HCMV Specific CD4+ T Cells are poly-functional and can respond to HCMV Infected Dendritic Cells *in vitro.*

HCMV specific CD4+ T cell Responses (36 max: 54 characters)

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Abstract (limit 250 words)

Human cytomegalovirus (HCMV) infection and periodic re-activation is generally well controlled by the HCMV-specific T cell response in healthy people. While the CD8+ T cell response to HCMV has been extensively studied, the HCMV-specific CD4+ T cell effector response is not as well understood, especially in the context of direct interactions with HCMV infected cells. We screened the IFNγ and IL-10 response to 6 HCMV peptide pools (selected as the most frequently responded to in our previous studies: pp65, pp71, IE1, IE2, gB and US3) in 84 donors, aged 23 – 74 years. Predominantly the HCMV specific CD4+ T cell response to pp65, IE1, IE2 and gB was Th1 biased with neither loss nor accumulation of these responses with increasing age. A larger proportion of donors produced an IL-10 response to pp71 and US3 but the IFNγ response was still dominant. CD4+ T cells specific to the HCMV proteins studied were predominantly effector memory cells and produced both cytotoxic (CD107a expression) and cytokine (MIP1β secretion) effector responses. Importantly, when we measured the CD4+ T cell response to CMV infected Dendritic Cells *in vitro*, we observed that the CD4+ T cells produced a range of cytotoxic and secretory effector functions, despite the presence of CMV encoded immune evasion molecules. CD4+ T cell responses to HCMV infected dendritic cells were sufficient to control the dissemination of virus in an *in vitro* assay. Together the results show that HCMV-specific CD4+ T cell responses are highly functional even from elderly individuals and are directly anti-viral.

Importance (limit 150 words) *(non-technical explanation of significance)*

Human cytomegalovirus (HCMV) infection is carried for a lifetime and in healthy people is kept under control by the immune system. HCMV has evolved many mechanisms to evade the immune response, possibly explaining why the virus is never eliminated during the hosts' lifetime. Dysfunction of immune cells associated with long-term carriage of HCMV has been linked with poor responses to new pathogens and vaccines when older. In this study we have investigated the response of a subset of immune cells (CD4+ T cell) to HCMV proteins in healthy donors of all ages demonstrating that the functionality of the CD4+ T cells is maintained. We have also shown that CD4+ T cells produce effector functions in response to HCMV infected cells and can prevent virus spread. Our work demonstrates that these HCMV-specific immune cells retain many important functions and help to prevent deleterious HCMV disease in healthy older people.

Introduction

Human cytomegalovirus (HCMV), a β herpes virus, is a ubiquitous pathogen found worldwide (1). Infection with this virus is characterised by the establishment of life-long persistence, in part, because HCMV can establish a latent infection in bone marrow stem cells and cells of the myeloid lineage (2). Infection with HCMV is asymptomatic for most individuals, however when the immune system is compromised by other infections or treatments (such as HIV/AIDS or transplant patients) or is immature (such as the foetus in utero), it can cause significant morbidity and mortality (3, 4). During primary infection with HCMV both the innate and adaptive branches of the immune system respond (1, 3, 4) and evidence from mouse studies has shown the important role CD4+ T cells play in controlling CMV infection (reviewed in(5)). Studies in humans undergoing bone marrow, stem cell and solid organ transplantations have confirmed the role CMV specific CD4+ T cells have in abrogating reactivating infection (6-9) and studies in primary infection in adults have also clearly shown the requirement for functional CD4+ T cells in the resolution of symptomatic disease (10-12). In healthy subjects, persistent shedding of the virus into urine and saliva is associated with a lack of CD4+ T cell response directed towards CMV, which is particularly observed in CMV infection in young children (13).

Identification of HCMV specific CD4+ T cells has mainly been by intracellular cytokine production, predominantly measuring IFNγ production in response to stimulation, these studies have shown large responses to both pp65 and IE proteins of the virus (work

summarised in (3, 4, 14)). CD4+ T cell responses specific to the gB protein (UL55) (15, 16) have also been described. Analysis of the CD4+ T cell response to the whole HCMV proteome also identified numerous responses towards many different ORFs, this study suggested that an individual donor, has on average, CD4+ T cells specific to 12 different HCMV ORFs (17). A meta-analysis of published studies has identified the 10 most frequently recognised HCMV ORFs by CD4+ T cells these were TRL14, UL16, UL55, UL83, UL85, US3, UL25, US18, UL45 and UL32 (3). Many more studies have investigated the frequency, phenotype and function of CD4+ T cells specific to HCMV using whole viral lysate stimulation (10, 12, 18-22), these have estimated that up to 5% (19) of the CD4+ T cell peripheral blood compartment can be directed towards the virus. This dominance of the CD4+ T cell compartment has also been observed as high as 10% when the whole virus proteome was used (17). Additionally, the application of MHC Class II tetramers has also observed as high as 5% of the CD4+ T cell pool responding to one HCMV gB protein Class II epitope (23).

CMV specific CD4+ T cells, mainly identified by IFNγ secretion following stimulation with whole CMV viral lysate, have been shown to be enriched for phenotypes linked to terminal differentiation and dysfunctional responses characterised by CD45RA re-expression (11, 21, 24) and the loss of expression of the co-stimulatory molecules CD28 and CD27 (10, 20, 22, 25, 26) and these cells have also been associated with a loss of cytokine secretion ability and limited proliferation capacity (19, 22, 27). These previous studies have led to the hypothesis that enlarged “dysfunctional” HCMV specific CD4+ T cell populations accumulate with age and that these HCMV induced changes

105 may become detrimental to individuals (27-29). However, it is noteworthy that in other
106 studies HCMV specific CD4+ T cells have also been shown to produce a range of anti-
107 viral effector functions including production of multiple anti-viral cytokines and to have
108 cytolytic effector functions (30-32), functional CMV specific CD4+ T cells were also
109 confirmed in the Rhesus Macaque ageing model (33) and murine ageing models
110 looking at latent MCMV infection (34, 35). The poly-functional capacity of CMV specific
111 CD4+ T cells were also maintained in the more differentiated memory phenotypes seen
112 in older CMV sero-positive donors (20, 21, 26, 30, 36). A number of longitudinal
113 studies have linked HCMV sero-positivity and the associated changes to the T cell
114 repertoire with older individuals being more susceptible to infections, responding poorly
115 to vaccinations and increased risk of mortality compared to age matched HCMV sero-
116 negative individuals (systematically reviewed in (37)). However, a poor response to
117 influenza vaccination in CMV sero-positive older people is not seen in all studies, and
118 there is evidence that certainly in the young being CMV positive can be beneficial in
119 mediating responses to vaccination (34). There is a body of evidence that over the age
120 of 65 there are changes to the immune response that increase morbidity and mortality in
121 responses to infection and autoimmune disease (38, 39). Analysis of a number of large
122 population cohorts recruited for cancer, dementia and nutritional studies in the UK and
123 USA have shown a significant association between CMV sero-positivity and mortality
124 from cardiovascular related disease (40-43). Despite these observations older CMV
125 sero-positive individuals do not appear to suffer from overt HCMV disease from
126 reactivating virus or super infection, suggesting that the HCMV specific T cells retain the
127 ability to control the virus (44). There is also evidence that does not support the role of

CMV sero-positivity in causing a decline in immune responses to novel infections in the elderly (45, 46).

Many studies investigating the functionality of CD4+ T cell responses to HCMV infection have relied on using whole viral lysate or focusing only on the seemingly immune-dominant pp65 or gB viral proteins as stimuli. However, few studies have interrogated the different contributions of the many HCMV proteins that CD4+ T cells respond to (17), to the functional activity of the CMV specific CD4+ T cell response. Previously, we have shown that CD4+T cell responses to the limited number of proteins expressed during HCMV latency produce the immunosuppressive cytokine IL-10 which differed from the CD4+ T cell response to HCMV proteins only expressed during lytic infection (47). Using only peptide pools or viral lysate also ignores the impact of the large number of immune evasion molecules encoded by the virus during active lytic infection on the immune response and the effector functions of CD4+ T cells. We have measured the effect of donor age on CD4+ T cell responses to 6 HCMV ORF encoded proteins (UL83 (pp65), UL82 (pp71), UL123 (IE1), UL122 (IE2), UL55 (gB) and US3), measuring IFNγ and IL-10 responses by Fluorospot. We did not observe an accumulation of CD4+ T cell IFNγ responses to the 6 HCMV proteins with increasing donor age and there were limited IL-10 responses to pp65, gB, IE1 and IE2 stimulation within this donor cohort. The IL-10 response to pp71 and US3 stimulation was more frequently observed however the magnitude of the response was maintained regardless of donor age. CD4+ T cells responding to the 6 HCMV proteins examined had both cytotoxic and inflammatory effector functions and were mostly effector memory T cells,

with pp65 specific CD4+ T cells exhibiting a more differentiated phenotype than the other HCMV specific CD4+ T cells. We next assessed the effector capacity of CD4+ T cells when stimulated by HCMV infected moDCs, CMV specific CD4+ T cells isolated directly *ex-vivo* produced both cytotoxic and secretory effector functions. Using an *in vitro* model of lytic CMV infection, where moDCs were infected with CMV for 7 days prior to co-incubation with CD4+ T cells, we demonstrated that CMV specific CD4+ T cells are able to prevent viral dissemination. This study shows that healthy people of all ages can maintain highly functional HCMV-specific CD4+ T cell responses that can respond to HCMV infected cells.

Materials and Methods

Ethics and Donor Cohort information

Healthy CMV sero-positive and negative donors were recruited locally with ethical approval from the Addenbrookes National Health Service Hospital Trust institutional review board (Cambridge Research Ethics Committee); informed written consent was obtained from all volunteers in accordance with the Declaration of Helsinki (LREC 97/092). 22 HCMV sero-positive donors (5/17 Female/Male) aged 23 – 77 years were recruited. A second healthy donor cohort was recruited from the National Institute of Health Research (NIHR) Cambridge BioResource. 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). A cohort of 84 HCMV sero-positive donors (48/36 Female/Male) aged 23 – 74 years and 24 sero-negative donors (14/10 Female/Male) aged 29 – 78 years were included in this study. CMV sero-status of all donors was confirmed by serological assessment of CMV IgG levels using Captia CMV IgG EIA test (Trinity Biotech, Ireland) following the manufacturer's instructions.

Peripheral Blood Mononuclear cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated from heparinised blood samples using Lymphoprep (Axis-shield, Alere Ltd, Stockport, UK) density gradient centrifugation. PBMC were either used fresh or frozen in a 10% DMSO (Sigma Aldrich, Poole, UK) and 90% Fetal Bovine Serum (FBS) (Gibco – Thermofisher Scientific,

Paisley, UK) solution at a high cell concentration. Frozen PBMC were resuscitated before use in pre-warmed serum-free media in the presence of 10U/ml DNase I (Roche Diagnostics Ltd, Burgess Hill, UK) or Benzonase nuclease (Millipore, Watford, UK), followed by 1 hour incubation in warmed serum-free media and DNase I or Benzonase nuclease at 37°C then further rested in X-VIVO 15 media (Lonza, Slough, UK) at 37°C before use in subsequent assays.

HCMV ORF peptide mixes

10 HCMV ORFs (UL28, UL48, UL55 (gB), UL82 (pp71), UL99, UL122 (IE2), UL123 (IE1), US3, US29 and US32) were selected and consecutive 15mer peptides overlapping by 10 amino acid libraries were synthesised by ProImmune PEPScreen (Oxford, UK) from sequences detailed in the Sylwester *et. al*. study (17). A UL83 (pp65) ORF 15mer peptide library was synthesised by JPT Peptide Technologies GmbH (Berlin, Germany). The individual lyophilised peptides from each ORF library were reconstituted and used as previously described (48).

Virus

HCMV strain TB40/e UL32-GFP (gift of Christian Sinzger, Universitätsklinikum Ulm Institut für Virologie, Germany) was used in this study. The infectious titre of the endothelial tropic passaged strain was determined using ARPE-19 cells; the pfu/ml (plaque forming units) was used to calculate the Multiplicity of Infection used to infect monocyte derived dendritic cells. Ultra-violet (UV) treatment of TB40/e UL32-GFP was performed for 60 minutes to inactivate the virus stock.

Dual FLUOROSPOT assays

PBMC were depleted of CD8+ T cells by MACS using anti-CD8+ direct beads (Miltenyi Biotech), according to manufacturer's instructions and separated on either LS columns with VarioMACS stand (Miltenyi Biotech) or 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 analysed by flow cytometry. Depletions performed in this manner resulted in 0.2 – 4.3% residual CD8+ T cells (n=40). Triplicate wells of 2 x 10^5 CD8+ T cell depleted PBMC suspended in X-VIVO 15 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)) with ORF mix peptides (final peptide concentration 2µg/ml/peptide) and an unstimulated and positive control mix (containing anti-CD3 antibody (αCD3) (Mabtech AB), Staphylococcus Enterotoxin B (SEB), Phytohaemagglutinin (PHA), 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 (Autoimmun Diagnostika (AID) GmbH, Strassberg, Germany) and counted using EliSpot v7 software (Autoimmun Diagnostika). 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 ORFs and the positive control response after background correction. This analysis determined that the positive response for IFNγ was greater

than 100 sfu/million (spot forming units per million cells) (Fig. 1B) and for IL-10 was greater than 50 sfu/million (Fig. 2A).

Measurement of Degranulation, cytokine secretion and phenotyping of antigen specific CD4+ T cells

2.5 x 10^6 PBMC suspended in X-VIVO 15 + 5% Human AB serum were stimulated with ORF peptide mixes in the presence of CD107a Alexa fluor 647 (BioLegend), unstimulated or Positive Control mix (SEB, αCD3, PHA, PWM and LPS) for one hour and then 5μg/ml Brefeldin A and 2μM Monensin (both BioLegend) were added and incubated overnight at 37ºC in a humidified CO_2 atmosphere. Cells were then washed, stained with a combination of surface antibodies including CD3 Brilliant Violet 650, CD45RA PE-Cy7, CD27 APC-eFluor 780 (eBioscience), CD14 and CD19 FITC – dump channel (eBioscience) and LIVE/DEAD Fixable Yellow Dead cell stain (Invitrogen) at 4ºC. Cells were fixed and permeabilised using FIX&PERM (Nordic-MuBio, Susteren, Holland) and stained intracellularly with CD69 Pacific Blue, 4-1BB PE-Cy5, CD8 AlexaFluor 700, CD4 PE Dazzle (BioLegend), CD40L PerCPeFluor 710 (eBioscience) and MIP-1β PE (BD Biosciences) at 4ºC in the dark. Samples were washed and fixed in a final 1% paraformaldehyde solution and acquired on a BD LSR Fortessa cytometer using FACSdiva software. Data was analysed using FlowJo software, antigen specific CD4+ T cell populations were identified as CD40L+ and $CD69^{high}$ above the background expression observed in the unstimulated control following elimination of doublets, removal of monocytes and B cells and dead cells from the analysed population (example of the CD40L+ and $CD69^{high}$ expression is shown in Fig. 3A), CD69

253 expression is lower due to the overnight incubation as opposed to 6hrs, which is why a
254 CD69^{high} criteria was used to identify the activated cell populations. The levels of
255 CD107a staining was set based on the expression measured in activated CD8+ T cells
256 compared to unstimulated cells for each donor, the positive control sample verified the
257 expression of the activation markers, CD107a and MIP1β for each donor.
258
259 **Measurement of Poly-functional T cell responses to HCMV infected dendritic**
260 **cells.**
261 Monocytes were isolated from donor PBMC by MACS using anti-CD14+ direct beads
262 (Miltenyi Biotech), according to manufacturer's instructions and separated on LS
263 columns with VarioMACS system. Purified monocytes were adhered to a 48 well tissue
264 culture plate at 0.3 x 10^6 cells per well density and then incubated in X-VIVO 15
265 supplemented with 2.5mM L-Glutamine (Sigma Aldrich) and 1000 IU/ml IL-4 and 1000
266 IU/ml GM-CSF (Miltenyi Biotec) for 6 days at 37°C in a humidified CO_2 atmosphere.
267 The differentiated monocytes were matured by exchanging media for X-VIVO 15
268 supplemented with 2.5mM L-Glutamine and 50ng/ml Lipopolysaccharide (LPS) for 24
269 hours. The dendritic cells (moDCs) were then infected with HCMV strain TB40\e-UL32-
270 GFP at an MOI of 0.1 or the equivalent amount of UV-virus for 3 hours in L-glutamine
271 supplemented X-VIVO 15. Media was then replaced and the infected cells were
272 incubated in fresh supplemented X-VIVO 15 for 7 days at 37°C in a humidified CO_2
273 atmosphere. Infection was confirmed by observation of GFP expression in dendritic
274 cells by fluorescent microscopy compared to mock and UV-virus treatment and qRT-
275 PCR. CD4+ T cells were purified from defrosted autologous PBMC by MACS using

anti-CD4+ beads following the manufacturer's instruction using LS columns and VarioMACS. 0.5 x 10^6 CD4+ T cells suspended in X-VIVO 15 + L-glutamine were added to each well of uninfected, infected, UV infected, positive control mix pulsed and pp65 and gB proteins pulsed moDCs in the presence of CD107a Alexa fluor 647 (BioLegend) for one hour and then 5µg/ml Brefeldin A and 2µM Monensin (both BioLegend) were added and incubated overnight at 37ºC in a humidified CO_2 atmosphere. Cells were harvested, washed and stained with a combination of surface antibodies; CD45RA PE-Cy7, CD27 APC-eFluor 780 (eBioscience), CD3 Brilliant Violet 650, CD57 PE-Dazzle, CD28 Alexa Fluor 700, CD14 and CD19 Brilliant Violet 510 (BioLegend) and LIVE/DEAD Fixable Aqua Dead cell stain (Invitrogen) – dump channel at 4ºC. Cells were fixed and permeabilised using FIX&PERM and stained intracellularly with CD69 Pacific Blue, 4-1BB PE-Cy5, CD8 Brilliant Violet 570, CD4 Brilliant Violet 605, Granzyme A FITC (BioLegend), Granzyme B FITC (Miltenyi Biotec), CD40L PerCPeFluor 710 (eBioscience), IFNγ Brilliant Violet 786 and MIP-1β PE (BD Biosciences) at 4ºC in the dark. Samples were washed and fixed in a final 1% paraformaldehyde solution and acquired on a BD LSR Fortessa cytometer using FACSdiva software. Data was analysed using FlowJo software, CD4+ T cells were identified following elimination of doublets, removal of monocytes and B cells and dead cells from the analysed population. Antigen specific CD4+ T cell populations were identified by the expression of CD40L and 4-1BB above the background expression observed in the unstimulated sample. The percentage of antigen specific CD4+ T cells producing combinations of the following functional markers CD107a, Granzymes A and B, IFNγ, MIP1β above background were identified (gating of populations was

299 determined by the use of Fluorescence Minus One samples and the unstimulated
300 control).
301
302 **Measurement of viral dissemination control by CD4+ T cells.**
303 Monocytes were isolated and differentiated and matured to moDCs in 96 well plates at a
304 density of 0.1x 10^6 cells per well, as described in the previous section. The moDC's
305 were then infected with HCMV strain TB40\e-UL32-GFP at an MOI of 0.007 or the
306 equivalent amount of UV-virus for 3 hours in L-glutamine supplemented X-VIVO 15.
307 Media was then replaced and the infected cells were incubated in fresh supplemented
308 X-VIVO 15 for 7 days at 37°C in a humidified CO_2 atmosphere. Infection was confirmed
309 by observation of GFP expression in moDCs by fluorescent microscopy compared to
310 mock and UV-virus treatment wells. Autologous CD4+ T cells were purified from
311 frozen PBMC as described and then added to the wells with infected moDCs at a range
312 of E:T ratios (1.2:1, 0.6:1 and 0.3:1) in supplemented X-VIVO 15. The CD4+ T cells
313 were co-incubated with infected moDCs, after 7 days indicator dermal fibroblasts were
314 added to moDCs monolayer following removal of the well supernatant and non-adherent
315 cells. The fibroblast co-culture was maintained in DMEM (Gibco) supplemented with
316 20% FBS (Gibco) for up to 21 days at 37°C in a humidified CO_2 atmosphere. The
317 spread of TB40\e-UL32-GFP in to fibroblasts after 21 days was measured by flow
318 cytometry acquisition on a BD Accuri C6 flow cytometer following fibroblast harvest with
319 trypsin and fixing with 2% PFA.
320
321 **Statistics**

Statistical analysis was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, CA, USA). The correlation between age and the T cell response to CMV was assessed by Spearman rank correlation for non-normally distributed data. CD107a, MIP-1β and memory phenotype peptide specific analyses were compared using a 1 way ANOVA Kruskall-Wallis test with post hoc Dunn's multiple comparisons and Wilcoxon matched ranks paired test. In the cases of repeated analyses of the same donor cohort results were only considered significant if $p \leq 0.01$.

Results

The magnitude of the HCMV specific CD4+ T cell response to 6 different HCMV ORF encoded proteins is maintained in older donors.

Previous work investigating the HCMV specific CD4+ T cell response, using whole viral lysate as the stimulus, has shown that the frequency of the HCMV specific CD4+ T cell response increases with donor age (19, 21, 22) by measuring IFNγ production by intra-cellular flow cytometry methods. In order to measure the CD4+ T cell response to individual HCMV proteins we performed an initial screen of the CD4+ T cell response to 11 HCMV proteins using pools of overlapping peptides to each HCMV protein, in a small cohort of 18 sero-positive and 4 sero-negative donors. The 11 selected HCMV protein peptide pools included the highest frequency CD4+ T cell responses previously measured in a whole proteome screen (17), measurement of the frequency of the CD4+ T cell response to the selected HCMV proteins was performed by IFNγ ELISPOT assay. Using 100 spot forming units per million cells (sfu/million) as the positive response cut off for CD4+ T cell responses we ranked the HCMV proteins according to the number of responding donors (Fig. 1A). This ranking enabled identification of the HCMV proteins gB, pp71, pp65, IE1, IE2 and US3 as being the most commonly responded to peptide pools in our donor cohort.

In order to assess whether the frequency of CD4+ T cell responses to different HCMV proteins change with increasing donor age, we recruited a large CMV sero-positive donor cohort (n=84) aged 23 – 74 years and also 13 CMV sero-negative donors aged

37 – 72 years to act as background response controls. We measured the frequency of the CD4+ T cell IFNγ response to the 6 highest ranked HCMV encoded proteins detailed in Fig. 1A, using an IFNγ Fluorospot assay. The results from the entire donor cohort for all 6 HCMV proteins and the Positive control are summarised (Fig. 1B). A positive response threshold of 100 sfu/million was determined as being above the distribution of any responses measured in the sero-negative cohort to each HCMV peptide pool, whilst below the response of both sero-positive and negative donors to the positive control, the threshold is indicated on the graph with negative responses falling below the line. The proportion of the donor cohort responding to each protein are shown on the graph, a majority of the sero-positive donor cohort responded to all six proteins studied, with 91.9% of donors generating a positive response to pp65, 70.6%, 69.8%, 65.1%, 56.5% and 52.3% of donors responding to gB, IE2, pp71, US3 and IE1 CMV peptide pools respectively. Analysis of the frequency of the sero-positive cohort responding to 1 or more CMV proteins revealed that all donors made a response to at least 1 of 6 HCMV proteins and 63.1% of donors examined produced an IFNγ response to 4 or more proteins (Fig. 1C).

To assess whether donor age had an impact on the frequency of the CD4+ T cell response to HCMV within this cohort, the sum of the IFNγ responses to all 6 HCMV proteins for each donor with respect to age was analysed (Fig. 1D), overall there was no significant change in the magnitude of the response as donor age increased (Spearman rank correlation). The magnitude of the donor responses to each of the 6 HCMV proteins is also illustrated individually for sero-positive donors (Fig. 1E – 1J). Spearman

rank correlation tests of the data showed that the relationship between magnitude of HCMV protein response and age was not significant and the spearman r values for each ORF (indicated on each graph) are all close to zero for pp65, to correct for repeated measures results were only considered significant if $p \leq 0.01$. These results suggest that there is no obvious change in the size of the response to these HCMV proteins with increasing age.

CD4+ T cells specific for HCMV ORFs expressed during lytic infection predominantly have a Th1 cytokine profile.

CD4+ T cells can be characterised by the expression of certain transcription factors and the cytokines they secrete into different T helper cell populations (49). We have previously shown that CD4+ T cells specific to the HCMV ORF encoded proteins UL138 and LUNA have the capacity to secrete the immunomodulatory cytokine IL-10, as well as having distinct UL138 and LUNA specific CD4+ T cell populations able to secrete IFNγ, a Th1 defined cytokine (47). Others have identified CMV specific CD4+ T cells which secrete IL-10 and their experiments suggested the generation of iTreg cells specific for HCMV (pp65 and IE ORFs) was related to frequent exposure to CMV antigen (50). This suggests that an older CMV seropositive donor may have an increased numbers of CD4+ T cells secreting IL-10 following CMV stimulation due to potentially being exposed to the viral antigens for a longer time period. We measured the ability of CD4+ T cells to secrete IL-10 and or IFNγ in response to the 6 HCMV proteins using a dual fluorospot method in 59 sero-positive donors and 8 sero-negative donors. We assessed the IL-10 responses of the donor cohort to the 6 HCMV proteins

399 analysed and the positive control alone and used the distribution of the response from
400 the sero-negative cohort to the 6 HCMV protein peptide pools and Positive control to
401 derive a positive response threshold for IL-10 responses as 50 sfu/million (line shown
402 on graph in Fig. 2A). The proportion of donors responding above the positive cut-off for
403 each HCMV protein are also shown, the US3 and pp71 proteins produced an IL-10
404 response from 44.1% of the cohort, 28.8% responded to pp65, 25.4% to IE2, 20.3% to
405 IE1 and 15.3% responding to gB. Confirming the reduced number of IL-10 responses to
406 the 6 HCMV proteins in this cohort the number of proteins that triggered an IL-10
407 response above the positive threshold for each donor were analysed, 40.7% of the
408 cohort did not make an IL-10 response to any of these 6 HCMV proteins (Fig. 2B), no
409 donor produced an IL-10 response to all 6 proteins in comparison to 20.2% of donors
410 producing an IFNγ response to all 6 proteins analysed (Fig. 1C).
411
412 The fluorospot technology used allowed the simultaneous assessment of the IFNγ and
413 IL-10 responses to each HCMV protein, enabling the contribution of IFNγ and IL-10
414 secretion to the overall response for each donor to be assessed. The data is
415 summarised (Fig. 2C – 2H) for the entire sero-positive cohort, only donors that
416 responded above the positive threshold cut-off for either IFNγ or IL-10 are shown. For
417 each donor the size of the IFNγ (grey bar) and IL-10 (clear bar) response in sfu/million
418 is shown in increasing donor age order along the x-axis, the rarely observed cell
419 population secreting both IFNγ and IL-10 are indicated by a red bar. The data shows
420 that overall the majority of the T cells produce IFNγ (grey bars) and that for pp65, IE1,
421 gB and IE2 the IL-10 responses are limited. The combined IFNγ and IL-10 graphs for

422 pp71 and US3 clearly show more IL-10 white bars for each donor with no impact on the
423 frequency with increasing donor age, however the IFNγ response (grey bars) still
424 predominates for the majority of the donors analysed. Spearman rank correlation of
425 age with the magnitude of the IL-10 response to the 6 HCMV ORFs for the donor cohort
426 revealed that there was a significant decline in IL-10 production in response to IE1
427 stimulation (r_s= -0.4185, p=0.01), the IL-10 response to the other 5 ORFs did not reveal
428 any significant changes in magnitude with increasing donor age.
429
430 **HCMV specific CD4+ T cells have a predominantly effector memory phenotype**
431 **and cytotoxic capacity**
432 We and others have previously reported that CMV specific CD4+ T cells can have direct
433 effector functions both cytotoxic and by secreting inflammatory cytokines (16, 24, 26,
434 30, 47). To examine the functional capacity of CD4+ T cells specific to the 6 different
435 HCMV proteins studied here, we determined CD107a expression, a well-defined marker
436 of degranulation and indicative of cytotoxic capacity in CD4+ T cells (30, 51) and
437 secretion of the pro-inflammatory chemokine macrophage inflammatory protein (MIP)-
438 1β which has been shown to be secreted by CD4+ T cells (30) in response to
439 stimulation by HCMV protein peptide pools. CMV specific CD4+ T cells were identified
440 as CD40L+ and $CD69^{high}$ compared to the background unstimulated population (52) and
441 the proportion of CD107a or MIP-1β positive cells measured, a representative analysis
442 of gB specific CD4+ T cell response is shown (Fig. 3A). The results of CD107a
443 expression for n=12 donors (Fig. 3B) and MIP-1β secretion for n=9 donors (Fig. 3C) by
444 CMV specific CD4+ T cells for each of the 6 HCMV ORFs are summarised. CD4+ T

445 cells stimulated directly *ex vivo* are capable of degranulation as shown by the proportion
446 of antigen specific CD107a expressing cells present (Fig. 3B). The ability of gB specific
447 CD4+ T cells to produce cytotoxic function has previously been established (16, 47, 53),
448 however this analysis indicates that CD4+ T cells specific to the other 5 proteins also
449 possess this capacity. All the donors examined expressed CD107a in CMV specific
450 CD4+ T cells in response to at least one of the six HCMV proteins. A proportion of
451 CD4+ T cells specific to all 6 HCMV proteins studied were also able to secrete MIP-1β
452 following stimulation extending previous reports of this phenomenon in pp65 specific
453 CD4+ T cells (30).
454
455 The memory phenotype of CMV specific CD4+ T cells in previous studies has been
456 shown to be a differentiated memory phenotype, characterised by the downregulation of
457 the co-stimulatory molecule CD27 (21, 24) and re-expression of CD45RA (11, 21, 24),
458 and the loss of CD28 and expression of CD57 (20, 22, 27, 30). To assess whether the
459 memory T cell phenotype differs between the 6 HCMV protein specific CD4+ T cell
460 populations the proportion of antigen specific cells with one of 4 memory populations
461 defined by expression of CD27 and CD45RA molecules, and the proportion that have
462 lost expression of CD28 and upregulated CD57 expression was measured.
463
464 Antigen specific CD4+ T cells were again identified by upregulation of CD69 and CD40L
465 (52), an example of the phenotype of the total CD4+ T cell population and an exemplar
466 HCMV specific population from one donor is shown (Fig. 4A). The proportion of HCMV
467 protein specific CD4+ T cells with the different memory populations are compared in

summary graphs for CD27- CD45RA+; T_{EMRA} (EMRA, Effector Memory CD45RA expressing) (Fig. 4B), CD27+ CD45RA+; T_{NL} (NL, naive like) (Fig. 4C), CD27- CD45RA- ; T_{EM} (EM, Effector Memory) (Fig. 4D), CD27+ CD45RA-; T_{CM} (CM, Central Memory) (Fig. 4E), CD28- CD57+, Highly Differentiated Memory cells (Fig. 4F) and CD28+ CD57-; less differentiated Memory cells (Fig. 4G). Additionally the proportion of total CD4+ T cells from age-matched CMV sero-negative donors for the 4 memory populations defined by CD27 and CD45RA expression is also shown (Fig. 4B – E). The comparison of the CMV sero-negative and positive total CD4+ populations for each of the 4 memory populations revealed that CMV sero-positive donors have significantly more T_{CM} (Fig. 4E), T_{EM} (Fig. 4D) and T_{EMRA} (Fig. 4B) differentiated CD4+ T cells (Mann Whitney U test, significant results $p<0.01$ $^{\#\#}$ and $p<0.0001$ $^{\#\#\#\#}$ are shown). This confirms previous observations of the impact of CMV infection on the phenotype of CD4+ T cells (summarised in (37)). The distribution of the CMV sero-positive donors CD4+ T cell responses to the 6 HCMV proteins and total CD4+ T cells for the 6 different memory phenotype populations was analysed using a non-parametric Kruskall-Wallis 1-way ANOVA test (results are shown Fig. 4B – 4G). Where there was significant variance a Wilcoxon rank pairs test was used as a post-test to pairwise compare the proportion of the antigen specific population expressing each phenotype with the total CD4+ population and the other HCMV proteins for each donor, significant differences of $p\leq0.01$ (to account for repeated measures) between the populations are indicated on the graphs. The analysis of memory phenotypes showed that CMV protein specific CD4+ T cells have a significant decrease in the less differentiated memory phenotypes compared to the total CD4+ population (Fig. 4C and 4E). There was a corresponding

significant enrichment in the differentiated effector memory sub-populations for all 6 HCMV protein specific populations (Fig. 4B). Comparison of the proportion of CMV specific CD4+ T cells exhibiting different memory population phenotypes revealed that US3 specific CD4+ T cells had a significantly greater proportion of T_{EMRA} cells compared to gB and IE2 (Fig. 4B; gB specific CD4+ T cells were enriched in the T_{CM} population compared to 4 other HCMV proteins (Fig. 4E). There was no significant changes in the proportion of CMV specific CD4+ T cells with a highly differentiated (Fig. 4F) or undifferentiated (Fig. 4G) memory cell populations, although the antigen specific populations were enriched for the highly differentiated population compared to the total CD4 T cells.

The HCMV specific CD4+ T cells produce poly-functional responses to virally infected cells

The use of CMV viral lysate or overlapping peptide pools to characterise CD4+ T cell responses to CMV does not allow the effect of CMV encoded immune evasion molecules during infection, particularly the downregulation of MHC Class II expression on antigen presenting cells (54). To assess the CD4+ T cell response in the presence of viral encoded immune evasion molecules we used an *in vitro* infection of autologous dendritic cells using a clinical isolate of CMV method. Autologous dendritic cells derived from individual donor monocytes (moDCs) were infected with a UL32 GFP tagged HCMV strain TB40\E (TB40\e-UL32-GFP). The CMV infected moDCs were incubated for 7 days prior to co-incubation with autologous CD4+ T cells overnight and

514 measuring functional responses in responding CD4+ T cells by flow cytometry. In total
515 the functional responses of 12 donors to virus infected moDCs were analysed, the size
516 of the HCMV specific response (identified by the co-upregulation of the activation
517 markers CD40L and 4-1BB) above background by the CD4+ T cells and the fraction of
518 those cells producing each of the 4 individual functional markers (MIP-1β, Granzymes A
519 & B, IFNγ and CD107a) or none were compared (Fig. 5A). The breakdown of the
520 antigen specific response equates to 49.2% (±8.9 S.E.M.) of responding CD4+ T cells
521 not producing any of these effector markers, of the remaining antigen specific CD4+ T
522 cells 41.7% (±8.6% S.E.M.) produced IFNγ and 16.3% (±4.0% S.E.M.) expressed
523 CD107a, with a minority of virus specific CD4+ T cells producing MIP-1β (4.9% ± 2.4
524 S.E.M.) or Granzymes A and B (4.6% ±2.1 S.E.M.). The poly-functionality of the virus
525 specific CD4+ T cell response in 12 donors was assessed and the mean proportion of
526 virus specific cells producing 1 or more functions was compared (Fig. 5B), 14.6% of
527 responding cells produced 2, 3 or 4 functions, and a further 36.2% produced 1
528 functional response. The proportion of virus specific CD4+ T cells responding within the
529 16 different categories created by combinations of the 4 functional markers for all 12
530 donors is shown (Fig. 5C), this breakdown analysis of the HCMV specific CD4+ T cells
531 that produce one or more functional response (comprising 50.8% of the activated cells
532 response) confirms the dominance of IFNγ production alone (28.8% ± 5.3 S.E.M.) and
533 in combination with CD107a expression (7.9% ± 2.3 S.E.M.). The other notable
534 populations are expression of CD107a only (4.8% ± 1.3 S.E.M, the population
535 producing IFNγ and MIP-1β (2.2% ± 1.1 S.E.M.), the Granzymes A and B only
536 producing cells (2.1% ± S.E.M.) and triple functional cells producing IFNγ, MIP-1β and

537 expressing CD107a (1.8% ± 1.2 S.E.M.). Phenotype analysis of the virus specific CD4+
538 T cells reveals an undifferentiated (CD28+ CD57-, 58.9% ± 7.5 S.E.M.) effector memory
539 (CD27- CD45RA-, 35.6% ± 2.9 S.E.M.) population (Fig. 5D) similar to that seen in
540 peptide stimulated cells (Fig. 4).
541
542 In 3 donors we compared the CD4+ T cell response to TB40\e-UL32-GFP infected cells
543 with moDCs infected with an identical MOI of UV inactivated virus. The size of the
544 corrected antigen specific response measured by upregulation of CD40L and 4-1BB is
545 shown for each donor (Fig. 5E), this comparison shows that for all three donors the
546 response to live virus was greater than for the UV inactivated virus. There was however
547 a notable CD4+ T cell response to the UV virus treated cells, suggesting that inactive
548 viral particle proteins were still being presented by the moDCs 7 days after the initial
549 infection. We confirmed that the UV treatment of the virus was inactive by fluorescent
550 microscope analysis of GFP expression in the moDCs (an example from donor CMV320
551 is shown Fig. 5F), which clearly shows that the late gene UL32 which is tagged with
552 GFP in the HCMV viral strain used for these studies is only expressed in the infected
553 cells at day 7 post infection and is not observed in the UV inactivated virus treated
554 moDCs.
555
556 **HCMV specific CD4+ T cells control the dissemination of virus *in vitro***
557
558 The evidence that live cytomegalovirus infection was able to stimulate functional CD4+
559 T cell responses to a greater extent that UV-inactivated virus led to an investigation of

560 whether these CMV specific CD4+ T cells could directly target an active lytic infection.
561 We have previously established a viral dissemination assay for CD8+ T cells (48) using
562 autologous fibroblasts infected with a low MOI of TB40\e-UL32-GFP strain to measure
563 the ability of the CD8+ T cell subset to abrogate viral spread. To be able to interrogate
564 the role of CMV specific CD4+ T cells in the same way required an adaptation of this
565 experimental model because fibroblasts do not constitutively express MHC Class II
566 molecules (55). Autologous *in vitro* differentiated moDCs was chosen as these cells
567 both constitutively express MHC Class II (55) and are permissive for lytic CMV infection
568 (56). Dendritic cells derived from each donor were infected with TB40\e-UL32-GFP at a
569 low MOI, after 7 days culture CD4+ T cells isolated directly *ex-vivo* were added at
570 effector (CD4+ T cells) to target (moDCs) (E:T) ratios of 1.2:1, 0.6:1 and 0.3:1 in
571 triplicate. The CD4+ T cells were co-incubated with the infected moDCs for a further 7
572 days and then indicator fibroblasts were added, to be infected by virus released from
573 any remaining infected dendritic cells, and co-incubated for 14 – 21 days prior to
574 analysis by flowcytometry for GFP expressing (virus infected) fibroblasts.
575
576 We have measured viral dissemination in 5 HCMV sero-positive donors and 1 HCMV
577 sero-negative donor as a control. The dissemination of TB40\e-UL32-GFP to
578 fibroblast cells was assessed by fluorescent microscopy and flow cytometry, an
579 example from donor CMV320 is illustrated following 14 days incubation with indicator
580 fibroblasts (Fig. 6A), this clearly shows a lack of GFP expression in the uninfected wells
581 and the wells treated with CD4+ T cells at all 3 E:T ratios compared to that observed in
582 the infected only control. The results from the flow cytometry analysis for 5 sero-

positive donors (Fig. 5B – 5F) and 1 sero-negative donor (Fig. 5G) are summarised, the data for each treatment was corrected for background and then expressed as a proportion of the Infected control (which is therefore set at 100% for all 6 donors). The graphs from all 5 HCMV sero-positive donors show that the addition of CD4+ T cells stopped the dissemination of HCMV into the fibroblast layer, the results from CMV425 (Fig. 5G) the sero-negative donor clearly demonstrate that this is the action of CMV specific CD4+ T cells as the proportion of fibroblasts infected with the GFP tagged virus was observed at similar levels to the infected control. The evidence from this functional assay together with the poly-functional responses described in Fig. 5 clearly shows that resting HCMV specific CD4+ T cells isolated directly *ex-vivo* have direct anti-viral activity producing inflammatory cytokines and cytotoxic responses which enable these cells to prevent viral dissemination.

Discussion

Within ageing, studies have implicated CMV as being associated with increased risk of all-cause mortality in older people (37) and causing detrimental changes to the immune response (27-29). The paradox with the association of CMV sero-positivity with the loss of immune function in older people is that overt CMV disease from reactivation or new infections is not observed, however there is an increase in detectable virus in urine in the old (44). This strongly suggests that the immune response to HCMV itself retains sufficient functionality within the older immunocompetent population, but that immunomodulation as a consequence of lifelong carriage of HCMV may alter the immune response (57). Secretion of the immunomodulatory cytokine IL-10 (58) by CMV specific CD4+ T cells is a candidate for mediating immunomodulation of the CMV specific T cell response during ageing. Previously we have identified populations of CD4+ T cells specific for the HCMV proteins UL138 and LUNA that secrete IL-10 (47). Others have observed secretion of IL-10 by CMV specific CD4+ T cells in response to stimulation by pp65 and IE1 and they demonstrated that frequent exposure to CMV antigens drove the generation of an iTreg CD4+ T cell population specific to HCMV (50). We hypothesised that older CMV sero-positive donors may have increased numbers of CD4+ T cells secreting IL-10 following CMV antigen stimulation due to longer periods of exposure to viral antigens and that this subset of CMV specific CD4+ T cells may inhibit efficient recognition of the virus by IFNγ secreting CMV specific CD4+ T cells.

We did not see any influence of donor age on the magnitude of the total CMV specific CD4+ T cell IFNγ or IL-10 responses to gB, pp65, pp71 and IE2 stimulations. There was a significant decline in the IE1 IL-10 specific CD4+ T cell response in older donors, but the relationship of the magnitude of the total IFNγ and IL-10 responses to all 6 proteins for each donor was not affected by donor age. We have therefore demonstrated that a proportion of CD4+ T cells specific to the 6 different HCMV proteins examined here produced IL-10 but overall this response is limited compared to the IFNγ response observed. However, we did observe some donors (approximately 1 in 6) within the cohort who did have an equal or higher frequency IL-10 sfu/million response compared to the IFNγ response to 3 or more of the CMV protein responses examined. Overall there was no alteration in the balance of IL-10 and IFNγ secretion with increasing donor age and putative increased length of viral carriage and exposure to viral antigens in this study. It would be interesting to examine whether donors in a suitably sized cohort with an IL-10 bias in the CD4+ T cell response to CMV antigens differ in other aspects of their CMV immune response, such as CMV IgG titres or viral carriage.

The observations from this cohort regarding the impact of donor age on CMV specific CD4+ T cell responses are in contrast to some other studies which have shown an accumulation of IFNγ secreting CD4+ T cells in older donors (19, 21, 22). These studies used viral lysate to stimulate HCMV specific CD4+ T cells rather than focussing on responses to particular HCMV proteins and intracellular flow cytometry to measure the IFNγ response. The current study used fluorospot assays to measure IFNγ

production by CD4+ T cells; Fluorospot is a development of enzyme-linked immunospot (ELISPOT) assays which enables measurement of multiple cytokines simultaneously (59). Experience from our own studies and other groups suggests that ELISPOT is more sensitive for detecting T cell responses to HCMV antigens (60), so the contrasting observations in this study compared to previously published work are not explained by the use of different techniques to measure IFNγ responses. The studies demonstrating increased frequency of CMV specific CD4+ T cells in older donors (19, 21, 22) did not investigate the absolute size of the T cell compartment in peripheral blood. This is important as the increased percentage measured in these studies may only equate to the same numbers of IFNγ producing CD4+ T cells if the total CD4+ T cell compartment size decreases for instance. It is therefore difficult to compare the conclusions from studies where the methods of reporting results differ between frequency and percentages, particularly when the information required for interpreting the presented percentage data are absent. It has been previously observed that donor cohorts from different geographical locations, e.g. a study of older Sicilians with 70% CMV sero-positivity (61), did not show an accumulation of CMV specific CD8+ T cells in the older donor group compared to young donors. Understanding that HCMV infection does not have the same effect on all older donor cohorts is important when interpreting studies which propose medical intervention in CMV sero-positive older people as necessary to improve immune response and promote a healthy ageing phenotype.

It is becoming increasingly clear in both CMV infection (26, 31, 53) and other viral infections, e.g. influenza, West Nile virus, rotavirus and sendai virus (reviewed in (62))

663 that CD4+ T cells can exhibit direct effector functions, including cytotoxicity and the
664 secretion of pro-inflammatory effector molecules that help to control or resolve viral
665 infections. We saw that CD4+ T cells specific to all 6 HCMV proteins upregulated
666 expression of CD107a, a marker of degranulation used as a surrogate indicator of
667 potential cytotoxic activity (30), and produced the pro-inflammatory chemokine MIP-1β.
668 Our observations on the memory phenotype of CMV specific CD4+ T cells do confirm
669 previous studies using pp65 peptides and viral lysate stimulation which have described
670 CMV specific CD4+ T cells as having an effector memory phenotype (19, 21, 30). The
671 use of *in vitro* stimulation of CD4+ T cells with HCMV peptide pools or viral lysate
672 allows the determination of T cell effector functions but this is in the absence of immune
673 evasion molecules expressed by CMV during its lytic lifecycle. Examining CMV specific
674 T cell responses in the absence of viral immunomodulation is not representative of the
675 situation during CMV infection or reactivation in the host (57). CMV encoded proteins
676 target many aspects of the immune response including evading natural killer cell
677 responses, the interferon response and perturbation of immunomodulatory pathways
678 (reviewed in (54). Pertinent to affecting host CD4+ T cell effector responses, the
679 proteins encoded by CMV US3 (63) and US2 (64) interfere with MHC Class II
680 presentation at the cell surface, UL82 which encodes the phosphoprotein pp65 can
681 mediate destruction of HLA-DR molecules (65), and loss of MHC Class II expression by
682 CMV infected dendritic cells (66, 67). The combined effect of these immune evasion
683 and modulatory proteins in an active infection or reactivation event by CMV in the host
684 could lead to very different effector behaviour by CMV specific CD4+ T cells than
685 observed in response to isolated viral proteins. This problem has been addressed to an

686 extent in the murine model with MCMV infection, a recent paper using novel epitope
687 Class II restricted tetramers *in vivo* have observed direct killing of infected cells (68).
688 With respect to HCMV infections, Sinzger et al have shown that IE1 specific CD4+ T cell
689 clones are able to produce IFNγ in response to stimulation by TB40\E infected
690 macrophages which have downregulated MHC Class II expression (69).
691
692 We used an experimental model of lytic infection *in vitro* to measure the effector
693 functions of CD4+ T cells isolated directly *ex vivo* in response to HCMV infected
694 monocyte derived dendritic cells. The predominant effector function produced was IFNγ
695 followed by CD107a expression, low levels of the cytolytic enzymes Granzymes A & B
696 and MIP-1β were also detected. The poly-functional CD4+ T cell responses observed
697 are important as they have been shown to be better effector cells (51) and reduced
698 frequencies of poly-functional CMV specific CD4+ T cells are associated with the
699 occurrence of congenital CMV infections (70). However these assays still just measure
700 effector mechanisms and does not give any indication if the T cells could mediate direct
701 anti-viral activity. In order to measure the effectiveness of these CMV specific CD4+ T
702 cells we performed a viral dissemination assay using the non-attenuated clinical strain
703 TB40\e-UL32-GFP. We performed the assay in 5 HCMV sero-positive donors and
704 clearly observed the CD4+ T cells preventing viral dissemination from the virus infected
705 dendritic cells. By performing the assay with a CMV sero-negative donor we confirmed
706 that this was a CMV specific CD4+ T cell effect, as there was no control of viral
707 dissemination in the presence of non-specific CD4+ T cells. This assay convincingly
708 shows that CD4+ T cells can respond directly to CMV infected cells probably using both

cytokine and cytotoxicity mechanisms observed in the poly-functional flow cytometry experiments. The ability of CMV specific CD4+ T cells to control viral dissemination is inspite of the large array of immune evasion mechanisms encoded by the virus, and confirms the observations made *in vivo* in the murine model (68) and previously in infected macrophages in HCMV *in vitro* models (69). Further interrogation of the CMV specific CD4+ T cell response using this *in vitro* model will be able to determine whether cytokines or cytotoxicity produced by CD4+ T cells are more important in resolving CMV infection or re-activation in the host.

In summary, we have shown that the CD4+ T cell response to lytic HCMV antigens and infection is not obviously attenuated in older donors. CD4+ T cells specific to HCMV have cytotoxic capability and secrete MIP-1β and IFNγ which are known to be essential to control viral replication (13, 20, 70) and can control viral dissemination *in vitro*. Previous studies focussing solely on T cell responses to limited HCMV ORF encoded proteins or inactive viral lysate in the ageing immune response (71) may have resulted in too narrow a perspective on understanding the aetiology of the CMV infection and diseases in healthy older people. In order to understand why older donors may reactivate virus more frequently compared to younger donors (44) will require further study of CD4+ T cell responses in the context of viral infection models. By interrogating the immune response to the entire HCMV proteome expressed during both lytic and latent infection using direct anti-viral assays instead of relying on responses to isolated peptide stimulation will help to identify whether the HCMV specific T cell response is impaired in ageing or immunocompromised patients. This will also enable the

development of effective immunotherapeutic treatments for HCMV infection and widen our knowledge of the functional capacity of CD4+ T cells in response to virus infection.

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991 FIGURE LEGENDS

992

993 **FIG 1 – The magnitude of IFNγ secreting CD4+ T cell responses to 6 HCMV**

994 **proteins is maintained with increasing donor age.**

995 The frequency of the CD4+ T cell responses to 11 HCMV protein peptide pools was

996 determined in 18 donors by IFNγ ELISPOT, the number of donors with a positive

997 response (<100 spot forming units/million cells (sfu/million) after background count

998 correction) to each protein is tallied and ranked (A). The IFNγ secreting CD4+ T cell

999 response to 6 HCMV proteins: pp65, gB, IE2, pp71, US3 and IE1 was measured in a

1000 cohort of 84 HCMV sero-positive and 13 sero-negative donors using an IFNγ Fluorospot

1001 technique. The results were converted to sfu/million T cells with background counts

1002 subtracted, the response to each protein and the positive control by the entire cohort is

1003 summarised (B) with both CMV seropositive donors (dark grey data points) and CMV

1004 seronegative donors (white data points) illustrated. The distribution of the CMV

1005 seronegative donor's responses to each HCMV protein peptide pool and the response

1006 to the positive control determined the positive HCMV peptide pool response threshold

1007 cut-off of 100 sfu/million (dashed line), the proportion of donors responding above the

1008 threshold to each protein and the positive control are shown. The proportion of the 84

1009 sero-positive donors producing a positive response to 1 or more of the 6 HCMV protein

1010 peptide pools is summarised (C). Within the sero-positive cohort the total IFNγ

1011 response to all six proteins is shown as a correlation of donor age with the size of the

1012 donor response (D) and for each individual protein; pp65 (E), IE1 (F), gB (G), IE2 (H),

1013 pp71 (I) and US3 (J). The correlation of the CMV proteins response with age was

analysed using Spearman rank correlation (Spearman r_s with 95% Confidence Intervals (CI) and p value are indicated on each graph); a line of best fit (solid) and the 95% CI (dotted lines) are also shown; due to the repeated analyses performed results were only considered significant if $p \leq 0.01$.

FIG 2 – The HCMV specific CD4+ T cell response is predominantly Th1.

The frequency of the IL-10 secreting CD4+ T cells in response to 6 HCMV proteins and positive control stimulation in 59 sero-positive and 8 sero-negative donors is shown. IL-10 secretion was measured using a Fluorospot method, the results were converted to spot forming units/million cells (sfu/million) with background counts subtracted, the response to each protein and the positive control by the entire cohort is summarised (A) with both CMV sero-positive donors (dark grey data points) and CMV sero-negative donors (white data points) illustrated. The distribution of the CMV sero-negative donor's responses to each protein and the response to the positive control determined the positive HCMV protein response threshold cut-off of 50 sfu/million (dashed line), the proportion of donors responding above the threshold to each protein and the positive control are indicated (ranging from 44% sero-positive donors responding to pp71 and US3 stimulation to 15% responding to gB stimulation). The frequency of sero-positive donors producing a positive response to none, 1 or more of the 6 proteins is summarised (B). The frequency of CD4+ T cells that secrete IFNγ or IL-10 in response to 6 HCMV proteins were measured simultaneously using a dual IFNγ/IL-10 fluorospot assay. The response to 6 HCMV proteins; pp65 (UL83) (C), IE1 (UL123) (D), gB (UL55) (E), IE2 (UL122) (F), pp71 (UL82) (G) and US3 (H) are summarised for the 59

donors arranged along the x-axis in age order (donor ages 23 – 74 years). For each HCMV protein graph, only donors with positive responses above the threshold for either IFNγ (> 100 sfu/million) [dark grey bars] or IL-10 (> 50 sfu/million) [clear bars] are shown; dual secreting cells are also indicated when present [hatched yellow bars]. There was no significant change in the magnitude of the IFNγ response with donor age measured using Spearman rank correlation, there was a significant decrease in IE1 specific cells secreting IL-10 with donors age (Spearman r_s= -0.4185, (Confidence Interval: -0.6595, -0.0994), p=0.01 **, n=37), there was no significant changes in IL-10 secretion with donor age for the other 5 proteins.

FIG 3 – A proportion of HCMV specific CD4+ T cells have cytotoxic capacity and can secrete MIP-1β

PBMC were stimulated overnight with HCMV peptide pools in the presence of αCD107a antibody, Brefeldin A and Monensin to measure degranulation and production of MIP-1β. Identification of HCMV specific CD4+ T cell responses was as described in the methods; antigen specific CD4+ populations were identified as CD40L+ and CD69 ^{high} compared to the background unstimulated population and the proportion of antigen specific CD4+ T cells upregulating CD107a or producing MIP-1β was measured (a representative example of the response to gB is shown (A)). The results from all the donors examined are summarised for CD107a expression n=12 (B) and MIP-1β production n=9 (C). There were no significant differences in the proportion of CMV protein specific CD4+ T cells upregulating CD107a or producing MIP-1β (Kruskall-Wallis 1-way ANOVA with post-hoc Dunn's multiple comparisons).

FIG 4 – HCMV specific CD4+ T cells have a predominantly effector memory phenotype and are not highly differentiated.

PBMC were stimulated overnight with HCMV protein peptide pools in the presence of Brefeldin A. HCMV specific CD4+ T cell responses were identified by expression of CD40L and CD69 above background and 4 memory phenotype sub-sets defined according to the expression of CD27 and CD45RA: T_{NL} (NL; Naïve like) CD27+CD45RA+, T_{CM} (CM; Central Memory) CD27+CD45RA-, T_{EM} (EM; Effector Memory) CD27-CD45RA- and T_{EMRA} (EMRA; Effector Memory CD45RA+) CD27-CD45RA+, and 2 memory differentiation phenotype populations defined according to the expression of CD57 and CD28: undifferentiated (CD28+ CD57-) and highly differentiated (CD28- CD57+) were measured. A representative example illustrating the expression of these six phenotypes in total CD4+ and pp65 specific T cells is shown (A). The results are summarised for each phenotype population of interest comparing the responses to 6 HCMV proteins with the total CD4+ T cell population for n=15 CMV sero-positive donors and the total CD4+ T cell population for n=15 age-matched CMV sero-negative donors for the following populations; T_{EMRA} (B), T_{NL}(C), T_{EM} (D), T_{CM} (E). The responses to 6 HCMV proteins with the total T cell population for n=15 CMV sero-positive donors only are summarised for these populations; CD28- CD57+ (F) and CD28+ CD57- (G). A non-parametric Kruskall-Wallis 1-way ANOVA test was performed for the CMV sero-positive donors for each memory population (results indicated on each graph). Where significant variation was observed a Wilcoxon matched-pairs post-test was performed to compare the different proportion of CMV specific CD4+ T cells to total

CD4+ T cells and each of the other CMV specific protein populations; significant results for each individual comparison are indicated on the appropriate graph (** p<0.01; *** p<0.001). Lastly the CMV sero-positive donors total CD4+ T cell CD27 and CD45RA defined memory populations were compared to the CMV sero-negative donors using a Mann Whitney U test; significant results are indicated on the appropriate graph ($^{\#\#}$ p<0.01; $^{\#\#\#\#}$ p<0.0001).

FIG 5 – The HCMV specific CD4+ T cell response to HCMV infected cells is poly-functional.

Monocyte derived dendritic cells (moDCs) were prepared from each donor and then mock or lytically infected with HCMV strain TB40\e UL32-GFP at an MOI 0.1 for 7 days. Autologous CD4+ T cells were incubated overnight with either uninfected, HCMV infected or UV irradiated HCMV infected moDCs in the presence of αCD107a antibody, monensin and brefeldin A. CD4+ T cells were then stained with a poly-functional flow cytometry antibody panel, acquired and analysed. Virus specific CD4+ T cells were identified by the upregulation of CD40L and 4-1BB above background. The total specific response to CMV virus and the proportion of the specific response composed of MIP-1β, Granzymes A and B, CD107a and IFNγ production or no functional marker in n=12 donors are shown (A). The mean proportion of virus specific CD4+ T cells generating poly-functional responses from all donors is summarised as a pie chart indicating the proportion of HCMV specific CD4+ T cells producing 4, 3, 2, 1 or no functions (B). The composition of the HCMV specific CD4+ T cell response as a proportion of antigen specific population for all donors is illustrated (C). The proportion

of virus specific cells expressing different memory cell phenotype markers (CD27, CD45RA, CD28 and CD57) is shown (D). A direct comparison of the size of the specific T cell response to live virus vs UV inactivated response in 3 donors is shown (E). A representative example of UL32 (late gene) tagged GFP expression in moDCs infected with live virus vs UV inactivated virus (F) indicating GFP expression in live virus infected moDCs only.

FIG 6 – HCMV specific CD4+ T cells are able to prevent dissemination of virus *in vitro.*

Monocyte derived dendritic cells (moDCs) were prepared from each donor and then mock or lytically infected with HCMV strain TB40\e-UL32-GFP at MOI 0.007 for 7 days. CD4+ T cells were co-incubated with the infected moDCs at a range of E:T ratios for a further 7 days. Indicator fibroblasts were then added to the post CD4+ T cell treated infected moDCs for up to 28 days and then the percentage of fibroblasts expressing GFP tagged virus were measured by flow cytometry. Representative dot plots showing the GFP expression from one well of triplicates and a corresponding fluorescent microscope image are shown (A). The bar charts from 5 HCMV sero-positives (B – F) and 1 sero-negative donor (G) summarise the percentage of TB40\e-UL32-GFP expressing fibroblasts corrected for background and as a percentage of the infected only control.

FIG 1

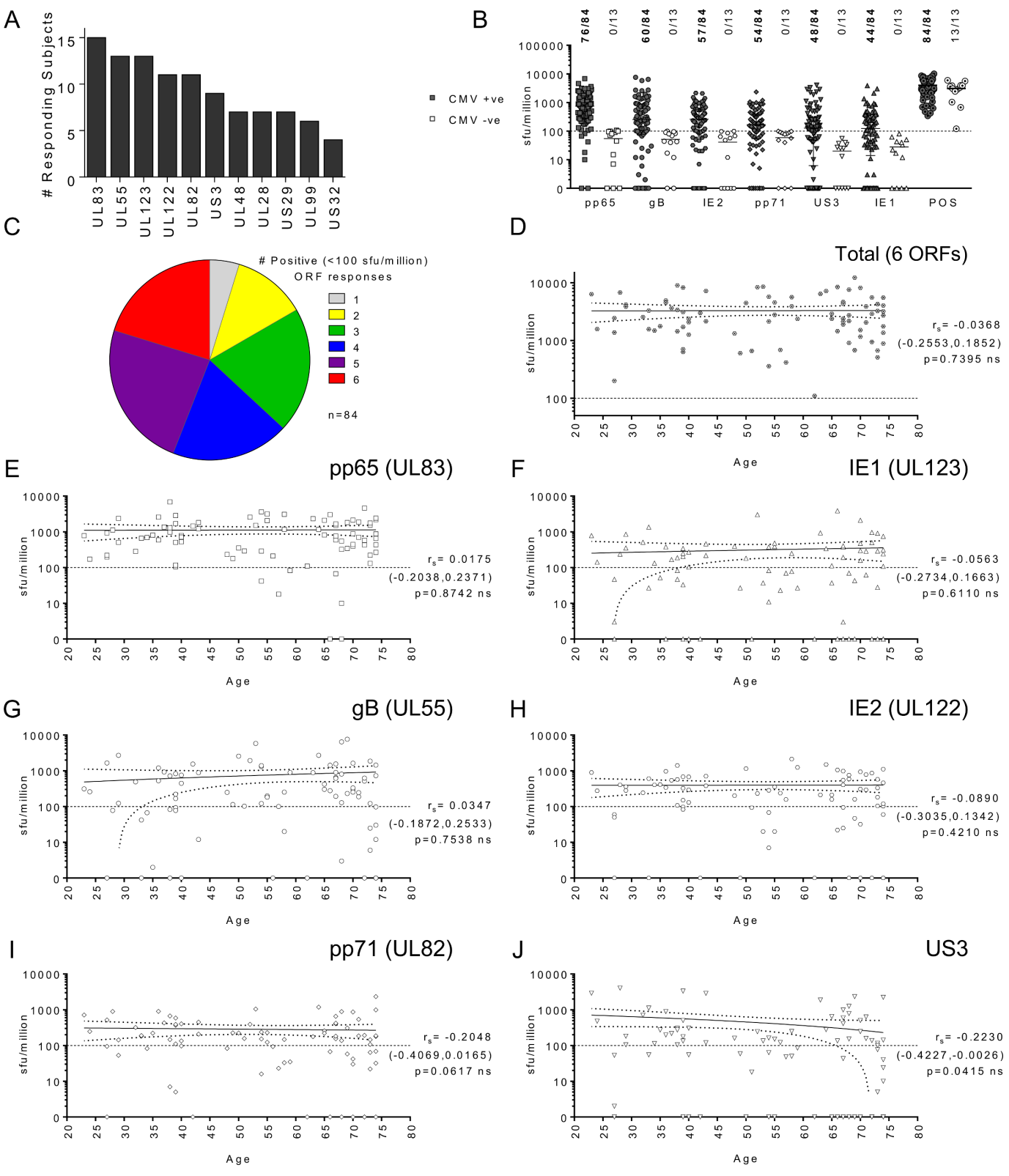

FIG 2

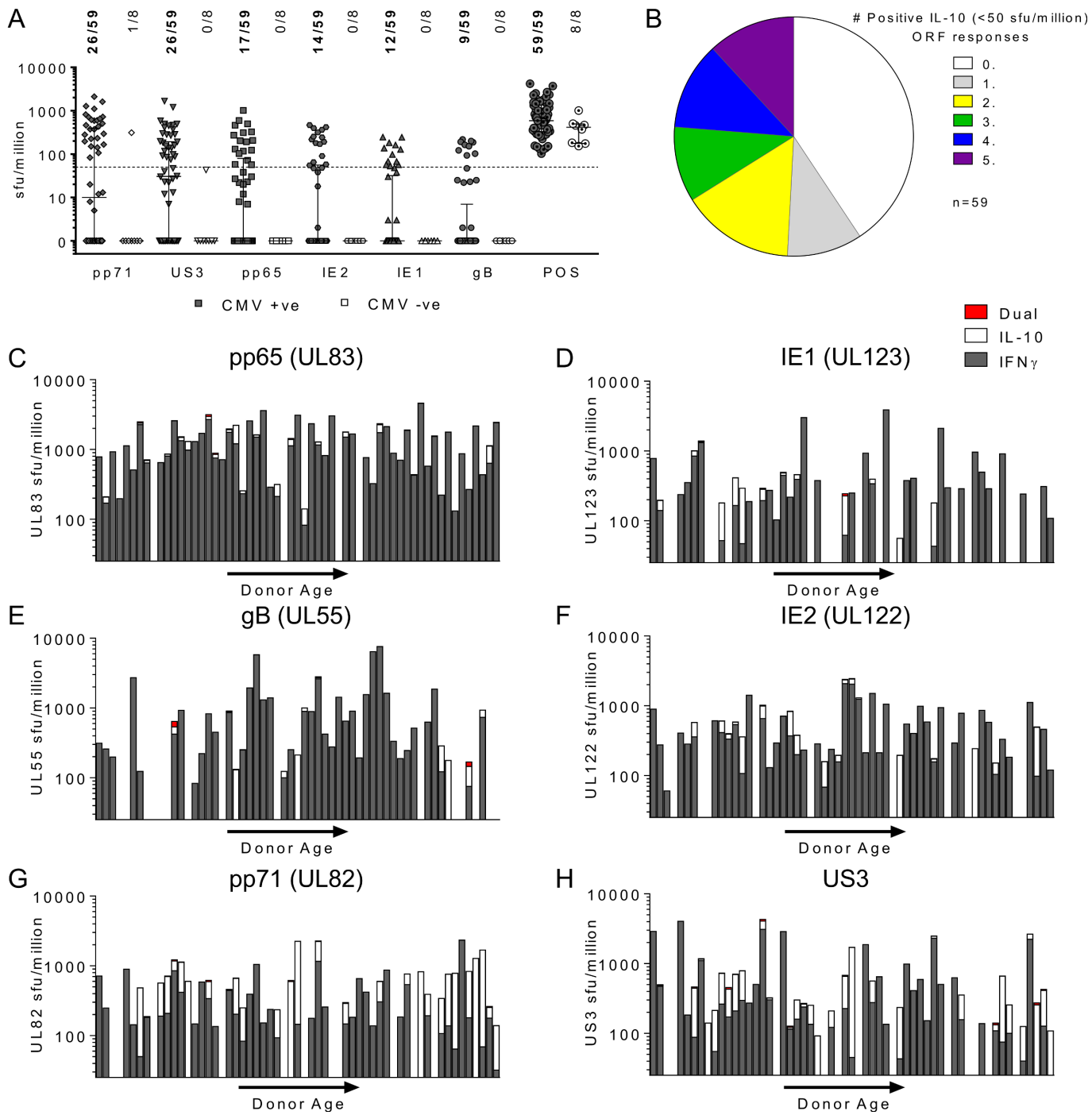

FIG 3

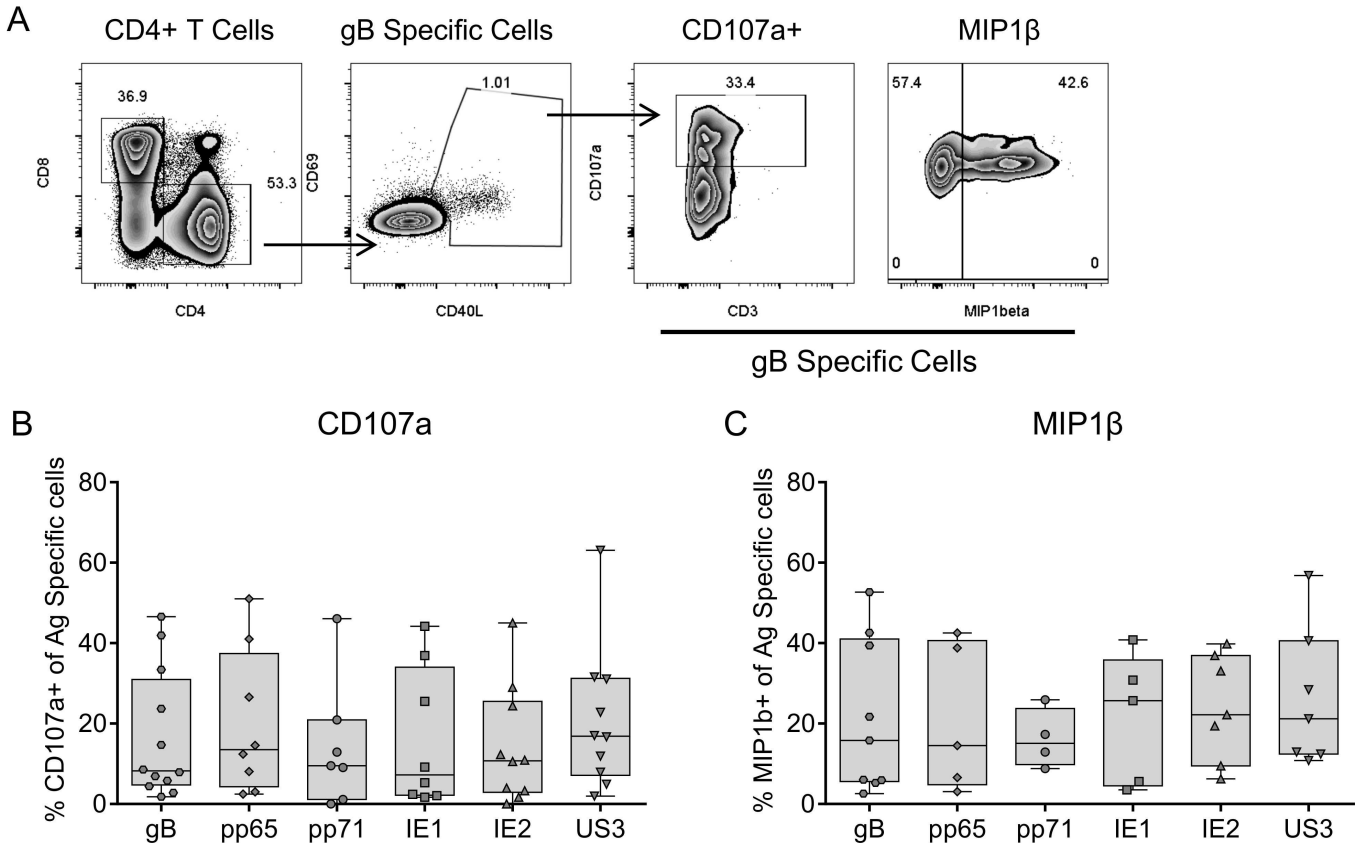

FIG 4

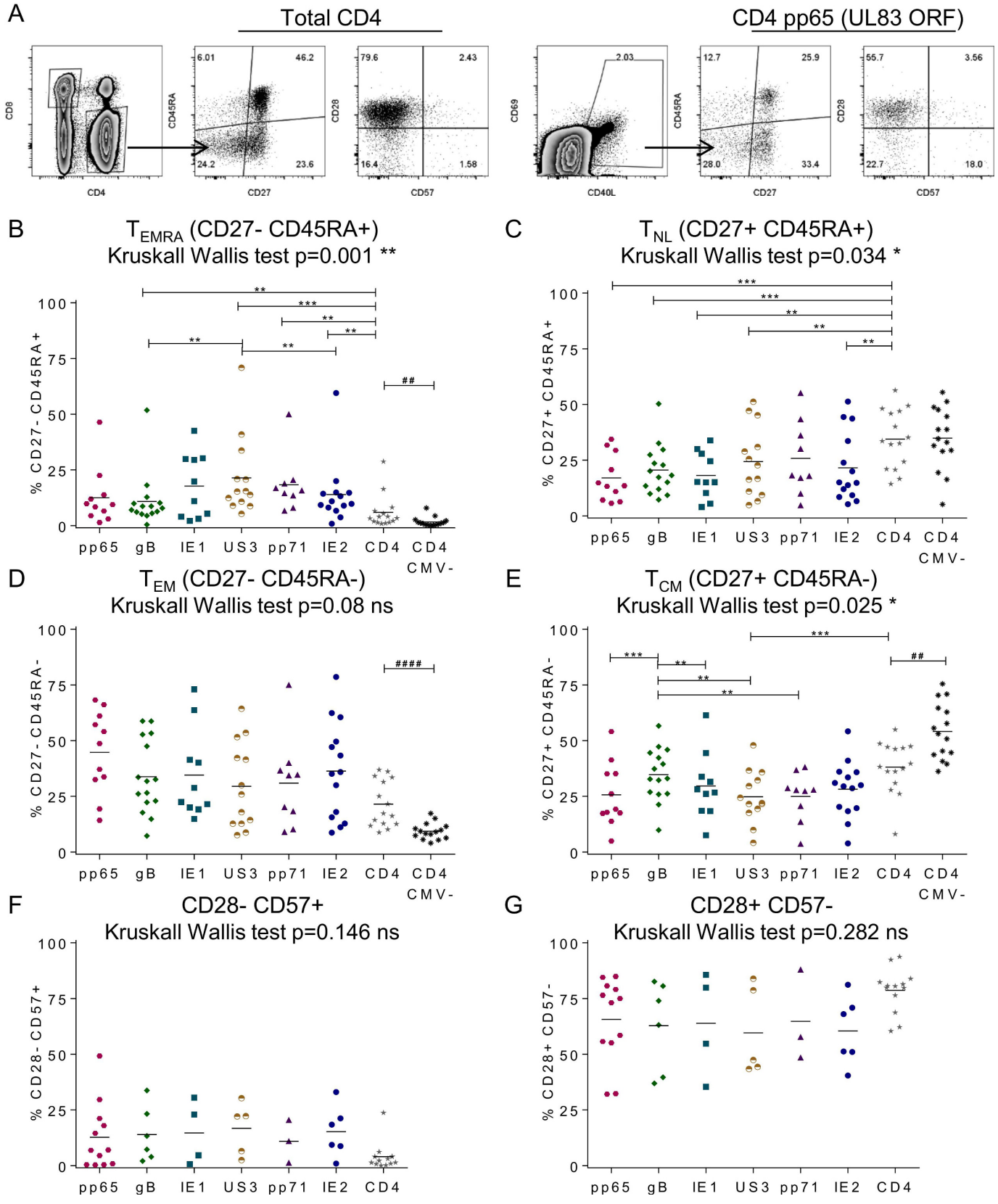

FIG 5

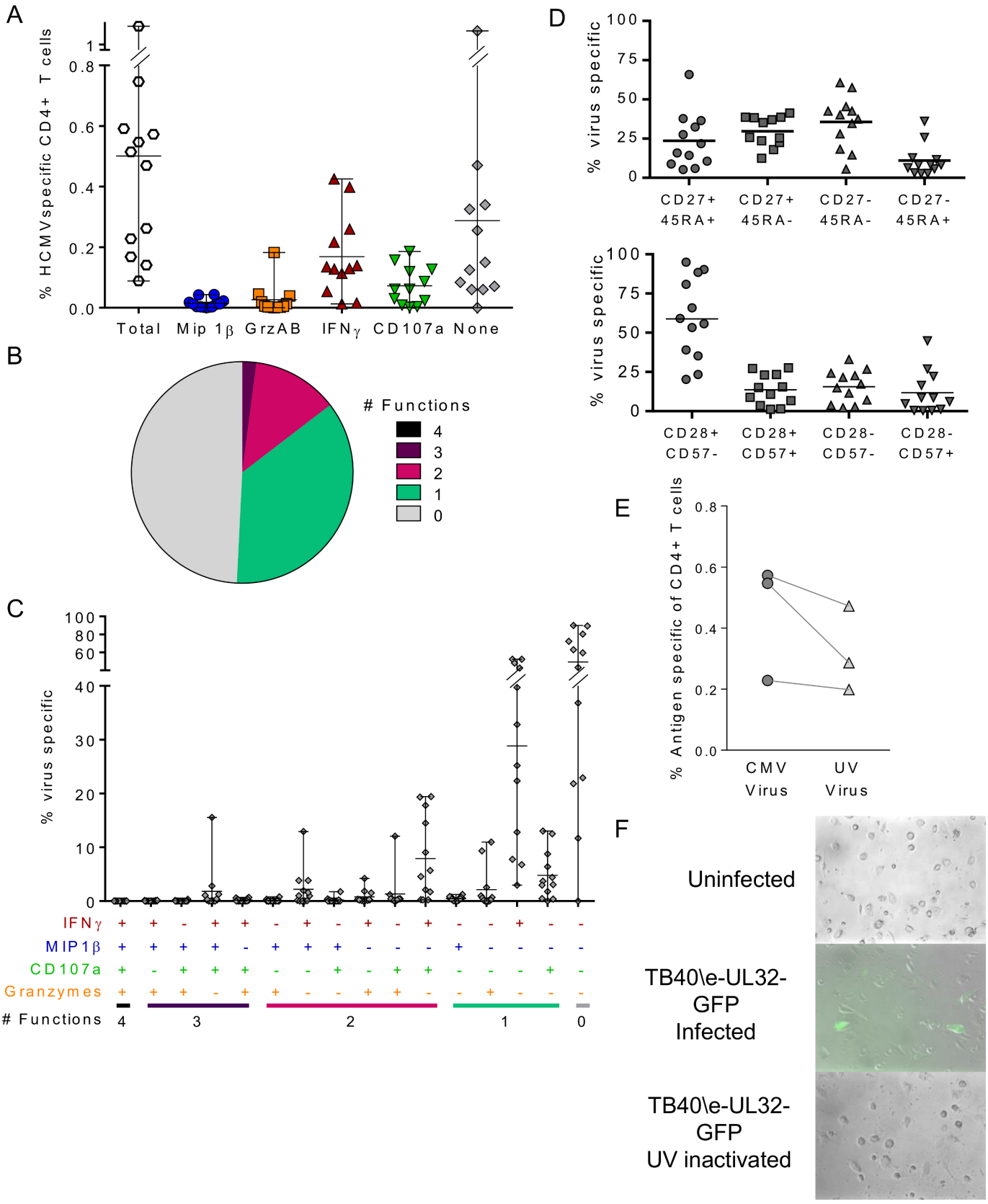

FIG 6