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T-cell Responses to Human Cytomegalovirus in Immune-Impaired Individuals

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by

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Abstract

HCMV is a near-ubiquitous β–herpesvirus that asymptomatically infects the majority of immunocompetent adults. However, in the immunodeficient or the immunosuppressed, HCMV becomes a major cause of morbidity. Solid organ transplant recipients require immunosuppressive regimens prior to organ transplantation; the resultant reduction in their T-cell responses leads to an increased risk of uncontrolled HCMV replication, which left unchecked is associated with HCMV disease.

In this thesis we observed fluctuations of HCMV-specific CD4 and CD8 T-cell populations in 20 renal transplant patients during the six months following transplant using flow cytometry. The frequencies of these populations were related to incidences of viraemia. Patients suffering viraemic episodes post-transplant had significantly decreased HCMV-specific CD8+ cell responses when compared to individuals who remained HCMV PCR negative and a link was established between the absolute numbers of HCMV-specific CD4 cells and the size of the HCMV-specific CD8 response.

Temporal increases in virus-specific CD8 responses were also observed, which corresponded with decreases in the incidences of HCMV viraemia

after immunosuppressive regimens were reduced. Further work focused on the emergence of CTL-escape mutants in individuals who, despite having detectable HCMV epitope-specific CD8 responses failed to prevent viraemic episodes. Although no mutations were detected in the patients analysed, a substitution analysis was performed on a defined epitope to determine the possible effects of any substitutions on MHC I molecules binding and recognition by CTLs. Results showed a variety of substitutions could abrogate IFNγ production by CTLs whilst binding and stabilising MHC I complexes.

I also demonstrated for the first time that individuals suffering from common variable immunodeficiency (CVID) - who lack the capacity to mount their own B-cell responses, had detectable HCMV-specific T-cell responses and did not suffer symptomatic HCMV infection, indicating that control of HCMV replication could be mediated by the T-cell arm of the adaptive immune response.

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Open Reading Frame	ORF
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Peripheral Blood Mononuclear Cell	PBMC
Precursor assembly protein protease	pAP
Phosphate Buffered Saline	PBS
Phycoerythrin	PE

Polymerase Chain Reaction	PCR
Protein Kinase C	PKC
Pre-emptive therapy	PET
Retinal pigment epithelium	RPE
Reverse transcriptase	RT
Ribonucleic acid	RNA
Small capsid protein	SCP
Thermus Aquaticus	Taq
T-Cell Receptor	TCR
Toll-like receptor	TLR
Transporter associated with antigen processing	TAP
Tumour necrosis factor alpha	TNFα
UL16-binding protein	ULBP
Unique Long Region	U_L
Unique Short Region	U_S

Amino acids

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Chapter 1

Introduction

1. 1 Classification

Human cytomegalovirus (HCMV), or human herpes virus 5 (HHV5), is the prototype member of the *Betaherpesvirinae* subgroup of the family *herpesviridae*, which includes two other human viruses: human herpes viruses 6 and 7 (HHV6 and HHV7). Cytomegaloviruses were so-named because of their distinctive cytopathic effect (CPE); causing "Owl's-eye" intranuclear occlusions in infected cells [1, 2]. HCMV is a complex virus that contains a large double stranded (ds) DNA genome, which encodes more than 200 open reading frames (ORFS). Similar to other cytomegaloviruses, HCMV infection is highly species-specific - being confined either to the human population or to human-derived cells in culture, indicative of a long period of co-evolution with the host [3].

1. 2 Structure and Morphology

HCMV possess the morphology of a typical herpesvirus in that it is a large enveloped virus, of 150-200nm diameter, with an amorphous tegument that surrounds an icosahedral capsid [4]. The capsid enclosing the HCMV genome is composed of 162 hexagonal capsomeres [5, 6]. The surrounding tegument is closely associated with a lipid bilayer, which is thought to derive from the host's nuclear membrane [4]. Through this lipid bilayer protrude viral glycoprotein spikes, these are incorporated during the budding process from both the nuclear and cytoplasmic membranes [4, 7]. Two further virus-like particles can be detected following *in vitro* virus culture; dense bodies, lacking nucleocapsids are composed solely of viral tegument proteins and an

envelope, and non-infectious enveloped particles, which consist of enveloped capsids lacking the electron-dense genome core [8].

1. 3 HCMV life cycle

HCMV is able to infect a wide variety of tissues and cell types, although attachment and penetration of the virus can occur in permissive and non-permissive cells alike. Initial attachment is thought to be mediated through glycoprotein B (gB) binding to poly-anionic heparin sulphate proteoglycan (HSPG) moieties that are found distal to the cell surface [9], although the heterodimic complex of glycoproteins M and N (gM and gN) also both contains heparin binding sites [9]. Further interactions subsequently occur with other cellular receptors of between 30 and 36kd in size, which are found at closer proximity to the cell surface [10], together these interactions are believed to result in virus penetration.

Because HCMV infection stimulates the activation of similar signalling pathways to those induced by ligation of epidermal growth factor (EGF) to its receptor (EGFR), Wang *et al.* chose to investigate EGFR as a possible receptor for HCMV, and gB was indeed discovered to be the ligand [11, 12]. However, other cells permissive for HCMV infection, such as monocytes, macrophages and dendritic cells, lack EGFR expression, from which it can be surmised that presence of EGFR is dispensable for HCMV entry [13]. Yet the gB/EGFR interaction may induce a signalling cascade in target cells priming them for HCMV replication [12]. Glycoprotein B's ability to bind toll-like receptor 2 (TLR2) is also thought to initiate another signalling cascade,

which leads to the activation of innate immune responses such as the induction of type I interferons [14]. The binding of the heterotrimeric HCMV glycoprotein complex gCIII, consisting of gH/gL, gO and gB to integrins is believed to be necessary for membrane fusion and subsequent internalisation of HCMV [12]. Other receptors have also been implicated in this process, although the principal one required for entry has not yet been elucidated. Penetration occurs through a pH-independent fusion event between the virus envelope and the cell membrane [15]. Once inside the cytoplasm the nucleocapsids make their way towards the nucleus; on arrival, approximately 20-30 minutes post-infection [16], expression of immediate early genes can be detected.

1.3. 1 HCMV genome

At 235kb, the HCMV genome is the largest of any virus known to infect man. Similar to other human betaherpesviruses, HCMV contains a G/C-rich, linear bi-partite, E-class genome; this means that HCMV has two constituent genome parts, the unique short (U_S) and unique long (U_L) regions, which are themselves both flanked by direct and inverted repeat (IR) sequences. These are in turn capable of isomerization producing four different linear genome arrangements [17-19]. The direct repeat sequences are found at the termini of both segments, within these regions are contained cleavage and packaging cis-acting sequences *pac-1* and *pac-2* [20].

1.3. 2 DNA Replication

HCMV replication was initially thought to be relatively slow, with production of progeny virus requiring between 48 and 72 hours *in vivo*. However, more recently the *in vivo* doubling time has been predicted to be nearer 24 hours [21].

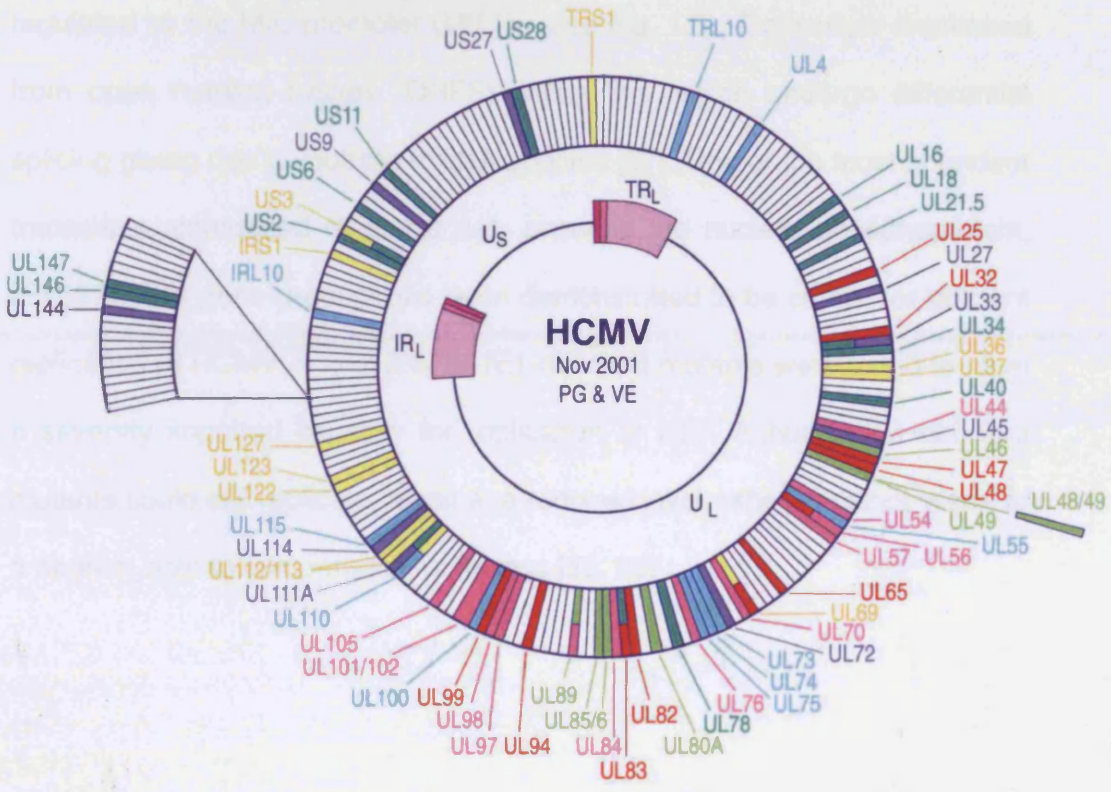


Figure 1. 1 HCMV genome structure kindly donated by Professors Griffiths and Emery (2001).

Similar to other herpes viruses, HCMV genes are expressed in a temporal, hierarchical manner. These are stratified according to the appearance of virus transcripts and consequently proteins in infected cells, namely: immediate-early (IE), early (E) and late (L) genes (Fig. 1.2) [22-24]. The first

group of genes to be expressed are known as the immediate early (alpha or IE) genes. Their expression occurs in the absence of *de novo* protein synthesis, as expression is unaffected by the presence of cyclohexamide and anisomycin [25] and is believed to be initiated by the tegument proteins ppUL82 and ppUL69 [26]. The most important of these genes are encoded by the major IE (MIE) locus of the HCMV genome, and their expression is regulated by the MIE promoter (MIEP – see Fig. 1.3). Transcripts expressed from open reading frames (ORFS) within this region undergo differential splicing giving rise to multiple mRNA species [27]. One of the most abundant transcripts, composed of exons 1-4, encodes the nuclear phosphoprotein, IE1 [27]. This gene product has been demonstrated to be critical for efficient replication of HCMV *in vitro* [28] as IE1-deficient mutants were found to have a severely impaired capacity for replication *in vitro.* Although IE1-deficient mutants could still replicate, albeit at a reduced level, other IE genes were, to a degree, able to compensate for its loss [28, 29] .

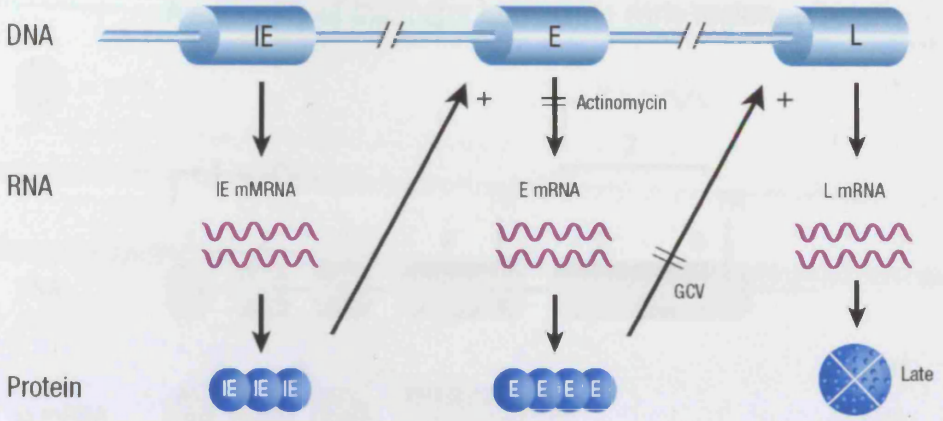


Figure 1. 2 Temporal Hierarchy of HCMV-expressed genes, picture provided by Professor Vincent Emery

IE gene products are required for the transactivation of the Early-phase HCMV genes (Beta or E genes) [30-32] and certain other host genes. Furthermore, they are also able regulate their own expression through binding MIEP [33]. The loci encoding IE regulatory proteins are UL36-38, UL122/123 (IE2 and IE1 respectively, both are encoded by the MIE region), UL84, UL112/113 and IRS1/TRS. UL112/113 gene products are transactivator proteins, which bind to and activate the UL54 promoter in association with other MIE proteins [34, 35]. Early-phase genes (E-genes) encode proteins necessary for viral DNA replication. Late phase (L genes) genes are only expressed following the onset of viral DNA replication and include genes encoding the structural components of the virion.

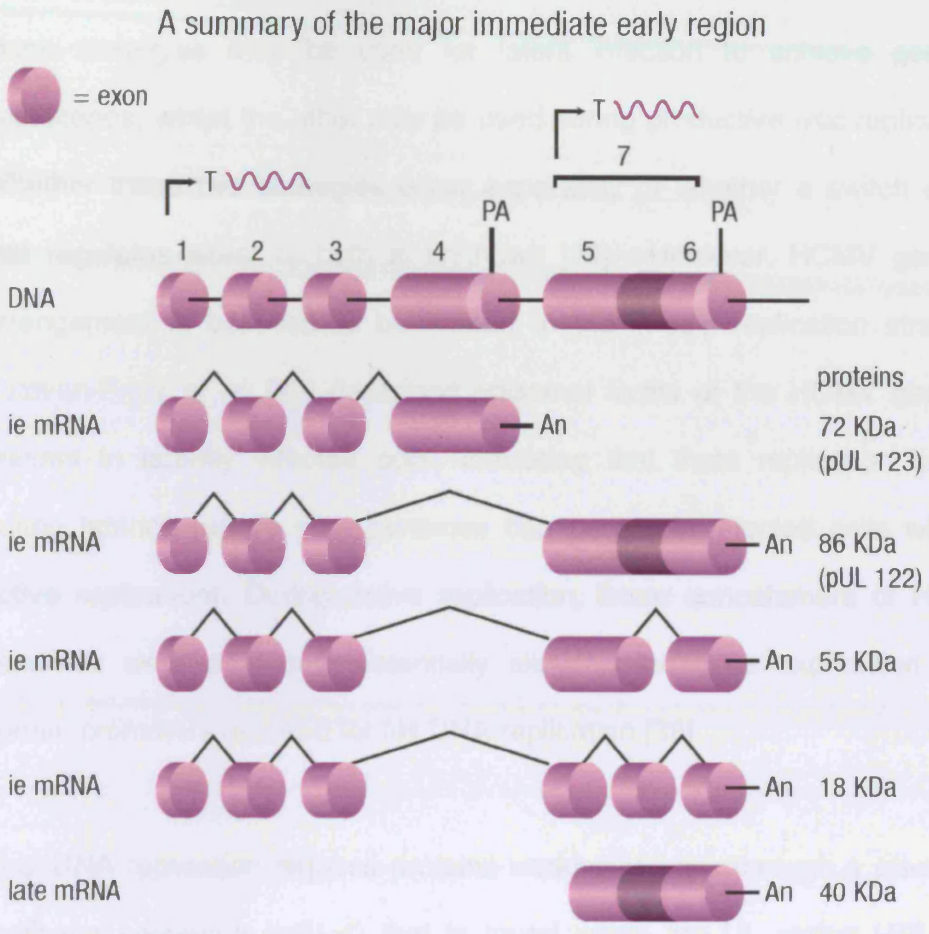


Figure 1. 3 The structure of the Major Immediate Early Promoter (MIEP), image provided by Professor Vincent Emery.

HCMV DNA replication occurs through both theta and rolling circle mechanisms, depending on such circumstances as the cell type and its differentiation state [36]. Theta replication would result in the production of closed-circular episomes, whereas rolling circle replication results in the formation of linear, unit-length, concatameric genomes, which can subsequently be packaged, cleaved and incorporated into virions [37]. These systems are analogous to those employed by certain bacteriophages, which

also have both lytic and lysogenic lifecycles; it is conceivable that one of these strategies may be used for latent infection to achieve genome persistence, whilst the other may be used during productive lytic replication. Whether these two strategies occur separately or whether a switch exists that regulates either or both is unknown [38]. However, HCMV genome arrangement is believed to be related to the virus's replication strategy. Bolovan-Fritts *et al.* [39] described episomal forms of the HCMV genome present in latently infected cells, indicating that theta replication occurs during latency (where viral genomes can persist in infected cells without active replication). During active replication, linear concatamers of HCMV genomes were produced, potentially allowing the gene expression from certain promoters required for full DNA replication [39].

Viral DNA replication requires proteins working in *trans* through a *cis*-acting replicator sequence (*ori*-Lyt) that is found within the UL region [40]. The expression of eleven genes is required for *ori*-Lyt-mediated replication. These include an origin-binding protein, pUL84, which associates with $IE2_{579}$ [41], a single stranded DNA-binding protein (pUL57) [42], a helicase-primase complex encoded by the genes UL70, UL102, UL105 [42] and the DNA polymerase itself UL54 with its accessory factor, a double stranded DNA-binding protein pUL44, which also co-precipitates with the uracil-DNA glycosylase homolog ppUL144 [43].

The replication mechanism used by HCMV is believed be analogous to that of other herpes viruses. Initial binding of pUL84 to the origin of replication initiates the cycle [44], where it is subsequently joined by the helicase-primase complex and pUL57 [45], which respectively unwind helical DNA at the replication fork, whilst priming for synthesis of the lagging strand and also preventing the re-annealing of unwound single-stranded DNA sequences [42]. These complexes create a bubble in the episome that, when joined at either end by the polymerase complex, proceeds to unwind the double stranded DNA and replicate the single strands in both directions; once this cycle is complete one strand is displaced and replication continues using the remaining strand [42].

Rolling circle replication occurs when the circularised genome is cut producing a 3' hydroxyl group and a 5' phosphate group [46]. Nucleotides are added to the 3' end synthesizing a new DNA strand using the inner circle of DNA as a template; this displaces the 5' end causing it to form linear double-stranded DNA through lagging-strand DNA synthesis, which is then cleaved forming genome-length concatamers [46]. Cleavage occurs through a terminase complex that is composed of pUL56 [47] and pUL89 cutting at the *pac1* site [48]. The pUL89 gene product was shown to be a part of this complex as BDCRB (2-bromo-5,6-dichloro-1-beta-D-ribofuranosyl benzimidazole – a potent inhibitor of HCMV DNA processing and packing) resistance maps to it [49, 50]. pUL56 also associates with linear DNA and viral nucleocapsids *in vivo*, and it has been suggested that pUL56 first associates with concatameric DNA through recognition of splice motifs

known as *pac* sequences [51]. Binding at these sites recruits pUL89, which cleaves the linear DNA into genome length copies; pUL56 is then thought to drive the translocation of the concatamer-terminase complexes in to procapsids using an ATP-dependent process [52, 53]. Because pUL56 is known to interact with procapsids this may be an early phase of DNA processing and packing for the assembly of mature virions by a mechanism analogous to that found in bacteriophages [49].

1.3. 3 DNA packaging and virus assembly

Upon cleavage at the *pac* sites, unit-length HCMV DNA, in a complex with pUL56, pUL89 and UL104, then associates with the preformed HCMV capsids. UL104 is believed to act as a portal protein that allows the viral DNA into the capsid [54]. *Pac-2*-directed cleavage causes the end of the sequence, denoted the S-terminal fragment, to be preferentially inserted into the maturing capsid rather than the L-terminal fragment near the *pac-1* element [55, 56].

The assembly of procapsids is controlled by the major capsid protein (MCP or UL86 [57]) and the precursor assembly protein protease (pAP or UL80), which is auto- cleaved to yield AP complexes [58]. These proteins associate in the cytoplasm and their interaction allows the translocation of the resultant complex into the nucleus [58, 59]. Once there, AP precursors form a scaffold around which minor capsid protein (mCP), along with mc and bp, form trimeric complexes, which in turn produce hexons and pentons. Together these form the dense core ring inside the pro-capsids. The small capsid

protein (SCP, UL48/49 [60]) then binds to the surfaces of procapsids and the MCP-AP complex dissociates through proteolytic cleavage mediated by UL80 [61]. Following this, viral DNA is packaged into the pro-capsids, displacing AP, this process is mediated by the UL97-encoded protein kinase [62, 63]. The virus particle then buds from the nuclear membrane, incorporating several virus glycoproteins during the process [4]. However, although some of the mature virions incorporate glycoproteins and lipid envelopes that are initially derived from the nuclear membrane, the tegument proteins are incorporated while this virus in the cytoplasm. This indicates that there is a process of re-envelopment occurs that allows incorporation of tegument proteins [64, 65]. Transport of the virus particle to the cell surface is inhibited by the Golgi-disrupting agent brefeldin A, indicating that it occurs via the Golgi [66, 67], which would consequently allow the N- and O-linked glycosylation of the virion constituents. The accumulation of virus particles in the golgi causes the formation large inclusion bodies causes a distortion in the shape of the nucleus giving it a kidney bean-like appearance [4]. Together these phenomena can be observed under a light microscope and give the characteristic Owls eye cytopathology associated with cytomegalovirus infection [68].

1. 4 Epidemiology and Transmission

HCMV infection, which is usually assessed through the presence of HCMV-specific IgG antibodies, is almost ubiquitous with seroprevalence ranging between 70-100% [69]. Seropositive individuals shed HCMV in most bodily fluids including blood, saliva, tears, breast milk, cervico-vaginal secretions and urine [4]. Numerous factors also influence the prevalence of HCMV such as: socio-economic conditions, hygiene and proximity of contact with others [70]. Prevalence generally increases with age and transmission can occur both horizontally and vertically [4]. Vertical transmission arises as a consequence of either intrauterine infection, perinatal infection [71] or through ingestion of breast milk [72, 73], which itself accounts for the bulk of vertical infections [74]. Transplacental HCMV transmission is most frequently associated with neonatal morbidity although only 5% of congenitally infected babies display symptoms [75].

Horizontal transmissions occur throughout life but are most commonly acquired during childhood [76]. Child-to-child transmission in day care centres is responsible for many early infections [76], possibly through 'mouthing' of hands and toys by infants [77-79] . Sexual contact is an important means of virus spread in later life, where an individual is exposed to bodily fluids from an infected partner [80].

The majority primary infections following horizontal transmission are asymptomatic in the immunocompetent, although a minority of cases are associated with an infectious mononucleosis-like syndrome often mistaken

for EBV mononucleosis [81]. The incubation period of HCMV, which takes into account the date of primary infection and the onset of virus shedding, has been predicted to be anywhere between four and eight weeks [82].

Following primary infection, HCMV achieves life-long latency in the immunocompetent. Latency can be defined as the persistence of the viral genome that cannot be detected in leucocytes or secretions taken from patients by conventional laboratory techniques [83]. Latency is punctuated throughout life by asymptomatic sporadic shedding normally controlled by immune responses [83], this can be shown through detection of infectious virus particles in both urine and saliva [82]. Consequently, HCMV-associated diseases usually occur in the immunocompromised, where the immune response is either insufficient or inappropriate. Examples of patient groups most likely to be affected by HCMV-associated diseases are - neonates, HIV-infected individuals or transplant patients who require immunosuppression to prevent rejection of transplanted organs.

1. 5 Latency

Following the resolution of primary infection, HCMV is able to establish lifelong latency. One site of latency is the bone marrow [84] where HCMV is thought to reside in $CD34^+$ haematopoietic progenitor cell populations [84-86]. These cells differentiate into $CD33^+$ $CD10^+$, $CD1a^+$ and $CD14^+$ or $CD15^+$ myeloid-committed dendritic cell populations on infection, this population supports HCMV latency while allowing the restricted expression of several latency-associated transcripts [86].

Full reactivation of HCMV from latency can be observed when latently-infected monocyte-derived DC progenitor cells are stimulated either with fibroblast-conditioned medium or with IFNγ, TNFα, IL-4 or GM-CSF; this process leads to further differentiation and/or activation events in these cells [87-90]. Reactivation of murine cytomegalovirus (MCMV) has also been shown in monocyte that were induced to differentiate into mature macrophages [91-94].

Such events induce the MIEP to express IE genes through activation of protein kinase C (PKC) and nuclear factor kappa B (NF-κB) [95]. These are involved in the remodelling of the differentiation-dependent chromatin structures associated with the promoter [96, 97], as histone acetylation has been shown to control lytic gene expression, thus resulting in HCMV lytic gene expression [32]. In fully permissive cells the MIEP associates with acetylated histones, while in non-permissive cells, such as monocyte derived macrophages (MDMs), the MIEP associates with heterochromatin protein 1,

which has been associated with a gene silencing activity. Differentiation-dependent expression patterns of HCMV has also been shown in other cell types that may similarly act as latency reservoirs [98].

These issues suggest that an environment where the latency reservoir is exposed to inflammatory cytokines, which frequently occurs as a result of physiological stress to the host, induces the differentiation and consequently activation of latently infected cells that in turn reactivates of HCMV [87].

Other findings suggest that whole virus particles can accumulate, and therefore achieve latency, in the vacuoles of monocyte-derived macrophages (MDMs) [68]. These vacuoles are believed to derive from the golgi complex but do not form part of either the early or the late endosomal compartments, as they lack endosome-associated markers such as rab5 and transferring receptor and lamp 1 (CD107a) and lamp2 (CD107b) respectively [68]. The kinetics of HCMV replication have been shown to be slower in HCMV-infected MDMs; with the appearance of early and late gene transcripts peaking at 5-7 days post-infection compared with infection of fibroblast lines which peaks at 5-48 hours [92].

Perturbations in the microtubule transport networks have been shown to occur in MDMs 13-15 days post-infection [99]. This would have several consequences: vacuoles would be prevented from fusing normally with lysosomes, thus enabling virus particles to avoid the immune response while preventing virus egress [99] and the fusion of vacuoles with the plasma

membrane may also result in cytopathic effect [68]. HCMV is also known to infect "immune privileged sites" [97, 100] such as the retinal pigment epithelium (RPE), infection of such sites would render the virus protected from immune responses [97]. Furthermore, HCMV has developed multiple immune-evasion strategies that would render many infected cells undetectable by immune surveillance (see below).

1. 6 Infection of Solid Organ Transplant Patients

Immunosuppressive therapies are necessary to prevent the rejection of donated organs in patients requiring solid organ transplants. Without such regimens the cellular immune responses of the recipient would reject the donor organ on the basis of it being "non-self". However, the resultant decrease in T-cell populations would predispose patients to certain previously-controlled infections, such as CMV - these in turn can cause disease, and in the context of solid organ transplants the major cause of morbidity is HCMV.

Several factors affect the risk and severity of HCMV-associated disease, such as the level and duration of immunosuppressive treatments, the organ's source (i.e. live or cadaveric donor), poor HLA-matching of the organ to the recipient, the type of organ transplant and, most crucially, the donor/recipient HCMV serostatus [101, 102]. As mentioned previously, HCMV remains latent in the majority of individuals but can be reactivated by immunosuppressive therapies, cytotoxic drugs and inflammation. Inflammation would result in the release of inflammatory cytokines such as tumour necrosis factor-alpha

(TNFα); these, in turn, activate nuclear transcription factors such as NF-κB and PKC, which stimulate the HCMV major immediate early promoter (MIEP) and allow HCMV to reactivate from latency [103-105]. It has been demonstrated in numerous patient groups that the extent to which the virus replicates, as determine by the viral load, can also be a determining factor of determining the risk of disease [106-108].

In transplant patients HCMV replication may result either from the primary infection of a seronegative recipient by the organ from a seropositive donor (D^+/R^-), a reinfection from the seropositive donor organ in a seropositive recipient (D^+/R^+) [109] or through reactivation of virus in the seropositive recipient (D^+/R^+ or D^-/R^+) [110]. Because 60-80% of adults are seropositive for HCMV the likelihood that either the donor or the recipient harbours the virus is high. In the context of renal transplant patients, 15-50% excrete virus post-transplant and of these 30-40% develop HCMV-associated disease [106, 111]. Patients can be divided into high and low-risk categories based on their susceptibility to developing HCMV-associated disease. Individuals at highest risk of developing HCMV-associated disease are patients who were seronegative (D^-R^-)prior to transplant and receiving organs from seropositive donors (D^+R^-) [70].

Low-risk patients, such as those who are either HCMV-seropositive prior to transplant or those that receive an organ from a seronegative donor, can still experience febrile illness and end-organ disease through virus reactivation, although this is less likely to cause serious illness [109]. HCMV-seropostivity

consequently affords a level of protection against HCMV-disease; this may be due to pre-existing humoral or virus-specific cellular component of the immune responses, which may be retained to some degree despite immunosuppression. Seropositive patients can still suffer from HCMV disease should they receive an organ from a seropositive donor, these incidences of disease probably occur as a result of reinfection from the seropositive organ rather than through reactivation of the virus by the recipient [109].

Determination of HCMV serostatus prior to transplant therefore gives valuable prognostic information on the potential risk of HCMV-associated disease. It also allows the incidence of disease to be drastically reduced by avoiding the transplantation of organs from seropositive donors to seronegative individuals; although this is not always possible due to the low number of donors, consequently high risk individuals need to be identified and may be placed on antiviral regimens to reduce the potential for infection and possible subsequent disease.

1. 7 HCMV Pathology

Without treatment up to 60% of high-risk D^+R^- renal transplant patients will develop HCMV disease from the resultant primary infection [105, 112]. This can either be a self-limiting syndrome, which includes symptoms such as leukocytopenia, fever, malaise and febrile illness manifested during virus excretion - both are resolved in the majority of patients [4]. Yet some of these patients progress to develop more severe HCMV-associated diseases such as pneumonitis, retinitis, nephritis, gastrointestinal ulceration, severe hepatic dysfunction and impaired graft function [4]. Individuals in this high-risk group have no pre-existing virus-specific immunity; their requirement for immunosuppression also limits the number of T-cells available to establish a subsequent HCMV-specific adaptive response. Immunosuppression also predisposes individuals to secondary infection by pathogens such as *Pneumocystis jiroveci* and *carinii*, *Candida*, *Nocardia asteroids, Cryptococcus neoformans* and *Aspergillus* along with other bacterial pathogens [113, 114]. In fact >90% of solid organ transplant patients have been reported to have developed these infections while they were infected with either HCMV of other immuno-modulating viruses, indicating that HCMV could result in further immune-suppression [115].

Primary HCMV infection has also been linked with allograft injury (through fibrosis), dysfunction and rejection, although the exact mechanisms mediating rejection are unclear. It is possible that HCMV can do this either directly through expression of MHC-I homologues on infected donor cells or indirectly through the altered expression patterns of MHC molecules that

occurs as a direct result of infection, these would render infected cells more vunerable to recognition and subsequent rejection as "non-self" [116, 117]. Other indirect effects of HCMV that may mediate rejection could include the virus' ability to upregulate the expression of proinflammatory cytokines and adhesion molecules. Indeed, HCMV infection has been shown to upregulate expression of adhesion molecules ICAM-1 (CD54) and LFA-3 (CD58) *in vitro* despite the presence of ganciclovir and foscarnet, indicating that this process is mediated by early virus transcripts and may also exacerbate organ rejection [118]. HCMV infection of fibroblasts has been shown to directly upregulate secretion of the proinflammatory cytokines IL-6 and fractalkine [119], both of which have antiviral activities in addition to being able to cause vascular inflammation and may also result in rejection [120].

Although it occurs infrequently in solid organ transplants, HCMV retinitis is common in other immunocompromised groups. HCMV-infected retinal pigment epithelial (RPE) cells are exposed to higher levels of both IFNγ and TNFα. These cause HCMV reactivation, via a NF-κB-independent mechanism [121] and immunopathology ensues through upregulation of FasL by immediate early/early viral transcripts on infected cells [122, 123]. Ligation of Fas expressed on activated leucocytes by FasL on infected cells results in their apoptosis, an acute infection can normally be resolved in this way but chronic virus replication may result in the prolonged destruction of the RPE and hence retinitis.

Some pathologies that occur as a result of HCMV infection may be immune-driven where virus specific immune responses result in the destruction of HCMV-infected tissues. Pneumonitis is one such example; detection of NK cells and $CD4^+$ and $CD8^+$ T-cells has been shown in the bronchoalveolar lavage (BAL) of bone marrow transplant patients and proposed to contribute to inflammation through destruction of HCMV-infected lung tissue [124]. Yet increased frequencies of infiltrating $CD4^+$ and $CD8^+$ T-cell have previously been shown to be protective against pneumonitis, and frequencies of both of these T-cell populations are much decreased in these individuals compared to normal healthy controls [125]. It was therefore suggested that a possible cause of this inflammation was the secretion of high levels of TNFα by alveolar macrophages stimulated by NK cells through IFNγ [125]. HCMV-driven immunopathology through virus-specific $CD8^+$ T-cell has also been suggested as a cause of gastrointestinal disease in HIV-1 co-infected individuals. Here gut-associated lymphoid tissues (GALT) from two HIV-1-infected individuals with chronic diarrhoea were shown to contain high frequencies of HCMV-specific $CD8^+$ T-cells [126].

1. 8 Drugs

1.8. 1 Immunosuppressive Drugs for Solid Organ Transplants

Cyclosporin

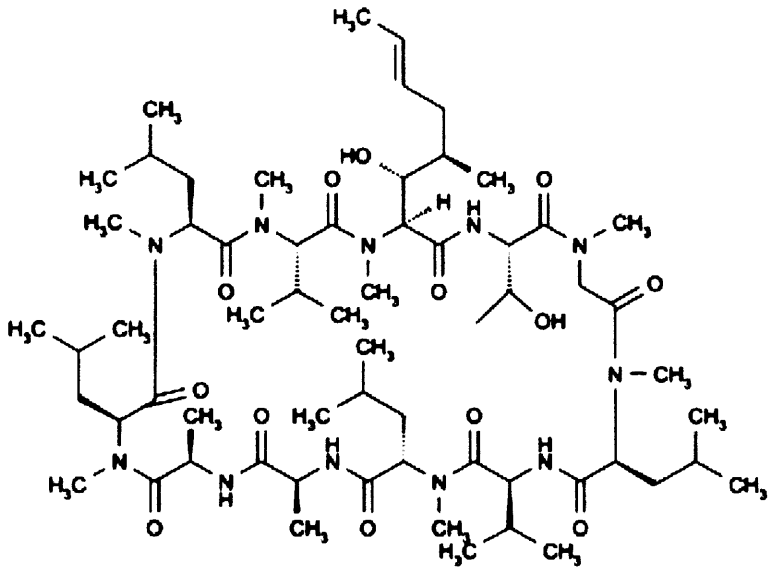


Figure 1. 4 Cyclosporin

Cyclosporin is cyclic peptide of 11 amino acids that was isolated in Norway in 1970 from two species of fungus [127, 128]. It functions by associating with cyclophilin and this heterodimeric complex then inhibits the dephosphorylase activity of calcineurin, which prevents the translocation of the nuclear factor for activated T-cells (NFAT) into the nucleus [129, 130], where it would otherwise activate the transcription of IL-2 and other cytokines [131-134]. Since IL-2 major growth factor for T-cells a lack of IL-2 results in the inhibition of T-cell proliferation.

Tacrolimus

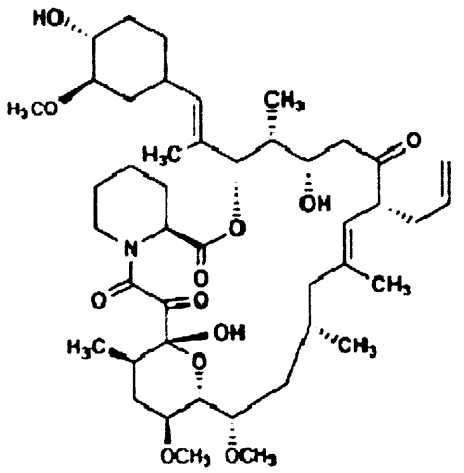


Figure 1. 5 Tacrolimus

Similar to cyclosporine, Tacrolimus (FK506) is a fungal metabolite; in this case it was isolated from *Actinomyces* spp38 [130, 135]. Together with FKBP (FK506-binding protein) it forms a complex that inhibits the dephosphorylation activity of calcineurin [128], thereby preventing the transcription of IL-2 and thus prevents lymphoproliferation [128, 136].

Mycophenolate mofetil

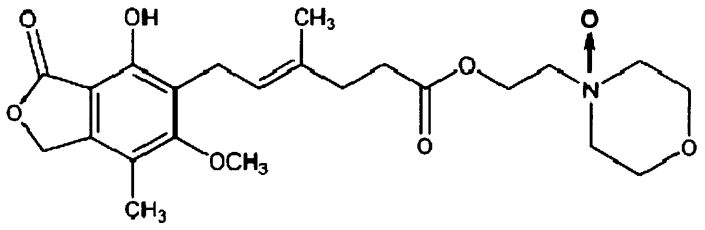


Figure 1. 6 Mycophenolate mofetil

Mycophenolate mofetil (MMF or RS-61443), is the ester derivative of mycophenolic acid (MPA). Once ingested, MMF is hydrolyzed in the intestine and blood by esterases to become MPA, which reversibly inhibits eukaryotic inosine monophosphate dehydrogenases (IMPDH) [137]. This prevents the *de novo* synthesis of guanosine monophosphate (GMP) [138-140], which consequently leads to decreased levels of guanosine triphosphate (GTP) and hence reduces deoxyguanosine triphosphate (dGTP) [141]. This has a cytostatic effect on cells, which is more pronounced in lymphocytes than in other cell types as they require *de novo* synthesis of purine nucleotides whereas other cells do not [142].

Corticosteroids

Prednisolone is one of a family of immunosuppressive corticosteroids used in the transplantation milieu. These are believed to associate with intracellular glucocorticoid receptors (GRs). Together these complexes move into the nucleus where they dimerize and then bind GR-binding motifs at target gene promoters and block expression of various genes encoding cytokines and enzymes [130, 143, 144]. There is also evidence that corticosteroids can suppress transcription factors such as the Jun/Fos complex (AP1) and NF-κB, thereby preventing the transcription of genes such as IL-2 and other cytokines [130, 145, 146].

1.8. 2 Antiviral Therapy

There are two main strategies for preventing HCMV disease in transplant patients: prophylactic and pre-emptive therapy. Prophylactic therapy can be further subdivided into universal prophylaxis - where antivirals are administered universally to all patients post-transplant, and targeted prophylaxis - where antiviral drugs are administered to all individuals deemed to be at high risk of developing HCMV-associated disease, such as those that are D^+/R^-, commencing immediately after transplantation. Incidences of HCMV replication in liver transplant patients undergoing prophylaxis with oral ganciclovir has been shown to be significantly reduced [147]. Prophylactic administration of three daily doses of oral ganciclovir or a once-daily dose of valganciclovir for 100 days post-transplant were compared in >350 D^+R^- solid organ transplant. Results demonstrated valganciclovir to be better at preventing HCMV disease during the period of prophylaxis than patients treated pre-emptively [148, 149]. Prophylaxis is also preferable for the treatment of other herpes virus infections and in reducing the risk of HCMV mediated graft rejection in transplant patients [150].

However, several studies have established that a large proportion of D^+R^- individuals on prophylaxis display HCMV disease in the proceeding months following cessation of treatment, therefore prophylaxis could be argued only to delay the onset of HCMV disease and not prevent it [114, 147, 148]. Other potential problems include the unnecessary treatment of individuals who would not develop HCMV with antiviral drugs; not only would this be expensive, but it would result in the patients being exposed to the potentially

toxic side-effects of the antiviral drugs. Indeed, prolonged prophylaxis with ganciclovir and foscarnet is myelosuppressive and may result in mortality due to fungal infection and HCMV disease [151, 152]. There is also an increased risk of the emergence of ganciclovir resistant mutants, with mutations predominantly occurring in UL97 and UL54 genes, in patients that are subjected to prolonged treatment with suboptimal levels of ganciclovir [153].

Pre-emptive therapy (PET) is in many ways a preferable strategy as it relies on frequent monitoring of transplant patients for HCMV replication by either antigenemia or frequent measurements of viral load [154]. Treatment is initiated only when virus replication is detected, thus preventing exposure of non-viraemic patients to the toxic side-effects of the drugs, whilst also minimizing the costs [155] . However, this method relies on the ability to rapidly and reliably detect systemic virus replication and frequent and prolonged monitoring of individuals at high risk of developing disease.

Studies have shown pre-emptive therapy with ganciclovir to be more effective in preventing HCMV disease in liver transplant patients than prophylaxis with aciclovir, with 4% patients on pre-emptive therapy suffering HCMV disease compared to 29% on aciclovir prophylaxis [156]. Another comparison between prophylactic and pre-emptive therapy, using ganciclovir on lung transplant patients, showed pre-emptive therapy to be as safe but also more cost-effective [155]. One important advantage of PET is that high-risk D^+R^- patients are exposed to small amounts of virus antigen prior to

initiation of therapy, these would be available to the immune system during the initial replicative burst [151, 154]. Such antigen exposure should be sufficient to prime adaptive HCMV-specific humoral and cell-mediated responses that would be useful for controlling future episodes of HCMV replication [157]. This might explain why a large proportion of individuals subjected to prophylactic treatment suffer HCMV viraemia in the 6 months after cessation of prophylaxis [157]. Studies in bone marrow transplant patients comparing the use of pre-emptive ganciclovir to prophylactic ganciclovir showed an increase in both the incidence of invasive fungal infections and the incidence of other virus infections in individuals receiving ganciclovir prophylaxis [158], although this is probably due to the neutropenia associated with prolonged use [158].

Ganciclovir (and Valganciclovir)

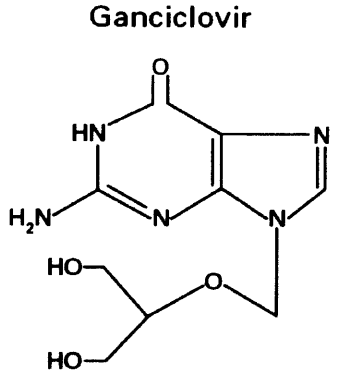


Figure 1. 7 Ganciclovir

Ganciclovir (9-(1,3-Dihydroxy-2-propxy)-methylguanine, GCV) is a deoxyguanosine analogue. It has a much improved (one hundred-fold) efficacy against HCMV infection compared to HSV infection [159]. Although HCMV lacks a thymidine kinase, mono-phosphorylation of GCV is achieved by the HCMV-encoded protein kinase, UL97 [160, 161]. GCV is successive di- and tri-phosphorylated by cellular kinases [161]. The tri-phosphorylated form of ganciclovir selectively inhibits the viral DNA polymerase by competing with dGTP for incorporation into the elongating viral DNA. However, unlike acyclovir it is not an obligate chain terminator because of the presence of a 3' hydroxyl group, this also results in some cell toxicity [162]. GCV resistance maps to at least one mutation at codons 460, 520 or 591-607 [163-165] within the UL97 locus, as this gene product alone controls phosphorylation of GCV in HCMV-infected cells [166-168]. Other, rarer, resistance mutants also map to the viral DNA polymerase UL54 [163]. These

may occur as a result of the prolonged, sub-optimal use of GCV during maintenance therapies or possible as a result of prophylaxis.

In infected cells GCV triphosphate accumulates to levels that are one hundred-fold higher than in uninfected cells [169]. Ganciclovir has proven useful in the treatment of HCMV retinitis in HIV patients [170], although side-effects such as granulocytopenia and thrombocytopenia can occur as a result of its use [171]. Furthermore oral administration of GCV has the increased benefit in retinitis treatment of having fewer side-effects, such as sepsis and neutropenia, which are associated with intravenous administration [172]. Treatment of pneumonitis with ganciclovir has been shown to be a success in renal transplant patients [173], although not in BMT patients [174].

Valganciclovir is a valine ester prodrug of ganciclovir, which increases the oral bioavailability of ganciclovir [175]. Activation of valganciclovir into ganciclovir occurs on its hydrolysis by intestinal and hepatic esterases; its subsequent mode of action is therefore the same as ganciclovir. Plasma drug levels achieved with oral 900mg valganciclovir once-daily are 1.7-fold greater than to 1000mg of ganciclovir taken thrice-daily and is as effective as three daily does of 5mg/kg ganciclovir taken intravenously (i.v.) [175, 176].

Foscarnet

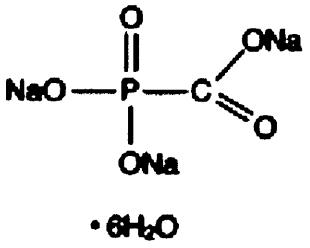


Figure 1. 8 Foscarnet

Foscarnet (Trisodium phosphoformate) is a organic analogue of inorganic pyrophosphate [177, 178]. Foscarnet functions by non-competitively inhibiting the virus DNA polymerase by blocking the pyrophosphate-binding site [179]. Foscarnet is able to the inhibit replication of all herpes virus DNA *in vitro* and also has been shown to have antiretroviral activity. Furthermore, at treatment concentrations foscarnet does not bind to eukaryotic DNA polymerases. It is used as an alternative treatment in individuals with ganciclovir resistance, and is particularly effective in the treatment of HCMV and HIV retinitis in AIDS patients, where it benefits from its antiretroviral activity [180, 181]. The main draw-backs of its use are its high nephrotoxicity and its poor oral bioavailability, which requires it to be administered intravenously three times daily [182].

Cidofovir

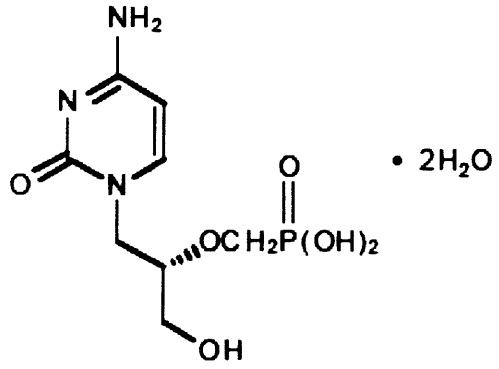


Figure 1. 9 Cidofovir

Unlike other nucleoside analogues Cidofovir does not require phosphorylation by viral thymidine kinases or HCMV UL97 to achieve its antiviral effect. It is instead phosporylated to its active form (Cidofovir-diphosphate) by cellular enzymes, which are present in both infected and non-infected cells [183]. Cidofovir-diphosphate competitively inhibits dCTP incorporation into the elongating DNA by DNA polymerase [183, 184]. Cidofovir also has a prolonged half-life when compared to ganciclovir and foscarnet; this enables less frequent administration of treatment which is required to be administered intravenously (either once weekly or every other week) [171]. Like foscarnet cidofovir is highly nephrotoxic and must therefore be administered with probenecid to prevent irreversible kidney damage [185]. It has been used successfully in the treatment of HCMV-retinitis in AIDS patients [186].

1. 9 Immune Responses to HCMV

As mentioned before, HCMV establishes a life-long latent infection in myeloid-lineage cells in immunocompetent individuals [87], with sporadic virus shedding occurring at mucosal surfaces. Innate immune responses (such as those provided by natural killer cells and complement) and humoral immune responses both play important roles in controlling HCMV infection. Adaptive cellular immune responses are crucial in controlling HCMV replication and any disruption in T-cell responses; either through immunosuppressive treatments required by transplant recipients or becoming immunocompromised following infection with HIV leads to uncontrolled virus replication and HCMV-associated disease.

1.9. 1 Innate immune responses to HCMV

1.9.1. 1 NK cells

Natural killer (NK) cells provide protection against primary HCMV infection, clinical evidence supporting this comes from reports of a NK cell-deficient patient who succumbed to severe HCMV infection [187]. NK cells are recruited to infected tissues and activated by macrophage-derived cytokines, such as IL-12, TNFα, IFNα and IFNβ, induced by HCMV infection between 2 to 4 days post-infection [188]. Synergistic stimulation of NK cells by IL-12 and TNFα also increases the ability of NK cells to produce IFNγ [189]. NK cells are able to recognise virus-infected cells through alterations in MHC class I expression or glycosylation of other cell-surface glycoproteins that occur as a result of viral infection, leading to the "missing-self" hypothesis [190]. On ligation with a further 'activation signal', provided by ligation of

molecules such as NKG2D, NK cells are able to lyse infected cells through release of cytotoxic granules [191]. Following stimulation with IL-12 (in synergy with TNFα) NK cells are also able to secrete large quantities of IFNγ, which attract and activate components of both innate and naïve adaptive immune responses [188].

Avoidance of NK responses by HCMV

HCMV-mediated down-regulation of MHC I molecules on infected cells serves as a mechanism for avoidance of the CD8 T-cell response (see below). However, it renders infected cells more susceptible to NK cell recognition. HCMV has therefore developed multiple strategies for NK cell-avoidance; these include encoding decoy inhibitory molecules on infected cells, which are able to ligate inhibitory receptors on NK cell surfaces [192]. One HCMV decoy molecule is the MHC I homologue - UL18, which associates with β-microglobulin [193]. This complex migrates to the cell surface where it prevents NK cell-mediated lysis by associating with the NK-inhibitory receptor CD94 [194, 195]. Expression of UL18 has been shown previously in PBMCs isolated from immunocompromised organ transplant patients suffering active HCMV replication, this suggests that UL18 expression occurs coincidently at a time of potential NK-mediated lysis, possibly preventing recognition of infected cells [196]. The cellular receptors for UL18 have since been found to be related to killer inhibitor receptors (KIRs) found on the NK cells [197, 198].

HCMV infection also upregulates the expression of a non-classical MHC I molecule - HLA-E, on the surface of infected cells [199, 200] through the expression of the leader peptide of gpUL40. The product of the UL16 gene has been found to bind UL16-binding proteins (ULBPs) [201]; these are a family of MHC class I-like molecules that bind NK cells by interacting with NKG2D molecules on their surfaces. Through intracellular sequestration of NKG2D-binding proteins [202], UL16 prevents them overriding the inhibitory signals provided by NK cell recognition of MHC I molecules thereby allowing NK-mediated cytolysis [203]. More recently UL141 and UL142 have been shown to block NK cell activity. UL141 is thought to achieve this by blocking the surface expression of CD155 on infected cells; this prevents it ligating NK receptors CD96 and CD226 and thus blocks NK cell activation [204]. While expression of the MHC I-related HCMV-encoded UL142 gene has also been shown to selectively inhibit lysis by certain NK cell clones *in vitro* [205] .

1.9.1. 2 Complement-mediated immune responses

Complement proteins are another facet of the innate immune response and can function in a variety of ways. The 'alternative' complement pathway results in deposition of complement complexes on surfaces of virus-infected cells causing cell lysis; although HCMV can circumvent this through upregulation of complement control proteins (CCPs) on the surfaces of infected cells [206]. Complement also interacts with the humoral response, mediating antibody-dependent cytotoxicity (ADCC) via the Fc portions of virus-associated antibodies. HCMV is able to avoid this through encoding its own Fc receptor homologues, which are capable of binding and "mopping

up" opsonizing antibodies [207]. Furthermore, virus particles have been shown to incorporate complement control proteins, CD46, CD55 and CD59, into their envelopes, thus reducing complement-mediated lysis of virus particles [206, 208, 209].

1.9.2 Adaptive immune responses

1.9.2. 1 Humoral immune response

Individuals known to be seronegative prior to solid organ transplant are more susceptible to HCMV-associated disease than those who are seropositive; Indicating that circulating HCMV-specific antibodies, along with other adaptive immune responses confer some degree of protection against HCMV disease [4]. Moreover, infusion of high-titre HCMV-specific sera into pre-transplant patients reduces the incidence of disease associated with primary infection [210]. Antibody responses are normally directed against viral glycoproteins B (gB), gN, gM, and gH [211], the tegument phosphoprotein pp150 and the phosphoprotein pp52 [212]. Predictably, the majority of neutralising responses are focused towards viral envelope glycoproteins, specifically gB, gH [211] and the heterodimeric gN/gM complex to which 62% of seropositive donors mount a humoral response [213, 214] . Within gB there is linear antigen determining region (AD-1) against which the majority of seropositive individuals mount their gB-specific humoral responses towards. This region lies between codons 552-635 [215] and is highly conserved possibly because it has a functional role as an oligomerisation site required for the formation of gB multimers essential for the production of infectious viruses [216]. In addition to being able to directly

neutralise HCMV, humoral responses can also work in tandem with complement, as mentioned above.

1.9.2. 2 $CD8^+$ cell response

Virus-specific $CD8^+$ cells recognise short peptide stretches of 8-10 amino acid residues in length, presented to them on the surfaces of infected cells in the context of MHC class I molecules, which are expressed on all nucleated cells [217]. The peptides are normally derived from endogenously synthesised proteins, such as those produced by intracellular parasites like viruses [218]. Proteins are cleaved by the 20S/26S proteasome [219, 220] and transported into the lumen of the endoplasmic reticulum (ER) by the heterodimeric transporter associated with antigen processing (TAP) proteins [221]. Here the peptides are further trimmed to between 8-10 amino acids in length by an ER aminopeptidase [222-224]. The newly-formed MHC class I molecules are partially folded and transported to the lumen of the endoplasmic reticulum where they associate with the chaperone protein calnexin [189]. When the $Beta_2$-microglobulin associates with the MHC I molecule calnexin is displaced by a complex of different chaperone proteins, calreticulin and Erp57 [189]. The unloaded MHC I molecules then associate with TAP proteins through the adaptor protein tapasin [217]. Peptide fragments synthesised in the cytosol are then transported into the lumen of the ER through the TAP proteins and loaded into the MHC class I complex and complete the folding process at which point the stabilised MHC I/peptide complex is transported to the surface of infected cell via the golgi apparatus. At the surfaces they can be recognised by the TCR of $CD8^+$ T-cells [189].

However, the interaction between the peptide/MHC I complex and the TCR alone is insufficient for the activation of naïve T-cells, and would result in the formation of anergic cell populations. Subsequent co-stimulatory signals are required in order for their full activation [225] (Fig. 1.10). These co-stimulatory molecules are present only on the surfaces of antigen presenting cells (APCs), such as dendritic cells (DCs). Following antigen uptake, APCs home to secondary lymphoid organs where they encounter naïve CD8 and CD4 T-cell populations [189].

Co-stimulatory molecules include CD40L, which interacts with CD40 on the surface of the naïve T-cells [226], and members of the B7 family, which bind CD28 or CTLA-4 [227, 228]. Following these ligations, T-cells become activated and undergo clonal expansion; giving rise to a population specific for the presented antigen. In the case of $CD8^+$ cells, activation allows recognition of specific epitopes on the surfaces of infected cells and results in their subsequent lysis. This results either through release of perforin [229-231] and other lytic granules or through ligation of the Fas Ligand [232-235]. Ligation of the TCR with a cognate antigen in the context of MHC class I results in the mobilisation of intracellular Ca^{2+} ions, this in turn signals the mobilisation of secretory granules [236-238]. Pre-formed granules are then transported, in a polarised manner to the site of stimulation via microtubules using a kinesin family motor towards the microtubule organizing centre (MTOC). The MTOC is polarised towards the point of contact at the immunological synapse and therefore re-orientates the cytoskeleton towards the target cell [238-241]. The lysosome then uses actin-based movement to

travel the short distance from the periphery of the cell surface to the cell surface itself, whereupon the endosome and cellular membranes fuse using SNARE and Rab proteins and the contents of the lysosome are released into the periplasmic space adjacent to the target cell [189]. Both the release of cytolytic granules and the ligation of Fas receptors on target cell surfaces induces an apoptotic response and thus destruction of the target cell [231, 242, 243].

CTLs are also effective at controlling virus replication in parts of the body which would either be at risk of damage from a large cytolytic response, such as the brain or where infected cells would outnumber the CTLs by several orders of magnitude, such as in the liver. Here CTLs can control virus infection through the secretion of IFNγ and TNFα cytokines, as shown through adoptive CTL transfer experiments in HBV transgenic mice, where virus clearance is not just mediated by lysis of infected cells on recognition of cognate peptide [244-247]. These cytokines are not released in the stringent polarised manner that would be focused on an individual target cell; they are released into the general environment where they can affect a much greater number of infected cells without needing to destroy them directly [248]. IFNγ also functions by activating macrophages and DCs; enabling them to better phagocytose debris from virus infected cells and present them more efficiently by upregulating presentation by of MHC class I and II molecules, which would in turn permit better recognition by other CD8 and CD4 T-cell populations. Both IFNγ and TNFα can also activate NK cells, enabling them

to better recognise infected cells and produce cytokines such as IFNγ themselves [189].

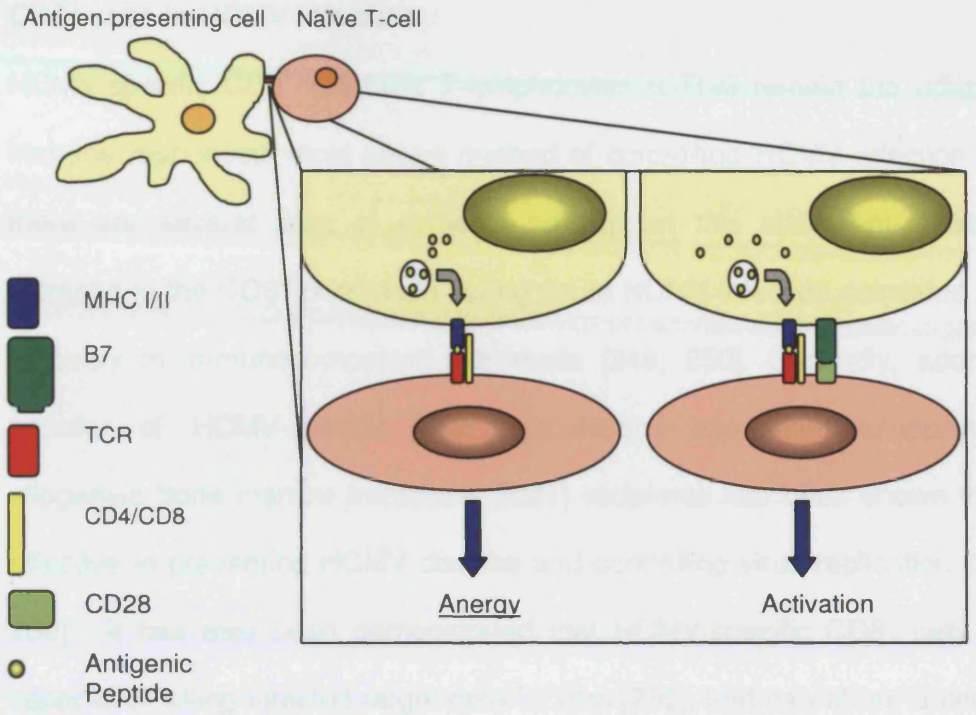


Figure 1. 10 Activation of Naïve T-cells by antigen presenting cells (APCs), these take up the virus or viral proteins and present them either in the context of MHC I or II, to $CD8^+$ or $CD4^+$ cells respectively. However, this is insufficient for activation of the T-cells and causes anergy. Co-stimulatory signals provided by B7/CD28 interactions provide signals for full activation and expansion of antigen-specific T-cell populations.

$CD8^+$ cells in HCMV infection

HCMV-specific $CD8^+$ cytotoxic T-lymphocytes (CTLs) remain the adaptive immune responses' most potent method of controlling HCMV infection and there are several lines of evidence to support this statement. First an increase in the $CD8^+$ population during acute HCMV infection coincides with recovery in immunocompetent individuals [249, 250]. Secondly, adoptive transfer of HCMV-specific $CD8^+$ populations into immunosuppressed allogeneic bone marrow transplant (BMT) recipients has been shown to be effective in preventing HCMV disease and controlling virus replication [251, 252] . It has also been demonstrated that HCMV-specific $CD8^+$ cells are capable of killing infected target cells *in vitro* [250], and can afford a certain degree of protection from HCMV infection in stem-cell and renal transplant patients [253-256]. Immunodeficiency that results either from HIV infection or through immunosuppression decreases the absolute number of $CD8^+$ T-cells, these in turn lead to a reduction in the ability of the patients to control HCMV replication thereby predisposing them to associated diseases.

Targets of the HCMV-specific $CD8^+$ response

The majority (70-90%) of HCMV-specific $CD8^+$ T-cells responses were initially believed to be targeted against several epitopes in the major late lower matrix phosphoprotein, pp65 (UL83) [257], with frequencies commonly reaching 10% of the total $CD8^+$ populations in certain healthy immunocompetent and immunocompromised individuals [258]. Indeed Lang *et al.* [259] described one healthy seropositive individual with 50% of their total $CD8^+$ response directed against the NLVPMVATV epitope within pp65.

These $CD8^+$ populations were found to be functionally more heterogenous in healthy than in immunocompromised individuals, with only small subpopulations having any functional HCMV-specific cytolytic activity [260].

Being a structural component of the virus, pp65 is processed and presented to the alternative MHC-I pathway after virus penetration and in the absence of virus gene expression [261]. Because pp65 is present prior to the expression of immediate early genes it is also able to phosphorylate another potent CTL target, IE1, thereby preventing its proteosomal processing and subsequent association with MHC I molecules [262]. This might enable pp65 to remain the immunodominant protein in the majority of infected individuals. However, in certain immunocompromised patient groups IE-1 has been found to be immunodominant [263]. Reddehasse [261] suggested two main reasons for this observation, first, mutations occurring in the kinase domain of pp65 prevents it from being able to phosphorylate IE1, and thus abrogating its processing. Second, several clinical isolates of HCMV have been found to express lower levels of pp65; which could render them less able to phosphorylate IE1. It is also conceivable that pp65-specific $CD8^+$ cell populations become exhausted due to the strong immunogenicity of the antigen, leading to increases in populations specific for other targets (see fig 2 for a list of known CD8 and CD4 epitopes). The presence of pp65 in infected cells prior to viral gene expression may also indicate that MHC I and II down-regulation by US2-11 gene products (see below) would not affect pp65 recognition and processing.

Recent work has demonstrated that the $CD8^+$ T-cell response is far broader than previously thought [264, 265]. Using overlapping peptide sets spanning the entire HCMV proteome other targets elucidated in this study included UL48 and UL122 (IE2), which, along with IE1 and pp65, form the targets to which the majority of individuals mount detectable HCMV-specific responses. Although the largest responses were directed against IE1, pp65, UL82 (pp71), UL36 and UL32, which together appeared to form the bulk of global HCMV-specific $CD8^+$ responses a range of other proteins were also targeted, although the role of that the other responses play in the control of HCMV is not yet fully understood.

$CD8^+$ epitopes				
pp65	**Start**	**Length**	**HLA Type**	**Reference**
NLVPMVATV	495	9	A2	[266]
VLGPISGHV	14	9	A2	[267]
MLNIPSINV	120	9	A2	[267]
QYDPVAALF	328	9	A24	[268]
VYALPLKML	113	9	A24	[269]
RPHERNGFTV	265	10	B7	[266]
TPRVTGGGAM	417	10	B7	[266]
EFFWDANDIY	511	10	B12	[262]
IPSINVHHY	123	9	B35	[270]
YYTSAFVFPTKDVAL	181	15	B35	[266]
VFPTKDVAL	187	9	B35	[266]
DDVWTSGSDSDEELV	397	15	B35	[266]
IHASGKQMWQARLTV	148	15	B52	[271]
GKQMWQARLTVSGLA	152	15	B52	[271]
TRQQNQWKEPDVYYT	169	15	?	[271]
NQWKEPDVYYTSAFV	173	15	?	[271]
IE1				
YILEETSVM	315	9	A2	[272]
ELKRKMIYM	199	9	B18	[273]
CVETMCNEY	279	9	B18	[273]
DEEDAIAAY	379	9	B18	[273]
EEEEGAQEER	354	10	A2	[274]
VLEETSVML	316	9	A2	[275]

CD4 epitopes				
pp65	**Start**	**Length**	**HLA Type**	**Reference**
LLQTGIHVRVSQPSL	41	15	DR15 (DQ6)	[271]
IIKPGKISHIMLDV	281	14	DR53 (DQ3)	[271]
GKISHIMLDVAFTSH	285	15	DR53 (DQ3)	[271]
PQYSEHPTFTSQYRI	361	15	DR11	[276]
EHPTFTSQYRIQGKL	365	15	DR11	[276]
FTSQYRIQGKLEYRH	369	15	DR11	[276]
KYQEFFWDANDIYRI	508	15	DR52	[276]
FFWDANDIYRIFAEL	513	15	DR52	[276]
PPWQAGILARNLVPMV	485	16	?	[276]
AGILARNLVPMVATV	489	15	?	[276]
ARNLVPMVATVQGQN	493	15	?	[276]
IE1				
DKREMWMACIKELH	162	14	DR8	[277]

Table 1. 1 List of known CD4 and CD8 epitopes identified in pp65 and IE1 proteins

$CD8^+$ responses during primary infection

Shortly after primary infection (~1 month), a powerful, focused HCMV-specific $CD8^+$ response arises. The bulk of this $CD8^+$ population has a lymphocyte-homing $CD62L^+$ phenotype and expresses high levels of granzyme (GrA), which is needed for induction of apoptosis in infected cells [278]. The increase of this population corresponds with a decrease in HCMV-DNAemia [279]. These populations control the initially infection and then differentiate into different memory subpopulations that are maintained at high levels throughout life.

Memory $CD8^+$ cells

In normal healthy individuals HCMV-specific $CD8^+$ T-lymphocyte responses are capable of controlling virus replication [265]. Several studies have demonstrated that large, heterogenous HCMV-specific $CD8^+$ populations are maintained throughout life, yet questions remain as to how these populations arise, how they differ functionally to populations focused against other viruses and how they are retained [260, 280, 281].

Because such populations are virus-specific and remain able to react to sporadic virus reactivation indefinitely, they are often referred to as "memory" T-cells. The initial paradigm for T-cell memory proposed that naïve cells and memory cells could be differentiated through their phenotypes. Naïve T-cells expressed the RA isoform of the molecule CD45, while memory T-cells displayed the RO isoform. These isoforms arise from differential splicing of the CD45 transcript [282, 283]. However, it is now clear that $CD45RO^{High}$

populations can revert back into a $CD45RA^{High}$ phenotype. Furthermore, it was initially believed that revertant cells, which were also $CD28^-$ and $CCR7^-$, were terminally differentiated and would thus die off. CCR7 is a chemokine receptor required for homing to secondary lymphoid tissues [284] therefore $CCR7^-$ T-cell subsets are been termed "effector-memory" populations because they are found in peripheral tissues and at sites of viral replication [284]. Similarly, $CCR7^+$ memory T-cells are termed "central memory" due to their presence in secondary lymphoid organs. During persistent HCMV infection the proportion of $CD8^+$ cells expressing the co-stimulatory receptor $CD28^+$ diminishes [285] and the proportion of these cells that become $CD57^+/CD85j^+$ increase [286, 287]. This phenotype also increases with age [288], from this one can surmise that the older the individual, the less naïve, or more antigen-experienced, the T-cell population and that this can be driven by HCMV infection [289].

Wills *et al.* reported that different $CD8^+$ sub-populations arise as a consequence of HCMV infection [290]. Naïve T-lymphocytes tend to display a $CD45RA^{High}/CD28^+/CD27^+/CCR7^+$ phenotype, while during acute primary HCMV infection pp65-specific $CD8^+$ populations display a $CD45RO^{High}/CD28^-/CD27^-/CCR7^-$ phenotype, and after recovery most of this population switches to a $CD45RA^{High}/CD28^-/CD27^-/CCR7^-$ phenotype; this was previously thought to be the phenotype for terminally differentiated T-cells [292] . Appay *et al.* [291] described a similar HCMV-specific $CD8^+$ population in healthy seropositive individuals; demonstrating that the same populations to exhibit shortened telomere length and contain high levels of

GMP, granzyme A (GrA) and perforin. Following *in vitro* stimulation with HCMV peptides this "terminally differentiated" population regained the ability to undergo clonal expansion, reverted back to the RO isoform, up-regulated CCR5 (expression of which is associated with homing to inflamed tissues, and retained strong cytolytic activity [290].

T-cell Memory in the Immunocompromised Host

Increases in the proportion of HCMV-specific $CD8^+$ cells in HCMV-seropositive liver transplant patients were noted 6 to 33 months post-transplantation when compared with healthy seropositive individuals [293] . These cells, which were predominantly of a $CD28^-CD27^-$ phenotype, also expressed high levels of granzyme B, perforin, and CD95 ligand and were thus classified as being "effector cells". These populations are likely to be the same as described in healthy individuals [291]. However, such populations would increase in magnitude with each corresponding bout of virus replication, this would be likely to result in increased frequencies of these populations in the immunocompromised host. Such antigen-experienced "effector-memory" populations with increased cytolytic potential were termed "late-memory" and contrast with the phenotypes of $CD8^+$ memory cell populations that become enriched following chronic infection with other viruses such as EBV, HCV and HIV [291].

Gamadia *et al.* [294] demonstrated that the majority of HCMV-specific $CD8^+$ cells found within renal transplant patient groups on immunosuppressive therapies were also of a $CD45RA/RO/CD27^-/CCR7^-/perforin^+/granzyme\ B^+$

late-stage phenotype. The suggestion being that the proportion of these HCMV-specific "effector-memory" cells rises with time in immunocompromised patients whilst the remainder of the $CD8^+$ population remains phenotypically stable. The same paper [294] reported that healthy seropositive patients and donors had a lower proportion of HCMV-specific $CD8^+$ cells to renal-transplantation recipients and that these were mostly of a "central" memory phenotype that resided within secondary lymphoid organs (i.e. $CCR7^+/CD27^+/CD28^-$).

In HCMV seropositive, immunocompromised HIV1-infected patients, both HIV-1-specific $CD4^+$ and $CD8^+$ populations steadily decrease while HCMV-specific $CD4^+$ and $CD8^+$ populations continue to expand [295]. One possible explanation is that virus-specific "effector-memory" T-cell populations are found at the site of viral replication. Since HIV-1 predominantly replicates in the lymphoid tissues, targeting $CD4^+$ cells that are $CCR7^+$, whilst also expressing CCR5, a co-receptor for HIV-1, $CD4^+$ cells become depleted and consequently HIV-specific $CD8^+$ populations cannot be maintained [296, 297]. HCMV-specific "effector-memory" $CD4^+$ and $CD8^+$ populations, which are $CCR7^-$, home to HCMV target areas such as the salivary glands, the retina and the lungs, thereby avoiding depletion in the lymphoid tissues through HIV1 replication [295].

Clonality of HCMV-specific CD8 populations

Within both immunosuppressed and immunocompetent seropositive populations, HCMV-specific $CD8^+$ populations tend to become oligoclonal, a phenomenon common to infection with other persistent viral infections such as Epstein Barr virus (EBV) and HIV-1 [298, 299]. These $CD8^+$ populations, which also tend to express the senescence marker CD57 [300] while being $CD28^-$, are believed to undergo massive expansion with each viral reactivation [301]. High viral loads frequently occur during immunosuppression and would therefore account for the large numbers of HCMV-specific cells in those individuals. The proportion of these cells also increases throughout life, albeit probably at a slower rate, with the average healthy adults having 5-20% $CD57^+$ CD28- T-cells [299, 302-304]. These cells are usually $perforin^+$ and yet show a decreased ability to proliferate on stimulation with cognate antigens [305].

Peggs and colleagues [306], confirmed work by Gillespie *et al.* [260], by demonstrating that HCMV-specific $CD8^+$ populations remain relatively high in immunocompetent patients following resolution of primary infection, this is consistent with the theory that there is a constant restimulation of the immune response throughout life by the virus. Paradoxically, viral loads, at least in the whole blood and plasma of immunocompetent individuals remain very low [307]. Such a phenomenon could be explained by the partial expression of early-phase HCMV proteins on the surfaces of monocytic cells which may continually stimulate CTL populations [298, 306] instead of active viral replication where all proteins would be expressed widening the targets

for the CTL response. In this scenario one would expect a large proportion of these $CD8^+$ T-cells to be IE1-specific yet this are not always the case.

Efficacy of the HCMV-specific CD8 response

One major consideration must be made of the HCMV-specific $CD8^+$ response, this is: are such large $CD8^+$ populations functionally active? Hassan-Walker *et al.* [308] reported that non-viraemic liver transplant patients undergoing tacrolimus regimens displayed 70% activation in their pp65-specific CTL population, this contrasted to 5% activation in healthy patients. In this study median expression levels of the activation marker CD38 were used to quantify the activation status of the CD8 population. Although tacrolimus regimens have previously been shown to reduce levels of both $CD4^+$ and $CD8^+$ T-cell activation, HCMV replication was still controlled [308]. The concept of a protective threshold level of HCMV-specific $CD8^+$ populations being required in order for control of HCMV infection was hence proposed. When $CD8^+$ population levels fall below this threshold the balance would tip in the favour of virus replication, which would consequently become uncontrolled. This balance is also influenced by viral load thresholds; in hepatic [108], renal [106] and bone-marrow [309] transplants, where viral loads may supersede this critical point possibly due of the lack of an effective T-cell response; this would potentially result in an increased risk of disease development.

As mentioned previously, high frequencies of HCMV-specific $CD8^+$ T-cells accumulate over time in healthy individuals. The highest frequencies would therefore be expected to occur in the elderly, where reactivations have occurred most frequently [310-312]. Studies investigating epitope-specific responses, using MHC class I tetramers, in the elderly show that although the percentages of CD8 cells able to recognise specific epitopes was highest in elderly populations [313], the proportions of this population that were able to secrete IFNγ in response to stimulation with the cognate peptide was much reduced when compared with younger individuals, rendering them dysfunctional [314]. Indeed, the sheer numbers of virus-specific cells that accumulate during the course of life might well result in a lack of circulating naïve $CD8^+$ T-cells in later years, potentially predisposing the elderly to other lethal infections [315-317]; diseases would therefore not occur as a direct consequence of the lower proportion of functional cells, but rather the decreased numbers of remaining naïve cells would be unable to cope with new pathogens [317, 318].

Some immunosuppressed individuals also still suffer episodes of HCMV replication, and therefore might be prone to associated diseases, in spite of large virus-specific $CD8^+$ populations. This suggests that the size of the HCMV-specific response might not be as critical as its antiviral quality. Studies have demonstrated that CTLs specific for HCMV contained perforin and could therefore be classed as being potentially cytolytic and yet these same populations are less able to produce IFNγ on stimulations [319]. These cells may correspond to senescent $CD57^+$ populations that are potentially

cytolytic although they have a decreased capacity to proliferate [305]. While cytotoxicity is important in the control of most virus infections, it can sometimes result in an inappropriate response that causes immunopathology, especially when the magnitude of antigen-specific CTLs is so large. This suggests that a heterogenous $CD8^+$ T-cell population would be the best way of controlling HCMV infection.

1.9.2. 3 $CD4^+$ cell function

$CD4^+$ cells recognise peptides of between 13 and 18 residues in length presented in the context of MHC II molecules found solely on the surface of antigen-presenting cells (APCs). Once fully activated, a process also requiring co-stimulation, $CD4^+$ cells differentiate further into either of two distinct populations, these can be defined by their cytokine profiles, Th1 and Th2. The basis for this polarisation is influenced by several factors such as the cytokine milieu in which activation occurs, antigen dose, the ratio of dendritic cells (DCs) to T-cells and the polarity of DC response (either DC1 or DC2). During primary infections a rise in the Th2-polarised $CD4^+$ T-cell population, which secretes cytokines such as IL-4, IL-5 and IL-13, drives antibody production. During the peak of acute infections Th1 responses tend to predominate, these can be characterised through their secretion of IFNγ, IL-2, IL-12 and TNFα. These cytokines can activate APCs, NK cells and help drive the proliferation and maintenance of $CD8^+$ T-cells responses. As mentioned previously, CD4 populations are unquestionably essential for the maintenance of functional virus-specific $CD8^+$ responses [320] , indeed $CD8^+$

T-cell populations specific to LCMV in adult mice could not be sustained in their absence [321, 322].

HCMV-specific CD4 response

The role played by $CD4^+$ T-lymphocytes during HCMV infection is less well defined than with $CD8^+$ cells yet both are crucial for control of HCMV; several line of evidence support this view. HCMV replication in the salivary glands, a major site of HCMV persistence and an organ where $CD8^+$ CTLs have little or no effect, has previously been shown to be controlled by IFNγ and TNFα-secreting Th1 CD4 cells [323, 324]. Active HCMV end-organ disease in seropositive patients was also seen to correlate with low frequencies of TNFα-secreting $CD4^+$ cells. Furthermore, $CD8^{-/-}$ knock-out mice are seen to prevent MCMV disease with similar kinetics to wild-type mice; this suggests a compensatory role for the $CD4^+$ population in HCMV control [325]. Moreover, the control of HCMV replication through adoptive transfer of HCMV-specific $CD8^+$ clones in BMT patients correlated with levels of HCMV-specific $CD4^+$ cell help [251, 326]. Furthermore, dysfunction and poor reconstitution of HCMV-specific $CD8^+$ T-cells has been demonstrated in allogeneic stem cell transplant patients with low frequencies of CMV-specific $CD4^+$ cells [327].

Targets of HCMV-specific CD4$^+$ response

Using overlapping synthetic peptides, Kern *et al.* [271] , demonstrated that more than 60% of normal healthy HCMV seropositive patients had a pp65-specific CD4$^+$ T-cell population with a frequency of approximately 2 cells per 1000. This surprisingly high proportion of the CD4$^+$ cell population being directed towards pp65 was explained by its being an abundantly expressed tegument protein that would likely be presented to CD4$^+$ cells in the context of MHC II following either endocytosis or phagocytosis of the virus. Glycoprotein B (gB) of HCMV has also been shown to be a target for CD4$^+$ cells. Using cytotoxicity assays Gyulai *et al.* [328] and Hopkins *et al.* [329] showed gB-specific MHC II-restricted lysis in around 30% of their seropositive patients. More recent studies have also shown other targets for the CD4$^+$ response such as UL86, UL32, UL99 and UL153 in addition to pp65 and gB constitute the majority of global HCMV-specific CD4 response [264].

CD4$^+$ responses during HCMV infection

Analysis of HCMV-specific CD4 and CD8 populations of seronegative patients during primary infection following renal allografts shows that CD8$^+$ levels dominate during primary infection and upon ganciclovir treatment, decrease along with viral load [330]. However, as viral loads decrease the HCMV-specific CD4$^+$ response increases; this correlates with findings of large HCMV-specific CD4$^+$ populations in patients many years after seroconversion. It has therefore been suggested that whilst CD8$^+$ cells are important in controlling HCMV infection when there are levels of high

viraemia, $CD4^+$ cell populations are necessary during persistent infection in order to maintain antiviral immunity [330].

Recently Essa *et al.* [331] reported that levels of total Th1 $CD4^+$ lymphocytes were decreased in HCMV-positive patients following renal transplantation, and proposed that this may be attributed to an immunological impairment that predisposed those patients to HCMV disease. Such a reduction in the frequency of HCMV-specific $CD4^+$ cells has also been shown to be a predictive factor for HCMV-associated disease [332], as lowest $CD4^+$ cell frequencies were found 2-6 months post-transplantation, a period of time that coincides with most HCMV-associated complications. Moreover, $CD4^+$ cell frequencies of below 0.25% were shown to correlate with a increased susceptibility to HCMV-associated, end organ disease [332]. High doses of immunosuppressive drugs administered following transplantation have been proposed to disrupt the immune response to HCMV to different extents between individuals and permit higher levels of viral replication and hence a greater chance of disease. Furthermore, Komanduri *et al.* [333] reported that two HIV-1-infected patients with recurrent retinitis had high total $CD4^+$ levels but reconstitution of HCMV-specific $CD4^+$ populations was deficient. These data reinforce the idea that large HCMV-specific $CD4^+$ populations are essential for control of HCMV replication and disease prevention.

CD4 Memory and Clonality

De Vries *et al.* [334] described a rapid reconstitution of the $CD4^+$ population in HCMV seropositive children following BMT compared to seronegative patients, this may have been due to a clonal expansion of existing $CD45RO^{High}$ populations, as CD45RA populations were replenished at a much slower rate. Most of the replenished $CD45RO^{High}$ population also lacked CCR7 indicating a more "effector-memory" phenotype. $CD4^+/CCR7^-$ populations tend to secrete IFNγ in response to antigen, whereas the $CD4^+/CCR7^+$ populations preferentially secrete IL-2 and, as mentioned above, remain in secondary lymphoid tissues. After the total $CD4^+$ population has recovered to levels similar to those prior to immunosuppression HCMV replication is again controlled in the majority of patients indicating that a balance between the immune response and viral replication has again been reached [332].

Research by Bitmansour *et al.* [335] suggested a hierarchical pattern of clonal populations $CD4^+$ specific for certain HCMV epitopes and that as few as 3 of these dominant epitopes could constitute up to 50% of the total HCMV-specific $CD4^+$ response within an individual. Pp65-specific clonotypes alone constituted almost one-third of the total CD4 response in two individuals studied from a group of four healthy patients. Khattab [276] and Beninga *et al.* [336] suggest that in addition to being the major target for $CD8^+$ cell responses in the majority of individuals pp65 also acts as a major target for $CD4^+$ cells. The oligoclonality of these populations together with pp65 being a major $CD4^+$ cell target implies that pp65 may shape both $CD8^+$

and $CD4^+$ responses towards HCMV. The relatively low frequency of these dominant populations suggests that, in the context of a genetically stable virus pathogen such as HCMV, the low diversity of $CD4^+$ population is sufficient to control the infection [335].

CD4 cells and HCMV Latency

As mentioned previously, $CD4^+$ lymphocytes recognise antigens in the context of MHC II expressed solely on APCs, suggesting that HCMV latency may be affected by HCMV-specific $CD4^+$ populations. Le Roy *et al.* [337] showed that IE1-specific $CD4^+$ cells were able to lyse a HCMV-infected astrocytoma cell line expressing MHC class II transactivator protein, suggesting that $CD4^+$ cells can control HCMV infection in APCs. Certain subsets of $CD4^+$ cells are known to secrete TNFα, which was shown by Prosch *et al.* [338] and Hummel *et al.* [339] to enhance the activity of the IE1 activator/promoter through induction of the transcription factor NF–κB. IE1 expression may allow latently infected cells to be recognised by IE1-specific CTLs, demonstrating that secretion of TNFα by $CD4^+$ cells could cause reactivation of latent HCMV or by the same rational, prevent latency.

1. 10 Evasion of cellular immune responses

HCMV encodes a variety of proteins that are able to interfere with the immune response. US2 and US11 target the heavy chain of MHC I for degradation [340] , US3 is able to retain peptide-loaded MHC I complexes within the ER and US6 inhibits TAP [341]. US2 and US3 have also been shown to be associated with the down-regulation of MHC class II molecules [342, 343] . Indeed both HLA-DRα and DMα degradation have been demonstrated in astroglial cells with IFNγ-inducible MHC II expression [344], which would abrogate HCMV antigen recognition by $CD4^+$ cells, thereby preventing CD4-mediated lysis and precluding IFNγ-induced upregulation of co-stimulatory molecules on APCs by $CD4^+$ cells. Furthermore, HCMV is able to prevent the IFNγ signalling cascade thus preventing activation of macrophages and hence precluding up-regulation of MHC I and MHC II. HCMV has developed another way of achieving evasion of CD8 and CD4 Th1 responses, through expression of an IL-10 homologue UL111a [345], IL-10 is also known to have a strong immunosuppressive capacity and may in turn down- regulate T-cell responses. Therefore, it is important to note that although the CD4 and CD8 responses are able to control HCMV replication and prevent disease in healthy, immunocompetent patients it is not usually sufficient to clear the virus; which may be due to the virus being able to evade the CD8 response.

1. 11 CVID patients

Common variable immunodeficiency is a heterogenous disease that affects approximately 1 in 25000 Caucasians and is characterised by an inability to produce antibodies which results in low serum-immunoglobulin concentrations [346]. Symptoms of hypogammaglobulinaemia are usually manifested after the age of 20 years [347] and they correspond with an increased susceptibility to bacterial infections of the respiratory and gastrointestinal tract. Between 10-23% of CVID patients also suffer from a range of auto-immune diseases such as thrombocytopenia and haemolytic anaemia [348, 349], these patients also frequently suffer inflammatory bowel disorders with large lymphocyte infiltration into the gut.

Individuals suffering CVID have also been shown to have defects in the differentiation state of their DCs, which also have decreased levels of co-stimulatory molecules on their surfaces along with a decreased capacity to produce IL-12 [350], which coincides with impaired T-cell activation and proliferation. Conversely, monocytic expression of IL-12 in some CVID patients may polarise CD4 cells to a Th1 response and hence lead to the induction in IFNγ production [351].

Despite these abnormalities, the majority of CVID patients cope well with virus infections. Their $CD8^+$ T-cells are predominantly of a CCR7- "effector-memory" phenotype and have an increase in levels of intracellular perforin, granzyme A and also an increased capacity to produce IFNγ on stimulation [352]. A subgroup of the patients also have raised levels of CDR7- $CD8^+$

cells in the peripheral blood that could be concomitant with raised levels of the lympho-proliferative cytokine IL-7 [353]. Virus-specific T-cell responses in these individuals have not been extensively investigated.

1. 12 Aims of project

This PhD thesis aims to further investigate the role played by HCMV-specific $CD4^+$ and $CD8^+$ T-cell responses in renal transplant patients. It also seeks to determine whether virus-specific T-cell responses are produced in CVID patients, who despite lacking endogenous Ig, are able control the many virus infections asymptomatically. This is presumably due to other facets of their immune responses; therefore the focus of part of this work thus deals with analysing their HCMV-specific T-cell responses and comparing them to healthy HCMV seropositive individuals and patients who are unable to control HCMV replication. The final aim of the thesis is to elucidate whether the magnitude of functional T-cell responses, as ascertained by their ability to produce IFNγ on stimulation with HCMV antigens, contributes to protection from HCMV-associated disease.

Previous work that focused on HCMV-specific CD4 and CD8 responses in renal transplant patients has not conclusively determined whether these responses are protective against HCMV replication. In D^+R^- patients the emergence of Th1-polarised HCMV-specific CD4 responses was found a median of 7 days after the detection of HCMV genomes in the blood, and was proceeded by the detection of HCMV-specific humoral responses [354]. The presence of HCMV-specific $CD4^+$ T-cells is also associated with the subsequent appearance of CD8 responses, and although $CD4^+$ cells were deemed to be required for the control of initial infection, no protective benefit was seen in renal transplant patients with virus-specific CD8 responses in the absence of CD4 responses [355]. The same group also demonstrated

that the emergence of HCMV-specific CD4 responses was delayed until after antiviral treatment in symptomatic patients [356]. A decrease in the HCMV-specific CD4 response of symptomatic renal transplant patients also coincided with the appearance of virus replication and subsequent disease [332].

There have been relatively few comprehensive studies on the role of HCMV-specific CD8 responses in renal transplant patients. During the first three months post-transplant HCMV-specific CTL responses, as determined through cytotoxicity assays, were found to inversely correlate with HCMV virus load as quantified by antigenaemia [254]. The percentages of HCMV tetramer$^+$ CD8$^+$ T-cells were also shown to be higher in renal transplant patients than healthy controls [294]. This was complimented by findings of Radha *et al.* [357] who also observed elevated frequencies of CD8$^+$ T-cells in seropositive renal transplant patients when compared to healthy controls although the proportion of these that were HCMV-specific was not determined.

The principal disadvantage of these studies is that they only focus of the percentages of T-cell populations able to respond to antigen stimulation and not the absolute numbers of these cells in the peripheral blood; this measurement would give a better indication of how many cells are capable of reacting and potentially controlling infection. Furthermore, HCMV-specific CD8 responses were measured using specific HLA-restricted epitopes instead of whole antigens, responses to specific epitopes would greatly

underestimate the global CD8 response. It is also unclear as to what constitutes functionality, in this thesis functionality is determined also by IFNγ production by activated CD69 expressing T-cells although this might itself not be representative and neither accounts for cytolytic ability nor ability to proliferate in response to appropriate antigen stimulation. In chapter 6 of this thesis a comparison of the phenotypes of epitope-specific CD8 populations within three groups of individuals is performed using cytolytic potential, gauged by intracellular perforin levels, as an alternative measurement of functionality.

These are the aims of the individual results chapters of the thesis:

1) To evaluate the fluctuations in both the percentages and absolute numbers of IFNγ-producing, HCMV-specific and pp65-specific $CD4^+$ T-cell populations in renal transplant patients and to determine whether there is any impairment in the $CD4^+$ responses in those individuals suffering HCMV-viraemia.

2) To evaluate the fluctuations in both the percentages and absolute numbers of IFNγ-producing, IE1-specific and pp65-specific $CD8^+$ T-cell populations in renal transplant patients and to determine whether there is any impairment in the $CD8^+$ responses in those individuals suffering HCMV-viraemia.

3) To ascertain whether CTL-escape mutants arise in patients that remain HCMV PCR-positive for long periods post transplant despite the presence of an epitope-specific CD8 response.

4) To investigate whether individuals suffering CVID have detectable T-cell responses towards HCMV and to compare the frequencies and phenotypes of HCMV tetramer+ CD8 populations in these individuals with age-matched healthy controls.

Chapter 2

Materials and Methods

2. 1 Cell Culture

2.1. 1 Growth and Maintenance of T-2 Cell Lines

TAP-deficient T2 cells (lacking genes encoding TAP-1 and TAP-2) were grown in suspension using upright vented T75 flasks (Sarstedt, Leicester) with RPMI 1640 medium supplemented with 10% FCS and penicillin streptomycin (Gibco, see appendix) in a humidified incubator at 37^0C in 5% CO_2. Cells were split 1:5 with fresh supplemented RPMI 1640 medium every three to five days. Cell density and viabilities were ascertained by staining with 4% trypan blue in PBS (Sigma) and counting under a disposable haemocytometer.

2.1. 2 Isolation of PBMCs from Whole Blood

20ml of fresh whole blood was taken from patients and volunteers obtained using sodium-heparin syringes (Sarstedt, Leicester). The blood was then carefully overlaid onto Ficoll-Paque Plus (Amersham Biosciences) at a 1:1 volume ratio in 50ml sterile Falcon® universal tubes (Sarstedt, Leicester); these were centrifuged at 700g for 20min with slow acceleration and without braking. The tubes were removed from the centrifuge and the interphase, created between the plasma layer and the lymphocyte separation medium (Fig. 2.1), was carefully removed to avoid contamination with either adjacent layer and placed into a fresh universal tube. The volume of the interphase was made up to 30ml with unsupplemented RPMI 1640; this was then centrifuged at 550g for 10 minutes (with fast acceleration and brake) to remove any residual Ficoll. Then supernatant was discarded and the

resultant pellet was resuspended in an additional 20ml of RPMI 1640. Cells were washed twice by centrifugation at 450g for 5 minutes, after the second wash cells were resuspended in 10 ml of supplemented RPMI 1640. Viable cells were then stained with 4% trypan-blue and then counted using a haemocytometer.

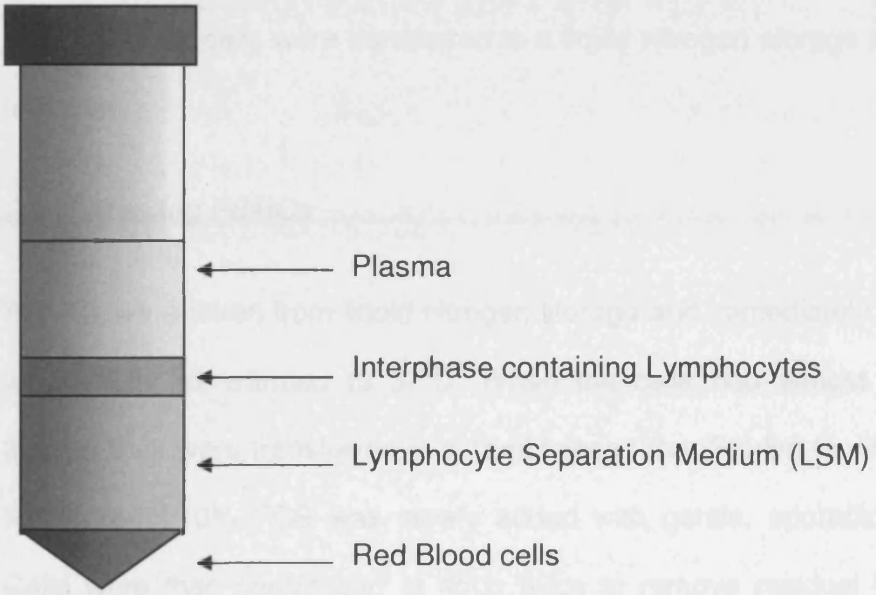


Figure 2. 1 Sedimented layers formed after centrifugation of whole blood over a Ficoll-paque plus gradient

2.1. 3 Freezing PBMCs for ex vivo tetramer staining

Isolated PBMCs were centrifuged at 450g for 5 minutes and the pellets were resuspended at a density of >5 x 10^6 cells/ml with 90% FCS (sterile filtered, Labtech) 10% DMSO (Sigma) that had been pre-chilled to 4^0C. The suspensions were transferred to labelled 1.5ml cryovials (Alpha Laboratories) and placed in a 4^0C isopropanol-containing freezing chamber (Fisher). Freezing chambers were transferred to a -80^0C freezer, which allowed cells to cool at a rate of 1^0C/minute, thereby preserving cell viability. After 48 hours cells were transferred to a liquid nitrogen storage unit and left until use.

2.1. 4 Thawing PBMCs

PBMCs were taken from liquid nitrogen storage and immediately placed in a water bath pre-warmed to 37^0C. When the cells had almost completely thawed they were transferred to a 15ml falcon tube (Sterlin) to which 9 ml of RPMI 1640 10% FCS was slowly added with gentle, sporadic vortexing. Cells were then centrifuged at 450g twice to remove residual DMSO and assessed for number and viability. Cells were then used for subsequent immune staining.

2. 2 PCR

2.2. 1 Primers for amplification of sequences encoding CD8 epitopes

Design of primers

Primers for the amplification of pp65 HLA-A*0201 and -*B35 epitopes by nested polymerase chain reaction (PCR) were designed using Primer3 software available at:

http://www.genome.wi.mit.edu/genome_software/other/primer3.html)

All primers were synthesised and purified commercially by MWG-Biotech.

VLGPISGHV – epitope 1

External Primers

Forw

Rev

Internal Primers

Forw

Rev

PCR amplicon size 409bp

MLNIPSINV – epitope 2

External Primers

Forw

Rev

Internal Primers

Forw

Rev

PCR amplicon size 194bp

NLVPMVATV – epitope 3

External Primers

Forw

Rev

Internal Primers

Forw

Rev

PCR amplicon size 200bp

2.2. 2 Nested polymerase chain reaction (PCR)

PCR reactions were performed using a Bio-X-act Short high-fidelity polymerase (Bioline) to minimise the potential emergence of random sequence changes during the amplification process. DNA was isolated from PCR-positive liver transplant patients, of known HLA types (8 x HLA-A02, 1 x B35, with viral loads >3 log genome copies/ml of whole blood. DNA sequences were initially amplified using the external primers and 2µl of resultant DNA was amplified using inner sense and antisense primers.

MasterMixes

10X OptiBuffer (Bioline)	5µl
50mM $MgCl_2$ Solution (Bioline)	2.5µl
100mM dNTP (Promega)	1µl
Template DNA	0.5µl
External or internal Primer mix 100µM	1µl
BIO-X-ACT Short 4u/µl (Bioline)	1µl
Water (ddH_20) Up to 50µl	

Cycling Conditions for first stage PCR

Step	Temperature (^{0}C)	Time (m)	Number of Cycles
1	95	5	1
2	55	30	20
3	70	30	20
4	95	30	20
5	70	10	1
6	4	Hold	1

Cycling Conditions for nested PC

Step	Temperature (^{0}C)	Time (m)	Number of Cycles
1	95	5	1
2	55	30	20
3	70	30	20
4	95	30	20
5	70	10	1
6	4	Hold	1

Thermal cycling was performed using a Hybaid thermal cycler. Following amplification the DNA was mixed with loading dye and visualised on 1.5% TAE agarose gel.

2.2. 3 Agarose Gel Electrophoresis and Purification of DNA Product

1.5% agarose gels were prepared by adding 1.5g of multipurpose agarose (Bioline) to 100ml of 1x TAE (40mM Tris-acetate, 1mM EDTA buffer, Sigma-Aldrich). Agarose was dissolved by heating in a 850W microwave and cooled to 50^{0}C using a water-bath. 2µl of ethidium bromide (10mg/ml Sigma) was then added to the solution and mixed. The solution was carefully poured into gel frames and combs were inserted to make sample loading wells. The gels were allowed to cool and solidify, combs were removed and gel frames were submerged in an Embi Tec RunOne® electrophoresis cell. DNA samples were diluted 1:1 in blue dye sample loading (Bioline) and loaded into separate wells alongside Hyperladder IV (Bioline) and were run at 100V for approximately 15 minutes.

Amplified gene products were visualised under UV light on a transilluminator and sizes were determined by comparison with Hyperladder IV (Bioline). Bands of interest were purified from 1.5% w/v low melting-point agarose gel (Sigma) run in 1 x TAE (40mM Tris-acetate, 1mM EDTA buffer). Using a low powered UV light to visualise the bands of interest, the gels were placed on to a Perspex tray to minimize exposure of DNA to UV to reducing the formation of thymidine dimers. The bands were excised with a sterile scalpel and purified using the standard QIAquick gel extraction protocol (Qiagen). Briefly, bands of interest were excised and weighed in a pre-weighed sterile 1.5ml centrifuge tube, 3 times (w/v) QG buffer was then added to the tubes and gently mixed. Tubes were then incubated in a 50^{0}C water bath for 10 minutes, with periodic vortexing, until the gel had dissolved. 1 gel volume of

isopropanol was added to the tubes and mixed thoroughly and the solution was then added to a QIAquick spin column, which was centrifuged at 13000g for 1 minute on a standard desktop microcentrifuge. The flow-through was discarded and the spin column was washed, to remove residual agarose, with 0.5 ml QG buffer for 1 minute. The flow-through was discarded and the column was washed again using PE buffer. Once more the flow-through was discarded and the column was centrifuged again to remove residual PE buffer. The column was then placed in a new sterile 1.5 ml microfuge tube. To elute the DNA 50µl of nuclease-free water was carefully added to the DNA-binding filter of the column, and columns were left to stand for one minute before centrifugation. The eluate was collected at the bottom of the microfuge tube and stored until use at 4^0C.

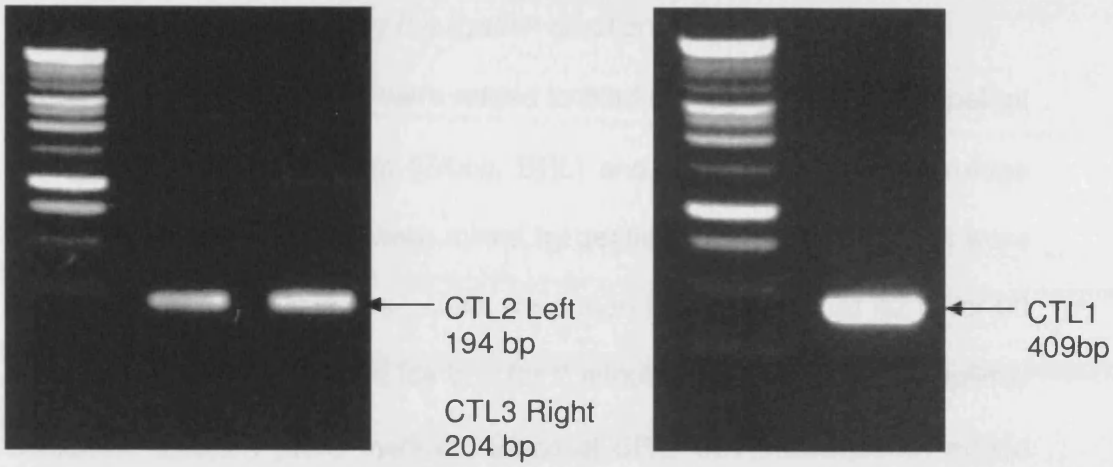


Figure 2. 2 1.5% agarose gel, run in 1X TAE buffer showing nested PCR products

2.2. 4 Ligations of purified Sequences into pGem-T®-easy Vector

Purified DNA products were ligated into pGem-T®-easy vector (Promega) using a 3:1 molar ratio using the following equation to calculate amounts of insert required.

$$\left(\frac{ng\ of\ vector \times kb\ size\ of\ insert}{kb\ size\ of\ vector}\right) \times insert{:}vector\ molar\ ratio = ng\ of\ insert$$

Ligation mixes consisted of 5µl of 2X Rapid Ligation Buffer, 1µl (50ng) of pGEM-T® Easy Vector, 1µl (3 units) T4 DNA, *x*ng of DNA insert and deionized water taking the final volume to 10µl. Ligations mixtures were incubated for one hour at room temperature or overnight at 4^0C to increase the efficiency.

2.2. 5 Transformations using the ligation reactions

2µl of the ligation reactions were added to 50µl of freshly-thawed competent *E.coli* strain DH5-alpha cells (Gibco, BRL) and placed in 1.5 ml microfuge tubes (Sarstedt). Contents were mixed by gentle flicking and the tubes were placed on ice for 20 minutes. Cells were then heat-shocked at 42^0C for 90 seconds and returned to the ice box for 2 minutes. 950µl of Super Optimal Catabolite medium (SOC medium, Gibco at BRL) was subsequently added and tubes were incubated at 37^0C in a shaking incubator for a further hour.

100µl of each transformation culture was plated onto the amp, IPTG, X-gal LB agar plates (see appendix) and which were inverted and incubated overnight at 37^0C. The next day individual white colonies were picked using a sterile pipette tip and dipped into 25ml falcon universal tubes containing 5ml LB broth supplemented with ampicillin at 50µg/ml and these were then incubated overnight with agitation at 37^0C. The following day, 800µl of ampicillin-resistant bacteria suspension was added to 200µl of sterile glycerol (Sigma) in sterile 1.5ml centrifuge tubes, these were vortexed, snap-frozen using dry ice and stored at -70C. The remaining suspension was centrifuged at 13000g for 10 minutes and DNA from the pellet was purified using a QIAprep MiniPrep Kit (Qiagen) following standard protocols.

Briefly, cell pellets were resuspended in 250µl of P1 buffer (with RNase A added) and transferred into a 1.5 ml microfuge tube. 250µl of P2 buffer was then added and the mixture was inverted 4-6 times before addition of 350µl of P3 precipitation buffer. Solutions were mixed quickly by inverting 4-6 times, to avoid localised protein precipitation. Tubes were then centrifuged for 10 minutes at 13000g on a standard desktop microcentrifuge. The resultant supernatants were then added to Qiaprep spin columns, which were subsequently centrifuged at 13000g for 1 minute. The flow-through was discarded and the column was washed with 500µl of PB wash buffer. The spin column was washed again using 750µl of PE buffer. The flow-through was also discarded and the columns were spun again for 1 minute to remove any residual PE buffer. The column was then transferred to a new, clean 1.5 ml microfuge tube and DNA was eluted by adding 50µl of nuclease free water. Samples were then stored at 4^0C until they were used for DNA sequencing. DNA sequencing was performed on an ABI Prism® capillary-based sequence analyser using a BigDye® terminator v3.1 cycle sequencing kit (Applied Biosystems). Sequences were analysed and graphics were generated utilising Chromas 2.23 software available at:

http://www.technelysium.com.au

2. 3 Peptides

Variants of the pp65 HLA-A*0201 epitope NLVPMVATV are summarised in table 2.1. Peptide variants were designed based on individual glycine substitutions or like-for-like based on charge and shape, anchor residues were conserved in all. (Blue indicates anchor residues Red indicates modified residues). Peptides were synthesised commercially by Altabiosciences (University of Birmingham) and purified to >90% purity. Lyophilised peptides were resuspended in DMSO to 5mg/ml.

Peptide sequences	Name	Comments
N L V P M V A T V	Wildtype (wt)	
G L V P M V A T V	G1N	
N L G P M V A T V	G3V	
N L V G M V A T V	G4P	
N L V P G V A T V	G5M	
N L V P M G A T V	G6V	
N L V P M V G T V	G7A	
N L V P M V A G V	G8T	
Q L V P M V A T V	Q1N	
N L L P M V A T V	L3V	
N L V P I V A T V	I5M	Described in [358]
N L V P C V A T V	C5M	
N L V P M L A T V	L6V	
N L V P M V A S V	S8T	

Table 2. 1 List of NLVPMVATV variants with conservative and non-conservative substitutions

2. 4 Enzyme-Linked Immuno Spot (ELISpot) using modified peptides

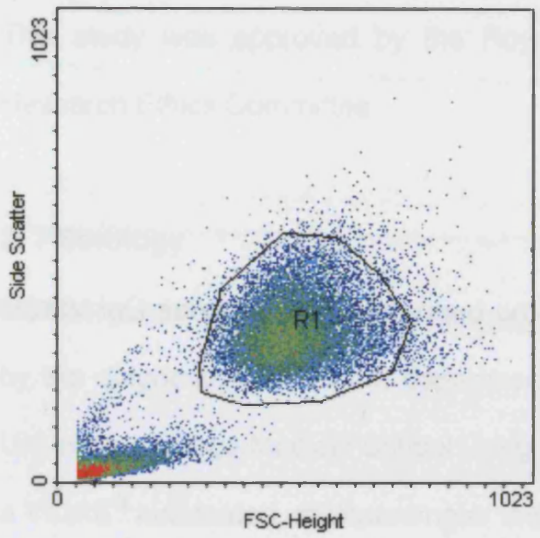
Mouse anti-human-IFNγ mAb (1-D1K, MabTech, Sweden) was diluted in sterile-filtered PBS (Sigma) and used to block 96-well nitrocellulose plates (MabTech, Sweden) overnight at 4^0C. Plates were subsequently washed six times in sterile, filtered PBS and blocked with RPMI 1640 (supplemented with 10% FCS). PBMCs isolated from HCMV seropositive and control (seronegative) volunteers were re-suspended to 5 x 10^4 cells/well in supplemented RPMI 1640. 20μM of the wild-type NLVPMVATV and variant peptides were added in triplicate to each well along with 1μg/ml of co-stimulatory anti-CD28 (BD Biosciences, Oxford) and diluted in RPMI to make volumes up to 200μl/well. Cell/peptide mixes were incubated overnight at 37^0C in an atmosphere of 5% CO_2. Cell suspensions were then discarded from the plate and wells were washed six times with sterile, filtered PBS/Tween-20 (0.05% v/v). Plates were then coated with biotinylated mouse anti-human-IFNγ mAb (7-B6-1, MabTech, Sweden), diluted 1:1000 in PBS/1% FCS, for 3 hours at room temperature. Plates were washed again, as above, and incubated with streptavidin-alkaline phosphatase diluted 1:1000 using PBS 1% FCS for 2 hours at room temperature. Plates were washed as before, substrate was then added and spots were allowed to develop for approximately 15 minutes. Reactions were quenched using tap water and dried overnight prior to counting using light microscope.

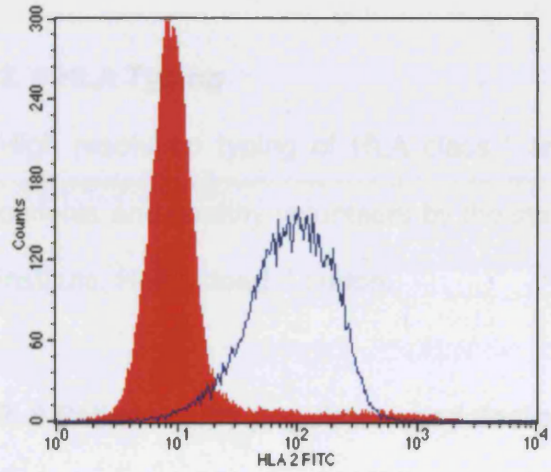
2. 5 Peptide Stabilization Assay

T2 cells are a somatic cell hybrid of human B and T lymphoblastoid lines deficient in TAP proteins and expressing low levels of surface HLA-A*0201. HLA-A*0201 molecules can be stabilised and therefore upregulated on the cell surfaces on binding to exogenous HLA-restricted peptides. T2 cells were seeded in 48 well plates at a density of 5 x 10^5 cells/ ml in fresh RPMI 1640 and were left at room temperature for 1 hour to equilibrate. They were subsequently incubated with the peptides for 16h at 37^0C 5% CO_2, after which the suspensions were centrifuged at 400g for 5 minutes and washed three times in 5ml PBS (4^0C) to remove any unbound peptides. Cell pellets were resuspended and incubated with mouse anti-human IgG2b HLA-A*0201 FITC conjugate (BB7.2 SeroTec), diluted 1/10 in PBS/1% FCS, for 30 min at 4^0C. The cells were centrifuged and washed, as before, and were finally resuspended in PBS 0.01% sodium azide (Sigma-Aldricht). Isotype control (IgG2b) antibodies were also used to stain cells using the same conditions. Increase in mean fluorescence intensity was analysed using CellQuest™ software on a FACSCAN™ (Becton Dickinson Biosciences).

The percentage increase in fluorescence intensity was measured calculated as:

(MFI (peptide)/MFI (control)) x 100 = increase in MFI





Red fill – Isotype control

Blue line – anti A2-FITC binding to unstimulated T2 cells

Figure 2. 3 (Upper panel) Contour plot of T2 cells with R1 gate to identify viable cells (Lower panel) Histogram showing HLA-A*0201 expression on unstimulated T2 cells using isotype control (IgG2b) and anti HLA-A*0201-FITC antibody (BB6.72 SeroTec).

2. 6 Ethical approval

This study was approved by the Royal Free and Medical School Local Research Ethics Committee.

2. 7 Serology

HCMV IgG serology was performed on both patient and volunteer samples by the diagnostic staff at the department of virology at the Royal Free and University College Medical School using the Biomerieux® CMV IgG assay on a VIDAS® automated, mulitparameter immunoassay system.

2. 8 HLA Typing

High resolution typing of HLA class I and II alleles was performed on all patients and healthy volunteers by the staff at of the Anthony Nolan research institute, Hampstead, London.

2. 9 Patient study population and design

To investigate the role of HCMV-specific $CD4^+$ and $CD8^+$ T-cell responses in the control of HCMV replication post renal transplant a total of 20 renal transplant patients of either D^+/R^-, D^+/R^+ or D^-/R^+ were monitored at monthly intervals for six months post-transplant. Patients were from a variety of different HLA backgrounds. For investigation of CTL-escape emergence in chapter 5, DNA was extracted from the peripheral blood of 4 persistently-viraemic liver transplant patients of known HLA backgrounds that had previously-characterised HCMV tetramer populations, and five further liver transplant patients with acute phase viraemia.

Patient Number	Sex	Age (at Transplant)	Donor/Recipient serostatus	HLA type	Organ Source	Immunosuppression	Rejection	Viraemia	Antiviral
1	M	76	D- R+	A19 B08	Cadaveric	Pred, CycA, MMF, Simulect	3 x methypred	y	Valganciclovir
2	M	31	D+ R+	A01 B07	Live related	Tac	1 x methypred	n	
3	M	47	D+ R+	A28 B17	Cadaveric	Pred, CycA, MMF		y	
4	M	63	D- R+	A01 B07	Cadaveric	Pred, Tac, MMF		n	
5	F	18	D- R+	A02 B35	Cadaveric	Pred, Tac, MMF		n	
6	M	57	D+ R-	A02 B27	Live related	Pred, Tac, MMF, Simulect		n	
7	F	48	D- R+	A01 ?	Live related	Pred, Tac		y	
8	M	53	D+ R+	A19 B16	live	Pred, Tac, MMF, Simulect	1 x ATN rejection	n	
9	M	58	D+ R+	A01 B08	Cadaveric	Pred, Tac, Simulect		y	
10	F	42	D+ R+	A02 B12	Cadaveric	Pred, Tac, Simulect		n	
11	M	46	D- R+	? ?	?	?		y	
12	M	66	D- R+	A02 B16	Cadaveric	Pred, Tac, MMF, Simulect		y	
13	F	51	D- R+	A01 B08	Cadaveric	Pred, Tac, MMF		y	
14	F	39	D+ R-	? ?	Cadaveric	Pred, Tac, MMF, Simulect		n	
15	M	31	D+ R+	A01 B08	Cadaveric	Pred, Tac, MMF		n	
16	M	47	D- R+	A02 B05	Cadaveric	Pred, Tac, MMF, Simulect		n	
17	M	44	D- R+	A01 B12	Cadaveric	Pred, CycA, MMF, Simulect	1 x ATN rejection	y	
18	M	40	D- R+	A09 B07	Cadaveric	Pred, Tac, Simulect		n	
19	F	70	D+ R-	A09 B12	?	?		y	
20	F	48	D- R+	A01 B07	Cadaveric	Pred, Tac, MMF		n	

Table 2.2. Table shows information of renal transplant patients used for FACS-based studies in chapters 3, 4 and 6. Viral load kinetics of the 9 viraemic individuals can be found in the appendix. Note: Patient 14 was removed from data of Figs 3.7 and 3.8 as a potential non-seroconverter.

2. 10 Immunosuppression

Initial immunosuppression of renal transplant patients consisted of prednisolone plus tacrolimus or cyclosporine, with an additional combination of Mycophenolate and Simulect in certain patients.

2. 11 Routine HCMV

DNA was extracted from 200µl of whole using a Qiagen extraction kit (Minden, Germany) according to the manufacturer's instructions. Quantification of HCMV was performed using a TaqMan (ABI)-based method adapted from previously published protocol [359].

2. 12 Intracellular Cytokine Staining of Stimulated $CD4^+$ and $CD8^+$ cells

Within 2 hours of isolation PBMCs, taken from CMV-seropositive and seronegative volunteers, were seeded at a density of 1 x 10^6cells/ml in sterile vented, capped 12 x 75mm polypropylene tubes (Elkay). Cells were stimulated with either IE1 or pp65 PepMixes (Jerini), HLA-restricted class I epitopes (ProImmune) or CMV whole virus lysate and control lysate (both Autogen Bioclear Ltd) at a density of 1 x 10^6 cells/ml in the presence of co-stimulatory CD28 and CD49d antibodies both at 1µg/ml (BD Biosciences) in a humidified incubator at 37^0C with 5% CO_2. Two hours after stimulations, suspensions were mixed with 2µl/ml of Brefeldin A (GolgiPlug, BD PharMingen) and incubated overnight.

The next day cells were washed twice with PBS 0.1% sodium azide (Sigma) by centrifugation at 450g for 5min. Pellets were resuspended and stained with fluorophore-conjugated cell surface antibodies CD4-PerCP (5µl) and CD3-APC (1µl), in the dark at room temperature for 15 minutes. They were washed, as before, and fixed using 100µl of Medium A fixative (Caltag) for 15 minutes. Cells were washed again and then resuspended in 100µl of Medium B permeabilization buffer (Caltag) for 15 minutes in the dark, along with antibodies against CD69 (5µl) and IFNγ (5µl), after this were washed and resuspended in 200µl of PBS 2% paraformaldehyde and stored at 4^0C for no longer than 48 hours before acquisition using a four-colour FACSCalibre (Becton Dickinson) and CellQuest software. Gates were set on lymphocyte-positive (low SSC, medium FSC) and $CD3^+IFN\gamma^+$ populations. $CD3^+$ $CD4^+$ populations were then analysed for $CD69^+IFN\gamma^+$ populations, and $CD8^+$ populations were taken as being those lymphocytes that were $CD3^+$ but $CD4^-$. Although there are certain subpopulations of $CD3^+$ cells that are $CD4^-$ that are not $CD8^+$, such as NK T-cells, they do not contribute more that 3% of the population in the peripheral blood.

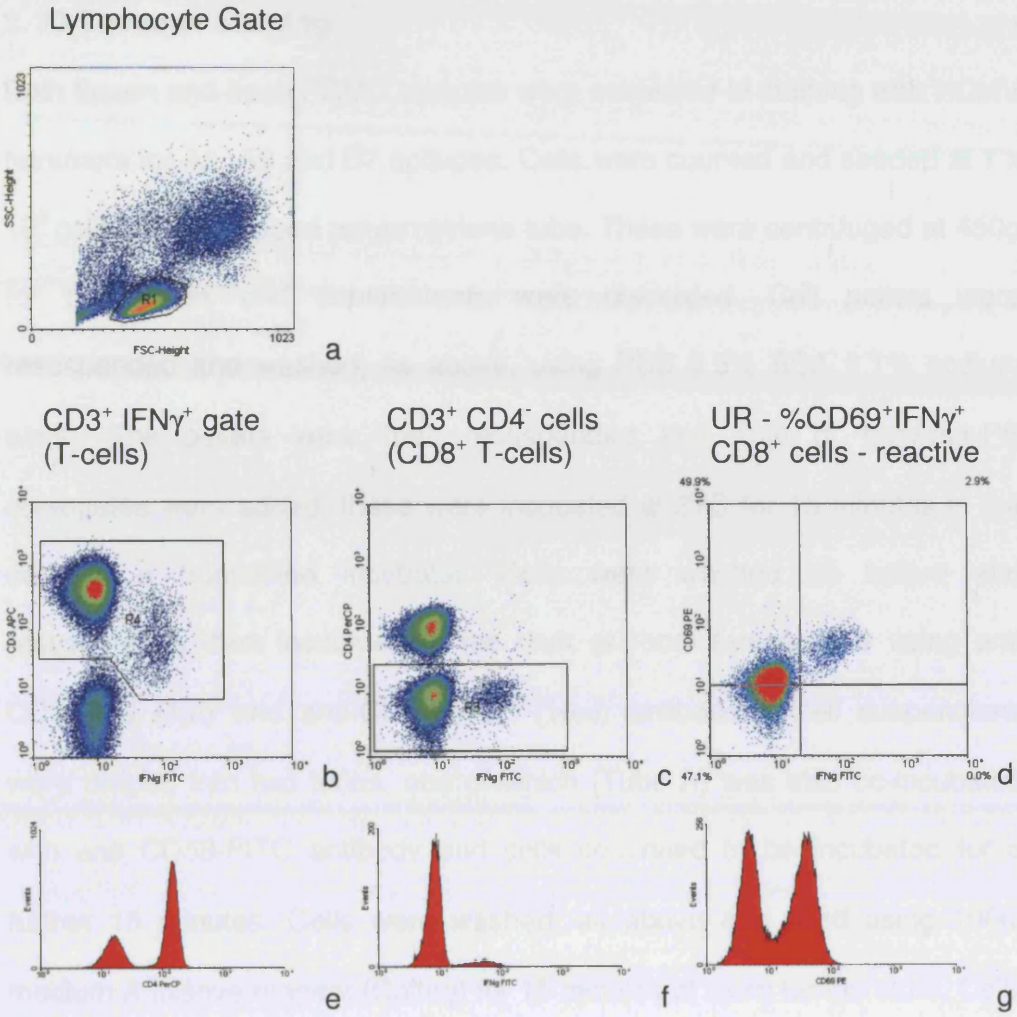


Figure 2. 4 FACS density plots and histograms showing staining on stimulated PBMCs

2. 13 Tetramer staining

Both frozen and fresh PBMC samples were subjected to staining with HCMV tetramers for A1, A2 and B7 epitopes. Cells were counted and seeded at 1 x 10^6 cells/ml in a capped polypropylene tube. These were centrifuged at 450g for 5 minutes and supernatants were discarded. Cell pellets were resuspended and washed, as above, using PBS 0.5% BSA 0.1% sodium azide. The pellets were then resuspended and 10µl of tetramer-PE conjugates were added, these were incubated at 37C for 15 minutes in the dark in a humidified incubator. Cells were washed as before and resuspended, then incubated in the dark at room temperature using anti CD3-APC (1µl) and anti-CD8-PerCP (10µl) antibodies. Cell suspensions were divided into two tubes, one of which (Tube A) was also co-incubated with anti CD58-FITC antibody and cells continued to be incubated for a further 15 minutes. Cells were washed, as above and fixed using 100µl medium A fixative reagent (Caltag) for 15 minutes at room temperature. Cells were washed again and permeabilized using 100µl medium B reagent, in Tube B was co-incubated with 10µl of anti-perforin-FITC, for 15 minutes. Cells were washed again and resuspended in 100µl 4% paraformaldehyde and were stored at 4C until acquisition. >50,000 cells were acquired using the lymphocyte and $CD3^+$ gates (see Fig. 2.4)

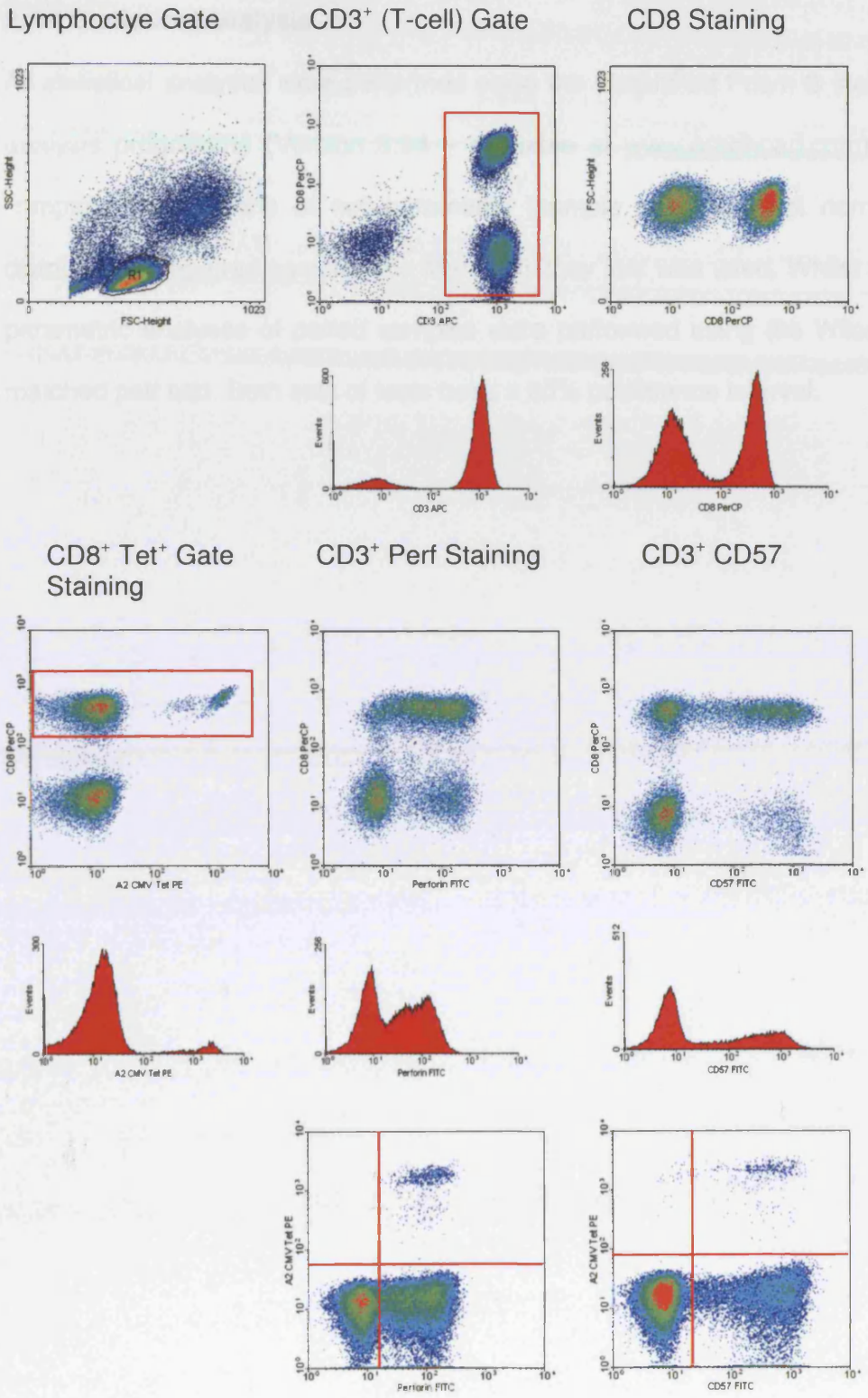


Figure 2. 5 Staining to determine Perforin and CD57 phenotypes of $CD3^+$ $CD8^+$ HCMV $Tetramer^+$ populations Red Boxes/lines indicate gated/quadranted populations respectively.

2. 14 Statistical analysis

All statistical analyses were performed using the GraphPad Prism ® statistic analysis programme (Version 3.04 – available at www.graphpad.com). To compare the medians of non-parametric (sample data was not normally distributed) un-paired samples the Mann-Whitney test was used. Whilst non-parametric analyses of paired samples were performed using the Wilcoxon matched pair test. Both sets of tests used a 95% confidence interval.

Chapter 3

$CD4^+$ Responses to HCMV in Renal Transplant Patients

3.1 Introduction

Previously work by Hassan-Walker *et al.* [107] described how high HCMV loads in immunosuppressed patients correlate with an increase risk of HCMV-associated disease. Immunosuppression results in a reduction in T-cell populations and causes a disruption in the balance between an effective immune response and virus replication. The control of HCMV replication by T-cell responses and the potential for the development of HCMV-associated diseases in the context of immunosuppressive therapies which decrease these populations needs to be fully addressed.

There are several lines of evidence supporting the importance of $CD4^+$ T-cells in the control of HCMV infection. First, an increased risk of HCMV associated-disease occurs when $CD4^+$ T-cell levels fall below 50 cells/μl^{-1} in AIDS patients [360-362]. The persistence and effectiveness of infused HCMV-specific $CD8^+$ T-cell clones in a BMT setting is increased when co-infused with HCMV-specific CD4 cell populations; indicating that reductions in HCMV-specific $CD4^+$ T-cell levels has dramatic downstream effects on other facets of the adaptive immune response [251]. Sester *et al.* [332] noted that HCMV-specific CD4 frequencies below 0.25% were predictive of an increased risk of reactivation and disease in renal transplant patients. While work by Gamadia et al. 2003 [355] demonstrated that although HCMV-specific $CD8^+$ cells were found in both symptomatic and non-symptomatic renal transplant patients, the emergence of $IFN\gamma^+$ HCMV-specific $CD4^+$ T-cell populations were necessary for the prevention of HCMV-associated disease in seronegative individuals transplanted with organs from seropositive

donors. Indeed, individuals suffering HCMV disease had a delay in the emergence of their HCMV-specific CD4 responses.

This chapter aims to further investigate the role played by HCMV-specific $CD4^+$ cells in controlling HCMV replication in the renal transplant setting. To do this, longitudinal analyses of both the percentages and the absolute numbers of HCMV-specific $CD4^+$ cells in twenty renal transplant patients were performed using a whole virus lysate or peptide pools for pp65 [271, 276, 335, 363] and IE1 [277, 324, 364-366], both targets for the $CD4^+$ immune response.

3.2 Kinetics of HCMV-specific $CD4^+$ T-cell re-emergence in Renal Tx Patients

Blood was taken from 20 renal transplant patients of varying donor/recipient HCMV serostatus using sodium heparin syringes. PBMCs were isolated and stimulated within three hours of extraction, with either a commercially available HCMV lysate or peptide pools that spanned either the IE1 or pp65 sequences, along with co-stimulatory antibodies against CD28 and CD49d. After 2 hours incubation, brefeldin A was added and stimulations were allowed to proceed for an additional 12 hours. HCMV-specificity was enumerated through FACs by measuring the frequency of $CD4^+$ $CD3^+$ cells able to produce IFNγ while expressing the activation marker CD69 (Fig. 3.1).

Figure 3.2 shows the relative contributions of IE1 and pp65 to the global HCMV-specific $CD4^+$ T-cell response as ascertained by responses to the HCMV lysate. Median responses towards the HCMV lysate were, predictably, the largest elicited by any of the three antigens, with responses ranging from <0.05 to 10.4% (median 1.1%), while responses towards pp65 ranged between <0.05 and 2% (median 0.26%), the lowest responses where towards IE1 and these ranged between <0.05 and 0.8% (with a median of below the lower limit of detection <0.05%). Because of the relatively small contribution of IE1 to the CD4 response of the patients included, this chapter primarily focused on the CD4 responses to the HCMV lysate and pp65.

$CD4^+$ T-cell responses to HCMV lysate were analysed at monthly intervals for six months post-transplant and compared with responses obtained from

healthy seropositive individuals (Fig. 3.3). The results show increased frequencies of HCMV-specific $CD4^+$ cells in renal patients compared to healthy controls although this increase was not significant (Mann-Whitney p = >0.05). A two-tier increase in HCMV-specific $CD4^+$ responses occurred between the first and third months (median frequencies 0.65% and 0.87% respectively, Willcoxon test p = 0.03) and the third and sixth (median frequencies 0.87% and 1.45%, p = 0.01) post-transplant, although the CD4 responses peaked at month 4 (median 1.55%).

Since data on percentages of HCMV-specific $CD4^+$ do not give an accurate representation of the absolute numbers of circulating cells in an individual, the results were recalculated based upon the absolute number of lymphocytes from each individual at each time point.

Figure 3.4 demonstrates that, as with percentages of HCMV-specific $CD4^+$ cells, the absolute numbers of circulating HCMV-specific $CD4^+$ cells showed a significant increase over the first three months post-transplant, and that these populations continued to rise until month four when they peaked (median 7.84 x 10^6 cells/l) and remained constant for the next two months. Results correlate well with evidence suggesting that the majority of HCMV-seropositive patients becoming viraemic during the first three months after transplant (all viraemic patients did see appendix patients 1 to 9). Should increases in $CD4^+$ populations be protective, it would be conceivable that frequencies of these cells would be decreased in viraemic patients relative to non-viraemic patients. Interestingly, the highest responses (68.3 x 10^6 cells/l)

actually occurred in an individual who suffered repeated viraemic episodes, although this individual did not have the highest percentages of $CD4^+$ cells that are HCMV-specific.

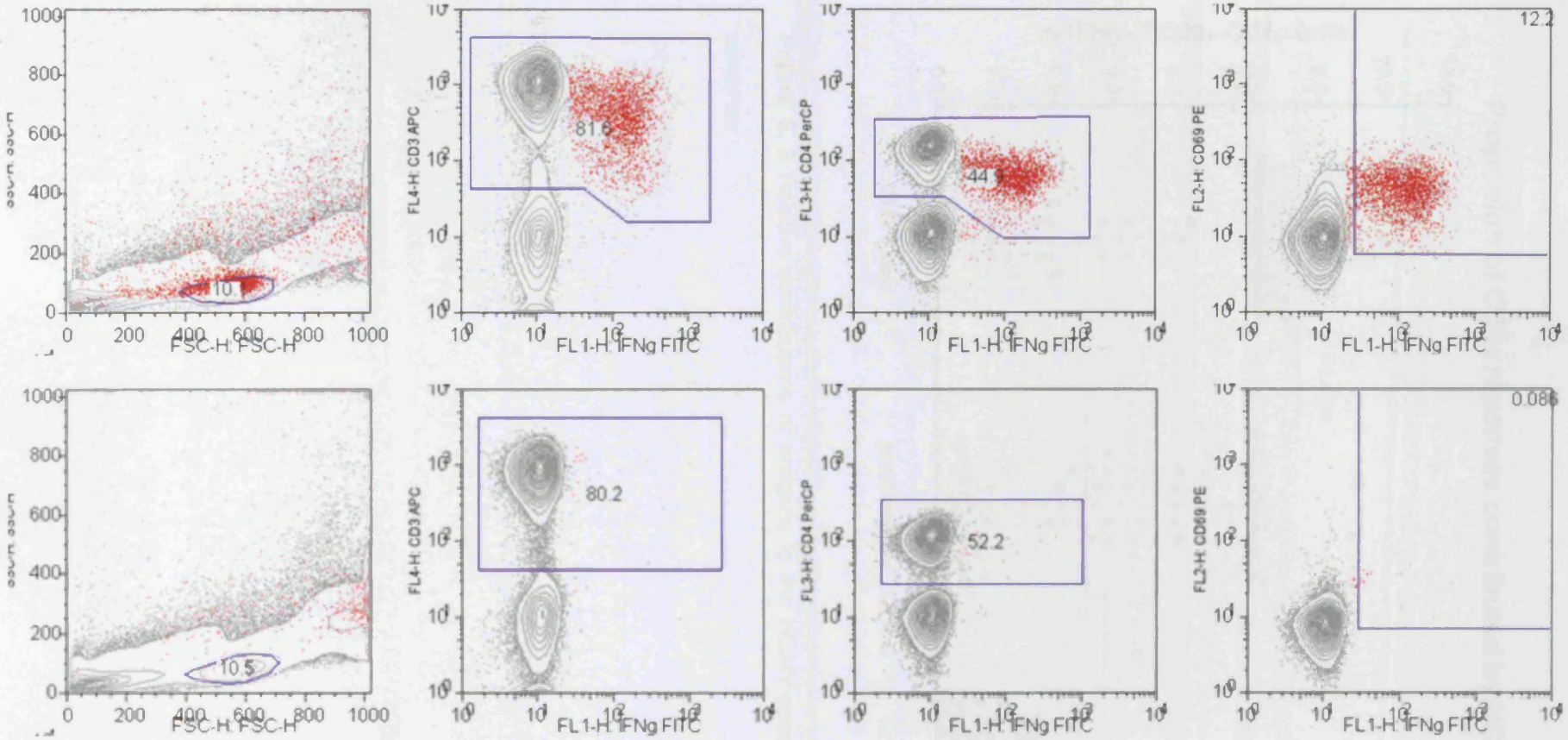


Figure 3. 1 FACS analysis plots of IFNγ production by a renal transplant patient in response to HCMV and control lysate. Populations are gated on lymphocyte $^{+}$ $CD3^{+}$ CD4- populations

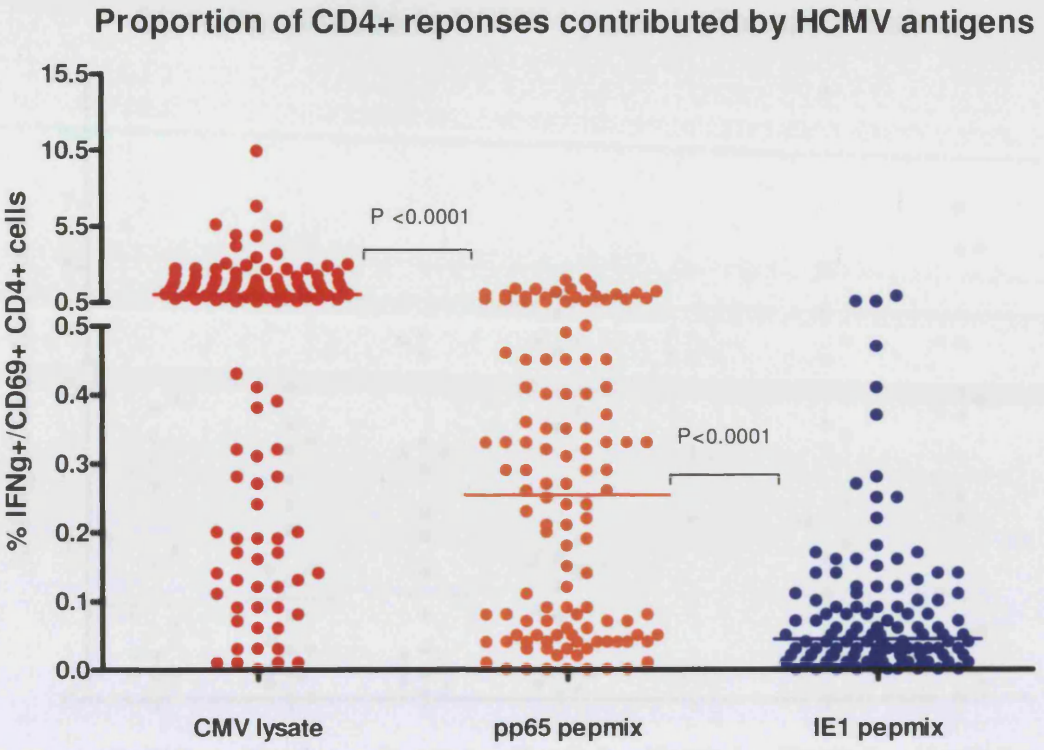
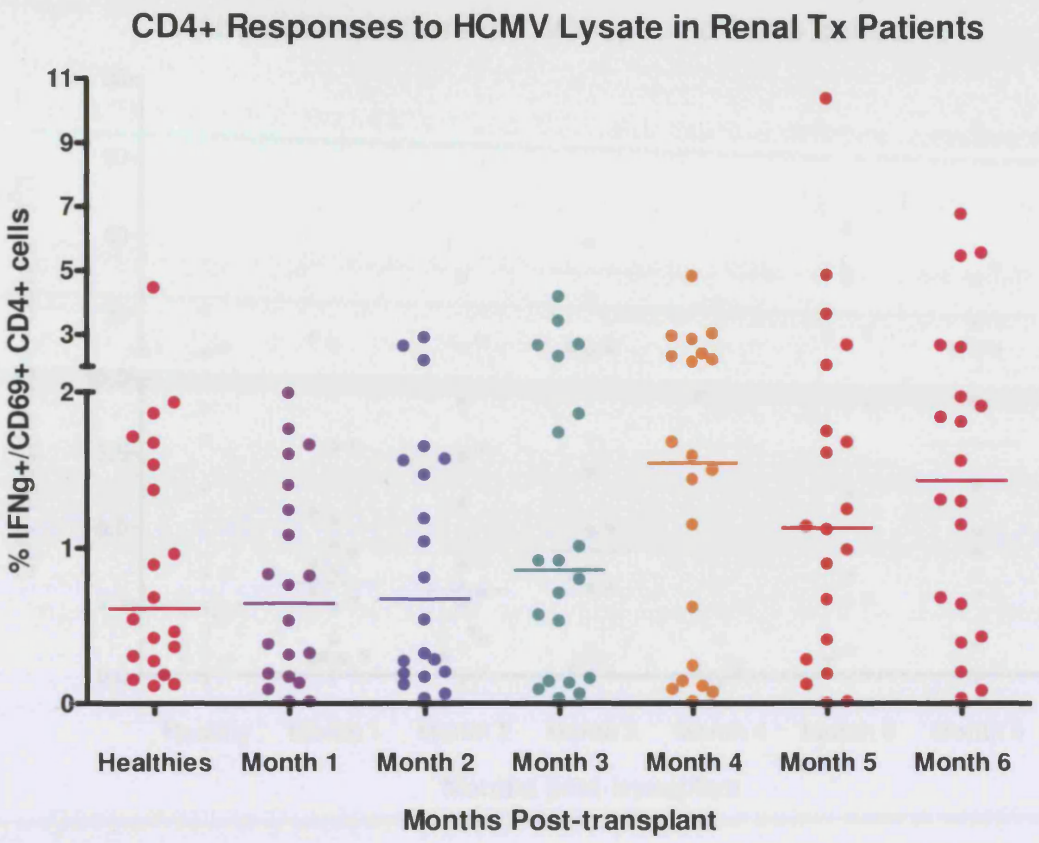
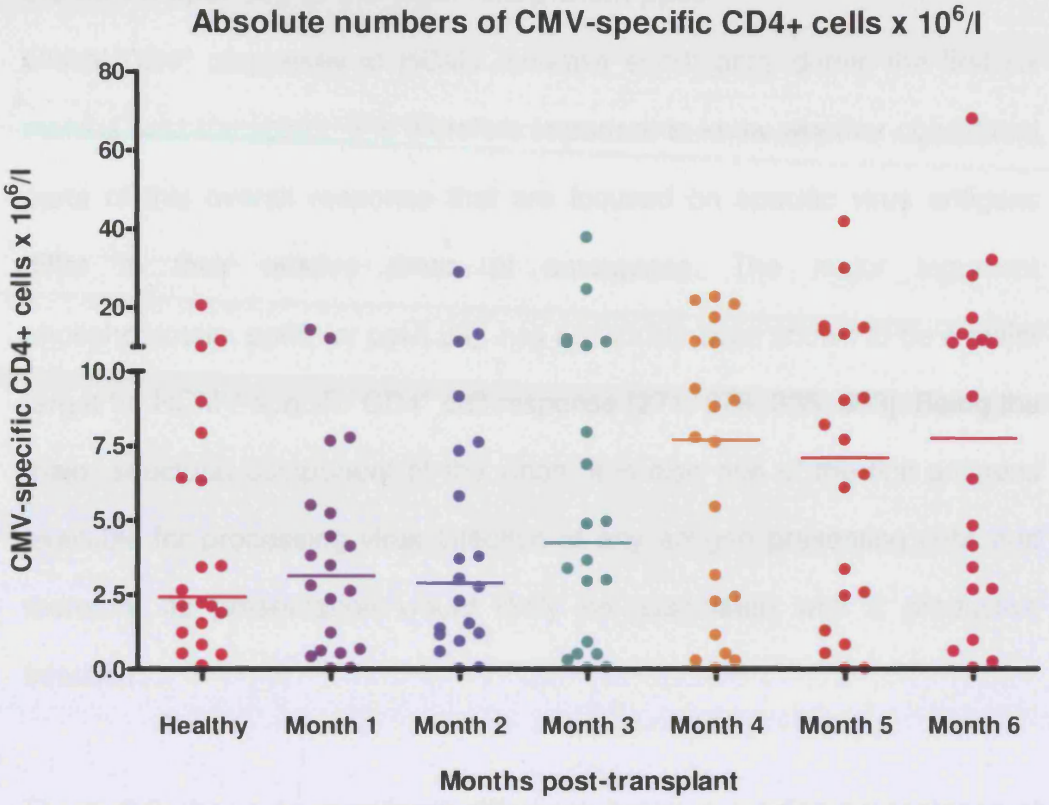


Figure 3. 2 Relative contributions of antigens to the HCMV-specific $CD4^+$ T-cell response



	median	healthy						
healthy	0.62		month 1					
month 1	0.65	0.42		month2				
month 2	0.69	0.65	0.38		month 3			
month 3	0.87	0.72	0.03	0.36		month 4		
month 4	1.55	0.25	0.004	0.006	0.21		Month 5	
month 5	1.14	0.37	0.004	0.011	0.16	0.69		month 6
month 6	1.45	0.1	0.001	0.003	0.009	0.21	0.11	

Figure 3. 3 Fluctuations in HCMV-specific $CD4^+$ populations as quantified by the percentage of cells expressing IFNγ and CD69 over a six month period post-transplant. Dotted line denotes the lower limit of detection (0.1%). The table below shows median percentages of each data set and P values as calculated using Willcoxon test for comparison between matched pairs and Mann-Whitney test for unmatched pairs. Significant values are highlighted in red.



	median	month 1					
month 1	3.27		month2				
month 2	2.94	0.26		month 3			
month 3	4.36	0.03	0.30		month 4		
month 4	7.84	0.002	0.10	0.34		month 5	
month 5	7.19	0.002	0.01	0.22	0.79		Month 6
month 6	7.84	0.003	0.02	0.05	0.15	0.04	

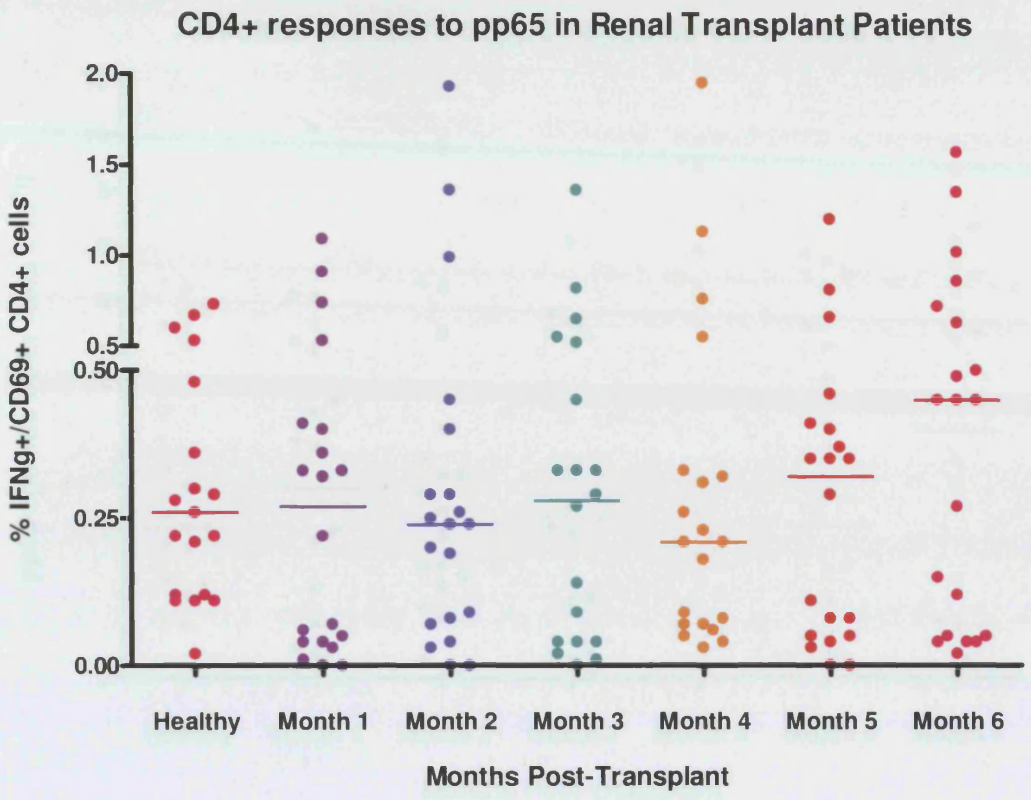
Figure 3. 4 Longitudinal analysis of the absolute number of circulating HCMV-specific $CD4^+$ cells, which were able to express CD69 and IFNγ on stimulation. Table shows median number of cells x 10^6/l of each time-point, and p-values for the significance of differences between samples using a paired T-test. Significant values are highlighted in red.

3.3 CD4 responses to the tegument protein pp65

Global $CD4^+$ responses to HCMV increase significantly during the first six months post-transplant; it is therefore important to know whether constituent parts of this overall response that are focused on specific virus antigens differ in their relative times of emergence. The major tegument phosphoprotein, pp65 (or ppUL83), has previously been shown to be a major target for HCMV-specific $CD4^+$ cell response [271, 276, 335, 363]. Being the major structural component of the virion, it is also one of the first antigens available for processing virus infection of any antigen presenting cells and therefore its presentation would likely be associated with a productive infection.

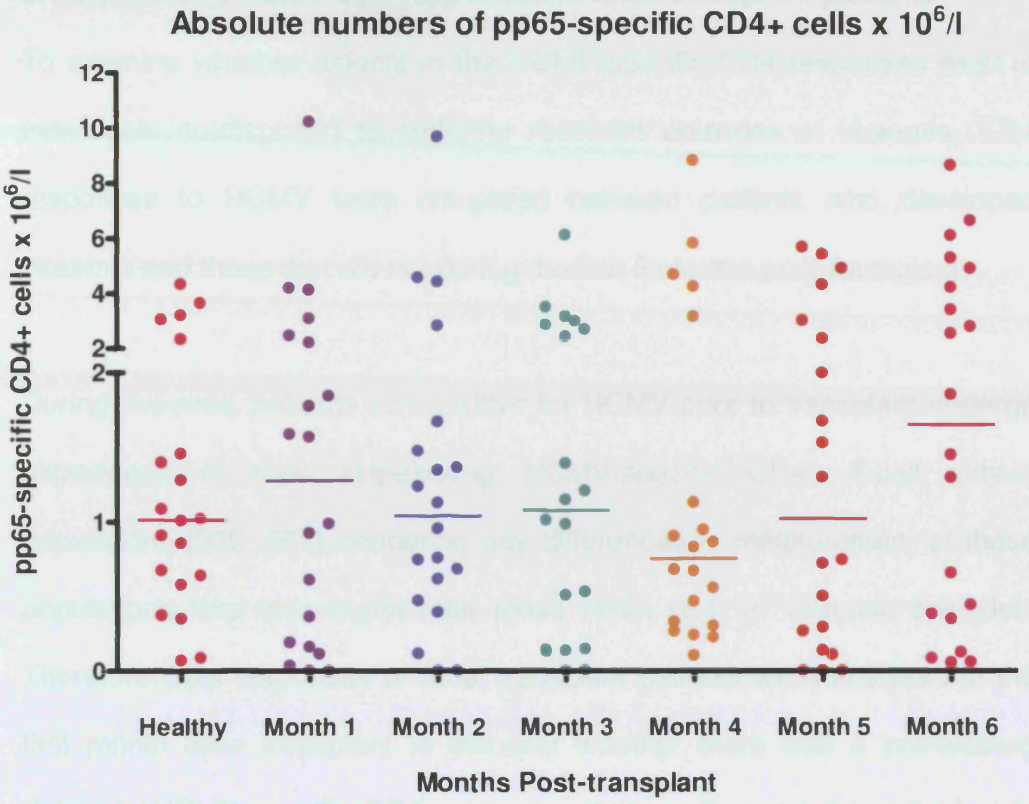
Figure 3.5 shows no significant difference between median percentages of pp65-specific $CD4^+$ T-cells was found between healthy seropositive individuals (median 0.27%) and renal transplant patients (highest median month 6, 0.46%, Mann-Whitney $p = 0.59$). Longitudinal analysis of pp65-specific CD4 responses in renal transplant patients, for the 6 months proceeding transplant, shows that they are fairly stable throughout with percentages ranging between <0.1% (below the lower limit of detection) and 1.95%. Median responses are lowest in the fourth month (0.21%) and peak in the sixth month post-transplant (0.46%). Significant increases occurred between months three and six (medians 0.28% to 0.46% $p = 0.04$), four and six (0.21% to 0.46% $p = 0.05$) and month five and six (0.33 to 0.46% $p = 0.003$).

When absolute responses are compared over the same period (Fig. 3.6), the number of circulating pp65-specific $CD4^+$ cells ranged from 0.05 x 10^6 cells/l to 10.23 x 10^6 cells/l. While the responses fluctuated during the six months period the only significant elevation occurred during the sixth month post-transplant.



	median	Healthy						
healthy	0.27		month 1					
month 1	0.27	0.53		month2				
month 2	0.25	0.43	0.56		month 3			
month 3	0.28	0.62	0.66	0.79		month 4		
month 4	0.21	0.37	0.41	0.85	0.44		month 5	
month 5	0.33	0.58	0.30	0.80	0.84	0.84		month 6
month 6	0.46	0.59	0.06	0.11	0.04	0.05	0.003	

Figure 3. 5 Fluctuations in pp65-specific $CD4^+$ populations as quantified by the percentage of cells expressing IFNγ and CD69 over a six month period post-transplant. Dotted line denotes the lower limit of detection (0.1%). The table below shows median percentages of each data set and P values as calculated using Willcoxon test for comparison between matched pairs and Mann-Whitney test for unmatched pairs. Significant values are highlighted in red.



	median	month 1					
month 1	1.31		month2				
month 2	1.06	0.66		month 3			
month 3	1.14	0.43	0.72		month 4		
month 4	0.77	0.77	0.44	0.53		month 5	
month 5	1.06	0.99	0.45	0.43	0.88		month 6
month 6	1.69	0.04	0.05	0.006	0.04	0.001	

Figure 3. 6 Longitudinal analysis of the absolute number of circulating IE1-specific $CD8^+$ cells that were able to express CD69 and IFNγ on stimulation. Table shows median number of cells x 10^6/l of each timepoint, and p-values for the significance of differences between samples using a paired T-test. Significant values are highlighted in red.

3.4 Comparing HCMV CD4 responses in renal transplant patients

To examine whether defects in the HCMV-specific CD4 responses exist in individuals predisposed to suffering recurrent episodes of viraemia, CD4 responses to HCMV were compared between patients who developed viraemia and those that did not during the first 6 months post-transplant.

During viraemia, patients seropositive for HCMV prior to transplant undergo expansions of their pre-existing HCMV-specific $CD4^+$ T-cell subset populations [335, 367], rendering any differences in measurement of these populations less meaningful than those taken prior to viraemic episodes. Therefore CD4 responses of renal transplant patients were analysed in the first month after transplant to discover whether there was a pre-existing defect in HCMV-specific CD4 responses that predisposed the individual to viraemic episodes post-transplant.

There was a non-significant decrease in the frequency of HCMV-specific $CD4^+$ cells in viraemic patients (median 0.53% and 0.80%, $p = 0.66$) during the first month post-transplant compared to transplant recipients who remain PCR negative for HCMV throughout the six months post-transplant (Fig. 3.7). This is consistent with data obtained when absolute numbers of circulating HCMV-specific $CD4^+$ cells were compared; viraemic patients had a median of 2.65×10^6 cells/l while patients who remained free from viraemia had a median of 4.69×10^6 cells/l ($p = 0.21$).

Several patients suffered from recurrent HCMV viraemia throughout the six month period post-transplant (see appendix). To investigate whether this was due to a defect in the overall HCMV-specific $CD4^+$ response during that time period, a comparison was performed using data on HCMV-specific $CD4^+$ T-cell populations accumulated from viraemic individuals and non-viraemic individuals over the six month period post-transplant (Fig. 3.8). Results again showed a non-significant decrease in the percentages of HCMV-specific $CD4^+$ T-cells in the peripheral blood of viraemic individuals when compared with non-viraemic individuals (median 0.97% and 1.25% respectively, $p = 0.08$). Although when absolute numbers were analysed the decrease was more pronounced (medians 3.43 and 7.00 x 10^6 cells/l respectively, $p = 0.02$), indicating that the responses were significantly elevated in those individuals capable of controlling virus replication.

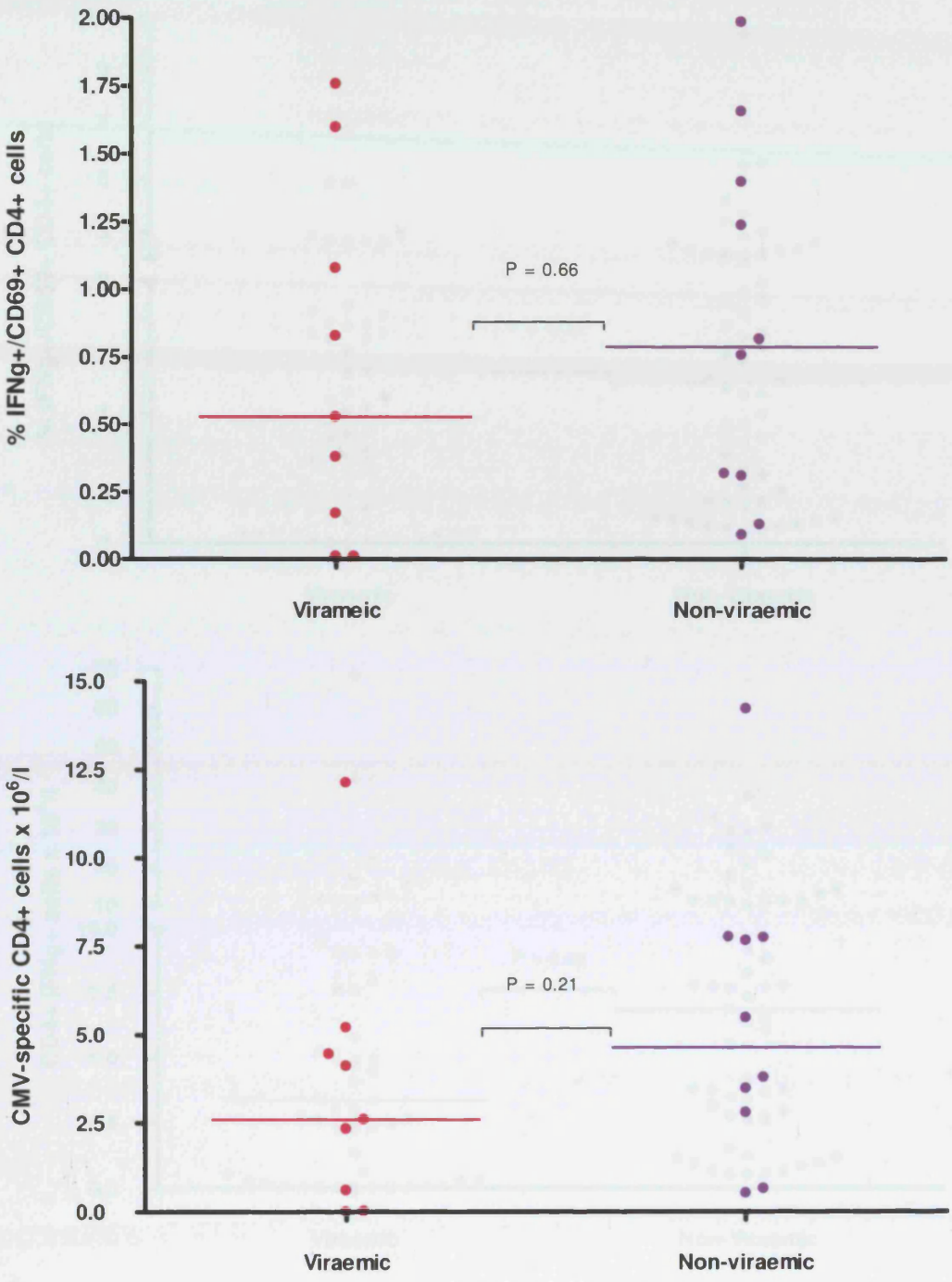


Figure 3. 7 Comparison of global $CD4^+$ responses to HCMV between viraemic and non-viraemic renal transplant patients. Top panel shows a comparison of $CD4^+$ T-cell percentages while the bottom panel has a comparison of absolute HCMV-specific $CD4^+$ T-cell populations in the peripheral blood.

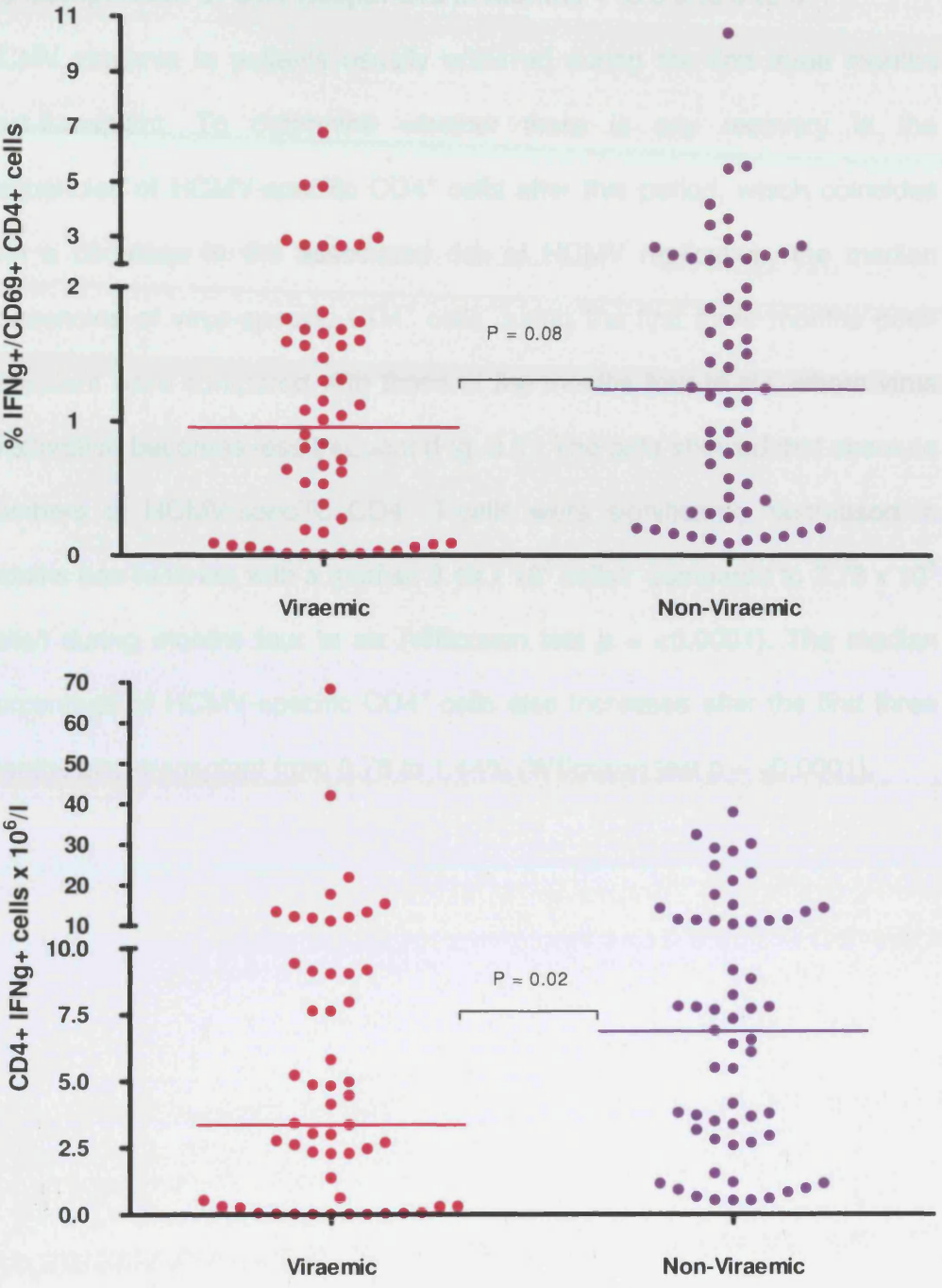


Figure 3. 8 Comparison of global $CD4^+$ responses to HCMV between viraemic and non-viraemic renal transplant patients over the entire six month period post-transplant. Upper panel shows a comparison of $CD4^+$ T-cell percentages while the lower panel shows absolute HCMV-specific $CD4^+$ T-cell populations in the peripheral blood.

3.5 Comparison of CD4 Responses in Months 1 to 3 and 4 to 6

HCMV viraemia in patients usually occurred during the first three months post-transplant. To determine whether there is any recovery in the frequencies of HCMV-specific $CD4^+$ cells after this period, which coincides with a decrease in the associated risk of HCMV replication, the median frequencies of virus-specific $CD4^+$ cells during the first three months post-transplant were compared with those of the months four to six, where virus reactivation becomes less frequent (Fig. 3.9). The data showed that absolute numbers of HCMV-specific $CD4^+$ T-cells were significantly decreased in months one to three, with a median 3.48×10^6 cells/l compared to 7.73×10^6 cells/l during months four to six (Willcoxon test $p = <0.0001$). The median percentage of HCMV-specific $CD4^+$ cells also increases after the first three months post-transplant from 0.78 to 1.44% (Willcoxon test $p = <0.0001$).

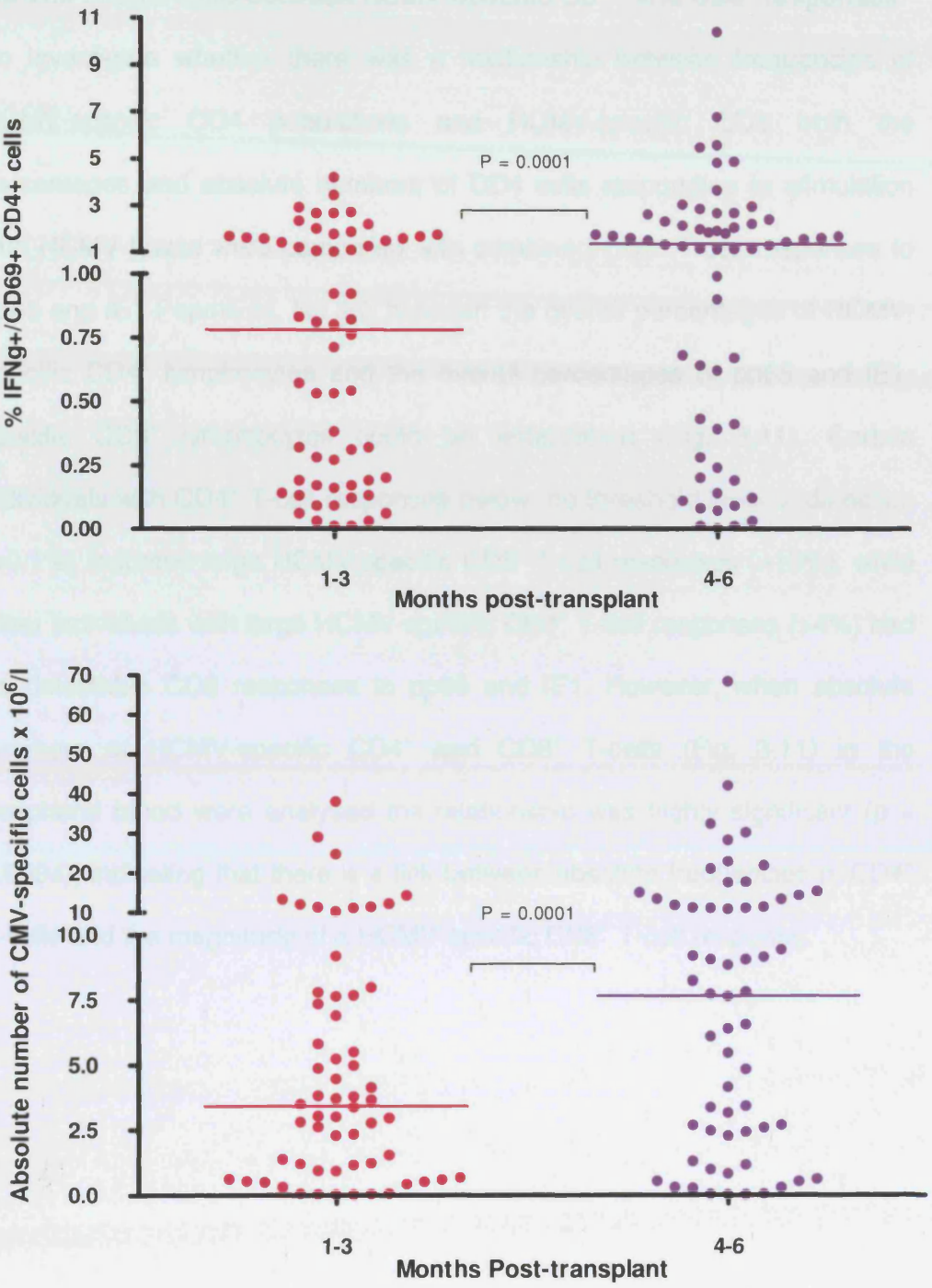


Figure 3. 9 A comparison of the absolute number of HCMV-specific $CD4^+$ responses of 20 renal transplant patients in months one to three and months four to six post-transplant. Top panel shows the percentages of HCMV-specific $CD4^+$ T-cells bottom panel shows the absolute number of HCMV-specific $CD4^+$ T-cells/ml in the peripheral blood.

3.6 The relationship between HCMV-specific $CD4^+$ and $CD8^+$ responses

To investigate whether there was a relationship between frequencies of HCMV-specific CD4 populations and HCMV-specific CD8 both the percentages and absolute numbers of CD4 cells responding to stimulation with HCMV lysate were compared with combined $CD8^+$ T-cell responses to pp65 and IE1 Pepmixes. No link between the overall percentages of HCMV-specific $CD4^+$ lymphocytes and the overall percentages of pp65 and IE1-specific $CD8^+$ lymphocytes could be established (Fig. 3.11). Certain individuals with $CD4^+$ T-cell responses below the threshold level of detection (<0.1%) mounted large HCMV-specific $CD8^+$ T-cell responses (>10%), while other individuals with large HCMV-specific $CD4^+$ T-cell responses (>4%) had no detectable CD8 responses to pp65 and IE1. However, when absolute numbers of HCMV-specific $CD4^+$ and $CD8^+$ T-cells (Fig. 3.11) in the peripheral blood were analysed the relationship was highly significant ($p = 0.0004$), indicating that there is a link between absolute frequencies of $CD4^+$ T-cells and the magnitude of a HCMV-specific $CD8^+$ T-cell response.

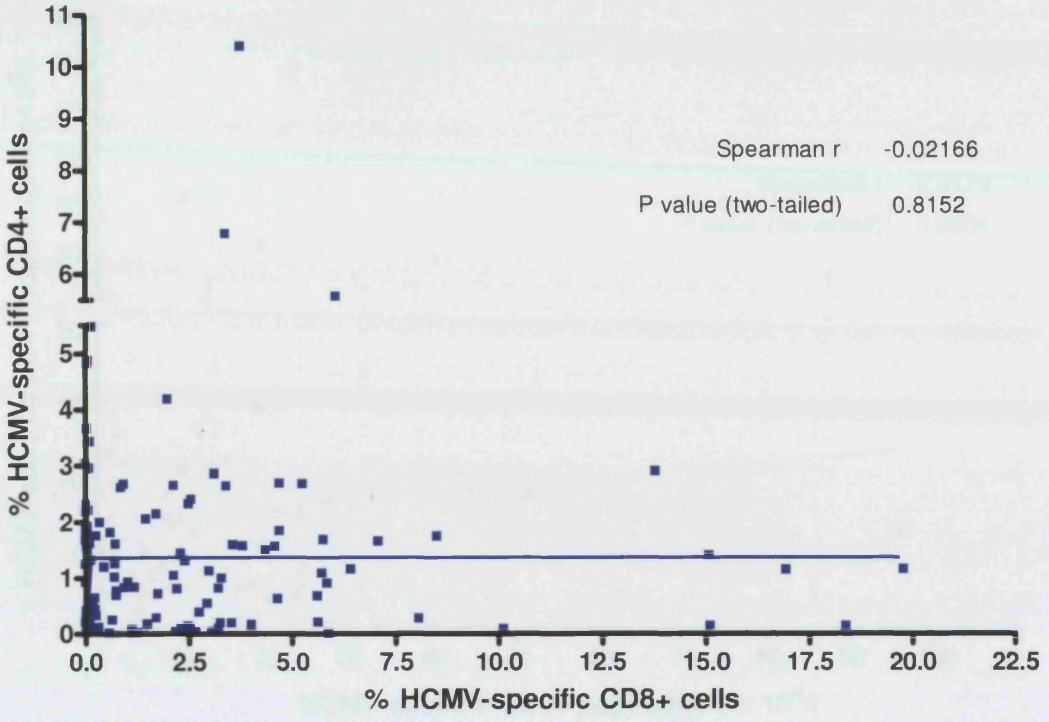


Figure 3. 10 Relationship between the percentages of HCMV-specific $CD4^+$ and $CD8^+$ T-cell populations taken from patients at one month intervals for six months post-transplant.

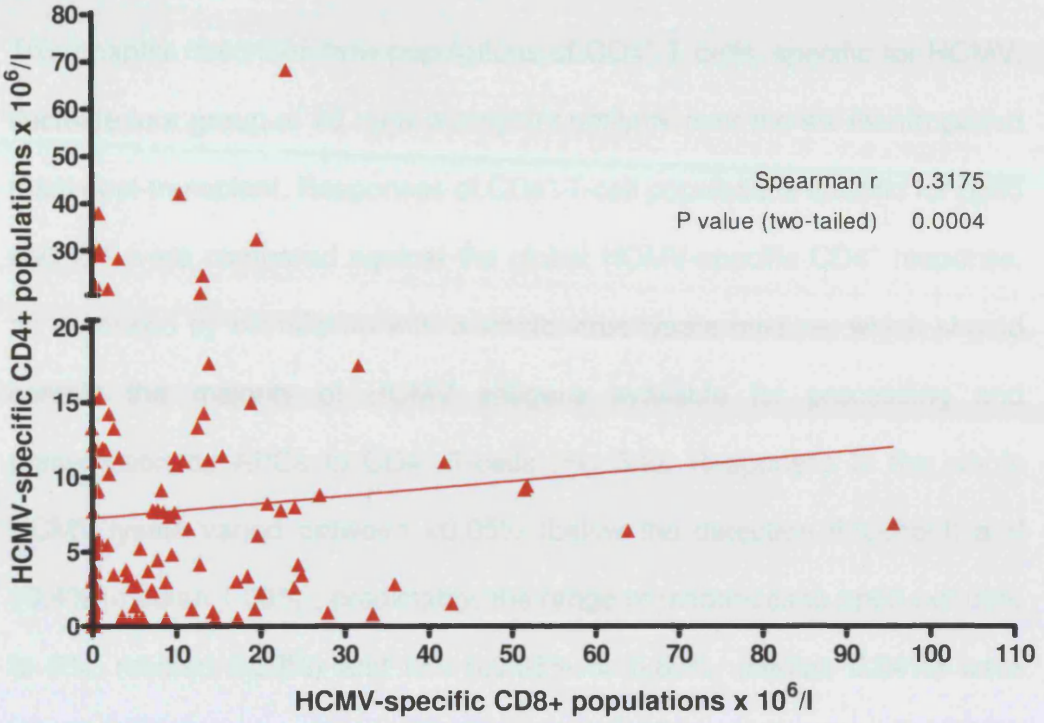


Figure 3. 11 Relationship between the absolute number of HCMV-specific $CD4^+$ and $CD8^+$ T-cell populations in the peripheral blood of twenty renal transplant patients taken at one month intervals for six months post-transplant.

3.7 Discussion

This chapter describes how populations of $CD4^+$ T-cells, specific for HCMV, fluctuate in a group of 20 renal transplant patients over the six month period renal post-transplant. Responses of $CD4^+$ T-cell populations specific for pp65 and IE1 were compared against the global HCMV-specific $CD4^+$ response, as assessed by stimulation with a whole virus lysate mixture, which should contain the majority of HCMV antigens available for processing and presentation by APCs to $CD4^+$ T-cells (Fig 3.2). Responses to the whole HCMV lysate varied between <0.05% (below the detection threshold) and 10.4% (median 1.08%); predictably, the range of responses to pp65 (<0.05% to 2%, median 0.26%) and IE1 (<0.05% to 0.89%, median 0.04%) were significantly lower, only contributing around 25% of the overall HCMV-specific $CD4^+$ response. These data indicate there must be other targets of the $CD4^+$ response to HCMV such as gB (the UL55 gene product) and UL32 [264]. Despite previously being shown to be a major target for the HCMV-specific $CD4^+$ T-cells [277, 324, 364-366], responses to IE1 were only above the lower level of detection (<0.05%) in two individuals analysed over the entire six month period analysed. Because of this, responses to pp65 were used as a more appropriate measure of $CD4^+$ T-cell responses to a constituent of the virion.

Figure 3.3 shows a longitudinal analysis of changes in the percentages of HCMV-specific $CD4^+$ T-cells; a general increase in HCMV-specific $CD4^+$ T-cell populations in the six months post-transplant that peaks during month four (median 1.56%), the populations then stabilised during the two

subsequent months. A two-tier increase in HCMV-specific populations was observed during this period, the first increase occurred between months one and three ($p = 0.03$) and the second between months three and six post-transplant ($p = 0.01$). Contrary to work performed by Sester et al. [332], there was no significant difference between the percentages of HCMV-specific $CD4^+$ T-cells in renal-transplant patients and healthy individuals.

Figure 3.4 shows that fluctuations in the absolute numbers of HCMV-specific $CD4^+$ T-cell in the peripheral blood follow a similar pattern, this being a gradual increase in the magnitude of HCMV-specific responses until month four, where they peak (median 7.84×10^6 cells/l) and then stabilise in months five and six. This once more shows a two-tier shape to the rise in the HCMV-specific CD4 population, which may coincide with previous observations that HCMV reactivation in renal transplant patients usually occurs for the first time within the three months immediately after transplant when T-cell populations are decreased because of immunosuppressive therapies [101, 368, 369]. Together these data indicate that $CD4^+$ T-cell levels may inversely correlate with the incidence of viraemia.

To determine whether the frequencies of $CD4^+$ populations directed against constituent parts of the virus follow similar kinetics for re-emergence, a similar longitudinal analysis was performed on populations specific to the major structural phosphoprotein pp65. Pp65 would be available for processing by APCs following initial infection as it is one of the most abundant components of the virion; also because it is a late-phase gene

product it would also be available for processing at late stages in the virus replication cycle. Both of these observations indicate that responses to pp65 could arise as a consequence of a productive virus infection. Data showed that $CD4^+$ T-cell responses to pp65 ranged from <0.05% and 1.95%; median responses towards pp65 fluctuated during the six month period post-transplant, being lowest during month four (0.21%) and highest in month six (0.46%) (Fig. 3.5). One significant rise in median CD4 populations occurred in month six. Fluctuations in the absolute frequencies of pp65-specific $CD4^+$ cells followed a similar pattern with median populations are again lowest during month four (0.76×10^6 cells/l) and highest during month six (1.67×10^6 cells/l) (Fig. 3.6). Because responses to pp65 fluctuate so much and do not mirror global responses as elicited by HCMV lysate, they would appear not to influence HCMV-specific responses as much as other antigens, such as glycoprotein B (gB) [370].

Several individuals monitored during the course of this study developed HCMV viraemia, which could predispose them to HCMV-associated disease. A possible reason for viraemia would be that T-cell responses in these individuals are for some reason unable to control virus replication, either because they are of insufficient magnitude or functionally deficient and therefore unable control virus replication. The frequencies of virus-specific IFNγ-producing $CD4^+$ T-cells were therefore compared in viraemic and non viraemic patients during the first month post-transplant when most incidences of viraemia occur. These measurements would give an indication of any predisposing defect in their $CD4^+$ responses rendering the individuals

more susceptible to viraemic episodes. Data suggests that although a slight increase in the percentages (Fig. 3.7 upper panel, median viraemics 2.65% median non-viraemics 3.84% p = 0.29) and the absolute levels (Fig. 3.7 lower panel, median viraemics 0.53 x 10^6 cells/l median non-viraemics 0.76 x 10^6 cells/l p = 0.76) of HCMV-specific $CD4^+$ T-cells occurred in the patients able to control virus replication in the six month period post-transplant, these elevations were not significant.

Because these patients almost invariably suffered repeated bouts of viraemia during the post-transplant period, overall responses to HCMV lysate over the entire period monitored were combined and compared to the responses of individuals able to control virus replication. A comparison of the median percentages of HCMV-specific $CD4^+$ T-cells between these two groups showed no significant difference (Fig. 3.8 upper panel 0.97% and 1.16% p = 0.08). Whilst the same analysis on median absolute numbers of HCMV-specific $CD4^+$ T-cells similarly showed an elevation (Fig. 3.8 lower panel 3.43 x 10^6 cells/l compared to 7.00 x 10^6 cells/l) which was significant (p = 0.02). Together these data indicate that $CD4^+$ T cell responses to HCMV play a crucial role in the control of viraemia.

The number of HCMV-specific $CD4^+$ T-cells seems to correlate directly with protection from HCMV viraemia - there also seems to be a two-tier increase in magnitude of HCMV-specific $CD4^+$ T-cell responses. A comparison of HCMV-specific $CD4^+$ T-cell responses was performed between months one to three and months four to six post-transplant (Fig. 3.9). Median absolute

numbers rose significantly ($p = 0.0001$) from months one to three (median 3.48×10^6 cells/l) to months four to six (median 7.78×10^6 cells/l). This was consistent with absolute numbers of HCMV-specific CD4 cells in the peripheral blood, which rose from 0.78% during the months one to three to 1.44% in months four to six ($p = 0.0001$). One possible explanation for this increase is that a decrease in the immunosuppressive regimens administered to the patients occurs in the months post-transplant; indeed, levels of calcineurin inhibitors tacrolimus and cyclosporine A are decreased during the third month post-transplant, from 10-15ng/ml to 6-10ng/ml and 250-350ng/ml to 150-250ng/ml, respectively. Furthermore administration of the corticosteroid prednisolone is decreased every two weeks post-transplant and stopped in the majority of patients after 2-3 months. This would allow recovery of T-cell populations and the resultant increases would correlate well with a reduced frequency of viraemia three months post-transplant. Therefore the increase in $CD4^+$ T-cell populations could have indirect effects that help control viraemia such as by increasing the frequency of HCMV-specific $CD8^+$ populations.

To determine whether increases in HCMV-specific $CD4^+$ T-cells has an effect on the populations of HCMV-specific $CD8^+$ T-cells, the relationship between HCMV-specific $CD4^+$ cells and combined $CD8^+$ T-cells responses elicited against IE1 and pp65 was compared within individuals. Figure 3.10 showed that there was no direct correlation between the frequencies of HCMV-specific $CD4^+$ and $CD8^+$ T-cells in terms of the percentages of each population. Whilst certain individuals had large $CD4^+$ T-cell responses

against HCMV their CD8 responses were undetectable. Similarly, certain individuals who had large $CD8^+$ responses in some cases had no detectable $CD4^+$ responses. Conversely, a comparison between the absolute numbers of HCMV-specific $CD4^+$ T-cell in the peripheral blood and the corresponding numbers of circulating HCMV-specific $CD8^+$ T-cells a correlation could be seen (Fig. 3.11 Spearman's $r = 0.32$, $p = 0.0004$). This data indicates that the magnitude of individuals' $CD4^+$ responses to HCMV has a bearing on the same individuals' responses to targets of the HCMV-specific $CD8^+$ T-cell response. Although certain individuals may still have high frequencies of pp65 and/or IE1-specific $CD8^+$ T-cell populations in the absence of HCMV-specific CD4 responses. This chapter demonstrates that, while there is a link between the absolute magnitude of HCMV-specific $CD4^+$ and $CD8^+$ T-cell populations in renal transplant patients, $CD4^+$ T-cells are not themselves directly protective in viraemic individuals. It may therefore be possible to conclude that virus-specific CD4 populations have an *in trans* effect on HCMV-specific $CD8^+$ T-cell populations, which may themselves be protective.

Chapter 4:

HCMV-specific $CD8^+$ Responses in Renal Transplant Recipients

4. 1 Introduction

This chapter focuses on HCMV-specific $CD8^+$ T-cell responses in renal transplant patients. Post-transplant, these individuals require immunosuppressive regimens to prevent the rejection of a donor organ. The resultant immunosuppression causes depletion of T-cell populations, thus increasing the chance of reactivation, reinfection or primary infection of the patient with HCMV.

$CD8^+$ T-cells recognise target antigens presented to them on the surface of all infected nucleated cells in the context of MHC class I molecules. Their principal function is to lyse infected cells, a process mediated through the polarised release of lytic proteins such as perforin [371-373], granzymes A and B and granulozymes, which are released on ligation of the MHC class I/cognate antigen complex with the TCR of the $CD8^+$ T-cell [230, 374, 375].

In immunocompetent individuals, a fine balance is maintained between HCMV and virus-specific $CD8^+$ T-cells; any reduction in the CD8 response would disrupt this equilibrium and this may consequently result in uncontrolled HCMV replication, which left unchecked could lead to HCMV-associated disease. Patients who are seronegative prior to transplantation and receive an organ from a seropositive donor have an increased risk of HCMV-associated disease on virus reactivation from the donor organ due to the lack of an, albeit suppressed, pre-existing virus-specific immune response being able to prevent high level replication.

Adoptive transfer of HCMV-specific $CD8^+$ T-cell clones in haematopoietic stem cell transplant recipients (SCT) has been shown to be successful for preventing of HCMV-associated disease [376, 377], although this is improved by the presence of HCMV-specific $CD4^+$ T-cells . Several studies have focused on the relationship between HCMV replication and $CD8^+$ T-cell frequencies in the various solid organ transplant patient groups; although few of these have addressed the absolute numbers of responsive cells or have looked longitudinally at the responses to the two major immunodominant targets for the $CD8^+$ T-cell response, namely pp65 and IE1.

High frequencies of $CD8^+$ T-cells specific for the structural phosphoprotein pp65 have previously been detected in a number of individuals [257, 266]. pp65 is a major constituent of the virion and would be immediately available for processing by professional antigen presenting cells (APCs) after infection. Its expression as a late stage protein during the HCMV replication cycle could therefore allow it to be viewed as a marker for production replication following virus reactivation.

This chapter aims to quantify the temporal magnitude of functional $CD8^+$ T-cells responses to the two immunodominant targets of HCMV-specific CD8 response, IE1 and pp65, longitudinally in renal transplant patients. Co-expression of the activation marker CD69 with the Th1 cytokine IFNγ in response to stimulation of PBMCs with overlapping 15-mer peptide mixes spanning IE1 and pp65 proteins was used as a measure of functionality of these $CD8^+$ T-cell populations [378].

These functional responses were also used in parallel with lymphocyte counts to determine the absolute numbers of functional virus-specific $CD8^+$ T-cells in the blood of these renal transplant patients; measured over six months post-transplant.

A subset of these patients still suffered from virus reactivation in the months following transplant despite the presence of HCMV-specific $CD8^+$ T-cell responses. To determine whether this phenomenon occurred as a result of a deficiency in the magnitude of HCMV-specific $CD8^+$ responses, a comparison of these responses was performed between viraemic and non-viraemic individuals.

4. 2 CD8$^+$ responses to pp65 in renal transplant patients

To examine the fluctuations in populations of HCMV-specific CD8$^+$ T-cells over the six months following renal transplant, PBMCs isolated from 20 renal transplant patients at monthly intervals were stimulated with overlapping synthetic peptide pools spanning the entire sequences of IE1 and pp65 proteins [378]. HCMV-specific responses towards these two pools were measured using FACS to determine the percentage of CD3$^+$ CD4$^-$ cells able to express CD69 and IFNγ (Fig. 4.1). Frequencies of CD69$^+$ IFNγ$^+$ CD8 cells were compared to those of healthy HCMV-seropositive individuals and analysed over time to determine whether there were any significant fluctuations in these populations in the six month period post-transplant.

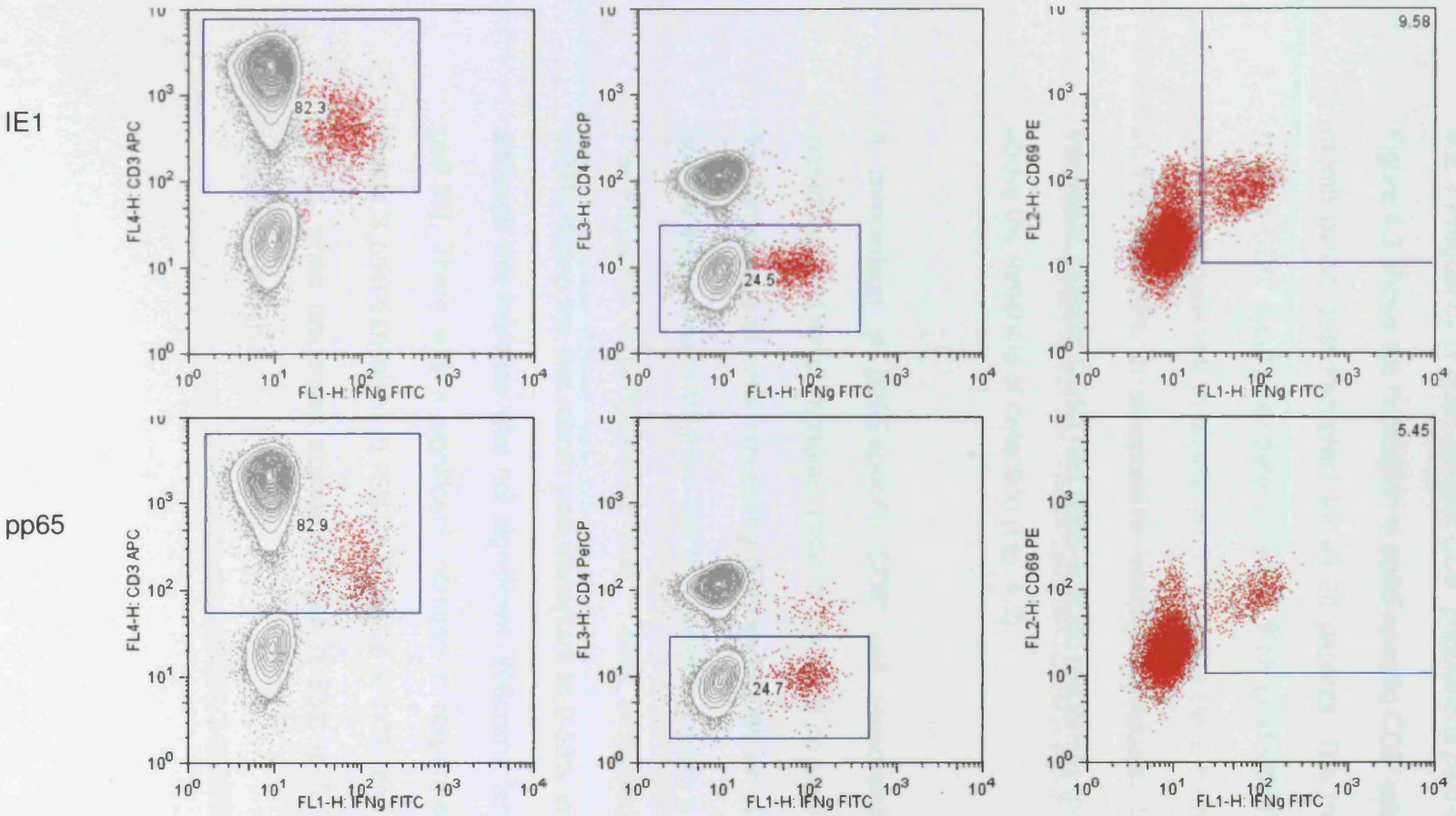


Figure 4. 1 FACS analysis plots of IFNγ production by a renal transplant patient in response to overlapping synthetic peptide pools spanning IE1 and pp65 sequences. Populations are gated on lymphocyte $^{+}$ $CD3^{+}$ $CD4^{-}$ populations. Left panels show gating of $CD3^{+}$ $IFN\gamma^{+}$ cells (T-cells), central panels show gating on $CD3^{+}$ $CD4^{-}$ T-cell populations (used here as CD8) the right panels show the proportion of the $CD3^{+}$ $CD4^{-}$ cells expressing CD69 and IFNγ in response to stimulation.

4.2. 1 Frequency of pp65-specific CD8⁺ populations post-transplant

Figure 4.3 shows the fluctuation in pp65-specific $CD8^+$ populations over the 6 month period post transplant for all 20 patients. The frequencies of pp65-specific $CD8^+$ T-cells, as determined by IFNγ production, range from <0.1% (below the detection threshold) to 6.7% in the renal transplant patients, and <0.1% to 5.69% in seropositive healthy individuals. 14 of the 20 renal transplant patients (70%) had pp65-specific responses that were consistently above the threshold of detection (Fig. 4.2).

A comparison of pp65-specific $CD8^+$ cell responses between healthy individuals and renal transplant patients revealed no significant differences in the percentage of cells expressing IFNγ and CD69 on stimulation with pp65 peptide mixes (Mann-Whitney test for unmatched pairs p = >0.05) (Fig. 4.3). The frequency of pp65-specific $CD8^+$ T-cells increased from a median of 0.25% during the first month post-transplant to 0.53% in the second month, although this increase was not significant (Willcoxon test for matched pairs p>0.05). There was a significant increase in responses from month 1 to month 3 post-transplant (0.25% to 0.53% p = 0.03) and between months 2 and 5, when responses appear to peak (0.53% to 0.94% p = 0.03) and stabilise at month 6.

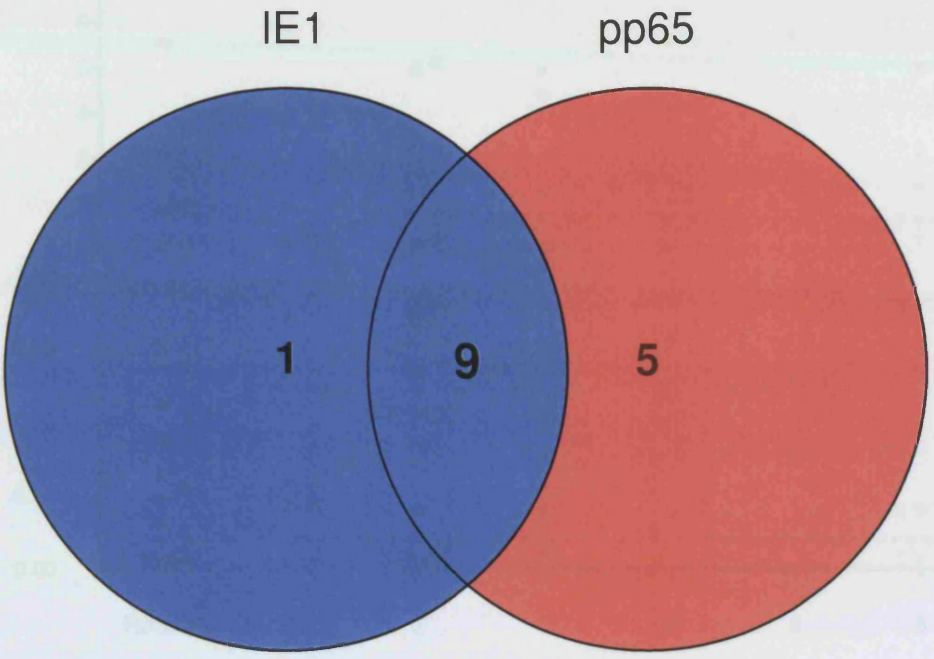
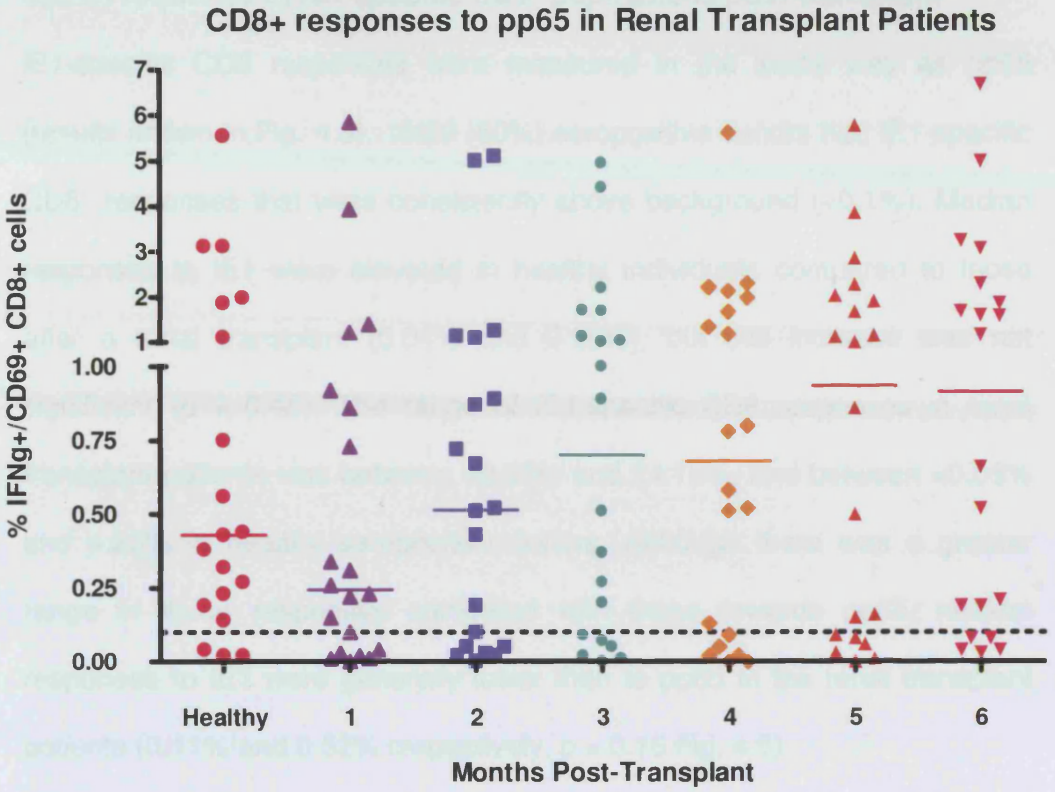


Figure 4. 2 Numbers of HCMV seropositive individuals with consistent detectable (>0.1%) $CD8^+$ T-cell responses against IE1 and/or pp65



	median %	healthy						
healthy	0.44		month 1					
month 1	0.25	0.23		month2				
month 2	0.53	0.70	0.26		month 3			
month 3	0.70	0.93	0.03	0.24		month 4		
month 4	0.69	0.88	0.07	0.10	0.32		month 5	
month 5	0.94	0.83	0.02	0.03	0.32	0.17		month 6
month 6	0.92	0.63	0.01	0.02	0.11	0.06	0.16	

Figure 4. 3 Fluctuations in pp65-specific CD8+ populations as quantified by the percentage of cells expressing IFNγ and CD69 over a six month period post-transplant. The dotted line denotes the lower limit of detection (0.1%). The table below shows median percentages of each data set and p values as calculated using Willcoxon test for comparison between matched pairs and Mann-Whitney test for unmatched pairs. Significant values are highlighted in red.

4.2. 2 Frequency of IE1-specific CD8⁺ populations post-transplant

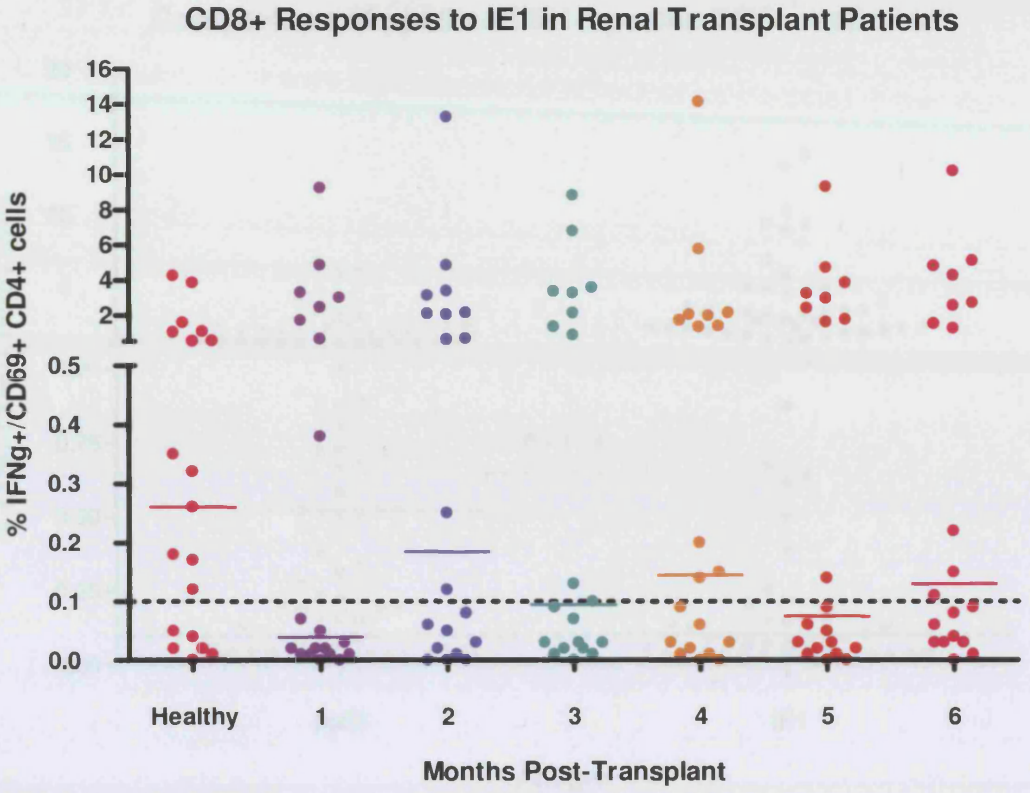
IE1-specific CD8 responses were measured in the same way as pp65 (results shown in Fig. 4.3). 10/20 (50%) seropositive donors had IE1-specific $CD8^+$ responses that were consistently above background (<0.1%). Median responses to IE1 were elevated in healthy individuals compared to those after a renal transplant (0.04% and 0.26%), but this increase was not significant ($p = 0.46$). The range of IE1-specific CD8 responses in renal transplant patients was between <0.05% and 14.19%, and between <0.05% and 4.23% in healthy seropositive donors. Although there was a greater range in these responses compared with those towards pp65, median responses to IE1 were generally lower than to pp65 in the renal transplant patients (0.11% and 0.52% respectively, $p = 0.16$ Fig. 4.5).

Fig 4.4 shows that median immune responses to IE1 were lowest in the first month post-transplant (0.04%) and peaked during the second month post-transplant (0.19%) and this increase was significant (Willcoxon $p = 0.007$). This peak could correspond to viral reactivation in either previously seropositive patients or the organs from seropositive donors early after transplantation driving the IE1-specific $CD8^+$ response. Frequencies continued to fluctuate in the subsequent four months although the only other significant increase in these populations occurred between the first and sixth month post-transplant (0.04% and 0.14% $p = 0.025$).

4.2. 3 Responses of combined pp65/IE1-specific CD8⁺ populations post-transplant

Because IE1 and pp65 are believed to represent the principal targets for the CD8-T-cell response, combining the CD8 responses to both of these antigens may give a more accurate representation of global HCMV-specific $CD8^+$ T-cell responses. Figure 4.6, shows combined responses to these two antigens to vary between <0.05% to 19.8% following renal transplant. 15/20 (75%) of renal transplant had T-cell responses to either or both of these antigens (Fig. 4.2) while some lacked detectable $CD8^+$ responses to both of these antigens. Interestingly, the highest percentages of antigen-specific $CD8^+$ responses consistently came from one individual who suffered repeated episodes of viraemia during the 6 months post-transplant.

Figure 4.6 shows $CD8^+$ responses to pp65 and IE1 in renal transplant patients over the 6 month period post-transplant. Lowest median responses, as before, occurred during the first month post-transplant (median 0.74%) and peaked during the fourth month post-transplant (2.25%), with a significant difference in median values between months one and two (rising from 0.74 to 1.17% Willcoxon $p = 0.02$). These percentages continued to rise significantly, with respect to the first month, until month four when the percentage of HCMV-specific $CD8^+$ T-cells against pp65 and IE1 peaked at a median of 2.25% and stabilised in the months thereafter.



	median %	healthy						
healthy	0.26		month 1					
month 1	0.04	0.46		month2				
month 2	0.19	0.92	0.01		month 3			
month 3	0.1	0.84	0.09	0.89		month 4		
month 4	0.15	0.97	0.28	0.77	0.68		Month 5	
month 5	0.08	0.87	0.13	0.59	0.50	0.93		month 6
month 6	0.14	0.78	0.02	0.92	0.41	0.71	0.12	

Figure 4. 4 Fluctuations in IE1-specific CD8+ populations as quantified by the percentage of cells expressing IFNγ and CD69, over a six month period post-transplant. The dotted line denotes background (0.1%). The table below shows median percentages of each data set and p values as calculated using Willcoxon test for comparison between matched pairs and Mann-Whitney test for unmatched pairs. Significant values are highlighted in red.

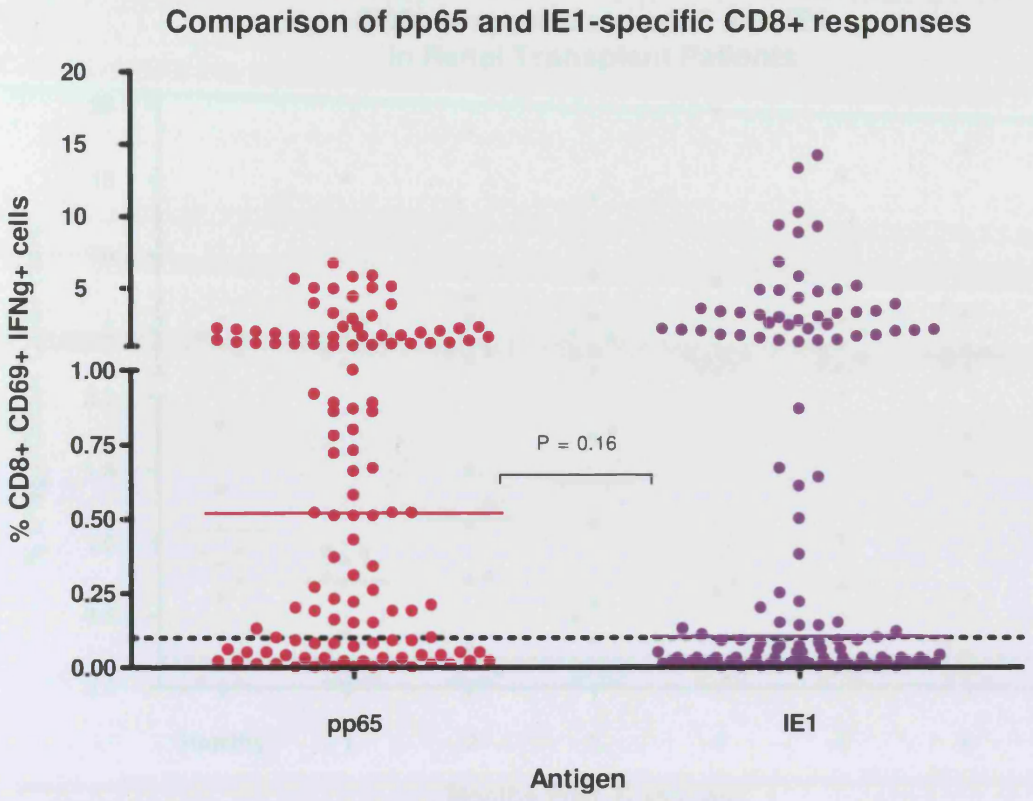
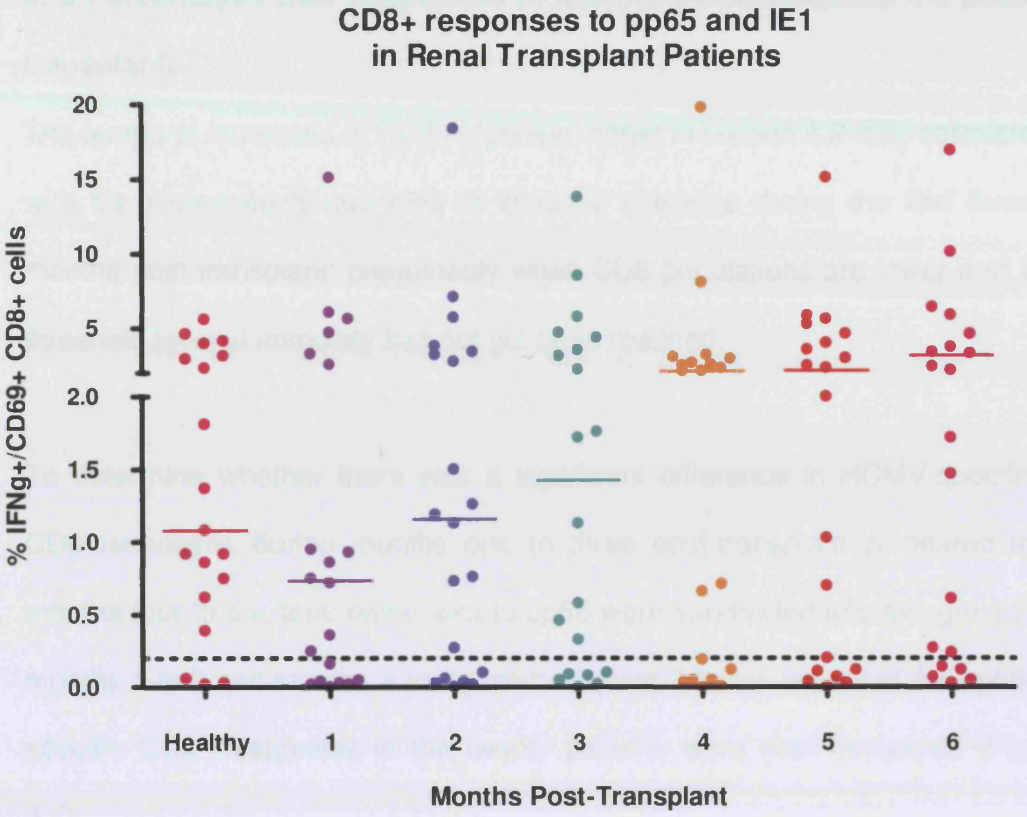


Figure 4. 5 The relative contributions of $CD8^+$ responses to pp65 and IE1 in renal transplant patients during the six months post-transplant. The dotted line indicates the lower limit of sensitivity.



	median %	healthy						
healthy	1.08		month 1					
month 1	0.74	0.31		month2				
month 2	1.18	0.78	0.02		month 3			
month 3	1.44	0.96	0.03	0.40		month 4		
month 4	2.25	0.75	0.16	0.57	0.79		month 5	
month 5	2.36	0.67	0.04	0.46	0.45	0.20		month 6
month 6	3.31	0.63	0.01	0.26	0.34	0.28	0.22	

Figure 4. 6 Fluctuations in IE1 and pp65-specific CD8+ populations, quantified by the percentage of cells expressing IFNγ and CD69, over a six month period post-transplant. The dotted line denotes background (0.2%). Table below shows median percentages of each data set and p values as calculated using Willcoxon test for comparison between matched pairs and Mann-Whitney test for unmatched pairs. Significant values are highlighted in red.

4. 3 Percentages CD8 Responses in Months 1-3 and Months 4-6 post-transplant.

The temporal increases in CD8 responses noted in section 4.2 may correlate with the increased frequencies of viraemic episodes during the first three months post-transplant; presumably when CD8 populations are lower and a threshold level of immunity has not yet been reached.

To determine whether there was a significant difference in HCMV-specific CD8 responses during months one to three post-transplant compared to months four to six, total responses to pp65 were subdivided into two groups, months 1 to 3 and months 4 to 6 post-transplant. Median values of the pp65-specific $CD8^+$ responses in the twenty patients were then compared (Fig. 4.7).

A significant rise in the median percentages of pp65-specific $CD8^+$ populations occurred after the third month post-transplant, with these populations increasing from 0.42% to 0.8% (Willcoxon test $p = 0.002$). The same analyses were performed using responses towards IE1 and combined IE1 and pp65 antigens. IE1 responses increased from a median of 0.09% to 0.15% but this increase was not significant (Fig 4.8). The combined median antigen-specific $CD8^+$ cell percentage increased from 0.93 to 2.18%, although this rise was not significant (Fig 4.9).

Determining the percentage of $CD8^+$ T-cells able to produce IFNγ in response to these immunodominant targets allowed the determination of the

proportions of CD8$^+$ populations specific to virus antigens, but it does not provide the absolute numbers of these functional HCMV-specific cells in the peripheral blood of the individuals. The absolute numbers of cells may give a more accurate measure of CD8$^+$ T-cells cells able to control virus replication.

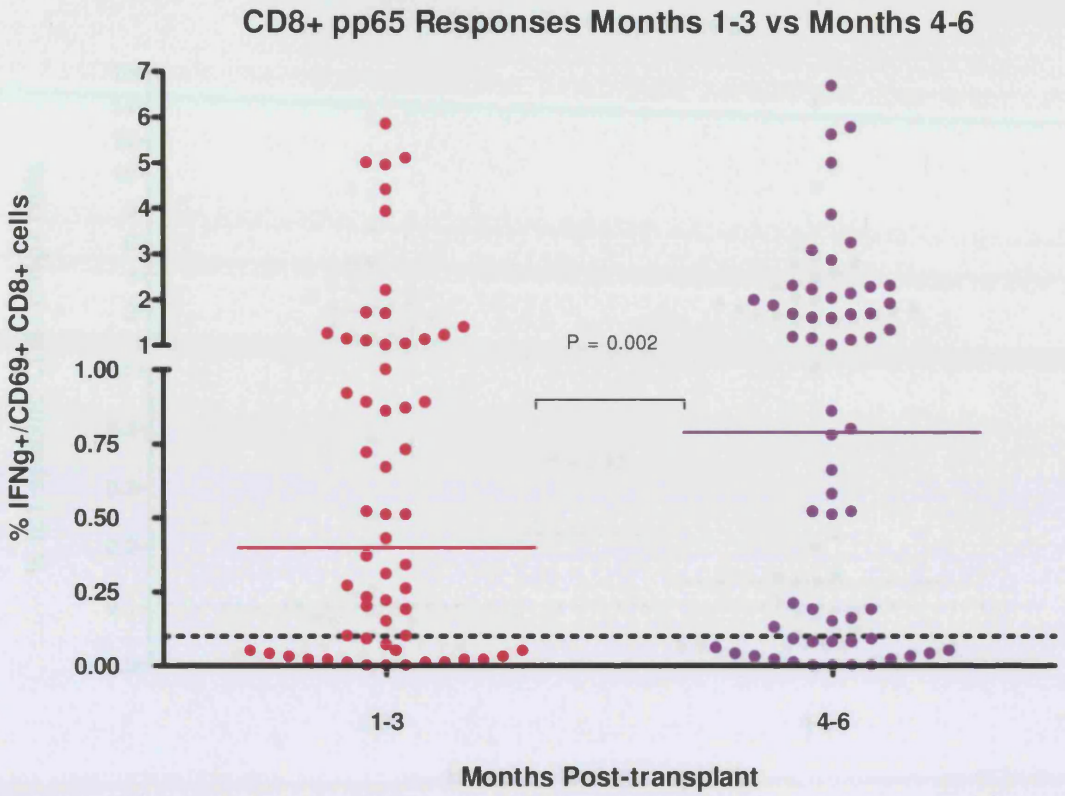


Figure 4. 7 Comparison of pp65-specific CD8+ responses in twenty renal transplant patients in months 1 to 3 and months 4 to 6 post-transplant. The dotted line denotes lower level of sensitivity (0.1%).

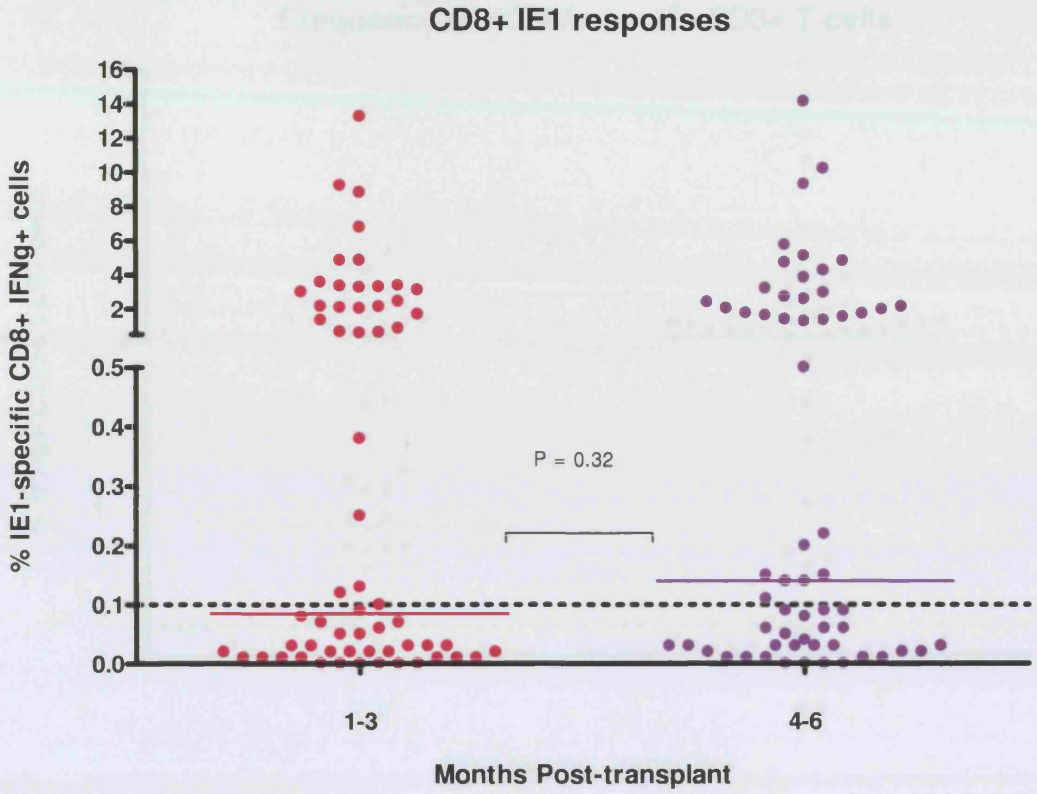


Figure 4. 8 A comparison of IE1-specific CD8+ responses in twenty renal transplant patients in the first three and second three months post-transplant. The dotted line denotes lower level of sensitivity (0.1%).

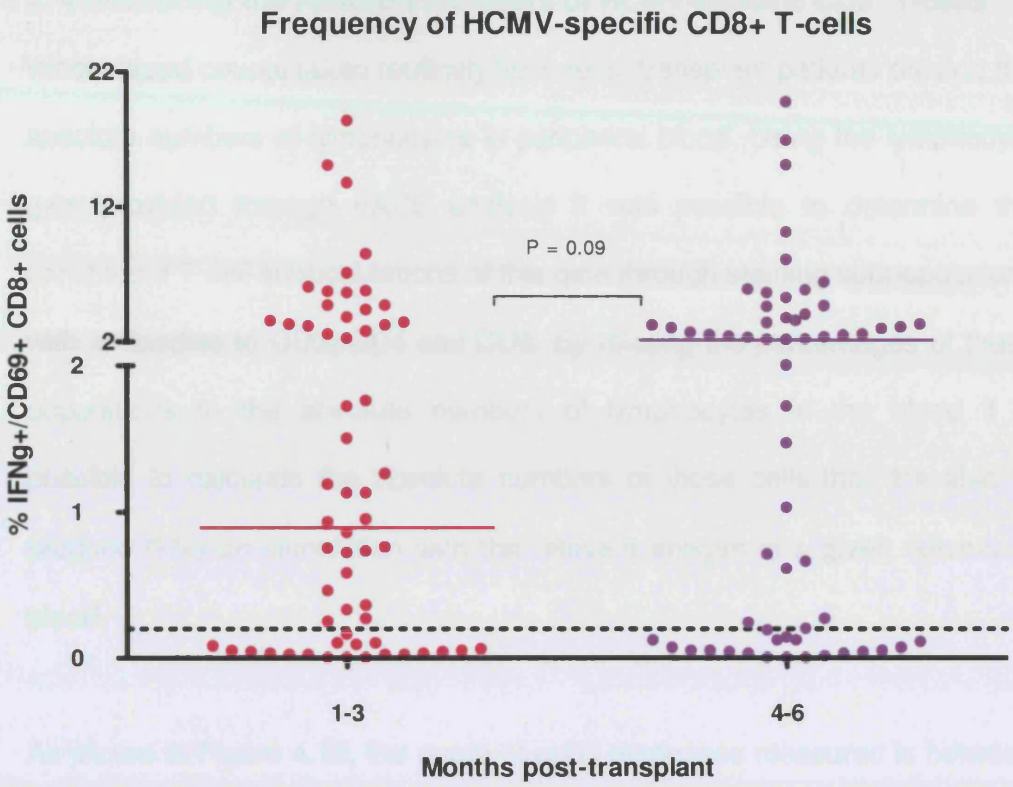


Figure 4. 9 Combined IE1 and pp65-specific CD8+ responses in twenty renal transplant patients in the first three and second three months post-transplant. The dotted line denotes lower level of sensitivity (0.2%).

4. 4 Measuring the Absolute Numbers of HCMV-specific CD8⁺ T-cells

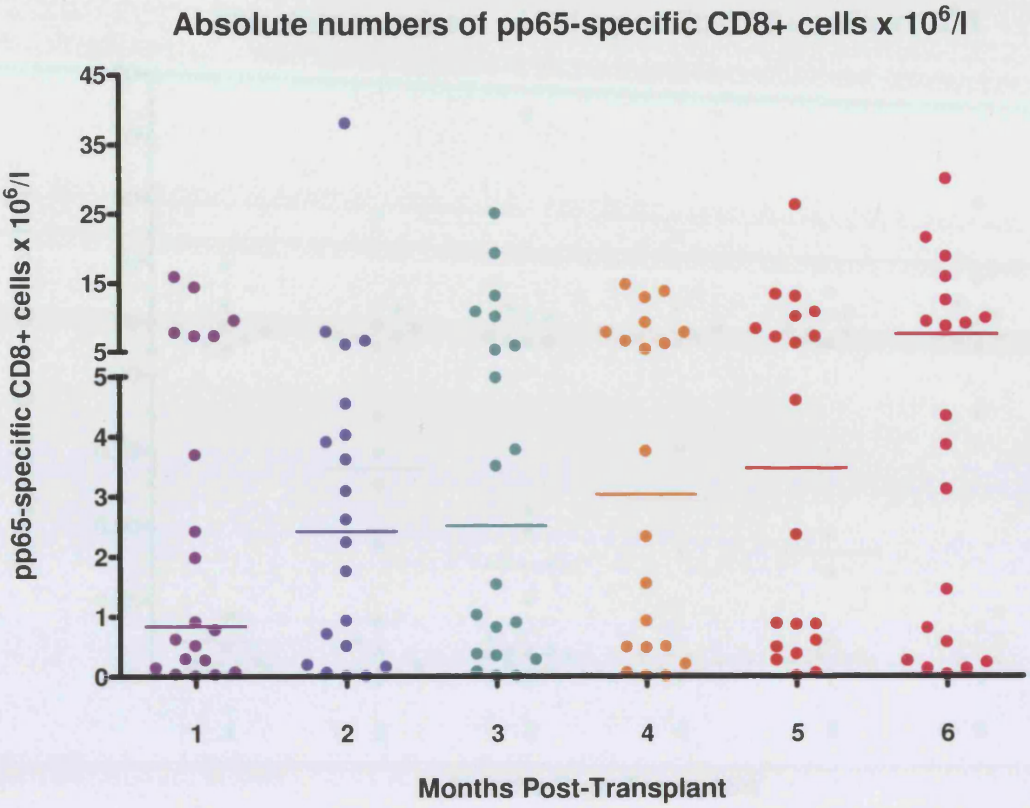
Whole blood counts taken routinely from renal transplant patients provide the absolute numbers of lymphocytes in peripheral blood. Using the lymphocyte gate provided through FACS analysis it was possible to determine the constituent T-cell subpopulations of this gate through staining subpopulations with antibodies to CD3, CD4 and CD8. By relating the percentages of these populations to the absolute numbers of lymphocytes in the blood it is possible to calculate the absolute numbers of these cells that are able to produce IFNγ on stimulation with the relevant antigen in a given volume of blood.

As shown in Figure 4.10, the range of pp65 responses measured is between 0 (below the lower level of detection) and 3.8 x 10^7 cells per litre, confirming that some seropositive individuals lack detectable pp65-specific $CD8^+$ responses. The absolute numbers of pp65-specific $CD8^+$ cells was lowest during the first month post-transplant (median 0.87 x 10^6 cells/l). This increased in the second (median 2.45 x 10^6 cells/l), third (median 2.53 x 10^6 cells/l), fourth (median 3.05 x 10^6 cells/l), fifth (median 3.48 x 10^6 cells/l) months and peaks in the sixth month (median 4.14 x 10^6 cells/l). Despite the consistent increases in these populations the first significant rise occurred between the first and the third months post-transplant (Willcoxon $p = 0.04$) and continued to increase, non-significantly, in the subsequent three months.

Absolute numbers of IE1-specific $CD8^+$ cells in this group of renal transplant patients range from 0 to 7.6 x 10^7 cells/l (Fig. 4.11). The lowest median

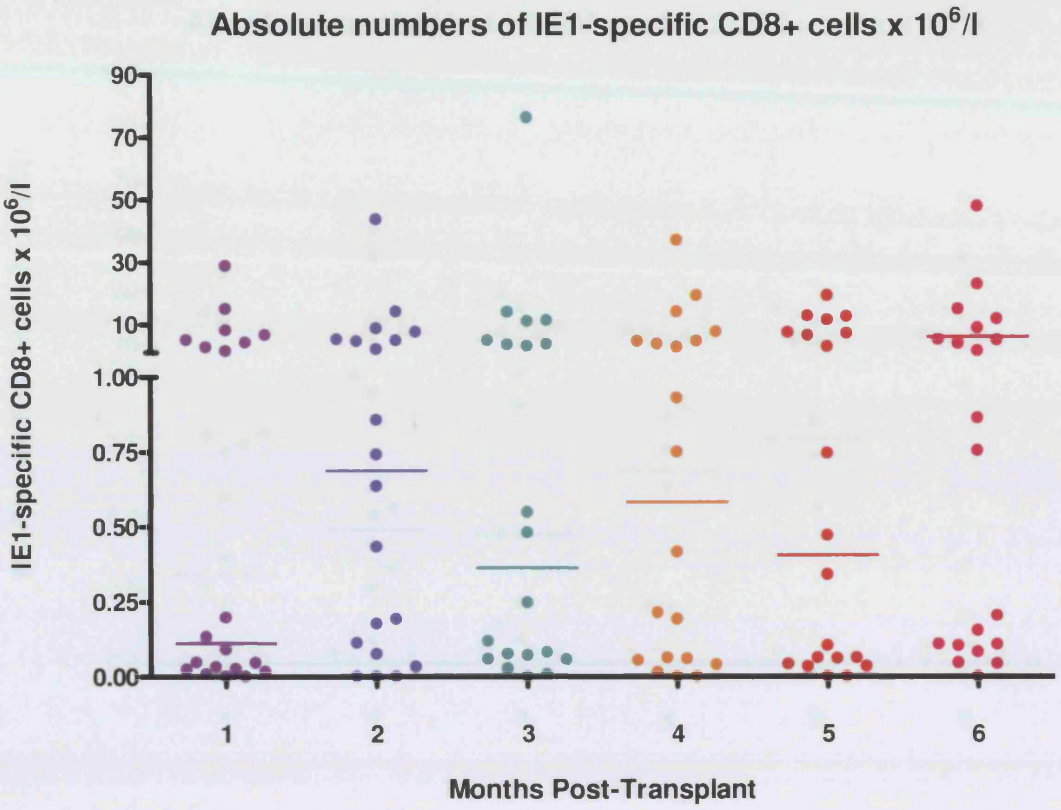
frequencies of IE1-specific CD8$^+$ cells are again found in the first month post-transplant (1.2 x 10^5cells/litre). This number increased during the second month to 6.9 x 10^5 cells/l and then fluctuated for the subsequent four months peaking at 6 months post-transplant at 8.2 x 10^5 cells/ml. The only significant increase in IE1-specific populations occurred between months one and six post-transplant (Willcoxon p = 0.001).

The changes in the absolute numbers of combined pp65 and IE1-specific CD8 cells are shown in Figure 4.12. The combined CD8$^+$ responses gradually increased over the 6 months post-transplant, rising from a median of 3.1 x 10^6 cells/ml in the first month to 9.8 x 10^6 cells/ml in month six (p = 0.0023), although the increase between month 1 to 6 and between month 2 and month 6 (4.57 to 9.8 x 10^6 cells/ml, p = 0.03) are the only statistically significant differences.



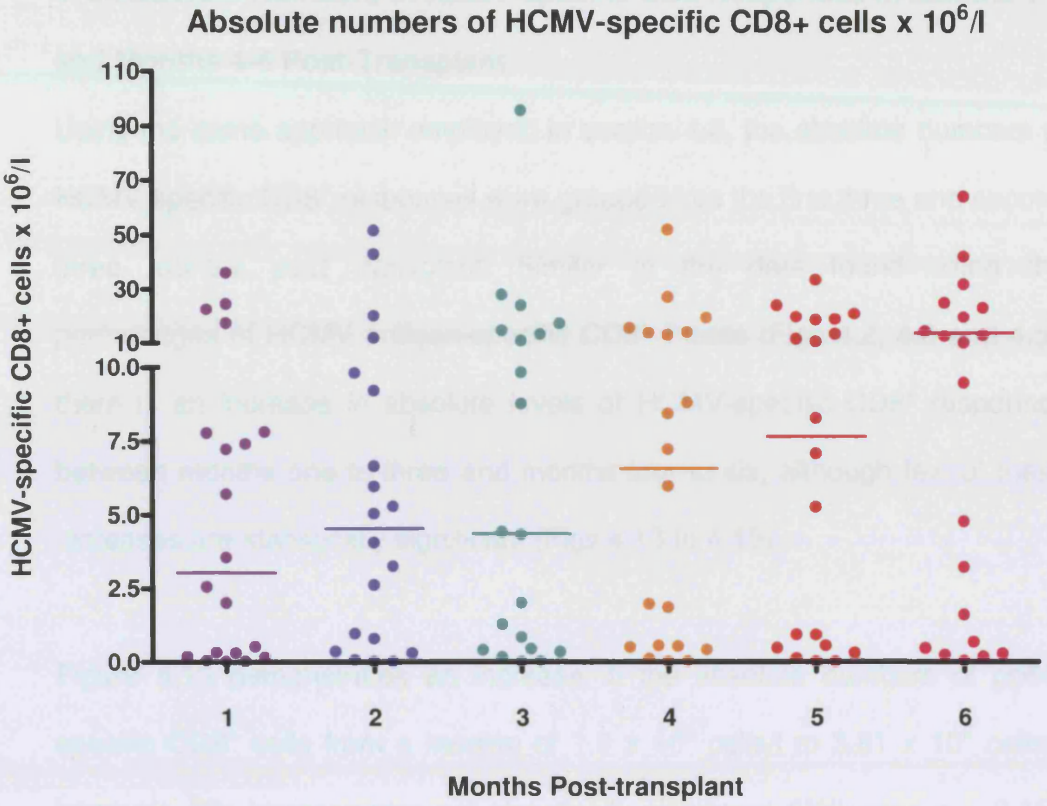
	median						
		month 1					
Month 1	0.87		month2				
Month 2	2.45	0.53		Month 3			
Month 3	2.53	0.04	0.20		month 4		
Month 4	3.05	0.07	0.11	0.58		month 5	
Month 5	3.48	0.03	0.07	0.23	0.72		month 6
Month 6	4.14	0.001	0.008	0.12	0.09	0.1108	

Figure 4. 10 Longitudinal analysis of the absolute number of circulating pp65-specific CD8+ cells that are able to express CD69 and IFNγ on stimulation. The table shows median number of cells x 10^6/l of each time-point, and p-values for the significance of differences between samples using a paired T-test. Significant values are highlighted in red.



	median						
Healthy		month 1					
Month 1	0.12		month2				
Month 2	0.69	0.08		month 3			
Month 3	0.37	0.12	0.67		month 4		
Month 4	0.59	0.23	0.85	0.25		month 5	
Month 5	0.41	0.20	0.82	0.32	0.56		month 6
Month 6	0.82	0.001	0.11	0.32	0.15	0.06	

Figure 4. 11 Longitudinal analysis of the absolute number of circulating IE1-specific CD8+ cells that are able to express CD69 and IFNγ on stimulation. The table shows median number of cells x 10^6/l of each timepoint, and p-values for the significance of differences between samples using a paired T-test. Significant values are highlighted in red.



	Median	month 1					
Month 1	3.1		month2				
Month 2	4.57	0.36		month 3			
Month 3	4.41	0.10	0.40		month 4		
Month 4	6.64	0.24	0.40	0.45		month 5	
Month 5	7.73	0.22	0.26	0.19	0.51		month 6
Month 6	9.8	0.002	0.03	0.19	0.13	0.07	

Figure 4. 12 Absolute number of circulating HCMV-specific CD8+ cells that are able to express CD69 and IFNγ on stimulation in renal transplant patients over six months. The table shows median number of cells x 10^6/l of each time-point, and p-values for the significance of differences between samples using a paired T-test. Significant values are highlighted in red.

4. 5 Absolute Numbers of HCMV-specific CD8 Responses in Months 1-3 and Months 4-6 Post-Transplant.

Using the same approach employed in section 4.3, the absolute numbers of HCMV-specific CD8$^+$ responses were grouped into the first three and second three months post transplant. Similar to the data found using the percentages of HCMV antigen-specific CD8$^+$ T-cells (Figs 4.2, 4.3 and 4.5), there is an increase in absolute levels of HCMV-specific CD8$^+$ responses between months one to three and months four to six, although few of these increases are statistically significant (Figs 4.13 to 4.15).

Figure 4.13 demonstrates an increase in the absolute numbers of pp65-specific CD8$^+$ cells from a median of 1.9 x 10^6 cells/l to 3.81 x 10^6 cells/l, however, this increase was not statistically significant (Willcoxon p = 0.15). Median numbers of IE1-specific CD8$^+$ T-cells also increased from 3.4 x 10^5 cells/l to 6.2 x 10^5 cells/l, but once again this increase was not statistically significant (p = 0.19) (Fig. 4.14).

A comparison of the absolute numbers of combined IE1 and pp65-specific CD8 responses illustrated in Figure 4.15 shows a significant increase in median CD8 populations after the first three months post-transplant. The median overall number of CD8$^+$ T-cells able to secrete IFNγ on stimulation with pp65 and IE1 in the peripheral blood increased significantly from 4.25 to 7.84% (p = <0.05) between months 1 to 3 and 4 to 6. These data correlate well with the observation that patients usually suffer viraemic episodes within the first three months post-transplant.

These data provide information on the kinetics of CD8$^+$ T-cell population fluctuations in the months proceeding renal transplantation, but they do not provide information on whether these populations can protect the recipient from HCMV replication.

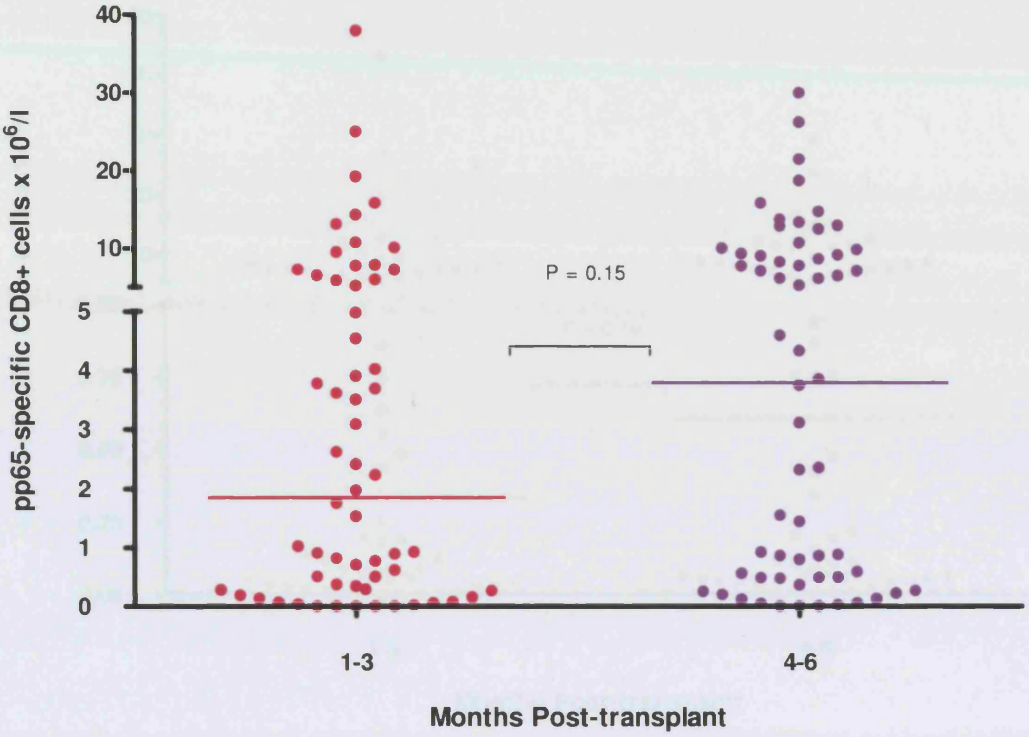


Figure 4. 13 Comparison of the absolute number of pp65-specific CD8+ responses in months 1-3 and 4-6 post-transplant.

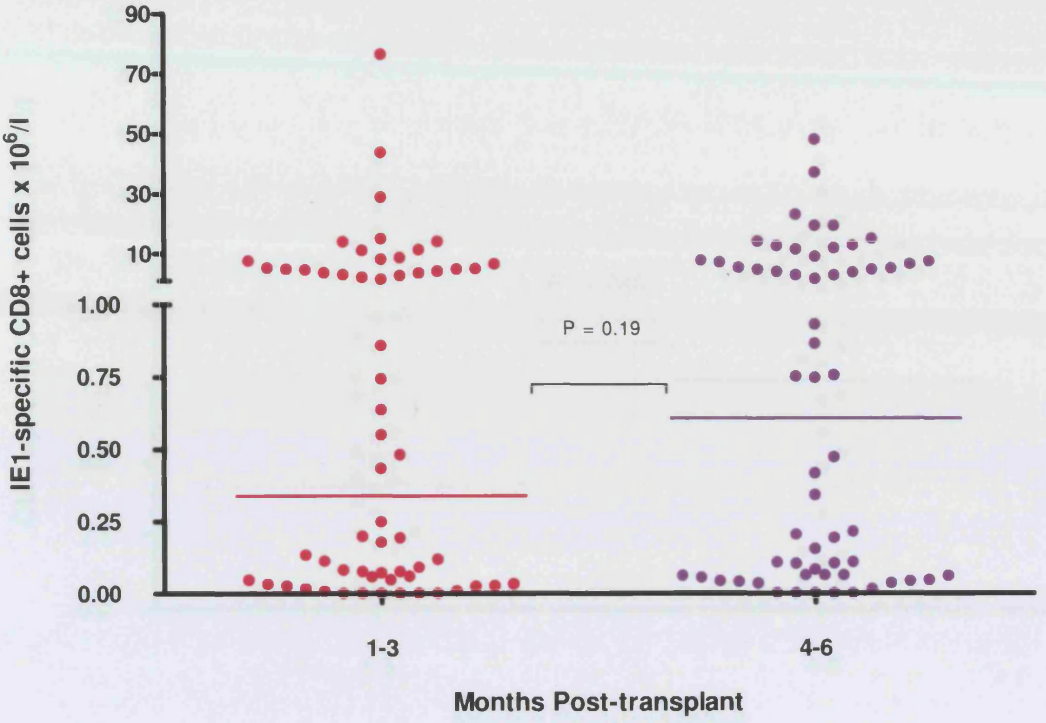


Figure 4. 14 Comparison of the absolute number of IE1-specific CD8⁺ responses in months 1-3 and 4-6 post-transplant.

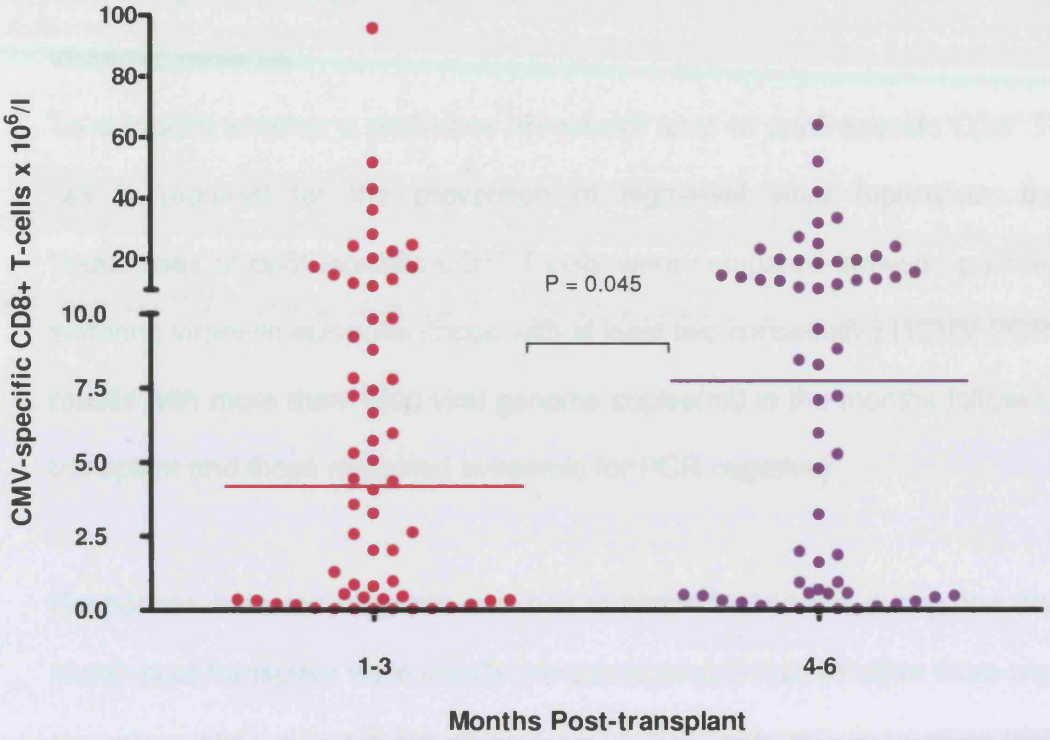


Figure 4. 15 Comparison of the absolute number of combined pp65 and IE1-specific CD8+ responses in the months 1-3 and 4-6 post-transplant.

4. 6 Comparison of pp65-specific CD8⁺ responses in viraemic and non-viraemic patients

To establish whether a protective "threshold" level of pp65-specific $CD8^+$ T-cell is required for the prevention of high-level virus replication, the frequencies of pp65-specific $CD8^+$ T-cells were compared between patients suffering viraemic episodes (those with at least two consecutive HCMV PCR^+ results with more than 1000 viral genome copies/ml) in the months following transplant and those remained aviraemic (or PCR negative)

Responses between viraemic and non viraemic individuals during the first month post-transplant were initially compared to ascertain whether there was any pre-existing defect in the proportions of $CD8^+$ cells able to produce IFNγ in response to pp65 in patients pre-disposed to viraemic episodes.

The results in the upper panels of Figures 4.16 and 4.17, indicate an increase in both the percentages (0.13% in viraemics and 0.37% in non-viraemics) and the absolute numbers of pp65-specific $CD8^+$ T-cells in those individuals who remain PCR-negative for HCMV after transplant, (median cell count of 0.11×10^6 cells/l in viraemics, 0.35×10^6 cells/l in non-viraemics), although this was not statistically significant using either percentages or absolute numbers (Mann Whitney test, $p = 0.22$ and $p = 0.24$, respectively).

There were also no significant differences between the pp65-specific responses of viraemic individuals and non-viraemic individuals during the

first month post-transplant, therefore no pre-existing defect in these responses could be demonstrated. Despite this, many of the patients with active HCMV replication have recurrent phases of viraemia over the six months; an analysis of pp65-specific responses was therefore performed which compared combined responses from patients with viraemic episodes to those of non-viraemic patients over the entire six month period post-transplant. The results are shown in the lower panels of Figures 4.16 and 4.17. Figure 4.16 shows there was a significantly higher overall percentage of pp65-specific $CD8^+$ T-cells in non-viraemic (median 0.9%) compared to viraemic (0.21%) individuals (Mann Whitney test $p = 0.019$).

A similar comparison was performed to resolve whether this increase also occurred in the absolute numbers of pp65-specific $CD8^+$ T-cells in the peripheral blood. When total responses over six months were compared between patients with viraemic episodes and patients without, a four-fold increase was observed (0.81×10^6 to 4.06×10^6 cells/l $p = <0.005$) (Lower panel Fig. 4.17). Taken together these data indicate that there was a significant increase in both the percentage of pp65-specific $CD8^+$ cells and the overall number of circulating HCMV-specific $CD8^+$ cells in the blood of patients who remain free from HCMV viraemia in the six months post-transplant.

Responses to IE1 were analysed in a similar way to those above. There were no significant differences ($p=0.97$) in the median percentage of IE1-specific $CD8^+$ cells in the peripheral blood of viraemic and non-viraemic renal

transplant patients during the first month post-transplant (upper panel, Fig. 4.18), indicating that there is no detectable difference in the frequency of IE1-specific $CD8^+$ cells of viraemic patients prior to viraemic episodes. Similarly, the overall percentage of IE1-specific $CD8^+$ cells during the first 6 months post-transplant shown in the lower panel of Figure 4.18, does not vary significantly between viraemics and non-viraemics ($p = 0.24$). Although the median percentages of the IE1-specific $CD8^+$ populations are not significantly different either in the first month post-transplant or for the entire six month duration post-transplant (Fig. 4.19). Nevertheless, there was a statistically significant increase in the absolute numbers of IE1-specific $CD8^+$ cells in non-viraemic patients (median 0.76 compared to 0.1×10^6 cells/l in viraemic individuals Mann-Whitney test $p = 0.01$) when analysed over six months compared to the viraemic patients.

When responses to both antigens were compared there again were no statistically significant differences detectable between percentages of antigen-specific T-cells either in the first month post-transplant or over the whole six month period (Fig. 4.20). Despite this, responses appeared elevated in the non-viraemic individuals in both time periods. Furthermore, during the comparison of response for whole 6 month period the difference between the two groups was of borderline significance ($p=0.08$). Overall frequencies of HCMV-specific $CD8^+$ cells as shown in Figure 4.21, are also not significantly different in the first month post-transplant; however, again there was a highly significant elevation in total $CD8^+$ responses over the six month period in non viraemic individuals ($2.32 - 8.9 \times 10^6$ cells/l Mann

Whitney test p = 0.005) compared to those individuals suffering from viraemic episodes.

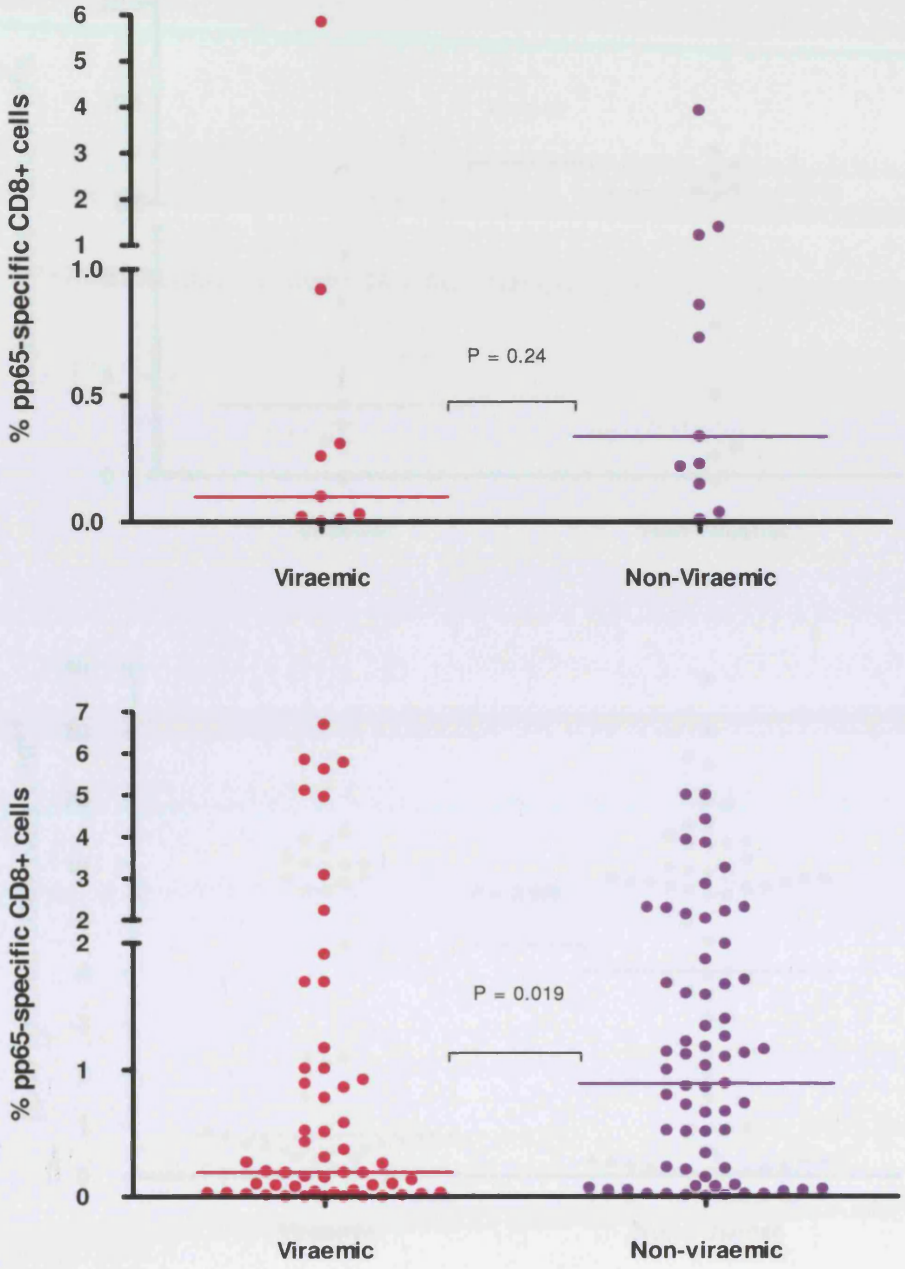


Figure 4. 16 Comparison of the percentages of pp65-specific CD8+ T-cells in patients suffering viraemic episodes and those that do not during the first month (upper panel) and total six month period post-transplant (lower panel).

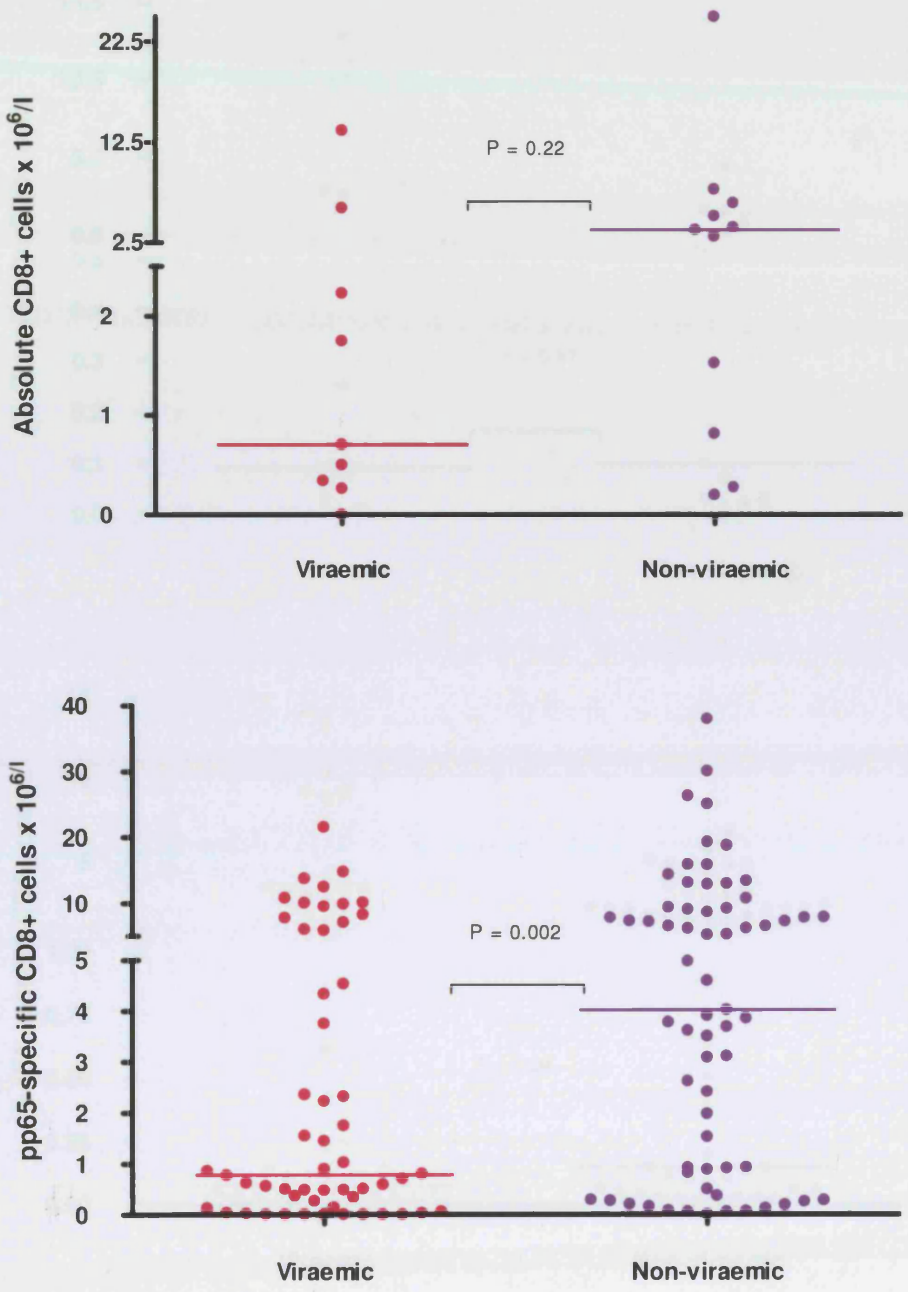


Figure 4. 17 Comparison of the absolute numbers of pp65-specific CD8+ T-cells in patients suffering viraemic episodes and those that do not during the first month (upper panel) and total six month period post-transplant (lower panel).

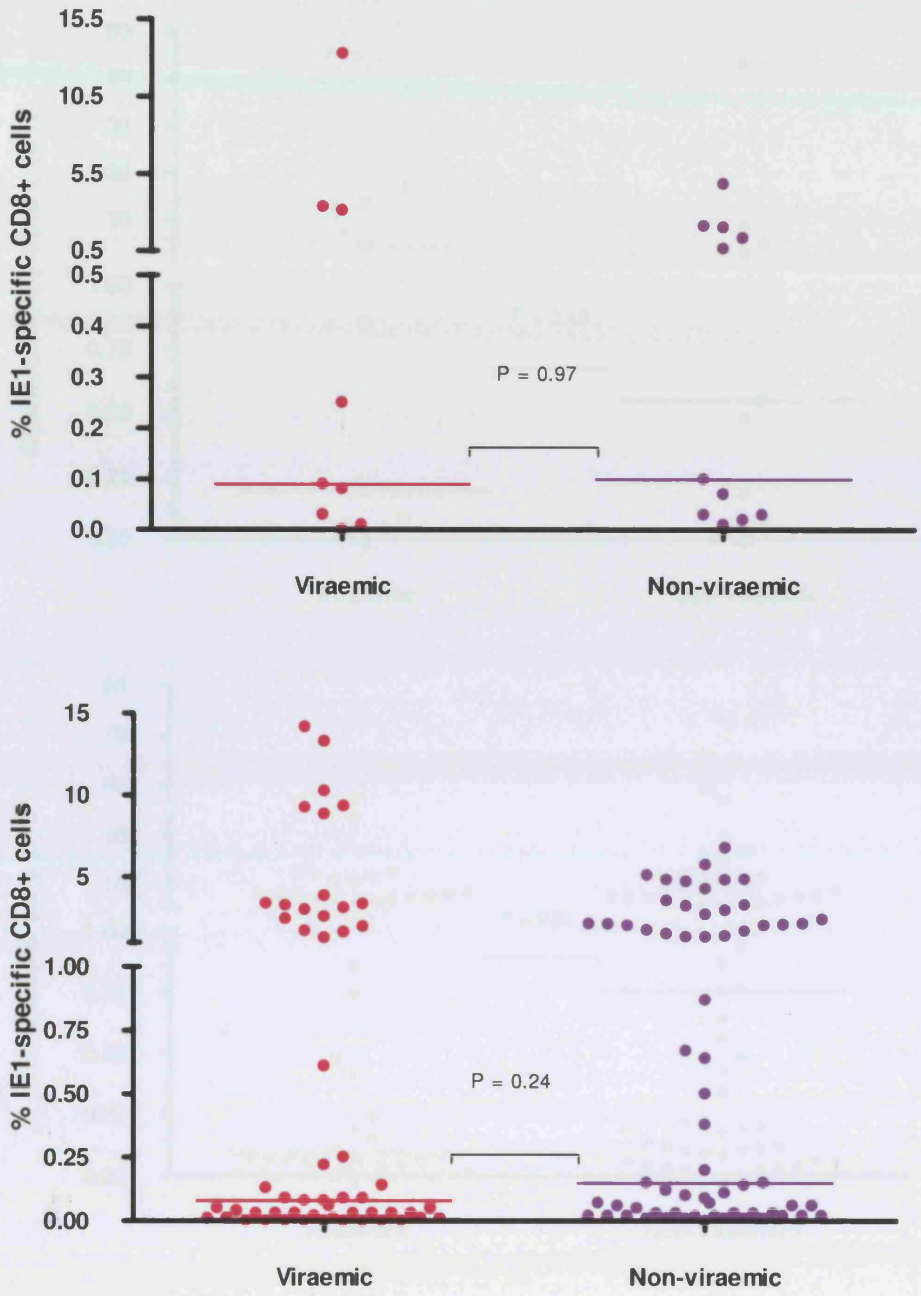


Figure 4. 18 Comparison of the percentages of IE1-specific $CD8^+$ T-cells in patients suffering viraemic episodes and those that do not during the first month (upper panel) and total six month period post-transplant (lower panel).

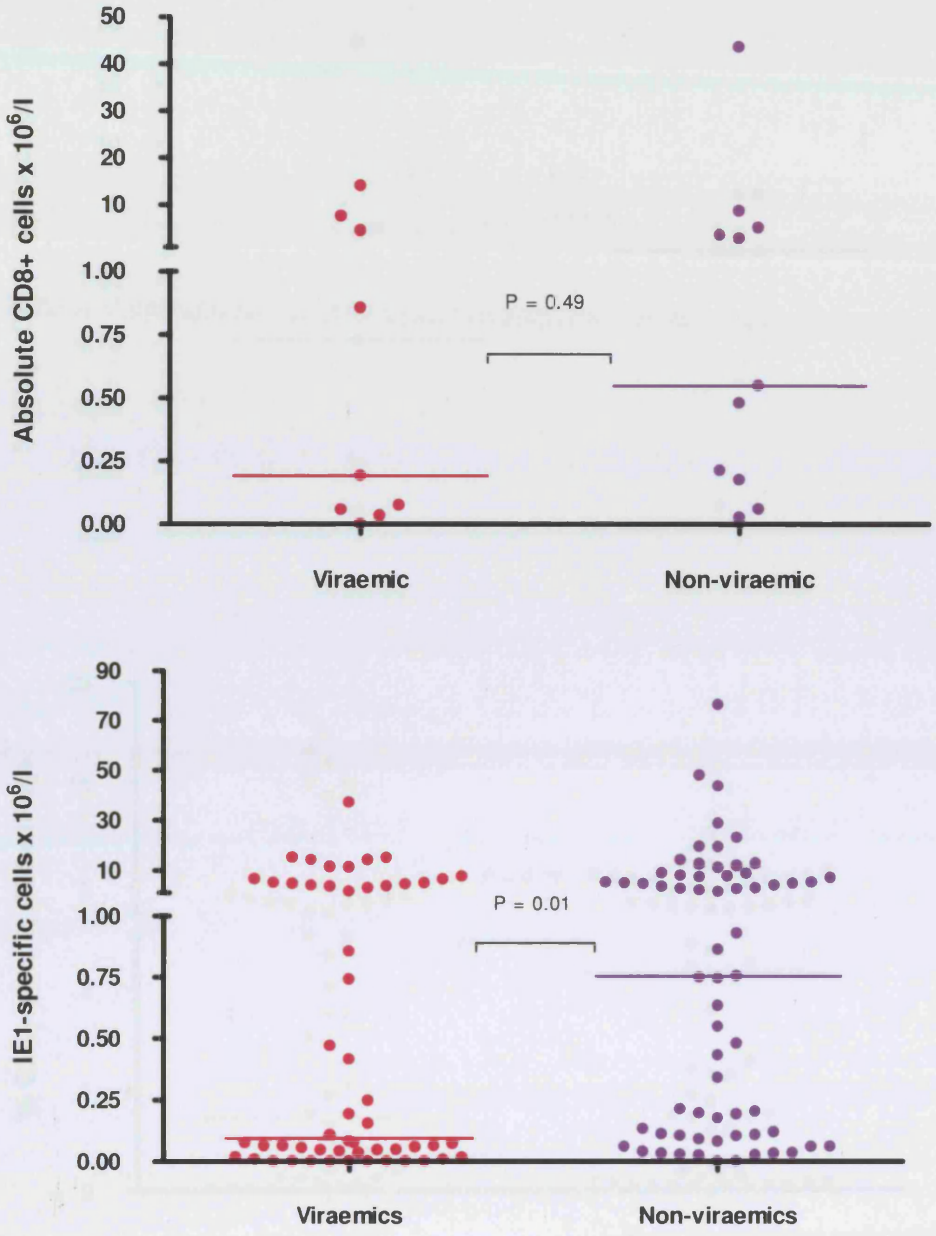


Figure 4. 19 Comparison of the absolute numbers of IE1-specific CD8+ T-cells in patients suffering viraemic episodes and those that do not during the first month (upper panel) and total six month period post-transplant (lower panel).

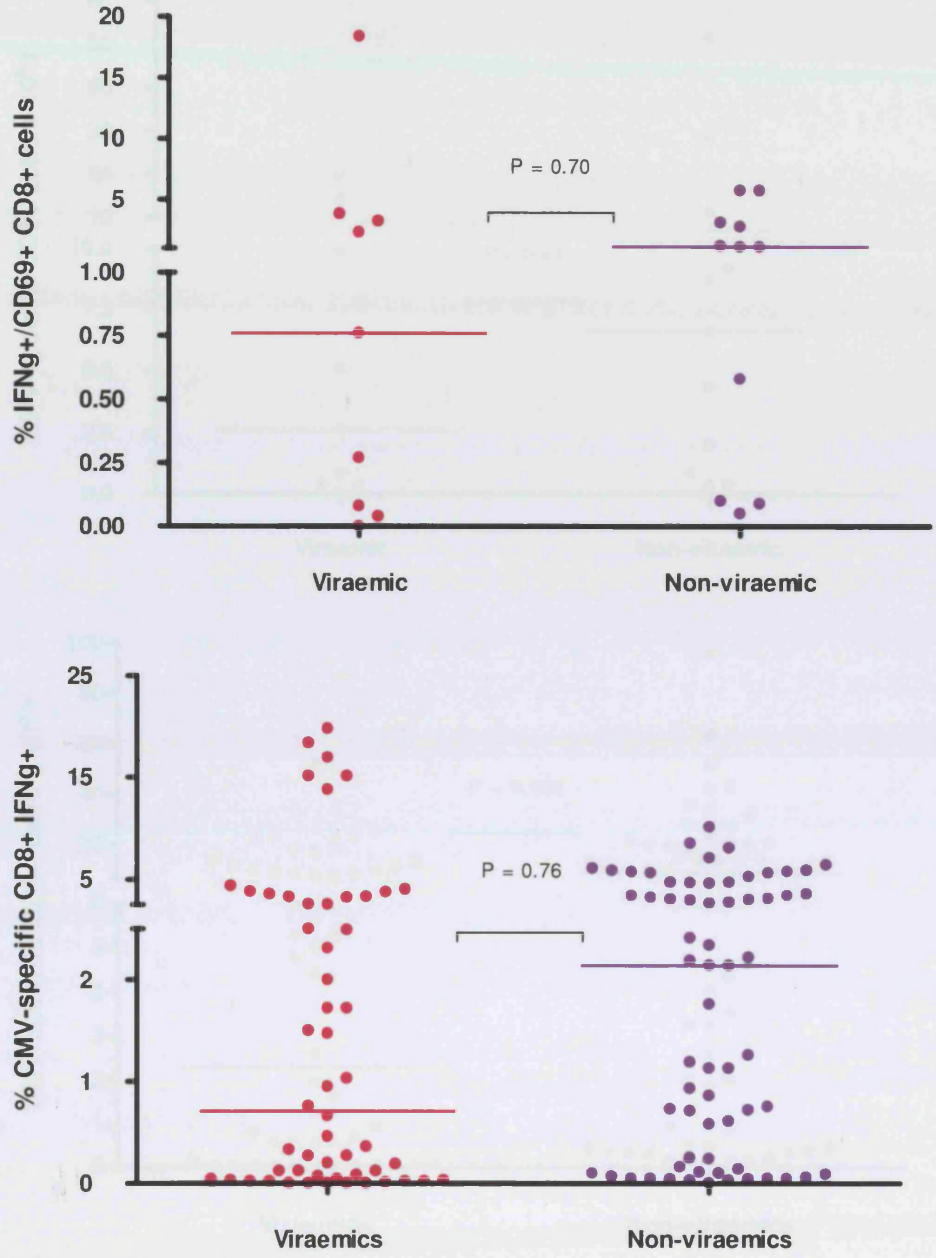


Figure 4. 20 Comparison of the percentages of total pp65 and IE1-specific CD8+ T-cells in patients suffering viraemic episodes and those that do not during the first month (upper panel) and total six month period post-transplant (lower panel).

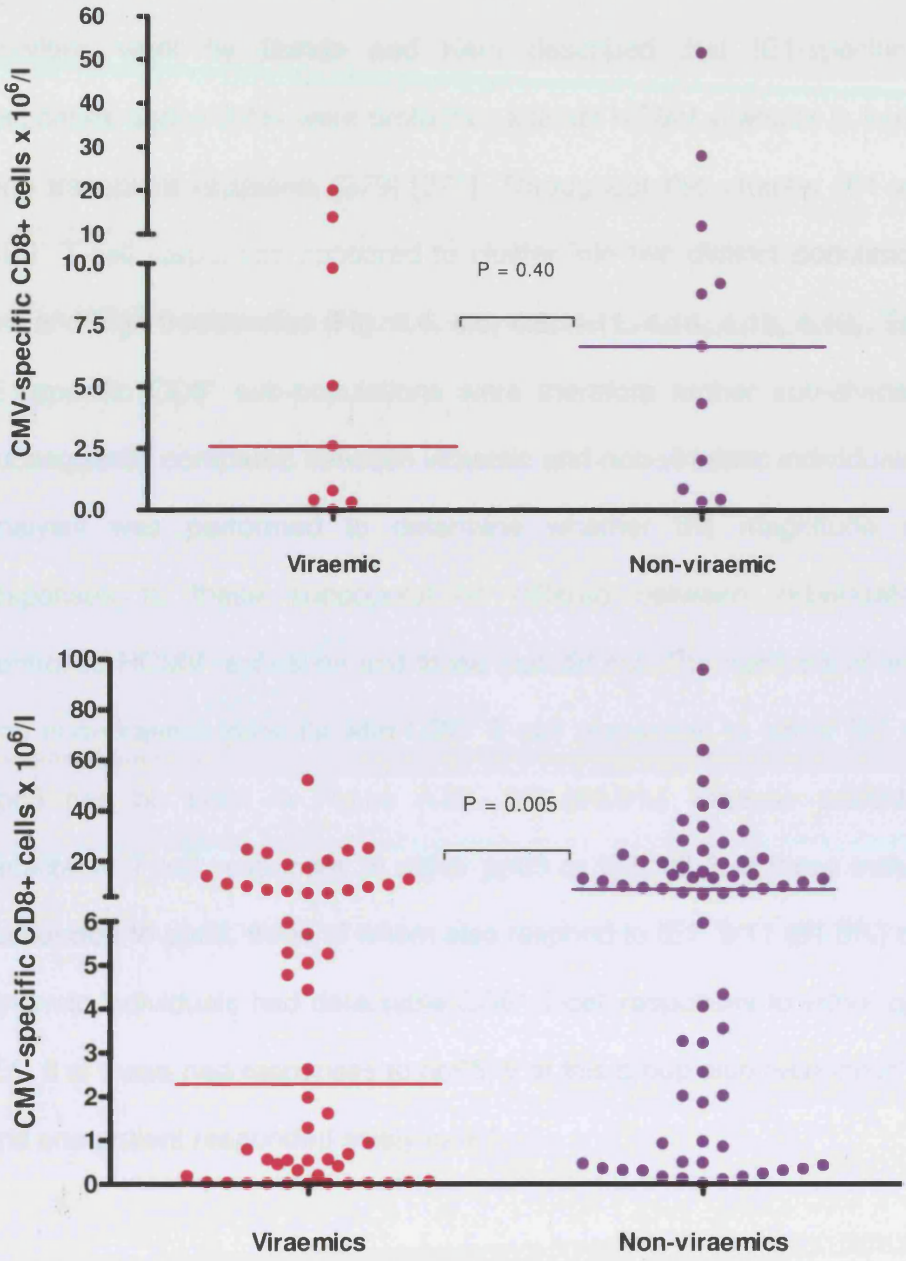


Figure 4. 21 Graph to compare the absolute numbers of combined HCMV-specific CD8+ T-cells in patients suffering viraemic episodes and those that do not during the first month (top panel) and total six month period post-transplant (bottom panel).

4. 7 Are responses towards IE1 protective at different thresholds?

Previous work by Bunde and Kern described that IE1-specific CD8 responses above 0.4% were protective against HCMV viraemia in heart and lung transplant recipients [379] [271]. Throughout this chapter IE1-specific $CD8^+$ T-cell responses appeared to cluster into two distinct populations, of low and high frequencies (Fig. 4.4, 4.5, 4.8, 4.11, 4.14, 4.18, 4.19). The two IE1-specific $CD8^+$ sub-populations were therefore further sub-divided and subsequently compared between viraemic and non-viraemic individuals. This analysis was performed to determine whether the magnitude of the responses to these subpopulations differed between individuals who controlled HCMV replication and those that did not. The numbers of viraemic and non-viraemic patients with $CD8^+$ T-cell responses to either IE1 and/or pp65 can be seen in Figure 4.22. 6/9 (66.6%) viraemic patients had detectable T-cell responses to either pp65 or IE1, all 6 of these individuals responded to pp65, three of whom also respond to IE1. 9/11 (81.8%) of non-viraemic individuals had detectable $CD8^+$ T-cell responses to either pp65 or IE1, 8 of these had responses to pp65, 5 of this group also responded to IE1 and one patient responded solely to IE1.

Samples from viraemic individuals with a consistently high (i.e. above threshold level of detection) IE1-specific CD8 response had significantly elevated frequencies when compared to non-viraemic individuals (median 3.35% viraemic and 2.1% non-viraemic, $p = 0.04$). Although there was no statistical difference in the percentages of $CD8^+$ T-cells in those individuals will low IE1 response.

When the absolute numbers of IE1-specific $CD8^+$ T-cells were analysed in the peripheral blood there was no significant difference between the frequencies of IE1 specific $CD8^+$ T-cells in either viraemic or non-viraemic patients with consistently high percentages of IE1-specific cells (medians 7 x 10^6 cells/ml and 8.56 x 10^6cells/ml, $p = 0.66$). Conversely, non-viraemic individuals with lower-tier responses to IE1 had an increase in the numbers of circulating $CD8^+$ T-cells specific for this antigen ($p = 0.01$).

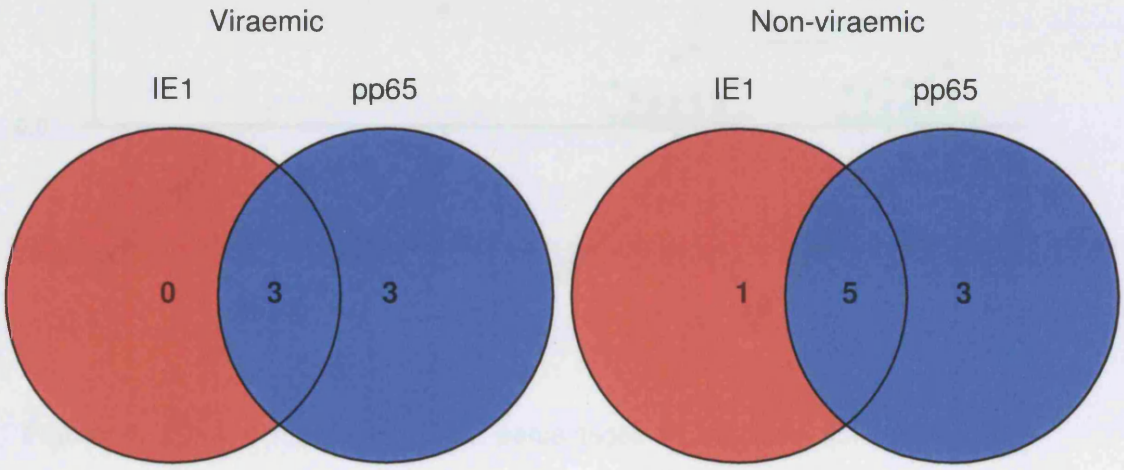


Figure 4. 22 Proportion of patients with $CD8^+$ T-cell responses to IE1 and/or pp65 in viraemic (left panel) and non-viraemic (right panel) patients.

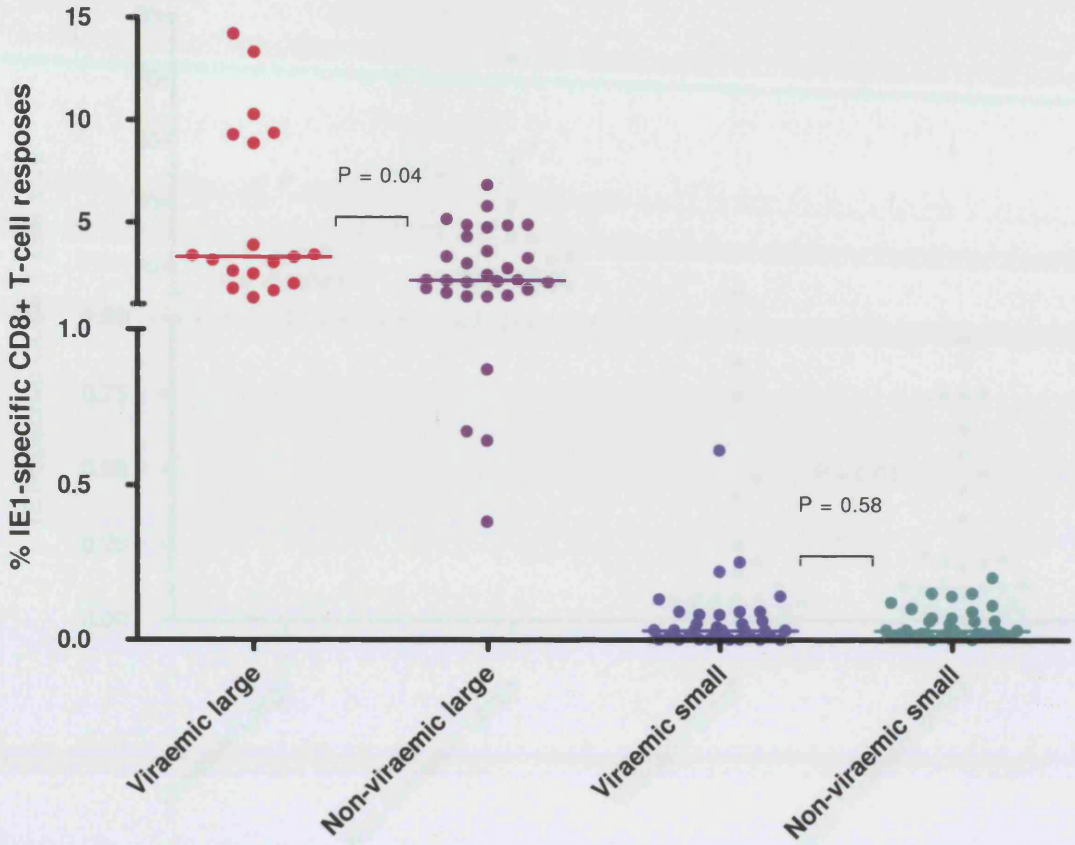


Figure 4. 23 A comparison of the percentages of stratified IE1-specific CD8 responses in viraemic and non-viraemic patients.

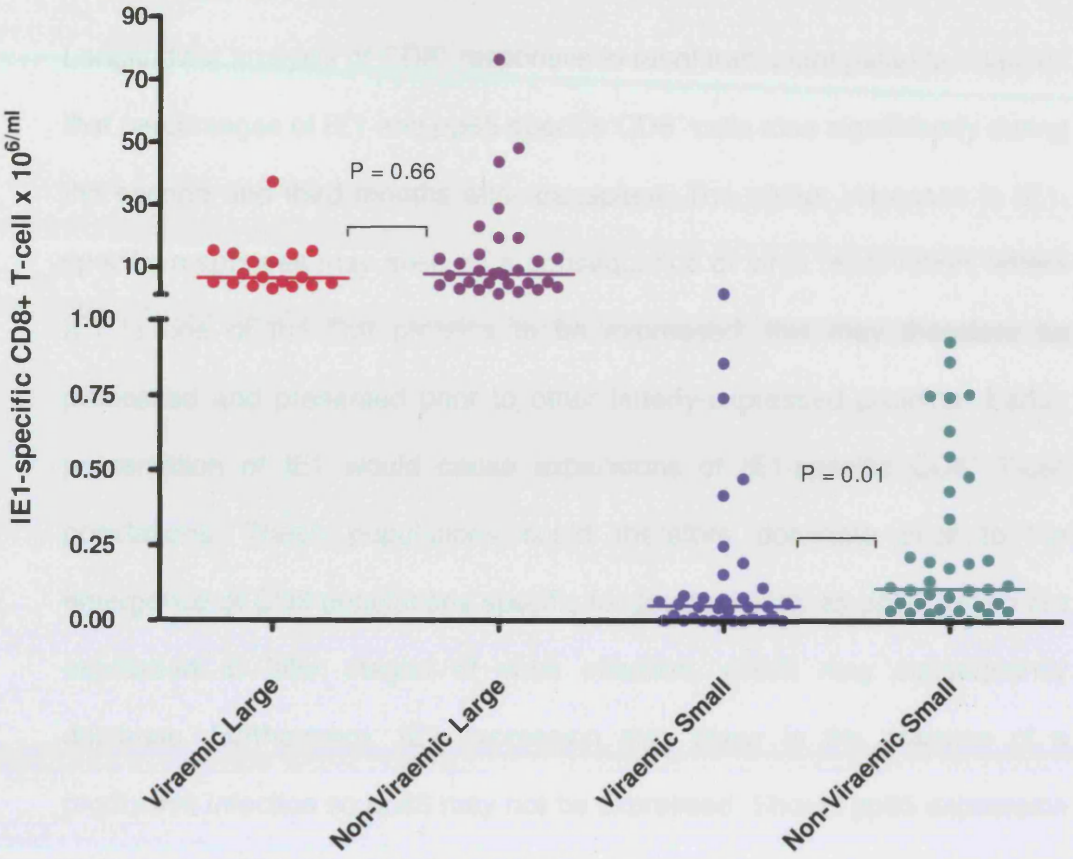


Figure 4. 24 A comparison of the absolute numbers of stratified IE1-specific CD8 responses in viraemic and non-viraemic patients.

4. 8 Discussion

Longitudinal analysis of $CD8^+$ responses in renal transplant patients revealed that percentages of IE1 and pp65-specific $CD8^+$ cells rose significantly during the second and third months after transplant. The earlier increases in IE1-specific responses may arise as a consequence of virus reactivation, where IE1 is one of the first proteins to be expressed; this may therefore be processed and presented prior to other latterly-expressed proteins. Earlier presentation of IE1 would cause expansions of IE1-specific $CD8^+$ T-cell populations. These populations could therefore dominate prior to the emergence of CD8 populations specific for proteins such as pp65, which are expressed at later stages of virus infection, which may subsequently dominate. Furthermore, IE1 expression may occur in the absence of a productive infection so pp65 may not be expressed. Should pp65 expression not occur, pp65 would thus be unable to abrogate processing of IE1 through phosphorylation [262].

Generally fewer renal transplant patients have responses to IE1 (10/20) than to pp65 (14/20), although most individuals who mount a response to pp65 also mount IE1-specific responses (9/14) (Fig. 4.2). This contradicts findings of Gilbert *et al.* 1996, who speculated that responses to one protein was dominant over the other because of pp65's ability to phosphorylate IE1 thereby preventing its processing and presentation [262].

When combined responses to both of these antigens were analysed it became apparent that the percentages of $CD8^+$ cells that recognise these

targets stabilised after three months, and the percentage of these $CD8^+$ T-cells were similar in magnitude to those found in healthy individuals.

Determining the absolute numbers of virus-specific $CD8^+$ T-cell populations in the periphery, instead of the analysis of percentages of these populations alone, gives a more accurate view of how these virus-specific $CD8^+$ populations may be effective in controlling HCMV. An example of where this might occur would be an individual possessing a high percentage of their $CD8^+$ T-cells focused on HCMV who may actually have substantially fewer total $CD8^+$ cells compared to an individual with a lower percentage of HCMV-specific cells but a higher overall level of $CD8^+$ cells in their peripheral blood.

Significant increases in absolute numbers of pp65-specific $CD8^+$ cells in the peripheral blood also occur during the third month post-transplant, although this is not borne out with IE1 responses, which increased significantly in absolute number only after six months post-transplant. This observation indicates that expansions of IE1-specific populations do occur during the first few months of transplant, but, in absolute terms, these increases are not significant. Similarly, significant increases in overall combined CD8 responses to these antigens only occur during the sixth month post-transplant. There does seem to be a two-tier level of responses between the first three months post-transplant and the next three months. The temporal pattern of CD8 T-cells against HCMV is consistent with previous observations that the majority of viraemic episodes in solid organ transplant patients occur within the first three month period immediately after transplant.

After this period, populations of $CD8^+$ cells may recover sufficiently to prevent the majority of subsequent reactivations. Results show elevated percentages of HCMV-specific $CD8^+$ cells during the second three months post-transplant but these elevations were not significant. Nevertheless the absolute numbers of these same populations are significantly elevated in during the months four to six post-transplant, this might explain why viraemic episodes have previously been shown to occur less frequent after the first three months post transplant.

To assess whether patients suffering viraemic episodes have a pre-existing defect in their HCMV-specific $CD8^+$ responses that renders them more susceptible to HCMV-associate disease, their CD8 responses to both IE1 and pp65 during the first month post-transplant were compared to those individuals who remained PCR negative throughout. Despite seeing no significant differences in either the percentage or absolute number of antigen-specific $CD8^+$ T-cells, non-viraemic patients consistently had higher median populations of antigen-specific $CD8^+$ cells than those who suffered viraemia.

Because the majority of this subgroup suffered repeated phases of viraemia over the six month period monitored, this analysis was repeated using combined samples taken from each group over the entire period the median percentages of $CD8^+$ cells specific for pp65 were increased from 0.82% to 4.03% ($p = 0.02$) and the median absolute numbers of pp65-specific cells also rose significantly from 0.81×10^6 cells/l to 4.06×10^6 cells/l ($p = <0.005$).

While there was no significant difference in the corresponding median percentage of IE1-specific CD8 cells (0.09 and 0.15% p = 0.24), the absolute numbers of IE1-specific cells also rose significantly from a median of 0.1 to 0.76 x 10^6 cell/l (p = 0.01). When these responses were combined a similar phenomenon was observed, non-viraemic patients had a slight but not significant increase in antigen-specific CD8$^+$ populations but the increase in the median absolute numbers of antigen-specific CD8$^+$ cells was again highly significant (medians 2.3 x 10^6 cells/l to 9 x 10^6 cells/l p = 0.005). Taken together, these data indicate that patients with elevated CD8$^+$ T-cell responses to pp65 and IE1 are less likely to suffer virus reactivation and therefore there are protective affects.

Fig. 4.20 illustrates that measuring the percentages of antigen-specific populations does not necessarily correlate with absolute levels these same populations in the peripheral blood. Also, high levels of antigen-specific CD8$^+$ T-cells do not always correlate with protection from viraemia; one patient with the highest percentages of virus-specific CD8$^+$ T-cells detected (median 16.03%) and absolute number of CD8$^+$ T-cells in the blood (median 22.2 x 10^6 cells/l) suffered repeated bouts of viraemia. Could this be due to IFNγ production not being an accurate indicator CD8 of functionality or could this be due to the possible expansion of HCMV CTL escape mutants?

In summary, the majority of individuals analysed in this chapter have detectable populations of CD8$^+$ T-cells that are able to secrete IFNγ in response to stimulation with the two immunodominant targets of the HCMV-

specific CD8 response. However, some individuals lack these responses and yet remain free from viraemia in the months post-transplant. Furthermore, high frequencies of antigen-specific $CD8^+$ populations do not necessarily confer complete protection against viraemia. There was also a significant increase in the median absolute $CD8^+$ T-cell responses towards pp65 and IE1 in individuals who were able to control virus infection compared with those who could not. This highlights the importance of high frequency HCMV-specific $CD8^+$ T-cell populations for the control of HCMV in renal transplant patients.

Chapter 5

Do HCMV CTL-Escape Mutants Occur in Viraemic Renal Transplant Patients?

5. 1 Introduction

Chapter 4 demonstrated that renal transplant patients with higher median numbers of $CD8^+$ T-cells in the peripheral blood capable of producing IFNγ in response to HCMV antigens pp65 (ppUL83) and IE1 (UL123), were less prone to viraemic episodes in the six month period post-transplant. However, several patients suffered recurrent viraemic episodes despite the presence of high-frequency ppUL83 and/or UL123IE1 responses. It can therefore be concluded that the CD8 response focused towards these antigens was inadequate to control virus replication in these individuals. The reason for this could be two-fold; either a defect may exists in the virus-specific CD8 populations, which could either be in terms of the magnitude or functionality of those responses, rendering them less functional than those found in other individuals or the virus itself may be able to escape from surveillance by the antigen-specific $CD8^+$ T-cells through mutation of the CTL targets.

HCMV has developed a wide variety of immune-evasion strategies; including mechanisms for the down-regulation of HLA class I molecules on the surface of infected cells thereby preventing them being recognised and subsequent lysis by virus-specific CD8+ cells. Down-regulation of HLA I molecules from the cell surface would render HCMV-infected cells more susceptible to NK cell-mediated lysis. Although HCMV has developed several strategies for circumventing the NK response, CTL-escape mutants could arise allowing HLA class I molecules on the cell surface to be bound by not recognised by the CTL response, thereby facilitating HCMV's ability to remain undetected by the CTL responses and thus continue replication without needing to

circumvent the NK cell response. CTL escape mutants have previously been described in several other viral infections such as LCMV , HIV [380] and HBV [381].

Given the large clonal expansions of HCMV-specific $CD8^+$populations during the natural course of HCMV infection and subsequent sporadic reactivations [299, 314], there should be a considerable selective pressure applied on HCMV to change its CTL epitopes. HCMV CTL escape mutants have previously been isolated from HCMV seropositive bone-marrow transplant (BMT) patients [358]; yet only one mutation was detected in this study. This mutations occurred in the A*0201-restricted NLVPMVATV epitope within pp65, where the M (Methionine) at position 5 was mutated into an I (Isoleucine). Although this mutant was able to decrease CTL-mediated lysis of cells presenting the peptide, amplification of the DNA sequence of interest encoding this epitope was performed using a non-proofreading DNA polymerase, which itself increases the likelihood of the emergence of artificial substitutions.

A situation where an individual who has repeated episodes of HCMV viraemia in spite of a detectable $CD8^+$ response, would provide an ideal setting for the detection of CTL-escape mutants. The aim of this chapter was to elucidate whether CTL-escape mutants arise in liver transplant patients (different to the renal transplant patients analysed in chapters 3, 4 and 6), of known HLA haplotypes, who suffer from repeated sustained viraemic episodes despite the presence of detectable epitope-specific CTL responses.

To complement these studies the effects of certain amino acid substitutions within the HLA-A*0201 epitope NLVPMVATV on HLA binding and recognition by the epitope-specific CTLs were investigated.

5.2 Do Mutations occur within ppUL83 CTL epitopes in response viraemic renal transplant patients?

DNA sequences encoding three HLA-A*0201 and one HLA-B*3501-restricted CTL epitopes present within ppUL83 were PCR-amplified from liver transplant patients with known HLA-haplotypes, who remained persistently HCMV PCR-positive for CMV for >2 months post-transplant despite the presence of epitope-specific CTL responses. The presence of epitope-specific $CD8^+$ T-cell responses were assessed by class I HLA tetramer staining. Sequences encoding the HLA-A*0201-restricted epitopes from patients with log-phase HCMV-replication where also assessed for mutations. Purified DNA was ligated into pGem-T-easy vector and used to transform competent bacteria. Multiple (5) recombinant clones were selected from each time point, DNA was extracted and sequencing was carried out using an ABI Prism® capillary-based sequence analyser using a BigDye® terminator v3.1 cycle sequencing kit (Applied Biosystems). One series of chromatograms depicting representative sequences are shown in Fig. 5.1.

No variations in the sequences of any of the epitopes analysed were detected in either the four long-term HCMV-PCR positive patients or the five transplant patients with log-phase HCMV replication (Table 5.1). These data indicate that mutations within HLA-A*0201 CTL epitopes of ppUL83 occur either infrequently or not at all. This compounds previous findings by Zaia *et al.* [358] , who found only one coding mutation in the HLA-A*0201 epitope $ppUL83_{(495\text{-}503)}$. In their study, sequences encoding this epitope were amplified using a regular Taq polymerase without high-fidelity proof-reading

capabilities as utilised here. Furthermore, neither patient epitope-specific CD8+ populations nor CMV-PCR status was established.

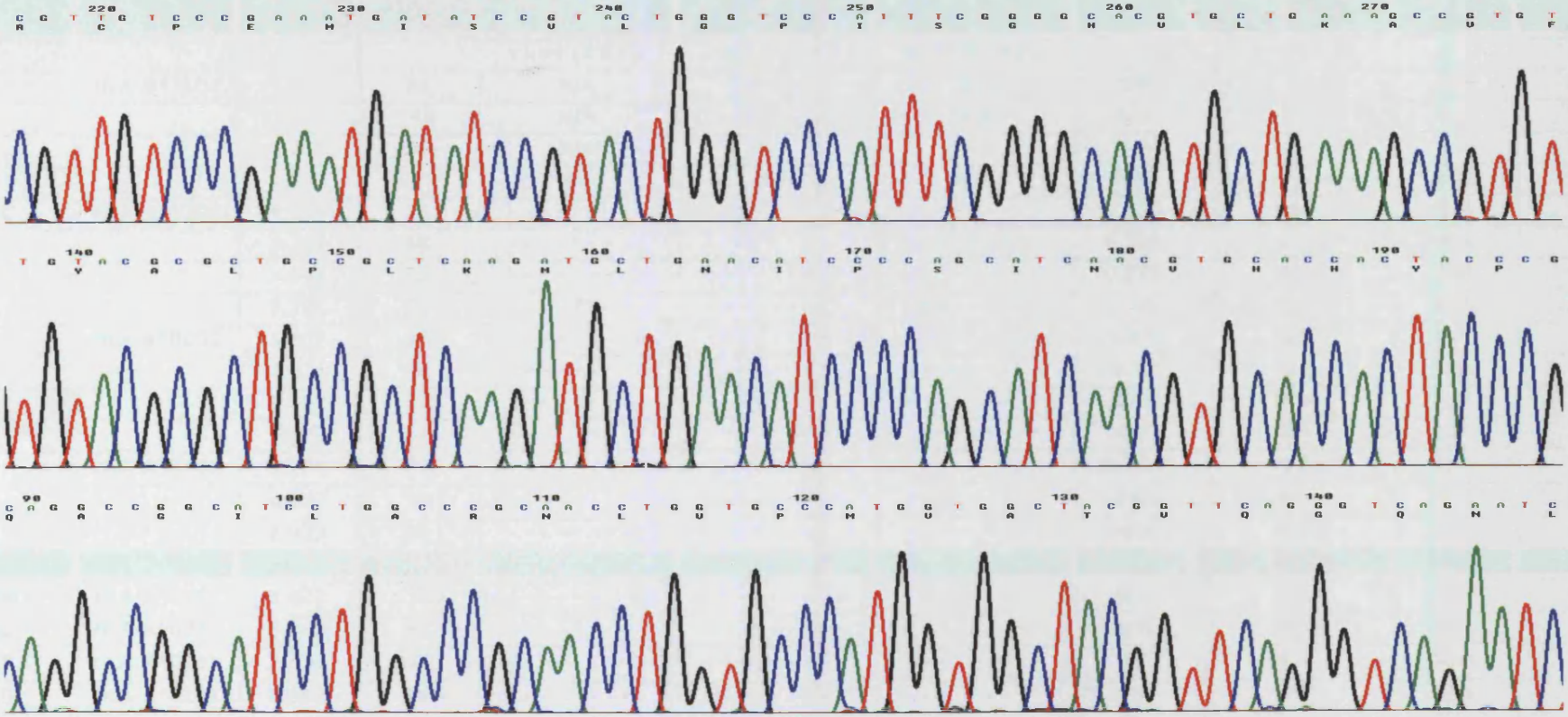


Figure 5. 1 Chromatogram showing the sequence of amplified DNA encoding (a) HLA-A*0201 epitope VLGPISGHV (b) HLA-A*0201 epitope MLNIPSINV and HLA-B*3501 epitope IPSINVHHY and (c) HLA-A*0201 epitope NLVPMVATV. Graphics were generated utilising Chromas 2.23 software available at: http://www.technelysium.com.au

Patients Long term PCR+	Haplotype	Viral Load (log)	Days PT	ppUL83(14-22) HLA-A*0201	ppUL83(120-8) HLA-A*0201	ppUL83(495-503) HLA-A*0201	% NLV-specific CD8	ppUL83(123-131) HLA-B*3501	% IPS-specific CD8	Clone Frequency
				VLGPISGHV	MLNIPSINV	NLVPMVATV		IPSINVHHY		
1 S	HLA-B*3501	3.397	54	N/A	N/A	N/A	N/A	- - - - - - - - -	N/A	5/5
		6.170	140	N/A	N/A	N/A	N/A	- - - - - - - - -	2.3	5/5
		4.260	372	N/A	N/A	N/A	N/A	- - - - - - - - -	4.1	5/5
		3.797	507	N/A	N/A	N/A	N/A	- - - - - - - - -	3.01	5/5
2 C	HLA-A*0201	4.954	26	- - - - - - - - -	- - - - - - - - -	- - - - - - - - -	0.395	N/A	N/A	5/5
		3.262	48	- - - - - - - - -	- - - - - - - - -	- - - - - - - - -	0.325	N/A	N/A	5/5
		3.647	53	- - - - - - - - -	- - - - - - - - -	- - - - - - - - -	0.275	N/A	N/A	5/5
		3.201	93	- - - - - - - - -	- - - - - - - - -	- - - - - - - - -	0.65	N/A	N/A	5/5
3 Y	HLA-A*0201	3.102	17	- - - - - - - - -	- - - - - - - - -	- - - - - - - - -	0.335	N/A	N/A	5/5
		3.425	38	- - - - - - - - -	- - - - - - - - -	- - - - - - - - -	0.32	N/A	N/A	5/5
		3.652	57	- - - - - - - - -	- - - - - - - - -	- - - - - - - - -	0.43	N/A	N/A	5/5
		2.76	67	- - - - - - - - -	- - - - - - - - -	- - - - - - - - -	0.4	N/A	N/A	5/5
4 C	HLA-A*0201	3.774	26	- - - - - - - - -	- - - - - - - - -	- - - - - - - - -	0.335	N/A	N/A	5/5
		4.022	30	- - - - - - - - -	- - - - - - - - -	- - - - - - - - -	0.65	N/A	N/A	5/5
		2.923	90	- - - - - - - - -	- - - - - - - - -	- - - - - - - - -	N/A	N/A	N/A	5/5
Log Phase										
5 M	HLA-A*0201	3.022	>180	- - - - - - - - -	- - - - - - - - -	- - - - - - - - -	N/A	N/A	N/A	5/5
6 G	HLA-A*0201	5.243	41	- - - - - - - - -	- - - - - - - - -	- - - - - - - - -	N/A	N/A	N/A	5/5
7 P	HLA-A*0201	3.331	102	- - - - - - - - -	- - - - - - - - -	- - - - - - - - -	N/A	N/A	N/A	5/5
8 S	HLA-A*0201	3.582	56	- - - - - - - - -	- - - - - - - - -	- - - - - - - - -	N/A	N/A	N/A	5/5
9 Sh	HLA-A*0201	3.858	>150	- - - - - - - - -	- - - - - - - - -	- - - - - - - - -	N/A	N/A	N/A	5/5

Table 5. 1 Table showing information on liver transplant patient samples and the sequence data obtained from clones of the amplified sequences encoding designated epitopes

5.3 Effect of amino acid substitutions in the immunodominant ppUL83 HLA-A*0201 epitope NLVPMVATV on recognition by CTLs.

To assess the impact that mutations in the immunodominant HLA-A*0201 epitope NLVPMVATV would have on CTL recognition, peptide variants of this sequence were designed based on substitutions of all residues (except anchor residues at P2 and P9) with either a glycine residue or, where possible, a conservative (like for like) amino acid substitution. Peptides were synthesised commercially and purified to >90% purity (Fig. 5.2).

Peptides were used to stimulate lymphocytes isolated from healthy controls overnight (three HCMV-seropositive, two of these HLA*A0201 the other HLA*B0701, and one seronegative HLA*A0201 control, see Fig. 5.3) in an IFNγ ELISPOT. Cells secreting IFNγ were counted and numbers were plotted as a proportion of those produced by the wild type peptide (Fig. 5.4).

Results from the IFNγ ELISPOT assays (Fig. 5.4) showed that key amino acid substitutions abrogated IFNγ production by epitope-specific $CD8^+$ lymphocytes. Substitutions that reduced IFNγ production most markedly occurred around the centre of the peptide, at residues 4 and 5; and these substitutions resulted in the production of less than 20% of the IFNγ response elicited by the wild-type peptide.

Interestingly, different substitutions at the same residues appeared to have varying effects on IFNγ induction. For example, substituting the G6V mutant effectively abolished IFNγ production whereas L6V still elicited a response,

albeit lower than the wild-type. Therefore, the nature of the amino acid substitution has a bearing on the ability of the peptide to be recognised. This can also be seen with L3V and G3V, and G8T and S8T substitutions.

Surprisingly, substitutions around the anchor residues seemed less important than those nearer the core. The G4P substitution significantly lowered the response (less than 5% that of wild-type peptide); similarly, none of G5M, C5M, I5M and G6V substitutions are were able to stimulated more than 20% of the IFNγ elicited by stimulation with the wild-type peptide.

These data demonstrate that certain substitutions have an effect on IFNγ production by epitope-specific $CD8^+$ cells, but it is unclear whether these function at the level of HLA I binding or TCR recognition. Thus, it was important to consider that certain substitutions may have resulted in the peptide being less able to bind the groove of the HLA class I molecule, and thus rendering it less stable and consequently less efficiently presented at the surface of the infected cell.

P	1	2	3	4	5	6	7	8	9		
	N	L	V	P	M	V	A	T	V	wild-type	
	G	L	V	P	M	V	A	T	V	G1N	
	N	L	G	P	M	V	A	T	V	G3V	
	N	L	V	G	M	V	A	T	V	G4P	
	N	L	V	P	G	V	A	T	V	G5M	
	N	L	V	P	M	G	A	T	V	G6V	
	N	L	V	P	M	V	G	T	V	G7A	
	N	L	V	P	M	V	A	G	V	G8T	
	Q	L	V	P	M	V	A	T	V	Q1N	
	N	L	L	P	M	V	A	T	V	L3V	
	N	L	V	P	I	V	A	T	V	I5M	[358]
	N	L	V	P	C	V	A	T	V	C5M	
	N	L	V	P	M	L	A	T	V	L6V	
	N	L	V	P	M	V	A	S	V	S8T	

Figure 5. 2 Peptide substitutions designed to conserve the anchor residues (blue) substitutions are highlighted in (red)

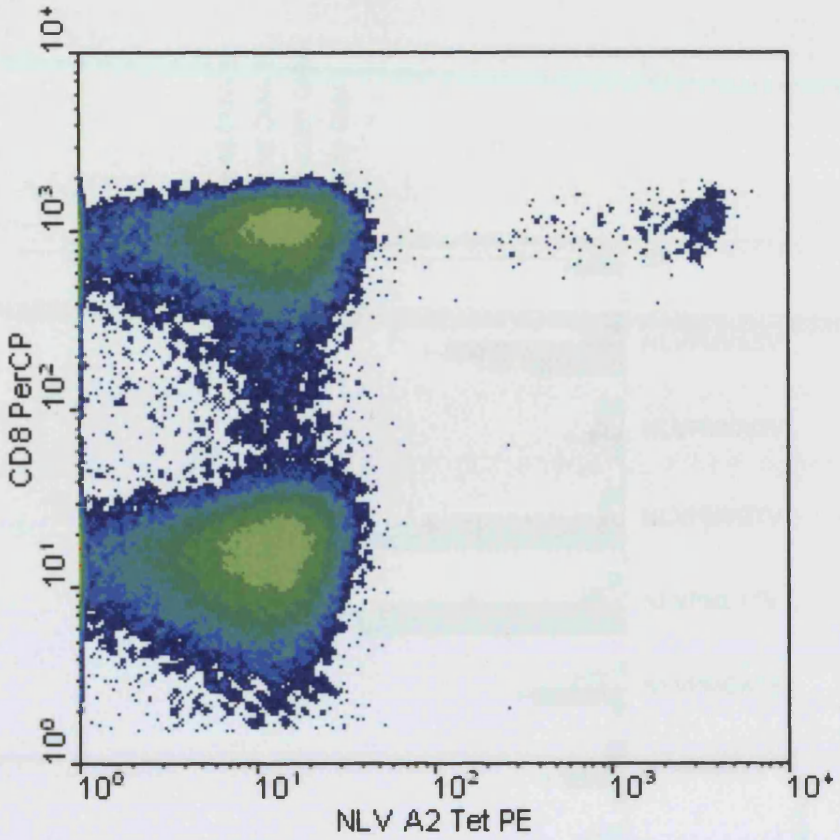


Figure 5. 3 FACS plot of one Healthy HCMV seropositive HLA-A*0201-restricted individual with responses to NLVPMVATV. Blot used gates of lymphocyte and $CD3^+$ populations

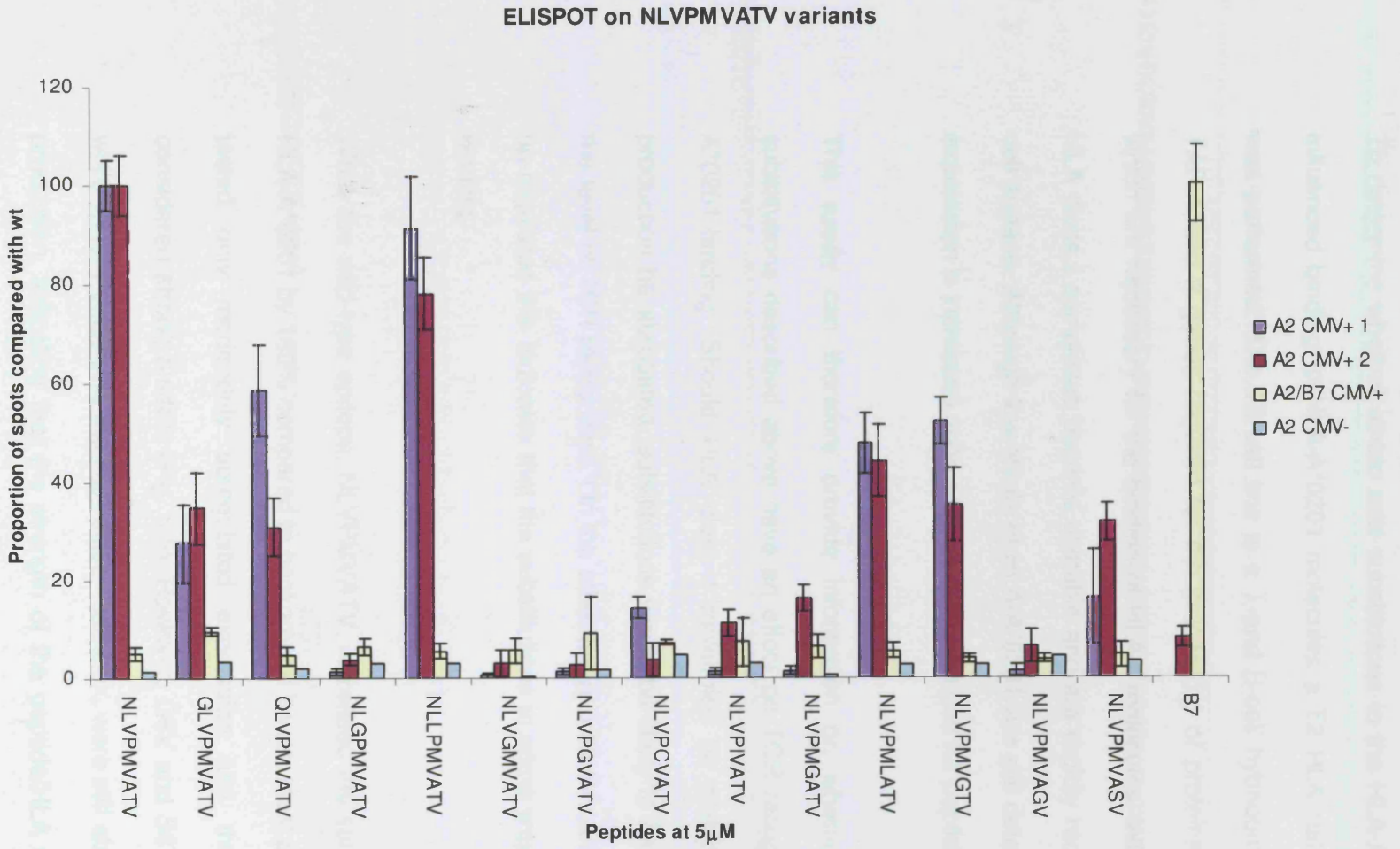


Figure 5. 4 IFNγ ELISPOT showing responses of four individuals to NLVPMVATV peptide variants

5.4 HLA-stabilisation assay

To determine whether amino acid substitutions in the HLA-A*0201 epitope influenced binding to HLA-A*0201 molecules a T2 HLA-stabilisation assay was performed. The T2 cell line is a T-and B-cell hybridoma lacking both TAP 1 and 2 genes required for the processing of proteins into peptides, which are necessary for the binding of HLA I molecules. Without peptides, HLA class I complexes become unstable and are rapidly recycled from the cell surface. Although low levels of HLA-A*0201 are still detectable, surface expression is increased upon addition of exogenous A2 peptides.

This assay can therefore provide information on whether the peptide substitutions described above have an effect on TCR recognition or HLA-A*0201-binding. Should HLA class I molecules be stabilised and IFNγ production be abrogated, substitutions would be likely to have an effect at the level of TCR recognition. On the other hand, if HLA-I complexes are not up-regulated this indicates that the substitutions in some way preclude HLA binding.

While the wild-type epitope, NLVPMVATV, increased the surface density of HLA-A*0201 by 180% compared to background levels, most peptide variants tested only moderately upregulated expression and thus cannot be considered strong binders (Fig. 5.5). However, G6V and S8T substitutions, while weakly stabilising the HLA class I complex, were still able to elicit IFNγ production, indicating that the strength of the peptide/HLA class I binding interaction does not necessarily correlate with an increased capacity to

induce IFNγ production by $CD8^+$ T-cells. Conversely, methionine for cysteine substitutions at position 5 (C5M) strongly upregulated HLA-A*0201 stability, by more than four times that of background, and yet were unable to elicit IFNγ production (Fig. 5.5).

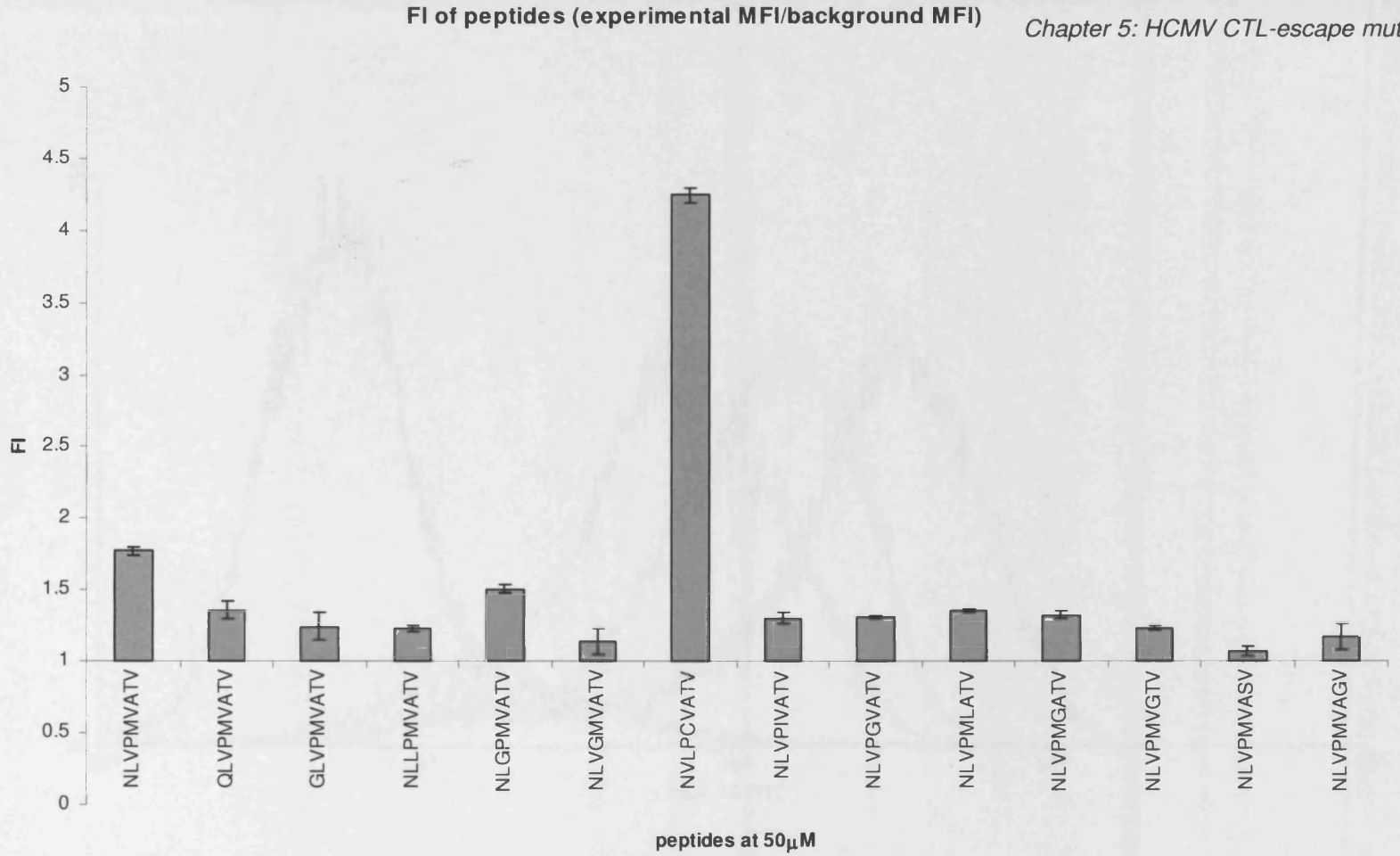


Figure 5. 5 Increase in HLA-A*0201 expression in T2 cells pulsed with NLVPMVATV variants. FI (fluorescence intensity) of 1 denoted the relative FI of T2 cells incubated compared with the background surface levels.

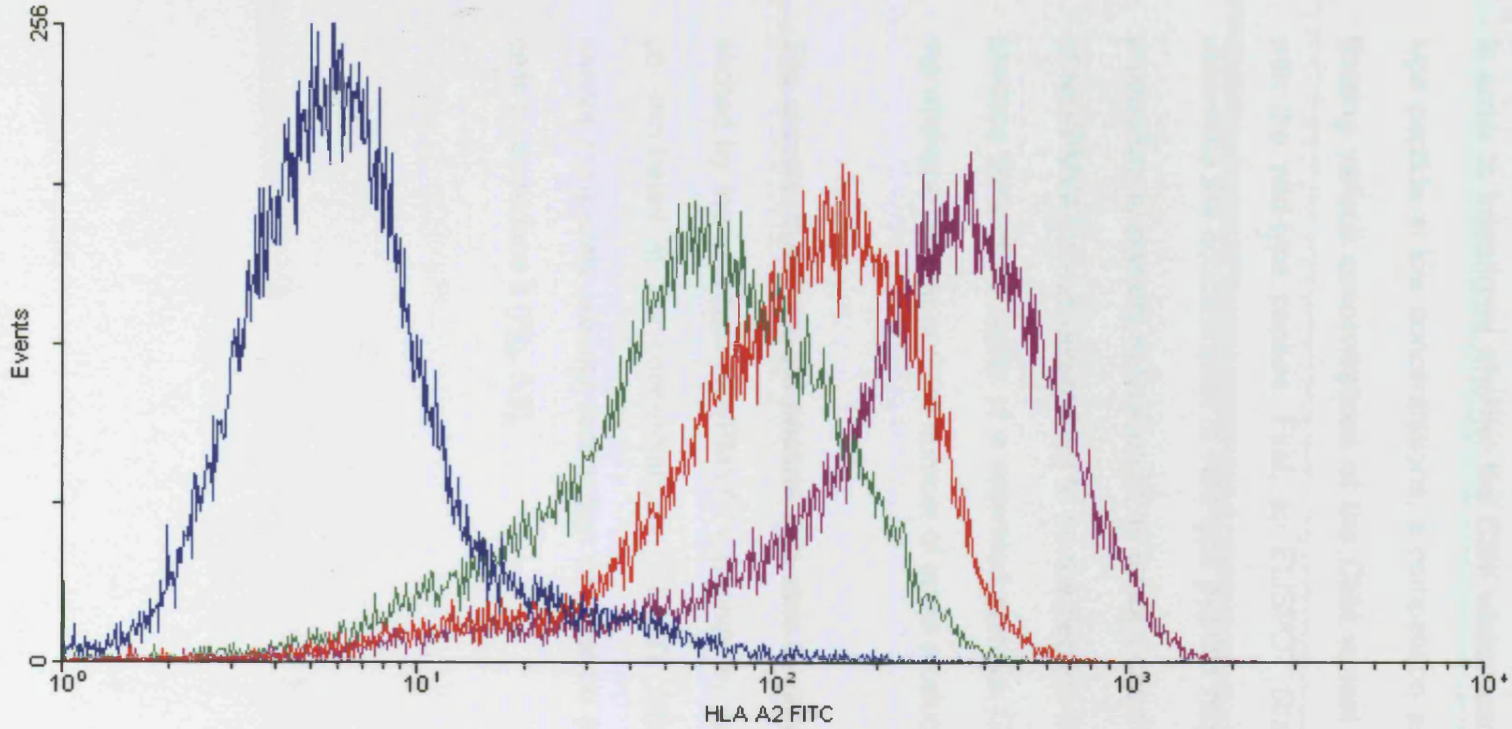


Figure 5. 6 Increase in HLA-A*0201 expression in T2 cells pulsed with NLVPMVATV variants. Blue line represents the isotype control, green line = background level of expression in absence of peptide, red line = stimulated with 50μM of wildtype (wt) peptide and the purple line = stimulation with the C5M variant.

5.5 Peptide competition assay using IFNγ ELISPOT

In order to investigate whether the C5M variant can out-compete the wild-type peptide at low concentrations, a competition assay was performed by titrating various concentrations of the C5M variant into cells co-incubated with the wild-type peptide. First, an ELISPOT titration was performed to determine the concentration of wild-type peptide required to elicit 50% IFNγ production; to prevent saturation of all epitope-specific TCRs. 0.75uM 75μM of NLVPMVATV was determined to be the peptide concentration required to produce 50% of the spots of a saturated sample. C5M was co-titrated with the wild-type peptide and the number of spots produced counted (Fig. 5.7).

The showed that the C5M peptide was able to reduce the amount of IFNγ elicited by the wild-type NLVPMVATV peptide by approximately 25% when co -incubated at low concentrations (10 nM). Although the C5M variant cannot completely prevent recognition of wildtype (wt) peptide by all $CD8^+$ cells it can reduce it (Fig. 5.8).

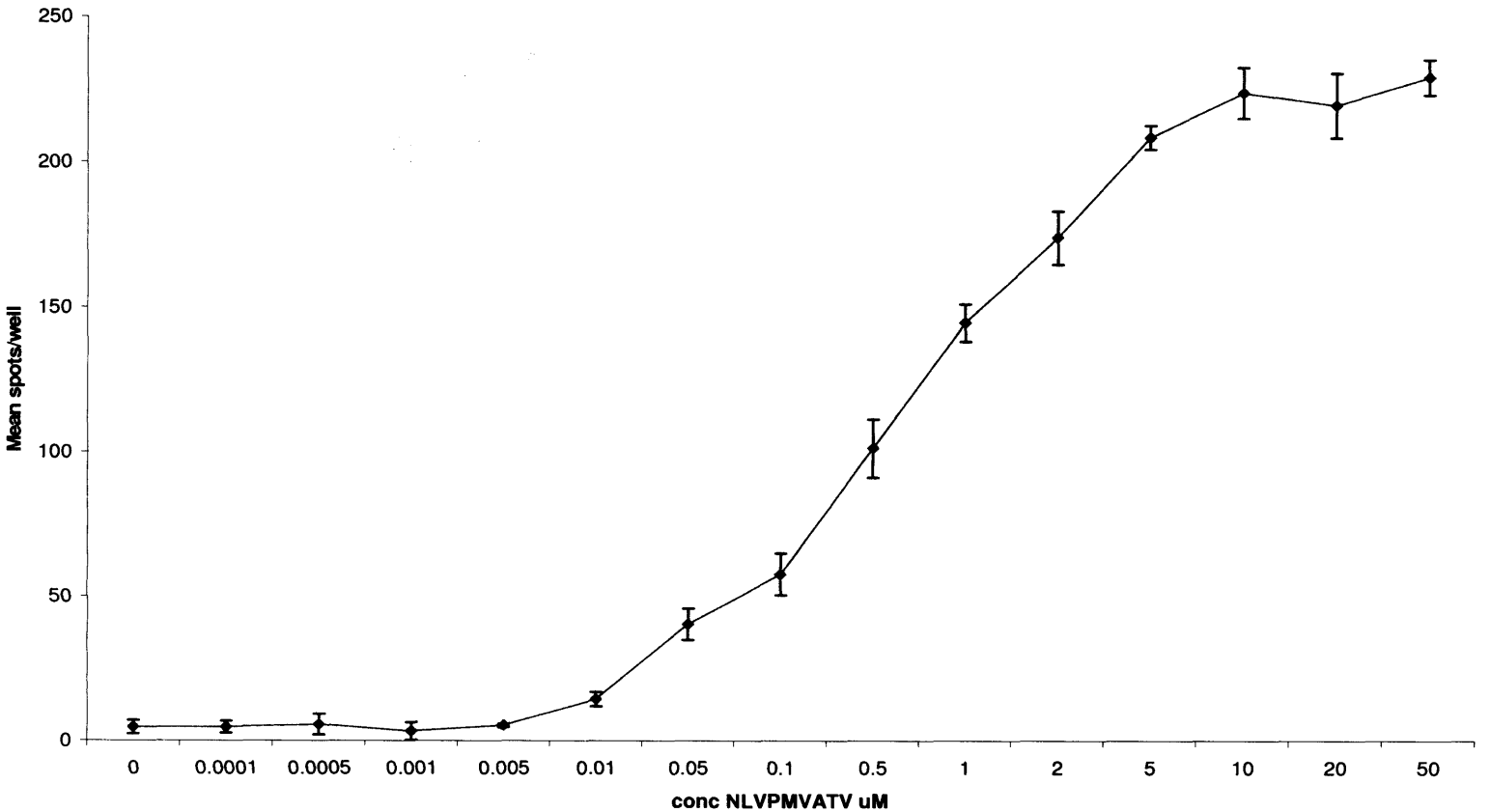


Figure 5. 7 ELISPOT to determine concentration of NLVPMVATV that elicits 50% IFNγ production, a peptide concentration of ~0.75μM was found to stimulate 50% of the maximum production of IFNγ.

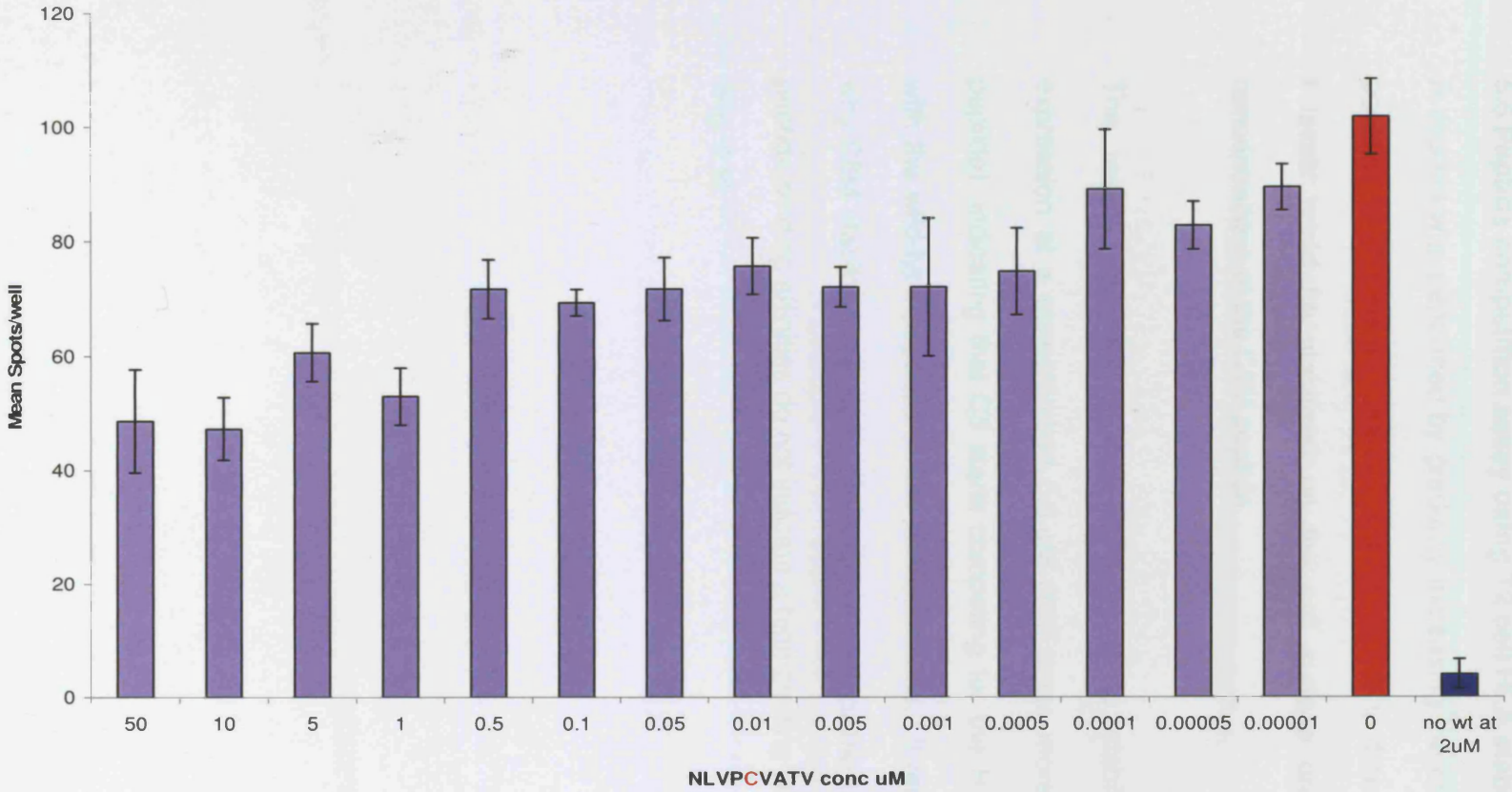


Figure 5. 8 Results of IFNγ ELISPOT titrating in the C5 variant against 0.75uM 75μM of wildtype peptide

5.6 **Peptide competition assay using T2 cell HLA stabilisation assay**

A titration was performed by gradually increasing the concentration of C5M peptide into T2 cells co-incubated with wt peptide to determine whether HLA-I levels could be stabilised on the cell surface on addition of lower concentrations of the C5M peptide.

The results demonstrate that C5M starts to stabilise HLA-I surface expression at a concentration 0.5 μM (125 times more dilute than the wt peptide), indicating that C5 starts competing for the HLA-I binding groove with the wild-type peptides at low concentrations. It remains unclear as to why C5M stabilises the HLA I molecule so well, online algorithms predicting peptide binding affinities do not indicate a high binding affinity for this peptide (Fig. 5.8).

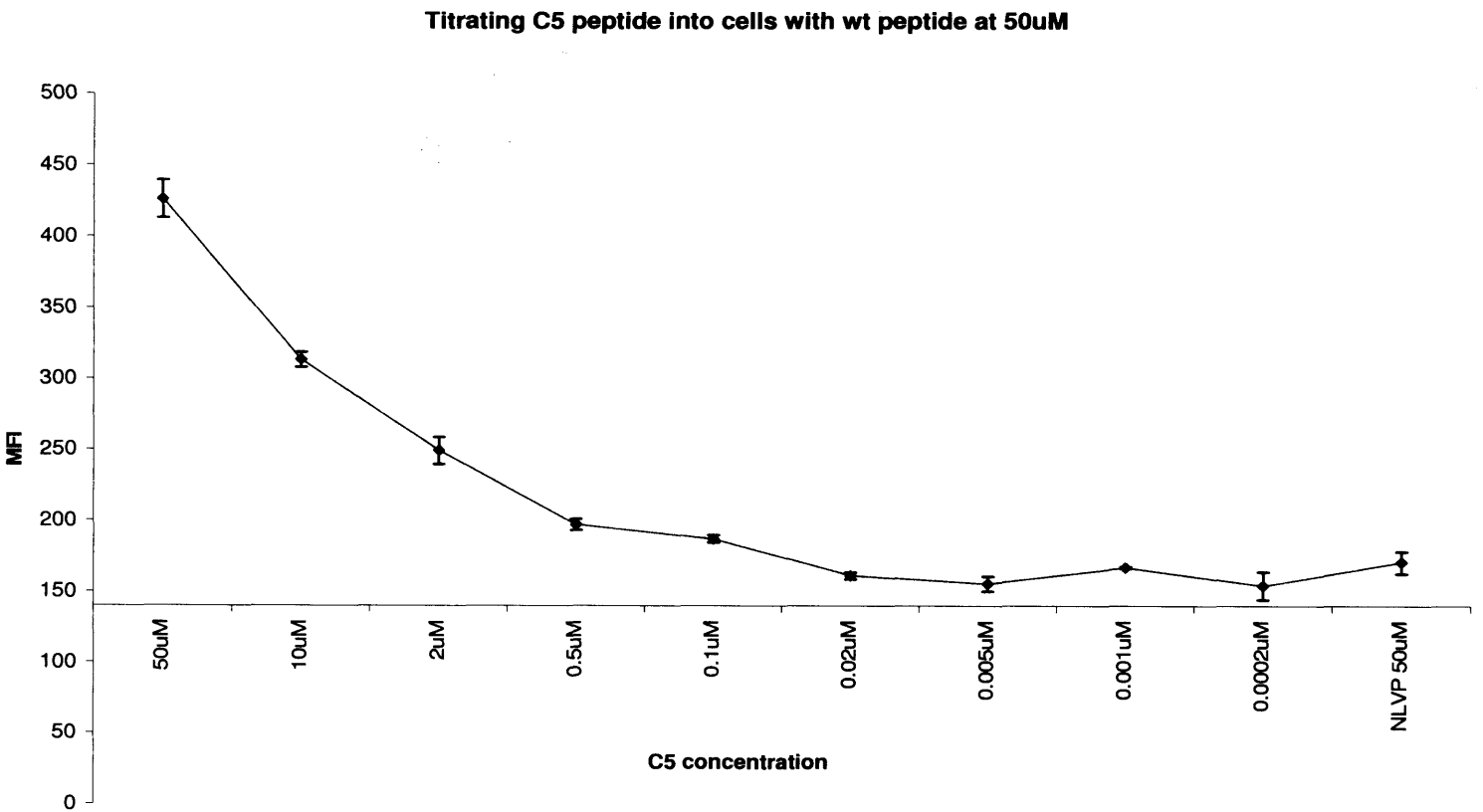


Figure 5. 9 FACS analysis showing mean fluorescence intensity (MFI) created by stabilisation of HLA class I complexes by co-incubation of T2 cells with wildtype peptide and titrated concentrations of the C5M variant.

5.7 Discussion

CTL-escape mutants have previously been shown to arise in response to several virus infections caused by strong selective pressure provided by large, virus-specific $CD8^+$ responses. Because $CD8^+$ T-cells are therefore less able to recognise these altered epitopes and their ability to lyse infected cells will be reduced, this enables the virus to persist and potentially allow it to replicate to higher levels.

Certain renal transplant patients suffer from repeated bouts of HCMV viraemia despite the presence of HCMV epitope-specific $CD8^+$ cells, as detected by HLA class I tetramers. These patients could provide the ideal environment for the generation of CTL escape mutants. Sequence analysis of regions encoding CTL epitopes was performed on DNA amplified from DNA extracted from several patients, of known haplotypes, who suffered phases of prolonged and/or acute viraemia, showed no detectable variation (Table 5.1). It is possible that CTL escape mutants arise within these individuals, but remain as a minority population enabling a small reservoir of HCMV to persist, while the vast majority of infected cells can be recognised and in many cases cleared. This compounds previous findings by Zaia *et al.* [358] , who found only one coding mutation in the HLA-A*0201 epitope $ppUL83_{(495\text{-}503)}$. In their study, sequences encoding this epitope were amplified using a regular Taq polymerase without high-fidelity proof-reading capabilities as utilised here. Furthermore, neither patient epitope-specific $CD8^+$ populations nor HCMV-PCR status was established.

Whether these mutants would be as replicatively fit as the wild type virus and the mutations result in a decreased capacity of the virus to replicate is unclear. It is also important to consider whether novel $CD8^+$ T-cell populations arise, which become specific for these novel mutant epitopes; should this occur it is reasonable to assume that the mutant populations would occur transiently. This might also result in the relative immunodominance of the wild-type epitope-specific response shifting towards another, previously less immunogenic, epitope.

It is also important to also mention that any such mutations would occur in the context of other well-defined CTL evasion strategies that would further facilitate virus escape.

Nevertheless, there is a large potential for CTL escape mutants to arise is this setting, although the exact nature of these mutations remains poorly-defined. To address the possible implications that amino acid substitutions of a known CTL epitope, NLVPMVATV, would have on CTL recognition a series of synthetic peptides, with both conservative and non-conservative substitutions, were designed (Fig. 5.2). Previous work used alanine walking [382] to ascertain the effect of substitutions in the NLVPMVATV on CTL recognition; nevertheless, this alone would not allow the determination of how flexible each of the amino acids are to change nor how accommodating the resultant CTL response would be.

Using an IFNγ ELISPOT the variant peptides were used to stimulate PBMCs isolated from HCMV seropositive individuals and the numbers of cells producing IFNγ were compared to responses elicited by the wildtype. Substitutions at positions 4, 5 and 6 of the epitope caused the most profound reductions in IFNγ production <20% that of wildtype, while substitutions around the anchor residues seemed to have a lesser effect. One possible reason for this is that the centre of the epitope is most likely to be the part most easily accessed by the TCR of the epitope-specific T-cell and therefore the part most important for recognition. Also, because proline residues, s (such as the one found at position P4,) introduce 'kinks' into protein structures, the effect of which cannot be mimicked by other amino acid substitutions, any variant at this position should greatly alter the peptide structure, thus presumably disrupting both the recognition of the epitope and its capacity to bind and stabilise HLA class I molecules.

Certain amino acid substitutions at specific residues seemed to have greater effects than others at the same residues, e.g. at residues 3, 6 and 8 where the conservative substitutions were able to induce a larger IFNγ response than the non-conservative glycine substitutions (Fig. 5.4).

To address whether the substitutions had a direct effect on TCR recognition or whether they affected the ability of the epitope to bind and therefore stabilise HLA class I complexes a T2 HLA stabilisation assay was performed. This assay showed that the wildtype NLVPMVATV epitope was only able to moderately increase the stability of the HLA class I complex compared to

background levels. This indicated that a highly stable complex between HLA class I/ and a peptide is not essential for induction of IFNγ production. Furthermore, a C5M substitution was able to strongly increase HLA class I stability, to more than four times that of the background, whilst simultaneously being unable to induce IFNγ production. Such a mutation would be advantageous to HCMV for it would enable the retention of HLA class I molecules on the surfaces of infected cells, preventing NK-mediated lysis whilst being able to circumvent the epitope-specific response.

Although The emergence of such a mutation would require three nucleotide substitutions and would therefore be unlikely to spontaneously arise. Given that HCMV is a DNA virus, one would assume that it would be more genetically stable than RNA viruses, such as HBV and HIV, which are prone to accumulating CTL-escape mutants. Nevertheless, the selective pressure applied by antivirals has resulted in the evolution of a variety of mutations in both UL54 and UL97 with antiviral resistance. Furthermore, these resistant mutants evolve when the viral load is low; as a result of the antiviral therapies used. In this scenario the selective pressure would be exerted by high numbers of epitope-focused CTLs when the virus is replicating at much higher frequencies; thus resulting in a higher probability of the emergence of such mutants.

Nevertheless, should this mutation occur it is possible that even at low concentrations this peptide could out-compete the wild-type through the

strength of its interaction with HLA-A*0201 and possibly allow mutant virus to escape the CTL response.

By co-titrating the C5M variant with the wild-type peptide it was evident that the C5M could reduce the absolute IFNγ production elicited by the wildtype peptide in an IFNγ ELISPOT by 25% even at low concentrations. When both were present at a 1 μM concentration, IFNg production was approximately halved, although titrating in the C5M variant at high concentrations could never inhibit IFNγ production completely. Similarly, co-incubation of T2 cells with these two peptides showed an increase in the surface expression of HLA class I with only 0.5 μM of the C5M, indicating that competition for that HLA class I binding site starts at a low concentration and eventually C5M seems to upregulate HLA class I on most cells to levels normally reached in the presence of wildtype peptide at 50 μM.

One explanation for the strong C5M/HLA-A*0201 association could be the formation of a disulphide bond between the Cysteine at position 5 and any cysteines present in the HLA-A*0201 peptide-binding groove. Such an interaction alone would probably be insufficient to stabilise the HLA complex, as anchor residues are still required for stabilisation of the complex, but it could increase the avidity of the interaction. This would depend on the environment in which any reactions occur and the concentration of cysteines in the medium. RPMI 1640 used during the stimulations contains cysteine so there is the possibility that disulphide bonds may form prior to interactions with the HLA molecule and furthermore, formation of the bond usually

requires a basic environment which is unlikely to be the situation in the present case.

Although cells may still express some wildtype peptide on their surfaces, because IFNγ is still produced during stimulation, it is clear that competition for the HLA complexes occurs. Therefore, whilst C5 may not be able to abrogate IFNγ production in a heterogeneous population completely, should HCMV alter a major CTL epitope to obtain a similar mutant, this would render cells infected by this variant less susceptible to CTL lysis. These mutants could conceivably occur in patients suffering repeated viraemic episodes as described above, and although no such mutants could be detected in the sequence analysis their frequency may have been below the detection threshold of the assay <20% of the total virus population.

In addition to the potential for CTL escape mutants arising during HCMV infection, there is also the possibility of $CD8^+$ T-cell responses to HCMV being functionally defective in individuals unable to prevent viraemia. IFNγ secretion has indirect effects on infected cells, causing up-regulation of HLA molecules and allowing infected cells to be more easily recognised by other $CD8^+$ cells as well as activating other aspects of the immune response; but it is not directly cytolytic.

Is it possible that these epitope-specific CD8+ T-cell populations have a defect in their cytolytic potential or that they may be replicatively senescent and thus less able to react to a virus infection?

Chapter 6

T-cell Responses to HCMV in Patients with Common Variable Immunodeficiency (CVID)

6. 1 Introduction

Individuals suffering from common variable immunodeficiency syndrome (CVID) are an important clinical model for the study of various aspects of the adaptive immune response. During the third decade of life these individuals cease to be able to produce immunoglobulins against invading pathogens [346, 347]. This increases their susceptibility to a multitude of predominantly bacterial infections; yet despite this, they are still able to control and resolve many acute virus infections without complications. Because they are unable to mount B-cell responses it is not possible to ascertain the serostatus of the patients towards previously-encountered pathogens. However, using T-cell-based immune assays, such as ICS analysed by FACS, it may be possible to establish which organisms have been encountered at an earlier stage of life and could therefore be used as a crude determination of previous infection.

The majority of healthy HCMV-seropositive individuals mount large, HCMV-specific T-cell responses that expand as a result of sporadic low-level, asymptomatic reactivations throughout life. Data from previous chapters indicated that renal transplant patients, unable to control HCMV replication whilst on immunosuppressive regiments in the months post transplant, had a significant decrease in the frequencies of their HCMV-specific $CD8^+$ T-cell populations compared to those that were able control HCMV replication.

The aim of this chapter was to use FACS analysis of intracellular cytokine staining to determine whether CVID patients mount detectable $CD4^+$ and $CD8^+$ T-cell responses to HCMV; these virus-specific responses were

subsequently compared between these patients and age-matched healthy controls. Because none of the CVID patients in this group has ever suffered a symptomatic HCMV infection, even prior to CVID being diagnosed, it is possible to conclude that although immunoglobulins afford some level of protection during the resolution of primary HCMV infection, long-term control, in these patients, may be the result of some other facet of the immune response; namely the T-cell responses.

Phenotypic analyses were also performed on epitope-specific $CD8^+$ T-cell populations using HLA-restricted class I tetramers. Phenotypic analyses focused on the proportions of epitope-specific cells that displayed the senescence marker CD57 [303, 383, 384] and the intracellular lytic protein perforin. Together, these markers would give an indication to the proportion of late-stage, replicatively-senescent, potentially cytolytic cells in the $CD8^+$ T-cell populations. These populations were compared in CVID patients, healthy age-matched controls and also renal transplant patients who suffered repeated bouts of viraemia to determine whether there was a functional aspect to these cell populations that rendered certain individuals less susceptible to HCMV replication.

6. 2 CVID responses to HCMV antigens

Similar to previous chapters, $CD4^+$ and $CD8^+$ T-cell responses to HCMV lysate, pp65 and IE1 were measured in CVID patients by ICS using FACS, by overnight stimulation of freshly isolated PBMCs with antigens in the presence of CD28 and CD49d. This assay was used to provide a crude determination of past HCMV infection, which would previously have been unidentified due to the lack of any detectable B-cell responses. HCMV-specific T-cell responses above the lower limit of detection for either CD8 and/or CD4 responses were found to be present in sixteen of the nineteen patients analysed (84%).

The median $CD8^+$ T-cell response of these sixteen individuals towards IE1 was 0.55% (range 0.02% to 4%), while towards pp65 it was 3.22% (range 0.31% to 14.41%) (Fig. 7.1). Interestingly, this means that all CVID patients analysed with detectable CD4 and/or CD8 response towards HCMV also had $CD8^+$ T-cell responses against pp65 that were above the lower limit of detection.

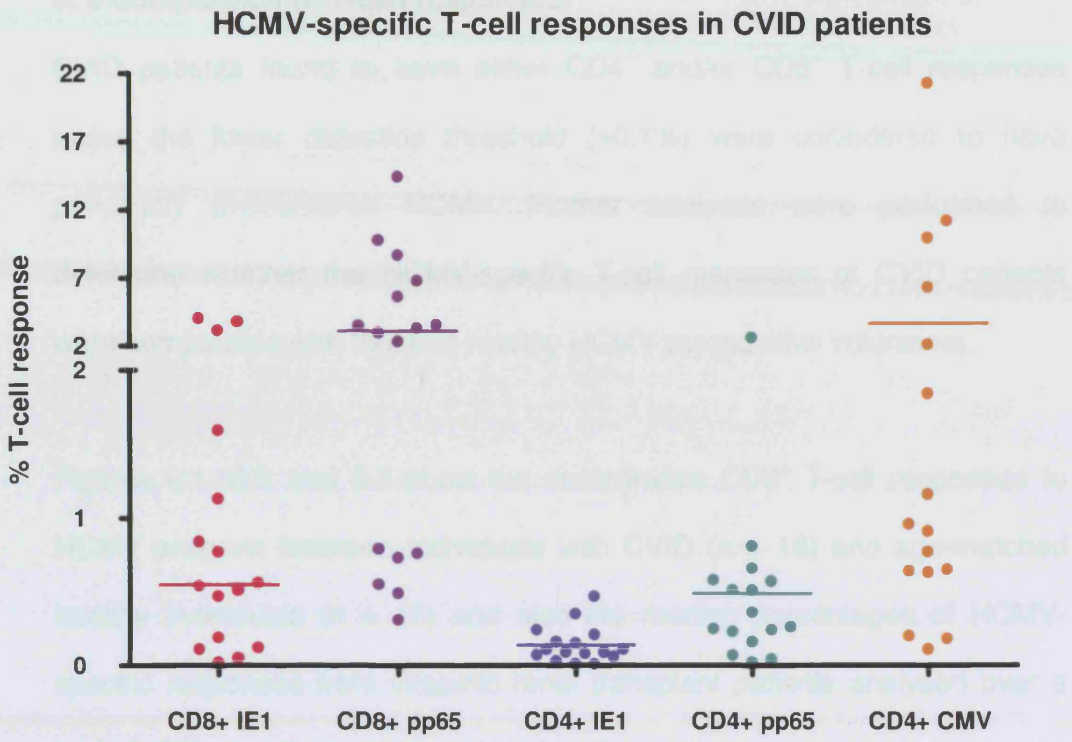


Figure 6. 1 Analysis of $CD4^+$ and $CD8^+$ T-cell responses to HCMV antigens in CVID patients

6. 3 Comparison of T-cell responses

CVID patients found to have either $CD4^+$ and/or $CD8^+$ T-cell responses above the lower detection threshold (>0.1%) were considered to have previously encountered HCMV. Further analyses were performed to determine whether the HCMV-specific T-cell responses of CVID patients were comparable with those of healthy HCMV seropositive volunteers.

Figures 6.1, 6.2 and 6.3 show the comparative $CD8^+$ T-cell responses to HCMV antigens between individuals with CVID (n = 16) and age-matched healthy individuals (n = 15) and also the median percentages of HCMV-specific responses from viraemic renal transplant patients analysed over a period of six months post-transplant (n = 8).

The frequency of $CD8^+$ T-cell populations specific for pp65 were significantly elevated in individuals with CVID compared to those of healthy controls (median 2.97% and 0.44% respectively, p = 0.01). The same responses were compared between CVID patients and viraemic renal transplant (median 2.97% and 0.25% p = 0.009). $CD8^+$ T-cell responses towards IE1 were also elevated in CVID individuals compared to healthy controls (median 0.56% and 0.22% p = 0.02). However, there was no significant increase when compared to viraemic individuals (median 0.56% and 0.08% p = 0.22). When $CD8^+$ T-cell responses to pp65 and IE1 were combined there was again a significant elevation in the HCMV-specific responses of CVID patients compared to healthy controls (median 4.27% and 0.74% p = 0.003) and also over viraemic individuals (median 4.27% and 0.65% p = 0.02). Such

increases in the percentages of HCMV-specific $CD8^+$ populations in CVID populations when compared to healthy individuals may be related to the increase in the frequencies of HCMV-specific $CD4^+$ T-cell or possibly increased exposure to HCMV through ongoing replication, which cannot be detected in the peripheral blood.

A similar comparison was performed to ascertain whether there are any differences in the frequencies of global HCMV-specific $CD4^+$ T-cells between these two groups; to do this, responses towards HCMV lysate were measured (as in chapter 3). Figure 6.4 shows that the HCMV-specific $CD4^+$ T-cell populations of CVID patients were not significantly different to those of healthy age-matched controls. Median HCMV-specific CD4 responses were 0.95% in CVID patients, 0.79% in healthy age-matched controls and 1.33% in viraemic renal transplant patients; none of these differed significantly.

6. 4 Analysis and comparison of HCMV-specific Tet$^+$ populations

In order to ascertain whether the epitope-specific CD8$^+$ T-cell populations of CVID patients were similar to those found in healthy individuals and viraemic transplant patients, tetramers specific for HCMV A1, A2 and B7-restricted epitopes were analysed for intracellular levels of perforin, the presence of which would be an indicator of the cytolytic potential of the cells, and the senescence marker CD57 expression of which would correlate an effector memory phenotype and a decreased capacity to proliferate in response to antigen stimulation.

A significant increase was apparent in the percentage of tetramer$^+$ CD8$^+$ T-cells in CVID patients when compared with healthy age-matched controls (CVID n = 9, median 4.65% and healthy n = 9, 0.84% P = 0.03) and also when compared to viraemic renal transplant patients (Fig. 7.11 CVID n = 9, median 4.65% and viraemic n = 7, 0.95% P = 0.03).

When expression levels of CD57 in HCMV epitope-specific CD8 populations were compared (Fig. 7.12), there was an elevation in CVID patients (n = 9, median 79%) compared to healthy controls (n = 9, median 58%) but this was not significant (p = 0.06). There was also no significant difference in the median percentages of tetramer-positive cells expressing CD57 between CVID patients and viraemic renal transplant patients (n = 7, median 55.8%, p = 0.07).

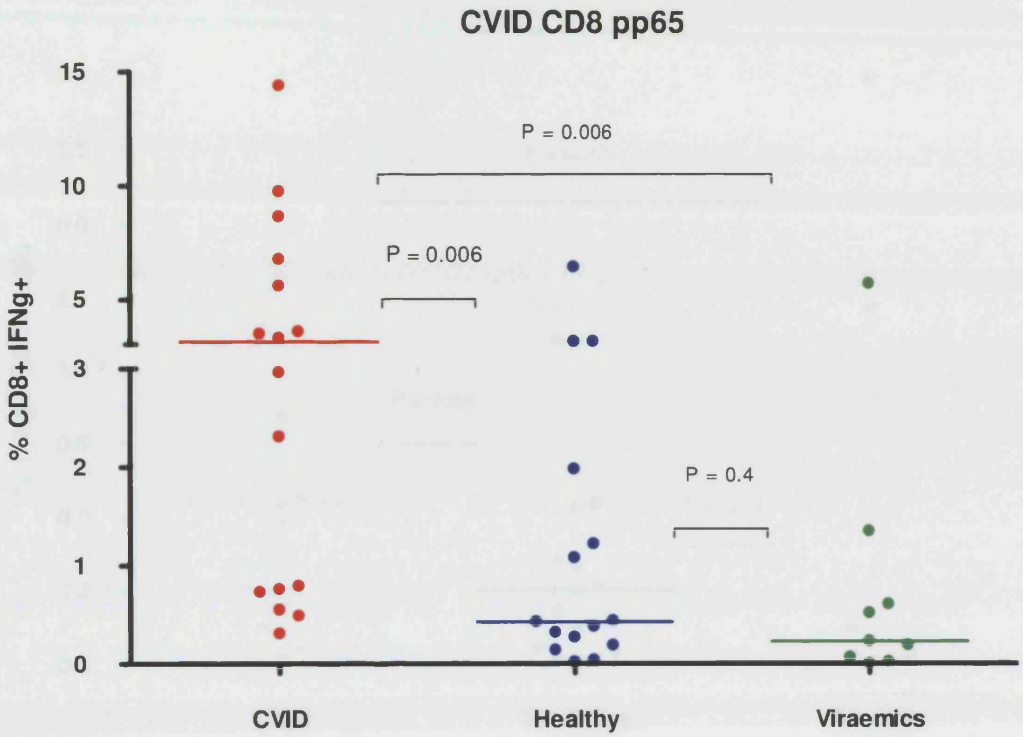


Figure 6. 2 Comparison of pp65-specific $CD8^+$ T-cell responses between CVID patients, healthy age-matched controls and viraemic renal transplant patients.

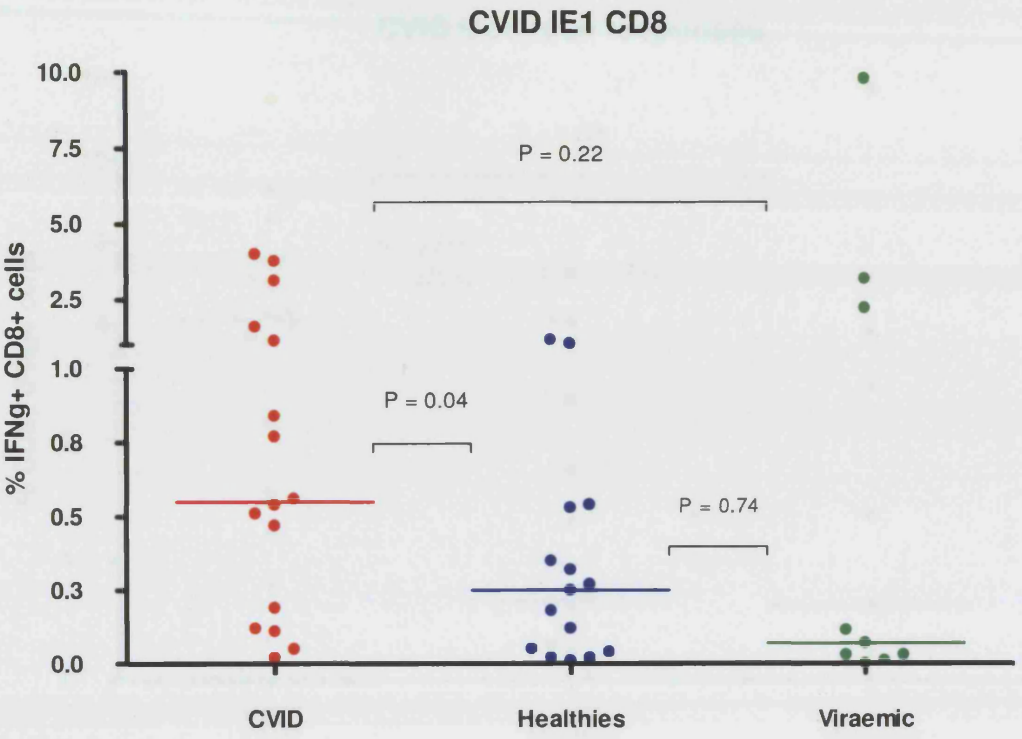


Figure 6. 3 Comparison of IE1-specific $CD8^+$ T-cell responses between CVID patients, healthy age-matched controls and viraemic renal transplant patients.

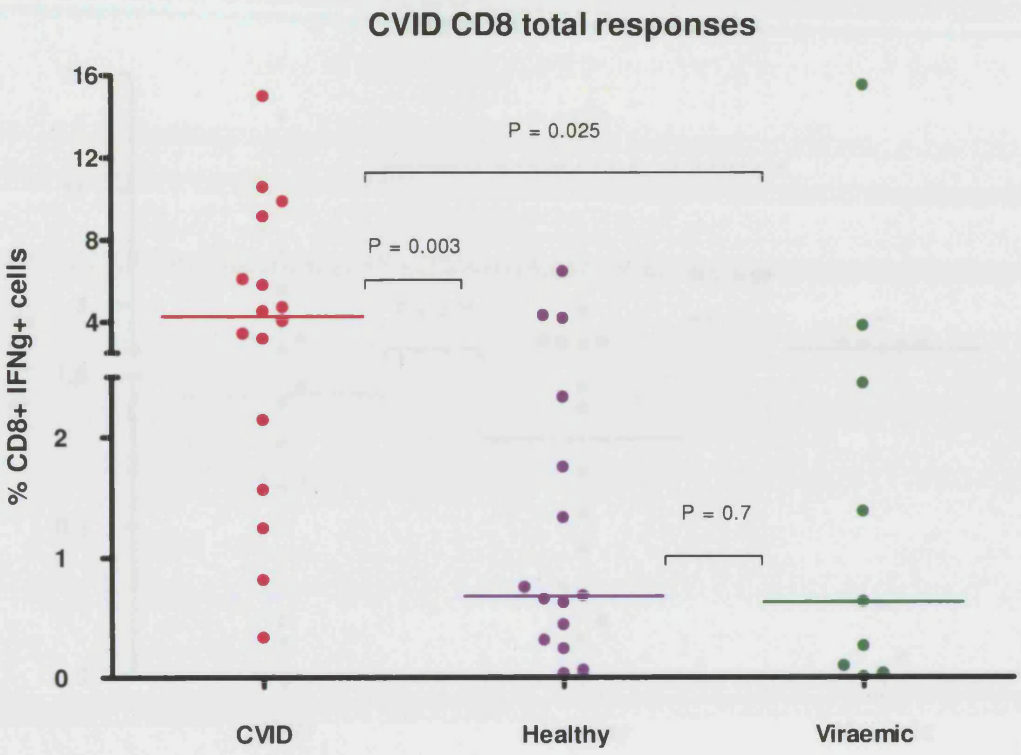


Figure 6. 4 Comparison of combined IE1 and pp65-specific $CD8^+$ T-cell responses between CVID patients, healthy age-matched controls and viraemic renal transplant patients.

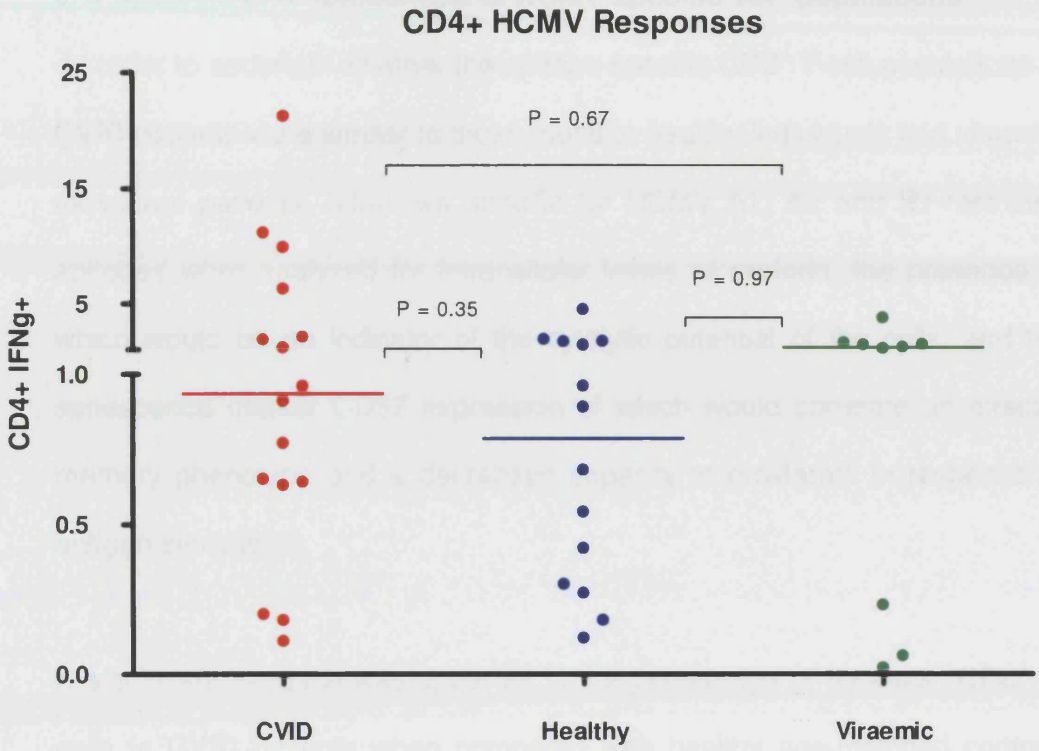


Figure 6. 5 Comparison of global $CD4^+$ T-cell responses to whole virus lysate between CVID patients, healthy age-matched controls and viraemic renal transplant patients.

6. 4 Analysis and comparison of HCMV-specific Tet$^+$ populations

In order to ascertain whether the epitope-specific CD8$^+$ T-cell populations of CVID patients were similar to those found in healthy individuals and viraemic transplant patients, tetramers specific for HCMV A1, A2 and B7-restricted epitopes were analysed for intracellular levels of perforin, the presence of which would be an indicator of the cytolytic potential of the cells, and the senescence marker CD57 expression of which would correlate an effector memory phenotype and a decreased capacity to proliferate in response to antigen stimulation.

A significant increase was apparent in the percentage of tetramer$^+$ CD8$^+$ T-cells in CVID patients when compared with healthy age-matched controls (CVID n = 9, median 4.65% and healthy n = 9, 0.84% P = 0.03) and also when compared to viraemic renal transplant patients (Fig. 7.11 CVID n = 9, median 4.65% and viraemic n = 7, 0.95% P = 0.03).

When expression levels of CD57 in HCMV epitope-specific CD8 populations were compared (Fig. 7.12), there was an elevation in CVID patients (n = 9, median 79%) compared to healthy controls (n = 9, median 58%) but this was not significant (p = 0.06). There was also no significant difference in the median percentages of tetramer-positive cells expressing CD57 between CVID patients and viraemic renal transplant patients (n = 7, median 55.8%, p = 0.07).

Similarly, a greater proportion of intracellular perforin was evident in the tetramer$^+$ cells of CVID patients (90%) compared to healthy age-matched controls (77.5%) although this again was not significant ($p = 0.06$). The difference between intracellular perforin levels of CVID patients and viraemic (median 78%) individuals was also not significant (Fig. 7.13 $p = 0.6$).

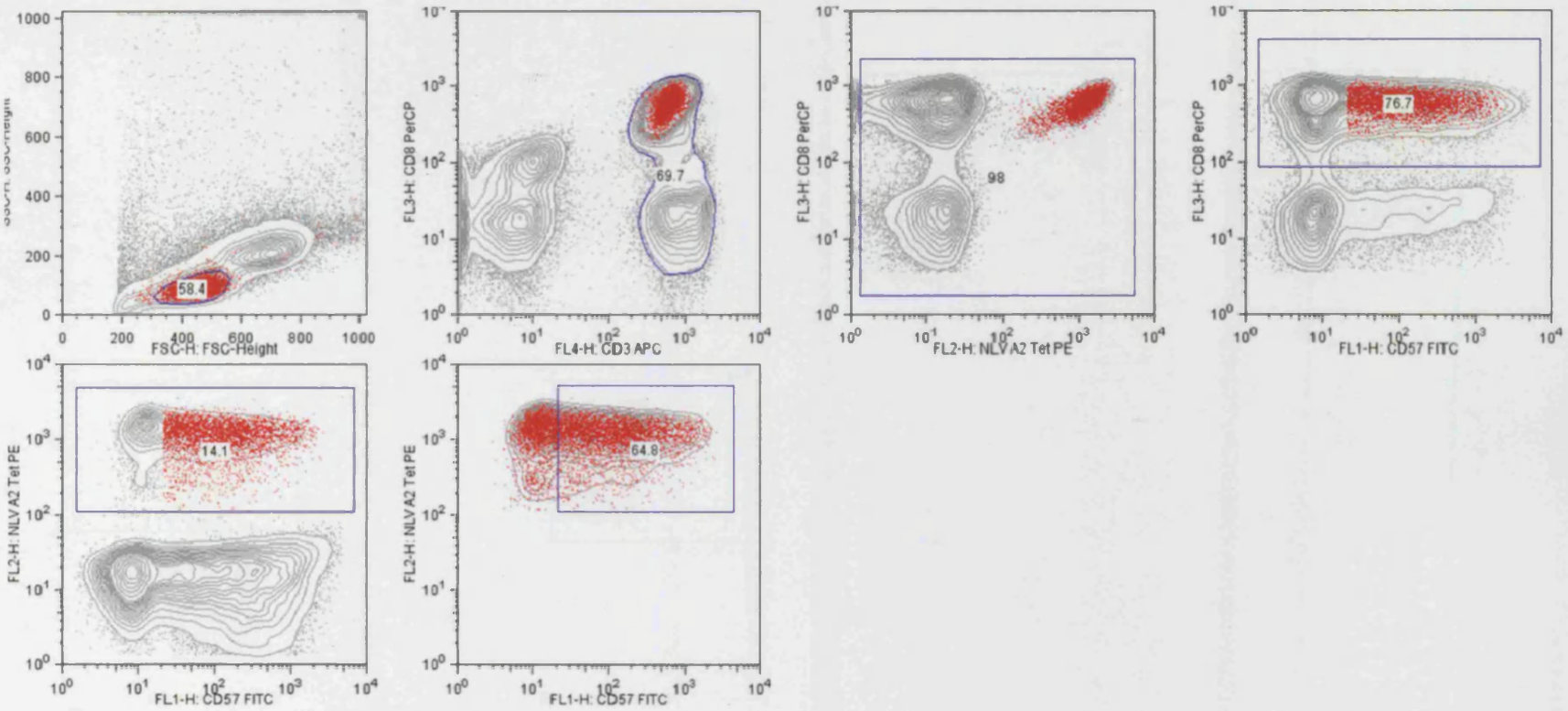


Figure 6. 6 FACs analysis of CD57 staining in $CD8^+$ $CD3^+$ Tetramer populations. Upper panels show (from left to right) the forward scatter (x) against side scatter (y), CD3 (x) against CD8 (y), HLA-A*0201 HCMV tetramer (x) against CD8 (y), CD57 (x) against CD8 (y). Lower Panels show CD57 (x) against HLA-A*0201 HCMV tetramer (y) and the proportion of these HCMV $tetramer^+$ cells expressing CD57 (x).

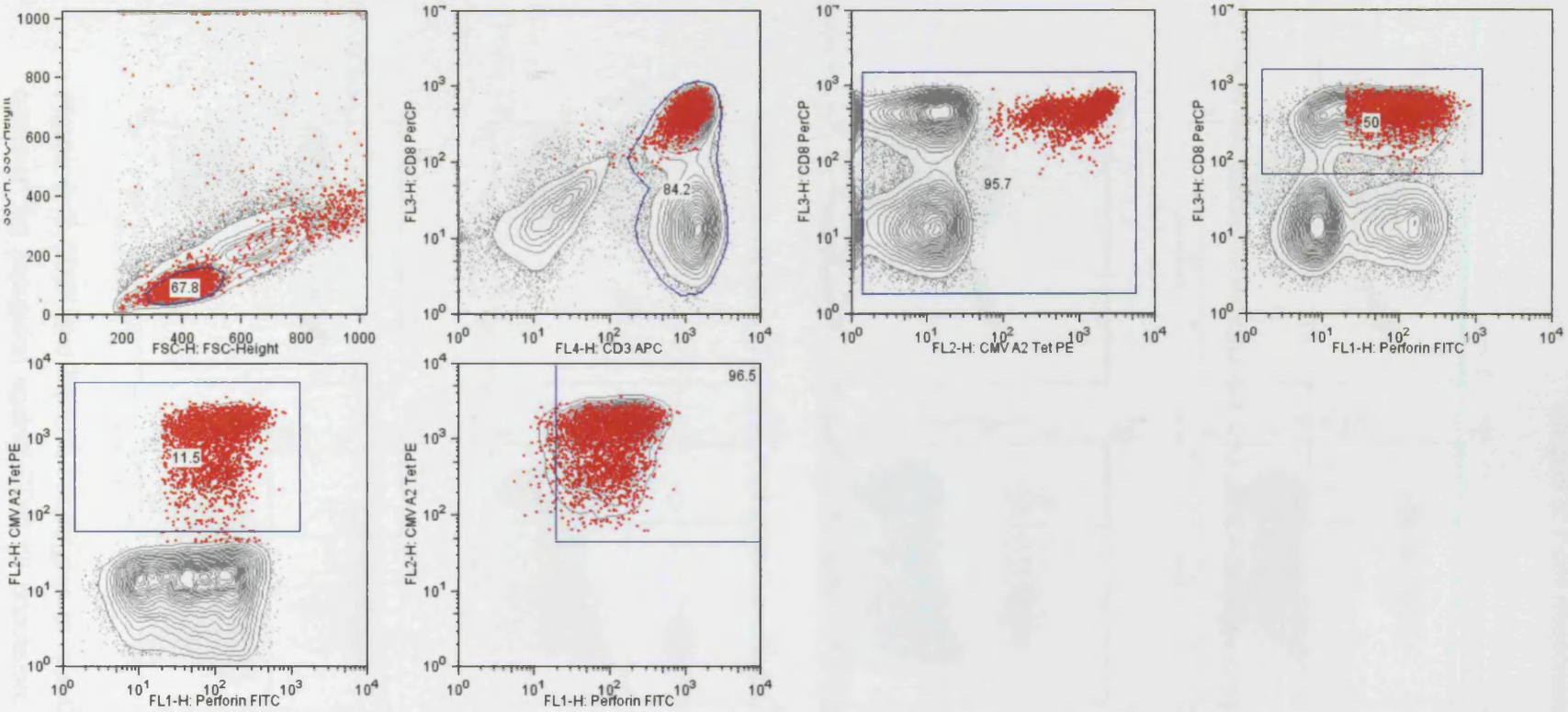


Figure 6. 7 FACs analysis of perforin staining in $CD8^+$ $CD3^+$ Tetramer populations. Upper panels show (from left to right) the forward scatter (x) against side scatter (y), CD3 (x) against CD8 (y), HLA-A*0201 HCMV tetramer (x) against CD8 (y), perforin (x) against CD8 (y). Lower Panels show perforin (x) against HLA-A*0201 HCMV tetramer (y) and the proportion of these HCMV $tetramer^+$ cells expressing perforin (x).

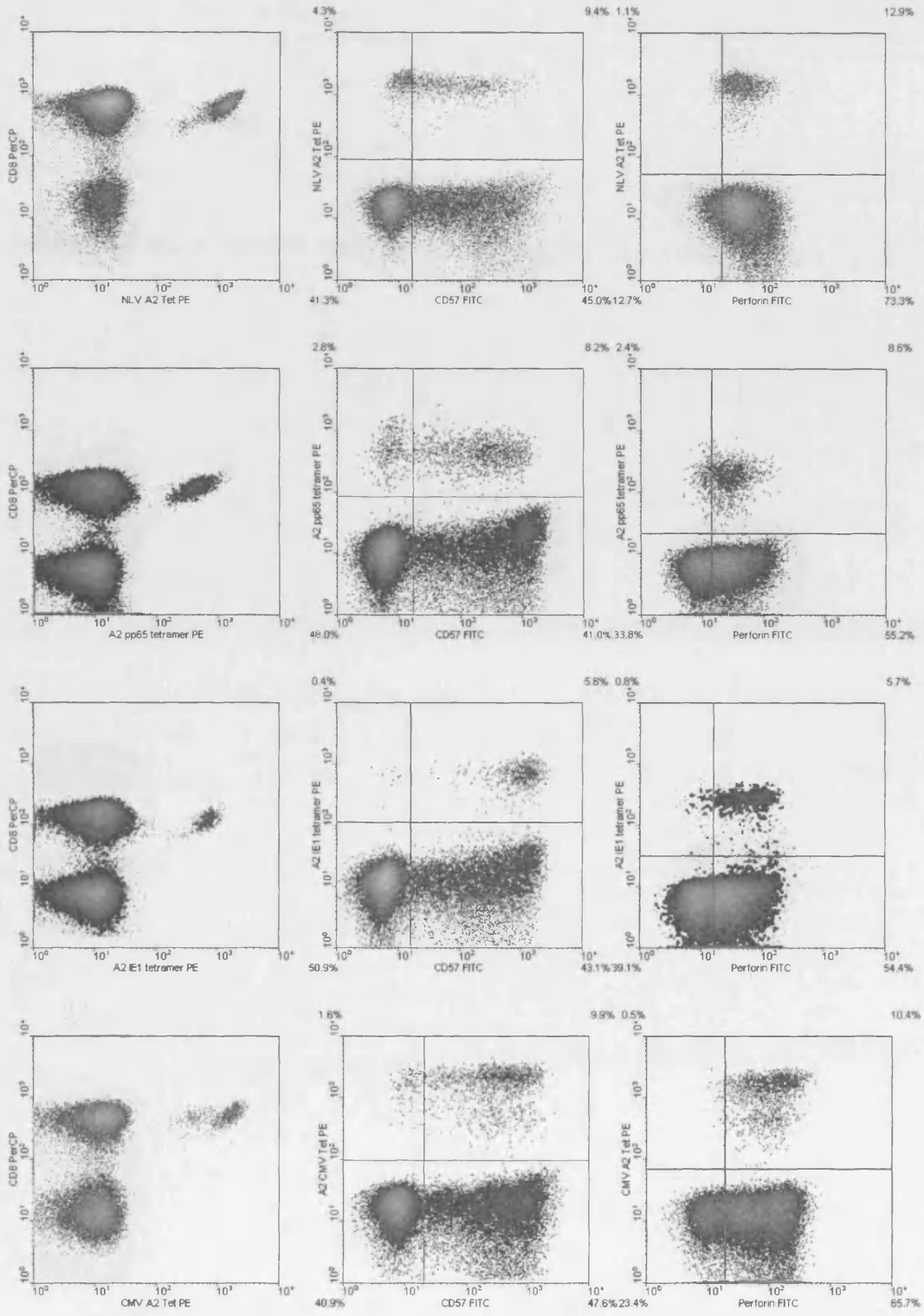


Figure 6. 8 CD57 and Perforin Phenotypes of four CVID patients, left panels show tetramer$^+$ (x) population against CD8$^+$ (y) population, central panels show CD57 (x) against tetramer (y), right panels show perforin (x) against tetramer (y).

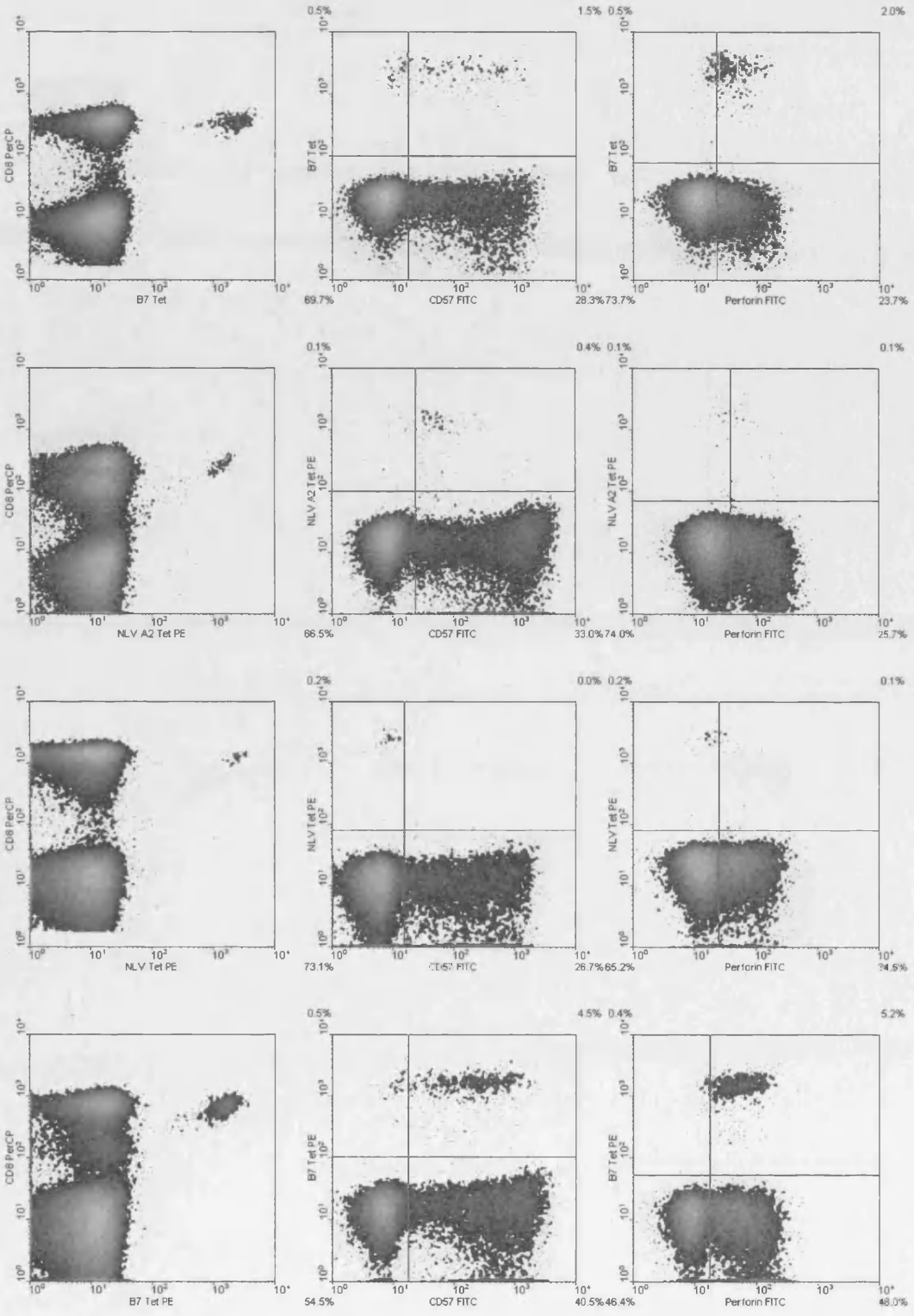


Figure 6. 9 $CD8^+$ Phenotypes of four age-matched healthy controls, left panels show $tetramer^+$ (x) population against $CD8^+$ (y) population, central panels show CD57 (x) against tetramer (y), right panels show perforin (x) against tetramer (y).

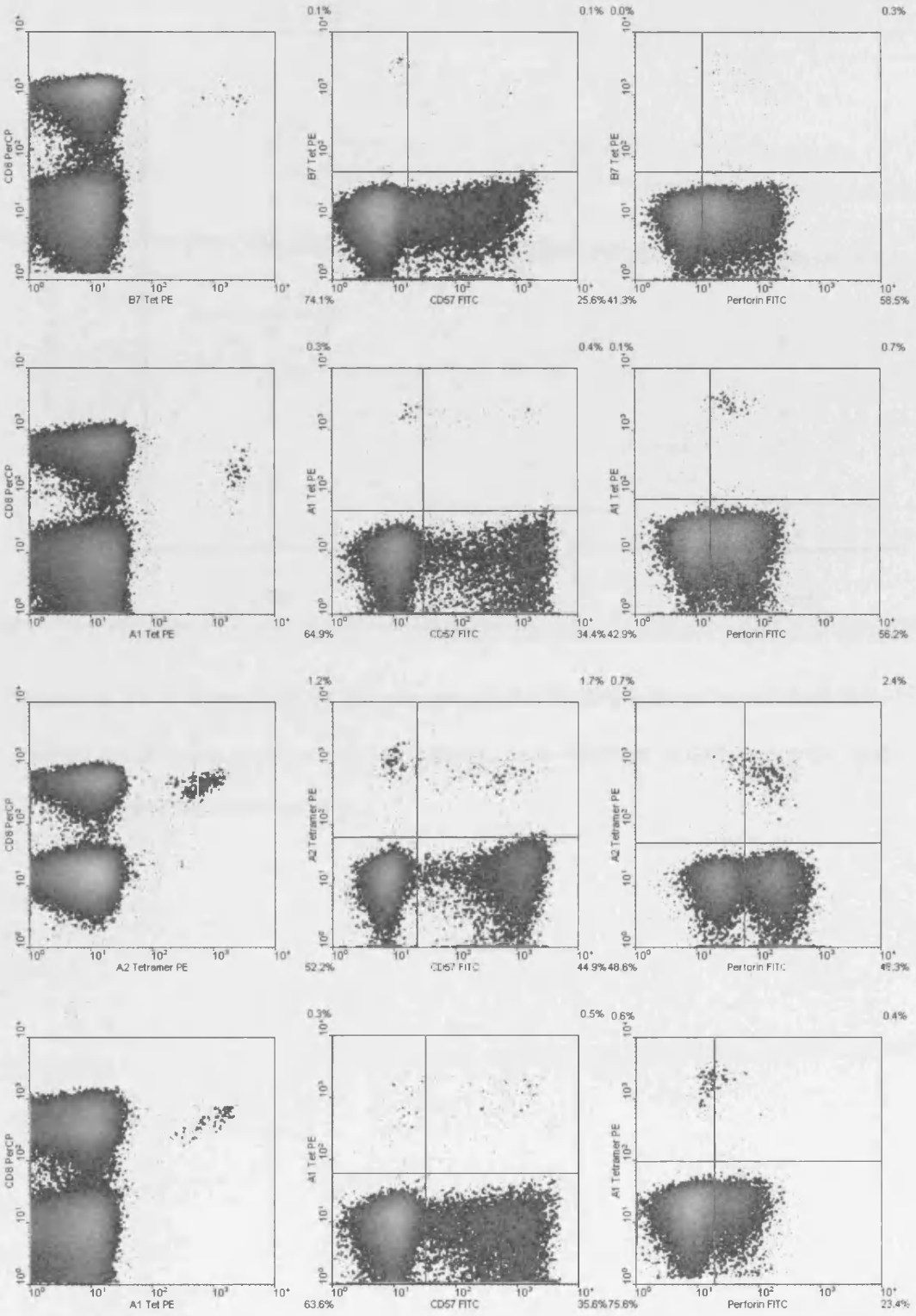


Figure 6. 10 Examples $CD8^+$ Phenotypes of four viraemic renal transplant patients left panels show $tetramer^+$ (x) population against $CD8^+$ (y) population, central panels show CD57 (x) against tetramer (y), right panels show perforin (x) against tetramer (y).

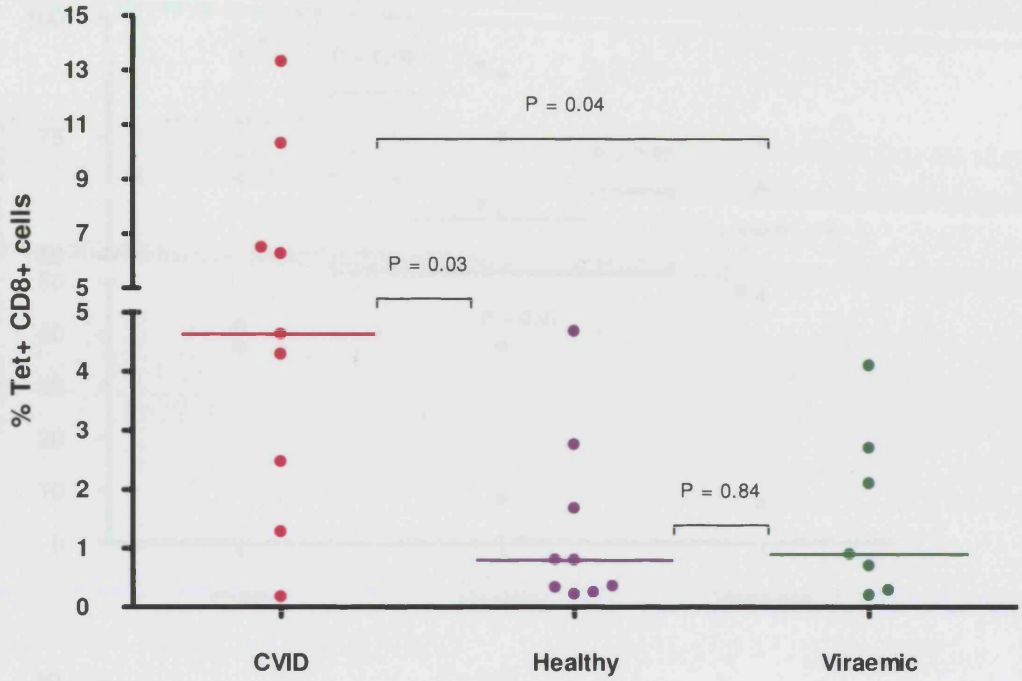


Figure 6. 11 A comparison of the percentages of HCMV epitope-specific tetramer stained $CD8^+$ cells between CVID patients, age-matched healthy controls and viraemic renal transplant patients.

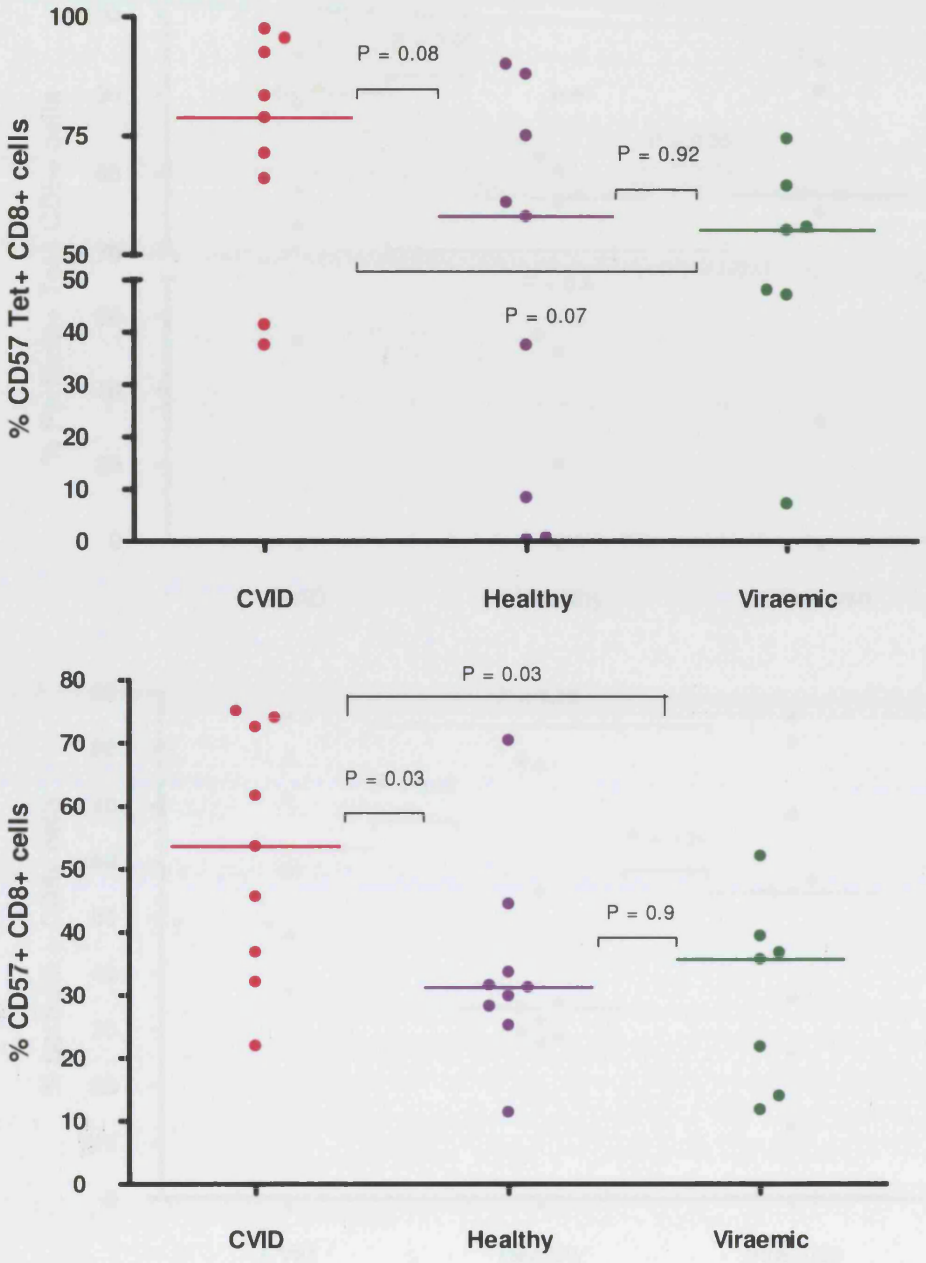


Figure 6. 12 A comparison of the percentages of HCMV epitope-specific tetramer stained $CD8^+$ cells (upper panel) and $CD8^+$ cells that express the senescence marker CD57 (lower panel).

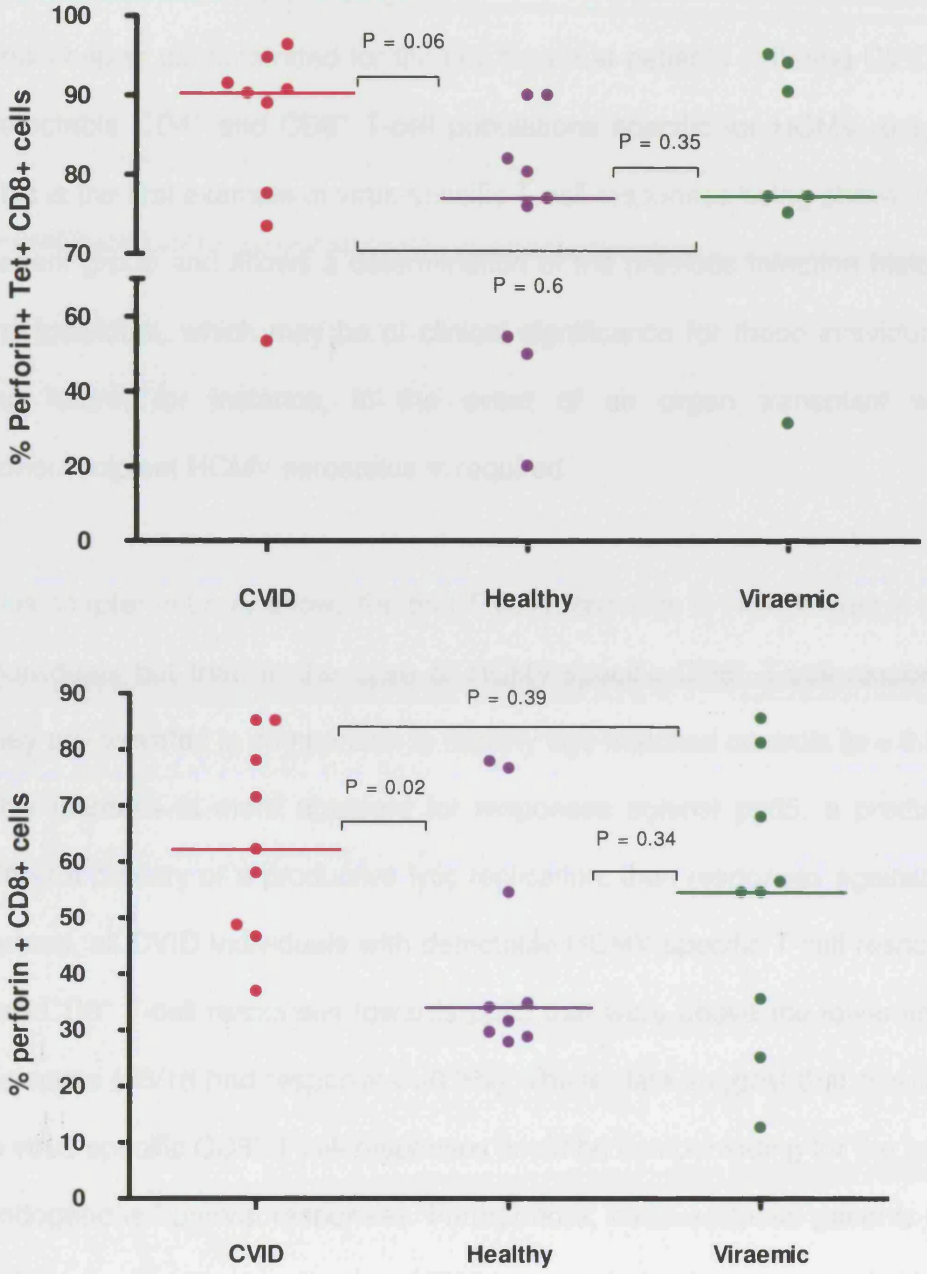


Figure 6. 13 A comparison of the percentages of HCMV epitope-specific tetramer stained $CD8^+$ cells (upper panel) and $CD8^+$ cells (lower panel) that express intracellular perforin.

6. 5 Discussion

This chapter demonstrated for the first time that patients suffering CVID had detectable $CD4^+$ and $CD8^+$ T-cell populations specific for HCMV antigens. This is the first example of virus-specific T-cell responses being shown in this patient group and allows a determination of the previous infection history of the individual, which may be of clinical significance for these individuals in the future; for instance, in the event of an organ transplant where donor/recipient HCMV serostatus is required.

This chapter not only shows the that T-cell responses to HCMV exist in these individuals but that, in the case of HCMV-specific $CD8^+$ T-cell responses, they are elevated in comparison to healthy age-matched controls ($p = 0.006$). This increase is more apparent for responses against pp65, a product of either a primary or a productive lytic replication, than responses against IE1; indeed, all CVID individuals with detectable HCMV-specific T-cell responses had $CD8^+$ T-cell responses towards pp65 that were above the lower limit of detection (16/16 had responses >0.3%). These data suggest that elevations in virus-specific $CD8^+$ T-cell responses could be compensating for the lack of endogenous humoral responses. Furthermore, because these patients have neither suffered any known HCMV-associated disease nor had any detectable episodes of viraemia indicates that their humoral responses were not as critical for the control of HCMV infection as cell-mediated responses. Although these individuals do receive regular infusions of polyclonal sera, which might alleviate any potential illness, there was a stage prior to

diagnosis in which Ig levels were low and no incidences of CMV disease were reported.

Because of the elevation in HCMV-specific $CD8^+$ responses, it was reasonable to suggest that there would be a concomitant rise in the HCMV-specific $CD4^+$ T-cell responses of those same individuals. This assumption proved to be unfounded as $CD4^+$ T-cell responses to HCMV lysate were not significantly different between either the healthy controls or the viraemic individuals tested. This phenomenon was also apparent in chapter 3, where there was no significant variation in the $CD4^+$ T-cell responses to HCMV between viraemic and non-viraemic transplant patients. Taken together these data indicate that there is no apparent link between the frequencies of HCMV-specific $CD4^+$ T-cell responses and an increase in the susceptibility of an individual to suffering HCMV viraemia.

$CD8^+$ T-cells specific for immunodominant HLA class I-restricted CMV epitopes were compared between individuals of known haplotypes for each group. Only individuals of known haplotypes, with detectable HCMV tetramer-positive populations were selected from each group. Median frequencies of tetramer-positive cells were found to be highest in the CVID patients (n = 9, median 4.65%); these responses were significantly higher than found in age-matched healthy controls (n = 9, median 0.84%, p = 0.03) and also to renal transplant patients suffering repeated viraemic episodes (n = 7, 0.91% p = 0.04). Although responses to HLA-restricted CTL episodes are higher in CVID patients, they are not representative of total $CD8^+$ T-cell

responses to HCMV. Measuring the percentage of $CD8^+$ T-cells that can recognise a given epitope would also not allow the determination of the proportion of those cells that are potentially functional.

The proportion of tetramer-positive cells expressing the senescence marker CD57 was also measured and compared between the three groups. $CD57^+$ T-cell populations have been postulated to arise as a direct consequence of repeated bouts of T-cell proliferation, brought about by sporadic virus reactivation; this results in a decreased ability to proliferate in response to in vitro stimulation with cognate antigen in vitro, a decrease in telomere length, which would correlate with past expansions, and an increased cytolytic ability [384]. These cells are generally thought of as late-stage effector memory T-cells as they also tend to have a higher cytolytic ability.

Although the median percentages of $tetramer^+$ $CD8^+$ cells expressing CD57 were highest in CVID patients (median 79%), median percentages of the same population in healthy individuals (58%) were not significantly different ($p = 0.08$), this was also the case with viraemic renal transplant patients (median 55%, $p = 0.07$). CD57 levels were elevated in CD8 populations of CVID patients as a whole with a median of 54% compared to 31% in healthy individuals ($p = 0.03$), and 36% in viraemic transplant patients ($p = 0.03$). There was no significant differences between median levels of CD57 expression between the viraemic individuals and healthy controls ($p = 1$). Higher frequencies of these $CD57^+$ CD8 T-cells in the CVID group may have occurred as a direct consequence of repeated episodes of past virus and/or

bacterial replication which, although not symptomatic, may have resulted because of the lack of immunoglobulins. This may have led to larger expansions of end-stage populations necessary for control of the infection.

Analysis of intracellular perforin levels of $CD8^+$ T-cell populations also yielded similar results, in that there was an elevation in the percentages of tetramer-positive cells with intracellular perforin in CVID patients (median 90%) compared to healthy controls (median 77.5% $p = 0.06$) but not when compared with viraemic transplant patients (78% $p = 0.6$). These results were slightly altered when analysed in the context of overall CD8 populations; the median percentage of $CD8^+$ cells containing perforin in CVID patients was 62%, which was significantly higher than that found in healthy patients (34%, $p = 0.02$), although not significantly different from viraemic renal transplant patients (55%, $p = 3.4$).

Together these data, while looking at narrowly-focused $CD8^+$ T-cell subsets of all three groups, show that epitope-specific responses for HCMV tend to be of a late-stage, potentially-cytolytic, effector memory phenotype with the majority of cells expressing perforin and CD57. Levels of these markers are increased in epitope-specific $CD8^+$ T-cells in comparison to overall $CD8^+$ T-cell populations, indicating that HCMV does influence the type of $CD8^+$ T-cell responses it elicits. Tetramer-positive populations were significantly elevated in CVID patients when compared to healthy individuals and viraemic renal transplant patients. Intracellular levels of perforin were increase in the tetramer-positive populations of CVID patients when compared to healthy

controls but not when compared to viraemic patients and this phenotypic phenomenon was consistent when CD57 expression levels were analysed.

Although the high percentages HCMV-specific effector-memory $CD8^+$ T-cells in the peripheral blood may prevent viraemia in CVID patients, as none of the CVID patients were PCR-positive (data not shown), and thus circumvent the need for an autologous humoral response, these seem insufficient for controlling virus replication in individuals who become viraemic and retain normal B-cell responses. Previous chapters have demonstrated a decreased absolute number of virus-specific $CD8^+$ T-cells in viraemic individuals compared to non-viraemic individuals, indicating it there is a protective threshold of CD8 responses required for control of HCMV replication.

As a possible caveat for this, a number of CVID patients in this study suffered from an inflammatory colitis-like syndrome with frequent bloody diarrhoea similar to that seem in certain immunocompromised HCMV-infected individuals. Furthermore, biopsies examined showed the presence of HCMV particles in the gut tissues, interestingly these symptoms lessened on administration of ganciclovir. This possibly indicates that although large numbers of T-cells may control virus replication in the peripheral blood, the expression of virus transcripts in various tissues may be recognised by a powerful potentially cytolytic CD8 response which may destroy infected tissues preventing full virus replication whilst causing immunopathologies.

Chapter 7

Discussion

In this thesis longitudinal analyses of HCMV-specific T-cell responses were performed in twenty renal transplant patients of different donor/recipient serostatus for 6 months post-transplant (see Appendix). This study serves improved the understanding of the natural history of HCMV-specific T-cell response dynamics in the context of the renal transplant patient group. Moreover, it gives clues as to how the HCMV-specific $CD4^+$ and $CD8^+$ subpopulations relate to the incidences of HCMV replication, which themselves may correlate with the progression of HCMV-associated diseases. Previous studies have looked at HCMV-specific CD4 and CD8 populations although T-cell responses may have been misrepresented because they focused solely on percentages of IFNγ-producing T-cells rather than the absolute numbers of these cells in the peripheral blood [294, 332, 355, 357, 385-387].

The thesis then sought to discover whether HCMV CD8-escape mutants evolve in a highly selective environment, where high viral loads persist in certain renal transplant patients despite the presence of large epitope-specific CD8 responses. Finally, this work tried to elucidate whether the potential cytolytic phenotype and the magnitude of HCMV-specific $CD8^+$ populations helps to prevent disease by comparing epitope-specific CD8 populations in renal transplant patients suffering repeated bouts of HCMV viraemia, healthy adults and patients with CVID, who despite lacking an endogenous B cell response are able to control virus infections, presumably through their T-cell responses.

Chapter 3

Chapter 3 focused on how Th1-polarised $CD4^+$ T-cell responses against HCMV fluctuated in 20 renal transplant patients over the 6 months post-transplant. Results indicated that during the first six months post-transplant, functional HCMV-specific CD4-responses, as measured by their capacity to produce the Th1 cytokine IFNγ in response to HCMV antigens, rose gradually. This rise was significant in terms of both the percentage (Fig. 3.3) and absolute number (Fig 3.4) of circulating $CD4^+$ T-cells able to produce IFNγ following stimulation with virus lysate. There was an association between the magnitude of CD4 responses and the likelihood of this patient group to suffer episodes of HCMV viraemia (Fig. 3.8) and this compounds findings of Sester et al. [332] who stated that CD4 responses were crucial for the control of HCMV replication. CD4 responses increased three months post-transplant, when immunosuppressive therapies were reduced, and the rise between months 1 to 3 and months 4 to 6 was statistically significant (Fig. 3.9). All incidences of HCMV replication occurred during the first three months post-transplant (see appendix), this corresponds to previous findings which indicate show that HCMV reactivation usually occurs during the first three months post-transplant.

To determine whether the there was a link between the magnitude of HCMV-specific CD4 responses and CD8 responses, which would also be protective against HCMV-replication, a correlation between both the percentages and absolute numbers of $CD4^+$ T-cell responsive the HCMV lysate and the percentages and absolute numbers of $CD8^+$ T-cells responses to two

immunodominant targets, IE1 and pp65 was performed. No relationship between the percentages of HCMV-specific CD4 cells and CD8 cells could be detected (Fig. 3.10), yet there was a correlation between the absolute numbers of HCMV-specific $CD4^+$ T-cells and $CD8^+$ T-cells (Fig. 3.11). This indicates that CD4 responses are important in controlling virus replication either directly or indirectly, possibly by influencing the size of virus-specific CD8 responses which may be able to lyse virus-infected cells.

Chapter 4

The aim of chapter 4 was to further investigate the kinetics of the HCMV-specific CD8 response after renal transplantation and the role it plays in the control of HCMV replication. Similar to chapter 3, the dynamics of the $CD8^+$ responses in the 6 months post-transplant was measured, this time to IE1 and pp65, two targets that have previously been shown to be immunodominant in the HCMV-specific CD8 response. Of the twenty individuals included in this study only fifteen had $CD8^+$ responses to either antigen that were consistently above the detection threshold, fourteen had responses to the tegument phosphoprotein pp65, ten of which had responses to the immediate early protein IE1 and nine who had responses to both (Fig. 4.2). Moderate increases in both the percentages and absolute numbers of pp65 and/or IE1-specific $CD8^+$ T-cell responses occur over the six months post-transplant (Figs 4.6 and 4.12), and again, this increase follows the pattern of the CD4 populations where responses rise significantly after the third month post-transplant. To determine whether CD8 responses protected against a susceptibility to viraemia, frequencies of CD8 responses

to both IE1 and pp65 were combined and compared between individuals suffering viraemic episodes and those that remained free from episodes of HCMV replication in the six months following transplant. Both the percentages and the absolute numbers of combined pp65 and IE1-specific CD8 responses were shown to be significantly decreased in individuals who suffer viraemic episodes post-transplant; demonstrating that the magnitude of HCMV-specific IFNγ-producing $CD8^+$ T-cell responses can be protective against HCMV replication (Figs. 4.16-4.21). The frequencies of CD8 responses to IE1 and/or pp65 were also not significantly different to those of healthy controls (Figs, 4.3, 4.4 and 4.6); this is contrary to previous reports [355, 357] where viraemic post-transplant patients had increased HCMV-specific CD8 responses when compared to healthy controls, although these studies focused solely on HLA-restricted epitope-specific responses rather than more representative responses to entire antigens as shown here.

This discovery may help determine which patients are more susceptible to HCMV viraemia and therefore more predisposed to associated diseases after renal transplant and further allow antiviral therapy to focus on a high disease-risk group. Previous reports have described IE1-specific CD8 responses to be protective against HCMV disease in the first three months post lung and liver transplant [379]. In work presented here there was no association between the magnitude of IE1-specific responses alone and decreased incidences of viraemia (Figs. 4.23 and 4.24). However, since pre-emptive therapy was given to all patients with high level replication it is possible that while such responses were unable to control replication, they

may have been sufficient to reduce the peak viral loads attained in the absence of therapy and thereby prevent disease.

One of the individuals presented in this chapter had the highest frequencies of pp65 and IE1-specific CD8 cells and yet still suffered viraemic episodes. One explanation for this may be that this same individual had a decreased amount of "help" as would normally be provided by virus-specific CD4+ cells. Janssen and colleagues previously demonstrated that antigen-specific CD8+ populations we more susceptible to trail-mediated apoptosis in the absence of helper CD4 responses [388].

One other explanation for this may be that CD8 responses are "helpless" [388] this occurs when CD8 responses occur in the absence of helper CD4 responses and would result in possible apoptosis of antigen-stimulated virus-specific CD8 cells. More recently it has been shown that during chronic virus replication T-cell apoptosis is be driven through expression of PD-1 (programmed death 1) on the surfaces of repeatedly-exposed ,exhausted $CD8^+$ cells [389].

Chapter 5

Although a link was found between the decreased frequencies of pp65 and IE1-specific $CD8^+$ T-cells and an increase in the incidence of HCMV viraemia, certain patients suffering viraemic episodes did so in spite of large $CD8^+$ T-cell responses. Chapter 5 addressed the question of whether mutations within CTL epitopes may be able to explain this phenomenon.

DNA isolated from the peripheral blood of four individuals who suffered prolonged episodes of HCMV viraemia despite the presence of detectable tetramer-specific $CD8^+$ responses, was analysed for the presence of CTL escape mutants. No mutations were found within the HLA-restricted epitopes of these individuals nor were they found in sequences from individuals with acute-phase high-level viraemia (Table 5.1). This might indicate that mutations either do not arise in these epitopes, or that mutant populations do not make up the bulk of the HCMV populations or that the CTL populations directed against the epitope are insufficient for control of virus replication. Also there is the possibility that transient mutants arise in the virus population, which allow restricted escape but are not maintained [390]. Although a mutation in this epitope has previously been described [358] sequence amplification was performed using a non-proof-reading polymerase which may have introduced artificial mutants [358]. Because the possibility remained that a subpopulation of HCMV in these individuals contained mutant epitopes that would allow virus persistence through being able to circumvent the CTL responses, a series of mutations in the immunodominant HLA-A0201 epitope NLVPMVATV were synthesised (Fig. 5.2) and analysed in their capacity to elicit immune responses, as measured by IFNγ production, and to stabilise MHC class I molecules.

ELISPOT assays using the both the wt and variant epitopes demonstrated that the wt peptide was best capable of eliciting IFNγ production when used to stimulate PBMCs isolated from HLA-A0201-restricted healthy HCMV seropositive individuals (Fig. 5.4). Variants with mutations around the anchor

regions were also capable of eliciting IFNγ production, albeit to a lower level than the wt peptide, and were generally better recognised by epitope-specific CTLs than variants with mutations within the core of the peptide. Using the peptides to stabilise MHC I molecules on TAP-deficient T2 cells it was demonstrated that high avidity interactions with MHC I molecules were not essential for recognition by the TCR of epitope-specific $CD8^+$ T-cells (Fig. 5.5). Interestingly the C5M variant peptide was better capable of stabilising cell-surface MHC I molecules whilst simultaneously being unable to elicit IFNγ production. The possible emergence of such a mutant in the HCMV population would enable CTL evasion whilst not rendering infected cells susceptible to NK-mediated lysis [391].

Chapter 6

Because no CTL-escape mutants could be detected in patients with high viral loads in spite of large focused CTL responses chapter 6 sought to establish whether a functional impairment could be ascribed to the inability of $CD8^+$ T-cell responses to control virus replication. This chapter focused on a patient group who usually resolve most virus infections asymptomatically; these individuals suffer from CVID, which renders them unable to produce normal levels of immunoglobulins. Although this makes them more susceptible to bacterial infections, which can be overcome through infusions of polyclonal antibodies, this defect does not appear to have any noticeable effect on their capacity to control virus infections. The HCMV serostatus of these patients could obviously not be defined unless it was known prior to the manifestation of any CVID-associated symptoms, therefore the capacity

of their T-cell responses towards HCMV was measured using FACS analysis. The majority of these individuals were found to have high frequencies of HCMV-specific CD4 and CD8 cell populations indicative of prior infection. Furthermore, their CD8 responses to HCMV antigens pp65 and IE1 were significantly increased when compared to healthy age-matched controls (Figs. 6.2 and 6.3). The magnitude of these responses could then give a clue as to the mechanism by which they are able to control HCMV replication.

When epitope-specific subpopulations of the CD8 response were compared between CVID patients, healthy age-matched controls and viraemic renal transplant patients, epitope-specific responses were also elevated in CVID compared to the other groups. Phenotypic analyses of these populations showed that, of the tetramer$^+$ populations there was an elevation in the percentage of these cells that were CD57 positive in the CVID patients, although this was not significant. However, looking at overall CD8$^+$ populations there was a significant increase in the percentages of CD8$^+$ cells expressing CD57 when compared to those found in both healthy age-matched controls and viraemic renal transplant patients. Expression of the activation marker CD57 has previously been shown to be associated with a late-stage, replicatively-senescent, cytotoxic CD8$^+$ phenotype, therefore the percentages of these same populations containing the cytolytic granule perforin were compared [392]. In the epitope-specific population levels of intracellular perforin were highest in the CVID patients although this was again not a significant increase compared to the other two groups (Fig. 6.13).

However, perforin levels were significantly increased in CVID $CD8^+$ populations as a whole when compared to healthy age-matched controls. Interestingly, the perforin levels in viraemic renal transplant patients were also significantly higher than those of healthy individuals (Fig. 6.13); this suggests that cytolytic potential alone is not protective alone should the magnitude of the response be insufficient. The fact that, in terms of percentages of the overall $CD8^+$ T-cell populations, the healthy age-matched controls had phenotypically similar epitope-specific responses to the viraemic transplant patients indicates that any defect in the viraemic individuals may lie in the absolute number of these cells in circulation or in their functional capacity.

One caveat arising from the assumption that large virus-specific, potentially-cytolytic CTL responses are capable of controlling HCMV replication comes from an interesting phenomenon demonstrated in the CVID patients. Many individuals with CVID suffer a variety of inflammatory disorders and a large proportion of these endure a colitis-like syndrome, a similar phenomenon has been described as an HCMV-associated disease. Biopsies taken from the gut of several CVID patients at the Royal Free Hospital have shown the presence of HCMV particles in the gut issue along with large T-cell infiltrate, despite none of these patients having HCMV DNAemia. Furthermore, the symptoms displayed in these patients have been shown to ameliorate on administration of ganciclovir. It can therefore be speculated that the large infiltrates of HCMV-specific $CD8^+$ cells in these individuals, expressing higher than normal levels if perforin and CD57 may home to the gut tissue in

response to HCMV replication. The resultant cytolytic response may subsequently cause the destruction of the gut epithelium, which in turn leads to gut inflammation. Further work could therefore focus on the role that HCMV-driven immunopathology has in certain inflammatory diseases and whether the magnitude and the phenotype of such a response has any bearing on symptoms.

References

1. Plummer, G., *Cytomegaloviruses of man and animals.* Prog Med Virol, 1973. **15**: p. 92-125.
2. Weller, T.H., J.B. Hanshaw, and D.E. Scott, *Serologic differentiation of viruses responsible for cytomegalic inclusion disease.* Virology, 1960. **12**: p. 130-2.
3. Weller, T.H., *The cytomegaloviruses: ubiquitous agents with protean clinical manifestations. I.* N Engl J Med, 1971. **285**(4): p. 203-14.
4. Mocarski, E.C., C., *Cytomegaloviruses and their replication.* Lippincott, Williams and Wilkins, Philadelphia, Pa., 2001: p. 2629-2673.
5. McGavran, M.H. and M.G. Smith, *Ultrastructural, Cytochemical, And Microchemical Observations On Cytomegalovirus (Salivary Gland Virus) Infection Of Human Cells In Tissue Culture.* Exp Mol Pathol, 1965. **76**: p. 1-10.
6. Wright, H.T., Jr., C.R. Goodheart, and A. Lielausis, *Human Cytomegalovirus. Morphology By Negative Staining.* Virology, 1964. **23**: p. 419-24.
7. Emery, V.C. and P.D. Griffiths, *Molecular biology of cytomegalovirus.* Int J Exp Pathol, 1990. **71**(6): p. 905-18.
8. Sarov, I. and I. Abady, *The morphogenesis of human cytomegalovirus. Isolation and polypeptide characterization of cytomegalovirions and dense bodies.* Virology, 1975. **66**(2): p. 464-73.
9. Compton, T., D.M. Nowlin, and N.R. Cooper, *Initiation of human cytomegalovirus infection requires initial interaction with cell surface heparan sulfate.* Virology, 1993. **193**(2): p. 834-41.

10. Adlish, J.D., R.S. Lahijani, and S.C. St Jeor, *Identification of a putative cell receptor for human cytomegalovirus.* Virology, 1990. **176**(2): p. 337-45.

11. Wang, X., et al., *Epidermal growth factor receptor is a cellular receptor for human cytomegalovirus.* Nature, 2003. **424**(6947): p. 456-61.

12. Compton, T., *Receptors and immune sensors: the complex entry path of human cytomegalovirus.* Trends Cell Biol, 2004. **14**(1): p. 5-8.

13. Ewald, J.A., et al., *Ligand- and kinase activity-independent cell survival mediated by the epidermal growth factor receptor expressed in 32D cells.* Exp Cell Res, 2003. **282**(2): p. 121-31.

14. Compton, T., et al., *Human cytomegalovirus activates inflammatory cytokine responses via CD14 and Toll-like receptor 2.* J Virol, 2003. **77**(8): p. 4588-96.

15. Compton, T., R.R. Nepomuceno, and D.M. Nowlin, *Human cytomegalovirus penetrates host cells by pH-independent fusion at the cell surface.* Virology, 1992. **191**(1): p. 387-95.

16. Dal Monte, P., et al., *Expression of human cytomegalovirus ppUL83 (pp65) in a stable cell line and its association with metaphase chromosomes.* J Gen Virol, 1996. **77 (Pt 10)**: p. 2591-6.

17. Kilpatrick, B.A. and E.S. Huang, *Human cytomegalovirus genome: partial denaturation map and organization of genome sequences.* J Virol, 1977. **24**(1): p. 261-76.

18. Hayward, G.S., et al., *Anatomy of herpes simplex virus DNA: evidence for four populations of molecules that differ in the relative*

orientations of their long and short components. Proc Natl Acad Sci U S A, 1975. **72**(11): p. 4243-7.

19. Wilkie, N.M. and R. Cortini, *Sequence arrangement in herpes simplex virus type 1 DNA: identification of terminal fragments in restriction endonuclease digests and evidence for inversions in redundant and unique sequences.* J Virol, 1976. **20**(1): p. 211-21.
20. Kemble, G.W. and E.S. Mocarski, *A host cell protein binds to a highly conserved sequence element (pac-2) within the cytomegalovirus a sequence.* J Virol, 1989. **63**(11): p. 4715-28.
21. Emery, V.C., *Viral dynamics during active cytomegalovirus infection and pathology.* Intervirology, 1999. **42**(5-6): p. 405-11.
22. Wathen, M.W. and M.F. Stinski, *Temporal patterns of human cytomegalovirus transcription: mapping the viral RNAs synthesized at immediate early, early, and late times after infection.* J Virol, 1982. **41**(2): p. 462-77.
23. Wathen, M.W., D.R. Thomsen, and M.F. Stinski, *Temporal regulation of human cytomegalovirus transcription at immediate early and early times after infection.* J Virol, 1981. **38**(2): p. 446-59.
24. DeMarchi, J.M., C.A. Schmidt, and A.S. Kaplan, *Patterns of transcription of human cytomegalovirus in permissively infected cells.* J Virol, 1980. **35**(2): p. 277-86.
25. Tenney, D.J. and A.M. Colberg-Poley, *Human cytomegalovirus UL36-38 and US3 immediate-early genes: temporally regulated expression of nuclear, cytoplasmic, and polysome-associated transcripts during infection.* J Virol, 1991. **65**(12): p. 6724-34.

26. Winkler, M., S.A. Rice, and T. Stamminger, *UL69 of human cytomegalovirus, an open reading frame with homology to ICP27 of herpes simplex virus, encodes a transactivator of gene expression.* J Virol, 1994. **68**(6): p. 3943-54.

27. Stenberg, R.M., et al., *Promoter-specific trans activation and repression by human cytomegalovirus immediate-early proteins involves common and unique protein domains.* J Virol, 1990. **64**(4): p. 1556-65.

28. Mocarski, E.S., et al., *A deletion mutant in the human cytomegalovirus gene encoding IE1(491aa) is replication defective due to a failure in autoregulation.* Proc Natl Acad Sci U S A, 1996. **93**(21): p. 11321-6.

29. Greaves, R.F. and E.S. Mocarski, *Defective growth correlates with reduced accumulation of a viral DNA replication protein after low-multiplicity infection by a human cytomegalovirus ie1 mutant.* J Virol, 1998. **72**(1): p. 366-79.

30. Malone, C.L., D.H. Vesole, and M.F. Stinski, *Transactivation of a human cytomegalovirus early promoter by gene products from the immediate-early gene IE2 and augmentation by IE1: mutational analysis of the viral proteins.* J Virol, 1990. **64**(4): p. 1498-506.

31. Schwartz, R., B. Helmich, and D.H. Spector, *CREB and CREB-binding proteins play an important role in the IE2 86-kilodalton protein-mediated transactivation of the human cytomegalovirus 2.2-kilobase RNA promoter.* J Virol, 1996. **70**(10): p. 6955-66.

32. Stenberg, R.M., *The human cytomegalovirus major immediate-early gene.* Intervirology, 1996. **39**(5-6): p. 343-9.

33. Jupp, R., et al., *Human cytomegalovirus IE86 protein interacts with promoter-bound TATA-binding protein via a specific region distinct from the autorepression domain.* J Virol, 1993. **67**(12): p. 7539-46.

34. Gebert, S., et al., *The UL84 protein of human cytomegalovirus acts as a transdominant inhibitor of immediate-early-mediated transactivation that is able to prevent viral replication.* J Virol, 1997. **71**(9): p. 7048-60.

35. Kerry, J.A., et al., *Multiple regulatory events influence human cytomegalovirus DNA polymerase (UL54) expression during viral infection.* J Virol, 1996. **70**(1): p. 373-82.

36. St Jeor, S.C. and R. Hutt, *Cell DNA replication as a function in the synthesis of human cytomegalovirus.* J Gen Virol, 1977. **37**(1): p. 65-73.

37. Lehman, I.R. and P.E. Boehmer, *Replication of herpes simplex virus DNA.* J Biol Chem, 1999. **274**(40): p. 28059-62.

38. Courcelle, C.T., et al., *Requirement for uracil-DNA glycosylase during the transition to late-phase cytomegalovirus DNA replication.* J Virol, 2001. **75**(16): p. 7592-601.

39. Bolovan-Fritts, C.A., E.S. Mocarski, and J.A. Wiedeman, *Peripheral blood CD14(+) cells from healthy subjects carry a circular conformation of latent cytomegalovirus genome.* Blood, 1999. **93**(1): p. 394-8.

40. Anders, D.G. and S.M. Punturieri, *Multicomponent origin of cytomegalovirus lytic-phase DNA replication.* J Virol, 1991. **65**(2): p. 931-7.

41. Spector, D.J. and M.J. Tevethia, *Protein-protein interactions between human cytomegalovirus IE2-580aa and pUL84 in lytically infected cells.* J Virol, 1994. **68**(11): p. 7549-53.

42. Pari, G.S. and D.G. Anders, *Eleven loci encoding trans-acting factors are required for transient complementation of human cytomegalovirus oriLyt-dependent DNA replication.* J Virol, 1993. **67**(12): p. 6979-88.

43. Prichard, M.N., et al., *Human cytomegalovirus uracil DNA glycosylase associates with ppUL44 and accelerates the accumulation of viral DNA.* Virol J, 2005. **2**: p. 55.

44. Anders, D.G. and L.A. McCue, *The human cytomegalovirus genes and proteins required for DNA synthesis.* Intervirology, 1996. **39**(5-6): p. 378-88.

45. Penfold, M.E. and E.S. Mocarski, *Formation of cytomegalovirus DNA replication compartments defined by localization of viral proteins and DNA synthesis.* Virology, 1997. **239**(1): p. 46-61.

46. Anders, D.G., et al., *Boundaries and structure of human cytomegalovirus oriLyt, a complex origin for lytic-phase DNA replication.* J Virol, 1992. **66**(6): p. 3373-84.

47. Bogner, E., et al., *Identification of the gene product encoded by ORF UL56 of the human cytomegalovirus genome.* Virology, 1993. **196**(1): p. 290-3.

48. Spaete, R.R. and E.S. Mocarski, *The alpha sequence of the cytomegalovirus genome functions as a cleavage/packaging signal for herpes simplex virus defective genomes.* J Virol, 1985. **54**(3): p. 817-24.

49. Scheffczik, H., et al., *The terminase subunits pUL56 and pUL89 of human cytomegalovirus are DNA-metabolizing proteins with toroidal structure.* Nucleic Acids Res, 2002. **30**(7): p. 1695-703.

50. Krosky, P.M., et al., *Resistance of human cytomegalovirus to benzimidazole ribonucleosides maps to two open reading frames: UL89 and UL56.* J Virol, 1998. **72**(6): p. 4721-8.

51. Bogner, E., K. Radsak, and M.F. Stinski, *The gene product of human cytomegalovirus open reading frame UL56 binds the pac motif and has specific nuclease activity.* J Virol, 1998. **72**(3): p. 2259-64.

52. Scholz, B., et al., *Identification of the ATP-binding site in the terminase subunit pUL56 of human cytomegalovirus.* Nucleic Acids Res, 2003. **31**(5): p. 1426-33.

53. Hwang, J.S. and E. Bogner, *ATPase activity of the terminase subunit pUL56 of human cytomegalovirus.* J Biol Chem, 2002. **277**(9): p. 6943-8.

54. Dittmer, A. and E. Bogner, *Analysis of the quaternary structure of the putative HCMV portal protein PUL104.* Biochemistry, 2005. **44**(2): p. 759-65.

55. McVoy, M.A., et al., *The ends on herpesvirus DNA replicative concatemers contain pac2 cis cleavage/packaging elements and their formation is controlled by terminal cis sequences.* J Virol, 2000. **74**(3): p. 1587-92.

56. McVoy, M.A., et al., *Sequences within the herpesvirus-conserved pac1 and pac2 motifs are required for cleavage and packaging of the murine cytomegalovirus genome.* J Virol, 1998. **72**(1): p. 48-56.

57. Chee, M., et al., *Identification of the major capsid protein gene of human cytomegalovirus.* J Virol, 1989. **63**(3): p. 1345-53.

58. Wood, L.J., et al., *Human cytomegalovirus capsid assembly protein precursor (pUL80.5) interacts with itself and with the major capsid protein (pUL86) through two different domains.* J Virol, 1997. **71**(1): p. 179-90.

59. Plafker, S.M. and W. Gibson, *Cytomegalovirus assembly protein precursor and proteinase precursor contain two nuclear localization signals that mediate their own nuclear translocation and that of the major capsid protein.* J Virol, 1998. **72**(10): p. 7722-32.

60. Gibson, W., et al., *Human cytomegalovirus (HCMV) smallest capsid protein identified as product of short open reading frame located between HCMV UL48 and UL49.* J Virol, 1996. **70**(8): p. 5680-3.

61. Lai, L. and W.J. Britt, *The interaction between the major capsid protein and the smallest capsid protein of human cytomegalovirus is dependent on two linear sequences in the smallest capsid protein.* J Virol, 2003. **77**(4): p. 2730-5.

62. Wolf, D.G., et al., *Distinct and separate roles for herpesvirus-conserved UL97 kinase in cytomegalovirus DNA synthesis and encapsidation.* Proc Natl Acad Sci U S A, 2001. **98**(4): p. 1895-900.

63. Marschall, M., et al., *Cellular p32 recruits cytomegalovirus kinase pUL97 to redistribute the nuclear lamina.* J Biol Chem, 2005. **280**(39): p. 33357-67.

64. Sanchez, V., et al., *Accumulation of virion tegument and envelope proteins in a stable cytoplasmic compartment during human*

cytomegalovirus replication: characterization of a potential site of virus assembly. J Virol, 2000. **74**(2): p. 975-86.

65. Homman-Loudiyi, M., et al., *Envelopment of human cytomegalovirus occurs by budding into Golgi-derived vacuole compartments positive for gB, Rab 3, trans-golgi network 46, and mannosidase II.* J Virol, 2003. **77**(5): p. 3191-203.
66. Eggers, M., et al., *Inhibition of human cytomegalovirus maturation by brefeldin A.* J Gen Virol, 1992. **73 (Pt 10)**: p. 2679-92.
67. Tooze, J., et al., *Progeny vaccinia and human cytomegalovirus particles utilize early endosomal cisternae for their envelopes.* Eur J Cell Biol, 1993. **60**(1): p. 163-78.
68. Soderberg-Naucler, C., K.N. Fish, and J.A. Nelson, *Growth of human cytomegalovirus in primary macrophages.* Methods, 1998. **16**(1): p. 126-38.
69. Britt, W.J.A., C.A., *Cytomegalovirus, in Fields Virology.* Lippincott, Williams and Wilkins, Philadelphia, Pa., 1996: p. 2495-2523.
70. Pass, R., *Cytomegalovirus, in Fields Virology.* Lippincott, Williams and Wilkins, Philadelphia, Pa., 2001: p. 2675-2685.
71. Stagno, S., et al., *Factors associated with primary cytomegalovirus infection during pregnancy.* J Med Virol, 1984. **13**(4): p. 347-53.
72. Numazaki, K., *Human cytomegalovirus infection of breast milk.* FEMS Immunol Med Microbiol, 1997. **18**(2): p. 91-8.
73. Stagno, S., et al., *Maternal cytomegalovirus infection and perinatal transmission.* Clin Obstet Gynecol, 1982. **25**(3): p. 563-76.

74. Dworsky, M., et al., *Cytomegalovirus infection of breast milk and transmission in infancy.* Pediatrics, 1983. **72**(3): p. 295-9.

75. Preece, P.M., K.N. Pearl, and C.S. Peckham, *Congenital cytomegalovirus infection.* Arch Dis Child, 1984. **59**(12): p. 1120-6.

76. Adler, S.P., *The molecular epidemiology of cytomegalovirus transmission among children attending a day care center.* J Infect Dis, 1985. **152**(4): p. 760-8.

77. Reddehase, M.J., *Antigens and immunoevasins: opponents in cytomegalovirus immune surveillance.* Nat Rev Immunol, 2002. **2**(11): p. 831-44.

78. Pass, R.F., et al., *Increased frequency of cytomegalovirus infection in children in group day care.* Pediatrics, 1984. **74**(1): p. 121-6.

79. Pass, R.F., et al., *Cytomegalovirus infection in day-care center.* N Engl J Med, 1982. **307**(8): p. 477-9.

80. Kumar, M.L., et al., *Primary cytomegalovirus infection in adolescent pregnancy.* Pediatrics, 1984. **74**(4): p. 493-500.

81. Jordan, M.C., et al., *Spontaneous cytomegalovirus mononucleosis. Clinical and laboratory observations in nine cases.* Ann Intern Med, 1973. **79**(2): p. 153-60.

82. Griffiths, P., *Chapter 1.iii Cytomegalovirus.* Principles and Practice of Clinical Virology (2nd Edition), 1990: p. 69-102.

83. Toro, A.I. and J. Ossa, *PCR activity of CMV in healthy CMV-seropositive individuals: does latency need redefinition?* Res Virol, 1996. **147**(4): p. 233-8.

84. Mendelson, M., et al., *Detection of endogenous human cytomegalovirus in CD34+ bone marrow progenitors.* J Gen Virol, 1996. **77 (Pt 12)**: p. 3099-102.

85. Kondo, K., J. Xu, and E.S. Mocarski, *Human cytomegalovirus latent gene expression in granulocyte-macrophage progenitors in culture and in seropositive individuals.* Proc Natl Acad Sci U S A, 1996. **93**(20): p. 11137-42.

86. Taylor-Wiedeman, J., et al., *Monocytes are a major site of persistence of human cytomegalovirus in peripheral blood mononuclear cells.* J Gen Virol, 1991. **72 (Pt 9)**: p. 2059-64.

87. Hahn, G., R. Jores, and E.S. Mocarski, *Cytomegalovirus remains latent in a common precursor of dendritic and myeloid cells.* Proc Natl Acad Sci U S A, 1998. **95**(7): p. 3937-42.

88. Soderberg-Naucler, C., K.N. Fish, and J.A. Nelson, *Reactivation of latent human cytomegalovirus by allogeneic stimulation of blood cells from healthy donors.* Cell, 1997. **91**(1): p. 119-26.

89. Soderberg-Naucler, C., et al., *Reactivation of latent human cytomegalovirus in CD14(+) monocytes is differentiation dependent.* J Virol, 2001. **75**(16): p. 7543-54.

90. Soderberg-Naucler, C., K.N. Fish, and J.A. Nelson, *Interferon-gamma and tumor necrosis factor-alpha specifically induce formation of cytomegalovirus-permissive monocyte-derived macrophages that are refractory to the antiviral activity of these cytokines.* J Clin Invest, 1997. **100**(12): p. 3154-63.

91. Hanson, L.K., et al., *Replication of murine cytomegalovirus in differentiated macrophages as a determinant of viral pathogenesis.* J Virol, 1999. **73**(7): p. 5970-80.

92. Fish, K.N., et al., *Growth kinetics of human cytomegalovirus are altered in monocyte-derived macrophages.* J Virol, 1995. **69**(6): p. 3737-43.

93. Ibanez, C.E., et al., *Human cytomegalovirus productively infects primary differentiated macrophages.* J Virol, 1991. **65**(12): p. 6581-8.

94. Sinclair, J.H., et al., *Repression of human cytomegalovirus major immediate early gene expression in a monocytic cell line.* J Gen Virol, 1992. **73 (Pt 2)**: p. 433-5.

95. Stein, J., et al., *Tumour necrosis factor alpha stimulates the activity of the human cytomegalovirus major immediate early enhancer/promoter in immature monocytic cells.* J Gen Virol, 1993. **74 (Pt 11)**: p. 2333-8.

96. Murphy, J.C., et al., *Control of cytomegalovirus lytic gene expression by histone acetylation.* Embo J, 2002. **21**(5): p. 1112-20.

97. Scholz, M., H.W. Doerr, and J. Cinatl, *Human cytomegalovirus retinitis: pathogenicity, immune evasion and persistence.* Trends Microbiol, 2003. **11**(4): p. 171-8.

98. Poland, S.D., et al., *The extent of human cytomegalovirus replication in primary neurons is dependent on host cell differentiation.* J Infect Dis, 1994. **170**(5): p. 1267-71.

99. Fish, K.N., W. Britt, and J.A. Nelson, *A novel mechanism for persistence of human cytomegalovirus in macrophages.* J Virol, 1996. **70**(3): p. 1855-62.

100. Medawar, P.B., *Immunity to homologous grafted skin. III. The fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye.* British Journal of Experimental Pathology, 1948. **29**: p. 58-69.

101. Betts, R.F., et al., *Clinical manifestations of renal allograft derived primary cytomegalovirus infection.* Am J Dis Child, 1977. **131**(7): p. 759-63.

102. Chou, S.W., *Acquisition of donor strains of cytomegalovirus by renal-transplant recipients.* N Engl J Med, 1986. **314**(22): p. 1418-23.

103. Koffron, A., et al., *Immunosuppression is not required for reactivation of latent murine cytomegalovirus.* Transplant Proc, 1999. **31**(1-2): p. 1395-6.

104. Fietze, E., et al., *Cytomegalovirus infection in transplant recipients. The role of tumor necrosis factor.* Transplantation, 1994. **58**(6): p. 675-80.

105. Rubin, R.H., *Impact of cytomegalovirus infection on organ transplant recipients.* Rev Infect Dis, 1990. **12 Suppl 7**: p. S754-66.

106. Cope, A.V., et al., *Quantity of cytomegalovirus viruria is a major risk factor for cytomegalovirus disease after renal transplantation.* J Med Virol, 1997. **52**(2): p. 200-5.

107. Hassan-Walker, A.F., et al., *Quantity of human cytomegalovirus (CMV) DNAemia as a risk factor for CMV disease in renal allograft recipients: relationship with donor/recipient CMV serostatus, receipt of augmented methylprednisolone and antithymocyte globulin (ATG).* J Med Virol, 1999. **58**(2): p. 182-7.

108. Cope, A.V., et al., *Interrelationships among quantity of human cytomegalovirus (HCMV) DNA in blood, donor-recipient serostatus, and administration of methylprednisolone as risk factors for HCMV disease following liver transplantation.* J Infect Dis, 1997. **176**(6): p. 1484-90.

109. Grundy, J.E., et al., *Symptomatic cytomegalovirus infection in seropositive kidney recipients: reinfection with donor virus rather than reactivation of recipient virus.* Lancet, 1988. **2**(8603): p. 132-5.

110. Chou, S.W., *Reactivation and recombination of multiple cytomegalovirus strains from individual organ donors.* J Infect Dis, 1989. **160**(1): p. 11-5.

111. Balfour, H.H., Jr., et al., *A randomized, placebo-controlled trial of oral acyclovir for the prevention of cytomegalovirus disease in recipients of renal allografts.* N Engl J Med, 1989. **320**(21): p. 1381-7.

112. Fishman, J.A. and R.H. Rubin, *Infection in organ-transplant recipients.* N Engl J Med, 1998. **338**(24): p. 1741-51.

113. Kotton, C.N. and J.A. Fishman, *Viral infection in the renal transplant recipient.* J Am Soc Nephrol, 2005. **16**(6): p. 1758-74.

114. Lowance, D., et al., *Valacyclovir for the prevention of cytomegalovirus disease after renal transplantation. International Valacyclovir Cytomegalovirus Prophylaxis Transplantation Study Group.* N Engl J Med, 1999. **340**(19): p. 1462-70.

115. Rubin, R.H., *Cytomegalovirus in solid organ transplantation.* Transpl Infect Dis, 2001. **3 Suppl 2**: p. 1-5.

116. Fernando, S., et al., *Association of cytomegalovirus infection with post-transplantation cardiac rejection as studied using the polymerase chain reaction.* J Med Virol, 1994. **42**(4): p. 396-404.

117. Lautenschlager, I., et al., *Cytomegalovirus DNA persists in various structures of liver allografts with chronic rejection.* Transplant Proc, 1995. **27**(6): p. 3513-4.

118. Craigen, J.L. and J.E. Grundy, *Cytomegalovirus induced up-regulation of LFA-3 (CD58) and ICAM-1 (CD54) is a direct viral effect that is not prevented by ganciclovir or foscarnet treatment.* Transplantation, 1996. **62**(8): p. 1102-8.

119. Carlquist, J.F., et al., *Cytomegalovirus induction of interleukin-6 in lung fibroblasts occurs independently of active infection and involves a G protein and the transcription factor, NF-kappaB.* J Infect Dis, 1999. **179**(5): p. 1094-100.

120. Visseren, F.L., et al., *Interleukin-6 production by endothelial cells after infection with influenza virus and cytomegalovirus.* J Lab Clin Med, 1999. **134**(6): p. 623-30.

121. Cinatl, J., Jr., et al., *Human cytomegalovirus circumvents NF-kappa B dependence in retinal pigment epithelial cells.* J Immunol, 2001. **167**(4): p. 1900-8.

122. Cinatl, J., Jr., et al., *Decreased neutrophil adhesion to human cytomegalovirus-infected retinal pigment epithelial cells is mediated by virus-induced up-regulation of Fas ligand independent of neutrophil apoptosis.* J Immunol, 2000. **165**(8): p. 4405-13.

123. Chiou, S.H., et al., *Up-regulation of Fas ligand expression by human cytomegalovirus immediate-early gene product 2: a novel mechanism in cytomegalovirus-induced apoptosis in human retina.* J Immunol, 2001. **167**(7): p. 4098-103.

124. Milburn, H.J., et al., *Pneumonitis in bone marrow transplant recipients results from a local immune response.* Clin Exp Immunol, 1990. **81**(2): p. 232-7.

125. Barry, S.M., Johnson, M.A., Janossy, G, *Cytopathology or immunopathology? The puzzle of cytomegalovirus pneumonitis revisited.* Bone Marrow Transplant, 2000. **26**(6): p. 591-7.

126. Shacklett, B.L., et al., *Isolation of cytomegalovirus-specific cytotoxic T-lymphocytes from gut-associated lymphoid tissue (GALT) of HIV type 1-infected subjects.* AIDS Res Hum Retroviruses, 2000. **16**(12): p. 1157-62.

127. Borel, J.F., et al., *Effects of the new anti-lymphocytic peptide cyclosporin A in animals.* Immunology, 1977. **32**(6): p. 1017-25.

128. Liu, J., et al., *Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes.* Cell, 1991. **66**(4): p. 807-15.

129. Shibasaki, F., et al., *Role of kinases and the phosphatase calcineurin in the nuclear shuttling of transcription factor NF-AT4.* Nature, 1996. **382**(6589): p. 370-3.

130. Takatsuka, H., et al., *Intestinal graft-versus-host disease: mechanisms and management.* Drugs, 2003. **63**(1): p. 1-15.

131. O'Keefe, S.J., et al., *FK-506- and CsA-sensitive activation of the interleukin-2 promoter by calcineurin.* Nature, 1992. **357**(6380): p. 692-4.

132. Borel, J.F., *Mechanism of action of cyclosporin A and rationale for use in nephrotic syndrome.* Clin Nephrol, 1991. **35 Suppl 1**: p. S23-30.

133. Fruman DA, K.C., BIERER BE, BURAKOFF SJ, *Calcineurin phosphatase activity in T lymphocytes is inhibited by FK 506 and cyclosporin A.* Proc. Nati. Acad. Sci. USA, 1992. **89**: p. 3686-3690.

134. Schreiber SL, C.G., *The mechanism of action of cyclosporin A and FK506.* Immunol Today, 1992. **13**: p. 136-42.

135. Kino, T., et al., *Effect of FK-506 on human mixed lymphocyte reaction in vitro.* Transplant Proc, 1987. **19**(5 Suppl 6): p. 36-9.

136. Peters, D.H., et al., *Tacrolimus. A review of its pharmacology, and therapeutic potential in hepatic and renal transplantation.* Drugs, 1993. **46**(4): p. 746-94.

137. Carr, S.F., et al., *Characterization of human type I and type II IMP dehydrogenases.* J Biol Chem, 1993. **268**(36): p. 27286-90.

138. Allison, A.C. and E.M. Eugui, *Inhibitors of de novo purine and pyrimidine synthesis as immunosuppressive drugs.* Transplant Proc, 1993. **25**(3 Suppl 2): p. 8-18.

139. Allison, A.C., et al., *Mechanisms of action of mycophenolic acid.* Ann N Y Acad Sci, 1993. **696**: p. 63-87.

140. Franklin, T.J. and J.M. Cook, *The inhibition of nucleic acid synthesis by mycophenolic acid.* Biochem J, 1969. **113**(3): p. 515-24.

141. Allison, A.C. and E.M. Eugui, *Mycophenolate mofetil and its mechanisms of action.* Immunopharmacology, 2000. **47**(2-3): p. 85-118.

142. Allison, A.C. and E.M. Eugui, *Mechanisms of action of mycophenolate mofetil in preventing acute and chronic allograft rejection.* Transplantation, 2005. **80**(2 Suppl): p. S181-90.

143. Bamberger, C.M., H.M. Schulte, and G.P. Chrousos, *Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids.* Endocr Rev, 1996. **17**(3): p. 245-61.

144. Pratt, W.B. and D.O. Toft, *Steroid receptor interactions with heat shock protein and immunophilin chaperones.* Endocr Rev, 1997. **18**(3): p. 306-60.

145. Yang-Yen, H.F., et al., *Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction.* Cell, 1990. **62**(6): p. 1205-15.

146. Ray, A. and K.E. Prefontaine, *Physical association and functional antagonism between the p65 subunit of transcription factor NF-kappa B and the glucocorticoid receptor.* Proc Natl Acad Sci U S A, 1994. **91**(2): p. 752-6.

147. Gane, E., et al., *Randomised trial of efficacy and safety of oral ganciclovir in the prevention of cytomegalovirus disease in liver-transplant recipients. The Oral Ganciclovir International Transplantation Study Group [corrected].* Lancet, 1997. **350**(9093): p. 1729-33.

148. Paya, C., et al., *Efficacy and safety of valganciclovir vs. oral ganciclovir for prevention of cytomegalovirus disease in solid organ transplant recipients.* Am J Transplant, 2004. **4**(4): p. 611-20.

149. Razonable, R.R., et al., *Herpesvirus infections in solid organ transplant patients at high risk of primary cytomegalovirus disease.* J Infect Dis, 2005. **192**(8): p. 1331-9.

150. Hart, G.D. and C.V. Paya, *Prophylaxis for CMV should now replace pre-emptive therapy in solid organ transplantation.* Rev Med Virol, 2001. **11**(2): p. 73-81.

151. Li, C.R., et al., *Recovery of HLA-restricted cytomegalovirus (CMV)-specific T-cell responses after allogeneic bone marrow transplant: correlation with CMV disease and effect of ganciclovir prophylaxis.* Blood, 1994. **83**(7): p. 1971-9.

152. Aubert, G., et al., *Cytomegalovirus-specific cellular immune responses and viremia in recipients of allogeneic stem cell transplants.* J Infect Dis, 2001. **184**(8): p. 955-63.

153. Emery, V.C. and P.D. Griffiths, *Prediction of cytomegalovirus load and resistance patterns after antiviral chemotherapy.* Proc Natl Acad Sci U S A, 2000. **97**(14): p. 8039-44.

154. Ljungman, P., et al., *Use of a semi-quantitative PCR for cytomegalovirus DNA as a basis for pre-emptive antiviral therapy in allogeneic bone marrow transplant patients.* Bone Marrow Transplant, 1996. **17**(4): p. 583-7.

155. Kelly, J., D. Hurley, and G. Raghu, *Comparison of the efficacy and cost effectiveness of pre-emptive therapy as directed by CMV*

antigenemia and prophylaxis with ganciclovir in lung transplant recipients. J Heart Lung Transplant, 2000. **19**(4): p. 355-9.

156. Singh, N., et al., *High-dose acyclovir compared with short-course preemptive ganciclovir therapy to prevent cytomegalovirus disease in liver transplant recipients. A randomized trial.* Ann Intern Med, 1994. **120**(5): p. 375-81.

157. Emery, V.C., *Prophylaxis for CMV should not now replace pre-emptive therapy in solid organ transplantation.* Rev Med Virol, 2001. **11**(2): p. 83-6.

158. Boeckh, M., et al., *Cytomegalovirus pp65 antigenemia-guided early treatment with ganciclovir versus ganciclovir at engraftment after allogeneic marrow transplantation: a randomized double-blind study.* Blood, 1996. **88**(10): p. 4063-71.

159. Crumpacker, C.S., *Ganciclovir.* N Engl J Med, 1996. **335**(10): p. 721-9.

160. Sullivan, V., et al., *A protein kinase homologue controls phosphorylation of ganciclovir in human cytomegalovirus-infected cells.* Nature, 1992. **359**(6390): p. 85.

161. Littler, E., A.D. Stuart, and M.S. Chee, *Human cytomegalovirus UL97 open reading frame encodes a protein that phosphorylates the antiviral nucleoside analogue ganciclovir.* Nature, 1992. **358**(6382): p. 160-2.

162. Hamzeh, F.M., et al., *Identification of the lytic origin of DNA replication in human cytomegalovirus by a novel approach utilizing ganciclovir-induced chain termination.* J Virol, 1990. **64**(12): p. 6184-95.

163. Noble, S. and D. Faulds, *Ganciclovir. An update of its use in the prevention of cytomegalovirus infection and disease in transplant recipients.* Drugs, 1998. **56**(1): p. 115-46.

164. Erice, A., *Resistance of human cytomegalovirus to antiviral drugs.* Clin Microbiol Rev, 1999. **12**(2): p. 286-97.

165. Boivin, G., et al., *Rate of emergence of cytomegalovirus (CMV) mutations in leukocytes of patients with acquired immunodeficiency syndrome who are receiving valganciclovir as induction and maintenance therapy for CMV retinitis.* J Infect Dis, 2001. **184**(12): p. 1598-602.

166. Baldanti, F., et al., *A three-nucleotide deletion in the UL97 open reading frame is responsible for the ganciclovir resistance of a human cytomegalovirus clinical isolate.* J Virol, 1995. **69**(2): p. 796-800.

167. Chou, S., et al., *Analysis of the UL97 phosphotransferase coding sequence in clinical cytomegalovirus isolates and identification of mutations conferring ganciclovir resistance.* J Infect Dis, 1995. **171**(3): p. 576-83.

168. Hanson, M.N., et al., *Novel mutation in the UL97 gene of a clinical cytomegalovirus strain conferring resistance to ganciclovir.* Antimicrob Agents Chemother, 1995. **39**(5): p. 1204-5.

169. Biron, K.K., et al., *Metabolic activation of the nucleoside analog 9-[(2-hydroxy-1-(hydroxymethyl)ethoxy]methyl)guanine in human diploid fibroblasts infected with human cytomegalovirus.* Proc Natl Acad Sci U S A, 1985. **82**(8): p. 2473-7.

170. Buhles, W.C., Jr., et al., *Ganciclovir treatment of life- or sight-threatening cytomegalovirus infection: experience in 314 immunocompromised patients.* Rev Infect Dis, 1988. **10 Suppl 3**: p. S495-506.

171. Reusser, P., *Antiviral therapy: current options and challenges.* Schweiz Med Wochenschr, 2000. **130**(4): p. 101-12.

172. Drew, W.L., et al., *Oral ganciclovir as maintenance treatment for cytomegalovirus retinitis in patients with AIDS. Syntex Cooperative Oral Ganciclovir Study Group.* N Engl J Med, 1995. **333**(10): p. 615-20.

173. Hecht, D.W., et al., *Ganciclovir for treatment of renal transplant-associated primary cytomegalovirus pneumonia.* J Infect Dis, 1988. **157**(1): p. 187-90.

174. Shepp, D.H., et al., *Activity of 9-[2-hydroxy-1-(hydroxymethyl)ethoxymethyl]guanine in the treatment of cytomegalovirus pneumonia.* Ann Intern Med, 1985. **103**(3): p. 368-73.

175. Cvetkovic, R.S. and K. Wellington, *Valganciclovir: a review of its use in the management of CMV infection and disease in immunocompromised patients.* Drugs, 2005. **65**(6): p. 859-78.

176. Pescovitz, M.D., *Oral ganciclovir and pharmacokinetics of valganciclovir in liver transplant recipients.* Transpl Infect Dis, 1999. **1 Suppl 1**: p. 31-4.

177. Helgstrand, E., et al., *Trisodium phosphonoformate, a new antiviral compound.* Science, 1978. **201**(4358): p. 819-21.

178. Jacobsen, M.A., et al., *In vivo additive antiretroviral effect of combined zidovudine and foscarnet therapy for human immunodeficiency virus infection (ACTG Protocol 053).* J Infect Dis, 1991. **163**(6): p. 1219-22.

179. Chrisp, P. and S.P. Clissold, *Foscarnet. A review of its antiviral activity, pharmacokinetic properties and therapeutic use in immunocompromised patients with cytomegalovirus retinitis.* Drugs, 1991. **41**(1): p. 104-29.

180. Walmsley, S.L., et al., *Treatment of cytomegalovirus retinitis with trisodium phosphonoformate hexahydrate (Foscarnet).* J Infect Dis, 1988. **157**(3): p. 569-72.

181. Ganly, P.S., et al., *Foscarnet as treatment for cytomegalovirus retinitis following bone marrow transplantation.* Postgrad Med J, 1988. **64**(751): p. 389-91.

182. Nyberg, G., et al., *Tubulointerstitial nephritis caused by the antiviral agent foscarnet.* Transpl Int, 1989. **2**(4): p. 223-7.

183. Balzarini, J., L. Naesens, and E.D. Clercq, *New antivirals - mechanism of action and resistance development.* Curr Opin Microbiol, 1998. **1**(5): p. 535-46.

184. Polis, M.A., et al., *Anticytomegaloviral activity and safety of cidofovir in patients with human immunodeficiency virus infection and cytomegalovirus viruria.* Antimicrob Agents Chemother, 1995. **39**(4): p. 882-6.

185. Lalezari, J.P., *Cidofovir: a new therapy for cytomegalovirus retinitis.* J Acquir Immune Defic Syndr Hum Retrovirol, 1997. **14 Suppl 1**: p. S22-6.

186. Kirsch, L.S., et al., *Intravitreal cidofovir (HPMPC) treatment of cytomegalovirus retinitis in patients with acquired immune deficiency syndrome.* Ophthalmology, 1995. **102**(4): p. 533-42; discussion 542-3.

187. Biron, C.A., K.S. Byron, and J.L. Sullivan, *Severe herpesvirus infections in an adolescent without natural killer cells.* N Engl J Med, 1989. **320**(26): p. 1731-5.

188. Carr, J.A., et al., *Interleukin-12 exhibits potent antiviral activity in experimental herpesvirus infections.* J Virol, 1997. **71**(10): p. 7799-803.

189. Janeway C.A, T.P., Walport M, Shlomchik M., *Chapter 5: Antigen Presentation to T Lymphocytes.* Immunobiology 5th Edition, 2001: p. 155-184.

190. Ljunggren, H.G. and K. Karre, *In search of the 'missing self': MHC molecules and NK cell recognition.* Immunol Today, 1990. **11**(7): p. 237-44.

191. Lanier, L.L., *NK cell receptors.* Annu Rev Immunol, 1998. **16**: p. 359-93.

192. Farrell, H.E., M.A. Degli-Esposti, and N.J. Davis-Poynter, *Cytomegalovirus evasion of natural killer cell responses.* Immunol Rev, 1999. **168**: p. 187-97.

193. Browne, H., et al., *A complex between the MHC class I homologue encoded by human cytomegalovirus and beta 2 microglobulin.* Nature, 1990. **347**(6295): p. 770-2.

194. Reyburn, H., et al., *Human NK cells: their ligands, receptors and functions.* Immunol Rev, 1997. **155**: p. 119-25.

195. Chapman, T.L., A.P. Heikeman, and P.J. Bjorkman, *The inhibitory receptor LIR-1 uses a common binding interaction to recognize class I MHC molecules and the viral homolog UL18.* Immunity, 1999. **11**(5): p. 603-13.

196. Hassan-Walker, A.F., et al., *Transcription of the human cytomegalovirus natural killer decoy gene, UL18, in vitro and in vivo.* J Gen Virol, 1998. **79 (Pt 9)**: p. 2113-6.

197. Cosman, D., N. Fanger, and L. Borges, *Human cytomegalovirus, MHC class I and inhibitory signalling receptors: more questions than answers.* Immunol Rev, 1999. **168**: p. 177-85.

198. Cosman, D., et al., *A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules.* Immunity, 1997. **7**(2): p. 273-82.

199. Tomasec, P., et al., *Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40.* Science, 2000. **287**(5455): p. 1031.

200. Braud, V.M., P. Tomasec, and G.W. Wilkinson, *Viral evasion of natural killer cells during human cytomegalovirus infection.* Curr Top Microbiol Immunol, 2002. **269**: p. 117-29.

201. Cosman, D., et al., *ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor.* Immunity, 2001. **14**(2): p. 123-33.

202. Welte, S.A., et al., *Selective intracellular retention of virally induced NKG2D ligands by the human cytomegalovirus UL16 glycoprotein.* Eur J Immunol, 2003. **33**(1): p. 194-203.

203. Sutherland, C.L., N.J. Chalupny, and D. Cosman, *The UL16-binding proteins, a novel family of MHC class I-related ligands for NKG2D, activate natural killer cell functions.* Immunol Rev, 2001. **181**: p. 185-92.

204. Tomasec, P., et al., *Downregulation of natural killer cell-activating ligand CD155 by human cytomegalovirus UL141.* Nat Immunol, 2005. **6**(2): p. 181-8.

205. Wills, M.R., et al., *Human cytomegalovirus encodes an MHC class I-like molecule (UL142) that functions to inhibit NK cell lysis.* J Immunol, 2005. **175**(11): p. 7457-65.

206. Spear, G.T., et al., *Host cell-derived complement control proteins CD55 and CD59 are incorporated into the virions of two unrelated enveloped viruses. Human T cell leukemia/lymphoma virus type I (HTLV-I) and human cytomegalovirus (HCMV).* J Immunol, 1995. **155**(9): p. 4376-81.

207. Keller, R., et al., *An IgG-Fc receptor induced in cytomegalovirus-infected human fibroblasts.* J Immunol, 1976. **116**(3): p. 772-7.

208. Spiller, O.B., et al., *Neutralization of cytomegalovirus virions: the role of complement.* J Infect Dis, 1997. **176**(2): p. 339-47.

209. Spiller, O.B., et al., *Altered expression of host-encoded complement regulators on human cytomegalovirus-infected cells.* Eur J Immunol, 1996. **26**(7): p. 1532-8.

210. Snydman, D.R., et al., *Use of cytomegalovirus immune globulin to prevent cytomegalovirus disease in renal-transplant recipients.* N Engl J Med, 1987. **317**(17): p. 1049-54.

211. Britt, W.J., *Recent advances in the identification of significant human cytomegalovirus-encoded proteins.* Transplant Proc, 1991. **23**(3 Suppl 3): p. 64-9, discussion 69.

212. Greijer, A.E., et al., *Molecular fine-specificity analysis of antibody responses to human cytomegalovirus and design of novel synthetic-peptide-based serodiagnostic assays.* J Clin Microbiol, 1999. **37**(1): p. 179-88.

213. Mach, M., et al., *Complex formation by human cytomegalovirus glycoproteins M (gpUL100) and N (gpUL73).* J Virol, 2000. **74**(24): p. 11881-92.

214. Mach, M., et al., *Complex formation by glycoproteins M and N of human cytomegalovirus: structural and functional aspects.* J Virol, 2005. **79**(4): p. 2160-70.

215. Wagner, B., et al., *A continuous sequence of more than 70 amino acids is essential for antibody binding to the dominant antigenic site of glycoprotein gp58 of human cytomegalovirus.* J Virol, 1992. **66**(9): p. 5290-7.

216. Britt, W.J., et al., *Antigenic domain 1 is required for oligomerization of human cytomegalovirus glycoprotein B.* J Virol, 2005. **79**(7): p. 4066-79.

217. Heemels, M.T. and H. Ploegh, *Generation, translocation, and presentation of MHC class I-restricted peptides.* Annu Rev Biochem, 1995. **64**: p. 463-91.

218. Townsend, A.R., et al., *Cytotoxic T lymphocytes recognize influenza haemagglutinin that lacks a signal sequence.* Nature, 1986. **324**(6097): p. 575-7.

219. Baumeister, W., et al., *The proteasome: paradigm of a self-compartmentalizing protease.* Cell, 1998. **92**(3): p. 367-80.

220. Coux, O., K. Tanaka, and A.L. Goldberg, *Structure and functions of the 20S and 26S proteasomes.* Annu Rev Biochem, 1996. **65**: p. 801-47.

221. Lankat-Buttgereit, B. and R. Tampe, *The transporter associated with antigen processing: function and implications in human diseases.* Physiol Rev, 2002. **82**(1): p. 187-204.

222. Roelse, J., et al., *Trimming of TAP-translocated peptides in the endoplasmic reticulum and in the cytosol during recycling.* J Exp Med, 1994. **180**(5): p. 1591-7.

223. Serwold, T., et al., *ERAAP customizes peptides for MHC class I molecules in the endoplasmic reticulum.* Nature, 2002. **419**(6906): p. 480-3.

224. Serwold, T., S. Gaw, and N. Shastri, *ER aminopeptidases generate a unique pool of peptides for MHC class I molecules.* Nat Immunol, 2001. **2**(7): p. 644-51.

225. Shahinian, A., et al., *Differential T cell costimulatory requirements in CD28-deficient mice.* Science, 1993. **261**(5121): p. 609-12.

226. Bennett, S.R., et al., *Help for cytotoxic-T-cell responses is mediated by CD40 signalling.* Nature, 1998. **393**(6684): p. 478-80.

227. Harding, F.A., et al., *CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones.* Nature, 1992. **356**(6370): p. 607-9.

228. Chen, L., et al., *Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4.* Cell, 1992. **71**(7): p. 1093-102.

229. Kagi, D., et al., *CD8+ T cell-mediated protection against an intracellular bacterium by perforin-dependent cytotoxicity.* Eur J Immunol, 1994. **24**(12): p. 3068-72.

230. Kagi, D., et al., *Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice.* Nature, 1994. **369**(6475): p. 31-7.

231. Berke, G., *The CTL's kiss of death.* Cell, 1995. **81**(1): p. 9-12.

232. Kagi, D., et al., *Lymphocyte-mediated cytotoxicity in vitro and in vivo: mechanisms and significance.* Immunol Rev, 1995. **146**: p. 95-115.

233. Kagi, D., et al., *The roles of perforin- and Fas-dependent cytotoxicity in protection against cytopathic and noncytopathic viruses.* Eur J Immunol, 1995. **25**(12): p. 3256-62.

234. Carter, L.L. and R.W. Dutton, *Relative perforin- and Fas-mediated lysis in T1 and T2 CD8 effector populations.* J Immunol, 1995. **155**(3): p. 1028-31.

235. Depraetere, V. and P. Golstein, *Fas and other cell death signaling pathways.* Semin Immunol, 1997. **9**(2): p. 93-107.

236. Radoja, S., et al., *CD8(+) tumor-infiltrating T cells are deficient in perforin-mediated cytolytic activity due to defective microtubule-*

organizing center mobilization and lytic granule exocytosis. J Immunol, 2001. **167**(9): p. 5042-51.

237. Podack, E.R. and A. Kupfer, *T-cell effector functions: mechanisms for delivery of cytotoxicity and help.* Annu Rev Cell Biol, 1991. **7**: p. 479-504.

238. Sedwick, C.E., et al., *TCR, LFA-1, and CD28 play unique and complementary roles in signaling T cell cytoskeletal reorganization.* J Immunol, 1999. **162**(3): p. 1367-75.

239. Stinchcombe, J.C. and G.M. Griffiths, *The role of the secretory immunological synapse in killing by CD8+ CTL.* Semin Immunol, 2003. **15**(6): p. 301-5.

240. Kupfer, A. and S.J. Singer, *Cell biology of cytotoxic and helper T cell functions: immunofluorescence microscopic studies of single cells and cell couples.* Annu Rev Immunol, 1989. **7**: p. 309-37.

241. Dustin, M.L. and J.A. Cooper, *The immunological synapse and the actin cytoskeleton: molecular hardware for T cell signaling.* Nat Immunol, 2000. **1**(1): p. 23-9.

242. Peters, P.J., et al., *Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes.* J Exp Med, 1991. **173**(5): p. 1099-109.

243. Bossi, G. and G.M. Griffiths, *Degranulation plays an essential part in regulating cell surface expression of Fas ligand in T cells and natural killer cells.* Nat Med, 1999. **5**(1): p. 90-6.

244. Guidotti, L.G., et al., *Cytotoxic T lymphocytes inhibit hepatitis B virus gene expression by a noncytolytic mechanism in transgenic mice.* Proc Natl Acad Sci U S A, 1994. **91**(9): p. 3764-8.

245. Guidotti, L.G., et al., *Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes.* Immunity, 1996. **4**(1): p. 25-36.

246. Guidotti, L.G., et al., *Viral clearance without destruction of infected cells during acute HBV infection.* Science, 1999. **284**(5415): p. 825-9.

247. Gilles, P.N., G. Fey, and F.V. Chisari, *Tumor necrosis factor alpha negatively regulates hepatitis B virus gene expression in transgenic mice.* J Virol, 1992. **66**(6): p. 3955-60.

248. Guidotti, L.G. and F.V. Chisari, *Noncytolytic control of viral infections by the innate and adaptive immune response.* Annu Rev Immunol, 2001. **19**: p. 65-91.

249. Quinnan, G.V., Jr., et al., *Cytotoxic t cells in cytomegalovirus infection: HLA-restricted T-lymphocyte and non-T-lymphocyte cytotoxic responses correlate with recovery from cytomegalovirus infection in bone-marrow-transplant recipients.* N Engl J Med, 1982. **307**(1): p. 7-13.

250. Riddell, S.R., et al., *Class I MHC-restricted cytotoxic T lymphocyte recognition of cells infected with human cytomegalovirus does not require endogenous viral gene expression.* J Immunol, 1991. **146**(8): p. 2795-804.

251. Walter, E.A., et al., *Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of*

T-cell clones from the donor. N Engl J Med, 1995. **333**(16): p. 1038-44.

252. Riddell, S.R. and P.D. Greenberg, *Therapeutic reconstitution of human viral immunity by adoptive transfer of cytotoxic T lymphocyte clones.* Curr Top Microbiol Immunol, 1994. **189**: p. 9-34.

253. Reusser, P., et al., *Cytomegalovirus-specific T-cell immunity in recipients of autologous peripheral blood stem cell or bone marrow transplants.* Blood, 1997. **89**(10): p. 3873-9.

254. Reusser, P., et al., *Cytomegalovirus (CMV)-specific T cell immunity after renal transplantation mediates protection from CMV disease by limiting the systemic virus load.* J Infect Dis, 1999. **180**(2): p. 247-53.

255. Gratama, J.W., et al., *Tetramer-based quantification of cytomegalovirus (CMV)-specific CD8+ T lymphocytes in T-cell-depleted stem cell grafts and after transplantation may identify patients at risk for progressive CMV infection.* Blood, 2001. **98**(5): p. 1358-64.

256. Gratama, J.W. and J.J. Cornelissen, *Diagnostic potential of tetramer-based monitoring of cytomegalovirus-specific CD8+ T lymphocytes in allogeneic stem cell transplantation.* Clin Immunol, 2003. **106**(1): p. 29-35.

257. McLaughlin-Taylor, E., et al., *Identification of the major late human cytomegalovirus matrix protein pp65 as a target antigen for CD8+ virus-specific cytotoxic T lymphocytes.* J Med Virol, 1994. **43**(1): p. 103-10.

258. Jin, X., et al., *High frequency of cytomegalovirus-specific cytotoxic T-effector cells in HLA-A*0201-positive subjects during multiple viral coinfections.* J Infect Dis, 2000. **181**(1): p. 165-75.

259. Lang, K.S., et al., *High frequency of human cytomegalovirus (HCMV)-specific CD8+ T cells detected in a healthy CMV-seropositive donor.* Cell Mol Life Sci, 2002. **59**(6): p. 1076-80.

260. Gillespie, G.M., et al., *Functional heterogeneity and high frequencies of cytomegalovirus-specific CD8(+) T lymphocytes in healthy seropositive donors.* J Virol, 2000. **74**(17): p. 8140-50.

261. Reddehase, M.J., *The immunogenicity of human and murine cytomegaloviruses.* Curr Opin Immunol, 2000. **12**(4): p. 390-6.

262. Gilbert, M.J., et al., *Cytomegalovirus selectively blocks antigen processing and presentation of its immediate-early gene product.* Nature, 1996. **383**(6602): p. 720-2.

263. Kern, F., et al., *Target structures of the CD8(+)-T-cell response to human cytomegalovirus: the 72-kilodalton major immediate-early protein revisited.* J Virol, 1999. **73**(10): p. 8179-84.

264. Sylwester, A.W., et al., *Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects.* J Exp Med, 2005. **202**(5): p. 673-85.

265. Elkington, R., et al., *Ex vivo profiling of CD8+-T-cell responses to human cytomegalovirus reveals broad and multispecific reactivities in healthy virus carriers.* J Virol, 2003. **77**(9): p. 5226-40.

266. Wills, M.R., et al., *The human cytotoxic T-lymphocyte (CTL) response to cytomegalovirus is dominated by structural protein pp65: frequency,*

specificity, and T-cell receptor usage of pp65-specific CTL. J Virol, 1996. **70**(11): p. 7569-79.

267. Solache, A., et al., *Identification of three HLA-A*0201-restricted cytotoxic T cell epitopes in the cytomegalovirus protein pp65 that are conserved between eight strains of the virus.* J Immunol, 1999. **163**(10): p. 5512-8.

268. Kuzushima, K., et al., *Efficient identification of HLA-A*2402-restricted cytomegalovirus-specific CD8(+) T-cell epitopes by a computer algorithm and an enzyme-linked immunospot assay.* Blood, 2001. **98**(6): p. 1872-81.

269. Masuoka, M., et al., *Identification of the HLA-A24 peptide epitope within cytomegalovirus protein pp65 recognized by CMV-specific cytotoxic T lymphocytes.* Viral Immunol, 2001. **14**(4): p. 369-77.

270. Gavin, M.A., et al., *Alkali hydrolysis of recombinant proteins allows for the rapid identification of class I MHC-restricted CTL epitopes.* J Immunol, 1993. **151**(8): p. 3971-80.

271. Kern, F., et al., *Cytomegalovirus (CMV) phosphoprotein 65 makes a large contribution to shaping the T cell repertoire in CMV-exposed individuals.* J Infect Dis, 2002. **185**(12): p. 1709-16.

272. Prod'homme, V., et al., *Modulation of HLA-A*0201-restricted T cell responses by natural polymorphism in the IE1(315-324) epitope of human cytomegalovirus.* J Immunol, 2003. **170**(4): p. 2030-6.

273. Retiere, C., et al., *Generation of cytomegalovirus-specific human T-lymphocyte clones by using autologous B-lymphoblastoid cells with*

stable expression of pp65 or IE1 proteins: a tool to study the fine specificity of the antiviral response. J Virol, 2000. **74**(9): p. 3948-52.

274. Frankenberg, N., et al., *Identification of a conserved HLA-A2-restricted decapeptide from the IE1 protein (pUL123) of human cytomegalovirus.* Virology, 2002. **295**(2): p. 208-16.

275. Khan, N., et al., *Comparative analysis of CD8+ T cell responses against human cytomegalovirus proteins pp65 and immediate early 1 shows similarities in precursor frequency, oligoclonality, and phenotype.* J Infect Dis, 2002. **185**(8): p. 1025-34.

276. Khattab, B.A., et al., *Three T-cell epitopes within the C-terminal 265 amino acids of the matrix protein pp65 of human cytomegalovirus recognized by human lymphocytes.* J Med Virol, 1997. **52**(1): p. 68-76.

277. Gautier, N., et al., *Characterization of an epitope of the human cytomegalovirus protein IE1 recognized by a CD4+ T cell clone.* Eur J Immunol, 1996. **26**(5): p. 1110-7.

278. Wever, P.C., et al., *Expression of granzyme B during primary cytomegalovirus infection after renal transplantation.* J Infect Dis, 1999. **179**(3): p. 693-6.

279. Rentenaar, R.J., et al., *CD8(POS) lymphocyte dynamics in primary CMV infection.* Transplant Proc, 2001. **33**(1-2): p. 1867-9.

280. Borysiewicz, L.K., et al., *Human cytomegalovirus-specific cytotoxic T cells: their precursor frequency and stage specificity.* Eur J Immunol, 1988. **18**(2): p. 269-75.

281. Borysiewicz, L.K., et al., *Human cytomegalovirus-specific cytotoxic T cells. Relative frequency of stage-specific CTL recognizing the 72-kD*

immediate early protein and glycoprotein B expressed by recombinant vaccinia viruses. J Exp Med, 1988. **168**(3): p. 919-31.

282. Akbar, A.N., et al., *Loss of CD45R and gain of UCHL1 reactivity is a feature of primed T cells.* J Immunol, 1988. **140**(7): p. 2171-8.

283. Merkenschlager, M., et al., *Limiting dilution analysis of proliferative responses in human lymphocyte populations defined by the monoclonal antibody UCHL1: implications for differential CD45 expression in T cell memory formation.* Eur J Immunol, 1988. **18**(11): p. 1653-61.

284. Sallusto, F., et al., *Two subsets of memory T lymphocytes with distinct homing potentials and effector functions.* Nature, 1999. **401**(6754): p. 708-12.

285. Weekes, M.P., et al., *Human CD28-CD8+ T cells contain greatly expanded functional virus-specific memory CTL clones.* J Immunol, 1999. **162**(12): p. 7569-77.

286. Northfield, J., et al., *Does memory improve with age? CD85j (ILT-2/LIR-1) expression on CD8 T cells correlates with 'memory inflation' in human cytomegalovirus infection.* Immunol Cell Biol, 2005. **83**(2): p. 182-8.

287. Wang, E.C. and L.K. Borysiewicz, *The role of CD8+, CD57+ cells in human cytomegalovirus and other viral infections.* Scand J Infect Dis Suppl, 1995. **99**: p. 69-77.

288. Azuma, M., J.H. Phillips, and L.L. Lanier, *CD28- T lymphocytes. Antigenic and functional properties.* J Immunol, 1993. **150**(4): p. 1147-59.

289. Pawelec, G., et al., *Human immunosenescence: is it infectious?* Immunol Rev, 2005. **205**: p. 257-68.

290. Wills, M.R., et al., *Identification of naive or antigen-experienced human CD8(+) T cells by expression of costimulation and chemokine receptors: analysis of the human cytomegalovirus-specific CD8(+) T cell response.* J Immunol, 2002. **168**(11): p. 5455-64.

291. Appay, V., et al., *Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections.* Nat Med, 2002. **8**(4): p. 379-85.

292. Sallusto, F., A. Lanzavecchia, and C.R. Mackay, *Chemokines and chemokine receptors in T-cell priming and Th1/Th2-mediated responses.* Immunol Today, 1998. **19**(12): p. 568-74.

293. Benz, C., et al., *Activated virus-specific T cells are early indicators of anti-CMV immune reactions in liver transplant patients.* Gastroenterology, 2002. **122**(5): p. 1201-15.

294. Gamadia, L.E., et al., *Differentiation of cytomegalovirus-specific CD8(+) T cells in healthy and immunosuppressed virus carriers.* Blood, 2001. **98**(3): p. 754-61.

295. Harari, A., et al., *Analysis of HIV-1- and CMV-specific memory CD4 T-cell responses during primary and chronic infection.* Blood, 2002. **100**(4): p. 1381-7.

296. Bronke, C., et al., *Progression to CMV end-organ disease in HIV-1-infected individuals despite abundance of highly differentiated CMV-specific CD8+ T-cells.* Immunol Lett, 2005. **97**(2): p. 215-24.

297. Chen, G., et al., *CD8 T cells specific for human immunodeficiency virus, Epstein-Barr virus, and cytomegalovirus lack molecules for homing to lymphoid sites of infection.* Blood, 2001. **98**(1): p. 156-64.

298. Peggs, K., et al., *Characterization of human cytomegalovirus peptide-specific CD8(+) T-cell repertoire diversity following in vitro restimulation by antigen-pulsed dendritic cells.* Blood, 2002. **99**(1): p. 213-23.

299. Weekes, M.P., et al., *Large clonal expansions of human virus-specific memory cytotoxic T lymphocytes within the CD57+ CD28- CD8+ T-cell population.* Immunology, 1999. **98**(3): p. 443-9.

300. Sze, D.M., et al., *Clonal cytotoxic T cells are expanded in myeloma and reside in the CD8(+)CD57(+)CD28(-) compartment.* Blood, 2001. **98**(9): p. 2817-27.

301. Kern, F., et al., *The enigma of CD57+CD28- T cell expansion--anergy or activation?* Clin Exp Immunol, 1996. **104**(1): p. 180-4.

302. Phillips, J.H. and L.L. Lanier, *Lectin-dependent and anti-CD3 induced cytotoxicity are preferentially mediated by peripheral blood cytotoxic T lymphocytes expressing Leu-7 antigen.* J Immunol, 1986. **136**(5): p. 1579-85.

303. Wang, E.C., et al., *Subsets of CD8+, CD57+ cells in normal, healthy individuals: correlations with human cytomegalovirus (HCMV) carrier status, phenotypic and functional analyses.* Clin Exp Immunol, 1993. **94**(2): p. 297-305.

304. Wang, E.C., et al., *CD8highCD57+ T lymphocytes in normal, healthy individuals are oligoclonal and respond to human cytomegalovirus.* J Immunol, 1995. **155**(10): p. 5046-56.

305. Chochi, K., et al., *The increase of CD57+ T cells in the peripheral blood and their impaired immune functions in patients with advanced gastric cancer.* Oncol Rep, 2003. **10**(5): p. 1443-8.

306. Peggs, K.S. and S. Mackinnon, *Clinical trials with CMV-specific T cells.* Cytotherapy, 2002. **4**(1): p. 21-8.

307. Hebart, H., et al., *Monitoring of CMV infection: a comparison of PCR from whole blood, plasma-PCR, pp65-antigenemia and virus culture in patients after bone marrow transplantation.* Bone Marrow Transplant, 1996. **17**(5): p. 861-8.

308. Hassan-Walker, A.F., et al., *CD8+ cytotoxic lymphocyte responses against cytomegalovirus after liver transplantation: correlation with time from transplant to receipt of tacrolimus.* J Infect Dis, 2001. **183**(6): p. 835-43.

309. Gor, D., et al., *Longitudinal fluctuations in cytomegalovirus load in bone marrow transplant patients: relationship between peak virus load, donor/recipient serostatus, acute GVHD and CMV disease.* Bone Marrow Transplant, 1998. **21**(6): p. 597-605.

310. Ouyang, Q., et al., *Age-associated accumulation of CMV-specific CD8+ T cells expressing the inhibitory killer cell lectin-like receptor G1 (KLRG1).* Exp Gerontol, 2003. **38**(8): p. 911-20.

311. Vescovini, R., et al., *Different contribution of EBV and CMV infections in very long-term carriers to age-related alterations of CD8+ T cells.* Exp Gerontol, 2004. **39**(8): p. 1233-43.

312. Fagnoni, F.F., et al., *Expansion of cytotoxic CD8+ CD28- T cells in healthy ageing people, including centenarians.* Immunology, 1996. **88**(4): p. 501-7.

313. Ouyang, Q., et al., *Large numbers of dysfunctional CD8+ T lymphocytes bearing receptors for a single dominant CMV epitope in the very old.* J Clin Immunol, 2003. **23**(4): p. 247-57.

314. Ouyang, Q., et al., *Dysfunctional CMV-specific CD8(+) T cells accumulate in the elderly.* Exp Gerontol, 2004. **39**(4): p. 607-13.

315. Fagnoni, F.F., et al., *Shortage of circulating naive CD8(+) T cells provides new insights on immunodeficiency in aging.* Blood, 2000. **95**(9): p. 2860-8.

316. Luciani, F., et al., *A stochastic model for CD8(+)T cell dynamics in human immunosenescence: implications for survival and longevity.* J Theor Biol, 2001. **213**(4): p. 587-97.

317. Ouyang, Q., et al., *An age-related increase in the number of CD8+ T cells carrying receptors for an immunodominant Epstein-Barr virus (EBV) epitope is counteracted by a decreased frequency of their antigen-specific responsiveness.* Mech Ageing Dev, 2003. **124**(4): p. 477-85.

318. Olsson, J., et al., *Age-related change in peripheral blood T-lymphocyte subpopulations and cytomegalovirus infection in the very*

old: the Swedish longitudinal OCTO immune study. Mech Ageing Dev, 2000. **121**(1-3): p. 187-201.

319. Appay, V., et al., *HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function.* J Exp Med, 2000. **192**(1): p. 63-75.
320. Matloubian, M., R.J. Concepcion, and R. Ahmed, *CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection.* J Virol, 1994. **68**(12): p. 8056-63.
321. Battegay, M., et al., *Enhanced establishment of a virus carrier state in adult CD4+ T-cell-deficient mice.* J Virol, 1994. **68**(7): p. 4700-4.
322. Cardin, R.D., et al., *Progressive loss of CD8+ T cell-mediated control of a gamma-herpesvirus in the absence of CD4+ T cells.* J Exp Med, 1996. **184**(3): p. 863-71.
323. Lucin, P., et al., *Gamma interferon-dependent clearance of cytomegalovirus infection in salivary glands.* J Virol, 1992. **66**(4): p. 1977-84.
324. Davignon, J.L., et al., *Anti-human cytomegalovirus activity of cytokines produced by CD4+ T-cell clones specifically activated by IE1 peptides in vitro.* J Virol, 1996. **70**(4): p. 2162-9.
325. Jonjic, S., et al., *Efficacious control of cytomegalovirus infection after long-term depletion of CD8+ T lymphocytes.* J Virol, 1990. **64**(11): p. 5457-64.
326. Greenberg, P.D., et al., *Genetic modification of T-cell clones for therapy of human viral and malignant diseases.* Cancer J Sci Am, 1998. **4 Suppl 1**: p. S100-5.

327. Ozdemir, E., et al., *Cytomegalovirus reactivation following allogeneic stem cell transplantation is associated with the presence of dysfunctional antigen-specific CD8+ T cells.* Blood, 2002. **100**(10): p. 3690-7.

328. Gyulai, Z., et al., *Cytotoxic T lymphocyte (CTL) responses to human cytomegalovirus pp65, IE1-Exon4, gB, pp150, and pp28 in healthy individuals: reevaluation of prevalence of IE1-specific CTLs.* J Infect Dis, 2000. **181**(5): p. 1537-46.

329. Hopkins, J.I., et al., *Cytotoxic T cell immunity to human cytomegalovirus glycoprotein B.* J Med Virol, 1996. **49**(2): p. 124-31.

330. Sester, M., et al., *Sustained high frequencies of specific CD4 T cells restricted to a single persistent virus.* J Virol, 2002. **76**(8): p. 3748-55.

331. Essa, S., et al., *CD4(+) T cell levels are decreased during active CMV infection in kidney transplant recipients.* FEMS Immunol Med Microbiol, 2002. **34**(1): p. 17-22.

332. Sester, M., et al., *Levels of virus-specific CD4 T cells correlate with cytomegalovirus control and predict virus-induced disease after renal transplantation.* Transplantation, 2001. **71**(9): p. 1287-94.

333. Komanduri, K.V., et al., *Loss of cytomegalovirus-specific CD4+ T cell responses in human immunodeficiency virus type 1-infected patients with high CD4+ T cell counts and recurrent retinitis.* J Infect Dis, 2001. **183**(8): p. 1285-9.

334. de Vries, E., et al., *Reconstitution of lymphocyte subpopulations after paediatric bone marrow transplantation.* Bone Marrow Transplant, 2000. **25**(3): p. 267-75.

335. Bitmansour, A.D., et al., *Clonotypic structure of the human CD4+ memory T cell response to cytomegalovirus.* J Immunol, 2001. **167**(3): p. 1151-63.

336. Beninga, J., B. Kropff, and M. Mach, *Comparative analysis of fourteen individual human cytomegalovirus proteins for helper T cell response.* J Gen Virol, 1995. **76 (Pt 1)**: p. 153-60.

337. Le Roy, E., et al., *Infection of APC by human cytomegalovirus controlled through recognition of endogenous nuclear immediate early protein 1 by specific CD4(+) T lymphocytes.* J Immunol, 2002. **169**(3): p. 1293-301.

338. Prosch, S., et al., *Stimulation of the human cytomegalovirus IE enhancer/promoter in HL-60 cells by TNFalpha is mediated via induction of NF-kappaB.* Virology, 1995. **208**(1): p. 197-206.

339. Hummel, M., et al., *Allogeneic transplantation induces expression of cytomegalovirus immediate-early genes in vivo: a model for reactivation from latency.* J Virol, 2001. **75**(10): p. 4814-22.

340. Wiertz, E.J., et al., *Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction.* Nature, 1996. **384**(6608): p. 432-8.

341. Ahn, K., et al., *The ER-luminal domain of the HCMV glycoprotein US6 inhibits peptide translocation by TAP.* Immunity, 1997. **6**(5): p. 613-21.

342. Hegde, N.R., et al., *Inhibition of HLA-DR assembly, transport, and loading by human cytomegalovirus glycoprotein US3: a novel mechanism for evading major histocompatibility complex class II antigen presentation.* J Virol, 2002. **76**(21): p. 10929-41.

343. Hegde, N.R. and D.C. Johnson, *Human cytomegalovirus US2 causes similar effects on both major histocompatibility complex class I and II proteins in epithelial and glial cells.* J Virol, 2003. **77**(17): p. 9287-94.

344. Tomazin, R., et al., *Cytomegalovirus US2 destroys two components of the MHC class II pathway, preventing recognition by CD4+ T cells.* Nat Med, 1999. **5**(9): p. 1039-43.

345. Kotenko, S.V., et al., *Human cytomegalovirus harbors its own unique IL-10 homolog (cmvIL-10).* Proc Natl Acad Sci U S A, 2000. **97**(4): p. 1695-700.

346. Bayry, J., et al., *Common variable immunodeficiency: the immune system in chaos.* Trends Mol Med, 2005. **11**(8): p. 370-6.

347. Webster, A.D., *Primary immunodeficiency.* Hum Exp Toxicol, 1995. **14**(1): p. 99-100.

348. de Gracia, J., et al., *Immunoglobulin therapy to control lung damage in patients with common variable immunodeficiency.* Int Immunopharmacol, 2004. **4**(6): p. 745-53.

349. Cunningham-Rundles, C., *Hematologic complications of primary immune deficiencies.* Blood Rev, 2002. **16**(1): p. 61-4.

350. Bayry, J., et al., *Common variable immunodeficiency is associated with defective functions of dendritic cells.* Blood, 2004. **104**(8): p. 2441-3.

351. Cambronero, R., et al., *Up-regulation of IL-12 in monocytes: a fundamental defect in common variable immunodeficiency.* J Immunol, 2000. **164**(1): p. 488-94.

352. Holm, A.M., et al., *Gene expression analysis of peripheral T cells in a subgroup of common variable immunodeficiency shows predominance of CCR7(-) effector-memory T cells.* Clin Exp Immunol, 2004. **138**(2): p. 278-89.

353. Holm, A.M., et al., *Abnormal interleukin-7 function in common variable immunodeficiency.* Blood, 2005. **105**(7): p. 2887-90.

354. Rentenaar, R.J., et al., *Development of virus-specific CD4(+) T cells during primary cytomegalovirus infection.* J Clin Invest, 2000. **105**(4): p. 541-8.

355. Gamadia, L.E., et al., *Primary immune responses to human CMV: a critical role for IFN-gamma-producing CD4+ T cells in protection against CMV disease.* Blood, 2003. **101**(7): p. 2686-92.

356. Gamadia, L.E., et al., *Properties of CD4(+) T cells in human cytomegalovirus infection.* Hum Immunol, 2004. **65**(5): p. 486-92.

357. Radha, R., et al., *Cellular immune responses to cytomegalovirus in renal transplant recipients.* Am J Transplant, 2005. **5**(1): p. 110-7.

358. Zaia, J.A., et al., *Infrequent occurrence of natural mutations in the pp65(495-503) epitope sequence presented by the HLA A*0201 allele among human cytomegalovirus isolates.* J Virol, 2001. **75**(5): p. 2472-4.

359. Mattes, F.M., et al., *Kinetics of cytomegalovirus load decrease in solid-organ transplant recipients after preemptive therapy with valganciclovir.* J Infect Dis, 2005. **191**(1): p. 89-92.

360. Riddell, S.R. and P.D. Greenberg, *T cell therapy of human CMV and EBV infection in immunocompromised hosts.* Rev Med Virol, 1997. **7**(3): p. 181-192.

361. Gerard, L., et al., *Cytomegalovirus (CMV) viremia and the CD4+ lymphocyte count as predictors of CMV disease in patients infected with human immunodeficiency virus.* Clin Infect Dis, 1997. **24**(5): p. 836-40.

362. Williams, P.L., J.S. Currier, and S. Swindells, *Joint effects of HIV-1 RNA levels and CD4 lymphocyte cells on the risk of specific opportunistic infections.* Aids, 1999. **13**(9): p. 1035-44.

363. Gallot, G., et al., *Purification of Ag-specific T lymphocytes after direct peripheral blood mononuclear cell stimulation followed by CD25 selection. I. Application to CD4(+) or CD8(+) cytomegalovirus phosphoprotein pp65 epitope determination.* J Immunol, 2001. **167**(8): p. 4196-206.

364. Davrinche, C., et al., *Expression of human cytomegalovirus immediate early protein IE1 in insect cells: splicing of RNA and recognition by CD4+ T-cell clones.* Biochem Biophys Res Commun, 1993. **195**(1): p. 469-77.

365. Le Roy, E., et al., *Escape of human cytomegalovirus from HLA-DR-restricted CD4(+) T-cell response is mediated by repression of gamma interferon-induced class II transactivator expression.* J Virol, 1999. **73**(8): p. 6582-9.

366. Davignon, J.L., et al., *Analysis of the proliferative T cell response to human cytomegalovirus major immediate-early protein (IE1):*

phenotype, frequency and variability. Scand J Immunol, 1995. **41**(3): p. 247-55.

367. Fletcher, J.M., et al., *Cytomegalovirus-Specific CD4+ T Cells in Healthy Carriers Are Continuously Driven to Replicative Exhaustion.* J Immunol, 2005. **175**(12): p. 8218-25.

368. Fiala, M., et al., *Epidemiology of cytomegalovirus infection after transplantation and immunosuppression.* J Infect Dis, 1975. **132**(4): p. 421-33.

369. Morris, P.J., et al., *A controlled trial of cyclosporine in renal transplantation with conversion to azathioprine and prednisolone after three months.* Transplantation, 1983. **36**(3): p. 273-7.

370. Ludwig, B., et al., *Cytomegalovirus-specific CD4 T-cell and glycoprotein B specific antibody response in recipients of allogenic stem cell transplantation.* J Clin Virol, 2005.

371. Podack, E.R., H. Hengartner, and M.G. Lichtenheld, *A central role of perforin in cytolysis?* Annu Rev Immunol, 1991. **9**: p. 129-57.

372. Podack, E.R., *Perforin: structure, function, and regulation.* Curr Top Microbiol Immunol, 1992. **178**: p. 175-84.

373. Podack, E.R., J.D. Young, and Z.A. Cohn, *Isolation and biochemical and functional characterization of perforin 1 from cytolytic T-cell granules.* Proc Natl Acad Sci U S A, 1985. **82**(24): p. 8629-33.

374. Podack, E.R., et al., *Cytolytic T-cell granules: biochemical properties and functional specificity.* Adv Exp Med Biol, 1985. **184**: p. 99-119.

375. Edwards, K.M., et al., *Anti-viral strategies of cytotoxic T lymphocytes are manifested through a variety of granule-bound pathways of apoptosis induction.* Immunol Cell Biol, 1999. **77**(1): p. 76-89.

376. Reusser, P., et al., *Cytotoxic T-lymphocyte response to cytomegalovirus after human allogeneic bone marrow transplantation: pattern of recovery and correlation with cytomegalovirus infection and disease.* Blood, 1991. **78**(5): p. 1373-80.

377. Kagi, D., et al., *Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity.* Science, 1994. **265**(5171): p. 528-30.

378. Kern, F., et al., *Analysis of CD8 T cell reactivity to cytomegalovirus using protein-spanning pools of overlapping pentadecapeptides.* Eur J Immunol, 2000. **30**(6): p. 1676-82.

379. Bunde, T., et al., *Protection from cytomegalovirus after transplantation is correlated with immediate early 1-specific CD8 T cells.* J Exp Med, 2005. **201**(7): p. 1031-6.

380. Borrow, P., et al., *Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus.* Nat Med, 1997. **3**(2): p. 205-11.

381. Bertoletti, A., et al., *Natural variants of cytotoxic epitopes are T-cell receptor antagonists for antiviral cytotoxic T cells.* Nature, 1994. **369**(6479): p. 407-10.

382. La Rosa, C., et al., *Enhanced immune activity of cytotoxic T-lymphocyte epitope analogs derived from positional scanning synthetic combinatorial libraries.* Blood, 2001. **97**(6): p. 1776-86.

383. Wang, F., et al., *Continuous detection of CMV DNA in plasma of patients with advanced HIV infection implies the poorest prognosis.* Clin Diagn Virol, 1995. **3**(4): p. 371-5.

384. Brenchley, J.M., et al., *Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8+ T cells.* Blood, 2003. **101**(7): p. 2711-20.

385. ten Berge, I.J., et al., *Differentiation of CMV-specific CD8(POS) T lymphocytes in primary CMV infection after renal transplantation.* Transplant Proc, 2001. **33**(7-8): p. 3630.

386. Sester, U., et al., *Differences in CMV-specific T-cell levels and long-term susceptibility to CMV infection after kidney, heart and lung transplantation.* Am J Transplant, 2005. **5**(6): p. 1483-9.

387. Hilton, R.M., et al., *Direct visualisation of cytomegalovirus-specific CD8+ T cells in renal transplant recipients.* Transplant Proc, 2002. **34**(4): p. 1171-3.

388. Janssen, E.M., et al., *CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death.* Nature, 2005. **434**(7029): p. 88-93.

389. Barber, D.L., et al., *Restoring function in exhausted CD8 T cells during chronic viral infection.* Nature, 2006. **439**(7077): p. 682-7.

390. Whalley, S.A., et al., *Evolution of hepatitis B virus during primary infection in humans: transient generation of cytotoxic T-cell mutants.* Gastroenterology, 2004. **127**(4): p. 1131-8.

391. Bertoletti, A., et al., *Cytotoxic T lymphocyte response to a wild type hepatitis B virus epitope in patients chronically infected by variant*

viruses carrying substitutions within the epitope. J Exp Med, 1994. **180**(3): p. 933-43.

392. Isa, A., et al., *Prolonged Activation of Virus-Specific CD8(+)T Cells after Acute B19 Infection.* PLoS Med, 2005. **2**(12): p. e343.

List of Companies and Reagents used

Caltag Medsystems

Fax Number	0800 2799114
GAS0025-100	Medium A
GAS0015-100	Medium B

Jerini Peptide Technologies

Fax Number	(+49) 3097 893105
PepMix IE1	
PepMix pp65	

Gibco (at Invitrogen)

Fax Number	0800 243485
21875-034	RPMI-1640 (with L-glutamine)
18265-017	Library-efficiency DH5-alpha competent cells

ProImmune Limited, Oxford, England

Fax Number	0870 7120588	
F008-2B-G	HLA A*0201 NLVPMVATV-PE	Pentamer
F045-2B-G	HLA B*0702 TPRVTGGGAM-PE	Pentamer
P008-0B-G	2mg NLVPMVATV	peptide
P045-0B-G	2mg TPRVTGGGAM	peptide

Sarstedt, Leicester, England

Fax Number	0116 2366099
01.1613.100	S-Monovette® Na-Heparin syringes 7.5ml
83.1813.302	75cm^3 vented yellow cell culture flasks
86.1254.001	10ml Serological pipettes
86.1685.001	25ml Serological pipettes
86.1171.001	3.5ml sterile wide-bore transfer pippette
62.547.254	50ml sterile Falcon® tubes
62.554.002	15ml sterile tubes

Labtech

Fax Number	
4-101-500	Foetal Calf Serum

Mabtech, Sweden

Fax Number	(+46) 87162701
3420-3-250	Anti-Hu IFNg mAb 1-D1K
3420-6-250	Anti-Hu IFNg mAb 7-B6-1 Biotin
3310-10	Streptavidin ALP-PQ

Elkay

Fax Number	01256 811116
0002005STR	12 x 75mm sterile polypropylene tubes and caps

Becton Dickinson Biosciences, Oxford, England

Fax Number	01865 781627
555029	GolgiPlug (Brefeldin A)
345770	anti-CD4 PerCP
345774	anti-CD8 PerCP
340449	anti-IFNg FITC
341652	anti-CD69 PE
348040	anti-CD28-leu
333169	anti-CD57 FITC
345767	anti-CD3 APC
342003	FACS Flow buffer
340345	FACS Clean buffer
340976	anti-CD49d
556377	anti-perforin FITC

Sigma Aldricht

Fax Number	0800 378785
D2650	DiMethylSulphOixde (DMSO)
D8537	Phosphate Buffered Saline (PBS)
S4881	Staphylococcus Enterotoxin B (SEB)
B3928	Blue-White Select screening reagent
T9650	10X TAE buffer
D3937	Direct-Load 1kb DNA ladder
T8154	1X Trypan Blue solution (0.4%)
G1146	Glutamine-Penicillim-Streptomycin
L3522	Luria Broth (Miller's LB)
A6560	Agarose (type VII) Low gelling temperature

Fisher

Cry-180-020	CryoBoxes

Amersham Biosciences

Fax Number	01494 544350
17-1440-03	Ficoll Paque Plus (Sterile, endotoxin-tested)

MWG from world-wide web

All Oligonucleotide primers were purchased here

Autogen Bioclear Limited

Fax Number	01249 817266
10-144-000	1mg CMV virus lysate
10-595-001	1mg control lysate

BDH

Fax Number	
10224BQ	Propan-2-ol (iso-propyl-alcohol)

Promega

Fax Number	
A1360	pGem-T Easy Vector system I
U1240	40 umol dNTPs (dATP, dCTP, dGTP, dTTP)

Bioline

BIO-33029	Hyperladder IV DNA marker
BIO-41025	Agarose Multi-purpose molecular grade
BIO-21065	500 units Bio-X-Act (short) DNA polymerase

Oxoid

LP0011	Agar bacterial (agar No.1)

Qiagen

27106	Qiaprep Spin MiniPrep kit
28106	Qiaquick PCR purification kit

ISL

Fax Number	01803 526776

BV5100	Disposable Counting Chambers

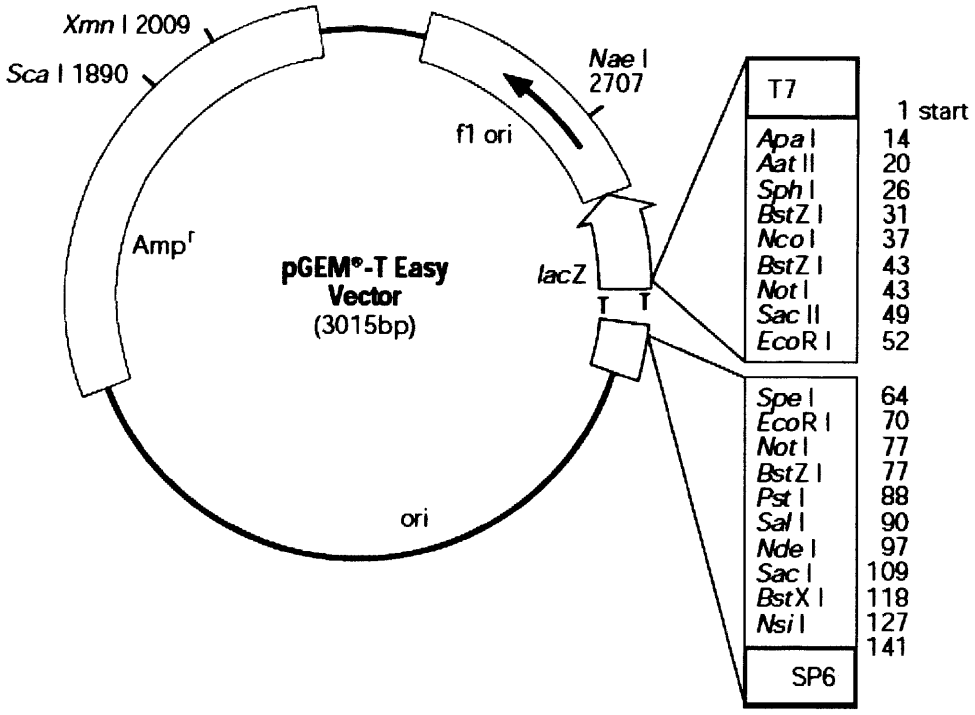
Alpha Laboratories

Fax Number

LW3332 V	1.2ml Free-standing Cryovials

Appendix

pGem-T Easy Vector system I

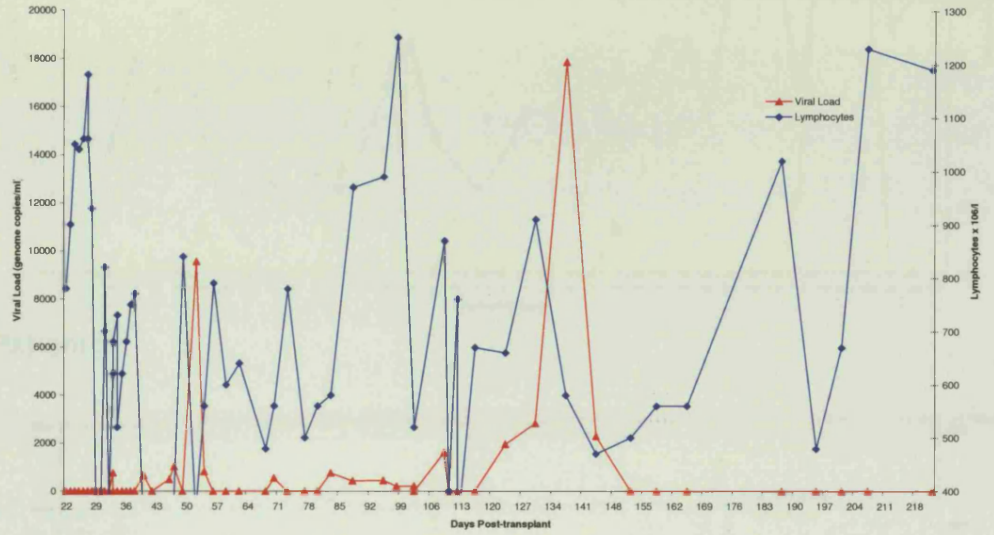


Preparation of LB plates with ampicillin

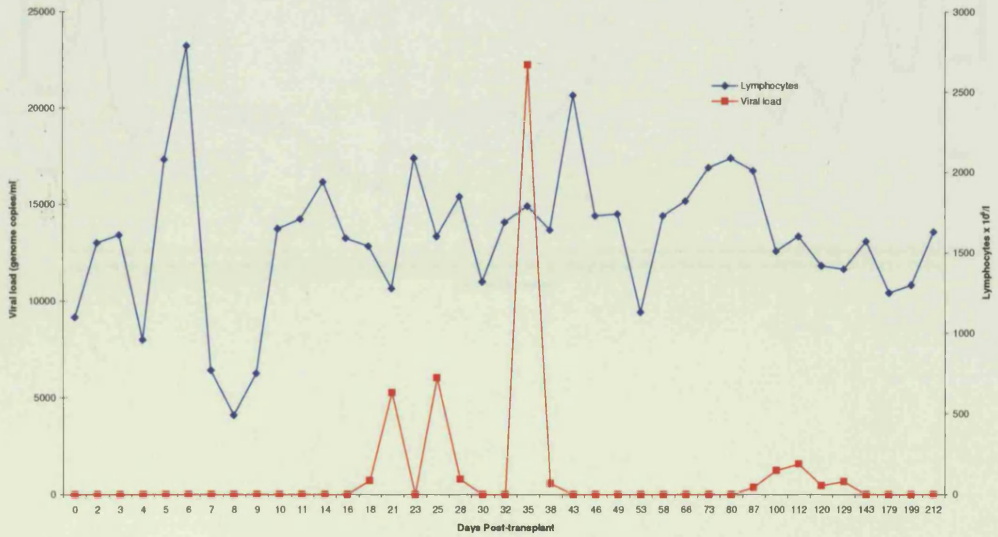
15 grams of agar were added to 1l of LB medium, which was subsequently autoclaved and allowed to cool to 50℃ before being supplemented with ampicillin to a final concentration of 100µg/ml. 30 ml of LB agar ampicillin mix was then quickly added to a 90mm Petri dish and allowed to set. 40µl of Blue-White Select™ screening seagent (Sigma-Aldrich) containing X-Gal and IPTG was then spread onto the surface of the set agar and allowed to absorb for 30 minutes at 37℃ prior to use. Plates were stored at 4℃ for up to two weeks.

Lymphocytes counts and CMV viral loads from 9 viraemic renal transplant patients **(See Table 2.2)**

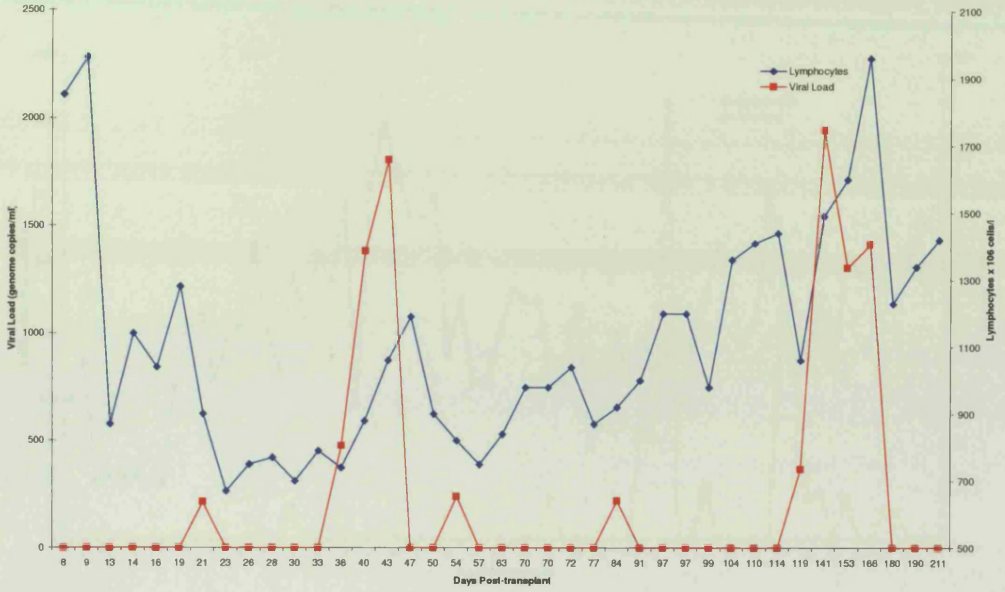
Patient 1



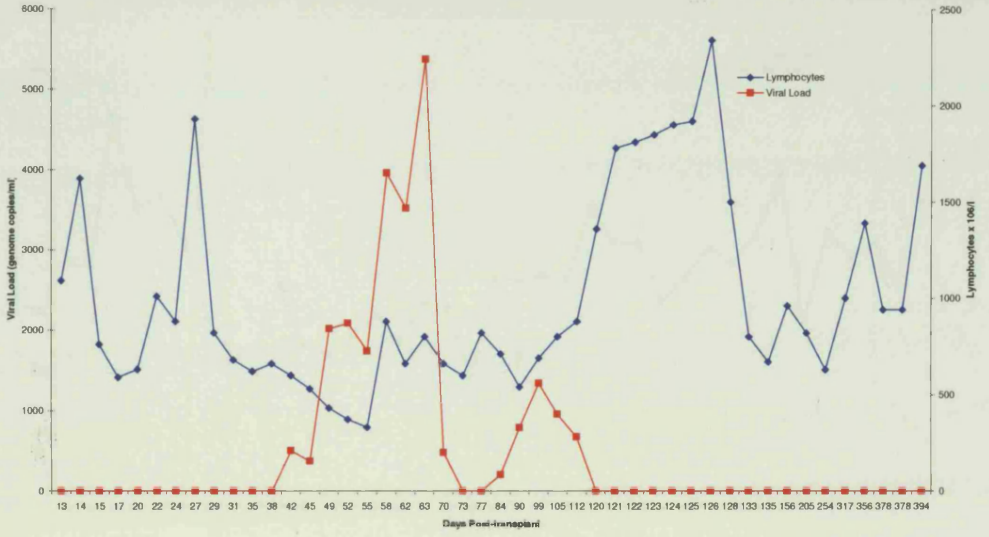
Patient 7



Patient 3



Patient 9



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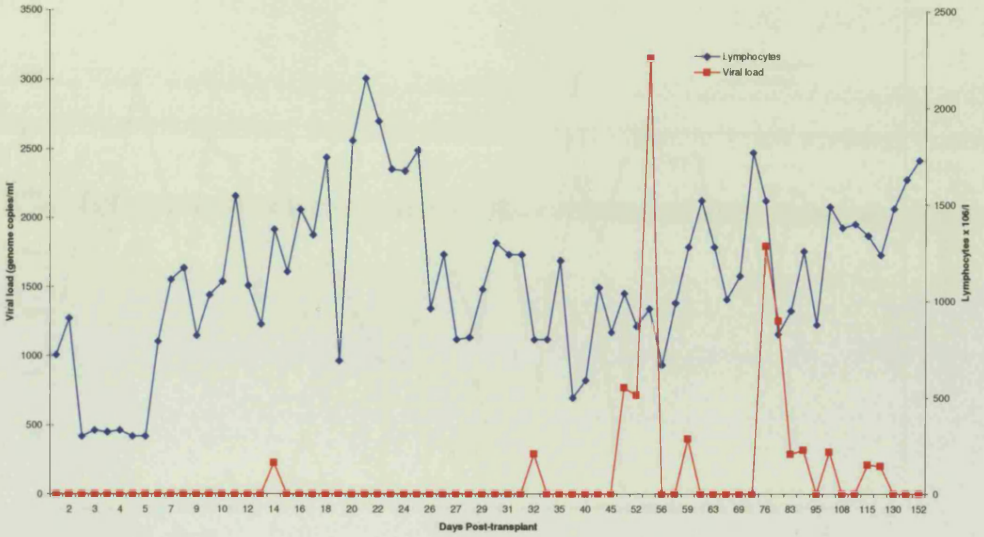

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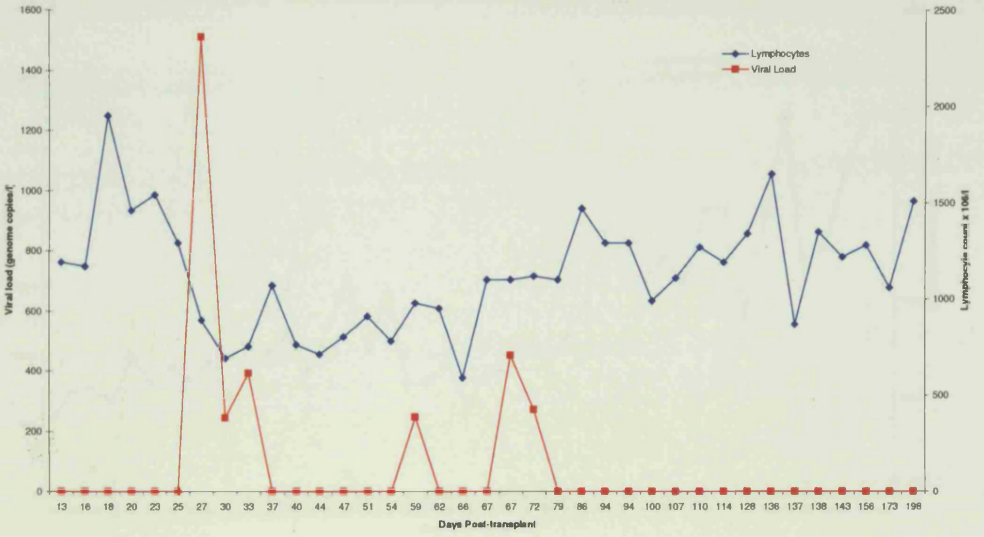
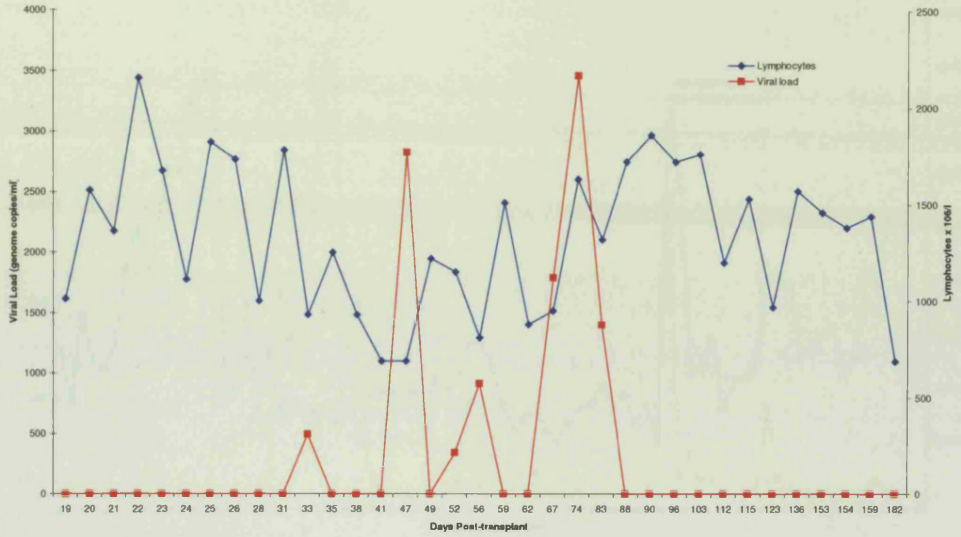
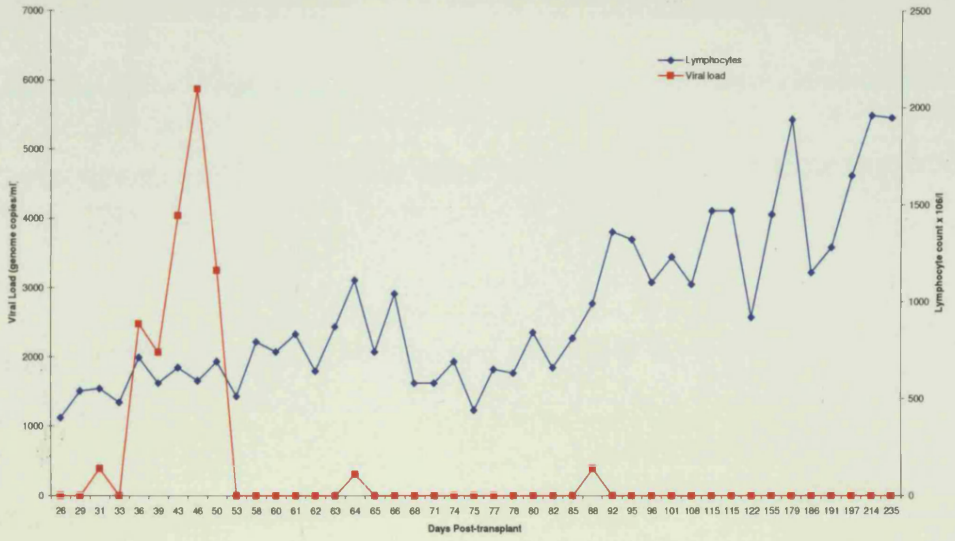


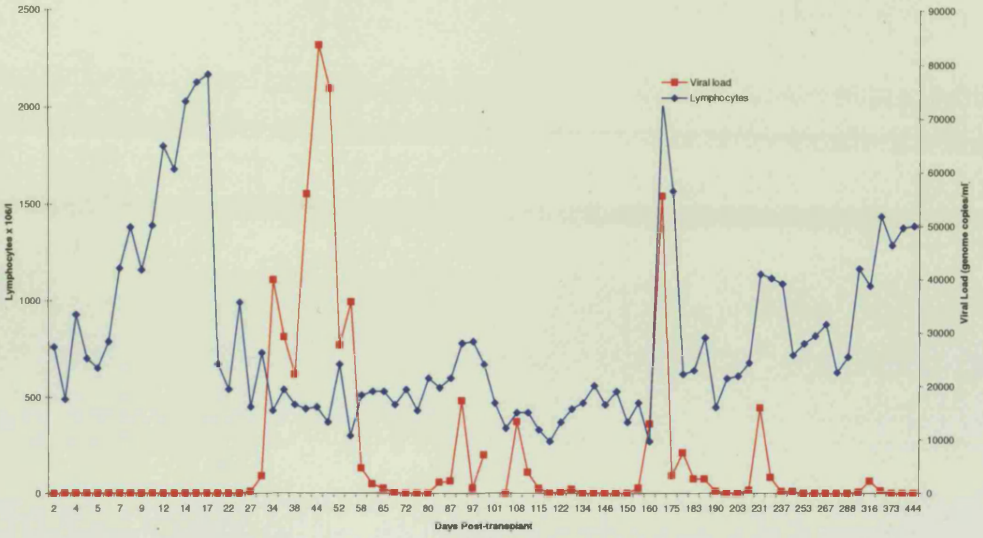
Patient 13



Patient 17



Patient 19


Viral load
Lymphocytes
Lymphocytes x 10e9
Viral Load (genome copies/ml
Days Post-transplant
2500
2000
1500
1000
500
0
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
2 4 5 7 9 12 14 17 22 27 34 38 44 52 58 65 72 80 87 97 101 108 115 122 134 146 150 160 175 183 190 203 231 237 253 267 288 316 373 444