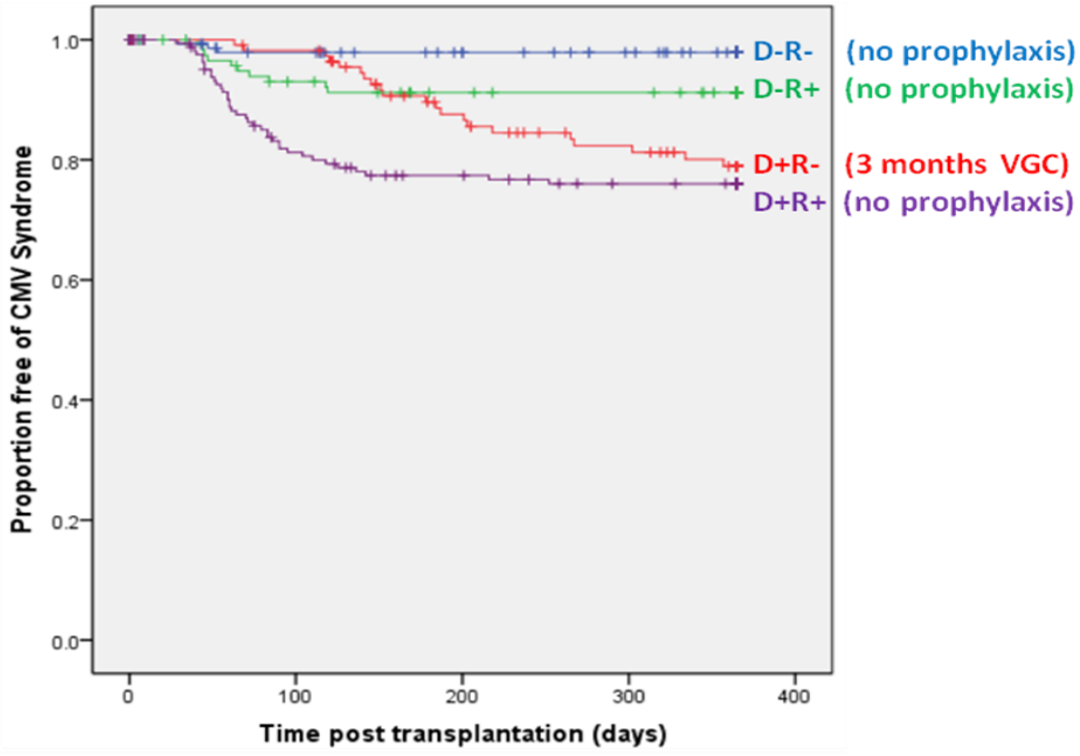

Figure 2.1 CMV disease based on donor-recipient CMV serostatus

Development of CMV disease by donor-recipient risk based on serostatus combination**.** Data shown for first 12 months in light of few episodes of CMV disease beyond this point.

Median peak viral loads (copies/ml) during infections were also associated with serostatus: D-R-: $2.2x10^7$ (range: $1.6x10^6$-$3.1x10^7$); D+R-: $3.1x10^5$ (571-$7.7x10^6$); D+R+: $1.3x10^4$ (504-$1.7x10^8$); D-R+: $1.1x10^4$ (1025-$1.9x10^5$) ($p<0.001$). Histological evidence of CMV infection was gastro-intestinal in all cases but was uncommonly sought and proven in only 5 patients.

2.4.2 Multivariate analysis confirms donor serostatus influences CMV disease rates in seropositive recipients

Table 2.2 shows the univariate relationships between CMV serostatus (and other demographic variables) and time to CMV disease.

Table 2.2 Risk factors for CMV disease

Variable	Category	Hazard ratio (95% CI)	p-value
CMV Serostatus	D-R+	1	
	D-R-	0.20 (0.05-0.70)	<0.001
	D+R+	2.61 (1.36-5.01)	
	D+R-	2.03 (1.10-4.08)	
Recipient Age	per 10 year increase	1.18 (0.98-1.41)	0.08
Recipient Sex	Male	1.10 (0.70-0.74)	0.67
Recipient Ethnicity	White	1	
	Indo-Asian	2.22 (1.31-3.76)	0.01
	African-Caribbean	1.50 (0.69-3.22)	
	Other	1.32 (0.39-4.47)	
Cause of ESRF	Glomerular	1	
	Hereditary/Cystic	1.15 (0.62-2.15)	
	Diabetes	3.22 (1.46-7.08)	0.07
	Vascular	2.10 (0.84-5.26)	
	Interstitial	1.93 (0.57-6.52)	
	Other	1.43 (0.76-2.67)	
Source	DBD	1	
	DCD	0.92 (0.46-1.85)	0.62
	Live donor	0.73 (0.38-1.43)	
Donor Age	per 10 year increase	1.19 (1.01-1.41)	0.04
Donor Sex	Male	0.95 (0.61-1.48)	0.82
Class I Mismatch	per Ag	1.16 (0.96-1.40)	0.12
Class II Mismatch	per Ag	0.97 (0.69-1.35)	0.84

Compared with D-R+ transplantation (selected as the reference group), D-R- transplantation was associated with reduced risk, and D+R- with increased risk for CMV disease (Table 2.2).

In addition, increased risk of CMV was seen when comparing D+R+ and D-R+ serostatus groups (HR:2.61[95%CI: 1.36-5.01]; p=0.004 for this specific comparison).

In the multivariate model, the only independent factor associated with time to CMV disease was donor-recipient serostatus, and therefore the effect identical to that in the univariate analysis (Table 2.2). Adjusting the model for post-transplant delayed graft function and acute rejection resulted in no material difference in the relationship (HR [D+R+ versus D-R+]:2.42 [95%CI:1.31-4.47]; p=0.007).

2.4.3 Total HLA class I mismatch between donor and recipient increases CMV disease risk in D+R+ transplantation

No association was evident between HLA mismatch at either Class I (combined HLA-A and HLA-B) or Class II (HLA-DR) loci and time to CMV disease (Table 2.2). However, a significant statistical interaction was seen between serostatus group and total HLA Class I mismatch (p=0.02).

To illustrate this, the association between total HLA Class I mismatch and time to CMV was evaluated for each serostatus risk group. In D+R+ transplantation (n=179), a significant relationship was evident between HLA Class I mismatch and time to CMV disease, and this remained significant in the multivariate model (HR [per mismatch]:1.43[95%CI:1.12-1.82]; p=0.02). Kaplan-Meier estimates of CMV-free survival in the D+R+ cohort are shown in Figure 2.2 (p=0.03).

The effect of HLA Class I mismatch was not evident in the other three serostatus groups (p>0.3 for all). In addition, no statistical interaction was seen between serostatus group and Class II mismatch (HLA-DR only; p=0.13).

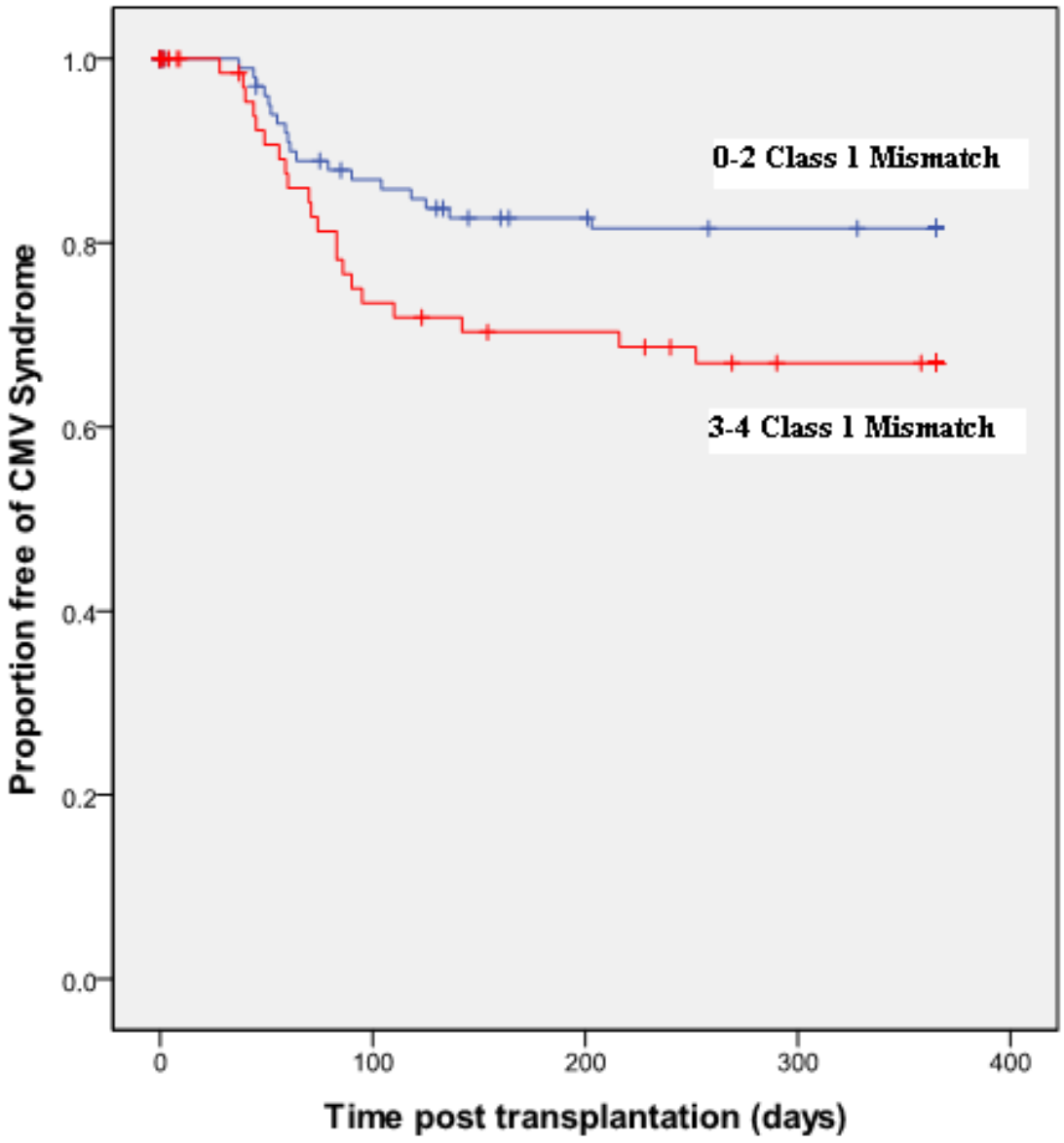

Figure 2.2 Donor-recipient HLA mismatch and time to CMV disease.
Influence of donor-recipient HLA mismatch on time to CMV disease in the D+R+ serostatus pairing. Data are shown for first 12 months in light of few episodes of CMV disease beyond this point.

2.4.4 Transplant patients fail to mount a CD8⁺ T-cell response to immunodominant CMV epitopes selectively presented by donor MHC

We next investigated the $CD8^+$ T-cell response to CMV peptides presented exclusively by either donor or recipient HLA alleles. We prospectively identified 11 instances of transplantation from CMV seropositive donors where both the donor and recipient expressed at least one unique HLA Class I allele and where appropriate HLA-peptide tetramers were available to study T-cell immune responses against immunodominant CMV peptides presented through these two alleles. In 8 cases the recipient was also CMV seropositive; in 3 cases the recipient was seronegative.

Following transplantation, 4 of the 8 recipients in the D+R+ group displayed $CD8^+$ T-cell responses to peptides presented by recipient-specific HLA alleles. Representative results for one individual (and gating strategy) are shown in Figure 2.3A and the upper row of Figure 2.3B, demonstrating the HLA A1-restricted $CD8^+$T-cell response to the VTEHDTLLY peptide (recipient type: HLA-A1, A8; donor: HLA-A2, A3). In the remaining 4 patients no such $CD8^+$ T-cell response was detected. However, and of particular interest, no immune response restricted through a *donor-specific* HLA allele was detected in any of the 8 recipients at any point during the first-year post transplantation (p=0.02 comparing recipient and donor-restricted responses). Similarly, none of the 3 patients in the D+R- group mounted a $CD8^+$ T-cell response to CMV peptides restricted by a donor-specific HLA allele. However, one recipient did develop a response to a peptide restricted by a *recipient-specific* allele, which was not evident early post transplantation, but was then evident at 6 months and persisted to 18 months post-transplantation (HLA-A1 restricted YSE peptide; Figure 2.3B lower row).

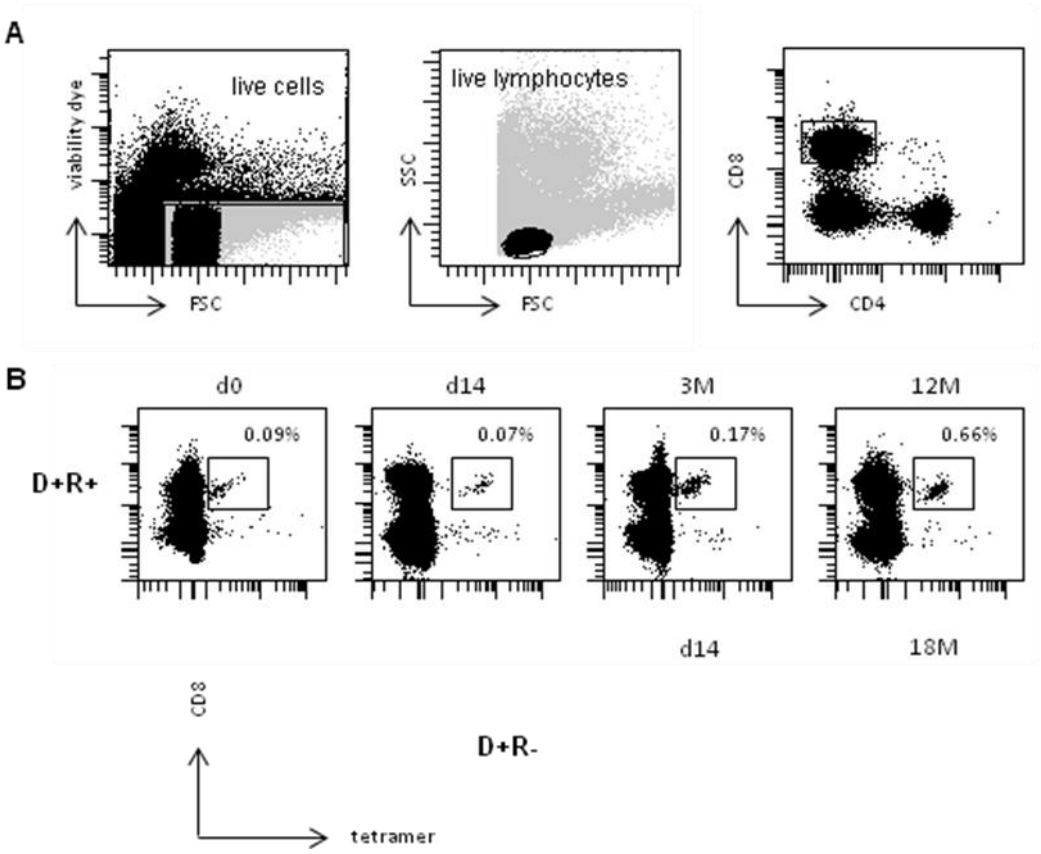

Figure 2.3 CD8⁺ T-cell responses against recipient-HLA-presented CMV epitopes

CD8⁺ T-cell responses against recipient-HLA-presented CMV-derived epitopes can be detected in both CMV-seropositive recipients of seropositive donor organs (D+R+) and CMV-negative recipients of seropositive organs (D+R-). Gating strategy is shown in (A). The upper row of (B) shows an example of a serial evaluation of the CD8⁺ T-cell response to the HLA-A1 restricted epitope VTE (pp50) in the D+R+ setting at time of transplantation (d0), day 14, 3 months and 12 months post transplantation (B; top row). The lower row of (B) shows the T-cell response to the HLA-A1 restricted epitope YSE (pp65) in a CMV-seronegative recipient of a CMV-seropositive donor organ, which was not evident early post-transplantation, but then emerged and persisted through to 18 months post-transplantation.

Taken together across 11 transplant procedures, 5 recipients developed a CD8⁺ T-cell response to CMV peptides restricted by recipient-specific HLA alleles, whereas none demonstrated a response to epitopes restricted by donor-specific alleles (p=0.01).

2.4.5 CMV within transplanted tissue from seropositive kidney transplant donors is common

Histological examination of implantation biopsies from 10 seropositive kidney transplant donors revealed evidence of CMV protein expression in all cases (HCMV-IE, HCMV-LA and HCMV-pp65; Figure 2.4). Staining was absent in seronegative controls.

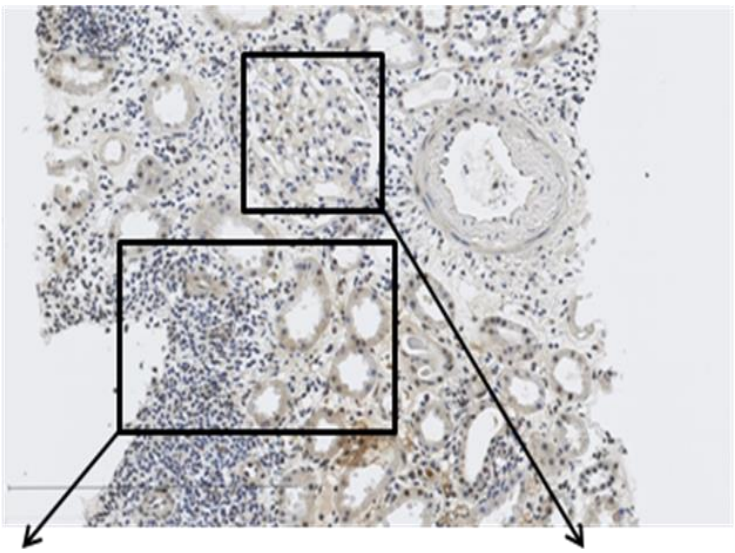

Figure 2.4A HCMV IE immunostaining of implantation biopsy from seropositive donor

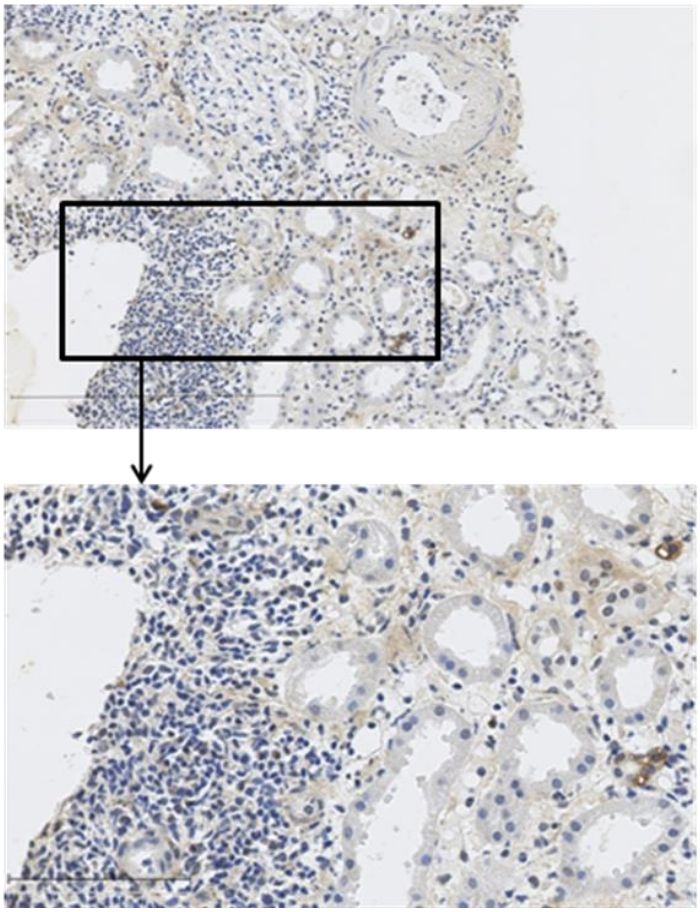

Figure 2.4B HCMV LA immunostaining of implantation biopsy from seropositive donor

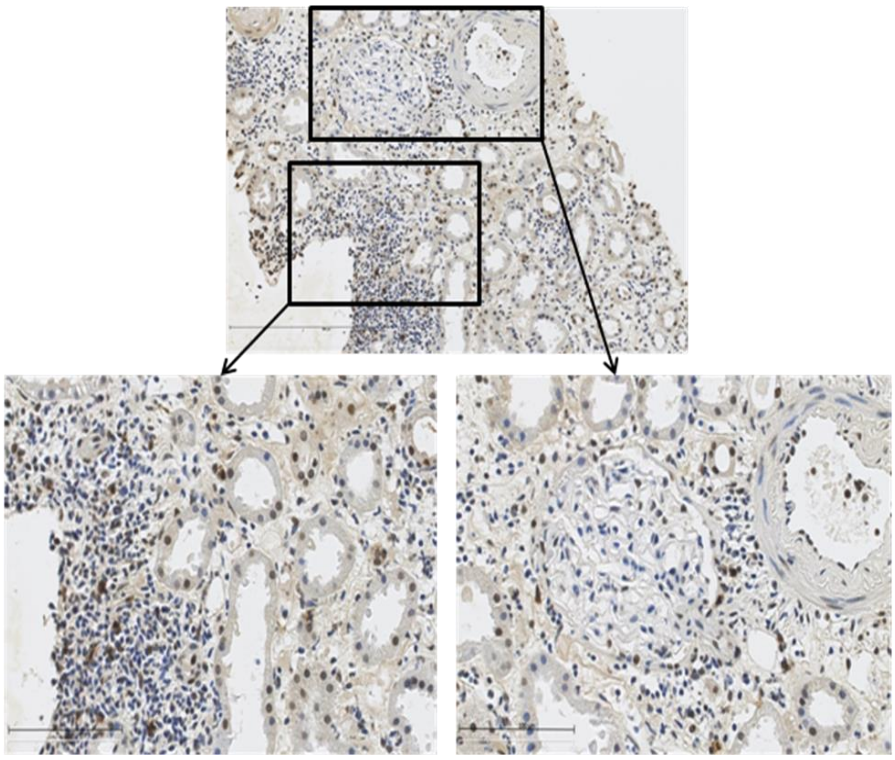

Figure 2.4C HCMV pp65 immunostaining of implantation biopsy from seropositive donor

2.4.6 The predictive utility of assessment of the pre-transplant CMV-specific T-cell immune response differs between D+R+ and D-R+ transplantation

Finally, the prognostic value of CMV-specific cellular immunity immediately prior to transplantation was evaluated in CMV seropositive recipients. In D-R+ transplantation (n=19), a median 0.13% of total $CD8^+$T cells responded to an IE-1 peptide mix as measured by IFN-γ production (range 0-8.18%), and 0.06% (range 0-4.35%) of total $CD4^+$ T cells responded to pp65-derived peptides. Six patients (31%) in this group developed detectable CMV replication during the first year. In D+R+ transplantation (n=19), a median 0.13% $CD8^+$ T cells responded to IE-1 epitopes (range 0-23.05%), and 0.05% $CD4^+$ T cells (range 0-1.43%) responded to pp65 epitopes, with 9 patients (47%) displayed CMV replication.

ROC curve analysis defined the optimal prognostic cut-off, and then sensitivity, specificity, and positive and negative predictive values at that cut-off were calculated. Results are shown in Table 2.3.

Table 2.3 Prognostic performance of pre-transplant assays of cellular immunity to CMV.

Endpoint of CMV infection during first 12 months post transplantation. NPV=Negative Predictive Value; PPV=Positive Predictive Value

	c-statistic	Cut-off (percentage IFN-γ releasing cells)	Sensitivity	NPV	Specificity	PPV
D-R+ transplantation						
IE-1 (CD8)	0.76	0.16	0.83	0.86	0.50	0.45
pp65 (CD4)	0.54	0.40	1.00	1.00	0.08	0.35
D+R+ transplantation						
IE-1 (CD8)	0.56	0.82	0.67	0.50	0.38	0.55
pp65 (CD4)	0.52	0.05	0.56	0.50	0.50	0.56

In D-R+ transplantation, a $CD8^+$ T-cell response of 0.16% (i.e. when 0.16% total $CD8^+$ cells produced IFN-γ following IE-1 peptide stimulation) was associated with moderate to good prediction (c-statistic 0.76) for CMV replication. From a clinical perspective, in 83% of patients experiencing CMV replication less than (or equal to) 0.16% total $CD8^+$ T cells responded to IE-1 peptide stimulation prior to transplantation; 86% of patients with greater than 0.16% total $CD8^+$ T cells responding to IE-1 did not develop CMV replication within 12 months post-transplantation. The $CD8^+$ T-cell response to pp65 demonstrated identical prognostic performance to the IE-1 $CD8^+$ T-cell response, although the optimal cut-off value was 0.08% total $CD8^+$ T cells.

Conversely, the pre-transplant $CD8^+$ T-cell response did not demonstrate prognostic utility in D+R+ transplantation (Table 2.3). In fact, of 9 recipients in this subgroup displaying an IE-1 response ≥0.16% total $CD8^+$ T cells, 5 developed CMV replication, and 4 did not. Interestingly,

and despite the small numbers studied, there was some suggestion that these 5 patients developing post-transplant CMV replication displayed increased HLA Class I mismatch in comparison to the 4 patients in whom this percentage of CMV-responding $CD8^+$ T cells was "protective" (Class I mismatch: 4,3,3,2,2 versus 3,2,1,1 respectively; p=0.1).

The relationship between the IE-1 $CD8^+$ T-cell response and risk of CMV replication in D-R+ and D+R+ transplantation is shown in Figure 2.5.

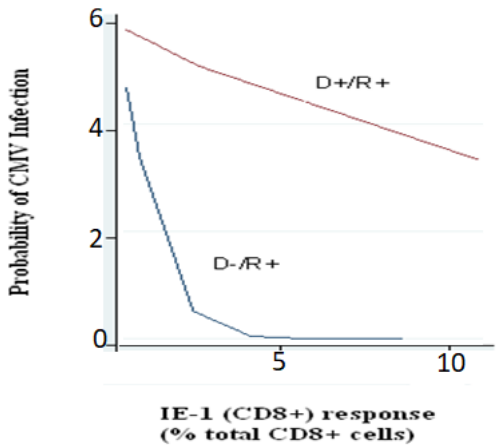

Figure 2.5 IE-1 $CD8^+$ T-cell response in predicting post-transplant CMV infection
The relationship between the IE-1 $CD8^+$ response and the probability of CMV infection over the first post-transplant year, comparing D^-R^+ and D^+R^+ serostatus pairings.

The $CD4^+$ response (pp65) did not demonstrate prognostic utility in either D^-R^+ or D^+R^+ transplantation (Table 2.3).

2.5 DISCUSSION

This study demonstrates important characteristics of CMV infection in kidney transplant recipients. First, CMV seropositive recipients receiving contemporary immunosuppression are at increased risk of clinical CMV disease when receiving kidneys from CMV seropositive compared with seronegative donors. This observation represents a "real world" scenario, dealing specifically with patients experiencing clinically manifest CMV disease, thereby extending data from previous clinical trials addressing asymptomatic CMV infection/replication. [5,7] Although "strain-specific" mismatch is cited as an underlying mechanism for this phenomenon, [9] substantial data exist which question this hypothesis (discussed further below). We therefore investigated whether donor-recipient HLA mismatch impaired development of cognate cellular immunity to CMV within transplanted donor tissue. Indeed, further analysis demonstrated increased risk of CMV disease with greater HLA Class I mismatch. To investigate the underlying mechanism, we utilised HLA-peptide tetramers to interrogate the development of $CD8^+$ T-cell immunity against peptides presented through HLA alleles specific for either recipient or donor. Notably we observed that although cellular responses to CMV peptides presented through recipient-specific HLA alleles were frequently detected, $CD8^+$ T-cell responses to peptides presented solely by donor-specific HLA alleles were not generated within 12 months post-transplantation.

The latter observation is relevant to current theories of the relative roles of different antigen presentation pathways in viral infection. These include direct presentation by infected donor antigen presenting cells (APCs), presentation by recipient APCs "cross dressed" with donor-derived MHC: viral peptide complexes transferred from donor immune or parenchymal cells,

and cross-presentation by recipient APCs which have engulfed and processed circulating virions. [14,15] Recipients' failure to develop $CD8^+$ immune responses through donor-specific HLA alleles suggests that priming and expansion of immune responses to donor-transmitted CMV does not result from direct presentation or "cross dressing". This novel finding with regard to transplantation-associated viral transmission resonates with data from models of murine CMV, suggesting cross-presentation as the primary pathway to antiviral immunity, [16-18] with direct priming inhibited due to CMV-mediated downregulation of activating costimulatory molecules (such as CD86) and upregulation of inhibitory ligands (such as PD-L1) on APCs, [19-21] along with inhibition of dendritic cell maturation and antigen presentation [22,23].

Absence of directly primed $CD8^+$ T-cell immune responses raises questions regarding how infection is ultimately controlled within donor tissue. Several possibilities include $CD4^+$ T-cell immunity, natural killer cells and humoral immunity. However, sensitive DNA-based techniques commonly detect CMV persistence post-transplantation in kidneys from seropositive donors, [24] suggesting donor tissue may act as a 'reservoir' for infection, which may later become clinically manifest. [25] Certainly the pre-implantation histological data from the current study suggests CMV transmission peri-transplantation is very common. Following CMV reactivation, HLA Class I upregulation occurs in infected and surrounding cells, [26-29] with subsequent presentation of CMV antigens to $CD8^+$ T -cells. [30,31] In D+R+ transplantation, pre-existing cellular immunity may control spread of donor-derived virus, but donor-recipient mismatching at Class I loci may impair the efficacy of this response, and so increase the likelihood of clinical disease, as seen in the current study. HLA Class I mismatch may be less important in the setting of D+R- or D-R+ transplantation, because control of CMV

infection in the former is primarily contingent on a de novo immune response (rather than a pre-existing one), and the latter depends on responses to recipient HLA-presented virus.

This study highlights differences in risk of CMV when seropositive recipients receive kidneys from donors either with or without latent CMV infection, i.e. between D+R+ and D-R+ transplantation. This finding opposes the view that these groups be considered together as an “intermediate risk” category, and supports policies of antiviral prophylaxis or heightened surveillance in D+R+ transplantation, particularly in instances of increased donor-recipient HLA Class I mismatch. The introduction of a novel CMV strain has been implicated as enhancing the risk of CMV disease in D+R+ transplantation [32]. However, it is noteworthy that the study showing this effect incorporated ’high level antigenemia’ without symptoms within the definition of CMV disease and did not show increased CMV infection rates with strain-specific donor-recipient serological mismatch [32]. Also, it is known that human immunity to CMV is broadly targeted [33], suggesting “redundancy” in the cellular repertoire. Furthermore, mismatching for immunodominant $CD8^+$ epitopes in murine models of CMV has minimal effect on disease severity [34]. A primate model also shows that hepatitis C re-infection rates are similar irrespective of whether the re-infecting strain was homologous or heterologous to the original infection [35]. Therefore ‘strain-mismatch’ may not necessarily fully explain increased CMV rates in D+R+ transplantation. Indeed, combinations of viral variation and HLA mismatch may be important and future study is required to clarify their relative contributions. In light of the extreme difficulty in isolating latent viral DNA from transplant donors, we were unable to directly address this issue of genotype mismatch in this study.

Assays of cellular immunity to CMV are emerging research tools. However, this study suggests that such *in vitro* assessments, whereby CMV peptides are presented by *recipient* HLA (using *recipient* PBMCs) may be misleading, considering that *in vivo* the virus may be harboured within *donor* tissue, and therefore presented upon reactivation by *donor* HLA. This may account for the inferior prognostic utility of these assays in D+R+ (compared with D-R+) transplantation. There was also some suggestion that HLA mismatch played a role in this context, and future evaluation of this phenomenon in larger cohorts may be worthwhile. To our knowledge this is only the second study to evaluate the prognostic utility of pre-transplant cellular immunity to CMV. The first study, published recently [36] also identified an association between pre-transplant immunity and post-transplant viral replication in 55 kidney or lung transplant recipients undergoing either antiviral prophylaxis or pre-emptive therapy, but did not compare D+R+ and D-R+ groups specifically, or present metrics of prognostic utility.

It can be speculated that these findings may be relevant to other viruses that are transmitted in allograft tissue such as Epstein-Barr virus (EBV) or polyoma virus. They may also be relevant when considering targets for adoptive cellular transfer therapy in the treatment of viral infection where the infection is of donor origin. The requirement for a cellular response against donor-presented epitopes may also have implications for vaccine development and efficacy in the context of viruses transmitted by transplantation, as identified in the context of anti-tumour vaccination [37].

In conclusion, clinically relevant CMV disease is more common in non-prophylaxed seropositive recipients of kidneys from seropositive (compared with seronegative) donors;

donor-recipient HLA Class I mismatch modifies this risk. We suggest that $CD8^+$ T-cell immune responses against CMV transmitted within grafted tissue fail to develop, although cannot yet claim this as definitive pending further study in other cohorts. Nevertheless, these preliminary findings have considerable implications for the clinical management and immunological understanding of viral infection in transplantation.

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Chapter 3: CMV-associated $CD4^{+}CD28^{null}$ cells in NKG2D-dependent glomerular endothelial injury and kidney allograft dysfunction

This chapter appears as published in the American Journal of Transplantation and reflects work undertaken as part of my PhD. (Featured in the editorial)

Cytomegalovirus-Associated CD4(+) CD28(null) Cells in NKG2D-Dependent Glomerular Endothelial Injury and Kidney Allograft Dysfunction.
Shabir S, Smith H, Kaul B, Pachnio A, Jham S, Kuravi S, Ball S, Chand S, Moss P, Harper L, Borrows R.
Am J Transplant. 2016 Apr;16(4):1113-28.

3.1 ABSTRACT

Emerging data suggests that expansion of a circulating population of atypical, cytotoxic $CD4^+$ T cells lacking costimulatory CD28 ("$CD4^+CD28^{null}$" cells) is associated with latent CMV infection. The purpose of the current study was to increase the understanding of the relevance of these cells in 100 unselected kidney transplant recipients followed prospectively for a median of 54 months. Multicolour flow cytometry of PBMCs prior to transplantation and serially post-transplantation was undertaken. $CD4^+CD28^{null}$ cells were found predominantly in CMV-seropositive patients, and expanded in the post-transplant period. These cells were predominantly effector-memory phenotype, and expressed markers of endothelial homing (CX3CR1) and cytotoxicity (NKG2D and perforin). Isolated $CD4^+CD27^-CD28^{null}$ cells proliferated in response to PBMCs previously exposed to CMV-derived (but not HLA-derived) antigens, and following such priming incubation with glomerular endothelium resulted in signs of endothelial damage and apoptosis (release of fractalkine and von Willebrand factor; increased caspase 3 expression). This effect was mitigated by NKG2D-blocking antibody. Increased $CD4^+CD28^{null}$ cell frequencies were associated with delayed graft function, and lower eGFR at end follow-up. This study suggests an important role for this atypical cytotoxic $CD4^+CD28^{null}$ cell subset in kidney transplantation, and points to strategies that may minimize the impact on clinical outcomes.

3.2 INTRODUCTION

Cytomegalovirus (CMV) is a ubiquitous β-herpesvirus which may cause significant clinical disease in transplanted patients. It is also now appreciated that CMV can exert a range of "indirect effects", including allograft dysfunction, vasculopathy and glomerulopathy, although the mechanisms underlying these phenomena are unclear (1-3).

Latent CMV infection in patients with end-stage renal disease (ESRD) is associated with expansion of circulating, late-differentiated, cytotoxic $CD4^+$ T cells. These are characterised by absence of the costimulatory molecule CD28 from the cell surface ("$CD4^+CD28^{null}$")(4). Although also recognised in patients with autoimmune conditions such as multiple sclerosis, rheumatoid arthritis and vasculitis (5-7), these $CD4^+CD28^{null}$ cells are unresponsive to candidate autoantigens (5, 6), but rather respond to CMV-derived proteins. Furthermore, one study demonstrated emergence of $CD4^+CD28^{null}$ cells in 4 kidney transplant recipients following primary CMV infection (8), although evaluation of these cells' reactivity to alloantigen has not yet been studied.

In contrast to $CD28^-$expressing $CD4^+$ cells ("$CD4^+CD28^+$"), $CD4^+CD28^{null}$ cells express cytotoxic mediators such as perforin (4, 5, 8), and cells isolated from patients with acute coronary syndromes promote damage to human umbilical vein endothelial cells (HUVEC) *in vitro* (9). Interestingly, exposure of $CD4^+$ cells to CMV antigens (but not other viral antigens) promotes expansion of $CD4^+$ cells expressing the killer lectin-like receptor NKG2D (10, 11), also uncommonly expressed on $CD4^+$ T cells (10). Upon ligation, NKG2D initiates an intracellular cascade culminating in perforin exocytosis and consequent cytotoxicity.

The purpose of this study was to gain greater understanding of the epidemiology, biology, and clinical consequences of these cytotoxic $CD4^+$ cells in the relatively understudied field of kidney transplantation. We evaluated kidney transplant patients from the time of transplantation, undertaking serial examination of circulating $CD4^+CD28^{null}$ cell frequencies, demonstrating a close relationship with CMV-serostatus. These cells proliferated to CMV-derived antigens, but not to HLA-derived antigens, and demonstrated toxicity to glomerular endothelial cells *in vitro*, highlighting the role of NKG2D. Elevated cell frequencies were associated with relevant clinical outcomes to 5 years post-transplantation.

3.3 CONCISE METHODS

3.3.1 Clinical Cohort

One hundred unselected recipients of solitary kidney transplants were enrolled into this prospective study between 2009 and 2010. All patients underwent transplantation and follow-up at Queen Elizabeth Hospital Birmingham. Local ethics committee approval was granted for the study.

Prior to transplantation, baseline donor and recipient information was collected: donor and recipient age and sex, inflammatory cause of renal failure (glomerulonephritis; vasculitis; lupus), HLA mismatch (class I and class II), source of transplant (live related; live unrelated; deceased donor following brain death [DBD]; deceased donor following cardiac death [DCD]); dialysis modality prior to transplantation (pre-emptive versus haemodialysis versus

peritoneal dialysis). Transplantation proceeded provided the cross match between donor and recipient was negative by flow cytometry and cytotoxicity.

Immunosuppression regimen was identical for all participants, and consisted of Basiliximab induction followed by maintenance tacrolimus (Prograf; trough level 5-8ng/ml initially, measured by liquid chromatography-tandem mass spectrometry), mycophenolate mofetil (Cellcept; 2g daily initially) and prednisolone (20mg daily, reducing to 5mg maintenance by 3 months post transplantation). Data collected subsequently in the course of standard clinical care was evaluated: Post-operative events of delayed graft function (requirement for dialysis during the first post-operative week) and biopsy proven acute rejection (any time; any histological grade) were collected.

Estimated glomerular filtration rate (eGFR) was calculated using the 4 variable MDRD (Modification of Diet in Renal Disease) equation, with IDMS (Isotope Dilution Mass Spectrometry) aligned creatinine measurements; early morning urine albumin: creatinine ratio (UACR) measurement on a “spot” urine sample was used as the measure of proteinuria, and high sensitivity C-reactive protein (hsCRP) as the marker of inflammation. These were collected at each clinic review following transplantation.

3.3.2 Assessment of CMV serostatus, infection, and disease

Pre-transplant CMV serostatus of the recipients and their donors was collected. CMV prophylaxis with 100 days of valganciclovir was given to the D+R- group only, with dose adjustment for renal function. Serial whole blood samples were taken for CMV DNA PCR in all

patients at day 0 (prior to transplantation), and then weeks 1, 2, 3, 4, 6, 8, 10, 12, 16, 20, 24, 28, 34, 40, 46, and 52. The clinical team remained unaware of these results and no changes in clinical management ensued (it is not unit policy to undertake viral load testing and pre-emptive therapy in the context of asymptomatic CMV infection, as is the case in most centres, and so these assessments were undertaken for research purposes only). Additional sampling was undertaken at the time of clinical suspicion of CMV disease, which was then diagnosed according to international guidelines and was based on one or more of the following in association with the finding of CMV viraemia: fever; new onset severe malaise; leucopenia; thrombocytopaenia; hepatitis (alanine transaminase or aspartate transaminase levels greater than twice the upper limit of normal); tissue invasive disease proven by histology. For our laboratory, a copy rate of >500 CMV genome copies/ml of whole blood represents significant CMV viraemia.

Patients who were CMV seronegative at the time of transplantation underwent repeat serological testing at 12 months post transplantation to identify those who have developed asymptomatic infection within the first year, for whom no DNAemia was detected by protocolised testing as above.

3.3.3 Immunophenotyping

Multicolour flow cytometry was used to undertake detailed phenotyping of patients' peripheral blood mononuclear cells (PBMCs) prior to transplantation and then 14 days, 3 months, and 12 months post-transplantation. PBMCs were isolated from heparinised blood of the transplant recipient by density gradient centrifugation using Ficoll-Paque (Fisher, UK)

and cryopreserved in fetal calf serum (FCS) containing 10% DMSO. This was performed within 6 hours following venepuncture.

Based on surface expression of CD27 and CD45RA, the broad differentiation status of $CD4^+CD28^{null}$ cells and their $CD4^+CD28^+$ counterparts were defined as follows. Naïve-like: $CD27^+CD45RA^+$; Central memory-like (CM): $CD27^+CD45RA^-$; Effector memory-like (EM): $CD27^-CD45RA^-$; Effector memory cells re-expressing RA ("EMRA"): $CD27^-CD45RA^+$. The panels of antibodies used for flow cytometric analyses are outlined in the supplementary data.

3.3.4 Isolation of $CD4^+CD27^-CD28^{null}$ and $CD4^+CD27^-CD28^{pos}$ Cells

At 12-months post transplantation, heparinised blood was taken from patients shown to have a high $CD4^+CD27^-CD28^{null}$ cell count (>10% total $CD4^+$ T cells) and PBMCs were isolated by Ficoll-Paque (Fisher, UK) gradient. $CD4^+$CD27-CD28null and $CD4^+CD27^-CD28^{pos}$ cells were isolated first by positive selection using a $CD4^+$ T-cell Isolation Kit, followed by selection of CD27 negative cells (using anti-CD27 human microbeads) and separation of $CD28^+$ and $CD28^{null}$ cells within this population using a CD28 Microbead Kit (Miltenyl Biotec, UK), in accordance to manufacturer's instructions. These cells were used in 2 experiments to investigate i] their proliferative capacity in response to CMV-antigen and HLA-derived peptides, and ii] their ability to induce damage on purified glomerular endothelial cells, as described next.

3.3.4.1 Proliferation Assays (CMV- and HLA-derived peptides)

$CD4^+CD27^-CD28^{null}$ and $CD4^+CD27^-CD28^{pos}$ sorted cells were labelled with 0.5µM CFSE (Molecular Probes/Invitrogen) in PBS for 5 min at 37°C followed by 5 min in ice-cold RPMI-1640 containing 5% human AB serum (Sigma, UK). Cells were then seeded at 10^5 with 10^5 antigen-pulsed irradiated autologous PBMCs in RPMI-1640 containing 5% human AB serum and incubated at 37^0C with 5% CO_2 for 5 days. These autologous PBMCs had been pulsed for 4h prior to irradiation with either i) CMV lysate [from fibroblasts infected with CMV strain AD69], ii) α_3-domain derived Class-I HLA peptides (also used in ELISPOT assay described below; Thermo, UK;) or iii) control culture media. Cells were surface stained with CD3-APC, CD4-APC-Cy7 (eBiosicences) and Yellow Dead Cell Stain Kit ((Molecular Probes/Invitrogen), then analysed using a Cyan Flow Cytometer (Beckman Coulter).

The α_3-domain derived Class-I HLA peptides used in the second part of this experiment are known targets for cellular alloresponses, and their use allows inter-patient assay standardization (20,21). The responding cells are $CD28^-$ expressing effector-memory $CD4^+$ cells which recognise cryptic, autologous HLA-derived peptide epitopes, such that inter-patient standardisation can be undertaken without using distinct HLA peptides tailored to the donor and recipient HLA type (20).

3.3.4.2 Analysis of Damage to Glomerular Endothelial Cells

Conditionally immortalized human glomerular endothelial cells (GEnC) were kindly gifted by Dr S. Satchell, Bristol, UK. These were maintained in supplemented endothelial basal medium-

2 (Lonza, UK) as described previously (26). GEnC cells were grown until 90% confluence at 33^0C, then at 37^0C for at least 24h prior to addition of T cells.

PBMCs were isolated from patients and incubated for 16h with either CMV lysate, an α3-domain derived HLA Class I peptide, or culture media. $CD4^+CD27^-CD28^{null}$ and $CD4^+CD27^-CD28^{pos}$ cells were then isolated as described above, with 10^5 cells per well incubated with GEnC, and seeded in 24 well plates for 1 hour using transwell inserts to determine the role for chemokines involved in lymphocyte migration (specifically fractalkine in this study). One well per patient was incubated with either 1µg/ml antiNKG2D antibody or isotype control (BD Biosciences) for 2h prior to addition of T cells. Cells were stained for activated caspase 3 (as a marker of apoptosis; BD Biosciences) following T-cell migration, whereby GEnC and T cells were differentiated by staining for cell-specific markers anti-CD31-APC and anti-CD4-PE (eBiosciences) respectively, and analysed using Cyan flow cytometer (Beckman Coulter); In addition, fractalkine production was measured in the supernatants collected from GEnC incubated with T cells by Recombinant Human CX3CL1/Fractalkine ELISA Kit according to manufacturer's instructions (R & D Systems, UK); Von Willebrand Factor (vWF) was also measured in supernatants by sandwich ELISA, using unconjugated (capture) and conjugated (detection) vWF antibodies (DAKO, UK).

3.3.5 Enzyme Linked Immunosorbent Spot Assay to detect anti-HLA cellular response

A γ-interferon ELISPOT assay to evaluate the cellular immune response of transplant recipients to non-polymorphic HLA-derived peptides was undertaken as previously described (20, 21), and described briefly above.

3.3.6 Statistical analysis

Data are presented as mean ± standard deviation, unless otherwise stated; the figures demonstrate data distribution as mean, standard error, median, range, interquartile range and percentiles as described in figure legends. Non-normal distributed data underwent transformation as necessary prior to analysis. Of note, $CD4^{+}CD27^{-}CD28^{null}$ cell frequencies displayed heavily positive skewing, such that straightforward attempts (such as logarithmic transformation) to transform into a more normally distributed scale were not possible. Instead, these data were evaluated using negative binomial regression analysis. Other continuously distributed end-point data was evaluated using linear regression analysis; categorical endpoints were evaluated by logistic regression; time to event outcomes were analysed using a Cox proportional hazards model. For all analyses, initially the effect of each predictor variable on the outcome was considered separately in a series of univariate analyses. Post-transplant events of acute rejection and CMV infection were analysed as time-dependent variables as required. Variables showing some effect in the univariate analysis ($p<0.15$) were included in a subsequent multivariable analysis, with a stepwise backwards

selection procedure to retain only the statistically significant variables in the final model. For all analyses, a type 1 error rate below 5% ($p<0.05$) was considered statistically significant.

Another feature of the data was the fact that there were several measurements for each subject. To allow for the non-independence of the data, multilevel statistical methods were used for data analysis in the linear regression and negative binomial models. Two-level models were used with individual measurements nested within patients. This was implemented using the xtnbreg procedure with the Stata statistical software package. For time to event analyses, predictor variable was analysed as time-dependent covariates; for analyses of single endpoints (either categorical or continuously distributed), averaged values (over the course of the study) for predictor variables were entered.

For other analyses, continuously distributed parametric data was compared using Pearson's correlation coefficient and Student's t-test; non-parametric data was analysed by Mann-Whitney testing (with Bonferroni correction for multiple comparisons where appropriate); multiple-group independent non-parametric data was analysed by Kruksall-Wallis testing, with Friedman testing used for multiple-group non-independent (multiple comparisons over time) non-parametric data. Categorical data was compared using Chi-square testing. Inter-test concordance was evaluated using the kappa (κ) statistic.

3.4 RESULTS

3.4.1 Clinical outcomes

The clinical cohort is described in Table 3.1. Over the study duration, three patients died (from sepsis), and four lost their grafts (primary non-function with nephrectomy 2 months post-transplantation, early acute rejection, recurrent disease, late [14 months] acute rejection); all patients underwent serial sampling (as described in "methods") until death or graft failure (95 patients were alive with graft function at 12 months post-transplantation). For the entire cohort, median follow-up was 54 months (range 2-60 months). For the 93 patients alive with graft function at the end of the study, mean eGFR at the end of follow-up was 52±17ml/min/1.73m^2, and median UACR was 2.6 mg/mmol (IQR:1.0-6.6; range 0.3-280). Twenty-three patients experienced DGF, and twenty-five acute rejection (≥Banff grade 1).

In the D-R- group, 1 patient developed detectable CMV DNAemia (with clinical disease) and 1 seroconverted without prior detectable DNAemia. In the D+R- group (prophylaxed for 100 days), 5 patients developed detectable DNAemia (2 clinical disease) and 3 seroconverted without detectable DNAemia. In the D-R+ group, 6 patients developed detectable DNAemia (1 clinical disease). In the D+R+ group, 9 patients developed detectable DNAemia (3 clinical disease).

Table 3.1 Patient demographics

Recipient Age	48±14 years
Recipient Sex	55 male
Recipient ethnicity White Indo-Asian African-Caribbean	 74 15 11
Cause of Renal Failure Glomerular Cystic Diabetes Hypertension Other	 29 19 14 19 19
Pre Transplant Modality Haemodialysis Peritoneal Dialysis Pre-emptive	 38 35 27
Repeat transplantation	23
Donor Age	47±15 years
Transplant Source Deceased donor (DBD[1]) (DCD[2]) Live donor	 56 (44) (12) 44
Donor-Recipient HLA Mismatch Class I (HLA-A+B) Class II (HLA-DR)	 2.1±0.9 Ag 0.9±0.7 Ag
Donor-Recipient CMV Serostatus D-R- D-R+ D+R+ D+R-	 25 22 32 21
Biopsy proven Acute rejection	25
Delayed graft Function[3]	23
Cytomegalovirus Asymptomatic infection[4] Disease[5]	 18 7

[1] Donation after Brain Death

[2] Donation after Cardiac death

[3] Requirement for dialysis within the first week post transplantation

[4] Denotes the development of asymptomatic CMV viremia on protocol sampling, or the *de novo* development of anti-CMV antibody in those patients who were seronegative at the time of transplantation – none of these patients displayed evidence of clinical disease (see text for details)

[5] Denotes the development of symptomatic CMV disease requiring clinical intervention

3.4.2 $CD4^+CD28^{null}$ cell frequencies, expansion and CMV status

Circulating $CD4^+CD28^{null}$ cell frequencies (expressed as a percentage of total $CD4^+$ cells), and the flow cytometry gating strategy these results are based on, are shown in Figure 3.1 (A and B).

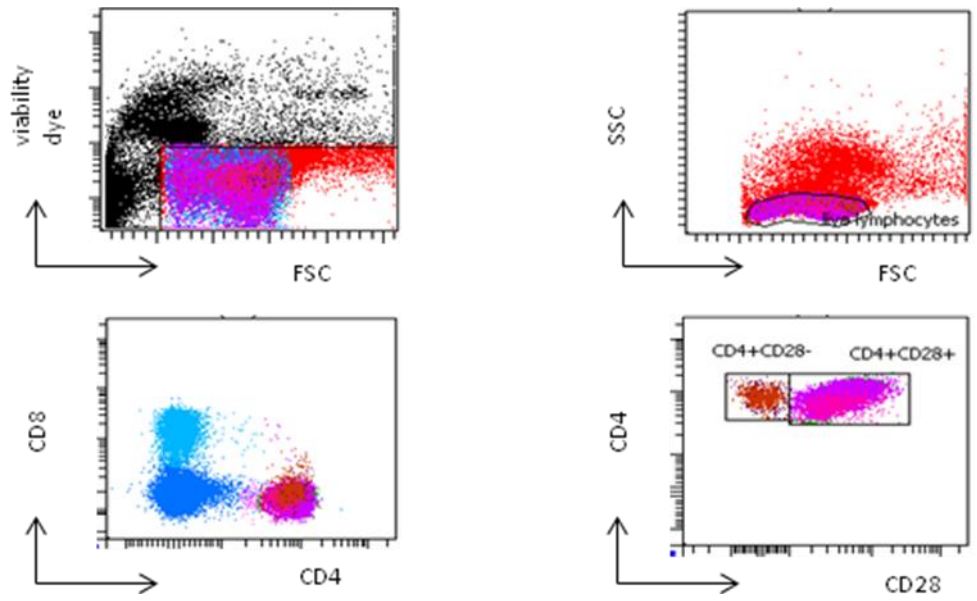


Figure 3.1A Flow cytometry gating strategy for identification of $CD4^+CD28^{null}$ cells

The gating strategy (Figure 3.1A) and results (Figure 3.1B) for the flow cytometry identification of $CD4^+CD28^{null}$ cells is shown. $CD4^+CD28^{null}$ cell frequencies (expressed as a proportion of total CD4 cells) over time are shown as median (line), interquartile range (boxes) and range (whiskers). This demonstrates significant differences in cell frequencies across CMV-serostatus subgroups at all timepoints (Kruksall-Wallis $p<0.05$ for all) and evidence of expansion of $CD4^+CD28^{null}$ cells in CMV seropositive recipients over the first-year post-transplantation, which was not see in their CMV-seronegative counterparts (Friedman test $p<0.001$ for D-/R+ and D+/R+ groups). Samples available and analysed for 100 patients pre-transplant, 94 patients at 14 days, 92 patients at 3 months, and 90 patients at 12 months. Figure 3.1C demonstrates the expansion of $CD4^+CD28^{null}$ cells in 10 patients who were seronegative at the time of transplant and who developed CMV infection during the first 12 months post transplantation (2 received kidneys from CMV-seronegative donors, and 8 from CMV-seropositive donors).

$CD4^+CD28^{null}$ frequencies were higher in seropositive recipients than seronegative recipients at all timepoints ($p<0.05$ for all; Figure 3.1B). This was particularly evident at the 12-month

timepoint, as there was expansion of $CD4^+CD28^{null}$ frequencies in CMV-seropositive individuals over time that was not seen in the (overall) CMV-seronegative patients. Multivariable analysis (Table 3.2) showed recipient serostatus and time post transplantation as the significant independent predictors of $CD4^+CD28^{null}$ cell frequencies. In the entire cohort, although development of CMV infection (detection of DNAemia or "silent" seroconversion) was associated with $CD4^+CD28^{null}$ expansion on univariate analysis, this did not hold in the multivariate analysis.

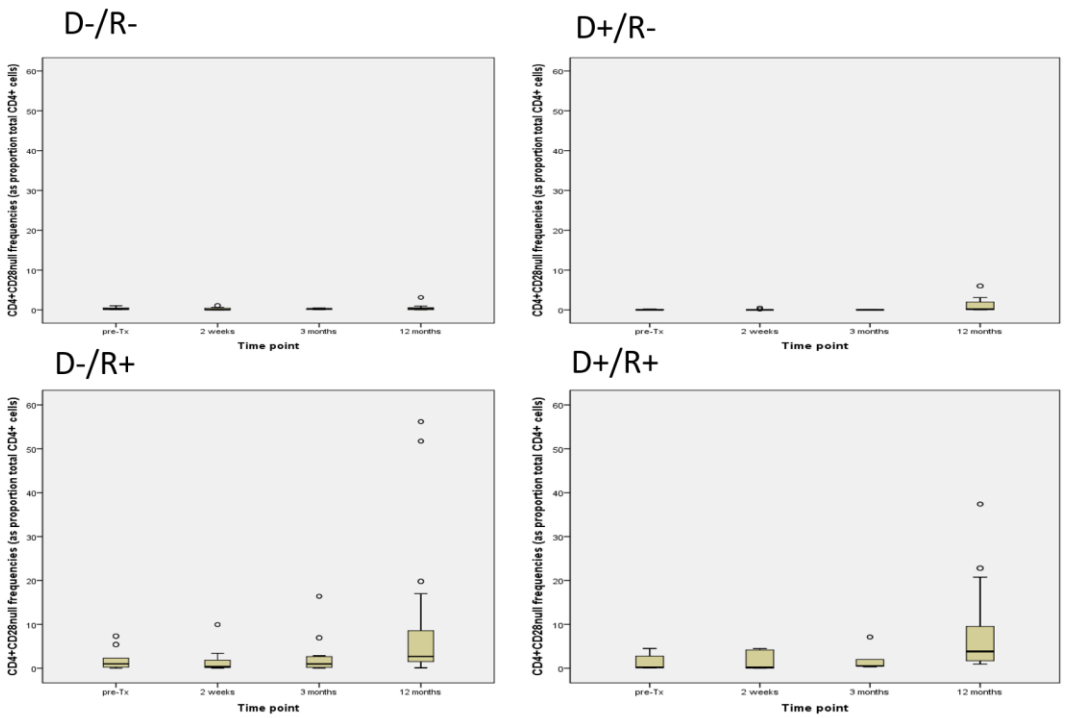
Table 3.2 Predictors of $CD4^+CD28^{null}$ cell frequencies

Variable	Category	Univariate Ratio (95%CI)	p-value	Multivariate Ratio (95%CI)	p-value
Time	Baseline 14 days 3 months 12 months	1 0.64 (0.33, 1.29) 0.90 (0.47, 1.75) 2.31 (1.36, 3.92)	<0.001	1 0.58 (0.31, 1.09) 0.75 (0.41, 1.37) 1.92 (1.17, 3.14)	<0.001
CMV Status	D-R- D+R- D-R+ D+R+	1 0.53 (0.21, 1.36) 3.16 (1.39, 7.19) 4.46 (1.86, 10.70)	<0.001	1 0.65 (0.25, 1.68) 4.45 (1.90, 10.40) 4.28 (1.78, 10.30)	<0.001
CMV Infection*	Yes	4.40 (1.77, 10.9)	0.001		
Recipient Age	Per 10 years	0.9 (0.76, 1.28)	0.91		
Recipient Sex	Male vs Female	1.76 (0.88, 3.55)	0.11		
Inflammatory Diagnosis**	Yes	1.53 (0.69, 3.38)	0.30		
Dialysis Modality	Haemodialysis Peritoneal Dialysis Pre-emptive	1 0.54 (0.23, 1.25) 0.46 (0.19, 1.10)	0.16		
Repeat Transplantation	Yes	1.75 (0.67, 4.58)	0.25		
Class I HLA Mismatch	Per antigen	0.93 (0.65, 1.33)	0.67		
Class II HLA Mismatch	Per antigen	0.92 (0.54, 1.57)	0.76		
Delayed Graft Function***	Yes	1.30 (0.60, 2.83)	0.51		
Rejection****	Yes	1.37 (0.62, 3.05)	0.44		

Table 3.2 shows the predictors of $CD4^+CD28^{null}$ cell frequencies (expressed as a proportion of total $CD4^+$ cells) in the study population. Results from univariate and multiple regression models displayed. The effect sizes are summarised in the form of ratios. For the categorical variables these represent the ratio of $CD4^+CD28^{null}$ cell frequencies in each category relative to a baseline category. For the continuous variables, these represent the change in ratio for a one-unit increase in that variable (unless otherwise indicated).

*denotes either i] detection of CMV DNAemia (either clinically manifest or silent) prior to sampling time or ii] recipient seroconversion prior to sampling time
**denotes primary renal disease due to inflammatory nephritis (e.g. vasculitis, lupus, glomerulonephritis)
***defined as dialysis requirement in first week post transplantation
****Biopsy proven rejection (any grade) prior to sampling time

Figure 1B



	Pre-Transplant	14 days	3 months	12 months
-/-	0.20 (0.0-1.1)	0.3 (0.0-1.1)	0.3 (0.1-0.5)	0.3 (0.0-3.1)
+/-	0.1 (0.0-0.2)	0.1 (0.0-0.5)	0.1 (0.0-0.3)	0.2 (0.0-6.0)
-/+	1.1 (0.0-7.3)	0.3 (0.0-9.9)	1.0 (0.0-16.4)	2.7 (0.1-56.2)
+/+	0.7 (0.0-4.5)	0.2 (0.0-4.5)	0.5 (0.3-7.1)	3.8 (0.9-37.4)

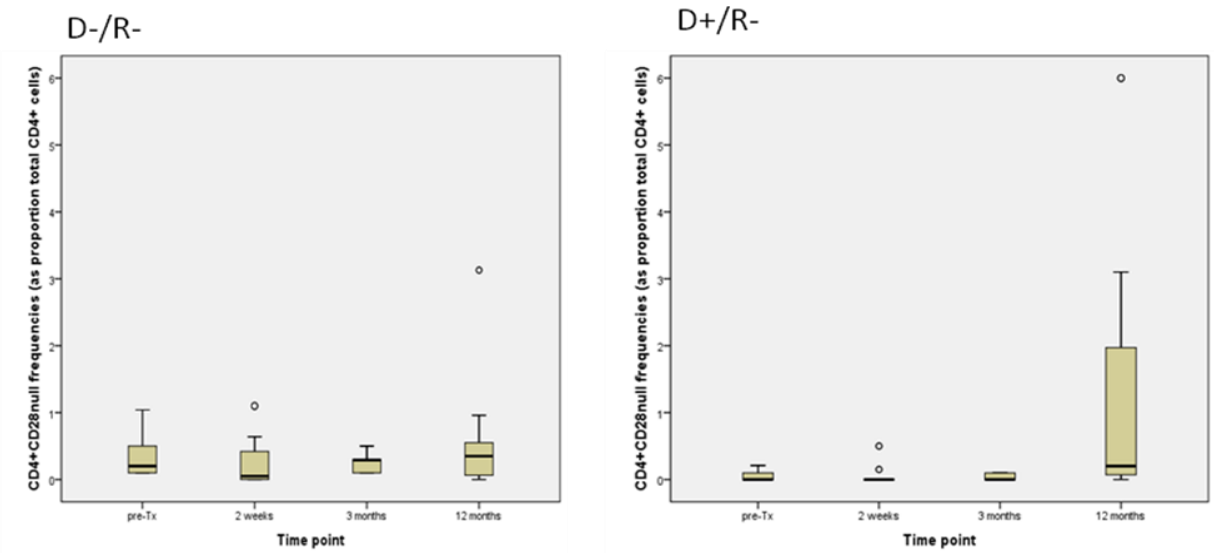


Figure 3.1B **$CD4^{+}CD28^{null}$ frequencies over time in the donor-recipient CMV serostatus groups**

Although as mentioned above, there was no expansion of $CD4^{+}CD28^{null}$ cells in CMV-seronegative recipients, the individuals who did experience primary CMV infection within the first year (either with or without clinical disease as described above; n=10), displayed increases in $CD4^{+}CD28^{null}$ frequencies from 0.2±0.15% pre-transplant to 2.5±1.8% at 12 months post-transplantation (p=0.009). Multiple regression analysis (adjusted for variables in Table 3.2) demonstrated prior CMV infection as the only predictor of $CD4^{+}CD28^{null}$ frequencies in these patients (ratio:9.67 (95%CI:3.30-28.40; p<0.001). Figure 3.1C demonstrates the evolution of $CD4^{+}CD28^{null}$ cells in these 10 specific patients.

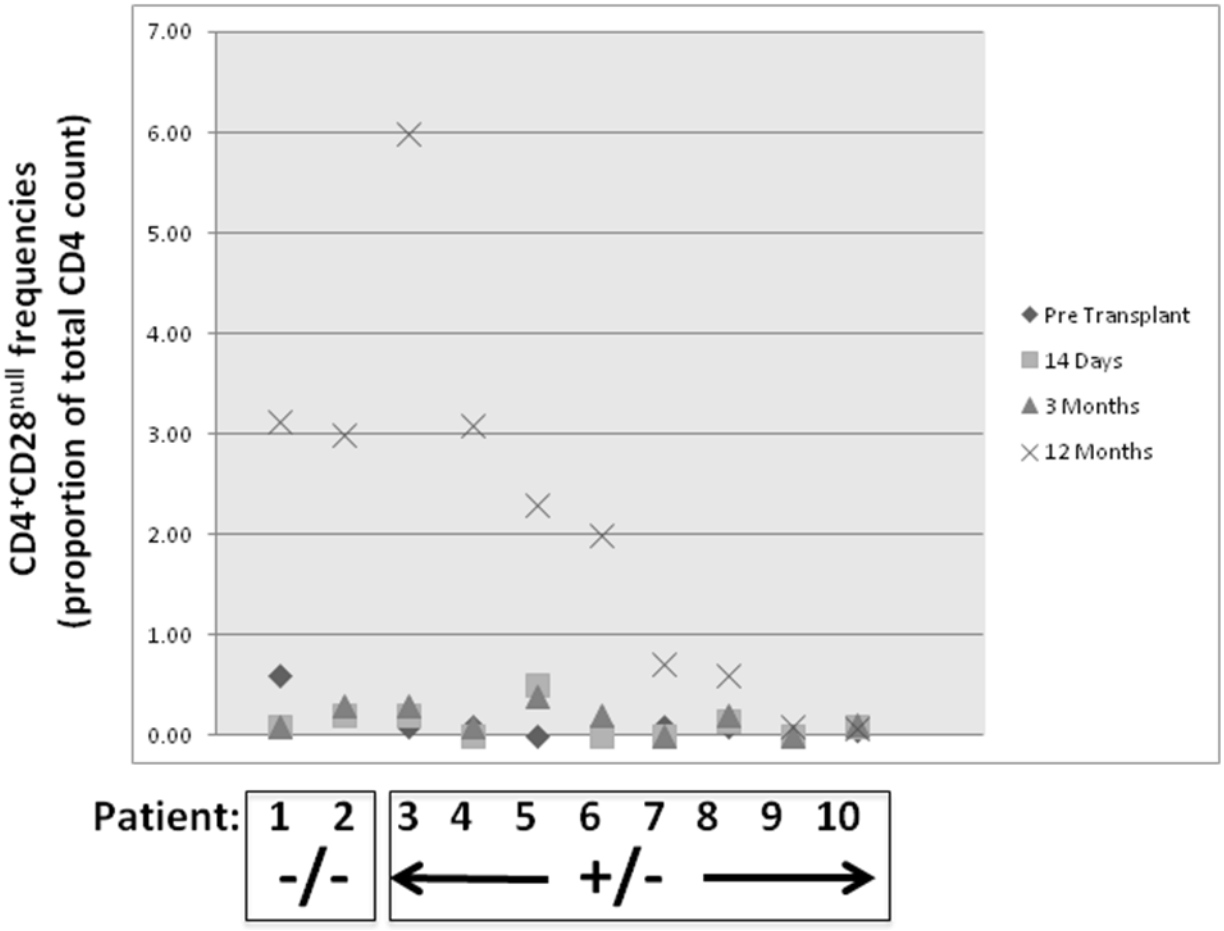


Figure 3.1C Evolution of $CD4^{+}CD28^{null}$ cells following primary CMV infection

3.4.3 Episodes of inflammation are associated with expansion of $CD4^{+}CD28^{null}$ cells in CMV-seropositive recipients

In vitro work and clinical data from patients with critical illness suggests importance of inflammation in driving CMV reactivation (12-15). Because information was lacking in regard to inflammatory events or markers *prior to* transplantation, we undertook a separate "delta analysis" identifying factors associated with changes ("deltas") in cell frequencies between sampling timepoints (i.e. baseline to 14 days; 14 days to 3 months; 3 months to 12 months). Analysis focused on the relationship between the highest recorded (peak) hsCRP measurement during these timepoints and the delta $CD4^{+}CD28^{null}$ frequencies between these timepoints. Peak hsCRP values demonstrated positive skewing (median:21mg/L; range 0.3-140mg/L); delta $CD4^{+}CD28^{null}$ frequencies were normally distributed (mean:1.0±3.8; range -11.1 to 16.7). In the entire cohort, some association between hsCRP and delta $CD4^{+}CD28^{null}$ cells was seen (coefficient:0.5;95%CI:0.1-1.1; p=0.07). A significant statistical interaction between peak hsCRP and recipient CMV-serostatus was seen ($p<0.05$), with a significant relationship between hsCRP and delta count only in CMV-seropositive recipients (coefficient:1.0; 95%CI:0.2-1.8; p=0.02).

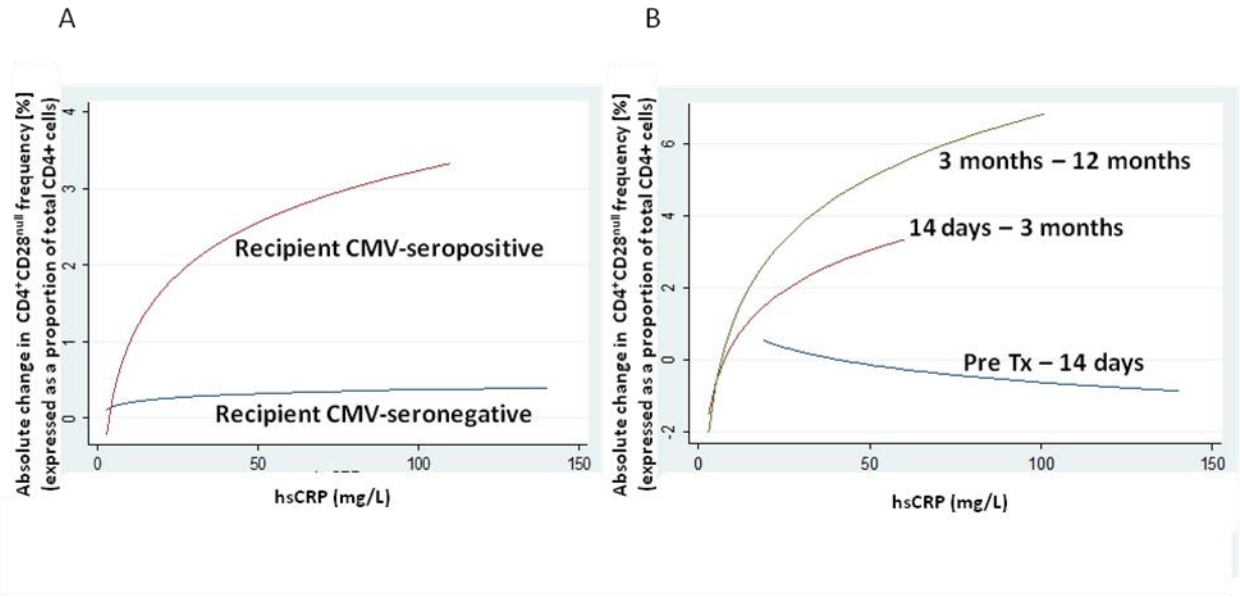


Figure 3.2 Association between delta $CD4^+CD28^{null}$ cell count and peak hsCRP across sampling time points

Sampling time points were baseline to 14 days; 14 days to 3 months; and 3 months to 12 months. The "delta count" (as described in "methods") represents the absolute change in $CD4^+CD28^{null}$ frequency (expressed as the percentage of total $CD4^+$ cells) between time points. Curves shown represent lines of best fit from the regression analyses undertaken. Figure 3.2A demonstrates the relationship between delta count (all time points considered) and hsCRP in all studied patients. This shows the influence of inflammation on $CD4^+CD28^{null}$ cell expansion was only evident in CMV-seropositive recipients. Further analysis of this CMV-seropositive group (only) is represented in Figure 3.2B. This shows the influence of time post-transplantation on the relationship between hsCRP and delta $CD4^+CD28^{null}$ cell count, whereby this effect of inflammation-associated $CD4^+CD28^{null}$ cell expansion is only seen beyond 14 days post-transplantation in this CMV-seropositive group.

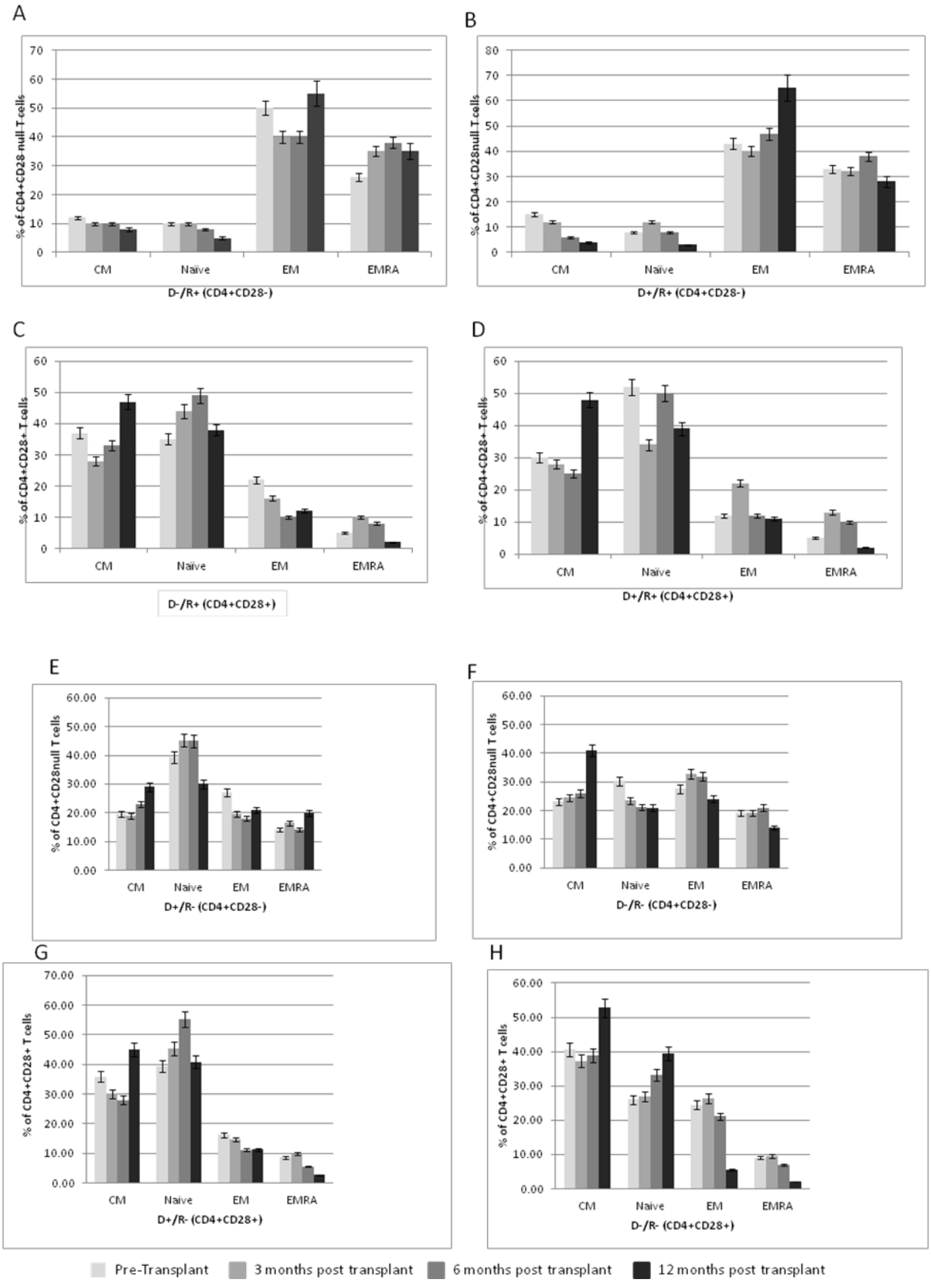
Figure 3.2A shows the line of best fit demonstrating the relationship between hsCRP and expansion of $CD4^+CD28^{null}$ cells (based on the output from the regression analysis) in both serostatus groups. Indeed, hsCRP was the only independent predictor of delta count in seropositive recipients when adjusted for variables in Table 3.2, including the development of CMV viremia between timepoints. Furthermore, in subgroup analysis of these CMV-seropositive patients in whom $CD4^+CD28^{null}$ cell expansion was seen, a further interaction between hsCRP and timepoint was evident (p=0.007), and suggested episodes of inflammation had a greater effect upon $CD4^+CD28^{null}$ cell expansion at times beyond 14 days

post-transplantation. This relationship is demonstrated in Figure 3.2B. In summary, this suggests episodes of inflammation may drive expansion of $CD4^+CD28^{null}$ cells in CMV-seropositive recipients, particularly beyond 14 days post-transplantation.

3.4.4 In CMV-seropositive recipients $CD4^+CD28^{null}$ cells display predominant effector memory-like or EMRA-like phenotype

In CMV-seropositive individuals the majority of $CD4^+CD28^{null}$ cells displayed either an EM or EMRA phenotype compared to $CD4^+CD28^+$ cells (Figure 3.3; Panels A/B versus Panels C/D; $p<0.001$). This confirms $CD4^+CD28^{null}$ cells in CMV-seropositive individuals as a "late-differentiated", antigen-experienced population; this data is compatible with the concept that $CD4^+$ cells lose surface CD27 and then CD28 during differentiation (4-8).

CMV-seronegative recipients' $CD4^+CD28^{null}$ cells more commonly expressed CD27 than those from seropositive individuals, thereby displaying a 'naïve-like' or 'CM-like' phenotype. This pattern of loss of CD28 without loss of CD27 may represent recent $CD4^+$-cell activation and temporary downregulation of CD28 in isolation, as previously suggested (16). This is shown in Figure 3.3 (panels E and F) which show data from CMV-seronegative patients excluding patients who experienced CMV infection in the first 12 months post transplantation (as described above); these data therefore demonstrate the 'natural history' of the (lack of) evolution of $CD4^+CD28^{null}$ cells across the first-year post-transplantation in recipients who


A
% of CD4+CD28-null T cells
CM
Naive
EM
EMRA
D-/R+ (CD4+CD28-)
B
% of CD4+CD28null T cells
D+/R+ (CD4+CD28-)
C
% of CD4+CD28+ T cells
D-/R+ (CD4+CD28+)
D
D+/R+ (CD4+CD28+)
E
% of CD4+CD28null T cells
D+/R- (CD4+CD28-)
F
D-/R- (CD4+CD28-)
G
% of CD4+CD28+ T cells
D+/R- (CD4+CD28+)
H
D-/R- (CD4+CD28+)
Pre-Transplant
3 months post transplant
6 months post transplant
12 months post transplant

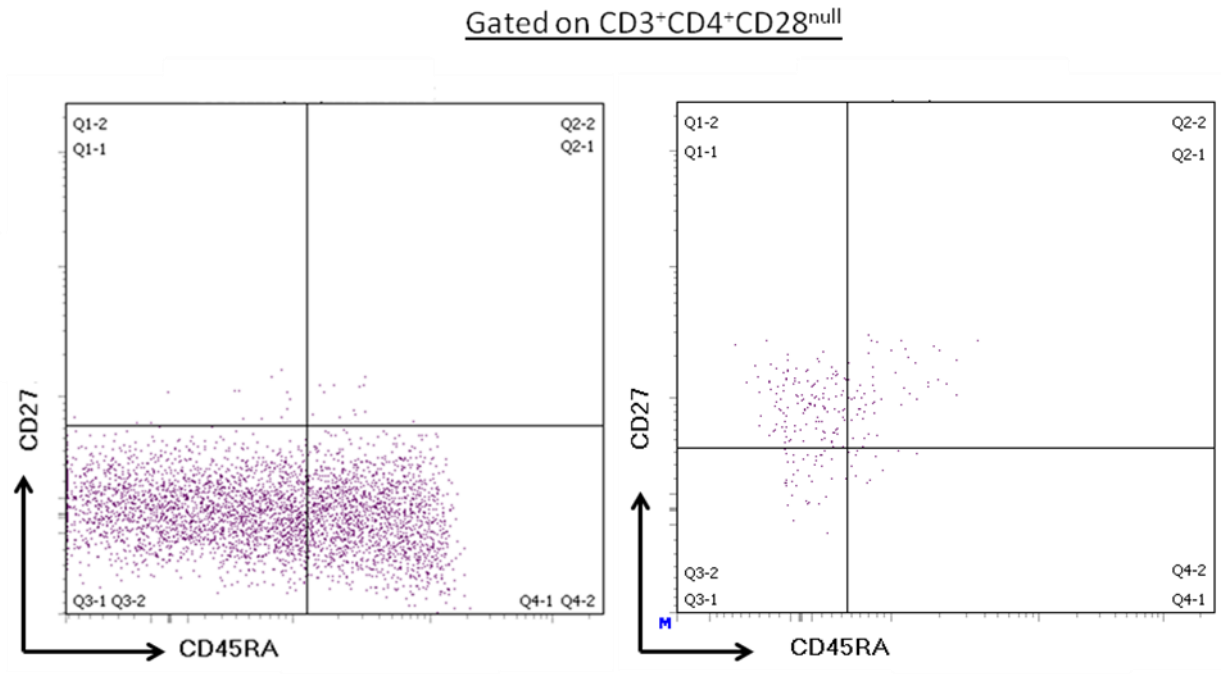


Figure 3I

Figure 3J

Figure 3.3 Phenotype of CD4⁺CD28^{null} and CD4⁺CD28⁺ cells according to donor-recipient CMV serostatus pairing

Frequencies of naïve, central memory (CM), effector memory (EM) and effector memory re-expressing RA (EMRA) cells within the CD4⁺CD28^{null} and CD4⁺CD28⁺ cell populations are shown (as defined by CD27 and CD45RA co-expression; see methods). In CMV-seropositive recipients the majority of CD4⁺ CD28^{null} cells display an EM-like or EMRA-like phenotype (A and B), contrasting with their phenotype in CMV-seronegative recipients (E and F), and also with the phenotype of CD4⁺ CD28⁺ cells from either seropositive or seronegative recipients (C, and D and G and H). Samples available and analysed for 100 patients pre-transplant, 94 patients at 14 days, 92 patients at 3 months, and 90 patients at 12 months. Data normally distributed and displayed as mean and standard error. To demonstrate the "natural history" of the evolution of cell frequencies in CMV-seronegative patients, the data shown in Panels E through H exclude patients who were CMV-seronegative at the time of transplant and who developed evidence of CMV infection during the first-year post transplantation (total n=10 as described further in text). "Representative" flow cytometry readouts for the evaluation of CD4⁺CD28^{null} cell phenotype are shown in Figures 3I and 3J. Figure 3.3I shows data from a CMV-seropositive patient sampled at month 12 (CD4⁺CD28^{null} cells comprised 4% of total CD4⁺ population); Figure 3.3J shows data from a CMV-seronegative patient also sampled at month 12 (donor CMV-seronegative; no CMV disease, infection or seroconversion by 12 months; CD4⁺CD28^{null} cells comprised 0.6% of total CD4⁺ population). Although the results from CMV-seronegative patients are interpretable, results are not as robust as for the case of seropositive patients displaying higher CD4⁺ CD28^{null} cell frequencies.

were CMV-seronegative at the time of transplantation. It should be specifically highlighted that the low CD4⁺CD28^{null} cell frequencies in CMV-seronegative patients means that the surface phenotyping results in these patients require cautious interpretation (representative

flow cytometry readouts for seropositive and seronegative patients are shown in Figures 3I and 3J respectively).

We next analysed those CMV-seronegative patients who developed primary CMV infection during the first-year post-transplantation (described above and Figure 3.1B). In these patients, phenotype characteristics resembled those of CMV-seropositive patients, with an EM or EMRA phenotype in 44±8% and 31±6% of these patients at 12 months respectively. These proportions were greater than the proportion of EM or EMRA seen at baseline in these patients, and also greater than the proportions in CMV-seronegative patients who did not experience CMV infection during the first year ($p<0.05$ for all comparisons).

$CD4^+CD28^+$ cells from CMV-seronegative and CMV-seropositive individuals displayed a similar phenotype as shown in Figure 3.3 Panels C/D and G/H (the latter again excluding patients experiencing CMV infection in the first 12 months post-transplantation). These described phenotype characteristics were stable during the first-year post-transplantation.

3.4.5 $CD4^+CD27^-CD28^{null}$ cells display markers of endothelial homing and cytotoxic potential

Further detailed immune phenotyping of $CD4^+CD28^{null}$ and their comparator $CD4^+CD28^+$ cells were then undertaken. As $CD4^+CD28^{null}$ cells were essentially limited to CMV-seropositive patients, phenotyping was undertaken 12 months post-transplantation in this group (49 patient samples available from the 52 CMV-seropositive patients alive with graft function at 12 months). As also described above, the majority of $CD4^+CD28^{null}$ cells in CMV-seropositive

individuals display an effector-memory phenotype, whereas many $CD4^+CD28^+$ cells display naïve-or CM-like characteristics. Therefore, we compared specifically the flow cytometry characteristics of the effector-memory subsets of both cell types, i.e. $CD4^+CD27^-CD28^{null}$ versus $CD4^+CD27^-CD28^+$ cells, with $CD4^+CD27^-CD28^{null}$ cells representing a later differentiated population of effector-memory cells. We did not attempt to differentiate EM and EMRA in these analyses.

Of particular interest, $CD4^+CD27^-CD28^{null}$ cells displayed increased expression of the natural killer (NK) cell marker NKG2D and also of perforin. Figure 3.4A shows the gating strategy for these phenotype characteristics. Figure 3.4B shows the summary data for these and other surface phenotype characteristics for which differences between $CD4^+CD27^-CD28^{null}$ and $CD4^+CD27^-CD28^+$ cells were seen. $CD4^+CD27^-CD28^{null}$ cells also displayed increased expression of the late-differentiation marker CD57. Another NK receptor, CD56, was also expressed at higher levels although the difference was not as marked as for NKG2D.

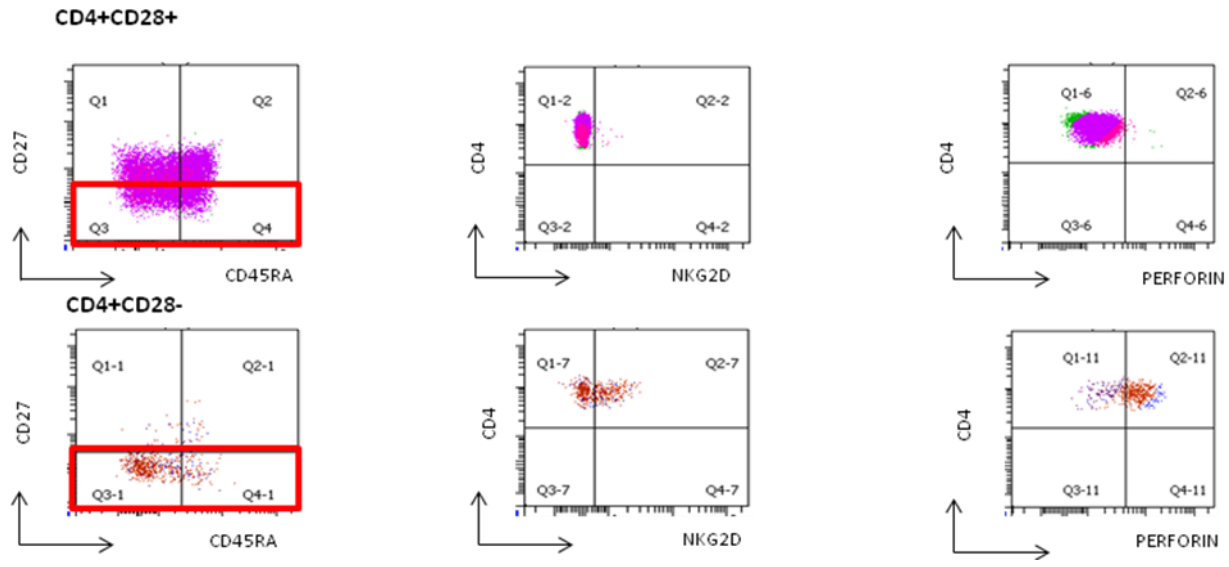


Figure 3.4A NKG2D expression, perforin release in $CD4^{+}CD28^{null}$ and $CD4^{+}$ $CD28^{+}$ cells

Example gating strategy and summary data demonstrating phenotypic comparison between $CD4^{+}CD27^{-}CD28^{null}$ and $CD4^{+}CD27^{-}CD28^{+}$ cells in 49 CMV seropositive recipients alive with graft function at 12 months post-transplantation. Initial gating to define $CD4^{+}CD27^{-}CD28^{null}$ and $CD4^{+}CD27^{-}CD28^{+}$ cells was conducted as shown in Figure 3.1A. Figure 3.4A then demonstrates surface staining for NKG2D and intracellular staining for perforin in $CD4^{+}CD27^{-}CD28^{null}$ cells (lower panel), which was absent in the $CD4^{+}CD27^{-}CD28^{+}$ compartment (upper panel).

Furthermore, expression of the fractalkine receptor CX3CR1 was almost exclusive to $CD4^{+}CD27^{-}CD28^{null}$ cells, suggesting capability of homing to fractalkine-releasing inflamed endothelium. Of relevance to endothelial homing and tissue invasion potential was increased expression of CD11a (component of LFA-1) and CD49d (component of VLA-4) in the $CD4^{+}CD27^{-}CD28^{null}$ population. It is worth noting that CD11a and CD49d may also act as co-stimulation molecules as well as purely adhesion molecules (17, 18). $CD4^{+}CD27^{-}CD28^{null}$ cells also expressed higher levels of other co-stimulation receptors CD134 (OX-40) and CD137 (4-1BB), albeit at low absolute levels in the resting state.

In comparison, Figure 3.4C shows surface characteristics which did not differ between $CD4^{+}CD27^{-}CD28^{null}$ and $CD4^{+}CD27^{-}CD28^{+}$ cells ($p>0.05$ for all comparisons). Specifically,

despite increased expression of NK markers on $CD4^+CD27^-CD28^{null}$ cells, expression of the Vα24Vβ11 TCR, which is characteristically expressed on iNKT cells, was low and comparable to that in $CD4^+CD27^-CD28^+$ cells. No difference in expression of the lymph node homing molecule CD62L was seen between $CD4^+CD27^-CD28^{null}$ and $CD4^+CD27^-CD28^+$ cells, in keeping with the effector-memory characteristics of both these cell subsets. Despite the "late differentiation" status of $CD4^+CD27^-CD28^{null}$ cells, expression of the inhibitory co-stimulation molecules KLRG-1 or PD-1, considered markers of T-cell "exhaustion", was not increased.

Figure 4B

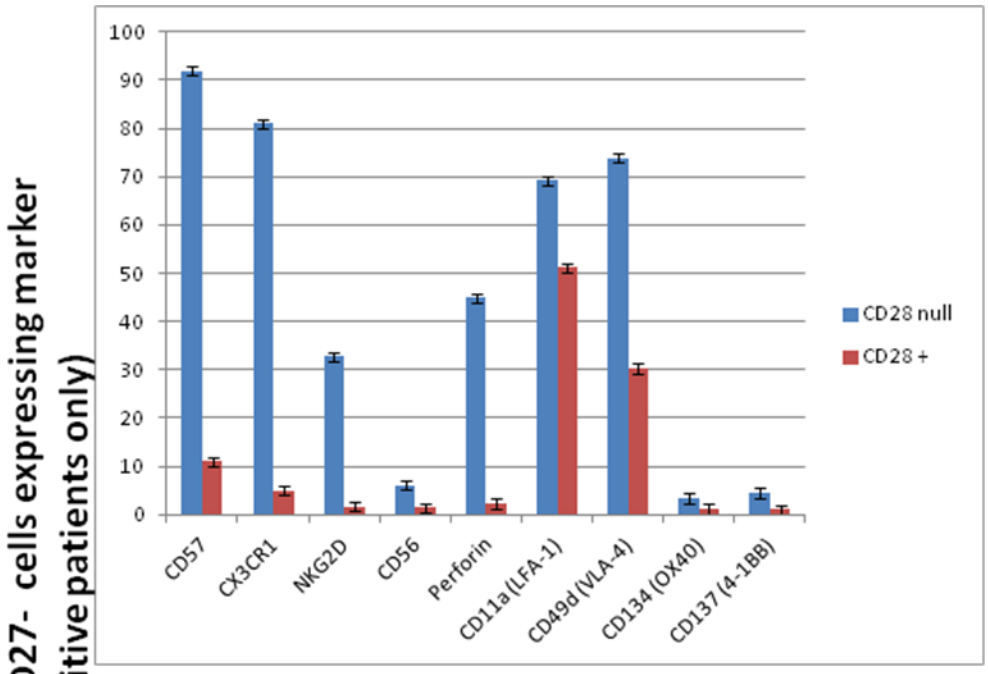


Figure 4C

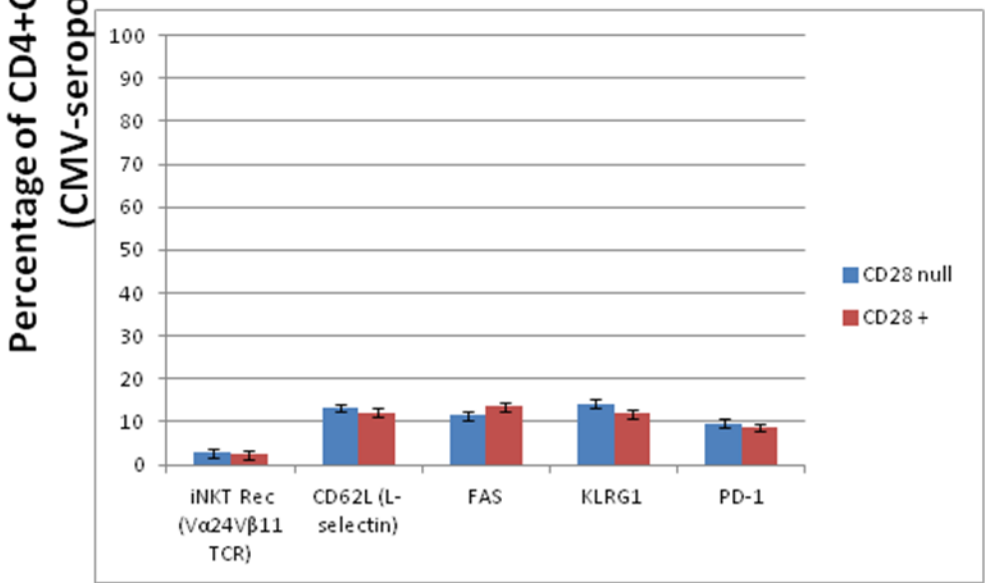


Figure 3.4B Phenotypic differences and similarities between $CD4^{+}CD28^{null}$ and $CD4^{+}CD28^{+}$ cells

demonstrates summary data for those phenotype characteristics which differed between $CD4^{+}CD27^{-}CD28^{null}$ and $CD4^{+}CD27^{-}CD28^{+}$ cells ($p<0.05$ for all); Figure 3.4C demonstrates phenotyping characteristics where no difference was observed between these cell subsets. Data normally distributed and displayed as mean and standard error.

3.4.6 $CD4^+CD27^-CD28^{null}$ cells from CMV-seropositive transplant recipients proliferate in response to CMV lysate but not HLA-derived peptide

$CD4^+CD27^-CD28^{null}$ and $CD4^+CD27^-CD28^+$ cells were isolated from CMV-seropositive recipients 12 months post-transplantation (n=10), and examined for their proliferative responses to irradiated autologous PBMCs alone ("control"), or PBMCs previously exposed to either CMV-derived or HLA-derived peptides. Figure 3.5 demonstrates representative flow cytometry analyses (left panel) and summary data (right hand panels).

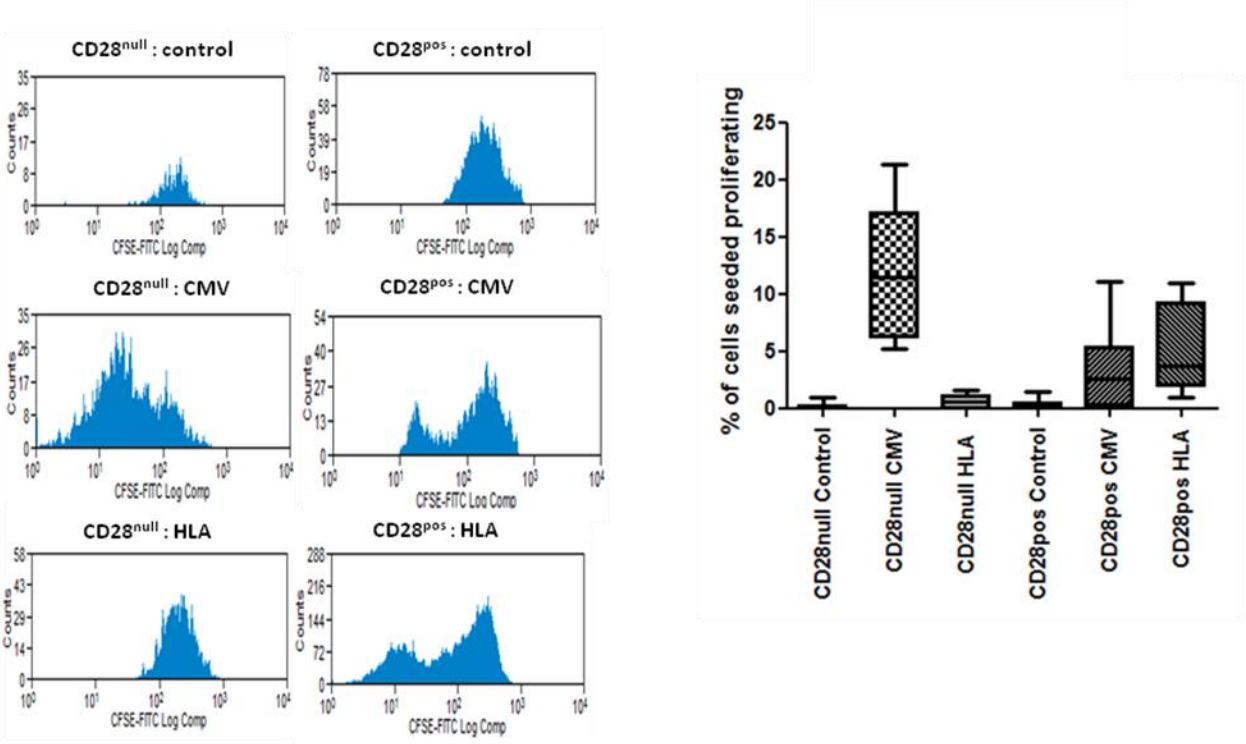


Figure 3.5 Proliferation of $CD4^+CD28^{null}$ and $CD4^+CD28^+$ cells from CMV positive patients

Flow cytometric analysis demonstrating proliferation of $CD4^+CD27^-CD28^{null}$ and $CD4^+CD27^-CD28^+$ cells from CMV positive renal transplant patients (n=10). Proliferation of $CD4^+CD27^-CD28^{null}$ and $CD4^+CD27^-CD28^+$ cells was evaluated by CFSE dilution. Cells were pulsed with irradiated autologous PBMCs previously exposed to either control culture media, CMV lysate, or HLA peptide. Example plots of flow cytometric analysis are displayed in left hand panel with summary data from the 10 subjects shown in right hand panel. Differences in proliferation across groups was seen ($p<0.005$ by Kruksall-Wallis), with proliferation of $CD4^+CD27^-CD28^{null}$ cells pulsed with CMV lysate significantly increased compared to either control (no antigen), HLA pulsed $CD4^+CD27^-CD28^{null}$ cells, or $CD4^+CD27^-CD28^+$ cells pulsed with CMV lysate ($p<0.01$ for all post hoc comparisons; Mann-Whitney testing with Bonferroni correction). Data displayed as median (bars), interquartile range (boxes) and 10th-90th percentile (whiskers).

$CD4^+CD27^-CD28^{null}$ proliferation in response to CMV antigen was evident in all patients, whereas no response to HLA peptide was observed in any ($p<0.01$). Differences in proliferation across groups was seen ($p<0.005$), with proliferation of $CD4^+CD27^-CD28^{null}$ cells pulsed with CMV lysate significantly increased compared to either control (no antigen), HLA pulsed $CD4^+CD27^-CD28^{null}$ cells, or $CD4^+CD27^-CD28^+$ cells pulsed with CMV lysate ($p\leq0.01$ for all comparisons).

The lack of proliferation of $CD4^+CD27^-CD28^{null}$ cells to HLA peptide was not a reflection of assay sensitivity, as $CD4^+CD27^-CD28^+$ cells from 50% (5/10) of patients demonstrated proliferative responses to HLA-derived peptides ($p=0.01$). Interestingly, there was complete inter-patient concordance between this assay of $CD4^+CD27^-CD28^+$ cell proliferation to HLA-derived peptides and the responses in the IFN-γ ELISPOT assay ("raw" data not shown), with all 5 patients responding in one assay also responding in the other, and all 5 patients unresponsive in one assay being similarly unresponsive in the other (κ-statistic=1.0; $p=0.008$).

3.4.7 Glomerular endothelial cell (GEnC) injury following exposure to $CD4^+CD27^-CD28^{null}$ cells

PBMCs from CMV-seropositive transplant recipients (n=4) were incubated for 16h in media alone (control) or with either CMV lysate or HLA peptide. Thereafter, 10^5 $CD4^+CD27^-CD28^{null}$ or $CD4^+CD27^-CD28^+$ cells were isolated and incubated with GEnC for 6 hours.

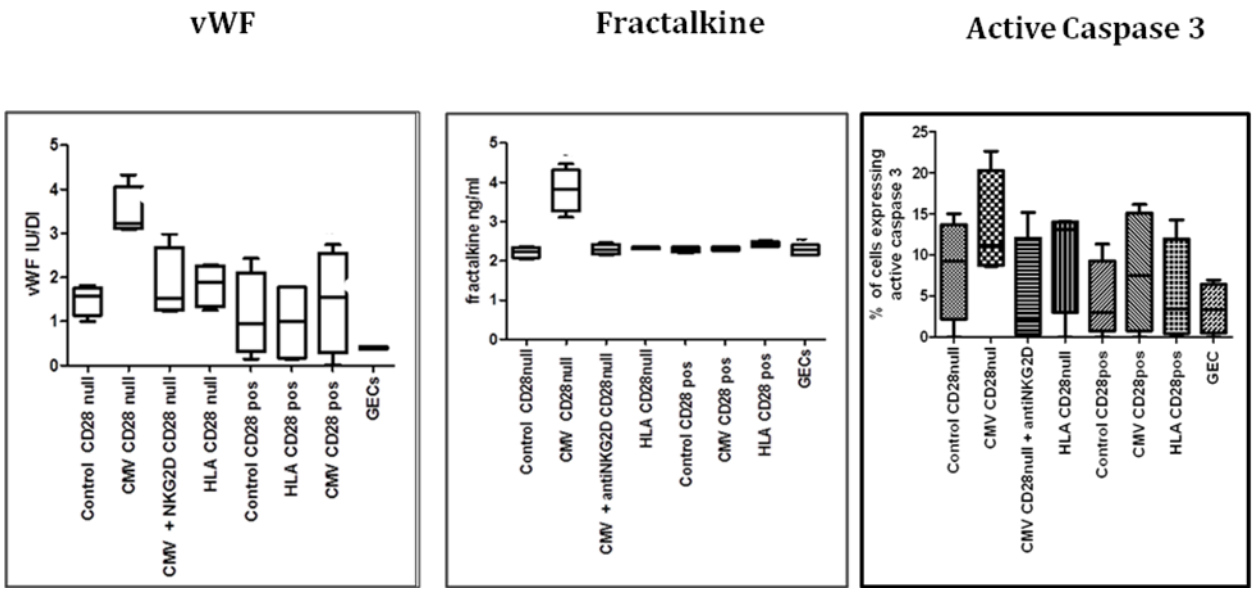


Figure 3.6 Glomerular endothelial cell (GEnC) injury following exposure to $CD4^+$ $CD28^{null}$ cells

Glomerular Capillary Endothelial cells (GEnC) were incubated with 105 $CD4^+CD27^-CD28^{null}$ or $CD4^+CD27^-CD28^+$ cells isolated from CMV positive renal transplant patients (n=4), incubated for 16h in media alone (Control) or stimulated with CMV lysate or HLA peptide prior to sorting. At 6 hours, release of vWF, release of fractalkine, and frequency of GEnCs expressing active caspase 3 was significantly different across experiments (Kruksall-Wallis p=0.001), and specifically increased following prior incubation of $CD4^+CD27^-CD28^{null}$ cells with CMV lysate compared to either control media, HLA peptide ($p<0.05$ for both comparisons in all experiments; Mann-Whitney testing with Bonferroni correction), and also compared with $CD4^+CD27^-CD28^+$ cells pre-treated with either CMV lysate or HLA peptide ($p<0.05$ for both comparisons in all experiments; Mann-Whitney testing with Bonferroni correction). This effect was inhibited by incubation with an NKG2D blocking antibody during and two hours prior to the addition of $CD4^+CD27^-CD28^{null}$ cells ($p<0.05$ for all experiments; Mann-Whitney testing with Bonferroni correction). Data displayed as median (bars), interquartile range (boxes) and 10^{th}-90^{th} percentile (whiskers).

Figure 3.6 demonstrates release of vWF and fractalkine from GEnC, and increased intracellular active caspase 3 expression within GEnC following incubation with $CD4^+CD27^-CD28^{null}$ cells pre-exposed to CMV lysate. This was not seen with $CD4^+CD27^-CD28^{null}$ cells pre-incubated with HLA-derived peptide, nor with $CD4^+CD27^-CD28^+$ cells pre-incubated with either CMV-derived HLA-derived peptide ($p<0.05$ for all comparisons). Of particular note, all effects were attenuated by addition of NKG2D-blocking antibody to GEnC for 2 hours prior to, and then during their incubation with $CD4^+CD27^-CD28^{null}$ cells ($p<0.05$ for all comparisons).

3.4.8 Increased circulating $CD4^+CD28^{null}$ cells are independently associated with delayed graft function and inferior medium-term allograft function

$CD4^+CD28^{null}$ frequencies immediately prior to transplantation were significantly associated with post-operative DGF. This relationship held when adjusted for baseline demographics, including donor/recipient CMV serostatus (multivariate model shown in Table 3.3).

Graft failure was uncommon, and so not investigated as an outcome. However, eGFR at the end of follow up was associated with increased $CD4^+CD28^{null}$ cell frequencies (averaged over follow-up), with 7.3ml/min lower eGFR for each $\log_{10}$ increase in $CD4^+CD28^{null}$ cells in the multivariate analysis which included adjustment for DGF and also for peak hsCRP averaged over time (p=0.03; Table 3.3). No statistical interaction between $CD4^+CD28^{null}$ cells and either DGF or acute rejection was seen for the eGFR endpoint. Results were similar when $CD4^+CD28^{null}$ cell frequencies specifically at 12 months were analysed as the predictor variable (Beta = -7.8 (-14.9, -0.7); p=0.02).

No association was found between $CD4^+CD28^{null}$ cells (averaged over follow-up) and log-transformed UACR at end of follow-up, or between baseline $CD4^+CD28^{null}$ cells and time to acute rejection (final multivariate models shown in Table 3.3).

Table 3.3 Predictors of clinical endpoints

Variable	Category	Effect Size	p-value
eGFR at end of follow up[1]			
$CD4^+CD28^{null}$ count	Expressed as proportion of total CD4 cells; log10 scale	-7.3 (-15.2, -0.6)	0.03
Biopsy-proven acute rejection	Yes (any grade)	-9.9 (-18.8, -0.9)	0.03
Donor Age	Per 10 years	-6.9 (-11.4, -2.4)	0.01
Delayed Graft function[2]			
$CD4^+CD28^{null}$ count	Expressed as proportion of total CD4 cells; log_{10} scale	1.81 (1.11, 2.95)	0.02
Recipient Age	Per 10 years	1.53 (1.04, 2.24)	0.03
Donor Age	Per 10 years	1.39 (1.05, 1.84)	0.03
UACR at end of follow up[1]			
Biopsy-proven acute rejection	Yes (any grade)	3.02 (1.58, 5.81)	0.001
Delayed Graft Function	Yes	2.15 (1.13, 4.12)	0.02
Time to Biopsy Proven Acute Rejection[3]			
Class II HLA Mismatch	Per Antigen	1.93 (1.04, 3.59)	0.04
CMV infection*	Yes	9.91 (2.94, 33.4)	<0.001
dnDSA**	Yes	3.42 (1.69, 6.92)	0.01

Table 3.3 shows final multivariate model describing relationships between predictor variables and clinical outcomes.

Effect size reported as [1] beta coefficient; [2] odds ratios; [3] hazards ratios

*denotes detectable CMV DNAemia
**de novo anti HLA donor-specific antibody

3.5 DISCUSSION

This study represents a detailed, prospective, longitudinal examination of $CD4^{+}CD28^{null}$ cells in unselected kidney transplant recipients. Our work demonstrates $CD4^{+}CD28^{null}$ T-cell expansion is driven by inflammation on a background of latent CMV infection. $CD4^{+}CD28^{null}$ cells isolated from kidney transplant recipients responded to CMV-derived antigens *in vitro*, but not to HLA-derived antigens. Detailed phenotyping of $CD4^{+}CD28^{null}$ cells confirmed their late differentiation status and potential for endothelial adhesion, tissue invasiveness, and cytotoxicity. In addition, exposure of $CD4^{+}CD28^{null}$ cells to glomerular endothelial cells *in vitro* resulted in NKG2D-dependent endothelial cell activation and apoptosis. This mechanism might plausibly explain the association between increased $CD4^{+}CD28^{null}$ cells and important clinical endpoints such as DGF and eGFR 5 years post-transplantation. Taken together these data point to the importance of this cell subset in kidney transplantation, particularly in regard to "indirect" effect of CMV on kidney allograft function and outcome (1-3).

Expansion of circulating $CD4^{+}CD28^{null}$ cells is recognized for CMV-seropositive patients with ESRD (4). The current study extends the available evidence in kidney transplantation, again showing this circulating subset as "pathognomonic" of latent CMV at the time of transplantation. A novel finding is that episodes of significant inflammation are associated with subsequent expansion of $CD4^{+}CD28^{null}$ cells. This resonates with increased $CD4^{+}CD28^{null}$ cell frequencies in CMV-seropositive patients with ESRD (a state of chronic inflammation) (4), and in other autoinflammatory conditions (5-7). In CMV-seropositive recipients, no independent association between $CD4^{+}CD28^{null}$ cell expansion and the development of prior CMV DNAemia was seen, suggesting the latter was not a "pre-requisite" for expansion of

these cells. One plausible explanation for this might be that episodes of inflammation drive "abortive" viral replication, whereby inflammation-induced dendritic cell expression of early viral antigens is not followed by overt viraemia (13), but might nevertheless incite an immune response (in this case CMV-specific $CD4^{+}CD28^{null}$ cells). Regarding this, knowledge of specific viral proteins to which CMV-specific $CD4^{+}$ cells respond would be informative, but is not currently available and was beyond the current study's scope. But of relevance many healthy CMV-seropositive individuals control CMV effectively with a $CD8^{+}$ T-cell repertoire predominantly directed against early-expressed viral proteins, rather than later structural components(19).

The relationship between $CD4^{+}CD28^{null}$ cells and CMV-serostatus is likely more than mere association, as purified $CD4^{+}CD28^{null}$ cells (specifically $CD4^{+}CD27^{-}CD28^{null}$ cells) isolated from patients proliferated to CMV antigens *in vitro*. Importantly, $CD4^{+}CD27^{-}CD28^{null}$ cells demonstrated no evidence of HLA-alloreactivity, this being confined to the CD28-expressing $CD4^{+}CD27^{-}CD28^{+}$ subset. The robustness of this important and novel finding is supported by the perfect concordance between this proliferation assay and IFN-γ release in a "confirmatory" ELISPOT assay, which is specifically relevant because previous studies showed the responding cells in this ELISPOT assay also demonstrate an EM-like $CD4^{+}CD28^{+}$ phenotype (20, 21).

Further detailed immune phenotyping was undertaken on samples available 12 moths post transplantation. And so, although the temporal dynamics of the findings cannot be assessed (and was not a primary aim of the study), we can confirm $CD4^{+}CD27^{-}CD28^{null}$ cells as late-differentiated (expressing CD57), effector memory cells with increased cytotoxic potential

(expressing NKG2D and perforin). They express very low levels of the lymph node homing receptor CD62L, but high levels of the receptor for fractalkine (a chemokine of endothelial origin) along with increased expression of adhesion molecules, collectively suggesting capabilities of endothelial homing and tissue-invasiveness.

Increased $CD4^+CD27^-CD28^{null}$ expression of fractalkine receptor (CX3CR1) makes biological sense regarding CMV cellular immunity, as endothelium is a key site of CMV latency and reactivation (22). However, the current study suggests that an unwanted consequence of CMV immunosurveillance may be development of endothelial injury, which was mediated by $CD4^+CD27^-CD28^{null}$ cells *in vitro*. Preincubation of PBMCs with CMV antigen was required to induce this injury, which was mitigated by addition of NKG2D-blocking antibody. This NK receptor represents an integral component of CMV immunosurveillance and immunoevasion (23), was upregulated on $CD4^+CD27^-CD28^{null}$ cells isolated from patients in the current study, and we propose as an important component of the cytotoxic effects (either protective or pathogenic) of these cells. Indeed HUVEC injury by $CD4^+CD28^{null}$ cells in the context of coronary disease is *independent* of conventional T-cell receptor ligation (9), and so the current study extends these observations specifically to glomerular endothelial injury, highlighting the importance of CMV and NKG2D in the process. Although not specifically addressed in the current study, it is recognized that human endothelial cells express NKG2D ligands (24), and it is likely (albeit unproven) that the same holds for GEnC. These results also align with other broader observations (from non-transplant cohorts) including induction of NKG2D on $CD4^+$ cells exposed to CMV antigen (10, 11), degranulation of NKG2D-expressing $CD4^+$ cells in the presence of endothelial cells (24), endothelial fractalkine release following exposure to $CD4^+$ cells from CMV-seropositive individuals (albeit not further characterised phenotypically) (25),

and CMV-induced endothelial damage in a rodent model which is independent of endothelial infection (26).

The use of GEnC is relevant as these glomerular cells display unique structural and functional properties which are not shared by endothelial cells of different vessels, anatomical locations, or species (27). The conditionally immortalized cells ("ciGEnC") used in this study retain similar morphological and physiological characteristics as primary culture human GEnC (27), and therefore give informative insights into events occurring *in vivo*. Indeed a major component in the pathogenesis of both DGF and chronic allograft dysfunction is endothelial injury (28, 29), and therefore the mechanism of endothelial injury described above may explain the independent associations between $CD4^{+}CD28^{null}$ cell expansion at baseline and the development of DGF, and also between $CD4^{+}CD28^{null}$ cell numbers during the first year post-transplantation and renal function at 5 years post-transplantation. Further insight into this may be gained from detailed histological evaluation, which might demonstrate precise anatomic localization of $CD4^{+}CD28^{null}$ cells, and also further characterize their phenotype, specifically in regard to whether they represent a subpopulation of CD4 tissue-resident memory cells. This will be the focus for future work.

Although sampling was not continued beyond the first year, the intensive protocol and detailed phenotyping conducted during this critical period adds robustness and relevance to these findings, which are novel to transplantation but resonate with the inverse association between circulating $CD4^{+}CD28^{null}$ cells and renal function in a cross sectional study of stable patients with renal vasculitis (6). This link between CMV, inflammation (discussed above) cytotoxic $CD4^{+}CD28^{null}$ cell expansion, endothelial injury and allograft dysfunction may

contribute to explaining the well-recognised but incompletely understood association between inflammation and graft outcome (30, 31), and may even go towards explaining the so-called “transplant paradox” whereby improvements in acute rejection rates have not been mirrored by long-term improvement in graft survival (32).

In summary, this study reveals that expansion of CMV-related, cytotoxic $CD4^{+}CD28^{null}$ cells is an important biomarker for, and potential mediator of, adverse events following kidney transplantation. Interventions to avoid and reduce CMV infection (33) and potentially novel strategies to interfere with $CD4^{+}CD28^{null}$ cell toxicity (34) may result in further improvements in allograft outcome.

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Chapter 4 Immunosenescence in kidney transplantation: relationship with CMV, infection, and neutrophil dysfunction

This chapter has been submitted to the Journal of American Society of Nephrology and is currently undergoing external peer review.

The chapter has been prepared as a manuscript for publication.

4.1 **ABSTRACT**

Increasing human longevity has prompted increasing attention towards the clinical importance of T-cell immunosenescence, and it has been suggested that cytomegalovirus (CMV) infection may drive this phenomenon. In this study we longitudinally and repeatedly examined a cohort of one hundred kidney transplant recipients from the time of transplantation, focussing on identifying the most informative phenotypic markers of immunosenescence, establishing the potential link between CMV and immunosenescence and determining the impact of immunosenescence upon the incidence of infectious complications. We also examined the relationship between T-cell immunosenescence and innate immunity (neutrophil function) *in vitro*. Our results show $CD8^{+}CD28^{null}$ cell frequencies as a robust, plausible and convenient marker of "immunosenescence", with CMV latency and post-transplant CMV infection associated with increased $CD8^{+}CD28^{null}$ cell frequencies. In turn, increased $CD8^{+}CD28^{null}$ cell frequencies were associated with increased risk of clinically-relevant infection episodes post-transplantation, with some evidence of an association with reduced rejection rates; this marker of T-cell immunosenescence was associated with impaired neutrophil migration *in vivo*, suggesting a potential mechanism for these relationships. In summary, this work suggests an important role of CMV in shaping the underlying immune architecture of transplant recipients, and impacting on 'downstream' clinical events. Further understanding of these complex virological and immunological relationships may ultimately lead to informed improvements in clinical care.

4.2 INTRODUCTION

With increases in human longevity, aging of the immune system ('immunosenescence') has received increased attention. Although no consensus definition based on such cell phenotypes has been developed to date, immunosenescence is characterised by accumulation of memory T cells, including late differentiated $CD8^+$ cells which as a response to chronic antigenic stimulation may lose expression of the costimulatory molecule CD28 ('$CD8^+CD28^{null}$' cells) or regain expression of CD45RA (effector memory re-expressing RA; 'EMRA')[1, 2]. Because this effect of chronic antigenic stimulation is quantitatively far greater in the CD8 compartment then the CD4 compartment, a further phenotypic feature of T-cell immunosenescence is an inverted CD4:CD8 ratio. Finally, the accumulation of memory cells relative to naïve cells, coupled with reduced thymic output of naïve cells with aging, may result in an increased memory: naïve cell ratio within the CD8 compartment [1-3].

These markers of immunosenescence have clinical correlates in the general population, and specifically are associated with increased risk for infection and mortality, and a reduced vaccine response [4-8]. Perhaps best-recognised are a series of studies of elderly Scandinavian individuals where a so-called 'immune risk profile' ('IRP'), a key component of which is accumulation of circulating $CD8^+CD28^{null}$ cells, is associated with these adverse clinical sequelae [5, 9-11].

Human cytomegalovirus (CMV) is characterised by lifelong latency following initial infection, and during that time periods of intermittent (but limited) reactivation. CMV elicits a particularly profound CD8 immune response, with expansion of CMV-specific memory CD8

cells, such that in certain individuals up to 50% of circulating CD8 cells may display CMV specificity, and up to 20% may be specific even for a single viral protein, sometimes demonstrating clonal expansion [12]. This dramatic immune response, known as 'memory inflation' is not shared by other chronic viral infections [13, 14], and is also characterised by accumulation of late-differentiated $CD8^{+}CD28^{null}$ and CD8 EMRA subsets as part of this ongoing chronic antigenic stimulation [15].

It has therefore been suggested and increasingly accepted, that CMV infection is a major driver of the immune phenotype characteristic of immunosenescence, and this topic has been the subject of a number of reviews and International workshops [14–18]. It is also realised that children and young adults infected with CMV may also display similar cell phenotypes, thereby extending the concept of immunosenescence from aging *per se* to a more generalised phenomenon [19]. Yet despite this, data within the field of transplantation remains limited. The link between CMV and the immunosenescence phenotype is not clearly established in transplantation, although a recent study demonstrated expansion of $CD8^{+}CD28^{null}$ cells in 11 kidney transplant recipients experiencing primary CMV infection [20]. And whilst the effect of age in determining outcomes of kidney transplantation has been detailed in numerous epidemiological studies, the role of immunosenescence (based on cell phenotype characteristics) has received little attention. Increased frequencies of CD8 EMRA and $CD8^{+}CD28^{null}$ cells at the time of transplantation have been associated with freedom from early biopsy-proven acute rejection [21, 22]; CD8 EMRA frequencies were also associated with (a trend towards) increased risk of graft dysfunction over the long term in prevalent patients [23]. In a cross sectional 'extended phenotype' study, $CD8^{+}CD28^{null}$ cell frequencies were higher in patients with histological lesions of 'chronic rejection' in comparison to those with

drug-free operational tolerance [24]. Surprisingly only one (very recent) study addressed infection as an endpoint, with no association found between $CD8^{+}CD28^{null}$ cell frequencies or CD4:CD8 ratio and episodes of infection [25]. These studies of transplant cohorts understandably addressed the 'transplant-specific' end measures as described; the potential role of CMV in driving the studied markers of immunosenescence was not a focus in most of these particular studies.

Finally, an increasingly recognised phenomenon is immunosenescence of the innate immune system, in particular abnormalities of neutrophils which represent the most abundant circulating immune cell and a first line of defence against pathogens [26, 27]. Yet far less work has been undertaken in this area compared with T-cell immunosenescence, and is lacking in the field of transplantation. Overviews of this subject in transplantation to date have necessarily extrapolated data from non-transplant settings [28-30].

In this study we longitudinally and repeatedly examined a cohort of kidney transplant recipients from the time of transplantation, focussing on identifying the most informative single cell-phenotype marker of immunosenescence, establishing the link between CMV and immunosenescence, and then the relationship between immunosenescence and episodes of infection as an important and relevant endpoint in transplantation. We conclude that $CD8^{+}CD28^{null}$ cells represented the best marker of immunosenescence in the studied cohort, and that pre-transplant CMV latency, and post-transplant CMV infection are associated with such increased $CD8^{+}CD28^{null}$ cell frequencies. Furthermore, increased $CD8^{+}CD28^{null}$ cell frequencies are associated with subsequent episodes of clinically relevant infection and also with impaired neutrophil migration characteristics *in vitro*. This work has informed the

understanding of the risk factors for, biomarkers of, and potential mechanism of immunosenescence and infection in the context of transplantation.

4.3 RESULTS

4.3.1 Clinical outcomes

The clinical cohort is described in Table 4.1; median follow-up from transplantation was 54 months (range 2-60 months). Three patients died (from sepsis), and four lost their grafts (primary non-function, early acute rejection, recurrent disease, late [14 months] acute rejection). For the remaining patients, mean eGFR at the end of follow-up was 52±17ml/min/1.73m^2, and median UACR was 2.6 mg/mmol (IQR:1.0-6.6; range 0.3-280). Twenty-three patients experienced DGF, and twenty-five biopsy-proven acute rejection (≥Banff grade 1).

Clinical and standard laboratory data pertinent to the study was available for all patients at all timepoints; immunophenotyping analyses were complete for >95 of patients (who were alive with graft function) across timepoints; CMV cell-mediated immunity assays were undertaken at 12-months post-transplantation, and were available in 70 patients (some samples unavailable for analysis for technical reasons related to collection and storage).

Table 4.1 Patient demographics and clinical outcomes

Recipient Age	48±14 years
Recipient Sex	55 male
Recipient ethnicity	
White	74
Indo-Asian	15
African-Caribbean	11
Cause of Renal Failure	
Glomerular	29
Cystic	19
Diabetes	14
Hypertension	19
Other	19
Pre Transplant Modality	
Haemodialysis	38
Peritoneal Dialysis	35
Pre-emptive	27
Repeat transplantation	23
Donor Age	47±15 years
Transplant Source	
Deceased donor	56
(DBD[1])	(44)
(DCD[2])	(12)
Live donor	44
Donor-Recipient HLA Mismatch	
Class I (HLA-A+B)	2.1±0.9 Ag
Class II (HLA-DR)	0.9±0.7 Ag
Donor-Recipient CMV Serostatus	
D-R-	25
D-R+	22
D+R+	32
D+R-	21
Biopsy proven Acute rejection	25
Delayed graft Function[3]	23
Cytomegalovirus	
Asymptomatic infection[4]	18
Disease[5]	7
Respiratory Tract Infection	17
Intestinal Infection	5
Fungal Infection	2
Other	20
[Urinary Tract Infection][6]	[46]

[1] Donation after Brain Death

[2] Donation after Cardiac death

[3] Requirement for dialysis within the first week post transplantation

[4] Denotes the development of anti-CMV antibody in patients who were seronegative at the time of transplantation with or without the detection of CMV DNA in blood on either protocol or indication sampling (see text for details)

[5] Denotes the development of symptomatic CMV disease requiring clinical intervention

[6] Urinary Tract Infection shown for completeness, but excluded from analysis due to rationale described in 'methods', i.e. likelihood that UTI is more related to surgical/ureteric/anatomical factors rather than the immunological architecture

4.3.1.1 Immunophenotyping shows $CD8^{+}CD28^{null}$ cell frequencies as a robust marker of "immunosenescence"

Representative flow cytometry results for the investigation of the candidate markers of immunosenescence are shown in Figure 4.1. Cluster analysis using samples from patients 12 months following transplantation is shown in Table 4.2 (similar results for the cluster analyses were observed for other timepoints; data not shown). This revealed $CD8^{+}CD28^{null}$ cell frequency as the most robust marker of immune senescence (Table 4.2). In turn, recipient CMV seropositivity was associated with frequencies of $CD8^{+}CD28^{null}$ cells (p=0.02; Table 4.3). In-vitro T-cell response to the CMV-derived peptide IE-1 was also significantly (p=0.013) associated with the frequency of $CD8^{+}CD28^{null}$ cells. There was no significant association with the CMV-derived peptides pp50 and pp565 (p=0.91, p=0.32 respectively).

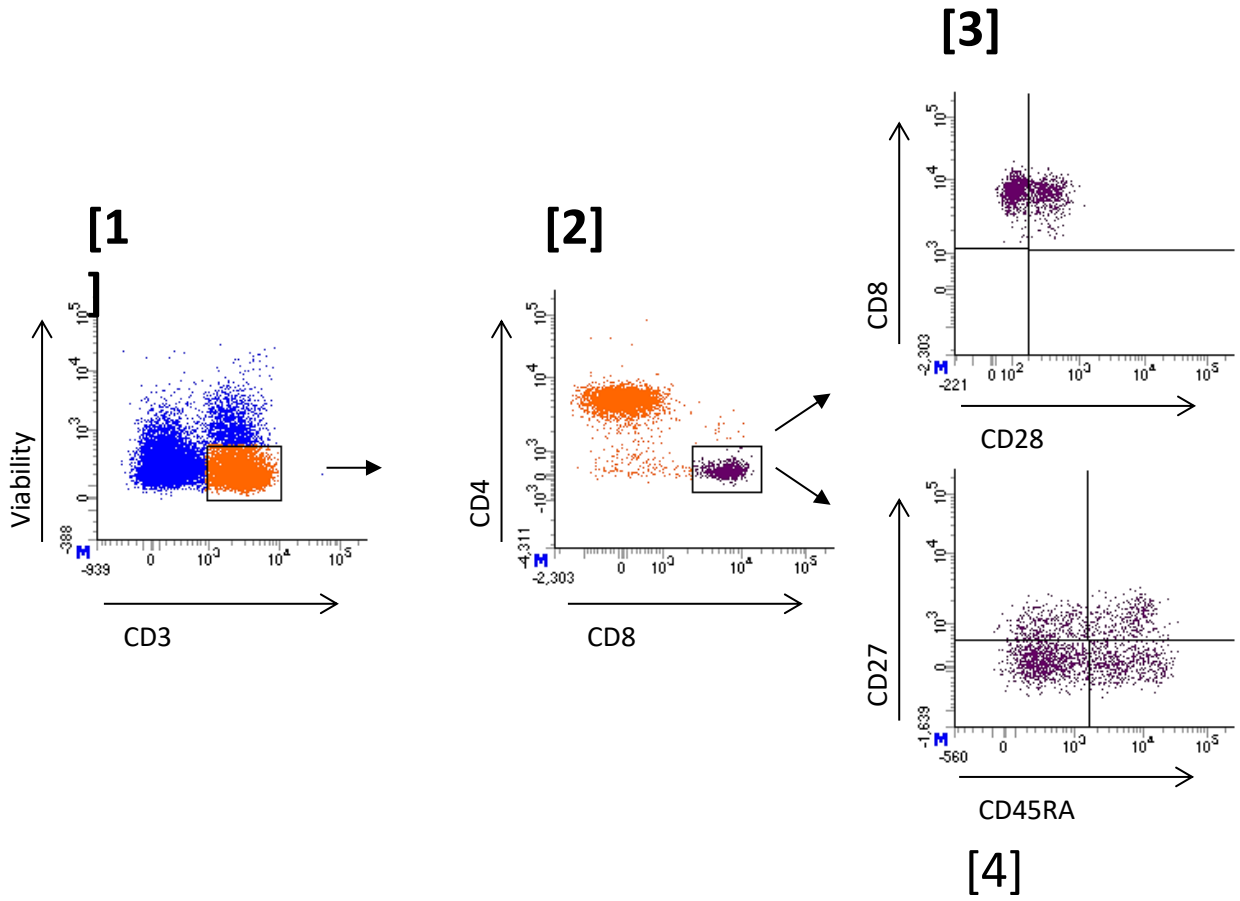


Figure 4.1 Flow cytometry gating strategy for $CD8^{+}CD28^{nulls}$ cells

Representative flow cytometry plots displaying gating strategy to determine cell surface phenotype-based characteristics of putative markers of immunosenescence. Lymphocyte population identified within total PBMC population based on viability and CD3 staining (panel 1). $CD4^{+}$ and $CD8^{+}$ subsets identified from total lymphocyte population based on CD4 and CD8 surface staining (panel 2). $CD4^{+}$:$CD8^{+}$ ratio calculated accordingly (panel 2). Gating for CD28 within $CD8^{+}$ population identifies $CD8^{+}$ CD28null population of interest (panel 3). Identification of $CD8^{+}$ differentiation status based on CD27 and CD45RA surface staining allows calculation of i] $CD8^{+}$ memory: naïve ratio and ii] $CD8^{+}$ EMRA frequencies (panel 4)

Table 4.2. Cluster analysis identifying most relevant marker of immune-senescence

Factor	Relative Importance	Cluster 1 (N=66)	Cluster 2 (N=24)
$CD8^+CD28^{null}$ Freq.[1]	1.00	31.6 (27.2 - 36.5)	6.9 (3.9 - 12.2)
$CD8^+$ TEMRA Freq.[1]	0.68	26.7 (22.0 - 32.4)	7.1 (4.0 - 12.6)
B Cell Freq.[2]	0.62	4.1 (3.1 - 5.5)	0.8 (0.4 - 1.6)
$CD4^+$:$CD8^+$ Ratio	0.30	1.4 (1.1 - 1.7)	2.6 (2.1 - 3.1)
$CD8^+$ Memory:Naïve Ratio	0.20	2.0 (1.5 - 2.6)	1.0 (0.6 - 1.7)

Data shown for 90 patients with immunophenotyping samples available 12 months post transplantation; results reported as Geometric Mean (95% Confidence Interval); [1] $CD8^+CD28$null and CD8 TEMRA frequencies reported as percentage of total CD8 cells; [2] B-cell frequencies reported as percentage of total PBMCs

Table 4.3. Relationship between immunosenescence clusters and CMV antibody and cell-mediated responses.

Factor	Cluster 1 (N=52)	Cluster 2 (N=18)	p-Value
CMV Antibody Positive	44 (84.6%)	10 (55.6%)	0.02
CMV CMI Assays			
IE1	0.08 (0.04 - 0.14)	0.04 (0.00 - 0.08)	0.01
pp65	0.08 (0.04 - 0.21)	0.07 (0.00 - 0.24)	0.32
pp50	0.13 (0.04 - 1.15)	0.11 (0.00 - 1.57)	0.91

Data reported in 70 patients with available samples (immunophenotyping coupled with measures of CMV antibody and cell-mediated immune [CMI] response assays) 12 months post-transplantation; CMI assay results presented as median (interquartile range); p-values from Fisher's Exact test or Mann-Whitney test for antibody and CMI assay results respectively.

4.3.1.2 CMV latency and post-transplant infection are associated with Increased $CD8^{+}CD28^{null}$ cell frequencies

Having established $CD8^{+}CD28^{null}$ frequencies (as a proportion of total CD8 cells) as the marker of immune senescence carrying most 'weight' in the cluster analysis, we proceeded to evaluate the determinants of these cell frequencies over time, paying particular attention in the first instance to the relationship with CMV serostatus.

$CD8^{+}CD28^{null}$ frequencies were normally distributed, and in all tested samples in all patients the mean cell frequencies were 44.7±20.9, expressed as a proportion of total CD8 PBMCs (median 43%; range 8-94%). Table 4.4 demonstrates the evolution of this cell subset in the entire population, and then in subgroups according to donor/recipient CMV-serostatus pairing. The key features of this data are summarised as follows:

i] Pre-transplantation, $CD8^{+}CD28^{null}$ frequencies are higher in transplant recipients who are CMV-seropositive (ANOVA $p=0.001$).

ii] This relationship with recipient CMV-seropositive status is maintained over time (i.e. these differences are observed at all subsequent timepoints; $p<0.01$ for all)

iii] There is a statistically significant, although relatively modest, increase in $CD8^{+}CD28^{null}$ cell frequencies in CMV-seropositive recipients in the first 2 weeks following transplantation, with subsequent stabilisation of cell frequencies at later time points ($p=0.03$ for -/+ and +/+ CMV-serostatus pairings; repeated measures ANOVA)

iv] No such effect is seen in CMV-seronegative recipients of kidneys from CMV-seronegative donors (-/-), where cell frequencies do not change over time.

v] In CMV-seronegative recipients of kidneys from CMV-seropositive donors (+/-), and considering this group in entirety, $CD8^{+}CD28^{null}$ cell frequencies increase over time ($p=0.01$, repeated measures ANOVA). More specifically, cell frequencies do not change over the first 3 months post-transplantation, but then rise by 12 months post-transplantation (post hoc $p<0.01$ for all comparisons with 12-month values)

vi] This effect is related to whether these CMV-seronegative recipients experience CMV seroconversion (i.e. whether they develop clinically evident or subclinical CMV infection). Specifically, in patients not experiencing CMV-seroconversion, cell frequencies do not change over time; serial cell frequencies in this group are no different to those seen in the -/- group. In contrast, CMV-seronegative patients (at the time of transplantation) experiencing subsequent CMV-seroconversion display a marked increase in $CD8^{+}CD28^{null}$ cell frequencies by 12 months post-transplantation, with values not distinguishable from (and in fact numerically slightly higher than) those seen in CMV-seropositive individuals at the equivalent timepoint ($p<0.01$ for all analyses).

Regression analysis was undertaken to evaluate the independent relationship between CMV serostatus and $CD8^{+}CD28^{null}$ frequencies, and to investigate other potential predictors. The results are shown in Table 4.5, and demonstrate that when compared with D-/R- transplantation, the other serostatus combinations were associated with increased cell frequencies. This effect was especially marked in the CMV-seropositive recipients. In addition,

the development of CMV infection (i.e. detectable viraemia with or without clinically evident CMV disease, or in the case of CMV-seronegative recipients evidence of seroconversion at 12 months post-transplant) was also associated with increased $CD8^{+}CD28^{null}$ cell frequencies. These associations both held in the analysis adjusted for the other predictor variables shown in Table 4.5.

Renal function (eGFR) was not modelled in this primary analysis in light of the difficulties with ascribing values to patients with ESRD prior to transplantation. However, secondary analyses in which eGFR values of 0ml/min or 5ml/min were entered for patients requiring dialysis (prior to or early after transplantation) showed no association between renal function and $CD8^{+}CD28^{null}$ cell frequencies (univariate $p>0.05$ for both). In addition, post-transplant delayed renal allograft function and time post-transplant (other possible 'surrogates' for renal function) were associated with increased cell frequencies on univariate analysis in the primary model, but these did not hold in the adjusted model.

Table 4.4 Evolution of $CD8^+CD28^{null}$ cell frequencies according to donor/recipient CMV-serostatus pairing

Donor/Recipient CMV serostatus pairing (at time of transplantation)	Pre-Transplant	Day 14	Month 3	Month 12	p-value [1]
All	37.8 (18.3)	45.8 (22.2)	39.7 (19.4)	47.4 (20.6)	0.05
-/+	42.7 (15.0)	56.7 (19.1)	50.1 (16.5)	54.6 (15.6)	0.03
+/+	46.8 (17.7)	59.0 (21.8)	51.6 (16.5)	54.6 (17.9)	0.03
-/-	28.2 (8.8)	30.8 (12.3)	25.0 (12.8)	28.9 (15.8)	NS
+/-	31.7 (11.9)	33.0 (17.2)	29.2 (11.3)	44.7 (20.5)	0.01
+/- (no seroconversion)	29.9 (11.5)	30.5 (16.9)	28.1 (11.1)	34.2 (16.4)	NS
+/- (seroconversion)	34.3 (12.0)	36.7 (17.7)	30.8 (11.4)	61.8 (13.9)	0.001

$CD8^+CD28^{null}$ frequencies described as a proportion of total $CD8^+$ cells; data normally distributed and presented as mean (standard deviation); [1] repeated measures ANOVA across serostatus/seroconversion groupings

Table 4.5 Regression analysis describing determinants of $CD8^+CD28^{null}$ cell frequencies

	Univariate Analysis		Adjusted Analysis	
Variable	Coefficient (95%CI)	p-value	Coefficient (95%CI)	p-value
Donor/Recipient serostatus D-/R- (baseline risk category) D+/R- D-/R+ D+/R+	 - 13.4 (3.0, 23.8) 24.5 (14.6, 34.5) 28.1 (18.1, 38.1)	 <0.001	 - 10.1 (0.1, 20.) 24.5 (15.1, 33.9) 27.4 (18.0, 36.9)	 <0.001
CMV infection post-transplant	21.4 (14.8, 27.9)	<0.001	22.8 (16.4, 29.2)	<0.001
Delayed Graft Function	10.0 (2.6, 17.4)	0.008		
Time Post Transplant (per month)	0.6 (0.2, 1.0)	0.005		
Prior Transplant	10.9 (-0.4, 22.3)	0.06		
Recipient Age (per 10 years)	2.3 (-0.6, 5.3)	0.11		
Recipient Sex (male)	1.3 (-7.0, 9.7)	0.76		
Dialysis Modality PD (baseline risk category) HD PRE	 - 1.3 (-8.3, 10.9) -8.9 (-20.2, 2.5)	 0.14		
Recipient Ethnicity White (baseline risk category) South Asian Black	 - 9.1 (-1.4, 19.6) 8.8 (-4.6, 22.2)	 0.14		
Biopsy proven acute rejection	3.8 (-2.6, 10.2)	0.25		
HLA Class I Mismatch (per Ag)	1.5 (-2.5, 5.6)	0.45		
HLA Class II Mismatch (per Ag)	0.1 (-5.9, 6.1)	0.98		
Inflammatory Renal Disease	1.9 (-6.8, 10.7)	0.66		
hsCRP (per 10mg/L)	1.03 (0.76, 1.38)	0.87		

Determinants of $CD8^+CD28^{null}$ cell frequencies (expressed as proportion of total $CD8^+$ cells) investigated by linear regression analysis adjusted for repeated measures; PD=peritoneal dialysis; HD=haemodialysis; PRE=pre-emptive transplantation; HLA=human leucocyte antigen; Ag=antigen; hsCRP=high sensitivity C-reactive protein

In summary, the CMV-serostatus of recipients at the time of transplantation, and the development of CMV infection following transplantation, have a profound effect on $CD8^{+}CD28^{null}$ cell frequencies, with strong and independent associations of positive recipient serostatus and subsequent infection and increased cell frequencies.

4.3.1.3 Higher $CD8^{+}CD28^{null}$ cell frequencies are associated with increased risk of infection

A significant number of patients developed clinically-relevant infection following transplantation (Table 4.1). Episodes of infection within 2 weeks of transplantation, and urinary tract infections at any time, were excluded for reasons described in 'methods'. The clinical events of this analysis therefore represented 17 patients experiencing lower respiratory tract infections, 5 with gastrointestinal infections, 2 with fungal infections, and 20 with other miscellaneous infections (these included pilonidal abscess, skin infections, breast abscess and bacterial peritonitis). Asymptomatic cytomegalovirus infection occurred in eighteen patients whilst seven patients developed clinical disease. As discussed in 'methods' these CMV infections were considered potential predictors of infection, rather than episodes of infection in themselves.

On multivariate analysis (Cox model with time-dependent modelling), higher $CD8^{+}CD28^{null}$ cell frequencies were independently associated with increased risk of infection (p=0.005; Table 4.6). Delayed graft function was also associated with increased risk of infection in the final model (p=0.006). Recipients who were CMV-seropositive at the time of transplantation were at increased risk of infection (compared with CMV-seronegative individuals, and irrespective

of donor CMV antibody status) on univariate analysis (p=0.03), but this did not hold in the final model adjusted for cell phenotyping characteristics.

Although $CD8^{+}CD28^{null}$ frequencies were analysed as a time-dependent variable in the formal statistical analysis, a more intuitive representation of the relationship with infection is shown in Figure 4.2, which demonstrates the relationship between mean $CD8^{+}CD28^{null}$ frequencies over the duration of the study and the risk of infection. Higher mean cell frequencies were associated with greater risk of clinically-relevant infection at any time following transplantation when considered as a continuous measure (Figure 4.2A), and with reduced time to infection with mean values above the lowest tertile ($CD8^{+}CD28^{null}$ frequency>30% of total CD8 cells; Figure 4.2B).

Figure 4.2a

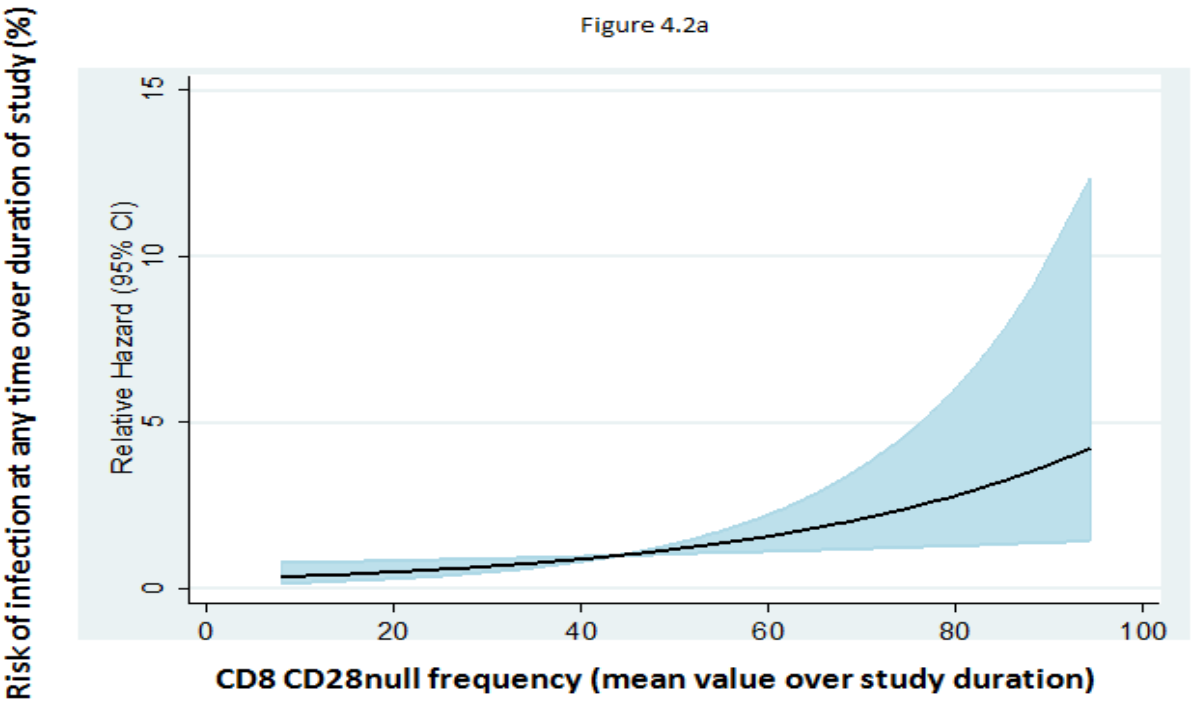


Figure 4.2b

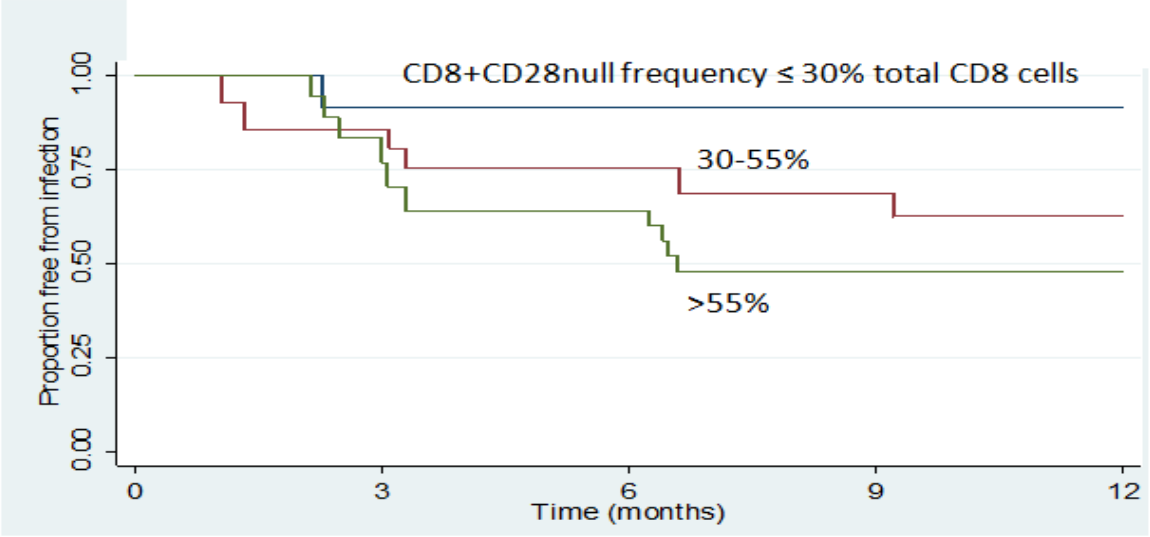


Figure 4.2 $CD8^{+}CD28^{null}$ cell frequencies and risk of clinically relevant infection

Relationship between mean $CD8^{+}CD28^{null}$ cell frequencies (average value for all results in an individual across the duration of the study and expressed as a proportion of total $CD8^{+}$ cells), and the risk of clinically relevant infection at any time during the duration of the study. Figure 4.2A shows the regression line representing the relationship between risk of infection and mean $CD8^{+}CD28^{null}$ cell frequencies. Shaded area represents 95% confidence interval around the point estimate. Average $CD8^{+}CD28^{null}$ cell frequency of 44% (of total PBMC) used as 'baseline' value. Figure 4.2B demonstrates relationship between time to first infection and tertiles of mean $CD8^{+}CD28^{null}$ cell frequencies (averaged across the duration of the study)

Table 4.6 Regression analysis describing relationship between CD8CD28^{null} cell frequencies and time to clinically-relevant episodes of infection following transplantation.

	Univariate Analysis		Adjusted Analysis	
Variable	Hazard Ratio (95%CI)	p-value	Hazard Ratio (95%CI)	p-value
Recipient CMV-seropositive (versus CMV-seronegative)	4.06 (1.17, 14.0)	0.03		
CD8CD28null frequency (per 10% as proportion of total CD8$^+$ cells)	1.33 (1.07, 1.65)	0.009	1.40 (1.11, 1.78)	0.005
Delayed Graft Function	2.65 (1.05, 6.73)	0.04	3.87 (1.48, 10.1)	0.006
Prior Transplant	3.33 (1.18, 9.39)	0.02		
Recipient Age (per 10 years)	1.16 (0.84, 1.62)	0.37		
Recipient Sex (male)	0.86 (0.34, 2.23)	0.76		
Dialysis Modality CAPD (baseline risk category) HD PRE	 - 1.32 (0.44, 3.94) 0.72 (0.22, 2.35)	 0.67		
Recipient Ethnicity White (baseline risk category) South Asian Black	 - 2.17 (0.72, 6.48) 3.86 (1.19, 12.6)	 0.06		
BPAR	2.38 (0.89, 6.34)	0.08		
HLA Class I Mismatch (per Ag)	1.16 (0.71, 1.88)	0.55		
HLA Class II Mismatch (per Ag)	0.96 (0.50, 1.84)	0.90		

Time to first clinically-relevant infection following kidney transplantation investigated by Cox regression adjusted for repeated measures for time-dependent variables; PD=peritoneal dialysis; HD=haemodialysis; PRE=pre-emptive transplantation; HLA=human leucocyte antigen; Ag=antigen; hsCRP=high sensitivity C-reactive protein

4.3.1.4 Higher $CD8^+CD28^{null}$ cell frequencies are associated with impaired neutrophil migration but not with neutrophil function

Higher $CD8^+CD28^{null}$ cell frequencies were associated with impairment of the four neutrophil migration parameters of speed, velocity, chemotactic index, and persistence ($p<0.02$ for all). Figure 4.3 (A-D) shows the relationship between increased $CD8^+CD28^{null}$ cell frequencies and impairment of these 4 parameters of neutrophil migration. Recipient age was not associated with these migration parameters ($p>0.05$ for all).

No association between $CD8^+CD28^{null}$ cell frequencies and either bactericidal function (phagoburst test) or phagocytic function (phagotest) was evident ($p=0.92$ and 0.67 respectively). Neither was there an association between $CD8^+CD28^{null}$ cell frequencies and the production of NETS ($p=0.72$).

Figure 4.3a

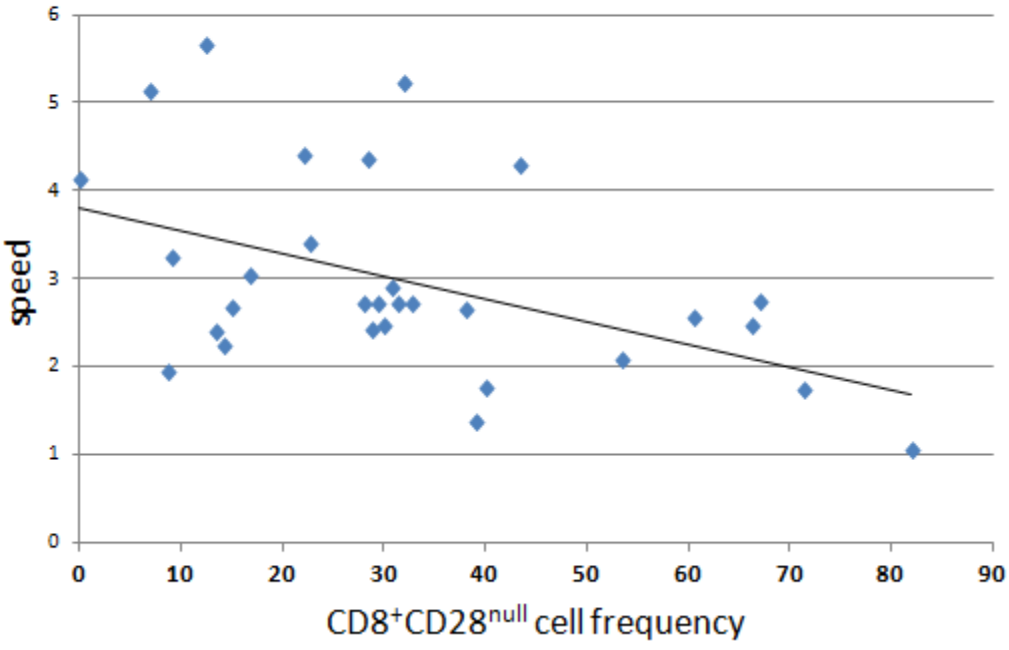


Figure 4.3b

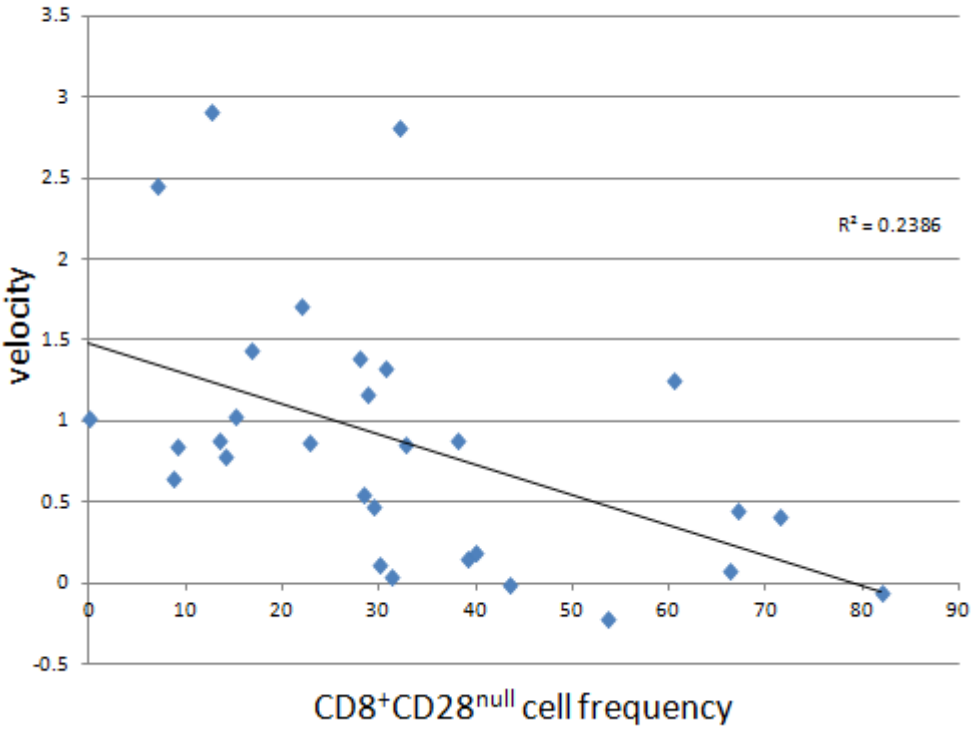
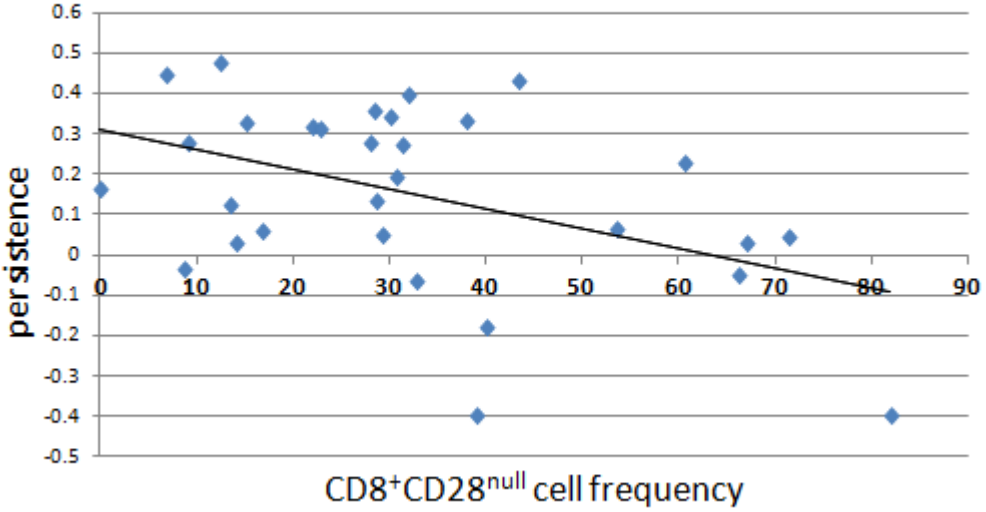


Figure 4.3c



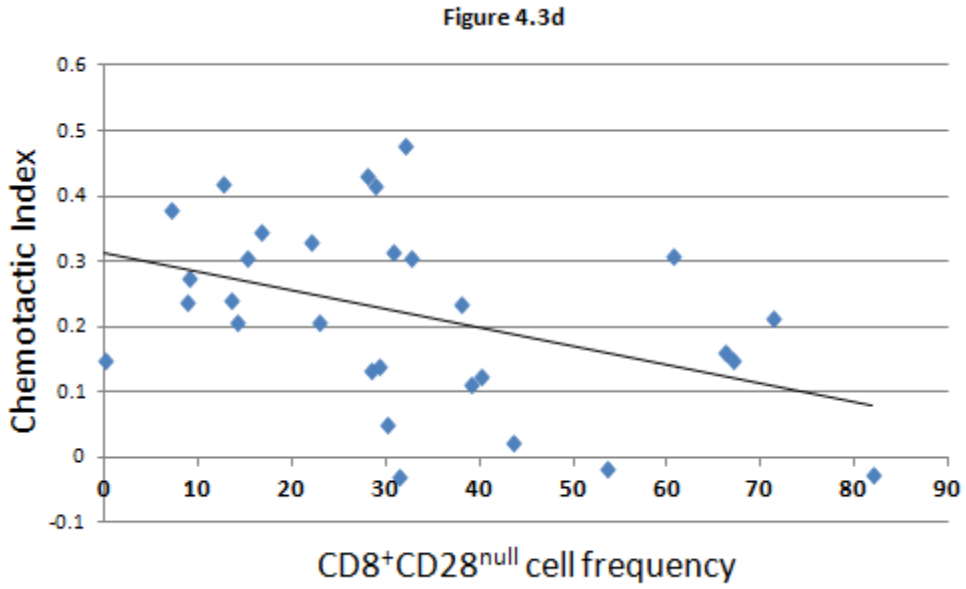


Figure 4.3 $CD8^{+}CD28^{null}$ cell frequencies and *in vitro* neutrophil migration characteristics

Figure 4.3 displays the relationship between circulating $CD8^{+}CD28^{null}$ cell frequencies and *in vitro* neutrophil migration characteristics in a subset of 30 patients studied 20±3 months post-transplantation. Neutrophil migration characteristics were evaluated as (A) average cell speed of the distance travelled between frames in any direction over time, (B) average cell velocity in a consistent direction toward the chemoattractant, (C) persistence, and (D) chemotactic index (accuracy).

4.3.2 Other clinical endpoints

Secondary analyses investigated the relationship between $CD8^{+}CD28^{null}$ frequencies and time to biopsy-proven acute rejection (BPAR), and also with renal function (eGFR) and proteinuria (UACR) at the end of the study. Cox regression revealed some evidence of an effect, with higher cell frequencies associated with lower risk of BPAR (HR: 0.80, 95%CI: 0.62-1.05; p=0.09). For the analysis of eGFR and UACR at the end of study follow-up, linear regression was used with mean $CD8^{+}CD28^{null}$ frequencies over the duration of the study as the predictor variable. No association between cell frequencies and either of these end measures was evident (p=0.98 and p=0.99 respectively).

4.4 DISCUSSION

The increase in human longevity has prompted closer evaluation of the aging immune system, and has resulted in the concept of immunosenescence and its clinical sequelae. However, immunosenescence is not necessarily only relevant to aging populations, but also for younger individuals and those with a variety of chronic conditions. The current study addresses the determinants and consequences of immunosenescence in the context of kidney transplantation, extending the limited data available hitherto. Specifically, we demonstrate the importance of circulating $CD8^{+}CD28^{null}$ frequencies as a valuable marker of immunosenescence in this context. By tracking patients serially, we identify the importance of CMV latency prior to transplantation, and then CMV infection post-transplantation, as major determinants of progressive immunosenescence. We show a temporal association between elevated $CD8^{+}CD28^{null}$ frequencies (the immunosenescence phenotype) and the subsequent development of clinically relevant episodes of infection following kidney transplantation. Finally, we demonstrate dysfunction of the innate immune system (specifically abnormal neutrophil migration) as a potential mechanism by which immunosenescence predisposes to such infective episodes.

There is no established optimal marker for T-cell immunosenescence, and previous studies in the transplant and non-transplant cohorts have used different cell subsets as markers of this phenomenon. We performed cluster analysis to define (at least in our cohort) which cell subset(s) were associated with, and contributed most to the overarching immunosenescence phenotype. Of the previously described markers of immunosenescence (the rationale for which are described in the 'introduction'), it was frequencies of 'late differentiated'

$CD8^{+}CD28^{null}$ cells which contributed most information. Whilst we cannot attest to the generalisability of this specific result, it certainly aligns with data from non-transplant cohorts, where the same subset represents a key component of the immune risk profile of elderly people [10, 11]. It is also this subset that has received attention in transplantation in regard to its potential association with rejection and with tolerance [22, 24]. We therefore contend it is an intuitive and reasonable cell subset to study in regard to the drivers towards, and consequences of immunosenescence in transplantation.

"Indirect' effects of CMV, as opposed to the 'direct' clinical syndrome(s) of CMV infection and disease, are well recognised but ill-understood [31], and the current study suggests that CMV-driven immunosenescence may represent a pathway towards such effect(s). Cluster analysis demonstrated an association between immunosenescence and both antibody and cell-mediated responses to CMV and its peptides. It is recognised that CMV IE-1 peptides, which in vivo are expressed only intracellularly, elicit a particularly strong CD8 response [32], probably resulting from frequent 'abortive reactivation' and repeated presentation of intracellular antigens such as IE-1 [33, 34]. Expansion of cells specific for such 'inflationary' epitopes results in expansion and accumulation of late-differentiated cells, notably including $CD8^{+}CD28^{null}$ cells [35]. Furthermore, when patients in the current study were serially evaluated, it was pre-transplant CMV latency (i.e. recipient CMV-seropositivity) and post-transplant CMV infection that were associated with $CD8^{+}CD28^{null}$ cell expansion. Consequently, we observed $CD8^{+}CD28^{null}$ frequencies rise during the first-year post transplantation, in particular between 3 and 12 months, and especially (but not exclusively) in CMV-seronegative recipients of CMV-seropositive donors. This kinetic is similar to that seen in a previous study by Meijer's [36], although the emphasis there was the lack of improvement

in immunosenescence phenotype post-transplantation, and the role of CMV was not examined. However the same authors more recently went on to describe the expansion of circulating $CD8^{+}CD28^{null}$ cells in 11 kidney transplant recipients experiencing primary CMV infection [20], a finding which aligns with the results from the current study. Whilst we do not suggest that immunosenescence is an entirely CMV-driven process, it is clear that CMV is an important contributor to the phenotype in this context. We found no association between $CD8^{+}CD28^{null}$ cell frequencies and other potential determinants, in particular renal function and age, albeit in a cohort with a mean age of 47 years and a (relatively) narrow range in comparison with previous studies across age groups.

In the next analysis we demonstrated increased $CD8^{+}CD28^{null}$ frequencies were associated with development of clinically relevant episodes of infection following the initial post-transplant period, with a 'dose-dependent' association between this biomarker of immunosenescence and outcome. This effect was independent of other clinically relevant covariates, including CMV-serostatus, which showed an association with infection on univariate analysis, but not in the model adjusted for this biomarker of immunosenescence. We acknowledge the described associations only strictly pertain to the time-course of the study, and cannot state whether the relationship would hold at later times following transplantation. We also accept that episodes of significant infection might have been missed if the patients presented elsewhere to our unit. However, in light of the geographical architecture and follow-up arrangements within our practice, we suggest that most episodes of hospitalisation would be at our parent unit, and it is unlikely that minor ascertainment bias would influence the conclusions of the study. Finally, in regard to the clinical outcome data in the current study, it was interesting to see a relationship between immunosenescence and

reduced risk of biopsy-proven acute rejection. This association did not reach statistical significance in the current study, but is worthy of mention in light of the similar results from Betjes suggesting immunosenescence may also impact on the allo-immune response [21]. We must also highlight the seemingly contrasting results of the current study, and the most recent study from the same group, the latter demonstrating no association between $CD8^{+}CD28^{null}$ frequencies and post-transplant infection [25]. The 2 studies (which together represent the first examinations of the subject) are similar in many regards (year of enrolment; immunosuppression; infection frequency; $CD8^{+}CD28^{null}$ frequencies) that simplifies comparison. But a major difference is that Dedeoglu and colleagues considered all infections (including many mild infections), urinary tract infection (which we excluded with the rationale described previously), CMV viraemia and disease (which we consider a major *determinant of,* rather than a consequence of immunosenescence). Patients with severe rejection, who received lymphodepletion were excluded, thereby removing one source of bias, but perhaps introducing another. We also believe our statistical approach with time-dependent modelling is a more robust and powerful way to investigate such clinical series. Ultimately what seems clear is that further study is required for this important aspect of transplantation.

It is proposed, and widely accepted, that immunosenescence exerts its deleterious consequences as a result of expansion of the circulating CD8 memory compartment, thereby restricting the 'space' for new memory cells ('memory cell attrition'), and also by constraining the naïve CD8 cell compartment [16, 37-39]. But there are conceptual issues, and also clinical discrepancies, which question this paradigm: of the 10^{11} T cells within the human body, only a small fraction circulate within blood [40]; the CMV-associated immune phenotype in blood is not mirrored within lymphoid tissue, the major site of the immune response [41]**;** the

memory response to repeated antigenic challenge may be additive rather than fixed [42], and is characterised by considerable cross-reactivity to varied antigens and epitopes [43]; clinical data from the Leiden Longitudinal Study of unselected individuals aged >85 years demonstrated *lower* (rather than higher) naïve CD8 frequencies were associated with improved subsequent survival [44], and it should also be remembered that reduced naïve cell frequencies was *not* associated with the immune risk phenotype of a general aging population [5, 9-11].

On that basis, we examined the relationship between T-cell immunosenescence and innate immunosenescence, the latter in specific regard to neutrophil function for the first time in transplantation. Innate immunosenescence is characterised by defects in neutrophil migration and (less consistently) neutrophil cytotoxicity [26, 27]. We found no association between $CD8^{+}CD28^{null}$ frequencies and any studied marker of neutrophil degranulation or NET formation, but did find associations with all four studied neutrophil migration characteristics. This is relevant in light of the association between abnormal neutrophil migration *in vitro* and adverse clinical outcome in other settings [45, 46]. Larger series involving repeated neutrophil analysis are now required to expand and refine this pilot data from transplantation. The mechanism for impaired neutrophil migration, whilst outside the scope of the current study, may involve epigenetic changes secondary to chronic neutrophil activation through pattern recognition and cytokine receptors [47, 48], resulting in changes to the PI3 kinase pathway which is crucial for directional migration [49, 50]. Finally, preliminary data suggesting impaired neutrophil migration may be modifiable [50, 51], sets the scene for further investigation in transplantation, where infection is such a key determinant of outcome.

In summary, we suggest an important role of CMV (one component of the 'virome') in shaping the underlying immune architecture, and specifically contributing to the phenomenon of immunosenescence in the context of transplantation. We identify $CD8^{+}CD28^{null}$ cell frequencies as a robust, plausible and straightforward biomarker for this complex phenotype, and demonstrate clinical relevance in its association with transplant-associated infection. We propose the potential importance of neutrophil dysfunction in this context, and envisage that further understanding of these complex relationships will inform and improve clinical care.

4.5 CONCISE METHODS

4.5.1 Clinical Cohort

One hundred unselected recipients of solitary kidney transplants were enrolled into this prospective study during 2009. All patients underwent transplantation and follow-up at Queen Elizabeth Hospital Birmingham. Local ethics committee approval was granted for the study (08/H1204/103), which was performed in accordance with the Declaration of Helsinki.

4.5.2 Demographic data and protocol

Baseline donor and recipient information was collected prior to transplantation (Table 4.1): donor and recipient age, sex and CMV (cytomegalovirus) status, recipient race, recipient cause of renal failure, donor-recipient HLA mismatch (class I and class II), source of transplant (live related; live unrelated; deceased donor following brain death [DBD]; deceased donor

following cardiac death [DCD]); dialysis modality prior to transplantation (pre-emptive versus haemodialysis versus peritoneal dialysis).

Transplantation proceeded provided the cross match between donor and recipient was negative by cytotoxicity and flow cytometry. Immunosuppression regimen was identical for all participants, and consisted of Basiliximab induction followed by maintenance tacrolimus (Prograf; pre-dose level 5-8ng/ml measured by liquid chromatography-tandem mass spectrometry), mycophenolate mofetil (Cellcept; 2g daily) and prednisolone (20mg daily; reducing to 5mg maintenance by 3 months post transplantation).

4.5.3 Clinical data post-transplantation

Data on delayed graft function, acute rejection, infection, estimated glomerular filtration rate (eGFR) and proteinuria evaluation were collected post-operatively and at each clinic review. Delayed graft function (DGF) was defined as dialysis requirement during the first post-operative week; acute rejection was biopsy proven in all cases (BPAR) and classified according to the Banff schema; significant infection was defined as requirement for hospitalisation with clinical symptoms of infection and either microbiological identification or histological/radiological evidence of tissue invasion. We excluded episodes of infection within the first 2 weeks post transplantation, and also urinary tract infections at any time, as we contend such episodes are more related to the anaesthetic and surgical procedure/complications than the overarching competence of the immune system which was the focus of this investigation; estimated glomerular filtration rate (eGFR) was calculated using the 4 variable MDRD (Modification of Diet in Renal Disease) equation; proteinuria was

evaluated by early morning albumin: creatinine measurements on a “spot” urine sample (‘UACR’).

4.5.4 Assessment of CMV serostatus, infection, and disease

Pre-transplant CMV serostatus of the recipients and their donors was collected. CMV prophylaxis with 100 days of valganciclovir was given to the D+R- group only, with dose adjustment for renal function. Serial whole blood samples were taken for CMV DNA PCR in all patients at day 0 (prior to transplantation), and then weekly for first month, twice weekly during months 2-3, monthly between months 4-12. However, the clinical team remained unaware of these results and no changes in clinical management ensued. Additional sampling was undertaken at the time of clinical suspicion of CMV disease, which was then diagnosed according to international guidelines and was based on one or more of the following in association with the finding of CMV viraemia: fever; new onset severe malaise; leucopenia; thrombocytopaenia; hepatitis (alanine transaminase or aspartate transaminase levels greater than twice the upper limit of normal); tissue invasive disease proven by histology. For our laboratory, a copy rate of >500 CMV genome copies/ml of whole blood represents significant CMV viraemia.

Patients who were CMV seronegative at the time of transplantation underwent repeat serological testing at 12 months post transplantation to identify those who have developed asymptomatic infection within the first year, for whom no DNAemia was detected by protocolised testing as above.

Because the aim of this study was to evaluate the relationship between CMV infection and immunosenescence, and then whether the latter predicts episodes of other infections, the development of CMV infection was considered a predictor variable in these analyses, rather than an infective episode *per se*.

4.5.5 Immunophenotyping

Multicolour flow cytometry was used to undertake detailed phenotyping of patients' peripheral blood mononuclear cells (PBMCs) prior to transplantation and then 14 days, 3 months, 6 months, 9 months and 12 months post-transplantation. PBMCs were isolated from heparinised blood of the transplant recipient by density gradient centrifugation using Ficoll-Paque (Fisher, UK) and cryopreserved in fetal calf serum (FCS) containing 10% DMSO. This was performed within 6 hours following collection with lithium-heparin anticoagulation. PBMC samples were stored in liquid nitrogen until analysis.

The following cell types were defined: $CD4^+$T-lymphocytes ($CD3^+/CD4^+$); $CD8^+$T-lymphocytes ($CD3^+/CD8^+$). $CD8^+$ T-lymphocytes were further characterised into the following subsets: Naïve-like: $CD8^+CD27^+CD45RA^+$; Central memory-like (CM): $CD8^+CD27^+CD45RA^-$; Effector memory-like (EM): $CD8^+CD27^-CD45RA^-$; Effector memory cells re-expressing RA ('EMRA'): $CD8^+CD27^-CD45RA^+$. The proportion of $CD8^+$ T-lymphocyte cells lacking CD28 expression ('$CD28^{null}$') was evaluated. In addition, frequencies of B-lymphocytes ($CD19^+$) were assessed. The following panel was used for this evaluation: CD8 AmCyan (BD Biosciences UK), CD4-PerCPCy5.5, CD28Cy7, Fixable Viability Dye eFluor® 450, CD3-APC, CD27-eFluor®780 (eBiosciences UK), CD45RA-AlexaFluor700 (Biolegend), CD19-PE (eBiosciences).

Samples were processed in batches of 5-10, with a healthy blood donor PBMC sample included with each batch as a quality control. Immediately upon thawing samples were washed by dilution with calcium and magnesium-free PBS, pelleted by centrifugation, stained at RmT for 30' in PBS with 2.5% fetal calf serum (Gibco, Paisley, UK), then washed in PBS before running on a FacsDiva flow cytometer (Becton Dickinson, Oxford, UK) and analysed using Infinicyte software (Cytognos, Salamanca, Spain). Gating was performed manually by a single operator within an accredited laboratory, with lymphoid populations defined by forward and side scatter. Throughout, the flow cytometry laboratory and personnel were blinded to the clinical course of the patients within the study.

4.5.6 Assessment of T-cell mediated immune response to CMV

The frequency of circulating CMV-reactive T cells was determined by antigen stimulation and subsequent detection of cytokine production, with the aim to correlate this data with the presence of immunosenescence based on cell phenotyping as above. Briefly, fresh PBMCs were stimulated with CMV-derived peptides for 16 hours. Peptide pools derived from CMV IE-1, pp65 and pp50 proteins were used at a final concentration of 1 µg/ml per peptide (all Alta Biosciences, Birmingham, UK). 10µg/ml Brefeldin A (Sigma-Aldrich, Gillingham, UK) was added to block cytokine secretion after 1 hour of incubation. As a positive control, cells were stimulated with staphylococcus enterotoxin B (SEB) (0.2µg/ml final concentration; Sigma-Aldrich) and unstimulated cells served as a negative control. Cells were then stained with anti-CD4 and anti-CD8 antibodies (BD Biosciences, Oxford, UK), fixed using 4% paraformaldehyde, and permeabilised with 0.5% Saponin. Intracellular Interferon (IFN)-γ was detected with anti-

IFN-γ-FITC (BD Biosciences). Analysis was performed on a Beckton-Dickinson LSRII flow cytometer with FlowJo software.

4.5.7 Neutrophil function analyses

In vitro neutrophil function assays were performed to quantify the ability of neutrophils to i] engulf bacteria (phagocytosis; phagotest), ii] produce reactive oxygen species (phagoburst test), iii] to lay Neutrophil Extracellular Traps (NETS) to kill bacteria extracellularly, and iv] to evaluate the directed migration of neutrophils.

These investigations were conducted in a subset of 30 patients selected from the wider cohort. These patients were 'hand selected' based on the results of the cell phenotyping data from their 12-month samples, specifically to provide a range of immunosenescence characteristics, and in particular a range of $CD8^{+}CD28^{null}$ cell frequencies in light of the results of the cluster analysis at month 12 showing this to be the marker which contributed most to the measures of immunosenescence (see 'results'). Because of this lag time between the first and last enrolled patients reaching 12 months post-transplantation, these neutrophil function analyses were undertaken 20±3 months post-transplantation. To allow correlation between neutrophil function results and $CD8^{+}CD28^{null}$ frequencies, the latter were repeated at the time of neutrophil function testing. None of these selected patients had experienced clinically overt infection or inflammation within the month prior to neutrophil function testing. The mean age of these participants was 47±17years (range 20-70yr); 11 of the 30 participants were women.

4.5.8 Measurement of neutrophil phagocytosis (Phagotest)

The assessment of phagocytosis was performed using the commercial kit Phagotest (BD Biosciences, UK) containing the fluorescein-labelled opsonized Escherichia coli (*E. coli* - FITC). 100µL of heparinised whole blood were cooled in an ice bath for 10 minutes, followed by incubation with 20µL E.coli or control (RPMI) for 10 minutes at 37°C. A negative control was represented by the same suspension incubated at 0°C. 100µL of ice cold quenching solution was added to stop phagocytosis. After two washing steps, erythrocytes were lysed and 200µL of DNA staining solution was added. The samples were analysed within sixty minutes by flow cytometry (BD Accuri C6 Flow Cytometer) and the phagocytic index was determined. The phagocytic index was calculated as the percentage of the cells having ingested bacteria (FITC positive cells), multiplied by the mean fluorescence intensity (MFI) of the FITC positive population and divided by 100.

4.5.9 Measurement of neutrophil bactericidal activity (Phagoburst)

Neutrophil oxidative burst was determined quantitatively with the Phagoburst Kit (BD Biosciences, UK). 100µL of heparinised whole blood were cooled in an ice bath for 10 minutes, followed by incubation with 20µL non-labelled opsonized *E. coli*, 20µL fMLP (peptide *N*-formyl-MetLeuPhe) as low control, 20µL PMA (phorbol 12-myristate 13-acetate) as high control and 20µL of wash solution as negative control for 10 minutes at 37°C. Following the addition of 20µL of substrate solution (dihydrorhodamine (DHR) 123), all tubes were incubated again for another 10 minutes at 37°C. The erythrocytes were removed using lysing

solution and the samples washed and stained with 200µL of DNA staining solution. The percentage of cells having produced reactive oxygen metabolites (on phagocytizing E.coli) as well as their mean fluorescence intensity (MFI) were analysed by flow cytometry.

4.5.10 Quantification of Neutrophil Extracellular Traps (NETS) production

Peripheral blood neutrophils were isolated within two hours from sample collection. The method used has been previously described [52-54]. Briefly, 2% dextran solution was added to heparinized peripheral blood, the leukocyte layer was collected, and neutrophils were purified by Percoll density gradient centrifugation. Giemsa staining (Diff-Qik; Gentaur Europe) and trypan blue staining was used to determine the purity and viability of isolated neutrophils. The purity and cell viability was routinely greater than 97%. The neutrophils were resuspended at 1-5 $x10^6$/ml in RPMI-1640 media (Sigma-Aldrich, UK) containing 2mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin.

The methods used to quantify extracellular DNA in cell-free supernatants have been previously described by Hazeldine et al 2014. 1 x 10^5 neutrophils in assay media were treated with 25nM PMA (Sigma-Aldrich) (treated arm), or assay media (untreated arm) for 3 hours at 37°C in a humidified 5% CO_2 chamber. Post-incubation, 1U/mL of micrococcal nuclease (MNase; Sigma-Aldrich) and 1µM of the cell impermeable DNA binding dye SYTOX Green (Life Technologies, UK) were added to cells in order to digest and stain extracellular DNA, respectively. After a 10-minute incubation in the dark at room temperature, cells were pelleted, and extracellular DNA content was determined in the cell-free supernatant by measuring fluorescence in a BioTek Synergy 2 fluorometric plate reader (NorthStar Scientific

Ltd, Leeds, UK), at an excitation wavelength of 485nm and emission at 530nm. Experiments were performed in quadruplicate, and background fluorescence values of neutrophils treated with assay media alone were subtracted from test samples.

4.5.11 Neutrophil migration assays

Neutrophils were isolated as described above. The isolated neutrophils were resuspended in RPMI-1640 medium containing 0.15% bovine albumin. The method used for this assay has been previously described by Sapey et al, 2014.

An Insall Chamber (Weber Scientific International Ltd., United Kingdom) was used to assess the three parameters of neutrophil migration (speed, velocity, and persistence). Coverslips were sterilised by washing once in 0.4M H_2SO_4, and twice in deionised water followed by coating with 400µl of 7.5% culture tested bovine serum albumin (Sigma-Aldrich). 400µl of neutrophil solution (suspended at 2 x 10^6/mL) was pipetted onto the albumin-coated coverslips, and left to incubate for thirty minutes. The chemotaxis chamber was prepared by washing thrice with 400µl RPMI-1640. After tipping off excess neutrophil solution from the coverslip, the slide was inverted onto the chemotaxis chamber. The chemoattractant well was slowly filled with 60µl of 100nM IL-8 (R&D systems) and RPMI was used for negative control.

The slides were analysed using Leica DMI6000 Video Capture Microscope. One frame every 20 seconds for a total of 36 frames (12 minutes) was taken. The data was analysed using image J software (Wayne Rasband, National Institutes of Health, Bethesda, MD). All analyses were carried out by a single analyst. Four measures of neutrophil migration characteristics were

tested: **i]** chemokinesis (average cell speed of the distance travelled between frames in any direction over time); **ii]** chemotaxis (average cell velocity in a consistent direction toward the chemoattractant) was measured in micrometres per minute; **iii]** accuracy, termed 'chemotactic index', was calculated by the cosine of the angle between the cell's direction and the orientation of the chemoattractant gradient at each time frame forming a vector analysis of movement, expressed in a comparative scale (cs) ranging from -1 to 1, where 1 represents movement directly toward the chemoattractant, and -1 represents movement directly away from the chemoattractant source in all frames; **iv]** persistence represents the duration and distance a neutrophil moves in one direction before stopping or turning. It is calculated by the cosine of the angle between cellular orientations in consecutive frames, expressed with reference to the y direction and expressed as a score between 0 to 1. Cells that tend to move in a straight line, or cells that execute slow changes in direction have a high persistence value (~1); whereas cells that move randomly and rapidly change direction do not (~0); [50].

4.5.12 Statistical analysis

Data are presented as mean ± standard deviation, or as median (interquartile range; IQR). Continuously distributed parametric data was compared using Student's t-test. Multiple independent group comparisons were evaluated by ANOVA; repeated measures ANOVA compared multiple non-independent datasets (in this case due to repeated results from the same patients over time). Categorical data was compared using Chi-square testing.

Cluster analysis was performed to segregate patients into groups based on the values of a range of potential markers of immune senescence. Two-Step cluster analysis in IBM SPSS 19 was used, with the number of clusters determined automatically. Demographic factors were then compared between the resulting clusters, using Mann-Whitney or Fisher's Exact test, as applicable.

The independent relationship between continuously distributed end-measures and potential predictors was evaluated using linear regression analysis. Time to event analysis was undertaken using Cox regression. Non-normal distributed data underwent transformation as necessary prior to analysis. For all analyses variables showing some effect in the univariate analysis ($p<0.15$) were included in a subsequent multivariable analysis, with a stepwise backwards selection procedure to retain only the statistically significant variables ($p\leq0.05$) in the final model.

A feature of the data was that there were several measurements for each subject over the course of the study. To allow for the non-independence of such repeated data, multilevel statistical methods were used for data analysis in the regression models. Two-level models were used with individual measurements nested within patients. This was implemented using the xtnbreg procedure with the Stata statistical software package. For time to event analyses, post-transplant predictor variables, in particular $CD8^{+}CD28^{null}$ cell frequencies, were analysed as time-dependent covariates. For analyses of single continuously-distributed endpoints at study end (specifically renal function and proteinuria), averaged values (over the course of the study) were analysed by linear regression.

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Chapter 5: Transitional B lymphocytes are associated with protection from kidney allograft rejection: a prospective study.

This chapter appears as published in the American Journal of Transplantation and reflects work undertaken as part of my PhD.

Due to the lack of association between CMV and B-cell numbers (including lack of association between CD8+CD28null cells), this chapter was prepared as a manuscript for publication and focused on the positive findings of the study.

Transitional B lymphocytes are associated with protection from kidney allograft rejection: a prospective study.

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5.1 ABSTRACT

Recent cross-sectional studies suggest an important role for transitional B-lymphocytes ($CD19^{+}CD24^{hi}CD38^{hi}$) in promoting transplant tolerance, and protecting from late antibody mediated rejection. However prospective studies are lacking. This study enrolled 73 de novo transplant recipients, and collected serial clinical, immunological and biochemical information over 48±6 months. Cell phenotyping was conducted immediately prior to transplantation, and then on 5 occasions during the first-year post-transplantation. When modelled as a time-dependent covariate, transitional B-cell frequencies (but not total B cells or "regulatory" T cells) were associated with protection from acute rejection (any Banff grade; HR:0.60;95%CI:0.37-0.95; p=0.03). No association between transitional B-cell proportions and either de novo donor-specific or non-donor-specific antibody (dnDSA; dnNDSA) formation was evident, although preserved transitional B-cell proportions were associated with reduced rejection rates in those patients developing dnDSA. Three episodes of antibody mediated rejection (ABMR) occurred, all in the context of non-adherence, and all associated with *in vitro* anti-HLA T-cell responses in an Elispot assay (p=0.008 versus antibody-positive patients not experiencing ABMR). This prospective study supports the potential relevance of transitional ("regulatory") B cells as a biomarker and therapeutic intervention in transplantation, and highlights relationships between humoral immunity, cellular immunity, and nonadherence.

5.2 INTRODUCTION

While the immunoregulatory properties of B lymphocytes have been recognised for many years (1,2) their importance in human autoimmune and allo-immune disease has developed more recently. A subset of IL-10 secreting cells with regulatory properties has been identified within the transitional B-cell compartment, characterised by the surface phenotype $CD19^{+}CD24^{hi}CD38^{hi}$, which may protect from autoimmune disease such as systemic lupus and multiple sclerosis (3,4). In kidney transplantation, the importance of $CD19^{+}CD24^{hi}CD38^{hi}$ cells is highlighted by patients who are "tolerant" to their graft, who display increased frequencies of these cells (5-8), although proof as to their direct role in this phenomenon is lacking. Following these landmark studies, 2 subsequent case-control, cross-sectional investigations (9,10) revealed associations between reduced $CD19^{+}CD24^{hi}CD38^{hi}$ frequencies and transplant rejection, albeit mainly in the context of antibody-mediated rejection (ABMR) many years following transplantation.

Although these reports highlight the potential importance of these cells in clinical transplantation, prospective studies are required to track the evolution of these cells post-transplantation, and to evaluate the temporal relationship with subsequent rejection episodes. In the current study we prospectively followed an incident kidney transplant cohort, with sequential, frequent clinical monitoring and immuno-phenotyping to track the temporal relationship between these regulatory, transitional B cells and clinical events. This showed that patients displaying higher transitional B-cell frequencies experience reduced rejection rates, suggesting potential utility of these cells as biomarkers and stratifiers predictive of outcome, and for targeting regulatory pathways to improve transplant outcomes.

5.3 MATERIALS AND METHODS

Seventy-three unselected recipients of solitary kidney transplants were enrolled into this prospective study during 2009 in a single centre. Local ethics committee approval was granted for the study (08/H1204/103), which was performed in accordance with the Declaration of Helsinki.

5.3.1 Demographic data and protocol

Information collected included (Table 5.1): donor and recipient age, sex and CMV (cytomegalovirus) status, recipient cause of renal failure, donor-recipient HLA mismatch, source of transplant (live related; live unrelated; deceased donor following brain death [DBD]; deceased donor following cardiac death [DCD]).

Transplantation proceeded provided the cross match between donor and recipient was negative by cytotoxicity and flow cytometry. Immunosuppression was identical for all participants, and consisted of Basiliximab induction followed by maintenance tacrolimus (Prograf; pre-dose level 5-8ng/ml), mycophenolate mofetil (Cellcept; 2g daily) and prednisolone (20mg daily; reducing to 5mg by 3 months post transplantation). Valganciclovir prophylaxis (100 days) was administered to CMV seronegative recipients of seropositive donors; co-trimoxazole prophylaxis was administered to all for 12 months, and isoniazid prophylaxis for 12 months to those of South-Asian ethnicity or previous tuberculosis.

Table 5.1. Patient demographics

Recipient Age	49±14 years
Recipient Sex	39 male
Recipient ethnicity	
White	54
Indo-Asian	12
African-Caribbean	7
Cause of Renal Failure	
Glomerular	23
Cystic	12
Diabetes	10
Hypertension	14
Other	14
Pre Transplant Modality	
Haemodialysis	28
Peritoneal Dialysis	25
Pre-emptive	20
Repeat transplantation	17
Median peak PRA (range)	4% (0-89%)
Donor Age	46±14 years
Transplant Source	
Deceased donor	41
(DBD[1])	(34)
(DCD[2])	(7)
Live donor	32
Donor-Recipient HLA Mismatch	
Class I (HLA-A+B)	2.2±0.9 Ag
Class II (HLA-DR)	1.0±0.7 Ag
Donor-Recipient CMV Serostatus	
D-R-	17
D-R+	17
D+R+	23
D+R-	16
Biopsy Proven Acute Rejection	17
Delayed Graft Function[3]	15

[1] Donation after Brain Death
[2] Donation after Cardiac death

5.3.2 Clinical data post-transplantation

Delayed graft function was defined as dialysis requirement during the first post-operative week; acute rejection was biopsy proven in all cases (BPAR) and classified according to the

Banff schema; significant infection was defined as requirement for hospitalisation with clinical symptoms of infection and either microbiological identification or histological/radiological evidence of tissue invasion; estimated glomerular filtration rate (eGFR) was calculated using the 4 variable MDRD (Modification of Diet in Renal Disease) equation; proteinuria was evaluated by early morning albumin: creatinine measurements on a "spot" urine sample.

5.3.3 HLA antibody screening

For evaluation of anti-HLA antibodies, samples were taken prior to transplantation, and then weekly for first month, twice weekly during months 2-3, monthly between months 4-12, and then three monthly thereafter. Antibody screening was undertaken using mixed screen beads; all screen positive samples were tested for HLA specificity using single antigen beads (One Lambda). HLA Fusion software was used to obtain raw median fluorescence intensity of different alleles, with a positive cut-off defined as MFI ≥ 500 as per manufacturer's instructions.

5.3.4 Cellular Immunophenotyping

Multichannel flow cytometry of patients' peripheral blood mononuclear cells (PBMCs) was undertaken prior to transplantation (within 6 hours before the procedure, and prior to immunosuppression dosing) and then 14 days, 3 months, 6 months, 9 months, and 12 months post-transplantation. PBMCs were isolated from heparinised blood of the transplant recipient by density gradient centrifugation over Ficoll using RPMI-1640 medium (Sigma) and

cryopreserved in fetal calf serum (FCS) containing 10% DMSO. This was performed within 6 hours following collection with lithium-heparin anticoagulation. PBMC samples were stored in liquid nitrogen until analysis (interval approximately 10-24 days)

The following flow cytometry panel was used to define: B-lymphocytes ($CD19^{+}$); transitional ("regulatory") B lymphocytes ($CD3^{+}CD19^{+}CD24^{hi}CD38^{hi}$); memory B lymphocytes ($CD19^{+}CD27^{+}$); and by way of comparison regulatory T-lymphocytes ($CD3^{+}CD4^{+}CD25^{+}$CD127low) (all antibodies from Biolegend, London, UK). Samples were processed in batches of 5-10, with a healthy blood donor PBMC sample included with each batch as a quality control. Immediately upon thawing samples were washed by dilution with calcium and magnesium-free PBS, pelleted by centrifugation, stained at RmT for 30' in PBS with 2.5% fetal calf serum (Gibco, Paisley, UK), then washed in PBS before running on a FacsDiva flow cytometer (Becton Dickinson, Oxford, UK) and analysed using Infinicyte software (Cytognos, Salamanca, Spain). Gating was performed manually by a single operator (JG) within an accredited laboratory, with lymphoid populations defined by forward and side scatter. The healthy donor control was used to set the gate for transitional B-cell frequency evaluation. Throughout, the flow cytometry laboratory and personnel were blinded to the clinical course of the patients within the study.

5.3.5 Enzyme Linked Immunosorbent Spot Assay to detect anti-HLA cellular response

A γ-interferon ELISPOT assay to evaluate the cellular immune response of transplant recipients to non-polymorphic HLA-derived peptides was undertaken as previously described

(11-13). The responding cells in this assay are effector-memory $CD4^+$ cells (12,13) which recognise cryptic, autologous HLA-derived peptide epitopes, such that inter-patient standardisation can be undertaken without using distinct HLA peptides tailored to the donor and recipient HLA type. PBMCs were isolated from peripheral blood by Ficoll density gradient centrifugation and immune responses to peptides at 11.4μmol/L were assayed in triplicate using $4x10^5$ PBMCs per well in a 96-well ELISPOT plate (Mabtech, Nacka Strand, Sweden) and cultured for 44 hours at 37^0C and 5%CO_2. Plates were developed according to manufacturer's instructions and the number of spots per well counted using an AID ELISPOT plate reader (Strassberg, Germany). A positive response was defined as >7 spots per well in single peptide based studies which was >99.8th centile when responses to a wide range of HLA-derived peptides are analysed. (This is also the definition of a positive response to tuberculin-derived peptides using similar methodology).

5.3.6 Statistical analysis

Data are presented as mean ± standard deviation, or as median (interquartile range; IQR). Continuously distributed data was compared using Student's t-test with multiple comparisons evaluated by one-way ANOVA followed by Tukey's Multiple Comparison Test; categorical data was compared using Chi-square and Fisher's exact testing.

Time to event analyses was undertaken using Weibull regression, an example of a parametric survival analysis. This is preferable to the more commonly used Cox regression (semi-parametric) analysis when exposure variables are absent at early timepoints (14). In the case of the current investigation this pertained to de novo anti-HLA antibodies which (by

definition) appeared sometime after transplantation. Repeated measures of immunophenotyping results and HLA antibody results were modelled as time-dependent covariables. The endpoints of UACR and eGFR at the end of follow up were analysed using linear regression analysis. Averaged values for the immunophenotyping results were used for these analyses, and other time-dependent variables such as biopsy-proven acute rejection and the presence of dsHLA antibodies were modelled as categorical (yes/no) variables. Non-normal distributed data underwent transformation as necessary prior to analysis. For all analyses variables showing some effect in the univariate analysis ($p<0.15$) were included in a subsequent multivariable analysis, with a stepwise backwards selection procedure to retain only the statistically significant variables ($p\leq0.05$) in the final model. Due to collinearity specifically between transitional B-cell frequencies and total numbers, only cell frequencies were analysed as a predictor variable to avoid statistical error.

5.4 RESULTS

5.4.1 Clinical Outcomes

Mean follow up was 48±6 months. 15 patients experienced DGF, and 17 developed (BPAR) at a median of 15 days post-transplantation (range 7-450 days). Of these, 3 patients displayed biopsy features of microcirculation inflammation (glomerulitis, peritubular capillaritis and C4d deposition) in the presence of donor-specific anti HLA antibody. Over the course of follow-up there were 3 deaths (all sepsis) and 3 further graft failures (antibody mediated rejection n=2; recurrent disease n=1).

5.4.2 Transitional B cells and Biopsy-Proven Acute Clinical Rejection

Figure 5.1 shows a representative plot demonstrating transitional B cells as a $CD19^{+}CD24^{hi}CD38^{hi}$ population; Figure 5.2 shows the evolution of transitional B-cell frequencies (expressed as a percentage of total B cells) over the first year post-transplantation year. Significant differences in transitional B-cell frequencies were evident across time points (ANOVA $p<0.001$), which represented a decrease from pre-transplant levels of 3.66±2.30% to 1.49±1.60% at 3 months post-transplantation (post hoc $p<0.001$), and then a rise to 2.66±1.82% by 6 months post-transplantation ($p=0.02$ compared with 3 months), and then stabilisation.

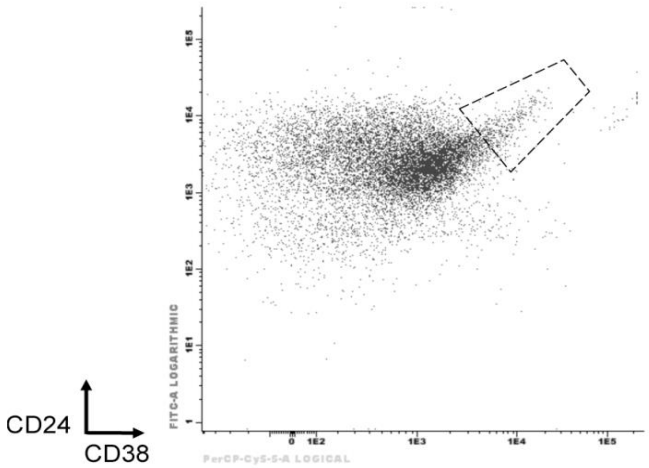

Figure 5.1 Flow cytometry readout of $CD24^{hi}CD38^{hi}$ transitional cell population within $CD19^{+}$ B lymphocytes.
Lymphocytes were gated according to forward and side scatter, then $CD19^{+}$ cells were analysis for CD24/CD38.

Univariate analysis, with transitional B cells modelled as a time-dependent covariate, revealed a significant association between transitional B cells and the subsequent development of BPAR of any grade, with increased transitional B-cell frequencies protective from BPAR (Hazard Ratio [HR]: 0.60; 95% CI: 0.37-0.95; p=0.03).

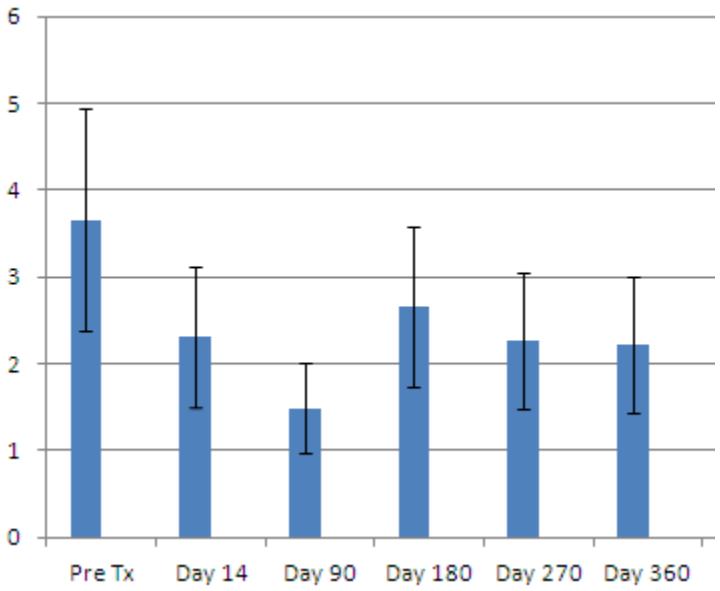

Figure 5.2 Evolution of transitional B cells over time.
Transitional B-cell frequencies expressed as percentage of total $CD19^+$ B-cell population. Bars represent standard deviation. Significant differences in transitional B-cell frequencies were evident across time points (ANOVA $p<0.001$).

This relationship held in the multiple regression model following adjustment for the variables shown in Table 5.2, and indeed was the only predictor of acute rejection in the final model. The relationship between transitional B-cell frequencies and BPAR is shown in different ways in Figure 5.3. Figure 5.3A represents the regression plot for the relative hazard of BPAR versus transitional B-cell frequencies (derived from the statistical analysis and modelling cell

frequencies as a time-dependent variable); Figure 5.3B displays time-to-BPAR survival estimates based on tertiles of transitional B cells.

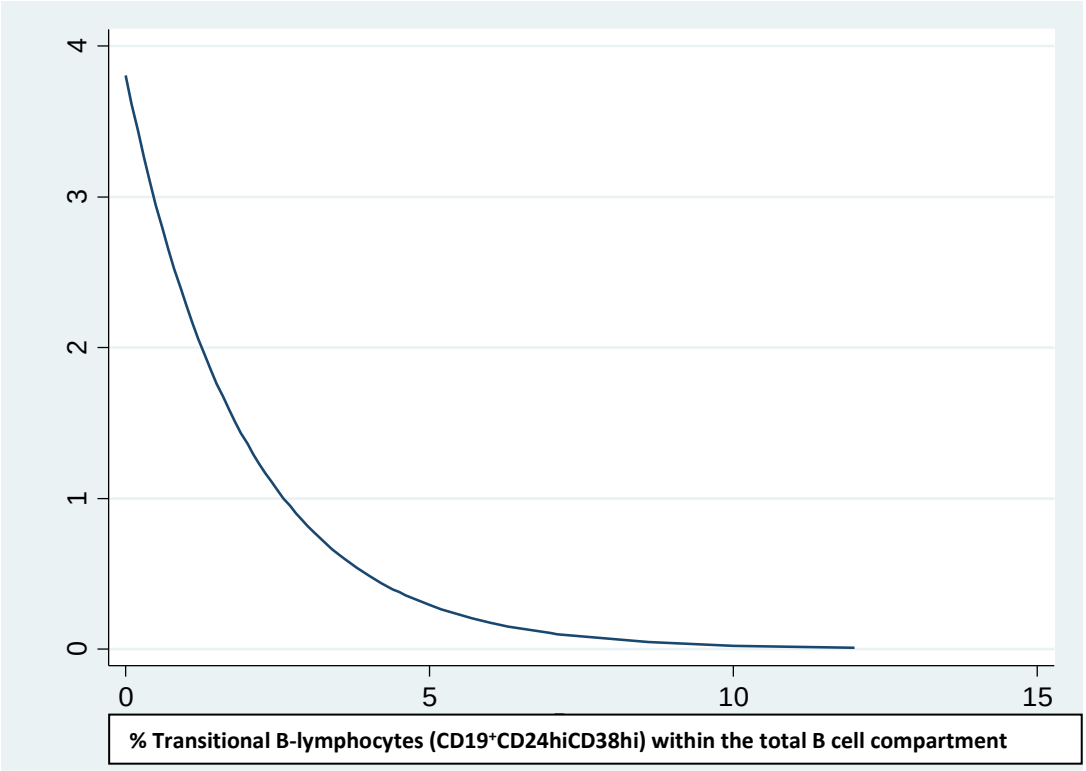

Figure 5.3A Risk of BPAR versus transitional B-cell frequency

B-cell frequency expressed as percentage of total $CD19^+$ B-cell population. Regression line represents output from Weibull model, with cell frequencies modelled as a time-dependent variable and model adjusted for repeated measures.

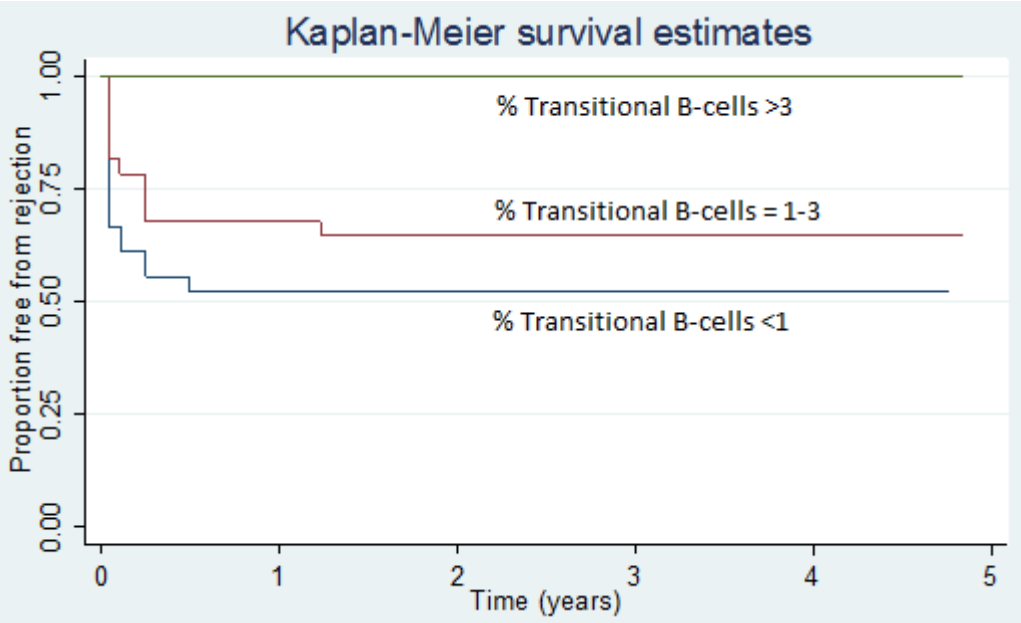

Figure 5.3B Rejection-free survival estimates for tertiles of transitional B cells (log rank p=0.02)

cell frequencies averaged over follow-up; Figure 5.3C demonstrates increased transitional B-cell frequencies in non-rejectors compared to rejectors when sampled immediately pre-transplant and 14 days post-transplantation (the latter data applying to patients experiencing BPAR beyond day 14; n=9).

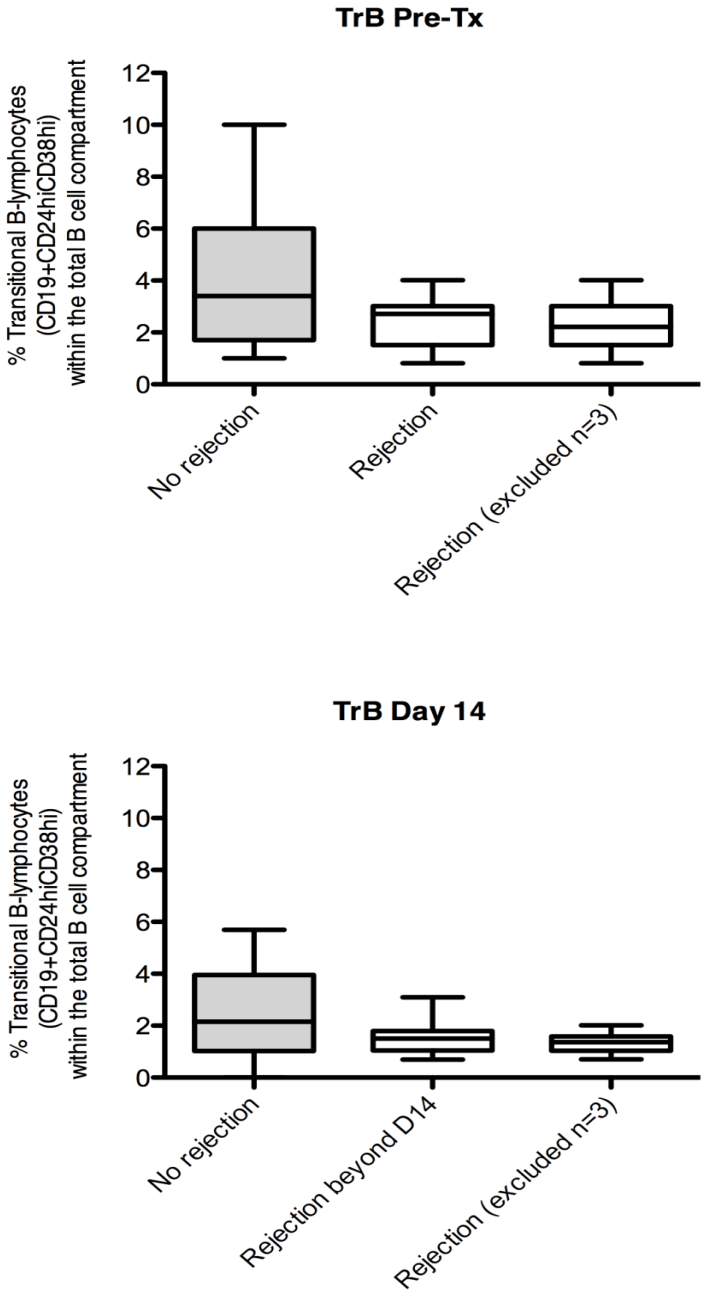

Figure 5.3C Transitional B-cell frequencies at baseline and 14 days post-transplant in the presence and absence of rejection.

Transitional B-cell frequencies at baseline and Day 14 post-transplantation in patients with and without episodes of biopsy-proven acute rejection. For the latter group, acute rejection episodes beyond day 14 are considered events. Data shown as median (line), interquartile range (box) and range (whiskers). Non-rejectors displayed higher transitional B-cell frequencies than rejectors at both timepoints, including comparison with rejectors following exclusion of patients with non-adherence (n=3) ($p<0.05$ for all rejection versus non-rejection analyses).

Table 5.2 Association between predictor variables and time to biopsy-proven acute rejection

	Univariate analysis		Multiple regression model	
Variable	**Hazard ratio (95% CI)**	**p-value**	**Hazard ratio (95% CI)**	**p-value**
Transitional B cells [1]	0.60 (0.37-0.95)	0.03	0.60 (0.37-0.95)	0.03
Recipient Ethnicity (versus white)		0.03		
South Asian	0.74 (0.16-3.38)			
African-Caribbean	3.92 (1.33-11.6)			
dnDSA	2.72 (0.59-12.6)	0.20		
dnNDSA	1.58 (0.49-5.09)	0.65		
Recipient Age [2]	1.06 (0.76-1.48)	0.74		
Recipient Male	0.48 (0.18-1.24)	0.13		
Donor Age [2]	0.99 (0.59-1.64)	0.95		
Repeat Transplant	1.59 (0.46-5.53)	0.47		
Dialysis Modality (versus haemodialysis)		0.39		
Peritoneal-	0.58 (0.16-2.16)			
Pre-emptive-	1.39 (0.47-4.14)			
HLA Mismatch				
Class I	1.41 (0.84-2.39)	0.20		
Class II	1.50 (0.79-2.87)	0.22		
CMV serostatus (versus D-/R-)		0.82		
D-/R+	1.42 (0.38-5.28)			
D+/R+	1.10 (0.31-3.88)			
D+/R-	0.64 (0.12-3.51)			
Total B cells [3]	1.11 (0.84-1.45)	0.46		
Memory B cells [4]	1.21 (0.94-1.56)	0.19		
"Regulatory" T cells [5]	1.09 (0.92-1.31)	0.33		

Table 5.2 demonstrates association between predictor variables and time to biopsy-proven acute rejection (Weibull regression). Multiply-sampled data modelled as time-dependent covariate, and model adjusted for repeated measures.

[1] Effect shown for each percentage increase in transitional B cells expressed as a proportion of total B cells

[2] Effect shown per 10-year increase in age

[3] Effect shown for each 5% increase in B cells expressed as a proportion of total mononuclear cells
[4] Effect shown for each 5% increase in memory B cells expressed as a proportion of total $CD19^+$ cells

[5] Effect shown for each percentage increase in regulatory T cells expressed as a proportion of total $CD3^+CD4^+$ cells

dnDSA=de novo donor-specific anti HLA antibody detection

dnNDSA=de novo non-donor-specific anti HLA antibody detection

No association with time to BPAR was seen with time-dependent measurements for frequencies of “regulatory” T cells (expressed as a proportion of total T cells), total B cells (expressed as a proportion of total mononuclear cells), or memory B cells (expressed as a proportion of total $CD19^+$ cells). Nevertheless, it should be noted that the confidence intervals around the point estimates for the effect of these variables was such that an effect could not be excluded.

5.4.3 Transitional B cells in patients developing dnDSA and ABMR

Prior to transplantation, only 4 patients displayed anti-HLA donor specific antibodies (DSA) above the pre-defined cut off (MFI>500; flow cytometry crossmatch negative as per unit protocol described above); none developed BPAR. During follow-up, 10 patients developed dnDSA, 23 patients developed dnNDSA, and 9 developed both dnDSA and dnNDSA.

No relationship between time-dependent transitional B-cell frequencies and time to appearance of either dnDSA (HR: 1.09; CI: 0.85-1.41; p=0.50) or dnNDSA (HR: 0.88; 95%CI: 0.70-1.12; p=0.30) was seen. When adjusted for the covariates shown in Table 5.2 (and also including BPAR as a predictor variable), HLA-DR mismatch was the sole independent predictor of time to dnDSA appearance (HR per mismatch: 2.01; 95%CI: 1.12-3.60; p=0.008); independent predictors of time to dnNDSA were prior development of dnDSA (modelled as a time-dependent covariate; HR: 13.6; 95%CI: 4.42-41.5; p<0.001) and repeat transplantation (HR: 3.73; 95%CI: 1.33-10.5; p=0.01).

As described above, of the 10 patients who developed dnDSA, 3 developed graft dysfunctions with microvascular inflammation on biopsy, and 7 retained stable function. Although no association between the development of dnDSA and rejection of any grade was seen (Table 5.2), there was a strong association between the development of dnDSA and microvascular inflammation (HR: 7.23; 95%CI: 1,37-38.1; p=0.02). The characteristics of the patients with dnDSA who either did or did not experience graft dysfunction (i.e. "antibody mediated rejection"; ABMR) differed markedly. All 3 patients with ABMR were nonadherent to prescribed medication, whereas none of the 7 rejection-free patients were nonadherent. Interestingly, at the time of antibody development all 3 patients developing ABMR displayed a positive response in the Elispot test (measuring "indirect" cellular immunity), but none of the 7 rejection-free patients displayed such a response (Fisher's p=0.008). At the end of follow up 2 of the 3 patients with ABMR had experienced graft failure, and a marked reduction in eGFR (30ml/min) was seen in the other. In contrast all 7 non-rejecting patients displayed stable graft function with eGFR 60.1± 17.1ml/min and urine albumin: creatinine ratio 2.0±1.5 mg/mmol at end of follow-up.

Transitional B-cell frequencies were not associated with development of ABMR in these 3 nonadherent patients. But following exclusion of these 3 patients, the relationship between transitional B-cell frequencies and rejection held for patients with dnDSA (p=0.3 for interaction), suggesting that preserved transitional B cells is also associated with protection from rejection even in patients with dnDSA. As shown in Table 5.3, up to day 90 post-transplantation transitional B-cell frequencies were similar in rejection-free patients who did and did not develop dnDSA, and in fact were then higher in the former group at later timepoints (ANOVA p=0.005; post hoc p<0.05 for timepoints beyond 90 days).

Table 5.3 B-cell frequencies in patients with and without dnDSA and no BPAR

	dnDSA+; No Rejection	dnDSA-; No Rejection	p-value
Pre-Transplant	4.2±2.3	4.0±2.2	0.76
Day 14	1.9±1.5	2.6±1.6	0.35
Day 90	2.4±1.1	1.9±1.0	0.30
Day 180	4.2±1.8	1.6±1.1	0.02
Day 270	3.0±1.4	2.2±1.6	0.04
Day 360	2.8±1.7	1.7±1.5	0.04

Table 5.3 compares transitional B-cell frequencies in patients developing and not developing de novo donor-specific anti-HLA antibody without experiencing biopsy-proven acute rejection. ANOVA p=0.001; p-values in last column of table refer to post-hoc inter-group comparison.

5.4.4 Transitional B cells and other outcomes

No statistically significant relationships between transitional B-cell frequencies (averaged over follow-up) and either eGFR (reported as change in ml/min) or UACR (reported as change per 1mg/mmol [10mg/g]) at the end of follow-up (in patients alive with graft function at end follow-up; n=67) were seen (Effect: -4.3; 95%CI: -9.9, 1.2; p=0.12 and Effect: 0.79; 95%CI: 0.51-1.22; p=0.22 respectively). Similarly, transitional B-cell frequencies were not associated with eGFR at 12 months post transplantation in patients alive with graft function at 12 months (n=68; Effect: -4.1; 95%CI: -9.7, 1.5; p=0.14). Repeating these analyses for the entire cohort (and including patients who had died or suffered graft failure as displaying eGFR 10ml/min) did not influence these results (Effect for eGFR at end follow-up: -4.5; 95%CI: -9.8, 0.8; p=0.09; Effect for eGFR at 12 months: -4.4; 95%CI: -10.0, 1.2; p=0.12). Clinically significant infection (as defined in "methods") was diagnosed in 22 patients over follow-up, but no association between transitional B-cell frequencies (modelled as time-dependent variable) and time to infection was evident (HR: 1.03; 95%CI: 0.83-1.28; p=0.76). It should be noted that due to

sample size, these results do not exclude a potential effect of transitional B-cell frequencies on these outcomes. Death and graft failure numbers were low, but with no clear difference in transitional B-cell frequencies in patients experiencing these events compared to the overall cohort (data not shown).

5.5 DISCUSSION

B cells with regulatory properties are of increasing interest in kidney transplantation, in particular clinical outcomes and phenotypes associated with transitional B cells characterised by surface $CD19^{+}CD24^{hi}CD38^{hi}$ expression (5-10). This study shows that increased frequencies of these transitional B cells are associated with protection from episodes of BPAR in unselected low/medium immune risk transplant recipients followed longitudinally from the time of transplantation. The results of this prospective study, the first to date, are biologically plausible and extend the current literature, thereby supporting efforts to further clarify the role these cells in clinical transplantation, either as biomarkers of rejection risk, or in potential cell-based therapies.

The majority of episodes of BPAR in this study represented cellular rejection, with a minority representing ABMR in the context of non-adherence (discussed further below). Although not now a common cause of direct and immediate graft failure, cellular rejection remains a potent and relevant risk factor for subsequent graft failure (15-17), thereby highlighting the relevance of the current findings to contemporary transplantation.

Unsurprisingly, no association between transitional B cells and ABMR was seen, as these rejection episodes were driven by nonadherence rather than a predisposing immune profile. However, the relationship between transitional B cells and rejection held in adherent patients who developed dnDSA, suggesting that peripheral cellular regulation may reduce the risk of rejection in those patients demonstrating circulating HLA antibodies. This is reminiscent of recent work showing absence of immune-related gene expression in peripheral blood of "antibody-positive/rejection-negative" kidney transplant recipients (18). The current finding also resonates with the inverse relationship between transitional B-cell frequencies and ABMR in recent studies (9,10), perhaps suggesting that the presence of both antibody *and* lack of regulation are required for rejection to occur. It should be noted that these case-control studies investigated patients undergoing indication biopsies, usually many years post-transplantation, and as such their designs differ from the current investigation. The results do raise the possibility that immunoregulation, and specifically transitional B cells with regulatory properties, may explain the reproducible observation that many patients with circulating HLA antibody display freedom from rejection and experience clinical stability over prolonged follow-up (19-22).

Although not a primary aim of the study, a further interesting finding was the link between cellular and humoral allo-immunity in patients developing ABMR. All 3 such patients displayed a positive response to HLA peptide in an Elispot assay of indirect cellular immunity, whereas none of the patients developing dnDSA but not experiencing BPAR (and who displayed stable renal function and negligible proteinuria over follow-up) displayed a positive test at the time of antibody formation. This supports the concept that damage as a result of cellular activation and infiltration may be more important to graft outcome then the presence of anti-donor

antibody *per se* (18,23,24), thereby extending previous work to the specific situation of nonadherence-associated ABMR. We do acknowledge however that this observation, whilst interesting, remains preliminary and over-interpretation of this finding based on the low numbers of patients with ABMR in the current study should be avoided.

Recent studies have suggested that the development of dnDSA (and then later ABMR) following episodes of rejection, are responsible for the link between cellular rejection and outcome (15,21,25). This association was not seen in the current study, although this may be unsurprising in light of the study size, and the recognition that despite the above association, most patients developing dnDSA (generally around 80%) do not have a history of prior BPAR (15,21,25). Worthy of note in passing though is that the current study, which carefully evaluated dnDSA in a time-dependent fashion, does confirm previous established (26,27) and recent (28) data demonstrating that in a low immune-risk population the development of dnDSA does not predict acute rejection episodes (although it does predict the development of microvascular inflammation).

The prospective, longitudinal design of the current investigation, coupled with time-dependent statistical analysis in an unselected cohort, represent obvious strengths of the study. Although based on a single-centre experience, the results are biologically plausible and extend the currently limited understanding of the field. Nevertheless, these data should be considered preliminary, and further investigation in separate and larger cohorts with longer follow-up duration are required to validate the robustness of the findings, in particular in regard to the interplay between cellular immunity, humoral immunity, nonadherence and regulation. A major challenge for the future will be inter-laboratory standardisation, a pre-

requisite for widespread clinical application. Further work is also required to evaluate the stability of these cell subsets during storage. Understanding cytokine profiles of transitional B cells may refine the predictive utility of immunophenotyping (9), but was beyond the scope of this pilot study. However, the findings of the current study set the scene for further clinical investigation, contribute to the understanding, and provide a rationale for the use of this immune biomarker and potential intervention in clinical transplantation.

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Chapter 6: GENERAL DISCUSSION

Indirect Effects of Cytomegalovirus in Kidney Transplantation

6.1 Overview of the Thesis

Despite the availability of effective therapy, cytomegalovirus remains the most important infection in kidney transplantation. Response to CMV and the presence of large populations of CMV-specific T cells that can be easily measured ex vivo provided an ideal model for not only studying CMV but also examining the immune response to persistent viruses in solid organ transplantation.

In a longitudinal prospective study serial clinical, immunological and biochemical information was studied to gain a deeper understanding of the indirect effects of cytomegalovirus in kidney transplantation. The findings of the studies are presented in Chapters 2 to 5 of this thesis.

In Chapter 2, we show CMV re-infection is clinically relevant. Our preliminary data shows we might be able to identify a high-risk subgroup in D^-/R^+ transplantation by pre-transplant assessment of cellular immunity. This may pave the way for a more logical and effective prophylactic strategy in these patients. We provide previously undescribed and direct evidence of immune hypo- responsiveness to latent CMV. This has implications for how we best deal with CMV in this setting, and also wider implications for vaccination programmes. In chapter 3, we focus on the mechanism of kidney transplant damage associated with CMV infection. In chapter 4, we focus on the role of CMV in causing immunosenescence within the kidney transplant cohort. We demonstrate changes to the adaptive and innate immune system with resultant effect on post-transplant outcomes.

6.2 Summary of the major findings

6.2.1 CMV re-infection is clinically relevant

In 2008, a study published by Kliem et al demonstrated a greater incidence of CMV infection in $D^{+/}R^{+}$ transplantation. However, this lacked data on CMV disease. In chapter 2, we highlight the differential risk of clinically relevant CMV disease between D+/R+ and D-/R+ transplantation. In clinical transplantation, D^{+}/R^{+} and D^{-}/R^{+} groups are considered "intermediate risk" for CMV infection. As such, CMV prophylaxis is not advised for these two serostatus groups in the current British Transplant Society guidelines (with the exception of patients treated with T-cell depleting antibody therapy). This study demonstrates the increased incidence of CMV disease in non-prophylaxed seropositive recipients of kidneys from seropositive donors (D^{+}/R^{+}) compared with seronegative donors (D^{-}/R^{+}). This risk was modified by donor-recipient HLA class I mismatch.

These findings have important implications for CMV risk management in transplantation and certainly our clinical practice has changed, such that, the D^{+}/R^{+} group has been re-classified as 'high risk' with resultant changes in CMV prophylaxis strategy.

6.2.2 Mechanism of viral infection in kidney transplantation

As mentioned above, we observed a modified risk of CMV disease with HLA Class I mismatch. In D^{+}/R^{+} transplantation, a significant relationship was evident between HLA Class I mismatch and time to CMV disease. This effect was not evident in the other three serostatus groups.

It is possible that donor-recipient HLA mismatch impairs development of cellular immunity to CMV within transplanted donor tissue. We utilised HLA-peptide tetramers to investigate the underlying mechanism. We have shown, in D+/R+ transplantation, CMV-specific CD8+ T-cell immunity does not develop against peptides presented solely by donor-specific HLA alleles.

The relevance of this is that although there is a wealth of data describing mechanisms by which latent CMV is proposed to reduce immune responsiveness (↓ HLA presentation**,** ↓ Antigen presentation, ↓ T-cell proliferation, ↓ Production of IL-2, INF-c, PD-1, ↑ Fc receptor expression, ↑ Complement inhibitors, ↓ Macrophage migration) there is little evidence to directly demonstrate that effect in vivo.

This physiological model of transplantation provides novel insight into antigen presentation pathways in viral infection. Absence of directly primed $CD8^+$ T-cell immune responses raises questions regarding how infection is ultimately controlled within donor tissue.

6.2.3 Prognostic utility of pre-transplant cellular immunity assays

The pre-implantation histological data from the current study suggests CMV transmission peri-transplantation is very common. Early detection of patients at increased risk of developing CMV disease could allow targeted CMV prophylaxis/treatment and avoid potential toxic side effects of unnecessary exposure to anti-viral drugs. Assays of cellular immunity to CMV are emerging research tools. These in-vitro assays use recipient PBMCs, thus CMV peptides are presented by recipient HLA. In our in-vivo model, we have already demonstrated that CMV may be harboured within donor tissue and presented by donor HLA

on re-activation (section 6.6.2). This has important implications for the prognostic utility of these assays in D+R+ (compared with D-R+) transplantation.

This is the first study to look at the prognostic utility of pre-transplant cellular immunity assays in predicting CMV infection between D^+/R^+ and D^-/R^+ transplantation. We have shown the pre-transplant CMV-specific $CD8^+$ T-cell is predictive of CMV infection in D^-/R^+ but not in D^+/R^+ transplantation. This suggests that targeted prophylaxis to such seropositive patients without cell mediated immunity may be a clinically effective strategy. However, the same cannot be said for the D^+/R^+ group and our local approach to these patients is now universal prophylaxis.

6.2.4 Mechanism of kidney transplant damage by CMV

Cytomegalovirus infection produces a profound immune system with resultant generation and clonal expansion of CMV-specific T cells. In 2003, a cross sectional study published by Pawlik [1] demonstrated higher frequencies of $CD4^+CD28^{null}$ cells in patients with chronic kidney allograft dysfunction. In 2004, Van Leeuwen [2] reported emergence of $CD4^+CD28^{null}$ cells following primary CMV disease in four kidney transplant patients. These cells proliferated to CMV antigens in the four patients studied. These rare and unusual subset of T helper cells release cytolytic molecules perforin and Granzyme B and behave like NK cells as they express NK cell receptors [3]. The suggestion has arisen that these cells may target and damage endothelium in auto-inflammatory diseases. In fact, the few studies reporting endothelial damage in autoimmune disease have generally demonstrated T-cell activation in the presence of endothelium – usually HUVEC – rather than damage to endothelium by these cells [4].

In chapter 3, we confirm $CD4^{+}CD27^{-}CD28^{null}$ cells are atypical and bear markers of potential cytotoxicity including perforin and NKG2D. These cells are pathognomonic of prior CMV exposure. But the rest of our findings are novel to kidney transplantation. The $CD4^{+}CD27^{-}CD28^{null}$ cells proliferate in vitro to CMV antigens. We found no evidence that these cells mediate an allo-immune response (no in vitro proliferation to HLA antigens). We have demonstrated a role for these cells in glomerular endothelial cell damage, an effect which may be mediated by NKG2D. We have shown higher $CD4^{+}CD27^{-}CD28^{null}$ cell counts at 12 months post-transplantation predict a steeper decline in kidney allograft function thereafter. This has important implications for the role of CMV in kidney transplantation and strategies to ameliorate its damaging effect.

6.2.5 Role of CMV in immunosenescence and transplant outcome

Primary CMV infection produces a profound $CD8^{+}$ T-cell immune response. These CMV-specific $CD8^{+}$ T cells lose expression of the co-stimulatory molecule CD28 ($CD8^{+}CD28^{null}$). CMV establishes lifelong latency following primary infection. Aging and the immunocompromised state give rise to low level, repetitive viral re-activation of the latent CMV, with resultant clonal expansion of terminally differentiated $CD8^{+}CD28^{null}$ cells. A role for CMV in immunosenescence is suggested by the Swedish studies of healthy elderly donors. $CD8^{+}CD28^{null}$ phenotype and CMV seropositive status form part of the immune-risk profile (IRP) for immunosenescence [5, 6]. This has been shown to be associated with increased risk of infection, mortality, and reduced vaccine response in octo- and nonagenarians (the Swedish OCTO/NONA immune study) [7, 8]. Recently, this IRP has been demonstrated in hexagenerians in the Swedish HEXA immune study [9]. In 2014, Yap reported a 2-fold higher

risk (p=0.06) of long-term kidney allograft dysfunction in patients who had increased levels of $CD8^{+}CD28^{null}$ cells [10]. It has been argued that CMV contributes materially to immunosenescence [11] but there are no studies addressing the potential role of CMV in driving immunosenescence.

In chapter 4, we describe the immune-risk profile of the kidney transplant recipients in our study. We confirm the importance of circulating $CD8^{+}CD28^{null}$ cell frequencies as an informative marker of immunosenescence. The temporal relationship between CMV serostatus, primary infection, CMV re-activation and $CD8^{+}CD28^{null}$ cell frequencies provides novel insight into the 'indirect' effect of CMV in the pathogenesis of $CD8^{+}CD28^{null}$ cells. We report a temporal association between elevated $CD8^{+}CD28^{null}$ cell frequencies and subsequent development of clinically relevant episodes of infection. Our work suggests CMV drives immunosenescence, and that immunosenescence increases risk of infections. In the setting of kidney transplantation, his is the first study to explore mechanism for the increased risk of infection by means of in-vitro neutrophil function assays.

6.2.6 CMV infection, B cells and kidney transplant outcomes

In our study, we did not find an association between B-cell numbers and CMV. However, retrospective, cross-sectional studies report a protective role for a B-cell subset in kidney transplantation [12, 13]. This transitional (regulatory) B-cell subset is defined by the phenotype $CD19^{+}CD24^{hi}CD38^{hi}$. These studies focused on antibody-mediated rejection and were conducted as case control studies many years after transplantation. The novelty of our study is the ability to track the evolution of these transitional B cells post transplantation and

evaluate the temporal relationship between cell counts, rejection, and graft function from the time of transplantation. The main findings of the study are summarised below.

6.2.6.1 Transitional B-cell numbers fall following transplantation

A significant fall in transitional B-cell numbers was evident during the first three months following transplantation. Although cell numbers subsequently rose, these in general represented a fall from the baseline levels.

Our findings are supported by two prospective studies published by Kamburova [14] and Svachova [15]. Kamburova reports a decrease in the percentage of transitional B cells in fourteen kidney transplant patients receiving standard triple maintenance immunosuppression (steroids, tacrolimus, mycophenolate mofetil) [14]. This study did not look at clinical transplant outcomes (rejection or graft function). In a recent study involving 98 kidney transplant patients, Svachova [15] reports a significant reduction in transitional B-cell numbers up until the third post-transplant month, with partial repopulation in the first year.

6.2.6.2 Increased transitional B-cell numbers protect against onset of biopsy-proven acute rejection (BPAR)

Both univariate and multivariate regression analysis revealed a significant association between transitional B-cell frequency and the subsequent development of BPAR, with increased transitional B cells protecting against the onset of BPAR.

This is supported by the findings from Svachova's study [15] where lower numbers of transitional B cells at three months post-transplantation were associated with higher risk of graft rejection.

6.2.6.3 Pre-transplant transitional B-cell numbers associated with rejection risk

A novel finding from our study is the significant association between pre-transplant transitional B-cell numbers and subsequent risk of biopsy-proven acute rejection, with lower numbers associated with increased risk of rejection. This is contrary to Svachova's study in which pre-transplant transitional B-cell numbers were not predictive of kidney transplant rejection [15]. However, larger prospective studies are required to further clarify the role of these cells as biomarkers of rejection risk in clinical transplantation.

6.2.6.4 Rejection-free survival optimum in patients with >3% transitional B cells

Our study is the first to use Kaplan-Meier rejection-free survival estimates to quantify risk of kidney transplant rejection in relation to specific transitional B-cell frequencies. We have shown patients with <1% transitional B cells have the worst rejection free survival and those with >3% have the best outcome. This is now supported by findings from Svachova's study [15].

6.2.6.5 Preserved transitional B cells associated with protection from rejection in patients with de-novo Donor Specific Antibody (dnDSA)

This is the first study to look at the association between transitional B-cell frequencies, de-novo Donor Specific Antibodies and subsequent rejection. Our study suggests preserved transitional B-cell frequency is also associated with protection from rejection even in patients with dnDSA. This suggests that peripheral regulation may reduce the risk of rejection in patients with circulating HLA antibodies.

6.3 Limitations of the study

The studies described in Chapters 2 to 5 were observational in nature and represent single-centre experience. Nonetheless, associations have been established and supported by statistically significant *p*-values. Although type I statistical errors may exist, the probability remains low. Due to the small study size, absences of associations between certain variables give rise to the probability of type II statistical error.

As freshly-isolated PBMCs were used for a significant proportion of the laboratory work undertaken, we are not able to reproduce assay results using the same cell material.

Future studies with larger cohorts are necessary to validate the conclusions.

6.4 Scope for future work

The studies presented in this thesis add to the current knowledge of T-cell immunity against CMV in controlling viral latency and susceptibility to CMV disease. Oxford Immunotec have recently developed a T-SPOT.CMV test for measuring T-cell mediated CMV response with ELISPOT technology. A clinical trial is necessary to evaluate its therapeutic role in solid organ transplantation. Larger studies are necessary to further evaluate CMV cell mediated immunity in D+/R+ transplantation. A randomised controlled trial to evaluate the impact of CMV prophylaxis on the development and evolution of CMV cell mediated immunity and transplant outcomes will take forward the preliminary findings of our small observational study.

6.5 Conclusions

The studies presented in this thesis provide novel insight into the differential risk of CMV infection, mechanism of viral infection and subsequent allograft damage following kidney transplantation. The role of CMV in driving immunosenescence with subsequent increases in infection is explored. Prognostic utility of cellular immune assays is examined in respect to predicting post-transplant CMV infection. The findings from this thesis set the scene for future interventional research and therapeutic strategies.

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Chapter 7: Appendix

7.1 Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

1) Blood samples should be fresh (no more than 6 hours old) with anticoagulant.

2) Dilute blood 6 in 1 with RPMI1640 and add 10units of sodium heparin per ml of undiluted blood.

3) Add 15ml of Ficoll-Paque per Accuspin tube and bring to below frit by centrifuging at 1000 x g for 30 seconds.

4) Pour in diluted blood (do not exceed 43ml per tube).

5) Centrifuge for 15min at 1000g.

6) Carefully remove tubes, as tops can work loose.

7) Aspirate plasma carefully to within 2cm of PMBCs.

8) Using a filtered tip carefully transfer PMBC layer to a fresh sterile universal.

9) Fill tube to 30ml with RPMI1640 and spin at 800 x g for 10min.

10) Aspirate supernatant and resuspend pellet as above.

11) Wash pellet twice with RPMI1640.

12) On final wash resuspend cells in RPMI1640 supplemented with 5% human AB serum.

7.2 Protocol Heat Inactivation of AB

1) Thaw serum rapidly in a water bath at 37^0C and check all proteins are dissolved by shaking when defrosted.
2) Heat a circulating water bath to 56 ^{0}C.
3) Place the thawed serum bottles in the water bath so that each bottle is completely immersed up to the level of the serum. Do not immerse the cap.
4) Monitor the temperature in the water bath. When the temperature of the water bath returns to 56 ^{0}C, start timing the process for 30 minutes. Agitate the bottles approximately every 5 minutes during the heat inactivation process to prevent gelling of the serum proteins and to promote more uniform heating of the serum.
5) After 30 minutes of heat inactivation at 56 ^{0}C, remove the serum bottles from the water bath and rapidly cool them in an ice bath. Prolonged treatment of the serum at elevated temperatures will cause deterioration of serum components critical for growth of cells.

7.3 Enzyme-Linked Immunosorbent Spot Assay (ELISPOT).

7.3.1 Point of Emphasis

- After initial washing of ELISPOT plates (x4 with PBS) do not allow membranes to dry out.
- When developing, wash plates thoroughly with PBS before adding antibody or substrate.
- AB serum should be heat inactivated. Use Heat Inactivation Protocol, Appendix 7.2.

7.3.2 Methods

1) Prewash plate with x4 washes of PBS.
2) Block for at least 30min and no more than 5h with 10% human AB serum.
3) Isolate PBMCs using protocol outlined in Appendix 7.1.
4) Using haemocytometer determine cell number and adjust to 8 x 1^{06} cells per ml
   (do not use cells below 6 x 1^{06} cells per ml)
5) Add 50µl of cell suspension per well to give 4 x10^5 cells per well.
   (NB prewash plate with PBS and block as describe below).
6) Add 50µl of peptide/antigen stock as described in Appendix 7.4.

Incubate cells in a humid atmosphere of 5% CO_2 for 48h.

7.3.3 Developing ELISPOT Plate

1. Wash plates thoroughly x5 with PBS.
2. Dilute alkaline phosphatase-conjugated detection mAb 1:200 in filtered (using 0.45µm filter) PBS containing 0.5% FCS.
3. Add 100µl of antibody per well and leave for 2h at room temp.
4. Wash plate again x5 with PBS.
5. Filter BCIIP/NBT-plus with a 0.45µm filter.
6. Add 100µl of substrate per well and leave spots to develop (max time 15 min to reduce background).
7. Wash thoroughly x5 in tap water.
8. Remove underdrain and wash again.
9. Leave plate to dry in dark for at least 12h.

7.2.2.3 Reading Plates

Read plates using ELISPOT reader on 3 floors of Cancer Studies Building.

1. Calibrate stage (either recalibrate or use previous settings).
2. Read plate.
3. Reset background if necessary.
4. Check well for debris/false counts.

7.4 Dissolving and Diluting Antigens and Peptides for ELIPOT Assay

7.4.1 Points of Emphasis

- Stock peptides should be stored at -20^{0}C in glass vials and repeat freeze thawing kept to a minimum.
- Peptide aliquots can be frozen at 1mg/ml in microfuge tubes for up to six weeks.
- Once peptides are diluted with complete media they can be kept at 4^{0}C for a week. Do not Freeze.
- PPD and antiCD3 both stock solution and dilutions in complete media should be kept at 4^{0}C.

7.4.2 Method

1) Peptides should be dissolved initial in 1ml of 0. 1mM HCL (conc HCl 16M (1ml to 16ml gives 1M solution, 100µl to 9.9µl 10^{-2}M and another 100µl to 9.9µl gives 10^{-4}M or 0.1mM.)

2) Add RPMI1640 supplemented with 1% Pen/Streps 1% glutamine, no serum. These are stock solutions that should be stored in glass vials at -20^{0}C. Avoid excessive freeze thawing. Peptides at 1mg/ml can be aliquoted into 1.5ml microfuge tubes and frozen for up to 6 weeks.

3) Peptide mixes

Once diluted peptides should be discarded after 1 week. Store at 4^{0}C.

4) PPD (1mg/ml stock) 50µl to 2450µl complete medium to give final 20µg/ml, and 1µl of antiCD3 mab (1mg/ml) to 999µl complete medium per ml, to give 1ng/ml. Make up fresh every week from stocks. Store both stocks and dilutions at 4^0C.

7.5 Protocol for antigen stimulation and intracellular cytokine staining to enumerate CMV-reactive T cells

- Isolate PBMCs using Ficoll paque density gradient, washed x2 in RPMI-1640 medium, resuspended in RPMI-1640/10% FCS
- Add $2x10^5$ cells per tube (10 tubes)
- Unstim, CMV lysate (10µl), mix 1 (30µl), mix 2 (30µl), mix 3 (30µl), SEB (10µl)
- Make up Unstim, Compensation and SEB tubes to 500µl with media
- Incubate for 1 hour
- Make up remaining tubes to 500µl with media
- Add 0.5ul of BFA to each tube (not compensation) and incubate overnight

Day 2

- Add MACS buffer (1%PBS, 0.05%BSA, EDTA) and centrifuge (2000rpm, 5 minutes)
- Pour off supernatant and resuspend cells
- Surface stain 2.5µl of Ab mix per tube ($CD4^-$PE Texas Red 0.5µl, $CD8^-$PE Cy5 2µl of 1/5 dilution)

- Compensation tubes-2µl CD27-FITC, 0.5µl CD4-PE Texas Red, 2µl of 1/5 dilution CD8-PE Cy5
- Incubate 30 minutes in fridge
- Wash
- Add 100µl of 4% PFA to each tube, incubate 15 minutes in the dark at room temp
- Wash
- Add 100µl of 0.5% saponin to each tube, incubate 5 minutes in the dark at room temp
- Add 4µl IFNg-FITC per tube, incubate 30 minutes in the dark at room temp
- Wash

Intracellular Cytokine Stimulation and Staining

Day 1

- Add $2x10^5$ cells per tube (I have cells at $2x10^6$/ml and add 100µl cells per tube).
- Unstim, CMV lysate (10µl LyS), mix 1 (IE1 30µl), mix 2 (pp65 30µl), mix 3 (30µl), SEB (10µl).
- Make up Unstim and SEB tube to 500µl with media (RPMI, 10% FCS, 1% P/S, 1% Glutamine).
- Incubate for 1 hour.
- Make up CMV lysate stimulated, peptide stimulate tubes, etc. to 500µl with media.
- Add 0.5µl of undiluted BfA to each tube and incubate overnight (do not add to Comp tubes).
- Retain 500µl ($1x10^6$ cells) for Comp tube (100µl cells and 400µl media).

Day 2

- Prepare Comp tubes (Unstain, FITC, PE, and TC). Do not add antibody to these mixes.
- Add 4µl MACS buffer and centrifuge (2000rpm, 5 minutes).
- Pour off supernatant and resuspend cells.
- Surface stain 4µl of Ab mix per tube (CD4-PE 2µl, and CD8-TC 2µl of 1/5 PBS dilution). Add to Unstimulated tubes too.
- Compensation tubes – 2µl CD27-FITC, 2µl CD4-PE 1:5, and 2µl of 1/5 dilution PBS CD8-TC. Do not add to Unstained tube.
- Incubate 30 minutes in the fridge.
- Wash with 4ml MACS buffer and resuspend. Spin 2000rpm for 5 minutes.
- Add 100µl of 4% PFA to each tube including Comp tubes.
- Vortex and incubate 15 minutes at room temp in the dark.
- Wash with MACS buffer. Spin 5 minutes at 2000rpm. Por out buffer, resuspend.
- Add 100µl of 0.5% saponin to each tube including Comp tubes (4% stock diluted to 0.5% in MACS buffer at room temperature).
- Mix gently (DO NOT VORTEX) and incubate 5 minutes in the dark at room temperature.
- Add 4µl IFNy-FITC per tube (do not add to Comp tubes).
- Incubate 30 minutes at room temperature in dark.
- Wash with 4ml MACS buffer, spin and pour supernatant off.
- Put in fridge and run samples.

7.6 Protocol for HLA: peptide tetramer complexes and staining for flow cytometry

- $2x10^5$ PBMCs incubated with VLE and NLV tetramer at 37^0C for 30 minutes.
- Wash cells with PBS containing 2% FCS.
- Incubate cells with monoclonal antibodies for 30 minutes on ice.
- Wash cells and fix in PBS containing 2% PFA and 2% FCS.

Tetramer Staining

- Count cells, need $1x10^6$ per 100µl per tube (50µl per plate). Can use $1\text{-}2x10^5$ for Comp tubes.
- Mix 1ml cells with 10mls PBS.
- Spin at 2000rpm for 5 minutes, pour off supernatant and resuspend.
- Add 1µl of tetramers to tubes then vortex and incubate at 37^0C for 15 minutes.
- Was with MACS buffer.
- Make up master mixes for tetramers and phenotyping (combined).
- Add master mixes and antibodies to tubes including Comp tubes.
- Incubate 20-40 minutes at 4^0C.
- Wash with MACS buffer and read on LSR.
- Use following antibodies for phenotyping: CD4 PerCp-Cy5, CD8 AmCyan, CD3 pacific blue, CD57 FITC, CD127 PE, CD45RO ECD (Texas red), CD28 PE-Cy7, CD45RA AF700, CD27 APC-Cy7, tetramer APC.

7.7 **Cell sorting and 5,6-carboxyfluorescein succinimidylester (CFSE) proliferation assays**

1. Isolate PBMCs and count.

2. Divide into **a**. Cells to be irradiated 1 x 10^5 per well

b. Compensations tube well for after proliferation 1 x 10^5 (x10 wells)

c. Cells to be sorted – remaining cells.

a. Cells to be Irradiated

1. Add 4 x 10^5 cells in Facs tube and antigen pulse.

   X2 tubes containing complete media

   CMV lysate (as for lysate assay)

   PPD and/or HLA peptide mix (as for ELISPOT assay).
2. Antigen pulse for x3 hours.
3. Irradiate cells with 10 grays.
4. Add cells to wells at concentration of 1 x 10^5

b. Compensation Tubes

1. Add 1 x 10^5 cells to x 10 well and stimulate with antigen (i.e. PPD or CMV lysate).

c. Cells to be sorted

1. Count remaining cells and resuspend at 2 x 10^7 cells per ml.

2. Add 100µl of stain per 10^6 cells

CD4 PerCP Cy5.5	5µl
CD28 PE-Cy7	7µl
CD8 APC	5µl
CD27 APC-Cy7	4µl
	21 add 79µl MACs Buffer

3. Sort CD4 positive CD27 negative, CD28 positive, CD28 negative

   CD8 positive CD27 negative CD28 positive, CD28 negative

4. Spin at 800g for 10 minutes and resuspend at 1 x 10^5 cells per 200µl in PBS.
5. Dilute CFSE 1µl to 999µl in PBS and add 1µl per 200µl of cells.
6. Incubate at 37^0C for 10min.
7. Quench reaction with 4ml complete media for 5min on ice.
8. Spin at 800g for 10 minutes.
9. Resuspend cells in 100µl of media at 40 000 to 100 000 cells per ml.

   Incubate all for x 5 days and stain.

Staining Cells

1. Remove 100µl of supernatant, spin at 800xg for 10 min, then freeze at -80^{0}C for Luminex assay.
2. Resuspend cells using pipette and transfer to Facs tube.
3. Top up to 4ml with MACs buffer spin at 1000g for 5min.
4. Resuspend cells in 100µl of MACs buffer add stain:

Compensation tubes:

FITC CD4	12.5µl
PE CD2	2.0µl
Texas Red CD4	5.0µl
PE-PerCP Cy5.5 CD4	5.0µl
PE-Cy7 CD28	7.0µl
eFluoro®450 CD45RA	4.0µl
Pacific Orange CD3	6.0µl
APC CD8	5.0µl
APC-Cy7	4.0µl

Experimental Wells

PE CD2	2.0µl
PE-PerCCy5.5CD4	5.0µl

PE-Cy7 CD28	7.0µl
eFluoro®450 CD45RA	4.0µl
Pacific Orange CD3	6.0µl
APC CD8	5.0µl
APC-Cy7	4.0µl
	33µl vs 67µl

5. Incubate at 4^0C for 30 minutes.
6. Top up tube with MACs buffer and spin at 1000g for 5 min.
7. Aspirate and repeat step 6
8. Resuspend in 500µl of MACs buffer.
9. Analyze on Cyan.

7.8 Purifying $CD4^+CD27^-CD28^+$ or $CD28^{null}$ Cells using MACs Columns

7.8.1 MACs Buffer

100ml x10 PBS, 4ml 0.5M EDTA solution, make up to 1litre and add 5g BSA

Filter steriliser before use.

Do not put more than 2 x 10^8 cells on columns

7.8.2 Isolation of $CD4^+$ Cells

1. Isolate PBMCs wash with MACs buffer and count.
2. Remove supernatant and resuspend in 40µl of MACs buffer per 1 x 10^7 cells.
3. Add 10µl of Biotin Antibody Cocktail per 1 x 10^7 cells.
4. Mix well and incubate for 5 min at 4-8^{0}C.
5. Add 30µl MACs buffer per 1 x 10^7 cells.
6. Add 20µl of Anti-Biotin Microbeads per 10^7 cells.
7. Mix well and incubate for an additional 10 min at 4-8^{0}C.
8. Wash with MACs buffer, completely remove supernatant and resuspend up to 1 x 10^8 per 500µl.
9. Place MACs column on magnetic field and rinse with 3ml of MACs buffer.
10. Add cells to column and wash through with 3x3ml MACs buffer.

7.8.3 Isolation of $CD4^+CD27^-$ Cells

1. Wash cells with MACs buffer and resuspend in 80µl of MACs buffer per 1 x 10^7 cells.
2. Add 20µl of CD27 microbeads per 1 x 10^7 cells.
3. Mix well and incubate for 15 min at 4-8^{0}C.
4. Wash with MACs buffer and remove supernatant.
5. Resuspend at 1 x 10^8 cells per 500µl.
6. Place MACs column on magnetic field and rinse with 3ml of MACs buffer.
7. Add cells to column and wash through with 3x3ml MACs buffer.

NB. It is the cells passing through the column that need to be collected.

7.8.4 Isolation of $CD4^+CD27^-$ $CD28^+$ or $CD28^{null}$ Cells

1. Wash cells with MACs buffer and resuspend at 1 x 10^7 cells per 40µl.
2. Add 10µl of CD28-PE per 1 x 10^7 cells.
3. Mix well and incubate for an additional 15 min at 4-8^{0}C.
4. Wash cells with MACs buffer and remove supernatant.
5. 80µl of MACs buffer per 1 x 10^7 cells.
6. Add 20µl of AntiPE Microbeads per 1 x 10^7 cells.
7. Mix well and incubate for an additional 15 min at 4-8^{0}C.
8. Wash cells with MACs buffer and remove supernatant
9. Resuspend up to 1 x 10^8 cells in 500µl of MACs buffer.
10. Place MACs column on magnetic field and rinse with 3ml of MACs buffer.

11. Add cells to column and wash through with 3x3ml MACs buffer.

NB. These are the $CD4^{+}CD27^{-}CD28^{null}$ cells.

12. For CD27+ cells remove from magnet and wash through with 5ml MACs buffer.

To further purify the $CD28^{+}$ cells repeat 9 to 12 with a fresh column. NB. Any cells washed through the column will be $CD4^{+}CD27^{-}CD28^{null}$ cells.

7.9 Protocol for stimulating $CD4^+CD28^{null}$ and $CD4^+CD28^+$ cells with CMV peptide, HLA peptide

CMV Lysate x2

Tra34 x2

Media x2

Irradiated Cells x2

PPD x2

Compensation tubes x5 unstained

CD4-FITC 2 $x10^5$ cells in x5 wells and stimulate with PPD

CD4-PECP5.5

CD28-PECy7

CD27-APC-Cy7 100µl to 100µl 1 x 10^6 cells

Irradiated Cells

X6 tubes of 3 x 10^5 cells 1.8 x 10^6 cells

Staining Cells

Take out 1.8 x 10^6 cells for irradiating and 2 x 10^6 cells for compensation tubes

2 x 10^5 cells per tube: 8 x 10^5

Unstained

CD4-PECP5.5 0.5

CD28-PECy7 1µl

CD27-APC-Cy7 2µl

Stains to be added per 1 x 10^6 cells:

CD4-PECP5.5 1.3µl

CD28-PECy7 2.5µl

CD27-APC-Cy7 5µl

Pulsing Cells with CFSE

1. Wash cells and resuspend at 1x 10^5 cells per 200µl in PBS.
2. Dilute CFSE 1µl to 1000 in PBS and add 1µl per 200µl of cell suspension.
3. Incubate for 5min at 37^0C.
4. Top up with 5% AB Serum Complete.
5. Wash and resuspend at 3-5 x 10^4 cells per 100µl in Complete media.
6. Add irradiated antigen pulsed cells and incubate.

	CMV CD28null	Tra34 CD28null	Media CD28null	Irradiated CD28null	PPD CD28null
	CMV CD28+	Tra34 CD28+	Media CD28+	Irradiated CD28+	PPD CD28+
	Comp CMV	Comp CMV	Comp CMV	Comp CMV	Comp CMV

Irradiated cells:

1. 1.8 x 10^6 cells divide into x5 tubes
2. Stimulate with CMV lysate in Complete

   Tra34 in Complete

   -ve Control (media) in Complete

   Irradiated cells (media) Complete
3. In 1ml for x3 hours
4. Irradiate at 50 grays
5. Spin and resuspend in 100µl of Complete media and add to test wells.

Compensation wells:

1. 1 x 10^6 cells, resuspend and add to CMV lysate at 2 x 10^5 cells per 100µl
2. Seed x5 well as per sheet.

7.10 Culturing Immortalised Glomerular Endothelial Cells (iGECs)

1. Seed 24 well plates at 6 x 10^4 cells per ml (or well).
2. Incubate for 72h until confluent at 33^0C.
3. Replace media and incubate at 37^0C for 24h to differentiate.

Purifying T cells

1. Isolate PBMCs and incubate in 6 well multi-dishes at 2 x 10^7 cells per well.

   Do x3 wells control, x3 wells with HLA, x4 wells CMV (current lysate add 10µl per well).
2. Incubate overnight.
3. Aspirate cells and supplement sup wells with supernatant at 10% (i.e. 150µl)

Isolation of $CD4^+$ Cells

1. Wash isolated cells in MACs buffer.
2. Remove supernatant and resuspend in 40µl of MACs buffer per 1 x 10^7 cells.
3. Add 10µl of Biotin Antibody Cocktail per 1 x 10^7 cells.
4. Mix well and incubate for 5 min at 4-80C.
5. Add 30µl MACs buffer per 1 x 10^7 cells.
6. Add 20µl of Anti-Biotin Microbeads per 10^7 cells.
7. Mix well and incubate for an additional 10 min at 4-8^0C.

8. Wash with MACs buffer, completely remove supernatant and resuspend up to 1 x 10^8 per 500µl.

9. Place MACs column on magnetic field and rinse with 3ml of MACs buffer.

10. Add cells to column and wash through with 3x3ml MACs buffer.

Isolation of $CD4^+CD27^-$ Cells

1. Wash cells with MACs buffer and resuspend in 80µl of MACs buffer per 1 x 10^7 cells.

2. Add 20µl of CD27 microbeads per 1 x 10^7 cells.

3. Mix well and incubate for 15 min at 4-8^{0}C.

4. Wash with MACs buffer and remove supernatant.

5. Resuspend at 1 x 10^8 cells per 500µl.

6. Place MACs column on magnetic field and rinse with 3ml of MACs buffer.

7. Add cells to column and wash through with 3x3ml MACs buffer.

NB. It is the cells passing through the column that need to be collected.

Isolation of $CD4^+CD27^-$ $CD28^+$ or $CD28^{null}$ Cells

1. Wash cells with MACs buffer and resuspend at 1 x 10^7 cells per 40µl.

2. Add 10µl of CD28-PE per 1 x 10^7 cells.

3. Mix well and incubate for an additional 10 min at 4-8^{0}C.

4. Wash cells with MACs buffer and remove supernatant.

5. 80µl of MACs buffer per 1 x 10^7 cells.

6. Add 20µl of AntiPE Microbeads per 1 x 10^7 cells.

7. Mix well and incubate for an additional 15 min at 4-8^{0}C.

8. Wash cells with MACs buffer and remove supernatant

9. Resuspend up to 1 x 108 cells in 500µl of MACs buffer.

10. Place MACs column on magnetic field and rinse with 3ml of MACs buffer.

11. Add cells to column and wash through with 3x3ml MACs buffer.

NB. These are the $CD4^+CD27^-CD28^{null}$ cells.

12. For CD27+ cells remove from magnet and wash through with 5ml MACs buffer.

To further purify the CD28+ cells repeat 9 to 12 with a fresh column.

NB. Any cells washed through the column will be $CD4^+CD27^-CD28^{null}$ cells.

24 well plates:

Add same number of T cells to well each well in 500µl of GEC media.

Add 2µl of TNF stock to give 2ng/ml to one well

Add 4µl of PR3 stock to give 2µg/ml.

Add 2µl of camptothecin to give 2µm.

Cells Counts

	Control	HLA	CMV
PBMCs			
CD4			
CD4CD27neg			
CD4CD27negCD28null			
CD4CD27negCD28pos			

	1	2	3	4	5	6
A	Control CD28 null	HLA CD28 null	CMV CD28 null	CMV antiNKG2D	T-cells +ve	GECs
B	Control CD28 pos	HLA CD28 pos	CMV CD28pos		T-cells -ve	GECs + TNF
C	10% Control sup	10% HLA sup	10% CMV sup		Comp	GECs + PR3
D					Comp	GECs + Camptothecin

cells with inserts

Leave cells for 4h and 12h.

Supernatants

1. Remove media from insert and spin, split into x3 150µl.
2. Resuspend cell pellet in 100µl of PBS and count.
3. Remove supernatant from bottom layer and other wells, replacing with 1ml PBS.

4. Spin media and freeze x3 300µl aliquots (NB make sure label bottom layer).

DAPI Staining

1. Wash cells three times with PBS (1ml per well).
2. Replace with 1ml ethanol and leave for 10 minutes.
3. Allow to air dry.
4. Add 100µl of 1µg/ml DAPI per well (dilute 1µl to 5000µl).
5. Stain for 2 minutes.
6. Wash x3 with PBS.
7. Add 500µl of PBS and view under fluorescence microscope.

Caspase Staining

1. Save supernatants as describe above.
2. Wash twice with ice-cold MACs buffer.
3. Trypsinise Cells with 100µl of Trypsin.
4. Stop reaction with MACs Buffer.
5. Spin at 1000xg for 5 min
6. Resuspend in residual volume and add 100µl of surface antibodies 5µl of CD31 and 4µl of CD4.
7. Incubate in dark for 10 min at room temperature
8. Wash with MACs buffer.

9. Add 50µL of Reagent A (Fixation Medium) and incubate for 15 minutes in the dark at room temperature.
10. Wash once with 3mL of MACs buffer
11. Centrifuge for 5 minutes at 800x g, aspirate supernatant, resuspend the cell pellet.
12. Add 50µL of Reagent B (Permeabilization Medium), add 10µl of Caspase -3 stain or 2.5µl of Isotype Control incubate for 20 minutes in the dark at 4^0C.
13. Wash once with 3mL MACs buffer.
14. Centrifuge for 5 minutes at 800 x g and aspirate the supernatant.
15. Resuspend in 250µl MACs buffer.

7.11 Fractalkine

1. Dilute capture antibody to 4.0µl per ml.

   Dilute 1µl to 179µl

   1 plate 100µl to 17900µl (17.9ml)

2. Incubate 100µl per well overnight at 4^0C.
3. Wash with 3 x 400µl PBS-Tween
4. Block plate for 1h with 1% BSA
5. Wash x3 with PBS-Tween
6. Standards: 20ng/ml stock dilute 250µl to 250µl in 1% BSA

   10, 5, 2.5,1.25, 0.6 and 0.3ng/ml

   Add 100µl of supernatant and incubate at room temperature for 2 hour
7. Wash x3 PBS Tween
8. Dilute detection antibody 1: 179 i.e. 50µl to 8,950µl

   To do x1 full plate dilute 100µl to 17.9ml
9. Incubate for 2h at room temperature.
10. Wash x 3 with PBS-Tween.
11. Dilute HRP dilute 1: 100 (100µl to 10ml)
12. Incubate x20 minutes.
13. Wash x3 PBS
14. Develop as for vWF.

7.12 vWF ELISA

Solutions

Carbonate Buffer Na_2CO_3 0.4g (19mM final concentration)

$NaHCO_3$ 0.74g (28mM final concentration) add 250ml

PBS tween -1 litre PBs to 1ml of Tween20

NB use charged flat bottomed plates.

Method

1. Dilute polyclonal Rb anti-human vWF (DAKO A0082) 1:500 in carbonate buffer. Add 100µl to each well (samples in doublet + column 1 and 2 for standards) of a medium/high bind ELISA plate.
2. Cover (or use humid box) and incubate overnight in the fridge/cold room

(day2)

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.25	1.25	Sample1	Sample1								
B	0.63	0.63	Sample2	Sample2								
C	0.31	0.31	Sample3	Sample3								
D	0.16	0.16	Sample4	Sample4								
E	0.08	0.08	Sample5	Sample5								
F	0.04	0.04										
G	0.02	0.02										
H	PBS	PBS										

3. Wash 3x in PBS+0.05%tween20

4. Block plate with 5% BSA for minimum of 1h (can leave as long as you like)

5. Make up standards – stock 100IU/dL. Use a top dilution of 1.25IU/dL in PBS and serial dilute down columns 1 and 2. Leave row H (bottom row) with just PBS and no standard to act as a blank (see example layout below).

NB Use supernatant samples neat (non-diluted) – NB serum and plasma will need to be diluted should you ever use these.

6. Wash plate in PBS-tween
7. Add samples and standards and incubate for 1 hour at room temperature
8. Wash in PBS-tween
9. Dilute secondary antibody, Rb anti-human vWF-HRP (DAKO P0226) 1:500 in PBS-tween and add 100µl per well.
10. Incubate at room temp for 1 hour.
11. Prepare OPD solution (1silver+1gold tablet in 20ml ddH2O)
12. Wash plate in PBS tween
13. Add 100µl of OPD to each well and read on kinetic plate reader at 0, 5, 10, 15, 20, 25, 30, 45min and 1hour at 450nm. Use the reading at which the standard curve is at its most linear for all future experiments.

Using plate reader:

Log on as normal

Gen S Software

Calculate samples using Prism (spread sheet set up).

7.13 Modified Protocol for B Regs

1. Coat wells with 50µl of 2µg/ml antiCD3 for 1h. (Add 2µl of 1mg/ml antiCD3 per 1ml media) 1ml will do 5 patients (20wells), incubate for 1h at 37^0C.

2. Add 150µl of PBMCs, gives 2.5 x10^5 cells per well, at least x8 per patient. (Gives a final [c] of antiCD3 of 0.5µg/ml).
3. Incubate for 72h.
4. Stimulate for last 6h with PMA (50ng/ml) and Ionomycin (500ng/ml).

   PMA 1mg/ml stock: add10µl to 490µl to give 500µg/ml stock.

   add 15µl to1485µl to give 5µg/ml

   add 2µl per well.

   Ionomycin 1mg/ml stock: add 10µl to 490µl to give 500µg/ml stock

   add 150µl to 1350µl to give 50µg/ml

   add 2µl per well

   BFA stock 1mg/ml add 100µl to 900µl of media and add 2µl per well. (Treat x2 compensation wells to check staining).
5. Add 6µl containing all 3 per well.
6. Defrost x2 samples of transplantation PBMS and seed a 1 x10^7 cells per ml in Facs tubes. (Do isotype on x1 and use spare cells for comp tubes)
7. Stimulate with PMA for 4h. Process as for 72h stimulate
8. Transfer cells to Facs tubes: x2 tubes per patient, wash with ice cold PBS and adjust [c] to 1 x 10^7 cells per ml. (Any spare use as for Isotype control)
9. Stain with live dead stain.
10. Wash and resuspend in MACs buffer.
11. Surface stain with

Stain	[C] per 1 x 10^6 (µl)	For 5 samples (µl)	For 1 x 10^7
CD24FITC	2	10	20

CD19PE	1	5	10
CD38 PE-Cy7	1	5	10
Volume MACs	96	1444	1960

for 30 minutes at -20^{0}C.

Compensation Tubes CD4 use 1 well per tubes (spare tubes for unstained)

Stain	[C] per 2 x 10^5 cells (µl)
CD4 FITC	2
CD3 PE	2
CD4 PE-Cy7	2
CD3 APC	2

Stains for Experiment

Stain	[C] per 2 x 10^5 cells (µl)
CD24 FITC	1
CD19PE	1
CD38PE-Cy7	1
IL-10 APC* (stain PMA treated wells)	1

(Do x1 stimulated well with isotype control)

12. Wash with MACs buffer

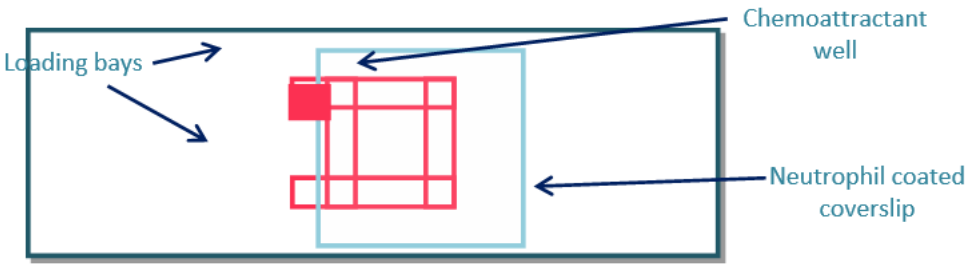
13. Add 50µl of Reagent A (Fixation Medium) and incubate for 15 min in dark.

14. Wash with MACs buffer (500µl 1 x 10^7 cells).

15. Add 2µl of IL-10 stain to one tube and isotype control to one tube per patient in 50µl of Reagent B (Permeabilization Media), leave for 30 min. (x dilute to 500µl stain x 250µl of stain 20µl
16. Wash with MACs buffer.
17. Resuspend in 200µl MACs buffer and read.

7.14 Chemotaxis Assay: Insall Chamber

1. Isolate neutrophils using Percoll gradient.
2. Suspend **neutrophils at 2x10^6/ml in RPMI-1640**, and add 0.15% by volume of 7.5% Bovine Serum **Albumin** (Sigma)
3. **Sterilize coverslips**: Wash 1x in 0.4M H_2SO_4, and 2x in deionized water. Place in covered filter paper lined petri dish.
4. Remove one coverslip to a second petri dish. Pipette **400µl albumin** onto coverslip surface and tilt coverslip to cover the whole surface in solution. Tip off excess albumin and leave to dry in a covered petri dish for ~30seconds.
5. Pipette **400µl of neutrophil** solution onto albumin-coated coverslip. **Incubate for 30 minutes**.
6. Prepare chemotaxis chamber by **washing 3x with 400µl RPMI-1640**. After final wash, fill the wells with RPMI and leave.
7. Lift coverslip out of petri dish and tip off excess neutrophil solution. **Invert the slide onto the chemotaxis chamber** leaving loading bays uncovered, as shown below:



8. **Mop up excess RPMI** with filter paper. Draw up remaining RPMI from chemoattractant wells using a 200µl pipette.
9. Slowly **fill chemoattractant well** with 70µl of chemoattractant/RPMI, ensuring there are no bubbles.
10. **Analyze slide** using microscope. Take one frame every 20 seconds for a total of 36 frames (12 minutes).

Materials

- **7.5% Bovine Serum Albumin**: Sigma A8412
- **Coverslips**: IBR Stores: IBR-COVER1. Micro Cover glass 22x22mm
- **Inverted forceps:** Sigma Z168769
- **fMLP:** Sigma F3506
- **IL8:** R&D Systems 208-IL

Microscope Booking: Technology Hub Leica DMI6000 Video Capture Microscope

7.15 Buffers, Media, Solutions and Reagents

(Sigma- Aldrich, Dorset, UK)	
Tissue Culture Medium (Complete Medium)	
Roswell Park Memorial institute (RPMI) 1640 (Sigma, Poole, UK)	50ml
L-glutamine (Sigma, Poole, UK)	2mM
Penicillin (Sigma, Poole, UK)	100IU/ml
Streptomycin (Sigma, Poole, UK)	100IU/ml
Foetal calf serum (PAA labs, Somerset, UK)	5% v/v
Ficoll Plaque (Sigma- Aldrich, Dorset, UK)	
RPMI-1640 (Sigma, Poole, UK)	
Phosphate Buffered Saline (PBS)	
PBS tablet (Oxoid)	1 tablet
Sterile distilled water	100ml
Magnetic Activated Cell Sorting (MACs) Buffer	
Sterile PBS (pH 7.2)	500ml
Bovine Serum Albumin	0.50%
EDTA	2mM
Lipopolysaccharide (LPS) (Sigma- Aldrich, Dorset, UK)	100ng/ml
Brefeldin A (eBioscience, ID 00-4506-51)	1:1000