# Characterisation of T cell responses to proteins expressed during Human Cytomegalovirus latency

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I would like to dedicate this thesis to my parents, I hope my eight years in full time education was worth it, and to the memory of my grandmother, who most certainly would not have understood a word, but would have been proud nonetheless.

# Declaration

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text. It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. It does not exceed the prescribed word limit for the Degree Committee of the Faculties of Clinical Medicine and Veterinary Medicine.

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# Abstract

Human cytomegalovirus (HCMV) is a betaherpesvirus that establishes a lifelong infection in hosts. In the majority of cases, the immune response to primary HCMV infection limits viral replication and dissemination, such that overt clinical disease is prevented. However, the immune system cannot prevent the virus establishing a latent infection, which enables lifelong persistence. Historically, a long-standing view was that viral gene expression during latency was largely absent, thus facilitating the avoidance of immune detection. However, it has now been established that viral activity in latency is far from quiescent, and the expression of a number of viral genes is known to occur. Therefore, an important question arises: are T cells specific to these proteins generated, and, if so, why are HCMV latently infected cells maintained in the face of these potential T cell responses? Previous work has shown that two such viral gene products, UL138 and LUNA, are recognised by CD4+ T cells, with a subpopulation of these cells secreting the immunosuppressive cytokines interleukin (IL)-10 and transforming growth factor beta ($\beta$). However, little is known about the host immune response to other key latency-associated viral proteins; US28, UL111A, and UL144.

Using overlapping peptide pools designed to cover the whole of the predicted amino acid sequence of these HCMV proteins, in combination with fluorescent ELIspot (FluoroSpot), ELISA, and intracellular cytokine staining, I have determined the frequency, cytokine secretion profile, effector function, and memory phenotype of CD4+ and CD8+ T cells in a large cohort of HCMV seropositive healthy donors. My results show that these viral gene products are also recognised by CD4+ T cells and are composed of distinct cellular populations secreting either IFN$\gamma$ or IL-10. The high sensitivity of this assay has also revealed previously uncharacterised CD8+ T cell responses to US28, UL111A, and UL144, as well as responses to LUNA and UL138. Intriguingly, IL-10 secretion by a distinct population of latency-specific CD8+ T cells was also observed. T cell responses to latency-associated ORF products were found to be composed of greater proportions of IL-10 secreting cells compared to responses to the lytic ORFs pp65, IE1, and gB. The frequencies of IL-10 secreting T cells specific to HCMV latency-associated proteins did not increase

with greater time of viral carriage, as measured indirectly by donor age. Although IFN$\gamma$ secreting T cells specific to latency-associated proteins were detected in kidney transplant recipients immediately following primary HCMV infection, there were no such IL-10 secreting sub-populations, despite IL-10 T cell responses being detected to two lytic proteins, US3 and pp71.

Given that latency-specific IL-10 secreting T cells were found to be a separate population to those secreting IFN$\gamma$, it was hypothesised that depleting the IL-10 secreting cells could improve the recognition and killing of latently infected cells by the specific but non-IL-10 producing T cell population. Classic regulatory T cells (Tregs) can be defined as CD4+CD127$^{lo}$CD25$^{hi}$, and depleting CD4+CD25$^{hi}$ cells was investigated as a means to remove the latent-specific IL-10 secreting T cells. The results showed that this had a variable effect, suggesting that HCMV-specific IL-10 secreting cells of this Treg phenotype represent only a proportion of the IL-10 secreting CD4+ T cell population. As this strategy could not provide a consistent method to remove IL-10 secreting CD4+ T cells, neutralising the effect of IL-10 and TGF$\beta$ using anti-cytokine and anti-receptor antibodies was tested. My initial data suggest that treatment of latently infected cells with these neutralising antibodies in the presence of CD4+ and CD8+ T cells reduced latent viral carriage.

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# Abbreviations

| | |
|---|---|
| APC | Antigen presenting cell |
| BMMNC | Bone marrow derived mononuclear cells |
| CDV | Cidofivir |
| cGAS | Cyclic GMP-AMP synthase |
| cmvIL-10 | Cytomegalovirus-encoded human interleukin-10 |
| DC | Dendritic cell |
| ELISA | Enzyme-linked immunosorbent assay |
| ELISpot | Enzyme-linked immunospot assay |
| FACS | Fluorescence activated cell sorting |
| FluoroSpot | Fluorescent ELISpot |
| gB | Glycoprotein B |
| GCV | Ganciclovir |
| GFP | Green fluorescent protein |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| HAART | Highly active antiretroviral therapy |
| HCMV | Human cytomegalovirus |
| HIV | Human immunodeficiency virus |
| HSCT | Hematopoietic stem cell transplantation |
| HSV | Herpes simplex virus |
| ICS | Intracellular cytokine staining |
| IE | Immediate early |
| IFN | Interferon |
| IgG | Immunoglobulin G |
| IL | Interleukin |
| LAcmvIL-10 | Latency-associated viral interleukin 10 |
| LPS | Lipopolysaccharide |
| LUNA | Latency unique natural antigen |
| MACS | Magnetic-activated cell sorting |
| MAPK | Mitogen-activated protein kinase |
| MCMV | Murine cytomegalovirus |
| MHC | Major histocompatibility complex |
| MIEP | Major immediate early promoter |
| NK cells | Natural killer cells |
| OAS | 2',5'-Oligoadenylate synthetase |

| | |
|---|---|
| ORF | Open reading frame |
| PAMP | Pathogen-associated molecular pattern |
| PBMC | Peripheral blood mononuclear cells |
| PBS | Phosphate buffered saline |
| PKR | Protein kinase R |
| PMA | Phorbol 12-myristate 13-acetate |
| pp | Phosphoprotein |
| PRR | Pattern recognition receptor |
| vIL-10 | Viral interleukin-10 |
| $T_{CM}$ | T central memory |
| TCR | T cell receptor |
| $T_{EM}$ | T effector memory |
| $T_{EMRA}$ | T effector memory expressing CD45RA |
| TGF$\beta$ | Transforming growth factor beta |
| Th | T helper |
| TNF$\alpha$ | Tumour necrosis factor alpha |
| $T_{NL}$ | T naive-like |
| Treg | T regulatory |
| $T_{RM}$ | T resident memory |
| UL | Unique long |
| US | Unique short |

**Table 1** List of common abbreviations

# Chapter 1

# Introduction

## 1.1 Herpesviruses and HCMV

The *Herpesviridae* are a family of viruses all known to establish a lifelong latent infection within their host. Herpesviruses are large double stranded DNA viruses with worldwide seroprevalence that infect many animal species. The *Herpesviridae* can be grouped into three subfamilies, the $\alpha$-, $\beta$-, or $\gamma$-herpesviruses, which vary in host specificity, cell tropism, rates of replication, and cytotoxicity [1]. Herpesviruses capable of infecting mammals are present in each of the subfamilies. To date nine herpesviruses have been identified for which humans are the primary host (Table 1.1). Human cytomegalovirus (HCMV) is a member of the subfamily betaherpesvirinae and is the largest human herpesvirus [2].

| **Subfamily** | **Herpesvirus** | **Abbreviation** |
|---|---|---|
| *Alphaherpesvirinae* | Herpes Simplex Virus 1 | HSV-1 / HHV-1 |
| | Herpes Simplex Virus 2 | HSV-2 / HHV-2 |
| | Varicella Zoster Virus | VZV / HHV-3 |
| *Betaherpesvirinae* | Human Cytomegalovirus | HCMV / HHV-5 |
| | Human Herpesvirus 6A | HHV-6A |
| | Human Herpesvirus 6B | HHV-6B |
| | Human Herpesvirus 7 | HHV-7 |
| *Gammaherpesvirinae* | Epstein Barr Virus | EBV / HHV-4 |
| | Kaposi's sarcoma-associated Herpesvirus | KSHV / HHV-8 |

**Table 1.1** Classification of the human herpesviruses, adapted from Crough (2009) [2, 3]

### 1.1.1 HCMV seroprevalence and transmission

HCMV infection can be measured by the presence of anti-HCMV antibodies in the blood, which informs the seroprevalence rates of the virus across populations. The proportion of HCMV infected individuals varies throughout the world, and although its presence is globally ubiquitous, prevalence is known to vary among different geographic regions. The highest seroprevalence rates are found in less developed countries such as those in Africa, South America, and Asia, where seroprevalence reaches 100%, and virus acquisition occurs from early childhood [4–6]. This contrasts with the rates observed in more developed regions, including certain areas of North America and Western Europe, where seroprevalence can be as low as 30 to 40% [4], and are much lower in children and young adults [5, 6]. Seroprevalence rates are inversely correlated with socio-economic status, with individuals of lower socio-economic status being more likely to be seropositive [4]. In addition, seroprevalence is directly correlated with age, reaching 60% or more in people over 50 [4]. Age can therefore be a strong predictor of HCMV serostatus in areas where seroprevalence is moderate to low. For example, in North America the seroprevalence of HCMV has been shown to rise steadily between childhood and those over 60 years old [4], and it increases by around 2% per year post-adolescence in North America and Northern Europe [7, 8]. A cross-sectional study conducted in the USA found that HCMV seroprevalence rates within defined age groups were fairly stable over time, indicating the age-related trends of rising seroprevalence rates were not due to earlier cohorts having a higher rate of infection during early life [9].

Infectious virus is shed in blood, semen, urine, breast milk, saliva, cervical secretion, and tears. It is thought that spread of the virus occurs naturally through close contact via saliva, or sexual exposure for example, but can also iatrogenically following blood, stem cell, and solid organ transplantation [2]. Additionally, vertical transmission can occur *in utero*, during birth or postnatally through breastfeeding.

## 1.2 HCMV virology

### 1.2.1 HCMV genome and virion structure

HCMV is the largest human herpesvirus with a genome of approximately 235Kbp, thought to encode between 175-750 open reading frames (ORFs) and at least 24 microRNAs [10–16]. The HCMV genome consists of two segments known as the unique long (UL) and the unique short (US) sequences, which are separated by repeating sections. The UL region is flanked by

terminal repeat long (TRL) and internal repeat long (IRL), whereas the US region is flanked by internal repeat short (IRS) and terminal repeat short (TRL) regions. The order of the HCMV genome is therefore TRL-UL-IRL-IRS-US-TRS.

The linear double stranded viral genome is packaged within an icosahedral capsid that is enclosed within a protein complex called the viral tegument. The capsid and tegument layers are composed of virally-encoded proteins in addition to the tegument layer containing viral and cellular RNAs, which are incorporated during the generation of new virions [17–19]. The viral tegument is composed of many proteins, the most abundant of which is the phosphoprotein 65 (pp65) (UL83) [20, 21], which carry out a diverse array of functions during initial infection and at the final stages of virion assembly. Surrounding the thick viral tegument is a lipid bilayer envelope that is formed from the ER-Golgi intermediate compartment or endosomal membranes and is modified by the insertion of viral-encoded glycoproteins, many of which mediate entry into susceptible host cells. The viral envelope is made up of both cellular proteins and virally-encoded glycoproteins, and is formed during viral budding from the endoplasmic reticulum (ER) and Golgi apparatus [22–24].

### 1.2.2 HCMV cell tropism and viral entry

During primary HCMV infection, the virus is able to spread to the majority of tissues throughout the host due to its ability to infect an extensive range of cells, including monocytes, macrophages, dendritic cells (DCs), smooth muscle, neuronal, fibroblast, epithelial, and endothelial cells [25–27]. The infection of such a diverse array of tissues underlies its pathogenesis in immunocompromised hosts (see section 1.4.1 for more detail) and aids the virus in its spread. For example, infection of epithelial cells will facilitate spread to new hosts, whereas infection of endothelial and haematopoietic cells will promote systemic spread within the host [28]. Furthermore, the tropism HCMV exhibits for such a wide range of cells, demonstrates its utilisation of multiple cell-specific receptors and/or the use of cellular receptors that are broadly expressed for entry. Indeed, HCMV has several different envelope glycoprotein complexes that facilitate this broad cell tropism.

Infection is initiated by the tethering of HCMV virions to extracellular heparin sulphate proteoglycans via one of the envelope glycoproteins, such as gB or the gM/gN complex, which stabilises the virus at the cell surface and facilitates further receptor interactions [29–31]. Subsequently, it is thought that more stable and specific interactions take place between one of the gH/gL complexes, either the trimeric gH/gL/gO or the pentameric gH/gL/UL128/130/131A envelope glycoprotein complex, with cellular entry receptors. A

number of cellular receptors have been proposed to be important for viral entry including the epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor-$\alpha$ (PDGFR-$\alpha$), and integrins, which have all been shown to bind gB or gH, in addition to PDGFR-$\alpha$ binding the trimeric gH/gL/gO complex [32–38]. Despite this, questions remain over the role these receptors have in viral entry [39–41]. Following this stable interaction, the gH/gL complex, together with gB, is thought to mediate fusion of the viral envelope with the cellular membrane, most likely through integrin-mediated interactions, for entry into fibroblasts [32, 42–45]. In contrast, entry into epithelial and endothelial cells is dependent on the pentameric gH/gL/UL128/130/131A complex [46, 47].

The use of HCMV in cell culture has revealed its propensity to rapidly acquire mutations during *in vitro* passage. Such mutations are known to occur in these glycoprotein complexes. Mutants that are unable to form the gH/gL/UL128/130/131A pentamer lose their broad cell tropism, rendering them unable to infect epithelial, endothelial, monocytic, and DCs *in vitro*, but still retain the ability to infect fibroblasts [48–52]. Thus, as a consequence of passage through fibroblasts, certain laboratory strains of HCMV, which have resulting mutations in the ULb' locus containing the UL128-131 genes, will be varied in their tropism for epithelial and myeloid lineage cells [48, 49, 53]. Although propagation of HCMV in fibroblasts does not select for viruses with endothelial tropism, passage through endothelial cells can maintain the wide range cellular tropism [54, 55].

### 1.2.3 The lifecycle of HCMV

Following membrane fusion, the tegument is lost from the viral capsid. The liberated tegument proteins then facilitate the microtubule-mediated trafficking of the capsid to the nucleus, the release of the viral genome from the capsid, and the activation of viral gene expression [56–60]. Genome replication can now take place. Lytic infection is characterised by a temporal cascade of gene expression in three phases: the immediate early (IE) phase, the early phase, and the late phase. During the IE phase, the most abundant gene transcription occurs from the Major Immediate-Early promoter (MIEP). Alternative splicing of the UL123 and UL122 regions give rise to the IE72 (IE1) and IE86 (IE2) proteins respectively, which act as transcriptional regulators by transactivating many HCMV genes and promoters, allowing a progression into early and late phase [61–63]. IE2 is the main transcriptional activator of HCMV early genes and has been shown to synergise with IE1 at low multiplicity of infection (MOI) [61, 64–66]. Additionally, IE genes products can alter cell cycle progression [67] and prevent expression of certain cellular antiviral genes [68–71]. Moreover, IE1 is able

to disrupt ND10, also known as promyelocytic leukemia protein (PML) nuclear bodies to counter cellular restriction factors [72–79]. During the early phase of lytic cycle genes which are critical for successful viral replication are expressed, including those involved in DNA synthesis and immune modulation [80]. Finally, from around 48 hours post infection, late genes are expressed, the products of which are predominantly structural proteins of the virus required for the assembly of infectious virions and egress from the cell [24].

Recent work utilising a temporal viromics approach, where mass spectrometry was performed following the administration of protein synthesis or viral DNA synthesis inhibitors, has extended the phases of viral gene expression into 5 temporal protein profiles, which map well to the established phases of viral gene expression [81].

## 1.3 HCMV latency

### 1.3.1 Sites and establishment of HCMV latency

All herpesviruses can establish a latent infection, which enables the virus to persist for the life of the host. Latency is defined as the maintenance of viral genome in an infected cell without the production of infectious virions, but with the ability to reactivate given certain stimuli into a full lytic infection. Lytic infection is then characterised by the expression of all viral genes and the production of infectious virus. Herpesviruses have particular sites of latency, which often differ between, and within, subfamilies [82–86].

Leukocytes were identified as one potential reservoir of HCMV infection as a result of the link between unscreened blood donations and viral transmission, which then led to findings showing such transmission is reduced when transfused blood has been leukocyte depleted [87–90]. It was suspected that the carriage of virus in leukocytes was latent because infectious virus could not be isolated from healthy donors [91]. However, attempts to detect HCMV DNA, RNA, or protein were not reproducible, in part because the methods used were not sensitive enough [92]. The advent of PCR enabled the amplification of specific DNA sites, and thus partially overcame the problem of very low copy number of the virus in peripheral blood, where during latency between every 100-5000 monocytes is infected, therefore enabling the interrogation of the latent sites of HCMV infection [91–93].

The broad cell tropism of HCMV enables it to lytically infect a wide range of cells. Sites of latent infection are more restricted however. The use of highly-sensitive DNA PCR has revealed that one site of latent viral carriage is in cells of the early myeloid lineage, such as

CD34+ progenitor cells and CD14+ monocytes, which in the peripheral blood is the principle cell type carrying HCMV DNA [94–96]. Crucially, the presence of viral genomes in CD34+ cells and monocytes was shown to be in the absence of extensive lytic gene transcription, a necessity for true sites of latency [95, 94, 97]. CD34+ cells are a self-renewing population of stem cells present in the bone marrow, which are the progenitors of all blood cell lineages [98]. CD34+ cells can differentiate down the myeloid lineage, to monoblasts followed by promonocytes in the bone marrow, before entering the bloodstream where they lose their CD34 cell-surface antigen and develop into monocytes [99]. The progress of this lineage development is dependent on exposure to haematopoietic growth factors, in particular GM-CSF and IL-3 [100]. Despite the ability of CD34+ cells to differentiate into lymphoid cells and polymorphonuclear leukocytes, the viral genome has not been detected in these cells [95, 101]. Similarly, CD34+ cells are able to differentiate into endothelial cells, and it has been suggested these could also be a site of latency, especially given the HCMV-associated pathologies observed in these cells [102–104]. Currently however, the evidence for this is somewhat inconclusive. HCMV was not detected in endothelial cells isolated from saphenous vein tissue of healthy seropositive individuals [105], but *in vitro* studies suggest that certain subsets of endothelial cells, such as aortic, could potentially support a latent infection [106]. Unfortunately, carrying out this research is made difficult by the ethical issues of analysing such tissues, in addition to the unsuitability of cadaverous tissue for study due to reactivation of latent virus at death [107].

### 1.3.2 Reactivation from latency

The ability of HCMV to reactivate from sites of latency is crucial to its strategy of lifelong persistence in the infected host, where subclinical reactivation events could enable the virus to replenish the pool of latently infected cells [108]. It is thought that the key trigger for viral reactivation from latency is cellular differentiation. Insights into this process came from the observations that while monocytes were non-permissive to experimental infection, differentiation of monocytes to macrophages led to a fully permissive phenotype [109, 110]. As a result, it was suggested that differentiation of myeloid lineage cells induces viral reactivation. Data from *ex vivo* studies have shown that differentiation of monocytes to macrophages leads to reactivation of viral IE gene expression [111] and production of infectious virus [112]. Likewise, *ex vivo* differentiation of CD34+ cells to DCs leads to full reactivation of infectious virus from naturally infected individuals [113]. Consistent with the view that differentiated myeloid cells reactivate latent HCMV, *in vivo* differentiated alveolar macrophages and DCs were found to express IE, whereas isolated monocytes that carried

genome did not, in addition to recovering infectious virus from the alveolar macrophages [114, 115].

### 1.3.3 The role of chromatin in latency and reactivation

Lytic HCMV infection is characterised by a temporal cascade of viral gene expression. The initial genes expressed in this cascade, the IE genes, are under the control of the Major Immediate Early Promoter (MIEP). During latency, the constraint on gene expression is thought to be achieved in large part through the suppression of the MIEP, as repression of IE genes will prevent E and L genes from being expressed. Repression of the MIEP is mediated by a number of mechanisms, all functioning to keep the virus in latency. A shift in the balance between repressive and activatory factors will subsequently lead to an active promoter state on the MIEP, allowing lytic gene expression to occur. One such factor is the chromatin structure of the MIEP, which is closely linked to the viral state within the infected host cell. In early myeloid lineage cells (CD34+ progenitors and monocytes), the MIEP is associated with repressive chromatin that suppresses lytic gene expression, and keeps the virus in latency. Such repressive post-translational modifications of MIEP-associated histones found during latent infection include trimethylation of histone H3 (lysine 9 and 27), the recruitment of heterochromatin protein-1 (HP-1), and a lack of acetylation on histone H4 [116–118]. The repressive chromatin structure however, is altered following the differentiation of myeloid progenitor cells into terminally differentiated macrophages or DCs, allowing activation of the MIEP and viral IE gene expression, with subsequent reactivation of lytic infection and production of infectious virus [114, 116, 118, 119]. Thus, the differentiation dependent presence of repressive or activatory chromatin marks around the MIEP is strongly related to the latent or lytic phenotype of the virus. The chromatin-mediated suppression of lytic gene expression has consequently been exploited to target latent HCMV. Inhibition of the enzyme histone deacetylase (HDAC)-4 resulted in transient IE expression in latently infected cells without full lytic reactivation, which then enabled these cells to be targeted by cytotoxic T lymphocytes (CTLs) [120].

### 1.3.4 Gene expression during latency

The extent of HCMV activity during latency has been subject to much debate, but there is growing evidence that the virus can carry out considerable cellular modifications in this

state. Gene expression during latency is thought to function to maintain the carriage of viral genomes, resist intrinsic cell death responses, and evade the host immune system [121–123].

During latent infection, it might be desirable for the virus to be in a completely quiescent state, where viral proteins are not expressed, to prevent the processing and presentation of viral proteins to the immune system and allow the virus to remain undetected by T cells. Evidence now suggests that this is not the case for HCMV, and there is routine expression of a several viral transcripts resulting in protein expression, as has been well established for other herpesviruses such as EBV [124]. Gene expression during latency was thought to be severely restricted in comparison to during lytic infection. A considerable body of work has shown that only a small subset of viral genes are expressed, with an absence of expression of the major lytic genes such as IE, caused by the repression of the MIEP [122]. The work to identify these has resulted in the consistent detection of a handful of genes in naturally latent cells, and include *US28* [125], *LUNA* [126], *UL111A* [127], *UL144* [125], and *UL138* [128]. Although the full roles of the products of these genes are still to be elucidated, a variety of functions for their gene products have been described, both during latent and lytic infection. However, more recent work utilising highly sensitive RNA-sequencing techniques has unbiasedly detected expression of a broader set of transcripts during natural and experimental latency that posits a contrasting view on the level of transcriptional activity during latent infection [129, 130]. The analysis of the transcriptome from single cells in this manner revealed a gene expression profile during latency similar to that found during the late stage of lytic viral infection, only with lower levels of expression [130].

### UL138

The sequential knockout of genes in the ULb' region of HCMV revealed the importance of UL138 for the establishment of a latent infection [128], and subsequent work has shown that UL138 contributes to the transcriptional repression of IE gene expression to achieve this [131]. UL138 does this by inhibiting the recruitment of cellular lysine demethylases to the MIEP, therefore preventing the removal of repressive histone methylation and subsequent activation of viral IE gene expression [131]. Recently, UL138 was shown to be expressed as two isoforms that have overlapping functions in IE repression and prevention of infectious virion production, but might act at different time points during latency [132].

UL138 is also known to possess a number of other functions. UL138 encodes a transmembrane protein that interacts with the tumour necrosis factor (TNF) receptor 1 (TNFR1), increasing and extending its expression on the cell surface [133, 134]. The

upregulation of TNFR1 and resulting increase in sensitivity of latently infected cells to TNF$\alpha$, which has cell-death, as well as immunostimulatory and antiviral properties, might seem to be counter-productive to the virus. However, it is hypothesised that this could be a mechanism to aid in reactivation of the virus from latency given the ability of TNF$\alpha$ to trigger monocytic differentiation, a known site of latency *in vivo*, into macrophages [135]. Additionally, TNF$\alpha$ can activate NF$\kappa$B, and as the MIEP of HCMV is known to have several NF$\kappa$B target sites, TNF$\alpha$ could be directly stimulating IE expression, as has been shown *in vitro* [136]. By increasing the sensitivity of a latently infected cell to TNF$\alpha$, the expression of UL138 during latency could therefore be acting at the level of the cell, by promoting differentiation and therefore viral reactivation, and at the level of the virus, by inducing MIEP activation and an associated switch to lytic infection. UL138 can also reduce the surface expression of multidrug resistance-associated protein-1 (MRP1) by targeting it for lysosomal degradation [137]. The loss of MRP1 reduces the infected cell's ability to export certain MRP1 specific substrates, and in the case of leukotriene C4, this might function to prevent the migration of infected DCs to secondary lymphoid organs where HCMV-specific immune responses would be generated [137].

### UL144

The UL144 ORF encodes a transmembrane glycoprotein with structural homology to the TNF receptor Herpesvirus Entry Mediator (HVEM/TNFRSF14) and is expressed early during lytic infection [138]. There is considerable sequence variation in UL144 between clinical isolates of HCMV [139], and polymorphisms within UL144 have been linked to worse clinical outcomes following congenital HCMV infection [140, 141], although this link has been disputed [142]. UL144 is responsible for the upregulation of CCL22 during lytic infection, via potent NF$\kappa$B activation [143, 144]. CCL22 acts as a chemoattractant for Th2 and regulatory cells T cells (Tregs) [145, 146], and this could be a mechanism employed by the virus to dampen Th1-mediated responses. UL144 also acts by binding the B and T lymphocyte attenuator (BTLA), and this could inhibit T cell proliferation [147].

Much less is known about the role of UL144 during latent HCMV infection. UL144 has been shown to be dispensable for the establishment and reactivation from latency, and its expression during latency occurs in an isolate specific manner [125]. A comparison of the promoter regions of UL144 from different viral isolates, both experimental strains and those from natural infection, suggested this was partially due to the presence or absence of GATA-2 binding sites [125].

### LUNA

The UL81-82 locus which encodes the tegument protein pp71 also gives rise to an antisense transcript, predicted to code for a 133 amino acid long protein termed Latency Unique Nuclear Antigen (LUNA) [148]. Experimental models of latency point to a role for LUNA in latent viral carriage and virus reactivation. The promoter of LUNA contains functional GATA-2 binding sites, similar to those found in the UL144 promoter, that enhance expression of LUNA during latency, perhaps pointing to a common mechanism of gene regulation as GATA-2 is upregulated during latent infection [125]. During lytic infection, LUNA was found to be dispensable for virus replication, as a LUNA-deficient virus produced similar levels of infectious particles to wild-type [149]. Furthermore, the expression kinetics and levels of an IE gene UL123 (IE1) and a late gene UL82 (pp71) were comparable to wild-type during lytic infection of fibroblasts.

The expression of LUNA was found to be required for the reactivation of HCMV from latency, as infection of CD14+ monocytes with a LUNA-deficient virus resulted in an inability to reactivate following differentiation, despite the maintenance of viral genomes [149]. Additionally, there were considerable reductions in the expression levels of UL138 during latent infection of CD14+ monocytes and lytic infection of fibroblasts with the LUNA-deficient virus [149]. Consistent with these findings, use of a virus that was mutated to prevent LUNA expression had little impact on lytic replication or carriage of latent viral genomes in CD34+ cells, and UL138 expression was also decreased on infection with this LUNA-disrupted virus [125]. Similarly, there was a considerable defect in the reactivation of virus from CD34+ cells latently infected with the LUNA-mutated virus [150]. Investigations into the mechanisms by which LUNA facilitates viral reactivation uncovered the ability of the LUNA encoded gene product to act as a deSUMOylase [150]. The formation of antiviral ND10 bodies is dependent on PML SUMOylation [151], and LUNA was found to mediate the dispersal of ND10 through its deSUMOylation of PML. In doing so, HCMV therefore ensures that reactivating virus, which is re-entering lytic cycle, is not subject to the antiviral effects of ND10 and can reactivate efficiently [150].

### UL111A

Viruses capable of expressing homologues of immunomodulatory cellular cytokines can use these to deflect immune responses and increase virus survival. IL-10 is one such immunosuppressive cytokine that has been exploited by HCMV, in addition to other viruses, such as EBV [152, 153], to subvert host immune responses. In HCMV, the UL111A gene

encodes two homologues of human IL-10 (hIL-10) that have slightly different properties. During lytic infection, the homologue expressed from UL111A is the 175-amino acid protein cmvIL-10, which shares 27% sequence homology with hIL-10, and can to bind to, and signal through, the hIL-10R to mediate many of the same immunomodulatory effects as hIL-10 [154]. This includes suppression of proinflammatory cytokine production and inhibition of PBMC proliferation [155], as well as downregulation of MHC class I and II expression [155, 156] and stimulation of B cells [157]. cmvIL-10 can also upregulate hIL-10 secretion from certain myeloid cells, giving it a mechanism to potentially amplify its immunosuppressive effects [158].

During latent infection however, alternative splicing results in the expression of an alternate transcript from UL111A, termed latency-associated cmvIL-10 (LAcmvIL-10). LAcmvIL-10 is collinear with cmvIL-10 at the amino terminus, but a lack of splicing of a second intron results in the coding of an in-frame stop codon, generating a predicted 139-amino acid protein that is truncated at its C terminus [127]. LAcmvIL-10 is expressed during both lytic and latent infection, and is similar to cmvIL-10 in its ability to downregulate MHC class II in latently infected granulocyte/macrophage progenitor cells [127, 156, 159]. Recently, an assay capable of detecting both cmvIL-10 and LAcmvIL-10, although unable to distinguish between them, found both isoforms in the peripheral blood of healthy HCMV seropositive donors [160]. Furthermore, LAcmvIL-10 also suppresses transcription of the master regulator of MHC class II components, the class II transactivator (CIITA), indicating that this downregulation of MHC class II occurs mainly at the level of mRNA transcription [156]. The accumulation of MHC class II molecules in cytoplasmic vesicles in a small percentage of monocytes treated with LAcmvIL-10 also hints at the ability of LAcmvIL-10 to target MHC class II expression post-translationally [156]. Although the ability to downregulate MHC class II expression on myeloid cells is shared between cmvIL-10 and LAcmvIL-10, LAcmvIL-10 does not appear to exhibit the full range of functions that cmvIL-10 possesses. For example, the inhibition of LPS-induced maturation of DCs treated with cmvIL-10 [161, 162] cannot be replicated with LAcmvIL-10 [156]. Furthermore, unlike hIL-10 and cmvIL-10, LAcmvIL-10 does not stimulate proliferation of B cells [157] or increase Fc$R\gamma$ mediated phagocytosis by human monocytes [163]. It is unclear why the differences between LAcmvIL-10 and cmvIL-10 functionality exist, but they could in part be due to the differences in signalling induced upon receptor binding. The mechanism by which LAcmvIL-10 signals through the human IL-10R is unknown, and it is thought it either does not bind, or it engages the receptor in a different way to both cmvIL-10 and hIL-10 [157]. This would be in contrast to cmvIL-10, which mediates its immunomodulatory effects via signalling through the hIL-10R. In this respect, cmvIL-10 induces the phosphorylation of

Stat3, whereas LAcmvIL-10 does not, but still causes a down-regulation of MHC class II, even in the presence of anti-human IL-10R antibodies [156].

UL111A has been shown to be important for the establishment of latency in CD14+ monocytes and CD34+ progenitor cells, most likely through enhancing infected cell survival [164]. UL111A functions as an immunomodulatory agent during latency to evade CD4+ T cell recognition by downregulating MHC class II on infected cells, therefore suppressing the ability of these cells to present viral peptides [164, 165]. In addition to this, UL111A can inhibit the production of proinflammatory cytokines such as TNF$\alpha$ and IL-1$\beta$ from latently infected CD34+ myeloid progenitor cells and to suppress their differentiation to DCs [166]. Given that DCs are the most potent antigen presenting cells (APCs), preventing differentiation of latently infected cells to DCs could be another mechanism to limit the presentation of viral antigens to the immune system [166]. Moreover, LAcmvIL-10 has also been shown to be involved in the regulation of cellular miRNA expression, which results in changes to secreted cellular proteins. For example, LAcmvIL-10, via a decrease in the cellular microRNA hsa-mir-92a, upregulates the expression of hIL-10 in CD14+ monocytes, which could be a mechanism of amplifying the effects of LAcmvIL-10 [164]. A similar effect of cmvIL-10 on hIL-10 expression has also been reported, although it is likely to act via a different mechanism to LAcmvIL-10 [158].

In addition, the UL111A gene products can modulate myeloid cell differentiation. Exposure of CD14+ monocytes to a combination of cmvIL-10 and LAcmvIL-10, or supernatant from productively infected fibroblasts, induced polarisation to the immunosuppressive M2c macrophage phenotype that reduced CD4+ T cells activation and proliferation [167].

### US28

US28 is a cell surface G protein-coupled receptor and viral chemokine receptor homologue that can bind both CC (e.g., CCL2 [MCP-1], CCL5 [RANTES]), and CX3C (e.g. CX3CL1 [Fractalkine]) chemokines. US28 is expressed early during lytic infection [168, 169] and is thought to be mainly localised to endocytic vesicles, with around 20% present on the plasma membrane [170]. Ligand binding induces internalisation of US28, endocytosis, and recycling from the cell surface [168, 171]. Following this, US28 is then recycled back to the cell surface [168, 171]. Given the ability of US28 to bind and internalise multiple cytokines, it has been proposed that this receptor acts as a chemokine sink, therefore reducing the local concentration of inflammatory and chemotactic cytokines, which aids in immune evasion

through the inhibition of antiviral immune responses. In support of this, HCMV infected fibroblast have reduced production of cytokines CCL2 and CCL5, suggested to be caused by US28 expression [171, 172]. This model of chemokine sequestration has been challenged however, as the overexpression of US28 in endothelial cells was found to be insufficient to prevent chemokine mediated adhesion of monocytes to endothelial monolayers, suggesting that the local concentrations of cytokine are too high for US28 to have a significant effect [173].

During lytic infection, US28 signals constitutively, resulting in the promotion of proliferative signals such as NF-$\kappa$B and MAP kinase (MAPK) which are known to transactivate the MIEP. US28 is known to be expressed during latency [174, 121, 125], and it has been shown that US28 is required for the establishment and maintenance of a latent infection [175]. In this study, a US28 knockout virus was unable to establish latency in CD34+ progenitor cells due to its inability to repress MIEP-driven transcription [175]. Although these roles of US28 contrast, where US28 activates the MIEP during lytic infection, but inhibits the MIEP during latent infection, recent work has shown that US28 has divergent effects on cell signalling in undifferentiated compared to differentiated cells [176]. In undifferentiated cells, where the virus establishes a latent infection, US28 attenuates NF-$\kappa$B and MAPK signalling, which supports the epigenetic silencing of the MIEP, thus preventing lytic infection. In differentiated cells however, US28 activates these signalling pathways, to help drive IE gene expression and productive viral reactivation [176].

## 1.4 Pathogenesis and treatment of HCMV infection

### 1.4.1 HCMV infection and disease

Primary infection of an immunocompetent host with HCMV is generally asymptomatic or in some cases results in a mononucleosis syndrome [2]. This is a result of the immune response to HCMV which is capable of limiting lytic viral replication and preventing clinical disease in the majority of cases. Although in a healthy host HCMV infection is typically asymptomatic, it is of great clinical concern for those with an immature immune system, or an immune system that is suppressed, either by drugs or by co-infection with another pathogen. It is in these individuals, where the immune system is unable to control HCMV replication, either during primary infection or during viral reactivation, where disease occurs [2]. The broad

cell tropism of HCMV leads to a wide array of symptoms, which can culminate in organ failure [28].

Congenital HCMV infection can lead to severe morbidity and mortality in newborns. Of congenitally infected babies that are symptomatic at birth, there is a mortality rate of around 30% in the most severely affect infants [177]. Surviving newborns can exhibit any of a number of serious neurological, hearing, and developmental impairments [177]. Both the risk and severity of symptomatic congenital HCMV infection are highest following primary infection in a seronegative mother during the first trimester [178]. However, the reactivation of latent HCMV, or infection with a different viral strain, during pregnancy can still lead to symptomatic congenital infection [179, 180]. Around 85-90% of congenitally infected babies are asymptomatic at birth [177], and 10-15% of these go on to develop one or more long-term neurological sequelae, including sensorineural hearing loss [181].

The impairment of the adaptive immune system due to HIV infection can also lead to HCMV disease. Prior to the introduction of highly active antiretroviral therapy (HAART) to treat HIV, approximately 40% of HIV-infected patients with advanced stage disease suffered from HCMV disease over their lifetime [182]. The use of HAART has led to a significant decline in the incidence of HCMV disease in this group through its ability to suppress HIV viraemia and reconstitute CD4+ T cell immunity [182]. Despite this being the case, there is evidence suggesting that HCMV infection can accelerate the progression to AIDS and death [183–186]. HCMV retinitis remains a problem in HIV-infected individuals despite a decline of between 80 and 90% in the HAART era [182, 187], and the use of HAART has been linked to a new clinical problem, "immune recovery uvitis" (IRU). IRU is characterised by an increase in intraocular inflammation following HAART, thought to be due to the recovered immune responses to accumulated HCMV antigens in the eye [188, 189].

Following transplantation, the requirement to suppress T cells capable of mediating graft rejection has the undesired effect of inhibiting antiviral T cells. In solid organ transplantation such as the kidney, the greatest risk of developing HCMV disease arises in seronegative recipients (R-) transplanted with an organ from a seropositive donor (D+) [190]. In these D+R- cases, the donor organ is highly likely to contain latent virus, which is then transplanted into a recipient with no prior antiviral immunity. HCMV has also been linked to a greater risk of graft rejection or long-term graft dysfunction, although this remains controversial in the absence of causative evidence [191–195].

During haematopoietic stem cell transplantation (HSCT), the recipient immune system is ablated and reconstituted with the donor immune system. As a result, the greatest risk

for HCMV disease arises in situations where the donor is seronegative, but the recipient is seropositive (D-R+). This is because the donor does not possess HCMV-specific T cells, whereas the recipient harbours latent virus and is receiving immunosuppressive treatment [196]. Primary infection or reactivation of latent virus can therefore lead to serious morbidity and mortality, with viral reactivations in the seropositive recipient accounting for the majority of HCMV infections [197–199].

### 1.4.2 Treatments for HCMV disease

Ganciclovir (GCV) is the current therapy of choice for HCMV infections, and is used routinely for treatment, in addition to prophylactic and preemptive therapy [200]. GCV is a nucleoside analogue that ultimately targets the HCMV DNA polymerase (encoded by the UL54 gene), following a series of stepwise phosphorylations, to ganciclovir triphosphate. The initial phosphorylation step is catalysed by the HCMV-encoded UL97 kinase followed by subsequent phosphorylation steps mediated by cellular kinases [201–203]. Once ganciclovir triphosphate is formed, it acts as a substrate for HCMV DNA polymerase, competitively inhibiting viral DNA elongation [204, 205]. As the first phosphorylation event is dependent on the viral kinase UL97, GCV is able to selectively target infected cells. Resistance to GCV is observed following prolonged use and even after several months of therapy, with characteristic mutations commonly arising in UL54 and UL97 [206–208]. Valganciclovir, a prodrug of GCV, is also used to treat HCMV, and was developed with a greater oral bioavailability in comparison to GCV [209–211]. Second-line drug therapies include foscarnet (FOS) and cidofivir (CDV), which also inhibit HCMV DNA polymerase activity [212–214], although nephrotoxicity, and low oral bioavailability in the case of CDV, limit their clinical utility, often to when resistance to GCV is suspected [200]. Treatment with FOS and CDV can also lead to drug resistant HCMV infections [200]. Recently, letermovir, which is an HCMV DNA terminase complex inhibitor, and therefore acts to inhibit the packaging of DNA into the virion, was approved for use as a prophylactic in adult seropositive recipients of allogeneic HSCT [215–217]. As the mechanism of action of letermovir differs from GCV, CDV, and FOS, which are DNA polymerase inhibitors, its use does not result in the development cross-resistance to these other drugs [216].

### 1.4.3 Vaccines against HCMV

HCMV can cause severe morbidity, and even mortality, in the immunosuppressed and immunonaive populations. Currently there are no licensed vaccines for HCMV, despite it being ranked at the highest priority in a review by the Institute of Medicine in 1999, which was based on the economic costs and prospective years of life and disability that would be saved by a successful vaccine [218].

Initial efforts in the 1970s to develop a vaccine were focused on live attenuated viruses derived from strains AD-169 and Towne [219, 220]. The Towne strain of HCMV was generated following 125 passages in human diploid fibroblasts [220]. The Towne vaccine was found to be immunogenic [221, 222], and reduced the severity of HCMV disease in kidney transplant recipients, but did not reduce the rate of infection post-transplantation, generated a limited neutralising antibody response compared to wild-type infection, and also failed to prevent vaccinated mothers from acquiring HCMV [223–226]. In an effort to improve the immunogenicity of Towne while maintaining its safety profile, recombinants between Towne and the low passage Toledo isolate were constructed [227]. Four recombinant virus vaccines have been tested, first in seropositive [228], and seronegative [229] subjects, demonstrating safety and tolerability that warrant further trials. In HCMV seronegative individuals, no viral shedding was observed in urine or saliva, and two chimeras showed superior abilities to induce seroconversion [52]. CD4+ and CD8+ T cell responses were also detected in a handful of vaccine recipients [52].

Development has also focused on recombinant vaccines utilising the highly immunogenic viral antigen gB. Several trials of a gB subunit vaccine utilising recombinant gB in conjunction with the oil in water adjuvant MF59 have been carried out at phase II [230–234]. The vaccine showed 50% efficacy against primary HCMV infection in seronegative mothers [235, 231], a 45% efficacy in adolescent girls (although this did not reach significance) [232], and reduced viraemia in kidney and liver transplant patients with a corresponding drop in GCV treatment [233].

DNA vaccines are also in development, which have garnered interest for their potential to elicit CD8+ T cell responses. These consist of plasmids containing immunogenic proteins such as gB and pp65, and have been trialled with varying successes so far [236–238]. Other potential vaccine candidates in earlier stages of development include RNA-based vaccines utilising self-replicating RNA [239, 240], and vaccines based on dense bodies, which are noninfectious enveloped particles that possess viral glycoproteins and tegument proteins but no viral DNA, making them safer vaccine candidates [241–243].

A successful vaccine against HCMV will likely need to induce robust cellular and humoral responses to provide protection [244]. Given the observed ability of HCMV to superinfect an already infected host [245], which can lead to a multi-strain infection [179], such a development could be even more challenging, as vaccination would need to function to elicit protective immunity in HCMV-naive individuals, and boost immunity in those already infected to provide protection against superinfection and viral reactivation. Antivirals will therefore continue to play an important role in treating HCMV infections, and deepening our understandings of the mechanisms of HCMV persistence during latency could generate novel targets against the virus.

## 1.5 The immune response to HCMV infection

### 1.5.1 The innate immune response to HCMV

The infection of host cells by HCMV triggers rapid intracellular immune responses which attempt to establish an antiviral state in the infected and surrounding cells. Sensing of HCMV is largely initiated by pattern recognition receptors (PRRs) detecting evolutionarily conserved pathogen-associated molecular patterns (PAMPs) from HCMV. Toll-like receptor (TLR) 2 expression at the plasma membrane has been shown to recognise the HCMV glycoproteins gB and gH, resulting in the activation of the NF-$\kappa$B pathway and subsequent production of inflammatory cytokines including IL-6, IL-12, and interferon-(IFN)$\beta$ [246–248]. Intracellularly, multiple sensors are capable of recognising HCMV DNA including cyclic GMP/AMP synthase (cGAS) and Z-DNA-binding protein 1 (ZBP1). Studies on vascular endothelial cells and monocyte-derived DCs and macrophages have reported that recognition of HCMV DNA in the cytosol by cGAS leads to the expression of type I IFNs via the transcription factor IFN regulatory factor 3 (IRF3) [249, 250]. HCMV also activates IRF3 following the sensing of cytoplasmic dsDNA by ZBP1, leading to the transcription of IFN-stimulated genes (ISGs) and the expression of type I IFNs such as IFN$\beta$ [251, 252]. When secreted, IFN$\beta$ acts in an autocrine and paracrine manner to induce the expression of ISGs. The proteins encoded by ISGs are broad acting and serve to impair intracellular viral activities necessary for replication, and promote a general antiviral state. Such proteins include protein kinase R (PKR) and 2',5'-Oligoadenylate synthetases (OAS), which act by blocking host and viral protein translation and causing the cleavage of mRNA and ribosomal RNA respectively [253, 254].

NK cells are innate lymphocytes and are important effector cells in the immune response to HCMV. Their importance has been highlighted by cases of patients with a rare NK cell deficiency resulting in enhanced susceptibility to HCMV infection [205, 255, 256], and by a case describing a patient with T-B+NK+ Severe Combined Immune Deficiency (SCID) who recovered from HCMV disease after an expansion of IFN$\gamma$ producing NK cells without antiviral therapy [257]. The reconstitution of NK cell responses in HSCT recipients was also associated with protection against HCMV viraemia [258]. NK cells can rapidly respond on encountering virally infected cells [259, 260] by carrying out effector functions such as secreting antiviral cytokines including IFN$\gamma$ and TNF$\alpha$, and lysing infected cells [261–265]. NK cell activity is regulated by the balance between multiple germ-line encoded activatory and inhibitory receptors. Activatory receptors can recognise stress-induced ligands on virus-infected cells while inhibitory receptors can bind self molecules such as MHC class I [266]. HCMV infection is associated with the expansion and persistence of CD94/NKG2C expressing NK cells capable of enhanced effector functions on re-exposure to HCMV [267–273]. Additionally, *in vitro* experiments have highlighted the important role of IL-12 in the expansion of CD94/NKG2C expressing NK cells [274], providing further evidence that this subset could represent a type of memory NK cell [275].

### 1.5.2 The humoral response to HCMV

Although antibodies against HCMV are generated shortly after primary infection [276], their protective capacity is debated. Antibodies to a diverse array of HCMV proteins have been found in infected individuals, and many of these are specific for viral glycoproteins. Among these are antibodies to gB, which constitute a large fraction of HCMV-neutralising antibodies [277, 278] and gH which is part of the trimeric gH/gL/gO complex and pentameric gH/gL/gO/UL128/UL131 complex [279, 280]. Antibodies generated against other components of the gH-pentameric complex have potent neutralising ability as well [281, 282], and many of these antibodies have been shown to neutralise HCMV *in vitro* [281, 283].

Pre-existing antibodies in seropositive mothers are thought to be an important factor in the prevention of congenital HCMV infection [284]. In a study following a large group of 3000 women, the rate of congenital infection was three times higher in cases where the mother was initially seronegative [285]. In addition, infants born prematurely and requiring a blood transfusion were protected from transfusion-acquired infectious complications when their mothers were seropositive [87]. Work on murine CMV (MCMV) has also shown that

B cells, and therefore likely antibodies, are critical in preventing viral dissemination and limiting disease severity [286].

### 1.5.3 The CD8+ T cell response to HCMV

The robust T cell response that is generated following primary HCMV infection is well documented, with a high proportion of the total peripheral blood pool composed of CMV-specific CD8+ T cells [287, 288]. In addition to the high frequencies of CD8+ T cells directed against lytic antigens such as pp65 and IE [289, 290], the T cell responses to HCMV are extensively diverse, with CD8+ and/or CD4+ T cells being directed to 70% of viral ORFs, encompassing different replication stages and functions [288]. The T cell responses also have considerable variation between individuals [288, 291]. Whereas this evidence points to the effective ability of the immune system to limit viral replication during primary infection and prevent serious disease, latency is always established and the virus is not eliminated.

HCMV-specific CD8+ T cells have a distinct phenotype, characterised by the expression of markers associated with T cell maturation. The majority of HCMV-specific CD8+ T cells in the blood during persistent phases of infection are phenotypically similar to terminally differentiated effector memory T cells. Typically, HCMV-specific T cells downregulate expression of the co-receptors CD27 and/or CD28, upregulate CD57 and killer cell lectin-like receptor subfamily G member 1 (KLRG1), and express effector molecules such as perforin and granzyme B [287, 292–295]. A large proportion of HCMV-specific CD8+ T cells also re-express CD45RA, which is typically associated with naive T cells, from CD45RO, which is associated with memory T cells [292, 293, 296–298]. Such cells are termed $T_{EMRA}$ (effector memory expressing CD45RA) cells. Transcriptionally, features of mature effector T cells are present from early following primary infection [299]. Furthermore, CD45RA+ HCMV-specific CD8+ T cells are also detectable soon after primary infection and persist into the latent phase of infection, expanding with age [300–303]. It is possible that this phenotype arises as a result of repeated antigenic stimulation [304–306]. However, unlike infections with other chronic viruses including HIV-1 [307–314], HCMV-specific CD8+ T cells are not exhausted and maintain functionality, as exemplified by their low expression of the inhibitory receptor PD-1, their cytotoxic ability, and the secretion of antiviral cytokines such as IFN$\gamma$ and TNF$\alpha$ [287, 292–294, 299, 315, 316].

IFN$\gamma$ is a proinflammatory cytokine with a range of direct and indirect antiviral functions (such as upregulating MHC class I and II), in addition to its role in the activation and differentiation of immune cells including macrophages, NK cells, and T cells [317–323].

Similarly, the cytokine TNF$\alpha$ can act directly to induce inducing apoptosis in virally infected cells, and indirectly to counter virus infection [317–319, 321, 324–327]. The emergence of polyfunctional memory CD8+ T cells capable of carrying out multiple effector functions has been associated with better control of HCMV [328–330]. Polyfunctional CMV-specific CD8+ T cells are able to secrete a combination of TNF$\alpha$, IFN$\gamma$, CCL4 (MIP1$\beta$), and upregulate CD107a, an established marker of degranulation [287, 329, 331, 332].

The CD8+ T cell responses to HCMV remain highly stable over time, and deuterium-labelled glucose experiments have demonstrated that CD45RA+ CD8+ memory T cells have a long lifespan [315]. The clonal response against HCMV does not contract following primary infection. Clones detected early after infection are maintained during the latent phase and can be detected many years later, with hardly any new clones appearing [298, 333, 334]. Analysing the clonal composition of memory CD8+ T cells specific for pp65 and IE1 epitopes by T cell receptor (TCR) sequencing revealed a high degree of clonal focusing, where in many donors CD8+ T cells specific to certain epitopes are dominated by a few clones [294, 335–337]. Following the memory response to two different pp65 epitopes from primary infection showed that, although the CD8+ TCR usage was diverse initially, there was a rapid focusing as CD8+ T cells bearing certain TCR V$\beta$ segments became undetectable [333]. The cells selected into the memory phase were a subset of the CD8+ T cell responding originally, and focusing occurred during the resolution of primary infection [333]. The selection of particular CD8+ T cell clones during the course of HCMV infection may be dependent on TCR avidity, which results in repertoire focusing on higher-avidity T cells [298, 333, 336, 338]. Certain studies though, reported the opposite [339, 340], with proposed antigen-independent expansion of low avidity clones occurring due to IL-15 [339].

HCMV-specific CD8+ T cells are detected at high frequencies in HCMV seropositive donors [288], and it has been reported that these frequencies are higher in older individuals [297, 341, 342]. It is presumed that longer term carriage of the virus is associated with the expansion of the HCMV-specific CD8+ memory T cell pool because of repeated antigen stimulation serving to boost T cell responses following viral reactivations. This phenomenon was originally observed to occur in MCMV infection and has been termed ‘memory inflation’ [343–346]. Despite the detected expansions in memory CD8+ T cells from HCMV seropositive individuals, the majority of the evidence for memory inflation in humans comes from cross-sectional age studies [302, 347], rather than longitudinal studies examining the size of the HCMV-specific CD8+ T cell responses over a long period. It has been hypothesised that the accumulation and maintenance of memory CD8+ T cells specific for HCMV over time could reduce the pool of T cells needed for protection against other

pathogens, which might explain the observations of increased all-cause mortality and susceptibility to new infections in elderly HCMV seropositive individuals [348–352]. However, this hypothesis is disputed by numerous studies showing that poor responses to vaccination in the elderly occur independently of HCMV seropositivity [353, 354], and that HCMV infection does not impair the ability of older people to respond to new infections [355, 356].

### 1.5.4 The CD4+ T cell response to HCMV

CD4+ T cells are composed of several functional subsets that can be grouped based on cytokine secretion and transcription factor expression [357]. These subsets include: Th1 cells, which secrete the proinflammatory cytokine IFN$\gamma$, express the transcription factor T-bet, and provide help to CD8+ T cells and macrophages; Th2 cells which express the transcription factor GATA-3, secrete IL-4 and provide help to B cells; and Tregs, which will be discussed below (Section 1.5.7) [357]. Naive CD4+ T cells become polarised toward an effector subtype based on several factors including the signalling induced by exposure to certain cytokines, the duration and strength of TCR signalling following antigen recognition, which can be affected by the antigen dose and TCR affinity for the peptide-MHC class II complex, and by the engagement of specific co-stimulatory molecules [357–360]. CD4+ T cells possess a diverse array of antiviral effector functions. They exert their role in orchestrating immune responses through the secretion of cytokines and via the expression of cell-surface markers that regulate immune cell function, such as CD40L [361–365]. In this manner, CD4+ T cells activate APCs, recruit other lymphoid cells, and help in CD8+ T cell priming as well as the generation of high-affinity antibody responses [365]. Although these helper functions are critical in protective immune responses, CD4+ T cells are also capable of more direct antiviral functions such as cytotoxicity or secretion of antiviral cytokines [366–368].

CD4+ T cells play a diverse role in the immune response to HCMV. Their importance in HCMV infection is evident from human studies and inferentially from experiments in animal models. In mice, long-term depletion of CD4+ T cells is associated with persistent replication of MCMV in the salivary gland [369]. Similar experiments, where CD8+ T cells were depleted before infecting with MCMV, demonstrated that the remaining CD4+ T cells could control virus in these mice with almost identical kinetics as immunocompetent mice [370]. The secretion of IFN$\gamma$ has also been demonstrated to be important in the control of MCMV, where neutralisation of IFN$\gamma$ reduced the antiviral activity of CD4+ T cells to a level comparable with CD4+ cell depletion [371]. A more recent study also highlighted

the necessity of CD4+ T cells secreting IFN$\gamma$ to control MCMV replication in the salivary gland, while showing the helper functions of CD4+ T cells for CD8+ T cells or B cells to be dispensable [372]. In this study, IFN$\gamma$R expression on non-haematopoietic cells was required for the control of MCMV, pointing to the role of IFN$\gamma$ secreted by CD4+ T cells in signalling to infected non-hematopoietic cells, or bystander cells, to exert control of the virus, rather than activating other immune cells [372]. Clinical data from transplant patients also illustrates the importance of CD4+ T cells secreting IFN$\gamma$ to control HCMV, with higher levels of these cells correlating with increased viral control and better clinical prognosis [373, 374]. Moreover, patients with a deficiency in IFN$\gamma$R expression have an increased susceptibility to HCMV and associated disease [375–377].

In renal transplant recipients, a delay in the generation of HCMV-specific CD4+ T cell responses was associated with symptomatic infection and prolonged viral replication, whereas asymptomatic infection was associated with the emergence of CD4+ T cells capable of secreting IFN$\gamma$ prior to the emergence of HCMV-specific CD8+ T cell responses [373]. Likewise, in healthy young children with primary HCMV infection, an impaired HCMV-specific CD4+ T cell response was associated with prolonged viral shedding in urine and saliva compared to adults with primary infection [378]. The presence of HCMV-specific CD4+ T cells is also correlated with viral control and better clinical outcomes in several other studies in the transplant setting [379–381], and the adoptive transfer of autologous *ex vivo* expanded CD4+ T cells into stem cell transplant patients led to a significant decrease in HCMV load associated with the reconstitution of their HCMV-specific CD4+ T cell response [382].

Further evidence for the importance of CD4+ T cells in the control of HCMV infection is provided by studies undertaken on HIV infected individuals. The reduction in CD4+ T cell numbers caused by HIV infection is associated with a higher risk of developing HCMV-related disease [383, 384]. Additionally, HIV infected individuals with quiescent end-organ disease had unimpaired anti-HCMV CD4+ T cell responses, whereas those with active CMV-associated end-organ disease correlated strongly with a loss of this HCMV-specific CD4+ T cell response [385].

Much like CD8+ T cells, the CD4+ T cell responses to HCMV lytic ORFs, including pp65 and IE1, are widespread amongst seropositive individuals [386–389]. The use of peptide libraries spanning the entire HCMV proteome has extended these findings and demonstrated that CD4+ T cells from seropositive donors are capable of recognising a very broad array of HCMV ORFs [288]. In this study on 33 seropositive subjects, the median number of HCMV ORFs each donor had a CD4+ T cell response to was 12, and over half of donors

had a response to pp65, IE, gB, UL86, and pp28 [288]. CD4+ T cells specific for HCMV have also been estimated to reach up to 5% of peripheral blood CD4+ T cells [390], and have been reported to even be as high as 10% [288]. The phenotypes of HCMV-specific CD4+ T cells have been investigated during both primary and chronic infection stages. The study of healthy seropositive donors and renal transplant patients undergoing primary infection indicated that during primary infection the HCMV-specific CD4+ T cell population expressed CD38, both CD45RA and CD45RO, and a large proportion of cells maintained expression of CD27 [391, 392]. Furthermore, CD4+ T cells obtained during both primary and latent infection stages expressed CD11a, CD49d, CD40L, and were negative for CD62L and CCR7 [379, 391]. During latency, HCMV-specific CD4+ T cells lose expression of CD38, CD27, and CD45RA, and the population becomes enriched for CD45RO expressing cells [379, 391–395]. In seropositive individuals, a population of HCMV-specific CD4+ $T_{EMRA}$ cells, which re-express CD45RA+ [393, 394, 396–399], and high frequencies of CD27-CD28- CD4+ T cells, which produce IFN$\gamma$ and express granzyme B and perforin, can also be detected [392, 400–403].

In addition to mediating antiviral effects by cytokine secretion, CD4+ T cells capable of cytotoxicity have been documented in HCMV infection. The emergence of CD4+ T cells from HCMV infected individuals that are able to express cytotoxic molecules, such as granzyme B, is an indicator that these cells possess cytotoxic capacity [379]. These populations of cells have been widely detailed. As mentioned previously, following primary HCMV infection in renal transplant recipients, there was an emergence of a subpopulation of CD4+ T cells that lost expression of CD28 and expressed granzyme B [400]. HCMV-specific CD4+ T cells have also been shown to express the pore-forming protein perforin [401], and show surface mobilisation of CD107a, a marker for degranulation [393, 404]. From the same study, the expression of granzymes A and B, in addition to perforin, was elevated on highly differentiated CD4+ T cells, which had lost expression of CD27 and gained expression of CD57, giving weight to the idea that these CD4+ T cells develop cytotoxic capabilities with increased maturation [404]. Further to this, a recent study confirmed that HCMV-specific CD4+ T cells possessed a highly differentiated phenotype, and by utilising MHC class II tetramers revealed these cells had a considerable cytotoxic profile, further supported by microarray analysis revealing an upregulation of genes associated with cytotoxicity [405]. The ability of CD4+ T cells bearing these phenotypic markers for cytotoxicity have been verified by *in vitro* killing assays where *ex vivo* isolated CD4+ T cells could lyse EBV-transformed B lymphoblastoid cell lines loaded with HCMV antigens [404–407].

The size of the HCMV-specific CD4+ T cell population has been observed to expand with age, although to a lesser extent than CD8+ T cells [408, 409], and the documented expansion of cells with phenotypes linked to terminal differentiation and dysfunctional responses mentioned previously, has led to the hypothesis that dysfunctional CD4+ T cells accumulate with age and may have negative consequences for the host [390, 410–412]. However, the generation of polyfunctional CD4+ T cells capable of multiple simultaneous antiviral effector functions, such as production of IL-2, TNF$\alpha$, and IFN$\gamma$, has been reported for HCMV-specific CD4+ T cells [393, 413, 414]. Polyfunctional CD4+ T cells have been shown to be superior effector cells [316, 415], and are associated with better control of HCMV [374, 413]. Importantly, in older HCMV seropositive donors, polyfunctional HCMV-specific CD4+ T cells are found, and can be detected within CD4+ T cell populations possessing highly differentiated phenotypes [316, 394, 400, 401, 404, 416]. CD4+ T cells specific for HCMV therefore do not appear to undergo functional exhaustion with age.

### 1.5.5 HCMV, the immune system, and ageing

It has been suggested that HCMV infection enhances the age-related decline in immune function (immunosenescence). Several lines of evidence have been drawn on to support the detrimental effects of HCMV carriage on immune ageing, including the above mentioned phenomenon of memory T cell inflation, reported expansions of dysfunctional T cells in old age, and impairment of responses newly encountered pathogens and vaccines [341, 350, 348, 417–419]. These observations have led to the hypothesis that HCMV could be the main driver of immunosenescence, with long term viral carriage being linked to an increase in all cause mortality.

This decrease in life expectancy associated with HCMV infection has been reported alongside a number of other immune parameters to generate the ‘immune risk phenotype’ (IRP) [420]. In addition to HCMV seropositivity, the parameters in the IRP include reduced B cell numbers, an inverted CD4+:CD8+ T cell ratio, an increased frequency of CD8+CD28- T cells, and poor proliferative response to mitogen stimulation [348, 349, 351, 352, 421]. Further studies aiming to address the association between HCMV and increased mortality have also been carried out, with some studies finding evidence supporting such a link [422–426], and others not [427, 428].

As discussed previously, in HCMV seropositive individuals, there is an expansion of highly differentiated HCMV-specific CD8+ T cells that re-express CD45RA, and gain expression of CD57 as well as KLRG1, with a concurrent loss of expression of CD27 and/or

CD28. It has been suggested that these T cells could be dysfunctional, with reports of a loss of proliferative capacity and ability to secrete cytokines [341, 350, 429, 430]. However, separate studies examining the ability of these cells to proliferate have found no evidence of a defect when given appropriate co-stimulation [293, 315, 431, 432], and HCMV-specific T cells with a highly differentiated phenotype have been shown to produce multiple antiviral cytokines and possess cytotoxic potential [315, 433, 414]. Despite the presence of these functional cells however, an age-related increase in HCMV DNA detected in the urine and blood has been reported, suggesting a possible alteration in HCMV-specific responses with age [434, 435]. Another potential reported manifestation of HCMV-enhanced immunosenescence is a weakened response to immunisation. Several studies have shown an association between HCMV seropositivity, or a high anti-HCMV IgG titre, with decreased antibody responses to influenza vaccination in older age [419, 436–439], although numerous studies reported no such effect, or even an enhanced response to vaccination in HCMV seropositive individuals [351, 353, 354, 440–442]. Therefore, the evidence for a direct link between HCMV and immunosenescence remains inconclusive.

### 1.5.6 Tissue-resident memory T cells

The view that memory T cells are grouped into either central memory ($T_{CM}$) or effector memory ($T_{EM}$), with $T_{CM}$ cells circulating between the secondary lymphoid organs and the blood, and $T_{EM}$ through the blood, lymphoid and non-lymphoid tissues, has recently been expanded on to include a population of tissue-resident memory T cells ($T_{RM}$) [443–445]. $T_{RM}$ cells have been identified in barrier tissues and secondary lymphoid organs where they are maintained without recirculating into the blood and are not replenished by the circulating memory T cell population [446–451]. Their localisation at sites of pathogen entry and reactivation allows them to mount rapid immune responses and restrict infection to the tissue site [448–455]. On antigen encounter, $T_{RM}$ cells are able to rapidly produce IFN$\gamma$ and carry out cytotoxicity against infected cells. The production of IFN$\gamma$ along with cytokines such as TNF$\alpha$ and IL-2 also acts to promote an antiviral state in the tissue by recruiting recirculating CD4+ and CD8+ T cells, and B cells, as well as induce DC maturation, and NK cell activation [448, 456–458].

Studies examining the development of $T_{RM}$ have demonstrated a level of tissue- and context-dependency. For example, the induction of CD8+ $T_{RM}$ cells has been demonstrated to occur independently of local antigen in the gut, skin, and female reproductive tract [455, 459–462], whereas in the brain this has been shown to be dependent on antigen [451]. The surface

markers CD103 and CD69 have commonly been used to identify $T_{RM}$ over other memory T cells [451, 463–465], although there are reports of CD103- and/or CD69- $T_{RM}$ [466–468]. In a thorough examination of memory T cell distribution in the circulation and tissues from deceased organ donors, memory T cell isolated from the lymphoid and mucosal tissue had constitutive CD69 expression compared to circulating memory T cells which were mainly negative for CD69 [465]. Furthermore, CD103 was shown to be exclusively expressed on mucosal memory CD8+ T cells isolated from the colon and small intestine, with large proportions of CD103+ memory CD8+ T cells also found in the lung and intestinal-draining lymph node, whereas blood, spleen, and peripheral lymph node CD8+ memory T cells were mainly CD103- [465].

In the context of herpesvirus infection, the presence of $T_{RM}$ cells at sites of latency and viral entry could provide a rapid defence against reactivation and superinfection compared to circulating memory T cells that might not have access to these tissue sites in non-inflammatory conditions. Indeed, HSV-1 specific CD8+ $T_{RM}$ cells rapidly detect virus at neuronal sites of latency and provide superior protection compared to circulating memory T cells [455, 469–471]. CD4+ and CD8+ $T_{RM}$ cells have also been detected in response to MCMV infection [460, 461]. Although CD8+ T cells are unable to control lytic MCMV replication during primary infection in the salivary gland [369, 372], following re-infection, the population of salivary gland CD8+ $T_{RM}$ can confer immediate protection due to the tropism of the virus for cells that resist complete MHC class I downregulation [460]. In humans, CD103+ CD8+ T cells specific for EBV, and CD103+ CD4+ and CD8+ T cells specific for HSV-2, have been detected in the tonsils and female genital mucosa, respectively [472–474], and a large proportion of HCMV-specific CD8+ T cells in the lung and spleen were found expressing CD69, indicative of a $T_{RM}$ phenotype [475].

### 1.5.7 HCMV and regulatory T cells

CD4+ regulatory T cells (Tregs) are an indispensable subset of cells with immunosuppressive properties that negatively regulate immune-mediated inflammation. A deficiency of Tregs results in spontaneous autoimmune disease, which in humans is fatal [476–478]. CD4+ Tregs are defined by their expression of the transcription factor forkhead box protein 3 (Foxp3) [479], which is required for their suppressor functions [480, 481], but they can also be identified by a number of cell surface markers including the low expression of CD127 and high expression of CD25 [482–484]. One population of Tregs is generated in the thymus, termed natural Tregs (nTregs). The differentiation of nTregs is dependent on

the high-avidity interaction between the TCR and peptide/MHC class II complexes in the presence of IL-2 [485, 486]. Thus, nTregs are largely self-reactive and contribute to the prevention of autoimmunity through tolerance to self antigens. Tregs can also differentiate in the periphery, known as induced Tregs (iTregs), predominantly when naive T cells encounter antigen under certain tolerogenic conditions. Factors that favour the generation of iTregs include strong TCR signalling and suboptimal costimulation in the presence of high levels of TGF$\beta$ and retinoic acid [485, 487, 488]. Differentiation of iTregs is therefore likely to occur in response to non-self antigens, which iTregs can act on to restrict excessive immune responses.

Tregs mediate their suppressive effects via several mechanisms such as the secretion of immunosuppressive cytokines IL-10 and TGF$\beta$ [485, 489, 490]. Tregs also express surface molecules involved in their suppressive functions, including CTLA-4, which may allow Tregs to suppress DC-mediated activation of T cells by downregulating the co-stimulatory molecules CD80 and CD86 [485, 491]. Additionally, the constitutively high expression of CD25 (IL-2R$\alpha$ chain), which binds IL-2, can act to reduce IL-2 availability for CD8+ T cells, limiting their activation [492].

Although Tregs can mediate a protective effect against excessive inflammatory responses during certain viral infections [493], they have also been demonstrated to interfere with protective immune responses [494–500]. Multiple studies have reported the presence of HCMV-specific Tregs [403, 494, 501–505] and demonstrated their ability to secrete IL-10 and TGF$\beta$, and to inhibit cytokine secretion and T cell proliferation [403, 502–504]. Attempts to target these Tregs have found that depletion of CD25+ expressing cells can increase the frequency of pp65- and IE-1-specific CD8+ T cells secreting IFN$\gamma$ [494, 502]. There is also evidence that the frequency of HCMV-specific iTregs is greater in older individuals [503].

In addition to FoxP3+ Tregs, a population of regulatory CD4+ T cells generated in the periphery lacking expression of FoxP3 has been described, termed type 1 regulatory (Tr1) cells. Tr1 cells were reported to be induced following the chronic activation of CD4+ T cells by antigen in the presence of IL-10, and were able to suppress antigen-specific CD4+ T cell proliferation and prevent colitis in a mouse model of inflammatory bowel disease [506]. Tr1 cells exert their suppressive effects mainly through elevated secretion of IL-10 and TGF$\beta$, which can act to suppress T cell and DC function [506–509]. DCs modulated in this manner, could upregulate tolerogenic surface molecules and inhibitory factors, and thus indirectly suppress T cells [509]. Tr1 cells can also inhibit T cell responses by cell-to-cell contact dependent mechanisms, metabolic disruption, and cytolysis, where Tr1 cells were shown to express granzyme B and lyse myeloid APCs, representing another potential indirect

mechanism to suppress T cells [509–511]. Tr1 cells have a distinct cytokine expression profile and are defined by their secretion of IL-10, TGF$\beta$, and IL-5, as well as low levels of IFN$\gamma$ and IL-2, in the absence of IL-4 [506–508]. In addition, several surface marker have been associated with Tr1 cells such as inducible co-stimulatory molecule (ICOS), CD18, CD49b, and the lymphocyte activation gene-3 (LAG-3) [512–515].

Various methods have been used to generate Tr1 cells *in vitro*. To do so, CD4+ T cells have been stimulated with different subsets of APC, including plasmacytoid DCs, immature DCs, or tolerogenic DCs [506, 516–520], as well as with different cytokines, such as IL-10, IL-6, or IL-27 [521–524]. Furthermore, engagement of a range of co-stimulatory molecules have been reported to be important for Tr1 cell generation such as CD46, CD55, ICOS, and CD2 [521, 525–527]. It is unclear how representative these methods are of the complex environment *in vivo*, and the different protocols employed to generate Tr1 cells has resulted in a heterogeneous population of cells being described, making characterisation more difficult. To date, no lineage defining transcription factor has been identified for Tr1 cells, and it has not been conclusively demonstrated that they are a distinct cellular population [528].

### 1.5.8 The T cell responses during latent HCMV infection

Although the primary immune response to HCMV is usually effective at resolving primary infection in immunocompetent hosts, HCMV is never cleared and can establish a latent infection. During latency, the products of viral gene expression serve mainly to maintain latency and assist in immune evasion. As the genes expressed during latency are also expressed during lytic infection, it might be expected that T cells specific to latency-associated gene products are also generated, potentially rendering latently infected cells vulnerable to T cell detection and killing.

An early study examining T cell responses to 213 HCMV ORFs by peptide stimulation and intracellular cytokine staining (ICS) found CD4+ T cell responses to UL138 in one donor and to UL144 in four donors out of 33, but failed to detect responses to US28 or UL111A [288]. Likewise, CD8+ T cell responses were only detected in one donor out of 33 in response to UL138 and US28, and in none of the donors in response to UL144 and UL111A [288]. An additional study utilising ICS following peptide stimulation, did not detect CD4+ T cell responses to LUNA and UL138, or LUNA-specific CD8+ T cell responses, while only a CD8+ T cell response to one UL138-derived peptide presented on HLA-B35 was reported [529]. A more recent study however, measured T cell responses to viral proteins expressed during latency over a longer period of 48 hours by ELISpot [501]. T

cell responses to UL138 and LUNA were detected, and these were primarily CD4+ T cell mediated, with only low frequencies of CD8+ T cell responses detected if at all [501]. A proportion of these CD4+ T cells specific for proteins expressed during latency secreted the immunomodulatory cytokines IL-10 and TGF$\beta$, which contrasted with CD4+ T cells specific for lytic HCMV ORF products [501]. Furthermore, in CD4+ T cell lines specific for UL138 and LUNA, but not gB, a subset of cells was found to possess phenotypic characteristics of Tregs, as they were CD4+CD25$^{hi}$FoxP3+ [501]. The supernatant from UL138-stimulated PBMC, but not gB-stimulated PBMC, was also able to inhibit the proliferation of CD4+ T cells, and the use of neutralising antibodies against IL-10 and TGF$\beta$ confirmed they were the mediators of this inhibition as proliferation was restored [501]. Based on these findings, it was hypothesised that the secretion of IL-10 and TGF$\beta$ by latency-associated ORF specific CD4+ T cells was contributing to the inhibition of antiviral IFN$\gamma$/TNF$\alpha$ secreting cytotoxic Th1 cells during latency.

## 1.6 Immune evasion

There is a complex interplay between HCMV and the host immune system. HCMV has evolved a variety of strategies to effectively modulate both innate and adaptive immune responses. HCMV encodes an array of proteins that are capable of subverting immune surveillance, and these often have overlapping and redundant functions [530, 531].

### 1.6.1 Immune evasion of the innate immune response

As the first line of defence against infection, innate immunity plays an important role in preventing the establishment of viral infections and initiating antiviral immune responses. HCMV disrupts the actions of PRRs in a number of ways. For example, the virus is able to target the TLR2 pathway at late stages of infection with two microRNAs, miR-US5-1 and miR-UL112-3p [532, 533]. The expression of miR-UL112-3p downregulates TLR2 expression, and both of these microRNAs inhibit downstream NF$\kappa$B signalling by inhibition of IKK$\alpha$ and IKK$\beta$, resulting in a decrease in proinflammatory cytokine secretion [532, 533]. HCMV encoded UL31 also interacts directly with cGAS and inhibits its enzymatic activity, resulting in a decrease in cGAMP production [534]. Additionally, knockdown or knockout of UL31 was found to increase production of type I IFNs and antiviral genes induced by HCMV [534].

IFNs play a key role in the defence against viruses, and HCMV employs several additional mechanisms to evade IFN-dependent responses. UL83-encoded pp65 has a main role in the inhibition of type I IFN responses, although the level at which it acts is still under debate [535–538]. The tegument protein pp71 has been shown to interfere with stimulator of interferon genes (STING) mediated signalling resulting in an inhibition of IFN$\beta$ transcription, amongst other antiviral genes [539]. The viral glycoprotein US9 also interferes with STING-mediated signalling pathways to block nuclear translocation of IRF3, resulting in an inhibition of the IFN$\beta$ response [540], as does the IE2 protein IE86 by blocking NF$\kappa$B binding to the IFN$\beta$ promoter [68, 69, 541]. Furthermore, HCMV inhibits IFN signalling and ISG expression by interfering with signalling components, such as Jak1, that are downstream of the stimulated IFN-receptors [531]. In addition, HCMV impairs the induction of IFN-responses through the secretion of a viral homologue of cmvIL-10 encoded by UL111A [154], and inhibits the actions of PKR and OAS [531].

Cellular restriction factors represent a first line of defence against viruses by counteracting viral replication. In defence against a nuclear DNA virus such as HCMV, the components of ND10, which include hDaxx, Sp100, and PML, act as restriction factors and exert their antiviral functions mainly by epigenetic mechanisms to repress HCMV IE gene expression [542–547], with PML acting as a structural platform for the recruitment of other proteins [548–550]. To replicate successfully, HCMV has evolved several mechanisms that help evade these restriction factors. As mentioned previously (Section 1.2.3), IE1 disrupts ND10 by preventing its SUMOylation and therefore its oligomerisation [73, 74, 76, 551]. Additionally, the tegument protein pp71 can displace the chromatin-associated factor ATRX from ND10, and cause the degradation of hDaxx by the proteasome, therefore relieving hDaxx-dependent repression of the MIEP [552, 553, 544, 554].

As discussed subsequently HCMV downregulates MHC class I molecules on infected cells to evade CD8+ T cell recognition (Section 1.6.2). Doing so however, should render infected cells vulnerable to NK mediated lysis, which would normally receive inhibitory signals from MHC class I molecules [266]. To prevent this, HCMV encodes multiple genes dedicated to suppressing NK cell activation and cytotoxicity, the majority of which aim to either prevent signalling from activating receptors, or trigger inhibitory signals [555]. HCMV attempts to maintain or increase inhibitory signals in a number of ways, including the expression of MHC class I decoys, and selective retention or upregulation of MHC class I molecules (such as the non-classical MHC molecule HLA-E) that are less adept at stimulating extensive CD8+ T cell responses. HLA-E, for example, is restricted in its binding of peptides, as its natural ligands are the leader sequences of HLA-A, -B, -C and -G alleles

[556]. At the cell surface, HLA-E provides an inhibitory signal to NKG2A/CD94 on NK cells [557]. HCMV takes advantage of this by expressing UL40 which contains a nonameric peptide able to bind HLA-E and increase its cell surface expression in a TAP-independent manner following processing in the ER [558–560]. HCMV also expresses a homologue of MHC class I, UL18, which can bind the inhibitory NK receptor LIR-1 with greater affinity than cellular MHC class I and inhibit LIR-1+ NK cells [560–563]. Additionally, the UL40 nonameric peptide is capable of upregulating UL18, in addition to HLA-E [560].

The potent activating receptor NKG2D, which is expressed on all human NK cells [564], can bind ligands such as MICA, MICB, and ULBP1-3 and ULBP6 [555]. HCMV infection results in an upregulation of these stress-induced ligands, which the virus can counteract through binding of ULBP1, 2, and 6, and MICB, via its glycoprotein UL16, retaining them intracellularly [555]. Downregulation of MICA and ULBP3 has been shown to be mediated by the glycoprotein UL142, again via intracellular retention [565–567]. MICA can also be targeted by the US12 gene family member US18 and US20, which promote MICA proteolysis by lysosomal degradation [568]. A recent multiplex proteomic analysis followed these results up to uncover additional member of the US12 family with roles in targeting activating NK cell ligands, such as the contribution of US13 in downregulation of MICB [569]. An HCMV encoded microRNA, miR-UL112-1, is also involved downregulating MICB by reducing MICB mRNA translation [570].

### 1.6.2 Immune evasion of the adaptive immune response

HCMV can diminish the ability of CD4+ and CD8+ T cells to recognise viral antigens by interfering with the MHC class I and II processing pathways. The viral US2-11 region is integral to this. The immune evasion genes US2 and US11 code for proteins that retrotranslocate newly synthesised MHC class I heavy chains from the ER to the cytosol, where they are targeted for degradation by the proteasome [571–573]. Similarly US3 binds to MHC class I peptide complexes and causes them to be retained in the ER [574–576], whereas US6 prevents the transport of peptides from the cytosol into the ER by binding to the cytosolic side of the transporter associated with antigen processing, thereby inhibiting ATP binding [577–579]. US3 is expressed as an immediate-early protein, whereas US2 and US11 as early proteins, and US6 is synthesised during both early and late times of infection [580]. Antigen presentation via MHC class II is also interfered with by US2, which targets HLA-DR$\alpha$ and HLA-DM$\alpha$ for proteasomal degradation [581, 582]. In addition, US3 can bind MHC class II $\alpha/\beta$ complexes in the ER, and disrupt their association with the invariant

chain [583]. This leads to inefficient sorting of MHC class II molecules into the peptide loading compartment and a reduction in the loading of antigenic peptides onto MHC class II [583]. Finally, viral pp65 can downregulate the expression of HLA-DR by mediating the transport and accumulation of HLA class II molecules in lysosomes, resulting in the degradation of the HLA-DR$\alpha$ chain [584].

Although MHC class II expression is usually restricted to professional APCs, such as macrophages and dendritic cells, IFN$\gamma$ stimulation can also induce expression of MHC class II on non-professional APCs such as fibroblasts, endothelial, and epithelial cells [585, 586]. However, HCMV can target this IFN$\gamma$-mediated upregulation of MHC class II following infection by interfering with the expression of the class II transactivator, CIITA, which is essential for MHC class II transcription and expression [587]. The virus achieves this by directly inhibiting the expression of CIITA mRNA, as has more recently been demonstrated in a human dendritic cell subset [588, 589]. Moreover, HCMV interferes with IFN$\gamma$-mediated signalling by inhibiting JAK expression, which results in reduced CIITA activation and MHC class expression [590, 591]. These mechanisms serve to decrease the amount of viral peptides presented to T cells, thereby reducing the potency of anti-viral T cell immunity.

The viral gene products of UL111A and UL144 can also interfere with T cell mediated immune responses. As mentioned previously, the viral gene UL111A encodes two homologues of hIL-10 which have immune evasion properties (Section 1.3.4). During lytic infection these homologues (cmvIL-10 and LAcmvIL-10) can downregulate MHC class I and II, inhibit the proliferation of PBMC, and suppress the production of proinflammatory cytokines [127, 155, 156]. Furthermore, cmvIL-10 can upregulate the secretion of hIL-10 from certain myeloid cells, possibly amplifying its suppressive effects [160]. In addition, expression of UL144 could also interfere with T cell responses, both through binding to BTLA to inhibit T cell proliferation, and through its ability to induce expression of the Th2 chemoattractant CCL22 to dampen Th1 responses [143, 144, 147].

The use of microRNAs by HCMV is an effective way of avoiding immune surveillance by T cells, given the lack of peptide component that could be presented on MHC molecules. microRNAs implicated in the modulation of the host immune system during lytic infection include miR-UL112-5p. miR-UL112-5p was shown to downregulate the aminopeptidase ERAP1, which is required for peptide trimming prior to MHC class I presentation, and reduce presentation of an immunodominant pp65 peptide to CD8+ T cells [592]. Finally, miR-UL148D is responsible for the degradation of CCL5 mRNA, thereby impairing T cell chemoattraction to infected cells [593].

Despite the plethora of immune evasion functions encoded by HCMV, in the majority of cases, the immune system is able to resolve primary infection. However, the immune response to primary infection cannot prevent the establishment of latency, allowing the virus to persist for the lifetime of the host. During primary infection, immune evasion mechanisms targeting innate and intrinsic immunity could therefore be responsible for giving the virus a window of opportunity where it can replicate and disseminate to cell types that support a latent infection. As these events likely happen prior to the generation of the adaptive immune response, immune evasion mechanisms targeting the components of adaptive immunity might thus be more important in protecting the virus during reactivation to enable replication and the production of infectious virions.

### 1.6.3 Immune evasion during latency

During latency, the known expression of a number of viral proteins should be expected to result in the recognition of infected cells by HCMV-specific T cells. However, multiple immune evasion mechanisms operate during latency to suppress T cell mediated responses. The expression of non-immunogenic microRNAs during latency provide one such mechanism. miR-UL148D targets the cellular receptor ACVR1B in latently infected monocytes, and limits secretion of the proinflammatory cytokine IL-6 in response to activin A [594]. IL-6 has several effects on T cells, including interference with the generation and suppressive effects of Tregs [595–598]. Additionally, HCMV miR-UL112-1 was shown to target viral IE72 transcripts in latently infected cells to prevent IE RNA expression and subsequent recognition by IE-72 specific CD8+ T cells [599].

HCMV is known to induce changes in the secreted proteins (secretome) of infected cells during lytic infection. Such secreted proteins of both cellular and viral origin include chemokines, cytokines, and growth factors [600–602]. HCMV also modulates the secretome of latently infected cells. In this manner, HCMV can influence cellular migration, for example by increasing the secretion of the monocyte-attracting cytokine CCL2 in latently infected granulocyte macrophage progenitors, which was linked to the migration of CD14+ monocytes toward the site of latency [603]. Similarly, short-term latent infection of CD14+ monocytes resulted in the secretion of CCL2 and CCL8, which was linked to the recruitment of both CD14+ monocytes and CD4+ T cells to latently infected cells [604]. In addition, analysis of the supernatants from experimentally latent CD34+ cells revealed the secretion of many cellular factors during latent infection [605]. Among these were chemokines such as CCL2, CCL8, and CXCL12, but also the immunomodulatory cytokines IL-10 and

TGF$\beta$ [605]. In this study, latently infected CD34+ cells were found to induce CD4+ T cell migration, which was dependent on CCL8 binding to CCR3 and CCR5 receptors on the T cells. The latent secretome was then able to inhibit the secretion of Th1 cytokines (IFN$\gamma$, TNF$\alpha$, and TNF$\beta$) from CD4+ T cells, as well as inhibit CD4+ T cell mediated cytotoxicity. Furthermore, the latent secretome upregulated the secretion of IL-10 and TGF$\beta$ from bystander CD34+ cells [605]. These results led to the hypothesis that latently infected cells generate an immunosuppressive microenvironment that can inhibit CD4+ T cell recognition and clearance of latently infected cells, even those recruited by CCL8 secretion [605].

Alongside cellular protein secretion being regulated during latency, viral proteins are also secreted. One such protein is an isoform of viral IL-10, LAcmvIL-10, which is capable of downregulating MHC class II molecules on latently infected cells [127, 156]. LAcmvIL-10 can also upregulate the transcription of cellular IL-10 via the downregulation of the cellular microRNA hsa-mir-92a [164]. Thus, LAcmvIL-10 expression could additionally impact the immunosuppressive microenvironment created by latently infected cells via upregulation of cellular IL-10. Further to its function in immune evasion, IL-10 acts as a survival factor for early myeloid lineage cells such as CD34+ progenitor cells and CD14+ monocytes by increasing resistance to apoptotic signals. IL-10 drives the expression of anti-apoptotic proteins such as BCL-2 and PEA-15 [606–608]. PEA-15 was recently shown to be upregulated following IL-10 signalling in CD34+ cells, which resisted extrinsic FAS-mediated death [607]. Therefore, the increase in cellular IL-10 expression induced by LAcmvIL-10 during latency could also act to prevent apoptosis by increasing the resistance of latently infected cells to pro-death signals, thus helping to maintain viral genome carriage.

## 1.7 Aims for this thesis

It was hypothesised that T cells specific to HCMV latency-associated gene products could be contributing to the maintenance of viral latency through the secretion of immunosuppressive cytokines. In this manner, these T cells could be suppressing antiviral responses during latency, enabling the virus to persist. To test this hypothesis, the following aims were set out for this project:

- Determine if CD4+ and CD8+ T cells from HCMV seropositive donors respond to the latency-associated ORFs UL138, LUNA, US28, UL111A, and UL144

- Determine if T cells specific for latency-associated gene products produce IL-10
- Investigate the influence of age on the frequencies of cytokine secreting T cells specific for latency-associated ORFs
- Characterise the phenotype and effector functions of latency-specific CD4+ T cells
- Characterise the phenotype and effector functions of latency-specific CD8+ T cells
- Improve recognition and clearance of latently infected cells by modulating immunosuppressive cytokines

# Chapter 2

# Materials and Methods

## 2.1 Donors, Testing HCMV seropositivity, Data collection

Heparinised peripheral blood was collected from 104 healthy HCMV-seropositive donors, and 24 HCMV-seronegative donors (Appendix TableD.1). HCMV serostatus was verified from serum by enzyme-linked immunosorbent assay (ELISA), using a commercial HCMV specific IgG ELISA kit (Trinity Biotech) according to the manufacturer's instructions. Donors were recruited from the National Institute of Health Research (NIHR) Cambridge BioResource, with ethical approval obtained from University of Cambridge Human Biology Research Ethics Committee (HBREC.2014.07). Informed written consent was obtained from all donors in accordance with the Declaration of Helsinki. The age range of seropositive donors tested was 23 to 76 years, 60 donors were female and 44 donors were male. The age range of seronegative donors was 38 to 78 years, 13 donors were female and 11 donors were male. Full donor information is shown in Appendix D. Four donors were also HLA typed by genotyping, which was carried out by the NHS Tissue Typing Service, Addenbrooke's Hospital, Cambridge.

The collection of FluoroSpot data from this large donor cohort, transplant recipients, and bone marrow mononuclear cell donors (contained within Chapters 3, 4, and 5) was carried out in collaboration with Dr Sarah Jackson, a Postdoctoral Research Associate whose work focused on T cell responses to proteins expressed during lytic HCMV infection. The collected raw data was then analysed independently

## 2.2 Cell lines and culture media

All cell types were incubated at 37°C in 5% $CO_2$. Primary dermal fibroblasts were maintained in 175cm$^2$ flasks in Dulbecco modified Eagle medium (DMEM) (Gibco), supplemented with 20% inactivated foetal calf serum (FCS) (Gibco) and 5% penicillin/streptomycin (Sigma-Aldrich). Human foreskin fibroblast cells (HFFs) were grown in DMEM supplemented with 10% inactivated FCS and 5% penicillin/streptomycin in 175cm$^2$ flasks. Fibroblasts were split at a 1:2 ratio, twice a week. Media was removed and the fibroblasts washed with phosphate-buffered saline (PBS) before the addition of 5ml trypsin (Promega) for 5 minutes. The enzymatic activity of trypsin was then stopped by the addition of DMEM supplemented with FCS as appropriate. PBMC were maintained in RPMI-1640 (Sigma-Aldrich) supplemented with 10% FCS and 5% penicillin/streptomycin, while T cells were maintained in X-VIVO 15 (Lonza) supplemented with 5% Human AB serum (Sigma-Aldrich).

## 2.3 Isolation of PBMC from whole blood

Fresh PBMC were isolated from venous blood obtained by venipuncture. Varying amounts of blood was collected into 50ml tubes containing 2.5ml (100IU/ml) heparin sodium (Fannin) diluted in PBS (Sigma-Aldrich). Blood was then diluted 1:2 in PBS before isolating PBMC via Lymphoprep (Axis-shield) density gradient centrifugation. 12.5ml of Lymphoprep was overlayed with 25ml of diluted blood then centrifuged at 500G for 25 minutes with the brake off. The PBMC layer was extracted using a Pasteur pipette and resuspended in 50ml PBS before being centrifuged at 500G for 5 minutes with the brake on. Finally, the supernatant was poured off, and the PBMC resuspended in 50ml PBS and centrifuged at 300G for 10 minutes. PBMC numbers were then enumerated by hemocytometer counting of cells stained with trypan blue (Sigma-Aldrich).

## 2.4 Cryopreservation and defrosting of cells

Cell lines and donor derived PBMCs that required storage in liquid nitrogen were cryopreserved by washing in their constituent media and chilling the resulting cell pellet in ice for 2 minutes before being resuspended at up to 4x10$^7$ cells per ml in chilled freezing medium (10% dimethylsuphoxide, 90% foetal calf serum) and transferred to 1ml cryovials

(Corning). Cryovials were placed in a freezing container (Thermo Fisher Scientific) at -80°C overnight before transfer to liquid nitrogen for long term storage the following day.

To defrost frozen cells, cryovials were removed from liquid nitrogen and hand warmed until partially defrosted. Each 1 ml aliquot of cells in freezing media was then resuspended in 10ml pre-warmed 37°C serum-free RPMI-1640 made up with 10U/ml RNase free DNase (Sigma-Aldrich). Cells were then centrifuged for 10 minutes at 300G before being resuspended in 10ml RPMI-1640 with 10U/ml RNase free DNase and incubated for 1 hour at 37°C. Cells were then washed again, by centrifuging for 5 minutes at 500G, and re-suspended in the appropriate media. Defrosted PBMC or T cells were rested overnight at 37°C in media before use in assays.

## 2.5 Cell separations by Magnetic Associated Cell Sorting (MACS)

### 2.5.1 Negative selection

To deplete an unwanted population(s) from PBMC and leave desired cells untouched, negative selection using the MACS system was used. To isolate CD3+ T cells the Pan T cell isolation kit was utilised (Miltenyi). PBMC were first washed twice in MACS buffer (PBS without calcium and magnesium supplemented with EDTA (2nM) (Thermo Fisher Scientific) and 0.5% FCS), and then resuspended in MACS buffer at the correct concentration as per the manufacturer's instructions, before the addition of direct beads against the cell population(s) to be removed (Miltenyi). Cells were incubated for 15 minutes at 4°C, washed in MACS buffer once, before being resuspended in MACS buffer at desired concentration. Depletions were then performed on LS columns (Miltenyi) or using automated cell separation with autoMACS (Miltenyi) and autoMACS columns (Miltenyi) in accordance with the manufacturer's instructions.

### 2.5.2 Positive selection

Again, using the MACS system to obtain a pure population of cells where labelling the cells of interest is not a concern, direct beads (Miltenyi) were used. For example, this was used to isolate CD14+ monocytes from PBMC. Whole PBMC were first washed twice in

MACS buffer, then resuspended in MACS buffer at the correct concentration according to the manufacturer's instructions. Direct beads (Miltenyi) against the target population were then added and incubated for 15 minutes at 4°C, washed in MACS buffer once, before being resuspended in MACS buffer finally. Depletions were carried out on LS columns (Miltenyi) and labelled cells eluted, or using automated cell separation with autoMACS (Miltenyi) and autoMACS columns (Miltenyi) in accordance with the manufacturer's instructions.

## 2.6 Peptides

Peptides obtained from Proimmune were constructed as sequential 15 amino acid peptides that overlap by 10 amino acids, spanning UL55 (gB), UL123 (IE1), UL122 (IE2), UL83 (pp65), UL82 (pp71), US3, UL138, US28, UL81-82as (LUNA), UL111A (vIL-10), and UL144. The sequences of all peptides are shown in Appendix B. Peptides were reconstituted in 80% dimethylsulphoxide and stored according to the manufacturer's instructions to a concentration of 40 mg/ml. Each individual peptide was further diluted in RPMI-1640 to give a stock of 1 mg/ml and a working concentration of 40$\mu$g/ml for each peptide. Peptides were stored at -80°C. Peptide pools spanning entire ORFs, or smaller regions of an ORF were constructed from single peptides to a final concentration of 2$\mu$g/ml of each individual peptide. To test T cell responses from patients undergoing kidney transplantation, peptide pools were generated encompassing multiple ORFs, maintaining each peptide from each pool at a concentration of 2$\mu$g/ml. Pools were made up from the peptides of UL138, LUNA, US28, UL111A, and UL144, pp65 and UL144, IE1 and IE2, and pp71 and US3.

## 2.7 Flow Cytometry Analysis

### 2.7.1 Quantifying efficiency of cell depletions

PBMC alone, or PBMC subsets following cell separations by MACS, were stained to ensure efficient separation had occurred. Cell suspensions were washed twice in MACS buffer before being labelled with a mix of the relevant antibodies (Table 2.1) in MACS buffer for 45 minutes at 4°C in the dark at the manufacturer's recommended concentration.

| Target | Species | Clone | Fluorophore | Supplier |
|---|---|---|---|---|
| CD3 | Mouse | UCHT1 | FITC | BioLegend |
| CD4 | Mouse | RPA-T4 | PE | BioLegend |
| CD8 | Mouse | HIT8a | PerCP/Cy5.5 | BioLegend |
| CD14 | Mouse | HCD14 | APC | BioLegend |

**Table 2.1** Antibodies used for post separation purity analysis

### 2.7.2 Measuring T cell proliferation by flow cytometry

Freshly isolated PBMC were labelled with CellTrace violet proliferation kit, for flow cytometry (Thermo Fisher Scientific), to a final dilution of dye at 1:2000 in PBS, according to the manufacturer's instructions. Labelled PBMC were then resuspended with supernatant from CD4+ T cells stimulated for 48 hours with whole ORF US28 peptides (Section 2.13.1), media alone, or 2ng/ml IL-10 (Peprotech) at a concentration of $1.0\times10^5$ cells per well of a round bottom 96 well plate and incubate for one hour at 37°C, 5% $CO_2$. Post incubation cells were stimulated with Dynabeads® Human T-Activator CD3/CD28/CD137 (Thermo Fisher Scientific) at a bead to cell ratio of 1:100. Alternatively, cells were left unstimulated or stimulated with 1ng/ml PHA (Sigma-Aldrich, U.K.) with 2ng/ml recombinant IL-2 (National Institute for Biological Standards and Control, Potters Bar, U.K.) as a proliferation control. All wells were adjusted to 200$\mu$l total volume and incubated for 5 days at 37°C, 5% $CO_2$. Following the 5-day incubation, cells were stained with LIVE/DEAD® Fixable Green Dead Cell Stain Kit (Thermo Fisher Scientific) at 1$\mu$l/test of a 1:10 stock dilution, along with surface staining for CD4 and CD8. Cells were then acquired on the BD LSR-FORTESSA and analysed with FlowJo software.

### 2.7.3 Identifying regulatory T cell populations

The antibodies used to phenotype regulatory T cells are shown in Table 2.2. Surface staining was carried out in 100$\mu$l total volume of MACS buffer for 45 minutes at 4°C. Staining of CD25 was done using two different clones of antibody, both conjugated to PE, to provide a brighter signal. A mix of CD25, CD4, CD3, CD8 and CD127 antibodies was made and used to stain the cell surface before proceeding to intracellular staining. FoxP3 staining was carried out using the FoxP3 Staining Buffer Set (Miltenyi) together with two different clones of anti-FoxP3 antibody, or their isotype controls. The fixation/permeabilisation solutions and permeabilisation buffer were freshly diluted before each experiment as per the manufacturer's instructions. Cells were incubated in fixation/permeabilisation buffer for 1

hour at 4°C followed by a wash with permeabilisation buffer before being resuspended in permeabilisation buffer with anti-FoxP3 antibodies (5$\mu$l/antibody/test) for an additional 1 hr. Cells were then acquired on the BD LSR-FORTESSA and analysed with FlowJo software.

| Target | Species | Clone | Fluorophore | Concentration | Supplier |
|---|---|---|---|---|---|
| CD3 | Mouse | UCHT1 | FITC | 1:100 | BioLegend |
| CD4 | Mouse | RPA-T4 | PE/Cy7 | BioLegend | BioLegend |
| CD8 | Mouse | HIT8a | APC | BioLegend | BioLegend |
| CD25 | Mouse | M-A251 | PE | BioLegend | BioLegend |
| CD25 | Mouse | 2A3 | PE | BD Biosciences | BD Biosciences |
| CD127 | Mouse | A019D5 | Alexa Fluor 647 | BioLegend | BioLegend |
| FoxP3 | Mouse | PCH101 | eFluor 450 | eBioscience | eBioscience |
| FoxP3 | Mouse | 259D/C7 | V450 | BioLegend | BD Biosciences |
| IgG1 $\kappa$ isotype | Rat | MOPC-21 | Pacific Blue | eBioscience | BioLegend |
| IgG2a $\kappa$ isotype | Rat | eBR2a | eFluor 450 | BioLegend | eBioscience |

**Table 2.2** Antibodies used for regulatory T cell staining

### 2.7.4 Intracellular cytokine staining and cell phenotyping

Polyfunctional T cell responses to HCMV ORF peptides were determined by intracellular cytokine staining. In addition, memory and effector phenotypes of HCMV specific T cells was determined. The antibodies used are shown in Table 2.3. Between 2-3x$10^6$ PBMC were resuspended in 200$\mu$l X-VIVO 15 in individual polypropylene FACS tubes (Falcon). 5$\mu$l anti-CD107a antibody was added per tube and incubated at 37°C for one hour. 245$\mu$l of either X-VIVO 15 only, positive control mixture (anti-CD3 antibody (Mabtech AB), Staphylococcus enterotoxin B (SEB), phytohemagglutinin (PHA), pokeweed mitogen (PWM), and lipopolysaccharide (LPS) (all from Sigma-Aldrich)), or the appropriate HCMV ORF mixes were then added to the FACS tubes. Cells were then incubated overnight at 37°C. 20 hours later, monensin (BioLegend) was added at a 1:1000 dilution to each tube and incubated at 37°C for four hours. Cells were then washed in MACS buffer and stained for surface antigens (Table 2.3), in addition to a live cell discriminator (LIVE/DEAD$^{TM}$ Fixable Aqua Dead Stain Kit (Thermo Fisher Scientific)) at a 1:1000 dilution, for 20 minutes at 4°C before the addition of 100$\mu$l solution A (Nordic-MUbio) to fix the cells. Cells were then washed in MACS buffer and resuspended in a mix of intracellular antibodies in solution B (Nordic-MUbio) (Table 2.3) for up to 45 minutes at 4°C. Cells were again washed in MACS buffer before being resuspended at a final concentration of 2% PFA and acquired on the BD LSR-FORTESSA and analysed with FlowJo software.

| Target | Species | Clone | Fluorophore | Dilution | Supplier |
|---|---|---|---|---|---|
| CD107a | Mouse | H4A3 | AF647 | 1:200 | BioLegend |
| CD14 | Mouse | M5E2 | BV510 | 4:200 | BioLegend |
| CD19 | Mouse | HIB19 | BV510 | 3:200 | BioLegend |
| CD45RA | Mouse | H1100 | PECy7 | 1:200 | BioLegend |
| CD3 | Mouse | OKT3 | BV650 | 2.5:200 | BioLegend |
| CD27 | Mouse | 0323 | APC-Cy7 | 2:200 | BioLegend |
| CD40L | Mouse | 24-31 | PerCP-eFluor710 | 2.5:200 | Thermo Fisher Scientific |
| CD69 | Rat | FN50 | Pacific Blue | 2:200 | BioLegend |
| 4-1BB | Rat | 4B4-1 | PE/Cy5 | 3:200 | BioLegend |
| IL-10 | Mouse | JES3-9D7 | PE | 5:200 | BioLegend |
| IFNgamma | Mouse | 4S.B3 | BV786 | 3:200 | BioLegend |
| Granzyme A | Rat | CB9 | FITC | 3:200 | BioLegend |
| Granzyme B | Rat | REA226 | FITC | 5:200 | Miltenyi |
| Granzyme K | Mouse | SC-56125 | FITC | 3:200 | Santa Cruz Biotech |
| CD4 | Mouse | 259D/C7 | BV605 | 2.5:200 | BD Biosciences |
| CD8 | Rat | MOPC-21 | BV570 | 2.5:200 | BioLegend |
| TNF | Rat | MAB 11 | BUV395 | 5:200 | BD Biosciences |

**Table 2.3** Antibodies used for intracellular cytokine staining and phenotyping of HCMV-specific T cells. Antibodies used for surface staining are shaded in grey while those used for intracellular staining are not shaded. AF = Alexa Fluor, BV = Brilliant Violet.

## 2.8 Quantifying cytokine secretion by flow cytometry

The quantitative detection of secreted cytokines was achieved through the use of the LEGENDplex$^{TM}$ Human Anti-Virus Response Panel (BioLegend) which allowed for multi-analyte detection from the same sample. This assay was utilised to quantify secretion of IFN$\gamma$, TNF$\alpha$, IL-10, and TGF$\beta$ by HCMV peptide stimulated T cells. TGF$\beta$ secretion was analysed using the same system but as a single-plex assay however, as supernatants required treatment with acidification solution as per the manufacturer's instructions.

### 2.8.1 Cytokine secretion inhibition assay

The inhibitory effects of supernatant derived from latency-specific CD4+ T cells (Section 2.13.1), or exogenous IL-10 and TGF$\beta$, on anti-viral cytokine secretion was determined in this assay system. 1x10$^5$ PBMC were incubated for 24 hours with varying concentrations of IL-10 (Peprotech) and TGF$\beta$ (Miltenyi), or 150$\mu$l CD4+ T cell supernatants, either neat or diluted 1:2, at 37°C in a total volume of 200$\mu$l in 96 well round bottom plates. In certain

experiments, neutralising antibodies to IL-10 (R & D Systems) and TGF$\beta$ 1, 2, and 3 (R & D Systems), were added at a concentration of 2$\mu$g/ml concurrently with the addition of IL-10 and TGF$\beta$ to block cytokine activity. Following this 18 hour incubation, a 1:250 dilution of a 1:1 mix of anti-CD3 and anti-CD28 antibodies (Mabtech) was added to the PBMC. PBMC were then incubated overnight at 37°C with the anti-CD3 and anti-CD28 antibody mix. The following day, the PBMC were centrifuged for 5 minutes at 500G and the supernatants collected, either to be used fresh or frozen at -80°C. Supernatants were then assessed in duplicate on the LEGENDplex$^{TM}$ Human Anti-Virus Response Panel (BioLegend) according to the manufacturer's instructions for a combination of TNF$\alpha$, IL-10, and IFN$\gamma$. Supernatants were acquired on the BD Accuri C6 cytometer and analysed using the LEGENDplex$^{TM}$ Data Analysis Software.

## 2.9 Virus Preparations

Virus preparations were carried out by myself, Georgina Brown, or Ian Groves. Virus stocks were expanded in HFFs. Fibroblasts were infected when they reached 90% confluency in 175cm$^2$ cell culture flasks (Corning). HFFs were infected at a multiplicity of infection (MOI) of 0.1 and rocked for 30 minutes at room temperature before being incubated at 37°C + 5% $CO_2$. The supernatants were removed when cytopathic effect (CPE) was apparent in 80% of cells or infection determined by GFP expression by fluorescent microscopy if a GFP tagged virus was used. Supernatants were removed every three days until the breakdown of the cellular monolayer. Supernatants were frozen at -80°C after each harvest. After the final harvest, all frozen supernatants were defrosted and clarified by centrifugation (15 minutes at 1000G). Following this, the supernatants were centrifuged at 12000G for 2 hours to pellet the virus. The virus was then resuspended in RPMI-1640 and stored in 100$\mu$l aliquots at -80°C. To determine the infectivity of viruses they were each titrated on HFFs and retinal pigmented epithelial cells. The HCMV strains used were:

- TB40e, a gift from Christian Sinzger, University of Ulm [609]
- Merlin-UL32-GFP (RCMV 1172), a gift from Richard Stanton, University of Cardiff [610]
- TB40e-UL32-GFP, a gift from Christian Sinzger, University of Ulm [611]
- TB40e-IE2-YFP (RV1164), a gift from Michael Winkler [612]

For adherent cells such as HFFs, cell culture media was removed before the addition of media containing virus diluted to the desired MOI with enough media to ensure cells are well covered. The cells were then incubated for 3 hours at 37°C before removing the virus containing media, washing twice in PBS, and resuspending with the relevant fresh culture media for the cell type.

For CD14+ monocytes in suspension, cells were centrifuged, and resuspended at 1ml per $1x10^6$ cells with the desired virus at the desired MOI. Cells were then incubated for 3 hours at 37°C and shaken once an hour before removing the virus containing media, washing in PBS, and resuspending with the relevant fresh culture media for the cell type.

Latent infection of CD14+ monocytes was carried out following their isolation by positive selection from seropositive donors or apheresis cones (Section 2.5.2). Isolated monocytes were resuspended in PBS with calcium and magnesium and plated at $2.5x10^6$ per ml on the desired size of flat well plates. Monocytes were then incubated for 1 hour 37°C. The PBS was then removed, the cells resuspended in X-VIVO 15 supplemented with 2.5mM $_L$-Glutamine and incubated overnight at 37°C. The subsequent day, the desired virus type was diluted to the appropriate MOI in X-VIVO 15. The media was removed from the monocytes and replaced with virus-containing media before incubating at 37°C for 3 hours. Monocytes were then washed twice in PBS and incubated at 37°C in X-VIVO 15 supplemented with 2.5mM $_L$-Glutamine for between 4 and 7 days.

## 2.10 Latency-associated secretome generation

To generate latent secretomes, PBMC from an apheresis cone (designated CMV180308) were first isolated. CD34+ cells were then positively selected, using CD34+ microbeads (UltraPure) (Miltenyi), by MACS. The CD14+ cells were then obtained from the CD34+ cell negative fraction by MACS using CD14+ microbeads (Miltenyi). Isolated CD14+ and CD34+ cells were plated in separate 24 well plates at a concentration of $1x10^6$ in X-VIVO 15 supplemented with 2.5mM $_L$-Glutamine in a total volume of 1ml, and incubated overnight at 37°C. The following day, cells were infected at with wild type TB40e at an MOI of 5 to establish latency. Supernatants were harvested without disturbing the cellular monolayer on day 14 post infection, pooled, and clarified by centrifugation at 1000G for 5 minutes and then stored at -80°C.

## 2.11 Statistical Testing

Statistical testing was performed using GraphPad Prism. All statistical tests carried out were non-parametric, having assessed that the data were not normally distributed by D'Agostino and Pearson normality testing. The tests used are mentioned in the text. Where multiple experimental replicates have been tested the spread of the data is measured by standard error of the mean.

## 2.12 Detection of cytokine production by FluoroSpot assay

Pre-coated human IFN$\gamma$ and IL-10, or IFN$\gamma$, IL-10, and TNF$\alpha$ FluoroSpot plates (Mabtech AB) were used to simultaneously detect IFN$\gamma$ and IL-10, or IFN$\gamma$, IL-10, and TNF$\alpha$ secretion respectively following peptide stimulation. PBMC depleted of CD4+ or CD8+ T cells by MACS using direct beads (Miltenyi) according to the manufacturer's instructions were plated in triplicate wells at a concentration of between 1x×$10^5$ and 2×$10^5$ cells per well in X-VIVO 15 supplemented with 5% Human AB serum (Sigma-Aldrich), together with peptide ORF mixes or individual peptides at a final concentration of 2$\mu$g/ml/peptide, to a total volume of 150$\mu$l. Cells were tested for ability to produce cytokine by stimulating with a polyclonal mix of mitogens (anti-CD3 antibody (Mabtech AB) PHA, PWM, SEB, LPS (all Sigma-Aldrich)), at a concentration of 1$\mu$g/ml for each mitogen. Cells were also left unstimulated by the addition of extra media. Plates were incubated for 48 hours at 37°C and 5% $CO_2$ without motion. Following incubation, plates were developed according to the manufacturer's instructions and dried overnight before being read on the AID iSpot reader (Autoimmun Diagnostika [AID] GmbH) and counted using EliSpot (version 7) software (Autoimmun Diagnostika) to detect number of cytokine secreting cells. The efficiencies of depletions were determined by surface staining cells with anti-CD4 or anti-CD8 antibodies (Section 2.7.1), then analysed by flow cytometry.

## 2.13 Cytokine Quantification by ELISA

### 2.13.1 FluoroSpot Validation and generation of CD4+ T cell secretomes

To generate CD4+ T cell secretomes, PBMC were depleted of CD8+ T cells by MACS and plated at $1\times10^6$ cells/ml in X-VIVO 15 in 48 well flat bottom plates. Peptides pools of each HCMV ORF were added at a concentration of $2\mu$g/ml/peptide, to a total volume of $500\mu$l per well. Cells were also left unstimulated and supplemented with extra media or stimulated with a positive control mix of mitogens (PHA, PWM, SEB, LPS (all Sigma-Aldrich)) at concentration of $1\mu$g/ml for each mitogen. Cells were incubated for 48 hours at 37°C. The supernatants were then isolated, clarified by centrifuging at 1000G for 5 minutes, and frozen at -80°C or used immediately.

To validate the FluoroSpot results by ELISA, high binding 96 well flat bottom plates (Greiner Bio-One) were coated 18 hours prior to use with anti-IFN$\gamma$ or anti-IL-10 antibody capture antibody, as per the manufacturer's instructions (Mabtech AB), and incubated overnight at 4°C. Supernatant from stimulated cells was then added, and the ELISA plates developed according to the manufacturer's instructions before being read at 405nm. Results were converted to pg/ml using a standard curve generated from known amounts of IL-10 or IFN$\gamma$.

### 2.13.2 Inhibiting ORF-specific IFN$\gamma$ secretion

PBMC depleted of CD8+ T cells were plated at $1\times10^6$ cells/ml in X-VIVO 15 in 48 well flat bottom plates. Cells were either incubated with a range of IL-10 (Peprotech) and TGF$\beta$ (Miltenyi) concentrations, or with $120\mu$l of latency-associated secretome (Section 2.10), for 2 hours prior to the addition of peptides. Alternatively, anti-IL-10 (R & D Systems), anti-TGF$\beta$ 1, 2, 3 (R & D Systems), and anti-IL-10R antibodies (BioLegend), or IgG1 and IgG2b isotype control antibodies (both R & D Systems), were added at a concentration of $5\mu$g/ml, simultaneously with peptides. Peptides pools of HCMV ORFs were utilised at a concentration of $2\mu$g/ml/peptide, to a total volume of $300\mu$l per well, or cells were left unstimulated and supplemented with extra media. Cells were incubated for 48 hours at 37°C. The supernatants were then isolated and clarified by centrifuging at 1000G for 5 minutes, then used immediately. High binding 96 well flat bottom plates (Greiner Bio-One) were coated 18 hours prior to use with anti-IFN$\gamma$ capture antibody, as per the manufacturer's

instructions (BioLegend IFN$\gamma$ ELISA MAX$^{TM}$ Deluxe), and incubated overnight at 4°C. Supernatant from stimulated cells was then added, and the ELISA plates developed according to the manufacturer's instructions before being read at 450nm. Results were converted to pg/ml using a standard curve generated from known amounts of IFN$\gamma$.

## 2.14 Viral dissemination assay

Primary, donor-derived, fibroblasts, isolated by skin punch biopsy, were plated in 96-well flat bottom plates to 80% confluency and incubated at 37°C overnight. The same day, donor-matched PBMC were defrosted and incubated overnight at 37°C. The following day, the fibroblasts were infected with Merlin-UL32-GFP at a MOI of between 0.01 and 0.03 and incubated at 37°C overnight. The same day, CD8+ T cells were isolated from whole PBMC by negative selection using the MACs system as above (Section 2.5.2). Desired numbers of CD8+ T cells, for the appropriate effector to target ratios, were then incubated overnight in a 96 well plate with fresh media alone, IL-10 (Peprotech) and TGF$\beta$ (Miltenyi), or CD4+ T cell supernatants (Section 2.13.1). The following day, to remove the CD4+ T cell supernatants, the CD8+ T cells were washed twice by transferring to 96 well V-bottom plates before being centrifuged and resuspended in PBS. CD8+ T cells were then resuspended in X-VIVO 15 and the desired numbers of T cells were transferred to the 96-well flat bottom plate containing infected fibroblasts. CD8+ T cells incubated in media alone or IL-10 and TGF$\beta$ were transferred without washing. Fibroblasts were then monitored daily by fluorescence microscopy for the expression of GFP, and the experiment terminated between 9 and 12 days post infection. To test for the ability of CD4+ T cells supernatant alone to control virus spread, these supernatants were added 24 hours post infection and viral spread monitored over 9 to 12 days as above. Fibroblasts were then isolated for analysis. The media was removed, and cells were washed twice in PBS without calcium and magnesium. Fibroblasts were then detached by the addition of 75$\mu$l trypsin/EDTA per well. Cells were incubated at 37°C for 10 minutes or until the cells detached. The wells were then quenched by the addition of 75$\mu$l DMEM containing 10% FCS. Cells were then acquired in the 96 well plate using a BD Accuri C6 cytometer with automatic C-sampler. Data was then analysed using FlowJo and the percentage of GFP positive fibroblasts in test wells was expressed as a proportion of the GFP positive fibroblasts in infected only control wells.

## 2.15 Reactivation of latent virus

### 2.15.1 Differentiation of monocytes to dendritic cells

CD14+ monocytes were differentiated to reactivate virus by one of two ways. Differentiation to a dendritic cell (iDC) phenotype was carried out as previously described [613] [614]. Media was removed and replaced with X-VIVO15 supplemented with 2.5mM L-Glutamine and 1000U/ml of GM-CSF (Peprotech) and 1000U/ml of IL-4 (Peprotech). Cells were then incubated at 37°C for five days and then matured by removing the media and replacing with X-VIVO15 supplemented with 2.5mM L-Glutamine and 50ng/ml lipopolysaccharide (LPS) (Sigma-Aldrich) for two days.

### 2.15.2 PMA treatment of monocytes

Alternatively, the media of latently infected monocytes was removed and replaced with X-VIVO15 supplemented with 2.5mM L-Glutamine supplemented with 20ng/ml phorbol 12-myristate 13-acetate (PMA) to reactivate HCMV [615]. Infected cells were monitored over a period of three days for the peak of virus reactivation.

## 2.16 Killing of latently infected monocytes

To attempt to improve T cell killing of latently infected cells by neutralising IL-10 and TGF$\beta$, monocytes were first isolated from a seropositive donor by CD14+ positive MACS separation (Section 2.5.2). CD14+ monocytes were plated onto 96 well flat bottom plates at a density of 1.25x$10^5$ cells per well and incubated at 37°C overnight. Monocytes were then infected with TB40E/UL32-GFP at an MOI of 1.25 and incubated at 37°C for 3 hours before washing twice with PBS and resuspending with media. Monocytes were then incubated at 37°C overnight. The following day, infected monocytes were washed to remove any anti-viral cytokines and resuspended in media and incubated at 37°C for an additional 6 days. On the penultimate day, matched donor PBMC were defrosted (Section2.4) and incubated at 37°C overnight. The subsequent day, anti-TGF$\beta$ 1, 2, 3 (R & D Systems) and anti-IL-10 antibodies (R & D Systems) or relevant isotype controls (IgG1, and IgG2b (both R & D Systems)) were added to appropriate wells of infected monocytes at 5$\mu$g/ml/antibody and incubated for 1 hour at room temperature. CD3+ T cells were then isolated from defrosted PBMC by MACS isolation and

these pure T cells were added to appropriate wells of infected monocytes following IL-10 and TGF$\beta$ neutralisation. The cells were then incubated at 37°C for three days, after which the T cells were removed by aspirating off the media and washing twice with PBS. The X-VIVO 15 media supplemented with 2.5mM $_L$-Glutamine was replaced with or without 5$\mu$g/ml purified NA/LE mouse anti-Human HLA-DR, DP, DQ and 5$\mu$g/ml purified NA/LE mouse anti-Human HLA-ABC antibodies (both BD Biosciences) to wells that had T cells added. Latently infected monocytes were then differentiated to iDC (Section 2.15.1).

# Chapter 3

# Identifying Latency-specific CD4+ T cells

## 3.1 Introduction

Primary infection with HCMV induces antiviral immune responses that encompass both the innate and adaptive arms of the immune system and lead to the generation of a high frequency of antiviral T cells. [2, 616]. Despite these responses against HCMV, the virus is not eliminated from the host, and a latent infection is established. CD4+ T cells are known to play an important role during antiviral responses to HCMV, as has been shown in transplant recipients and healthy young children [373, 378, 381, 382]. Additionally, it has been reported that CD4+ T cells from seropositive individuals can recognise a median of 12 HCMV ORFs, with responding CD4+ T cells making up on average 4% of the total peripheral CD4+ T cell compartment [288].

The majority of studies on T cells have focused on lytic antigens such as pp65 and IE, and therefore little is known about the T cell responses to proteins expressed during latent HCMV infection, where gene expression is restricted. During latent infection, proteins known to be expressed during latency include UL138, LUNA, US28, UL111A, and UL144 [125–128, 148, 149]. Recent work has also shown that viral gene expression during latency might not be as highly restricted as previously thought [129, 130]. One study found no evidence of highly restricted gene expression in an experimental latency model, with gene expression resembling a late lytic profile with lower levels of gene expression [130]. A study utilising intracellular cytokine staining (ICS) of T cells stimulated with overlapping peptide pools found CD4+ T cell responses to UL138 in one donor and to UL144 in four of 33 donors tested, but did not find any responses in donors to US28, or UL111A, and did not test

responses to LUNA [288]. However, a subsequent ELISpot-based study on a relatively small number of donors did find CD4+ T cells specific for the latency-associated proteins UL138 and LUNA in seropositive individuals [501]. Whole PBMC were also stimulated with US28 and UL111A peptide pools to reveal responses to these ORFs, but whether CD4+ T cells were the responding cells was not determined [501].

The presence of CD4+ T cells specific for proteins expressed during latency therefore raises the question of why latency is maintained in the face of a potential anti-viral T cell response specific these viral antigens. It is even more intriguing given that UL138- and LUNA-specific CD4+ T cell responses were Th1-like, characterised by the secretion of IFN$\gamma$ and by the ability of UL138-specific CD4+ T cells to mediate MHC class II restricted cytotoxicity against peptide-loaded target cells [501]. However, as well as secreting IFN$\gamma$, a proportion of these CD4+ T cells were also found to secrete the immunomodulatory cytokines IL-10 and TGF$\beta$, which together with the immunosuppressive microenvironment generated by latently infected cells, could help explain why such antigen-specific T cells do not eliminate latently infected cells [501, 605].

It is now recognised that UL138 and LUNA are only two of a number of latency-associated proteins that are expressed during HCMV latency (as well as being expressed during lytic infection), and these therefore could also be T cell targets. Consequently, I initially set out to determine if CD4+ T cells specific for additional proteins known to be expressed during latency, UL144, UL111A and US28 are also generated [125–128, 148, 149]. To accomplish this, I utilised Fluorescent ELISpot (FluoroSpot) to simultaneously detect the secretion of IFN$\gamma$ and IL-10 by CD4+ T cells isolated from seropositive donors in response to HCMV proteins. The FluoroSpot provided a highly sensitive assay that could capture responses over a long period (48 hours), and was therefore superior to the previously utilised ELISpot assays. It was hoped that an examination of the T cell responses to proteins expressed during latency could therefore yield important insights into the ability of the virus to persist, and how these responses could then be modulated to clear a latent infection.

The large number of donors in our cohort also enabled an initial examination into the effect age, as an indicator for time of latent carriage, might have on T cell responses to HCMV. The normal age-related decline in T cell functions, characterised by a loss of CD28 expression and progressive accumulation of highly differentiated memory T cells, is thought to be exacerbated by HCMV infection [408]. It has been suggested that long-term carriage of the virus results in T cell dysfunction, with HCMV serostatus being linked to an increased susceptibility to infection, poor responses to vaccines, and an increased risk of all-cause mortality compared to seronegative individuals [351, 408, 410–412, 617]. I therefore wanted

to test how T cell responses to the latency-associated proteins varied across a donor cohort of 23 to 74 years of age, and if age was an important factor in anti-viral responses.

The aims of this chapter were therefore to identify the presence of latency-specific CD4+ T cells and determine if they secrete the immunosuppressive cytokine IL-10, which could be inhibiting anti-viral T cell responses during latency. In addition, I aimed to examine the balance of IL-10 and IFN$\gamma$ secreting cells to determine the extent IL-10 secreting cells might dominate CD4+ T cell responses to latency-associated proteins. Finally, I wanted to investigate the impact age and sex might have on these CD4+ T cell responses.

## 3.2 Results

### 3.2.1 Serostatus determination

Peripheral blood was taken from healthy donors for serological screening. CMV serostatus was determined by the presence or absence of HCMV specific IgG antibodies as measured by ELISA (Table D.1). Donors were grouped depending on their seropositivity and seronegative donors used as controls for many of the analyses.

### 3.2.2 Detection of UL138- and LUNA-specific T cell responses by FluoroSpot

Previous work has found that CD4+ T cells are able to recognise the latency-associated proteins UL138 and LUNA [501]. In this previous study, the quantification of IFN$\gamma$ and IL-10 secreting cells was limited by the need to use separate ELISpot plates for the detection of each cytokine. Data on the secretion of both of these cytokines from the same cell could therefore not be collected. The FluoroSpot assay however allows for the detection of both IFN$\gamma$ and IL-10 secretion simultaneously on the same plate, and can thus detect dual secreting CD4+ T cells. I therefore utilised the FluoroSpot assay to detect and quantify HCMV-specific CD4+ T cells responses. To do this, *ex vivo* isolated CD4+ T cells from seropositive donors were stimulated with overlapping peptide pools spanning HCMV ORFs and IFN$\gamma$ and IL-10 secreting CD4+ T cells were quantified.

To confirm previous findings, CD4+ T cell responses were first tested to LUNA and UL138. PBMC were isolated from four seropositive donors and depleted of CD8+ expressing

cells by Magnetic-Activated Cell Sorting (MACS). The efficiencies of these depletions were assessed by staining PBMC before and after depletion for expression of CD3, CD4, and CD8. Representative plots and the gating strategies used are shown in Figure 3.1. Doublets were excluded first, followed by gating for lymphocytes and CD3+ cells (Figure 3.1A), then CD4+ and CD8+ cells, for PBMC pre-depletion (Figure 3.1B), post CD8+ cell depletion (Figure 3.1C) or post CD4+ isolation (Figure 3.1D). The percentages of residual CD8+ T cells following CD8+ cell depletion were calculated following gating on CD3+ cells. The mean residual percentage of T cells that were CD8+ following CD8+ cell depletion was 1.8% (standard deviation 1.36, n=57), leaving a mean percentage of 83.5% CD4+ T cells (standard deviation 13.1, n=55) (Figure 3.1E). The CD8+ depleted PBMC was then incubated with the overlapping peptide pools spanning the predicted ORFs of gB, IE1, pp65, UL138, and LUNA for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates to assess the CD4+ T cell response to these ORFs. The individual peptides in the pools were 15 amino acids long and overlapped by 10 amino acids.

The T cell responses to proteins expressed during lytic infection have been extensively studied. The lytically-expressed proteins gB, pp65, and IE1 were therefore included in the analysis to provide a comparison between T cell responses to these ORFs and to latency-associated proteins. Of the four seropositive donors tested, CD4+ T cells were found to secrete IFN$\gamma$ in response to at least two of these three lytic ORFs (Figure 3.2). CD4+ T cell responses to UL138 were found in three of the four donors (75%), while positive responses to LUNA were found in 2 of the four donors (50%) (Figure 3.2).

The same analysis was then carried out for IL-10 secreting cells. A relatively high frequency of CD4+ T cells were found to secrete IL-10 in response to pp65 in three of the four donors (75%), while low responses to gB were found in two donors (50%), and no responders detected to IE1 (Figure 3.3). CD4+ T cells capable of secreting IL-10 in response to UL138 were found in three of the four donors (75%) (Figure 3.3), while in only one of the four donors (25%) was there a high frequency of CD4+ T cells that secreted IL-10 (Figure 3.3B).

The results from four HCMV seropositive donors therefore show that CD4+ T cells secreting IFN$\gamma$ and IL-10 in response to the latency-associated proteins UL138 and LUNA can be detected, as previously reported [501].

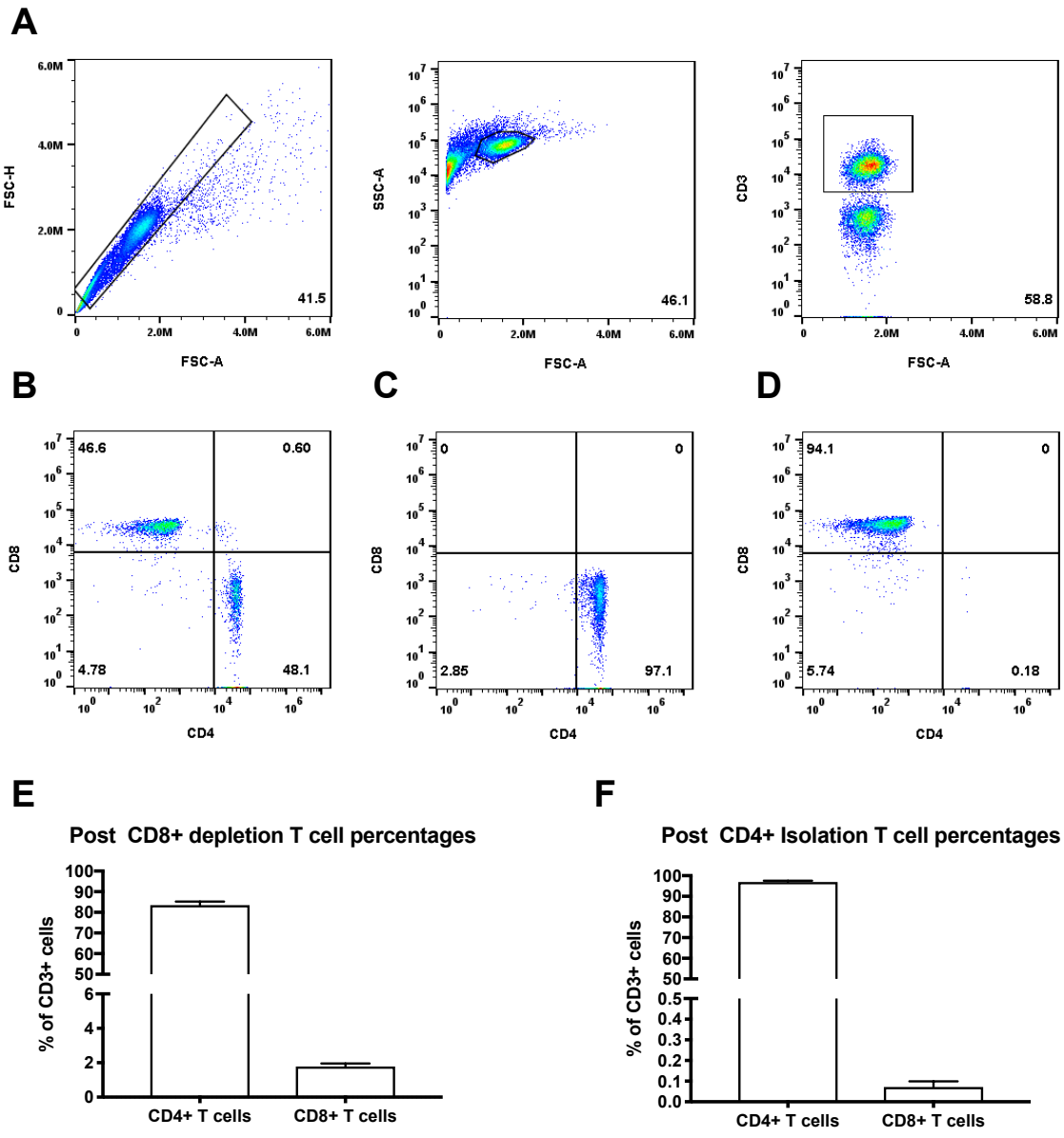



**Fig. 3.1 Magnetic-activated Cell Sorting to deplete CD8+ cells, and to isolate untouched CD4+ T cells, from PBMC.** (A) Gating strategy to identify CD4+ and CD8+ T cells from whole blood. Cells were gated to exclude doublets by forward scatter (FSC)-Height (FSC-H) and FSC-Area (FSC-A), before gating for lymphocytes by FSC-A against side scatter area (SSC-A). CD3+ cells were then gated to identify T cells by CD4 and CD8 expression. Representative plots of (B) CD4+ and CD8+ T cells from whole PBMC, (C) CD8+ cell depleted PBMC, and (D) CD4+ depleted PBMC. (E) Whole PBMC from 57 donors were depleted of CD8+ cells using direct MACS CD8 microbeads or (F) used to isolate CD4+ T cells untouched using an indirect MACS CD4+ isolation kit, and then stained for CD3, CD4, and CD8 expression to determine the residual proportions of CD4+ and CD8+ T cells of total CD3+ cells.

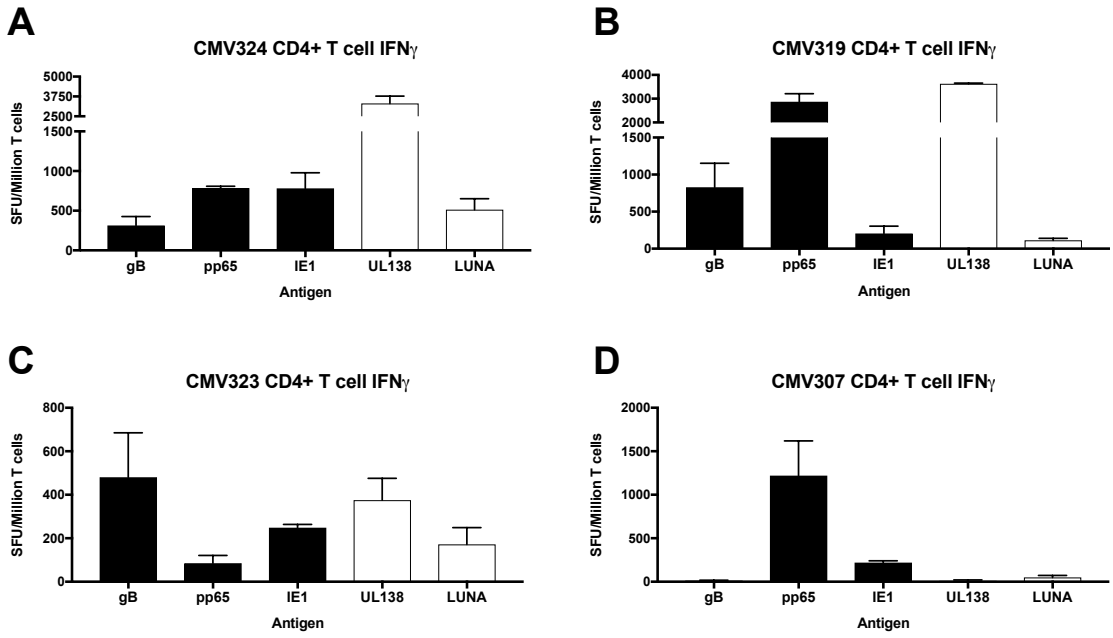



**Fig. 3.2 CD4+ T cells secrete IFN$\gamma$ in response to UL138 and LUNA.** PBMC from four HCMV seropositive donors, (A) CMV324, (B) CMV319, (C) CMV323, and (D) CMV307, were depleted of CD8+ expressing cells and stimulated with HCMV ORF peptides spanning gB, p65, IE1, UL138, and LUNA, for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. IFN$\gamma$ secretion was then stained for to give a number of positive spots that were counted, and converted to spot forming units (SFU) per million cells. All samples were run in triplicate and the frequencies of IFN$\gamma$ positive cells was determined by subtracting values in all test wells from the background number of positive cells in unstimulated well. The solid black bars indicate proteins expressed during lytic HCMV infection, while the white bars show proteins that are also expressed during latency.

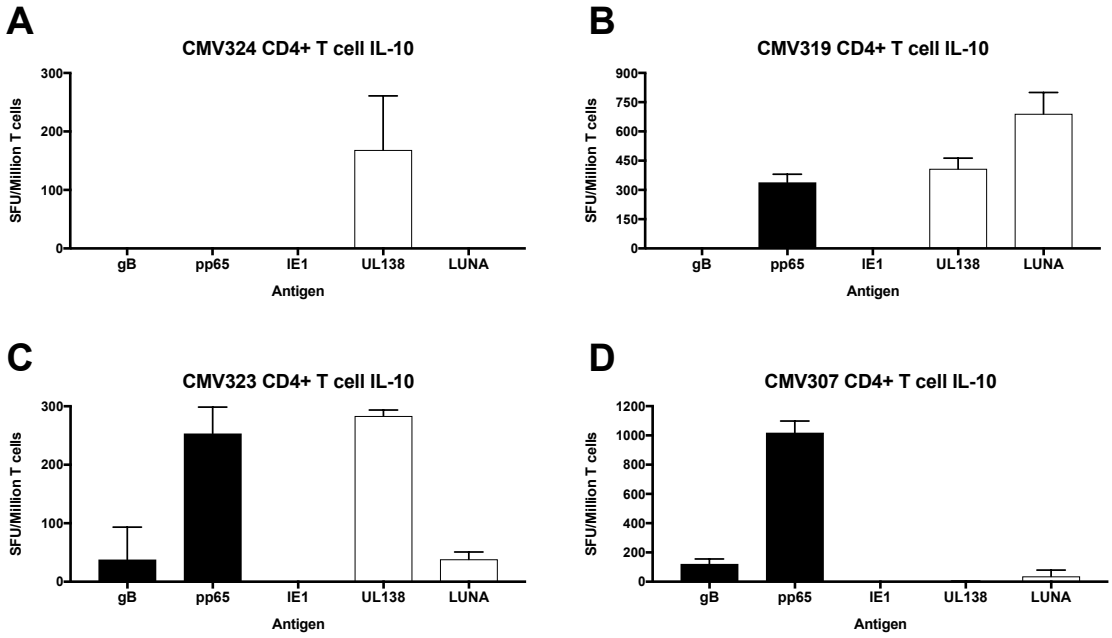



**Fig. 3.3 CD4+ T cells secrete IL-10 in response to UL138 and LUNA.** PBMC from four HCMV seropositive donors, (A) CMV324, (B) CMV319, (C) CMV323, and (D) CMV307, were depleted of CD8+ expressing cells and stimulated with HCMV ORF peptides spanning gB, p65, IE1, UL138, and LUNA, for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. IL-10 secretion was then stained for to give a number of positive spots that were counted, and converted to spot forming units (SFU) per million cells. All samples were run in triplicate and the frequencies of IL-10 positive cells was determined by subtracting values in all test wells from the background number of positive cells in unstimulated well. The solid black bars indicate proteins expressed during lytic HCMV infection, while the white bars show proteins that are also expressed during latency.

### 3.2.3 Validation of the FluoroSpot Assay

The confirmation of previous research showing CD4+ T cell responses to UL138 and LUNA using the FluoroSpot assay was encouraging, although further work was required to ensure that cytokine secreting cells detected in this manner were indeed valid. A number of assays were therefore carried out to validate this system.

Cytokine secretion was assessed by ELISA, in parallel to FluoroSpot, on the supernatants derived from stimulating PBMC depleted of CD8+ cells for 48 hours with HCMV ORF peptides. The expectation was that greater frequencies of detected cytokine secreting cells would also be apparent from increased cytokine concentration detected in the supernatants of similarly stimulated cells. As expected, data from two seropositive donors showed that ELISA results were highly comparable to the results obtained from the FluoroSpot assay. Individuals with high frequencies of IFN$\gamma$ (Figure 3.4A and B) or IL-10 (Figure 3.4C and D) positive spots generally had elevated levels of IFN$\gamma$ or IL-10 production following stimulation, and vice versa where there were low frequencies of cytokine producing cells. This was not the case for all responses however. The IL-10 responses to gB from donor CMV319 (Figure 3.4D) and the frequencies of cytokine positive cells detected by the FluoroSpot did not correlate well with the cytokine secretion detected by ELISA.

In addition, the use of ELISA enabled the reliable quantification of cytokine secretion, which could not be achieved by FluoroSpot alone. Two further donors were also analysed for the secretion of IFN$\gamma$ and IL-10 from their CD4+ T cells by ELISA. IFN$\gamma$ and IL-10 secretion was detected in response to many of the ORF peptide pools (Figure 3.5), and together with the ELISA data from two previous donors (Figure 3.4), show that secretion of these cytokines from CD4+ T cells can be reliably quantified. This lends further support to the validity of the results obtained via the FluoroSpot assay.

As a result of the experimental approach utilised with the FluoroSpot assay, the CD8+ depleted PBMC populations being stimulated with HCMV ORF peptides contained a very small fraction of unwanted CD8+ T cells due to slight inefficiencies in the depletion (Figure 3.1E). To ensure that the cytokine responses being detected from PBMC depleted of CD8+ cells was indeed originating from CD4+ T cells, a modified approach to the FluoroSpot assay was carried out. CD4+ T cells were isolated with high purity by MACS using a CD4+ T cell isolation kit that removes all non-CD4+ cells (Figure 3.6A), and stimulated with HCMV peptides ORFs for 48 hours in tandem with FluoroSpot utilising the depletion protocol as before (where PBMC depleted of CD8+ cells were used) (Figure 3.6A). Using this CD4+ T cell isolation method, the mean residual CD8+ T cell proportion of total CD3+

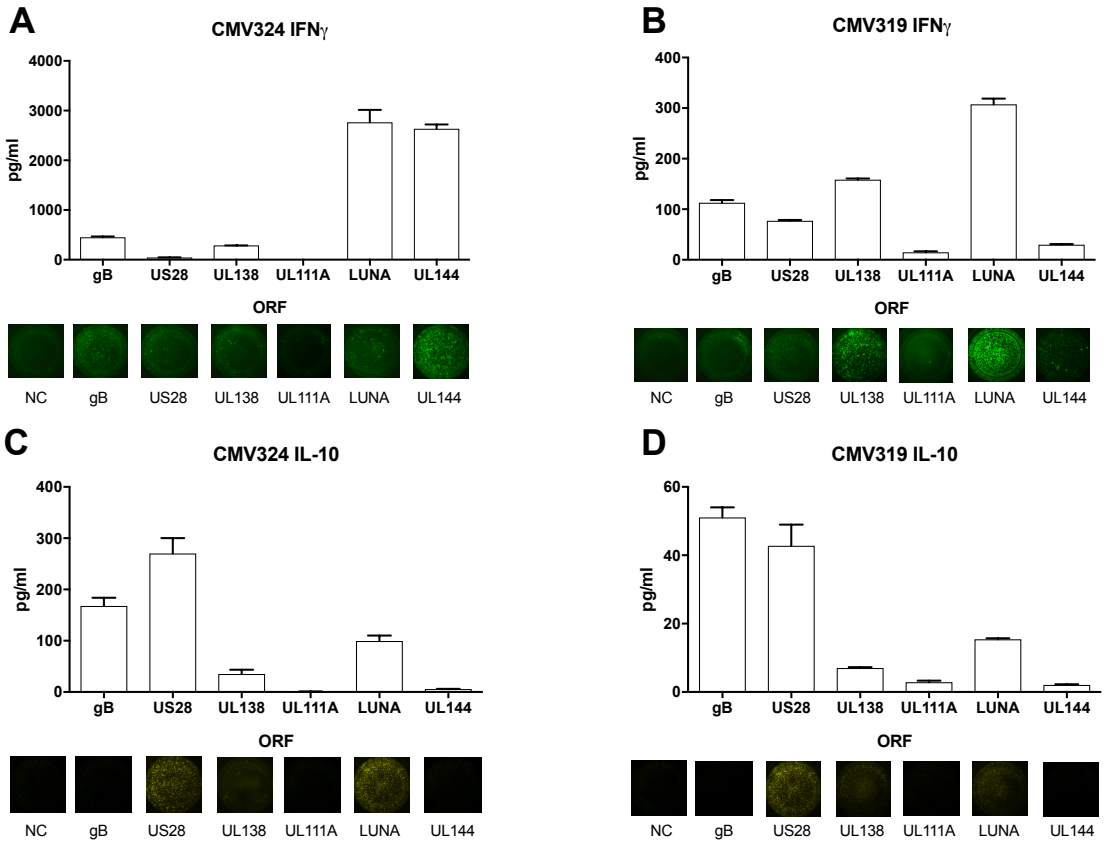



**Fig. 3.4 Validating FluoroSpot results for cytokine secretion from CD4+ T cells with ELISA.** PBMC isolated from two seropositive donors were depleted of CD8+ cells and incubated for 48 hours with pools of overlapping 15 amino acid peptides spanning each of the HCMV ORFs tested (gB, US28, UL138, UL111A, LUNA, and UL144). This was simultaneously carried out on dual IFN$\gamma$/IL-10 FluroSpot plates, and on 48 well plates. Following this stimulation, the supernatant was harvested and IFN$\gamma$ and IL-10 secretion was measured by ELISA (quantification was achieved using a standard curve of known amounts of cytokine), and IFN$\gamma$ captured on the FluoroSpot plates was stained to visualise cytokine positive spots. All samples were run in triplicate with the mean value shown here. CD4+ T cell IFN$\gamma$ secretion was detected by ELISA from donors (A) CMV324, and (B) CMV319, and IL-10 secretion from donors (C) CMV324, and (D) CMV319 and compared to the images showing the frequencies of IFN$\gamma$ positive spots obtained from the FluoroSpot for each HCMV ORF peptide pool tested (NC = negative control).

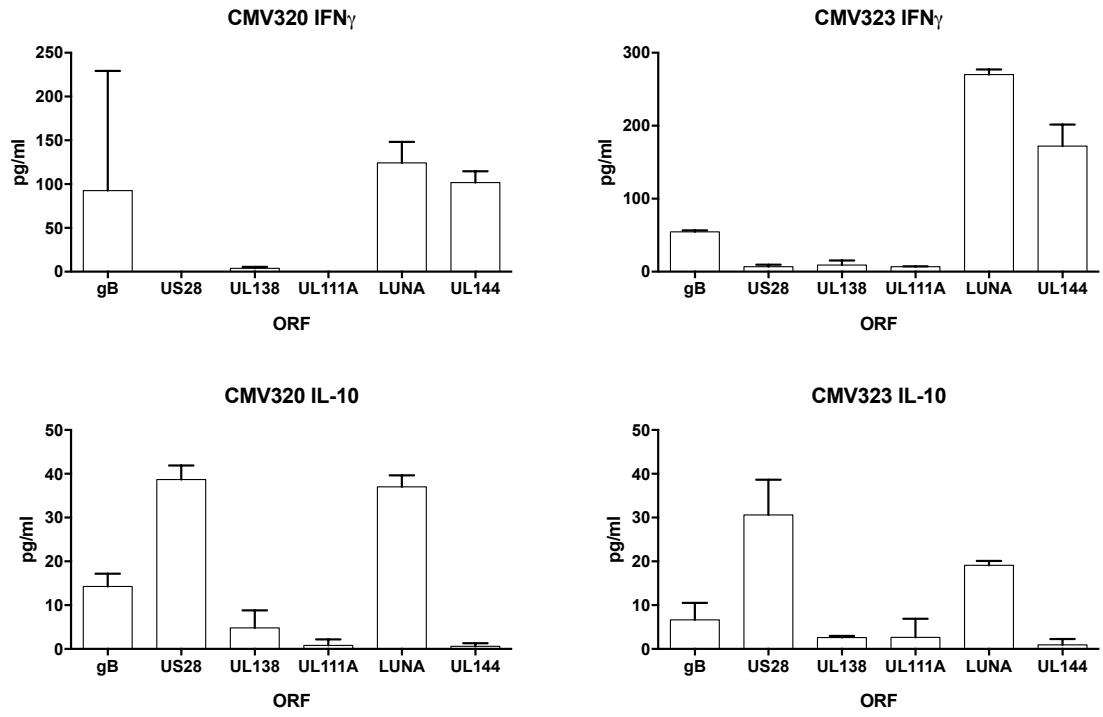



**Fig. 3.5 Quantifying IFN$\gamma$ and IL-10 secretion from CD4+ T cells by ELISA.** PBMC isolated from two seropositive donors (CMV320 and CMV323) were depleted of CD8+ cells and incubated for 48 hours with overlapping peptide pools spanning each of the HCMV ORFs tested (gB, US28, UL138, UL111A, LUNA, and UL144). Following this stimulation, the supernatant was harvested and IFN$\gamma$ and IL-10 secretion was measured by ELISA (quantification was achieved using a standard curve of known amounts of cytokine). All samples were run in triplicate with the mean value shown here.

cells was 0.072% (standard deviation=0.12, n=19), while the mean proportion of CD3+ cells that were CD4+ T cells was 96.97% (standard deviation=2.71, n=19) following the isolation (Figure 3.1F). The results show a high degree of similarity in the frequency of detected IFN$\gamma$ or IL-10 secreting cells between the two CD4 preparation methods (Figure 3.6B and C). The higher frequencies of cytokine secreting cells detected in the CD4+ T cell isolation condition in response to many of the HCMV ORF peptide pools could be explained by the higher proportion of CD4+ T cells present in each well. This results from the higher purity of the isolation method and absence of other PBMC subsets in this condition, such as monocytes. However, this effect was not seen with IL-10 secreting CD4+ T cells responding to gB, although no differences were detected between the conditions (Figure 3.6C). I am therefore confident that the small frequency of unwanted CD8+ T cells present in our depletion experiments does not have a large effect on the overall frequency of responding cells detected, and neither does some other PBMC subset, such as monocytes or NK cells.

For further validation in a different assay system, I stimulated PBMC from 18 seropositive donors with HCMV ORF peptides pools from each ORF for 24 hours and subsequently carried out ICS on these cells. The typical gating strategy to define cytokine secreting cells is shown in Figure 3.7 for the CD4+ T cell response to UL144 ORF peptides. Live cells were first gated on, followed by the exclusion of doublets and gating of lymphocytes (Figure 3.6A). CD3+ cells and CD4+ T cells were then defined, and, to detect cytokine secretion from antigen-specific T cells only, cells positive for 4-1BB and/or CD40L were gated on, before IFN$\gamma$ and IL-10 gates were placed (Figure 3.6A). IFN$\gamma$ and IL-10 production from CD4+ T cells was readily detected in response to all the HCMV ORF peptide pools tested with variations in the proportions of cytokine positive cells responding to different ORFs (Figure 3.7). The ability to delineate cytokine secretion to defined cellular phenotypes, in this case CD4+ T cells, also lends further support that the cytokine secretion detected in the FluoroSpot assay is indeed originating from CD4+ T cells and not another PBMC subset. Given these validation experiments, I continued using the FluoroSpot assay with this depletion protocol, where PBMC were depleted of CD8+ cells to measure CD4+ T cell responses, as this proved to be a relatively quick and effective method to achieve reliable results.

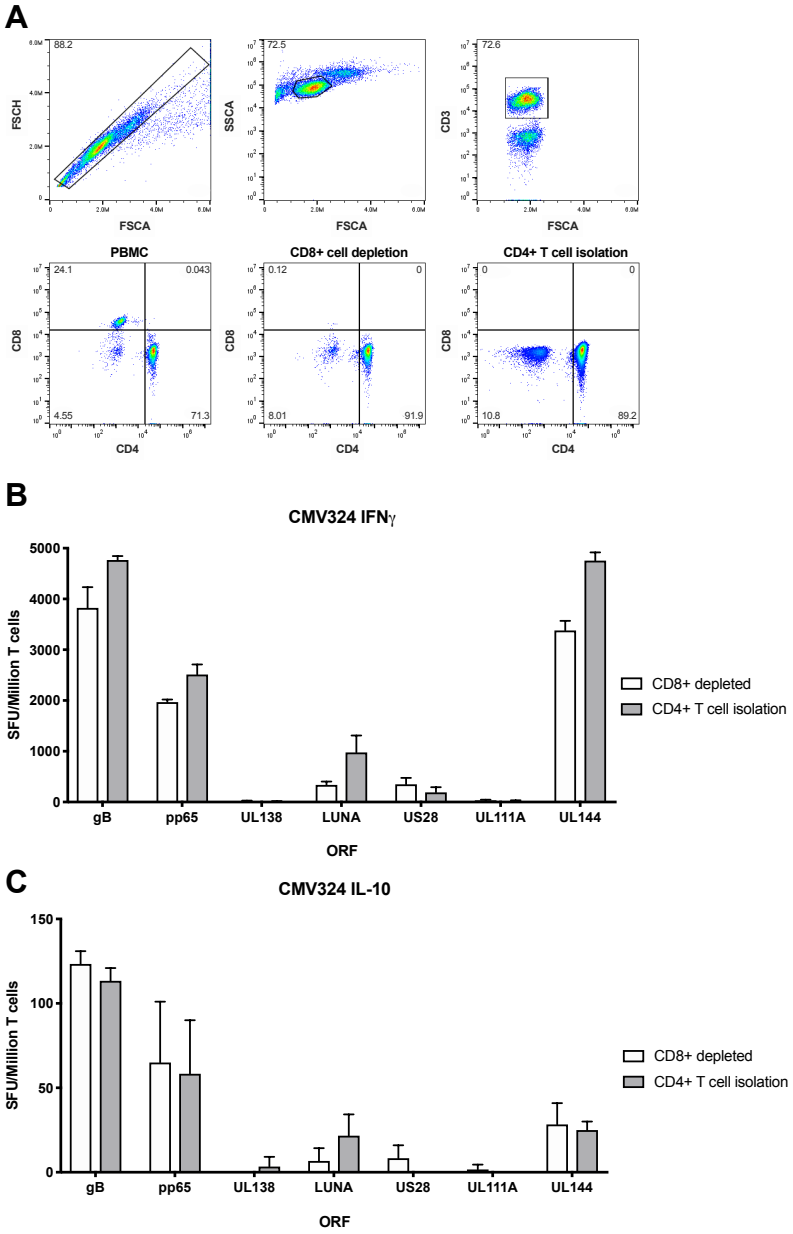



**Fig. 3.6 Comparison of the frequencies of cytokine secreting cells detected following CD8+ cell depletion, or CD4+ T cell isolation, from PBMC using a negative selection or isolation kit.** In parallel, whole PBMC were either depleted of CD8+ cells by direct MACS, or CD4+ T cells were isolated untouched using a CD4+ T cell isolation kit, before being stimulated with HCMV ORF peptide pools for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. IFN$\gamma$ and IL-10 secretion was then stained for to give a number of positive spots that were counted and converted to spot forming units (SFU) per million cells. All wells were tested in triplicate and the background number of cytokine secreting cells observed in unstimulated wells was deducted from test wells. (A) The gating strategy to identify T cells prior to, and following, MACS depletion and isolation. (B) IFN$\gamma$ secretion from CD8+ depleted PBMC (shown with white bars) and isolated CD4+ T cells (shown with grey bars) (C) IL-10 secretion from CD8+ depleted PBMC and isolated CD4+ T cells.

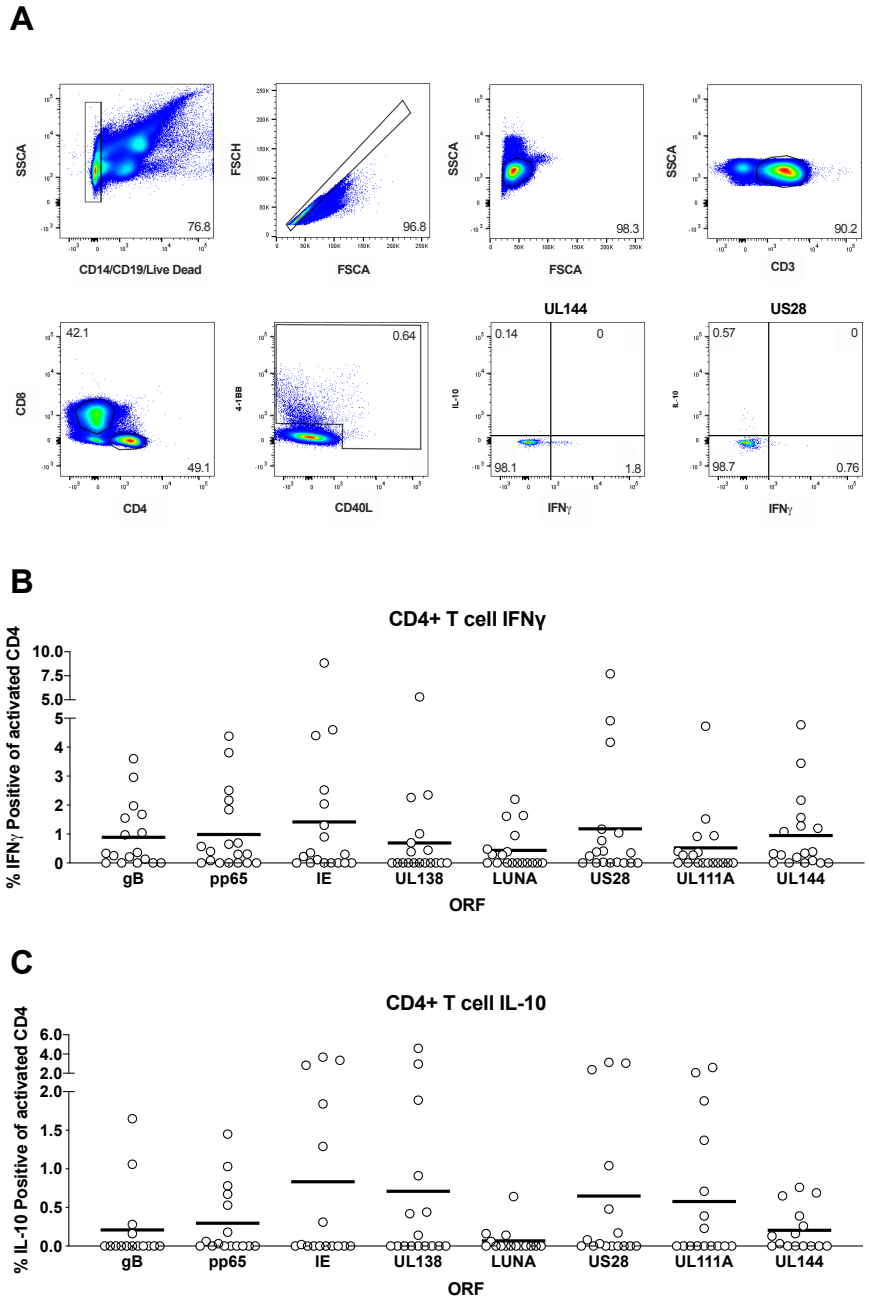



**Fig. 3.7 Intracellular cytokine staining of CD4+ T cells following HCMV ORF peptide pool stimulation.** Whole PBMC isolated from 18 seropositive donors were stimulated overnight with HCMV peptide pools spanning the ORFs of gB, pp65, IE1, UL138, LUNA, US28, UL111A, and UL144, in the presence of monensin. Stimulated PBMC were then stained intracellularly. (A) The gating strategy to identify IFN$\gamma$ and IL-10 secretion from CD4+ T cells in response to the UL144 and US28 peptide pool is shown. Dead cells, CD14+ and CD19+ cells were first excluded, followed by doublets. Lymphocytes were then gated on by FSCA and SSCA and CD3+ cells identified. CD4+ and CD8+ cells were then gated, followed by activated CD4+ T cells, identified by gating for CD40L or 4-1BB positive cells, and finally IFN$\gamma$ and IL-10 positive cells. (B) CD4+ T cells positive for IFN$\gamma$ and (C) CD4+ T cells positive for IL-10 are illustrated as proportions of CD3+CD4+ cells following the gating shown in (A).

### 3.2.4 The frequency of responding CD4+ T cells detected fluctuates over time

Of total T cells, those present in the blood represent a low proportion, with one estimate placing this figure at 2.5% for CD4+ T cells and 2.3% for CD8+ T cells [618]. I therefore wanted to examine how sampling of PBMC at different time points might affect the T cell responses detected. The small group of four seropositive donors used previously was tested for their CD4+ T cell responses to HCMV ORF peptide pools multiple times over a period of three years using the FluoroSpot assay. The sampling of donors was not uniform over time, and certain donors were only sampled twice. The most comprehensive analysis was undertaken on one donor (CMV324), with eight time points analysed for their CD4+ T cell responses.

The CD4+ T cell responses to certain ORFs were very stable over the eight time points, where positive responses were detected at all time points in response to gB, pp65, IE1, LUNA, US28, and UL144 (Figure 3.8A). In contrast, responses to UL138 and UL111A fluctuated over the eight time points. At certain sampling times there were effectively no CD4+ T cells that secreted IFN$\gamma$ in response to the latency-associated proteins UL138 and UL111A (Figure 3.8A). The CD4+ T cell responses can therefore fluctuate to the extent that positive responses are lost. In the case of UL138 responses for example, the first two time points showed a considerably high number of IFN$\gamma$ secreting CD4+ T cells, which was not seen again over the next six time points (Figure 3.8A). This is in contrast to the UL111A-specific CD4+ T cell responses, which were observed to oscillate between positive and no responses over the sample period (Figure 3.8A). Furthermore, the frequency of IFN$\gamma$ positive CD4+ T cells was observed to vary considerably between certain time points (Figure 3.8A).

Overall, IL-10 responses were much less consistent than the IFN$\gamma$ responses, and this could partially be attributed to time points when the background IL-10 responses were too high in the unstimulated wells, resulting in data that was not assessable. Positive responses to US28 were the most frequently observed over the eight time points, while positive responses to gB, pp65, and UL138, were the next most frequent, and UL111A, UL144, and IE1 responses the least frequent (Figure 3.8B). Of note, there were two time points months apart which had considerably high US28 IL-10 responses that were much higher than any other response at any time point (Figure 3.8B), and for which the IFN$\gamma$ US28 responses were the lowest of all the eight time points (Figure 3.8A).

As will be shown in Section 3.2.5, the threshold values for positive IFN$\gamma$ and IL-10 responses were determined based on the number of cytokine positive spots detected from

HCMV seronegative donors in response to HCMV ORF peptide pools. These values (100 SFU/$10^6$ for IFN$\gamma$ secreting cells and above 50 SFU/$10^6$ for IL-10 secreting cells) provide a threshold to identify responses as positive, in addition to the deduction of background cytokine secreting cells from all test values. Based on these thresholds, examining how the number of positive responses to HCMV ORFs changes over time in donor CMV324 revealed that the number of ORFs responded to with IFN$\gamma$ secretion oscillated between responding to all eight, and to five, ORFs over the eight time points (Figure 3.9A). These fluctuations were mainly in the responses to latency-associated proteins, where at all time points except for one, all three lytically expressed proteins were responded to (Figure 3.9A).

Although the CD4+ IL-10 T cell responses were less frequent, they were still highly variable. The number of positive CD4+ IL-10 T cell responses oscillated over time considerably, from zero to three ORFs responded to, of eight total (Figure 3.9). There were also two time points where there no CD4+ IL-10 T cell responses to any latency-associated proteins were detected, highlighting the greater variation in sampling IL-10 responses (Figure 3.9B) compared to IFN$\gamma$ responses (Figure 3.9A).

Longitudinal data collected in the same way from different donors also illustrated the fluctuations in T cell responses detected over time. As expected, the CD4+ IFN$\gamma$ T cell responses to the lytically expressed proteins were very consistent, where positive responses were registered at nearly all sampling times (Figure 3.10A, 3.11A, and 3.12A). Interestingly, the CD4+ IFN$\gamma$ T cell responses to UL144 were the most consistent and often composed of similar frequencies of IFN$\gamma$ secreting cells at different time points (Figure 3.10A, 3.11A, and 3.12A). Responses to the latency-associated proteins were slightly more variable, although it was still the case that if a donor made a response to an ORF, then they responded again to that ORF at most time points. Donor CMV319, who was sampled across three time points, possessed CD4+ IFN$\gamma$ T cell responses at all time points to LUNA, US28, and UL111A, while at two of three time points responded to UL138 (Figure 3.10A). On the other hand, donor CMV323 had greater variability in responses over the three time points, with responses to UL138 and LUNA only detected on two occasions, in addition to the pronounced fluctuations in the frequencies of IFN$\gamma$ secreting CD4+ T cells at different time points (Figure 3.11A).

In these two donors the CD4+ IL-10 T cell responses were fairly consistent despite there being fewer ORFs that were responded to, although there were ORFs that were responded to at only one time point. Donor CMV319 had IL-10 responses at all three time points to pp65 and UL138, and at two time points to LUNA, and US28, while only at one time point to UL111A and UL144 (Figure 3.10B). Similarly donor CMV323 had IL-10 responses at

two points to UL138 and US28, but only at one in response to pp65, gB, and LUNA (Figure 3.11B).

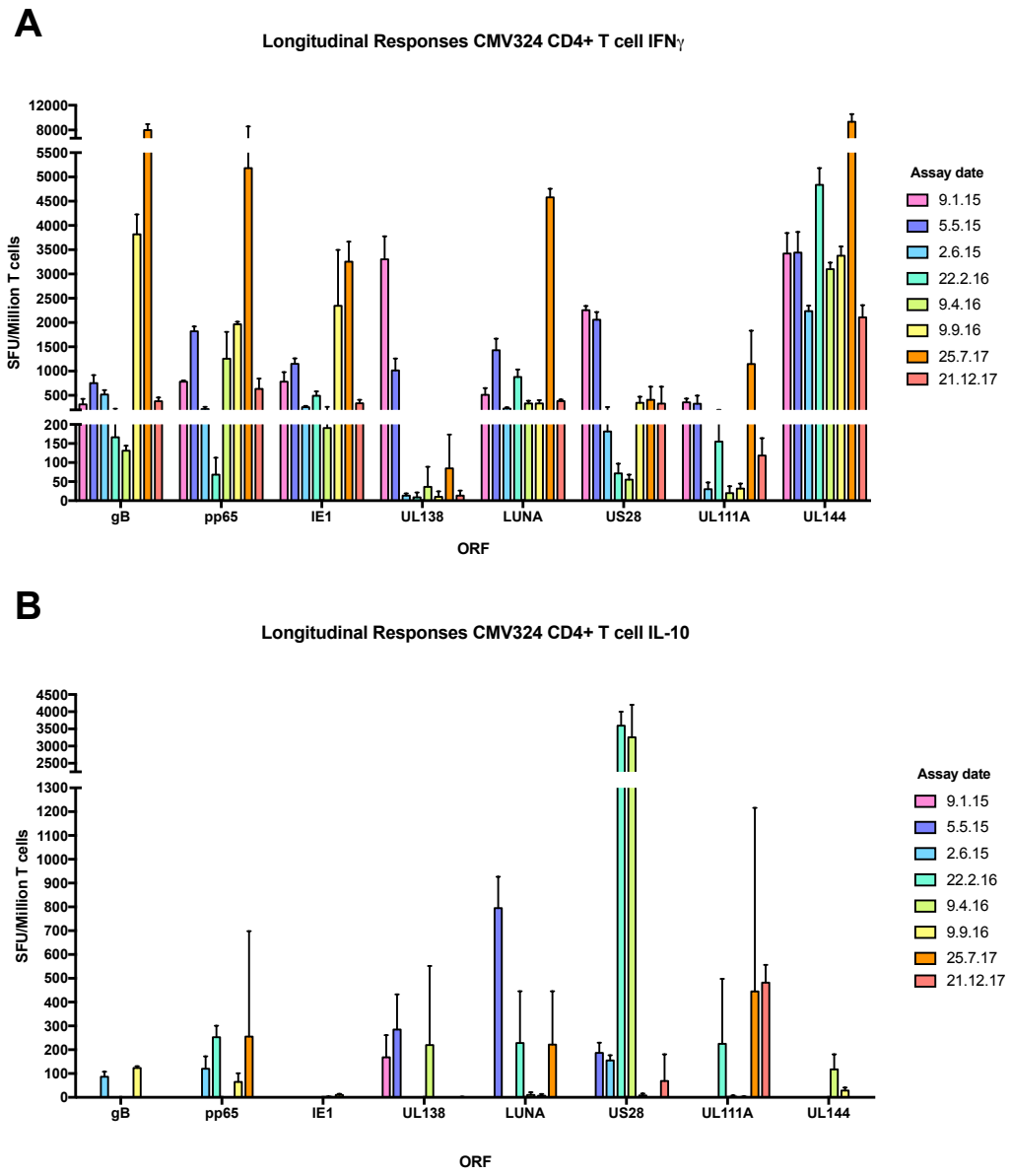
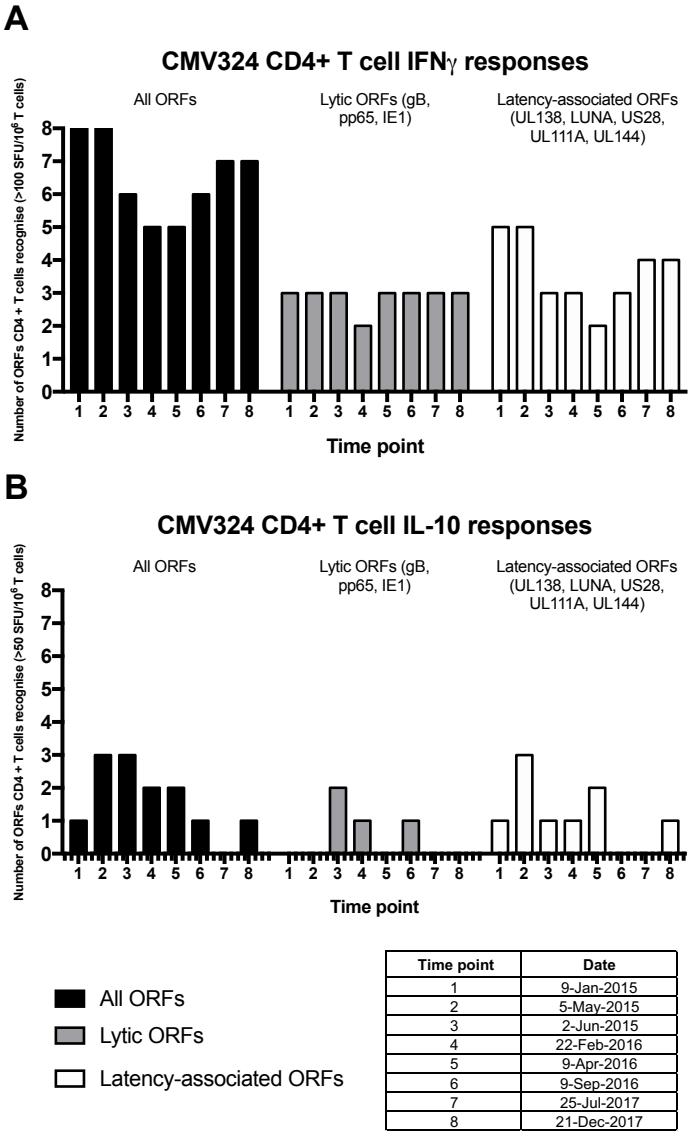



**Fig. 3.8 Longitudinal IFN$\gamma$ and IL-10 CD4+ T cell responses to HCMV ORFs from donor CMV324.** CD4+ IFN$\gamma$ and IL-10 T cell responses from donor CMV324 were tested at eight separate time points over 35 months. PBMC were depleted of CD8+ expressing cells and stimulated with overlapping peptide pools of HCMV peptides spanning gB, p65, IE1, UL138, LUNA, US28, UL111A, and UL144, for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. (A) IFN$\gamma$ and (B) IL-10 secretion was then stained for to give a number of positive spots that were counted, and converted to spot forming units (SFU) per million cells. All samples were run in triplicate and the frequencies of positive cells were determined by subtracting values in all test wells from the background number of positive cells in unstimulated well. Each different colour of bar indicates a different sampling time point. The thresholds for positive responses of 100 SFU/$10^6$ cells for IFN$\gamma$ and 50 SFU/$10^6$ for IL-10 are indicated by the dotted line.




| Time point | Date |
|---|---|
| 1 | 9-Jan-2015 |
| 2 | 5-May-2015 |
| 3 | 2-Jun-2015 |
| 4 | 22-Feb-2016 |
| 5 | 9-Apr-2016 |
| 6 | 9-Sep-2016 |
| 7 | 25-Jul-2017 |
| 8 | 21-Dec-2017 |

**Fig. 3.9 Longitudinal analysis of the number of positive CD4+ IFN$\gamma$ and IL-10 T cell responses in donor CMV324.** (A) Positive IFN$\gamma$ CD4+ T cell responses (responses over 100 SFU/$10^6$ cells) and (B) Positive CD4+ IL-10 T cell responses (threshold above 50 spot forming units/$10^6$ cells) are shown at 8 time points over three years from one donor. The dates of each time point are shown in the table. PBMC were depleted of CD8+ expressing cells and stimulated with overlapping peptide pools of HCMV peptides spanning gB, p65, IE1, UL138, LUNA, US28, UL111A, and UL144, for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. The number of IFN$\gamma$ and IL-10 positive spots was determined and converted to SFU/$10^6$ cells. All samples were run in triplicate and the frequencies of positive cells were determined by subtracting values in all test wells from the background number of positive cells in unstimulated well. The black bars indicate responses to all 8 ORFs, both lytic and latency-associated, the grey to lytic ORFs gB, pp65, and IE, and the white to latency-associated ORFs UL138, LUNA, US28, UL111A, and UL144.

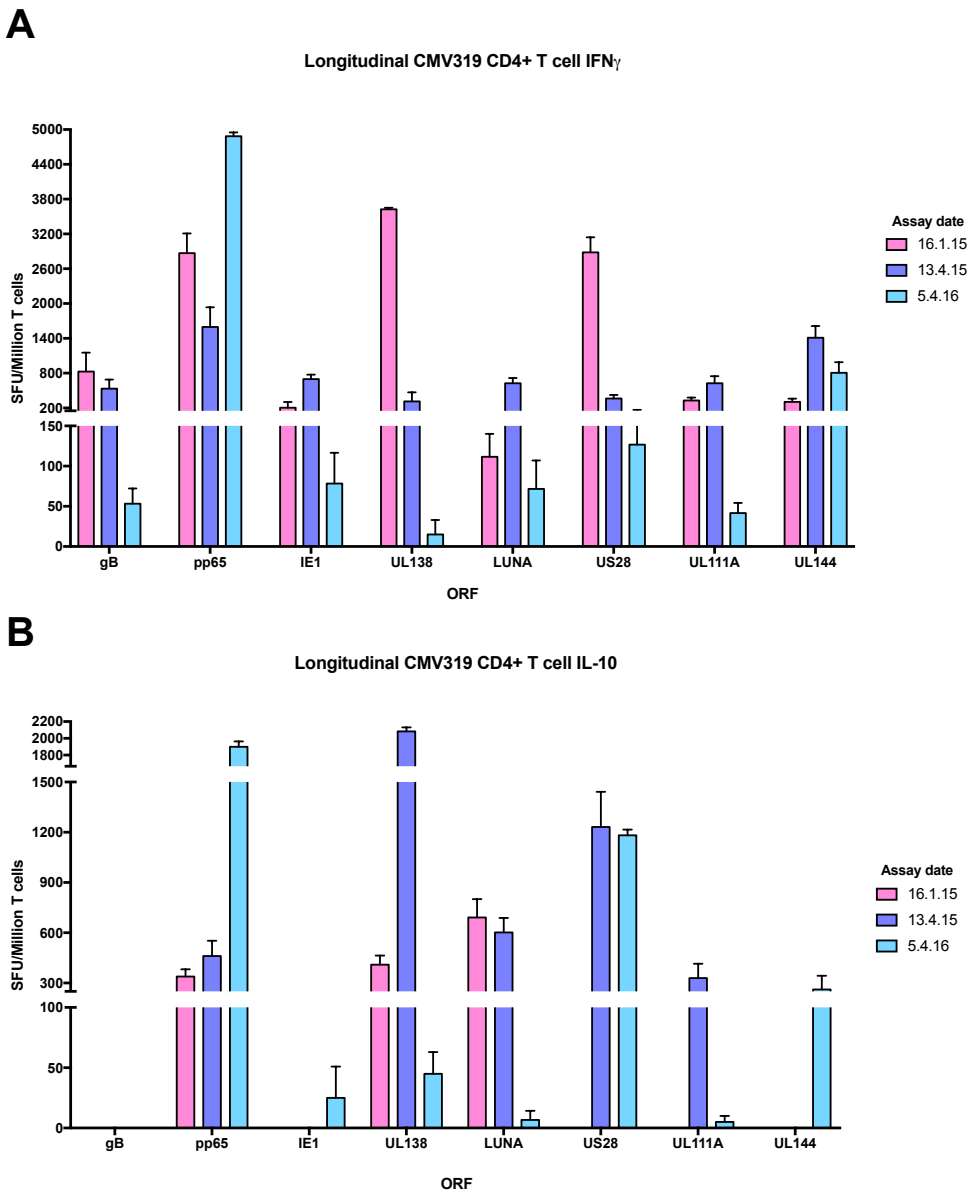



**Fig. 3.10 Long term IFN$\gamma$ and IL-10 CD4+ T cell responses to HCMV ORFs from donor CMV319.** CD4+ IFN$\gamma$ and IL-10 T cell responses from donor CMV319 were tested at three separate time points. PBMC were depleted of CD8+ expressing cells and stimulated with overlapping peptide pools of HCMV peptides spanning gB, p65, IE1, UL138, LUNA, US28, UL111A, and UL144, for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. (A) IFN$\gamma$ and (B) IL-10 secretion was then stained for to give a number of positive spots that were counted, and converted to spot forming units (SFU) per million cells. All samples were run in triplicate and the frequencies of positive cells were determined by subtracting values in all test wells from the background number of positive cells in unstimulated well. Each different colour of bar indicates a different sampling time point. The thresholds for positive responses of 100 SFU/$10^6$ cells for IFN$\gamma$ and 50 SFU/$10^6$ for IL-10 are indicated by the dotted line.

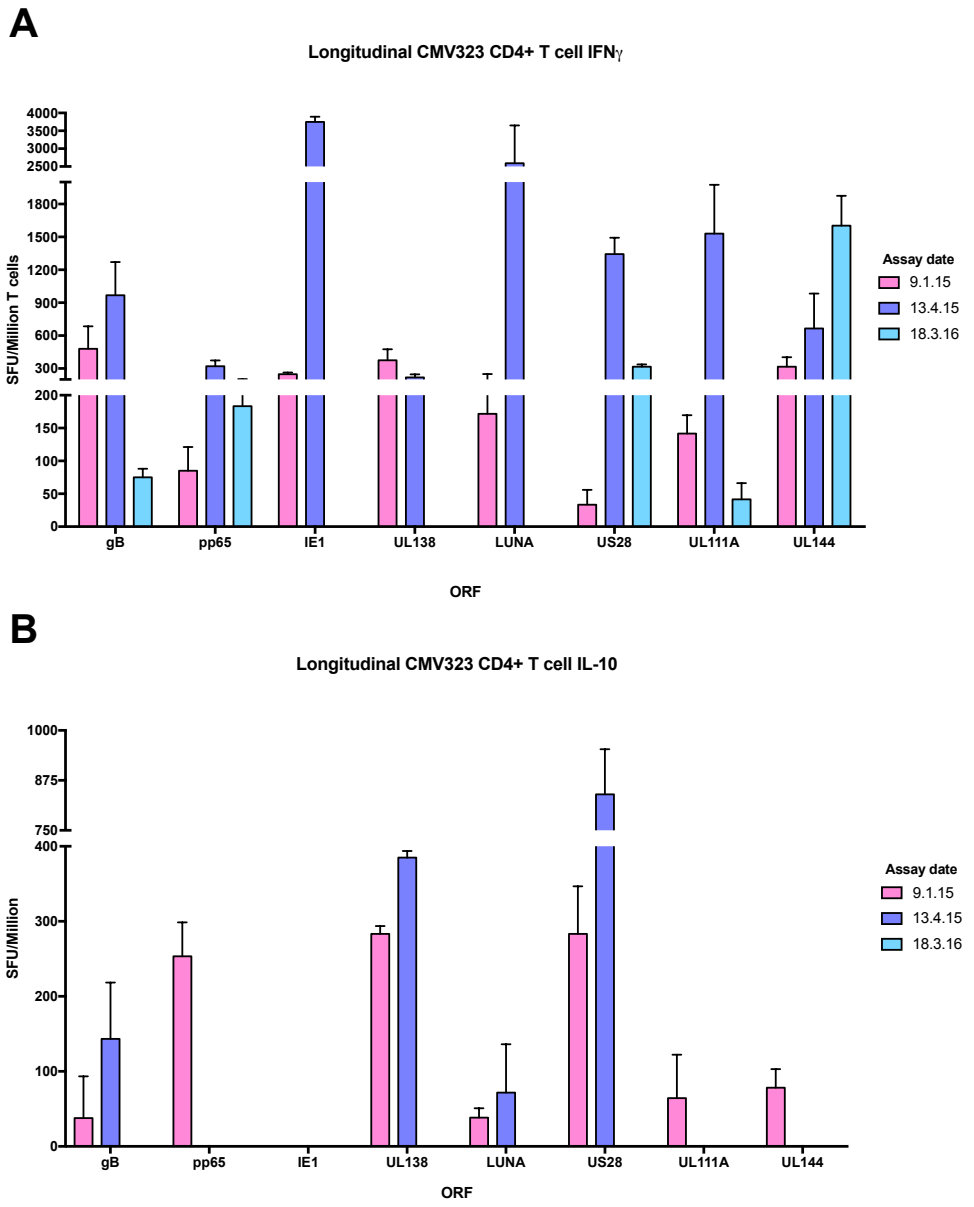



**Fig. 3.11 Long term IFN$\gamma$ and IL-10 CD4+ T cell responses to HCMV ORFs from donor CMV323.** CD4+ IFN$\gamma$ and IL-10 T cell responses from donor CMV323 were tested at three separate time points. PBMC were depleted of CD8+ expressing cells and stimulated with overlapping peptide pools of HCMV peptides spanning gB, p65, IE1, UL138, LUNA, US28, UL111A, and UL144, for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. (A) IFN$\gamma$ and (B) IL-10 secretion was then stained for to give a number of positive spots that were counted, and converted to spot forming units (SFU) per million cells. All samples were run in triplicate and the frequencies of positive cells were determined by subtracting values in all test wells from the background number of positive cells in unstimulated well. Each different colour of bar indicates a different sampling time point. The thresholds for positive responses of 100 SFU/$10^6$ cells for IFN$\gamma$ and 50 SFU/$10^6$ for IL-10 are indicated by the dotted line.

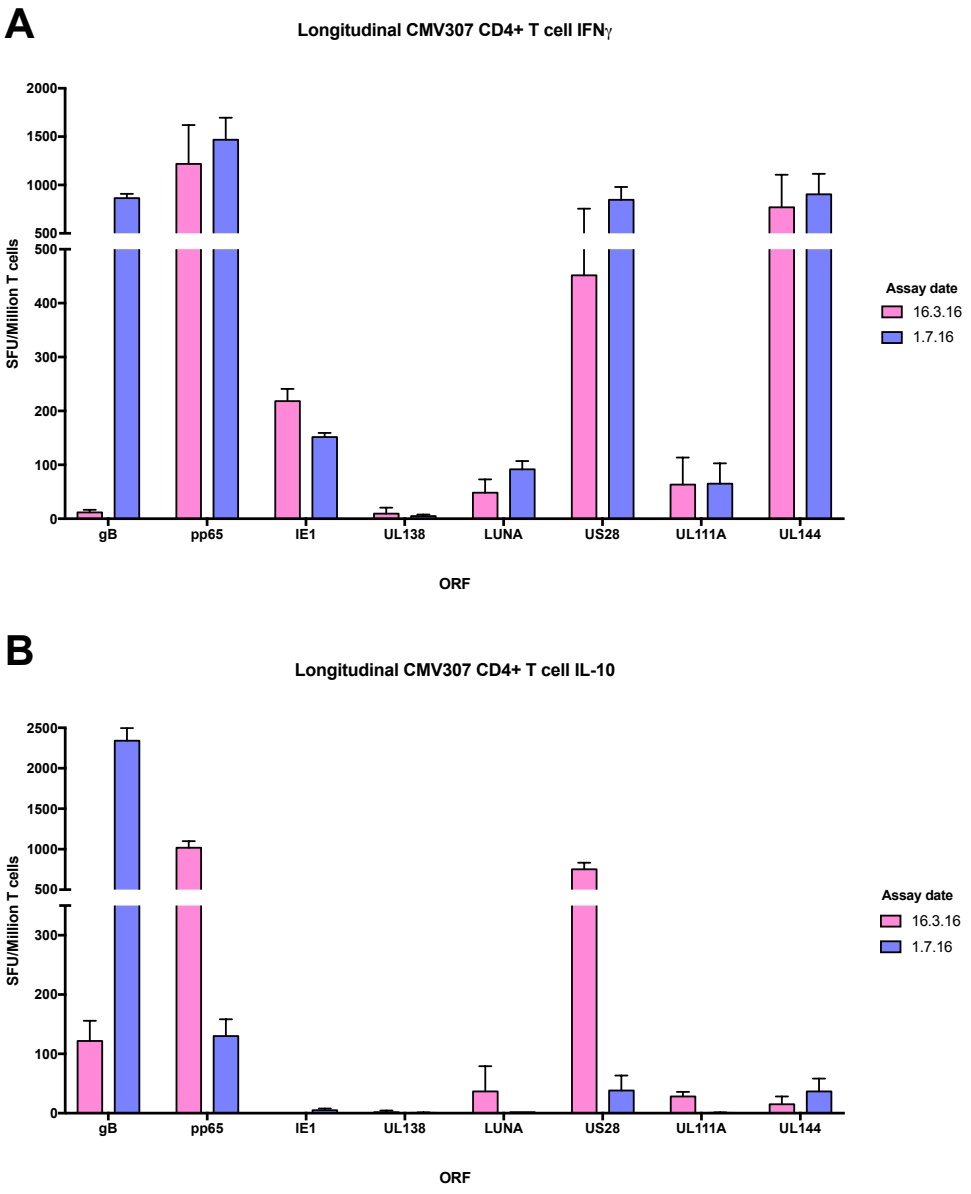



**Fig. 3.12 Long term IFN$\gamma$ and IL-10 CD4+ T cell responses to HCMV ORFs from donor CMV307.** CD4+ IFN$\gamma$ and IL-10 T cell responses from donor CMV307 were tested at two separate time points. PBMC were depleted of CD8+ expressing cells and stimulated with overlapping peptide pools of HCMV peptides spanning gB, p65, IE1, UL138, LUNA, US28, UL111A, and UL144, for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. (A) IFN$\gamma$ and (B) IL-10 secretion was then stained for to give a number of positive spots that were counted, and converted to spot forming units (SFU) per million cells. All samples were run in triplicate and the frequencies of positive cells were determined by subtracting values in all test wells from the background number of positive cells in unstimulated well. Each different colour of bar indicates a different sampling time point. The thresholds for positive responses of 100 SFU/$10^6$ cells for IFN$\gamma$ and 50 SFU/$10^6$ for IL-10 are indicated by the dotted line.

### 3.2.5 CD4+ T cells can recognise and secrete IFN$\gamma$ in response to US28, UL111A, and UL144

Having tested a small group of seropositive donors for the ability of their CD4+ T cells to respond to the latency-associated proteins UL138 and LUNA, the same methodology was applied to a larger cohort to confirm these responses in the wider population, and to investigate the CD4+ T cell responses to several other proteins known to be expressed during HCMV latency, namely US28, UL111A, and UL144.

Once again, 15mer overlapping peptide pools spanning each ORF were utilised in conjunction with the FluoroSpot assay to do this. All samples were run with unstimulated control wells composed of the donor's cells alone without any HCMV peptides. This enabled the determination of the background level of cytokine secretion that each donors' cells possessed and this value was subtracted from all test conditions. To ensure stringent identification of positive cytokine responses, several quality control checks were put in place. These were particularly important to assess IL-10 responses, as many donors possessed high frequencies of background IL-10 secretion in their unstimulated samples. Donors with responses in their background, unstimulated samples of above 1000 spots were excluded. For IFN$\gamma$ responses, the difference between the unstimulated samples and the positive control samples had to be greater than 100 spots. For IL-10 responses, this difference was 50 spots, given the relatively weaker ability of our positive control to elicit strong IL-10 secretion, in addition to simultaneous IFN$\gamma$ secretion. These imposed thresholds resulted in IL-10 response data from 33 donors being discarded, while for IFN$\gamma$ responses, 10 donors were excluded.

Positive responses from the FluoroSpot assay were counted as greater than 100 SFU/$10^6$ cells for IFN$\gamma$ responses and greater than 50 SFU/$10^6$ cells for IL-10 responses. These thresholds were chosen based on the non-specific responses obtained when stimulating cells from seronegative donors with the HCMV ORF peptide pools (Figure 3.13 and 3.16) to ensure the responses being observed in seropositive donors were specific to HCMV.

Alongside measuring the responses to the latency-associated proteins UL138, LUNA, US28, UL111A, and UL144, I also tested ORF pools from the lytically expressed proteins gB, pp65, and IE1 to allow for comparison between responses against proteins only expressed during lytic infection and those also expressed during latency.

Of 91 donors tested that passed quality control, positive CD4+ T cell IFN$\gamma$ responses were detected against all ORFs and the number of responding donors varied according to ORF.

Positive CD4+ T cell IFN$\gamma$ responses were registered against UL138 (27 donors, 29.7%), LUNA (26 donors, 28.6%), US28 (54 donors, 59.3%), UL111A (27 donors, 29.7%), and UL144 (69 donors, 75.8%), in addition to responses against the lytically expressed gB (61 donors, 67%), IE1 (46 donors, 50.5%), and pp65 (78 donors, 85.7%) (Figure 3.13).

The results presented here confirm previously reported findings for the presence of UL138 and LUNA specific CD4+ T cells in seropositive individuals from a much larger donor cohort. Additionally, these findings are extended to the latency-associated proteins US28, UL111A, and UL144, where responses against all of these ORFs are reported here.

There were many individuals who have a high frequency of CD4+ T cells that secrete IFN$\gamma$ in response to the HCMV ORFs tested. Grouping these high responders as those who have a T cell response greater than 250 SFU/$10^6$ cells reveals that of 91 donors, CD4+ T cell responses to the lytically expressed proteins tested were made up of 47 high responders to gB (51.6%), 68 to pp65 (74.7%), and 31 to IE1 (34.1%) (Figure 3.13). Of the CD4+ T cell responses to the latency-associated proteins tested, the greatest number of high responders were found to UL144 (55/91, 60.4%), and to US28 (34/91, 37.4%), whereas high responders to LUNA (17/91 18.7%), UL111A (15/91, 16.5%), and UL138 (12/91, 13.2%) were much less frequent (Figure 3.13).

These results demonstrate that T cell responses to the latency-associated proteins tested are present, and in certain cases comparable to the frequencies of T cells responding to lytically expressed proteins. There were clear differences in the CD4+ T cell responses to each latency-associated protein, where UL144 and US28 were found to have the highest number of donors with a positive CD4+ response, in addition to the highest frequency of T cells responding to these proteins within individuals. Less common, were donors with a positive IFN$\gamma$ CD4+ T cell response to UL111A, LUNA, and UL138.

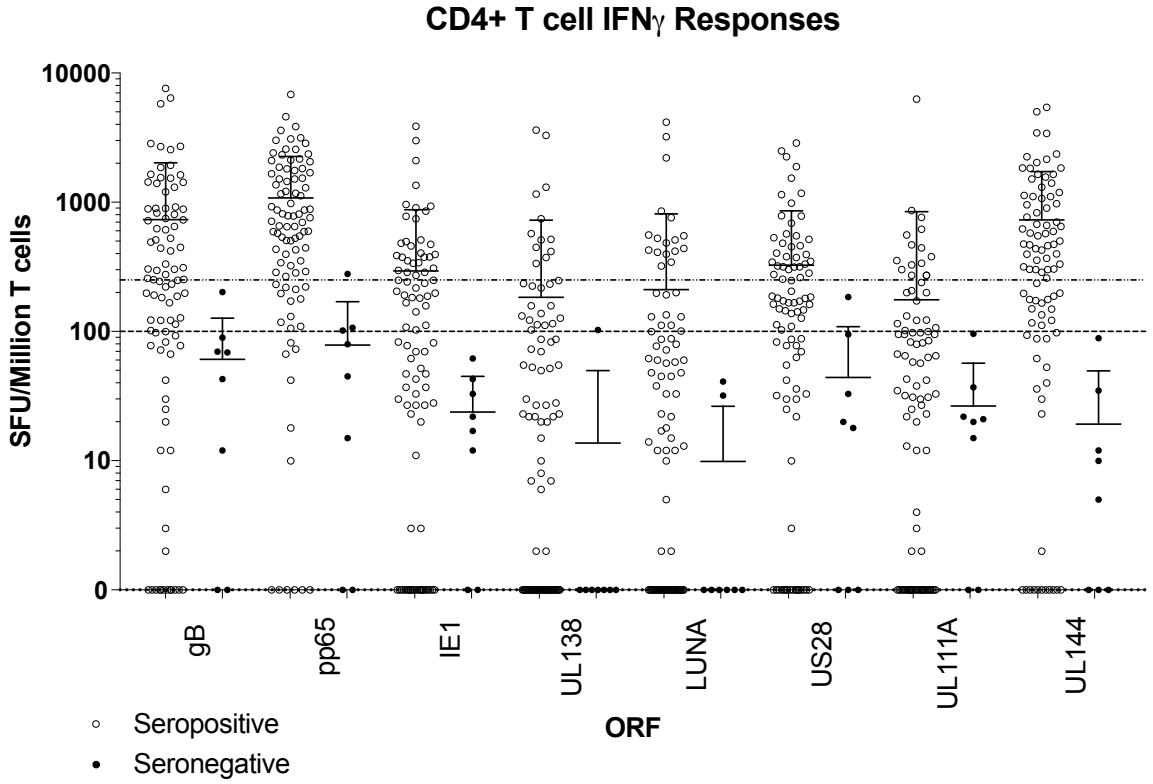



**Fig. 3.13 The frequency of CD4+ T cells from HCMV seropositive and seronegative donors that recognise HCMV ORF pools.** PBMC from 91 seropositive and eight seronegative donors were depleted of CD8+ T cells and stimulated with HCMV ORF peptide pools spanning gB, pp65, IE1, UL138, LUNA, US28, UL111A, and UL144 for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. Each peptide pool was tested in triplicate and the values from the number of background cells secreting cytokines in the unstimulated wells was deducted from all test wells. Following stimulation, the number of IFN$\gamma$ positive spots was enumerated by the automated spot counter and converted to a spot forming unit (SFU) value per million cells. The dotted line at 100 SFU/$10^6$ cells indicates the threshold for positive responses, while the line at 250 SFU/$10^6$ cells shows the threshold for high responding donors. Seropositive donors are shown with open circles (○) while seronegative donors are illustrated with filled black circles (●).

### 3.2.6 The frequency of latency-specific IFN$\gamma$ secreting CD4+ T cells does not change with age

The large donor cohort used here enabled data to be obtained from a range of ages, which could therefore give an indication of whether the strength of T cell responses to latency-associated ORFs vary with age - given an assumed longer term carriage of the virus. Ideally, a long-term study from the point of primary HCMV infection, with regular testing of T cell responses, would be carried out, but this was not feasible. Consequently, the age of donors was used as an indirect measure for time of HCMV carriage, where the use of a large donor cohort was necessary to overcome the inability to determine time of primary infection.

Although the T cell responses to each latency-associated protein tested varied in magnitude across all ages, Spearman rank correlation analysis yielded no significant correlation between the strength of CD4+ T cell responses (SFU/$10^6$ cells) and the age of donors for responses to any of the lytic or latency-associated proteins tested (Figure 3.14). CD4+ T cell IFN$\gamma$ responses were also not found to be affected by age when grouping the donor cohort according to sex, with the exception of responses to US28 in male donors which showed a weak negative correlation with donor age (rs = -0.3454, P = 0.0313) (Appendix A.1)

Although the magnitude of responses to latency-associated proteins was not found to vary with age, it could still be possible that age might affect the number of ORFs responded to, as longer term carriage of the virus and resulting viral reactivations could be responsible for the generation of responses to additional ORFs. However, when looking at the relationship between the ages of donors and the number of latency-associated ORFs each individual had a CD4+ T cell response to, there were no significant differences following a multiple comparisons test (Kruskal-Wallis) (Figure 3.15).

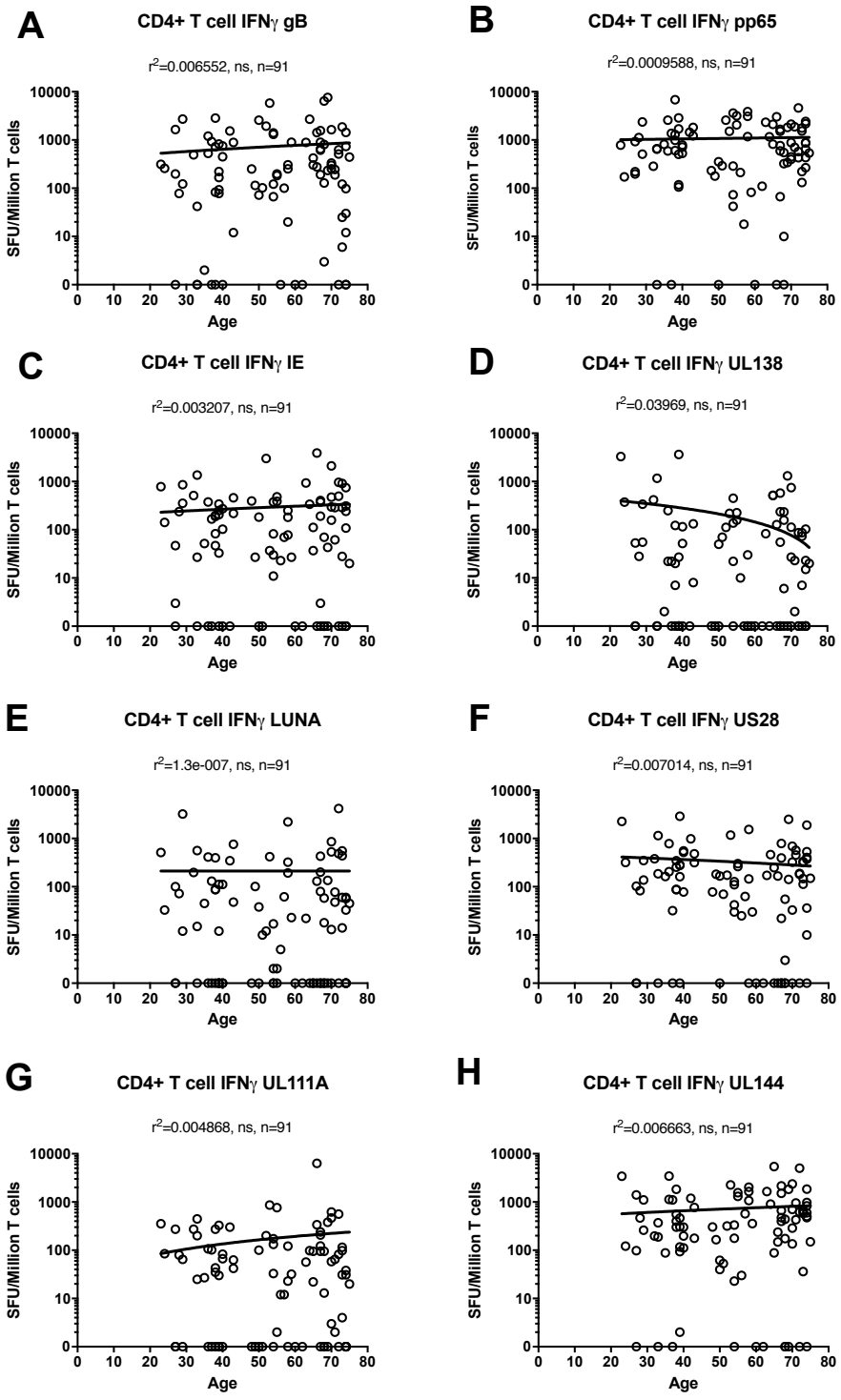



**Fig. 3.14 Analysis of the magnitudes of CD4+ T cell IFN$\gamma$ responses to 8 HCMV ORF peptide pools correlated with donor age.** CD8+ depleted PBMC from 91 seropositive donors were stimulated with HCMV ORF peptide pools for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates and the frequencies of IFN$\gamma$ positive spots within each seropositive donor enumerated and converted to Spot Forming Units (SFU)/$10^6$ cells. The SFU/$10^6$ cells of IFN$\gamma$ secreting CD4+ T cells is shown against donor age for responses to (A) gB, (B) pp65, (C) IE1, (D) UL138, (E) LUNA, (F) US28, (G) UL111A, and (H) UL144. The correlation (Spearman $r_s$ valus and p values shown on each graph), and line of best fit are shown.

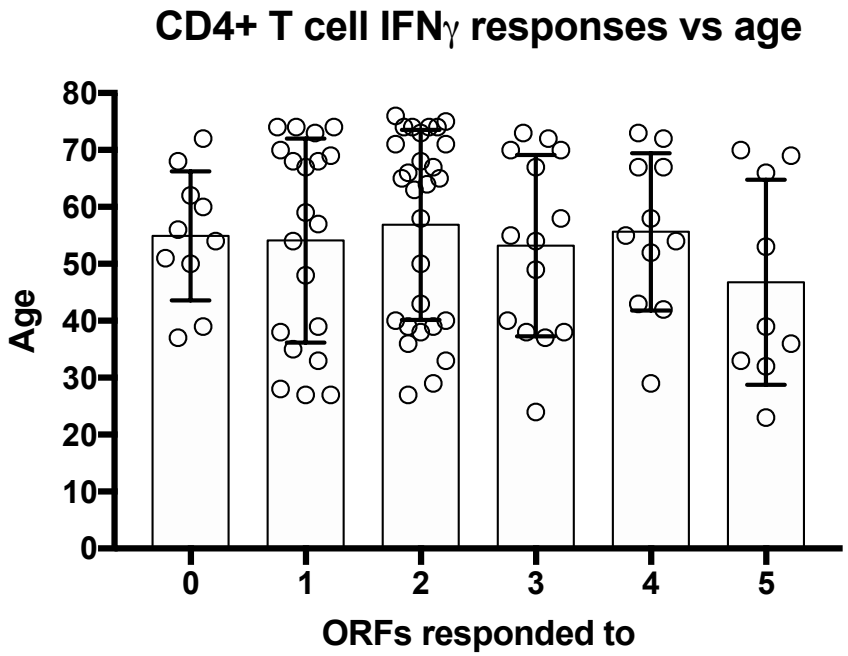



**Fig. 3.15 The number of latency-associated HCMV ORF peptide pools that CD4+ T cells respond to with IFN$\gamma$ secretion across donor age.** CD8+ depleted PBMC were stimulated with HCMV ORF peptide pools for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates and the frequencies of CD4+ T cells secreting IFN$\gamma$ enumerated and converted to Spot Forming Units (SFU)/$10^6$ cells. The number of latency-associated ORFs out of the five tested (UL138, LUNA, US28, UL111A, and UL144) that each donor responds to (considered a positive response if above the threshold 100 SFU/$10^6$ cells) is shown against the age of donor. There were no significant differences between the number of ORFs responded to by CD4+ T cells and the ages of donors according to Kruskal-Wallis multiple comparisons test.

### 3.2.7 CD4+ T cells can secrete the immunosuppressive cytokine IL-10 in response to HCMV latency-associated antigens

Having identified novel CD4+ T cell responses to the latency-associated HCMV proteins US28, UL111A, and UL144 by IFN$\gamma$ secretion, the ability of these T cells to secrete the immunosuppressive cytokine IL-10 was assessed. Although CD4+ T cell responses to lytically expressed HCMV proteins are characteristically Th1-like with high frequencies secreting IFN$\gamma$, CD4+ T cells specific for latency-associated proteins have previously been shown to secrete the immunosuppressive cytokine IL-10 [501]. Through the use of the FluoroSpot assay, I was able to simultaneously examine the secretion of both IFN$\gamma$ and IL-10 in response to the HCMV ORF peptide pools. In this manner, the frequency of T cells capable of secreting IL-10, in addition to those able to secrete both IL-10 together with IFN$\gamma$ was determined.

As previously, when utilising the FluoroSpot assay, depleting PBMC of CD8+ cells enabled the examination of CD4+ T cell responses to HCMV ORF peptide pools. By doing so, CD4+ T cells capable of secreting IL-10 in response to HCMV proteins were found to be present in a proportion of seropositive donors, with positive responses counted as those above 50 SFU/$10^6$ (Figure 3.16). In comparison to the number of donors with a positive CD4+ T cell IFN$\gamma$ response, there were considerably fewer donors with such CD4+ T cell IL-10 responses, which can be partially attributed to the stringent selection criteria used that excluded 33 donors. Although fewer donors had a CD4+ T cell IL-10 response, it is clear that there were differences in the frequencies of CD4+ T cells secreting IL-10 in response to different HCMV ORFs (Figure 3.16). Typically, from the 68 donors tested, there were fewer CD4+ T cells secreting IL-10 in response to lytically-expressed proteins, especially gB (10 donors, 14.7%) and IE1 (13 donors, 19.1%), although these were present, and in response to pp65 relatively frequent (19 donors, 27.9%) (Figure 3.16). CD4+ T cell IL-10 responses were most frequently seen in response to US28 (30 donors, 44.1%), LUNA (29 donors, 42.6%), and UL138 (29 donors, 42.6%), followed by UL144 (17 donors, 25%) and UL111A (13 donors, 19.1%), which were the least frequent (Figure 3.16).

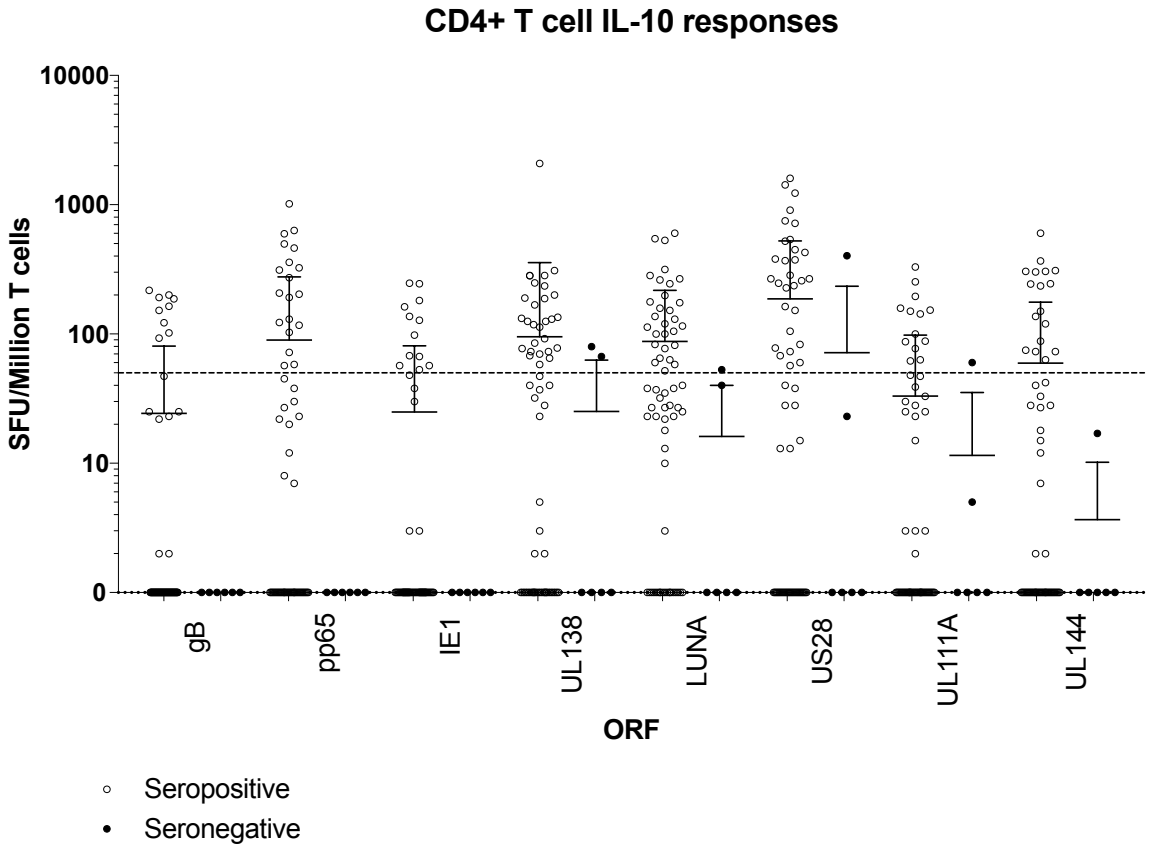



**Fig. 3.16 The frequency of CD4+ T cells from HCMV seropositive and seronegative donors secreting IL-10 in response to HCMV ORF pools.** PBMC from 68 seropositive and 6 seronegative donors were depleted of CD8+ T cells and stimulated with HCMV ORF peptide pools spanning gB, pp65, IE1, UL138, LUNA, US28, UL111A, and UL144 for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. Each peptide pool was tested in triplicate and the values from the number of background cells secreting cytokines in the unstimulated wells was deducted from all test wells. Following stimulation, the number of IL-10 positive spots was enumerated by the automated spot counter and converted to a spot forming unit (SFU) value per million cells. The dotted line at 50 SFU/$10^6$ cells indicates the threshold for positive responses. Seropositive donors are shown with open circles (○) while seronegative donors are illustrated with filled black circles (●).

### 3.2.8 The frequency of latency-specific IL-10 secreting CD4+ T cells responding to IE1 and UL111A changes with donor age

Although the frequency of IFN$\gamma$ secreting T cells within donors did not change with age in response to any of the HCMV ORF pools tested (Figure 3.14), this might not be the case for CD4+ T cells secreting IL-10. To investigate this, I examined how the frequencies of IL-10 secreting T cells that had been stimulated with HCMV ORF overlapping peptide pools changed with the age of seropositive donors (an indirect measure of time of viral carriage). In response to the HCMV ORF peptide pools tested, I found CD4+ T cells secreting IL-10 across all ages, with varying magnitudes. Spearman rank correlation analysis was used to test the correlation between the age and magnitude of CD4+ T cell IL-10 responses. There were marginally significant decreases in the number of IL-10 secreting CD4+ T cells with increasing donor age in response to IE1 (Spearman $r_s$ = -0.2427, $P$ = 0.0461) (Figure 3.17C), and to UL111A (Spearman $r_s$ = -0.2602, $P$ = 0.0321) (Figure 3.17F), which were found to be significant negative correlations in male rather than female donors when splitting the cohort by sex (Appendix A.2). There were no significant changes in the frequencies of CD4+ T cells secreting IL-10 in response to any of the ORFs tested with increasing age (Figure 3.17), although a significant negative correlation was found in UL138 responses in male donors only (Appendix A.2).

Long-term viral carriage could result in IL-10 secreting CD4+ T cells recognising an increasing number of latency-associated ORFs, however no such increases were observed across the ages tested (Kruskal- Wallis multiple comparisons test) (Figure 3.18). Although the age of the donors was not related to the number of ORFs T cells secreted IL-10 in response to, it cannot be ruled out that these results could change with an increase in the number of donors that passed IL-10 selection criteria.

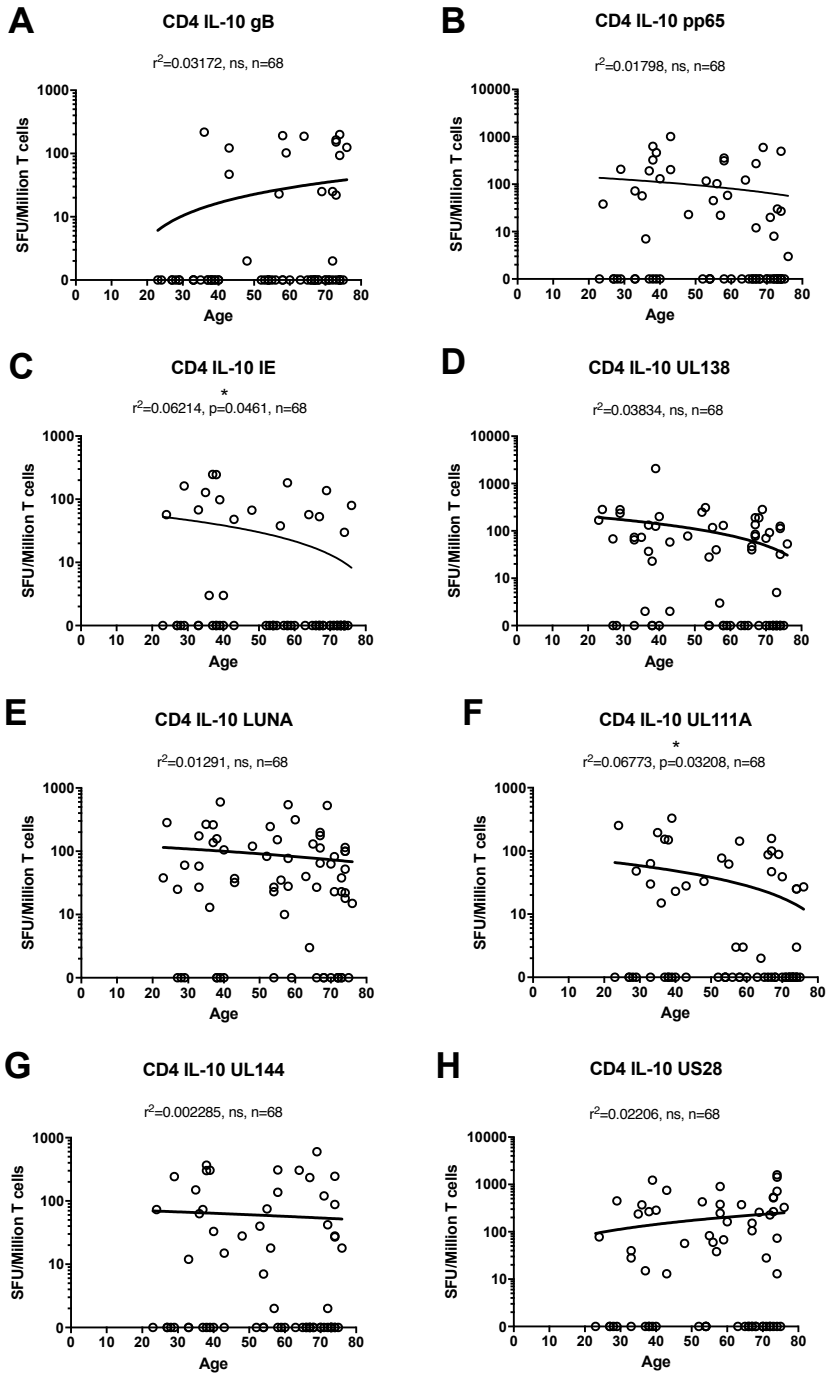



**Fig. 3.17 Analysis of the magnitudes of CD4+ T cell IL-10 responses to 8 HCMV ORF peptide pools correlated with donor age.** CD8+ depleted PBMC from 68 seropositive donors were stimulated with HCMV ORF peptide pools for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates and the frequencies of CD4+ T cells secreting IL-10 within each seropositive donor enumerated and converted to Spot Forming Units (SFU)/$10^6$ cells. The SFU/$10^6$ cells of IL-10 secreting CD4+ T cells is shown against donor age for responses to (A) gB, (B) pp65, (C) IE1, (D) UL138, (E) LUNA, (F) US28, (G) UL111A, and (H) UL144. The correlation (Spearman $r_s$ valus and p values are shown on each graph), and line of best fit are shown.

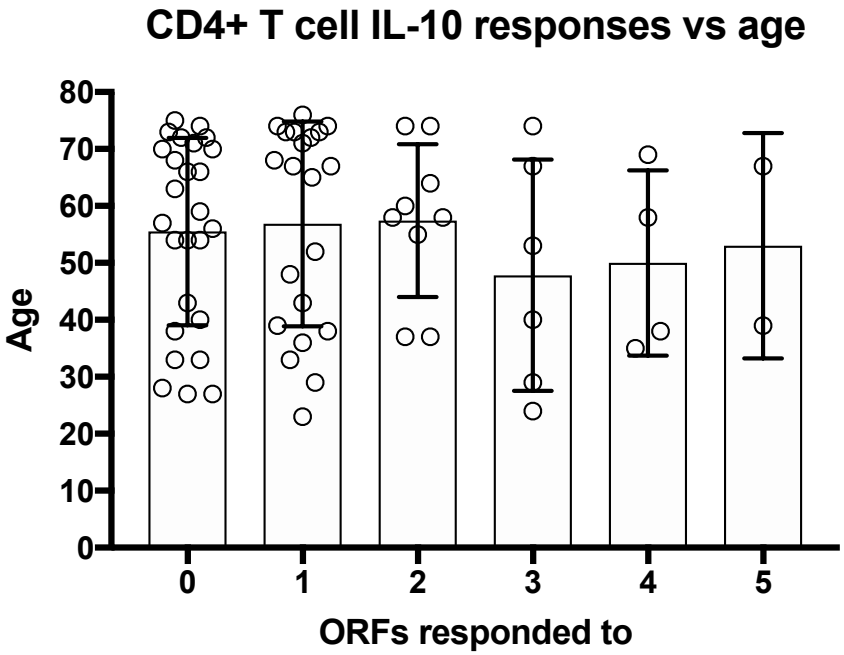



**Fig. 3.18 The number of latency-associated HCMV ORF peptide pools that CD4+ T cells respond to with IL-10 secretion across donor age.** CD8+ depleted PBMC were stimulated with HCMV ORF peptide pools for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates and the frequencies of CD4+ T cells secreting IL-10 enumerated and converted to Spot Forming Units (SFU)/$10^6$ cells. The number of latency-associated antigens out of the five tested (UL138, LUNA, US28, UL111A, and UL144) that each donor responds to (considered a positive response if above the threshold 50 SFU/$10^6$ cells) is shown against the age of donor. There were no significant differences between the number of ORFs responded to by CD4+ T cells and the ages of donors according to Kruskal-Wallis multiple comparisons test.

### 3.2.9 CD4+ T cell response magnitudes to individual HCMV ORFs vary depending on sex

In addition to age, the immune response to virus infection has been shown to be influenced by the sex of the individual [619, 620]. The frequency of IFN$\gamma$ and IL-10 secreting CD4+ T cells in response to HCMV ORFs was compared between male and female donors. There were significantly greater numbers of CD4+ T cells in male donors than female donors that secreted IFN$\gamma$ in response to UL144 ($P = 0.0183$, Kruskal-Wallis) (Figure 3.19A), in addition to significantly greater frequencies of CD4+ T cells that secreted IL-10 in response to pp65 ($P = 0.0171$, Kruskal-Wallis) and US28 ($P = 0.0330$, Kruskal-Wallis) in male compared to female donors (Figure 3.19B). There was also an appreciably greater frequency of CD4+ T cells secreting IFN$\gamma$ in male donors in response to LUNA, although this did not reach significance (Figure 3.19A).

Subsequently, the cumulative CD4+ T cell response to all HCMV ORFs within each donor was compared between male and female donors. When taking into account all eight HCMV ORFs tested (gB, pp65, IE1, UL138, LUNA, US28, UL111A, UL144), there were significantly more cells secreting IFN$\gamma$ ($P = 0.0134$, Mann-Whitney U) and IL-10 ($P = 0.007$, Mann-Whitney U) in male compared to female donors (Figure 3.20A and B). The differences in total response magnitudes was also apparent when considering only the three lytic ORFs (gB, pp65, and IE1), where male donors had significantly greater number of IFN$\gamma$ ($P = 0.037$, Mann-Whitney U) and IL-10 ($P = 0.0006$, Mann-Whitney U) secreting cells compared to female donors (Figure 3.20C and D). Additionally, total responses to latency-associated proteins (UL138, LUNA, US28, UL111A, and UL144) were also significantly higher in male compared to female donors for both IFN$\gamma$ ($P = 0.0111$, Mann-Whitney U) and IL-10 ($P = 0.0.0272$, Mann-Whitney U) secreting CD4+ T cells (Figure 3.20E and F). Thus, when taking into account responses to all ORFs, male donors have higher frequencies of cytokine secreting cells than female donors.

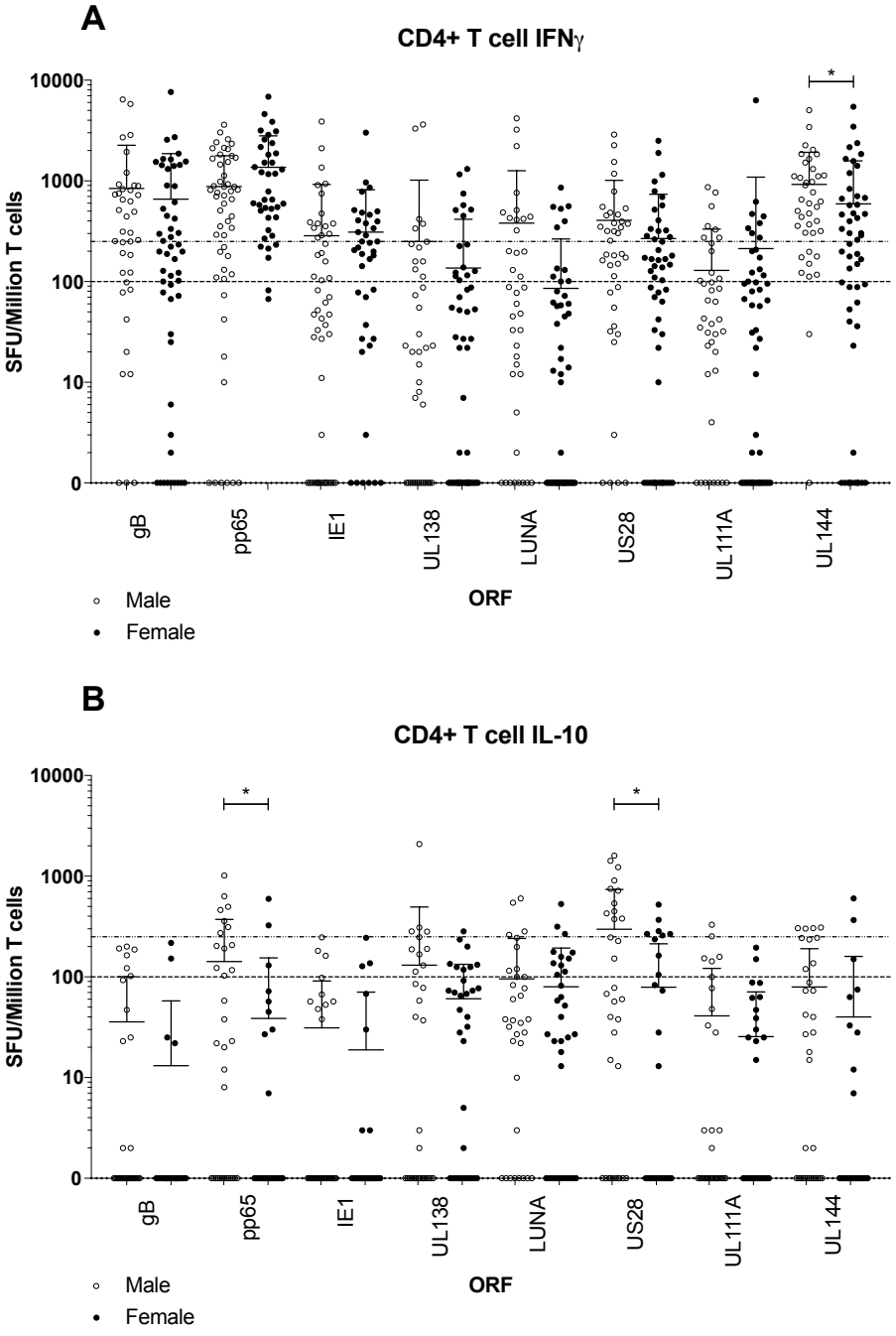



**Fig. 3.19 The frequency of CD4+ T cells secreting IFN$\gamma$ or IL-10 in response to HCMV ORF pools from HCMV seropositive donors grouped by sex.** PBMC depleted of CD8+ T cells were stimulated with overlapping HCMV ORF peptide pools spanning each antigen of interest for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. Each peptide pool was tested in triplicate and the values from all test wells were deducted from the number of background cells secreting cytokines in the unstimulated wells. Following stimulation, the number of cells secreting (A) IFN$\gamma$ and (B) IL-10 was enumerated by the automated spot counter and converted to a spot forming unit (SFU) value per million cells. Male responses are illustrated with open circles (○), and female responses with filled circles (●). Kruskal-Wallis multiple comparisons test was carried out between male and female responses to each ORF.

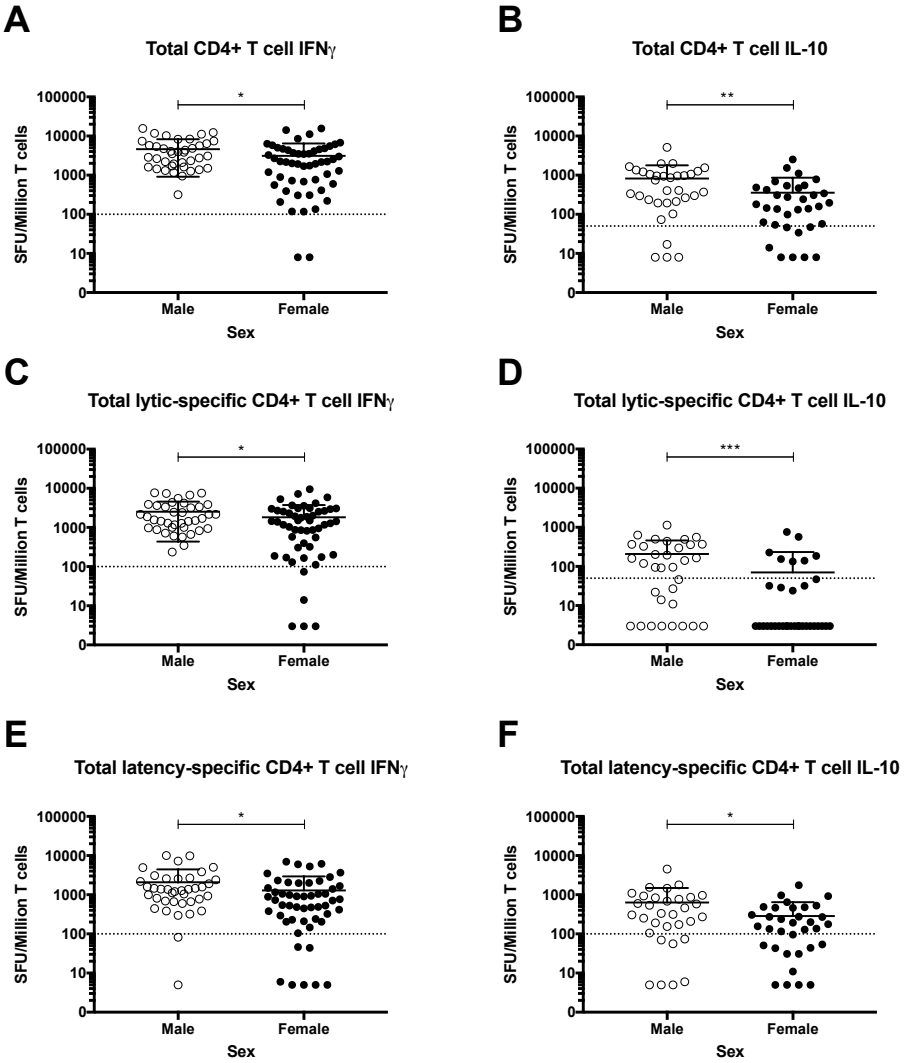



**Fig. 3.20 A comparison of the total frequencies of CD4+ T cells within individual donors that respond to HCMV ORFs when donors are grouped by sex.** The total frequency of CD4+ T cells responding with IFN$\gamma$ or IL-10 secretion within individual donors is shown in male and female donors. PBMC depleted of CD8+ T cells were stimulated with overlapping HCMV ORF peptide pools spanning each HCMV ORF (gB, pp65, IE, UL138, LUNA, US28, UL111A, UL144) for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. Each peptide pool was tested in triplicate and the values from all test wells were deducted from the number of background cells secreting cytokines in the unstimulated wells. Following stimulation, the number of cells secreting IFN$\gamma$ and IL-10 was enumerated by the automated spot counter and converted to a spot forming unit (SFU) value per million cells. Within in each donor (A) the total number of CD4+ T cells secreting IFN$\gamma$ or (B) IL-10 to all ORFs is shown. Additionally, (C) the total number of CD4+ T cells secreting IFN$\gamma$ or (D) IL-10 in response to lytic ORFs only (gB, pp65, IE) and the total secreting (E) IFN$\gamma$ and (F) IL-10 in response to the latency-associated ORFs (UL138, LUNA, US28, UL111A, UL144) is shown. Male responses are illustrated with open circles (○), and female responses with filled circles (●). Mann-Whitney U tests were utilised to compare between male and female responses to ORFs.

### 3.2.10 The secretion of IFN$\gamma$ and IL-10 originates from distinct CD4+ T cell populations

Having shown that CD4+ T cells from seropositive donors responding to latency-associated proteins are composed of IFN$\gamma$ and IL-10 secreting cells, it was important to determine to what extent responses within individuals were dominated by each cytokine secreting population, and if overall responses to certain ORFs are composed of greater IFN$\gamma$ or IL-10 secreting cells. Furthermore, the added benefit of the FluoroSpot assay, through its ability to count cells that secrete both IFN$\gamma$ and IL-10, enabled me to subsequently determine the extent to which cytokine secretion from HCMV-specific T cells was originating from the same population, or if these secreted cytokines separated cells into distinct populations.

To do this, the frequencies of each cytokine secreting population (CD4+ T cells secreting IFN$\gamma$, IL-10, or both cytokines) was calculated as a proportion of the total number of responding T cells within each donor. CD4+ T cell responses to the lytic proteins gB, pp65, and IE1 were all composed of the highest percentages of IFN$\gamma$-secreting T cells, where the majority of donors had CD4+ T cell responses dominated by IFN$\gamma$ secretion (Figure 3.21A, B, and C). I also observed several donors with CD4+ T cell IL-10 responses to these lytic ORFs that were the dominant response, although these were much more infrequent than dominant IFN$\gamma$ responses. With the exception of the responses to UL144, CD4+ T cell responses to the latency-associated proteins were composed of much higher proportions of IL-10 secreting cells compared to the responses to the lytically-expressed proteins gB, pp65, and IE1. Responses to UL144 had the lowest proportions of IL-10 secreting CD4+ T cells of the responses to the latency-associated proteins, where the vast majority of individuals had a dominant IFN$\gamma$ response, resembling responses to gB, pp65, and IE1 (Figure 3.21D). The CD4+ T cell responses to US28, UL111A, UL138, and LUNA were all composed of much higher proportions of IL-10 secreting cells (Figure 3.21E, F, G, and H). To illustrate these differences in the dominant cytokine responses, the proportion of donors with a given dominant response to each ORF (where over 55% of all cytokine secreting CD4+ T cells secrete IFN$\gamma$ or IL-10) is shown (Figure 3.22). There is a clear hierarchy in dominant cytokine responses to certain ORFs. As expected, over 85% of responding donors had a dominant IFN$\gamma$ response to the lytically expressed proteins gB (90.3%), pp65 (96.1%), or IE1 (86.2%) (Figure 3.22). Responses to latency-associated proteins were more mixed and highlight differences in dominant responses to different ORFs. The responses to UL144 for example were IFN$\gamma$ biased in 95.7% of all donors, which decreases steadily in response to UL111A (74.3%), US28 (53.8%), LUNA (43.5%), and UL138 (40.5%) (Figure 3.22).

Examining the CD4+ T cells that secreted both IL-10 and IFN$\gamma$ in response to HCMV ORFs revealed that these cells make up very small proportions of the total CD4+ T cell response, indicating that cytokine secretion is originating from two separate populations (Figure 3.21). This was the case for CD4+ T cell responses to all the HCMV proteins tested, both lytically expressed and latency-associated, where very few donors possessed CD4+ T cells secreting both cytokines, and in the donors that did have dual responders, these made up very small proportions of the overall CD4+ T cell response (Figure 3.21).

The proportions of CD4+ T cells secreting IFN$\gamma$ or IL-10 therefore varies in response to different ORFs, but it could also be possible that these proportions were affected by the age of the donor. Although no changes were observed in the frequencies of CD4+ T cells secreting IFN$\gamma$ in response to HCMV ORFs across the ages of donors (Figure 3.14), CD4+ T cell IL-10 responses were less frequent in older ages in response to UL111A and IE1, which might also be indicative of a change in the overall balance of cytokine secreting cells (Figure 3.17C and F). Donors were grouped into three age categories, young (18-39), middle (40-64), and old (64+), and each individual's corresponding dominant cytokine response analysed across these age groups. Dominant responses were defined where over 50% of responding CD4+ T cells secreted IFN$\gamma$ or IL-10. As expected, in response to UL111A, the proportion of CD4+ T cells secreting IFN$\gamma$ compared to IL-10 increased across each age group from 62% in the young, to 80% in the middle, and 91% in the old group (Figure 3.23D). While this was by far the greatest change in proportion of cytokine secreting cells with age, CD4+ T cell responses to LUNA decreased from 53% IFN$\gamma$ biased, to 40% from young to old age (Figure 3.23B). The responses to US28 changed in the opposite direction, where the proportion of donors with an IFN$\gamma$ dominated response increased from 53 to 63% (Figure 3.23B). Rather surprisingly, responses to UL138 and US28 had a characteristic shift in the middle age group that did not follow the overall trend from young to old. CD4+ T cell responses to UL138 for example increased from 36% in the young to 56% in the middle, before reducing to 31% IFN$\gamma$ dominated in the old age group (Figure 3.23A). Similarly responses to US28 decreased from 53% to 31% at middle age, before increasing to 63% IFN$\gamma$ dominated in the old age group (Figure 3.23C).

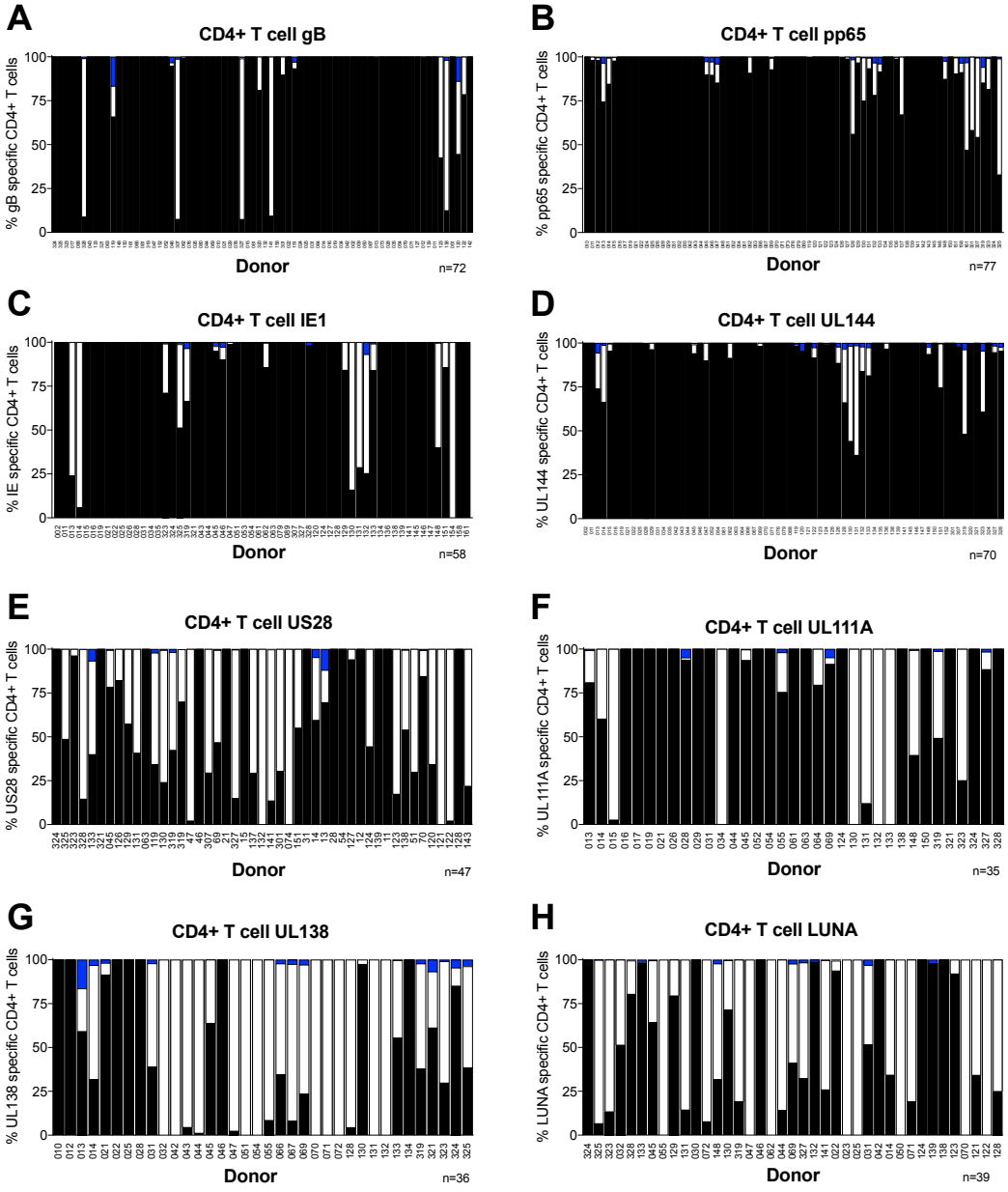



**Fig. 3.21 The proportions of CD4+ T cells secreting IL-10 and IFN$\gamma$ in response to latency-associated HCMV ORF pools from individual seropositive donors.** PBMC from 91 seropositive donors were depleted of CD8+ cells and stimulated with overlapping peptide pools from HCMV ORFs (A) gB, (B) pp65, (C) IE, (D) UL144, (E) US28, (F) UL111A, (G) UL138, and (H) LUNA, for 48 hours. Following stimulation, IFN$\gamma$ (black bars) and IL-10 (white bars) secretion was detected by FluoroSpot and enumerated to spot forming units (SFU), where each cytokine response was calculated as a proportion of the total CD4+ T cell response. The proportion of CD4+ T cells of total cytokine secreting cells that secreted both IFN$\gamma$ and IL-10 is also shown (blue bars). All samples were run in triplicate with the mean value shown here. Donors without an IL-10 or IFN$\gamma$ responses were omitted from each ORF analysis.

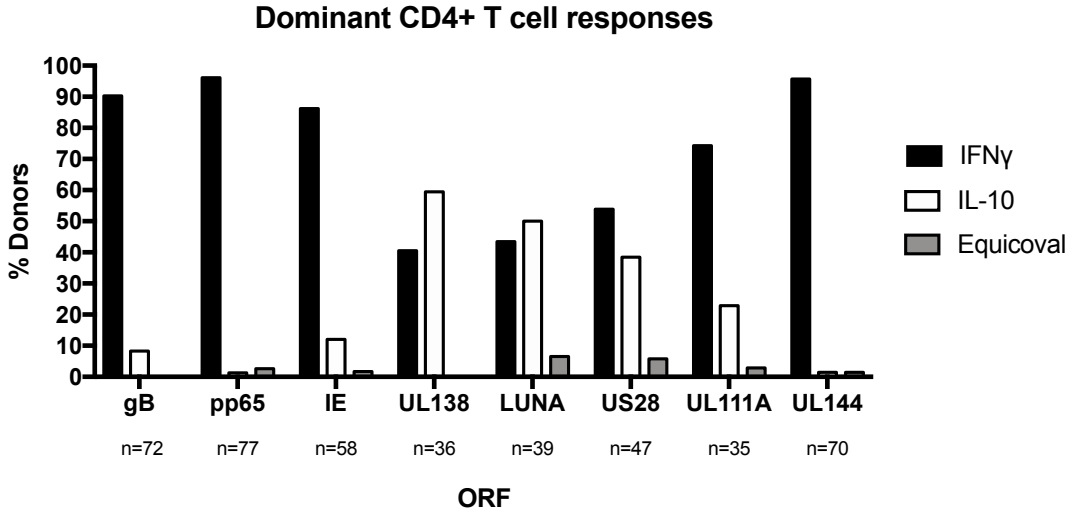



**Fig. 3.22 The distribution of dominant CD4+ T cell IFN$\gamma$ and IL-10 responses to HCMV ORF peptide pools.** PBMC from seropositive donors were depleted of CD8+ cells and stimulated with overlapping peptide pools from HCMV ORFs gB, pp65, IE, UL138, LUNA, US28, UL111A, and UL144 for 48 hours. Following stimulation, IFN$\gamma$ and IL-10 secretion was detected by dual IFN$\gamma$/IL-10 FluoroSpot and enumerated to spot forming units (SFU), which allowed the proportion of each cytokine response to be calculated of the total CD4+ T cell response. Dominant responses are classified when over 55% of the cytokine secreting cells secreted (A) IFN$\gamma$ (black bars), or (B) IL-10 (white bars). CD4+ T cell responses where none of the cytokine secreting population was greater than 55% was classed as equivocal (grey bars). Donors without an IL-10 or IFN$\gamma$ responses were omitted from each ORF analysis and the number of donors analysed for each antigen-specific response is shown.

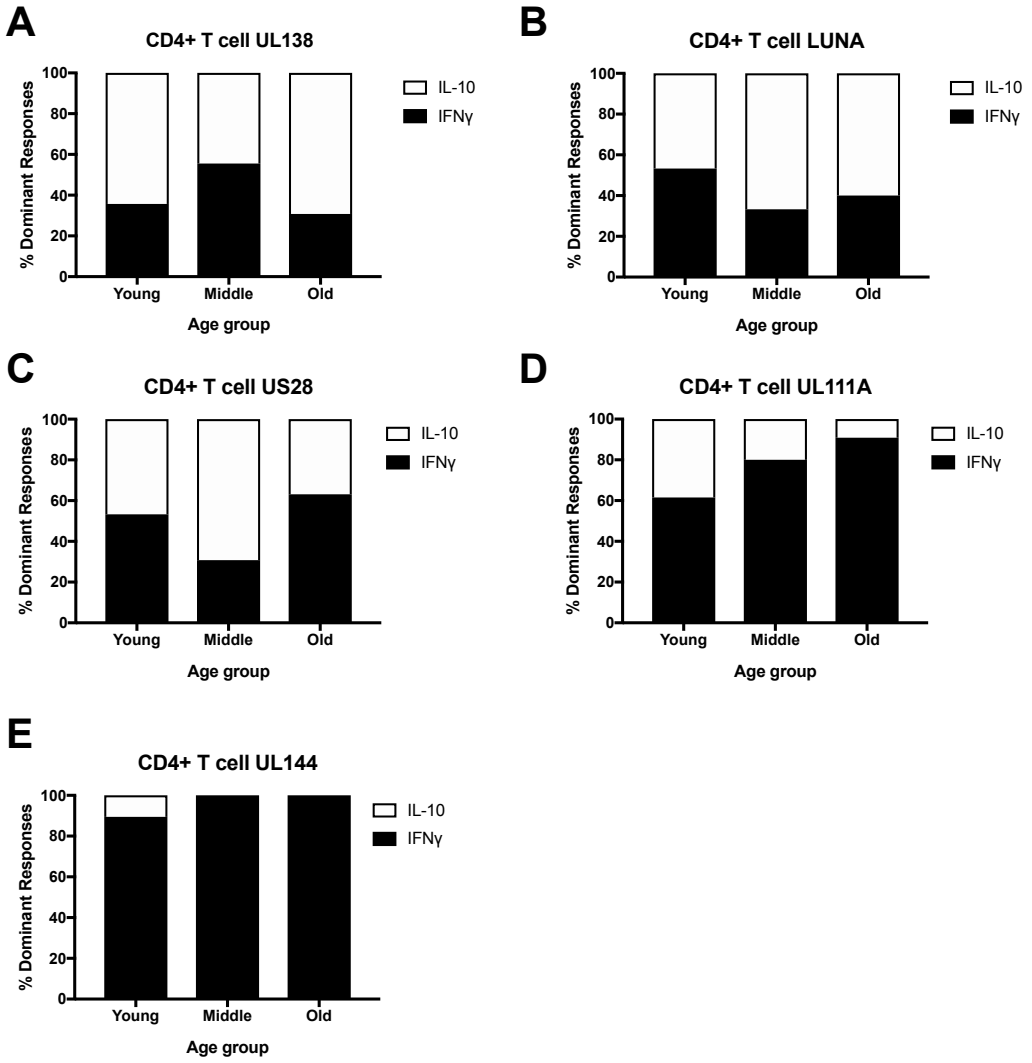



**Fig. 3.23 The distribution of dominant CD4+ T cell IFN$\gamma$ and IL-10 responses to HCMC ORF peptide pools across age groups.** PBMC from seropositive donors were depleted of CD8+ cells and stimulated with overlapping peptide pools from HCMV ORFs for 48 hours. Following stimulation, IFN$\gamma$ and IL-10 secretion was detected by dual IFN$\gamma$/IL-10 FluoroSpot and enumerated to spot forming units (SFU), which allowed the proportion of each cytokine response to be calculated of the total CD4+ T cell response. Dominant responses are classed as such when over 50% of the cytokine secreting cells secreted IFN$\gamma$ (black bars), or IL-10 (white bars). The percentage of donors with dominant IFN$\gamma$ and IL-10 responses to (A) UL138, (B) LUNA, (C) US28, (D) UL111A, and (E) UL144 is shown for three age groups. Donors were separated into young (18-39), middle (40-64), and old (64+) age groups. Donors without an IL-10 or IFN$\gamma$ responses were omitted from each ORF analysis and the number of donors analysed is shown.

### 3.2.11 CD4+ T cells can also secrete TNF$\alpha$ in response to HCMV latency-associated ORFs

FluoroSpot plates capable of detecting three cytokines simultaneously were then utilised to examine the secretion of an additional antiviral cytokine, TNF$\alpha$, together with IFN$\gamma$ and IL-10 secretion in response to HCMV ORFs. Eight seropositive donors were tested for their CD4+ T cell TNF$\alpha$ responses. As previously, the frequency of responding CD4+ T cells was measured by stimulating PBMC depleted of CD8+ cells with HCMV ORF peptides for 48 hours. Again, to eliminate donors with high background TNF$\alpha$ secretion in their unstimulated wells, data from donors was only used if the positive control elicited twice the number of secreting cells as in unstimulated wells. Unfortunately due to high levels of background TNF$\alpha$ secretion, five donors were excluded from the CD4+ T cell analysis.

Despite the exclusion of a large proportion of the donors tested, TNF$\alpha$ secretion by CD4+ T cells was observed in response to many of the HCMV ORFs tested. Although only three donors were included for their CD4+ T cell responses, I did observe responses to gB (3 donors), pp65 (2 donors), LUNA (2 donors), UL138 (1 donor), and UL144 (1 donor), but not to IE1, US28, and UL111A (Figure 3.24). Given the low number of donors, it is not possible to determine if there are differences between responses to the lytically-expressed proteins compared to the latency-associated proteins, however, it can be seen that all three donors that passed the CD4+ T cell selection criteria had very high frequencies of CD4+ T cell that secrete TNF$\alpha$ in response to gB, much higher than responses to the other ORFs (Figure 3.24).

ICS for TNF$\alpha$ was also carried out on five donors to ensure the FluoroSpot results were reproducible in a different assay system. PBMC stimulated overnight with HCMV ORF peptides were stained intracellularly for TNF$\alpha$ in addition to IFN$\gamma$ and IL-10. CD4+ T cell TNF$\alpha$ responses were found to all ORFs tested, including IE1, US28, and UL111A, which were not detected by FluoroSpot (Figure 3.24), and these responses were found in nearly every donor (Figure 3.25). Overall, there was no major difference in the number of donors with a positive TNF$\alpha$ response to lytically-expressed proteins compared to latency-associated proteins (Figure 3.25). However IE1 and US28 were responded to with TNF$\alpha$ production in all donors tested, and had the greatest percentages of CD4+ T cells positive for TNF$\alpha$ as well (Figure 3.25).

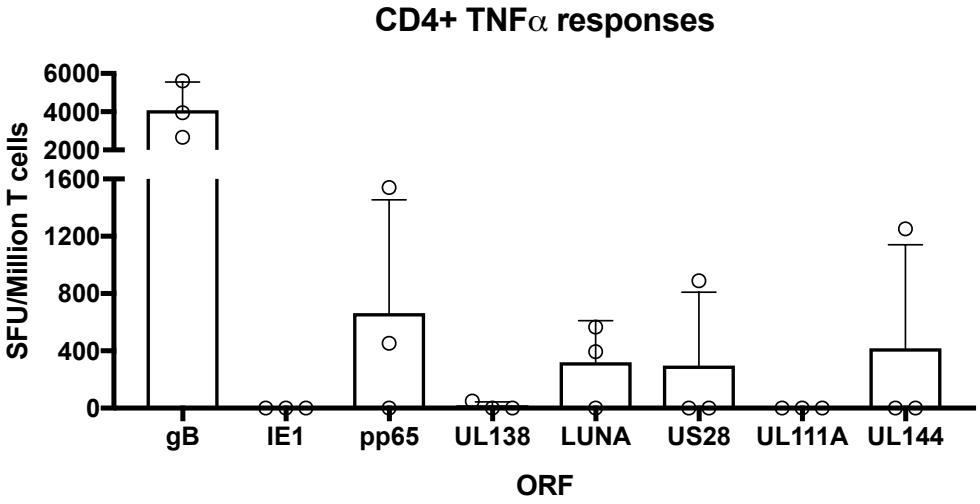



**Fig. 3.24 The secretion of TNF$\alpha$ by CD4+ T cells from seropositive donors in response to HCMV ORF peptide pools.** PBMC from three seropositive donors were depleted of CD8+ cells and stimulated with overlapping HCMV ORF peptide pools spanning gB, pp65, IE1, UL138, LUNA, US28, UL111A, and UL144, for 48 hours on triple IFN$\gamma$/IL-10/TNF$\alpha$ FluoroSpot plates. Each peptide pool was tested in triplicate and the values from all test wells were deducted from the number of background cells secreting cytokines in the unstimulated wells. Following stimulation, the number of cells secreting TNF$\alpha$ was enumerated by the automated spot counter and converted to a spot forming unit (SFU) value per million cells.

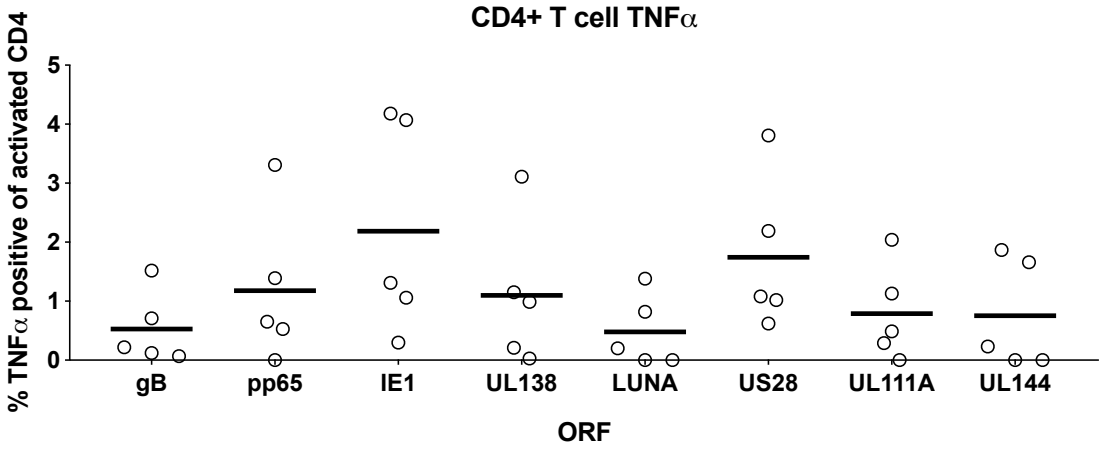



**Fig. 3.25 Detection of TNF$\alpha$ production by CD4+ T cells in response to HCMV ORF peptide pools by intracellular cytokine staining.** PBMC depleted of CD8+ cells were stimulated with overlapping HCMV peptide pools overnight spanning the ORFs of gB, pp65, IE1, UL138, LUNA, US28, UL111A, and UL144. The following day, the PBMC were incubated in monensin before staining intracellularly for TNF$\alpha$. CD4+ T cells were identified by gating on CD3+ and CD4+ cells, and ORF-specific T cells by expression of 4-1BB and/or CD40L. The data shown is from five seropositive donors where TNF$\alpha$ positive cells are shown as a proportion of CD3+CD4+ cells.

### TNF$\alpha$ secreting CD4+ T cells are generally a separate population to those secreting IFN$\gamma$ or IL-10

Having established the ability of CD4+ T cells from seropositive donors to secrete TNF$\alpha$ in response to HCMV ORFs, the secretion of IFN$\gamma$ and IL-10 in combination with TNF$\alpha$ secretion was then examined. The proportions of responding CD4+ T cells within donors that secreted each cytokine alone, or secreted combinations of IFN$\gamma$ or IL-10 together with TNF$\alpha$ were determined from 3 HCMV seropositive donors. Donors with cytokine responses above the background secretion from unstimulated samples were included in the analysis resulting in the omission of several donor responses. All three TNF$\alpha$/IFN$\gamma$ responses to UL138, and one such donor response to UL111A were not included. TNF$\alpha$/IL-10 responses from one donor were excluded to pp65, LUNA, and US28, while two of these responses were removed from responses to UL138, UL111A, and UL144. CD4+ T cells that were dual TNF$\alpha$/IFN$\gamma$ producers were detected mainly in one donor (Donor 181) but were never greater than 25% of the total response (Figure 3.26). The only other donor with detected dual secretors were from donor 310 in response to pp65, which made up nearly 12% of the total responding CD4+ T cell population (Figure 3.26B). The dual secreting CD4+ T cells from donor 181 were found to be greater than 10% of the total response to gB (Figure 3.26A), pp65 (Figure 3.26B), LUNA (Figure 3.26D), and UL144 (Figure 3.26G). The dual TNF$\alpha$/IFN$\gamma$ CD4+ T cells therefore do not appear to be a major population that is generated in response to these HCMV ORFs, and are not generated preferentially in response to particular ORFs, although these results could change with the further addition of donors.

CD4+ T cells that secreted both TNF$\alpha$ and IL-10 were even more infrequent, with almost no T cells found to be such dual producers, with the exception of CD4+ T cell responses to gB present in two of the three donors (Figure 3.27A).

Data from ICS of stimulated PBMC also corroborates these findings, where dual TNF$\alpha$ secreting cells were most commonly found from CD4+ T cells co-producing IFN$\gamma$ (Figure 3.28A). Here, individuals with dual TNF$\alpha$/IFN$\gamma$ producing CD4+ T cells were also found in response to IE1, US28 and UL111A (Figure 3.28A) which were not seen in the FluoroSpot assay (Figure 3.26). However, just as the results from the FluoroSpot, there were comparatively few donors that possessed dual secreting TNF$\alpha$/IFN$\gamma$ CD4+ T cells (Figure 3.28A) compared to the number of donors with a TNF$\alpha$ response alone (Figure 3.25), illustrating that these are mainly two separate populations. There were also very few donors with CD4+ T cells that produced both TNF$\alpha$ and IL-10 (Figure 3.28B). Furthermore, there

were no cells found to secrete all three cytokines in response to HCMV ORFs (data not shown).

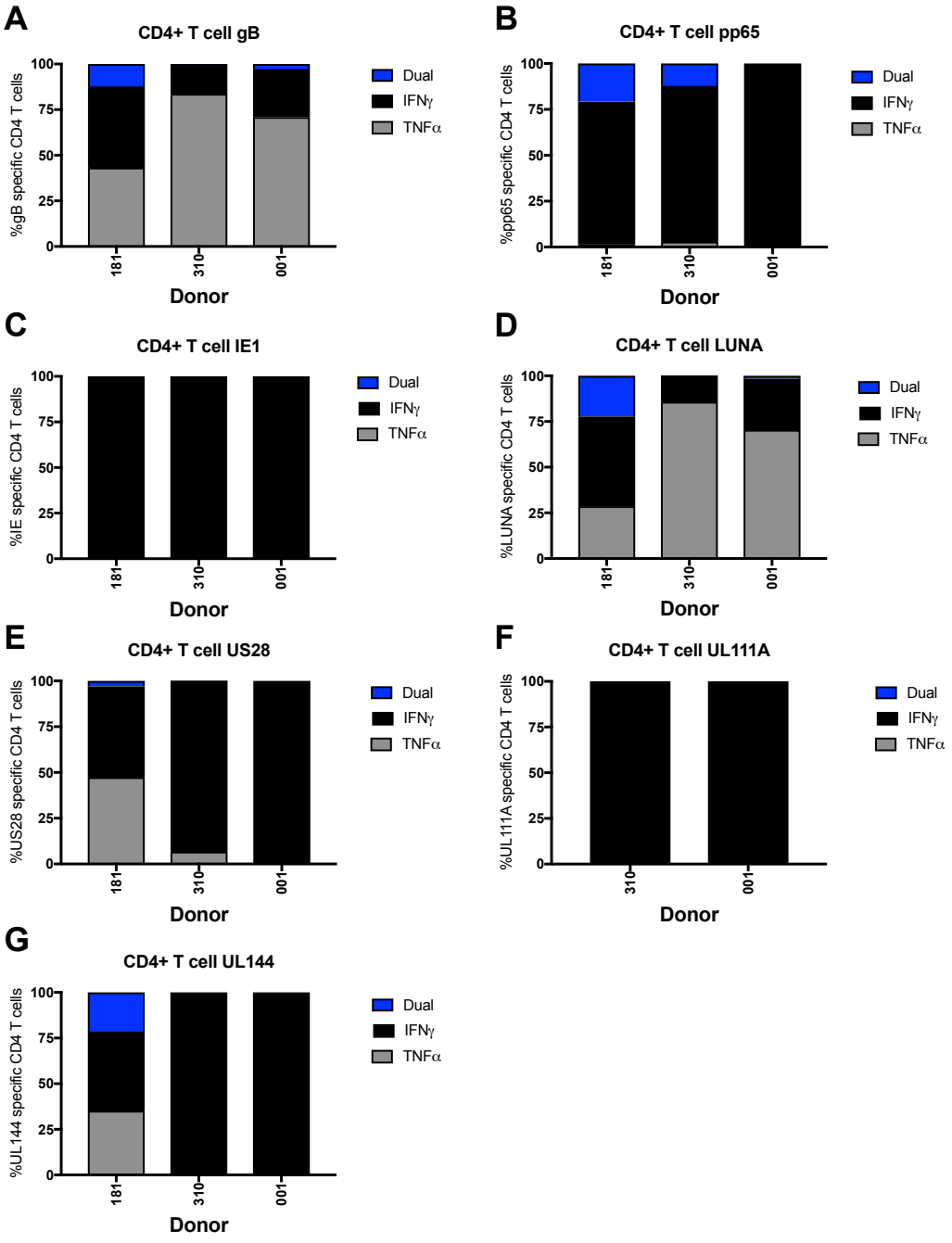



**Fig. 3.26 Proportions of CD4+ T cells secreting TNF$\alpha$ and IFN$\gamma$ in response to latency-associated HCMV ORF peptide pools from seropositive donors.** PBMC from 3 seropositive donors were depleted of CD8+ cells and stimulated with overlapping peptide pools from HCMV ORFs (A) gB, (B) pp65, (C) IE, (D) LUNA, (E) US28, (F) UL111A, and (G) UL144 for 48 hours. Following stimulation, IFN$\gamma$ (black bars) and TNF$\alpha$ (grey bars) secretion was detected by FluoroSpot and enumerated to spot forming units (SFU), where each cytokine response was calculated as a proportion of the total CD4+ T cell response. The proportion of CD4+ T cells of total cytokine secreting cells that secreted both IFN$\gamma$ and IL-10 is also shown (blue bars). All samples were run in triplicate and background spots from unstimulated wells were deducted from the values obtained from test wells. The mean value from each triplicate is shown here. Donors without an IL-10 or IFN$\gamma$ responses were omitted from each ORF analysis.

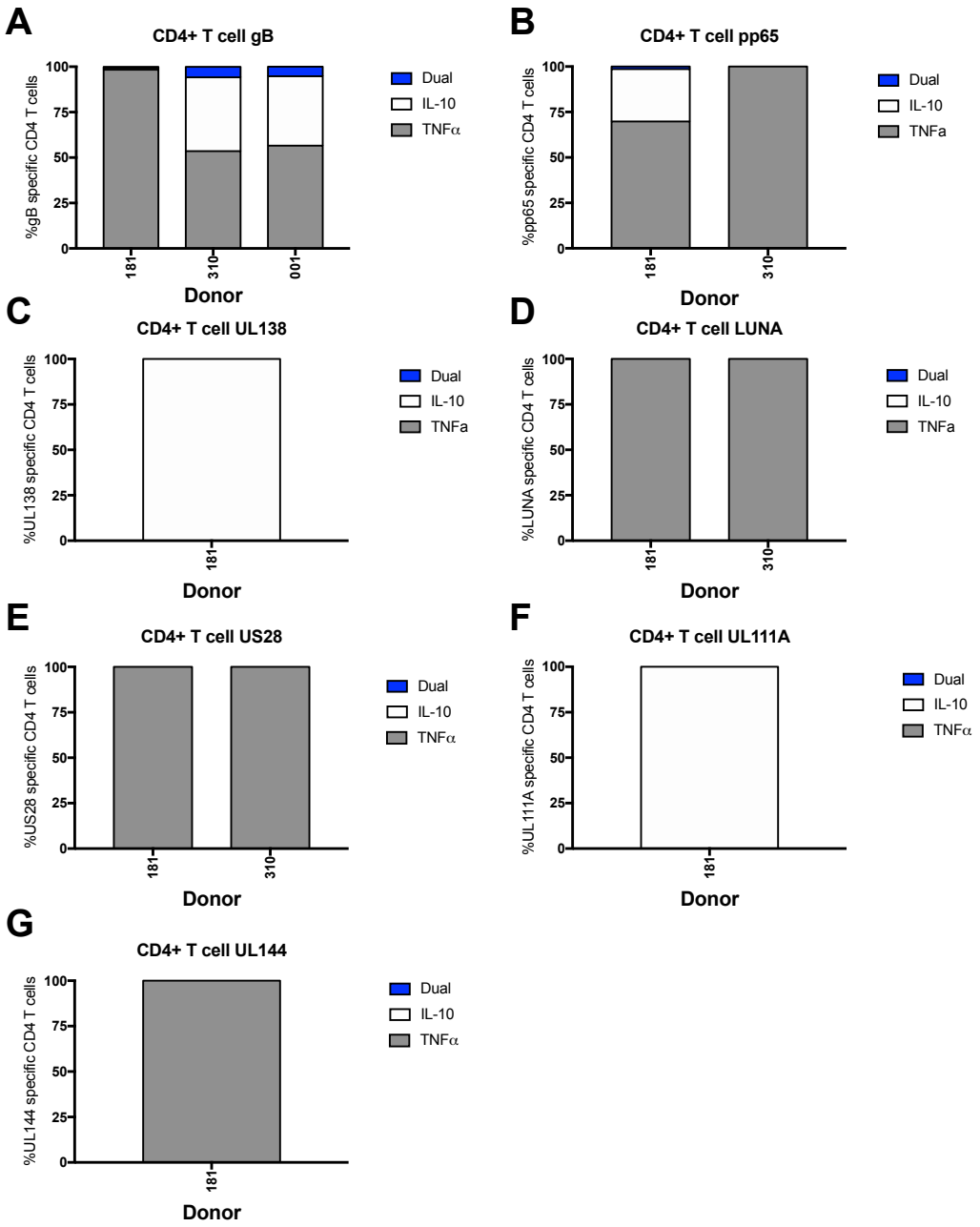



**Fig. 3.27 Proportions of CD4+ T cells secreting TNF$\alpha$ and IL-10 in response to latency-associated HCMV ORF peptide pools from seropositive donors.** PBMC from 3 seropositive donors were depleted of CD8+ cells and stimulated with overlapping peptide pools from HCMV ORFs (A) gB, (B) pp65, (C) UL138, (D) LUNA, (E) US28, (F) UL111A, and (G) UL144 for 48 hours. Following stimulation, IL-10 (white bars) and TNF$\alpha$ (grey bars) secretion was detected by FluoroSpot and enumerated to spot forming units (SFU), where each cytokine response was calculated as a proportion of the total CD4+ T cell response. The proportion of CD4+ T cells of total cytokine secreting cells that secreted both TNF$\alpha$ and IL-10 is also shown (blue bars). All samples were run in triplicate and background spots from unstimulated wells were deducted from the values obtained from test wells. The mean value from each triplicate is shown here. Donors without an IL-10 or TNF$\alpha$ responses were omitted from each ORF analysis.

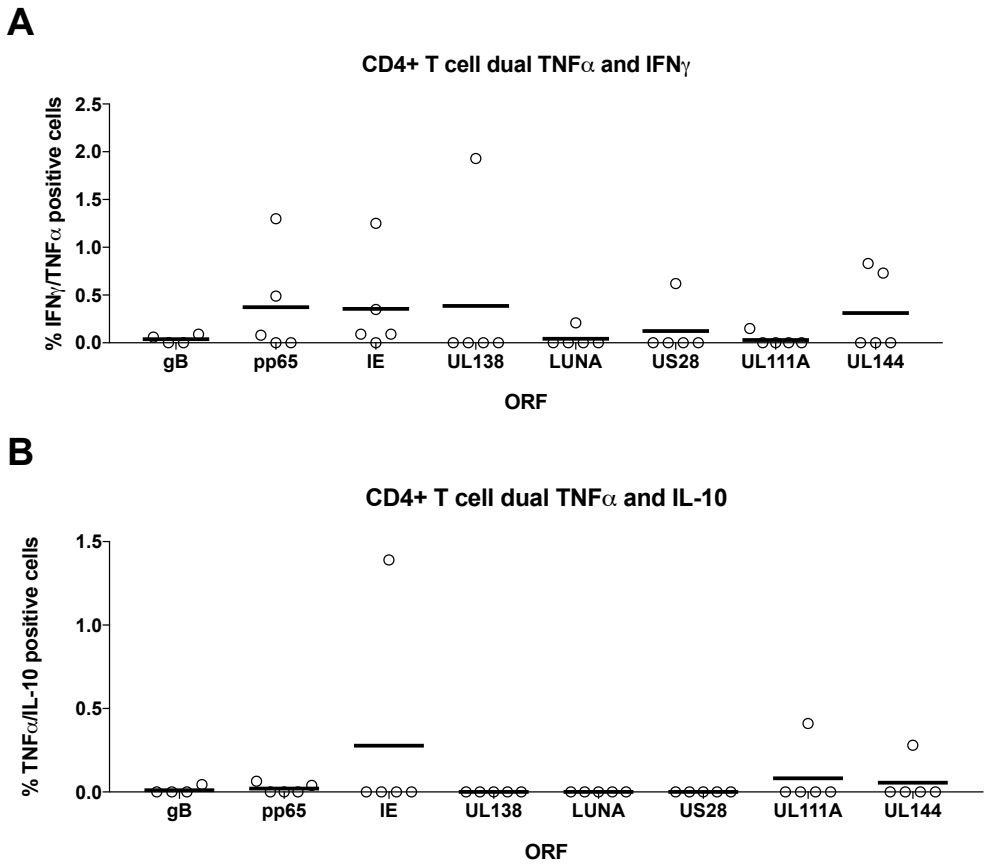



**Fig. 3.28 Detection of CD4+ T cells capable of co-producing TNF$\alpha$ together with IFN$\gamma$ or IL-10 in response to HCMV ORF peptide pools by intracellular cytokine staining.** Whole PBMC isolated from 5 seropositive donors were stimulated overnight with HCMV peptide pools spanning each ORF of interest (gB, pp65, IE, UL138, LUNA, US28, UL111A, UL144), in the presence of monensin. Stimulated PBMC were then stained intracellularly for IFN$\gamma$, IL-10 and TNF$\alpha$ production from CD4+ T cells. CD4+ T cells positive for both (A) TNF$\alpha$ and IFN$\gamma$, and (B) TNF$\alpha$ and IL-10 are shown. T cells were identified after gating for live and CD3+ cells, and ORF-specific T cells by expression of 4-1BB and/or CD40L.

## 3.3 Discussion

The data presented in this chapter, which was mainly obtained from studies on a large donor cohort, cover the CD4+ T cell responses to HCMV. In the interest of clarity, the analogous data gathered simultaneously on CD8+ T cell responses is presented in the following chapter.

### 3.3.1 CD4+ T cell responses to latency-associated HCMV ORFs

Little is known about the T cell responses to proteins expressed during HCMV latency. Therefore, one aim of this work was to determine if CD4+ T cells isolated from seropositive donors could recognise latency-associated ORFs. Using an overlapping peptide pool approach to stimulate CD4+ T cells *ex vivo*, cytokine secretion was determined by FluoroSpot assay. The FluoroSpot assay was first validated to ensure the cytokine secretion being detected was robust, reliable, and was originating from CD4+ T cells. An analysis of 91 HCMV seropositive donors revealed CD4+ T cells recognised overlapping peptide pools from the latency-associated ORFs UL138, LUNA, US28, UL111A, and UL144. The recognition of UL138 and LUNA by CD4+ T cells has been reported previously [501], but responses to US28, UL111A, and UL144 have not been reported at such prevalence before or from such a large donor cohort [288].

Intriguingly, the frequencies of CD4+ T cells responding to UL144 and US28 were comparable to those responding to the lytic ORFs gB, and IE1 respectively. While donors with a positive CD4+ T cell response to UL138, LUNA, and UL111A were not as common, these still represented a large minority of sampled individuals. In addition, 67% of donors sampled were capable of responding to two or more of the five latency-associated ORFs tested (data not shown). CD4+ T cells specific for proteins expressed during latency are therefore widely present in HCMV seropositive donors. However, it is not clear why the frequencies of CD4+ T cells secreting IFN$\gamma$ in response to US28 and UL144 were more prevalent, and of higher frequency, than those to UL138, LUNA, and UL111A.

The generation of CD4+ T cell responses to latency-associated ORFs is perhaps not surprising given the expression of these proteins during the lytic infection cycle. However, the extent to which HCMV seropositive donors have CD4+ T cells capable of recognising viral proteins expressed during latency raises numerous questions regarding the ability of the virus to persist in the face of such responses. One possible explanation could be that these CD4+ T cells, or a subset of these cells, also secrete immunosuppressive factors that inhibit

the anti-viral immune response during latency. One such factor is IL-10, which has been shown to be produced by UL138- and LUNA-specific CD4+ T cells [501].

The capacity of CD4+ T cells from HCMV seropositive donors to secrete IL-10 in response to proteins expressed during latency was examined. CD4+ T cells were capable of secreting IL-10 in response to all ORFs tested, including UL138 and LUNA as previously described [501]. Generally, IL-10 responses to the latency-associated ORFs, in particular UL138, LUNA, and US28 were of a higher magnitude than those to the lytic ORFs gB, IE1, and pp65. A higher frequency of CD4+ T cells secreting IL-10 in response to pp65 were however found compared to gB, IE1, and UL111A responses. The detection of IL-10 response to lytic ORFs might not be surprising as CD4+ T cells secreting IL-10 have been previously reported to be generated in response to pp65, as well as gB [502, 621]. The highest frequencies of IL-10 secreting cells were detected in response to US28, LUNA, and UL138, followed by UL144 and UL111A. Of note, the frequencies of responding CD4+ T cells secreting IL-10 were lower than those secreting IFN$\gamma$ for all ORFs.

Examining the proportions of responding CD4+ T cells secreting IFN$\gamma$ and IL-10 within individuals revealed that a higher percentage of donors had dominant CD4+ T cell IL-10 responses to UL138 and LUNA than dominant IFN$\gamma$ responses, while CD4+ T cell responses to US28, UL111A, and UL144 were found to be dominated by IFN$\gamma$ secreting cells. CD4+ T cell responses to UL144 in particular resembled responses to the lytically expressed ORFs gB, pp65, and IE1, in that the vast majority of CD4+ T cells responding to these ORFs secreted IFN$\gamma$.

TNF$\alpha$ is a known pro-inflammatory and antiviral cytokine [622, 623]. In HCMV infection, TNF$\alpha$ has been shown to repress viral IE expression in differentiated cells [624], and to inhibit virus production [317, 625]. CD4+ T cell responses from a small number of HCMV seropositive donors were tested for the secretion of TNF$\alpha$ by FluoroSpot and ICS in response to overlapping HCMV peptide pools. TNF$\alpha$ secreting CD4+ T cells were found in nearly all donors when measured by ICS, but these were not found reliably when measured by FluoroSpot. One problem was the high background levels of TNF$\alpha$ secretion detected in the unstimulated control wells which meant several donors had to be excluded from the FluoroSpot analysis. It is not clear why the FluoroSpot and ICS results are not in alignment for the TNF$\alpha$ secretion when those for IFN$\gamma$ and IL-10 secretion are. The addition of further donors to the FluoroSpot assessment and carrying out both ICS and FluoroSpot assays simultaneously on the same donor will help rule out the small sample size and donor specific effects. Examining the secretion of TNF$\alpha$ in combination with IFN$\gamma$ and IL-10 by triple FluoroSpot assays revealed that there was a small minority of cells that co-produced TNF$\alpha$

and IFN$\gamma$, but these were only highly prevalent in one donor, and otherwise were not widely detected. Dual IFN$\gamma$/TNF$\alpha$ producing cells were also not detected by ICS, contrary to several reports which carried out ICS following stimulation with CMV lysate, IE1, pp65, or gB peptides [405, 414, 415]. IL-10/TNF$\alpha$ co-producers were even less common with almost none detected. It appears that the majority of the CD4+ T cells responding to latency-associated ORFs are therefore single producers from one of three sub-populations of IL-10, IFN$\gamma$, or TNF$\alpha$ secreting cells. Furthermore, no cells secreting all three cytokines were detected in any donor. More donors need to be tested for TNF$\alpha$ responses however before these results can be verified.

The extensive CD4+ T cell IL-10 responses detected in response to all of the latency-associated ORFs tested further lends weight to the hypothesis that these IL-10 producing T cells are suppressing antiviral T cells, and therefore providing a mechanism that facilitates the maintenance of viral latency. This hypothesis is further supported by the finding that CD4+ T cells secreting IFN$\gamma$ and those secreting IL-10 were separate cellular populations, as has previously been reported for gB- and pp65-specific CD4+ T cells [621]. It could therefore be possible that CD4+ T cells recognising proteins expressed during latency secrete IL-10, and inhibit the population of IFN$\gamma$ secreting CD4+ T cells, which might otherwise be capable of eliminating latently infected cells.

Work carried out in mice on MCMV provides support that this could be the case. MCMV is known to persistently replicate in the salivary glands of infected mice, and CD4+ T cells secreting IL-10 have been observed to accumulate as a direct result of infection [626]. Blockade of IL-10R signalling results in a decrease in viral replication, and an increase of IFN$\gamma$ secreting CD4+ T cells known to be protective at this site [371, 626]. Furthermore, in mice lacking CD4+ T cell IL-10 production, there was an increase in MCMV-specific CD4+ T cells secreting IFN$\gamma$ in the salivary glands and periphery [621]. The resulting increase in Th1 cells led to a reduction in replication and shedding of virus from the salivary gland [621]. Thus, during MCMV infection, IL-10 secreting CD4+ T cells act to limit the differentiation of Th1 cells, which modulates anti-viral immune responses. Similarly, IL-10 derived from CD4+ T cells contributed to restricting the expansion of MCMV-specific memory T cells, and reducing the production of antiviral cytokines [627]. MCMV infection of IL-10 knockout mice or IL-10R blockade during latent infection resulted in a reduction in viral load [627].

The exploitation of the IL-10 pathway to evade host immune responses is not unique to HCMV. IL-10 secreting CD4+ T cells are also generated in response to a latent protein from EBV. CD4+ T cells specific to EBV latent membrane protein 1 were shown to secrete IL-10

and suppress T cell proliferation and IFN$\gamma$ secretion, which could contribute to the evasion of antiviral immune responses during latency [628].

In addition to IL-10 production by a subset of latency-associated ORF specific CD4+ T cells, HCMV has further mechanisms that could act together to suppress anti-viral T cell responses during latent infection. During latency, HCMV induces a number of modifications to the infected cell that result in a reduction of T cell recognition and activation. The expression of the UL111A gene product LAcmvIL-10, which is a cellular IL-10 homologue, can downregulate the expression of MHC class II on granulocyte macrophage progenitor cells and monocytes [156]. Furthermore, the ability of CD4+ T cells to recognise latently infected myeloid progenitor cells was enhanced upon infection with a UL111A deletion virus, in line with the resulting increase in MHC class II expression [165]. Therefore, UL111A acts to modulate the presentation of viral peptides via MHC class II, in an attempt to limit CD4+ T cell recognition of latently infected cells.

UL111A could also be contributing to the evasion of T cell immunity through its ability to affect the differentiation of myeloid progenitor cells. Latent infection of myeloid progenitor cells with a UL111A deletion virus results in the higher production of pro-inflammatory cytokines, and the differentiation of a greater proportion of myeloid DCs compared to infection with wild-type virus [166]. As DCs are the most potent antigen presenting cell, restricting their differentiation from myeloid progenitor cells could act to limit the presentation of latency-associated viral peptides to virus-specific T cells, contributing to the evasion of immune detection by latently infected cells.

Latent infection with HCMV is also associated with changes to the cellular secretome that contribute to the evasion of T cell immunity. Latently infected CD34+ progenitor cells secrete both IL-10 and TGF$\beta$, and upregulate CCL8 expression, a chemokine that induces migration of CD4+ T cells [605]. These factors then act in combination to modulate the effector functions of CD4+ T cells attracted to sites of latent infection, resulting in a decrease in CD4+ T cell mediated cytotoxicity and in the production of inflammatory Th1 cytokines [605]. This upregulation of CCL8 and IL-10 is at least in part caused by the expression of LAcmvIL-10, which acts by suppressing cellular hsa-miR-92a [164]. In addition, the secretome from latently infected CD34+ cells induces the expression of IL-10 from uninfected bystander cells [605]. Thus, the virus is able to create an immunosuppressive microenvironment capable of preventing T cell mediated effector functions that might otherwise act to eliminate latently infected cells.

The effects of the latent secretome on anti-viral T cell responses could therefore be compounded by the secretion of IL-10 from both uninfected bystander cells and from latency-specific CD4+ T cells entering the microenvironment of latently infected cells. By suppressing anti-viral T cell responses, such concerted action could act to prevent the elimination of latently infected cells. The inhibition of T cells by secreted immunosuppressive factors is also known to occur during cancer. The secretion of IL-10 and TGF$\beta$ into the tumour microenvironment by tumour cells or tumour-associated macrophages has been shown to contribute to the suppression of tumour-specific T cell responses [629–637]. Crucially, it has been demonstrated that in certain cases interference with these factors results in improved T cell immune responses against cancer cells [638–641].

It is unclear why CD4+ T cell responses to lytically-expressed proteins are dominated by IFN$\gamma$ secreting cells while responses to the proteins expressed during latency are composed of much greater proportions of IL-10 secreting cells. In the case of responses to UL138 and LUNA for example, more donors had dominant CD4+ T cell IL-10 responses than IFN$\gamma$ responses. Interestingly, UL144, which is hypervariable [139], was not found to be expressed in all donors during natural latency [125]. However, whether this influences the highly IFN$\gamma$ biased CD4+ T cell response to UL144 remains to be determined.

It could be possible that chronic activation of CD4+ T cells recognising cognate antigen in the presence of the latent cell microenvironment, which contains the immunosuppressive cytokines IL-10 and TGF$\beta$, results in the generation of latency-specific CD4+ T cells that secrete immunosuppressive cytokines themselves [506]. As lytic proteins are not expressed to the same extent during latency, this could lead to the generation of a greater number of IL-10 secreting CD4+ T cells specific to latency-associated ORFs in comparison. It is therefore important to determine whether IL-10 secreting CD4+ T cells arise shortly after primary infection, or are generated following the establishment of latency. Preliminary work to address this question will be presented in a later chapter.

### 3.3.2 Fluctuations in the CD4+ T cell responses detected over time

The frequencies of CD4+ T cells secreting IFN$\gamma$ and IL-10 in response to HCMV ORFs were found to fluctuate across independent sampling points over a three year period in the same individuals. The extent of these fluctuations was in part ORF-dependent. Responses to the lytically expressed proteins and to UL144 were the most consistent across all time points, while the T cell responses to latency-associated ORF products had a greater degree of variability. However, because only one donor was sampled on more than three occasions,

it is not possible to ascertain if responses to latency-associated ORFs do indeed vary to a greater extent than responses to lytically-expressed proteins. The detection of CD4+ T cell IL-10 responses was also more inconsistent than IFN$\gamma$ responses, and there were time points where no IL-10 secreting cells were detected to any of the latency-associated ORFs tested. It is also not possible to determine if the variation in detected cytokine secreting CD4+ T cells over time is more common in certain donors over others, given the limited sampling points from three of the four donors.

Fluctuations in CD4+ T cell responses to lytic HCMV proteins have been reported previously, although over a much shorter 9- to 25-week period with frequent sampling [642]. The observed fluctuations in the frequencies of cytokine secreting CD4+ T cells over time could be a direct result of isolating CD4+ T cells from peripheral blood. The peripheral blood compartment contains a small proportion of an individual's total T cells and given its dynamic and transient nature, this could lead to differences in the frequencies of cytokine secreting cells detected between sampling time points [618, 643, 644]. Furthermore, CD4+ T cell numbers in peripheral blood could be drastically affected by active immune responses. HCMV reactivation events that might drive more CD4+ T cells into the blood could result in an over-representation of responses at that time point, or if immune responses to other pathogens were occurring, this might result in a lymphocytosis, and resulting dilution of HCMV-specific CD4+ T cells in the peripheral blood. As total lymphocyte counts in combination with CD3+/CD4+ phenotyping were not carried out, which would provide an absolute value for CD4+ T cells numbers at different time points, the possibility that fluctuations in HCMV-specific CD4+ T cells were caused by overall fluctuations in CD4+ T cells in the peripheral blood cannot be excluded. It is interesting to note that there are certain time points where responses to many of the ORFs were much higher than other time points, which could point to such a change in the overall blood compartment. Furthermore, ORF-specific CD4+ T cell responses of low frequency might be disproportionately affected by such fluctuations in peripheral blood CD4+ T cells composition. Large differences in the number of IFN$\gamma$ secreting cells in response to UL144 for example, never resulted in a time point where positive cells could not be detected. These relatively high frequency responses, such as to UL144, might therefore remain detectable when the peripheral blood compartment changes, whereas lower frequency responses might drop to below positive.

Another contributing factor to the observed fluctuations in CD4+ T cell responses over time could be incomplete binding of released cytokines by the capture antibodies of the FluoroSpot assay. Following peptide pool stimulation, ORF-specific CD4+ T cells secreting IFN$\gamma$ might therefore be capable of inhibiting the release of IL-10, and vice versa. There

are certain time points from the longitudinal sampling of within donor responses where ORF-specific IL-10 secretion is high but IFN$\gamma$ secretion is low, as well as the reverse being observed. Further work could assay the supernatant for IFN$\gamma$ and IL-10 by ELISA following the completion of the assay to determine levels of free cytokines, as well as introducing neutralising IL-10 antibodies concurrently with peptide pool stimulation to determine if this increases IFN$\gamma$ secretion.

### 3.3.3 CD4+ T cell responses and age

A number of studies have drawn a link between lifelong carriage of HCMV and an age-related decline in immune functions, such as an increased susceptibility to infections and poor responses to vaccinations [419, 645], although many studies have also found no such associations [353, 355]. Older HCMV infected individuals seem to retain the ability to control HCMV to the extent that they do not suffer from overt clinical disease from viral reactivations or re-infection [434]. There does however appear to be an age-related increase in the levels of viral DNA detected in the blood [434] and urine [435]. The immune responses of older individuals might therefore undergo changes with age, which could be caused by lifelong viral carriage. In this manner, HCMV might directly or indirectly modulate anti-HCMV immune responses to the extent that there is reduced control of reactivating virus. As IL-10 possesses inhibitory properties, it could be possible that long-term viral carriage results in a skewing of CD4+ T cell responses from pro-inflammatory, IFN$\gamma$ secreting, to IL-10 secreting cells. Such immunosuppressive cells might then reduce the ability to control lytic HCMV replication and account for the apparent decreases in anti-HCMV immune responses with age. The breadth (number of ORFs donors responded to), and frequencies of CD4+ T cell responses were examined in relation to donor age using a cross-sectional methodology on donors ranging from 23 to 74 years of age. ORF-specific responses were compared across donor ages for both IFN$\gamma$ and IL-10 secreting CD4+ T cells.

No association between the frequencies of IFN$\gamma$ secreting CD4+ T cells and donor age was found when taking into account our cohort as a whole and interestingly, marginally significant decreases were detected in the frequencies of CD4+ T cells secreting IL-10 in response to IE1 and UL111A with increasing donor age. Additionally, the breadth of responses did not seem to be affected by the age of donor either, where it might be predicted that longer viral carriage and resulting increase in reactivation events might be expected to drive immune responses to additional ORFs. Therefore when considering the donor cohort

as a whole, it seems unlikely that potential declines in anti-HCMV immunity with age are caused by increases in HCMV-specific IL-10 secreting CD4+ T cells.

The unchanging number of ORFs responded to by CD4+ T cells with increasing donor age could indicate that primary infection is responsible for generating ORF-specific T cell responses against HCMV. Latent viral carriage and periodic reactivations into productive infection might then serve to stimulate existing T cells rather than generate responses to new antigens. Indirect evidence for this arises from the observations that CD4+ T cell clonal diversity contracts and becomes severely restricted in the persistent stages of infection compared to the acute phase [406]. As the donors used here have not been followed from primary infection, and it is not known when this occurred, it cannot be determined how the number of ORF-specific responses changes immediately following acute infection. Furthermore, questions still remain over the kinetics of IL-10 secreting CD4+ T cell generation, as these could arise immediately following primary infection, or could be induced during the latent phase. Work seeking to address this by utilising multiple samples from transplant recipients before and after primary infection will be presented in a subsequent chapter.

An important caveat to the results from the longitudinal studies, and those measuring responses at different ages, is that antigen-specific T cell response frequencies are being detected by IFN$\gamma$/IL-10 FluoroSpot assays, which rely on antigen-specific cells possessing these functions. As such, changes in antigen-specific T cell frequencies with age or over time could be due to changes in the proportions of T cells which possess these functions, rather than in the absolute sizes of these populations.

### 3.3.4 CD4+ T cell responses and sex

When grouping the donor cohort based on sex, differences in CD4+ T cell responses were found when comparing the frequencies of cytokine secreting cells responding to individual ORFs. CD4+ T cell IFN$\gamma$ responses to UL144 were found to be of higher magnitude in male compared to female donors. Likewise male CD4+ T cell IL-10 responses to pp65 and US28 were significantly higher than female CD4+ T cell responses to these ORFs. Furthermore, when considering the total CD4+ T cell responses within individuals to either all ORFs, latency-associated ORFs only, or lytic ORFs only, male donors exhibit greater frequencies of both IFN$\gamma$ and IL-10 secreting cells than female donors.

It is not clear why certain ORF-specific CD4+ T cell response frequencies differ between sexes. Sex has been shown to have an influence on a number of aspects of adaptive immunity [619]. In humans, females have higher numbers of CD4+ T cells and higher CD4/CD8 T cell ratios than age-matched males [619]. Additionally, females have greater antibody responses than males and higher numbers of B cells [619]. Differences in cytokine production between sexes has also been observed for CD4+ T cells. Naive CD4+ T cells isolated from the peripheral blood of female donors were found to proliferate to a greater extent in response to anti-CD3 and anti-CD28 stimulation than those isolated from men [646]. Female CD4+ T cells also produced more IFN$\gamma$, while male CD4+ T cells had a bias towards IL-17A production following stimulation [646].

There is currently a lack of evidence on the sex-specific immune responses to HCMV. However, seroprevalence is known to be higher among females than males [9, 647], with higher reported HCMV IgG titres in elderly females [423], although this might not be the case for middle aged individuals, where in a separate study no sex-specific difference was found [648]. Middle aged male HCMV infected individuals were also shown to have lower absolute numbers of CD4+ T cells than females [648], while a study assessing the frequency of CD4+ T cells within the T cell compartment found no difference between men and women in old and young HCMV positive seropositive donors [649]. Further work could assess the CD4+ T cell compartment sizes in males and females by absolute counts in conjunction with FluoroSpot response data for CD4+ T cells. This could assess whether differences in HCMV-specific CD4+ T cell numbers are proportional to any differences in the overall CD4+ T cell compartment between male and female donors. Interestingly, the absolute numbers of CD8+ T cells have been found to be relatively unaffected by HCMV infection and sex [648], and the data from CD8+ T cells presented in the subsequent chapter, found no differences in total donor CD8+ T cell responses to either all ORFs, latency-associated ORFs only, or lytic ORFs only, between sexes (Section 4.2.8).

A major limitation of these experiments is that they only take into account CD4+ T cells isolated from the peripheral blood compartment. As fewer than 2.5% of all T cells have been estimated to reside in the peripheral blood [618], the results obtained here ideally need to be paired with responses from CD4+ T cells isolated from other tissue sites. This will enable a more complete picture of CD4+ T cell responses to proteins expressed during latency to be generated. In this manner, any differences in CD4+ T cell antigen-specificities and effector-functions between tissue-sites and the peripheral blood could be determined. One such site to investigate could be the bone marrow, where resident CD34+ progenitor cells are known to harbour latent HCMV [94]. Polyfunctional pp65-specific CD4+ T cells have

been found to be considerably enriched in the bone marrow compared to peripheral blood [650], and carrying out a paired comparison with peripheral blood CD4+ T cell responses to latency-associated ORFs would be highly beneficial. Examining the CD4+ T cell responses to HCMV from tissues such as bone marrow could also provide a means to reduce the fluctuations in the frequencies of detected cytokine secreting cells when sampling from the peripheral blood. The characterisation of CD4+ T cell responses from bone marrow derived T cells will be elaborated on in a subsequent chapter.

# Chapter 4

# Identifying Latency-specific CD8+ T cells

## 4.1 Introduction

CD8+ T cell responses to HCMV can reach a large proportion of total T cells in the peripheral blood compartment, especially to highly immunogenic antigens such as pp65 and IE1 [297, 288]. An extensive analysis utilising overlapping peptide pools spanning 213 predicted HCMV ORFs revealed that three ORFs (UL48, pp65, and IE1) were recognised by over half of the 33 donors tested, where recognition was determined by IFN$\gamma$ production via ICS [288]. Furthermore, CD8+ T cells from donors recognised a median of 8 ORFs, although this was highly heterogeneous between individuals [288]. Subsequent work has since highlighted the considerable variability in the size of these responses between individuals, the reasons for which are not fully understood [291].

Studies examining CD8+ T cell responses have failed to detect high frequencies of CD8+ T cells specific for latency-associated ORFs. Out of 33 HCMV seropositive donors, responses to US28 and UL138 were found in one donor, while no responses to UL144 and UL111A were detected by ICS [288]. Work examining CD8+ T cell responses to the latency-associated HCMV ORFs UL138 and LUNA by ELISpot did find low frequencies of responding cells, but these were below the set positive threshold [501]. The relative simplicity of the depletion system used to test CD4+ T cell responses, and the ease of the FluoroSpot assay, which allowed for both CD4+ and CD8+ T cell responses to be tested on the same plate, provided the rationale to test the CD8+ T cell responses to proteins expressed during latency.

As both CD4+ and CD8+ T cell responses to HCMV ORFs were being tested from a large donor cohort, this gave the opportunity to assess the effects, if any, of age on CD8+ T

cell responses to HCMV. As with the effect of HCMV on CD4+ T cells, HCMV has been observed to be associated with age-related alterations in the CD8+ T cell compartment, and thought to be a driver of immunosenescence [651]. These include the expansion of CD8+ T cells with a proposed highly differentiated phenotype (loss of CD28 expression, gain of CD57 expression), and diminished replicative ability, which many report to be dysfunctional [352, 417, 652–654]. As such, it has been suggested that the accumulation of these highly differentiated T cells during HCMV infection could have negative consequences for responses to vaccination, responses to other pathogens, and on mortality [348, 418, 655, 656]. Utilising the FluoroSpot assay, I therefore wanted to assess the effect of age on the magnitudes of CD8+ T cell responses to HCMV proteins.

This chapter therefore aimed to determine whether CD8+ T cells specific for latency-associated ORFs US28, UL111A, and UL144 are present in seropositive individuals, if there was any association in the frequencies of HCMV-specific CD8+ T cells and the age of donor, and to examine the cytokines secreted by HCMV-specific CD8+ T cells.

## 4.2 Results

### 4.2.1 CD8+ T cells can recognise proteins expressed during HCMV latency

A FluoroSpot based approach was utilised to examine the CD8+ T cell responses to proteins expressed during latency. Overlapping peptide pools spanning HCMV ORFs were used to stimulate CD8+ T cells from HCMV seropositive donors, and the frequency of cytokine secreting cells determined by FluoroSpot. To obtain a dominant CD8+ T cell population, CD4+ cells were depleted from PBMC by MACS, which resulted in a mean residual CD4+ T cell proportion of 5.42% (standard deviation=3.77, n=47) of all CD3+ cells (Figure 4.1A). Consequently, the mean CD8+ T cell proportion of CD3+ cells following CD4+ cell depletion was 68.22% (standard deviation=14.39, n=47) (Figure 4.1A).

Previous work carried out by Mason et al. [501] using ELISpot assays did not find donors with CD8+ T cell responses to UL138 and LUNA greater than 100 SFU/$10^6$ cells. In the experiments here, the FluoroSpot assay was employed in part because of its greater sensitivity compared to the ELISpot. To validate this increase in sensitivity, CD8+ T cells were polyclonally stimulated and the frequencies of IFN$\gamma$ secreting cells were compared between FluoroSpot and ELISpot. Substantially higher frequencies of CD8+ T cells secreting

IFN$\gamma$ were detected by FluoroSpot compared to ELISpot in three donors (Figure 4.2), illustrating the greater sensitivity of the FluoroSpot assay.

The FluoroSpot was then utilised to test CD8+ T cell responses to latency-associated ORF products in a large donor cohort. To do this, PBMC depleted of CD4+ cells by MACS were stimulated with overlapping peptide pools spanning the HCMV ORFs of gB, pp65, IE1, UL138, LUNA, US28, UL111A, and UL144 on dual IFN$\gamma$/IL-10 FluoroSpot plates. Following this, the frequencies of cytokine secreting CD8+ T cells were determined. As previously, selection criteria were put in place to exclude potentially erroneous results. Initially, donors were excluded if the background responses in the unstimulated control wells were greater than 1000 spots. Subsequently, positive IFN$\gamma$ responses were only considered if the difference between the unstimulated samples and positive control samples was greater than 100 spots. These selection criteria resulted in 11 donors being excluded from the IFN$\gamma$ secretion analysis.

Examining the secretion of IFN$\gamma$ from 90 seropositive donors revealed previously uncharacterised CD8+ T cell responses against all latency-associated ORFs tested. As with the data from CD4+ T cells, the number of donors with above threshold CD8+ T cell IFN$\gamma$ responses (>100 SFU/$10^6$ cells as determined by HCMV seronegative donor responses), was found to vary in response to different ORFs. CD8+ T cells from a number of donors had positive IFN$\gamma$ responses to UL138 (27 donors, 30%), LUNA (29 donors, 32.2%), US28 (66 donors, 73.3%), UL111A (41 donors, 45.6%), and UL144 (70 donors, 77.8%) (Figure 4.3). CD8+ T cell responses to lytically-expressed proteins were expectedly frequent, with the majority of all donors possessing a positive response to each of gB (62 donors, 68.9%), IE1 (62 donors, 68.9%), and pp65 (82 donors, 91.1%) (Figure 4.3).

Out of 90 donors, there were 43 CD8+ T cell high responders (with above 250 SFU/$10^6$ responding cells) to gB (47.8%), 74 to pp65 (82.2%), and 49 to IE1 (54.4%) (Figure 4.3). Interestingly, CD8+ T cell responses to the latency-associated ORFs were composed of a similar number of donors who were high responders compared to CD4+ T cell responses (Figure 3.13). There were CD8+ T cell high responding donors most frequently found to UL144 (56/90, 62.2%) and US28 (44/90, 48.9%), and least frequently to UL138 (12/90, 13.3%), LUNA (14/90, 15.6%), and UL111A (14/90, 15.6%) (Figure 4.3).

Similarities were also found between the number of donors with a positive CD4+ or CD8+ T cell response to certain latency-associated proteins. UL144 and US28 had the highest number of donors with a positive CD4+ or CD8+ T cell response, in addition to the highest frequencies of T cells responding to these ORFs within individuals (Figures 3.13 and 4.3).

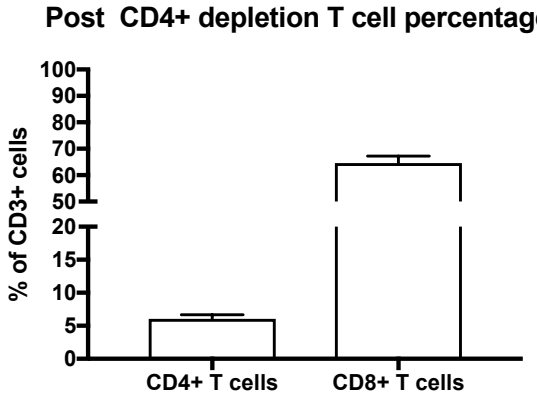



**Fig. 4.1 Magnetic-activated Cell Sorting to deplete CD4+ cells from PBMC and to isolate untouched CD8+ T cells from PBMC.** Whole PBMC from 47 donors were depleted of CD4+ cells using direct MACS CD4+ microbeads and stained for CD3, CD4, and CD8 expression to determine the residual proportions of CD4+ and CD8+ T cells of total CD3+ cells.

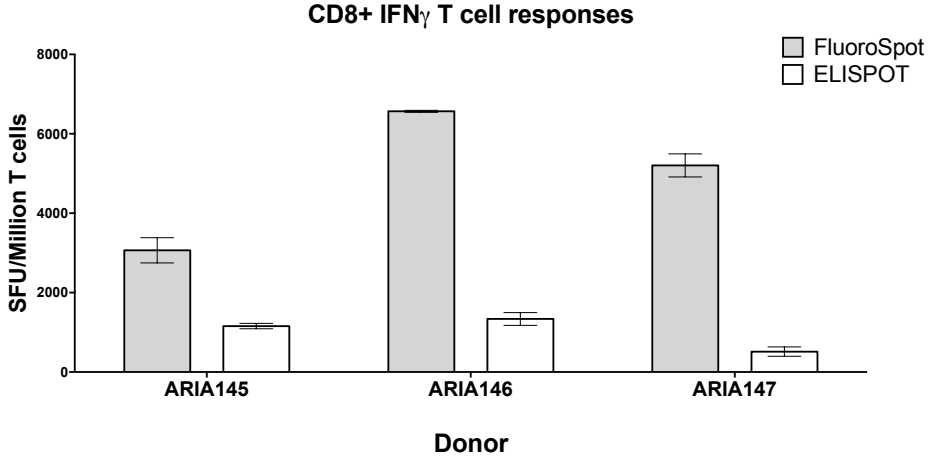



**Fig. 4.2 Comparison of the detected frequencies of CD8+ T cells secreting IFN$\gamma$ between FluoroSpot and ELISpot assays**. PBMC from three donors (ARIA145, ARIA146, and ARIA147) were depleted of CD4+ cells by MACS to obtain a dominant CD8+ T cell population. The CD4+ cell depleted PBMC were then stimulated with a mix of anti-CD3 and anti-CD28 monoclonal antibodies for 48 hours and the secretion of IFN$\gamma$ assessed by FluoroSpot (grey bars) and ELISpot (white bars) concurrently. The frequency of IFN$\gamma$ positive spots was counted and converted to spot forming units (SFU) per million cells.

Likewise, the lowest number of donors with a positive IFN$\gamma$ T cell response was found to UL111A, LUNA, and UL138 in both CD4+ and CD8+ T cells.

To ensure the detected IFN$\gamma$ secretion was mediated specifically by CD8+ T cells and the responses detected by the FluoroSpot were reproducible, these responses were also examined by ICS. PBMC from 18 donors were stimulated with HCMV ORF overlapping peptide pools overnight, and the secretion of IFN$\gamma$ identified from CD8+ T cells. IFN$\gamma$ production was readily detected in the majority of donors in response to the lytically-expressed proteins gB, pp65, and IE1, whereas responses to the latency-associated proteins were more variable, both in the number of donors with positive responses, and in the proportions of CD8+ T cells secreting IFN$\gamma$ within donors (Figure 4.4). The proportion of donors with CD8+ T cell responses detected by ICS were similar to those obtained by the FluoroSpot, with positive responses to UL138 and UL111A the least common, while those to US28 and UL144 were the most common (Figure 4.4). Interestingly, responses to LUNA were more frequently detected by ICS (72% of donors) (Figure 4.4) than by FluoroSpot (31.9% of donors) (Figure 4.3). The results from the ICS assay are therefore in agreement with the cytokine secretion detected by the FluoroSpot assay and show the responses are CD8+ T cell specific.

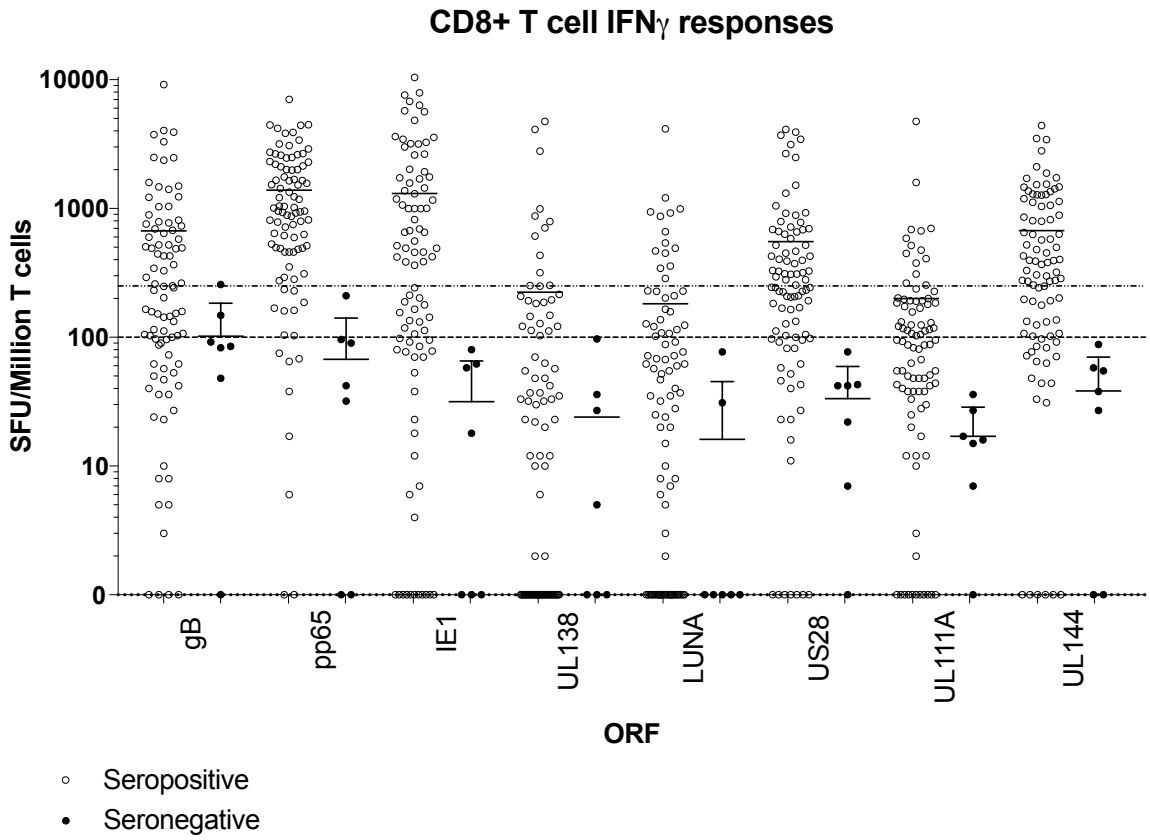



**Fig. 4.3 The frequency of CD8+ T cell responses from HCMV seropositive and seronegative donors stimulated with HCMV ORF pools.** PBMC from 90 seropositive and seven seronegative donors were depleted of CD4+ T cells and stimulated with HCMV ORF peptide pools spanning gB, pp65, IE1, UL138, LUNA, US28, UL111A, and UL144 for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. Each peptide pool was tested in triplicate and the values from the number of background cells secreting cytokines in the unstimulated wells was deducted from all test wells. Following stimulation, the number of IFN$\gamma$ positive spots was enumerated by the automated spot counter and converted to a spot forming unit (SFU) value per million cells. The dotted line at 100 SFU/$10^6$ cells indicates the threshold for positive responses, while the line at 250 SFU/$10^6$ cells shows the threshold for high responding donors. Seropositive donors are shown with open circles (○) while seronegative donors are illustrated with filled black circles (●).

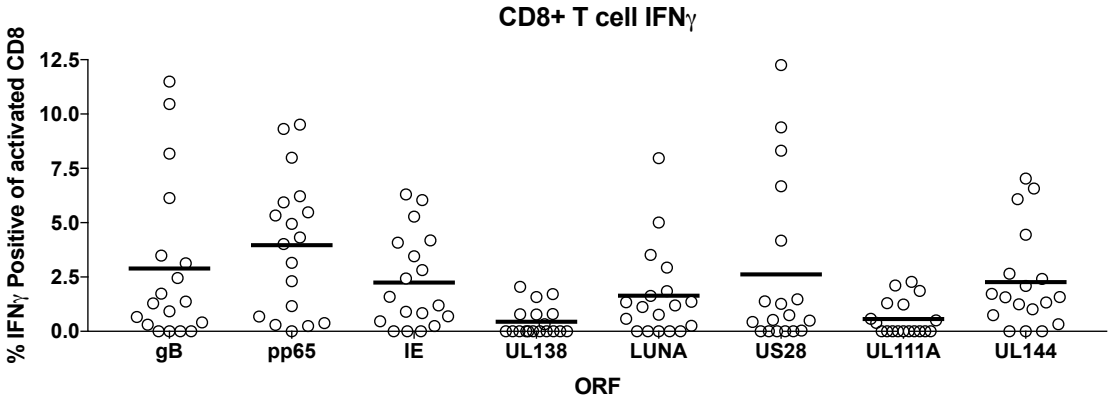



**Fig. 4.4 Intracellular cytokine staining for IFN$\gamma$ production from CD8+ T cells following HCMV ORF peptide pool stimulation.** Whole PBMC isolated from 18 seropositive donors were stimulated overnight with overlapping HCMV peptide pools spanning the ORFs of gB, pp65, IE1, UL138, LUNA, US28, UL111A, and UL144, in the presence of monensin. Stimulated PBMC were then stained intracellularly for IFN$\gamma$ from CD8+ T cells. T cells were identified after gating for live and CD3+ cells, and ORF-specific T cells by expression of 4-1BB and/or CD69.

### 4.2.2 The frequency of IFN$\gamma$ secreting CD8+ T cells detected fluctuates over time

The CD4+ T cell responses to latency-associated HCMV ORFs within four donors were found to vary across independent sampling points taken over a three year period (Section 3.3.2). In parallel, the CD8+ T cell responses to HCMV ORFs were examined from the same four donors at various time points over three years using the FluoroSpot assay. The most extensive analysis was carried out on one donor (CMV324), who had CD8+ T cell responses measured on seven occasions over three years. Similar to the CD4+ T cell responses, there were responses to certain ORFs that were relatively consistent over time, such as to the lytically-expressed proteins gB, pp65, and IE1, which were composed of a high frequency IFN$\gamma$ secreting cells at nearly all times points (Figure 4.5). Likewise, consistently elevated CD8+ T cell responses were observed to US28 and UL144 at all seven time points (Figure 4.5). Although subject to greater variation, CD8+ T cell responses to LUNA were also above background at all time points (Figure 4.5). The responses to UL138 and UL111A were the most inconsistent, with responses fluctuating from positive to no response at various time points (Figure 4.5), much like the CD4+ IFN$\gamma$ T cell responses over time in this donor (Figure 3.8A). Even for the most consistent above-threshold ORF-specific responses, there were often substantial fluctuations in the frequencies of IFN$\gamma$ secreting CD8+ T cells detected between time points (Figure 4.5). Examining the number of ORFs responded to across these time points (responses greater than 100 SFU/$10^6$ T cells) highlighted the greater degree of fluctuations in response to the latency-associated ORFs compared to the lytic ORFs. At all but one time point all three lytic ORFs were responded to by CD8+ T cells, while the number of positive responses to latency-associated ORFs varied from five to two across the sampling times (Figure 4.6).

Three additional donors were also examined on more than one occasion for their CD8+ T cell responses over time. Donor CMV319 had their CD8+ T cell responses tested at four time points, and again the results obtained highlighted that responses to certain ORFs were more consistent than others. High frequency IFN$\gamma$ secreting CD8+ T cells were present in response to the lytically-expressed proteins gB, pp65, and IE1 at each time point, as were responses to US28, UL111A, and UL144 (Figure 4.7). CD8+ T cell responses to UL138 and LUNA were the least consistent, where positive responses were registered at the first two time points but these were not observed again (Figure 4.7). Additionally, frequencies of detected IFN$\gamma$ positive CD8+ T cells were relatively similar across all time points for which a positive response was registered for this donor (Figure 4.7).

Although donor CMV323 was only sampled at two time points, there was also variation in their CD8+ T cell IFN$\gamma$ responses. Donor CMV323 had inconsistent IFN$\gamma$ responses to pp65 and US28 across the two time points, and there were considerable differences in the frequencies of IFN$\gamma$ secreting cells between sampling times in response to all ORFs, with the exception of UL138 (Figure 4.8).

Donor CMV307 was also only sampled on two occasions and had relatively consistent responses between these time points, although the frequencies of CD8+ T cells secreting IFN$\gamma$ did vary in response to gB (Figure 4.9).

Therefore, the CD8+ T cell responses tended to vary over time, often considerably, in the frequencies of IFN$\gamma$ secreting cells detected. The detection of positive responses to particular ORFs, such as UL138 and LUNA were found to fluctuate across the sampling times to a greater extent than responses lytically-expressed proteins such as gB. Furthermore, these results are comparable to the fluctuations observed in CD4+ T cell responses to HCMV ORFs.

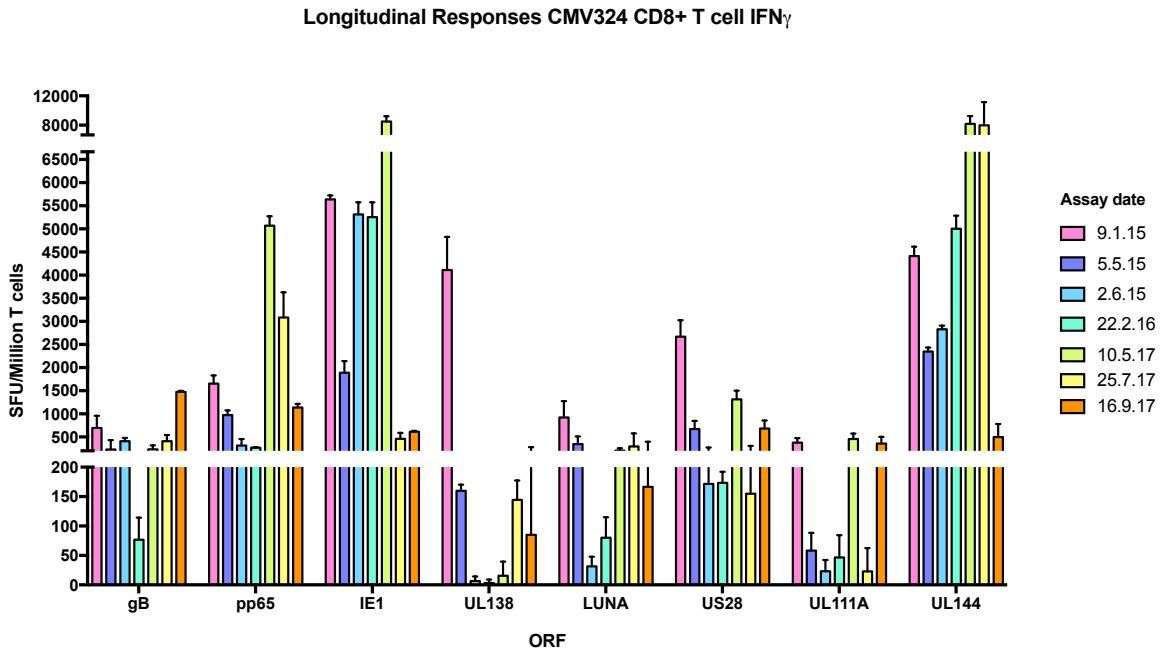
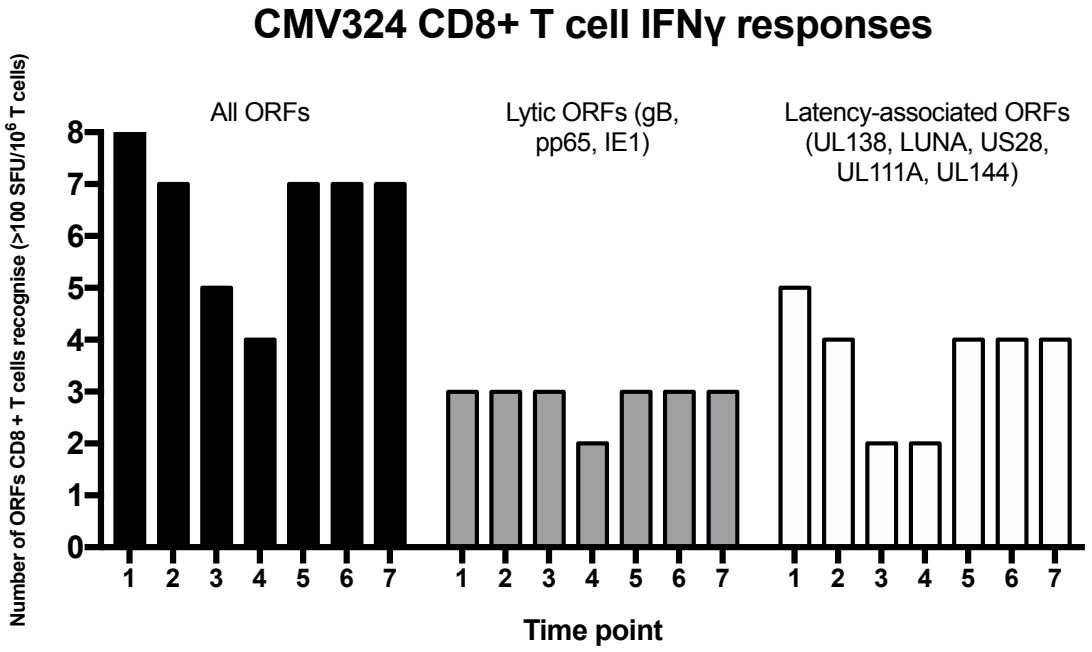



**Fig. 4.5 Longitudinal CD8+ T cell IFN$\gamma$ responses to HCMV ORF peptide pools from donor CMV324.** CD8+ T cells from one donor were tested at seven separate time points over three years. PBMC were depleted of CD4+ expressing cells and stimulated with overlapping peptide pools of HCMV peptides spanning gB, p65, IE1, UL138, LUNA, US28, UL111A, and UL144, for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. IFN$\gamma$ secreting CD8+ T cells were then stained for, counted, and converted to spot forming units (SFU) per million cells. All samples were run in triplicate and the frequencies of positive cells was determined by subtracting values in all test wells from the background number of positive cells in unstimulated well. Each different colour of bar indicates a different sampling time point.




| Time point | Date |
|---|---|
| 1 | 9-Jan-2015 |
| 2 | 5-May-2015 |
| 3 | 2-Jun-2015 |
| 4 | 22-Feb-2016 |
| 5 | 10-May-2017 |
| 6 | 25-Jul-2017 |
| 7 | 16-Sep-2017 |

**Fig. 4.6 Longitudinal analysis of the number of positive CD8+ T cell responses in donor CMV324.** Positive IFN$\gamma$ CD8+ T cell responses (responses over 100 SFU/$10^6$ cells) are shown at 7 time points over three years from one donor. The dates of each time point are shown in the table. PBMC were depleted of CD4+ expressing cells and stimulated with overlapping peptide pools of HCMV peptides spanning gB, p65, IE1, UL138, LUNA, US28, UL111A, and UL144, for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. The number of IFN$\gamma$ positive CD8+ T cells was determined and converted to SFU/$10^6$ cells. All samples were run in triplicate and the frequencies of positive cells was determined by subtracting values in all test wells from the background number of positive cells in unstimulated well. The black bars indicate responses to all 8 ORFs, both lytic and latency-associated, the grey to lytic antigens gB, pp65, and IE, and the white to latency-associated antigens UL138, LUNA, US28, UL111A, and UL144.

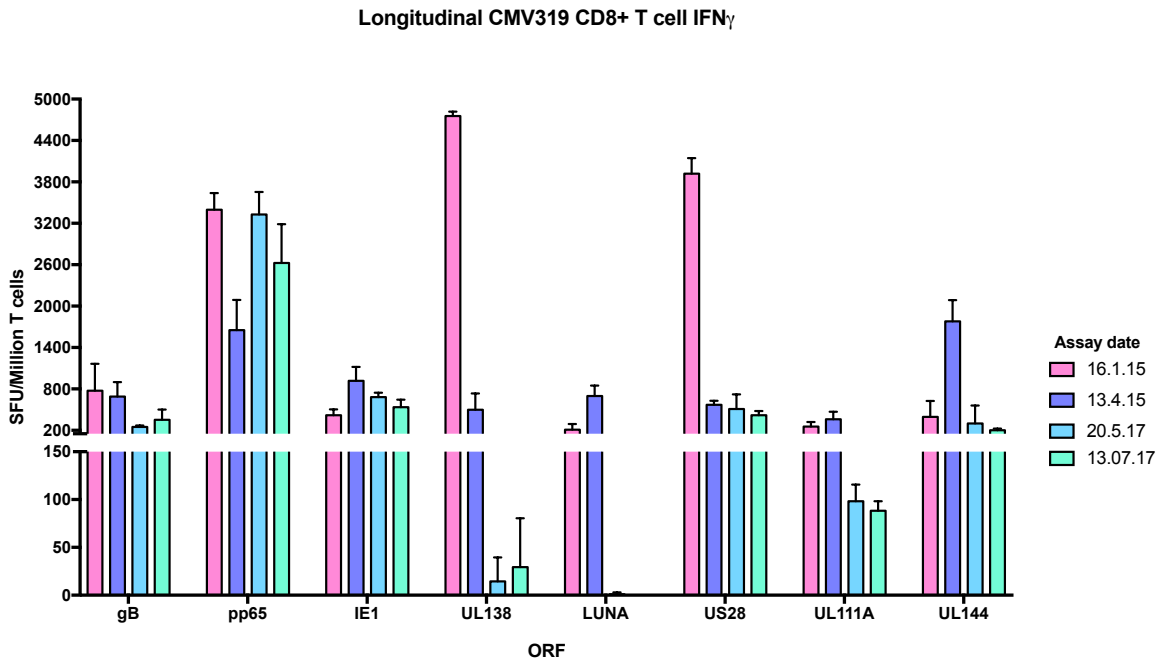



**Fig. 4.7 Longitudinal CD8+ T cell IFN$\gamma$ responses to HCMV ORF peptide pools from donor CMV319.** CD8+ T cell responses from one donor were tested at four separate time points over three years. PBMC were depleted of CD4+ expressing cells and stimulated with overlapping peptide pools of HCMV peptides spanning gB, p65, IE1, UL138, LUNA, US28, UL111A, and UL144, for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. IFN$\gamma$ secreting CD8+ T cells were then stained for, counted, and converted to spot forming units (SFU) per million cells. All samples were run in triplicate and the frequencies of positive cells was determined by subtracting values in all test wells from the background number of positive cells in unstimulated well. Each different colour of bar indicates a different sampling time point.

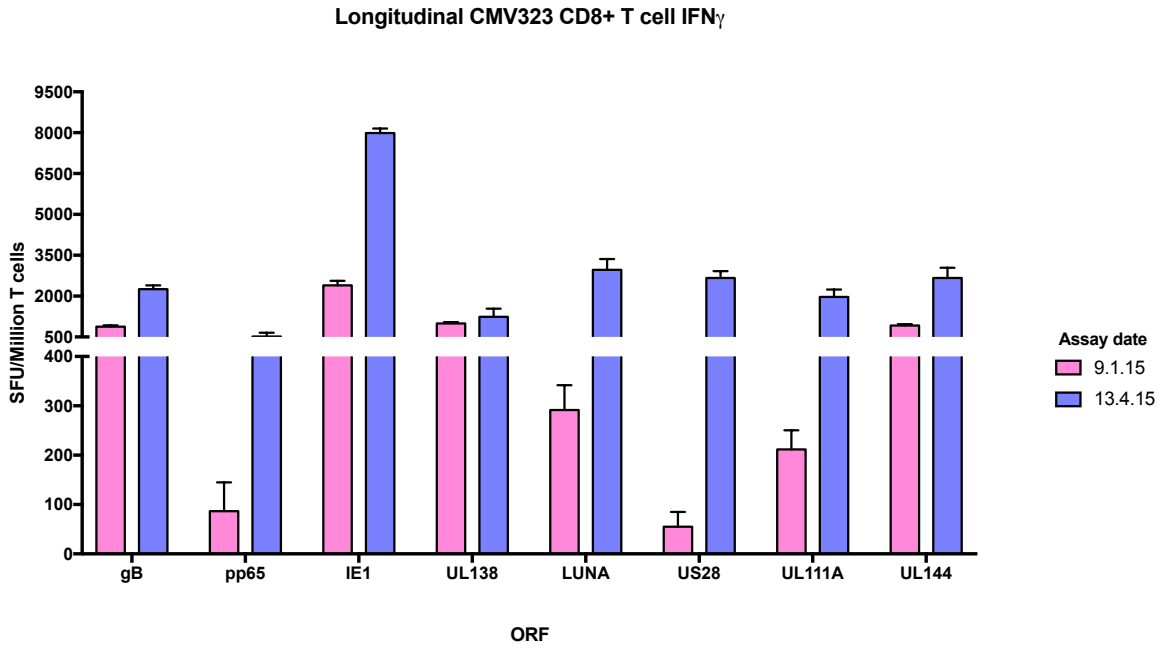



**Fig. 4.8 Longitudinal CD8+ T cell IFN$\gamma$ responses to HCMV ORF peptide pools from donor CMV323.** CD8+ T cell responses from one donor were tested at two separate time points over three years. PBMC were depleted of CD4+ expressing cells and stimulated with overlapping peptide pools of HCMV peptides spanning gB, p65, IE1, UL138, LUNA, US28, UL111A, and UL144, for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. IFN$\gamma$ secreting CD8+ T cells were then stained for, counted, and converted to spot forming units (SFU) per million cells. All samples were run in triplicate and the frequencies of positive cells was determined by subtracting values in all test wells from the background number of positive cells in unstimulated well. Each different colour of bar indicates a different sampling time point.

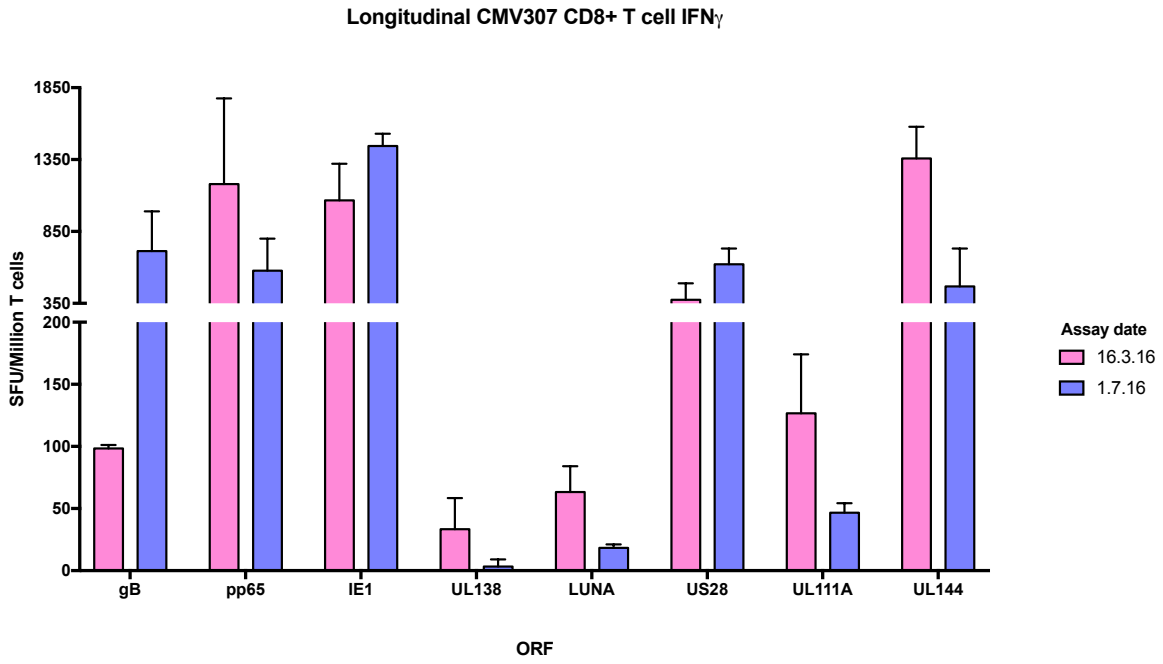



**Fig. 4.9 Longitudinal CD8+ T cell IFN$\gamma$ responses to HCMV ORF peptide pools from donor CMV307.** CD8+ T cell responses from one donor were tested at two separate time points over three years. PBMC were depleted of CD4+ expressing cells and stimulated with overlapping peptide pools of HCMV peptides spanning gB, p65, IE1, UL138, LUNA, US28, UL111A, and UL144, for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. IFN$\gamma$ secreting CD8+ T cells were then stained for, counted, and converted to spot forming units (SFU) per million cells. All samples were run in triplicate and the frequencies of positive cells was determined by subtracting values in all test wells from the background number of positive cells in unstimulated well. Each different colour of bar indicates a different sampling time point.

### 4.2.3 The frequency of latency-specific IFN$\gamma$ secreting CD8+ T cells does not change with age

Age could be in an important factor affecting the magnitude of CD8+ T cell responses to HCMV, as age provides an indirect measure for the time of latent viral carriage. The frequencies of CD8+ T cells that secreted IFN$\gamma$ in response to each HCMV ORF was therefore examined in relation to donor age in the cohort of 90 individuals. CD8+ T cell responses were found to vary in magnitude across all ages but there was no significant correlation (Spearman rank correlation analysis) between the frequency of IFN$\gamma$ secreting CD8+ T cells and the age of donor for responses to any of the latency-associated ORFs tested (Figure 4.10). However, there was a significant negative correlation between CD8+ T cell IFN$\gamma$ responses to UL138 and the age of donor in males when the donor cohort was grouped by sex (Spearman $r_s$ = -0.3805, $P$ = 0.0169) (Appendix A.3). The frequency of CD8+ T cells responding to the lytically-expressed proteins gB, pp65, and IE1 with IFN$\gamma$ secretion also did not change with increasing donor age (Figure 4.10).

Although the frequencies of IFN$\gamma$ secreting CD8+ T cells did not vary with increasing donor age, older donors might be expected to respond to a greater number of HCMV ORFs. This would be the case given a presumed longer time of viral carriage, and therefore exposure to more viral reactivation events, which could serve to generate new responses. The number of latency-associated HCMV ORFs each donor responded to out of the five tested were compared to the ages of donors. In agreement with data from CD4+ T cells (Figure 3.15), there was no change in the number of ORFs responded to with increasing donor age following a Kruskal-Wallis multiple comparisons test, and there was a relatively consistent distribution of ages responding to each number of ORFs (Figure 4.11).

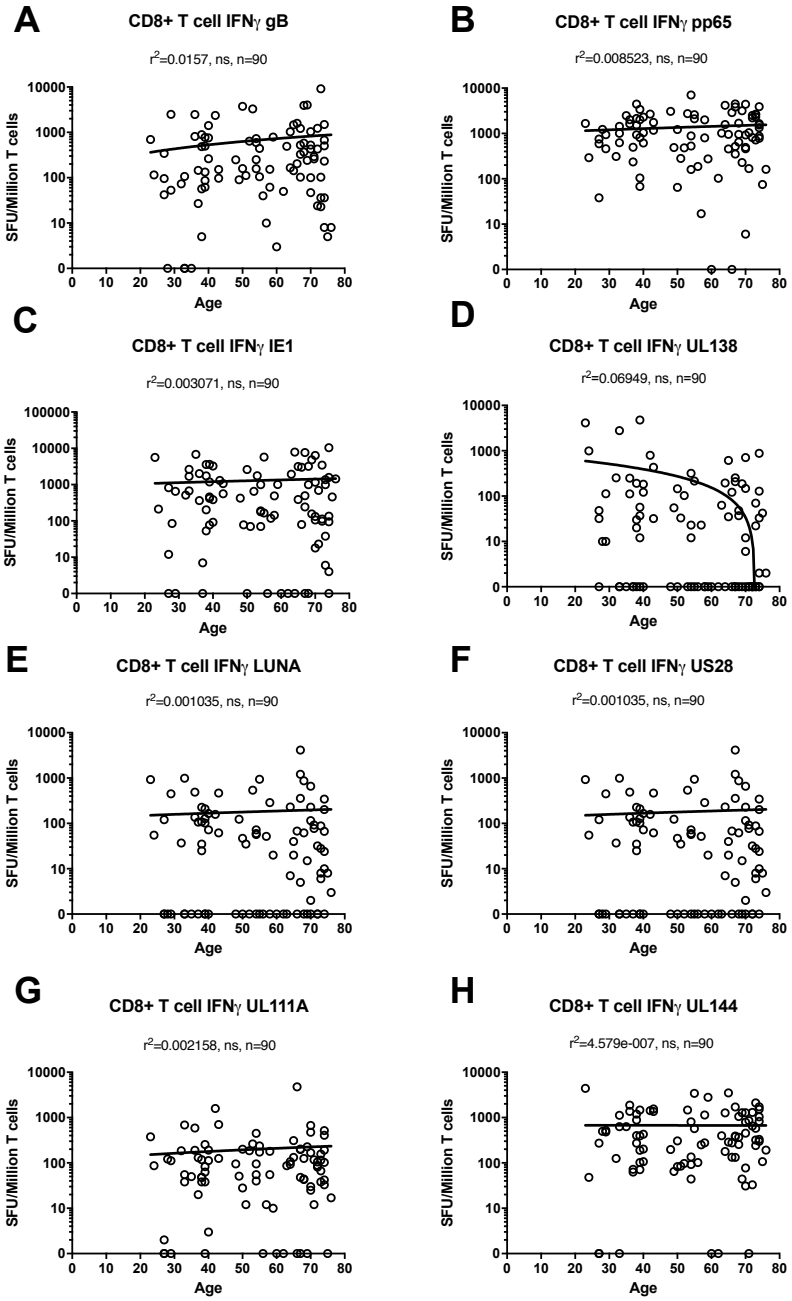



**Fig. 4.10 Analysis of the magnitudes of CD8+ T cell IFN$\gamma$ responses to 8 HCMV ORF peptide pools correlated with donor age.** CD4+ depleted PBMC from 90 seropositive donors were stimulated with HCMV ORF peptide pools for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates and the frequencies of CD8+ T cells secreting IFN$\gamma$ within each seropositive donor enumerated and converted to Spot Forming Units (SFU)/$10^6$ cells. The SFU/$10^6$ cells of IFN$\gamma$ secreting CD8+ T cells is shown against donor age for responses to (A) gB, (B) pp65, (C) IE1, (D) UL138, (E) LUNA, (F) US28, (G) UL111A, and (H) UL144. The correlation (Spearman $r_s$ values and p values shown on each graph), and line of best fit are shown.

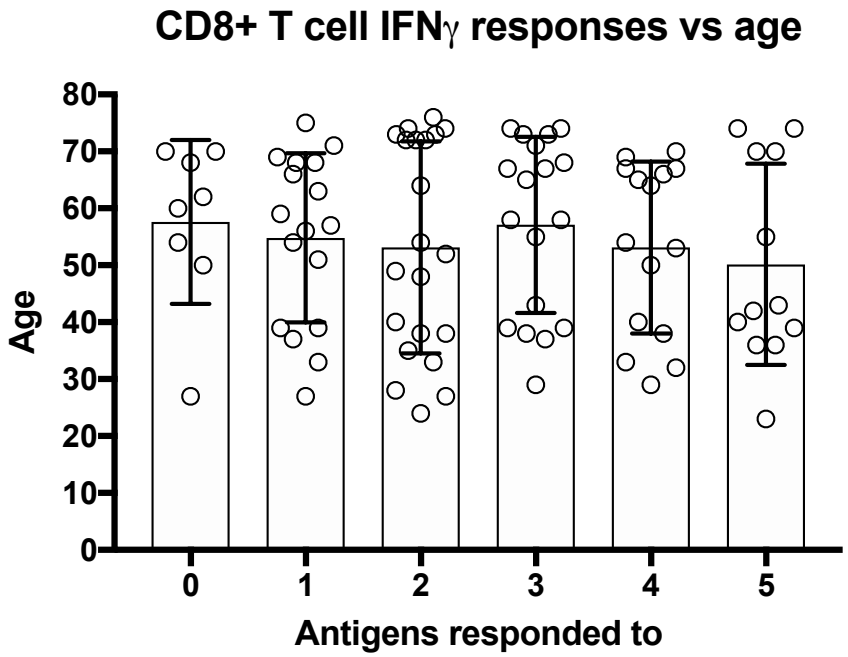



**Fig. 4.11 The number of latency-associated HCMV ORF peptide pools that CD8+ T cells respond to with IFN$\gamma$ secretion across donor age.** CD4+ depleted PBMC were stimulated with HCMV ORF peptide pools for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates and the frequencies of CD8+ T cells secreting IFN$\gamma$ enumerated and converted to Spot Forming Units (SFU)/$10^6$ cells. The number of latency-associated ORFs out of the five tested (UL138, LUNA, US28, UL111A, and UL144) that each donor responds to (considered a positive response if above the threshold 100 SFU/$10^6$ cells) is shown against the age of donor. There were no significant differences between the number of ORFs responded to by CD8+ T cells and the ages of donors according to Kruskal-Wallis multiple comparisons test.

### 4.2.4 CD8+ T cells can secrete the immunosuppressive cytokine IL-10 in response to HCMV latency-associated ORFs

CD8+ T cells secreting IL-10 in response to latency-associated HCMV ORFs have not been reported previously. IL-10 has several immunosuppressive effects and could therefore be an important factor contributing to the evasion of host immune responses. As the FluoroSpot plates capture both IFN$\gamma$ and IL-10 secretion simultaneously, IL-10 secretion from CD8+ T cells following HCMV ORF peptide pool stimulation was also assessed from the large donor cohort. As previously, PBMC from 45 seropositive donors were depleted of CD4+ cells and stimulated with overlapping peptide pools spanning HCMV ORFs for 48 hours then analysed for IL-10 secretion by FluoroSpot assay. CD8+ T cell responses to gB, pp65, IE1, UL138, LUNA, US28, UL111A, and UL144 were tested. Unexpectedly, the use of this highly sensitive FluoroSpot assay has revealed previously uncharacterised IL-10 responses to HCMV, as CD8+ T cells were found to respond with IL-10 secretion to all latency-associated ORFs tested (Figure 4.12). As with the detected CD4+ IL-10 T cell responses (Figure 3.16), the frequencies of IL-10 secreting CD8+ T cells varied in response to different ORFs (Figure 4.12).

Applying the selection criteria for positive IL-10 responses resulted in the inclusion of data from only 45 donors. Responses were excluded if the background frequency of IL-10 positive spots in the unstimulated wells was greater than 1000 or the difference between the unstimulated samples and the positive control samples was less than 50 spots. Using these selection criteria, 56 donors were excluded. Of these donors, responses to the lytically-expressed proteins were typically the least frequent, as they were only detected in 11 donors in response to gB (24.4%) and 9 donors in response to IE1 (20%). However, similar to CD4+ T cell IL-10 responses (Figure 3.16), CD8+ T cell IL-10 responses were found quite frequently to pp65, in 21 of 45 donors (46.7%) (Figure 4.12). CD8+ T cell IL-10 responses to the latency-associated proteins tested were most frequently found in response to US28 (30 donors, 66.7%), followed by LUNA (21 donors, 46.7%), and UL138 (20 donors, 44.4%), with the least frequent responses detected against UL144 (16 donors, 35.6%), and UL111A (14 donors, 31.1%) (Figure 4.12).

To ensure the CD8+ T cell IL-10 responses to latency-associated ORFs detected by FluoroSpot were reproducible, ICS was also carried out as previously. PBMC from 16 seropositive donors were stimulated with overlapping HCMV ORF peptide pools for 18 hours before staining for IL-10. CD8+ T cells capable of producing IL-10 were detected consistently by ICS. CD8+ T cells producing IL-10 were detected in a number of donors

(Figure 4.13), although these were generally less frequent and were a lower proportion of total CD8+ T cells compared to those secreting IFN$\gamma$ (Figure 4.4). These data are in agreement with the data obtained from the FluoroSpot assay, where IL-10 responding CD8+ T cells were not found as often, and were of lower frequency than those secreting IFN$\gamma$. The ability to detect IL-10 secretion from CD8+ T cells by ICS therefore validates the data obtained by FluoroSpot, and confirms that IL-10 secretion was CD8+ T cell specific and not other PBMC subsets.

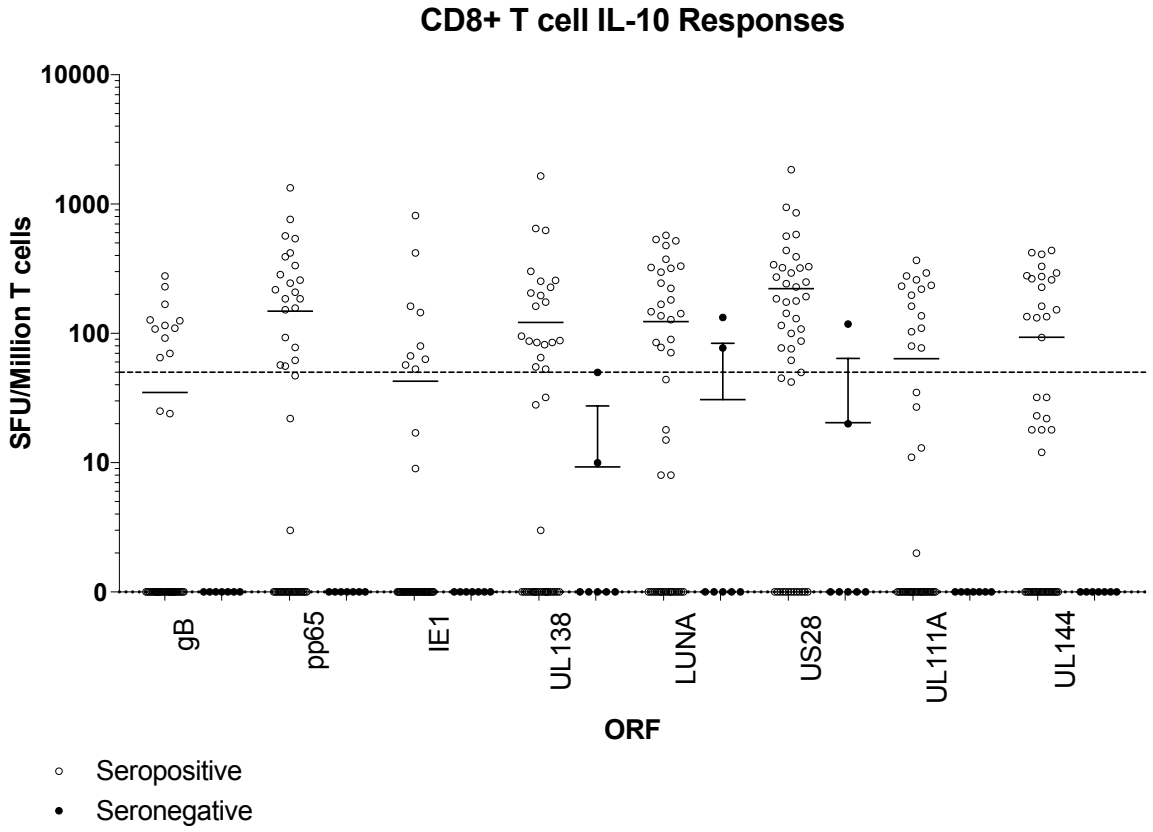



**Fig. 4.12 The frequency of CD8+ T cells from HCMV seropositive and seronegative donors secreting IL-10 in response to HCMV ORF pools.** PBMC from 45 HCMV seropositive and 6 seronegative donors were depleted of CD4+ T cells and stimulated with HCMV ORF peptide pools spanning gB, pp65, IE1, UL138, LUNA, US28, UL111A, and UL144 for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. Each peptide pool was tested in triplicate and the values from the number of background cells secreting cytokines in the unstimulated wells was deducted from all test wells. Following stimulation, the number of IL-10 positive spots was enumerated by the automated spot counter and converted to a spot forming unit (SFU) value per million cells. The dotted line at 50 SFU/$10^6$ cells indicates the threshold for positive responses. Seropositive donors are shown with open circles (○) while seronegative donors are illustrated with filled black circles (●).

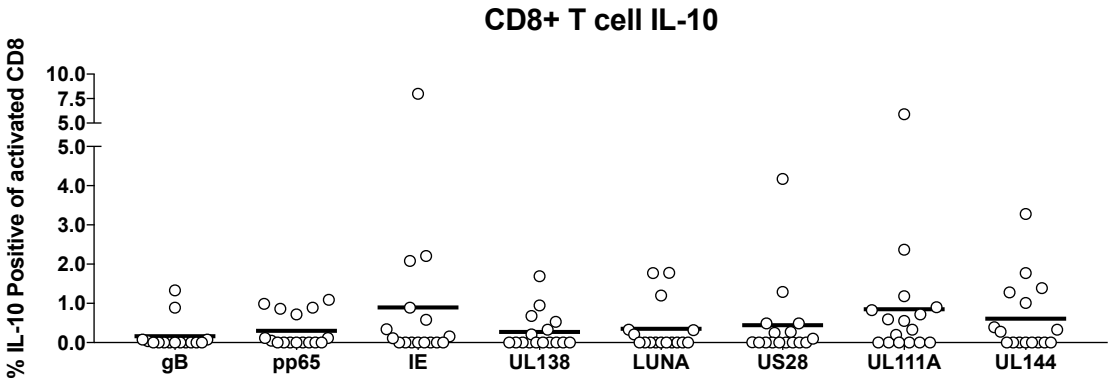



**Fig. 4.13 Intracellular cytokine staining of CD8+ T cells for IL-10 following HCMV ORF peptide pool stimulation.** Whole PBMC isolated from 16 seropositive donors were stimulated overnight with HCMV peptide pools spanning the ORFs of gB, pp65, IE1, UL138, LUNA, US28, UL111A, and UL144, in the presence of monensin. Stimulated PBMC were then surface stained for CD3, CD4, and CD8, and intracellularly stained for IL-10. T cells were identified after gating for live cell, CD3+ cells, CD8+ cells, and ORF-specific T cells by expression of 4-1BB and/or CD69.

### 4.2.5 The frequency of IL-10 secreting CD8+ T cells detected fluctuates over time

A longitudinal analysis to assess the stability of CD8+ T cell IL-10 responses to HCMV ORFs was carried out in four seropositive donors. Donor CMV324 was sampled at seven time points over three years. PBMC depleted of CD4+ T cells were stimulated with overlapping peptide pools spanning the ORFs of gB, pp65, IE1, UL138, LUNA, US28, UL111A, and UL144 and analysed for IL-10 secretion by FluoroSpot assay. There was very little consistency in the positive CD4+ T cells responses over time to certain ORFs, for example clear positive responses to gB, pp65, and US28 were present at the same three of the seven sampled time points, but were absent at the other four time points (Figure 4.14). IL-10 responses to UL138, LUNA, UL111A, and UL144 were consistently negative, although there was one time point (10.5.17), with positive responses to these ORFs. An additional response was also observed to UL138, although this was absent for the further five time points. (Figure 4.14). This time point was also one that was positive for responses to gB, pp65, and US28 (Figure 4.14).

The fluctuations in CD8+ T cell IL-10 responses can be further illustrated by the changes in the number of positive responses (those over 50 SFU/$10^6$) across the seven sample time points. Taking into account responses to all 8 ORFs, the number of positive responses varied from one to seven (Figure 4.15). Although there were positive responses to two of the three lytic ORFs at only three of the seven time points, there was at least one positive response to the latency-associated ORFs at every time point, although these varied from one to all five (Figure 4.15).

Donor CMV319 was sampled four times over a two and half year period. There was no consistent detection of ORF-specific responses over the four sampling points to any ORF (Figure 4.16). Of the responses detected at two or more time points, there were often large differences in the frequencies of IL-10 secreting CD8+ T cells detected between sampling times. This was especially true of pp65, UL138, and US28 responses (Figure 4.16). There was also a high frequency gB response that was detected on only one of the four time points (Figure 4.16).

Two donors were tested at two time points for their CD8+ T cell IL-10 responses. Differences in the ORF-specific responses and the frequencies of IL-10 secreting cells were detected between the two time points. For donor CMV323, clear responses to LUNA and UL138 were detected on both occasions, while clear responses to IE1, US28, and UL111A were only detected on one occasion (Figure 4.17). Large differences in the frequencies of IL-10 positive cells were also observed from the first to the second time point in this donor

(Figure 4.17). Likewise donor CMV307 exhibited extensive variation in the frequencies of IL-10 positive CD8+ T cells responding to gB, pp65, and US28 between the two sampling points (Figure 4.18).

HCMV ORF-specific CD8+ T cell responses were therefore observed to vary considerably over time in four donors. This variation in the frequencies of IL-10 secreting CD8+ T cells between time points often resulted in fluctuations above or below the positive response threshold for ORF-specific responses.

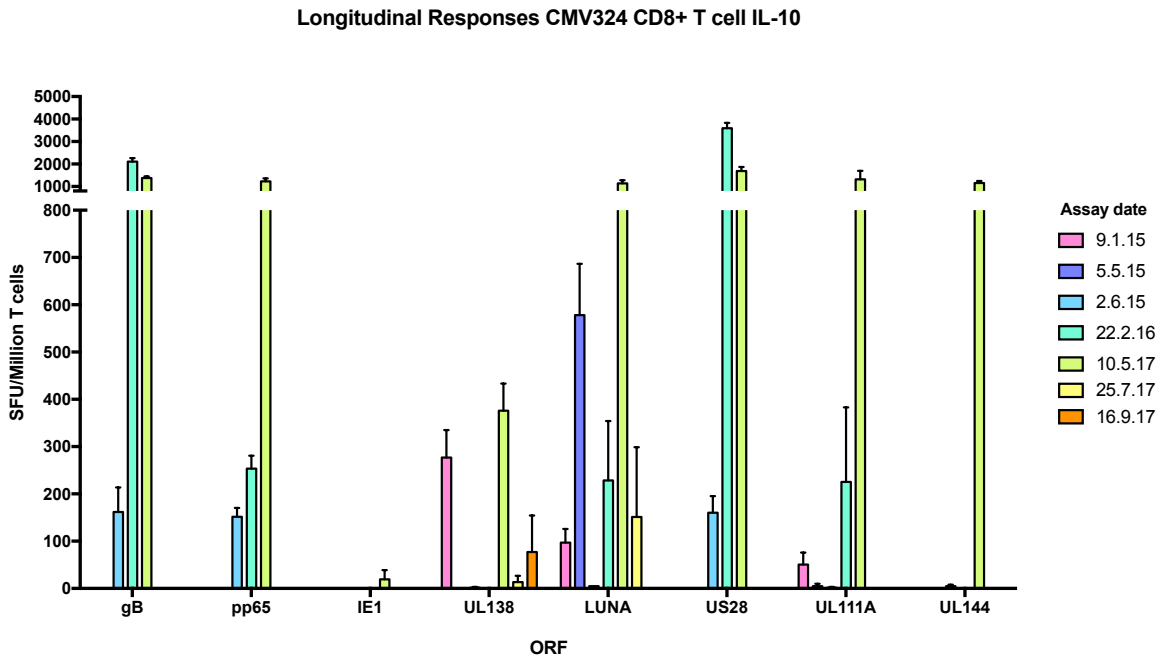
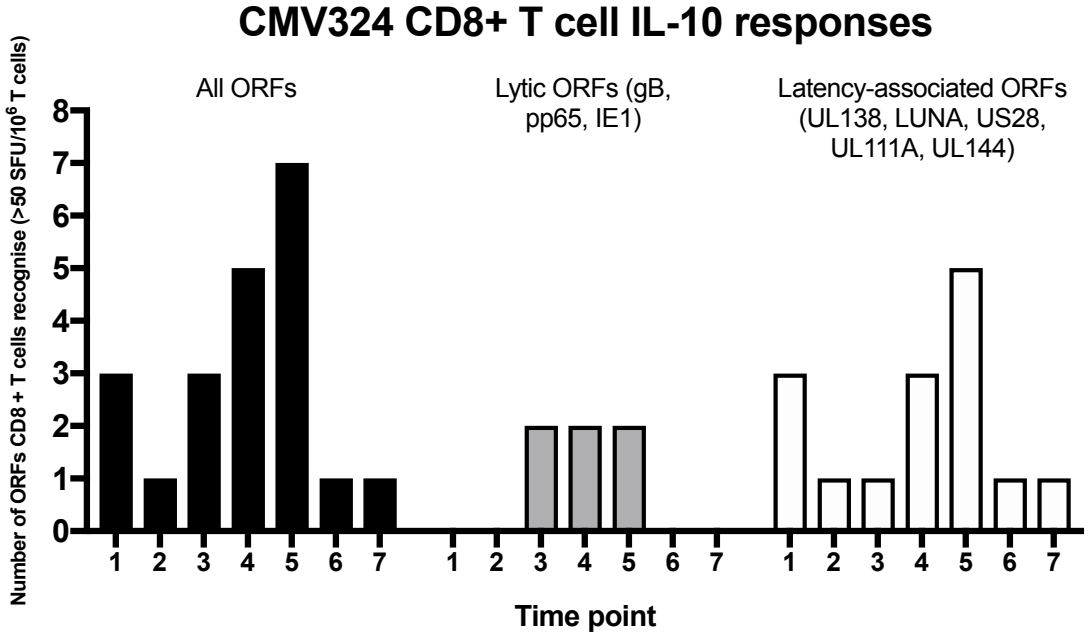



**Fig. 4.14 Longitudinal CD8+ T cell IL-10 responses to HCMV ORFs from donor CMV324.** CD8+ T cell responses from one donor were tested at seven separate time points over three years. PBMC were depleted of CD4+ expressing cells and stimulated with overlapping peptide pools of HCMV peptides spanning gB, p65, IE1, UL138, LUNA, US28, UL111A, and UL144, for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. IL-10 secreting CD8+ T cells were then stained for, counted, and converted to spot forming units (SFU) per million cells. All samples were run in triplicate and the frequencies of positive cells was determined by subtracting values in all test wells from the background number of positive cells in unstimulated well. Each different colour of bar indicates a different sampling time point.




All ORFs
Lytic ORFs
Latency-associated ORFs

| Time point | Date |
|---|---|
| 1 | 9-Jan-2015 |
| 2 | 5-May-2015 |
| 3 | 2-Jun-2015 |
| 4 | 22-Feb-2016 |
| 5 | 10-May-2017 |
| 6 | 25-Jul-2017 |
| 7 | 16-Sep-2017 |

**Fig. 4.15 Longitudinal analysis of the number of positive CD8+ T cell responses in donor CMV324.** Positive IL-10 CD8+ T cell responses (responses over 50 SFU/$10^6$ cells) are shown at 7 time points over three years from one donor. The dates of each time point are shown in the table. PBMC were depleted of CD4+ expressing cells and stimulated with overlapping peptide pools of HCMV peptides spanning gB, p65, IE1, UL138, LUNA, US28, UL111A, and UL144, for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. The number of IL-10 positive CD8+ T cells was determined and converted to SFU/$10^6$ cells. All samples were run in triplicate and the frequencies of positive cells was determined by subtracting values in all test wells from the background number of positive cells in unstimulated well. The black bars indicate responses to all 8 ORFs, both lytic and latency-associated, the grey to lytic antigens gB, pp65, and IE, and the white to latency-associated antigens UL138, LUNA, US28, UL111A, and UL144.

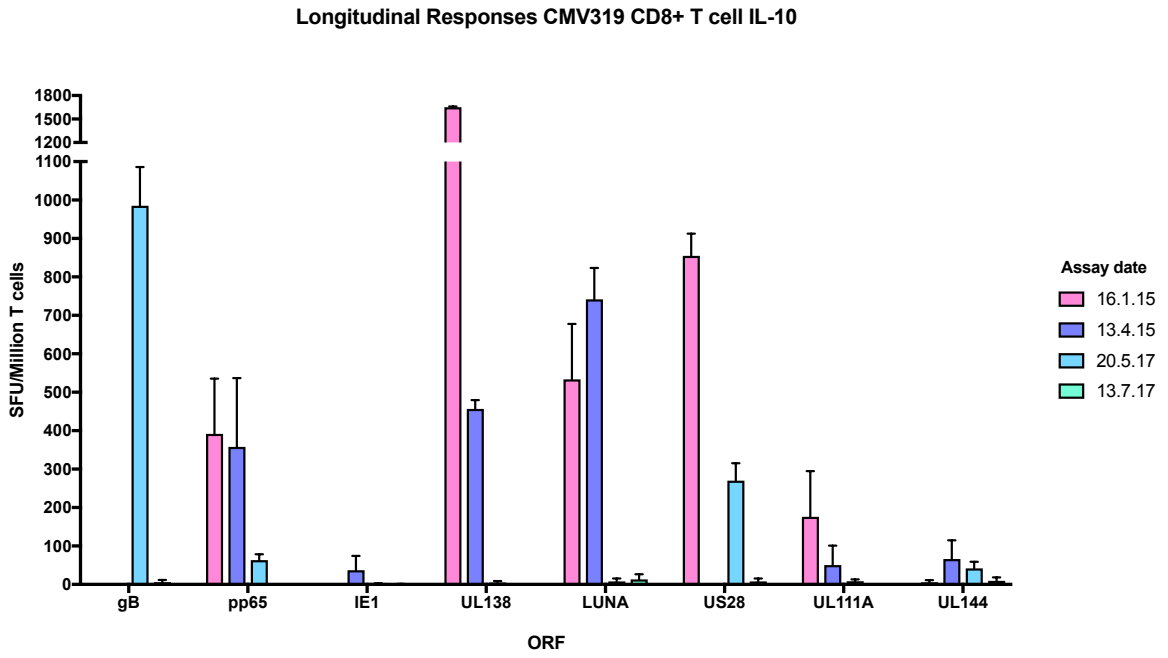



**Fig. 4.16 Longitudinal CD8+ T cell IL-10 responses to HCMV ORFs from donor CMV319.** CD8+ T cell responses from one donor were tested at four separate time points over 30 months. PBMC were depleted of CD4+ expressing cells and stimulated with overlapping peptide pools of HCMV peptides spanning gB, p65, IE1, UL138, LUNA, US28, UL111A, and UL144, for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. IL-10 secreting CD8+ T cells were then stained for, counted, and converted to spot forming units (SFU) per million cells. All samples were run in triplicate and the frequencies of positive cells was determined by subtracting values in all test wells from the background number of positive cells in unstimulated well. Each different colour of bar indicates a different sampling time point.

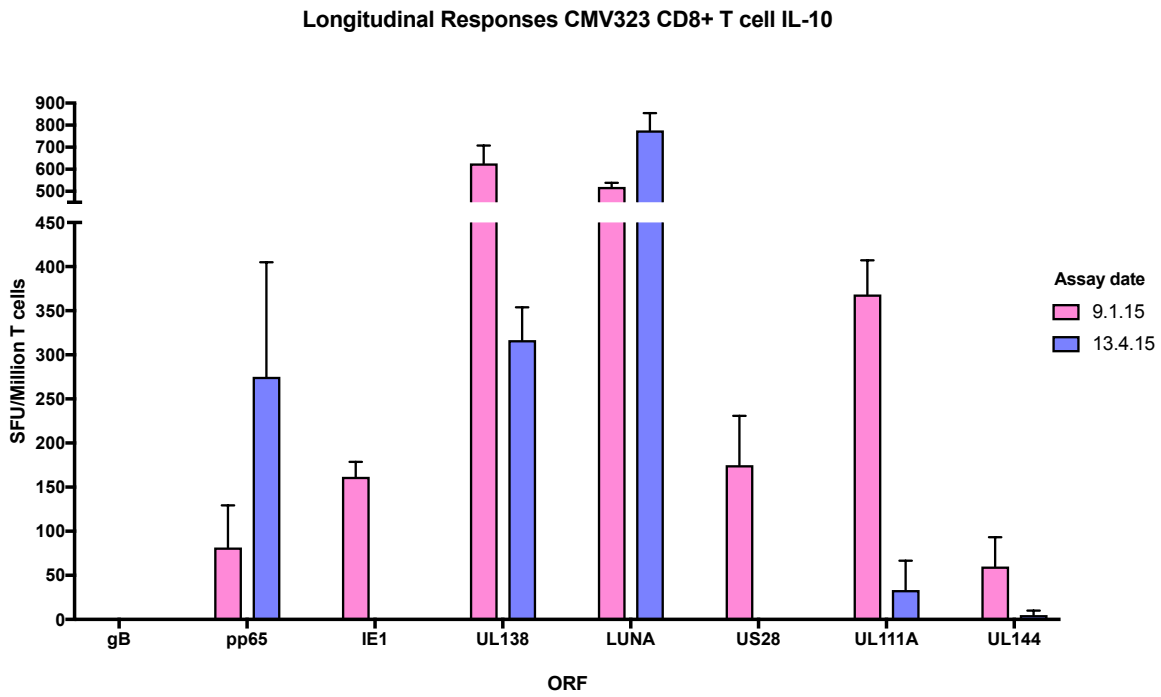



**Fig. 4.17 Longitudinal CD8+ T cell IL-10 responses to HCMV ORFs from donor CMV323.** CD8+ T cell responses from one donor were tested at two separate time points. PBMC were depleted of CD4+ expressing cells and stimulated with overlapping peptide pools of HCMV peptides spanning gB, p65, IE1, UL138, LUNA, US28, UL111A, and UL144, for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. IL-10 secreting CD8+ T cells were then stained for, counted, and converted to spot forming units (SFU) per million cells. All samples were run in triplicate and the frequencies of positive cells was determined by subtracting values in all test wells from the background number of positive cells in unstimulated well. Each different colour of bar indicates a different sampling time point.

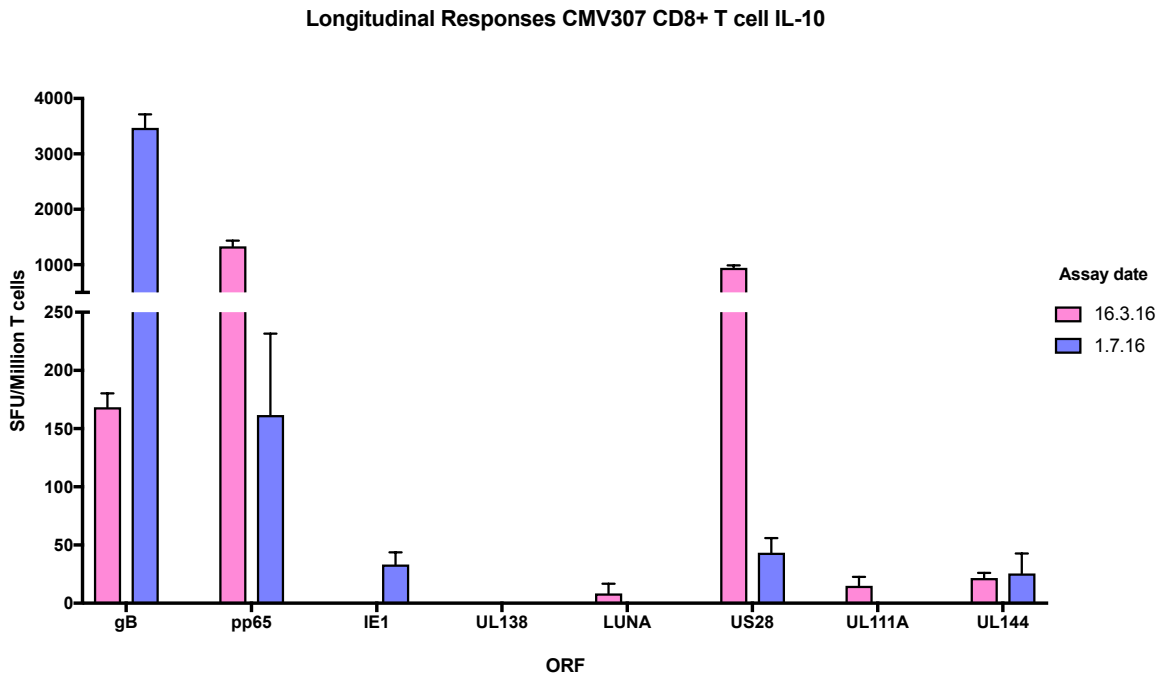



**Fig. 4.18 Longitudinal CD8+ T cell IL-10 responses to HCMV ORFs from donor CMV307.** CD8+ T cell responses from one donor were tested at two separate time points. PBMC were depleted of CD4+ expressing cells and stimulated with overlapping peptide pools of HCMV peptides spanning gB, p65, IE1, UL138, LUNA, US28, UL111A, and UL144, for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. IL-10 secreting CD8+ T cells were then stained for, counted, and converted to spot forming units (SFU) per million cells. All samples were run in triplicate and the frequencies of positive cells was determined by subtracting values in all test wells from the background number of positive cells in unstimulated well. Each different colour of bar indicates a different sampling time point.

### 4.2.6 The frequency of latency-specific IL-10 secreting CD8+ T cells does not change with age

The frequencies of detected IL-10 secreting CD8+ T cells could change with increasing donor age given an assumed longer time of viral carriage. CD8+ T cells secreting IL-10 were therefore examined across a large donor cohort in relation to the age of donor. CD4+ depleted PBMC from 45 HCMV seropositive donors ranging in age from 23 to 74 were stimulated with overlapping peptide pools spanning the ORFs of gB, pp65, IE1, UL138, LUNA, US28, UL111A, and UL144 and analysed for IL-10 secretion by FluoroSpot assay. CD8+ T cells secreting IL-10 were found across all ages tested and were of varying magnitudes. There was no significant correlation between the frequency of IL-10 secreting CD8+ T cells and the age of donors, according to Spearman rank correlation analysis, in response to any of the HCMV ORFs tested. However, grouping donors by sex did reveal a significant negative correlation in males between the frequency of IL-10 secreting CD8+ T cells and increasing donor age in response to pp65 (Spearman $r_s$ = -0.5288, $P$ = 0.0055) (Figure A.4B)

The number of latency-associated ORFs each donor had a positive IL-10 response to (those greater than 50 SFU/$10^6$) was then examined in relation to the age of donors. It could be expected that older donors, assuming they have been infected for longer periods of time, might have generated responses to a greater number of ORFs than younger donors. However, there was no significant difference in the number of ORFs responded to across donor ages following a Kruskal-Wallis multiple comparisons test, although it is possible that with more donors these results could change (Figure 4.20).

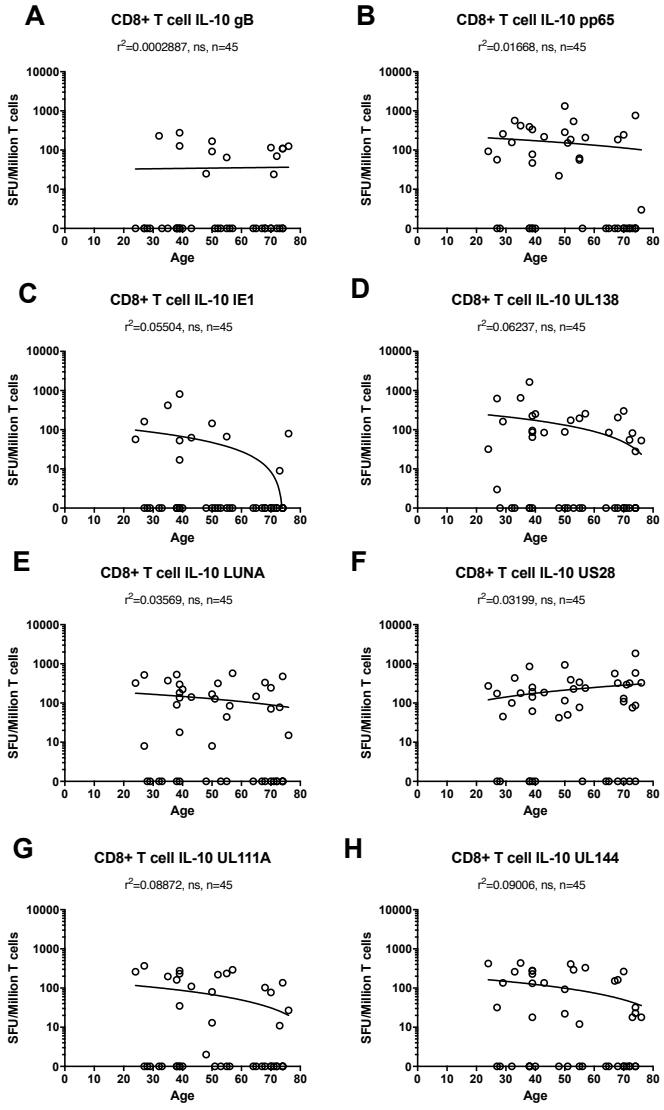



**Fig. 4.19 Analysis of the magnitudes of CD8+ T cell IL-10 responses to 8 HCMV ORF peptide pools correlated with donor age.** CD4+ depleted PBMC from 45 seropositive donors were stimulated with HCMV ORF peptide pools for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates and the frequencies of CD8+ T cells secreting IFN$\gamma$ within each seropositive donor enumerated and converted to Spot Forming Units (SFU)/$10^6$ cells. The SFU/$10^6$ cells of IFN$\gamma$ secreting CD8+ T cells is shown against donor age for responses to (A) gB, (B) pp65, (C) IE1, (D) UL138, (E) LUNA, (F) US28, (G) UL111A, and (H) UL144. The correlation (Spearman $r_s$ values and p values shown on each graph), and line of best fit are shown.

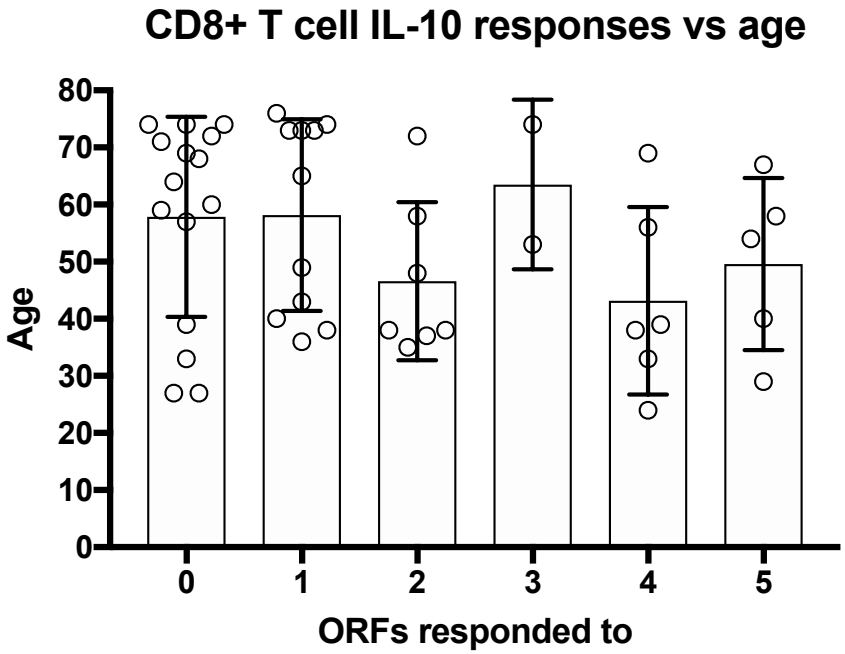



**Fig. 4.20 The number of latency-associated HCMV ORF peptide pools that CD8+ T cells respond to with IL-10 secretion across donor age.** CD4+ depleted PBMC were stimulated with HCMV ORF peptide pools for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates and the frequencies of CD8+ T cells secreting IL-10 enumerated and converted to Spot Forming Units (SFU)/$10^6$ cells. The number of latency-associated ORFs out of the five tested (UL138, LUNA, US28, UL111A, and UL144) that each donor responds to (considered a positive response if above the threshold 50 SFU/$10^6$ cells) is shown against the age of donor. There were no significant differences between the number of ORFs responded to by CD8+ T cells and the ages of donors according to Kruskal-Wallis multiple comparisons test.

### 4.2.7 The secretion of IL-10 and IFN$\gamma$ is from distinct CD8+ T cell populations

CD8+ T cells from HCMV seropositive donors can secrete IFN$\gamma$ and IL-10 in response to HCMV ORFs, but whether these were two distinct cellular populations, as was shown with CD4+ T cells, remained to be determined. By utilising the ability of the FluoroSpot assay to detect both IFN$\gamma$ and IL-10 secretion from HCMV specific cells, the frequencies of such dual cytokine producing CD8+ T cells responding to HCMV ORFs were investigated. Additionally, this enabled the distribution between IFN$\gamma$ and IL-10 secreting CD8+ T cells within individuals to be determined. Of the total responding CD8+ T cells within donors, there were only low proportions of cells that secreted IL-10 in response to the lytically-expressed proteins gB, pp65, and IE1, with the majority of donors possessing a dominant IFN$\gamma$ response to these ORFs (Figure 4.21A, B, and C). However, CD8+ T cell responses to the latency-associated ORFs were composed of higher proportions of IL-10 secreting cells (Figure 4.21 D, E, F, G), with the exception of responses to UL144 which were composed predominantly of IFN$\gamma$ secreting cells (Figure 4.21H).

To quantify these differences in the distribution of cytokine responses within individuals, the dominant cytokine response to each ORF (where over 55% of all cytokine secreting CD8+ T cells secrete IFN$\gamma$ or IL-10) was calculated for every donor. The proportions of donors with an IFN$\gamma$ or IL-10 dominant response was then plotted for responses to each ORF (Figure 4.22). Using this analysis, a clear hierarchy in dominant cytokine responses to the different ORFs was revealed. IFN$\gamma$ dominant CD8+ T cell responses were most common to the lytically-expressed proteins, where over 84% of responding donors had a dominant CD8+ T cell IFN$\gamma$ response to gB (84.2%), pp65 (88.1%), or IE1 (87%) (Figure 4.22). Responses to latency-associated proteins were more variable and highlighted differences in dominant responses to different ORFs. The responses to UL144 for example were similar to those against the lytically-expressed proteins, where IFN$\gamma$ dominant responses were very prevalent, and observed in 86% of all donors (Figure 4.22). Compared to UL144, no other latency-associated ORF elicited such a high percentage of donors with a dominant IFN$\gamma$ response. The proportion of donors with a dominant IFN$\gamma$ response to US28 and UL111A was considerably lower, with 67% and 57% of donors respectively (Figure 4.22). Donors responding to UL138 and LUNA however were much more likely to have a dominant IL-10 CD8+ T cell response, where the proportions of donors with an IFN$\gamma$ dominant response were 36% and 27% respectively, the lowest of all responses to latency-associated ORFs (Figure 4.22). This is also in agreement with the data obtained from CD4+ T cell responses (Figure 3.22), where UL138- and LUNA-specific responses were the most IL-10 dominant.

Proportionally, of all the CD8+ T cells that secreted IFN$\gamma$ or IL-10, there were very few that secreted both of these cytokines, and this was the case for responses to all the ORFs tested (Figure 4.21). In the small number of individuals that did have dual responding CD8+ T cells, these cells were a very small proportion of the total CD8+ T cell response (Figure 4.21). Similar to the CD4+ T cell responses, the CD8+ T cells that secreted IFN$\gamma$ and those that secreted IL-10 therefore appear to be separate cellular populations.

Long-term carriage of HCMV is associated with changes in the CD8+ T cell compartment, a number of which are linked to immune dysfunction [340, 348, 350, 656]. It might therefore be expected that long-term infection with HCMV could result in a shift in the balance between IFN$\gamma$ and IL-10 secreting CD8+ T cells, with either a decrease in IFN$\gamma$ secreting cells occurring concurrently with an increase in IL-10 secreting cells, or either of these occurring in isolation. Age can provide a surrogate measure for the duration of HCMV carriage. To determine if there was a shift towards an IL-10 CD8+ T cell responses with age, the balance between dominant IL-10 and IFN$\gamma$ ORF-specific CD8+ T cell responses were stratified by age group. Each donor's dominant cytokine response, where over 50% of all responding CD8+ T cells secreted IFN$\gamma$ or IL-10, was grouped by age as young (18-39), middle (40-64), and old (64+). The proportions of IL-10 dominant CD8+ T cell responses detected in response to UL138 (Figure 4.23A) and US28 (Figure 4.23C) showed small increasing shifts with age, while responses to UL111A (Figure 4.23D) and UL144 (Figure 4.23E) showed the reverse, with small shifts towards IFN$\gamma$ bias with age. CD8+ T cell responses to LUNA showed a decreasing trend with increasing age. The proportions of donors with an IL-10 dominant CD8+ T cell responses decreased from 50% in the young age group, to 80% in the middle age group, to 90% in the old age group (Figure 4.23B). Therefore, taking into account CD8+ T cell responses to all latency-associated proteins, large shifts in dominant cytokine responses do not seem to occur with increasing age.

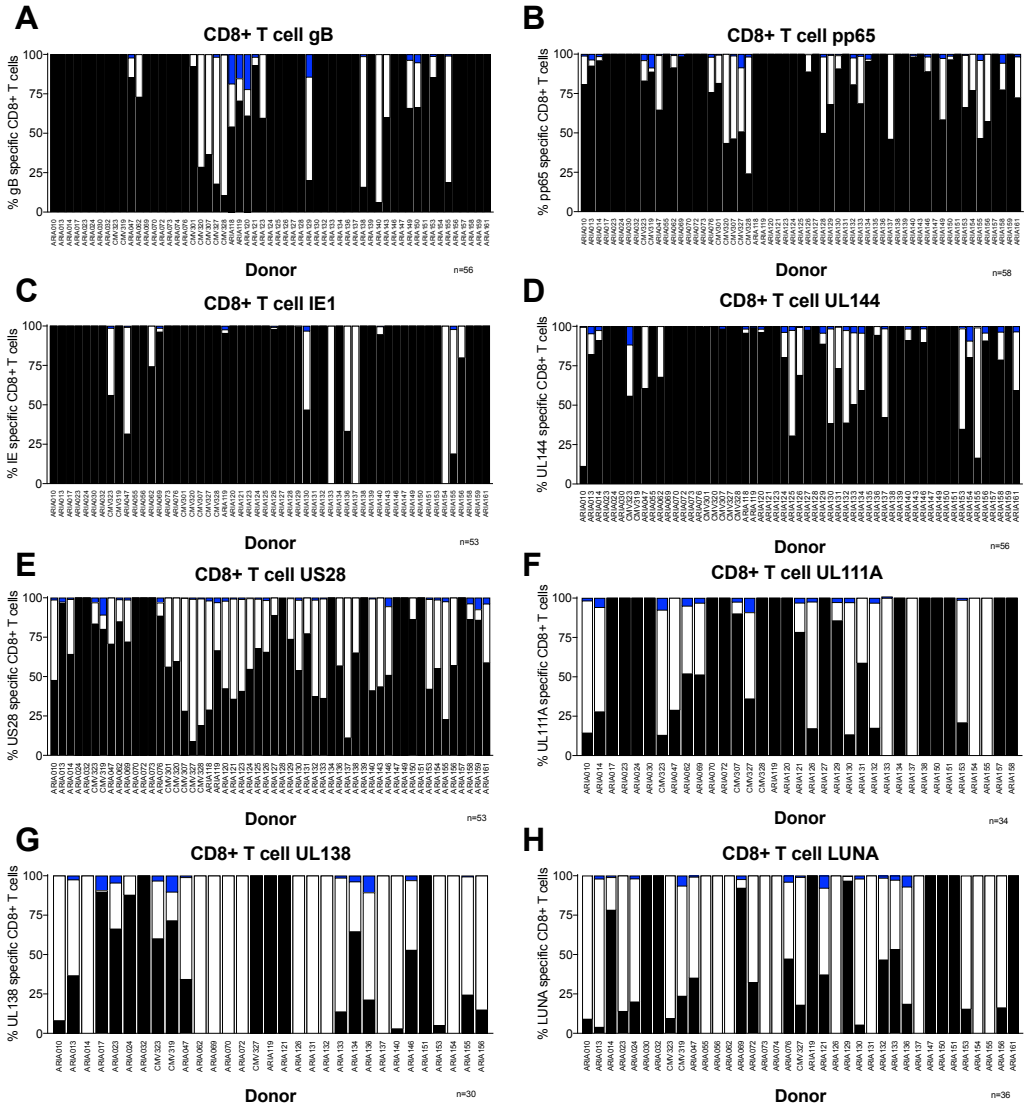



**Fig. 4.21 Proportions of CD8+ T cells secreting IL-10 and IFN$\gamma$ in response to latent HCMV antigens from individual seropositive donors.** PBMC from seropositive donors were depleted of CD4+ cells and stimulated with overlapping peptide pools from HCMV ORFs (A) gB, (B) pp65, (C) IE, (D) UL144, (E) US28, (F) UL111A, (G) UL138, and (H) LUNA, for 48 hours. Following stimulation, IFN$\gamma$ (black bars) and IL-10 (white bars) secretion was detected by FluoroSpot and enumerated to spot forming units (SFU), where each cytokine response was calculated as a proportion of the total number of responding CD8+ T cells. The proportion of CD8+ T cells of total cytokine secreting cells that secreted both IFN$\gamma$ and IL-10 is also shown (blue bars). All samples were run in triplicate with the mean value shown here. Donors without an IL-10 or IFN$\gamma$ responses were omitted from each ORF analysis.

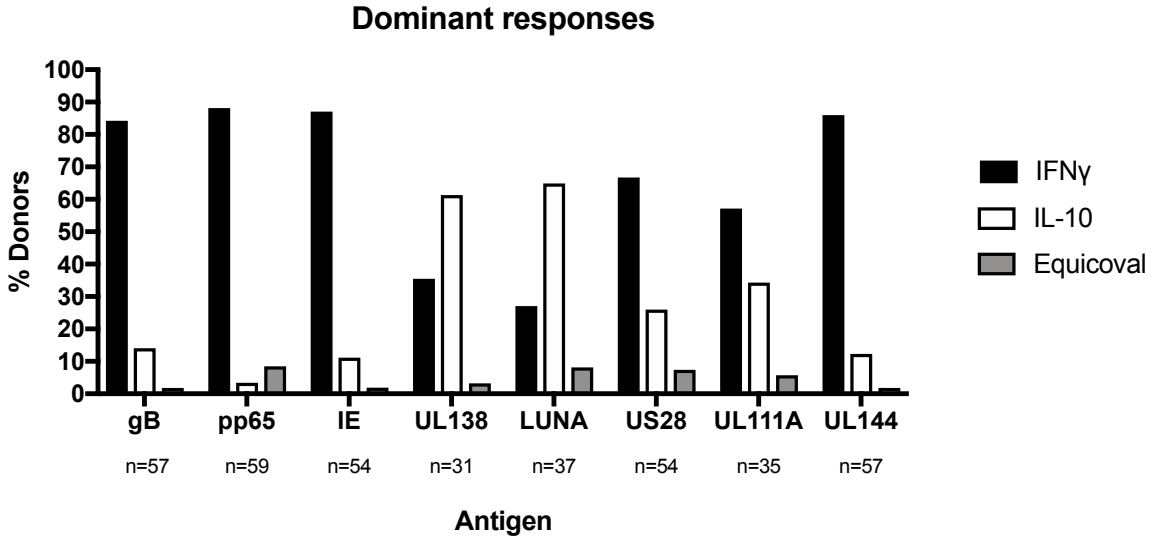



**Fig. 4.22 The distribution of dominant CD8+ T cell IFN$\gamma$ and IL-10 responses to HCMV ORF peptide pools.** PBMC from seropositive donors were depleted of CD4+ cells and stimulated with overlapping peptide pools from HCMV ORFs gB, pp65, IE, UL138, LUNA, US28, UL111A, and UL144 for 48 hours. Following stimulation, IFN$\gamma$ and IL-10 secretion was detected by dual IFN$\gamma$/IL-10 FluoroSpot and enumerated to spot forming units (SFU), which allowed the proportion of each cytokine response to be calculated of the total CD4+ T cell response. Dominant responses are classed as such when over 55% of the cytokine secreting cells secreted (A) IFN$\gamma$ (black bars), or (B) IL-10 (white bars). CD4+ T cell responses where none of the cytokine secreting population was greater than 55% was classed as equivocal (grey bars). Donors without an IL-10 or IFN$\gamma$ responses were omitted from each ORF analysis and the number of donors analysed for each ORF-specific response is shown.

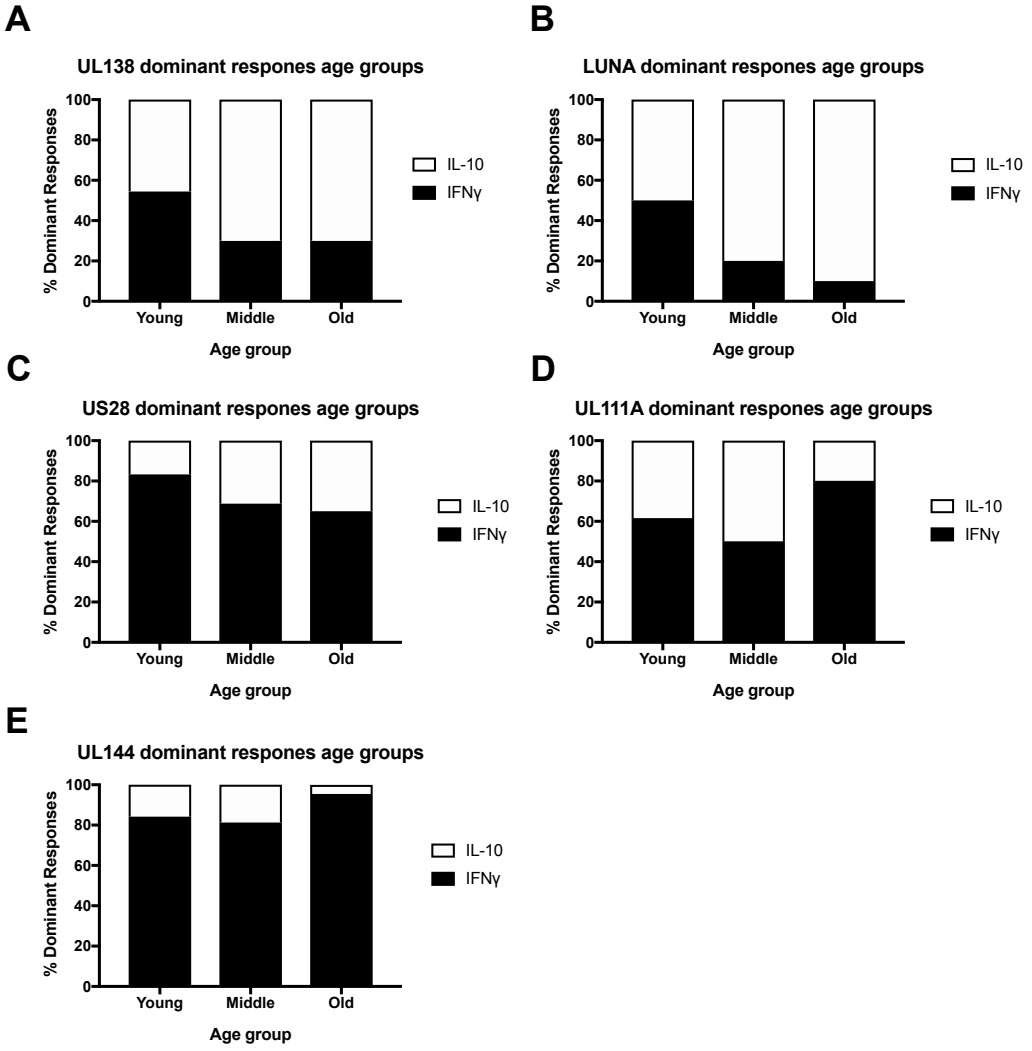



**Fig. 4.23 The distribution of dominant CD8+ T cell IFN$\gamma$ and IL-10 responses to HCMV ORF peptide pools across age groups.** PBMC from seropositive donors were depleted of CD4+ cells and stimulated with overlapping peptide pools from HCMV ORFs for 48 hours. Following stimulation, IFN$\gamma$ and IL-10 secretion was detected by dual IFN$\gamma$/IL-10 FluoroSpot and enumerated to spot forming units (SFU), which allowed the proportion of each cytokine response to be calculated of the total CD4+ T cell response. Dominant responses are classed as such when over 50% of the cytokine secreting cells secreted IFN$\gamma$ (black bars), or IL-10 (white bars). The percentage of donors with dominant IFN$\gamma$ and IL-10 responses to (A) UL138, (B) LUNA, (C) US28, (D) UL111A, and (E) UL144 is shown for three age groups. Donors were separated into Young (18-39), Middle (40-64), and Old (64+) age groups. Donors without an IL-10 or IFN$\gamma$ responses were omitted from each ORF analysis and the number of donors analysed is shown.

### 4.2.8 CD8+ T cell response magnitudes to individual HCMV ORFs vary depending on sex

The frequencies of IFN$\gamma$ and IL-10 secreting CD8+ T cells in response to HCMV ORFs was compared with donor age between males and females, as sex has been shown to be a factor affecting the immune response to virus infection [619, 620]. The frequencies of CD8+ T cells secreting IFN$\gamma$ and IL-10 in response to HCMV ORF peptide pools was grouped according to sex. CD8+ T cells secreting IFN$\gamma$ in response to gB were found to be of higher frequency in male compared to female donors ($P$ = 0.0003, Kruskal-Wallis, Dunn's multiple comparisons) (Figure 4.24A). No other significant differences between male and female donors were found for ORF-specific CD8+ T cell IFN$\gamma$ responses. Examining IL-10 responses however showed CD8+ T cells secreting IL-10 in response to LUNA to be of higher frequency in female donors compared to male donors ($P$ = 0.0376, Kruskal-Wallis, Dunn's multiple comparisons) (Figure 4.24B). No other differences in IL-10 response magnitudes were found.

The total within-donor CD8+ T cell response to HCMV ORFs was then compared between males and females. For each donor, the frequencies of CD8+ T cells that secreted IFN$\gamma$ or IL-10 in response to each HCMV ORF was summed and then compared between males and females. No differences were found in the total IFN$\gamma$ or IL-10 CD8+ T cell responses to all eight HCMV ORFs tested (gB, pp65, IE1, UL138, LUNA, US28, UL111A, and UL144) (Figure 4.25A and B), the lytic ORFs only (gB, pp65, IE1) (Figure 4.25 C and D), or the latency-associated ORFs (UL138, LUNA, US28, UL111A, and UL144) (Figure 4.25 E and F).

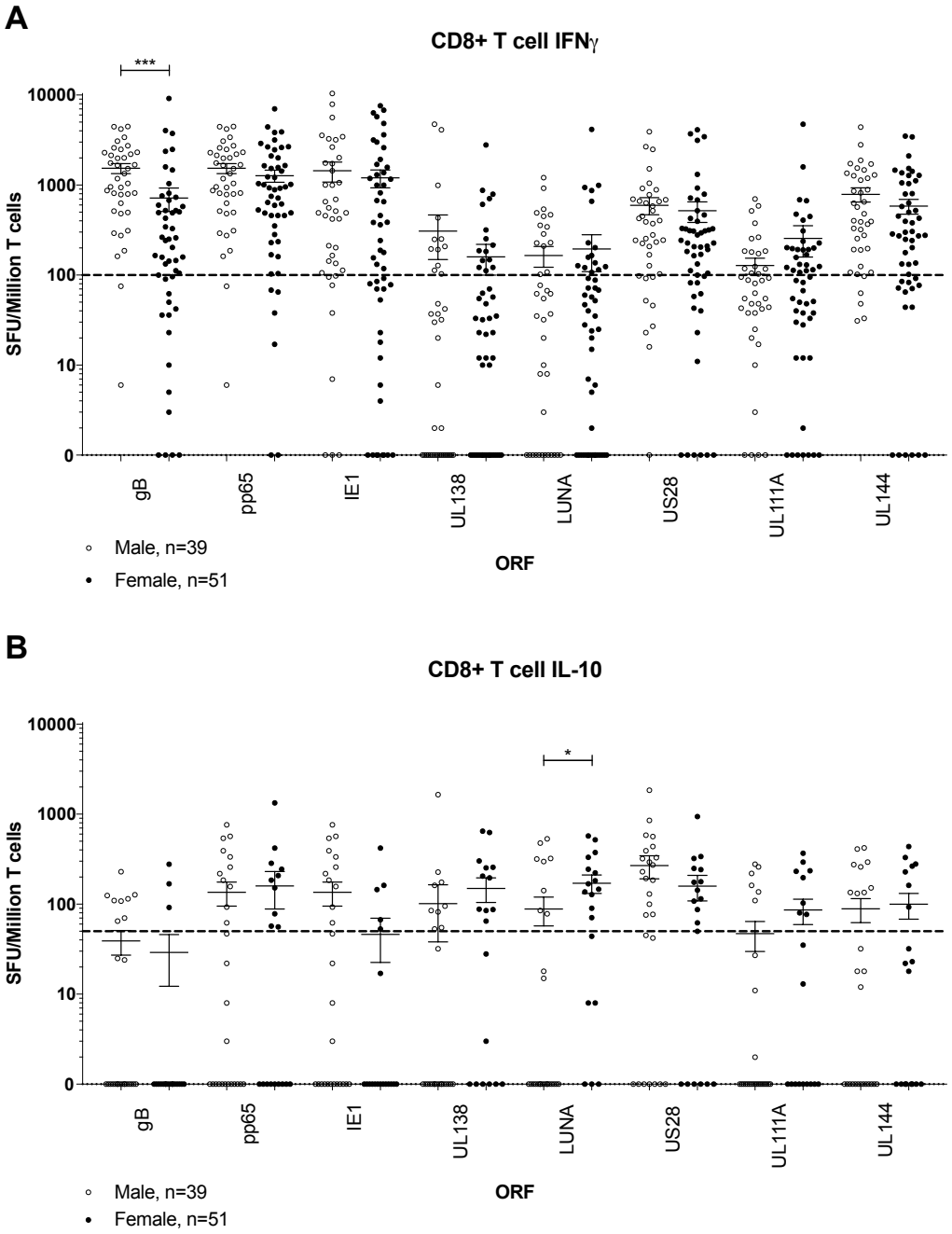



**Fig. 4.24 The frequency of CD8+ T cells secreting IFN$\gamma$ or IL-10 in response to HCMV ORF pools from HCMV seropositive donors grouped by sex.** PBMC depleted of CD4+ T cells were stimulated with overlapping HCMV ORF peptide pools spanning each antigen of interest for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. Each peptide pool was tested in triplicate and the values from all test wells were deducted from the number of background cells secreting cytokines in the unstimulated wells. Following stimulation, the number of cells secreting (A) IFN$\gamma$ from 90 HCMV seropositive donors and (B) the number of cells secreting IL-10 from 45 HCMV seropositive donors was enumerated by the automated spot counter and converted to a spot forming unit (SFU) value per million cells. Male responses are illustrated with open circles (○), and female responses with filled circles (●). Kruskal-Wallis multiple comparisons test was carried out between male and female responses to each ORF and the standard error of the mean shown for each response.

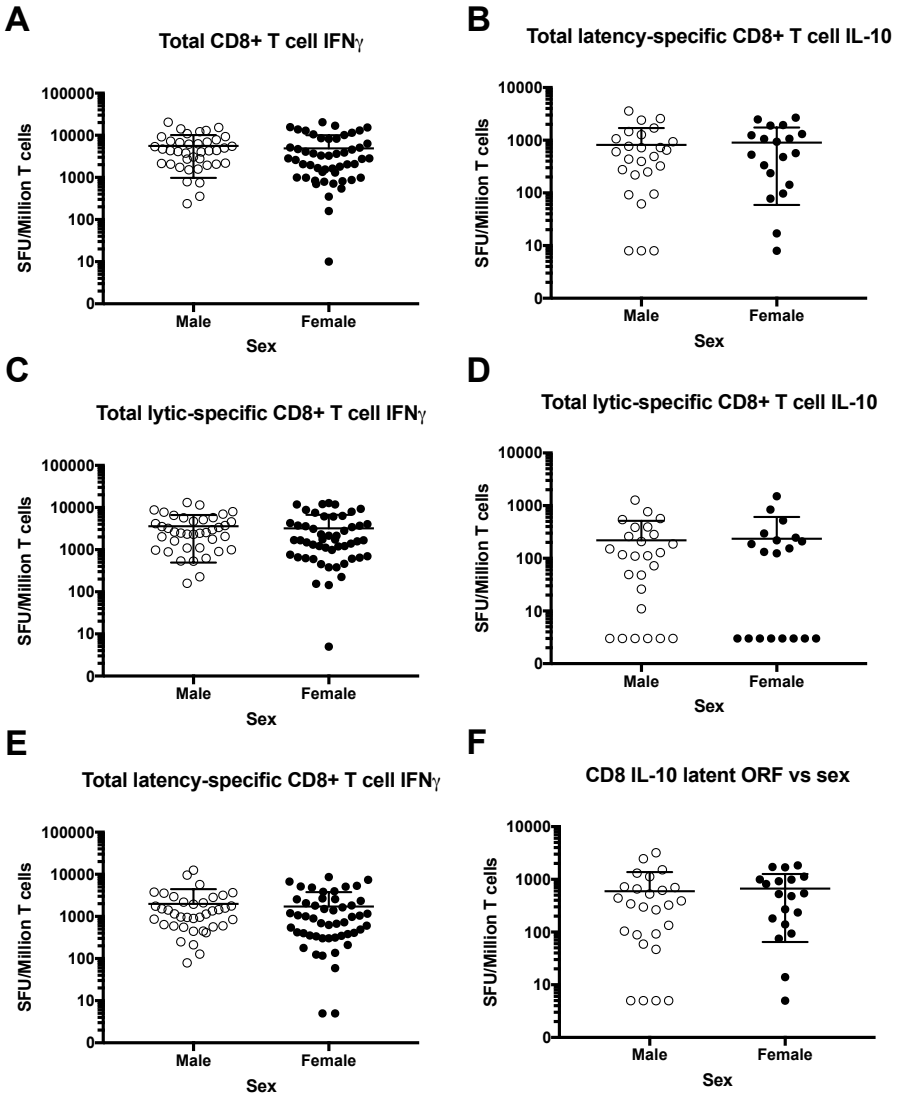



**Fig. 4.25 A comparison of the total frequencies of CD8+ T cells within individual donors that respond to HCMV ORFs when donors are grouped by sex.** The total frequency of CD8+ T cells responding with IFN$\gamma$ or IL-10 secretion within individual donors is shown in male and female donors. PBMC depleted of CD4+ T cells were stimulated with overlapping HCMV ORF peptide pools spanning each HCMV ORF (gB, pp65, IE, UL138, LUNA, US28, UL111A, UL144) for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. Each peptide pool was tested in triplicate and the values from all test wells were deducted from the number of background cells secreting cytokines in the unstimulated wells. Following stimulation, the number of cells secreting IFN$\gamma$ from 90 HCMV seropositive donors and the number secrering IL-10 from 45 HCMV seropositive donors was enumerated by the automated spot counter and converted to a spot forming unit (SFU) value per million cells. Within in each donor (A) the total number of CD8+ T cells secreting IFN$\gamma$ or (B) IL-10 to all ORFs is shown. Additionally, (C) the total number of CD4+ T cells secreting IFN$\gamma$ or (D) IL-10 in response to lytic ORFs only (gB, pp65, IE) and the total secreting (E) IFN$\gamma$ and (F) IL-10 in response to the latency-associated ORFs (UL138, LUNA, US28, UL111A, UL144) is shown. Male responses are illustrated with open circles (○), and female responses with filled circles (●). Mann-Whitney U tests were utilised to compare between male and female responses to ORFs.

### 4.2.9 CD8+ T cells can also secrete TNF$\alpha$ in response to HCMV latency-associated antigens

Having examined the secretion of IFN$\gamma$ and IL-10 from CD8+ T cells in response to HCMV ORF peptide pools, the analysis was extended to include TNF$\alpha$ production. CD8+ T cells can secrete TNF$\alpha$ in response to lytic HCMV ORF products, but such responses have not been tested to latency-associated proteins [316, 414, 657]. Using FluoroSpot plates capable of detecting IFN$\gamma$, IL-10, and TNF$\alpha$ secretion simultaneously, the CD8+ T cell responses to HCMV ORF peptide pools were investigated. CD4+ cell depleted PBMC were stimulated with overlapping peptide pools spanning the ORFs of gB, pp65, IE1, UL138, LUNA, US28, UL111A, and UL144, on FluoroSpot plates for 48 hours before assessing the number of cytokine positive spots. Donors with high background TNF$\alpha$ secretion in their unstimulated wells were excluded from the data if their positive control did not elicit above twice the number of TNF$\alpha$ secreting cells than in unstimulated wells. Due to high levels of background TNF$\alpha$ secretion, eight donors were excluded, leaving five donors for the analysis.

Despite the exclusion of a large proportion of sampled donors, TNF$\alpha$ secretion from CD8+ T cells was found in response to all of the HCMV ORFs tested. Of the five donors, responses to gB (5 donors), IE1 (2 donors), pp65 (3 donors), UL138 (2 donors), LUNA ( 1 donor), US28 (5 donors), UL111A (2 donors), UL144 (3 donors) were observed (Figure 4.26). CD8+ T cell responses to gB were composed of the highest frequencies of cytokine secreting cells (Figure 4.26). Responses to the latency-associated ORFs were variable in the number of cytokine positive cells detected. UL138 and LUNA responses were composed of the lowest frequencies of TNF$\alpha$ positive CD8+ T cells, compared to UL111A and UL144 responses which were intermediate, and to US28 which were the highest (Figure 4.26).

ICS was also carried out to detect TNF$\alpha$ production from CD8+ T cells. PBMC from five HCMV seropositive donors were stimulated with viral ORF peptide pools overnight and stained for TNF$\alpha$. CD8+ T cells producing TNF$\alpha$ were only detected at high proportions in one donor, who had positive responses to all the ORFs tested excluding gB and LUNA (Figure 4.27 illustrated with black filled circles). Low level TNF$\alpha$ responses that were detectable by ICS were also found in one donor in response to pp65 and UL144, and in another to IE1 (Figure 4.27). The results obtained by ICS were therefore not consistent with those obtained by the FluoroSpot assay.

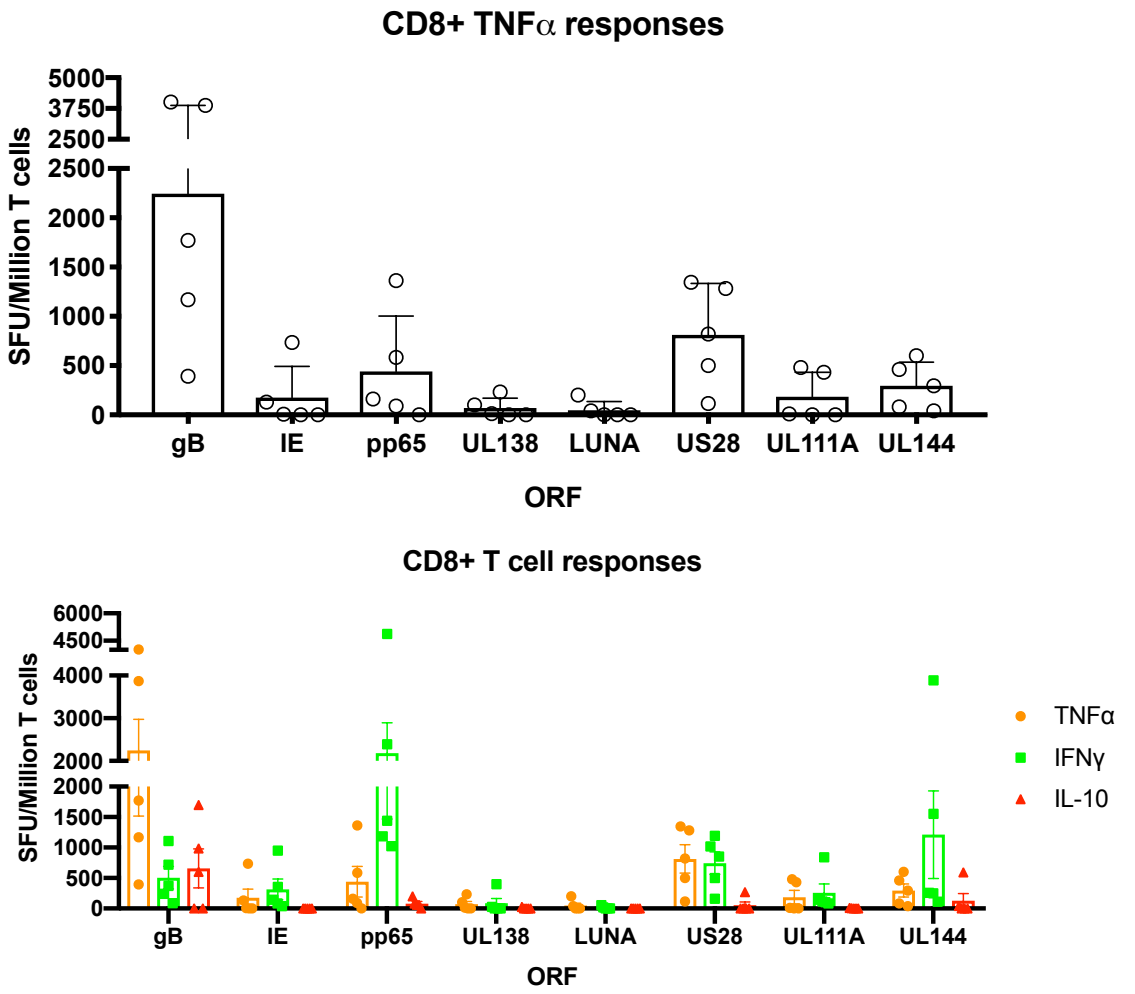



**Fig. 4.26 The secretion of TNF$\alpha$ by CD8+ T cells from seropositive donors in response to HCMV ORF peptide pools.** PBMC from five seropositive donors were depleted of CD4+ cells and stimulated with overlapping HCMV ORF peptide pools spanning gB, pp65, IE1, UL138, LUNA, US28, UL111A, and UL144, for 48 hours on triple IFN$\gamma$/IL-10/TNF$\alpha$ FluoroSpot plates. Each peptide pool was tested in triplicate and the values from all test wells were deducted from the number of background cells secreting cytokines in the unstimulated wells. Following stimulation, the number of cells secreting TNF$\alpha$, IFN$\gamma$, and IL-10 was enumerated by the automated spot counter and converted to a spot forming unit (SFU) value per million cells.

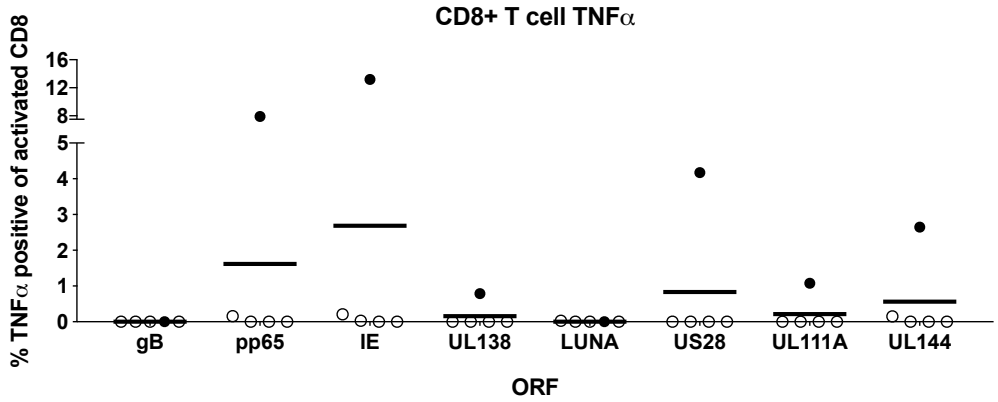



**Fig. 4.27 Detection of TNF$\alpha$ production by CD8+ T cells in response to HCMV ORF peptide pools by intracellular cytokine staining.** PBMC depleted of CD4+ cells were stimulated with HCMV ORF peptide pools overnight. The following day, cells were incubated in monensin before staining intracellularly for TNF$\alpha$. CD8+ T cells were identified by gating on CD3+CD8+ cells, and ORF-specific T cells by expression of 4-1BB and/or CD69. TNF$\alpha$ positive cells are shown as a proportion of these CD3+CD8+ cells. The data shown are from five seropositive donors and the filled black circles (•) represent the proportions of TNF$\alpha$ CD8+ T cells from donor, CMV310.

### TNF$\alpha$ secreting CD8+ T cells are generally a separate population to those secreting IFN$\gamma$ or IL-10

Having detected CD8+ T cells secreting IFN$\gamma$, IL-10, and TNF$\alpha$ in response to HCMV ORF peptide pools, it remained to be established if CD8+ T cells could secrete these cytokines in combination. The data obtained from the stimulation of CD8+ T cells from five seropositive donors with overlapping HCMV ORF peptide pools on triple FluoroSpot plates enabled this analysis. Following this, the proportions of responding CD8+ T cells within donors that secreted one cytokine, or combinations of IFN$\gamma$/IL-10 together with TNF$\alpha$ were established. Only donors with cytokine responses above the background frequencies observed in the unstimulated samples were included in the analysis, which resulted in the omission of several donor responses. Two donor TNF$\alpha$/IFN$\gamma$ responses to UL138 were excluded, while two donor TNF$\alpha$/IL-10 responses to IE1 and UL138, and three such responses to LUNA and UL111A were not included.

CD8+ T cells that secreted both IFN$\gamma$ and TNF$\alpha$ were only found in two of the five donors tested (donors 181 and 321) and were never greater than 5% of all cytokine secreting CD8+ T cells (Figure 4.28). There were also no ORF-specific responses that had characteristically higher frequencies of such dual cytokine secreting cells. Of note, there were no dual secreting cells detected in response to IE1, UL138, or LUNA (Figure 4.28C, D, G).

Similarly, there were almost no CD8+ T cells that secreted both TNF$\alpha$ and IL-10 in response to HCMV ORFs, with the exception of responses to gB and pp65 (Figure 4.29A and B). CD8+ T cells secreting both TNF$\alpha$ and IL-10 in response to gB accounted were found in three donors (Figure 4.29A). In two of these donors, dual TNF$\alpha$/IL-10 secreting CD8+ T cells accounted for approximately 11% of all cytokine secreting cells (Figure 4.29A). This was also the case for responses to pp65, which were found in three of the five donors tested, two of which had between 5-10% dual TNF$\alpha$/IL-10 secreting CD8+ T cells (Figure 4.29B). Although dual TNF$\alpha$/IL-10 secreting CD8+ T cells were found in response to UL144 in two donors, these cells were less than 3% of total UL144-specific cytokine secreting population (Figure 4.29H).

Dual cytokine secreting CD8+ T cells were also assessed by ICS following HCMV ORF peptide stimulation. IFN$\gamma$/TNF$\alpha$ secreting cells could only be detected in two donors in response to LUNA and UL144, and these were a very small proportion of responding CD8+ T cells (Figure 4.30A). Only one donor was found to possess a dual IL-10/TNF$\alpha$ response to IE1 (Figure 4.30B).

The FluoroSpot and ICS methods utilised were therefore not able to find significant populations of CD8+ T cells secreting combinations of IL-10, IFN$\gamma$, and TNF$\alpha$, although firm conclusions cannot be drawn from this limited set of five donors.

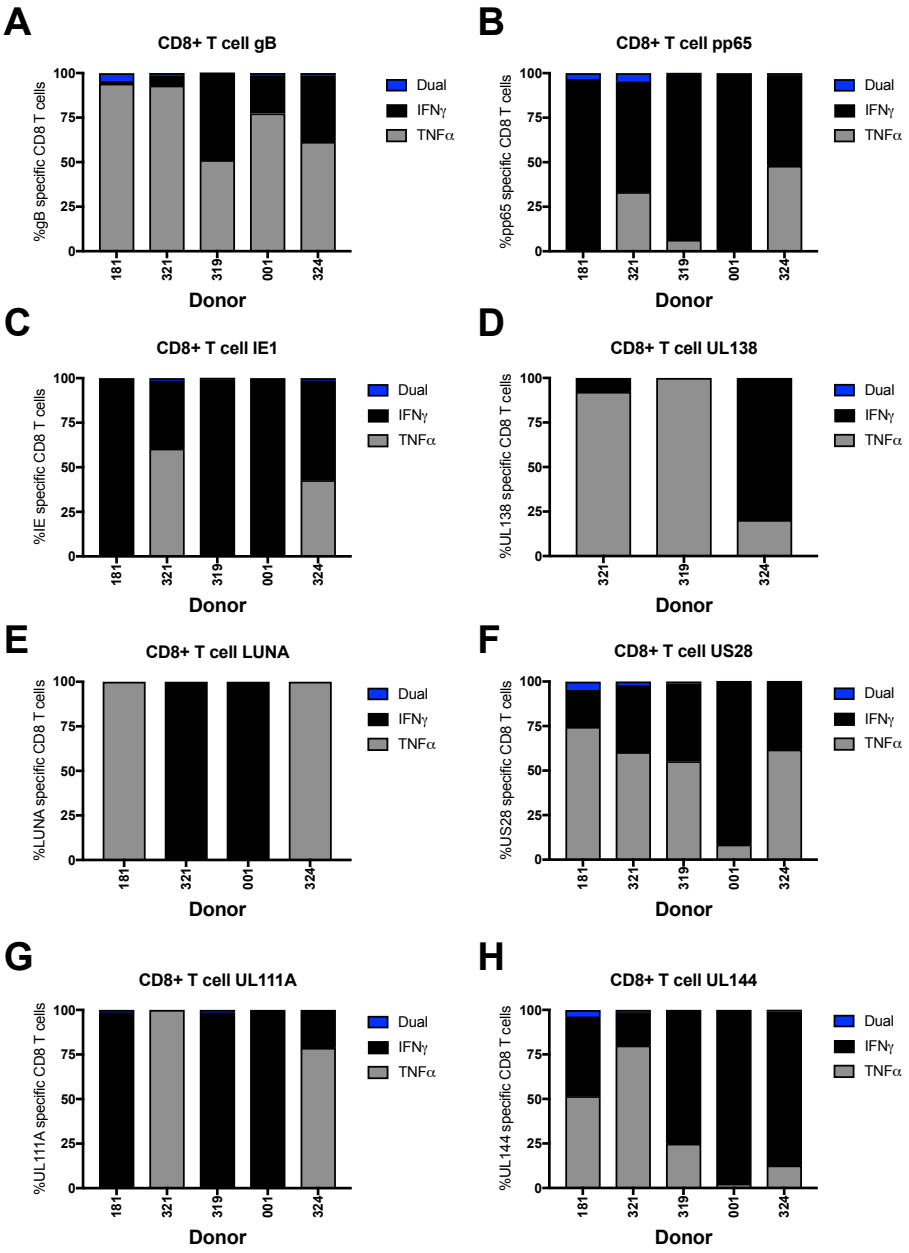



**Fig. 4.28 Proportions of CD8+ T cells secreting TNF$\alpha$ and IFN$\gamma$ in response to latency-associated HCMV ORF peptide pools from seropositive donors.** PBMC from three HCMV seropositive donors were depleted of CD4+ cells and stimulated with overlapping peptide pools from HCMV ORFs (A) gB, (B) pp65, (C) IE, (D) LUNA, (E) US28, (F) UL111A, and (G) UL144 for 48 hours. Following stimulation, IFN$\gamma$ (black bars) and TNF$\alpha$ (grey bars) secretion was detected by FluoroSpot and enumerated to spot forming units (SFU), where each cytokine response was calculated as a proportion of the total CD8+ T cell response. The proportion of CD8+ T cells of total cytokine secreting cells that secreted both IFN$\gamma$ and IL-10 is also shown (blue bars). All samples were run in triplicate and background spots from unstimulated wells were deducted from the values obtained from test wells. The mean value from each triplicate is shown here. Donors without an IL-10 or IFN$\gamma$ responses were omitted from each ORF analysis.

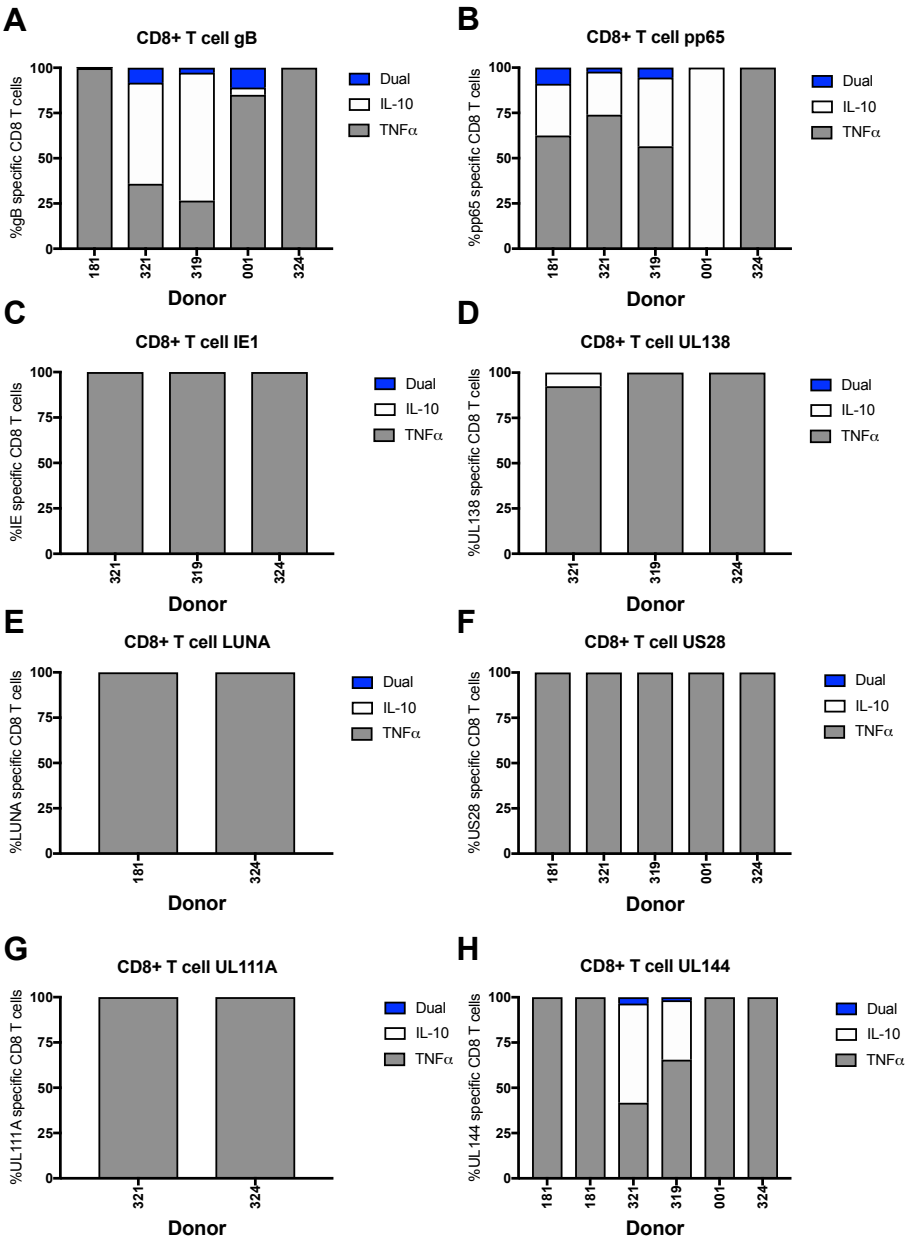



**Fig. 4.29 Proportions of CD8+ T cells secreting TNF$\alpha$ and IL-10 in response to latency-associated HCMV ORF peptide pools from seropositive donors.** PBMC from three HCMV seropositive donors were depleted of CD4+ cells and stimulated with overlapping peptide pools from HCMV ORFs (A) gB, (B) pp65, (C) UL138, (D) LUNA, (E) US28, (F) UL111A, and (G) UL144 for 48 hours. Following stimulation, IL-10 (white bars) and TNF$\alpha$ (grey bars) secretion was detected by FluoroSpot and enumerated to spot forming units (SFU), where each cytokine response was calculated as a proportion of the total CD8+ T cell response. The proportion of CD8+ T cells of total cytokine secreting cells that secreted both TNF$\alpha$ and IL-10 is also shown (blue bars). All samples were run in triplicate and background spots from unstimulated wells were deducted from the values obtained from test wells. The mean value from each triplicate is shown here. Donors without an IL-10 or TNF$\alpha$ responses were omitted from each ORF analysis.

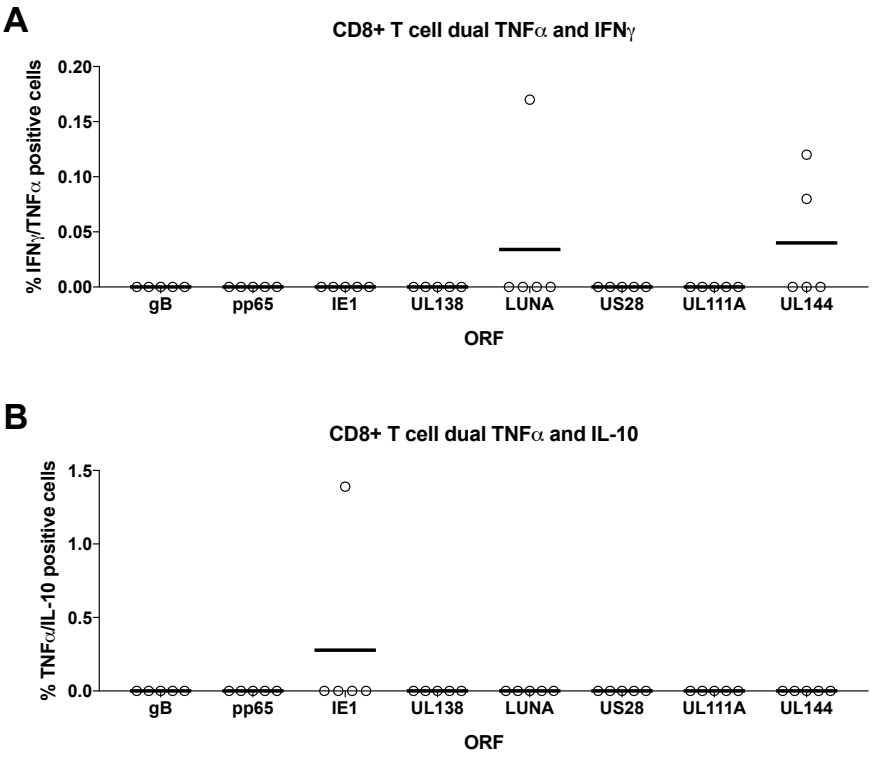



**Fig. 4.30 Detection of CD8+ T cells capable of co-producing TNF$\alpha$ together with IFN$\gamma$ or IL-10 in response to HCMV ORF peptide pools by intracellular cytokine staining.** Whole PBMC isolated from five HCMV seropositive donors were stimulated overnight with HCMV peptide pools spanning each ORF of interest (gB, pp65, IE, UL138, LUNA, US28, UL111A, UL144), in the presence of monensin. Stimulated PBMC were then stained intracellularly for IFN$\gamma$, IL-10 and TNF$\alpha$ production from CD4+ T cells. CD4+ T cells positive for both (A) TNF$\alpha$ and IFN$\gamma$, and (B) TNF$\alpha$ and IL-10 are shown. T cells were identified after gating for live and CD3+ cells.

## 4.3 Discussion

### 4.3.1 CD8+ T cell responses to latency-associated HCMV ORFs

Previous work examining CD8+ T cell responses to latency-associated ORF products have either been unable to detect responses, or have detected CD8+ T cell responses at frequencies below a set positive threshold [288, 501]. While low frequency CD8+ T cell responses to UL138 and LUNA were detected [501], US28, UL111A, and UL144 responses were not tested directly. The CD4+ and CD8+ T cell responses to these additional latency-associated ORFs were therefore examined in parallel using the highly sensitive FluoroSpot assay. The Fluorospot plates used were dual IFN$\gamma$/IL-10, chosen to be able to test the IL-10 secretion from CD4+ T cells simultaneously to IFN$\gamma$. The use of these plates however also generated data for IL-10 secreting CD8+ T cells, which was assessed across the large donor cohort of 90 individuals ranging in ages from 23 to 74. Donors were assessed by stimulating CD4+ depleted PBMC with overlapping peptide pools spanning the ORFs of gB, pp65, IE1, US28, LUNA, UL138, UL111A, and UL144 on dual IFN$\gamma$/IL-10 FluoroSpot plates and the frequencies of cytokine secreting cells were then determined. The threshold for positive responses in these experiments was set based on the frequencies of cytokine secreting cells detected following whole peptide ORF stimulation of CD8+ T cells from HCMV seronegative donors, which was not done previously [501]. Several of these seronegative donors were found to possess relatively high responses to peptide pool stimulation however, and while it is entirely plausible that these responses were non-HCMV specific, perhaps due to T cell cross-reactivity [658], HCMV-specific T cell responses have been observed from individuals deemed to be seronegative, which could also explain these responses [659, 660].

Unexpectedly, CD8+ T cell responses to all the HCMV ORFs tested were detected, and this can be largely attributed to the highly sensitive nature of the FluoroSpot assay. There were differences in the number of donors responding to each ORF, and in the frequencies of IFN$\gamma$ secreting CD8+ T cells specific for each ORF. Of the 90 donors tested for CD8+ T cell responses, the vast majority recognised US28 and UL144, and these responses were more prevalent than responses to the lytic ORFs gB and IE1. Additionally, responses to these ORFs were composed of the highest frequencies of IFN$\gamma$ secreting CD8+ T cells. Around a third of donors recognised UL138 and LUNA, while nearly half recognised UL111A. Two-thirds of donors were also capable of responding to two or more of the five latency-associated ORFs tested (data not shown), making these responses widespread amongst the cohort. The extent to which these CD8+ T cell responses were present was surprising given the lack of such

responses reported in the literature. Given the potent antiviral effectors functions of CD8+ T cells, such widespread recognition of proteins expressed during latency without subsequent elimination of the virus raises further questions regarding the ability of HCMV to persist when latent.

The ability of the FluoroSpot to detect both IFN$\gamma$ and IL-10 secretion simultaneously enabled an analysis of IL-10 secretion from CD8+ T cells recognising latency-associated ORF products. Surprisingly, this has uncovered the presence of widespread CD8+ T cell IL-10 responses. Of the donors that passed the selection criteria, 80% were found to have an IL-10 response to at least one of the five latency-associated ORF products, and 56% to at least two (data not shown). These CD8+ T cells were a separate population from those secreting IFN$\gamma$, in the same way HCMV-specific CD4+ T cells were found to be, and were most frequently found in response to US28, where 67% of donors had a positive IL-10 response. CD8+ T cell IL-10 responses to UL138 and LUNA were also observed relatively frequently, with a response detected in around 45% of donors. IL-10 responses to UL144 and UL111A were the least frequently detected of the latency-associated ORFs. In general there were low responses to the lytic ORFs compared to the latency-associated ORFs, although pp65 was the exception to this. The ability of pp65-specific CD8+ T cells to produce IL-10 has been reported previously in a study comparing the effects of ageing on the balance between proinflammatory and anti-inflammatory cytokine secretion [350]. In young HCMV seropositive donors, pp65-specific CD8+ T cells that secreted IL-10 following *in vitro* peptide stimulation made up an average of 2.77% of the pp65-specific CD8+ T cell population, while this figure was 0.82% in the old group.

It should be noted that in the donor cohort analysed, a large number of participants (56) had to be excluded from the IL-10 FluoroSpot analysis because they did not pass the selection criteria, usually due to the levels of background IL-10 secretion being too high. The high level of IL-10 positive spots in unstimulated control wells could be the result of non-antigen specific IL-10 secretion and was considerably higher than that observed for IFN$\gamma$ secretion.

Examining the distribution of IFN$\gamma$ and IL-10 secretion by CD8+ T cells in response to HCMV ORFs within individuals revealed similarities with the CD4+ T cell responses. The responses to the lytic ORFs gB, pp65, and IE1 were dominated by IFN$\gamma$ secreting CD8+ T cells, while responses to latency-associated ORFs were composed of much higher proportions of IL-10 secreting CD8+ T cells, with the exception of UL144 responses. This IL-10 bias was particular for responses to UL138 and LUNA, where the majority of donors possessed a dominant CD8+ T cell IL-10 response to these ORFs. Thus, the subset of CD8+ T cells capable of secreting IL-10 on recognition of proteins expressed during latency could

be aiding virus persistence through the inhibition of antiviral T cell responses, including the CD8+ T cells capable of secreting IFN$\gamma$ detected here.

IL-10 secreting CD8+ T cells have been shown to be generated in response to infection with various acute and chronic viruses. These CD8+ T cells secreting IL-10 have been described as both regulatory CD8+ T cells, expressing FoxP3 and CD25, in EBV- and Human immunodeficiency virus (HIV)-infected individuals for example [661–664], as well as FoxP3-negative effector CD8+ T cells, expressing the transcription factor T-bet and effector molecules such as granzyme B, CD107a, and TNF$\alpha$, in animal models of influenza, respiratory syncitial virus (RSV), and vaccinia virus [665–668]. Several studies have also demonstrated that IL-10 secreting effector CD8+ T cells were generated transiently and their decline coincided with viral control [665, 669–671].

During acute virus infection, CD8+ T cells secreting IL-10 are thought to act in a regulatory capacity by contributing to the prevention of excess inflammation and immunopathology. This has been demonstrated using mouse models of influenza virus and RSV where IL-10 secreting CD8+ T cells were found in the airways and/or lungs of infected animals [665, 666, 672, 673]. These cells acted to regulate the levels of inflammation and inflammatory cell infiltrate, as demonstrated by the blockade of the IL-10R in infected mice [665, 666, 672, 673]. While IL-10R blockade resulted in increased pulmonary inflammation and morbidity it did not have an effect on viral clearance [665, 666, 672]. A protective role of IL-10 produced by CD8+ T cells has also been demonstrated in a mouse coronavirus model where these cells reduced the severity of coronavirus-induced acute encepahilitis [669]. In these studies showing a protective role for IL-10 secreting CD8+ T cells during viral infection, the phenotypes of the CD8+ T cells were either not examined or possessed markers of effector T cells. Interestingly, one report identifying regulatory FoxP3-positive CD8+ T cells that secreted IL-10 in response to influenza infection found that these cells were responsible for the suppression of CD8+ T cell effector responses and increased the mortality of infected mice [662]. The researchers also found a population of FoxP3-negative IL-10 secreting CD8+ T cells that were induced but did not test if these acted to control lung inflammation and improve mouse morbidity and mortality [662].

CD8+ T cells that secrete IL-10 in response to chronic viruses such as HIV and Hepatitis C virus have also been described [674–680]. In HIV-1 infected individuals who were antiretroviral naive, the presence of IL-10 secreting CD8+ T cells, as detected by ICS following Gag peptide pool stimulation, was associated with a lower CD4+ T cell count, higher levels of HIV-1 plasma RNA, and a decreased frequency of Gag-specific CD107a/b-positive CD8+ T cells [674]. HIV Gag- and Nef-specific CD8+ T cells that secreted IL-10

did not express FoxP3 and removal of these cells resulted in an increase in the frequency of HIV-specific CD8+ T cells expressing CD107a/b and IL-2 [675, 676]. A further study also found the majority of the IL-10 secreting CD8+ T cells specific for HIV-1 to be negative for FoxP3 [678].

During chronic viral infection, the immunosuppressive functions of IL-10 that act to prevent immunopathology from chronic inflammation could also aid the persistence of the virus. During HIV-1 infection, the IL-10 produced by CD8+ T cells is thought to contribute to the avoidance of the host's immune system as evidenced by their ability to suppress HIV-specific CD8+ T cell mediated cytolysis [674, 675]. However, it has also been shown that patients with greater proportions of CD8+ T cells positive for IL-10 and negative for IFN$\gamma$ had better control of viraemia compared to those with more dual producing CD8+ T cells. This suggests that these cells arise as a protective response to inflammation resulting from chronic antigenic stimulation [678]. Similarly, there is evidence that during chronic HCV infection virus-specific CD8+ T cells secreting IL-10 are involved in reducing liver immunopathology [680] by protecting against hepatocellular necrosis and liver fibrosis caused by chronic inflammation [679]. However, a recent report claims that IL-10 producing CD8+ T cells contribute to liver immunopathology by preventing the antigen-dependent apoptosis of IFN$\gamma$ secreting effector CD8+ T cells [681].

Few investigations have focused on IL-10 secreting CD8+ T cells during HCMV infection. From one such report, carried out on donors co-infected with HIV [678], it was not clear what role these cells have during infection and if they are generated protectively and/or by the virus to aid persistence [678]. Ten donors were tested for HCMV responses and eight of these possessed a CD8+ T cell IL-10 response. Of these individuals, dual IFN$\gamma$/IL-10 secreting cells, detected by flow cytometry following enrichment of cytokine secreting cells stimulated with pp65 peptides, made up the majority of responding CD8+ T cells in most donors [678]. In a number of previous studies carried out on a range of viruses, CD8+ T cells secreting IL-10 have also mainly been reported as dual producers of IL-10 and IFN$\gamma$, where IFN$\gamma$ was also examined [665, 669, 670, 672, 673, 678]. These studies run contrary to the work presented here, where very low proportions of dual IL-10/IFN$\gamma$ secreting CD8+ T cells were detected in response to HCMV. However, one important difference between the previously published work and the results presented here is that the previous studies were carried out in mouse models, or in HIV/HCMV co-infected individuals. Data from humans do report the presence of CD8+ T cells from HIV and HCV infected individuals that secreted IL-10 without IFN$\gamma$ [677–680], which supports the findings that single IL-10 producing CD8+ T cells can be generated from virus infection.

Carrying out in depth phenotypic and functional analyses of IL-10 secreting CD8+ T cells in response to HCMV will determine if these cells express markers of regulatory or effector T cells, whether these cells are capable of anti-viral effector functions, and advance our understanding of the role these cells have during infection. Furthermore, the conditions which lead to the generation of these cells should be investigated. It has been demonstrated that IL-10 expression from CD8+ T cells can been induced by IL-4 alone [682] or together with IL-12 [683], by blockade of TNF$\alpha$ [684], and by IL-27 [668, 673, 685, 686]. The role of IL-27 in promoting IL-10 expression from CD8+ T cells has also been studied in the context of viral infection. In a mouse model of influenza, optimal IL-10 expression from CD8+ T cells required the synergy of CD4+ T cell derived IL-2 with IL-27, and CD8+ T cells that expressed IL-10 also expressed high levels of the transcription factor Blimp-1 [673]. Although IL-27 was shown to be required for CD8+ T cell IL-10 expression in response to acute virus infection of the lung [668, 673], memory CD8+ T cells upon rechallenge were found to be unresponsive to IL-27 and were unable to produce IL-10 due to the downregulation of surface gp130 [668]. It could therefore be possible that during primary HCMV infection, IL-27 is involved in the initial generation of IL-10 secreting CD8+ T cells, which arise as a protective response against immunopathology. These cells might then be maintained by the virus following the establishment of the immunosuppressive latent microenvironment. To begin to answer these questions, initial work on the generation of HCMV-specific IL-10 secreting T cells following primary infection will be presented in the following chapter.

CD8+ T cells were also tested for their ability to secrete the anti-viral cytokine TNF$\alpha$ in response to HCMV ORF peptide pools by FluoroSpot. TNF$\alpha$ responses to all ORFs tested were observed from CD8+ T cells but due to high background TNF$\alpha$ secretion the majority of donors had to be excluded from the analysis. The highest frequency responses were detected in response to gB and US28 while the lowest in response to UL138 and LUNA. ICS was also utilised to validate these TNF$\alpha$ responses but robust responses were found from only one of the five donors. TNF$\alpha$ secretion by HCMV-specific CD8+ T cells has been detected by ICS by many others [287, 414, 687], and the failure to detect them here could be partly due to the ICS protocol used. Better optimisation might therefore be required in order to capture TNF$\alpha$ secretion.

Of the limited number of donors analysed for TNF$\alpha$, IL-10, and IFN$\gamma$ simultaneously by FluoroSpot, none were found to possess cells that secreted all three cytokines, and very few had CD8+ T cells secreting both IL-10 and TNF$\alpha$, or IFN$\gamma$ and TNF$\alpha$. Although this preliminary work might indicate these are three distinct cellular populations within the

HCMV-specific CD8+ T cell pool, several investigations have found polyfunctional HCMV-specific dual secreting IFN$\gamma$ and TNF$\alpha$ CD8+ T cells by ICS [316, 414, 657]. Such dual secreting CD8+ T cells were only found in two of five donors tested by ICS here, and none were found responding to lytic ORFs such as pp65 and IE1, as previous research has shown [316, 414, 657]. The absence of these dual secreting cells as measured by FluoroSpot or ICS here could be due to the very small donor cohort and problems associated with background TNF$\alpha$ secretion in the FluoroSpot, in addition to sub-optimal staining protocols for ICS, especially as dual TNF$\alpha$/IFN$\gamma$ secreting cells were also not detected in positive control (CD3/CD28 MAb mix) treated cells (data not shown), although they were in the FluoroSpot assay.

The finding that IFN$\gamma$ secreting CD8+ T cells were a separate population to those secreting IL-10, and possibly TNF$\alpha$, raises the question of whether epitope-specific CD8+ T cells from each of these populations originate from the same parent cell or are distinct T cell lineages. The experiments conducted here used peptides derived from whole ORFs to stimulate cells. However, individual peptides could be used to map CD8+ T cell responses, where it might be expected that CD8+ T cells secreting IFN$\gamma$ or IL-10 in response to a single peptide would be the same T cell clonotype. Doing so could therefore give an indication of whether T cell clones diverge from their parent cell into two distinct cytokine secreting populations specific for the same peptide; this is addressed in a subsequent chapter.

### 4.3.2 Fluctuations in the frequency of CD8+ T cell responses over time

The CD8+ T cell responses to individual HCMV ORFs were found to vary across independent sampling points spanning a three year period, with responses to certain ORFs found to be more variable than others. CD8+ T cell IFN$\gamma$ responses were highly consistent across time points in response to the lytically-expressed proteins gB, pp65, and IE1, and to the latency-associated proteins US28 and UL144. Greater variation across time points was observed in responses to UL138, LUNA, and UL111A. The CD8+ T cell IL-10 responses displayed much greater variability across the sampled time points with the frequencies of IL-10 secreting cells detected fluctuating below and above the seronegative threshold point frequently, resulting in the respective loss or gain of positive responses. There were no ORFs that were consistently responded to at all time points and these fluctuations often resulted in very large differences in the frequencies of IL-10 secreting CD8+ T cells detected between time points.

As only two donors could be sampled on more than three instances it is not possible to generalise these differences in ORF-specific fluctuations to the broader population. It is

however clear that there were differences in the magnitudes of IFN$\gamma$ and IL-10 secreting CD8+ T cells in response to HCMV ORFs when the same donors were re-challenged multiple times, and these differences were considerably greater for IL-10 secreting CD8+ T cells.

Fluctuations in the CD8+ T cell response to HCMV over time have been reported previously in studies utilising ELISpot assays [291, 642]. One of these, using IFN$\gamma$ ELISpot, examined the CD8+ T cell responses to HCMV ORFs, including IE1 and pp65, at multiple time points over a 40 month period, and also found variation in the donor specific response frequencies over time [291]. A number of factors could be driving these fluctuations in detected cytokine secreting CD8+ T cell frequencies, such as periodic reactivations of HCMV from latency serving to expand certain antigen-specific CD8+ T cells. Exercise has also been shown to affect the peripheral blood CD8+ T cell compartment, and it was demonstrated that the mobilisation of effector memory CD8+ T cells was greatest in HCMV seropositive individuals, compared to seronegative participants, during a one hour period of exercise [688]. It is also possible that such fluctuations were observed because of the nature of sampling CD8+ T cells from the peripheral blood compartment. As the peripheral blood contains a small percentage of total T cells, and is more prone to fluctuations in T cell composition, caused for example by active infections and environmental perturbations, it is more likely to lead to differences in HCMV ORF-specific responses detected across many time points [618, 643, 644]. Furthermore it has been suggested that the blood could act as a repository for newly activated CD8+ T cells generated in response to infection [398], such as observed following yellow fever virus and smallpox vaccination [689]. From the experiments carried out here, there were particular time points where high frequency IFN$\gamma$ or IL-10 responses to many HCMV ORFs were detected, which could be indicative of a greater infiltration of HCMV-specific CD8+ T cells into the blood or a decrease in non-HCMV specific CD8+ T cells in the blood. To investigate this possibility, total lymphocyte counts in combination with CD3+/CD8+ phenotyping could be carried out which would also allow for changes in the size of the CD8+ T cell compartment to be taken into account over time. The extent of the fluctuations in T cell responses observed also raises the question of how reproducible the data are from studies that take a snapshot approach of T cell responses. This might especially be a problem for studies using smaller donor cohorts as such sampling dependent variations would be amplified, particularly those looking for correlations, as the fluctuations in frequencies of responding T cells can be very high across time points.

### 4.3.3 CD8+ T cell responses and age

Previous investigations into the effects of long-term HCMV carriage on age-related immunosenescence have reported a number of changes in the CD8+ T cell compartment. Relatively early following HCMV infection, the CD8+ memory T cell pool increases [297, 690]. Characteristic of HCMV infection, an expansion of CD8+ T cells considered to be highly differentiated occurs. These cells exhibit features such as a loss of CD27 and CD28 expression, upregulated expression of CD57 and killer cell lectin-like receptor subfamily G member 1 (KLRG1), and the re-expression of CD45RA [292, 300, 304]. Such T cells could potentially be dysfunctional as they often lose the ability to secrete cytokines such as IFN$\gamma$, and display limited proliferative capacity [340, 341, 348, 350, 429, 656]. The expansion of highly differentiated CD8+ T cells with age, and subsequent loss of naive CD8+ T cells, has been linked to mortality [351, 352, 617], however such highly differentiated CD8+ T cells have also been found amongst young HCMV-positive individuals [301], and were shown to be proliferative given correct co-stimulation [432, 433]. Additionally, despite viral reactivation occurring more frequently in older HCMV infected individuals, these did not result in overt clinical disease, which could indicate a retained ability to control HCMV in the aged [434]. However, the increase in HCMV DNA detected in urine and blood of elderly individuals [434, 435] could be indicative of a reduced ability to control reactivating virus, perhaps caused by the effects of the lifelong carriage of HCMV on the immune system.

HCMV possesses a multitude of immune evasion strategies to facilitate viral persistence. The secretion of immunosuppressive cytokines by latently infected cells, bystander cells, and HCMV-specific CD4+ T cells could all contribute to this immune evasion by suppressing T cell function [501, 605]. It might be expected that longer carriage of HCMV could result in more pronounced effects of such immunomodulatory cytokines on antiviral CD8+ T cell responses and lead to immune dysfunction. One manifestation of immune dysfunction could be a decrease in HCMV-specific IFN$\gamma$ secreting CD8+ T cells. Given the difficulty in following individuals from the time of primary infection, the effects of age on IFN$\gamma$ secreting CD8+ T cells was examined as an indirect measure for time of viral carriage. The use of the dual IFN$\gamma$/IL-10 FluoroSpot plates to do this also enabled the examination of changes in IL-10 secreting CD8+ T cells with increasing age, which could be predicted to increase. The breadth and magnitude of HCMV ORF-specific CD8+ T cell responses in relation to donor age were thus studied by a cross-sectional approach on donors ranging from 23 to 74 years of age.

No significant association between the frequency of CD8+ T cells secreting IFN$\gamma$ or IL-10 and age was found for any of the ORF-specific responses examined to gB, pp65, IE1, UL138, LUNA, US28, UL111A, or UL144. Age also did not have an affect on the breadth of CD8+ T cell IFN$\gamma$ or IL-10 responses to HCMV ORFs as might be expected from a presumed longer time of virus carriage. Previous work from our group on a smaller subset of donors also found the breadth of CD8+ T cell IFN$\gamma$ responses to be independent of age [291].

The balance between IFN$\gamma$ and IL-10 secretion within individuals was largely unaffected by age. Small changes in the proportion of donors with dominant CD8+ T cell IL-10 responses were observed for UL138 and US28, which went up with age, and UL111A and UL144, which went down with age, while a large shift in the balance towards IL-10 producing LUNA-specific CD8+ cells was observed with increasing age. It is unclear whether such shifts have any impact on immune response to HCMV, especially given the persistence of detectable IFN$\gamma$ responses into old age, particularly for US28, UL111A, and UL144. Any potential immune dysfunction caused by HCMV with increasing age therefore does not seem to affect the frequencies of HCMV-specific CD8+ T cells secreting IFN$\gamma$ or IL-10. One possible explanation for not finding a decline in IFN$\gamma$ secreting CD8+ T cells as has been reported previously [350] is due to the whole ORF approach that was utilised here. By stimulating T cells with peptide pools spanning the entire predicted ORFs, this has likely captured a broader set of HCMV ORF-specific CD8+ T cell responses compared to the single peptide tetramer-based approach used in this study [350].

### 4.3.4 CD8+ T cell responses and sex

The ORF-specific CD8+ T cell responses were also examined according to sex. Widespread differences between male and female donors were not observed, however the frequency of IFN$\gamma$ secreting CD8+ T cells was significantly higher in male compared to female donors in response to gB, and CD8+ T cell IL-10 responses were found to be significantly higher in female donors in response to LUNA. Furthermore, no differences were found between males and females when total individual responses to all ORFs, lytic ORFs only, or latency-associated ORFs only were compared. This contrasts with CD4+ T cell responses where male donors had greater frequencies of IFN$\gamma$ and IL-10 secreting cells when taking into account total responses to all ORFs, lytic ORFs only, or latency-associated ORFs only.

# Chapter 5

# The Generation of HCMV-specific IL-10 Secreting T cells

## 5.1 Introduction

In previous chapters I have demonstrated that T cells specific to a number of HCMV ORFs can secrete IL-10 [350, 501]. Of the HCMV-specific T cell responses identified, IL-10 secreting T cells were most commonly found in response to latency-associated proteins of HCMV. This IL-10 secretion, in addition to the secretion of TGF$\beta$, could be suppressing antiviral T cell responses during latency and therefore aiding viral persistence. It is possible that these T cells are being generated as a result of lifelong viral carriage which could be driving T cell differentiation towards an IL-10 phenotype. However, in a large donor cohort, no differences were observed in the magnitudes of peripheral blood derived CD4+ or CD8+ T cell IL-10 responses with increasing age, demonstrating that IL-10 secreting T cells do not accumulate with longer viral carriage during the latent phase of infection (Chapter 3 and 4) [691].

Whether responses to latency-associated proteins are detectable during primary infection remains to be established, as prior studies have mainly focused on responses to lytic ORF products such as pp65 [333, 373]. IL-10 secreting T cells could therefore be generated during the primary immune response to HCMV infection, as latency-associated proteins are also expressed during lytic infection. A well established model of primary immune responses using seronegative renal transplant patients receiving a seropositive kidney was used to investigate these T cell responses [373, 400]. This work was carried out with material from

Dr Ester Remmerswaal and Professors Ineke ten Berge and Rene Van Lier (Academisch Medisch Centrum, AMC Amsterdam) who provided the blood samples from transplant recipients and data for viral loads.

## 5.2 Results

### 5.2.1 T cell responses to pp71 and US3

The donor cohort used to investigate CD4+ and CD8+ T cell responses to latency-associated HCMV ORFs was also tested for their ability to respond to the lytic ORFs US3 and pp71. US3 and pp71 (UL82) were found to be in the top 15 most recognised ORFs by T cells in a whole HCMV ORF screen [288], warranting their further study. US3 is expressed at IE time points and has a role in immune evasion, while the tegument protein pp71 is expressed at late time points [574, 692, 693]. PBMC were depleted of either CD4+ or CD8+ cells then stimulated with overlapping peptide pools spanning the ORFs of pp71 and US3 on dual IFN$\gamma$/IL-10 FluoroSpot plates. After 48 hours the frequencies of cytokine positive cells were then determined. Extensive CD4+ and CD8+ T cell IFN$\gamma$ responses were observed in response to US3 (CD4+ T cell responses in 51/91 donors [56%], CD8+ T cell responses in 60/91 donors [66%]), and pp71 (CD4+ T cell responses in 57/91 donors [63%], CD8+ T cell responses in 66/91 donors [73%]) (Figure 5.1A and 5.2A). However, high background levels of non-specific IFN$\gamma$ secretion in response to pp71 was observed in HCMV seronegative individuals in both CD4+ and CD8+ T cells, which could indicate that the threshold for positive responses to pp71 is greater than 100 SFU/$10^6$ T cells. Examining the number of donors with high frequency T cell responses, using a threshold of 250 SFU/$10^6$ T cells as previously, revealed CD4+ T cell high responders to US3 in 26/91 donors (29%) (Figure 5.1A), and in 42/91 donors (46%) for CD8+ T cell responses (Figure 5.2A). High frequency responses to pp71 were detected in 28/91 donors (31%) for CD4+ T cell responses (Figure 5.1A), and in 40/91 donors (44%) for CD8+ T cell responses (Figure 5.2A). The responses from all HCMV seronegative donors fell below this threshold for high frequency responses.

Surprisingly, a large proportion of seropositive donors possessed IL-10 secreting T cells in response to US3 (CD4+ T cell responses in 27/67 donors [40%], CD8+ T cell responses in 26/46 donors [57%]), and pp71 (CD4+ T cell responses in 28/67 donors [42%], CD8+ T cell responses in 30/46 donors [65%]) (Figure 5.1B and 5.2B). These IL-10 responses were the most commonly detected in comparison with responses to the other lytic ORFs gB, pp65,

and IE1 (Figure 3.16 and 4.12), with the exception of CD8+ T cell IL-10 responses to pp65 which were more prevalent than those to pp71.

The relative proportions of IFN$\gamma$ and IL-10 secreting T cells within individuals was then determined using the ability of the FluoroSpot to count dual secreting cells. CD4+ T cell responses to US3 and pp71 were composed of high proportions of IL-10 secreting cells with many individuals possessing a dominant IL-10 response to these ORFs (Figure 5.3). Comparatively, CD4+ T cell responses to other lytic ORFs gB, pp65, and IE1 were composed of considerably lower propotions of IL-10 secreting cells and were dominated by IFN$\gamma$ secreting cells (Figure 3.21). Similarly, a large number of donors possessed CD8+ T cell responses to US3 and pp71 that were predominantly composed of IL-10 secreting cells (Figure 5.4), which is in contrast to the corresponding responses to gB, pp65, and IE1 (Figure 4.21). Low proportions of dual IFN$\gamma$ and IL-10 secreting T cells were found in response to US3 and pp71, with the highest proportions reaching around 10% of total responses to US3 (Figure 5.3A and 5.4A), although these were not observed in all donors (Figure 5.3 and 5.4). The T cells responding to US3 and pp71 therefore also seem to be separate IFN$\gamma$ and IL-10 secreting populations.

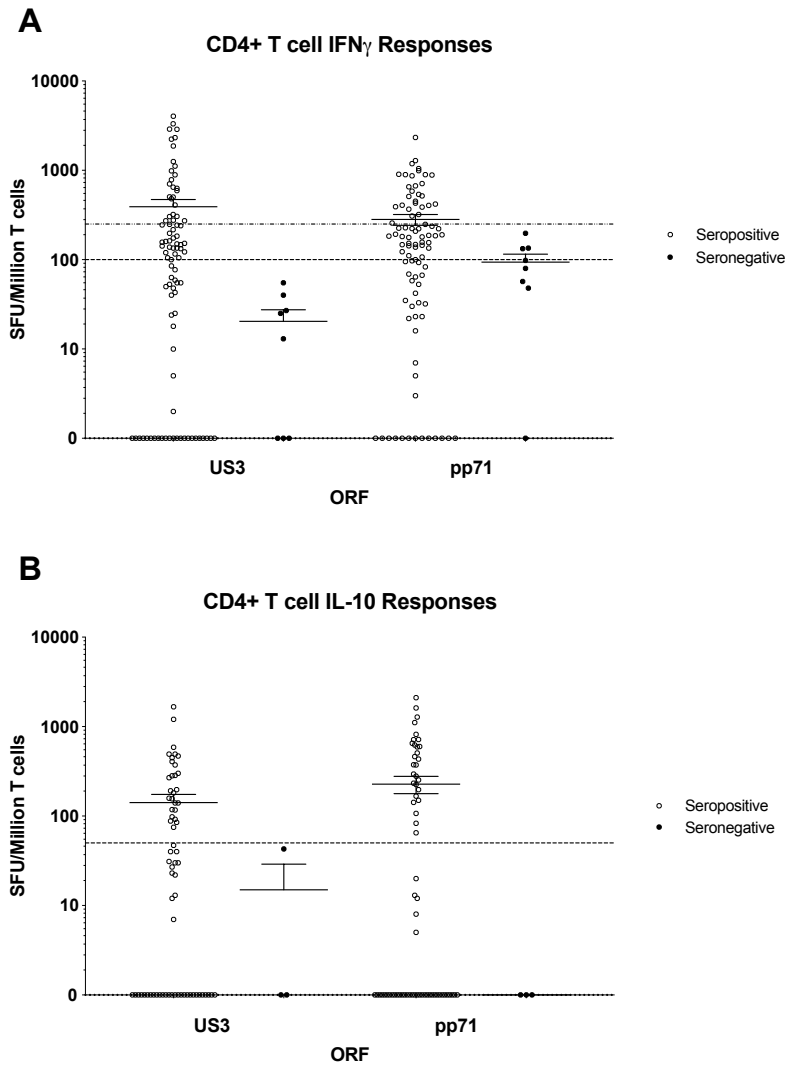



**Fig. 5.1 The frequency of CD4+ T cells from HCMV seropositive and seronegative donors that secrete IFN$\gamma$ or IL-10 in response to US3 and pp71.** PBMC were depleted of CD8+ T cells and stimulated with HCMV peptide pools spanning the ORFs of US3 and pp71 for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. Each peptide pool was tested in triplicate and the values from the number of background cells secreting cytokines in the unstimulated wells was deducted from all test wells. Following stimulation, the number of (A) IFN$\gamma$ or (B) IL-10 positive spots was enumerated by the automated cell counter and converted to a spot forming unit (SFU) value per million cells. Results from 91 seropositive and eight seronegative donors displayed for IFN$\gamma$ responses while results from 67 seropositive and three seronegative donors displayed for IL-10 responses. The dotted line at 100 SFU/$10^6$ cells indicates the threshold for positive responses. Seropositive donors are shown with open circles (○) while seronegative donors are illustrated with filled black circles (●).

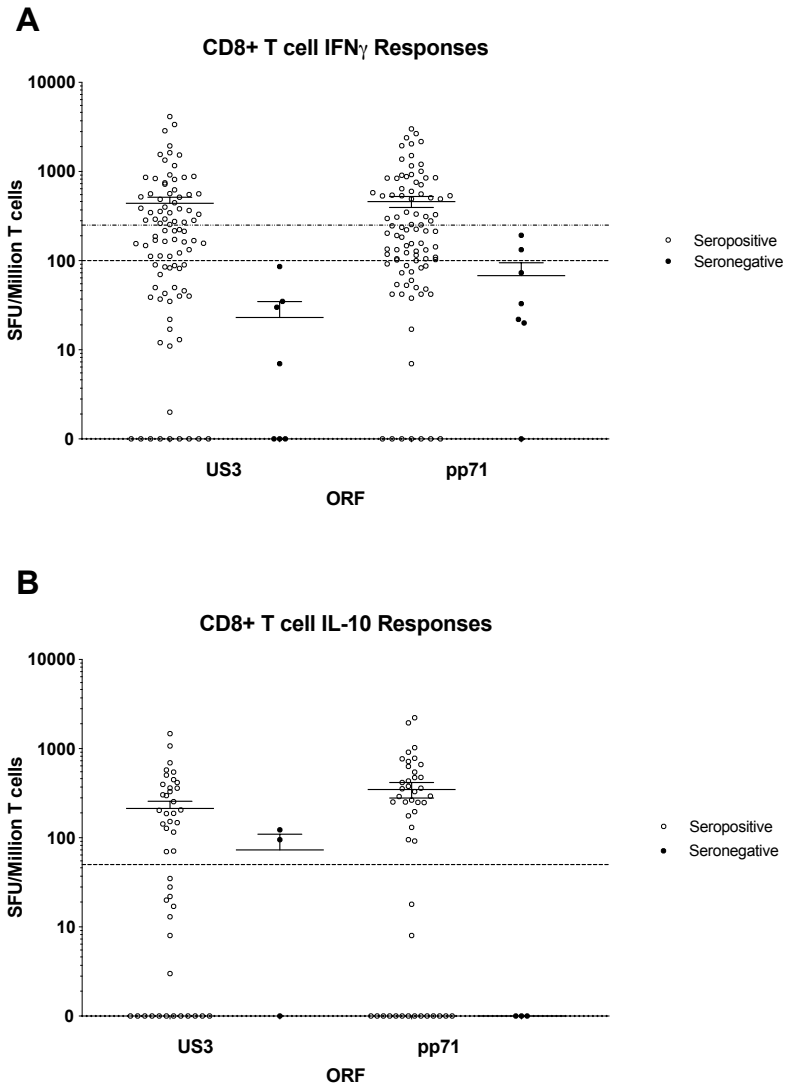



**Fig. 5.2 The frequency of CD8+ T cells from HCMV seropositive and seronegative donors that secrete IFN$\gamma$ or IL-10 in response to US3 and pp71.** PBMC were depleted of CD4+ T cells and stimulated with HCMV peptide pools spanning the ORFs of US3 and pp71 for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. Each peptide pool was tested in triplicate and the values from the number of background cells secreting cytokines in the unstimulated wells was deducted from all test wells. Following stimulation, the number of (A) IFN$\gamma$ or (B) IL-10 positive spots was enumerated by the automated cell counter and converted to a spot forming unit (SFU) value per million cells. Results from 91 seropositive and eight seronegative donors displayed for IFN$\gamma$ responses while results from 46 seropositive and three seronegative donors displayed for IL-10 responses. The dotted line at 100 SFU/$10^6$ cells indicates the threshold for positive responses. Seropositive donors are shown with open circles (○) while seronegative donors are illustrated with filled black circles (●).

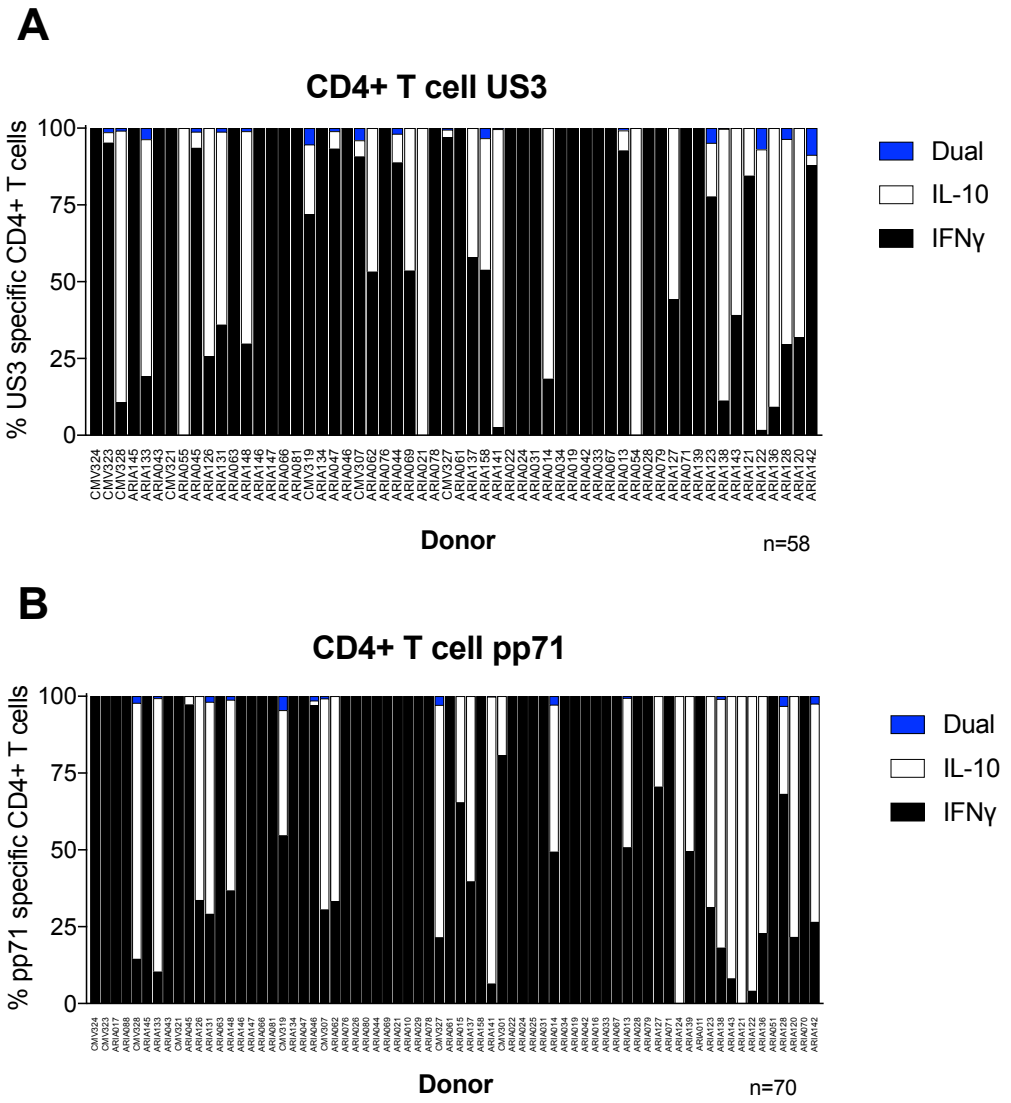



**Fig. 5.3 The proportions of CD4+ T cells secreting IL-10 and IFN$\gamma$ in response to US3 and pp71 from individual seropositive donors.** PBMC seropositive donors were depleted of CD8+ cells and stimulated with overlapping peptide pools from HCMV ORFs (A) US3, and (B) pp71, for 48 hours. Following stimulation, IFN$\gamma$ (black bars) and IL-10 (white bars) secretion was detected by FluoroSpot and enumerated to spot forming units (SFU), where each cytokine response was calculated as a proportion of the total CD4+ T cell response. The proportion of CD4+ T cells of total cytokine secreting cells that secreted both IFN$\gamma$ and IL-10 is also shown (blue bars). All samples were run in triplicate with the mean value shown here. Donors without an IL-10 or IFN$\gamma$ response were omitted from each ORF analysis.

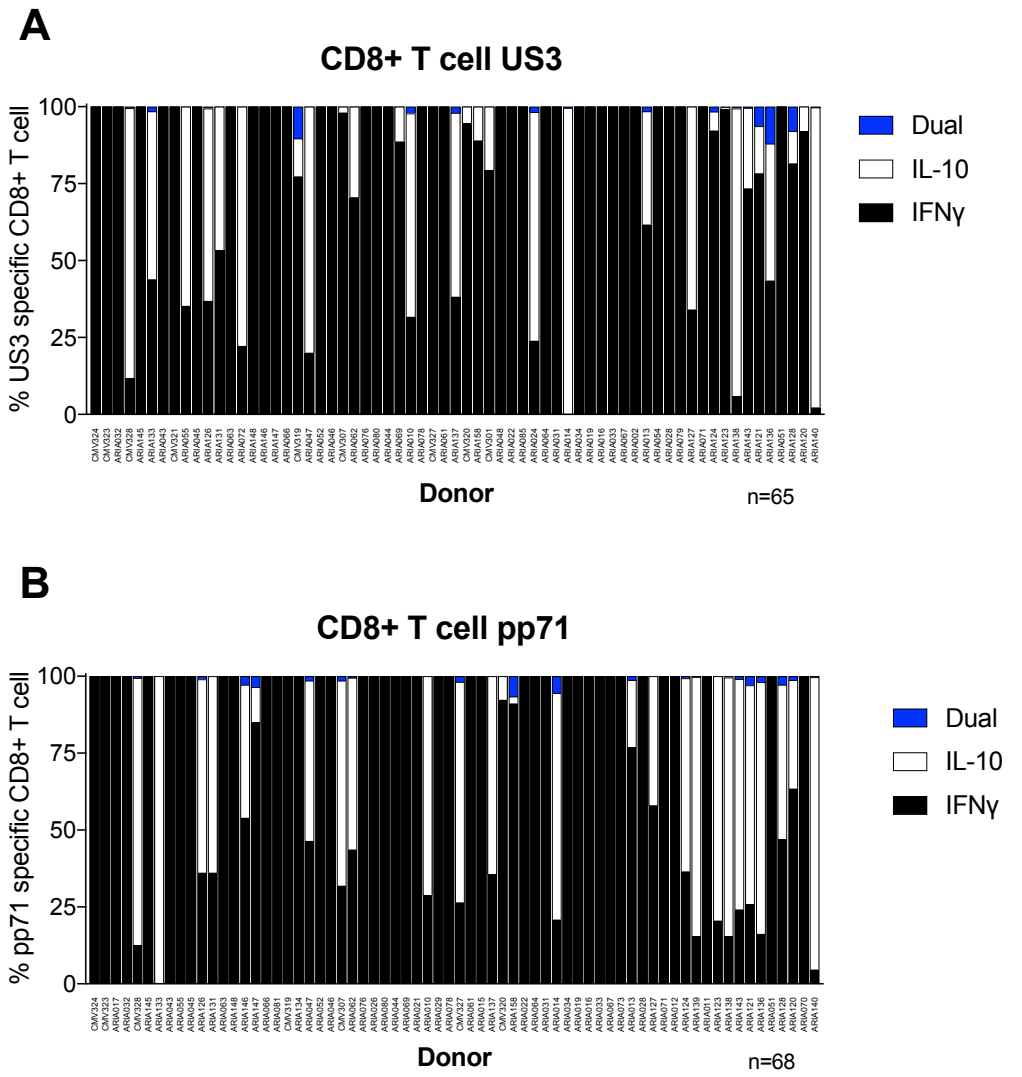



**Fig. 5.4 The proportions of CD8+ T cells secreting IL-10 and IFN$\gamma$ in response to US3 and pp71 from individual seropositive donors.** PBMC seropositive donors were depleted of CD4+ cells and stimulated with overlapping peptide pools from HCMV ORFs (A) US3, and (B) pp71, for 48 hours. Following stimulation, IFN$\gamma$ (black bars) and IL-10 (white bars) secretion was detected by FluoroSpot and enumerated to spot forming units (SFU), where each cytokine response was calculated as a proportion of the total CD4+ T cell response. The proportion of CD4+ T cells of total cytokine secreting cells that secreted both IFN$\gamma$ and IL-10 is also shown (blue bars). All samples were run in triplicate with the mean value shown here. Donors without an IL-10 or IFN$\gamma$ response were omitted from each ORF analysis.

### 5.2.2 T cell responses to HCMV following primary infection

The identification of IL-10 secreting CD4+ and CD8+ T cells responding to HCMV ORFs in a large donor cohort led to the question of when in the course of infection these cells are generated. The secretion of immunosuppressive IL-10 could be further aiding HCMV persistence during latency, and it remains to be determined if T cells secreting IL-10 are generated early during primary infection or during longer term carriage of the virus. The sampling of responses from a large donor cohort of varying ages did not find any significant differences in IL-10 secreting T cell frequencies with age, but as these experiments were cross-sectional, even donors in the youngest age groups could have been infected for over 20 years if infection occurred in early life. Therefore, there was a lack of data from primary infection and the weeks following from this.

To investigate the generation of IFN$\gamma$ and IL-10 secreting HCMV-specific T cells following primary infection, peripheral blood samples from seven individuals who underwent kidney transplantation were analysed. Prior to transplantation, the recipients were seronegative for HCMV, but were longitudinally studied after receiving kidneys from seropositive donors, as the likelihood of primary HCMV infection is very high in these situations [373, 400]. Blood samples were collected at multiple time points beginning from between one day prior, to two days post, transplantation, with subsequent samples collected at varying times points up to a maximum time point of 158 weeks post-transplantation. The patient details, including their immunosuppressive drug regimes, are summarised in Table 5.1.

| **Patient Number** | **Age at Tx** | **Immunosuppressive Drugs** | **Peak Viraemia (weeks post Tx)** | **HCMV Seroconversion (weeks post Tx)** |
|---|---|---|---|---|
| 133 | 66 | P, CSA, MMF | 12.14 | 15.71 |
| 136 | 31 | P, CSA, MMF | 8.57 | 10.57 |
| 197 | 31 | P, MMF, FK, CD25MAb | 14.00 | 14.00 |
| 352 | 26 | P, CSA, MMF, CD25MAb | 7.14 | 7.14 |
| 365 | 54 | P, CSA, MMF, CD25MAb | 6.86 | 8.57 |
| 439 | 21 | P, MMF, FK, CD25MAb | 9.00 | 10.00 |
| 574 | 50 | P, MMF, FK, CD25MAb | 6.29 | 25.43 |

**Table 5.1** Transplant recipient information. Tx = transplantation, immunosuppressive drugs P = prednisolone, CSA = cyclosporin A, MMF = mycophenolate mofetil, FK = Tacrolimus, CD25MAb = anti-CD25 monoclonal antibodies

Due to the limited lymphocyte numbers available from the clinical samples, HCMV responses were investigated by combining particular ORF peptide pools together instead

of testing each ORF individually. Similarly, whole PBMC were stimulated instead of CD4+ or CD8+ T cell depleted populations. The background cytokine secretion from unstimulated PBMC was deducted from peptide stimulated samples. To control for variation in lymphocyte numbers over time, the frequencies of spot forming units detected were expressed as a proportion of the total CD3+ cell population, as determined by lymphocyte absolute count data and flow cytometry. In this way the number of CD4+ and CD8+ T cells responding to HCMV ORF peptide pools together was determined. Pools composed of UL138, LUNA, US28, and UL111A peptides were made to determine the T cell responses to latency-associated ORFs without the highly IFN$\gamma$ biased responses to UL144. UL144 and pp65 ORF pools were combined, as were IE1 and IE2, while the gB pool was used in isolation. Additionally, as widespread CD4+ and CD8+ T cell IL-10 responses to US3 and pp71 were detected, these ORF pools were combined and tested together. PBMC were defrosted in batches at each time point and stimulated with these ORF pools on FluoroSpot plates capable of detecting IFN$\gamma$ and IL-10 for 48 hours before the frequencies of cytokine positive cells were determined. Representative individual wells obtained from the FluoroSpot assay of patient 133 show IFN$\gamma$ responses for each time point and highlight the low background secretion of IFN$\gamma$ observed in unstimulated T cells (Figure 5.5). Data for HCMV DNAemia at each time point was provided by our collaborators to give a measure of viral load.

Across the seven donors, detectable IFN$\gamma$ responses arose immediately following transplantation in response to nearly all ORF pools. Individual patient IFN$\gamma$ secretion followed one of a number of patterns in their immune response in the weeks following transplantation. In contrast to IFN$\gamma$ responses, IL-10 secreting T cells were detected much less frequently and were of lower magnitudes in response to most of the ORF pools tested. The most consistent IL-10 T cell responses were found to the US3 and pp71 pool, as well as to gB. Each patient's results are presented individually below.

### Patient 133

The viral load of patient 133 was first detected at week 6, and peaked at week 13, following transplantation (Figure 5.6). Viral load remained elevated until week 35, following which virus was not detected in the peripheral blood again (Figure 5.6). Seroconversion was detected at week 16 post transplantation (Figure 5.6). Patient 133 had very early rising IFN$\gamma$ secreting T cells which were maintained at a relatively stable frequency over the regular sampling period, remaining relatively unaffected by peak viral load (Figure 5.6) and a decrease in total peripheral blood lymphocytes at weeks 5 and 15 post transplantation (Figure 5.6F). This

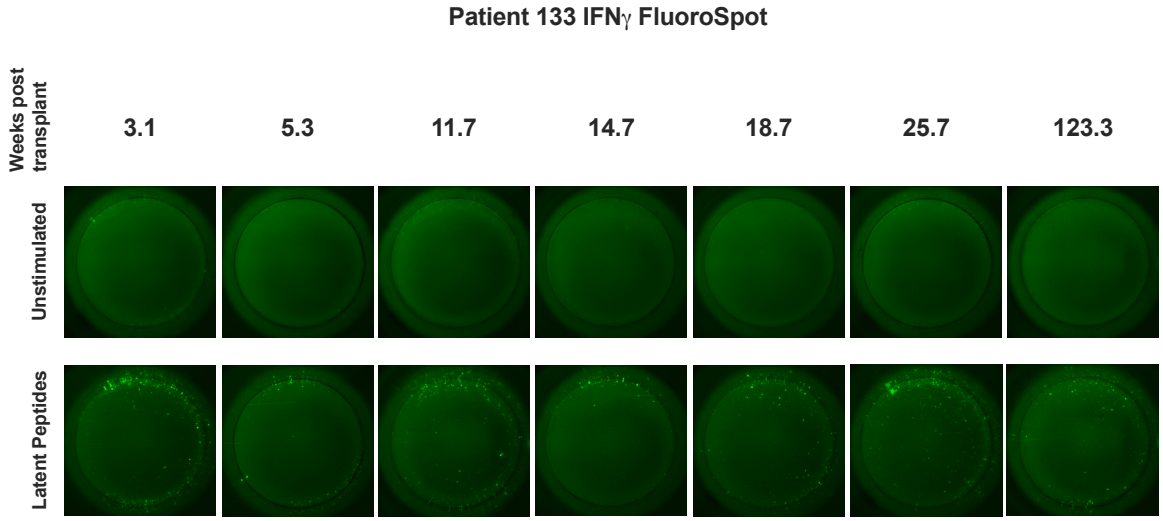



**Fig. 5.5 The individual FluoroSpot wells showing the frequencies of T cells secreting IFN$\gamma$ following primary infection in response to HCMV latency-associated peptides** Blood samples were collected at several time points from seronegative transplant patient 133 following the transplantation of a seropositive kidney. These samples were analysed for T cell secretion of IFN$\gamma$ in response to HCMV latency-associated ORFs. PBMC were incubated for 48 hours with an ORF peptide mix composed of UL138, LUNA, US28, and UL111A (latent peptides), or with media only (unstimulated), on FluoroSpot plates capable of detecting IFN$\gamma$. Each ORF peptide pool was tested in triplicate and one well of each triplicate is represented here for each time point.

pattern was the same for responses to all five ORF pools tested, although the frequencies of cytokine secreting cells detected did differ. IL-10 T cell responses on the other hand were not observed to all ORF pools. T cells secreting IL-10 in response to gB were found to fluctuate. Immediately following transplantation IL-10 secreting cells were detected, but these preceded to drop below the positive threshold just after the peak of viral load, before increasing again and dropping below threshold again by the last time point (Figure 5.6B). IL-10 secreting cells were only found at one time point in response to pp65 and UL144, coincidental with peak viral load (Figure 5.6C). T cell IL-10 responses to US3 and pp71 showed a similar oscillating pattern as those responding to gB, although the frequencies of IL-10 positive cells detected were higher at each time point and there was only one drop below the positive threshold, following peak viral load (Figure 5.6D).

### Patient 439

Patient 439 had detectable viral load from week 7 post transplantation, which peaked at week 9 and gradually decreased to undetectable levels at week 14 (Figure 5.7). Seroconversion in patient 439 was detected at week 10 post transplantation (Figure 5.7). Patient 439 T cell responses to ORF pools could also be detected immediately after transplantation and these responses exhibited a peak in the frequencies of IFN$\gamma$ secreting T cells coinciding with the the peak of viral load (Figure 5.7) and drop in total lymphocyte numbers (Figure 5.7F). The frequencies of IFN$\gamma$ secreting T cells then decreased, most dramatically in response to gB which fell below the positive threshold (Figure 5.7B), before stabilising to around the same levels as before the peak by week 17 (Figure 5.7). Positive IL-10 T cell responses were first detected in response to gB (Figure 5.7B), pp65 and UL144 (Figure 5.7C), and UL138, LUNA, US28, and UL111A (Figure 5.7E) 7 weeks post transplantation, concurrent with the rise in virus load and decrease in total lymphocyte numbers (Figure 5.7F). These responses then dropped below threshold and either rebounded for responses to gB and the pp65 and UL144 pool, or remained below threshold. T cell IL-10 responses to US3 and pp71 were detected immediately post transplantation and then began decreasing from 7 weeks post transplant as virus load increased (Figure 5.7D).

### Patient 574

Virus present in the peripheral blood of patient 574 was first detected 7 weeks post transplantation and remained detectable until week 16 (Figure 5.8). Viraemia then recurred at week 21 and lasted until week 35, with seroconversion detected in week 25 (Figure 5.8).

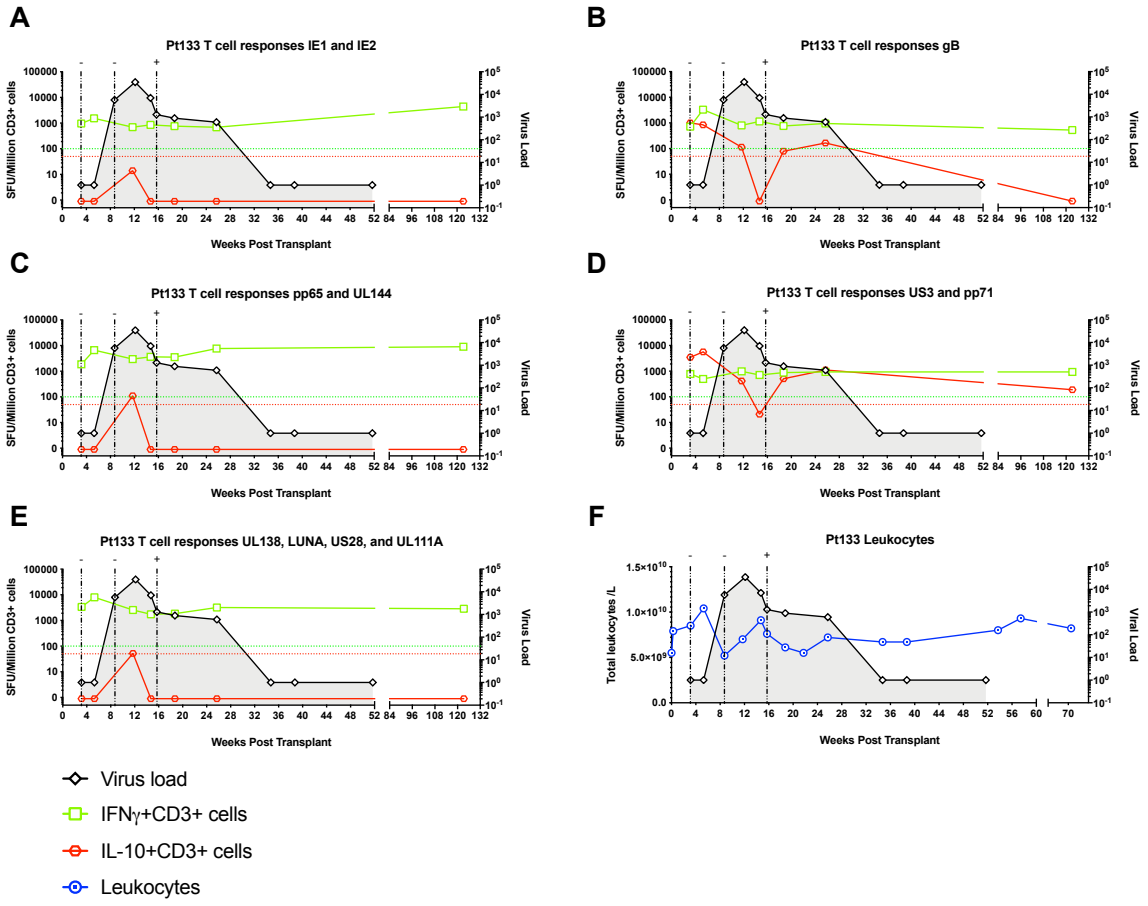



**Fig. 5.6 The frequencies of HCMV-specific T cells secreting IFN$\gamma$ or IL-10 following primary infection** Blood samples were collected at various time points from seronegative transplant patient 133 following the transplantation of a seropositive kidney. These samples were analysed for T cell secretion of IFN$\gamma$ and IL-10 in response to HCMV ORF peptide pools. PBMC were incubated for 48 hours with ORF peptide mixes composed of (A) IE1 and IE2, (B) gB, (C) pp65 and UL144, (D) US3 and pp71, and (E) UL138, LUNA, US28, and UL111A on FluoroSpot plates capable of detecting IFN$\gamma$ and IL-10 secretion. Each ORF peptide pool was tested in triplicate and the values from the number of background cells secreting cytokines in the unstimulated wells was deducted from all test wells. Following stimulation, the number of cytokine positive spots was enumerated by the automated cell counter and converted to a spot forming unit (SFU) value per million cells. PBMC were also analysed for their composition of CD3+, CD4+, and CD8+ cells by flow cytometry, and the values for cytokine secreting cells adjusted as a proportion of CD3+ T cells per donor. Virus load expressed as viral copies/ml of blood. The black vertical dotted lines indicate the time points sampled for serum anti-HCMV IgG, the outcome of which is denoted above the line as negative (-) or positive (+) for the time of seroconversion. The horizontal dotted red line indicates the 50 SFU/$10^6$ cell threshold for positive IL-10 responses, while the green dotted line at 100 SFU/$10^6$ illustrated the threshold for positive IFN$\gamma$ responses. (F) Total leukocyte counts per litre of blood were also carried out over a range of time points.

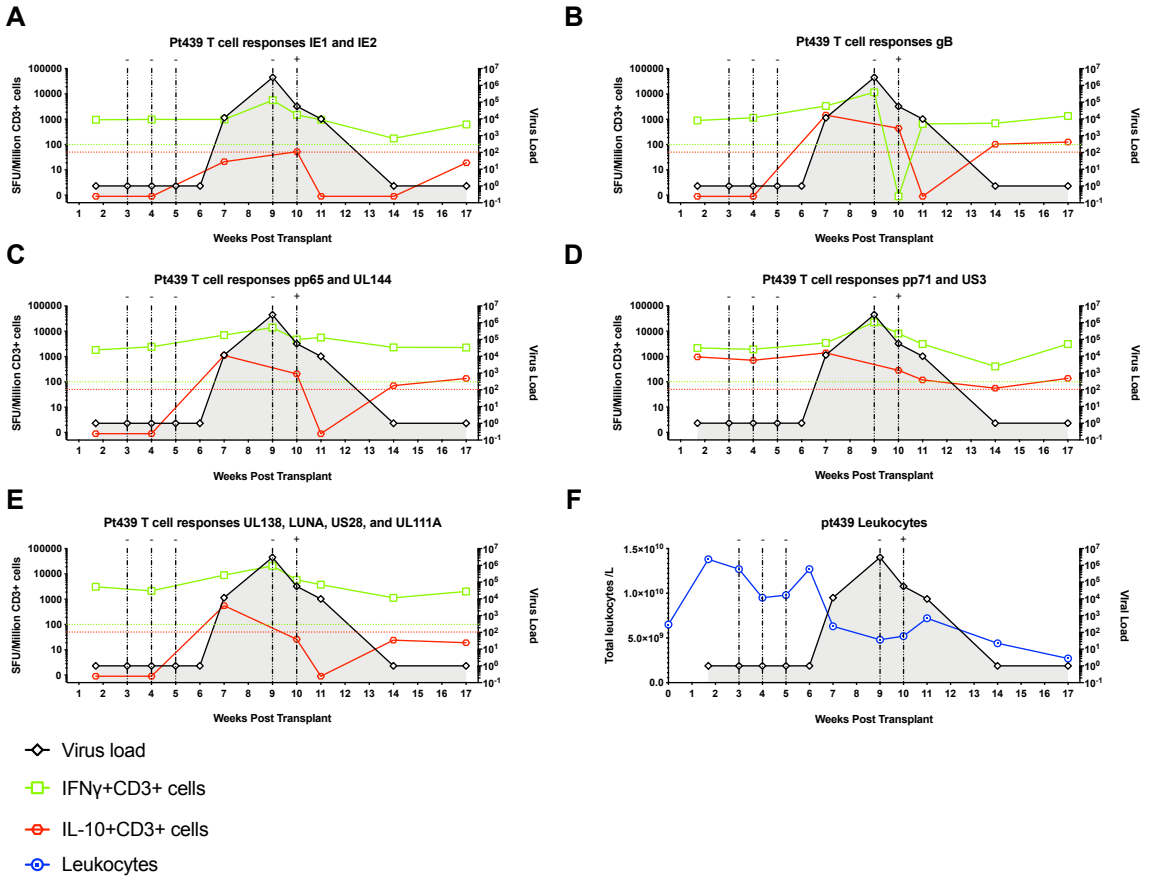



**Fig. 5.7 The frequencies of HCMV-specific T cells secreting IFN$\gamma$ or IL-10 following primary infection** Blood samples were collected at various time points from seronegative transplant patient 439 following the transplantation of a seropositive kidney. These samples were analysed for T cell secretion of IFN$\gamma$ and IL-10 in response to HCMV ORF peptide pools. PBMC were incubated for 48 hours with ORF peptide mixes composed of (A) IE1 and IE2, (B) gB, (C) pp65 and UL144, (D) US3 and pp71, and (E) UL138, LUNA, US28, and UL111A on FluoroSpot plates capable of detecting IFN$\gamma$ and IL-10 secretion. Each ORF peptide pool was tested in triplicate and the values from the number of background cells secreting cytokines in the unstimulated wells was deducted from all test wells. Following stimulation, the number of cytokine positive spots was enumerated by the automated cell counter and converted to a spot forming unit (SFU) value per million cells. PBMC were also analysed for their composition of CD3+, CD4+, and CD8+ cells by flow cytometry, and the values for cytokine secreting cells adjusted as a proportion of CD3+ T cells per donor. Virus load expressed as viral copies/ml of blood. The black vertical dotted lines indicate the time points sampled for serum anti-HCMV IgG, the outcome of which is denoted above the line as negative (-) or positive (+) for the time of seroconversion. The horizontal dotted red line indicates the 50 SFU/$10^6$ cell threshold for positive IL-10 responses, while the green dotted line at 100 SFU/$10^6$ illustrated the threshold for positive IFN$\gamma$ responses. (F) Total leukocyte counts per litre of blood were also carried out over a range of time points.

At the first sample time point, one day prior to transplantation, although patient 574 was negative for HCMV according to serology, positive IFN$\gamma$ T cell responses were detected to all ORF pools (Figure 5.8). The IFN$\gamma$ secreting T cells were then observed to decrease following transplantation before increasing contemporaneously with viral load (Figure 5.8). Although the magnitude of T cell IFN$\gamma$ responses to all ORF pools remained elevated, the virus was not being controlled, as evidenced by the elevated viral load across the majority of time points (Figure 5.8). T cells secreting IL-10 were found to exhibit a similar response pattern as those secreting IFN$\gamma$ in response to gB (Figure 5.8B ) and to the US3 and pp71 pool (Figure 5.8D). These high frequency IL-10 responses were detected immediately post transplant and peaked coincidentally with the peak virus load. HCMV-specific IFN$\gamma$ and IL-10 secreting T cell frequencies were unaffected by the fluctuations in total lymphocyte number (Figure 5.8F).

**Patient 352**

Patient 352 exhibited a similar inability to control viral replication with periodic spikes of high virus load detected in these post transplantation blood samples which persisted over the majority of the sampled time points (Figure 5.9). Seroconversion was detected in week 7 post transplantation (Figure 5.9). IFN$\gamma$ secreting T cells were detected in response to all ORF pools from the first post transplant sample, and these increased to a peak just after the peak of virus load at 11 weeks post transplantation (Figure 5.9), despite a decrease in total lymphocyte numbers at this point (Figure 5.9F). IFN$\gamma$ positive T cells were maintained at a high level but showed oscillations over the remaining sample points (Figure 5.9). IL-10 T cell responses were only detected to gB, and to US3 and pp71. IL-10 responses to gB were present at the first two time points within 5 weeks post transplantation but then no positive response was observed again until week 54 and week 97 (Figure 5.9B). T cell IL-10 responses to US3 and pp71 on the other hand were detectable at all time points and followed a similar pattern and frequency of positive cells as those secreting IFN$\gamma$ (Figure 5.9D).

**Patient 197**

The virus load of patient 197 showed one peak at week 14 and remained detectable until week 22, with seroconversion also detected at week 14 (Figure 5.10). T cell responses from patient 197 showed two different patterns over the sampling period. Responses to gB (Figure 5.10B), pp65 and UL144 (Figure 5.10C), and UL138, LUNA, US28, and UL111A (Figure 5.10E) all displayed a high frequency of IFN$\gamma$ positive T cells immediately following

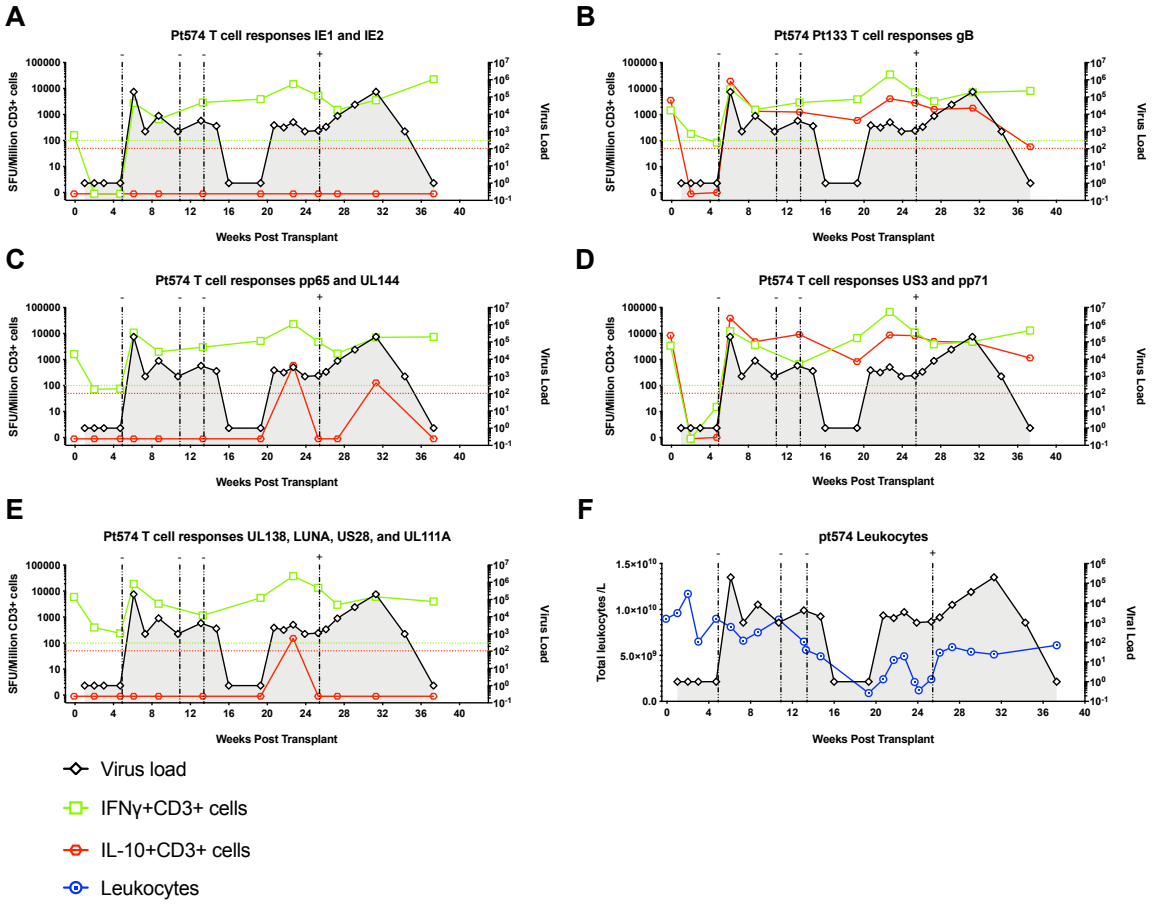



**Fig. 5.8 The frequencies of HCMV-specific T cells secreting IFN$\gamma$ or IL-10 following primary infection** Blood samples were collected at various time points from seronegative transplant patient 574 following the transplantation of a seropositive kidney. These samples were analysed for T cell secretion of IFN$\gamma$ and IL-10 in response to HCMV ORF peptide pools. PBMC were incubated for 48 hours with ORF peptide mixes composed of (A) IE1 and IE2, (B) gB, (C) pp65 and UL144, (D) US3 and pp71, and (E) UL138, LUNA, US28, and UL111A on FluoroSpot plates capable of detecting IFN$\gamma$ and IL-10 secretion. Each ORF peptide pool was tested in triplicate and the values from the number of background cells secreting cytokines in the unstimulated wells was deducted from all test wells. Following stimulation, the number of cytokine positive spots was enumerated by the automated cell counter and converted to a spot forming unit (SFU) value per million cells. PBMC were also analysed for their composition of CD3+, CD4+, and CD8+ cells by flow cytometry, and the values for cytokine secreting cells adjusted as a proportion of CD3+ T cells per donor. Virus load expressed as viral copies/ml of blood. The black vertical dotted lines indicate the time points sampled for serum anti-HCMV IgG, the outcome of which is denoted above the line as negative (-) or positive (+) for the time of seroconversion. The horizontal dotted red line indicates the 50 SFU/$10^6$ cell threshold for positive IL-10 responses, while the green dotted line at 100 SFU/$10^6$ illustrated the threshold for positive IFN$\gamma$ responses. (F) Total leukocyte counts per litre of blood were also carried out over a range of time points.

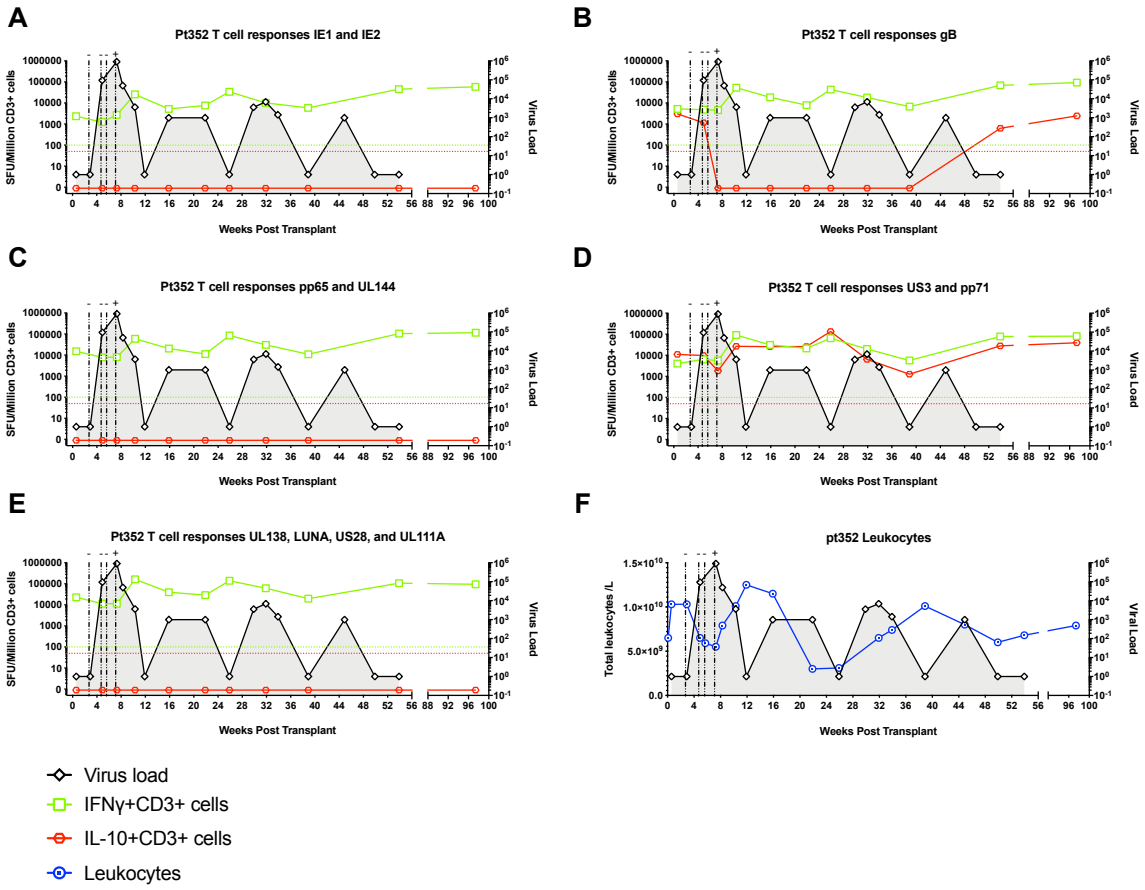



**Fig. 5.9 The frequencies of HCMV-specific T cells secreting IFN$\gamma$ or IL-10 following primary infection** Blood samples were collected at various time points from seronegative transplant patient 352 following the transplantation of a seropositive kidney. These samples were analysed for T cell secretion of IFN$\gamma$ and IL-10 in response to HCMV ORF peptide pools. PBMC were incubated for 48 hours with ORF peptide mixes composed of (A) IE1 and IE2, (B) gB, (C) pp65 and UL144, (D) US3 and pp71, and (E) UL138, LUNA, US28, and UL111A on FluoroSpot plates capable of detecting IFN$\gamma$ and IL-10 secretion. Each ORF peptide pool was tested in triplicate and the values from the number of background cells secreting cytokines in the unstimulated wells was deducted from all test wells. Following stimulation, the number of cytokine positive spots was enumerated by the automated cell counter and converted to a spot forming unit (SFU) value per million cells. PBMC were also analysed for their composition of CD3+, CD4+, and CD8+ cells by flow cytometry, and the values for cytokine secreting cells adjusted as a proportion of CD3+ T cells per donor. Virus load expressed as viral copies/ml of blood. The black vertical dotted lines indicate the time points sampled for serum anti-HCMV IgG, the outcome of which is denoted above the line as negative (-) or positive (+) for the time of seroconversion. The horizontal dotted red line indicates the 50 SFU/$10^6$ cell threshold for positive IL-10 responses, while the green dotted line at 100 SFU/$10^6$ illustrated the threshold for positive IFN$\gamma$ responses. (F) Total leukocyte counts per litre of blood were also carried out over a range of time points.

transplantation but subsequently declined and settled at a lower level than the initial response. T cell IFN$\gamma$ responses to IE1 and IE2 (Figure 5.10A), and US3 and pp71 (Figure 5.10D) were also detected from the initial time point, but these gradually increased and peaked at over 100 weeks post transplantation. T cells secreting IL-10 were only detected in response to pp65 and UL144 (Figure 5.10C), and UL138, LUNA, US28, and UL111A (Figure 5.10E) at the final time point (157 weeks post transplantation), while IL-10 responses to gB fluctuated, with responses above threshold at two time points (50 and 115 weeks post transplant) (Figure 5.10B). IL-10 T cell responses to US3 and pp71 were detected early post transplantation and showed a gradual increase to 115 weeks post transplant before declining slightly (Figure 5.10D). Despite initial decreases in total lymphocyte numbers, the frequencies of cytokine secreting HCMV-specific T cells were not affected (Figure 5.10F).

### Patient 136

The viral load of patient 136 was first detected at week 7, peaked at week 9, and remained elevated until week 15 post transplantation (Figure 5.11). At week 51, virus was again detected in the peripheral blood but was not present at any later time point (Figure 5.11). Seroconversion was measured at 11 weeks post transplantation (Figure 5.11). Unfortunately, PBMC samples from patient 136 were only obtained from 7 weeks post transplantation so T cell responses prior to this are unknown. T cell IFN$\gamma$ responses to IE1 and IE2 (Figure 5.11A), and pp65 and UL144 (Figure 5.11C) were found to be relatively constant over the first 36 weeks, with a slight decrease in the IE1 and IE2 pool response magnitude, but after 36 weeks the frequency of IFN$\gamma$ positive cells was found to increase into the 80 week time point. T cell IFN$\gamma$ responses to gB (Figure 5.11B), US3 and pp71 (Figure 5.11D), and UL138, LUNA, US28, and UL111A (Figure 5.11E) pools decreased following the peak of viral load, but where response magnitudes to gB, and UL138, LUNA, US28, and UL111A increased to around the initial post transplant levels, response to US3 and pp71 stabilised to below their initial level. Positive T cell IL-10 responses were sporadically found in this patient. Several relatively low frequency responses to gB observed (Figure 5.11B) and responses to US3 and pp71 were found to decline from initially positive responses to below threshold before increasing again to positive responses consistently after 50 weeks post transplantation (Figure 5.11D). T cell responses were again maintained despite decreased lymphocyte numbers from four weeks post transplantation (Figure 5.11F).

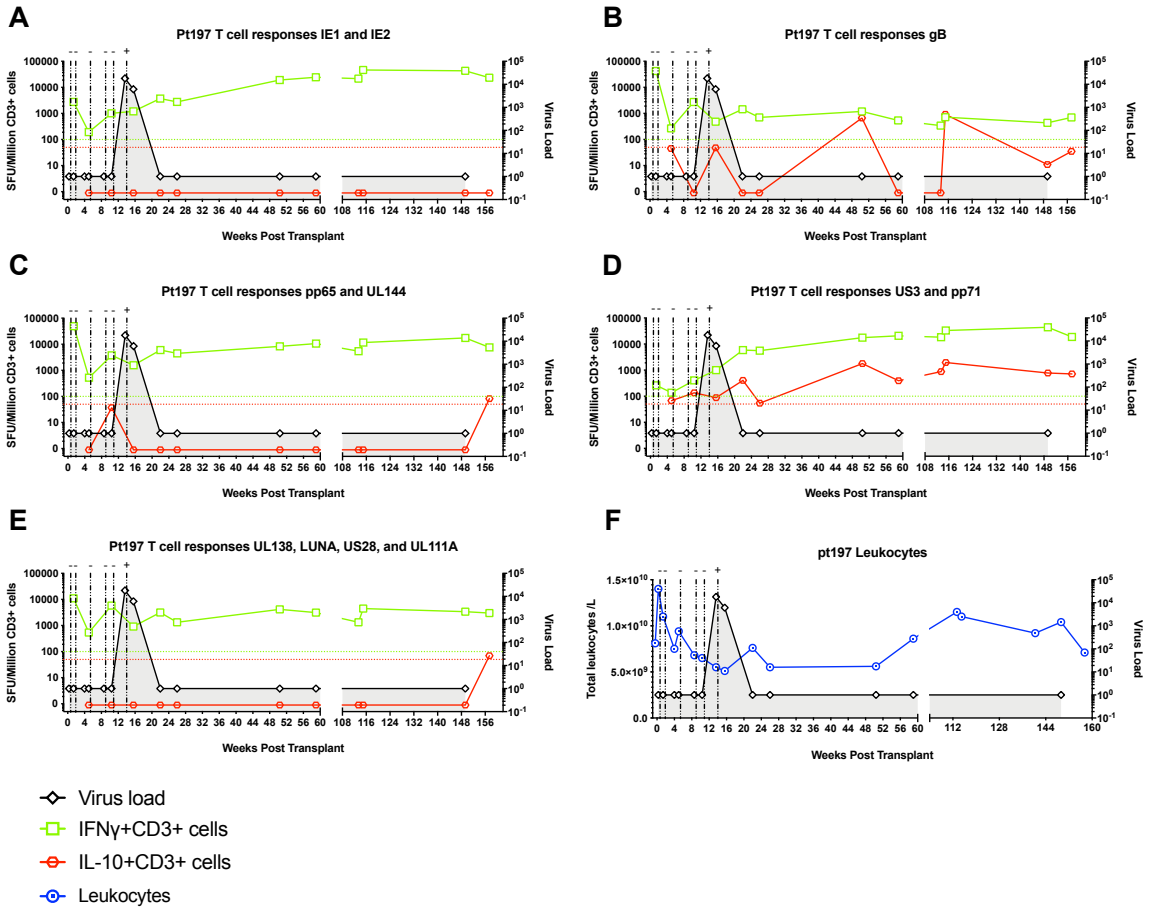



**Fig. 5.10 The frequencies of HCMV-specific T cells secreting IFN$\gamma$ or IL-10 following primary infection** Blood samples were collected at various time points from seronegative transplant patient 197 following the transplantation of a seropositive kidney. These samples were analysed for T cell secretion of IFN$\gamma$ and IL-10 in response to HCMV ORF peptide pools. PBMC were incubated for 48 hours with ORF peptide mixes composed of (A) IE1 and IE2, (B) gB, (C) pp65 and UL144, (D) US3 and pp71, and (E) UL138, LUNA, US28, and UL111A on FluoroSpot plates capable of detecting IFN$\gamma$ and IL-10 secretion. Each ORF peptide pool was tested in triplicate and the values from the number of background cells secreting cytokines in the unstimulated wells was deducted from all test wells. Following stimulation, the number of cytokine positive spots was enumerated by the automated cell counter and converted to a spot forming unit (SFU) value per million cells. PBMC were also analysed for their composition of CD3+, CD4+, and CD8+ cells by flow cytometry, and the values for cytokine secreting cells adjusted as a proportion of CD3+ T cells per donor. Virus load expressed as viral copies/ml of blood. The black vertical dotted lines indicate the time points sampled for serum anti-HCMV IgG, the outcome of which is denoted above the line as negative (-) or positive (+) for the time of seroconversion. The horizontal dotted red line indicates the 50 SFU/$10^6$ cell threshold for positive IL-10 responses, while the green dotted line at 100 SFU/$10^6$ illustrated the threshold for positive IFN$\gamma$ responses. (F) Total leukocyte counts per litre of blood were also carried out over a range of time points.

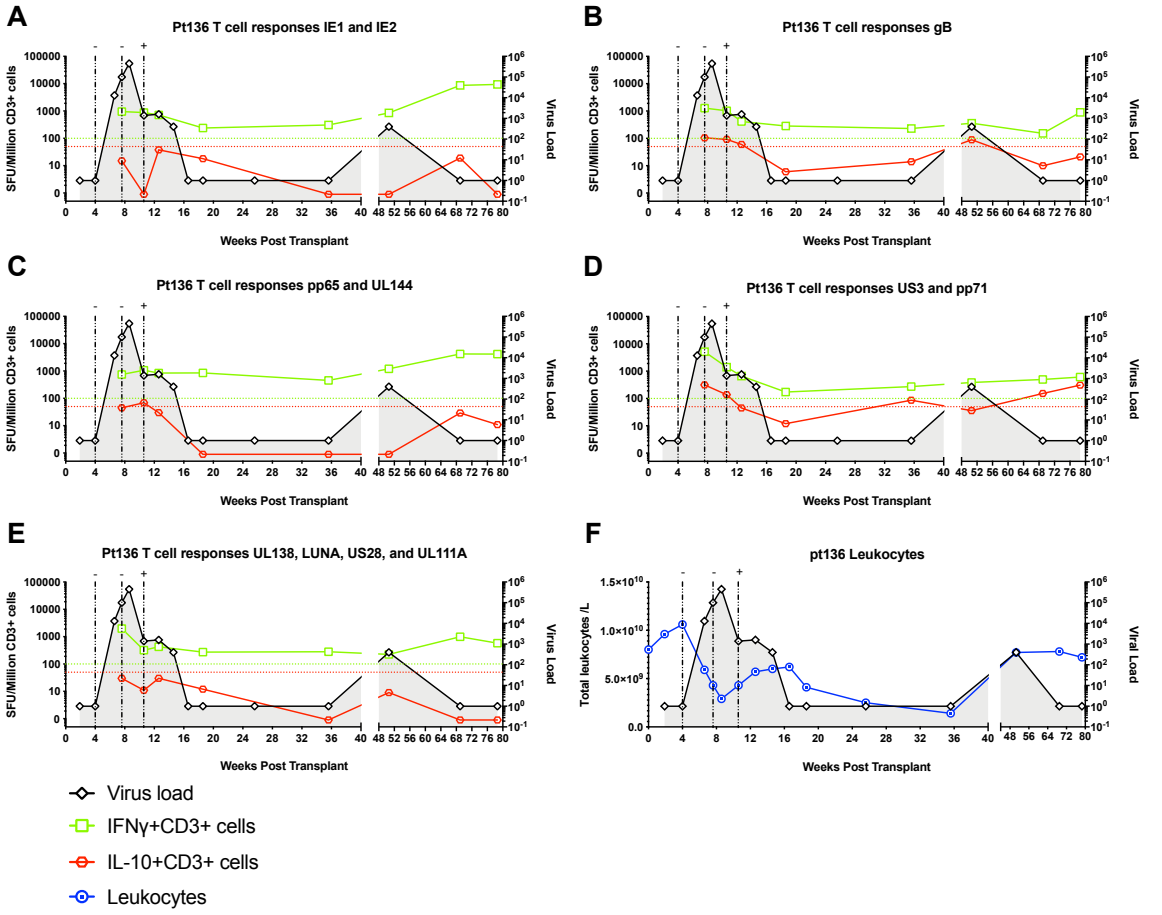



**Fig. 5.11 The frequencies of HCMV-specific T cells secreting IFN$\gamma$ or IL-10 following primary infection** Blood samples were collected at various time points from seronegative transplant patient 136 following the transplantation of a seropositive kidney. These samples were analysed for T cell secretion of IFN$\gamma$ and IL-10 in response to HCMV ORF peptide pools. PBMC were incubated for 48 hours with ORF peptide mixes composed of (A) IE1 and IE2, (B) gB, (C) pp65 and UL144, (D) US3 and pp71, and (E) UL138, LUNA, US28, and UL111A on FluoroSpot plates capable of detecting IFN$\gamma$ and IL-10 secretion. Each ORF peptide pool was tested in triplicate and the values from the number of background cells secreting cytokines in the unstimulated wells was deducted from all test wells. Following stimulation, the number of cytokine positive spots was enumerated by the automated cell counter and converted to a spot forming unit (SFU) value per million cells. PBMC were also analysed for their composition of CD3+, CD4+, and CD8+ cells by flow cytometry, and the values for cytokine secreting cells adjusted as a proportion of CD3+ T cells per donor. Virus load expressed as viral copies/ml of blood. The black vertical dotted lines indicate the time points sampled for serum anti-HCMV IgG, the outcome of which is denoted above the line as negative (-) or positive (+) for the time of seroconversion. The horizontal dotted red line indicates the 50 SFU/$10^6$ cell threshold for positive IL-10 responses, while the green dotted line at 100 SFU/$10^6$ illustrated the threshold for positive IFN$\gamma$ responses. (F) Total leukocyte counts per litre of blood were also carried out over a range of time points.

**Patient 365**

Peak viraemia in patient 365 occurred at week 7 post transplantation with virus remaining detectable in the peripheral blood until week 12 (Figure 5.12). Seroconversion was measured at week 9 post transplantation (Figure 5.12). Initially, T cell IFN$\gamma$ secretion was detected in response to all ORFs, although the frequencies of IFN$\gamma$ positive cells then decreased to below the positive threshold following peak viral load (Figure 5.12). This was most dramatically seen in T cell responses to IE1 and IE2 (Figure 5.12A), gB (Figure 5.12B), and US3 and pp71 (Figure 5.12D). IFN$\gamma$ secreting T cells then rebounded and increased in frequency to a peak at 28 or 40 weeks post transplantation before contracting (Figure 5.12). IL-10 T cell responses to gB (Figure 5.12B), and US3 and pp71 (Figure 5.12D), were similar to IFN$\gamma$ responses in that a decline in IL-10 secreting cells to below threshold was observed after peak viral load which then rebounded by week 27 to positive. There was then a decline in IL-10 positive cells. IL-10 responses to pp65 and UL144 also showed a similar pattern but lower frequencies of cytokine secreting cells were detected (Figure 5.12C). The decline in cytokine secreting T cells following peak viral load at week 7 was also coincidental with a decrease in total lymphocyte numbers (Figure 5.12F). Furthermore, the increase in cytokine secreting cells from week 16 parallels the increase in total lymphocytes present in the peripheral blood from this time point (Figure 5.12F).

In summary, IFN$\gamma$ T cell responses to lytic and latency-associated proteins were detectable immediately post transplantation and these were generally maintained throughout the sampling period with some fluctuations. These T cell responses were also detectable prior to viraemia and in one case prior to transplantation, despite the donor being HCMV-negative when tested by serology. IL-10 T cell responses to latency-associated proteins were inconsistently seen or were of low frequency in this time period. Likewise no T cell IL-10 responses were observed to IE1 and IE2. T cells secreting IL-10 were however consistently found in response to US3 and pp71, and these were maintained across the time period tested in most donors.

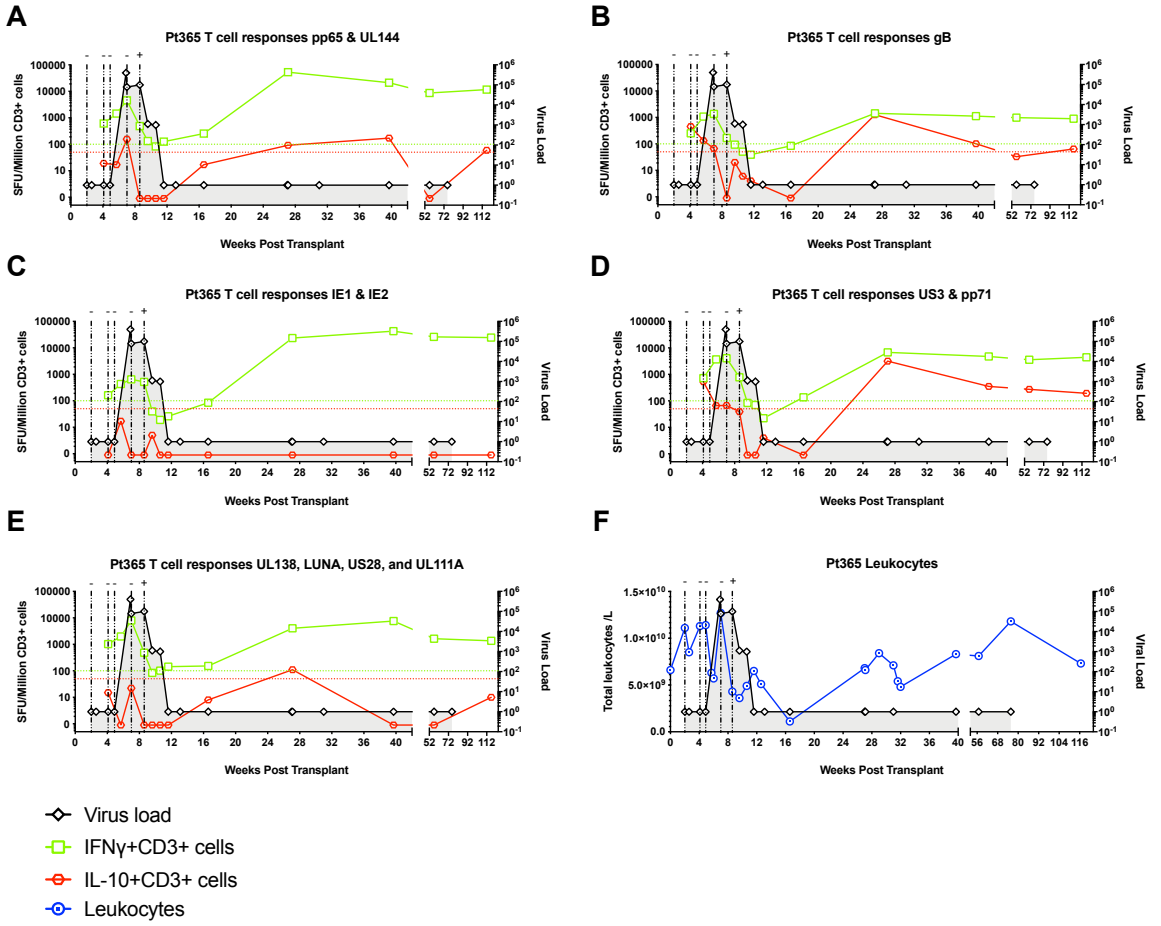



**Fig. 5.12 The frequencies of HCMV-specific T cells secreting IFN$\gamma$ or IL-10 following primary infection** Blood samples were collected at various time points from seronegative transplant patient 365 following the transplantation of a seropositive kidney. These samples were analysed for T cell secretion of IFN$\gamma$ and IL-10 in response to HCMV ORF peptide pools. PBMC were incubated for 48 hours with ORF peptide mixes composed of (A) IE1 and IE2, (B) gB, (C) pp65 and UL144, (D) US3 and pp71, and (E) UL138, LUNA, US28, and UL111A on FluoroSpot plates capable of detecting IFN$\gamma$ and IL-10 secretion. Each ORF peptide pool was tested in triplicate and the values from the number of background cells secreting cytokines in the unstimulated wells was deducted from all test wells. Following stimulation, the number of cytokine positive spots was enumerated by the automated cell counter and converted to a spot forming unit (SFU) value per million cells. PBMC were also analysed for their composition of CD3+, CD4+, and CD8+ cells by flow cytometry, and the values for cytokine secreting cells adjusted as a proportion of CD3+ T cells per donor. Virus load expressed as viral copies/ml of blood. The black vertical dotted lines indicate the time points sampled for serum anti-HCMV IgG, the outcome of which is denoted above the line as negative (-) or positive (+) for the time of seroconversion. The horizontal dotted red line indicates the 50 SFU/$10^6$ cell threshold for positive IL-10 responses, while the green dotted line at 100 SFU/$10^6$ illustrated the threshold for positive IFN$\gamma$ responses. (F) Total leukocyte counts per litre of blood were also carried out over a range of time points.

### 5.2.3 Detection of IL-10 responses to latency-associated proteins from tissue resident T cells

It is possible that IL-10 secreting T cells specific for HCMV latency-associated proteins were not detected following primary infection because these T cells were confined to tissue sites and had not yet entered the peripheral blood. To investigate the presence of HCMV-specific T cells in tissue sites, preliminary work was carried out using bone marrow samples obtained from two HCMV positive donors.

Bone marrow derived mononuclear cells (BMMNC) were tested for IFN$\gamma$ and IL-10 secretion by FluoroSpot assay. BMMNC were incubated with HCMV ORF peptide pool mixes for 48 hours on FluoroSpot plates and the frequencies of IFN$\gamma$ and IL-10 positive cells determined. BMMNC were also stained for CD3 to determine the proportion of T cells present. The frequencies of cytokine secreting cells detected was then expressed as a proportion of all T cells. An initial screen was carried out with BMMNC from one donor to test if responses to HCMV ORF products were present. IFN$\gamma$ or IL-10 secreting T cells were detected to all of the HCMV ORFs tested (Figure 5.13). The frequencies of cytokine secreting T cells in response to certain ORFs were very low, such as to UL138 (Figure 5.13). As it was not possible to test bone marrow T cell responses from HCMV seronegative donors, a threshold for positive responses could not be determined. It is therefore possible that these low frequency responses were due to non-HCMV specific T cell activation. However, as the background frequencies of cytokine secreting cells in unstimulated cells were exceptionally low, these responses were likely not due to a general activation of the donor's T cells.

Having demonstrated that bone marrow derived T cells could respond to HCMV ORFs, paired bone marrow and blood mononuclear cell samples were obtained from one donor to test if these different compartments possessed ORF-specific T cells of different frequencies. BMMNC and PBMC samples were depleted of CD4+ or CD8+ cells by MACS, to test CD8+ and CD4+ T cell responses respectively, and then analysed in parallel on the FluoroSpot. Doing so enabled the frequencies of IFN$\gamma$ and IL-10 secreting T cells specific to HCMV ORF products to be determined.

Considerable differences in the frequencies of T cells secreting IFN$\gamma$ or IL-10 were found between bone marrow and peripheral blood derived mononuclear cells. High frequencies of ORF-specific IFN$\gamma$ secreting CD4+ T cells were detected in both the bone marrow and peripheral blood. However, greater frequencies of IFN$\gamma$ secreting CD4+ T cells were detected in the bone marrow compared to the peripheral blood for the majority of responses, with the exception of responses to LUNA, which were only detected in the blood (Figure 5.14A).

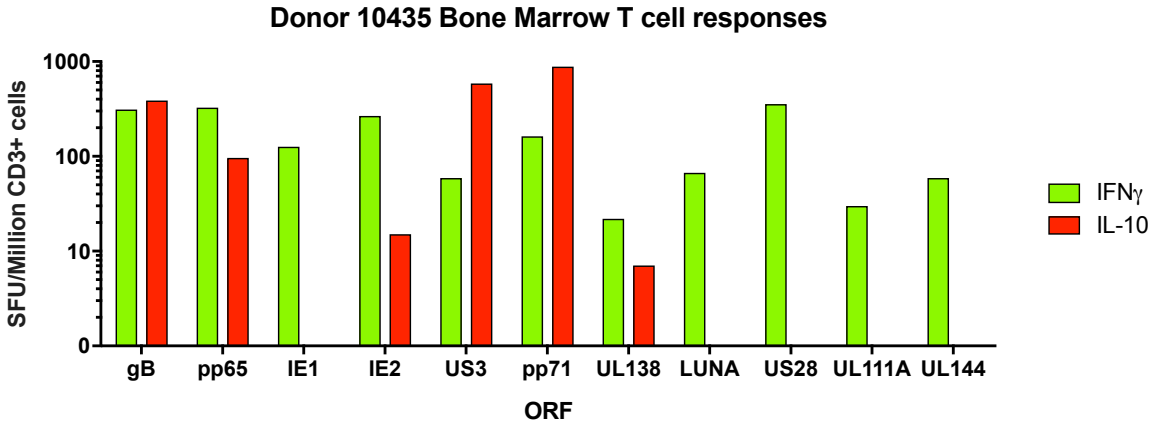



**Fig. 5.13 Bone marrow resident T cell responses to HCMV ORF peptide pools** Bone marrow mononuclear cells (BMMNC) from donor 10435 were incubated for 48 hours with HCMV ORF peptide pools on FluoroSpot plates capable of detecting IFN$\gamma$ and IL-10 secreting cells. Peptide pools composed of HCMV ORFs gB, pp65, IE1, IE2, US3, pp71, UL138, LUNA, US28, UL111A, and UL144 were tested. Each ORF peptide pool was tested in triplicate and the values from the number of background cells secreting cytokines in the unstimulated wells was deducted from all test wells. Following stimulation, the number of IFN$\gamma$ (green bars) and IL-10 (red bars) positive spots was enumerated by the automated cell counter and converted to a spot forming unit (SFU) value per million cells. BMMNC were also analysed for their composition of CD3+ cells by flow cytometry, and the values for cytokine secreting cells adjusted as a proportion of CD3+ T cells.

IFN$\gamma$ responses to IE1 and UL138 were also only detected in the bone marrow (Figure 5.14A). Of the ORF-specific responses detected in both compartments (excluding LUNA), the proportions of peripheral blood CD4+ T cells secreting IFN$\gamma$ was reduced by up to 80% compared to bone marrow derived CD4+ T cells (Figure 5.16A). High frequency US3 and pp71 IL-10 T cell responses were detected in both the bone marrow and blood, although these were of greater frequency in the bone marrow by around 25% and 35% respectively (Figure 5.14B and 5.16B). IL-10 responses were also greater in the bone marrow in response to UL111A and IE1, where they were not detected in the blood, as well as to LUNA (Figure 5.14B). Conversely, IL-10 secreting CD4+ T cells responding to gB, pp65, and UL138 were only detected in the peripheral blood (Figure 5.14B).

Although the bone marrow harboured mostly greater frequencies of IFN$\gamma$ secreting CD4+ T cells compared to the peripheral blood, differences in the CD8+ T cell responses to HCMV were more varied between the two compartments (Figure 5.16C). Greater frequencies of IFN$\gamma$ secreting CD8+ T cells responding to gB, pp65, IE2, UL111A, and UL144 were detected in the peripheral blood compared to the bone marrow, while the reverse was true for responses to IE1, US3, pp71, and US28 (Figure 5.15A). CD8+ T cell IFN$\gamma$ responses were also only detected to UL138 and LUNA in the peripheral blood (Figure 5.15A). In contrast, greater frequencies of CD8+ T cells secreting IL-10 were generally found in the bone marrow as evidenced by responses to IE1, IE2, and US28, which were absent from the peripheral blood, and to pp65, US3, pp71, UL138, LUNA, UL111A, which were nearly twofold higher on average in the bone marrow (Figure 5.15B and 5.16D).

In summary, this preliminary data has highlighted differences in the frequencies of CD4+ and CD8+ T cells responding to HCMV ORFs between T cells derived from the bone marrow and peripheral blood. In particular, in CD4+ T cells secreting IFN$\gamma$ and CD8+ T cells secreting IL-10, seemed to be retained in the bone marrow in higher frequencies compared to the peripheral blood.

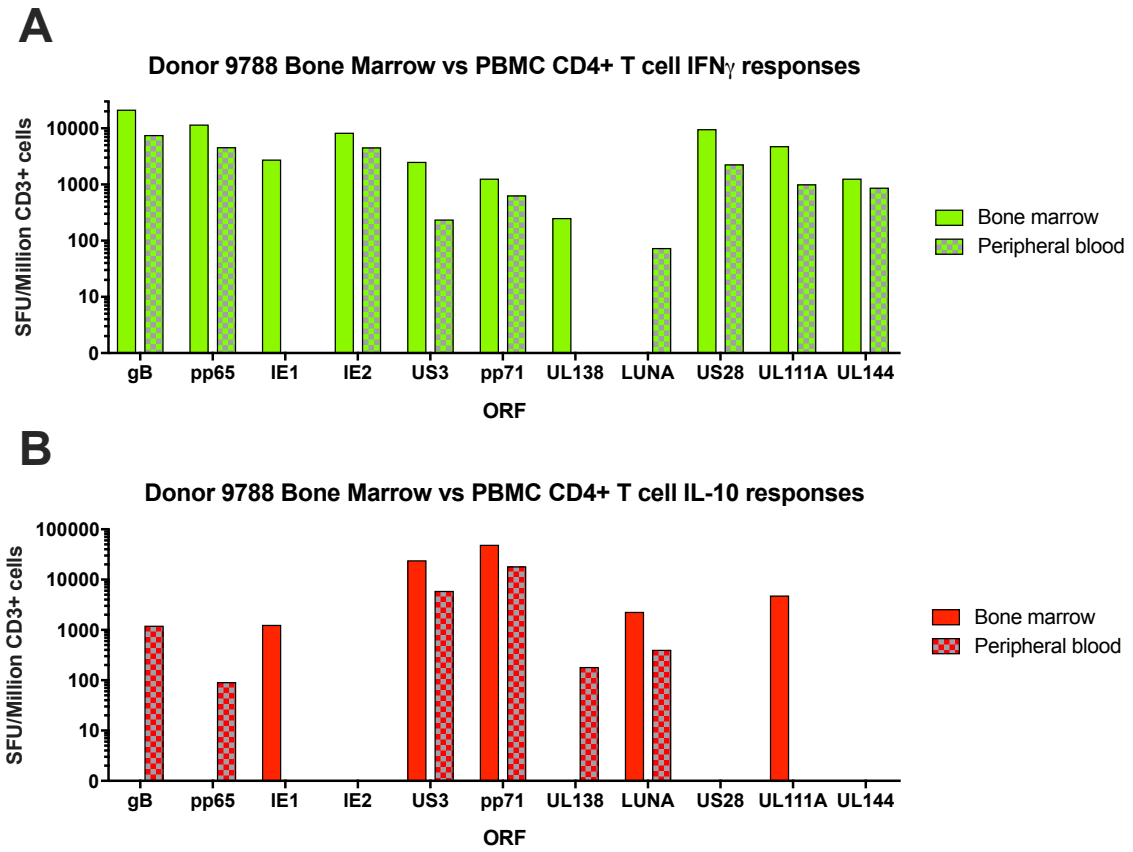



**Fig. 5.14 Bone marrow and peripheral blood derived CD4+ T cell responses to HCMV ORF peptide pools** Bone marrow mononuclear cells (BMMNC) and peripheral blood mononuclear cells (PBMC) from donor 9788 were depleted of CD8+ cells and incubated for 48 hours with HCMV ORF peptide pools on FluoroSpot plates capable of detecting IFN$\gamma$ and IL-10 secreting cells. Peptide pools composed of HCMV ORFs gB, pp65, IE1, IE2, US3, pp71, UL138, LUNA, US28, UL111A, and UL144 were tested. Each ORF peptide pool was tested in triplicate and the values from the number of background cells secreting cytokines in the unstimulated wells was deducted from all test wells. Following stimulation, the number of (A) IFN$\gamma$ positive spots from BMMNC (solid green bars) and PBMC (patterned green bars) and (B) IL-10 positive spots from BMMNC (solid red bars) and PBMC (patterned red bars) was enumerated by the automated cell counter and converted to a spot forming unit (SFU) value per million cells. BMMNC were also analysed for their composition of CD3+ cells by flow cytometry, and the values for cytokine secreting cells adjusted as a proportion of CD3+ T cells.

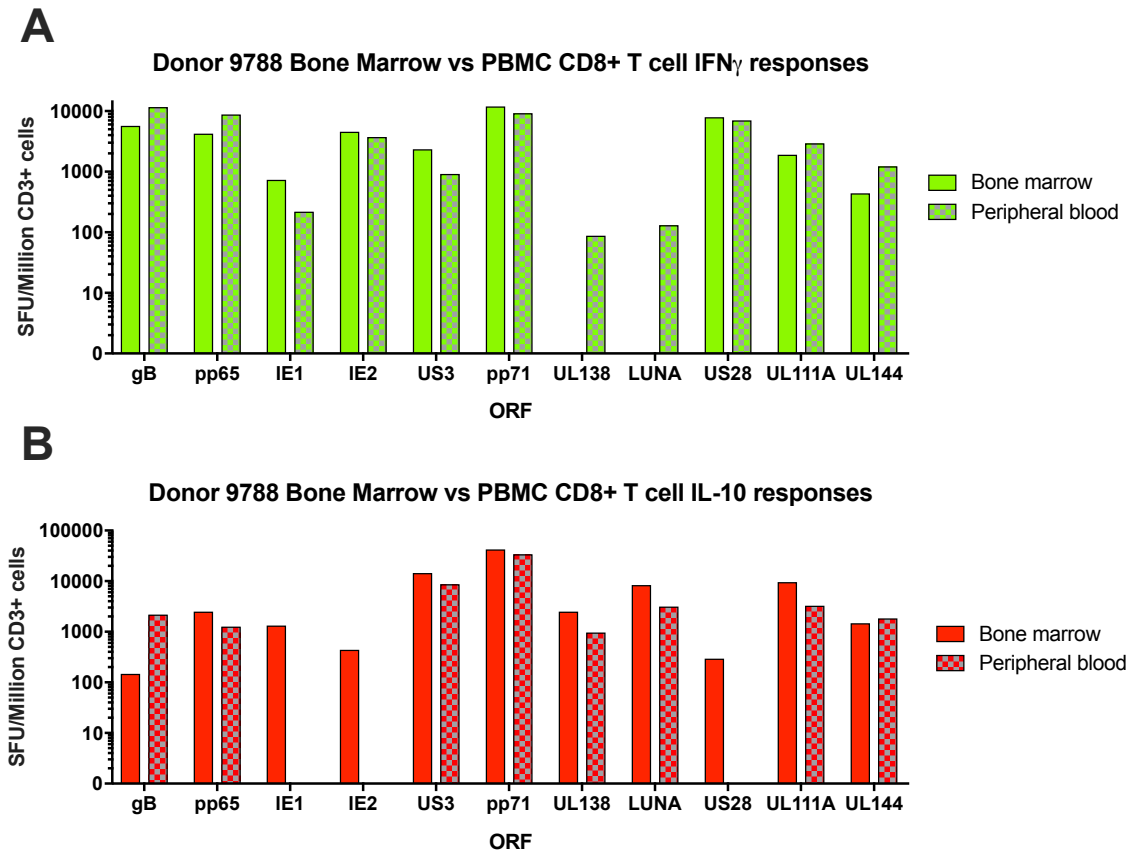



**Fig. 5.15 Bone marrow and peripheral blood derived CD8+ T cell responses to HCMV ORF peptide pools** Bone marrow mononuclear cells (BMMNC) and peripheral blood mononuclear cells (PBMC) from donor 9788 were depleted of CD4+ cells and incubated for 48 hours with HCMV ORF peptide pools on FluoroSpot plates capable of detecting IFN$\gamma$ and IL-10 secreting cells. Peptide pools composed of HCMV ORFs gB, pp65, IE1, IE2, US3, pp71, UL138, LUNA, US28, UL111A, and UL144 were tested. Each ORF peptide pool was tested in triplicate and the values from the number of background cells secreting cytokines in the unstimulated wells was deducted from all test wells. Following stimulation, the number of (A) IFN$\gamma$ positive spots from BMMNC (solid green bars) and PBMC (patterned green bars) and (B) IL-10 positive spots from BMMNC (solid red bars) and PBMC (patterned red bars) was enumerated by the automated cell counter and converted to a spot forming unit (SFU) value per million cells. BMMNC were also analysed for their composition of CD3+ cells by flow cytometry, and the values for cytokine secreting cells adjusted as a proportion of CD3+ T cells.

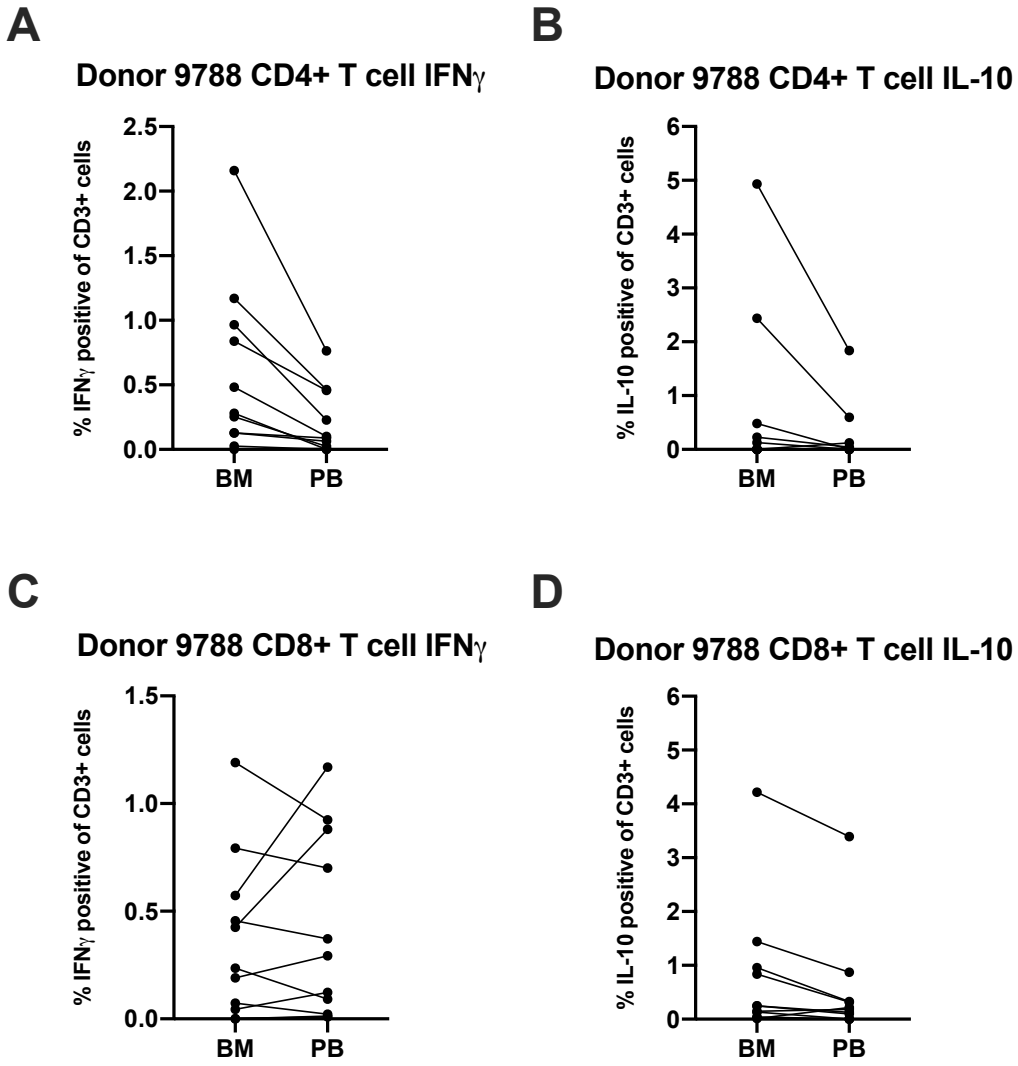



**Fig. 5.16 Proportions of bone marrow and peripheral blood derived CD4+ and CD8+ T cells secreting IFN$\gamma$ in response to HCMV ORF peptides pools** Paired bone marrow mononuclear cell (BM) and peripheral blood mononuclear cell (PB) samples from donor 9788 were depleted of CD8+ or CD4+ cells, to test (A and B) CD4+ and (C and D) CD8+ T cell responses respectively, and incubated for 48 hours with HCMV ORF peptide pools on FluoroSpot plates capable of detecting IFN$\gamma$ and IL-10 secreting cells. Peptide pools composed of HCMV ORFs gB, pp65, IE1, IE2, US3, pp71, UL138, LUNA, US28, UL111A, and UL144 were tested, and the responses to each ORF are represented as individual points. Each ORF peptide pool was tested in triplicate and the values from the number of background cells secreting cytokines in the unstimulated wells was deducted from all test wells. Following stimulation, the number of cytokine positive cells was enumerated by the automated cell counter. Samples were analysed for their composition of CD3+ cells by flow cytometry, and the values for cytokine secreting cells adjusted as a proportion of CD3+ T cells.

## 5.3 Discussion

The generation of IL-10 secreting T cells specific for HCMV latency-associated proteins following primary infection was investigated using blood samples collected from patients undergoing kidney transplantation (D+R-). Blood samples from seven initially seronegative transplant recipients were obtained over a period of several weeks post transplantation. T cell IFN$\gamma$ and IL-10 responses to HCMV ORF pools were analysed by FluoroSpot assay and the frequencies of cytokine secreting T cells determined. IFN$\gamma$ responses were deemed positive if they exceeded a 100 SFU/$10^6$ T cell threshold, while this was set at 50 SFU/$10^6$ T cells for IL-10 responses. However, this threshold was set using data from healthy HCMV seronegative donors for each ORF peptide mix, and to CD4+ and CD8+ T cells separately. It is therefore possible that the threshold values used here are not appropriate, and future work should test seronegative D-R- transplant patients with these peptide mixes to determine the frequencies of CD4+ and CD8+ T cells secreting IFN$\gamma$ and IL-10 non-specifically. Doing so is also important to determine if the transplant process has an effect on the background cytokine secretion from T cells. In several transplant patients studied here, at the initial time points following transplantation, IFN$\gamma$ secreting T cells could be detected in response to peptide stimulation, weeks before detectable viral load. The frequencies of IFN$\gamma$ secreting cells at these first time points were often between 0.1% and 1% of all T cells, or even higher for patients 197 and 352. In some cases these cells decreased in the subsequent weeks, before increasing again after, or in line with, peak viral load. These initial responses therefore seem erroneous given how quickly after transplantation many of these have been detected, especially as several previous studies examining T cell responses to primary HCMV infection found virus specific T cells to emerge only after viral load was detectable [333, 373, 381, 391, 392, 400, 406, 694]. Although low background cytokine secretion was detected from unstimulated T cells, indicating T cells were not spontaneously producing IFN$\gamma$, it could be possible that T cells isolated at such early time points post transplantation non-specifically produce IFN$\gamma$ in response to peptide stimulation.

The presence of T cell responses one day prior to transplantation in one donor (574) was also surprising, given their negative HCMV infection status as measured by serology. No additional patient information is available so it is not known what the health status of this individual was, which could influence non-specific T cell activity. However, recent work has revealed HCMV-specific T cell responses in HCMV seronegative individuals [659, 660]. One such study reported the presence of HCMV-specific CD4+ and CD8+ T cells capable of secreting IFN$\gamma$ in seronegative pretransplant patients [660]. HCMV seronegative transplant recipients with IFN$\gamma$ secreting CD4+ T cells were also observed to have a lower

risk of developing an HCMV viraemia after transplantation with a seropositive donor kidney compared to those without such T cells [660]. It could therefore be possible that the sole reliance on serology to test HCMV infectivity is not adequate, and this recipient had been exposed to the virus prior to transplantation. Likewise, this could also be possible for the other transplant patients who displayed IFN$\gamma$ responses shortly after transplantation. The detection of high frequency T cell responses so early before the peak of viral load could also be the result of viral replication being confined to the donor organ. In this manner, HCMV replicating in the kidney soon after transplantation would not be detected in the blood, which was sampled here, and could thus result in the delivery of viral antigens to the draining lymph nodes and the initiation of virus-specific T cell responses prior to detectable viraemia.

IFN$\gamma$ secreting T cells were found to arise immediately post transplantation in response to both HCMV lytic and latency-associated proteins. These positive IFN$\gamma$ responses were mostly maintained throughout the sampling period with a relative degree of fluctuation in the frequencies of cytokine secreting cells detected. Generally, CD4+ and CD8+ T cells secreting IFN$\gamma$ in response to latency-associated proteins made up less than 1% of total CD3+ cells within individuals, although these did reach around 5% in some patients (197, 365, and 574). Patient 352 was the exception to this, where T cell frequencies were mostly between 1% and 16% of total CD3+ cells. Patient 352 had recurrent viraemia for the majority of the sampled time points however, which could explain these high frequency T cell responses. The frequencies of IFN$\gamma$ secreting T cells responding to latency-associated ORFs following primary infection were comparable to those observed from healthy donors, which were also nearly entirely below 1% of total CD3+ cells (Mean=0.54, Std. deviation=0.73, Std. error of mean=0.17, n=19) (data not shown). Additionally, these frequencies of IFN$\gamma$ positive T cells detected following primary infection are comparable to the frequencies of HCMV-specific T cells reported in studies utilising tetramers or HCMV peptide/lysate stimulation and ICS during primary infection [333, 373, 381, 391, 392, 400, 406]. The ability to generate IFN$\gamma$ responses to latency-associated ORF products following primary infection therefore does not seem to be affected by immunosuppressive drug therapy. However, the data generated here do not distinguish between CD4+ and CD8+ T cells. It is therefore possible that the frequencies of CD4+ and CD8+ T cells specific to latency-associated ORFs in these immunosuppressed transplant recipients could differ compared to healthy individuals.

IL-10 T cell responses were considerably less common and exhibited more dramatic fluctuations than IFN$\gamma$ responses following primary infection. No consistent IL-10 T cell responses were detected to the latency-associated proteins during this initial period following primary infection. Many donors did have IL-10 responses to gB early following

transplantation, but the most consistent responses were found to US3 and pp71. The detection of IL-10 T cell responses to US3 and pp71 so soon after transplantation, and the consistency with which these were maintained over the subsequent weeks was surprising. While T cells secreting IFN$\gamma$ in response to US3 and pp71 have been reported previously [288], analogous IL-10 responses have not. When testing a large HCMV seropositive donor cohort composed of healthy individuals for CD4+ and CD8+ T cell responses to US3 and pp71, high frequencies of IL-10, and IFN$\gamma$, secreting T cells were detected in a substantial proportion of donors. These IL-10 secreting T cells were also mainly a separate population from T cells secreting IFN$\gamma$. Age was not a factor influencing the frequencies of IL-10, or IFN$\gamma$, secreting T cells responding to US3 or pp71 (data not shown), and it therefore appears that these IL-10 responses are generated immediately following primary infection. While expression of US3 and pp71 has been well characterised during lytic infection [574, 692, 693], recent evidence suggests they could also be expressed during latent infection [130, 604], although the expression of US3 detected by single-cell RNA-sequencing could have originated from virus in the process of reactivating from latency. Of note, the kidney transplant recipients were taking a range of immunosuppressive drugs which could also have had an effect on the T cell responses detected. Further details beyond which drugs each patient was prescribed, such as the duration of drug use, was not available. However, despite the administration of these drugs, the consistent detection of IL-10 secreting T cells to US3 and pp71 does illustrate that the absence of latency-associated ORF specific responses was not the result of a defect in IL-10 secreting T cells caused by immunosuppression.

The lack of T cells secreting IL-10 specific for latency-associated proteins following primary infection suggests that these T cells could be generated as a result of longer term viral carriage beyond the time points sampled here, or that they are not found in the peripheral blood at such early stages after primary infection. It is therefore possible that IL-10 secreting T cells are resident in certain tissue sites which are not accessible by sampling from the peripheral blood. To support this idea, T cells secreting IL-10 specific to gB and pp65 have been detected in the mucosal tissue [621], and a recent study analysing multiple tissue sites and peripheral blood from deceased organ donors found a heterogeneous distribution of HCMV-specific CD8+ T cells across several tissues in individual donors, including the bone marrow, lymph nodes, and lungs [398]. Additionally, the frequency of HCMV-specific CD8+ T cells detected in the peripheral blood was not representative of their frequencies in the tissues [398]. Such a discordance between HCMV-specific T cells in the peripheral blood and in the tissues has also been reported in several studies carrying out a paired analysis between a tissue site and the peripheral blood. One study using samples from

kidney transplant recipients found pp65- and IE-specific CD8+ T cells to be less abundant in lymph nodes, which also had fewer IFN$\gamma$+ CD8+ T cells, compared to the peripheral blood [695]. A separate study utilising samples from lung transplant recipients however found that pp65-specific IFN$\gamma$+ CD8+ T cell frequencies did not differ between peripheral blood and lung mononuclear cells during the chronic phase of infection [380]. The frequencies of pp65-specific IFN$\gamma$+ CD4+ T cells on the other hand were preferentially distributed in the lung compared to the peripheral blood during chronic infection stages [380].

To begin to investigate the presence of T cells specific for HCMV latency-associated proteins from tissue sites, samples from BMMNC were tested for T cell responses. The bone marrow has been identified as an important site for the maintenance of memory T cells [696–698], and several studies have examined HCMV-specific memory T cells at this site [650, 699, 700]. In an initial experiment testing BMMNC responses to a range of HCMV ORFs, IFN$\gamma$ and IL-10 responses were detected. IFN$\gamma$ secreting T cells were detected in response to both lytic and latency-associated ORFs, including pp65 and IE1, which is in agreement with previous studies [650, 699, 700]. Conversely, IL-10 responses were only found in response to UL138 of the latency-associated ORFs, and these were at a very low frequency. High frequencies of IL-10 secreting T cells were detected in response to US3 and pp71, mirroring responses seen from PBMC.

A further preliminary experiment was carried out using paired BMMNC and PBMC samples from one donor, enabling a test of CD4+ and CD8+ T cell HCMV ORF responses between the two compartments. Considerable differences in the CD4+ and CD8+ T cells responses were found between BMMNC and PBMC. The frequencies of CD4+ T cells secreting IFN$\gamma$ were found to be higher in BMMNC in response to nearly every ORF tested, with the exception of LUNA. CD4+ T cells secreting IL-10 and CD8+ T cells secreting IFN$\gamma$ were more variable in their distribution, with responses to certain ORFs detected in only one of the two compartments. However, CD8+ T cells secreting IL-10 were found in response to every HCMV ORF tested in the bone marrow, where there were generally higher frequencies of IL-10 secreting T cells compared to the peripheral blood. There were also several ORF-specific responses from both CD4+ and CD8+ T cells that were only detected in one of the two compartments, including CD4+ T cell IL-10 responses to UL111A, and CD8+ T cell IL-10 responses to US28. This preliminary evidence suggests that bone marrow could be one reservoir site for IL-10 secreting HCMV-specific T cells, although further work will have to be undertaken to determine if responses to certain latency-associated ORF products are present in higher frequencies in the bone marrow compared to the peripheral blood, and when during infection they appear.

Comparisons between blood and bone marrow have examined T cells specific for lytic HCMV ORFs such as pp65 and IE1 with somewhat conflicting results. One study found similar frequencies of pp65-specific CD8+ T cells in both the bone marrow and peripheral blood when examining tetramer positive CD8+ T cells [699]. In the same study however, when assessing the frequencies of pp65-specific CD8+ T cells by IFN$\gamma$ production following peptide stimulation and ICS analysis, there were slightly lower frequencies of pp65-specific CD8+ T cells in the bone marrow compared to the peripheral blood [699], which is in agreement with the results presented here. Contrarily, a report using blood and bone marrow samples from cancer patients found considerably fewer tetramer positive pp65-specific CD8+ T cells in the bone marrow than blood [700]. This study also reported finding fewer IFN$\gamma$ positive CD8+ T cells in the bone marrow compared to peripheral blood, in three donors, following IE1 peptide stimulation and analysis by ICS [700], of which the opposite was found here. In agreement with the data presented here, one study examining CD4+ T cells found higher frequencies, as well as higher absolute numbers, of memory CD4+ T cells specific for pp65 in the bone marrow after stimulation with pp65 protein and analysis by ICS [650]. Additionally, considerably higher frequencies and absolute numbers of polyfunctional CD4+ T cells secreting two or more of TNF$\alpha$, IL-2, or IFN$\gamma$ were also found resident in the bone marrow compared to the blood [650]. As the experiments presented here were only carried out on one donor, the inclusion of additional donors is required to be able to fully compare the findings to previous research.

Within the three individuals tested, the presence of bone marrow derived T cells specific to nearly every HCMV ORF examined was surprising. CD34+ progenitor cells, which are one cellular site for HCMV latency, are also known to be resident in the bone marrow [94]. The presence of T cells specific for the full array of lytic and latency-associated ORFs in the bone marrow could therefore be indicative of considerable viral activity at this site, where T cells might be required to control reactivating virus. An uncontrolled lytic infection in the bone marrow could be extensively damaging, and the presence of so many HCMV-specific T cells might be necessary to prevent this. Although these experiments were only performed in two donors, the presence of high frequencies of IL-10 secreting T cells in the bone marrow capable of responding to a broad array of ORFs, including IE1 and IE2, which were not detected in the peripheral blood in paired samples, or at high frequencies in healthy individuals (Section 3.2.7 and 4.2.4) [691], suggests a functional role for IL-10 at this site. It is therefore plausible that IL-10 secretion from HCMV-specific T cells acts as a balance against pro-inflammatory anti-viral responses, to prevent damaging immunopathology in the bone marrow.

These results are somewhat limited however by the lack of information on the donor health and infection status at the time of sampling, and because the time points between the sampling of BMMNC and PBMC were several months apart. As was shown in a previous chapter (Sections 3.2.4, 4.2.2, and 4.2.5), fluctuations in detected T cell responses from peripheral blood occur over time, which could result in an unrepresentative comparison between the two compartments.

Although the conclusions that can be drawn from these data are limited given the small sample size, differences in HCMV-specific responses between peripheral blood derived T cells and bone marrow derived T cells were detected. These results provide the basis for further experiments to be carried out in this area to determine if differences exist between latency-associated ORF-specific bone marrow and PBMC T cell responses for example. Furthermore, these results highlight the limitations of previous studies which only examined T cell responses to lytic ORFs, such as pp65 or IE1, and therefore only provide a restricted view of HCMV-specific T cell responses.

# Chapter 6

# *In vitro* Characterisation of Latency-specific CD4+ T cells

## 6.1 Introduction

CD4+ T cells isolated from HCMV seropositive donors can recognise viral proteins expressed during latency (Chapter 3) [501]. These CD4+ T cells were composed of two separate populations, which either secreted IFN$\gamma$ or IL-10 (Section 3.2.10). Previous work has demonstrated that PBMC stimulated with UL138 ORF peptides can suppress CD4+ T cells proliferation and that a subset of these UL138-specific CD4+ T cells express phenotypic markers of Tregs [501]. These CD4+ T cells could therefore be involved in the prevention of antiviral T cell responses during latency.

The aims of this chapter were to investigate in more detail the CD4+ T cells found to be specific for the latency-associated proteins US28, UL111A, UL144, UL138, and LUNA, both phenotypically and functionally. Comparing such CD4+ T cells specific for HCMV latency-associated ORFs to those specific for lytic ORFs could reveal factors which prevent antiviral responses during latency. To do this, CD4+ T cells were phenotypically characterised to determine their memory sub-populations, in addition to assessing the expression of markers associated with cytotoxicity. The effector functions of CD4+ T cells specific for latency-associated HCMV ORFs was also examined by assessing the effects of the secretomes from these cells on the proliferation of bystander CD4+ T cells and on the secretion of anti-viral cytokines. The finding that CD4+ T cells secreting IFN$\gamma$ in response to HCMV ORF peptide pools were a separate sub-population from those secreting IL-10 led to the hypothesis that

if these IL-10 secreting cells could be selectively removed, those secreting IFN$\gamma$ should be able to recognise and clear latently infected cells. Given that previous work has shown that UL138-specific CD4+ express markers characteristic of Tregs [605], and these cells are known producers of IL-10, selective removal of these cells was attempted to remove CD4+ T cells expressing IL-10. Finally, an initial investigation into the peptide specificity of CD4+ T cells specific for HCMV latency-associated ORFs was carried out with the aim of assessing whether CD4+ T cells responding to the same individual peptide were composed of cells secreting either IFN$\gamma$ or IL-10, or IFN$\gamma$ and IL-10 secreting cells together. Additionally, the results of this peptide mapping could provide valuable information which could be used in the design of an MHC class II tetramer.

## 6.2 Results

### 6.2.1 Latency-specific CD4+ T cells secrete TGF$\beta$ in addition to IL-10

In addition to the previously described ability of UL138 and LUNA-specific CD4+ T cells to secrete IL-10, it was also reported that these CD4+ T cells could secrete the immunomodulatory cytokine TGF$\beta$ [501]. To determine if US28-, UL111A-, and UL144-specific CD4+ T cells also secrete TGF$\beta$, PBMC depleted of CD8+ cells were stimulated with overlapping peptide pools spanning the ORFs of pp65, IE1, UL138, LUNA, US28, UL111A, and UL144 for 48 hours. The resulting supernatants were evaluated for TGF$\beta$ secretion by flow cytometry using a bead-based assay. TGF$\beta$ secretion was found to vary in response to different ORFs between individuals. The responses to UL138 were either very low or absent across the five donors tested (Figure 6.1). However, TGF$\beta$ was consistently detected in response to the remaining latency-associated ORFs (Figure 6.1). TGF$\beta$ secretion was consistently elevated in response to pp65, while IE1 responses were more variable although they were also detected from every donor (Figure 6.1). TGF$\beta$ secretion did not differ substantially in response to the lytic ORFs pp65 and IE1, compared to the latency-associated ORFs tested, with TGF$\beta$ secretion in response to pp65 being consistently raised (Figure 6.1). CD4+ T cells are therefore capable of secreting TGF$\beta$ in response to HCMV ORFs, both to those expressed during lytic infection and those that are latency-associated.

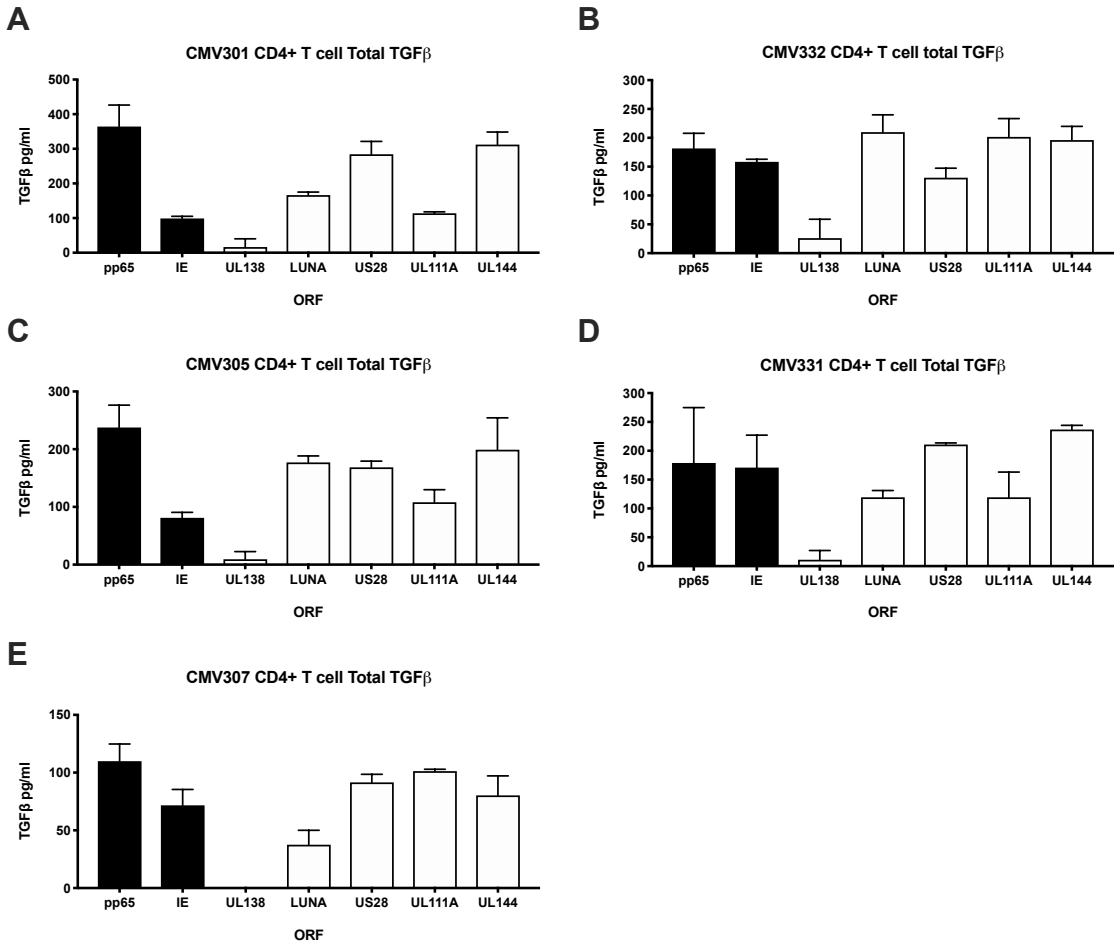



**Fig. 6.1 Detecting TGF$\beta$ secretion from CD4+ T cells in response to HCMV ORF peptide pools** PBMC from 5 HCMV seropositive donors were depleted of CD8+ cells and stimulated with overlapping peptide pools spanning the ORFs of pp65, IE1, UL138, LUNA, US28, UL111A, and UL144 for 48 hours. Following this, the supernatant was harvested and the concentrations of total TGF$\beta$ assessed by flow cytometry. Samples were run in duplicate and the total TGF$\beta$ present in the supernatant from unstimulated CD8+ depleted PBMC was deducted from all test samples. The lytic ORFs are illustrated with black bars and the latency-associated ORFs with white bars.

### 6.2.2 Phenotypic characterisation of latency-specific CD4+ T cells

HCMV-specific CD4+ T cells are reported to have direct effector functions such as cytokine secretion (as shown in Section 3.2), and cytotoxicity [396, 401, 404, 501]. To investigate the potential cytotoxic capacity of CD4+ T cells specific to latency-associated ORFs, the expression of CD107a on CD4+ T cells following stimulation with overlapping peptide pools spanning the HCMV ORFs gB, pp65, IE1, UL138, LUNA, US28, UL111A, and UL144 was determined. CD107a is a well-defined marker of degranulation and an indicator of the cytotoxic potential of CD4+ T cells [404, 415]. In addition to CD107a, the levels of expression of several granzymes (A, B, and K), and the expression of both CD107a and granzymes together on CD4+ T cells was also determined.

HCMV-specific CD4+ T cells were identified through the expression of CD40L and 4-1BB [404, 701, 702] above the background level of expression on unstimulated CD4+ T cells following peptide stimulation (Figure 6.2A). The expression of CD107a and granzymes on CD4+ T cells with an ORF-specific response was then determined from 18 seropositive donors. As has previously been described [393], a number of donors had CD4+ T cells capable of degranulating in response to the lytic ORFs gB (7/14, 50%), pp65 (12/16, 75%), and IE1 (5/16 31%) when stimulated *ex vivo*, although not all ORF-specific CD4+ T cells possessed this ability, and the proportion of cells expressing CD107a was variable between donors (Figure 6.2B). CD4+ T cell CD107a degranulation was also observed in response to latency-associated ORFs. The number of donors with CD4+ T cells capable of degranulating was highest in response to US28 (7/13 54%) and UL144 (6/13 46%), followed by LUNA (4/13 31%) and UL111A (3/10, 30%), while none of the four donors with a UL138-specific CD4+ T cell response had detectable CD107a upregulation (Figure 6.2B).

In general, CD4+ T cells expressing granzymes A, B, and K only were detected in approximately one-third of donors who possessed an ORF-specific response, with the exception of UL111A-specific CD4+ T cells which were found in 70% of such donors (7/10) (Figure 6.2C). There were also donors with a small proportion of ORF-specific CD4+ T cells that were positive for both CD107a and granzymes A, B, and K, which were found in response to pp65 (7/15, 53%), gB (8/14, 57%), IE1 (9/16, 56%), UL144 (5/13, 38%), US28 (5/12, 42%), and LUNA (5/13, 38%) (Figure 6.2D). ORF-specific CD4+ T cells positive for CD107a and granzyme expression in response to UL138 (1/4, 25%) and UL111A (2/10, 20%) were detected in one and two donors respectively (Figure 6.2D).

In addition to these effector functions, the memory phenotypes of CD4+ T cells specific for proteins expressed during latency was also examined. Previous work has found that a

subset of HCMV-specific CD4+ T cells possessed a differentiated memory cell phenotype, where costimulatory molecules such as CD27 and CD28 were downregulated and there was reexpression of CD45RA [390, 394, 396, 408]. To determine if the memory T cell phenotypes differed between lytic- and latent-specific CD4+ T cells, cells were stimulated with HCMV ORF peptide pools for 18 hours and then stained for expression of memory markers. Antigen-specific CD4+ T cells were again determined by the upregulation of CD40L and 4-1BB. The CD4+ T cells were grouped into four memory subsets based on the expression of CD45RA and CD27 to give naive-like T cells ($T_{NL}$) (CD27+ CD45RA+), central memory T cells ($T_{CM}$) (CD27+ CD45RA-), effector memory T cells ($T_{EM}$) (CD27- CD45RA-), and effector memory CD45RA-expressing T cells ($T_{EMRA}$) (CD27- CD45RA+). The typical strategy for gating the flow cytometry plots and a representative phenotype from one donor are shown in Figure 6.3A. Data from 19 seropositive donors shows that antigen-specific CD4+ T cells were predominantly $T_{CM}$ and $T_{EM}$ subsets, with a small proportion expressing markers characteristic of ($T_{EMRA}$) cells (Figure 6.3B). Despite CD4+ T cells specific for US28 and UL111A having slightly greater proportions of cells with a naive-like phenotype, there was also no significant difference between CD4+ T cell memory subsets specific for lytic ORFs and those specific for latency-associated ORFs. The data for UL138-specific CD4+ T cells reflects the low number of individuals with a positive response.

CD4+ T cells specific for latency-associated ORF products therefore expressed markers associated with cytotoxic potential, and were mainly composed of $T_{CM}$ and $T_{EM}$ memory subsets, which did not differ significantly from CD4+ T cells specific to HCMV lytic ORFs.

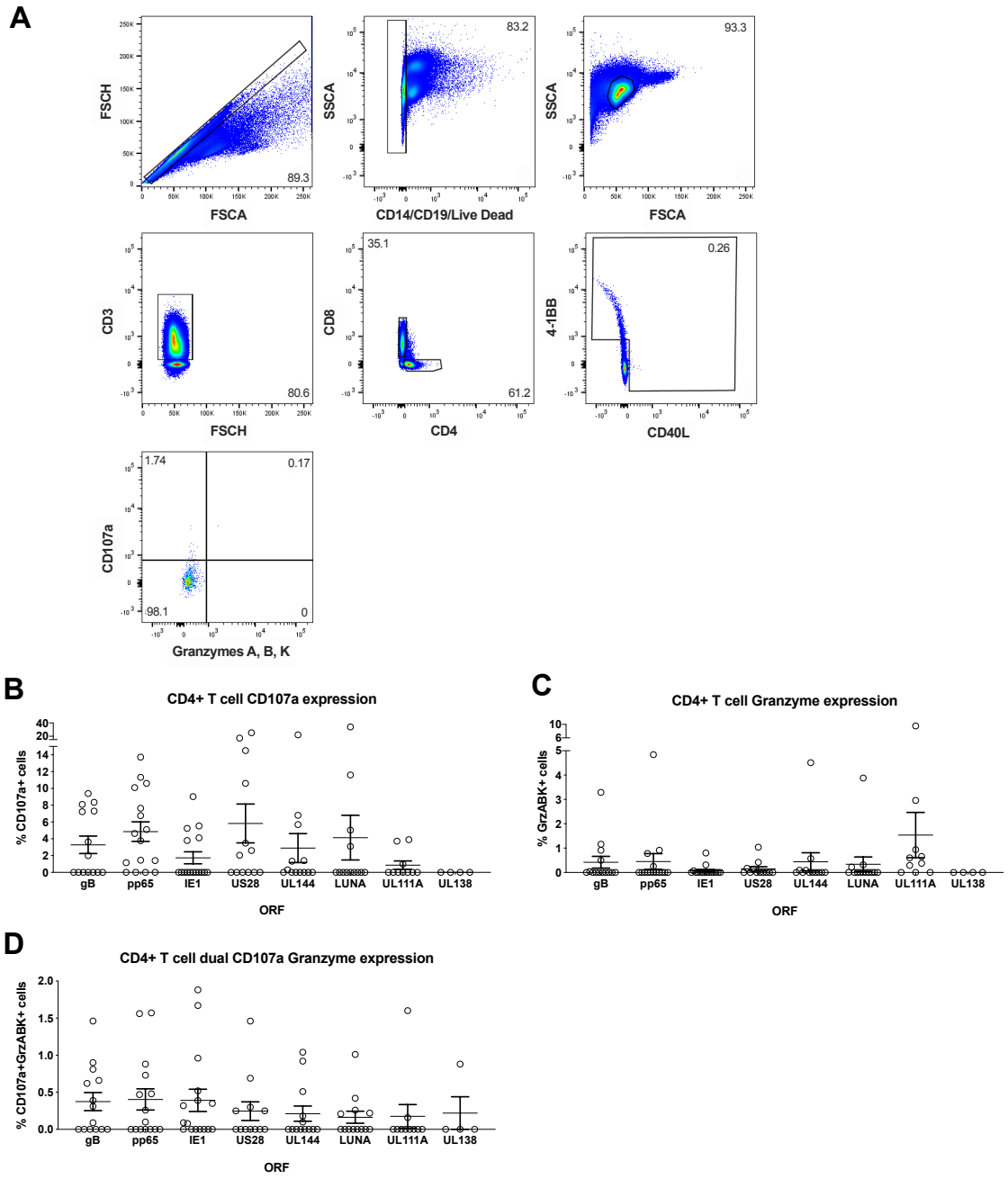



**Fig. 6.2 Assessing the expression of CD107a and Granzymes A, B, and K on CD4+ T cells specific to HCMV ORF peptide pools by flow cytometry.** PBMC from 18 HCMV seropositive donors were stimulated with overlapping peptide pools spanning the ORFs of gB, pp65, IE1, UL138, LUNA, US28, UL111A, and UL144 for 18 hours, in the presence of monensin. PBMC were then stained intracellularly for CD107a and the granzymes A, B, and K. (A) The representative gating strategy to identify ORF-specific CD4+ T cells is shown for UL144-specific CD4+ T cells from donor CMV305. Dead cells, CD14+ and CD19+ cells were first excluded, followed by doublets. Lymphocytes were then gated on by forward scatter (FSCA) and side scatter (SSCA) and CD3+ cells identified. CD4+ and CD8+ cells were then gated, followed by activated CD4+ T cells, identified as CD40L or 4-1BB positive cells. CD4+ T cells with above background expression of CD40L or 4-1BB were considered ORF-specific. The percentages of ORF-specific CD4+ T cells expressing (B) CD107a, (C) granzymes A, B, or K, (D) or both CD107a and granzymes A, B, or K following ORF peptide pool stimulation is shown.

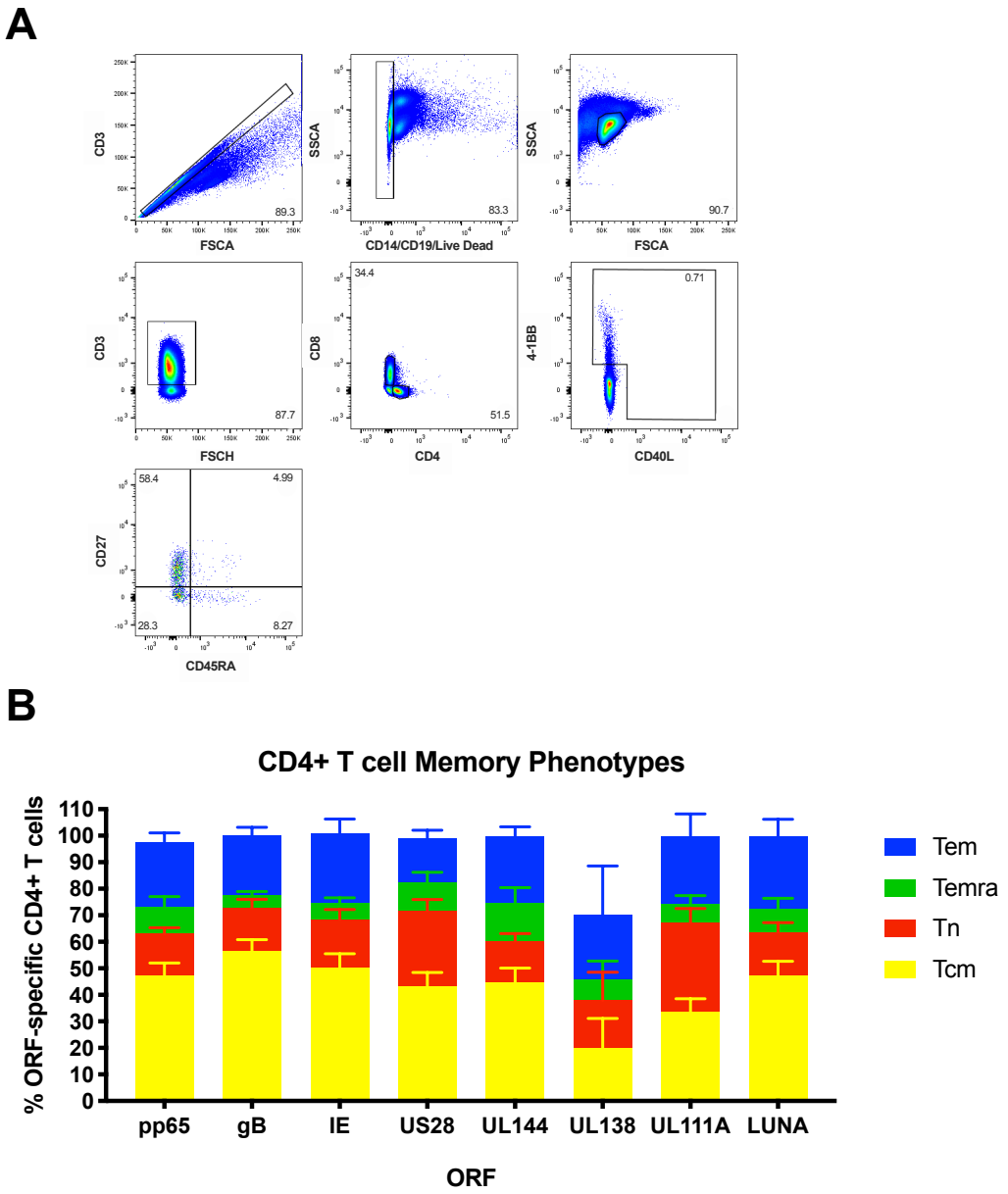



**Fig. 6.3 Memory phenotypes of CD4+ T cells specific for HCMV antigens.** PBMC isolated from 19 HCMV seropositive donors were stimulated for 18 hours with HCMV peptide pools spanning the ORFs of gB, pp65, IE1, UL138, LUNA, US28, UL111A, and UL144, in the presence of monensin. Stimulated PBMC were then stained intracellularly. (A) Representative gating strategy to identify the memory populations of CD4+ T cells specific for IE1 from donor CMV324 is shown. Dead cells, CD14+ and CD19+ cells were first excluded, followed by doublets. Lymphocytes were then gated on by FSCA and SSCA and CD3+ cells identified. CD4+ and CD8+ cells were then gated, followed by activated CD4+ T cells, identified by gating for CD40L or 4-1BB positive cells, and finally CD27 and CD45RA to identify the memory phenotype. (B) The proportions of each CD4+ T cell memory population responding to each ORF grouped by CD27 and CD45 expression. Naive-like ($T_{NL}$) (CD27+ CD45RA+), central memory (central memory ($T_{CM}$) (CD27+ CD45RA-), effector memory ($T_{EM}$) (CD27- CD45RA-), and effector memory CD45RA-expressing T cells ($T_{EMRA}$) (CD27- CD45RA+).

### 6.2.3 The secretome from latency-specific CD4+ T cells inhibits T cell proliferation and secretion of antiviral cytokines

The production of IL-10 and TGF$\beta$ by latency-specific CD4+ T cells, which are cytokines known to inhibit T cell proliferation and effector functions, could be affecting the ability of anti-viral T cells to eliminate latently infected cells. Given that within individuals a segregation of latency-specific CD4+ T cells into two populations was found, one which produces the anti-viral cytokine IFN$\gamma$, and one which produces the immunosuppressive cytokine IL-10 (Section 3.2.10), it was important to determine the overall effect of the secretome from latency-specific CD4+ T cells on effector functions. To investigate a range of effects the secretome from latency-specific CD4+ T cells could be having on other T cells, a proliferation assay and a cytokine secretion inhibition assay were used.

In the first instance, the ability of T cells to proliferate following a polyclonal stimulus, in the presence of CD4+ T cell secretomes, was assessed. PBMC were labelled with a proliferation dye (CellTrace) and stimulated with anti-CD3/CD28/CD137 antibody coated beads in the presence of supernatant derived from CD4+ T cells stimulated with US28 ORF peptides. After five days, the proliferation of T cells was assessed by flow cytometry. The proliferation assay was optimised so that the stimulus to proliferate was strong enough that proliferation was observed robustly, but was not so potent as to prevent the inhibition from recombinant IL-10. Proliferation was induced over a range of bead concentrations, before a bead-to-cell ratio of 1:100 was chosen (Figure 6.4A). The ability of IL-10 to inhibit the proliferation induced by this bead concentration was then confirmed and subsequently used as a control in further experiments (Figure 6.4B). Using this assay, the supernatant derived from US28-stimulated CD4+ T cells was found to inhibit the proliferation of both CD4+ and CD8+ T cells by over 70% in comparison to anti-CD3/CD28/CD137 stimulated T cells only (Figure 6.4C and D).

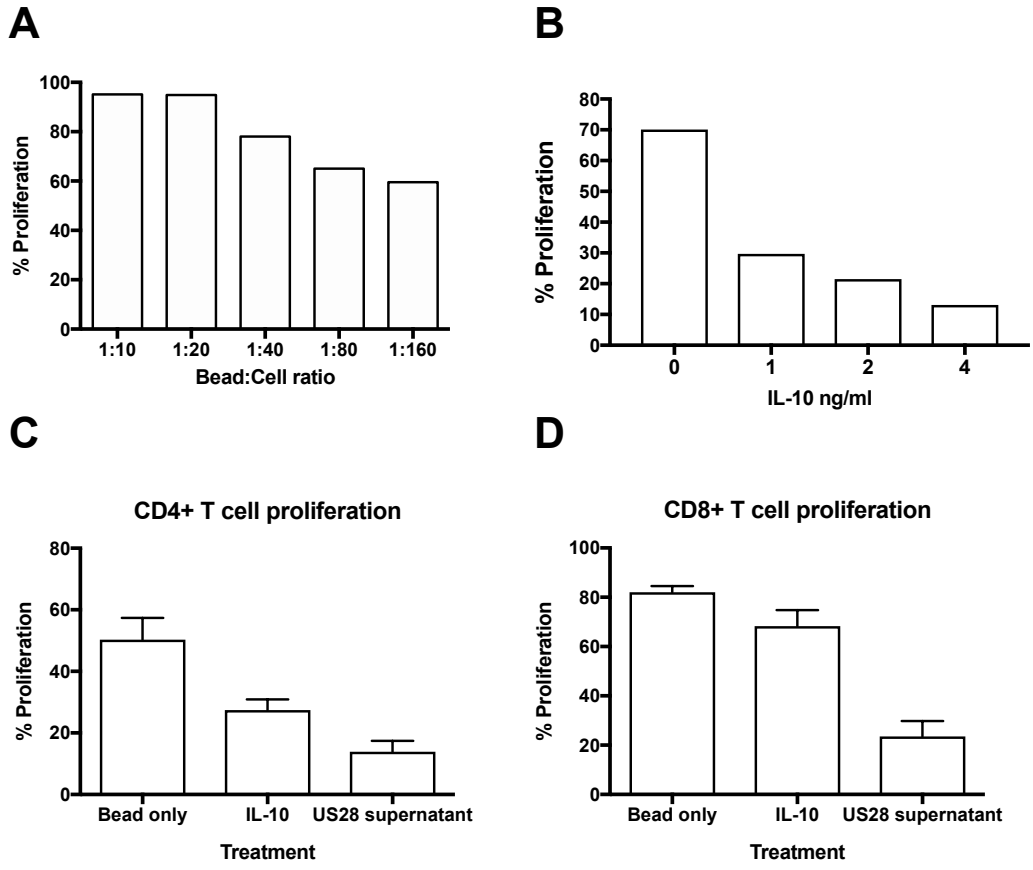



**Fig. 6.4 Assessing the proliferation of CD4+ T cells following incubation with supernatant dervied from PBMC stimulated with US28 ORF peptides.** (A) To determine the concentration of polyclonal stimulus to use in the proliferation assays, PBMC from one donor were stained with a proliferation dye then stimulated polyclonally with Dynabeads® Human T-Activator CD3/CD28/CD137 at a range of concentrations. Following a five day incubation, PBMC were phenoypted to determine the levels of proliferation of CD4+ T cells. (B) The ability to inhibit this proliferation was confirmed using IL-10. A range of IL-10 concentrations was utilised. PBMC were incubated in IL-10 prior to the addition of the polyclonal stimulus before assessing proliferation after five days. PBMC from one HCMV seropositive donor were stimulated with the US28 ORF peptide pool for 48 hours and the supernatant was harvested. Separately isolated PBMC were stained with a proliferation dye then incubated in this supernatant and stimulated polyclonally with Dynabeads® Human T-Activator CD3/CD28/CD137 at a ratio of 1:100 beads per cell. IL-10 was used as a positive control for inhibition at 2ng/ml. After five days, PBMC were analysed by flow cytometry for the levels of proliferation of (C) CD4+ T cells and (D) CD8+ T cells.

**Development of the cytokine secretion inhibition assay**

The ability of latency-specific CD4+ T cells secreting IL-10 and TGF$\beta$ to affect the production of anti-viral cytokines from PBMC was then assessed using a cytokine secretion inhibition assay. In this assay, PBMC were incubated in supernatant derived from HCMV ORF peptide pool stimulated CD4+ T cells, before being stimulated with an anti-CD3/CD28 monoclonal antibody mix. The secretion of anti-viral IFN$\gamma$ and TNF$\alpha$ was then quantified by a flow cytometry based bead array. Initial experiments to develop this assay were carried out using a mixture of recombinant IL-10 and TGF$\beta$, in the place of CD4+ T cell secretomes, at a range of concentrations. This positive control mixture of TGF$\beta$ and IL-10 was utilised to ensure that anti-CD3/CD28 antibody induced IFN$\gamma$ and TNF$\alpha$ secretion from PBMC could be inhibited. A range of IL-10 and TGF$\beta$ concentrations was tested, with all showing strong inhibition of TNF$\alpha$ (Figure 6.5A) and IFN$\gamma$ (Figure 6.5B) secretion. The extent of the inhibition of anti-viral cytokine secretion can be illustrated by taking the relative percentage of inhibition at every IL-10 and TGF$\beta$ concentration in comparison to the cytokine secretion from the anti-CD3/CD28 antibody stimulated PBMC only. Doing so for this donor showed that the secretion of IFN$\gamma$ from PBMC was inhibited by approximately 95% for all four IL-10 and TGF$\beta$ concentrations used compared to anti-CD3/CD28 stimulated PBMC only (Figure 6.5C). The secretion of TNF$\alpha$ was also highly inhibited, even at the lowest concentrations of IL-10 and TGF$\beta$ (Figure 6.5C).

A cohort of donor PBMC that could be used in subsequent experiments for these assays was sought. The ability of TGF$\beta$ and IL-10 to inhibit IFN$\gamma$ and TNF$\alpha$ secretion from PBMC was therefore tested in several donors. Utilising a greater range of IL-10 and TGF$\beta$ concentrations that extended lower than those used previously (Figure 6.5), donor CMV401 was found to exhibit a high level of inhibition of both TNF$\alpha$ and IFN$\gamma$ secretion from CD3/CD28 stimulated PBMC across the range of IL-10 and TGF$\beta$ concentrations tested (Figure 6.6). The secretion of TNF$\alpha$ was consistently inhibited by around 70% at all IL-10 and TGF$\beta$ concentrations, with IFN$\gamma$ secretion found to be inhibited by between approximately 65% and 75% at most concentrations (Figure 6.6A and C).

When carrying out the same analysis on additional donors, a donor specific variation in the ability of IL-10 and TGF$\beta$ to inhibit the secretion of IFN$\gamma$ and TNF$\alpha$ was observed across the concentrations tested. For example, the inhibition of TNF$\alpha$ secretion from the PBMC of donor CMV331 was low, even at the highest concentration of TGF$\beta$ and IL-10 (Figure 6.7A), where there was around a 20% inhibition (Figure 6.7C). Furthermore, at concentrations of IL-10 and TGF$\beta$ below this, TNF$\alpha$ secretion actually increased above

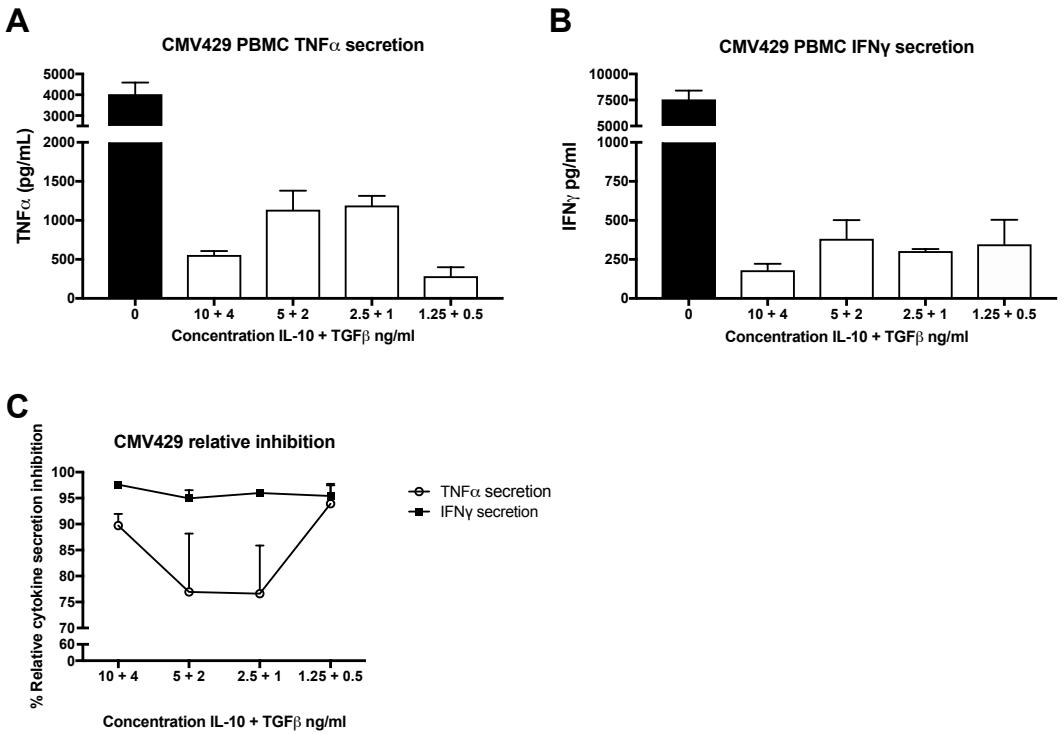
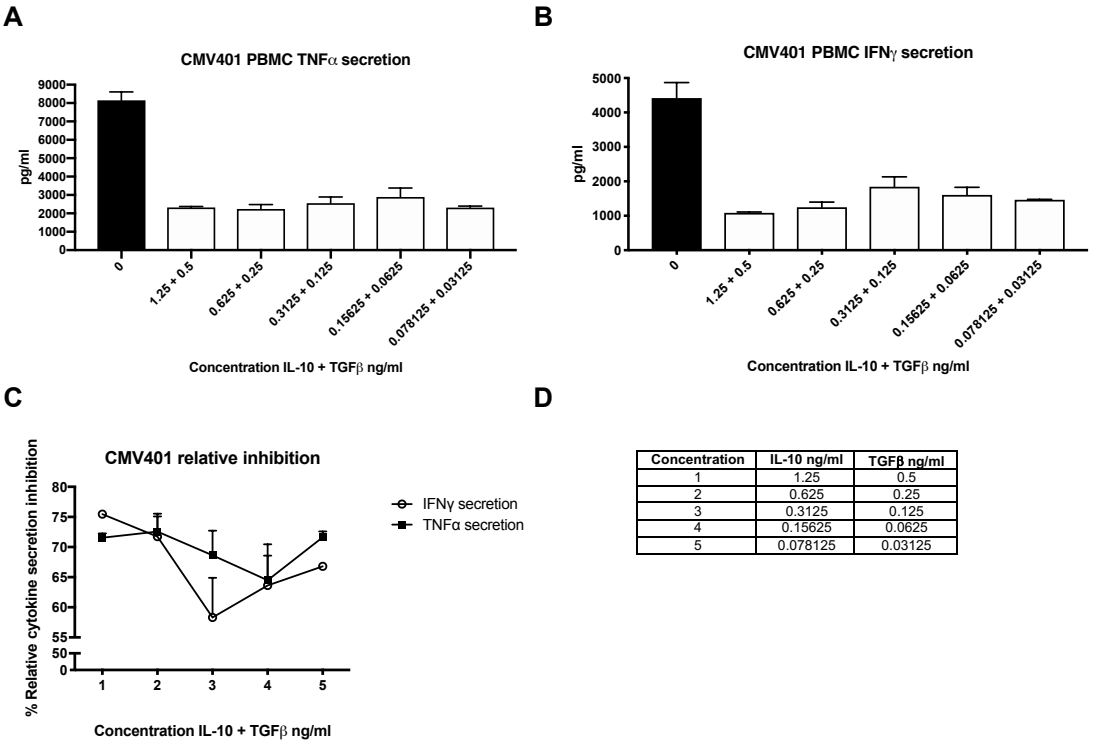



**Fig. 6.5 IFN$\gamma$ and TNF$\alpha$ production from PBMC following IL-10 and TGF$\beta$ treatment**. PBMC from one donor, CMV429, were incubated with media, or with combinations of different IL-10 and TGF$\beta$ concentrations for 24 hours. The PBMC were then stimulated with anti-CD3/CD28 monoclonal antibodies for 18 hours. Following this, the supernatants were harvested and analysed for the secretion of (A) TNF$\alpha$ and (B) IFN$\gamma$ by flow cytometry. (C) The ability of different concentrations of IL-10 and TGF$\beta$ to inhibit cytokine secretion was then expressed as a percentage of the TNF$\alpha$ and IFN$\gamma$ secretion observed from the anti-CD3/CD28 stimulated only control. Supernatant samples were tested in triplicate.



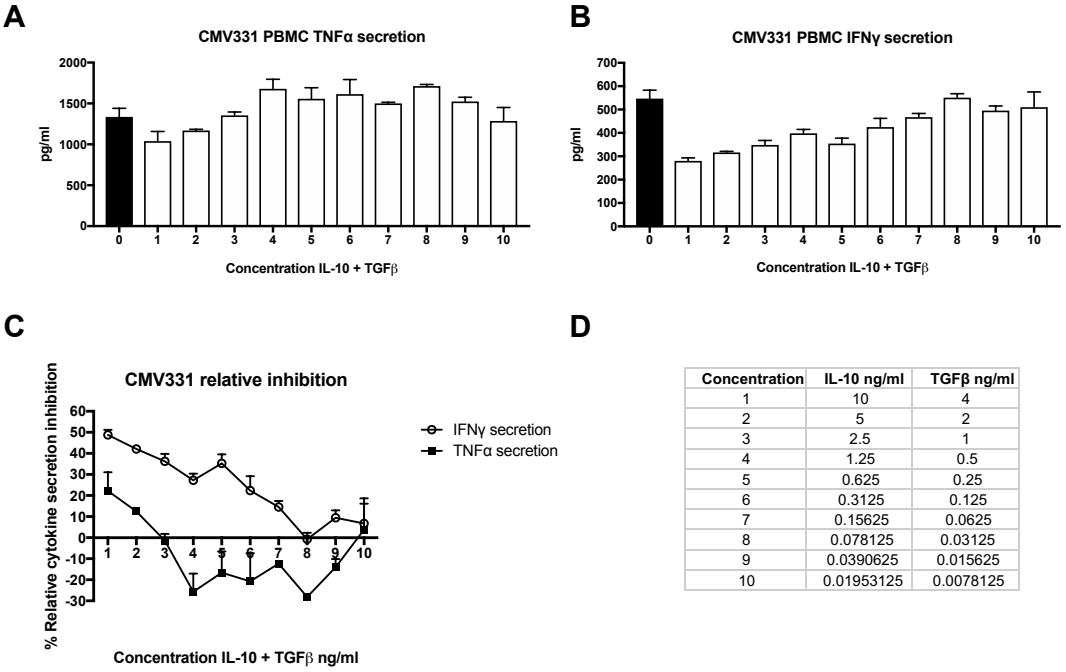

| Concentration | IL-10 ng/ml | TGFβ ng/ml |
|---|---|---|
| 1 | 1.25 | 0.5 |
| 2 | 0.625 | 0.25 |
| 3 | 0.3125 | 0.125 |
| 4 | 0.15625 | 0.0625 |
| 5 | 0.078125 | 0.03125 |

**Fig. 6.6 IL-10 and TGF$\beta$ inhibit the production of IFN$\gamma$ and TNF$\alpha$.** PBMC from donor CMV401 were incubated with media, or with combinations of different IL-10 and TGF$\beta$ concentrations for 24 hours. The PBMC were then stimulated with anti-CD3/CD28 monoclonal antibodies for 18 hours. Following this, the supernatants were harvested and analysed for the secretion of (A) TNF$\alpha$ and (B) IFN$\gamma$ by flow cytometry. (C) The ability of different concentrations of IL-10 and TGF$\beta$ to inhibit cytokine secretion was then expressed as a percentage of the TNF$\alpha$ and IFN$\gamma$ secretion observed from the anti-CD3/CD28 stimulated only control. Supernatant samples were tested in triplicate. (D) The concentrations of IL-10 and TGF$\beta$ used are shown in the table.

that observed in the stimulation only control (Figure 6.7C). In contrast to the TNF$\alpha$, IFN$\gamma$ secretion exhibited a 50% inhibition at the highest TGF$\beta$ and IL-10 concentration (Figure 6.7B and C).

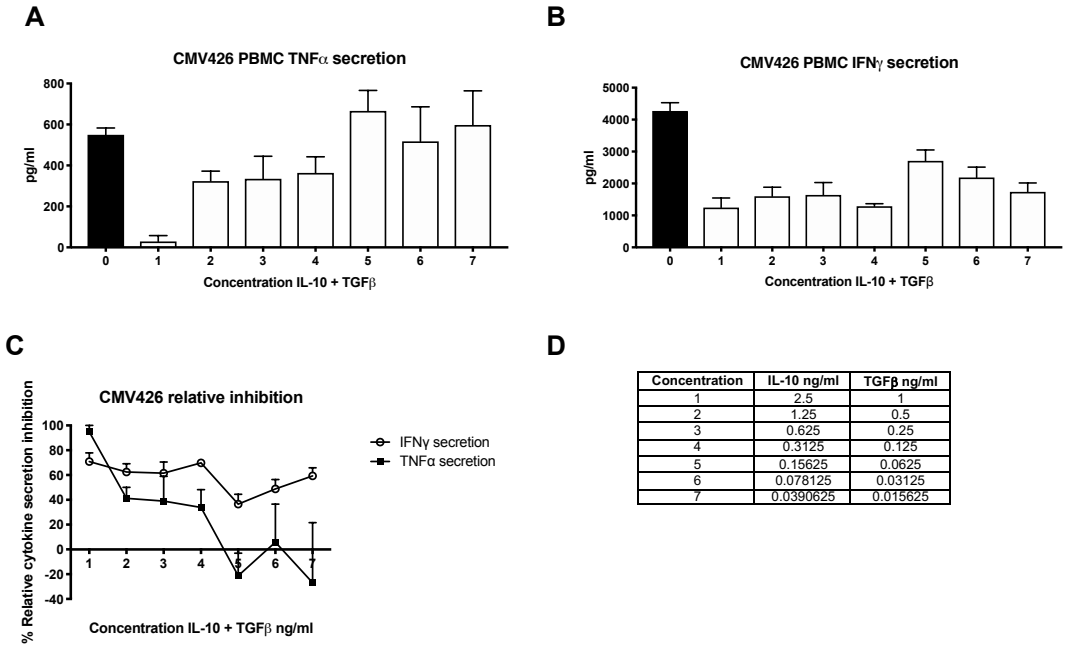
Donor CMV426 showed near complete inhibition of TNF$\alpha$ secretion at the highest concentration of IL-10 and TGF$\beta$, which decreased consistently with decreasing concentrations of IL-10 and TGF$\beta$ (Figure 6.8A and C). At the lowest concentrations, there was a stimulatory effect of IL-10 and TGF$\beta$ on TNF$\alpha$ and production (Figure 6.8C), as seen with donor CMV331 (Figure 6.7C). On the other hand, the inhibition of IFN$\gamma$ secretion remained elevated across all IL-10 and TGF$\beta$ concentrations, with around a 60% inhibition observed at the lowest concentrations (Figure 6.8B and C).

The data from these four individuals revealed considerable variation between donors in several characteristics that are important for the design of a reliable assay to detect the inhibition of cytokine secretion. The inhibition of TNF$\alpha$ and IFN$\gamma$ were differentially affected by IL-10 and TGF$\beta$ in certain donors. Donor CMV331 for example exhibited inhibition of IFN$\gamma$ at many IL-10 and TGF$\beta$ concentrations where there was either no inhibition of TNF$\alpha$, or an observed increase in TNF$\alpha$ production (Figure 6.7). There was also extensive variation in the TNF$\alpha$ and IFN$\gamma$ secretion induced by the positive control mix of anti-CD3/CD28 antibodies between donors. Additionally, the abilities of IL-10 and TGF$\beta$ to inhibit TNF$\alpha$ and IFN$\gamma$ differed dramatically between donors when comparing across the same concentrations. Initially therefore, two donors were selected (CMV401 and CMV426) for use in subsequent assays as TNF$\alpha$ and IFN$\gamma$ secretion could be inhibited from their PBMC by IL-10 and TGF$\beta$, in addition to producing enough quantities of these cytokines to give a wide range of inhibition.




| Concentration | IL-10 ng/ml | TGFβ ng/ml |
|---|---|---|
| 1 | 10 | 4 |
| 2 | 5 | 2 |
| 3 | 2.5 | 1 |
| 4 | 1.25 | 0.5 |
| 5 | 0.625 | 0.25 |
| 6 | 0.3125 | 0.125 |
| 7 | 0.15625 | 0.0625 |
| 8 | 0.078125 | 0.03125 |
| 9 | 0.0390625 | 0.015625 |
| 10 | 0.01953125 | 0.0078125 |

**Fig. 6.7 IFN$\gamma$ and TNF$\alpha$ production from PBMC following IL-10 and TGF$\beta$ treatment**. PBMC from donor CMV331 were incubated with media, or with combinations of different IL-10 and TGF$\beta$ concentrations for 24 hours. The PBMC were then stimulated with anti-CD3/CD28 monoclonal antibodies for 18 hours. Following this, the supernatants were harvested and analysed for the secretion of (A) TNF$\alpha$ and (B) IFN$\gamma$ by flow cytometry. (C) The ability of different concentrations of IL-10 and TGF$\beta$ to inhibit cytokine secretion was then expressed as a percentage of the TNF$\alpha$ and IFN$\gamma$ secretion observed from the anti-CD3/CD28 stimulated only control. Supernatant samples were tested in triplicate. (D) The concentrations of IL-10 and TGF$\beta$ used are shown in the table.




| Concentration | IL-10 ng/ml | TGFβ ng/ml |
|---|---|---|
| 1 | 2.5 | 1 |
| 2 | 1.25 | 0.5 |
| 3 | 0.625 | 0.25 |
| 4 | 0.3125 | 0.125 |
| 5 | 0.15625 | 0.0625 |
| 6 | 0.078125 | 0.03125 |
| 7 | 0.0390625 | 0.015625 |

**Fig. 6.8 IL-10 and TGF$\beta$ inhibit the production of IFN$\gamma$ and TNF$\alpha$.** PBMC from donor CMV426 were incubated with media, or with combinations of different IL-10 and TGF$\beta$ concentrations for 24 hours. The PBMC were then stimulated with anti-CD3/CD28 monoclonal antibodies for 18 hours. Following this, the supernatants were harvested and analysed for the secretion of (A) TNF$\alpha$ and (B) IFN$\gamma$ by flow cytometry. (C) The ability of different concentrations of IL-10 and TGF$\beta$ to inhibit cytokine secretion was then expressed as a percentage of the TNF$\alpha$ and IFN$\gamma$ secretion observed from the anti-CD3/CD28 stimulated only control. Supernatant samples were tested in triplicate. (D) The concentrations of IL-10 and TGF$\beta$ used are shown in the table.

**The secretome from latency-specific CD4+ T cells can inhibit the secretion of TNF$\alpha$ and IFN$\gamma$ from activated bystander T cells**

The cytokine secretion inhibition assay was then used to investigate the ability of supernatants from HCMV ORF stimulated CD4+ T cells to inhibit anti-viral cytokine production (illustrated in Figure 6.9). Initially, PBMC from three HCMV seropositive donors were depleted of CD8+ cells and stimulated with HCMV ORF peptide pools for 48 hours, and the levels of IL-10, IFN$\gamma$, and TNF$\alpha$ determined by a flow cytometry based bead array (Figure 6.9A and Appendix B.1). Of note, IL-10 was only detected from CMV319 CD4+ T cell supernatants, and these were below 10pg/ml (Appendix B.1). The resulting three sets of CD4+ T cell supernatants were then tested for their ability to inhibit the secretion of IFN$\gamma$ and TNF$\alpha$ from CD3/CD28 stimulated PBMC. Initially, these three sets of CD4+ T cell ORF supernatants were tested for their abilities to inhibit cytokine secretion from the PBMC of two non-autologous donors (CMV401 and CMV426). PBMC were incubated in CD4+ T cell ORF supernatants for 24 hours before the addition of anti-CD3/CD28 antibodies. The following day, the resulting CD3/CD28 stimulated supernatants were analysed for the secretion of TNF$\alpha$ and IFN$\gamma$ by a flow cytometry based bead array (Figure 6.9C). Cytokine secretion in these test samples was compared to the secretion observed from CD3/CD28 stimulated PBMC following incubation with supernatant obtained from unstimulated CD4+ T cells. As a control, donor PBMC were left incubated with supernatants for 42 hours without the addition of the anti-CD3/CD28 monoclonal antibody mix to determine the effect of the supernatants alone on cytokine secretion (Figure 6.9B). Any IFN$\gamma$ and TNF$\alpha$ secretion induced by the supernatant alone (Appendix B.2 and B.3) was treated as background and deducted from the test values.

There were very few CD4+ T cell ORF supernatants that inhibited IFN$\gamma$ and TNF$\alpha$ secretion from CMV401 CD3/CD28 stimulated PBMC when comparing against the control supernatant derived from unstimulated CD4+ T cells (Figure 6.10). The majority of CD4+ T cell supernatants resulted in no change, or an increase, in IFN$\gamma$ and TNF$\alpha$ secretion from PBMC (Figure 6.10). CD4+ T cell supernatants derived from donor CMV324 did not have any inhibitory effect on TNF$\alpha$ or IFN$\gamma$ secretion on donor CMV401 PBMC, while there were considerable increases in the secretion of these cytokines incubated with pp65 and UL144 supernatants (Figure 6.10A and B, and 6.12C). The UL111A and UL144 stimulated supernatants from donor CMV328 did have an inhibitory effect of around 50% on TNF$\alpha$ secretion on PBMC from donor CMV401 (Figures 6.10C and 6.12A). Conversely, IFN$\gamma$ secretion was increased by incubation with all CMV328 CD4+ T cell ORF supernatants (Figures 6.10D and 6.12A). The largest increase, of around double the IFN$\gamma$ secretion of the

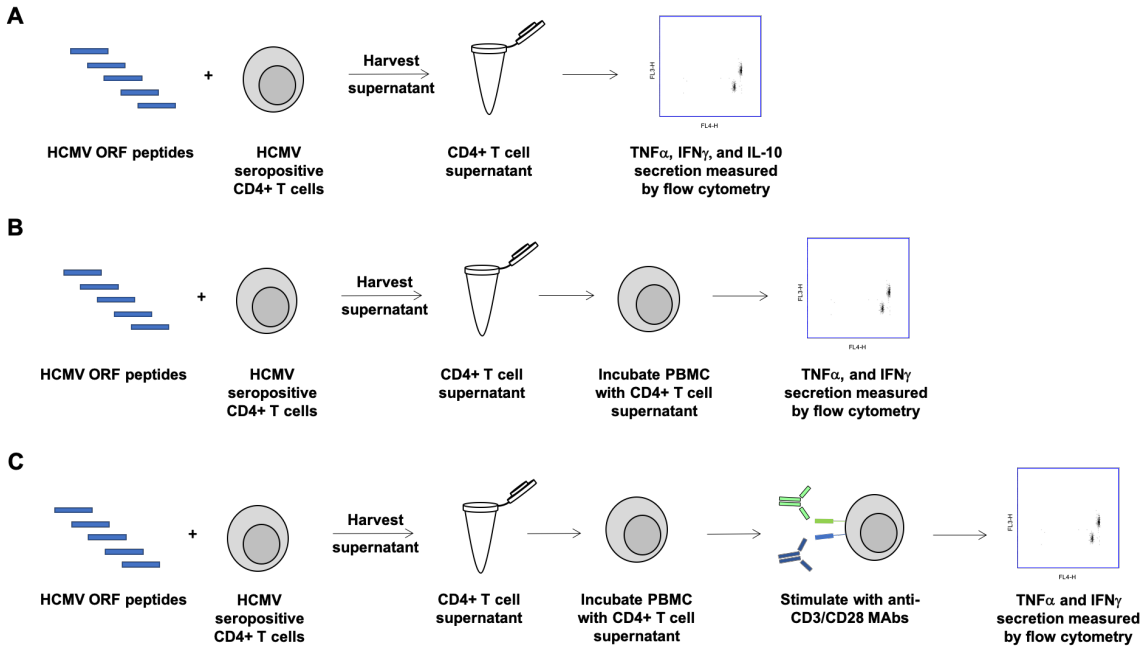



**Fig. 6.9 Representation of the stages in the cytokine secretion inhibition assay.** (A) PBMC depleted of CD8+ cells were stimulated with HCMV ORF peptide pools for 48 hours, the supernatants harvested, and examined for IL-10, IFN$\gamma$, and TNF$\alpha$ secretion by a flow cytometry based bead array. (B) PBMC were incubated in these HCMV ORF stimulated CD4+ T cell supernatants for 24 hours, and as a measure of background cytokine secretion, IFN$\gamma$ and TNF$\alpha$ was quantified by a flow cytometry based bead array. (C) In test conditions, a mix of anti-CD3 and anti-CD28 monoclonal antibodies (MAbs) were added to PBMC following the incubation in (B), and 18 hours later the supernatants were harvested. IFN$\gamma$ and TNF$\alpha$ secretion was then determined by a flow cytometry based bead array.

CD4+ T cell unstimulated supernatant control, was observed from the US28 ORF supernatant (Figures 6.10D and 6.12A). Finally, there was only a small inhibitory effect on TNF$\alpha$ and IFN$\gamma$ secretion from donor CMV401 CD3/CD28 stimulated PBMC when incubated with pp65 and UL144 CD4+ T cell supernatants from donor CMV319 (Figure 6.10E and F, and 6.12E). In contrast, there was an increase in IFN$\gamma$ secretion from LUNA and US28 ORF CD4+ T cell supernatant incubation, and an increase in TNF$\alpha$ following LUNA and UL111A supernatant incubation (Figure 6.10E and F, 6.12E).

The same three sets of CD4+ T cell ORF stimulated supernatants were then tested on CD3/CD28 stimulated PBMC from donor CMV426, highlighting considerable differences in their effects on PBMC IFN$\gamma$ and TNF$\alpha$ secretion between CMV401 and CMV426. None of the CMV324 derived CD4+ T cell supernatants showed an inhibitory effect on CMV401 PBMC (Figure 6.10A and B, and 6.12C), but on CMV426 PBMC, all were able to mediate inhibition of both IFN$\gamma$ and TNF$\alpha$ secretion, excluding US28, and UL111A CD4+ T cell supernatant on TNF$\alpha$ secretion (Figure 6.11A and B, and 6.12D). While UL111A and UL144 CD4+ T cell supernatants from donor CMV328 inhibited TNF$\alpha$ secretion from CMV401 CD3/CD28 stimulated PBMC (Figure 6.10C and 6.12A), these supernatants had a stimulatory effect on CMV426 CD3/CD28 stimulated PBMC (Figure 6.11C and 6.12B). All of the CD4+ T cell ORF supernatants from CMV328 also increased IFN$\gamma$ secretion from donor CMV426 PBMC to a much greater extent, reaching around 7.5 times higher than the unstimulated control supernatant in several cases (Figure 6.11D and 6.12B). Similarly, differential abilities of these supernatants to inhibit cytokine secretion between donors was observed for CMV319 supernatants. The pp65 and UL144 derived CD4+ T cell ORF supernatants showed a slight inhibitory effect on IFN$\gamma$ and TNF$\alpha$ secretion on donor CMV401 PBMC (Figure 6.10E and F, and 6.12E), but this was not replicated for donor CMV426, where cytokine secretion was actually increased (Figure 6.11E and F, and 6.12F). Inhibition was however observed from CM426 PBMC for LUNA and US28 CD4+ T cell ORF supernatants (Figure 6.11E and F, and 6.12F), which was not seen for donor CMV401 PBMC (Figure 6.10E and F, and 6.12E).

The discrepancies in cytokine secretion inhibition observed between PBMC from donors CMV401 and CMV426 were considerable. It was hypothesised that this could be due to the non-autologous nature of these experiments where CD4+ T cell supernatants were not matched to the PBMC donor. Further work was therefore carried out using an autologous system where supernatants were tested for their abilities to inhibit PBMC from the same donor. CD4+ T cell supernatants were generated from one donor (CMV332) and assayed for the presence of IL-10, IFN$\gamma$, and TNF$\alpha$ as before (Figure 6.9A and Appendix B.4), and for the quantities of total TGF$\beta$ (Figure 6.1B). In the CD4+ T cell supernatants, IL-10 was

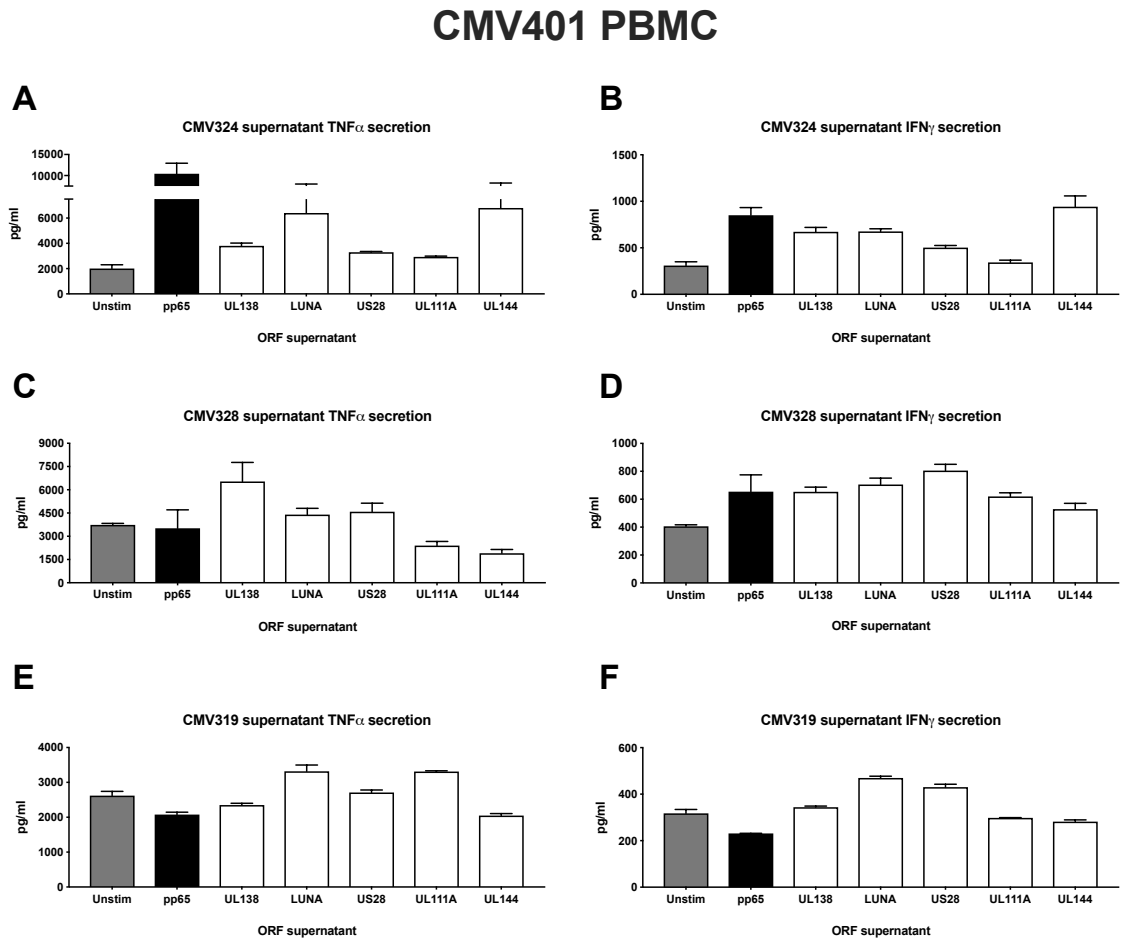



**Fig. 6.10 IFN$\gamma$ and TNF$\alpha$ secretion by PBMC stimulated following incubation with supernatant derived from CD4+ T cells stimulated with HCMV ORF peptide pools** PBMC from donor CMV401 were incubated with three sets of supernatant generated from the HCMV seropositive donors CMV328, CMV324, and CMV319 for 24 hours. These supernatants were derived from the stimulation of CMV328, CMV324, and CMV319 CD4+ T cells with overlapping HCMV ORF peptide pools spanning pp65, UL138, LUNA, US28, UL111A, and UL144 ORFs, or media alone (Unstim), for 48 hours. Following this incubation, PBMC were then stimulated with a mix of anti-CD3/CD28 monoclonal antibodies for 18 hours. Supernatants were then assayed for the secretion of (A, C, E) TNF$\alpha$ and (B, D, F) IFN$\gamma$ by flow cytometry in duplicate.

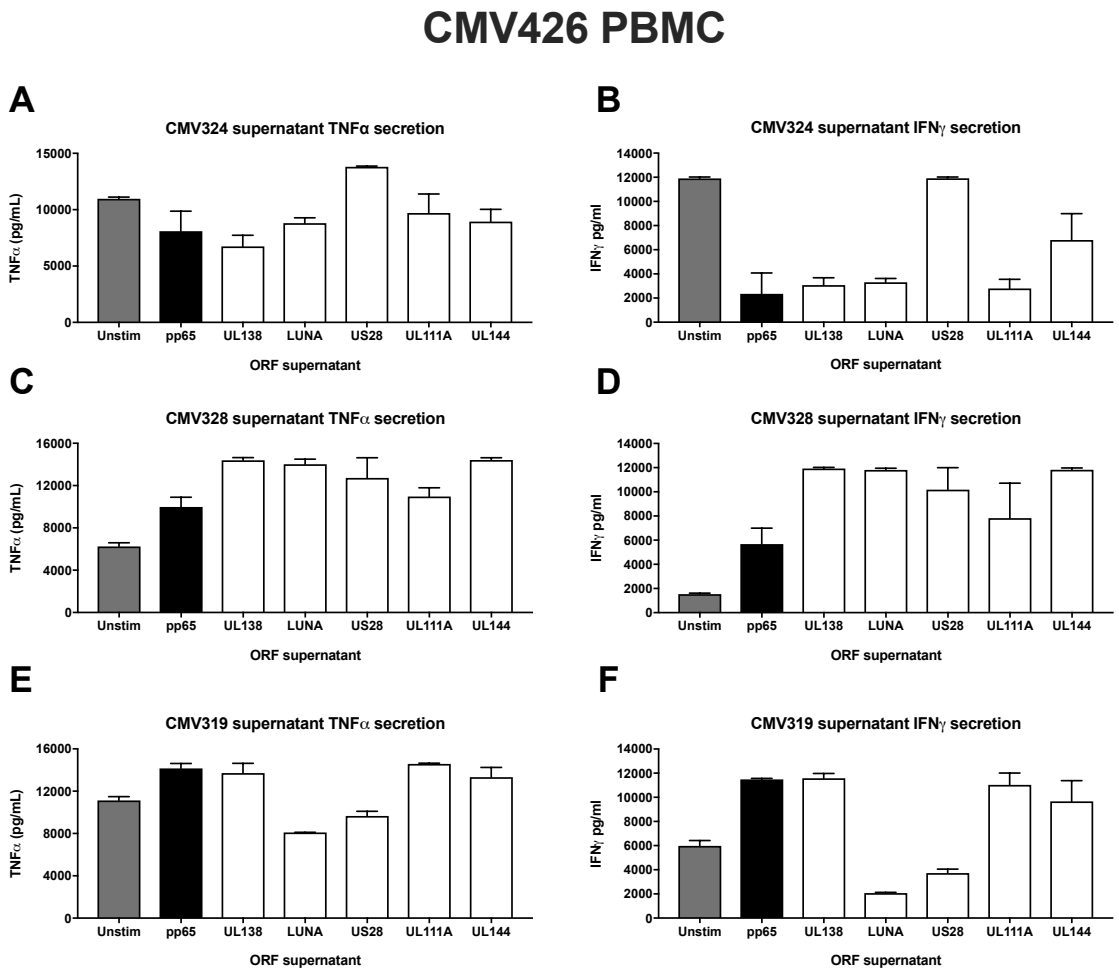



**Fig. 6.11 IFN$\gamma$ and TNF$\alpha$ secretion by PBMC stimulated following incubation with supernatant derived from CD4+ T cells stimulated with HCMV ORF peptide pools** PBMC from donor CMV426 were incubated with three sets of supernatant generated from the HCMV seropositive donors CMV328, CMV324, and CMV319 for 24 hours. These supernatants were derived from the stimulation of CMV328, CMV324, and CMV319 CD4+ T cells with overlapping HCMV ORF peptide pools spanning pp65, UL138, LUNA, US28, UL111A, and UL144, or left unstimulated in media, for 48 hours. Following this incubation, PBMC were then stimulated with a mix of anti-CD3/CD28 monoclonal antibodies for 18 hours. Supernatants were then assayed for the secretion of (A, C, E) TNF$\alpha$ and (B, D, F) IFN$\gamma$ by flow cytometry in duplicate.

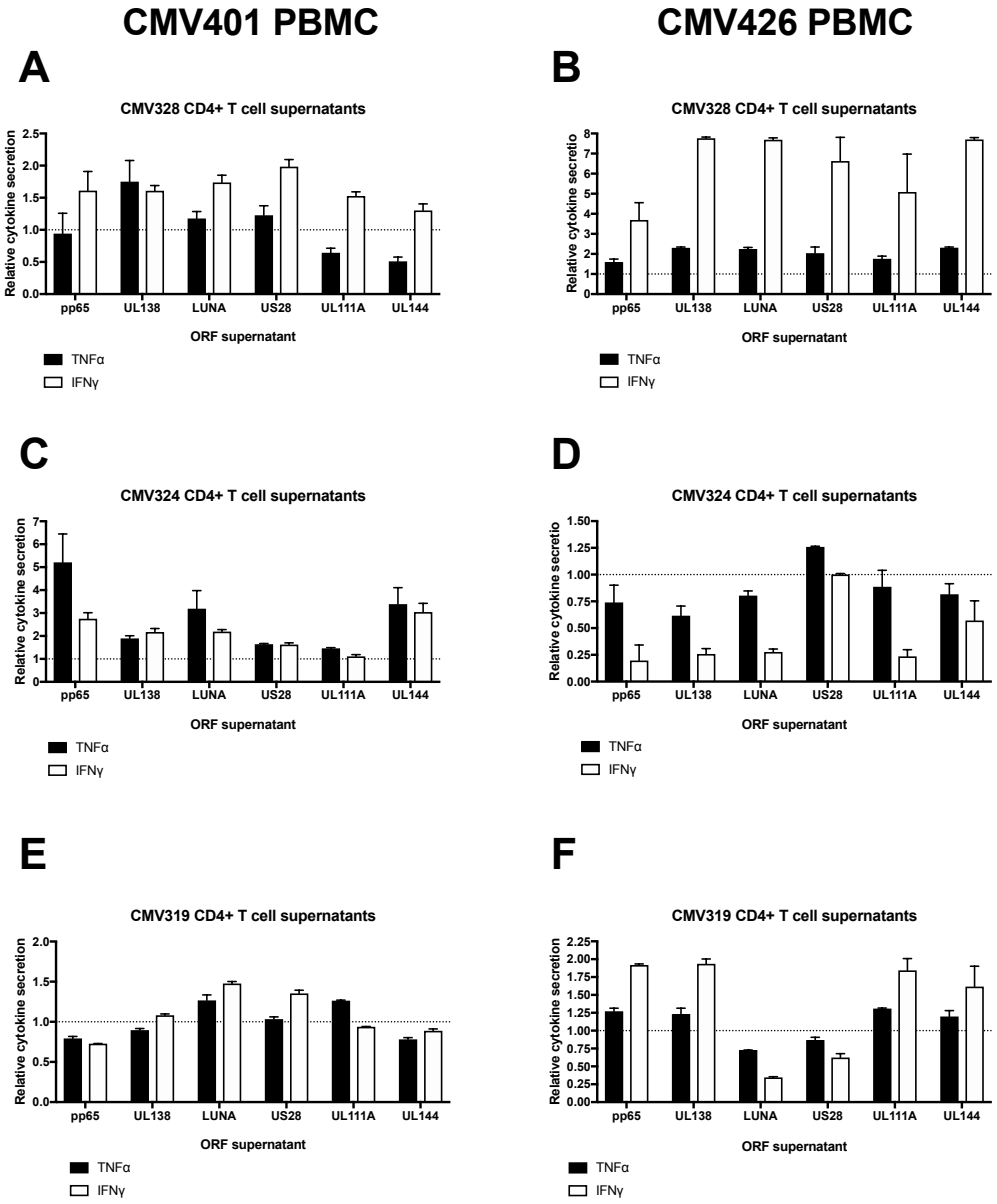



**Fig. 6.12 Relative IFN$\gamma$ and TNF$\alpha$ secretion by PBMC stimulated following incubation with supernatant derived from CD4+ T cells stimulated with HCMV ORF peptide pools** PBMC from donors CMV401 and CMV426 were incubated with three sets of supernatant generated from the HCMV seropositive donors CMV328, CMV324, and CMV319 for 24 hours. These supernatants were derived from the stimulation of CMV328, CMV324, and CMV319 CD4+ T cells with overlapping HCMV ORF peptide pools spanning pp65, UL138, LUNA, US28, UL111A, and UL144, or left unstimulated in media, for 48 hours. Following this incubation, PBMC were then stimulated with a mix of anti-CD3/CD28 monoclonal antibodies. The next day, supernatants were assayed for the secretion of TNF$\alpha$ and IFN$\gamma$ by flow cytometry. Secretion of IFN$\gamma$ and TNF$\alpha$ is displayed relative to the secretion of these cytokines from PBMC incubated in CD4+ T cell unstimulated supernatant and stimulated with anti-CD3/CD28 antibodies (shown with the dotted line).

detected at very low levels, around 1pg/ml, in response to US28 from CMV332 (Appendix B.4). Total TGF$\beta$ was also elevated in response to all HCMV ORFs, with the exception of UL138 (Figure 6.1B). PBMC from donor CMV332 were then incubated with these CD4+ T cell ORF supernatants for 42 hours to determine the background levels of IFN$\gamma$ and TNF$\alpha$ secretion when PBMC were not stimulated, and these quantities were deducted from the cytokine secretion observed in the test conditions (Appendix B.4).

The autologous PBMC were then stimulated with anti-CD3/CD28 antibodies in the presence of the CD4+ T cell supernatants. Inhibition of cytokine secretion was observed from the majority of CD4+ T cell ORF supernatants, although there were differential effects on TNF$\alpha$ and IFN$\gamma$ secretion from several ORF pools. IFN$\gamma$ secretion was inhibited by all of the ORF supernatants tested except for the UL138 ORF stimulated supernatant (Figure 6.13A), suggesting this effect could be due to the presence of TGF$\beta$ in these CD4+ T cell supernatants (Figure 6.1B). The greatest inhibition of IFN$\gamma$ secretion was observed from PBMC stimulated in the presence of supernatant from UL144 ORF stimulated CD4+ T cells, reaching nearly 42% inhibition compared to the unstimulated supernatant (Figure 6.13A and C). Low levels of IFN$\gamma$ were detected in the pp65 ORF CD4+ T cell supernatant, while no IFN$\gamma$ was detected in the IE1 ORF CD4+ T cell supernatant (Appendix B.4). The TGF$\beta$ present in both of these supernatants (Figure 6.1B) could therefore have been the mediator of the IFN$\gamma$ secretion from PBMC.

The effects of the CD4+ T cell ORF supernatants on TNF$\alpha$ secretion were more variable in comparison to IFN$\gamma$ secretion, as has been observed in previous experiments. The UL138, LUNA, and UL111A CD4+ T cell ORF supernatants caused a low level of inhibition on TNF$\alpha$ secretion from CD3/CD28 stimulated PBMC, while supernatants from pp65, IE1, and US28 increased TNF$\alpha$ secretion, and the UL144 supernatant had no effect (Figure 6.13 B and C).

In summary, the use of an autologous system for the cytokine secretion inhibition assay has provided initial data showing that supernatant from HCMV-specific CD4+ T cells can inhibit anti-viral cytokine production, although the extent of inhibition of TNF$\alpha$ secretion was less than for IFN$\gamma$. Furthermore, certain CD4+ T cell ORF supernatants that inhibited the secretion of IFN$\gamma$ were found to increase secretion of TNF$\alpha$ from stimulated PBMC. This inhibition was observed in the absence of elevated levels of IL-10 and could be mediated by TGF$\beta$.

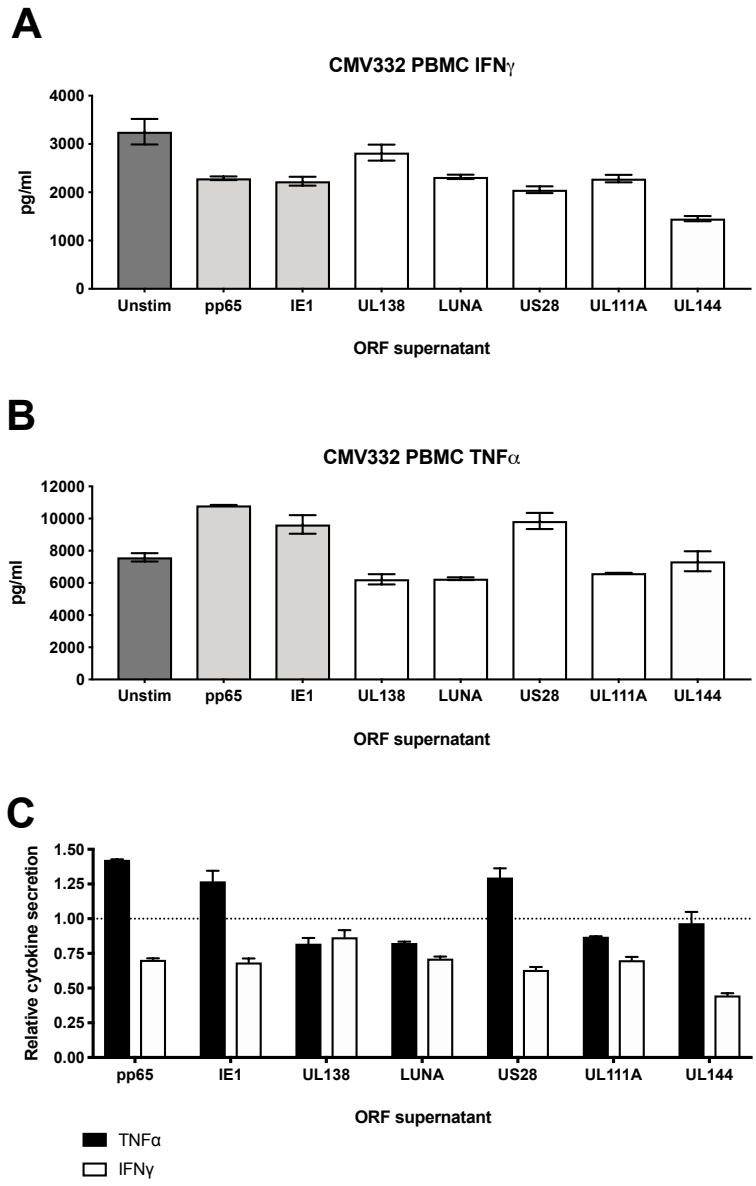



**Fig. 6.13 IFN$\gamma$ and TNF$\alpha$ secretion by PBMC stimulated following incubation with supernatant derived from autologous CD4+ T cells stimulated with HCMV ORF peptide pools** CD4+ T cells from donor CMV332 were stimulated with overlapping HCMV ORF peptide pools spanning pp65, UL138, LUNA, US28, UL111A, and UL144, or left unstimulated in media (Unstim), for 48 hours. Autologous PBMC were then incubated with these supernatants for 24 hours and then stimulated with a mix of anti-CD3/CD28 monoclonal antibodies. The next day, supernatants were assayed for the secretion of (A) TNF$\alpha$ and (B) IFN$\gamma$ by flow cytometry. (C) The secretion of TNF$\alpha$ (black bars) and IFN$\gamma$ (white bars) from PBMC following stimulation in ORF-specific supernatants relative to the secretion of these cytokines from stimulation in the unstimulated supernatant.

### 6.2.4 Assessing the ability of HCMV-specific CD4+ T cell secretomes to inhibit CD8+ T cell control of viral spread

The secretome from CD4+ T cells stimulated with HCMV ORF peptide pools was then investigated for their ability to inhibit CD8+ T cell control of an active lytic infection in fibroblasts. As CD4+ T cells specific for HCMV latency-associated proteins secrete IL-10 and TGF$\beta$, it was hypothesised that these immunosuppressive factors could reduce the ability of CD8+ T cells to control HCMV lytic infection. To do this, a well described viral dissemination assay was used [291]. Preliminary experiments were carried out using autologous fibroblasts infected at low multiplicity of infection (MOI) with a Merlin virus carrying a UL32-GFP tag. One day post infection, decreasing concentrations of CD8+ T cells were added to the infected fibroblasts. The infected cells were then monitored for the spread of virus over 10 days and the percentage of GFP-expressing fibroblasts determined by flow cytometry. As expected, the control of HCMV spread was dependent on the effector-to-target (E:T) ratio, where the greatest levels of control were seen at the highest E:T ratios, with a gradual decline in control with decreasing E:T ratio observed (Figure 6.14). CD8+ T cells were then incubated with recombinant IL-10 and TGF$\beta$ for two hours prior to their addition onto the infected fibroblasts and the spread of the virus monitored as before. CD8+ T cells treated with recombinant IL-10 and TGF$\beta$ were unable to control viral spread as well as CD8+ T cells alone, although this effect was most pronounced at the lowest E:T ratio tested (Figure 6.15). It is likely that the relatively weak inhibition of CD8+ T cell responses here were due to the ability of this donor's CD8+ T cells to overcome the effects of IL-10 and TGF$\beta$ at a high E:T ratio.

CD4+ T cell secretomes generated from the stimulation of CD8+ cell depleted PBMC with HCMV ORF peptide pools were then tested for their abilities to inhibit CD8+ T cell mediated control of viral spread. As these secretomes could contain antiviral factors such as IFN$\gamma$ and TNF$\alpha$, in addition to any inhibitory cytokines like IL-10 and TGF$\beta$, they were first tested for their ability to inhibit viral spread in isolation, in two donors. One day post infection, fibroblasts were incubated with CD4+ T cell secretomes and were not removed for the remainder of the assay. Several of the CD4+ T cell secretomes were able to inhibit viral spread compared to the effect of fresh media and the secretome from unstimulated CD4+ T cells. Donor CMV301 displayed the greatest inhibition of viral spread from the pp65 and UL144 stimulated supernatants (Figure 6.16A). IE1, LUNA, and UL111A stimulated supernatants also inhibited viral spread to a large extent, while UL138 and US28 stimulated supernatants did not have an effect (Figure 6.16A). Likewise, many CD4+ T cell supernatants from donor CMV305 had an inhibitory effect on viral spread in isolation. In particular,

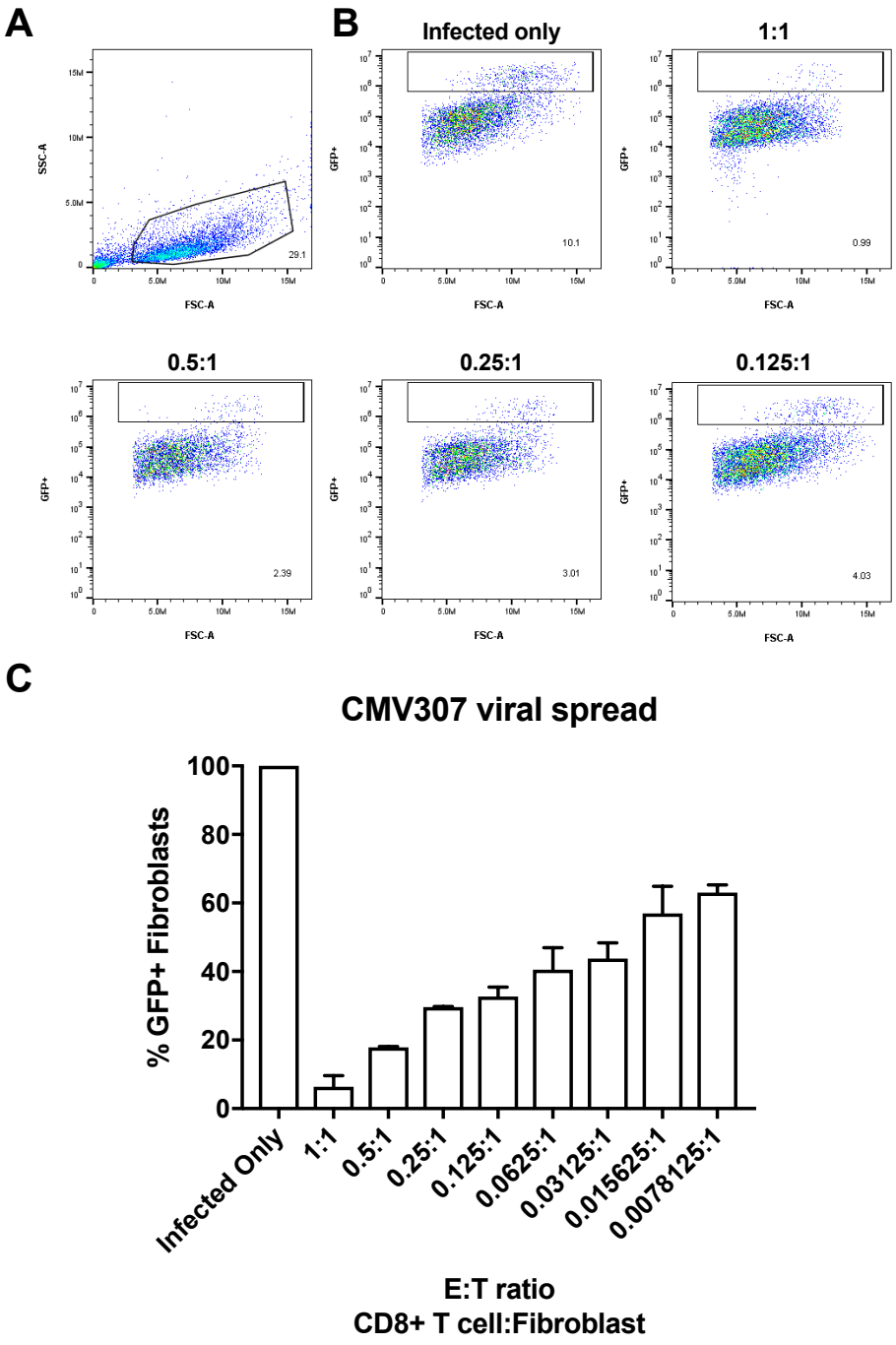



**Fig. 6.14 *In vitro* spread of HCMV in the presence of CD8+ T cells** Autologous dermal fibroblasts from donor CMV307 were infected at low MOI with a Merlin-GFP virus. The following day, CD8+ T cells isolated from the same donor by MACS were added at a range of effector-to-target (E:T) ratios to the infected cells. The spread of the virus was then monitored over 10 days and analysed for the percentage of GFP expressing fibroblasts by flow cytometry compared to uninfected fibroblasts. The percentage of GFP expressing fibroblasts in test conditions with added CD8+ T cells was normalised to the infected only control samples to give a relative measure of infection.

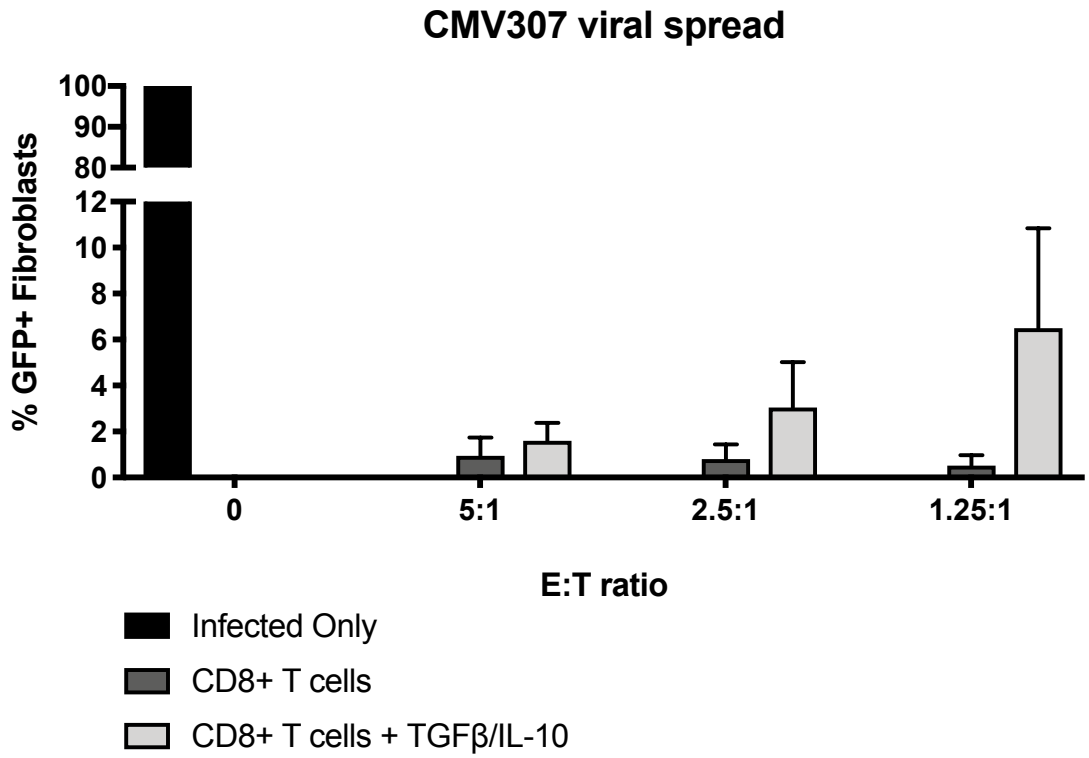



**Fig. 6.15 *In vitro* spread of HCMV in the presence of CD8+ T cells treated with TGF$\beta$ and IL-10** Autologous dermal fibroblasts from donor CMV307 were infected at low MOI with a Merlin-GFP virus. The following day, CD8+ T cells isolated from the same donor were incubated with a mix of 1.25ng/ml IL-10 and 0.5ng/ml TGF$\beta$ and added at a range of effector-to-target (E:T) ratios to the infected cells. The spread of the virus was then monitored over 10 days and analysed for the percentage of GFP expressing fibroblasts by flow cytometry compared to uninfected fibroblasts. The percentage of GFP expressing fibroblasts in test conditions with added CD8+ T cells was normalised to that in fibroblasts infected without added CD8+ T cells to give a relative measure of infection.

IE1, LUNA, UL111A, and UL144 stimulated supernatants, which inhibited virus spread considerably (Figure 6.16B).

CD8+ T cells were then incubated for 18 hours in the donor-matched CD4+ T cell secretomes before their addition to infected autologous fibroblasts. The CD8+ T cells were washed prior to their addition onto lytically infected fibroblasts to remove the potentially antiviral CD4+ T cell supernatants. When comparing the ability of CD8+ T cells to control viral spread following incubation with ORF secretomes or with the control unstimulated secretomes, no inhibitory effect was found for any of the ORF secretomes (Figure 6.17). However, compared to CD8+ T cells alone, incubation with UL138 CD4+ T cell supernatant resulted in a reduction in the ability of CD8+ T cells to control viral spread by nearly 25% (Figure 6.17). It was thought that the inability of the CD4+ T cell secretomes to inhibit CD8+ T cells was due to the removal of the secretomes, and with it the removal of any inhibitory cytokines. It could therefore be the case that these secretomes need to be present for the duration of the assay to mediate inhibition.

Despite initial data showing IL-10 and TGF$\beta$ could inhibit the ability of CD8+ T cells to control a lytic HCMV infection in fibroblasts, the same effect with HCMV latency-associated ORF-specific CD4+ T cell supernatants could not be replicated. The factors present in CD4+ T cell supernatants from two donors were unable to inhibit the ability of CD8+ T cells to control viral spread in autologous fibroblasts following a 18 hour incubation with the supernatants. Incubating infected fibroblasts with the CD4+ T cell supernatants alone confirmed the presence of anti-viral factors that in many cases reduced viral spread considerably.

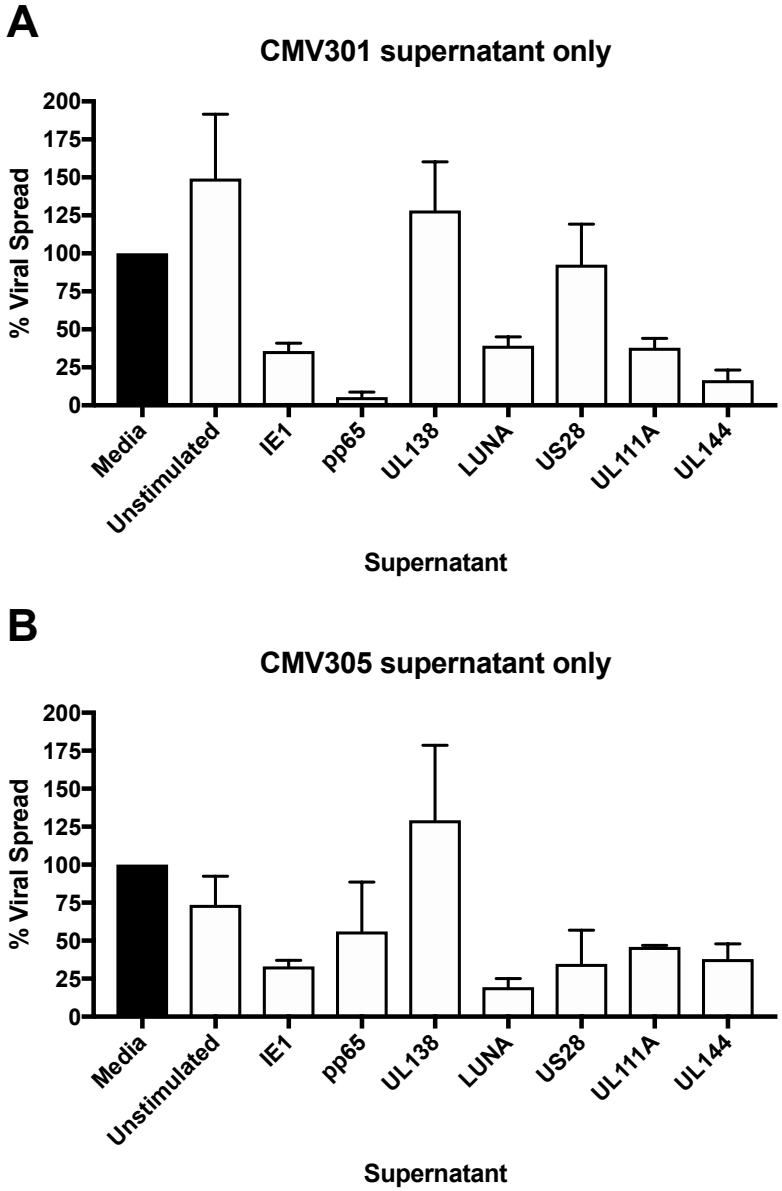



**Fig. 6.16 *In vitro* spread of HCMV in the presence of CD4+ T cell secretomes** Autologous dermal fibroblasts from donors (A) CMV301 and (B) CMV305 were infected at low MOI with a Merlin-GFP virus. The following day, infected fibroblasts were incubated in supernatant derived from autologous CD4+ T cells left unstimulated for 48 hours, or stimulated with overlapping peptide pools spanning the ORFs of IE1, pp65, UL138, LUNA, US28, UL111A, and UL144 or with fresh media. The spread of the virus was then monitored over 10 days and analysed for the percentage of GFP expressing fibroblasts by flow cytometry compared to uninfected fibroblasts. The percentage of GFP expressing fibroblasts in test conditions with added CD8+ T cells was normalised to the infected only control samples to give a relative measure of infection.

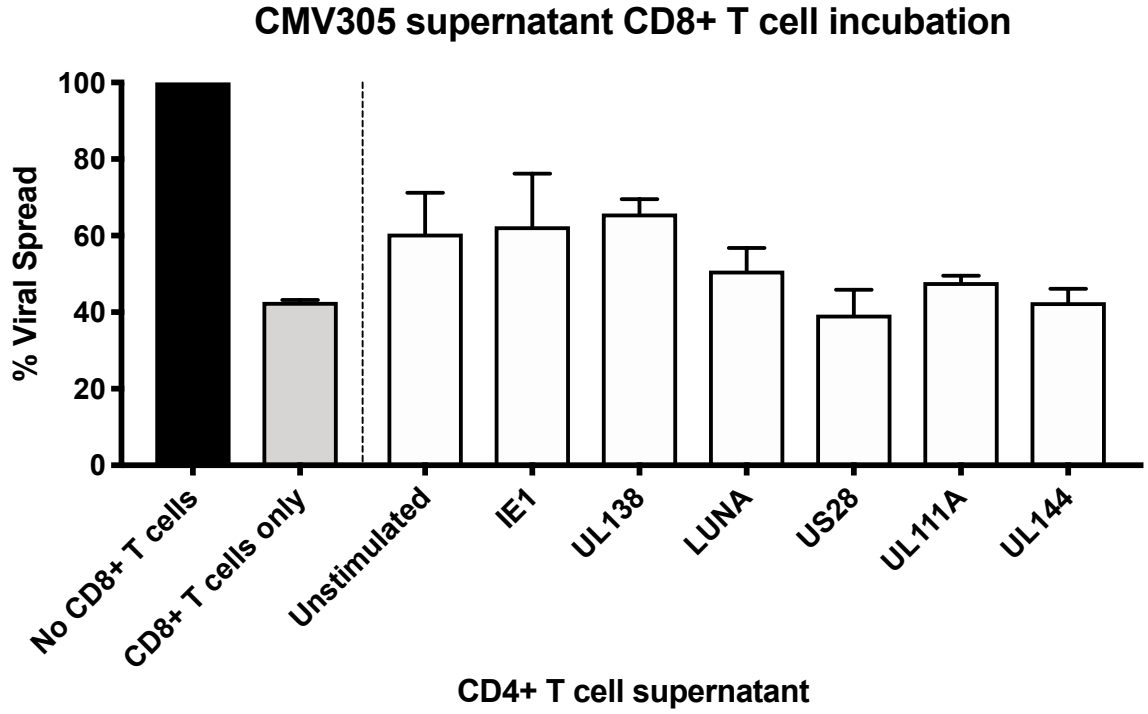



**Fig. 6.17 *In vitro* spread of HCMV in the presence of CD8+ T cells pre-incubated with CD4+ T cell secretomes** Autologous dermal fibroblasts from donor CMV305 were infected at low MOI with a Merlin-GFP virus. The same day, CD8+ T cells were isolated from the donor and incubated for 18 hours in supernatant derived from this donor's CD4+ T cells left unstimulated for 48 hours, or stimulated with overlapping peptide pools spanning the ORFs of IE1, UL138, LUNA, US28, UL111A, and UL144 or with fresh media. The following day, CD8+ T cells were washed in fresh media were added at an effector-to-target (E:T) ratio of 2:1 to the infected fibroblasts. The spread of the virus was then monitored over 10 days and analysed for the percentage of GFP expressing fibroblasts by flow cytometry compared to uninfected fibroblasts. The percentage of GFP expressing fibroblasts in test conditions with added CD8+ T cells was normalised to the infected only control samples to give a relative measure of infection.

### 6.2.5 Depletion of CD25+ expressing CD4+ T cells does not consistently reduce the frequency of IL-10 secreting cells

The secretion of IL-10 is a well described function of Tregs. Tregs mediate their immunosuppressive effects in part through IL-10 and to TGF$\beta$ secretion. Previous work identified expression of phenoyptic markers associated with Tregs in a subset of LUNA- and UL138-specific CD4+ T cells, which were absent from gB-specific CD4+ T cells [501]. Having also found extensive CD4+ T cell IL-10 responses to UL138, LUNA, US28, and UL111A, it was hypothesised that these cells could be Tregs. The presence of two separate populations of CD4+ T cells specific for the latency-associated HCMV ORFs, those secreting IFN$\gamma$ and those secreting IL-10, could enable the selective depletion of the IL-10 secreting CD4+ T cells if these cells could be identified phenotypically. Thus, if such IL-10 secreting cells are predominantly Tregs, their selective depletion could be an effective and simple approach to remove IL-10 secreting CD4+ T cells and enable the CD4+ T cells that secrete IFN$\gamma$, which might be more effective anti-viral cells, to predominate.

Tregs can be identified based on a number of different phenotypic markers. As has been described, CD4+ T cells with low expression of CD127 and high expression of CD25 were found to express FoxP3 and have the characteristics of Tregs [482–484]. Therefore, the elevated expression of the IL-2 receptor alpha chain (CD25) on Tregs could provide a convenient target for their selective depletion. Indeed, previous work targeting Tregs by CD25+ cell depletion led to an increase in IE1- and pp65-specific IFN$\gamma$ secretion [494, 502]. The expression of FoxP3 by CD4+ T cells expressing high levels of CD25 was confirmed using an intracellular staining procedure. The gating strategy to identity these cells is shown in Figure 6.18. PBMC were stained and CD3+CD4+ cells identified following the exclusion of doublets and gating for lymphocytes on forward and side scatter (Figure 6.18A). The $CD127^{lo}CD25^{hi}$ population was then gated on and the expression of FoxP3 within this population compared to the $CD127^{hi}$ expressing CD4+ T cells, confirming that FoxP3 expression was elevated on the $CD127^{lo}CD25^{hi}$ CD4+ T cells, which are therefore likely to be Tregs (Figure 6.18B).

The depletion of $CD25^{hi}$ CD4+ T cells was then tested in an attempt to remove the HCMV-specific IL-10 expressing CD4+ T cells. PBMC were either depleted of CD8+ cells alone, or depleted of both CD8+ and CD25+ high expressing cells, by MACS to test the CD4+ T cell responses with and without $CD25^{hi}$ cells. These depleted populations were then stimulated with overlapping peptide pools spanning the ORFs of gB, pp65, IE1, UL138, LUNA, US28, UL111A, and UL144 on dual IFN$\gamma$/IL-10 FluoroSpot plates. The frequencies

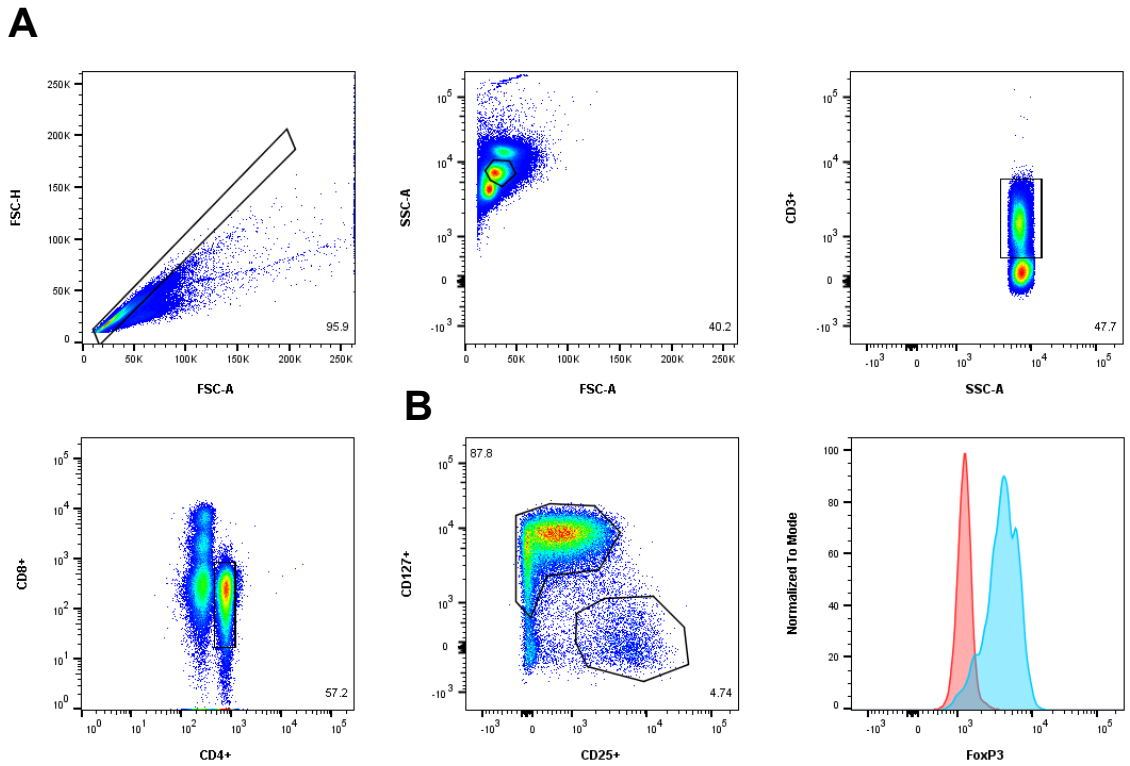



**Fig. 6.18 Identifying regulatory CD4+ T cells by FoxP3 expression** PBMC from donor CMV327 were phenotyped to identify Tregs by the markers CD127, CD25, and FoxP3. (A) Doublets were first excluded, following by gating for lymphocytes by forward (FSC-A) and side scatter (SSC-A). CD3+ cells and CD4+ cells were then gated on. (B) Tregs were identified as $CD127^{lo}CD25^{hi}$, and the expression of FoxP3 from this population confirmed to be elevated (blue histogram) compared to other CD4+ T cells expressing CD127+ (red histogram).

of IFN$\gamma$ and IL-10 secreting CD4+ T cells was then compared between the CD8+ depleted and CD8+CD25$^{hi}$ depleted conditions. It might be expected that in addition to a decrease in the frequency of IL-10 producing cells when CD25$^{hi}$ cells are depleted, there could also be an increase in the frequency of IFN$\gamma$ secreting cells, as they should occupy a higher proportion of the total input cell number, and because of the decrease in immunosuppressive IL-10 secretion. CD25+ cell depletions were carried out on PBMC from six HCMV seropositive donors.

The depletion of CD25$^{hi}$ cells was verified by flow cytometry to ensure that cells with the highest expression of CD25+ had been depleted. Depleting CD25$^{hi}$ CD4+ T cells from donor CMV319 (Figure 6.19A) resulted in a decrease in the frequency of IFN$\gamma$ secreting cells responding to pp65 and UL144, which were the only two ORFs responded to by a high frequency of IFN$\gamma$ secreting cells (Figure 6.19B). Following the same pattern, a decrease in the frequency of IL-10 secreting cells was also observed for responses to pp65 and UL144 after CD25$^{hi}$ cell depletion (Figure 6.19C). Additionally, CD4+ T cells secreting IL-10 in response to gB and US28 were also reduced following this depletion (Figure 6.19C). Therefore, while the depletion of CD25$^{hi}$ cells in this donor did not remove all IL-10 secreting CD4+ T cells, a proportion of ORF-specific CD4+ T cells secreting IL-10 were CD25$^{hi}$.

Depletion of CD25$^{hi}$ cells from donor CMV321 (Figure 6.20A) resulted in a decrease in the frequency of IFN$\gamma$ secreting CD4+ T cells responding to UL144, but had no effect on IFN$\gamma$ responses to other ORFs (Figure 6.20B). As with donor CMV319, this decrease in IFN$\gamma$ secreting cells responding to UL144 following CD25$^{hi}$ cell depletion was also observed for CD4+ T cells secreting IL-10 (Figure 6.20C). Therefore, approximately two-thirds of the IL-10 secreting CD4+ T cell population responding to UL144 were CD25$^{hi}$ (Figure 6.20C). However, this was not the case for IL-10 secreting CD4+ T cells responding to LUNA, which were not appreciably changed following this depletion (Figure 6.20C).

Depletion of CD25$^{hi}$ cells (Figure 6.21A) from donor CMV328 resulted in an increase in the frequency of IFN$\gamma$ secreting CD4+ T cells for those responding to gB, pp65, and US28 (Figure 6.21B), which was associated with a decrease in the frequencies of IL-10 secreting cells responding to gB and US28 (Figure 6.21B). However, a decrease in the frequency of IFN$\gamma$ secreting CD4+ T cells was observed for responses to IE1, UL111A, and UL144 following CD25$^{hi}$ cell depletion (Figure 6.21B). No change was observed in the IL-10 secreting CD4+ T cell frequencies responding to pp65, LUNA, and UL144 (Figure 6.21C). Therefore, depletion of CD25$^{hi}$ cells from this donor removed a minor proportion of IL-10 secreting CD4+ T cells, and resulted in an increase in the frequencies of IFN$\gamma$ secreting CD4+ T cells, responding to gB and US28.

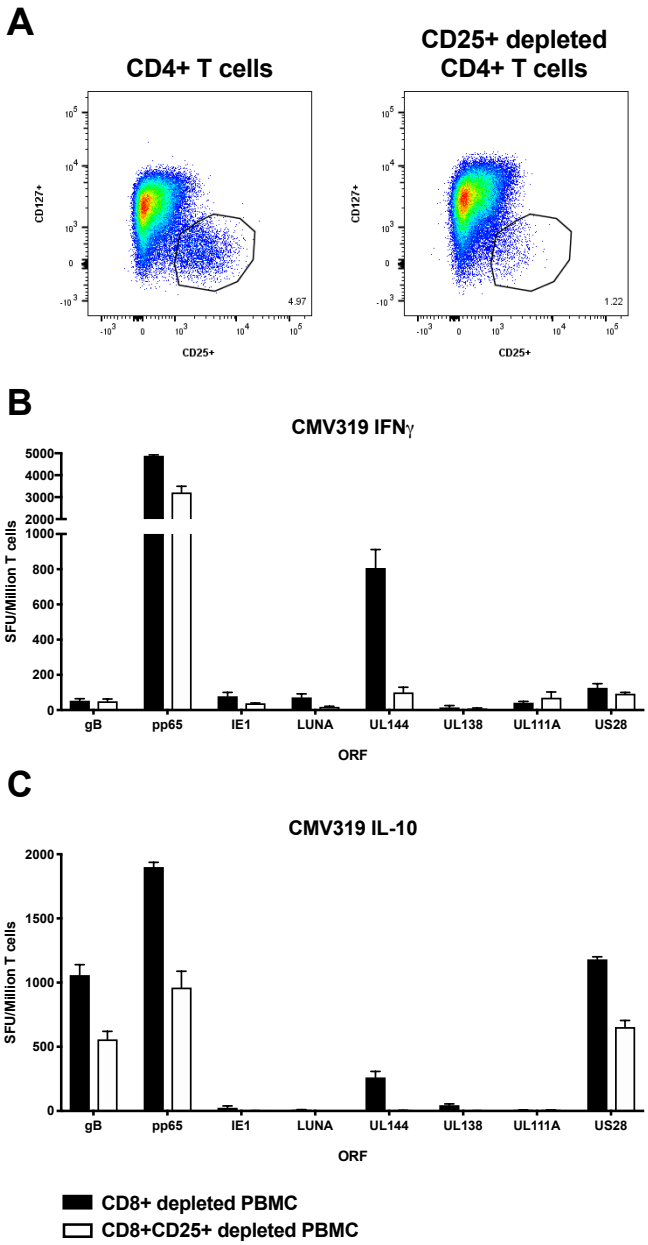



**Fig. 6.19 The frequency of IFN$\gamma$ and IL-10 secreting CD4+ T cells and CD25$^{hi}$ depleted CD4+ T cells from donor CMV319 following stimulation with HCMV ORF peptide pools** (A) PBMC from donor CMV319 were depleted of CD8+ cells and high expressors of CD25 by MACS to remove Tregs. Pre-depletion and post-depletion cells were stained for CD3, CD4, CD127, and CD25 to verify this depletion. PBMC depleted of CD8+ cells or CD8+CD25$^{hi}$ cells were then stimulated with overlapping peptide pools spanning the ORFs of gB, pp65, IE1, LUNA, UL144, UL138, UL111A, and US28 for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. The secretion of (B) IFN$\gamma$ and (C) IL-10 was then stained for and the number of positive spots enumerated by the automated cell counter and converted to a spot forming unit (SFU) value per million cells. Each peptide pool was tested in triplicate and the values from the number of background cells secreting cytokines in the unstimulated wells was deducted from all test wells.

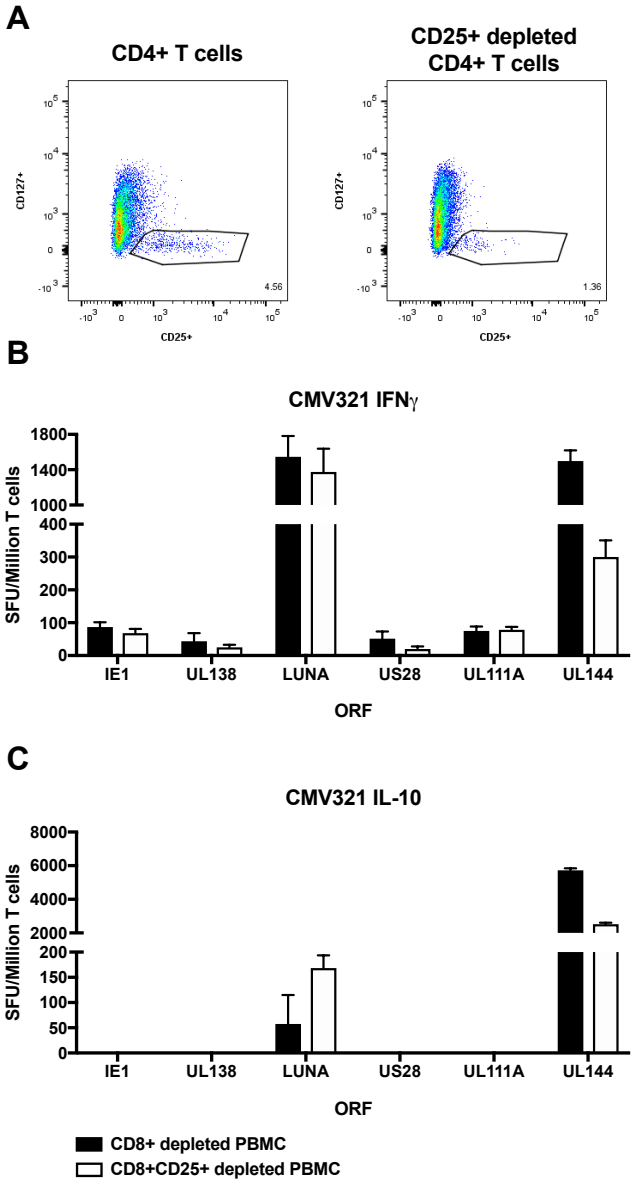



**Fig. 6.20 The frequency of IFN$\gamma$ and IL-10 secreting CD4+ T cells and CD25$^{hi}$ depleted CD4+ T cells from donor CMV321 following stimulated with HCMV ORF peptide pools** (A) PBMC from donor CMV321 were depleted of CD8+ cells and high expressors of CD25 by MACS to remove Tregs. Pre-depletion and post-depletion cells were stained for CD3, CD4, CD127, and CD25 to verify this depletion. PBMC depleted of CD8+ cells or CD8+CD25$^{hi}$ cells were then stimulated with overlapping peptide pools spanning the ORFs of gB, pp65, IE1, LUNA, UL144, UL138, UL111A, and US28 for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. The secretion of (B) IFN$\gamma$ and (C) IL-10 was then stained for and the number of positive spots enumerated by the automated cell counter and converted to a spot forming unit (SFU) value per million cells. Each peptide pool was tested in triplicate and the values from the number of background cells secreting cytokines in the unstimulated wells was deducted from all test wells.

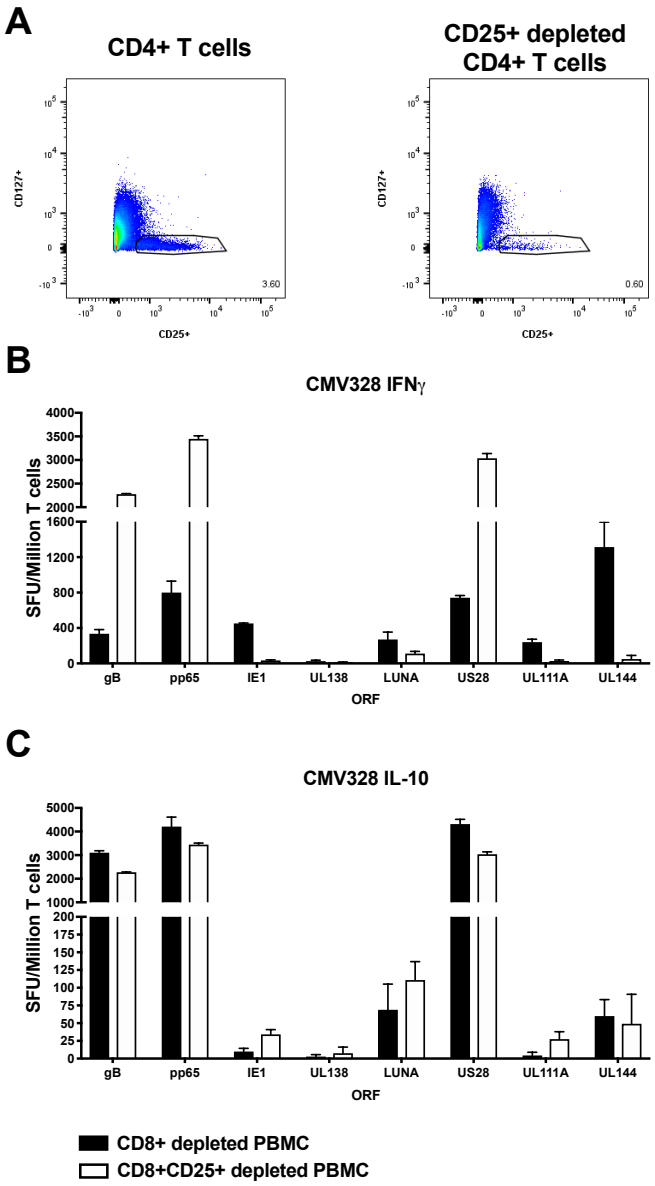



**Fig. 6.21 The frequency of IFN$\gamma$ and IL-10 secreting CD4+ T cells and CD25$^{hi}$ depleted CD4+ T cells from donor CMV328 following stimulated with HCMV ORF peptide pools** (A) PBMC from donor CMV328 were depleted of CD8+ cells and high expressors of CD25 by MACS to remove Tregs. Pre-depletion and post-depletion cells were stained for CD3, CD4, CD127, and CD25 to verify this depletion. PBMC depleted of CD8+ cells or CD8+CD25$^{hi}$ cells were then stimulated with overlapping peptide pools spanning the ORFs of gB, pp65, IE1, LUNA, UL144, UL138, UL111A, and US28 for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. The secretion of (B) IFN$\gamma$ and (C) IL-10 was then stained for and the number of positive spots enumerated by the automated cell counter and converted to a spot forming unit (SFU) value per million cells. Each peptide pool was tested in triplicate and the values from the number of background cells secreting cytokines in the unstimulated wells was deducted from all test wells.

There were a number of inconsistent changes following depletion of $CD25^{hi}$ cells from donor CMV324 (Figure 6.22A). Very small increases in CD4+ T cells secreting IFN$\gamma$ in response to gB, LUNA, UL144, and UL111A were detected, while no changes were observed for responses to pp65, and a small decrease was detected for the response to IE1 (Figure 6.22B). While no IL-10 secreting CD4+ T cells were detected in response to LUNA and UL111A prior to $CD25^{hi}$ cell depletion, there was a considerable increase in IL-10 secreting cells after this depletion (Figure 6.22B and C). A small decrease in IL-10 secreting CD4+ T cells was observed in response to pp65 post depletion, and there was no change in responses to gB and US28 (Figure 6.22C). $CD25^{hi}$ CD4+ T cells from donor CMV324 were therefore not part of the IL-10 secreting population for responses to most ORFs.

To ensure that the IL-10 secreting CD4+ T cells thought to be Tregs were not contained within the moderate and lower CD25+ expressing cells, a more comprehensive depletion of the CD25+ population was carried out. To do this, a higher concentration of CD25 specific microbeads was used to deplete these cells. Two donors were tested in this way and the depletion of CD25+ CD4+ T cells (Figure 6.23A) from donor CMV327 decreased the frequencies of CD4+ T cells secreting IFN$\gamma$ in response to gB, pp65, IE1, LUNA, and UL144 (Figure 6.23B). CD4+ T cells secreting IFN$\gamma$ in response to US28 were increased however (Figure 6.23B). The frequencies of IL-10 secreting CD4+ T cells were observed to decrease for responses to gB, pp65, LUNA, UL144, and US28 following CD25+ cell depletion (Figure 6.23C). Of note, CD25+ cell depletion decreased the frequencies of both IFN$\gamma$ and IL-10 secreting cells responding to gB, pp65, LUNA, and UL144, while for response to US28 this depletion increased IFN$\gamma$ secreting cells and decreased IL-10 secreting cells (Figure 6.23B and C). An increase in IL-10 secreting cells was observed for responses to IE1 and there was no change for responses to UL111A (Figure 6.23C). Although not the case for all ORF-specific responses, CD25+ cells made up a considerable proportion of IL-10 secreting CD4+ T cells in this individual.

Following CD25+ cell depletion, (Figure 6.24A), donor CMV323 had a decrease in CD4+ T cells secreting IFN$\gamma$ in response to UL144, while there was no such change for responses to pp65 and US28 (Figure 6.24B). CD25+ cell depletion increased the frequencies of CD4+ T cells secreting IL-10 in response to the majority of ORFs, including pp65, LUNA, UL111A, and US28, while there was no change for responses to gB, and UL144 (Figure 6.24C). Therefore in this donor, CD25+ cells were not responsible for HCMV ORF-specific IL-10 secretion.

Across the six donors tested, depletion of CD25 expressing cells had inconsistent effects on the frequencies of IFN$\gamma$ and IL-10 secreting CD4+ T cells. It is therefore likely that Tregs

of this phenotype (CD4+CD127$^{lo}$CD25$^{hi}$) are not the only CD4+ T cells secreting IL-10 in response to proteins expressed during HCMV latency, and the presence and size of this population can depend on the individual and on the ORF-specific response.

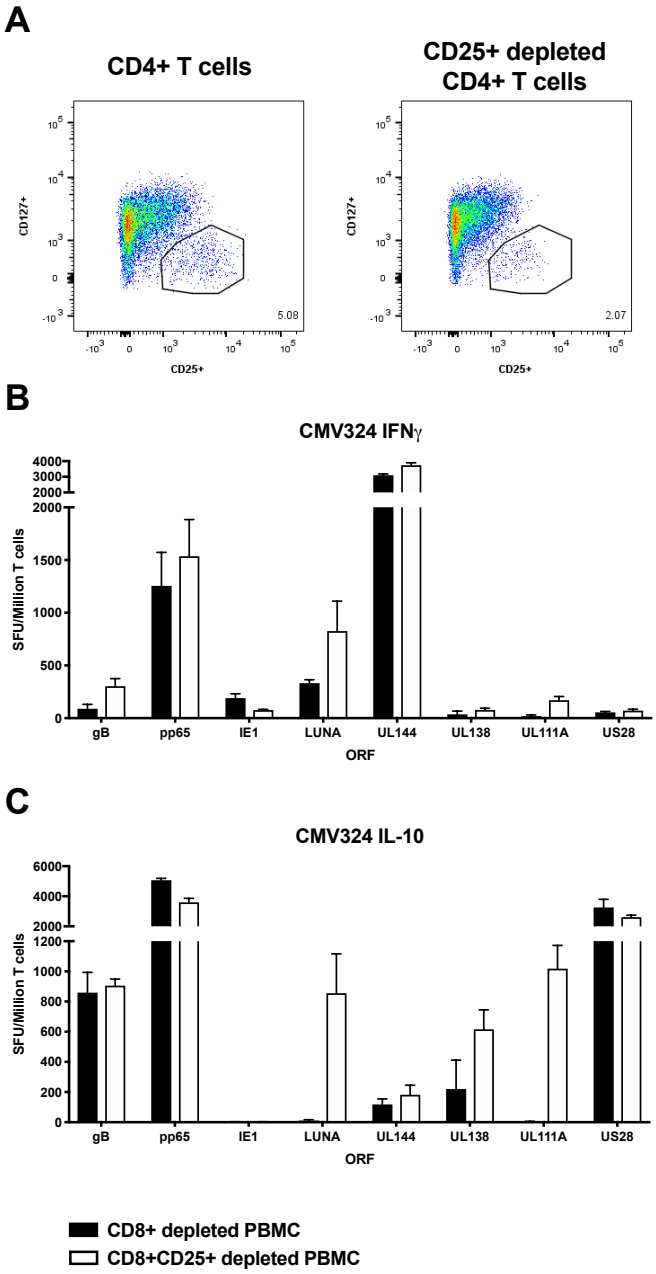



**Fig. 6.22 The frequency of IFN$\gamma$ and IL-10 secreting CD4+ T cells and CD25$^{hi}$ depleted CD4+ T cells from donor CMV324 following stimulated with HCMV ORF peptide pools** (A) PBMC from donor CMV324 were depleted of CD8+ cells and high expressors of CD25 by MACS to remove Tregs. Pre-depletion and post-depletion cells were stained for CD3, CD4, CD127, and CD25 to verify this depletion. PBMC depleted of CD8+ cells or CD8+CD25$^{hi}$ cells were then stimulated with overlapping peptide pools spanning the ORFs of gB, pp65, IE1, LUNA, UL144, UL138, UL111A, and US28 for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. The secretion of (B) IFN$\gamma$ and (C) IL-10 was then stained for and the number of positive spots enumerated by the automated cell counter and converted to a spot forming unit (SFU) value per million cells. Each peptide pool was tested in triplicate and the values from the number of background cells secreting cytokines in the unstimulated wells was deducted from all test wells.

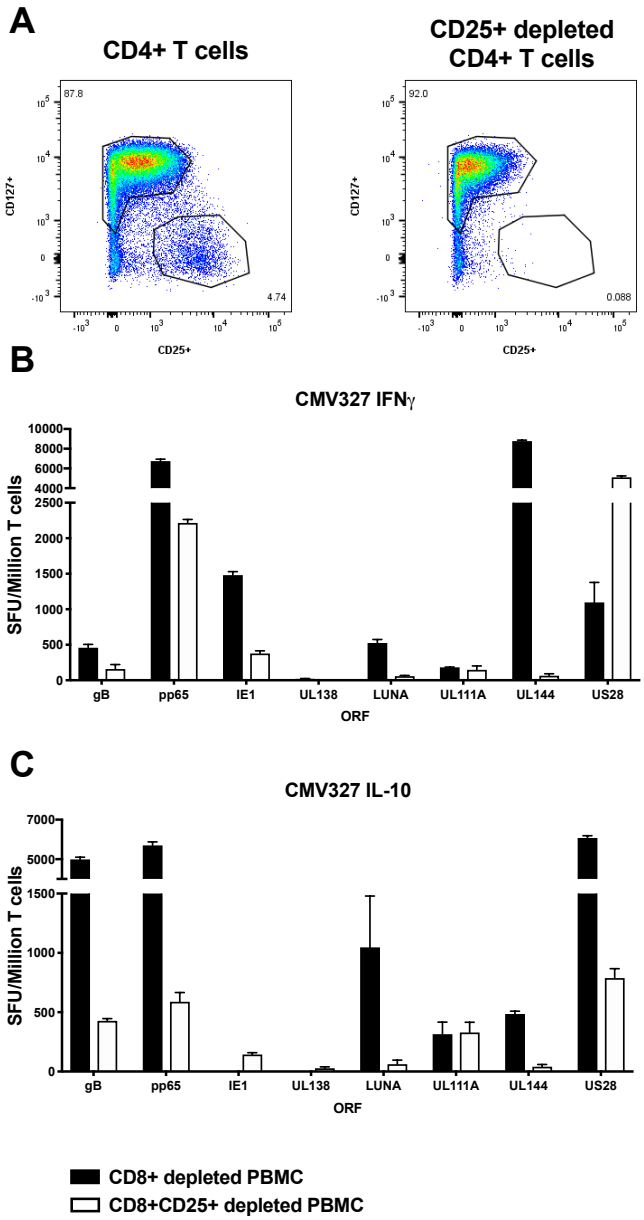



**Fig. 6.23 The frequency of IFN$\gamma$ and IL-10 secreting CD4+ T cells and CD25+ depleted CD4+ T cells from donor CMV327 following stimulated with HCMV ORF peptide pools** (A) PBMC from donor CMV327 were depleted of CD8+ cells and moderate to expressors of CD25+ by MACS to remove Tregs. Pre-depletion and post-depletion cells were stained for CD3, CD4, CD127, and CD25 to verify this depletion. PBMC depleted of CD8+ cells or CD8+CD25$^{hi}$ cells were then stimulated with overlapping peptide pools spanning the ORFs of gB, pp65, IE1, LUNA, UL144, UL138, UL111A, and US28 for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. The secretion of (B) IFN$\gamma$ and (C) IL-10 was then stained for and the number of positive spots enumerated by the automated cell counter and converted to a spot forming unit (SFU) value per million cells. Each peptide pool was tested in triplicate and the values from the number of background cells secreting cytokines in the unstimulated wells was deducted from all test wells.

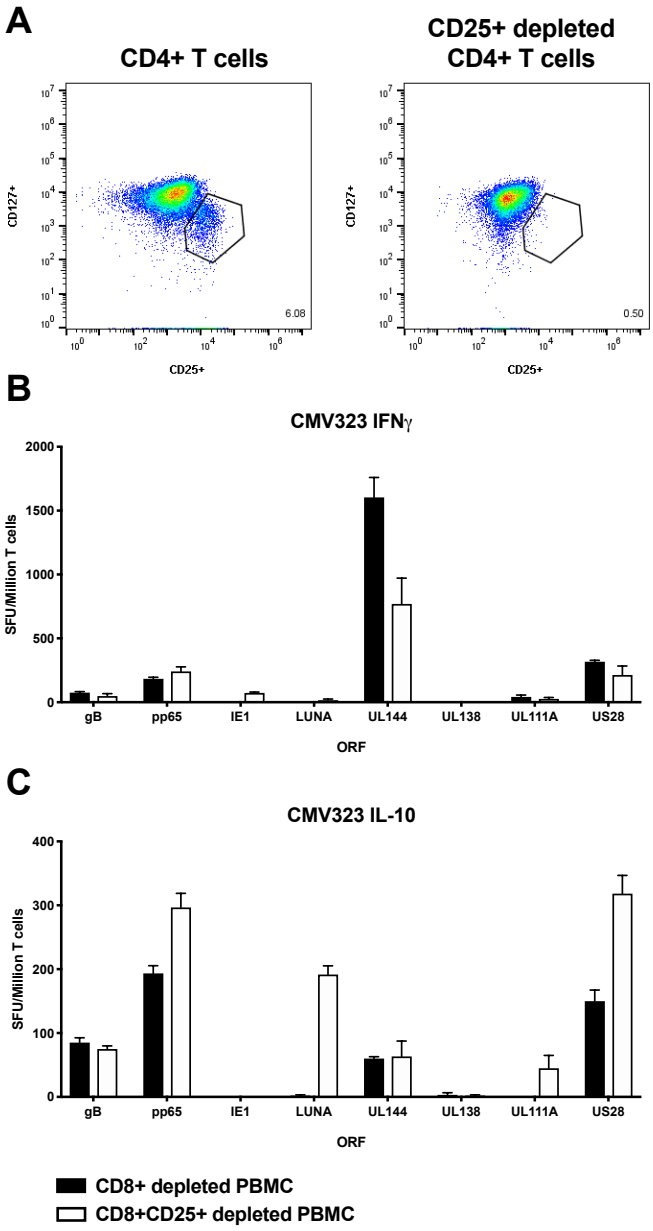



**Fig. 6.24 The frequency of IFN$\gamma$ and IL-10 secreting CD4+ T cells and CD25+ depleted CD4+ T cells from donor CMV323 following stimulated with HCMV ORF peptide pools** (A) PBMC from donor CMV323 were depleted of CD8+ cells and moderate to high expressors of CD25 by MACS to remove Tregs. Pre-depletion and post-depletion cells were stained for CD3, CD4, CD127, and CD25 to verify this depletion. PBMC depleted of CD8+ cells or CD8+CD25$^{hi}$ cells were then stimulated with overlapping peptide pools spanning the ORFs of gB, pp65, IE1, LUNA, UL144, UL138, UL111A, and US28 for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. The secretion of (B) IFN$\gamma$ and (C) IL-10 was then stained for and the number of positive spots enumerated by the automated cell counter and converted to a spot forming unit (SFU) value per million cells. Each peptide pool was tested in triplicate and the values from the number of background cells secreting cytokines in the unstimulated wells was deducted from all test wells.

### 6.2.6 Characterisation of CD4+ T cell peptide specific cytokine secretion

CD4+ T cells were found to either secrete IL-10 or IFN$\gamma$ in response to HCMV ORF peptide pools (Section 3.2.10). Whether CD4+ T cells specific to individual HCMV peptides were composed entirely of one of these sub-population of cells, which secrete either IL-10 or IFN$\gamma$, was investigated. By mapping CD4+ T cell responses to individual peptides, this could also be useful in determining minimal peptide epitopes for the generation of MHC class II tetramers. To begin characterising the specificity of the CD4+ T cell responses to latency-associated HCMV ORFs, small peptide pools were used to stimulate CD8+ cell depleted PBMC, and the frequencies of IFN$\gamma$ and IL-10 secreting cells determined by FluoroSpot. The peptide pools consisted of five individual overlapping peptides of 15 amino acids in length spanning the ORFs of US28, LUNA, UL138, and UL111A. Two donors were tested for their IFN$\gamma$ and IL-10 responses to small peptide pools.

Donor CMV329 exhibited a number of responses to the small peptide pools (Figure 6.25). Although the responses to small LUNA, UL138, and UL111A peptide pools were not very common in this donor, the CD4+ T cell responses did exhibit a mix of cytokine responses. For example, CD4+ T cells responding to US28 pools 2 and 8 were exclusively IL-10 secreting cells, while those responding to pool 9 were all IFN$\gamma$ secreting (Figure 6.25A). A mix of IFN$\gamma$ and IL-10 secreting cells responded to pools 3 and 6, although there were many more cells secreting IL-10 in response to pool 6 (Figure 6.25A).

There were several pools that donor CMV324 had exclusive IFN$\gamma$ responses to, such as those to pool 1 and 7 of US28 (Figure 6.26A), pool 2 of LUNA (Figure 6.26B), pool 1 and 3 of UL138 (Figure 6.26C), and pool 4 of UL11A (Figure 6.26D). This donor did not have CD4+ T cell responses to any small peptide pools that were composed entirely of IL-10 secreting cells. However, a small number of pools did elicit both IFN$\gamma$ and IL-10 secreting CD4+ T cells, including UL138 pool 2 and LUNA pool 1 (Figure 6.26C and B).

Therefore, several peptide pools were found that elicited a dominant IFN$\gamma$ or IL-10 CD4+ T cell response, as well as a number that induced a combination of cells secreting both cytokines. The individual 15 amino acid peptides from these peptide pools can subsequently be tested individually to determine which 15 amino acid peptides are responded to.

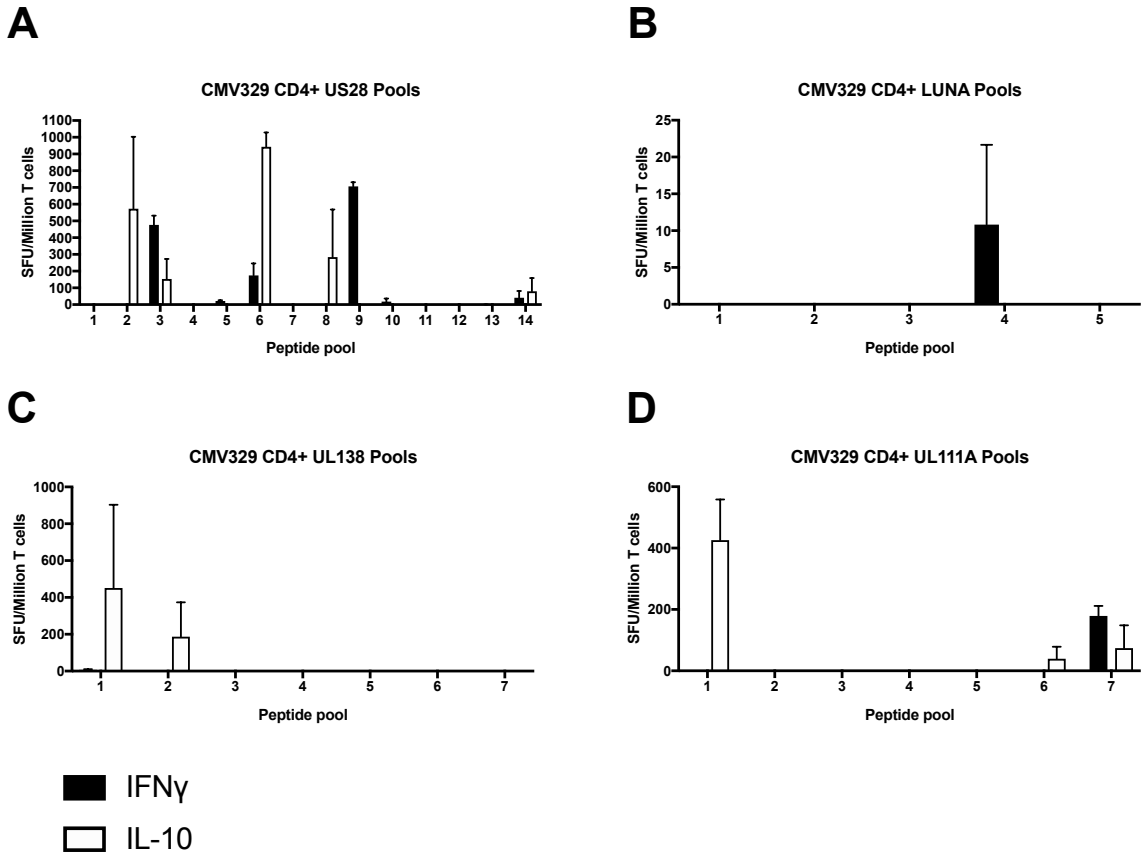



**Fig. 6.25 Specificity of CD4+ T cells from donor CMV329 to small pools of latency-associated ORF peptides.** PBMC from donor CMV329 were depleted of CD8+ cells and stimulated with pools composed of five individual overlapping peptides for 48 hours. These small pools spanned the latency-associated ORFs of (A) US28, (B) LUNA, (C) UL138, and (D) UL111A. IFN$\gamma$ secretion was then stained for to give a number of positive spots that were counted, and converted to spot forming units (SFU) per million cells. All samples were run in triplicate and the frequencies of IFN$\gamma$ positive cells was determined by subtracting values in all test wells from the background number of positive cells in unstimulated well.

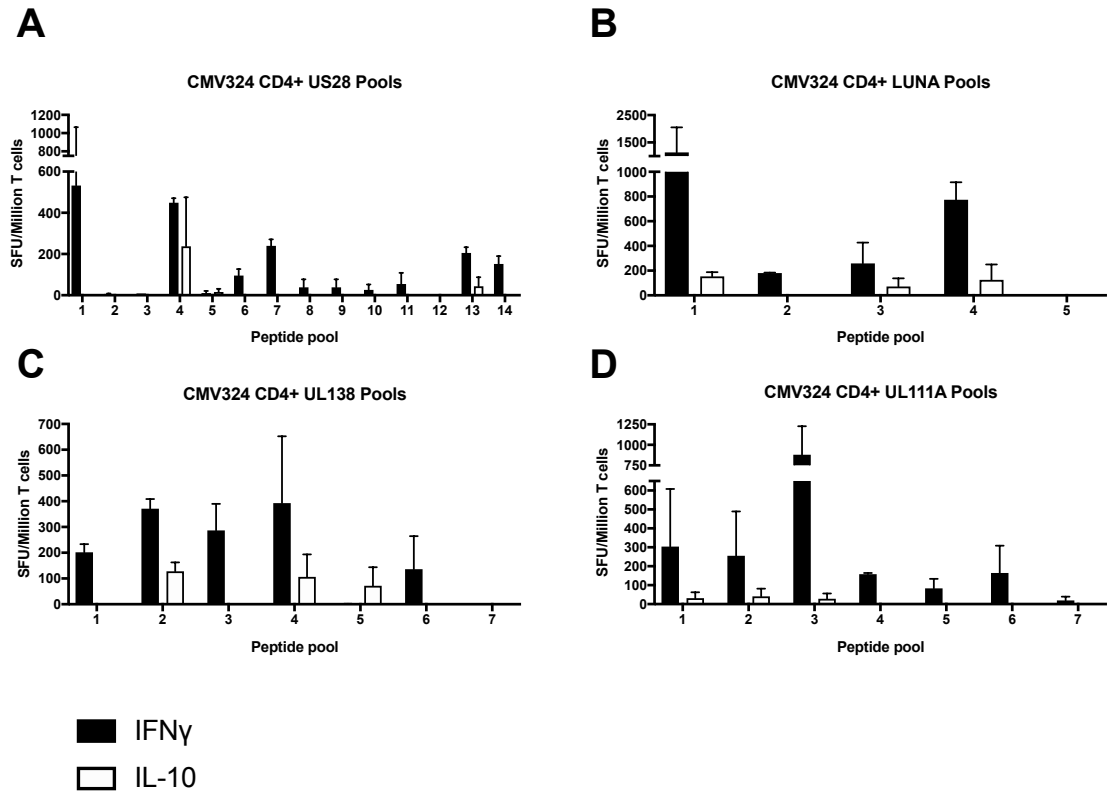



**Fig. 6.26 Specificity of CD4+ T cells from donor CMV324 to small pools of latency-associated ORF peptides.** PBMC from donor CMV324 were depleted of CD8+ cells and stimulated with pools composed of five individual overlapping peptides for 48 hours. These small pools spanned the latency-associated ORFs of (A) US28, (B) LUNA, (C) UL138, and (D) UL111A. IFN$\gamma$ secretion was then stained for to give a number of positive spots that were counted, and converted to spot forming units (SFU) per million cells. All samples were run in triplicate and the frequencies of IFN$\gamma$ positive cells was determined by subtracting values in all test wells from the background number of positive cells in unstimulated well.

## 6.3 Discussion

### 6.3.1 CD4+ T cell functional and memory phenotype analysis

CD4+ T cells specific for HCMV latency-associated ORF products were further characterised phenotypically and functionally by a range of methods. Having identified the ability of CD4+ T cells to secrete the immunosuppressive cytokine IL-10 in response to HCMV latency-associated ORF products UL138, LUNA, US28, and UL111A, the secretion of TGF$\beta$ was then examined. Previous work identified CD4+ T cells capable of secreting TGF$\beta$ following UL138 and LUNA peptide pool stimulation, while this did not occur following gB peptide pool stimulation [501]. The experiments carried out here have extended the HCMV ORFs examined to include the additional latency-associated ORFs US28, UL111A, and UL144. In these experiments, TGF$\beta$ was found to be produced by CD4+ T cells in response to all latency-associated ORFs tested, with the exception of UL138 responses which were very low or absent. Surprisingly, pp65 and IE1 peptide pool stimulated CD4+ T cells were also capable of producing TGF$\beta$.

TGF$\beta$ is a pleiotropic regulator of immune responses that has been shown to have a number of effects on T cells. Immunomodulatory effects include the suppression of T cell proliferation, Th1 cell differentiation, and IFN$\gamma$ secretion, the promotion the iTreg differentiation, and suppression of cytotoxic CD8+ T cell effector functions [487, 630, 703–711]. TGF$\beta$ is also able to induce IL-10 expression via activation of the IL-10 promoter [712]. The effects of TGF$\beta$ have also been shown to be amplified by IL-10 and there is evidence for the synergistic action of these cytokines on T cells [713]. Treatment of activated T cells with IL-10 increases the expression of TGF receptor type-II and restores responsiveness to TGF$\beta$ [710], while IL-10 is also thought to downregulate Th1 cytokines such as IFN$\gamma$ and IL-12 which would otherwise inhibit TGF$\beta$ secretion [714]. This evidence suggests that because CD4+ T cells specific for lytic HCMV ORFs are predominantly IFN$\gamma$ producers, concomitant TGF$\beta$ production by these cells might not have as much of an inhibitory effect in the absence of IL-10 and in the presence of Th1 cytokines such as IFN$\gamma$. The production of TGF$\beta$ in addition to IL-10 by HCMV latency-associated ORF specific CD4+ T cells could therefore be acting together to inhibit antiviral T cell responses.

HCMV-specific CD4+ T cells were examined for their cytotoxic potential to determine if differences existed among T cells specific for latency-associated ORFs, and in comparison to lytic ORF specific T cells. CD4+ T cells possessing direct anti-viral effector functions such as cytotoxicity have been identified in HCMV infection in response to lytic ORFs

including gB and pp65 [393, 401, 405, 715, 716], but a comparison with responses to latency-associated ORFs has not been carried out previously. In agreement with reports showing CD4+ T cell CD107a or granzyme expression in response to lytic ORFs such as gB, pp65, and IE1 [393, 405, 715, 716], expression of these markers was also observed in response to these ORFs, with degranulation in response to pp65 being particularly common. CD4+ T cells were also capable of CD107a degranulation and granzyme expression in response to latency-associated ORFs. CD107a expression was most commonly detected in response to US28 and UL144, which were comparable to gB, and IE1 responses. CD4+ T cells were least commonly detected in response to UL111A and UL138, although few donors had UL138-specific CD4+ T cell responses that could be assessed. Whether these differences in the expression of cytotoxic markers translates into a decreased ability to control HCMV infection has not been examined, although it is interesting that UL138- and LUNA-specific CD4+ T cells were shown to be dominated by IL-10 secreting T cells (Section 3.2.10). CD4+ T cells specific for HCMV proteins expressed during latency therefore possess the potential to carry out cytotoxic effector functions. This remains to be confirmed using a direct viral control assay, such as the viral dissemination assay, to test the cytotoxic ability of latency-associated ORF specific CD4+ T cells in the presence of virally encoded immunoevasins such as those causing downregulation of MHC class II [127, 165, 581, 582, 717].

An analysis of the memory phenotypes of latency-associated ORF specific CD4+ T cells was then carried out. In general, there were no differences in the CD4+ T cell memory subsets of cells specific to lytic ORFs compared to latency-associated ORFs, although US28- and UL111A-specific CD4+ T cells did display slightly higher proportions of cells with a naive-like phenotype. The majority of HCMV ORF-specific CD4+ T cells were found to express markers characteristic of $T_{CM}$ and $T_{EM}$ memory T cells. CD4+ memory T cells which had re-expressed CD45RA were also detected, and these generally made up a small proportion of ORF-specific cells, as has been reported previously [390, 715]. CD4+ $T_{CM}$ cells were in many cases the dominant ORF-specific memory population. Several previous studies performed using viral lysate or gB/pp65 peptide stimulation have described HCMV-specific CD4+ T cells as mainly having an effector memory phenotype [379, 394, 404, 715]. This discrepancy could be due to the different methods for identifying HCMV-specific T cells, as the expression of IFN$\gamma$ was often used in these studies to do this, compared to the expression of activation markers CD40L and/or 4-1BB as carried out here. Furthermore, one recent study using HCMV peptide pools from lytic ORFs, including gB and pp65, identified antigen-specific CD4+ T cells as those positive for CD40L and CD69 [393]. 4-1BB was chosen over CD69 as a marker for activation in the work presented here as it was found to

maintain higher levels of expression on CD4+ T cells for the duration of the assay (data not shown).

An analysis of the memory phenotypes of CD4+ T cells specific for latency-associated ORFs derived from tissue sites is also required as the results presented here were performed on peripheral blood CD4+ T cells only. Differences in the proportions of CD4+ T cell memory subsets between HCMV seronegative and seropositive individuals have been described in a number of different tissues [398], but how the memory phenotypes of ORF-specific T cells in tissues compared to peripheral blood remains to be determined. In one study, despite finding CD4+ $T_{EM}$ and $T_{EMRA}$ cell subsets to be generally elevated in the bone marrow compared to peripheral blood, no differences in the HCMV-specific $T_{EM}$ or $T_{EMRA}$ cell subsets between the two compartments was detected [394].

Due to the low numbers of HCMV ORF-specific T cells in the peripheral blood, a very large number of PBMC were required for these assays. Unfortunately, this meant it was not possible to determine the expression of cytotoxic markers or the memory phenotypes of IFN$\gamma$ and IL-10 expressing CD4+ T cells, which was an original intention. IL-10 secreting CD4+ T cells specific for latency-associated HCMV ORFs might show reduced expression of markers associated with cytotoxic effector functions for example, and previous work has shown minimal granzyme and perforin expression in naive-like and central memory CD4+ T cells [394]. As the analysis of cytotoxic marker expression and memory phenotypes were carried out on the entire population of CD4+ T cells deemed to be antigen-specific, it is possible that delineating cytotoxic and memory phenotypes to IL-10 or IFN$\gamma$ secreting cells could reveal differences between lytic and latency-associated ORF specific CD4+ T cells. Further work should seek to address these questions and could be vastly aided by the generation of HLA class II-peptide tetramers specific to latency-associated ORF products, as has been carried out for gB and pp65 [405].

### 6.3.2 CD4+ T cell functional analysis

HCMV-specific CD4+ T cells secreting IL-10 and TGF$\beta$ could be inhibiting antiviral T cell responses. Several experiments were carried out examining the effects of the secretomes of latent-specific CD4+ T cells on T cell effector functions. As two populations of CD4+ T cells specific for latency-associated ORFs were identified, one secreting predominantly IFN$\gamma$ and one IL-10, it was not known if the secretomes containing a mixture of both cytokines would be suppressive. Initially, the proliferation of CD4+ and CD8+ T cells stimulated in the presence of CD4+ T cell secretomes was investigated. The secretome from US28

peptide pool stimulated CD4+ T cells was found to inhibit the proliferation of both CD4+ and CD8+ T cells by over 70%. This agrees with results from the secretome of UL138 peptide stimulated CD4+ T cells, which were shown to inhibit CD4+ T cell proliferation [501].

CD4+ T cell secretomes were then examined for their ability to inhibit anti-viral cytokine production. A cytokine secretion inhibition assay was designed and optimised to do this. Using mismatched PBMC and CD4+ T cell supernatants derived from different donors was found to result in considerable differences in cytokine secretion between different donor PBMC incubated with the same supernatants. In an attempt to reduce this variability, matched PBMC and CD4+ T cell secretomes were tested from one donor. Many of the ORF-specific secretomes from this donor were able to inhibit cytokine secretion, although the inhibitory effects were more pronounced for IFN$\gamma$ compared to TNF$\alpha$ secretion. This inhibition could have been mediated by TGF$\beta$ in isolation, as elevated levels of IL-10 were not detected in the CD4+ T cell supernatants. Future experiments neutralising IL-10 and TGF$\beta$ could confirm this. The results from optimisation experiments and from testing CD4+ T cell supernatants with PBMC from mismatched donors highlighted the differential effects of IL-10 and TGF$\beta$ on the inhibition of IFN$\gamma$ and TNF$\alpha$ secretion in certain donors, and this could explain why the inhibition of TNF$\alpha$ was minor in comparison to the inhibition of IFN$\gamma$.

This differential ability of CD4+ T cell supernatants to inhibit IFN$\gamma$ and TNF$\alpha$ secretion made it difficult to obtain a consistent system across donors. The donor-specific variation in the abilities of IL-10 and TGF$\beta$ to inhibit PBMC secretion of IFN$\gamma$ and TNF$\alpha$, as well as variation between donors in the levels of IFN$\gamma$ and TNF$\alpha$ PBMC secreted when stimulated with the same concentration of anti-CD3/CD28 antibody, also limited the reproducibility of this assay. The inability of the flow cytometry based system to detect elevated secretion of IL-10 was unexpected, especially as IL-10 secretion of peptide stimulated CD4+ T cells was detected by both FluoroSpot and ELISA from donors CMV324 and CMV319 (Section 3.2.3 and 3.2.4). While it could be possible that fluctuations in the peripheral blood CD4+ T cell frequencies as described in section 3.2.4 resulted in no IL-10 producing CD4+ T cells being present at this time point, this seems unlikely to have occurred for all the supernatants tested. The inhibition observed by CD4+ T cell supernatants from donor CMV332 could therefore be due to the production of low levels of IL-10 not detected by the flow cytometry based bead array. Additionally, when quantifying IL-10 secretion by ELISA (Section 3.2.3), twice as many cells were stimulated with HCMV ORF peptides compared to experiments here. It could also be the case that the inhibition mediated by CD4+ T cell was due to other inhibitory factors that have not been sampled for. Further work in this area should attempt to

measure IL-10 secretion in the supernatants by ELISA concurrently with the flow cytometry based bead array.

The effect of CD4+ T cell secretomes on anti-viral CD8+ T cell control of a lytic HCMV infection was then examined. It was hypothesised that the immunosuppressive cytokines in the CD4+ T cell latency-associated ORF secretomes would inhibit the antiviral functions of CD8+ T cells. Using an experimental model of lytic infection, the spread of a GFP-tagged virus was measured following incubation with CD8+ T cells that had been incubated with CD4+ T cell secretomes. While preliminary work indicated that CD8+ T cells incubated with IL-10 and TGF$\beta$ had a reduced ability to control virus spread, this could not be replicated with any of the ORF-specific CD4+ T cell supernatants. This could have been due to the requirement to remove the CD4+ T cell supernatants before the addition of CD8+ T cells to the infected fibroblasts, as these secretomes possessed antiviral activity alone. However, the CD4+ T cell secretomes used in these assays were not analysed for their cytokine content, and this should be done in future to quantify the levels of pro-inflammatory and immunosuppressive cytokines.

These limited experiments have given an initial indication that the secretomes from CD4+ T cells specific for HCMV latency-associated ORF products are capable of inhibiting T cell proliferation, the secretion of antiviral cytokines IFN$\gamma$ and TNF$\alpha$, and can control viral dissemination *in vitro*. Further work should build on these experiments through repeating them in additional donors and confirming the roles of IL-10 and TGF$\beta$ in these suppressive effects through direct measurement and the use of neutralising antibodies where possible.

### 6.3.3 Depleting CD25$^{hi}$ cells to remove IL-10 secreting cells

The cytokines IL-10 and TGF$\beta$ have been shown to be secreted by Tregs as a potential mechanism to inhibit immune activity [629, 718–726]. The characteristic transcription factor FoxP3 is central to the function of Tregs [727] and many studies have shown the presence of virus-specific FoxP3 expressing CD4+ T cells in HCMV infection [403, 502, 728]. Furthermore, previous work has shown that a subset of CD4+ T cells specific for UL138 and LUNA, but not gB, expressed FoxP3 [501]. While FoxP3 is the hallmark of Tregs, its intracellular expression makes it unsuitable as a means to isolate or remove FoxP3+ cells. A number of cell surface markers have been used as surrogates to identify Tregs. One such combination of markers identifies Tregs as CD4+CD127$^{lo}$CD25$^{hi}$ [483, 484, 729]. The constitutively high expression of CD25 on Tregs therefore provided a relatively simple target to selectively deplete these cells. It was reasoned that because the latency-associated

CD4+ T cells secreting IL-10 were a separate population from those secreting IFN$\gamma$ (Section 3.2.10), removal of CD4+CD25+$^{hi}$ T cells would deplete the IL-10 producing cells and leave the IFN$\gamma$ secreting subset intact. If Tregs of this phenotype were the source of IL-10 then their depletion might abrogate a source of immunosuppression on IFN$\gamma$ secreting Th1 cells, potentially enabling them to carry out more effective antiviral functions during latency.

Depletion of CD25+ cells has been utilised with some success to remove suppressive regulatory populations [495, 730, 731]. In the context of HCMV, one study found that depletion of CD25+ cells from PBMC resulted in an increase in the proportion of pp65-specific CD8+ T cells secreting IFN$\gamma$ [494]. Likewise, the depletion of CD25$^{hi}$ cells from PBMC of kidney transplant patients with HCMV infection resulted in an increase in HCMV-specific IFN$\gamma$ secretion in those with recurring infection [502]. When testing this approach, the depletion of CD4+CD25$^{hi}$ cells prior to stimulation with HCMV ORF peptide pools did not consistently result in a decrease in the IL-10, or increase in IFN$\gamma$, secreting CD4+ T cell frequencies. This was also the case when depletion of moderate and lower CD25 expressing CD4+ T cells was carried out. In certain donors, CD25+ cell depletion was able to reduce the frequencies of IL-10 secreting CD4+ T cells, but even this was found to vary when comparing different ORF-specific responses within individuals. Therefore, in certain donors, and in certain ORF-specific CD4+ T cell populations, CD25+ expressing cells, likely to be Tregs, were a proportion of the IL-10 secreting CD4+ T cell population. CD25+ cell depletion was also found to have varying effects on the ORF-specific IFN$\gamma$ secreting CD4+ T cell populations, which often resulted in a decrease in this population. This could be due to the removal of activated CD4+ T cells known to express CD25 [458, 732, 733]. Additionally, certain resting memory CD4+ T cells that are Foxp3- have been shown to express intermediate levels of CD25, which could also have been depleted [734, 735]. Although the majority of late differentiated memory cells (CD28-GranzymeB+), which included the CD4+ T cells secreting IFN$\gamma$ in response to HCMV lysate, were found to be contained in the CD25- population [734], it is possible that less differentiated memory CD4+ T cells specific to HCMV also express intermediate levels of CD25. Given that depletion of CD25+ cells did not consistently remove HCMV-specific IL-10 secreting CD4+ T cells, and also often removed IFN$\gamma$ secreting CD4+ T cells, which might be antiviral, this did not present a viable strategy to target immunosuppressive T cell responses during latent HCMV infection.

As the population of IL-10 secreting CD4+ T cells that were CD25+ was found to be variable within donors, it could be possible that these remaining IL-10 secreting cells are part of a separate regulatory T cell population. Other potential regulatory T cell populations that could be responsible for this IL-10 secretion include Tr1 cells. Tr1 cells do not typically

express CD25 [528], and therefore would not have been depleted in these experiments. Tr1 cells express IL-10 and TGF$\beta$, which are critical to their suppressive abilities, as well as co-stimulatory markers such as ICOS, CD18, and LAG-3 [506–509, 512–515]. Expression of these cell surface markers therefore provides potential targets which could be used to deplete such cells. Assessing transcription factor expression and carrying out further phenotypic characterisation of latency-associated ORF specific CD4+ T cells could also reveal differences between the IL-10 and IFN$\gamma$ secreting populations, and determine if IL-10 secreting cells possess markers of Tr1 cells, thus enabling the targeting of IL-10 positive cells. The ability to study latency-ORF specific CD4+ T cells without *in vitro* culture, which could alter their phenotypes, would be highly useful. MHC class II-peptide tetramers provide this opportunity, as have been synthesised to pp65 and gB [405].

### 6.3.4 CD4+ T cell peptide mapping

While designing an MHC class II tetramer specific to a latency-associated peptide was beyond the scope of this work, small pools of five peptides from latency-associated ORFs were used to stimulate CD4+ T cells to begin to map these T cells to a minimal peptide epitope. In addition, this work provided an initial step in characterising the cytokine secretion of single-peptide specific CD4+ T cells to begin to address the question of whether CD4+ T cells specific for a single ORF peptide secreted IFN$\gamma$ or IL-10 in isolation, or whether epitope-specific CD4+ T cell populations were composed of a combination of cytokine secreting cells.

CD4+ T cells from three donors were stimulated with small pools of five peptides spanning the ORFs of UL138, LUNA, US28, and UL111A. This revealed several pools that elicited an IFN$\gamma$ or IL-10 dominant response, as well as responses composed of both cytokine secreting cells. Therefore, data from this initial work can be used to test CD4+ T cell responses to the individual peptides that composed these pools, to determine peptide-specificity and cytokine secretion.

# Chapter 7

# *In vitro* Characterisation of Latency-specific CD8+ T cells

## 7.1 Introduction

In HCMV seropositive donors, the presence of high-frequency CD8+ T cell responses against lytic HCMV proteins such as pp65 and IE1 are well established [287–290, 294]. The ability of CD8+ T cells to recognise proteins expressed during latency has now also been demonstrated (Chapter 4) [501, 691]. CD8+ T cells specific for lytic ORF products have been well characterised by numerous groups showing these cells can have a distinct phenotypic composition associated with T cell maturation, including downregulation of CD27 and CD28, upregulation of CD57, and expression of effector molecules such as granzyme B and perforin [287, 291, 292, 294, 295]. In addition, a population of HCMV-specific memory CD8+ T cells can be defined by their reversion of the expression of CD45RA, which is associated with naive T cells, from CD45RO, which is associated with memory T cells [291–293, 296, 297].

The memory phenotypes and cytotoxic potential of CD8+ T cells specific for HCMV proteins expressed during latency have not been extensively studied. The aims of this chapter were therefore to further characterise these CD8+ T cells, in an effort to reveal differences between lytic ORF and latency-associated ORF specific T cells, and to begin to map their peptide-specificities, which could inform the generation of MHC class I tetramers to enable latency-specific CD8+ T cells to be isolated for further study.

## 7.2 Results

### 7.2.1 Phenotypic characterisation of latency-specific CD8+ T cells

To investigate the cytotoxic potential of CD8+ T cells recognising HCMV latency-associated ORFs, the expression of CD107a and granzymes A, B, and K was determined on CD8+ T cells following overnight stimulation with overlapping peptide pools spanning the ORFs of gB, pp65, IE1, UL138, LUNA, US28, UL111A, and UL144. Cytotoxic degranulation, and subsequent surface expression of CD107a has been well documented for CD8+ T cells and is an established marker for cytotoxicity [736].

Following peptide pool stimulation, HCMV-specific CD8+ T cells were identified by gating for the expression of the activation markers CD69 and 4-1BB [702, 737] above the expression observed on unstimulated CD8+ T cells (Figure 7.1A). The expression of CD107a and granzymes A, B, and K were then determined on CD8+ T cells positive for these markers (Figure 7.1A). The data from 18 HCMV seropositive donors shows that the proportions of CD8+ T cells capable of degranulating in response to HCMV ORF peptide pool stimulation were higher among ORF-specific CD8+ T cells (Figure 7.1B) compared to ORF-specific CD4+ T cells (Figure 6.2B). Of the lytically-expressed ORFs, donors had the highest percentages of CD8+ T cells specific for pp65 that expressed CD107a but also the most number of donors with CD8+ T cells that showed any degranulation (17/18, 94%) (Figure 7.1B). The proportion of pp65-specific CD8+ T cells expressing CD107a was significantly higher than gB- and UL111A-specific CD8+ T cells (Kruskal-Wallis with Dunn's multiple comparisons). Where donors had US28- and UL144-specific CD8+ T cell responses, a proportion of these cells were always found to express CD107a, and the proportion of US28-specific CD8+ T cells expressing CD107a was comparable to that of pp65-specific CD8+ T cells (Figure 7.1B). UL111A- and UL138-specific CD8+ T cells expressing CD107a were the least frequently observed, and fewer antigen-specific CD8+ T cells expressed CD107a in comparison to other responses, although only three donors had detectable UL138-specific CD8+ T cells (Figure 7.1B). Interestingly, CD4+ T cells specific to UL111A and UL138 also displayed the lowest expression of CD107a (Figure 6.2B).

In general, there were lower proportions of antigen-specific CD8+ T cells positive for the expression of granzymes A, B, and K alone compared to those expressing CD107a. CD8+ T cells specific to gB had the highest expression of granzymes amongst responses to lytic ORFs (Figure 7.1C). Interestingly, where pp65-specific CD8+ T cells expressing CD107a were detected in 94% (17/18) of donors, expression of granzymes by pp65-specific CD8+ T cells

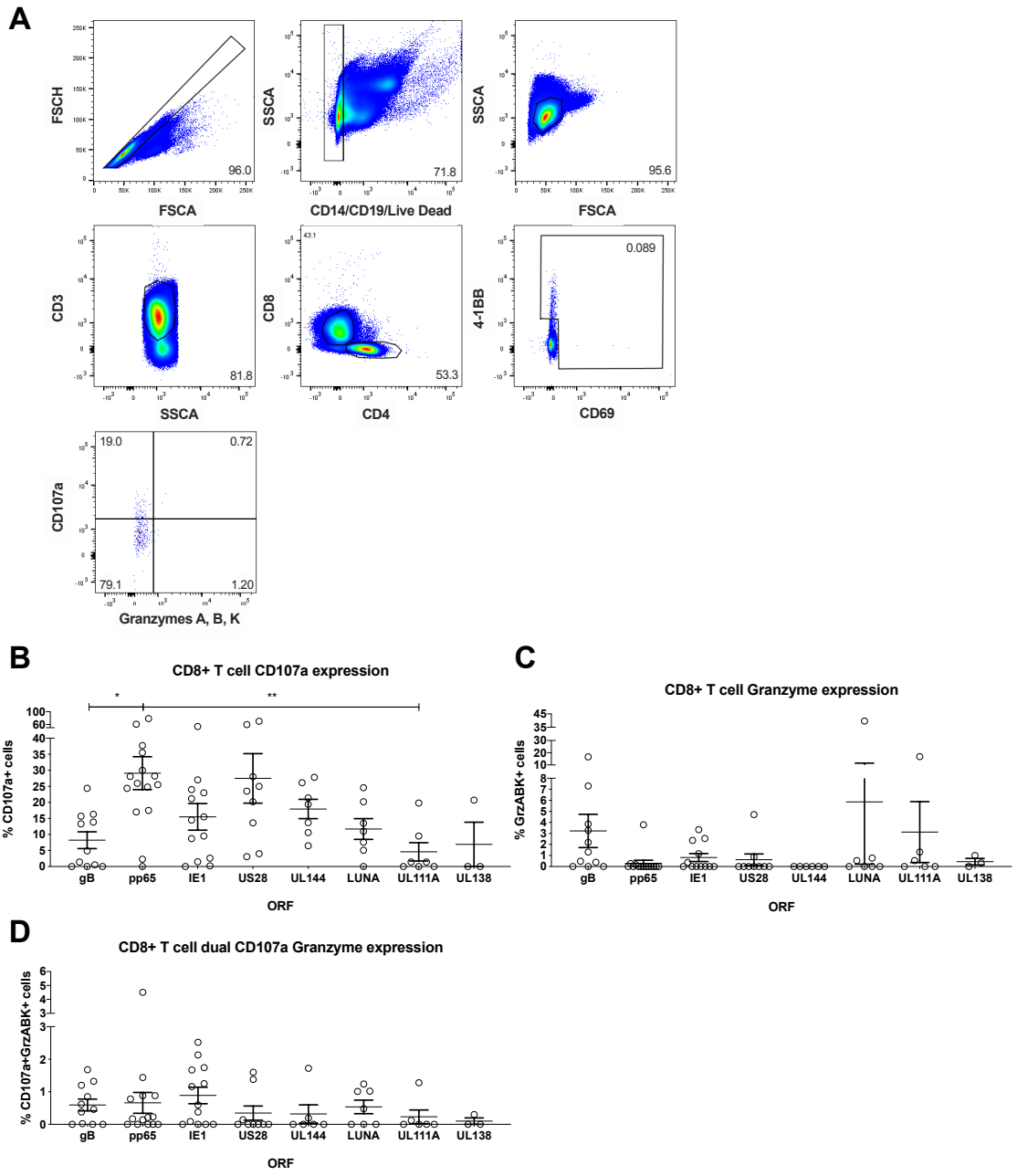



**Fig. 7.1 Assessing the expression of CD107a and granzymes A, B, and K on CD8+ T cells in response to HCMV ORF peptide pools by flow cytometry.** PBMC from 18 HCMV seropositive donors was stimulated with overlapping peptide pools spanning the ORFs of gB, pp65, IE1, UL138, LUNA, US28, UL111A, and UL144 overnight, in the presence of monensin. PBMC were then stained intracellularly for CD107a and the granzymes A, B, and K. (A) The representative gating strategy to identify ORF-specific CD8+ T cells is shown for LUNA-specific CD8+ T cells from donor CMV211. Dead cells, CD14+ and CD19+ cells were first excluded, followed by doublets. Lymphocytes were then gated on by forward scatter (FSCA) and side scatter (SSCA) and CD3+ cells identified. CD4+ and CD8+ cells were then gated, followed by activated CD8+ T cells, identified as CD69 or 4-1BB positive cells. CD8+ T cells with above background expression of CD69 or 4-1BB were considered ORF-specific. The percentages of ORF-specific CD8+ T cells expressing (B) CD107a, (C) granzymes A, B, or K, (D) or both CD107a and granzymes A, B, or K following ORF peptide pool stimulation is shown. Differences in CD107 and granzyme expression was assessed by Freedman test with Dunn's multiple comparisons with significant results set at $p<0.05$.

was only detected in 20% (3/15) of donors (Figure 7.1C). The proportions of CD8+ T cells expressing granzymes alone was also low on CD8+ T cells specific for latency-associated ORFs but these cells could be detected in response to US28, LUNA, UL111A, and even UL138, where no CD107a degranulation was detected (Figure 7.1C). No CD8+ T cells specific to UL144 express granzymes in isolation (Figure 7.1C). CD8+ T cells expressing both CD107a+ and granzymes was also determined. Low percentages of HCMV-specific CD8+ T cells were found to express both CD107a and granzymes together in a subset of individuals, with these cells tending to be make up higher proportions for CD8+ T cells specific to the lytic ORFs gB, pp65, and IE1 (Figure 7.1D). Dual CD107a and granzyme expressing CD8+ T cells were also identified in response to all latency-associated ORFs, although these were less commonly detected than to lytic ORFs (Figure 7.1D).

The memory phenotypes of CD8+ T cells specific for proteins expressed during latency was also examined. CD8+ T cells were stimulated with overlapping peptide pools and the ORF-specific CD8+ T cells were grouped based on memory phenotypes as defined by the expression of CD27 and CD45RA: naive-like ($T_{NL}$) (CD27+ CD45RA+), central memory (central memory ($T_{CM}$) (CD27+ CD45RA-), effector memory ($T_{EM}$) (CD27- CD45RA-), and effector memory CD45RA-expressing T cells ($T_{EMRA}$) (CD27- CD45RA+). The representative gating strategy to identify these four memory subsets is shown in Figure 7.2A. This phenotypic analysis was carried out on 17 HCMV seropositive donors. HCMV-specific CD8+ T cells were found in each memory subset, although $T_{EMRA}$ cells tended to be the dominant memory population, with the exception of gB- and UL144-specific CD8+ T cells, which had a smaller proportion of $T_{EMRA}$ cells and larger $T_{EM}$ and/or $T_{CM}$ subset (Figure 7.2B). This is in contrast to the HCMV-specific CD4+ T cells, where $T_{CM}$ cells were the largest memory subset (Figure 6.3B). On the whole, there were no significant differences in memory subset composition between CD8+ T cells specific for lytic or latency-associated proteins (Figure 7.2B). Unfortunately, only two donors possessed UL138-specific CD8+ T cells with above background responses, so the memory composition of these cells could not accurately be assessed.

In summary, CD8+ T cells specific for latency-associated ORF proteins express markers consistent with the potential for cytotoxic activity. With the exception of pp65, there do not appear to be large differences in expression of these cytotoxic markers between CD8+ T cells specific for lytically-expressed ORFs gB and IE1, and latency-associated ORFs, especially for US28-, LUNA-, and UL144-specific CD8+ T cells which were highly comparable in CD107a expression. Likewise, no difference in the memory phenotypes of CD8+ T cells specific for lytic proteins compared to latency-associated proteins was observed.

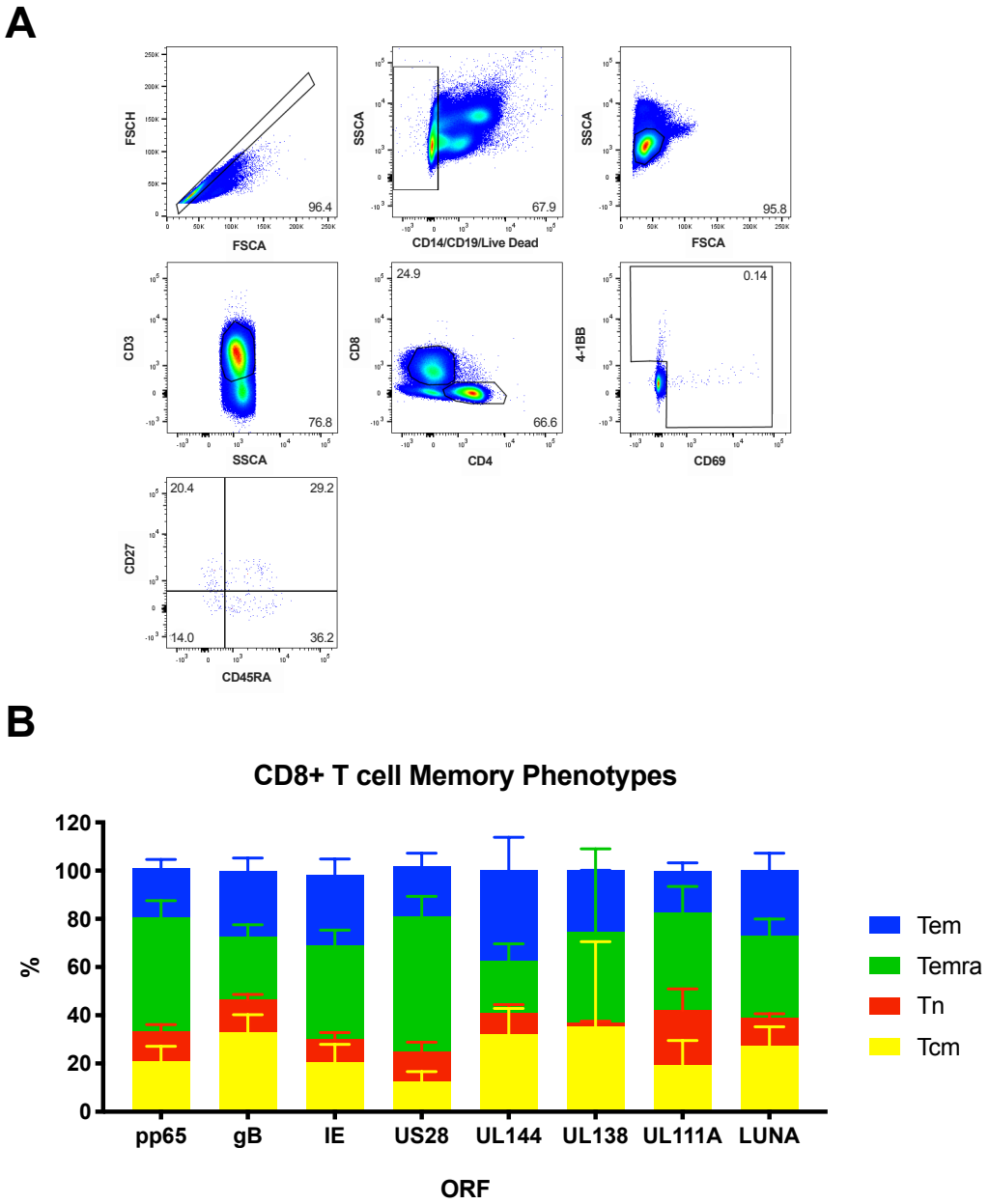



**Fig. 7.2 Memory phenotypes of CD8+ T cells specific for HCMV antigens.** PBMC isolated from 17 HCMV seropositive donors were stimulated overnight with HCMV peptide pools spanning the ORFs of gB, pp65, IE1, UL138, LUNA, US28, UL111A, and UL144, in the presence of monensin. Stimulated PBMC were then stained intracellularly. (A) Representative flow cytometry dot plots the gating strategy to identify the memory populations of CD8+ T cells is shown. Dead cells, CD14+ and CD19+ cells were first excluded, followed by doublets. Lymphocytes were then gated on by FSCA and SSCA and CD3+ cells identified. CD4+ and CD8+ cells were then gated, followed by activated CD8+ T cells, identified by gating for CD69 or 4-1BB positive cells, and finally CD27 and CD45RA to identify the memory phenotype. (B) The proportions of each CD8+ T cell memory population responding to each ORF grouped by CD27 and CD45 expression. Naive-like ($T_{NL}$) (CD27+ CD45RA+), central memory (central memory ($T_{CM}$) (CD27+ CD45RA-), effector memory ($T_{EM}$) (CD27- CD45RA-), and effector memory CD45RA-expressing T cells ($T_{EMRA}$) (CD27- CD45RA+).

## 7.3 Characterisation of the peptide specificity of US28-specific CD8+ T cells

In response to HCMV ORF peptide pools, CD8+ T cells capable of secreting IL-10 were found to be a separate population to those secreting IFN$\gamma$ (Section 4.2.7). However, whether individual CD8+ T cell clones all secrete either IL-10 or IFN$\gamma$, or are a mix of IL-10 and IFN$\gamma$ secreting cells, remained an open question. To begin to address this, CD8+ T cells were stimulated with individual overlapping peptides spanning the US28 ORF. In this manner, the cytokine secretion from CD8+ T cells specific for individual US28 peptides could give an indication of whether certain peptide responses were composed of one cytokine (IFN$\gamma$ or IL-10), or both. Additionally, this would act as an initial step in mapping CD8+ T cell responses to a minimal US28 peptide epitope, which could be used in the design of an MHC class I tetramer. The US28 ORF was focused on because in the vast majority of donors tested, high frequency IFN$\gamma$ and IL-10 CD8+ T cell responses were found to this ORF (Section 4.2.1 and 4.2.4). Furthermore, a number of donors were found to have CD8+ T cells specific to US28 capable of upregulating CD107a (Figure 7.1B).

CD4+ cell depleted PBMC from four HCMV seropositive donors were stimulated with individual 15 amino acid peptides spanning the US28 ORF. The frequencies of IFN$\gamma$ and IL-10 secreting CD8+ T cells was then determined in response to each peptide. The HLA type of these four donors was also determined, which could enable mapping of distinct peptide-specific responses to certain HLA types (Table 7.1).

| **Donor** | **HLA Type** | | | | | |
|---|---|---|---|---|---|---|
| | **HLA-A** | | **HLA-B** | | **HLA-C** | |
| | a | b | a | b | a | b |
| **CMV319** | 34 | 43 | 07 | 39 | 10 | 12 |
| **CMV320** | 01 | 32 | 14 | 27 | 08 | 03 |
| **CMV321** | 03 | 25 | 27 | 35 | 02 | 04 |
| **CMV324** | 01 | 24 | 07 | 37 | 06 | 07 |

**Table 7.1** HLA typing of four HCMV seropositive donors.

Donor CMV324 CD8+ T cells were tested on three separate occasions for their responses to individual US28 derived peptides. The first test of the CD8+ T cell responses from donor CMV324 yielded only two peptides that elicited a relatively high frequency of CD8+ T cells secreting IFN$\gamma$, peptides 18 and 58 (Figure 7.3A). In contrast, there were high frequency IL-10 responses to many of the peptides tested, which also included peptides 18 and 58

(Figure 7.3B). Additionally, there were many peptides that elicited only IL-10 secreting CD8+ T cell responses, such as peptides 8, 9 and 38 (Figure 7.3).

A second test of this donor's responses was undertaken a month later and several peptides (7, 8, 11, 12, 16, 18, 30, 55, 57, and 58) that elicited high frequencies of cytokine secreting CD8+ T cells in the first test (Figure 7.3) were selected and tested in duplicate. In addition, peptides 31, 67, and 69 were tested in duplicate as negative controls because there were either no CD8+ T cell responses to these peptides, or the responses were very low. In this experiment, CD8+ T cell IFN$\gamma$ responses were higher frequency, and much more prevalent than those detected in the initial test (Figure 7.4A). The opposite was the case for the IL-10 responses. Although there were several clusters of two or three peptides overlapping that elicited CD8+ T cell IL-10 responses, which could indicate that the T cell epitopes are contained across the 5 amino acid overlap from adjacent 15 amino acid peptides, overall the responses were less common and of lower frequency compared to the first test (Figure 7.4B). There were also broad inconsistencies in the responses elicited by the peptides tested in duplicate. Although the IFN$\gamma$ responses to peptides 18 and 58 were only slightly lower than the first test, responses to numerous other peptides far exceeded these responses, highlighting the variability in responses over time. Moreover, none of the peptides selected because of the high frequency CD8+ IL-10 T cell responses detected in the initial test elicited responses in the second test (Figure 7.4B).

A further peptide screen was carried out on this donor's CD8+ T cell responses. Peptides 18 and 58 again elicited IFN$\gamma$ responses, and these were therefore the only peptides to do so on all three tests (Figure 7.5A). There were several peptide-specific responses detected on this third test that were also present on the second test (Figure 7.4) but absent from the first (Figure 7.3). Similar frequencies of IFN$\gamma$ secreting CD8+ T cells were found in response to peptides 4, 21, 24, 33, 46, 50, 61, and 66 (Figure 7.5A).

Donor CMV319 CD8+ T cell responses were tested on two separate occasions. Considerable differences in the frequencies of cytokine secreting cells were observed between these two time points. Markedly lower frequencies of IFN$\gamma$ secreting CD8+ T cells were detected in response to individual US28 peptides on the first sampling point (Figure 7.6A) compared to the second (Figure 7.7A), with only one of the relatively high frequency responses, to peptide 11, observed at both time points. This inconsistency was also detected in IL-10 responses across the two time points, where none of the higher frequency IL-10 responses were observed on both occasions despite high frequency responses to peptide 8 and 11 at the first sampling time point (Figure 7.6B) and peptides 18, 51, 62, and 68 on the second (Figure 7.7B).

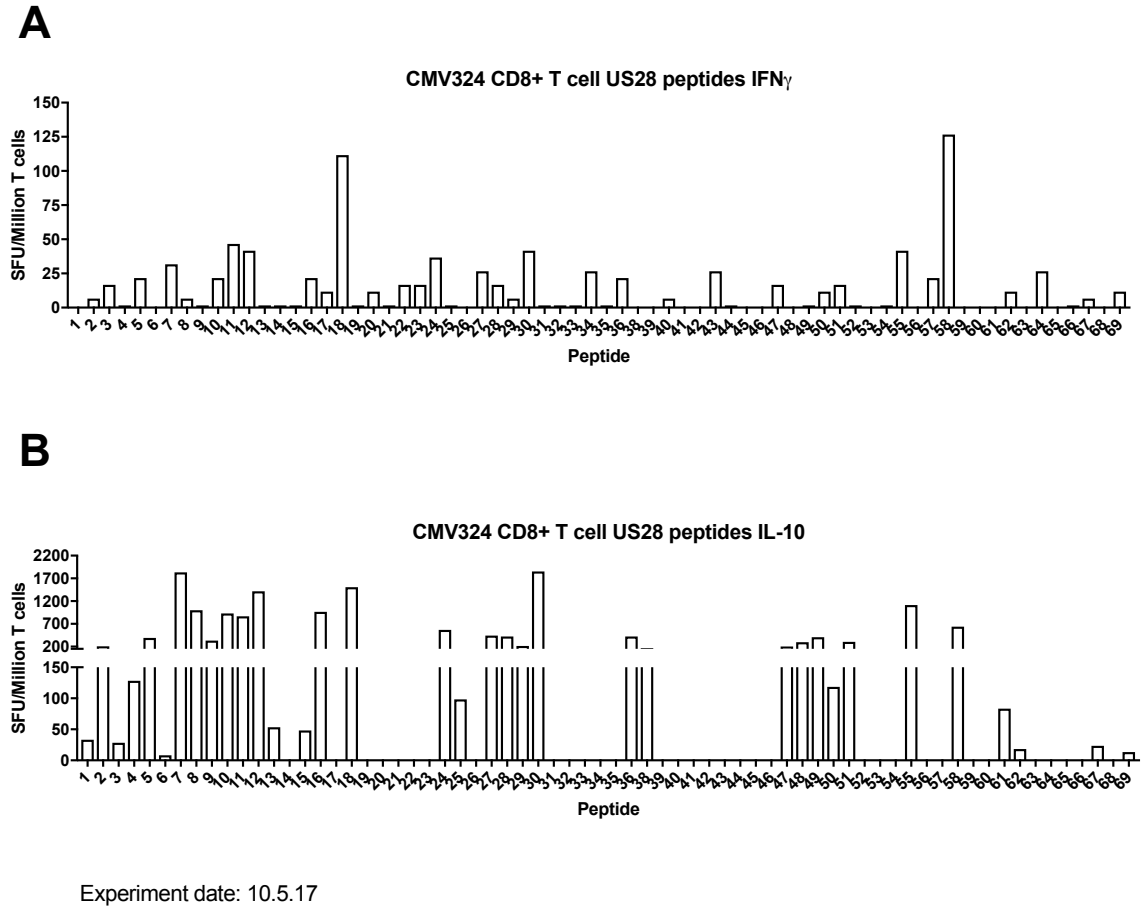



**Fig. 7.3 CD8+ T cell responses from donor CMV324 to individual peptides spanning the US28 ORF.** PBMC from donor CMV324 were depleted of CD4+ cells and stimulated with individual 15 amino acid peptides spanning the ORF of US28 for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. (A) IFN$\gamma$ and (B) IL-10 secretion was then stained for to give a number of positive spots that were counted, and converted to spot forming units (SFU) per million cells. The frequencies of positive cells was determined by subtracting values in all test wells from the background number of positive cells in unstimulated well.

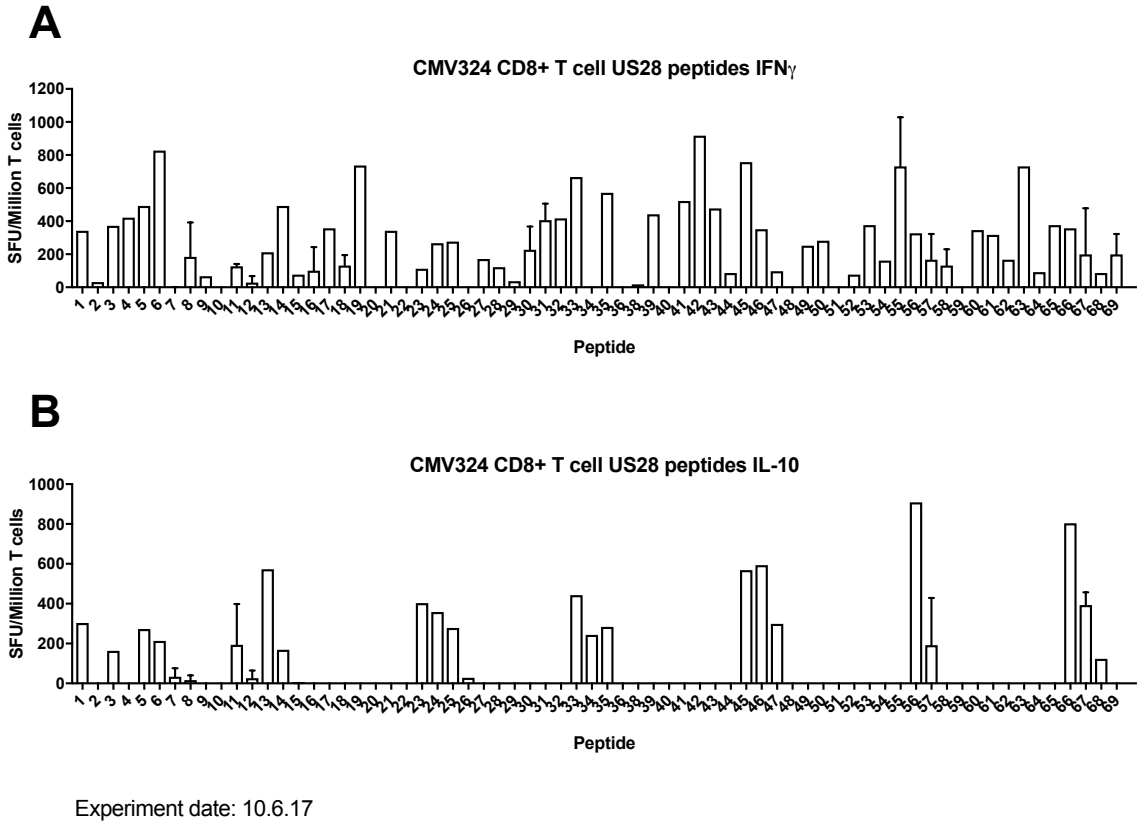



**Fig. 7.4 CD8+ T cell responses from donor CMV324 to individual peptides spanning the US28 ORF.** PBMC from donor CMV324 were depleted of CD4+ cells and stimulated with individual 15 amino acid peptides spanning the ORF of US28 for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. (A) IFN$\gamma$ and (B) IL-10 secretion was then stained for to give a number of positive spots that were counted, and converted to spot forming units (SFU) per million cells. Peptides 7, 8, 11, 12, 16, 18, 30, 31, 55, 57, 58, 67, and 69 were tested in duplicate. The frequencies of positive cells was determined by subtracting values in all test wells from the background number of positive cells in unstimulated well.

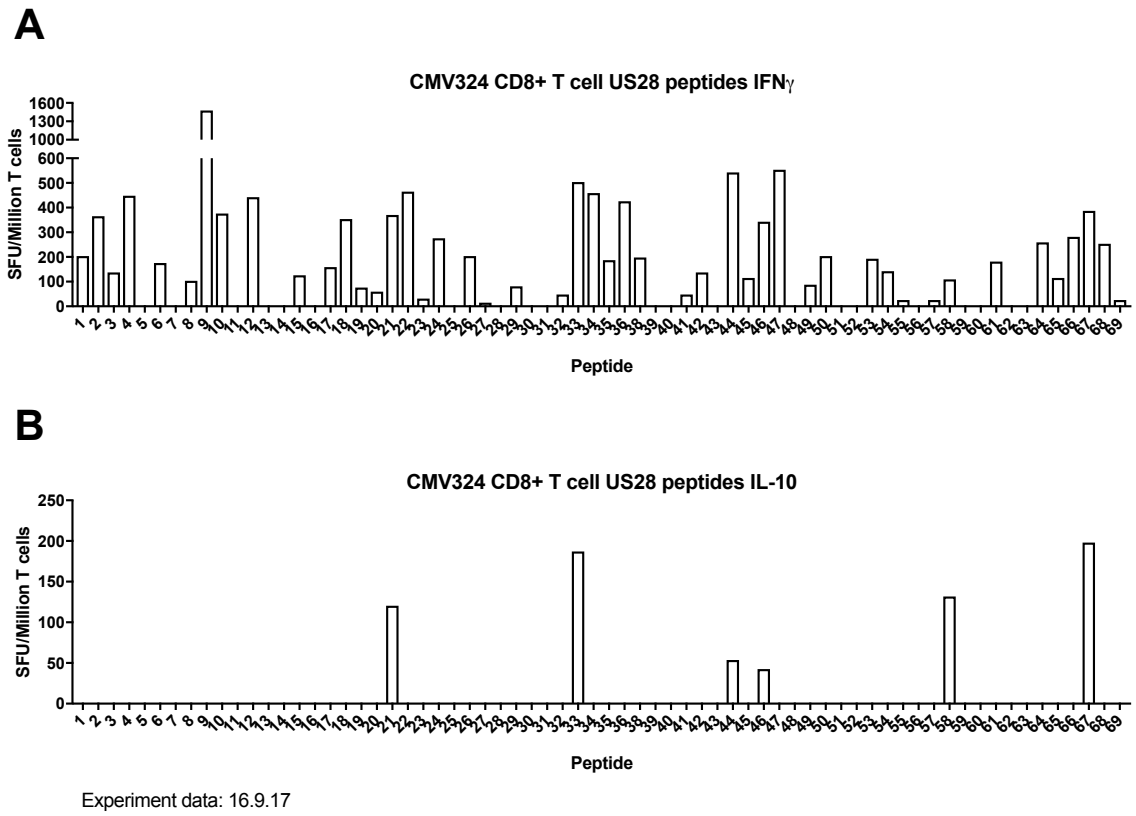



**Fig. 7.5 CD8+ T cell responses from donor CMV324 to individual peptides spanning the US28 ORF.** PBMC from donor CMV324 were depleted of CD4+ cells and stimulated with individual 15 amino acid peptides spanning the ORF of US28 for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. (A) IFN$\gamma$ and (B) IL-10 secretion was then stained for to give a number of positive spots that were counted, and converted to spot forming units (SFU) per million cells. The frequencies of positive cells was determined by subtracting values in all test wells from the background number of positive cells in unstimulated well.

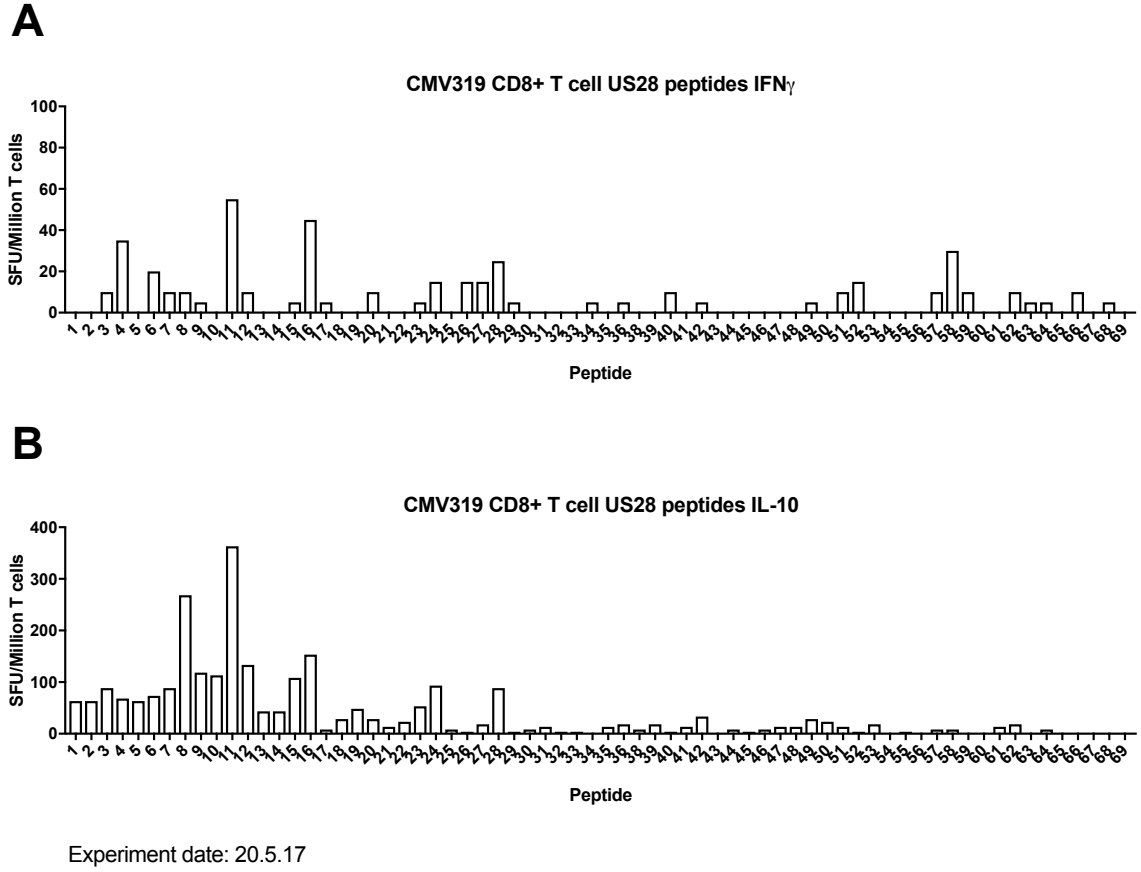



**Fig. 7.6 CD8+ T cell responses from donor CMV319 to individual peptides spanning the US28 ORF.** PBMC from donor CMV319 were depleted of CD4+ cells and stimulated with individual 15 amino acid peptides spanning the ORF of US28 for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. (A) IFN$\gamma$ and (B) IL-10 secretion was then stained for to give a number of positive spots that were counted, and converted to spot forming units (SFU) per million cells. The frequencies of positive cells was determined by subtracting values in all test wells from the background number of positive cells in unstimulated well.

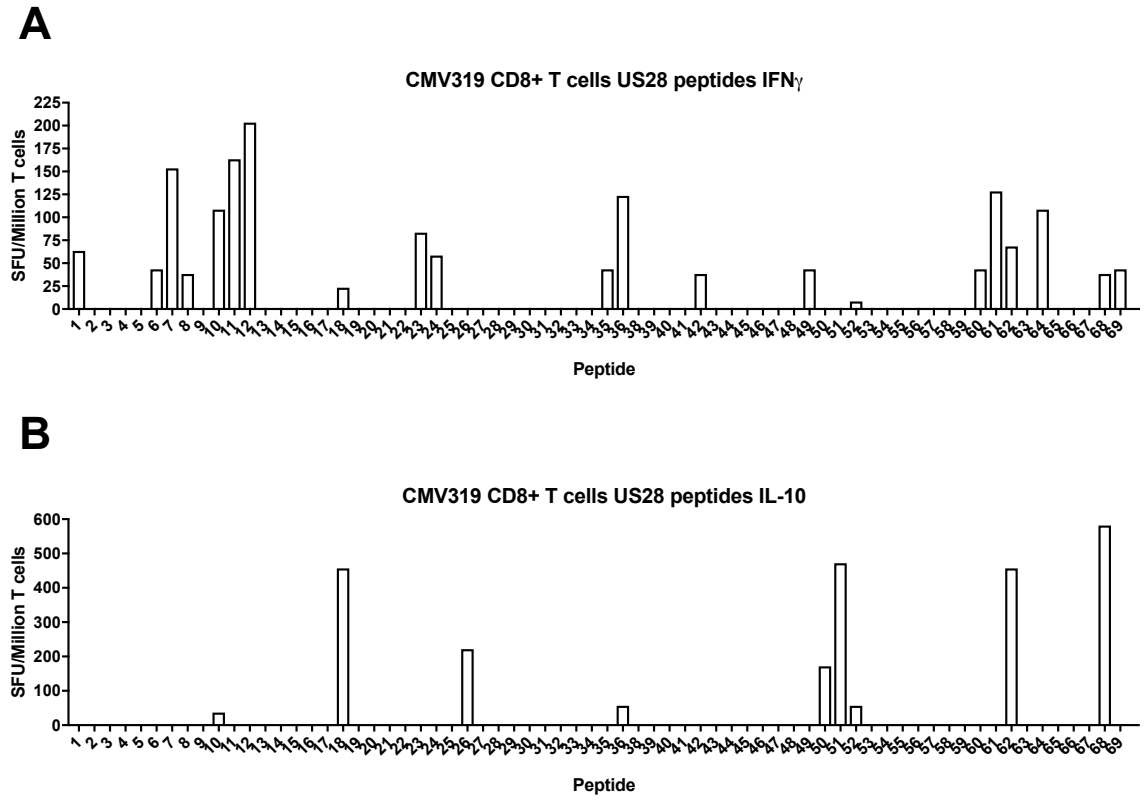



**Fig. 7.7 CD8+ T cell responses from donor CMV319 to individual peptides spanning the US28 ORF.** PBMC from donor CMV319 were depleted of CD4+ cells and stimulated with individual 15 amino acid peptides spanning the ORF of US28 for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. (A) IFN$\gamma$ and (B) IL-10 secretion was then stained for to give a number of positive spots that were counted, and converted to spot forming units (SFU) per million cells. The frequencies of positive cells was determined by subtracting values in all test wells from the background number of positive cells in unstimulated well.

Donor CMV321 was sampled once. Several individual peptides elicited IFN$\gamma$ secretion from a moderate number of CD8+ T cells, with peptide 61 showing the greatest responses (Figure 7.8A). IL-10 responses were composed of very high numbers of cytokine secreting cells which were organised into clusters (Figure 7.8A). All the peptides which elicited appreciable IFN$\gamma$ responses also displayed high frequency IL-10 responses, including peptides 12, 25, 29, 38, 49, and 61, while many peptides showed only IL-10 responses (Figure 7.8).

Donor CMV320 was also sampled on only one occasion. Few peptides elicited an IFN$\gamma$ response, with only moderate frequencies of IFN$\gamma$ secreting CD8+ T cells detected in response to peptides 40, 57, and 58 (Figure 7.9A). No IL-10 secreting cells were detected in response to any of the peptides tested (Figure 7.9B).

Testing individual CD8+ T cell responses to 15-mer peptides has highlighted several peptides that elicited either an IFN$\gamma$ or IL-10 response alone, or a combination of both cytokine secreting cells. However, testing donors at multiple time points revealed that these responses to individual peptides are often detected inconsistently.

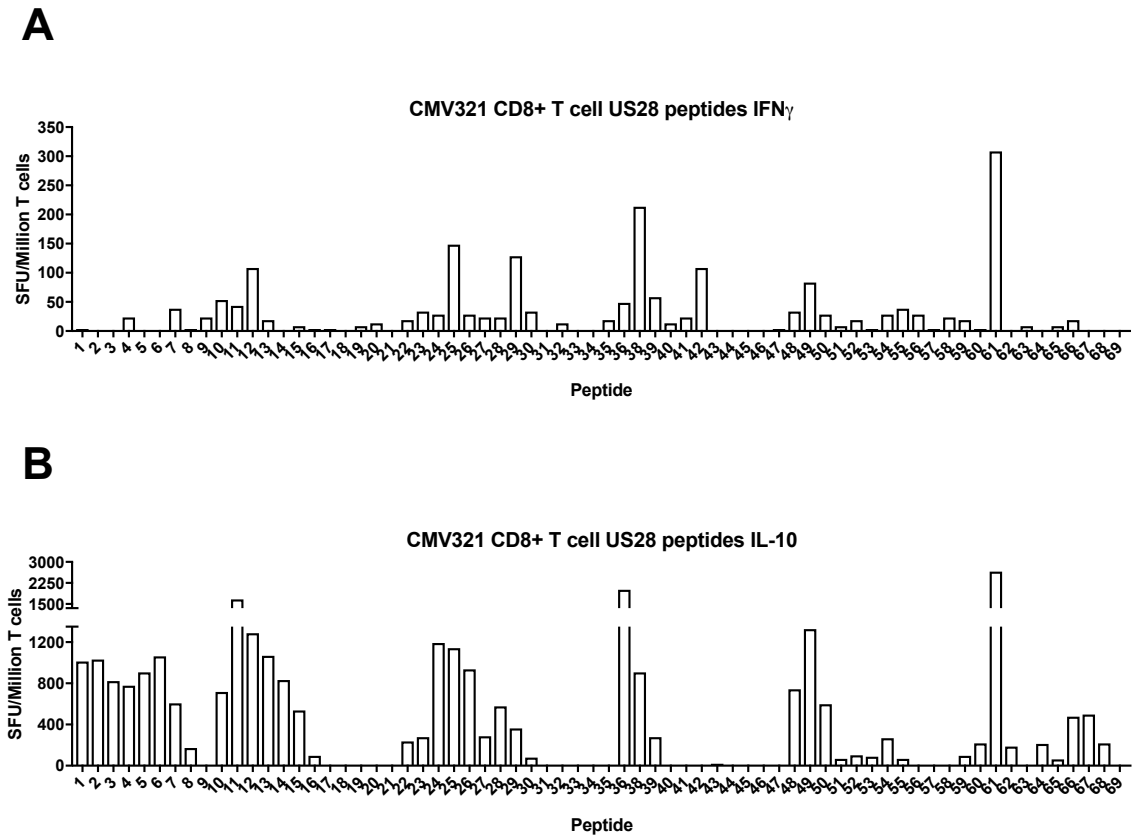



**Fig. 7.8 CD8+ T cell responses from donor CMV321 to individual peptides spanning the US28 ORF.** PBMC from donor CMV321 were depleted of CD4+ cells and stimulated with individual 15 amino acid peptides spanning the ORF of US28 for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. (A) IFN$\gamma$ and (B) IL-10 secretion was then stained for to give a number of positive spots that were counted, and converted to spot forming units (SFU) per million cells. The frequencies of positive cells was determined by subtracting values in all test wells from the background number of positive cells in unstimulated well.

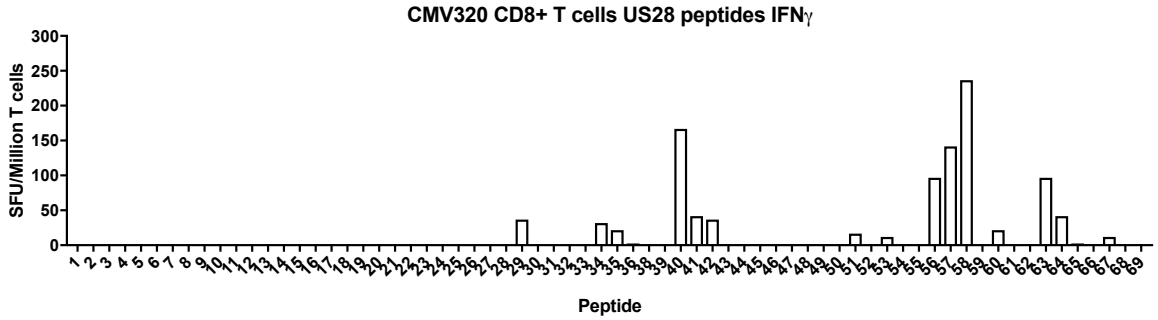



**Fig. 7.9 CD8+ T cell responses from donor CMV320 to individual peptides spanning the US28 ORF.** PBMC from donor CMV320 were depleted of CD4+ cells and stimulated with individual 15 amino acid peptides spanning the ORF of US28 for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates. IFN$\gamma$ secretion was then stained for to give a number of positive spots that were counted, and converted to spot forming units (SFU) per million cells. The frequencies of positive cells was determined by subtracting values in all test wells from the background number of positive cells in unstimulated well.

## 7.4 Discussion

CD8+ T cells specific for proteins expressed during latency were phenotypically characterised to investigate differences in cytotoxic potential and memory phenotypes in comparison to lytic ORF specific CD8+ T cells. As has been well documented, CD107a degranulation and granzyme expression in response to proteins expressed during lytic infection, such as pp65 and IE1 was observed [304, 315, 316, 398, 738–740]. However, antigen-specific CD8+ T cell CD107a expression was also detected in response to latency-associated ORFs, which has not been previously described. The expression of CD107a and granzymes were found to generally be similar between lytic and latency-associated ORF specific CD8+ T cells, although pp65-specific CD8+ T cells did have significantly higher expression of CD107a compared to gB- and UL111A-specific CD8+ T cells. Interestingly, a proportion of ORF-specific CD8+ T cells that expressed CD107a was always detected in donors that possessed UL144- and US28-specific CD8+ T cells. In addition, the high proportion of cells expressing CD107a on US28-specific CD8+ T cells, which was comparable to that detected on pp65-specific CD8+ T cells, was also observed for US28-specific CD4+ T cells (Section 6.2.2). CD8+ T cells specific to latency-associated ORF products therefore possess the potential to mediate cytotoxic responses. In addition, the memory T cell phenotypes of latency-associated ORF specific CD8+ T cells did not differ substantially compared to lytic ORF specific T cells, with large proportions of HCMV-specific CD8+ T cells occupying the $T_{EMRA}$ cell subset. These results are in agreement with previous research carried out on responses to lytic ORFs such as pp65 [287, 292, 294, 296, 301].

The potential of latency-associated ORF specific CD8+ T cells to mediate cytotoxic responses raises questions as to why they are not effective against latently infected cells *in vivo*. Whether this is due to inhibition mediated by suppressive cytokines released by the virus, or by CD4+ and CD8+ T cells, requires investigation. The results presented here are limited by the inability to determine the expression of cytotoxic and memory markers on latent-specific CD8+ T cells that expressed IFN$\gamma$, IL-10, or neither cytokine, by ICS. It was therefore not possible to examine whether latent-specific CD8+ T cells (defined here as CD69+ and/or 4-1BB+) that were positive or negative for CD107a expressed IFN$\gamma$ or IL-10, due to the low frequency of IL-10 positive cells and insufficient number of acquired events. These separate cytokine secreting populations could have different cytotoxic abilities and could display different memory phenotypes to lytic ORF specific T cells, and this would be important to determine given that responses to HCMV latency-associated ORFs are composed of higher proportions of IL-10 secreting cells compared to lytic ORF-specific responses (Section 4.2.7). As the results presented here represent the entire population of

CD8+ T cells deemed to be antigen-specific, this information is lacking however. Future work could approach this problem through the use of MHC class I tetramers. The use of tetramers would enable the targeted sorting of CD8+ T cell specific to latency-associated ORFs without the requirement to stimulate them. Whole populations of latency-associated ORF specific CD8+ T cells could therefore be phenotyped and could also allow for more in depth study, such as through single cell transcriptomics.

The inability of cytotoxic CD8+ T cells to target latently infected cells could also be due to direct viral immune evasion. HCMV is known to interfere with antigen processing and presentation, with the US2-US11 genomic region playing an important role in MHC class I downregulation [571–574, 577, 741, 742], and these could also be active during latent infection. The impact these genes have on MHC class I expression during latency is yet to be fully established. Expression of the US2 and US3 genes, but not US11, have been reported in an experimental model of latency [604], with recent RNA-sequencing analyses showing detectable US2, and US11 [129], or US3 expression [130], although it is speculated that US3 expression was originating from virus in the process of reactivating to lytic infection. As US3 is an IE gene, and can be detected from one hour post infection in lytically infected fibroblasts, this explanation is plausible [743]. The cytotoxic capacities of CD8+ T cells specific for latency-associated proteins expressed could be examined first using *in vitro* viral dissemination assays to initially confirm they can prevent the spread of a lytic HCMV infection. This is important because the use of overlapping peptide pool stimulation does not account for the effects of virally encoded immune evasion molecules expressed during infection, particularly those responsible for the downregulation of MHC class I molecules.

Differences between the cytotoxic and memory phenotypes of CD8+ T cells specific for latency-associated ORFs compared to lytic ORFs could also be revealed if CD8+ T cells derived from tissue sites are analysed. CD8+ T cells specific to HCMV are broadly distributed across tissues in addition to the peripheral blood compartment [398]. A recent study found the frequencies of CD8+ T cells specific for pp65, IE1, and pp50 epitopes to be heterogeneously distributed across different tissue sites and peripheral blood within individuals [398]. Interestingly, CD8+ T cells specific for varying epitopes of pp65 have been shown to differ in functional capacities and memory phenotype [744]. Sampling from the peripheral blood only might therefore not be representative of the CD8+ T cell responses to HCMV latency-associated ORFs. In support of this, a comparison of HCMV-specific CD8+ T cells between the lymph nodes and peripheral blood found both lower proportions of $T_{EMRA}$ cells and granzyme B positive cells in the lymph nodes [695]. Additionally while two studies examining pp65-specific CD8+ T cells between bone marrow and peripheral blood reported

either no difference in frequencies [699], or a lower frequency in the bone marrow [700], the bone marrow was found to be enriched for $T_{CM}$ cells [699]. However, an analysis of CD27, CD28, and IL-7R$\alpha$ expression on pp65-specific CD8+ T cells found similar phenotypes between blood and lung T cells [745]. Differences between latency-associated and lytic ORF specific CD8+ T cells might therefore be detected if certain tissue sites are enriched for latency-associated ORF specific CD8+ T cells, or for CD8+ T cells specific to a particular epitope within the latency-associated protein.

With a view towards the generation of an MHC class I tetramer, four donors were utilised in the analysis of CD8+ T cell single-peptide responses to US28. As well as providing initial data for the identification of a minimal CD8+ T cell peptide epitope, testing the responses to individual peptides could provide an indication of whether CD8+ T cells specific for a certain peptide are IL-10 or IFN$\gamma$ producers only, or a mix of both. Across these four donors, several peptides were found to elicit responses that were composed of either entirely IFN$\gamma$ or IL-10 secreting cells, or were a mixture of both. However, these responses were found to be inconsistently detected in the donors sampled on multiple occasions. The previously described results from the longitudinal sampling of CD4+ (Section 3.2.4) and CD8+ (Section 4.2.2) T cells showing fluctuations in detected responses over time provide a possible explanation for these results. Furthermore, the CD8+ T cell responses to individual peptides might be expected to result in greater inconsistencies over time compared to whole ORF responses as examined previously (Section 3.2.4 and 4.2.2). As with all T cell responses being measured by FluoroSpot, it is also possible that the positive responses detected are due to T cell cross-reactivity [658], necessitating the validation of positive responses. The sampling of donors on multiple occasions might therefore be required to determine the stability of peptide-specific responses, as shown for peptides 18 and 58 of US28 from donor CMV324. Once peptides eliciting consistent responses are chosen, these can then be re-synthesised to confirm the response, before mapping the minimal epitopes based on the CD8+ T cell responses to the overlapping peptides. This will enable a minimal peptide epitope to be determined which can then be validated and used to synthesise an appropriate tetramer.

# Chapter 8

# Neutralising IL-10 and TGF$\beta$ to improve T cell responses against latently infected monocytes

## 8.1 Introduction

It has been demonstrated that cells latently infected with HCMV are capable of secreting the immunosuppressive cytokines IL-10 and TGF$\beta$, in addition to inducing secretion of these cytokines from uninfected bystander cells [605]. Additionally, the expression of the LAcmvIL-10 isoform of UL111A during latency is able to upregulate the transcription of cellular IL-10 [164]. Investigations into the effect the secretome from latently infected cells has on T cell effector function found it was able to inhibit CD4+ T cell production of Th1 cytokines and CD4+ T cell mediated cytotoxicity [605]. These observations led to the hypothesis that HCMV creates an immunosuppressive microenvironment during latency to avoid recognition and clearance by T cells.

The findings from this research project, that CD4+ and CD8+ T cells specific for latency-associated ORF products are capable of secreting IL-10, and TGF$\beta$ in the case of CD4+ T cells, support such a hypothesis where immunosuppressive factors secreted into the microenvironment of latently infected cells inhibit antiviral T cell responses during latency. The observations that IL-10 secreting T cells were a separate sub-population to those secreting IFN$\gamma$ also raised the possibility of selectively targeting the suppressive population in an attempt to boost Th1-type immune responses. However, efforts to deplete CD25+

expressing cells which could be Tregs, in an attempt to target the ORF-specific IL-10 secreting CD4+ T cell population, were not able to consistently remove these cells. It was therefore hypothesised that neutralising the immunosuppressive effects of IL-10 and TGF$\beta$ could have the desired outcome of improving antiviral T cell responses against latently infected cells. The aim of this chapter was therefore to determine if T cell responses could be modulated to improve the clearance of latently infected cells by interfering with the immunosuppressive factors IL-10 and TGF$\beta$.

## 8.2 Results

### 8.2.1 The latency-associated secretome inhibits CD4+ T cell IFN$\gamma$ production in response to HCMV ORF peptide pools

The secretion of IL-10 and TGF$\beta$ into the cellular microenvironment by latently infected cells, myeloid lineage bystander cells, and potentially latency-associated ORF specific T cells could be preventing antiviral T cell responses during latency (Figure 8.1). Previous work has shown that the secretome of latently infected CD34+ cells can inhibit the secretion of Th1-associated cytokines [605]. To build on this, the inhibitory effects of the secretome from latently infected cells on HCMV-specific T cell responses were examined. Initially, whether CD4+ T cells exposed to IL-10 and TGF$\beta$, prior to stimulation with HCMV peptides, would result in a decrease in HCMV-specific IFN$\gamma$ production was investigated. The IL-10 and TGF$\beta$ were used as a proxy for the latent cell secretome. CD8+ cell depleted PBMC from one donor were incubated with increasing concentrations of recombinant IL-10 and TGF$\beta$ for two hours, then stimulated with HCMV ORF peptide pools. After incubation for 48 hours, the supernatants were analysed for IFN$\gamma$ production by ELISA. CD4+ T cells that had been pre-incubated with IL-10 and TGF$\beta$ were found to have reduced IFN$\gamma$ production in response to pp65 and US28, although for pp65 peptide stimulated cells, this was only at the lowest IL-10 and TGF$\beta$ concentration (Figure 8.2). However, the concentrations of IL-10 and TGF$\beta$ tested were unable to mediate the inhibition of UL144-specific CD4+ T cell IFN$\gamma$ secretion (Figure 8.2)

The secretome of latently infected cells, which has been shown to contain the immunosuppressive cytokines IL-10 and TGF$\beta$ [605], could therefore have a similar effect on HCMV-specific CD4+ T cell IFN$\gamma$ secretion. To test this, CD8+ cell depleted PBMC from one donor were incubated with supernatants from latently infected CD14+ and CD34+

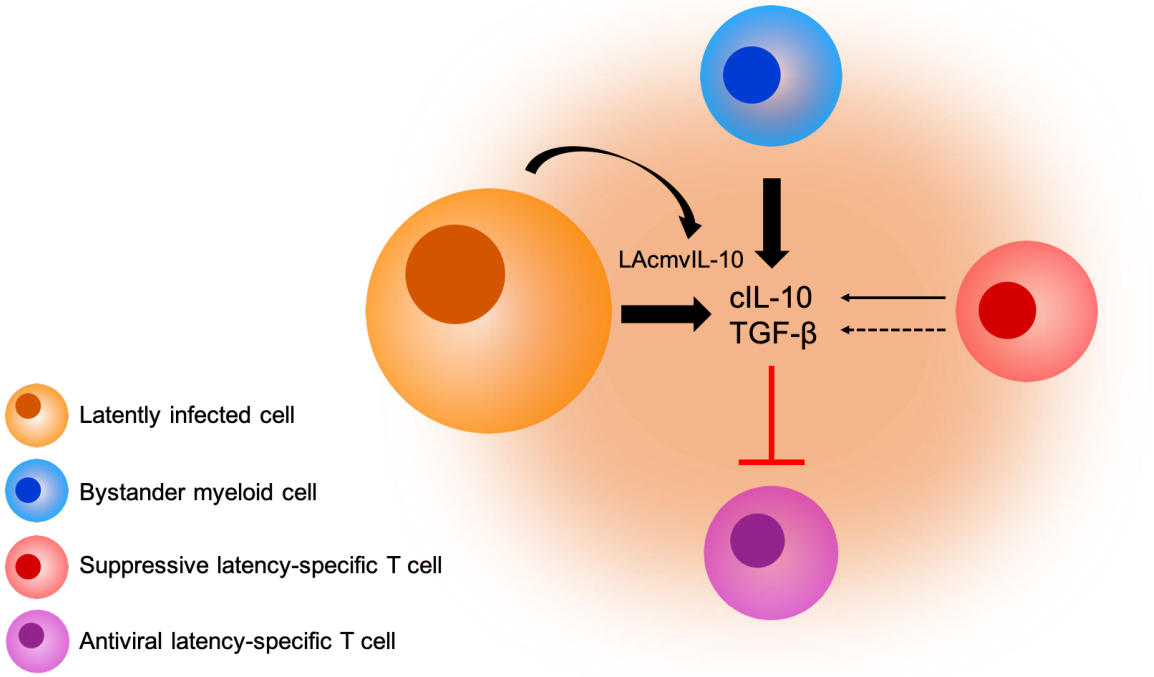



**Fig. 8.1 Model for inhibition of T cell responses during latency.** Antiviral T cells specific for proteins expressed during latency could be inhibited by LAcmvIL-10 secreted by latently infected cells, the secretion of cellular IL-10 (cIL-10) and TGF$\beta$ by latently infected cells, bystander myeloid cells, and latency-associated ORF specific CD4+ T cells, in addition to cIL-10 secreted by latency-associated ORF specific CD8+ T cells [127, 165, 501, 605]

cells (generated by Sarah Jackson) prior to HCMV ORF peptide stimulation. Analysing IFN$\gamma$ secretion by ELISA revealed that for this donor, ORF peptide pool stimulation resulted in levels of IFN$\gamma$ greater than the upper limit of detection in response to pp65 (not shown), LUNA, and US28, while responses to UL111A were within the limits (Figure 8.3). CD4+ T cells incubated with latent secretomes prior to peptide stimulation were found to have a considerable reduction in IFN$\gamma$ secretion for responses to LUNA, US28, and UL111A (Figure 8.3).

### 8.2.2 Inhibition of IL-10 and TGF$\beta$ increases CD4+ T cell IFN$\gamma$ secretion in response to UL111A peptides

T cells responding to HCMV ORF peptide pools were found to be composed of distinct IFN$\gamma$ and IL-10 secreting sub-populations (Section 3.2.10 and 4.2.7). TGF$\beta$ secretion was also detected from CD4+ T cells in response to HCMV ORFs. Whether the secretion of these immunosuppressive cytokines from one sub-population of CD4+ T cell could be inhibiting IFN$\gamma$ secretion from the other was initially examined. CD4+ T cells from one donor were stimulated with ORF peptide pools in the presence of anti-IL-10, anti-TGF$\beta$, and anti-IL-10R antibodies, or isotype control antibodies, and the secretion of IFN$\gamma$ examined by ELISA. The IFN$\gamma$ secretion in response to pp65, LUNA, and US28 all exceeded the upper limits of detection of the assay so were not included in the analysis. The addition of anti-IL-10, anti-TGF$\beta$ and anti-IL-10R antibodies was found to increase IFN$\gamma$ secretion in response to UL111A compared to no treatment by approximately threefold, and by nearly twofold in comparison to treatment with isotype control antibodies (Figure 8.4).

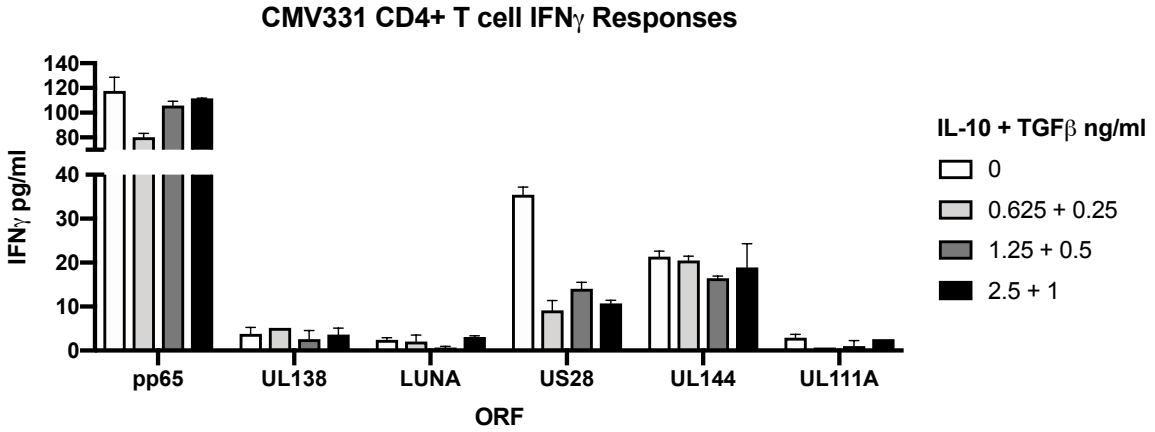



**Fig. 8.2 IFN$\gamma$ secretion in response to HCMV latency-associated ORF peptides following pre-incubation with IL-10 and TGF$\beta$.** CD8+ cell depleted PBMC were incubated with increasing concentrations of IL-10 and TGF$\beta$, or with media alone, for two hours prior to the addition of HCMV ORF peptide pools spanning the ORFs of pp65, UL138, LUNA, US28, UL144, and UL111A. After 48 hours, supernatants were harvested and assessed for IFN$\gamma$ secretion by ELISA in duplicate. Background IFN$\gamma$ secretion from unstimulated CD4+ T cells was deducted from test values.

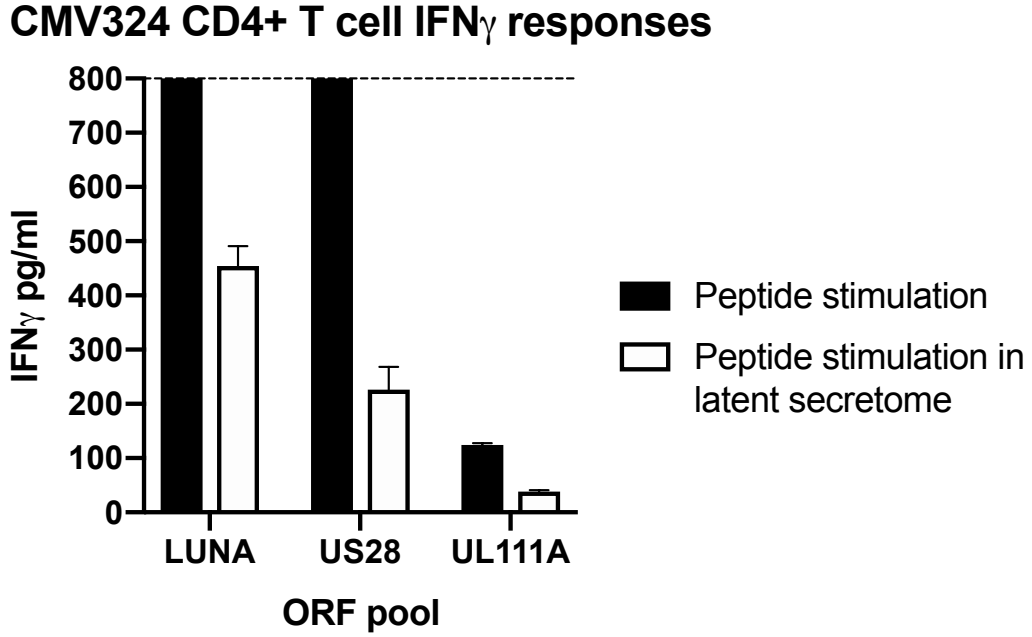



**Fig. 8.3 IFN$\gamma$ secretion in response to HCMV latency-associated ORF peptides following pre-incubation with latency-associated secretome.** CD8+ cell depleted PBMC from donor CMV324 were incubated with the secretome harvested at day 14 from donor CMV180308 CD14+ cells and CD34+ cells latently infected with the TB40e strain of HCMV (white bars), or with media alone (black bars), for two hours prior to the addition of HCMV ORF peptide pools spanning the ORFs of LUNA, US28, and UL111A. After 48 hours, supernatants were harvested and assessed for IFN$\gamma$ secretion by ELISA in duplicate. Background IFN$\gamma$ secretion from unstimulated CD4+ T cells was deducted from test values. The dashed line indicates the limit of detection of the assay.

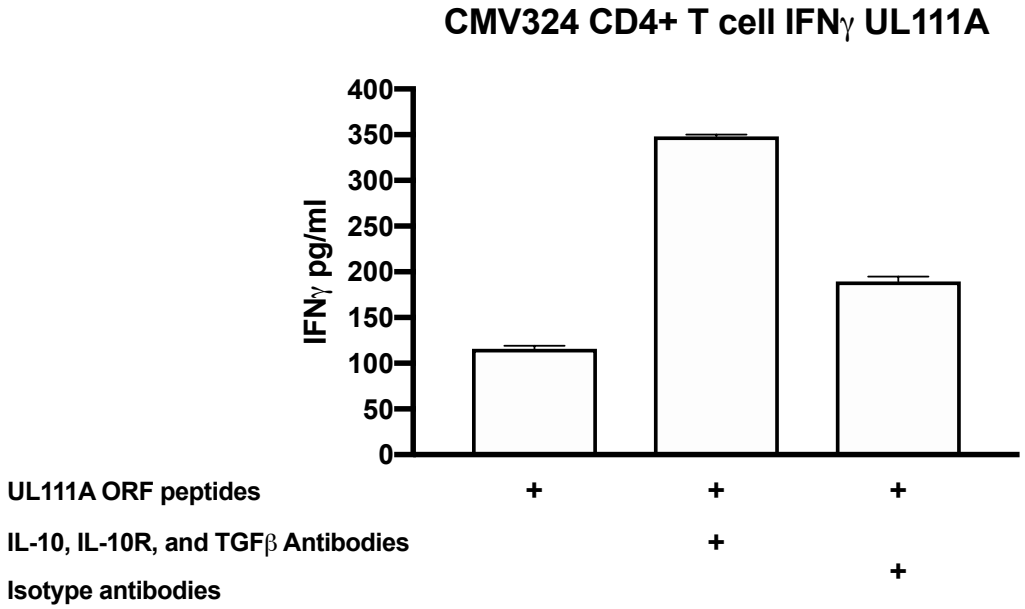



**Fig. 8.4 IFN$\gamma$ secretion in response to HCMV latency-associated ORF peptides in the presence of neutralising IL-10 and TGF$\beta$ antibodies.** CD8+ cell depleted PBMC were incubated with media alone, or stimulated with HCMV ORF peptide pools spanning the UL111A ORF, in the presence or absence of a cocktail of anti-IL-10 and anti-TGF$\beta$, and anti-IL-10 receptor antibodies at 5$\mu$g/ml, or a mix of IgG1 and IgG2b isotype control antibodies at 5$\mu$g/ml. After 48 hours, supernatants were harvested and assessed for IFN$\gamma$ secretion by ELISA in duplicate. Background IFN$\gamma$ secretion from unstimulated CD4+ T cells was deducted from test values.

### 8.2.3 Modulation of IL-10 and TGF$\beta$ during latency to improve T cell recognition and clearance of infected cells

Prior work attempting to remove the CD4+ T cell population responsible for IL-10 production by CD25+ cell depletion was not consistently able to do so, as the size of the CD25+ cell population secreting IL-10 varied between donors and between ORF-specific CD4+ T cell populations. As this population could not be removed, the neutralisation of the immunosuppressive activity of IL-10 and TGF$\beta$ was tested. From the work presented in this thesis and by others, IL-10 and TGF$\beta$ are known to be expressed by HCMV latency-associated ORF specific T cells, and cells in the latent microenvironment [501, 605]. Furthermore, IL-10 and TGF$\beta$ expressed by latently infected CD34+ cells was also demonstrated to inhibit cytotoxic gB-specific CD4+ T cells [605]. An *in vitro* system was utilised to examine the effects of IL-10 and TGF$\beta$ neutralisation on T cell killing of latently infected cells.

Initially, CD14+ monocytes from an HCMV seropositive donor were latently infected with TB40e-UL32-GFP for a period of 7 days to determine the optimal MOI for use in subsequent experiments. A MOI of 1.25 was chosen as this produced high numbers of GFP+ cells at a lower concentration of virus (Figure 8.5A). Following this, the immunosuppressive latent microenvironment was targeted by the addition of anti-IL-10 and anti-TGF$\beta$ antibodies, before the addition of autologous CD3+ T cells isolated at high purity by MACS (mean=92.9% CD3+ cells, Std. deviation=3.8, n=6) (data not shown). Following the incubation of T cells with latently infected cells for three days with or without antibodies, T cells were removed and monocytes were differentiated with GM-CSF and IL-4, followed by LPS, to reactivate the latent virus, and the number of infected cells counted by microscopy (Figure 8.5B). The cytotoxic effects of residual T cells that could not be removed prior to reactivation were blocked by the addition of MHC class I and II antibodies. The selective targeting of T cells was attempted through the use of cyclosporin A, but this was found to reduce GFP expression in fibroblasts infected with TB40e-UL32-GFP at all concentrations at 7 days post infection (Figure 8.6A), independently of cell death (Figure 8.6B). In this manner, the effects of neutralising IL-10 and TGF$\beta$ on the ability of T cells to kill latently infected cells was assessed compared to T cell killing with these cytokines present.

The addition of T cells to latently infected monocytes without antibodies was found to reduce the number of infected cells by around 50%, compared to a 70% reduction when T cells were added following IL-10 and TGF$\beta$ neutralisation (Figure 8.7). The addition of isotype control antibodies prior to T cell addition was not found to have any additional

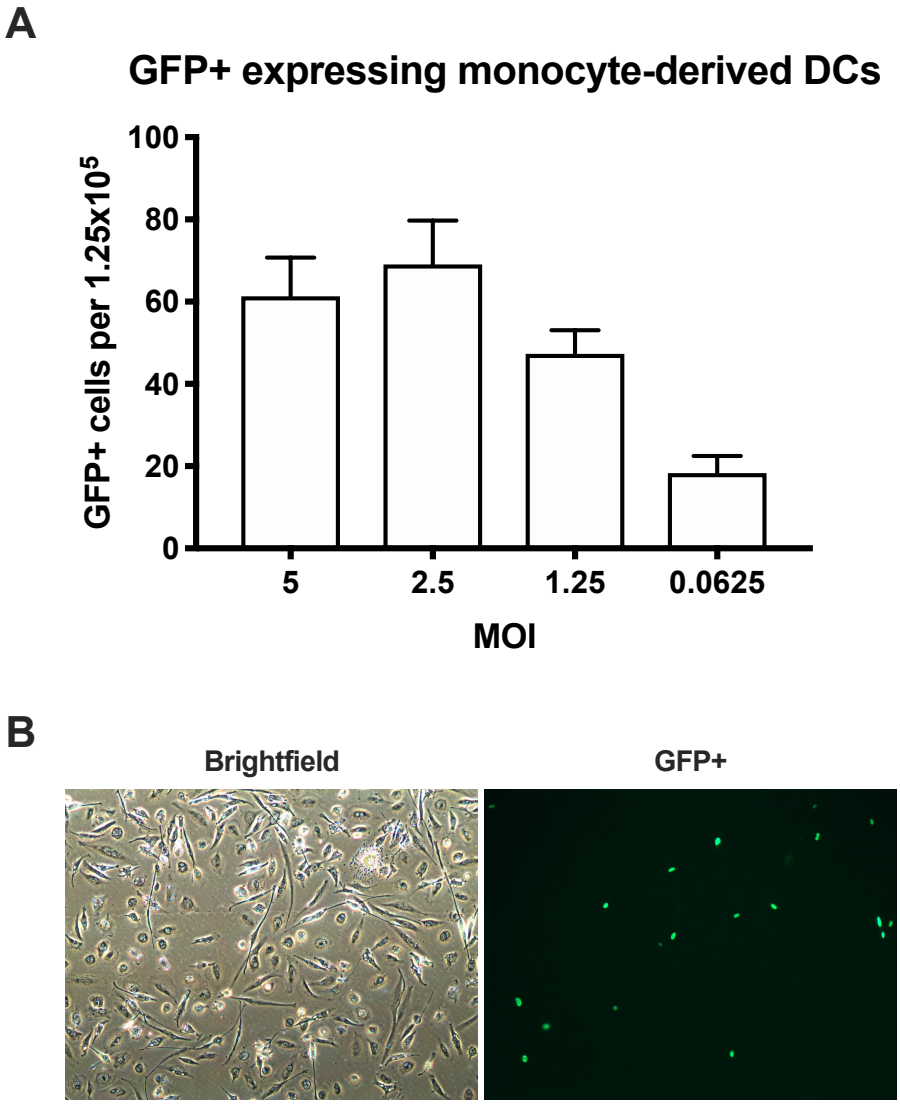



**Fig. 8.5 TB40e-UL32-GFP titration.** CD14+ monocytes were latently infected over a range of multiplicities of infection (MOI) with TB40e-UL32-GFP and incubated for five days in 96 well plates. To differentiate monocytes to dendritic cells (DCs), and reactivate latent virus, latently infected cells were incubated with GM-CSF and IL-4 for five days and LPS was added for 24 hours. (A) GFP positive DCs for each MOI were then counted under the microscope. (B) A well of cells infected at an MOI of 1.25 is visualised. Each MOI was tested in quadruplicate.

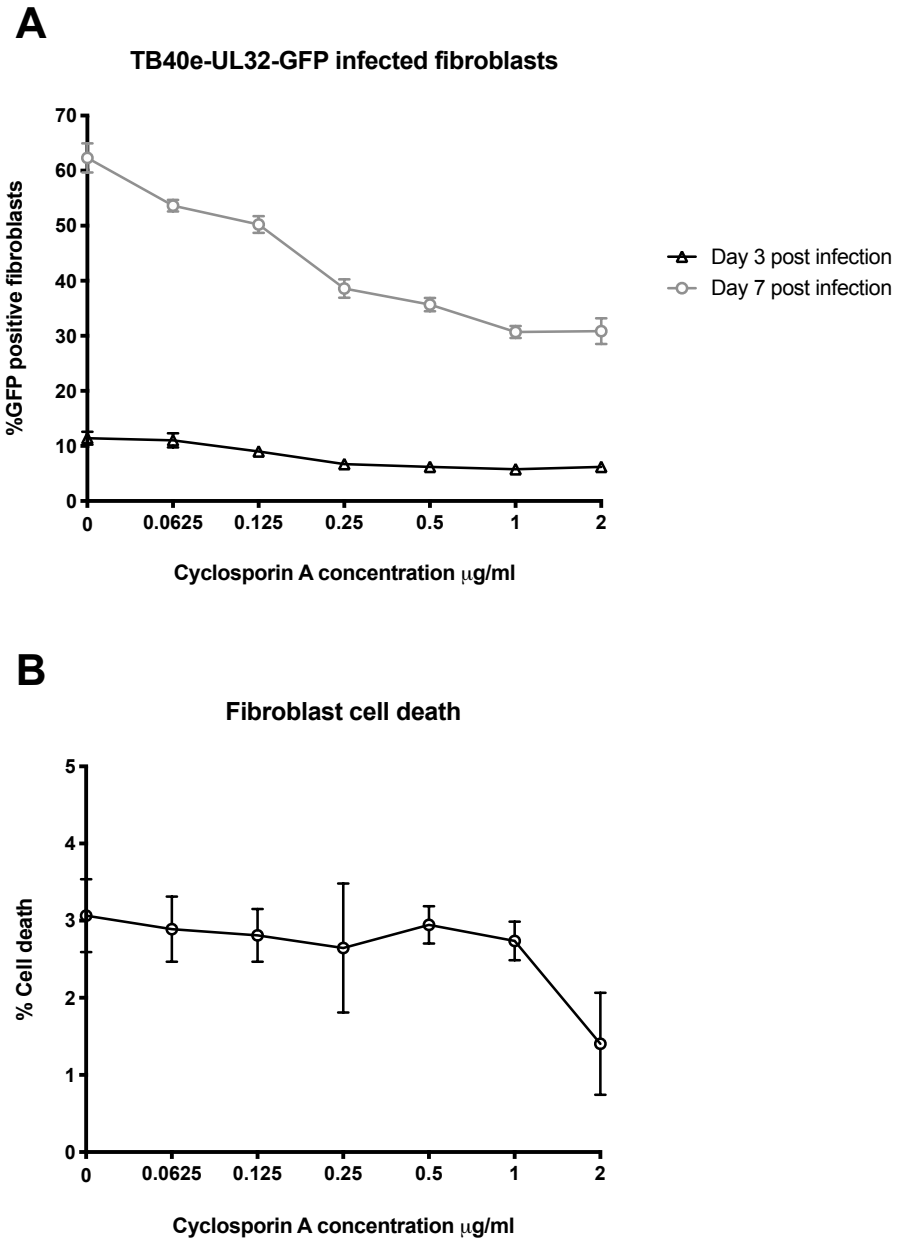



**Fig. 8.6 Effect of cyclosporin A on viral gene expression.** Fibroblasts were infected with TB40e-UL32-GFP at an MOI of 0.1 before the addition of cyclosporin A at a range of concentrations. (A) Fibroblasts were harvested at 3 days (black line), and 7 days (grey bar) post infection, and the percentages of GFP+ fibroblasts determined by flow cytometry. (B) Cell death of fibroblasts at each cyclosporin A concentration was determined by Annexin V staining and flow cytometry at 7 days post infection. Fibroblasts were tested in triplicate at each cyclosporin A concentration.

effect on latent monocyte killing over T cells alone (Figure 8.7). This experiment was then repeated as above with the inclusion of conditions to test the effects of neutralising antibodies, or their isotype controls, on latent viral carriage. In these additional conditions, latently infected monocytes were incubated with anti-IL-10 and anti-TGF$\beta$ or IgG1 and IgG2b isotype antibodies for 72 hours at 7 days post infection. As before, a decrease in the number of GFP+ cells was observed, although by approximately 66%, following the addition of T cells alone to latently infected monocytes (Figure 8.8). A comparable reduction in GFP+ cells was also observed on addition of anti-IL-10 and anti-TGF$\beta$ antibodies or their isotype control antibodies in isolation, and on addition of isotype control antibodies together with T cells (Figure 8.8). The addition of T cells following neutralisation of IL-10 and TGF$\beta$ however did lead to a further reduction in GFP+ cell numbers of around 83% compared to the addition of T cells to latent monocytes alone (Figure 8.8).

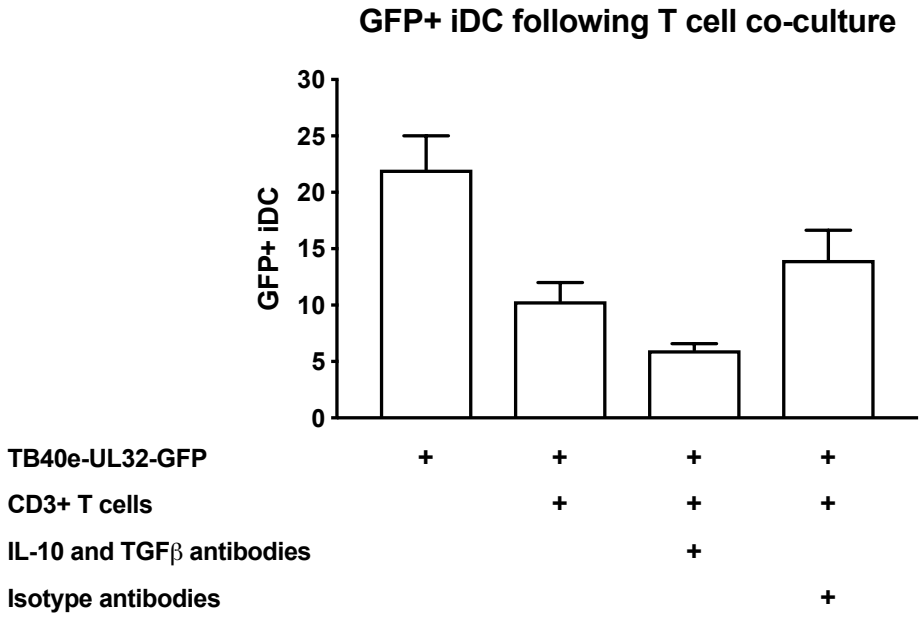



**Fig. 8.7 Effect of anti-IL-10 and anti-TGF$\beta$ antibodies on T cell killing of latently infected monocytes.** CD14+ monocytes were latently infected with TB40e-UL32-GFP at an MOI of 1.25 for 7 days. Following this, anti-IL-10 and anti-TGF$\beta$ antibodies, or IgG1 and IgG2b isotype antibodies were added for two hours prior to the addition of isolated CD3+ cells. CD3+ T cells were also added to latently infected cells without antibodies. T cells were removed after 72 hours by PBS wash and replaced with media containing GM-CSF and IL-4 to differentiate monocytes, in addition to anti-MHC class I and class II antibodies. After 5 days, LPS was added and GFP+ cells counted after 2 days by microscopy. Each experimental condition was carried out in triplicate.

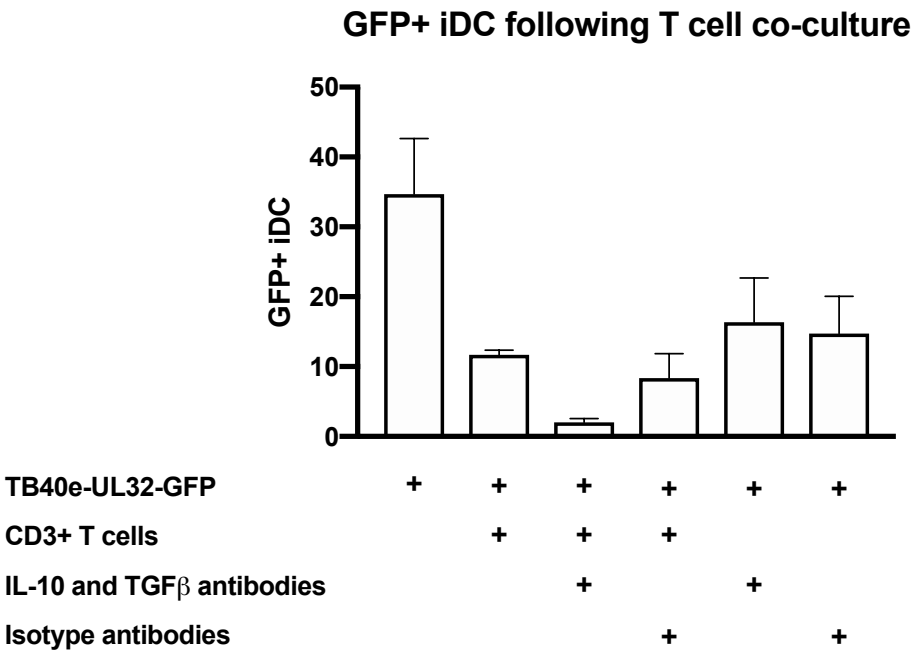



**Fig. 8.8 Effect of anti-IL-10 and anti-TGF$\beta$ antibodies on T cell killing of latently infected monocytes.** CD14+ monocytes were latently infected with TGF40e-UL32-GFP at an MOI of 1.25 for 7 days. Following this, anti-IL-10 and anti-TGF$\beta$ antibodies, or IgG1 and IgG2b isotype antibodies were added for two hours prior to the addition of isolated CD3+ cells. CD3+ T cells or antibodies alone were also added to latently infected cells in isolation. T cells were removed after 72 hours by PBS wash and replaced with media containing GM-CSF and IL-4 to differentiate monocytes, in addition to anti-MHC class I and class II antibodies. After 5 days, LPS was added and GFP+ cells counted after 2 days by microscopy. Each experimental condition was carried out in triplicate.

## 8.3 Discussion

The secretion of IL-10 and TGF$\beta$ by latently infected cells and uninfected bystander cells [605], CD4+ T cells specific for HCMV latency-associated proteins [501], and IL-10 secretion by CD8+ T cells specific to latency-associated proteins, could be inhibiting antiviral T cell responses during latency. To investigate the effects of the latent secretome on CD4+ T cell responses to HCMV proteins expressed during latency, CD4+ T cells were incubated in latent secretome prior to the addition of HCMV ORF peptide pools, and IFN$\gamma$ secretion was measured by ELISA. IFN$\gamma$ secretion from CD4+ T cells incubated with latent secretome was found to be inhibited in response to LUNA, US28, and UL111A, which is in agreement with a previous study showing that CD4+ T cells given a polyclonal stimulus of PHA in the presence of latent secretomes had reduced IFN$\gamma$ secretion [605]. As the experiment here was only carried out in one donor, who produced levels of IFN$\gamma$ greater than the upper limit of detection of this assay in response to LUNA and US28, it was not possible to see the precise levels of inhibition for those responses. These results are also limited by the use of latent secretomes without comparison to secretomes from mock infected, and UV-inactivated virus infected, CD14+ and CD34+ cells. Future work should seek to address this, and include neutralising antibodies to IL-10 and TGF$\beta$, in addition to vIL-10, to confirm these are the mediators of IFN$\gamma$ secretion inhibition. Additionally, the experiments should be repeated to investigate the inhibitory potential of latent secretomes on CD8+ T cell IFN$\gamma$ secretion. Finally, to confirm that the latent secretomes are not inducing IFN$\gamma$ secretion from another cell type, these experiments should be repeated using ICS to confirm IFN$\gamma$ production is originating from CD4+ T cells.

The inhibition of HCMV-specific CD4+ T cell IFN$\gamma$ secretion mediated by latent secretomes could also have been augmented by the IL-10 and TGF$\beta$ secretion from CD4+ T cells. Given that two distinct sub-populations of T cells responding to HCMV latency-associated ORF products were detected (Section 3.2.10 and 4.2.7), secreting either IFN$\gamma$ or IL-10, targeting inhibitory cytokines by neutralising antibodies could improve the antiviral effector functions of the IFN$\gamma$ secreting T cell population. An initial test was carried out examining IFN$\gamma$ secretion from CD4+ T cells treated with or without anti-IL-10, anti-TGF$\beta$, and anti-IL-10R antibodies in the presence of HCMV ORF peptides. Use of the antibodies was found to increase IFN$\gamma$ secretion in response to UL111A peptides, giving an indication that the population of CD4+ T cells specific to HCMV latency-associated ORFs that secreted IL-10 and TGF$\beta$ can suppress the IFN$\gamma$ secreting population. In MCMV infection, IL-10R blockade was associated with an increase in CD4+ T cells producing IFN$\gamma$ [626], and it would be interesting to examine whether targeting IL-10 and TGF$\beta$ results in

an increase in the number of cells secreting IFN$\gamma$ and/or an increase in the quantity of IFN$\gamma$ secreted per cell in response to HCMV peptides. As this was carried out in only one donor, and IFN$\gamma$ responses to LUNA and US28 were above the upper limit of detection of the ELISA for all conditions, this experiment needs to be repeated to determine if this effect can be observed for all ORF-specific IL-10 responses. Additionally, extending this analysis to CD8+ T cells is important.

Having shown the inhibitory effects of latent secretomes on IFN$\gamma$ secretion from CD4+ T cells stimulated with HCMV latency-associated peptides, and shown that treating CD4+ T cells with antibodies against IL-10, TGF$\beta$, and the IL-10R increased IFN$\gamma$ secretion following HCMV peptide stimulation, these sources of inhibitory cytokines were then targeted in the context of the latent microenvironment. The production of IL-10 and TGF$\beta$ by latently infected cells has been shown to inhibit the cytotoxicity of gB-specific CD4+ T cells [605], providing a promising approach to assess the ability of T cells to eliminate latently infected monocytes in the presence of IL-10 and TGF$\beta$ neutralising antibodies. This approach has also been used in a cancer setting, where neutralisation of IL-10 and TGF$\beta$ improved T cell cytotoxicity against tumour cells in mice [746].

In two experiments, T cells were added to latently infected monocytes, with and without IL-10 and TGF$\beta$ neutralising antibodies, to examine whether T cell responses against latently infected cells could be improved when the inhibitory effects of these cytokines were abrogated. A considerable decrease in infected monocyte numbers was observed following co-incubation with T cells alone. A previous study has also utilised a similar *in vitro* latency system to approach T cell responses to latently infected cells. An histone deacetylase inhibitor (MC1568) was used to transiently upregulate IE expression on latent monocytes, making them susceptible to IE-specific T cells [120]. A reduction in infected monocyte numbers was observed when such T cells were incubated with infected monocytes alone, however, this was only about a 25% decrease [120] in comparison to the 50% and 66% decrease observed in experiments here. It was hypothesised that this could be due to the virus preparation containing incoming lytic proteins such as gB and pp65, which could be processed and presented by infected monocytes, in the absence of lytic gene expression, to activate lytic-specific T cells. Future work could purify virus by sorbitol density gradients to reduce this input of lytic proteins.

The addition of neutralising or isotype control antibodies alone was also found to reduce the number of latently infected monocytes. Previous work has shown that latently infected CD34+ cells treated with an anti-IL-10 antibody for seven days had increased levels of apoptosis [606]. It might therefore be expected that the neutralisation of IL-10 might have

the same effect on the survival of latently infected monocytes, but it was surprising to see that treatment with isotype control antibodies resulted in a comparable decrease in virus-infected cell numbers. However, it could be the case that the addition of anti-IL-10 antibody for seven days is required to see this effect [606], compared to the three days tested here. Additionally, in this previous experiment, an isotype control antibody was not used to compare the levels of apoptosis between latent CD34+ cells treated with and without antibody [606]. Repeating the experiment carried out here with a greater number of replicates is also important to determine if this effect is reproducible.

In both experimental repeats, despite the large decrease in latently infected monocytes observed when T cells were added in isolation, the addition of IL-10 and TGF$\beta$ neutralising antibodies was found to give an additional level of monocyte-specific T cell killing, in comparison to T cells added alone. Therefore, taking the results presented in this chapter as a whole suggests that neutralising the suppressive effects of IL-10 and TGF$\beta$ will promote the recognition and killing of latently infected cells.

# Chapter 9

# General Discussion

## 9.1 Discussion

The work presented in this thesis has provided novel insights into T cells specific for proteins that are expressed during latent HCMV infection. Both CD4+ and CD8+ T cell responses to the latency-associated ORFs UL138, LUNA, US28, UL111A, and UL144 were consistently detected across a large donor cohort. CD4+ T cells, and surprisingly CD8+ T cells, from HCMV seropositive donors were also capable of producing the immunosuppressive cytokine IL-10 in response to HCMV ORF products, and these responses were generally more prevalent to latency-associated ORFs. The IL-10 secreting T cells were found to be separate sub-population than to those secreting IFN$\gamma$, and CD4+ T cells specific to latency-associated ORFs inhibited proliferation and antiviral cytokine secretion. Taking the evidence presented here in combination with previous research [501, 605], suggests that HCMV infection manipulates that antiviral T cell response to a more suppressive phenotype, which could be aiding the ability of the virus to persist during latency.

Alongside IL-10 secreting T cells specific for latently expressed HCMV proteins, latent-specific IFN$\gamma$ secreting T cells were also detected together with latent-specific T cells expressing markers associated with cytotoxicity. Such T cells could therefore represent a population of antiviral T cells that are capable of eliminating latently infected cells but are being inhibited from doing so. The inhibition of these T cells could be in part mediated by the populations of IL-10 secreting CD4+ and CD8+ T cells, which, by secreting immunosuppressive cytokines alongside latently infected cells and their bystander cells, prevent antiviral T cells from targeting latently infected cells. Indeed, when IL-10 and TGF$\beta$

were targeted with neutralising antibodies, both IFN$\gamma$ secretion from CD4+ T cells, and T cell killing of latently infected cells were increased, highlighting the ability of these suppressive cytokines to modulate the interactions between antiviral T cells and latently infected cells for the benefit of viral persistence.

Given these observed increases in antiviral T cell functions following the neutralisation of IL-10 and TGF$\beta$, future work should also aim to identify and target the latent-specific T cell populations responsible for immunosuppressive cytokine secretion, as Tregs were found to only represent a proportion of this population. Further work targeting the immunosuppressive cytokines directly to improve T cell recognition and clearance of latently infected cells should also be undertaken. The initial results from two experiments showing an increase in T cell killing of latently infected cells following the addition of IL-10 and TGF$\beta$ neutralising antibodies emphasise the potential for such an intervention to reduce latent viral loads. Whether targetting the microenvironment in this manner represents an effective therapeutic option will require considerable experimental investigation in the future.

To improve on the *in vitro* system utilised in these experiments, virus could be purified by sorbitol density gradients to reduce the input of lytic proteins on infection, and thus reduce T cell killing of latently infected cells when T cells are added in isolation. Additionally, the use of T cell lines specific to single or multiple latency-associated proteins could result in a lower background level of infected monocyte killing. Virus preparations could also be titrated on CD14+ monocytes from each donor prior to testing them, to provide a more accurate MOI for infection. Similarly, such titrations could be carried out for the differentiation stimuli, with GM-CSF and IL-4, M-CSF and IL-1$\beta$, and PMA, by titrating these on each donor's infected monocytes to determine which is most effective.

The use of TB40e-UL32-GFP virus was a also limitation due to the time required for GFP expression to occur [611]. As UL32 is a late gene, GFP was usually detected at around three days post differentiation of latently infected monocytes. To improve on this, initial experiments were carried out using a TB40e-IE2-YFP virus with PMA as the reactivation stimulus (Appendix C.1), as this provided a much quicker system for detecting infected cells, given that YFP expression occurs from IE time points. However, this virus is missing the US2-US6 genomic region [612], and future research should seek to generate a repaired IE reporter virus with these genes intact. Finally, follow-up investigations could examine the effect of IL-10 and TGF$\beta$ neutralisation on T cell killing in the context of a naturally latent infection. Although such an approach has its difficulties, it has been used previously to examine T cell responses to latently infected monocytes [120], and doing so could eliminate

the problem of introducing lytic viral proteins on infection, which was encountered in this experimental latency system.

The potential to identify immunosuppressive cytokine secreting latent-specific T cell populations could also enable those secreting pro-inflammatory cytokines, that might be antiviral during latency, to be identified. This could then enable an adoptive T cell therapy approach to be taken if the T cells capable of targeting latently infected cells could be isolated reliably and expanded *ex vivo* prior to infusion. Numerous studies have shown successes in such an approach, in both HSCT and solid organ transplantation settings, to restore HCMV immune responses, reduce viral load, and thus reduce the risk of HCMV disease [382, 747–751]. An increased understanding of the distinct IL-10 and IFN$\gamma$ producing latency-specific T cell populations might therefore enable antiviral T cells to be expanded based on certain HCMV-derived peptides or TCRs. The expanded T cells capable of targetting latently infected cells, or strategies to target inhibitory T cells, could then be used in the immunosuppressed transplant setting to reduce latent viral load and improve clinical outcomes. The need to develop new therapeutic options to combat HCMV is especially important given the limitations of antivirals and vaccines. Given the emergence of resistance, novel therapeutics could alleviate the reliance on currently available drugs and prolong their effectiveness [752]. The lack of an effective vaccine to HCMV capable of conferring protection to naive individuals, and protecting against superinfection and reactivation in infected individuals, is also an issue, and one that could also be partially addressed through developing the means to target latently infected cells.

Several questions remain unanswered regarding the phenotypes and capabilities of the IFN$\gamma$ and IL-10 secreting populations of T cells. Phenotypic analysis indicated that T cells specific for latency-associated ORFs possessed cytotoxic potential and did not differ in memory phenotype composition from lytic-ORF specific T cells. The ICS techniques used in this work however could not delineate the cytotoxic potential and memory phenotypes of these two separate populations, and thus it is not known whether IFN$\gamma$ secreting T cells differ from IL-10 secreting T cells in these aspects. Moreover, in initial examinations, a proportion of latent-specific CD4+ T cells secreting IL-10 expressed CD25, and could thus be Tregs, but further characterisation of surface markers and transcription factor expression of these cells could help determine whether these cells fit into a defined T helper cell subset. Likewise, the widespread IL-10 secreting CD8+ T cells found in response to the latency-associated ORFs should be examined in more detail with similar analyses, especially by determining if these cells can secrete TGF$\beta$. To address these issues, future research could make use of MHC class I and II tetramers. The use of tetramers will allow for the sorting of epitope specific T

cells, which will enable in-depth phenotypic characterisation, such as via single cell RNA sequencing and transcription factor expression analysis [334, 337, 405, 753, 754]. In addition, through the use of tetramers, T cells can be sorted to high purity for use in downstream functional assays as well as grown out as lines. Further phenotypic characterisation of IL-10 secreting T cells could reveal markers that are differentially expressed compared to IFN$\gamma$ secreting T cells. Doing so could then enable the selective depletion of IL-10 secreting T cells to both elucidate their role during infection and to remove suppression on antiviral T cell responses during latency.

With a view towards generating tetramers, I carried out the initial mapping of CD4+ and CD8+ T cell responses to minimal peptide epitopes. For CD8+ T cells this highlighted several 15mer peptides able to elicit either sole IFN$\gamma$ and IL-10 responses, or in several cases separate populations of cells secreting both cytokines to the same peptide. Knowledge of the cytokine secretion of single peptide-specific T cells can give an initial indication as to whether the IFN$\gamma$ and IL-10 secreting T cell clones diverged from the same parent cell, but this can be confirmed by TCR sequencing once tetramers have been made. Having mapped responses to individual peptides, these peptides could also be used to stimulate T cells and generate specific clonal lines, which could enable the expansion of peptide-specific IL-10 or IFN$\gamma$ secreting T cells. This could provide a relatively simple way of determining if CD4+ and CD8+ T cells specific to certain latency-associated ORF products are cytotoxic. Additionally, using an *in vitro* latency system, expanded latent-specific IFN$\gamma$ secreting T cells could be tested for their ability to clear a latent infection. However, the manipulations required to expand T cells could impact on their functional capacity and phenotypes so it would be advantageous to use tetramers to sort cells for phenotyping and functional assays without manipulation.

The unexpected findings that IL-10 secreting CD8+ T cells specific to latent proteins were present and so widespread could be indicative of an additional axis by which HCMV interferes with antiviral immunity during latency to aid viral persistence. CD8+ T cell-derived IL-10 could act together with IL-10 and TGF$\beta$ produced by latently infected cells and their bystander cells, and by latent-specific CD4+ T cells to inhibit pro-inflammatory latent-specific antiviral T cells. While experiments were carried out showing the ability of latency-associated ORF specific CD4+ T cell secretomes to inhibit secretion of antiviral cytokines, future work should also examine the effects of CD8+ T cell secretomes on antiviral T cell functions, as this could represent another key part of the inhibitory latent microenvironment. Investigations into the relative importance of CD4+ versus CD8+ T cell derived inhibitory factors on latent carriage could also be undertaken. Furthermore, the

experiments carried out on CD4+ T cell secretomes should be extended to additional donors to validate the inhibition of IFN$\gamma$ and TNF$\alpha$ by supernatants, as well as to investigate the differential inhibitory effects on these cytokines supernatants can have.

The generation of these IL-10 secreting CD4+ and CD8+ T cells was investigated at the point of primary HCMV infection. To do this, samples from patients undergoing kidney transplantation were utilised, where blood had been collected over a number of time points before and after seroconversion. Although T cells secreting IFN$\gamma$ were observed immediately following transplantation, IL-10 secreting T cells were not detected consistently to latency-associated ORF products. Surprisingly, T cells secreting IL-10 could be detected in response to the lytic proteins US3 and pp71 soon after transplantation and these responses were maintained. It is therefore possible that IL-10 secreting T cells specific to latency-associated proteins are generated following longer term viral carriage, perhaps influenced by T cell activation in the established latent microenvironment which contains immunosuppressive cytokines [506, 755, 756]. It could also be possible that IL-10 secreting latent-specific T cells are generated immediately following primary infection, but they are not present in the blood at such early stages. Initial experiments were then carried out on paired bone marrow and peripheral blood T cells to begin to address whether T cells secreting IL-10 were preferentially localised to tissue sites rather than peripheral blood. Differences in the localisation of ORF-specific IFN$\gamma$ and IL-10 secreting latent-specific T cells were found between the blood and bone marrow, with IL-10 secreting T cells found to be enriched in the bone marrow for many ORF-specific responses. The enrichment of IL-10 secreting T cells in the bone marrow could be indicative of a functional role for these immunosuppressive cells at this site, such as the prevention of immunopathology caused by the pro-inflammatory T cells which were also present. Further work should seek to address this question as the results could have implications for the feasibility of any intervention that targets inhibitory cytokines or cells at this site.

Initial additional experiments should include more donors, and test paired tissue/blood samples that have been isolated from the same time points to validate the findings presented here. T cells should also be sampled from additional tissues to enable a greater degree of comparison between sites, especially from tissues serving as potential entry routes during transmission, such as oral and lung. In MCMV infection, for example, CD4+ and CD8+ $T_{RM}$ cells are induced in the salivary gland, of which the CD8+ T cells provided immediate protection against re-infection [460]. Furthermore, future research could attempt to determine the phenotypes of HCMV-specific T cells found in the bone marrow and other tissue sites. Phenotyping of such T cells to identify if they are truly resident memory T cells, using well

defined markers such as CD69 and CD103 [465], and detecting IL-10 production from these cells, will be important in elucidating the role of virally induced immunomodulation, and the role of HCMV-specific T cells, at these sites. Building on the research presented here, further work is therefore required to increase our understanding of the immunobiology of HCMV, which could inform attempts to target the virus and lead to the ability to remove latently infected cells in a clinical setting.

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# Appendix A

# T cell responses grouped by sex

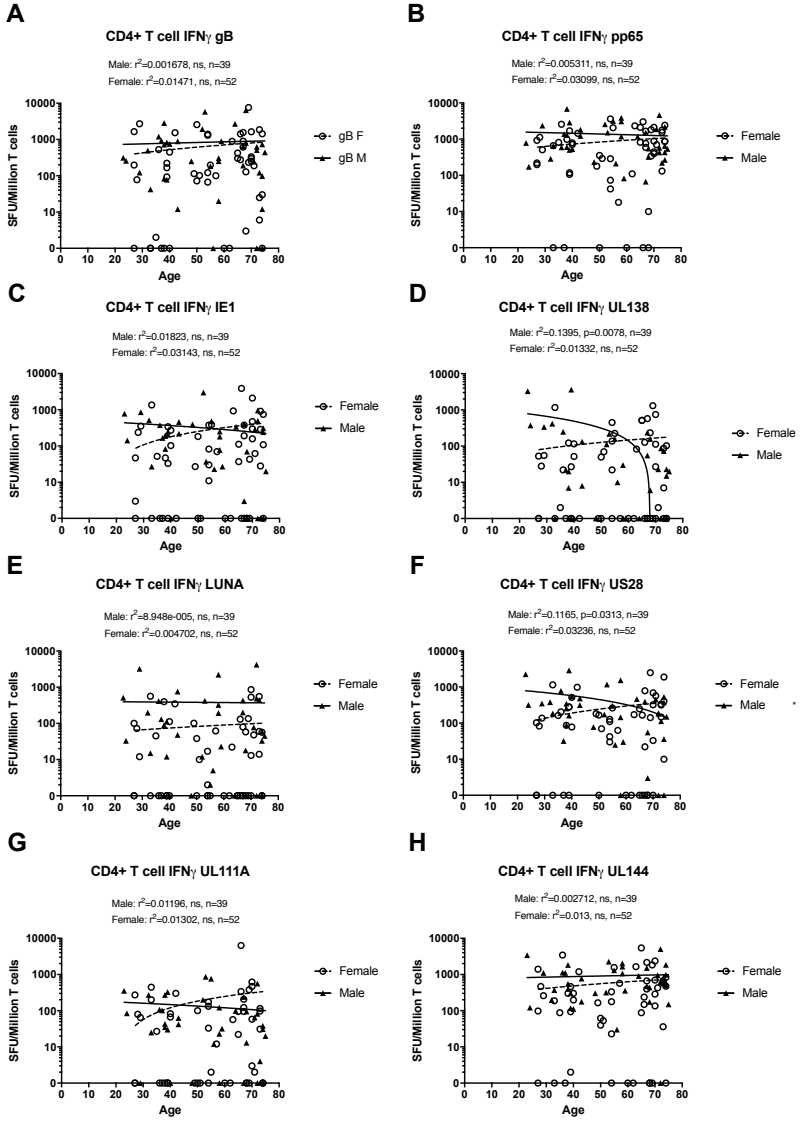


**Fig. A.1 Analysis of the magnitudes of CD4+ T cell IFN$\gamma$ responses to 8 HCMV ORF peptide pools correlated with donor age after donors have been separated by sex.** CD8+ depleted PBMC from 91 seropositive donors were stimulated with HCMV ORF peptide pools for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates and the frequencies of CD4+ T cells secreting IFN$\gamma$ within each seropositive donor enumerated and converted to Spot Forming Units (SFU)/$10^6$ cells. The SFU/$10^6$ cells of IFN$\gamma$ secreting CD4+ T cells is shown against donor age for responses to (A) gB, (B) pp65, (C) IE1, (D) UL138, (E) LUNA, (F) US28, (G) UL111A, and (H) UL144 with male responses illustrated by the filled triangles (▲) and female responses with open circles (○). The correlation (Spearman $r_s$ values and p values shown on each graph), and line of best fit are shown. Spearman rank correlation analysis of these responses indicates that CD4+ IFN$\gamma$ T cell responses to (F) US28 from male donors significantly decrease with donor age ($r_s$ = -0.3454, $P$ = 0.0313).

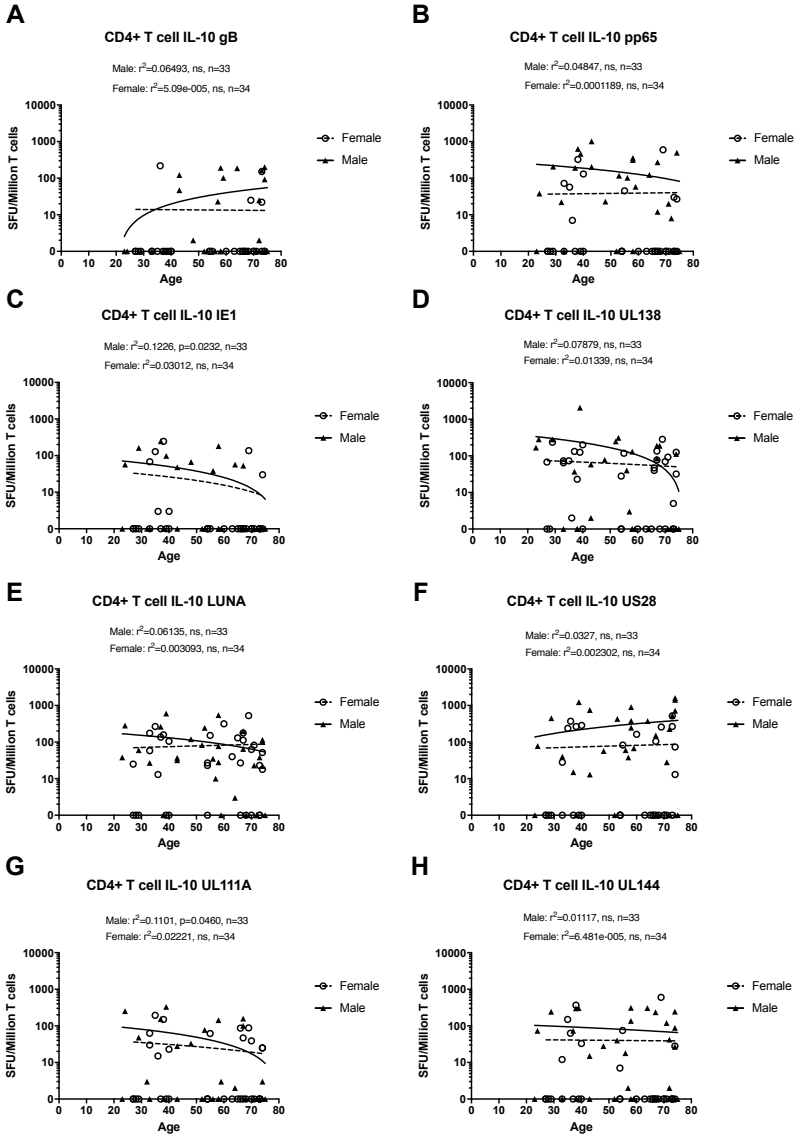


**Fig. A.2 Analysis of the magnitudes of CD4+ T cell IL-10 responses to 8 HCMV ORF peptide pools correlated with donor age after donors have been separated by sex.** CD8+ depleted PBMC from 68 seropositive donors were stimulated with HCMV ORF peptide pools for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates and the frequencies of CD4+ T cells secreting IL-10 within each seropositive donor enumerated and converted to Spot Forming Units (SFU)/$10^6$ cells. The SFU/$10^6$ cells of IL-10 secreting CD4+ T cells is shown against donor age for responses to (A) gB, (B) pp65, (C) IE1, (D) UL138, (E) LUNA, (F) US28, (G) UL111A, and (H) UL144 with male responses illustrated by the filled triangles (▲) and female responses with open circles (○). The correlation (Spearman $r_s$ values and p values shown on each graph), and line of best fit are shown. There were significant negative correlations in male donors between the magnitude of CD4+ T cells secreting IL-10 and age in response to (C) IE1 ($r_s$ = -0.3941, $P$ = 0.0232), (D) UL138 ($r_s$ = -0.4553, $P$ = 0.0078), and (G) UL111A ($r_s$ = -0.3498, $P$ = 0.0460) by Spearman rank correlation analysis.

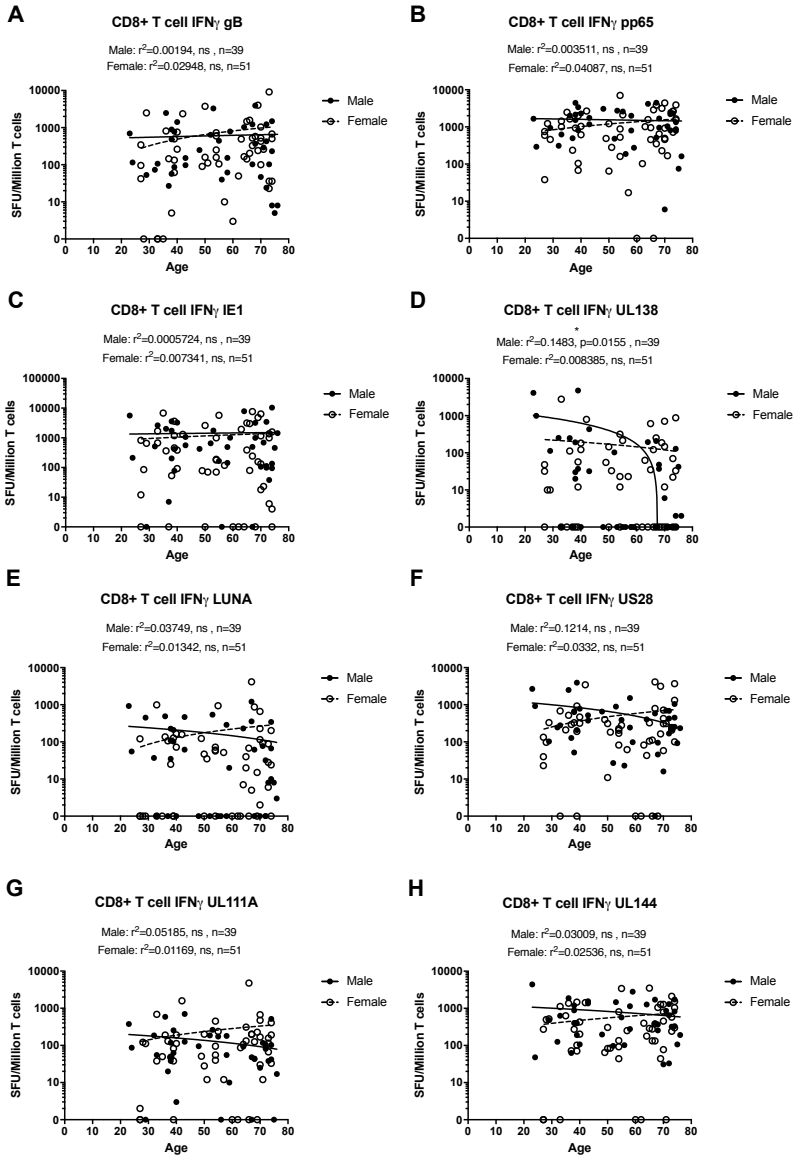


**Fig. A.3 Analysis of the magnitudes of CD8+ T cell IFN$\gamma$ responses to 8 HCMV ORF peptide pools correlated with donor age after donors have been separated by sex.** CD4+ depleted PBMC from 91 seropositive donors were stimulated with HCMV ORF peptide pools for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates and the frequencies of CD8+ T cells secreting IFN$\gamma$ within each seropositive donor enumerated and converted to Spot Forming Units (SFU)/$10^6$ cells. The SFU/$10^6$ cells of IFN$\gamma$ secreting CD4+ T cells is shown against donor age for responses to (A) gB, (B) pp65, (C) IE1, (D) UL138, (E) LUNA, (F) US28, (G) UL111A, and (H) UL144 with male responses illustrated by the filled triangles (▲) and female responses with open circles (○). The correlation (Spearman $r_s$ values and p values shown on each graph), and line of best fit are shown. A significant negative correlation between the frequency of IFN$\gamma$ secreting CD8+ T cells and increasing donor age in response to (D) UL138 in male donors was observed (Spearman $r_s$ = -0.3805, $P$ = 0.0169).

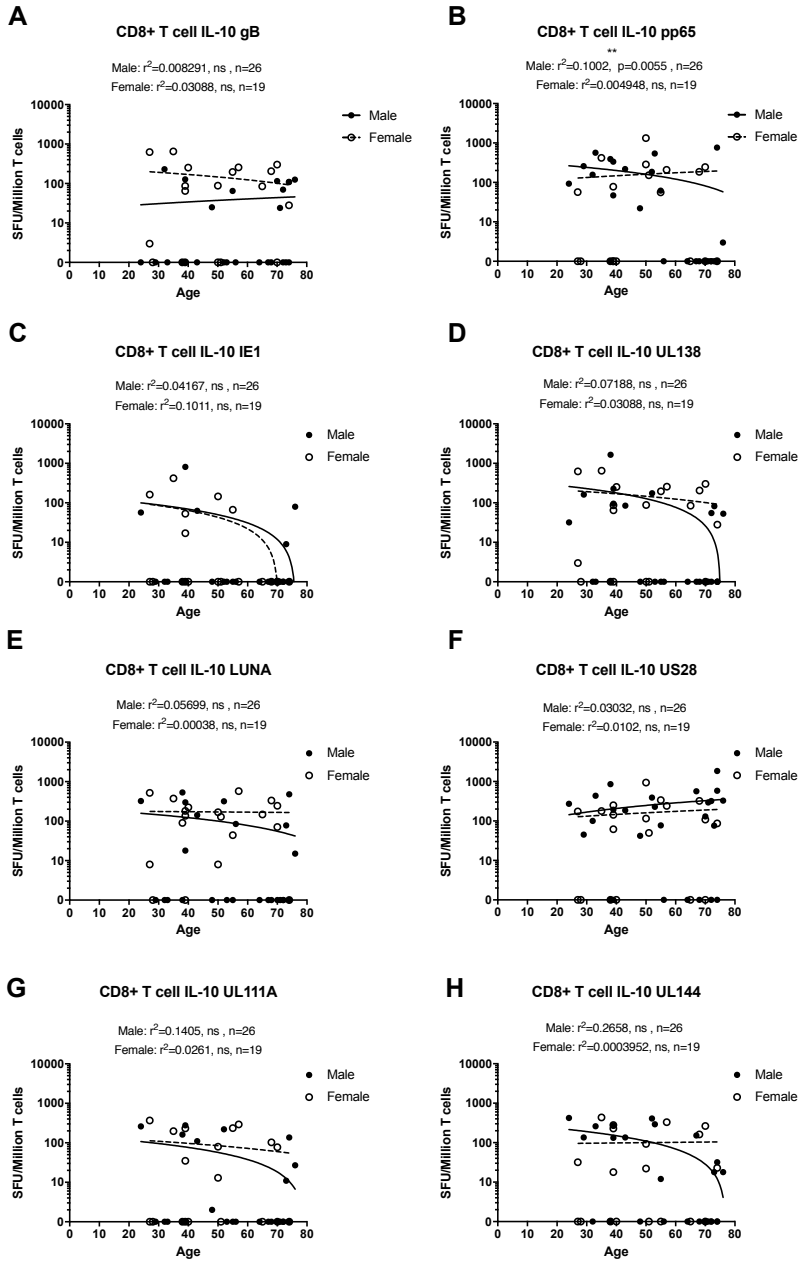


**Fig. A.4 Analysis of the magnitudes of CD8+ T cell IL-10 responses to 8 HCMV ORF peptide pools correlated with donor age after donors have been separated by sex.** CD4+ depleted PBMC from 45 seropositive donors were stimulated with HCMV ORF peptide pools for 48 hours on dual IFN$\gamma$/IL-10 FluoroSpot plates and the frequencies of CD8+ T cells secreting IFN$\gamma$ within each seropositive donor enumerated and converted to Spot Forming Units (SFU)/$10^6$ cells. The SFU/$10^6$ cells of IFN$\gamma$ secreting CD4+ T cells is shown against donor age for responses to (A) gB, (B) pp65, (C) IE1, (D) UL138, (E) LUNA, (F) US28, (G) UL111A, and (H) UL144 with male responses illustrated by the filled triangles (▲) and female responses with open circles (○). The correlation (Spearman $r_s$ values and p values shown on each graph), and line of best fit are shown. There was a significant negative correlation between CD8+ T cell IL-10 responses and increasing donor age in response to (B) pp65 in male donors (Spearman $r_s$ = -0.5288, $P$ = 0.0055).

# Appendix B

# Cytokine secretion inhibition assay supplementary data

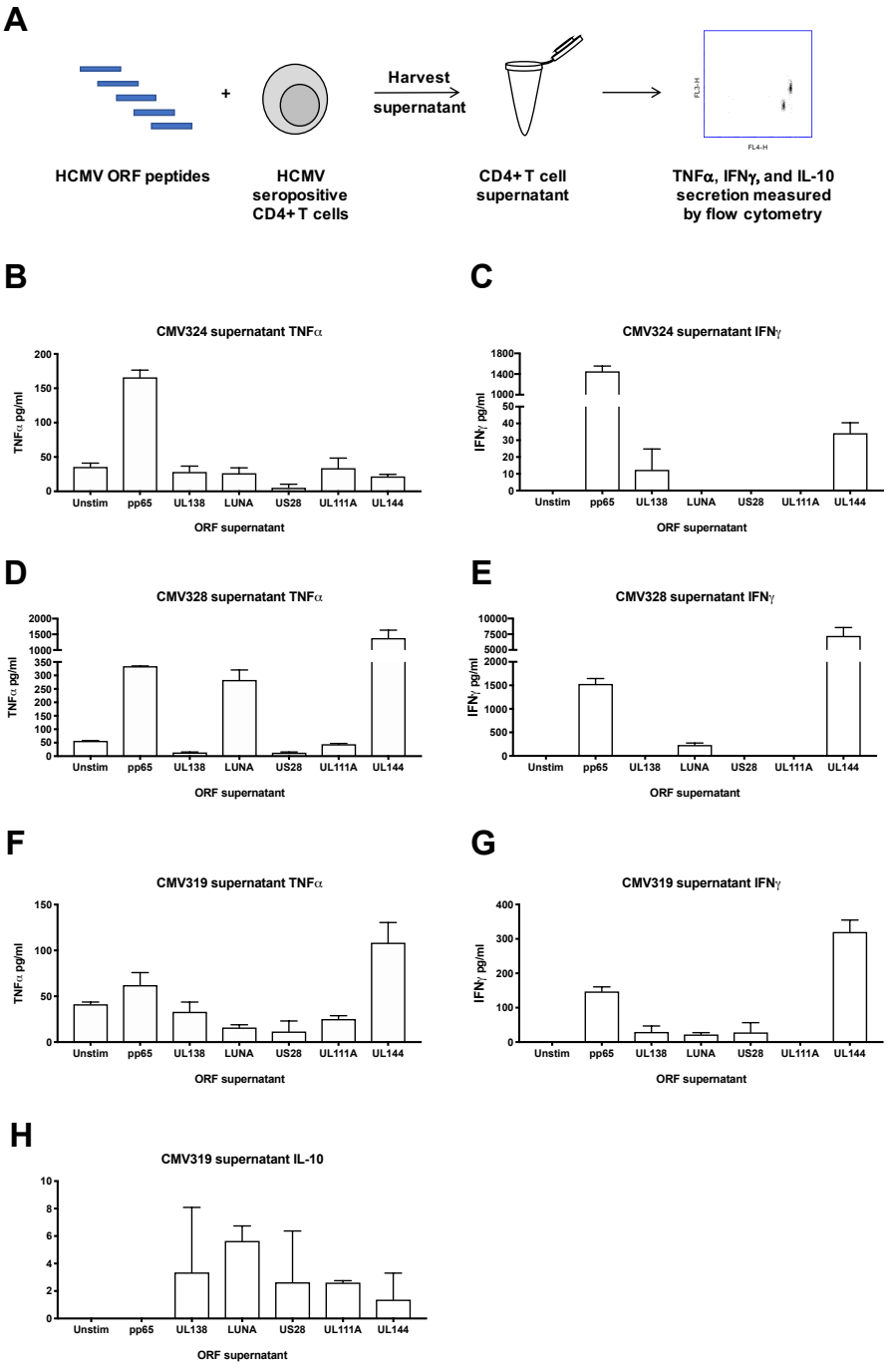


**Fig. B.1 Secretion of IFN$\gamma$, TNF$\alpha$, and IL-10 from CD4+ T cells stimulated with HCMV ORF peptide pools** (A) PBMC depleted of CD8+ cells from donors CMV324, CMV328, and CMV319 were stimulated with overlapping HCMV ORF peptide pools spanning pp65, UL138, LUNA, US28, UL111A, and UL144, or left unstimulated (Unstim) in media, for 48 hours. Following this incubation, supernatants were harvested and assayed for the secretion of (B, D, F) TNF$\alpha$, (C, E, G) IFN$\gamma$, and (H) IL-10 by flow cytometry. Samples were tested in duplicate.

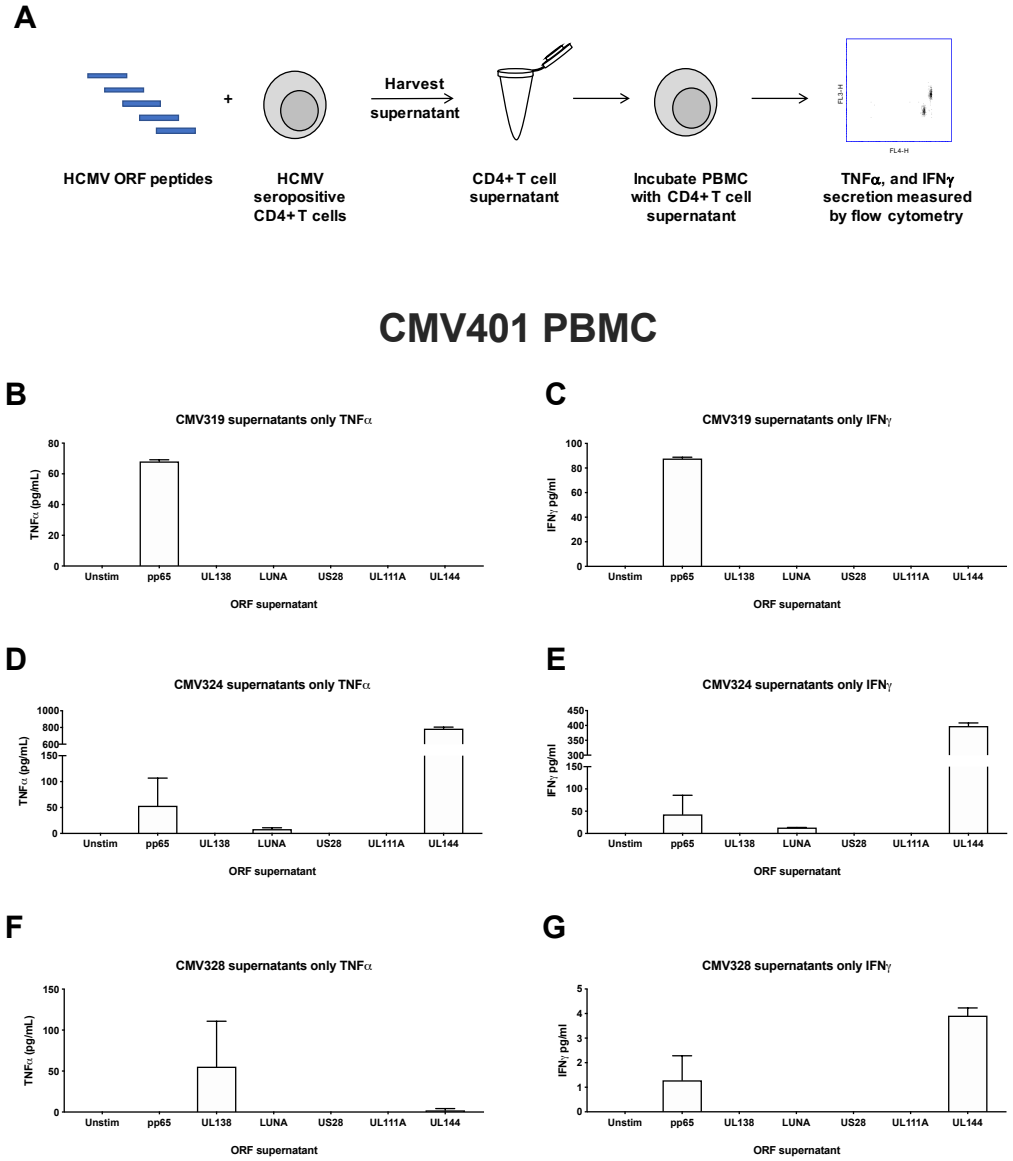


**Fig. B.2 IFN$\gamma$ and TNF$\alpha$ secretion by PBMC following incubation with supernatant derived from CD4+ T cells stimulated with HCMV ORF peptide pools** (A) PBMC depleted of CD8+ cells from donors CMV328, CMV324, and CMV319 were stimulated with overlapping HCMV ORF peptide pools spanning pp65, UL138, LUNA, US28, UL111A, and UL144, or left unstimulated (Unstim) in media, for 42 hours. Following this incubation, the supernatants were harvested. PBMC from donors CMV401 were then incubated in these supernatants for 36 hours and assayed for the secretion of (B, D, F) TNF$\alpha$ and (C, E, G) IFN$\gamma$. Samples were tested in duplicate.

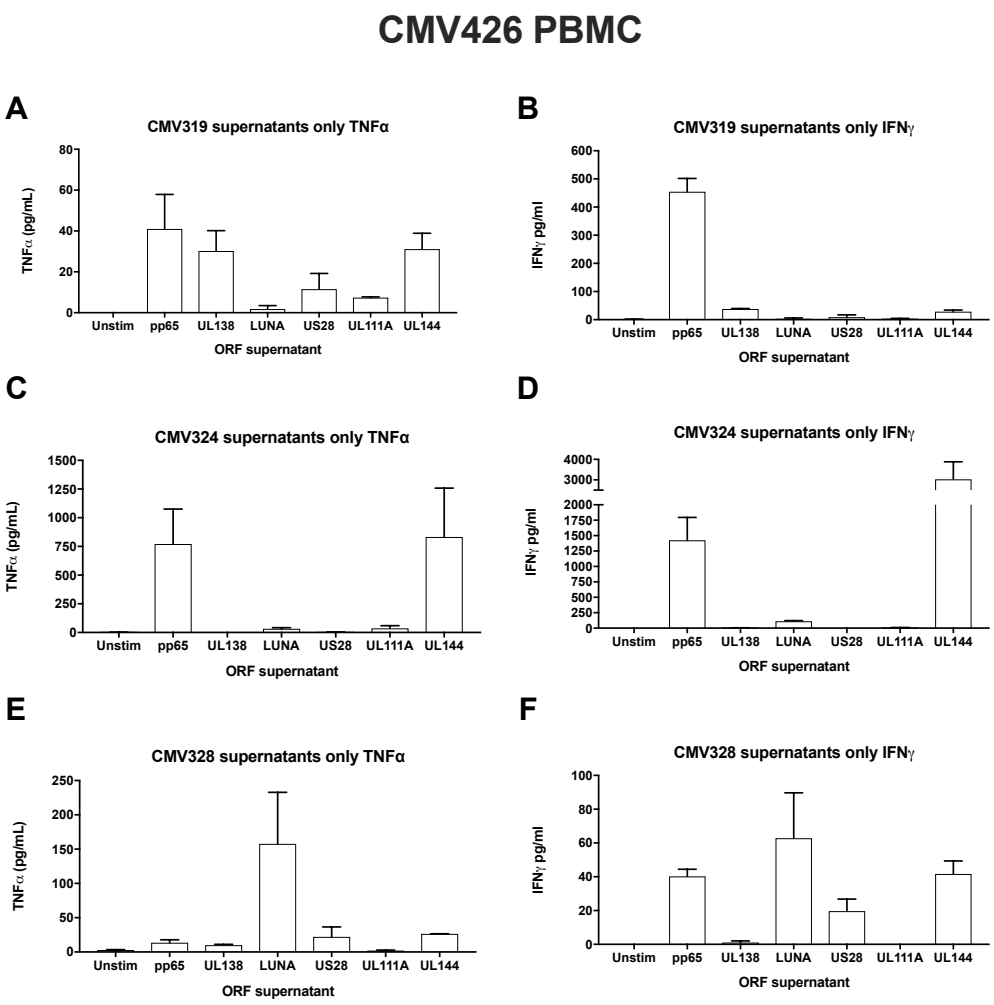


**Fig. B.3 IFN$\gamma$ and TNF$\alpha$ secretion by PBMC following incubation with supernatant derived from CD4+ T cells stimulated with HCMV ORF peptide pools** PBMC depleted of CD8+ cells from donors CMV328, CMV324, and CMV319 were stimulated with overlapping HCMV ORF peptide pools spanning pp65, UL138, LUNA, US28, UL111A, and UL144, or left unstimulated (Unstim) in media, for 42 hours. Following this incubation, the supernatants were harvested. PBMC from donors CMV426 were then incubated in these supernatants for 36 hours and assayed for the secretion of (A, C, E) TNF$\alpha$ and (B, D, F) IFN$\gamma$. Samples were tested in duplicate.

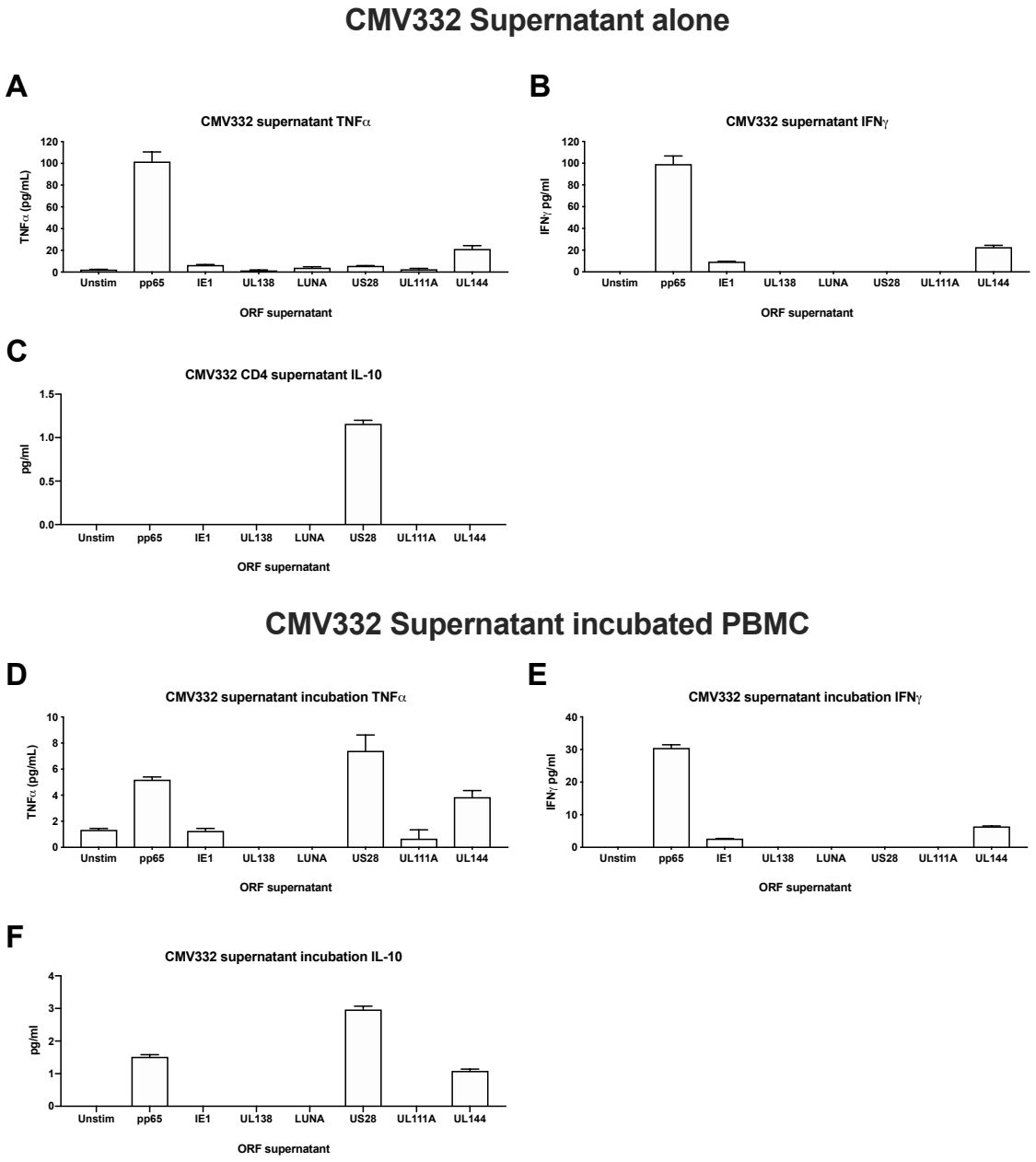


**Fig. B.4 IFN$\gamma$ and TNF$\alpha$ secretion by PBMC following incubation with supernatant derived from CD4+ T cells stimulated with HCMV ORF peptide pools** PBMC depleted of CD8+ cells from donor CMV332 were stimulated with overlapping HCMV ORF peptide pools spanning pp65, UL138, LUNA, US28, UL111A, and UL144, or left unstimulated (Unstim) in media, for 48 hours. Following this incubation, the supernatants were harvested and assessed for the secretion of (A) TNF$\alpha$, (B) IFN$\gamma$, and (IL-10) by a flow cytometry based bead array. PBMC from donor CMV332 were then incubated in these supernatants for 36 hours and assayed for the secretion of (D) TNF$\alpha$, (E) IFN$\gamma$, and (F) IL-10 by a flow cytometry based bead array. Samples were tested in duplicate.

# Appendix C

# Latent infection of monocytes with TB40e-UL32-GFP virus

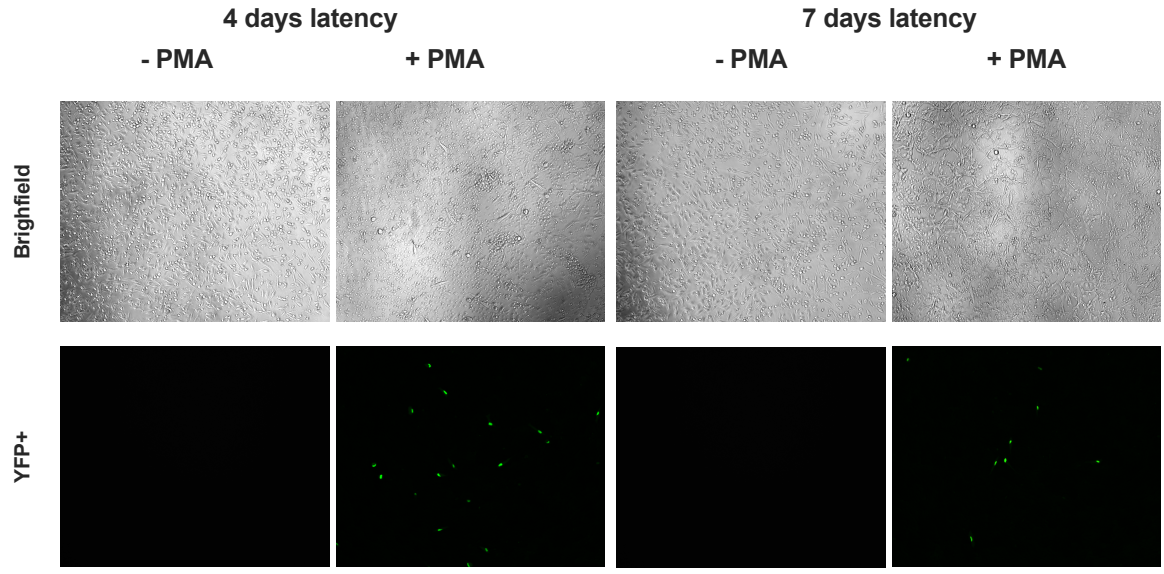


**Fig. C.1 Reactivation of HCMV following PMA treatment.** CD14+ monocytes were latently infected with TB40e-IE2-YFP for a period of 4 or 8 days before the addition of 20ng/ml Phorbol 12-myristate 13-acetate (PMA) for 48 hours. Infected monocytes were also left untreated. YFP+ cells were then examined by microscopy.

# Appendix D

# Donor data

| Donor ID | Age | Sex | HCMV Serostatus | Donor ID | Age | Sex | HCMV Serostatus |
|---|---|---|---|---|---|---|---|
| CMV324 | 23 | Male | Positive | ARIA014 | 67 | Male | Positive |
| CMV323 | 24 | Male | Positive | ARIA034 | 67 | Male | Positive |
| ARIA133 | 29 | Male | Positive | ARIA002 | 68 | Male | Positive |
| CMV321 | 32 | Male | Positive | ARIA067 | 68 | Male | Positive |
| ARIA126 | 33 | Male | Positive | ARIA086 | 70 | Male | Positive |
| ARIA063 | 36 | Male | Positive | ARIA127 | 71 | Male | Positive |
| ARIA148 | 37 | Male | Positive | ARIA012 | 72 | Male | Positive |
| ARIA146 | 38 | Male | Positive | ARIA124 | 72 | Male | Positive |
| ARIA150 | 38 | Male | Positive | ARIA139 | 72 | Male | Positive |
| ARIA161 | 38 | Male | Positive | ARIA123 | 73 | Male | Positive |
| CMV319 | 39 | Male | Positive | ARIA120 | 74 | Male | Positive |
| ARIA147 | 39 | Male | Positive | ARIA121 | 74 | Male | Positive |
| ARIA039 | 40 | Male | Positive | ARIA122 | 74 | Male | Positive |
| ARIA152 | 40 | Male | Positive | ARIA128 | 74 | Male | Positive |
| ARIA046 | 43 | Male | Positive | ARIA142 | 75 | Male | Positive |
| CMV307 | 43 | Male | Positive | ARIA140 | 76 | Male | Positive |
| ARIA005 | 46 | Male | Positive | ARIA160 | 38 | Male | Negative |
| ARIA062 | 48 | Male | Positive | ARIA125 | 38 | Male | Negative |
| ARIA044 | 52 | Male | Positive | ARIA049 | 40 | Male | Negative |
| ARIA069 | 53 | Male | Positive | ARIA087 | 45 | Male | Negative |
| ARIA061 | 55 | Male | Positive | ARIA149 | 51 | Male | Negative |

| Donor ID | Age | Sex | HCMV Serostatus | Donor ID | Age | Sex | HCMV Serostatus |
|---|---|---|---|---|---|---|---|
| ARIA137 | 56 | Male | Positive | ARIA075 | 55 | Male | Negative |
| ARIA132 | 58 | Male | Positive | ARIA053 | 68 | Male | Negative |
| ARIA141 | 58 | Male | Positive | ARIA020 | 71 | Male | Negative |
| ARIA158 | 58 | Male | Positive | ARIA155 | 72 | Male | Negative |
| CMV301 | 59 | Male | Positive | ARIA023 | 72 | Male | Negative |
| ARIA065 | 62 | Male | Positive | ARIA118 | 78 | Male | Negative |
| ARIA151 | 64 | Male | Positive | ARIA024 | 65 | Female | Positive |
| ARIA017 | 27 | Female | Positive | ARIA025 | 65 | Female | Positive |
| ARIA032 | 27 | Female | Positive | ARIA003 | 66 | Female | Positive |
| ARIA088 | 27 | Female | Positive | ARIA031 | 66 | Female | Positive |
| ARIA145 | 28 | Female | Positive | ARIA064 | 66 | Female | Positive |
| ARIA043 | 29 | Female | Positive | ARIA016 | 67 | Female | Positive |
| ARIA144 | 32 | Female | Positive | ARIA019 | 67 | Female | Positive |
| ARIA045 | 33 | Female | Positive | ARIA042 | 67 | Female | Positive |
| ARIA055 | 33 | Female | Positive | ARIA033 | 68 | Female | Positive |
| ARIA131 | 35 | Female | Positive | ARIA013 | 69 | Female | Positive |
| ARIA119 | 36 | Female | Positive | ARIA018 | 69 | Female | Positive |
| ARIA072 | 37 | Female | Positive | ARIA037 | 69 | Female | Positive |
| ARIA130 | 38 | Female | Positive | ARIA073 | 69 | Female | Positive |
| ARIA006 | 39 | Female | Positive | ARIA028 | 70 | Female | Positive |
| ARIA066 | 39 | Female | Positive | ARIA035 | 70 | Female | Positive |
| ARIA081 | 39 | Female | Positive | ARIA054 | 70 | Female | Positive |
| ARIA089 | 39 | Female | Positive | ARIA079 | 70 | Female | Positive |
| ARIA091 | 39 | Female | Positive | ARIA071 | 71 | Female | Positive |
| ARIA047 | 40 | Female | Positive | ARIA011 | 73 | Female | Positive |
| ARIA134 | 40 | Female | Positive | ARIA138 | 73 | Female | Positive |
| ARIA052 | 42 | Female | Positive | ARIA143 | 73 | Female | Positive |
| ARIA076 | 49 | Female | Positive | ARIA051 | 74 | Female | Positive |
| ARIA026 | 50 | Female | Positive | ARIA070 | 74 | Female | Positive |
| ARIA084 | 50 | Female | Positive | ARIA136 | 74 | Female | Positive |
| ARIA080 | 51 | Female | Positive | ARIA159 | 29 | Female | Negative |
| ARIA004 | 54 | Female | Positive | ARIA040 | 32 | Female | Negative |
| ARIA010 | 54 | Female | Positive | ARIA030 | 37 | Female | Negative |
| ARIA021 | 54 | Female | Positive | ARIA068 | 39 | Female | Negative |

| Donor ID | Age | Sex | HCMV Serostatus | Donor ID | Age | Sex | HCMV Serostatus |
|---|---|---|---|---|---|---|---|
| ARIA029 | 54 | Female | Positive | ARIA082 | 48 | Female | Negative |
| ARIA078 | 54 | Female | Positive | ARIA036 | 48 | Female | Negative |
| ARIA015 | 55 | Female | Positive | ARIA154 | 48 | Female | Negative |
| CMV320 | 57 | Female | Positive | ARIA083 | 58 | Female | Negative |
| ARIA008 | 60 | Female | Positive | ARIA007 | 60 | Female | Negative |
| ARIA074 | 60 | Female | Positive | ARIA056 | 66 | Female | Negative |
| ARIA048 | 62 | Female | Positive | ARIA156 | 73 | Female | Negative |
| ARIA022 | 63 | Female | Positive | ARIA157 | 73 | Female | Negative |
| ARIA085 | 64 | Female | Positive | ARIA038 | 75 | Female | Negative |

**Table D.1 Donor data.** Serum from 128 healthy donors was tested for HCMV serostatus by CMV IgG ELISA and grouped according to age and sex.

# Appendix E

# Peptide library sequences

## E.1 UL138

| Peptide Number | Peptide Sequence | Peptide Number | Peptide Sequence |
|---|---|---|---|
| 1 | MDDLPLNVGLPIIGV | 17 | RFSERPDEILVRWEE |
| 2 | LNVGLPIIGVMLVLI | 18 | PDEILVRWEEVSSQC |
| 3 | PIIGVMLVLIVAILC | 19 | VRWEEVSSQCSYASS |
| 4 | MLVLIVAILCYLAYH | 20 | VSSQCSYASSRITDR |
| 5 | VAILCYLAYHWHDTF | 21 | SYASSRITDRRVGSS |
| 6 | YLAYHWHDTFKLVRM | 22 | RITDRRVGSSSSSSV |
| 7 | WHDTFKLVRMFLSYR | 23 | RVGSSSSSSVHVASQ |
| 8 | KLVRMFLSYRWLIRC | 24 | SSSSVHVASQRNSVP |
| 9 | FLSYRWLIRCCELYG | 25 | HVASQRNSVPPPDMA |
| 10 | WLIRCCELYGEYERR | 26 | RNSVPPPDMAVTAPL |
| 11 | CELYGEYERRFADLS | 27 | PPDMAVTAPLTDVDL |
| 12 | EYERRFADLSSLGLG | 28 | VTAPLTDVDLLKPVT |
| 13 | FADLSSLGLGAVRRE | 29 | TDVDLLKPVTGSATQ |
| 14 | SLGLGAVRRESDRRY | 30 | LKPVTGSATQFTTVA |
| 15 | AVRRESDRRYRFSER | 31 | GSATQFTTVAMVHYH |
| 16 | SDRRYRFSERPDEIL | 32 | QFTTVAMVHYHQEYT |

**Table E.1** Amino acid sequences of individual overlapping 15mer peptides spanning the predicted UL138 ORF

## E.2 LUNA

| Peptide Number | Peptide Sequence | Peptide Number | Peptide Sequence |
|---|---|---|---|
| 1 | MTSVRAPLLPLRRLC | 14 | SSSSVGGNPGDCGRN |
| 2 | APLLPLRRLCPVRIS | 15 | GGNPGDCGRNSETAP |
| 3 | LRRLCPVRISARDSP | 16 | DCGRNSETAPRMTLL |
| 4 | PVRISARDSPAWVSE | 17 | SETAPRMTLLRGKRP |
| 5 | ARDSPAWVSESSSPL | 18 | RMTLLRGKRPARSCT |
| 6 | AWVSESSSPLASSKP | 19 | RGKRPARSCTWGRLI |
| 7 | SSSPLASSKPANMAS | 20 | ARSCTWGRLILSGLP |
| 8 | ASSKPANMASDRGVG | 21 | WGRLILSGLPGVRVQ |
| 9 | ANMASDRGVGVGVEE | 22 | LSGLPGVRVQNPRRK |
| 10 | DRGVGVGVEERSSSS | 23 | GVRVQNPRRKKWMRP |
| 11 | VGVEERSSSSSSSSS | 24 | NPRRKKWMRPSGCRC |
| 12 | RSSSSSSSSSSSSSV | 25 | RRKKWMRPSGCRCSK |
| 13 | SSSSSSSSSVGGNPG | | |

**Table E.2** Amino acid sequences of individual overlapping 15mer peptides spanning the predicted LUNA ORF

## E.3 US28

| Peptide Number | Peptide Sequence | Peptide Number | Peptide Sequence |
|---|---|---|---|
| 1 | MTPTTTTAELTTEFD | 36 | DYDYLEVSYPIILNV |
| 2 | TTAELTTEFDYDEDA | 37 | EVSYPIILNVELMLG |
| 3 | TTEFDYDEDATPCVF | 38 | IILNVELMLGAFVIP |
| 4 | YDEDATPCVFTDVLN | 39 | ELMLGAFVIPLSVIS |
| 5 | TPCVFTDVLNQSKPV | 40 | AFVIPLSVISYCYYR |
| 6 | TDVLNQSKPVTLFLY | 41 | LSVISYCYYRISRIV |
| 7 | QSKPVTLFLYGVVFL | 42 | YCYYRISRIVAVSQS |
| 8 | TLFLYGVVFLFGSIG | 43 | ISRIVAVSQSRHKGR |
| 9 | GVVFLFGSIGNFLVI | 44 | AVSQSRHKGRIVRVL |
| 10 | FGSIGNFLVIFTITW | 45 | RHKGRIVRVLIAVVL |
| 11 | NFLVIFTITWRRRIQ | 46 | IVRVLIAVVLVFIIF |
| 12 | FTITWRRRIQCSGDV | 47 | IAVVLVFIIFWLPYH |
| 13 | RRRIQCSGDVYFINL | 48 | VFIIFWLPYHLTLFV |
| 14 | CSGDVYFINLAAADL | 49 | WLPYHLTLFVDTLKL |
| 15 | YFINLAAADLLFVCT | 50 | LTLFVDTLKLLKWIS |
| 16 | AAADLLFVCTLPLWM | 51 | DTLKLLKWISSSCEF |
| 17 | LFVCTLPLWMQYLLD | 52 | LKWISSSCEFERSLK |
| 18 | LPLWMQYLLDHNSLA | 53 | SSCEFERSLKRALIL |
| 19 | QYLLDHNSLASVPCT | 54 | ERSLKRALILTESLA |
| 20 | HNSLASVPCTLLTAC | 55 | RALILTESLAFCHCC |
| 21 | SVPCTLLTACFYVAM | 56 | TESLAFCHCCLNPLL |
| 22 | LLTACFYVAMFASLC | 57 | FCHCCLNPLLYVFVG |
| 23 | FYVAMFASLCFITEI | 58 | LNPLLYVFVGTKFRQ |
| 24 | FASLCFITEIALDRY | 59 | YVFVGTKFRQELHCL |
| 25 | FITEIALDRYYAIVY | 60 | TKFRQELHCLLAEFR |
| 26 | ALDRYYAIVYMRYRP | 61 | ELHCLLAEFRQRLFS |
| 27 | YAIVYMRYRPVKQAC | 62 | LAEFRQRLFSRDVSW |
| 28 | MRYRPVKQACLFSIF | 63 | QRLFSRDVSWYHSMS |
| 29 | VKQACLFSIFWWIFA | 64 | RDVSWYHSMSFSRRG |
| 30 | LFSIFWWIFAVIIAI | 65 | YHSMSFSRRGSPSRR |

| | | | |
|---|---|---|---|
| 31 | WWIFAVIIAIPHFMV | 66 | FSRRGSPSRRETSSD |
| 32 | VIIAIPHFMVVTKKD | 67 | SPSRRETSSDTLSDE |
| 33 | PHFMVVTKKDNQCMT | 68 | ETSSDTLSDEVCRVS |
| 34 | VTKKDNQCMTDYDYL | 69 | DTLSDEVCRVSQIIP |
| 35 | NQCMTDYDYLEVSYP | | |

**Table E.3** Amino acid sequences of individual overlapping 15mer peptides spanning the predicted US28 ORF

## E.4 UL111A

| Peptide Number | Peptide Sequence | Peptide Number | Peptide Sequence |
|---|---|---|---|
| 1 | MLSVMVSSSLVLIVF | 19 | RYLEIVFPAGDHVYP |
| 2 | VSSSLVLIVFFLGAS | 20 | VFPAGDHVYPGLKTE |
| 3 | VLIVFFLGASEEAKP | 21 | DHVYPGLKTELHSMR |
| 4 | FLGASEEAKPATTTT | 22 | GLKTELHSMRSTLES |
| 5 | EEAKPATTTTIKNTK | 23 | LHSMRSTLESIYKDM |
| 6 | ATTTTIKNTKPQCRP | 24 | STLESIYKDMRQCPL |
| 7 | IKNTKPQCRPEDYAT | 25 | IYKDMRQCPLLGCGD |
| 8 | PQCRPEDYATRLQDL | 26 | RQCPLLGCGDKSVIS |
| 9 | EDYATRLQDLRVTFH | 27 | LGCGDKSVISRLSQE |
| 10 | RLQDLRVTFHRVKPT | 28 | KSVISRLSQEAERKS |
| 11 | RVTFHRVKPTLQRED | 29 | RLSQEAERKSDNGTR |
| 12 | RVKPTLQREDDYSVW | 30 | AERKSDNGTRKGLSE |
| 13 | LQREDDYSVWLDGTV | 31 | DNGTRKGLSELDTLF |
| 14 | DYSVWLDGTVVKGCW | 32 | KGLSELDTLFSRLEE |
| 15 | LDGTVVKGCWGCSVM | 33 | LDTLFSRLEEYLHSR |
| 16 | VKGCWGCSVMDWLLR | 34 | DTLFSRLEEYLHSRK |
| 17 | GCSVMDWLLRRYLEI | 35 | RQCVSVSVAALSAQR |
| 18 | DWLLRRYLEIVFPAG | | |

**Table E.4** Amino acid sequences of individual overlapping 15mer peptides spanning the predicted UL111A ORF

## E.5 UL144

| Peptide Number | Peptide Sequence | Peptide Number | Peptide Sequence |
|---|---|---|---|
| 1 | MKPLVMLICFGVILL | 43 | SGLYNCTNCTECNDT |
| 2 | MKPLVMLILLSMLLA | 44 | TGLYNCTNCTQCNDT |
| 3 | MKPLIMLICFAVILL | 45 | CTDCTQCNVTQVMIR |
| 4 | MLICFGVILLQLGVT | 46 | CTNCTECNDTEVTIR |
| 5 | MLILLSMLLACIGKT | 47 | CTNCTQCNDTQITVR |
| 6 | MLICFGVFLLQLGGS | 48 | QCNVTQVMIRNCTST |
| 7 | GVILLQLGVTKVCQ OH | 49 | ECNDTEVTIRNCTST |
| 8 | SMLLACIGKTEICKP | 50 | QCNDTQITVRNCTST |
| 9 | GVFLLQLGGSKMCKP | 51 | QVMIRNCTSTNNTVC |
| 10 | QLGVTKVCQHNEVQL | 52 | EVTIRNCTSTNNTVC |
| 11 | CIGKTEICKPEEVQL | 53 | QITVRNCTSTNNTIC |
| 12 | QLGGSKMCKPDEVKL | 54 | NCTSTNNTVCAPKN OH |
| 13 | KVCQHNEVQLGNECC | 55 | NCTSTNNTVCASKNY |
| 14 | EICKPEEVQLGNQCC | 56 | NCTSTNNTICASKN OH |
| 15 | KMCKPDEVKLGNQCC | 57 | NNTVCAPKNHTYFST |
| 16 | NEVQLGNECCPPCGL | 58 | NNTVCASKNYTSFSV |
| 17 | EEVQLGNQCCPPCKQ | 59 | NNTICASKNHTSFSS |
| 18 | DEVKLGNQCCPPCGS | 60 | APKNHTYFSTPGVQ OH |
| 19 | GNECCPPCGLGQRVT | 61 | ASKNYTSLSVPGVQ OH |
| 20 | GNQCCPPCKQGYRVT | 62 | ASKNYTSFSISGVQ OH |
| 21 | PPCGLGQRVTKVCTE | 63 | ASKNHTSFSSPGVQ OH |
| 22 | PPCKQGYRVTGQCTQ | 64 | TYFSTPGVQHHKQRQ |
| 23 | PPCGSGQRVTKVCTD | 65 | TSLSVPGVQHHKQRQ |
| 24 | GQRVTKVCTERTSVT | 66 | PGVQHHKQRQQNHTA |
| 25 | GYRVTGQCTQYTSTT | 67 | PGVQHHKQRQNHTA |
| 26 | GQRVTKVCTDYTSVT | 68 | HKQRQQNHTAHITVK |
| 27 | GQKVTKVCTEISGIT | 69 | HKQRQNHTAHVTVK |
| 28 | KVCTERTSVTCTPCP | 70 | QNHTAHITVKQGKSG |
| 29 | GQCTQYTSTTCTLCP | 71 | NHTAHVTVKQGKSG |
| 30 | KVCTDYTSVTCTPCP | 72 | QNHTAHVTVKQRKSG |
| 31 | KVCTEISGITCTLCP | 73 | HITVKQGKSGRHTLA |

| | | | |
|---|---|---|---|
| 32 | RTSVTCTPCPNGTYV | 74 | HVTVKQGKSGRHTLA |
| 33 | YTSTTCTLCPNGTYV | 75 | HVTVKQRKSGRHTLA |
| 34 | YTSVTCTPCPNGTYV | 76 | QGKSGRHTLAWLSLF |
| 35 | ISGITCTLCPNGTYL | 77 | RHTLAWLSLFIFLVG |
| 36 | CTPCPNGTYVSGLYN | 78 | WLSLFIFLVGIILLI |
| 37 | CTLCPNGTYVSGLYN | 79 | IFLVGIILLILYLIA |
| 38 | CTLCPNGTYLTGLYN | 80 | IILLILYLIAAYRSE |
| 39 | NGTYVSGLYNCTDCT | 81 | LYLIAAYRSERCQQC |
| 40 | NGTYVSGLYNCTNCT | 82 | AYRSERCQQCCSIGK |
| 41 | NGTYLTGLYNCTNCT | 83 | RCQQCCSIGKIFYRT |
| 42 | SGLYNCTDCTQCNVT | 84 | CSIGKIFYRTL |

**Table E.5** Amino acid sequences of individual overlapping 15mer peptides spanning the predicted UL144 ORF

## E.6 gB

| Peptide Number | Peptide Sequence | Peptide Number | Peptide Sequence |
|---|---|---|---|
| 1 | MESRIWCLVVCVNLC | 91 | LNITHRTRRSTSDNN |
| 2 | WCLVVCVNLCIVCLG | 92 | RTRRSTSDNNTTHLS |
| 3 | CVNLCIVCLGAAVSS | 93 | TSDNNTTHLSSMESV |
| 4 | IVCLGAAVSSSSTSH | 94 | TTHLSSMESVHNLVY |
| 5 | AAVSSSSTSHATSST | 95 | SMESVHNLVYAQLQF |
| 6 | SSTSHATSSTHNGSH | 96 | HNLVYAQLQFTYDTL |
| 7 | ATSSTHNGSHTSRTT | 97 | AQLQFTYDTLRGYIN |
| 8 | HNGSHTSRTTSAQTR | 98 | TYDTLRGYINRALAQ |
| 9 | TSRTTSAQTRSVYSQ | 99 | RGYINRALAQIAEAW |
| 10 | SAQTRSVYSQHVTSS | 100 | RALAQIAEAWCVDQR |
| 11 | SVYSQHVTSSEAVSH | 101 | IAEAWCVDQRRTLEV |
| 12 | HVTSSEAVSHRANET | 102 | CVDQRRTLEVFKELS |
| 13 | EAVSHRANETIYNTT | 103 | RTLEVFKELSKINPS |
| 14 | RANETIYNTTLKYGD | 104 | FKELSKINPSAILSA |
| 15 | IYNTTLKYGDVVGVN | 105 | KINPSAILSAIYNKP |
| 16 | LKYGDVVGVNTTKYP | 106 | AILSAIYNKPIAARF |
| 17 | VVGVNTTKYPYRVCS | 107 | IYNKPIAARFMGDVL |
| 18 | TTKYPYRVCSMAQGT | 108 | IAARFMGDVLGLASC |
| 19 | YRVCSMAQGTDLIRF | 109 | MGDVLGLASCVTINQ |
| 20 | MAQGTDLIRFERNII | 110 | GLASCVTINQTSVKV |
| 21 | DLIRFERNIICTSMK | 111 | VTINQTSVKVLRDMN |
| 22 | ERNIICTSMKPINED | 112 | TSVKVLRDMNVKESP |
| 23 | CTSMKPINEDLDEGI | 113 | LRDMNVKESPGRCYS |
| 24 | PINEDLDEGIMVVYK | 114 | VKESPGRCYSRPVVI |
| 25 | LDEGIMVVYKRNIVA | 115 | GRCYSRPVVIFNFAN |
| 26 | MVVYKRNIVAHTFKV | 116 | RPVVIFNFANSSYVQ |
| 27 | RNIVAHTFKVRVYQK | 117 | FNFANSSYVQYGQLG |
| 28 | HTFKVRVYQKVLTFR | 118 | SSYVQYGQLGEDNEI |
| 29 | RVYQKVLTFRRSYAY | 119 | YGQLGEDNEILLGNH |
| 30 | VLTFRRSYAYIYTTY | 120 | EDNEILLGNHRTEEC |
| 31 | RSYAYIYTTYLLGSN | 121 | LLGNHRTEECQLPSL |

| | | | |
|---|---|---|---|
| 32 | IYTTYLLGSNTEYVA | 122 | RTEECQLPSLKIFIA |
| 33 | LLGSNTEYVAPPMWE | 123 | QLPSLKIFIAGNSAY |
| 34 | TEYVAPPMWEIHHIN | 124 | KIFIAGNSAYEYVDY |
| 35 | PPMWEIHHINKFAQC | 125 | GNSAYEYVDYLFKRM |
| 36 | IHHINKFAQCYSSYS | 126 | EYVDYLFKRMIDLSS |
| 37 | KFAQCYSSYSRVIGG | 127 | LFKRMIDLSSISTVD |
| 38 | YSSYSRVIGGTVFVA | 128 | IDLSSISTVDSMIAL |
| 39 | RVIGGTVFVAYHRDS | 129 | ISTVDSMIALDIDPL |
| 40 | TVFVAYHRDSYENKT | 130 | SMIALDIDPLENTDF |
| 41 | YHRDSYENKTMQLIP | 131 | DIDPLENTDFRVLEL |
| 42 | YENKTMQLIPDDYSN | 132 | ENTDFRVLELYSQKE |
| 43 | MQLIPDDYSNTHSTR | 133 | RVLELYSQKELRSSN |
| 44 | DDYSNTHSTRYVTVK | 134 | YSQKELRSSNVFDLE |
| 45 | THSTRYVTVKDQWHS | 135 | LRSSNVFDLEEIMRE |
| 46 | YVTVKDQWHSRGSTW | 136 | VFDLEEIMREFNSYK |
| 47 | DQWHSRGSTWLYRET | 137 | EIMREFNSYKQRVKY |
| 48 | RGSTWLYRETCNLNC | 138 | FNSYKQRVKYVEDKV |
| 49 | LYRETCNLNCMLTIT | 139 | QRVKYVEDKVVDPLP |
| 50 | CNLNCMLTITTARSK | 140 | VEDKVVDPLPPYLKG |
| 51 | MLTITTARSKYPYHF | 141 | VDPLPPYLKGLDDLM |
| 52 | TARSKYPYHFFATST | 142 | PYLKGLDDLMSGLGA |
| 53 | YPYHFFATSTGDVVY | 143 | LDDLMSGLGAAGKAV |
| 54 | FATSTGDVVYISPFY | 144 | SGLGAAGKAVGVAIG |
| 55 | GDVVYISPFYNGTNR | 145 | AGKAVGVAIGAVGGA |
| 56 | ISPFYNGTNRNASYF | 146 | GVAIGAVGGAVASVV |
| 57 | NGTNRNASYFGENAD | 147 | AVGGAVASVVEGVAT |
| 58 | NASYFGENADKFFIF | 148 | VASVVEGVATFLKNP |
| 59 | GENADKFFIFPNYTI | 149 | EGVATFLKNPFGAFT |
| 60 | KFFIFPNYTIVSDFG | 150 | FLKNPFGAFTIILVA |
| 61 | PNYTIVSDFGRPNAA | 151 | FGAFTIILVAIAVVI |
| 62 | VSDFGRPNAAPETHR | 152 | IILVAIAVVIITYLI |
| 63 | RPNAAPETHRLVAFL | 153 | IAVVIITYLIYTRQR |
| 64 | PETHRLVAFLERADS | 154 | ITYLIYTRQRRLCTQ |
| 65 | LVAFLERADSVISWD | 155 | YTRQRRLCTQPLQNL |
| 66 | ERADSVISWDIQDEK | 156 | RLCTQPLQNLFPYLV |
| 67 | VISWDIQDEKNVTCQ | 157 | PLQNLFPYLVSADGT |

| | | | |
|---|---|---|---|
| 68 | IQDEKNVTCQLTFWE | 158 | FPYLVSADGTTVTSG |
| 69 | NVTCQLTFWEASERT | 159 | SADGTTVTSGSTKDT |
| 70 | LTFWEASERTIRSEA | 160 | TVTSGSTKDTSLQAP |
| 71 | ASERTIRSEAEDSYH | 161 | STKDTSLQAPPSYEE |
| 72 | IRSEAEDSYHFSSAK | 162 | SLQAPPSYEESVYNS |
| 73 | EDSYHFSSAKMTATF | 163 | PSYEESVYNSGRKGP |
| 74 | FSSAKMTATFLSKKQ | 164 | SVYNSGRKGPGPPSS |
| 75 | MTATFLSKKQEVNMS | 165 | GRKGPGPPSSDASTA |
| 76 | LSKKQEVNMSDSALD | 166 | GPPSSDASTAAPPYT |
| 77 | EVNMSDSALDCVRDE | 167 | DASTAAPPYTNEQAY |
| 78 | DSALDCVRDEAINKL | 168 | APPYTNEQAYQMLLA |
| 79 | CVRDEAINKLQQIFN | 169 | NEQAYQMLLALARLD |
| 80 | AINKLQQIFNTSYNQ | 170 | QMLLALARLDAEQRA |
| 81 | QQIFNTSYNQTYEKY | 171 | LARLDAEQRAQQNGT |
| 82 | TSYNQTYEKYGNVSV | 172 | AEQRAQQNGTDSLDG |
| 83 | TYEKYGNVSVFETSG | 173 | QQNGTDSLDGQTGTQ |
| 84 | GNVSVFETSGGLVVF | 174 | DSLDGQTGTQDKGQK |
| 85 | FETSGGLVVFWQGIK | 175 | QTGTQDKGQKPNLLD |
| 86 | GLVVFWQGIKQKSLV | 176 | DKGQKPNLLDRLRHR |
| 87 | WQGIKQKSLVELERL | 177 | PNLLDRLRHRKNGYR |
| 88 | QKSLVELERLANRSS | 178 | RLRHRKNGYRHLKDS |
| 89 | ELERLANRSSLNITH | 179 | KNGYRHLKDSDEEEN |
| 90 | ANRSSLNITHRTRRS | 180 | NGYRHLKDSDEEENV |

**Table E.6** Amino acid sequences of individual overlapping 15mer peptides spanning the predicted gB ORF

## E.7 pp65

| Peptide Number | Peptide Sequence | Peptide Number | Peptide Sequence |
|---|---|---|---|
| 1 | MESRGRRCPEMISVL | 57 | IIKPGKISHIMLDVA |
| 2 | RRCPEMISVLGPISG | 58 | KISHIMLDVAFTSHE |
| 3 | MISVLGPISGHVLKA | 59 | MLDVAFTSHEHFGLL |
| 4 | GPISGHVLKAVFSRG | 60 | FTSHEHFGLLCPKSI |
| 5 | HVLKAVFSRGDTPVL | 61 | HFGLLCPKSIPGLSI |
| 6 | VFSRGDTPVLPHETR | 62 | CPKSIPGLSISGNLL |
| 7 | DTPVLPHETRLLQTG | 63 | PGLSISGNLLMNGQQ |
| 8 | PHETRLLQTGIHVRV | 64 | SGNLLMNGQQIFLEV |
| 9 | LLQTGIHVRVSQPSL | 65 | MNGQQIFLEVQAIRE |
| 10 | IHVRVSQPSLILVSQ | 66 | IFLEVQAIRETVELR |
| 11 | SQPSLILVSQYTPDS | 67 | QAIRETVELRQYDPV |
| 12 | ILVSQYTPDSTPCHR | 68 | TVELRQYDPVAALFF |
| 13 | YTPDSTPCHRGDNQL | 69 | QYDPVAALFFFDIDL |
| 14 | TPCHRGDNQLQVQHT | 70 | AALFFFDIDLLLQRG |
| 15 | GDNQLQVQHTYFTGS | 71 | FDIDLLLQRGPQYSE |
| 16 | QVQHTYFTGSEVENV | 72 | LLQRGPQYSEHPTFT |
| 17 | YFTGSEVENVSVNV OH | 73 | PQYSEHPTFTSQYRI |
| 18 | EVENVSVNVHNPTGR | 74 | HPTFTSQYRIQGKLE |
| 19 | SVNVHNPTGRSICPS | 75 | SQYRIQGKLEYRHTW |
| 20 | NPTGRSICPSQEPMS | 76 | QGKLEYRHTWDRHDE |
| 21 | SICPSQEPMSIYVYA | 77 | YRHTWDRHDEGAAQG |
| 22 | QEPMSIYVYALPLKM | 78 | DRHDEGAAQGDDDVW |
| 23 | IYVYALPLKMLNIPS | 79 | GAAQGDDDVWTSGSD |
| 24 | LPLKMLNIPSINVH OH | 80 | DDDVWTSGSDSDEEL |
| 25 | LNIPSINVHHYPSAA | 81 | TSGSDSDEELVTTER |
| 26 | INVHHYPSAAERKHR | 82 | SDEELVTTERKTPRV |
| 27 | YPSAAERKHRHLPVA | 83 | VTTERKTPRVTGGGA |
| 28 | ERKHRHLPVADAVI OH | 84 | KTPRVTGGGAMAGAS |
| 29 | HLPVADAVIHASGKQ | 85 | TGGGAMAGASTSAGR |
| 30 | DAVIHASGKQMWQAR | 86 | MAGASTSAGRKRKSA |
| 31 | ASGKQMWQARLTVSG | 87 | TSAGRKRKSASSATA |

| | | | |
|---|---|---|---|
| 32 | MWQARLTVSGLAWTR | 88 | KRKSASSATACTSGV |
| 33 | LTVSGLAWTRQQNQW | 89 | SSATACTSGVMTRGR |
| 34 | LAWTRQQNQWKEPDV | 90 | CTSGVMTRGRLKAES |
| 35 | QQNQWKEPDVYYTSA | 91 | MTRGRLKAESTVAPE |
| 36 | KEPDVYYTSAFVFPT | 92 | LKAESTVAPEEDTDE |
| 37 | YYTSAFVFPTKDVAL | 93 | TVAPEEDTDEDSDNE |
| 38 | FVFPTKDVALRHVVC | 94 | EDTDEDSDNEIHNPA |
| 39 | KDVALRHVVCAHELV | 95 | DSDNEIHNPAVFTWP |
| 40 | RHVVCAHELVCSMEN | 96 | IHNPAVFTWPPWQAG |
| 41 | AHELVCSMENTRATK | 97 | VFTWPPWQAGILARN |
| 42 | CSMENTRATKMQVIG | 98 | PWQAGILARNLVPMV |
| 43 | TRATKMQVIGDQYVK | 99 | ILARNLVPMVATVQG |
| 44 | MQVIGDQYVKVYLES | 100 | LVPMVATVQGQNLKY |
| 45 | DQYVKVYLESFCEDV | 101 | ATVQGQNLKYQEFFW |
| 46 | VYLESFCEDVPSGKL | 102 | QNLKYQEFFWDANDI |
| 47 | FCEDVPSGKLFMHVT | 103 | QEFFWDANDIYRIFA |
| 48 | PSGKLFMHVTLGSDV | 104 | DANDIYRIFAELEGV |
| 49 | FMHVTLGSDVEEDLT | 105 | YRIFAELEGVWQPAA |
| 50 | LGSDVEEDLTMTRNP | 106 | ELEGVWQPAAQPKRR |
| 51 | EEDLTMTRNPQPFMR | 107 | WQPAAQPKRRRHRQD |
| 52 | MTRNPQPFMRPHERN | 108 | QPKRRRHRQDALPGP |
| 53 | QPFMRPHERNGFTVL | 109 | RHRQDALPGPCIAST |
| 54 | PHERNGFTVLCPKNM | 110 | ALPGPCIASTPKKHR |
| 55 | GFTVLCPKNMIIKPG | 111 | LPGPCIASTPKKHRG |
| 56 | CPKNMIIKPGKISHI | | |

**Table E.7** Amino acid sequences of individual overlapping 15mer peptides spanning the predicted pp65 ORF

## E.8 IE1

| Peptide Number | Peptide Sequence | Peptide Number | Peptide Sequence |
|---|---|---|---|
| 1 | CNENPEKDVLAELVK | 42 | TCVETMCNEYKVTSD |
| 2 | EKDVLAELVKQIKVR | 43 | MCNEYKVTSDACMMT |
| 3 | AELVKQIKVRVDMVR | 44 | KVTSDACMMTMYGGI |
| 4 | QIKVRVDMVRHRIKE | 45 | ACMMTMYGGISLLSE |
| 5 | VDMVRHRIKEHMLKK | 46 | MYGGISLLSEFCRVL |
| 6 | HRIKEHMLKKYTQTE | 47 | SLLSEFCRVLCCYVL |
| 7 | HMLKKYTQTEEKFTG | 48 | FCRVLCCYVLEETSV |
| 8 | YTQTEEKFTGAFNMM | 49 | CCYVLEETSVMLAKR |
| 9 | EKFTGAFNMMGGCLQ | 50 | EETSVMLAKRPLITK |
| 10 | AFNMMGGCLQNALDI | 51 | MLAKRPLITKPEVIS |
| 11 | GGCLQNALDILDKVH | 52 | PLITKPEVISVMKRR |
| 12 | NALDILDKVHEPFEE | 53 | PEVISVMKRRIEEIC |
| 13 | LDKVHEPFEEMKCIG | 54 | VMKRRIEEICMKVFA |
| 14 | EPFEEMKCIGLTMQS | 55 | IEEICMKVFAQYILG |
| 15 | MKCIGLTMQSMYENY | 56 | MKVFAQYILGADPLR |
| 16 | LTMQSMYENYIVPED | 57 | QYILGADPLRVCSPS |
| 17 | MYENYIVPEDKREMW | 58 | ADPLRVCSPSVDDLR |
| 18 | IVPEDKREMWMACIK | 59 | VCSPSVDDLRAIAEE |
| 19 | KREMWMACIKELHDV | 60 | VDDLRAIAEESDEEE |
| 20 | MACIKELHDVSKGAA | 61 | AIAEESDEEEAIVAY |
| 21 | ELHDVSKGAANKLGG | 62 | SDEEEAIVAYTLATA |
| 22 | SKGAANKLGGALQAK | 63 | AIVAYTLATAGVSSS |
| 23 | NKLGGALQAKARAKK | 64 | TLATAGVSSSDSLVS |
| 24 | ALQAKARAKKDELRR | 65 | GVSSSDSLVSPPESP |
| 25 | ARAKKDELRRKMMYM | 66 | DSLVSPPESPVPATI |
| 26 | DELRRKMMYMCYRNI | 67 | PPESPVPATIPLSSV |
| 27 | KMMYMCYRNIEFFTK | 68 | VPATIPLSSVIVAEN |
| 28 | CYRNIEFFTKNSAFP | 69 | PLSSVIVAENSDQEE |
| 29 | EFFTKNSAFPKTTNG | 70 | IVAENSDQEESEQSD |
| 30 | NSAFPKTTNGCSQAM | 71 | SDQEESEQSDEEEEE |
| 31 | KTTNGCSQAMAALQN | 72 | SEQSDEEEEEGAQEE |

| | | | |
|---|---|---|---|
| 32 | CSQAMAALQNLPQCS | 73 | EEEEEGAQEEREDTV |
| 33 | AALQNLPQCSPDEIM | 74 | GAQEEREDTVSVKSE |
| 34 | LPQCSPDEIMAYAQK | 75 | REDTVSVKSEPVSEI |
| 35 | PDEIMAYAQKIFKIL | 76 | SVKSEPVSEIEEVAP |
| 36 | AYAQKIFKILDEERD | 77 | PVSEIEEVAPEEEED |
| 37 | IFKILDEERDKVLTH | 78 | EEVAPEEEEDGAEEP |
| 38 | DEERDKVLTHIDHIF | 79 | EEEEDGAEEPTASGG |
| 39 | KVLTHIDHIFMDILT | 80 | GAEEPTASGGKSTHP |
| 40 | IDHIFMDILTTCVET | 81 | TASGGKSTHPMVTRS |
| 41 | MDILTTCVETMCNEY | 82 | KSTHPMVTRSKADQ |

**Table E.8** Amino acid sequences of individual overlapping 15mer peptides spanning the predicted UL123 (IE1) ORF

## E.9 IE2

| Peptide Number | Peptide Sequence | Peptide Number | Peptide Sequence |
|---|---|---|---|
| 1 | MESSAKRKMDPDNPD | 58 | GASVTSSHHGRGGFG |
| 2 | KRKMDPDNPDEGPSS | 59 | SSHHGRGGFGGAASS |
| 3 | PDNPDEGPSSKVPRP | 60 | RGGFGGAASSSLLSC |
| 4 | EGPSSKVPRPETPVT | 61 | GAASSSLLSCGHQSS |
| 5 | KVPRPETPVTKATTF | 62 | SLLSCGHQSSGGAST |
| 6 | ETPVTKATTFLQTML | 63 | GHQSSGGASTGPRKK |
| 7 | KATTFLQTMLRKEVN | 64 | GGASTGPRKKKSKRI |
| 8 | LQTMLRKEVNSQLSL | 65 | GPRKKKSKRISELDN |
| 9 | RKEVNSQLSLGDPLF | 66 | KSKRISELDNEKVRN |
| 10 | SQLSLGDPLFPELAE | 67 | SELDNEKVRNIMKDK |
| 11 | GDPLFPELAEESLKT | 68 | EKVRNIMKDKNTPFC |
| 12 | PELAEESLKTFEQVT | 69 | IMKDKNTPFCTPNVQ |
| 13 | ESLKTFEQVTEDCNE | 70 | NTPFCTPNVQTRRGR |
| 14 | FEQVTEDCNENPEKD | 71 | TPNVQTRRGRVKIDE |
| 15 | EDCNENPEKDVLAEL | 72 | TRRGRVKIDEVSRMF |
| 16 | NPEKDVLAELGDILA | 73 | VKIDEVSRMFRNTNR |
| 17 | VLAELGDILAQAVNH | 74 | VSRMFRNTNRSLEYK |
| 18 | GDILAQAVNHAGIDS | 75 | RNTNRSLEYKNLPFT |
| 19 | QAVNHAGIDSSSTGP | 76 | SLEYKNLPFTIPSMH |
| 20 | AGIDSSSTGPTLTTH | 77 | NLPFTIPSMHQVLDE |
| 21 | SSTGPTLTTHSCSVS | 78 | IPSMHQVLDEAIKAC |
| 22 | TLTTHSCSVSSAPLN | 79 | QVLDEAIKACKTMQV |
| 23 | SCSVSSAPLNKPTPT | 80 | AIKACKTMQVNNKGI |
| 24 | SAPLNKPTPTSVAVT | 81 | KTMQVNNKGIQIIYT |
| 25 | KPTPTSVAVTNTPLP | 82 | NNKGIQIIYTRNHEV |
| 26 | SVAVTNTPLPGASAT | 83 | QIIYTRNHEVKSEVD |
| 27 | NTPLPGASATPELSP | 84 | RNHEVKSEVDAVRCR |
| 28 | GASATPELSPRKKPR | 85 | KSEVDAVRCRLGTMC |
| 29 | PELSPRKKPRKTTRP | 86 | AVRCRLGTMCNLALS |
| 30 | RKKPRKTTRPFKVII | 87 | LGTMCNLALSTPFLM |
| 31 | KTTRPFKVIIKPPVP | 88 | NLALSTPFLMEHTMP |

| | | | |
|---|---|---|---|
| 32 | FKVIIKPPVPPAPIM | 89 | TPFLMEHTMPVTHPP |
| 33 | KPPVPPAPIMLPLIK | 90 | EHTMPVTHPPEVAQR |
| 34 | PAPIMLPLIKQEDIK | 91 | VTHPPEVAQRTADAC |
| 35 | LPLIKQEDIKPEPDF | 92 | EVAQRTADACNEGVK |
| 36 | QEDIKPEPDFTIQYR | 93 | TADACNEGVKAAWSL |
| 37 | PEPDFTIQYRNKIID | 94 | NEGVKAAWSLKELHTH |
| 38 | TIQYRNKIIDTAGCI | 95 | AAWSLKELHTHQLCP |
| 39 | NKIIDTAGCIVISDS | 96 | KELHTHQLCPRSSDY |
| 40 | TAGCIVISDSEEEQG | 97 | HQLCPRSSDYRNMII |
| 41 | VISDSEEEQGEEVET | 98 | RSSDYRNMIIHAATP |
| 42 | EEEQGEEVETRGATA | 99 | RNMIIHAATPVDLLG |
| 43 | EEVETRGATASSPST | 100 | HAATPVDLLGALNLC |
| 44 | RGATASSPSTGSGTP | 101 | VDLLGALNLCLPLMQ |
| 45 | SSPSTGSGTPRVTSP | 102 | ALNLCLPLMQKFPKQ |
| 46 | GSGTPRVTSPTHPLS | 103 | LPLMQKFPKQVMVRI |
| 47 | RVTSPTHPLSQMNHP | 104 | KFPKQVMVRIFSTNQ |
| 48 | THPLSQMNHPPLPDP | 105 | VMVRIFSTNQGGFML |
| 49 | QMNHPPLPDPLGRPD | 106 | FSTNQGGFMLPIYET |
| 50 | PLPDPLGRPDEDSSS | 107 | GGFMLPIYETAAKAY |
| 51 | PLGRPDEDSSSSSSS | 108 | PIYETAAKAYAVGQF |
| 52 | EDSSSSSSSSCSSAS | 109 | AAKAYAVGQFEQPTE |
| 53 | SSSSSCSSASDSESE | 110 | AVGQFEQPTETPPED |
| 54 | CSSASDSESESEEMK | 111 | EQPTETPPEDLDTLS |
| 55 | DSESESEEMKCSSGG | 112 | TPPEDLDTLSLAIEA |
| 56 | SEEMKCSSGGGASVT | 113 | LDTLSLAIEAAIQDL |
| 57 | CSSGGGASVTSSHHG | 114 | LAIEAAIQDLRNKSQ |

**Table E.9** Amino acid sequences of individual overlapping 15mer peptides spanning the predicted UL122 (IE2) ORF

## E.10 US3

| Peptide Number | Peptide Sequence | Peptide Number | Peptide Sequence |
|---|---|---|---|
| 1 | MKPVLVLAILAVLFL | 19 | VLAILAVLFLRLADS |
| 2 | AVLFLRLADSVPRPL | 20 | RLADSVPRPLDVVVS |
| 3 | VPRPLDVVVSEIRSA | 21 | DVVVSEIRSAHFRVE |
| 4 | EIRSAHFRVEENQCW | 22 | HFRVEENQCWFHMGM |
| 5 | ENQCWFHMGMLYFKG | 23 | FHMGMLYFKGRMSGN |
| 6 | LYFKGRMSGNFTEKH | 24 | RMSGNFTEKHFVNVG |
| 7 | FTEKHFVNVGIVSQS | 25 | FVNVGIVSQSYMDRL |
| 8 | IVSQSYMDRLQVSGE | 26 | YMDRLQVSGEQYHHD |
| 9 | QVSGEQYHHDERGAY | 27 | QYHHDERGAYFEWNI |
| 10 | ERGAYFEWNIGGHPV | 28 | FEWNIGGHPVTHTVD |
| 11 | GGHPVTHTVDMVDIT | 29 | THTVDMVDITLSTRW |
| 12 | MVDITLSTRWGDPKK | 30 | LSTRWGDPKKYAACV |
| 13 | GDPKKYAACVPQVRM | 31 | YAACVPQVRMDYSSQ |
| 14 | PQVRMDYSSQTINWY | 32 | DYSSQTINWYLQRSM |
| 15 | TINWYLQRSMRDDNW | 33 | LQRSMRDDNWGLLFR |
| 16 | RDDNWGLLFRTLLVY | 34 | GLLFRTLLVYLFSLV |
| 17 | TLLVYLFSLVVLVLL | 35 | LFSLVVLVLLTVGVS |
| 18 | VLVLLTVGVSARLRF | 36 | LVLLTVGVSARLRFI |

**Table E.10** Amino acid sequences of individual overlapping 15mer peptides spanning the predicted US3 ORF

# E.11 pp71

| Peptide Number | Peptide Sequence | Peptide Number | Peptide Sequence |
|---|---|---|---|
| 1 | MSQASSSPGEGPSSE | 56 | RRLPDNGFQLLIPKS |
| 2 | SSPGEGPSSEAAAIS | 57 | NGFQLLIPKSFTLTR |
| 3 | GPSSEAAAISEAEAA | 58 | LIPKSFTLTRIHPEY |
| 4 | AAAISEAEAASGSFG | 59 | FTLTRIHPEYIVQIQ |
| 5 | EAEAASGSFGRLHCQ | 60 | IHPEYIVQIQNAFET |
| 6 | SGSFGRLHCQVLRLI | 61 | IVQIQNAFETNQTHD |
| 7 | RLHCQVLRLITNVEG | 62 | NAFETNQTHDTIFFP |
| 8 | VLRLITNVEGGSLEA | 63 | NQTHDTIFFPENIPG |
| 9 | TNVEGGSLEAGRLRL | 64 | TIFFPENIPGVSIEA |
| 10 | GSLEAGRLRLLDLRT | 65 | ENIPGVSIEAGPLPD |
| 11 | GRLRLLDLRTNIEVS | 66 | VSIEAGPLPDRVRIT |
| 12 | LDLRTNIEVSRPSVL | 67 | GPLPDRVRITLRVTL |
| 13 | NIEVSRPSVLCCFQE | 68 | RVRITLRVTLTGDQA |
| 14 | RPSVLCCFQENKSPH | 69 | LRVTLTGDQAVHLEH |
| 15 | CCFQENKSPHDTVDL | 70 | TGDQAVHLEHRQPLG |
| 16 | NKSPHDTVDLTDLNI | 71 | VHLEHRQPLGRIHFF |
| 17 | DTVDLTDLNIKGRCV | 72 | RQPLGRIHFFRRGFW |
| 18 | TDLNIKGRCVVGEQD | 73 | RIHFFRRGFWTLTPG |
| 19 | KGRCVVGEQDRLLVD | 74 | RRGFWTLTPGKPDKI |
| 20 | VGEQDRLLVDLNNFG | 75 | TLTPGKPDKIKRPQV |
| 21 | RLLVDLNNFGPRRLT | 76 | KPDKIKRPQVQLRAG |
| 22 | LNNFGPRRLTPGSEN | 77 | KRPQVQLRAGLFPRS |
| 23 | PRRLTPGSENNTVSV | 78 | QLRAGLFPRSNVMRG |
| 24 | PGSENNTVSVLAFAL | 79 | LFPRSNVMRGAVSEF |
| 25 | NTVSVLAFALPLDRV | 80 | NVMRGAVSEFLPQSP |
| 26 | LAFALPLDRVPVSGL | 81 | AVSEFLPQSPGLPPT |
| 27 | PLDRVPVSGLHLFQS | 82 | LPQSPGLPPTEEEEE |
| 28 | PVSGLHLFQSQRRGG | 83 | GLPPTEEEEEEEEED |
| 29 | HLFQSQRRGGEENRP | 84 | EEEEEEEEEDDEDDL |
| 30 | QRRGGEENRPRMEAR | 85 | EEEEDDEDDLSSTPTP |
| 31 | EENRPRMEARAIIRR | 86 | DEDDLSSTPTPTPLS |

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|---|---|---|---|
| 32 | RMEARAIIRRTAHHW | 87 | SSTPTPTPLSEAMFA |
| 33 | AIIRRTAHHWAVRLT | 88 | PTPLSEAMFAGFEEA |
| 34 | TAHHWAVRLTVTPNW | 89 | EAMFAGFEEASGDED |
| 35 | AVRLTVTPNWRRRTD | 90 | GFEEASGDEDSDTQA |
| 36 | VTPNWRRRTDSSLEA | 91 | SGDEDSDTQAGLSPA |
| 37 | RRRTDSSLEAGQIFV | 92 | SDTQAGLSPALILTG |
| 38 | SSLEAGQIFVSQFAF | 93 | GLSPALILTGQRRRS |
| 39 | GQIFVSQFAFRAGAI | 94 | LILTGQRRRSGNNGA |
| 40 | SQFAFRAGAIPLTLV | 95 | QRRRSGNNGALTLVI |
| 41 | RAGAIPLTLVDALEQ | 96 | GNNGALTLVIPSWHV |
| 42 | PLTLVDALEQLACSD | 97 | LTLVIPSWHVFASLD |
| 43 | DALEQLACSDPNTYI | 98 | PSWHVFASLDDLVPL |
| 44 | LACSDPNTYIHKTET | 99 | FASLDDLVPLTVSVQ |
| 45 | PNTYIHKTETDERGQ | 100 | DLVPLTVSVQHAALR |
| 46 | HKTETDERGQWIMLF | 101 | TVSVQHAALRPTSYL |
| 47 | DERGQWIMLFLHHDS | 102 | HAALRPTSYLRSDMD |
| 48 | WIMLFLHHDSPHPPT | 103 | PTSYLRSDMDGDVRT |
| 49 | LHHDSPHPPTSVFLH | 104 | RSDMDGDVRTAADIS |
| 50 | PHPPTSVFLHFSVYT | 105 | GDVRTAADISSTLRS |
| 51 | SVFLHFSVYTHRAEV | 106 | AADISSTLRSVPAPR |
| 52 | FSVYTHRAEVVARHN | 107 | STLRSVPAPRPSPIS |
| 53 | HRAEVVARHNPYPHL | 108 | VPAPRPSPISTASTS |
| 54 | VARHNPYPHLRRLPD | 109 | PSPISTASTSSTPRS |
| 55 | PYPHLRRLPDNGFQL | 110 | STASTSSTPRSRPRI |

**Table E.11** Amino acid sequences of individual overlapping 15mer peptides spanning the predicted pp71 ORF