Does Cytomegalovirus accelerate human ageing and the epigenetic clock?

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Abstract

Background: Human Cytomegalovirus (HCMV) is ubiquitous and associated with chronic T cell activation, immunosenescence, and age-related co-morbidities including cardiovascular disease, Alzheimer's disease, and bacterial and viral pneumonia infections. Additionally, HCMV is a component of the immune risk phenotype (IRP), which has been shown to be predictive of mortality in elderly. HCMV causes oligoclonal expansion of T cells that have a pro-inflammatory phenotype, which is thought to drive systemic inflammation. The seroprevalence of HCMV increases with age, thus very few studies have been carried out in older individuals. This makes it difficult to determine if immune dysregulation is due to HCMV infection, the natural ageing process, or a combination of the two. Therefore, we studied HCMV infection in healthy, older adults to elucidate immune phenotypic and functional changes, and how these changes are associated with the ageing process.

Methods: Healthy adults over 60 years old were screened and enrolled based on a strict exclusion criteria: active or known chronic infection, history of malignancy, autoimmune disease, diabetes, cardiovascular disease, or use of immune modulators at any time. A one-time blood sample was collected and used for complete blood count, flow cytometric analysis, and DNA methylation analysis. Flow cytometry was utilized to phenotype T cell subsets and characterize functional responses to HCMV antigens. DNA methylation analysis was used to measure biological ageing of each sample through specific epigenetic patterns, termed EpiAge.

Results: A total of 520 individuals were screened for health status, and 87 subjects met the enrollment criteria. 65 subjects were HCMV-seropositive (HCMV+) and 22 HCMV-seronegative (HCMV-). The HCMV-positive population in our cohort had higher lymphocyte counts ($p=0.02$), decreased CD4/CD8 T cell ratio ($p=0.049$)**,** higher absolute numbers of CD8 T cells ($p=0.0064$) and significant increases in CD8+ CD45RA+ CD57+ T cells ($p<0.0001$). The CD4 and CD8 CD45RA+ CD57+ T cell subsets in those HCMV+ had decreased expression of the exhaustion marker TOX, as compared to those HCMV- ($p<0.0001$, $p=0.013$). Additionally, the CD4 and CD8 CD45RA+ CD57+ subsets were both multi-cytokine producers when stimulated with HCMV antigens, with the CD4+ CD45RA+ CD57+ having the highest proportion of TNF-alpha+ IFN-gamma+ producing cells ($p=0.0096$, $p=0.011$). Strikingly, HCMV+ individuals had a higher

average EpiAge (65.34 years) than those HCMV- (59.53 years) (p=0.0042) and a 5.1-year age acceleration (p=0.0042). Interestingly, cytokine production and expansion of CD45RA+ CD57+ subsets were not associated with increasing chronological age or EpiAge. However, decreases in percent (p=0.010) and absolute numbers of CD4 T cells (p=0.0099) were associated with increasing EpiAge when analysing the study population as a whole.

Conclusions: This study shows that HCMV infection in healthy individuals, 60 years and older, is associated with accelerated EpiAge. Additionally, we found an expansion of CD45RA+ CD57+ T cells that lacked exhaustion markers and are capable of producing pro-inflammatory cytokines when stimulated with HCMV antigens. It is possible that this cell subset is contributing to systemic inflammation, thus increasing the risk of age-related diseases associated with HCMV infection. Our novel findings better define age as a biologic variable, and distinguish altered immunity in the elderly based on HCMV status. Additional studies exploring the mechanisms by which HCMV causes systemic inflammation and promotes age-related diseases, particularly looking at CD45RA+ CD57+ T cells, should be initiated.

Résumé

Introduction: Le cytomégalovirus humain (HCMV) est omniprésent et associé avec l'activation chronique des lymphocytes T, l'immunocenescence, ainsi qu'avec les comorbidités liées à l'âge tels la maladie cardiovasculaire, la maladie d'Alzheimer's et les pneumonies bactériennes ou virales. De plus, le HCMV est l'une des composantes du phénotype de risque immunitaire (PRI), un facteur prédictif de mortalité chez les personnes âgées. Le HCMV provoque l'expansion des lymphocytes T qui ont un phénotype pro-inflammatoire, pouvant contribuer à l'inflammation systémique. La séroprévalence du HMCV augmente avec l'âge, et à date, très peu d'études ont évalué l'impact du HMCV sur le système immunitaire des personnes âgées en santé. Cela rend difficile de déterminer si la dérégulation du système immunitaire observé chez les patients avec le PRI est en lien avec l'infection au HMCV, le vieillissement naturel ou une combinaison des deux. Pour cette raison, nous avons étudié l'infection au HCMV chez des adultes âgés en bonne santé pour décrire les changements immunitaires phénotypiques et fonctionnels et déterminer comment ces changements s'apparentent au vieillissement naturel.

Méthodes: Des adultes plus de 60 ans en bonne santé vivant dans la communauté ont été dépistés et recrutés selon des critères d'inclusion stricts. Un échantillon de sang a été prélevé pour obtenir une formule sanguine complète, une analyse par cytométrie de flux et une analyse de la méthylation de l'ADN. Nous avons fait un phénotypage de la sous-population de lymphocytes T et avons caractérisé leur réponse fonctionnelle au HMCV par cytométrie de flux. Nous avons ensuite procédé à l''analyse de la méthylation de l'ADN pour mesurer le vieillissement biologique de chaque échantillon en étudiant les motifs épigénétiques trouvés sur l'ADN.

Résultats: Les patients ayant une infection chronique au HCMV avaient un nombre de lymphocytes plus élevé ($p=0.02$), un ratio de lymphocytes CD4/CD8diminué ($p=0.049$), un nombre absolu de lymphocyte CD8 plus élevé ($p=0.0064$) et une augmentation significative de lymphocytes T CD8+ CD45RA+ CD57+ ($p<0.0001$) par rapport aux patients HCMV -. Les lymphocytes CD4 et CD8 CD45RA+ CD57+ chez les patients HCMV+ avaient une diminution de l'expression du marqueur d'épuisement TOX par rapport à ceux qui étaient HCMV- ($p<0.0001$, $p=0.013$). De plus, chez les patients HCMV +, les sous-populations CD4 et

CD8+CD45RA+ CD57+ produisent une multitude de cytokines pro-inflammatoires lorsque stimulés avec des antigènes de HCMV, avec le sous-population CD4+ CD45RA+ CD57+ ayant la plus forte proportion de cellules TNF-alpha+ IFN-gamma+ (p=0.0096, p=0.011). Les patients HCMV+ ont un EpiAge moyen plus élevé (65.34 ans) que les patients HCMV- (59.53 ans) (p=0.0042), et une accélération d'âge de 5.1 ans (p=0.0042). Curieusement, la production de cytokine et l'expansion des CD45RA+ CD57+ n'étaient pas associés à l'augmentation de l'âge chronologique ni de l'EpiAge. Cependant, la diminution en pourcentage (p=0.010) et nombre absolu des lymphocyte T CD4 (p=0.0099) étaient associés avec l'augmentation de l'EpiAge lors de l'analyse de la population totale de l'étude.

Conclusions: Cette étude démontre que l'infection avec le HCMV chez les individus âgés en bonne santé, est associée avec une accélération de l'âge chronologique et à une augmentation de l'EpiAge. De plus, nous avons trouvé une expansion de T lymphocytes CD45RA+ CD57+ sans marqueurs d'épuisement, capables de produire des cytokines pro-inflammatoires lorsque stimulés avec des antigènes du HCMV. Il est possible que cette sous-population de cellules contribue à l'inflammation systémique, augmentant ainsi le risque de maladies liées à l'âge. Nos nouvelles découvertes définissent mieux l'âge en tant que variable biologique et distinguent l'immunité altérée chez les personnes âgées en fonction du statut du HCMV. Ces résultats justifient des études futures explorant les mécanismes par lesquels le HCMV provoque une inflammation systémique et des maladies liées à l'âge, en particulier en ce qui concerne les lymphocytes T CD45RA+ CD57+.

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Preface and Author Contributions:

The work presented in this thesis is a combination of two studies currently in preparation for publication:

Healthy Cytomegalovirus seropositive individuals over the age of 60 have age acceleration, immune dysregulation and a higher epigenetic age than those who are seronegative - implications for infectious disease susceptibility and mortality risk during pandemics.

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HCMV-infection is associated with an increased percentage of late-differentiated T cells that lack exhaustion markers and are capable of pro-inflammatory, multi-cytokine production in older, healthy adults.

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Chris Tsoukas conceptualized, supervised the studies and recruited the patients. Chris Tsoukas and Moshe Szyf provided study concepts, resources, methodology, data, and analyses. Chad Poloni conducted investigations, interpreted data and lead the drafting the manuscripts, as well as wrote the introduction and literature review and summary and conclusions chapters. Chris Tsoukas interpreted data and contributed to drafting the manuscripts, as well as reviewed

and edited the final thesis. Moshe Szyf and David Cheishvili provided data and investigations. Chad Poloni performed statistical analyses. Dr. Geneviève Genest provided help in drafting and editing the French abstract. All authors reviewed the manuscripts.

Chapter 1: Introduction and Literature Review

Cytomegalovirus is a common, human, double-stranded DNA virus that is part of the *Herpesviridae* family (HCMV) [1]. The seroprevalence of HCMV is very high and increases with age. In the United States, the seroprevalence of those over the age of 6 is about 59%, with HCMV infection rates peaking in the elderly at 90% in those over 80 years of age [2]. Additionally, HCMV infections rates differ by ethnicity and socioeconomic status. When controlling for household income, there are increased infection rates in non-Hispanic black persons and Mexican Americans [2]. HCMV seroprevalence is increased in those within the bottom income quartile as compared to the top income quartile, starting during early life (6 years old) and persisting into late adulthood, where the differences are less striking [3].

Chronic HCMV infection is asymptomatic in most cases. The virus primarily infects monocytes, macrophages, salivary glands, and colonic epithelial tissue, where it undergoes sporadic reactivation [1]. In most individuals, a sufficient T cell response is generated that eliminates the lytic cycle, however the latent virus is never cleared [4]. In a clinical setting, HCMV is most notable for its ability to cause complications during pregnancy, in the immunocompromised, and during transplantation of organs. It is a common congenital infection and the leading cause of non-genetic hearing loss in children [5]. Additionally, infection at birth has been linked to cognitive deficits and vision loss [5]. In the immunocompromised, specifically those co-infected with HIV, HCMV infection can lead to retinitis, neurological disorders, and hepatitis [6]. HCMV co-infection has also been associated with increased all-cause mortality in those living with HIV [7]. In the case of solid organ transplantation, a mismatch between donor (HCMV+) and recipient (HCMV-), paired with immunosuppressive medication, can lead to active HCMV infection. This can lead to pneumonia, hepatitis, and death [8]. Although HCMV infection

remains subclinical in healthy individuals, chronic HCMV infection leads to dysregulation of the immune system. In turn, this dysregulated immune phenotype, which thought to be pro-inflammatory, contributes to the ageing process known as "inflam-ageing" [9].

HCMV has evolved closely with humans, being shown to cross between hominid lineages millions of years ago [10]. The human immune system has been uniquely adapted to deal with this long-term infection. HCMV is transmitted via bodily fluids, and has been shown to take up latency in a variety of tissue types, notably the salivary glands and colonic tissue [11]. T cell responses are critical in controlling acute HCMV infection and maintaining lifelong suppression of the virus. HCMV antigens are presented to corresponding T cells, generating an anti-viral T cell response and the production of T effector memory (Tem) cells [12]. In other viral infections, such as influenza, the antigen-specific Tem cells contract after the virus is cleared [13]. However, in the case of HCMV, the Tem subset expands, causing an oligoclonal expansion of HCMV-specific CD8 T cells [12].

In healthy HCMV+ adults, HCMV-specific T cells account for an average of 10% of all CD8 T cells circulating in the peripheral blood [14]. Expansion of HCMV-specific CD4 T cells also occurs, but to a lesser extent [15]. Oligoclonal expansion of T cells is primarily driven by two immunodominant HCMV antigens: pp65 and IE-1 [16]. pp65 acts as a tegument protein and is thought to have immune evasion functions, while the IE-1 protein is a nuclear phosphoprotein that has been shown to activate the lytic cycle of the virus [17, 18]. IE-1 specific T cells increase overtime, and have been shown to re-express CD45RA, a cell surface marker typically seen on naïve T cells [16, 19]. It is thought that this modified phenotype is due to sporadic reactivation of HCMV in infected cells, as the IE-1 protein is one of the first antigens to be recognized during

this process [16]. There are a variety of other HCMV proteins capable of generating robust T cell responses, with 91.9% immunocompetent donors generating CD4 T cell responses to pp65, gB, pp71, IE-1, IE-2 and US3 proteins [20]. Additionally, CD8 T cells have been shown to respond to 11 HCMV reading frames (ORFs), with a highly differentiated phenotype consisting of CD45RA+, CD57+ and CD28- [21]. In both CD4 and CD8 T cells, the cytokine response is polyfunctional and mostly Th1 [22, 23]. CD4 and CD8 T cells have been shown to produce IL-2, IFN-gamma, TNF-alpha, IL-10, MIP-1beta, and upregulate CD107a [20, 24]. Although the immune system generates a robust response, the virus is never cleared.

Overtime, constant antigenic stimulation via murine CMV (MCMV) and HCMV proteins lead to an expansion of CD4 and CD8 T cells, known as memory inflation. This was first termed in a murine study, showing a steady accumulation of MCMV-specific T cells overtime, reaching up to 20% of all peripheral CD8 T cells [25]. However, this phenomenon was reported earlier in humans, with 33% more clonal expansions of CD8 T cells being found in HCMV+ individuals as compared to HCMV- individuals [26]. Another study published around the same time reported up to 65% of CD8 T cells being specific for HCMV and capable of IFN-gamma production, while viral DNA was still detected [27]. Surprisingly, CMV-specific T cells lack the inhibitory molecule PD-1, which is usually upregulated during chronic infection [28]. These cells are capable of producing pro-inflammatory cytokines, and are maintained by short-lived functional T cells [29]. Inflationary MCMV memory cells can be characterized as CD27- CD28- CD62L- CD127- KLRG-1+ PD-1- and IL-2 +/- [30]. It is also important to note the lack of CD28 surface expression on the inflationary T cell subset from both MCMV and HCMV, which is a crucial co-stimulating molecule in T cell activation [31]. Other co-stimulatory receptors, OX40 and 41-BB,

have been shown to activate MCMV-specific T cells in the absence of CD28, and both promote accumulation of MCMV-specific CD8 T cells [32, 33].

Memory inflation is maintained independent of CMV replication, which was demonstrated in a murine model [34]. It has been shown that oligoclonal expansion of T cells is driven by random CMV antigen encounters, solely dependent on infected non-hematopoietic cells [35, 36]. It is thought that partial gene transcription during viral latency is sufficient to drive memory inflation. Furthermore, reinfection with additional strains of HCMV can lead to an increase in memory inflation, which is maintained by IL-15 [37, 38].

In humans, it is unknown at what point after acute infection inflationary T cells can be detected. Immunodominant CMV-clonotypes have been shown to be absent in young HCMV+ individuals, indicating the absence of an inflationary T cell subset [39]. However, other cross-sectional studies have shown no change in CMV-specific T cells between young and older individuals [40]. It is important to note that these studies do not take into account how long each individual was infected, as well as the varying disease states of each donor. These two factors can be easily controlled using a murine model. A small-scale, longitudinal study of 25 healthy, seropositive adults indicated an increase in memory T cells, as well as a decrease in CD4/CD8 T cell ratio after a 5-year follow-up [41]. This study implies HCMV acts in a similar manner as MCMV, where T cell inflation is time dependent.

Memory inflation eventually leads to a dysregulated immune system, which ultimately has deleterious effects. This is an age-related process, and is known as immunosenescence [42]. The most prominent studies looking at ageing and the immune system are the longitudinal Swedish OCTA and NONA studies, which were first published in 1995 and 2002. The OCTA study was

carried out on octogenarians, and was originally designed to examine psychosocial parameters [43]. An immune sub-study identified immune parameters associated with an increased 2-year mortality, making the immune portion a major part of the original study. Specifically, poor T-cell proliferation, increased CD8 T cell percentage, and low CD4 T cell percentage were predictive of 2-year mortality [43]. These parameters were only predictive in combination, and did not predict mortality when analyzed separately [43]. A two-year follow up was performed on the same immune parameters, confirming the original findings [44]. The impact of HCMV infection was not noted in the early published results. Interestingly, the decrease in CD4 T cells and expansion of CD8 T cells were both discussed as potential contributors to the T cell ratio. We now know that the decreased ratio is due entirely to the expansion of CD8 T cells driven by HCMV infection.

Additional data from the OCTA cohort were published in 2000 from the final follow up (T-8 years). T cell subsets were analyzed in subsequent studies to characterize the immune system of those with the risk factors described in the original OCTA study. New parameters were discovered: increased CD8+ CD28- and CD57+ T cell subsets [45]. Additionally, these subsets were associated with HCMV infection, and HCMV was suggested to cause oligoclonal expansion of CD8 T cells [45]. The NONA study was a continuation of the OCTA study, looking at nonagenarians. The data associated a CD4/CD8 ratio <1 with increased CD8 T cells with either CD28-, CD57+, or CD45RA+ [46]. These markers were not looked at in combination, possibly due to technical limitations at the time. Additionally, these data were significantly associated with HCMV seropositivity [46]. From these data, the immune risk phenotype (IRP) was generated, defined as a CD4/CD8 T cell ratio <1 and a peripheral blood expansion of CD8+

CD28- T cells in those HCMV seropositive [47]. In addition to the IRP being a predictor of all-cause mortality, it has been proposed as a biomarker of overall health in the elderly [46].

The same research group that conducted the OCTA study found that the IRP is associated with decreased interleukin 2 (IL-2) [48]. Furthermore, the IRP is associated with nosocomial bacterial and viral pneumonia, specifically within the elderly [49]. The IRP is further amplified in those living with HIV, occurring at an earlier age and causing more immune dysregulation, as measured by CD8 T cell cytokine production [47]. These studies highlight the potential use for the IRP in a clinical setting, as each component of the IRP can be easily measured in a standard hospital laboratory.

It is likely that components one and two, CD4/CD8 ratio <1 and CD8 expansion, of the IRP are a consequence of HCMV infection itself, as HCMV infection has been shown to drive oligoclonal expansion of CD8 T cells. HCMV alone has been associated with several diseases. A recent study done on 849 individuals revealed an association between CMV infection and risk of Alzheimer disease [50]. Furthermore, A meta-analysis of cardiovascular disease that analyzed 10 articles, including over 34,000 individuals, revealed a significant association between HCMV and risk of cardiovascular disease [51]. Additionally, HCMV DNA has been found at a higher rate in arterial tissue from atherosclerosis patients [52]. The mechanisms directly linking HCMV infection to these diseases is not yet known. However, it seems that the expansion of HCMV-specific T cells is a key component.

We hypothesized that infection of HCMV potentiates immune ageing marked by significant age-related changes to the T cell phenotype and T cell function.

We undertook a cross-sectional study of HCMV+ and HCMV- healthy, older adults to better understand the impact of HCMV on the immune system, both in terms of its function and phenotype. 520 individuals between ages 60-90 were screened at two McGill University Health Centre affiliated sites and excluded if they had active and chronic infection based on clinical evaluation, cardiovascular disease, history of malignancy, autoimmune disorder, diabetes, and medication that would modulate the immune system. A total of 87 individuals were successfully enrolled and blood was taken for immune phenotypic and functional assays, and HCMV serology. It is important to note that we carefully selected participants based on modified SENIEUR criteria, as described above, in order to eliminate as many confounding variables as possible [53]. The inclusion criteria were defined as: healthy individuals, determined by a physician at the time of enrollment, over the age of 60, who agreed to a one-time blood draw. The exclusion criteria were defined as: active or chronic infection determined by a physician at the time of enrollment, history of cardiovascular disease, history of malignancy, autoimmune disorder, diabetes, and/or currently taking immune modulating medication defined as medication that is known to alter immune function.

The main objectives of the study were: 1) Evaluate memory inflation by assessing T cell phenotypic changes associated with ageing in HCMV- and HCMV+ individuals, 2) determine the functional changes of T cells associated with ageing in HCMV- and HCMV+ individuals, 3) Determine if these changes correlate with chronological age or epigenetic age.

In order to achieve these aims, two separate sub-studies were conducted. The first one was to characterize memory inflation as a function of chronological and epigenetic ageing. This was done by flow cytometry, analyzing the CD4 and CD8 T cell subsets in HCMV- and HCMV+

individuals. Ageing was measured by CpG methylation patterns in the DNA, known as EpiAge. The methylation clock used in this study is a novel biological age predictor that uses methylation patterns around the *ELOVL2* gene, and has been validated via comparison to the Horvath clock using publicly available data. This has been previously done in HIV patients, revealing a 5.1-year increase in EpiAge in those living with HIV [54]. Associations between EpiAge, chronological age, and T cell phenotypes were investigated.

The second study built on the first by further characterizing the phenotypes of expanded T cell subsets via flow cytometry. T cell function was measured by cytokine production. EpiAge and chronological age were used to determine if there were any age-related changes to cytokine production. The second and third chapters of this thesis report the findings.

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Chapter 2: Healthy cytomegalovirus-seropositive individuals over the age of 60 have age acceleration, immune dysregulation and a higher epigenetic age than those who are seronegative - implications for infectious disease susceptibility and mortality risk during pandemics

Introduction

Age is a major risk factor for increased susceptibility to infectious diseases and decreased vaccine efficacy [1, 2]. Immune dysregulation and chronic subclinical inflammation contribute substantially to this risk [3]. Notably, dysregulation of the immune response was recently described in patients with SARS-CoV-2, which disproportionately affects the elderly [4]. Cytomegalovirus (CMV), an almost ubiquitous human herpesvirus is strongly associated with chronic subclinical inflammation, age-associated comorbidities, and immunosenescence [5-7]. Large studies in octa- and nonagenarians have linked a CMV-dysregulated immune profile to an increased two-year mortality [8]. The profile is defined by a low CD4:CD8 T-cell ratio, an expanded population of $CD8^{+}CD28^{-}$ T cells and CMV seropositivity, and is known as the immune risk phenotype (IRP) [9]. Herpesviruses, and in particular CMV, have an ability to initiate and maintain chronic immune activation and dysregulation, characterized by a progressive oligoclonal expansion of CD4+ and CD8+ effector memory T cells (Tem) [10]. In acute viral infections, once the infection clears, these T cell populations contract [10]. In contrast, the Tem cell pool following CMV infection increases, creating Tem inflation and a concomitant peripheral naïve T cell decline, characterizing a state of chronic immune dysregulation [11-12].

Chronic viral infections can accelerate biological ageing, as determined by epigenetic changes. This was first shown in those with HIV, where an average age-acceleration of 5.2 years was described [13]. Epigenetic age (EpiAge) was determined by measuring methylation profiles of sites in the human genome whose methylation state correlates with age [14]. The EpiAge can

also be used to calculate epigenetic age acceleration, which is a measurement of the difference between the observed EpiAge and a predicted EpiAge, from a linearized control model [13]. This tool may be useful as a clinical marker of disease progression. Additionally, hyper methylated *ELOVL2* CpG islands have been shown to be a marker of ageing and cell replication in peripheral blood [15]. DNA methylation of two CpG sites proximal to the gene exhibit high correlation with age [15]. In the elderly and those with HIV, CMV seroprevalence is exceedingly high. It remains unclear to what extent latent CMV infection, rather than HIV, accounts for the epigenetic age acceleration, and if it predisposes to co-morbidities frequently found in ageing.

CMV-specific T cells account for a significant portion of the Tem pool in the elderly, with estimates ranging from 10-45% of the total $CD8^+$ T and 10% of the total $CD4^+$ T cell populations [16-18]. These CMV-specific T cells have an altered immune phenotype, characterized by decreased cell surface expression of CD28 and increased expression of KLRG-1 and CD57. These markers identify cell populations that have undergone repeated antigen stimulation [19, 20]. Furthermore, CMV-specific $CD8^+$ T cells re-express CD45RA, a marker usually seen on naïve T cells [21]. Cells with this profile of surface markers are found in increasing numbers with age in the bone marrow and peripheral blood [22]. The immunopathology contributing to the dysregulated T cell phenotype is unknown.

CMV-associated T cell alterations may have important consequences for immunosenescence, rates of ageing, and age-associated co-morbidities. Despite the challenge of identifying CMV uninfected seniors without co-morbidities, we undertook a comparative study of healthy, older CMV+ and CMV- adults to identify phenotypic and epigenetic changes associated with ageing.

Methods

Adults over the age of 60, attending annual healthcare evaluations, were examined by a physician at two McGill University Health Centre (MUHC) affiliated family medicine clinical sites. MUHC Research Institute Review Board study approval was obtained. Patients were screened for enrollment. Subjects were enrolled if determined to be in good health, were 60 years or older, and willing to provide a single 40 ml blood sample. Individuals were excluded if they were symptomatic, had an active or known chronic infection, history of malignancy, autoimmune disease, diabetes, cardiovascular disease, or used immune modulators at any time. Informed consent was obtained from all study participants.

White count and differential were performed on fresh blood as a standard of care. Absolute CD4 and CD8 counts were determined from white count and differentials. Flow cytometric analyses of lymphocyte subsets and serological assays for CMV were performed using from serum and peripheral blood mononuclear cell (PBMC) samples. PBMC samples were divided in three aliquots, frozen in 10% DMSO, and stored in liquid nitrogen immediately after being drawn. Plasma was stored at -80 C for CMV serology that was determined using a qualitative anti-CMV IgG ELISA (ABCAM), with duplicates run for each sample.

Targeted DNA methylation assays: A Pearson correlation between states of methylation of cytosine/guanine sites across the genome in blood cells from publicly available Illumina450K arrays and age (GSE61496), revealed that two sites residing proximal to the *ELOVL2* gene (cg16867657 and cg21572722) exhibit a strong Pearson product-moment correlation coefficient ($r=0.934$, $p<0.0001$) and ($r=0.81004$, $p<0.0001$). We developed a targeted DNA methylation assay to this region. A weighted "EpiAge" value was calculated for the 13 CGs in this region using a linear regression model.

1 million PBMC cells were placed in DNA stabilization buffer (SDS 0.5%, EDTA 20mM, TritonX100 1%, Tris-HCl 20 mM pH8.0). Bisulfate conversion was performed using EZ-96 DNA Methylation MagPrep (D5041, Zymo Reasearch), which was followed by two rounds of polymerase chain reaction (PCR); the first round targeted the *ELOVL2* and the second PCR introduced barcode sequences for multiplex amplification (the primers are available upon request). The pooled library was purified and sequenced on Illumina MiSeq, fast Q files were aligned with reference genome using BisMark, and percentage of DNA methylation at each of the 13 CG positions was computed. EpiAge was calculated with a linear regression equation predicting age in the control group as a function of the weighted methylation levels of all CG sites.

Lymphocyte Phenotyping: Batches of cryopreserved PBMCs were thawed and washed twice in PBS. One million cells were stained with the following antibodies: BV650-CD3, BUV737-CD4, APC R700-CD8, BUV395-CD38, BV785-HLA-DR (Biolegend), and Indo-1-Live (Invitrogen). All surface stains were done at room temperature for 30 min in the presence of human Fc block (BD Biosciences). All samples were analysed on a BD LSR Fortessa X-20 (BD Biosciences). Fluorescence data from at least 50,000 lymphocytes were acquired. Analysis of data was performed using FlowJo V10.

Statistical analysis:

A multilinear regression model was generated to control for sex, age, weight, height, BMI, smoking status, hypertension, dyslipidemia, hypothyroidism, osteoporosis, benign prostatic hypertrophy, depression, and chronic gastritis. The least squares method, in tandem with normality tests, was used via Prism 8, assuming a Gaussian distribution of residuals. This model

was used to test for significant differences between CMV+ and CMV- groups. The following equation defines the model used to investigate each dependent variable investigated in this study:

Dependent (e.g. CD4/CD8 ratio): $Y = \beta0 + \beta1*CMV + \beta2*Sex + \beta3*Age + \beta4*Hypertension + \beta5*Dyslipidemia + \beta6*Hypothyroidism + \beta7*Osteoporosis + \beta8*Benign Prostatic Hypertrophy + \beta9*Depression + \beta10*Chronic Gastritis + \beta11*Smoking + \beta12*Weight + \beta13*Height + \beta14*BMI + random scatter$

Where, female = 0, male = 1
CMV negative = 0, CMV positive = 1
Disease states: no = 0, yes = 1
Smoking: no = 0, yes = 1
Beta-0: intercept parameter

P values were considered significant at <0.05. All significance tests were carried out using GraphPad Prism 8.

Results

CMV-seropositive individuals had higher lymphocyte counts.

87 subjects were enrolled. Ages range from 60-90 years old, with 65 subjects CMV-seropositive (CMV+) and 22 CMV-seronegative (CMV-) (Table 1). The two groups did not significantly differ in sex, age, weight, height, BMI, smoking status, hypertension, dyslipidemia, hypothyroidism, osteoporosis, benign prostatic hypertrophy, depression, or chronic gastritis as determined by drug regimen (Table 1). There were complete blood counts carried out on each individual, which revealed no significant differences in red blood cell, hemoglobin, leukocyte, or neutrophil counts between the CMV+ and CMV- groups. The CMV+ group, however, had a significantly higher lymphocyte count than the CMV- group ($p=0.029$) (Fig 2).

CMV+ individuals had a decreased CD4/CD8 T cell ratio.

CMV-infection was associated with a decrease in the CD4/CD8 T cell ratio (p=0.049) (Fig 1)(Supp. Table 1). Our established, normal CD4/CD8 ratios are 1.8-3.4 at the MUHC, and we defined ratios of <1 indicative of relevant dysregulation based on the OCTA and NONA studies. To assess the degree of T cell phenotypic dysregulation, the CMV groups were stratified on very low <1.0, extended normal 1.1-4.9 and very high > 5.0 ratios. The CMV-seronegative population had a lower proportion of individuals with a very low T cell ratio <1 (4.6%) compared to the seropositive population (16.9%).

Differences in T cell subsets between CMV+ and CMV- populations

The percentages of CD4 and CD8 T cells were calculated from live, CD3+ lymphocytes. There were no differences in CD4 percentage and absolute numbers between the two groups. The CMV+ had a significantly higher percentage (p=0.014) and absolute number (p=0.0064) of CD8 T cells, as compared to the seronegative population (Fig 2)(Supp. Table 2). In CMV-seropositives, a significant increase in the percentage of CD8+ CD28- T cells (p<0.0001) was noted (Fig 3)(Supp. Table 3). There was no difference in the percentage of CD4+ CD28- T cells between groups. There were no significant differences in the activation markers CD38 and HLA-DR between CMV+ and CMV-.

Increased EpiAge is associated with CMV-infection.

EpiAge was calculated for each donor. There was 1 CMV- and 11 CMV+ samples that failed to sequence for the EpiAge analysis. The CMV+ group had a significantly higher EpiAge (65.34 years) than the CMV- control group (59.53 years) (p=0.0042) (Fig. 4). We calculated age acceleration as previously described [13]. EpiAge (X) and chronological age (Y) in the CMV- group were plotted and the linear regression equation (Y=0.5527*X + 20.43). We then computed

the residual between the regression line and EpiAge in the CMV+ group to calculate age acceleration. The result revealed a 5.1-year age acceleration in the CMV+ group (p=0.0042) (Fig. 5).

Increasing EpiAge is associated with decreased percentage and absolute CD4 T cells

A multilinear regression was carried out to determine the relationships between EpiAge and absolute and percent CD4, absolute and percent CD8, IRP, percent CD4+ CD28- and CD8+ CD28-, and T cell ratio. EpiAge was associated with decreased absolute (p=0.0099) and percent CD4 T cells (p=0.010). Linear regressions were generated to visualize the relationship (Fig. 6). Interestingly, hypertension (p=0.028) and dyslipidaemia (0.039) significantly impacted IRP status, and sex, and BMI impacted percent CD8+ CD28- T cells. CMV was the only variable that had significant effects on T cell ratio and absolute and percent CD8.

Discussion

A decrease in the CD4/CD8 T cell ratio has been established in the context of chronic CMV infection [23]. Our study not only confirms this finding, but it also provides insight into the T cell subsets causing the alteration in the ratio. We found that CMV infection is associated with proportional increases in CD8 T cells. Although this has been reported during active CMV infection in kidney transplant recipients, it has not yet been reported in healthy, human individuals [24]. Of importance, there was no decrease in the absolute number of CD4 T cells, but a significant increase in the absolute number of CD8 T cells. We found that CMV infection was associated with an increased proportion of T cell ratios of <1. As previously shown by others, low T cell ratios are largely due to an oligoclonal expansion of CMV-specific CD8 T cells [25]. We have also shown an increase in the total lymphocyte count in the CMV+ group. A similar finding was noted in human renal transplant recipients and in baboons [26, 27]. In CMV+

individuals, an increase in lymphocytes is a function of age, however we show it is also a function of CMV infection, as our CMV+ group had a higher lymphocyte count than the CMV- group [28].

The effect of CMV infection on epigenetic ageing was first described by Kananen et al, which found accelerated ageing in CMV+ individuals between the ages of 20-30 and 90+ (nonagerians). This study only had 6 CMV- controls in the 90+ group, and did not include anyone between the ages of 60-90. Our study expands on these findings by analysing the missing age group, as well as making correlations between epigenetic age, a dysregulated T cell phenotype, and the IRP. As such, the findings have relevance regarding the use of EpiAge as a risk factor for many co-morbidities and infectious diseases. The SARS CoV-2 pandemic has highlighted the importance of mortality risk stratification based on age, where the elderly account for 20% of those infected, but 80% of deaths [31].

In healthy individuals over 60 numerical years of age, our model indicated an age acceleration of 5.1 years solely due to CMV infection. Almost all HIV patients have CMV co-infections (>93%), leading us to dispute that the 5.2-year average-age acceleration is attributed to HIV-1 alone, since the contribution of CMV was not considered in those studies [13]. A CMV-/HIV+ population is needed to truly elucidate the age-accelerating effects of HIV. Additionally, we showed that decreased percent and absolute number of CD4 T cells were associated to increases in EpiAge, identifying potential contributors to increased EpiAge. A cohort spanning additional chronological ages is needed to accurately investigate T cell dysregulation and EpiAge.

It is important to note that this study did not phenotype CMV-specific T cells, rather we looked at T cell subsets as a whole. Moving forward, it is important to determine if phenotypic changes

occur in CMV-specific T cells, and if changes in CMV-specific T cells are correlated with chronological and EpiAge.

In conclusion, we identified new biomarkers indicating increased epigenetic age, accelerated ageing and immunosenescence in healthy, asymptomatic, older adults with CMV. Since the COVID-19 pandemic has identified age as a major risk factor for infectious disease severity, our novel findings may have prognostic relevance. Further studies are needed to determine their clinical significance.

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Potential conflicts of interest: The authors have submitted the ICMJE form. CP and CT report no conflicts of interest, **MS and DC** have applied for patents on EpiAge and MS owns shares in HKG epitherapeutics.

Figures:

	Human Cytomegalovirus (CMV) Negative		Human Cytomegalovirus (CMV) Positive		P-value
Donors (n)		22		65	
	Male	13 (59.1%)	Male	32 (49.2%)	0.46
	Female	9 (40.9%)	Female	34 (51.5%)	
Covariables					
Age		72.7 +/- 8.2		70.3 +/- 8.9	0.23
Hypertension		6 (27.3%)		22 (33.8%)	0.64
Dyslipidemia		7 (30.4%)		15 (23.4%)	0.58
Hypothyroidism		1 (4.5%)		13 (20%)	0.11
Osteoporosis		6 (27.3%)		23 (35.4%)	0.60
Benign Prostatic hypertrophy		1 (4.5%)		7 (10.8%)	0.67
Depression		1 (4.5%)		8 (12.3%)	0.44
Chronic Gastritis		4 (18.2%)		11 16.9%)	>0.99
Smoking		0 (0%)		5 (6.8%)	0.32
Weight		169.2 +/- 35.6 lbs		160.7 +/- 30.9 lbs	0.31
Height		66.2 +/- 3.4 inches		64.3 +/- 2.8 inches	0.33
BMI		26.6 +/- 4.8		26.4 +/- 4.1	0.85

Table 1. Study population characteristics
Fisher's exact test was used to determine significant differences in sex, hypertension, dyslipidemia, hypothyroidism, osteoporosis, benign prostatic hypertrophy, depression, chronic gastric, and smoking between the study groups, and the student's t test was used to test for significant differences in age, weight, height, and BMI. *P* values below 0.05 were considered significant.

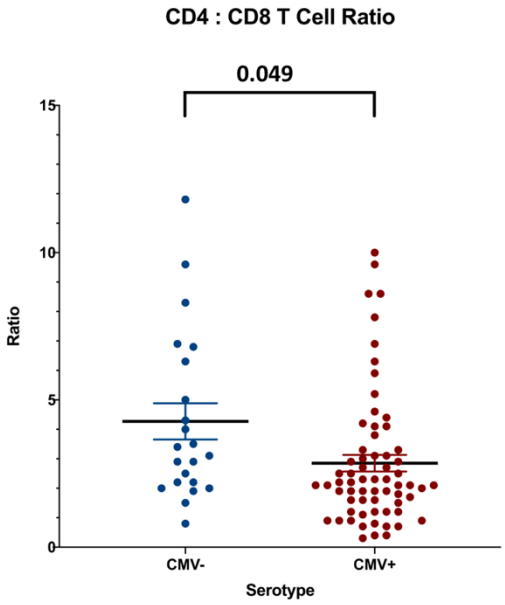


Figure 1. The CD4/CD8 T cell ratio is decreased in the CMV+ population
The CD4/CD8 T cell ratio was determined for each participant using flow cytometry, gating on the CD3+ population. The CMV+ (n=65) and CMV- (n=22) populations were compared using a multivariable linear regression. *P* values below 0.05 were considered significant.

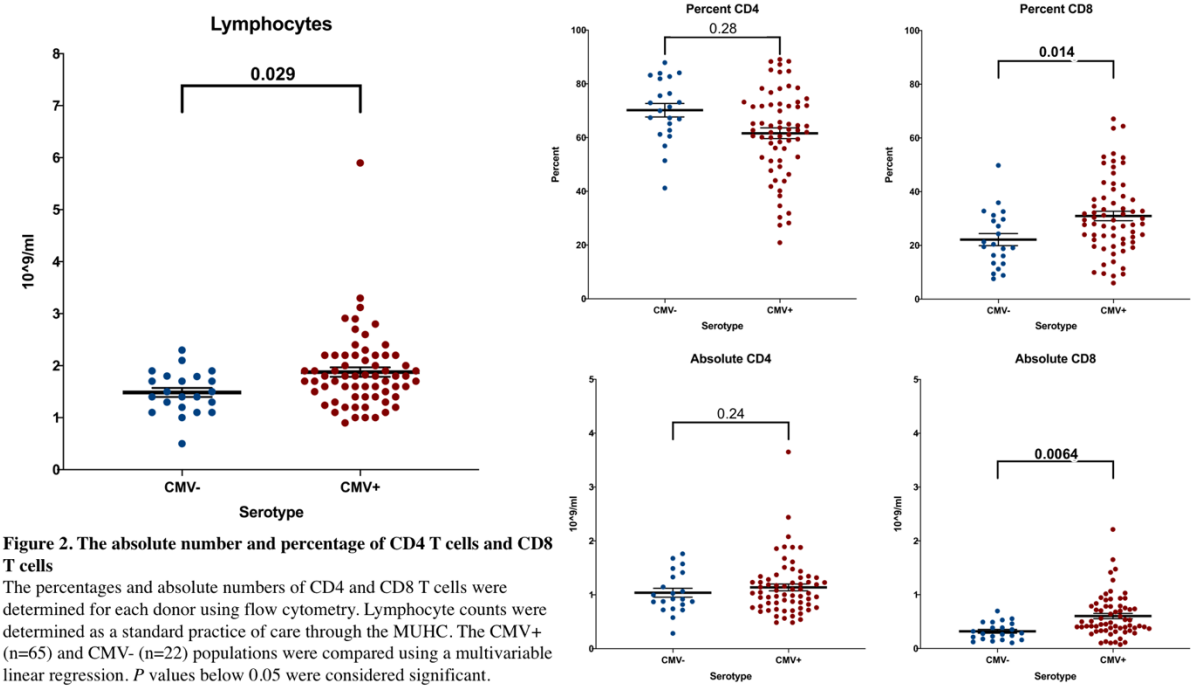


Figure 2. The absolute number and percentage of CD4 T cells and CD8 T cells
The percentages and absolute numbers of CD4 and CD8 T cells were determined for each donor using flow cytometry. Lymphocyte counts were determined as a standard practice of care through the MUHC. The CMV+ (n=65) and CMV- (n=22) populations were compared using a multivariable linear regression. *P* values below 0.05 were considered significant.

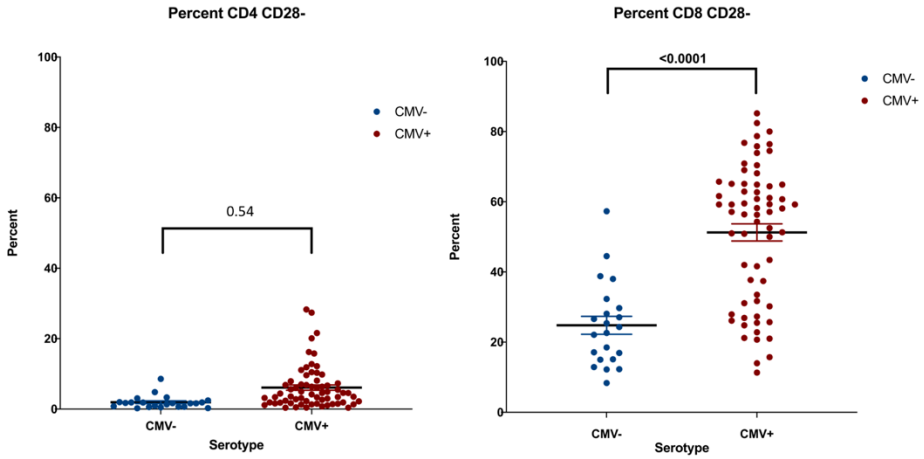


Figure 3. The CD8 T cell phenotype is altered in CMV-infected donors
Peripheral blood mononuclear cells were isolated from CMV+ and CMV- individuals. Flow cytometry was used to phenotype CD8-positive T cells from each individual donor, specifically analyzing CD4, CD8, and CD28. The proportions of CD28 for CMV+ (n=65) and CMV- (n=22) of the CD4 and CD8-positive T cell populations were calculated. Multivariable linear regression models were used to test for significance, with *P* values below 0.05 were considered significant.

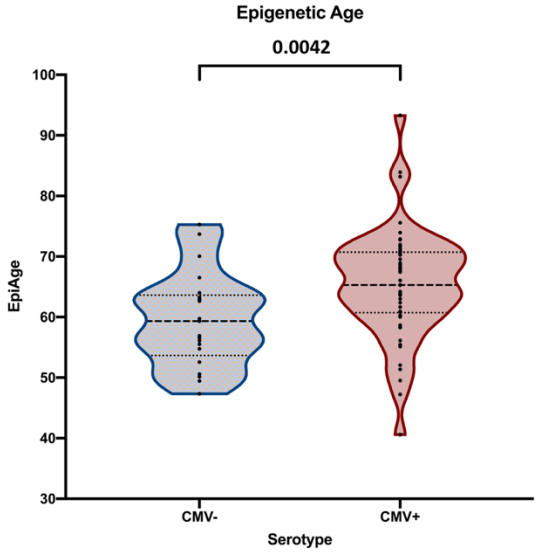
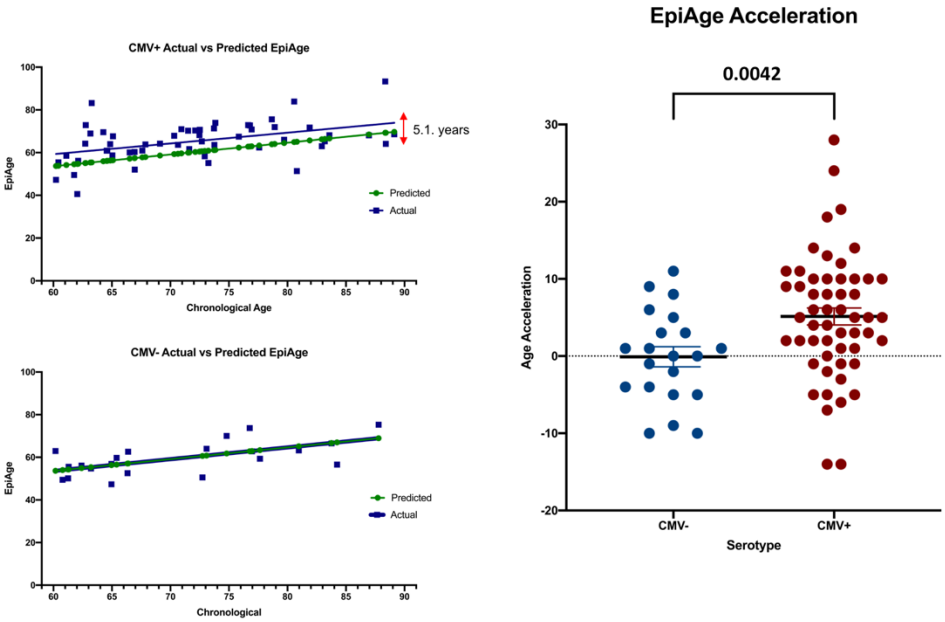


Figure 4. EpiAge is accelerated in those with CMV infection.
DNA was isolated from study participants and the methylation status near the Elovl2 gene was measured using bisulfite-sequencing. An EpiAge was calculated for each individual, and the CMV- (n=21) and CMV+ (n=54) groups were compared using a multilinear regression analysis. A violin plot was generated for each group, with the median and quartiles represented by dashed lines. *P* values below 0.05 were considered significant.

Fig 5. CMV-associated age acceleration
The age acceleration due to CMV infection was determined by calculating the difference in the experimental age and the predicted age. The predicted age was generated from a linear model based on the control CMV- group. The age acceleration was calculated for both CMV- (n=21) and CMV+ (n=54) groups. *P* values below 0.05 were considered significant.



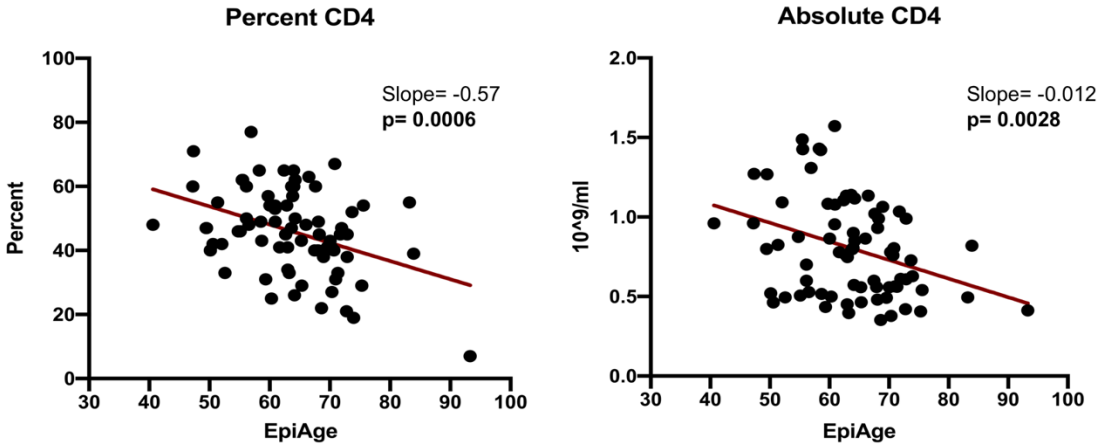


Fig 6. Increased Epi-Age is associated with decreased CD4 T cells
Flow cytometry was used to phenotype T cells from each individual included in the EpiAge analysis. Percent and absolute number of CD4 T cells were plotted against EpiAge and linear regressions were generated for the entire study population. Slopes with p-values less than 0.05 were considered significant. A red line indicates a negative slope and a green line indicates a positive slope.

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Supplementary Data:

Supplemental Table 1. Multilinear regression analysis for figure 1

CD4/CD8 T cell ratio		
Variable	P value	
Intercept	0.0255	
B : CMV	0.0499	
C : Sex	0.094	
D : Age	0.6995	
E : Hypertension	0.3489	
F : Dyslipidemia	0.8267	
G : Hypothyroidism	0.9964	
H : Osteoporosis	0.523	
I : Bebign Prostatic Hypertrophy	0.8971	
J : Depression	0.8241	
K : Chronic Gastritic	0.4748	
L : smoker	0.0574	
M : weight	0.026	
N : height	0.0318	
O : BMI	0.0478	

Supplemental Table 2. Multilinear regression analysis for figure 2							
Lymphocyte count			**CD4 Percent**			**CD8 Percent**	
Variable	P value		Variable	P value		Variable	P value
Intercept	0.6093		Intercept	0.3973		Intercept	0.2735
B : CMV	0.0289		E : CMV	0.2833		E : CMV	0.0136
C : Sex	0.6018		F : Sex	0.0473		F : Sex	0.0926
D : Age	0.6659		G : Age	0.0049		G : Age	0.7576
E : Hypertension	0.7024		H : Hypertension	0.8334		H : Hypertension	0.092
F : Dyslipidemia	0.5221		I : Dyslipidemia	0.3046		I : Dyslipidemia	0.1422
G : Hypothyroidism	0.922		J : Hypothyroidism	0.4902		J : Hypothyroidism	0.7379
H : Osteoporosis	0.515		K : Osteoporosis	0.1629		K : Osteoporosis	0.8789
I : Bebign Prostatic Hypertrophy	0.3687		L : Bebign Prostatic Hypertrophy	0.4834		L : Bebign Prostatic Hypertrophy	0.1047
J : Depression	0.5038		M : Depression	0.6496		M : Depression	0.4489
K : Chronic Gastritic	0.8993		N : Chronic Gastritic	0.7765		N : Chronic Gastritic	0.6034
L : smoker	0.5607		O : smoker	0.1755		O : smoker	0.1815
M : weight	0.6276		P : weight	0.6341		P : weight	0.1809
N : height	0.6178		Q : height	0.7232		Q : height	0.1741
O : BMI	0.2684		R : BMI	0.7656		R : BMI	0.2031
Absolute CD4			**Absolute CD8**				
Variable	P value		Variable	P value			
Intercept	0.7375		Intercept	0.4935			
E : CMV	0.2364		E : CMV	0.0064			
F : Sex	0.0846		F : Sex	0.1087			
G : Age	0.002		G : Age	0.5036			
H : Hypertension	0.6501		H : Hypertension	0.1143			
I : Dyslipidemia	0.1315		I : Dyslipidemia	0.1871			
J : Hypothyroidism	0.4288		J : Hypothyroidism	0.846			
K : Osteoporosis	0.7164		K : Osteoporosis	0.6828			
L : Bebign Prostatic Hypertrophy	0.6479		L : Bebign Prostatic Hypertrophy	0.0693			
M : Depression	0.9242		M : Depression	0.4381			
N : Chronic Gastritic	0.9318		N : Chronic Gastritic	0.6048			
O : smoker	0.7589		O : smoker	0.2231			
P : weight	0.48		P : weight	0.4099			
Q : height	0.7235		Q : height	0.4182			
R : BMI	0.941		R : BMI	0.3343			

Supplemental Table 3. Multilinear regression analysis for figure 3				
CD4 CD28-			**CD8 CD28-**	
Variable	P value		Variable	P value
Intercept	0.381		Intercept	0.4256
C : CMV	0.544		C : CMV	<0.0001
D : Sex	0.7655		D : Sex	0.2435
E : Age	0.391		E : Age	0.8539
F : Hypertension	0.0917		F : Hypertension	0.1441
G : Dyslipidemia	0.7063		G : Dyslipidemia	0.8268
H : Hypothyroidism	0.6764		H : Hypothyroidism	0.4705
I : Osteoporosis	0.7274		I : Osteoporosis	0.801
J : Bebign Prostatic Hypertrophy	0.6314		J : Bebign Prostatic Hypertrophy	0.1152
K : Depression	0.9437		K : Depression	0.5425
L : Chronic Gastritic	0.7256		L : Chronic Gastritic	0.2734
M : smoker	0.4177		M : smoker	0.1589
N : weight	0.7357		N : weight	0.9138
O : height	0.3836		O : height	0.4592
P : BMI	0.815		P : BMI	0.6904

Chapter 3: CMV-infection is associated with increased percentage of late-differentiated T cells that are capable of pro-inflammatory, multi-cytokine production in older, healthy adults

Introduction

Cytomegalovirus is the most prevalent of the human herpes viruses [1]. It is the causal agent of most congenital infections and an important cause of morbidity and mortality in those with primary and secondary immune deficiencies [2]. Although it establishes persistent infection early in life and infects the majority of the world population, most individuals remain asymptomatic. Human CMV utilizes numerous genes for host evasion and adaptation, allowing it to remain in a lifelong state of latency. Despite the seemingly long-term inconsequential infection in otherwise healthy humans, CMV has long-term negative health consequences. It is associated with multiple diseases that are seen with increased frequency in the elderly, specifically cardiovascular disease and Alzheimer's [3, 4]. However, it is unknown if CMV plays a causative role in these diseases. It is well established that chronic, latent CMV infection leads to a dysregulated immune state. First described in the OCTA- and NONA-studies conducted in Sweden, a CMV-dysregulated immune phenotype contributes to ageing co-morbidities, and is known as the immune risk profile (IRP) [5, 6]. It consists of three components: CMV-seropositivity, a CD4/CD8 T cell ratio <1, and increased levels of CD28- CD8 T cells. It remains unknown at what age CMV-associated immune dysregulation first occurs in healthy adults, how frequent it is, if it is associated with accelerated ageing, and if immune dysregulation is manifested by functional changes in cytokine production in otherwise healthy individuals.

CMV infection drives an oligoclonal expansion of CD8 T cells that are late differentiated and have altered cytokine production [7] [8]. CMV antigens induce IFN-gamma, TNF-alpha, and IL-2 production from CD4 and CD8 T cells [8]. This pro-inflammatory cytokine profile has been

implicated in COVID-19-induced ARDS, atherosclerosis, and graft rejection [9-11]. It is not well understood to what extent CMV-infection contributes to pro-inflammatory cytokine production, nor the immune functional impact on ageing and age-related diseases. Most studies on CMV have focused on unselected human populations, and have not eliminated many confounding variables from an already complicated ageing model. A set of heath defining criteria known as the SENIEUR criteria were proposed to reduce the effect of concurrent comorbidities in immune ageing research [12]. We identified the need to better characterize the terminally effector T cell phenotype known to be related to mortality in the elderly. We chose to study a cohort of healthy individuals aged 60 years and over, using modified strict SENIEUR criteria, in order to determine the phenotype and cytokine profile in those with and without CMV. Additionally, we employed the use of a relatively novel "biological age" marker termed EpiAge, which utilizes CpG patterns in the DNA to predict age to measure age-related changes.

Methods

Study Population

Healthy, older adults attending annual health care evaluations were enrolled from two McGill University Health Centre (MUHC) affiliated clinical sites. MUHC Research Institute Review Board study approval was obtained. All individuals were evaluated by a physician and enrolled if determined to be healthy using a strict version of the SENIEUR criteria and met the predetermined inclusion and exclusion criteria. Contrary to the SENIEUR criteria, subjects were excluded if they had previous cardiac events such as myocardial infarction or stroke. Subjects were included if they were 60 years of age or over, and willing to provide a single blood sample. They were excluded if they were 59 years of age or younger, had an active or chronic infection,

history of malignancy, autoimmune disease, diabetes, cardiovascular disease, or had ever used immune modulators. Informed consent was obtained from all study participants.

A one-time blood draw of 40 ml was collected in tubes containing acid citrate dextrose (BD Biosciences) from each individual for white count and differential, as well as flow cytometric analyses of lymphocyte subsets and serological assays for CMV. Peripheral blood mononuclear cell (PBMC) samples were divided in three aliquots, frozen and stored in liquid nitrogen immediately after being drawn. Plasma was stored at -80 C for CMV serology that was determined using an qualitative anti-CMV IgG ELISA (ABCAM), with duplicates run for each sample.

Targeted DNA methylation assays done in partnership with HKTherapeutics: A Pearson correlation between states of methylation of cytosine/guanine across the genome in blood cells in publicly available Illumina450K arrays and age (GSE61496), revealed that two sites residing proximal to the *ELOVL2* gene (cg16867657 and cg21572722) exhibit a strong Pearson product-moment correlation coefficient ($r=0.934$ $p<0.0001$) and ($r=0.81004$, $p<0.0001$). We developed a targeted DNA methylation assay to this region. A weighted "EpiAge" value was calculated for the 13 CGs in this region using a linear regression model.

1 million PBMC cells were placed in DNA stabilization buffer (SDS 0.5%, EDTA 20mM, TritonX100 1%, Tris-HCl 20 mM pH8.0). Bisulfate conversion was performed using EZ-96 DNA Methylation MagPrep (D5041, Zymo Reasearch), which was followed by two rounds of polymerase chain reaction (PCR); the first round targeted the *ELOVL2* and the second PCR introduced barcode sequences for multiplex amplification (the primers are available upon request). The pooled library was purified and sequenced on Illumina MiSeq, fast Q files were aligned with reference genome using BisMark and percentage of DNA methylation at each of the

13 CG positions was computed. EpiAge was calculated with a linear regression equation predicting age in the control group as a function of the weighted methylation levels of all CG sites.

Lymphocyte Phenotyping: Batches of cryopreserved PBMCs were thawed and washed twice in PBS and stained with the following fluorescently labelled antibodies: BV650-CD3, BUV737-CD4, APC-R700-CD8, BV711-CD28, BUV395-CD38, PE-Cy7-CD45RA, APC-Cy7-CD45RO, FITC-CD57, APC-CD103, BV785-HLA-DR, PE-TOX, BV605-PD-1, Indo-1-Live. All surface stains were done at room temperature for 30 min in the presence of human Fc block (BD Biosciences). All samples were analysed on a BD LSR Fortessa X-20 (BD Biosciences). Fluorescence data from 50,000 lymphocytes were acquired. Analysis of data was performed using FlowJo V10.

Cytokine Measurement: Cryopreserved PBMCs were thawed and washed in PBS. Cells were rested overnight prior to stimulation. Cells were stimulated for 6-hours with PMA+Ionomycin (Biolegend), CMV (Microbix), or unstimulated in the presence of a golgi complex inhibitor (Biolegend). Anti-CD49d (BD Biosciences) was added to all culture conditions in order enhance cytokine production. Production of IL-2, TNF-alpha, and INF-gamma were measured via intracellular staining using the following fluorochrome-labelled antibodies: Pacific Blue-CD3, BUV737-CD4, APC-H7-CD8, FITC-CD28, BV650-CD45RA, APC-CD57, Indo-1-Live, PE-CF594-TNF-alpha, PE-IL-2, PE-Cy7-IFN-gamma. Data from at least 50,000 cells were acquired. Analysis of data was performed using FlowJo V10.

Statistical analysis: A multilinear regression model was generated to control for sex, age, weight, height, BMI, smoking, hypertension, dyslipidemia, hypothyroidism, osteoporosis, benign prostatic hypertrophy, depression, and chronic gastritis as determined by drug regimen. The least

squares method was used via Prism 8, assuming a Gaussian distribution of residuals. This model was used to test for significant differences between CMV+ and CMV- groups for each parameter investigated. The following equation defines the model used to evaluate each dependent variable investigated in this study:

Dependent variable (e.g. %CD4 T cells) = Y = β0 + β1*CMV + β2*Sex + β3*Age + β4*Hypertension + β5*Dyslipidemia + β6*Hypothyroidism + β7*Osteoporosis + β8*Benign Prostatic Hypertrophy + β9*Depression + β10*Chronic Gastritis + β11*Smoking + β12*Weight + β13*Height + β14*BMI + random scatter

Where, female = 0, male = 1
CMV- = 0, CMV+ = 1
Disease states no = 0, yes = 1
Smoking no = 0, yes = 1

One-way ANOVA tests were carried out to determine significant differences in cytokine production between cell phenotypes and stimulation conditions within the CMV+ group via Prism 8. Tukey's multiple comparisons test was used for post-hoc analysis.

P values were considered significant at <0.05. All statistical tests were carried out using GraphPad Prism 8.

Results

A total of 520 individuals were screened for health status, and 87 subjects met the enrollment criteria. They ranged in age from 60 to 90 years old. Of these, 65 were CMV seropositive (CMV+) and 22 CMV-seronegative (CMV-). A complete blood count and clinical exam was carried out on each individual to evaluate health status. There were no significant differences in red blood cell, hemoglobin, leukocyte, or neutrophil counts between the CMV+ and CMV- groups. There were no significant differences between age, weight, height, BMI, smoking, hypertension, dyslipidemia, hypothyroidism, osteoporosis, benign prostatic hypertrophy, depression, and chronic gastritis between the two groups (Table 1).

Differences in T cell subsets between CMV+ and CMV- individuals

There were no differences in TOX expression, an intracellular marker of exhaustion, between CMV+ and CMV- groups when comparing the entire CD4 and CD8 populations. There was a significant increase in the percentage of CD4+ CD57+ PD-1- T cells ($p=0.0039$), and a significant decrease in CD57- PD-1- T cells ($p=0.0083$), when comparing CMV+ to CMV- (Fig 1). The CD8 T cell subset displayed similar trends, with an increased percentage of CD8+ CD57+ PD-1- T cells ($p<0.0001$), and a decreased percentage of CD8+ CD57- PD-1- ($p<0.0001$) when comparing CMV+ to CMV- (Fig 1). Additionally, the CMV+ group had a decreased percentage of CD8+ CD57- PD-1+ T cells ($p=0.0002$), as compared to the CMV- group (Fig 1).

Increased proportion of T cells with late-differentiated phenotype in CMV infection

The CMV+ population had significantly higher levels of CD8+ CD45RA+ CD57+ cells ($p<0.0001$), as compared to the CMV- population (Fig 2). Additionally, the CMV+ group had significantly decreased expression of TOX, as measured by MFI, in both CD4+ CD45RA+ CD57+ ($p<0.0001$) and CD8+ CD45RA+ CD57+ ($p=0.013$) subsets, as compared to the CMV- group (Fig 3). The CD8 CD45RA+ CD57+ subset from the CMV+ population had a significantly increased percentage of CD28- T cells ($p=0.037$) and decreased percentage of PD-1+ T cells ($p=0.0005$), when compared to CMV- (Fig 4). Percent of CD4+ CD45RA+ CD57+, CD8+ CD45RA+ CD57+, and TOX expression were not significantly impacted by EpiAge.

Multi-cytokine production of CD4 and CD8 T cell subsets via PMA stimulation

The CMV+ group had a higher MFI of IFN-gamma in CD4+ IL-2 IFN-gamma+ multi-producing T cells and in CD4+ TNF-alpha+ IFN-gamma+ multi-producing T cells ($p=0.0443$, $p=0.0394$) (Supp. Table 1). Additionally, the CMV+ group had increased MFI of TNF-alpha from CD4+

CD28- TNF-alpha+ IL-2+ multi-producing T cells (p=0.0355) and had higher percentage of CD4+ CD28- TNF-alpha+ IFN-gamma+ multi-producing T cells compared to the CMV- group (p=0.0118) (Supp. Table 1).

There were no significant differences in percentage of cytokine producing CD8 T cells. CD8+ CD28- IFN-gamma+ IL-2+ T cells had an increased IFN-gamma MFI, and CD8 CD45RA+ CD57+ T cells had an increased IFN-gamma MFI and TNF-alpha MFI (p=0.0451, p=0.0409, p=0.0322) (Supp. Table 2).

Differences in cytokine production between T cell subsets when stimulated with CMV

The multi-cytokine producing ability of each T cell subset was measured within the CMV+ group. The CD4+ CD28- subset had an increased percentage of IL-2+ IFN-gamma+ (p=0.0007), IL-2+ TNF-alpha+ (p=0.0424), and IFN-gamma+ TNF-alpha+ (p=0.0002) producing cells as compared to the CD4+ subset (Fig 5). The IFN-gamma MFI and TNF-alpha MFI from the first two phenotypes was higher in the CD4+ CD28- subset (p=0.0424, p=0.0002). The CD4+ CD45RA+ CD57+ subset had a higher percentage of IFN-gamma+ TNF-alpha+ producing T cells as compared to the CD4+ subset (p=0.0114) (Fig 5). There were no significant differences in CD8 T cell cytokine production (Table 1). EpiAge did not significantly impact cytokine production of any T cell phenotype analyzed.

Discussion

Using a stricter version of the SENIEUR criteria, we show that CMV-infection is associated with significant changes to CD4 and CD8 T cell phenotypes in healthy, older individuals. Our study differed from the others in that we strictly defined good heath, by excluding any history or presence of active comorbidities. PD-1 expression has been reported to increase in those CMV+ [13]. These findings were reported in those with active CMV disease and graft-versus-host-disease. In our study of healthy, elderly individuals, we noted a decrease in PD-1 expressing CD4 and CD8 T cells. A similar finding was reported in CMV-specific CD4 T cells, where the intensity and distribution of PD-1 was decreased in elderly individuals [14]. Additionally, there was a significantly higher percentage of CD8+ CD45RA+ CD57+T cell subset, a relatively novel T cell subset that's expansion has been inversely related to mortality to octogenarians [15]. We found this expansion occurs in individuals as young as 60. This subset has been previously shown to be expanded in healthy, CMV-infected individuals and produces pro-inflammatory cytokines IL-2, IL-7, IL-15, IFN-gamma, and TNF-alpha via stimulation of the TCR or IL-15 in media [16]. We expanded upon this finding by showing this subset had a decreased percentage of PD-1 expressing cells and decreased expression of TOX in those CMV+. Recently, the transcription factor TOX has been identified as having a major role in the reprogramming of effector memory cells into exhausted T cells. TOX is upregulated in response to stimulation via antigen presenting cells and acts via the NFAT pathway to cause chromatin remodelling within the cell, which eventually leads to upregulation of PD-1, decreased cytokine production, and increased survival signals [17]. In the context of CD4+ CD45RA+ CD57+ and CD8+ CD45RA+ CD57+ T cells and CMV, TOX does not seem to be upregulated. We see a decrease of T cell

exhaustion markers both intracellularly with the transcription factor TOX, as well as at the cell surface with PD-1 in those CMV+.

The mechanism by which CMV infection is involved in TOX expression and PD-1 is unknown. However, CMV has developed a variety of mechanisms to evade the immune response, which ultimately may be contributing to this specific cell fate. Notably, human CMV has been shown to produce a variety of microRNA that modulate T cell responses. In the case of CD8 T cells, microRNA miR-US4-1 has been shown to modulate MHC-1 presentation on the surface of infected cells, which has implications for CD8 T cell activation [18]. Additionally, the secretome profile of CMV infected cells has been shown to recruit CD4 T cells and modulate their function [19]. The CMV genome encodes a homolog for IL-10, which has been shown to be important for long-term viral survival in a human host [20]. Further research is needed to identify the specific mechanism(s) that contribute to a lack of cell exhaustion that we show in the CMV+ group.

Additionally, we show significant differences in the cytokine-production profile of those CMV+ and CMV-, as well as between the various T cell subsets when stimulated with CMV. Notably, we show that PMA stimulates a significantly higher percentage of CD4+ CD28- TNF-alpha+ IFN-gamma+ T cells in the CMV+ population. Additionally, we show that within in the CMV+ population, the CD4+ CD28- and CD4+ CD45RA+ CD57+ subpopulations have a significantly higher percentage of TNF-alpha, IFN-gamma multi-producing cells, as compared to the whole CD4 population. Interestingly, we show that these subpopulations within the CD8 compartment are capable of strong cytokine production, however they do not significantly differ from the CD8 T cell subset as a whole. This indicates that both the CD4 and CD8 terminally differentiated subsets from CMV+ individuals are capable of pro-inflammatory multi-cytokine production in response to CMV antigens. It is already known that CMV infection induces systemic immune

activation characterized by a type 1 cytokines [21]. Our results highlight possible cell types that are contributing to CMV-induced systemic inflammation.

We have recently shown that CMV-infection is associated with epigenetic changes that correlate with an increased age. Our study revealed a 5.1-year age acceleration due to chronic CMV infection, which was similar to the 5.2-year age acceleration seen with HIV infection [22]. We associated this finding to a significant expansion of lymphocytes, primarily due to an increase in absolute CD8 T cell count. Here, we find no associations with EpiAge and CD8+ CD45RA+ CD57+, indicating this cell type is already inflated prior to the age of 60 and remains constant with increasing EpiAge. Additionally, we show that cytokine production from PMA stimulation is not associated to EpiAge. There is no association found between EpiAge and the cytokine producing capability of the CD45RA+ CD57+ subsets, suggesting a constant ability of this cell subset to produce pro-inflammatory cytokines independent of EpiAge. This is similar to what has been previously reported on late-differentiated CD45RA+ cells, which show this subset to be functionally competent by expressing high levels of IFN-gamma and TNF-alpha [23]. It is possible that the CD45RA+ CD57+ subsets have long-term negative health impacts due to their ability to produce pro-inflammatory cytokines in the elderly.

One important note to take into consideration is the lack of CMV-specific T cell analysis in this study. Our study focused on cell subsets as a whole, rather than phenotyping CMV-specific cells. We therefore cannot conclude that the differences in cytokine production are due to CMV-specific cells alone. Additionally, a combination of three CMV antigens were used for stimulation, namely pp52, pp65, and glycoprotein B. This combination generated robust cytokine responses, however it is not completely representative of T cell responses to CMV in vivo.

Our findings strongly implicate late-differentiated T cells as a potential source of pro-inflammatory cytokines during chronic CMV infection. Specifically, CD4+ CD45RA+ CD57+ and CD8+ CD45RA+ CD57+, found in those CMV+, are capable of pro-inflammatory cytokine secretion in response to CMV-antigen stimulation. Additionally, these subsets have decreased TOX expression, which may be contributing to the cytokine-producing capabilities. It is important to take note of both CD4 and CD8 late-differentiated cell types and their implications in CMV-induced immune ageing. Further studies are needed to fully understand how these cell subsets contribute to systemic inflammation, and ultimately age-related diseases.

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Potential conflicts of interest: The authors have submitted the ICMJE form. CP and CT report no conflicts of interest, **MS and DC** have applied for patents on EpiAge and MS owns shares in HKG epitherapeutics.

Figures:

	Human Cytomegalovirus (CMV) Negative		Human Cytomegalovirus (CMV) Positive		P-value
Donors (n)		22		65	
	Male	13 (59.1%)	Male	32 (49.2%)	0.46
	Female	9 (40.9%)	Female	34 (51.5%)	
Covariables					
Age		72.7 +/- 8.2		70.3 +/- 8.9	0.23
Hypertension		6 (27.3%)		22 (33.8%)	0.64
Dyslipidemia		7 (30.4%)		15 (23.4%)	0.58
Hypothyroidism		1 (4.5%)		13 (20%)	0.11
Osteoporosis		6 (27.3%)		23 (35.4%)	0.60
Benign Prostatic hypertrophy		1 (4.5%)		7 (10.8%)	0.67
Depression		1 (4.5%)		8 (12.3%)	0.44
Chronic Gastritis		4 (18.2%)		11 16.9%)	>0.99
Smoking		0 (0%)		5 (6.8%)	0.32
Weight		169.2 +/- 35.6 lbs		160.7 +/- 30.9 lbs	0.31
Height		66.2 +/- 3.4 inches		64.3 +/- 2.8 inches	0.33
BMI		26.6 +/- 4.8		26.4 +/- 4.1	0.85

Table 1. Study population characteristics
Fisher's exact test was used to determine significant differences in sex, hypertension, dyslipidemia, hypothyroidism, osteoporosis, benign prostatic hypertrophy, depression, chronic gastric, and smoking between the study groups, and the student's t test was used to test for significant differences in age, weight, height, and BMI. *P* values below 0.05 were considered significant.

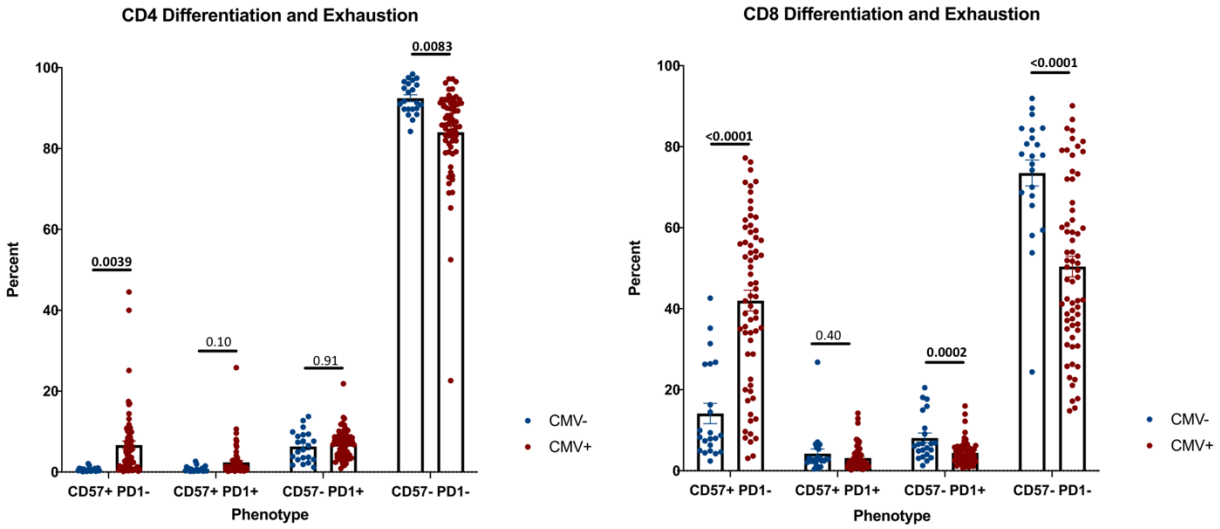


Figure 1. The CD4 and CD8 T cell phenotype is altered in CMV-infected donors
Peripheral blood mononuclear cells were isolated from CMV+ (n=65) and CMV- (n=22) individuals. Flow cytometry was used to phenotype T cells from each individual donor, specifically analyzing CD4, CD8, CD57, and PD-1. CD57 and PD-1 were graphed on the X and Y axis, resulting in the phenotypes above. Multivariable linear regression models were used to test for significance, with *P* values below 0.05 were considered significant.

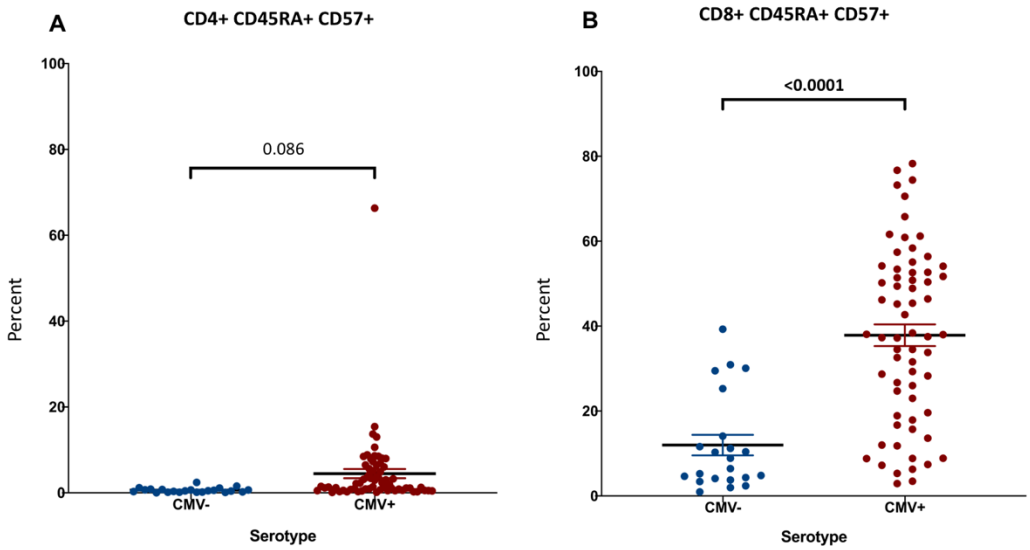


Figure 2. The CD8+ CD45RA+ CD57+ subset is increased in CMV+
Peripheral blood mononuclear cells were isolated from CMV+ and CMV- individuals. Flow cytometry was used to phenotype CD4 and CD8-positive cells, using CD57+ CD45RA+. The percentages of double positive cells within the CD4 and CD8-positive T cell populations were determined for the CMV+ (n=65) and CMV- (n=22) groups, using a multivariable linear regression model to determine significance. *P* values below 0.05 were considered significant.

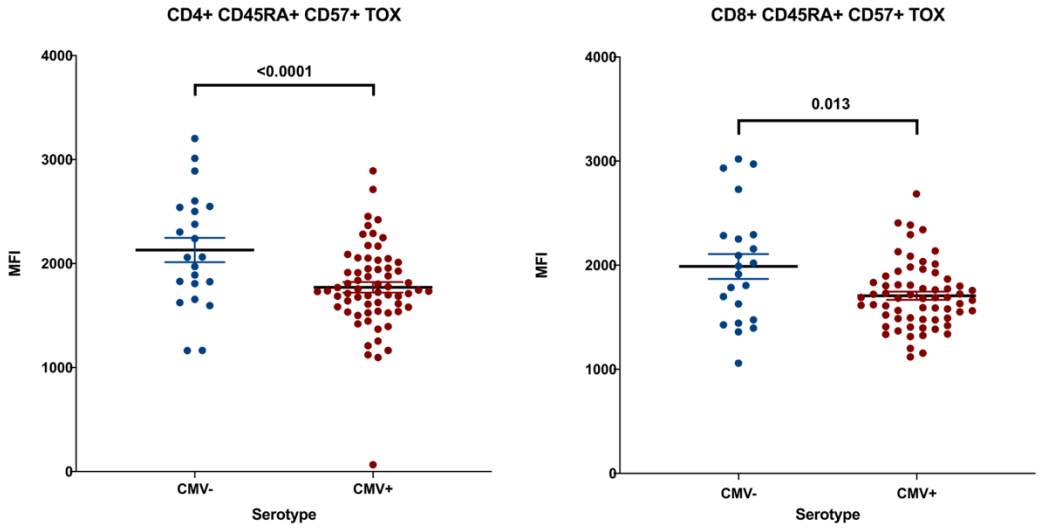


Figure 3. CD8, CD45RA cell subsets are altered in CMV-infection
Peripheral blood mononuclear cells were isolated from CMV+ and CMV- individuals. Flow cytometry was used to phenotype CD4 and CD8-positive cells. TOX expression was analyzed using MFI (geometric mean) in CD45RA+ CD57+ cells, and was determined for the CMV+ (n=65) and CMV- (n=22) populations. A multivariable linear regression model was used to test for significance. *P* values below 0.05 were considered significant.

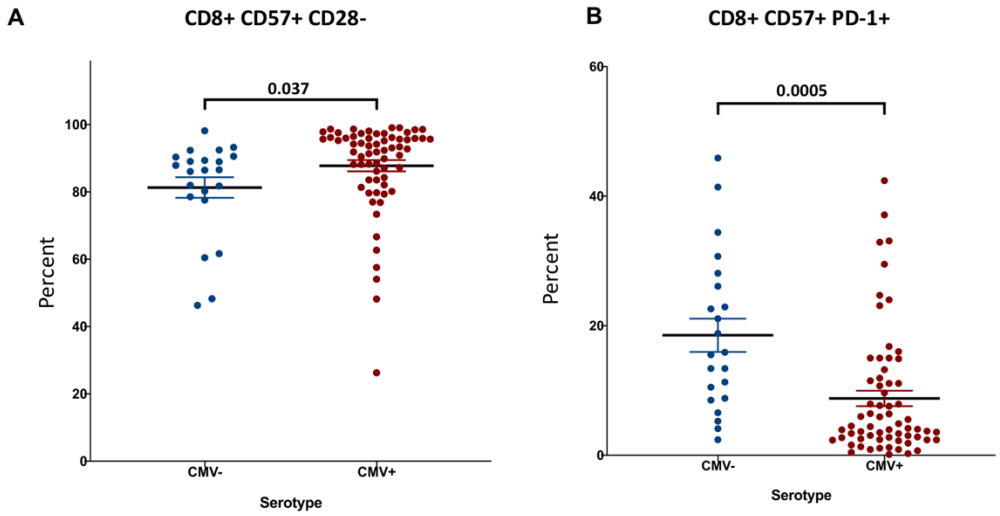
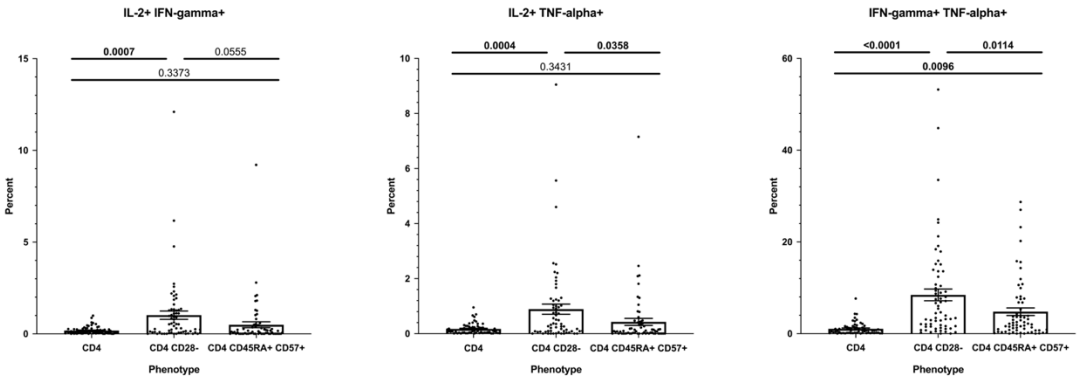


Figure 4. CD8, CD45RA cell subsets are altered in CMV-infection
Peripheral blood mononuclear cells were isolated from CMV+ and CMV- individuals. Flow cytometry was used to phenotype CD8-positive cells. The percentage of CD28- and PD-1+ were determined for the CMV+ (n=65) and CMV- (n=22) populations. A multivariable linear regression model was used to test for significance. *P* values below 0.05 were considered significant.



	CD8 (%)	CD8 CD28-(%)	CD8 CD45RA+ CD57+ (%)	% p-value
IL-2+ IFN-gamma+	0.045	0.051	0.051	0.975
IL-2 + TNF-alpha+	0.037	0.038	0.042	0.956
IFN-gamma+ TNF-alpha+	0.92	1.46	1.47	0.579

Figure 5. CMV stimulation causes increased percentage of multi-cytokine producing CD4- CD28+ and CD4+ CD45RA+ CD57+ T cells
One million cells from each donor were stimulated with 4 ug/ml of CMV antigens for 6 hours at 37 C in the presence of a golgi inhibitor. Cells were intracellularly stained for IL-2, TNF-alpha, and IFN-gamma to measure cytokine production. The percentage of double positive cells for each cell subset was measured. Significance between all groups was measured using a one-way ANOVA, Tukey's multiple comparisons test was used to generate p-values when ANOVA analysis revealed significance.

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Supplementary Data

T cell subset		CMV-	CMV+	P-value
CD4				
IFN-gamma+ IL-2+ (%)		1.5	1.8	0.440
	IL-2 (MFI)	412.3	416.1	0.822
	IFN-gamma (MFI)	1078.3	1332.9	**0.0443**
TNF-alpha+ IL-2+ (%)		8.4	7.9	0.843
	IL-2 (MFI)	484	482.7	0.974
	TNF-alpha (MFI)	1648.9	1696.7	0.769
IFN-gamma+ TNF-alpha+ (%)		4.1	6.5	0.110
	TNF (MFI)	1906.9	2001.0	0.581
	IFN (MFI)	952.5	1173.0	**0.0394**
CD4 CD28-				
IFN-gamma+ IL-2+ (%)		1.5	1.8	0.290
	IL-2 (MFI)	423	369.3	0.104
	IFN-gamma (MFI)	1585.6	1682.0	0.473
TNF-alpha+ IL-2+ (%)		2.7	2.3	0.624
	IL-2 (MFI)	469.7	411.8	0.110
	TNF-alpha (MFI)	1857.2	2828.8	**0.0355**
IFN-gamma+ TNF-alpha+ (%)		16.42	29.0	**0.0118**
	TNF (MFI)	2006.2	2332.7	0.115
	IFN (MFI)	996.5	1153.2	0.0817
CD4 CD45RA+ CD57+				
IFN-gamma+ IL-2+ (%)		2.1	2.1	0.781
	IL-2 (MFI)	349.7	381.5	0.638
	IFN-gamma (MFI)	1633.4	1822.6	0.412
TNF-alpha+ IL-2+ (%)		3.0	2.4	0.448
	IL-2 (MFI)	378.7	404.7	0.403
	TNF-alpha (MFI)	2336.1	2924.6	0.226
IFN-gamma+ TNF-alpha+ (%)		31.0	36.7	0.227
	TNF (MFI)	1976.5	2239.8	0.168
	IFN (MFI)	1008.6	1121.4	0.238

Supplementary Data Table 1. PMA Stimulation of CD4 subsets
One million cells from each donor were stimulated with PMA for 6 hours at 37 C in the presence of a golgi inhibitor. Cells were intracellularly stained for IL-2, TNF-alpha, and IFN-gamma to measure cytokine production. The percentage of double positive cells for each cell subset was measured. Significance between groups was measured using a multilinear regression model, with p-values less than 0.05 considered significant.

T cell subset		CMV-	CMV+	P-value
CD8				
IFN-gamma+ IL-2+ (%)		3.8	3.6	0.937
	IL-2 (MFI)	374.7	358.4	0.401
	IFN-gamma (MFI)	1833.2	2145.4	0.074
TNF-alpha+ IL-2+ (%)		7.5	7.7	0.887
	IL-2 (MFI)	441.6	441.6	0.978
	TNF-alpha (MFI)	1969.9	1958.3	0.898
IFN-gamma+ TNF-alpha+ (%)		22.9	28.3	0.151
	TNF (MFI)	1744.2	1922.7	0.236
	IFN (MFI)	1269.6	1487.1	0.0967
CD8 CD28-				
IFN-gamma+ IL-2+ (%)		2.3	2.3	0.891
	IL-2 (MFI)	383.5	340.99	0.129
	IFN-gamma (MFI)	1779.9	2208.9	**0.0451**
TNF-alpha+ IL-2+ (%)		2.1	2.0	0.932
	IL-2 (MFI)	372.4	377.6	0.770
	TNF-alpha (MFI)	2270.4	2665.3	0.272
IFN-gamma+ TNF-alpha+ (%)		35.7	42.9	0.169
	TNF (MFI)	1843.2	2180.5	0.0753
	IFN (MFI)	1136.7	1290.6	0.154
CD8 CD45RA+ CD57+				
IFN-gamma+ IL-2+ (%)		2.8	2.3	0.590
	IL-2 (MFI)	355.6	322.6	0.0870
	IFN-gamma (MFI)	1948.8	2423.3	**0.0409**
TNF-alpha+ IL-2+ (%)		2.0	1.8	0.953
	IL-2 (MFI)	383.9	379.4	0.946
	TNF-alpha (MFI)	2051.1	3223.5	**0.0322**
IFN-gamma+ TNF-alpha+ (%)		48.8	54.9	0.167
	TNF (MFI)	2052.8	2140.7	0.672
	IFN (MFI)	1089.5	1259.6	0.110

Supplementary Data Table 2. PMA Stimulation of CD8 subsets
One million cells from each donor were stimulated with PMA for 6 hours at 37 C in the presence of a golgi inhibitor. Cells were intracellularly stained for IL-2, TNF-alpha, and IFN-gamma to measure cytokine production. The percentage of double positive cells for each cell subset was measured. Significance between groups was measured using a multilinear regression model, with p-values less than 0.05 considered significant.

Chapter 4: Summary and Final Conclusions

The findings presented in this thesis reflect the older (60-90 years) population of Montreal, Canada. It is important to note the recruitment stage of this study was one of the major obstacles, with the enrollment and recruiting process starting four years prior to completion of the study. 520 adults over the age of 60 were screened, with only 85 (16.3%) being successfully enrolled. Of these, 25.3% were HCMV-, making up only 4.2% of the total population screened. This highlights a common struggle when researching HCMV in the elderly. It is known that infection rates of the virus increase with age, and 74.4% of our study population was HCMV+, which matches other studies [1]. The OCTA study, with an even older population, had a seropositivity of 90% at the first time point and 95% seropositivity at the fourth time point [2]. Only one individual was HCMV- at the end of the OCTA study, making it near impossible to show significance in regard to HCMV [2]. Our study included 22 elderly HCM-seronegative, which allowed for comparisons with age-matched seropositive individuals.

We found that 10.3% of our population was IRP+, which is comparable to the 15% IRP+ in the OCTA studies [2]. Additionally, we found that, in those HCMV+, 16.9% had a CD4/CD8 T cell ratio <1. This is in line with the KRIS, OCTA, and NONA studies, which reveal 14.7% in those 60-70, 15.5% in those 86-92, and 15.9% in those 86-94 have a ratio <1 [3]. Our findings also confirm that HCMV infection is associated with an expansion of CD8 T cells that drives the dysregulated ratio, as we show increases in the absolute counts of lymphocytes and CD8 T cells. Interestingly, we show a significant percentage of a CD4/CD8 T cell ratio >5 in our population. This may be indicative of immune dysregulation, as the established normal range for T cell ratios at the McGill University Health Centre is 1.8-3.4. This is not a novel finding, as it was first reported in the BEFRAIL study, which documented T cell ratios >5 in the very elderly [4]. The

BEFRAIL study reported having 33% of their >81.5 year old population having a ratio >5, while we show 20% of our population (60-90 years) with a >5 ratio [4]. It was noted that those with higher ratios had decreased amounts of differentiated T cells and weaker HCMV-IgG reactivity [4]. The decrease in differentiated T cells was shown in both HCMV- and HCMV+ elderly individuals. Age, regardless of HCMV status, is implicated in immune dysregulation, and the effect of age must be factored in as a covariate when performing statistical analyses.

Our analysis outperforms those done in the BEFRAIL, OCTA, and NONA studies, as all of these studies explicitly mention that unknown disease states may have been a factor in their results [4-6]. Our strict screening allowed us to produce a more robust analysis. We included sex, age, weight, height, BMI, smoking status, hypertension, dyslipidemia, hypothyroidism, osteoporosis, benign prostatic hypertrophy, depression, and chronic gastritis in our multivariate linear analysis, while eliminating diabetes, malignancy, infection, immune modulating drugs, cardiovascular disease, and autoimmune disorders through the selection process. We were still able to confirm the findings of the BEFRAIL, OCTA, and NONA studies, specifically an altered T cell ratio and expansion of CD8 T cells.

The novelty of our work appears when phenotyping T cell subsets in those HCMV- and HCMV+. Interestingly, we found significant expansions of CD8+ CD45RA+ CD57+ T cells. This subset is terminally differentiated and re-expresses the naïve T cell marker CD45RA [7]. It was termed terminally differentiated effector memory cells re-expressing CD45RA (TEMRA) [8]. These cells can be detected through a variety of different cell surface markers, most notably CCR7- CD45RA+ [8]. Using CD57 as a marker only includes the most differentiated cells in this subset [9]. This panel of surface markers may have excluded a proportion of “younger” more

functionally active TEMRAs in our functional assays. We therefore did not define this subset as TEMRA in our study, rather TEMRA-like or terminally-differentiated CD45RAs.

TEMRA cells have been linked to chronic inflammation and negative health outcomes. Recent studies linked the TEMRA subset to an increased risk of acute kidney allograft rejection and HIV disease progression [5, 10]. Additionally, CD8 TEMRA cells have also been shown to be capable of producing high levels of IFN-gamma and TNF-alpha, both type 1 pro-inflammatory cytokines [11]. Furthermore, increased numbers of TEMRA cells have been associated with Alzheimer's disease and have been negatively associated with cognition [12]. Most notably, those with severe calcified aortic stenosis have increased absolute numbers of TEMRA cells, as compared to moderate aortic stenosis and healthy controls [13]. It is clear that TEMRA cells play a role in age-related diseases and negative health outcomes. However, this role is not always clear. A recent study regarding octogenarians indicated an inverse relationship between increased CD8+ CD27- CD28+ TEMRA cells and non-cardiovascular and cardiovascular deaths, while increased total CD8+ TEMRAs had the opposite effect [14]. This was independent of HCMV status. Interestingly, elevated levels of total CD28- T cells have been linked to negative cardiac outcomes [15]. It is unknown if increased CD28- TEMRA cells increases the risk of cardiovascular deaths, and if the effect is dependent on HCMV serostatus.

We showed that a higher percentage of CD4 TEMRA-like cells are multi-cytokine producers and produce more cytokines when compared to the total T cell population. We analyzed three combinations of pro-inflammatory cytokine profiles: IL-2+ IFN-gamma+, IL-2+ TNF-alpha+, and IFN-gamma+ TNF-alpha+. There was a higher percentage of multi-cytokine producers for each cytokine profile analyzed. Additionally, the CD8 TEMRA-like subset produced equal levels

of cytokines as compared to the other T cell subsets analyzed. There was a significantly higher percentage of IFN-gamma+ TNF-alpha+ TEMRA-like cells than the other cytokine profiles for both CD4 and CD8 subsets. Interestingly, the CD4 subset produced significantly more pro-inflammatory cytokines than the CD8 subset, highlighting CD4 TEMRAs as potential contributors to harmful inflammation. CD4 TEMRAs have been shown to produce cytotoxic molecules in response to dengue virus [16]. However, this is the first time we see a strong pro-inflammatory response from CD4 TEMRA-like cells in response to HCMV antigen stimulation. It is possible this is a response to control HCMV. In fact, this function has been demonstrated in the CD8 T cell subset, where HCMV viremia controllers have increased frequencies of IFN-gamma+ TNF-alpha+ CD8 TEMRA cells [17]. It is unknown if this holds true for CD4 TEMRA-like cells as well.

Our study also showed significant alterations in the phenotype of TEMRA-like cells between those HCMV- and HCMV+. The CD8+ TERMA-like cells from the HCMV+ group had a significantly decreased percentage of PD-1 positive cells, as compared to the HCMV- group. Studies of other chronic infections, such as HCV, show increases in PD-1 expression on TERMA cells [18]. Furthermore, PD-1 expression on CD8 T cells has been reported to increase in HCMV infection [19]. However, that study did not account for confounding variables and was conducted in kidney transplant recipients. We also show a decrease in the intracellular transcription factor TOX within CD4+ and CD8+ TEMRA cells. TOX is a relatively novel transcription factor that is responsible for programming effector T cells into an exhausted fate and is directly correlated with PD-1 surface expression [20]. Our findings of decreased TOX corroborate our findings of decreased PD-1 surface expression. Additionally, the lack of exhaustion programming within the TERMA cell subset may be a possible mechanism by which the subset is still capable of

producing pro-inflammatory cytokines. It is unknown why chronic HCMV stimulation does not drive TEMRA-like cells into an exhausted fate. It is possible that HCMV-specific TEMRA cells have been epigenetically programmed to remain functional, which has been reported in TEMRA cell subsets [21].

One potential explanation is that TEMRA cells are short-lived and continually regenerated from memory effector cells. It is possible that HCMV-specific TEMRA cells die before reaching an exhausted fate. These could then be replaced via sporadic antigen presentation, driving differentiation of Tem. This has not yet been shown. Additionally, HCMV has developed a variety of different mechanism that evade the immune system, many of which are immunomodulatory. HCMV can produce an IL-10 homolog, which has been shown to alter dendritic cell function [22]. Additionally, HCMV can modulate MHC 1 and 2 processing and presentation [23, 24]. These mechanisms have the capability of interfering with CD4 and CD8 T cell activation, and thus providing insufficient signaling to promote an exhausted T cell phenotype. The mechanisms by which HCMV modulates the immune system need to be further explored.

The most important finding presented in our study is the increase in EpiAge in healthy adults with HCMV+. We used a model of methylated CpG islands in the DNA to accurately predict biological age. This was first carried out by Horvath and was shown to be useful as a biomarker of ageing [25]. Epigenetic age has been shown to outperform other diagnostic markers in predicting all-cause mortality, cancers, and Alzheimer's disease [25]. We became interested in using EpiAge when a 5.2-year increase was found in those living with HIV [26]. A majority of people living with HIV are co-infected with HCMV, bringing into question the claim that HIV

alone causes this increase. Our analysis shows a 5.1-year age acceleration in those HCMV+ as compared to HCMV-, with an average EpiAge of 65 and 60 respectively. This finding further implicates HCMV as a major contributor to age-related diseases.

Interestingly, we did not find any significant associations between increasing EpiAge and cytokine production. This association has been reported by Horvath, who showed that increased Epigenetic Age is associated with increased activation of pro-inflammatory pathways [25]. This study was conducted using a national database of almost 10,000 unselected individuals. We were able to make significant associations between increased EpiAge and decreased percent and absolute CD4 T cells. This association has been previously reported using chronological age [6].

HCMV has been implicated as a major contributor to age-related diseases. It has been associated with increased risk of cardiovascular disease, Alzheimer's disease, and all-cause mortality [27-30]. It is likely that HCMV, along with the natural ageing process, exacerbates natural disease progression. Further mechanistic research is needed to delineate changes in T cell function and phenotype that are noted with chronic HCMV infection.

Our study highlights promising avenues of research to link HCMV infection to disease-causing mechanisms. Specifically, we identify terminally differentiated CD45RA expressing T cells as possible contributors to age-related diseases. We show that this T cell subset is a multi-cytokine producer, capable of contributing systemic inflammation. In fact, it has been shown that HCMV causes continuous systemic immune activation, as measured by serum Th1 cytokines [31]. The specific cells that are the source of cytokines remains unknown. Furthermore, we show that those with HCMV have advanced EpiAge, a novel biomarker which has emerged as a reliable

predictor for health outcomes. As more data connecting HCMV infection to negative health outcomes is published, the development of a HCMV vaccine will become paramount.

More research is needed to further elucidate the impact of HCMV on immune dysregulation and disease. It remains unknown why HCMV generates a robust expansion of TEMRA-like cells, and why these cells do not enter an exhausted phenotype. Our study also falls short of identifying these cells as HCMV-specific, something that must be done moving forward. Additionally, we fail to link our findings to any clinical outcomes, such as cardiovascular disease. It is clear a longitudinal study investigating negative health outcomes, T cell expansion and function, and HCMV infection is needed. However, the findings of this project provide a strong base to continue HCMV research, by supporting claims made by previous studies, identifying novel T cell phenotypes in those HCMV+, and identifying EpiAge as a potential biomarker in chronic viral infection.

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