

Physical Function Impairment of Older, HIV-Infected Adults Is Associated with Cytomegalovirus Immunoglobulin Response

Kristine M. Erlandson,¹ Amanda A. Allshouse,² Eric Rapaport,¹ Brent E. Palmer,¹ Cara C. Wilson,¹ Adriana Weinberg,^{1,3,4} Samantha MaWhinney,² and Thomas B. Campbell¹

Abstract

Cytomegalovirus (CMV) is associated with poor outcomes, including physical function impairment, in older HIV-uninfected adults. Whether CMV is associated with physical functional impairment in HIV-infected adults is unknown. The primary objective of this study was to determine the relationship between CMV-specific humoral and cell-mediated immune responses with functional impairment in well-controlled HIV infection. In a case-control study, low-function cases were matched by age, gender, and time from HIV diagnosis to high-function controls. Quantitative CMV IgG and %CMV-specific CD8⁺ and CD4⁺ T cells (interferon- γ expression following CMV pp65 stimulation) were used to estimate physical function. Among 30 low-function cases and 48 high-function matched controls, CMV IgG ranged from <10 to 8,830 EU/ml, including four controls with results <10 EU/ml. Each log₁₀ increase in CMV IgG was associated with 5-fold greater odds of low function ($p=0.01$); these findings were robust to adjustment for concomitant CD4⁺ count, tobacco use, and age; to exclusion of subjects with CMV IgG <10 EU/ml; and to adjustment for hepatitis C viremia. %CMV-specific CD4⁺ or CD8⁺ T cells were not associated with low function. In bivariable models, the relationship between CMV IgG and physical function was attenuated and was no longer significant when including IL-6, CD4/CD8 ratio, or the Veterans Aging Cohort Study Index score. High levels of CMV-specific IgG were associated with impaired physical function. Attenuation of the strength of this association in bivariable models suggests an indirect relationship mediated by systemic inflammation and immune suppression.

Introduction

CYTOMEGALOVIRUS (CMV) INFECTION is ubiquitous among adults with seroprevalence of up to 95% in HIV-uninfected elderly populations,^{1,2} and as high as 99% in HIV-infected populations.³ After primary infection, CMV typically establishes latency, but periodically reactivates, particularly in the presence of host immune suppression. In contrast to other infections such as influenza or pneumococcus, the immune response to CMV is greater in older age, with increases in both humoral [CMV-specific immunoglobulin G (IgG)] and in the cell-mediated (proportions of CMV-specific T cells) immune responses, possibly reflecting repeated reactivations or reinfections. In healthy middle-aged persons, 4–5% of the total CD4⁺ or CD8⁺ T cells produce interferon- γ in response to *in vitro* CMV antigenic stimula-

tion.⁴ In elderly persons, CMV-specific T cells can comprise up to 40% of CD8⁺ T cells,^{5,6} have shortened telomeres, have little to no proliferative capacity to new antigens, and are resistant to apoptosis.⁷

CMV seropositivity and high proportions of terminally differentiated CMV-specific T cells characterize an “immune risk profile” that is associated with increased mortality risk among elderly populations.⁸ An association between an increased proportion of CMV-specific CD4⁺ T cells or CMV IgG and neurofibrillary tangles in Alzheimer’s disease provides evidence of a relationship between immune responses to CMV and cognitive changes.⁹ Among HIV-uninfected elderly populations, CMV-specific IgG levels are also associated with institutionalization,⁷ poor functional status,^{1,10} frailty,^{2,11,12} and mortality.^{12–15} Whether CMV directly contributes to complications of aging or whether CMV leads to

¹Department of Medicine, University of Colorado, Aurora, Colorado.

²Colorado School of Public Health, Aurora, Colorado.

³Department of Pediatrics, University of Colorado, Aurora, Colorado.

⁴Department of Pathology, University of Colorado, Aurora, Colorado.

immune senescence, or immune activation and inflammation, which subsequently lead to physical function impairment and frailty, is unclear. Prior studies have demonstrated associations between CMV IgG and mortality, and CMV IgG and interleukin (IL)-6 and tumor necrosis factor (TNF)- α ^{12,14} suggesting that chronic CMV infection contributes to age-related complications through heightened inflammation.

Both CMV IgG and the percentage of CMV-specific T cells are higher in persons with HIV infection compared to HIV-uninfected controls,^{3,16} and CMV-specific T cells are higher among HIV-infected persons on antiretroviral therapy (ART) compared to ART-naïve persons.^{3,17} CMV-specific cell-mediated immune responses increase with age such that the highest percentages of CMV-specific CD4⁺ or CD8⁺ T cells are seen among HIV-infected older adults on ART.¹⁸ These CMV-specific humoral and cell-mediated immune responses are associated with comorbid disease markers among virologically suppressed HIV-infected persons.^{19–21} Furthermore, reductions in immune activation, as measured by the percentage of CD38⁺HLA-DR⁺CD8⁺ T cells, among HIV-infected, CMV-seropositive participants after treatment with valganciclovir, an inhibitor of CMV replication, provide direct evidence that CMV drives immune activation during chronic HIV infection.²²

We have previously demonstrated a strong association between markers of inflammation and immune activation with physical function impairment among middle-aged HIV-infected persons on effective ART.²³ The goals of the present study were to (1) determine the relationship between CMV-specific humoral and cell-mediated immune responses and functional impairment in well-controlled HIV infection and (2) explore the impact of clinical characteristics, inflammation, and immune activation on those relationships.

Materials and Methods

Study population

Details of the study population, clinical assessments, and measurement of markers of inflammation, immune activation, and immune senescence have been previously published.^{23–25} Briefly, individuals with HIV-1 infection on antiretroviral therapy for a minimum of 6 months and no plasma HIV-1 RNA >200 copies/ml within the prior 6 months underwent physical function testing by the Short Physical Performance Battery and Fried's frailty assessment. Cases with low physical function, defined by a combination of deficits on both functional assessments,²³ were matched by age, gender, and time since HIV diagnosis to controls with high physical function (no deficits on either functional assessment). Stored samples and existing data were used for the current study. Veterans Aging Cohort Study (VACS) Index scores were calculated as previously described.²⁶ Approval was obtained by the Colorado Multiple Institutional Review Board and informed consent was obtained from all participants.

Quantitative immunoglobulins

Plasma IgG antibodies against CMV, varicella zoster virus (VZV), and combined herpes simplex virus (HSV) 1 and 2 were measured in stored specimens using enzyme immunoassay kits (Diamedix Corp., Miami, FL). All assays were performed according to the manufacturer's instructions, with

the following exception: the VZV IgG kit was modified to include a standard curve consisting of the World Health Organization Biological Standard #90/690 (National Institute for Biological Standards and Control, NIBSC, Hertfordshire, UK) diluted from 20 milli-international units per milliliter (mIU) to 0.625 mIU/ml.

CMV-specific T cell responses

Cryopreserved peripheral blood mononuclear cells (PBMCs) were thawed and resuspended in RPMI 1640 plus 10% human Ab serum with the addition of anti-CD28 and -CD49d monoclonal antibodies (mAbs) (1 μ g/ml; BD Biosciences, San Jose, CA). Samples with >12% nonviable cells were excluded (one sample). Cells were stimulated with a pool of 15 overlapping CMV pp65 peptides (2 μ g/ml of each peptide; NIH AIDS Reference and Reagent Program), staphylococcal enterotoxin B (SEB, 1 μ g/ml; Toxin Technologies), or medium control. Cells were incubated at a 45° slant for a total of 6 h at 37°C in a humidified 5% CO₂ atmosphere with the addition of 1 μ g/ml brefeldin A (BD Biosciences, San Jose, CA) after 2 h of stimulation. Stimulated PBMCs were washed with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and stained for viability 30 min at 4°C (Live/Dead Fixable Aqua Dead Cell Stain for 405 nm; Invitrogen, Life Technologies, Grand Island, NY). Cells were then washed and surface stained with fluorochrome-conjugated mAbs anti-CD4 (Cy5.5), anti-CD3 (Brilliant Violet 605), and anti-CD8 (Alexa 405, all antibodies purchased from BD Biosciences) for 30 min at 4°C. Cells were washed, fixed, permeabilized (Invitrogen), and intracellularly stained with anti-interferon (IFN)- γ (PE Cy7) and anti-TNF- α (Alexa 700) for 30 min at 4°C. Finally, cells were again washed and fixed with 1% formaldehyde.

Fluorescence minus one (FMO) controls were used in all staining. Cells were analyzed using an LSR-II flow cytometer (BD Immunocytometry Systems, San Jose, CA). Electronic compensation was performed with Ab capture beads (BD Biosciences) stained separately with individual mAbs used in the test samples. The data files were analyzed using Diva software (BD Biosciences) and FlowJo Software (Tree Star, Ashland, OR). Lymphocytes were gated by their forward and side scatter profile. CD3⁺ cells were selected, and expression of CD4 and CD8 was analyzed in a bivariate dot plot with CD8 to exclude CD4/CD8 double-positive T cells (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/aid).

Study design and data analysis

This study used a matched case-control design as previously described,²³ except that one case and the matched control were excluded from the current study because adequate specimens were not available. Data were collected and managed with Research Electronic Data Capture (REDCap) hosted at the University of Colorado.²⁷ Comparisons were considered significant if $p \leq 0.05$ and there was no correction for multiple comparisons. Demographics were summarized by low/high-function group using frequency with percent for categorical and mean with standard error (or geometric mean and geometric 95% confidence interval) for continuous variables; differences were tested with chi-square tests or two-

sample *t*-tests, respectively. Odds ratios (OR) and 95% confidence intervals (CI) are presented from conditional logistic regression for the primary analysis where conditional odds of low function was estimated for each measure, with adjustment for most recent CD4⁺ T cell count, age, and smoking status. Skewed measures were log transformed prior to analysis.

The monotonic relationships between immune responses to CMV and markers of inflammation, immune activation, or senescence were assessed with the Spearman correlation coefficient. We chose IL-6 for model inclusion among the inflammatory markers, consistent with known relationships between IL-6 and CMV.^{12,28} The outcome of low function was modeled using bivariable conditional logistic regression models with CMV IgG and one additional effect variable (smoking, IL-6, %CD38⁺HLA-DR⁺CD8⁺ T cells, VACS Index scores, age, CD4⁺ T cells, and CD4/CD8 ratio) for each model. Analyses were performed in SAS v.9.3 (Cary, NC).

Results

Study population

Among 30 low-function cases and 48 high-function age- and gender-matched controls,²³ 77% were male and 76% were white. The mean age was 53 years and 96% had an HIV-1 RNA below the limits of detection of 48 copies/ml. No subjects were taking corticosteroids, ganciclovir, or valganciclovir; one case and no controls had a history of CMV end-organ disease. The low- and high-function groups were similar by baseline demographic characteristics with the exception that low-function cases were more likely to be current tobacco users, have a lower CD4/CD8 ratio, and have a higher VACS Index Score (Table 1).

Humoral response to CMV and association with physical function

CMV IgG ranged from <10 to 8,830 EU/ml, including four participants (5%), all controls, with concentrations below the

manufacturer’s cutoff for positive results of 10 EU/ml. CMV IgG was significantly greater among the low-function than the high-function group (*p*=0.002; Fig. 1A), with a nearly 5-fold greater odds of low function (OR 4.8; 95% CI 1.4, 16.7) per log₁₀ increase in CMV IgG EU/ml (*p*=0.015). These findings were robust to adjustment for concomitant CD4⁺ count, tobacco use, and age (OR 4.0; 95% CI 1.2, 12.8; *p*=0.021) or to exclusion of subjects with CMV IgG <10 EU/ml (OR 4.8; 95% CI 1.3, 17.2; *p*=0.016). Similarly, the relationship between physical function and CMV IgG remained robust after adjusting for hepatitis C viremia (OR 5.1; 95% CI 1.4, 18.4; *p*=0.001).

A weak, negative correlation between CMV IgG and the concomitant CD4⁺ lymphocyte count (*r* = -0.24; *p*=0.04) and a trend toward a positive correlation with the VACS Index (*r*=0.21; *p*=0.06) were detected. No correlations between CMV IgG and age or CD4⁺ nadir were detected (Fig. 2A). Weak to moderate correlations that reached statistical significance were detected between CMV IgG and IL-6, TNF-α, and hs-CRP (*r* ≥ 0.23; *p* < 0.05). Correlations between CMV IgG and markers of T cell or monocyte activation were not significant, although a trend toward a positive correlation with the %CD8⁺CD38⁺HLA-DR⁺ was detected (*r*=0.20; *p*=0.07). CMV IgG correlated negatively with the CD4/CD8 ratio (*r* = -0.32; *p*=0.004) and positively with the %CD8⁺CD28⁻ T cells (*r*=0.27, *p*=0.02) and %CD4⁺CD28⁻ T cells (*r*=0.19; *p*=0.09).

Humoral response to other herpesviruses and association with physical function

Twelve participants had negative responses for HSV-1/2 antibody and five were equivocal. The mean HSV-1/2 antibody was not significantly different between the low-function and high-function groups (Fig. 1B; *p*=0.062). The odds of being low function per 1 log increase in HSV IgG did not reach statistical significance (OR 3.72; 95% CI 0.98, 14.16; *p*=0.054), and was further attenuated after adjustment for CD4⁺ count, age, and tobacco use (OR 3.09; 95% CI 0.79, 12.06; *p*=0.10). All participants were seropositive for VZV.

TABLE 1. BASELINE CHARACTERISTICS OF THE CASE-CONTROL POPULATION

Characteristic	Low-function cases N = 30	High-function controls N = 48	p value
Age (years) ^{a,b}	52.8 (0.8)	52.9 (0.7)	0.94
Female ^{b,c}	8 (27)	10 (21)	0.56
White ^c	22 (73)	37 (77)	0.71
Hispanic ^c	7 (23)	6 (13)	0.22
Current tobacco use ^c	15 (50)	10 (21)	0.009
Current CD4 count (cells/μl) ^d	474 (369, 609)	598 (531, 673)	0.096
Nadir CD4 count (cells/μl) ^d	55 (33, 93)	84 (52, 136)	0.23
Ratio CD4:CD8 T lymphocytes ^d	0.6 (0.5, 0.8)	1.0 (0.8, 1.1)	0.001
Detectable HIV-1 RNA ^c	1 (3)	2 (4)	0.85
Time since HIV diagnosis (years) ^{b,d}	11.6 (8.3, 16.3)	13.1 (10.6, 16.1)	0.55
Detectable plasma HCV RNA ^c	6 (20)	4 (9)	0.15
VACS Index Score ^b	23 (19, 28)	17 (15, 19)	0.007

^aMean (standard error).

^bCase-control matching criteria.

^cFrequency (percentage).

^dGeometric mean and 95% confidence interval.

VACS, Veterans Aging Cohort Study; HCV, hepatitis C virus.

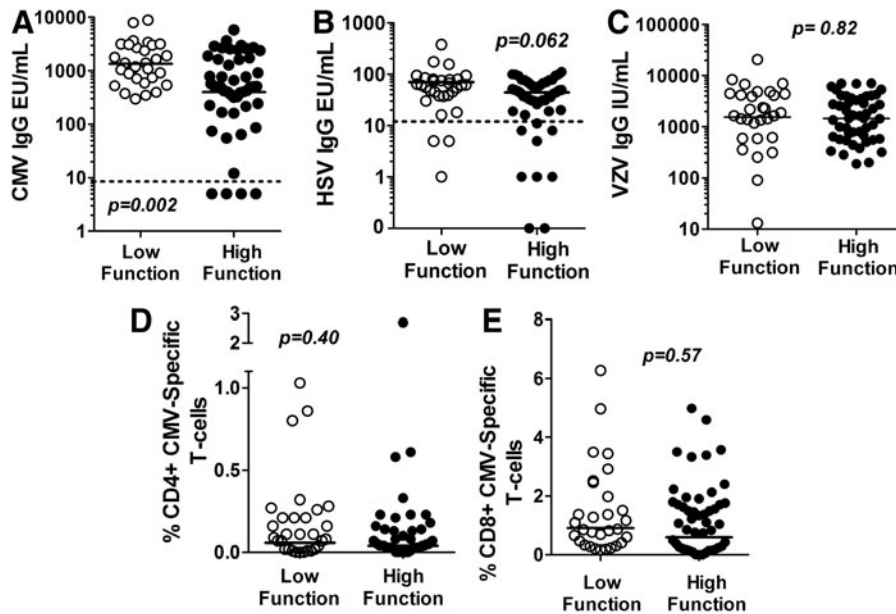


FIG. 1. Distribution of cytomegalovirus IgG (A), herpes simplex virus 1 and 2 IgG (B), varicella zoster virus IgG (C), % CMV-specific CD4⁺ T cells (D), and % CMV-specific CD8⁺ T cells (E) between low and high physical function groups. Group geometric means are indicated by the *solid line*. The assay limits of detection for the immunoglobulin assays are indicated by the *dotted line*, with the exception of varicella zoster virus where all participants were positive.

The mean VZV IgG was not significantly different in the low-function compared to the high-function group (Fig. 1C; $p=0.82$). Similarly, the odds of having low physical function did not change with a change in VZV IgG (OR 1.02; 95% CI 0.43, 2.44; $p=0.97$).

Cell-mediated response to CMV and association with physical function

The percentages of CMV-specific IFN- γ -producing CD4⁺ or CD8⁺ T cells were not significantly different between the low-function and high-function groups (Fig. 1D and E). Similarly, low function was not associated with the % of CD4⁺ or CD8⁺ CMV-specific T cells producing IFN- γ , TNF- α , or both (Supplementary Table S1).

CMV-specific CD4⁺ or CD8⁺ T cells did not correlate with age, CD4⁺ nadir, or current CD4⁺ count (Fig. 2B and C). CMV-specific CD4⁺ and CD8⁺ T cells were not correlated with markers of inflammation or lymphocyte or monocyte activation (Fig. 2). The CMV-specific CD4⁺ T cells correlated moderately with the %CD4⁺CD28⁻ T cells ($r=0.37$; $p<0.001$) and weakly with %CD4⁺CD28⁻ T cells ($r=0.22$; $p=0.058$). No significant associations were seen between CMV cell-mediated immune responses and other markers of immune senescence (Fig. 2). CMV-specific CD8⁺ T cells weakly correlated with CMV IgG ($r=0.24$; 95% CI 0.02, 0.44; $p=0.033$), but CMV-specific CD4⁺ T cells did not ($r=0.13$; 95% CI -0.10, 0.35; $p=0.26$).

Effect of clinical characteristics, inflammation, and immune activation on the relationship between CMV and low physical function

Results of univariable and bivariable conditional logistic regression models are shown in Fig. 3A and B. The relationship between CMV IgG and low physical function remained robust to inclusion of smoking, %CD8⁺CD38⁺ HLA-DR⁺, age, and current CD4⁺ T cells, but was attenuated

and no longer significant when IL-6, VACS index, or the CD4/CD8 ratio was included in the model.

Discussion

Among HIV-infected middle-aged adults on effective ART, we found that higher CMV humoral immune responses were associated with greater odds of functional impairment. The absence of an association between physical function and VZV or HSV-1/2 antibodies provided evidence that the relationship was specific to CMV, and not a heightened generalized humoral response to and/or a higher rate of reactivations of herpesviruses. In contrast, the odds of physical function impairment were not significantly associated with greater CMV cell-mediated responses. In bivariable regression models, however, the association of CMV IgG with physical function did not remain significant when other factors including inflammation, the VACS index, or CD4/CD8 ratio were included, suggesting that one or more of these markers might mediate the effect of CMV on physical function.

The association between CMV humoral immune responses and impaired physical function has been previously described in HIV-uninfected cohorts: among 724 women (87% CMV-seropositive) between ages 70 and 79 years in the Women's Health and Aging Studies I and II, CMV seropositivity was independently associated with prevalent frailty after adjusting for additional comorbid and demographic characteristics.² Of the 635 women from this cohort with longitudinal data, those in the highest quartile of CMV IgG had a greater incidence of frailty compared to CMV-seronegative participants (hazard ratio 3.46).¹² Similarly, among 295 older (mean age 78 years), community dwelling men and women, both CMV seropositivity (77%) and higher CMV IgG titers were associated with increased relative risk of frailty.¹¹ In contrast, in a cohort of 567 men and women aged 80 years and older (74% CMV seropositive), CMV seropositivity was associated with a lower prevalence of frailty in adjusted analyses, and higher CMV IgG titers were not associated with frailty or

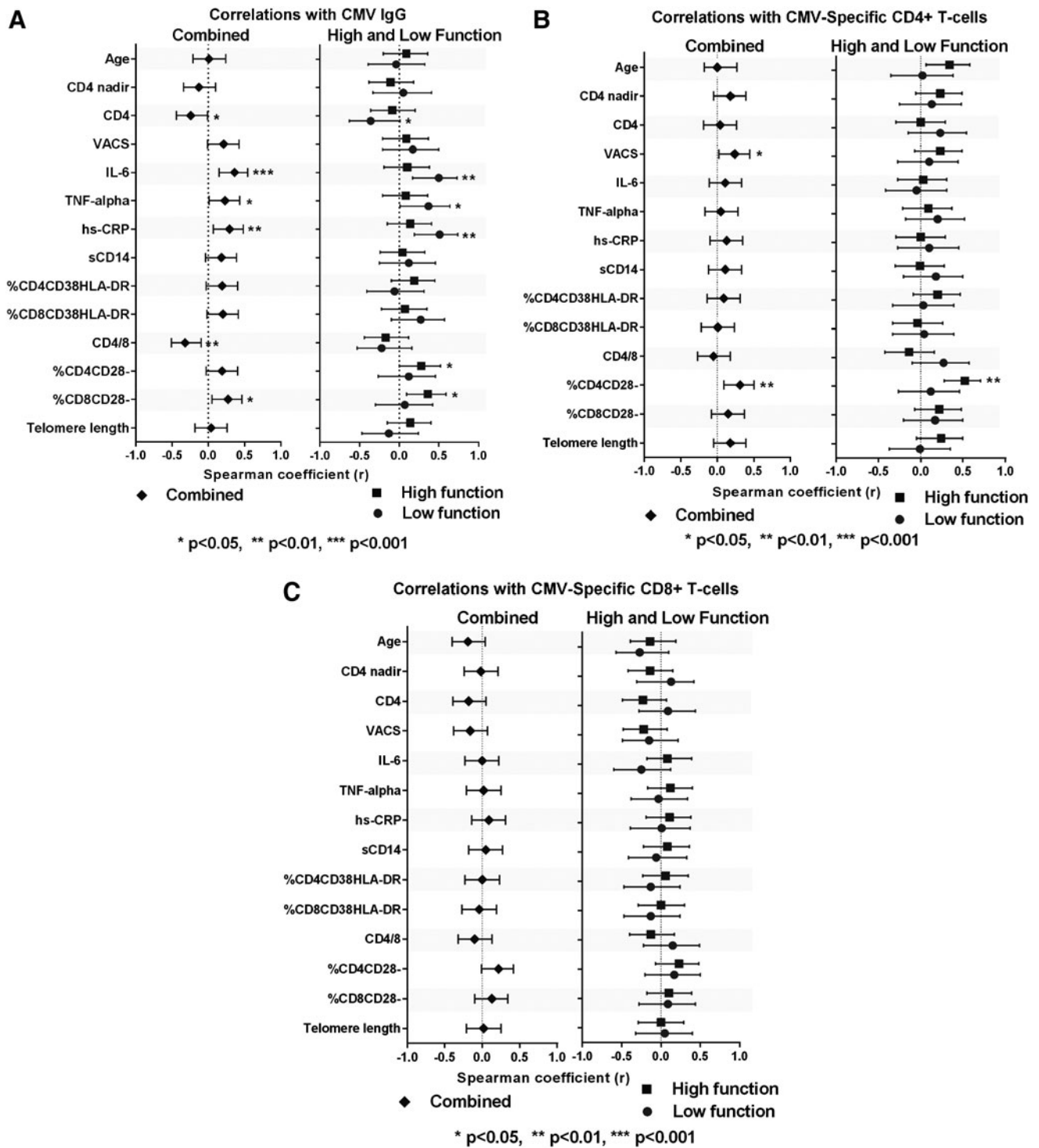


FIG. 2. Correlations between cytomegalovirus (CMV) humoral or cell-mediated immune response and baseline characteristics, inflammation, activation, and senescence markers. (A–C) Correlations including all subjects (*diamond*) are shown in the *left panels*; correlations separated by high (*square*) and low (*circle*) physical function are shown in the *right panels*.

physical function impairment, after adjustments for age, sex, and education.²⁹

These findings suggest that the relationship between CMV seropositivity and frailty or physical function impairment may differ by gender or by age, with a survival effect among

the oldest populations. To the best of our knowledge, the results we have presented herein are the first description of the association between impaired physical function and CMV in either a middle-aged or an HIV-infected cohort, suggesting that the relationship between CMV and impaired physical

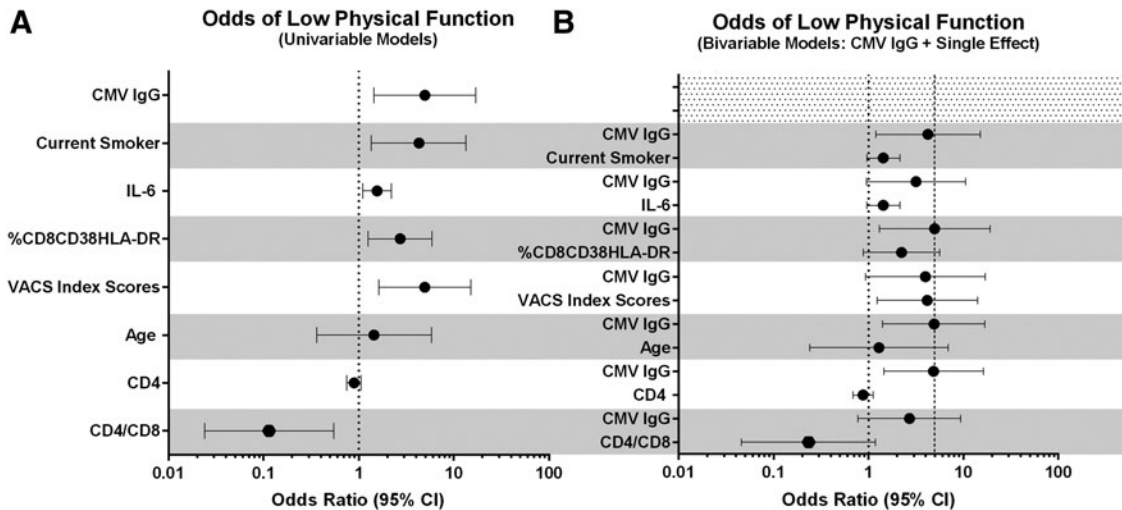


FIG. 3. (A) The odds of having low physical function by each variable listed on the y-axis (univariable models). The *dot* indicates the increase in the odds of low function per 1 unit increase in CMV IgG and the CD4/CD8 ratio per 0.2 pg/ml increase in interleukin (IL)-6, per 10% increase in %CD8CD38HLA-DR, per 10 point increase in the VACS Index score, per 5 year increase in age, and per 100 cells/ μ l increase in CD4⁺ count. The *dotted line* marks an odds ratio of 1. (B) The odds of low physical function per 1 unit increase in CMV IgG, with each bivariable model including CMV and the single corresponding variable. The *dotted line* marks an odds ratio of 1 and the *dashed line* marks the unadjusted odds of low function per increase in CMV IgG, for visual comparison.

function may be apparent many years earlier among HIV-infected adults with a high prevalence of CMV seropositivity.

CMV humoral responses were associated with heightened markers of inflammation suggesting a role of active CMV infection in the ongoing inflammation of HIV-infected adults despite long-term effective ART and virologic suppression. Although not statistically significant, we detected a similar trend toward an association between CMV IgG and markers of T cell activation (%CD8⁺CD38⁺HLA-DR⁺). A recently published transcriptome analysis exploring pathways associated with high CMV IgG titers found that multiple metabolic (androgens, cholesterol, inositol processing) and immune response-associated pathways were increased, thus providing potential direct or indirect mechanistic links between CMV IgG and physical function.³⁰ The association between CMV IgG and the “immune risk profile” was apparent even in our middle-aged population, with a strong association between lower CD4/CD8 ratio and higher CMV IgG.

The results of our bivariable model suggest a complex relationship between CMV IgG, inflammation or T cell activation, and physical function. Hunt *et al.* previously demonstrated that administration of the anti-CMV therapy vanganciclovir led to a significant reduction in T cell activation but not IL-6 or other markers of inflammation, supporting a key role of CMV as a driver of immune activation, but not inflammation, in HIV-1 infection.²² We detected only a weak correlation between CMV IgG and immune activation that suggested CMV contributed but was not the only driver of immune activation in our population. Similarly, the strong association between CMV IgG and physical function impairment was attenuated when adjusted for IL-6 but not for T cell activation, suggesting that IL-6, but not %CD8⁺CD38⁺HLA-DR⁺, partially explained the association between CMV IgG and impaired physical function.³¹

We found discordance between the cell-mediated and humoral CMV responses in persons with HIV-1. This discordance was also previously demonstrated in healthy blood donors.³² The most likely explanation is that while CMV antibodies increase with each reactivation, the relationship between cell-mediated responses is more complex: strong cell-mediated immune responses limit CMV reactivations, but when the virus escapes immune control and reactivates it also boosts cell-mediated responses. Several lines of evidence support this model: (1) CMV antibody titers are higher in older people, suggesting multiple and/or prolonged reactivations³³ and (2) in individuals with AIDS, robust CMV-specific cell-mediated responses are associated with a lower risk of developing CMV retinitis or end-organ disease.^{34,35}

It should also be noted that a small study of HIV-infected persons found that detectable CMV DNA was negatively correlated with CMV IgG¹⁷ and among 12 HIV-uninfected CMV-seropositive women from the Women’s Health and Aging Study, CMV IgG titers were minimally different between two time points 12 years apart, despite detectable CMV DNA at both time points in seven participants.³⁶ Furthermore, CMV cell-mediated immune responses to the pp65 peptide pool may underestimate total T cell responses against CMV.³⁷ Thus, the reason for discordance between cell-mediated and humoral responses in our HIV-infected participants cannot be entirely explained and further investigation is needed.

Our study has several limitations, including the cross-sectional design. Because of the lack of longitudinal samples and limitations of CMV detection at a single time point, we chose not to include a measure of CMV DNA. pp65 is an immunodominant CMV antigen and the majority of CMV-infected individuals would be expected to mount a CD4 and CD8 T cell response; however, other epitopes not included in our studies may have elicited a greater immune response

among some individuals.^{38–40} The age range of our study population is relatively narrow, and findings at higher and lower extremes of age may contrast the findings we have presented herein. The small sample size may have also limited (1) the ability to find significant associations of CMV cell-mediated responses with activation and/or inflammation; (2) the number of variables that could be considered in the regression models; and (3) the ability to detect associations within low- or high-function groups. Lastly, no corrections for multiple comparisons were done and associations should be considered exploratory.

Associations between CMV and complications of aging have been demonstrated in multiple cohorts of both HIV-infected and -uninfected persons.^{15,36,41–44} Our study provides the first demonstration of a strong relationship between CMV IgG and impaired physical function in HIV infection. Whether CMV alone plays a causative role in the deterioration of physical function or is a marker of another variable that is associated with decreased physical function could not be determined in this study. In the absence of an adequate animal model for human CMV infection, additional studies in human subjects are needed to further clarify the significance of these associations. Longitudinal studies including close monitoring of CMV reactivation and perhaps therapeutic interventions that suppress CMV reactivation may clarify the relationships between immune activation, inflammation, and CMV with physical function impairment in HIV-infected persons.

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Kristine M. Erlandson
 University of Colorado-Anschutz Medical Campus
 12700 East 19th Avenue
 Mail Stop B168
 Aurora, Colorado 80045

E-mail: kristine.erlandson@ucdenver.edu