The age-related increase in low grade systemic inflammation (Inflammaging) is not driven by cytomegalovirus infection


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Abstract

Background
Ageing is accompanied by the development of low grade systemic inflammation, termed ‘inflammageing’, characterised by raised serum C-reactive protein (CRP) and pro-inflammatory cytokines. Importantly, inflammageing is implicated in the pathogenesis of several age-related diseases including cardiovascular disease, type 2 diabetes and dementia and is associated with increased mortality. The incidence of infection with the persistent herpes virus cytomegalovirus (CMV) also increases with age. Cross-sectional studies have proposed CMV infection as a significant driver of inflammageing, but a definitive case for CMV as a causative agent in inflammageing has not been made.

Methods
We studied longitudinally 249 subjects (153 men, 96 women) who participated in the Hertfordshire Ageing Study at baseline (1993/5, mean age 67.5 years) and at 10 year follow up. At both times anthropometric measurements were made, subjects provided blood samples for analysis of inflammatory status and CMV seropositivity and completed lifestyle questionnaires.

Findings
In the cohort as a whole, serum CRP (p<0.02) and pro-inflammatory cytokines TNFα (p<0.001) and IL-6 (p<0.001) were increased between baseline and follow up sampling whereas levels of the anti-inflammatory cytokine IL-10 were decreased (p<0.001). These changes to cytokine status over time occurred equally in the 60% of subjects who were seropositive for CMV at baseline and follow up, the 8% who were CMV negative at baseline but who became CMV positive by the follow up, and also in the 32% who were CMV
seronegative throughout. Factors that positively associated with inflammeing were a sedentary lifestyle and a higher BMI.

**Interpretation**

Inflammeing occurs independently of CMV infection. Vaccination against CMV is unlikely to prevent age-related inflammation and lifestyle factors are the key modifiable variables to be addressed to reduce inflammeing.

**Funding**

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Introduction

Physiological ageing is associated with a chronic sub-clinical systemic inflammatory state, termed inflammageing\(^1\), which is characterised by elevated levels of serum pro-inflammatory cytokines such as interleukin 6 (IL-6) and TNF\(\alpha\) and acute phase proteins such as C-reactive protein (CRP)\(^1,2\). Further, the levels of cytokines which counteract the inflammatory state, such as IL-10, are reduced with age\(^3\) compounding the inability to maintain immune homeostasis. Importantly, inflammageing is a predictor of frailty\(^4\), is associated with increased mortality\(^5\), and chronic low grade inflammation is now accepted as a key pathogenic factor in the development of several age-related pathologies including cardiovascular disease\(^6\), dementia\(^7\) and type 2 diabetes\(^8\). Further, studies in centenarians\(^9\) show that these extremely long lived individuals maintain the cytokine profile of younger adults and do not develop inflammageing. Understanding the causes of inflammageing is therefore important for developing interventions to prevent its occurrence and extend the healthy lifespan of our ageing population.

Various factors have been proposed to drive inflammageing including increased adiposity with age, as adipose tissue is a significant source of pro-inflammatory cytokines\(^10\), and decreased production of sex steroids\(^11\), many of which are anti-inflammatory. However one of the dominant theories is that inflammageing is driven in large part by the sustained efforts of the immune system to control infections with persistent herpes viruses, most notably cytomegalovirus\(^12,13\). Cytomegalovirus (CMV) is a prevalent \(\beta\)-herpes virus infecting 60-85% of the Western population\(^14\) and approximately 80-90% of those over 65 years\(^15\). Infection is usually asymptomatic in immunocompetent individuals and the virus is thought to be latent within myeloid leukocytes but undergoes intermittent reactivation\(^16\). CMV infection has been shown to induce IL-6 and TNF\(\alpha\) production by leukocytes\(^17\) and CMV seropositivity is in turn associated with increased all-cause mortality\(^12,18\). These observations have led to the
suggestion that CMV infection is a major driver of inflammaging and that this effect contributes to the increased morbidity and mortality associated with infection. Moreover, if correct, this would suggest that vaccination against CMV in early life would help to prevent inflammaging and therefore improve health in old age. To date no longitudinal studies have been reported to confirm a link between CMV infection and inflammaging.

The aim of this study was to determine the impact of CMV infection on serum pro- and anti-inflammatory cytokine levels in a 10 year longitudinal study of older adults, comparing individuals who were CMV seronegative throughout, those who acquired CMV during the 10 year period, and those who were CMV seropositive at baseline and follow up. The relative contribution made by lifestyle and anthropomorphic influences were also considered.

**Methods**

The Hertfordshire Ageing Study (HAS) has been described previously\(^\text{19}\). In brief, 717 men and women who were born in Hertfordshire, UK between 1920 and 1930, attended a home interview and clinic in 1993/5 for the first phase of the HAS where a wide range of markers of ageing were characterised and a blood sample was taken. 10 years later in 2003/5, a second phase of follow-up was conducted and 249 men and women provided a follow up blood sample. Serum was aliquoted and stored at -80°C on both occasions. The HAS follow-ups had ethical approval from the Hertfordshire and Bedfordshire Local Research Ethics Committee.

**Serological Analysis**

Serum was thawed and cytokines IL-6, IL-10 and TNFα were simultaneously measured using commercially available multiplex luminometry whilst C-reactive protein was measured using singleplex luminometry (Invitrogen, UK). Samples were analysed on a Luminex\(^{100}\) instrument (Luminex Corp., Austin, TX, USA) with the acquisition software StarStation.
IFN\(\gamma\) was measured using a commercial ELISA kit and following manufacturer’s instructions (eBiosciences, Hatfield, UK).

CMV serostatus was determined using an in-house pre-validated ELISA. Briefly, MRC-5 fibroblasts were infected with AD169 or Towne125 human CMV strains at a multiplicity of infection (MOI) of 4:1 for 4 hours. Mock infections were also conducted to eliminate non-specific IgG binding. Supernatants were harvested and centrifuged to pellet the virus before quantification by stimulation of PBMC’s from CMV positive donors. Viral or mock lysate was then bound to an ELISA NUNC plate overnight. CMV standards and donor serum samples were then incubated for 1 hour in both viral and mock lysate wells. Following washing, samples were then incubated with an anti-IgG-Horseradish Peroxidase conjugated secondary antibody (DAKO Cytomation Ltd, Cambridge, UK) for 1 hour before addition of TMB solution for 10 minutes. Reactions were stopped using 2N H\(_2\)SO\(_4\) and the plate read at 450nm using an ELISA plate reader. Quantification of anti-CMV titre was determined by subtraction of mock lysate from CMV lysate data.

**Statistical methods**

Statistical analyses were performed using PASW version 18.0 (Chicago, IL, USA) and all data were summarised as mean ± standard error of the mean (SEM). Three categorical groups were created based on CMV infection over time and represented as either being negative (CMV-/−), positive (CMV+/+) or having converted from negative to positive (CMV-/+) over time. Data were then analysed using repeated measures ANOVA to assess the effect of time on inflammation and then its interaction with CMV status and gender. Following this, BMI and smoking status were included in analyses as covariates to adjust for potential confounding variables. The assumption of sphericity was tested using Mauchley’s method and any violations corrected for using the Greenhouse-Geisser method. Partial \(\eta^2\) is reported as a measure of effect size. Post-hoc pairwise analysis with Bonferroni correction was
conducted to assess inflammatory variable differences over time in each CMV group. Linear regression analysis of the log-normalised data was used to test for significant associations between lifestyle factors of the follow-up and inflammatory mediator’s percentage change over time. Individual lifestyle components were entered individually as independent variables (model 1); following this, any significant related lifestyle factors were entered together (model 2) to assess their mutually adjusted associations with cytokine levels. Statistical significance was accepted at p<0.05.

Results

Sera were available from 249 subjects at both baseline and 10 year follow up and the mean age at study entry was 67.5 ± 2.4 years. CMV antibody status was determined on serum at both time points and revealed that 149 (60%) of donors were CMV seropositive at entry and follow up (CMV+/+), 80 (32%) remained CMV seronegative throughout the study (CMV-/−) and 20 subjects (8%) converted from being CMV negative to CMV positive (CMV-/+/) during the 10 year study period.

Analysis of serum CRP and pro- and anti-inflammatory cytokines revealed a profile of increased systemic inflammation during ageing in line with previous observations\(^1\) (Fig. 1). IL-6 (Fig. 1A) concentrations increased 2.3 fold over time (F(1,154) = 117.3, p < 0.001; \(\eta^2 = 0.432\)) whilst TNFα (Fig. 1B) showed a dramatic 4.3 fold increase over the 10 year period (F(1,104) = 126.5, p < 0.001; \(\eta^2 = 0.549\)). CRP (Fig. 1C) levels were 1.2 fold higher between follow up and baseline (F(1,240) = 5.0, p = 0.026; \(\eta^2 = 0.021\)). In addition, levels of the anti-inflammatory cytokine IL-10 (Fig. 1E) decreased by 65% between the baseline and follow up samplings (F(1,53) = 42.8, p < 0.001; \(\eta^2 = 0.447\)). However we saw no change in IFN\(\gamma\) (F(1,199) = 1.7, p = 0.188; \(\eta^2 = 0.009\)) (Fig. 1D).
When the analysis was repeated for the three different CMV groupings we found that similar increases in both IL-6 and TNFα occurred between baseline and follow up in all three groups (Fig. 2A and B). In particular, IL-6 showed a 2.4, 2.2 and 2.4 fold increase in the CMV-/+ (p = 0.03), CMV-/ (p < 0.001) and CMV+/+ (p < 0.001) groups respectively. A corresponding 6.0, 4.2 and 4.1 fold increase in TNFα was seen in each of these subsets over time (all p < 0.001). Interestingly, the CRP level did not change over time in the CMV-/ group (p = 0.736) but did show a significant increase in the CMV+/+ (p = 0.004) group (Fig. 2C) suggesting that CMV infection may contribute to increased CRP levels with ageing. However there was also no change in the CRP levels for the donors that seroconverted for CMV infection during the study period (p = 0.541), indicating that factors other than CMV infection may also be influencing increases in CRP in the CMV+/+ group.

In our cohort IL-10 showed a 0.8 fold and 0.5 fold reduction in the CMV-/ (p = 0.001) and CMV+/+ (p = 0.01) groups respectively and a trend towards a reduction in the CMV-/+ (p = 0.07) group (Fig. 2E). Interestingly, these changes were seen only in male subjects (data not shown). Gender differences for IL-10 production have been suggested previously and may reflect an increased incidence of autoimmunity in females. The serum level of IFNγ did not increase over the 10 year period in the group as a whole (p = 0.188) and no change was seen in any of the three CMV groupings (Fig. 2D), CMV-/+ (p = 0.928), CMV-/ (p = 0.401) and CMV+/+ (p = 0.474).

Taken together these data suggest that mechanisms other than the immune response to CMV infection are driving the increase in systemic inflammation that is observed with normal physiological ageing.

Table 1 shows the relationships between lifestyle factors, BMI and waist to hip ratio and serum IL-6, IL-10, TNFα and CRP levels at baseline, and reveals that the increase in CRP levels were associated with lifestyle factors consisting of sedentary behaviours (hours
watching TV, \( p < 0.001 \); lower distance walked \( p < 0.001 \); higher BMI, \( p < 0.001 \); higher waist to hip ratio, \( p < 0.001 \)). Raised IL6 and TNFα were also associated with a sedentary lifestyle (hours watching TV, \( p < 0.002 \); increased car travel, \( p = 0.014 \) for IL6 and hours watching TV, \( p = 0.034 \) for TNFα), whilst reduced IL10 was influenced by waist to hip ratio (\( p < 0.001 \)) and alcohol consumption (\( p = 0.042 \)). As the \( R^2 \) values in table 1 show around 7% of elevated IL-6 and 8% of elevated CRP can be attributed to a more sedentary lifestyle and a higher BMI and waist to hip ratio contributed 6.2% to the increase in CRP. Similar associations between lifestyle factors and body mass were found if comparisons were made with the levels of inflammatory markers at the follow up sampling (data not shown).

Discussion

The longitudinal OCTO-Immune and NONA-Immune studies followed small cohorts of the very elderly (>85 years) for up to 6 years and correlated various immune markers with mortality. These studies were the first to define a set of parameters termed the “immune risk profile” which was associated with increased mortality during follow up. Interestingly, these included CMV seropositivity and a profile of raised pro-inflammatory markers\(^{18,21}\). However, these studies did not compare the inflammatory status of individuals who remained CMV-seronegative or who seroconverted in the 6 year study period. Similarly, Roberts \textit{et al}\(^{22}\) conducted a prospective analysis of CMV titres and found that increased titre was associated with increased TNFα, IL-6 and raised mortality but did not consider longitudinal differences between CMV seronegative and seropositive individuals. Our study is thus the first to address the impact of CMV serostatus on the increase in inflammatory markers during ageing and reveals that chronic systemic inflammation occurs independently of CMV infection and is driven by other factors including a sedentary lifestyle and a raised BMI.
Several genetic and environmental factors may also contribute to inflammageing. IL-6 polymorphisms (174G>C) are associated with elevated IL-6 levels and increase in prevalence in the over 65 year olds, possibly reflecting an evolutionary benefit of a robust pro-inflammatory response in early life which is not conducive to a healthy old age. Indeed raised inflammatory cytokines such as IL-6 are not seen in centenarians or members of families with extended longevity, supporting the notion that an anti-inflammatory genotype is beneficial in reaching extreme old age. Amongst lifestyle factors that could influence inflammageing are the age-related increase in adiposity, reduced physical activity, the loss of sex hormones during menopause and andropause which are all known to increase systemic inflammation. Indeed, additional analyses of our data suggested that lifestyle factors such as level of inactivity (hours watching TV, distance walked daily, level of car travel) and a higher BMI were contributing to raised levels of circulating pro-inflammatory cytokines. Current health recommendations regarding the benefit of an active lifestyle and maintaining a healthy bodyweight thus hold true for old age and extend to reducing the age-related increase in systemic inflammation.

Our study has some limitations. Firstly, we cannot completely exclude the effects of co-existing sub-clinical infections at the time of cytokine analysis. However, participants were presumed fit and able to attend clinic appointments for data collection and results were screened prior to analysis for patterns suggestive of acute infection or haematological malignancy and four results removed from the data set. Secondly, we did not consider the impact of other latent viral infections such as Epstein Barr virus and Varicella Zoster Virus, which might possibly contribute to inflammation in the CMV negative grouping. Also, due to the relatively low seroconversion rate for CMV during the study period the size of the CMV-/-+ group is quite small and the impact of seroconversion on serum markers should be addressed in a larger study. Finally, study participants were lost to follow-up between the
1993/5 baseline and 2003/5 follow-up clinics due to a variety of reasons (including 121 to mortality, loss to follow-up, refusal to participate) and we have previously shown that a healthy participant effect is, unsurprisingly, evident in HAS. In the current study, the 153 men who went on to have CMV status classified at the 2003/5 HAS follow-up were significantly (p<0.05) younger, less likely to be current smokers, were of higher social class, and had lower IL-6 and CRP levels at the baseline clinic than the 258 men who only participated in the baseline study. Selection effects were less evident for women; the 101 women who were sampled at both time points were significantly (p<0.05) less likely to be current smokers than the 205 women who only participated in the baseline study, but were otherwise similar. These selection effects have the potential to bias our results. However, our analyses were internal to the HAS sample; bias would only be introduced if the associations between CMV status and inflammageing were systematically different among those who participated in our study, and those who did not; this seems unlikely.

In conclusion, CMV infection does not appear to determine the age-related increase in serum markers of inflammation. This suggests that interventions to control CMV infection incidence in the population would have little or no impact on the inflammatory profile of ageing individuals. However, it is well established that the cellular immune response against CMV is extremely immunodominant and increases further in older adults, occupying valuable “immune space” and potentially impairing the response to other pathogens and to vaccination in old age. Further epidemiological analyses of the impact of CMV infection on health status in the elderly thus remain warranted.

Panel: Research in context

Interpretation: The age-related increase in low grade systemic inflammation (inflammageing) is well documented and infection with the persistent virus CMV had been suggested as one of the factors promoting this process. However, prior to our study
longitudinal data supporting a causative role for CMV in inflammageing were lacking. Our data refute this association and instead suggest that lifestyle factors and a high BMI are more likely to drive age-related increases in systemic inflammation, thus contributing to major conditions known to be driven by the inflammatory process.

**Conflicts of interest**

None declared.

**Author contributions**

D Bartlett and CF performed the cytokine and CMV analyses, D Bartlett, AC, D Baylis and HS performed the statistical analyses, AAS and CC carried out the HAS and collected the primary data, D Bartlett, PM and JML wrote the manuscript.

**Role of funding source**

The funders took no part in the study itself, data analysis, manuscript preparation or the decision to submit the manuscript.
Legends to Figures and Table

Figure 1. Cytokine and CRP concentrations at baseline and 10 year follow up in subjects in the Hertfordshire Ageing Study. Significant increases over time were observed for serum concentrations of IL-6 (A), TNFα (B) and CRP (C) whilst a decrease was observed for IL-10 (E). No differences over time were observed for IFNγ (D). Results are mean ± SEM analysed by mixed model repeated measures ANOVA. *p<0.05, **p<0.01 for baseline compared with 10 year follow up.

Figure 2. Impact of CMV serostatus and ageing on serum cytokine and CRP concentrations. Subjects were grouped by CMV serostatus as either negative at baseline and positive at 10-year follow-up (CMV-/+), negative at both time points (CMV-/-), or positive at both time points (CMV+/+). Serum IL-6 (A) and TNFα (B) concentrations increased in all 3 CMV groups. CRP (C) was only increased in the CMV+/+ group, while the anti-inflammatory cytokine IL-10 (E) was decreased in the CMV/- and CMV+/+ groups and a trend (p=.07) towards a reduction was observed in the CMV-/+. IFNγ (D) was unchanged for all groups. Results are mean ± SEM analysed by mixed model repeated measures ANOVA with Bonferroni corrected pair wise comparisons. * p<0.05, **p<0.01, ***p<0.001 for baseline compared with 10 year follow up.

Table 1. Relationship between lifestyle factors at baseline and the level of serum cytokines and CRPs at baseline. Linear regression analysis of the log-normalised data was used to test for significant associations between lifestyle factors at baseline sampling and the level of inflammatory markers. Individual lifestyle components were entered individually as independent variables (model 1); following this, any significant related lifestyle factors were entered together (model 2) to assess their mutually adjusted associations with cytokine levels.
Figure 1.
Figure 2.
Table 1

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