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Rudimentary signs of immunosenescence in Cytomegalovirus seropositive healthy young adults

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Running head: Immunosenescence in early adulthood

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Abstract

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

One hundred and fifty eight individuals (mean ± SD; age 21 years ± 3, body mass index 22.7 $kg \cdot m^2 \pm 2.7$) were assessed for CMV-serostatus, the numbers/proportions of $CD4^+$ and $CD8^+$ late-differentiated/effector-memory cells (i.e., $CD27^-CD28^-/CD45RA^+$), plasma interleukin-6 (IL-6), and antibody responses to an *in vivo* antigen challenge (half-dose influenza vaccine). Thirty per-cent (48/158) of participants were CMV^+. A higher lymphocyte and $CD8^+$ count (both $p<.01$), and a lower CD4:CD8 ratio ($p<.03$) was observed in CMV^+ people. Eight per-cent (4/58) of CMV^+ individuals exhibited a CD4:CD8 ratio <1.0, whereas no CMV^- donor showed an inverted ratio ($p<.001$). The numbers of $CD4^+$ and $CD8^+CD27^-CD28^-/CD45RA^+$ cells were ~4-fold higher in CMV^+ people ($p<.001$). Plasma IL-6 was higher in CMV^+ donors ($p<.05$) and showed a positive association with the numbers of $CD8^+CD28^-$ cells ($p<.03$). Finally, there was a significant negative correlation between vaccine-induced antibody responses to the A/Brisbane influenza strain and CMV-specific Immunoglobulin-G titres ($p<.05$). This reduced vaccination response was associated with greater numbers of total $CD8^+$, and $CD4^+$ and $CD8^+CD27^-CD28^-/CD45RA^+$ cells ($p<.05$).

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

Introduction

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

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

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

Methods

Participants

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

Procedures

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

Vaccination

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

Blood sample processing

Serum was obtained by allowing blood to clot in plain Vacutainer tubes (Becton-Dickinson, UK), centrifuging at 3400 *g* for 5 min at 21 °C, and was stored at −20 °C. Plasma was obtained by centrifuging blood in potassium ethylene-diamine-tetra-acetic acid (K_3EDTA) Vacutainer tubes at 3400 *g* for 10 min at 1 °C, and was stored at −80 °C.

Assays

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

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

Flow cytometry and immunophenotyping

Leukocyte differential was assessed in K_3EDTA blood 24-h post-vaccination and repeated 28-d later (Coulter ACT^{diff} haematology analyser; Beckman-Coulter, High Wycombe, UK). These samples were processed for flow cytometric measurements as previously described (Turner et al. 2010). Fixed cell preparations were read on a multi-parameter flow cytometer (BD FACS CANTO II, BD Biosciences). Lymphocytes were gated on the forward *versus* side-scatter. Sub-populations of $CD3^+CD4^+$and $CD3^+CD8^+$ T cells were identified using two analytical strategies. First, expression of CD27 in combination with CD45RA identified naïve ($CD27^+CD45RA^+$), central memory ($CD27^+CD45RA^-$; CM), effector memory ($CD27^-CD45RA^-$; EM), 'revertant' effector memory cells which re-express CD45RA ($CD27^-CD45RA^+$; EMRA). Second, CD27 expression was analysed in combination with CD28 to identify early- ($CD27^+CD28^+$), intermediate- ($CD27^+CD28^-$) and late-differentiated ($CD27^-CD28^-$) sub-populations (Hamann et al. 1997; Appay et al. 2002). Data were analysed with FlowJo (Treestar, Ashland, OR).

Statistical analyses

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

Results

CMV serostatus

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

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

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

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

CMV infection is associated with an expansion of late-differentiated effector memory $CD4^+$ and $CD8^+$ T cells

The numbers and proportions of $CD4^+$ and $CD8^+$ EM ($CD27^-CD45RA^-$), EMRA ($CD27^-CD45RA^+$), 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 × 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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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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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567

568

Figure legends

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

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

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

Figure 1

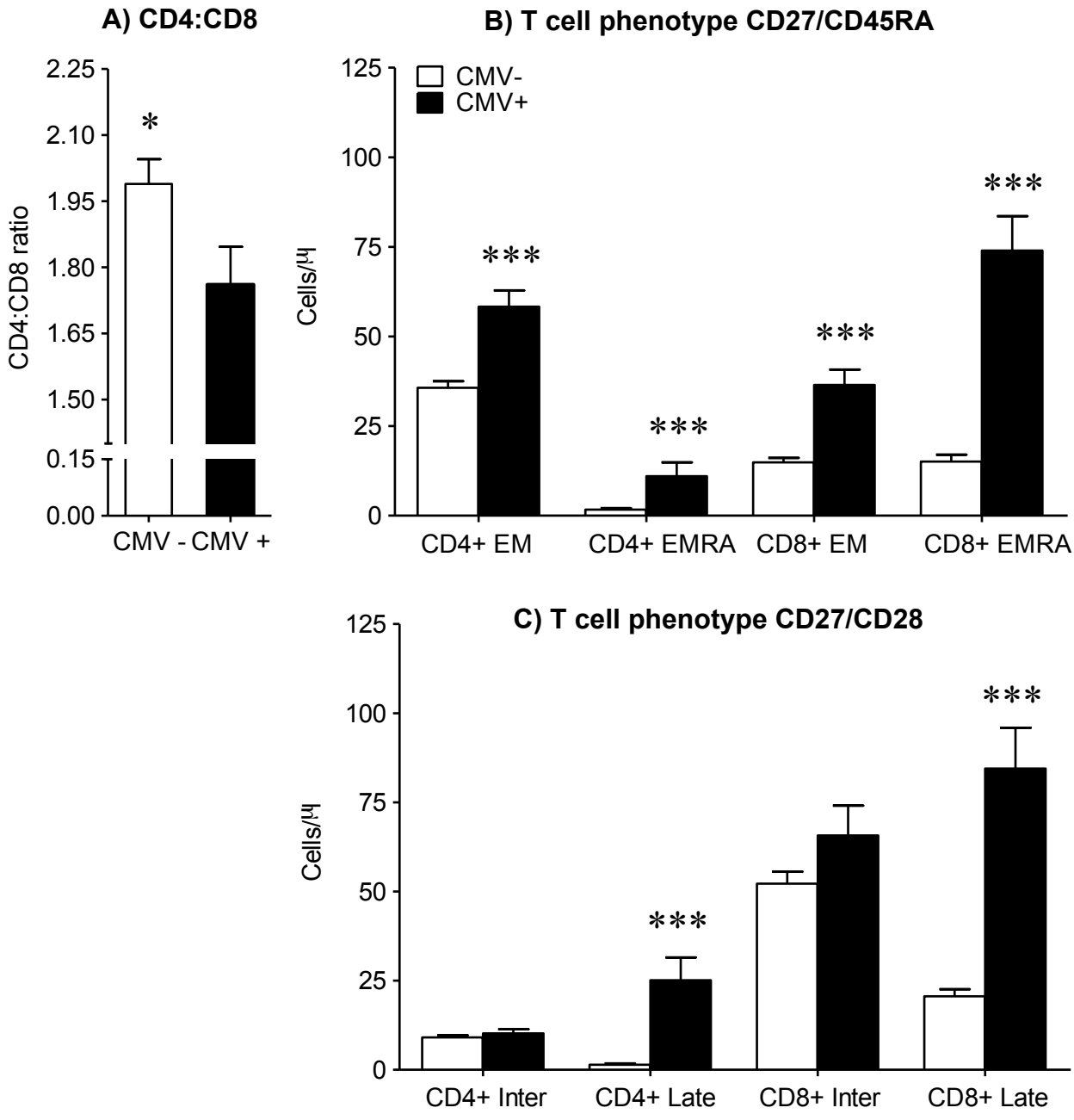

A) CD4:CD8
CD4:CD8 ratio
2.25
2.10
1.95
1.80
1.65
1.50
0.15
0.00
*
CMV - CMV +
B) T cell phenotype CD27/CD45RA
CMV-
CMV+
Cells/µl
125
100
75
50
25
0
***
***
***
***
CD4+ EM
CD4+ EMRA
CD8+ EM
CD8+ EMRA
C) T cell phenotype CD27/CD28
Cells/µl
125
100
75
50
25
0
***
***
CD4+ Inter
CD4+ Late
CD8+ Inter
CD8+ Late

Figure 2

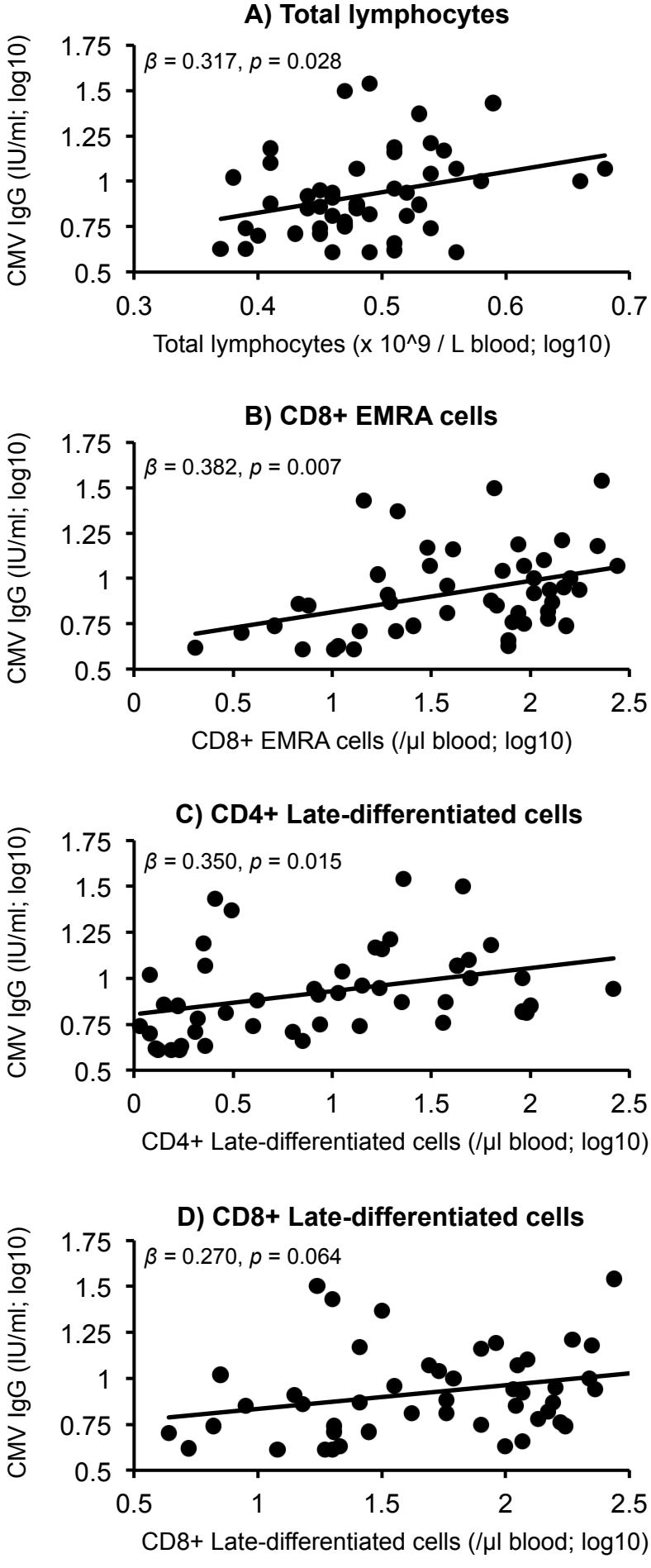
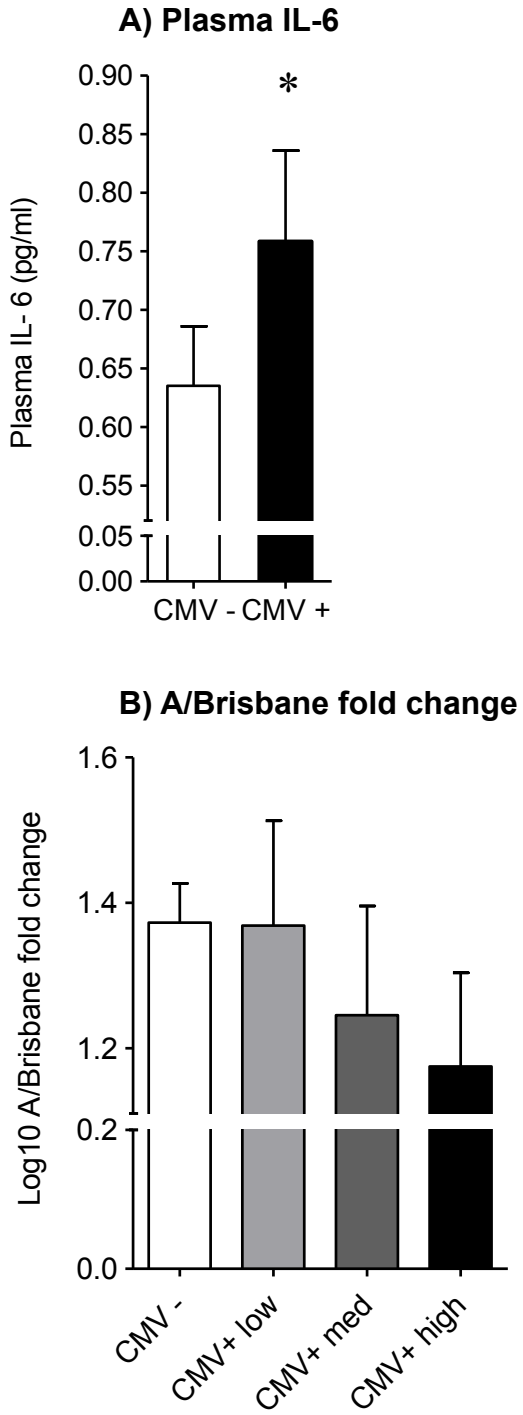

A) Total lymphocytes
β = 0.317, p = 0.028
CMV IgG (IU/ml; log10)
Total lymphocytes (x 10^9 / L blood; log10)
B) CD8+ EMRA cells
β = 0.382, p = 0.007
CMV IgG (IU/ml; log10)
CD8+ EMRA cells (/µl blood; log10)
C) CD4+ Late-differentiated cells
β = 0.350, p = 0.015
CMV IgG (IU/ml; log10)
CD4+ Late-differentiated cells (/µl blood; log10)
D) CD8+ Late-differentiated cells
β = 0.270, p = 0.064
CMV IgG (IU/ml; log10)
CD8+ Late-differentiated cells (/µl blood; log10)

Figure 3


A) Plasma IL-6
Plasma IL- 6 (pg/ml)
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.05
0.00
*
CMV - CMV +
B) A/Brisbane fold change
Log10 A/Brisbane fold change
1.6
1.4
1.2
0.2
0.0
CMV -
CMV+ low
CMV+ med
CMV+ high