Diverse specificity, phenotype and anti-viral activity of cytomegalovirus specific CD8+ T cells.

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HCMV specific T cells phenotype and function

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ABSTRACT (224 words)

CD8+ T cells specific for pp65, IE1 and IE2 are present at high frequencies in HCMV seropositive individuals and these have been shown to have phenotypes associated with terminal differentiation, as well as both cytokine and proliferative dysfunctions, especially in the elderly. However, more recently, T cell responses to many other HCMV proteins have been described but little is known about their phenotype and function. Consequently, in this study, we chose to determine the diversity of HCMV specific CD8+ T cell responses to eleven HCMV ORFs in a cohort of donors aged 20 – 80 years old as well as their ability to secrete IFNγ. Finally, we also tested their functional anti-viral capacity using a novel viral dissemination assay.

We identified substantial CD8+ T cell responses by IFNγ ELISPOT assays to all eleven of these HCMV proteins and, across the cohort, individuals displayed a range of responses from the tightly focused to highly diverse which were stable over time. CD8+ T cell responses to the HCMV ORFs were highly differentiated and predominantly CD45RA+, CD57+ and CD28-, across the cohort. These highly differentiated cells had the ability to inhibit viral spread even following direct ex-vivo isolation. Taken together, our data argue that HCMV specific CD8+ T cells have effective anti-viral activity irrespective of the viral protein recognized across the whole cohort and despite viral immune evasion.

IMPORTANCE

Human cytomegalovirus (HCMV) is normally carried without clinical symptoms and is widely prevalent in the population, however, it often causes severe clinical disease in individuals with compromised immune responses. HCMV is never cleared after

primary infection but persists in the host for life. In HCMV carriers, the immune response to HCMV includes large numbers of virus-specific immune cells and the virus has evolved many mechanisms to evade the immune response. While this immune response seems to protect healthy people from subsequent disease the virus is never eliminated. It has been suggested that this continuous surveillance by the immune system may have deleterious effects in later life. The data presented in this paper examines immune responses from a cohort of donors and shows that these immune cells are effective at controlling the virus and can overcome the viruses lytic cycle immunevasion mechanisms.

INTRODUCTION

The β herpes virus human cytomegalovirus (HCMV) is a common infection worldwide (1). After primary infection, the virus establishes life-long persistence in individuals at least in part due to its ability to undergo latent infection in pluripotent CD34+ stem cells in the bone marrow and the myeloid cell lineages derived from them (2). Both primary infection with HCMV and its long-term persistence is largely sub-clinical for the majority of individuals. However, infection whether due to primary infection, reactivation from latency or superinfection in the immunocompromised or immature (such as HIV/AIDS patients, transplant patients or the foetus in utero, respectively) can be life threatening (1). Primary HCMV infection of an otherwise healthy host elicits responses from both the innate and adaptive arms of the immune system and evidence from patients undergoing bone marrow and stem cell transplantations has shown that the generation of HCMV specific CD4+ and CD8+ T cell responses is crucial for successful control of virus infection (3-7). This importance of T cell responses is further supported by evidence from the murine model of MCMV infection, where transfer of immediate early (IE) antigen specific CD8+ T cells into animals with an ablated immune system was protective from viral challenge (8). Other murine studies have also shown that removal of CD4+ T cells from mice leads to reactivation of virus resulting in disease (9). Additionally, CD8+ T cells also prevent lethal infection of mice with MCMV in the absence of CD4+ T cells (10, 11).

It is now well established that there are high frequency cytomegalovirus specific CD8+ T cell responses directed towards the pp65 (UL83) and IE1 (UL123) viral proteins in the majority of HCMV seropositive individuals (4, 12-15). Although the

responses to pp65 and IE1 are immunodominant and large, in most individuals there are CD8+ specific T cell responses to numerous other HCMV proteins (16) such that the frequency of the total CD8+ T cell response to HCMV in infected individuals has been estimated to comprise up to 10% of the total CD8+ T cell compartment in peripheral blood (16).

As a consequence of HCMV infection, it has been established that the composition of the T cell repertoire is altered resulting from virus specific cells undergoing major expansions which has been linked to the concept of “memory inflation” described in the murine model of MCMV (17) (18). The CD8+ IE and pp65 specific T cells that have been studied have a phenotype that has been linked to terminal differentiation as well as dysfunction defined by the re-expression of CD45RA (19-24); the loss of expression of the co-stimulatory molecules CD27 and CD28 (13, 21, 25-27) and expression of CD57, a marker of activation associated with differentiated T cells (21, 28-30). It has also been concluded, from these studies, that enlarged HCMV specific CD8+ T cell populations accumulate with age which include increasing numbers of “dysfunctional” T cells as defined by their highly differentiated phenotype; loss of cytokine secretion ability and limited proliferation capacity (21, 27, 31). These observations have led to the suggestion that the HCMV induced changes to the T cell immune system may become detrimental to individuals during their lifetime. These HCMV influenced changes to the phenotypes of host CD8+ T cells have been used to suggest on the basis of association that older seropositive individuals are more susceptible to infection, respond poorly to vaccinations and have an increased risk of mortality compared to age-matched HCMV sero-negative individuals (32-36). However, older individuals do not appear to suffer from overt HCMV disease

resulting from reactivating virus or re-infection which suggests that HCMV specific T cells in these elderly donors do retain the ability to control the virus infection (37). Other studies have also challenged the dogma that these expansions of HCMV specific T cells are dysfunctional and shown that the CD45RAhi HCMV specific CD8+ T cells found in elderly donors proliferate well when given the correct co-stimulation signals (38) and that the accumulating HCMV-specific CD8+ population tend to be polyfunctional as they secrete multiple anti-viral cytokines and are highly cytotoxic (39).

In this study, we wished to analyse the diversity of HCMV specific CD8+ T cell responses to eleven of the highest ranked T cell ORFs in a cohort of donors with wide age variation and to determine the stability of these responses over a period of years. Additionally, we wished to determine if T cells specific to HCMV antigens, other than pp65 and IE, displayed alternative differentiation phenotypes and, if so, how this correlated with IFNγ production, cytotoxicity and anti-viral activity. To address this, we screened a donor cohort aged 24 – 80 years old and measured the CD8+ T cell response to UL83 (pp65), UL82 (pp71), UL123 (IE-1), UL122 (IE-2), UL99, UL28, UL48, US29, US32, UL55 (gB), and US3 by IFNγ ELISPOT assays. The results showed substantial CD8+ T cell responses to all of these proteins and that, over a period of three years, the diversity of HCMV protein responses in individual donors were stable. Of the 11 ORFs analysed, we identified 6 which were recognized by the majority of the donor cohort (UL83, UL82, UL123, UL122, UL28 and US3) and performed both a functional and phenotypic analysis of the CD8+ T cells responses to these ORFs. Whilst many of the HCMV specific CD8+ T cells identified secreted IFNγ, there were a large proportion of antigen specific T cells that

did not secrete the cytokine. These analyses also revealed a highly differentiated memory T cell population common to all the ORFs studied and displayed by all individuals in the cohort. Finally, all the HCMV T cell specificities tested were able to prevent dissemination of a clinical isolate of HCMV through indicator fibroblasts..

MATERIALS AND METHODS

Donor sample collection and isolation

Heparinized peripheral blood was collected from healthy donors, HCMV serostatus was determined using an IgG enzyme-linked immunosorbent assay (Trinity Biotech, Didcot, United Kingdom). Eighteen HCMV-seropositive, and 4 HCMV-seronegative donors were included in this study. Ethical approval was obtained from the Addenbrookes National Health Service Hospital Trust institutional review board (Cambridge Research Ethics Committee) for this study. Informed written consent was obtained from all recipients in accordance with the Declaration of Helsinki (LREC 97/092). The age range of the HCMV-seropositive donors was 24 – 80 years, 5 donors were female (37.2 ± 12.3 years) and 13 male (47.5±16.9 years). All donors were HLA typed by genotyping by the Diabetes and Inflammation Laboratory, CIMR, University of Cambridge or the NHS Tissue Typing Service, Addenbrookes Hospital, Cambridge. Peripheral blood mononuclear cells (PBMC) were isolated using Lymphoprep (Axis-shield, Oslo, Norway) density gradient centrifugation. PBMC were either used fresh or frozen in liquid Nitrogen in a 10% DMSO (Sigma Aldrich, Poole, UK) and 90% Fetal Calf Serum (FCS) (PAA, Linz, Austria) solution. Frozen PBMC were rapidly thawed in a 37°C water bath and the freezing medium diluted into 25mls of fresh RPMI-10, centrifuged and resuspended in fresh RPMI-10 before use.

HCMV ORF peptide mixes

11 HCMV ORFs (UL28, UL48, UL55 (gB), UL82 (pp71), UL83 (pp65), 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 (16). The individual lyophilised peptides from each ORF library were reconstituted in 80% DMSO 20% RPMI 1640 (PAA laboratories, Austria) to give 40mg/ml master stock, the individual peptides were then diluted 1/40 in RPMI 1640 (unsupplemented) to give a 1mg/ml (2% DMSO) working stock. Peptide pools were used as either entire ORF mixes at a concentration of 5µg/ml/peptide or in the case of UL48 ORF) 10 pools of 48 peptides at a concentration of 20µg/ml/peptide was used in this study. Additionally ProMix HCMVA (pp65) and Promix HCMVA (US3) peptide pools (ProImmune) were diluted to the equivalent of 20µg/ml/peptide were also used.

Individual HLA typed HCMV and EBV peptides

Individual HLA restricted peptides from HCMV pp65 and IE1 used in this study were HLA-A2 NLVPMVATV (pp65 495 -504aa), HLA-A1 YSEHPTFTSQY (pp65 363 - 373aa), HLA-A3 KLGGALQAK (IE1 184 -192aa), HLA-B7 TPRVTGGGAM (pp65 417-426aa), HLA-B8 QIKVRVDMV (IE1 88-96aa) and HLA-A2 VLEETSVML (IE1 316 – 324aa). Additionally individual HLA restricted peptides from EBV BMLF-1, EBNA 3A and BZLF-1 were HLA-A2 GLCTLVAML (BMLF-1 259-267aa), HLA-A3 RLRAEAQVK (EBNA 3A 603-611aa), HLA-B7 RPPIFIRRL (EBNA-3A 247-255aa) and HLA-B8 RAKFKQLL (BZLF-1 190-197aa) (all ProImmune) were also used in the study.

ELISPOT assays

$2 \times 10^5 – 3 \times 10^5$ PBMC or PBMC depleted of CD4+ T cells, using magnetic cell separation and anti-CD4 conjugated beads (Miltenyi Biotech, Bisley, UK), were resuspended in supplemented RPMI 1640 + 10% FCS and IFNγ ELISPOT assays performed according to manufacturer's instructions (eBioscience, San Diego, USA), in 96-well PVDF membrane plates (Millipore, Billerica, USA). Cells were stimulated with entire ORF mix peptides (final peptide concentration 2μg/ml/peptide) or ORF pool mix peptides (final peptide concentration 5μg/ml/peptide) at 37ºC in a humidified CO_2 atmosphere for 48 hours. All the ELISPOT assays were performed on fresh PBMC the same day as taking the blood, assays were all performed to a fixed protocol and using antibody sets from the same company as well as ELISPOT plates with identical incubation periods. PHA was used as a positive control in every assay. Developed plates were recorded by ELISPOT plate reader (AID, Strassberg, Germany) and counted using ImageJ software (National Institutes of Health, USA).

Intracellular cytokine and phenotype staining

2×10^6 PBMC resuspended in RPMI + 10% FCS were stimulated with ORF peptide mixes or individual mapped peptides overnight. After 2 hours incubation 5μg/ml Brefeldin A (BD Biosciences, San Jose, USA) was added for the remainder of the incubation period. Cells were then washed and stained with a combination of surface antibodies including CD3 eFluor 650NC (eBioscience) or CD3 Qdot655 (Invitrogen, Paisley, UK), CD45RA PE-Cy7, CD27 APC-eFluor 780 (eBioscience), CD8 AlexaFluor 700, CD57 AlexaFluor 647 (BioLegend, San Diego, USA), CD28 PerCPCy5.5, CD45RO FITC (BD Biosciences) and LIVE/DEAD Fixable Yellow Dead cell stain (Invitrogen). Cells were then fixed and permeabilised using FIX&PERM (ADG, Kaumberg, Austria). Peptide specific T cells were identified by the co-

expression of CD69 and 41BB according to established published protocols (40-43) in brief, cells were stained intracellularly with CD69 Pacific Blue, 4-1BB PE-Cy5 (BioLegend) and IFNγ 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 (BD Biosciences). Data was analysed using FlowJo software (Treestar, Oregon, USA).

Virus

HCMV strain TB40/e UL32-GFP (gift of Christian Sinzger, University of Tübingen, Tübingen, Germany) grown in HFFs as previously described (44) was used in this study.

Expansion of HCMV specific CD8+ T cells

CD8+ T cells were isolated from PBMC using MACS anti-CD8 direct beads (Miltenyi Biotec, Bisley, United Kingdom) magnetic separation and then resuspended in supplemented RPMI + 10% Fetal Bovine Serum (FBS) (Invitrogen) + 10% heat inactivated autologous donor serum. Cells were stimulated with peptide pulsed irradiated autologous PBMC in the presence of 2.5IU/ml human recombinant IL-2 (National Institute for Biological Standards and Control, Potters Bar, United Kingdom) in round bottom 96 well plates at 37ºC +5% CO_2 for 10 – 14 days, fresh media was replenished every five days. Specificity of expanded CD8+ T cell cultures using mapped peptides was determined by specific pentamer staining, cells were harvested and washed and then stained with the specific unlabelled pentamer (ProImmune), washed and then further stained with pentamer specific PE

fluorophore (ProImmune) and anti-CD8 and anti-CD3 antibodies conjugated to PerCP-Cy5.5 and FITC respectively, cells were then fixed and acquired on a FACS Sort using CellQuest software (BD Biosciences) and data analysed using FlowJo software. All expanded T cell lines were also tested for specificity using IFNγ ELISPOT assays. Briefly, resting CD8+ T HCMV specific cells were harvested and washed and then 2000 T cells were incubated in IFNγ coated PVDF membrane plates with 50 000 peptide pulsed autologous B-lymphoblastoid cell lines (LCL) and with unpulsed and mitogen stimulated controls for 48 hours at 37ºC +5% CO_2. ELISPOT assays were again developed according to manufacturer's instruction.

Measurement of cytotoxicity

The cytotoxic capability of the expanded HCMV specific CD8+ T cell lines was assessed using a chromium release assay. Briefly, the rested CD8+ T cell lines were harvested, washed in acidified RPMI-10 and plated at between 15 – 80:1 E:T ratios in triplicate. Target cells used comprised autologous LCL lines, the cells were washed in PBS and $Na_2Cr^{57}O_4$ (Perkin Elmer) added to the cell pellet, and incubated at 37°C for 45 min, appropriate peptide or no peptide was then added to the target cells and incubated for an additional 45 min at 37°C. Target cells were washed three times in acidified RPMI + 10% FBS medium and added to the assay plate (containing effectors, medium-only wells, and wells containing Nonidet P-40, as described above) at between 2000 – 4000 cells/well in acidified medium. Plates were incubated at 37°C for 5 h, after which 70 µl of supernatant was harvested from each well and used to quantify radioactive emission. Percent specific lysis was calculated using the following formula: percent specific lysis = 100 x (target release – spontaneous release)/(maximum release – spontaneous release).

In vitro viral dissemination assay

The ability of specific HCMV CD8 T cells to control the spread of HCMV virus *in vitro* was measured. Autologous or partial HLA matched dermal fibroblasts were seeded in a 24 or 48 well plate to be 80-90% confluent when they were infected with TB40e UL32-GFP virus at an MOI of 0.03. Rested HCMV specific CD8+ T cell lines were harvested, washed and resuspended in supplemented RPMI 1640 + 10% FBS then added to the infected fibroblasts 24 hours post infection at a series of T cell:Fibroblast ratios of 5, 2.5, 1.2, 0.6, 0.3:1, each experiment included a CD8+ T cell line specific to a HLA matched individual peptide from EBV (listed earlier) as a control. In further experiments total CD8+ T cells isolated directly *ex vivo* from HLA matched CMV seropositive and seronegative donors were added to infected fibroblasts 24 hours post infection at a series of T cell: Fibroblast ratios of 5, 2.5, 1.2 or NLV and VLE MHC Class I pentamer FACS-sorted CD8+ T cells from PBMC directly ex vivo, at T cell: Fibroblast ratios of 0.14 and 0.08. The viral dissemination assay was incubated at 37ºC +5% CO_2, assessment of viral dissemination was performed at 14 and 21 days by detection of GFP expression by both fluorescent microscopy and flow cytometry, the data was analysed and the percentage of GFP positive fibroblasts was expressed as a proportion of the Infected Control for each time point.

Statistics

Statistical analysis was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). The correlation between age and the T cell

response to HCMV was assessed by Pearson or Spearman correlation according to the distribution of the data. The Wilcoxon matched pairs test was used to compare two groups of matched data and 1 way ANOVA paired Friedman test compared three groups of matched data.

RESULTS

HCMV specific T cell responses vary widely in their diversity between individuals.

The whole proteome screen performed by Sylwester et al in 2005 (16) examined the CD8+ and CD4+ T cell responses in a cohort of 33 individuals chosen to represent a wide variety of ethnic and HLA backgrounds. Based on this analysis, we selected 11 of the most frequently recognized HCMV ORFs by CD8+ T cells which included 6 of the highest frequency CD8+ T cell responses derived from a meta-analysis of data from a number of independent studies (45). We determined the frequency of the CD8+ T cell response to each of these ORFs in a cohort of 18 CMV sero-positive donors and 4 CMV sero-negative donors using IFNγ ELISPOT assays. The 18 CMV seropositive donors represented a wide range of ages (24-77 years at the start of the study) and included 5 older donors aged between 56-77 years. Table 1 summarises the age, gender and HLA information for each donor, and details the number of HCMV ORFs responded to by CD8+ T cells (defined by a response > 100 sfu/million above background (unstimulated) by IFNγ ELISPOT).

The results showed considerable inter-donor variation in the pattern of HCMV ORFs which CD8+ T cells responded to, and that some donors mounted a more diverse response while other donors had a more focused HCMV ORF response (Figure 1A and B). Comparison of donor age and the number of HCMV ORFs recognised also showed that the age of the donor had little impact on the number of HCMV ORFs recognised (Figure 1C). We also calculated the cumulative T cell response in each donor and correlated this with the donor age. There was a trend towards an increase

in the magnitude of the CD8+ T cell response with age, but this did not reach significance for this cohort (Spearman r=0.43, n=18) (Figure 1D). However, when the analysis was performed on the number of ORFs that were high frequency (>1000 SFU/million) (Figure 1E) this strongly correlated with age (Pearson r=0.53, n=18, p=0.02).

The frequency of the CD8+ T cell response of each individual donor to each HCMV ORF was also tallied and ranked, and then subdivided into responders (> 100 sfu/million by IFNγ Elispot) (Figure 1F) and the sub-set of high frequency responders (> 1000 sfu/million by IFNγ Elispot) (Figure 1G). We identified 4 HCMV ORFs, which the majority of the donors in the cohort responded to, UL123 (IE1), UL83 (pp65), UL122 (IE2), UL82 (pp71), which many donors also recognized at high frequency in addition to UL28 and US3 which were also seen at high frequency (Figure 1F and G). The CD8+ T cell responses to these selected ORFs are further characterised in this study.

The diversity and magnitude of HCMV specific T cell responses were not significantly changed over time.

T cell responses to HCMV in some individuals were clearly less diverse compared to the HCMV-specific T cell responses of others in the cohort. In order to determine if donors with less diverse responses generated responses to additional HCMV ORFS or if donors with diverse responses could lose responses we determined the stability of the CD8+ T cell responses by repeating the HCMV ORF screen analysis at 24 months (n=11) (Figure 2A) and 36 months (n=7) (Figure 2B) after the original

analysis. While the magnitude of individual ORF responses does vary with individual donors over the three time points at 24 months, 9/10 ORFs showed no statistically significant differences in the magnitude of the response for each donor. A decrease in the magnitude of the IE1 (UL123) response was observed over this 2 year period ($p=0.01$, Wilcoxon matched pairs test), a further examination of this response at 36 months (1 way ANOVA paired Friedmans test) revealed that, overall, there was no statistically significant difference in the size of the IFNγ response in any of the ORFs examined. The data also shows that no new CD8+ T cell ORF responses were observed for any individual donor in the cohort at either 24 or 36 months following the original analysis.

We noted that within the data presented in Figure 2 there was an obvious change in the frequency of the response to individual HCMV ORFs for some donors. To clarify this observation we further analysed the responses to UL83 (pp65), UL82 (pp71), UL123 (IE1) and US3 HCMV ORFs in 5 donors within the cohort which had been sampled at multiple time points over a 40 month period by IFNγ Elispot. (Figure 3A), there was a 4-fold difference in the magnitude of the detectable response to the HCMV ORF in an individual over this time period. The specific ORF responses for donors CMV 305, CMV 300 and CMV 301 were also collated (Figure 3B). We observed a decrease or increase across a number of HCMV ORF responses at selected time points for each donor.

HCMV specific T cells are predominantly T_{EMRA} cells irrespective of their antigen specificity

353 Numerous studies of pp65 (UL83) and IE1 (UL123) specific T cells in the peripheral
354 blood of healthy donors have reported that HCMV pp65 specific CD8+ T cells have a
355 differentiated memory phenotype compared to other virus specific T cells; this is
356 characterised by the loss of expression of the co-stimulatory molecule CD27 and re-
357 expression of CD45RA (19-23, 28, 46). Within our donor cohort, we identified 4
358 additional HCMV ORFs (UL122, UL28, UL82 and US3), besides UL83, UL123,
359 which generated CD8+ T cell responses in a large proportion of subjects examined
360 (Figure 1F and G). Consequently, we asked whether these ORF specific CD8+ T
361 cells directed against these other HCMV ORFs had a similar memory phenotype to
362 that previously described for pp65 and IE specific T cells. The phenotype of HCMV
363 specific CD8+ T cells from a range of donors (encompassing the age range of the
364 donor cohort) was determined by co-expression of 4-1BB, CD69, CD45RA and
365 CD27 after ORF stimulation. Following the exclusion of doublet and dead cells, four
366 memory T cell subsets were defined (Figure 4A). A representative activation and
367 phenotype analysis following UL28 stimulation is illustrated (Figure 4A). To
368 demonstrate that CD69 and 4-1BB co-expression identifies antigen specific T cells in
369 combination with CD45RA and CD27 as memory subset markers we analysed the
370 CD8+ T cell response from three donors taken from the cohort, in parallel cells were
371 stained with MHC Class I pentamers containing either pp65 epitope NLV or IE
372 epitopes (VLE and QIK) or stimulated with these peptides and identified antigen
373 specific cells by CD69 and 4-1BB expression in combination with CD45RA and
374 CD27 expression. The results show that the phenotypic distribution of Naïve, T_{EM},
375 T_{CM} and T_{EMRA} identified by MHC Class I pentamers was very similar to the
376 proportions identified by CD69 and 4-1BB expression (Figure 4B). The size of the
377 antigen specific T cell population identified by pentamer staining compared to that

identified by CD69/4-1BB co expression was variable between the donors, the NLV response was very similar in donor CMV324 (pentamer v CD69/4-1BB, 2.5% v 2.3%), in donor CMV307 the size of the pentamer population was larger than the CD69/4-1BB population (pentamer v CD69/4-1BB, 6% v 2.4%) and in donor CMV301 the pentamer population was smaller than the CD69/4-1BB population e.g. NLV (pentamer v CD69/4-1BB, 1% v 1.9%).

Six donors were analysed using 6 HCMV ORF stimulations (Figure 4C). As expected, the results confirmed previous analyses that pp65 and IE specific T cells were predominantly T_{EMRA} cells (CD45RA+ CD27-) (22, 38, 47, 48). However, we were also able to show that T cells specific for another immediate early antigen, US3, and another late antigen, pp71, were also dominated by T_{EMRA} cells as well as subpopulations of T_{EM} (CD45RA- CD27-) and T_{CM} (CD45RA- CD27+) cells. CD8+ T cells specific for two further ORFs, UL28 and IE2, also had a very similar pattern of T cell subset distribution.

HCMV specific CD8+ T cells can secrete cytokines and are cytotoxic

We also assessed the ability of the different antigen specific CD8+ T cell populations we had identified in our cohort to secrete IFNγ following ORF stimulation. ORF reactive T cells were, again, identified by expression of 4-1BB and CD69 following the exclusion of doublet and dead cells; in addition the T cells were permeabilized and stained for intracellular IFNγ expression as a control the % IFNγ expression in cells not expressing 4-1BB and CD69 was also determined in most cases this was negative or <0.1% . The results show that all ORF specific T cells from all donors,

had populations of T cells which were IFNγ positive as well as negative. In every donor, we could identify individual ORF responses in which the T cells had predominantly lost the ability to make IFNγ as well as other ORF responses where the ability to make IFNγ was predominantly retained (Table 2).

As T_{EMRA} cells are considered to be more highly differentiated, we also determined the ability of UL82, UL83, UL28, US3, UL123 and UL122-specific T_{EMRA} (CD27-CD45RA+) cells to secrete IFNγ following stimulation (Figure 5). The results show that only a proportion of these cells retained the ability to make IFNγ, however this was observed in all the donors tested.

The gain of CD57 and loss of CD28 expression are also well recognized markers of the late differentiation status of T_{EMRA} cells. Consequently, we determined the percentage of CD28- and CD57+ HCMV ORF specific T_{EMRA} cells in each of the donors (Table 3). The results clearly show that, irrespective of antigen specificity, the majority of the T_{EMRA} cells had lost CD28 expression and gained CD57 expression and that this occurred to the same extent in all of the donors.

In addition to assessing IFNγ function of T cells specific to different HCMV ORFs, we also measured their cytotoxic capability. T cells lines specific to UL82, UL28, US3 and UL122 encoded proteins were expanded *in vitro*. The specificity of these T cell lines was confirmed by IFNγ ELISPOT or MHC Class I pentamer specific staining (data not shown) and then they were used in chromium release cytotoxicity assays. As expected, CD8+ T cells specific for pp65 and IE1 from all donors tested elicited

cytotoxicity (Figure 6). Similarly, UL82, UL28, US3 and UL122 specific CD8+ T cells also demonstrated good cytotoxic function.

HCMV specific CD8+ T cells are able to control viral dissemination *in vitro*

We next wanted to assess the ability of individual HCMV ORF specific CD8+ T cells to recognize virus infected cells. Consequently, we developed a novel *in vitro* assay to measure the ability of virus specific CD8+ T cells to control the spread of HCMV virus in culture. Primary dermal fibroblasts derived from specific donors were infected with a low MOI of a GFP-tagged clinical strain of HCMV (TB40e UL32GFP) and then co-cultured with individual ORF specific CD8+ T cells at a range of effector:target cell ratios. After 10 to 28 days of co-culture, the spread of the virus through the fibroblast layer was measured by flow cytometic analysis of GFP expression in the indicator fibroblasts. The specificity of inhibition of HCMV spread by HCMV specific T cells was controlled for by including T cell lines specific for EBV derived from each donor in the assays. Additionally, we included a positive control of infected fibroblasts with no T cell co-culture as well as a negative control of uninfected fibroblasts in each assay.

A representative assay comparing UL83 (pp65) and UL123 (IE1) specific CD8+ T cells derived from donor 307 is illustrated (Figure 7A and B) and shows that both IE and pp65 specific T cells are highly effective at controlling spread of HCMV even at the lowest E:T ratio of 0.6:1. Importantly, EBV specific T cells from the same donor could not inhibit HCMV spread. We repeated this analysis on pp65 and IE1 CD8+ T cell lines derived from four additional donors with similar results (Table 4).

449 The effector T cells used in these assays were generated from resting memory cells,
450 previously expanded *in vitro*, and whilst the analysis clearly shows that these *in vitro*
451 generated cell lines have specificity and were able to prevent viral dissemination, the
452 *in vitro* manipulation might not represent effector cells generated *in vivo*. To address
453 this, we isolated total CD8+ T cells directly *ex vivo*, from two different HCMV
454 seropositive and two HCMV seronegative donors for which we had also derived an
455 autologous fibroblast cell line. Total CD8 T cells were added at E:T ratios of 5:1 and
456 2.5:1 to the viral dissemination assays. The results show that T cells from HCMV
457 seropositive donors prevented viral dissemination, while T cells from HCMV
458 seronegative donors did not (Figure 7C). We also performed this experiment using
459 defined antigen specific T cells, NLV pp65 specific or VLE (IE) specific T cells were
460 isolated directly *ex vivo* using MHC Class I pentamers and FACS, from a HLA-A2
461 seropositive donor and total CD8 T cells from HLA-A2 seroponegative donor and the
462 T cells used in a viral dissemination assays. The results again show that the direct
463 ex vivo isolated HCMV antigen specific T cells exhibited direct anti-viral function,
464 preventing viral dissemination in these assays (Figure 7D).

465

466 Finally, we also examined the ability of CD8+ T cells specific for UL82 (Figure 8A)
467 and US3 (Figure 8B) to prevent viral dissemination and clearly observed both US3
468 and UL82 specific CD8+ T cells were also able to control the spread of a non-
469 attenuated, clinical strain of HCMV. It was noted that the EBV specific T cell line did
470 show some non-specific viral control at the higher E:T ratio but this was not observed
471 at the lower E:T ratio (Figure 8B).

DISCUSSION

In this study, we have investigated the T cell response to 11 HCMV ORFs in a cohort of 18 donors ranging from 20 to 80 years of age. Within this cohort, some donors had only a small diversity of responses to HCMV ORFs whereas other donors responded to a much broader range of HCMV antigens. There was a significant correlation between age and the number of ORFs that elicited high frequency (>1000 SFU/million) T cell responses. The memory phenotype of CD8+ T cells specific for 6 HCMV ORFs recognized by most donors, including pp65 and IE, showed that the predominant phenotype of CD8+ T cells for all the HCMV ORFs was CD27-CD45RA+ T_{EMRA} cells across the cohort tested. Likewise, the distribution of IFNγ expressing T cells and markers of late differentiation (presence of CD57 and loss of CD28). Similarly, pp65 and IE specific T cells were both effective anti-viral T cells.

Memory T cell "inflation" was originally reported in MCMV infection of mice, where the magnitude of memory and long term memory T cell responses was measured following primary MCMV infection (17, 49, 50). Based on these studies, it has been suggested that the same phenomenon also occurs in the latently infected human host. However, there have been no extensive longitudinal studies on latently infected individuals and, as such, memory T cell inflation in the human host has only been inferred by analysis of donors over a cross section of ages and, almost exclusively, by examining pp65 and IE specific T cell responses (21, 31, 34, 51, 52). It is clear that elderly individuals often have high frequency pp65 and IE specific T cell responses (21, 47, 53-57) although it is not unusual to also observe this in the young (39). Clearly, an important issue is the use of the age of a donor to infer the

length of time that individual has been carrying HCMV. Yet it is important to note that infection in early childhood would mean that a 30 year old (classified as young) would have been carrying virus for just as long as a 75 year old who was infected in their mid-forties. Given this caveat, our data shows that the cumulative T cell frequency including HCMV ORF specificities other than pp65 and IE tends to increase with age and that the number of ORFs that are at high frequency (>1000 SFU/million) was strongly correlated with age, thus supporting the idea that periodic HCMV reactivation provides antigenic stimulation to drive increased T cell responses.

Analysis of the T cell frequency to 11 different HCMV ORFs, in a number of donors at multiple time points for up to 36 months, showed that the frequency of T cells which recognised these ORFs did not show inflation and in fact demonstrated both increased and decreased frequency. A detailed analysis of some of the cohort sampled at multiple time points over 40 months confirmed that the frequency to an individual ORF in an individual donor varies with time. These observations are in complete agreement with a study published by Crough and colleagues (58) who first described this periodic fluctuation in both HCMV and EBV specific CD8+ T cells over a 9-25 week period and provided evidence of sub-clinical viral reactivation driving expansions. Clearly this fluctuation continues to occur over years, however as an important caveat, the antigen specific frequency was determined using a functional assay (IFNγ ELISPOT) as such the fluctuations noted could be due to changes in the proportion of cells with this function rather than changes in the absolute size of the population. Interestingly, ORFs that were not recognized in the original analysis were not recognised in analyses at later time points. Consequently, a longitudinal study over much longer periods of time will be required in humans if the process of

522 “memory inflation” is to be observed and the use of a more absolute measure of
523 frequency such as MHC Class I multimers in conjunction with functional measures
524 would be most informative .

525

526 The expansion of a T_{EMRA} CD8+ HCMV specific population has been previously
527 reported for pp65 and IE specific T cells (14, 19, 20, 22, 23, 28, 46). Our
528 observations also show that T cells for other HCMV ORFs show a similar expansion
529 of this T cell phenotype across the cohort tested. It was also noted that the
530 distribution of memory T cell phenotypes in a given donor was very similar
531 irrespective of the ORF specificity, this was also observed with in the two donors
532 who were analysed by MHC Class I pentamers to either IE or pp65 specific peptides
533 both donors had similar T cell subset distributions regardless of the epitope
534 specificity (Figure 4). Although accumulation of T_{EMRA} CD8+ T cells in HCMV has
535 been associated in some studies with a loss of T cell functionality (21, 34), more
536 recent studies from ourselves and others have shown that these T cells are actually
537 polyfunctional as long as they are correctly co-stimulated and are able to proliferate
538 and secrete multiple anti-viral cytokines as well as markers of cytotoxic potential (38,
539 39).

540

541 The T_{EMRA} CD8+ HCMV specific cells for all the HCMV ORF specificities identified in
542 this study, despite having a highly differentiated CD57+ CD28- phenotype, were also
543 able to secrete IFNγ following antigen stimulation. The low percentage of IFNγ
544 secretion by CMV ORF specific CD8+ T cells by some donors has been previously
545 observed in other studies (34, 59, 60). The changing pattern of cytokine secretion by

546 antigen specific T cells has been proposed as a measure of the differentiation status
547 of the memory T cell population (61-64). In particular the loss of IFNγ secretion
548 capacity coupled with the secretion of CC Chemokines (MIP-1beta, MIP-1alpha, and
549 RANTES) has been associated with very late stage differentiated CD8+ T cells and
550 has been observed principally in CMV specific CD8+ T cells (65).

551

552 The relationship between HCMV T cell antigen specificity, the frequency of highly
553 differentiated T cells and their overall anti-viral functionality with respect to the
554 changes that are observed in elderly individuals remains unclear. HCMV infection in
555 the elderly has been associated with a detrimental impact on the health of elderly
556 individuals (32-36) and it is suggested that this may result from the T cell response
557 to HCMV, itself, becoming dysfunctional, with a subsequent loss of control of the
558 virus, as well as a concomitant degradation of the host immune system, in general,
559 to the point when the generation of new responses are impaired (66). Despite the
560 association of HCMV with a loss of immune function, older sero-positive individuals
561 do not appear to suffer from overt HCMV disease from either reactivating virus or
562 new infections, suggesting that HCMV specific T cells do retain the ability to control
563 the virus (37). T cell functional fitness is assessed as the ability of T cells to mount
564 appropriate polyfunctional responses by multi-analyte analysis or even mediate
565 cytotoxicity. However, this is an indirect measure of anti-viral activity and previously
566 published assays have not, so far, used HCMV infected cells as targets.
567 Consequently, they do not assess functionality in a background of, for instance, the
568 well described MHC Class I evasion mechanisms which occur during HCMV lytic
569 infection.

570

In our analyses, we have developed a viral dissemination assay, with T cell co-culture, in order to provide a direct measure of anti-viral activity. This now allows an assessment of different HCMV ORF specificities from the same donor for their anti-viral potential. Interestingly, such assays showed that T cells specific for the late antigen pp65 were as effective at preventing viral dissemination as IE specific T cells. All the pp65 and IE specific T cells derived from individual members of the cohort were effective at preventing viral dissemination.

HCMV is a paradigm for viral immune evasion of T cell recognition, yet it has always been somewhat of a paradox that a virus with such an extensive array of immune evasion mechanisms elicits a very strong T cell response following primary infection (25, 28) and, likely, reactivation as immunosuppressed HCMV seropositive patients often lose the ability to control viral replication resulting in end organ disease. These observations suggest that the T cell immunity must also be effective at preventing disease following reactivation in normal healthy individuals. The data presented in this manuscript supports this conjecture as T cells specific for both early and late gene products are highly effective at preventing viral dissemination in an *in vitro* model and the viral immunevasion strategies appear unable to allow avoidance of T cell immune responses in the context of lytic infection. Our view is that the real value of the T cell immune evasion functions of HCMV is to allow a latent virus in an individual cell sufficient time to undergo reactivation and assemble new virus rather than to allow unrestricted viral replication that ends up causing disease in the host upon a primary infection or following reactivation.

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TABLE AND FIGURE LEGENDS

Table 1 - Donor information and summary of HCMV CD8+ T cell responses.

The age, gender and HLA allele typing data for 18 HCMV sero-positive and 4 sero-negative donors examined in this study. The number and identity of HCMV ORFs recognized (>100 sfu/million cells) and the high frequency ORFs (>1000 sfu/million are summarized.

Table 2 Frequency of HCMV ORF specific T cells and proportion that secrete IFN γ

ORF specific T cells were identified by 4-1BB and CD69 co-expression in 6 donors, IFNγ secretion was determined by intracellular staining. The percentage of HCMV ORF specific CD8+ T cells which secrete IFNγ for each donor was determined. IFNγ secretion by cells not expressing 4-1BB and CD69 unactivated (unact) was also determined.

Table 3 Percentage of HCMV ORF specific CD8+ T_{EMRA} cells with a CD28- and CD57+ phenotype

ORF specific CD8+ T cells were identified by 4-1BB and CD69 expression in 6 donors. The proportion of HCMV specific T_{EMRA} cells that are CD57+ and CD28- were enumerated.

Table 4 pp65 and IE1 specific CD8+ T cells control viral dissemination in an *in vitro assay*

The percentage of TB40eUL32-GFP+ fibroblasts (expressed as a percentage of the Infected Control) present at Day 21 for UL83 (pp65) and UL123 (IE1) specific cells compared to EBV specific cells for 5 different donors examined are shown.

Figure 1 – The HCMV ORF specific diversity of CD8+ T cell responses varies widely between donors.

Frequency of the CD8+ T cell response to 11 HCMV ORFs in 18 donors is shown. The responses were measured by IFNγ ELISPOT and shown as sfu/million cells (spot forming unit/million cells) following the subtraction of background counts from unstimulated cells. The donor cohort is arranged according to the age of the donor and the size of the response to each HCMV ORF is shown as a heat map (A). The number of ORFs each donor responded to (>100 sfu/million) (B), and also plotted according to the age of the donor (C). There was no statistical correlation between age and the number of ORFs an individual recognised by Pearson correlation. The cumulative IFNγ response to all HCMV ORFs for each donor was plotted according to donor age and this was not significantly correlated with age by Spearman correlation (D). The number of ORFs each donor responded to at high frequency (>1000 sfu/million) was also correlated with age by Pearson correlation, there is a significant increase in high frequency ORFs in older donors p=0.02 (E). The frequency of recognition by CD8+ T cells of each HCMV ORF is shown for all responses >100 sfu/million (F) and the high frequency responses (>1000 sfu/million) (G) (a subset of the data in (F)), the HCMV ORFs were ranked according to the number of subjects responding.

Figure 2 – The frequency and diversity of HCMV ORF CD8+ T cell responses was not significantly changed over time.

HCMV ORF specific T cell frequency was determined for 18 donors in 2009 by IFNγ ELISPOT and then repeated for 11 donors in 2011 (24 months) and again on 7

donors in 2012 (36 months). The responses for each ORF are shown for the 24 month (A) and the 36 month (B) data (2009, black; 2011, white; 2012 grey points). The positive response (100 sfu/million) cut off line is indicated on each graph. The 24 month paired data was tested using a Wilcoxon matched pairs test, significant results * ($p<0.05$) are indicated. The 3 time points in the 36 month data group were tested using a 1 way ANOVA paired Friedman test which showed no significant change in the variance of the data.

Figure 3 – There is fluctuation in the magnitude of an individual donor's CD8+ T cell response to particular HCMV ORFs.

HCMV ORF specific T cell frequency was determined by IFNγ ELISPOT at multiple time points over a 40 month period. The responses of 5 different donors for UL83 (pp65), UL82 (pp71), UL123 (IE1) and US3 HCMV ORFs (A) show that for an individual the magnitude of the response both decreases and increases of the period of time observed. Also the responses of donors CMV 305, CMV 300 and CMV 301 to selected HCMV ORFs are shown for the same period of time (B). The positive response (100 sfu/million) cut off line is indicated on each graph.

Figure 4 – HCMV specific CD8+ T cells have a predominantly CD45RA+ CD27- (T_{EMRA}) phenotype.

PBMC were stimulated overnight with mapped HCMV ORF peptides or HCMV ORF pools in the presence of Brefeldin A. Identification of HCMV specific CD8+ T cell responses was as shown in the example gating strategy (A); antigen specific CD8+ populations were identified by expression of 4-1BB and CD69 and 4 memory populations defined according to the expression of CD27 and CD45RA

(CD27+CD45RA+, T Central Memory (T_{CM}) (CD27+CD45RA-), T Effector Memory (T_{EM}) (CD27-CD45RA-) and T Effector Memory CD45RA+ cells (T_{EMRA}) (CD27-CD45RA+). Comparison of the CD27 and CD45RA phenotype of mapped peptide stimulated 4-1BB and CD69 positive CD8+ T cells with matched pentamer identified CD8+ T cells (B). The memory phenotype of 6 HCMV ORFs (pp65, pp71, UL28, IE1, IE2 and US3) identified by expression of CD69 and 4-1BB for 6 donors was examined the data for each ORF is arranged in increasing age order from left to right on the x-axis (C).

Figure 5 – HCMV specific CD8+ T cells from 6 different ORFs had a very similar pattern of IFNγ secretion.

PBMC from 6 donors were stimulated overnight with mapped HCMV ORF peptides or HCMV ORF pools in the presence of Brefeldin A. Antigen Specific populations were identified as described in Figure 5 and briefly shown in (A), the IFNγ production by the T_{EMRA} antigen specific CD8+ T cell subset was determined. The proportion of the HCMV specific T_{EMRA} population which secreted IFNγ (black) as a proportion of the total percentage of antigen specific T_{EMRA} cells (grey) for the different HCMV ORFs examined: pp65, pp71, UL28, IE1, IE2 and US3; the data for each ORF was arranged in increasing age order from left to right on the x-axis (B). The proportion of HCMV specific T_{EMRA} cells which secrete IFNγ varies between donors but there was no relationship with either age or HCMV ORF specificity.

Figure 6 – HCMV specific CD8+ T cells specific for pp65, IE1, pp71, UL28, US3 and IE2 mediated cytotoxicity.

HCMV specific CD8+ T cells were used as effector cells in chromium release assays to determine the cytotoxicity function of cells specific for pp65, IE1, pp71, UL28, US3 and IE2 HCMV ORFs. Target cells were donor matched lymphoblastoid B cell lines which were pulsed or not with mapped peptides for each donors known HCMV ORF responses. CD8+ T cells specific to all the HCMV ORFs examined show specific lysis of peptide pulsed target cells compared to activity against unpulsed target cells.

Figure 7 – Both pp65 and IE1 specific CD8+ T cells control viral dissemination in an *in vitro assay*.

Donor matched dermal fibroblasts were infected with TB40e-UL32-GFP virus at a low MOI, HCMV specific *in vitro* expanded CD8+ T cells were co-cultured with the virus infected fibroblasts at a range of effector:target ratios. The percentage of GFP+ fibroblasts was measured by flow cytometry at 14, 21 and 28 days in comparison to an uninfected, infected control and a non-specific CD8+ T cell line (EBV specific). A representative example from Donor 307 at Day 14 of the assay, show dot plots (A) and a summarised bar chart (B) of data expressed as a proportion of the Infected control for pp65 and IE1 specific CD8+ T cells which are both equally able to control dissemination of virus. CD8+ T cells directly *ex vivo* from HLA matched CMV seropositive and seronegative donors were co-cultured with the virus infected fibroblasts, the percentage of GFP+ fibroblasts as a proportion of the Infected control at Day 21 of the assay are summarised (C). Directly *ex* vivo HCMV specific CD8+ pentamer sorted cells at 0.14:1 and 0.08:1 effector:target ratios were able to control the dissemination of virus, data shown at Day 21 of the assay (D).

Figure 8 – UL82 and US3 specific CD8+ T cells control viral dissemination in an *in vitro* assay.

Donor matched dermal fibroblasts were infected with TB40e-UL32-GFP virus at a low MOI, HCMV specific *in vitro* expanded CD8+ T cells were co-cultured with the virus infected fibroblasts at a range of effector:target ratios as described (Figure 6). Summary bar chart show the percentage of GFP+ fibroblasts present at Day 21 for UL82 (A) and US3 (B) specific CD8+ T cells compared to UL83 (pp65) and UL123 (IE1) specific cells respectively (expressed as a proportion of the Infected Control). Both UL82 and US3 specific CD8+ T cells are able to control dissemination of virus in this assay.

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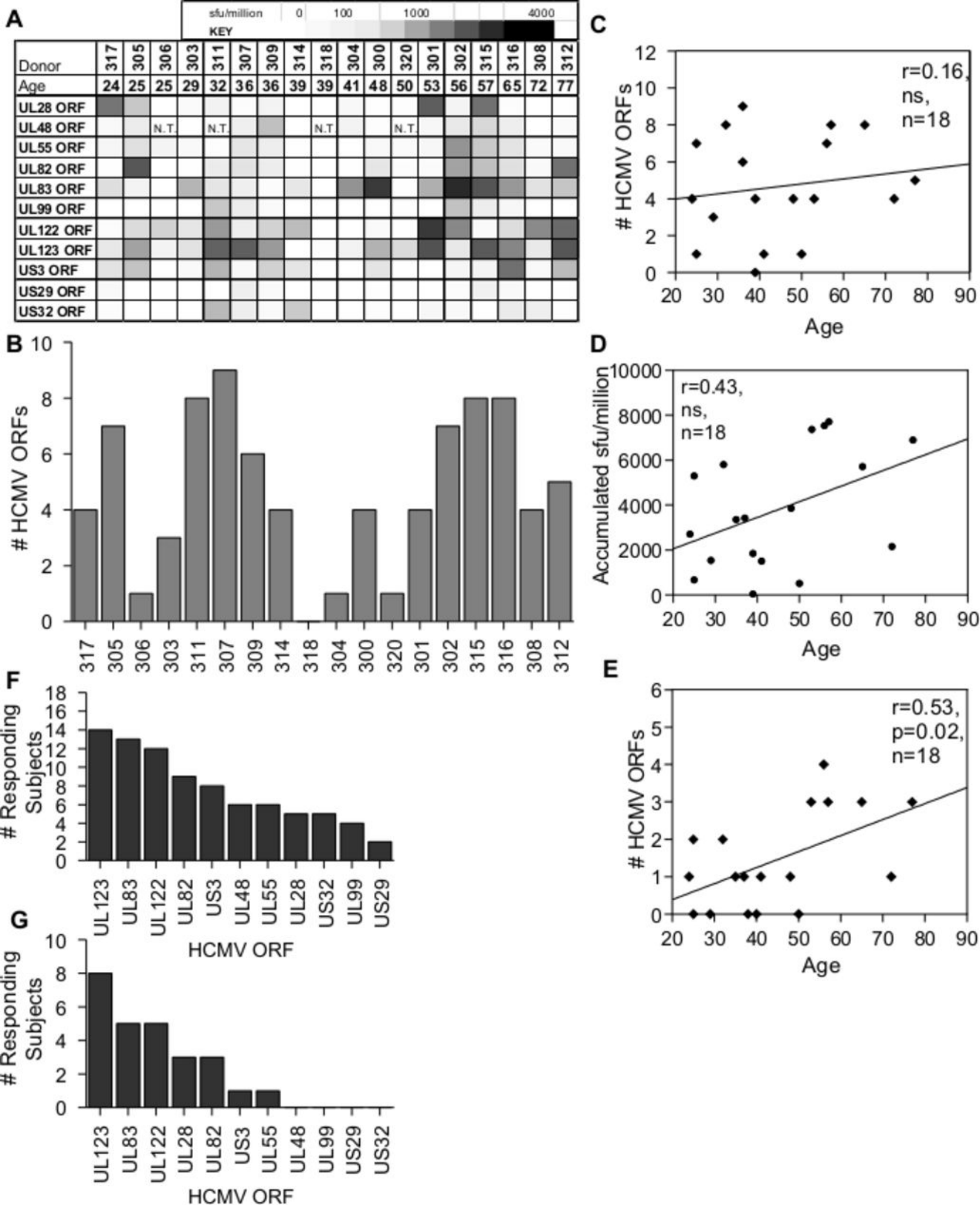
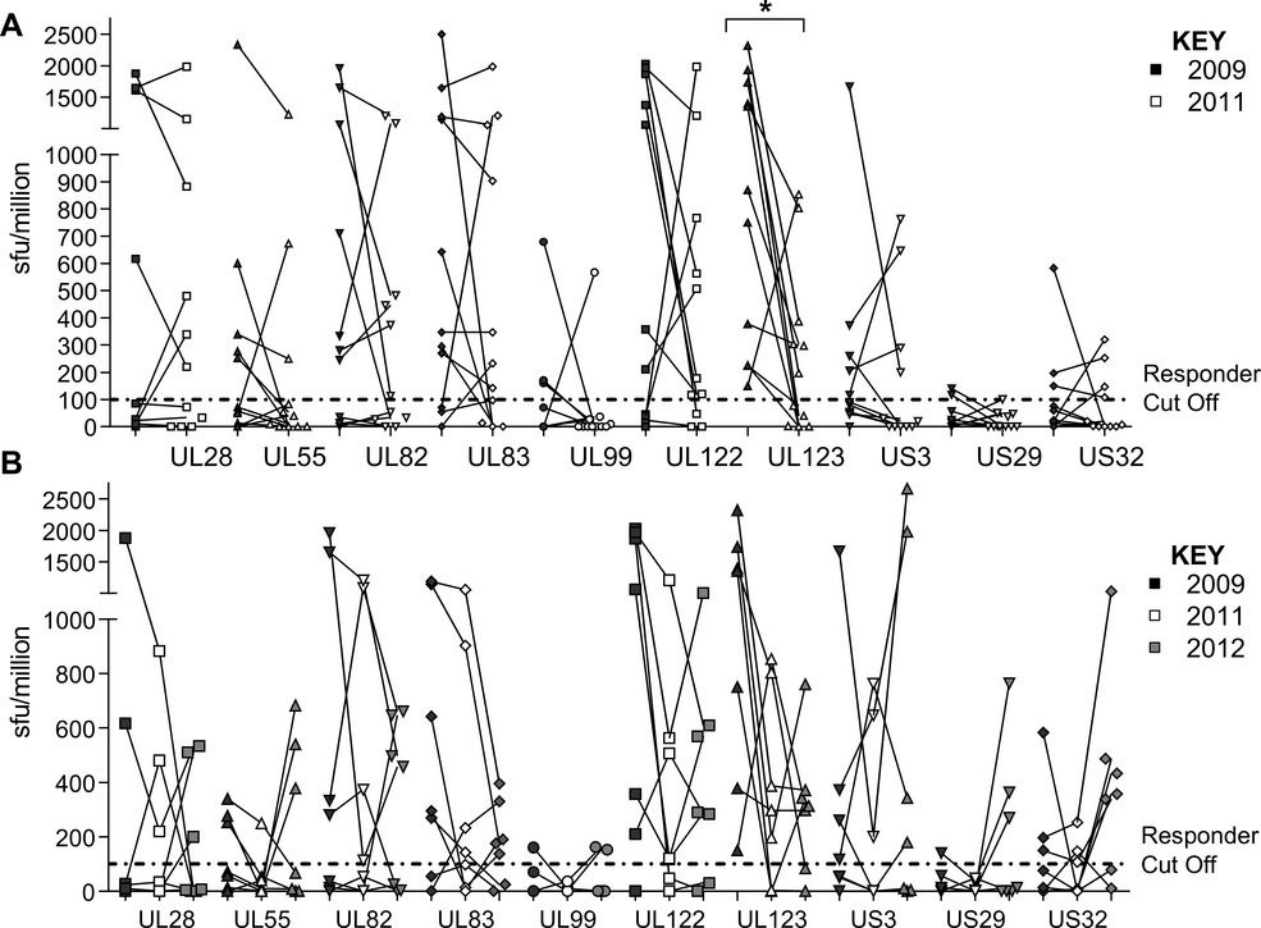
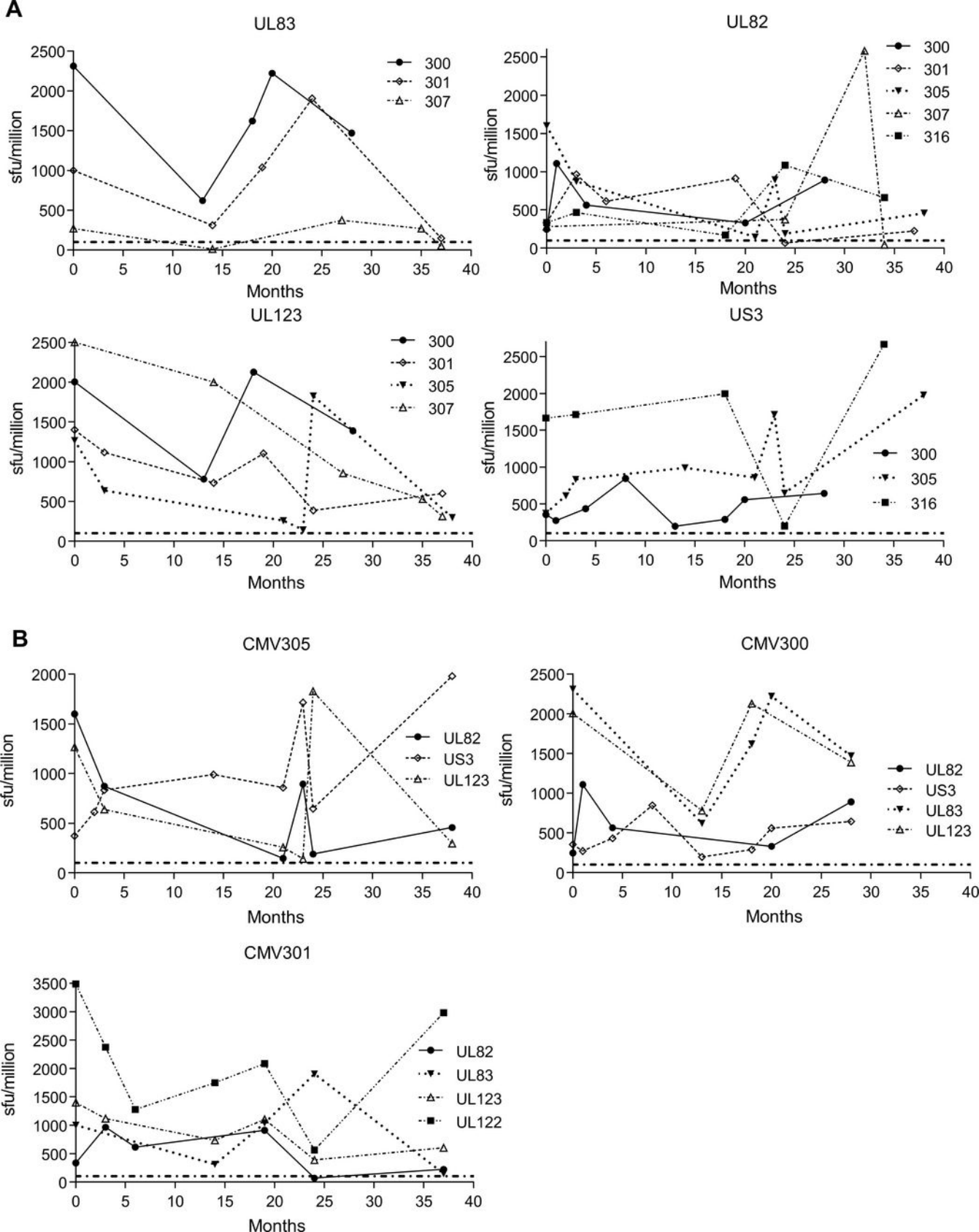
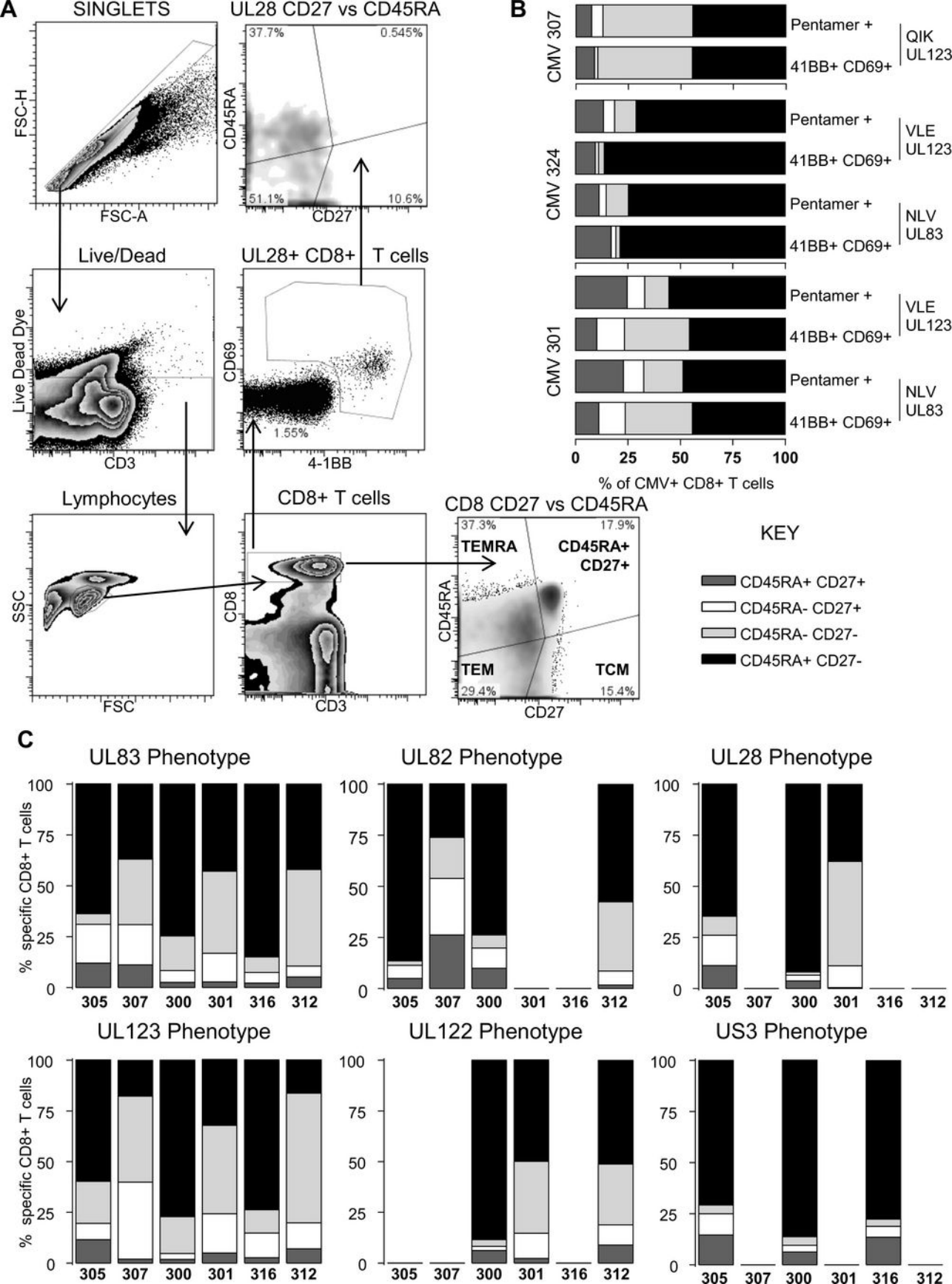
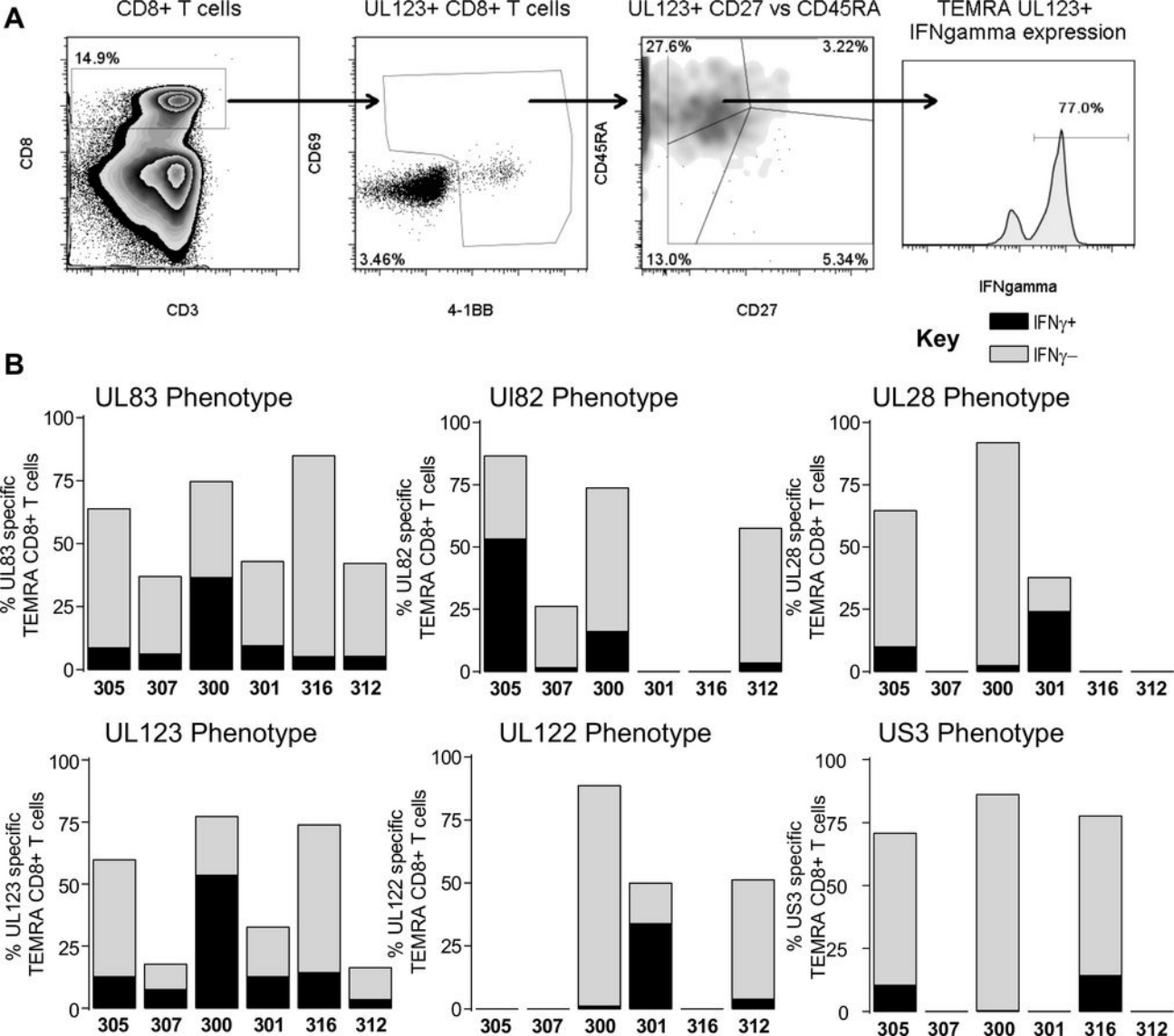
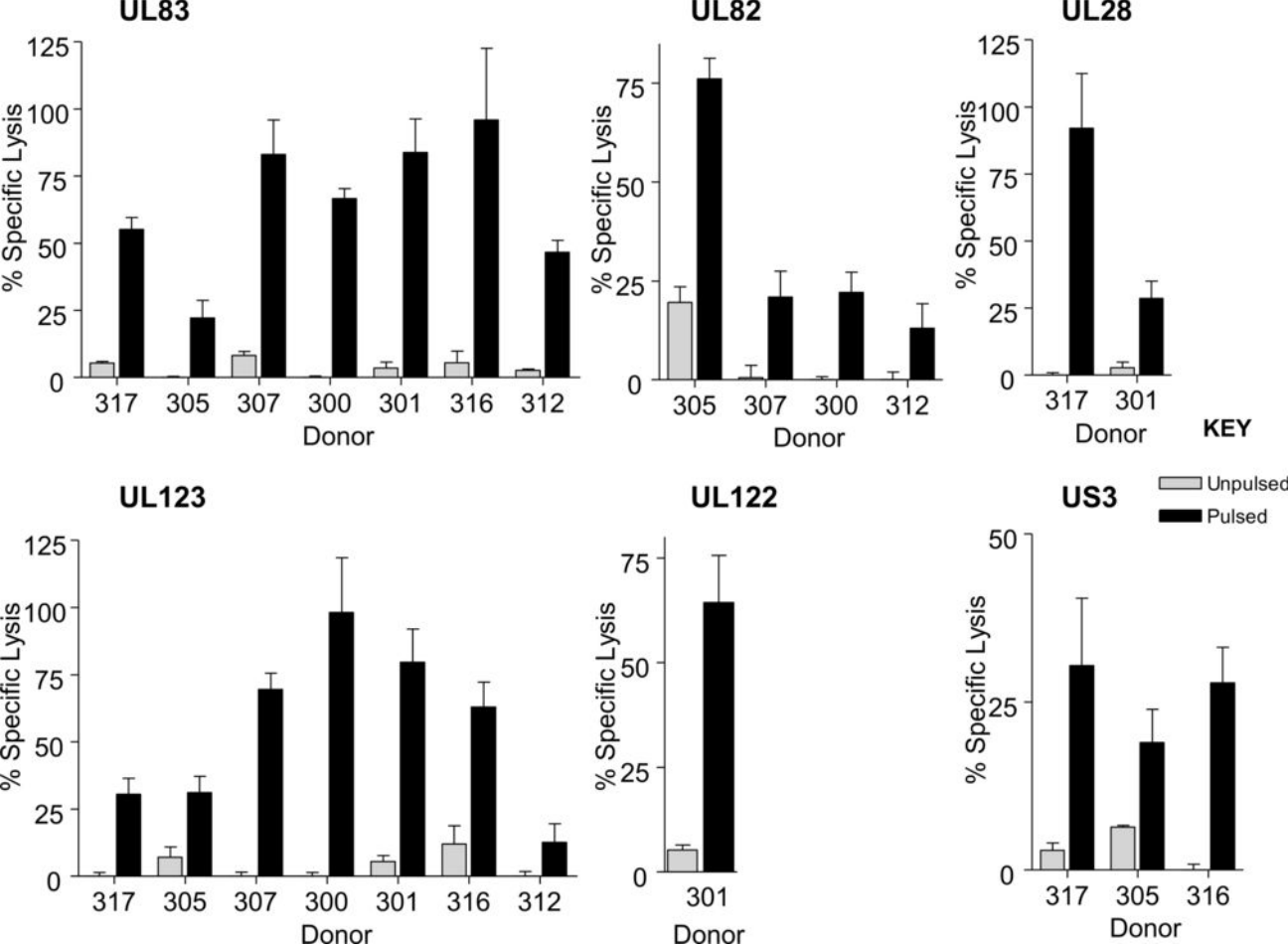

A
sfu/million
0
100
1000
4000
KEY
Donor
317 305 306 303 311 307 309 314 318 304 300 320 301 302 315 316 308 312
Age
24 25 25 29 32 36 36 39 39 41 48 50 53 56 57 65 72 77
UL28 ORF
UL48 ORF
UL55 ORF
UL82 ORF
UL83 ORF
UL99 ORF
UL122 ORF
UL123 ORF
US3 ORF
US29 ORF
US32 ORF
N.T.
B
HCMV ORFs
317 305 306 303 311 307 309 314 318 304 300 320 301 302 315 316 308 312
C
# HCMV ORFs
r=0.16,
ns,
n=18
Age
D
Accumulated sfu/million
r=0.43,
ns,
n=18
Age
E
# HCMV ORFs
r=0.53,
p=0.02,
n=18
Age
F
Responding Subjects
UL123 UL83 UL122 UL82 US3 UL48 UL55 UL28 US32 UL99 US29
HCMV ORF
G
Responding Subjects
UL123 UL83 UL122 UL28 UL82 US3 UL55 UL48 UL99 US29 US32
HCMV ORF

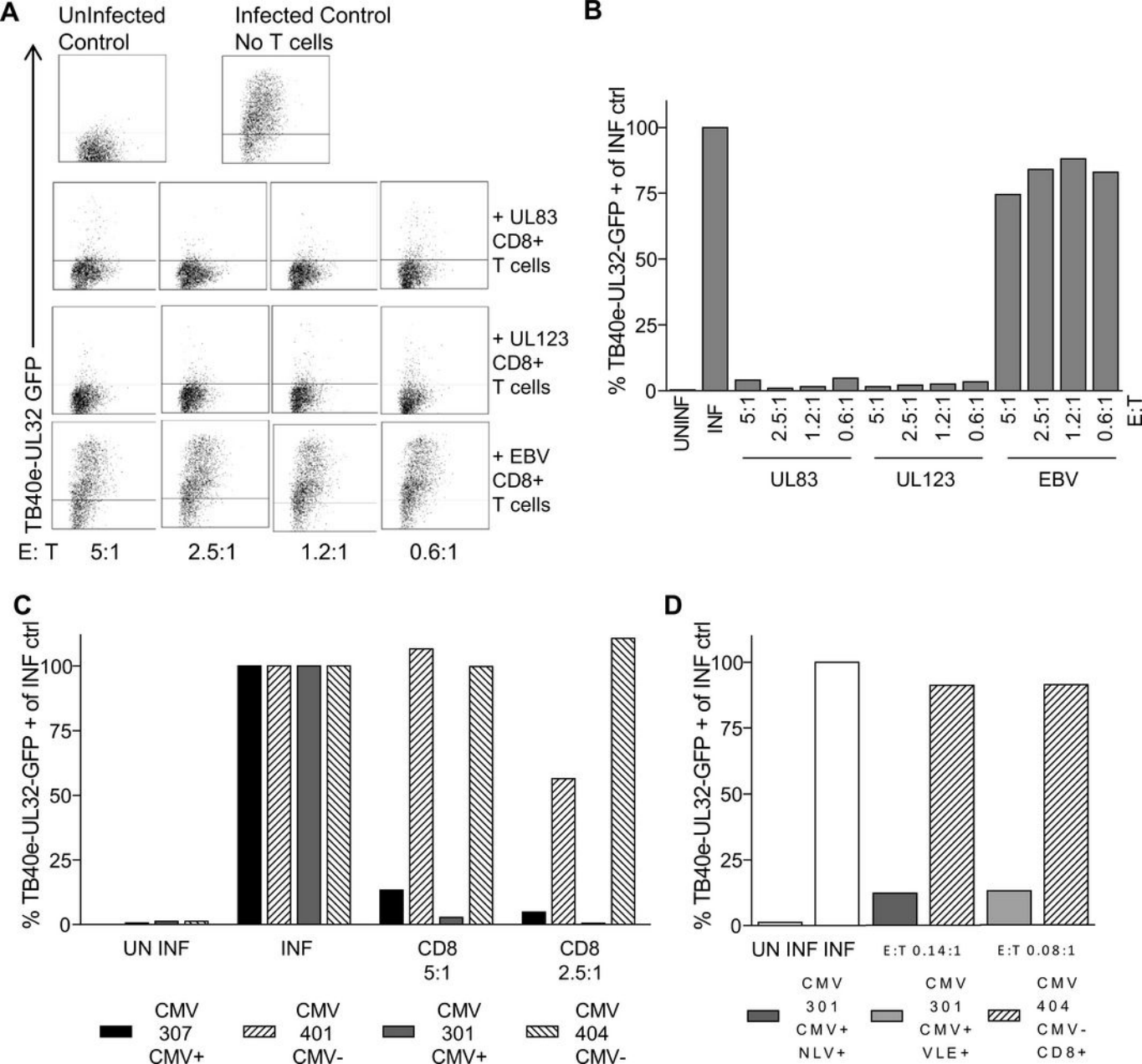

A
B
sfu/million
KEY
2009
2011
2012
Responder
Cut Off
*
UL28
UL55
UL82
UL83
UL99
UL122
UL123
US3
US29
US32

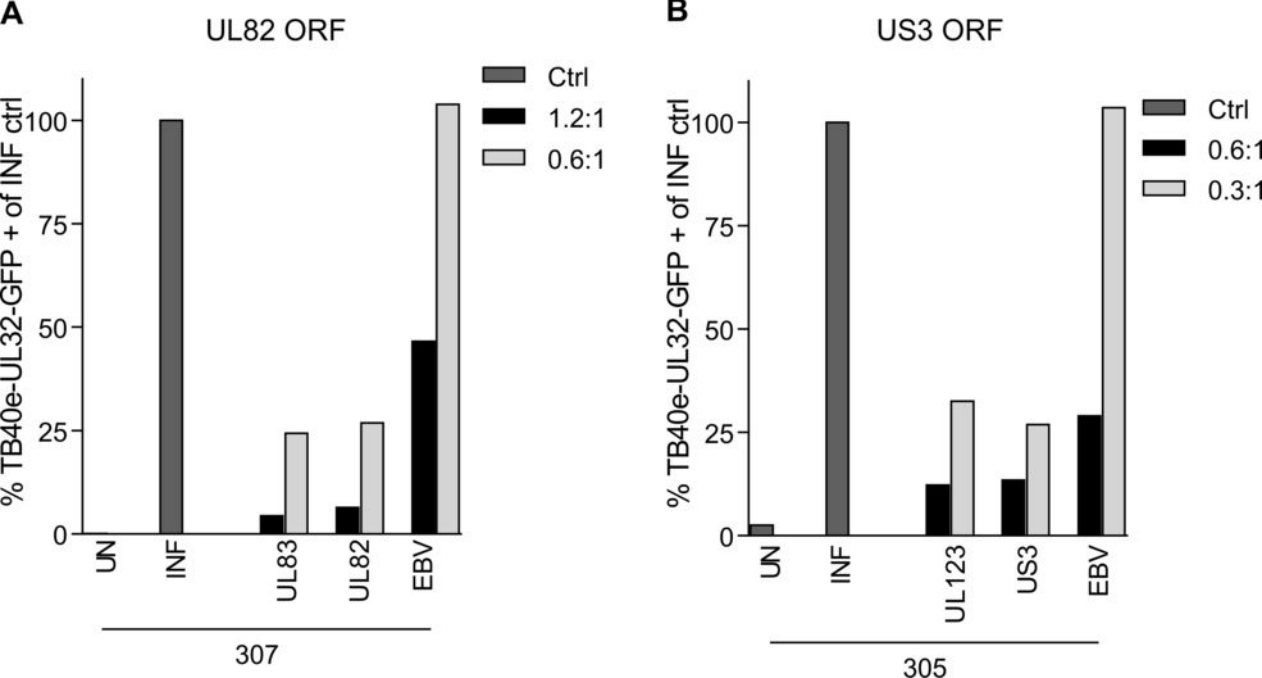

A
UL83
sfu/million
Months
300
301
307
UL82
300
301
305
307
316
UL123
300
301
305
307
US3
300
305
316
B
CMV305
UL82
US3
UL123
CMV300
UL82
US3
UL83
UL123
CMV301
UL82
UL83
UL123
UL122


A
SINGLETS
FSC-H
FSC-A
UL28 CD27 vs CD45RA
37.7%
0.545%
CD45RA
51.1%
10.6%
CD27
Live/Dead
Live Dead Dye
CD3
UL28+ CD8+ T cells
CD69
1.55%
4-1BB
Lymphocytes
SSC
FSC
CD8+ T cells
CD8
CD3
CD8 CD27 vs CD45RA
37.3%
17.9%
TEMRA
CD45RA+
CD27+
CD45RA
TEM
TCM
29.4%
15.4%
CD27
B
CMV 307
CMV 324
CMV 301
Pentamer +
41BB+ CD69+
QIK
UL123
VLE
UL123
NLV
UL83
VLE
UL123
NLV
UL83
0
25
50
75
100
% of CMV+ CD8+ T cells
KEY
CD45RA+ CD27+
CD45RA- CD27+
CD45RA- CD27-
CD45RA+ CD27-
C
UL83 Phenotype
UL82 Phenotype
UL28 Phenotype
% specific CD8+ T cells
0
25
50
75
100
305
307
300
301
316
312
UL123 Phenotype
UL122 Phenotype
US3 Phenotype


A
CD8+ T cells
UL123+ CD8+ T cells
UL123+ CD27 vs CD45RA
TEMRA UL123+ IFNgamma expression
14.9%
CD8
CD3
CD69
3.46%
4-1BB
27.6%
3.22%
CD45RA
13.0%
5.34%
CD27
77.0%
IFNgamma
Key
IFNγ+
IFNγ–
B
UL83 Phenotype
% UL83 specific TEMRA CD8+ T cells
UI82 Phenotype
% UL82 specific TEMRA CD8+ T cells
UL28 Phenotype
% UL28 specific TEMRA CD8+ T cells
UL123 Phenotype
% UL123 specific TEMRA CD8+ T cells
UL122 Phenotype
% UL122 specific TEMRA CD8+ T cells
US3 Phenotype
% US3 specific TEMRA CD8+ T cells
0
25
50
75
100
305
307
300
301
316
312


UL83
% Specific Lysis
125
100
75
50
25
0
317
305
307
300
301
316
312
Donor
UL82
% Specific Lysis
75
50
25
0
305
307
300
312
Donor
UL28
% Specific Lysis
125
100
75
50
25
0
317
301
Donor
KEY
Unpulsed
Pulsed
UL123
% Specific Lysis
125
100
75
50
25
0
317
305
307
300
301
316
312
Donor
UL122
% Specific Lysis
75
50
25
0
301
Donor
US3
% Specific Lysis
50
25
0
317
305
316
Donor


A
UnInfected Control
Infected Control No T cells
+ UL83 CD8+ T cells
+ UL123 CD8+ T cells
+ EBV CD8+ T cells
TB40e-UL32 GFP
E: T 5:1 2.5:1 1.2:1 0.6:1
B
% TB40e-UL32-GFP + of INF ctrl
100
75
50
25
0
UNINF
INF
5:1
2.5:1
1.2:1
0.6:1
UL83
UL123
EBV
E:T
C
% TB40e-UL32-GFP + of INF ctrl
UN INF
INF
CD8 5:1
CD8 2.5:1
CMV 307 CMV+
CMV 401 CMV-
CMV 301 CMV+
CMV 404 CMV-
D
% TB40e-UL32-GFP + of INF ctrl
UN INF INF
E:T 0.14:1
E:T 0.08:1
CMV 301 CMV+ NLV+
CMV 301 CMV+ VLE+
CMV 404 CMV- CD8+


A
UL82 ORF
% TB40e-UL32-GFP + of INF ctrl
100
75
50
25
0
Ctrl
1.2:1
0.6:1
UN
INF
UL83
UL82
EBV
307
B
US3 ORF
% TB40e-UL32-GFP + of INF ctrl
100
75
50
25
0
Ctrl
0.6:1
0.3:1
UN
INF
UL123
US3
EBV
305

Donor	Age	Gender	Serostatus	HLA Alleles			HCMV ORFs which elicit positive CD8+ T cell responses		
				HLA-A	HLA-B	HLA-C	# ORF	> 100 sfu/million	> 1000 sfu/million
CMV300	48	F	+	02 02	44 44	05 07	4/11	UL82, UL123, US3	UL83
CMV301	53	M	+	02 02	07 40	03 07	4/11	UL83	UL28, UL122, UL123
CMV302	56	M	+	02 03	07 37	07 06	7/11	UL48, UL99, UL123	UL55, UL82, UL83, UL122
CMV303	29	M	+	01 24	08 35	04 07	3/11	UL83, UL122, UL123	
CMV304	41	M	+	02 03	15 56	01 15	1/11		UL83
CMV305	25	M	+	03 26	44 51	05 14	7/11	UL28, UL48, UL55, UL122, US3	UL82, UL123
CMV306	25	F	+	01 29	08 27	07 16	1/10	UL122	
CMV307	36	M	+	01 26	08 27	01 07	9/11	UL48, UL55, UL82, UL83, UL99, UL122, US29	UL123
CMV308	72	M	+	01 01	40 58	03 07	4/11	UL83, UL123, US32	UL122
CMV309	36	M	+	01 23	35 37	04 06	6/11	UL48, UL55, UL82, UL122, US3	UL123
CMV311	32	M	+	25 25	18 18	12 12	8/10	UL28, UL82, UL83, UL99, US3, US32	UL122, UL123
CMV312	77	M	+	02 29	35 44	04 16	5/11	UL83, US3	UL82, UL122, UL123
CMV314	39	F	+	30 74	14 50	08 06	4/11	UL83, UL122, US3, US32	
CMV315	57	M	+	01 32	07 13	07 06	8/11	UL48, UL55, UL82, UL99, US29	UL28, UL83, UL123
CMV316	65	M	+	01 03	08 14	07 08	8/11	UL48, UL55, UL82, Ul122, US32	UL83, UL123, US3
CMV317	24	F	+	01 31	35 51	07 14	4/11	UL83, UL123, US3	UL28
CMV318	39	M	+	03 30	13 38	06 12	0/10		
CMV320	50	F	+	01 32	14 27	08 03	1/10	UL123	
CMV400	55	M	-	02 33	44 44	05 07	0/10		
CMV401	45	M	-	24 25	08 35	07 12	0/10		
CMV405	35	F	-	02 03	14 40	08 03	0/10		
CMV406	24	M	-	02 26	14 40	08 03	0/10		

CMV ORF specific CD8+ T cell Responses (4-1BB+ and CD69+)

	UL28			UL82			UL83		
Donor	% CD8+ UL28+	% unact IFNγ+	% UL28+ IFNγ+	% CD8+ UL82+	% unact IFNγ+	% UL82+ IFNγ+	% CD8+ UL83+	% unact IFNγ+	% UL83+ IFNγ+
305	0.24	0.05	11.80	0.24	0.00	59.60	0.08	0.07	10.30
307	-	-	-	0.06	0.00	4.62	0.19	0.00	17.30
300	1.00	0.00	2.67	0.97	0.29	19.40	1.45	0.08	54.80
301	1.18	0.00	59.80	-	-	-	0.16	0.00	24.80
316	-	-	-	-	-	-	0.11	0.02	12.10
312	-	-	-	0.54	0.00	11.10	0.03	0.03	13.20

	UL122			UL123			US3		
Donor	% CD8+ UL122+	% unact IFNγ+	% UL122+ IFNγ+	% CD8+ UL123+	% unact IFNγ+	% UL123+ IFNγ+	% CD8+ US3+	% unact IFNγ+	% US3+ IFNγ+
305	-	-	-	0.08	0.00	21.80	0.07	0.05	10.40
307	-	-	-	4.06	0.04	25.60	-	-	-
300	0.68	0.00	1.90	2.73	0.09	71.70	0.63	0.00	0.41
301	0.59	0.00	45.40	0.06	0.00	32.60	-	-	-
316	-	-	-	0.87	0.09	33.20	4.79	0.05	17.80
312	0.34	0.00	8.90	0.09	0.02	9.33	-	-	-

	UL28		UL82		UL83		UL122		UL123		US3	
Donor	CD28-	CD57+	CD28-	CD57+	CD28-	CD57+	CD28-	CD57+	CD28-	CD57+	CD28-	CD57+
305	90.40	19.20	69.70	32.80	59.50	40.50	-	-	65.40	32.70	90.40	35.30
307	-	-	94.10	29.40	96.70	60.00	-	-	89.10	64.10	-	-
300	73.90	77.50	73.70	61.40	78.60	58.80	76.60	80.40	75.90	86.50	75.70	68.10
301	86.10	53.50	-	-	80.00	71.10	79.30	83.50	88.10	69.50	-	-
316	-	-	-	-	62.00	75.10	-	-	61.10	77.90	62.30	68.80
312	-	-	0.86	100.00	0.00	81.20	2.08	97.90	7.14	92.90	-	-

	% Fibroblasts TB40e-UL32-GFP+ at Day 21								
				pp65		IE1		EBV	
Donor	UNINF	INF	E:T	1.2:1	0.6:1	1.2:1	0.6:1	1.2:1	0.6:1
305	0.10	100.00		0.18	13.47	0.52	12.18	104.15	96.63
307	0.57	100.00		2.66	14.76	0.74	0.71	28.46	63.05
300	0.28	100.00		4.08	19.17	8.04	8.14	79.72	88.75
316	0.87	100.00		20.22	26.05	7.87	0.41	93.08	96.74
312	0.05	100.00		5.90	1.98	44.00	23.75	104.25	88.25