Relationship between cytomegalovirus (CMV) IgG serology, detectable CMV DNA in peripheral monocytes, and CMV pp65_{495–503}-specific CD8⁺ T cells in older adults

Sean X. Leng · Tao Qu · Richard D. Semba · Huifen Li · Xu Yao · Tricia Nilles ·
Xi Yang · Bhavish Manwani · Jeremy D. Walston · Luigi Ferrucci ·
Linda P. Fried · Joseph B. Margolick · Jay H. Bream

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Abstract In immunocompetent individuals, cytomegalovirus (CMV) is thought to persist in a latent state in monocytes and myeloid progenitor cells, establishing a lifelong infection. In CMV-seropositive older adults, aging has been associated with both expansion of CMV pp65_{495–503}-specific CD8⁺ T cell clones and shrinkage of the T cell repertoire that characterize T

cell immunosenescence. In fact it has been suggested that chronic CMV infection is a driving force in age-related T cell immunosenescence. In older adults, chronic CMV infection is conventionally diagnosed by positive IgG serology which does not distinguish between past and persistent infections. To better define the relationship between chronic CMV infection and expansion of CMV pp65_{495–503}-specific CD8⁺ T cells, we directly assessed CMV viral DNA in monocyte-enriched peripheral blood mononuclear cells in 16 HLA-A2-positive elderly volunteers (mean age=83 years). While all participants had positive CMV IgG serology by enzyme-linked immunosorbent assays, only nine (56%) had detectable CMV DNA by nested polymerase chain reaction. These nine individuals had significantly higher percentages of CMV pp65_{495–503} tetramer-positive CD8⁺ T cells (median=1.3%) than those without detectable CMV DNA (median=0.1%; $p<0.001$). Absolute CMV IgG antibody titers did not differ between these two groups (median=54.6 vs 44.2 EU/ml, respectively, $p=0.4$). CMV IgM titers were negative for all 16 participants, suggesting that recent primary CMV infection was unlikely. These results demonstrate a strong association between the presence of CMV DNA in peripheral monocytes and the expansion of CD8⁺ T cells specific for the CMV immunodominant epitope pp65_{495–503}. Although the sample size in this study is relatively small, these findings provide initial evidence suggesting the heterogeneity of CMV IgG-seropositive older adult population and CMV viral DNA detection

S. X. Leng (✉) · T. Qu · H. Li · X. Yao · X. Yang ·
B. Manwani · J. D. Walston
Division of Geriatric Medicine and Gerontology,
Department of Medicine,
Johns Hopkins University School of Medicine,
5505 Hopkins Bayview Circle,
Baltimore, MD 21224, USA
e-mail: sleng1@jhmi.edu

R. D. Semba
Department of Ophthalmology,
Johns Hopkins University School of Medicine,
Baltimore, MD, USA

T. Nilles · J. B. Margolick · J. H. Bream (✉)
Department of Molecular Microbiology and Immunology,
Johns Hopkins Bloomberg School of Public Health,
Baltimore, MD 21205, USA
e-mail: jbrema@jhsph.edu

L. Ferrucci
Clinical Research Branch, Longitudinal Studies Section,
National Institute on Aging,
Baltimore, MD, USA

L. P. Fried
Mailman School of Public Health, Columbia University,
New York, NY, USA

in peripheral monocytes as an informative tool to better understand the relationship between chronic CMV infection and T cell immunosenescence.

Keywords Monocytic CMV DNA · CMV pp65_{495–503}-specific CD8⁺ T cells · CMV IgG serology · Older adults

Introduction

In immunocompetent individuals, cytomegalovirus (CMV) is thought to persist in a latent state with its DNA genome harbored primarily in monocytes and myeloid progenitor cells, establishing a chronic infection with intermittent reactivations (Sissons et al. 2002; Limaye et al. 2008; Osawa and Singh 2009; Bolovan-Fritts et al. 1999; Taylor-Wiedeman et al. 1991, 1993; Hahn et al. 1998; Sinclair 2008). The predominant adaptive immune response to CMV infection is mediated by T lymphocytes. A recent study in CMV IgG-seropositive young adults found that up to 5% of CD4⁺ and CD8⁺ T cells were CMV specific, suggesting that a significant portion of the T cell repertoire is devoted to this virus (Sylwester et al. 2005).

In older adults, chronic CMV infection is typically diagnosed by positive IgG serology, and CMV seroprevalence is estimated to be as high as 70–99% (Staras et al. 2006; Dowd et al. 2009). Khan and colleagues have identified significant age-related clonal expansion of CD8⁺ T cells specific for the CMV proteins pp65 and IE-1 in CMV IgG-seropositive older individuals (Khan et al. 2002, 2004). This observation has been extended to the CD4⁺ T cell compartment and linked to shrinking of the T cell repertoire (Vescovini et al. 2007; Pourghesari et al. 2007; Hadrup et al. 2006; Vescovini et al. 2010). These and other studies (Olsson et al. 2000; Wikby et al. 2002; Ouyang et al. 2003; Pawelec et al. 2005; Koch et al. 2007) suggest that chronic CMV infection may contribute to age-related T cell immunosenescence. In addition, the combination of CMV IgG seropositivity, poor T cell proliferation, and inverted CD4/CD8 ratio, termed the “immune risk phenotype”, was associated with greater numbers of CMV pp65_{495–503}-specific T cells and predicted mortality in those 85 and above (Olsson et al. 2000; Pawelec et al. 2005; Wikby et al. 2002; Nilsson et al. 2003). Nonetheless, because a positive

CMV IgG titer merely indicates prior exposure to the virus, it is not known if seropositive older individuals continue to harbor CMV and, if so, whether the presence of CMV is associated with the expansion of CMV pp65_{495–503}-specific CD8⁺ T cells.

To gain more insight into this question, we evaluated the presence of CMV DNA in peripheral monocytes, one of the known host cell types for latent CMV infection, and its relationship with the frequency of CMV pp65_{495–503}-specific CD8⁺ T cells in 16 HLA-A2-positive older individuals with positive CMV IgG serology.

Materials and methods

Human subjects

Study participants were selected from a previous study assessing responses to influenza vaccination among community-dwelling adults over 70 years old who were recruited from outpatient clinics, senior centers, and residential retirement communities in Baltimore, Maryland. Exclusion criteria applied in that study included chronic inflammatory conditions (e.g., rheumatoid arthritis and inflammatory bowel disease), active malignancy, acute illness such as bacterial or viral infections or acute exacerbation of chronic conditions, or use of immune modulating agents (oral steroids, methotrexate, etc.) or chemotherapy. These exclusion criteria were intended to minimize the impact of potential immune activation or suppression from the listed acute or chronic systemic conditions or immune modulating agents. A detailed medical history and brief physical examination were performed by a physician investigator to ensure enrollees' eligibility and ascertain clinical diagnoses and medication use. The Johns Hopkins Institutional Review Board approved the study protocol, and written informed consent was obtained from all participants.

Preparation of sera and monocyte-enriched peripheral blood mononuclear cells and determination of HLA-A2 status

Peripheral venous blood samples were collected prior to vaccination. Sera were obtained after a 20-min centrifugation in serum separation tubes (Becton Dickinson, Mountain View, CA, USA). They were aliquoted and stored at −80°C until analysis. Periph-

eral blood mononuclear cells (PBMCs) were isolated from peripheral blood samples by centrifugation over Ficoll–Hypaque density gradient (specific density, 1.077 g/ml) for 10 min at 600×g at room temperature, washed three times with phosphate-buffered saline containing 2 mM EDTA and 0.5% bovine albumin (pH 7.4), and stored in liquid nitrogen. Monocytes were enriched from freshly thawed PBMC samples via 2-h incubation in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (Gibco, Gaithersburg, MD, USA) at 37°C in a humidified 5% CO₂ incubator, after which non-adherent cells were removed by repeated rinsing with serum-free RPMI 1640. The number of CD14⁺ monocytes were assessed by flow cytometry and standardized as previously described (Rodriguez et al. 1999; Qu et al. 2009a, b). DNA was extracted from monocyte-enriched PBMCs using a Qiagen kit (Qiagen, Valencia, CA, USA) and quantified using standard laboratory protocol. HLA-A2 status (positive/negative) was determined by polymerase chain reaction (PCR) as described (Liang et al. 2006).

CMV viral DNA detection by nested polymerase chain reaction

Nested PCR with primers targeted to the CMV UL123 gene (first set: forward 5'-CAATACACTT CATCTCCTCGAAAGG-3' and reverse 5'-ATG GAGTCCTCTGCCAAGAGAAAGATGGAC-3'; second set: forward 5'-TCTGCCAGGA CATCTTTCTC-3' and reverse 5'-GTGACCAAGGC CACGACGTT-3') as previously reported (Roback et al. 2001; Slobedman and Mocarski 1999) was performed using Tapbead hot start polymerase (Promega, Madison, WI, USA) with 1.5 mM MgCl₂. Sample DNA (50 ng) extracted from the monocyte-enriched PBMCs described above was added to the first-round PCR from which 2 µl of the product mix was added to the second-round PCR with a thermal cycling program of enzyme activation for 5 min at 95°C and 40 cycles of 1 min denaturation at 94°C, 1 min annealing at 45°C, and 2 min extension at 72°C for both PCR reactions. A 167-bp CMV viral DNA fragment was visualized by gel electrophoresis and confirmed by DNA sequencing. The quality of input sample DNA was confirmed by amplification of a cellular housekeeping gene glyceraldehydes 3 phosphate dehydrogenase (GAPDH).

All negative results for CMV DNA detection were confirmed by increasing the amount of input sample DNA to 500 ng. For participants with positive results in the CMV gene UL123 region, another set of nest PCR with primers targeted to the CMV gene UL93 (first set: forward 5'-GGCAGCTATCGTGACTGGGA-3' and reverse 5'-GATCCGACCCATTGTCTAAG-3'; second set: forward 5'-TTAGCGCGTGACCTGTTACG-3' and reverse 5'-TCTAAGTTATTACGCAGTCCG-3') were performed under the same experimental conditions for the detection of a 113-bp CMV DNA fragment.

Measurement of serum anti-CMV IgG and IgM antibody titers

Serum anti-CMV IgG and IgM titers were determined by commercially available enzyme-linked immunosorbent assays (ELISA; United Biotech Inc., Mountain View, CA, USA) with an interassay coefficient of variance of 5.2% and 5.7%, respectively. A titer of 15 ELISA units (EU)/ml of IgG or greater was pre-determined by the manufacturer as CMV IgG seropositive and that of 100 EU/ml of IgM or greater as CMV IgM seropositive.

Determination of frequency of CMV pp65_{495–503}-specific CD8⁺ T cells by tetramer analysis

CMV pp65_{495–503}-specific CD8⁺ T cells were identified using an HLA-A2 class I tetramer loaded with CMV pp65_{495–503} (NLVPMVATV) peptide (Beckman Coulter, Inc. Miami, FL, USA). This CMV pp65 tetramer was conjugated to allophycocyanin (APC) and was used with conjugated antibodies (Becton Dickinson) to CD3 (Am Cyan), CD4 (Pacific Blue), and CD8 (APC-Cy7) and analyzed on an LSR2 flow cytometer (Becton Dickinson). The percentage of nonspecific tetramer binding to CD4⁺ T cells was subtracted from the percentage of CMV pp65_{495–503}-specific tetramer binding.

Statistical analysis

Data on CMV DNA detection were presented as a categorical variable (positive vs negative). Serum anti-CMV IgG antibody titers were expressed as a continuous (absolute titers) or categorical variable (seropositive vs seronegative) based on the criteria pre-determined by

the manufacturer. Results from tetramer analysis were expressed as a percentage of CMV pp65_{495–503}-specific CD8⁺ T cells in total CD8⁺ T cells. The Kruskal–Wallis test was employed to determine the statistical significance of differences between participants with and without detectable CMV DNA.

Results

Of 71 subjects in the vaccination study, 23 were HLA-A2 positive. Sixteen of the HLA-A2-positive participants had PBMCs and sera available for testing and were included in the present study. Table 1 summarizes major demographic and clinical characteristics of the study participants. Their mean age was 83.1 years (range 72–90). Most participants were Caucasian females who graduated from high school or beyond. Typical for a geriatric population, these individuals had multiple chronic conditions, including hypertension, other cardiovascular diseases (coronary artery disease, congestive heart failure, atrial fibrillation, or stroke), and diabetes mellitus, and were taking multiple medications. All participants had positive anti-CMV IgG titers and negative anti-CMV IgM serology. There were no significant differences in above demographic and clinical characteristics between the study groups (Table 1).

CMV DNA detection in peripheral monocytes

Of the 16 subjects studied, only nine (56%) had detectable CMV DNA in their monocyte-enriched PBMCs, even though all had positive anti-CMV IgG serology. In all detectable cases, a 167-bp DNA sequence in the CMV UL123 gene was amplified by the nested PCR with an input of 50 ng of sample DNA (Fig. 1a). In addition, these individuals had a 113-bp DNA sequence detected in a separate CMV gene region, the UL93 gene (data not shown). The undetectability of CMV DNA was confirmed in the remaining seven cases by increasing the input to 500 ng of sample DNA. All samples yielded good quality of genomic DNA as confirmed by amplification of the cellular gene *GAPDH* (Fig. 1a). As shown in Fig. 1b and Table 1, absolute CMV IgG antibody titers were similar in CMV DNA-positive and DNA-negative participants (medians=54.6 vs 44.2 EU/ml, respectively, $p=0.4$). CMV IgM titers were negative for all 16 participants, indicating that recent primary CMV infection was unlikely.

Frequency of CMV pp65_{495–503}-specific CD8⁺ T cells and its association with positive CMV DNA detection

All participants had measurable percentages of CD8⁺ T cells that bound CMV pp65 tetramer; Fig. 2a shows a representative participant with easily detectable

Table 1 Major demographic, clinical, and study variables of the study participants

Study variables	All participants ($N=16$)	CMV DNA (+) ($N=9$)	CMV DNA (–) ($N=7$)	p value
Age (years): mean (SD)	83.1 (5.5)	82.1 (5.3)	84.3 (6.0)	NS
Race (white), %	94	100	86	NS
Sex (female), %	81	78	86	NS
Education (high school or above), %	93.9	93.3	94.7	NS
Body mass index (kg/m ²), mean (SD)	23.2 (4.6)	22.4 (5.1)	23.9 (6.1)	NS
Total # of diagnoses, mean (SD)	4.2 (1.6)	4.4 (1.6)	4.1 (1.8)	NS
Hypertension, %	68.8	66.7	71.4	NS
Other cardiovascular diseases, %	43.8	44.4	43.0	NS
Diabetes mellitus, %	12.5	11.1	14.3	NS
Total # of medications, mean (SD)	4.4 (1.7)	4.7 (1.9)	4.2 (1.5)	NS
CMV IgG seropositivity (%)	100	100	100	NS
Serum CMV IgG titers (EU/ml), median (range)	49.6 (16.7–61.3)	54.6 (31.9–61.2)	44.2 (16.7–61.3)	NS
CMV IgM seropositivity (%)	0	0	0	NS
CMV-specific CD8 ⁺ T cells (%), median (range)	0.39 (0.03–22.01)	1.3 (0.28–22.01)	0.09 (0.03–0.15)	<0.001

Fig. 1 a CMV viral DNA detection in monocyte-enriched PBMCs from all 16 participants by nested PCR targeted to the CMV UL123 gene. *Upper panel:* lane 1 positive control, 167-bp PCR product from purified genomic DNA of human CMV strain AD169 CMV_{AD169} (Advanced Biotech Inc., Paterson, NJ, USA); lanes 2–3 negative controls with no input sample DNA (lane 2, primers for both rounds of PCR, lane 3, primer for first round only); lanes 4–19 participant samples. Nine

participants had detectable CMV viral DNA (top CMV DNA band, lanes 4–12), and seven participants had no detectable CMV DNA (lanes 13–19); *lower panel:* PCR amplification of GAPDH. **b** Distribution of serum anti-CMV IgG titers as determined by ELISA (UBI, Mountain View, CA, USA); with a titer of ≥ 15 EU/ml considered as seropositive, all participants were seropositive. Horizontal bars indicate median values

CMV pp65_{495–503}-specific CD8⁺ T cells. Surprisingly, the percentages of CD8⁺ T cells that bound to this tetramer differed markedly between the CMV DNA-positive and DNA-negative groups (Fig. 2b). Specifically, this percentage was low (median=0.09%, range 0.03–0.15%) in subjects with no detectable CMV DNA but was over 10-fold higher (median=1.3%, range 0.28–22.01%) in those with detectable CMV DNA ($p<0.001$). In fact, the two groups did not overlap at all (Table 1; Fig. 2b). Absolute CD8⁺ T cell counts were similar in both groups (medians=273 vs 201 cells/mm³, respectively, $p=0.5$)

Discussion

To the best of our knowledge, this study is the first to evaluate the presence of CMV DNA in peripheral monocytes in an elderly population. Not all CMV IgG-seropositive individuals in this study had detectable CMV DNA. However, those who did have

detectable CMV DNA had significantly more CMV pp65_{495–503}-specific CD8⁺ T cells than those who did not, regardless of their CMV IgG antibody titers. These results are consistent with a recent report which failed to identify an association between higher CMV IgG titers and CMV pp65_{495–503}-specific CD8⁺ T cell responses in very old individuals (Vescovini et al. 2010). In the same study, however, Vescovini et al. observed a correlation between higher CMV IgG titers and CMV pp65_{495–503}-specific CD4⁺ T cell responses. We did not evaluate CMV pp65_{495–503}-specific CD4⁺ T cell responses in this study, but it will be important to determine if monocytic CMV DNA detection will help to clarify the relationship between CMV IgG seropositivity and T cell responsiveness in the elderly.

Many quantitative real-time PCR assays are available for the assessment of CMV viral load in immunocompromised individuals, primarily HIV-infected patients with AIDS. However, nested PCR, which is a highly sensitive and specific assay, appears

Fig. 2 a Flow cytometric dot plots from CMV pp65_{495–504} tetramer analysis of a representative participant with detectable CMV viral DNA. *Left panel:* CD4⁺ and CD8⁺ T cells gated on CD3⁺ cells; *right panel:* tetramer binding to CD8^{bright+} (blue or magenta) T cells, but not CD8^{dim} (green) or CD8⁻ (yellow) T cells. **b** Distribution of percentages of CD8⁺ T cells that were CMV pp65_{495–503} specific in participants with detectable (+) or no detectable (-) CMV DNA in monocyte-enriched PBMCs. Horizontal bars indicate median values

to be more sensitive in cell-associated CMV DNA detection and has been successfully applied in CMV DNA detection in the PBMCs in large numbers of apparently immunocompetent blood donors (Zhang et al. 2010; Roback et al. 2001). In addition, CMV DNA has not been detected by quantitative real-time PCR in the serum or plasma in more than 70 community-dwelling older persons from the Baltimore area (George Wang, personal communication), suggesting that clinical CMV infection with detectable CMV viremia in our study population is unlikely. While all participants were CMV IgG seropositive, none had positive CMV IgM serology, making the possibility of recent primary CMV infection very unlikely. Nonetheless, we acknowledge the semi-quantitative/categorical nature of our CMV DNA detection data as a limitation of the nested PCR assay employed in the present study.

Human CMV is a beta herpesvirus with a DNA genome of approximately 230 kb. It has been shown that CMV persists in peripheral monocytes in a

circular conformation episomally (Bolovan-Fritts et al. 1999). Amplification of DNA sequences from both the UL123 and UL93 genes, which are 37.8 kb apart in the CMV genome, makes the possibility of detection of a random CMV DNA fragment in this study highly unlikely. Whether the presence of CMV viral DNA in monocytes is a marker of CMV reactivation requires further investigation. In addition, Stowe et al. reported detection of CMV DNA in the urine of older adults (Stowe et al. 2007). Here we focused on CMV DNA detection in peripheral monocytes, since this is a well-established cell reservoir for CMV infection. However, we cannot exclude the possibility that CMV DNA may be present in other tissues (kidney, salivary gland, etc.).

While pp65_{495–503} is a dominant CMV T cell epitope and is widely used to assess anti-CMV T cell responsiveness (Wills et al. 1996; Heijnen et al. 2004; Brooimans et al. 2008; Pita-Lopez et al. 2009), additional CMV-specific epitopes within the CD8⁺ T cell pool were not evaluated in this study. In addition,

we acknowledge the need to expand our analysis to include larger cohorts of older adults. Likewise, it will be important to extend these findings to include functional studies of CMV-specific T cell responses in both CD4⁺ and CD8⁺ T cells and in other HLA haplotype groups to determine the relationship between CMV DNA detection and T cell responsiveness. Despite these limitations, our findings suggest that the CMV IgG-seropositive elderly population is heterogeneous at least with respect to CD8⁺ T cell response to a CMV immunodominant epitope pp65_{495–503}. These data also suggest that detection of CMV DNA in peripheral monocytes could shed light on the relationship between chronic CMV infection and T cell immunosenescence.

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