CHARACTERISATION OF T CELL RESPONSES TO HUMAN CYTOMEGALOVIRUS (HCMV) IN DIFFERENT AGE GROUPS

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ABSTRACT

Infection with human cytomegalovirus (HCMV) is thought to promote the expansion of low affinity, terminally differentiated HCMV-specific memory T cells with increasing age, thereby resulting in reduced vaccine responsiveness and poor responses to novel pathogens. This project sought to characterise and explore the relationship between the size of the HCMV-specific immune-response and general health in older age. A multi-parametric flow cytometry assay was used to characterise ex-vivo activation induced T cell responses against 19 immunogenic HCMV proteins in three groups of healthy volunteers aged between 18 to 85 years old. This approach is different to that utilised by most studies investigating the role of HCMV-specific immunity and ageing whereby focus is often limited to T cell responses against a few 'dominant' peptides, or proteins.

The response size against the 19 frequently recognized HCMV proteins were assessed by means of intracellular cytokine staining (ICS) assay based on these functional markers; CD40L, CD107a, TNFα, IFNγ, and IL-2. The memory phenotype with respect to each protein was also explored using these markers; CD45RA and CD27.

A significant increase in pp65-specific $CD4^+$ T cells in the Oldest group was observed compared to the Young group. Also, a significant increase was observed in the size of the summated (sum of all protein-specific responses) $CD8^+$ HCMV-specific T cell responses in the Older group compared to Young group, but no further increase was seen in the Oldest group indicating no further expansion with age.

Differences in levels of HCMV-specific T cell polyfunctionality was observed between target proteins, however level of polyfunctionality was neither reduced with increasing age nor in those with very large responses.

In this study no significant differences were observed in the number of infections and health related problems reported by HCMV seropositive and seronegative aged individuals keeping a health diary; however, a significant trend was observed between the size of the summated HCMV-specific $CD8^+$ T cells and the proportion of months unwell.

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Last but never least, a very special thanks to my husband Moyo, for his words of encouragement and support. I dedicate this thesis in its entirety to my lovely Isabelle, you have given me plenty of reasons to always give my best.

AUTHOR'S DECLARATION

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. This thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.

Signed:

Date:

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 6

CHAPTER 8

LIST OF ABBREVIATIONS

aa	Amino acids
Ab	Antibody
APC	Antigen presenting cell
APC	Allophycocyanin
APC-Cy7	Allophycocyanin cyanin7
BFA	Brefeldin A
BSA	Bovine serum albumine
CD40L	CD40-ligand
CFSE	Carboxyfluorescein succinimidyl ester
CI	Confidence interval
CTL	Cytotoxic T lymphocyte
DMSO	Dimethyl sulfoxide
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
FSC	Forward scatter
g	Centrifugal force
HCMV	Human Cytomegalovirus
HHV	Human herpesviruses
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen

ICS	Intracellular cytokine staining
IE	Immediate early protein
IFNγ	Interferon gamma
IL	Interleukin
IRP	Immune risk profile
IQR	Interquartile range
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
ng	Nanogram
NK cells	Natural killer cells
PB	Pacific blue
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PECy7	Phycoerythrin cyanine dye
PerCP	Peridinin chlorophyl protein
PFA	Paraformaldehyde
PMT	Photomultiplier
pp65	Phosphoprotein 65
PPD	Purified protein derivate of M. tuberculosis
R_S	Spearmans correlation coefficient
ROC curve	Receiver operating characteristics curve
RPMI medium	Roswell Park Memorial Institute Medium
SEB	Staphylococcal enterotoxin B

SE	Standard error
SSC	Sideward scatter
SD	Standard deviation
T_{CM}	Central memory T cell
TCR	T cell receptor
T_{EM}	Effector memory T cell
T_{EMRA}	Effector memory revertant T cell
T_N	Naïve T cell
TNF	Tumour necrosis factor
Treg	Regulatory T cell
µg	Microgram
µl	Microliter

1 INTRODUCTION

1.1 IMMUNE SYSTEM

The immune system constitutes a wide array of cell types that have evolved to mount an effective response to a plethora of potentially harmful pathogens (bacteria, fungi, parasites and virus), eliminating tumours and allergens, whilst maintaining tolerance to antigens derived from hosts. These cell types can be divided into two arms; namely, the innate and adaptive immune system.

The innate immune system acts as the first line of defence recognizing conserved specialized pathogen associated molecular patterns (PAMPs) on invading pathogens. The production of molecules including inflammatory cytokines ensues to clear up both host and pathogen derived debris. In the event that an infection is not successfully contained, cells of the adaptive immune system are recruited to clear up microbial insult.

The hallmark of the adaptive immune system which comprises both humoral (B cell) and cellular (T cell) mediated immunity is the ability to respond to a wide array of pathogens through recognition of specific pathogen derived antigens and generate immunological memory in the event of re-encounter with the same pathogen. The sections (1.1.1 – 1.1.8) outlined below will give a brief insight into the development of T cells, activation of T cells, generation and maintenance of the memory T cells. The focus of my thesis is characterizing T cell responses; as a result there will be no further elaboration on the humoral responses to cytomegalovirus.

1.1.1 T cell development

The thymus is a primary lymphoid organ that plays a crucial role in the development of T cells. Bone-marrow derived committed progenitor T cells migrate from circulation via a

chemotactic gradient to the specialized thymic micro-environment where they interact with non-lymphoid stromal cells providing secreted growth factors and Notch1 signalling to aid the generation of immature thymocytes and drive T cell lineage commitment (1, 2). Each individual immature thymocyte has a unique T cell receptor (TCR) which enables recognition of a specific antigen when presented by major histocompatibility complex (MHC) on antigen presenting cells. The majority of T cells express TCR alpha beta (αβ) heterodimer chain on their cell surface alongside either CD4 or CD8 molecules; however a small minority (1-10%) display a gamma delta (γδ) chain. Studies in mouse have shown a substantial proportion of γδ T cells reside in the intraepithelial lymphocyte (IEL) compartments of skin, intestine and the genitourinary tract (3, 4). In a recent study by Sell et al 2015, they demonstrate the role of γδ T cells in providing effective protection against murine cytomegalovirus (MCMV) infection in the absence of other conventional adaptive immune responses by αβ T cell and B cells.

Thymocytes displaying a functional TCR are further subjected to stringent checks referred to as positive and negative selection which is based on their strength of interaction between the self-antigen and self-MHC. The ability to distinguish "self" from foreign antigen is particularly crucial, as such, only thymocytes with low avidity interactions with self-MHC presenting self-antigen on thymic stromal cells are positively selected. This ensures that all positively selected cells can interact with self MHC.

Selected thymocytes migrate towards the thymic medulla where they are presented with self-antigen in complex with MHC on antigen presenting cells such as bone marrow derived dendritic cells. Those immature cells that display strong affinity interactions undergo apoptosis and are said to be negatively selected (5). These thymocytes further differentiate into mature $CD4^+$ or $CD8^+$ naïve T cells that eventually leave the thymus.

Naïve T cells recirculate between the blood stream and secondary lymphoid tissues such as the spleen and lymph nodes.

A large proportion of thymocytes that interact strongly with self-antigens are purged from the thymus thereby minimizing, but not completely eradicating these cells in the periphery. The result is a fully functional, MHC restricted, mostly self-tolerant naïve T cell repertoire that is diverse and capable of responding to a wide array of foreign pathogens.

1.1.2 Antigen presenting cells

Naïve T cells are divided into two major subsets based on surface expression of either $CD4^+$ or $CD8^+$ co- receptor, which interact with MHC molecules class II and class I respectively.

MHC class 1 molecules are present on all nucleated cells and present peptides derived from intracellular pathogens on their peptide binding groove to specific $CD8^+$ T cells. $CD4^+$ T cells recognise peptide antigen tightly bound in the groove of MHC class II molecules. MHC II molecules are constitutively expressed on 'professional' antigen presenting cells (APC) such as dendritic cells (DCs), and are up regulated under inflammatory conditions. Although mature DCs are the most potent APCs expressing high levels of MHC class II and co-stimulatory molecules, other cell types such as B cells and macrophages are also able to act as APCs to present antigen to their cognate T cells.

Immature DCs are efficient at capturing antigen by taking up microbial particles using various methods including phagocytosis of apoptotic cell debris and macropinocytosis. They can also engage directly with microbes through PRR such as the Toll-like receptors on pathogen cell surface. This triggers the maturation and migration of the DC to secondary lymph nodes where antigen processing ensues and the resultant peptides are displayed on MHC molecules which are presented on the cell surface. DCs in lymph nodes

produce small chemotactic proteins known as chemokines to attract circulating specific T and B cells (6).

1.1.3 T cell activation (Signal 1)

The successful engagement of naïve TCR and cognate peptide:MHC molecules on the surface of DCs or other APCs results in downstream biochemical changes and effector functions.

It is therefore important to consider the three signals needed to ensure successful activation, expansion and differentiation of naïve T cells into effector T cells. The specific recognition and interaction between the TCR and peptide: MHC complex on APC constitutes the first signal. This is essential for activating a naïve T cell. Additional signals are provided by molecules such as the co-stimulatory signals (signal 2) which promote the survival and expansion of the T cells and secretion of cytokines such as IL-12 (signal 3) that are involved in directing differentiation of T cells into subsets of effector T cells. Optimal T cell activation results in the differentiation of $CD8^+$ T cells into cytotoxic T lymphocytes (CTLs) and $CD4^+$ T cells into T helper 1 (T_H1) , T_H2, T_H17 and regulatory T cells.

The differentiation of an activated T cell into a specific subset depends greatly on the cocktail of cytokines it is exposed to (7). For example, $CD4^+$ T cells can differentiate into T_H1 cells in the presence of Interferon gamma and Interleukin-12 and are mostly involved in immune responses against extracellular pathogens and bacterial infection in macrophages. Upon activation they secrete the following cytokines; Interferon gamma (IFNγ), Interleukin-2 (IL-2), Granulocyte macrophage colony stimulating factor (GM-CSF), Tumour necrosis factor-alpha (TNFα) and Tumour necrosis factor- beta (TNFβ).

The binding of TCR to peptide: self MHC complexes on APCs results in a clustering of the TCR and associated proteins such as CD3 (TCR-CD3 complex), the co-receptors $CD4^+$ or $CD8^+$ including co-stimulatory molecules at the site of cell to cell contact, forming a dynamic complex described as the immunological synapse (IS).

CD3 dimer (γ, δ, ε) and its associated ζ chains are identical in all T cells irrespective of antigen specificity. They contain a single copy of the conserved sequence motif immune receptor tyrosine-based activation motif (ITAM) in their cytoplasmic tail. This becomes phosphorylated by the src family of kinases such as Lck, which associates with the cytoplasmic tail of $CD4^+$ or $CD8^+$ molecules or Fyn, which is linked to CD3. ZAP-70 (70-KD ζ associated protein) is a tyrosine kinase that is recruited to the ζ chain, phosphorylation of which results in downstream activation of LAT (linker of activated T cells) molecules and SLP-76 adaptor proteins. These events trigger the activation of various distinct signalling pathways which ultimately leads to changes in gene expression of the T cell. This eventually results in the production of effector molecules such as cytokines to clear up pathogens and provide anti-tumour immunity(8).

1.1.4 Co-stimulatory molecules (Signal 2)

The combination of all three signals promotes activation and proliferation of T cells, whilst the delivery of just one signal in the absence of co-stimulation results in anergy or dysregulation, whereby T cells are unable to initiate a productive response.

One of the best characterised family of co-stimulatory molecules are the B7 family of molecules comprising of CD80 (B7-1) and CD86 (B7-2) on APCs. These molecules interact with CD28 which is the principal co-stimulatory receptor on T cells. The interaction results in the augmentation of signal transduction by contributing to the

increased synthesis of phosphatidylinositol 4,5-bisphosphate (PIP_2) in T lymphocytes subsequently promoting the up-regulation of cytokines and chemokine signalling pathways as well as promoting T cell survival and proliferation (9).

Costimulatory signals can also be provided by alternative molecules such as the Tumour necrosis factor receptor superfamily (TNFRSF). Although structurally diverse, they consist of co-stimulatory molecules namely CD27, CD40, 4-IBB and OX40. For example, CD70 on DCs binds to CD27 receptor which is constitutively expressed on naïve T cells delivering a strong co-stimulatory signals early on in the activation cascade (10).

Cytotoxic T lymphocyte antigen 4 (CTLA-4), is a member of the Immunoglobulin super family (IgSF) and a receptor for B7 molecules. It delivers an inhibitory signal, dampening the immune response, often protecting against the development of autoimmune disease. In an experimental model of CTLA-4 deficient mice, the animals died within 3 weeks of age, as a result of tissue infiltration and organ destruction (11).

1.1.5 Effector functions of T cells (Signal 3)

The successful activation of the naïve TCR is followed by 4-5 days of rapid clonal expansion induced by IL-2, T cells undergo more than 15 consecutive division [Reviewed in (12)]. This results in the generation of effector T cells producing cytokines (soluble proteins secreted by cells which can alter the properties of another cell or itself) to clear up pathogens or tumour cells. Signal 3 aids the cytokine mediated differentiation of T cells into different effector T cell subsets. Once activated, naïve $CD8^+$ T cells differentiate into cytotoxic effector $CD8^+$ T cells targeting intracellular pathogens especially viruses by producing cytotoxic molecules such as perforin which enables the entry of granzymes into infected cells to promote apoptosis. Cytokines such as TNFα and IFNγ are also produced; they play an important role in the inhibition of viral replication.

Naïve $CD4^+$ T cells have the potential to differentiate into several subsets of effector T cells with distinct functions [T_H1, T_H2, T_H17 and T regulatory T cells] based on the signals provided by the micro-environment during naïve T cell activation. $CD4^+$ T cells can differentiate into T_H1 cell subsets in the presence of IFNγ and IL-12. Effector $CD4^+$ T cells act mainly via the production of cytokines and membrane associated proteins such as TNFα, FAS ligand (FASL), CD40L. Notably, they provide "help" to $CD8^+$ and B cells via effector cytokines to augment the activation of these cells. This cell subset is also involved in the immune response to extracellular pathogens and bacterial infection in macrophages. Typically $CD4^+$ T cells differentiate into T_H2 T cells in the presence of IL-4 secreting cytokines such as IL-3, IL-4, IL-5, IL9 and IL-13 and are involved in immune responses against parasites and allergens.

The resolution of an infection leads to the majority of effector T cells undergoing massive clonal contraction via apoptosis with some of these activated T cells becoming long lived memory T cells.

1.1.6 Pathways of effector and memory T cells

Various models of memory T cell development have been proposed as it remains unclear whether effector and memory subsets develop independently. Two of the models proposed are the "linear differentiation model" and "divergent differentiation model" (Figure 1.1) (13). It is important to note that models of differentiation may differ for CD4+ and $CD8^+$ T cells.

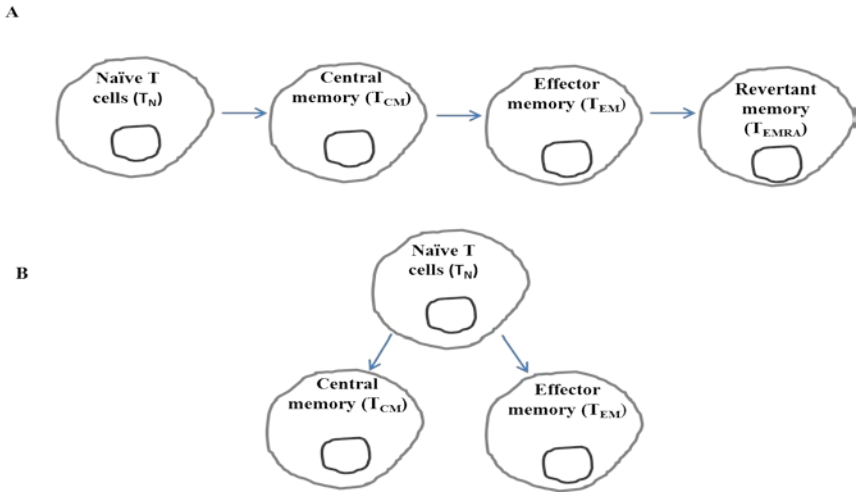


Figure 1.1. The schematic outline of different models of T cell differentiation.

The linear model of differentiation (A) depicts the progression of a single naïve T cell through to revertant memory T cell while the divergent differentiation model (B) provides a divergent pathway whereby a single naïve T cell upon exposure to different signals and upregulation/downregulation of transcription factors can develop into an effector memory T cell or central memory T cell.

The linear differentiation pathway proposes a direct lineage from naive to effector and memory T cells, contrary to this model is the divergent pathway which proposes that both effector and memory cells are generated as daughter cells from a naïve predecessor (14, 15).

This becomes further convoluted as initial studies reviewed by Chang and colleagues suggest that a single activated T cell has a remarkable ability to yield a heterogeneous progeny, however, the individual T cells do so with a high degree of variability. Mechanisms controlling this variability are not yet known but it could be down to a number of factors such as TCR signalling, abundance of antigen and cytokines present in the microenvironment (16). A growing list of transcription factors including t-bet and blimp-1 have been described which are involved in regulating the differentiation of terminal effector and memory cells (17, 18).

1.1.7 Phenotypic and functional delineation of memory T cells

Memory T cells are able to mount a faster immune response upon re-encounter with the same strain of pathogen as they require lower levels of antigen exposure and co-stimulation compared to naïve T cells. They form the basis of protective vaccination against infectious diseases.

Memory T cells are typically delineated based on the expression levels of markers such as the lymph node homing molecules CCR7, CD62L, and the leukocyte common Ag (CD45) isoform RA and RO.

A wide array of cell surface markers including expression levels of co-stimulatory molecules and receptors (e.g. CD27, CD28), chemokine receptors (e.g. CCR7), and adhesion molecules [e.g. CD62 Ligand,(CD62L)] are often used in the characterisation of the different T cell memory subsets [Rev in (19)] as detailed in Table 1.1 These markers give insight into the stage of differentiation, effector functions and migration of the T cell.

Cytokines produced by DCs and stromal cells both in the early and late phase of the immune response have been shown to be essential for the generation of memory T cells. IL-15 and IL-7 are both members of the common γ chain family of cytokines and several studies have described their importance in the maintenance of $CD8^+$ and $CD4^+$ memory T cells respectively, even, in the absence of antigens [(20), Rev in (21)].

MEMORY SUBSETS	**PHENOTYPIC MARKERS**
NAÏVE	$CD45RA^+$, $CCR7^+$, $CD28^+$, $CD27^+$
EFFECTOR	$CD45RO^+$, $CCR7^-$,$CD62L^-$, $CD28^{+/-}$, $CD27^{+/-}$
CENTRAL	$CD45RO^+$, $CCR7^+$, $CD62L^+$
REVERTANT	$CD45RA^+$, $CCR7^-$, $CD57^+$

Table 1.1 Phenotypic markers of memory T cells.

A detailed list of some of the markers used in delineating T cell memory subsets.

1.1.7.1 Naïve T cells (TN)

These are cells that have never encountered their cognate antigen and so retain full length of protein tyrosine phosphatase, denoted CD45RA, unlike antigen-experienced memory T cells that express the shorter form, CD45R0 on their surface. T_N are further characterised by the expression of CCR7 and co-stimulatory receptors CD28 and CD27 and the lack of expression of cytolytic molecules. The presence of CD27 on naïve CD8 T cells promotes the proliferation, survival and differentiation of activated T cells (22)

1.1.7.2 Central Memory T cells (T_{CM}) and Effector memory T cells (T_{EM})

The memory T cell compartment is heterogeneous and has typically been conventionally divided into two subsets on the basis of the expression of the lymph node homing molecules CD62L and CCR7.

T_{CM} cells express the lymph node homing receptors CCR7 and CD62L; they possess limited effector function but high proliferative capacity. T_{EM} cells on the other hand lack CCR7 and CD62L and show immediate effector function in peripheral tissues. Depending on their state of differentiation, T_{EM} cells express either of the co-stimulatory molecules CD27 or CD28 on their cell surface. They also possess immediate effector functions (cytotoxicity and IFNγ secretion) with reduced ability to proliferate and secrete IL-2.

1.1.7.3 Effector memory revertant T cells ($_{TEMRA}$)

T_{EMRA} re-express CD45RA, but lack expression of CCR7. They have limited proliferative capacity and display potent effector functions (23). The proportion of this highly differentiated subset is significantly higher in older individuals compared to young. It is also more prominent in $CD8^+$ T cell compartment compared to $CD4^+$ (24). When

compared to naïve CD45RA expressing T cells, T_{EMRA} cells show signs of repeated stimulation with relatively low expression of CD27 and CD28 co-stimulatory molecules which is indicative of repeated cell division (23).

In this study, the division of memory subset was based on two markers, CD45RA and CD27. This is frequently done due to limitations when assembling a multi-parameter assay with multiple activation markers. Although this division is not perfect, studies have demonstrated homology in CD27, CCR7 and CD62L expression, therefore making a fair comparison with other studies possible (25-27).

1.1.7.4 T memory stem cells (T_{SCM})

T_{SCM} are a recently described memory T cell subset at the early stage of memory T cell development displaying an enhanced capacity for self-renewal, and a multipotent ability to derive T_{CM}, T_{EM} and effector T cells, with a gene profile nestled between T_N and T_{CM} subset (28-30). T_{SCM} subset have a $CD44^{low}$ $CD62L^{high}$ phenotype which is similar to that of the T_N subset profile, however , they co-express stem cell antigen -1 (Sca-1), high levels of anti-apoptotic molecule B cell lymphoma 2 (BCL-2), the β chain of the IL-2 and IL-15 receptor (IL-2R β), and the chemokine (CXC motif) (28). T_{SCM} are phenotypically characterised as, $CD45RO^{-}$, $CD45RA^{+}$, $CCR7^{+}$, $CD127^{+}$, and $CD95^{+}$ T cells and are thought to represent about 2-4% of all circulating $CD8^{+}$ and $CD4^{+}$ T lymphocytes according to recent studies (28, 31, 32).

Recent phenotypic analyses revealed that expression levels of BCL-2, LFA-1, CXCR3 and CXCR4 are higher in T_{SCM} cells compared to T_N cells, whilst levels of CD38 and CD31 are less than that observed in T_N cells (30).

Studies have also shown that T_{SCM} subset possess the memory capability of rapid acquisition of effector functions by secreting cytokines such as TNF-α, IFN-γ and IL-2

after antigenic TCR stimulations and homeostatic stimuli unlike T_N cells (33).

1.2 HUMAN HERPESVIRUSES AND CYTOMEGALOVIRUS

Human herpesviruses (HHVs) are large, ubiquitous, double stranded DNA viruses capable of establishing lytic and latent infections. There are 8 HHVs which are further divided into 3 sub-groups namely, α, β and γ herpesviruses. The β herpesvirus family consists of HHV5/Human cytomegalovirus (HCMV), HHV6 and HHV7, of which HCMV is the largest.

1.2.1 Transmission of HCMV

There is no single defined mode of transmission in HCMV infection; instead studies have shown that the virus can be acquired in a number of ways primarily through bodily fluids including saliva, urine, blood, breast milk and semen. Pregnant women infected for the first time with HCMV can also transmit it to their unborn babies via the placenta [Rev in (34, 35)]. HCMV infection can occur at any point, with the majority of individuals infected in early childhood. Initial infection often results in a mild self-limiting fever, with most people remaining asymptomatic. A unique trait of HHV infections is their ability to maintain life-long persistence in all infected persons. In most healthy individuals, the immune system is able to suppress and/or minimize the effect of the virus with little or no symptoms exhibited. However, HCMV infection poses a huge threat to immunocompromised individuals especially those with HIV infection or in organ transplant recipients. Infection in this group of individuals can result in end organ diseases such as hepatitis, pneumonia and retinitis etc. Primary infection with HCMV in pregnant women can also result in congenital birth defects such as hearing loss and developmental delays (36).

1.2.2 Seroprevalence of HCMV

HCMV seroprevalence rates vary greatly depending on socio-economic status, with some parts of Asia and Africa recording rates as high as 95-100%. However, developed countries such as Japan and Sweden have recorded high seroprevalence rates which are suggestive of a non-direct relationship between HCMV seroprevalence and socio-economic conditions (36, 37). In developed countries such as the UK, child to child transmission of HCMV is reportedly common in a child care setting. In a random stratified study of 949 pregnant women enrolled in the Bradford birth cohort, there was a reported HCMV seroprevalence rate of 49% in White British women, 89% in South Asian UK born women and 98% in South Asian women born in South Asia (38).

1.2.3 Viral structure and gene expression

HCMV virion consists of an icosahedral nucleo-capsid enclosing a 230kbp double stranded linear DNA genome. This is enveloped by a proteinaceous layer defined as the tegument or matrix, which is in turn enclosed by a phospolipid bi-layer containing a large number of viral glycoproteins (Rev in (39)). The HCMV genome is the largest of all HHVs, with more than 700 identified open reading frames (ORF) (40). Approximately 151 of the proteins translated from some of these ORFs were immunogenic for both $CD4^+$ and/or $CD8^+$ T cells as demonstrated in a highly cited ground breaking study (41). The expression of the HCMV genome follows on shortly after infection of host cells resulting in an abundance of viral protein products. Gene expression is controlled by a cascade of transcriptional events that leads to the synthesis of three categories of viral proteins designated, “Immediate early” (IE), “Early” (E) and “Late” (L), based on the order of gene synthesis (42, 43).

1.2.4 Viral Entry

HCMV can be shed in nearly all body fluids, illustrating its broad cellular tropism and capacity to spread to, and infect most cells in the host namely, stromal cells, smooth muscle cells, epithelial cells, endothelial cells, fibroblasts, hepatocytes and leucocytes (44). Although finding a single host receptor for HCMV has proved elusive, several cellular receptors including the epidermal growth factor receptor (EGFR), the platelet-derived growth factor α receptor (PDGFRα), β1 and β3 integrins have been reported to play a role in HCMV attachment and entry (45, 46). The ability of HCMV to infect a vast array of cell types suggests a common host receptor or a complex entry pathway utilizing a combination of host receptors with commonality amongst infected cells.

HCMV expresses several types of glycoprotein H complexes, different combinations of which enable attachment to, and infection of biologically relevant cells. The HCMV pentameric glycoprotein H (gH) complex comprising of viral proteins (gH, gL, UL128, UL130, and UL131A) is known to be crucial for receptor mediated viral entry into endothelial/epithelial cells and monocytes, as shown by several independent studies (47-49). However, the exact mechanisms of viral entry into host cells are not fully elucidated.

1.2.5 Immune response to HCMV

In addition to virus specific $CD4^+$ and $CD8^+$ T cells, neutralizing antibodies play a major role in the defence against HCMV (50, 51). Recent findings described by Ciferri and colleagues demonstrate the importance of HCMV gH/gL/gO and pentamer epitopes for generating strong neutralizing human monoclonal antibodies (52). Natural killer (NK) cells are one of the key components of the innate immune system and are thought to be important in the control of HCMV infection however, the majority of these studies have been described in murine cytomegalovirus (MCMV) infection (53). Guma and colleagues

described an expansion of a subset of NK cells expressing CD94/NKG2C$^+$ receptor in a study of HCMV seropositive individuals, although its role and the mechanisms utilized in the control of infection is not clear at present (54).

Virus specific CD4$^+$ T cells are detectable a week after HCMV infection in recipients of kidney grafts from seropositive HCMV donors (55). Anti-HCMV immunoglobulin M (IgM) antibodies are also produced after primary infection with HCMV followed by the production of anti HCMV IgG antibodies which can last a lifetime.

A robust cellular immune response is vital for controlling the latency and viral replication in infected hosts. Although T cell responses are sufficient to control the virus in healthy individuals, HCMV is never completely eradicated in those infected.

1.2.6 Establishment and maintenance of latency in permissive cells

The hallmark of all HHVs is their ability to maintain latency which is defined as the persistence of viral genome in the absence of infectious virions. HCMV is no exception; it persists throughout the lifetime of an infected individual reactivating sporadically. Reactivation is often successfully controlled by the host; however, reactivation in immuno-suppressed hosts can result in complications.

HCMV has developed a wide range of mechanisms for evading recognition by the host immune system, for example interference with antigen processing and presentation through production of HCMV immune-evasins, coding for 'decoy' class-I major histocompatibility complex (MHC) receptors that are unable to present peptide (56, 57).

Following HCMV infection, latency is established in cells of the myeloid lineage, such as CD14$^+$ monocytes and bone marrow derived CD34$^+$ progenitor cells (58). Although the mechanisms and genes governing latency are not fully elucidated, a number of HCMV-specific latency associated transcripts (LATs) have been described. Three viral genes widely described as important in maintaining latency and reactivation are as follows;

UL138 (a TNF modulator), UL111A (variant cmvIL-10 homologue), and UL81-82 antisense transcript encoding HCMV protein, LUNA (latency unique natural antigen) (59, 60).

MicroRNAs (miRNAs) are small (approximately 18-22 nucleotides) non-coding ribonucleic acids (RNAs) regulating gene expression at the post transcriptional level by translational repression and/or mRNA degradation (61). In a recent study by Fu et al., 2014, miRNA from HCMV infected THP-1 cells were sequenced in an effort to understand genes responsible for the lytic and latent phase of HCMV infection. They identified 8 HCMV encoded miRNAs that are expressed during latency, of which miR-US25-1 and miR-US25-2 constituted the majority of the total miRNA read out (62). These two miRNAs have also been shown to inhibit viral DNA synthesis. Other groups have also demonstrated that miR-US25-1 targets various cellular genes associated with cell cycle control (63).

1.2.7 Immunogenic peptides

Immunogenic peptides elicit a strong immune response in infected hosts resulting in long lasting protection upon re-infection with the same pathogen. Several research groups have described $CD4^+$ and/or $CD8^+$ T cell responses to whole viral lysates, virally infected cells, and select HCMV epitopes/peptides; however, the extent to which this reflects overall T cell responses to HCMV is not clearly defined (64-66). The results from various studies have led to focus on HCMV proteins pp65 (lower matrix phospho-protein, the most abundant structural protein upon infection) and IE-1 (Immediate-Early-1, a non-structural protein), as these two proteins have been shown to be highly immunogenic (67-69). However, the focus on these two proteins ignores the potential impact of other less dominant HCMV proteins and will undoubtedly skew understanding of how the virus subverts the host immune system.

1.2.8 HCMV protein spanning peptide pools

Protein spanning peptide pools (PSPPs) are mixtures of overlapping peptides (typically 15-amino acids (aa) in length), spanning the entire length of a protein of interest. A number of studies evaluating the immunologic responses to proteins of interests have successfully used PSPPs to measure both CD4 and CD8 T cell responses (69-73). The advantage of this approach is that it can be used in T cell stimulation assays irrespective of study participants Human Leukocyte Antigen (HLA) type ["Tissue type"].

In a 2005 study by Sylwester and colleagues, overlapping PSPPs comprising 213 HCMV proteins were used to characterise the T cell responses in 33 HCMV seropositive adults (41). 151 of these proteins were found to be immunogenic for $CD4^+$ and/or $CD8^+$ T cells. In order to establish if fewer than all proteins could be used to predict the size of the summated response to all proteins, all individual proteins were ranked by summated response (i.e. all responses from all donors were added up per protein). This was done separately for $CD4^+$ and $CD8^+$ T cells. Next, the sum for the top ranked protein, the sum for the top 2 ranked proteins, the sum for the top 3 ranked proteins and so on were correlated with the summated response to all proteins until the Pearson correlation coefficient was above 0.9, which allows prediction of the size of the overall summated response with reasonable accuracy.

This required the top 6 ranked $CD4^+$ target proteins for the $CD4^+$ response and 15 ranked $CD8^+$ target proteins for the $CD8^+$ response. Two proteins, UL83 (pp65) and UL99, were contained both in the top 6 $CD4^+$ and 15 $CD8^+$ target proteins, so that a total of 19 proteins were selected. These 19 HCMV PSPPs formed the basis of our study interrogating the size of the HCMV-specific T cell responses in individuals aged 19 to 85 years old.

1.2.9 Detecting Antigen Specific T cells

There are numerous tools currently available for the detection and enumeration of antigen specific T cells. Different techniques using Multimers (Tetramers, Dextramer etc.), ELISPOT, Proliferation assays and Intracellular cytokine assays enable the detection of antigen specific T cells. The advantage of analysing T cells using flow cytometry is the ability to simultaneously evaluate multiple characteristics at a single cell level using several markers thereby allowing the assessment of the magnitude, phenotypic attributes and functional capabilities of T cells of interest.

Flow cytometry enables the measurement of different parameters such as size, granularity and fluorescence of a single cell in a flow stream. Each cell passes through a laser causing the light to be scattered, this results in the excitation of the fluorescently-conjugated monoclonal antibody attached to the cell and emission of fluorescence. Highly sensitive photomultiplier tubes detect the scattered light, providing information on the size, granularity and fluorescence emission of cells analysed. It is important to note that increasing the number of fluorescent dyes in a multi-colour assay invariably causes widespread spectral overlap between detectors due to the combination of multiple fluorochromes. This can be minimised by careful selection of fluorochromes and appropriate compensation controls.

1.3 AGEING OF THE IMMUNE SYSTEM - IMMUNOSENESCENCE

As humans age, there is a decline in naive T cell output due to thymic involution –a decrease in size, weight and activity of the thymus gland (74). TCR diversity becomes restricted and cell numbers are increasingly maintained through long lived memory T cells and T cell division outside the central lymphoid organs.

Immunosenescence is the gradual weakening of cellular and molecular functions of the

aging immune system. It is characterized by the presence of low numbers of naive T cells and accumulation of memory T cells coupled with chronic low grade inflammation. Although homeostatic mechanisms are in place to ensure turnover of existing memory T cells and retention of immune characteristics, HCMV is thought to skew this mechanism by promoting expansion of low affinity, terminally differentiated memory T cells thereby "crowding out" other memory T cells possibly leaving the host vulnerable to new pathogens particularly in old age (64). In a study of seronegative renal transplant recipients experiencing a primary HCMV infection, the researchers observed no change in absolute numbers of pre-existing memory T cells against EBV and influenza in peripheral blood of study participants in the presence of CMV-specific $CD8^+$ memory T cells (75).

1.3.1 HCMV and the immune risk phenotype

The Swedish OCTO and follow on NONA longitudinal studies identified a set of factors termed the 'Immune risk phenotype' (IRP) which was predictive of 2-year mortality in elderly people aged 86-94 years in their cohort (76, 77). The IRP was associated with HCMV sero-positivity amongst other factors such as an inverted CD4:CD8 ratio, accumulation of differentiated $CD8^+CD28^-$ T cells, and poor proliferative responses. However, a direct correlation between HCMV infection and increased mortality was not established in this study.

1.3.2 Expansion of HCMV-specific T cells

It is widely speculated that the large expansion of HCMV-specific $CD8^+$ T cell populations in aged individuals could potentially have deleterious effects on the maintenance of a diverse TCR repertoire, by crowding out $CD8^+$ T cells of other specificities. In a 5-year longitudinal study of individuals with a primary HCMV infection the authors analysed the diversity of T cell clones against immuno-dominant

pp65 peptides from early primary response through to memory differentiation. They reported the disproportionate contraction of certain clones which were abundant in the primary response, and late expansion of clones that were subdominant in the primary response. Interestingly, all dominant clones selected into the memory pool showed similar high functional avidity of their TCR whereas the clones that contracted substantially had lower avidity (78).

A number of studies have described clonally-expanded HCMV-specific memory T cells as being predominantly dysfunctional, with poor proliferative capacity, and inability to produce cytokines such as IFNγ upon challenge (79, 80). However, previous work by our group did not find large HCMV-specific T cell responses to be any more dysfunctional than small responses (81).

It is likely that the manner in which the host immune system deals with HCMV is of more importance and has more effect on longevity than simply being HCMV seropositive. This was suggested in a recent study by Derhovanessian et al., 2010 who analyzed samples from the off-spring of long-lived families and their spouses. It was revealed that although HCMV infection was associated with an age related reduction in the frequency of naive T cells and accumulation of terminally differentiated T cell subsets in the spouses, this was not the case in offspring of long lived families, indicating that their immune systems dealt with the HCMV infection differently (82). It would seem that genetic factors must play a big role in the way the immune system deals with HCMV.

1.3.3 CD57-marker of terminal differentiation

Human natural killer (HNK-1) epitope was first described on HNK cells more than two decades ago; further research has however highlighted its importance as a marker of terminal differentiation or clonal exhaustion on T lymphocytes (83). This marker is now commonly referred to as $CD57^{+}$ antigen and is expressed on a subset of $CD8^{+}$ and to a

lesser extent $CD4^+$ T lymphocytes, in healthy individuals. The frequency of $CD8^+$ $CD57^+$ T cells has been shown to increase with age, possibly a result of life long exposure to pathogens. Indeed, at birth all T cells express CD28 ($CD28^+$) and are CD57 negative ($CD57^-$), whereas in those aged 80 and above, more than half of $CD8^+$ T cells lack CD28 ($CD8^+$ $CD28^-$). Some studies have observed an increase in $CD8^+CD57^+$ T cells particularly in individuals with chronic viral infections such as HCMV, Human immunodeficiency virus (HIV), Epstein Barr virus (EBV) and Hepatitis C virus [Reviewed in (84)].

It has been observed that $CD8^+$ $CD57^+$ T cells are mostly specific for HCMV and EBV antigens in otherwise healthy individuals, thereby indicating that chronic infection and not necessarily age may be promoting the accumulation of these T cells (85, 86).

$CD8^+$ $CD57^+$ T cells are thought to be highly cytotoxic expressing molecules such as perforin and granzymes, whilst exhibiting reduced proliferative capacity and reduced telomerase activity, hence their association with senescence. However, the senescent status conferred on this T cell subset remains contentious as a few groups have demonstrated their proliferative capabilities under certain stimulation conditions.

$CD8^+CD57^+$ T cells are thought to be highly resistant to apoptosis which could possibly explain why high frequencies of this subset are observed in those with a persistent viral infection such as HCMV (87).

1.3.4 HCMV and Inflammation

Aside from the reported changes in the adaptive immune system which are characteristic of the immunosenescence process, infection with HCMV has also been linked to conditions with an inflammatory component such as cardiovascular diseases, cognitive decline and impairment (88, 89). This could be a cumulative effect due to

reactivation of HCMV from its latent state in infected individuals driving up levels of pro-inflammatory cytokines.

The association between HCMV infection and cardiovascular pathology has been observed by several researchers; recently our group reported a link between the size of the HCMV specific $CD8^+$ T cells and resting blood pressure in aged (60-85 years) HCMV seropositive individuals (90-93). In support of the role of HCMV in vascular pathology, Bentz & colleagues observed HCMV infection of endothelial cell induces angiogenesis (formation of blood vessels from pre-existing ones) potentially showing a link between viral infection and vascular disease (94).

1.3.5 Does HCMV infection have a role in cognitive decline?

The role of HCMV in predicting lower cognitive functioning in otherwise healthy older individuals has been of great interest (89, 95-97). Using a wide range of standardized cognitive measures, Gow and colleagues reported an association between high HCMV antibody levels and lower general cognitive abilities in a cohort of healthy HCMV seropositive individuals with a mean age of 70. The association still remained after a number of potential confounding factors including background and early social environmental factors were considered (98).

A recent study analysing samples from young (mean age 32.8 years) HCMV infected individuals reported an association between HCMV antibody levels and reduced cognitive functioning based on a plethora of cognitive assessment tools (99).

HCMV infection has also been implicated in depression and wellbeing as demonstrated in a study investigating the role of HCMV in older people with depression and anxiety. It was observed that individuals with higher HCMV IgG titres reported symptoms of depression and anxiety (100).

However, this could be an association with inflammation as another study of community

dwelling individuals aged more than 81 years, reported no association between HCMV infection, cognitive functioning as measured by mini mental state examination (MMSE) or suspected depression (101).

1.4 STUDY AIMS

The central aim of this project was to explore the size of the HCMV-specific T cell immune response in different age groups and to evaluate the general health and wellbeing of study participants.

For this purpose, we have used mainly flow cytometry assays for enumerating and characterizing HCMV-specific T cells. To determine the effect of HCMV infection on health related quality of life, all participants were asked to complete the RAND-36 questionnaire at the initial assessment with research nurse. Additionally, participants aged 60 and above were asked to keep a monthly health diary for a period of 2 years, reporting acute infections such as head cold, urine infection, other infection, and other health related problems experienced within that period.

The specific aims of this project are outlined below:

1. To compare the magnitude of HCMV-specific T cell responses in groups of Young, Older and Oldest HCMV seropositive individuals
2. To characterise and compare HCMV- specific memory subset distribution in all three age groups
3. To determine the level of T cell polyfunctionality of HCMV-specific T cells in these groups
4. To establish the effect of HCMV infection on the health related quality of life in HCMV seropositive individuals using data from the widely used survey instrument RAND-36 and comparing data from health diary records collected over a 2 year period in the Older and Oldest age groups

1.4.1 Hypotheses

1. There is at least a doubling of the proportion of HCMV-specific T cells in the Oldest group compared to the Young.
2. There are significant differences in the memory subset distribution between all three age groups
3. There is a decrease in polyfunctionality of HCMV-specific T cells with increasing age
4. HCMV infection has significant effects on the health related quality of life based on the RAND-36 scores and data from monthly health diaries

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Laboratory equipment

a. Sterile hood (Heraeus, Buckinghamshare, UK)
b. Incubator (Heraeus, Buckinghamshare, UK)
c. Haemocytometer (Neubauer Improved)
d. Centrifuge Multifuge 3 (Heraeus, Buckinghamshare, UK)
e. Flow cytometer LSR II (BD, Biosciences, USA)
f. FACS tubes (BD, Biosciences, USA)

2.1.2 Buffers and Solutions

FACS wash buffer: Sterile water, Phosphate buffered saline (PBS, Fisher Scientific), 0.5% Bovine Serum Albumine (BSA, Acros Organics), and 0.1% Sodium azide (Sigma Aldrich, USA).

Fixing solution: 0.5% Paraformaldehyde [(PFA), Biochrom, Germany] dissolved in FACS wash buffer stored at 4°C until use.

Ethylenediaminetetraacetic acid (EDTA): 500mM stock solution (Sigma Aldrich, USA) stored at room temperature until use; diluted to 20mM in FACS wash buffer and stored at 4°C.

Lysing Solution: 10x concentration (BD, Biosciences, USA) is diluted 1 in 10 (1X) according to manufacturer's instructions using distilled water and stored at room temperature.

FACS Permeabilisation II Solution: 10x concentration (BD, Biosciences, USA) is diluted 1 in 10 (1X) according to manufacturer's instructions using distilled water and stored at

room temperature.

2.1.3 Cell culture reagents

Brefeldin A (BFA): BFA (Sigma Aldrich, USA) dissolved in 1ml dimethyl sulphoxide (DMSO) to 5mg/ml and stored at -20°C until use. 1µl of BFA is used per 500ml of stimulation to give a final concentration of 5µg/ml.

Complete Media: Roswell Park Memorial Institute (RPMI) 1640 media (Gibco, Invitrogen), 100 U/ml Penicillin (Invitrogen), 100 U/ml Streptomycin (Invitrogen), 2mM L-glutamine (Invitrogen), 10% FCS (Invitrogen). The media was stored at 4°C until use and discardedafter 2 weeks.

Ficoll-Paque PLUS: Ficoll PM400 5.7g, Diatrizoate Sodium 9.0g plus Edetate Calcium Disodium in Purified Water (GE Healthcare Life Science) stored at room temperature.

Monensin (BD Biosciences, USA) stored at -4°C

Sterile Phosphate Buffered Saline (PBS): Sterile PBS (Fisher Scientific) 140mM NaCl, 2.7mM KCl, 10mM Na2HPO4, 1.8mM KH2PO4, pH 7.4 stored at room temperature.

2.1.4 Antibody panels

Laser	Short pass (SP)	Long Pass (LP)	Antibody	Clone	Amount (µl)/test	Company
BLUE 488nm	530/30	505	IL-2 FITC	5344.111	12	BD Biosciences, USA
	575/26	550	CD27 PE	L128	3	BD Biosciences, USA
	610/20	600	CD45RA ECD	2H4	3	Beckman Coulter
	670/14	635	CD4 PerCp	OKT4	2	Biolegend
	780/60	755	IFNγ PeCy7	B27	3	Biolegend
RED 633nm	660/20		CD107a APC	H4A3	1.5	BD Biosciences, USA
	730/45	710	TNF-α Alexa 700	Mab11	1.5	BD Biosciences, USA
	780/60	755	CD8 APC H7	SK1	1.5	BD Biosciences, USA
VIOLET 405nm	450/50		CD40L PB	24-31	0.6	Biolegend
	525/50	505	CD3 v500	UCHT1	5	BD Biosciences, USA
	585/42	560	Live/dead yellow stain	-	0.1	Invitrogen

Table 2.1. List of antibodies for intracellular cytokine staining (ICS)

Laser	Fluorochrome	Antigen	Clone	Amount (µl)	Company
BLUE 488nm	FITC	CD57	NC1	5	Beckman Coulter
	PE	CD45	J33	1	Beckman Coulter
	PerCP	CD4	OKT3	3	Biolegend
RED 633nm	APC	CD8		3	BD
VIOLET 405nm	PB	CD3	UCHT1	3	Biolegend

Table 2.2: List of antibodies for whole blood assay

2.1.5 Antigens

HCMV PSPP: PSPP were synthesised using solid-phase peptide synthesis method (JPT, Berlin, Germany). Peptide sequence is 15mers, over-lapping by 10 or 11 amino acids. Each freeze dried aliquot of PSPPs was reconstituted in 100µl of DMSO and stored in -20°C freezer until use. Final concentrations were 1µg /ml per stimulation tube. Quality control (QC) included Mass spectroscopy and high performance liquid chromatography (HPLC), synthesized PSPPs were at least 80% pure, but generally > 90% pure. Antigens listed from 15 to 18 in Table 2.3 contain 2 HCMV PSPPs. These PSPPs were combined to minimize reagent costs and to reduce the number of peripheral blood mononuclear cells needed to set up antigen stimulation assay due to limited amount of blood samples collected from participants (approximately 25ml per donor).

Antigen	Protein ID	Protein ID (Swiss prot)	Number of peptides	Company
1	SEB (Positive control)	-	-	Sigma Alrich, USA
2	DMSO (negative control)	-	-	Fisher, UK
3	UL 55	P06473	224	JPT, Germany
4	UL 83(pp65)	P06725	138	JPT, Germany
5	UL 86		340	JPT, Germany
6	UL 122(IE-2)	P19893	143	JPT, Germany
7	UL 123 (IE-1)	P19893	120	JPT, Germany
8	UL 99	P13200	45	JPT, Germany
9	UL 153	P19893	67	JPT, Germany
10	UL 32	P08318	260	JPT, Germany
11	UL 28	P16847	92	JPT, Germany
12	UL 48A	P16785	281	JPT, Germany
13	UL 48B	P16785	281	JPT, Germany
14	US 3	P09712	44	JPT, Germany
15	UL 151 + UL 82	Q68405 / P06726	82 + 137	JPT, Germany
16	UL 94 + US 29	P16800 / P09705	84 + 113	JPT, Germany
17	UL 103 + US 32	P16734 / P09708	60 + 43	JPT, Germany
18	US24 + UL36	P09700 / P16767	123 + 117	JPT, Germany

Table 2.3. List of antigens including HCMV PSPP used for stimulation assay.

Purified Protein Derivative (PPD): PPD (SSI, Denmark) from mycobacterium tuberculosis reconstituted in 1ml of sterile PBS and stored at -20°C until use. It was used at a final concentration of 5µg/ml.

Staphylococcus Enterotoxin B (SEB): SEB (Sigma Aldrich, USA) was reconstituted in sterile PBS to 1mg/ml and stored at -20°C until use.

2.2 METHODS

2.2.1 Ethical Approval

The study was approved by the UK National Research Ethics Service (09/H1102/84). This project was conducted according to the Declaration of Helsinki; all participants gave written informed consent.

2.2.2 Donor Recruitment

Healthy Young volunteers aged between 18 and 35 years were recruited from Brighton & Sussex Medical School (BSMS) through advertisements on student notice boards and e-mail. Elderly volunteers aged between 60 and 85 were recruited from General Practices in South East England, with assistance from the Primary Care Research Network (PCRN). This cohort was further divided into two distinct groups based on age, classified as "Older" (60-70 years) and "Oldest" (75-85 years) (Refer to Table 2.4).

2.2.2.1 Inclusion Criteria

Healthy individuals aged between 18 to 85 years

2.2.2.2 Exclusion Criteria

Please note that young participants were directly asked by research nurse if they met any of the exclusion criteria before enrollment into the study, whereas the GP records of all old (combination of 'Older' and 'Oldest' cohort) volunteers were assessed to ensure they met study criteria before being enrolled into the study. The exclusion criteria includes the following;

- Any recorded diagnosis of immunodeficiency (including HIV-infection)
- Organ transplantation, use of immunosuppressive or immune modulating drugs within the last year

- Cancer diagnosis or treatment for cancer within the previous 5 years of study start date
- Diagnoses of insulin dependent diabetes mellitus
- Moderate to advanced renal failure (estimated GFR < 20 ml/min)
- Severe liver disease (known hepatitis or cirrhosis)
- Endocrine disorders
- Autoimmune disease
- Dementia or mental incompetence
- Alcohol or other form of drug abuse
- Participants with acute infections or recent infections had their appointments rescheduled for at least a minimum of 4 weeks after full recovery

Groups	n	HCMV Seropositive n=95 (51.1%)		TOTAL	HCMV Seronegative n=91 (48.9 %)		TOTAL	Median age (Range)
		Male	Female		Male	Female		
Young	55	8	18	**26**	11	18	**29**	27 (19-35)
Older	71	20	20	**40**	16	15	**31**	64 (60-70)
Oldest	60	15	14	**29**	18	13	**31**	78 (75-85)
Total	186	43	52	**95**	45	46	**91**	

Table 2.4. Participant demographics including HCMV IgG serology status

2.3 EXPERIMENTAL PROTOCOLS

2.3.1 Peripheral Blood Mononuclear Cell (PBMC) Isolation

Heparinized blood was diluted with an equal volume (1:1 dilution) of sterile PBS (Fisher Scientific). Using a sterile Pasteur pipette, diluted blood was gently layered onto Ficoll-Paque PLUS (GE Healthcare Life Sciences) in a 50ml Falcon tube. PBMC was separated by centrifugation at 1000xg with no brakes for 20 minutes at room temperature. The PBMC layer was gently aspirated using a Pasteur pipette and transferred to a 50ml falcon tube. Sterile PBS was added up to 50ml and centrifuged at 300xg for 10 minutes at room temperature to eliminate red cell debris. The supernatant was decanted before re-suspending the pellet in sterile PBS up to 50ml and centrifuged at 200xg for 10 minutes at room temperature. The supernatant was decanted, and the 'clean' PBMC pellet was re-suspended in 1ml of complete media. Cells were counted using a haemocytometer at a 1:10 dilution in acetic acid to exclude dead cells. PBMCs were finally re-suspended at $5x10^6$ per ml in complete media. Cells were incubated at 37°C and 5% humidified CO_2 at a slant position before antigen stimulation.

2.3.2 Granulocyte Isolation

After isolation of PBMC from blood samples, a thin white layer of granulocytes above the red blood pellet was collected with the aid of a sterile Pasteur pipette, and transferred to a 50ml falcon tube. The cells were re-suspended up to 50ml of FACS wash buffer and centrifuged at 400xg for 8 minutes. After supernatant was decanted, 5ml of 1X lysing solution (BD Biosciences, USA) was added to the tube, sample was vortexed and incubated at room temperature for 10 minutes. FACS wash buffer was added up to the 50 ml mark and the sample was centrifuged at 400g for 8 minutes. Once the supernatant was decanted, pellet was vortexed and the sample was transferred to a labelled cryovial and stored in -80°C freezer.

2.3.3 Serum Sample Preparation

Whole blood samples were collected in red topped vacutainers (5ml vacuette, Clot activator (spray dried), BD Bioscience, USA). The vacutainer was inverted a couple of times and left upright at room temperature for at least an hour. Tubes were centrifuged at 1000xg for 10 minutes at room temperature. Serum sample was pipetted carefully into labelled cryovials (2x-250µl and 1x-50µl) and stored in –80°C freezer until use.

2.3.4 HCMV IgG Serology

Labelled frozen serum samples were thawed on ice and transported to the Brighton and Sussex University Hospital (BSUH) virology laboratory where levels of HCMV-IgG were measured (Architect CMV IgG, Abbot, Maidenhead, UK) according to hospital protocols.

2.3.5 Specimen storage

In addition to blood samples, urine and saliva samples were collected as part of study protocol from consented participants aged 60-85 years old, by the research nurse. Granulocytes were isolated from most volunteers for downstream DNA extraction and stored at -80°C. Urine samples were collected in 20ml universal pots and left at room temperature. Urine samples were transferred into labelled cryovial tubes (2X, 1ml) and stored at –80°C. Saliva samples were initially collected in sterile universal pots, these were transferred into labelled eppendorf tubes and stored in –80°C freezer.

2.3.6 White blood count (WBC)

5 µl of heparinised blood sample was added to 95µl (1:20) of 3% acetic acid. The diluted sample was mixed thoroughly using a pipette to lyse red cells. 10µl of the sample was loaded onto a haemocytometer and white blood cells were counted. The absolute cell number per ml of blood was recorded for each sample.

2.3.7 Whole blood staining assay

50 µl of heparinized whole blood was added to a labelled FACS tube. Antibodies for cell surface markers (listed in table 2.2) were added to the FACS tube and mixed thoroughly by gently pipetting. The tube was covered with aluminum foil and incubated at 4°C for 30 minutes. After incubation, 1ml of 1X lysing solution (BD Biosciences, USA) was added to the tube and pipetted gently to lyse red cells. Tube was vortexed and incubated at room temperature in the dark for 10 minutes. Following lysis, cells were re-suspended in FACS wash buffer and centrifuged at 400xg for 8 minutes. Supernatant was decanted, and cells were acquired on the flow cytometer immediately.

2.3.8 PBMC Stimulation

5ml polypropylene FACS tubes were labelled prior to experimental set up. Stimulation of PBMC was set up in 250µl volume. PBMC antigen stimulation was set up by adding 2µl of each HCMV PSPP (0.5 µg) listed in Table 2.3 (row 3-18), once vortexed vigorously. In addition to this, 1.5µl of CD107a (BD Bioscience, USA), 0.5µl of Monensin (BD Bioscience, USA) and varying amounts of complete media up to 50 µl volume were added to corresponding tube.

For the control tubes, 1µl of SEB and 2µl of DMSO was added to the appropriately labelled tube, in addition to this, 1.5µl of CD107a (BD Bioscience, USA), 0.5µl of Monensin (BD Bioscience, USA) and varying amounts of complete media up to 50 µl volume were added to each tube (Table 2.5).

200µl of cell suspension (1 x 10^6 PBMC/ml) was added to each stimulation tube, resulting in a final volume of 250µl. FACS tubes were vortexed and placed in a slant position at 37°C in a 5% humidified CO_2 incubator.

250 µl of BFA suspension (1µl BFA + 249µl of complete media) was added to each stimulation tube after 2 hours. Samples were vortexed and incubated for a further 14 hours overnight at 37°C in a 5% humidified CO_2 incubator.

Tube number	Antigen	Volume of antigen (µl)	Volume of CD107a (µl)	Volume of Monensin ((µl)	Volume of complete media (µl)
1	SEB (Positive control)	1	1.5	0.5	47
2	DMSO (Negative control)	2	1.5	0.5	46
3	UL-55	2	1.5	0.5	46
4	UL-83 (pp65)	2	1.5	0.5	46
5	UL-86	2	1.5	0.5	46
6	UL-122 (IE-2)	2	1.5	0.5	46
7	UL-123 (IE-1)	2	1.5	0.5	46
8	UL-99	2	1.5	0.5	46
9	UL-153	2	1.5	0.5	46
10	UL-32	2	1.5	0.5	46
11	UL-28	2	1.5	0.5	46
12	UL-48A	2	1.5	0.5	46
13	UL-48B	2	1.5	0.5	46
14	US-3	2	1.5	0.5	46
15	UL-151 + UL-82	2 + 2	1.5	0.5	44
16	UL-94 + US-29	2 + 2	1.5	0.5	44
17	UL-103 + US-32	2 + 2	1.5	0.5	44
18	US-24 + UL-36	2 + 2	1.5	0.5	44

Table 2.5: Layout and volumes of antigen for overnight stimulation of PBMC

2.3.9 Surface and Intracellular Staining (ICS) Assay

Following overnight 14 hour incubation, 100µl of EDTA was added to each stimulation tube. Samples were vortexed and incubated at room temperature for 10 minutes.

3ml of FACS wash buffer was added to each tube and centrifuged at 400xg for 8 minutes at 4°C; supernatant was decanted completely by blotting on paper. Once surface staining antibodies (CD3 v500, CD4 PerCP, CD8 APC-H7, CD45RA ECD, CD27 PE and live/dead yellow as outlined in Table 2.1) were added to each tube, samples were vortexed, covered with aluminium foil and, incubated at 4°C for 30 minutes. Following surface staining, 1ml of IX lysing solution (BD Biosciences, USA) was added to each tube. Samples were vortexed and incubated at room temperature for 10 minutes in the dark. 3ml of FACS wash buffer was added to each tube and centrifuged at 400xg for 8 minutes at 4°C; the supernatant was decanted and all sample tubes were vortexed. 1ml of 1X FACS Permeabilisation II Solution was added to each tube, and vortexed. Tubes were covered in the dark and incubated at room temperature for 10 minutes.

3ml of FACS wash buffer was added to each tube and centrifuged at 400xg for 8 minutes at 4°C; the supernatant was decanted completely by blotting on paper. Intracellular antibodies (CD40L PB, IFNγ PeCy 7, TNFα Alexa 700 and IL-2 FITC as outlined in Table 2.1) were added to the tubes and cells were incubated, covered with aluminium foil at 4°C for 30 minutes. Cells were re-suspended in 3ml of FACS wash buffer and centrifuged (at 4°C for 8 minutes at 400xg) before incubating with 1ml of fixing solution (0.5% PFA) for 5 minutes covered at room temperature. PBMCs were re-suspended in FACS wash buffer and centrifuged (at 4°C for 8 minutes at 400xg). Supernatant was decanted and tubes were covered with aluminium foil at 4°C ready for acquisition.

2.3.10 Compensation controls

FACS tubes were labelled for all fluorochromes used in experimental set up - one tube for each fluorochrome used. All compensation control beads (BD Biosciences, USA) were vortexed before use. A drop of positive and a drop of negative mouse IgG1 compensation control beads was added to each labelled tube. The appropriate antibody (half of the volume used in the staining protocol was added, for example 6ul of IL-2 FITC) was added to each labelled tube was vortexed.

The FACS tubes were covered with aluminium foil and incubated for 30 minutes at 4°C. 3ml of FACS wash buffer was added to each tube and centrifuged (at 4°C for 8 minutes at 400xg). Supernatant was decanted and the tubes were covered with aluminium foil and stored at 4°C before use.

2.4 FLOW CYTOMETRIC GATING STRATEGY

Flow cytometry acquisition was carried out on BD LSR II using FACSDIVA v6.1.3. An average of 200, 000 events was acquired for each sample. The data from experiments were analysed using FlowJo 9.3.3 software. $CD4^+$ and $CD8^+$ positive T cells were selected using the strategy highlighted in Figure 2.1. Briefly, lymphocytes were gated in a SSC vs. FSC plot, and doublets were eliminated from downstream analysis using FSC-H vs. FSC-A gate, with single events presented in a diagonal display. All the single cells were plotted against the live dead yellow stain; "live cells" which stained negative for this dye were selected. $CD3^+$ T cells were gated in a $CD3^+$ vs $CD4^+$ and $CD3^+$ vs $CD8^+$ plot to increase resolution and allow for inclusion of $CD3^+$ cells down-regulating surface expression of $CD4^+$ or $CD8^+$ (This is essential when gating activated cells). A Boolean gate combination of $CD3^+CD8^+$ and $CD3^+CD4^+$ subsets was generated from which $CD4^+$ T cells and $CD8^+$ T cells were then gated.

The memory subsets were delineated on both $CD4^+$ and $CD8^+$ T cells based on CD45RA and CD27 expression; these are surrogate markers of memory compartment. We generated four memory subsets (T_N - $CD45RA^+$ $CD27^+$, T_{CM} - $CD45RA^-$ $CD27^+$, T_{EM} - $CD45RA^-$ $CD27^-$ and T_{EMRA} - $CD45RA^+$ $CD27^-$) and analysed the functional profile of all memory subsets on $CD4^+$ and $CD8^+$ T cells.

Individual activation markers (IL-2, IFNγ, CD107a, TNFα and CD40L) used in our ICS assay were plotted against $CD4^+$ and $CD8^+$ T cells to generate a functional profile of the HCMV specific T cells for further analysis (Figure 2.2). All functional gates were visually inspected to ensure positive events were truly positive.

The boolean gate function in FlowJo automatically generates all possible combinations (plus and minus existing gates of interest) of subsets of interest. In total, 32 subsets were generated based on a combination of the five activation markers used in our assay (Appendix, Figure 8.1). A non-functional subset with no responsive T cells to any of the activation markers was excluded. All presented data are background subtracted, this was achieved by subtracting unstimulated samples (DMSO-negative control) from stimulated sample subsets (For example DMSO-specific $CD107a^+$ $CD40L^+$ IL-2^+ $TNF\alpha^+$ $IFN\gamma^+$ subset was subtracted from pp65-specific $CD107a^+$ $CD40L^+$ IL-2^+ $TNF\alpha^+$ $IFN\gamma^+$ subset). The frequencies of T cells with the reference population are indicated where appropriate. A cut off value of 1 in 10,000 was applied after background subtraction to limit analyses of biologically irrelevant responses. This did not exclude any HCMV-specific T cells responses in seropositive donors.

Due to the large number of data generated from our experiments, we designed a database to house approximately 11,500 variables.

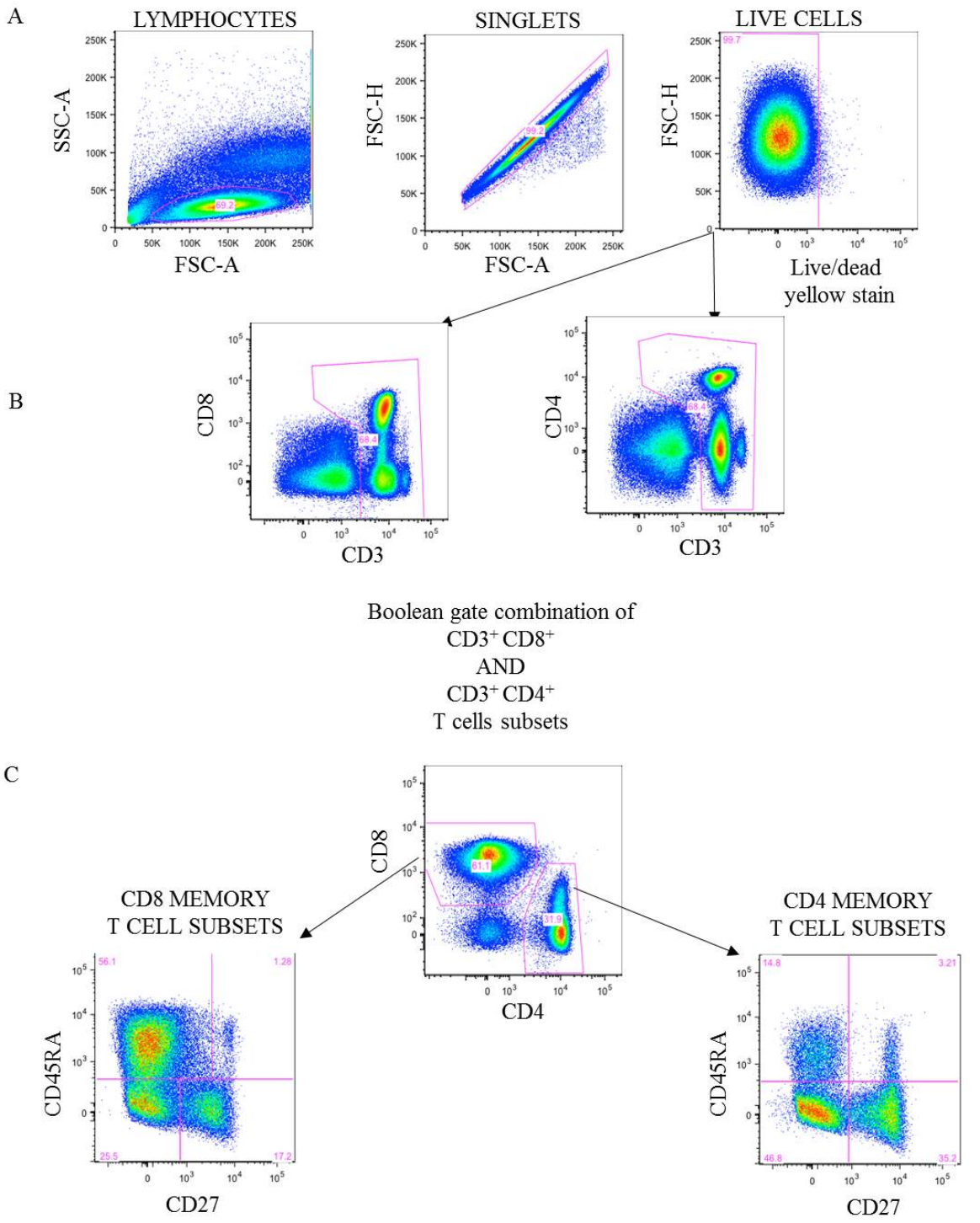


Figure 2.1. Gating strategy for characterising T cells.

The FlowJo plots above are from a pp65 stimulated sample of a seropositive elderly volunteer (HMC_130). (A)Lymphocytes were gated in a SSC vs. FSC gate and doublets were excluded using FSC-height vs. FSC-area. Live cells were gated using a live/dead yellow dye. (B) $CD4^+$ vs $CD3^+$ plots, $CD8^+$ vs $CD3^+$ plots were gated on live cells and both gates were automatically combined using the Boolean gate function in FlowJo. (C) $CD4^+$ T cells and $CD8^+$ T cells were subsequently gated and Memory T cell subsets were gated on both $CD4^+$ and $CD8^+$ T cells. Black arrow represents flow of plots.

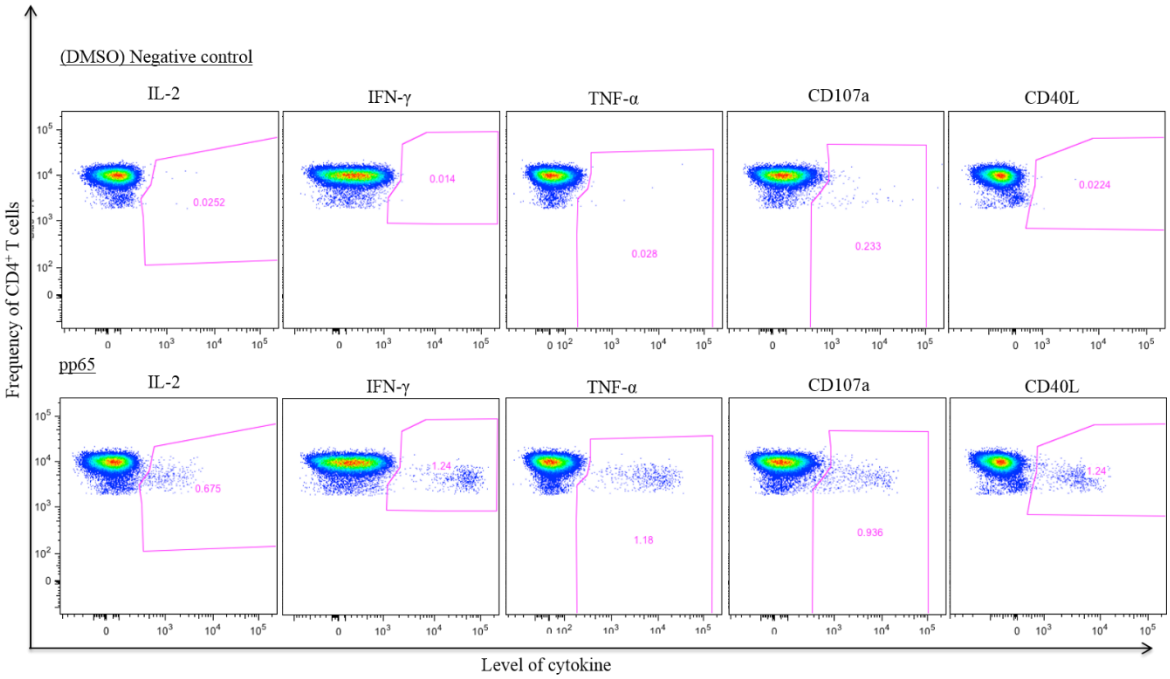


Figure 2.2. Frequency of HCMV-specific CD4$^+$ T cell with effector functions.

The figure above is a representative pseudo-colour plot of CD4$^+$ T cell responses in a seropositive individual. The top row depicts the responses against DMSO (negative control) and bottom row are plots of the pp65 specific responses. CD4$^+$ T cells are plotted against 5 individual activation markers (IL-2, IFNγ, TNFα, CD107a and CD40L).

2.5 DATA STORAGE

Raw data exported from Flowjo were processed in a MySQL database designed specifically for the study by Dr David Thomas. A Java script was written to aid the processing of raw flow cytometry data in a flexible computational pipeline to generate required reports and aid further analysis in a statistical package. Java is a computational language gaining widespread popularity due to the growth of bioinformatics and analyses of large datasets derived from gene sequencing. This language was used to create reports due to its speed of processing large datasets. The simplified flow of information into database is set out in Figure 2.3.

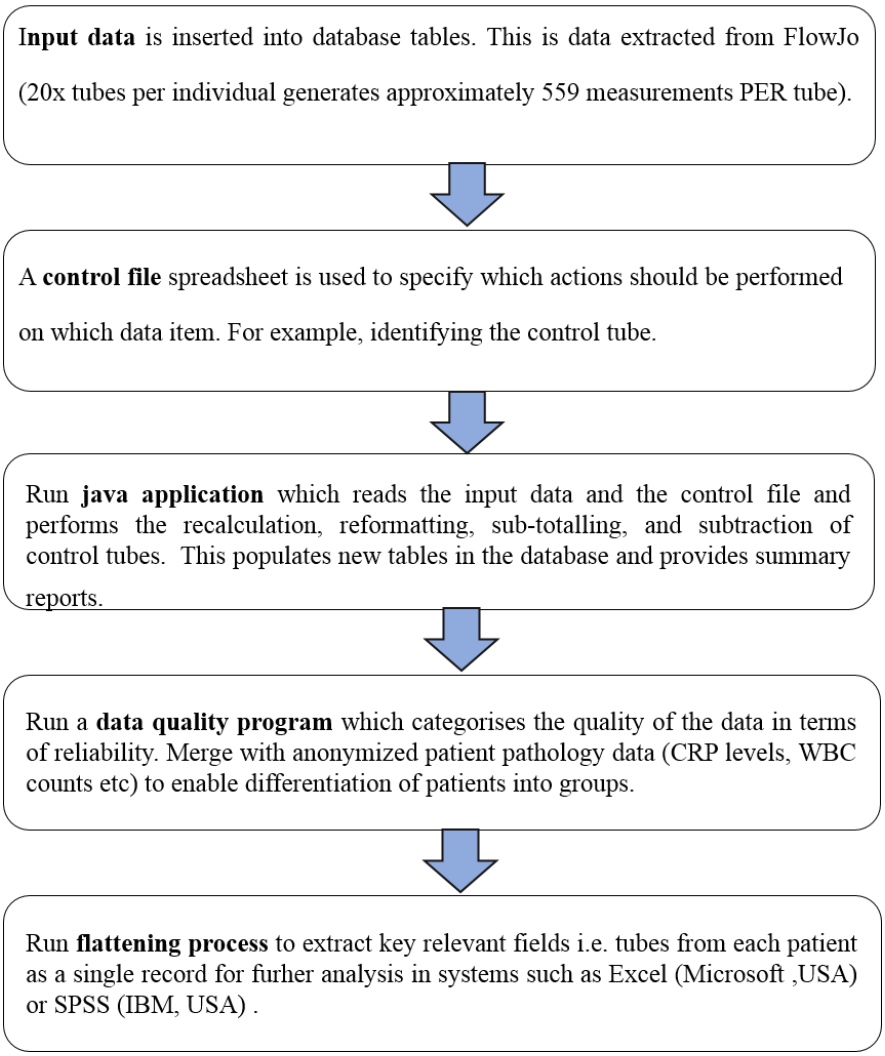


Figure 2.3 Flow diagram of data management and storage.

The flow diagram above represents a simplified flow of data from FlowJo to our study specific database to accommodate the large datasets derived from multi-parametric flow cytometry assay and additional pathology reports for each participant.

2.6 ASSESSING HEALTH-RELATED QUALITY OF LIFE USING RAND-36

The health-related quality of life (QOL) was assessed by means of a self-completed survey instrument, the RAND-36 questionnaire. All questionnaires were completed and returned to the research nurse on the same day, after consent was given.

Scores from RAND-36 questionnaire provides information on multiple aspects of health-related QOL. The questionnaire comprises of 36 items assessing 8 aspects of health listed in Table 2.6 (102).

No.	Health sub-scales	No. of items within each subscale	Scoring items (Average)
1	Physical functioning	10	3,4,5,6,7,8,9,10,11, 12
2	Role limitations due to physical health	4	13,14,15,16
3	Role limitations due to emotional problems	3	17,18,19
4	Energy/Fatigue	4	23,27,29,31
5	Emotional well-being	5	24,25,26,28,30
6	Social functioning	2	20, 32
7	Pain	2	21,22
8	General health	5	1,33,34,35,36

Table 2.6. The different sub-scales derived from the RAND-36 questionnaire.

Each item was scored on a range of 1 to 100, with higher scores indicating higher level of functioning. The score for each health sub-scale was derived by calculating the average score (for example the final value for the "Pain" sub-scale was derived by averaging the scores for item 21 and 22).

2.7 HEALTH DIARIES

Participants within the Older and Oldest groups were sent a monthly health diary (Appendix 8.8) to keep track of health related developments. Each participant was sent a total of 24 diary pages to complete over a 2 year period. Information captured in the diary

includes; number of days unwell within each calendar month, health related problems and/or infections (pain, chest infection, urine infection, head cold, and other infections) experienced. A free text box was also included to encourage participants to record any additional information. The variables listed below were derived from the diary pages:

a. Total number of days unwell

b. Total number of months with unwell days

c. Total number of months with days unwell from October to March

d. Number of months with pain

e. Number of months with chest infection

f. Number of months with urine infection

g. Number of months with head cold

h. Number of months with other infections

i. Number of months with other health problems

The proportion of health problems experienced per month was determined by dividing each variable of interest by the total number of diary pages returned to give the proportion variable per month.

For example:

The proportion of pain per month = (Number of months with pain /Total number of diary pages returned)

2.8 STATISTICAL ANALYSIS

SPSS v21 and Graphpad Prism (v6.0) software were used to construct all graphs displayed. The Gaussian distribution of the data was evaluated using Kolmogorov-Smirnov test, the majority (>80%) of which was not normally distributed. As a result, non-parametric tests were used to assess statistical significance. All p-values <0.05 were considered significant for single end-points. Bonferroni correction was applied where appropriate for multiple end point analysis.

Recent developments in polychromatic intracellular flow cytometry have enabled the simultaneous measurement of multiple activation markers per experimental set up. The use of multiple markers has consequentially resulted in researchers generating large sums of data points for individual samples. However there are no set guidelines or consensus outlining the best possible statistical methods to analyse the data generated.

The multi-parametric flow cytometry assay used in our study involved the simultaneous measurement of 5 activation markers in addition to other T cell phenotypic and memory T cell markers. $CD4^+$ and $CD8^+$ T cells responses were gated against each activation marker. Using the Boolean gate function in FlowJo, a total of 32 subsets were generated (Section 2.4).

All presented data are background-subtracted on a subset by subset basis. Responses not identifiable as a visible cluster on inspection and/or less than 0.0001 (1 in 10,000 T-cells) following background subtraction were considered absent. The analyses of the T cell response was complex due to the multiple outputs on $CD4^+$, $CD8^+$ and memory T cell subsets in both compartment.

Statistical significances between the three age groups were evaluated using Kruskal Wallis test for non-parametric data and values are expressed as medians. Analysis restricted to 2

age groups (Young and Old) were statistically evaluated using a Mann Whitney test. Correlations between variables were determined using the non-parametric Spearman rank correlation (Rs). T-cell frequencies were log-transformed where required. A p value of less than 0.05 (<0.05) was considered significant.

The polyfunctional profiles of HCMV-specific T cells were analysed using the recently described polyfunctionality index and SPICE (v5.3) software (103). The index is determined using "FunkyCells Boolean Dataminer" software (www.FunkyCells.com) (104). This evaluates the degree and variation of polyfunctionality on specific T cells of interest. The advantage of using the index is the ability to statistically compare differences between groups using both parametric and non-parametric tests. Median values were compared between the three age groups using Kruskal Wallis test for non-parametric data, the confidence interval indicated where appropriate. Where appropriate, median values were compared between two age groups using Mann Whitney U test for non-parametric data.

Statistical significance of data from the health diaries of HCMV seropositive and seronegative individuals were determined using Mann Whitney U test. The correlation between HCMV-specific CD4+ and CD8+ T cell responses and individual variables was assessed using Spearman rank correlation (Rs). A p value of less than 0.05 (<0.05) was considered significant.

Quality of life was assessed in Young, Older and Oldest groups using RAND-36 survey instrument. Significances between the three age groups were determined using Kruskal Wallis test. The correlation of HCMV- specific T cells and all 8 health sub-scales was determined using Spearman rank correlation (Rs). A p value of less than 0.05 (<0.05) was considered significant.

3 CHARACTERISING T CELL RESPONSES TO HCMV PROTEINS

3.1 OPTIMISATION OF FLOW CYTOMETRY ASSAY

A multi-parameter antibody panel was developed to enable the characterisation of T cell responses in our cohort of Young, Older and Oldest individuals. The LSRII flow cytometer with 3 lasers (Blue, Violet and Red) together with FACSDiva v6.1 software was used to detect activation markers (IL-2, IFNγ, CD107a, TNFα and CD40L) expressed by $CD4^+$ and $CD8^+$ T cell subsets. Several factors were taken into consideration before assembling the multi-colour assay used in this study. The brightest fluorochromes were assigned to weak/rarely expressed markers of interest and weak fluorochromes reserved for highly expressed, and therefore easily distinguishable markers (e.g. lineage markers such as CD3, CD4) (105, 106). CD27 was used as a phycoerythin (PE) conjugate, for ease of comparison.

Initial laboratory work involved titration of all antibody solutions under assay conditions to determine the concentration at which there was optimal signal-to-noise ratio with minimal background staining. Antibody solutions for phenotypic markers such as $CD3^+$, $CD4^+$ etc. were titrated on un-stimulated cells whereas antibodies for activation markers used were titrated on polyclonally (SEB) stimulated and un-stimulated cells (Negative Control-DMSO) in parallel. Live/dead (L/D) yellow viability dye was used to exclude dead cells from our analyses. This amine-reactive viability dye works by penetrating the damaged cell membranes of dead cells, binding to internal components, forming stable fluorescent aggregates [41].

It is important to note that increasing the number of fluorescent dyes in a multi-colour assay invariably causes widespread spectral overlap between detectors due to the combination of multiple dyes. As a result, once we identified the optimal concentration for each fluorochrome-antibody (Ab) conjugate based on noise to signal ratio and

calculation of the stain index, the full antibody panel was assembled to check for fluorescence overspill.

Fluorescence minus one (FMO) controls was performed on polyclonally stimulated cells to determine the sensitivity and level of overspill of our panel. The FMO assay was set up by excluding a single stain/marker from the panel, one at a time. This is helpful for setting positivity thresholds and to ascertain that populations positive for one marker are removed when the antibody staining for that marker is removed (107). No overspill was evident after compensation (Figure 3.1).

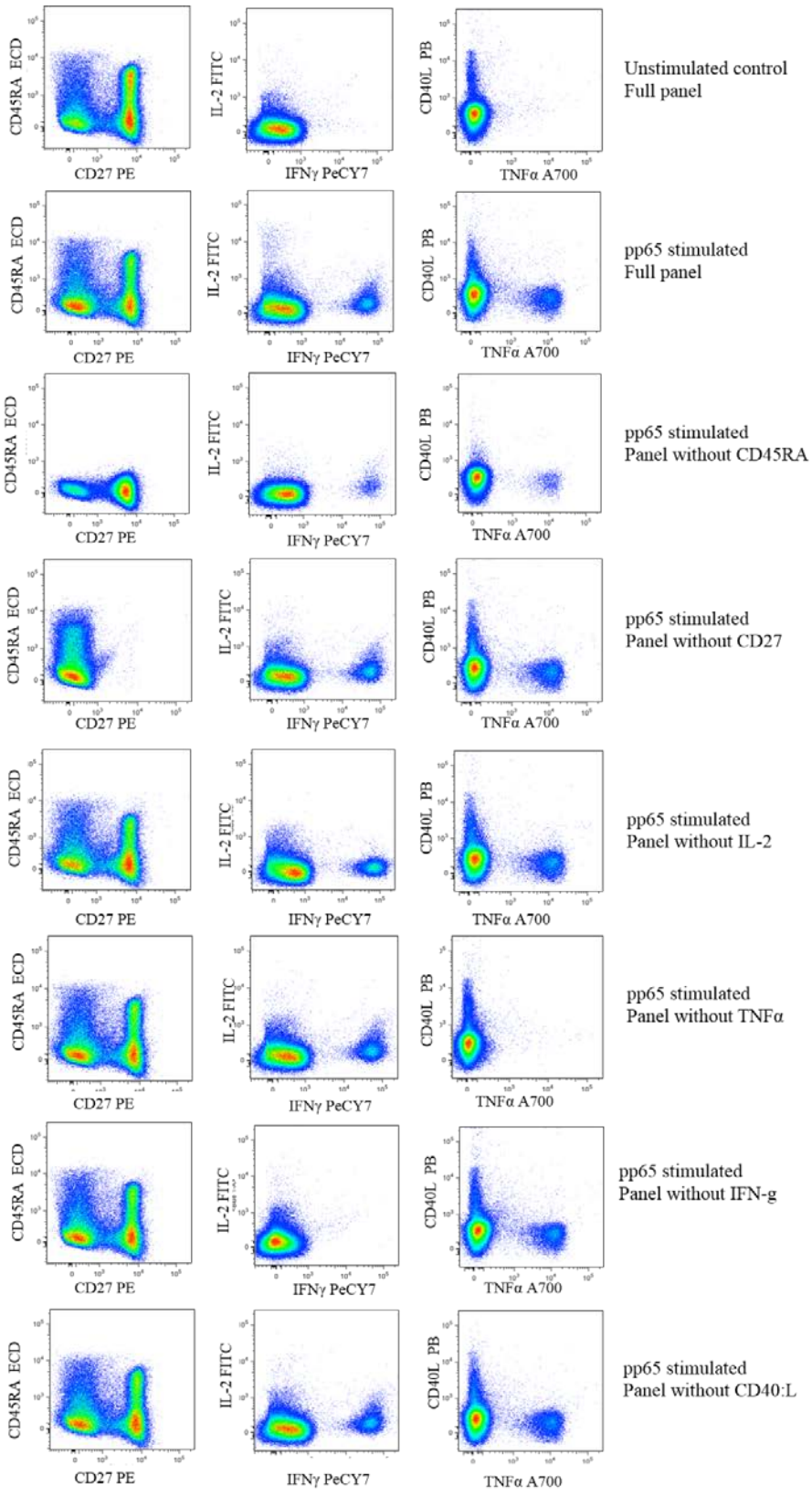


Figure 3.1. FMO controls of multi-parametric flow cytometry panel.

The pseudo-colour plot shown above is from a healthy HCMV seropositive Young volunteer. PBMCs were stimulated with pp65 antigen except where stated otherwise. The left plots display CD45RA ECD vs CD27PE, the middle plots display IL-2 FITC vs IFNγ PeCY7 and, the right plots display CD40L PB vs TNFα A700.

3.1.1 Quality Control

A multi-colour flow cytometry assay requires quality checks to be put in place to ensure consistency and normalisation in the data sets acquired between experiments set up on different days. Quality assurance checks such as the use of cytometer setting and tracking beads (CS & T, BD, Biosciences) which measures mean fluorescence intensity(MFI) target values for each channel among other relevant parameters was run before sample acquisition ensued. Photomultiplier tube (PMT) voltages suitable for each channel were noted as part of the assay optimisation. To ensure consistency in the measurements for the duration of the study, 8 peak 'rainbow beads' (BD, biosciences) were run and PMT voltages were manually adjusted so that each peak was in the same target channel prior to every experimental run [42].

3.1.2 Measuring frequency of $CD8^+CD57^+$ T cell subset in whole blood to determine HCMV serological status

At the point of blood collection and processing, it was unknown whether participants recruited to the study were HCMV seropositive or negative. HCMV status is often determined by serology, viral culture or PCR. We sought to use a surrogate marker based on frequency of $CD8^+CD57^+$ T cells to identify HCMV infected individuals. Several groups have shown a close association between increase in this T cell subset and HCMV sero-positivity (108, 109). The primary goal was to save on reagent costs and minimize time spent processing samples from HCMV seronegative individuals. Whole blood stained with the panel listed in Table 2.2 was gated initially on $CD45^+$ positive cells to include all white blood cells. The $CD4^+$ and $CD8^+$ subsets were gated on $CD3^+CD45^+$ T cells. The frequency of $CD57^+$ cell was further defined on $CD4^+$ and $CD8^+$ T cells for each participant (Figure 3.2).

Due to the logistics of the study, we were unable to use this approach until we had recruited a sufficient number of individuals. This was necessary to establish the correct cut-off using receiver operating curve (ROC curve) analysis. This is an effective method for evaluating the performance of diagnostic tests and to determine suitable cut-off values to provide the desired sensitivity or specificity. The HCMV IgG serology results (Positive or Negative, Table 3.1) were used as gold standard in order to determine the optimum threshold for our purpose. The ROC curves generated for discriminating anti-HCMV IgG seropositive from seronegative individuals is shown in Figure 3.3.

Participants categorized as 'Older' (aged 60 to 70 years) and 'Oldest' (aged 75 to 85 years) based on our initial age cut off limit were combined to generate a single cohort titled 'Old' group (aged 60 to 85 years).

The IgG serology results and $CD8^+CD57^+$ T cell frequencies from the first 99 'Old' participants (IgG negative n=44, IgG positive, n=55) and 41 Young participants (IgG negative n=22, IgG positive, n=19) were used in the ROC analysis. The analyses was performed separately in the Young and 'Old' cohort to allow for differences in frequencies of $CD8^+CD57^+$ T cell as various groups have shown an increase in the frequency of this subset with age .

The frequency of $CD8^+CD57^+$ T subset was a good predictor of HCMV seropositivity compared to "gold standard" IgG serology in both cohorts. The closer the area under the curve (AUC) value is to 1, the better the test. The AUC was 0.83 and 0.795 in the 'Old' and Young cohort respectively, equating to a very good test.

The primary goal of this exercise was to minimize the processing of samples from HCMV seronegative individuals without losing more than 10% of HCMV seropositive individuals that would ordinarily be included in the study (Table 3.1a & 3.1b). A

suitable cut off value of 12% $CD8^+CD57^+$ T cell frequency in Young and 25% $CD8^+CD57^+$ T cell frequency in the 'Old' cohort was considered of good sensitivity and specificity.

A cut off value of 12% $CD8^+CD57^+$ T cell frequency for Young (aged between 19 years and less than 60 years old) and 25% $CD8^+CD57^+$ T cell frequency for 'Old' (aged between 60 years and equal to or less than 85 years old) participants was applied henceforth to discriminate between HCMV seropositive and negative participants before commencing with full ICS analysis.

Although the cut off values were calculated with the intention of applying it to prospective study recruits, it was used in conjunction with validated HCMV IgG results. Addition of CD28 marker to the whole blood panel would have enabled better discrimination of $CD57^+$ and $CD57^-$ T cells as expressions of CD28 and CD57 are almost reciprocal on $CD8^+$ T cells.

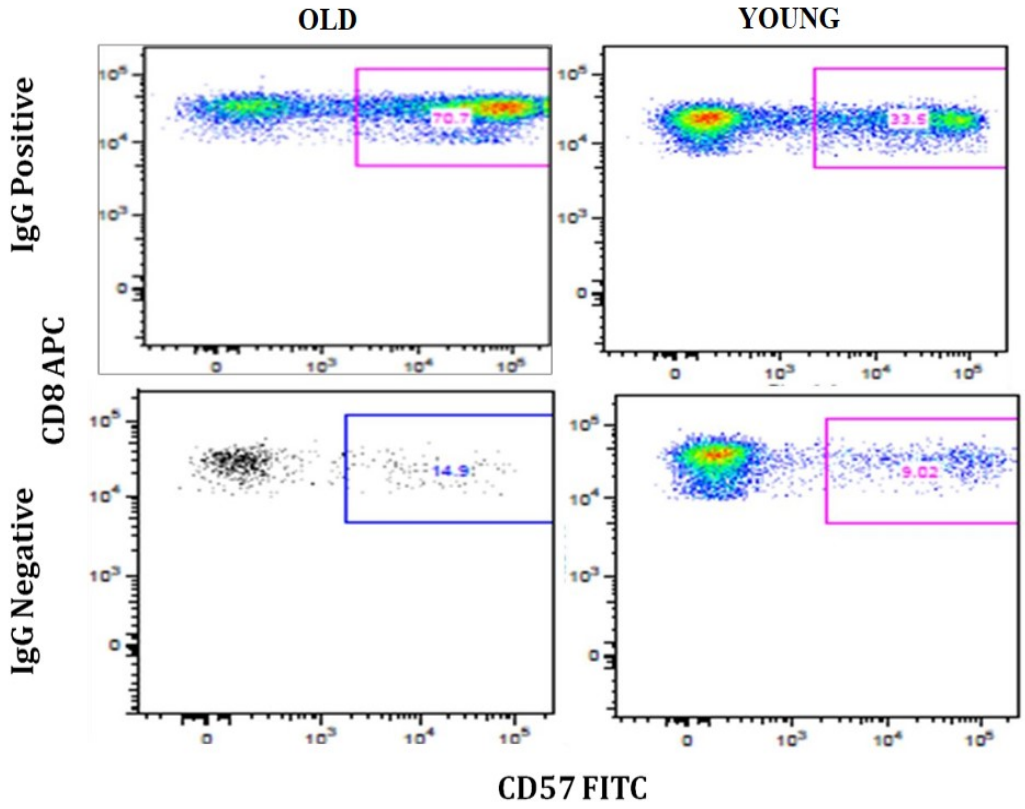


Figure 3.2. Representative plot of $CD8^{+}CD57^{+}$ T cell.

The frequency of $CD8^{+}CD57^{+}$ T cells depicted above is a pseudo colour plot of responses in 2 'Old' (Example of one HCMV IgG seropositive and one HCMV IgG seronegative donor) and 2 Young (Example of one HCMV IgG seropositive and one HCMV IgG seronegative donor) participants.

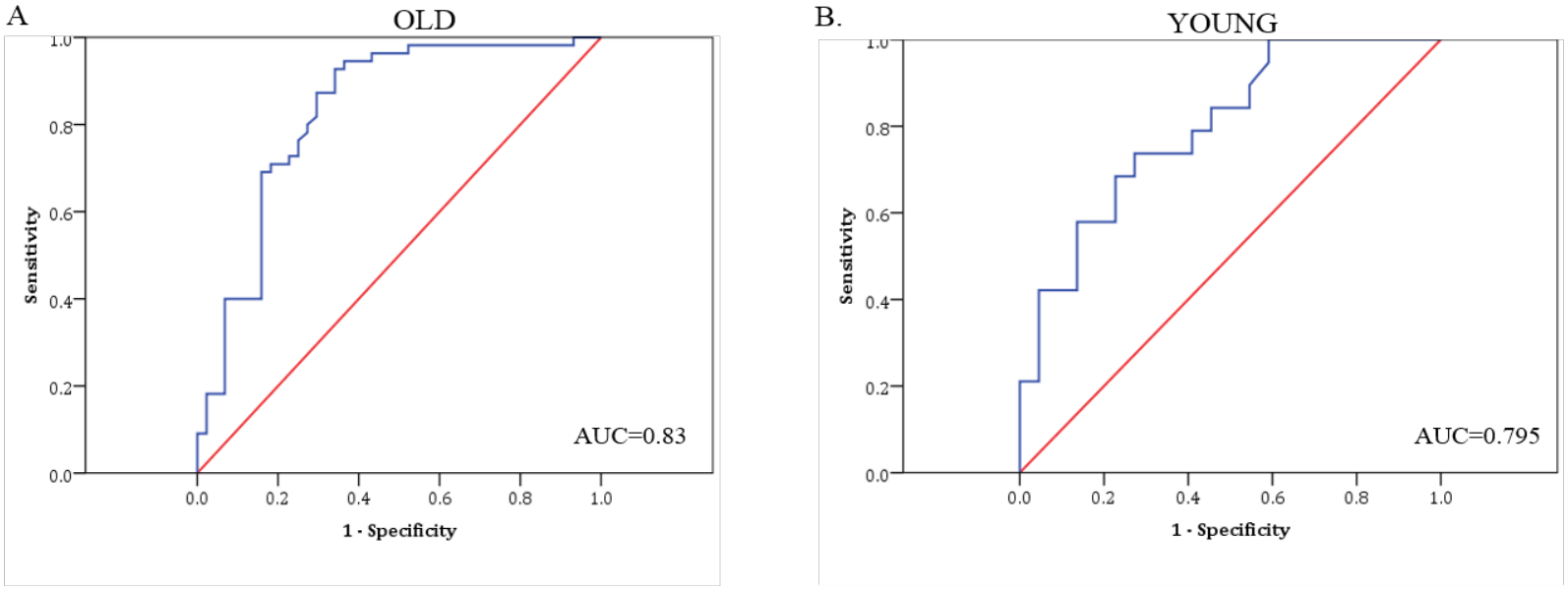


Figure 3.3. ROC curves for determining suitable frequencies of $CD8^+$ $CD57^+$ T cell cut-off.
The ROC curves depicted above were generated based on frequencies from 'Old' (A) and Young (B) cohort (Blue line) with HCMV serology defined as "Gold standard". The red line represents the line of no-discrimination. Area under the curve (AUC) for each group is shown within the plot area.

A.

		Threshold_25 (Old)		Total(n)
		0	1	
HCMV Serology	Negative	29	15	44
	Positive	4	51	55
Total		33	66	99

B.

		Threshold_12 (Young)		Total(n)
		0	1	
HCMV Serology	Negative	9	13	22
	Positive	1	18	19
Total		10	31	41

Table 3.1. $CD8^+$ $CD57^+$ threshold values for the Old and Young groups. The cross-tabs above depict the number of HCMV IgG positive and negative identified when a threshold [25 % (A) and 12 % (B)] on $CD8^+CD57^+$ T cell frequencies was applied.

3.2 T CELL RESPONSES TO HCMV PROTEINS

Persistent viruses such as HCMV have evolved immune evasive strategies to maintain latency in infected individuals. This long lasting evolutionary relationship between host and HCMV results in a fine balance of controlling virus replication whereby most infected but otherwise healthy individuals remain symptom free. However, this otherwise benign infection is thought to result in a progressive expansion of the HCMV-specific T cell with age, often leading to the "crowding out" and attrition of memory T cells of other specificities due to competition for space and growth factors (Rev. in (110) (24). These reported changes to the immune repertoire in HCMV infected aged individuals have been associated with various pathologies and a reduced lifespan (77).

$CD4^+$, and to a large extent $CD8^+$ specific T cells are important in the control of HCMV infection, they secrete cytokines such as TNFα and IFNγ with antiviral effects. The frequency of cytokine producing $CD4^+$ or $CD8^+$ specific T cells are often used as parameters to measure HCMV-specific responses after antigen stimulation

The aim of this chapter is to determine the breadth (number of responses) and magnitude (size of the responses) of T cell responses HCMV PSPPs in our study cohort (Figure 3.4).

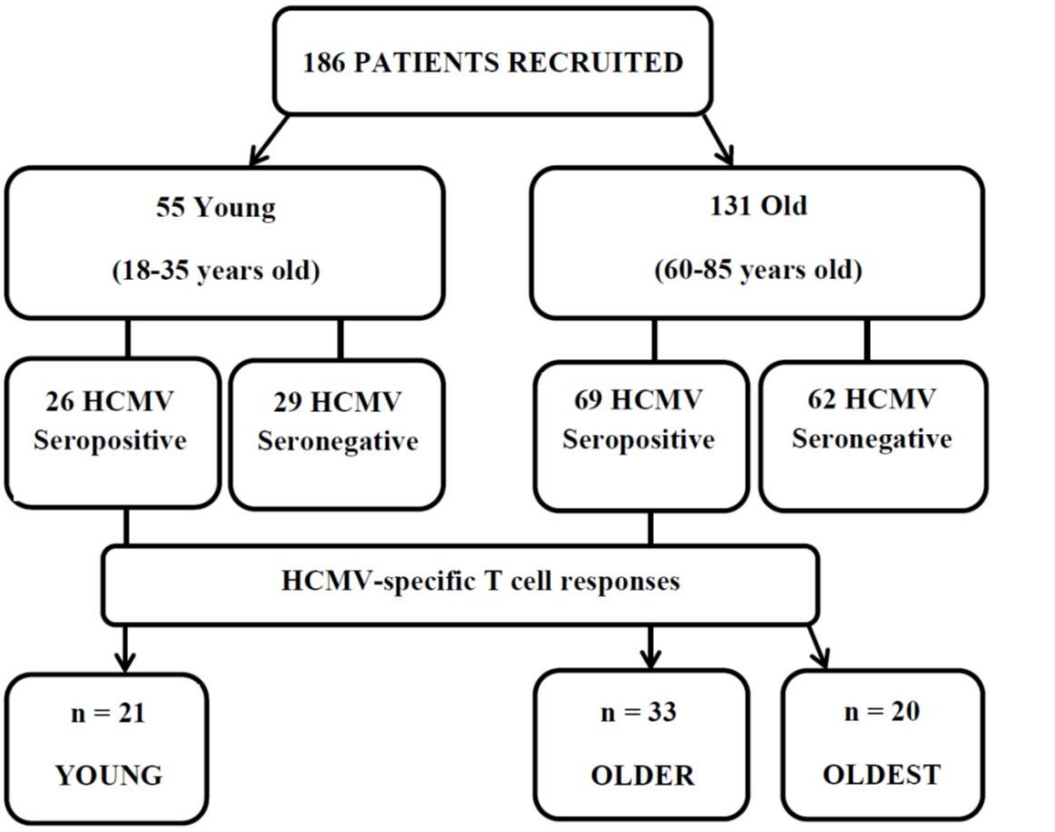


Figure 3.4. Flowchart of age, gender and HCMV serostatus of study participants.

The flowchart above outlines the number of participants recruited to this study. Individuals are initially grouped based on age, and subsequently on HCMV serostatus. As a result of some technical problems during preparation of antigen stimulation tubes resulting in the swapping of US32 and UL32 antigen stimulated tubes, and positive un-stimulated responses in some individuals , the evaluation of HCMV specific T cell responses was limited to n=21 young , n= 33 older and n= 20 oldest individuals.

This cross sectional observational study is designed to characterize HCMV-specific T cells in different age groups. Seropositive participants were stratified into three groups based on age (Table 3.2), to evaluate possible changes in response size with increasing age.

Groups	n	Gender		Median age (range)
		Male	Female	
Young	21	8	13	27 (19-35)
Older	33	16	17	64 (60-70)
Oldest	20	11	9	78 (75-85)

Table 3.2: Age groups and gender distribution of eligible HCMV seropositive study participants.

3.2.1 Number of responses to HMCV PSPPs

Blood from participants meeting study inclusion criteria were processed according to an optimized standard protocol (see methods and materials). Briefly, PBMCs from study participants were stimulated using 19 HCMV PSPPs. The frequency of $CD4^+$ and $CD8^+$ HCMV-specific T cells (defined based on the expression of at least one of the five activation markers (IL-2, IFNγ, TNFα, CD107a and CD40L) was compared between the three age groups (See flow gating strategy and Boolean subsets in Appendix Figure 8.1). Positive responses had to have at least one of these activation markers - IL-2, TNFα, or IFNγ. A representative plot of $CD4^+$ T cell responses to DMSO (negative control) and pp65 PSPP in a HCMV seropositive older individual is shown in Figure 2.2. Activated T cells are referred to as name/protein specific T cell, for instance, T cells responding to pp65 PSPP are referred to as pp65-specific T cell.

Previous studies analysing HCMV-specific T cell responses were typically limited to characterising T cell responses against HCMV immuno-dominant proteins namely pp65 and IE-1, or epitopes derived from these proteins (80, 111, 112). One of the novel aspects of this study is that a comprehensive approach was employed when characterising HCMV-specific T cell responses ($CD4^+$ and $CD8^+$) by analysing responses to 19 immunogenic HCMV targets.

The products of HCMV ORF are diverse with different T cell targets, to assess the frequency of recognition by $CD4^+$ and $CD8^+$ T cells the proportion of responses against HCMV proteins in all study participants within each group (Young , Older , and Oldest group) was explored (Figure 3.5A & 3.5B). The plot ranges in descending order, starting with HCMV proteins with high proportion of responders to the least. The proportion of responses to each HCMV protein of interest was derived by dividing the number of HCMV specific T cell responses ($CD4^+$ and $CD8^+$) by number of individuals within each respective group. Initial analyses focused on HCMV-specific $CD4^+$ T cell responses, results show that pp65 remains a top immunogenic target (proportion of responders in Young = 0.57, Older = 0.58, Oldest = 0.5). UL-103 + US-32 (Young = 0, Older = 0, Oldest = 0) and IE-2 (Young = 0, Older = 0.03, Oldest = 0) were the least recognised HCMV PSPPs in our cohort (Appendix, Table 8.1) .

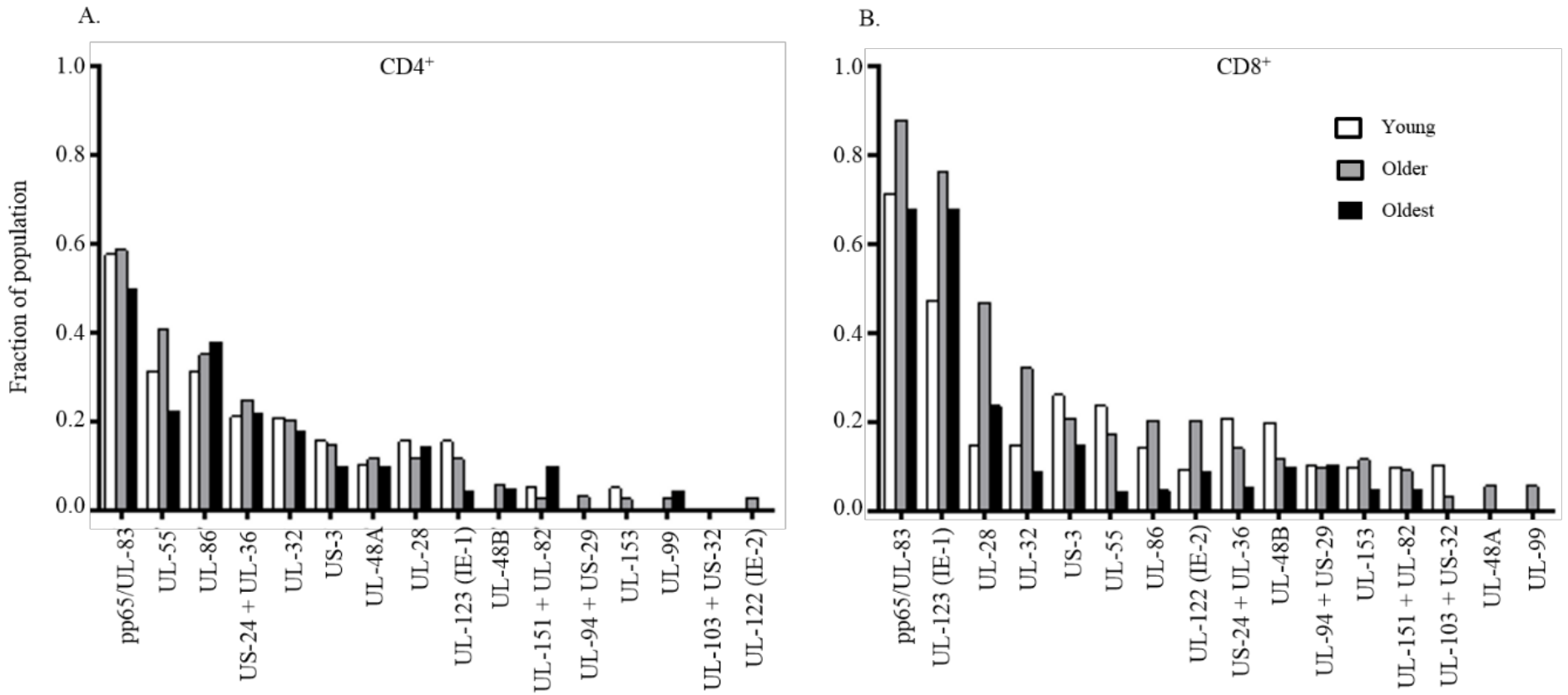


Figure 3.5. The proportion of responses to HCMV proteins.

Histogram plot above depicts the (A) proportion of responses to HCMV proteins based on HCMV-specific $CD4^+$ T cell responses and (B) proportion of responses to HCMV proteins based on HCMV-specific $CD8^+$ T cell responses. Bars appear in decreasing order based on proportion of response. White bar represents responses in Young , Grey bar represents responses in the Older group and Black bar depicts responses in the Oldest

On the otherhand, the analysis based on HCMV-specific $CD8^+$ T cell responses revealed the following HCMV proteins to be the top 4 targets based on the proportion of responders; pp65 (Young = 0.71, Older= 0.88, Oldest = 0.68), IE-1 (Young = 0.48, Older = 0.76, Oldest = 0.68), UL28 (Young = 0.15, Older = 0.47, Oldest = 0.23) and UL-32 (Young = 0.15, Older = 0.32, Oldest = 0.09). Interestingly, HCMV proteins pp65 and IE-1 maintain their immunodominance in our study cohort, as previously shown by other studies. Surprisingly, we observed small $CD8^+$ T cell responses to IE-2 across all age groups, despite being one of the highly recognised ORF in the Sylwester study (41). This could be due to HLA disparities reflected by the wider ethnic mix in Sylwester's study compared to ours, which in contrast, featured a larger caucasian group (Old= 100% White, Young= 71% White).

To determine if there is a difference in the number of HCMV proteins recognised with increasing age, we summed the number of proteins recognised by each seropositive participant in both T cell compartments ($CD4^+$ & $CD8^+$) (Figure 3.6A & 3.6B) and a scatterplot of the total HCMV specific response count was constructed. The histogram of the total HCMV specific response counts for both $CD4^+$ and $CD8^+$ is also displayed below in Figure 3.6C & 3.6D. We observed no significant differences in the total number of HCMV specific $CD4^+$ response counts between the Young, Older and Oldest groups, however participants in the Older group responded to a median of 2.5 HCMV proteins [min.total response count (n=1) - max. total response count (n=9)] compared to those in the Young group who responded to a median of 2 HCMV proteins [min.total response count (n=1) - max. total response count (n=4)].

Comparison of the total HCMV specific $CD8^+$ response counts showed a non-significant increase in the total number of proteins recognised by the Older group [Median=4, minimum total response count (n=1) - maximum total response count (n=14)] compared to the Oldest group [Median=2, minimum total response count (n=1) - maximum total response count (n=6)]. There was no significant difference when compared to the Young [Median=3, minimum total response count (n=1) – maximum total response count (n=7)]. There were notable outliers in the the Older group, with one individual responding to 14 HCMV Proteins.

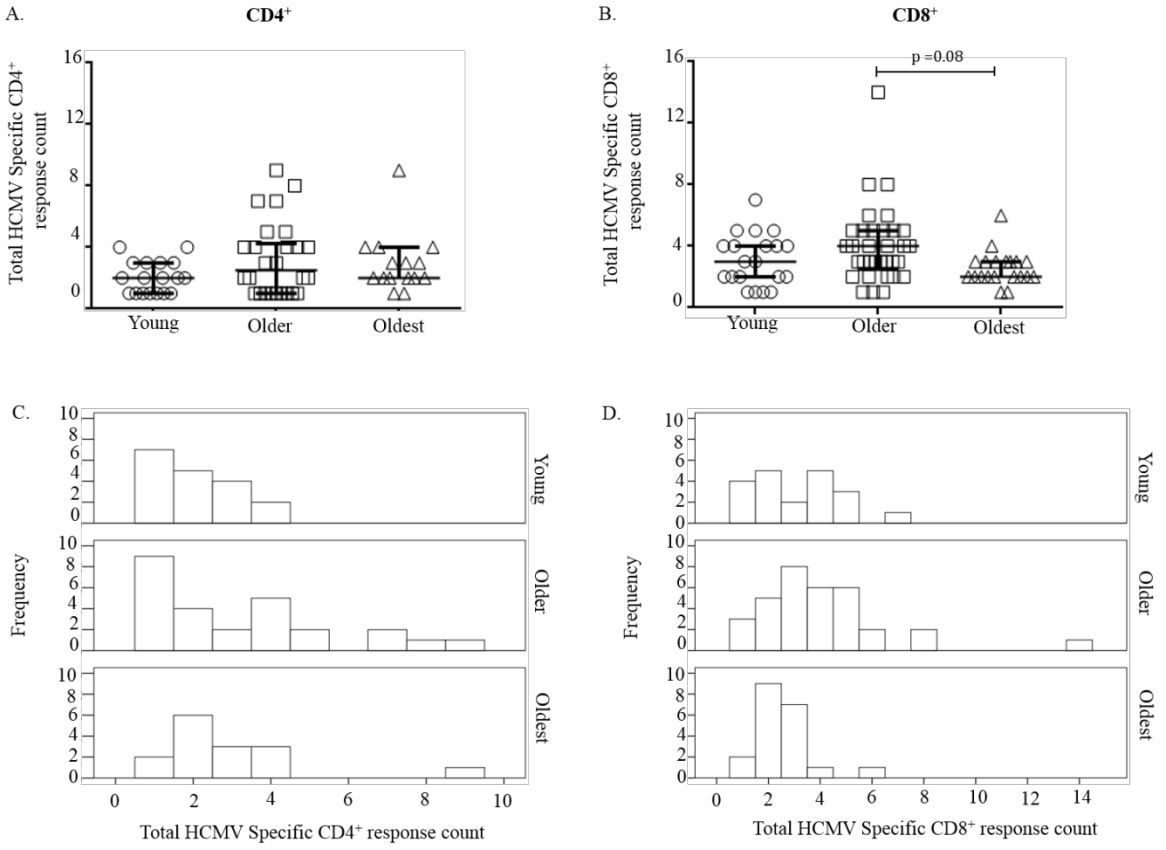


Figure 3.6. Response counts to HCMV PSPPs in different age groups.

Scatter plots depict the number of $CD4^+$ (A) and $CD8^+$ (B) HCMV-specific T cell responses per individual with their corresponding age. Each dot represents the number of responses based on $CD4^+$ or $CD8^+$ count per person. The frequency of total HCMV specific response counts in all three age groups are displayed in histograms based on $CD4^+$ (C) and $CD8^+$ (D) responses.

3.2.2 Response size to HCMV PSPPs

To investigate the quality and size of the T cell response to HCMV Proteins in our cohort, the frequencies of HCMV-specific $CD4^+$ and $CD8^+$ T cells were analysed for each group, and plotted. Although all T cell responses to HCMV proteins were analysed, only some of the proteins identified as top targets for $CD4^+$ (UL-55, pp65, UL86 and US-24 + UL-36) and $CD8^+$ T cells (pp65, IE-1, UL-32 and UL-28) had sufficient responses to be statistically analysed.

The frequencies of UL-55 specific, pp65-specific, UL-86 specific and US-24 + UL-36 specific $CD4^+$ T cells were compared between our 3 groups of Young, Older and Oldest individuals (Figure 3.7A-D). There were no significant differences observed when frequencies of UL-55 specific, UL-86 specific and US-24 + UL-36 specific $CD4^+$ T cells were compared between the three age groups.

There was a significant increase ($p=0.023$) observed when the size of the pp65-specific $CD4^+$ T cells in the Oldest group was compared to that of the Young (Figure 3.7B). An increase in size of the pp65-specific $CD4^+$ T cells was observed in the Older group compared to the Young, however this was not statistically significant. The responses against top recognised HCMV proteins based on $CD4^+$ and $CD8^+$ T cell responses were limited to two groups of Young and Old (Appendix Figure 8.3). There was a significant increase in pp65-specific CD4 T cell response in the Old group compared to the Young group.

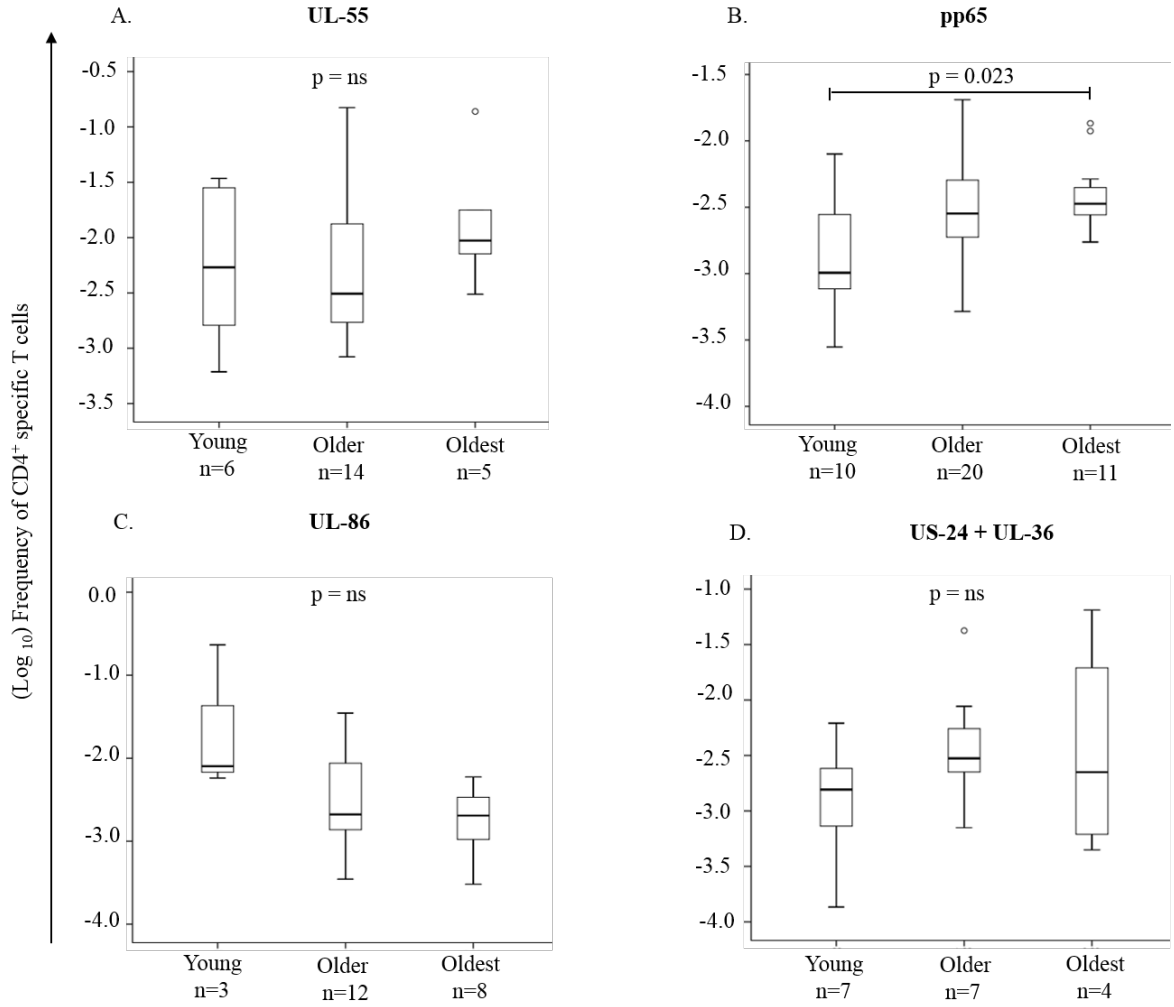


Figure 3.7. HCMV-specific CD4$^+$ T cell responses against top proteins.

Box plots displaying the median, 25th and 75th percentile depict the frequencies of top recognised targets (A) UL-55 specific (B) pp65 specific (C) UL-86 specific and (D) US-24 + UL-36 specific CD4$^+$ T cells in Young, Older and Oldest groups. p value <0.05 was considered significant. (^{O} represents outliers).

Subsequent analyses focused on the size of the CD8$^+$ T cell response to select HCMV Proteins (pp65, IE-1, UL-28 and UL-32). The frequencies of CD8$^+$ T cells against individual proteins were compared between the Young, Older and Oldest groups (Figure 3.8 A-D).

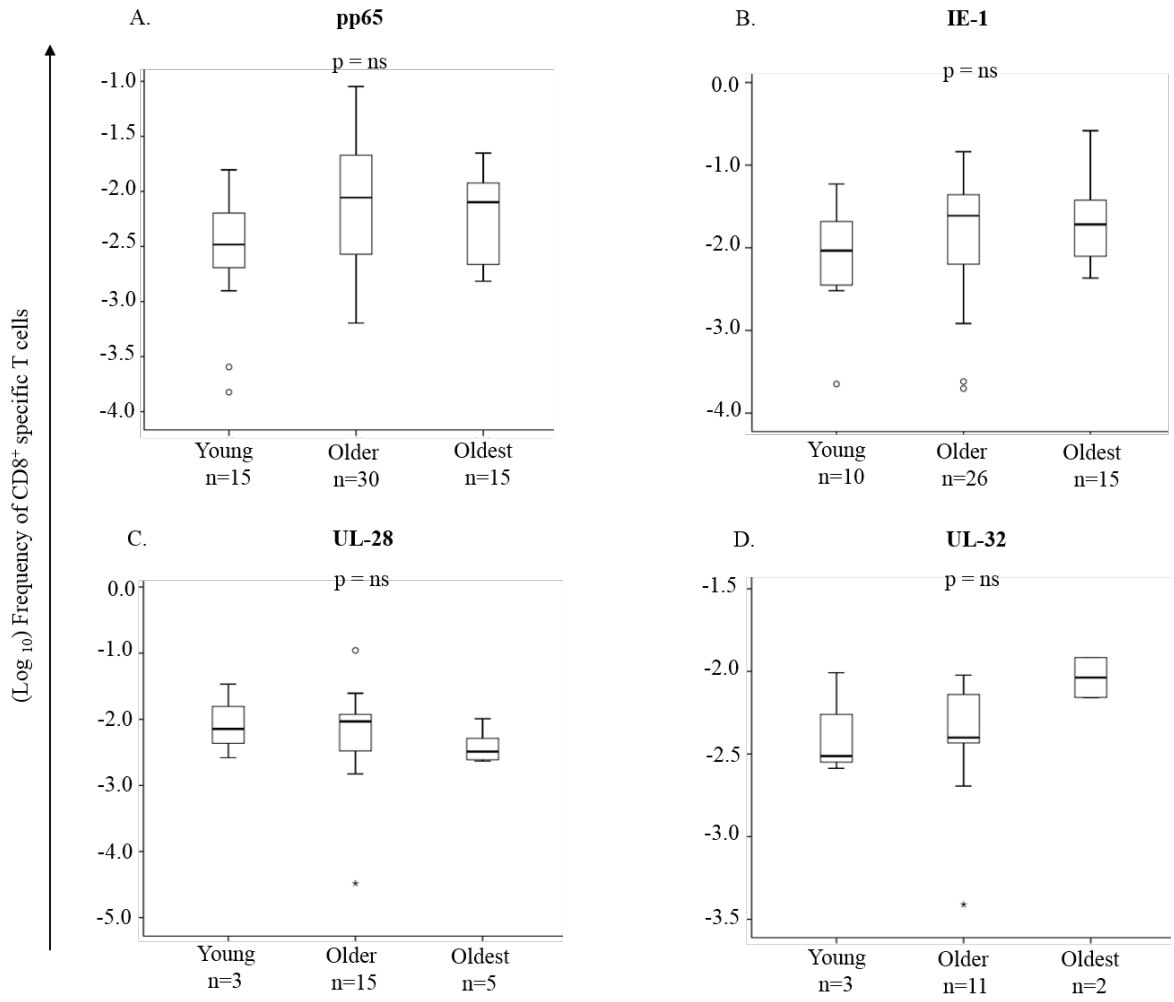


Figure 3.8. HCMV-specific CD8$^+$ T cell responses against top proteins.

Box plots depict the frequencies of top recognised proteins (A) pp65 specific (B) IE-1 specific (C) UL-28 specific and (D) UL-32 specific CD8$^+$ T cells in Young, Older and Oldest groups. p value <0.05 was considered significant.

Surprisingly, we observed no significant increase in the frequency of pp65-specific CD8$^+$ T cells with age, however, there appears to be a modest increase in the size of pp65-specific CD8$^+$ T cells in the Older group (Median = 0.009) compared to Young (Median = 0.003) and a decrease when compared to the Oldest group (Median =0.008).

Although no significant differences were seen when the size of IE-1 specific CD8$^+$ T cells was compared between the three age groups, there was a trend showing increased frequencies of IE-1 specific CD8$^+$ T cells in the Older group (Median = 0.024) compared to Young (Median = 0.009). There was a slight decrease in size of IE-1 specific CD8$^+$ T

cells in the Oldest group (Median = 0.019) compared to the Older group, however this was not statistically significant. There were no significant differences in the size of the UL-32 specific and UL-28 specific $CD8^+$ T cells in the Young, Older and Oldest groups.

To determine the specificity of HCMV PSPPs, pp65-specific $CD4^+$ and $CD8^+$ T cell responses in HCMV seropositive and HCMV seronegative individuals were evaluated (Appendix Figure 8.2). There was a significant pp65-specific CD4 and CD8 T cell responses in HCMV seropositive participants compared to the HCMV seronegative donors.

Responses to a single protein does not fully represent the total cellular responses to HCMV. As a result, we combined the sum of responses to all 19 HCMV proteins analysed to gain a better understanding of the total T cell response size in our different age groups (Figure 3.9).

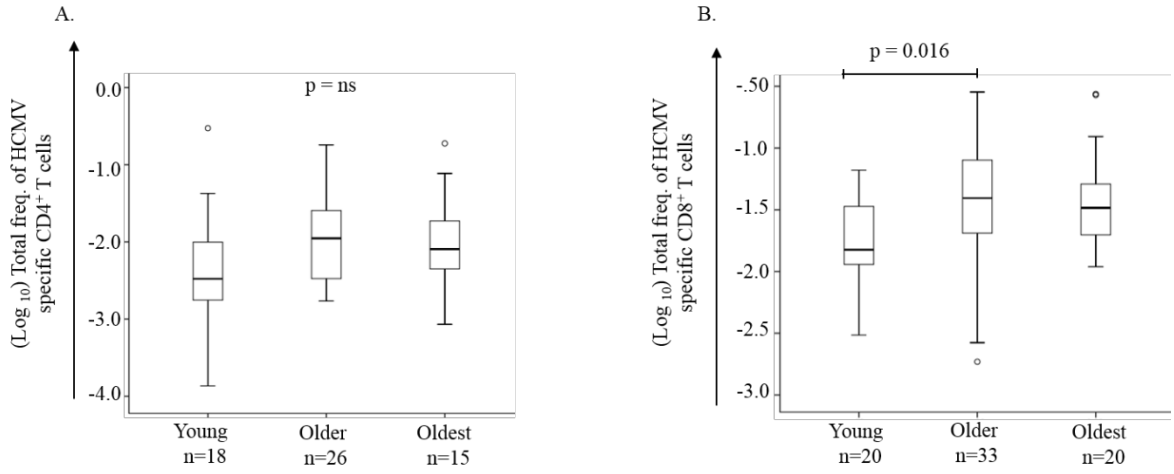


Figure 3.9. Summated HCMV-specific T cell responses to all 19 PSPPs.

Box plots above depict the summated frequencies of $CD4^+$ (A) and $CD8^+$ (B) HCMV-specific T cells in the Young, Older and Oldest groups. A p value <0.05 was considered significant.

Although no significant differences were observed in the size of the summated HCMV-specific $CD4^+$ T cells in Young, Older and Oldest groups, there was a marginal increase in

the size of HCMV-specific $CD4^+$ T cells in the Older and Oldest groups compared to the Young (Figure 3.9A).

The size of the summated HCMV-specific $CD8^+$ T cell responses were significantly increased ($p=0.016$) in the Older group compared to the Young (Figure 3.9B). A slight decrease in the size of the summated HCMV-specific $CD8^+$ T cell responses was observed in the Oldest compared to the Older group, although this was not significant.

3.3 DISCUSSION

HCMV has a considerable impact on the host immune system. It is a persistent virus subject to intense research due to its association with increased mortality and frailty in old age (113, 114).

Most published studies investigating HCMV-specific T-cell response size have used the CMV AD169 laboratory strain derived sequences same as that utilized in this study, but those investigations were often limited to one or two proteins. One of the primary aims of this study was to characterise T cell responses using 19 HCMV proteins encompassing both $CD4^+$ and $CD8^+$ T cell top targets as defined in an earlier study (41).

In an effort to evaluate the immunodominance of less characterised HCMV proteins, and to determine if there is a significant change in the number of responses to HCMV proteins with age, the proportion of responses to a single HCMV protein in all participants was calculated based on frequencies of $CD4^+$ and $CD8^+$ specific T cell.

All proteins were recognized by at least one participant in this study, however, they were recognized in a different hierarchical order than in the original study by Sylwester et al. For instance, UL48 was the most highly recognized target protein by $CD8^+$ T cells in the previous study cited, but the least frequently recognized one in this study. As evident in the original study, the hierarchy of protein recognition is not related to the size of the responses. The most frequently recognized proteins did not give rise to the biggest responses.

Our results showed considerable variation in the number of HCMV proteins recognized based on $CD8^+$ T cell responses; thereby emphasizing the fact that certain participants mounted a diverse response whilst responses in other participants were much more focused (Figure 3.6). For example, one of the subjects in the Older group mounted $CD8^+$ T cell

responses to 14 HCMV PSPPs whereby on average most individuals responded to a total of 2 HCMV PSPPs. Importantly, there appears to be no significant increase in the number of HCMV PSPPs recognized with increasing age based on $CD4^+$ T cell responses.

The participants in this study are predominantly White British; however 8 of the 21 young participants are of Asian and/ or African descent. The different protein recognition hierarchies observed in this group of individuals begs the questions whether the same array of proteins would have been selected for stimulation had the original study been performed on mainly Caucasian participants.

In support of the earlier observations by several groups HCMV proteins pp65 and IE-1 were immuno-dominant targets against which most of our partipants developed $CD4^+$ and $CD8^+$ responses (115, 116).

In conclusion, we observed no significant differences in the number of response counts to all HCMV proteins between all three age groups based on $CD4^+$ T cell response . An increase in the total number of $CD8^+$ T cell response counts in the Older group was observed when compared to the Oldest group however this was not statistically significant. This observation is further corroborated by a recent finding evaluating the diversity of $CD8^+$ T cell responses to 11 frequently recognised HCMV ORF in which the authors demonstrate that the age of the donor had minimal impact on the number of HCMV ORF products recognised. Furthermore, the authors did not observe new CD8 T cell responses to HCMV ORF products for any of the subjects analysed 2 years and 3 years post the original analysis which supports the stability of these HCMV specific T cell responses with time (115).

The evolution of HCMV-specific immune responses remains an intense area of research, several reports have described large accumulation of dysfunctional memory T cells, primarily $CD8^+$ T cells in aged individuals, with potentially deleterious effects due to

production of low grade inflammatory markers (117-119). Charting the impact of HCMV infection and distinguishing this from the normal physiological ageing of the immune system (i.e alterations to T cell subsets and reduction in the naïve T cells) is often difficult (120). It requires knowing the time course of primary infection which is almost impossible, except in a transplant setting.

One of the key aims of my project was to evaluate the magnitude of HCMV-specific T cell responses in the 3 groups (age range 18-85) using 19 HCMV proteins encompassing both $CD4^+$ and $CD8^+$ T cell top targets. To determine the size of HCMV-specific responses in our cohort, we compared the frequencies of $CD4^+$ and $CD8^+$ specific T cells against individual HCMV protein between the three age groups. Our results confirm that there is no expansion of pp65-specific $CD8^+$ T cells with age. Although we observed an increase in frequency of pp65-specific $CD8^+$ T cells in the Older cohort compared to Young, there was no statistically significant difference in size of pp65-specific $CD8^+$ T cell responses in Oldest group compared to the Older group. This is in line with a recent finding by Vescovini et al. who observed no correlation of anti-CMV $CD8^+$ T cells with age in participants aged above 60 years (116).

A significant increase in pp65-specific $CD4^+$ T cell response in the Oldest group was observed when compared to the Young. This is in line with other studies demonstrating the use of pp65 protein as an important target for $CD4^+$ T cells (118, 121). However, it is important to note that these studies have focused on select dominant epitopes such as pp65 peptide "NLVPMVATV", and also used different measures of functional output (80).

In addition to the widely recognised HCMV immuno-dominant proteins pp65 and IE-1, a large proportion of individuals in this study cohort also responded to UL-86, US-24 and UL-36 based on $CD4^+$ T cell responses as well as UL-28 and UL-32 HCMV proteins based

on $CD8^+$ T cell responses. Jackson et al. also identified UL-28 as one of the top recognised HCMV ORF based on $CD8^+$ T cell responses in their cohort.

To gain a reliable measure of the total size of the HCMV-specific $CD4^+$ and $CD8^+$ T responses, the summated responses to all 19 different HCMV PSPPs between our three age groups were compared. Overall, our results demonstrate no significant differences in magnitude of summated HCMV-specific $CD4^+$ T cells between the Young, Older and Oldest groups. Interestingly, although there was a significant increase in the magnitude of summated HCMV-specific $CD8^+$ T cell responses in the Older group compared to Young no further expansion was observed with increasing age.

Results generated from this study demonstrate that although HCMV-specific T-cells does increase in older age, such expansions do not occur invariably, and do not always lead to large expansions in older people.

4 MEMORY SUBSET DISTRIBUTION IN HCMV INFECTED INDIVIDUALS

It is widely speculated that large expansions of HCMV-specific T cell populations in aged individuals could potentially have deleterious effects on the maintenance of a diverse TCR repertoire, by crowding out T cells of other specificities (Reviewed in (122). It is therefore vital to assess the impact of this ubiquitous beta-herpesvirus on the immune system of seropositive individuals with increasing age.

To determine the effect of HCMV and possible alteration of memory subset distribution, PBMC stimulated with HCMV PSPPs were surface stained with CD45RA and CD27 in addition to other T cell phenotypic and activation markers. Memory subsets were delineated based on the expression (+) or lack (-) of CD45RA and CD27 phenotypic markers (Figure 2.1C). This is frequently done due to limitation when putting together a multi-parameter assay including activation markers.

To elucidate the memory distribution of HCMV-specific T cells in both $CD4^+$ and $CD8^+$ T cell compartment, the size of the 4 different subsets - T_N [$CD45RA^+$ $CD27^+$], T_{CM} [$CD45RA^-$ $CD27^+$], T_{EM} [$CD45RA^-$ $CD27^-$] and T_{EMRA} [$CD45RA^+$ $CD27^-$] - were compared between the 3 groups. The nomenclature adopted is in accordance with published scientific papers.

4.1.1 Memory subset distribution with age

The ageing process results in reduced thymic output, this coupled with multiple exposures to different pathogens over the course of a life time, contributes to changes in the memory subset distribution. There are several studies with often conflicting results demonstrating the inflation of certain memory subsets (T_{CM} and T_{EM}) with a decline of T_N in aged individuals infected with HCMV (123, 124). However, in a recent study by Wertheimer et al.,2014, investigating the effect of age and HCMV on circulating T cell homeostasis in a large study cohort totalling 391 (aged 21-101), the researchers observed a decline in

absolute numbers of $CD8^+$ T_N cells with increasing age, independent of HCMV infectivity (125).

In this study, the frequencies of memory subsets [T_N, T_{CM}, T_{EM} and T_{EMRA}] within the $CD4^+$ and $CD8^+$ T cell subset were compared between the three age groups (Figure 4.1). Analyses of $CD4^+$ T_N, T_{CM}, T_{EM} and T_{EMRA} subsets did not reveal any significant differences with age in the 3 groups.

Comparison of the frequencies of $CD8^+$ T_N subset showed a significant decrease in the size of the T_N memory subset with age, with a significant decrease observed in the Older group ($p = 0.0003$) compared to Young. A further decrease ($p<0.000$) in the frequencies of $CD8^+$ T_N subset was evident in the Oldest group when compared to the frequencies in the Young.

The analyses of the frequencies of $CD8^+$ T_{EMRA} subset demonstrated a significant increase ($p<0.0001$) in the frequencies of $CD8^+$ T_{EMRA} subsets in the Older group compared to the Young group. A significant increase ($p = 0.0009$) in the frequencies of $CD8^+$ T_{EMRA} subset was also observed in the Oldest group compared to Young.

There were no differences observed when the frequencies of $CD8^+$ T_{EM} and T_{CM} subsets were compared between the Young, Older and Oldest groups.

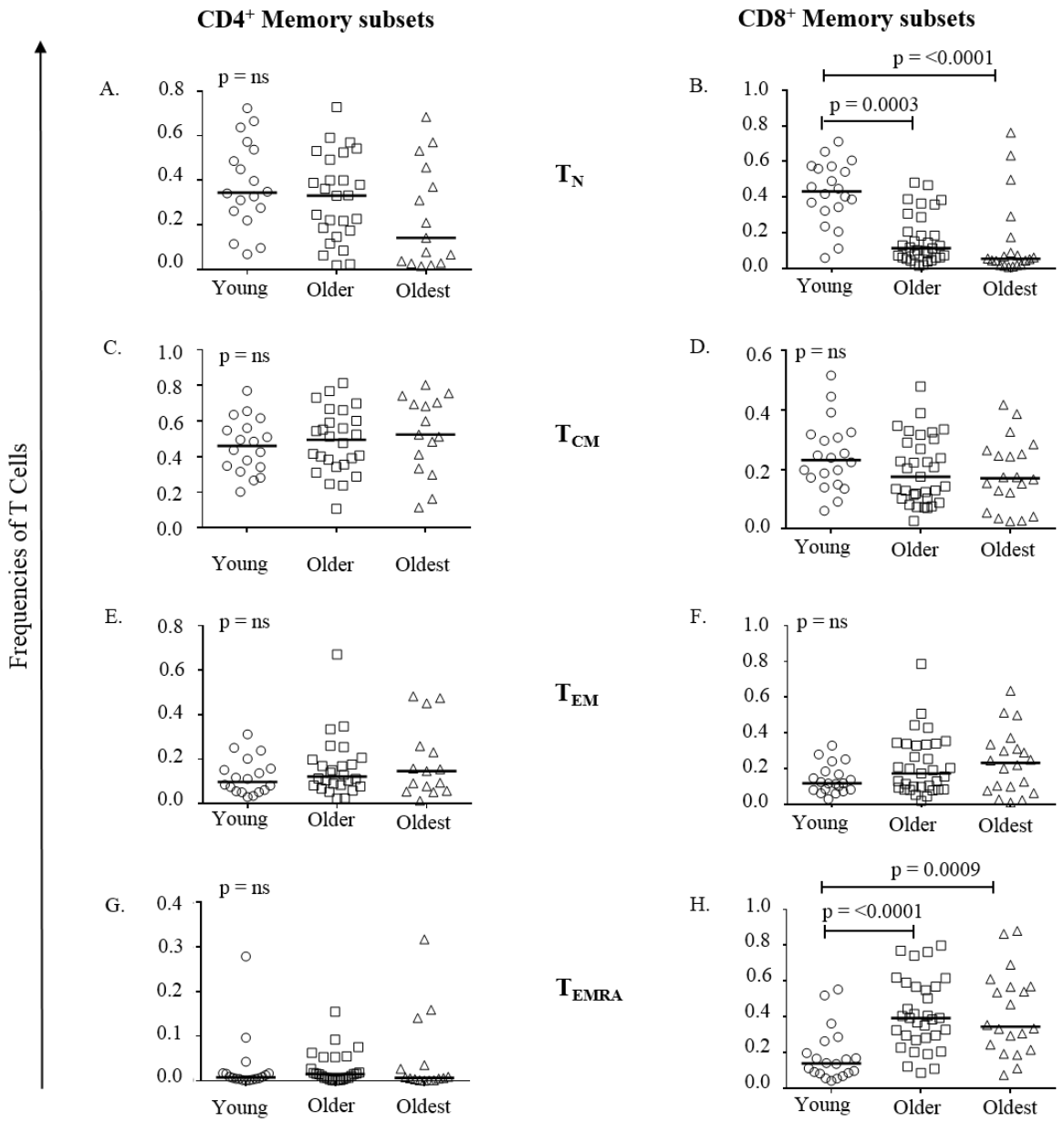


Figure 4.1. Memory subset distribution in different age groups.

The scatter plots above depict the frequencies of $CD4^+$ T_N (A),T_{CM} (C),T_{EM} (E) and T_{EMRA} (F) subsets in Young (n=18), Older (n=26) and Oldest (n=15) groups. Figures B,D,F,H represent the frequencies of $CD8^+$ T_N,T_{CM},T_{EM} and T_{EMRA} subsets respectively in the Young (n=20), Older (n=33) and Oldest (n=20) group. Each symbol (circle, square, and triangle) represents an individual participant. The median is indicated with a black line for each group. P<0.05 was considered significant.

4.1.2 Distribution of HCMV-specific memory subsets

The memory subset distribution of HCMV specific T cells was analysed to determine if differences exist with increasing age in the Young, Older and Oldest groups. Although all memory T subsets for the 19 HCMV proteins studied were analysed, focus will initially be on the top recognised HCMV PSPPs based on $CD4^+$ and $CD8^+$ T cell responses. The memory subset distribution of pp65-specific and UL-55-specific $CD4^+$ T cells (Top $CD4^+$ Targets) can be found in Figure 4.2 and Figure 4.3 respectively.

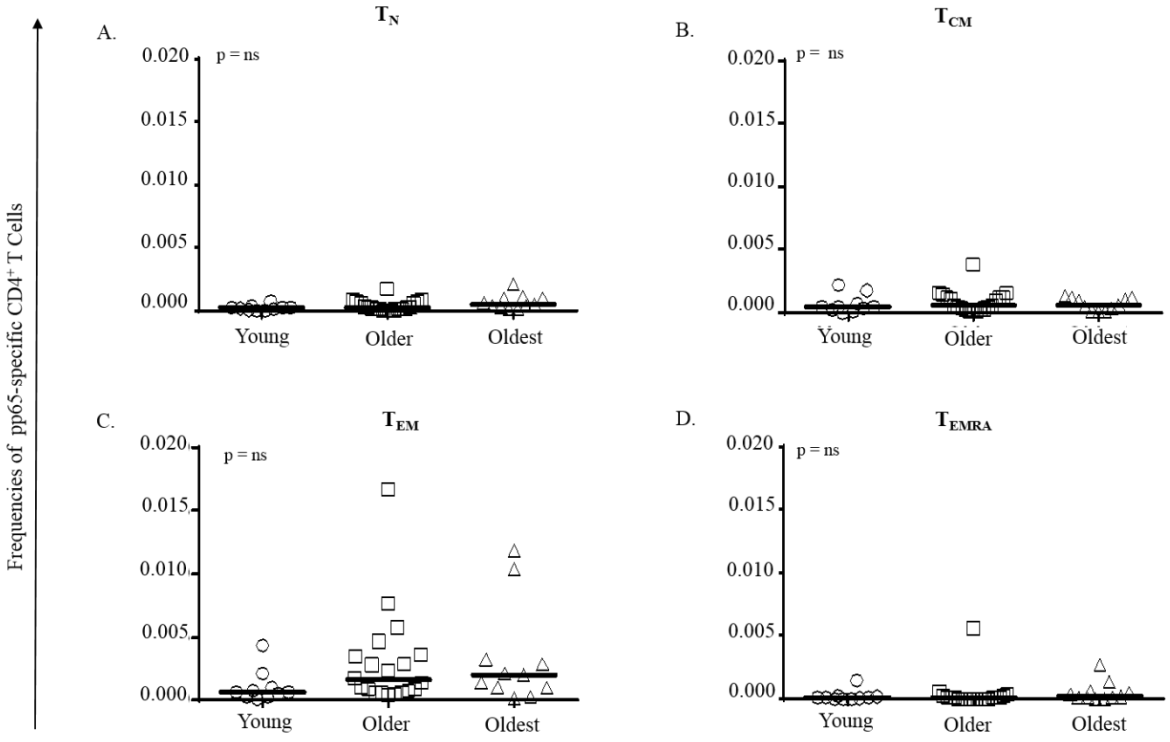


Figure 4.2. No significant change in pp65-specific $CD4^+$ T cell memory distribution with increasing age

Scatter plot of frequencies of pp65-specific T cells T_N (A), T_{CM} (B), T_{EM} (C) and T_{EMRA} (D) are depicted above for the Young (n=10), Older (n=20) and Oldest group (n=11). Each symbol (circle, square, and triangle) represents an individual participant. The median is indicated with a black line for each group. $P<0.05$ was considered significant.

The comparison of pp65-specific $CD4^+$ T_N, T_{CM}, T_{EM}, and T_{EMRA} subsets did not yield any significant differences in the Young, Older and Oldest groups.

The frequencies of UL-55 specific $CD4^+$ memory subsets (T_N, T_{CM}, T_{EM}, and T_{EMRA}) were also compared between the Young, Older and Oldest groups. There were no significant differences observed when all memory subsets were considered.

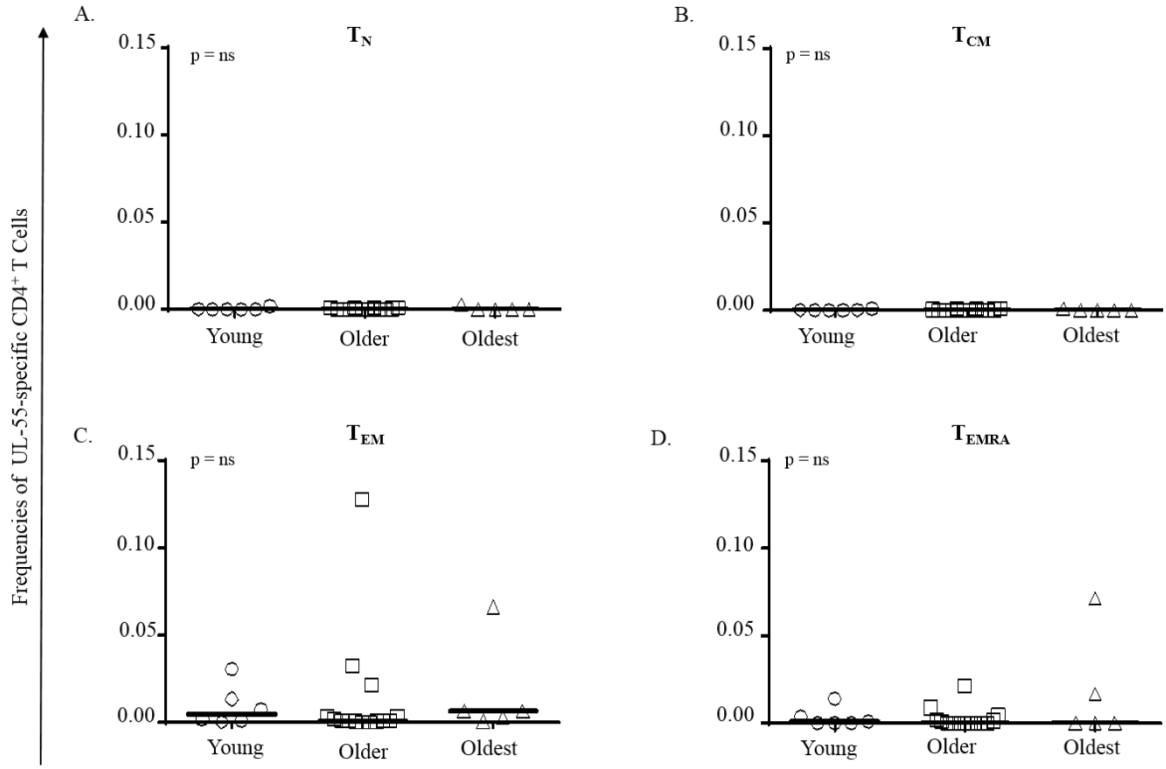


Figure 4.3. UL-55 specific $CD4^+$ T cell memory distribution.

Scatter plot of frequencies of UL-55-specific T cells T_N (A), T_{CM} (B), T_{EM} (C) and T_{EMRA} (D) are depicted above for the Young (n=6), Older (n=14) and Oldest group (n=5). Each symbol (circle, square, and triangle) represents an individual participant. The median is indicated with a black line for each group. $P < 0.05$ was considered significant.

Equivalent analyses of pp65-specific and IE-1 specific $CD8^+$ memory subset distribution in Young, Older and Oldest groups can be found in Figures 4.4 and 4.5 respectively.

Surprisingly, there were no significant differences observed when pp65-specific $CD8^+$ T_N, T_{CM}, T_{EM} and T_{EMRA} subsets were compared between the 3 groups. However, there were notable outliers observed in the Older and Oldest group.

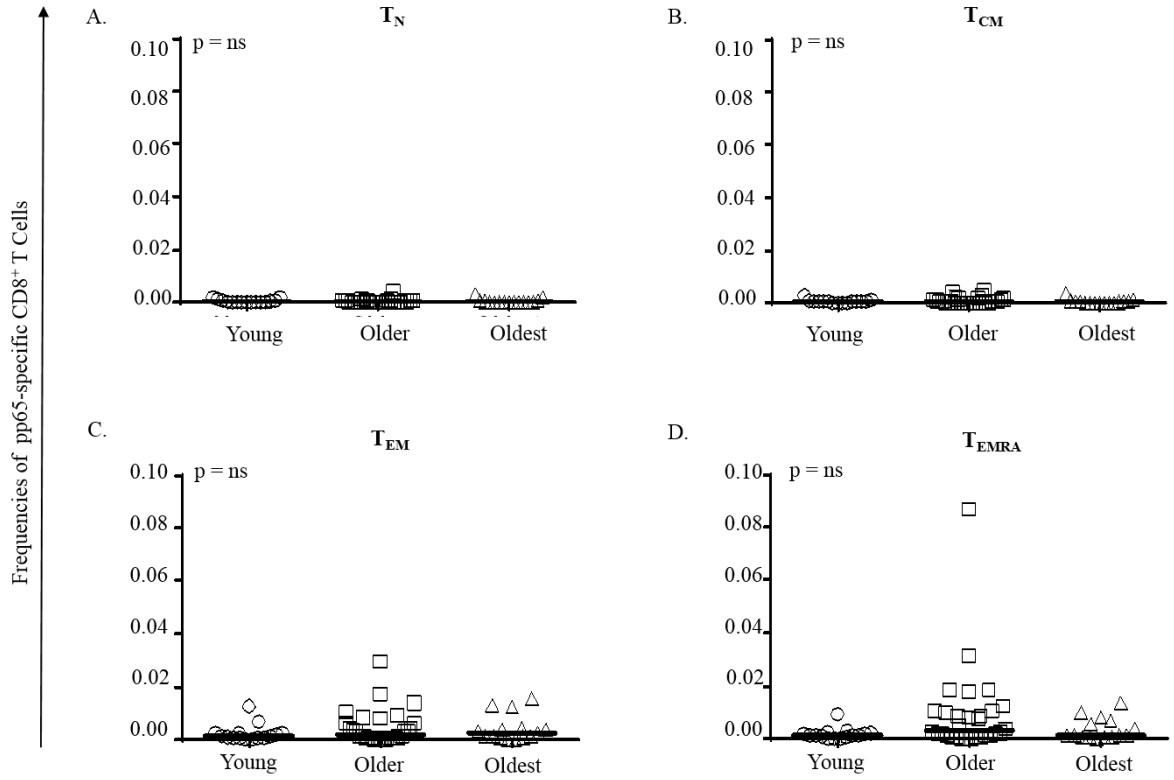


Figure 4.4. pp65 specific $CD8^+$ T cell memory distribution.

Scatter plot of frequencies of pp65-specific $CD8^+$ T cells T_N (A), T_{CM} (B), T_{EM} (C) and T_{EMRA} (D) are depicted above for the Young (n=15), Older (n=30) and Oldest group (n=15). Each symbol (circle, square, and triangle) represents individual participant. The median is indicated with a black line for each group. $P < 0.05$ was considered significant.

Conversely, the comparison of IE-1-specific $CD8^+$ T cell T_N, T_{CM}, T_{EM} and T_{EMRA} subsets in Young, Older and Oldest did not reveal any significant differences.

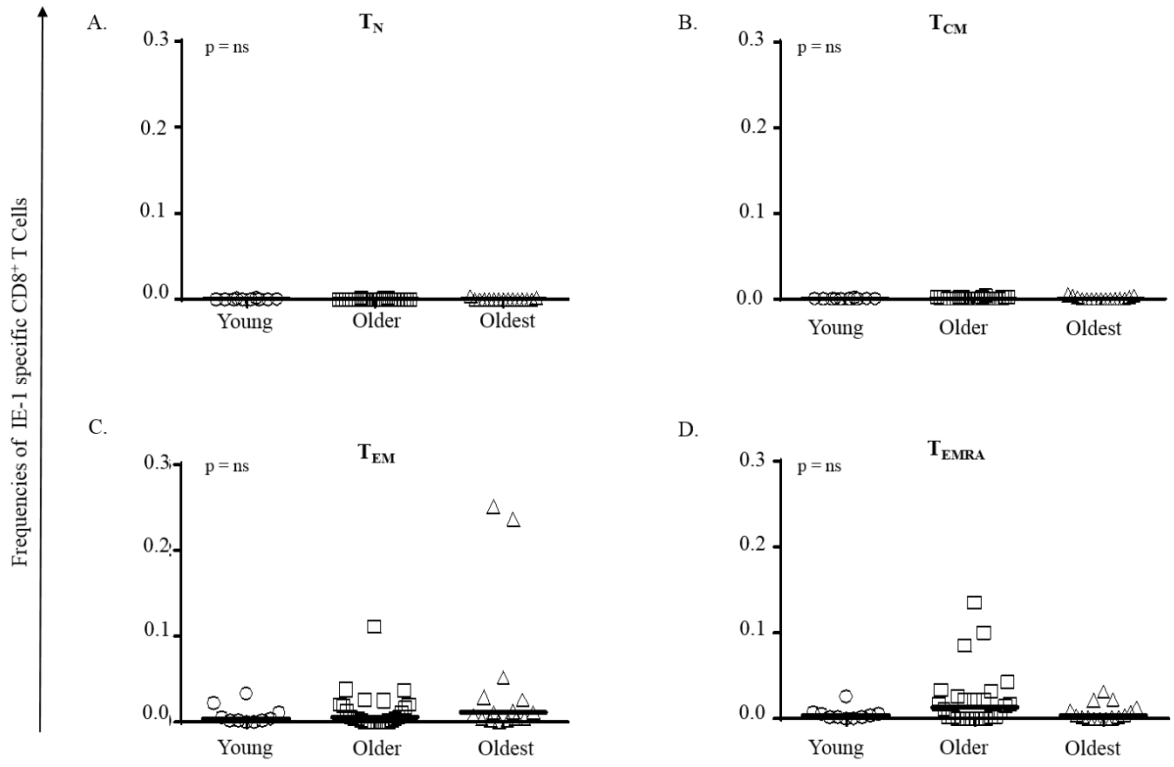


Figure 4.5. IE-1 specific $CD8^+$ T cell memory distribution.

Scatter plot of frequencies of IE-1-specific $CD8^+$ T cells T_N (A), T_{CM} (B), T_{EM} (C) and T_{EMRA} (D) are depicted above for the Young (n=10), Older (n=26) and Oldest group (n=15). Each symbol (circle, square, and triangle) represents an individual participant. The median is indicated with a black line for each group. $P < 0.05$ was considered significant.

The focus on single peptides or select immuno-dominant HCMV proteins does not fully capture the effect of the HCMV associated alterations in the memory T cell compartment. As such, the summated HCMV specific $CD4^+$ and $CD8^+$ T cell responses were analysed to determine the memory subset distribution in the three age groups. This is displayed in Figure 4.6 and Figure 4.7 respectively.

To determine the summated HCMV-specific T cell memory subset distribution, the HCMV specific responses within each memory compartment were summed, for example, the summated HCMV specific $CD4^+$ T_N was determined by summing the Naïve subsets for all HCMV proteins analysed, the effector subsets for all HCMV proteins analysed were also

summed (T_{EM}). The same procedure was repeated to determine the summated central (T_{CM}) and revertant T cells (T_{EMRA}).

Initial evaluation of memory subset distribution of summated HCMV-specific $CD4^+$ T cells revealed a significant increase (p=0.04) in the size of the $CD4^+$ T_{EM} subsets in the Older group compared to the Young. No further increase in frequencies of $CD4^+$ T_{EM} subset was seen in the Oldest group. However, significance was not maintained once the multiple endpoints due to number of groups analysed was taken into account (p=0.123). Overall, there were no statistically significant differences in the summated HCMV-specific $CD4^+$ T_N, $T_{CM,}$ T_{EM}, and T_{EMRA} subsets in the three age groups.

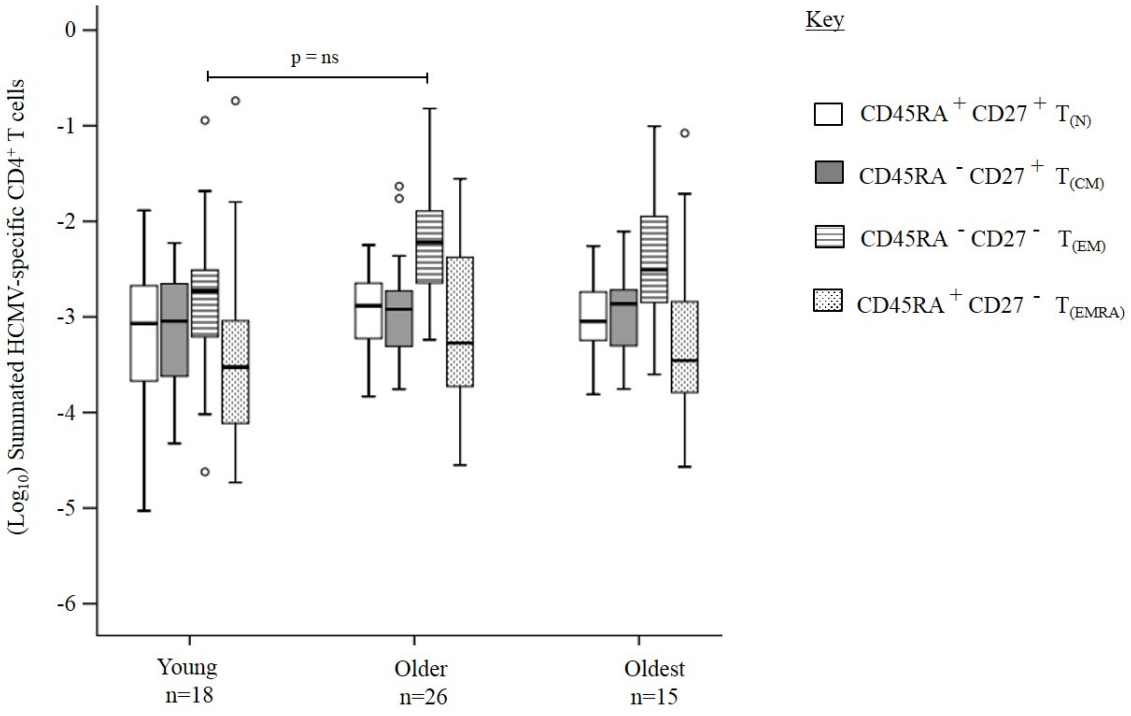


Figure 4.6. Summated HCMV-specific $CD4^+$ T cell memory subset distribution in the 3 age groups.

Box plots displaying the median, 25^{th} and 75^{th} percentile depict the frequencies of T_N, T_{CM}, T_{EM} and T_{EMRA} in Young, Older and Oldest groups. The frequencies of summated HCMV specific $CD4^+$ T cells were log transformed to aid visualisation of data. A p value <0.05 was considered significant. (^{O} represents outliers).

Analysis of summated HCMV-specific $CD8^+$ memory subset distribution in the three age groups highlighted a significant increase ($p = 0.015$) in the size of the $CD8^+$ T_{EMRA} cells in the Older group compared to the Young. No significant differences were observed when the frequencies of the summated HCMV-specific $CD8^+$ T_N, T_{EM}, and T_{CM} memory subsets were considered.

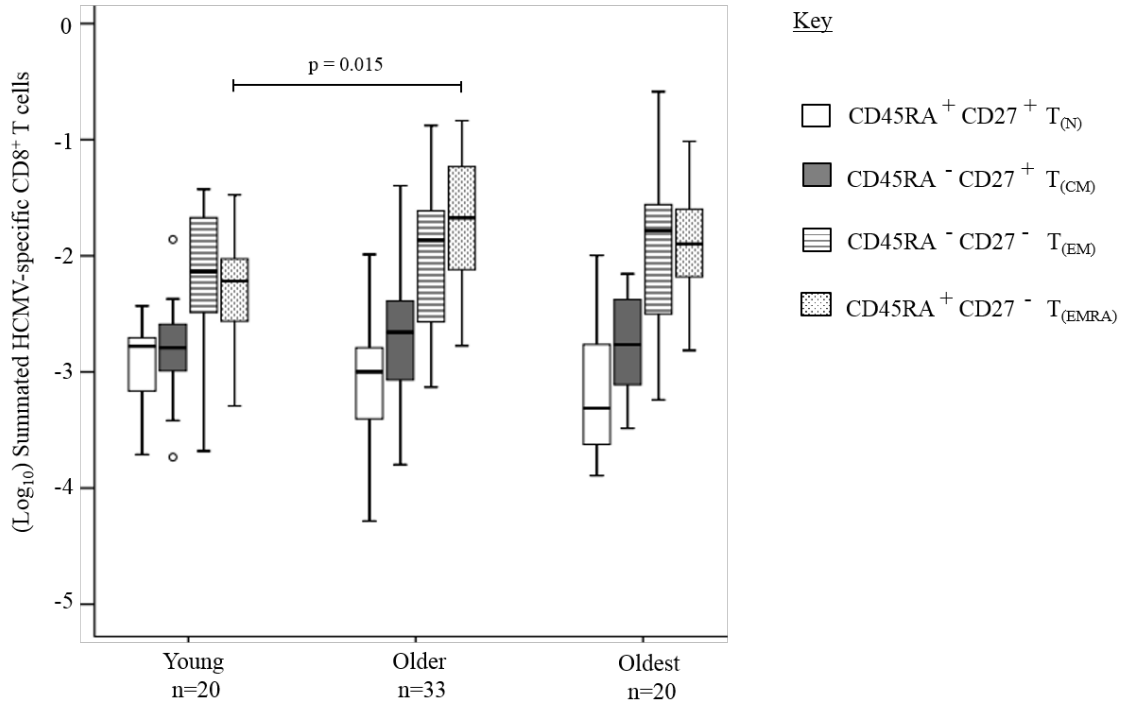


Figure 4.7. Summated HCMV-specific $CD8^+$ T cell memory subset distribution in the three age groups.

Box plots displaying the median, 25th and 75th percentile depict the frequencies of T_N, T_{CM}, T_{EM} and T_{EMRA} in Young, Older and Oldest groups. A p value <0.05 was considered significant. (^{O} represents outliers).

4.1.3 The degree of memory subset differentiation is correlated with HCMV-specific response size.

"Memory inflation" is a term coined to describe the delayed expansion and long term maintenance of CMV-specific memory subsets after initial infection (126). The majority of studies describing this phenomenon have been mostly in longitudinal mouse models infected with murine cytomegalovirus (MCMV) (127, 128). The extent of this phenomenon in humans is not clear cut due to various factors. Typically, the point of HCMV infection is unknown making it difficult to conduct longitudinal studies mapping out the evolution of HCMV-specific T cells over the course of time. Indeed, most studies describing the inflation of HCMV-specific T cells have used a cross-sectional design covering a range of age groups to take into account the effect of infection with time. Notably in these studies, T cell responses were mostly against select epitopes derived from immunodominant HCMV proteins such as pp65 and IE-1.

To explore the relationship between HCMV-specific response size and advanced differentiation of memory subsets, the frequencies of HCMV-specific T cells responding to select HCMV PSPPs were plotted against the corresponding (T_N, T_{CM}, T_{EM} and T_{EMRA}) memory subsets. Figure 4.8 is a scatterplot depicting the size of pp65-specific $CD4^+$ T cells in all HCMV seropositive donors (n=41) against pp65-specific (T_N, T_{CM}, T_{EM} and T_{EMRA}) $CD4^+$ memory subsets. There were no significant associations when the frequencies of pp65-specific $CD4^+$ T cells and pp65-specific $CD4^+$ T_N and T_{EMRA} memory subsets were considered. However, the frequencies of pp65-specific $CD4^+$ T_{CM} (Rs=0.499, p=0.005) and T_{EM} (Rs=0.859, p=0.000) were positively correlated with an increase in pp65-specific $CD4^+$ response size.

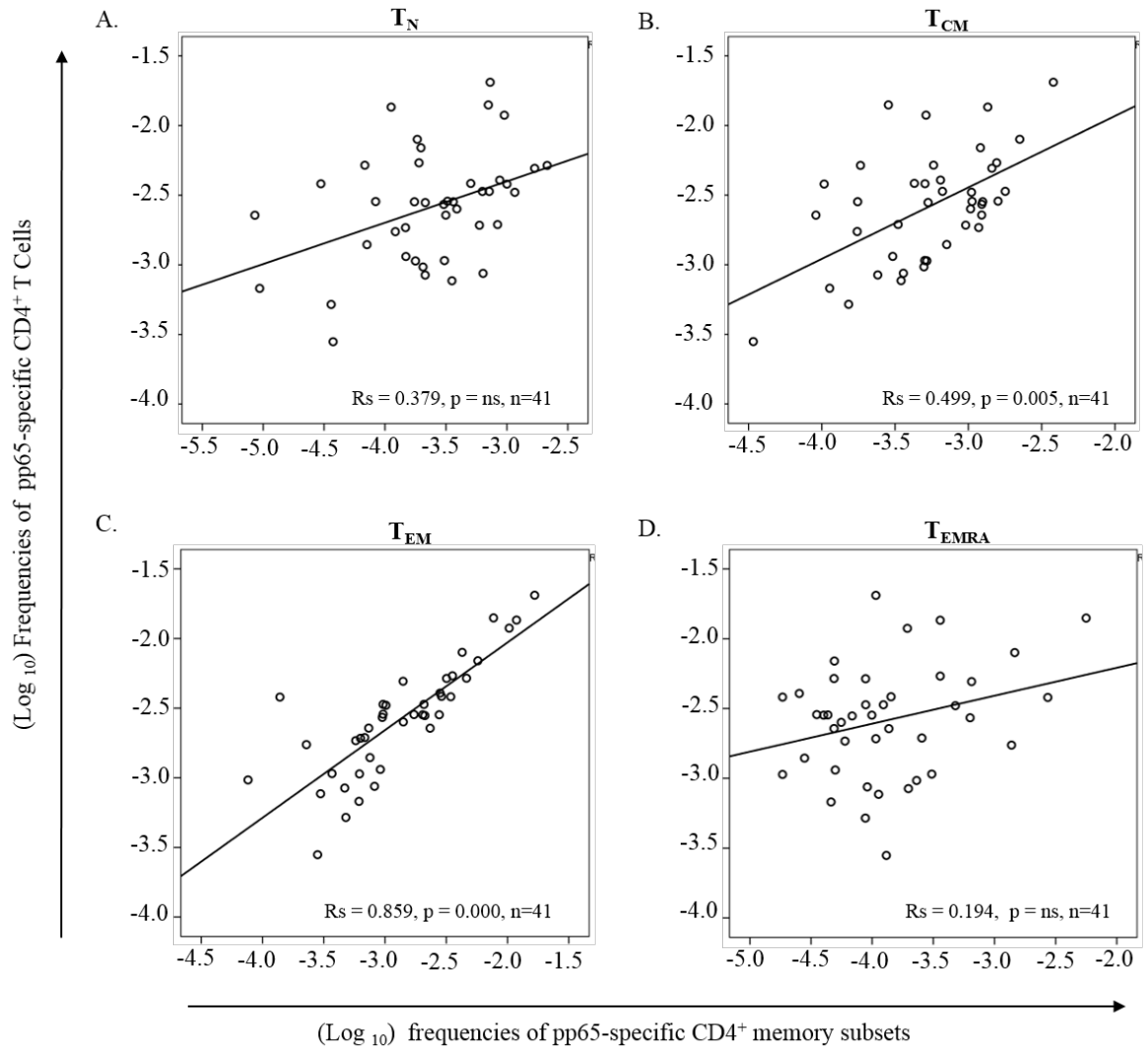


Figure 4.8. Correlation of pp65-specific $CD4^+$ T cell memory subsets with pp65-specific $CD4^+$ response size.

The scatterplots depict the frequencies of pp65-specific $CD4^+$ T cells against pp65-specific memory subsets T_N (A) ,T_{CM} (B), T_{EM} (C), and T_{EMRA} (D).$p < 0.05$ was considered significant.

UL-55 is another top recognised HCMV glycoprotein in our cohort, based on the number of $CD4^+$ T cell responses. The characteristics of UL-55 specific memory subset differentiation is not widely reported, as most studies have focused on the immunodominant pp65 protein. The relationship between UL-55 specific $CD4^+$ T cell response size and memory subset differentiation was further elucidated as depicted in Figure 4.9.

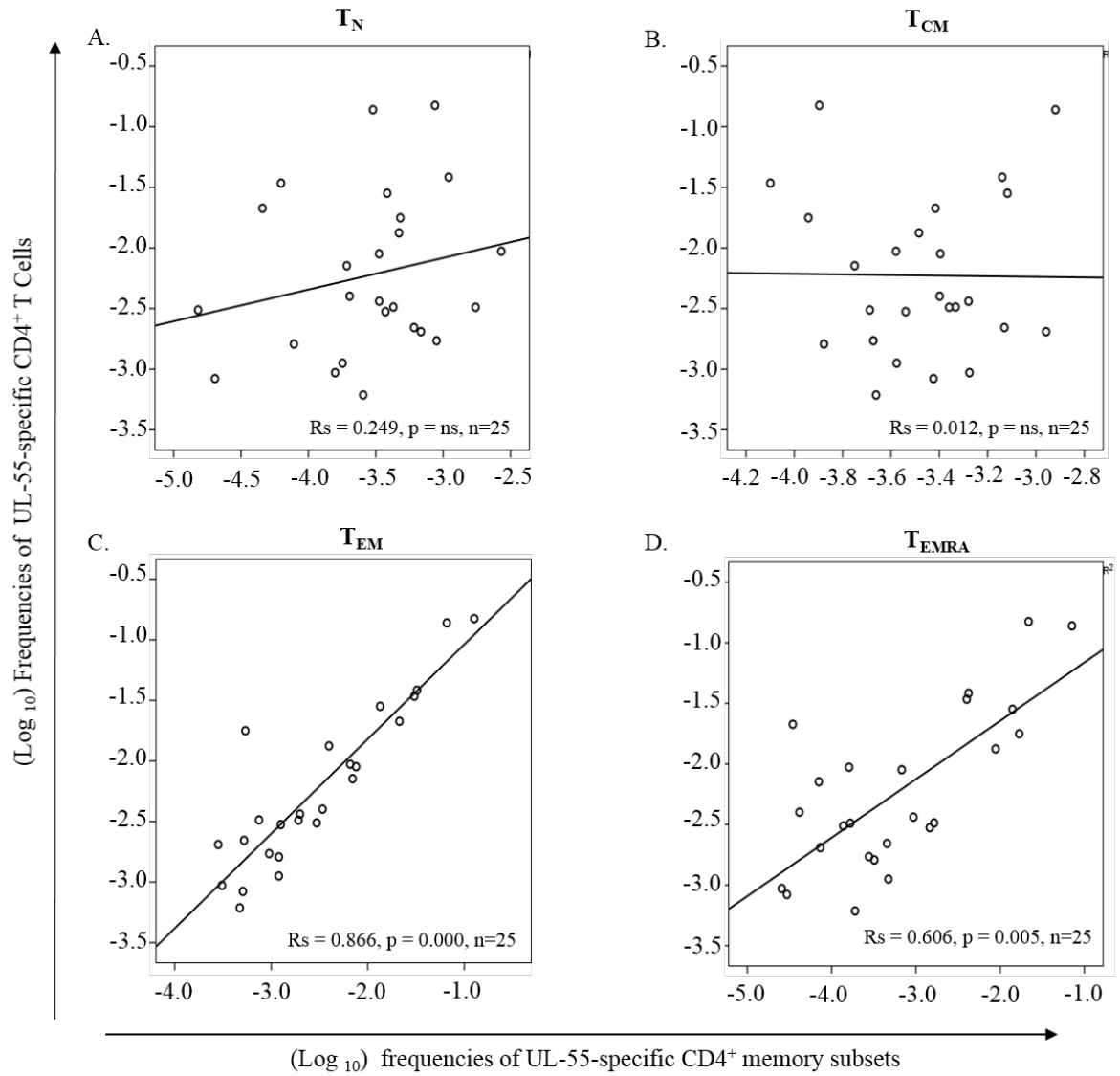


Figure 4.9. Correlation of UL-55-specific $CD4^+$ T cell memory subsets with increasing response size.

The scatterplots depict the frequencies of UL-55-specific $CD4^+$ T cells against UL-55-specific memory subsets T_N (A) ,T_{CM} (B), T_{EM} (C), and T_{EMRA} (D). $p < 0.05$ was considered significant.

There were no significant associations observed when the frequencies of UL-55-specific $CD4^+$ T cells and corresponding T_N and T_{CM} memory subsets were considered. However, the frequencies of UL-55-specific $CD4^+$ T_{EM} (Rs=0.866, p=0.000) and T_{EMRA} (Rs=0.606,

p=0.005) memory subsets were significantly correlated with increasing UL-55 specific $CD4^+$ T cell response size.

The analyses of UL-86 specific and US-24 & UL-36 specific $CD4^+$ T cell response size and the differentiation of the respective memory subsets shed further light on the relationship between response size and degree of differention of memory subsets (Figure 4.10 and Figure 4.11 respectively). UL-86 specific $CD4^+$ T cell response size was significantly correlated to increasing frequencies of UL-86 specific $CD4^+$ T_{EM} (Rs=0.858, p=0.000) and UL-86 specific $CD4^+$ T_{EMRA} (Rs=0.667, p=0.005) subsets. No significant relationship was established for the UL-86 specific $CD4^+$ T_N and UL-86 specific $CD4^+$ T_{CM} subsets.

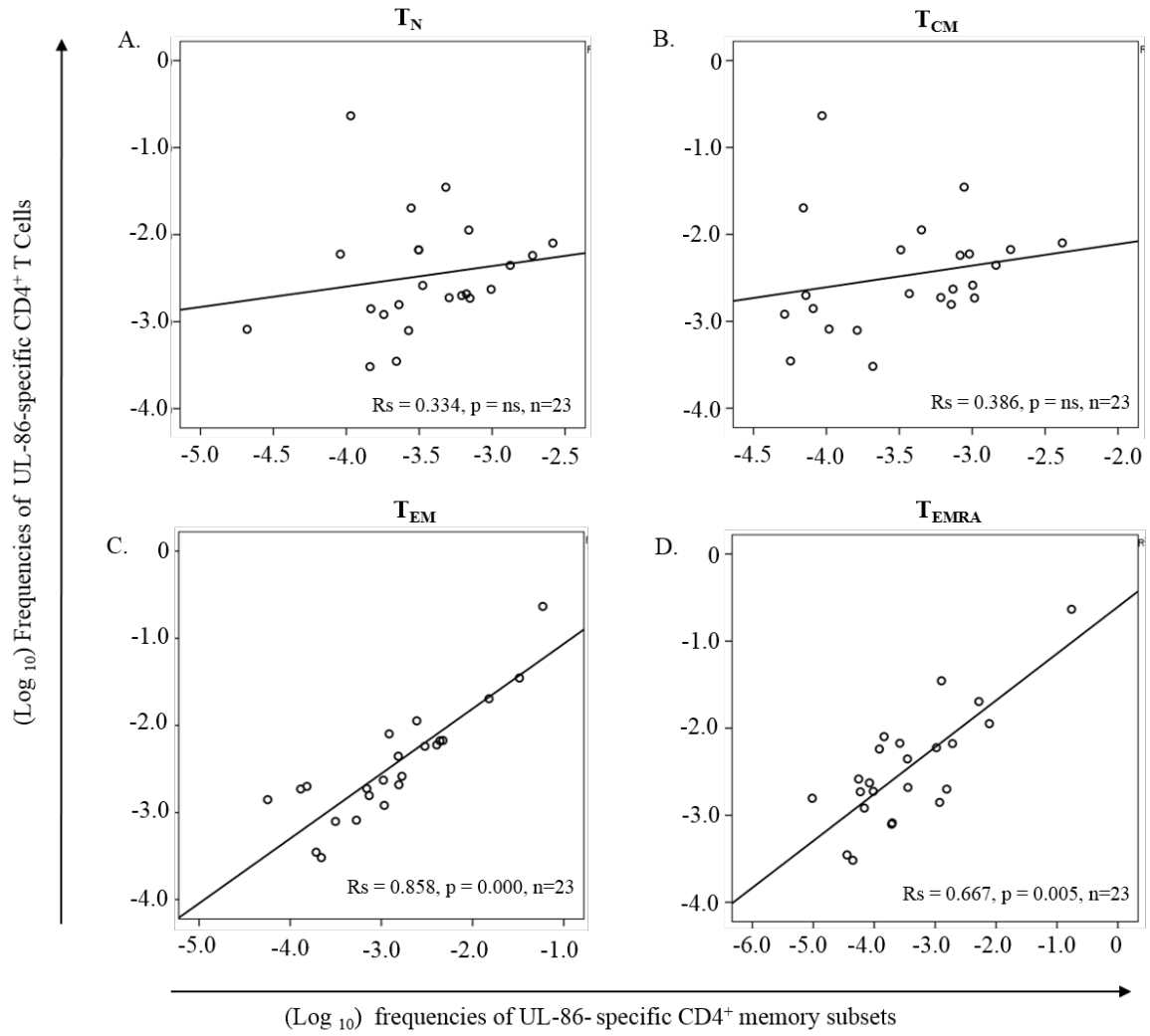


Figure 4.10. Correlation of UL-86 specific $CD4^+$ T cell memory subsets with UL-86 specific response size.

The scatterplots depict the frequencies of UL-86 specific $CD4^+$ T cells against UL-86 specific memory subsets T_N (A) ,T_{CM} (B), T_{EM} (C), and T_{EMRA} (D).$p < 0.05$ was considered significant.

The relationship between the US-24 & UL-36 specific $CD4^+$ T cell response size and US-24 & UL-36 specific $CD4^+$ T cell memory subsets deviated slightly from that observed for UL-86 specific responses. Notably, a significant positive correlation was observed between the US-24 & UL-36 specific $CD4^+$ T cell response size and all 4 memory subsets [US-24 & UL-36 specific $CD4^+$ T_N (Rs=0.647, p=0.02), US-24 & UL-36 specific $CD4^+$ T_{CM} (Rs=0.697, p=0.005), US-24 & UL-36 specific $CD4^+$ T_{EM} (Rs=0.876, p=0.000), US-24 & UL-36 specific $CD4^+$ T_N (Rs=0.691, p=0.02)]. The US-24 & UL-36 specific $CD4^+$ T_N

results can be dismissed in this instance, as they do not reflect a physiological phenomenon.

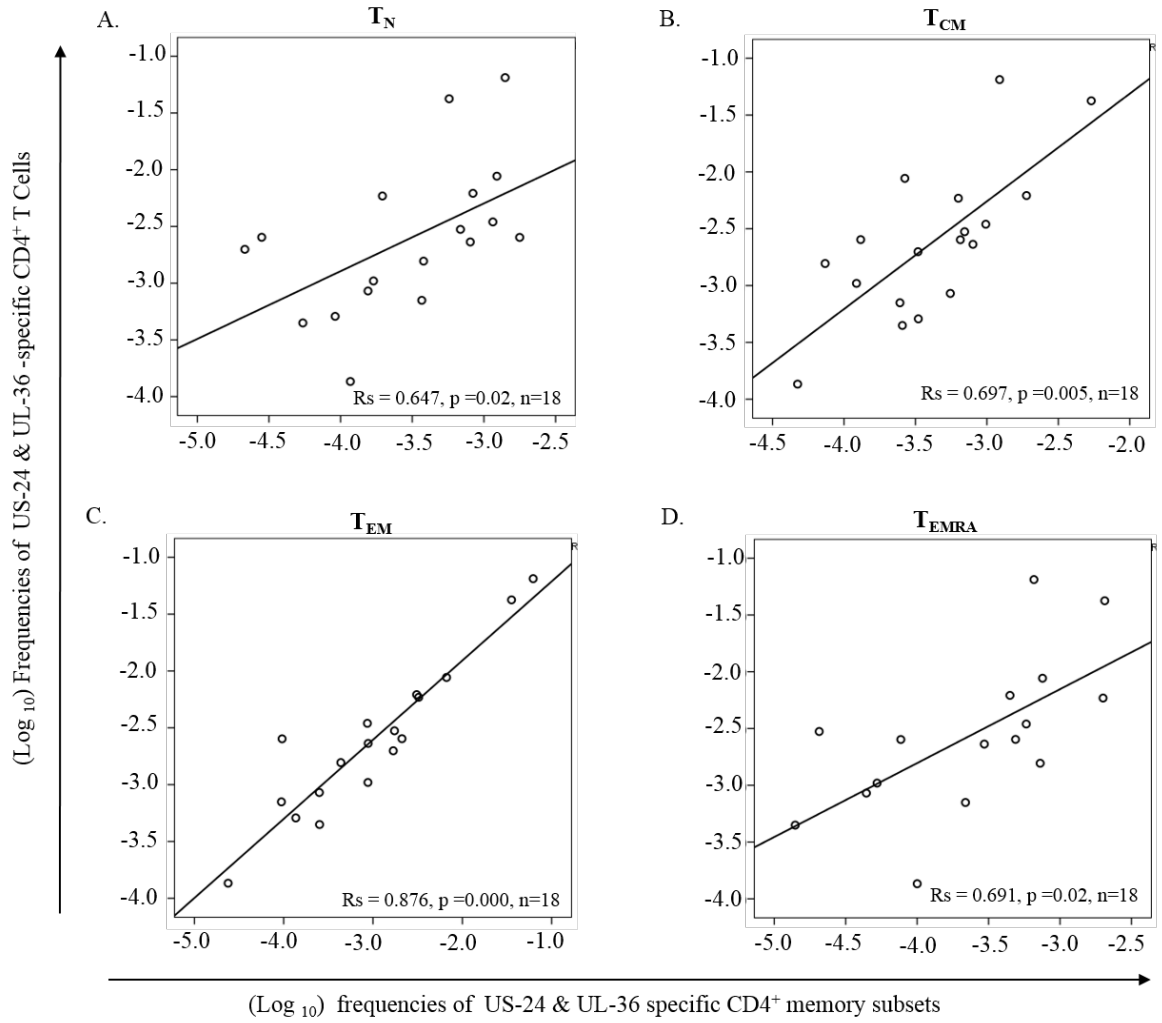


Figure 4.11. Correlation of US-24 & UL-36-specific CD4$^+$ T cell memory subsets with increasing response size.

The scatterplots depict the frequencies of US-24 & UL-36-specific CD4$^+$ T cells against US-24 & UL-36 specific memory subsets T_N (A) ,T_{CM} (B), T_{EM} (C), and T_{EMRA} (D).p <0.05 was considered significant.

Further analysis of pp65-specific CD8$^+$ T cell response size and its effect on the degree of memory subset differentiation was explored. Figure 4.12 depicts the scatterplots of pp65-specific CD8$^+$ T cell response size and the pp65-specific CD8$^+$ memory subsets (T_N, T_{CM},T_{EM}, and T_{EMRA}). There was a significant positive correlation between the frequencies of pp65-specific CD8$^+$ T cells and pp65-specific CD8$^+$ T_{EM} (Rs=0.686, p=0.000).

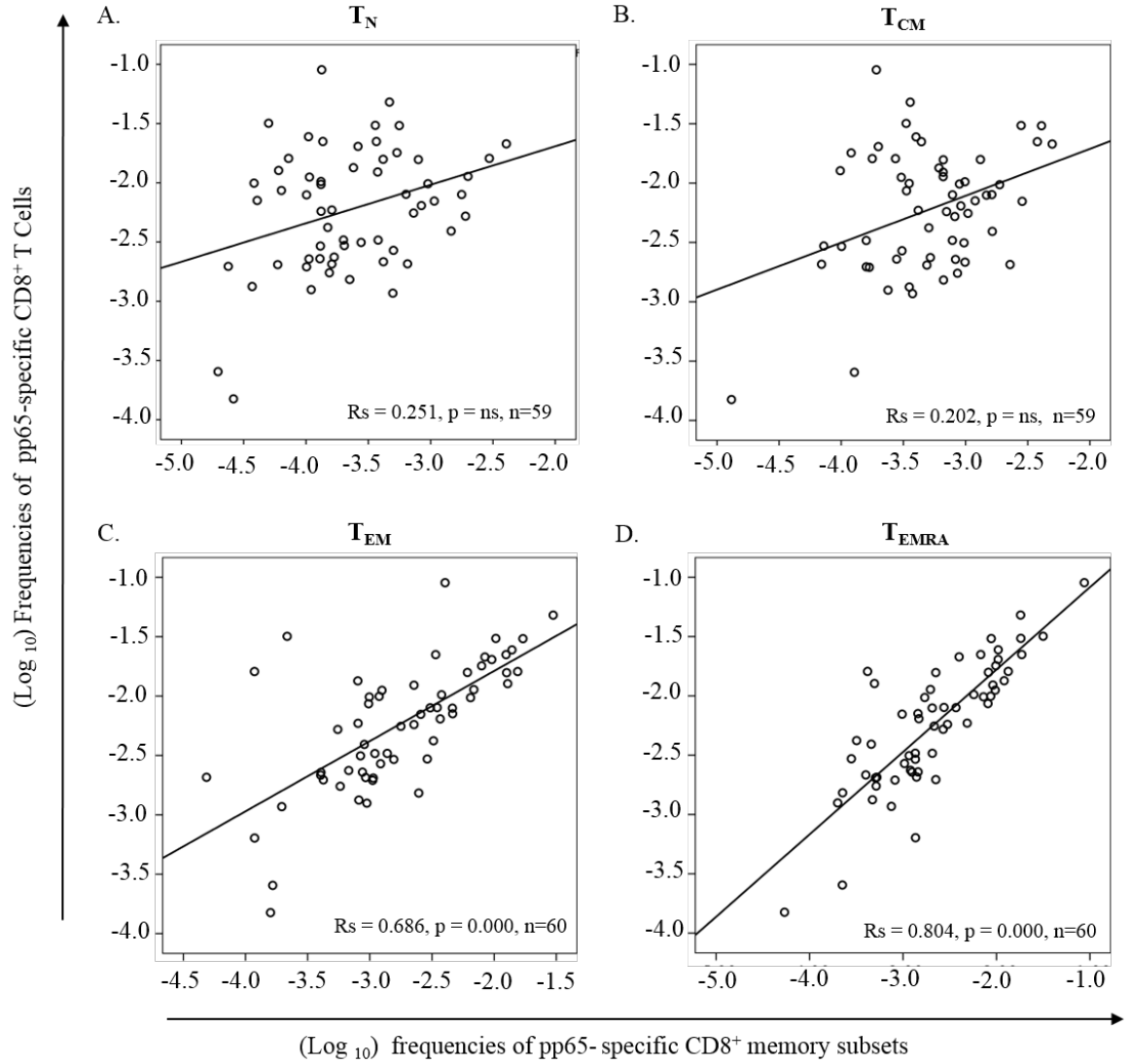


Figure 4.12. Correlation of pp65-specific $CD8^+$ T cell memory subsets with increasing response size.

The scatterplots depict the frequencies of pp65-specific $CD8^+$ T cells against pp65-specific memory subsets T_N (A) ,T_{CM} (B), T_{EM} (C), and T_{EMRA} (D).p <0.05 was considered significant.

The significant correlation was maintained when the size of pp65-specific $CD8^+$ T cells and pp65-specific $CD8^+$ T_{EMRA} (Rs=0.804, p=0.000) memory subsets were investigated. This is unlike the result observed with the pp65-specific $CD4^+$ T_{EMRA} memory subset which did not significantly change with increasing pp65-specific $CD4^+$ response size. In

essence, an increase in pp65-specific $CD8^+$ T cell significantly corresponds to a more advanced memory phenotype.

Focus turned to evaluating the relationship between the IE-1 specific $CD8^+$ T cell response size and IE-1 specific $CD8^+$ (T_N, T_{CM}, T_{EM} and T_{EMRA}) memory subsets (Figure 4.13). There was no signficant correlation observed between the size of IE-1 specific $CD8^+$ T cells and frequencies of IE-1 specific $CD8^+$ T_N memory subset.

However, It was observed that the frequencies of IE-1 specific $CD8^+$ T_{CM} (Rs=0.367, p=0.04), IE-1 specific $CD8^+$ T_{EM} (Rs=0.820, p=0.000) and IE-1 specific $CD8^+$ T_{EMRA} (Rs=0.768, p=0.000) increased significantly with increasing IE-1 specific $CD8^+$ T cell response size.

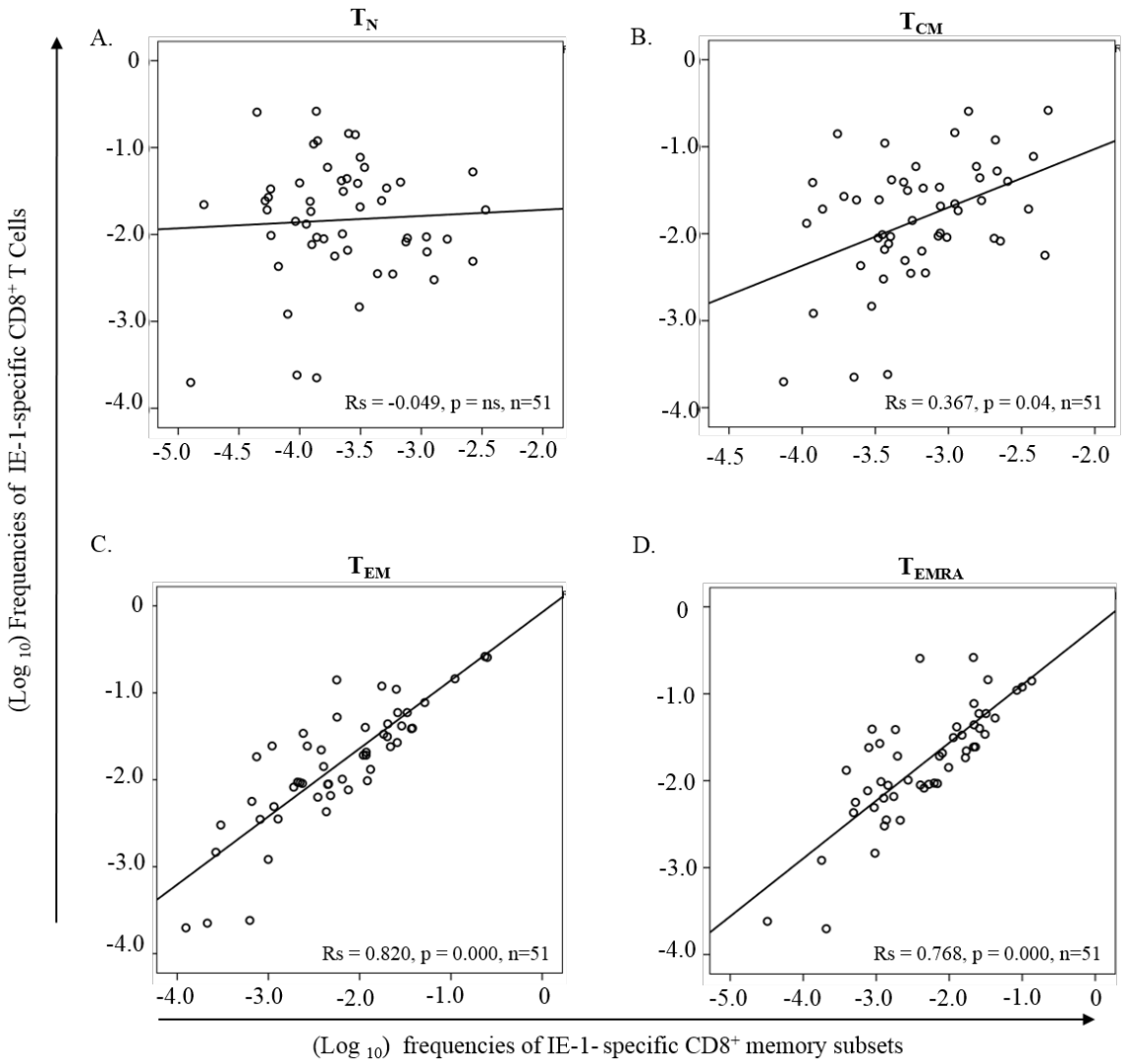


Figure 4.13. Correlation of IE-1-specific $CD8^+$ T cell memory subsets with increasing response size.

The scatterplots depict the frequencies of IE-1-specific $CD8^+$ T cells against IE-1-specific memory subsets T_N (A), T_{CM} (B), T_{EM} (C), and T_{EMRA} (D).$p < 0.05$ was considered significant.

Analyses of the third (UL-28) and fourth (UL-32) most recognised HCMV PSPPs based on the number of $CD8^+$ T cell responses was conducted. Scatterplots of UL-28 specific response size (Figure 4.14) against specific memory subsets revealed no relationship between the size of UL-28 specific $CD8^+$ T cell response and UL-28 specific $CD8^+$ T_N and UL-28 specific $CD8^+$ T_{CM} memory subsets. However, a similar trend to that observed with with pp65-specific $CD8^+$ response size emerged, an increase in UL-28 specific response

size significantly correlated with increasing size of UL-28 specific $CD8^+$ T_{EM} (Rs=0.783, p=0.000) and UL-28 specific $CD8^+$ T_{EMRA} (Rs=0.872, p=0.000) memory subsets.

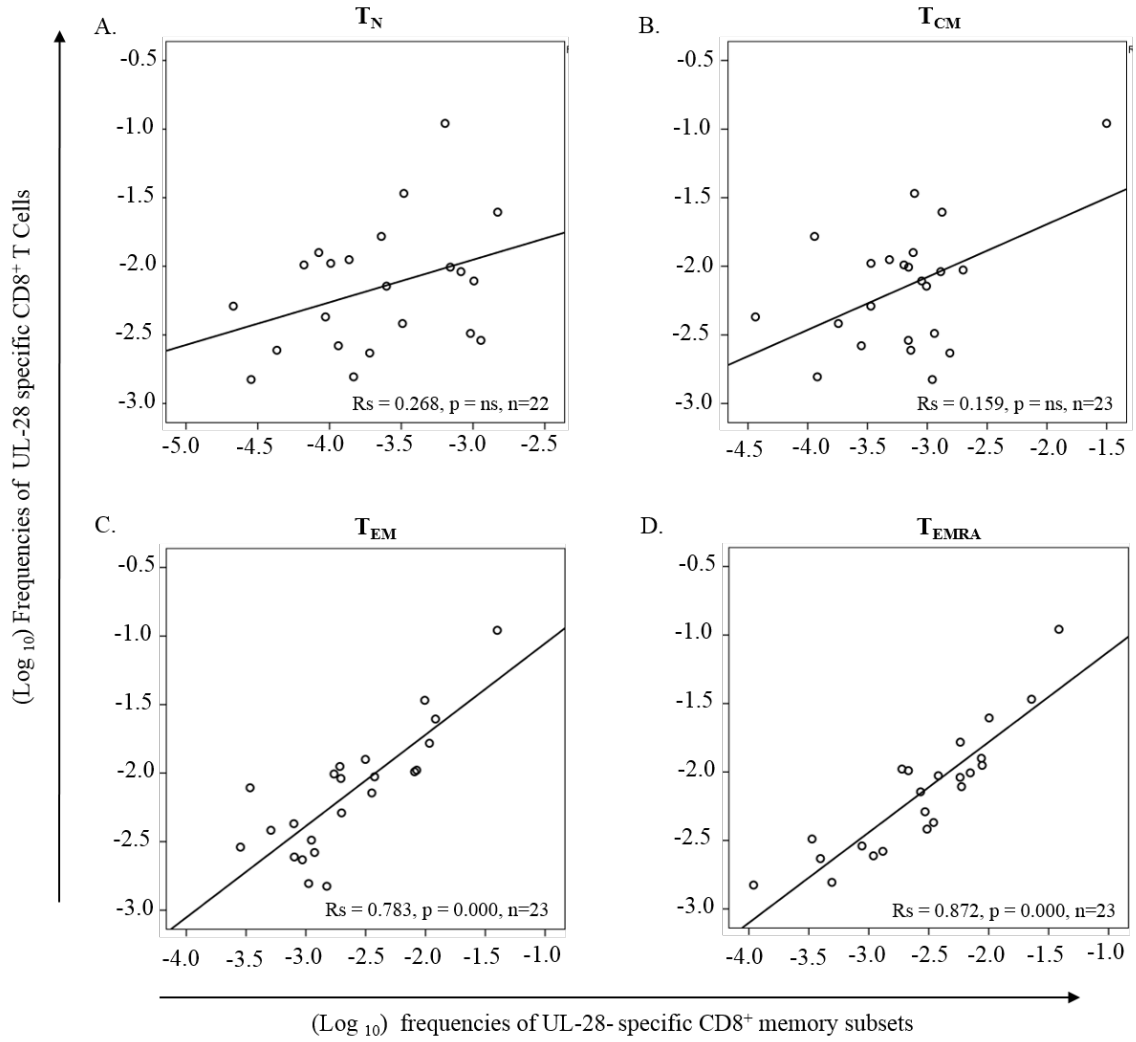


Figure 4.14. Correlation of UL-28 specific $CD8^+$ T cell memory subsets with UL-28 specific $CD8^+$ T response size.

The scatterplots depict the frequencies of UL-28 specific $CD8^+$ T cells against UL-28 specific memory subsets T_N (A), T_{CM} (B), T_{EM} (C), and T_{EMRA} (D).p <0.05 was considered significant.

Figure 4.15 depicts the scatterplot of the UL-32 specific $CD8^+$ T cell response size and the corresponding specific memory subsets. There was a significant correlation between the increasing size of the UL-32 specific $CD8^+$ T cell response and the UL-32 specific $CD8^+$

T_{EMRA} memory subset (Rs=0.729, p=0.005). No further significance was observed with the UL-32 specific $CD8^+$ T_N, T_{CM} and T_{EM} memory subsets.

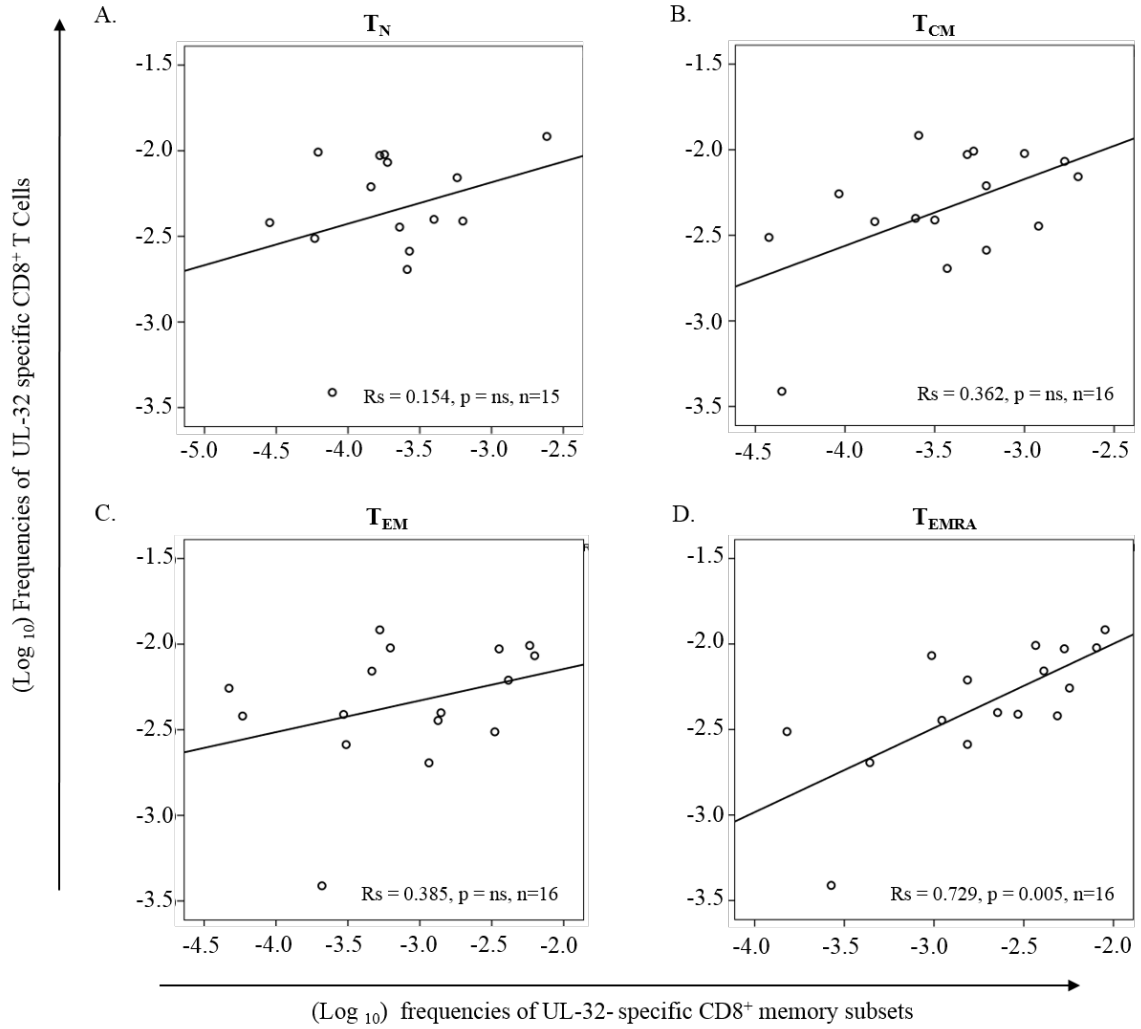


Figure 4.15. Correlation of UL-32 specific $CD8^+$ T cell memory subsets with UL-32 specific $CD8^+$ T response size.

The scatterplots depict the frequencies of UL-32 specific $CD8^+$ T cells against UL-32 specific memory subsets T_N (A), T_{CM} (B), T_{EM} (C), and T_{EMRA} (D).$p < 0.05$ was considered significant.

4.2 DISCUSSION

The impact of HCMV infection on general health and wellbeing in aged individuals is of great importance. Numerous publications investigating the effect of HCMV infection in aged individuals have reported increased mortality, and susceptibility to developing cardiovascular pathology (93, 129). The latent nature and frequent reactivation of HCMV means that vast immune resources are dedicated to dampening and maintaining efficient memory responses in healthy individuals.

Various studies characterising T cell responses against frequently recognised HCMV proteins pp65 and IE-1 have reported an expansion of HCMV-specific memory $CD8^+$ T cells with age. This is often dominated by the revertant memory subset (T_{EMRA}) when compared to other virus specific T cells (79, 130-132).

Questions such as why these large expansions exist , how they are maintained, and the functional profile of terminally differentiatied memory T cells, remain the focus of a number of studies .

One key issue that arises when using CD45RA and CD27 phenotypic markers to delineate the memory subsets of HCMV-specific T cells is deciphering the 'true' phenotypic nature of the T_N ($CD45RA^+$ $CD27^+$) memory subset. Limited numbers of available flourescence channels often results in stringent selection of multiparametric panels, this study was no different. In this instance, our memory subset panel was limited to two widely used markers, CD45RA and CD27. Admittedly, the additional use of CD45RO marker, which identifies activated T cells could have would have been useful in distinguishing the effector and reverting subsets. Responses to HCMV PSPPs were evident in the T_N memory subset. One possibility is that this subset contains T_{CM} memory T cells reverting to the T_N phenotype which could account for some of the large frequencies. Notably, a successful

combination of CD45RA and CD27 has been used to enumerate HCMV specific memory T cell subsets by various studies (115, 133-136).

A newly described memory T cell subset with stem cell like properties (Stem cell memory T cell- T_{SCM}) has been identified in humans with the phenotypic profile, $CD45RO^{-}CD45RA^{+}CCR7^{+}CD62L^{+}CD28^{+}CD127^{+}CD95$ (28-30, 32). It is highly probable that the high T_N memory subset observed in this study are not true 'Naïve' memory T cells but inclusive of T_{SCM} subset population. A couple of recently published studies identified HCMV specific T_{SCM} susbet within the T_N compartment. The authors demonstrated that the majority of the HCMV-tetramer binding T_N cell subsets from the HCMV seropositive cohort analysed, expressed high levels of CD95.

The low frequency of T_{SCM} subset (<1% of total T cells) has limited the characterisation of Ag-specific T_{SCM} until recently. In a study by Schmueck-Henneresse et al. in which expanded T_{SCM} subset were stimulated with HCMV pp65/IE-1 peptide pool, they observed that the T_{SCM} subset was able to mount effector functions upon antigenic stimulation. A substantial proportion of the HCMV-specific $CD8^{+}$ T_{SCM} subset produced IFNγ and TNFα after antigen stimulation compared to the $CD4^{+}$ T_{SCM} subset. The effector functions observed in the TSCM subset were specific as there were no effector cytokines secreted in response to HCMV antigenic stimulants in HCMV seronegative donors.

One of the aims of this work was to characterise memory subset distribution against a wide array of select HCMV proteins to determine how HCMV-specific memory T cell subsets changes with age and if this depends on the protein target.

Initial analysis focused on the influence of age on memory subset distribution in HCMV seropositive individuals. Results from this study found that while the total $CD8^{+}$ T_N pool significantly decreased with age, the memory compartment was increasingly dominated by

the $CD8^+$ T_{EMRA} subset with age, in HCMV seropositive individuals. This supports previous findings by our group, and other studies (123, 137, 138).

Using a set of different phenotypic markers to delineate memory subsets [(CD28 & CD95), (CD45 & CCR7)] in a large cross sectional study, Wertheimer and colleagues observed that ageing in HCMV seronegative individuals (n=152, 21-101 years) resulted in the absolute and relative loss of $CD8^+$ T_N subset, but no accumulation of $CD8^+$ T_{CM} and T_{EM} with increasing age (125). These observations support the findings that declining $CD8^+$ T_N, and stable memory $CD8^+$ population, are both normal features of physiological ageing.

Much has been published about the expansion of memory subsets in HCMV infected individuals which could be detrimental to long term health by limiting the immunological repertoire and resulting in a clonal expansion of select T cells in aged individuals (77, 80, 111, 139-143)

Indeed the persistent nature of HCMV means it is never eradicated from the host, which leads to alteration of the memory subset distribution due to chronic antigenic stimulation and proliferation of HCMV-specific T cells as described by several studies. This is unlike other persistent viruses such as Herpes simplex virus or Epstein barr virus (111, 144-147).

The unique aspect of our work is the use of a wide set of HCMV proteins to gain a wider perspective on immune response with age. The different experimental set up, breadth of antigenic stimulants (e.g whole lysate, immunodominant epitopes and single proteins and/or PSPP) and measured output (limited cytokines) makes direct comparison with other studies somewhat difficult. However, useful information can still be gleaned from such studies.

Murine cytomegalovirus (MCMV), a homologue of the human cytomegalovirus and a natural pathogen in mice has been a useful model for deciphering the effect of HCMV

infection with age (127, 128, 148). However, care must be taken when extrapolating findings from such studies to humans due to notable differences such as life-span, viral load, antigen, genetic background and course of infection etc.

Infection with MCMV follows a sequence starting with the transcription of IE genes through to synthesis of Late (L) gene products, which would indicate a higher preponderance of IE products dominating the immune responses, characteristics which are not so clear from human studies. A study exploring memory inflation in mouse models (BALB/c and C57BL/6) infected with a recombinant strain of MCMV describe the importance of gene expression kinetics and the availability of antigens/epitopes in determining the size of antigen specific memory responses (149).

In a study by Khan et al 2002 assessing the effect of CMV seropositivity on $CD8^+$ T cell repertoire with increasing age, the authors analysed $CD8^+$ epitope specific T cell responses to pp65 immuno-dominant epitopes (NLVPMVATV presented by HLA-A*0201 and TPRVTGGGAM presented by HLA-B*0702). They observed a significant increase in CMV epitope specific $CD8^+$ T cells in the older age group compared to healthy young controls (111).

Also, a previous finding by Pourgheysari and colleagues, reported an accumulation of HCMV specific $CD4^+$ T cells with age in healthy HCMV seropositive donors (aged between 22 to 84) (150). However, the $CD4^+$ response was measured against CMV lysate, the composition of which in terms of proteins is not entirely clear.

Our results demonstrate no significant differences in the frequencies of HCMV-specific T_N, T_{CM}, T_{EM}, T_{EMRA} memory subset against top $CD4^+$ (UL-55, pp65, UL-86 and US-24 + UL-36) and $CD8^+$ (pp65, IE-1, UL-28, and UL-32) targets in the Young, Older and Oldest groups. Although this study did not directly investigate the memory inflation of HCMV

specific memory T cells in humans, the findings from this study would firmly suggest that it is not a common wide phenomenon in humans. Infact, a recent study of HCMV seropositive individuals by Jackson et al. analysing the frequency of T cells responding to multiple HCMV ORF products at various time points over a 3 year period reported that the frequency of HCMV specific $CD8^+$ T cells did not show any attributes related to inflation , rather a fluctuation in T cell frequencies was observed (115).

Further analyses of the total (sum of all 19 HCMV proteins) HCMV-specific memory subset distribution did reveal a signficant increase in the size of the $CD8^+$ T_{EMRA} subset in the Older group (median age=64) compared to the Young (median age=27). However no significant increase was observed in the Oldest group (median age=74).

In conclusion, our findings reveal that the size of the response to HCMV proteins plays a greater role in the degree of advanced T cell memory subset differentiation, than age. This corroborates earlier findings by our group, and a recent publication by Vescovini et al., 2014 focusing on pp65 specific and IE-1 specific T cell responses in HCMV seropositive aged individuals (aged 60-100 years) (116, 151). This finding is not unique to the more established HCMV PSPPs (pp65 and IE-1), analyses of other top recognised PSPPs in this study based on $CD4^+$ (UL-86 and US-24 & UL-36) and $CD8^+$ (UL-32 and UL-28) T cell responses also support this observation.

5 POLYFUNCTIONAL PROFILE OF HCMV-SPECIFIC T CELLS

Most studies of functional HCMV-specific T cells are typically based on the frequency of $CD4^+$ and $CD8^+$ T cells producing IFNγ and or TNFα upon stimulation (150).

However, several studies have since shown that the protective capacity of T responses is proportional to the number of cytokines produced at the same time (152, 153). Indeed, vaccination experiments have demonstrated that T cell polyfunctionality correlates with protection. Although protective effect of different combinations of effector functions does appear to be disease specific.

Polyfunctional T cells have the ability to exhibit multiple effector functions (namely production of cytokines and chemokines whilst also promoting cytolysis) simultaenously upon antigenic stimulation (154). They produce greater quantities of cytokines compared to single cytokine producing cells. However, the combination of cytokines produced has been shown to be of importance in infection control. Cells producing a combination of activation markers such as IL-2, IFNγ and TNFα simultaneously are thought to play a very important role in the effective control of infection. In a previous study of HCMV seropositive individuals aged between 20-84 years by our group, the different polyfunctional profiles on pp65-specific $CD4^+$ and $CD8^+$ T cells were described. An increase in the subsets with the highest number of activation markers produced simultaeneously was observed in those with a large pp65-specific $CD4^+$ / $CD8^+$ T cell response size, irrespective of age (151).

The level of polyfunctionality can be assessed ex-vivo by means of multi-parametric flow cytometry. For this study, 5 activation markers (IFNγ, TNFα, IL-2, CD40L and, CD107a) were measured on stimulated T cells. Frequencies of activated T cells producing 1 of 5 listed markers, 2 of 5 activation markers, 3, 4 and 5 activation markers were captured.

These are henceforth described as having 1, 2, 3, 4, or 5 functions. Using the boolean gate function in FlowJo which automatically generates subsets based on the different combination of markers utilised, a functional profile of different combinations of activation markers for each HCMV protein of interest was devised. The subsets of activated T cells producing a combination of the activation markers were further analysed using "FunkyCells data mining" software (www.FunkyCells.com). This software utilises the functional subsets generated from boolean gating and provides a polyfunctionality index score. The index captures polyfunctionality as a (single) numeric parameter enabling statistical comparisons with other parameters of interest. This is unlike the conventional means of analysing polyfunctionality using tools such as SPICE which assess, and compare subsets that are positive for a defined number of effector functions (Appendix Figure 8.6).

The aim of this chapter was to determine if polyfunctionality of HCMV-specific T cells changed with increasing age in our cohort using the 'polyfunctionality index' of HCMV-specific T cell responses. Level of polyfunctionality was also assessed by comparing the number of functional markers produced in response to HCMV antigen stimulation in the Young, Older and Oldest group.

5.1 CHANGES IN POLYFUNCTIONALITY WITH AGE

The number of activation markers produced in response to select HCMV proteins was plotted for Young, Older and Oldest groups to assess changes in level of polyfunctionality with age.

Figures 5.1 and 5.2 depicts the polyfunctionality profile of HCMV-specific activated T cells against select proteins producing 1, 2, 3, 4 and 5 functions in all 3 groups. Briefly, the subsets of pp65-specific $CD4^+$ T cells (Figure 5.1A) with 1 function, 2 functions , 3

functions, 4 functions and 5 functions were compared in Young, Older and Oldest. The results from this analysis indicates no change in the level of polyfunctionality with age as we observed no significant differences in the number of functions produced by different subsets of pp65-specific $CD4^+$ T cells across the age groups.

Interestingly, significant differences emerged when subsets of pp65-specific $CD4^+$ T cells in Young and 'Old' (combination of the "Older" and "Oldest" groups) groups producing up to five functions simultaenously were compared.

There was a significant increase ($p=0.046$) in polyfunctionality observed in pp65-specific $CD4^+$ T cells producing 4 functional markers in the 'Old' cohort compared to Young (Appendix, Figure 8.4). Contrary to the trend observed with the pp65-specific $CD4^+$ T cells with 4 functions, there was a significant decrease ($p=0.046$) in polyfunctionality in pp65-specific $CD4^+$ T cells producing 2 functions in the 'Old' group compared to Young.

The percentage of UL55-specific $CD4^+$ T cells (Figure 5.1B) producing up to five functions were compared in Young, Older and Oldest. This did not yield any significance, indicating that there is no difference in the polyfunctionality profile with age.

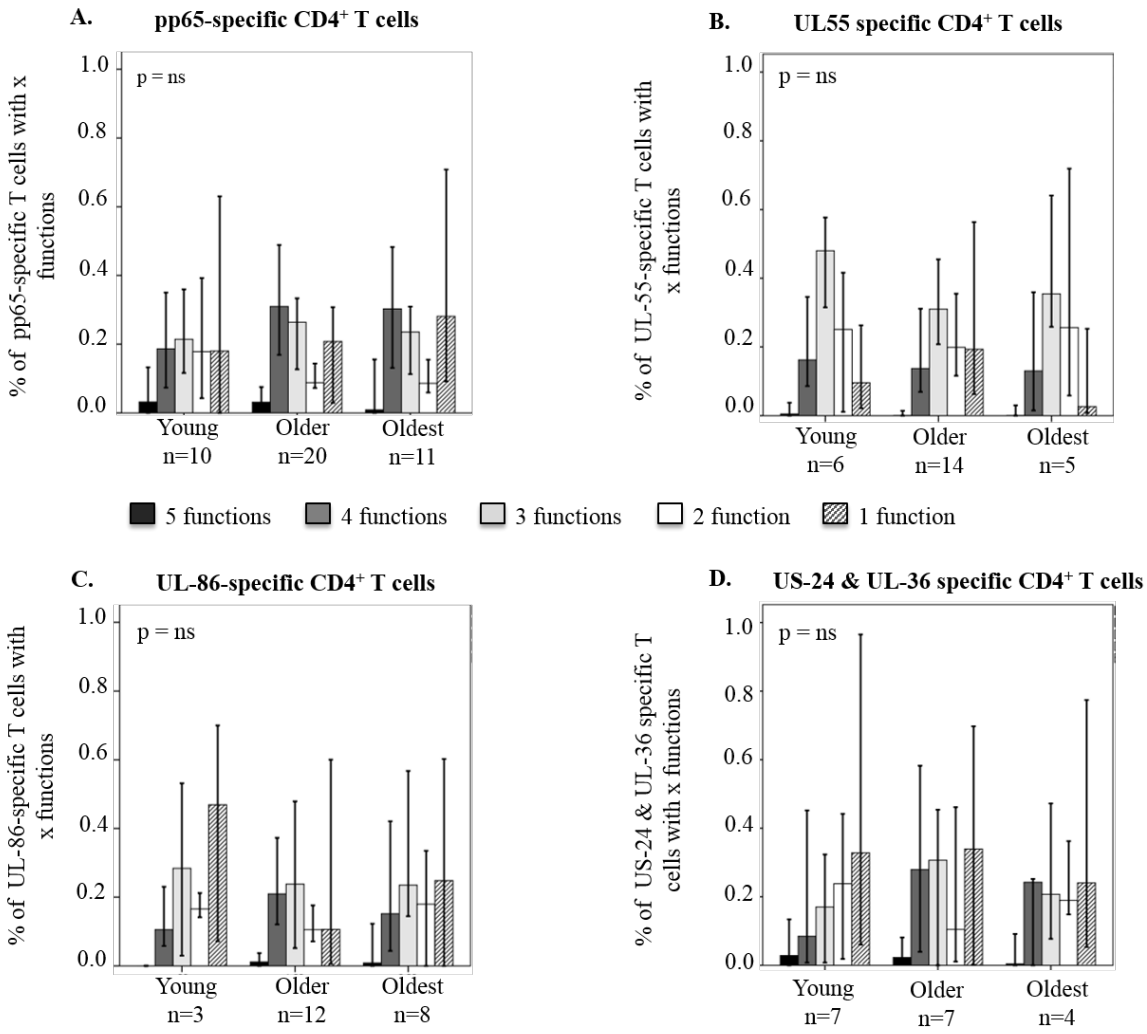


Figure 5.1. Subsets of HCMV-specific CD4$^+$ T cells with multiple functions does not change with increasing age.

The percentage of HCMV-specific CD4$^+$ T cells producing up to five activation markers upon antigenic stimulation in the 3 age groups are plotted in the bar charts above (A-D). pp65 specific (A), UL-55 specific (B), UL-86 specific (C), US-24 & UL-36 specific (D) CD4$^+$ T cells. Each bar ranging from black to white represents the number of cytokines produced, 5 functions down to 1 function. Median values with CI are displayed. n numbers displayed beneath each group. $p < 0.05$ was considered significant

There were no significant differences in level of polyfunctionality between the 3 groups when the UL-86 specific and US-24 & UL-36 specific CD4$^+$ T cell responses were compared in the Young, Older and Oldest groups (Figure 5.1C and 5.1D respectively).

Analyses of the polyfunctionality profile of the top 4 recognised HCMV proteins based on CD8$^+$ T cell responses is depicted in Figure 5.2A-D.

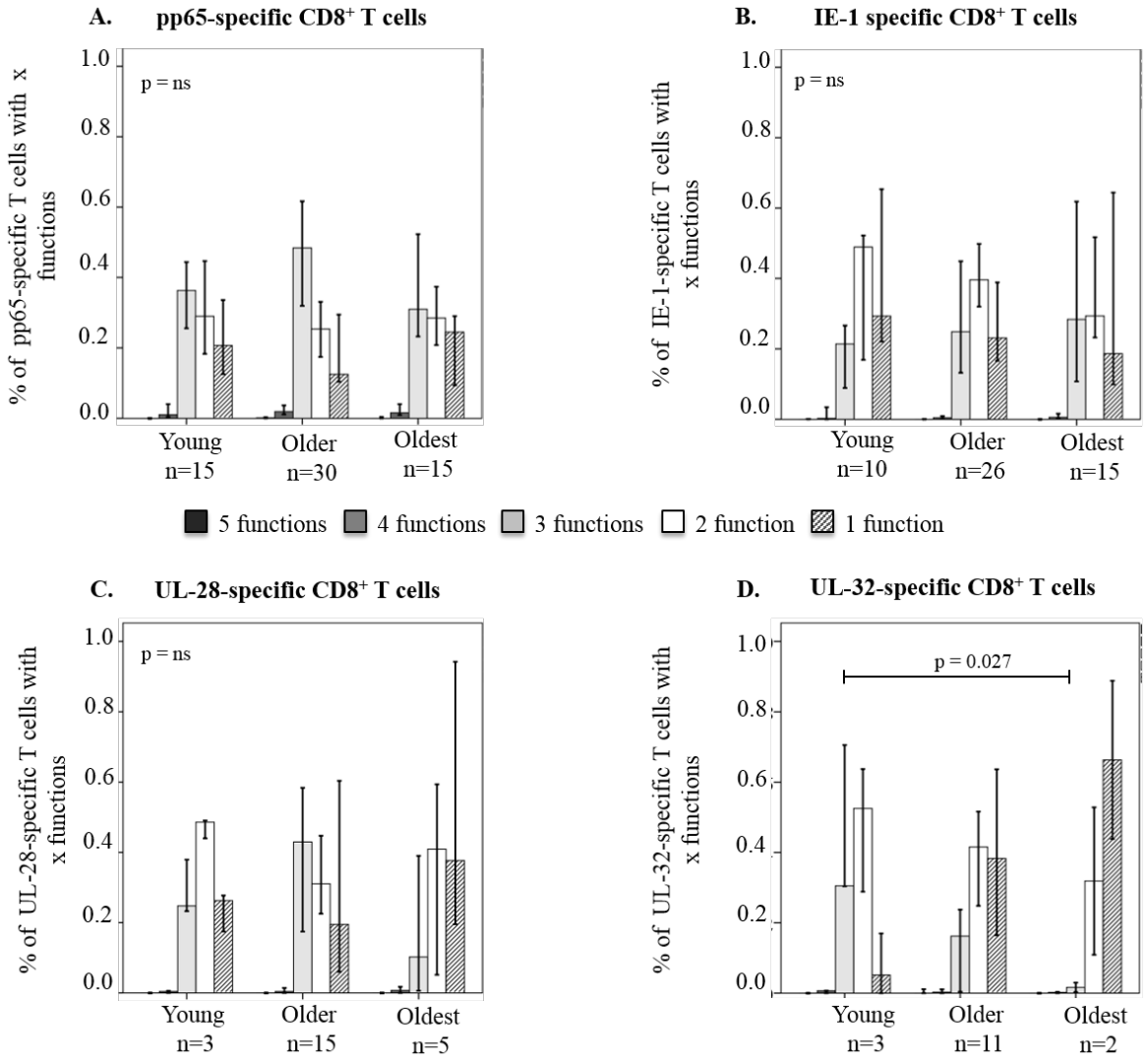


Figure 5.2. Subsets of HCMV-specific CD8$^+$ T cells with multiple functions does not change with increasing age.

The percentage of HCMV-specific CD8$^+$ T cells producing up to five activation markers upon antigenic stimulation in the 3 age groups are plotted in the bar charts above (A-D). pp65-specific (A), IE-1 specific (B), UL-28 specific (C), and UL-32 specific (D) CD8$^+$ T cells for Young, Older and Oldest. Each bar ranging from black to white represents the number of cytokines produced, 5 functions down to 1 function. Median values with CI are displayed. n numbers displayed beneath each group. $p < 0.05$ was considered significant.

The percentage of pp65-specific CD8$^+$ T cells producing up to 5 activation markers did not differ significantly across the 3 groups (Figure 5.2A). The same trend was observed when IE-specific and UL-28 specific CD8$^+$ T cells in Young, Older and Oldest groups were compared (Figure 5.2B and 5.2C respectively).

Interestingly, there was a significant decrease in the percentage of UL-32 specific $CD8^+$ T cells with 3 functions in the Oldest group compared to the Young (Figure 5.2D). However, it is worth noting that the comparison is between 2 individuals in the Oldest group and 3 individuals in the Young group. The analyses was once again restricted to two groups of Young and Old (This is a combination of the "Older" and "Oldest" groups). The proportion of pp65-specific $CD8^+$ T cells producing 5 functions was significantly increased in the Old group ($p= 0.037$) compared to the Young (Appendix, Figure 8.5).

There were no significant differences observed in the level of polyfunctionality in the IE-1 specific and UL-28 specific $CD8^+$ T cells between the Old and Young group. However, there was a significant decrease in the UL-32 specific $CD8^+$ T cells producing 3 functions in the Old group ($p=0.014$) compared to the Young. While a significant increase in the UL-32 specific $CD8^+$ T cells producing 1 function was observed in the Old compared to the Young group ($p=0.014$). However, due to the small sample size, no valid conclusions could be drawn.

5.2 LEVEL OF POLYFUNCTIONALITY CORRELATES WITH INCREASED RESPONSE SIZE

To determine the effect of response size on the level of polyfunctionality, the frequencies of T cells responding to top HCMV PSPPs based on $CD4^+$ and $CD8^+$ T cell responses were plotted against the corresponding polyfunctionality index (Figure 5.3 and Figure 5.4 respectively). There was a significant increase in polyfunctionality, as the size of pp65 and UL-55 specific $CD4^+$ T cells increased.

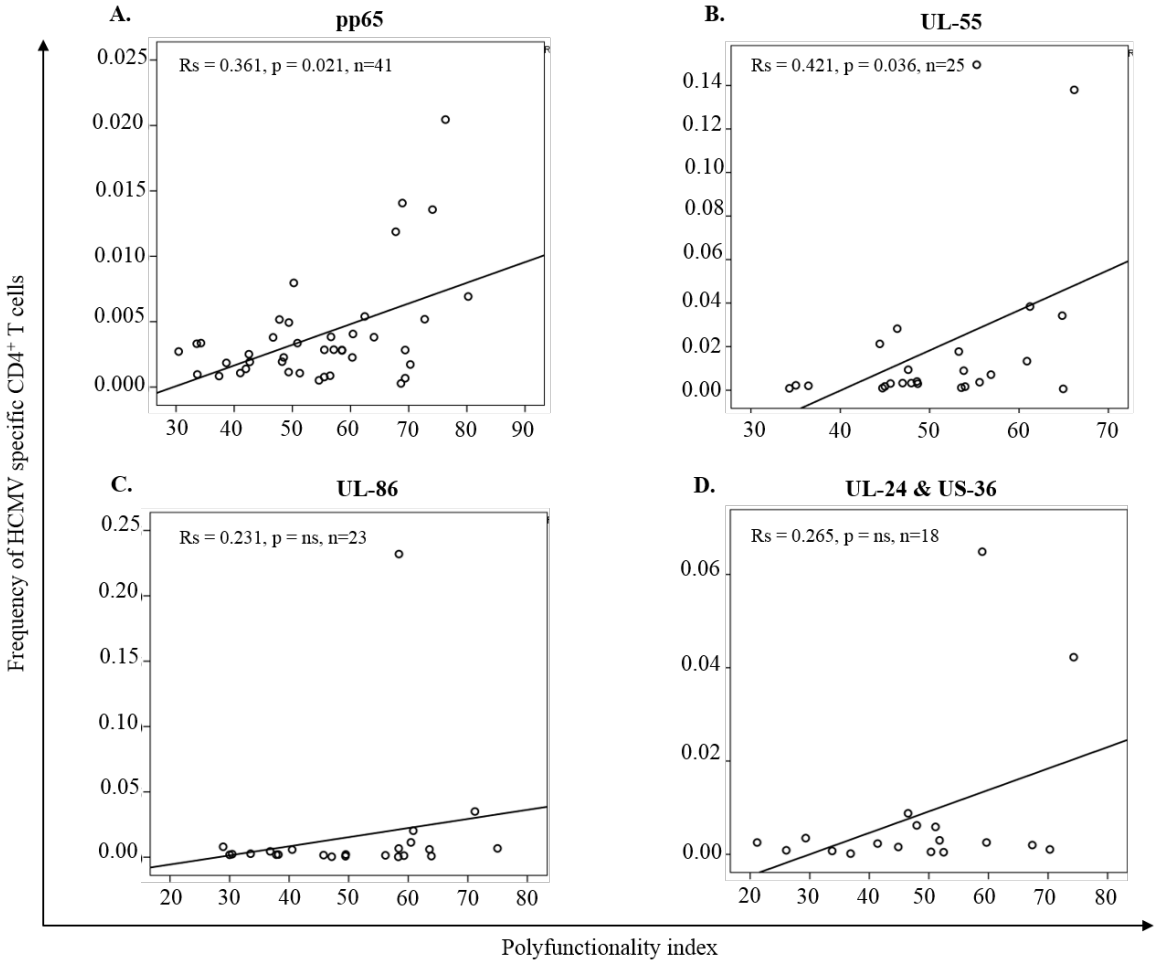


Figure 5.3. Correlation of response size and polyfunctionality index in top recognised HCMV PSPPs based on $CD4^+$ T cell responses.

The scatter plot displayed above represents the frequencies of pp65-specific (A), UL-55 specific (B), UL-86 specific (C) and UL-24 & US-36 (D) specific $CD4^+$ T cells against the corresponding polyfunctionality index. $p < 0.05$ was considered significant.

A significant increase in polyfunctionality was observed with increasing pp65-specific, IE-1 specific and UL-28 specific $CD8^+$ T cell response size.

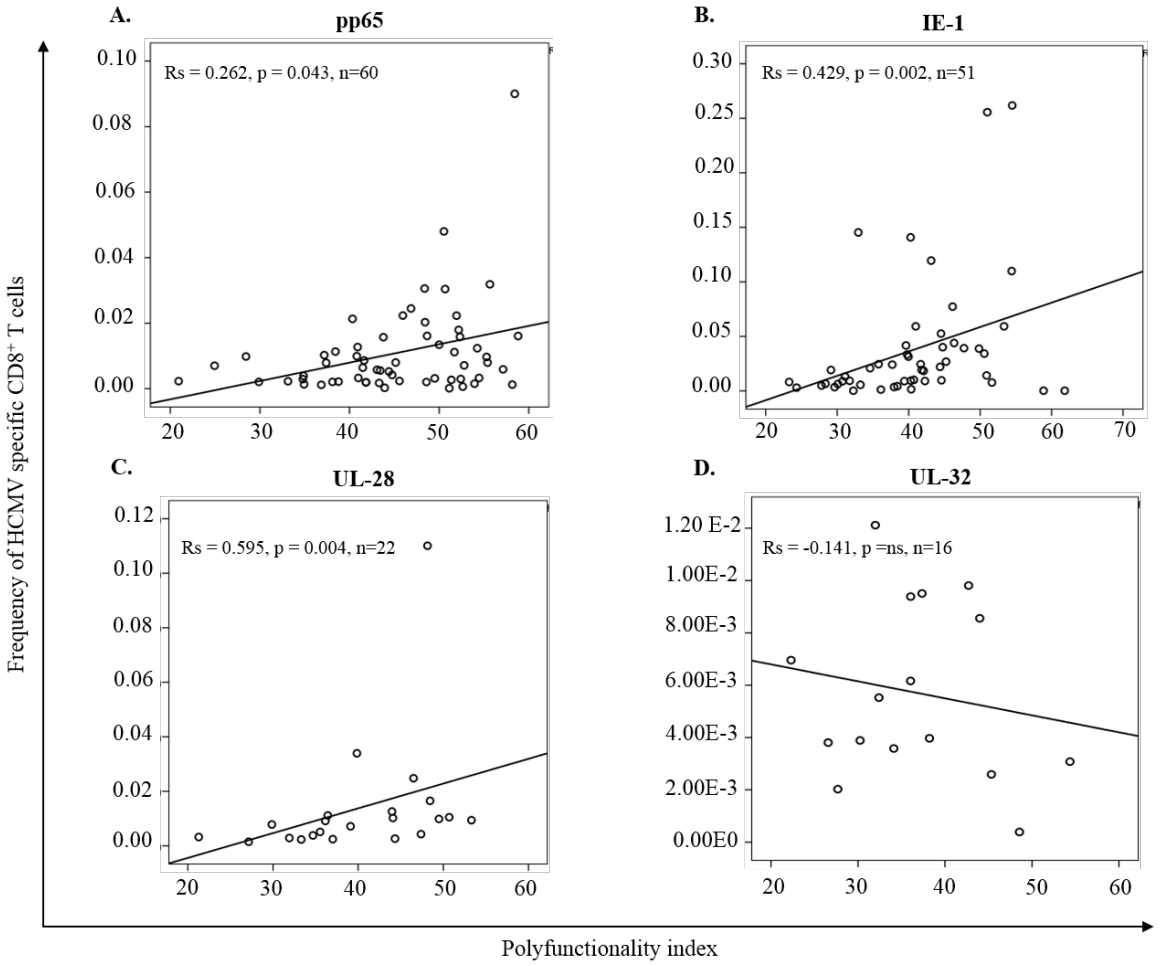


Figure 5.4. Correlation of response size and polyfunctionality index in top recognised HCMV PSPPs based on $CD8^+$ T cell responses.

The scatter plot displayed above represents the frequencies of pp65-specific (A), IE-1 specific (B), UL-28 specific (C) and UL-32 (D) specific $CD8^+$ T cells against the corresponding polyfunctionality index. $p < 0.05$ was considered significant.

5.3 FUNCTIONAL DOMINANCE OF ACTIVATION MARKERS PRODUCED DURING HCMV-SPECIFIC RESPONSES

The combination of functional markers selected for this study was done with the intention of capturing clearly detailed HCMV-specific T cell responses in our cohort.

The effector functions and other functional properties exhibited by activated T cells were further delineated to gain perspective on the dominance of each functional marker in response to HCMV proteins, within the 3 groups of interest.

To achieve this, the percentage of HCMV-specific T cell responses producing IL-2, IFNγ, TNFα, CD40L and CD107a against top recognised HCMV PSPPs based on $CD4^+$ and $CD8^+$ T cells responses in all age groups were analysed. The aim of this exercise was to determine if there are any significant differences in functional dominance of any of these cytokines against HCMV PSPPs between the Young, Older and Oldest groups.

Analyses of the top recognised HCMV PSPPs based on $CD4^+$ T cell responses was conducted as displayed in Figure 5.5. There were no significant, functionally dominant cytokines produced in response to UL-55, pp65, UL-86 and US-24 & UL-36 HCMV PSPPs in the three age groups.

Generally, the percentage of pp65-specific $CD4^+$ T cells producing CD40L and IFNγ appear to dominate responses in the Young group. This trend is also observed in the Older and Oldest groups. Additionally, there was an increase in the percentage of pp65-specific $CD4^+$ T cells producing TNFα in the latter groups, although this does not appear to be significant.

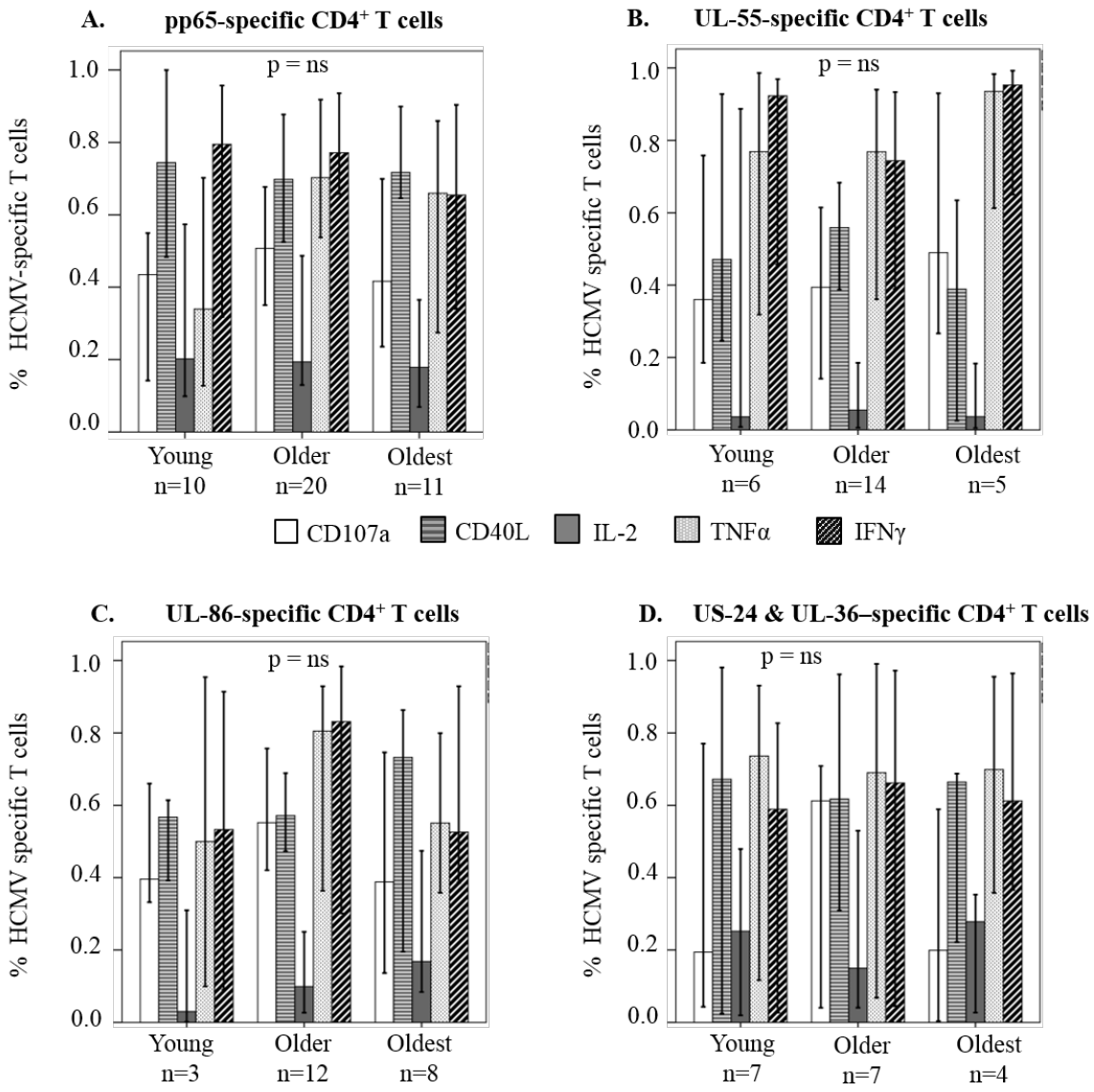


Figure 5.5. Functional dominance of activation markers upregulated by HCMV-specific CD4$^+$ T cells in response to antigen stimulation.

The bar charts of the functional dominance of any of the five cytokines utilized (CD107a, CD40L, IL-2, TNFα and IFNγ) based on UL-55 specific (A) , pp65 specific (B), UL-86 specific (C) and US-24 & UL-36 specific (D) CD4$^+$ T cell responses in Young, Older and Oldest groups. Median values with 95 % CI are indicated. P <0.05 was considered significant.

The percentage of UL-55 specific CD4$^+$ T cells producing IL-2 is low in all 3 groups compared to the percentage of T cells producing CD107a, TNFα, IFNγ, and CD40L. However, TNFα and IFNγ appear to be the dominant functional markers in all 3 groups based on the percentage of UL-55 specific CD4 $^+$ T cells producing these markers.

Although this was found to be non-significant, TNFα and IFNγ dominate the UL-86 specific CD4 T cell responses in the Older cohort compare to the other groups. There was a notable steady incline in the percentage of activated T cells producing IL-2 in the Oldest group compared to the Young, however, this was not significant.

The percentage of US-24 & UL-36 specific $CD4^+$ T cells degranulating which was measured using CD107a increased in the Oldest group compared to the Young, although this was not statistically significant. This was further reduced in the Oldest group, with percentages similar to that observed in the Young group.

The analyses was repeated focusing on pp65, IE-1, UL-28 and UL-32 HCMV PSPPs (Figure 5.6). These are the top recognised HCMV PSPPs based on $CD8^+$ T cell responses

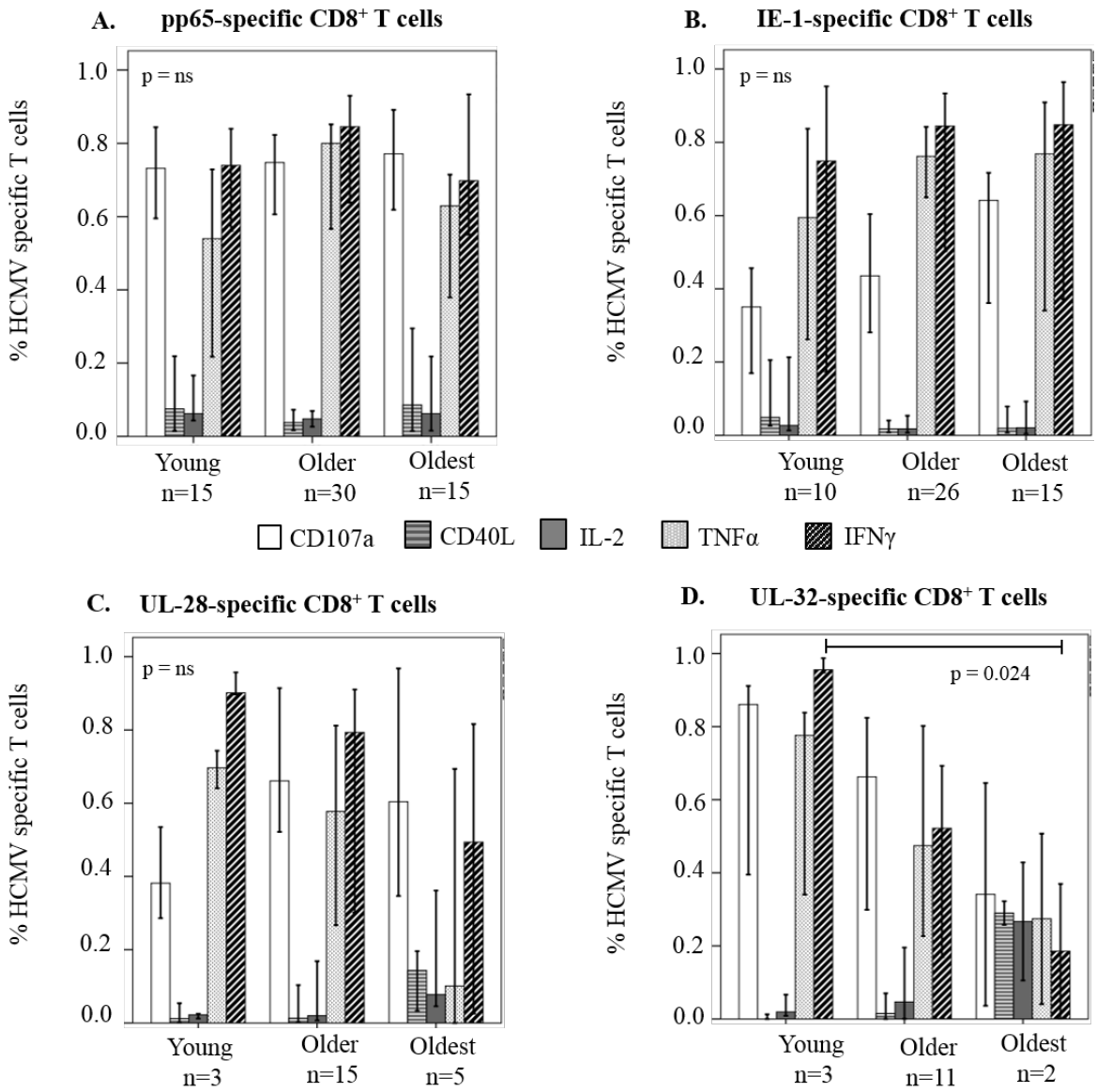


Figure 5.6. Functional dominance of activation markers upregulated by HCMV-specific CD8$^+$ T cell in response to antigen stimulation.

The bar charts are the functional dominance of any of the five cytokines utilized (CD107a, CD40L, IL-2, TNFα and IFNγ) based on pp65 specific (A), IE-1 specific (B), UL-28 specific (C) and UL-32 specific (D) CD8$^+$ T cell responses in Young, Older and Oldest groups. Median values with 95 % CI are indicated. P <0.05 was considered significant.

There were no significant differences in the percentage of activated CD8$^+$ T cells producing all 5 cytokines in response to pp65, IE-1 and UL-28 HCMV PSPPs between the 3 groups. However, there was a significant decrease (p=0.024) in the percentage of UL-32 specific T CD8$^+$ T cells producing IFNγ in the Oldest group compared to Young. Although

not significant, UL-32 specific $CD8^+$ T cells producing TNFα and activated T cells degranulating also appear to steadily decline in the Older and Oldest groups compared to the Young.

The pp65-specific $CD8^+$ T cell responses were dominated by TNFα, IFNγ and degranulating (CD107a) activation markers in all three groups. The percentage of specific T cells producing any of these markers did not differ statistically. This trend was also observed in IE-1 specific and UL-28 specific $CD8^+$ T cells in the three age groups.

5.4 DISCUSSION

It is impossible to deduce the breadth of functional capabilities of HCMV-specific T cells simply by measuring the magnitude of the response to individual HCMV PSPPs based on a single functional readout. This has heralded the use of multi-parametric flow cytometry to simultaneously measure the multiple functional outputs of antigen specific T cells at the single cell level, thereby contributing to the growing knowledge of polyfunctional profiles of antigen-specific T cells of interest.

Indeed polyfunctionality is one of the key measureable correlates of T cell efficacy as polyfunctional T cells exhibiting multiple effector functions such as degranulation and, production of other factors such as chemokines (MIP1β) etc. after stimulation have been associated with improved protective immunity (155).

It is interesting to note that although polyfunctional $CD8^+$ T cells are associated with good viral control, the reason(s) for this advantage is still being disentangled in an effort to identify the importance of factors such as strength of antigenic response, persistence of antigen, phenotypic heterogeneity of memory subsets and functional diversity (156).

Much has been published about the accumulation of functionally impaired, terminally differentiated $CD8^+$ T cells in HCMV seropositive older individuals and the effect this may have on immune responsiveness to novel infectious diseases and vaccines (157-160). Moreover, it is thought that polyfunctional profiles may differ depending on the state of memory differentiation as observed in a study of antiretroviral untreated, HIV infected individuals. One of the major findings from the study was that HCMV-specific $CD8^+$ T cells retained polyfunctional properties irrespective of the degree of memory differentiation unlike the HIV-specific $CD8^+$ T cells which demonstrated poor polyfunctional capabilities with advanced memory differentiation (161).

Distinguishing the impact of functional impairment of cellular responses due to normal physiological changes with increasing age and the role of pathogenic agents such as HCMV in promoting immunosenescence remains one of great importance. Several attempts have been made to understand functional changes in T cell responses with age (122, 162). A recent study evaluating the changes in polyfunctionality with increasing age did this by delineating the polyfunctionality of $CD4^+$ and $CD8^+$ T cell subtypes responding to the super-antigen, staphylococcal enterotoxin (SEB), in Young (median age =27) and Older (median age= 80) individuals. They observed that although the proportions of naïve T cells were lower in the Older cohort compared to Young, polyfunctionality was maintained (163) .

In line with one of the aims of this study, the polyfunctionality profiles of HCMV-specific T cells in all three age groups were evaluated based on the expression of any combination of 5 activation markers (IFNγ, TNFα, IL-2, CD40L and, CD107a) to better understand if polyfunctionality of HCMV-specific T cell responses changes with increasing age. It is important to bear in mind that these activation markers do not acccount for all possible functions as there are limitations in the number of measurable parameters.

The polyfunctionality profile based on $CD4^+$ and $CD8^+$ T cell responses to individual HCMV PSPP was derived using the polyfunctionality index described by Larsen et al.,2012 (104). Generally, there were no significant differences in polyfunctionality with increasing age against top $CD4^+$ targets. However, as outlined in the result section some significant differences were observed when participants categorized as 'Older' (aged 60 to 70 years) and 'Oldest' (aged 75 to 85 years) based on our initial age cut off limit were combined to generate a single cohort titled 'Old' group (aged 60 to 85 years) and compared to the Young group.

For instance, there was a significant increase in the frequency of pp65-specific $CD4^+$ T cells with 4 functions in the 'Old' group compared to Young. A decrease in the pp65-specific $CD4^+$ T cells with 2 functions was also observed in the 'Old' subjects compared to Young. No further significant differences were observed.

As outlined in preceding chapters, focus on 'immuno-dominant' pp65 and IE-1 specific T cell responses has overshadowed the impact of other HCMV PSPPs. Interestingly, a significant decrease in the polyfunctionality index of UL-32 specific $CD8^+$ T cells with 3 functions was observed in the Oldest group compared to the Young, albeit the sample size makes it difficult to draw any firm conclusions .

A recent study described the functional and phenotypic memory subset differentiation of pp65 and IE-2 specific T cells in infants with congenital HCMV infection and adults with primary and chronic HCMV infection. This report observed that although the infants were somewhat incapable of generating polyfunctional responses, when present, the patterns of functional expression were similar to adults (164). A previous study evaluating the polyfunctionality profile of $CD8^+$ T cell responses in individuals aged between 19 to 85 years acutely infected with West Nile Virus (WNV) also characterized their responses against chronic HCMV and Epstein Barr virus (EBV) infection. Their results demonstrated differences in functional profile depending on infectious agent but no significant changes in polyfunctionality with age based on WNV-specific $CD8^+$ T cell responses. Polyfunctionality was also maintained against chronic HCMV and EBV infections in the aged cohort (165).

This study has shown that age does not appear to have an effect on the level of polyfunctionality in our cohort. Data from this study demonstrates that the size of the response appears to be a better predictor of the level of polyfunctionality. The increase in

size of the pp65-specific $CD4^+$ T cells and pp65-specific $CD8^+$ was significantly correlated to increasing polyfunctionality index. This was true when the response size to other top recognized HCMV PSPPs such as UL-55, IE-1 and UL-28 were considered.

Inflammation is a recurring theme in HCMV studies, indeed TNF-α has been shown to be involved in the up-regulation and further exacerbation of the inflammatory response (166). As such, this feedback loop is thought to drive inflammation in elderly compared to young individuals prompting long lasting deleterious effects (167). As part of this study analysis, the functional dominance of all 5 cytokines against HCMV proteins was compared between the three age groups. No significance differences were observed across the 3 groups based on HCMV specific responses to top $CD4^+$ and $CD8^+$ targets.

In conclusion, although our result demonstrates an increase in the $CD8^+$ T_{EMRA} memory subset with age, these cells remain functional and no loss in polyfunctionality was observed with increasing age against HCMV PSPPs (Appendix Figure 8.7). The use of the polyfunctionality index enabled us to make this finding a more general one rather than focusing on single subsets. Indeed, the size of the response to HCMV proteins appears to play a prominent role in determining the level of polyfunctionality.

6 HEALTH RELATED QUALITY OF LIFE OF HCMV SEROPOSITIVE INDIVIDUALS WITH INCREASING AGE

The burden of HCMV infection is thought to have an impact on the health of infected aged individuals with studies reporting a predisposition to cardiovascular diseases and an association with cognitive decline and poor responsiveness to infections (89, 97).

All elderly individuals aged between 60 and 85 years of age meeting study inclusion criteria were sent a monthly health diary to complete over the course of 2 years (Appendix Figure 8.8). This was irrespective of HCMV serological status. The primary aim was to chart the health related changes in the number of infections (Head cold, Chest infections, Urine infection and other non-specified infection) and other health problems including pain experienced by these individuals.

In addition, the health-related quality of life (QoL) was assessed by means of a self-completed survey instrument, the RAND-36 questionnaire. This provides information on multiple aspects (8 aspects) including the physical, emotional and general well-being of individuals. All questionnaires were completed and returned to the research nurse on the same day, after consent was given.

Results from the health diaries and the RAND-36 questionnaire were correlated with HCMV-specific T cell responses to determine if any significant correlation exists between the size of the response and any aspect of general health assessed using the diaries and questionnaire.

6.1 SELF-REPORTED HEALTH STATUS OF AGED INDIVIDUALS

The exploratory analyses detailed below focused on the differences in the measured health variables captured in the monthly health diaries completed by HCMV seronegative and seropositive aged individuals. The aim was to evaluate if there are reported differences in the number of infections or health problems between the two groups. Table 6.1 outlines information such as the health related variables analysed, age and gender distribution of HCMV seropositive and seronegative aged individuals. A retrospective statistical power analysis was performed for sample size estimation using G*Power v3.1.9.2 with alpha set at 0.05, power =0.95 and the projected sample size calculated was n=25, therefore our sample size of 118 is sufficient for the objective outlined.

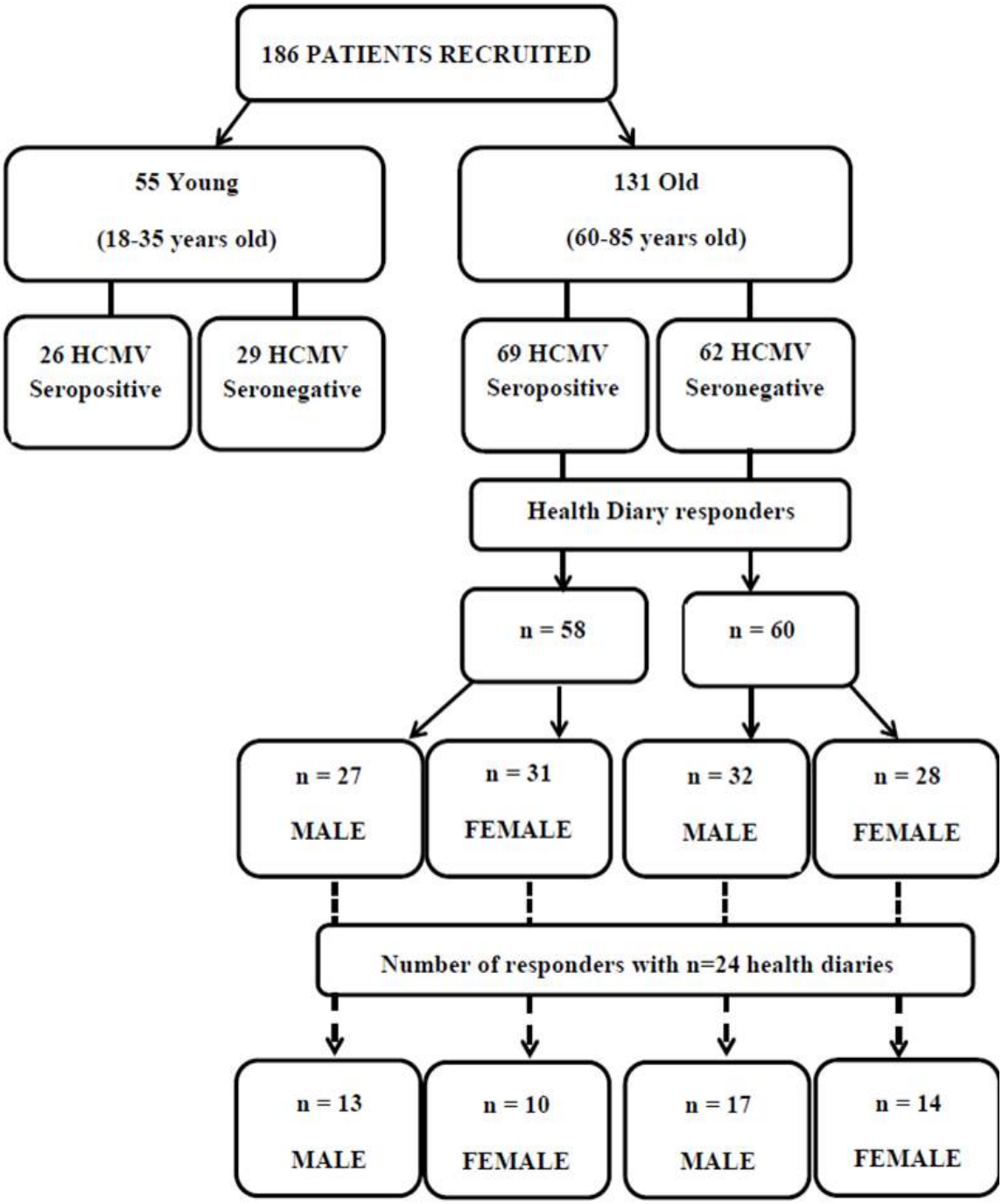


Figure 6.1. Flowchart of patients completing the self-reported health diaries

HCMV Serology	Positive			Negative		
Gender	Male	Female	**Total**	Male	Female	**Total**
n	27	31	58	32	28	60
Age (median age in years)	69	69	69	76	70	73
Total number of diary sheets returned	23	21	21	24	24	24
Proportion of pain per month	.0000	.0833	.0476	.0750	.0851	.0833
Proportion of chest infection per month	.0000	0000	.0000	.0000	.0000	.0000
Proportion of urine infection per month	.0000	.0000	.0000	.0000	.0000	.0000
Proportion of head cold per month	.0833	.1333	.0911	.1250	.1101	.1250
Proportion of other infections experienced	.0000	.0000	.0000	.0000	.0208	.0000
Proportion of other health problems	.0417	.1053	.0572	.0623	.1111	.1032
Proportion of months unwell	.2500	.2667	.2500	0.3125	.3542	.3333

Table 6.1. Baseline characteristics of HCMV seropositive and seronegative aged individuals.

The variables listed in the first column are further sub divided based on gender with a group total based on serology. Median values are depicted for all variables of interest.

The flowchart depicted in Figure 6.1 displays the number HCMV seropositive and seronegative old individuals (n=118) completing the health diary which formed the basis of these analyses. The initial focus was on the differences in the reported health status of HCMV seropositive and seronegative individuals. There were no significant differences observed in the proportion of infections (head cold, chest and other infections) reported per month in HCMV seronegative and HCMV seropositive old individuals (Figure 6.2). The boxplot of the proportion of urine infection per month in HCMV seropositive and seronegative individuals is not shown due to low numbers of events reported making graphical representation difficult.

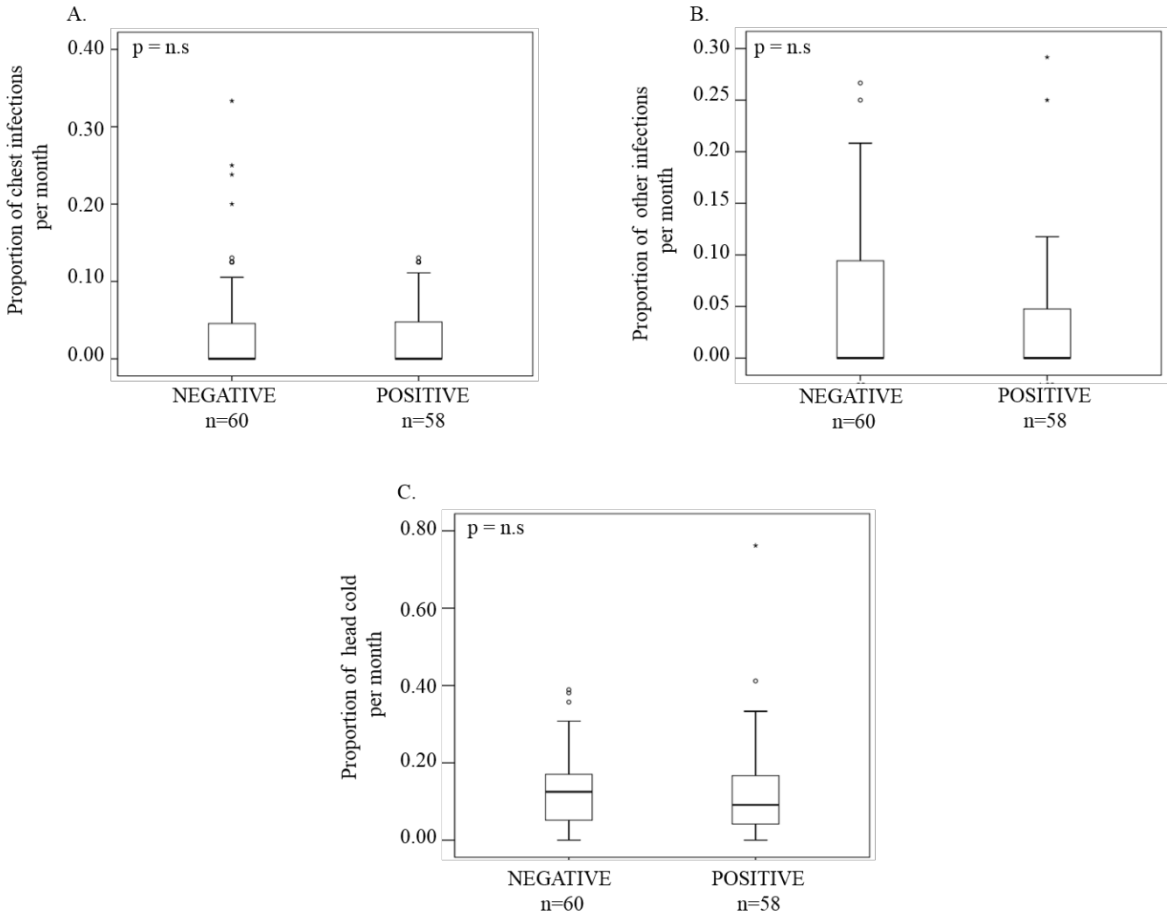


Figure 6.2. Proportion of all infections reported in aged individuals.

The box plots above depicts the average number of chest infections (A), other infections (B) and head cold (C) reported per month in HCMV seropositive negative and positive aged individuals. A p value < 0.05 was considered significant.

Other key measures of general well-being were deduced from proportion of other health problems experienced (Figure 6.3A), proportion of pain experienced on a monthly basis (Figure 6.3B) and proportion of months unwell (Figure 6.3C).

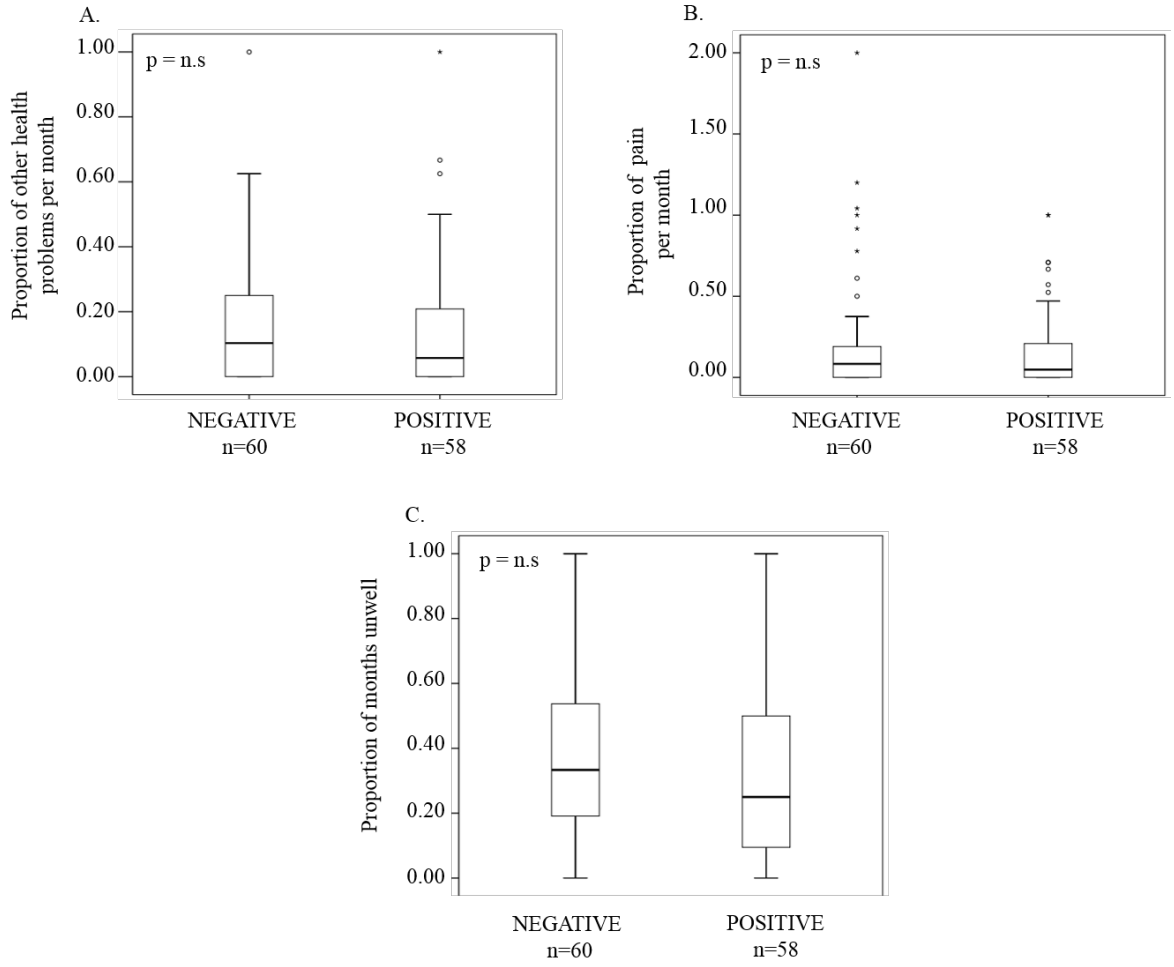


Figure 6.3. No significant differences in the proportion of health related problems in aged individuals.

The box plots above depicts the proportion of other health problems experienced per month (A), proportion of pain reported per month (B) and proportion of months unwell (C) in HCMV seropositive negative and positive aged individuals. Number of participants can be found beneath each group. A p value < 0.05 was considered significant.

There were no significant differences observed in self-reported health status between HCVM seropositive and HCMV-seronegative old individuals. The analysis was focused on HCMV seropositive old participants to determine if gender plays any role in the state of well-being and good health. There were no significant differences observed between HCMV seropositive male and HCMV seropositive females with regards to the proportion of infections (head cold, chest and other infections), proportion of other health problems experienced, and proportion of months unwell (Data not shown). Unsurprisingly, there was

a significant increase in the proportion of pain reported per month by HCMV seropositive females (old) compared to their male counterpart (Figure 6.4).

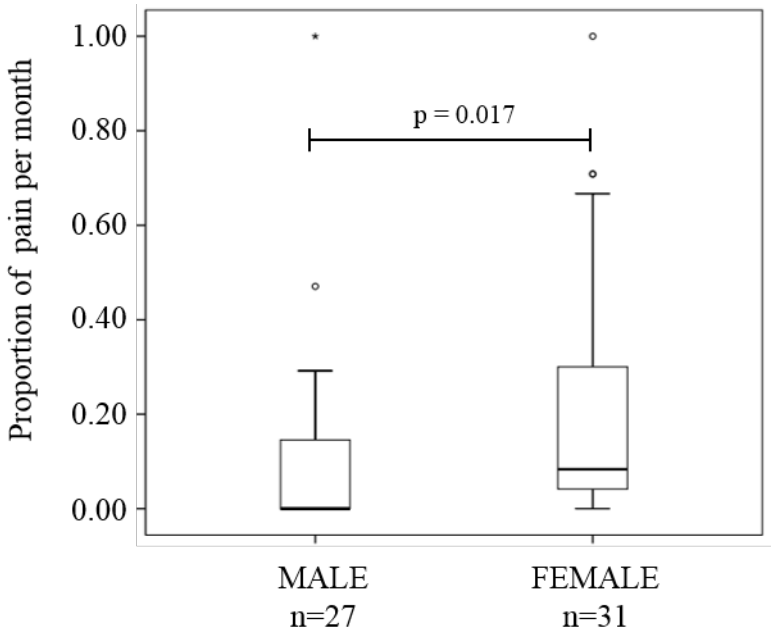


Figure 6.4. Significant number of pain reported in HCMV seropositive female individuals.

The box plot above depicts the proportion of pain reported per month in HCMV seropositive male and female individuals. A p value < 0.05 was considered significant.

Further analyses on responses from HCMV seropositive aged individuals was carried out to determine if there is an association between summated HCMV-specific T cell response size ($CD4^+$ and $CD8^+$) on health related QoL. For this analysis, overall health status was captured by a single measure, the proportion of months reported unwell. A scatterplot of the variables is depicted in Figure 6.5.

There was no significant correlation observed between the size of the summated HCMV-specific $CD4^+$ T cells and proportion of months unwell (Figure 6.5A). The evaluation of the relationship between summated HCMV-specific $CD8^+$ T cell responses and proportion

of months unwell demonstrated a significant correlation, with an increase in size of the response seemingly corresponding to an increase in the proportion of months unwell (Figure 6.5B).

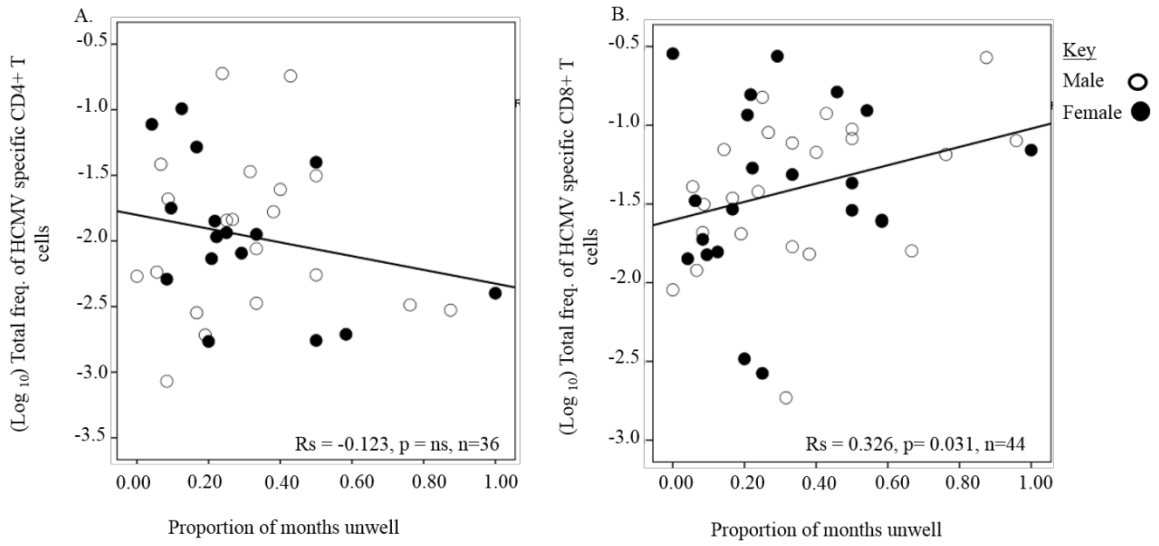


Figure 6.5. Significant correlation between summated HCMV-specific CD8$^+$ T cell responses and proportion of months unwell.

The scatter plots above depicts the correlation of the summated HCMV specific CD4$^+$ T cell responses (A), summated HCMV specific CD8$^+$ T cell responses (B) and the proportion of months unwell in HCMV seropositive aged individuals. Clear circles and closed circles represent male and female responses respectively. A p value <0.05 was considered significant.

6.2 EVALUATING QoL IN HCMV SEROPOSITIVE INDIVIDUALS BASED ON RAND-36 SCORES

The health related QoL has a wide reaching impact not only on the functional ability of the individual but the physical, social and emotional well-being. RAND-36 is one of the most widely used survey tools for assessing health related QoL. It captures QoL based on by measures 8 key aspects, namely physical conditioning (PC), role limitations due to physical health (RLPH), role limitations due to emotional problems (RLEP), energy and fatigue (EF), emotional well-being (EW), social functioning (SC), pain (P) and general

health (GH). Individual items are scored ranging from 0 which is associated with very poor functioning, to a maximum of 100. High scores indicate good health.

All eligible study participants were asked to complete the form as part of initial consultation with the research nurse (http://www.rand.org/health/surveys_tools/mos/mos_core_36item.html).

The results shown in Figure 6.6 is a simple comparison of all 8 measures of health related QoL between Young (n=21), Older (n= 33) and Oldest (n= 20) groups.

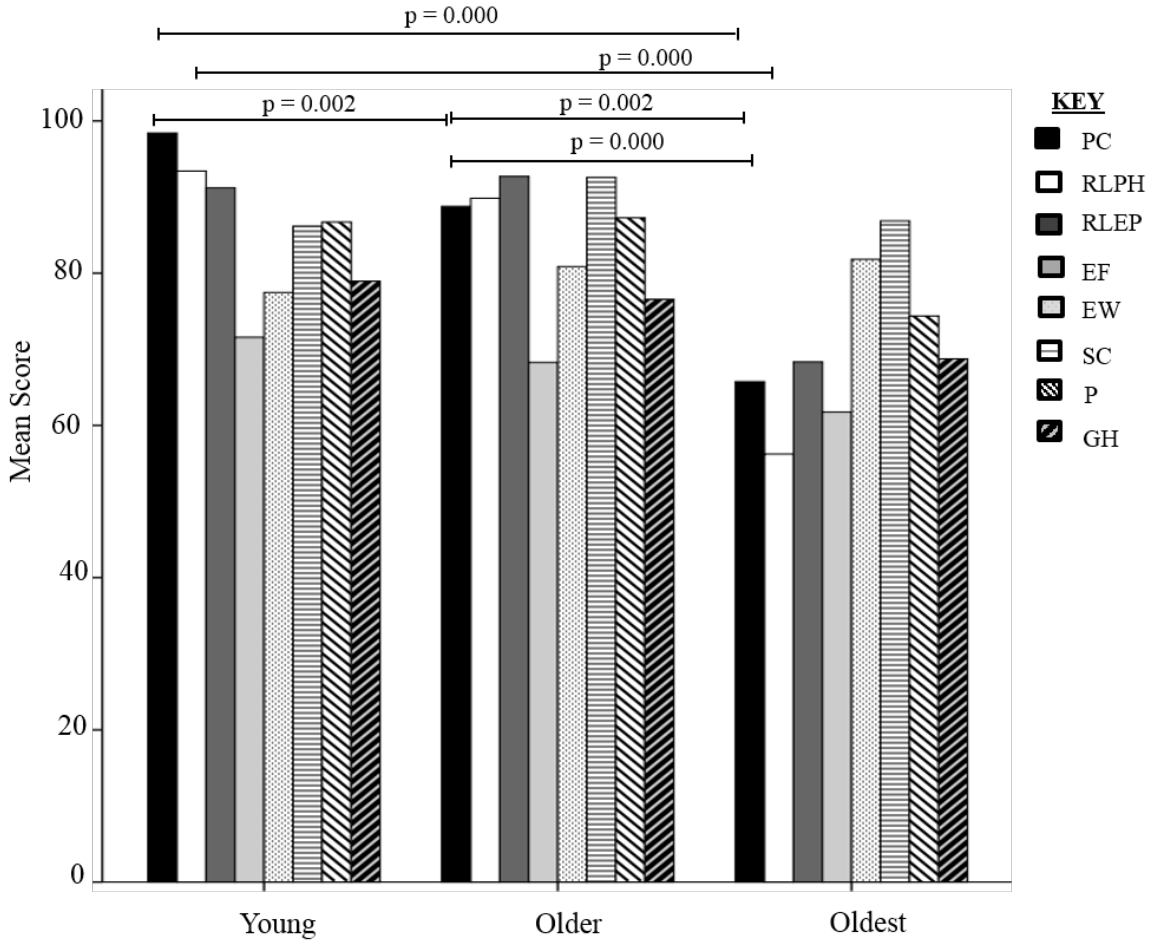


Figure 6.6. Evaluation of QoL based on RAND-36 scores in all three age groups.

The bar charts above depicts the mean scores of all 8 measures of health related quality of life captured by the RAND-36 survey tool. A p value < 0.05 was considered significant.

There was a significant decline in the physical functioning of HCMV seropositive individuals with age. RLEP is one of the 8 aspects measured by RAND-36, it captures the extent to which health interferes with working and other daily activities. There was a significant decrease in the RLEP with age.

The emotional impact on ability to work and perform daily activities is also captured by this QoL survey tool. There was a significant decrease ($p=0.02$) in the Older group compared to the Oldest. No further significant differences in the other measures was seen between the three groups.

To assess the impact of HCMV T cell responses on health related QoL, the summated HCMV specific $CD4^+$ and $CD8^+$ T cell response size was correlated with all 8 aspects measured by the RAND-36 item health questionnaire (Appendix Table 8.2). There was a significant negative correlation ($p=0.042$) between the size of the summated HCMV-specific $CD4^+$ and Energy/fatigue (EF) (Figure 6.7A). A negative correlation ($p=0.033$) of the summated HCMV-specific $CD8^+$ T cell response and physical functioning (PF) was also observed (Figure 6.7B).

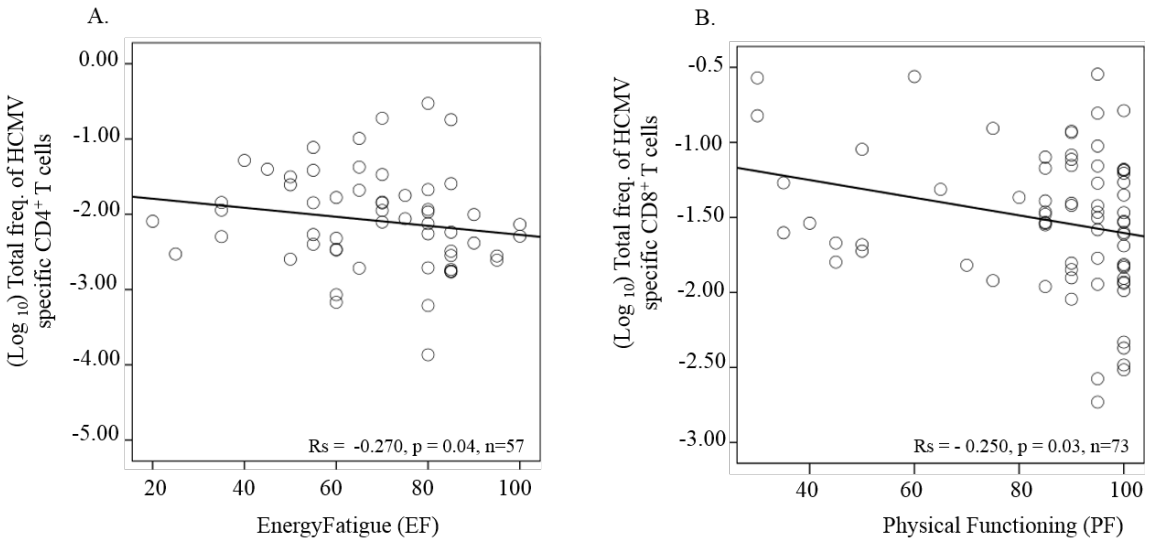


Figure 6.7. Correlation of HCMV-specific T cell responses and aspects of RAND-36 item survey.

The scatter plots above depict the correlation of the summated HCMV specific $CD4^+$ T cell responses and energy/fatigue scores (A) as well as the summated HCMV specific $CD8^+$ T cell responses and the physical functioning (B). A p value <0.05 was considered significant.

6.3 DISCUSSION

The long term burden of HCMV infection has been linked to reduced mortality and predisposition to conditions with a pro-inflammatory aetiology. The current reasoning is that the reduced number of naive $CD8^+$ T cells together with an accumulation of terminally differentiated memory $CD8^+$ T cells, one of the hallmarks of immunosenescence not only increases the risk but also the severity of infection.

The role of inflammation in aged individuals and the onset of age related diseases underpins the basis of numerous studies, with one such study describing the association between systemic inflammatory biomarkers, brain microstructure and visuospatial ability (168). Indeed, the relationship between infection, inflammation and risk of age related diseases has been established for chronic infections such as HIV and most recently for HCMV (169).

Working on this premise, the current study sought to investigate the health related status of HCMV seropositive Older and Oldest individuals to determine what impact, if any, it has on the number of infections and other health related problems experienced. This information was captured in a monthly health diary which was completed over a 2 year period.

A widely used, validated questionnaire tool, RAND-36 was also used to gauge the health related QoL in all eligible HCMV seropositive participant.

There were no significant differences in the number of infections and health related problems reported by HCMV seropositive and seronegative aged individuals. Interestingly though older female HCMV seropositive individuals experienced significantly more pain than male participants. The association between HCMV and predisposition to general ill health was another interesting area that was explored as part of this analysis. A significant

trend was observed between the size of the summated HCMV-specific $CD8^+$ T cells and the proportion of months unwell.

There was no significant correlation observed between the size of the summated HCMV-specific $CD4^+$ T cells and the proportion of months unwell.

In a recent study of older individuals investigating the impact of aging or persistent viruses such as HCMV on cellular responses to influenza, the authors observed that pre-existing $CD4^+$ memory T cells were associated with disease protection and limiting disease severity in an influenza challenge model in humans (160).

They observed that memory $CD4^+$ T cell responses to influenza virus core matrix protein were compromised in a subset of HCMV-seropositive aged individuals in contrast to HCMV-seronegative aged individuals who possessed influenza-reactive $CD4^+$ memory T cells. However, this was not due to CMV-associated immune senescence as individuals lacking a $CD4^+$ T cell response had significantly lower levels of late differentiated $CD4^+$ T cells expressing the CD57 and KLRG-1 markers.

Analysis of the RAND-36 validated questionnaire revealed an association between measures such as Physical Functioning (PF), Energy/Fatigue (EF) and size of the summated HCMV T cell responses, which are normal physiological changes associated with aging. Importantly, no significant associations were observed between size of the HCMV specific T cell response and measures of cognitive functioning. Indeed most conflicting studies reporting associations between HCMV seropositivity and cognitive decline have used measures such as MMSE (Mini mental state examination scores) and levels of HCMV IgG titres. In one such study characterizing cytokine responses in CMV seropositive and seronegative individuals with or without Alzheimer's disease observed little or no difference in cytokine profile at baseline in CMV seropositive (Alzheimer's

disease-AD) and (No disease- ND) groups. However, seropositive AD subjects showed a stronger IFN-g response to both anti-CD3/CD28 and CMV pp65 pepmix compared to ND subjects. Although this study provides some insight into the manner in which CMV specific immune response interacts with the aging immune system in AD, direct evidence of the role of CMV in AD pathogenesis is still not established (170).

7 CONCLUDING REMARKS

The results from this study represent the most comprehensive analysis of the magnitude of HCMV-specific T-cell responses in a sizeable number of participants aged from 19 to 85 years. Analysis was focused on a largely white British cohort, with the exception of 8 young participants of Asian and/or African descent.

In an effort to evaluate the immunodominance of less characterised HCMV proteins, and to determine if there is a significant change in the number of responses to HCMV proteins with age, the proportion of responses to a single HCMV protein in all participants was calculated based on frequencies of $CD4^+$ and $CD8^+$ specific T cell.

All proteins were recognized by at least one participant in this study, however, they were recognized in a different hierarchical order than in the original study by Sylwester et al. For instance, UL48 was the most highly recognized target protein by $CD8^+$ T cells in the previous study cited, but the least frequently recognized one in this study. As evident in the original study, the hierarchy of protein recognition is not related to the size of the responses. The most frequently recognized proteins did not give rise to the biggest responses.

Our results showed considerable variation in the number of HCMV proteins recognized based on $CD8^+$ T cell responses; thereby emphasizing the fact that certain participants mounted a diverse response whilst responses in other participants were much more focused. The different protein recognition hierarchies observed in this group of individuals begs the questions whether the same array of proteins would have been selected for stimulation had the original study been performed on mainly Caucasian participants.

Our initial goal was to determine if the number of HCMV proteins recognised increases with age. We observed no significant differences in the number of response counts to all HCMV proteins between all three age groups based on $CD4^+$ T cell response . An increase in the total number of $CD8^+$ T cell response counts in the Older group was observed when compared to the Oldest group however this was not statistically significant. One of the key aims of this project was to evaluate the magnitude of HCMV-specific T cell responses in the 3 groups ranging in age from 18 to 85 years using 19 HCMV proteins encompassing both $CD4^+$ and $CD8^+$ T cell top targets. Our results confirm that there is no expansion of pp65-specific $CD8^+$ T cells with age. Although we observed an increase in frequency of pp65-specific $CD8^+$ T cells in the Older cohort compared to Young, there was no statistically significant difference in size of pp65-specific $CD8^+$ T cell responses in Oldest group compared to the Older group.

In addition to the widely recognised HCMV immuno-dominant proteins pp65 and IE-1, a large proportion of individuals in this study cohort also responded to UL-86, US-24 and UL-36 based on $CD4^+$ T cell responses as well as UL-28 and UL-32 HCMV proteins based on $CD8^+$ T cell responses. Results generated from this study demonstrate that although HCMV-specific T-cells does increase in older age, such expansions do not occur invariably, and do not always lead to large expansions in older people.

The impact of HCMV infection on general health and wellbeing in aged individuals is of great importance. Numerous publications investigating the effect of HCMV infection in aged individuals have reported increased mortality, and susceptibility to developing cardiovascular pathology.Using a successful combination of CD45RA and CD27 to enumerate HCMV specific memory T cell subsets as utilised by various studies we characterised memory subset distribution against a wide array of select HCMV proteins to

determine how HCMV-specific memory T cell subsets changes with age, and if this depends on the protein target.

Initial analysis focused on the influence of age on memory subset distribution in HCMV seropositive individuals. Weobserved that while the total $CD8^+$ T_N pool significantly decreased with age, the memory compartment was increasingly dominated by the $CD8^+$ T_{EMRA} subset with age, in HCMV seropositive individuals. This supports previous findings by our group, and other studies (123, 137, 138).

Much has been published about the expansion of memory subsets in HCMV infected individuals which could be detrimental to long term health by limiting the immunological repertoire and resulting in a clonal expansion of select T cells in aged individuals. Our results demonstrate no significant differences in the frequencies of HCMV-specific T_N, T_{CM}, T_{EM}, T_{EMRA} memory subset against top $CD4^+$ (UL-55, pp65, UL-86 and US-24 + UL-36) and $CD8^+$ (pp65, IE-1, UL-28, and UL-32) targets in the Young, Older and Oldest groups. Although we did not directly investigate the memory inflation of HCMV specific memory T cells in humans as part of this study, our results would firmly suggest that it is not a common wide phenomenon in humans. Infact, a recent study of HCMV seropositive individuals by Jackson et al. analysing the frequency of T cells responding to multiple HCMV ORF products at various time points over a 3 year period reported that the frequency of HCMV specific $CD8^+$ T cells did not show any attributes related to inflation , rather a fluctuation in T cell frequencies was observed. In conclusion, our findings reveal that the size of the response to HCMV proteins plays a greater role in the degree of advanced T cell memory subset differentiation, than age which corroborates earlier findings by our group. This finding is not unique to the more established HCMV PSPPs (pp65 and IE-1) as analyses of other top recognised PSPPs in this study based on $CD4^+$

(UL-86 and US-24 & UL-36) and $CD8^+$ (UL-32 and UL-28) T cell responses also support this observation.

It is impossible to deduce the breadth of functional capabilities of HCMV-specific T cells simply by measuring the magnitude of the response to individual HCMV PSPPs based on a single functional readout. This has heralded the use of multi-parametric flow cytometry to simultaneously measure the multiple functional outputs of antigen specific T cells at the single cell level, thereby contributing to the growing knowledge of polyfunctional profiles of antigen-specific T cells of interest. Much has been published about the accumulation of functionally impaired, terminally differentiated $CD8^+$ T cells in HCMV seropositive older individuals and the effect this may have on immune responsiveness to novel infectious diseases and vaccines (157-160). Moreover, it is thought that polyfunctional profiles may differ depending on the state of memory differentiation.

This study has shown that age does not appear to have an effect on the level of polyfunctionality in our cohort. Data from this study demonstrates that the size of the response appears to be a better predictor of the level of polyfunctionality. The increase in size of the pp65-specific $CD4^+$ T cells and pp65-specific $CD8^+$ was significantly correlated to increasing polyfunctionality index. This was true when the response size to other top recognized HCMV PSPPs such as UL-55, IE-1 and UL-28 were considered. In conclusion, although our result demonstrates an increase in the $CD8^+$ T_{EMRA} memory subset with age, these cells remain functional and no loss in polyfunctionality was observed with increasing age against HCMV PSPPs. The use of the polyfunctionality index enabled us to make this finding a more general one rather than focusing on single subsets. Indeed, the size of the response to HCMV proteins appears to play a prominent role in determining the level of polyfunctionality.

The long term burden of HCMV infection has been linked to reduced mortality and predisposition to conditions with a pro-inflammatory aetiology. The current reasoning is that the reduced number of naive $CD8^+$ T cells together with an accumulation of terminally differentiated memory $CD8^+$ T cells, one of the hallmarks of immunosenescence not only increases the risk but also the severity of infection. Working on this premise, the current study sought to investigate the health related status of HCMV seropositive Older and Oldest individuals to determine what impact, if any, it has on the number of infections and other health related problems experienced. This information was captured in a monthly health diary which was completed over a 2 year period. A widely used, validated questionnaire tool, RAND-36 was also used to gauge the health related QoL in all eligible HCMV seropositive participant. There were no significant differences in the number of infections and health related problems reported by HCMV seropositive and seronegative aged individuals.

7.1 FUTURE WORK

This project has illustrated the importance of employing a comprehensive approach to determining HCMV-specific T cell responses. Although immunodominance was still maintained by well characterised HCMV proteins such as pp65 and IE-1 in our cohort, we successfully identified other top target based on $CD4^+$ (UL-86, US-24 and UL-36) and $CD8^+$ (UL-28 and UL-32) T cell responses. It will be interesting to perform epitope mapping on these HCMV proteins (UL-86, US-24, UL-36, UL-28 and UL-32). Enumerating responses against individual epitopes for each of the HCMV protein outlined will add more knowledge to HCMV T cell responses.

A number of HCMV PSPPs (Table 2.3) were combined to minimize reagent costs and to reduce the amount of PBMCs needed to set up antigen stimulation assay due to limited amount of blood samples collected from participants (approximately 25ml per donor). HCMV proteins US-24 and UL-36 were combined for the reasons outlined. A total number of 18 HCMV seropositive individuals responded to these PSPPs. It will be interesting to set up each of these proteins as individual antigen stimulation tubes to determine which of these proteins is responsible for the $CD4^+$ T cells responses observed in our cohort.

Our group is interested in the contribution of CMV infection to the vascular disease burden in the older population of the UK. In a previous paper published by this group, we established that while there is no general, massive increase of the HCMV-specific immune response in older age, very large $CD8^+$ T-cell responses are found in some older people (151). Using results from the cohort described in this thesis our group published a paper demonstrating a striking link between HCMV-specific cellular immunity and elevated blood pressure (BP) in generally healthy older people, however, the study was not large enough to justify a potential interventional study using antiviral medications to reduce the effect of CMV on vascular pathology (92). As part of on-going work in our group using

the established old cohort described in this thesis, we hope to provide supporting evidence showing the link between CMV infection, endothelial injury and vascular disease.

As part of the initial objective of this study, I wanted to carry out HLA typing on select individuals within our cohort to determine which HLA types were associated with large or small responses to select HCMV proteins; HLA typing will also provide information on the observed differences in the recognition hierarchy observed in this study. It is probable that differences in recognition could be as a result of the variation in protein sequences between the CMV strains that the participants were exposed.

8 APPENDIX

Number	Boolean derived subsets
1	$CD107a^{+}$ $CD40L^{+}$ $IL\text{-}2^{+}$ $TNF\alpha^{+}$ $IFN\gamma^{+}$
2	$CD107a^{+}$ $CD40L^{+}$ $IL\text{-}2^{+}$ $TNF\alpha^{+}$ $IFN\gamma^{-}$
3	$CD107a^{+}$ $CD40L^{+}$ $IL\text{-}2^{+}$ $TNF\alpha^{-}$ $IFN\gamma^{+}$
4	$CD107a^{+}$ $CD40L^{+}$ $IL\text{-}2^{+}$ $TNF\alpha^{-}$ $IFN\gamma^{-}$
5	$CD107a^{+}$ $CD40L^{+}$ $IL\text{-}2^{-}$ $TNF\alpha^{+}$ $IFN\gamma^{+}$
6	$CD107a^{+}$ $CD40L^{+}$ $IL\text{-}2^{-}$ $TNF\alpha^{+}$ $IFN\gamma^{-}$
7	$CD107a^{+}$ $CD40L^{+}$ $IL\text{-}2^{-}$ $TNF\alpha^{-}$ $IFN\gamma^{+}$
8	$CD107a^{+}$ $CD40L^{+}$ $IL\text{-}2^{-}$ $TNF\alpha^{-}$ $IFN\gamma^{-}$
9	$CD107a^{+}$ $CD40L^{-}$ $IL\text{-}2^{+}$ $TNF\alpha^{+}$ $IFN\gamma^{+}$
10	$CD107a^{+}$ $CD40L^{-}$ $IL\text{-}2^{+}$ $TNF\alpha^{+}$ $IFN\gamma$-
11	$CD107a^{+}$ $CD40L^{-}$ $IL\text{-}2^{+}$ $TNF\alpha^{-}$ $IFN\gamma^{+}$
12	$CD107a^{+}$ $CD40L^{-}$ $IL\text{-}2^{+}$ $TNF\alpha^{-}$ $IFN\gamma^{-}$
13	$CD107a^{+}$ $CD40L^{-}$ $IL\text{-}2^{-}$ $TNF\alpha^{+}$ $IFN\gamma^{+}$
14	$CD107a^{+}$ $CD40L^{-}$ $IL\text{-}2^{-}$ $TNF\alpha^{+}$ $IFN\gamma^{-}$
15	$CD107a^{+}$ $CD40L^{-}$ $IL\text{-}2^{-}$ $TNF\alpha^{-}$ $IFN\gamma^{+}$
16	$CD107a^{+}$ $CD40L^{-}$ $IL\text{-}2^{-}$ $TNF\alpha^{-}$ $IFN\gamma^{-}$
17	$CD107a^{-}$ $CD40L^{+}$ $IL\text{-}2^{+}$ $TNF\alpha^{+}$ $IFN\gamma^{+}$
18	$CD107a^{-}$ $CD40L^{+}$ $IL\text{-}2^{+}$ $TNF\alpha^{+}$ $IFN\gamma^{-}$
19	$CD107a^{-}$ $CD40L^{+}$ $IL\text{-}2^{+}$ $TNF\alpha^{-}$ $IFN\gamma^{+}$
20	$CD107a^{-}$ $CD40L^{+}$ $IL\text{-}2^{+}$ $TNF\alpha^{-}$ $IFN\gamma^{-}$
21	$CD107a^{-}$ $CD40L^{+}$ $IL\text{-}2^{-}$ $TNF\alpha^{+}$ $IFN\gamma^{+}$
22	$CD107a^{-}$ $CD40L^{+}$ $IL\text{-}2^{-}$ $TNF\alpha^{+}$ $IFN\gamma$-
23	$CD107a^{-}$ $CD40L^{+}$ $IL\text{-}2^{-}$ $TNF\alpha^{-}$ $IFN\gamma^{+}$
24	$CD107a^{-}$ $CD40L^{+}$ $IL\text{-}2^{-}$ $TNF\alpha^{-}$ $IFN\gamma^{-}$
25	$CD107a^{-}$ $CD40L^{-}$ $IL\text{-}2^{+}$ $TNF\alpha^{+}$ $IFN\gamma^{+}$
26	$CD107a^{-}$ $CD40L^{-}$ $IL\text{-}2^{+}$ $TNF\alpha^{+}$ $IFN\gamma$-
27	$CD107a^{-}$ $CD40L^{-}$ $IL\text{-}2^{+}$ $TNF\alpha^{-}$ $IFN\gamma^{+}$
28	$CD107a^{-}$ $CD40L^{-}$ $IL\text{-}2^{+}$ $TNF\alpha^{-}$ $IFN\gamma^{-}$
29	$CD107a^{-}$ $CD40L^{-}$ $IL\text{-}2^{-}$ $TNF\alpha^{+}$ $IFN\gamma^{+}$
30	$CD107a^{-}$ $CD40L^{-}$ $IL\text{-}2^{-}$ $TNF\alpha^{+}$ $IFN\gamma$-
31	$CD107a^{-}$ $CD40L^{-}$ $IL\text{-}2^{-}$ $TNF\alpha^{-}$ $IFN\gamma^{+}$
32	$CD107a^{-}$ $CD40L^{-}$ $IL\text{-}2^{-}$ $TNF\alpha^{-}$ $IFN\gamma^{+}$

"Activated effector" T cells are the sum of 31 Boolean derived subsets, representing the percentage of cells displaying at least one of the five activation markers.

NON FUNCTIONAL SUBSET

Figure 8.1. Boolean subsets derived from FlowJo analysis

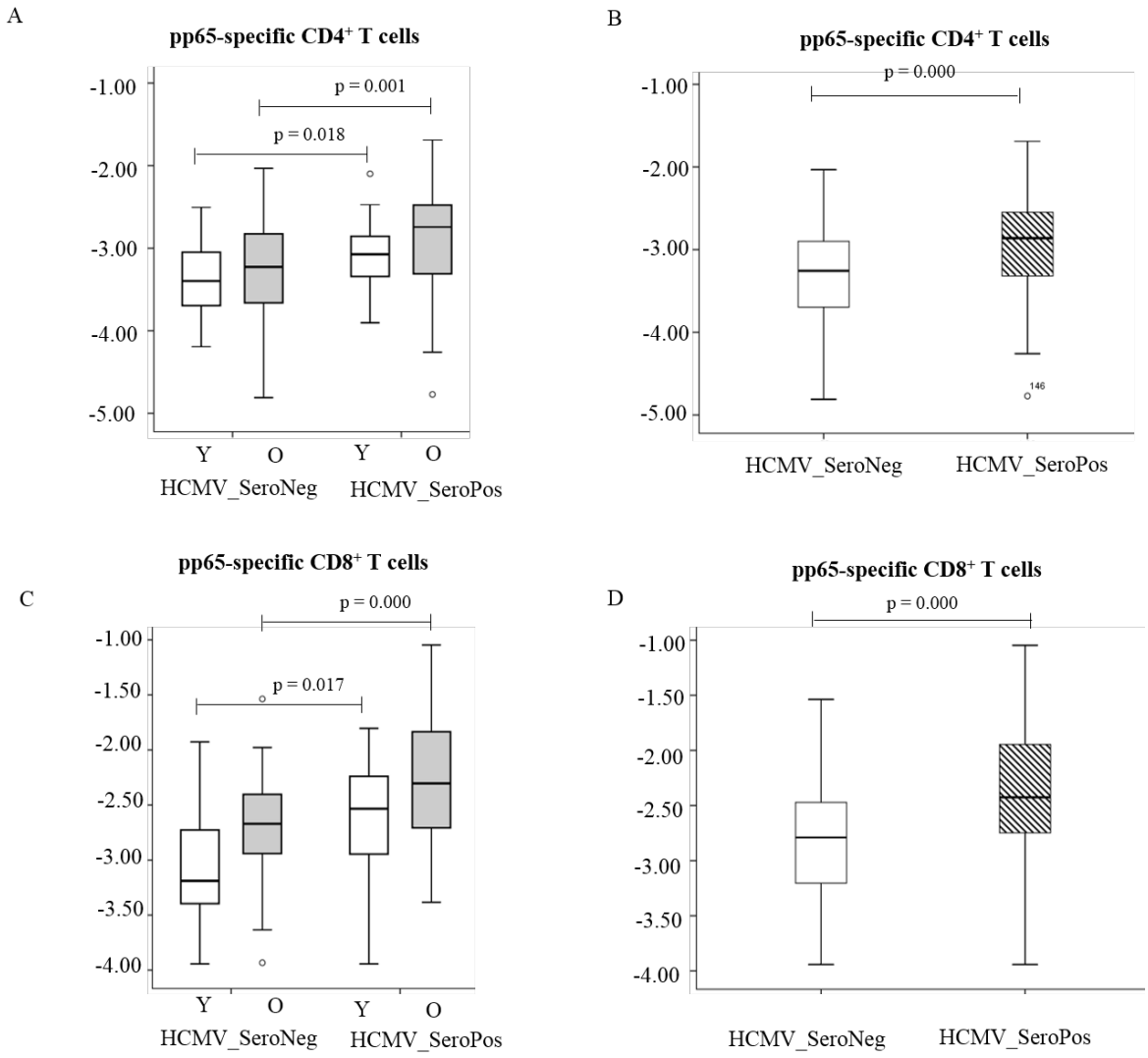
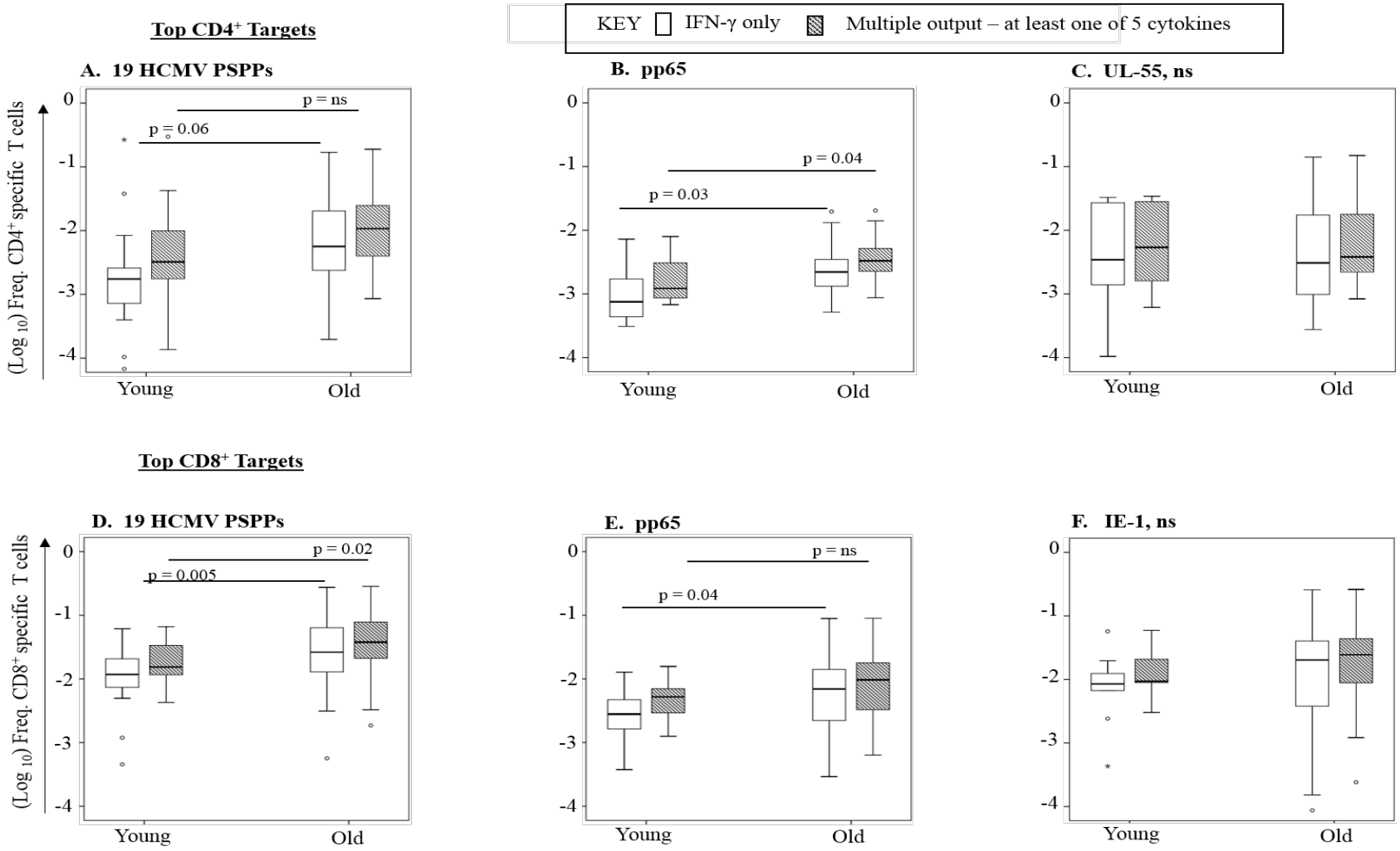


Figure 8.2. pp65 specific T cell responses increases significantly in old HCMV seropositive individuals.

Boxplots above depicts pp65 specific $CD4^+$ T cell in HCMV seronegative cohort (Young (Y, n=29) and Old (n=62) versus HCMV seropositive cohort (Young (Y, n=26) and Old (n=53) versus). Figure B is a combination of pp65-specific CD4 T cell responses in HCMV seronegative individuals versus HCMV seropositive individuals. Figure C depicts the pp65 specific $CD8^+$ T cell in HCMV seronegative participants (Young and Old) versus HCMV seropositive participants (Young and Old). Figure D is an amalgamation of all pp65-specific CD8 T cell responses in HCMV seronegative individuals versus HCMV seropositive individuals. $P < 0.05$ is significant. Significance was determined using Mann Whitney Test.

HCMV PSPPs	Number of $CD4^+$ Responses			Number of $CD8^+$ Responses		
	Young	Older	Oldest	Young	Older	Oldest
UL 55	6	14	5	5	6	1
UL 83(pp65)	10	20	11	15	30	15
UL 86	3	12	8	3	7	1
UL 122(IE-2)	0	1	0	2	7	2
UL 123 (IE-1)	0	4	1	10	26	15
UL 99	0	1	1	0	2	0
UL 153	1	1	0	2	4	1
UL 32	3	7	4	3	11	2
UL 28	0	3	3	3	15	5
UL 48A	1	4	2	0	2	0
UL 48B	1	2	1	4	4	2
US 3	3	5	2	5	6	3
UL 151+ UL 82	0	1	2	2	3	1
UL 94 + US 29	1	1	0	2	3	2
UL 103 + US 32	1	0	0	2	1	0
US24 + UL36	7	7	4	3	4	1

Table 8.1. $CD4^+$ and $CD8^+$ T cell responses to individual HCMV PSPPs. Positive $CD4^+$ and $CD8^+$ T cell responses to HCMV proteins were tallied for each group of Young, Older and Oldest individuals. For example, 6 out of the 21 Young individuals had a UL-55 specific $CD4^+$ T cell response.


KEY
IFN-γ only
Multiple output – at least one of 5 cytokines
Top CD4+ Targets
A. 19 HCMV PSPPs
B. pp65
C. UL-55, ns
(Log 10) Freq. CD4+ specific T cells
p = ns
p = 0.06
p = 0.04
p = 0.03
Young
Old
Top CD8+ Targets
D. 19 HCMV PSPPs
E. pp65
F. IE-1, ns
(Log 10) Freq. CD8+ specific T cells
p = 0.02
p = 0.005
p = 0.04

		HCMV PSPPs	IFN-g		Multiple output	
			Young (n)	Old (n)	Young (n)	Old (n)
$CD4^+$ responses	A	19 HCMV (summated)	17	41	18	41
	B	pp65	8	29	10	31
	C	UL-55	6	18	6	19
$CD8^+$ Responses	D	19 HCMV (summated)	19	51	20	53
	E	pp65	13	41	15	45
	F	IE-1	9	38	10	41

Figure 8.3. T cell responses to select HCMV proteins increase with age.

Box plots depict HCMV-specific T cell responses to the most recognised proteins in our cohort. Responses are based on the frequency of IFN-γ T cell responses only or, multiple outputs based on expression of one of these activation markers (IL-2, IFN-γ, TNF-α, CD107a and CD40L). The top panel shows (A) summated HCMV- specific $CD4^+$ T responses, (B) pp65 specific $CD4^+$ T responses, and (C) UL-55 specific $CD4^+$ T responses in Young and Old groups. The bottom panel is a plot of the (D) summated HCMV- specific $CD8^+$ T responses, (E) pp65-specific $CD8^+$ T, and (F) IE-1 specific $CD8^+$ T cells responses in Young and Old groups. Adjoining table lists all the n numbers for the comparative analyses between both groups. ^{o} represents outliers. $p<0.05$ is considered significant.

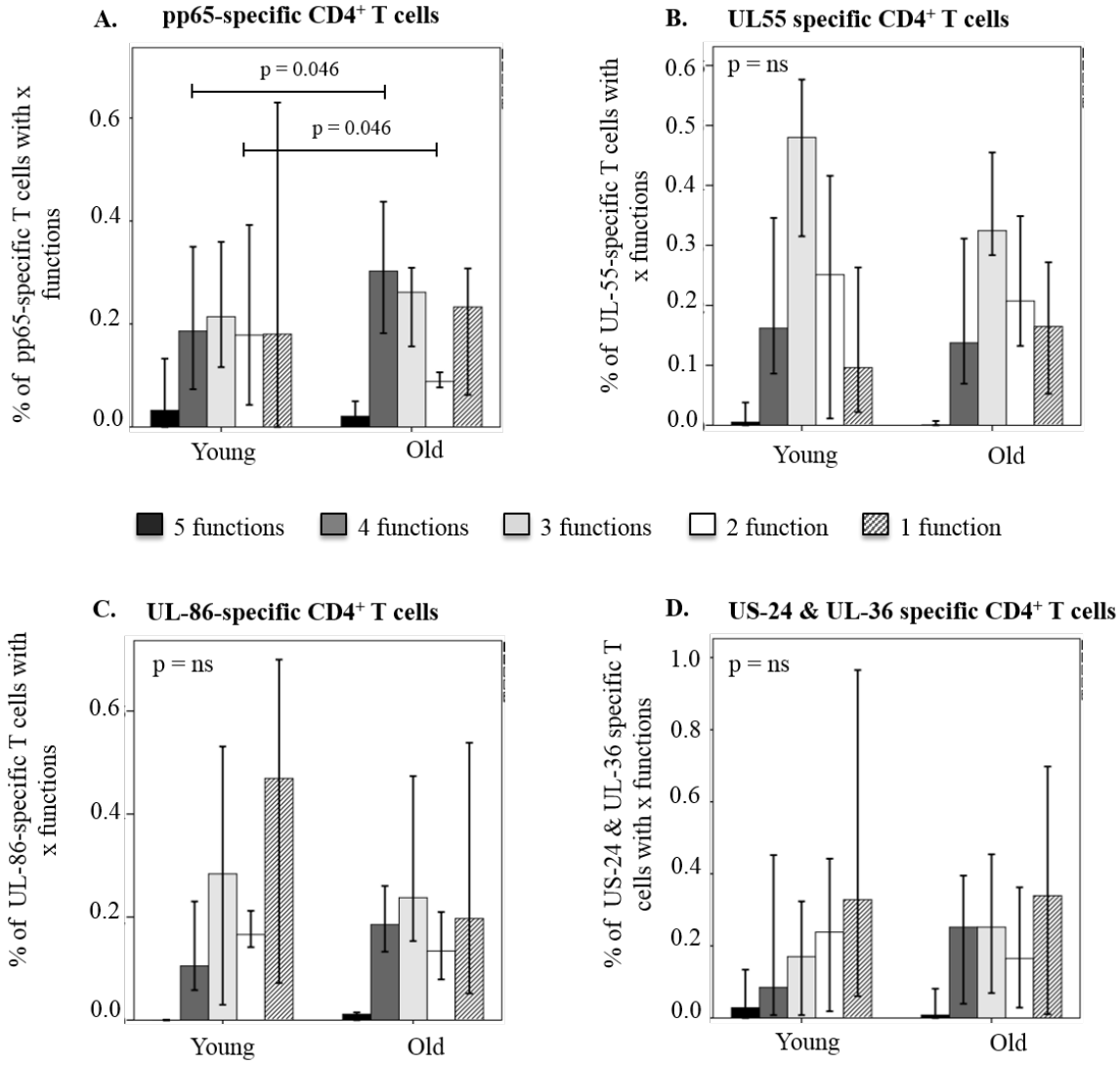


Figure 8.4. Subsets of HCMV-specific CD4$^+$ T cells with multiple functions does not change with increasing age.

The percentage of HCMV-specific CD4$^+$ T cells producing up to five activation markers upon antigenic stimulation in 2 age groups (Young n=18, Old n=41) are plotted in the bar charts above (A-D). (A) pp65 specific, (B) UL-55 specific, (C) UL-86 specific, (D) US-24 & UL-36 specific CD4$^+$ T cells. Each bar ranging from black to white represents the number of cytokines produced, 5 functions down to 1 function. Median values with CI are displayed. $p < 0.05$ was considered significant.

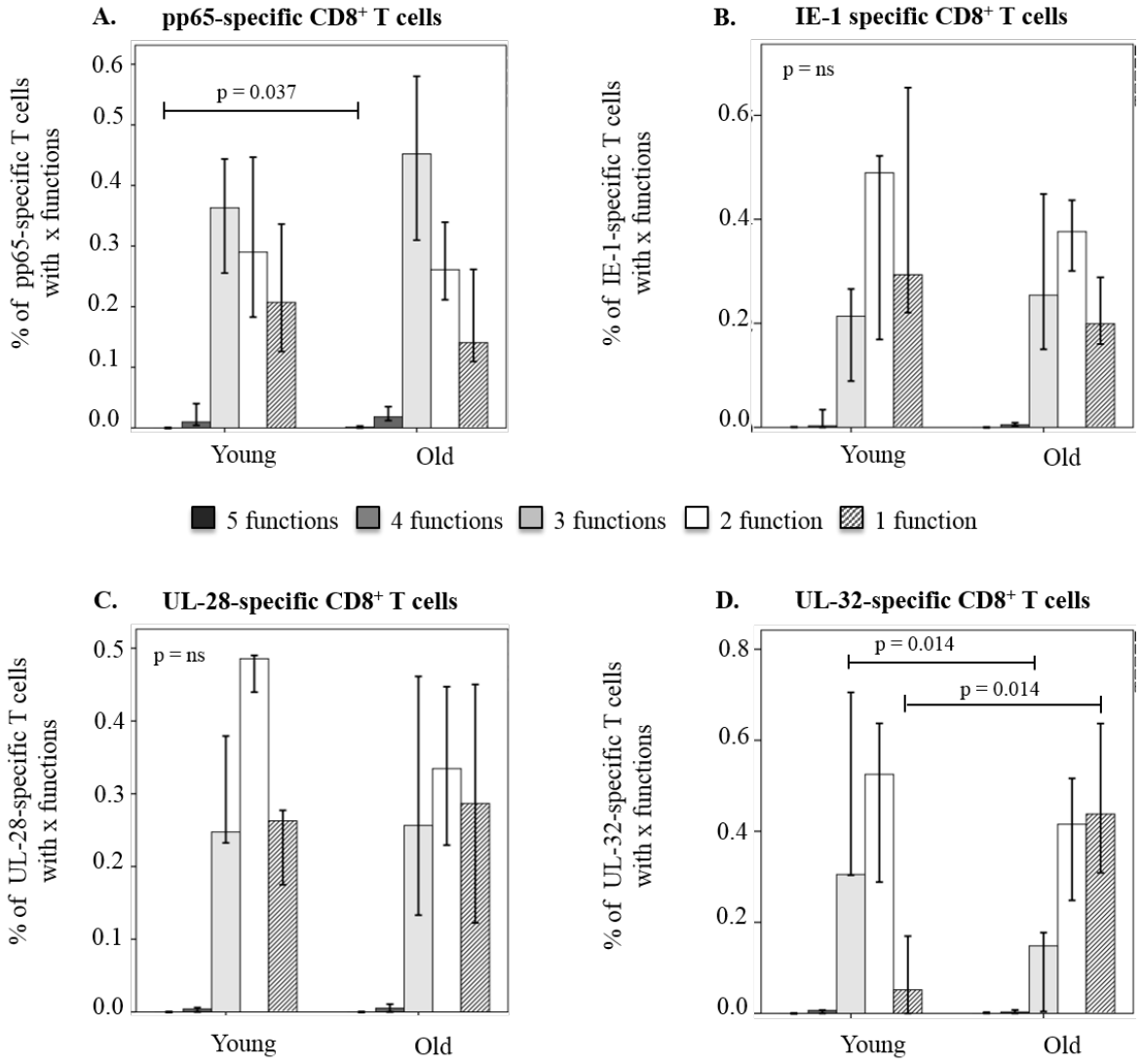


Figure 8.5. Subsets of HCMV-specific CD8$^+$ T cells with multiple functions does not change with increasing age.

The percentage of HCMV-specific CD4$^+$ T cells producing up to five activation markers upon antigenic stimulation in 2 age groups (Young n=20, Old n=53) are plotted in the bar charts above (A-D). (A) pp65 specific, (B) IE-1 specific, (C) UL-28 specific, (D) UL-32 specific CD8$^+$ T cells. Each bar ranging from black to white represents the number of cytokines produced, 5 functions down to 1 function. Median values with CI are displayed. $p < 0.05$ was considered significant

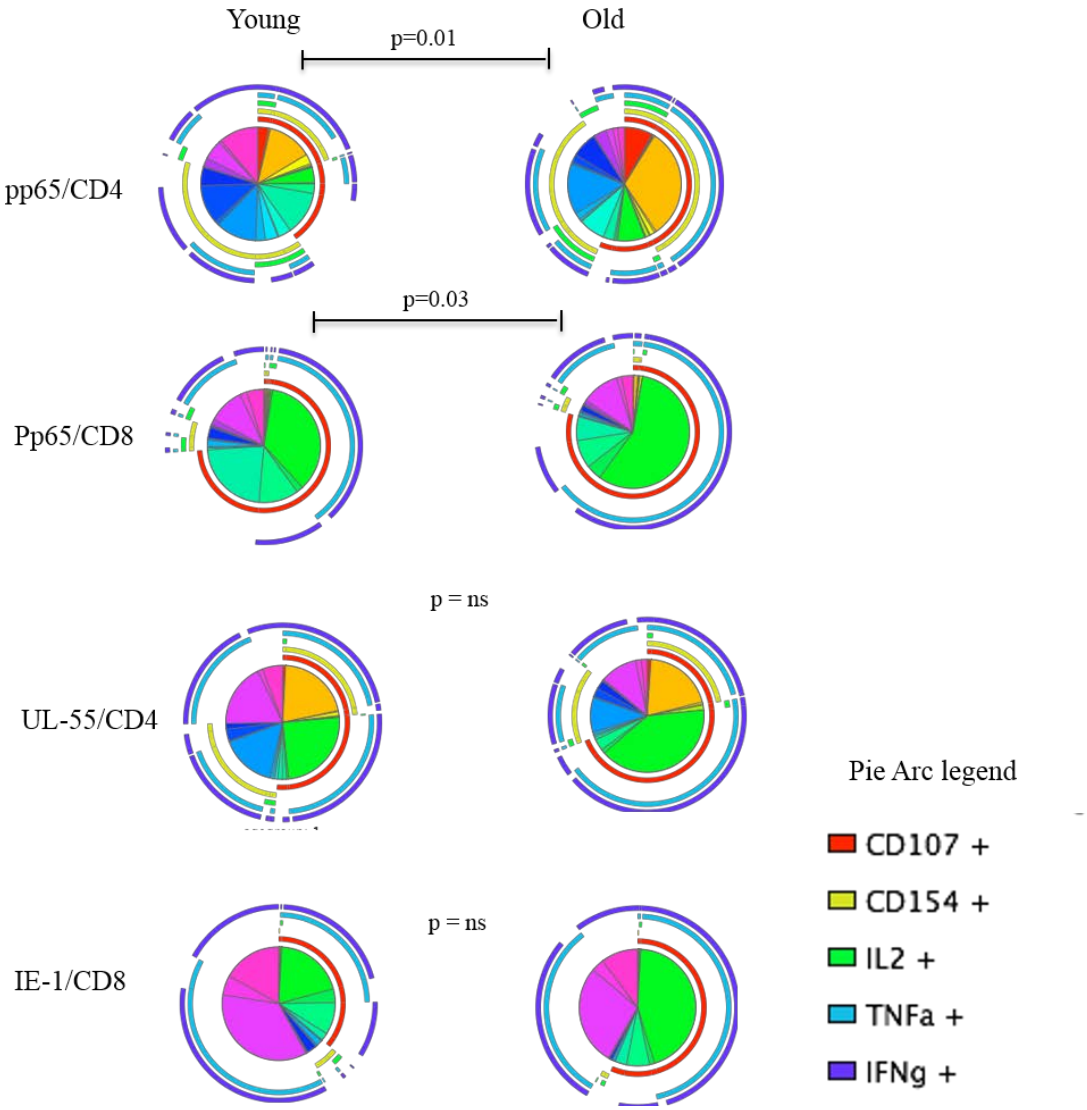


Figure 8.6. Comparison of polyfunctional profiles of top recognised HCMV proteins in Young and Old group.

The pie charts displayed above compares the proportion of pp65 specific $CD4^+$ T cells, pp65 specific $CD8^+$ T cells, UL-55 specific $CD4^+$ T cells and IE-specific $CD8^+$ T cells in Young and Old group. The arc around each pie denotes individual functional markers. Comparisons between both groups were performed using Monte Carlo permutation analysis. $P<0.05$ was considered significant.

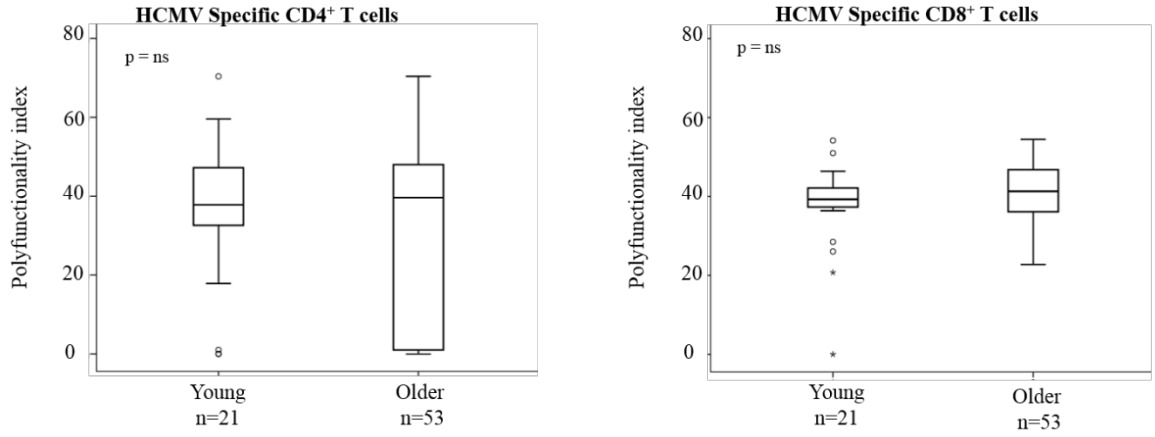


Figure 8.7. The polyfunctionality index reveals no significant change in level of polyfunctionality with increasing age among HCMV seropositive individuals.

The polyfunctionality index which captures functional subset distributions by weighting the number of functions in a subset as well as the size of a subset was calculated for total HCMV specific responses. The boxplots above depicts the polyfunctional index for all responses in Young versus Older groups in all (A) HCMV specific $CD4^+$ T cells and (B) HCMV specific $CD8^+$ T cells.

Diary page CMV study

INITIALS | | | DATE OF BIRTH | / | / | /

(Please circle the month and the year this diary sheet refers to)

YEAR		2009			2010	

MONTH	JAN	FEB	MAR	APR	MAY	JUNE
	JULY	AUG	SEP	OCT	NOV	DEC

DAYS OF THE MONTH

(Please circle any days on which your health has been worse than usual)

1	2	3	4	5	6	7
8	9	10	11	12	13	14
15	16	17	18	19	20	21
22	23	24	25	26	27	28
29	30	31				

WHAT HAVE BEEN THE PROBLEMS YOU HAVE ENCOUNTERED?

PAIN ☐

INFECTION
- CHEST INFECTION ☐
- URINE INFECTION ☐
- HEAD COLD ☐
- OTHER INFECTION ☐

OTHER HEALTH PROBLEM ☐

PLEASE DESCRIBE PROBLEMS IN MORE DETAIL (please use back of page if more space is required)

Diary page for 'The body's response to Cytomegalovirus in different age groups'

Version 1, 12th July 2009 (Ethics approval obtained from South East REC, Ref. 09/H1102/84)

Figure 8.8. Diary page for assessing health related quality of life.

(Version 1.0, 12th July 2009, Ethics approval obtained from south east REC, (REF. 09/H1102/84)

	PC	RLPH	RLEP	EF	EW	SC	P	GH
Total freq. of HCMV specific $CD4^+$ T cells	Rs = -0.212 ns n= 59	Rs= -0.158 ns n= 59	Rs= -0.09 ns n=59	Rs= -0.270* **0.042** n=57	Rs= -0.072 ns n=57	Rs= -0.092 ns n=58	Rs= 0.206 ns n=59	Rs= 0.001 ns n=58
Total freq. of HCMV specific $CD8^+$ T cells	Rs= -0.250* **p=0.033** n=73	Rs= -0.069 ns n=73	Rs= -0.095 ns n=73	Rs= -0.114 ns n=71	Rs= -0.038 ns n=71	Rs= -0.023 ns n=72	Rs= -0.132 ns n=73	Rs= -0.109 ns n=72

Table 8.2. Correlation of 8 different scales determined using the RAND-36 assessment form and summated HCMV-specific $CD4^+$ and $CD8^+$ T cell responses.

9 POSTERS , PRESENTATION AND PUBLICATIONS

9.1 POSTERS

a. 'Is age-corrected CD57 expression on CD8 T-cells highly correlated with CMV-serostatus?' 2011, British Society for Immunology (BSI), Liverpool, UK.

b. 'Characterisation of T cell specific responses to Cytomegalovirus in an elderly and young cohort', 2014, BSMS Research day, Brighton, UK.

c. 'Characterisation of T cell specific responses to human cytomegalovirus with age', 2014, British Society for Immunology (BSI) Brighton, UK..

9.2 ORAL

a. 'Change of immune response to Cytomegalovirus (CMV) in older age', 2013, BSMS Postgraduate research day, Brighton, UK

9.3 PUBLICATIONS

a. **Martha Bajwa Joseph**, Serena Vita, Susanna Vescovini, Paolo Sansoni, Nadia Terrazzini, Stefano Caserta, David Thomas, Kevin Davies, Helen Smith, and Florian Kern* (2015) *The impact of chronological age on the cellular immune response to cytomegalovirus: revisiting a paradigm* (*In progress*).

b. Terrazzini, Nadia, **Bajwa Joseph, Martha**, Vita, Serena, Thomas, David, Smith, Helen, Vescovini, Rosanna, Sansoni, Paolo and Kern, Florian (2014). *Cytomegalovirus infection modulates the phenotype and functional profile of the T-cell immune response to mycobacterial antigens in older life.* Experimental Gerontology, 54. pp. 94-100.

c. Terrazzini, Nadia, **Bajwa Joseph, Martha**, Vita, Serena, Cheek, Elizabeth, Thomas, David, Seddiki, Nabila, Smith, Helen and Kern, Florian (2014). *A novel*

cytomegalovirus-induced regulatory-type t-cell subset increases in size during older life and links virus-specific immunity to vascular pathology. Journal of Infectious Diseases, 209 (9). pp. 1382-1392.

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