Faculty of Science & Technology

Detecting and defining immunity to human cytomegalovirus (HCMV) in health; combining QuantiFERON®-CMV and flow cytometry

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Copyright Statement

Detecting and defining immunity to cytomegalovirus (CMV) in health; combining QuantiFERON®-CMV and flow cytometry

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Abstract

Human cytomegalovirus (CMV) is an archaic and ubiquitous member of the beta-herpesvirus family (*Herpesviridae),* infecting approx. 83% of adults worldwide. Once infection has resolved, CMV enters a life-long latent state, with potential to reactivate. Infection is usually asymptomatic in healthy individuals, while severe disease can occur in the immunosuppressed. Following allogeneic haematopoietic stem cell transplant (allo-HSCT), reactivation of CMV can cause severe morbidity and mortality. CMV DNA monitoring by polymerase chain reaction (PCR) and timely administration of pre-emptive antiviral treatment in the case of reactivation are vital to avert such outcomes. T-cell mediated immunity is key in controlling CMV infection and assays designed to measure it would improve the management of CMV infection in transplant patients; however, they are lacking in standard clinical practice.

This study aimed to evaluate the performance of QuantiFERON-CMV, a commercial assay that detects CMV T cell mediated immune responses by detecting IFNy produced by CD8+ T cells. To confirm results from the QuantiFERON-CMV assay and to provide additional information on CMV-specific T cell frequency and phenotype, blood samples were assessed in parallel by flow cytometry. Finally, serology (detection of CMV IgG) was used to identify if any volunteers had been previously exposed to CMV. We hypothesised that only volunteers with a positive CMV IgG result would have detectable CMV-specific T cells by QuantiFERON-CMV and/or flow cytometry. This is a preliminary project to a clinical study which aims to evaluate the QuantiFERON-CMV assay in monitoring CMV immune reconstitution in allo-HSCT recipients.

Preliminary investigations sought to validate the flow cytometry approach using a human T cell line and then primary human T cells. Together, these assays confirmed that flow cytometry can be used to identify activated T cells accumulating intracellular IFNy, and that T cell subsets could also be distinguished by the gating strategy employed. Blood samples from eleven healthy volunteers were tested for the frequency/phenotype of CMV-specific T cells by QuantiFERON-CMV and flow cytometry and for the presence and titre of anti-CMV IgG. Six of the eleven healthy volunteers were CMV IgG positive and five were CMV IgG negative. The flow cytometry assay detected a CD8+ IFNy+ T cell response in four of the six CMV IgG positive volunteers. Of these four volunteers 0.1-3.3% of the total CD8+ T cell population was specific to CMV. Phenotypic analysis showed that the majority of CMV-specific CD8+ T cells were of the T_{EMRA} subset.

Overall, the QuantiFERON-CMV levels positively correlated with CMV IgG antibody titres, and QuantiFERON-CMV results also correlated with the frequency of CMV-specific CD8+ T cells detected by flow cytometry. We also conclude that the QuantiFERON-CMV assay is more sensitive than flow cytometry. These data support the evaluation of QuantiFERON-CMV in the allo-HSCT setting.

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Author's declaration

All experimental work in this MRes, except for the flow cytometry assay for volunteer 1, was performed by me. The flow cytometry assay for volunteer 1 was performed with Dr Sarah Buchan. All data analyses in this study were performed by me, including graph production and statistical analyses. Where Figures/Tables are taken from other sources these are acknowledged in the legend. All writing is my own.

Data obtained as part of this MRes were presented as a poster presentation at the 14th annual postgraduate research conference at BU in November 2022 and as an oral and poster presentation at the Wessex Immunology Group conference for "*The ageing immune system"* in June 2023.

Abbreviations

Abbreviation	Definition
Allo-HSCT	Allogeneic haematopoietic stem cell transplant
BMT	Bone marrow transplant
CMV	Cytomegalovirus
HPC	Haematopoietic progenitor cell
HSCT	Haematopoietic stem cell transplant
IFNy	Interferon gamma
IgG	Immunoglobulin G
IgM	Immunoglobulin M
MCMV	Murine cytomegalovirus
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
SOT	Solid organ transplant

Section 1: Introduction to cytomegalovirus

1. Introduction

Human cytomegalovirus (CMV) is an archaic and ubiquitous member of the beta-herpesvirus family (*Herpesviridae)* and of high epidemiological relevance, infecting around 83% of adults worldwide according to age, socio-economic and demographic factors (Zuhair et al. 2019). There are 3 subfamilies of herpesviruses, alpha, beta and gamma which derived from a common ancestor approximately 400 million years ago (Davidson 2011). CMV infects healthy individuals mainly asymptomatically and the benign nature of infection is attributed to the co-evolution of CMV with human immune defences, reaching an equilibrium that allows the virus to persist sub-clinically (Griffiths et al. 2014). When this immune balance is disrupted, virulence can develop and individuals with profound immune suppression are at the greatest risk of disease (Ngai et al. 2018). Characteristic of herpesviruses, CMV enters a life-long latent state in restricted cell types including haematopoietic stem cells and leucocytes, with the potential to reactivate (Forte et al. 2020).

1.1 Viral structure and lifecycle

CMV is the largest herpesvirus, with a ~235,000 base-pair, linear double-stranded DNA genome encoding approximately 165 genes and estimates of more than 200 open reading frames (ORFs) (Davison et al. 2003). Approximately 44 CMV genes are involved in active replication, and 117 others are involved in various mechanisms including host immune evasion (Forte et al. 2020). The classification of herpesviruses and their relative sizes are shown in Table 1.

Table 1. The classification of herpesviruses (Information adapted from Sehrawat et al. 2018).

Family	Herpesvirus	Common Abbreviation	Size (Kb)
Alphaherpesvirinae			
Simplexvirus	Herpes simplex virus type 1	HSV-1	152
	Herpes simplex virus type 2	HSV-2	153
Varicellovirus	Varicella-zoster virus	VZV	125
Betaherpesvirinae			
Cytomegalovirus	Human cytomegalovirus	HCMV	227 - 237
Roseolovirus	Human herpesvirus type 6	HHV-6	159 - 162
	Human herpesvirus type 7	HHV-7	144 - 153
Gammaherpesvirinae			
Lymphocryptovirus	Epstein-Barr virus	EBV	172 – 173
Rhadinovirus	Human herpesvirus type 8	HHV-8	134 - 138

The CMV virion (Figure 1), consists of a DNA core inside an icosahedral nucleocapsid, enveloped with a proteinaceous matrix (comprising of tegument proteins) which is encapsulated in a lipid bilayer envelope containing several viral glycoproteins (Isaacson et al. 2009). Major proteins contained in the tegument compartment include, the abundant phosphoprotein 65 (pp65), also known as unique long 83 (UL83), often used in diagnostic assays (Varnum et al. 2004). Most tegument proteins are phosphorylated, denoted by the prefix ‘pp’ and are highly immunogenic (Chen et al. 1999s). Glycoproteins on the viral envelope mediate entry to host cells followed by the release of viral DNA and tegument proteins into the cell (Kalejta. 2008). The phospholipid envelope of the CMV virion contains 6 key virally encoded glycoproteins (glycoprotein B (gB), gH, gL, gM, gN and gO), which are important for entry, virion maturation and dissemination of virus (Landolfo et al. 2003).

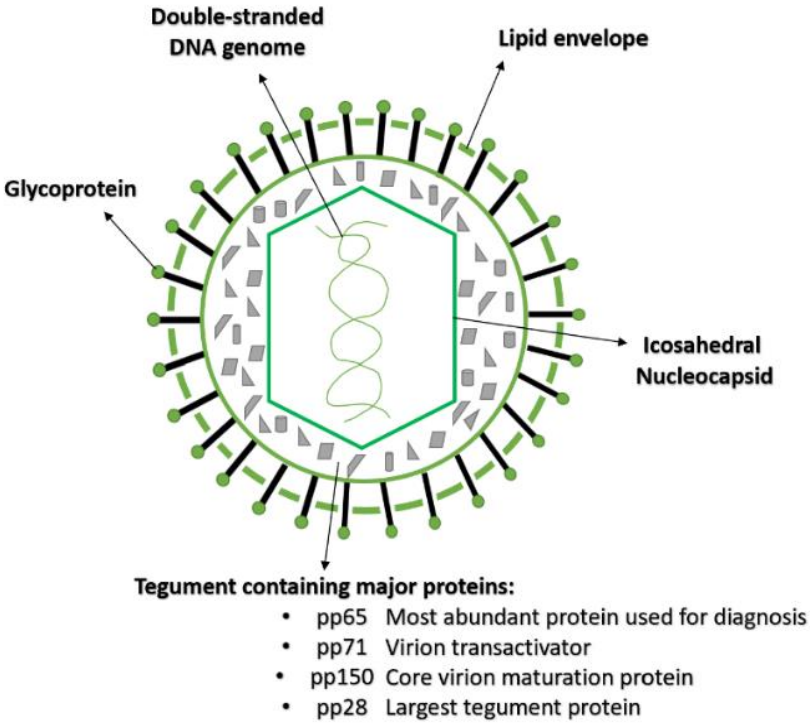


Figure 1. The structure of Cytomegalovirus (CMV) and its components. The diagram illustrates the structure of a typical infectious CMV virion and abundant tegument proteins are listed (Authors own. 2023).

Tegument proteins control gene expression, viral entry into host cells and immune evasion (Kalejta R. 2008). As well as establishing initial infection, tegument proteins accumulate to high levels during later stages of infection where they control the production of progeny virions, giving them a critical role in the lytic replication cycle (Kalejta R. 2008). Tegument proteins can be segregated into groups dependent on their function such as (1) proteins playing a structural role for virion assembly and (2) modulation of the host cell response to

infection (Isaacson et al. 2009). The function for less than half of the tegument proteins is known, some examples are shown in Table 2.

Table 2. The function of 4 major CMV tegument proteins (Adapted from Kalejta R. 2008 and Isaacson et al. 2019).

Tegument protein	Role in infection
pp65	Endogenous kinase activity Evasion of innate and adaptive immunity
pp71	Facilitates IE gene expression Stimulates cell cycle progression Prevents cell surface expression of MHC
pp150	Directs capsid to site of final envelopment
pp28	CMV DNA replication factor

1.2 Viral infection and latency

Infection of host cells leads to the synthesis of CMV proteins in 3 overlapping phases. Immediate-early (IE) proteins are synthesised within 0-2 hours of infection, delayed-early proteins within 2-24 hours and late viral proteins beyond 24 hours (Stinski. 1978). The expression of early IE proteins commits the virus to the lytic replication cycle and induces the host cell to express remaining viral early proteins which replicate viral DNA (Kalejta et al. 2008). Therefore, IE proteins are the master regulators to initiate lytic replication (Stenberg. 1996). Once DNA replication has taken place, late genes encoding structural proteins are expressed which dictate the assembly of newly formed lytic progeny virus (Stenberg. 1996).

In some cell lineages, IE genes are silenced upon CMV infection, which results in retention of the viral genome in the nuclei, without the production of infectious viral progeny (Sinclair et al. 2006; Wills et al. 2015). When the host immune system is suppressed, latent viruses can reactivate lytic infection, causing symptoms and disease (Porter et al. 1985). The life cycle of lytic and latent infection is shown in Figure 2.

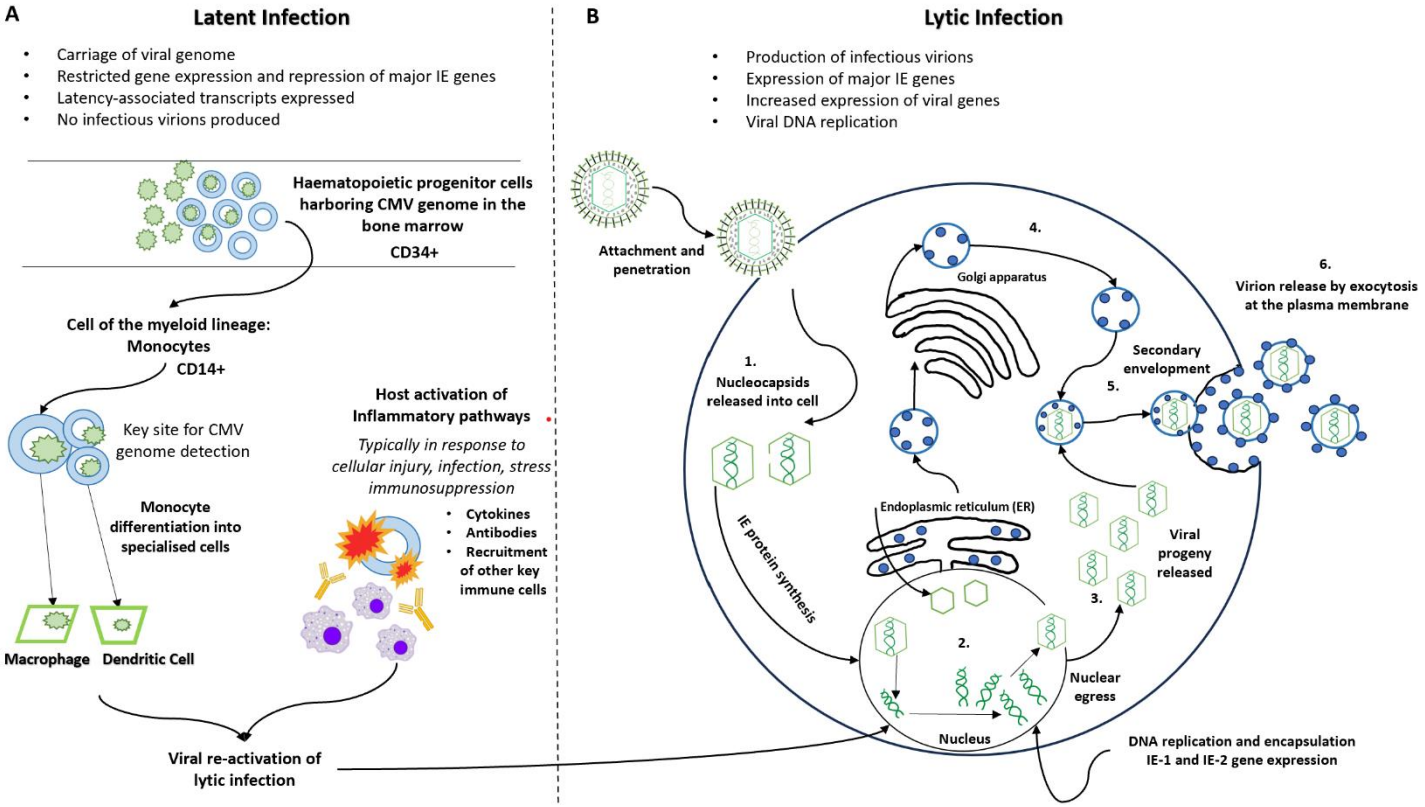


Figure 2. Overview of lytic and latent life cycles during CMV infection. (A) In latent CMV infection, CMV virions infect host cells causing viral dissemination and spread. In the bone marrow, latent genome is carried in CD34+ and CD14+ cells. Viral reactivation can occur due to normal host physiology e.g. upon CD14+ cellular differentiation into other cell types or physiological inflammatory pathways induced in the host in response to infection or cellular damage. (B) In lytic CMV infection, viral attachment to host cells occurs via interactions between the viral glycoproteins (e.g. gB and gH) and host cell surface receptors, followed by the fusion of the envelope with the cell membrane enabling (1) the release of nucleocapsids into the host cell cytoplasm. (2) The nucleocapsids translocate into the nucleus where viral DNA is released. The release of viral DNA initiates IE gene expression. Viral DNA replication occurs simultaneous to stimulation of the endoplasmic reticulum (ER) to produce viral capsids for DNA encapsulation. Once viral DNA is encapsulated, it can (3) be transported from the nucleus into the cytoplasm (nuclear egress). In parallel, (4) the golgi apparatus produces capsids to enable (5) secondary envelopment of viral progeny which enables (6) virion release by exocytosis at the plasma membrane (Authors own. 2023).

1.3 Transmission routes

CMV is known for its broad tropism for tissues and cell types which includes connective tissue, smooth muscle and vascular endothelial cells as well as hepatocytes (liver), alveolar epithelial cells (lungs) and neuronal cells (brain and retina) (Sinzger et al. 2008). In the bone marrow, CMV also infects hematopoietic progenitor cells (HPCs), which give rise to circulating blood cells and is thought to provide carriage for CMV infected cells around the body. Initial CMV infection is transmitted via close contact with infected individuals, via infectious bodily fluids such as blood, saliva, urine and breast milk (Forte. 2020) and infectious virus can be shed for months post infection (Forte. 2020).

1.4 Clinical manifestation of CMV

CMV infection is largely asymptomatic (Crough and Khanna 2009). Infrequently, individuals may present with mononucleosis-like symptoms characterised by extreme fatigue, muscle aches, sore throat and fever (Robbins and Cotran). 10% of all cases of infectious mononucleosis are caused by CMV infection (Crough and Khanna 2009). Clinical presentation of CMV infection varies across patient populations, as shown in Table 3.

Table 3. Symptoms associated with CMV infection and disease in various patient populations (Information adapted from Crough and Khanna. 2009).

Patient Population	Clinical Manifestation
Immunocompetent	• Majorly asymptomatic • Infrequently mononucleosis with: sore throat, muscle aches, fever, extreme fatigue, , splenomegaly
Immunocompromised	• Pneumonitis • Enterocolitis • Esophagitis or gastritis • Retinitis • Encephalitis (rare) • Hepatitis • Other tissue-invasive disease such as cystitis, myocarditis etc.
Congenital CMV	• Jaundice, hepatitis and hepatosplenomegaly • Multiorgan dysfunction • Neutropenia, pancytopenia (Petechiae) • Microcephaly and severe central nervous system damage • Seizures • Lethargy • Hearing impairment or hearing loss (the commonest clinical manifestation)

Section 2: Immune responses to CMV

There are 3 key stages to CMV infection: 1. primary infection and the innate immune response, 2. establishment of multi-site latency which promotes the immune 'inflation' over a lifetime and 3. Viral reactivation from latency (Basta et al. 2003). The phases of infection are shown in Figure 3.

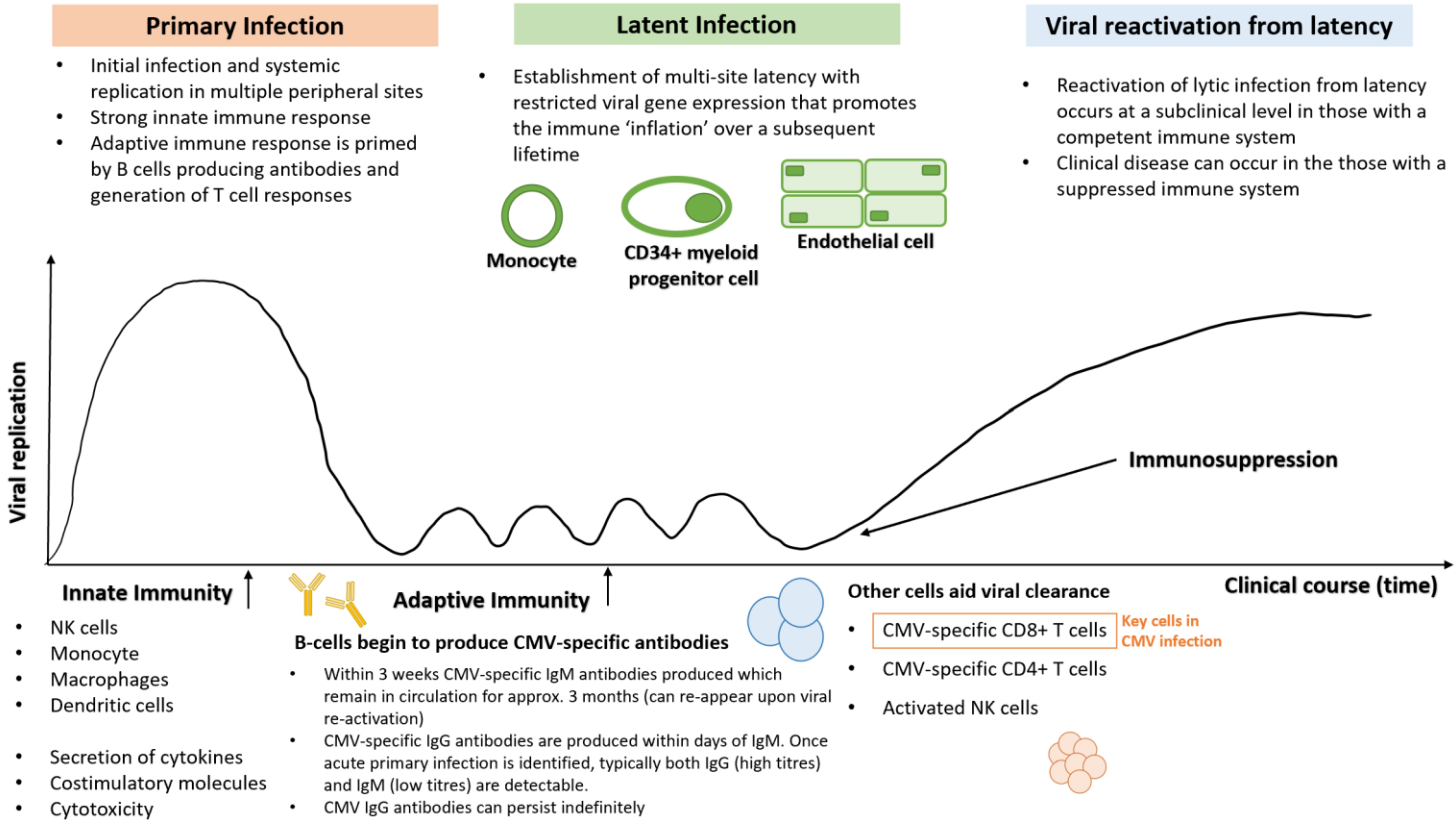


Figure 3. Overview of the phases of CMV infection. Innate responses are mediated by NK cells, monocytes and macrophages. CMV-specific IgM (and later on IgG) antibodies are present in circulation. Persistent infection occurs over months to years in which the virus is present at subclinical (asymptomatic) levels and remains under control due to robust innate and adaptive immune responses. Latency occurs in multiple cell types including the myeloid and endothelial lineages (see section 1.2; figure 2) and can also reside in endothelial cells. Viral reactivation occurs frequently from latency (see section 1.2; Figure 2) and can cause clinical disease in those with impaired immune function (Authors own. 2023).

1.5 Innate Immunity

CMV can be detected by toll-like receptors (TLRs) (Crough and Khanna. 2009) that induce the production of inflammatory cytokines which in turn, recruit innate immune cells and promote the upregulation of T cell co-stimulatory molecules such as CD80 and CD86 (Boehme and Compton. 2004).

Human innate immunity to CMV is poorly understood (Picarda et al. 2018). In murine CMV infection (MCMV), TLR3 and TLR9 become activated and induce macrophages and dendritic

cells to produce alpha/beta interferon (IFN- α/β) and activate natural killer (NK) cells (Moresco et al. 2011). NK cells are central in innate MCMV immunity and have demonstrated effective control of experimental MCMV infection (Bukowski et al. 1985). Despite their importance in MCMV, the role of NK cells during human infection is unclear, although solid organ transplant patients show increased NK cell activity during primary and recurrent CMV infection (Crough and Khanna. 2009). This suggests that NK cells may play a role in the recovery of lytic CMV infection (Crough and Khanna. 2009).

1.6 Adaptive Immunity

The adaptive immune system comprises B cells which secrete antibodies (known as humoral immunity), and T cells (which provide cellular immunity) which perform a variety of functions depending on T cell subtype.

1.6.1 Humoral Immunity

The establishment of lifelong immunity by production of neutralising antibodies is essential to prevent uncontrolled replication and CMV disease (Crough and Khanna. 2009). Post infection, B cells first produce CMV-specific IgM antibodies which remain present in circulation for approximately 3 months after viral clearance and can re-appear upon re-activation or re-infection (Azevedo et al. 2015). Shortly after IgM antibodies are produced, CMV-specific IgG antibodies are produced which can persist indefinitely (Azevedo et al. 2015).

The major target for CMV-specific neutralising antibodies is the cell surface glycoprotein-B (gB), with approximately 50% of all neutralising CMV antibodies showing gB specificity (Crough and Khanna. 2009). The importance of effective neutralising antibodies has been demonstrated in numerous animal and human studies. In human studies, CMV infected pregnant women with CMV antibodies (described as being ‘seropositive’), have a lower rate of foetal CMV transmission than seronegative women (Fowler et al. 1992). Additionally, mothers with low avidity antibodies have a higher rate of intrauterine CMV transmission to the foetus (Boppana et al. 1995).

1.6.2 T cell mediated immunity

T cells are critical in the control of CMV disease (Crough and Khanna. 2009). While the immune response to primary infection cannot eradicate infection entirely due to latency, CMV-specific CD8+ and CD4+ T cells are vital for control and restriction of viral replication.

1.6.2.1 CD8+ T cells

In mice, adoptive transfer of CMV-specific CD8+ cytotoxic T cells conferred protection from severe MCMV (Crough and Khanna. 2009). Additional T cell subset depletion experiments revealed CD8+ T cells as the most important subset in the control of MCMV (Crough and Khanna. 2009). In human CMV, recovery of CMV-specific CD8+ T cells is associated with increased control of CMV infection and recovery from disease after bone marrow transplant (BMT) (Li et al. 1994). From this study, over 50% of patients lacking a detectable anti-CMV CD8+ T-cell response later developed CMV disease (Li et al. 1994). Additionally, infusion of CMV-specific CD8+ T cells from donors effectively restored cellular immunity in BMT recipients and was coincident with the absence of CMV-associated complications (Rist et al. 2005). Similarly in renal and lung transplant settings, functional CD8+ T cells are associated with low levels of viremia (Radha et al. 2005). Studies in lung transplant patients have revealed that recipients with detectable CMV-specific CD8+ and CD4+ T cell immunity remain free from CMV disease and show higher preservation of allograft function compared to those without detectable CMV-specific T cells (Shlobin et al. 2006).

CD8+ T cells specific for CMV recognise diverse proteins at various stages of viral replication including structural, early and late antigens e.g, pp28, pp50, pp150, gH, gB, US2, US3, US6, US11, US16 and US18 (Elkington et al. 2003). CMV-specific CD8+ and/or CD4+ T cells are directed against more than 70% of the ORFs (Dunn et al. 2002). The model for establishment of T cell memory after CMV infection consists of initial expansion of CD8+ T cells, followed by a phase of apoptosis known as the contraction phase, leaving approximately 5% of the initially expanded CD8+ T cells once lytic infection has been cleared (Crough and Khanna. 2009). In CMV however, surviving antigen specific CD8+ T cells reach a high and stably maintained pool of functional memory cells, able to respond more quickly to subsequent viral replication events (Zangger et al. 2022). This secondary increase and maintenance of functional CMV-specific CD8+ T cells is a phenomenon termed ‘memory inflation’ (Zangger et al. 2022). Memory inflation may enable CMV-specific T cells to effectively scan the body for viral reactivation events to keep CMV replication under control; the ‘immune sensing hypothesis’ (Ynga-Durand et al. 2019). In support, the size of

the inflationary T cell pool correlates with protection from CMV disease (Baumann et al. 2019).

In latently infected individuals, the frequency of CMV-specific T cells can therefore be very high. On average, 10% of the total peripheral CD8+ T cell pool is specific to CMV, as observed in healthy CMV seropositive individuals with no viremia detectable by PCR (Sylwester et al. 2005). However, the variability in CMV-specific T cell frequencies in CMV seropositive individuals ranges from barely detectable to very high (over 40% in some outliers) (Pardieck et al. 2018). This variability is thought to be due to differences in infectious strains, number of re-infections, dose of viral exposure and host-intrinsic factors (Pardieck et al. 2018). Elderly individuals typically have a higher frequency of these circulating CMV-specific T cells, but their reactivity is reduced to a limited number of epitopes (Griffiths et al. 2013). This immune dominance of CMV-specific T cells also impacts the immune responses to other pathogens, however this is poorly understood (Crough and Khanna. 2009).

T cell responses against latent and persistent CMV infection display a pattern of continuous expansion and contraction in the acute phase of infection (Crough et al. 2005) and CMV-specific CD8+ T cell responses during persisting infection are highly dynamic, fluctuating in function and absolute number despite being part of a “stable” total T cell population (Dunn et al. 2002). In other chronic infections such as HIV and hepatitis C, long-term viral persistence is accompanied by T cell exhaustion, a broad term used to describe a dysfunctional T cell response (Dunn et al. 2002). However, T cell exhaustion is not observed in response to CMV. Fluctuating patterns of antigen production by CMV during the persistence phase (See Fig 3), may provide periods of relief from chronic antigen stimulation, thereby preventing T cell exhaustion (Dunn et al. 2002).

1.6.2.2 T cell memory

Memory (and other subsets) of CD8+ T cells can be divided based on expression of cell surface and intracellular markers (Crough and Khanna. 2009). A summary of commonly used CD8+ T cell markers and their characteristics is shown in Table 4.

Table 4. Common CD8+ T cell surface markers, their functions and the cell subsets with which they are associated (Information adapted from Carrasco et al. 2016, Martin et al. 2018 and Bottcher et al. 2015).

Surface Marker	Cell-subset	Function
CD45RA	Naïve	Initially identified naïve T cells lacking antigen exposure; the loss of CD45RA signifies antigenic stimulation. However, this marker may be re-expressed on e.g. T_{EMRA} cells.
CD45RO	Effector	Identifies T cells which can recall previous antigen exposure.
CCR7	Memory	Involved in entry of T cells to lymphoid organs.
CX3CR1	Memory	A marker of T-cell differentiation which indicates robust antiviral properties such as cytotoxicity.

These protein markers can be used to divide memory CD8+ T cells into effector memory (T_{EM}) and central memory (T_{CM}) cells which are both characterised by the absence of CD45RA and expression of CD45RO as shown in Figure 4 (Martin et al. 2018). T_{EM} cells are associated with immediate cytotoxic function after exposure to antigen, with limited proliferation, whereas T_{CM} show the opposite phenotype. These memory populations can be distinguished by the expression of the chemokine receptor CCR7 which enables T_{CM} (CCR7+) but not T_{EM} (CCR7-) to migrate and enter the lymph nodes. A third population of CD8+ memory cells, called T_{EMRA}, lacks CCR7 but expresses CD45RA (Zangger et al. 2019). T_{EMRA} cells persist in circulation, but have a low proliferative capacity and a high production of the cytokine IFN-y (Larbi. et al. 2014).

Most inflationary CMV-specific CD8+ T cells are T_{EMRA}, and the frequency of T_{EMRA} cells increases with age (Zangger et al. 2022). The frequency of CMV-specific CD8+ T_{EMRA} T cells is higher than the CD4+ T_{EMRA} T cell population, with CD4 T_{EMRA} T cells constituting less than 10% of the total CMV-specific IFNy+ CD4 T cell response (Tian et al. 2017). A small proportion of CMV memory inflated CD8+ T cells are T_{CM} cells which are mostly found in the lymph nodes (Zangger et al. 2022).

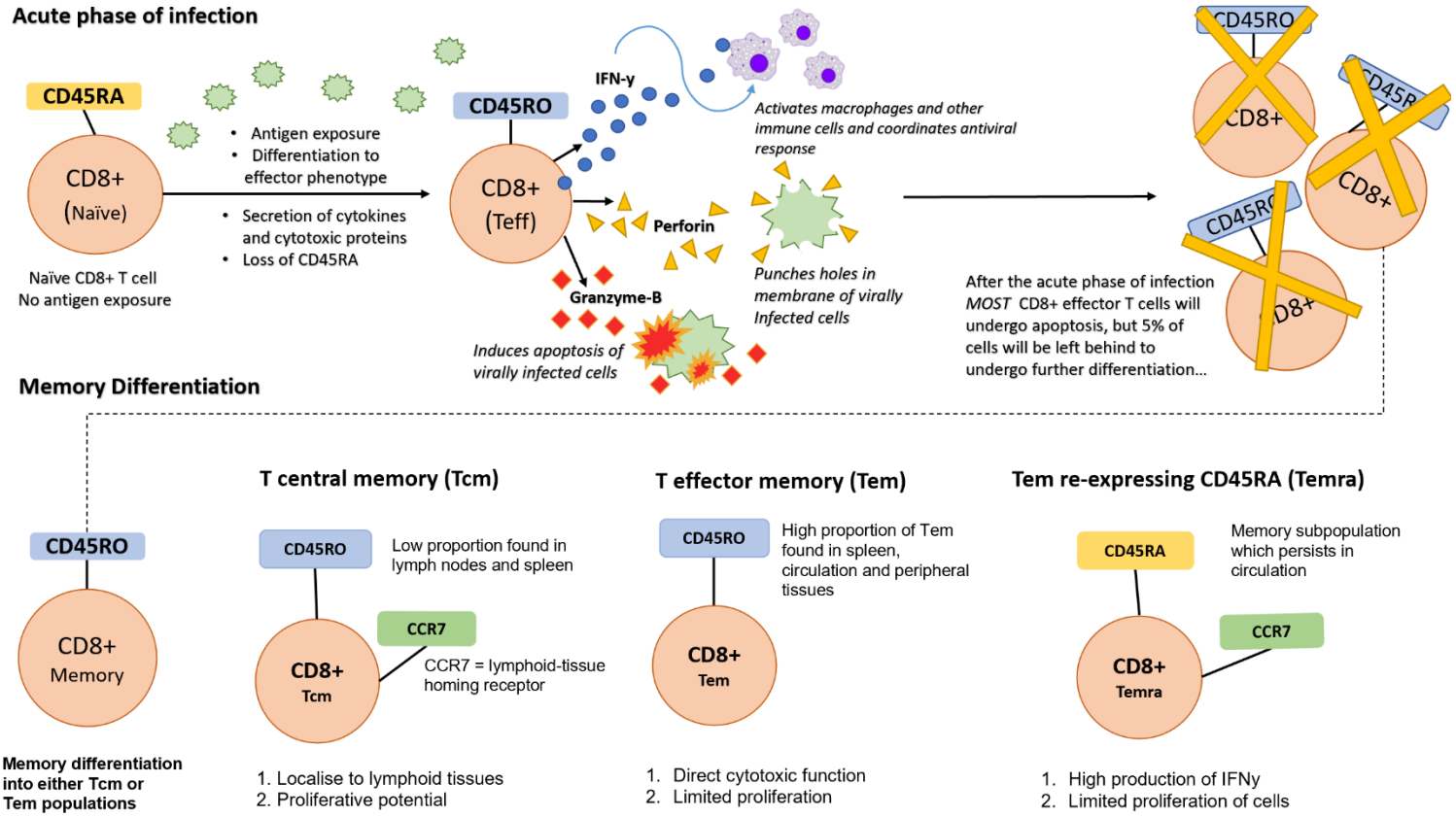


Figure 4. Overview of CD8+ T cell memory differentiation. In the acute phase of infection, naïve CD8+ T cells lose expression of surface marker CD45RA and become T effector (T_{EFF}) cells expressing CD45RO. T_{EFF} cells secrete cytokines and granule components to elicit an immune response to virally infected cells. Once the acute phase is over, wide-spread apoptosis eliminates most T_{EFF} CD8+ cells leaving approx. 5% behind to differentiate into memory cells. In brief, CD8+ T cells can differentiate into either T_{CM} or T_{EM}, defined by presence or absence of surface markers CCR7, CD45RA and CD45RO (Authors own. 2023).

Inflationary CMV-specific CD8+ T cells have also been reported to express unconventional markers, notably those associated with NK cells such as CD56, KIR and NKG2C (Picarda et al. 2018; Zangger et al. 2022). The anti-viral activity of CMV-specific CD8+ T memory cells has been attributed to CD8+ T_{EMRA} and T_{EM} cells, as well as an overlapping population expressing the fractalkine receptor CX3CR1 (Bottcher et al. 2015).

1.6.2.3 CMV immunity and ageing

CMV-specific T cell memory inflation may contribute to immune senescence and ageing of the immune system (Khan et al. 2004). Immune ageing is characterised by reduction in naïve T cells, accumulation of expanded CD28- memory T cells and a decline in overall immune responsiveness (Khan et al. 2004). The “immune-risk phenotype” is predictive of increased mortality in individuals aged 80+ years and includes CMV seropositivity (Day et al. 2007). CMV immunity may hinder the immune response to other pathogens. This assertion is supported by reports that CMV seropositivity is associated with lower success rate for influenza virus vaccination (Trzonkowski et al. 2003) and is a contributing factor to the enhanced progression of AIDS (Griffiths. 2006).

1.6.2.4 CD4+ T cells

Although CD8+ T cells are the prominent cells in CMV control, mice depleted of CD4+ T cells show increased incidence of MCMV infection, suggesting a critical role for CD4+ T cells (Polic et al. 1998; Einsele et al. 2002). In humans, children with primary CMV infection and CD4+ T cell deficiency had prolonged urinary and salivary shedding of CMV compared with matched CD4+ T cell normal counterparts (Szabolcs et al. 2008). In addition, low levels of CD4+ T cells have been shown coincident to CMV-associated complications in lung transplant recipients and with increasing viral load and symptomatic disease occurrence in renal transplant patients (Radha et al. 2005). Furthermore, individuals with low CD4+ T cell levels showed reduced viral control and were symptomatic, compared to asymptomatic individuals with higher levels of CD4+ T cells, implying that CD4+ T cells are critical for replicative control of CMV (Gamadia et al. 2003). After HSCT, CD4+ T-helper (Th) cell recovery is key to the reconstitution of CMV-specific CD8+ T cells and persistence of adoptively transferred T cells (Walter et al. 1995). Furthermore, adoptive transfer of CD4+ CMV-specific T cells reduces viral load in allo-HSCT patients (Einsele et al. 2002), and correlates with expansion of CMV-specific CD8+ T cells, suggesting that CD8+ effector T cells may be dependent on CD4+ T cell help in this context (Crough and Khanna. 2009).

CMV-specific CD4+ T cells, like CMV-specific CD8+ T cells, are high in frequency post infection, with 9-40% of the total CD4+ T cell pool being CMV-specific (Sylwester et al. 2005). CD4+ T cells show broad antigen recognition although the majority (>30%) are directed against gB in most individuals (Sylwester et al. 2005). In addition to providing help through aiding CD8+ T cell expansion, maintaining B-cells and promoting antibody

responses, CD4+ T cells may also kill CMV infected cells (Walter et al. 1995). Cytotoxic gB-specific CD4+ T cells have been expanded from healthy seropositive individuals and cytolytic activity by pp65-specific CD4+ T cells has also been described (Elkington et al. 2004).

1.7 Immune Evasion by CMV

During lytic infection, CMV expresses several viral proteins which mediate evasion from host immune defences (Crough and Khanna. 2009). This includes CMV encoded genes which interrupt antigen processing and presentation by MHC class-I and class-II (thereby hindering CD8+ and CD4+ antigen responses respectively), NK cell activation and host cell signalling (McSharry et al. 2012). The breadth of immune evasive mechanisms by CMV exceeds the scope of this MRes, although an overview is provided (Table 5).

Table 5. Overview of some immune evasive mechanisms employed by CMV (Information adapted from McSharry et al. 2012)

Immune mechanism disrupted by CMV	CMV encoded genes	Example Mechanisms
Major histocompatibility complex (MHC) expression	US2 US6 US11	MHC class-I and/or class-II are found on the majority of cells and enable antigen presentation to CD8+ and CD4+ T cells respectively to elicit T cell activation and recognition of virally infected cells. MHC can also inhibit NK activation. MHC class-I and class-II are downregulated on virally infected cells leading to reduced T cell and NK cell recognition.
Inhibition of antigen processing and presentation with MHC	US2 US3 US6 US10 US11	Antigens derived from pathogens are processed into short peptides for presentation with MHC for T cell recognition. During the immediate phase of CMV infection, the CD8+ T cell response is directed against peptides derived from the IE-1 transcription factor.
		MHC class-I/peptide presentation E.g. Matrix protein pp65 phosphorylates IE-1 to block presentation of the IE-1 derived peptides that CD8+ T cells are directed against.
		MHC class-II/peptide presentation US2 encoded proteins target MHC class-II to the proteasome for degradation.
Natural killer (NK) cell activation	UL16 UL18 UL40 UL141 UL142 US18 US20	Ligands (ULBP1 and ULBP2) once bound to NKG2D, activate NK cells. CMV encoded UL16 binds to ULBP1 and ULBP2 and blocks recognition by NKG2D, thus preventing NK cell activation. Matrix protein pp65 also inhibits another NK cell activating receptor NKp30.
Inhibition of IL-10	UL111a	Homologues of e.g. IL-10, mimic the shape of receptor ligands and have a higher affinity for their receptors. Once homologues are bound, the molecules cannot bind to their receptors to elicit their effect.

Section 3: The clinical burden of CMV

1.8 CMV in the HSCT setting

CMV infection is a frequent complication in the transplant setting. Primary infection can occur via transmission from a transplanted organ or stem cells from a CMV seropositive donor although more frequently, CMV complications arise from reactivation of latent virus (Azevedo et al. 2015). In hosts with depleted immunity such as HSCT recipients, uncontrolled viral replication leads to disseminated multi-organ disease and mortality (Emery et al. 2000). In HSCT, 60-70% of CMV seropositive patients suffer from CMV reactivation within the first 100 days post-transplant (Einsele et al. 2020) and without prompt treatment up to 10% of patients develop potentially fatal CMV disease (see Table 3 for symptoms). Studies into all-cause mortality in transplant setting have approximated a 28.9% mortality rate in HSCT recipients with disseminated CMV disease (Han et al. 2021).

Risk stratification for CMV disease uses CMV IgG status (serostatus) in the donor and recipient which forms the basis of transplant matching (Fishman 2017) (see Table 6). Due to the severe clinical consequences of CMV disease, at-risk transplant patients are treated with anti-viral prophylaxis (letermovir) for the defined period of immunosuppression (Owers et al. 2013). However, once letermovir is stopped, late CMV disease can occur which affects 25-40% of HSCT patients (Fishman 2017).

Despite the availability of effective anti-viral drugs, many patients develop significant side effects such as nephrotoxicity, neutropenia and increasing drug resistance (Leeaphorn et al. 2019). Despite CMV DNA monitoring post allo-HSCT, some patients develop CMV disease before CMV DNA becomes detectable in peripheral blood (Ruell et al. 2007). Additionally, HSCT recipients with detectable CMV DNA in peripheral blood do not always develop CMV disease (Ljungman et al. 2004). Several studies have demonstrated that frequency of IFN-y secreting CMV-specific CD8+ T cells correlates with protection from CMV infection following transplantation (Bunde et al. 2005). In one study, 80% of symptomatic patients showed a significant drop in the level of IFN-y expression by CD8+ T cells prior to clinical diagnosis of active disease, suggesting that CD8+ T cell changes might predict disease onset (Crough et al. 2007). Similar findings have been reported by others (Mattes et al. 2008). These key studies provided evidence that quantitative measures of functional T cells for CMV have potential to be an effective additional tool for disease monitoring in the transplant setting.

Table 6. Risk of CMV reactivation with respect to donor/recipient IgG status. (Illustrated by Dr Emanuela Pelosi).

CMV IgG status		Risk of CMV viremia and CMV disease*	
Donor (D)	Recipient (R)		
D^-	**R^-**	- The donor is not infected with CMV (CMV-IgG Not detected). - No risk of transmission of CMV infection from donor to recipient - Combination beneficial for the CMV susceptible recipient. - Should the recipient acquire primary infection post-HSCT, from a source different from the graft, the risk of CMV disease would be as high as for the combination D^-/R^+	Lowest
D^+	**R^-**	- The donor is infected with CMV (CMV-IgG Detected) - There is the risk of transmission of CMV from the graft to the HSCT recipient that would result in primary infection (the combination would become D^+/R^+) - There is transfer of CMV-specific $CD8^+$ T-cells to the recipient	
D^+	**R^+**	- The donor is infected with CMV (CMV-IgG Detected). - There is risk of transmission of CMV infection (superinfection) to the CMV infected transplant recipient - There is transfer of CMV-specific $CD8^+$ T-cells to the recipient - Combination beneficial for the recipient	
D^-	**R^+**	- The donor is not infected with CMV (CMV-IgG Not detected). - There is no risk of transmission of CMV infection (superinfection) to the CMV infected transplant recipient - There is no transfer of CMV-specific $CD8^+$ T-cells to the recipient - High risk for CMV reactivation and disease in the recipient	Highest

Section 4: Assays for detection of CMV and cellular mediated immunity (CMI)

Rapid detection of CMV is critical in enabling pre-emptive anti-viral therapy to prevent severe CMV disease (Griffiths et al. 2014). A number of techniques can be used for DNA detection and detecting immune responses to CMV.

1.9 Detection of CMV

Rapid detection of CMV is by antigenemia assays to detect pp65, by histology to detect morphological changes caused by the virus or by polymerase chain reaction (PCR). A summary of laboratory tests for CMV can be seen in Table 7.

Table 7. Overview of assays used for detection of CMV (Adapted from Razonable et al 2020).

Assay	Test Characteristic	Benefits and Clinical use	Disadvantages
Virus detection			
CMV QNAT (viral load)	• Detects and quantifies CMV nucleic acid. • Results reported in IU/mL	• Rapid & highly sensitive • Surveillance for pre-emptive therapy in HSCT • Monitoring of antiviral response • Testing of amniotic fluid and infant urine for congenital CMV	• Labour intensive
Antigenemia	• Monoclonal antibodies used to detect CMV pp65 antigen expressed in leukocytes during the early period of CMV replication. • Reported as number of pp65+ cells per number of leukocytes	• Sensitive diagnosis of CMV infection • Surveillance for pre-emptive therapy • Monitoring of antiviral response • Prognostic risk of CMV disease	• Lack of assay standardisation • Requirement of sufficient leukocytes (limits use in HSCT and neutropenia) • Labour intensive • Lack of automation • Subjective interpretation
Histopathology	• Detects CMV antigen and cytopathic changes in tissues	• Gold standard for diagnosis of end-organ CMV disease	• Highly invasive specimen collection • Long turnaround time for results, unsuitable for timely clinical management

Quantitative nucleic acid amplification tests (QNATs) are based on the PCR technique. These are rapid and sensitive approaches for detecting CMV in whole blood and plasma (Razonable et al. 2020). DNA can be extracted from whole blood, leucocytes, tissues, urine, amniotic fluid and many other tissue types (Beam et al 2018).

The antigenemia assay uses fluorescent monoclonal antibodies to detect pp65 in leukocytes in the early stages of HCMV replication (Ross et al. 2011). Interpretation of results in the immunocompromised is controversial however due to false-negative results in the absence of polymorphonuclear leucocytes such as those with neutropenia, which encompasses most transplant recipients (Boeckh et al. 1997). QNATs have replaced CMV antigenemia assays (Razonable et al. 2020).

Reverse transcriptase PCR (RT-PCR) can be used to detect viral mRNA transcripts independent of the presence of viral DNA in peripheral blood leucocytes. However, detection via Quantitative PCR is more sensitive than the RT-PCR method (Randhawa et al. 1994).

1.9.1 Detecting immune responses to CMV

1.9.1.1 Detecting antibodies to CMV

Assays have also been developed to detect immune responses to CMV, rather than the CMV virus itself (Table 7). Serology indicates previous or current CMV infection, determined by the presence of CMV specific immunoglobulin G (IgG) or immunoglobulin M (IgM) antibodies in plasma (Ross et al. 2011). Detection of CMV IgG antibodies is by enzyme-linked absorption assay (ELISA) (Ross et al. 2011). IgM assays lack specificity to determine primary infection due to the rate of false positive results, the persistence of IgM following resolution of infection and the presence of IgM in viral reactivation events (Bhatia et al. 2004).

IgG avidity assays may be used in populations who require the distinction of primary from non-primary infections (Prince et al. 2014). Over time, antibodies undergo affinity maturation and avidity of the antibody increases. Low avidity antibodies denote recent infection (within 3 or 4 months) whereas high avidity is defined as a historical infection i.e. matured for more than 3-4 months (Prince et al. 2014). Avidity tests cannot identify recent re-infections with different CMV strains. CMV-specific antibodies can be used to predict risk of HCMV disease after HSCT (Bruminhent et al. 2015); as described in Table 6.

1.9.1.2 Detecting T cells specific for CMV

Detection of T cells specific for CMV (cell mediated assays; CMI) can employ many *ex vivo* T cell assays such as peptide-MHC multimer binding, enzyme-linked immunospot (ELISPOT) and flow cytometric intracellular cytokine staining (ICS); see Tables 7 and 8. The latter is often used to detect and quantify cytokine-secreting cells such as IFN-y producing

CMV-specific T cells, in response to antigen stimulation (Bunde et al. 2005). Additional surface markers can be used to evaluate the phenotype of defined memory sub-populations of CMV-protective cells which provides a comprehensive characterisation of the CMV-specific T cell response (Appay et al. 2002).

Table *8*. Overview of CMI assays for detecting CMV-specific T cells (Information adapted from Crough and Khanna. 2009).

Assay	Cellular Targets	Technique/Method	Advantages	Disadvantages
Peptide-MHC multimers / tetramers	CD8+ T-Cells	Peripheral blood mononuclear cells (PBMCs) or whole blood incubated with MHC-peptide tetramer or synthetic peptide epitopes. After incubation, flow cytometric analysis detects CMV-specific T cells.	- Rapid and sensitive at risk predicting in stem cell transplant	- Flow cytometry is expensive and labour intensive - Sample limitations (must be processed immediately)
ELISPOT	CD8+ and CD4+ cells	Uses overlapping peptide pools to stimulate PBMCs. IFN-y is detected by HRP-labelled antibodies and spot-forming cells analysed using image analysis.	- High sensitivity - Assay performed on a fixed number of PBMCs from whole blood	- Lack of standard cut off values - PMBC isolation requires specialised staff
Flow Cytometry Intracellular stain (ICS)	Cytokine producing cells (mainly CD8+ T cells secreting IFN-y)	Lymphocytes isolated from whole blood are cell surface stained with fluorochrome conjugated antibodies and further intracellular stained following a fixation and permeabilisation step.	- Comprehensive characterisation of CMV protective cells - Quantitation of absolute number of CMV specific cells	- Complex - Labour intensive - Sample limitations (sample to be used immediately) - Expensive
QuantiFERON-CMV	CD8+ T cells	Stimulation of whole blood with 22 CMV peptides: pp50, pp65, gB, pp28, IE-1, IE-2 in blood collection tubes. Following incubation (16-24hrs), plasma is separated by centrifugation and standard ELISA is used to quantitate IFN-y in the plasma.	- Rapid turnaround - Highly sensitive - Simple sample processing - Sample can be stored following incubation - Amenable to automated ELISA	- Lack of assay standardisation - Detects only CD8+ T cells - Patients with uncommon HLA types may not be detected correctly

Despite the diagnostic potential of measuring CMI responses (Table 8) for CMV to define high risk patients, the utility of assays is restricted by several factors such as the level of complexity, limited standardisation and automation and absence of equipment and trained staff (Lilleri et al. 2008). The antigens used can also directly affect the efficiency and sensitivity of these assays (Lilleri et al. 2008). Several protein antigens may activate T cells and therefore analysing a single antigen or epitope alone may be insufficient to predict clinical CMV disease (Crough and Khanna. 2009). The focus of this MRes is on the QuantiFERON-CMV and flow cytometry assays. These are both described in more detail below.

1.9.1.2.1 The QuantiFERON-CMV assay

The QuantiFERON-CMV assay (Figure 5) includes 3 blood collection tubes, a nil tube containing to peptide, a mitogen tube containing phytohemagglutinin (PHA) as a positive control and a CMV peptide-coated blood tube which stimulates CD8+ T cells in whole blood to release IFNy which can then be quantified. The assay is simple, rapid and amenable to automation. The peptides used derive from proteins including pp65, pp50, gB and IE-1 antigens, and are binders for a broad range of HLA class-I alleles, which are recognised by approximately 98% of the human population (Elkington et al. 2003). The QuantiFERON-CMV is sufficient in detecting CMV-specific T-cell responses in infected healthy individuals and transplant recipients (Crough and Khanna. 2009). Furthermore, the QuantiFERON-CMV assay can detect suboptimal CMV immunity in peripheral blood prior to viral reactivation in transplant recipients (Westall et al. 2008). Therefore, this assay may be of clinical value in predicting the risk of CMV disease in transplant recipients, with potential to guide clinical management related to anti-CMV prophylaxis or therapies.

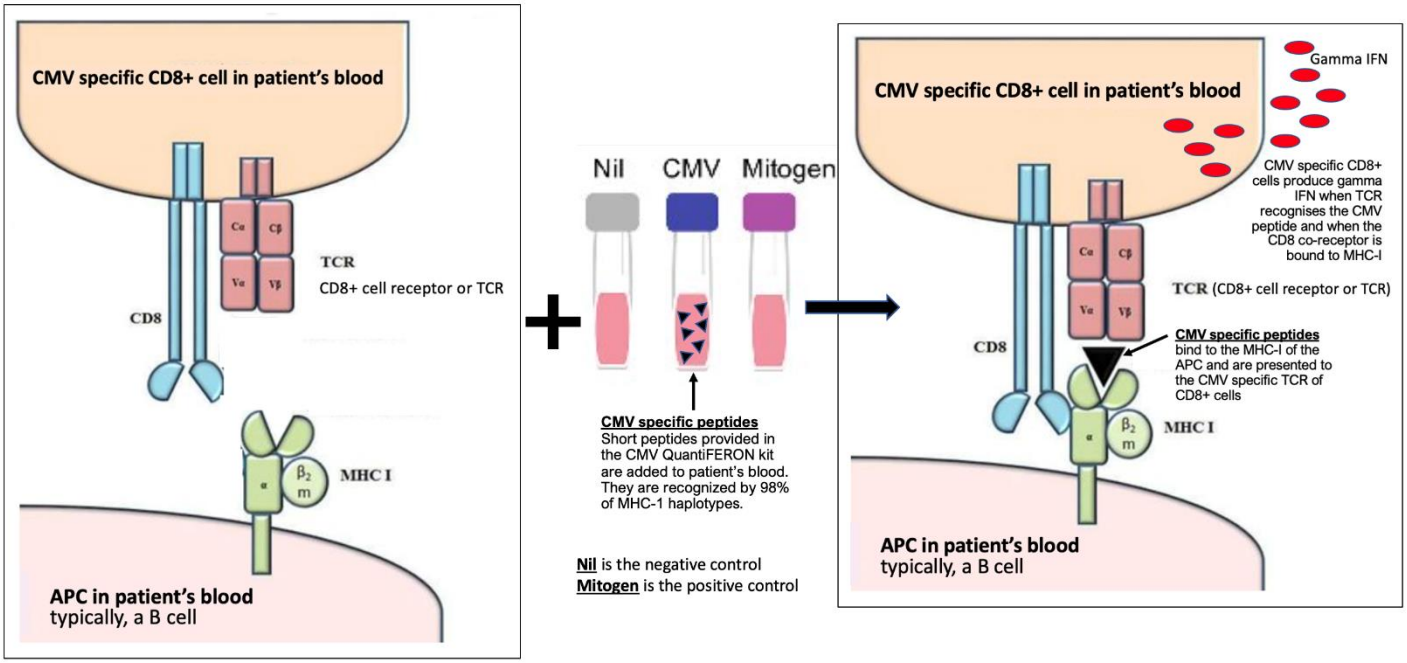


Figure 5. Overview of the QuantiFERON-CMV assay. 1ml of whole blood is introduced to the QuantiFERON-CMV tubes. Peptides coating the CMV tubes are expressed by MHC class-I in the sample. These peptide/MHC-I complexes are detected by CMV-specific CD8+ T cells in the sample. The nil tube acts as a negative control, with no peptide-coating. The mitogen tube acts as a positive control, stimulating a pan CD8+ T cell response, irrespective of antigen or CMV-specificity. In brief, the QuantiFERON-CMV result is calculated by subtracting the IFN-y present (measured in IU/mL) in the nil tube from the IFN-y present in the CMV tube after incubation. See methods for more information on result interpretation (Illustrated by Dr Emanuela Pelosi).

As outlined by the manufacturer, the QuantiFERON®-CMV assay is not a diagnostic tool for determining CMV infection and should not be used to exclude CMV infection in the clinical setting. It is an assay to monitor CMI to complement routine standard diagnostic methods for CMV infection (outlined in section 1.9). A theoretical model for clinical interpretation of the QuantiFERON-CMV assay is shown in Figure 6.

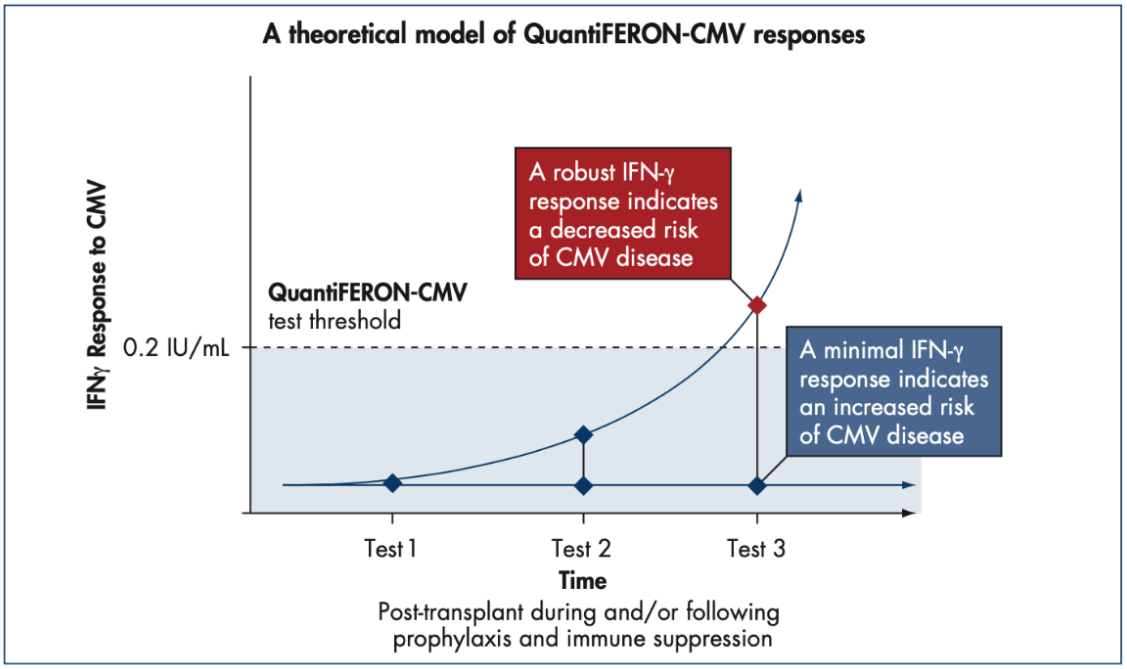


Figure 6. Theoretical model for clinical interpretation of the QuantiFERON-CMV assay. This model is based on measuring IFN-y responses to CMV peptides using the ELISA technique. A measurement of 0.2 IU/mL (CMV tube IFNy IU/mL minus Nil tube IFNy IU/mL) is indicative of a reactive immune response to CMV. An IFN-y value below 0.2 IU/mL is considered as an unreactive immune response. This model is theorised for use in the transplant setting and demonstrates QuantiFERON-CMV assay results at different timepoints during the immune reconstitution following immune suppression and/or anti-viral prophylaxis (Illustrated by and permissions for use granted by Qiagen Ltd).

1.9.1.2.2 Flow cytometry

Flow cytometry uses lasers to scrutinise individual cells within a mixed cell population. The angle of laser deflection provides information about cell size (forward scatter) and cell granularity (side scatter), to enable discrimination and quantification of broad cell types e.g. granulocytes, lymphocytes, erythrocytes.

Flow cytometry can also provide information on T cell subset frequencies if mixed cell populations (e.g. whole blood, isolated lymphocytes) are pre-incubated with fluorochrome-tagged antibodies specific for T cell subset markers of interest (see section 1.6.2.2). Multiple fluorochrome-tagged antibodies can be used to target several antigens at once. Individual fluorochromes are excited by specific wavelengths of light via lasers within the flow cytometer causing release of light at a specific wavelength which is detected by wavelength-specific detectors. Flow cytometry can be used to detect secreted proteins (such as IFN-y) by stimulating cells in the presence of inhibitors of secretion, thereby causing accumulation of proteins inside the cells. Fixation and permeabilization steps can then be added to the protocol to visualise antigen-specific T cells for example (i.e. those accumulating IFN-y after peptide restimulation).

1.10 Aims and objectives

The aim of this study is to evaluate the performance of the Qiagen QuantiFERON-CMV assay alongside flow cytometry in detecting CMV-specific CD8+ T cell responses in healthy individuals. In parallel, serology will assess whether CMV IgG titre correlates with cell mediated immunity to CMV in health. This Masters project will provide preliminary 'proof-of-principle' data to test the usefulness and clinical utility of the QuantiFERON®-CMV assay, coupled with flow cytometry to define CMV T cell responses in healthy individuals, prior to a clinical study evaluating CMV immune reconstitution in allogeneic-HSCT recipients at University Hospital Southampton (UHS).

Objectives:

1. To confirm that IFN-y production measured by Qiagen QuantiFERON-CMV assay can be used to quantify CMV-specific CD8+ T cells
2. Define CMV-specific $CD8^+$ lymphocyte phenotype and function by flow cytometry
3. Determine if there is a correlation between humoral and cellular immunity to CMV in healthy volunteers

Hypotheses

1. QuantiFERON-CMV will detect CMV-specific CD8+ T Cell responses
2. Flow cytometry will provide added value to defining CD8+ T cell responses
3. CMV IgG titre correlates with CMV T cell frequency as measured by QuantiFERON-CMV and flow cytometry

2. Methods

2.1 Reagents

Reagents and antibodies used in this study are listed in Table 9.

Table 9. Reagents and resources used in this MRes.

Reagent or Resource	Concentration in assay/volume per tube	Stock concentration	Source	Cat number/clone
Fluorescent Antibodies				
Anti-CD4 FITC	2.5ul	Lot specific	Biolegend	300538
Anti-CD8 PerCPCy5.5	5ul	Lot specific	Biolegend	301032
Anti-CD45RA APCCy7	2.5ul	Lot specific	Biolegend	304128
Anti-CCR7 PECy7	2.5ul	Lot specific	Biolegend	353226
Anti-CX3CR1 Af647	2.5ul	Lot specific	Biolegend	341608
Anti-IFNy PE	2.5ug/ml	200ug/ml	ThermoFisher/eBioscience	12-7319-82
Anti-CD45RA (Isotype control)	2.5ul	Lot specific	Biolegend	400328
Anti-CCR7 (Isotype control)	2.5ul	Lot specific	Biolegend	400232
Anti-CX3CR1 (Isotype control)	2.5ul	Lot specific	Biolegend	400626
Anti-CD3-PE	5ul	Lot specific	Biolegend	317308
Anti-IFN-y PE (Isotype control)	2.5ug/ml	200ug/ml	ThermoFisher/eBioscience	12-4714-82
Non-Fluorescent Antibodies				
Anti-CD3	1ug/ml	Lot specific	Biolegend	317326
Anti-CD28	50ng/ml	0.5mg/ml	Biolegend	302902
Reagents				
PMA	50ng/ml	1mg/ml	Sigma Aldrich	P8139
Ionomycin	1ug/ml	1mg/ml	Millipore	407950
Lymphoprep	N/A	N/A	Stemcell technologies	07801
Golgiplug	1/1000 dilution	Not specified	BD Biosciences (Fisher scientific)	BDB555029
Fc block	10ul	Not specified	Miltenyi biotech	130-059-901
Foxp3 staining buffer kit	N/A	N/A	ThermoFisher/eBioscience	00-5523-00
PBS tablets (Phosphate-buffered saline)	N/A	N/A	ThermoFisher	18912014

BSA (Bovine serum albumin)	0.5% w/w	N/A	Fisher scientific	9048-46-8
EDTA (Ethylenediaminetetraacetic acid)	2mM	0.5M	Sigma	E5134
FCS (Foetal calf serum)	10% in media	N/A	Fisher Scientific	11550356
Sodium pyruvate	1mM	100mM	Fisher Scientific	12539059
L-glutamine	1x solution	100x stock	Fisher Scientific	11500626
Penicillin and streptomycin	100U/ml	10,000U/ml	Fisher Scientific	11548876
RPMI media	N/A	N/A	GIBCO	21875-034
Blood collection tubes				
QIAGEN CMV-QuantiFERON blood collection tubes	N/A	N/A	QIAGEN	0350-0201

2.2 Cell Lines

The human Jurkat T cell line was used for experiments shown in Figures 7-12 was purchased from ATCC (www.atcc.org/products/tib-152). Cells were grown and maintained in a humidified incubator at 37C and 5% CO_2 in complete RPMI media supplemented with 10% heat inactivated FCS, 2mM L-glutamine, 1-mM pyruvate and 100 U/mL penicillin and 100 ug/mL streptomycin (henceforth called complete media). Cells were cultured for a maximum of 2 months before returning to a frozen stock in order to prevent drift. Cell lines were observed for contamination and visualised under a microscope to ensure cell viability and verification of expected phenotype.

2.2.1 Cell stimulation for human Jurkat T cells

Jurkat cells were counted and viability confirmed using a hemacytometer and plated at $5x10^6$ cells/ml in 1.2ml complete media at 100ul/well in duplicate. Complete media was supplemented or not with 50ng/ml PMA + 1ug/ml Ionomycin. Stimulation was in the presence of Golgiplug (see Table 9) at 1/1000 dilution or 10ul/well as indicated. Plates were incubated at 37C and 5% CO_2 and cells harvested after 4-16 hours as specified.

2.2.2 Flow cytometry of human Jurkat T cells

After stimulation, Jurkat cells were washed with PBS supplemented with 0.5% BSA + 1/1000 dilution Golgiplug (GP) by centrifugation at 1300rpm with the brake on. Fluorophore conjugated antibodies against surface markers (anti-CD4-FITC, anti-CD8-PerCPCy5.5 and anti-CD45RA-APCCy7 or isotype control (isotype-APCCy7 only) were added and incubated at 4C for 20mins. Cells were washed twice in PBS/0.5%/BSA/GP and fixed using the Foxp3 staining buffer kit according to manufacturer's instructions prior to intracellular staining with

antibodies targeting IFN-y (PE) or a similarly conjugated isotype control for 20 mins at 4C in permeabilization buffer. Cells were washed in permeabilization buffer and then twice in PBS before flow cytometric analysis on a BD Cytoflex. Appropriate single colour and fluorescence minus-one controls were included.

2.3 Human samples

2.3.1 Ethics and consent

The use of human blood samples was approved by Bournemouth University ethical committee. HTA approval was not required for the purpose of this study and samples were stored for no longer than 5 days. Informed consent for the use of human material was provided in accordance with the Declaration of Helsinki. All volunteers were supplied with a donor information sheet and signed a consent form (see Appendix).

2.3.2 Blood collection

For *in vitro* assays using human cells, 10mls of blood was collected into lithium-heparin containing vacutainers and processed for flow cytometry and QuantiFERON-CMV assay, within 1 hour of sample collection. Blood was collected by a trained phlebotomist and human PBMCs used in experiments shown in Figures 10-25 were obtained from healthy volunteers within Bournemouth University. Donated blood samples were only collected one time per participant. All samples were tested once per the methods below and subsequently discarded; no repeat testing was performed on any samples.

2.4 QuantiFERON-CMV assay

For the analysis of response to CMV using the Qiagen CMV-QuantiFERON assay as per manufacturer instructions, 1ml of freshly obtained whole human blood was aliquoted directly into three QIAGEN QuantiFERON tubes. The tubes contained a negative control with no antigens (Nil tube), a mix of 22 CMV peptides (CMV tube) and a positive mitogen control (Mitogen tube) containing phytohemagglutinin. Tubes were inverted 10 times prior to upright incubation at 37C in 5% CO_2 for 24 hours. Tubes were centrifuged at 2000g for 15mins and plasma collected. Plasma samples were transported from Bournemouth University to University Hospital Southampton and plasma was analysed for IFNy (IU/mL) by automated CLIA, on the DiaSorin Liaison® XL. Where results exceeded the upper limit of detection (i.e. >10 IU/mL), samples were manually diluted 1:10 and/or 1:100. Results were interpreted as per manufacturer's instructions and according to Table 10.

Table 10. Interpretation of QuantiFERON-CMV assay results as per manufacturer's instruction.

Nil (IU/mL)	**CMV minus Nil (IU/mL)**	**Mitogen minus Nil (IU/mL)**	**QF-CMV Result**	**Report/Interpretation**
Less than or the same as **8.0**	**Same or more than** 0.20 and 25% of the nil	Any	Reactive	Anti-CMV CD8+ immunity **detected**
	Less than **0.20** OR Same or **more** than **0.20** and **less** than **25%** of the nil	Same or **more** than **0.5**	Non-reactive	Anti-CMV CD8+ immunity **NOT detected**
		Less than 0.5	Indeterminate	Results are **indeterminate** for anti-CMV CD8+ responsiveness
More than **8.0**	Any	Any	Indeterminate	Results are **indeterminate** for anti-CMV CD8+ responsiveness

2.5 Determination of anti-CMV IgG and IgM antibodies

After QuantiFERON-CMV analysis, remaining serum from the 3 samples were combined and CMV-specific antibody (Ab) levels (both CMV IgG and IgM) were measured by semi-quantitative assay using a Beckmann UniCel DXI 800. Data are expressed as arbitrary units; AU/mL. Analyses for CMV IgG and IgM were performed in the Biochemistry Laboratory at University Hospital Southampton. Table 11 shows the cut-off values for CMV IgG and IgM by DXI, as determined for clinical use at University Hospital Southampton.

Table 11. Cut-off values for CMV antibody testing using a Beckmann UniCel DXI 800

CMV IgG (AU/mL)		**CMV IgM (AU/mL)**	
Negative	Less than 11.0	**Negative**	Less than 0.8
Equivocal	11.0 – 15.0	**Equivocal**	0.8 – 1.0
Positive	More than 15.0	**Positive**	More than 1.0

2.6 Flow cytometry of human blood samples

For analysis of blood samples by flow cytometry, leucocytes were isolated from the remaining 7ml of blood prior to activation and flow cytometry staining as follows.

2.6.1 Human lymphocyte isolation and quantification

Human lymphocytes from 7-10ml of blood were separated by centrifugation over density gradient using lymphoprep by centrifugation with the brake off at 2200rpm for 20mins at 4C. Lymphocytes were collected from the interface, cells diluted to a total volume of 50mls in PBS/2mM EDTA/10% FCS and centrifuged at 1600rpm for 5mins with the brake on. The cell pellet was then resuspended in 2mls complete media supplemented with 50uM 2-mercaptoethanol (2ME). For quantification, 10ul cells were diluted with 10ul trypan blue and counted using a haemocytometer. Cell count and viability were recorded. Cells were diluted to concentrations indicated in the text in 3mls complete media (supplemented with 2ME). Golgiplug was added to cells at a 1/1000 dilution prior to stimulation and incubation.

2.6.2 Human non-antigen-specific stimulation

In some assays, isolated human lymphocytes were plated in 100ul in triplicate in flat well 96-well plates at $5x10^6$ cells/ml in complete media alone or supplemented with 50ng/ml PMA + 1ug/ml Ionomycin, or with 1ug/ml plate-bound anti-CD3 + 50ng/ml soluble anti-CD28. Plates were incubated at 37C and 5% CO_2 and cells harvested after 4-16 hours as indicated.

2.6.3 Flow cytometry of non-antigen-specific human cells

Cells treated as 2.6.2 were transferred to flow cytometry tubes, washed twice in PBS/EDTA/GP and incubated in PBS/0.5%BSA/GP with 10ul Fc block reagent in the dark at 4C for 15mins. For cell surface staining, fluorochrome-conjugated antibodies were added as follows: anti-CD4-FITC, anti-CD8-PerCPCy5.5, anti-CD45RA-APCCy7, anti-CCR7-PECy7, anti-CX3CR1-AlexaFluor647 (all Biolegend). Isotype controls for anti-CD45RA-APCCy7, anti-CCR7-PECy7 and anti-CX3CR1-AlexaFluor647 were used in tubes where indicated. One tube incorporated a single colour control for PE (using anti-human-CD3-PE rather than anti-human IFNy to ensure a positive signal). Antibodies were incubated with cells in the dark for 20mins at 4C. Cells were then washed twice with PBS/0.5%-BSA/GP and fixed using a Foxp3 staining buffer kit (eBioscience) according to manufacturer's instructions. Cells were then incubated with anti-IFN-y-PE (eBioscience) or with a PE-conjugated isotype control antibody in permeabilization buffer and incubated for a further 20mins in the dark at 4C. Cells were washed twice with PBS prior to analysis on a CytoFLEX flow cytometer.

2.6.4 Human CMV-antigen stimulation

For the analysis of responses to CMV using flow cytometry, human lymphocytes were isolated as per section 2.6.1. 1ml isolated lymphocytes were then incubated in each of three QuantiFERON-CMV tubes in complete media supplemented with 2ME and 1/1000 dilution of GP for 4 hours at 37C and 5% CO_2.

2.6.5 Flow cytometry of CMV-antigen stimulated human cells

After activation in QuantiFERON-CMV tubes as section 2.6.4, cells were gently resuspended and transferred to flow cytometry tubes. Cells were then stained with fluorescent antibodies and analysed on a CytoFLEX flow cytometer as per section 2.6.3.

2.6.6 Determining CMV reactivity by flow cytometry

Following flow cytometric analysis, criteria in Table 12 were applied to define a positive or negative IFN-y response to CMV by flow cytometry.

Table 12. Criteria for definition of IFN-y response by flow cytometry. These values only apply when the mitogen tube gives a clear and strong positive value compared with the Nil tube.

Nil tube CD8+ IFNy+ T cells (%)	**CMV tube CD8+ IFNy+ T cells (%)**	**Interpretation**
Less than or the same as **0.5%** of the total CD8+ T cell population	>0.1%	Positive IFNy response by flow cytometry
	<0.1%	Negative IFNy response by flow cytometry
More than 0.5% of the total CD8+ T cell population	>0.2%	Positive IFNy response by flow cytometry
	<0.2%	Negative IFNy response by flow cytometry

2.7 Data analysis and statistics

Flow cytometry plots were analysed in CytXpert software and numerical data were exported to Excel. Data analyses were performed using Excel and GraphPad Prism 9.5.1 software. Correlations statistics used Pearson' correlation and pair-wise analyses used non-parametric Mann-Whitney *U* tests. Mean and medians (with SEM) are shown throughout. Values were considered statistically significant when the *p*-value <0.05. Graphical presentation was performed with GraphPad Prism 9.5.1.

3. Results

Part 1: Flow cytometry

3.1 Surface phenotyping of human Jurkat T cells

The flow cytometer (Beckman Coulter CytoFLEX) at Bournemouth University is new to the university and few flow cytometry staining protocols have been performed on site. It was therefore essential to confirm that surface staining with a basic panel of flow cytometric antibodies could successfully be performed on site. Therefore, initial experiments sought to confirm the binding of T-cell specific flow cytometric antibodies to surface antigens on human T cells. The human Jurkat T cell line (obtained fresh from ATCC; see methods) was used for this work. Cells were surface stained with fluorescent antibodies specific for CD4 (conjugated to FITC), CD8 (PerCPCy5.5) and CD45RA (APCCy7) (Figure 7). Staining profiles of unstained cells (absence of anti-CD4-FITC) or cells incubated with isotype control antibodies (for anti-CD8 and anti-CD45RA) were used to set the gates.

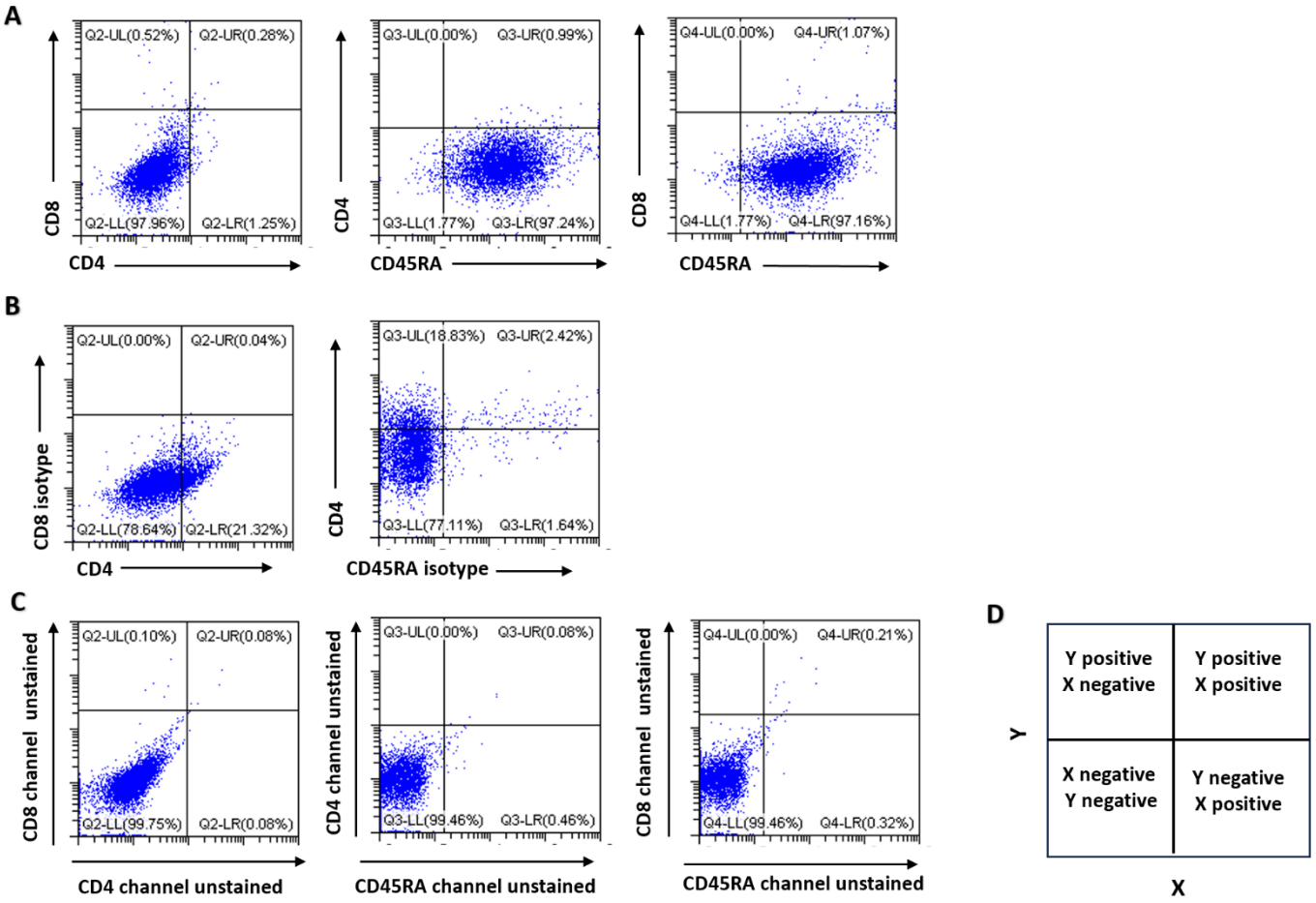


Figure 7. Flow cytometry surface staining of human Jurkat T cells. Human Jurkat T cells were grown in vitro and incubated with fluorochrome-conjugated antibodies for surface markers CD4, CD8 and CD45RA. (A) shows flow cytometry profile for Jurkat T cells stained with anti-CD4, anti-CD8 and anti-CD45RA as indicated. (B) shows isotype control staining's for CD8 and CD45RA used to set the gates in (A). No CD4 isotype was available and therefore in place of an isotype, cells were left unstained for this marker. (C) shows unstained Jurkat T cells (D) shows the interpretation of quadrant flow cytometry plots. Numbers in each quadrant indicate the percentage of Jurkat T cells expressing surface markers indicated.

Data show that Jurkat T cells are negative for CD8, express low levels of CD4 (Fig 7A and 7B) and highly express CD45RA (Fig 7B), consistent with a previous report showing that Jurkat cells express CD4 (Dong et al. 1999). These data confirm that Jurkat T cells express CD4 and CD45RA and that the surface staining protocol can be used to phenotype human Jurkat T cells using the new flow cytometer at BU.

3.2 Surface and intracellular phenotyping of human Jurkat T cells

This Masters project precedes a larger study which seeks to identify T cells intracellularly accumulating the cytokine IFN-y in response to stimulation. Following successful surface labelling of Jurkat T cells, we therefore sought to validate a protocol for intracellular cytokine staining (ICS) to detect intracellular IFN-y in activated T cells, again using Jurkat T cells initially. Jurkat T cells were therefore activated (or not; control) with PMA/Ionomycin (see methods). Cells were surface stained with anti-CD4-FITC, anti-CD45RA-APCCy7 or with isotype control conjugated to APCCy7. Anti-CD8-PerCPCy5.5 was not included as Jurkat T cells do not express CD8 (Figure 7). Cells were then fixed, permeabilised and incubated with anti-IFN-y-PE or with an isotype control antibody conjugated to PE. A control group of unstained cells was also processed similarly (not shown).

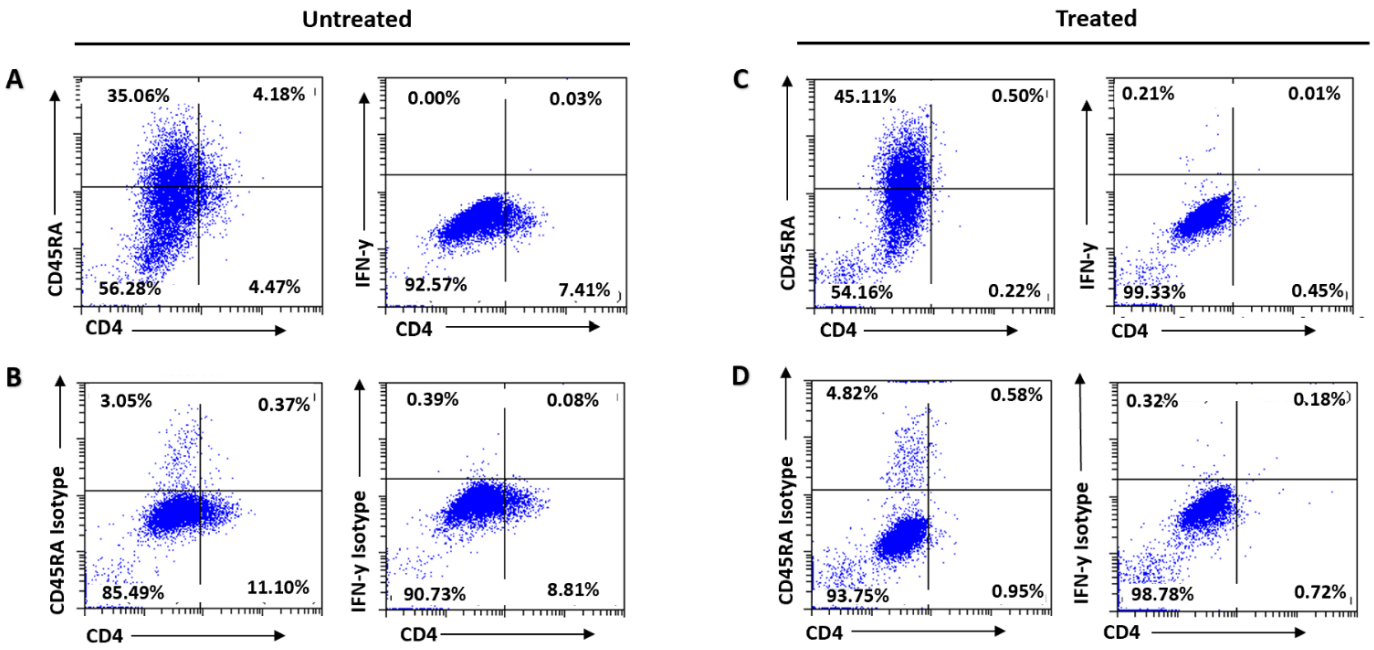


Figure 8. Jurkat T cells downregulate CD4 but fail to accumulate IFN-y after 4 hour PMA/Ionomycin activation. Human Jurkat T cells were either left un-treated (A and B) or were treated (C and D) with PMA/Ionomycin for 4 hours prior to antibody staining for flow cytometry. Stimulation was concurrent with blockade of protein secretion. Cells were surface stained for CD4 and CD45RA, fixed, permeabilised and intracellularly stained for IFN-y accumulation. (A and C) data from cells stained with anti-CD4, anti-CD45RA and anti-IFN-y or (B and D) with surface-labelling antibodies but an isotype control antibody in place of anti-IFN-y-PE. Numbers in each quadrant indicate the percentage of Jurkat T cells expressing surface markers indicated.

In confirmation of Figure 7, untreated Jurkat cells (Fig 8A) expressed high levels of CD45RA and low levels of CD4; no expression of IFN-y was observed beyond background (Figure 8B). PMA/Ionomycin activated cells (Fig 8C) expressed equally high levels of CD45RA as un-activated cells but downregulated CD4, confirming cell activation. Surprisingly, no IFN-y accumulation was observed in activated cells (Fig 8C and 8D). CD45RA isotype controls for both treated and untreated cells showed some positive staining due to non-specific binding to the isotype antibody.

Following the observation that Jurkat cells expressed no IFN-y even after cell activation, we hypothesised that a longer incubation period of 16 hours with PMA/Ionomycin may enable detection of IFN-y accumulation. Alternatively, absence of IFN-y accumulation after stimulation may be a characteristic of Jurkat cells. To distinguish between these possibilities, cells were stimulated (or not; control) with PMA/Ionomycin for 16 hours and incubated with fluorescent antibodies as per Figure 8.

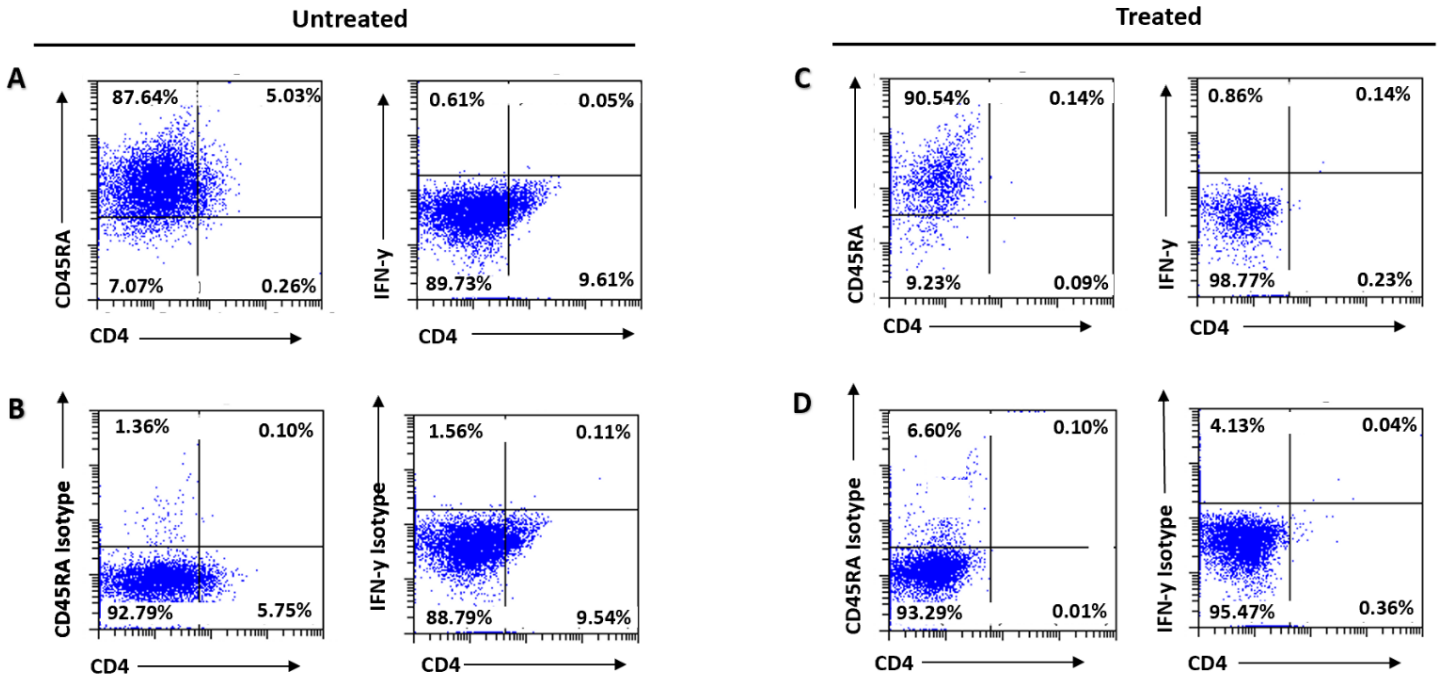


Figure 9. Jurkat T cells downregulate CD4 but fail to accumulate IFN-y after 16 hour PMA/Ionomycin activation. Human Jurkat T cells were plated in duplicate and were either (A and B) un-treated or (C and D) treated with PMA/Ionomycin for 16 hours prior to antibody staining for flow cytometry. Stimulation was concurrent with blockade of protein secretion. Cells were surface stained for CD4 and CD45RA, fixed, permeabilised and intracellularly stained for IFN-y accumulation. (A and B) data from untreated cells incubated with (A) all antibodies or (B) with surface antibodies but an isotype control antibody in place of anti-IFN-y-PE or in place of anti-CD45RA-APCCy7. (C+D) data from treated cells incubated with (C) all antibodies (i.e. anti-CD4-FITC, anti-CD45RA-APCCy7 and anti-IFNy-PE) or (D) with surface antibodies but an isotype control antibody in place of anti-IFN-y-PE or in place of anti-CD45RA-APCCy7. Numbers in each quadrant indicate the percentage of Jurkat T cells expressing surface markers indicated.

Data show (Figure 9), that as before (Figures 7 and 8), untreated cells (Fig 9A) highly expressed CD45RA, express low levels of CD4 and did not accumulate IFN-y. Treated cells (Fig 9C) expressed equally high levels of CD45RA as untreated cells, downregulated CD4 (again confirming cell activation) and did not accumulate IFN-y. After observing no expression of IFN-y in treated Jurkat cells, Jurkat cells are likely IFN-y negative, even after stimulation.

3.3 Phenotyping resting and activated human PBMCs

In previous assays, Jurkat T cells did not accumulate IFN-y (Fig 8 and 9). It was possible that this was due to a problem with the anti-IFN-y-PE antibody, or that Jurkat T cells do not upregulate IFN-y. To confirm whether the anti-IFN-y-PE antibody detected IFN-y inside cells, we moved to using human primary T cells rather than the Jurkat T cell line. Primary T cells are known to upregulate IFN-y in response to stimulation with PMA/Ionomycin or to the combination of agonist anti-CD3 and anti-CD28 (Nurieva et al. 2006). To ensure that IFN-y would be upregulated, primary PBMCs were therefore collected from a volunteer and left unstimulated or were stimulated with two separate stimulants; PMA/Ionomycin or anti-CD3/anti-CD28 for 4 hours in the presence of an inhibitor of protein secretion. Cells were then stained with a panel of surface antibodies and then intracellularly with anti-IFN-y-PE or with an isotype control. An example of the gating strategy used to determine cell populations is shown in Figure 10. For this assay, the surface staining panel was extended to include antibodies specific for CX3CR1 and CCR7 as well as CD4, CD8 and CD45RA. This enables T cell phenotype populations to be distinguished. Cells were gated based on a lymphocyte gate and singlets (Fig 10A). Cells were then further subdivided into those expressing CD4 or CD8 as shown and then naïve/memory populations within these gates were visualised (Fig 10B). Finally, cells accumulating IFN-y were gated for both CD4 and CD8 cell populations (Fig 10C).

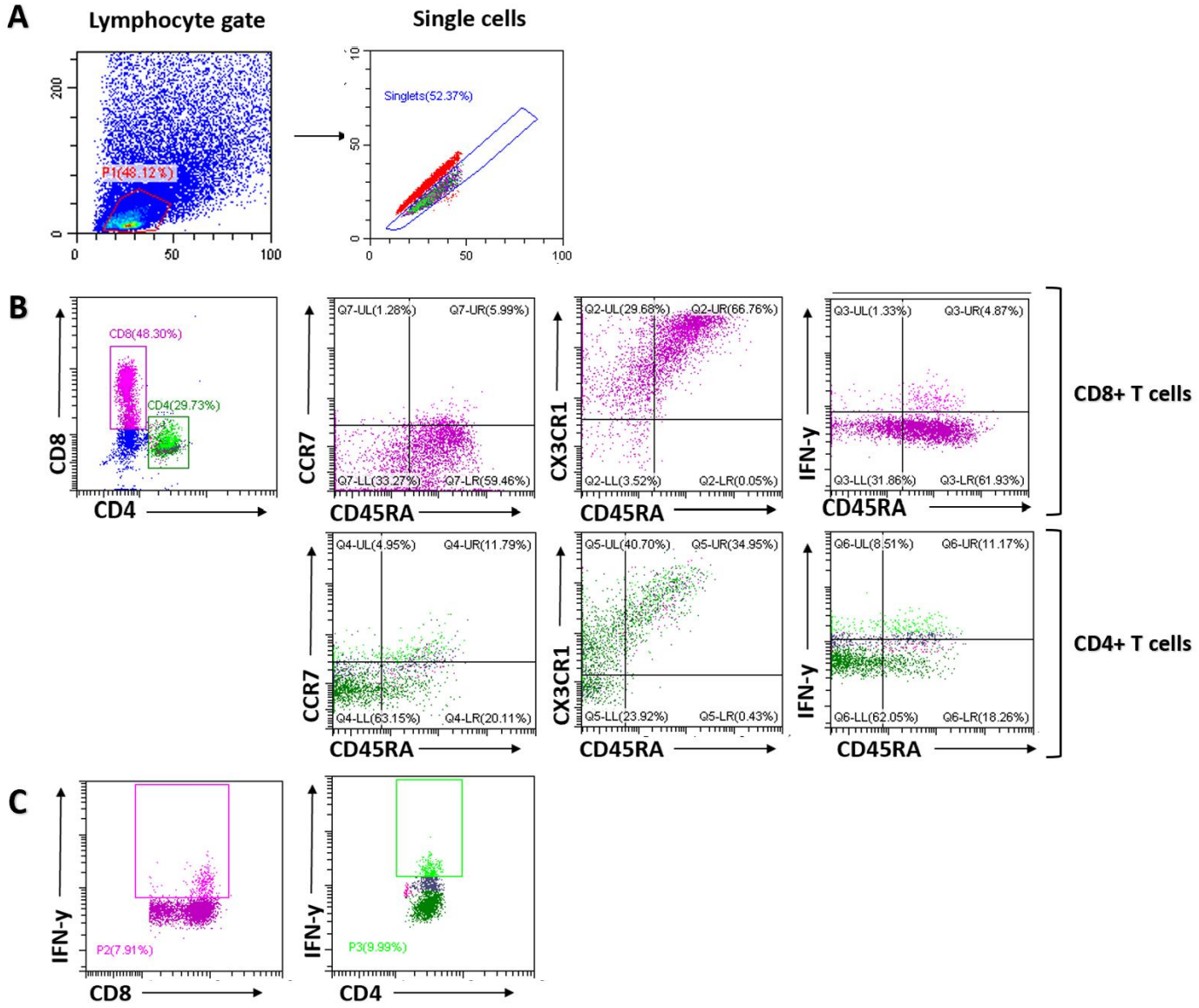


Figure 10. Flow cytometry gating strategy for evaluating IFNy accumulation and phenotype of human PBMCs across different stimulatory conditions. (A) Shows the lymphocyte gate and subsequent singlet gate. (B) Represents the subsequent gating for CD8+ and CD4+ singlet lymphocytes, followed by quadrant gates applied for distinguishing naïve/memory cell/activated populations for CD8+ and CD4+ cells. (C) Shows activated (IFNy+) cells within the CD8+ and CD4+ single lymphocytes. All gates and quadrants were set against single colour and fluorescence minus one controls. Staining shown is from non-stimulated cells. Selected plots from other conditions can be seen in Figure 11.

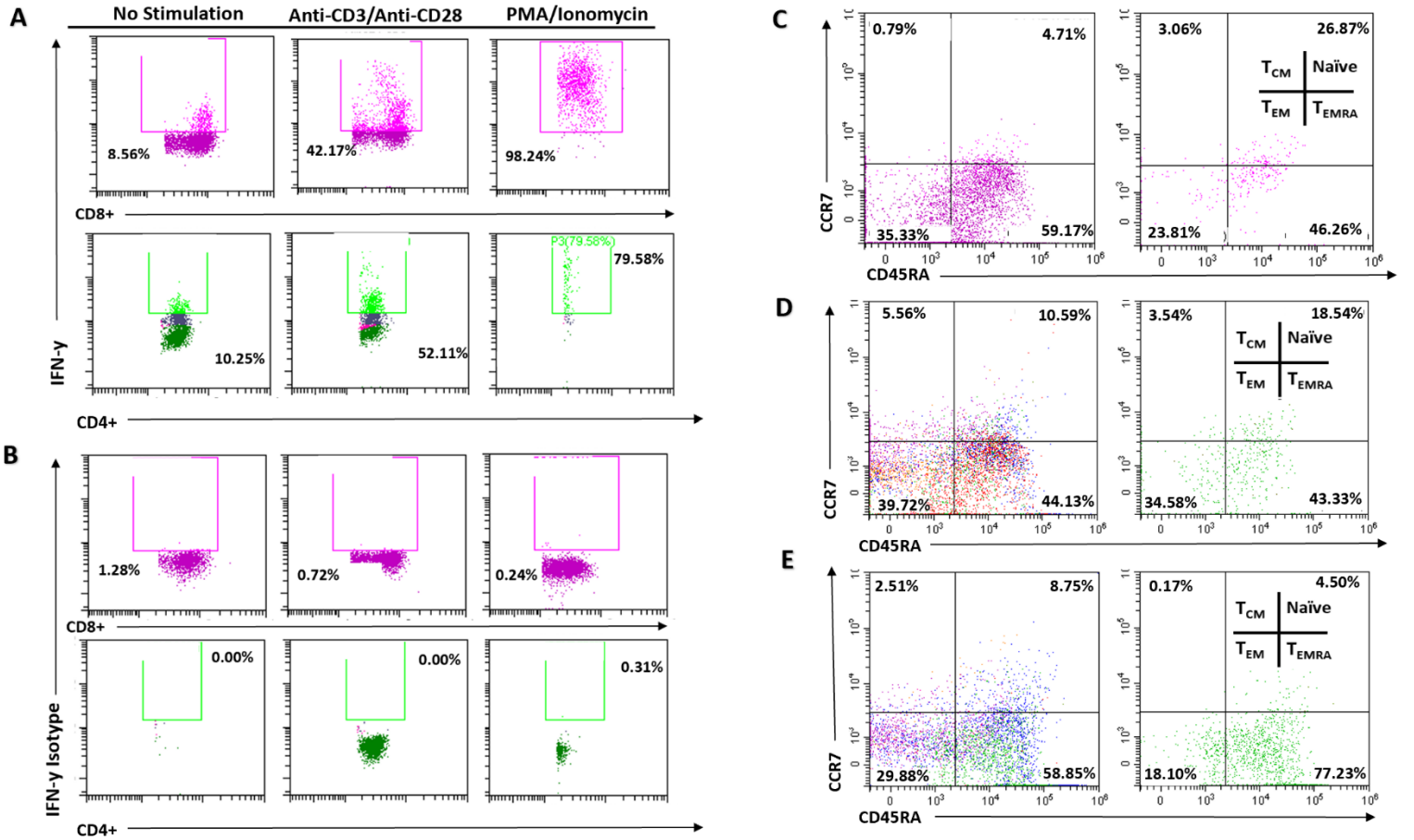


Figure 11. Immune reactivity and phenotype of human lymphocytes in various stimulatory conditions. Flow cytometric data (A and B) show both CD4+ and CD8+ cells gated for IFN-y positive cells as per Fig 10. Numbers indicate the percent of IFN-y+ cells in CD8+ or CD4+ T cell gates. (A) Cells surface stained with anti-CD4-FITC, anti-CD8-PerCPCy5.5, anti-CD45RA-APCCy7, anti-CCR7-PECy7, anti-CX3CR1-af647 and intracellular marker for IFN-y, anti-IFN-y-PE. (B) cells stained with surface markers anti-CD4-FITC, anti-CD8-PerCPCy5.5, anti-CD45RA-APCCy7, anti-CCR7-PECy7 and anti-CX3CR1-af647 and intracellularly stained with anti-IFN-PE. (B) cells stained as (A) but with an isotype control in place of IFN-y. (C) phenotype of non-stimulated total CD8+ T cells (left) and IFN-y+ CD8+ T cells (right). (D) phenotype of total CD8+ T cells (left) and IFN-y+ CD8+ T cells (right) stimulated by anti-CD3/anti-CD28. (E) phenotype of total CD8+ T cells (left) and IFN-y+ CD8+ T cells (right) stimulated by PMA/Ionomycin.

Data in Figure 11 show that stimulation of primary human CD8+ and CD4+ T cells with PMA/Ionomycin induced accumulation of intracellular IFN-y. While IFN-y also accumulated in anti-CD3/anti-CD28 stimulated CD4+ and CD8+ T cells, levels were lower than in equivalent cells activated with PMA/Ionomycin (Fig 11A). Non-stimulated cells accumulated less IFN-y compared with stimulated cells although background IFN-y was significant. While a small percent of both CD4+ and CD8+ T cells exhibited binding to the isotype control antibody (Fig 11B), there was a significantly higher proportion of both activated CD4+ and CD8+ T cells exhibiting florescence after incubation with the anti-IFN-y-PE antibody compared with the isotype-PE antibody (compare Fig 11 A and B). Also of note, unstimulated and anti-CD3/anti-CD28 stimulated CD4+ T cells accumulated more IFN-y compared to CD8+ T cells.

For this assay, an additional goal was to phenotype the naïve/memory characteristics of the CD8+ T cells. Therefore, in addition to surface staining with antibodies specific for CD4 and CD8, further antibodies were used to surface stain for CD45RA, CCR7 and CX3CR1 (see Fig 10 for gating strategy). Data show (Fig 11 C) that unstimulated total CD8+ T cells (Fig 11 C; left) in this donor were mainly of the T_{EMRA} and T_{EM} phenotype.

Data further show (Fig 11D) that anti-CD3/anti-CD28 stimulated CD8+ T cells (irrespective of IFN-y accumulation) were largely of the T_{EMRA} and T_{EM} phenotype. Similarly, PMA/Ionomycin stimulated CD8+ cells (Fig 11E), were predominantly of T_{EMRA} and T_{EM} phenotype, whether cells accumulated IFNy (Fig 11E; right) or not (Fig 11E; left). In the anti-CD3/anti-CD28 stimulated cells, the T_{EMRA} and T_{EM} populations (either in the total CD8+ (Fig 11D; left) or the IFN-y+ CD8+ T cells (Fig 11D; right)) were almost equal in frequency. However, after PMA/Ionomycin stimulation, the proportion of T_{EMRA} cells increased in the IFNy+ CD8+ T cell population (Fig 11D; right) compared to the total CD8+ T cell pool (Fig 11D; left).

In summary, the intracellular staining protocol detects IFN-y accumulating in primary T cells from a healthy individual. Furthermore, PMA/Ionomycin induced greater accumulation of intracellular IFN-y in CD4+ and CD8+ T cells compared with anti-CD3/anti-CD28. Anti-CD3/anti-CD28 stimulated more IFN-y accumulation than non-stimulated cells. Finally, a gating strategy incorporating detection of CD4, CD8, CD45RA and CCR7 allowed for discrimination of naïve/memory phenotypes and these phenotypes were largely unaltered between non-stimulated and anti-CD3/anti-CD28 stimulated cells (Fig 11C and 11D). However, in the PMA/Ionomycin stimulated cells, IFNy+ CD8+ T cells favoured the T_{EMRA} subset with a lower proportion of both naïve and T_{CM} populations (Fig 11E; right).

This assay also included an antibody specific for CX3CR1 (Fig 12).

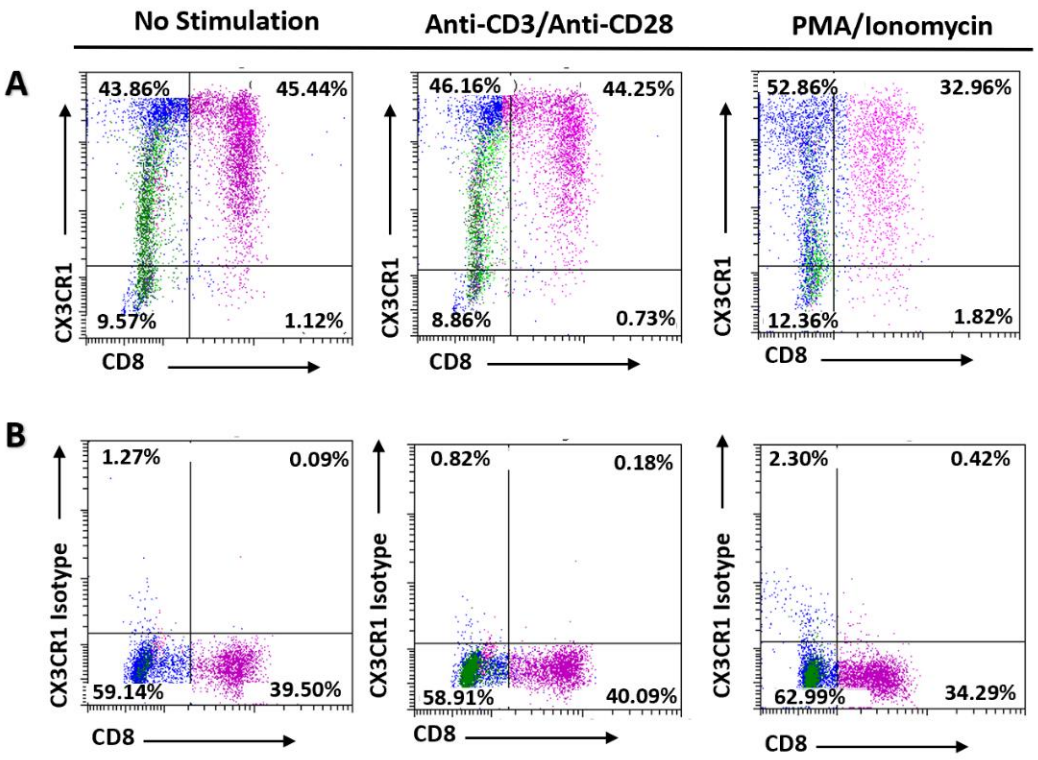


Figure 12. Overview of lymphocyte expression of CX3CR1 from one human donor across different stimulatory conditions. PBMCs were stimulated for 4 hours with the same stimulants indicated in the presence of an inhibitor of protein secretion. Cells were then stained as Fig 10. Flow cytometric data (A and B) show CD8+ cells gated on lymphocyte singlets as per Fig 10A. Numbers indicate the percent of CX3CR1+ cells in CD8+ T cell gates. (A) cells gated for CD8 and CX3CR1 in each stimulatory condition. (B) cells gated for CD8 and CX3CR1 using CX3CR1 isotype control tubes for each stimulatory condition.

Data show (Fig 12A) that the majority of CD8- and CD8+ T cells (gated on a lymphocyte gate) in each condition were CX3CR1+, expression of which has been associated with cytotoxic capacity in CD8+ T cells. There was little difference between the proportion of CD8+ CX3CR1+ T cells out of the CD8+ population in all three conditions (97.6%, 98.4% and 94.8% respectively for unstimulated, anti-CD3/anti-CD28 and PMA/Ionomycin stimulated cells respectively).

3.4 Flow cytometry gating strategies for analysing CMV responses in healthy volunteers

After confirming that the flow cytometry staining protocol can be used to phenotype primary human PBMCs and that primary CD8+ T cells produce IFN-y in response to cell activation, our aim was to evaluate T cell responses to CMV (detected by accumulation of IFN-y after CMV peptide stimulation by flow cytometry). For this, we made use of the tubes that come with the commercial QuantiFERON-CMV assay (see section 1.9.1.2.1; introduction) which stimulates whole blood with CMV peptides and then detects IFN-y secreted into the tube. For these assays, lymphocytes were isolated by density gradient centrifugation, counted, incubated in CMV tubes for 4 hours in the presence of an inhibitor of protein secretion and then surface stained with anti-CD4-FITC, anti-CD8-PerCPCy5.5, anti-CD45RA-APCCy7, anti-CCR7-PECy7, anti-CX3CR1-alexafluor647 and intracellularly stained with anti-IFN-y-PE. Single colour, isotype and fluorescence minus one controls were included. For each donor, information was therefore gained about frequencies of lymphocyte populations irrespective of specificity (e.g. CD4+, CD8+ T cells and different memory populations) and about the frequencies of CMV-specific subpopulations of cells (i.e those producing IFN-y after stimulation with CMV peptides).

In total, blood from 11 healthy donors was assessed by flow cytometry. A further two assays, each detecting an aspect of the immune response to CMV were also used from these same blood samples: conventional QuantiFERON-CMV assay (i.e. by the simple detection of soluble IFN-y secreted by CMV stimulated T cells) and the detection of CMV-specific IgG (see section 2.5; methods).

In order to analyse the flow cytometry data consistently for all 11 donors, a gating strategy was defined which could be used as a template to ensure analysis was as accurate as possible. The gates and quadrants for flow cytometry were set against single colour controls, isotype controls and fluorescence minus one controls. An example of the full gating strategy used throughout the analysis of the 11 healthy donors is shown in Figure 13 and is similar to that shown in Figure 10. An example of IFNy accumulation detected by flow cytometry for a CMV seronegative and a CMV seropositive individual (see section 3.10 for equivalent results from serology) is shown in Figure 14.

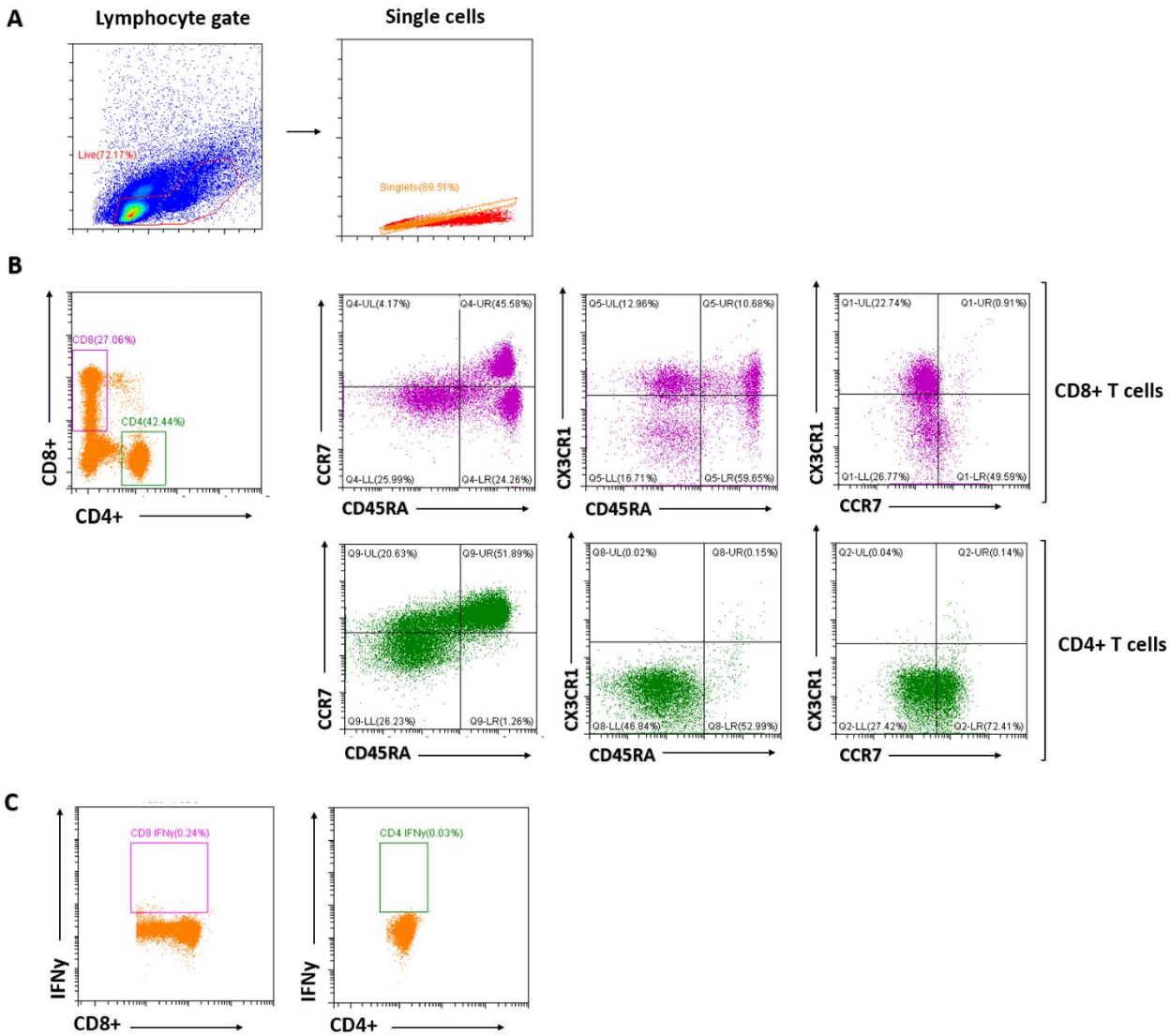


Figure 13. Flow cytometric gating strategy for evaluating CMV response in 11 healthy volunteers. This gating approach remained consistent for each sample analysis. 10mL of blood was collected from each volunteer. Lymphocytes were isolated by density gradient centrifugation and stimulated in QuantiFERON-CMV tubes for 4 hours in the presence of an inhibitor of protein secretion. Cells were then stained for surface CD4, CD8, CD45RA, CCR7 and CX3CR1 and intracellularly for IFN-y (or with appropriate controls) prior to analysis on the BU flow cytometer. Lymphocytes were gated on (A) forward scatter (FSC) and side scatter (SSC) to define lymphocytes, and then plotted on a FSC area and FSC height plot to distinguish single cell populations. From singlets, (B) CD8 and CD4 populations were identified and analysed for expression of CD45RA, CCR7 and CX3CR1. (C) CD4+ and CD8+ cells from (B) were additionally analysed for expression of IFNy. Cells within the gates in C are positive for IFNy. Gates were set according to isotype control tubes. Data are shown from a CMV seronegative volunteer and in which no CMV-reactive T cells were evident.

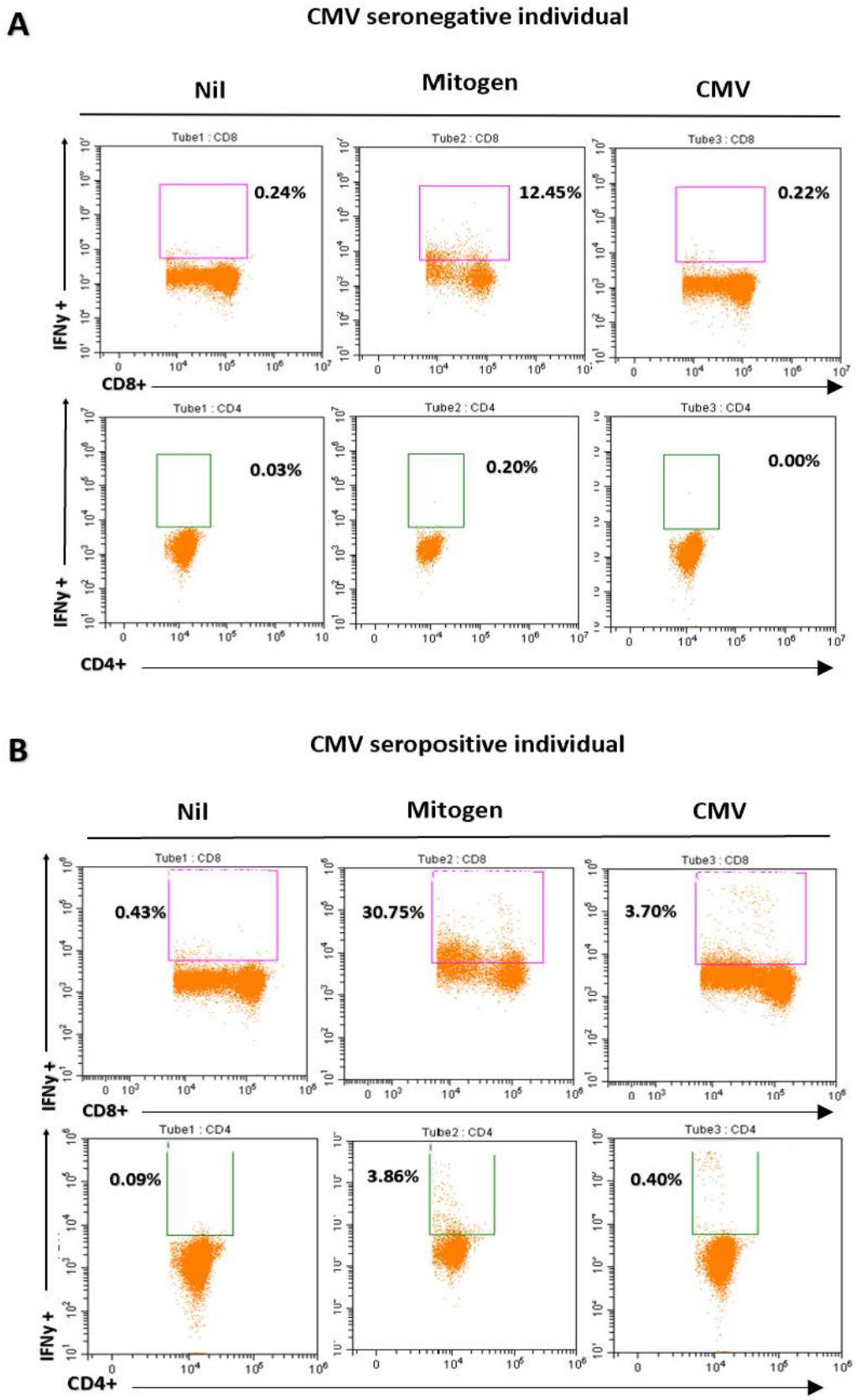


Figure 14. Gating strategy for IFNy detection in response to CMV stimulation. (A and B) cells were stimulated as Figure 13 and analysed by flow cytometry. Plots show data from each of the 3 tubes included in the QuantiFERON-CMV assay (Nil, mitogen and CMV). (A and B) Percentages (%) represent the proportion of IFNy+ cells out of the total CD8+ (top rows) or CD4+ (bottom rows) population. (A) represents data for a CMV seronegative individual and (B) represents data from a seronegative individual. The gating strategy is the same for both individuals.

3.5 The landscape of T cell populations in 11 healthy volunteers

To understand the breadth of T cell frequency among volunteers, initially the number of lymphocytes collected after the density gradient centrifugation were analysed. These data are shown in Figure 15A.

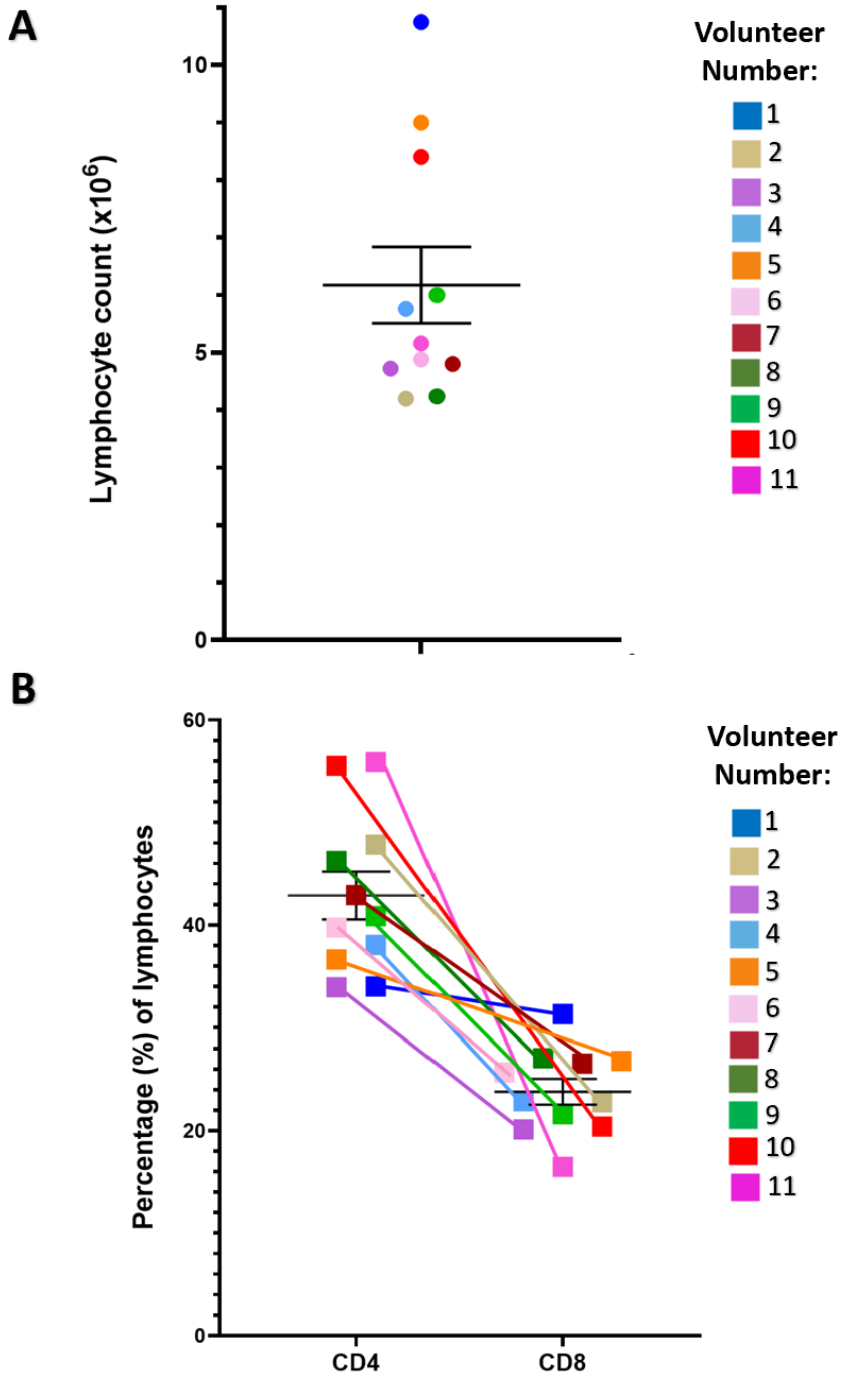


Figure 15. Overview of lymphocyte and T cell populations in 11 healthy volunteers. Lymphocytes were isolated from whole blood by density gradient centrifugation and counted using a haemocytometer. Data from each volunteer has been colour coded as shown. (A) The number of lymphocytes isolated from 7mL whole blood from each of 11 healthy volunteers is shown. Group mean +/- SEM is shown; each data point represents data from one individual. (B) After stimulation in QuantiFERON-CMV tubes and analysis by flow cytometry as Figure 13, the percent of CD4+ and CD8+ T cells within the singlet lymphocyte gate was calculated from the 'Nil' tubes. Paired data for each individual, and group means +/- SEM are shown.

The mean number of lymphocytes isolated from whole blood was 6.17 $x10^6$, although there was a considerable spread with a range of 4.2 - 10.74 $x10^6$; trypan blue exclusion showed that >90% of cells were live in every case (data not shown). After stimulation in QuantiFERON-CMV tubes, staining and appropriate gating (Figure 13) the percentage of singlet CD4+ and CD8+ lymphocytes was calculated from the Nil tubes. The average percentage of CD4+ T cells out of the lymphocyte singlet gate was 42% (+/- 1.25% SEM) and the average percentage of CD8+ T cells was 23% (+/- 2.3% SEM) (Fig 15B). As expected, the majority of cells in the lymphocyte gate were either CD4+ or CD8+ single expressors. Remaining cells were mainly negative for both CD4 and CD8 (likely predominantly B cells).

3.6 Overview of the CD8+ T cell landscape in healthy volunteers

Isolated lymphocytes from healthy donors represented in Figure 15, were either not stimulated (Nil tube), were stimulated by Mitogen (positive control) or by CMV peptides in the CMV tube prior to processing for flow cytometry. For each donor and stimulant the proportion of CD8+ T cells responding in each tube was detected by measuring the percentage (%) of CD8+ T cells with detectable accumulation of IFN-y (Fig 16).

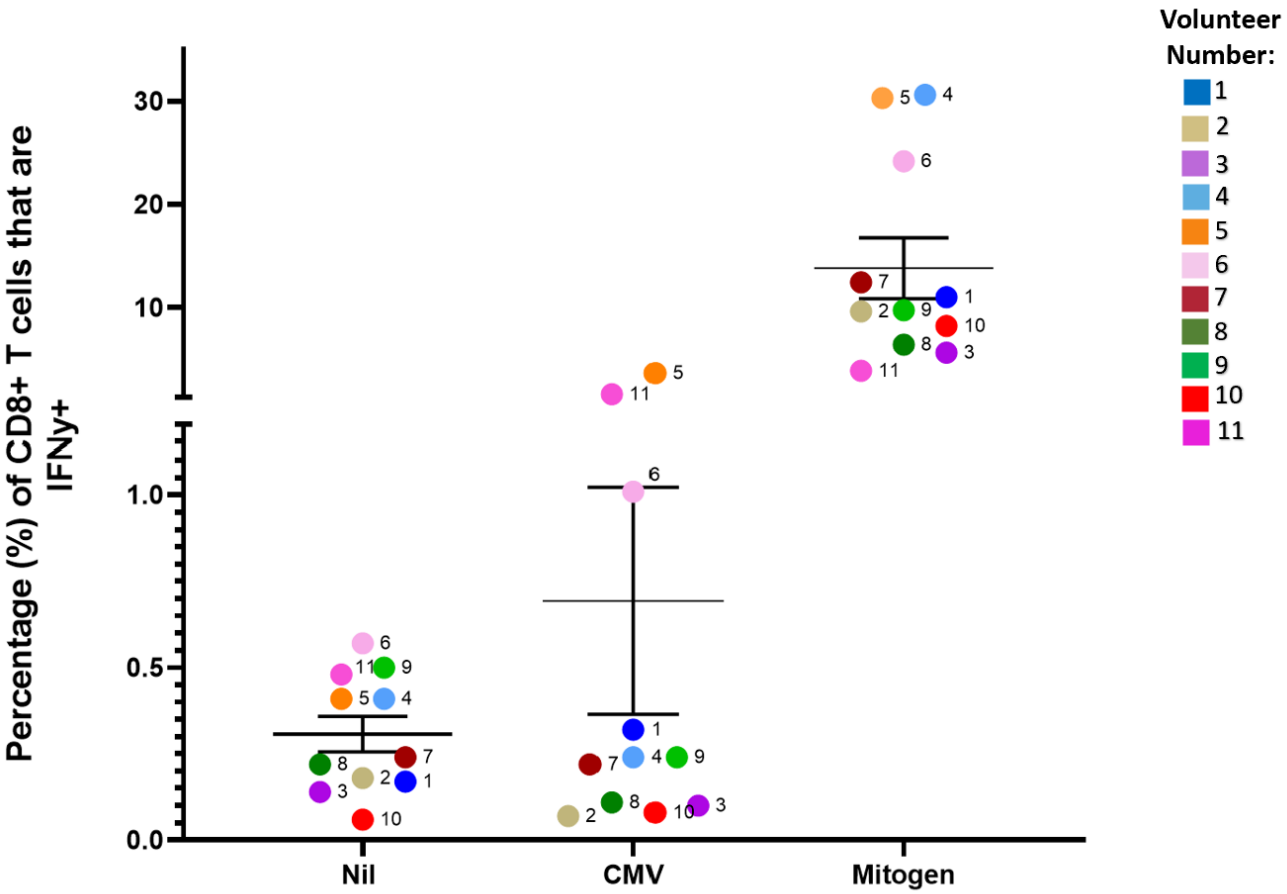


Figure 16. Overview of the CD8+ T cell response in 11 healthy volunteers following QuantiFERON-CMV stimulation. The x axis refers to the QuantiFERON-CMV tube cells were stimulated in. The y axis represents the percentage (%) of CD8+ T cells accumulating IFNy+ out of the total CD8+ T cell population per volunteer. Each volunteer was colour coded and identified as per the key. Mean +/- SEM is shown.

The mean frequency of IFN-y+ CD8+ cells out of total CD8+ T cells in the nil tube was 0.31% (+/- 0.0005% SEM) and didn't exceed 0.57% for any donor. The frequency of IFN-y+ CD8+ T cells out of the total CD8+ population in the mitogen positive control tube varied more widely (range 3.88 – 30.65%; Fig 16) and for every donor was the greatest stimulant of the three tubes. The mean frequency of CD8+ IFNy+ T cells out of the total CD8+ population in the CMV tube was 0.69% (+/- 0.003% SEM) of the total CD8+ population (range 0.11 – 3.65%).

Based on these data we assigned donors as either 'positive' or 'negative' for CMV reactivity by flow cytometry. Criteria for being assigned positive were: 1. %IFN-y (out of CD8+ T cells) in the Nil tube of <0.5% coupled with %IFN-y (out of CD8+ T cells in the CMV tube of >0.1% above the Nil value or 2. %IFN-y (out of CD8+ T cells) in the Nil tube of >0.5% and a %IFN-y (out of CD8+ T cells) in the CMV tube of >0.2% above the Nil value (also see Table 10; methods). On this basis, 4 of the donors (Fig 16: 1, 5, 6, 11) were positive for CMV reactivity as measured by flow cytometry.

3.7 Characteristics of the total CD8+ T cell population compared to CMV-specific CD8+ T cells

One of the aims of this MRes was to phenotype memory characteristics of CMV-specific CD8+ T cells. Therefore, in addition to surface staining with antibodies specific for CD4 and CD8, further antibodies were used to differentiate between 4 differentiation subsets (i.e. T_{EMRA}, T_{EM}, T_{CM} and Naïve). Figure 17A depicts the subpopulations found in the total non-stimulated (nil tube) CD8+ T cell population and in the IFNy+ CD8+ T cells stimulated by CMV peptides, for each CMV seropositive volunteer (see section 3.10 for details on seropositivity). Figure 17B depicts the average percentage of CD8+ T cells of each subtype across all 6 seropositive donors (which includes the 4 donors assigned as CMV reactive by flow cytometry).

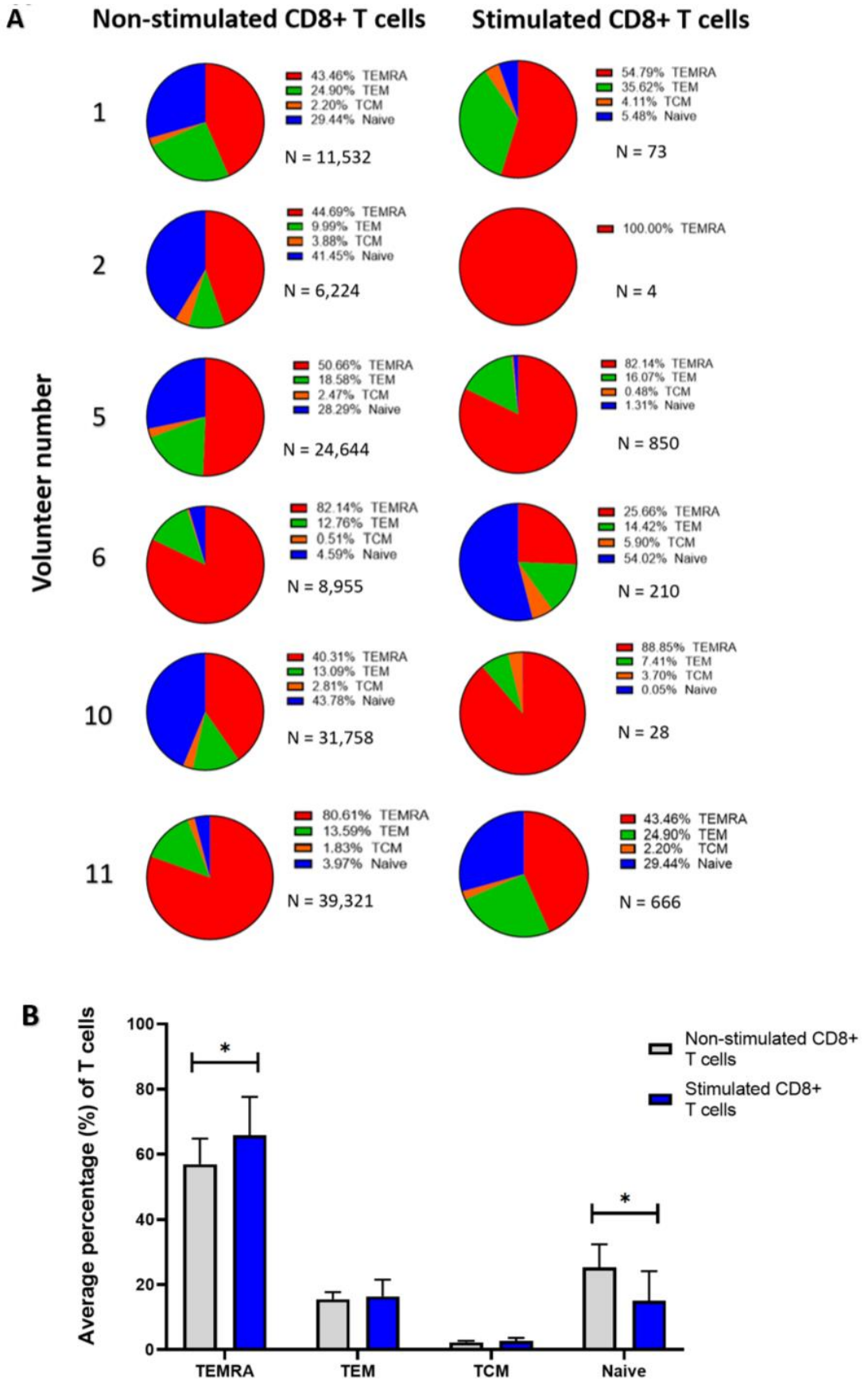


Figure 17. Phenotypes of non-stimulated vs stimulated CD8+ T cells in 6 CMV IgG+ volunteers (including 4 defined as positive to CMV by flow cytometry). (A) The phenotypes (T_{EMRA}, T_{EM}, T_{CM} or naïve as defined by Fig 11) of non-stimulated CD8+ T cells recovered from the 'Nil' QuantiFERON-CMV tubes, alongside matching and activated CMV-specific CD8+ T cells (IFNy+) from the 'CMV' QuantiFERON-CMV tubes. N values indicate the number of cells included in analysis in each case. (B) Represents the mean (+/-SEM) for each subpopulation in the non-stimulated CD8+ T cells and in the stimulated CMV-specific CD8+ T cells across all 6 volunteers. Means +/- SEM are shown. * $p < 0.05$.

Overall, (Fig 17) the most frequent phenotype among non-stimulated CD8+ T cells was T_{EMRA} (mean 44% +/- 7.80% SEM) and naïve (mean 35% +/- 7.09% SEM). While the trend was similar for the CMV-specific CD8+ T cells (i.e. dominance of the T_{EMRA} and naïve populations), there was a slight increase in the proportion of T_{EMRA} cells and a corresponding decrease in the naïve phenotype in the CMV-reactive cells. Significant differences were observed between the proportions of both T_{EMRA} and naïve populations when comparing non-stimulated and stimulated CD8+ T cells (Mann Whitney U test T_{EMRA}: $p < 0.043$; Mann Whitney U test Naive: $p < 0.017$) (Fig 17B).

In summary, this shows that the CD8+ T cell phenotype is different in activated CMV-specific cells compared with matched non-activated CD8+ T cells.

3.8 Analysis of CX3CR1 expression on CMV-specific CD8+ T cells

Some CMV-specific CD8+ T cells express surface marker CX3CR1, which is thought to identify cells with effective cytolytic protection against virally infected cells (Van de Burg et al. 2012). We hypothesised that CMV-responsive T cells (i.e. those accumulating IFN-y in CMV tubes) may therefore express CX3CR1. To address this, isotype control vs CX3CR1 staining was compared for the CMV-responding CD8+ T cells (i.e. IFNy+) and for CMV non-responding (IFNy-) CD8+ T cells from the same tube (Fig 18).

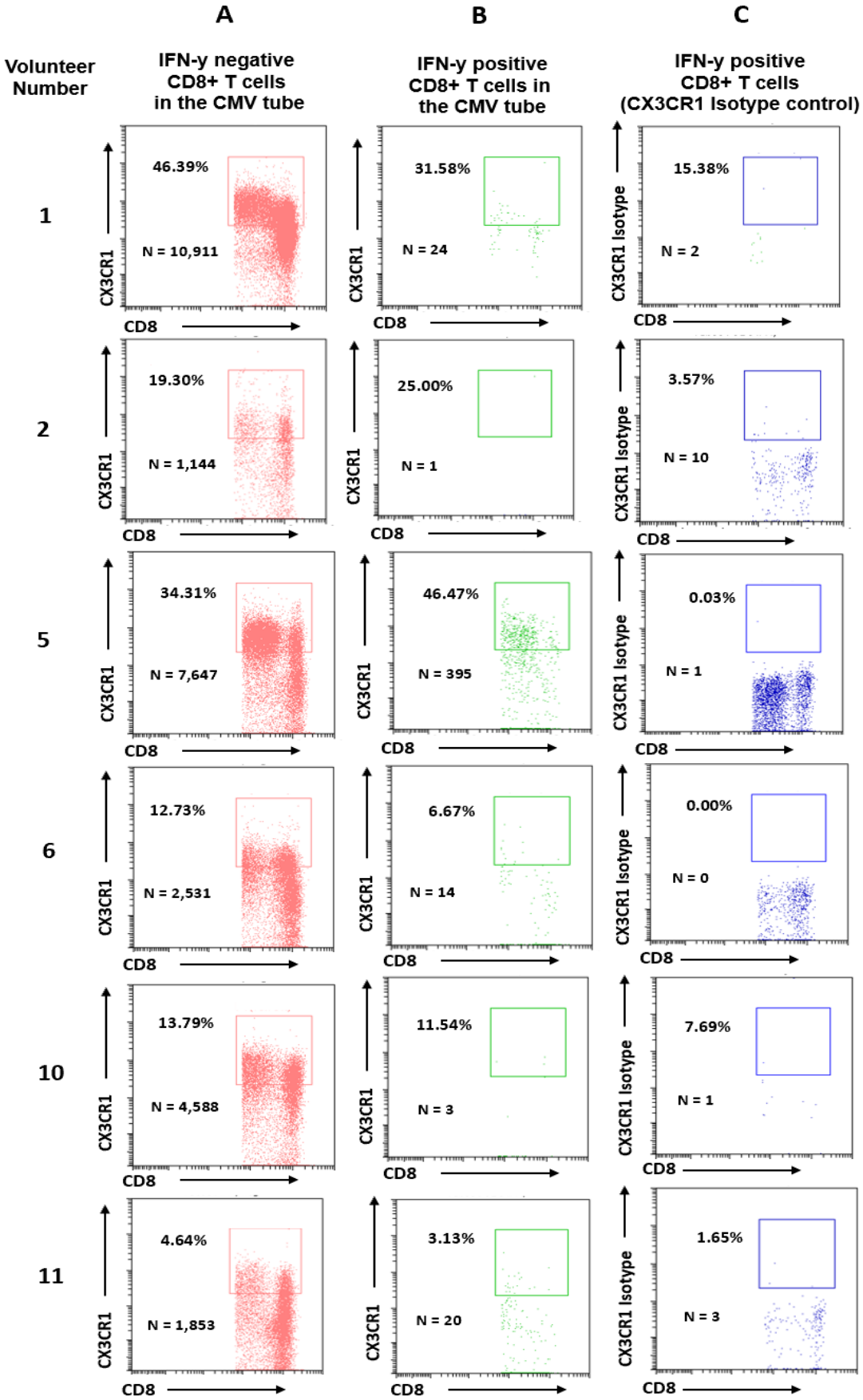


Figure 18. Overview of CD8+ T cell CX3CR1 expression in 6 CMV seropositive volunteers. Flow cytometry data is gated as per Figure 13. The CD8+ T cell populations are then split into two groups, those which are positive for IFNγ accumulation (green) and those which are negative for IFNγ accumulation (pink) within the CMV tube from the QuantiFERON-CMV assay. Each column beneath volunteer number represents that volunteer. (A) represents the CD8+ IFNγ- cells in the CMV QuantiFERON-CMV tube for each volunteer. (B) represents the CD8+ IFNγ+ cells in the CMV QuantiFERON-CMV tube for each volunteer. (C) represents the CD8+ IFNγ+ cells plotted against CX3CR1 but with CX3CR1 isotype control antibody. N = the number of cells within the CD8+ CX3CR1+ gate.

All volunteers showed some expression of CX3CR1 in CD8+ IFNy- T cells, although this varied considerably (Fig 18A) and, for those volunteers whom there were sufficient cells to comment, CX3CR1 cells were also detected within the IFNy+ CD8+ T cell populations (Fig 18B); when compared with isotype staining (Fig 18C).

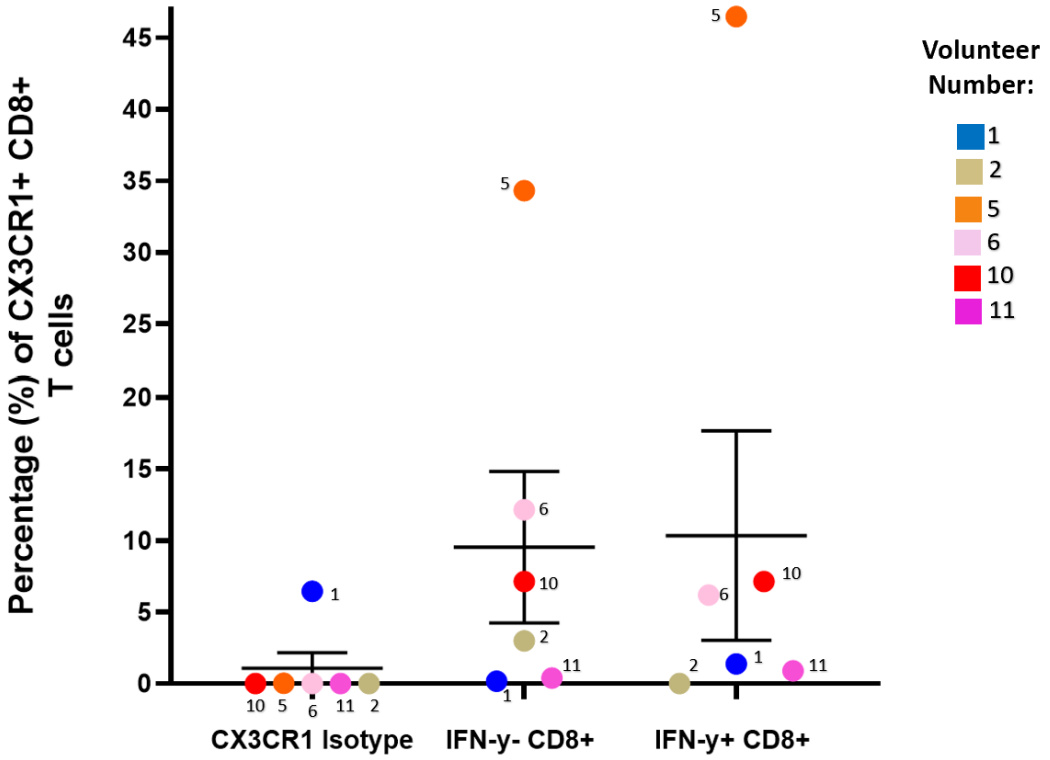


Figure 19. Average CD8+ T cell CX3CR1 expression in 6 CMV seropositive volunteers. The y axis depicts the percentage (%) of CD8+ CX3CR1+ T cells in the CMV QuantiFERON-CMV tube from the total CD8+ T cell population for each volunteer. Data for CX3CR1 isotype represents the % of CX3CR1+ CD8+ T cells incubated with the CX3CR1 isotype control, gated on the IFNy+ cells. CD8+ cells were further gated into IFNy- and IFNy+ populations and subsequently plotted against CX3CR1 for analysis. Mean +/- SEM is shown.

Analysis of the percentage of CX3CR1+ T cells (Figure 19) showed the proportion of CX3CR1+ T cells to be similar in CD8+ IFNy- and CD8+ IFNy+ populations from the CMV tube (means of 9.52% +/- 5.29% SEM and 10.34% +/- 7.34% SEM respectively). No significant difference was observed between the percentage of CX3CR1+ T cells in IFNy- and IFNy+ groups (Mann Whitney U test: $p > 0.9$).

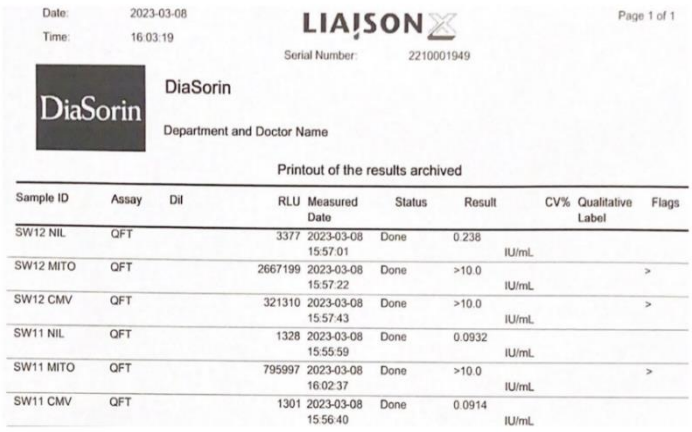
Part 2: QuantiFERON-CMV assay

3.9 Analysing the proportion of CMV-reactive CD8+ T cells in healthy volunteers

In addition to flow cytometry, T cell responses to CMV were also detected by QuantiFERON-CMV assay. The QuantiFERON-CMV assay has been commercially validated for detection of IFNy by ELISA and is used to identify *in vitro* responses by CD8+ T cells stimulated by CMV peptide antigens within the assay tubes. The University Hospitals Southampton (UHS) Virology laboratory is specialised in performing QuantiFERON-TB tests on the DiaSorin Liaison XL which uses the same sample processing and reagents as the QuantiFERON-CMV assay. Therefore, the QuantiFERON-CMV assay was performed on the same fully validated platform.

Representative data from 2 volunteers (4 and 5) are shown in Figure 20 A with results expressed in IU/mL for each of the 3 QuantiFERON-CMV tubes for each donor. Data were transferred to Excel for all 11 volunteers (Figure 20B). Three of the QuantiFERON-CMV results exceeded the upper limit of detection by Liaison testing and therefore required manual dilutions of 1:10 to obtain values (section 2.4; methods). A calculation enabled each donor to be defined as reactive or non-reactive by QuantiFERON-CMV assay (Table 10).

A



Date: 2023-03-08
Time: 16:03:19

LIAISON

Page 1 of 1

Serial Number: 2210001949

DiaSorin

DiaSorin

Department and Doctor Name

Printout of the results archived

Sample ID	Assay	Dil	RLU	Measured Date	Status	Result		CV%	Qualitative Label	Flags
SW12 NIL	QFT		3377	2023-03-08 15:57:01	Done	0.238	IU/mL			
SW12 MITO	QFT		2667199	2023-03-08 15:57:22	Done	>10.0	IU/mL			>
SW12 CMV	QFT		321310	2023-03-08 15:57:43	Done	>10.0	IU/mL			>
SW11 NIL	QFT		1328	2023-03-08 15:55:59	Done	0.0932	IU/mL			
SW11 MITO	QFT		795997	2023-03-08 16:02:37	Done	>10.0	IU/mL			>
SW11 CMV	QFT		1301	2023-03-08 15:56:40	Done	0.0914	IU/mL			

B

Volunteer Number	Liaison Result (IU/mL)			Interpreted result
	Nil	Mitogen	CMV	
1	0.17	>10.0	0.657	Reactive
2	0.0958	>10.0	0.464	Reactive
3	0.0542	>10.0	0.0556	Non-reactive
4	0.0932	>10.0	0.0914	Non-reactive
5	0.238	>10.0	>10.0	Reactive
6	0.0809	>10.0	>10.0	Reactive
7	0.0495	>10.0	0.0458	Non-reactive
8	0.0438	>10.0	0.0526	Non-reactive
9	0.354	>10.0	0.425	Non-reactive
10	0.119	>10.0	0.642	Reactive
11	0.0925	>10.0	>10.0	Reactive

Figure 20. Example of the output from the QuantiFERON-CMV assay. The QuantiFERON-CMV assay was performed as per manufacturer's instructions; see section 2.4. (A) represents an output from the Liaison XL of raw data for the QuantiFERON-CMV assay. (B) Table of summary QuantiFERON-CMV data for each volunteer. 'Interpreted result' is defined as reactive if the CMV value minus the Nil value is greater than 0.2 IU/mL (Table 10).

Of the 11 donors tested, 6 were reactive as defined by this assay. Within the non-reactive individuals, the IU/ml in response to CMV stimulation varied from 0.0458 - 0.425 compared to 0.3682 - >10.0 in reactive samples (Fig 20B). A sample with a IU/mL value of 0.425 in the

CMV tube was non-reactive despite a sample with a IU/mL value of 0.3682 in the CMV tube being reactive due to variance in values obtained in the Nil tube (Table 10).

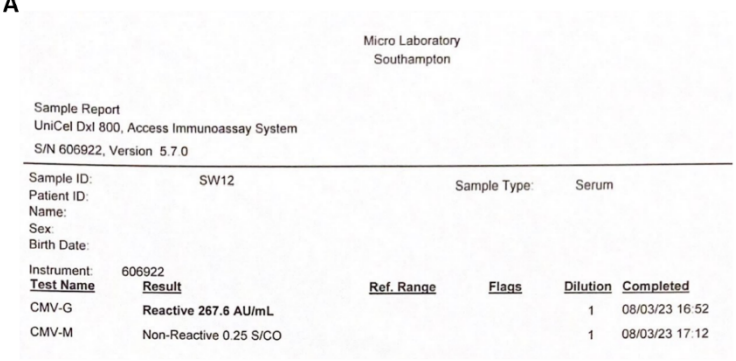
Part 3: CMV IgG antibodies

3.10 Assessing the breadth of CMV infection in healthy volunteers

Both flow cytometry and QuantiFERON-CMV detect T cell responses to CMV; neither can be used to determine whether an individual has an antibody response to CMV. Detection of CMV-specific IgG is validated and approved for determining an individual's previous viral infection. The UHS Virology department routinely uses an antibody assay performed on an automated platform (Beckmann's DXI; see section 2.5; methods), to determine prior infection to a range of pathogens, including CMV. Therefore, we made use of this platform to confirm whether healthy volunteers were CMV IgG positive or negative. Following QuantiFERON-CMV assay, remaining serum was immediately transferred to the DXI for CMV IgG and IgM antibody testing. An output of raw data from one volunteer (12) from the DXI is shown in Figure 21A.

Results are expressed in arbitrary units (AU/mL) and samples are automatically determined by the DXI as reactive or non-reactive for either CMV IgG or CMV IgM antibodies. For all 11 healthy volunteers, IgM results were negative (no serological evidence of active infection), and therefore only CMV IgG antibody data were used for analysis. Data were transferred to Excel for all 11 volunteers (Fig 21B).

A



Micro Laboratory
Southampton

Sample Report
UniCel DxI 800, Access Immunoassay System
S/N 606922, Version 5.7.0

Sample ID: SW12 Sample Type: Serum
Patient ID:
Name:
Sex:
Birth Date:

Instrument: 606922

Test Name	Result	Ref. Range	Flags	Dilution	Completed
CMV-G	Reactive 267.6 AU/mL			1	08/03/23 16:52
CMV-M	Non-Reactive 0.25 S/CO			1	08/03/23 17:12

B

Volunteer Number	CMV IgG Antibody (AU/mL)	CMV IgG -/+
1	151.1	Positive
2	143.8	Positive
3	1.4	Negative
4	0.0	Negative
5	267.6	Positive
6	206	Positive
7	0.2	Negative
8	0.3	Negative
9	0.1	Negative
10	56.4	Positive
11	186.4	Positive

Figure 21. Determining CMV IgG antibody titres in 11 healthy volunteers. (A) output from CMV IgG and IgM testing from one volunteer. (B) CMV IgG antibody results for all volunteers. Volunteers were considered CMV IgG positive if IgG >11.0 AU/mL (upper limit of detection of the assay 480 AU/mL); negative if IgG <7.0 AU/mL and indeterminate if IgG values lie between 7-11 AU/mL. No indeterminate results were observed in this study. See methods section 2.5 for more information on DXI result interpretation.

Of the 11 donors, 6 were positive for CMV IgG antibodies, meaning that those individuals had previous exposure to CMV infection. The 5 remaining donors were negative, indicating insignificant prior exposure to CMV infection.

Part 4: Comparison between assays

3.11 QuantiFERON-CMV assay and flow cytometry both detect CMV-reactive T cells

As the QuantiFERON-CMV and flow cytometry assays both detect IFNy produced by stimulated CD8+ T cells, results were further analysed to determine correlation between assays. For this, IU/mL values from the QuantiFERON-CMV assay CMV tubes (with background subtracted) were plotted against the % CD8+ IFNy+ T cells within CD8+ T cells from CMV tubes for the flow cytometry assay for each donor. Background IFNy values (nil tube) were not subtracted for the flow cytometry assay as this provided negative values in some cases. Thresholds for determining reactivity by QuantiFERON-CMV assay and positivity by flow cytometry are described in Tables 10 and 12 are indicated by the quadrant in Figure 22.

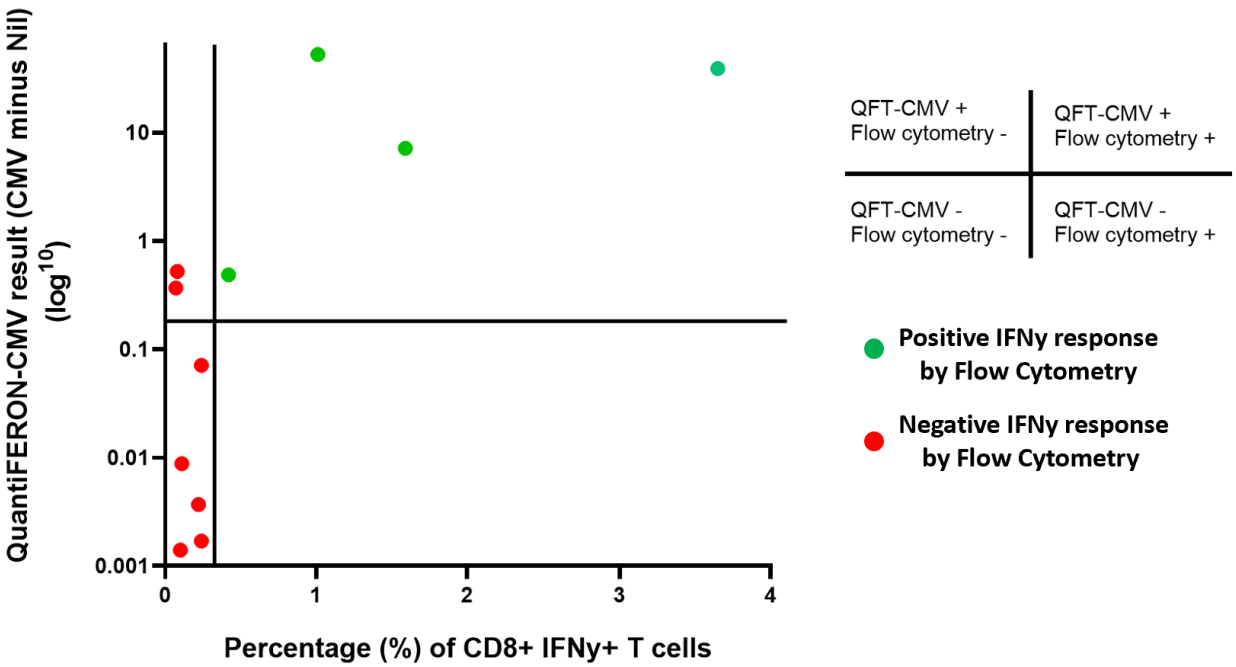


Figure 22. Correlation between QuantiFERON-CMV and flow cytometry results for detection of CMV-specific T cells. Data from the CMV tube from QuantiFERON-CMV assay (see Fig 20) and the % of IFNy+ cells out of the CD8+ T cell gate from the flow cytometry assay (also taken from the CMV tube; see Fig 16) were plotted for each volunteer. The QuantiFERON-CMV result represents the IFNy value in the CMV tube minus the IFNy value in the Nil tube. The quadrant represents the threshold for a positive/negative response by flow cytometry and for reactivity determined by QuantiFERON-CMV assay and can be interpreted by the key. Red plots represent a negative IFNy response by flow cytometry and the green plots represent a positive IFNy response by flow cytometry.

A significant positive correlation between the background adjusted QuantiFERON-CMV assay result and the percentage of CMV-reactive CD8+ IFNy+ T cells by flow cytometry was observed (Fig 22; Pearson correlation: $p < 0.0217$; $r^2 = 0.4603$; N = 11). Two volunteers with a negative IFNy response by flow cytometry had reactive QuantiFERON-CMV assay results. The discordance between these volunteers is likely due to higher sensitivity of the QuantiFERON-CMV assay compared to the flow cytometry assay.

The highest QuantiFERON-CMV results were observed in samples with over 0.32% of CD8+ IFNy+ T cells within the total CD8+ T cell population in response to CMV stimulation. As the %CD8+ IFNy+ T cells by flow cytometry increased above 0.3%, the corresponding QuantiFERON-CMV results required manual dilution of samples to obtain values for analysis.

3.12 Correlation between CMV IgG antibody titre and CMV T cell reactivity measured by QuantiFERON-CMV assay

To investigate whether there was a relationship between cellular immunity detected by QuantiFERON-CMV assay and humoral immunity (presence of CMV IgG), background adjusted IU/mL values from the QuantiFERON-CMV assay were plotted against CMV IgG antibody titre in AU/mL (Fig 23).

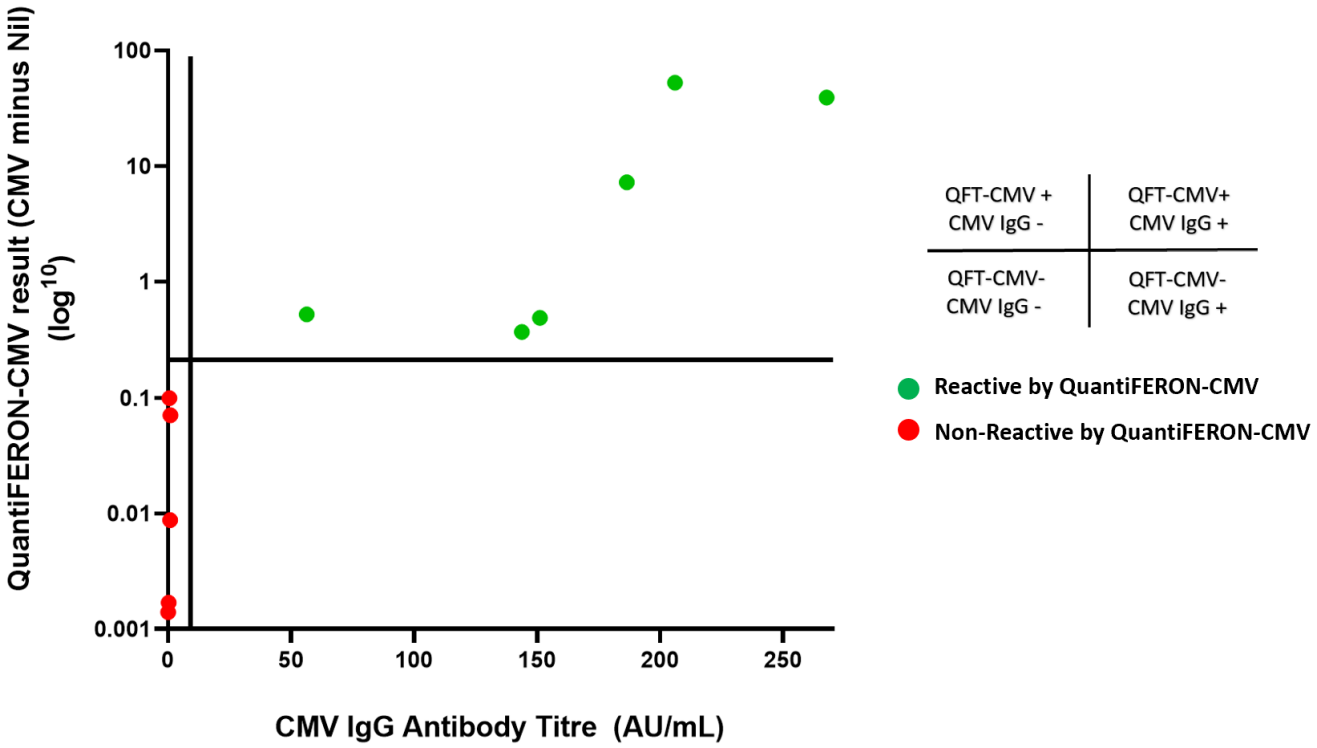


Figure 23. Correlation between CMV IgG antibody titre and CMV T cell immunity as detected by QuantiFERON-CMV assay. Data from the CMV tube from the QuantiFERON-CMV assay (see Fig 20) and CMV antibody titre (see Fig 21) were plotted for each volunteer. The QuantiFERON-CMV assay result represents the IFNy value in the CMV tube minus the IFNy value in the Nil tube. The quadrant represents the threshold for a positive CMV IgG titre and for reactivity determined by the QuantiFERON-CMV assay and can be interpreted by the key. Red plots represent a negative IFNy response by QuantiFERON-CMV and the green plots represent a positive IFNy response by QuantiFERON-CMV.

A positive correlation between antibody titre and QuantiFERON-CMV result was observed (Fig 23; Pearson correlation: $p < 0.012$; $r^2 = 0.5236$; $N = 11$). In all 5 non-reactive QuantiFERON-CMV individuals, the antibody titre was CMV IgG negative and all 6 individuals reactive by the QuantiFERON-CMV assay were positive for CMV IgG antibodies showing good concordance between humoral immunity and CMI detected by QuantiFERON-CMV.

3.13 Correlation between CMV IgG antibody titre and CMV T cell response detected by flow cytometry

To evaluate if CMI detected by flow cytometry similarly correlates with CMV IgG antibody titre, CMV IgG antibody titre was plotted against the percentage of CD8+ IFNy+ T cells within the CD8+ T cell gate after CMV peptide stimulation for each donor (Fig 24).

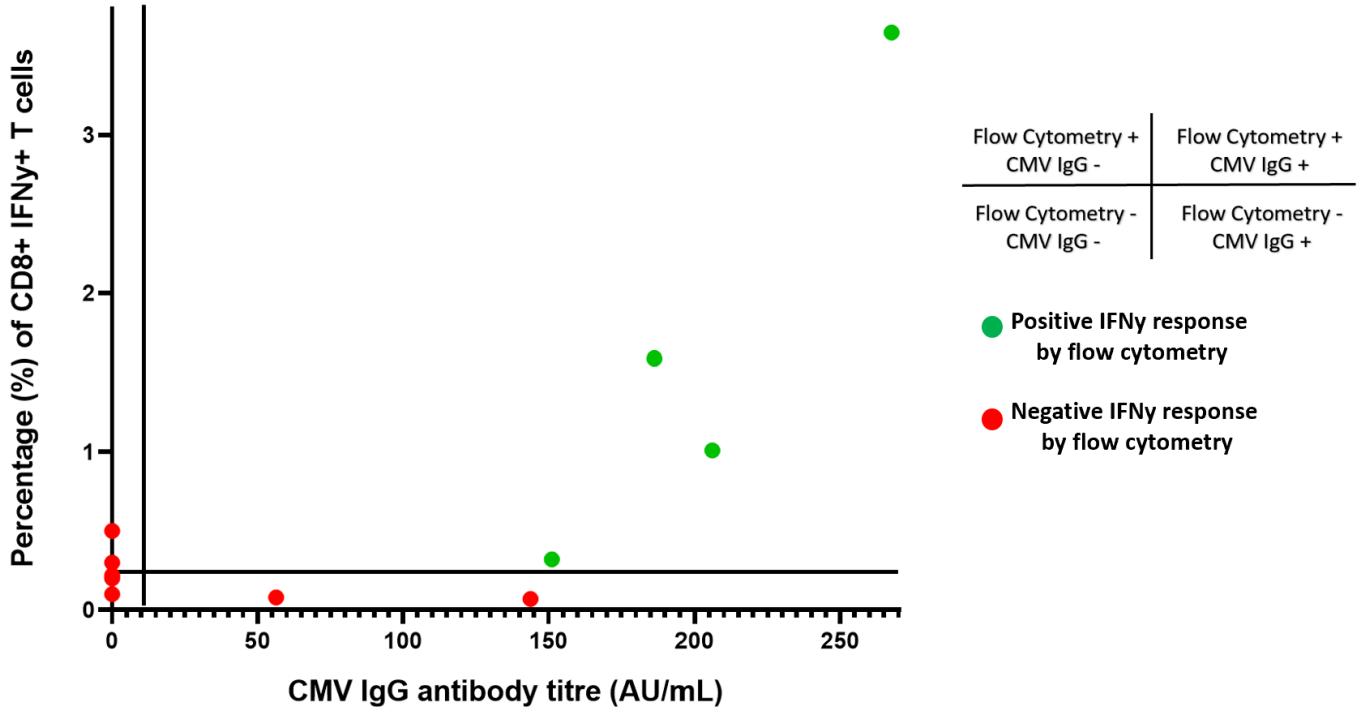


Figure 24. Correlation between CMV IgG antibody titre and flow cytometry response. The % of IFNy+ cells out of the CD8+ T cell gate from the flow cytometry assay (also taken from the CMV tube; see Fig 16) and CMV antibody titre (see Fig 21) were plotted for each volunteer. The quadrant represents the threshold for a positive/negative response by flow cytometry and positive/negative for CMV IgG antibodies and can be interpreted by the key. Red plots represent a negative IFNy response by flow cytometry and the green plots represent a positive IFNy response by flow cytometry.

Overall, there was a significant positive correlation between the %CD8+ IFNy+ T cells out of CD8+ T cells responding to CMV stimulation by flow cytometry and increasing antibody titre (Fig 24) (Pearson correlation: $p < 0.009$; $r^2 = 0.5474$; N = 11). Detectable CMV-reactive CD8+ IFNy+ T cells were only seen in samples from volunteers in which antibody titres were above 150 AU/mL. Of the 11 volunteers, 2 were CMV IgG positive but had a negative IFNy response by flow cytometry (Fig 24; red). As described in section 3.11 (Fig 22), this is likely due to the relatively low sensitivity of the flow cytometry assay.

3.14 CD4+ T cells respond to CMV peptides in only one donor

The QuantiFERON-CMV assay contains peptides designed to bind to MHC class-I and therefore to activate CD8+, but not CD4+ T cells. However, in 1 of the 11 samples (volunteer 5), a significant ('reactive') CD4+ T cell response was observed by flow cytometry after CMV peptide stimulation. This reactive response was defined in the same way as CD8+ T cell reactivity (see section 3.5 and Table 12 in methods). Representative data for the CD4+ T cell response in this volunteer are shown in Figure 25. The background adjusted %IFNy+ cells out of the CD4+ T cells in response to CMV stimulation for this sample was 0.31%, compared to a range of -0.6 to 0.02 in the other 10 samples (data not shown). CD4+ T cells from other volunteers mounted a similarly high response to the mitogen (data not shown). The phenotypes of CMV non-reactive (Fig 25B) and CMV reactive (Fig 25C) CD4+ T cells from this donor are also shown.

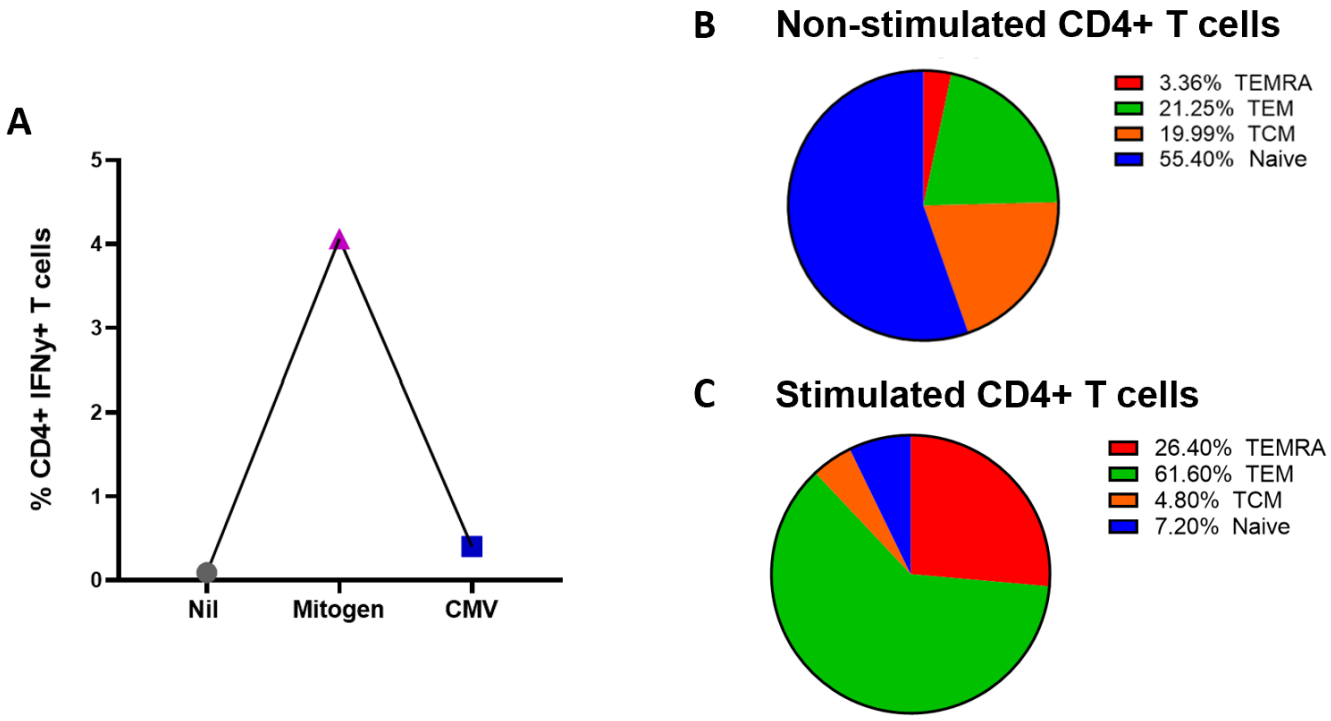


Figure 25. Overview of the CD4+ T cell response to CMV stimulation in volunteer 5. (A) The CD4+ T cell response to stimulation as detected by flow cytometry. The x axis represents the QuantiFERON-CMV tube used to stimulate cells prior to flow cytometric analysis. The y axis represents %CD4+ IFNy+ cells of the total CD4+ T cell population. (B) Phenotype of the total non-stimulated CD4+ T cell population in the 'nil' tube. (C) Phenotype of the IFNy+ CD4+ T cells in the CMV tube after stimulation.

Overall, (Fig 25) the most frequent phenotype among non-stimulated CD4+ T cells was Naive and T_{EM} with T_{EMRA} and T_{CM} populations accounting for the smallest proportion of phenotypes. In contrast, stimulated CD4+ T cells showed T_{EM} and T_{EMRA} dominance and a decrease in the naïve and T_{CM} population.

In summary, the one sample showing a CD4+ T cell response to CMV peptides in the QuantiFERON-CMV assay showed different phenotypes between CMV-reactive and non-reactive CD4+ T cell populations.

3.15 Results Summary

In summary, results from this MRes show that:

- Jurkat T cells are CD4+ and CD45RA+ but do not produce IFNy following stimulation with PMA/Ionomycin (Fig 7-9).
- Primary human PBMCs (both CD4+ and CD8+ T cells) produce IFNy following both PMA/Ionomycin and anti-CD3/anti-CD28 stimulation. CX3CR1 expression and cell phenotype (CD4+ and CD8+) of the cells show no significant change after 4 hour stimulation (Fig 10-12).
- The QuantiFERON-CMV assay tubes can be used to activate isolated lymphocytes and subsequent accumulated IFNy in CD4+ and CD8+ T cells can be detected by flow cytometry (Fig 13).
- CMV-specific CD8+ T cells stimulated by QuantiFERON-CMV trend towards T_{EMRA} enriched memory subset, and show a reduced naïve population in comparison to non-stimulated matched CD8+ T cells (Fig 17).
- There is no significant difference between CX3CR1 expression in CD8+ T cells which are CMV-specific and non-specific and CX3CR1 expression on CD8+ T cells varies considerably between individuals (Fig 18-19).
- The QuantiFERON-CMV assay is more sensitive than flow cytometry.
- CMI detected by both QuantiFERON-CMV and flow cytometry assays correlate with CMV IgG antibody titre (Fig 23 and Fig 24 respectively).
- CD4+ T cells rarely respond to CMV peptides in the QuantiFERON-CMV assay (Fig 25).

4. Discussion

This study demonstrates that QuantiFERON-CMV assay tubes can be utilised to stimulate IFNy accumulation in lymphocytes which can be detected by flow cytometry. Flow cytometry may also provide additional information regarding characteristics of CMV-specific CD8+ T cells activated by the QuantiFERON-CMV assay. Data show a significant correlation between IFNy levels measured by the QuantiFERON-CMV assay and %CMV-specific CD8+ T cells producing IFNy by flow cytometry (Fig 22). Results from both QuantiFERON-CMV and flow cytometry assays correlate with CMV IgG antibody titres (Fig 23 and Fig 24 respectively). The QuantiFERON-CMV assay had a rapid turnaround time and was simple to process in the laboratory.

This study provides 'proof of principle' data to support clinical evaluation of the QuantiFERON-CMV assay for allo-HSCT recipients at UHS. For this clinical study, prior to conditioning treatment, a blood sample will be collected from each patient and flow cytometry and QuantiFERON-CMV performed to establish the baseline CMV immune status. Samples from the donor will also be tested which will be relevant to assess CMV-specific T cell transfer from the donor to the recipient. Originally, we planned to take additional blood samples from patients at days 30, 60, 90, 120, 150 and 180 post-transplant for testing by QuantiFERON-CMV and flow cytometry to provide information on the rate and extent of CMV immune reconstitution. However, results from the current study provided support for changes to this schedule, as described below.

There are limited comparative data on CMV immune detection by QuantiFERON-CMV assay versus flow cytometry intracellular cell staining (ICS) (Clari et al. 2012). In many studies, flow cytometry assays use different stimulating antigens than the QuantiFERON-CMV assay (Clari et al. 2012). For this MRes, and unlike other similar studies, the QuantiFERON-CMV tubes were utilised as a source of antigen to stimulate isolated lymphocytes for the flow cytometry assay as well as the QuantiFERON-CMV assay. This enhances our assay by providing a parallel comparison of the same CMV-specific T cells the QuantiFERON-CMV assay is measuring. In the current study, six of the eleven volunteers were QuantiFERON-CMV reactive and four were non-reactive. These data are in line with epidemiological studies which demonstrate that more than 50% of the global adult population is infected with CMV (Fowler et al. 2022).

Although some studies report indeterminate QuantiFERON-CMV results in healthy CMV IgG positive individuals (Fleming et al. 2014; Clari et al. 2012; Valle-Arroyo et al. 2020), no volunteers were indeterminate in the current study, likely due to low sample number. Of the six QuantiFERON-CMV reactive volunteers, four showed a positive CD8+ IFNy response by

flow cytometry (see Table 12 for interpretation of flow positivity). Assays therefore yielded concordant results in 66.67% of samples, in line with similar studies (Clari et al. 2012; Valle-Arroyo et al. 2020), albeit these studies used different CMV peptides for stimulation. While studies state that ~10% of peripheral CD8+ T cells are CMV-specific (Sylwester et al. 2005; VanDen Burg et al. 2019) among CMV seropositive individuals, CMV-specific CD8+ T cells can range between barely detectable to over 40% in some individuals (Pardieck et al. 2018). We found no more than 3% of CD8+ T cells in peripheral blood were specific to CMV. Factors that affect the proportion of CMV-specific CD8+ T cells include the time of CMV infection, the dose of CMV upon primary infection, the number of re-infections and age of the individual (Smith et al. 2016; VanDen Burg et al. 2019), which weren't considered in these studies.

A reactive QuantiFERON-CMV result yet a negative IFNy response by flow cytometry in the same individual could be indicative of a false positive QuantiFERON-CMV result as suggested previously (Valle-Arroyo et al. 2020). After repeated QuantiFERON-CMV testing at later timepoints, positive samples have subsequently tested negative by QuantiFERON-CMV, indicating false positives are possible (Valle-Arroyo et al. 2020). As each sample in the current study was only tested once, we cannot exclude the possibility of false positive QuantiFERON-CMV results. However, the discrepancy of a reactive QuantiFERON-CMV result which is negative by flow cytometry was also in the context of the flow cytometry assay utilising different stimulating antigens to the QuantiFERON-CMV assay (Valle-Arroyo et al. 2020). Although two QuantiFERON-CMV reactive volunteers in the current study were negative for a CMV immune response when detected by flow cytometry, we suggest that the positive QuantiFERON-CMV assay results for these two individuals are unlikely to be false positivies, but rather that the QuantiFERON-CMV assay is more sensitive for IFNy detection than flow cytometry using our protocol. One rationale for this is that the QuantiFERON-CMV assay detects total IFNy accumulation over a 24 hour period whereas flow cytometry detects IFNy accumulation over a 4 hour timeframe. Secondly, each assay detects IFNy accumulation in different ways, i.e. the QuantiFERON-CMV assay is measuring total IFNy secreted by all CMV-specific CD8+ T cells into serum whereas flow cytometry detects single CMV-specific CD8+ T cells producing IFNy. Therefore, an individual could, in theory, have a low number of CMV-specific CD8+ T cells producing IFNy (i.e. below the threshold for positivity by flow cytometry) but those CD8+ T cells might produce enough IFNy to be detectable by the QuantiFERON-CMV assay.

Due to the postulated lower sensitivity of the flow cytometry assay in comparison to the QuantiFERON-CMV assay in the current study, performing flow cytometry may not be useful when there is a corresponding negative QuantiFERON-CMV result. Therefore, the plans for

the clinical study have been amended to delay testing by flow cytometry until day 90 or until a patient shows a positive QuantiFERON-CMV result. It may also be important to consider alternative ways to increase the sensitivity of flow cytometry for the detection of CMV immunity. We hypothesise that using more than 7ml blood would increase the number of cells for flow cytometric analysis and thereby increase the chance of detecting CMV-specific CD8+ T cells by flow cytometry. After searching the literature, 5 studies using flow cytometry for the detection of CMV-specific CD8+ T cells didn't disclose the volume of blood taken from volunteers for analysis (Clari et al. 2012; Valle-Arroyo et al. 2020; Rogers et al. 2020; Prakash et al. 2021; Yong et al. 2017).

A significant correlation between CMV IgG antibody titre (humoral immunity) and QuantiFERON-CMV assay result (cellular immunity) was observed in the current study. This confirms that CMV engages both branches of the immune system. Other studies with larger sample sizes have similarly seen an agreement between assays of 79-90% (Fleming et al. 2014; Valle-Arroyo et al. 2020). The QuantiFERON-CMV result varies considerably between individuals and is significantly higher in those with an antibody titre above 180 AU/mL in this study. In the clinical study, patients will be immunosuppressed with reduced humoral and cellular immunity, raising the question of whether assays will have sufficient sensitivity in this patient cohort. Nonetheless, studies utilising the QuantiFERON-CMV and flow cytometry assays in HSCT recipients have successfully detected CMV-specific CD8+ T cell responses in immunosuppressed patients from day 30 post-HSCT (Camargo et al. 2019; Clari et al. 2012). It is also important to question whether HSCT donors with low antibody titres may transfer relatively fewer CMV-specific CD8+ T cells to recipients, leading to negative QuantiFERON-CMV results in recipients.

CD8+ T cells stimulated by CMV peptides had a phenotype dominated by T_{EMRA} and T_{EM} but with only a small proportion of T_{CM} populations. Other studies have similarly observed that the major phenotype of peripheral CMV-specific CD8+ T cells is T_{EMRA}, closely followed by T_{EM} and only a minority of T_{CM} (Lilleri et al. 2008; VanDen Burg et al. 2019). These studies also used surface markers CCR7 and CD45RA to differentiate between memory phenotypes (Lilleri et al. 2008). The phenotype of CMV-specific T cells in the current study was not the same in all donors (although cell numbers were low); other factors that influence CD8+ T cell memory phenotype include the dose of primary infection and age of the individual (VanDen Burg et al. 2019).

The QuantiFERON-CMV assay cannot discriminate between functional subsets of CD8+ T cells and therefore we were interested to assess the levels of a marker, CX3CR1, which might predict the functional properties of CMV-specific CD8+ T cells (Gordon et al. 2018).

The fractalkine-receptor CX3CR1 identifies CD8+ T cells with cytotoxic effector function in both humans and mice and this CX3CR1+ CD8+ T cell population elicits direct effector function on virally infected cells by production of cytotoxic molecules granzyme B and perforin (Bottcher et al. 2015). CX3CR1+ CMV-specific CD8+ T cells are found in individuals with controlled CMV infection (Bottcher et al. 2015). CMV-specific CD8+ T cells can be separated into low (^{Lo}), intermediate (^{int}) and high (^{hi}) with respect to CX3CR1 expression; intermediate and high CD8+ T cells may correlate with memory inflated CMV-specific CD8+ T cells (Gordon et al. 2018). CX3CR1 expression patterns on CD8+ T cells from this MRes (Fig 18) are difficult to statistically analyse due to low cell numbers. However, a variable proportion of CMV-specific T cells (between 3-46% CD8+ T cells in the 'CMV' QuantiFERON-CMV tube) are CX3CR1+. After searching the literature, three human studies found that CX3CR1 was 'abundantly expressed' on effector cells, but lacked information on the proportion of these cells out of the total CD8+ T cell pool (Van De Burg et al. 2012; Hertoghs et al. 2010; Remmerswaal et al. 2012). However, conventional memory T cells in MCMV show that 40-51% are CX3CR1^{int}, compared to inflationary memory T cells, of which 50-90% are CX3CR1^{hi} (Gordon et al. 2018). Additionally, MCMV studies show that viral replication is correlated with CX3CR1 expression on CD8+ T cells, with high viral replication correlating with a high proportion of CX3CR1 expressing cells (Gordon et al. 2018).

Polyfunctional CMV-specific CD8+ T cells (those producing 2 or more cytokines) have more control over CMV infection in HSCT recipients than monofunctional CMV-specific CD8+ T cells (i.e. those producing IFNy only) (Gabanti et al. 2021). CMV-specific CD8+ T cells producing IFNy and IL-2 are present in the majority (>90%) of HSCT recipients with control of CMV infection, and are absent in those who do not have control of CMV infection (Krol et al. 2011; Lilleri et al. 2008). Similarly, CMV-specific CD8+ T cells producing combinations of IFNy/IL-2, IFNy/TNFa or IFNy/IL-2/TNFa are more prevalent in HSCT recipients with control of CMV infection and correlate with decreased duration of infection and a lower level of CMV DNA replication (Munoz-cobo et al 2011; Gimenez et al. 2015; Yong et al. 2017; Camargo et al 2019). Overall, results from these studies suggest that failure to control CMV reactivation in HSCT recipients is associated with loss in polyfunctionality within the CD8+ T cell population, despite the presence of monofunctional CMV-specific T cells (Camargo et al. 2019). The QuantiFERON-CMV assay only detects IFNy. As the flow cytometry assay in the current study was designed to corroborate data from QuantiFERON-CMV, this too also only detected IFNy. Assessing further cytokine profiles of CMV-specific CD8+ T cells in the clinical study may provide more accurate information of likely protection from CMV disease.

There are other cell types which are not detected by QuantiFERON-CMV. Of note, NK cells are also important in the control of CMV reactivation (Apiwattanakul et al. 2020). NK cell

reconstitution typically occurs prior to CD8+ T cell reconstitution in the post-HSCT setting, and their role in protection against infections in general during this period is well known (Buhlmann et al. 2011). Furthermore, although NK cells are classically considered innate-like, emerging evidence demonstrates that memory NK cells exist, indicative of an adaptive-like function (Min-Oo et al. 2014). In keeping with this adaptive-like function, CMV-specific NK cells recognise MHC class-I and may show a similar mechanism of cell activation to that of CD8+ T cells (Apiwattanakul et al. 2020). Individuals with prior CMV exposure have a higher number of NK cells expressing the NKG2C receptor and in HSCT patients, NKG2C+ NK cells are present in increased numbers during and after CMV infection (Lopez-Verges et al. 2011; Foley et al. 2012). NKG2C recognises HLA-E in combination with CMV-derived peptides (Sivori et al. 2019).

Interestingly, the proportion of CMV-specific NK cells (compared to the total NK cell population) exceeded that of the CMV-specific CD8+ T cells in patients post HSCT (Apiwattanakul et al. 2020). These CMV-specific NK cells also demonstrated excessive production of IFNy in response to IE1 and pp65 antigens which mimicked that of the CMV-specific CD8+ T cells (Apiwattanakul et al. 2020). It is not clear if these NK cells are activated by the QuantiFERON-CMV assay. NK cells, notably those expressing NKG2C, could potentially be a good additional surrogate marker of immune reconstitution against CMV (Parham et al.2013).

5. Limitations of the study

This MRes provides a platform for assessing the usefulness of QuantiFERON-CMV in the clinic. However, there are some limitations to the data as follows:

1. Some factors that determine outcome from CMV infection/reactivation and that shape the CMV-specific T cell response include age, gender and dose of CMV (VanDen Burg et al. 2019). Age and/or gender data could not be collected for this study due to ethical constraints. It is not possible to assess dose of prior CMV infection due to the asymptomatic nature of primary infection. In addition, a large proportion of CMV infections are acquired in childhood. Correlating age and gender with data obtained from the QuantiFERON-CMV assay may support improved risk-stratification.

2. Data may under represent the magnitude of the CMV-specific CD8+ T cell response as CMV-reactive cells were detected solely by production of IFNy in both the QuantiFERON-CMV and flow cytometry assays. It may be that cells not making IFNy are protective; it therefore may have been beneficial to look at polyfunctional CMV-specific CD8+ T cells.

3. Other cell types (e.g. NK cells) can produce IFNy in response to CMV peptide stimulation and these may have contributed to the QuantiFERON-CMV results. For flow cytometry, gating on CD8 alone in the absence of anti-CD3 also leaves the possibility of non-CD8+ T cells being included in the analysis. This could potentially include B cells, NK cells, monocytes and macrophages and of these, some subsets of NK cells might be expected to respond to CMV peptides.

4. Not all data could be statistically analysed due to low cell numbers.

5. Finally, as only one sample per volunteer was collected and each sample was only tested once, we cannot rule out the possibility of false positive QuantiFERON-CMV results. Indeed false positives/negatives cannot be ruled out for any of the assays because each assay has only been tested once per sample.

In the clinical study, we will be addressing the majority of the caveats highlighted above. For instance, we will have access to clinical data for every HSCT recipient including full transplant treatment plan, age, gender, HLA type, pre-treatment CMV IgG antibody levels, weekly CMV DNA PCR results and further test results should any CMV reactivation occur . We will also have access to limited information from the corresponding donors such as CMV IgG antibody status and potentially age and gender (point 1). This information will provide value to assess factors which may further influence CMV-specific immune reconstitution in HSCT recipients. Unfortunately, we cannot include detection of additional cytokines to look at polyfunctionality of CMV-reactive cells as we cannot obtain more blood from HSCT recipients (point 2). The flow cytometry protocol for the clinical study will include detection of the surface T cell marker CD3, which means we will only be identifying T cells in the analysis (point 3). This may enable identification of responding NK cells (IFNy+ but CD3- and CD8/CD4- in response to CMV stimulation). Finally, patient samples will be tested multiple times over several months. This longitudinal analysis is more likely to highlight false positive/negative assay results. It may also be possible to compile data from multiple time points to increase the number of reactive cells for meaningful analysis (points 4 and 5).

6. Conclusion

In conclusion, this MRes has confirmed that IFNy production measured by QuantiFERON-CMV can be used to quantify CMV-specific CD8+ T cells (aim 1) and that flow cytometry can additionally define the CD8+ lymphocyte phenotype (aim 2). Data have also shown a correlation between humoral and cellular immunity to CMV in healthy volunteers (aim 3). These data demonstrate the utility of both the QuantiFERON-CMV and flow cytometry assays for evaluation in the allo-HSCT setting. Overall, the current study has provided key

preliminary data resulting an the amended protocol for the planned clinical study. Ethical permissions and sponsorship by UHS has been granted for the clinical study and prospectively 14-20 HSCT patients will be recruited in Autumn 2023, including a follow up period of 6 months for each patient.

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Appendices

Appendix I: Ethical agreement

Bournemouth University

Research Ethics Checklist

About Your Checklist	
Ethics ID	45832
Date Created	04/10/2022 11:52:28
Status	Open
Risk	High

Researcher Details	
Name	Sarah Buchan
Faculty	Faculty of Science & Technology
Status	Staff
Course	Staff - FST
Have you received funding to support this research project?	Yes
Is this internal funding?	Yes
Please provide the Internal Funding Body	QR funding
Please list any persons or institutions that you will be conducting joint research with, both internal to BU as well as external collaborators.	Dr Emanuela Pelosi (Consultant Medical Virologist, Southampton NHS Trust), Sophie Willis (MRes student BU and Southampton NHS Trust staff,)

Project Details	
Title	Mapping the immune system in health
Start Date of Project	04/10/2022
End Date of Project	19/03/2024
Proposed Start Date of Data Collection	10/10/2022

Summary - no more than 600 words (including detail on background methodology, sample, outcomes, etc.)

This study aims to use a technique called *flow cytometry* and a *QuantiFERON®-CMV* test to characterize the immune system in healthy individuals. Immune cells (*white blood cells*) will be collected from the blood and the frequency of resting and activated cells quantified.

The immune system uses a variety of cells to protect the body from infections. For viruses, key cells conferring protection are a subset of white blood cells called "*T-cytotoxic cells*". These cells are heterogenous. Some have never been activated before while others have responded in the past to infection (called *memory cells*). There are many subtypes of memory cells, some of which may be better than others at killing infected cells and/or may be able to respond more quickly to infection. For T-cytotoxic cells to optimally combat infections they need support from other cell types in the blood including *CD4+ helper cells*. This study will tell us how many T cytotoxic cells, CD4+ T cells, and some other white blood cell types are present in health. Cytomegalovirus (CMV) is a common virus causing mild symptoms in most people. Most people have memory cells specific for CMV. Combining flow cytometry and the QuantiFERON®-CMV test will enable us to better define the memory cells present in healthy people and those that provide protection from CMV.

Filter Question: Does your study involve Human Participants?

Participants	
Describe the number of participants and specify any inclusion/exclusion criteria to be used	
It is anticipated that 10 participants will be initially included in this study. Inclusion criteria; volunteer has consented to be part of the study, is in good health and is aged over 18. Exclusion criteria; absence of consent, aged under 18.	
Do your participants include minors (under 16)?	No
Are your participants considered adults who are competent to give consent but considered vulnerable?	No
Is a Disclosure and Barring Service (DBS) check required for the research activity?	No

Recruitment	
Please provide details on intended recruitment methods, include copies of any advertisements.	
As the volume of blood required for this study, and the number of participants needed is low, participants will be recruited by word of mouth. It will be stressed to all participants that they are under no obligation to take part in the study.	
Do you need a Gatekeeper to access your participants?	No

Data Collection Activity	
Will the research involve questionnaire/online survey? If yes, don't forget to attach a copy of the questionnaire/survey or sample of questions.	No
Will the research involve interviews? If Yes, don't forget to attach a copy of the interview questions or sample of questions	No
Will the research involve a focus group? If yes, don't forget to attach a copy of the focus group questions or sample of questions.	No
Will the research involve the collection of audio materials?	No
Will your research involve the collection of photographic materials?	No
Will your research involve the collection of video materials/film?	No
Will the study involve discussions of sensitive topics (e.g. sexual activity, drug use, criminal activity)?	No
Will any drugs, placebos or other substances (e.g. food substances, vitamins) be administered to the participants?	No
Will the study involve invasive, intrusive or potential harmful procedures of any kind?	Yes
Please provide details and measures taken to minimise risks and explain why your research project does not require an ethical review by a NHS Research Ethics Committee	
Participants will be asked to donate a small blood sample (10 mls). To minimise risks, samples will be collected by a person trained and competent in the taking of blood. Blood samples will either be collected within the blood testing unit of an NHS hospital, or within a private and suitable space within private offices within Bournemouth University. Blood will be taken from participants while the participant is seated in a robust chair with an armrest and in a private and safe location (e.g. not in a laboratory or in an open area).	
Could your research induce psychological stress or anxiety, cause harm or have negative consequences for the participants or researchers (beyond the risks encountered in normal life)?	No

Will your research involve prolonged or repetitive testing?	No

Consent

Describe the process that you will be using to obtain valid consent for participation in the research activities. If consent is not to be obtained explain why.

Potential participants will be provided with Participant Information Sheet and a Participant Consent Form a minimum of 24 hours prior to blood collection. The Participant Information Sheet contains contact details should volunteers have any additional questions. Blood will not be collected from any volunteer without a signed Participant Consent Form. Participants will be under no pressure or obligation to join the study. Copies of the Participant Information Sheet and the Participant Consent Form are attached.

Do your participants include adults who lack/may lack capacity to give consent (at any point in the study)?	No
Will it be necessary for participants to take part in your study without their knowledge and consent?	No

Participant Withdrawal

At what point and how will it be possible for participants to exercise their rights to withdraw from the study?

Participants will be able to withdraw from the study up to the point that their blood begins to be processed. As no data about each participant is being collected and the samples are entirely anonymised, it will not be possible for participants sample to be identified and withdrawn after this point.

If a participant withdraws from the study, what will be done with their data?

As samples will be anonymised after collection it will not be possible for the data to be withdrawn from the study.

Participant Compensation

Will participants receive financial compensation (or course credits) for their participation?	No
Will financial or other inducements (other than reasonable expenses) be offered to participants?	No

Research Data

Will identifiable personal information be collected, i.e. at an individualised level in a form that identifies or could enable identification of the participant?	No
Will research outputs include any identifiable personal information i.e. data at an individualised level in a form which identifies or could enable identification of the individual?	No

Storage, Access and Disposal of Research Data

Where will your research data be stored and who will have access during and after the study has finished.

Research data will be fully anonymised. Samples will be identified by date of collection and an anonymous donor number only and data will be stored on the secure BU server. Fully anonymised flow cytometric data may be analysed using the web-based Cytobank platform but using 'private' rather than 'public' settings.

Once your project completes, will any anonymised research data be stored on BU's Online Research Data Repository "BORDaR"?	No

Please explain why you do not intend to deposit your research data on BORDaR? E.g. do you intend to deposit your research data in another data repository (discipline or funder specific)? If so, please provide details.

This is small scale dataset containing data in a format which is not accessable to those without a good grounding in flow cytometry and with access to the appropriate software tools to interpret the findings. It would not be appropriate for these data to be stored on BORDaR. In the longer term, should the data arising form this study contribute to a larger dataset then these data may be stored in BORDaR - this will be kept under review.

Dissemination Plans	
How do you intend to report and disseminate the results of the study?	
Peer reviewed journals,Conference presentation	
Will you inform participants of the results?	No
If Yes or No, please give details of how you will inform participants or justify if not doing so	
Data arising from this project will have no direct implications for the health/wellbeing of any participants.	

Final Review	
Are there any other ethical considerations relating to your project which have not been covered above?	No

Risk Assessment	
Have you undertaken an appropriate Risk Assessment?	Yes

Filter Question: Does your study involve the use of human tissue?

Additional Details	
What is the sample?	A small (10ml) volume of blood.
How will it be obtained?	Blood will be obtained by a trained phlebotomist. Blood will be collected directly into lithium/heparin vacutainers using sterile technique.
Where will the sample be stored and for how long?	Samples will not be stored. Samples will be held at room temperature for a maximum of 24 hours prior to processing and analysis. Processing and data collection will be completed within 5 days at which point all of the collected blood sample will be destroyed.
Does your research require NHS REC approval?	No
please explain why your research project does not require ethical review by a NHS REC	
The on line checklist tool has been completed which indicates that NHS REC is not required for this study. This document is attached. The samples are not being collected through the NHS, data will be anonymised, DNA is not being isolated and the sample is not being stored.	

Attached documents
Immunophenotyping of healthy human blood no IRAS needed 031022.pdf - attached on 04/10/2022 13:28:22
Participant Consent form - Mapping the immune system in health 001.docx - attached on 04/10/2022 13:28:29

Appendix II: Example of volunteer consent form

Ref & Version: Mapping the immune system in health 002
Ethics ID number: 45832
Date: 06/10/22

Participant Agreement Form

Full title of project: ("the Project") Mapping the immune system in health

Name, position and contact details of researchers:

Dr Sarah Buchan (Principal Academic in Immunology, Bournemouth University) sbuchan@bournemouth.ac.uk

Dr Emanuela Pelosi (Consultant Medical Virologist, University Hospital Southampton, NHS Foundation Trusts), Emanuela.Pelosi@uhs.nhs.uk

Sophie Willis (MRes student, Bournemouth University)

To be completed prior to data collection activity

Section A: Agreement to participate in the study

You should only agree to participate in the study if you agree with all of the statements in this table and accept that participating will involve the listed activities.

I have read and understood the Participant Information Sheet (Mapping the immune system in health version 002) and have been given access to the BU Research Participant Privacy Notice which sets out how we collect and use personal information (https://www1.bournemouth.ac.uk/about/governance/access-information/data-protection-privacy).
I have had an opportunity to ask questions.
I understand that my participation is voluntary. I can stop participating in research activities at any time without giving a reason and I am free to decline to answer any particular question(s).
I understand that taking part in the research will include the following activity as part of the research:
• Donation of a 10ml blood sample
I understand that, if I withdraw from the study, I will also be able to withdraw my data from further use in the study **except** where my data has been anonymised (as I cannot be identified) or it will be harmful to the project to have my data removed.
I understand that my data may be included in an anonymised form within a dataset to be archived at BU's Online Research Data Repository.
I understand that my data may be used in an anonymised form by the research team to support other research projects in the future, including future publications, reports or presentations.

	Initial box to agree
I consent to take part in the project on the basis set out above (Section A)	

Name of participant (BLOCK CAPITALS)	Date (dd/mm/yyyy)	Signature
Name of researcher (BLOCK CAPITALS)	Date (dd/mm/yyyy)	Signature

Once a Participant has signed, **please sign 1 copy** and take 2 photocopies:

- Original kept in the local investigator's file
- 1 copy to be kept by the participant (including a copy of PI Sheet)

Appendix III: Example of volunteer information sheet

Bournemouth University

Participant Information Sheet

The title of the research project

Mapping the immune system in health

Invitation to take part

You are being invited to take part in a research project. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether you wish to take part.

Who is organising/funding the research?

The study is being organized by doctors and scientists from Bournemouth University and the Southampton Specialist Virology Centre (University Hospital Southampton). Funding is through Bournemouth University and the Southampton Specialist Virology Centre (SSVC) fund aimed to support research and education for internal members of staff. This study is the Master of Research Project of a student who is also a member of the laboratory staff at the SSVC. The doctors conducting the study and the Master student performing the investigations are not being paid to perform the study.

What is the purpose of the project?

This study aims to use a technique called *flow cytometry* and a *QuantiFERON®-CMV* test to characterize the immune system in healthy individuals. Immune cells (*white blood cells*) will be collected from the blood and the frequency of resting and activated cells quantified.

What will we learn from this study?

The immune system uses a variety of cells to protect the body from infections. For viruses, key cells conferring protection are a subset of white blood cells called "*T-cytotoxic cells*". Some have never been activated before while others have responded in the past to infection (called *memory cells*). There are many subtypes of memory cells, some of which may be better than others at killing infected cells. For T-cytotoxic cells to optimally combat infections they need support from other cell types in the blood including *CD4+ helper cells*. This study will tell us how many T cytotoxic cells, CD4+ T cells, and some other white blood cell types are present in health. Cytomegalovirus (CMV) is a common virus causing mild symptoms in most people. Most people

have memory cells specific for CMV. This study will define the memory cells present in healthy people and those that provide protection from CMV.

What will happen to my blood sample?
We will collect approximately 10mls of blood. The red blood cells will then be removed from the sample either by adding chemicals which destroy only the red blood cells or by a process called density gradient centrifugation which removes the red blood cells due to their small size. This leaves only the white blood cells for further study. Your samples will not be stored.

The aims of this project are to:

- Quantify white blood cell subsets in health
- Define the frequency of CMV-specific memory cell subsets in healthy people

Why have I been chosen?

You have been chosen because you are:

- In good health
- You are above 18 years old.

We are looking to recruit 10 participants for this study.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part, you will be given this information sheet to keep and be asked to sign a participant agreement form. We want you to understand what participation involves, before you make a decision on whether to participate.

If you or any family member have an on-going relationship with BU or the research team, e.g. as a member of staff, as student or other service user, your decision on whether to take part (or continue to take part) will not affect this relationship in any way.

Can I change my mind about taking part?

Yes, you can stop participating in study activities at any time and without giving a reason.

If I change my mind, what happens to my information?

After you decide to withdraw from the study, we will not collect any further information from or about you.

As regards to the information we have already collected before this point, your rights to access, change or move that information are limited. This is because we need to manage your

information in specific ways in order for the research to be reliable and accurate. Further explanation about this is in the Personal Information section below.

It will not be possible to withdraw from this study once your blood sample has begun to be processed. This is because your blood will be processed anonymously and it will not be possible to trace the blood sample back to you.

What would taking part involve?

Taking part in the study involves donating 10 ml of blood. This will be collected by a person competent and trained to collect blood samples.

How will your samples be used?
Your blood samples will be used to perform flow cytometry and the QuantiFERON-CMV test in Bournemouth University laboratories. Some of your cells may be analysed in the Southampton Specialist Virology Centre, University Hospital Southampton, and using a flow cytometer at the University of Southampton (laboratory located within University Hospital Southampton). These studies will support a wider study to evaluate the clinical usefulness of the QuantiFERON®-CMV assay in patients undergoing transplant.

Will I be reimbursed for taking part?

No payment or any form of reimbursement will be available for participants in this study.

What are the advantages and possible disadvantages or risks of taking part?

Whilst there are no immediate benefits to you participating in the project, it is hoped that this work will provide important information about CMV-specific immunity in healthy individuals. The data gained from your blood sample will help us determine how to assess the risk of CMV disease in leukaemia patients after bone marrow transplantation. CMV reactivation in leukaemia patients can be severe and life threatening.

Whilst we do not anticipate any risks to you in taking part in this study, this study involves collection of blood which is an invasive procedure. To minimise these risks, blood will only be collected by a person competent and trained to collect blood.

What type of information will be sought from me and why is the collection of this information relevant for achieving the research project's objectives?

This study will collect information about how many white blood cells and which cell subsets are present in your body. This information will help us to characterise the healthy immune system and to determine which markers are expressed by memory cells specific for CMV. The data arising from this small study will inform a larger planned study in patients being treated for

leukaemia and other blood disorders, who are at serious risk from CMV disease. Study of your blood sample will help us define protocols to identify leukaemia patients at greater risk of CMV disease.

Will I be recorded, and how will the recorded media be used?

There will be no recordings of recorded media used as part of this study.

How will my information be managed?

Bournemouth University (BU) is the organisation with overall responsibility for this study and the Data Controller of your personal information, which means that we are responsible for looking after your information and using it appropriately. Research is a task that we perform in the public interest, as part of our core function as a university.

Undertaking this research study involves collecting and/or generating information about you. We manage research data strictly in accordance with:

- Ethical requirements; and
- Current data protection laws. These control use of information about identifiable individuals, but do not apply to anonymous research data: "anonymous" means that we have either removed or not collected any pieces of data or links to other data which identify a specific person as the subject or source of a research result.

BU's Research Participant Privacy Notice sets out more information about how we fulfil our responsibilities as a data controller and about your rights as an individual under the data protection legislation. We ask you to read this Notice so that you can fully understand the basis on which we will process your personal information.

Research data will be used only for the purposes of the study or related uses identified in the Privacy Notice or this Information Sheet. To safeguard your rights in relation to your personal information, we will use the minimum personally-identifiable information possible and control access to that data as described below.

Publication

You will not be able to be identified in any external reports or publications about the research; your information will only be included in these materials in an anonymous form, i.e. you will not be identifiable.

Research results will be published in peer-reviewed journals; published data will not identify you.

Security and access controls

BU will hold the information we collect about you in hard copy in a secure location and on a BU password protected secure network where held electronically.

Personal information which has not been anonymised will be accessed and used only by appropriate, authorised individuals and when this is necessary for the purposes of the research or another purpose identified in the Privacy Notice. This may include giving access to BU staff or others responsible for monitoring and/or audit of the study, who need to ensure that the research is complying with applicable regulations.

Your blood sample will be identified only by an anonymous number which will not be traced back to you after blood collection.

Sharing your personal information with third parties
No personal information will be collected in this study.

Further use of your information
The information collected about you may be used in an anonymous form to support other research projects in the future and access to it in this form will not be restricted. It will not be possible for you to be identified from this data. To enable this use, anonymised data may be added to BU's online Research Data Repository: this is a central location where data is stored, which is accessible to the public.

Keeping your information if you withdraw from the study
If you withdraw from active participation in the study we will keep information which we have already collected from or about you, if this has on-going relevance or value to the study. This will not include your personal identifiable information. As explained above, your legal rights to access, change, delete or move this information are limited as we need to manage your information in specific ways in order for the research to be reliable and accurate. However if you have concerns about how this will affect you personally, you can raise these with the research team when you withdraw from the study.

You can find out more about your rights in relation to your data and how to raise queries or complaints in our Privacy Notice.

Retention of research data

Project governance documentation, including copies of signed **participant agreements**: we keep this documentation for a long period after completion of the research, so that we have records of how we conducted the research and who took part. The only personal information in this documentation will be your name and signature, and we will not be able to link this to any anonymised research results.

Research results:

As described above, during the course of the study we will anonymise the information we have collected about you as an individual. This means that we will not hold your personal information in identifiable form after we have completed the research activities.

You can find more specific information about retention periods for personal information in our Privacy Notice.

We keep anonymised research data indefinitely, so that it can be used for other research as described above.

Contact for further information

If you have any questions or would like further information, please contact:

Dr Sarah Buchan (Principal Academic in Immunology, Bournemouth University)
sbuchan@bournemouth.ac.uk
Dr Emanuela Pelosi (Consultant Medical Virologist, University Hospital Southampton)

In case of complaints
Any concerns about the study should be directed to Dr Tiantian Zhang, Deputy Dean for Research & Professional Practice, Scitech, Bournemouth University by email to researchgovernance@bournemouth.ac.uk.

Finally

If you decide to take part, you will be given a copy of the information sheet and a signed participant agreement form to keep.

Thank you for considering taking part in this research project.

Appendix IV: Risk assessment: Handling of human blood

Risk Assessment Form

About You & Your Assessment	
Name	Sarah Buchan
Email	sbuchan@bournemouth.ac.uk
Your Faculty/Professional Service	Faculty of Science and Technology
Is Your Risk Assessment in relation to Travel or Fieldwork?	No
Status	Approved
Date of Assessment	27/01/2022
Date of the Activity/Event/Travel that you are Assessing	

What, Who & Where	
Describe the activity/area/process to be assessed	Handling of human blood and blood products
Locations for which the assessment is applicable	Christchurch House
Persons who may be harmed	Staff, Student, Contractors

Hazard & Risk	
Hazard	Breakage in centrifuge leading to aerosols
Severity of the hazard	Medium
How Likely the hazard could cause harm	Low
Risk Rating	Low
Control Measure(s) for Breakage in centrifuge leading to aerosols: Do not use glass tubes. If a tube containing blood-derived products breaks in the centrifuge, the centrifuge bucket lid should be kept closed, or if not detected prior to opening the lid should be closed gently to minimise aerosol dispersal. 30 mins should be allowed to elapse before opening the centrifuge bucket to allow aerosols to settle. The area should then be cleaned as above.	
With your control measure(s) in place - if the hazard were to cause harm, how severe would it be? Medium	
With your control measure(s) in place - how likely is it that the hazard could cause harm? Low	
The residual risk rating is calculated as: Low	
Hazard	COVID-19
Severity of the hazard	High
How Likely the hazard could cause harm	Medium
Risk Rating	High
Control Measure(s) for COVID-19: Lab coat and gloves should be worn throughout. No eating and drinking in the lab environment. Common areas should be cleaned thoroughly before and after use. Wash hands regularly. Wear masks when possible in line with current university and government guidance. Maintain distance with others in line with current university and government guidance.	

With your control measure(s) in place - if the hazard were to cause harm, how severe would it be? High	
With your control measure(s) in place - how likely is it that the hazard could cause harm? Low	
The residual risk rating is calculated as: Medium	
Hazard	Exposure to viral/bacterial pathogen
Severity of the hazard	Medium
How Likely the hazard could cause harm	Medium
Risk Rating	Medium
Control Measure(s) for Exposure to viral/bacterial pathogen: Wear gloves and lab coat. Cover all cuts and abrasions. Vaccination against hepatitis B should be considered by operators. Disinfectant should be kept nearby. Do not handle sharps or glassware while handling human blood. When preparing cell components from human blood work in a class 2 microbiological safety cabinet in a containment level 2 tissue culture room (C225). Keep tubes in trays/racks to minimise spillage. Keep samples capped whenever possible to minimise the risk of spillage. Use Virkon or equivalent at recommended concentration to clean up any spillages using paper towels, then clean area again with 70% ethanol. At completion of any experiment, wipe area with Virkon or equivalent and then 70% ethanol.	
With your control measure(s) in place - if the hazard were to cause harm, how severe would it be? Medium	
With your control measure(s) in place - how likely is it that the hazard could cause harm? Low	
The residual risk rating is calculated as: Low	
Hazard	Eye/skin irritation by disinfectant
Severity of the hazard	Low
How Likely the hazard could cause harm	Low

Risk Rating	Low
Control Measure(s) for Eye/skin irritation by disinfectant: Wear gloves and lab coat and dispense with care. Wash any exposed areas of skin with water.	
With your control measure(s) in place - if the hazard were to cause harm, how severe would it be? Low	
With your control measure(s) in place - how likely is it that the hazard could cause harm? Low	
The residual risk rating is calculated as: Low	
Hazard	Exposure to pathogens from waste disposal
Severity of the hazard	Medium
How Likely the hazard could cause harm	Medium
Risk Rating	Medium
Control Measure(s) for Exposure to pathogens from waste disposal: Residual waste from blood preparations should be treated with disinfectant prior to disposal down the sink for liquid waste. For solid waste (e.g. pipettes, paper, tubes) items should be double-bagged and disposed of in the lab bins. When using unfixed samples for flow cytometry, hypochlorite should be added to the waste bottle prior to disposal.	
With your control measure(s) in place - if the hazard were to cause harm, how severe would it be? Medium	
With your control measure(s) in place - how likely is it that the hazard could cause harm? Low	
The residual risk rating is calculated as: Low	

Review & Approval

Any notes or further information you wish to add about the assessment	This RA should be read in conjunction with all relevant SOPs as well as Risk Assessments that relate to any specific experiments to be conducted with the blood products.
Names of persons who have contributed	
Approver Name	Emilie Hardouin
Approver Job Title	Deputy Head of Department (LES)
Approver Email	EHardouin@bournemouth.ac.uk
Review Date	27/01/2023

Uploaded documents
DRAFT SOP Transport, storage and handling of human blood and blood-derived samples.docx - uploaded on 27/01/2022 15:28:46

Appendix V: Risk assessment: Isolating leucocytes and handling of reagents

Risk Assessment Form

About You & Your Assessment	
Name	Sarah
Email	sbuchan@bournemouth.ac.uk
Your Faculty/Professional Service	Faculty of Science and Technology
Is Your Risk Assessment in relation to Travel or Fieldwork?	No
Status	Approved
Date of Assessment	13/10/2022
Date of the Activity/Event/Travel that you are Assessing	

What, Who & Where	
Describe the activity/area/process to be assessed	Undergraduate teaching to isolate leucocytes and staff preparation of reagents
Locations for which the assessment is applicable	Christchurch House labs
Persons who may be harmed	Staff, Student

Hazard & Risk	
Hazard	Trips from spilllages
Severity of the hazard	Low
How Likely the hazard could cause harm	Low
Risk Rating	Low
Control Measure(s) for Trips from spilllages:	

All spillages to be cleaned promptly

With your control measure(s) in place - if the hazard were to cause harm, how severe would it be? Low

With your control measure(s) in place - how likely is it that the hazard could cause harm? Low

The residual risk rating is calculated as: Low

Hazard	Chemicals
Severity of the hazard	High
How Likely the hazard could cause harm	Medium
Risk Rating	High

Control Measure(s) for Chemicals:

When using trypan blue wear gloves at all times, including in the cleaning of the haemocytometer. Trypan blue is a suspected carcinogen; see COSHH form for more details. Women who are, or who suspect they may be pregnant should not handle trypan blue

With your control measure(s) in place - if the hazard were to cause harm, how severe would it be? Medium

With your control measure(s) in place - how likely is it that the hazard could cause harm? Low

The residual risk rating is calculated as: Low

Hazard	COVID
Severity of the hazard	High
How Likely the hazard could cause harm	Medium
Risk Rating	High

Control Measure(s) for COVID:

Social distancing should be maintained in the labs, masks should be encouraged. Hand washing should be performed regularly and benches and communal equipment cleaned after use. Testing for COVID should be carried out twice per week. Maximum occupancy restrictions should not be exceeded in rooms on campus. Anyone testing positive, experiencing symptoms of COVID, or who has been asked to isolate should not be attending campus.

With your control measure(s) in place - if the hazard were to cause harm, how severe would it be? High

With your control measure(s) in place - how likely is it that the hazard could cause harm? Low

The residual risk rating is calculated as: Medium	
Hazard	Risk of injury due to glass coverslip
Severity of the hazard	Low
How Likely the hazard could cause harm	Low
Risk Rating	Low
Control Measure(s) for Risk of injury due to glass coverslip: Students to wear gloves while handling coverslips and haemacytometers.	
With your control measure(s) in place - if the hazard were to cause harm, how severe would it be? Low	
With your control measure(s) in place - how likely is it that the hazard could cause harm? Low	
The residual risk rating is calculated as: Low	
Hazard	Biohazard
Severity of the hazard	Medium
How Likely the hazard could cause harm	Low
Risk Rating	Low
Control Measure(s) for Biohazard: Gloves, lab coat and safety goggles to be worn. Blood to be obtained from reputable source. Potential risk of infection by microbes in blood sample. Blood products to be animal derived, not human and supplied by reputable source. Gloves and labcoats to be worn at all times. Smallest possible volume of blood to be handled by students.	
With your control measure(s) in place - if the hazard were to cause harm, how severe would it be? Low	
With your control measure(s) in place - how likely is it that the hazard could cause harm? Low	
The residual risk rating is calculated as: Low	
Hazard	Electrical hazards - centrifuge
Severity of the hazard	Medium
How Likely the hazard could cause harm	Low

Risk Rating	Low
Control Measure(s) for Electrical hazards - centrifuge: Students to be supervised while centrifuges are in use. Liquids to be kept away from electrical equipment.	
With your control measure(s) in place - if the hazard were to cause harm, how severe would it be? Medium	
With your control measure(s) in place - how likely is it that the hazard could cause harm? Low	
The residual risk rating is calculated as: Low	
Hazard	Physical hazard due to unbalanced centrifuge
Severity of the hazard	Medium
How Likely the hazard could cause harm	Medium
Risk Rating	Medium
Control Measure(s) for Physical hazard due to unbalanced centrifuge: Students to be supervised to ensure they balance the centrifuges adequately. No centrifuge will be left unattended at any time	
With your control measure(s) in place - if the hazard were to cause harm, how severe would it be? Medium	
With your control measure(s) in place - how likely is it that the hazard could cause harm? Low	
The residual risk rating is calculated as: Low	

Review & Approval

Any notes or further information you wish to add about the assessment	RA amended to take COVID into account, RA reviewed as expired. 13/10/22 RA reviewed to take into account handling chemicals when pregnant
Names of persons who have contributed	
Approver Name	Emilie Hardouin
Approver Job Title	Deputy Head of Department
Approver Email	Ehardouin@bournemouth.ac.uk
Review Date	13/10/2027

Appendix VI: Risk assessment: Flow cytometry

Risk Assessment Form

About You & Your Assessment	
Name	Sarah Buchan
Email	sbuchan@bournemouth.ac.uk
Your Faculty/Professional Service	Faculty of Science and Technology
Is Your Risk Assessment in relation to Travel or Fieldwork?	No
Status	Approved
Date of Assessment	20/07/2021
Date of the Activity/Event/Travel that you are Assessing	

What, Who & Where	
Describe the activity/area/process to be assessed	Flow cytometry practical session and staff preparation of reagents
Locations for which the assessment is applicable	Christchurch House labs
Persons who may be harmed	Staff, Student

Hazard & Risk	
Hazard	COVID
Severity of the hazard	High
How Likely the hazard could cause harm	Medium
Risk Rating	High
Control Measure(s) for COVID:	

Social distancing should be adhered to and mask wearing is strongly encouraged. Hands should be washed regularly and all equipment/benches should be cleaned after use. Testing for COVID should be performed twice and week; anyone testing positive for COVID, experiencing COVID symptoms, or who has been asked to isolate should not be attending campus. Maximum occupancy levels in rooms on campus should not be exceeded.

With your control measure(s) in place - if the hazard were to cause harm, how severe would it be? High

With your control measure(s) in place - how likely is it that the hazard could cause harm? Low

The residual risk rating is calculated as: Medium

Hazard	Electrical hazards
Severity of the hazard	Medium
How Likely the hazard could cause harm	Low
Risk Rating	Low

Control Measure(s) for Electrical hazards:

Keep water away from electrical points when using the ovens, flow cytometer or centrifuge for the preparation of cells. Clean up any spills promptly.

With your control measure(s) in place - if the hazard were to cause harm, how severe would it be? Medium

With your control measure(s) in place - how likely is it that the hazard could cause harm? Low

The residual risk rating is calculated as: Low

Hazard	Harmful substances
Severity of the hazard	Medium
How Likely the hazard could cause harm	Low
Risk Rating	Low

Control Measure(s) for Harmful substances:

Lab coats and gloves should be worn throughout and good lab practice adhered to.

Some compounds used in this practical and hazardous (e.g. LPS and cyclohexamide). Students and staff should familiarise themselves with relevant COSHH assessment prior to the practical. Gloves should be worn throughout. In the even of contact with skin, skins should be washed with soap and water. Good lab practice should be adhered to at all times.

With your control measure(s) in place - if the hazard were to cause harm, how severe would it be? Medium	
With your control measure(s) in place - how likely is it that the hazard could cause harm? Low	
The residual risk rating is calculated as: Low	
Hazard	Slips/trips
Severity of the hazard	Medium
How Likely the hazard could cause harm	Low
Risk Rating	Low
Control Measure(s) for Slips/trips: Ensure any spillages are cleaned up promptly and appropriate signage displayed if required.	
With your control measure(s) in place - if the hazard were to cause harm, how severe would it be? Medium	
With your control measure(s) in place - how likely is it that the hazard could cause harm? Low	
The residual risk rating is calculated as: Low	

Review & Approval	
Any notes or further information you wish to add about the assessment	
Names of persons who have contributed	
Approver Name	Emilie Hardouin
Approver Job Title	Deputy Head of Department
Approver Email	Ehardouin@bournemouth.ac.uk
Review Date	20/07/2022

Appendix VII: Abstract for the Wessex Immunology Group conference

June 2023

Detecting and defining immunity to human cytomegalovirus (CMV) in health; combining QuantiFERON-CMV and flow cytometry

Authors: Sophie Willis, Rebecca Neal, Anna Mantzouratou, Emanuela Pelosi and Sarah Buchan.

Background: Human cytomegalovirus (CMV) is an archaic and ubiquitous member of the beta-herpesvirus family (*Herpesviridae),* infecting approx. 50% of adults worldwide. Once infection has resolved, CMV enters a life-long latent state, with potential to reactivate. Infection is usually asymptomatic in healthy individuals, while severe disease can occur in the immunosuppressed host.

After allogeneic haematopoietic stem cell transplant (HSCT), reactivation of CMV can cause severe morbidity and mortality. CMV DNA monitoring by Polymerase Chain Reaction (PCR) and timely administration of pre-emptive antiviral treatment in case of reactivation are vital to avert such outcomes. T-cell mediated immunity is key in controlling CMV infection and assays designed to measure it would further improve the management of CMV infection in transplant patients; however, they are lacking in standard clinical practice.

Aim: to evaluate the performance of QuantiFERON®-CMV, a commercial assay that detects CMV T-cell mediated immune response, to monitor CMV immune reconstitution post-allogeneic HSCT. This study additionally uses flow cytometry to detect and phenotype CMV-T cell responses with the aim to corroborate QuantiFERON®-CMV results.

Methods: 11 healthy volunteers who were tested for the presence/phenotype of CMV-specific T cells (by QuantiFERON®-CMV and flow cytometry) and for anti-CMV IgG to assess prior infection with CMV and ascertain that both assays can detect CMV T-cell immunity.

Results: Our preliminary data confirm that flow cytometry and QuantiFERON®-CMV both detect CMV reactive T-cells and that T-cell reactivity correlates with antibody titre, providing a platform to evaluate the clinical applicability of QuantiFERON®-CMV in the allogeneic-HSCT setting.

Evaluation of the clinical usefulness of CMV QuantiFERON assay in identifying when patients reconstitute their immunity against CMV after undergoing allogeneic HSCT

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Introduction

Cytomegalovirus (CMV) is a B-herpesvirus estimated to infect approximately 83% of the global population and a pathogen of primary importance in the setting of haematopoietic stem cell transplantation (HSCT). Despite advances in diagnostic measures and treatment modalities over the years, pervasive CMV disease post transplantation is a cause of significant non-relapse associated mortality. Previous CMV infection is pivotal in donor-recipient stem cell matching as it precludes several complications in allogeneic HSCT patients. Studies have shown that patients who have a cellular immune response to CMV have a significantly lower risk of developing CMV disease than those who do not have a detectable immune response to CMV.

Intended outcome

This research will add to current knowledge of CMV in HSCT by testing firstly a commercial assay (Qiagen QuantiFERON-CMV) which could be used as a predictor of risk of CMV disease and secondly by defining which T-cell subsets offer immunity to CMV in these patients. This information will enable personalised risk stratification and improve clinical management. Should Qiagen QuantiFERON®-CMV assay be successful in detecting CMV-specific immune reconstitution after allo-HSCT, it may be adopted for clinical use in allogeneic HSCT patients at University Hospital Southampton's bone marrow transplant unit.

Methods

We have performed preliminary experiments to confirm and refine the QuantiFERON-CMV assay and flow cytometric approaches. The principle of the QuantiFERON-CMV approach is shown in Figures 1 and 2.

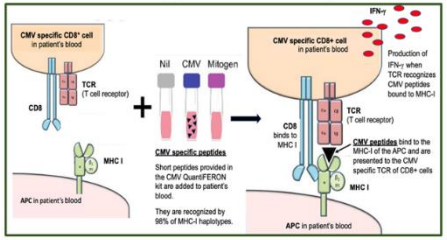

Figure 1. How the Qiagen CMV QuantiFERON assay works, illustrated by Dr Emanuela Pelosi.

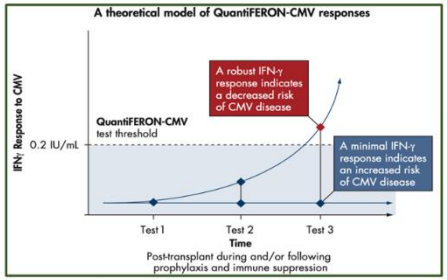

Figure 2. A theoretical model of QuantiFERON-CMV responses in a post-transplant setting during and/or following prophylaxis and immune suppression (from Qiagen IFU).

Preliminary Experiment 1

Objective: To confirm binding of flow cytometric antibodies to surface antigens on human T cells, using Jurkat T cells as a model. Figure 3 confirms that Jurkat T cells express CD45RA and CD4 , validating the staining approach. Jurkat cells expressed a small amount of CD8.

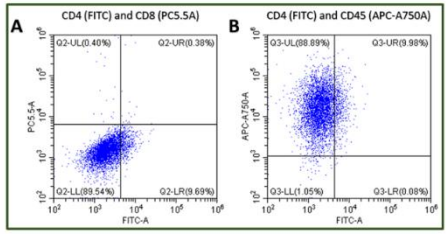

Figure 3. Antibodies successfully bind to human T cells in vitro. The Jurkat T cell line was stained with fluorescent antibodies specific for surface markers CD4 (FITC), CD8 (PC5.5) and CD45RA (APC-A750). Cells express (A+B) low levels of CD4, (A) but not CD8 and are (B) CD45RA positive.

Preliminary Experiment 2

Objective: To establish a protocol for detecting intracellular IFN-g in activated T-cells using Jurkat T cells as a model. Cells were treated or untreated for 4 hours with PMA/Ionomycin concurrent with blockade of protein secretion prior to surface and intracellular flow cytometry staining.

Figure 4 confirms that Jurkat T cells express CD4 and CD45RA, validating the staining approach, although Jurkat cells did not express IFN-g. CD4 expression is downregulated in treated cells, and CD45RA is expressed slightly more in treated cells.

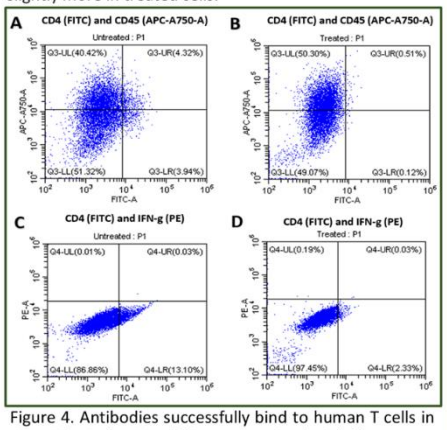

Figure 4. Antibodies successfully bind to human T cells in vitro. The Jurkat T cell line was stained with fluorescent antibodies specific for surface markers CD4 (FITC), CD45RA (APC-A750) and intracellular marker IFN-g (PE). Cells express CD4 (A+B+C+D), CD45RA (A+B) and did not express IFN-g (C+D). CD45RA is expressed more in treated cells (B) than untreated (A).

Pilot Study

A pilot study was conducted to determine the sensitivity of the CMV QuantiFERON assay and flow cytometry protocol in primary human cells from 1 volunteer where IgG level was known and to perform serial dilutions to mimic lymphopenia. We then tested our flow cytometry protocol to ascertain whether the surface and intracellular antibodies stained adequately.

For this, blood was collected from 1 healthy volunteer with circulating CMV IgG titres of 151 units/ml (data not shown). A QuantiFERON-CMV assay was performed using serially diluted blood. In addition leucocytes were isolated, stimulated for 4 hours in Nil/Mitogen/CMV QuantiFERON® tubes and accumulation of IFN-g in CD8+ and CD4+ T cell subsets quantified. Data (Figure 5A and B) show that IFN-g can be detected in CD8+ T cells by flow cytometry after stimulation in QuantiFERON-CMV tubes.

Fig. 5 also shows that CMV-reactive cells are largely of the TEMRA phenotype and that QuantiFERON-CMV can still detect CMV reactivity in blood diluted approximately 20 fold.

Pilot Study Results

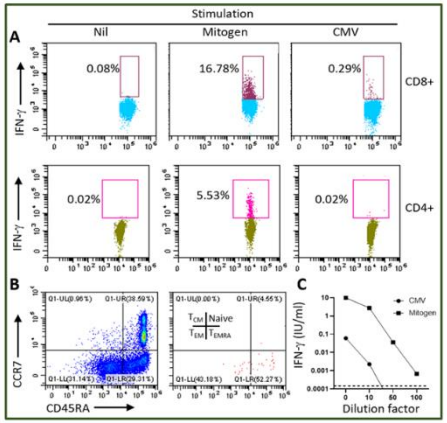

Figure 5. Results from pilot study data. (A) Numbers indicate percent IFN-g+ cells in CD8+ or CD4+ T-cell gates by flow cytometry. (B) Phenotype of whole (left) or CMV-reactive (right) CD8+ T cells by flow cytometry. (C) Quantification of CMV-specific immune response in diluted blood by QuantiFERON assay from a single volunteer.

Conclusion

- Surface and intracellular flow cytometry staining protocols can be used to phenotype human Jurkat T cells
- Intracellular staining for IFN-g detects CMV-reactive T cells in a heathy individual
- The QuantiFERON-CMV assay has a sensitivity sufficient to detect CMV reactivity in lymphopenia

References

1. Cui et al., *Diagnosis and treatment for the early stage of cytomegalovirus infection during hematopoietic stem cell transplantation* 2022
2. Ariza-Heredia et al., *Cytomegalovirus diseases after hematopoietic stem cell transplantation: A mini-review* 2014