Latent Cytomegalovirus (CMV) infection does not detrimentally alter T cell responses in the healthy old; but increased latent CMV carriage is related to expanded CMV specific T cells.

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author contribution statement

SEJ, MRW, ELP and JHS designed the project and experiments.
SEJ, GXS, GO and ELP carried out the experiments.
SEJ and MRW wrote the manuscript.
SEJ carried out statistical analysis and prepared figures.
SEJ and MRW submitted this paper.
All authors reviewed the manuscript.

Keywords

Human cytomegalovirus (hcmv), Immunology of Ageing, viral latency, HCMV specific T-cells, IFNγ production, cIL-10+ CD4+ T cells, Latent Viral Load

Abstract

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Human cytomegalovirus (HCMV) primary infection and periodic re-activation of latent virus is generally well controlled by T-cell responses in healthy people. In older donors, overt HCMV disease is not generally seen despite the association of HCMV infection with increased risk of mortality. However, increases in HCMV-DNA in urine of older people suggest that, although the immune response retains functionality, immunomodulation of the immune response due to lifelong viral carriage may alter its efficacy. Viral transcription is limited during latency to a handful of viral genes and there is both an IFNγ and cellular IL-10 CD4+ T-cell response to HCMV latency-associated proteins. Production of cIL-10 by HCMV-specific CD4+ T-cells is a candidate for ageing related immunomodulation. To address whether long-term carriage of HCMV changes the balance of cIL-10 and IFNγ secreting T-cell populations, we recruited a large donor cohort aged 23–78 years and correlated T-cell responses to 11 HCMV proteins with age, HCMV-IgG levels, latent HCMV-load in CD14+ monocytes and T-cell numbers in the blood. IFNγ responses by CD4+ and CD8+ T-cells to all HCMV proteins were detected, with no age-related increase in this cohort. IL-10 secreting CD4+ T cell responses were predominantly to latency-associated proteins but did not increase with age. Quantification of HCMV genomes in CD14+ monocytes, a known site of latent HCMV carriage, did not reveal any increase in viral genome copies in older donors. Importantly, there was a significant positive correlation between the latent viral genome copy number and the breadth and magnitude of the IFNγ T-cell response to HCMV proteins. This study suggests in healthy aged donors that HCMV specific changes in the T cell compartment were not effected by age and were effective, as viremia was a very rare event. Evidence from studies of unwell aged has shown HCMV to be an important co-morbidity factor, surveillance of latent HCMV load and low-level viremia in blood and body fluids, alongside typical immunological measures and assessment of the anti-viral capacity of the HCMV-specific immune cell function would be informative in determining if anti-viral treatment of HCMV replication in the old maybe beneficial.

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Ethical approval was obtained from University of Cambridge Human Biology Research Ethics Committee. Informed written consent was obtained from all donors in accordance with the Declaration of Helsinki (HBREC.2014.07)

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Abstract

Human cytomegalovirus (HCMV) primary infection and periodic re-activation of latent virus is generally well controlled by T-cell responses in healthy people. In older donors, overt HCMV disease is not generally seen despite the association of HCMV infection with increased risk of mortality. However, increases in HCMV-DNA in urine of older people suggest that, although the immune response retains functionality, immunomodulation of the immune response due to lifelong viral carriage may alter its efficacy. Viral transcription is limited during latency to a handful of viral genes and there is both an IFNγ and cellular IL-10 CD4+ T-cell response to HCMV latency-associated proteins. Production of cIL-10 by HCMV-specific CD4+ T-cells is a candidate for ageing related immunomodulation. To address whether long-term carriage of HCMV changes the balance of cIL-10 and IFNγ secreting T-cell populations, we recruited a large donor cohort aged 23–78 years and correlated T-cell responses to 11 HCMV proteins with age, HCMV-IgG levels, latent HCMV-load in CD14+ monocytes and T-cell numbers in the blood. IFNγ responses by CD4+ and CD8+ T-cells to all HCMV proteins were detected, with no age-related increase in this cohort. IL-10 secreting CD4+ T cell responses were predominantly to latency-associated proteins but did not increase with age. Quantification of HCMV genomes in CD14+ monocytes, a known site of latent HCMV carriage, did not reveal any increase in viral genome copies in older donors. Importantly, there was a significant positive correlation between the latent viral genome copy number and the breadth and magnitude of the IFNγ T-cell response to HCMV proteins. This study suggests in healthy aged donors that HCMV specific changes in the T cell compartment were not affected by age and were effective, as viremia was a very rare event. Evidence from studies of unwell aged has shown HCMV to be an important co-morbidity factor, surveillance of latent HCMV load and low-level viremia in blood and body fluids, alongside typical immunological measures and assessment of the anti-viral capacity of the HCMV-specific immune cell function would be informative in determining if anti-viral treatment of HCMV replication in the old maybe beneficial.

Introduction

A consequence of ageing in the human population is a decline in immune function, often described as immune senescence, which includes a loss of adaptive immune cells and an increase in inflammatory cytokines resulting in dysregulation of the immune response (McElhaney and Effros, 2009). There is now evidence from a number of studies that, after the age of 65, the age associated loss of immune function results in individuals becoming more susceptible to infectious diseases as well as increased morbidity and mortality from autoimmune disorders (Denkinger et al., 2015; Kline and Bowdish, 2016). Infection with human cytomegalovirus (HCMV) is characterized by its life-long persistence in the infected individual due, in part, to its ability to establish a latent infection in bone marrow stem cells and myeloid cells (Sinclair and Sissons, 2006). Despite a robust immune response to the primary infection, the large number of immune evasion molecules encoded by HCMV allows it to establish its latent life cycle (Wills et al., 2015). Primary HCMV infection and reactivation from latency is generally well controlled in healthy individuals; however, when the immune system is compromised, or under developed, it can become a significant problem (Crough and Khanna, 2009; Jackson et al., 2011). A potential impact of lifelong persistence of HCMV is its effect on the host immune response with ageing. A number of longitudinal and population cohort studies have suggested that HCMV sero-positivity was linked to age-related (i) increase in susceptibility to infections, (ii) poor response to vaccinations and (iii) increased risk of all-cause mortality compared to age matched HCMV sero-negative individuals – which has been termed the Immune Risk Phenotype (IRP) (Olsson et al., 2001; Wikby et al., 2002; Trzonkowski et al., 2003; Ouyang et al., 2004; Hadrup et al., 2006; Strindhall et al., 2013). Analysis of a number of large population cohorts recruited for cancer, dementia and nutritional studies in the UK and USA have also shown a significant association between HCMV sero-positivity and mortality from cardiovascular related disease (Simanek et al., 2011; Gkrania-Klotsas et al., 2012; Olson et al., 2013; Savva et al., 2013; Spyridopoulos et al., 2016). However, other studies have shown no such age-related correlation between HCMV sero-positivity and declines in immune responses to either novel infections (Lelic et al., 2012; Schulz et al., 2015) or responses to vaccination (Furman et al., 2015). Similarly, a study measuring frailty in older people saw a positive association with inflammatory cytokines but not HCMV infection (Collerton et al., 2012) perhaps consistent with studies that have shown that rises in inflammatory cytokines in the serum of older donors is not primarily driven by HCMV (Bartlett et al., 2012).

It has been observed that infection with HCMV changes the composition of the CD4+ and CD8+ memory T cell repertoires; this includes an expansion of the T cell population which have lost expression of the co-stimulatory molecules CD27 and CD28 but also show re-expression of CD45RA and co-expression of the carbohydrate HNK-1 (CD57) (reviewed in (Weltevrede et al., 2016)). Such T cells are considered to be a highly differentiated phenotype (Harari et al., 2004), and potentially dysfunctional as they often lose the ability to secrete cytokines and have limited proliferative capacity (Ouyang et al., 2004; Henson et al., 2009). It has been suggested that expanded populations of highly differentiated T cells in HCMV sero-positive older donors may be detrimental to the infected individual (Vescovini et al., 2010; Derhovanessian et al., 2014; Broadley et al., 2017). However, such increases in these highly differentiated T cells is also observed in young HCMV

positive individuals (Miles et al., 2007) and it is, also, now clear that these highly differentiated T cells are still functional and, with the correct co-stimulation, can proliferate (Waller et al., 2007; Riddell et al., 2015). Similarly, HCMV specific T cells have been shown to produce multiple anti-viral cytokines and have efficient cytotoxic capacity despite a highly differentiated phenotype (Casazza et al., 2006; Lachmann et al., 2012; Riou et al., 2012). Furthermore, older HCMV sero-positive individuals do not appear to suffer from overt HCMV disease from reactivating virus or HCMV re-infection which suggests that the immune response of older people retains the ability to control virus replication (Stowe et al., 2007). Despite older HCMV sero-positive donors having functional HCMV specific immune responses, there does appear to be age-related increases in levels of viral DNA detectable in urine (Stowe et al., 2007) and blood (Furui et al., 2013). This suggests that the immune response in older people may be altered, possibly due to lifelong carriage of the virus, and that immunomodulation of the HCMV specific immune response, as either a direct consequence of the viral infection or bystander effects, results in reduced clearance of reactivating virus in older people (Wills et al., 2015).

Latent carriage of HCMV in CD34+ progenitor cells and their myeloid derivatives is characterized by repression of viral immediate Early (IE) gene transcription with a restricted gene expression profile which cannot support production of infectious virus. A number of viral genes have been identified as being transcribed during HCMV latent infection, including UL138 (Goodrum et al., 2007), LUNA (latent undefined nuclear antigen; UL81-82as) (Bego et al., 2005; Reeves and Sinclair, 2010), US28 (Beisser et al., 2001), UL111A (vIL-10) (Jenkins et al., 2004) and UL144 (Poole et al., 2013). Analysis of the secreted cellular proteins (cell secretome) of experimentally latently infected CD34+ and CD14+ cells have identified the induced expression of chemokines which can recruit T cells as well as the cellular cytokines IL-10 and TGF-β, both of which can modulate the activity of T cells which have migrated to the environment surrounding the latent infection (Mason et al., 2012). HCMV specific CD4+ T cells have been identified that either secrete cIL-10 or have a regulatory cell phenotype (Tovar-Salazar et al., 2010; Schwele et al., 2012; Terrazzini et al., 2014; Derhovanessian et al., 2015; Clement et al., 2016) and, in the mouse, it has been shown that CD4+ T regulatory cells (Tregs) and IL-10 secretion can reduce viral clearance and increase persistence in murine cytomegalovirus (MCMV) (Jost et al., 2014; Clement et al., 2016). Additionally, there is evidence that the frequency of HCMV specific inducible Tregs is increased in older individuals (Terrazzini et al., 2014), alongside an overall increase in frequency of T regulatory cells in old age (Gregg et al., 2005; Chidrawar et al., 2009). Previously, we have identified CD4+ T cells specific for peptides to two of the latency-associated proteins, UL138 and LUNA which secrete cIL-10 and also possess Th1 anti-viral effector functions (Mason et al., 2013). We have also shown that the UL138 specific CD4+ T cells recognize experimentally latently infected CD14+ monocytes, secrete cIL-10 and suppress T cell function.

With these observations in mind, we hypothesized that the long term carriage of HCMV could create an immunomodulatory environment to help prevent clearance of the virus by skewing the CD4+ T cell compartment towards a suppressive or regulatory cIL-10 producing phenotype. We also wanted

to assess whether the same environment had an impact on the frequency of HCMV specific CD8+ T cells within a large old aged donor cohort, who have carried HCMV for longer compared to younger sero-positive donors. Additionally, within the study, we wanted to measure the levels of latent viral genome carriage and determine if infectious virus was detectable and relate this to changes in the T cell response. To address these questions, we conducted a study on a large healthy donor cohort which encompassed a broad age range (23 – 78 years) of both HCMV sero-positive and negative donors. We performed absolute cell counts, measured HCMV specific antibody levels, assayed viral genome copy number in total peripheral blood and in CD14+ cells as well as measuring the CD8+ specific production of IFNγ and CD4+ specific production of IFNγ and IL-10 in response to stimulation by overlapping peptide pools to eleven HCMV proteins (5 latency associated and 6 lytic only expressed proteins). The study group exhibited typical age-related decline in both absolute CD4+ and CD8+ naïve T cell numbers and HCMV sero-positive donors had increased absolute numbers of T cells with a differentiated phenotype compared to sero-negative donors. We did not see an inversion of the CD4:CD8 ratio within this donor cohort, a characteristic associated with the IRP, although CD4:CD8 ratio was decreased in HCMV sero-positive donors compared to sero-negative. In contrast to studies in other donor cohorts, we did not see an age related expansion of the HCMV IgG response or an influence of donor age on either the breadth or magnitude of the T cell responses (Parry et al., 2016; Weltevrede et al., 2016). We detected both CD4+ and CD8+ specific IFNγ responses to all 11 HCMV proteins analyzed and also detected more limited CD4+ specific IL-10 responses to the same proteins, we also confirmed our previous observations that CD4+ specific IL-10 responses are more common towards latency associated proteins. We were able to detect latent HCMV genomes in isolated peripheral blood CD14+ monocytes in 45% of donors but, in contrast to previous reports (Parry et al., 2016), we did not observe an increase in HCMV copy number in donors aged over 70 years old. Importantly, we did see a significant association between the levels of HCMV detected in CD14+ monocytes and both the breadth and magnitude of the CD8+ T cell responses to HCMV proteins, irrespective of donor age. Overall it is our opinion that larger latent HCMV reservoirs will lead to increased HCMV reactivation and dissemination events, which in normal healthy individuals will stimulate secondary HCMV specific T cell responses, thus driving increases in T cell frequency and differentiation status.

Materials and methods

Ethics and Donor Cohort information

The study donor cohort was recruited by the National Institute of Health Research (NIHR) Cambridge BioResource, using their Biobank of volunteers, who predominantly are local to Cambridge or live in the East Anglian Region of the United Kingdom. Ethical approval was obtained from University of Cambridge Human Biology Research Ethics Committee. Informed written consent was obtained from all donors in accordance with the Declaration of Helsinki (HBREC.2014.07). Known HCMV sero-positive and sero-negative donors were recruited in three age groups; Young (18 – 40 years), Middle (41 – 64 years) and Old (65+ years) were included in this study. Volunteers being treated with oral or intravenous immunomodulatory drugs (including steroids, tacrolimus, cyclosporins, azathioprines, Mycophenolate, Methotrexate, Rituximab, Cyclophosphamide) within the last 3 months, undergoing injected Rheumatoid Arthritis treatment including anti-TNFα agents and anyone actively, or within the last 24 months, being treated with cancer chemotherapy were excluded from the study. 119 HCMV sero-positive and sero-negative donors were included in this study, the age range of the recruited donor cohort was 23 – 76 years, 70 donors were female and 49 donors were male. Further characteristics of the studied donor cohort are detailed in Table 1. In total, a 50ml peripheral blood sample was collected from each donor, comprised of 1.2ml clotted blood, 1.2ml EDTA treated blood and 47.6ml Lithium Heparin treated blood samples.

Peripheral Blood Mononuclear cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated from the heparinized blood samples using Lymphoprep (Axis-shield, Oslo, Norway) density gradient centrifugation.

Absolute count Protocol

50µl of the EDTA treated whole blood sample was transferred to Becton Dickinson Trucount tubes (BD Biosciences, Oxford, UK) and stained with a pre-mixed antibody cocktail containing CD45-VioBlue, CD3-VioGreen (Miltenyi Biotec, Bisley, UK.), CD4-Brilliant Violet 605, CD8-PerCP-Cy5.5, CD28-PE, CD27-APC-Cy7, CD45RA-FITC, CD25-APC and CD127-PE-Cy7 (BioLegend, San Diego, USA). Following staining the red blood cells was lysed and the cells fixed using FACS Lysing solution (BD Biosciences). The samples were stored at -80°C until acquisition (Hensley-McBain et al., 2014). Samples were acquired on a LSR Fortessa (BD Biosciences) along with Fluorescence Minus One (FMO) controls using FACS Diva software (BD Biosciences). Samples were then analyzed using FlowJo software (Treestar, Oregon, USA), firstly the trucount bead population was identified and then the trucount bead negative population (i.e. cells) were analyzed by gating for single cells, then $CD45^{hi}$ lymphocytes, CD3+ T cells, CD4+ and CD8+ expressing cells.

The CD4+ and CD8+ T cell populations were further subdivided into 4 memory populations defined by expression of CD27 and CD45RA, and 4 differentiation populations defined by expression of CD27 and CD28 were identified and in CD4+ T cells a T_{reg} population defined as $CD25^{hi}$ and $CD127^{lo}$ were identified, gate and quadrant positions were identified using the FMO controls. A representative gating strategy and the formula used to calculate the absolute cell counts is illustrated in Sup. Fig. 1, the event number for all populations, and trucount beads were exported to an excel sheet where the number of cells per μl of blood for each T cell subset was calculated according to manufacturer instructions.

HCMV IgG Antibody levels Protocol

HCMV sero-status was confirmed using serum from the clotted blood sample and HCMV IgG levels determined using an IgG enzyme-linked immunosorbent (EIA) assay, HCMV Captia (Trinity Biotech, Didcot, UK) following manufacturer's instructions, on serum derived from clotted blood samples. The EIA assay is semi-quantitative, containing negative, positive and calibrator controls which allow the computation of an Immune Serum Ratio (ISR) value for the amount of HCMV IgG present in the sample. In addition to the manufacturer controls and quality control protocols, a known positive serum sample was also run to check inter-assay variability was acceptable.

HCMV ORF peptide mixes

8 HCMV ORF encoded proteins (UL55 (gB), UL82 (pp71), UL122 (IE2), UL123 (IE1), US3, UL138, US28 and UL111A(vIL-10)) were selected and peptide libraries comprising consecutive 15mer peptides overlapping by 10 amino acid were synthesized by ProImmune PEPScreen (Oxford, UK) from sequences detailed in the Sylwester *et. al.* study (Sylwester et al., 2005). A further 3 HCMV ORF encoded proteins (UL83 (pp65), UL144 (which incorporated known strain variants) and LUNA (UL81-82as)) 15mer peptide libraries were synthesized by JPT Peptide Technologies GmbH (Berlin, Germany). The individual lyophilized peptides from each ORF library were reconstituted and used as previously described (Jackson et al., 2014).

Depletion of CD4+ and CD8+ T cells from PBMC

PBMC were depleted of either CD4+ or CD8+ T cells by MACS using anti-CD4+ or anti-CD8+ direct beads (Miltenyi Biotech), according to manufacturer's instructions, and separated on either LS columns (Miltenyi Biotech) or by using an AutoMACS Pro (Miltenyi Biotech). Efficiency of depletion was determined by staining cells with a CD3-FITC, CD4-PE and CD8-PerCPCy5.5 antibody mix (all BioLegend) and analyzed by flow cytometry. Depletions performed in this manner resulted in mean 3.8% residual CD8+ T cells and 8.6% residual CD4+ T cells (from n=61 donors).

Dual FLUOROSPOT assays

2 x 10^5 PBMC depleted of either CD8+ or CD4+ T cells suspended in X-VIVO 15 (Lonza, Slough, UK) supplemented with 5% Human AB serum (Sigma Aldrich) were incubated in pre-coated Fluorospot plates (Human IFNγ and IL-10 FLUOROSPOT (Mabtech AB, Nacka Strand, Sweden)) in triplicate with ORF mix peptides (final peptide concentration 2µg/ml/peptide) and an unstimulated and positive control mix (containing anti-CD3 (Mabtech AB), Staphylococcus Enterotoxin B (SEB), Phytohaemagglutinin (PHA) and Pokeweed Mitogen (PWM) and Lipopolysaccharide (LPS) (all Sigma Aldrich)) at 37ºC in a humidified CO_2 atmosphere for 48 hours. The cells and medium were decanted from the plate and the assay developed following the manufacturer's instructions. Developed plates were read using an AID iSpot reader (Oxford Biosystems, Oxford, UK) and counted using AID EliSpot v7 software (Autoimmun Diagnostika GmbH, Strasberg, Germany) using distinct counting protocols for IFNγ and IL-10 secretion. Donor results were discounted from further analysis if there was greater than 1000 spot forming units (sfu) background secretion of IFNγ or IL-10 in the unstimulated wells, additionally the sfu response in the positive control wells had to be at least 100sfu (IFNγ) or 50sfu (IL-10) greater than the background sfu. All data were then corrected for background cytokine production and the positive response cut-off for IFNγ and the IL-10 responses was determined by comparing the distribution of the responses from HCMV sero-positive and sero-negative donors to all HCMV proteins and the positive control. This analysis determined that the positive response for IFNγ and IL-10 was greater than 100sfu/million, this threshold is indicated in Figures 3A, 4A and 5A (dashed line).

Measurement of HCMV DNAemia in whole blood

A 1ml EDTA treated whole blood sample was stored at -20°C for each donor. DNA was isolated from the whole blood sample using the QIAamp DNA Blood Midi Kit (Qiagen, Manchester, UK) following the manufacturer's instructions. Extracted DNA samples were stored at -20°C until required. The detection of HCMV by Real Time Quantitative PCR method using the StepOne Real-Time PCR system (Applied Biosystems, ThermoFisher Scientific) was performed using a method adapted from (Mattes et al., 2005). Real-time amplification of HCMV DNA used glycoprotein B-specific primers, (5'-GAGGACAACGAAATCCTGTTGGGCA-3' [gB1] and 5'-GTCGACGGTGGAGATACTGCTGAGG-3' [gB2] (Fox et al., 1995)), and detection with a TaqMan probe (5' 6-FAM- CAATCATGCGTTTGAAGAGGTAGTCCA-BHQ1 3' [gBP3] (Mattes et al., 2005)) mixed with ABI Universal Mastermix (Applied Biosystems, ThermoFisher Scientific), the final assay volume was 25µl, which includes a 5µl donor or control sample. PCR cycling conditions were 2 min at 50°C, 10 min at 95°C and 45 cycles of 15 s at 95°C and 60 s at 60°C, all donor samples were screened in duplicate with a high (50 000 copies/ml) and low (500 copies/ml) positive control samples (whole EDTA treated blood spiked with HCMV genomes from the World Health Organization (WHO) international standard (Fryer et al., 2010) (National Institute for Biological Standards and Control (NIBSC), Potters Bar, UK)), run in triplicate. Samples with detectable HCMV DNA were repeated in triplicate in a real-time amplification including a standard curve in triplicate of 1 – 10^4 HCMV genomes (WHO International Standard) in addition to the high

and low positive controls. The HCMV DNA load was calculated using the StepOne Software (Applied Biosystems, ThermoFisher Scientific) and reported as HCMV copies/ml blood.

Latent Viral Load Digital PCR

CD14+ Monocytes were extracted using CD14+ Magnetic beads and MS columns (Miltenyi Biotec) from PBMC isolated from 20ml of heparinized Peripheral Blood in a HCMV clean facility. The monocytes were enumerated, dry pelleted and stored at -80°C prior to DNA extraction. DNA was extracted from the cell pellet in a 1:1 mixture of PCR solutions A (100mM KCl, 10mM Tris-HCl pH8.3 and 2.5mM $MgCl_2$) and B (10mM Tris-HCl pH8.3, 2.5mM $MgCl_2$, 1% Tween 20, 1% Nonidet P-40 and 0.4mg/ml Proteinase K) at a final concentration equivalent to 10000 cells/µl, for 60 min at 60°C followed by a 10 min 95°C incubation (Roback et al., 2001), extracted DNA samples were stored at -80°C until required. Measurement of HCMV DNA in extracted CD14+ cells was assessed using a droplet digital PCR method (Parry et al., 2016). Using the QX200 droplet digital PCR system (Bio-rad, Watford, UK) a reaction mixture containing 2µl of donor CD14+ DNA (equivalent to 20000 cells) or positive control sample was mixed with PCR grade water, 2xddPCR supermix for probes (Bio-rad), FAM labeled HCMV primer and probe (from Human CMV HHV5 kit for qPCR using a glycoprotein B target, PrimerDesign, Southampton, UK) and HEX labeled RPP30 copy number assay for ddPCR (Bio-rad). Droplets were generated with droplet generation oil (Bio-rad) in the QX200 droplet generator (Bio-rad), then the sample was loaded into a 96 well PCR plate (Eppendorf, Stevenage, UK), sealed with a PX1 PCR Plate sealer (Bio-rad) and PCR amplification was performed using a C1000 Touch Thermocycler (Bio-rad), for 10 min at 95°C followed by 40 cycles of 30 s at 94°C and 60s at 60°C. Following PCR amplification the PCR plate was loaded onto the QX200 Droplet Reader (Bio-rad) where the presence or absence of PCR product in each droplet was read and analyzed by QuantaSoft software (Bio-rad) which gives the result of the number of virus copies per µl of PCR reaction. All donor CD14+ DNA samples were run in either quadruplicate or triplicate. The RPP30 copy number primer probe enabled the determination of the cell number included in the reaction and the HCMV viral load number was adjusted according to this and expressed as HCMV copies per million CD14+ cells.

Statistics

Statistical analysis was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, CA, USA). Correlation was assessed by Pearson or Spearman correlation according to the distribution of the data. Multiple data sets groups were compared using a 1 way ANOVA Kruskall-Wallis test with post hoc Dunn's multiple comparisons or selected Mann Whitney U comparisons using an adjusted p value ($p \leq 0.05/n$ comparisons) to correct for multiple testing false discovery.

Results

Characterization of the ARIA Study Donor Cohort

To determine whether long-term carriage of HCMV alters the HCMV specific T cell response, with respect to cytokine secretion or state of T cell differentiation, and whether any identified changes impact on latent HCMV viral carriage and/or levels of HCMV IgG, we designed an age cross-sectional study. Donors were placed into 3 age groups: Young (age ≤ 40 years), Middle aged (age 41 – 64 years) and Old (age ≥ 65 years) and also grouped on the basis of their HCMV sero-status. Potential donors were excluded from the study if they were currently taking, or had taken in the previous 3 months, any immunomodulatory or monoclonal antibody treatments or if they were currently cancer sufferers or had any form of cancer in the previous 24 months. In total, 119 individuals from the 3 age groups were included in this analysis: age range, virological and immunological parameters (HCMV IgG levels, HCMV DNA copies/ml whole blood and the CD4:CD8 Ratio) for the donor cohort are detailed in Table 1. Correlation of the levels of HCMV IgG (ISR) (summarized for the 3 age groups in Table 1) within HCMV sero-positive (HCMV+ve) donors with age did not show a significant accumulation with age (Pearson r=0.1012, (95% CI: -0.0923, 0.2873), p=0.3043). Neither was there a significant decrease in the CD4:CD8 ratio within the HCMV+ve donor group with age (Spearman r_s=0.08563, (95% CI:-0.1135, 0.2781), p=0.3851).

The composition of the CD8+ and CD4+ T cell compartments, in whole blood isolated directly *ex vivo,* were enumerated and compared between donor age and HCMV sero-status. Figure 1 summarizes the impact of increasing age on T cell numbers in the entire donor cohort. This analysis shows that both CD8+ and CD4+ T cell numbers significantly decrease with age (Figure 1B Spearman r_s=-0.255, p=0.005 and Figure 1D Spearman r_s=-0.207, p=0.024 respectively) which was likely due to the significant loss of naïve CD8+ and CD4+ T cells (Figures 1C and 1E) with no corresponding increase in numbers of memory T cell populations (Supplementary Figure 2). Enumeration of CD4+ T regulatory cells present in the peripheral blood of all donors, based on the expression of CD127 and CD25 (Hardy et al., 2013), showed that there was no effect of age on the size of this cell population (Figure 1F). When comparing the impact of HCMV infection, in donors of all ages, on the numbers of differentiated T cell subsets, we observed a significant expansion of the effector memory (T_{EM} – CD27-CD45RA-) population in both CD8+ (Figure 2B) and CD4+ T cells (Figure 2D). Within CD8+ T cells only, we also saw a significant increase in the highly differentiated T_{EMRA} (CD27-CD45RA+) and CD27-CD28- (LATE) populations (Figures 2B and 2C). A key component of the IRP which is associated with HCMV infection is the inversion of the CD4:CD8 ratio (<1), we only saw this phenomenon in 10% of the sero-positive donor group. However we observed that overall the CD4:CD8 ratio was significantly decreased in HCMV sero-positive donors compared to sero-negatives (Figure 2G).

Magnitude and Breadth of T cell responses to HCMV Proteins remain stable with donor age.

To establish whether HCMV latent and lytic protein specific T cells are maintained and are functional during long term carriage of the virus, we analyzed T cell responses to 5 viral genes known to be expressed during HCMV latent infection; UL138 (Goodrum et al., 2007), LUNA (Bego

et al., 2005; Reeves and Sinclair, 2010), US28 (Beisser et al., 2001), UL111A (vIL-10) (Jenkins et al., 2004) and UL144 (Poole et al., 2013), two of which (UL138 and LUNA), we have previously shown elicit both an IFNγ and IL-10 CD4+ T cell response (Mason et al., 2013). We also wanted to measure the range of T cell responses in a large donor cohort to a number of viral proteins expressed during lytic infection; we have previously identified both CD4+ and CD8+ T cells producing IFNγ from many donors to 6 HCMV lytic proteins pp65, IE1, IE2, gB, pp71 and US3 (Jackson et al., 2014; Jackson et al., 2017). Using Fluorospot methodology, we were able to measure CD8+ T cell IFNγ responses and both IFNγ and IL-10 CD4+ T cell responses to overlapping peptide pools of these 11 HCMV proteins. Both HCMV sero-positive and sero-negative donors of all ages were included in these antigen specific screens and, after discounting samples following quality control (high spontaneous cytokine spot forming unit (sfu) counts in unstimulated wells or failure of positive control stimulation), 98 donors were included in the CD8+ T cell analysis, 99 donors in the CD4+ T cell IFNγ analysis and 73 donors in the CD4+ T cell IL-10 analysis.

Figure 3 summarizes the results from the screen of 98 donors for CD8+ IFNγ T cell responses. A majority of the HCMV sero-positive donors analyzed had an above threshold (100 sfu/million) CD8+ IFNγ T cell response to the 6 lytic proteins analyzed as well as responses to the latency associated proteins UL144 and US28 proteins (Figure 3A). We noted positive CD8+ T cell responses to LUNA (31.8% of donors) and UL138 (29.6% of donors), which whilst present in our previous study, using an enzymatic ELISPOT method, were below the positive response threshold (Mason et al., 2013) because this was a much less sensitive detection system. The frequency of individual donors who produced CD8+ T cell responses to 1 or more HCMV proteins is presented as pie charts for the lytic expressed proteins (Figure 3B), latency associated proteins (Figure 3E) and for all HCMV proteins (Figure 3H). These analyses shows that a majority of the donors produced a response to 5 or 6 lytic proteins (51.6% - blue and deep pink segments Figure 3B), that 29.7% of the donor cohort responded to 4 or 5 of the latency associated proteins (green and blue segments Figure 3E) and, overall, 47.2% of the cohort responded to 8 or more HCMV proteins (orange, dark green, teal and purple segments, Figure 3H). The broad range of responses to lytic, latent and all HCMV proteins observed were also maintained with age (Figures 3C, 3F and 3I respectively). An analysis of whether increasing age alters the magnitude of the CD8+ T cell IFNγ response to HCMV revealed no impact on the 11 individual proteins (data not shown) or the summed responses to lytic (Figure 3D), latent (Figure 3G) or all (Figure 3J) HCMV proteins examined.

We also examined the CD4+ T cell responses of the donor cohort to the same 11 HCMV proteins in 99 donors. As observed for the CD8+ T cell responses, the majority of the HCMV seropositive donor cohort produced an above threshold IFNγ response to all the lytic expressed proteins but also latency-associated UL144 and US28 (Figure 4B). The responses to the lytic expressed proteins by CD4+ T cells have already been reported in a sub-set of this donor cohort (Jackson et al., 2017), however the observation that both UL144 and US28 proteins induce T cell responses in the majority of HCMV sero-positive donors has not previously been reported. Only 29.6% of the donor cohort examined produced an above threshold CD4+ IFNγ response to UL138, LUNA and vIL-10 latency associated proteins; this is a similar frequency to that seen in the CD8+ T cell compartment and not dissimilar to the percentage of responding donors for UL138 and LUNA CD4+ T cell responses

previously reported in a small scale study (Mason et al., 2013). The ability of individual donors to mount CD4+ IFNγ responses to multiple HCMV proteins is summarized as pie charts (Figure 4B, 4E, 4H). In contrast to the CD8+ T cell IFNγ response routinely seen to 5 or 6 lytic proteins, fewer donors were capable of mounting responses to 5 or 6 of the lytic expressed HCMV proteins (43.9% - blue and deep pink segments, Figure 4B). This trend was maintained in response to the latent proteins (22% responding to 4 or 5 proteins – green and blue segments, Figure 4E) and, overall, only 33% of the donor cohort responded to 8 or more of the examined HCMV proteins (Figure 4H - orange, dark green, teal and purple segments). Despite this lower proportion of HCMV sero-positive donors responding to many HCMV proteins, the overall breadth of the CD4+ IFNγ T cell response remained stable with increasing donor age which shows that there was no significant increase or decrease in the number of proteins an individual responded to within the lytic (Figure 4C) or latent group of proteins (Figure 4F) or to all 11 proteins examined (Figure 4I). Also, we did not observe an effect of donor age on the magnitude of the response to the individual HCMV proteins (data not shown) or to the summed responses to the 6 lytic proteins (Figure 4D), 5 latent proteins (Figure 4G) or to the summed response of all 11 proteins (Figure 4J).

We next examined the ability of CD4+ T cells to produce cIL-10 following stimulation with our 11 candidate HCMV proteins. Cellular IL-10 levels were measured in 73 HCMV donors from the cohort (these donors having passed the quality control thresholds outlined in the methods). Although we have already shown that lytically expressed proteins pp71 and US3 can induce cIL-10 production by CD4+ T cells in a small sub-set of this donor cohort (Jackson et al., 2017), in this larger donor cohort pp71 (38.8%), US3 (32.8%) and pp65 (23.8%) are the most common lytic proteins to trigger an above threshold cIL-10 CD4+ T cell response. The latency associated proteins, US28 (34.3%), LUNA (31.3%) and UL138 (26.8%) also frequently induced a CD4+ specific cIL-10 response in this donor cohort. In contrast to the ability of donors to produce IFNγ T cell responses to multiple HCMV proteins, a positive cIL-10 response to any one of the 11 HCMV proteins examined was absent in 19 of 67 seropositive donors (grey segment – Figure 5H) and no donors produced responses to more than 9 of the 11 HCMV proteins. When examining the response to the 6 lytic proteins, about half of the 67 donors (49.3%) did not produce a cIL-10 response (grey segment – Figure 5B). Despite this more limited breadth of the response, 70% of the donors examined produced an above threshold cIL-10 response to 1 or more HCMV protein. The ability of an individual donor to produce a cIL-10 response to HCMV proteins was not affected by age (Figures 5C, 5F, 5I) and neither was the magnitude of the responses to each of the 11 HCMV proteins (data not shown). The relationship of the total cIL-10 responses, for each donor, to the 6 lytic proteins (Figure 5D), 5 latent proteins (Figure 5G) and all 11 proteins (Figure 5J) was also stable with donor age. Overall, the data presented show that the breadth and magnitude of the IFNγ and cIL-10 HCMV specific T cell responses, within this donor cohort, do not show any impact of either increasing donor age or putative long term carriage of the virus on these HCMV specific T cell responses.

CD4+ T cells specific for LUNA, UL138, pp71, US3 and US28 proteins are more frequently biased towards expression of cIL-10 than IFN γ and this was not affected by donor age.

Using the fluorospot technology, we were able to ask whether CD4+ T cell responses to our candidate HCMV proteins was dominated by either IFNγ or IL-10 secretion or whether it was comprised of cells that secrete both cytokines. Figure 6 shows the relative cytokine composition of the CD4+ T cell response to each of the 11 HCMV proteins examined for donors who generated an above threshold response (> 100 sfu/million) for either cytokine. Overall, we found that IFNγ and cIL-10 are generally produced by distinct populations of CD4+ T cells, as dual secretors were very rare (red bars – Figure 6). The CD4+ T cell responses to UL144 (Figure 6D), gB (Figure 6J), pp65 (Figure 6H), IE1 (Figure 6K) and IE2 (Figure 6I) proteins were dominated by IFNγ secretion. In contrast, the donor cohort responses to the proteins UL138 (Figure 6C), LUNA (Figure 6B), US28 (Figure 6A), vIL-10 (Figure 6E), pp71 (Figure 6F) and US3 (Figure 6G) showed more cIL-10 secretors (white spotted bars). Although there was no significant change in the magnitude of the CD4+ T cell IL-10 response to HCMV proteins with age (summarized Figure 5), we were interested to see if there was a change in the proportion of IFNγ and IL-10 secretion by CD4+ T cells within individuals during long term viral carriage. The data presented in figure 6 are arranged with donor age along the x-axis and does not show any obvious changes in the composition of the positive CD4+ T cell response. Analysis of the proportion of donors in which the majority of the CD4+ T cell responses was secretion of cIL-10 (i.e. greater than 50% of the total CD4+ T cell response of the individual to each HCMV protein) revealed that for LUNA 48.5% of responding donors had a dominant cIL-10 response (Sup. Figure 3A). UL138, pp71, US3 and US28 also elicited a greater than 50% IL-10 response in more than a third of the donor cohort (42.8%, 38.4%, 34% and 33.3% respectively; Sup. Figure 3A). When looking at the breadth of the cIL-10 dominant responses with donor age, there was no significant increase in the breadth of HCMV proteins an individual produced a majority cIL-10 response towards for all proteins (Sup. Figure 3B), lytic proteins (Sup. Figure 3C) or latent associated proteins (Sup. Figure 3D).

The magnitude of latent HCMV DNA load in CD14+ monocytes is not affected by donor age in the ARIA Cohort.

In addition to assessing the effect of increasing age on the T cell response to HCMV lytic and latent expressed proteins, the other principle aim of this study was to determine if there was an age-related effect on latent viral load. Consequently, we screened whole blood of all donors in the study for the presence of HCMV DNA using a quantitative real time PCR assay. No viral DNA was detectable in the 14 HCMV sero-negative donors and of the 105 HCMV sero-positive donors, viral genome was only detected in 1 of these (274 copies/ml whole blood). The donor with detectable HCMV in whole blood also had an inverted CD4:CD8 ratio and above average numbers of differentiated memory CD8+ T cells, data summarized in supplementary Figure 4. During latent HCMV infection, virus is known to reside in CD34+ hematopoietic stem cells and derivative CD14+ monocytes (Reeves et al., 2005). Using a sensitive digital droplet PCR approach (Parry et al., 2016), we quantified the number of copies of HCMV present in isolated CD14+ monocytes from all donors. In

total we assessed 108 HCMV sero-positives and negatives for HCMV DNA present in CD14+ cells; of these, no copies of viral genome were detected in the 14 HCMV sero-negative donors. We did, however, detect HCMV genomes in 43 of 94 (45.7%) of CD14+ monocytes from HCMV sero-positive donors (51 of 94 were below the level of detection of this assay, 1 genome in 60 000 cells); the latent viral load (copies HCMV/million CD14+ cells) for the 94 sero-positive donors, relative to donor age, is summarized in figure 7. Within this ARIA donor cohort, we did not observe a significant relationship between age and the magnitude of the latent viral load.

High Latent viral loads in CD14+ Monocytes were associated with both increased breadth and frequency of IFNγ secreting HCMV specific T cells.

HCMV is latently carried in CD34+ hematopoietic progenitor cells and subsequently in the periphery by monocyte derivatives from these cells (Reeves and Sinclair, 2013). Virus reactivation from these myeloid lineage cells would activate HCMV specific T cells and could drive increased frequencies, as well as potentially seeding more cells in the latent reservoir. Theoretically, increased frequency of latently infected cells could result in increased virus reactivation events, potentially resulting in induction of more T cell stimulation and, possibly, an increase in HCMV specific antibody levels during life-long persistence. Consequently, we assessed whether there was an association between HCMV specific IgG levels and latent viral load, but these measures were unrelated (data not shown). We then assessed whether there was an association between the latent viral load and the CD8+ and CD4+ T cell responses to the individual HCMV proteins as well as to the magnitude and breadth of the total responses of each donor. We did not observe an association between latent load and the cIL-10 CD4+ response and there was only a significant association between the magnitude and breadth of the CD4+ IFNγ response to the subset of 6 lytic proteins and increased latent viral load (data not shown). There was a significant association with the summed total of the CD8+ T cell response to lytic (Figure 8B), latent (Figure 8D) and all proteins (Figure 8F). Also, high viral copy latent load correlated significantly to the breadth of the CD8+ T cell responses to lytic (Figure 8A) and all HCMV proteins (Figure 8E).

Discussion

The aims of this study were to determine whether HCMV specific CD4+ T cells secreting cIL-10 increase with age and long-term viral carriage and to determine whether there are changes in breadth and frequency of the IFNγ secreting T cell response to HCMV infection in healthy older donors. We also wanted to measure the latent viral load of HCMV DNA in a large donor cohort for the first time and assess whether donors aged over 65 years manifested changes in immune cell numbers indicative of immunosenescence. Using an age cross-sectional study methodology, we recruited a donor cohort spanning 6 decades (23 – 78 years) and measured virological and immunological parameters. The donors were recruited by the Cambridge Bioresource from their Biobank of volunteers who live predominantly in areas local to Cambridge and the East Anglian Region of the United Kingdom (UK). Donors were recruited based on HCMV sero-status and by excluding donors suffering from immune altering illnesses or under treatment for these conditions, such that all participants could be safely considered to be generally healthy.

We analyzed the CD4+ and CD8+ T cell compartments in peripheral blood and observed a loss of naïve CD4+ and CD8+ T cell numbers as well as a corresponding loss of total CD4+ and CD8+ T cell numbers with increasing age. The age-related loss of naïve T cells numbers is a well-established phenomenon due to the involution of the thymus and decreased T cell output (Lynch et al., 2009) and has been observed in most studies of ageing populations (Weltevrede et al., 2016). In our study, there was no accumulation of memory T cell populations (measured in absolute numbers) within this cohort, which has also been observed in other studies when using absolute numbers (Chidrawar et al., 2009; Wertheimer et al., 2014). However, when expressed as a percentage of the CD8+ T cell compartment, there was a significant age-related accumulation of differentiated T_{EMRA} (CD27-CD45RA+) and Late stage (CD27-CD28-) memory cell populations as has been previously reported (Weltevrede et al., 2016). It is likely that the increase in percentage (relative frequency) of differentiated memory T cell populations previously reported in aged cohorts, was due to the decrease in the absolute size of the overall CD8+ T cell compartment, which results in an increase in the proportion of memory cells even if the absolute numbers do not increase (Chidrawar et al., 2009; McElhaney and Effros, 2009; Wertheimer et al., 2014).

Previous investigations into the impact of HCMV persistence on immunosenescence in older people have reported a range of immune parameters and HCMV specific markers altering with age. These include the Immune Risk Phenotype (IRP), defined by a collection of markers which, taken together, were suggested to be indicative of increased mortality in the elderly and which included an inversion of the CD4:CD8 ratio, expansion of CD8+ $CD28^{null}$ and CD8+ T_{EMRA} memory T cells and HCMV sero-positivity (Olsson et al., 2001; Wikby et al., 2002; Hadrup et al., 2006; Strindhall et al., 2013). There have also been reports of HCMV specific IgG levels increasing in older donors (McVoy and Adler, 1989; Alonso Arias et al., 2013; Parry et al., 2016) as well as accumulation of HCMV specific T cells with age (summarized in (Weltevrede et al., 2016)). Similarly, it has been suggested that there is an age-related increase in levels of HCMV DNA in blood (Furui et al., 2013), urine (Stowe et al., 2007) and an increase in latent viral genome copy number in CD14+ cells of donors aged over 70 years (Parry et al., 2016). Overall, as our donor cohort exhibited a normal ageing immune

phenotype, we examined the impact of HCMV sero-positivity on T cell memory phenotype within the study group. There were no significant differences in naïve T cell numbers between aged HCMV sero-positive compared to aged HCMV sero-negative donors in our cohort and we only observed an inverted CD4:CD8 ratio in 10% of the sero-positive donor cohort; donors exhibiting this phenotype were distributed throughout the age categories. We did see an increase in the numbers of differentiated T cells in HCMV sero-positive donors of all ages compared to sero-negatives, confirming that our study participants have a similar T cell phenotype to that observed in many previous studies of HCMV infection (Weltevrede et al., 2016). There was, however, no association between increasing donor age and higher levels of HCMV IgG nor was there an increase in the breadth and frequency of the HCMV specific T cell IFNγ response or CD4+ cIL-10 response to the eleven HCMV proteins examined within the study group. We also did not detect increased copies of latent HCMV genome in CD14+ monocytes of our older donors. The separate impact of HCMV infection from ageing on the differentiation of T cells has been observed in other population studies (Lelic et al., 2012; Furman et al., 2015) and the kidney transplant primary infection model and reports from primary infection has shown a rapid acquisition of a more differentiated T cell phenotype in the months following initial infection (Gamadia et al., 2003; Day et al., 2007; Miles et al., 2007; Lilleri et al., 2008). Furthermore, we observed a significant association between high latent viral loads and higher frequency HCMV specific CD8+ T cell responses, which was again irrespective of donor age. These observations alongside the increased numbers of differentiated memory T cells suggest that, within this healthy donor cohort, it is HCMV infection, rather than the age of the donor, which leads to increased differentiation of the T cell population and expansion of HCMV specific T cells.

Work on donor cohorts from different geographical locations have reported different findings from the original Swedish studies which described the IRP (Olsson et al., 2001; Wikby et al., 2002; Hadrup et al., 2006; Strindhall et al., 2013), these have included a lack of "inflation" of HCMV specific T cells with age despite high HCMV sero-prevalence in the aged donor groups (Colonna-Romano et al., 2007) and the association of a naïve T cell phenotype in HCMV sero-positive old people with increased morbidity in Belgium (Adriaensen et al., 2015). HCMV sero-prevalence varies depending on geographical location and socio-economic status (Gandhi and Khanna, 2004; Crough and Khanna, 2009); in the developed world between 30 – 70% of populations are HCMV sero-positive, with acquisition of the virus increasing with age (Cannon et al., 2010). In contrast, in developing countries, sero-prevalence can be higher than 90% with acquisition of the virus commonly occurring in early childhood (Miles et al., 2007; Cannon et al., 2010). Consequently, the disparate observations reported as consequences of HCMV infection in different aged donor cohorts may be a result of geography as well as other biological parameters such as exposure to infectious diseases, vaccination history and the current health of the participants. It has also been shown in other studies of very old cohorts that increased HCMV IgG levels and differentiated CD4+ T cells are associated with elderly individuals in poor health (Vescovini et al., 2010) and there are also a number of studies associating HCMV sero-positivity and higher HCMV IgG titers with poor outcomes from cardiovascular disease (Simanek et al., 2011; Gkrania-Klotsas et al., 2012; Savva et al., 2013; Spyridopoulos et al., 2016). Our view is that, in some cohorts that have been studied, aged donors suffering from e.g. heart disease, cancer or neurodegenerative disorders may not control virus

efficiently leading to increased HCMV IgG levels or HCMV DNAemia and concomitant increased numbers of differentiated memory T cell populations and an inverted CD4:CD8 ratio thereby confounding some studies.

One of our aims was to address the production of cIL-10 by HCMV specific CD4+ T cells within a large donor cohort in order to assess how prevalent the production of this suppressive cytokine is by HCMV antigen specific T cells and whether this response increases in older donors. Evidence from mouse models of MCMV infection have shown that production of cIL-10 can result in reduced viral clearance and a reduction in production of IFNγ by MCMV specific T cells (Jost et al., 2014; Clement et al., 2016). This could provide an explanation for the observation that, despite a functional immune response preventing overt HCMV mediated disease, older donors have detectable HCMV DNA in blood and urine (Stowe et al., 2007; Furui et al., 2013). In some HCMV studies, increases in inducible regulatory CD4+ T cells have been reported in older people with this being associated with vascular pathology in these individuals (Terrazzini et al., 2014). Similarly, it has also been suggested that the HCMV specific CD4+ CD28-CD27- T cell population, reported as expanded in HCMV seropositive older people (Fletcher et al., 2005) contains a T regulatory population characterized by FoxP3 and $CD25^{hi}$ expression (Tovar-Salazar et al., 2010). As already discussed, there was no accumulation of the cIL-10 CD4+ T cell response with increasing donor age in this cohort; we were also interested to see if there was a shift in the bias of the responding CD4+ T cells to individual HCMV proteins from IFNγ to IL-10 or vice-versa. The results confirmed our previous observation that the production of cIL-10 by CD4+ T cells is more likely to be in response to latency associated proteins (Mason et al., 2013); in this cohort almost 50% and 40% of donors produced a majority cIL-10 response to stimulation by the LUNA and UL138 peptide pools respectively regardless of donor age. Similarly, other latency associated proteins included in this study, US28 and vIL-10, also showed a number of donors biased towards cIL-10 production, which is in contrast to the response towards many of the lytically expressed proteins included in this study.

The use of the digital droplet PCR (ddPCR) protocol (Parry et al., 2016) has, enabled better quantification of the levels of latent HCMV genomes in the CD14+ cell compartment. We were able to detect and quantify latent HCMV genomes in 45.7% of examined HCMV sero-positive donors comparing favorably to the 36% detection rate in HCMV positive donors described recently by ddPCR (Parry et al., 2016). Our ability to quantify latent HCMV load in our donor cohort led to a particularly interesting observation with respect to HCMV specific T cell response. As already noted, high copy numbers of latent HCMV detected in CD14+ monocytes significantly correlated with an increase in the breadth and magnitude of the HCMV specific CD8+ T cell response measured by IFNγ secretion. From this result, we hypothesize that higher viral genome copy number was a result of an accumulation of reactivation events over the time, resulting in viral replication and reseeding of the latent CD34+ cellular pool; consequently this production of viral proteins stimulates and activates HCMV specific memory T cell response leading to an increase in frequency of these cells. The virus most likely employs its immune evasion functions to create a window of opportunity to allow reactivation from latency and the production of new virions despite the presence of a primed anti-viral immune response (Wills et al., 2015). In older donors, uncontrolled reactivation of HCMV subsequently causing either disease or other medical complications has not been observed, and

HCMV DNA has not been routinely detected in the blood (Vescovini et al., 2004; Stowe et al., 2007), apart from in a Japanese cohort study, but the DNA positive detection rate was only 4.3% of donors aged 60 – 69 years (Furui et al., 2013). However, there is evidence that older people may not control virus replication as adequately as the young, as HCMV DNA has been detected in other bodily fluids in the old (Stowe et al., 2007). Within this study, our exclusion criteria may have precluded recruitment of donors who had less effective control of virus replication resulting in low level virus dissemination. In support of this conclusion, it is interesting to note that a single aged male donor with detectable HCMV DNA in whole blood did have an inverted CD4:CD8 ratio as well as an above average number of highly differentiated memory CD8+ T cell populations; they also had limited HCMV specific T cell responses to our 11 candidate HCMV proteins (Supplementary Figure 4).

We have demonstrated that, in an East Anglian-based donor cohort which has a typical healthy ageing profile, older HCMV sero-positive donors do not exhibit the hallmark features of the IRP, differences in the breadth and magnitude of their HCMV specific IFNγ production; or that latent viral load was affected by age. Importantly, though we did see a significant relationship between high latent viral load and increased breadth and magnitude of the functional HCMV specific CD8+ T cell responses, latent viral load did not correlate with increased numbers of differentiated memory T cell populations or HCMV specific IgG. This, we believe, reflects the importance of including measurement of viral load in studies on the impact of HCMV infection in older donors as opposed to inferring the impact of the virus from measuring a variety of other immune parameters as has previously occurred. In a previous study in a Birmingham based old aged cohort, the authors observed an increase in HCMV specific T cell responses alongside, an increase in latent viral carriage in donors aged over 70 years (Parry et al., 2016). Whilst the authors do not present data correlating latent viral load with the frequency of HCMV specific T cells, we think it possible in light of our findings, that in this older cohort study, the increase in HCMV specific T cell responses in older donors could be associated with increased latent viral carriage.

Detection of low level HCMV viremia in the blood of the old would be a strong indicator of a diminution of immune control, however the results from our study group and others (Vescovini et al., 2004; Stowe et al., 2007) suggests this is rarely observed, probably because it would represent a significant loss of control. However, the presence of virus in other bodily fluids e.g. saliva or urine could also indicate loss of immune control. It should be considered that chronic low level persistent HCMV replication and an associated inflammatory environment could be important in particular old patients groups; there is epidemiological evidence that HCMV comorbidity plays a role in exacerbating cardiovascular disease (Simanek et al., 2011; Gkrania-Klotsas et al., 2012; Savva et al., 2013; Spyridopoulos et al., 2016) and also with increasing impaired physical function and ill health (Vescovini et al., 2010; Haeseker et al., 2013; Adriaensen et al., 2015; Broadley et al., 2017). Future investigations into the impact of HCMV infection in older people should also monitor latent viral carriage of the virus alongside measuring whether low level viremia is present in the blood and other bodily fluids, e.g. urine or saliva; in order to improve our understanding of the impact of HCMV infection in the elderly.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

SEJ, MRW, ELP and JHS designed the project and experiments. SEJ, GXS, GO and ELP carried out the experiments. SEJ and MRW wrote the manuscript. SEJ carried out statistical analysis and prepared figures. SEJ and MRW submitted this paper. All authors reviewed the manuscript.

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940 **Tables**

		All Ages		Young (<40 years)	Middle (41-64 years)	Old (>65 years)
		HCMV +ve	HCMV -ve	HCMV +ve	HCMV +ve	HCMV +ve
Donors (n)	All	105	14	33	31	41
	M	44	5	14	14	16
	F	61	9	19	17	25
Age (Years) (Mean ± S.D.)	All	54.4 ± 15.6	51.4 ± 14.4	34.6 ± 5.1	54.5 ± 6.0	70.2 ± 3.1
	M	54.3 ± 16.1	46.2 ± 12.7	34.7 ± 5.5	53.9 ± 6.5	71.7 ± 2.8
	F	54.5 ± 15.3	54.3 ± 14.4	34.6 ± 4.8	54.9 ± 5.5	69.3 ± 2.8
HCMV IgG (ISR) (Mean ± S.D.)	All	3.78 ± 1.28	0.28 ± 0.14	3.66 ± 1.32	3.81 ± 0.99	3.85 ± 1.42
	M	3.67 ± 0.95	0.25 ± 0.10	3.15 ± 0.74	4.06 ± 0.74	3.78 ± 1.06
	F	3.86 ± 1.47	0.29 ± 0.15	4.03 ± 1.51	3.60 ± 1.12	3.90 ± 1.61
HCMV DNAemia (copies/ml blood) (Mean ± S.D.)	All	2.6 ± 26.7*	undetected	undetected	undetected	6.7 ± 42.4*
	M	6.3 ± 41.0*	undetected	undetected	undetected	17.2 ± 66.6*
	F	undetected	undetected	undetected	undetected	undetected
CD4:8 Ratio (Mean ± S.D.)	All	2.25 ± 1.61	3.60 ± 1.80	2.04 ± 0.85	1.96 ± 0.85	2.63 ± 2.29
	M	2.10 ± 1.00	4.00 ± 2.10	1.80 ± 0.70	2.00 ± 1.00	2.30 ± 1.20
	F	2.40 ± 1.90	3.40 ± 1.50	2.20 ± 0.90	1.90 ± 0.70	2.90 ± 2.80

941 * HCMV DNAemia detected in n=1 old male donor

942

Figure Legends

Table 1 – ARIA Cohort Donor Characteristics

Summary of the number of donors and age ranges, serum HCMV IgG levels (Immune Status Ratio – ISR), blood HCMV DNA copies and the CD4:CD8 ratio (generated from absolute count data).

Figure 1 – Impact of Ageing on T cell numbers

EDTA treated whole blood was stained with a panel of phenotyping antibodies in order to enumerate CD4+ and CD8+ T cells and their subsets. Representative dot plots from a young and old donor showing CD4+ and CD8+ T cell gates, naïve T cells subset were defined by CD27+ and CD45RA+ (T_{NAIVE}) expression and CD4+ T regulatory cells (T_{REG} – $CD25^{hi}$, $CD127^{lo}$); the number of cells/µl of whole blood present for each gated population of interest are also indicated (A). Graphs illustrating the numbers of total CD8+ T cells (B), T_{NAIVE} CD8+ T cells (C), total CD4+ T cells (D), T_{NAIVE} CD4+ T cells and CD4+ T_{REG} cells of the entire ARIA cohort (n=119) correlated to donor age. The relationship of T cell subset numbers with donor age was analyzed using Spearman rank correlation with the results indicated on each graph (r_s (95% Confidence Interval) and p value). There was a significant decrease in total and T_{NAIVE} CD4+ and CD8+ T cells with age, CD4+ T_{REG} numbers showed no significant difference.

Figure 2 – Impact of HCMV carriage on T cell numbers

EDTA treated whole blood was stained with a panel of phenotyping antibodies in order to enumerate CD4+ and CD8+ T cells and their subsets. Representative dot plots from a HCMV sero-positive (HCMV+ve) and HCMV sero-negative (HCMV-ve) age-matched donors are illustrated showing the memory (as defined by CD27 and CD45RA expression) and differentiation level (as defined by CD27 and CD28) phenotype of both CD4+ and CD8+ T cells; the number of cells/µl of whole blood for effector memory (T_{EM} – CD27-CD45RA-) CD4+ and CD8+ T cells and Intermediate (INT – CD27-CD28+) and Late (LATE – CD27-CD28-) differentiated CD8+ T cells are shown (A). Box and whisker plots comparing cell numbers of the memory (B, D) and differentiation phenotypes (C, E) of CD8+ T cells and CD4+ T cells respectively between HCMV+ve (red) and HCMV-ve (green) donors are shown. The differences between the two groups were analyzed by a Kruskall-Wallis one-way ANOVA test with post-hoc Mann Whitney U test performed with significant results set as $p \leq 0.015$ shown on each graph. A representative CD4 vs CD8 dot plot from the same donors with their respective CD4:CD8 ratio indicated are shown (F), the comparison of CD4:CD8 ratios for all sero-positive vs sero-negative donors are also shown (G) with the significant decrease in the CD4:CD8 ratio in HCMV positive donors indicated (Mann Whitney test).

Figure 3 – Magnitude and Breadth of CD8+ T cell IFNγ response to HCMV proteins.

The IFNγ secreting CD8+ T cell response to 6 HCMV proteins only expressed during lytic infection: pp65, IE2, pp71, IE1, gB, US3 and 5 HCMV latency associated proteins: UL144, US28, vIL-10, LUNA and UL138 were measured in a cohort of 91 HCMV sero-positive and 7 sero-negative donors. The production of IFNγ was measured using an IFNγ Fluorospot detection method; with the results

converted to spot forming units/million cells (sfu/million) with background counts subtracted. The response to the lytic expressed proteins (red), latency associated (blue) and the positive control by all 98 donors are summarized (A) with HCMV sero-positive donors (dark) and HCMV sero-negative donors (light) both illustrated. The positive response threshold cut-off of 100 sfu/million is shown (dashed line) and the proportion of donors with a positive response to each HCMV protein is indicated. The proportion of the 91 sero-positive donors producing a positive response to 1 or more of the 6 Lytic expressed proteins (B), 5 latency associated proteins (E) or all 11 HCMV proteins (H) are summarized as pie charts with the key to segments for each graph shown. Graphs illustrating the breadth of HCMV sero-positive donors response to HCMV proteins correlated with age are illustrated for lytic expressed (C), latency associated (F) and all 11 proteins (I); also shown is the summed IFNγ response to lytic (D), latent (G) and all proteins (J) correlated with age. Spearman rank correlation (Spearman r_s (95% Confidence Intervals (CI)) and p values) results are indicated on each graph.

Figure 4 – Magnitude and Breadth of CD4+ T cell IFNγ response to HCMV Proteins.

The IFNγ secreting CD4+ T cell response to 6 HCMV proteins only expressed during lytic infection: pp65, IE2, pp71, IE1, gB, US3 (red) and 5 HCMV latency associated proteins: UL144, US28, vIL-10, LUNA and UL138 (blue) were measured in a cohort of 91 HCMV sero-positive and 8 sero-negative donors. The production of IFNγ was measured using an IFNγ Fluorospot method; with the results converted to spot forming units/million cells (sfu/million) with background counts then subtracted. The response to the HCMV proteins and the positive control by all 99 donors are summarized (A) with HCMV sero-positive donors (dark) and HCMV sero-negative donors (light) both illustrated. The positive response threshold cut-off of 100 sfu/million (dashed line) and the proportion of donors with an above threshold response to each HCMV protein is indicated. The proportion of the 91 sero-positive donors producing a positive IFNγ response to 1 or more of the 6 Lytic expressed proteins (B), 5 latency associated proteins (E) or all 11 HCMV proteins (H) are summarized as pie charts with the key to segment color for each graph shown. Graphs illustrating the breadth of HCMV sero-positive donors IFNγ response to HCMV proteins correlated with age are illustrated for lytic expressed (C), latency associated (F) and all 11 proteins (I); also shown is the summed IFNγ response to lytic (D), latent (G) and all proteins (J) correlated with age. Spearman rank correlation (Spearman r_s (95% Confidence Intervals (CI)) and p values) results are indicated on each graph.

Figure 5 – Magnitude and breadth of CD4+ T cell IL-10 response to HCMV Proteins

The IL-10 secreting CD4+ T cell response to 6 HCMV proteins only expressed during lytic infection: pp65, IE2, pp71, IE1, gB, US3 (red) and 5 HCMV latency associated proteins: UL144, US28, vIL-10, LUNA and UL138 (blue) were measured in a cohort of 67 HCMV sero-positive and 6 sero-negative donors. The production of IL-10 was measured using an IL-10 Fluorospot method; with the results converted to spot forming units/million cells (sfu/million) with background counts then subtracted. The response to the HCMV proteins and the positive control by all 73 donors are summarized (A) with HCMV sero-positive donors (dark) and HCMV sero-negative donors (light) both illustrated. The positive response threshold cut-off of 100 sfu/million (dashed line) and the

proportion of donors responding to each HCMV protein is indicated. The proportion of the 67 sero-positive donors producing a positive IL-10 response to 1 or more of the 6 Lytic expressed proteins (B), 5 latency associated proteins (E) or all 11 HCMV proteins (H) are summarized as pie charts with the key to segment color for each graph shown. Graphs illustrating the breadth of HCMV sero-positive donors IL-10 response to HCMV proteins correlated with age are illustrated for lytic expressed (C), latency associated (F) and all 11 proteins (I); also shown is the summed IL-10 response to lytic (D), latent (G) and all proteins (J) correlated with age. Spearman rank correlation (Spearman r_s (95% Confidence Intervals (CI)) and p values) results are indicated on each graph.

Figure 6 – CD4+ T cell Donor responses to HCMV LUNA, UL138, pp71, US3 and US28 proteins were more frequently IL-10 biased.

The frequency of CD4+ T cells that secrete IFNγ or IL-10 or both in response to stimulation by HCMV proteins was measured simultaneously using a dual IFNγ/IL-10 Fluorospot assay. 67 HCMV sero-positive donors were analyzed, only donors with above threshold responses for either IFNγ or IL-10 (100 sfu/million) to each protein are shown. The IFNγ (dark grey), IL-10 (white spotted) and dual cytokine (red) responses of the donor cohort to US28 (A), LUNA (B), UL138 (C), UL144 (D), vIL-10 (E), pp71 (F) US3 (G), pp65 (H), IE2 (I), gB (J) and IE1 (K) are shown as a percentage of the total CD4+ T cell (IFNγ + IL-10) response of each donor, the donors are arranged along the x-axis in increasing age order. The Lytic expressed proteins axis label is in red (graphs F – K) and the latency associated protein responses are labelled in blue (graphs A – E).

Figure 7 – There was no effect of donor age on the magnitude of latent HCMV load in CD14+ Monocytes.

The DNA of purified CD14+ Monocytes was extracted and HCMV viral load detected using droplet digital PCR analysis. No HCMV was detected in 14 HCMV sero-negative donors tested. The HCMV viral load (copies/10^6 CD14+ cells) results from 94 HCMV sero-positive donors are shown correlated with donor age. Spearman rank correlation (Spearman r_s (95% Confidence Intervals (CI)) and p values) analysis is indicated on the graph.

Figure 8 – High levels of latent HCMV load in CD14+ monocytes correlates with increased frequency and breadth of HCMV specific IFNγ CD8+ T cell responses.

The HCMV viral load (copies/10^6 CD14+ cells) from 83 HCMV sero-positive donors was correlated with CD8+ HCMV specific T cell responses. Graphs illustrating the breadth (positive response) of individual donors CD8+ IFNγ response to the 6 lytic expressed (red) (A), 5 latency associated (blue) (C) and all 11 HCMV proteins (purple) (E) correlated with CD14+ cells HCMV viral load are shown. The magnitude of the CD8+ IFNγ response summed for all protein groups is correlated with HCMV viral load for lytic (red) (B), latent (blue) (D) and all proteins (purple) (F). Spearman rank correlation (Spearman r_s (95% Confidence Intervals (CI)) and p values) results are indicated on each graph.

Figure 1.TIFF

Figure 1 – Impact of Ageing on T cell numbers

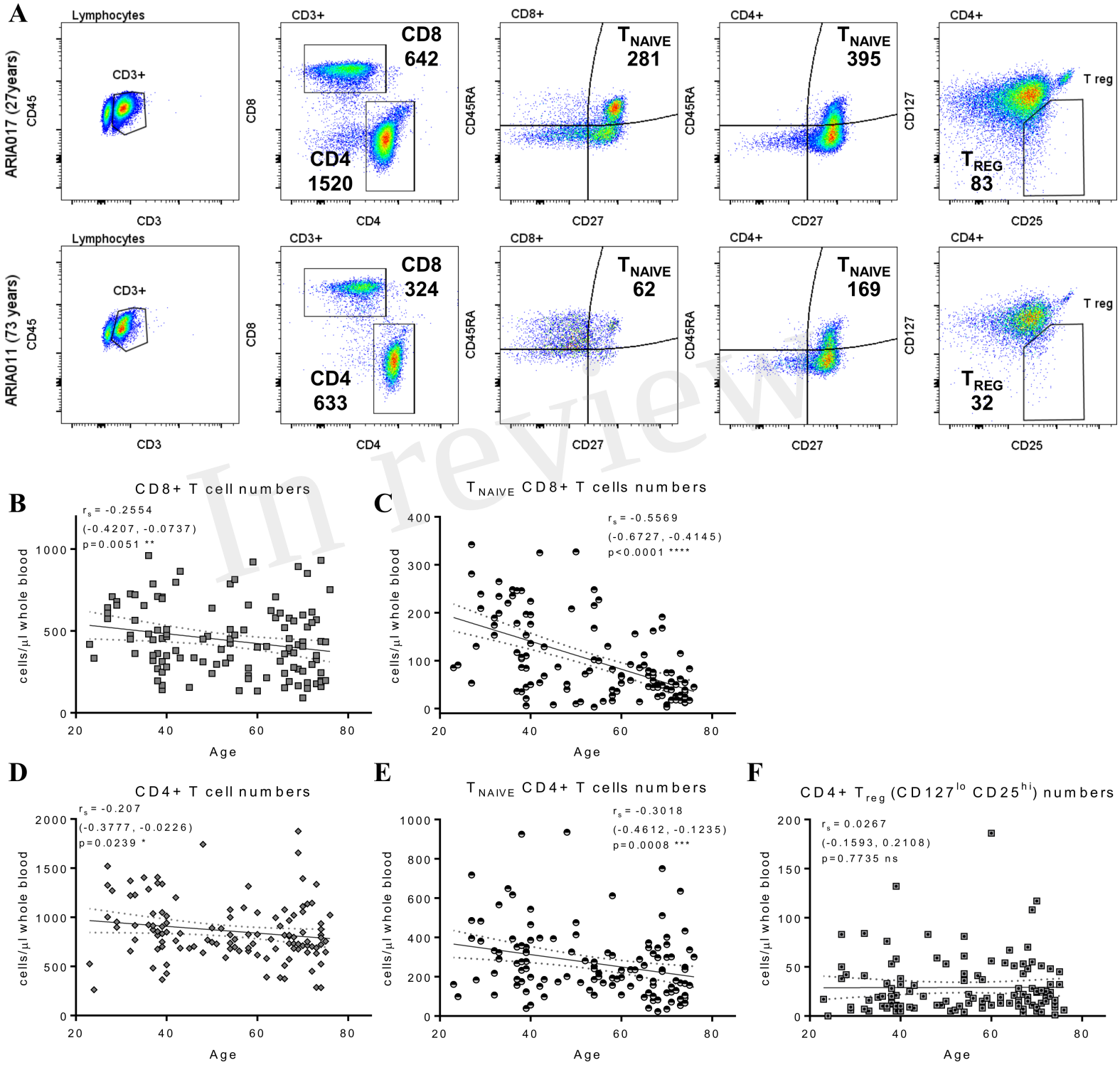

Figure 2 – Impact of CMV infection on T cell numbers

Figure 2.TIFF

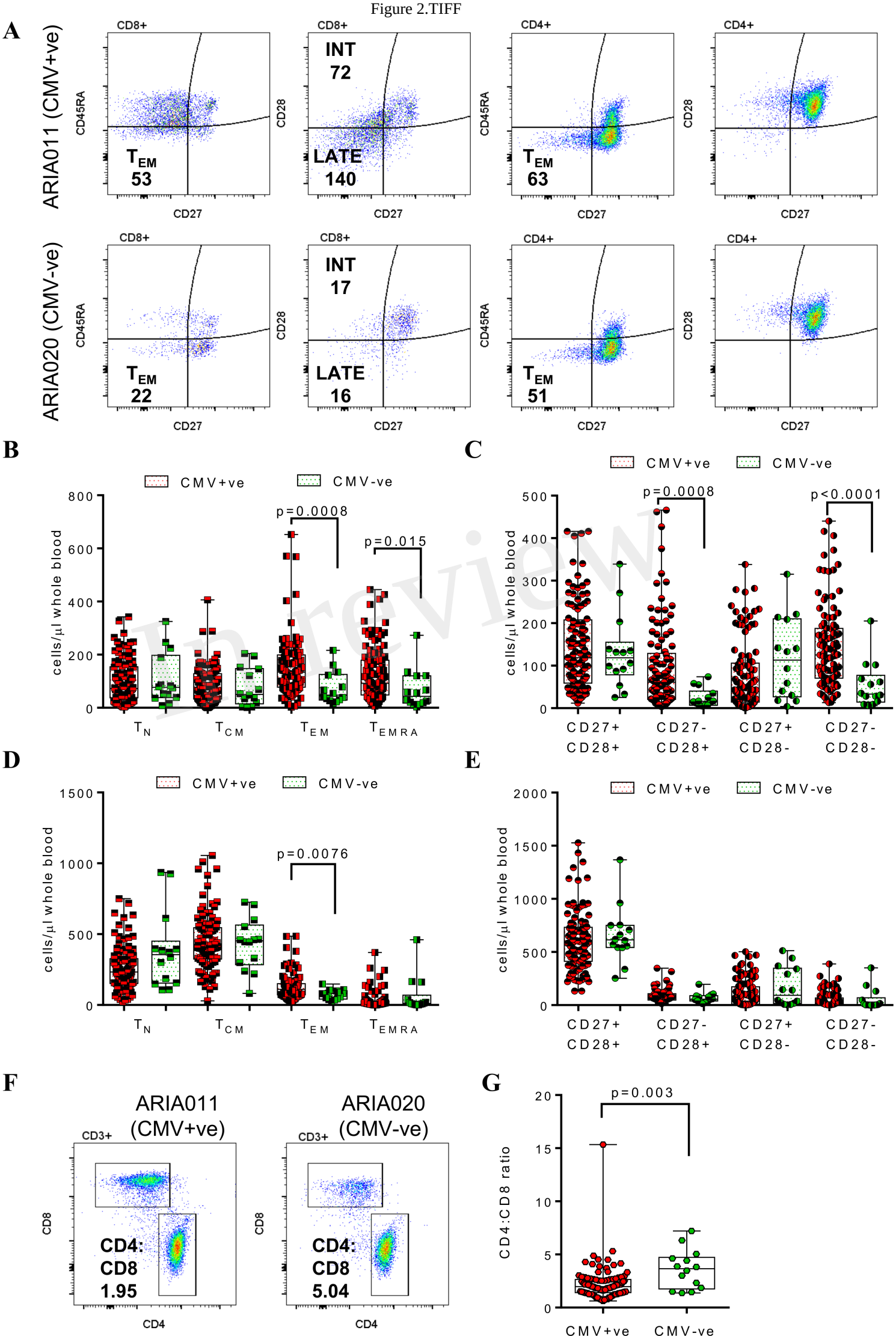

Figure 3.TIFF

Figure 3 – Magnitude and Breadth of CD8+ T cell IFNγ response to HCMV Proteins

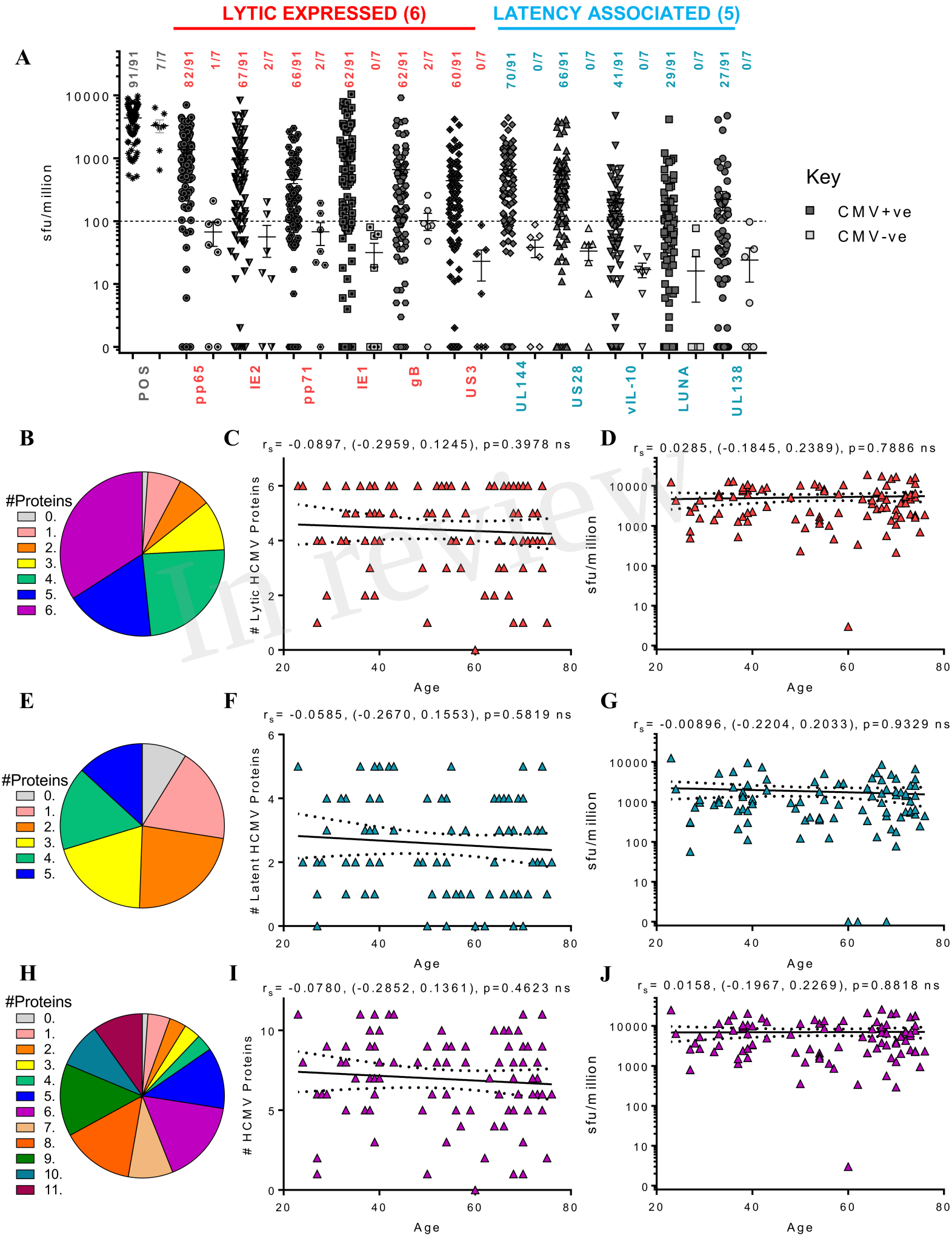

Figure 4.TIFF

Figure 4 – Magnitude and Breadth of CD4+ T cell IFNγ response to HCMV Proteins

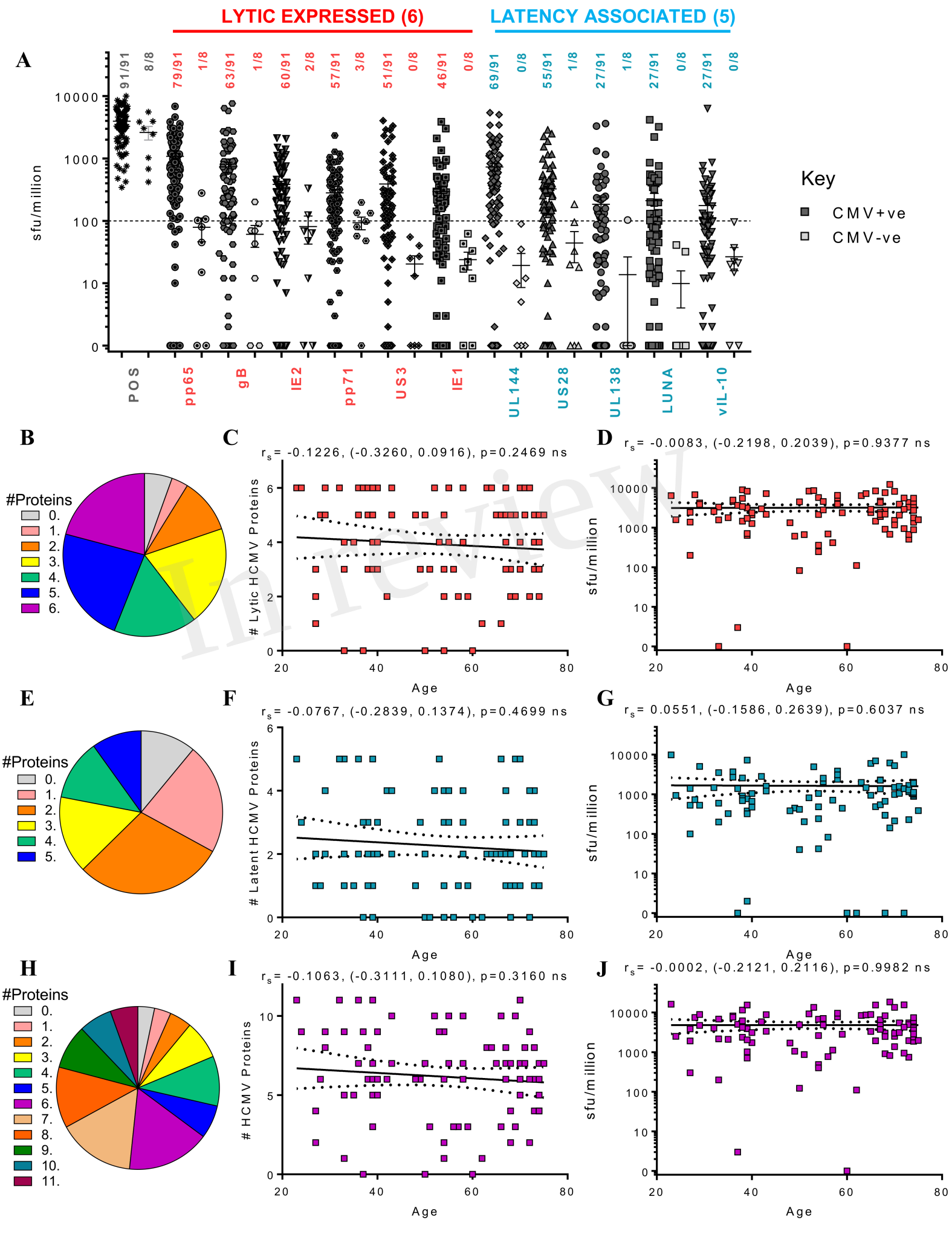

Figure 5.TIFF

Figure 5 – Magnitude and Breadth of CD4+ T cell IL-10 response to HCMV Proteins

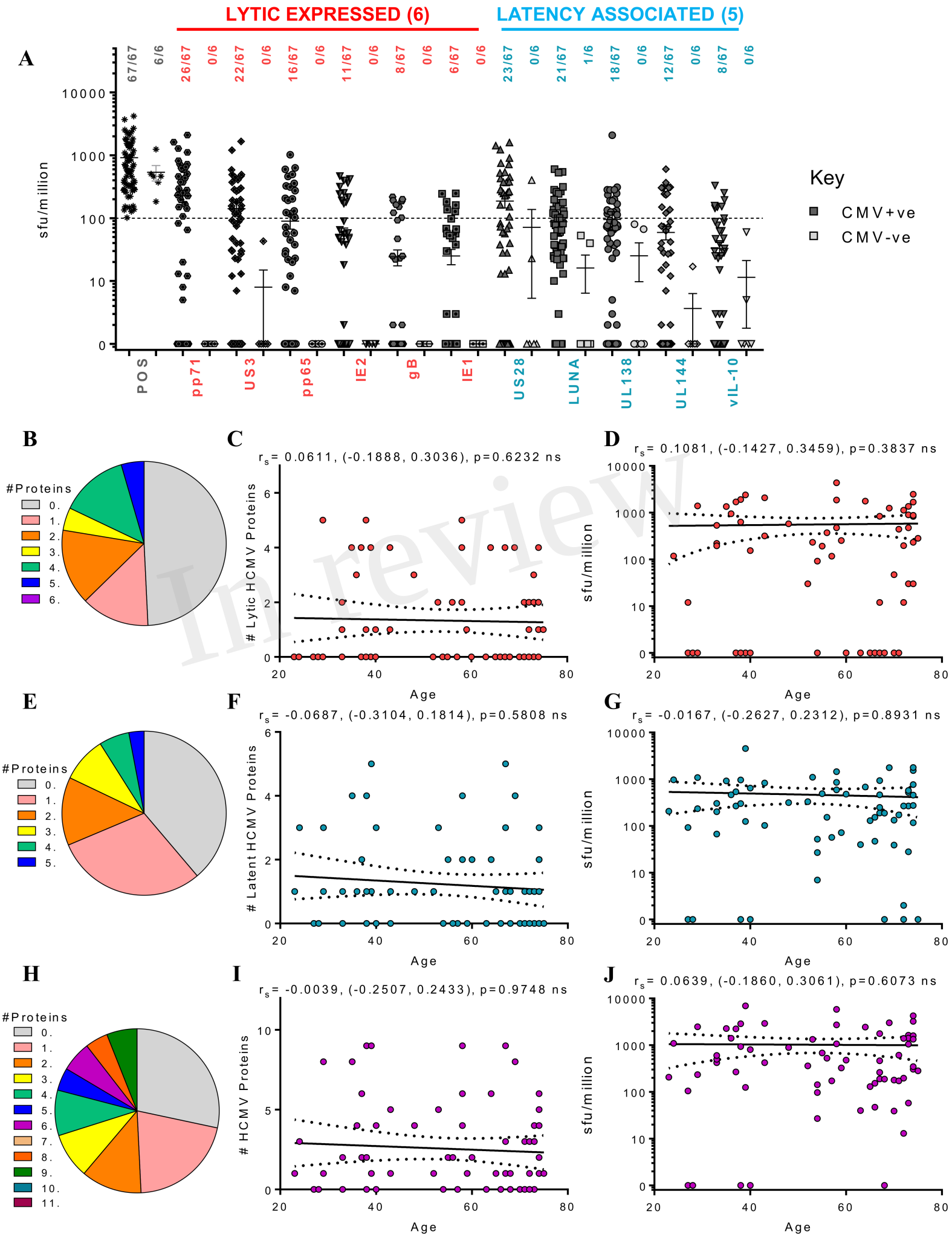

Figure 6.TIFF

Figure 6 – CD4+ T cell Donor responses to HCMV LUNA, UL138, pp71, US3 and US28 proteins more frequently express IL-10 instead of IFNγ.

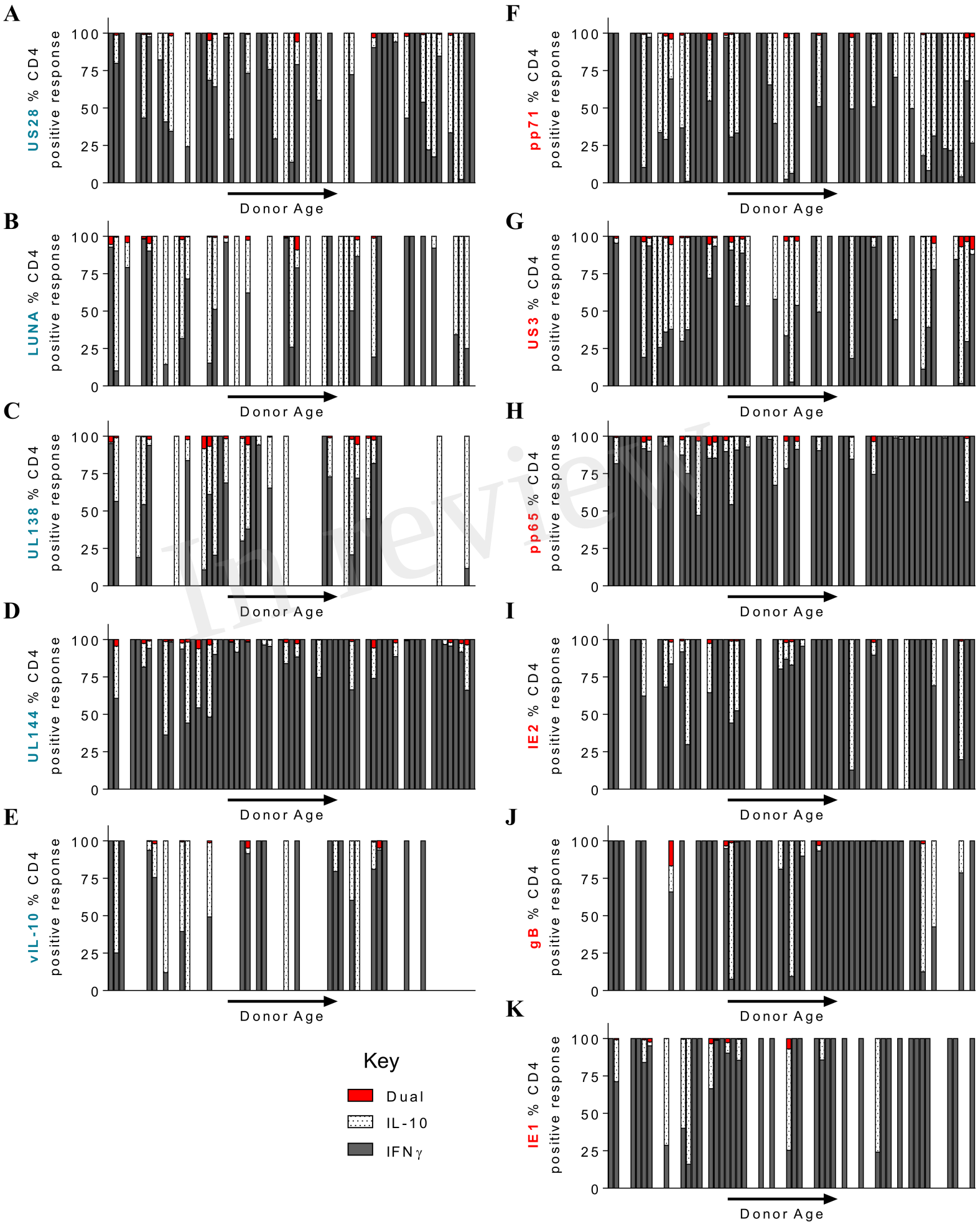

Figure 7.TIFF

Figure 7 – There is no impact of donor age on the carriage of latent CMV in CD14+ Monocytes

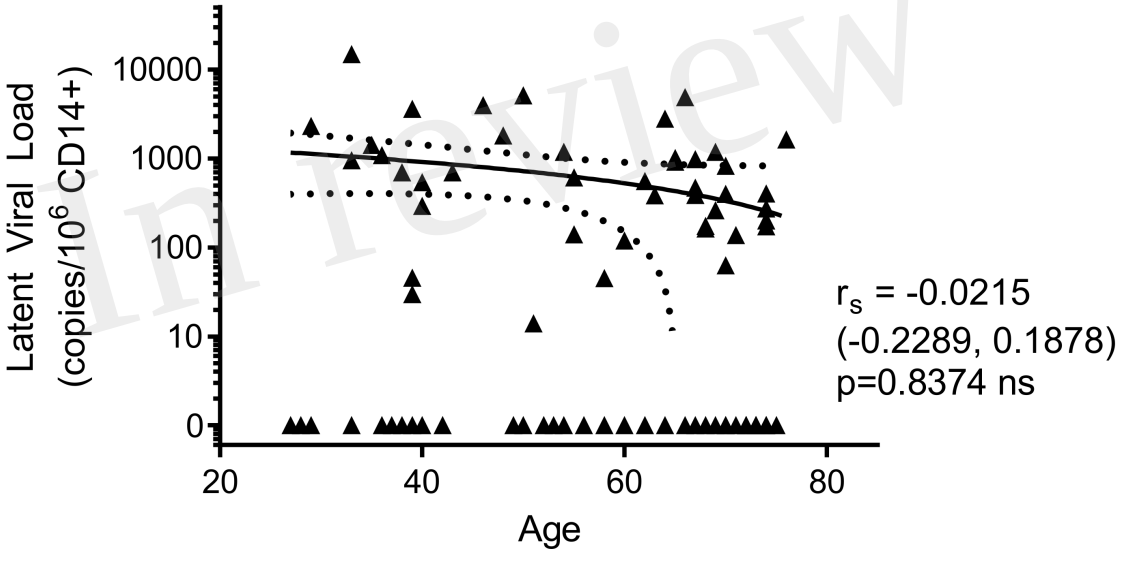

Figure 8 – High levels of latent CMV in CD14+ monocytes results in increased frequency and breadth of CMV specific IFNγ CD8+ T cell responses