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Rosanna Vescovini,* Claudia Biasini,* Anna Rita Telera,* Manuela Basaglia,* Adriano Stella,* Francesca Magalini,* Laura Bucci,*†,‡ Daniela Monti,‡ Tiziana Lazzarotto,* Paola Dal Monte,* Mario Pedrazzoni,* Maria Cristina Medici,‖ Carlo Chezzi,‖ Claudio Franceschi,‡,*,† Francesco F. Fagnoni,** and Paolo Sansoni*

Human aging is characterized by expanded and altered adaptive immune responses to human CMV (HCMV). It is unclear whether this expansion has its origins in age-related homeostatic disturbances or viral reactivation, whether anti-CMV immune surveillance may still be effective, and what are the consequences of this expanded immune response for health and longevity. We conducted an observational cross-sectional study in groups of HCMV-seropositive subjects aged ≥65 y of variable health status to compare the intensity of Ab responses against HCMV with those against EBV and with CD4+ and CD8+ T cell proinflammatory effector responses directed to HCMV-derived pp65 and immediate-early protein 1 synthetic peptides. Ab responses to HCMV, but not to EBV, and anti-HCMV CD4+, but not CD8+, T cell responses were more intense in elderly subjects aged ≥85 y in poor health and were inversely correlated with markers of functional activity and cognitive function. Therefore, humoral and CD4+ T cell anti-HCMV responses were specifically intensified in advanced aging associated with comorbidity and cognitive and functional impairments. Such a distinctive pattern of adaptive immunity indicates that immune responses targeting the extracellular phase of HCMV are increased in these elderly subjects and could represent an indirect effect of localized and undetectable HCMV reactivation. This study demonstrates that the oldest subjects in poor health with physical and mental impairment express intense functional immune responses to extracellular HCMV and suggests that they may be at risk for direct pathogenic effects by HCMV reactivation as well as indirect pathogenic effects linked to proinflammatory anti-HCMV effector responses. The Journal of Immunology, 2010, 184: 3242–3249.

The β-herpesvirus human CMV (HCMV) induces a widespread infection whose effects depend on the efficacy of immune surveillance (1). In immunocompetent subjects, HCMV induces a chronic subclinical infection, whereas in the absence of immune surveillance, HCMV replicates dynamically (2) and induces opportunistic effects, such as those occurring in congenitally infected neonates and late-stage AIDS (3, 4). Between these extremes there are other less well-understood possibilities where immune surveillance may be unable to prevent viral reactivation, but it still may be competent in inhibiting systemic uncontrolled infection. Indeed, recent reports showed that critically ill immunocompetent patients and patients with septic shock may undergo systemic, but self-limiting, reactivation of HCMV (5, 6). Furthermore, immunosuppressed patients transplanted with solid organs are susceptible to a statistical increase in mortality, transplant rejection, accelerated vascular diseases, and injury from other pathogens (7) that can be prevented by specific anti-HCMV therapy (8, 9). The mechanisms responsible for these indirect effects observed in the absence of HCMV detection by conventional histopathology do not seem to be dependent on systemic viral reactivation. The conditions associated with altered surveillance against HCMV, and characterized by self-limiting replication or indirect effects without detectable systemic reactivation, have been underestimated and require a better definition of the risk for direct or indirect pathology by HCMV.

Age is a significant cofactor for the risk for HCMV infection among different age groups (10), and HCMV seroprevalence increases with age, ranging from 40–50% in 18–24-y-olds to >90% in 75–80-y-old subjects (11, 12). An increasingly appreciated aspect of lifelong HCMV seropositivity is the amount of T cell memory devoted to its control in adulthood (13) and its further expansion that occurs in healthy aging (14–16). The age-related expansion of T cell reactivity to HCMV could have at least two distinct origins. The first could depend on episodes of viral reactivation disrupting the lifelong equilibrium with the host’s immune system and, thus, stimulating specific T cell responses (17). However, the evidence for reactivation occurring during aging is
very limited (18, 19). The second origin could depend on age-related homeostatic disturbances, as have been convincingly verified in experimental models of latent herpesvirus infections (20, 21). Irrespective of its possible origin(s), the age-related increase in anti-HCMV effector/memory T cells exhibits opposing functional aspects. Dysfunctional T cells emerging along with clonal restriction are considered leading contributors of immunosenescence in the healthy elderly (22–24) and are probably amplified further with approaching death (25). In contrast, functional anti-HCMV T cells competent for release of proinflammatory cytokines are also expanded with aging (26, 27). The expansion of these functional T cells could contribute to anti-HCMV surveillance, but it could also exert pathogenic effects upon cells and tissues in close proximity via recently suggested molecular mechanisms (28, 29). Accordingly, these functional T cells could contribute to the inflammation of unknown origin occurring in aging (30–32), as well as the pathogenesis and/or progression of inflammatory diseases (33). Finally, the emerging question concerning the age-related expansion of T cells reacting to HCMV is centered upon deciphering its potential negative role for health and diseases in seropositive elderly people.

Interestingly, previous studies suggested that HCMV seropositivity correlates with proinflammatory parameters in the context of the multifaceted frailty syndrome (34) and with the prevalence or incidence of various cardiac (35, 36), vascular (37), and neurologic diseases (38–40). However, a role for HCMV in such chronic diseases is far from proven by these associations, and Ab responses alone do not seem to justify pathogenesis (41), which could be related to disproportionate proinflammatory T cell accumulation, viral reactivation, immune system exhaustion, or other mechanisms. Therefore, the additional requirements for sustaining the hypothesis that HCMV may play a role in chronic diseases associated with aging should include the analysis of humoral responses, as well cellular anti-HCMV responses from elderly subjects with and without pathological conditions.

To contribute to this objective, we conducted an observational cross-sectional study in elderly subjects of variable health status, evaluating the intensity of humoral responses to HCMV and EBV, another ubiquitous persistent herpesvirus that can have effects in the elderly (42). Together with CD4+ and CD8+ T cell proinflammatory effector responses directed to HCMV-derived pp65 and immediate-early protein 1 (IE-1) synthetic peptides. The CD4+, but not CD8+, T cell proinflammatory effector responses and humoral anti-HCMV responses were more intense in the most elderly with comorbidity and cognitive and functional impairments. These results suggest that immune responses against extracellular HCMV increase when health status deteriorates in the elderly, and it could represent an indirect effect of systemically undetectable HCMV reactivation.

Materials and Methods

Subjects

The study was performed after approval from the Ethics Committee of the University of Parma. We contacted subjects aged ≥65 y living in Parma and Bologna (North Italy). After providing written, informed consent, all subjects received an extensive physical examination and answered standardized questionnaires to assess clinical history, current disease, current medication list, lifestyle, cognitive function by Mini-Mental State Examination (MMSE), and functional activity by the Katz Index of Activities of Daily Living (ADL) (44) and Instrumental Activities of Daily Living (IADL). Exclusion criteria were evidence of endocrine, autoimmune, neoplastic, or recent infectious diseases, renal or liver failure, and the use of medications known to modulate the immune responses (steroids, nonsteroidal anti-inflammatory agents, acetyl salicylic acid > 100 mg/dl, or other immunosuppressive drugs). All 83 subjects enrolled in this study (49 females and 34 males) had blood drawn for laboratory analyses. We divided the subjects into old subjects (65–84 y of age) and very old subjects (≥85 y of age), and the health status was assessed. Interview at enrolment, physical examination, current medication list, and results of laboratory analyses were used by trained clinicians to diagnose diseases, with the diagnosis based on established and widely recognized criteria. Each disease was considered as a dichotomous variable that could only have one of two values: present/absent. Overall, we stratified five different groups (Table I).

Determination of EBV- and HCMV-specific Ab levels

![Image](http://www.jimmunol.org/)

Surface staining was performed on heparinized whole blood using the following mAbs: FITC-, PE-, PE-Cy7-, or allophycocyanin-conjugated mAbs directed against CD3, CD4, CD8, CD95, and CD28 and isotype-matched irrelevant Abs (all from BD Biosciences, San Jose, CA). One hundred microliters of blood were incubated with saturating amounts of the mAbs for 20 min at room temperature and then were mixed with FACS Lysing Solution (BD Biosciences). Six-parameter flow cytometric acquisition and analysis were performed on a two-laser FACS Calibur instrument (BD Biosciences) using CellQuest software (BD Biosciences). Files were first gated on lymphocytes, identified by characteristic forward angle and side scatter profiles, and cells for each CD3+, CD3+CD4+, and CD3+CD8+ subsets were gated into monocytes (CD14+CD8+CD95+), lymphocytes (CD8+CD95+), and effector CD28+ (CD28+CD95+) subsets, as previously described (45). Isotype-matched irrelevant Abs were used to set fluorescence markers and to identify non-specific binding.

Assessment of pp65 and IE-1 HCMV-specific IFN-γ CD4+ and CD8+ T cells

PBMCs were obtained by Ficoll density gradient centrifugation (Biocell Separating Solution; Biochrom, Berlin, Germany) from freshly drawn venous blood. After washing with PBS, PBMCs were resuspended in RPMI 1640 medium supplemented with 10% FCS, 2 mmol/l L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin (complete medium). In all experiments, three tubes were immediately stimulated with two CMV-specific PepMixes (IPT Peptide Technologies, Berlin, Germany): the PepMix pp65 and the PepMix IE-1 spanning the 65-kDa lower matrix phosphoprotein and the 55-kDa intermediate protein 1, respectively. Stimulation and intracellular cytokine detection was performed in accordance with the protocol recommended by JPT Peptide Technologies and well described previously (26). Briefly, 106 PBMCs were placed in 15-ml conical polypropylene tubes (Coming Glass,Coming, NY) in a final volume of 500 μl complete medium and incubated with one test medium of each PepMix and 1 μg each of the costimulatory mAbs CD28 and CD49d (BD Biosciences, Immunocytometry Systems, San Jose, CA). Negative controls (incubation with anti-CD28/CD49d but not with PepMixes) were included in every experiment to detect the spontaneous production of cytokines. After 1 h of incubation in a standard incubator (37°C, humidified atmosphere), each tube received 500 μl complete medium containing the protein transport inhibitor monensin (BD Golgi Stop, BD Biosciences). After an additional 4 h, PBMCs were fixed, permeabilized, and stained with saturating amounts of the following Abs: FITC-conjugated IgG1 x isotype control Ig or anti–IFN-γ, PE-conjugated anti-CD4, or allophycocyanin-conjugated anti-CD8 (all from BD Biosciences). The samples were acquired on a FACSCalibur instrument (BD Biosciences), and ≥0.5 X 106 events were analyzed for each sample using CellQuest software (BD Biosciences). Files were gated on small lymphocytes (using forward versus side scatter), and cytokine-secreting populations were defined as the percentage of IFN-γ+ events gated on CD4+ and CD8+ bright T cell populations minus the percentage of events falling into the same region in the corresponding control sample. We identified the sum of the frequencies of anti-pp65 and anti–IE-1-IFN-γ-producing CD4+ T cells as the percentage of HCMV-specific CD4+ cells among total CD4+ cells, and the sum of the frequencies of anti-pp65 and anti–IE-1-IFN-γ-producing CD8+ T cells as the percentage of HCMV-specific CD8+ cells among total CD8+ cells.

![Image](http://www.jimmunol.org/)
Statistical analysis

The Student t test and ANOVA parametric tests were used to derive p values for data with a normal distribution; the Mann-Whitney U and Kruskal–Wallis nonparametric tests were used to derive p values for data without a normal distribution. The correlation between HCMV-IgG Ab levels and HCMV-specific CD4+ T cell-mediated responses was tested using the Spearman rank correlation coefficient. Statistical tests were performed with StatView and SPSS software. p values <0.05 were considered significant.

Results

Characteristics of the subject groups

The characteristics of the study subjects are shown in Table I. Overall, we stratified five groups of subjects with variable health status. Group I = healthy old, included 15 subjects aged 65–84 y (mean age, 70.6 ± 5.3 y) without evidence of disease, with the possible exception of hypertension or osteoarthritis; without severe cognitive impairment (MMSE > 21), functional impairment (no more than one compromised ADL), hemato-chemical alterations and not taking any medication; and, therefore, could not be assigned to group I. Group III = healthy very old, included 15 subjects aged ≥85 y (mean age, 90.4 ± 4.4 y) without evidence of disease, with the exception of hypertension or osteoarthritis; without severe cognitive impairment (MMSE > 21), functional impairment (no more than two compromised ADL), or hemato-chemical alterations; and not taking any medication, with the exception of antihypertensive therapy. Group IV = unhealthy old, included 15 subjects aged ≥85 y (mean age, 90.3 ± 3.1 y) who did not meet one or more of the previously indicated criteria and, therefore, could not be assigned to group III. Group V = institutionalized very old, included 18 subjects aged ≥85 y (mean age, 89 ± 3.9 y) who could not be included in group III and were not community dwelling. The subjects in groups IV and V seemed to have significantly lower MMSE, ADL, and IADL scores than subjects in group III. With regard to the laboratory analyses, there were significant differences in creatinine, albumin, and C-reactive protein (CRP) between healthy and unhealthy old subjects and in total proteins, albumin, iron, CRP, and RBC numbers between the groups of very old subjects. We also evaluated the immune phenotype; the T cell subset percentages are shown in Table II. The old, unhealthy subjects seemed to have significantly lower CD4+, higher CD8+, and lower naive CD8+ percentages and a lower CD4+/CD8+ ratio compared with healthy subjects. In the very old groups, unhealthy subjects had significantly higher CD3+ percentages compared with healthy subjects. We also tested the relationship between lymphocyte subsets and age, without the influence of health status, comparing healthy old and healthy very old subjects. Healthy very old subjects had significantly lower naive CD4+ and CD8+ percentages and a higher CD28-CD8+ percentage compared with healthy old subjects. Also, the CD28 CD4+ percentage was higher in healthy very old subjects compared with healthy old subjects, but this difference did not reach statistical significance. Collectively, these data confirm that many immune phenotype changes in elderly people (e.g., a decrease in naïve CD4+ and CD8+ T cells with an increase in CD28-CD8+ and CD28-CD4+ T cells) are related to physiological aging, but they also suggest an additional effect of the poor health status that seems to accelerate and/or intensify some changes.

Humoral responses to HCMV and EBV

Ab responses against HCMV and EBV were simultaneously quantified from frozen sera derived from all subjects enrolled in this study. All donors were HCMV IgM−, HCMV IgG−, EBV IgM−, and

Table I. Characteristics of the study subjects

<table>
<thead>
<tr>
<th>Group</th>
<th>Old (65–84 y)</th>
<th>Very Old (≥85 y)</th>
<th>( n )</th>
<th>( n )</th>
</tr>
</thead>
<tbody>
<tr>
<td>I: Healthy</td>
<td>II: Unhealthy</td>
<td>III: Healthy</td>
<td>IV: Unhealthy</td>
<td>V: Institutionalized</td>
</tr>
<tr>
<td>Age (y, mean ± SD)</td>
<td>70.6 ± 5.3</td>
<td>77.9 ± 4.3</td>
<td>90.4 ± 4.4</td>
<td>90.3 ± 3.1</td>
</tr>
<tr>
<td>Disease</td>
<td>No evidence of disease</td>
<td>Subjects who cannot be assigned to group I</td>
<td>No evidence of disease</td>
<td>Subjects who cannot be assigned to group III</td>
</tr>
<tr>
<td>Cardiovascular (%)</td>
<td>—</td>
<td>18/19 (94.7)</td>
<td>—</td>
<td>12/16 (75)</td>
</tr>
<tr>
<td>Cerebrovascular (%)</td>
<td>—</td>
<td>4/19 (21)</td>
<td>—</td>
<td>7/16 (43.7)</td>
</tr>
<tr>
<td>Pulmonary (%)</td>
<td>—</td>
<td>3/19 (15.8)</td>
<td>—</td>
<td>3/16 (18.7)</td>
</tr>
<tr>
<td>MMSE (mean [range])</td>
<td>27.5 (22.3–30)</td>
<td>24.3 (9–30)</td>
<td>27 (21.4–30)</td>
<td>17.5 (0–29.4)</td>
</tr>
<tr>
<td>ADL (mean [range])</td>
<td>6 (6–6)</td>
<td>5.8 (5–6)</td>
<td>5.6 (4–6)</td>
<td>2.9 (0–6)</td>
</tr>
<tr>
<td>IADL (mean [range])</td>
<td>18.8 (18–19)</td>
<td>18.1 (12–19)</td>
<td>15.2 (7–19)</td>
<td>7.7 (0–19)*</td>
</tr>
<tr>
<td>Serum values (mean ± SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>92.33 ± 9.84</td>
<td>92.94 ± 20.7</td>
<td>86.33 ± 10.6</td>
<td>85.81 ± 24.84</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.85 ± 0.13</td>
<td>1.17 ± 0.41*</td>
<td>1.10 ± 0.19</td>
<td>1.23 ± 0.65</td>
</tr>
<tr>
<td>AP (U/l)</td>
<td>62.26 ± 10.61</td>
<td>73.89 ± 23.83</td>
<td>69.8 ± 22.87</td>
<td>81.62 ± 26.37</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>22.4 ± 3.9</td>
<td>22.15 ± 7.29</td>
<td>20.66 ± 3.38</td>
<td>22.06 ± 7.25</td>
</tr>
<tr>
<td>Total proteins (g/dl)</td>
<td>6.86 ± 0.42</td>
<td>6.73 ± 0.28</td>
<td>6.77 ± 0.48</td>
<td>6.21 ± 0.41</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>3.97 ± 0.27</td>
<td>3.67 ± 0.44**</td>
<td>3.71 ± 0.28</td>
<td>3.23 ± 0.26</td>
</tr>
<tr>
<td>Iron (mg/dl)</td>
<td>79 ± 21.08</td>
<td>87 ± 40.78</td>
<td>84.2 ± 22.26</td>
<td>54.3 ± 21.83</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>1.69 ± 1.47</td>
<td>14.93 ± 22.09**</td>
<td>3.21 ± 3.13</td>
<td>24.81 ± 27.07</td>
</tr>
<tr>
<td>Hematology values (mean ± SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBCs (10^12/μl)</td>
<td>4.56 ± 0.44</td>
<td>4.85 ± 0.59</td>
<td>4.41 ± 0.70</td>
<td>3.92 ± 0.44</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>13.84 ± 1.31</td>
<td>14.35 ± 1.18</td>
<td>13.33 ± 1.56</td>
<td>12.14 ± 1.16</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>91.84 ± 6.22</td>
<td>87.47 ± 6.69</td>
<td>88.98 ± 8.81</td>
<td>90.80 ± 6.85</td>
</tr>
<tr>
<td>WBCs (10^3/μl)</td>
<td>5.72 ± 1.72</td>
<td>6.12 ± 1.49</td>
<td>6.65 ± 1.55</td>
<td>6.27 ± 1.10</td>
</tr>
<tr>
<td>Lymphocytes (10^3/μl)</td>
<td>1.74 ± 0.37</td>
<td>1.78 ± 0.58</td>
<td>1.97 ± 0.55</td>
<td>1.32 ± 0.52</td>
</tr>
</tbody>
</table>

*p < 0.01; **p < 0.05; calculated by Student t test between old groups and by ANOVA among very old groups.

ALT, alanine aminotransferase; AP, alkaline phosphatase; AST, aspartate aminotransferase; MCV, mean corpuscular volume; NA, not applicable.

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80 of 83 donors were EBV IgG+. Without considering health status, very old subjects had significantly higher HCMV IgG Ab levels than did old subjects by detection with indirect chemiluminescence immunoassays (medians: 15 and 8.8 UI/ml, respectively; Fig. 1A). This result was also confirmed by a conventional enzyme immunoassay (data not shown). When we analyzed anti-HCMV IgG levels among

![Figure 1](https://www.jimmunol.org/)

**FIGURE 1.** Humoral responses to HCMV and EBV. Box plots show the levels (median, 25th and 75th percentiles, range) of IgG Abs against CMV (CMV-IgG) and EBV VCA (EBV-IgG) determined in serum samples of all subjects. A and D, The magnitude of Ab levels in old and very old subjects. B, C, E, and F, The magnitude of Ab levels in subjects of variable health status. The Mann-Whitney U and Kruskal–Wallis nonparametric tests were used to derive p values.

### Table II. Immune phenotype of subjects studied

<table>
<thead>
<tr>
<th>Groups</th>
<th>I: Healthy</th>
<th>II: Unhealthy</th>
<th>p Valuea</th>
<th>III: Healthy</th>
<th>IV: Unhealthy</th>
<th>V: Institutionalized</th>
<th>p Valueb</th>
<th>Between Healthy Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+/Lymph</td>
<td>66.3 (56.6–74.6)</td>
<td>64.2 (54.7–82.5)</td>
<td>0.43</td>
<td>63.9 (51.2–87.9)</td>
<td>68.2 (43.7–77.5)</td>
<td>0.05</td>
<td>0.28</td>
<td>0.05</td>
</tr>
<tr>
<td>CD4+/CD3+</td>
<td>72.4 (46.3–87.3)</td>
<td>60.3 (43.7–80.2)</td>
<td>0.02</td>
<td>66.7 (41.1–79.5)</td>
<td>63.9 (30.5–83.3)</td>
<td>0.64</td>
<td>0.25</td>
<td>0.059</td>
</tr>
<tr>
<td>Naive/CD4+</td>
<td>43.3 (18.6–68.9)</td>
<td>34 (5–58.1)</td>
<td>0.39</td>
<td>26 (5.5–48.5)</td>
<td>33.9 (10.2–58.3)</td>
<td>2.11 (0.9–65)</td>
<td>0.53</td>
<td>0.019</td>
</tr>
<tr>
<td>Memory/CD4+</td>
<td>53.7 (30.9–75.4)</td>
<td>54.1 (33–90.4)</td>
<td>0.47</td>
<td>59.8 (34.6–88.4)</td>
<td>58 (31.6–82.8)</td>
<td>58 (34.1–85.7)</td>
<td>0.059</td>
<td>0.19</td>
</tr>
<tr>
<td>CD28+/CD4+</td>
<td>3.6 (0–32.3)</td>
<td>5.3 (0.07–55.6)</td>
<td>0.41</td>
<td>7.1 (0.26–57.2)</td>
<td>9.7 (0.19–21.7)</td>
<td>13.7 (0.1–34.3)</td>
<td>0.30</td>
<td>0.18</td>
</tr>
<tr>
<td>CD8+/CD3+</td>
<td>25.2 (10.5–51)</td>
<td>33.7 (17.9–52.5)</td>
<td>0.05</td>
<td>27.8 (19.5–57.8)</td>
<td>28.1 (16.7–63.5)</td>
<td>33.3 (12.4–95.9)</td>
<td>0.99</td>
<td>0.23</td>
</tr>
<tr>
<td>Naive/CD8+</td>
<td>19.6 (1.9–51.4)</td>
<td>8.8 (0.5–28.7)</td>
<td>0.03</td>
<td>3.3 (0.2–15.9)</td>
<td>5.6 (1.6–13.8)</td>
<td>2.1 (0.04–20.3)</td>
<td>0.15</td>
<td>0.0003</td>
</tr>
<tr>
<td>Memory/CD8+</td>
<td>14.4 (20.1–68.1)</td>
<td>35.4 (13.7–59.9)</td>
<td>0.24</td>
<td>40 (5.5–85.6)</td>
<td>42.4 (12.6–67.7)</td>
<td>34.6 (19.9–52.1)</td>
<td>0.74</td>
<td>0.66</td>
</tr>
<tr>
<td>CD28+/CD8+</td>
<td>39.7 (6.6–76.6)</td>
<td>55.8 (19.5–80.7)</td>
<td>0.54</td>
<td>56.2 (13.9–92)</td>
<td>52.3 (18.5–85.7)</td>
<td>60.7 (30.3–75.8)</td>
<td>0.66</td>
<td>0.04</td>
</tr>
<tr>
<td>Ratio CD4+/CD8+</td>
<td>2.7 (0.9–8.3)</td>
<td>1.81 (0.8–4.3)</td>
<td>0.02</td>
<td>2.5 (0.4–4)</td>
<td>2.18 (0.48–5)</td>
<td>1.87 (1.03–6.9)</td>
<td>0.89</td>
<td>0.19</td>
</tr>
</tbody>
</table>

Numbers of subjects in each group are the same as in Table I. Lymphocyte subsets are expressed as median percentage with range in parentheses. Naive, memory, and CD28+ subsets are defined in Materials and Methods.

aCalculated using the Mann-Whitney U nonparametric test between the two old groups and between the healthy old and healthy very old.

bCalculated using the Kruskal–Wallis nonparametric test among the very old groups.
groups stratified according to the health status (Fig. 1B, 1C), we observed that unhealthy and institutionalized very old subjects had significantly higher levels than healthy subjects of the same age (median: 107.8, 16.25, and 8.4 U/ml, respectively; Fig. 1C). Interestingly, the same analysis performed for anti-EBV VCA-IgG (Fig. 1D-F) and EBNA IgG (data not shown) levels did not reveal significant differences when considering age (Fig. 1D) or health status (Fig. 1E, 1F).

Collectively, these results indicate that impaired health in very old subjects is accompanied by an increase in anti-HCMV humoral responses. The contextual absence of a similar increase for another persistent viral infection (e.g., EBV) suggests that this phenomenon may be quite specific.

**HCMV-specific T cell responses**

We analyzed the cellular proinflammatory effector responses by CD4+ and CD8+ T cells against HCMV-derived pp65 and IE-1 synthetic peptides in freshly isolated PBMCs obtained from all study subjects. Again, without considering health status, very old subjects had significantly higher percentages of HCMV-specific CD4+ T cells than did old subjects (median: 0.58% and 0.25% of total CD4+ T cells respectively; Fig. 2A). When we analyzed HCMV-specific CD4+ T cells among groups stratified according to health status (Fig. 2B, 2C), we observed that unhealthy and institutionalized very old subjects had significantly higher proportions of IFN-γ-producing CD4+ T cells than did healthy subjects of the same age (median: 1.3%, 0.8%, and 0.31% of total CD4+ T cells, respectively; Fig. 2C). As with humoral responses, HCMV-specific CD4+ T cell responses were also increased only among very old subjects with comorbidity and cognitive and functional impairments. HCMV-specific CD8+ T cells showed a different response pattern; there was no significant difference between groups with regard to age (Fig. 2D) or health (Fig. 2E, 2F). Therefore, humoral and cellular responses directed to the extracellular phase of HCMV were increased in the same two groups of very old subjects.

**Correlation between anti-HCMV IgG levels and HCMV-specific CD4+ T cell responses**

The overlapping pattern of humoral and cellular CD4+ T cell responses prompted us to analyze their possible correlation. Representative plots of anti-pp65 CD4+ T cell responses of subjects with low and high HCMV IgG levels are shown in Fig. 3A and 3B, respectively. Considering all subjects, the HCMV-specific CD4+ T cell responses were analyzed among HCMV Ab levels divided into quartiles; the greatest CD4+ T cell responses were found in the highest HCMV IgG quartile (Fig. 3C). When the correlation between HCMV IgG Ab levels and HCMV-specific CD4+ T cell responses was tested using the Spearman rank correlation coefficient, a strong significant positive correlation was found ($p = 0.0001$; $r_s = +0.546$). This correlated increase in humoral and CD4+ T cell responses supports a common origin consisting of immune stimulation by HCMV Ags of extracellular origin, possibly produced by the emergence of HCMV in the extracellular environment.

**Correlation between antiviral immune responses and indices of cognitive function and functional activity**

The laboratory parameters were further compared with the geriatric multidimensional parameters referring to cognitive and functional assessments registered at the time when peripheral blood was drawn from all study subjects. As shown in Table III, we found that anti-HCMV IgG concentrations and anti-HCMV CD4+ T cell responses, but not anti-EBV IgG and anti-HCMV CD8+ T cell responses, were significantly different in relation to an MMSE cut-off score defining the absence ($\geq$21) or the presence ($\leq$21) of severe cognitive impairment (46). Greater anti-HCMV responses of humoral and cellular CD4+ T cell origins were related to cognitive impairment.

The same analysis was conducted for the ADL parameter, with a cut-off score defining the absence ($\geq$5) or the presence ($<5$) of functional impairment. Significantly higher anti-HCMV responses...
of humoral and cellular CD4+ T cell origin were found in subjects with functional impairment.

Taken together, anti-HCMV humoral and cellular CD4+ T cell responses, but not humoral anti-EBV responses and cellular CD8+ T cell response against HCMV Ags of intracellular origin, were increased in subjects with reduced indices of cognitive function and functional activity. This correlation indicates that elderly subjects with reduced cognitive function and functional activity expressed increased immune reactivity to extracellular HCMV Ags, possibly triggered by the emergence of HCMV in the extracellular environment.

Discussion
The consequences of the interplay between human aging, long-term HCMV latency/infection, anti-HCMV immune responses, and age-associated diseases are the subject of intense current research (41). This study reports data on overall humoral and cellular anti-HCMV adaptive immune responses, comparing them with regard to health status and cognitive and functional activity in subjects ≥65 y. In particular, we report for the first time an inverse correlation between the intensity of humoral (anti-HCMV IgG) and cellular (anti-HCMV functional proinflammatory T cells) anti-HCMV immune response and basic parameters of cognitive (MMSE) and functional status (ADL) in the elderly. In particular, humoral and CD4+ T cell-specific responses were not associated simply with aging and diseases; they were also associated with cognitive and functional impairment, confirming the evidence that HCMV infection may have an important role in these impairments (39, 47). Despite the limitations inherent to cross-sectional protocols, this study reinforces the consistency of the previously recognized association between HCMV seropositivity and frailty by adding a degree of specificity through comparison with anti-EBV response, by adding dose-response relationships through

Table III. Correlation between antivirus immune responses and MMSE and ADL

<table>
<thead>
<tr>
<th></th>
<th>MMSE</th>
<th>ADL</th>
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<tbody>
<tr>
<td>HCMV-IgG (IU/ml; median [range])</td>
<td>9.3 (0.9–652)</td>
<td>15.85 (2.4–1755)</td>
</tr>
<tr>
<td>EBV-IgG (U/ml; median [range])</td>
<td>173 (16.7–2002)</td>
<td>183 (23.4–750)</td>
</tr>
<tr>
<td>HCMV-specific CD4+ T cells (%; median [range])</td>
<td>0.37 (0–10.5)</td>
<td>0.81 (0–25.8)</td>
</tr>
<tr>
<td>HCMV-specific CD8+ T cells (%; median [range])</td>
<td>1.41 (0–14.8)</td>
<td>1.48 (0–9.77)</td>
</tr>
</tbody>
</table>

*aDefined as MMSE > 21.
*bDefined as MMSE ≤ 21.
*Calculated by Mann-Whitney U nonparametric test.
*Defined as ADL ≥ 5.
*Defined as ADL < 5.
evaluation of the intensity of immune responses, and by adding biologic plausibility to the pathogenic mechanisms driven by HCMV infection in diseased compared with healthy elderly.

Inflammation and viral reactivation are the two main mechanisms involved in HCMV-driven pathological conditions (17, 33). With regard to inflammation, our results showed that seropositive oldest (>85 y) patients in poor health contained more abundant anti-HCMV effector/memory CD4+ T cells producing IFN-γ than did seropositive healthy subjects of the same age (Fig. 2C). In contrast, the amount of Ag-specific effector/memory CD8+ T cells producing IFN-γ was not different, regardless of the health status and advanced age (Fig. 2F). This discrepancy between CD4+ and CD8+ T cells demonstrated that expansion of proinflammatory T cells related to diseases in the elderly was confined to CD4+ T cells and, therefore, was distinct from the general expansion of proinflammatory T cells observed when comparing aged and younger subjects, as reported elsewhere (26). Also, this discrepancy between CD4+ and CD8+ T cells suggests that the greater intensity of the cellular CD4+ T cell response may not be part of the general alteration of T lymphocytes homeostasis (21). In this study, the data about HCMV-specific cellular responses were collected on the basis of a functional test and, thus, we were not able to evaluate the degree of functional versus dysfunctional expansions, especially among CD8+ T cells. In this regard, other investigators reported that HCMV-seropositive elderly and nonagenarians may be characterized by an increase in dysfunctional CMV-epitope–specific CD8+ T cells (24, 25). If we consider the possibility that a similar increase in dysfunctional CD8+ T cells can take place in our elderly subjects in poor health, an intriguing hypothesis is that the increased numbers of circulating anti-HCMV effector/memory CD4+ T cells producing IFN-γ are recruited in these subjects to compensate for an insufficient surveillance by dysfunctional CD8+ T cells. This hypothesis deserves to be verified in the future.

A different explanation for the discrepancy between the levels of CD4+ and CD8+ HCMV-specific responses could be related to the antigenic stimulation induced by HCMV emerging in the extracellular microenvironment. This possibility was supported by the strong significant positive correlation ($p < 0.0001$; $r_s = +0.546$) between the numbers of CD4+ T cells and levels of anti-HCMV Abs. The age-related increase in anti-HCMV lgs was reported by other investigators (48, 49), but its relationship with subclinical reactivation is uncertain. In our study, the evaluation of Ab and T cell responses provided integrated information on anti-HCMV adaptive immune responses. The correlated greater intensities of humoral and cellular CD4+ T cell mediated responses reacting to Ags of common extracellular origin both contribute to suggest an increased exposure to HCMV in the extracellular phase (Fig. 3). Furthermore, Ab responses to HCMV, but not to EBV, were generally more intense in very old people in poor health compared with their age-matched healthy controls (Fig. 1), and they were inversely correlated with parameters of cognitive function (MMSE) and functional activity (ADL) (Table III). Thus, the greater intensity of humoral responses to HCMV was relatively specific, because it did not depend on generalized hyperreactivity against life-long persisting viral infection. Rather, these findings contribute to the hypothesis that HCMV may have emerged in the extracellular microenvironment from its latent phase in the peripheral tissues of the elderly with age-associated diseases.

In contrast, the increase in anti-HCMV Ab and CD4+ T cells was not simply related to diseases, because subjects <85 y of age did not show this effect. More precisely, there seemed to be an additive effect between age- and disease-dependent factors, because intensification of anti-HCMV responses was evident in groups IV and V but not in the other groups (Fig 1, 2). This observation raises the possibility that conflicting data and controversy surrounding an entire body of research on the association between vascular diseases and HCMV seropositivity (50) may be due, at least in part, to the aging factor; therefore, it may deserve further scrutiny by taking into account the age of patients included within various protocols. Apart from aging, it should be noted that the present study was not designed to investigate the role of HCMV in one specific disease. The purpose of this study was to distinguish between older adults with and without pathological conditions: the characteristics of the subjects in the different groups (Table I) show how the unhealthy old were impaired by various pathologies without reduced cognitive function and reduced functional activity, which became evident only in the oldest subjects. In particular, the subjects in groups IV and V might not be classified as frail on the basis of the more robust and validated determinations of the phenotypes (51, 52), however, at the same time, it is evident that our oldest subjects, with lower IADL and ADL scores, have an increased vulnerability to stressors and an increased risk for adverse events, such as disability, institutionalization, and death—all clinical conditions related to the frailty syndrome (53, 54). Based on this, it is realistic to assume that HCMV-seropositive elderly subjects are likely to be exposed to high levels of extracellular HCMV at advanced ages and after diseases have had their onset, as well as when functional activity and cognitive function are increasingly compromised within the framework of the frailty syndrome.

The most relevant implication of this study concerns the increased likelihood of HCMV reactivation derived from indirect immunological evidence in very old subjects with impaired health and cognitive and functional status. The study of HCMV localized reactivation from latency is beyond the reach of current laboratory methods (17), although recent data obtained by amplifying HCMV DNA from urine sediments showed that HCMV could be silently activated in the periphery (19). Given the expected relevance of HCMV reactivation, high Ab levels and CD4+ T cell responses could represent its most evident indirect biomarkers and provide guidance for more sensitive and detailed investigations. In accordance with this interpretation, the elderly in poor health might represent a major group of subjects at risk for HCMV reactivation in peripheral tissues and organs, and they may be candidates for direct or indirect HCMV-related pathology. The recent association observed between HCMV Ab levels and mortality in older adults supports this assumption (55). In conclusion, this study reinforces the association between anti-HCMV infection and chronic diseases in very old subjects, and it paves the way for antiviral interventional protocols designed to reduce biomarkers of indirect HCMV pathogenesis in selected elderly subjects with a high benefit/risk ratio.

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Disclosures
The authors have no financial conflicts of interest.

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23–35.


