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Massive Load of Functional Effector CD4⁺ and CD8⁺ T Cells against Cytomegalovirus in Very Old Subjects

Rosanna Vescovini,* Claudia Biasini,* Francesco F. Fagnoni,† Anna Rita Telera,* Luca Zanlari,* Mario Pedrazzoni,* Laura Bucci,‡ Daniela Monti,‡ Maria Cristina Medici,§ Carlo Chezzi,§ Claudio Franceschi,¶ and Paolo Sansoni2∗

A progressive, systemic, and low-grade proinflammatory status is one of the major characteristics of immunosenescence. Emerging data suggest a possible contribution of CMV, known to chronically infect a large proportion of humans, lifelong from newborns to centenarians. To test this hypothesis, we evaluated functional T cell responses to two CMV immunogenic proteins, pp65 and IE-1, in 65 chronically infected subjects aged 25–100 years. PBMC were stimulated with mixtures of peptides spanning the entire sequence of both proteins, and Ag specificity and magnitude of intracellular IFN-γ and TNF-α-positive cells were then analyzed within both CD4⁺ and CD8⁺ T cells. Results indicate that pp65 and, to a lesser extent, IE-1 constitute major Ags against which aged people target functionally efficient T cell effector responses with massive production of Th1 cytokines and exhibition of CD107a degranulation marker. As a result, the production of IFN-γ induced in T cells by both Ags was seven to eight times greater in very old than in young subjects. The comparative analysis of pp65-specific responses in these very long-term carriers revealed a reciprocal relationship between CD4⁺ and CD8⁺ producing IFN-γ in the same individuals. These results indicate that CMV represents an important pathogen responsible for a strong immune activation in human aging. Such a remarkable burden of effector CD4⁺ and CD8⁺ T cells may be necessary to protect the elderly from CMV endogenous reactivation, but can turn detrimental by giving a substantial contribution to the proinflammatory status that accompanies the main age-related diseases. The Journal of Immunology, 2007, 179: 4283–4291.

Inflammatory mechanisms play a prominent role in the pathogenesis of many age-related diseases and possibly in the primary process of aging itself. Some evidence—including a decrease in naive T cell, accumulation of memory T cells, loss of CD28 costimulatory signal, increased number of CD8⁺ T cells producing IFN-γ, and increased levels of proinflammatory cytokines—indicate that a peculiar activation of the immune system accompanies the aging process in humans (1–6). However, the basic mechanisms underlying this chronic inflammatory status in aged humans, we proposed to call inflam-aging (7), are still elusive.

The β-herpesvirus CMV is a common pathogen that infects more than half of the adult population. After primary infection, generally silent in immunocompetent hosts, it establishes a persistent chronic infection controlled by constant immune surveillance. The most important defense against CMV is provided by T cells, as indicated by the observation that uncontrolled viral replication and end-organ diseases occur in individuals with severely impaired T cell functions, including transplant recipients, late stage-HIV patients, and congenitally infected neonates. A remarkable study (8) demonstrated that healthy seropositive subjects aged 19–55 years maintain CMV under control by broadly targeting the overall CMV proteome with 4–5% of both CD4⁺ and CD8⁺ peripheral T cell producing intracellular IFN-γ. Such a surprisingly high burden of T cells producing IFN-γ in adulthood raises the possibility that the impact of CMV infection on effector T cells in aged subjects might be even greater, considering the persistence of the CMV infection over decades and the variety of molecular and cellular alterations occurring in immunosenescence.

In recent years, it has become evident that CMV chronic infection contributes to a number of modifications that characterize immunosenescence (9, 10). Several studies on CD8⁺ T lymphocytes with HLA class I tetramers reported that elderly subjects exhibit an increase in T cells specific for CMV-derived epitopes, constituting oligoclonal T cell expansions, with phenotype of highly differentiated effector cells (11–14). Functional analysis of these CMV-specific CD8⁺ T lymphocytes has shown that, after epitope-specific stimulation, old subjects have a lower proportion of IFN-γ-producing T cells than younger donors, thus suggesting accumulation of dysfunctional CD8⁺ T lymphocytes (12, 14, 15) and that aging and CMV lead to the Th1-oriented cytokine production profile of CD8⁺ T cells (16). As regards CD4⁺ T cells...
and CMV infection, few studies are available (17, 18). In particular, Fletcher et al. (17) showed that old carriers have higher percentages of CD4+ T cells producing IFN-γ in response to CMV lysate and bearing a late differentiated phenotype with very short telomeres. Apart from possible discrepancies among different studies, the emerging main concept from these results is that anti-CMV T cells in elderly have restricted clonality, terminally differentiated phenotype, and some dysfunctional aspects. However, several questions remain unanswered, including the contribution of anti-CMV responses to inflamm-aging and the Ags possibly selected after long-lasting infection, among others.

This study focused on the simultaneous evaluation of functional age-related CMV-specific CD8+ and CD4+ responses to two immunogenic CMV proteins: the major immediate early 1 gene product (IE-1)3 and the structural phosphoprotein pp65. To this end, we stimulated freshly drawn PBMC from subjects of different ages (age range: 25–100 years, subdivided into three groups aged 25–40, 65–85, and >85 years old) with mixtures of peptides spanning the entire sequence of both proteins and analyzed Ag specificity and magnitude of intracellular IFN-γ- and TNF-α-positive cells within both CD4+ and CD8+ T cells.

Materials and Methods

Subjects

We studied 65 CMV-seropositive subjects of three different age groups: young: 14 subjects 25–40 years old (mean age = 29.3 ± 3.9); old: 25 subjects 65–85 years old (mean age = 74 ± 6.6); oldest: 26 subjects >85 years old (mean age 90.6 ± 4). Young donors were recruited from healthy staff members. Elderly were recruited randomly from the general public or institutions and all met the following exclusion criteria: absence of diabetes, cancer, severe renal failure, severe liver disease, endocrine disorders, autoimmune diseases, or acute infectious disease; they were not using drugs whose activity is known to modify the functions of the immune system. The study was performed after approval from the Ethics Committee of the University of Parma.

CMV serology

Indirect chemiluminescence immunoassays (Liaison CMV IgG and IgM assays; DiaSorin) were used for the quantitative determination of serum-specific IgG and IgM Abs to human CMV. Samples were analyzed by a photomultiplier Liaison. All donors recruited were IgG positive and IgM negative.

Stimulation with PepMix, intracellular staining, and detection of CD107a expression

PBMC were obtained by Ficoll density gradient centrifugation (Biocoll separating solution; Biochrom) from freshly drawn venous blood. After washing with PBS, PBMC were resuspended in RPMI 1640 medium supplemented with 10% FCS, 2 mM l-glutamine, 100 µg/ml streptomycin, and 100 U/ml penicillin (complete medium). In all experiments, the cells were immediately stimulated with PepMixes (JPT Peptide Technologies). The PepMix pp65 contains 138 peptides (15-aa peptides with an overlap of 11 aa) spanning the 65-kDa lower matrix phosphoprotein. The PepMix IE-1 contains 120 peptides spanning the 55-kDa immediate-early protein 1.

Table I. Number of CMV-specific responders/donors tested within groups of different age

<table>
<thead>
<tr>
<th>T Cell Response</th>
<th>Viral Protein</th>
<th>Young (25–40 years)</th>
<th>Old (65–85 years)</th>
<th>Oldest Old (&gt;85 years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8+</td>
<td>pp65</td>
<td>6/14 (42.8%)</td>
<td>21/25 (84%)</td>
<td>21/26 (80.8%)</td>
</tr>
<tr>
<td></td>
<td>IE-1</td>
<td>2/13 (15.4%)</td>
<td>12/25 (48%)</td>
<td>12/26 (46%)</td>
</tr>
<tr>
<td>CD4+</td>
<td>pp65</td>
<td>8/14 (57%)</td>
<td>15/25 (60%)</td>
<td>22/26 (84%)</td>
</tr>
<tr>
<td></td>
<td>IE-1</td>
<td>1/13 (7.7%)</td>
<td>3/25 (12%)</td>
<td>4/25 (16%)</td>
</tr>
<tr>
<td>CD4+ and/or CD8+</td>
<td>pp65 and/or IE-1</td>
<td>11/14 (78.5%)</td>
<td>23/25 (92%)</td>
<td>25/26 (96%)</td>
</tr>
</tbody>
</table>

3 Abbreviation used in this paper: IE-1, immediate early 1 gene product.
FIGURE 1. Age-related functional CD8⁺ T cells responses to pp65 and IE-1 CMV proteins. A. Representative flow cytometry plots of anti-pp65 and anti-IE-1 CD8⁺ responses of donors of different age. PMBC were unstimulated (negative control) or stimulated with mixtures of peptides spanning the entire sequence of pp65 and IE-1 CMV proteins. Following in vitro Ag-specific stimulation, PBMC were stained as described in Materials and Methods. The cluster of events gated corresponds to the responder CD8⁺ T cells for each protein. The data are expressed as percentage of cells expressing intracellular IFN-γ within CD8⁺ bright T cell subset. The individual data of all donors of different age groups are also shown, both as percentage (B and C) and as absolute number (D and E) of circulating pp65- (green symbols) and IE-1- (magenta symbols) specific IFN-γ-producing CD8⁺ T cells and both as percentage (F and H) and as absolute number (G and I) of circulating pp65- and IE-1-specific TNF-α-producing CD8⁺ T cells. Bars indicate means. Values of p for comparing data between different donors groups are calculated by the Mann-Whitney U nonparametric test.
Statistical analysis

The Kruskal-Wallis nonparametric test was used to derive p values for comparing data among all three donor groups, whereas the Mann-Whitney U nonparametric test was used for comparing data between groups. The nonparametric Spearman rank correlation coefficient test was used to determine the correlation between anti-pp65 CD4+ vs CD8+ T cell responses. Statistical tests were performed with Statview software; p values <0.05 were considered significant.

Results

Functional T cell responses mediated by IFN-γ and TNF-α against pp65 and IE-1 proteins

A comparison among the three age groups confirmed that the frequency of subjects showing CD8+ T cell responses to pp65 and IE-1 proteins in people older than 65 years was about two and three times as much as that observed in young adults, respectively, but the number of responders did not increase further in the group of oldest old (Table I). A similar age-related pattern was observed when considering the intensity of CD8+ T cell response. The production of IFN-γ and TNF-α (Fig. 1) after stimulation with either pp65-derived or IE-1 peptides were increased in both groups of old subjects when compared with young adults. Representative plots of anti-pp65 and anti-IE-1 CD8+ responses mediated by IFN-γ of donors of different age are shown in Fig. 1A. Overall, functional CD8+ T cell responses against CMV in the three age groups revealed a significant increase in responder frequency as well as percentage (Fig. 1, B and C for IFN-γ, F and G for TNF-α), and absolute numbers of cells (Fig. 1, D and E for IFN-γ, H and I for TNF-α) from young to old subjects, whereas anti-CMV CD8+ T cells secreting IFN-γ and TNF-α were not increased in the oldest old when compared with old subjects. As regards CD4+ T cells, representative plots of anti-pp65 and anti-IE-1 CD4+ responses mediated by IFN-γ of donors of different age are shown in Fig. 2A. The pattern of CD4+ T cell responses to the same CMV Ags showed some distinctive aspects when compared with CD8+ T cell responses: 1) the frequency of subjects showing CD4+ T cell responses to pp65 was similar in the group of young and old subjects but increased in the oldest old (Table I); 2) both the percentage and absolute number of IFN-γ-producing CD4+ T cells in response to pp65 showed a significant increase only in the group of the oldest old, (Fig. 2, B and D); 3) both the percentage and absolute number of TNF-α-producing CD4+ T cells in response to pp65 tended to increase in both groups of old subjects, but this did not reach statistical significance; 4) we found uniformly low anti-IE-1 CD4+ T cell responses at all ages without any significant difference among the three groups for both cytokines (Fig. 2, C and E for IFN-γ, H and I for TNF-α). Nevertheless, CD4+ responses to IE-1 were present because a small but significant percentage of subjects had cell counts above the threshold of the detection method (Table I and Fig. 2, C and E). Thus, the results obtained by the analysis of distinct Ag-specific response indicated that, despite the reported broad Ag targeting, a significant amount of functional CD4+ T cell responses were directed against pp65, whereas the contribution of IE-1 to age-related accumulation of effector T cells appeared to be negligible. Finally, a comprehensive assessment of CD3+ T cell responses against both pp65 and IE-1 can be envisaged, to quantify the overall weight of functional cytokine-producing T cell in the peripheral blood of donors of different age. As shown in Fig. 3, a significant difference among the three age groups was found. In particular, the medians of percentage of IFN-γ-producing CD3+ T cells increased 3- and 8-fold in old and in very old subjects, respectively, in comparison with young adults (Fig. 3A), and this increment is also paralleled by the absolute number of circulating T cells (Fig. 3B). In all three age groups, percentages and absolute numbers of TNF-α-producing CD3+ T cells were considerably lower than IFN-γ-positive CD3+ T cells, but a significant increase (Fig. 3, C and D) was found in old and very old subjects in comparison with young adults. These data indicate that aged people are exposed to a heavy loading of effector T cells functionally competent for the production of proinflammatory cytokines in response to CMV proteins.

Functional cytotoxic profile of T cell responses against pp65 and IE-1 proteins

To get further insights into the functional profile of anti-CMV T cells, we measured the cell surface exposure of CD107a, a glycoprotein (LAMP-1) associated with the membrane of cytotoxic granules, as a reliable marker of fresh degranulation and cytotoxic potential (20, 21). Representative plots of CD107a expression, after stimulation with CMV proteins, on CD8+ and CD4+ T cell response of donors of different age, are shown in Fig. 4A. The proportion of cells expressing CD107a on CD4+ and CD8+ T cells producing IFN-γ in response to either pp65 or IE-1, within each group of subjects, is plotted in Fig. 4B. The fraction of CD8-mediated responses bearing a marker of cytotoxic capacity was higher in both elderly groups in comparison to young subjects. In particular, CD107a-positive cells reached approximately two-thirds and one-third of the CD8+ T cells producing IFN-γ against pp65 and IE-1, respectively. In contrast, the proportion of CD4+ T cells producing IFN-γ with coexpression of CD107a was significantly lower than that exhibited by CD8+ T cells for pp65 and was negligible for IE-1 (Fig. 4B). It is interesting to note that, despite the wide individual variability, the group of oldest subjects had approximately one-third of CD8+ T cells responding to pp65 that coexpressed CD107a, a proportion significantly higher than that found in the other two groups.

Relationship between functional pp65-specific CD4+ and CD8+ T cell responses

The difference in the magnitude of the CD8 and CD4 responses to IE-1 and the prevalence of responses to pp65 prompted us to verify the relationship between CD8 and CD4 T cell responses to this latter Ag. On the whole, the data regarding IFN-γ-producing T cells indicate that within the group of oldest subjects, both the percentage of responder CD4+ and CD8+ T cells (80.68% for CD8+ vs 84.6% for CD4+, Table I), as well as the magnitude of the response to pp65 are comparable (1633 cells/ml median for CD8+ T cells vs 2647 cells/ml median for CD4+ T cells, Fig. 5A). However, when comparison was made on an individual basis, in all subjects tested, a trend for a disproportion between specific CD4+ and CD8+ T cells response was observed. Indeed, subjects with high CD4+ T cell response exhibited low CD8+ T cells response and vice versa (Fig. 5B) and the statistical analysis by the nonparametric Spearman rank correlation coefficient test was found to be significant (p = 0.022).

Discussion

Human aging is characterized by expansion of effector T cells (1) and immune system activation of unknown origin (22–24). CMV is a candidate causative agent for this activation as it chronically infects people lifelong, from newborns to centenarians. Chronic CMV infection is also associated with prevalent frailty, a state with increased morbidity and mortality in older adults (25). Accordingly, we have evaluated functional T cell responses to peptides spanning two CMV immunogenic proteins, pp65 and IE-1, in 65 chronically infected subjects aged 25–100 years. Our results indicate that pp65 and, to a lesser extent, IE-1, constitute major Ags against which aged people target their T cell effector function with
FIGURE 2. Age-related functional CD4⁺ T cells responses to pp65 and IE-1 CMV proteins. A, Representative flow cytometry plots of anti-pp65 and anti-IE-1 CD4⁺ responses of donors of different age. PMBC were unstimulated (negative control) or stimulated for 5 h with mixtures of peptides spanning the entire sequence of pp65 and IE-1 CMV proteins. Following in vitro Ag-specific stimulation, PBMC were stained as described in Materials and Methods. The cluster of events gated corresponds to the responder CD4⁺ T cells for each protein. The data are expressed as percentage of cells expressing the intracellular IFN-γ within CD4⁺ T cell subset. The individual data of all donors of different age groups are also shown, both as percentage (B and C) and as absolute number (D and E) of circulating pp65- (green symbols) and IE-1- (magenta symbols) specific IFN-γ-producing CD4⁺ T cells and both as percentage (F and H) and as absolute number (G and I) of circulating pp65- and IE-1-specific TNF-α-producing CD4⁺ T cells. Bars indicate means. Values of $p$ for comparing data between different donors groups are calculated by the Mann-Whitney $U$ nonparametric test.
massive production of Th1 cytokines and increased presence of potential cytotoxic cells exhibiting the CD107a degranulation marker. Indeed, both Ags induced production of IFN-γ and TNF-α by a number of circulating T cells several times greater in old and very old subjects than in younger donors. Therefore, our data confirm that chronic infection by CMV engages a substantial fraction of T cells in infected people and, for the first time, demonstrate that CMV not only appears to drive differentiation of T cells toward the late effector phase (43), but–we can speculate—it may also induce concurrent quantitative expansion of both functional and dysfunctional elements.

When considering both CD4+ T cell, effector CD8+ T cell responses to pp65 and IE-1 were also found to be expanded in our aged subjects, both in terms of cytokine production and cytotoxic potential. Such proteins are indeed widely recognized by the CD8+ T cell subset in seropositive subjects at any age (37–40). The higher exhibition of CD107a on pp65- vs IE-1-specific CD8+ T cells (Fig. 4B) extends to long-term carriers the greater cytotoxic potential of pp65 over IE-1-specific CD8+ T cell, described in various clinical settings (41, 42). As regards CD8+ T cells, the main finding of our study is that we observed an age-related expansion of functional effector CD8+ T cells against pp65 and IE-1, despite the age-related impairment described in the largest CMV-specific CD8+ T cell clones (12, 14, 15). The overall picture emerging from previous studies on the response to CMV-specific peptides on HLA class I molecules (12, 14, 15), and the results of the present investigation on the response to peptides spanning entire proteins, is that CMV not only appears to drive differentiation of T cells toward the late effector phase (43), but–we can speculate—it may also induce concurrent quantitative expansion of both functional and dysfunctional elements.

The main observation from our study consisted of a several-fold age-related expansion of functional effector T cell responses to CMV which, in contrast, is targeted against many other Ags (8). Assuming that broad targeting of numerous CMV Ags may be conserved throughout aging, and that expansions similar to the one reported here may also occur for other numerous
CMV Ags, it can be estimated that the frequency of 4–5% of IFN-γ-producing T cells seen in adulthood may well rise to 20–50% in very old people. If this holds true, CMV would represent by far the most important agent of effector T cell expansion and probably one of the most important causes for persistent immune activation in human aging. Such an enormous and stable burden of effector CD8\(^+\) and CD4\(^+\) T cells producing IFN-γ, most of which is also producing TNF-α and showing potential cytotoxic activity,

**FIGURE 4.** Exposure of CD107a degranulation marker on anti-pp65 and anti-IE-1 CD8\(^+\) and CD4\(^+\) T cell responses in donors of different age. A. Representative flow cytometry plots of CD107a expression, after stimulation with pp65 and IE-1 CMV-proteins, on CD8\(^+\) and CD4\(^+\) T cells of donors of different age. In each plot, numbers indicate percentages of anti-pp65 and anti-IE-1 IFN-γ producing T cells positive (upper right quadrant) or negative (lower right quadrant) for the CD107a degranulation marker within the CD8\(^+\) or CD4\(^+\) subset. B. The individual data of all donors of different age groups are shown as proportion of cells expressing CD107a on IFN-γ-producing CD4\(^+\) and CD8\(^+\) T cells in response to either pp65 or IE-1. Bars indicate median; statistical analysis of data between different donors groups is calculated by the Mann-Whitney U nonparametric test (*1: >85 vs 25–40, \(p = 0.02\); *2: 65–85 vs 25–40, \(p = 0.02\); *3: >85 vs 25–40, \(p = 0.02\); *4: 65–80 vs 25–40, \(p = 0.03\)).

**FIGURE 5.** Relationship between anti-pp65 CD4\(^+\) and CD8\(^+\) T cells responses. A. Box plots show the magnitude (range, median, 25th and 75th percentiles) of anti-pp65 CD4\(^+\) and CD8\(^+\) T cells responses (number of circulating IFN-γ-positive T cells/ml of peripheral blood) in the oldest subjects group. B. Correlation between anti-pp65 CD4\(^+\) vs CD8\(^+\) T cells responses in all donors tested. Data are represented as dots corresponding to numbers of anti-pp65 IFN-γ-positive CD4\(^+\) and CD8\(^+\) T cells per milliliter of peripheral blood of each donor. \(r_s\) and \(p\) are calculated by nonparametric Spearman rank correlation coefficient test.
may be necessary to protect the elderly from CMV endogenous reactivation, but may also be detrimental at the systemic and/or tissue level. Consistent with this hypothesis, longitudinal studies tracking an immune risk phenotype in the elderly have already reported a cluster of immune parameters predictive for mortality including CMV seropositivity along with elevated serum levels of proinflammatory cytokines, and other parameters related to effector T cell expansion (45–48). These findings have been recently strengthened by the observation that CMV is one of the driving forces for acquiring immune risk phenotype (49). The present study adds a new piece to this picture by showing that expansion of functional effector T cell responses is a general age-related phenomenon in CMV-seropositive subjects that, according to the “inflamm-aging” theory, probably plays a relevant role in determining the inflammatory status in human aging.

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Disclosures

The authors have no financial conflict of interest.

References


