

**A STUDY OF CYTOMEGALOVIRUS INFECTION,
COGNITIVE ABILITY AND IMMUNOSENESCENCE IN
OLDER ADULTS**

By

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ABSTRACT

Cytomegalovirus (CMV) is ubiquitous and the incidence of human infection increases with age. CMV exacerbates immunosenescence and is associated with increased mortality and morbidity in older adults. Cognitive decline in older generations causes great personal and financial burden. Here, examination of the Lothian Birth Cohort (1936) establishes an association between higher CMV IgG titres and a decline in general cognitive ability. This work also further defines the influence of CMV and Epstein-Barr virus (EBV) infection upon immune repertoire.

Analysis of the 1000 Elders cohort in Birmingham demonstrates that CMV- and EBV-specific T cell responses remain stable over a period of ten years. However, CMV seronegative older adults display higher EBV viral loads compared to CMV seropositive individuals despite a similar frequency of EBV-specific T cell responses in both groups. In addition, CMV serostatus does not appear to influence the phenotype of EBV-specific T cell responses.

Collectively, this study defines an association between high CMV IgG titres and decreased cognitive ability in older adults and demonstrates differential control of EBV by CMV seropositive and negative adults. High EBV viral loads may impact negatively upon the health of older adults; this should be studied further in future work.

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LIST OF ABBREVIATIONS

ACS	Acute coronary syndromes
AF700	Alexa- Fluor 700
AIDS	Acquired immune deficiency syndrome
AP-1	Activator protein 1
APC (flow cytometry)	Allophycocyanin
APC-Cy7	Allophycocyanin-cyanine 7
APCs	Antigen presenting cells
AVT	Anti-viral therapy
BCR	B cell receptor
BSA	Bovine serum albumin
CCR	CC Chemokine receptor
CD	Cluster of differentiation
CM	Central memory T cell
CMV	Cytomegalovirus
CRP	C-reactive protein
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CVD	Cardiovascular disease
DCs	Dendritic cells
DLBCL	Diffuse large B cell lymphoma
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DVT	Deep vein thrombosis
EBNA	EBV nuclear antigen
EBV	Epstein Barr Virus
EDTA	Ethylenediaminetetracetic acid

ELISA	Enzyme linked immunosorbant assay
EM	Effector memory T cell
EMRA	Effector memory CD45RA revertants
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FSC	Forward scatter
FITC	Fluorescein isothiocyanate
GLM	General linear model
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigens
HSC	Haematopoietic stem cells
HSV	Herpes simplex virus
IE	Immediate early
IFN	Interferon
IL	Interleukin
IM	Infectious mononucleosis
KLRG-1	Killer-cell lectin like receptor G1
LAG-3	Lymphocyte-activation gene 3
LAT	Linker for activation of T cells
LCK	Lymphocyte-specific protein tyrosine kinase
LCMV	Lymphocytic choriomeningitis
MCMV	Murine cytomegalovirus
MHC	Major histocompatibility complexes
MMSE	Mini mental state examination
NART	National Adult Reading Test
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor-kappaB

NK	Natural killer
NKT	Natural killer T cell
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death 1
PE	R-phycoerythrin
PE-Cy7	Phycoerythrin cyanine 7
PerCPCy5.5	Peridinin chlorophyll protein complex-cyanine 5.5
PHA	Phytohaemagglutinin
PMA	Phorbol myristate acetate
pMHC	Peptide MHC complex
PTLD	Post transplant lymphoproliferative disorder
qPCR	Quantitative polymerase chain reaction
RA	Rheumatoid arthritis
SEM	Standard error of the mean
SEP	Socioeconomic position
SLE	Systemic lupus erythematosus
SLP-76	SH2 domain-containing leukocyte protein of 76 kDa
SSC	Side scatter
TAP	Transporter associated with antigen processing
TCR	T cell receptor
Th1/2/17	T helper 1/2/17
TIM-3	T cell immunoglobulin mucin-3
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TMT	Trail making test
TREC	T-cell receptor excision circles

Treg

Regulatory T cell

ZAP-70

Zeta-chain-associated protein kinase 70

Chapter 1. INTRODUCTION

1.1.AIMS OF THE THESIS

Three main questions are addressed in this thesis:

1. Is there an association between Cytomegalovirus (CMV) and cognitive function in older adults?
2. How do Cytomegalovirus (CMV) and Epstein-Barr Virus (EBV) contribute to overall immune phenotype in the Lothian Birth Cohort (1936)?
3. What influence do CMV- and EBV-specific responses have upon immunity in the 1000 Elders cohort?

1.2.BACKGROUND TO THE PROJECT

Cytomegalovirus is a common herpes virus. The virus is demonstrated to drive immunosenescence in older adults (Pawelec *et al.*, 2010b). Previous studies have demonstrated a role for this virus in mortality and morbidity, particularly in terms of cardiovascular health (Roberts *et al.*, 2010, Simanek *et al.*, 2011). There is also an association between CMV and lower socioeconomic status, thus suggesting that inequality may increase susceptibility to infection (Dowd *et al.*, 2009b). Studies of individuals with pre-existing conditions have demonstrated an association between CMV and cognition. (Blasko *et al.*, 2007, Shirts *et al.*, 2008). The aim of the current work is to assess whether there is an association between CMV and cognitive function in healthy older adults. In order for this to be investigated, members of the Lothian Birth Cohort

(1936) were studied. This cohort is a group of now older adults living in Scotland and is described in further detail in chapter 3. As the Lothian Birth Cohort (LBC) are a unique and well characterised cohort, samples taken from participants were used to assess the influence of CMV and EBV upon immunosenescence. The impact of CMV upon immunosenescence is quite well defined and is discussed further within the introduction (Wagner *et al.*, 2004). However, little is known about contribution of both CMV and EBV, a gamma herpes virus, upon immunosenescence. This is explored in chapter 4. The influence of CMV and EBV-specific responses upon immunity is discussed in chapter 5. For assessment of this relationship, a group of older adults living in Birmingham; the ‘1000 Elders’ cohort had blood samples taken from them and virus-specific immunity studied. In summary, this work examines two cohorts in order to address three main questions; the influence of CMV upon cognition and influence of CMV and EBV upon overall and virus-specific immunity. Using cohorts to answer these questions is vital to the work. An advantage of the Lothian Cohort, is that a large group of individuals are well characterised individuals can be studied, thus allowing appropriate statistical analysis with availability of relevant and important variables.

1.3. THE INNATE IMMUNE SYSTEM

The innate immune system is important for non-specific defence from pathogens and serves a number of functions, including complement, activating cytokines and recognising pathogens (Medzhitov *et al.*, 2000). The innate immune system also has a vital role in activating the adaptive immune system (Luster 2002). A number of cell types are important in terms of innate immunity, including phagocytes and mast cells. Immune responses are generated quickly post infection, however they do not result in immunological memory thus these responses are not long lasting (Tosi 2005).

1.3.1. NK cells

Natural Killer cells (NK cells) are cytotoxic lymphocytes that are involved with non-specific killing and form part of the innate immune system. NK cells kill tumour cells and react against pathogens during infection. These cells bind to various receptors which control the function of NK cells, some of which are involved in cytotoxicity and others which are inhibitory (Orr *et al.*, 2010). NK cells can be classified on the basis of differential expression of CD56 (cluster of differentiation 56) on the cell surface; NK CD56 dim cells are cytotoxic and NK CD56 bright cells are more regulatory in nature (Vivier *et al.*, 2008).

1.4.ADAPTIVE IMMUNITY

The specific recognition of antigens via T cells and B cells form part of the adaptive immune response. It takes time for effective adaptive immune responses to develop. Recognition takes place through receptors on the cell surface; thus enabling an immune response to take place (Parkin *et al.*, 2001). These interactions allow elimination of pathogens, as well as the development of immunological memory. This can be 'passive memory' which is short- term or active memory which is established via T and B cells. Vaccination relies upon active memory, as cells have to be able to respond upon subsequent encounters to an antigen for vaccination to be effective (Clem 2011).

1.4.1. T cell overview

T cells form an essential part of the adaptive immune system and have a number of functions; including helper function, cytotoxicity and regulation. T cells are derived from haematopoietic stem cells in the bone marrow and mature in the thymus, whereby they undergo positive and negative selection (Koch *et al.*, 2011). Positive selection involves selecting T cells which bind self- MHC which then go on to differentiate. Negative selection removes autoreactive T cells via apoptosis (Klein *et al.*, 2009).

1.4.2. CD8⁺ T cells

CD8⁺ T cells are described as ‘cytotoxic T cells’; they have a role in tumour immune surveillance and play an essential role in the elimination of virus infected cells. (Wherry *et al.*, 2004). The priming of naïve CD8⁺ T cells occurs in secondary lymphoid organs i.e. the lymph nodes and the spleen. Activation via antigen presenting cells (APCs) such as dendritic cells involves multiple signals; including antigen recognition through MHC class I, costimulation, cytokines and chemokines (Zhang *et al.*, 2011). T cells then proliferate and undergo a number of cell divisions between around five and eight days (Harty *et al.*, 2008). These cells can then perform effector function, such as cytotoxicity towards the target cell and inflammatory cytokine production (Zhang and Bevan 2011).

1.4.3. CD4⁺ T cells

CD4⁺ T cells are described as ‘helper T cells’ and are associated with antibody production, through maturation of B cells. CD4⁺ T cells also produce cytokines and chemokines and are involved in the recruitment of eosinophils, basophils and neutrophils to the site of inflammation (Zhu *et al.*, 2008). CD4⁺ T cells can be distinguished by their various cytokine profiles and are described as Th1, Th2, Th17 or iTreg (Zhu *et al.*, 2010). Th1 (T helper 1) cells are involved in autoimmunity and the generation of responses against intracellular parasites. These responses are triggered by IL-2 and IL-12; interferon gamma (IFN γ) secretion is an important element of the Th1 response. Th2 cytokines are initiated by IL-4 and drive humoral immunity. These responses involve IL-4, IL-5 and IL-15 secretion and result in eosinophil recruitment and antibody responses (Kidd 2003). In

contrast, Th17 cells produce IL-17, IL-17F and IL-22 (Korn *et al.*, 2009). Th17 cells tend to generate responses against extracellular pathogens and involves the recruitment of macrophages and neutrophils. Induced regulatory T cells (iTregs) have a role in immune tolerance and are suppressive in nature (Bilate *et al.*, 2012).

1.4.4. Gamma delta T cells

$\gamma\delta$ T cells are unique in that they bridge both the adaptive and innate system. They differ from $CD4^+$ and $CD8^+$ T cells as their T cell receptor (TCR) consists of a γ and δ chain. These cells are able to initiate cytokine responses in order to react against pathogens (Bonneville *et al.*, 2010). These cells can be further subdivided into vdelta 1 and vdelta 2 subsets which display differences in ligand specificity, effector responses and tissue locality (Bonneville *et al.*, 2010).

1.4.5. MHC Class I presentation

MHC class I molecules present peptides to $CD8^+$ T cells which then provide effector function (Hansen *et al.*, 2009). During MHC class I presentation endogenous antigens, including those derived from viral proteins, are processed and degraded by the proteasome. Peptides are then translocated into the endoplasmic reticulum (ER) via the transporter associated with antigen processing (TAP). ERp57 and calreticulum facilitate binding of the MHC class I molecule with the peptide (Hewitt 2003). Peptide-MHC class I complexes (pMHC) leave the ER and are subsequently transported to the cell membrane where they can be recognised by $CD8^+$ T cells (Neefjes *et al.*, 2011). Some pMHC

complexes are more ‘immunogenic’ than others and are therefore easily recognisable by CD8⁺ T cells (Calis *et al.*, 2013). Viruses often have different ways of avoiding detection via this mechanism; for example by preventing proteasomal processing or inhibiting TAP (Hansen and Bouvier 2009).

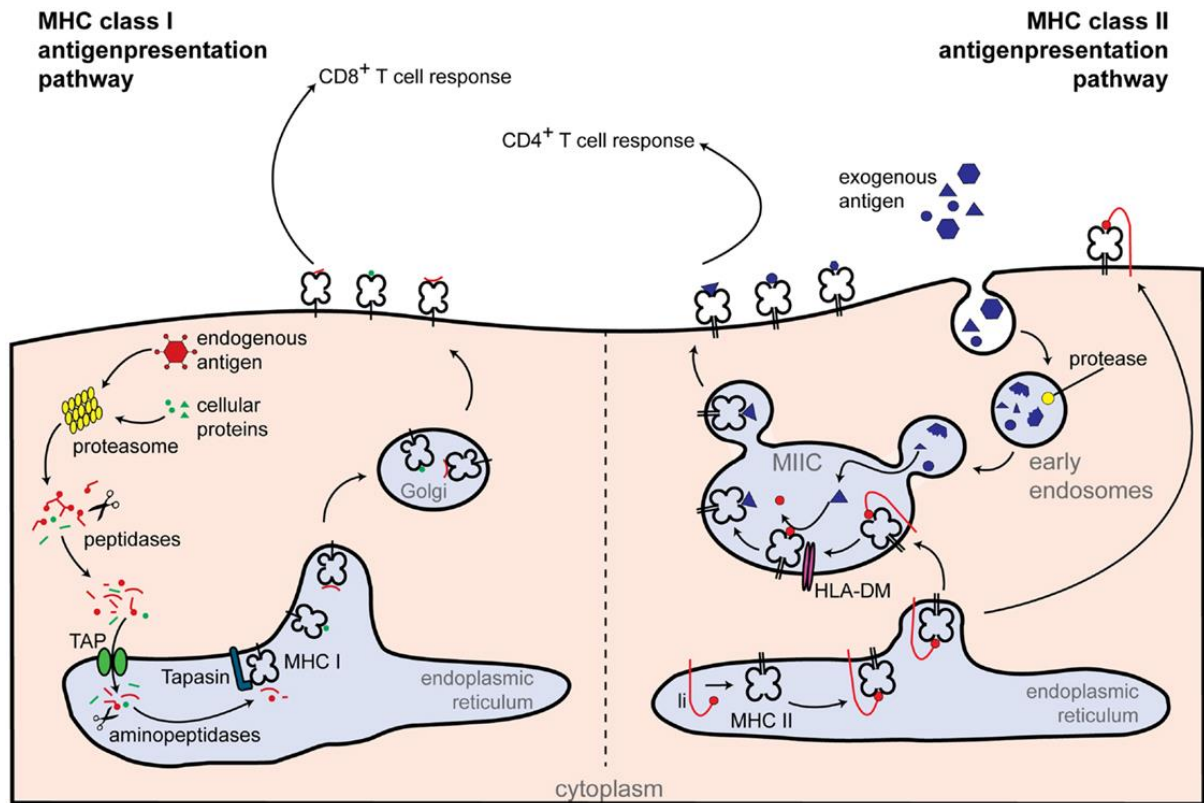


Figure 1-1 Classical routes of antigen presentation by MHC class I and II molecules.

Depiction of MHC class I (endogenous antigen) presentation and MHC class II (exogenous antigen) presentation

Adapted from Neerinx *et al* 2013 *Frontiers in Immunology*
(Neerinx *et al.*, 2013)

1.4.6. MHC Class II presentation

MHC class II molecules are found on the surface of professional antigen-presenting cells within the peripheral blood. They form complexes with peptides which are then recognised by CD4⁺ T cells (Jones *et al.*, 2006). MHC class II molecules are produced within the ER and are joined to an ‘invariant chain’ chaperone. This has a role in exporting MHC class II into the golgi whereby fusion with an endosome occurs. The class II associated invariant chain peptide (CLIP) blocks the peptide binding cleft in order to avoid premature binding (Chaturvedi *et al.*, 2000). Peptide binding the MHC class II molecule occurs via displacement of CLIP by HLA-DM, thus enabling the presentation of the MHC class II molecule on the cell surface (Kobayashi *et al.*, 2012).

1.4.7. The T cell receptor

The T cell receptor (TCR) recognises pMHC complexes. The TCR is a heterodimer comprising of two domains, either α and β or less commonly γ and δ which forms a complex alongside CD3. (Smith-Garvin *et al.*, 2009). The α and γ chains are generated via VJ recombination. In contrast the β and δ chains are generated by VDJ recombination.

TCRs are dependent on kinases, such as the SFK member lymphocyte-specific protein tyrosine kinase (LCK) in order to initiate signalling. LCK phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) of the various CD3 chains (Brownlie *et al.*, 2013). This causes activation of Zeta-chain-associated protein kinase 70 (ZAP-70) which phosphorylates scaffold proteins, linker for activation of T cells (LAT)

and SH2 domain-containing leukocyte protein of 76 kDa (SLP-76). These recruit and activate important signalling proteins. CD28 is an important co-receptor that provides the second signal for complete T cell activation. When bound to its ligands CD80/CD86 it mediates activation of transcription factors, including activator protein-1 (AP-1), nuclear factor kappa-B (NFκB) and nuclear factor of activated T cells (NFAT) to induce Interleukin-2 (IL-2) transcription, which is vital for T cell proliferation and differentiation (Huang *et al.*, 2004).

1.4.8. Defining T cell subsets

Naive T cells; cells that have not been challenged by antigen are characterised by lack of CD45RO. Their function is to rapidly respond to new pathogens. They display high levels of CD62L and can also be characterised by increased expression levels of CD45RA, CCR7, CD27 and CD28 (Larbi *et al.*, 2014, Mahnke *et al.*, 2013). CD28 and CD27 are co-stimulatory molecules that can serve as markers to determine the proliferative history of a cell (Dolfi *et al.*, 2007). Recent thymic emigrants can be identified by CD31 surface expression. Central memory T cells express homing markers such as CCR7, are CD45RO⁺ and upregulate CD40L. These cells lack effector function. In contrast, effector memory T cells do not express CCR7 or CD45RA and are able to respond to antigenic challenge (Sallusto *et al.*, 2004).

There is also a subset of 'late-stage' cells known as 'EMRA;' or 'TEMRA' these cells are CCR7⁻ but re-express CD45RA(Sallusto *et al.*, 2004). EMRA T cells tend to express high

levels of CD57 and killer-cell lectin like receptor G1 (KLRG-1) and low levels of the co-stimulatory molecule CD28 compared to effector memory cells (Larbi and Fulop 2014). Increased expression of CD57 and lack of expression of CD28 have been associated with lack of functionality in these cells (Larbi and Fulop 2014).

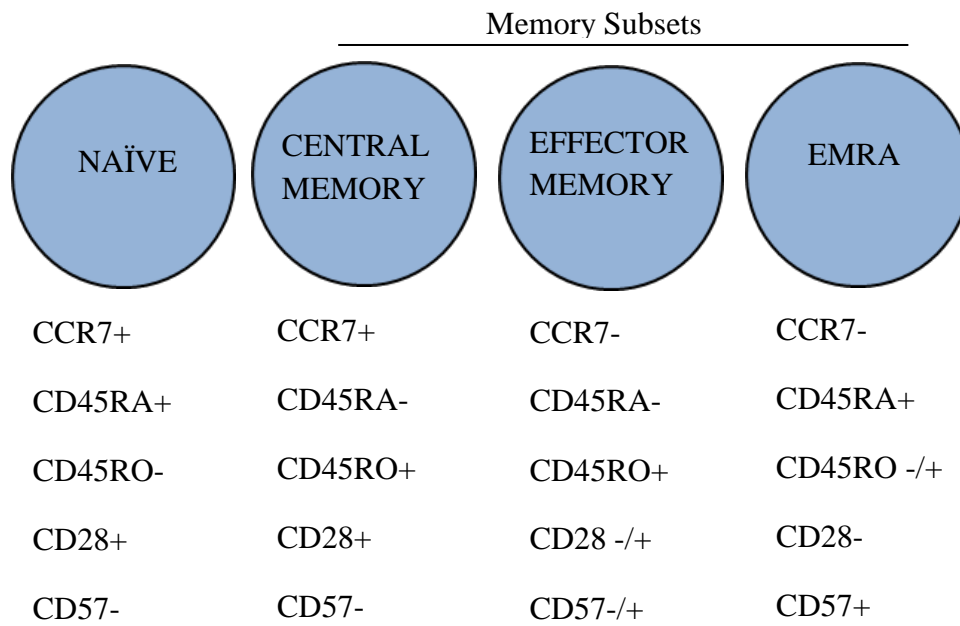


Figure 1-2 T cell differentiation status and expression of surface markers

Naïve, Central memory, Effector memory and EMRA T cells and expression of CCR7, CD45RA, CD45RO, CD28 and CD57. Data regarding T cell markers taken from (Larbi and Fulop 2014)

1.4.9. B cells

B cells are lymphocytes with key roles in humoral immunity and initiating T cell responses (Lebien *et al.*, 2008). Similarly to other antigen presenting cells, B cells display CD40 on their surface. B cells can recognise specific antigens via their B cell receptor (BCR); the antigen is processed and displayed on MHC class II molecules which can then present to CD4⁺ T cells (Yuseff *et al.*, 2013). Mature B cells are able to respond to antigen upon a second encounter. Plasma B cells are cells that have responded to antigen, proliferated and differentiated which are now able to secrete antibody; these are usually detected within tissue. B cells have also been demonstrated to have regulatory function, these are termed 'regulatory B cells' and have a role in the pathogenesis of autoimmune disease (Garraud *et al.*, 2012).

1.5. CYTOMEGALOVIRUS

Cytomegalovirus (CMV) is a member of the family *Herpesviridae*. Other viruses within this family include Epstein-Barr virus (EBV), Varicella Zoster virus (VZV) and Herpes Simplex (HSV). CMV, a beta herpesvirus is the largest of these viruses, the CMV genome is 236 kbp (Dolan *et al.*, 2004). The CMV virion consists of a double-stranded linear DNA core within a nucleocapsid, enveloped by the tegument. The capsid is surrounded by an outer envelope which contains viral glycoproteins (Crough *et al.*, 2009). CMV infection occurs when the virus comes into contact with a mucosal surface, allowing the virus to spread within the host (Wald *et al.*, 2007, Zanghellini *et al.*, 1999). A number of viral genes are expressed following infection with CMV; including early genes, delayed early genes and late genes which are not expressed until 24 hours post-infection (Isomura *et al.*, 2011).

CMV has the ability to establish life-long persistence in the host as the virus can remain latent. Reactivation, however can occur; which is particularly problematic in immunocompromised individuals (Sinclair *et al.*, 2006). Carriage of the virus can occur at a number of sites, cells of myeloid lineage are of importance. CMV DNA has been found in monocytes in peripheral blood mononuclear cells (PBMCs) as well as bone-marrow progenitors (CD34+) (Mendelson *et al.*, 1996, Smyth *et al.*, 1991). The ability of CMV to replicate within these cells relies on a number of genetic determinants. CMV can reside in myeloid-lineage progenitor cells within the bone marrow persists as these cells divide into PBMCs and dendritic cells (DCs). The differentiation of monocytes and DCs into

macrophages and mature DCs and the subsequent expression of viral IE genes is a cause of viral reactivation (Sinclair and Sissons 2006).

Glycoprotein B has been described as a virulence factor as this has a role in host entry, transmission and fusion (Humar *et al.*, 2003). A number of viral genes have been implicated in the spread of CMV including US28 and UL146. Studies in MCMV have found a number of viral genes that are important in viral replication (Britt 2007).

1.5.1. Epidemiology

CMV infection rates are affected by various factors, including sex, ethnicity and socioeconomic status (Bate *et al.*, 2010). Women of child-bearing age have CMV seroprevalence of between 45-100%. CMV infection rates vary greatly between different countries; with the highest rates noted in Africa, Asia and South America and reduced infection rates found in the United States and throughout Western Europe (Cannon *et al.*, 2010). It is well established that CMV infection rates increase with age with a greater proportion of CMV seropositive donors in older adults (Staras *et al.*, 2006, Vasto *et al.*, 2007). Women are also more likely to be seropositive than men (Cannon *et al.*, 2010).

The association between CMV infection and socioeconomic status has been greatly studied. The Third National Health and Nutrition Examination Survey (NHANES) in the US investigated a sample of over 10,000 CMV positive individuals aged 25+. As shown by other studies, non-white ethnicity was associated with elevated antibody levels to the

virus. Importantly, lower income and education were associated with increased antibody titres. A 0.05 reduction in the CMV 'ELISA index' was observed for each year of additional education, showing the influence of this on CMV status (Dowd *et al.*, 2009a). Bate *et al* have also published findings that low socioeconomic status increases the risk of CMV infection (Bate *et al.*, 2010). Children in the US of low socioeconomic status are also at risk from burden of other infections as well as CMV, such as *Helicobacter pylori*, Herpes simplex virus-1 and Hepatitis A and B (Dowd *et al.*, 2009b). Issues such as over-crowding and family size are thought to contribute to this (Staras *et al.*, 2006). It has also been argued that, after controlling for age, race, gender and education level CMV infection may be associated with the relationship found between socioeconomic position (SEP) and cardiovascular disease (CVD) (Simanek *et al.*, 2009). Recent work by Janicki-Deverts *et al* demonstrated that childhood environment, including less years of parental home ownership and having a parent who smoked was associated with an increased risk of infection (Janicki-Deverts *et al.*, 2014). This data demonstrates the vital role that socioeconomic status has on influencing disease status and mortality.

1.5.2. Transmission

CMV is present in saliva, urine, semen, tears and cervical secretions (Pass 2002). A CMV seropositive person will pass on the virus to an average of nearly two individuals during their life time (Colugnati *et al.*, 2007). Transmission of the virus can also occur either during pregnancy or post-natally. Seroconversion rates among pregnant women vary; and can range from 1 to 7%, increasing the risk of congenital CMV (Hyde *et al.*, 2010). Transplacental transmission normally occurs in women infected prior to conception or in

those who have primary infection when pregnant (Pass 2002). Transmission of CMV can also occur post-natally via breast milk; women who are seropositive can show viral reactivation during lactation causing shedding (Schleiss 2006). CMV infection in preterm infants is potentially very serious, and transmission of the virus in breast milk to infants has been studied widely. High CMV IgG titres in mothers blood has been identified as a risk factor for transmission to infants (Jim *et al.*, 2004). Due to the possibility of transmission of CMV through breast milk a number of prevention strategies to decrease risk of transmission have been studied, including pasteurisation and freezing of breast milk (Schleiss 2006).

CMV can be transmitted through urine and saliva. There is risk of transmission from young children to adults, as well as transmission between children. A meta-analysis concluded that day care centre workers are at a high risk of CMV infection (Joseph *et al.*, 2006). Hyde *et al* estimated the seroconversion rate of day care centre workers of around 0-12.4% (Hyde *et al.*, 2010). The risk of the offspring of pregnant women of having congenital infection is increased by caring for young children in the year before delivery (Fowler *et al.*, 2006b). CMV can be sexually transmitted; a study in the USA which investigated CMV prevalence in adolescent men found that number of sexual partners was associated with infection. (Stadler *et al.*, 2010). It has been argued that reduction of sexually transmitted CMV among women of child bearing age could be beneficial in terms of reducing congenital CMV infection (Staras *et al.*, 2008).

CMV transmission can occur through red blood cells, platelets or through whole blood (Blajchman *et al.*, 2001). Approximately 13-37% of immunocompromised people who are receiving blood products will contract the virus (Roback 2002) . To remove this risk, blood can be filtered to remove white blood cells or screened (Roback 2002). CMV can also be transferred in solid organ transplants; this will be discussed in more detail.

1.5.2.1. **Diagnosis**

As CMV is generally asymptomatic, infection often remains undiagnosed. However, diagnosis of CMV is particularly important for assessing risk of congenital CMV. High-risk individuals are also tested, such as people who have undergone a solid organ or stem cell transplant.

CMV can be detected through isolation from the urine, blood, bodily fluids or tissues and then culturing in fibroblasts. Staining with fluorescent antibodies confirms diagnosis of infection (De La Hoz *et al.*, 2002) This method can be used when investigating for foetal infection and this type of viral culture method has 100% specificity and is considered the gold standard for diagnosing fetal CMV through amniotic fluid (Mendelson *et al.*, 2006). One limitation is that it is time consuming as it can take up to 1-3 weeks for a result to be obtained (De La Hoz *et al.*, 2002). Antigen detection can confirm CMV seropositivity. The pp65 antigen can be detected in leukocytes; this is performed with use of fluorescently labelled monoclonal antibodies (Harari *et al.*, 2004). Another viral culture method used is the shell-vial assay. This is a quicker method and is more sensitive

compared with routine viral cultures (Chisholm *et al.*, 2014). Polymerase chain reaction (PCR) is a quick method for determining CMV serostatus as a result can be gained within 6-48 hours (De La Hoz *et al.*, 2002). Although a sensitive technique, contamination can be an issue (Mendelson *et al.*, 2006).

Dried blood spot tests are a quick way of screening for infection; this type of test is often used for diagnosis of congenital CMV by testing the blood of the neonate and has also been used to diagnose transplant patients via assessment of viral load (Barbi *et al.*, 2006a, Piccirilli *et al.*, 2014). Sensitivity of the method ranges from 71-100%, with a specificity between 99 and 100% (Barbi *et al.*, 2006a, Binda *et al.*, 2004).

Enzyme-linked immunosorbent assay (ELISA) for CMV IgM or IgG are commonly used for diagnosis. IgM assays can be used to diagnose primary infection, whereas IgG specific for CMV appears a few months post- infection and can increase over time (Barbi *et al.*, 2006b, Moss *et al.*, 2004). However, one negative aspect of the CMV ELISA assay is that arbitrary units are used (Mendelson *et al.*, 2006).

1.5.2.2. **CMV in immunocompromised individuals**

CMV infection can have a negative impact on the health of immunocompromised patients such as individuals who are HIV positive, those undergoing treatment for cancer and transplant patients. Successful diagnosis of CMV in immunocompromised patients is important as complications can be very serious (Kotton 2013).

1.5.2.3. **HIV/AIDS**

There is evidence to suggest that CMV viraemia is associated with mortality in this group of patients (Griffiths 2006). This is despite HAART therapy reducing end-stage organ disease caused by the virus (Griffiths 2006). Neurological complications due to CMV in AIDS patients are rare, however can be very serious (Silva *et al.*, 2010). CMV reactivation in AIDS patients can also lead to cytomegalovirus retinitis or CMV pneumonitis (Buffone *et al.*, 1993, Jabs).

1.5.2.4. **Transplant Patients**

Approximately 75% of individuals receiving an organ transplant will have CMV reactivation or be infected with the virus (Husain *et al.*, 2009). CMV infection normally occurs 4-8 weeks post-transplant however it can occur much later; approximately 30% of people may develop CMV disease in later stages (Husain *et al.*, 2009, Pass 2002). A study of over 200 patients receiving solid organ transplants found that a CMV positive donor transplant increased risk of infection for both CMV positive and negative recipients (Da Cunha-Bang *et al.*, 2011). Infection with CMV exposes patients to risks such as

rejection, development of other infections and decreased chance of survival (Fishman *et al.*, 2007). CMV pneumonitis is a common serious complication that occurs post haematopoietic stem cell transplant (Ljungman *et al.*, 2010). Preventing transplant-related CMV infection could cut costs for health care providers and be beneficial for patients. Universal anti-viral prophylaxis or pre-emptive treatment can be useful in preventing the negative health impacts of CMV infection within the transplant setting (Fishman *et al.*, 2007).

1.5.2.5. **CMV disease in immunocompetent patients**

CMV in immunocompetent hosts is usually asymptomatic. However, CMV has been linked with a number of conditions; including CMV mononucleosis-like illness, colitis, central nervous system infections, thrombosis, cardiovascular disease, frailty and anxiety and depression.

1.5.2.6. **CMV and Mononucleosis-like Illness**

Infectious Mononucleosis is a condition with symptoms such as fatigue, lymphadenopathy, pharyngitis and fever. This typically occurs in young adults and is often attributed to Epstein-Barr Virus, however CMV can also cause these symptoms (Bravender 2010). In this case the condition is usually termed a ‘mononucleosis-like illness.’ It has been estimated that 5-7% of these cases are caused by CMV infection (Hurt *et al.*, 2007).

1.5.2.7. **Cardiovascular disease**

CMV infection has been associated with CVD and therefore increased risk of mortality in immunocompetent individuals. Savva *et al* observed that cardiovascular death nearly doubled in a group of CMV seropositive older adults (Savva *et al.*, 2013). A study of older Latino individuals (aged 60-101 years) found that high levels of CMV IgG were associated with increased risk of 'all cause- mortality' as well as mortality related to CVD (Roberts *et al.*, 2010). This could occur through the action of pro-inflammatory cytokines, for example IL-6 and tumour necrosis factor (TNF) (Roberts *et al.*, 2010). Simanek et al 2011 found CMV positive individuals with high C-reactive protein levels (CRP) to display increased all-cause and cardiovascular mortality (Simanek *et al.*, 2011). CMV can modulate IL-6 production in endothelial cells *in vitro*, which is important in terms of cardiovascular health (Visseren *et al.*, 1999). Mouse models have shown CMV infection to correlate with a reduction in vascular function (Gombos *et al.*, 2009).

Liu *et al* investigated coronary plaques in individuals with and without acute coronary syndromes (ACS) and found an elevation in the number of CMV positive cells in those patients with ACS (Liu *et al.*, 2006).

CMV seropositivity has been associated with increased arterial stiffness in a group of patients with kidney disease as well as increased blood pressure in a group of young healthy adults (Haarala *et al.*, 2012, Wall *et al.*, 2013). This has also been demonstrated in an MCMV model (Cheng *et al.*, 2009).

1.5.2.8. **Thrombosis**

Thrombosis is a condition in which a blood clot forms within a blood vessel, which can subsequently cause problems by blocking blood flow. Deep vein thrombosis (DVT) and pulmonary embolism are associated with CMV infection. These are more common in immunocompromised patients.

It has been argued that CMV can cause endothelial damage which would increase risk of thromboembolisms; damage to the vascular endothelium could cause problems with expression of anticoagulation properties (Delbos *et al.*, 2007). Another possible cause of thrombosis in people with CMV infection is Hughes syndrome (antiphospholipid (aPL) antibody syndrome), a condition in which there are problems associated with coagulation (Youd *et al.*, 2003). CMV could contribute to production of these antibodies, thus having a role in causing thrombosis (Delbos *et al.*, 2007). It has also been argued that CMV infection could be associated with elevated factor VIII, von Willebrand factor, and lipoprotein(a), therefore increasing risk of coagulation and thrombosis (Youd *et al.*, 2003). Studies have concluded that it is important for clinicians to be aware of the risk of thrombosis in patients who have acute CMV infections (Atzmony *et al.*, 2010b, Justo *et al.*, 2011).

1.5.2.9. **Other complications associated with CMV infection**

Atzmony *et al* argued that infection of the GI tract or central nervous system with CMV can be severe and life threatening and that not enough attention is paid to CMV infection in immunocompetent individuals (Atzmony *et al.*, 2010a). A meta-analysis of individuals with CMV colitis found that male gender, advanced aged and other immune system problems decreased survival rates in these patients (Galiatsatos *et al.*, 2005). After colitis, central nervous system disorders are said to be the most common type of CMV infection on patients who are immunocompetent (Rafailidis *et al.*, 2008). CMV encephalitis is a particularly serious form of CMV infection; however, this is more common in immunocompromised patient.

1.5.2.10. **Frailty**

Frailty is defined by a number of characteristics, including weight loss, weakness of grip strength and exhaustion (Fried *et al.*, 2001). A prospective observational cohort of women aged 70-79 years (n=635) found that participants with higher CMV antibody titres had increase rates of this syndrome, with higher antibody titres being linked to mortality risk. Those in the highest quartile of CMV antibody ‘independently predicted a higher risk of 5 year mortality’(Wang *et al.*, 2010).Schmaltz *et al* observed a link between CMV infection and frailty in older women; it was suggested that IL-6 could exacerbate this through inflammation (Schmaltz *et al.*, 2005). Moro-Garcia *et al* observed CMV seropositivity to be associated with impairment of function in older adults (Moro-Garcia *et al.*, 2012). However, work by Mathei *et al* found that CMV seropositivity was not associated with

functional impairment, demonstrating a requirement for more studies in this area (Mathei *et al.*, 2011).

1.5.2.11. **Anxiety and Depression**

CMV infection has been linked to increased levels of anxiety and depression in older adults. CMV seropositive individuals with high CMV antibody titres were found to have increased levels of depression and anxiety compared with CMV seronegative individuals (Phillips *et al.*, 2008). Pro-inflammatory cytokines produced due to CMV infection could be implicated in the development of the symptoms of anxiety and depression and could be a causative factor for the association (Phillips *et al.*, 2008, Trzonkowski *et al.*, 2004). Rector *et al* recently demonstrated that higher CMV IgG titres were associated with increased psychological stress (Rector *et al.*, 2014).

1.5.2.12. **CMV and autoimmune diseases**

CMV has been implicated in various autoimmune diseases, including systemic lupus erythematosus (SLE) , rheumatoid arthritis (RA) sclerosis and vasculitis (Wolf *et al.*, 2001). There is data to suggest that EBV may be associated with autoimmune diseases, however the evidence for involvement of CMV is more limited (Barzilai *et al.*, 2007). A study of 21 children with SLE found that CMV infection was present in 28.6% of these, with none of the control group being CMV positive, demonstrating CMV infection to be more common in children with SLE (Zhang *et al.*, 2001). A number of mechanisms have been proposed for the involvement of herpes viruses in SLE. These include molecular

mimicry, encoding proteins related to cross-reactive immune responses and activation or apoptosis of T cells and B cells (Francis *et al.*, 2010). CMV IgM antibodies are more frequent in individuals with rheumatoid arthritis compared to healthy controls. Furthermore, CMV antibodies and CMV DNA have been found in the synovial membrane of patients with rheumatoid arthritis (Einsele *et al.*, 1992, Kurbanov *et al.*, 2009).

1.5.3. Congenital CMV

CMV is a cause of congenital infection; in serious cases the mortality rate of congenital CMV infection is approximately 30% (Malm *et al.*, 2007) Primary maternal infection carries greater risk to the foetus than secondary infection (Kenneson *et al.*, 2007) .There is a 30-40% risk of vertical transmission during maternal infection (Carlson *et al.*, 2010). Pembrey *et al* suggest there is a greater risk of congenital infection in children born to South Asian mothers; in this study of women in the Bradford area transmission was not usually via primary infection (Pembrey *et al.*, 2013). CMV infection can also be transmitted through breast milk. Infants with this infection can present with low birth weight, rash and seizures. Complications of congenital CMV include neurological impairment and ocular or auditory problems (Pass *et al.*, 2006). Hepatic dysfunction, bleeding, coagulation problems and secondary bacterial infections are causes of death in congenital CMV infections (Malm and Engman 2007). Congenital CMV infection is also a significant cause of hearing loss in children, possibly via changes that happen in the temporal bone structure due to the infection (Grosse *et al.*, 2008). Of children with symptoms following CMV infection, approximately 22-65% will develop hearing loss,

however, some may be initially asymptomatic but develop hearing problems later in life (Fowler *et al.*, 2006a). There is no difference between primary and secondary CMV infection and the risk of hearing loss in individuals with congenital CMV (Ross *et al.*, 2006). As a result of consequences of congenital CMV infection, screening programmes have been piloted aiming to reduce levels of this infection (Bale 2010, Koyano *et al.*, 2011).

1.5.4. CMV and Therapy

Anti-viral therapy (AVT) is sometimes used for the treatment of CMV infection post-transplant or to treat immunocompromised patients. In some circumstances, pre-emptive or prophylactic treatment can be used to prevent CMV infection from occurring at all. Immunotherapy, consisting of adoptive transfer of cells can also be used to prevent infection in at risk individuals.

Ganciclovir is the first line therapy used in transplant patients with CMV infection. This drug is a nucleoside and it inhibits CMV encoded DNA post-activation. This drug can also be used as part of pre-emptive or prophylactic therapy; prophylaxis can be given from the time of organ transplant or engraftment in order to prevent CMV disease (Griffiths *et al.*, 2007). Pre-emptive and prophylactic treatment with Ganciclovir has also caused a decrease in early CMV disease in haematopoietic stem cell transplant recipients, however late disease has increased due to this (Nichols *et al.*, 2000). Valganciclovir is a drug which is used either pre-emptively or to treat CMV infection in a transplant setting

(Len *et al.*, 2008). Foscarnet is sometimes used as a second line therapy (Torres-Madriz *et al.*, 2008). Despite these anti-viral therapies being generally effective, resistance can be a problem.

Administering CMV hyperimmunoglobulin can be used to treat maternal CMV. This is a form of passive immunisation against the virus. An advantage of this type of therapy is that it has lower costs than other therapies, such as AVT and this form of treatment is low in toxicity (Adler *et al.*, 2009). Hyper immunoglobulins can also be used to stop CMV disease after solid organ transplant. This treatment strategy has been found to lower CMV viral load, and is a good form of therapy for those who are resistant to AVT (Lutgens *et al.*, 2009). CMV-specific T cells have been used in order to reduce the need for drug therapy following allogeneic stem cell transplant (Blyth *et al.*, 2013).

1.5.5. CMV Vaccines

As congenital CMV can be potentially very serious, it has been argued that a vaccine would be worthwhile. Griffiths *et al* argued that a vaccine against CMV could be cost effective but for the vaccine to be successful in terms of preventing congenital CMV the uptake would have to be over 50-60% (Griffiths 2009). Another issue is that much testing would have to take place post-vaccination. Immunisation of young women prior to child-bearing age has been stated as being the most important time in which vaccination should take place to prevent congenital CMV (Schleiss 2007). A number of immunogenic CMV proteins have been proposed that could be used in a vaccine. These include UL55 (gB),

UL75 (gH), UL83(pp65) and UL123 (IE1) (Pass *et al.*, 2002). These proteins have roles in stimulating both humoral and cellular immunity to the virus; both of which are important so therefore makes them eligible to be used in a CMV vaccine (Pass and Burke 2002). Currently, CMV (gB) vaccines have been effective in clinical trials within the setting of congenital infection and post-transplant (Rieder *et al.*, 2014).

1.6.IMMUNOSENESCENCE

‘Immunosenescence’ refers to the decline in immune function with age, leading to increased infection rates in older adults. Changes occur to various cells including T cells, B cells and various innate cells and these can have an impact on the health of older individuals (Pawelec *et al.*, 2010b).

1.6.1. Stem Cells

A number of age-related changes occur within the haematopoietic stem cells (HSC) and progenitor cells which has an effect on the overall immune system of older individuals(Liu *et al.*, 2011). The number of HSC increases with age, however there is more dysfunction in the HSC of older adults (Geiger *et al.*, 2013). Changes that occur in the HSPCs include a decrease in homing efficiency, lymphoid production and adhesion to stromal cells as well as epigenetic changes and DNA damage (Waterstrat *et al.*, 2009). Changes in HSC causes issues with further cell types, for example impacting upon B cell lymphopoiesis (Geiger *et al.*, 2013).

1.6.2. Innate Cells

A number of changes occur in macrophages with age; including a reduction in toll-like receptor (TLR) function, reduced phagocytosis, reduction in super oxide and nitric oxide production and decrease in cytokine secretion (Plowden *et al.*, 2004). Similarly, some aspects of neutrophil function decline with age; there is a decrease in their free radical production as well as chemotaxis (Fulop *et al.*, 2004). In older adults, DCs display a decreased ability to phagocytose and migrate and display decreased ability to stimulate antigen specific cells (Agrawal *et al.*, 2011, Lang *et al.*, 2011a).

Numbers of NK cells increase with age, however subsets vary with an increase in NK cells which are CD56_{dim} and more mature, and a decrease CD56_{bright} cells (Camous *et al.*, 2012, Mcnerlan *et al.*, 1998). The increase in number of cytotoxic NK cells with age could be due to a 'compensatory mechanism' as there are changes in NK receptors (Almeida-Oliveira *et al.*, 2011) NK cytotoxicity also decreases on a per cell basis, with a decline in production of certain cytokines (Shaw *et al.*, 2010). This decline in NK cell function has been associated with an increase in infection in older adults (Hazeldine *et al.*, 2013).

1.6.3. T cells

As age increases the size of the thymus decreases, this is termed ‘thymic involution’ (Mcelhaney *et al.*, 2009). Thymic involution has a vital role in the changes that occur to the adaptive immune system with age and reversing thymic atrophy could have potential to reverse some aspects of immunosenescence (Aspinall *et al.*, 2010). TREC (T-cell receptor excision circles) are circles of DNA moved from the genome when $\alpha\beta$ T cells are produced. The TREC assay can be used to assess recent thymic emigrants and thymic output (Aspinall *et al.*, 2010).

Human studies and mouse models have shown that number of naïve T cells decline by up to 80% in older adults (Arnold *et al.*, 2011, Linton *et al.*, 2004). It has been argued that CD4⁺ naïve cells remain stable until later in life before declining in number; contrastingly CD8⁺ naïve T cells reduce in number earlier and the naïve pool is reduced significantly by the age of 65 (Goronzy *et al.*, 2007). Some older adults have been found to have almost no circulating naïve CD8⁺ T cells; and this may impact upon the life span of individuals (Fagnoni *et al.*, 2000). Lower numbers of naïve T cells has been associated with decreased thymic function (Ferrando-Martinez *et al.*, 2011). Other changes that occur in naïve T cells include loss of CD45RA in the lymph nodes (Lazuardi *et al.*, 2005). In addition, mouse and human studies have demonstrated that the TCR of CD8⁺ and CD4⁺ T cells become less diverse with age (Ahmed *et al.*, 2009, Naylor *et al.*, 2005, Yager *et al.*, 2008). This lack of diversity has been associated with increased risk of infection, cancer and various autoimmune diseases (Palmer 2013).

The age-related decline in CD4⁺ T cells can cause a decline in antibody production. Functional differences also occur, with naive CD4⁺ cells secreting less IL-2 and demonstrating decreased ability to proliferate, causing issues with Th1 to Th2 differentiation (Haynes *et al.*, 2009, Linton and Dorshkind 2004). In older adults, there is marked shift from Th1 to Th2 cytokine responses, involving production of cytokines such as TNF α , IL-6 and IL-1 (Mcelhaney and Effros 2009). Franceschi *et al* defined the phenomenon ‘inflamm-aging’; an increase in pro-inflammatory cytokines with age. ‘Inflamm-aging’ is hypothesised be caused by continuous antigenic stimulation and stress (Franceschi *et al.*, 2000). DNA damage causes some of the changes that occur in terms of T cells with age (Barnett *et al.*, 1998).

Memory T cells expand with age, this is due to antigenic stimulation over an extended period of time (Karrer *et al.*, 2003). Expansion of memory cells occurs in both CD4⁺ and CD8⁺ T cells, however is more marked in CD8⁺ T cells (Linton and Dorshkind 2004). Alongside this, older individuals often display increased frequencies of CD28 null T cells and an increase in cells expressing CD57 which are highly differentiated (Malaguarnera *et al.*, 2001, Weng *et al.*, 2009). Memory T cells have also display decreased telomere length with age (Weng *et al.*, 1997). The changes to memory cells will be discussed in more detail in the context of chronic antigenic stimulation by CMV.

1.6.4. B Cells

B cells work as antigen presenting cells, as well as having a role in antibody production. Generally, the number of circulating B cells (defined as CD19+) declines with age paralleled with a decline in the diversity of the B cell population as a whole (Dunn-Walters *et al.*, 2010) Responses to foreign antigens become less efficient and there is a decline in presence of natural antibodies (Weksler 2000). As well as contributing to ill health, these changes can cause protective immunity post-vaccination to become less efficient (Steger *et al.*, 1996). Presence of autoreactive antibodies can also increase in older adults (Lang *et al.*, 2011a). Furthermore, in older adults there are often dysfunctional T cell and B interactions (Lazuardi *et al.*, 2005).

1.7.T CELL EXHAUSTION

T cell exhaustion refers to the loss of function that occurs in T cells; including lack of ability to secrete cytokines and amount effector responses. T cell exhaustion differs to senescence, which instead refers to cell cycle arrest and the Hayflick limit (Schieteringer *et al.*, 2014). Secretion of IL-2 is generally the first effector function to decline during exhaustion (Yi *et al.*, 2010). During T cell exhaustion there is expression of inhibitory receptors such as PD-1, but may also involve lymphocyte-activation gene 3 (LAG-3), T cell immunoglobulin mucin-3 (TIM-3) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and other receptors(Wherry 2011). Chronic antigenic stimulation has been proposed as a contributing factor with LCMV, hepatitis C, hepatitis B and HIV infections being associated with exhaustion (Schieteringer and Greenberg 2014). Although generally being associated with these infections, there some evidence to suggest traits of T cell

exhaustion in primary infection of CMV, with CMV-specific CD4⁺ T cells demonstrating decreased proliferative ability and a decrease in IL-2 secretion (Antoine *et al.*, 2012). Exhaustion has also been examined in the context of renal transplantation; individuals with CMV viraemia displayed an increased proportion of PD-1 positive CMV-specific CD4⁺ T cells compared to patients without viraemia. The PD-1 specific T cells were also functionally impaired compared to cells lacking PD-1 expression (Sester *et al.*, 2008).

1.8. THE IMMUNE RESPONSE TO CMV INFECTION AND THE IMPACT UPON IMMUNOSENESCENCE

CMV elicits a very large T cell response; post infection there is expansion of CMV-specific CD8⁺ T cells. The immune response to CMV is largely directed against pp65 and IE-1 epitopes, however T cells specific for pp150, pp28, pp50 are also involved (Wills *et al.*, 2007). CMV-specific CD8⁺ T cells comprise a large part of the whole CD8⁺ T cell repertoire in CMV seropositive older adults (Khan *et al.*, 2002). These cells often have an effector memory phenotype (CD28⁻, CD57⁺, CCR7⁻). Older CMV seropositive individuals have also been found to have 33% more CD8⁺ T cell clonal expansions than those who are CMV seronegative, some of which are dysfunctional (Khan *et al.*, 2002). The persistence of CMV-specific responses is termed ‘memory inflation (Snyder *et al.*, 2011). This is because unlike with most other infections, some CMV epitope-specific T cells do not contract after expansion and instead accumulate (O’hara *et al.*, 2012). It has been argued that the increase of these in number could lead to a lack of available space for other T cells within the immune system (Ouyang *et al.*, 2004).

1.8.1. T cells responses

CMV infection influences the composition of the overall T cell pool; the number of naïve CD4⁺ and CD8⁺ T cells is reduced in CMV seropositive individuals (Chidrawar *et al.*, 2009, Weinberger *et al.*, 2007). This is particularly apparent in CMV seropositive older adults, as naïve T cells also decline with age and CMV exacerbates this (Almanzar *et al.*, 2005).

It is well established that the CMV-specific CD8⁺ T cell response is large in magnitude, the total CD8⁺ T cell count can increase in CMV seropositive individuals. The increase in CD8⁺ memory T cells in CMV positive individuals can be up to 60% (Chidrawar *et al.*, 2009). The increase in CD8⁺ T cells in CMV seropositive individuals can result in an inverted CD4/8 T cell ratio (Olsson *et al.*, 2000).

There are changes in CMV-specific CD4⁺ T cells with age, increasing from 2.2% of the total CD4 pool in people below the age of 50 to 4.7% in people aged 65 and over. These cells generally display a memory phenotype (Pourgheysari *et al.*, 2007). Wallace *et al.* suggest that this may be due to extended life span rather than accelerated proliferation (Wallace *et al.*, 2011).

The increased memory populations in CMV seropositive individuals can be characterised by a number of expression markers. CMV seropositive individuals display more T cells which are CD57⁺ CD28⁻ and CCR7⁻ (Vasto *et al.*, 2010). They are also found to have

more T cells expressing CD56 (Looney *et al.*, 1999). CD28 null cells are associated with replicative senescence (Effros *et al.*, 1994).

The frequency of EMRA T cells increase with age and with CMV seropositivity (Griffiths *et al.*, 2013). These are T cells which are thought to be terminally differentiated and lack CCR7 expression but re-express CD45RA (Ellefsen *et al.*, 2002). EMRA T cells will often be CD28 null and express high levels of CD57(Koch *et al.*, 2008).

1.8.2. The impact upon B cells and NK cells

CMV seropositivity also has an impact upon the B cell pool. In CMV seropositive younger individuals there is a 36% reduction in naive B cells and a 71% reduction in memory B cells, however this was found not to occur in older individuals (Chidrawar *et al.*, 2009). Evidence suggests that NK cells have an important role in controlling CMV. Studies from mouse models using NK cell depleted animals have shown that NK cells are important in reducing severity of CMV disease (Bukowski *et al.*, 1984). However, cells infected with the virus can be resistant to killing by NK cells (Wills *et al.*, 2007). Down regulation of NKG2D can occur decreasing immune surveillance (Guma *et al.*, 2006).

1.8.3. Immune System Function and Mortality Risk

There is evidence to suggest that CMV has a major influence on the immune system of older adults. The OCTO and NONA studies of ageing in Sweden described an ‘immune risk profile’ which was thought to predict mortality (Olsson *et al.*, 2000). It was observed that a decreased CD4/8 T cell ratio (characteristically associated with CMV infection) was related to an increase in 2 year mortality in this cohort (Wikby *et al.*, 2002). These studies also found an association between CMV antibody titre and changes in CD4⁺ and CD8⁺ T cells. This was found to be greater in individuals with a CD4:8 ratio of less than one (Wikby *et al.*, 2002). Furthermore, Moro-Garcia *et al* and Gkrania-Klotsas *et al* have established association between function of the immune system and functional ability (Gkrania-Klotsas *et al.*, 2013, Moro-Garcia *et al.*, 2012). This suggests the clinical importance of the association between CMV and immunosenescence. The humoral response to CMV in older adults has been studied. In individuals over the age of 85 who were in poorer health, the CMV antibody responses were found to be greater (Vescovini *et al.*, 2010).

1.8.4. CMV status in older adults- response to other antigens

Individuals infected with CMV may also have co-infection with Epstein- Barr virus (EBV) or other viruses. EBV is a common gamma herpes virus, it has been estimated that over 90% of older individuals are infected with this virus which is implicated in a number of conditions (Schmader *et al.*, 1989). Older adults with CMV infection have been found to have a stable EBV-specific immune response with increase in age. However, the

response to EBV increases with age in older individuals who are CMV seronegative (Khan *et al.*, 2004). This suggests that infection with CMV could impair the response to other antigens (Khan *et al.*, 2004). Libri *et al* demonstrated that CMV seropositive older donors have more CD4⁺ effector memory and EMRA cells specific for other antigens compared to CMV seronegative donors including EBV, HSV and VZV. It was suggested that CMV could ‘drive differentiation’ of other specific CD4⁺ T cells (Libri *et al.*, 2011). There is also evidence to suggest that CMV can influence the phenotype of T cell responses to tuberculin (Terrazzini *et al.*, 2013).

1.8.5. CMV and the Response to Vaccines

Vaccination stimulates both humoral and adaptive responses (Lang *et al.*, 2011a). Vaccination against influenza is particularly important for older adults who are more susceptible to complications from this infection and large-scale vaccination programs obviously have financial implications for health services. A T cell phenotype of CD8⁺ CD28⁻ has been put forward as a good marker for lack of response to vaccine in older adults; this is also associated with CMV infection (Goronzy *et al.*, 2001). As CMV accelerates the immune changes that occur with age, there have been a number of studies investigating the effect of CMV seropositivity on influenza vaccine responses in older people.

A study of 154 individuals receiving the influenza vaccination found that there were higher numbers of none-responders in the ‘elderly’ category than in the ‘young’ category

(Trzonkowski *et al.*, 2003). Importantly, in both age groups an association was made between lack of response to the vaccine, CMV seropositivity, increased levels of TNF α and IL-6 and decreased cortisol levels. Therefore, CMV could be increasing production of pro-inflammatory cytokines thus contributing to lack of response to the vaccine (Trzonkowski *et al.*, 2003). However, a study in the Netherlands of 731 older individuals in care facilities found that people who were CMV seropositive or seronegative both had similar responses to the vaccine (Elzen *et al.*, 2011).

1.9.EPSTEIN-BARR VIRUS BIOLOGY

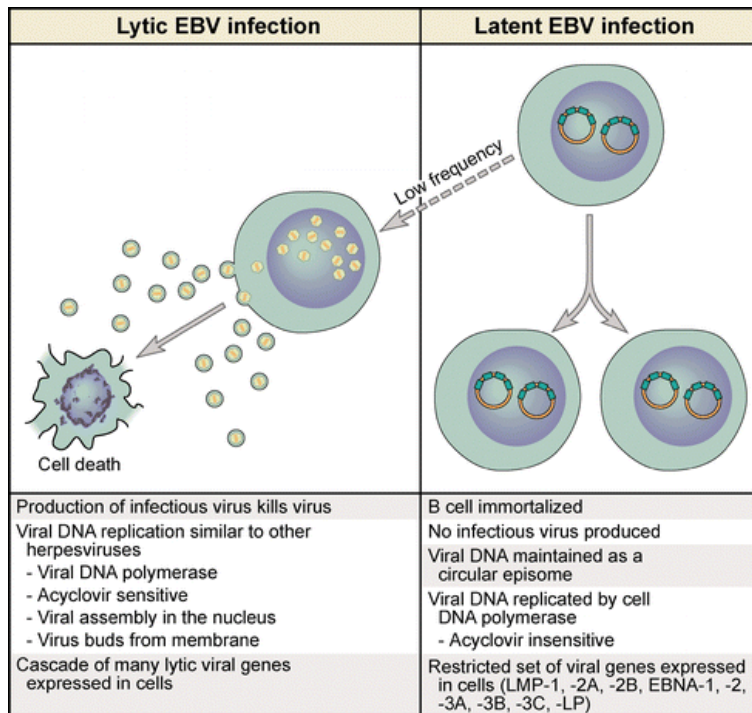
Epstein- Barr virus is a member of the herpesvirus family and is described as a γ -herpes virus. In terms of structure, the viral genome (~184-kb) is found within a nucleocapsid surrounded by a viral envelope (Cohen 2000, Kutok *et al.*, 2006). EBV primarily infects B cells, although other cell types such as epithelial cells, T cells and mesenchymal stem cells can be infected. EBV transmission occurs through the oral mucosa, with initial infection occurring in the tonsillar compartment (Odumade *et al.*, 2011). In order to infect B cells, EBV binds the major viral envelope glycoprotein gp350, to a receptor (CD21) which is found on the surface of B cells (Young *et al.*, 2004). HLA class II interacts with glycoprotein gp42 in order for internalisation to occur (Li *et al.*, 1997). The virus persists within the memory B cells. Epithelial cells do not possess CD21 or HLA II molecules, therefore infection occurs independently of these mechanisms (Shannon-Lowe *et al.*, 2009).

During lytic replication infectious viruses are produced, with approximately 80 viral proteins being expressed (Kutok and Wang 2006). For lytic cycle to be established the genome needs to become linearised. Within the lytic cycle, gene products are produced at one of three stages; immediate-early, early and late. These gene products have different functions depending on their stage; initial products are involved in replication, metabolism and stopping antigen processing. The later gene products have structural roles or help the virus to evade the immune system (Grinde 2013).

1.9.1. Latency

During viral latency the viral genome is circularized and there is no production of virions. In order to silence viral genes in latency, viruses rely on host mechanisms, however in the case of latent EBV infection a small proportion of its genes are expressed (Grinde 2013). The nuclear antigen EBNA-1 has an important role in maintaining viral genome in cells post-division (Chen 2011, Yates *et al.*, 1985) .

In addition, latent infection is associated with expression of other nuclear antigens including (EBNAs,2,3A,3B,3C and-LP) and three latent membrane proteins (LMPs 1,2A and 2B) (Young and Rickinson 2004). Latently infected cells can be reactivated, the cause of this is unknown; however experimental evidence suggests that it could be mediated by B-Cell receptor stimulation by other infections (Odumade *et al.*, 2011)



Kutok JL, Wang F. 2006.
 Annu. Rev. Pathol. Mech. Dis. 1:375-404

Figure 1-3 Comparison of major features of the lytic and latent cycles of Epstein-Barr virus (EBV) infection

During lytic infection there is viral DNA replication and assembly in the nucleus. In contrast no infectious virus is produced in latency, the B cell is immortalized and viral genes are restricted in expression.

Adapted from (Kutok and Wang 2006) Ann Rev. Pathol.

1.9.2. The immune response to EBV

EBV infection causes innate and adaptive immune responses. In terms of the innate response, there is evidence of TLR involvement and IFN secretion after infection (Odumade *et al.*, 2011). NK cells may also have a role in the control of proliferating B cells (Long *et al.*, 2011).

The magnitude of T cell responses differ at various stages of infection; during lytic cycle CD8⁺ T cell responses to lytic antigens are greater in magnitude than the CD4⁺ and CD8⁺ T cell responses to latent antigen (Amyes *et al.*, 2003, Odumade *et al.*, 2011). EBV-specific CD8⁺ T cells persist; there are differences in the CD8⁺ T cell phenotype of these responses with T cells against lytic cycle antigen being CD27⁺ and CD28⁺ and CD27⁺CD28⁻, whereas T cells specific for latent proteins tend to retain these molecules (Hislop *et al.*, 2002). A large proportion of latent CD8⁺ T cell responses are directed against EBNA 3 (Cohen 2000). The tonsils are the primary site of infection; long-term EBV-infected individuals presenting more reactivity to lytic and latent epitopes in the tonsils when compared with the peripheral blood (Hislop *et al.*, 2005). In terms of the humoral response to EBV, IgM and IgG levels can be used to detect stage of infection. Presence of VCA IgG and EBNA 1 IgG without VCA IgM confirms prior infection, whereas in primary infection VCA IgM and VCA IgG without EBNA 1 IgG will be found (De Paschale *et al.*, 2012).

1.9.3. Epidemiology

Infection rates of EBV are greater than 90% worldwide (Macswen *et al.*, 2003). There is evidence that individuals can acquire different strains of EBV throughout their lifetime (Walling *et al.*, 2003). A number of factors determine rates of infection; including gender, family income and ethnicity (Dowd *et al.*, 2013). Infection is usually asymptomatic or mild in children so often is undetected, infectious mononucleosis is more likely to occur in young adults who are therefore more likely to be tested for presence of the virus (Faulkner *et al.*, 2000). There is evidence suggesting a link between risk of EBV infection and HIV. Children who are HIV positive are more likely to shed the virus and more likely to acquire the virus if born to HIV-infected mothers (Jenson *et al.*, 1999).

1.9.4. Infectious mononucleosis

Infectious mononucleosis (IM) occurs in approximately 50% of primary EBV infections in adolescents and young adults (Faulkner *et al.*, 2000). During this primary infection there is viral replication within epithelial cells within the oropharynx, the virus is transmitted via infected saliva (Kutok and Wang 2006). The incubation period post-transmission can be between 30 and 50 days. Common symptoms of IM include lymphadenopathy, fever, pharyngitis, splenomegaly and hepatomegaly (Cohen 2000). Neurological complications occur within 1-5% of infected individuals (Luzuriaga *et al.*, 2010). A study of IM in university students found differences in symptoms between males and females, with females more likely to report prolonged fatigue (Macswen *et al.*, 2010).

In terms of the adaptive response, IM is characterised by large expansions of EBV-specific CD8⁺ T cells within peripheral blood; individual epitope responses can account for between 1-40% of the CD8⁺ T cell pool. This demonstrates that the T cell response in IM is dominated by few clones (Callan *et al.*, 1996, Hislop *et al.*, 2007). Some epitope responses remain post IM however others do not (Hislop *et al.*, 2002).

1.9.5. Burkitt's lymphoma

Burkitt's lymphoma is a B cell malignancy associated with EBV infection. There is high incidence of the disease in equatorial Africa; 5-10 cases occur per 1000,000 children (Young *et al.*, 2003). There is an association between this disease and malaria; BL is also more common in HIV-infected infants (Orem *et al.*, 2007). The transformation of B cells during infection has been suggested as a potential mechanism for the development Burkitt's lymphoma BL is associated with a chromosomal translocation of *Myc* to an immunoglobulin locus (God *et al.*, 2010).

1.9.6. Hodgkin's lymphoma

In Hodgkin's lymphoma there is an increase in Reed-Sternberg cells, of B-cell lineage (Thompson *et al.*, 2004). In approximately 40% of cases of this disease in the developed world there is an association with EBV infection (Jarrett *et al.*, 1996). Clonal expansion of EBV-infected cells may be involved in some cases of classical Hodgkin's lymphoma (Young and Murray 2003). There is evidence of increased incidence of HL post -IM and

high antibody titres to EBV viral capsid antigen in people with the disease, demonstrating further evidence to link the disease with EBV (Gutensohn *et al.*, 1980, Levine *et al.*, 1971).

1.9.7. Nasopharyngeal Cancer

Nasopharyngeal cancer (NPC), is a rare type of cancer which occurs in less than 1 in 100,000 people in the USA, however with increased incidence in South East Asia (Parkin *et al.*, 2005). Undifferentiated NPC has been associated with EBV infection. Expression of the latent EBV proteins LMP-1 and LMP-2 have been found in NPC tumours and lesions, these proteins may contribute to the behaviour of epithelial cells (Dawson *et al.*, 2012). As well as EBV infection, genetics, ethnicity and exposure to carcinogenic agents are associated with risk of NPC (Chu *et al.*, 2008).

1.9.8. PTLD

Post-transplant lymphoproliferative disease is characterised by increased proliferation of B cells after EBV infection/reactivation (Lacasse 2006). PTLD occurs more commonly due to primary infection post-transplant, which could be why there are more cases in children. Symptoms include night sweats, weight loss, malaise and swollen glands, in the stem-cell transplant setting patients may have a number of symptoms relating to the liver, kidneys, heart and lungs (Green *et al.*, 2013). PTLD has been highlighted as a relevant candidate for T-cell based therapy (Long *et al.*, 2010).

1.9.9. Diffuse Large B cell lymphoma

Diffuse large B cell lymphoma (DLBCL) is a type of non-Hodgkin lymphoma in adults and is seen more commonly in older individuals (Gurbuxani *et al.*, 2009). There are many subtypes of the disease including ‘diffuse large B cell lymphoma of the elderly.’ This has been associated with immunosenescence and EBV infection (Oyama *et al.*, 2003). Different types of DLBCL contain different gene expression patterns and vary morphologically so can be difficult to classify (Armitage 2007).

Chapter 2. METHODS

2.1. ISOLATION OF PBMC AND PLASMA FROM WHOLE

BLOOD

As PBMCs and plasma were needed for subsequent assays, these were isolated from whole blood. Heparinised blood was collected and processed in sterile conditions. Wash media was prepared containing RPMI-1640 media (Sigma-Aldrich), 100U/ml Penicillin (Gibco BRL) and 100µg/ml Streptomycin (GibCo BRL). Blood was diluted 1:1 with wash media before being layered onto lymphoprep™ (Nycomed) and then centrifuged at 800 x g for 30 minutes. PBMCs were aspirated with a pasteur pipette and 50ml of wash media added before centrifugation at 600 x g for ten minutes. Plasma was isolated by removing the top layer from the density gradient interface and frozen immediately. PBMCs were resuspended in 20 ml of RPMI media and counted using a haemocytometer, before centrifugation at 600 x g for 10 minutes. The supernatant was removed and the lymphocytes resuspended and either used for assays or cryopreserved. Cryopreservation involved resuspension of PBMCs in freezing media (10% DMSO –Sigma Aldrich, in RPMI). Cells were then frozen at -80°C in a Mr Frosty container (Nalgene) to allow constant cooling.

2.2. CMV IgG ELISA

An in-house CMV IgG ELISA assay was used in order to assess CMV serostatus, by presence or absence of CMV-specific antibodies. This assay also enabled semi-quantitative antibody titre to be measured which is why the method was selected.

Mock and viral-infected lysate was coated onto ELISA plates and incubated overnight. Plasma samples (1:600 dilution) and appropriate standards (a mixture of 3 CMV-positive plasma samples) were added to the plates for 1 hour. The plate was washed 3 times. An anti-human IgG-HRP (horseradish peroxidase) secondary antibody was then added to the plate for 1 hour. After washing, TMB (3, 3', 5, 5'-tetramethylbenzidine) substrate was added and the plate kept in the dark for 10 minutes before addition of 1 M HCl. The sample was assessed using an ELISA reader at 450 nm. To determine CMV titres, mock values were first subtracted from lysate values. The data were then analysed using GraphPad Prism Version 5.03 (GraphPad Software, San Diego, CA, USA), and CMV titres were calculated with reference to the standard curve. Values greater than 10 were considered to be seropositive. To ensure accuracy, all samples were tested in duplicate.

2.3. DNA EXTRACTION FROM PBMCs

DNA was required for qPCR experiments. DNA was extracted from isolated PBMCs using the GenElute™ Mammalian Genomic DNA Purification Kit according to manufacturer's instructions (Sigma Aldrich). Briefly, PBMCs were resuspended in 200µl of resuspension solution, then 20µl of Proteinase K solution added and vortexed to ensure mixing. 20µl of RNase A solution was added for two minutes at room temperature before addition of 200µl of lysis solution to the sample. In order to ensure a homogenous

mixture the sample was vortexed. 500µl of column preparation solution was added to GenElute Miniprep Binding Column and centrifuged at 12,000 x g for one minute; the flow through liquid was discarded. 200µl of 95% ethanol was added to the lysate and mixed by vortexing. The solution was then transferred into the binding column and centrifuged at 6500 x g for one minute. The flow-through liquid was discarded and the column placed into a new collecting tube. 500µl of was solution was added to the column and centrifuged for one minute at 6500 x g. After discarding of the flow-through and placing into a new collection tube the wash process was repeated, however centrifugation occurred for 3 minutes at 1500 x g. DNA was eluted by pipetting 200µl of Elution Solution into the binding column, incubating for five minutes and then centrifuging for one minute at 6,500 x g. Extracted DNA was then stored at -20°C.

2.4.FACS ANALYSIS

FACs analysis was performed in order to identify specific cell subsets of interest, both in the Lothian Birth Cohort and in the 1000 Elders cohorts. Antibody panels were designed and tested in order to identify these cell subsets whilst taking into consideration compensation issues on the flow cytometer. This method was selected as FACS analysis allows comparison of multiple markers of interest within one experiment, thus producing a comprehensive dataset. However, one limitation of the assay is that the analysis requires some element of subjectivity as to where specific cell subsets are gated.

PBMCs were thawed by transferral from a cryovial to a falcon tube containing 9ml of Phosphate buffered saline (PBS). The cells were then centrifuged at 600 x g for five minutes and the supernatant removed. 2ml of PBS was added to the cells which were then

counted using a haemocytometer. 10 ml of PBS was added to the cells, which were centrifuged at 600 x g for 5 minutes then the supernatant removed. PBS was added to make up the volume required (usually 200µl or 300µl). 100µl of the cells in PBS were then added into FACS tubes, including two tubes for each donor and a tube containing unstained cells as a control. Viability dye dilution was made up (1/100- 99µl PBS and 1µl of viability dye) and 2µl added to the donor cells and then incubated in the dark for 15 minutes. All tubes containing donor cells were then washed with PBS, centrifuged at 600 x g for five minutes and the supernatant removed. All tubes were washed with MACS buffer (PBS, 0.5% Bovine serum albumin (Sigma-Aldrich) and 2mM EDTA (Sigma-Aldrich), centrifuged at 600 x g for five minutes and the supernatant removed. The cells were then stained with antibodies (as referred to in chapter 2 and 3) and incubated for 30 minutes on ice. PBMCs were washed with MACS, centrifuged at 600 x g for five minutes and the supernatant removed and then refrigerated until analysis by the flow cytometer (LSR II, BD). Data was then analysed using FACS DIVA software (BD).

2.5.HLA TYPING

It was necessary to identify the HLA type of the 1000 Elders donors, as the tetramers used for CMV and EBV-specific T cell identification are specific for certain HLA types.

Buffers

PCR buffer, 670mM Tris base, 166mM ammonium sulphate, 1% tween-20

TDMH, 2.6 x PCR buffer (Bioline), 460 nM dNTPs, 6.25 mM MgCl₂

TBE buffer 800 ml dH₂O 108 g Tris Base, 55g Boric Acid, 9.3g EDTA

Gel for electrophoresis -of 1g agarose, 100mls of TBE.

In order to assess HLA type prior to tetramer staining, HLA typing was performed using 140ng of previously extracted DNA, this method has previously been described (Bunce *et al.*, 1995). A PCR master mix was prepared for this analysis including 35µl of nuclease free water, 70.87 µl of TDMH, 2µM of primers and 3.75 units of Taq polymerase (Bioline). A control tube was also prepared, with 8.5µl of master mix removed. DNA was added to the master mix and pipetted into PCR tubes with the primers for the HLA types A1, A2, B7 and B8. PCR was then carried out before the samples were run on a 1% agarose gel for 35 minutes at 140v.

2.6. TETRAMER ANALYSIS

Tetramer analysis enabled identification of CMV and EBV-specific T cell responses in the 1000 Elders cohort. MHC class I tetramers are peptide-MHC complexes which allow detection of antigen-specific T cell responses, thus allowing the magnitude of these responses to be assessed (Moss and Khan 2004). MHC Class I Tetramers were generated in-house at the University of Birmingham. PBMCs were thawed by transferral from the frozen sample tube to a tube containing 9ml of PBS. The cells were then centrifuged at 600 x g for 5 minutes and the supernatant removed. 2ml of PBS was added to the cells which were then counted using a haemocytometer. 10 ml of PBS was added to the cells, which were centrifuged at 600 x g for 5 minutes then the supernatant removed. PBS was added to make up the volume required (200 μ l). 100 μ l of the cells in PBS were then added into FACS tubes, including two tubes for each donor and a tube containing unstained cells as a control. Viability dye dilution was made up (1/100- 99 μ l PBS and 1 μ l of viability dye) and 2 μ l added to the donor cells and then incubated in the dark for 15 minutes. All tubes containing donor cells were then washed with PBS, centrifuged at 600 x g for five minutes and the supernatant removed. All tubes were washed with MACS buffer, centrifuged at 600 x g for five minutes and the supernatant removed. Tetramers were then added to the tubes (1 μ l) and then tubes left in the incubator at 37 °C for 15 minutes before being removed and washed with MACS buffer and centrifuged at 600 x g for five minutes. PBMCs were then stained with antibodies (chapter 3) and incubated for 30 minutes. Following this, cells were washed with MACS buffer, centrifuged at 600 x g for five minutes and the supernatant removed and then left in the fridge for a maximum of

two hours until analysis by the flow cytometer (LSR II, BD). Data was then analysed using FACS DIVA software (BD).

2.7. PEPTIDE STIMULATION AND INTRACELLULAR CYTOKINE STAINING USING PBMCs

In order to assess the function of T cell responses, a peptide stimulation assay was used. This enabled the frequency of CD8⁺ T cells which secrete IFN γ to be analysed after overnight stimulation with a mixture of EBV-lytic and EBV-latent peptides. Another method which could have been used would have been to stimulate cells with EBV lysate prior to assessment of IFN γ production (Amyes *et al.*, 2003).

PBMCs were thawed into warm GM media, consisting of RPMI-1640 media (Sigma Aldrich), 100 U/ml Penicillin (Gibco BRL), 100 μ g/ml Streptomycin, 2nM Glutamine (Gibco BRL) and 10% foetal calf serum (SPS biologicals). PBMCs were then centrifuged at 600 x g for 10 minutes. PBMCs were counted and 1 x 10⁶ added into FACS tubes in 100 μ l of media. One tube of cells was left as an unstimulated control and the other cells stimulated with CD8 lytic and latent peptides (1 μ g/ml) alongside a staphylococcal enterotoxin B (SEB) positive control. PBMCs were incubated at 37°C for one hour and 0.5 μ l of Brefeldin A (10 μ g/ml final concentration) added and incubated overnight. PBMCs were then washed with 4ml PBS and then centrifuged for five minutes at 600 x g. Viability dye was added to the cells (1 μ l, diluted 1/100) and left for fifteen minutes in the dark before a second wash with MACS buffer. Surface stain antibodies were added to the cells for analysis (CD8 Amcyan 5 μ l BD, CD3 PE 0.5 μ l E bioscience, CD4 PE-Cy7 0.5 μ l E bioscience) and to cells for compensation and left in the fridge for fifteen minutes

before washing with MACS buffer. 100µl of 4% PFA was added to each tube including compensation tubes and then vortexed and incubated for fifteen minutes at room temperature in the dark. MACS buffer was added and the cells centrifuged at 600 x g for five minutes and the supernatant discarded. 100µl of 0.5% saponin was added to each tube and mixed gently before incubating for five minutes in the dark at room temperature. Cytokine antibodies were then added to the cells (IFN γ FITC 2µl eBioscience) and cells incubated in the dark for 30 minutes at room temperature. Finally, cells were washed with MACS buffer and then run through the LSR II (BD)

2.8. EBV VIRAL CAPSID ANTIGEN (VCA) IgG ELISA

The EBV viral capsid antigen (VCA) IgG ELISA was used in order to assess EBV serostatus. Plasma samples were analysed an Epstein-Barr virus VCA IgG ELISA according to manufacturer's instructions (IBL international). 100µl of the standard, diluted sample and control were pipetted into the microtitre plate. The plate was incubated for 60 minutes at 37°C. Solution in the plate was discarded and the plate washed three times with 300µl of wash solution, using a multi -channel pipette. 100µl of TMB substrate solution was pipetted into each well using a multichannel pipette and incubated in the dark for 30 minutes. The reaction was stopped by addition of 100µl of stop solution. The data was measured at an absorbance wavelength of 450nm.

2.9. EBV IgG VCA IMMUNOFLUORESCENCE

The EBV IgG VCA immunofluorescence assay is widely used and provides a way in which to assess EBV serostatus and is described as the 'gold standard' (Gartner *et al.*, 2003). In the Lothian Birth Cohort this method was used to assess samples considered 'borderline' by the EBV VCA ELISA analysis. All of the samples in the 1000 Elders cohort (n=27) were assessed in this manner.

B95.8 cells and the B lymphoma BJAB cell line (negative control) as previously described were cultured in GM media (Lacy *et al.*, 1987, Skare *et al.*, 1982). Cells were then collected and centrifuged for five minutes at 600 x g before being resuspended in PBS. 10µl of this suspension was added onto slides with 12 chambers (Fisher Scientific). Slides were then air dried and fixed in cold acetone for ten minutes before storage at -20°C until use.

For staining, the cells were blocked with 30µl of heat-inactivated normal goat serum (HINGS). Plasma samples were diluted 1/20 and 1/40 and then placed in the relevant hole on each slide before incubation for 1 hour at 37 °C in a moist chamber. Slides were washed in PBS twice for ten minutes using a magnetic stirrer. 10µl (1/50 dilution) of FITC-conjugated anti human IgG (Invitrogen) was added to each hole and incubated for one hour at 37 °C. Slides were washed in PBS twice for ten minutes using a magnetic stirrer. DABCO (Sigma-Aldrich) was then added and the slides were examined under a UV microscope.

2.10. STATISTICAL METHODS

Data was initially tested for normality in order to ascertain which statistical tests to use. Two groups of normally distributed, un-paired data was analysed by T-tests, whereas nonparametric data was analysed using the Mann-Whitney U-test. When comparing three groups or more of nonparametric un-paired data the Kruskal-Wallis test was used, with Dunn's multiple comparison test to compare ranks between columns. GraphPad Prism presents approximate p. values for this i.e. $p > 0.05$, < 0.05 , < 0.01 , or < 0.001 and this is reported where appropriate. Paired nonparametric data was analysed Wilcoxon-signed rank test. Correlations were assessed by Pearson's correlation and the r^2 and p value stated.

In chapter 4, dot plots demonstrate the mean and the SEM. Box-and-whiskers plots show the median and the 5th and 95th percentiles. In chapter 5, figures demonstrate the mean and SEM.

Chapter 3. **THE IMPACT OF CMV INFECTION ON COGNITIVE ABILITIES IN THE LOTHIAN BIRTH COHORT (1936)**

3.1.INTRODUCTION

The term ‘cognition’ refers to various aspects of mental function including memory, thought processing and reasoning. With age there are gradual changes in cognitive function; some older individuals may display mild traits of cognitive decline whereas others may develop dementia (Deary *et al.*, 2007). Certain functions such as memory gradually decline with age (Deary *et al.*, 2007). The Lothian Birth Cohort (1936) is a group of older adults, who at aged 11 participated in the ‘Scottish Mental Survey,’ a test of childhood intelligence. The first Scottish Mental Survey took place in 1932, as arranged by the Scottish Council for Research and Education. In 1947 this survey took place again, the aim was to assess the intelligence of all children in Scotland born in 1936, who would be aged 11 at the time. This test was generally used for 11 year old children to assess the type of secondary school in which they would attend (Deary *et al.*, 2004). The total number of participants taking this test was 70,805(Deary *et al.*, 2012a).

Professor Deary and colleagues at the University of Edinburgh have followed up these individuals at various time points from the age of 70 in order to assess what variables contribute to changes in cognition with age. Participants have undertaken a number of tests of cognition and health checks. Importantly, there is a great deal of demographic

data available regarding both current and childhood background factors. The cohort has been extensively studied, with the impact of environment, lifestyle factors and genetics upon cognition being examined by our collaborators (Deary *et al.*, 2012a). However, the impact of chronic viral infection upon cognition had yet to be examined in this cohort; few studies currently exist examining the impact of viral infection on cognition in older adults. The Lothian Birth Cohort provides a unique opportunity to investigate the impact of CMV on normal cognitive ageing.

CMV establishes a life- long usually asymptomatic infection with periodic viral reactivation. There are a number of factors which may cause chronic viral infection to impact upon cognition. CMV has been implicated in increasing inflammatory responses; Gorelick *et al* argued that inflammation may be associated with cognitive impairment (Gorelick 2010, Strandberg *et al.*, 2003). The virus has also been described as neurotropic, and an increase in neuronal loss could be detrimental in terms of cognitive function (Strandberg *et al.*, 2003).

Current data assessing the association between CMV and cognition is limited. Much of the work focuses on individuals with pre-existing clinical conditions. Strandberg *et al* studied older adults with cardiovascular disease, it was observed that as viral burden increased, (HSV and CMV) there was a decrease in Mini Mental State Examination (MMSE) score. An association was also observed between CMV and HSV infection and dementia (Strandberg *et al.*, 2003). In schizophrenia patients CMV was associated with cognition, as measured by the Trail Making Test (TMT) (Shirts *et al.*, 2008). A recent study found an association between cognition and viral exposure (including CMV) in

schizophrenia patients and a control group of younger adults (Watson *et al.*, 2013). There is also evidence to suggest that the virus causes cognitive decline in Alzheimer's patients, it is speculated that the cognitive decline could be caused through the action of neopterin (Blasko *et al.*, 2007). There are very few studies investigating the association between CMV and cognition in healthy older adults. Aiello *et al* discovered that higher CMV IgG titres were associated with increased cognitive decline over a four year period (Aiello *et al.*, 2006). Word recall decline was also assessed and no associations between this and CMV were established. Mathei *et al* observed no association between CMV and MMSE scores in a group of adults aged eighty. It was suggested by the authors that the null results could be due to a survival affect (Mathei *et al.*, 2011).

As much the work in this area has focused on adults with pre-existing clinical conditions, or use limited ways in which to test cognition, the aim of the current work was to assess the association between CMV and cognitive function in a group of healthy older adults. The current work used a battery of tests to assess cognition; including general cognitive function, memory, processing speed and National Adult Reading Test. Due to the range of demographic, clinical and lifestyle data available, this allowed for a comprehensive analysis of the interaction between CMV and cognition, with consideration of relevant covariates.

3.2.METHODS

3.2.1. Recruitment to the Lothian Birth Cohort

Researchers at the University of Edinburgh were interested in studying the Lothian Cohort in order to assess factors which contribute to lifetime intelligence. In order for recruitment to take place, the Lothian Health Board identified potential participants through the Community Health Index (CHI), this list produced 3810 names. Most potential participants were currently living in the Lothian area, mainly within Edinburgh (Deary *et al.*, 2007). 3686 of these individuals were mailed, with 1703 responses. 1351 of were initially interested. From this group, participants were excluded due to medical reasons, lack of a Moray House Test score or withdrawal from the study. Of the 1226 then eligible, 85 withdrew and 50 were not tested. In total 1091 participants were tested in the Wellcome Trust Clinical Research Facility, Western General Hospital, Edinburgh (Deary *et al.*, 2007).

3.2.2. Medical Examination and Questionnaire

When participants were assessed at the Wellcome Trust Clinical Facility in Edinburgh, they underwent a physical examination and were questioned regarding their medical history. An in depth questionnaire was also completed, which provided useful information regarding their background including education and occupation, their personality plus daily activities and support networks (Gow *et al.*, 2011). This data was

later useful when assessing the relationship between cognitive function and Cytomegalovirus infection.

3.2.3. Measuring Cognitive Ability

At the age of 70, participants took a number of tests to assess cognitive function. These tests were set up and run by collaborators at the University of Edinburgh, prior to the start of this work. These included assessment of general cognitive ability, processing speed and memory. Participants also completed the Moray House test, the same verbal reasoning test taken at the age of 11. These results are described as age 11 and age 70 IQ. Other tests included the National Adult Reading Test (NART), in which participants were asked to pronounce irregular words and the Weschler Test of Adult Reading. Both of these were used to assess prior cognitive ability (Deary *et al.*, 2007). Participants were also given an MMSE score, often used clinically as a test for dementia. These measurements of cognitive function have been described in detail by Deary *et al* (Deary *et al.*, 2007). In terms of numbers of participants data was available for, this varied according to the type of test. This ranged from a maximum of 1060 regarding MMSE testing to 1001 participants having test scores available for age 11 IQ.

3.2.4. CMV IgG ELISA

CMV serostatus was assessed using a CMV IgG ELISA as described in chapter 2. Participants with missing plasma samples were excluded by list-wise deletion. 1061 of 1091 had plasma samples available. For this assay, plasma samples were used which were collected at the time in which participants were assessed at the Wellcome Trust Facility in Edinburgh. The assay also gave a semi-quantitative value for IgG titre.

3.2.4.1. Assessing the association between CMV and cognition

All statistical analysis was performed using IBM SPSS Statistics for Windows, Version 21.0. Associations between CMV infection and cognitive outcomes were firstly assessed by T-test. Correlation analysis (Pearson's) between CMV IgG titre and cognitive outcomes were also performed in CMV seropositive individuals only. Logistic regression was also used in order to investigate which categorical background factors significantly predicted CMV infection.

3.2.4.2. Assessing Covariates

Demographic information was available for the donors, as collected by researchers at the University of Edinburgh. This includes data for years of education. Levels of household childhood overcrowding were assessed by dividing the number of rooms in the house by the number of people living in the house. Age 11 toilet refers to whether the individual had access to an indoor toilet facility. Father's Social Class was assessed using a scale I-V (I being professional and V being unskilled manual work). Adult social class was

similarly assessed however in addition, class III was split into manual and non-manual. Information regarding health status at age 70 was also available including presence of hypertension, diabetes, cardiovascular disease or history of stroke. Any associations between CMV serostatus or CMV IgG titre and these variables were considered by either independent T-test for continuous variables or Chi Squared for dichotomous variables. This initial step was of importance in order to assess any factors associated with CMV that would need to be used in any further statistical models. These datasets, particularly regarding the health data were mostly complete. However there was data missing from some participants regarding overcrowding, presence or absence of an indoor toilet and adult social class.

Table 3-1 Covariates analysed in the LBC (1936) cognition studies

Gender

Age

Hypertension

Diabetes

Cardiovascular disease

redefined as '**adult health**'

History of stroke

C-Reactive Protein

Presence of indoor toilet

Father's social class

Years in education

Adult social class

overcrowding

* Variables in bold were found to be appropriate to use in the final general linear model.

3.2.4.3. **General Linear Models**

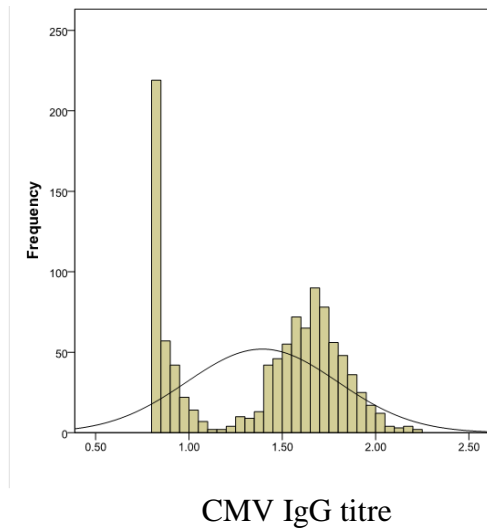
General linear models were then run to assess the effect of CMV infection or CMV IgG titre on cognitive function when a number of covariates were considered. Partial η^2 (h_p^2) shows the effect of CMV serostatus or CMV IgG titre (CMV positive only) on cognitive function. The baseline model included age and gender. Different covariates were added into the model, a reduction in the partial η^2 demonstrate how these factors attenuate the relationship between CMV infection or CMV IgG titre and cognitive ability.

3.3. RESULTS

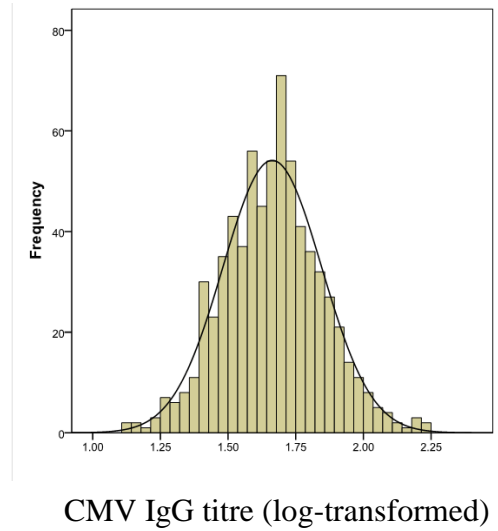
3.3.1. **CMV serostatus and IgG titres of 70 year old participants from the Lothian Birth Cohort**

CMV serostatus of 1061 donors were assessed by CMV IgG ELISA to detect presence of CMV IgG. Donors could then be classified as CMV seronegative or seropositive. Individuals with a titre above ten were considered seropositive and below this value were considered seronegative. Of the 1061 donors analysed, 65.5% (695) were CMV positive and 34.5% (366) seronegative. CMV titres were log-transformed in order to normalise the data for the subsequent correlation and general linear model analyses (figure 3.1 A-B). The distribution of titres is demonstrated in figure 3.1C.

A



B



C

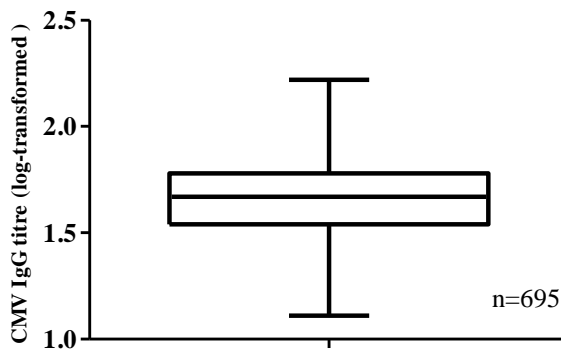


Figure 3-1 Distribution of CMV IgG titres in the Lothian Birth Cohort

(a) The distribution of CMV IgG titre in all 1061 participants of the cohort (b) The distribution of CMV IgG titre (CMV seropositive participants only,) log-transformed (x2) (c) Box and whiskers plot demonstrates the range of CMV IgG titres (median and bars showing min-max) in CMV seropositive individuals.

3.3.2. Background factors and CMV seropositivity

Data regarding demographic factors were available for the Lothian Birth Cohort donors. To determine if CMV infection was associated with different demographic factors, the association between CMV and these factors were assessed. Factors of interest included gender, overcrowding, presence or absence of an indoor toilet, father's social class, years in education or adult social class. This data is represented in table 3.2. Of the 1061 donors, 50.4% were male and 49.6% female. Within the CMV seropositive group a larger proportion were female. CMV seropositivity was significantly associated with a number of background factors such as childhood overcrowding, lack of access to indoor toilet facilities, lower father's social class, fewer years of education and lower adult social class. Logistic regression models displayed that background factors significantly predicted CMV seropositivity. Odds ratio for gender = 0.64 (95% C.I. = 0.50 to 0.83), overcrowding = 1.94 (1.58 to 2.38), indoor/outdoor toilet = 0.56 (0.36 to 0.86), father's social class = 1.51 (1.30 to 1.75) and education = 0.78 (0.70 to 0.87).

Table 3-2 Demographic details from Participants of the Lothian Birth Cohort

	Mean (SD) or frequencies (number/percentages)	CMV negative Participants Mean (SD)	CMV positive Participants Mean (SD)	Group differences (t- value/ χ^2)	Correlation CMV positive participants (Pearson's r)
<i>Gender (% male)</i>	50.40%	57.70%	46.60%	11.67 ***	0.17***
<i>Overcrowding (n= 1058)</i>	1.4 (0.79)	1.17 (0.64)	1.5 (0.84)	-7.13***	-0.08*
<i>Presence of indoor toilet (n=1059)</i>	88.50%	92.10%	86.60%	6.98 **	0
<i>Father's social class (I-V)</i>	I 7.1% II 20.1% III 55.7% IV 9.2% V 7.9%	2.68 (0.93) II/III	3.03 (0.93) III	-5.48 ***	0.08
<i>Years in Education</i>	10.7 (1.13)	11.0 (1.21)	10.6 (1.08)	4.36 ***	-0.04
<i>Adult social class (I-V) (n=1041)</i>	I 17.8% II 37.7% IIIN 22.8% IIIM 17.7% IV 3.6% V 0.6%	2.3 (0.91) II/IIIN	2.5 (0.91) II/IIIN	-2.88**	0.07

n=1061 unless otherwise stated. Figures are mean (SD) or percentages of categorical values. For continuous variables, t-tests were performed; for dichotomous variables, Chi-squared were performed. Overcrowding refers to the number of people in the house per room. Father's social class is measured I-V. (I ,professional- V, unskilled). Adult social class is measured I-V (I, professional- V, unskilled). Class III is split into two groups (N= non manual and M= manual). *p<0.05 **p<0.01 ***p<0.001

3.3.3. CMV infection and disease history

In order to identify potential covariates, the relationship CMV serostatus/ IgG titre and disease history were assessed by T-test or Chi-square (Table 3.3). Hypertension was the most commonly reported disease within the cohort (39.6% of donors reported this), followed by cardiovascular disease (24.8%), diabetes (8.4%) and history of stroke (5%). None of these diseases were significantly associated with CMV serostatus. Similarly CRP levels were also assessed and no association was found between CRP levels and CMV serostatus. Pearson's correlation was used to assess any association between CMV IgG titre (in CMV seropositive individuals only) and these measures. There was a significant association between CRP and CMV IgG titre.

3.3.4. CMV infection and cognition

Associations between CMV serostatus or IgG titre and cognition were assessed (Table 3.4). T-test analysis demonstrated that CMV seropositivity was associated with lower scores in all of the cognitive outcomes measured, including lower age 11 and 70 IQ, general cognitive ability, processing speed, memory, national adult reading test results (NART) and Weschler test of adult reading scores. Separate analysis demonstrated that age 11 IQ was very predictive of CMV serostatus, odds ratio= 0.99 (0.98 to 1.00).

Correlation data relating to CMV IgG titre is displayed in Table 3.4. For this analysis, only CMV positive IgG titres were used. There was a significant association between

higher CMV IgG titres and lower cognitive function scores across all of the cognitive measures, with the exception of age-70 IQ (R^2 ranging from 0.01-0.31).

Table 3-3 Health data from the Lothian Birth Cohort Participants

	Mean (SD) or frequencies	CMV negative Participants	CMV positive Participants	Group differences	Correlation
	(number/percentages)	Mean (SD)	Mean (SD)	(t- value/ χ^2)	<i>CMV positive participants</i> (Pearson's r)
<i>Hypertension</i>	39.6%	37.70%	40.60%	0.83	0.03
<i>Diabetes</i>	8.4%	6.80%	9.20%	1.76	0.04
<i>Cardiovascular disease history</i>	24.8%	24.00%	25.20%	0.17	-0.06
<i>History of stroke</i>	5%	5.20%	4.90%	0.05	-0.03
<i>CRP (mg/ml)</i>	5.3 (6.69)	5.2 (6.16)	5.3 (6.96)	-0.09	0.08*

*n=1061. Figures are percentages for categorical values and mean (SD) for continuous variables. In order to assess the influence of CMV upon variables chi-squared were performed for self-reported health data and t-tests were performed for the C-Reactive Protein (CRP) data. *p<0.05*

Table 3-4. Cognitive outcomes in the Lothian Birth Cohort

	Mean (SD)	CMV negative Participants	CMV positive Participants	Group differences	Correlation <i>CMV positive participants</i>
		Mean (SD)	Mean (SD)	(t- value)	(Pearson's r)
<i>MMSE (n=1060)</i>	28.8 (1.43)	29.0 (1.26)	28.7 (1.50)	2.58**	-0.08*
<i>Age 11 IQ (n=1001)</i>	100.1 (14.92)	101.8 (15.31)	99.2 (14.64)	2.65**	-0.09*
<i>Age 70 IQ (n=1050)</i>	100.2 (14.51)	102.4 (13.13)	99.1 (15.09)	3.65***	-0.07
<i>General Cognitive Ability (n=1043)</i>		0.18 (1.00)	-0.09 (0.97)	4.23***	-0.18***
<i>Processing Speed (n=1012)</i>		0.09 (1.02)	-0.05 (0.98)	2.23*	-0.13***
<i>Memory (n=1018)</i>		0.10 (1.01)	-0.05 (0.98)	2.43*	-0.10**
<i>National Adult Reading Test (n=1059)</i>	34.5 (8.16)	36.0 (7.89)	33.7 (8.20)	4.37***	-0.11**
<i>Weschler Test of Adult Reading (n=1059)</i>	41.03 (7.18)	42.4 (6.80)	40.3 (7.29)	4.46 ***	-0.01*

n=1061 unless otherwise stated. MMSE- Mini Mental State Examination. The influence of CMV upon cognitive outcomes was assessed by t-test. CMV IgG titres were correlated with cognitive outcome variables in CMV positive donors only. ***=p<0.001 **=p<0.01 *=p<0.05

3.3.5. General Linear Models assessing the impact of CMV serostatus and IgG on cognition

Initial analysis demonstrated that there was an association between CMV serostatus and CMV IgG titre and cognitive outcome; however it was important to consider potential confounders or mediators of this effect. A general linear model was developed in order to take these factors into account and understand how these variables attenuate the effect of CMV. Table 3.5 shows the general linear model, where CMV is the independent variable. The outcomes include age 70-IQ, general cognitive ability, memory, processing speed and NART. Weschler's Test of Adult Reading was removed from this analysis as it a very similar measure to NART. Relevant covariates, as described previously were added into the model. In tables 3.5-3.6 partial eta² represents the effect size that CMV has on the cognitive outcome. A change to this number when a variable is added demonstrates that variable is attenuating the effect of CMV.

The effect of CMV serostatus on cognitive outcomes is shown in table 3.5. When only age and gender were taken into account, CMV seropositivity was significantly associated with lower age 70-IQ, memory, general cognitive ability and NART scores. However, as different covariates were added into the model the effect size was attenuated. For example, when overcrowding was added, the effect of CMV on age 70 IQ and memory was attenuated to none significance. Age 11 IQ attenuated the effect of CMV to the point of none significance on all of outcomes with the exception of NART. However, when all

of the variables were added into a multivariate model there was no significant association between CMV infection and NART scores.

The relationship between CMV IgG titre and cognitive outcome were also assessed (table 3.6). For this analysis only CMV seropositive IgG titres were taken into consideration. When age and gender were added into the model, there were significant associations between a higher CMV IgG titre and general cognitive ability, processing speed and NART, accounting for 0.9-1.7% of the variance. Higher CMV IgG titre was significantly associated with general cognitive ability, even after addition of covariates into the model. Age 11 IQ reduced the partial η^2 to 0.009. When all factors were added into a multivariate model the partial η^2 was 0.008.

Table 3-5 General Linear Model of the association between CMV serostatus and Cognitive Outcomes in the Lothian Birth Cohort

MODEL COVARIATES	Age-70 IQ		General cognitive ability		Processing speed		Memory		National Adult Reading Test	
	<i>p</i>	h_p^2	<i>p</i>	h_p^2	<i>p</i>	h_p^2	<i>p</i>	h_p^2	<i>p</i>	h_p^2
<i>Age + gender</i>	0.001	0.011	<.001	0.012	0.062	0.003	0.034	0.004	<.001	0.017
<i>Age, gender + age-11 overcrowding</i>	0.097	0.003	0.037	0.004	0.546	0.00	0.287	0.001	0.007	0.007
<i>Age, gender + age-11 toilet</i>	0.005	0.008	0.001	0.011	0.104	0.003	0.051	0.004	<.001	0.016
<i>Age, gender + father's social class</i>	0.041	0.004	0.013	0.007	0.433	0.001	0.382	0.001	0.004	0.009
<i>Age, gender + age-11 IQ</i>	0.257	0.001	0.067	0.003	0.793	0.00	0.584	0.00	0.005	0.008
<i>Age, gender + years of education</i>	0.075	0.003	0.029	0.005	0.433	0.001	0.445	0.001	0.018	0.005
<i>Age, gender + adult social class</i>	0.034	0.004	0.014	0.006	0.375	0.001	0.229	0.001	0.002	0.009
<i>Age, gender + adult health</i>	0.001	0.01	0.001	0.011	0.099	0.003	0.045	0.004	<.001	0.016
<i>Age, gender + C-Reactive Protein</i>	0.001	0.011	<.001	0.013	0.042	0.004	0.046	0.004	<.001	0.018
<i>All variables</i>	0.631	0.000	0.091	0.002	0.725	0.000	0.838	0.000	0.078	0.004

P and h_p^2 show the effect of CMV in the model. Overcrowding refers to the number of people in the house per room. Age-11 toilet refers to presence of indoor toilet. Father's social class is measured I-V. (I, professional- unskilled). Adult social class is measured I-V (1, professional- V, unskilled). Class III is split into two groups (N= non manual and M= manual). Adult health is the presence or absence of each of hypertension, diabetes, or CVD. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

Table 3-6 General Linear Model of the Association between CMV IgG titre and Cognitive Outcomes in the Lothian Birth Cohort

MODEL COVARIATES	Age-70 IQ		General cognitive ability		Processing speed		Memory		National Adult Reading Test	
	<i>p</i>	h_p^2	<i>p</i>	h_p^2	<i>p</i>	h_p^2	<i>p</i>	h_p^2	<i>p</i>	h_p^2
<i>Age + gender</i>	0.107	0.004	0.001	0.017	0.01	0.01	0.095	0.004	0.01	0.009
<i>Age, gender + age-11 overcrowding</i>	0.034	0.007	<.001	0.022	0.003	0.014	0.074	0.005	0.000	0.013
<i>Age, gender + age-11 toilet</i>	0.117	0.004	0.001	0.017	0.012	0.01	0.104	0.004	0.02	0.008
<i>Age, gender + father's social class</i>	0.207	0.003	0.004	0.014	0.026	0.009	0.149	0.004	0.03	0.008
<i>Age, gender + age-11 IQ</i>	0.467	0.001	0.017	0.009	0.147	0.003	0.99	0.000	0.602	0.000
<i>Age, gender + education</i>	0.186	0.003	0.001	0.018	0.01	0.01	0.134	0.003	0.01	0.009
<i>Age, gender + adult social class</i>	0.45	0.001	0.01	0.01	0.038	0.007	0.36	0.001	0.08	0.005
<i>Age, gender + adult health</i>	0.104	0.004	<.001	0.018	0.009	0.011	0.094	0.004	0.01	0.009
<i>Age, gender + C-reactive protein</i>	0.146	0.003	0.001	0.017	0.016	0.009	0.101	0.004	0.02	0.009
<i>All variables</i>	0.530	0.001	0.040	0.008	0.122	0.005	0.844	0.000	0.442	0.001

P and h_p^2 show the effect of CMV in the model. Overcrowding refers to the number of people in the house per room. Age-11 toilet refers to presence of indoor toilet. Father's social class is measured I-V. (I, professional- V, unskilled). Adult social class is measured I-V (1, professional- V, unskilled). Class III is split into two groups (N= non manual and M= manual). Adult health is the presence or absence of each of hypertension, diabetes, or CVD. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

3.4.DISCUSSION

The current work demonstrates a significant association between CMV IgG titre and cognitive outcomes in this cohort. However, childhood factors such as overcrowding and father's occupation largely attenuate this effect to some extent (table 3.5-3.6). There is a very significant association between CMV infection and background factors, such as overcrowding or father's social class. Separate logistic regression analyses were also performed in order to see which factors significantly predicted CMV serostatus. Gender, overcrowding, presence of an indoor/outdoor toilet, father's social class and education all predicted CMV serostatus. This is consistent with previous studies, as it has been demonstrated that CMV infection is associated with similar background factors such as education and overcrowding (Bate *et al.*, 2010, Dowd *et al.*, 2009b). Overcrowding has an impact upon risk of infection as greater number of people sharing living space contributes to spread of infection (Staras *et al.*, 2006). Other studies published on participants of the Lothian Birth Cohort have shown that childhood/background factors, largely relating to socioeconomic status are also predictive of cognitive ability in adulthood (Johnson *et al.*, 2011).

At baseline, CMV seropositivity was significantly associated with age 70 IQ, general cognitive ability, memory and NART. Both overcrowding and age 11 IQ largely attenuated this affect in most of the outcomes, which is consistent with the logistic regression analysis that was performed. CMV seropositivity was significantly associated with lower NART scores, despite addition of covariates. Of all of the covariates,

education attenuated the effect the most. The second greatest attenuator of the effect was overcrowding, followed by age 11 IQ. This therefore established these factors, in particular education as potential confounding factors or mediators of the effect of CMV. When the variables were added into a full multivariate model, the effect of CMV upon NART was attenuated so that it was no longer significant.

An association between CMV IgG titre and cognition was established in this study. At baseline, higher IgG titres were associated with lower general cognitive ability, processing speed and NART, varying from the CMV serostatus analysis. Age 11 IQ attenuated the effect of CMV to that of none significance for processing speed, NART and age 70 IQ. This again demonstrates age 11 IQ to be an important factor relating to both risk of CMV infection and higher IgG titres. The current work establishes an association between higher CMV IgG titres and poorer general cognitive ability (Table 3.5). CMV accounted for 1.7% of the variance at baseline, which was attenuated to 0.9% with the addition of age 11 IQ. When all of the variables were added into a multivariate model, the effect of CMV IgG titre upon general cognitive ability was still significant accounting for 0.8% of variance. The association between CMV IgG titre and cognition is important as it suggests that it is the higher IgG titres that are having a detrimental effect on cognitive function, rather than serostatus alone. Aiello *et al* established an association between high CMV antibody titres and cognitive decline over a four year period as measured by the Modified Mini-Mental State Examination (3MSE), however word recall decline was not associated with the virus (Aiello *et al.*, 2006). The study focused on a large group of community-dwelling Mexican-American older adults. It has been suggested that higher CMV IgG titres can be detrimental in terms of other health outcomes. Roberts *et al* demonstrated an association between higher titres and all- cause

mortality, in a study of older adults over a nine year period. This affect was found to be mediated by pro-inflammatory cytokines (Roberts *et al.*, 2010). Wang *et al* demonstrated that individuals within the highest quartile of CMV antibody titre were at increased risk of frailty and being in the highest quartile of titre increased mortality risk by five years (Wang *et al.*, 2010). Higher CMV IgG titres have also been linked to increased mortality risk in older individuals with cardiovascular disease, independently of inflammation (Strandberg *et al.*, 2009). These studies suggest that higher antibody titres could be detrimental in terms of health and could contribute to mortality risk in older adults.

There are a number of factors which could cause higher CMV-specific IgG titres, such as chronic viral reactivation, or simply having acquired the virus at a younger age (Franceschi *et al.*, 2007). As it is unknown when the donors acquired CMV, it cannot be determined whether CMV is causal. As CMV infection risk is often associated with childhood background factors, it should be considered that a number of these individuals may have acquired the infection in childhood (Cannon *et al.*, 2010). However, in order to determine the time point an individual became infected with the virus, it would be necessary to continue antibody testing from a young age, throughout the lifetime.

The presence of higher titres could be due to failing functional ability, such as is seen with frailty. For example, individuals with high CMV antibody responses have been shown to have lower functional ability (as measured by Katz Index of Activities of Daily Living), poorer general health and decreased cognitive abilities (Vescovini *et al.*, 2010) . They are also described to be at further risk from CMV reactivation. As well as testing at

just one time-point another limitation to the study is that there may be other factors which may be able to cause increased CMV IgG titre which haven't been accounted for. For example, a link has been established between an increase in CMV IgG titres and psychological stress (Rector *et al.*, 2014).

Another factor to be considered is that due to the nature of recruitment, there may be some element of bias due to the fact that only a proportion of individuals who took the Moray House Test at age 11 were recruited. For example, individuals who were recruited were potentially healthier than those who did not respond, as they were well enough to be attend the Wellcome Trust Facility for testing. It must also be stressed that the study focusses on just the Lothian area of Scotland, and there may be variation within other areas of the UK. The analysis also involved testing of multiple variables. One way in which to correct for this would be the Bonferroni correction, in which the p. value is divided by the number of comparisons taking place (Bland *et al.*, 1995).

If CMV is causal there are a number of mechanisms that should be considered. CMV has been associated with atherosclerosis which could have an effect on cognition (Leng 2011). Blasko *et al* demonstrated that cognitive decline in patients with Alzheimer's disease was associated with an increase in levels of neopterin possibly due to CMV infection (Blasko *et al.*, 2007). This suggests a role for CMV infection causing cognitive decline through inflammatory processes; however it was not assessed in the study how this could contribute to disease progression over time. The virus has also been associated

with loss of neurons which could explain any impact on cognition (Strandberg *et al.*, 2003). Furthermore it has been found that CMV DNA is present in the brain tissue of individuals with vascular dementia, however causality was not determined in this particular study (Lin *et al.*, 2002).

Although other studies have demonstrated that there could be an association between CMV serostatus and cognitive function, these are limited. A recent study found an association between CMV, an inverted CD4/8 T cell ratio and cognition in a cohort of 360 young adults (Luz Correa *et al.*, 2014). The BELFRAIL cohort found no association between presence of the virus and cognition which could be due to the older age of the participants (aged eighty), which means that survival a affect should be considered (Mathei *et al.*, 2011) Many of the studies focus CMV infection only in participants with pre-existing conditions, such as Alzheimer's, schizophrenia or cardiovascular disease (Blasko *et al.*, 2007, Shirts *et al.*, 2008, Strandberg *et al.*, 2003).

A strength of the current work is that a variety of tests were used in order to assess cognition along with a range of covariates such as background and environmental factors. The work suggests that higher CMV antibody titres could be cognitively detrimental, accounting for almost 1% of the variance that occurs within general cognitive ability. The most significant factor that predicts cognition in old age is cognition in childhood, accounting for approximately 50% of the variance (Deary *et al.*, 2007) Genetic factors contribute to 24% of variance where as white matter abnormalities account for approximately 14% (Deary *et al.*, 2003, Deary *et al.*, 2012b) Although the effect of CMV

is small compared to these factors, CMV accounts for more variance than factors such as smoking (<1% in a study from ages 11 to 65) or levels of physical activity (Gow *et al.*, 2012, Whalley *et al.*, 2005). Future work to assess when individuals acquire CMV in order to establish causation and assessment of other contributing factors such as stress would be necessary before considering potential intervention, such as vaccination.

Chapter 4. **THE IMPACT OF CMV AND EBV INFECTION ON IMMUNOSENESCENCE IN THE LOTHIAN BIRTH COHORT**

4.1.INTRODUCTION

The aim of the current work is to assess the impact that CMV or EBV serostatus has upon the global lymphocyte repertoire in participants of the Lothian Birth Cohort. PBMC samples and plasma were available from participants taken at age seventy and were sent to the University of Birmingham by our collaborators at the University of Edinburgh. From the plasma samples CMV and EBV serostatus could be assessed; PBMCs were also analysed by flow cytometry in order to determine the immune repertoire of the donors. The current work aimed to investigate the impact that infection with CMV and EBV has on immunosenescence within these participants. Co-infection with CMV and EBV is an important aspect of this work.

‘Immunosenescence’ refers to the changes that occur to the immune system with age, this can include changes in both adaptive and innate immunity. Thymic involution is one cause of immunosenescence, thus decreasing the numbers of naive T cells that are available with age (Gruver *et al.*, 2007). This coupled with an increase in memory T cells, particularly those that are terminally differentiated causes the immune system of older

adults to be very different from that of younger individuals.(Arnold *et al.*, 2011). CMV infection has been found to significantly exacerbate immunosenescence in older adults (Pawelec *et al.*, 2010b). The OCTO and NONA studies of ageing in Sweden were instrumental in defining the role of CMV upon immunosenescence (Wikby *et al.*, 2002). Data from these studies found that an inverted CD4/8 T cell ratio and increased frequencies of highly differentiated T cells, commonly associated with CMV, were associated with increased mortality risk in older adults (Wikby *et al.*, 2002). Similarly an increased CMV antibody titre was associated with increased mortality risk. Data from a recent CMV meeting also suggested that an inverted CD4/8 T cell ratio was associated with risk of death in a cohort in Spain, (Ferrando-Martinez *et al.*, 2011, Solana *et al.*, 2012). Further studies have also investigated the influence of CMV upon immunity and it is well established that CMV seropositive individuals generally have increased EMRA T cells, which cells are highly differentiated and re-express CD45RA (Pawelec *et al.*, 2010b). Due to an aged immune system, vaccine responses are often poorer in older adults and there is some evidence to suggest that CMV can lead to lack of vaccine responsiveness, demonstrating further evidence as to why studying CMV and immunosenescence is of importance (Goodwin *et al.*, 2006, Trzonkowski *et al.*, 2003). Another issue that occurs with ageing is a pro-inflammatory environment, referred to as ‘inflammaging.’ In particular there is an increase in plasma IL-6 (Franceschi *et al.*, 2000).

It is known that CMV can alter the immune phenotype of older adults and has a potential role in terms of increasing mortality risk. However, the response to the virus can differ between cohorts. For example a study in Sicily found that older adults did not see typical features of immune phenotype, such as a greater expansion of CMV-specific T cells in

older adults(Colonna-Romano *et al.*, 2007). One aim of the current work is to assess the extent to which CMV alters immune phenotype in a large cohort of older adults in Scotland. The role of both CMV and Epstein-Barr virus upon immune phenotype is less well defined. Finding EBV seronegative donors for comparison is relatively difficult given that EBV infects around 90% of the population(Cohen 2000). This study aims to address the question of co-infection with both viruses and their influence upon immune phenotype by studying a large cohort in which participants vary in terms of their CMV and EBV infection status. Data assessing the impact of both viruses upon T cell subsets are the most novel and important element of this work.

4.2.METHODS

4.2.1. ANTIBODY PANELS FOR FLOW CYTOMETRIC ANALYSIS

Antibody panels were chosen in order to investigate cell subsets of interest, the panels are detailed in table 4.1. Panel 1 enabled T cells to be identified by CD3 expression, CD4⁺ and CD8⁺ T cell subsets could then be identified. Co-expression of CCR7 and CD4RA were used to identify cells that were naive (CCR7+CD45RA+), central memory (CCR7+CD45RA-), effector memory (CCR7-CD45RA-) or EMRA (CCR7-CD45RA+). This is of interest as age and CMV serostatus have been observed to change the frequencies of some of these cell types (Pawelec *et al.*, 2010a). Highly differentiated T cells were further identified as CD57+CD28-. This T cell subset in particular has been shown to be associated with immunosenescence which is why it was of importance that

these antibodies were included in the panel (Goronzy *et al.*, 2013). For additional information, CD45RO was investigated, CD45RO when expressed with other markers can identify memory T cells (Sallusto *et al.*, 2004). The gating strategy for T cells is demonstrated in figure 4.2.

A second panel was used to assess other lymphocyte subsets such as NK and NKT cells. NK cells were identified as CD3-CD56+. NK cells could be further phenotyped using an antibody against CD16, enabling identification of cells with different functional properties. NK CD56 bright CD16 dim cells are less cytotoxic than CD56 dim CD16 bright cells which are more cytotoxic in nature (Cooper *et al.*, 2001). Previous data has shown that NK cells increase with age, with an increase in particular within the CD56 dim NK cells (Gayoso *et al.*, 2011). NKT are cells which display features of both innate and adaptive immunity and can be identified as CD3+CD56+. All of the data was acquired using LSR II (BD) and analysed by BD FACSDIVA software, version 6.

Table 4-1 Panels of Antibodies for FACS analysis of PBMC samples from the 1000 Elders Cohort

Panel 1

Antigen	Clone	Fluorophore	Company	Concentration (µl/ml)
Viability	dye	Pacific Blue	Invitrogen	0.001
CD3	S4.1	APC	Invitrogen	0.01
CD4	RPA-T4	PE-Cy7	eBioscience	0.025
CD8	SK1	Amcyan	BD	0.05
CD28	CD28.2	Percpcy5.5	eBioscience	0.6
CD57	HCD57	PE	BioLegend	0.2
CCR7	150503	FITC	R&D systems	1.0
CD45RA	HI 100	AF700	BioLegend	0.05
CD45RO	UCHL1	ECD	Beckman Coulter	0.1

Panel 2

Antigen	Clone	Fluorophore	Company	Concentration (µl/ml)
Viability dye		Pacific Blue	Invitrogen	0.001
CD3	S4.1	APC	Invitrogen	0.01
CD4	RPA-T4	Percpcy5.5	eBioscience	0.025
CD8	SK1	Amcyan	BD	0.05
CD16	3G8	PE-Cy7	Biolegend	0.05
CD56	C5.9	PE	AbD serotec	0.25

4.2.2. Statistical Analysis

Data was analysed either by Mann-Whitney U Test or by the Kruskal-Wallis test (with Dunn's post-hoc test) in GraphPad Prism version 6.00 for Windows, GraphPad Software, San Diego California USA. The Mann-Whitney U Test compares two independent non-parametric groups and specific p-values are given. The Kruskal-Wallis with Dunn's post-hoc test compares three or more non-parametric groups. This test calculates approximate p-values for large data sets and these are displayed on the appropriate figures. Where shown CMV IgG titres were log-transformed twice. **** $p \leq 0.0001$, *** $p \leq 0.001$ ** $p \leq 0.01$ * $p < 0.05$

4.3. RESULTS

4.3.1. CMV and EBV serostatus in the Lothian Birth Cohort

CMV IgG antibody titres were determined by an in-house CMV IgG ELISA. EBV serostatus was also determined by an EBV VCA IgG ELISA. Borderline samples were analysed by immunofluorescence for EBV VCA anti-IgG for confirmation. These methods are discussed in more detail in chapter 2. In total 1061 donors were analysed this way, as detailed in the previous chapter. In a subset of participants PBMC samples were analysed by flow cytometry in order to assess the immune repertoire. Of these donors, 77 were CMV seronegative and 165 were CMV seropositive. EBV data was available for 240 of the participants. When donors were split into four groups according to CMV and EBV serostatus, 8 were CMV-/EBV-, 67 were CMV-/EBV+, 7 were CMV+/EBV- and

158 were CMV+/EBV+ (figure 4.1).

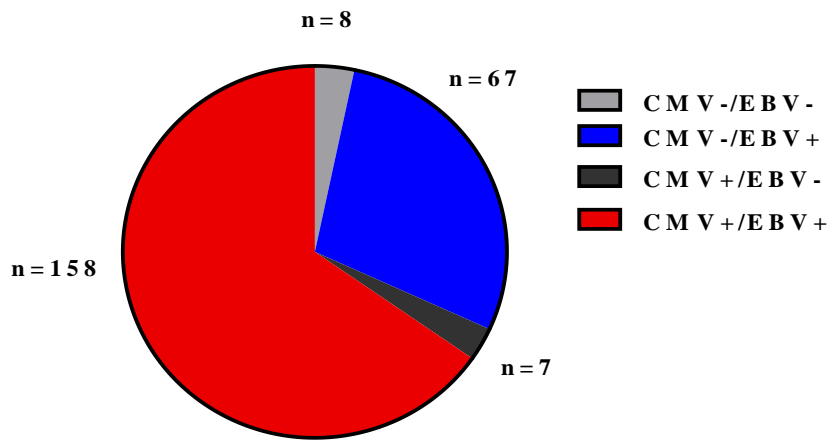


Figure 4-1 CMV and EBV serostatus in a subset of participants from the Lothian Birth Cohort

4.3.2. CMV seropositive individuals display lower CD4/8 T cell ratios

CD4⁺ and CD8⁺ T cells were identified by flow cytometry using the panels detailed in Table 4.1 and the gating strategy in Figure 4.2. CMV positive individuals displayed lower frequencies of CD4⁺ T cells compared with CMV negative individuals (median of 64.56% vs 54.49% $p=0.001$, $n=244$) (figure 4.3). On the other hand, CMV seropositivity was associated with higher frequencies of CD8⁺ T cells (median of 34.12 vs 23.98 for CMV negatives $p<0.0001$). As a consequence of this, CMV seropositive individuals had a significantly lower CD4/8 T cell ratio than CMV negative individuals (Figure 4.4, median of 2.51 vs 1.53 $p<0.0001$). 13/77 (16.9%) and 44/165 of CMV positive donors (26.7%) displayed a CD4/8 T cell ratio of less than one, demonstrating that a significant proportion of donors displayed an inverted T cell ratio.

Next, the association between anti-CMV IgG antibody titre and CD4/8 T cell ratio was analysed within the CMV seropositive donors ($n=166$). To do so CMV IgG titres were presented on a logarithmic scale and plotted against CD4/8 T cell ratio. There was an inverse correlation between CD4/8 T cell ratio and CMV IgG titre showing that the CD4/8 T cell ratio decreased with increasing antibody titre (figure 4.5, $R^2=0.253$, $p=0.0429$).

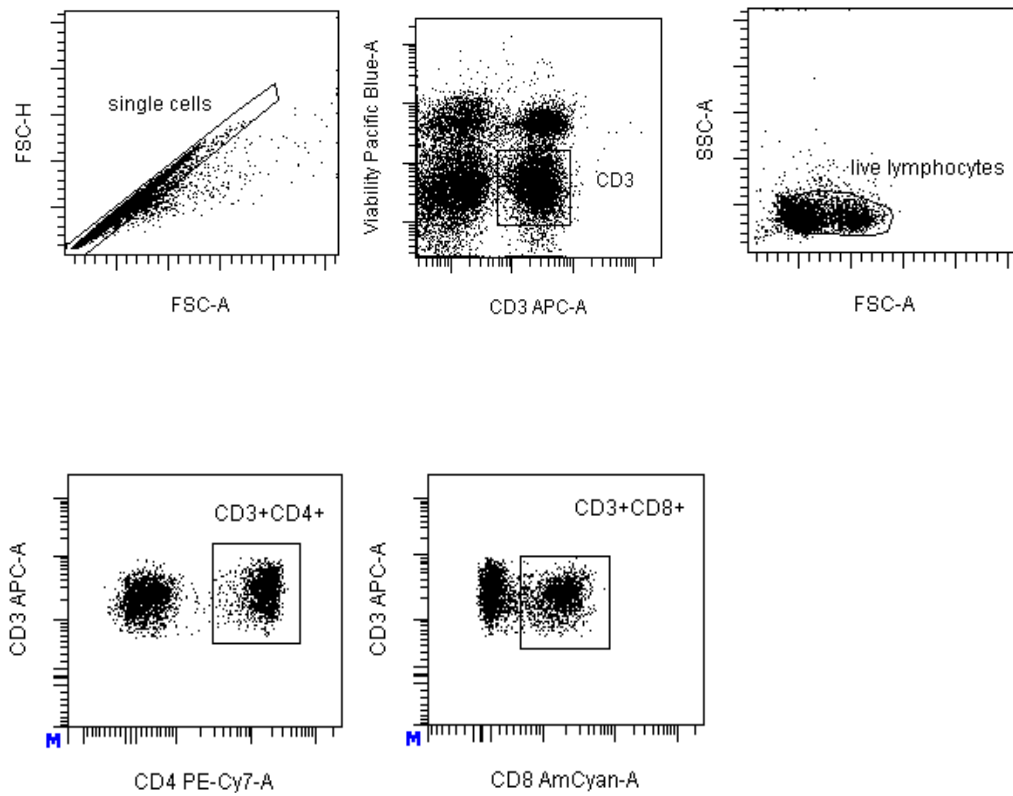


Figure 4-2 Gating strategy for $CD4^+$ and $CD8^+$ T cells.

Single cells were gated on followed by live $CD3^+$ T cells, live lymphocytes then $CD4^+$ and $CD8^+$ T cells

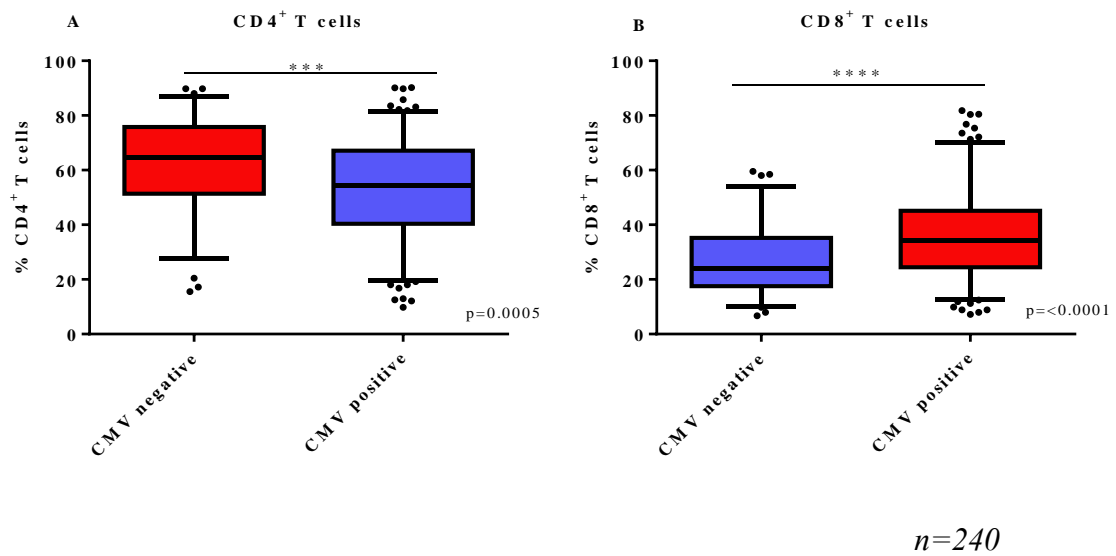


Figure 4-3. Frequencies of CD4⁺ and CD8⁺ T cells.

A. Frequencies of CD4⁺ T cells in CMV seronegative and CMV seropositive individuals.

B. Frequencies of CD8⁺ T cells in CMV seronegative and CMV seropositive individuals.

Data shown as box-and-whisker plots with 5th and 95th percentiles. Data analysed by the Mann-Whitney U Test.

**** $p \leq 0.0001$

*** $p \leq 0.001$

** $p \leq 0.01$

* $p \leq 0.05$

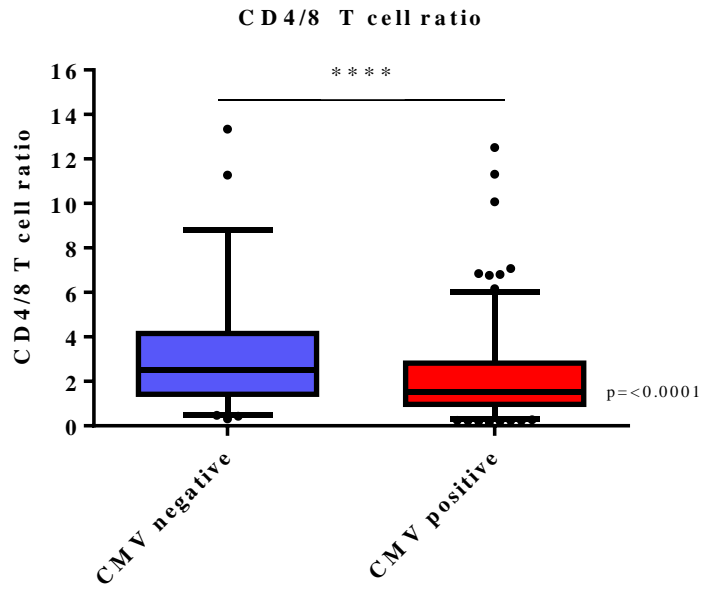


Figure 4-4. CD4/8 T cell ratio.

CD4/8 T cell ratio in CMV seronegative and CMV seropositive individuals. Data shown as box-and-whisker plots with 5th and 95th percentiles. Data analysed by the Mann - Whitney U Test.

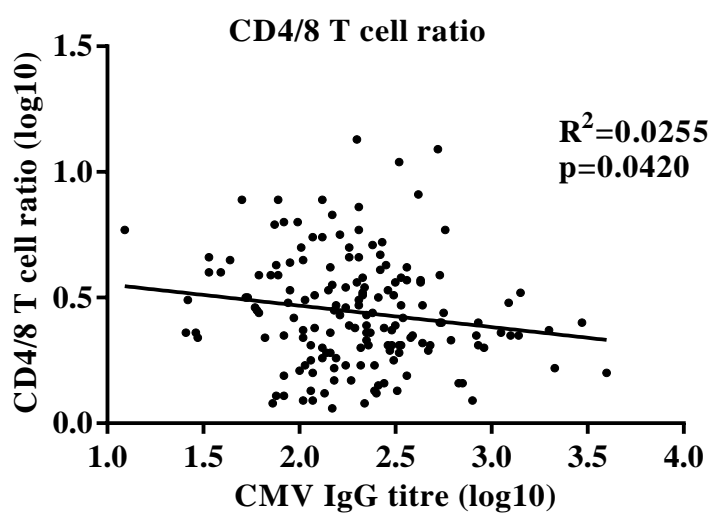


Figure 4-5. Correlation between CMV IgG antibody titre (log10) and CD4/8 T cell ratio.

Data was analysed by Pearson's correlation and the R² and p values displayed.

4.3.3. CMV influences the overall CD4⁺ and CD8⁺ T cell repertoire of Lothian Birth Cohort Donors

The impact of CMV upon the T cell repertoire of Lothian Birth Cohort participants was assessed. Initially expression levels of individual markers including CCR7, CD45RA, CD45RO, CD28 and CD57 were analysed on CD4⁺ T cells (Figure 4.6A). CMV seropositive individuals displayed a significantly lower proportion of CCR7⁺ CD4⁺ T cells, compared to CMV seronegative (median of 64.59% vs. 77.39%). CMV seropositive individuals also displayed lower proportions of CD28 (median of 85.42% vs. 96.33%). There was a significant difference in terms of frequencies of CD57⁺ T cells within the CD4⁺ T cells population, with CMV seropositive individuals displaying 9.09% and CMV seronegative individuals displaying a median of 1.64% CD57⁺ cells. There were no significant differences between frequencies of CD4⁺ CD45RA⁺ T cells in the CMV seronegative and seropositive groups. A trend towards an increase in CD4⁺ CD45RO⁺ T cells in the CMV seropositive group was observed, however this was not significant.

When studying the CD8⁺ T cell population CMV seropositivity was associated with lower frequencies of CD8⁺ T cells expressing CCR7, CMV positive individuals displaying a median of 14.99% of CCR7⁺ cells compared with 31.67% within the CMV seronegative group (Figure 4.6B). Similarly, CMV seropositive donors had lower frequencies of CD8⁺CD28⁺ T cells (34.82% vs. 53.48% for CMV seronegative).

CMV seropositive donors had increased frequencies of CD8⁺ CD57⁺ T cells compared with CMV seronegative donors (51.69% vs. 30.81%). This group also displayed increased proportions of CD8⁺ T cells expressing CD45RA compared with the CMV seronegative donors (61.92% vs. 58.83%). There was no difference in frequencies of CD8⁺ T cells expressing CD45RO⁺.

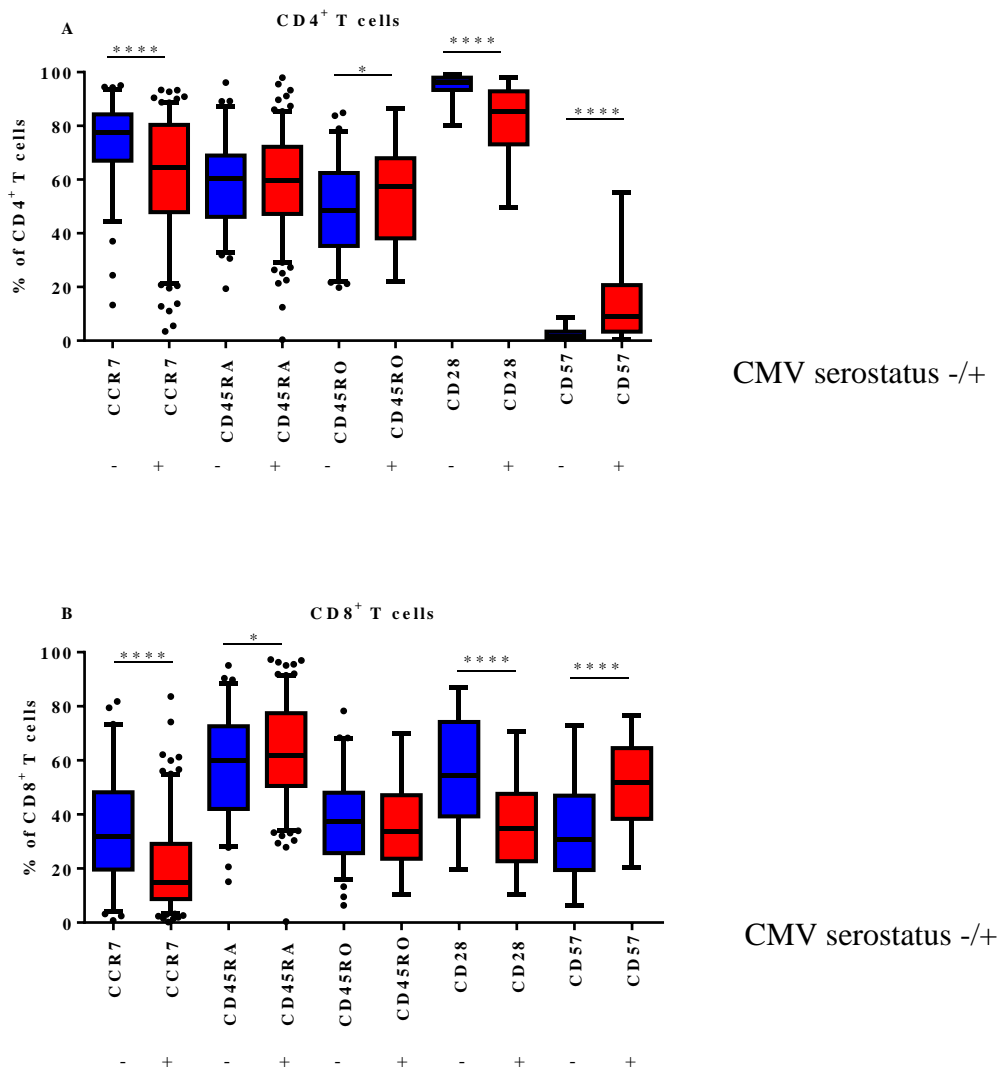


Figure 4-6 *The impact of CMV serostatus upon the T cell repertoire.*

A. The impact of CMV serostatus upon expression of markers within the CD4⁺ T cell compartment. B. The impact of CMV serostatus upon expression of markers within the CD8⁺ T cell compartment. Data shown as box-and-whisker plots with 5th and 95th percentiles. Data was analysed by Mann-Whitney U Test.

4.3.4. The impact of CMV serostatus upon frequencies of naïve, central memory, effector memory and EMRA T cells

Previous studies have shown CMV infection to impact upon frequencies of memory T cell subsets (Fulop *et al.*, 2013). Here we used CCR7 and CD45RA expression to address the distribution of naïve, central memory, effector memory and EMRA T cells within the CD4⁺ and CD8⁺ T cell compartments in this cohort (Figures 4.7 and 4.8).

Within the CD4⁺ T cell compartment, (Figure 4.8A) CMV positive individuals displayed lower frequencies of naïve T cells (41.12%) compared to CMV negative (51.36%) and similarly lower frequencies of central memory T cells in the CMV positive group (16.72% vs. 22.30%). CMV seropositivity was associated with increased proportions of effector memory T cells (18.78% vs. 13.85% CMV seronegative) and EMRA T cells (12.78% vs. 7.74%).

Within the CD8⁺ T cell compartment, (Figure 4.8B) CMV seropositive participants also showed a lower median of naïve T cells (8.25%) compared with CMV seronegative individuals (18.35%), overall this was lower than for CD4⁺ T cells. CMV seropositive individuals also demonstrated lower frequencies of central memory T cells (4.62 vs. 9.09%). There were no differences between frequencies of effector memory T cells between the two groups. However, CMV seropositivity was associated with strongly increased frequencies of CD8⁺ EMRA T cells (49.16% vs. 33.54%).

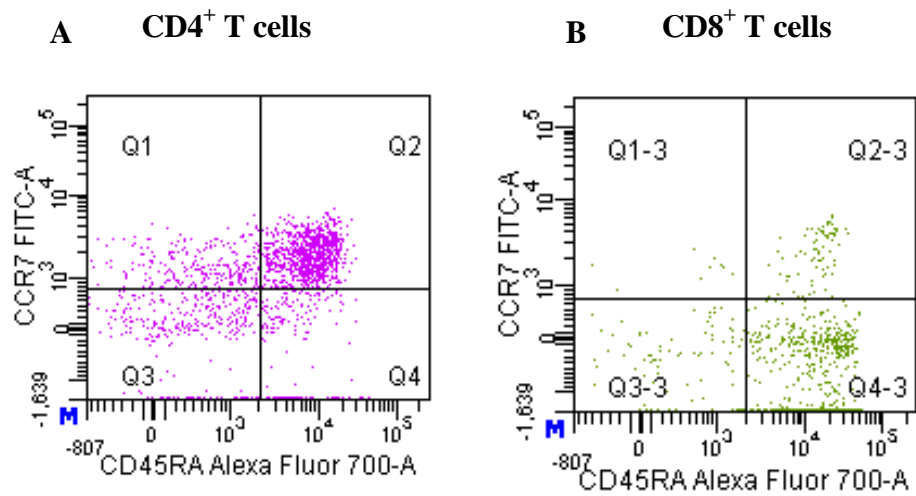


Figure 4-7 Gating Strategy for naïve, central memory effector memory and EMRA T cells.

T cells were defined by expression of CCR7 and CD45RA

A- CD4⁺ T cells. Q1 central memory, Q2 naïve, Q3 Effector memory Q4 EMRA

B- CD8⁺ T cells. Q1-3 central memory Q2-3 naïve, Q3-3 effector memory Q4-3 EMRA

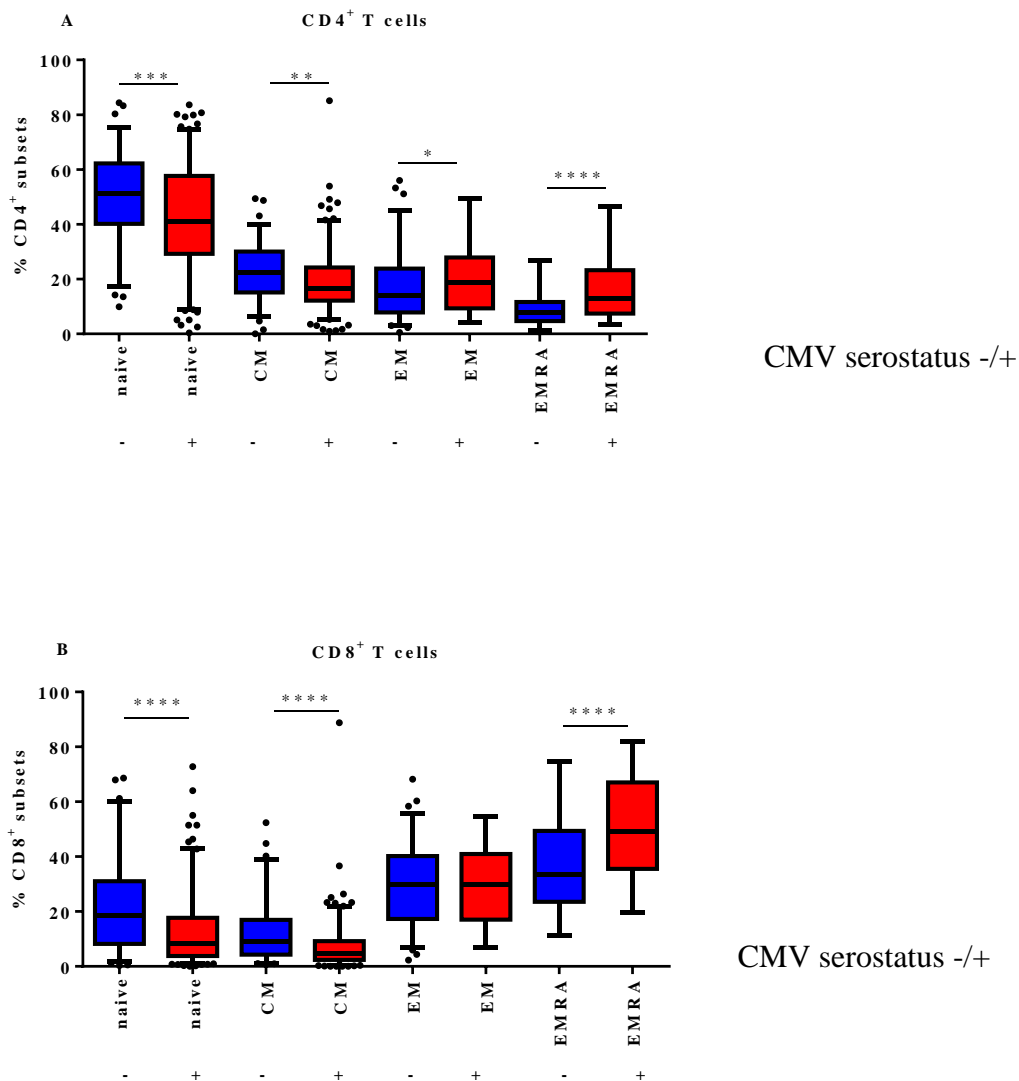


Figure 4-8 The impact of CMV serostatus upon frequencies of naïve, central memory, effector memory and EMRA T cells

CCR7 and *CD45RA* were used to identify different T cell subsets as naïve (*CCR7*+*CD45RA*+) , CM (*CCR7*+*CD45RA*-), EM (*CCR7*-*CD45RA*-) and EMRA (*CCR7*-*CD45RA*+) A. The impact of CMV serostatus upon frequencies of naïve CM, EM and EMRA CD4⁺ T cells. B. The impact of CMV serostatus upon frequencies of naïve, CM, EM and EMRA CD8⁺ T cells. Data shown as box-and-whisker plots with 5th and 95th percentiles .Data analysed using Mann-Whitney U-test.

4.3.5. The impact of CMV IgG titre upon naïve, central memory, effector memory and EMRA T cells

In order to assess the relationship of CMV IgG titre with the frequency of memory T cell subsets, CMV titre was log-transformed before being plotted against frequencies of naïve, central memory, effector memory and EMRA T cells (log10) (figure 4.9A-D).

There was no correlation between CMV IgG titre and naïve CD4⁺ ($R^2=0.0158$, $p=0.111$) or naïve CD8⁺ T cells (Figure 4.9 A-B, $R^2=0.0146$, $p=0.128$). However as CMV titre increased the proportion of central memory cells decreased within the CD4⁺ ($R^2=0.0634$, $p<0.0001$) and within the CD8⁺ T cell compartment (Figure 4.9 C-D, $R^2=0.0315$, $p=0.0264$).

There was no correlation between proportions of CD4⁺ or CD8⁺ effector T cells and CMV IgG titre (Figure 4.10 A-B). However, as CMV IgG titre increased the proportion of CD4⁺ EMRA T cells also increased (Figure 4.10C, $R^2=0.0550$, $p=0.028$). This was not observed within the CD8⁺ EMRA T cell subset (Figure 4.10D $R^2=0.0177$, $p=0.0934$).

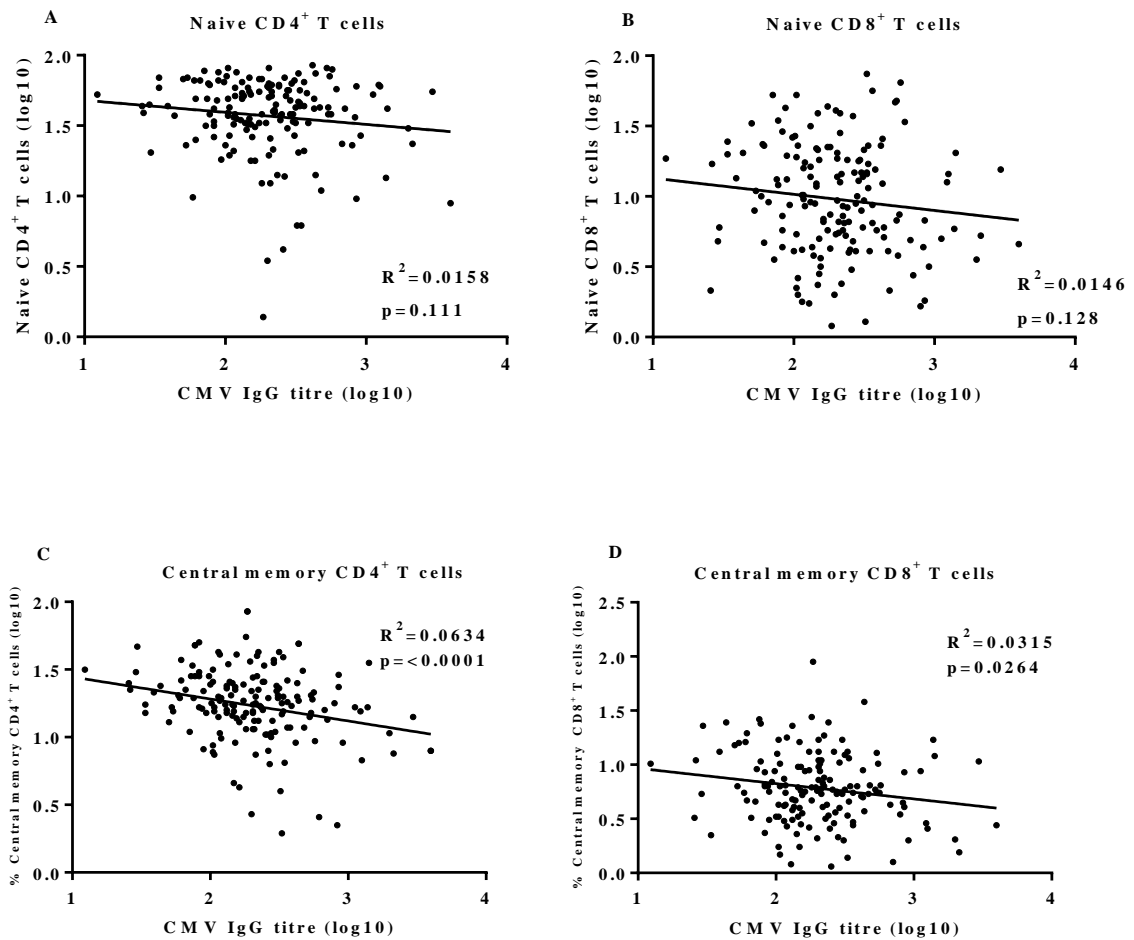


Figure 4-9 Assessing the relationship between CMV IgG titre (log10) and naive and central memory CD4⁺ and CD8⁺ T cells.

- A. Correlation between CMV IgG titre (log10) and naive (CCR7+CD45RA+) CD4⁺ T cells (log10). B. CMV IgG titre (log10) and naive CD8⁺ T cells (log10). C. Correlation between CMV IgG titre (log10) and central memory (CCR7+CD45RA-) CD4⁺ T cells (log10). D. CMV IgG titre (log10) and central memory CD8⁺ T cell (log10). Data was analysed by Pearson's correlation and the R^2 and p values displayed.

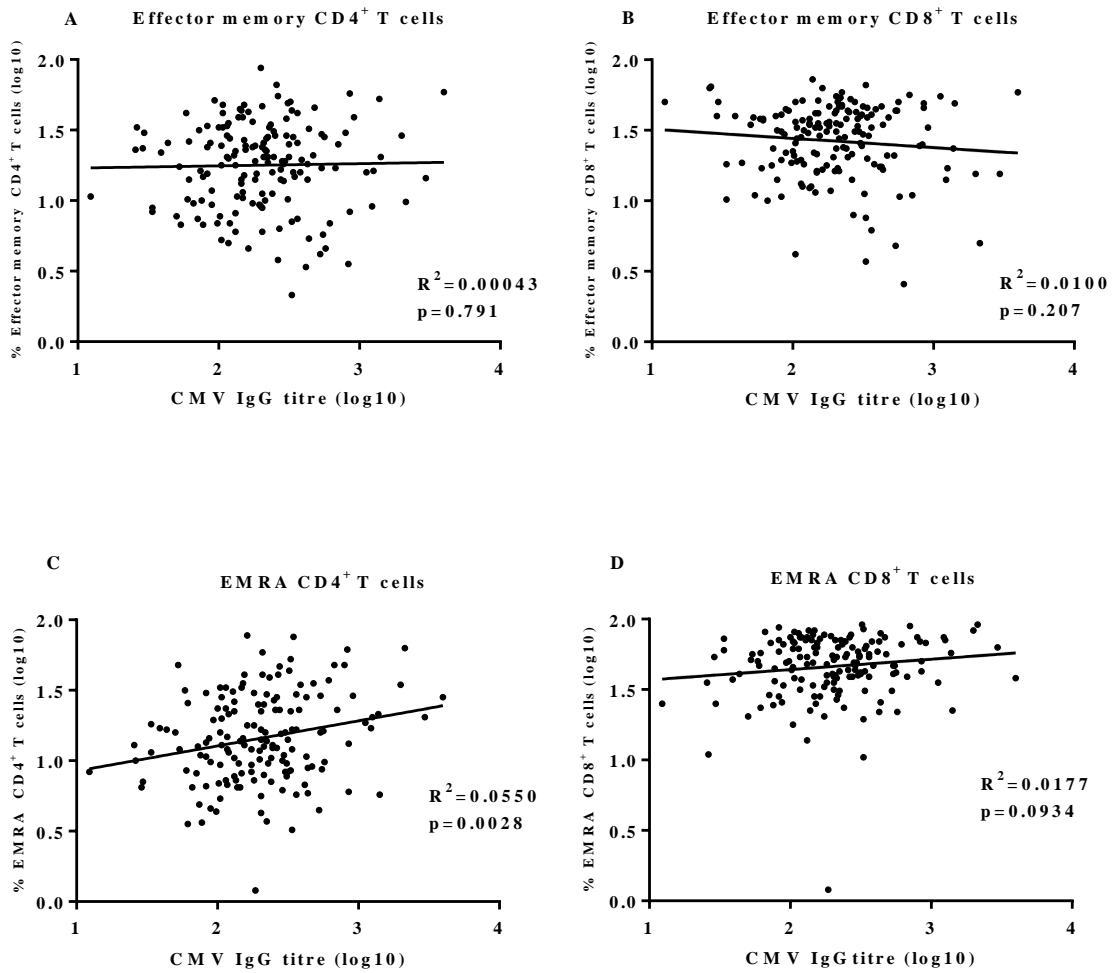


Figure 4-10 Correlation between CMV IgG titre (log10) and Effector memory (EM) and EMRA T cells

A. Correlation between CMV IgG titre (log10) and effector memory (CCR7-CD45RA-) CD4⁺ T cells (log10). B. CMV IgG titre and effector memory CD8⁺ T cells (log10). C Correlation between CMV IgG (log10) and EMRA (CCR7-CD45RA+) CD4⁺ T cells (log10). D. CMV IgG titre (log10) and EMRA CD8⁺ T cells (log10). Data was analysed by Pearson's correlation and the R^2 and p values displayed.

4.3.6. CMV seropositivity and IgG titres correlate with frequencies of highly differentiated T cells

CD57⁺CD28⁻ T cells are cells which are highly differentiated and often associated with CMV seropositivity (Kern *et al.*, 1996). Frequencies of these cells were greater within the CD8⁺ T cell compartment compared with the CD4⁺ T cell compartment (Figure 4.11).

CMV seropositivity greatly increased the frequencies of these cell types within the CD4⁺ T cell population (Figure 4.11A, 7.24% vs 0.28%). This was also apparent within the CD8⁺ T cell subset, with a nearly two-fold increase in frequencies of CD57⁺CD28⁻ T cells- in the CMV seropositive group of participants (Figure 4.11B 47.66% vs 23.99%).

When the relationship between anti-CMV IgG titre and the subset of CD57⁺CD28⁻ T cells was analysed, there was a significant correlation between CMV IgG titre (log₁₀) and an increase in CD4⁺ CD57⁺CD28⁻ T cells (Figure 4.12A, $R^2=0.0975$, $p<0.0001$). This was also the case within the CD8⁺ T cell compartment although it was less strong than within the CD4⁺ T cell compartment (Figure 4.12B, $R^2=0.0358$, $p=0.0169$).

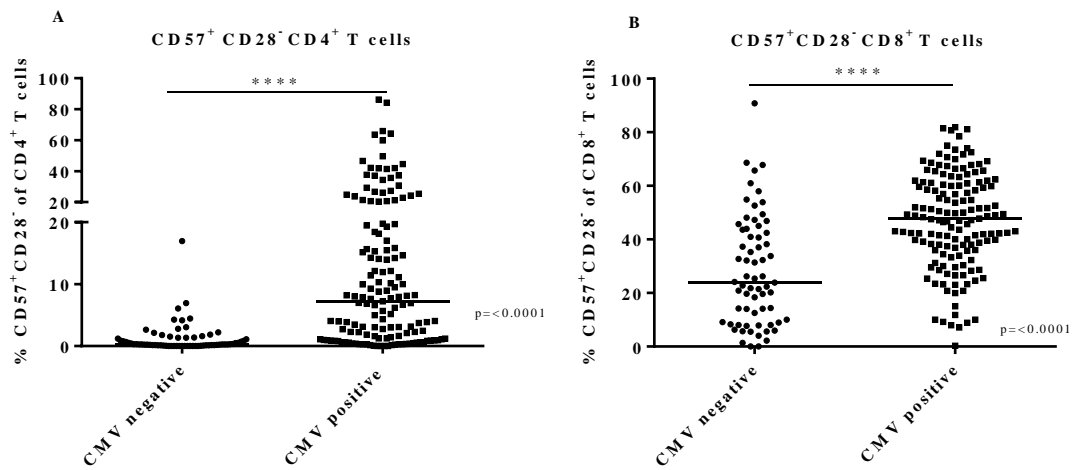


Figure 4-11. Frequencies of highly differentiated $CD57^+CD28^-$ T cells.

A. Frequencies of $CD57^+CD28^- CD4^+$ T cells in CMV seronegative and CMV seropositive groups. B. Frequencies of $CD57^+CD28^- CD8^+$ T cells in CMV seronegative and CMV seropositive groups. Data analysed by Mann-Whitney U-Test

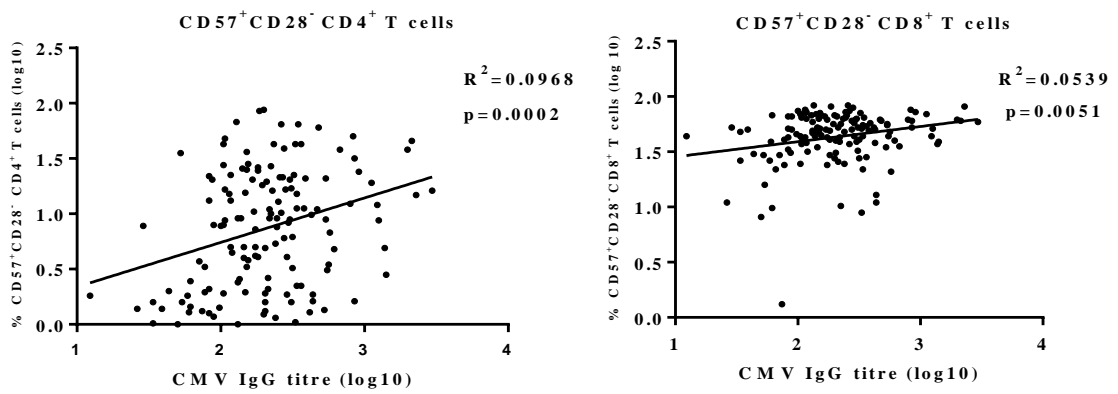


Figure 4-12. Correlation between CMV IgG titre (log10) and CD57+CD28- highly differentiated CD4⁺ and CD8⁺ T cells.

Data analysed by Pearson's correlation and the R^2 and p.values displayed

4.3.7. The impact of CMV and EBV upon the T cell repertoire of Lothian Birth Cohort donors

The data demonstrated that CMV has a significant impact upon the T cell repertoire, however as the majority of individuals carry other herpes viruses during their lifetime it was of interest to assess the potential impact that EBV might have on the immune repertoire. Here we studied the role of EBV in conjunction with CMV. Donors were split into four groups according to CMV and EBV serostatus; CMV-/EBV- (n=8), CMV-/EBV+ (n=67) and CMV+/EBV- (n=7) and CMV+/EBV+ (n=158) and T cell subsets analysed.

As demonstrated in the previous section, CMV seropositive individuals displayed lower frequencies of CD4⁺ T cells. When EBV serostatus was taken into account EBV does not appear to impact upon this. When donors were split into four groups lower proportions of CD4⁺ T cells were only seen in the CMV+/EBV+ compared with the CMV-/EBV+ group (median of 54.29% vs 64.28% respectively Figure 4.13). There were increased frequencies of CD8⁺ T cells in the CMV+/EBV+ compared to the CMV-/EBV- group (34.66% vs. 22.61%), and between the double positive group and the CMV-/EBV+ group (Figure 4.13 median 24.20%). However, one issue with the current work is that there were very few EBV negative donors.

Similarly, when the CD4/8 T cell ratio was examined the ratio was lowest in the CMV+/EBV+ group compared with the CMV-/EBV- group (Figure 4.14, 1.52% vs. 3.31%). There was also a lower ratio in the CMV+/EBV+ group compared with the

CMV-/EBV+ group (median of 1.52% vs 2.49%).

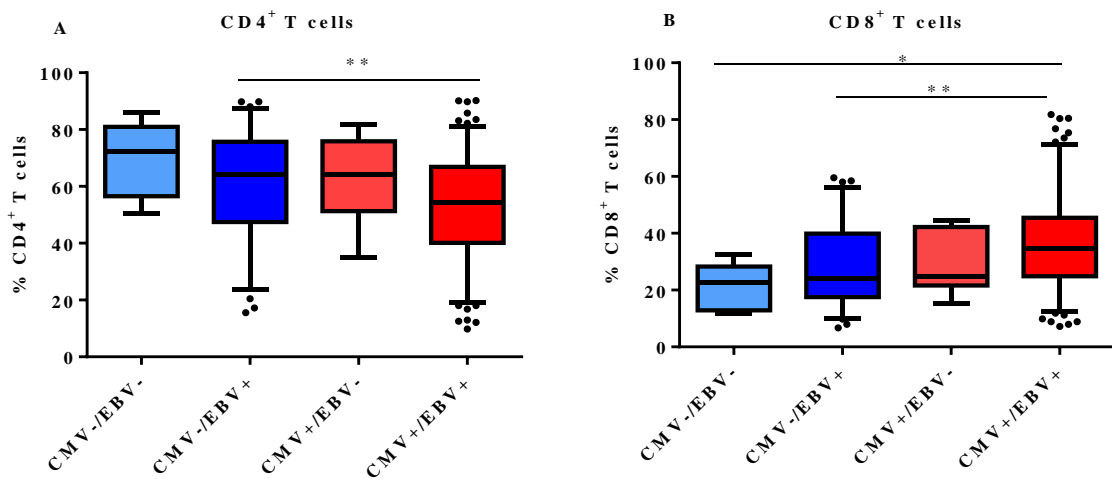


Figure 4-13 CD4⁺ and CD8⁺ T cells according to CMV and EBV serostatus

A. Frequency of CD4⁺ T cells in the four groups as defined by CMV and EBV serostatus.

B. Frequency CD8⁺ T cells in the four groups as defined by CMV and EBV serostatus.

Data shown as box-and-whiskers with 5th and 95th percentiles. Data analysed by Kruskal-Wallis with Dunn's post-hoc test.

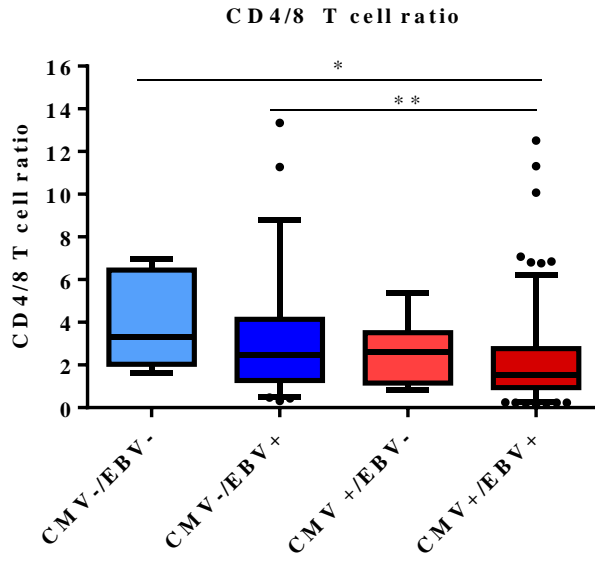


Figure 4-14 *CD4/8 T cell ratio according to CMV and EBV serostatus*

CD4/8 T cell ratio in the four groups as defined by CMV and EBV serostatus. Data shown as box-and-whiskers with 5th and 95th percentiles. Data analysed by Kruskal -Wallis with Dunn's post-hoc test.

Frequencies of CCR7⁺ CD4⁺ and CD8⁺ T cells were analysed in Figure 4.15A. In terms of CD4⁺ T cells, CMV but not EBV appeared to impact upon the frequencies of CCR7 positive T cells. With CMV⁻/EBV⁺ donors displaying higher frequencies of these cells compared with donors in the CMV⁺/EBV⁺ group (median of 77.39% vs 63.82%). Within the CD8⁺ T cell compartment the CMV⁺/EBV⁺ group displayed significantly lower frequencies of CCR7⁺ CD8⁺ T cells compared with the CMV⁻/EBV⁻ group (Figure 4.15B). There was an even greater difference between the CMV⁻/EBV⁺ and CMV⁺/EBV⁺ with a lower frequency of CCR7⁺ T cells in the latter group. (median 31.24% vs 14.41%).

CMV or EBV serostatus did not impact upon the frequency of CD4⁺ T cells expressing CD45RA (figure 4.16A). However, there were differences in frequencies of CD8⁺ T cells expressing CD45RA between the CMV⁻/EBV⁺ and CMV⁺/EBV⁻ groups with a higher percentage of CD45RA⁺ CD8⁺ T cells in the CMV⁺/EBV⁻ group (Figure 4.16B, median of 78.64% vs 59.49%). CD45RO expression was examined within the four serostatus groups. CMV⁺/EBV⁺ participants displayed significantly higher percentages of CD45RO⁺CD4⁺ T cells compared with CMV⁺/EBV⁻, suggesting a role for EBV in determining CD45RO expression (Figure 4.16C, median of 58.49% vs 35.77%). There were no differences between the four groups in terms of CD45RO⁺ CD8⁺ T cells (Figure 4.16D)

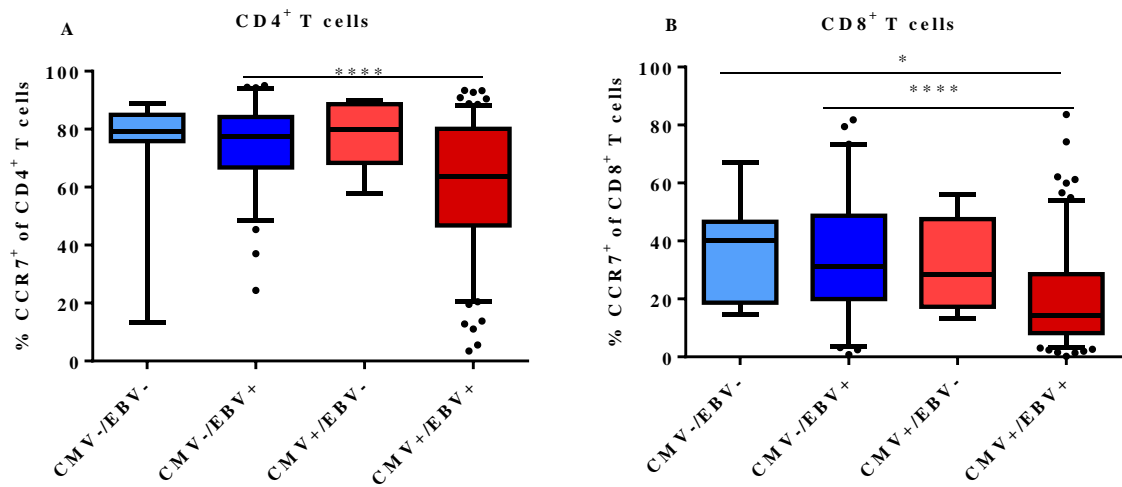


Figure 4-15. CCR7 expression on CD4⁺ and CD8⁺ T cells.

A. Frequency of CCR7⁺ of the CD4⁺ T cells in the four groups as defined by CMV and EBV serostatus. B. Frequency of CCR7⁺ of the CD8⁺ T cells in the four groups as defined by CMV and EBV serostatus. Data shown as box-and-whisker plots with 5th and 95th percentiles. Data analysed by Kruskal-Wallis with Dunn's post-hoc test.

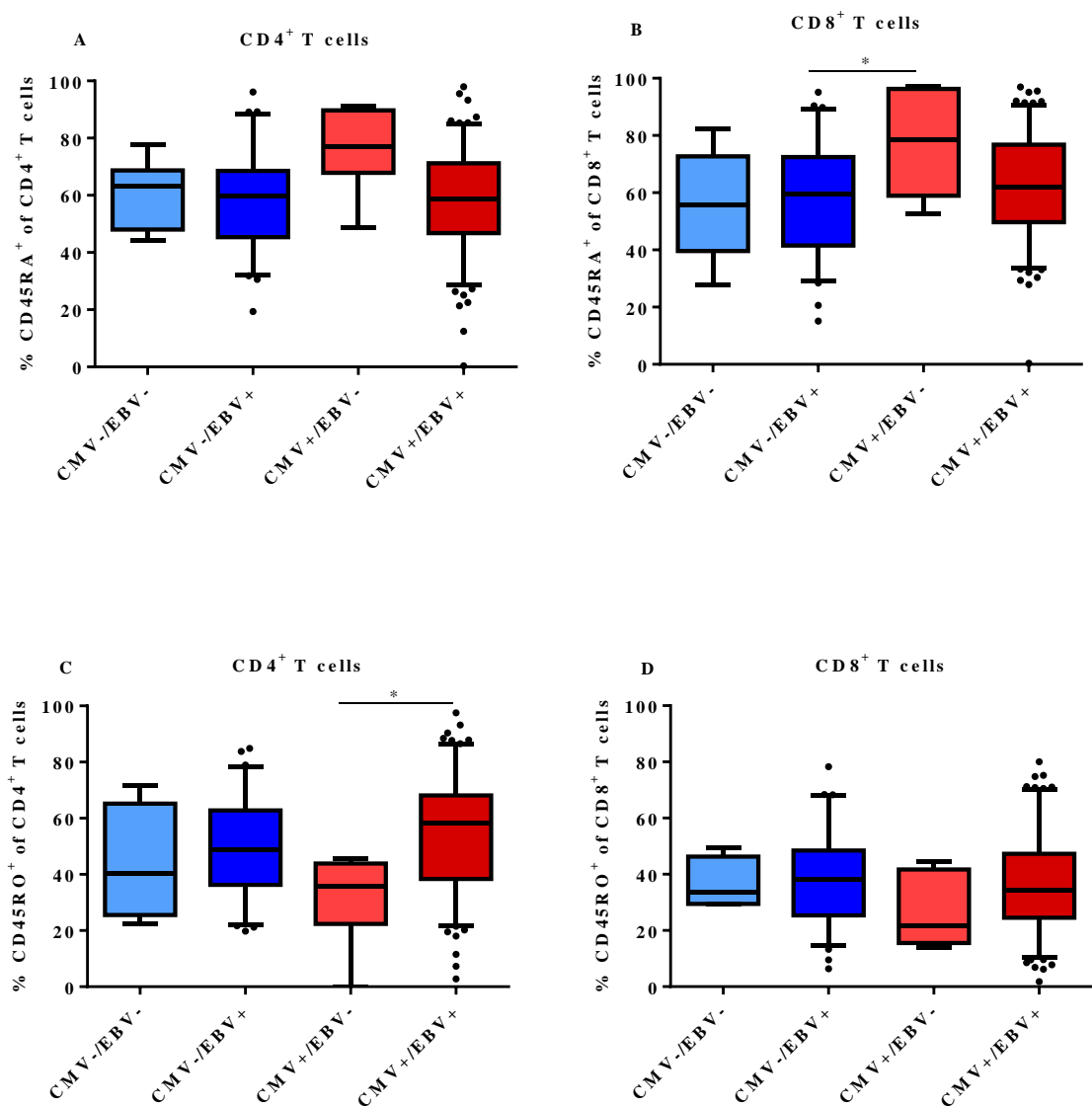


Figure 4-16. CD45RA and CD45RO expression on CD4⁺ and CD8⁺ T cells.

A. Frequencies of CD45RA⁺ of the CD4⁺ T cells in the four groups as defined by CMV and EBV serostatus. B. Frequencies of CD45RA⁺ of the CD8⁺ T cells in the four groups as defined by CMV and EBV serostatus. C. Frequencies of CD45RO⁺ of the CD4⁺ T cells in the four groups as defined by CMV and EBV serostatus. D. Frequencies of CD45RO⁺ of the CD8⁺ T cells in the four groups as defined by CMV and EBV serostatus. Data shown as box-and-whisker plots with 5th and 95th percentiles. Data analysed by Kruskal-Wallis with Dunn's post-hoc test.

When taking only CMV serostatus into account, CMV seropositive participants displayed increased frequencies of CD57⁺ T cells within the CD4⁺ T cell compartment (figure 4.11 $p < 0.0001$). Frequencies of these cells were higher in the CMV+/EBV+ (median of 9.64%) group compared with the other three serostatus groups including CMV+/EBV- (0.61%) CMV-/EBV+ (1.68%) and CMV-/EBV- (0.53%). (Figure 4.17A) CD57⁺ T cells within the CD8⁺ T cell compartment were also examined (figure 4.17B). Participants seropositive for both viruses displayed higher median frequencies of these cells compared to individuals in the CMV-/EBV+ group (52.61% vs. 31.42%). The greatest difference occurred between individuals CMV+/EBV+ and those who were CMV-/EBV- (52.61% vs 20.00%).

CD28 expression was examined within the different groups, individuals seropositive for both viruses displayed lower frequencies of CD28⁺ CD4⁺ T cells (85.21%) compared with CMV+/EBV- (97.40%), CMV-/EBV+ (96.29%) and CMV-/EBV- (97.76%), (Figure 4.17A). In terms of CD8⁺ T cells, participants in the double positive group displayed lower frequencies of CD28⁺ T cells compared with CMV-/EBV- individuals (34.37% vs 61.44%), (Figure 4.17B). There were also differences between the CMV+/EBV- and CMV-/EBV+ groups with the former displaying lower frequencies of this cell type (41.40% vs 54.48%).

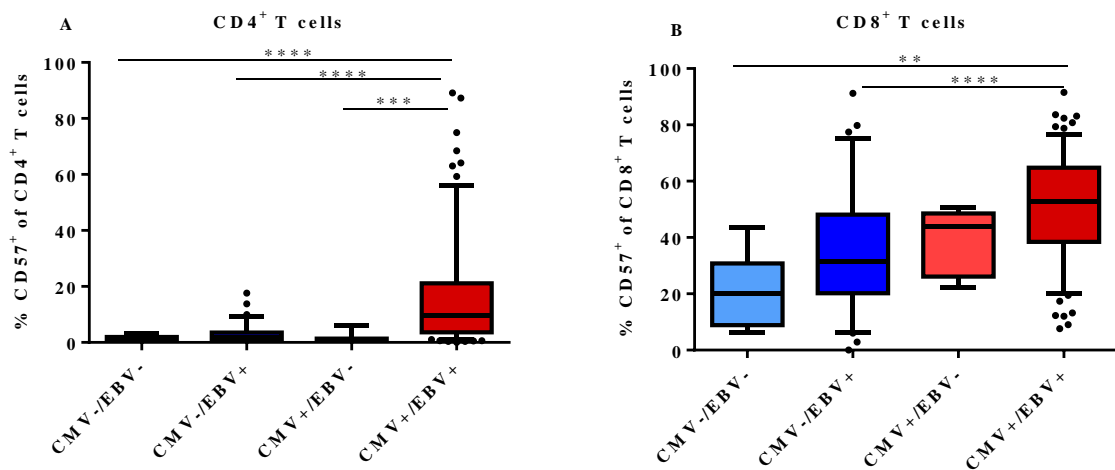


Figure 4-17. CD57 expression on CD4⁺ and CD8⁺ T cells.

A. Frequencies of CD57⁺ of the CD4⁺ T cells in the four groups as defined by CMV and EBV serostatus. B. Frequencies of CD57⁺ of the CD8⁺ T cells in the four groups as defined by CMV and EBV serostatus. Data shown as box-and-whisker plots with 5th and 95th percentiles. Data analysed by Kruskal -Wallis with Dunn's post-hoc test.

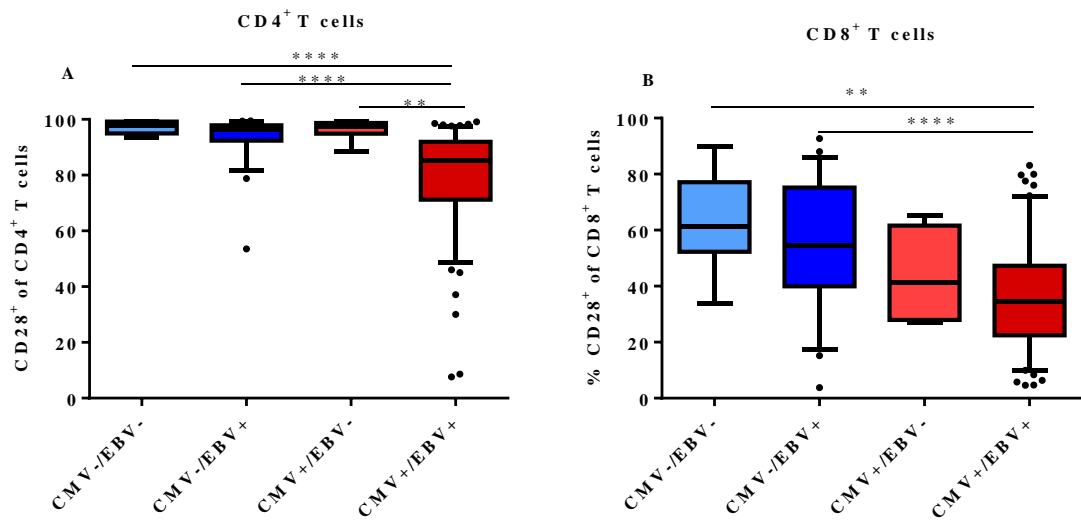


Figure 4-18. CD28 expression on CD4⁺ and CD8⁺ T cells.

A. Frequencies of CD28⁺ of the CD4⁺ T cells in the four groups as defined by CMV and EBV serostatus. B. Frequencies of CD28⁺ of the CD8⁺ T cells in the four groups as defined by CMV and EBV serostatus. Data shown as box-and-whisker plots with 5th and 95th percentiles. Data analysed by Kruskal -Wallis with Dunn's post-hoc test.

4.3.8. The impact of CMV and EBV upon naïve, central memory, effector memory and EMRA T cells

The impact of CMV and EBV upon frequencies of naïve, central memory, effector memory and EMRA T cells were examined. There are differences in the frequencies of naïve CD8⁺ T cells between the CMV+/EBV- and CMV+/EBV+ groups, with a lower proportion of naïve cells in individuals who were both CMV and EBV seropositive (figure 4.19, 59.94% vs 40.10%). The group of participants positive for both viruses also displayed lower frequencies of naïve CD4⁺ T cells compared with the CMV-/EBV+ group (median 51.36%). This was further exacerbated within the CD8⁺ T cell compartment (Figure 4.19B). The CMV+/EBV+ group has the lowest frequencies of naïve CD8⁺ T cells (median of 7.89%). This was significantly lower than the CMV-/EBV- group (27.94%), the CMV-/EBV+ group (16.79%) and the CMV+/EBV- group (25.58%).

CMV influences the frequencies of CD4⁺ and CD8⁺ central memory cells, there were lower percentages of central memory CD4⁺ T cells in the CMV+ and EBV+ groups compared with the CMV-/EBV+ group (figure 4.20A, median of 16.80% compared with 23.17%). There were also lower frequencies of CD8⁺ central memory T cells in the CMV+/EBV- group, compared with the CMV-/EBV+ group (Figure 4.20B, median of 3.55% vs 9.09%). There is a statistically significant difference between the CMV+/EBV+ and the CMV-/EBV+ group (4.80% vs. 9.09%).

There were also significantly lower frequencies of effector memory CD4⁺ T cells in the CMV+/EBV+ compared with the CMV+/EBV- group (Figure 4.21A, median of 19.31% vs 7.04%) ,suggesting a role for EBV as well as CMV in determining effector frequencies. There were no significant differences between effector CD8⁺ T cells when CMV or EBV serostatus were considered (Figure 4.21B).

Previous data showed frequencies of EMRA T cells to be greatly influenced by CMV serostatus, with higher frequencies of these cells occurring within the CD4⁺ T cell compartment of CMV positive individuals. When split into four groups according to serostatus, there were differences between CMV-/EBV- and CMV+/EBV+ groups with a higher frequencies in the latter (Figure 4.22A median of 5.39% vs 12.93%). There were lower frequencies in the CMV-/EBV+ group compared with the double positive group also (median of 8.20% in the CMV-/EBV+ group vs. 12.93%). When split according to CMV and EBV serostatus there were only significant differences between the CMV-/EBV+ and CMV+/EBV+ groups with the double positive group displaying a significantly higher percentage of EMRA CD8⁺ T cells (figure 4.22 B, 50.00% vs 31.03%).

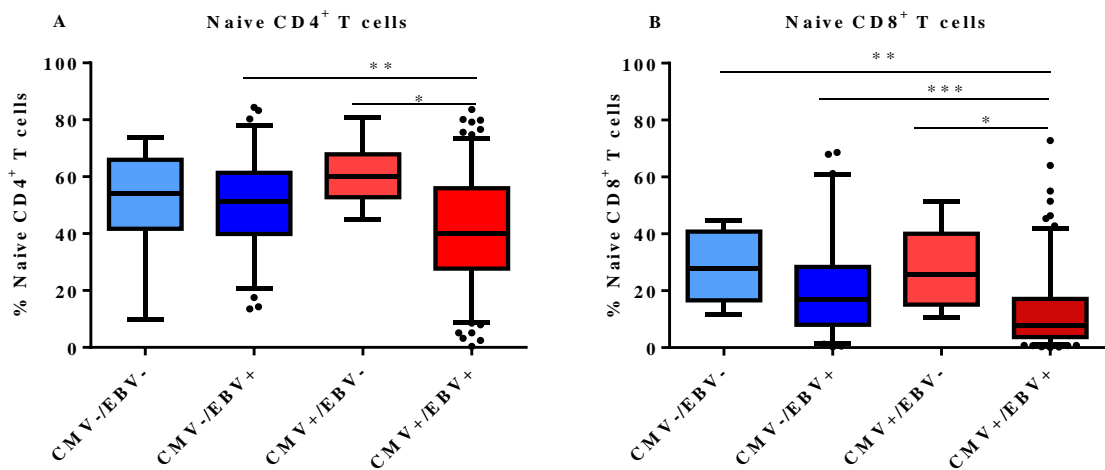


Figure 4-19 Frequencies of naïve CD4⁺ and CD8⁺ T cells

A. Frequencies of naïve CD4⁺ T cells in the four groups as defined by CMV and EBV serostatus. B. Frequencies of naïve CD8⁺ T cells in the four groups as defined by CMV and EBV serostatus. Data shown as box-and-whisker plots with 5th and 95th percentiles. Data analysed by Kruskal -Wallis with Dunn's post-hoc test.

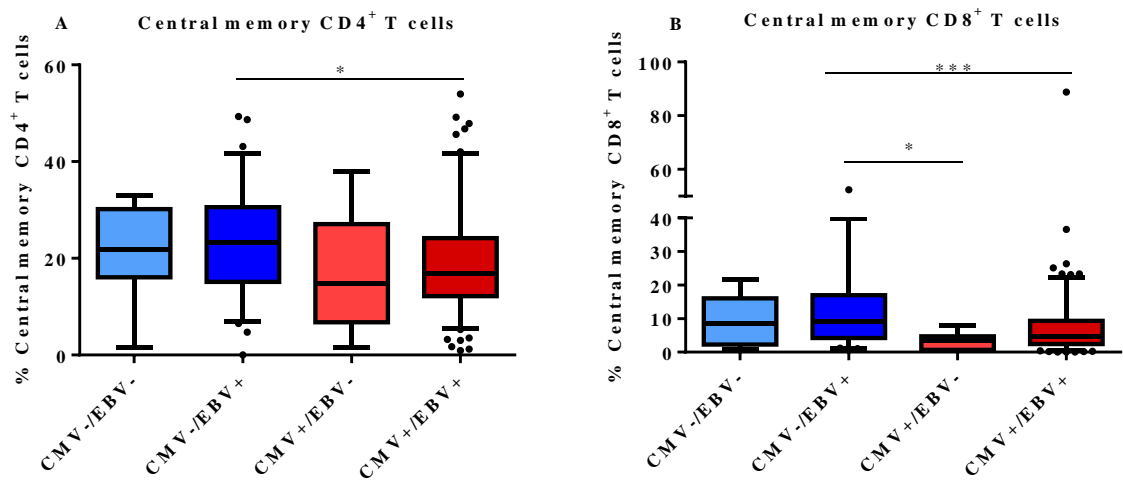


Figure 4-20 Frequencies of central memory CD4⁺ and CD8⁺ T cells

A. Frequencies of central memory CD4⁺ T cells in the four groups as defined by CMV and EBV serostatus. B. Frequencies of central memory CD8⁺ T cells in the four groups as defined by CMV and EBV serostatus. Data shown as box-and-whisker plots with 5th and 95th percentiles. Data analysed by Kruskal -Wallis with Dunn's post-hoc test.

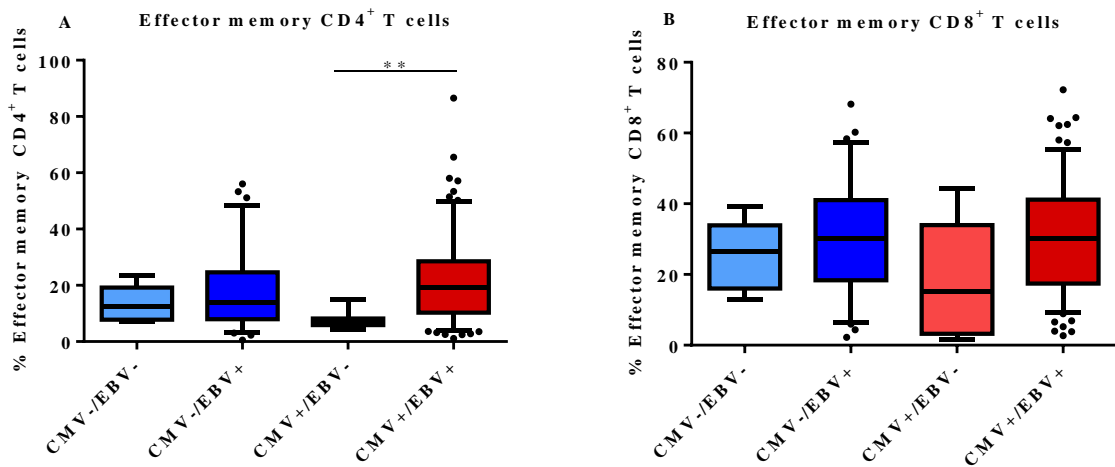


Figure 4-21 Frequencies of effector memory CD4⁺ and CD8⁺ T cells

A. Frequencies of effector memory CD4⁺ T cells in the four groups as defined by CMV and EBV serostatus. B. Frequencies of effector memory CD8⁺ T cells in the four groups as defined by CMV and EBV serostatus. Data shown as box-and-whisker plots with 5th and 95th percentiles. Data analysed by Kruskal- Wallis with Dunn's post-hoc test.

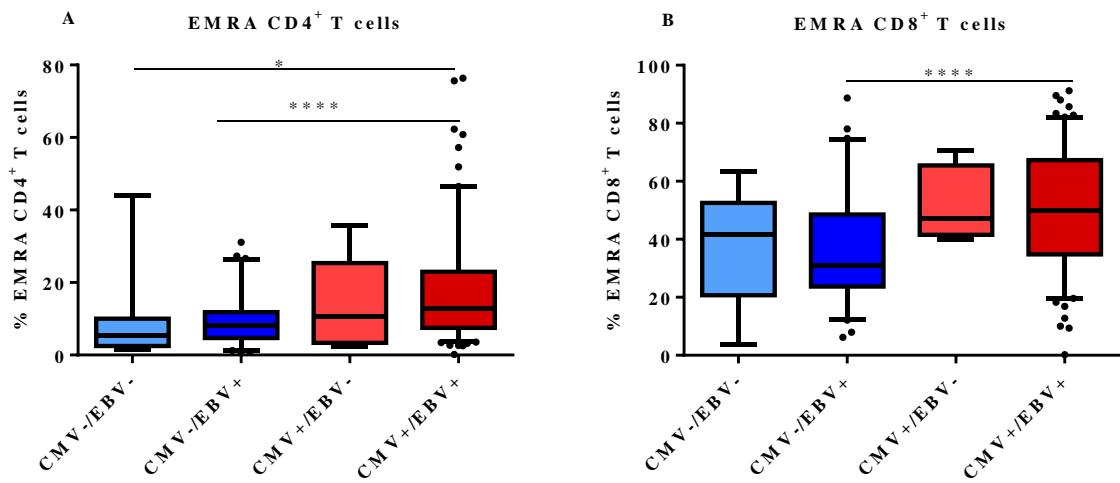


Figure 4-22 Frequencies of EMRA CD4⁺ and CD8⁺ T cells

A. Frequencies of EMRA CD4⁺ T cells in the four groups as defined by CMV and EBV serostatus. B. Frequencies of EMRA CD8⁺ T cells in the four groups as defined by CMV and EBV serostatus. Data shown as box-and-whisker plots with 5th and 95th percentiles. Data analysed by Kruskal -Wallis with Dunn's post-hoc test.

4.3.9. The influence of CMV and EBV infection upon CD57+CD28- T cells

CD57+CD28- T cell subsets were also examined (Figure 4.23 A-B). The highest frequencies were within the CMV+/EBV+ groups (7.91%) compared with those CMV-/EBV- (0.055%), CMV-/EBV+ (0.30%) and CMV+/EBV- (0.42%). There were differences in CD57+CD28- CD8⁺ T cell percentages between the CMV-/EBV+ group and the double positive group with the CMV+/EBV+ groups displaying higher frequencies of these cells (24.76% vs 48.89%). There were also differences between the CMV-/EBV- and CMV+/EBV+ groups with the latter displaying higher frequencies of these cells again (13.26% vs 48.89%).

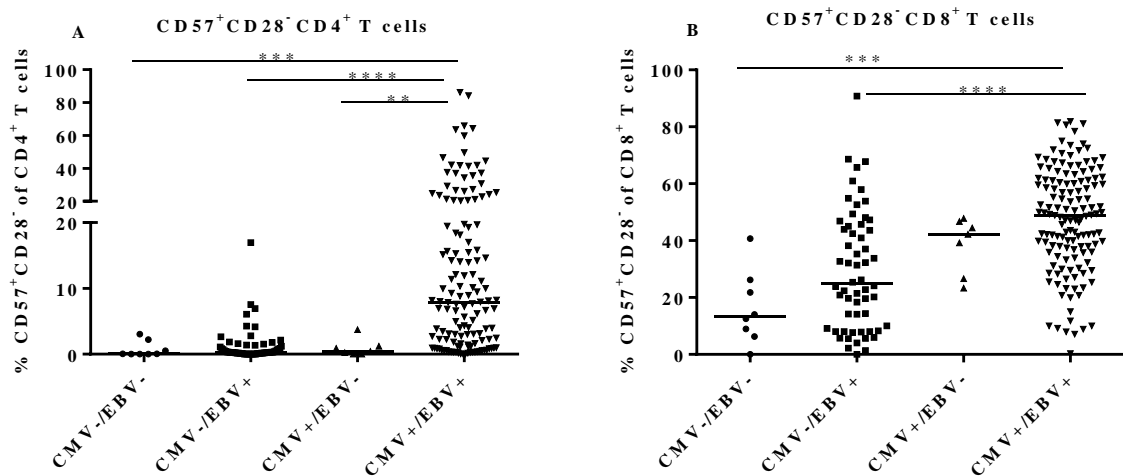


Figure 4-23 CD57⁺CD28⁻ highly differentiated CD4⁺ and CD8⁺ T cells.

A. Frequencies of CD57⁺CD28⁻ CD4⁺ T cells in four groups according to CMV and EBV serostatus. B. Frequencies of CD57⁺CD28⁻ T cells in four groups according to CMV and EBV serostatus. Data shown as box-and-whisker plots with 5th and 95th percentiles. Data analysed by Kruskal -Wallis with Dunn's post-hoc test.

4.3.10. **The impact of CMV and EBV upon other lymphocyte subsets**

NK cells have previously been found to increase in CMV seropositive individuals (Chidrawar *et al.*, 2009). A second antibody panel was used to determine proportions of NK and NKT cells in 218 participants. NK cells were defined as CD3⁻ CD56⁺, there was no difference in frequencies of this cell type between CMV negative and CMV positive individuals, EBV serostatus also did not influence this (Figure 4.24 A-B). NK cells can also be classified as bright and dim, with the NK bright cells being more regulatory and the NK dim cells more cytotoxic in nature (Cooper *et al.*, 2001). Similarly CMV or EBV serostatus did not influence proportions of these subsets (Figure 4.24C-F).

NKT cells were defined as CD3⁺CD56⁺. CMV positive individuals displayed significantly higher frequencies of this cell type compared with CMV negative individuals (Figure 4.25 median 5.25% vs 3.01% p=0.0021). When split into four groups there were significantly higher percentages of NKT cells in the CMV+/EBV+ group (median of 5.48%) compared with the CMV-/EBV- group (0.67%). CMV-/EBV+ participants also displayed a lower median percentage of NKT cells (3.46%) compared to the double positive group (5.48%).

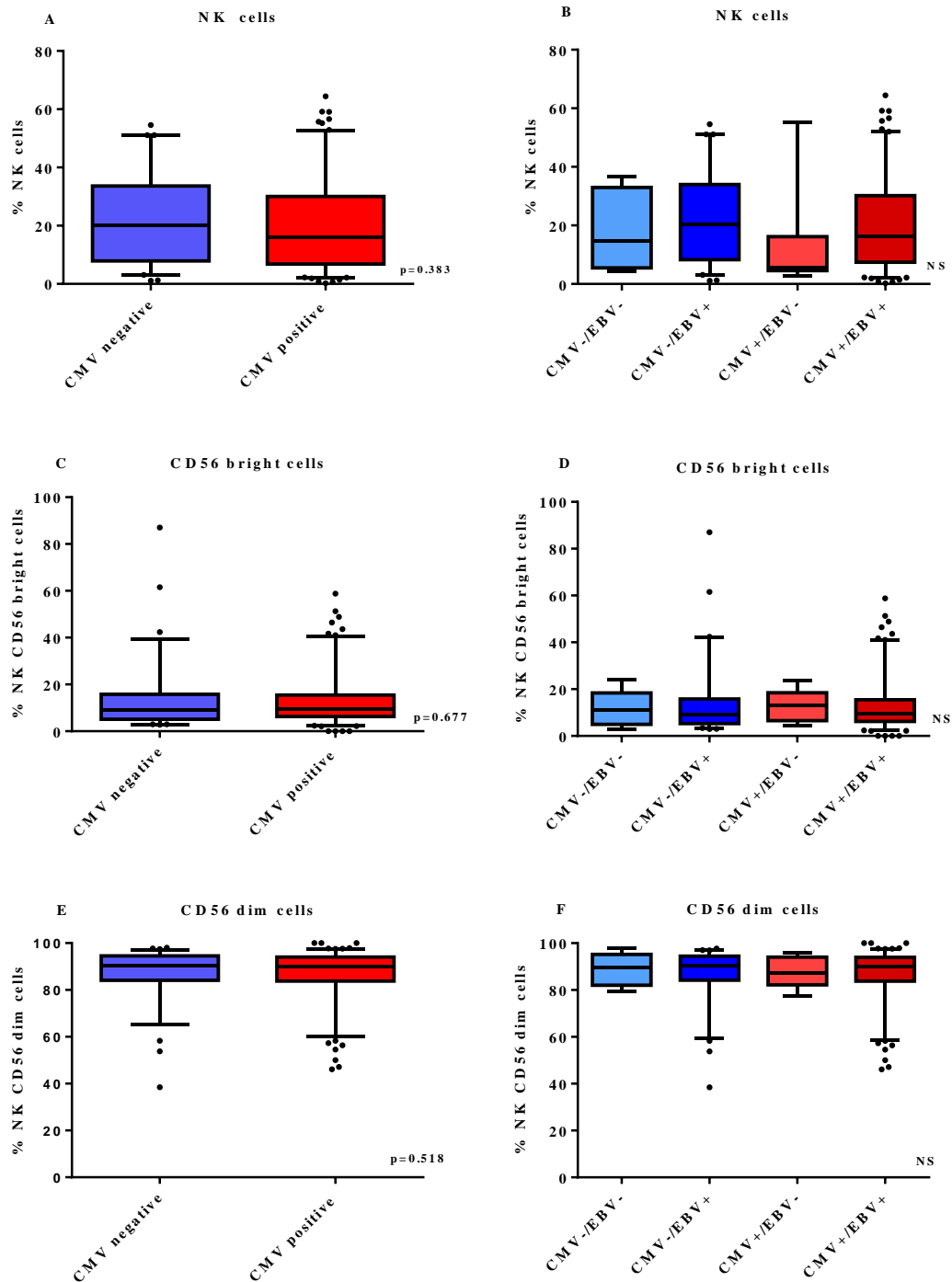


Figure 4-24. Frequencies of NK, NK CD56 bright and NK CD56 dim cells according to CMV and EBV serostatus

A. Frequencies of NK cells B. Frequencies of NK cells C. Frequencies of CD56 bright cells D frequencies of CD56 bright cells. E. CD56 dim cells. F. CD56 dim cells. Data shown as box-and-whisker plot with 5th and 95th percentiles. Data analysed by Mann-Whitney U-test or Kruskal -Wallis with Dunn's post-hoc test.

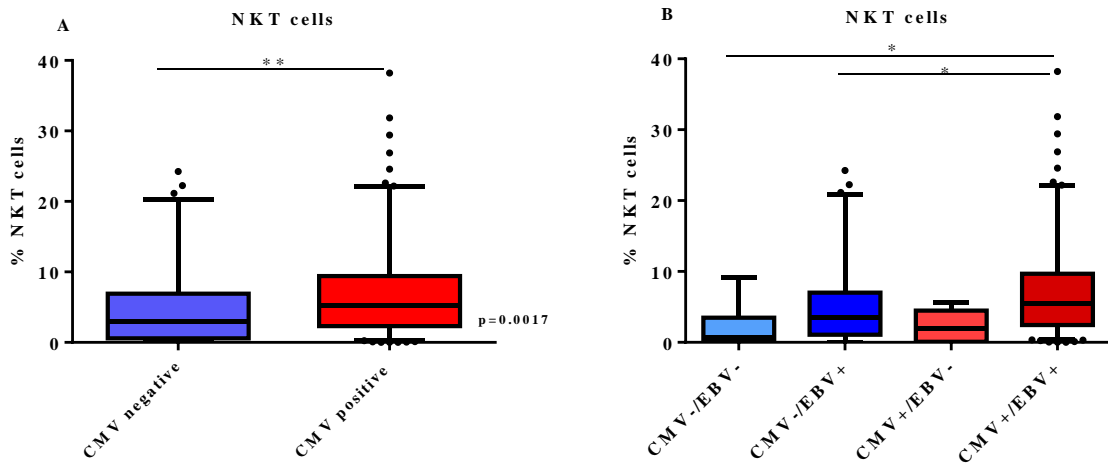


Figure 4-25. Frequencies of NKT cells.

A. Frequencies of NKT cells according to CMV serostatus. Frequencies of NKT cells according to CMV and EBV serostatus. Data shown as box-and-whiskers plot with 5th and 95th percentiles. Data analysed by Mann-Whitney U-Test and Kruskal- Wallis with Dunn's post-hoc test.

4.4.DISCUSSION

The current work examined the influence of CMV and EBV infection upon the immune repertoire in a unique cohort of older adults in the UK. For this analysis, 246 samples were analysed by flow cytometry and CMV and EBV serostatus examined. 165 CMV positive and 79 CMV negative individuals were studied, 15 of the participants studied were EBV negative within this group. CMV is of interest, particularly in older adults due to the large immune response to the virus; it has been observed that CMV-specific T cells can represent around 10% of the total effector memory CD4⁺ and CD8⁺ T cell repertoire (Sylwester *et al.*, 2005). The persistence of these responses are described as ‘memory inflation’ (Snyder *et al.*, 2011). Memory inflation is demonstrated via the MCMV model, where it has been observed that a few specific epitopes dominate inflationary responses (Munks *et al.*, 2006). In HCMV infection these specific cells tend to lack expression of CD27 and CD28 (O'hara *et al.*, 2012).

A number of established methods were used in order to produce these results. CMV serostatus was determined by an-in house IgG ELISA which has previously been published (Bartlett *et al.*, 2012). EBV serostatus was determined by a VCA ELISA, and where results were border-line this was repeated by immunofluorescence. Flow cytometry was used for most of this analysis; which gives a large data-set comparing multiple markers. However, one limitation of this method is that it has been described as partially subjective. A number of factors were put in place in order to ensure that the analysis was as reliable as possible. When samples were analysed by FACs, this was blinded so that the CMV or EBV serostatus of the samples were not known. Gating strategies were established at the start of the study, with discussion with experienced researchers within

the group. Standard templates were set-up in order to create consistency; gates were moved where appropriate. When necessary, this was also assessed by colleagues in order to ensure accuracy.

Similarly to other studies, CMV seropositive individuals displayed an inverted CD4/8 T cell ratio, involving an increase in CD8⁺ T cells and a decrease in CD4⁺ T cells. 58/246 of all donors (23.6%) and 44/165 of CMV positive donors (26.7%) displayed a CD4/8 T cell ratio of less than one. The OCTO and NONA studies investigated CMV serostatus and immune repertoire in a cohort of Swedish individuals; after the eight year follow up of the OCTO studies approximately 32% of individuals displayed an inverted ratio (absolute numbers) with CMV seropositivity contributing to decreasing the ratio (Olsson *et al.*, 2000, Wikby *et al.*, 2002). The HEXA study also observed a CMV related inverted CD4/8 T cell ratio in a group of individuals aged 66 (Strindhall *et al.*, 2012). HEXA participants are closer in age to those of the Lothian Birth Cohort; the present study confirms an association between CMV and an inverted ratio in adults aged 70. Recent evidence however suggests that this may not be limited to older adults. Turner *et al* demonstrated that 8% of a cohort of 48 healthy CMV positive university students displayed an inverted ratio (Turner *et al.*, 2013).

The impact of CMV upon proportions of T cell subsets were also examined. Frequencies of naïve T cells were much lower in the CMV seropositive group compared to the CMV negative group. This was particularly apparent within the CD8⁺ T cell compartment in which there was a two- fold decrease in naïve cells ($p < 0.0001$). Naïve T cells decline

with age, this decline is particularly apparent from middle age to old age (Almanzar *et al.*, 2005). Thymic involution is one reason for this, there is also evidence that adults thymectomized during childhood display an altered T cell compartment, with lower proportions of naïve T cells (Sauce *et al.*, 2009) It has been previously established that CMV seropositivity exacerbates the decline in naïve T cells that is observed in older adults (Pawelec *et al.*, 2010b, Weinberger *et al.*, 2007). Data from Derhovanessian *et al.* and the current study suggest that the decline in naïve T cells is more marked in the CD8⁺ T cell compartment compared with the CD4⁺ T cell compartments (Derhovanessian *et al.*, 2011). One reason for a more exaggerated decline in naïve CD8⁺ T cells with age could be that these cells are particularly susceptible to apoptosis compared to CD4⁺ T cells (Arnold *et al.*, 2011). Having fewer naïve T cells could be detrimental in terms of the ability to react to new pathogens (Fagnoni *et al.*, 2000). However, the Leiden 85 plus study demonstrated that a lower frequency of naïve CD8⁺ T cells was correlated with eight year survival within the cohort; suggesting a need for more studies in this area to establish the impact upon mortality and morbidity (Derhovanessian *et al.*, 2013a). In this work it was demonstrated that within the CMV seropositive group, the EBV seropositive donors had lower naïve T cells compared with EBV seronegative donors. This is an interesting finding as previous work has shown CMV to be a main driver of a decline in naïve T cells. Therefore more work is warranted in order to assess the influence of EBV upon the naïve repertoire.

In this study we found frequencies of CD4⁺ and CD8⁺ central memory T cells are lower in CMV positive individuals, this is particularly apparent in CD8⁺ T cell compartment. Other studies have demonstrated a decrease in central memory T cells in either CD4⁺ or

CD8⁺ T cells with CMV infection. (Chidrawar *et al.*, 2009) (Derhovanessian *et al.*, 2011).

The current work differs from other studies in terms of the effect of CMV upon effector memory T cells. In the current work, there was a slight increase in the frequencies of CD4⁺ effector memory T cells in CMV positive individuals; however CMV serostatus did not impact upon frequencies of CD8⁺ effector T cells. Other studies have found this subset to increase in CMV positive individuals in the CD4⁺ and CD8⁺ T cell compartments. (Chidrawar *et al.*, 2009, Derhovanessian *et al.*, 2013a). The population studied within the Lothian Cohort was significantly larger than work by most groups, which may explain the difference in results gained. However, CMV+/EBV+ participants displayed increased frequencies of CD4⁺ effector memory T cells compared with CMV+/EBV-, suggesting that there may be an association between EBV and frequencies of CD4⁺ effector memory T cells, which is a novel finding.

In the current study CMV seropositivity was associated with an increase in CD4⁺ and CD8⁺ EMRA T cells; which are CCR7⁻ but re-express CD45RA ($p < 0.0001$). These cells increase in frequency with age and chronic viral reactivation (Griffiths *et al.*, 2013). This work supports previous findings in which CMV seropositivity in older adults has been linked to an increase in these cells (Chidrawar *et al.*, 2009, Libri *et al.*, 2011). One reason for the accumulation of these cells could be that CD8⁺ EMRA T cells can be resistant to apoptosis (Gupta *et al.*, 2007). High proportions of EMRAs may be important in terms of immune function. EMRAs often express CD57 and KLRG1 and have previously been described as ‘exhausted T cells,’ (Fulop *et al.*, 2013). However, the description of ‘exhaustion’ is controversial, with some studies finding EMRA T cells to be functional, demonstrating cytotoxic potential through expression of granzyme B and perforin (Libri

et al., , Libri *et al.*, 2011).

In the current study, CMV seropositive individuals displayed higher frequencies of T cells which were CD57+CD28- compared with seronegative individuals and EMRA T cells will display this phenotype (Gupta *et al.*, 2004). Loss of CD28 is a factor associated with replicative senescence (Effros *et al.*, 1996). CD28 expression has also been found to be lower on CD4⁺ T cells in older adults and individuals with CMV infection (Pourghesari *et al.*, 2007, Solana *et al.*, 2012). Looney *et al* found that CD28 null CD4⁺ and CD8⁺ T cells accumulated in CMV positive individuals; when CMV serostatus was considered this was found to occur independently of age (Looney *et al.*, 1999) In contrast, cells positive for CD57 increase with age, and higher frequencies of both CD4⁺ and CD8⁺ T cells expressing CD57 occurred within the CMV positive group (Merino *et al.*, 1998) (Derhovanessian *et al.*, 2011, Wang *et al.*, 1993). Of interest, donors seropositive for both EBV and CMV displayed increased CD4⁺ CD57+CD28- T cells compared with CMV+/EBV- donors. It is difficult to draw conclusions from this data due to the low numbers of CMV+/EBV- participants. However, more work should be done to assess this association as it could be that EBV also has a role in driving T cell differentiation in older individuals.

The impact of CMV IgG titre upon the immune repertoire was examined. There was no association between CMV IgG titre and frequencies of either CD4⁺ or CD8⁺ naïve T cells. This is in contrast to previous findings; Alonso Arias *et al* observed a significant negative correlation between naïve CD4⁺ T cells and CMV titre (Alonso Arias *et al.*, 2013). The

current study did find a correlation for both CD4⁺ and CD8⁺ central memory cells. In contrast to previous work, there was no correlation found between effector memory CD4⁺ or CD8⁺ T cells and IgG titre; other studies have found an association between an increase in IgG titre and effector memory T cells (Alonso Arias *et al.*, 2013, Derhovanessian *et al.*, 2011). There was an association between increased CD4⁺ EMRA T cells and higher CMV antibody titre, but not CD8⁺ EMRA T cells. Data from the present study and from other studies collectively suggest that CMV antibody titres appear to influence various phenotypes of CD4⁺ T cells more so than CD8⁺ T cells. This could be due to the fact that CD4⁺ T cells can influence antibody producing cells (Zhu and Paul 2008). Although the CMV and EBV-specific T cell responses were not examined in this cohort inflation of EMRA T cells in CMV positive individuals could reflect the fact that these individuals may display high frequencies of CMV-specific T cells of this phenotype. CMV, HIV and HCV have all been implicated in driving an expansion in EMRAs, with EBV seemingly not having a role (Arnold *et al.*, 2011). This fits with the current study where EBV did not influence frequencies of EMRA T cells. As the EBV-specific T cell response is much smaller than that of CMV, it may not have the same impact that CMV-specific oligoclonal expansions have on the global T cell repertoire (Khan *et al.*, 2004).

Other lymphocyte subsets were also examined and no association was found between CMV or EBV infection and frequencies of NK cells. NK cells were defined as CD3-CD56+. No difference was found between total frequencies of NK cells according to CMV or EBV serostatus. There was also no influence of CMV or EBV on NK bright or dim cell subsets (data not shown). In contrast, a study by Chidrawer *et al* (2009) found an

increase in NK cells in the CMV positive participants which was attributable to an increase in the CD56 dim population (Chidrawar *et al.*, 2009). The HEXA cohort of older adults in Sweden found that CMV positive individuals displayed higher numbers of more highly differentiated NK cells, independent of changes to the T cell repertoire (Strindhall *et al.*, 2012). In the current study it was observed that CMV positive individuals displayed higher frequencies of NKT cells compared with CMV negative individuals. NKT cells possess features of both innate and adaptive immunity and are identified as CD3+CD56+. They are CD1 restricted T cells. (Terabe *et al.*, 2008). There is evidence to suggest that NKTs have anti-viral mechanisms which may explain why the frequencies of these cells are higher in the CMV seropositive group (Van Dommelen *et al.*, 2004).

There are consequences of an aged immune system. Some characteristics seen within this study, such as an inverted CD4/8 T cell ratio and highly differentiated T cells are parameters seen within the 'immune risk profile', associated with increased mortality risk in older adults (Olsson *et al.*, 2000). Previous studies have found higher antibody titres to be important clinically; with an association established between immune system function and functional ability as well as mortality (Gkrania-Klotsas *et al.*, 2013, Moro-Garcia *et al.*, 2012). Immunosenescence also has consequences in terms of infection and vaccine responses (Targonski *et al.*, 2007). This demonstrates the detrimental impact of CMV and immune senescence on older adults. EBV is another persistent herpes virus; the T cell response to EBV differs from that of CMV, with variation in terms of phenotype of T cells specific to lytic or latent epitopes of this virus (Hislop *et al.*, 2001).

A large and comprehensive data set was used in which to study the influence of CMV and EBV upon immunosenescence. CMV serostatus has been shown to alter the immune phenotype of the participants. However, explanations other than a causal relationship should be considered. Previous studies have demonstrated that CMV is more prevalent in lower socioeconomic groups, so it cannot be ruled out that socioeconomic status may confound the association between CMV and changes in immune repertoire (Dowd *et al.*, 2009b). Reverse causation has been demonstrated in other studies of health; in the case of this work, it would need to be considered whether immune phenotype was likely to influence the CMV serostatus or IgG titre of the participants (Gow *et al.*, 2012). Similar consideration should be applied to any relationship between EBV and alteration of immune phenotype.

An important aspect of the work is that both CMV and EBV were studied and that EBV may contribute to altering of immune repertoire. For example CMV+/EBV+ participants displayed an increase in CD4⁺ effector memory T cells, CD57⁺CD28⁻ T cells and a decline in both naïve CD4⁺ and CD8⁺ T cells. It may have been advantageous to analyse the EBV negative responses first as these are rarer and then design experiments from this data. Also, due to the rare nature of EBV seronegative donors it is important to acknowledge that a larger sample size would be needed to investigate co-infection in order to draw definite conclusions from the work.

In summary, the current work demonstrates that there is a relationship between CMV seropositivity and increased CMV IgG titres upon markers of immunosenescence. Also to some extent EBV infection may influence markers of immunosenescence in the Lothian Birth Cohort. It would be of interest in future to note how these factors impact upon mortality risk within the cohort.

Chapter 5. CMV- AND EBV-SPECIFIC IMMUNITY IN THE 1000 ELDERS COHORT

5.1.INTRODUCTION

CMV and EBV are both ubiquitous herpes viruses which persist in the host once infection has been established. Although CMV-specific immunity has been previously studied; the influence of co-infection with CMV and EBV is less well characterised (Harari *et al.*, 2004, Wallace *et al.*, 2011). The current work aimed to investigate immunity to CMV and EBV in a group of older adults the ‘1000 Elders’ cohort.

One known difference between the viruses is that CMV-specific T cell responses are large in magnitude and can constitute up to 10% of the peripheral CD8⁺ T cell pool, whereas EBV-specific T cell responses tend to be smaller (Khan *et al.*, 2004). An initial step was to determine whether these differences existed within the current cohort.

Differences have also been established in terms of the phenotype of CMV- and EBV-specific T cell responses. CMV-specific CD8⁺ T cells usually lack CD27 and CD28 expression (Pawelec *et al.*, 2011). In contrast, EBV- specific T cells generally retain CD28 and CD27, especially T cells specific for latent antigen (Hislop *et al.*, 2001). The highly differentiated phenotype of CMV-specific CD8⁺ T cells is particularly apparent in

older adults and is associated with immunosenescence (Pawelec and Derhovanessian 2011). However, this phenotype has also been established in CMV seropositive infants in the Gambia, demonstrating the ability of CMV infection to influence phenotype even in younger groups of individuals (Miles *et al.*, 2008). Previous data has shown that CMV seropositivity can influence phenotype and function of tuberculin-specific T cell responses (Terrazzini *et al.*, 2013). We wanted to assess whether CMV seropositivity impacted upon the phenotype of EBV-specific T cells. In order to do so, EBV tetramer responses were phenotyped using a panel of antibodies to cell surface markers of interest.

The stability of CMV and EBV-specific T cell responses over time is up for debate (Chidrawar *et al.*, 2009). At the clonal level, Klarenbeek *et al* demonstrated that CMV-specific CD8⁺ T cell clones were maintained over a five year period, with very few new clones occurring over this time point (Klarenbeek *et al.*, 2012). Neller *et al* used high throughput TCR sequencing of EBV-specific CD8⁺ T cell responses over an eighteen year period and found that clonotypes remained stable in magnitude (Neller *et al.*, 2013). In a study of EBV-specific T cell infection over a two year period, it has been demonstrated that EBV epitope-specific responses in acute infection do not always persist into latency and that some less dominant initial responses increase later on. However, in some individuals, the EBV-CD8⁺ T cell repertoire was maintained during a two year period, showing stability of responses (Catalina *et al.*, 2001).

The 1000 Elders cohort, which is a group of healthy older adults in Birmingham, presented a unique opportunity to monitor CMV and EBV MHC class I tetramer responses over a ten year period. In 2004 Khan *et al* assessed the frequencies of CMV-

and EBV- specific CD8⁺ T cells. Tetramer data from this time point was used for the current analysis, in addition PBMC samples were taken from the same individuals from the 1000 Elders cohort in order to assess the stability of CMV and EBV tetramer responses over time.

Studying EBV load enables assessment of the EBV virus host balance. EBV load is measured as EBV genome copies per million PBMC and presents another way of assessing EBV immunity. Higher EBV loads could suggest chronic reactivation; Stowe *et al* found EBV viral load to increase with age in the serum of individuals co-infected with CMV and EBV (Stowe *et al.*, 2007). Individuals in the current study had a median age of 81, there it was of use to assess whether in the setting of an aged immune system EBV loads were different compared to other donor groups and if CMV serostatus altered the EBV balance. In summary, this chapter aimed to assess CMV and EBV-specific T cell immunity in terms of magnitude, phenotype and stability. Immunity to EBV was also assessed in the context of EBV viral load, and the influence of CMV upon this examined.

5.1.1. The 1000 Elders Cohort recruitment

For this work we studied the 1000 Elders cohort; a group of older adults (65 years and older) from Birmingham. The cohort was initially formed in the 1980s and is currently run by Professor Janet Lord at the University of Birmingham. Participants who took part in a previous study in 2002-2003 were contacted as other donors who were on the 1000 Elders cohort database and who had not been bled within the past three months. In total 100 donors were contacted by letter. A total of 27 donors responded and were later consented and 50ml of blood taken at the Wellcome Trust Facility, Birmingham. Of the donors who consented, 14 had data available regarding their tetramer responses at the earlier time point. PBMCs and plasma were isolated for analysis in 27 donors. The median age of the group of individuals recruited was 81.

5.1.2. CMV and EBV infection status in the 1000 Elders Cohort

The CMV and EBV infection status was determined. Of the donors analysed by an in-house CMV IgG ELISA, 16/27 (59.3%) were CMV positive. The semi-quantitative CMV IgG titre was also determined (Figure 5.1). It was of interest to examine this in addition to CMV serostatus, as higher CMV antibody titres have been linked to mortality risk (Dollard *et al.*, 2011, Strandberg *et al.*, 2009). EBV serostatus was determined for all of the donors by anti-viral capsid antigen (VCA) immunofluorescence assay. Only one donor was considered to be EBV negative by this analysis. EBV load was also measured by qPCR and genome load per 10^6 PBMC established. EBV genome load could not be detected in 3 individuals.

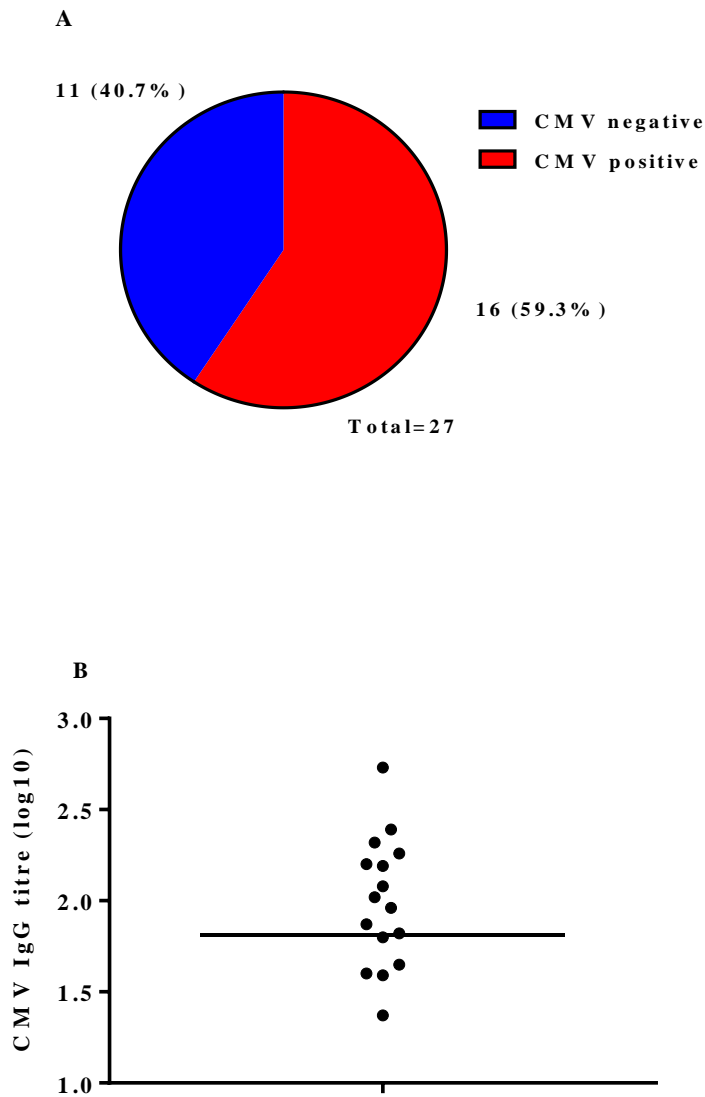


Figure 5-1. CMV serostatus and IgG titre in the 1000 Elders Cohort.

- A. Frequencies of CMV negative and CMV positive individuals as determined by an in-house CMV IgG ELISA. B. CMV IgG titre (log10) for CMV positive individuals only

5.2.RESULTS

5.2.1. Analysing CMV- and EBV-specific CD8⁺ T cell frequency by MHC class I tetramers

In order to assess the magnitude of CMV- and EBV-specific T cell responses in the cohort, MHC class I tetramers were used. A total of nine CMV and five EBV tetramers were available for use to analyse T cell responses (Table 5.1). PBMC samples were stained with the tetramers alongside an antibody panel and analysed by FACS. The gating strategy for CMV- and EBV-specific T cells is demonstrated in Figure 5.2. Firstly, doublets were gated out and single cells selected, followed by CD3 positive live cells and then lymphocytes. CD8⁺ T cells were then gated and tetramer responses reported as a percentage of total CD8⁺ T cells for each participant. Tetramer responses were validated by two researchers. In order to do this, all CD3 live lymphocytes were viewed to look at background staining and to decide whether the tetramer responses were genuine or whether there were no responses to that particular epitope. The smallest genuine responses recorded were 0.01%.

CMV and EBV tetramers are specific to certain HLA-alleles; the aim was to study a range of common HLA types within the groups where tetramers were available. For example, to analyse EBV-specific T cell responses, tetramers specific to two alleles, (A2 and B8) were available, covering three lytic (GLC, YVL,RAK) and two latent (CLG, FLR) peptides. There is the potential for bias, as there were no B7 tetramers available to analyse EBV-specific responses, so donors with this tissue type could not be studied.

Similarly, the CMV-specific responses were limited to certain tissue types; A1, A2, B7 and B8. Availability of tetramers meant that a number of pp65 epitopes (YSE, NLV, TPR, RPH) and IE1 epitopes (VLE, VLR, QIK, ELK) were studied with one pp50 epitope (VTE). Pp65 tetramers covered three HLA restrictions (A1, A2, B7) whereas IE-1 covered two (A2, B8). A notable limitation is that only one pp50 epitope was analysed (VTE), so therefore only A1 donors could be studied to investigate pp50 responses.

There were no significant differences between pp65 ,IE1 and pp50 (VTE) responses. The largest percentage of tetramer positive cells was against pp65 epitopes (3.25%) (Figure 5.3). IE1 responses represented 1.97% of the total CD8+ T cell pool. There were no significant differences in terms of the magnitude of EBV lytic compared with latent responses; however there was a trend towards a lower magnitude in terms of latent responses (Figure 5.4 mean of 1.2% vs 0.62%). It was noted that this was mainly due to small CLG responses, which are limited to A2 donors.

The association between CMV IgG titre and frequencies of CMV tetramer positive cells was examined; there was a significant correlation between these factors, suggesting that the higher the CMV IgG titre, the greater the magnitude of the CMV-specific T cell response (Figure 5.5 $R^2=0.406$ $p=0.0258$).

Table 5-1 CMV and EBV MHC Class I tetramers used for FACS analysis.

Antigen	HLA restriction	Peptide Sequence
pp65	A1	YSEHPTFTSQY
	A2	NLVPMVATV
	B7	TPRVTGGGAM
	B7	RIPHERNGFTVL
IE1	A2	VLEETSVML
	B8	QIKVRVDMV
	B8	ELRRKMMYM
	B8	ELKRKMIYM
pp50	A1	VTEHDTLLY
EBV lytic cycle		
BMLF1	A2	GLCTLVAML
BRLF1	A2	YVLDHLIVV
BZLF1	B8	RAKFKQLL
EBV latent cycle		
LMP2	A2	CLGGLLTMV
EBNA 3A	B8	FLRGRAYGL

* Epitope peptides are identified by the first three amino acids

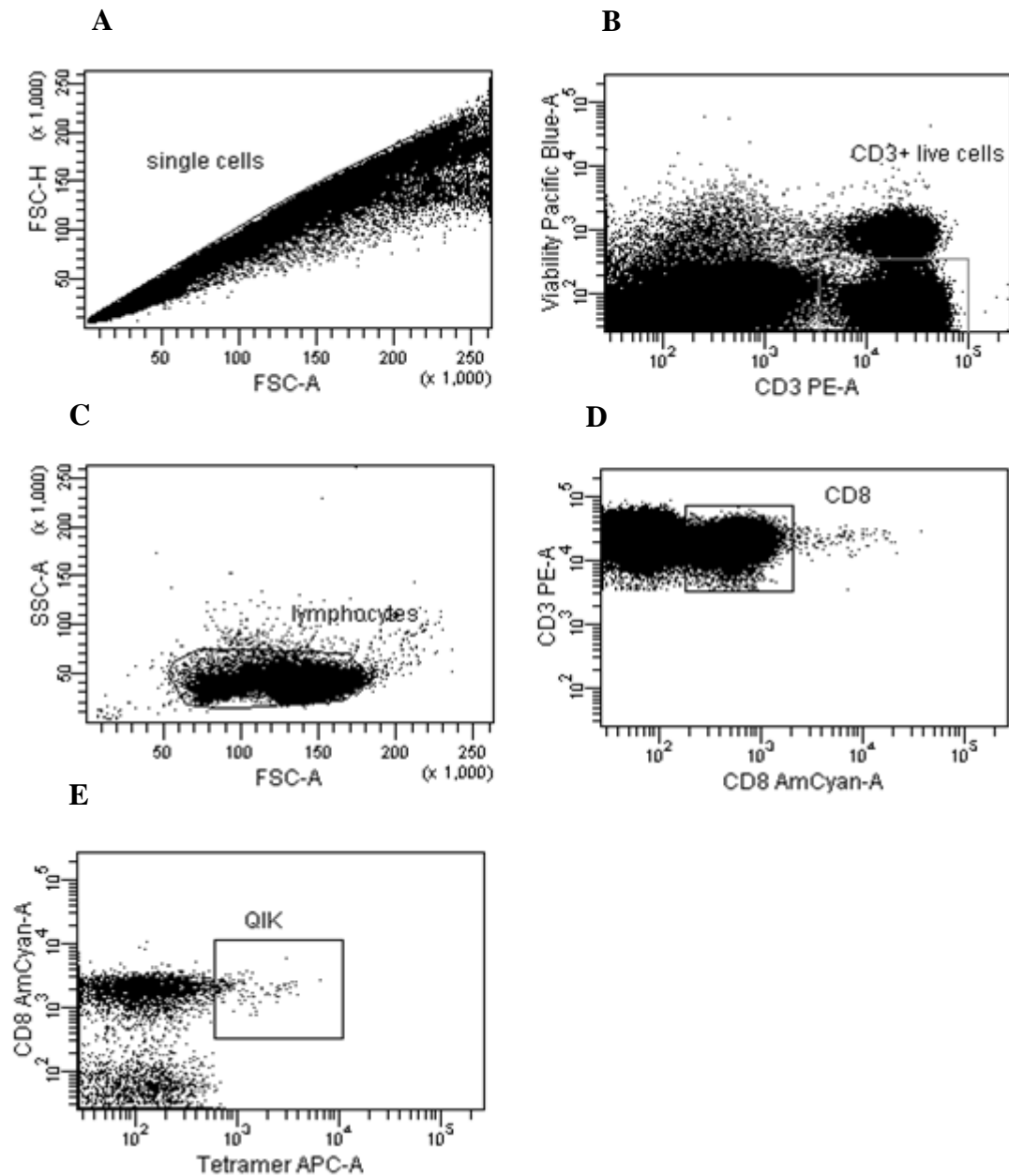


Figure 5-2. Gating strategy for tetramer positive cell analysis

PBMCs were thawed and stained with viability dye and MHC class I tetramers. PBMCs were then stained with an antibody panel including CD3, CD8, CD28, CD27, CCR7, CD45RA, CD45RO and CD38. PBMCs were then analysed by FACS (LSRII) and subsequently FACS DIVA (BD). A. doublets were excluded B. live CD3 positive cells were identified C. lymphocytes D. CD8⁺ T cells E. tetramer positive CD8⁺ T cell.

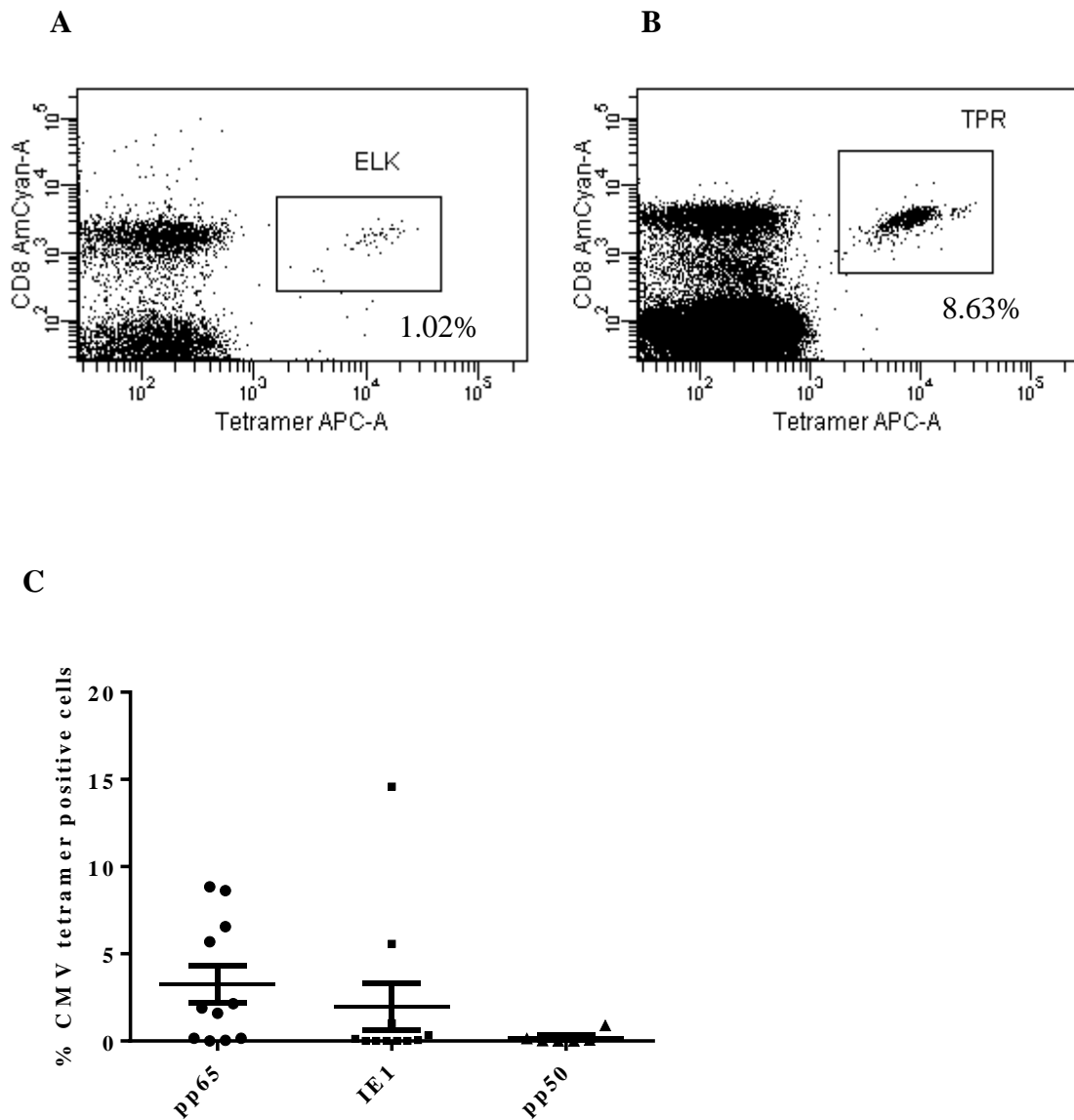


Figure 5-3. CMV-specific T cell responses, 2012-2013 data

A. ELK (pp65) tetramer response B. TPR (IE1) tetramer response. C. Responses shown are a sum of pp65, IE1 and pp50 tetramer responses per donor. Data analysed by one-way ANOVA (Kruskal-Wallis)

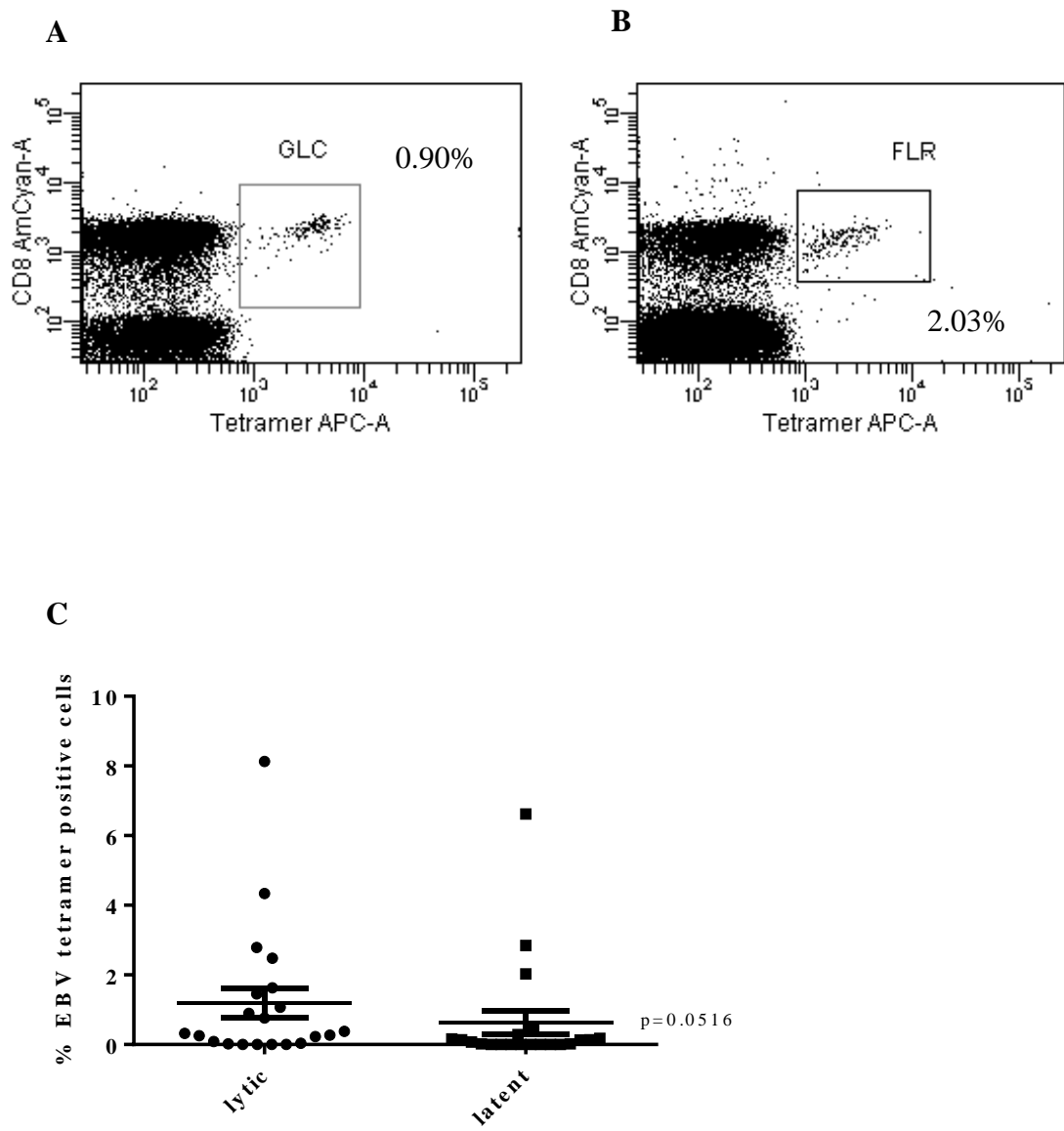


Figure 5-4. EBV-specific T cell responses 2012-2013 data

A. GLC, (EBV lytic) tetramer response B. FLR, (EBV latent) tetramer response C. Responses shown are a sum of lytic and latent responses per donor. Data analysed by Mann-Whitney U- Test.

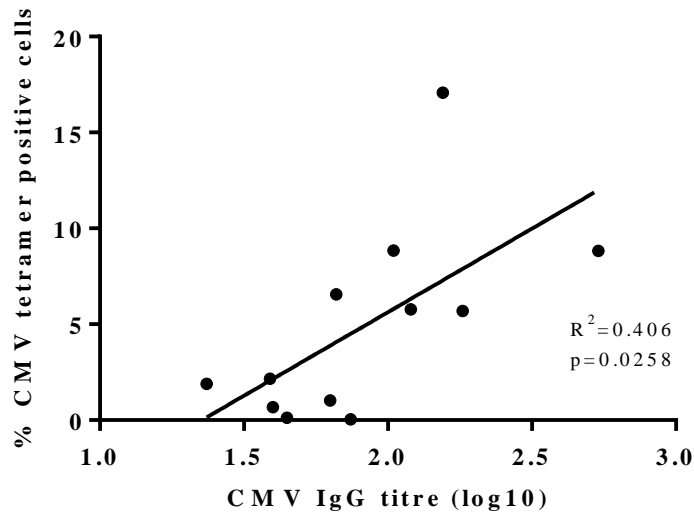


Figure 5-5. CMV IgG titre correlates with the frequency of CMV tetramer responses.

The relationship between CMV IgG titre (log10) as determined by an in-house CMV IgG ELISA, upon frequencies of CMV tetramer positive cells was assessed. Data was analysed by Pearson's correlation and the R² and p.values displayed

5.2.2. CMV serostatus does not influence the frequency of the EBV-specific T cell response

Representative lytic and latent EBV-specific T cell responses were measured using MHC Class I tetramers, to assess the influence of CMV serostatus upon the frequency of EBV-specific T cells. Although there was a trend towards an increased EBV-specific T cell frequency in CMV negative individuals (mean frequency of 2.59 vs. 1.12), this was not significant (Figure 5.6A). Similarly, there was no association between EBV-specific T cell responses to lytic or latent antigens and CMV serostatus (Figure 5.6 C, E). This was also reflected when absolute numbers of EBV tetramer positive cells were examined according to CMV serostatus (Figure 5.6 B, D, and F).

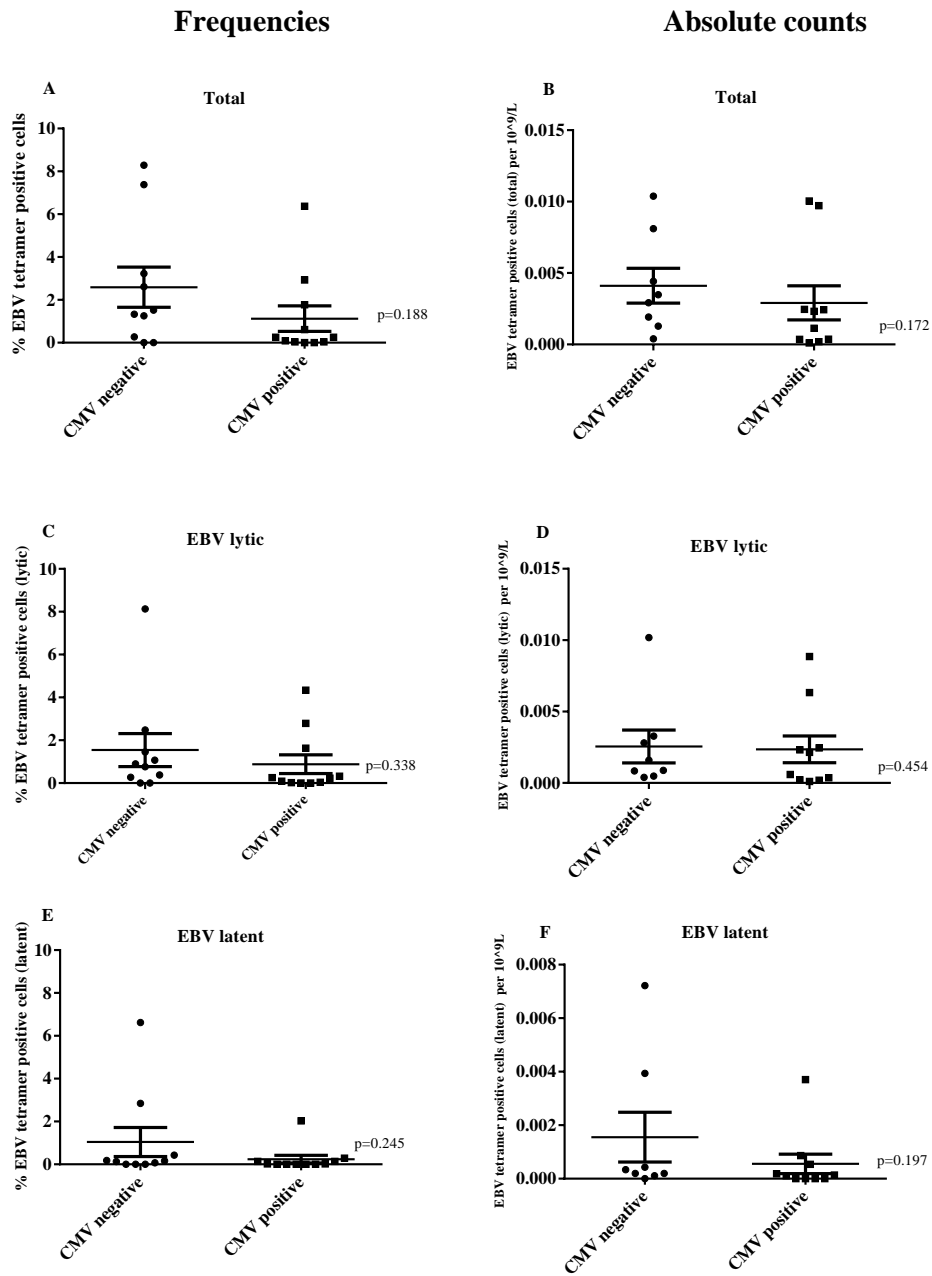


Figure 5-6 The relationship between CMV serostatus and EBV-specific T cell frequencies and absolute counts

PBMCS from CMV seronegative and CMV seropositive donors were stained with an antibody panel and MHC Class I tetramers for EBV-specific epitopes (lytic and latent) and analysed using LSR II (BD) and FACS DIVA (BD). A. frequencies of EBV-specific T cells B. absolute number of EBV-specific T cells C. frequencies of T cells specific to EBV lytic antigen D. absolute number of T cells specific to EBV lytic antigen E. frequencies of T cells specific to EBV latent antigen F. Absolute number of T cells specific to EBV latent antigen. Data analysed by Mann-Whitney U Test.

5.2.3. CMV- and EBV-specific CD8⁺ T cell responses display significantly different T cell phenotypes

The next aim was to establish the differences in terms of CMV- and EBV- specific T cell phenotype in the 1000 Elders. Differences in phenotype between CMV-specific T cell responses (pp65 and IE1) and EBV- lytic and latent-specific T cell responses were assessed by FACS using a panel of antibodies of interest (Table 5.2).

Results of the analysis are demonstrated in Figure 5.7. Differences were found between frequencies of CMV- specific and EBV- specific CD28⁺ T cells (Figure 5.7A). Frequencies of CD28⁺ pp65-specific T cells (mean of 31.40%) were lower than CD28⁺ EBV T cells specific for latent (72.91%) or EBV lytic antigen (67.74%). Similarly there were lower percentages of CD28⁺ IE1 specific T cells compared with CD28⁺ T cells specific for EBV latent antigen (Figure 5.7A) 28.51% vs. 72.91%).

A similar pattern was observed for CD27⁺ antigen specific cells (Figure 5.7B). Frequencies of CD27⁺pp65-specific T cells and CD27⁺ IE1-specific T cells (17.38%) were lower than CD27⁺ T cells specific for lytic (25.16% vs. 67.69%) and latent antigen (25.16% vs. 78.52%).

There were significantly lower proportions of pp65-specific CCR7⁺ T cells (1.72%) compared with CCR7⁺ T cells specific for EBV latent (16.12%), as well as lytic antigen (5.98%), (Figure 5.7 C). In contrast, there were higher frequencies of CD45RA⁺ pp65-

specific T cells and CD45RA+ IE-1 cells (60.83%) compared with CD45RA+ T cells specific for EBV latent antigen (54.00% vs. 13.44%), (Figure 5.7 D). There were lower frequencies of CMV-specific T cells which were CD45RO+ (pp65) compared with CD45RO+ cells within the EBV latent response (80.98% vs. 41.10%), (Figure 5.7E). CD38 is a marker of activation, there were increased frequencies of CD38+ T cells specific for EBV lytic antigen compared to IE1 (10.82% vs. 2.03%).

Table 5-2. Antibodies used for flow cytometric phenotyping of tetramer positive cells

Antigen	Clone	Fluorophore	Company	Amount used (μl)/ml
Viability dye		Pacific blue	Invitrogen	1 (1/100)
CD3	SK7	PE	Ebioscience	0.05
CD8	SK1	Amcyan	BD	0.05
CCR7	150503	FITC	R&D Systems	1.0
CD45RO	UCHL1	ECD	Beckman Coulter	1.0
CD45RA	HI 100	AF700	Biologend	0.05
CD38	HIT2	PE-Cy7	Biologend	0.5
CD28	CD28.2	Percpcy5.5	eBioscience	0.6
CD27	O323	APC-Cy7	eBioscience	0.4

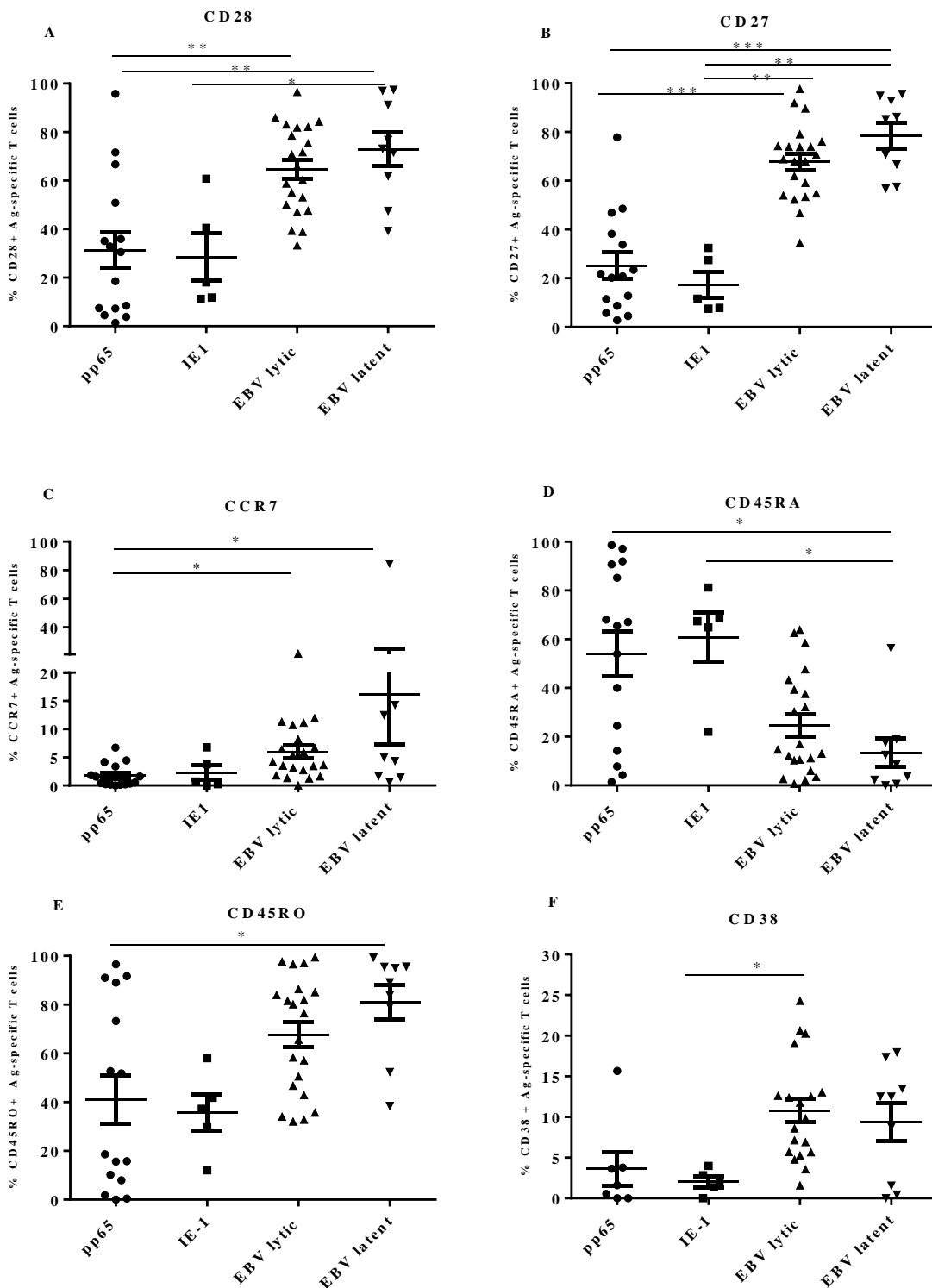


Figure 5-7. Analysis of cell subsets within CMV- and EBV-specific T cell responses
CD28 positive cells, B. CD27 positive cells C.CCR7 positive cells D. CD45RA positive cells, E. CD45RO positive cells. F. CD38 positive cells .Data analysed by Kruskal-Wallis with Dunn's post hoc test.

5.2.4. Distribution of CMV- and EBV-specific responses within naive, central memory, effector memory and EMRA T cell populations

The frequency of CMV- and EBV-specific T cell responses were then split into different memory populations (Figure 5.8). Significant differences in cell surface phenotype were observed between CMV-specific and EBV-specific T cell responses. A greater proportion of the CMV-specific T cells were classed as EMRA compared to the EBV-specific T cells (Figure 5.8E), with pp65 (mean of 53.48%) and IE1 (59.37%) responses containing higher frequencies of these cells compared with the EBV latent response (4.61%). There were higher frequencies of central memory EBV-specific T cells (for lytic antigen) compared with pp65-specific T cells (3.04% vs. 1.01%). The frequencies of central memory EBV-specific T cells for latent antigen appeared higher compared to the lytic epitope-specific T cells (Figure 5.8B) however this was not significant. There was a trend towards a higher frequency of naive cells specific for EBV-latent antigen compared with pp65-specific T cells (Figure 5.8A), however this was not significant (8.88% vs. 0.26%). CMV and EBV-specific effector memory T cells were also examined (Figure 5.8C). There were higher frequencies of effector memory T cells specific for EBV-latent antigen compared with frequencies of effector memory pp65-specific T cells (79.90% vs. 45.25%). Overall this data suggests that some CMV-specific T cell responses differ in terms of phenotype when compared to EBV-specific responses.

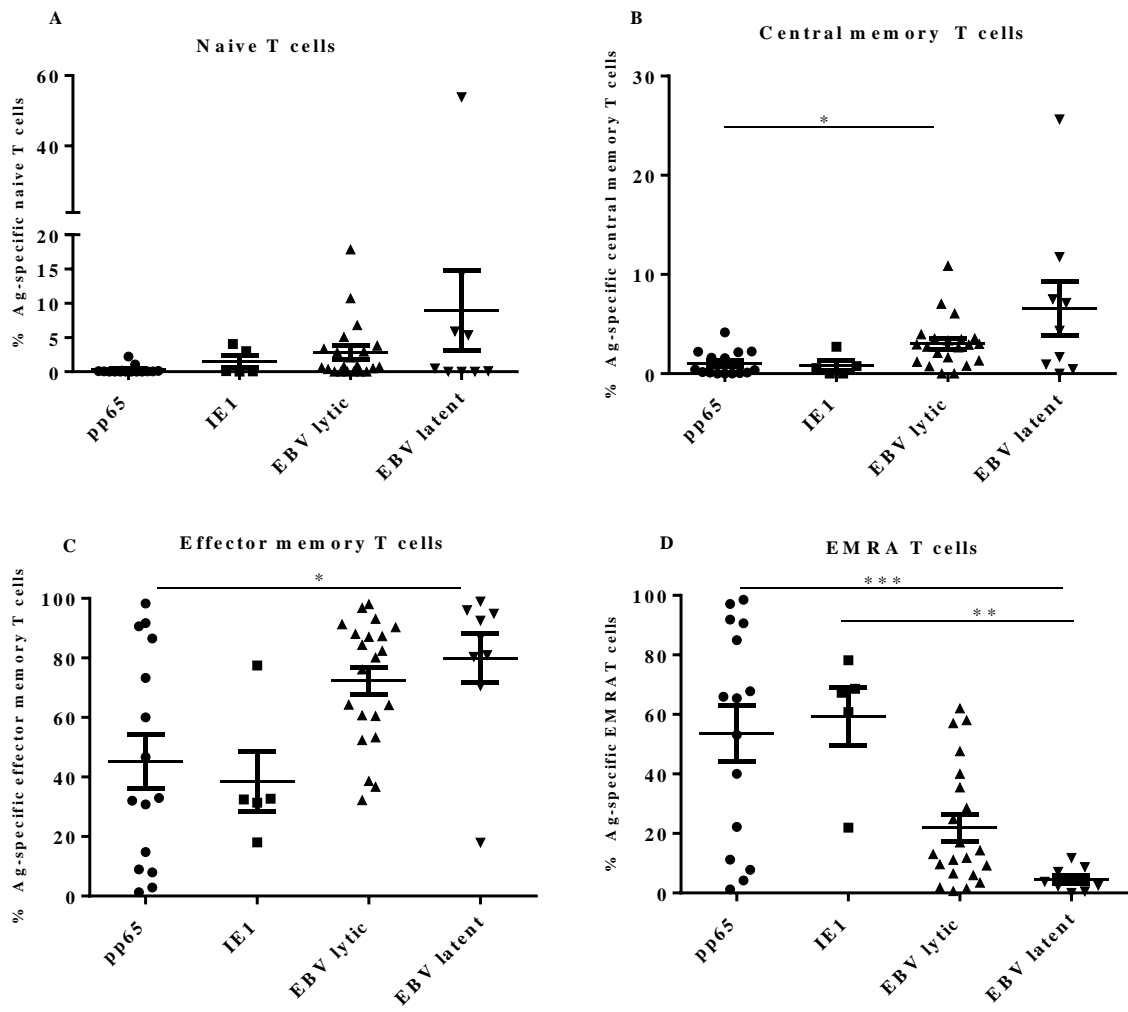


Figure 5-8 CMV- and EBV- specific T cell subsets.

A. CMV- and EBV-specific naive T cells (CCR7+CD45RA+) B. CMV and EBV-specific central memory T cells (CCR7+CD45RA-). C. CMV- and EBV-specific effector memory T cells (CCR7-CD45RA-). D CMV- and EBV- specific EMRA T cells (CCR7-CD45RA+).

Data analysed by Kruskal-Wallis with Dunn's post hoc test.

5.2.5. The impact of CMV infection upon the phenotype of EBV-Specific CD8⁺ T cell responses

It was clear from the previous analysis that the phenotype of CMV and EBV-specific responses differed, it was then of interest to assess the impact of CMV infection upon the phenotype of EBV-specific responses. CMV serostatus did not significantly impact upon the phenotype of EBV-specific responses. CMV serostatus did not significantly impact upon the phenotype of EBV lytic T cell responses (Figure 5.9). There was a trend towards lower frequencies of CD28⁺ T cells specific for latent antigen in the CMV seropositive group (Figure 5.9C, 56.79% vs. 76.45%), however this was not significant. Similarly there was a similar trend with the CD27⁺ T cells specific for latent antigen between CMV seropositive and negative (56.42% vs. 83.25%). There appeared to be higher frequencies of EMRA T cells specific for EBV latent antigen in the CMV seropositive group compared with the seronegative, however again this was not significant (Figure 5.9D, 15.69% vs. 5.09%).

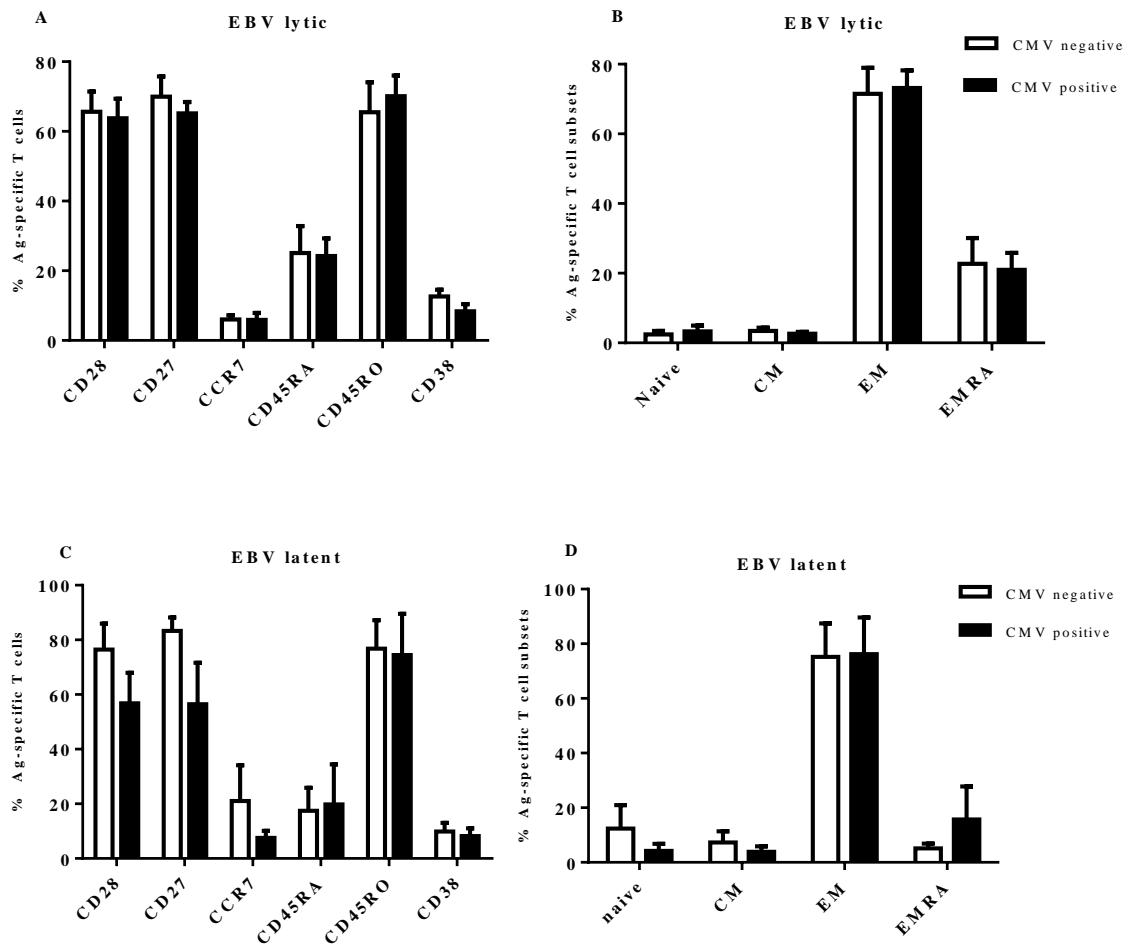


Figure 5-9 Frequencies of antigen-specific T cells defined by phenotype according to CMV serostatus.

A Frequencies of T cells specific for EBV-lytic antigens according to phenotype and CMV serostatus. *B*. Frequencies of naive, central memory, effector memory and EMRA T cells specific for EBV-lytic antigen. *C*. Frequencies of T cells specific for EBV-latent antigens according to phenotype and CMV serostatus. *D* Frequencies of naive, central memory, effector memory and EMRA T cells specific for EBV-latent antigens according to CMV serostatus. Data analysed by Kruskal-Wallis with Dunn's post hoc test.

5.2.6. CMV- and EBV-specific CD8⁺ T cell responses remain stable over time

We next asked if the frequency of CMV- and EBV-specific CD8⁺ T cells changes over a 10 year time course. The frequency of the CMV- and EBV-specific tetramer responses were compared at time-points; 2002-2003 and 2012-2013. The earlier data was generated by Khan *et al* and published in 2004 (Khan *et al.*, 2004). Although some differences were observed in individual participants, the pp65 and the IE1 responses remained stable across the time analysed, with no statistically significant difference between the two time points (figure 5.10 A-B). There was a trend towards a decrease in pp50 responses between 2002-2003 and 2012-2013, however this was not significant (Figure 5.10C). EBV lytic and EBV latent tetramer responses remained stable between the two time-points, with no statistically significant differences (Figure 5.11). These responses were much smaller in frequency compared with the CMV-specific T cell responses. It can be concluded from this data that CMV and EBV tetramer responses remain relatively stable over a ten year period within this cohort of older adults.

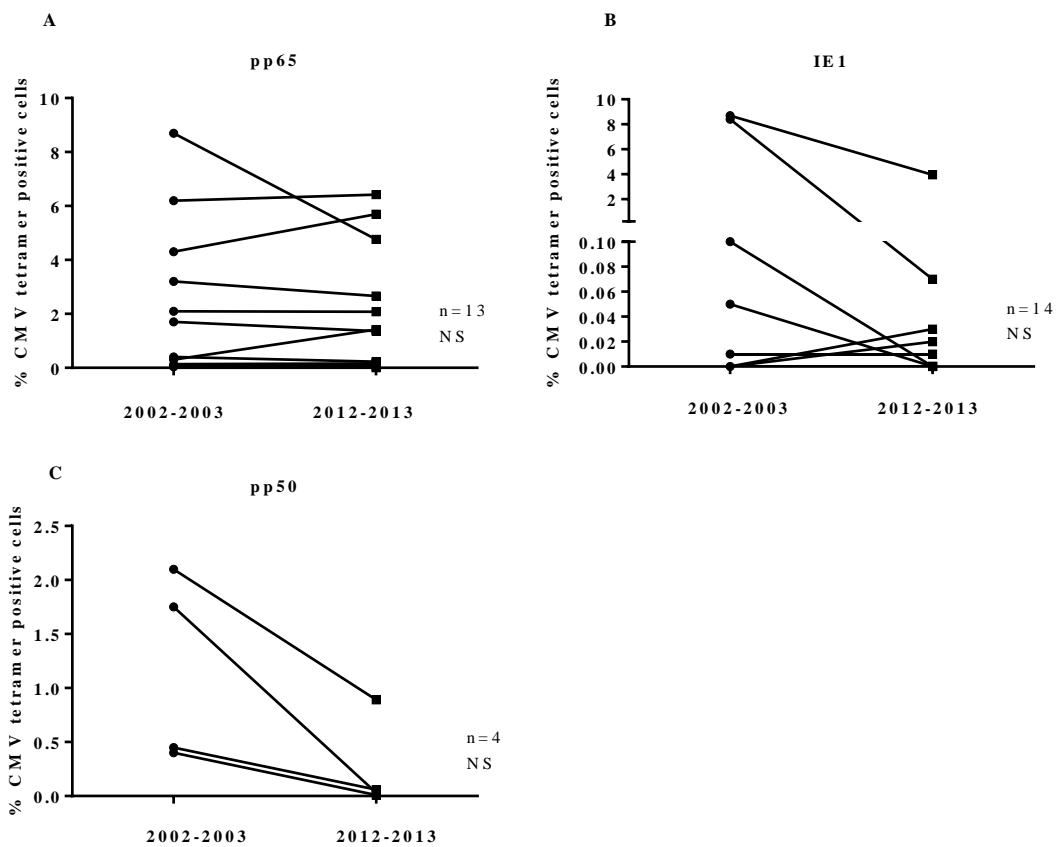


Figure 5-10 Stability of CMV-specific T cells over time.

PBMCs were stained with MHC class I tetramers along with an antibody panel as previously described. Data shown is from two time-points, 2002-2003 and 2012-2013A. pp65: sum of responses to NLV, YSE, TPR, RPH, B. IE1: sum of responses to VLE, QIK, ELR, ELK. C. pp50: responses to VLE. Data analysed by Wilcoxon matched-pairs signed rank test.

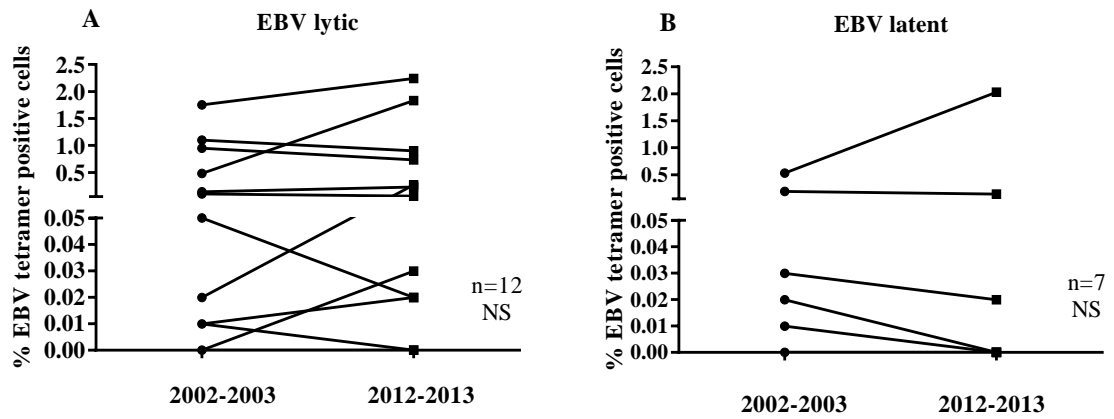


Figure 5-11 Stability of EBV-specific T cells over time.

PBMCs were stained with MHC class I tetramers along with an antibody panel. Data shown is from two time-points, 2002-2003 and 2012-2013 A. EBV lytic: Sum of responses to GLC, YVL, RAK . B. EBV latent: Sum of responses to CLG, FLR. Data analysed by Wilcoxon matched-pairs signed rank test.

5.2.7. Assessing EBV viral load in the 1000 Elders Cohort and younger donors

EBV load gives a good reflection of virus host balance; therefore EBV load was determined by qPCR and data shown as EBV genome per 10^6 PBMC (Figure 5.12). The mean viral load in this cohort was 831.8 per 1×10^6 PMBC/2.92 (log10) genome copies per 10^6 PBMC and a median load of 1122.0/3.05 (log10). This is considered a high load compared with younger donors, displaying a mean genome load of 1.7 (log10), however is lower than IM donors with a mean load of 14454.4/ 4.16 (log10) $\times 10^6$ PBMC. EBV load was then assessed with reference to age. Younger UK adults used for this analysis were aged 24-60 years and the 1000 Elders were aged 70-90 years. There is a strong correlation between an increase in age and EBV viral load (log10), ($R^2=0.46$, $p<0.0001$).

As EBV is found in the memory B cells it was of interest to assess whether 1000 Elders donors had normal percentages of B cells. This was assessed by FACs analysis using antibodies to CD19 and CD27. 5.1% of lymphocytes were demonstrated to be B cells in the cohort. A previous study found B cell frequencies to be between 6.6- 25.8% (Morbach *et al.*, 2010). Memory B cells (CD19+ CD27+) consist of 20-30% of B cells (Perez-Andres *et al.*, 2010). In this cohort the mean frequency of memory B cells (CD19+CD27+) was 19.8%. Naïve B cells (CD19+CD27-) have been shown to represent ~80% of B cells in older adults (Chong *et al.*, 2005). In this cohort the mean frequency of naïve B cells was 80.9% of total B cells.

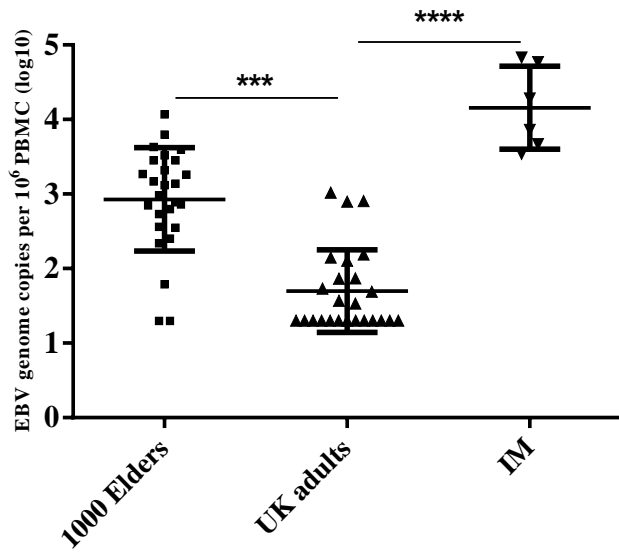


Figure 5-12 EBV viral load

From healthy lab donors, the 1000 Elders cohort and IM patients.

Data from UK adults and IM patients was generated by A.Hislop and permission given to use. Data analysed by Kruskal-Wallis with Dunn's post hoc test.

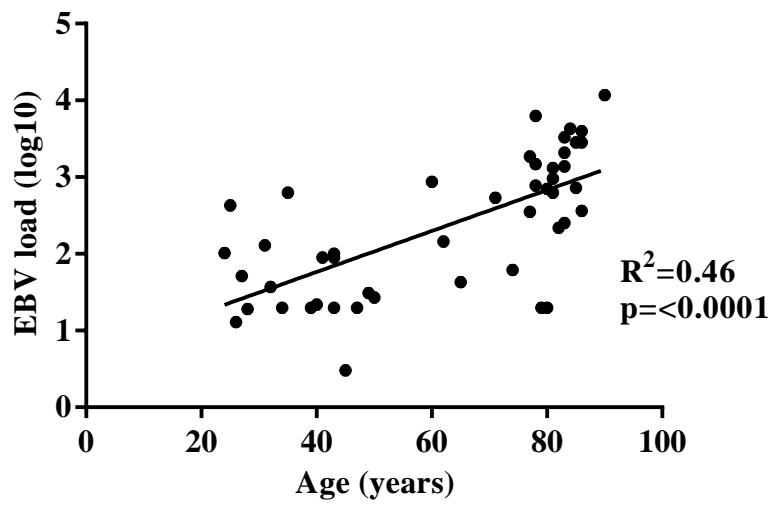


Figure 5-13 EBV viral and age

Data shown is from the 1000 Elders cohort and UK younger adults.

The data from UK adults (healthy laboratory donors) was generated by A.Hislop and permission given to use.

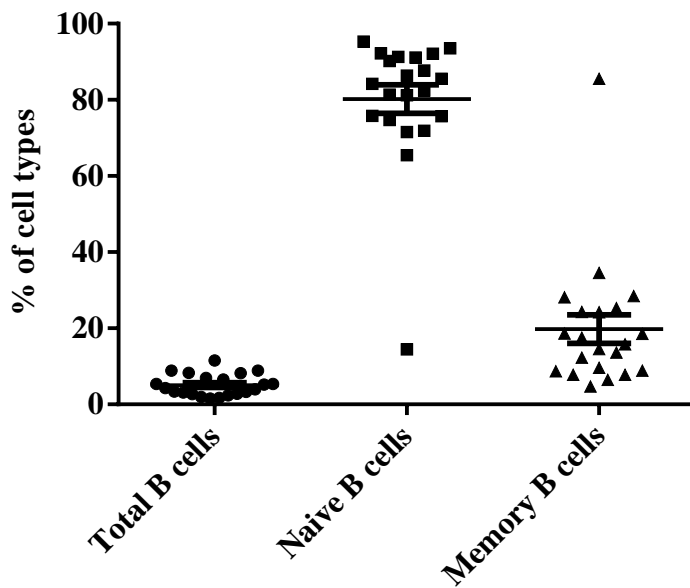


Figure 5-14. Frequencies of B cells in the 1000 Elders cohort

PBMCs were stained with viability dye and an antibody panel including CD3 to exclude T cells, CD19 and CD27 for identifying B cells. Frequencies of total B cells (CD19 positive cells are a percentage of all lymphocytes), naïve B cells (CD27⁻ cells as a proportion of CD19⁺ cells) and memory B cells (CD27⁺ cells as a proportion of CD19⁺ cells). Data analysed by Kruskal-Wallis with Dunn's post hoc test.

5.2.8. CMV negative participants display significantly higher EBV viral loads

Whether CMV infection had an impact upon EBV virus host balance was assessed by investigating the influence of CMV serostatus upon EBV viral loads. EBV loads were significantly higher in CMV negative individuals compared with seropositive individuals (Figure 5.15, mean of 3.27 (log10) compared with 2.68 (log10) $p=0.0245$). However, there was no significant correlation between EBV load and CMV-specific antibody titre within CMV positive individuals, demonstrating in this cohort that EBV load is affected by serostatus but not CMV IgG titre (Figure 5.16 $R^2= 0.184$, $p=0.818$).

As EBV resides in the B cells it was of use to investigate whether CMV serostatus influenced proportions of B cells. There is a trend towards lower proportions of Total B cells in CMV seropositive donors; however this did not reach significance. There were no differences between frequencies memory B cells or naïve B cells between CMV seronegative and CMV seropositive groups (figure 5.17).

EBV-specific T cell responses correlated with EBV viral load, in terms of total T cell response ($R^2= 0.186$, $p=0.073$). However, there was no correlation between EBV viral load or responses to lytic ($R^2= 0.241$, $p=0.104$) or latent ($R^2=0.0589$, $p=0.403$) antigens (Figure 5.18).

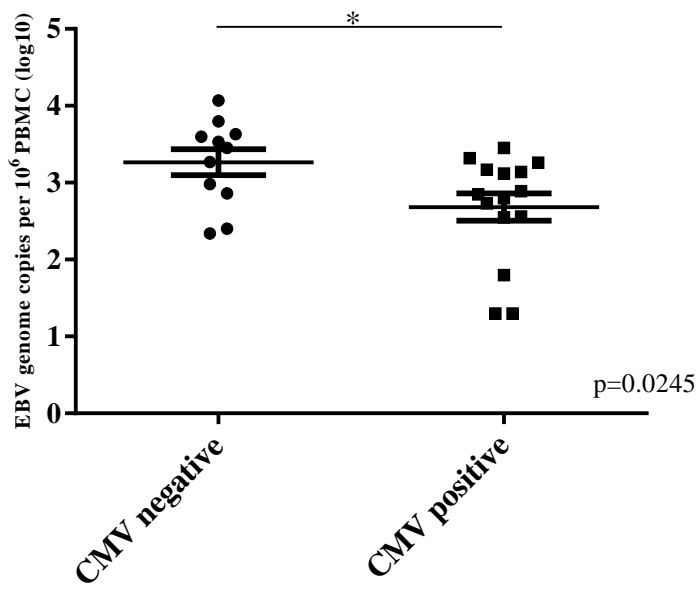


Figure 5-15 EBV load and CMV serostatus

EBV load was determined by qPCR .CMV serostatus was determined by an in-house CMV IgG ELISA. Data analysed by Mann-Whitney U-test

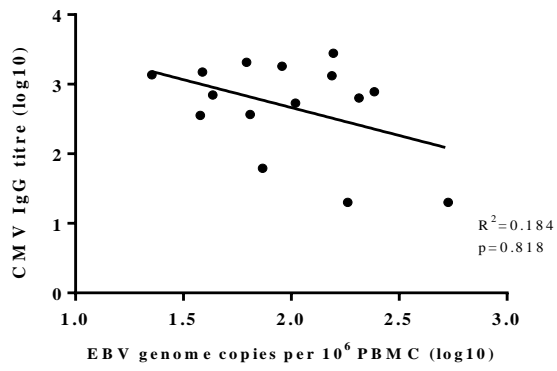


Figure 5-16. Correlation between EBV load and CMV IgG titre.
Correlation assessed using Pearson's correlation and R^2 and p -values stated.

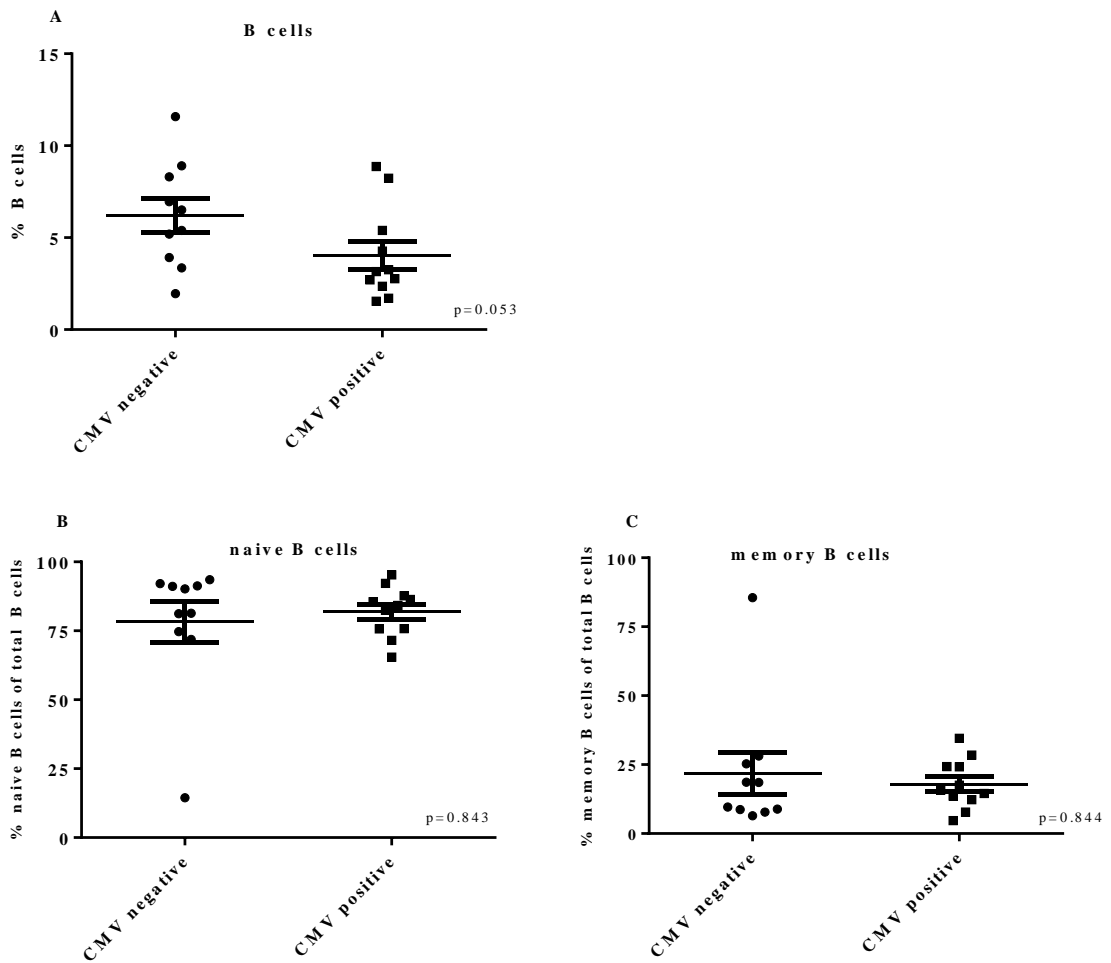


Figure 5-17. Frequencies of total, naive and memory B cells in CMV negative and CMV positive individuals.

PBMCs were stained with viability dye and an antibody panel including CD3, CD4, CD19 and CD27 for identifying B cells. A. B cells as a percentage of all lymphocytes B. percentage of naive B cells defined as (CD19+CD27-) of total B cells C. percentage of memory B cells defined as (CD19+CD27+) of total B cells

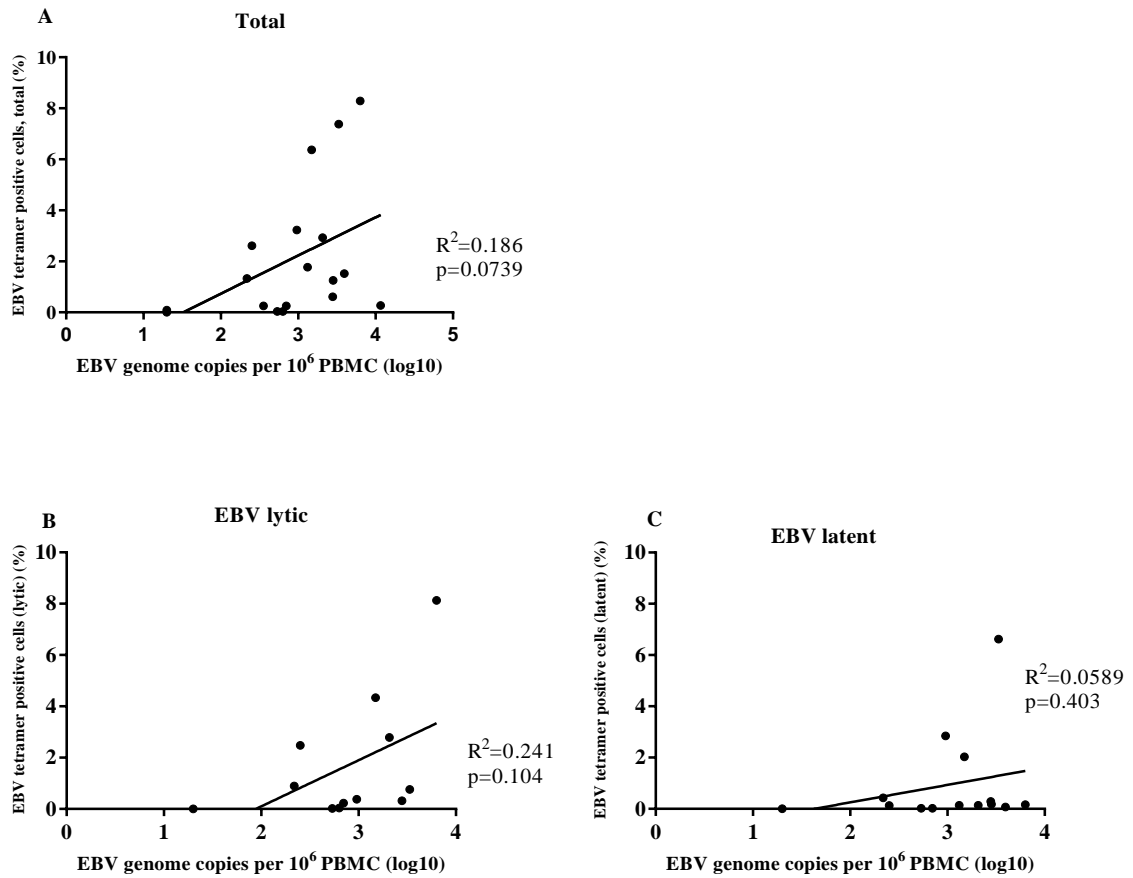


Figure 5-18. The association between EBV viral load and EBV -specific T cell responses.

EBV load was determined by qPCR. PBMCs were stained with MHC class I tetramers. A. all EBV-specific T cell responses (lytic and latent). B. total lytic responses C. total latent response. Data analysed by Pearson's correlation and R^2 and p.values displayed.

5.2.9. IFN γ secreting capacity of CD8⁺ T cells from 1000 Elders donors

It was demonstrated previously that there was no significant difference in terms of the frequency of the EBV-specific T cell response between CMV seronegative and CMV seropositive groups, despite infected donors displaying lower EBV viral loads. As there was no difference in frequency of EBV-specific T cells between these groups it was of interest to assess whether EBV-specific T cells from CMV infected donors displayed a greater capacity to make a functional response. The ability of CD8⁺ T cells from CMV seropositive and CMV seronegative donors to secrete IFN γ as a representative function was assessed when challenged with a range of EBV-specific peptides.

The gating strategy for CD8⁺ cells producing IFN γ after peptide stimulation is demonstrated in Figure 5.18. When PBMCs from all donors were analysed there was no difference between the frequency of CD8⁺ cells producing IFN γ in response to either lytic or latent stimulation (Figure 5.20 A-B, mean of. 0.60 vs 0.58 p=0.974). Similarly, CMV serostatus did not impact on functionality, as percentages of IFN γ secreting cells were similar between the two groups in terms of total IFN γ production (1.04 vs. 1.32 p=0.617), and when split into responses to lytic and latent peptides (Figure 5.20 C-D).

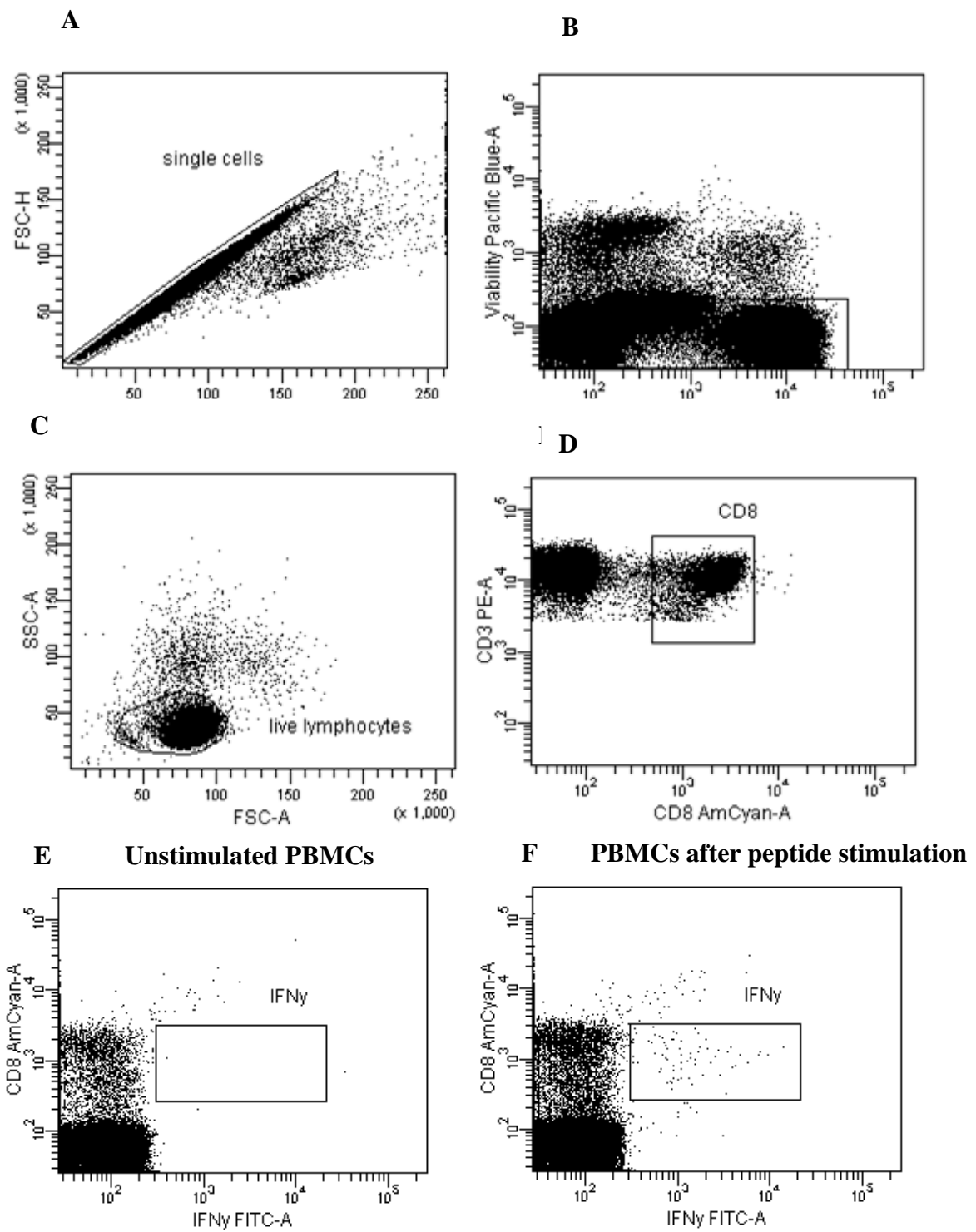


Figure 5-19 Gating strategy showing IFN γ producing CD8⁺ T cells after EBV peptide stimulation for 18 hours

PBMCs were stained with viability dye and an antibody panel (CD3, CD4, and CD8) then fixed and permeabilised before being stained with a cytokine antibody (IFN γ). Data was analysed using LSR II (BD) and FACS DIVA. A. Doublets were removed B. live CD3⁺ cells were identified C. live lymphocytes D. CD8⁺ T cells E. CD8⁺ producing IFN γ , unstimulated sample F. CD8⁺ T cell producing IFN γ after overnight stimulation with EBV lytic and latent peptides

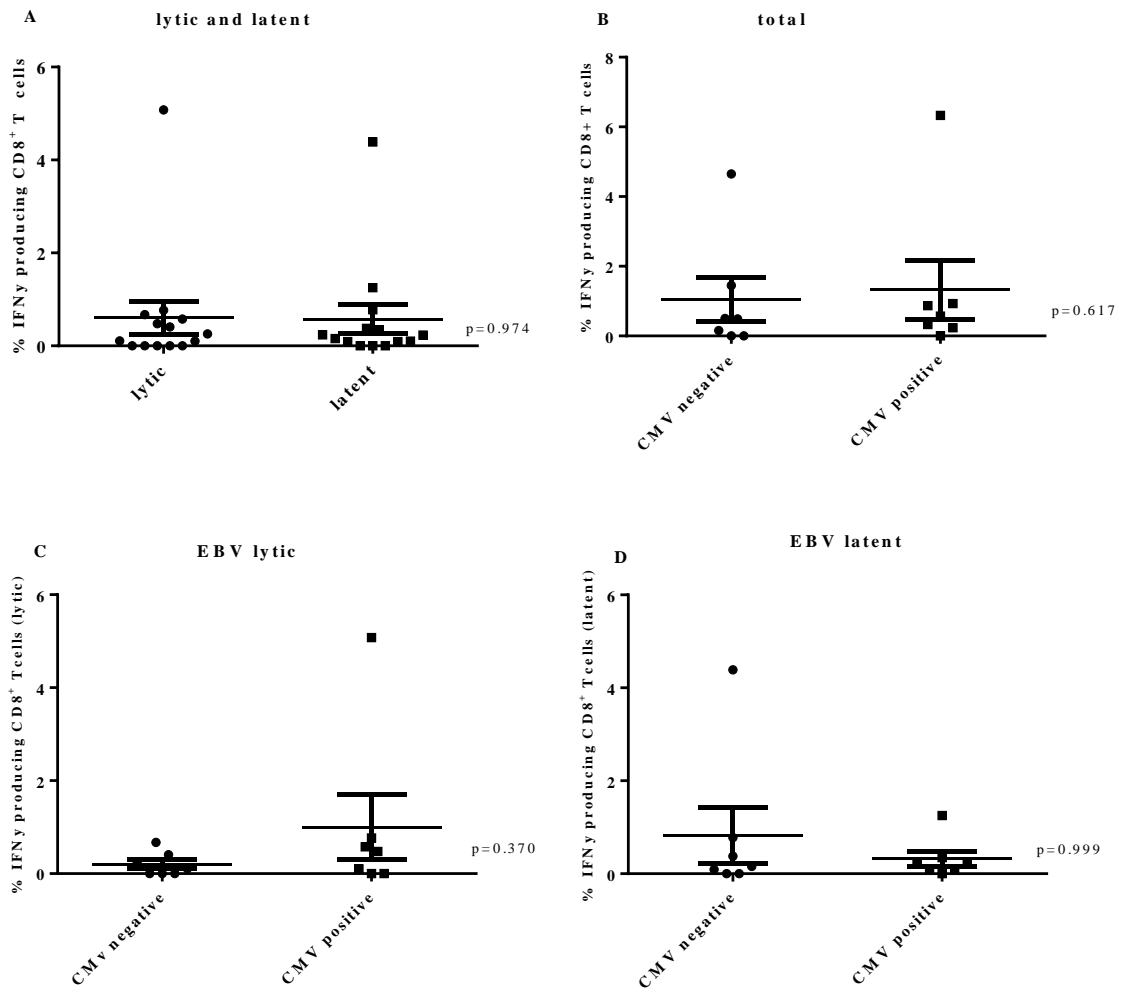


Figure 5-20 Frequencies of IFN γ producing CD8⁺ T cells after EBV peptide stimulation.

A. Frequencies of IFN γ producing CD8⁺ T cells in response to stimulation with lytic or latent peptides B. total percentage of IFN γ producing CD8⁺ T cells after EBV peptide stimulation according to CMV serostatus C. percentage of IFN γ producing CD8⁺ T cells after stimulation with lytic peptides D. percentage of IFN γ producing CD8⁺ T cells after stimulation with latent peptides. Data analysed by Mann-Whitney U Test.

There was no association between EBV load and frequencies of functional cells after peptide stimulation (Figure 5.21, $R^2= 0.0531$, $p=0.427$ total), this also included lytic and latent peptides.

The correlation between EBV-specific T cell response as measured by tetramer binding, and percentages of functional $CD8^+$ cells following peptide stimulation was then assessed. There was no correlation between percentages of tetramer positive cells and functionality when total responses or lytic responses were assessed (Figure 5.22). There was a positive correlation between frequencies of latent EBV-specific T cells and latent peptide responses although one donor made this correlation significant ($R^2=0.972$, $p=<0.001$, Figure 5.22C). The impact of CMV in this analysis could not be assessed due to low numbers of responders. The data demonstrates that there is no obvious correlation between EBV tetramer positive cells and $IFN\gamma$ producing $CD8^+$ T cells; in terms of lytic or total EBV responses.

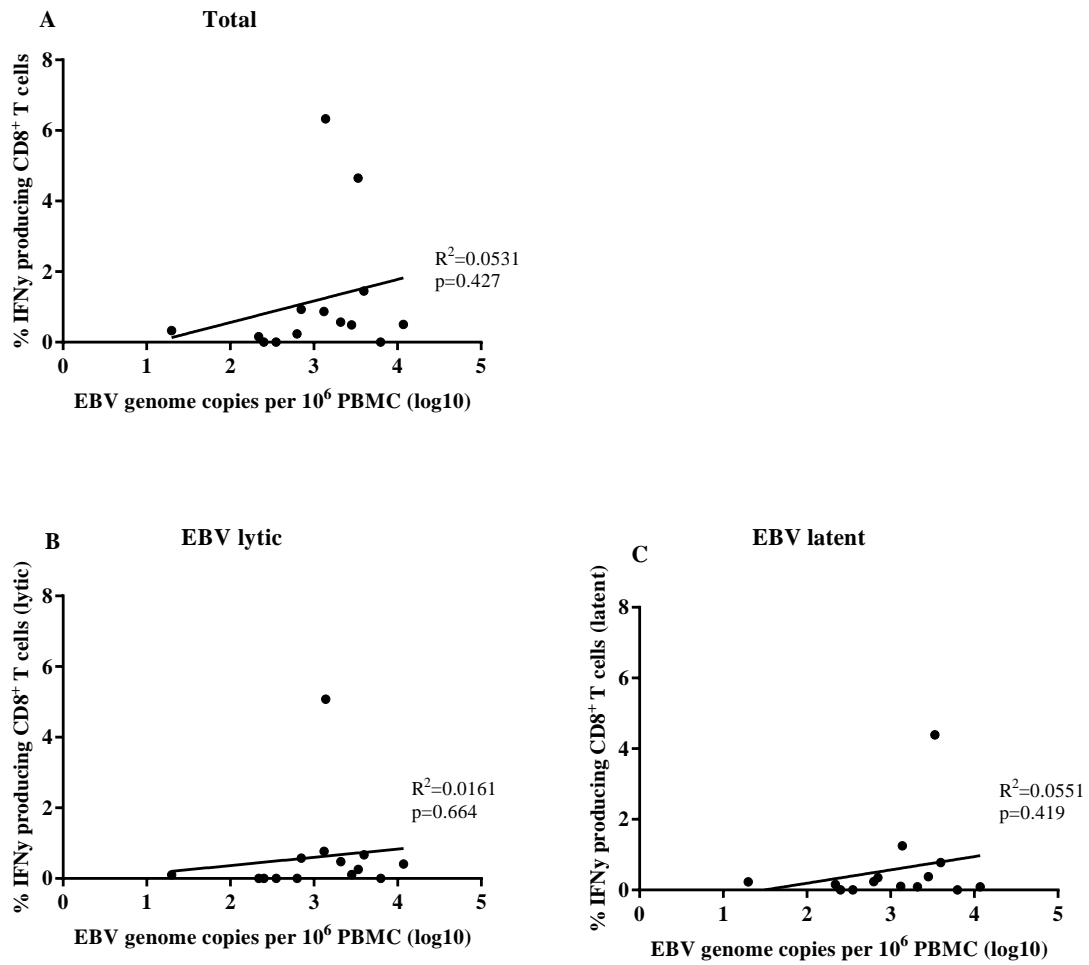


Figure 5-21 . Testing the correlation between EBV load and IFN γ producing CD8 $^{+}$ T cells following EBV peptide stimulation.

A. total IFN γ producing CD8 $^{+}$ T cells B. IFN γ producing CD8 $^{+}$ T cells following stimulation with lytic peptides C. IFN γ producing CD8 $^{+}$ T cells following stimulation with latent peptides. Data analysed by Pearson's correlation and R^2 and p .values displayed.

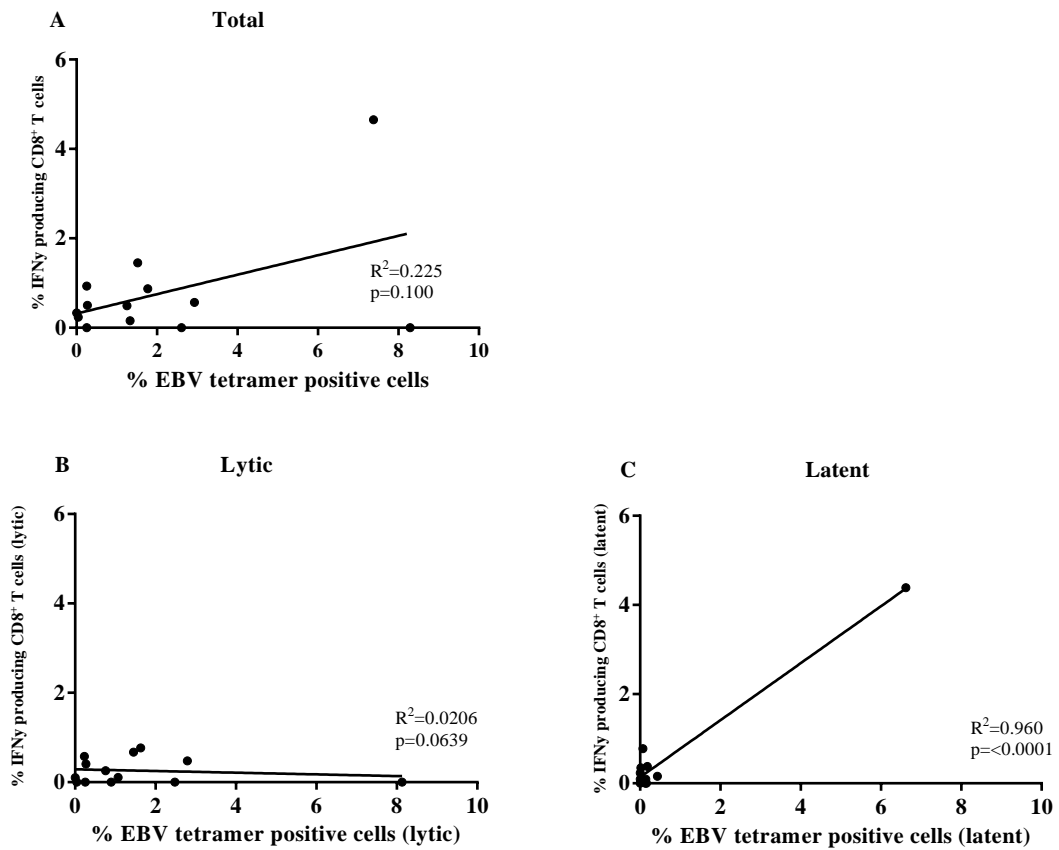


Figure 5-22 Testing the correlation between EBV- specific T cell response and IFN γ producing CD8 $^+$ T cells after stimulation with EBV peptides

PBMCs were stained as previously described for tetramer analysis and separately stimulated with EBV peptides overnight. A. EBV total response B. EBV lytic responses C. EBV latent responses

5.3.DISCUSSION

The current work aimed to assess the magnitude, phenotype and stability of CMV and EBV tetramer responses over time, with a view to investigating the impact of CMV serostatus upon immunity to EBV. It is known that the CMV-specific T cell response can be large in magnitude, often constituting up to 10% of the total CD8⁺ T cell pool (Chidrawar *et al.*, 2009). In the current work one individual displayed an IE1-specific response of over 10% of the total CD8⁺ T cell pool, whereas the EBV lytic and even more so latent CD8⁺ T cell responses were much smaller. This is similar to the current literature which suggests that lytic CD8⁺ T cell responses can constitute up to 3% whereas responses to the latent EBNA- 3 family of proteins consist of usually less than 0.5% of the CD8⁺ T cell pool (Hislop *et al.*, 2008). Age is an important factor in determining magnitude of some viral-specific responses; the median age of the 1000 Elders cohort was 81. Studies have found that both the CMV-specific CD8⁺ and CD4⁺ T cell responses increase with age, with older adults often having the largest expansions of these cells (Hadrup *et al.*, 2006, Pourgheysari *et al.*, 2007). There is conflicting evidence as to the impact of age upon the EBV-specific T cell responses; Ouyang *et al* demonstrated increased frequencies of EBV-specific T cells in older adults compared with younger donors however with impaired function (Ouyang *et al.*, 2003). In contrast, a study of older adults in West-Sicily demonstrated that frequencies of EBV tetramer positive cells was greater in younger individuals (Colonna-Romano *et al.*, 2007). The current work demonstrates that the EBV-specific T cell responses are smaller in magnitude than responses CMV in a cohort of older adults, with pp65 responses significantly greater in magnitude than EBV latent responses.

The influence of CMV serostatus upon the magnitude of EBV-specific T cell responses were examined; there were no significant differences between the magnitude of the EBV-specific T cell response in the two CMV serostatus groups. This is despite a trend towards an increase in EBV-specific T cell response in the CMV negative group (mean tetramer response of 2.59 vs. 1.12). In contrast, Khan *et al* (2004) demonstrated that EBV-specific T cell responses increased with age in a group of CMV negative individuals. In order to study this further in the 1000 Elders cohort it would be of interest to increase numbers of individuals studied.

As well as being different in magnitude, CMV- and EBV-specific T cell responses differ terms of phenotype and function (Lelic *et al.*, 2012). In order to assess this in the 1000 Elders cohort tetramer responses were phenotyped using a panel of antibodies. There were lower frequencies of CMV-specific T cells which were CD28+, CD27+, CCR7+ and CD45RO+ compared with T cell responses to some EBV antigens. There were higher frequencies of CD45RA+ CMV-specific T cells compared with CD45RA+ cells specific for EBV-latent epitopes. This data fits in with the current literature which suggests that CMV-specific T cells generally lack CD28 and CD27 expression, with CD45RA and CD45RO being variable (Vescovini *et al.*, 2004, Wallace *et al.*, 2011). T cells responding to EBV latent antigen tend to express CD28 and CD45RO, with variability in CD45RA and CD45RO expression by T cells against EBV lytic antigens. (Catalina *et al.*, 2002, Hislop *et al.*, 2001). Also, there were higher frequencies of effector memory and EMRA CMV-specific T cells compared with frequencies of these directed against EBV latent epitopes, with the opposite occurring for central memory T cells. This fits in with other studies which suggest that CMV-specific T cells have a very distinctive phenotype

(Ellefsen *et al.*, 2002, Harari *et al.*, 2004).

As the differences in phenotype of antigen-specific responses were established, it was then of interest to assess whether CMV serostatus impacted upon the phenotype of EBV-specific T cell responses. Terrazzini *et al* studied T cell responses to tuberculin in CMV seronegative and CMV seropositive individuals. CMV seropositive individuals display a memory T cell response to tuberculin which was more terminally differentiated compared to CMV seronegative individuals (Terrazzini *et al.*, 2013). This demonstrates that CMV seropositivity may impact upon T cell responses to other antigens. In the current study there was a trend towards lower frequencies of CD28⁺ or CD27⁺ T cells specific for EBV latent antigen in the CMV positive group compared with negative, however this did not reach significance. Similarly there was a trend towards higher frequencies of EMRA T cells specific for latent antigen in the CMV seropositive group. One issue within the current work was that some of the EBV-latent responses were too small in order for the phenotype to be assessed, so studying this in a larger group of donors could be of benefit.

Tetramer data from the study by Khan *et al* (2004) was used in conjunction with the current work to assess the stability of CMV and EBV tetramer response over a period of ten years. CMV responses were stable over time, with no significant difference in magnitude of pp65 and IE1 responses between the two time points. Other studies have sought to understand the stability of CMV-specific T cell responses. A study of Gambian infants found that in the first two years of infection, the percentages of tetramer positive cells remained stable (Miles *et al.*, 2008). Klarenbeek *et al* tetramer sorted and sequenced

TCR repertoire in order to assess the stability of CMV-specific CD8⁺ T cell clones. Most clones were maintained over a five year period, showing these responses to be stable over this period of time (Klarenbeek *et al.*, 2012). Although it is well established that CMV causes memory inflation, the current work suggests that CMV-specific expansions may occur at an earlier time point and then reach stability later in life. EBV-specific T cell responses to lytic and latent antigen were also stable between the two time-points analysed in the 1000 Elders. These results are similar to studies which found that EBV-specific T cell responses remained stable 6 months and 20 months post-IM (Catalina *et al.*, 2001, Scherrenburg *et al.*, 2008).

An interesting finding was that the median EBV viral load of 3.05 genome copies per 10⁶(log10) in the 1000 Elders was greater compared to younger and middle aged donors. The highest load recorded in the current study was over 11,000 EBV copies per 10⁶ PBMC, a load similar to that of individuals with infectious mononucleosis (Hislop *et al.*, 2005). Interestingly, the EBV loads demonstrated in the cohort is similar to EBV loads observed in healthy Gambian adults (Njie *et al.*, 2009). It has previously been observed that age can increase EBV load, Stowe *et al* found that EBV load in peripheral blood increased in older individuals, alongside detection of CD8⁺ T cells specific for EBV antigens and an increase in EBV DNA in the plasma, possibly due to recent activation (Stowe *et al.*, 2007). This was also demonstrated in the current study, when healthy UK donors and the 1000 Elders cohort were assessed in combination. This demonstrates that donors may have had recent episodes of EBV reactivation or may lack the ability to control the virus effectively.

A unique and important finding in the current study was that CMV seronegative individuals displayed significantly higher EBV loads compared with CMV seropositive participants (Figure 5.14, 3.27 (log₁₀) vs. 2.68). The function of EBV-specific T cells was examined in the present work by quantifying IFN γ production after stimulation with EBV peptides. Khan *et al* (2004) found that cells following EBV peptide stimulation were less functional in older adults compared with younger individuals, demonstrating that there could be a lack of functional EBV-specific T cells with age. There was no difference between CMV negative and CMV positive individuals and the function of EBV-specific T cells, so lack of T cell function was not considered a reason for the higher EBV viral loads in CMV negative individuals. It could be hypothesised that CMV may give protection from infection with EBV. Barton *et al* demonstrated that mice latently infected with CMV were resistant from infection with bacterial pathogens, thus demonstrating a protective role of CMV infection (Barton *et al.*, 2007).

In summary, data in this chapter demonstrates that CMV pp65 responses are greater in magnitude than EBV-latent responses and that CMV and EBV-specific T cell responses remain stable over time. CMV-specific T cells display a unique phenotype; however CMV seropositivity does not significantly impact upon the phenotype of EBV-specific T cell responses. The median EBV viral load of the 1000 Elders cohort participants was particularly high; CMV was demonstrated to influence this, with CMV negative individuals displaying higher loads compared with seropositive donors. Of particular interest, is the observation that individuals in this cohort display EBV loads similar to healthy adults in the Gambia, where EBV is acquired at a much earlier age (Njie *et al.*, 2009). It could be argued that older donors with very high EBV viral loads may be at risk

from EBV-related malignancies.

One limitation of the current work is that there were relatively small numbers of samples analysed; increasing numbers of participants would be worthwhile for future studies. This would be particularly useful if there were more tetramers available spanning a greater range of HLA-types. Due to the age of the participants, it should also be considered that there may be a survivor bias in the current work. The bias could arise from the fact that individuals with poor immunity and health, perhaps related to CMV infection may not still be alive or may have been too ill to respond to the request for blood samples. Therefore the data may represent a group of participants who are healthier than average or may not truly represent the detrimental influence of CMV upon immunity.

As well as increasing numbers of participants, it would be of interest for future work to assess whether the high EBV viral loads observed in this cohort are due to a high frequency of infected B cells or a higher EBV load per cell in these individuals. Although it is interesting to study peripheral blood, it may also be useful to obtain EBV viral load data from throat washings of donors and to study frequencies of cells within the tonsils as this is where EBV-specific T cells can be observed (Hislop *et al.*, 2005).

Chapter 6. GENERAL DISCUSSION

The acceleration of immunosenescence by CMV infection may have negative health consequences. Data from the OCTO and NONA studies described the ‘immune risk profile’ and the influence of CMV upon mortality risk (Wikby *et al.*, 2002). An age related reduction in naïve T cells and an increase in EMRA T cells have been observed in CMV seropositive individuals, but importantly this did not occur in long-lived individuals (Derhovanessian *et al.*, 2010). The influence of CMV and immunosenescence has also been observed in the context of functional status *i.e.* mobility and daily functioning/living; in a group of older adults. This has been associated with a lower CD4/8 T cell ratio (Moro-Garcia *et al.*, 2012). There is also a potential role for CMV and immunosenescence for lack of response to vaccination. There are 3-5 million severe cases of influenza worldwide each year and it has been observed that older adults have less efficient responses to the influenza virus, with only 30-40% efficacy for people aged 65 and over (Lang *et al.*, 2011b, Mcelhaney *et al.*, 2012). One reason for this could be the increase in CD8⁺ CD28 null T cells in older adults, with increased T cell dysfunction (Lang *et al.*, 2011b, Targonski *et al.*, 2007). Another factor contributing to this could be the increase in pro-inflammatory cytokines such as IL-6 and TNF α in older adults, termed ‘inflammaging’ (Franceschi *et al.*, 2007). However, there is conflicting data as to the role of CMV in ‘inflammaging’; some studies have demonstrated that CMV infection is also associated with a pro-inflammatory environment whereas Bartlett *et al* observed that this occurred in ageing adults independently of CMV infection (Bartlett *et al.*, 2012, Solana *et al.*, 2012). Trzonkowski *et al* demonstrated that non-responders to the influenza vaccine

tended to have higher CMV antibody titres and an increased frequency of CD57+CD27- T cells (Trzonkowski *et al.*, 2003). Another study observed that higher frequencies of CD4⁺ EMRA T cells and CMV seropositivity was associated with poor vaccine responses; suggesting a role for late differentiated T cells in poor vaccine responses (Derhovanessian *et al.*, 2013b). However, Elzen *et al* found no role for CMV in determining poor responsiveness in a group of older adults, demonstrating the need for further work in this area (Elzen *et al.*, 2011).

Aside from immunosenescence, the relationship between CMV infection and mortality has been demonstrated in a number of studies, often with reference to cardiovascular disease (Roberts *et al.*, 2010, Savva *et al.*, 2013, Simanek *et al.*, 2011). Higher CMV IgG titres, as well as CMV infection alone have also been associated with health and mortality risk (Gkrania-Klotsas *et al.*, 2013). Studies of older CMV seropositive adults have examined the relationship between CMV and frailty (Wang *et al.*, 2010, Moro-Garcia *et al.*, 2012). An association has also been established between CMV and IL-6 upon muscle size and grip strength (Kilgour *et al.*, 2013). However, knowledge regarding the influence of CMV upon cognition was limited prior to this study, with this often being studied in adults with pre-existing health conditions (Shirts *et al.*, 2008). The current work used a battery of tests to assess cognition in older adults. Remarkably participants with higher CMV IgG titres displayed lower general cognitive ability, even after accounting for potential confounding factors. However, it could not be demonstrated whether CMV had a causal role upon decline in cognitive function, as it is unknown when participants acquired CMV infection. Therefore, in order to establish causation it would be necessary for continuous testing for presence of the virus to occur throughout the lifetime.

Furthermore, as other herpes viruses have also been associated with a decline in cognitive function, for example HSV-1, it would be useful to examine the influence of these viruses upon cognition within the LBC (Thomas *et al.*, 2013, Watson *et al.*, 2013).

EBV-specific immunity was examined through studying the 1000 Elders cohort. Very high EBV viral loads were observed in this cohort; CMV seronegative individuals also displayed higher EBV loads compared to seropositive participants. Despite this, EBV-specific T cell responses as measured by MHC Class I tetramers remained the same between the CMV seronegative and seropositive groups. Higher EBV viral loads may demonstrate poor control of the virus in older adults; which could potentially lead to an increase in EBV-associated malignancies. Although the EBV viral load was higher in older donors compared with younger donors, it is unknown whether this was due to a high EBV genome load per cell or whether more cells were infected with the virus. This question could be addressed by performing a limiting dilution method by which frequencies of EBV infected B cells could be identified (Babcock *et al.*, 1998). It was difficult to immune phenotype small EBV-specific T cell responses in the current study, therefore it would be useful to examine EBV-specific immunity in a larger cohort in the future.

The influence of CMV infection upon immunosenescence and cognition has been examined in this thesis. It was observed that CMV seropositivity contributed towards an immune repertoire associated with immunosenescence; with higher CMV IgG titres also influencing immune parameters such as the CD4/8 T cell ratio. Furthermore, an

association between high CMV IgG titres and lower general cognitive ability was determined; demonstrating the role of CMV in aspects of decline in older adults. The data presented regarding the influence of CMV upon immunosenescence and cognitive function suggest that potential interventions to prevent CMV infection or reverse the negative influence that CMV has on T cell immunity may be beneficial. Work by Beswick *et al* has demonstrated via an MCMV model that anti-viral medication may reverse some aspects of immunosenescence, for example with a reduction in the MCMV-specific CD8⁺ T cell responses which also displayed a decrease in differentiation (Beswick *et al.*, 2013). This suggests the potential for antiviral drugs to reduce the burden of memory inflation in older adults. Vaccination against CMV is currently aimed at preventing congenital infection or CMV viraemia in the transplant setting, as these are significant public health issues, rather than tackling the issue of CMV and immunosenescence (Schleiss, 2008). CMV vaccines have shown promise in both of these settings thus far (Rieder and Steininger 2014). However, it may be important to consider the influence of CMV upon responses to other herpes viruses, as data here has shown that CMV may influence EBV-specific immunity. Such matters should therefore be taken into account when considering future therapeutic intervention strategies.

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