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2 Diverse specificity, phenotype and anti-viral activity of cytomegalovirus specific

3 CD8+ T cells.

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6 *HCMV specific T cells phenotype and function*

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18 ABSTRACT (224 words)

19 CD8+ T cells specific for pp65, IE1 and IE2 are present at high frequencies in HCMV
20 seropositive individuals and these have been shown to have phenotypes associated
21 with terminal differentiation, as well as both cytokine and proliferative dysfunctions,
22 especially in the elderly. However, more recently, T cell responses to many other
23 HCMV proteins have been described but little is known about their phenotype and
24 function. Consequently, in this study, we chose to determine the diversity of HCMV
25 specific CD8+ T cell responses to eleven HCMV ORFs in a cohort of donors aged 20
26 – 80 years old as well as their ability to secrete IFN γ . Finally, we also tested their
27 functional anti-viral capacity using a novel viral dissemination assay.

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

37

38 IMPORTANCE

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

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

51

52 INTRODUCTION

53 The β herpes virus human cytomegalovirus (HCMV) is a common infection
54 worldwide (1). After primary infection, the virus establishes life-long persistence in
55 individuals at least in part due to its ability to undergo latent infection in pluripotent
56 CD34+ stem cells in the bone marrow and the myeloid cell lineages derived from
57 them (2). Both primary infection with HCMV and its long-term persistence is largely
58 sub-clinical for the majority of individuals. However, infection whether due to primary
59 infection, reactivation from latency or superinfection in the immunocompromised or
60 immature (such as HIV/AIDS patients, transplant patients or the foetus in utero,
61 respectively) can be life threatening (1). Primary HCMV infection of an otherwise
62 healthy host elicits responses from both the innate and adaptive arms of the immune
63 system and evidence from patients undergoing bone marrow and stem cell
64 transplantations has shown that the generation of HCMV specific CD4+ and CD8+ T
65 cell responses is crucial for successful control of virus infection (3-7). This
66 importance of T cell responses is further supported by evidence from the murine
67 model of MCMV infection, where transfer of immediate early (IE) antigen specific
68 CD8+ T cells into animals with an ablated immune system was protective from viral
69 challenge (8). Other murine studies have also shown that removal of CD4+ T cells
70 from mice leads to reactivation of virus resulting in disease (9). Additionally, CD8+ T
71 cells also prevent lethal infection of mice with MCMV in the absence of CD4+ T cells
72 (10, 11).

73

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

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

82

83 As a consequence of HCMV infection, it has been established that the composition
84 of the T cell repertoire is altered resulting from virus specific cells undergoing major
85 expansions which has been linked to the concept of “memory inflation” described in
86 the murine model of MCMV (17) (18). The CD8+ IE and pp65 specific T cells that
87 have been studied have a phenotype that has been linked to terminal differentiation
88 as well as dysfunction defined by the re-expression of CD45RA (19-24); the loss of
89 expression of the co-stimulatory molecules CD27 and CD28 (13, 21, 25-27) and
90 expression of CD57, a marker of activation associated with differentiated T cells (21,
91 28-30). It has also been concluded, from these studies, that enlarged HCMV
92 specific CD8+ T cell populations accumulate with age which include increasing
93 numbers of “dysfunctional” T cells as defined by their highly differentiated phenotype;
94 loss of cytokine secretion ability and limited proliferation capacity (21, 27, 31). These
95 observations have led to the suggestion that the HCMV induced changes to the T
96 cell immune system may become detrimental to individuals during their lifetime.

97 These HCMV influenced changes to the phenotypes of host CD8+ T cells have been
98 used to suggest on the basis of association that older seropositive individuals are
99 more susceptible to infection, respond poorly to vaccinations and have an increased
100 risk of mortality compared to age-matched HCMV sero-negative individuals (32-36).

101 However, older individuals do not appear to suffer from overt HCMV disease

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

110

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

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

131 MATERIALS AND METHODS

132 **Donor sample collection and isolation**

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

151

152 **HCMV ORF peptide mixes**

153 11 HCMV ORFs (UL28, UL48, UL55 (gB), UL82 (pp71), UL83 (pp65), UL99, UL122
154 (IE2), UL123 (IE1), US3, US29 and US32) were selected and consecutive 15mer

155 peptides overlapping by 10 amino acid libraries were synthesised by ProImmune
156 PEPScreen (Oxford, UK) from sequences detailed in the Sylwester *et. al.* study (16).
157 The individual lyophilised peptides from each ORF library were reconstituted in 80%
158 DMSO 20% RPMI 1640 (PAA laboratories, Austria) to give 40mg/ml master stock,
159 the individual peptides were then diluted 1/40 in RPMI 1640 (unsupplemented) to
160 give a 1mg/ml (2% DMSO) working stock. Peptide pools were used as either entire
161 ORF mixes at a concentration of 5µg/ml/peptide or in the case of UL48 ORF) 10
162 pools of 48 peptides at a concentration of 20µg/ml/peptide was used in this study.
163 Additionally ProMix HCMVA (pp65) and Promix HCMVA (US3) peptide pools
164 (ProImmune) were diluted to the equivalent of 20µg/ml/peptide were also used.

165

166 **Individual HLA typed HCMV and EBV peptides**

167 Individual HLA restricted peptides from HCMV pp65 and IE1 used in this study were
168 HLA-A2 NLVPMVATV (pp65 495 -504aa), HLA-A1 YSEHPTFTSQY (pp65 363 -
169 373aa), HLA-A3 KLGGALQAK (IE1 184 -192aa), HLA-B7 TPRVTGGGAM (pp65
170 417-426aa), HLA-B8 QIKVRVDMV (IE1 88-96aa) and HLA-A2 VLEETSVML (IE1
171 316 – 324aa). Additionally individual HLA restricted peptides from EBV BMLF-1,
172 EBNA 3A and BZLF-1 were HLA-A2 GLCTLVAML (BMLF-1 259-267aa), HLA-A3
173 RLRAEAQVK (EBNA 3A 603-611aa), HLA-B7 RPPIFIRRL (EBNA-3A 247-255aa)
174 and HLA-B8 RAKFKQLL (BZLF-1 190-197aa) (all ProImmune) were also used in the
175 study.

176

177 **ELISPOT assays**

178 $2 \times 10^5 - 3 \times 10^5$ PBMC or PBMC depleted of CD4+ T cells, using magnetic cell
179 separation and anti-CD4 conjugated beads (Miltenyi Biotech, Bisley, UK), were
180 resuspended in supplemented RPMI 1640 + 10% FCS and IFN γ ELISPOT assays
181 performed according to manufacturer's instructions (eBioscience, San Diego, USA),
182 in 96-well PVDF membrane plates (Millipore, Billerica, USA). Cells were stimulated
183 with entire ORF mix peptides (final peptide concentration 2 μ g/ml/peptide) or ORF
184 pool mix peptides (final peptide concentration 5 μ g/ml/peptide) at 37°C in a humidified
185 CO $_2$ atmosphere for 48 hours. All the ELISPOT assays were performed on fresh
186 PBMC the same day as taking the blood, assays were all performed to a fixed
187 protocol and using antibody sets from the same company as well as ELISPOT plates
188 with identical incubation periods. PHA was used as a positive control in every assay.
189 Developed plates were recorded by ELISPOT plate reader (AID, Strassberg,
190 Germany) and counted using ImageJ software (National Institutes of Health, USA).

191

192 **Intracellular cytokine and phenotype staining**

193 2×10^6 PBMC resuspended in RPMI + 10% FCS were stimulated with ORF peptide
194 mixes or individual mapped peptides overnight. After 2 hours incubation 5 μ g/ml
195 Brefeldin A (BD Biosciences, San Jose, USA) was added for the remainder of the
196 incubation period. Cells were then washed and stained with a combination of
197 surface antibodies including CD3 eFluor 650NC (eBioscience) or CD3 Qdot655
198 (Invitrogen, Paisley, UK), CD45RA PE-Cy7, CD27 APC-eFluor 780 (eBioscience),
199 CD8 AlexaFluor 700, CD57 AlexaFluor 647 (BioLegend, San Diego, USA), CD28
200 PerCPCy5.5, CD45RO FITC (BD Biosciences) and LIVE/DEAD Fixable Yellow Dead
201 cell stain (Invitrogen). Cells were then fixed and permeabilised using FIX&PERM
202 (ADG, Kaumberg, Austria). Peptide specific T cells were identified by the co-

203 expression of CD69 and 41BB according to established published protocols (40-43)
204 in brief, cells were stained intracellularly with CD69 Pacific Blue, 4-1BB PE-Cy5
205 (BioLegend) and IFN γ PE (BD Biosciences) at 4°C in the dark. Samples were
206 washed and fixed in a final 1% paraformaldehyde solution and acquired on a BD
207 LSR Fortessa cytometer using FACSDiva software (BD Biosciences). Data was
208 analysed using FlowJo software (Treestar, Oregon, USA).

209

210 **Virus**

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

214

215 **Expansion of HCMV specific CD8+ T cells**

216 CD8+ T cells were isolated from PBMC using MACS anti-CD8 direct beads (Miltenyi
217 Biotec, Bisley, United Kingdom) magnetic separation and then resuspended in
218 supplemented RPMI + 10% Fetal Bovine Serum (FBS) (Invitrogen) + 10% heat
219 inactivated autologous donor serum. Cells were stimulated with peptide pulsed
220 irradiated autologous PBMC in the presence of 2.5IU/ml human recombinant IL-2
221 (National Institute for Biological Standards and Control, Potters Bar, United
222 Kingdom) in round bottom 96 well plates at 37°C +5% CO $_2$ for 10 – 14 days, fresh
223 media was replenished every five days. Specificity of expanded CD8+ T cell cultures
224 using mapped peptides was determined by specific pentamer staining, cells were
225 harvested and washed and then stained with the specific unlabelled pentamer
226 (Prolimmune), washed and then further stained with pentamer specific PE

227 fluorophore (ProlImmune) and anti-CD8 and anti-CD3 antibodies conjugated to
228 PerCP-Cy5.5 and FITC respectively, cells were then fixed and acquired on a FACS
229 Sort using CellQuest software (BD Biosciences) and data analysed using FlowJo
230 software. All expanded T cell lines were also tested for specificity using IFN γ
231 ELISPOT assays. Briefly, resting CD8 $^+$ T HCMV specific cells were harvested and
232 washed and then 2000 T cells were incubated in IFN γ coated PVDF membrane
233 plates with 50 000 peptide pulsed autologous B-lymphoblastoid cell lines (LCL) and
234 with unpulsed and mitogen stimulated controls for 48 hours at 37°C +5% CO $_2$.
235 ELISPOT assays were again developed according to manufacturer's instruction.

236

237 **Measurement of cytotoxicity**

238 The cytotoxic capability of the expanded HCMV specific CD8 $^+$ T cell lines was
239 assessed using a chromium release assay. Briefly, the rested CD8 $^+$ T cell lines
240 were harvested, washed in acidified RPMI-10 and plated at between 15 – 80:1 E:T
241 ratios in triplicate. Target cells used comprised autologous LCL lines, the cells were
242 washed in PBS and Na $_2$ Cr 57 O $_4$ (Perkin Elmer) added to the cell pellet, and incubated
243 at 37°C for 45 min, appropriate peptide or no peptide was then added to the target
244 cells and incubated for an additional 45 min at 37°C. Target cells were washed three
245 times in acidified RPMI + 10% FBS medium and added to the assay plate
246 (containing effectors, medium-only wells, and wells containing Nonidet P-40, as
247 described above) at between 2000 – 4000 cells/well in acidified medium. Plates were
248 incubated at 37°C for 5 h, after which 70 μ l of supernatant was harvested from each
249 well and used to quantify radioactive emission. Percent specific lysis was calculated
250 using the following formula: percent specific lysis = 100 x (target release –
251 spontaneous release)/(maximum release – spontaneous release).

252

253 ***In vitro* viral dissemination assay**

254 The ability of specific HCMV CD8 T cells to control the spread of HCMV virus *in vitro*
255 was measured. Autologous or partial HLA matched dermal fibroblasts were seeded
256 in a 24 or 48 well plate to be 80-90% confluent when they were infected with TB40e
257 UL32-GFP virus at an MOI of 0.03. Rested HCMV specific CD8+ T cell lines were
258 harvested, washed and resuspended in supplemented RPMI 1640 + 10% FBS then
259 added to the infected fibroblasts 24 hours post infection at a series of T
260 cell:Fibroblast ratios of 5, 2.5, 1.2, 0.6, 0.3:1, each experiment included a CD8+ T
261 cell line specific to a HLA matched individual peptide from EBV (listed earlier) as a
262 control. In further experiments total CD8+ T cells isolated directly *ex vivo* from HLA
263 matched CMV seropositive and seronegative donors were added to infected
264 fibroblasts 24 hours post infection at a series of T cell: Fibroblast ratios of 5, 2.5, 1.2
265 or NLV and VLE MHC Class I pentamer FACS-sorted CD8+ T cells from PBMC
266 directly *ex vivo*, at T cell: Fibroblast ratios of 0.14 and 0.08. The viral dissemination
267 assay was incubated at 37°C +5% CO₂, assessment of viral dissemination was
268 performed at 14 and 21 days by detection of GFP expression by both fluorescent
269 microscopy and flow cytometry, the data was analysed and the percentage of GFP
270 positive fibroblasts was expressed as a proportion of the Infected Control for each
271 time point.

272

273 **Statistics**

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

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

280

281 RESULTS

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

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

297

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

305 in the magnitude of the CD8+ T cell response with age, but this did not reach
306 significance for this cohort (Spearman $r=0.43$, $n=18$) (Figure 1D). However, when
307 the analysis was performed on the number of ORFs that were high frequency (>1000
308 SFU/million) (Figure 1E) this strongly correlated with age (Pearson $r=0.53$, $n=18$,
309 $p=0.02$).

310

311 The frequency of the CD8+ T cell response of each individual donor to each HCMV
312 ORF was also tallied and ranked, and then subdivided into responders (> 100
313 sfu/million by IFN γ Elispot) (Figure 1F) and the sub-set of high frequency responders
314 (> 1000 sfu/million by IFN γ Elispot) (Figure 1G). We identified 4 HCMV ORFs,
315 which the majority of the donors in the cohort responded to, UL123 (IE1), UL83
316 (pp65), UL122 (IE2), UL82 (pp71), which many donors also recognized at high
317 frequency in addition to UL28 and US3 which were also seen at high frequency
318 (Figure 1F and G). The CD8+ T cell responses to these selected ORFs are further
319 characterised in this study.

320

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

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

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

339

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

350

351 **HCMV specific T cells are predominantly T_{EMRA} cells irrespective of their**
352 **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

378 identified by CD69/4-1BB co expression was variable between the donors, the NLV
379 response was very similar in donor CMV324 (pentamer v CD69/4-1BB, 2.5% v
380 2.3%), in donor CMV307 the size of the pentamer population was larger than the
381 CD69/4-1BB population (pentamer v CD69/4-1BB, 6% v 2.4%) and in donor
382 CMV301 the pentamer population was smaller than the CD69/4-1BB population e.g.
383 NLV (pentamer v CD69/4-1BB, 1% v 1.9%).

384

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

393

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

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

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

406

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

412

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

419

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

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

428

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

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

443 A representative assay comparing UL83 (pp65) and UL123 (IE1) specific CD8+ T
444 cells derived from donor 307 is illustrated (Figure 7A and B) and shows that both IE
445 and pp65 specific T cells are highly effective at controlling spread of HCMV even at
446 the lowest E:T ratio of 0.6:1. Importantly, EBV specific T cells from the same donor
447 could not inhibit HCMV spread. We repeated this analysis on pp65 and IE1 CD8+ T
448 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 seronegative 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).

472 DISCUSSION

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

485

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

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

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

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

578

579 HCMV is a paradigm for viral immune evasion of T cell recognition, yet it has always
580 been somewhat of a paradox that a virus with such an extensive array of immune
581 evasion mechanisms elicits a very strong T cell response following primary infection
582 (25, 28) and, likely, reactivation as immunosuppressed HCMV seropositive patients
583 often lose the ability to control viral replication resulting in end organ disease. These
584 observations suggest that the T cell immunity must also be effective at preventing
585 disease following reactivation in normal healthy individuals. The data presented in
586 this manuscript supports this conjecture as T cells specific for both early and late
587 gene products are highly effective at preventing viral dissemination in an *in vitro*
588 model and the viral immune evasion strategies appear unable to allow avoidance of T
589 cell immune responses in the context of lytic infection. Our view is that the real value
590 of the T cell immune evasion functions of HCMV is to allow a latent virus in an
591 individual cell sufficient time to undergo reactivation and assemble new virus rather
592 than to allow unrestricted viral replication that ends up causing disease in the host
593 upon a primary infection or following reactivation.

594

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598 TABLE AND FIGURE LEGENDS

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

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

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

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

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

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

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

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

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

623 Frequency of the CD8+ T cell response to 11 HCMV ORFs in 18 donors is shown.
624 The responses were measured by IFN γ ELISPOT and shown as sfu/million cells
625 (spot forming unit/million cells) following the subtraction of background counts from
626 unstimulated cells. The donor cohort is arranged according to the age of the donor
627 and the size of the response to each HCMV ORF is shown as a heat map (A). The
628 number of ORFs each donor responded to (>100 sfu/million) (B), and also plotted
629 according to the age of the donor (C). There was no statistical correlation between
630 age and the number of ORFs an individual recognised by Pearson correlation. The
631 cumulative IFN γ response to all HCMV ORFs for each donor was plotted according
632 to donor age and this was not significantly correlated with age by Spearman
633 correlation (D). The number of ORFs each donor responded to at high frequency
634 (>1000 sfu/million) was also correlated with age by Pearson correlation, there is a
635 significant increase in high frequency ORFs in older donors $p=0.02$ (E). The
636 frequency of recognition by CD8+ T cells of each HCMV ORF is shown for all
637 responses >100 sfu/million (F) and the high frequency responses (>1000 sfu/million)
638 (G) (a subset of the data in (F)), the HCMV ORFs were ranked according to the
639 number of subjects responding.

640

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

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

645 donors in 2012 (36 months). The responses for each ORF are shown for the 24
646 month (A) and the 36 month (B) data (2009, black; 2011, white; 2012 grey points).
647 The positive response (100 sfu/million) cut off line is indicated on each graph. The
648 24 month paired data was tested using a Wilcoxon matched pairs test, significant
649 results * ($p < 0.05$) are indicated. The 3 time points in the 36 month data group were
650 tested using a 1 way ANOVA paired Friedman test which showed no significant
651 change in the variance of the data.

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

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

661

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

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

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

677

678 **Figure 5 – HCMV specific CD8+ T cells from 6 different ORFs had a very similar**
679 **pattern of IFN γ secretion.**

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

690

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

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

699

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

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

716

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

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

727

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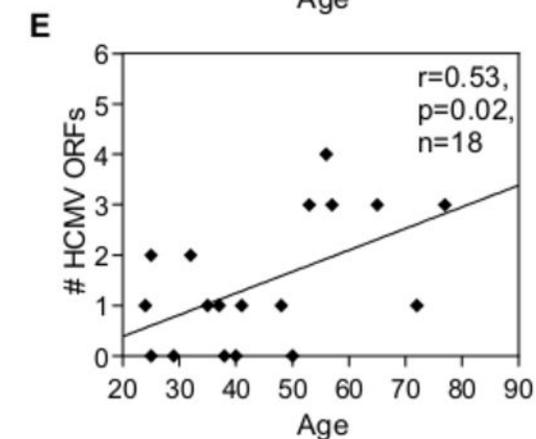
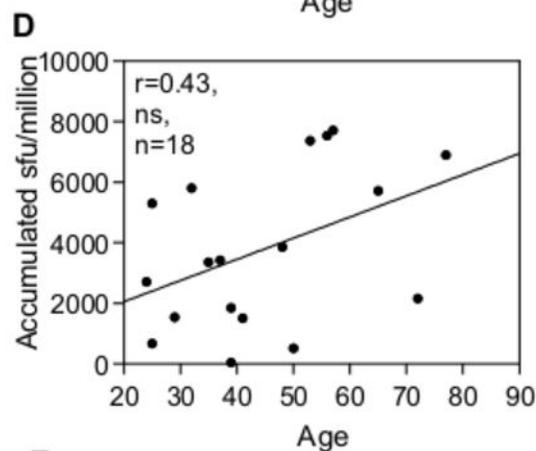
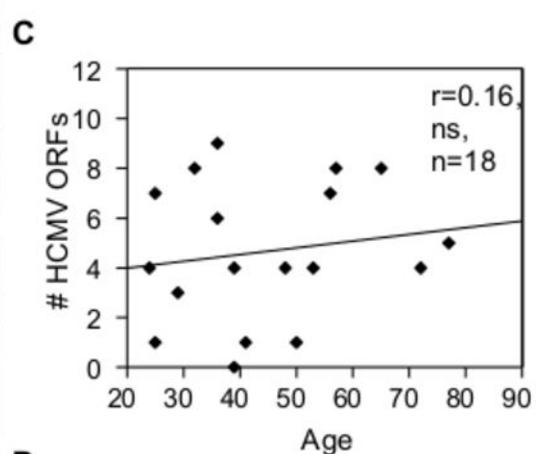
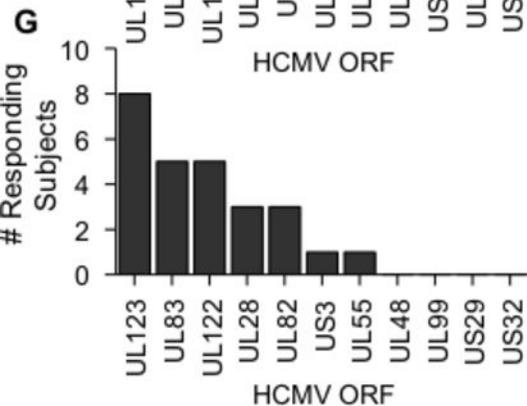
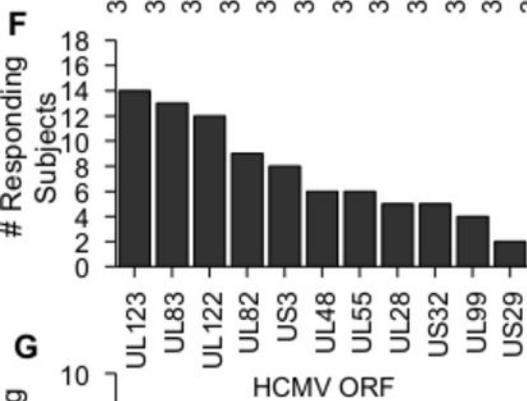
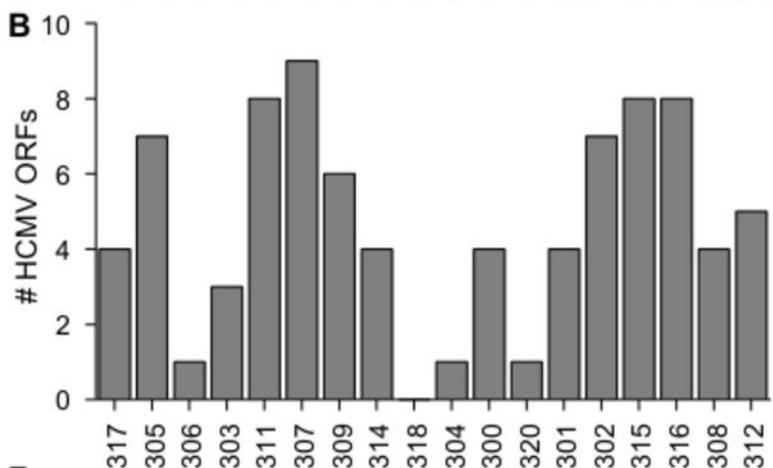
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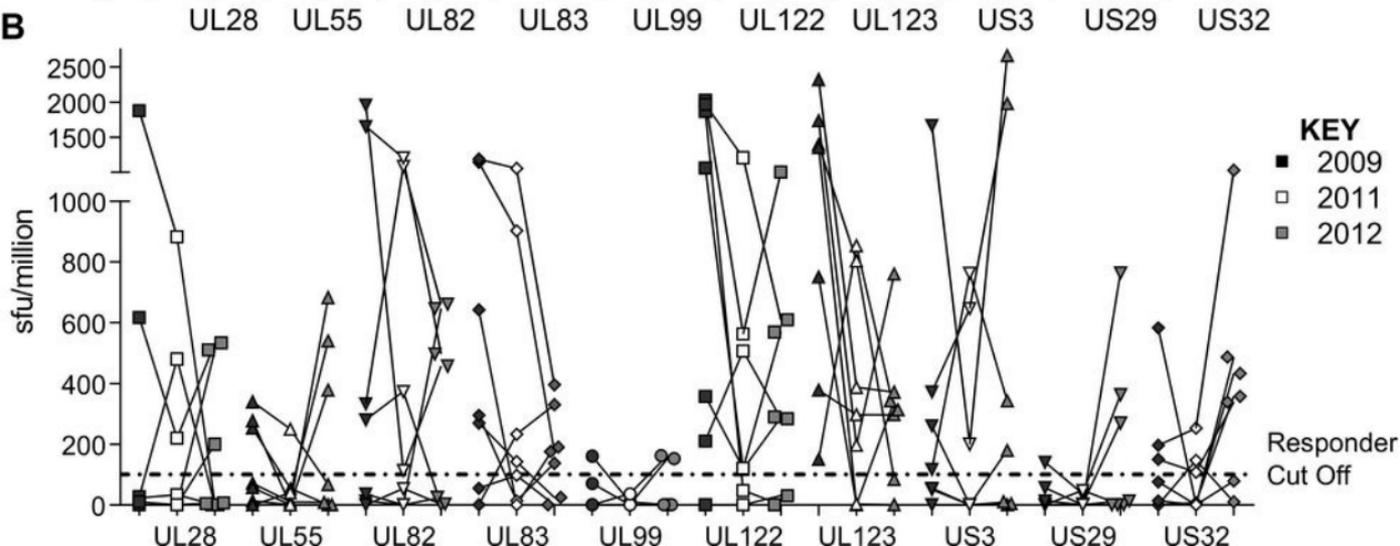
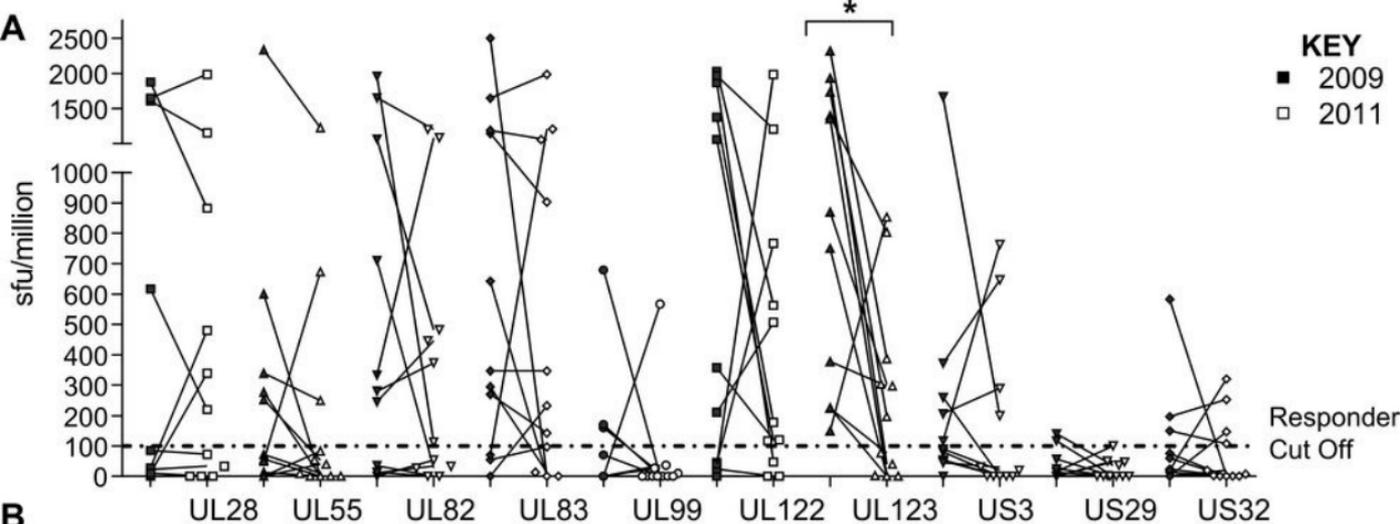
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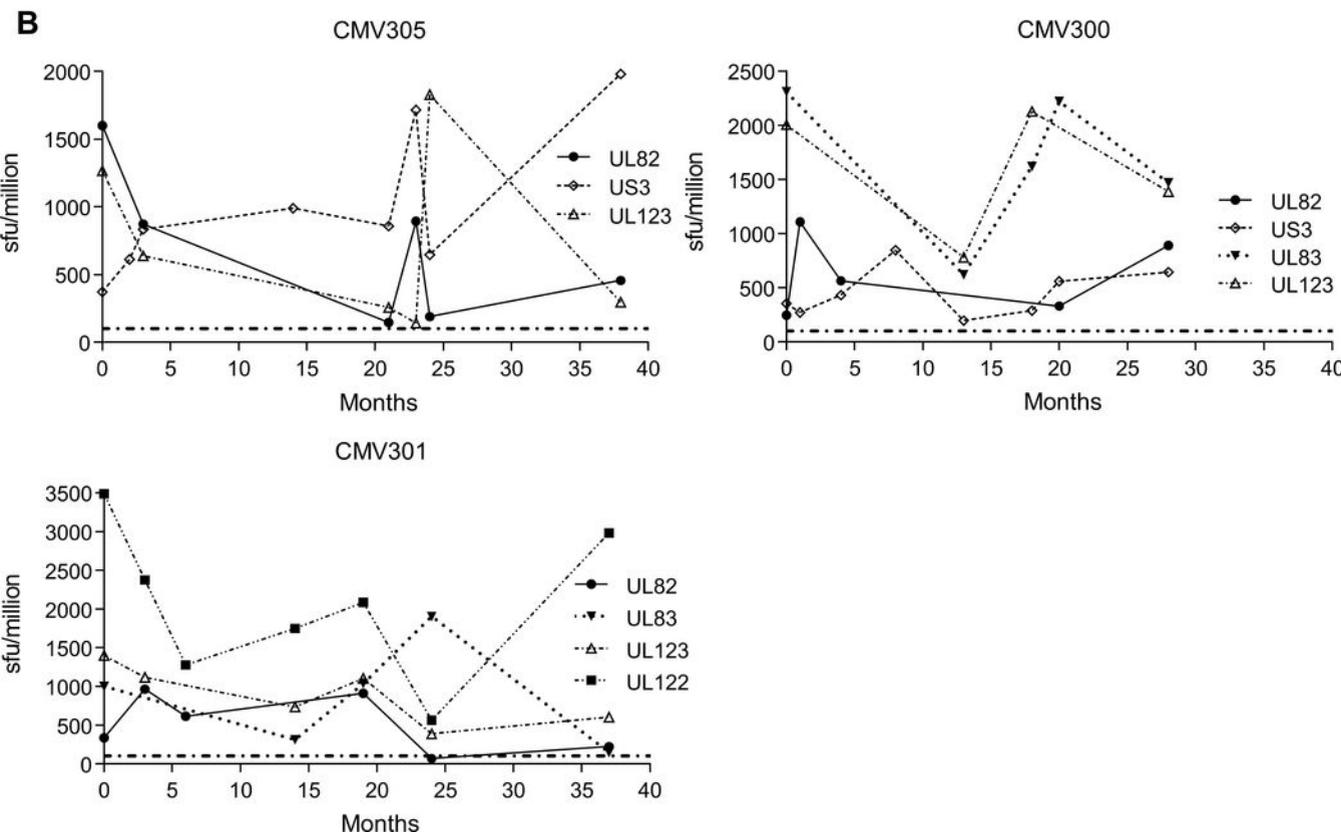
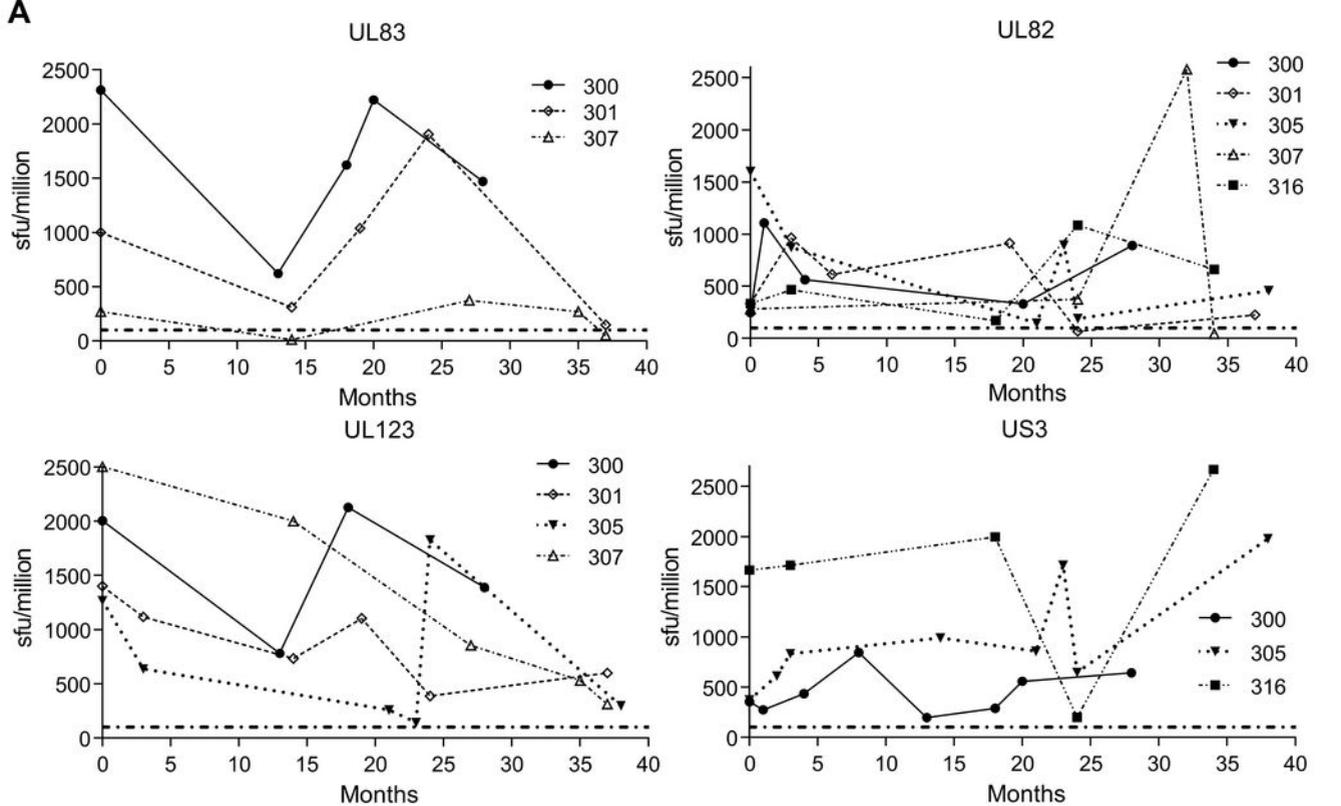
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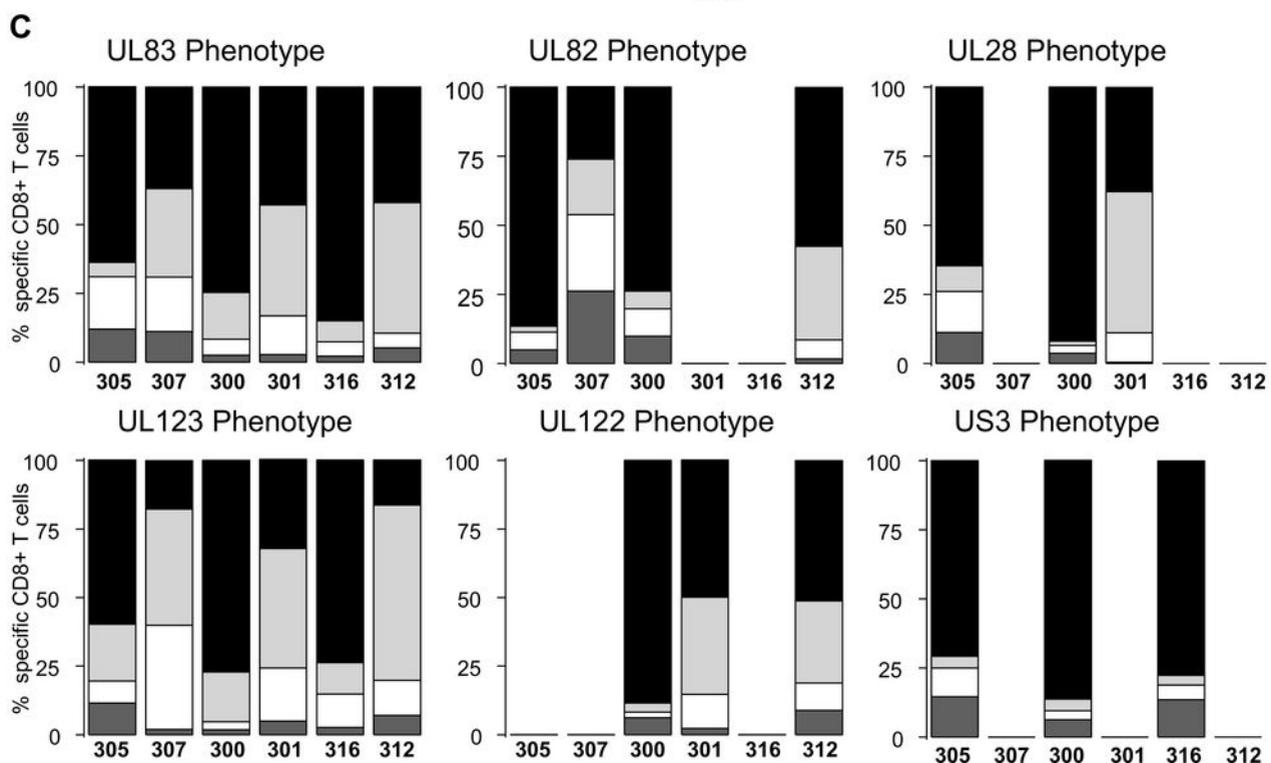
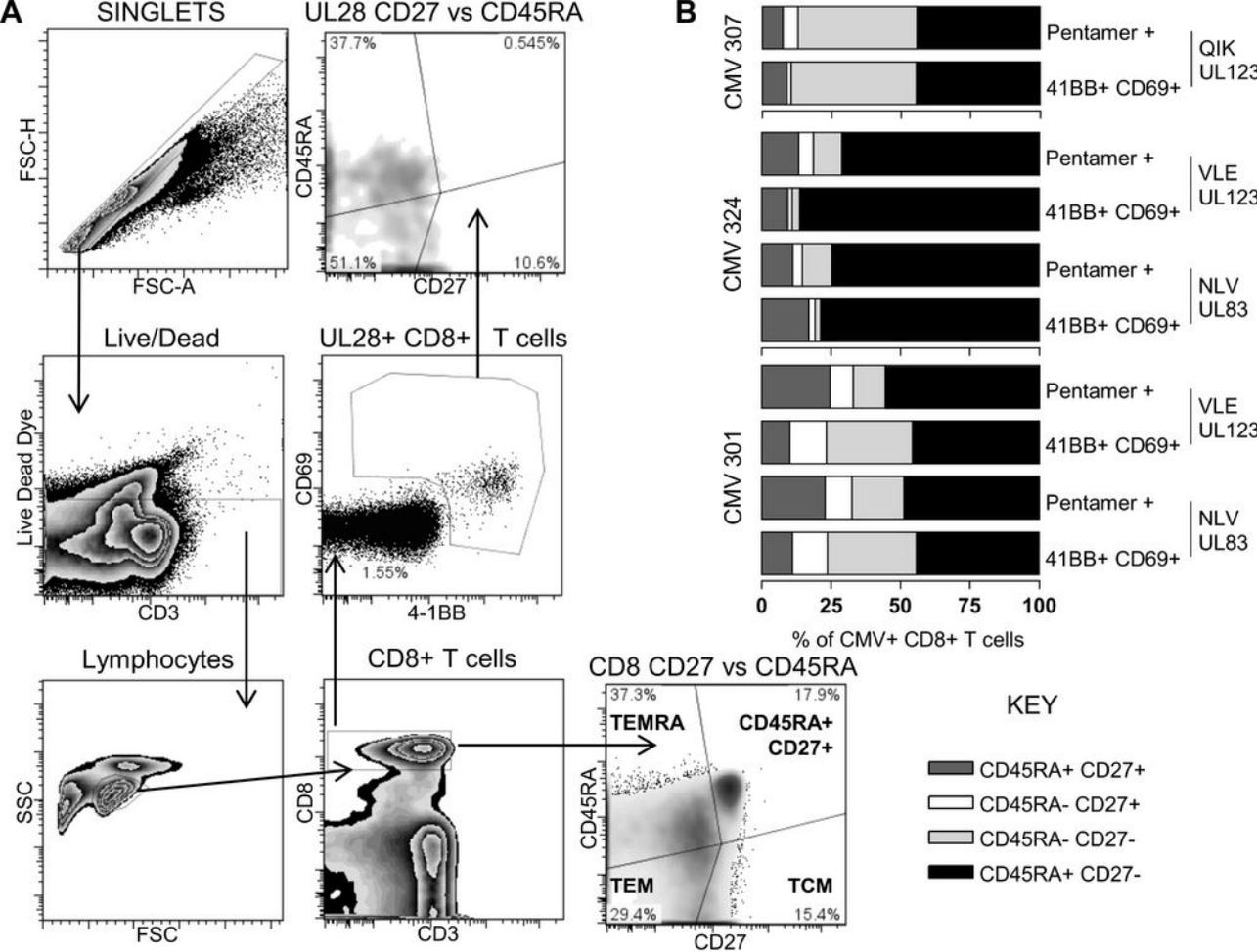
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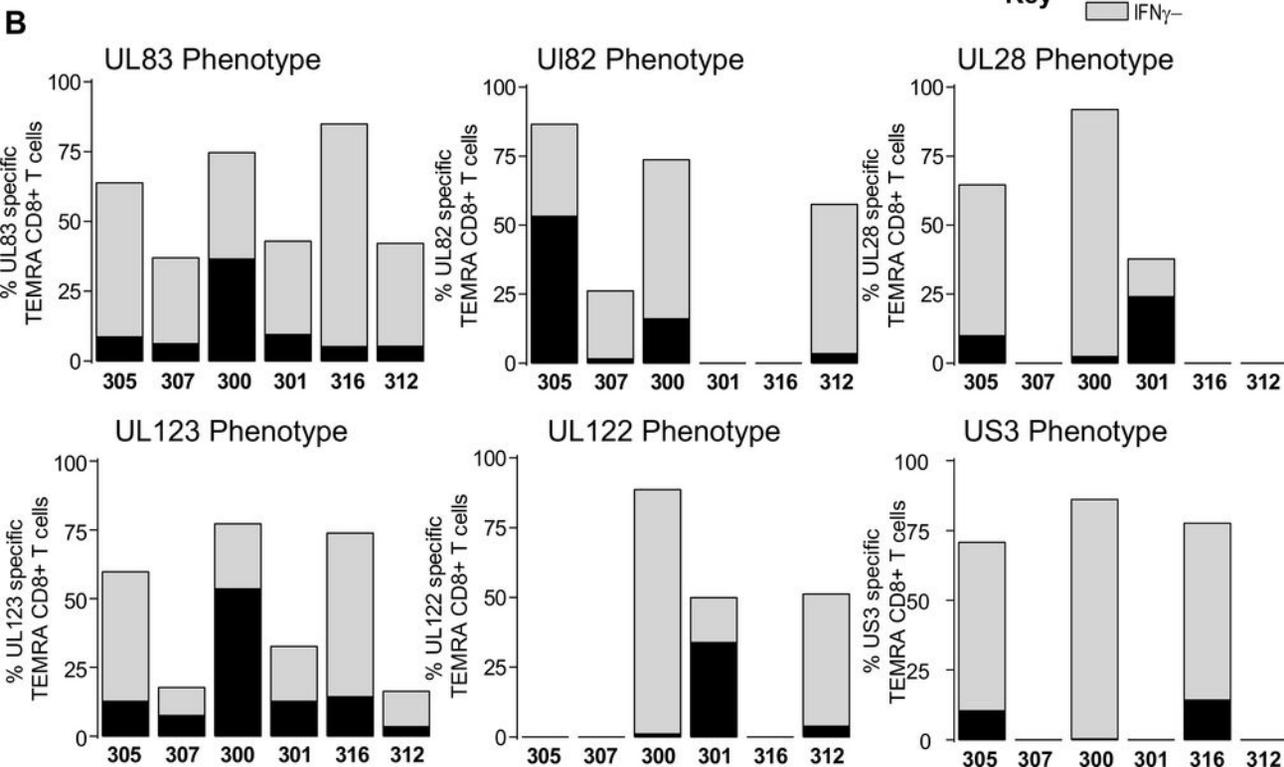
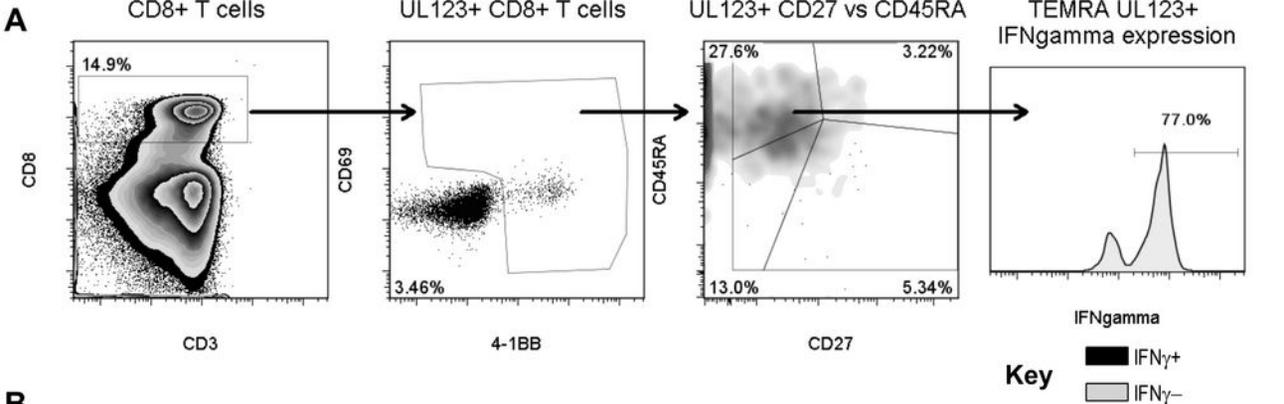
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Donor	317	305	306	303	311	307	309	314	318	304	300	320	301	302	315	316	308	312
Age	24	25	25	29	32	36	36	39	39	41	48	50	53	56	57	65	72	77
UL28 ORF																		
UL48 ORF			N.T.		N.T.				N.T.			N.T.						
UL55 ORF																		
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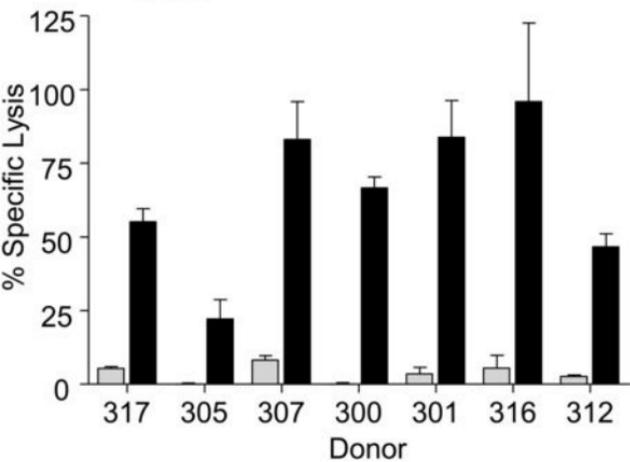
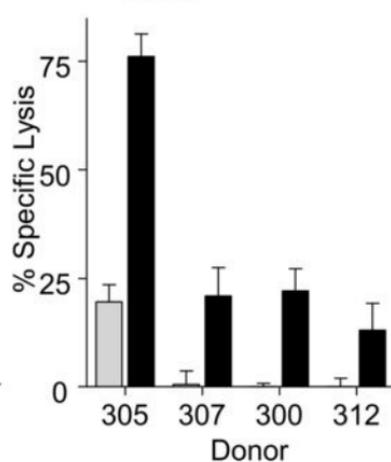
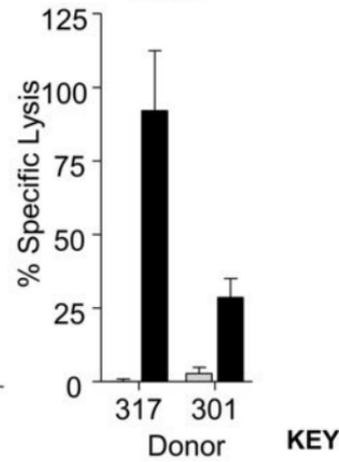
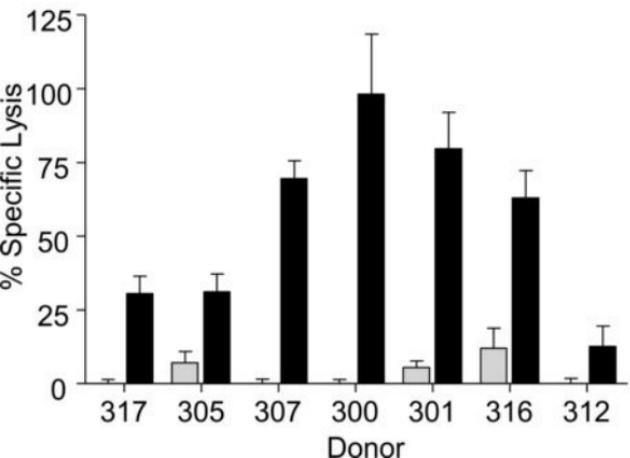
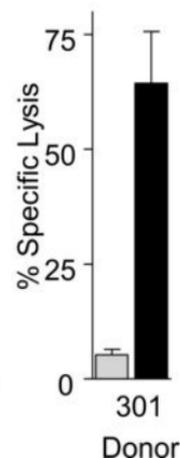
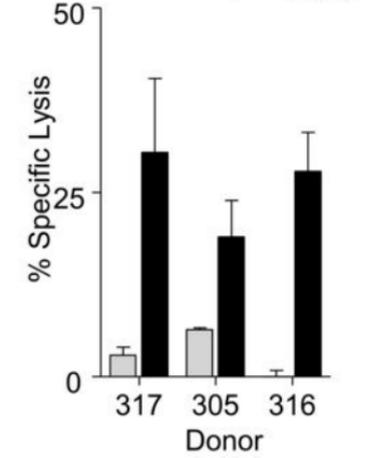






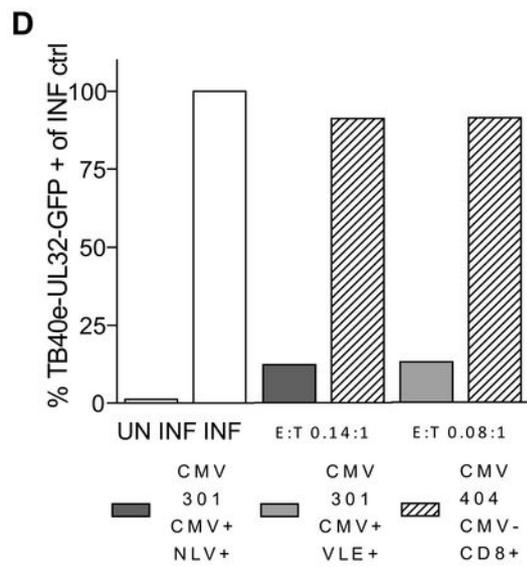
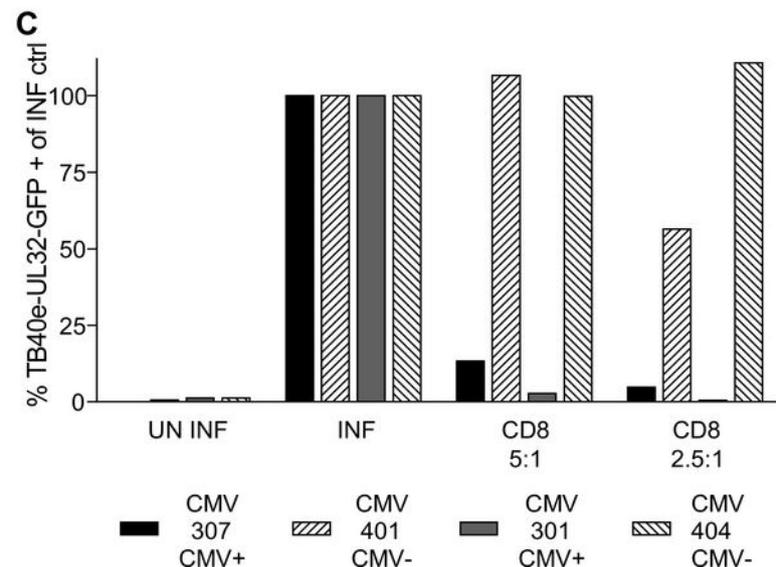
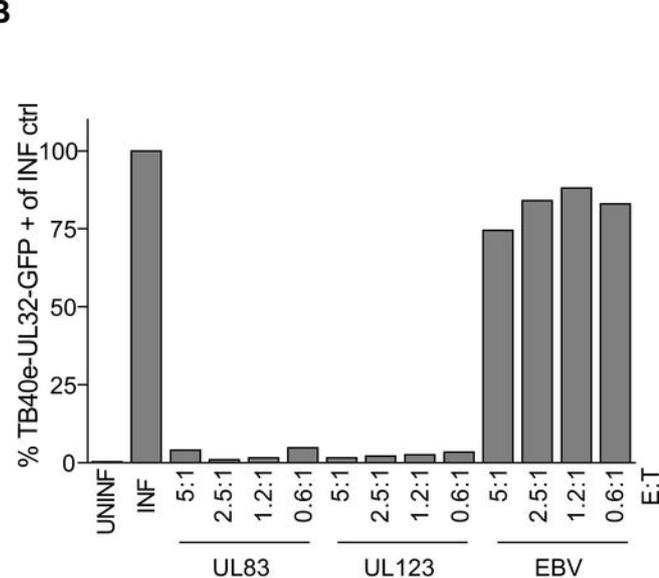
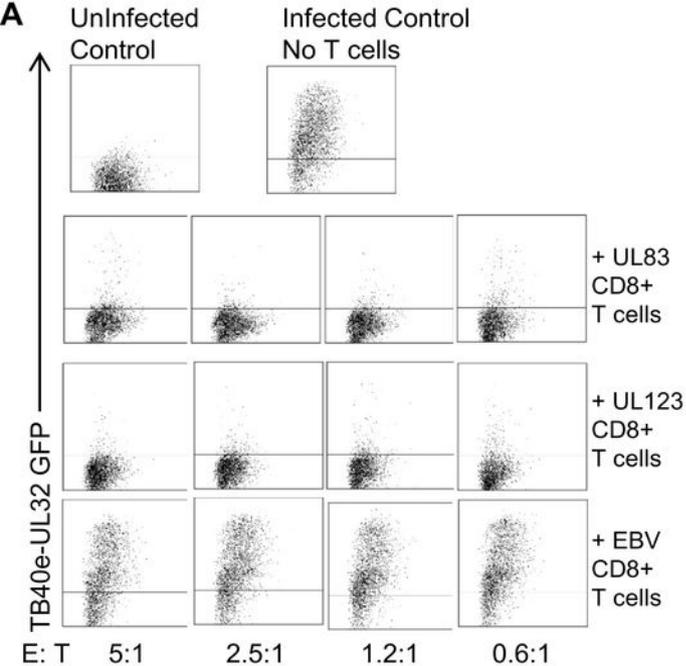




UL83**UL82****UL28****UL123****UL122****US3****KEY**

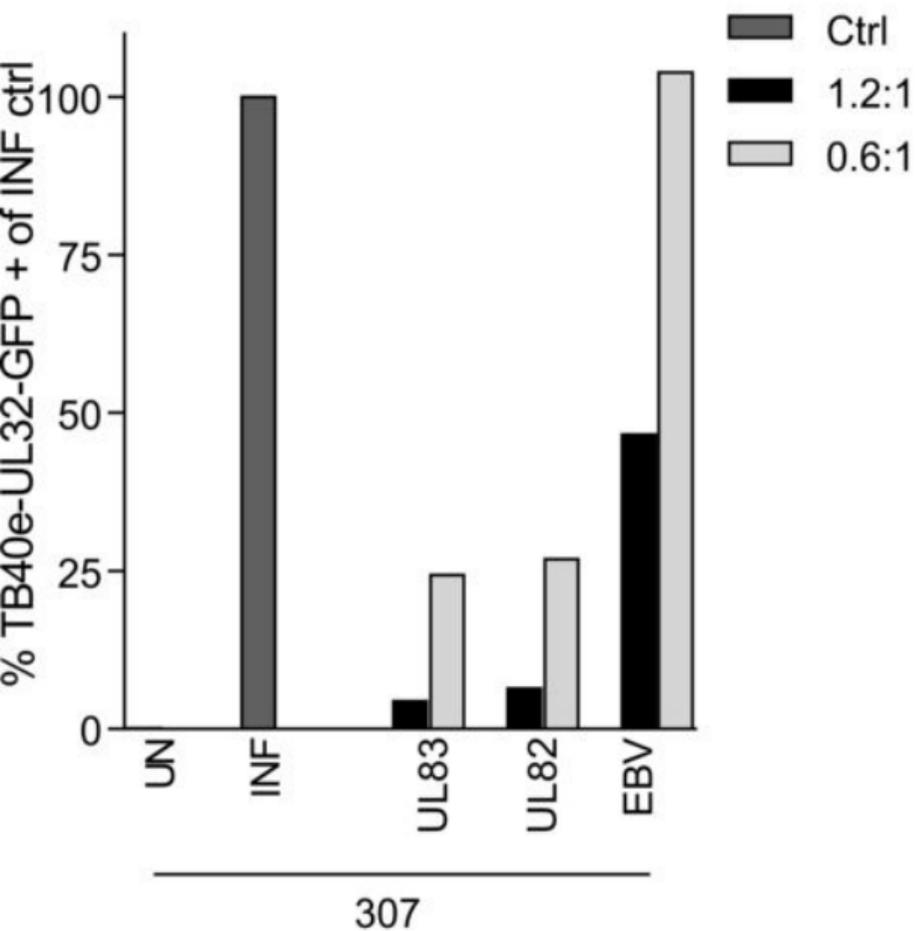
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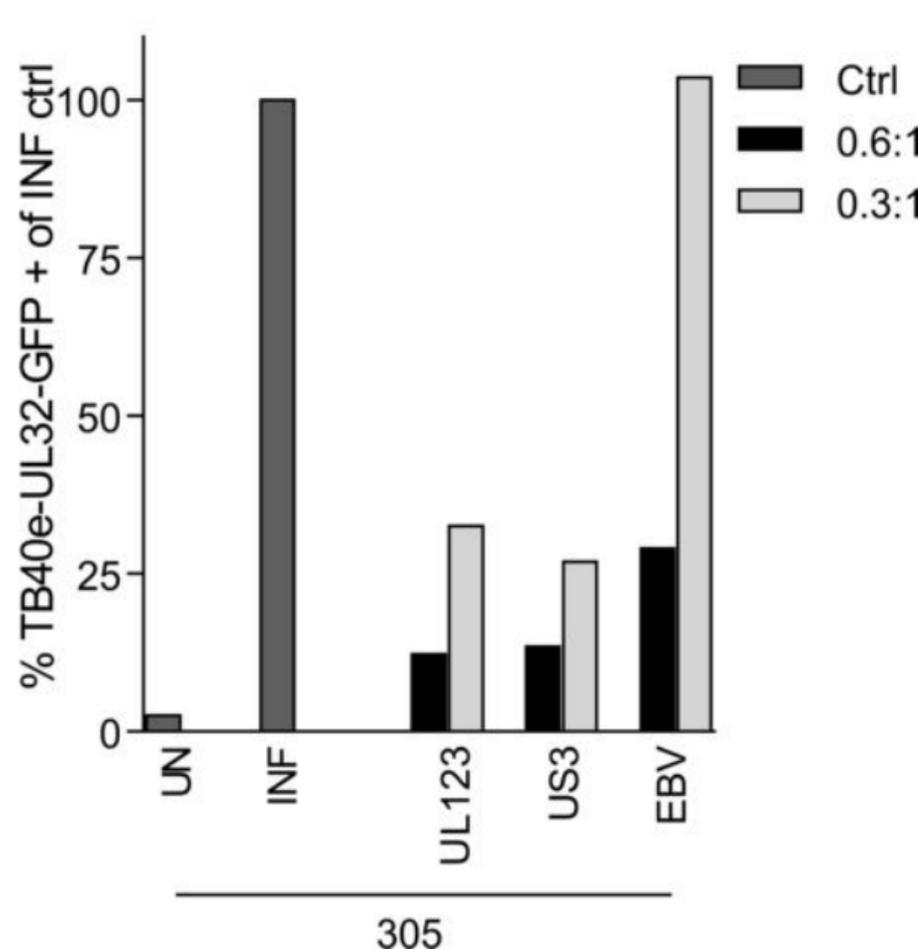


A

UL82 ORF

**B**

US3 ORF



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, UI122, 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+)									
Donor	UL28			UL82			UL83		
	%	%	%	%	%	%	%	%	%
	CD8+ UL28+	unact IFN γ +	UL28+ IFN γ +	CD8+ UL82+	unact IFN γ +	UL82+ IFN γ +	CD8+ UL83+	unact IFN γ +	UL83+ IFN γ +
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
Donor	UL122			UL123			US3		
	%	%	%	%	%	%	%	%	%
	CD8+ UL122+	unact IFN γ +	UL122+ IFN γ +	CD8+ UL123+	unact IFN γ +	UL123+ IFN γ +	CD8+ US3+	unact IFN γ +	US3+ IFN γ +
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	-	-	-

Donor	UL28		UL82		UL83		UL122		UL123		US3	
	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									
Donor	UNINF	INF	E:T	pp65		IE1		EBV	
				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