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3 **Rudimentary signs of immunosenescence in Cytomegalovirus seropositive healthy young**
4 **adults**

5

6 JAMES E. TURNER^{*,1,2,3}, JOHN P. CAMPBELL^{1,4}, KATE M. EDWARDS⁵, LAUREN J.

7 HOWARTH¹, GRAHAM PAWELEC⁶, SARAH ALDRED¹, PAUL MOSS², MARK T.

8 DRAYSON⁴, VICTORIA E. BURNS¹, JOS A. BOSCH^{7,8}

9

10 ¹ *School of Sport and Exercise Sciences, University of Birmingham, UK.*

11 ² *School of Cancer Sciences, University of Birmingham, UK.*

12 ³ *Department for Health, University of Bath, UK.*

13 ⁴ *School of Immunity and Infection, University of Birmingham, UK.*

14 ⁵ *Exercise Health and Performance Research Group, University of Sydney, Lidcombe, Australia*

15 ⁶ *Department of Internal Medicine II, Centre for Medical Research, University of Tübingen,*
16 *Tübingen, Germany.*

17 ⁷ *School of Psychology, University of Amdterdam, Amsterdam, The Netherlands.*

18 ⁸ *Mannheim Institute of Public Health, Social and Preventive Medicine (MIPH), Mannheim*
19 *Medical Faculty, University of Heidelberg, Germany.*

20

21 *Correspondence: James E Turner. Address: Department for Health, University of Bath, BA2

22 7AY, E-mail: j.e.turner@bham.ac.uk or turnerje1984@gmail.com (James E. Turner) or

23 j.a.bosh@uva.nl (Jos. A. Bosch)

24

25 **Running head:** Immunosenescence in early adulthood

26 **Key words:** Immunosenescence, Cytomegalovirus, Interleukin-6, CD8⁺ T cells, vaccination

27 efficacy.

28 **Abstract**

29 Ageing is associated with a decline in immune competence termed immunosenescence.
30 In the elderly, this process results in an accumulation of differentiated ‘effector’ phenotype
31 memory T cells, predominantly driven by Cytomegalovirus (CMV) infection. Here, we asked
32 whether CMV also drives immunity towards a senescent profile in healthy young adults.

33 One hundred and fifty eight individuals (mean \pm SD; age 21 years \pm 3, body mass index
34 22.7 kg·m² \pm 2.7) were assessed for CMV-serostatus, the numbers/proportions of CD4⁺ and
35 CD8⁺ late-differentiated/effector-memory cells (i.e., CD27⁻CD28⁻/CD45RA⁺), plasma
36 interleukin-6 (IL-6), and antibody responses to an *in vivo* antigen challenge (half-dose influenza
37 vaccine). Thirty per-cent (48/158) of participants were CMV⁺. A higher lymphocyte and CD8⁺
38 count (both $p < .01$), and a lower CD4:CD8 ratio ($p < .03$) was observed in CMV⁺ people. Eight
39 per-cent (4/58) of CMV⁺ individuals exhibited a CD4:CD8 ratio < 1.0 , whereas no CMV⁻ donor
40 showed an inverted ratio ($p < .001$). The numbers of CD4⁺ and CD8⁺CD27⁻CD28⁻/CD45RA⁺
41 cells were ~4-fold higher in CMV⁺ people ($p < .001$). Plasma IL-6 was higher in CMV⁺ donors
42 ($p < .05$) and showed a positive association with the numbers of CD8⁺CD28⁻ cells ($p < .03$).
43 Finally, there was a significant negative correlation between vaccine-induced antibody responses
44 to the A/Brisbane influenza strain and CMV-specific Immunoglobulin-G titres ($p < .05$). This
45 reduced vaccination response was associated with greater numbers of total CD8⁺, and CD4⁺ and
46 CD8⁺CD27⁻CD28⁻/CD45RA⁺ cells ($p < .05$).

47 This study observed marked changes in the immune profile of young adults infected with
48 CMV, suggesting that this virus may underlie rudimentary aspects of immunosenescence even in
49 a chronologically young population.

50 **Introduction**

51 Ageing is associated with a gradual decline in immune competence, termed
52 immunosenescence, which has been associated with increased susceptibility to infection,
53 accelerated cognitive decline, frailty, and increased mortality (Wikby et al. 2005). A hallmark of
54 immunosenescence is an accumulation of differentiated or ‘effector’ phenotype CD8⁺ T cells
55 (i.e., CD27⁻CD28⁻/CD45RA⁺). Other characteristics include a decline in the frequency and
56 number of naïve CD8⁺ T cells (i.e., CD27⁺CD28⁺/CD45RA⁺) and increased inflammatory
57 activity (e.g., plasma IL-6 levels) (Chidrawar et al. 2009; Wikby et al. 2005; Derhovanessian et
58 al. 2009). It has become clear that these features are to a significant extent determined by latent
59 Cytomegalovirus (CMV) infection (Derhovanessian et al. 2009; Olsson et al. 2000). CMV is a
60 beta-herpes virus that establishes lifelong latency after primary infection. Seroprevalence, as
61 determined by the presence of CMV-specific Immunoglobulin-G antibodies (anti-CMV IgG),
62 increases with age to reach levels of over 70% in many elderly populations (Staras et al. 2006).

63 It has been shown that the magnitude of the CMV-specific immune response can also
64 influence clinical outcomes in CMV seropositive individuals. High titres of anti-CMV IgG and
65 increased numbers of late-differentiated CD8⁺ T cells (i.e., CD27⁻CD28⁻) have been associated
66 with lower antibody responses to influenza vaccination and higher circulating levels of
67 inflammatory markers (Saurwein-Teissl et al. 2002; Goronzy et al. 2001; Trzonkowski et al.
68 2003; Wikby et al. 2006; Moro-Garcia et al. 2012). In contrast, a recent study observed
69 comparable antibody responses to influenza vaccination, irrespective of CMV serostatus, in
70 residents of long-term care facilities (den Elzen et al. 2011). Indeed, as with the latter study, most
71 observations of this kind have been made in elderly individuals that might already exhibit
72 immune impairment. Thus, it remains largely unexplored whether CMV infection also drives

73 immunity towards a senescent immune profile in healthy young adults. Here, in a
74 chronologically young population, we have examined the influence of CMV serostatus on
75 immune parameters including the CD4:CD8 ratio, the number of late-differentiated/effector
76 memory T cells and plasma IL-6 levels, as well as the *in vivo* functional response to antigen
77 challenge (a half dose influenza vaccine).

78

79

80

81 **Methods**

82 *Participants*

83 One hundred and fifty eight healthy university students were recruited by local campus
84 advertisement (Edwards et al. 2010). Mean \pm SD age and body mass index (BMI) were 21 years
85 \pm 3, and $22.7 \text{ kg}\cdot\text{m}^2 \pm 2.7$, respectively. An equal number of males and females were recruited,
86 and 90% were of White-British ethnicity. Exclusion criteria were smoking and self reported
87 history of inflammatory, autoimmune or cardiovascular disease, self-reported pregnancy or
88 suspected pregnancy, and use of prescription medication in the past month (excluding the
89 contraceptive pill). Participants self-reported no influenza-like illness in the year preceding this
90 investigation, and no symptoms of acute infection at the time of vaccination and follow up
91 measurements. All participants provided written informed consent and the study protocol was
92 approved by the Black Country Local Research Ethics Committee.

93

94 *Procedures*

95 Participants visited the laboratory between 12:00 and 16:00 to provide a baseline pre-
96 vaccine blood sample and to receive an influenza vaccination. Participants returned to provide
97 additional blood samples 24-h and 28-d after vaccination. Before arrival, participants were
98 instructed to abstain from vigorous exercise and over-the-counter medication for 24-h, alcohol
99 for 12-h, and food or caffeine for 2-h prior to their visit. Immediately after the baseline blood
100 sample, and prior to vaccination, the majority of participants ($n = 119$) undertook a 25-min
101 exercise intervention involving a series of weight lifting exercises. The results of this
102 manipulation are described elsewhere (Edwards et al. 2010) and did not influence the present
103 results.

104

105 *Vaccination*

106 Influenza vaccination was used as a marker of *in vivo* immune function (Burns and
107 Gallagher 2010). To increase sensitivity of this marker, a half-dose (0.25ml / 7.5µg; 50% adult
108 recommended dose) of the 2008/2009 northern hemisphere influenza vaccine (Fluarix,
109 GlasxoSmithKline, Inactivated Split Virion, Lot No. AFLUA384AB) was used. The vaccine
110 contained A/Brisbane- (both H3N2 and H1N1 strains), A/Uruguay- (H1N1 strain), and
111 B/Florida-like strains. A nurse administered the vaccine via intra-muscular injection into the
112 deltoid.

113

114 *Blood sample processing*

115 Serum was obtained by allowing blood to clot in plain Vacutainer tubes (Becton-
116 Dickinson, UK), centrifuging at 3400 g for 5 min at 21 °C, and was stored at –20 °C. Plasma was
117 obtained by centrifuging blood in potassium ethylene-diamine-tetra-acetic acid (K₃EDTA)
118 Vacutainer tubes at 3400 g for 10 min at 1 °C, and was stored at –80 °C.

119

120 *Assays*

121 Influenza antibody titre was determined in serum before vaccination (baseline) and at 28-
122 d using a haemagglutination inhibition test as previously described (Edwards et al. 2010). An
123 antibody titre represents the highest serum dilution to inhibit the agglutination of test
124 erythrocytes which bind together into a lattice-like structure upon exposure to influenza virus
125 particles (Burns and Gallagher 2010). Anti-CMV IgG and IL-6 were measured in plasma before
126 vaccination (baseline). CMV-seropositivity was defined as having an anti-CMV IgG titre > 3

127 IU/ml by ELISA, according to manufacturer instructions (Genesis Diagnostics, UK). IL-6 was
128 measured using a high sensitivity ELISA (Quantikine HS Human IL-6 ELISA, R&D Systems,
129 UK). Assay sensitivity was 0.1 IU/ml and 0.039 pg/ml for the CMV and IL-6 ELISAs
130 respectively. Only one individual fell below the sensitivity threshold for IL-6 (0.02 pg/ml) and
131 was included in analyses. Intra- and inter-assay precision (CV %) were <10% for both assays.

132

133 *Flow cytometry and immunophenotyping*

134 Leukocyte differential was assessed in K₃EDTA blood 24-h post-vaccination and
135 repeated 28-d later (Coulter ACT^{diff} haematology analyser; Beckman-Coulter, High Wycombe,
136 UK). These samples were processed for flow cytometric measurements as previously described
137 (Turner et al. 2010). Fixed cell preparations were read on a multi-parameter flow cytometer (BD
138 FACS CANTO II, BD Biosciences). Lymphocytes were gated on the forward *versus* side-scatter.
139 Sub-populations of CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells were identified using two analytical
140 strategies. First, expression of CD27 in combination with CD45RA identified naïve
141 (CD27⁺CD45RA⁺), central memory (CD27⁺CD45RA⁻; CM), effector memory
142 (CD27⁻CD45RA⁻; EM), ‘revertant’ effector memory cells which re-express CD45RA
143 (CD27⁻CD45RA⁺; EMRA). Second, CD27 expression was analysed in combination with CD28
144 to identify early- (CD27⁺CD28⁺), intermediate- (CD27⁺CD28⁻) and late-differentiated
145 (CD27⁻CD28⁻) sub-populations (Hamann et al. 1997; Appay et al. 2002). Data were analysed
146 with FlowJo (Treestar, Ashland, OR).

147

148 *Statistical analyses*

149 Data were inspected for normal distribution using the Kolmogorov-Smirnov test. Non-
150 normally distributed data were transformed logarithmically. Differences between CMV⁺ and
151 CMV⁻ participants were assessed with univariate analyses of variance (ANOVA) (continuous
152 variables) or Chi-squared tests (categorical variables). Relationships between IL-6 and
153 lymphocyte sub-populations were established using stepwise regression analysis. Anti-influenza
154 antibody titres were assessed between baseline and 28-d post-vaccination with repeated measures
155 ANOVA. To identify factors moderating the vaccination response, key variables (e.g., anti-CMV
156 IgG) were entered into ANOVAs as individual covariates and examined for significance
157 (Analysis of co-variance; ANCOVA). Significant covariates were further examined adjusting for
158 sex, CMV serostatus, baseline influenza antibody titre, and the pre-vaccine exercise-intervention.
159 Pearson's correlations established the direction of relationships between key variables (e.g., anti-
160 CMV IgG) and fold change of influenza antibody titre. Fold change was calculated by dividing
161 the geometric mean antibody titre by the baseline titre. Effect sizes are reported as eta-squared
162 (η^2). Conventionally, η^2 values of 0.01, 0.06, and 0.14 are considered small, medium and large
163 effect sizes respectively. For example, η^2 of 0.25 indicates that 25% of the observed effects (i.e.,
164 dependent variable) are accounted for by the experimental manipulation (i.e., the independent
165 variable). Data are presented as means \pm standard error of the mean (SEM) unless otherwise
166 stated. Data were analysed using SPSS statistical package version 18.0 for Windows (SPSS Inc.,
167 USA).

168

169 **Results**

170

171 *CMV serostatus*

172 Approximately 30% of individuals ($n = 48/158$) were CMV⁺ as determined by anti-CMV
173 IgG titres > 3 IU/ml. CMV-serostatus was not associated with age, sex, ethnicity or BMI ($p >$
174 .1).

175

176 *CMV seropositivity is associated with a higher lymphocyte count and lower CD4:CD8 ratio*

177 Lymphocyte and T cell sub-population data was averaged for the two collection days
178 (i.e., 24-h and 28-d post-vaccination). Analyses showed that for both days, the proportions of
179 cells were identical ($p > .38$), except for CD8⁺CD27⁻ CD45RA^{-/+} cells ($p < .01$) and correlated
180 between samples (average correlation $r = .72$, $p < .001$). Moreover, results reported below
181 remained the same when collection days were analysed separately.

182 CMV-seropositivity was associated with a higher lymphocyte count and a greater number
183 of CD8⁺ T cells ($F_{(1, 152)} > 8.1$; $p < .01$, $\eta^2 > .050$; data not shown). CMV-seropositivity was also
184 associated with a significantly lower CD4:CD8 ratio ($F_{(1, 152)} = 5.1$, $p < .03$, $\eta^2 = .032$; see Figure
185 1A). It is notable that a CD4:CD8 ratio < 1.0 was observed in 4/48 CMV⁺ individuals (8% of
186 CMV⁺) and none of the CMV⁻ individuals (independent samples t -test; $t_{(108)} 3.8$, $p < .001$).

187

188 *CMV infection is associated with an expansion of late-differentiated effector memory CD4⁺ and*
189 *CD8⁺T cells*

190 The numbers and proportions of CD4⁺ and CD8⁺ EM (CD27⁻CD45RA⁻), EMRA
191 (CD27⁻CD45RA⁺), and late-differentiated (CD27⁻CD28⁻) cells were significantly increased in

192 CMV⁺ individuals ($F_{(1, 152)} > 30.6$; $p < .001$, $\eta^2 > .167$, see Figure 1B, 1C and Table I). Further,
193 individuals with higher anti-CMV IgG titres (in CMV⁺ participants) had increased numbers of
194 total lymphocytes, CD8⁺ EMRA, and CD4⁺ late-stage differentiated cells (Pearson's correlations
195 $r = .32$ to $.38$, $p < .03$; CD8⁺ late-differentiated cells $r = .27$, $p = .064$; see Figure 2 A-D).

196

197 *Plasma IL-6 is increased with CMV infection and associated with the number of CD8⁺ CD28⁻*
198 *cells*

199 Modestly elevated plasma IL-6 was observed in CMV⁺ individuals, as compared to
200 CMV⁻ donors ($F_{(1, 152)} = 4.3$, $p < .05$, $\eta^2 = .027$; see Figure 3A). Individuals with higher IL-6 had
201 increased numbers of both intermediate (CD27⁺CD28⁻) and late-stage (CD27⁻CD28⁻)
202 differentiated CD8⁺ cells (Pearson's correlation $r > .18$, $p < .03$; data not shown). The
203 relationship between CD28⁻ cells and IL-6 was largely an effect of CMV infection. After
204 statistical adjustment for anti-CMV IgG, the association between IL-6 and late-stage
205 differentiated (CD27⁻CD28⁻) cells was lost. This association remained close to significance for
206 intermediate-stage (CD27⁺CD28⁻) cells ($r = .19$; $p < .05$).

207

208 *Higher anti-CMV IgG titres are associated with weaker antibody responses to A/Brisbane*
209 *antigen*

210 CMV serostatus did not predict vaccination responses (defined as a > 4 fold change in
211 antibody titre 28-d post-vaccination) to any of the influenza strains ($p > .1$). However, a higher
212 concentration of anti-CMV IgG was associated with a smaller increase in antibody titres against
213 the A/Brisbane antigen (anti-CMV IgG \times time interaction $F_{(1, 152)} = 4.1$, $p < .05$, $\eta^2 = .026$;
214 Pearson's correlation $r = .16$ $p < .05$; see Figure 3B). This effect remained significant after

215 statistical adjustment for sex, A/Brisbane baseline antibody titre, and the pre-vaccine exercise
216 intervention (Adjusted anti-CMV IgG \times time interactions; $F_{(1, 155)} > 3.9, p < .05, \eta^2 > .025$).
217 There were no relationships between anti-CMV IgG and antibody responses to the other
218 influenza strains.

219

220 *High numbers of total lymphocytes and late differentiated T cells are associated with smaller*
221 *antibody responses to A/Brisbane antigen*

222 A weaker A/Brisbane antibody response was associated with a higher total lymphocyte
223 and CD8⁺ T cell count, as well as increased numbers of late-differentiated CD4⁺ and CD8⁺ T
224 cells (CD27⁻CD28⁻), and CD8⁺ EM (CD27⁻CD45RA⁻) and EMRA (CD27⁻CD45RA⁺) cells
225 (cell population \times time interactions $F_{(1, 156)} > 4.4, p < .05, \eta^2 > .041$; Pearson's correlations $r =$
226 $-.16$ to $-.23, p < .05$). These effects remained significant after statistical adjustment for sex,
227 A/Brisbane baseline antibody titre, and the pre-vaccine exercise intervention (Adjusted analyses
228 all; $F_{(1, 155)} > 4.2, p < .05, \eta^2 > .019$). However, the observation that individuals with higher cell
229 counts exhibited smaller A/Brisbane antibody responses was largely an effect of CMV infection.
230 After statistical adjustment for anti-CMV IgG or CMV serostatus, most of these associations
231 were lost, except for total lymphocytes and total CD8⁺ T cells (Analyses adjusted for CMV
232 infection; $F_{(1, 155)} > 4.2, p < .05, \eta^2 > .026$). There were no relationships between lymphocyte and
233 lymphocyte sub-populations with antibody responses to the other influenza strains.

234

235 **Discussion**

236 The results of this study show that young adults infected with CMV exhibit mild signs of
237 immunosenescence, characterised by increased numbers of late-differentiated/effector memory T
238 cells and elevated inflammatory activity. The present study adds to the very sparse data on the
239 relationship between CMV and T cell differentiation in healthy young adults, and is the first to
240 confirm that the magnitude of the humoral response to CMV (i.e., anti-CMV IgG) is associated
241 with an accumulation of late-differentiated/effector memory T cells. Until now, to the best of our
242 knowledge, this phenomenon has only been shown in elderly subjects (Alonso Arias et al. 2013;
243 Vescovini et al. 2010). Moreover, donors with the strongest CMV-specific immune response,
244 characterised by high anti-CMV IgG titres and memory T cell numbers, exhibited a reduced
245 antibody response to a half-dose influenza vaccination.

246 Although the majority of individuals exhibited a > 4 fold change in antibody titre 28 days
247 post-vaccination to each influenza strain (i.e., individuals were World-Health-Organisation
248 defined “vaccine responders”), we found that an increased anti-CMV IgG titre was associated
249 with a smaller antibody response to the A/Brisbane influenza antigen only. Anti-CMV IgG was
250 also associated with increased numbers of late-differentiated/effector memory T cells. In turn, a
251 higher number of these cells also associated with lower vaccine responses to the A/Brisbane
252 antigen, but this was a consequence of CMV infection: when controlling statistically for CMV
253 serostatus, or anti-CMV IgG titre, most of these associations were lost.

254 It is unclear why our observations were limited to the A/Brisbane vaccine component,
255 although strain-specific differences in the magnitude of antibody response to influenza
256 vaccination have commonly been reported in a variety of contexts. For example, obese
257 individuals have been shown to exhibit larger antibody responses to single influenza vaccine

258 components (Sheridan et al. 2012; Talbot et al. 2012). Further, sexual dimorphism and diurnal
259 variation in vaccine efficacy has been observed with single influenza sub-types in trivalent
260 vaccines (Phillips et al. 2008; Langlois et al. 1995). Finally, the age-associated decline in
261 antibody response to vaccination is not uniform between strains. Differences in antibody
262 response between young and old are largest with H1N1 and B antigens, compared to H3N2
263 antigens (Goodwin et al. 2006). Indeed, our laboratory has repeatedly shown that weaker vaccine
264 antigens are the most sensitive to mild forms of immunomodulation, such as exercise or chronic
265 stress (Edwards et al. 2006; Edwards et al. 2007; Edwards et al. 2010; Edwards et al. 2008;
266 Burns and Gallagher 2010). We speculate that strain-specific variation in the immunogenicity of
267 vaccine components might explain contradictory findings in the literature (Burns and Gallagher
268 2010; Burns et al. 2003; den Elzen et al. 2011; Goodwin et al. 2006). In the present study, the
269 A/Brisbane strain was the most immunogenic vaccine component (Edwards et al. 2010)
270 consisting of both H1N1 and H3N2 subtypes, which show greatest age-related differences in
271 immunogenicity (Goodwin et al. 2006). Significantly, H3N2 was highly prevalent around the
272 time of this investigation (CDC 2008) and is the strain associated with most influenza-related
273 complications and deaths (Thompson et al. 2003). It is therefore fascinating to show that the
274 immunosuppressive effects of CMV infection can be observed with highly immunogenic
275 influenza vaccine components in such a young and healthy population. Thus, the present findings
276 provide further support for the concept that CMV is a major driving force behind
277 immunosenescence and might result in vaccine failure and increased infection risk in CMV
278 seropositive individuals (Derhovanessian et al. 2009).

279 It is currently unknown exactly how infection with CMV could promote lower antibody
280 responses to vaccination. Immunosenescence and chronological ageing might be associated with

281 impaired dendritic cell function (Panda et al. 2010). It is unclear whether this is a direct effect of
282 CMV infection on antigen presenting cells, or a secondary downstream consequence of infection
283 (e.g., indirect effects caused by the accumulation of memory T cells). Indeed, dendritic cell
284 function is impaired by co-incubation with CD8⁺CD28⁻ cells, which promote expression of
285 negative regulatory receptors such as immunoglobulin-like transcripts 3 and 4 (ILT3 and ILT4)
286 (Chang et al. 2002). In addition, CD8⁺CD28⁻ cells cause a down-regulation of CD40-ligand on
287 CD4⁺ T helper cells (Cortesini et al. 2001) and the ensuing impaired responsiveness of CD4⁺ T-
288 helper cells may in turn inhibit antibody production, which is a hallmark of B cell
289 immunosenescence (Siegrist and Aspinall 2009).

290 It is notable that 8% of CMV⁺ individuals (4/58) in the present study showed an inverted
291 CD4:CD8 ratio of less than 1.0. This marker of immunosenescence is a hallmark of the ‘Immune
292 Risk Profile’ (IRP), an immune phenotype observed in around 15% of people aged over 85 years
293 in the Swedish OCTO/NONA studies. The IRP is a cluster of immunological markers, including
294 the accumulation of CD8⁺ memory T cells (resulting in an inverted CD4:CD8 ratio) as well as
295 CMV infection, which predicts all cause mortality in the very old (Wikby et al. 2006;
296 Derhovanessian et al. 2009; Wikby et al. 2005). Further, a recent 10-year follow up of ~14,000
297 adults from the National Health and Nutrition Examination Survey (NHANES) provides
298 evidence that CMV infection alone predicts all cause mortality independently of inflammatory
299 activity (CRP level) (Simanek et al. 2011). The results of the present study suggest that a mild
300 IRP-like phenotype (which might be associated with mortality) can be observed in some adults
301 much earlier in life than has been reported previously.

302 To the best of our knowledge, we have shown for the first time that healthy young adults
303 latently infected with CMV exhibit increased levels of IL-6. Until now, interaction between IL-6

304 and pathogen burden has only been observed in the elderly (Nazmi et al. 2010; Schmaltz et al.
305 2005). This result seems mechanistically plausible considering the strong in vitro evidence that
306 CMV infection in a variety of cell types, including monocytes, epithelial cells, and adipocytes,
307 results in a marked increase of IL-6 gene and protein expression (Bouwman et al. 2009; Geist
308 and Dai 1996; Iwamoto and Konicek 1997; Visseren et al. 1999). Increased IL-6 is thought to
309 reflect viral reactivation (Bennett et al. 2011) due to a potential role for this cytokine in chronic
310 viral control (Harker et al. 2011). The present manuscript also shows that elevated IL-6 level is
311 associated with the numbers of CD8⁺ CD28⁻ T cells. Although our data suggest this relationship
312 is largely a direct effect of CMV infection, a causal relationship between CD8⁺ CD28⁻ T cells
313 and IL-6 is biologically plausible. For example, CD8⁺CD28⁻ are efficient producers of
314 inflammatory cytokines such as TNF- α , IFN- γ and IL-6 (Zanni et al. 2003; Lorre et al. 1994;
315 Effros et al. 2005). In addition, an association between systemic inflammation and an
316 accumulation of CD8⁺CD28⁻ cells has also been found in healthy older adults (Zanni et al. 2003;
317 Wikby et al. 2006), as well as in a variety of auto-immune diseases including rheumatoid
318 arthritis, multiple-sclerosis and Graves' disease (Sun et al. 2008; Schmidt et al. 1996; Markovic-
319 Plese et al. 2001). Together, our results extend the existing literature that links the concept of
320 'inflamm-ageing' and T cell senescence (Franceschi et al. 2000) by showing that such
321 associations may even be present in healthy young adults, likely driven by CMV infection.

322 In the present study, individuals infected with CMV exhibited plasma IL-6 levels
323 approximately 20% higher than CMV-negative individuals. This is comparable to the elevated
324 level of IL-6 seen with men and women who develop coronary heart disease later in life,
325 compared to those who remain healthy (Pai et al. 2004). The present finding may thus provide an
326 additional mechanism by which CMV has been implicated in the development of cardiovascular

327 disease (Soderberg-Naucler 2006; Strandberg et al. 2009). Individuals with a Guanine > Cytosine
328 single-nucleotide-polymorphism (SNP) at position -174 in the promoter region for the IL-6 gene
329 show ~60% increase in IL-6 levels, and are at greater risk of cardiovascular disease (Rafiq et al.
330 2007). It would be interesting to examine whether being CMV seropositive with this SNP carries
331 a further increased risk of disease (Soderberg-Naucler 2006; Pai et al. 2004).

332 This study does not provide data on specific T cell responses to CMV infection (e.g.,
333 IFN- γ production to CMV antigens and MHC-class I or II tetramer staining). However, in CMV-
334 seropositive individuals, it is well accepted that the majority of CD27⁻ and CD28⁻ T cells are
335 CMV-specific. Moreover, a substantial number of studies have shown positive correlations
336 between the number of late-differentiated/effector-memory T cells and the CMV-specific
337 immune response across a range of ages (Griffiths et al. 2013; Lachmann et al. 2012; Khan et al.
338 2002; Pita-Lopez et al. 2009). In future studies, such measurements of CMV-specific T cells
339 could be correlated with the antibody response to influenza vaccination in young adults. While
340 this data may not necessarily contribute to our understanding of the mechanisms underlying
341 diminished vaccine responses, it would broaden our understanding of CMV infection in young
342 adults, and might provide a potential predictor of vaccine efficacy in CMV infected people.
343 Based on the small amount of data on this topic, it might be expected that the magnitude of IFN-
344 γ production by CMV specific T cells in young adults would be negatively associated with
345 influenza vaccine responses, as has been shown in the elderly (Moro-Garcia et al. 2012).

346 The role of CMV in immunosenescence remains a topic of debate (Wills et al 2011). For
347 example, it has been shown that in residents of long-term care facilities, CMV does not impair
348 the antibody response to influenza vaccination (den Elzen et al. 2011). In light of these and other
349 inconsistent findings, it cannot be assumed that the effects of CMV shown in the elderly in some,

350 but not all studies, will translate into young adults. In the present study, by measuring multiple
351 biomarkers of immunosenescence, we confirm that the deleterious effects of CMV infection,
352 sometimes shown in the elderly, do indeed translate to young adults (Saurwein-Teissl et al. 2002;
353 Goronzy et al. 2001; Trzonkowski et al. 2003; Wikby et al. 2006). Our findings show that
354 healthy university students, infected with CMV, exhibit an accumulation of late-
355 differentiated/effector memory T cells, increased inflammatory activity, and poorer immune
356 responses to novel antigens early in life (i.e., well before overt signs of immune impairment
357 appear). It should be considered however, that the population examined in this work are a
358 socially homogenous group of young adults, the majority of whom were of White-British
359 ethnicity, and of high socio-economic status. Making identical measurements in a more
360 heterogenous group of individuals might result in larger and stronger effects of CMV on the
361 results presented.

362 The present observations are relevant to influenza control and might also apply to other
363 routine vaccination programmes (e.g., Hepatitis B). Likewise, these findings might warrant
364 investigation of vaccine efficacy in other contexts, such as yellow fever, which is important
365 considering the popularity of worldwide travel. Another implication is that the current findings
366 suggest a different theoretical approach to T cell immunosenescence, which might be relevant to
367 the type and timing of interventions that intend to ameliorate or prevent immunosenescence
368 (Adler 2008; Pawelec et al. 2010). Traditional thinking is that senescence is primarily driven by
369 thymic involution, whereby CMV infection could act as a secondary or amplifying, factor. The
370 current observations instead suggest a more primary role of CMV in establishing rudimentary
371 features of immunosenescence very early in life, whereby subsequent thymic involution may
372 potentially act as a secondary feature.

373

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383

384

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555 relationship between inflammation and immunosenescence. Exp Gerontol 38 (9):981-987.
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559

560 **Table I. Proportion of lymphocyte subpopulations in CMV⁻ and CMV⁺ participants (mean**
561 **± SD)**

Cells % [†]	CMV ⁻	CMV ⁺
CD4+ [#]	39.44 ± 6.18	38.53 ± 5.55
CD4+ naïve	46.26 ± 10.27	44.81 ± 10.96
CD4+ CM	48.61 ± 9.23	46.16 ± 9.54
CD4+ EM	4.90 ± 2.31	7.50 ± 4.22***
CD4+ EMRA	0.22 ± 0.49	1.53 ± 3.83***
CD4+ early	93.60 ± 2.60	89.68 ± 7.32*
CD4+ inter	1.27 ± 0.81	1.28 ± 1.03
CD4+ late	0.19 ± 0.43	3.29 ± 6.07***
CD8+ [#]	20.77 ± 4.12	23.42 ± 5.36**
CD8+ naïve	57.77 ± 10.52	48.69 ± 13.71***
CD8+ CM	34.38 ± 9.35	30.10 ± 11.97**
CD8+ EM	3.88 ± 2.68	7.23 ± 5.48***
CD8+ EMRA	3.96 ± 5.32	13.98 ± 11.12***
CD8+ early	79.38 ± 9.14	65.98 ± 14.88***
CD8+ inter	12.78 ± 6.44	12.81 ± 8.18
CD8+ late	5.39 ± 5.54	15.90 ± 12.87***

562

563 [†]Cells as a proportion of CD4⁺ or CD8⁺ T lymphocytes

564 [#]Cells as a proportion of Lymphocytes

565 *** *p* < .001, ** *p* < .01, * *p* < .05, Univariate ANOVA

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570 **Figure legends**

571 **Figure 1.** Composition of the T cell pool with CMV infection. **A)** Ratio of CD4⁺ to CD8⁺ T
572 lymphocytes. 4/58 CMV⁺ individuals (8%) exhibited a ratio < 1.0. **B)** CD4⁺ and CD8⁺ effector
573 memory (EM; CD27⁻CD45RA⁻) and ‘revertant’ effector memory (EMRA; CD27⁻CD45RA⁺) T
574 lymphocytes. **C)** CD4⁺ and CD8⁺ T intermediate-stage (CD27⁺CD28⁻) and late-differentiated
575 (CD27⁻CD28⁻) T lymphocytes. Data are means ± SEM. *** $p < .001$, * $p < .05$ CMV⁺ compared
576 to CMV⁻.

577

578 **Figure 2.** Significant* associations between anti-CMV IgG and lymphocyte populations. **A)**
579 Total lymphocytes. **B)** CD8⁺ ‘revertant’ effector memory (EMRA; CD27⁻CD45RA⁺) T
580 lymphocytes. **C)** CD4⁺ late-differentiated (CD27⁻CD28⁻) T lymphocytes. **D)** CD8⁺ late-
581 differentiated (CD27⁻CD28⁻) T lymphocytes (* $p = .064$). Statistical analyses were restricted to
582 CMV⁺ individuals (n=48) and were conducted on log10 data. β represents standardised
583 regression coefficient.

584

585 **Figure 3.** Elevated inflammatory activity and reduced antibody responses to antigen challenge
586 with CMV infection. **A)** Plasma IL-6 concentration. **B)** Data depicts association between anti-
587 CMV IgG titre and fold change in A/Brisbane antibody titre 28 d after influenza vaccination ($p <$
588 $.05$). For illustrative purposes, anti-CMV IgG is presented in quartiles; CMV⁻ < 3.0 IU/ml,
589 CMV⁺ low 3.0-5.1 IU/ml, CMV⁺ med 5.1-9.1 IU/ml, CMV⁺ high > 9.1 IU/ml. Statistical
590 analyses was conducted on continuous data (Altman and Royston 2006). Data are means ± SEM.
591 *** $p < .001$, * $p < .05$ CMV⁺ compared to CMV⁻.

Figure 1

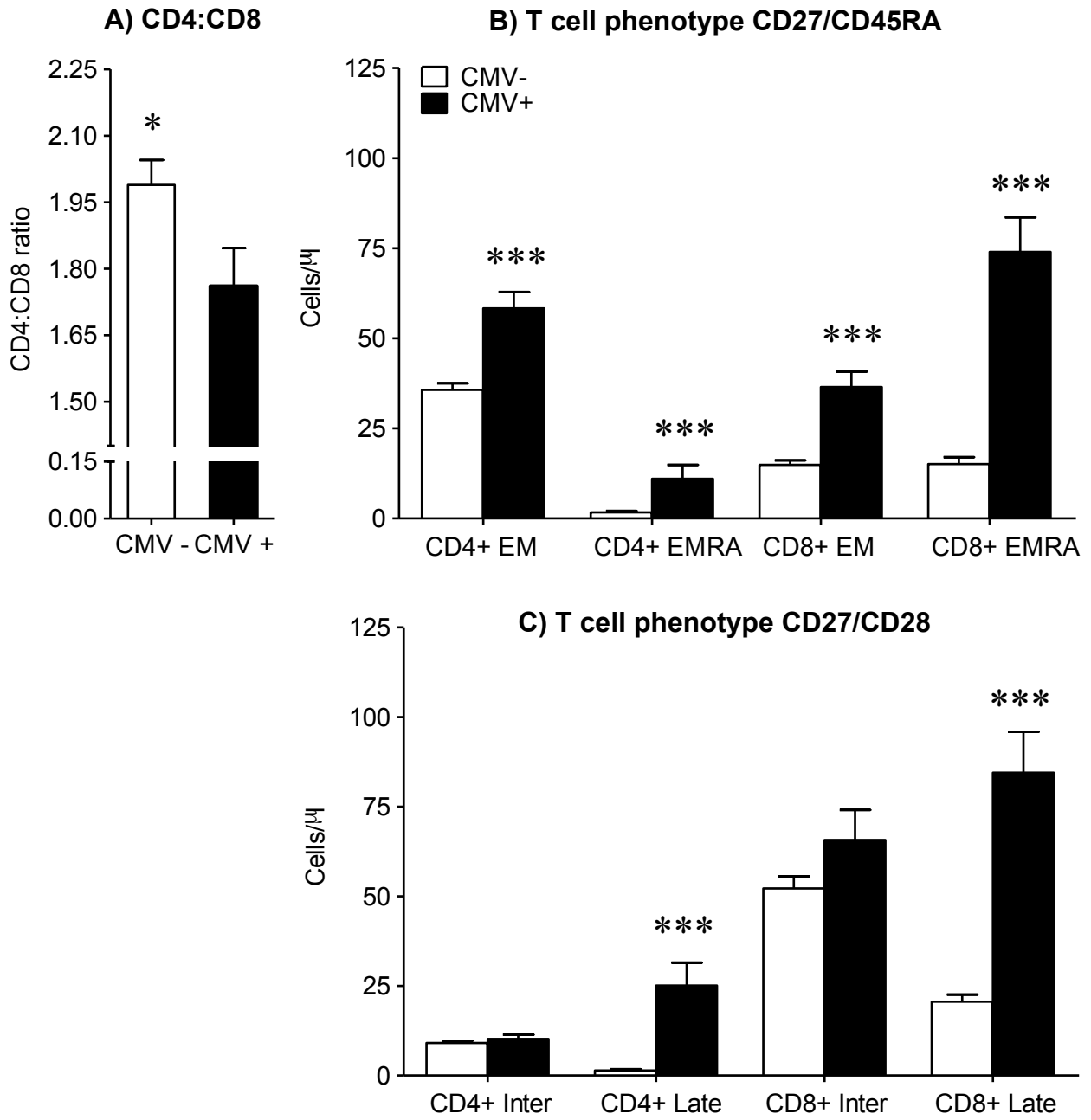


Figure 2

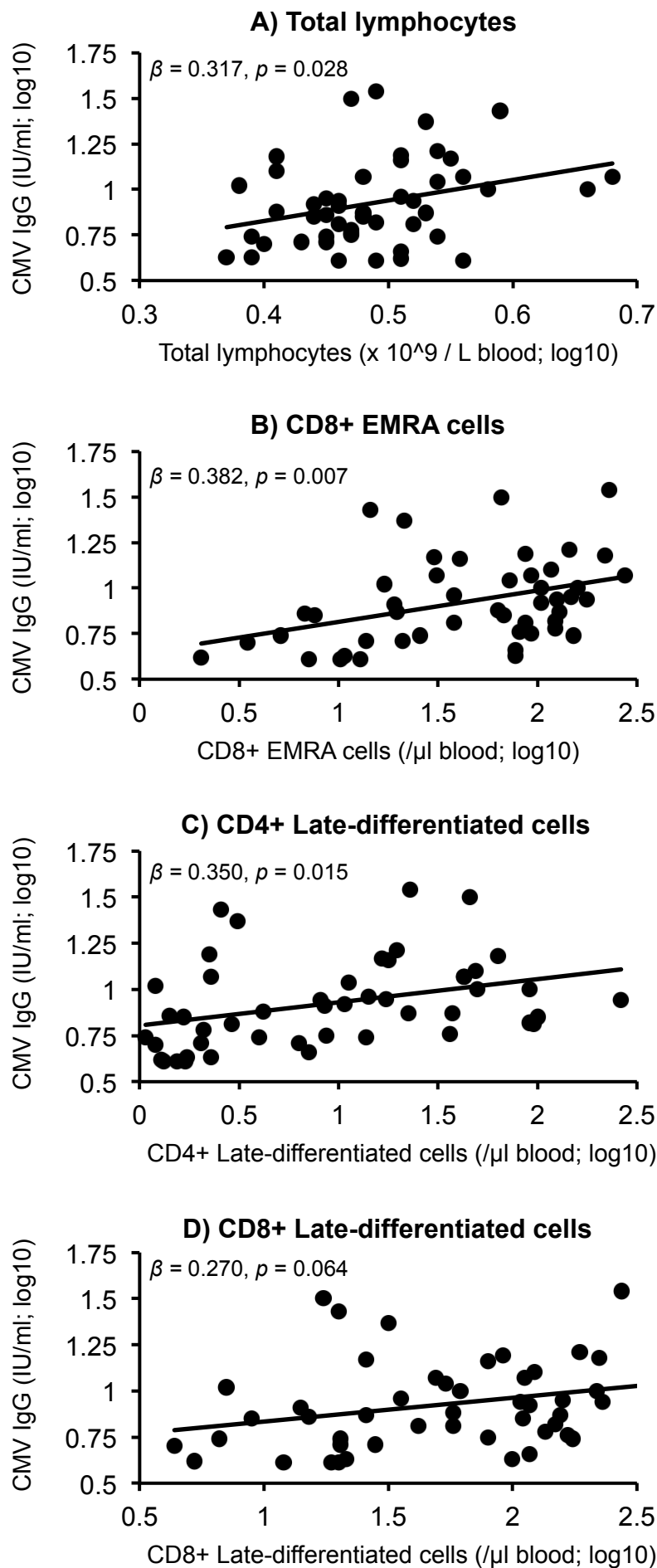


Figure 3

