Hallmark Features of Immunosenescence Are Absent in Familial Longevity

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Hallmark Features of Immunosenescence Are Absent in Familial Longevity

Evelyna Derhovanessian,* Andrea B. Maier,†‡ Robert Beck,§ Gerhard Jahn,§ Karin Hähnel,* P. Eline Slagboom,*‡ Anton J. M. de Craen,†‡ Rudi G. J. Westendorp,*§ and Graham Pawelec*  

Seropositivity for CMV is one of the parameters of the “immune risk profile” associated with mortality in longitudinal studies of the very elderly and may accelerate immunosenescence. Thus, any genetic factors influencing human longevity may be associated with susceptibility to CMV and CMV-accelerated immunosenescence. To test this, we analyzed long-lived families in the Leiden Longevity Study (LLS) in which offspring enjoy a 30% reduced standardized mortality rate, possibly owing to genetic enrichment. Serum C-reactive protein levels and the frequency of different T cell subsets were compared between 97 LLS offspring and 97 controls (their partners, representing the normal population). We also determined the capacity of T cells to respond against immunodominant Ags from CMV in a smaller group of LLS subjects and controls. CMV infection was strongly associated with an age-related reduction in the frequency of naïve T cells and an accumulation of CD45RA–re-expressing and late-differentiated effector memory T cells in the general population, but not in members of long-lived families. The latter also had significantly lower C-reactive protein levels, indicating a lower proinflammatory status compared with CMV-infected controls. Finally, T cells from a higher proportion of offspring mounted a proliferative response against CMV Ags, which was also of greater magnitude and broader specificity than controls. Our data suggest that these rare individuals genetically enriched for longevity are less susceptible to the characteristic CMV-associated age-driven immune alterations commonly considered to be hallmarks of immunosenescence, which might reflect better immunological control of the virus and contribute to their decreased mortality rate. The Journal of Immunology, 2010, 185: 4618–4624.

Data from one of the very few available longitudinal studies of immune parameters in aging free-living humans, the Swedish OCTO/NONA series, identified an immune risk profile (IRP) present in ∼15–20% of 85-year-olds at baseline, which was associated with 2, 4, and 6 y mortality at follow-up. The IRP was defined by a cluster of immunological parameters, primarily an inverted CD4:8 ratio, owing to accumulation of late-stage differentiated CD8 cells, and including low T cell proliferative responses to mitogens and low numbers of B cells. Seropositivity for human CMV was also found to be part of the IRP; 100% of 85-year-olds with an IRP were CMV seropositive at baseline, whereas this proportion was 85% in people without IRP (1).

CMV is a ubiquitous β herpesvirus with a seroprevalence rising with age and reported as ranging between 30 and 90% in industrialized countries (2). Primary CMV infection in immunocompetent individuals is usually asymptomatic, after which the virus establishes lifelong latency within the host and periodically Reactivates. It is not known whether reactivation occurs more frequently with age, but it does so with stress and immunodeficiency. The long-term consequences, if any, of such periodic reactivation are not known. CMV reactivation in response to immunosuppression or inflammation (3, 4) suggests an important role for the immune system in controlling the virus. Indeed, in an infected individual, a very large proportion of the immune system is committed to controlling the virus (even up to as much as 30% of CD4+ and CD8+ memory responses in some individuals) (5). Accordingly, ample evidence from many studies shows that CMV infection has an enormous impact on the distribution of T cell subsets (6–8), leading to an increasing realization that latent infection with CMV may directly contribute to emergence of cell phenotypes termed immunosenescent (9).

Despite the inclusion of CMV seropositivity in the IRP, no direct simple correlation between CMV infection and mortality has yet been shown. Nonetheless, with the possible exception of low B cell counts, all parameters of the IRP are potentially influenced by CMV infection. This observation might imply a major contribution of infection with this virus to a set of risk factors in some individuals, which does then correlate with mortality (reviewed in Refs. 1 and 9). In the OCTO/NONA studies, although all individuals with an IRP were CMV seropositive, the majority of CMV-seropositive elderly people did not have an IRP. What determines whether a CMV-infected individual falls into the IRP...
group or not is at present unclear. We hypothesize that it cannot be infection with the virus per se, but the manner in which the immune system of an individual handles the virus and how it is affected by it might be crucial in ensuring longevity. To begin testing this, we have established detailed immune phenotypes that we designate immune signatures in individuals from families enriched for longevity included in the Leiden Longevity Study (LLS) (10, 11). A 30% decreased mortality risk has been observed in three subsequent generations within these families. Our analysis was performed in the middle-aged offspring of one of at least a pair of nonagenarian siblings. This group, termed “offspring”, displays a decreased prevalence of metabolic disease (11) and allows for identification of healthy aging parameters at an earlier age that correlate with longevity and decreased mortality in later life.

In this paper, we show that CMV seropositivity is associated with a more “senescent” phenotype of the immune system in cross-sectional studies of the general population. However, this phenomenon is absent in individuals predisposed to longevity. We suggest that this finding may reflect a better immunological control of the virus in these people, and that one of the mechanisms by which longevity assurance genes contribute to extended lifespan could be via control of the response to CMV.

Materials and Methods

Subjects

PBMCs obtained from 97 offspring of one of at least a pair of nonagenarian siblings from the LLS were analyzed in this study and compared with those from their partners. Characteristics of these individuals are summarized in Table I. The details of the LLS study have been published previously (10, 11). The Medical Ethics Committee of the Leiden University Medical Center approved the study, and informed consent was obtained from all subjects.

Human serum C-reactive protein (CRP) levels were determined using the Dual Count Solid Phase No Boil Assay (Diagnostic Products, Los Angeles, CA). Because CRP levels were not normally distributed, they were log transformed.

Flow cytometry

All staining steps were performed in PFEA buffer (PBS, 2% FCS, 2 mM EDTA, and 0.01% azide). After thawing, PBMCs were treated with human IgG, GAMUNEX (Bayer, Leverkusen, Germany), and ethidium monoazide (EMA) (Invitrogen, Karlsruhe, Germany) for 10 min on ice to block FcRs and label nonviable cells. Cells were first stained with anti-KLRG-1 primary Ab (kindly provided by Prof. Hans-Peter Pircher, Freiburg, Germany) for 20 min at 4°C, followed by staining with Pacific Orange-conjugated goat anti-mouse IgG (Invitrogen) for another 20 min on ice. Mouse serum (Chemicon/Millipore, Schwalbach, Germany) was added for 15 min to block nonspecific binding to mouse secondary Ab, followed by addition of directly conjugated mAbs, CD3-PE (Calltag; Invitrogen), CD4-PerCP, CD8-allophycocyanin-Cy7, CCR7-PE-Cy7 (BD Biosciences, Heidelberg, Germany), CD27-allophycocyanin, CD45RA-Pacific Blue, CD28-Alexa Fluor 700. (BioLegend, San Diego, CA), and CD57-FITC (Immunotools, Freiburg, Germany). After 20 min incubation on ice, cells were washed and analyzed immediately on an LSR II cytometer with FACSDiva software (BD Biosciences). The spectral overlap between all channels was calculated automatically by the BD FACSDiva software, after measuring negative and single-color controls. Data were analyzed using FlowJo software (Tree Star, Portland, OR). For data analysis, EMA-positive dead cells were excluded. In the viable gate, lymphocytes were gated in a forward light scatter versus side scatter dot blot according to their size and granularity. T cells within the lymphocyte gate were characterized as CD3+ cells. T cell subsets were characterized according to previously published models (12–14). The gating strategy is shown in Supplemental Fig. 1. The absolute number of cells in each subset per unit of blood was calculated by multiplying the frequency of the subset within the lymphocytes by the absolute number of lymphocytes calculated during cyto-ometric analysis of fresh whole blood. Flow cytometry staining and data analysis were performed on blinded samples.

Virological methods

CMV serostatus was determined by ELISA, using the CMV-IgG-ELISA PKS assay (Medac GmbH, Wedel, Germany) according to the manufacturer’s instructions. These experiments were performed on blinded samples.

Proliferation assay

A CFSE dilution assay was used to measure proliferation of T cells. PBMCs were thawed and labeled with 5 μM CFSE (Molecular Probes/Invitrogen) according to the manufacturer’s instructions. Cells were then resuspended in X-Vivo 15 Medium (Lonza, Cologne, Germany) at a concentration of 2.5 × 10^6 cells/ml. The cells were either left untreated or stimulated with 1 μg/ml of a mixture of synthetic peptides spanning the whole sequence of pp65 or immediate early 1 (IE1) proteins (JPT Technologies, Berlin, Germany). PHA, 0.1%, was used as a positive control. After 7 d, cells were harvested, washed once in PFEA buffer, and stained with EMA, CD3-PE, CD4-Pacific Blue, and CD8-APC-Cy7, as described above. After gating out dead cells, CD4+CD8+ and CD8+CD4+ T cells from the CD3+ gate were analyzed for CFSE fluorescence levels, and proliferating cells were determined as CFSElo cells. The proliferation was considered peptide specific only when the stimulation index (frequency of CFSElo cells with peptide/frequency of CFSElo cells without peptide) was >2.

Statistical analysis

Statistical analyses were performed using Graphpad Prism v4. Mann–Whitney testing was used for comparison of two independent groups. Differences between groups for the categorical variables were assessed with the Chi-square test or McNemar’s test. To analyze correlation between the serum CRP levels and the frequency of different T cell subsets, the Spearman’s correlation analysis was performed. To control for the number of comparisons in T cell subset analysis, only a p value ≤0.001 was considered significant. In the experiments analyzing immune response against different CMV Ags, the α was set at 0.012 after Bonferroni correction. The p values <0.05 were considered strong trends and are also shown in the figures. For the remaining comparisons, the α was set at 0.05.

Results

Individuals from longevity families do not show CMV-associated changes to their immune signatures

To determine whether offspring enriched for longevity displayed different immune profiles compared with the general population, and to test whether the impact of CMV seropositivity was different in the two groups, we investigated the distribution of peripheral immune cell subsets in 97 offspring from long-lived families and their partners (n = 97), representing the general population. We found that 53% of the partners and 43% of the offspring were CMV seropositive (not significant). Demographical and medical characteristics of these individuals are summarized in Table I. The absolute number of lymphocytes and CD4+ T cells per microliter of blood did not differ between CMV-seropositive and -seronegative individuals, but those infected with CMV had higher numbers of CD8+ T cells (p = 0.0001 and p = 0.001 for partners and offspring, respectively, compared with CMV-seronegative persons [Table II]). However, such an accumulation of CD8+ T cells in CMV-seropositive individuals resulted in more total T cells per milliliter of blood only in partners, with marginal significance (p = 0.04), and not in offspring (Table II). The increase in the number of CD8+ T cells also led to a lower CD4/CD8 ratio in the CMV-seropositive group in both offspring and controls (Table II).

Because of the marked effect of CMV on the absolute number of CD8+ T cells, we analyzed their differentiation state in the offspring from long-lived families and their partners in detail. In partners, representing the normal population, the frequency of naive (CD45RA+CCR7+CD27+CD28+) T cells expressed as a percentage of all CD8+ T cells was lower in CMV-seropositive than -seronegative people, although as to be expected in human populations, there was a great deal of interindividual variability. Nonetheless, this difference was highly significant (p = 0.0001; Fig. 1A, circles). Reciprocally, in the normal population, there was an accumulation of late-differentiated effector memory (CD45RA-CCR7-CD27-CD28+) T cells in CMV-seropositive subjects.
(p < 0.0001; Fig. 1B, circles). Moreover, these late-differentiated cells expressed CD57 and KLRG1, sometimes referred to as “senescence-associated” markers, at high levels (Supplemental Fig. 1C). In marked contrast, in familial longevity, the frequency of naive CD8+ T cells was not decreased by CMV infection (Fig. 1A, squares). Consistent with this finding, neither was there an accumulation of late-stage effector memory T cells (CD45RA- CD62L+ KLRG1+) in this population (Fig. 1B, squares). Thus, there was a strong trend toward maintaining a higher frequency of naive CD8+ T cells as well as a lower frequency of late-stage effector memory T cells despite CMV seropositivity in long-lived offspring compared with their age-matched CMV-seropositive partners (p = 0.011 and 0.03, respectively; Fig. 2A, B), suggesting a more “youthful” phenotype in the former. Reciprocally, CMV-infected partners had higher levels of T cells expressing putative senescence markers CD57 and KLRG-1, compared with age-matched CMV-infected offspring (p = 0.024 and 0.018, respectively; Fig. 2C, D).

**Serum level of CRP is higher in CMV-infected partners than in offspring from longevity families**

Because chronic infection with agents such as CMV may be associated with higher levels of inflammation, we next determined the levels of CRP as a marker of inflammation in serum from these 194 individuals investigated for immune signatures. The mean level of serum CRP ± SE was calculated for each group. CMV-seronegative partners and offspring had similar levels of CRP (0.21 ± 0.18 mg/l and 0.25 ± 0.16 mg/l, respectively; p = 0.7). However, CMV-seropositive offspring had significantly lower levels of CRP compared with their CMV-seropositive partners (0.15 ± 0.09 mg/l and 0.75 ± 0.12 mg/l, respectively; p = 0.02) (Table I). CMV infection was associated with an increase in CRP levels in the partners (0.25 ± 1.1 mg/l in CMV-seronegative compared with 0.75 ± 1.2 mg/l in CMV-seropositive), but this did not reach statistical significance (p = 0.15). No such trend was evident in offspring, compared with their age-matched CMV-infected partners as controls (p = 0.011 and 0.03, respectively; Fig. 2A, B), suggesting a more “youthful” phenotype in the former. Reciprocally, CMV-infected partners had higher levels of T cells expressing putative senescence markers CD57 and KLRG-1, compared with age-matched CMV-infected offspring (p = 0.024 and 0.018, respectively; Fig. 2C, D).

**Table I. Comparison of demographics, comorbidity, and use of different medications in offspring and partners**

<table>
<thead>
<tr>
<th></th>
<th>Partners CMV- (n = 46)</th>
<th>CMV+ (n = 51)</th>
<th>p Value</th>
<th>Offspring CMV- (n = 56)</th>
<th>CMV+ (n = 41)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female, n (%)</td>
<td>29 (63.0)</td>
<td>30 (58.8)</td>
<td></td>
<td>20 (35.7)</td>
<td>20 (48.8)</td>
<td></td>
</tr>
<tr>
<td>Age, mean (SD), y</td>
<td>61 (8.2)</td>
<td>62 (8.0)</td>
<td></td>
<td>62 (6.4)</td>
<td>61 (6.5)</td>
<td></td>
</tr>
<tr>
<td>Prevalence of disease, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>1 (2.2)</td>
<td>2 (3.9)</td>
<td></td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>Stroke</td>
<td>2 (4.3)</td>
<td>1 (2.0)</td>
<td></td>
<td>0 (0.0)</td>
<td>1 (2.4)</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>10 (21.8)</td>
<td>11 (21.6)</td>
<td></td>
<td>11 (19.6)</td>
<td>8 (19.5)</td>
<td></td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>1 (2.8)</td>
<td>3 (5.9)</td>
<td></td>
<td>1 (1.8)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>Cancer</td>
<td>3 (6.5)</td>
<td>4 (7.8)</td>
<td></td>
<td>3 (5.4)</td>
<td>2 (4.9)</td>
<td></td>
</tr>
<tr>
<td>Chronic obstructive obstructive lung disease</td>
<td>1 (2.8)</td>
<td>2 (3.9)</td>
<td></td>
<td>1 (1.8)</td>
<td>2 (4.9)</td>
<td></td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>1 (2.8)</td>
<td>0 (0.0)</td>
<td></td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>Medication use, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertensive agents</td>
<td>6 (13)</td>
<td>13 (25.5)</td>
<td></td>
<td>10 (17.9)</td>
<td>8 (19.5)</td>
<td></td>
</tr>
<tr>
<td>Lipid-lowering agents</td>
<td>0 (0.0)</td>
<td>2 (3.9)</td>
<td></td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>Thyroid medication</td>
<td>0 (0.0)</td>
<td>1 (2.0)</td>
<td></td>
<td>1 (1.8)</td>
<td>1 (2.4)</td>
<td></td>
</tr>
<tr>
<td>Methotrexate</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td></td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>Level of education, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Only primary education</td>
<td>7 (15.6)</td>
<td>14 (28.6)</td>
<td></td>
<td>10 (18.2)</td>
<td>6 (15.4)</td>
<td></td>
</tr>
<tr>
<td>Secondary education</td>
<td>22 (48.9)</td>
<td>16 (32.7)</td>
<td></td>
<td>17 (30.9)</td>
<td>15 (38.5)</td>
<td></td>
</tr>
<tr>
<td>Higher education</td>
<td>16 (35.6)</td>
<td>19 (38.8)</td>
<td></td>
<td>28 (50.9)</td>
<td>18 (46.2)</td>
<td></td>
</tr>
<tr>
<td>Human serum CRP, mean (SE), mg/l</td>
<td>0.21 (0.18)</td>
<td>0.75 (1.2)</td>
<td></td>
<td>0.25 (0.16)</td>
<td>0.15 (0.9)</td>
<td></td>
</tr>
</tbody>
</table>

*Data from 188 individuals were available.

**Table II. Absolute number of lymphocytes and T cells in individuals representing the general population (partners) and offspring from longevity families**

<table>
<thead>
<tr>
<th></th>
<th>Partners CMV- (n = 46)</th>
<th>CMV+ (n = 51)</th>
<th>p Value</th>
<th>Offspring CMV- (n = 56)</th>
<th>CMV+ (n = 41)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.99 ± 0.07</td>
<td>2.63 ± 0.45</td>
<td>0.05</td>
<td>1.88 ± 0.07</td>
<td>2.04 ± 0.10</td>
<td>0.33</td>
</tr>
<tr>
<td>T cells&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.37 ± 0.06</td>
<td>1.86 ± 0.33</td>
<td>0.04</td>
<td>1.33 ± 0.06</td>
<td>1.42 ± 0.07</td>
<td>0.45</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt; T cells&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.98 ± 0.04</td>
<td>1.17 ± 0.20</td>
<td>0.96</td>
<td>0.92 ± 0.05</td>
<td>0.94 ± 0.05</td>
<td>0.74</td>
</tr>
<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt; T cells&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.25 ± 0.02</td>
<td>0.47 ± 0.08</td>
<td>0.0001</td>
<td>0.26 ± 0.02</td>
<td>0.34 ± 0.03</td>
<td>0.001</td>
</tr>
<tr>
<td>CD4/CD8 ratio</td>
<td>4.22 ± 0.26</td>
<td>3.08 ± 0.23</td>
<td>0.001</td>
<td>4.26 ± 0.29</td>
<td>3.09 ± 0.19</td>
<td>0.002</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean absolute number (×10<sup>7</sup>/μl blood) ± SEM. Significant differences are highlighted in bold.
offspring from longevity families \( (p = 0.96; \text{Table I}) \). These results therefore suggest that offspring, even when infected with CMV, do not display a proinflammatory status in the way the general population does.

To exclude the difference in proinflammatory status between the CMV-seropositive offspring and partners as a factor influencing the frequency of different T cell subsets analyzed, we performed a correlation analysis between serum CRP levels and T cell subsets in all of the individuals tested. No significant correlation was found between any of the T cell subsets tested and serum CRP levels (Supplemental Table I).

**Individuals from families enriched for longevity do not show age-associated changes to their immune signatures**

Next, we determined the effect of age on the distribution of the different CD8\(^+\) T cell subsets in CMV-seropositive and -seronegative groups. For this, we compared immune signatures in CMV-matched middle-aged \((<65 \text{ y})\) and older \((>65 \text{ y})\) individuals. There was a marked age-associated reduction in the frequency of naive T cells within the CD8\(^+\) compartment, but only in CMV-seropositive partners \( (p = 0.008; \text{Fig. 3A, white circles}); \) in CMV-seronegative partners, the frequency of naive CD8\(^+\) T cells was not significantly affected by chronological age in this extensive cross-sectional study \( (p = 0.63; \text{Fig. 3A, gray circles}) \). However, this highly significant age-associated decrease in the frequency of naive T cells seen in the general population was absent in CMV-seropositive offspring, where middle-aged and old individuals had similar levels of naive T cells in the periphery \( (p = 0.44; \text{Fig. 3A, white squares}) \). As expected, an age-associated decrease in naive cells was also absent in CMV-seronegative offspring (Supplemental Fig. 3A). In addition, we did observe a trend toward a higher frequency of naive cells in older CMV-infected offspring compared with old infected partners (Fig. 3A; \( p = 0.019 \)), whereas the frequency of these cells was similar between the two groups before the age of 65.

As well as reductions in naive cells, accumulation of late-differentiated memory T cells is another hallmark of immunosenescence. We documented a strong trend toward an increase in the frequency of memory T cells expressing CD45RA\(^{+}\)CD27\(^{+}\)CCR7\(^{+}\) in CMV-infected offspring from long-lived families, in contrast to their partners, the frequency of these memory T cells did not increase with age \( (p = 0.77); \text{Fig. 3B}) \). As in the general population, the frequency of memory T cells did not increase with age in CMV-seronegative offspring (Supplemental Fig. 3B).

**Individuals from families enriched for longevity tend to show higher proliferative responses to CMV-derived Ags**

Having established resistance to the acquisition of age-associated CMV-driven hallmarks of immunosenescence and a lower proinflammatory status in CMV-seropositive offspring from long-lived families, we hypothesized that this might be due to a better immunological control of the virus in this rare group of individuals. To begin testing this at the functional level, we measured reactivity of T cells against overlapping peptides representing the entire sequence of the pp65 and the IE1 proteins, two immunodominant Ags from CMV, using the CFSE dilution assay in CMV-seropositive offspring and partners. Fig. 4 shows a representative flow cytometry plot from an offspring with high frequencies of proliferating \( (\text{CFSE}\text{lo}) \) CD4\(^+\) and CD8\(^+\) T cells when stimulated with both IE1 and pp65 Ag \( (\text{upper panel}) \). Her partner did not show any specific immune response against IE1 and only a low
CD4 response against pp65 (Fig. 4, lower panel). A specific proliferative response against at least one Ag could be detected in 13/21 (61.9%) of the offspring and 12/23 (51.2%) of the partners tested (difference not significant). We then dissected the immune responses to each Ag and the type of T cell response induced (Table III). A pp65-specific CD4 response could be documented in a higher proportion of the offspring (28%) compared with their partners (8%), but this did not reach statistical significance (p = 0.08). A similar proportion of offspring or partners had an anti-pp65–specific CD8− or an anti-IE1–specific CD4+ T cell response as well. However, there was a strong trend toward a higher proportion of offspring (42%) with a cytotoxic T cell response against IE1 Ag, compared with controls (13%) (p = 0.02; Table III).

We then compared the type and frequency of Ag-specific T cells in those individuals with a detectable anti-CMV immune response (responders). The majority of offspring (9/13) had T cell responses against peptides from both pp65 and IE1, whereas only 4/12 partners had T cells reactive against both (p = 0.02). We also compared the frequency of CMV Ag-specific T cells and found that the frequency of CD4+ T cells specific for pp65 was similar between offspring and partners. However, the former tended to have a higher frequency of pp65-specific CD8+ T cells (7.60 ± 3.5%), compared with partners (0.94 ± 0.31%), although this difference was not statistically significant (Table IV). The two groups had similar levels of CD4 and CD8 T cells specific for IE1. CD4 and CD8 T cells from both groups responded equally well to PHA stimulation (Supplemental Fig. 4).

FIGURE 4. CFSE dilution assay for tracking CMV-specific proliferating T cells. PBMCs were stained with CFSE, as described in Materials and Methods, and either left untreated (left panel) or stimulated with pp65 (middle panel) or IE1 (right panel), two immunodominant Ags from CMV, for 7 d. FACS plots showing CD3+ cells from a 61-year-old female offspring (upper row) and her 65-year-old partner (lower row) are shown.

Table III. Proportion of individuals with CMV-specific T cell responses (responders) in offspring from long-lived families and their partners representing the general population

<table>
<thead>
<tr>
<th></th>
<th>Offspring (%)</th>
<th>Partners (%)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pp65-specific CD4+</td>
<td>6/21 (28)</td>
<td>2/23 (9)</td>
<td>0.08</td>
</tr>
<tr>
<td>pp65-specific CD8+</td>
<td>10/21 (47)</td>
<td>8/23 (34)</td>
<td>0.39</td>
</tr>
<tr>
<td>IE1-specific CD4+</td>
<td>4/21 (19)</td>
<td>5/23 (21)</td>
<td>0.82</td>
</tr>
<tr>
<td>IE1-specific CD8+</td>
<td>9/21 (42)</td>
<td>3/23 (13)</td>
<td>0.02</td>
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CFSE dilution assay was used to determine the proliferative response in CD4+ and CD8+ T cells against peptide pools from IE1 or pp65, two immunodominant Ags from CMV.

Discussion

In this study, we have demonstrated that individuals from families enriched for longevity, with a 30% decreased mortality risk compared to the general population, are resistant to CMV-driven, age-associated reduction in naive T cells and accumulation of CD45RA− re-expressing and late-differentiated effector memory T cells, hallmark features of immunosenescence, believed to contribute to the weakened immune status observed in the elderly (1). We have analyzed offspring from families with long-lived siblings in comparison with their age-matched partners, minimizing the likelihood that the differences observed in immune signatures between the two groups are due to differences in lifestyle (11), socioeconomic, or environmental factors, as these people shared the same environment for many years, even many decades. Moreover, considering the fact that probably only half of the offspring carry the longevity-assurance genes, the observed difference between these rare individuals and their partners becomes even more striking.

Our data, showing a marked effect of CMV infection on reduced levels of naive CD8+ T cells and accumulation of memory T cells in the general population, are in line with some other published reports (6–8), adding more evidence to the hypothesis that persistent infection with CMV and not chronological age alone may be accelerating immunosenescence (9). Indeed, late-differentiated memory T cells are induced very rapidly after primary CMV infection, even in infants, and persist over long periods (15–18). Chronological age may add to this effect by simply prolonging the time that the immune system is exposed to the virus. Although direct evidence regarding the pathological impact of such a differentiated T cell compartment is still lacking, individuals with a low frequency of naive T cells in the periphery are assumed to be more susceptible to novel infectious agents. Furthermore, high frequencies of memory T cells in the periphery in CMV-infected individuals might fill up the “immunological space”, displacing...
T cells specific for other Ags. Indeed, the presence of a latent infection with CMV impairs the immune response against EBV, suggesting that CMV-seropositive individuals may be at greater risk for morbidity and mortality from heterologous infections (19). In addition, CMV is considered a cofactor that enhances progression to AIDS (20, 21). Consistent with this, CMV infection has been associated with poor clinical response to influenza vaccination in the elderly (22). In this context, individuals with a poor humoral response against influenza vaccination had higher frequencies of lymphocytes expressing CD57 and lacking CD28 (22–24), which accumulate in CMV-seropositive individuals but were present at lower levels in offspring from long-living siblings despite latent infection with CMV.

Another important finding in our study is that CMV-infected offspring from long-lived families have significantly lower levels of CRP, a marker for systemic inflammation, compared with age-matched CMV-infected controls, suggesting a lower proinflammatory status in these rare individuals (representing 0.5% of the population). CMV-associated increased proinflammatory status has been reported in several other studies, contributing to increased risk for atherosclerotic and coronary artery disease (25–27). Thus, the lower seroprevalence of CMV and the significantly lower proinflammatory status observed in the offspring in our study might be associated with the lower incidence of myocardial infarction, hypertension, and use of cardiovascular medication observed in the larger cohort of the LLS (11). The association between CMV and serum CRP values is being currently analyzed in the whole LLS cohort (A.B. Maier, T. de Craen, E. Derhovanessian, G. Pawelec, P.E. Slagboom, R.G.J. Westendorp, manuscript in preparation).

Clonal expansion of memory T cells observed in CMV-seropositive individuals may be a result of repeated exposure to Ag during reactivation of the virus. Thus, the absence of a CMV-driven late-differentiated phenotype of the CD8 compartment in the offspring observed in our study might be due to a lower reactivation rate of the virus in these individuals, which can also be reflected in the lower proinflammatory status observed. Our data demonstrating a higher CD8+ T cell response against IE1, one of the T cell immunodominant Ags of the virus, are in line with this hypothesis. Indeed, ample evidence from immunocompromised transplant or HIV patients documents a crucial role of the immune system, particularly CD8+ T cells, in anti-CMV immunity and in controlling the virus and CMV disease in these individuals (2). In murine models, IE-1–specific CD8+ T cells protected mice from a lethal challenge dose of virus, both in acute infection and following bone marrow transplantation with simultaneous murine CMV infection (28, 29). The few available human studies comparing the protective capacity of pp65- or IE-1–specific immune responses tend to be contradictory. However, some suggest that the immune response against IE-1 might be more effective than that against pp65 in controlling reactivation of the virus and in occurrence of CMV disease in transplant patients (30). In contrast, low proliferative response against CMV Ags in the controls compared with the offspring might be due to higher levels of differentiated effector memory cells, as well as T cells lacking the costimulatory receptors CD27 and CD28 and expressing the negative receptors CD57 and KLRG1, with poor proliferative capacity in this group. Indeed, CMV-specific T cells lacking CD45RA and CD28 have been shown to proliferate poorly and fail to undergo clonal expansion in vitro, even in the presence of IL-2 (8). Clearly, it cannot be excluded that the time of primary infection with the virus might be different between CMV-infected offspring and partners, leading to the difference observed in CRP levels and immune signatures in these individuals. Currently, virological methods do not allow determination of the time point of the primary infection, so there is no way of establishing the validity of this notion. However, it is known that socioeconomic status is an important parameter associated with CMV prevalence (31). We compared the level of education as a marker that would reflect socioeconomic status in adult life as well as during childhood, when CMV seroconversion is expected to occur most frequently. In our study, the CMV-seropositive offspring and partners were not significantly different regarding their level of education (Table I). Neither was there any difference in the titer of anti-CMV IgG in CMV-seropositive offspring and partners. Finally, lack of correlation between the serum level of CRP and the immune signatures analyzed implies that the difference observed in the T cell signatures is probably due to the genetic background of the individuals rather than time of primary infection or lower proinflammatory status in CMV-infected offspring.

In conclusion, our data demonstrate that individuals genetically enriched for longevity, with a 30% decreased mortality risk, possess immune signatures different from those of the general population. They fail to show the CMV- (and age-) associated alterations to immune parameters that the CMV-seropositive general population does show. This observation may imply that they retain better responsiveness to neoantigens and heterologous infection. Furthermore, the lower proinflammatory status in CMV-infected offspring might protect them from cardiovascular morbidity and ensure their healthy longevity. Our data support the notion that these two phenomena may be due to a better immunological control of the virus in the offspring from long-lived families. We therefore hypothesize that one mechanism by which the genes enriched in this population, and which are responsible for its longevity, operate is via an effect on immune status, and that this is also reflected in the individual’s handling of CMV.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1. General gating strategy for analyzing different naïve and memory T-cell subsets.

A) CD3+CD8+CD4- T cells were first divided into four groups (numbered 1-4) according to the surface expression of CD45RA and CCR7 following a widely accepted model (12). B) These subsets were further divided according to the surface expression of CD27 and CD28 as described previously (12, 13). C) Expression of differentiation markers CD57 and KLRG1 on naïve and memory T-cell subsets. The subsets are arranged according to simultaneous expression of CD57 and KLRG1. Mean and standard deviation of data from 87 control subjects are shown. D) In old CMV-seropositive individuals, we observed an accumulation of a CCR7- memory subset carrying low levels of CD45RA (marked with an arrow). This population was termed CD45RAmidCCR7-. N, naïve; CM, central memory; EM, effector memory.
Supplemental Figure 2. Effect of CMV-infection on the frequency of different CD8+ T-cell subsets in males (M) and females (F). A,B) The frequency of naïve (CD45RA⁺CCR7⁺CD27⁺CD28⁺) and late-differentiated effector memory (CD45RA⁻CCR7⁻CD27⁻CD28⁻) from total CD8⁺ T-cells. C) The frequency of CD8⁺ T-cells carrying KLRG-1. The mean and standard error of mean for each group is shown.
Supplemental Figure 3. The influence of age on the frequency of naïve (A) and memory (B) CD8+ T-cell subsets in middle aged (MA: <65 years) and Old (>65 years) CMV-seronegative offspring from long-lived families. MA: N=35, mean age 58.5 (46-65 years), Old: N=21, mean age 69.3 (65-74 years).
Supplemental Figure 4. Percentage of proliferating T-cell in response to PHA. PBMC were stained with CFSE as described in the Methods and treated with 0.1% PHA. The frequency of dividing cells was calculated from total CD4+ or CD8+ T-cells. CMV+P: CMV-seropositive partner (n=23), CMV+O: CMV-seropositive offspring (n=21).
**Supplemental Table I.** Correlation analysis between the serum CRP levels and the frequency of different T-cell subsets analysed (n=194).

<table>
<thead>
<tr>
<th>T-cell subset</th>
<th>Spearman’s r</th>
<th>p value</th>
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<tr>
<td>CD45RA+CCR7+CD27+CD28+</td>
<td>-0.07</td>
<td>0.33</td>
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<tr>
<td>CD45RA-CCR7-CD27-CD28-</td>
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<td>0.43</td>
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<tr>
<td>CD57+</td>
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<td>KLRG1+</td>
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