CD4:8 Ratio >5 Is Associated With a Dominant Naive T-Cell Phenotype and Impaired Physical Functioning in CMV-Seropositive Very Elderly People: Results From the BELFRAIL Study

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A subset of older people is at increased risk of hospitalization and dependency. Emerging evidence suggests that immunosenescence reflected by an inverted CD4:8 ratio and cytomegalovirus (CMV) seropositivity plays an important role in the pathophysiology of functional decline. Nevertheless, the relation between CD4:8 ratio and functional outcome has rarely been investigated. Here, CD4:8 ratio and T-cell phenotypes of 235 community-dwelling persons aged ≥81.5 years in the BELFRAIL study and 25 younger persons (mean age 28.5 years) were analyzed using polychromatic flow cytometry. In the elderly persons, 7.2% had an inverted CD4:8 ratio, which was associated with CMV seropositivity, less naive, and more late-differentiated CD4+ and CD8+ T cells. However, 32.8% had a CD4:8 ratio >5, a phenotype associated with a higher proportion of naive T cells and absent in young donors. In CMV seropositives, this subgroup had lower proportions of late-differentiated CD4+ and CD8+ T cells and weaker anti-CMV immunoglobulin G reactivity. This novel naive T-cell-dominated phenotype was counterintuitively associated with a higher proportion of those with impaired physical functioning in the very elderly people infected with CMV. This underscores the notion that in very elderly people, not merely CMV infection but also the state of its accompanying immune dysregulation is of crucial importance with regard to physical impairment.

Key Words: CD4:8 ratio—T-cell subsets—Physical functioning—Very elderly people—Cytomegalovirus.

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OLDER persons show differences in both innate and adaptive immune measures with increasing age, generally interpreted in terms of declining immune competence. One of the most prominent changes is a profound remodeling of CD8+ and to a lesser extent CD4+ T-cell phenotypes characterized by an accumulation of late-differentiated effector memory and effector T cells potentially filling the “immunological space.” These changes are generally believed to be detrimental because they are associated with a decreased response to vaccination, higher incidence and severity of infectious diseases, and a low chronic inflammatory condition associated with frailty (1–3).

The β-herpesvirus human cytomegalovirus (CMV) has a major role in the remodeling of the T-cell compartment (4). CMV infection is also part of the original “Immune Risk Profile (IRP).” The concept of the IRP has emerged in an attempt to find predictive blood markers of deteriorating immune function based on the findings of the Swedish OCTO and NONA immune longitudinal studies (5–9). The IRP was associated with an elevated 2-, 4-, and 6-year mortality rate (10).

Essentially, the IRP could be defined using solely an inverted CD4:8 ratio as a surrogate marker, which was present in about 15% of free-living 85-year olds, caused by an accumulation of late-stage CD8+ memory cells. However, longitudinal follow-up showed that by the time the surviving OCTO and NONA participants had become nonagenarians and centenarians, individuals with an inverted CD4:8 were no longer present, suggesting selection against people with this risk factor (11). This implies that different risk factors may become prevalent at even more advanced age or under different conditions in different populations.

Along these lines, in the community-dwelling “Leiden 85+” population, we observed that a lower proportion of naive
CD8+ T cells, associated with a higher proportion of memory cells, predicted better survival from the age of 89 years over a 8-year follow-up (12). We postulated that the reason for this could be that in such a population, exposure to novel pathogens would be minimal, so that there would be no advantage to possessing more naive cells; in contrast, more memory cells, mostly CMV reactive, would be beneficial for more efficient immunosurveillance or control of CMV under these circumstances. In general, this higher frequency of effector memory-type cells could be beneficial because sessile elderly individuals are more likely to be challenged by previously encountered agents, or persistent viruses like CMV and other herpesviruses, rather than by novel pathogens.

Emerging evidence suggests that this immune remodeling also plays an important role in the pathophysiology of functional decline in the elderly people, in which a decreased CD4:8 ratio was associated with poor functioning in elderly people (13–15).

Similarly, a limited number of studies, though conducted in selected populations, have demonstrated a relationship between CMV infection and functional ability (16). Nonetheless, the identification of a subset of older people at increased risk of hospitalization, dependency, and a reduced life expectancy is important for recommending measures aimed at prevention and rehabilitation at the earliest possible time at the individual level and for planning adequate health policies at the societal level. This is crucial because the forthcoming so-called “grey epidemic” will pose a major challenge to our health care systems with an increased demand for social and medical care and economical costs (17).

Therefore, our objective in the BELFRAIL study was to investigate the T-cell phenotype distribution in a larger and less highly selected age group, compared with the Leiden 85+ study, of community-dwelling participants aged 80 and older and investigate their relationship with CMV infection and functional ability. We confirmed the presence of an IRP defined as an inverted CD4:8 ratio, at about half the frequency in these participants compared with the Swedish study (7% vs 15%). But strikingly, we found that a much higher proportion of the population had exceedingly high CD4:8 ratios and that this correlated with a weaker anti-CMV immunoglobulin G (IgG) reactivity and higher proportion of physically impaired elderly people when they were infected with CMV.

**METHODS**

**Study Design and Participants**

Data were derived at the second data point of the BELFRAIL study, a representative ongoing cohort study of community-dwelling individuals over 80 years old in Belgium. All participants in the study gave informed consent, and the Biomedical Ethics Committee of the Medical School of the Université Catholique de Louvain of Brussels approved the study. The study design, methods, recruitment of participants, and characteristics of the cohort were previously described in detail (18). Briefly, 29 general practitioner centers included 567 patients aged 80 and older at baseline (November 2, 2008, and September 15, 2009). Only three exclusion criteria were used: those with known severe dementia (Mini-Mental State Examination [MMSE] <15), those in palliative care, and those with medical emergencies. Eighteen months after baseline, T-cell subsets were measured for a study population of 235 participants (Supplementary Figure S1). This random subgroup with a mean age of 86.7 years had no significant difference with the total BELFRAIL population, except a slightly lower non-cardiovascular comorbidity and Geriatric Depression Scale (GDS)-15 score (Supplementary Table S1). A control group of 25 younger participants, with a mean age of 28.5 years, was recruited among among members and students.

**Flow Cytometry Analysis**

Between March 19, 2011, and May 10, 2011, peripheral blood mononuclear cells were isolated and cryopreserved from 276 older patients and 25 younger participants from the control group (isolated between January 10, 2012, and January 17, 2012). After thawing, 41 donors were excluded due to low quality of peripheral blood mononuclear cells. Cryopreserved peripheral blood mononuclear cells were treated with human immunoglobulin, GAMUNEX (Bayer, Leverkusen, Germany), and ethidium monoazide (Invitrogen, Karlsruhe, Germany) to block Fc receptors and label nonviable cells. Peripheral blood mononuclear cells were then stained indirectly with anti-CCR7 primary antibody (R&D Systems, Wiesbaden-Nordenstadt, Germany) and Pacific Orange–conjugated goat anti-mouse IgG (Invitrogen). After blocking with mouse serum (Chemicon/Millipore, Schwalbach, Germany), directly conjugated monoclonal antibodies, CD3-Alexa Fluor 700, CD4-PerCP, CD8-allophycocyanin-H7, CD45-RA-V450, CD28-PE (BD Biosciences, Heidelberg, Germany), CD27-allophycocyanin (BioLegend, San Diego, CA), and CD57-FITC (Immunotools, Freiburg, Germany) were added. After 20 minutes of incubation on ice, cells were washed and analyzed immediately on an LSR II cytometer with FACSDiva software (BD Biosciences). The BD FACSDiva software calculated the spectral overlap between all channels automatically after measuring negative and single-color controls. Data were analyzed using FlowJo Software (Tree Star, Portland, OR). For data analysis, ethidium monoazide–positive 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 according to surface expression of CD45RA, CCR7, CD27, and CD28 based on a model suggested by Romero and coworkers (19) (Supplementary Figure S2). Flow cytometry staining and data analysis were performed on blinded samples. Parent populations with counts below 150 were excluded from the analysis.
T-cell subsets were expressed as proportions and T-cell differentiation indexes were determined at the individual donor level by calculating the ratio of late-differentiated effecter memory and effector subsets divided by the frequency of naive T cells for each person (12). Absolute T-cell numbers (count/μL) were calculated by the formula: total lymphocyte count (cells/μL) × 1,000 × frequency of T-cell subset in decimal.

CMV IgG Status
The CMV IgG serostatus was determined by means of a recombinant CMV IgG immunoblot (Mikrogen, Neuried, Germany) using six different epitopes (IE-1, p150, CM2, p65, gB1, and gB2). An individual was defined to be CMV IgG positive, if the reactivity was at least weakly positive (+) for p150 or other antigens compared with a control and according to the information of the manufacturer. Based on the scores, high reactivity (++ and ++++) and very high reactivity (++++) were defined.

Performance Measures
The clinical research associate successfully carried out performance testing on 223 participants of the study population between December 9, 2010, and August 30, 2011 (Supplementary Figure S1).

Physical Performance
The activities of daily living (ADL) and short physical performance battery (SPPB) were assessed as a proxy for the participant’s physical dependence and performance, respectively. Physical limitations of daily living were assessed by asking the respondent to describe the degree of difficulty they had with six ADL: climbing stairs, walking 5 minutes outdoors without resting, getting up and sitting down in a chair, dressing and undressing oneself, using own or public transportation, and cutting one’s own toenails. The total score was calculated by summing the scores of all of the activities (range: 6–30). The lowest gender-adjusted quartiles of ≤18 or ≤17 for men and women were used as cutoff, respectively. The SPPB consists of timed measurements of walking speed, rising from a chair, putting on and taking off a cardigan, and maintaining balance in a tandem stand. Categories of performance were created for each set of performance measures to permit analyses that included those unable to perform a task. The summary performance scale, ranging from 0 to 14, has been used in several studies and has been shown to be a reliable and valid measure of physical functioning (20–22). The lowest gender-adjusted quartiles of ≤4 for men and women were used as cutoff to define a physical impairment.

Mental Performance
The MMSE score and GDS-15 were reported as a proxy for the participant’s cognitive functioning and suspected depression status, respectively. For the MMSE, a cutoff of 24 points (range: 6–30) was used for mild to severe cognitive impairment. The GDS-15 has been especially designed to screen for suspected depression in the elderly people (23). A cutoff of five of more points was used for a depressed status.

Comorbidity
Noncardiovascular comorbidities were defined as thyroid problems, anemia, asthma, chronic obstructive pulmonary disease, Parkinson’s disease, arthritis, osteoarthritis, documented osteoporosis, malignancy, and renal insufficiency. Cardiovascular comorbidities were defined as hypertension, diabetes mellitus, hyperlipidemia, a history of angina pectoris or myocardial infarction, known cardiomyopathy, a history of transient ischemic attack or cardiovascular accident, peripheral arterial disease, a history of decompensated heart failure, atrial fibrillation, valvular disease, or a history of edema of the lower extremities.

Statistical Analyses
Continuous data are presented as the mean and standard deviation (SD) or median and interquartile range (IQR). Categorical data are presented as numbers and frequencies. Comparisons between different categories of participants were performed using the chi-square test or Fisher exact test, if appropriate, and Student’s t test or Mann–Whitney U test for continuous data. The Kruskal–Wallis test and p for trend test were used to compare continuous and categorical data between three or more groups, respectively. p < .05 was considered to be statistically significant. Dot plots are shown with median and IQR.

The relationship between physical and mental functioning (dependent variable) and CD4:8 ratio phenotypes (independent variable) was assessed with multivariate logistic regression analyses. For all of the analyses, robust estimates were used because the assumption of constant variance of the error was violated (24). Two consecutive adjusted models were used to assess the relationship with physical and mental impairment. In the first model, age categories and gender were included to correct for potential confounding; in the second model, analyzed batch, number of cardiovascular comorbidities, and number of noncardiovascular comorbidities were added.

The statistical analyses were performed using STATA 11 (StataCorp, College Station, TX) and GraphPad prism 6 (GraphPad Software, San Diego, CA).

Results
Demographics and T-Cell Subset Distribution of the Old and Young Population With Regard to CMV Serostatus
The mean age of the 235 study participants was 86.7 years, of whom 67.2% were women. One hundred and three (74%) were CMV seropositive. None of these
participants was free of pathology of one type or another. Nine percent were institutionalized and significantly fewer women were present in the CMV-seronegative older persons, together with a trend toward a higher education level (Table 1). The control group, consisting of 25 young adults, had a mean age of 28.5 years, of whom 60% were women. Twelve percent were CMV seropositive; these individuals were significantly older compared with the seronegative individuals (Table 1).

In the aged population, CMV seropositivity was significantly associated with a lower CD4:8 ratio (R). Both, in the aged and young population, a higher CD8+ and CD4+ T-cell differentiation index were significantly associated with CMV seropositivity (Supplementary Table S2). Also, higher proportions of late-differentiated effector memory (EM3, defined as CD45RA−CCR7−CD28−CD27−) and late-differentiated effector (E, defined as CD45+CCR7−CD28−CD27−) CD4+ and CD8+ T cells were significantly associated with a CMV infection. No significant differences were found with respect to the proportion of naive (N, defined as CD45RA+CCR7+CD28+CD27+) T cells between the seropositive and seronegative individuals. Nonetheless, lower proportions of early differentiation markers CD27+ and CD28+ and higher proportions of late-differentiated CD57+ cells were seen in the CD4+ and CD8+ subsets in CMV-seropositive individuals.

All of the T-cell phenotypes and ratios were significantly different between the old and young groups.

**Distribution of T-Cell Differentiation Phenotypes Based on CD4:8 Ratios (R) Relative to CMV Infection**

Seventeen elderly individuals (7.2%) and one younger individual had an R < 1, which was significantly associated with a latent CMV infection in the older population (Table 2). Seventy-seven individuals (32.8%), of whom 62.3% were CMV seropositive, had an R > 5. This group showed no difference in terms of proportions or absolute numbers of CD4+ T cells but had a significantly lower median CD8+ T-cell content (5.49%; absolute numbers, Kruskal–Wallis: p < .001). The phenotype R > 5 was not observed in any of the younger individuals (Rmedian: 3.23). The R > 5 group was characterized by a significantly high proportion or absolute number of naive T cells and a low proportion or absolute number of EM3 CD4+ and E CD4+ and CD8+ T cells (Table 2 and Supplementary Figure S3).

When stratifying for CMV serostatus as a confounder, both the R > 5 CMV-seropositive and CMV-seronegative groups had a significantly higher frequency of naive CD4+ and CD8+ T cells compared with the 1 < R < 5 or R < 1 group (Figure 1). This difference was also observed when absolute cell numbers were analyzed (results not shown). Higher proportions of late-stage E CD4+ and CD8+ T cells were observed only in the seropositive R < 1 and 1 < R < 5 group compared with the R > 5 group (Figure 2A). Similarly, the EM3 CD4+ T-cell frequency was significantly higher in the seropositive R > 5 group only (Supplementary Figure S4A). The proportions of EM3 CD8+ T cells did not differ significantly across the three groups, independent of CMV infection. These exact differences for the late-differentiated E and EM3 CD8+ and CD4+ T cells were also observed in terms of absolute numbers (results not shown). Significantly higher proportions of CD4+ and CD8+ T cells expressing the costimulatory molecules CD27 were seen in the seropositive R > 5 group compared with the 1 < R < 5 or R < 1 seropositive group (Supplementary Figure S4B). Furthermore, a significantly higher number of CD27+CD4+ T cells was also seen in the seronegative R > 5 group. Figure 2B shows that a significantly lower proportions of the CD8+ and CD4+ T cells were late differentiated or potentially “senescent” (CD57+) in the seropositive R > 5 group compared with the 1 < R < 5 and R < 1 group. This difference was also observed in terms of absolute numbers (results not shown).

**Relationship Between Anti-CMV IgG Reactivity and CD4:8 Ratio**

A linear trend toward higher proportions of individuals with high anti-CMV IgG reactivity against five major viral proteins (pp65, IE-1, CM2, gB1, and gB2) with decreasing

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**Table 1. Demographics of the Study and Control Population, Based on Their Cytomegalovirus (CMV) Serostatus**

<table>
<thead>
<tr>
<th></th>
<th>General (n = 25)</th>
<th>CMV+ (n = 22)</th>
<th>CMV− (n = 22)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD</td>
<td>28.5 ± 6.1</td>
<td>37.8 ± 11.6</td>
<td>27.2 ± 9.0</td>
<td>.003</td>
</tr>
<tr>
<td>Women, n (%)</td>
<td>15 (60)</td>
<td>2 (66.7)</td>
<td>13 (59.1)</td>
<td>1</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>Non-CV comorbidity, median (interquartile range [IQR])</td>
<td>21 (8.94)</td>
<td>17 (9.83)</td>
<td>4 (6.45)</td>
<td>.424</td>
</tr>
<tr>
<td>CV comorbidity, median (IQR)</td>
<td>3 (2–4)</td>
<td>2 (2–3)</td>
<td>3 (2–4)</td>
<td>.306</td>
</tr>
</tbody>
</table>

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CD4:8 ratio was shown (Figure 3). The high IgG reactivity against tegument protein pp65 and very high IgG reactivity against pp65, IE-1, and gB1 were significantly less frequent with increasing CD4:8 ratio.

**Impairment of Physical and Mental Performance in Patients With a R > 5 or R < 1, Relative to CMV Serostatus**

This study population had a mean ADL score of 21.8 ± 5.9, a mean SPPB score of 7.06 ± 3.7, a median MMSE score of 28 (IQR: 26-29), and a median GDS-15 score of 2 (IQR: 1–3). In the R > 5 phenotype, a significant subgroup of 36.1% had a low SPPB score compared with the 23% in the 1 < R < 5 group (Figure 4). Likewise, a low ADL score was present in 33.3% of those with an R > 5 compared with 20.9% in the 1 < R < 5 group. After stratification for CMV infection, a significant impairment of ADL and SPPB was only observed in the R > 5 group of CMV-seropositive individuals but not in those who were CMV seronegative (Figure 4). In the CMV-seropositive R > 5 group, 38.6% and 36.4% had SPPB and ADL impairment compared with...

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**Table 2. CD4:8 Ratio Distribution in Older Persons**

<table>
<thead>
<tr>
<th>Variables</th>
<th>R &lt; 1 (n = 17, 7.2%)</th>
<th>1 &gt; R &lt; 5 (n = 141, 60%)</th>
<th>R &gt; 5 (n = 77, 32.8%)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD 87.7 ± 3.4</td>
<td>68.4 ± 3.7</td>
<td>68.4 ± 3.7</td>
<td>.24</td>
<td></td>
</tr>
<tr>
<td>Women, n (%)</td>
<td>12 (70.6)</td>
<td>96 (68.1)</td>
<td>50 (65)</td>
<td>.85</td>
</tr>
<tr>
<td>CMV IgG+, n (%)</td>
<td>15 (88.2)</td>
<td>110 (78)</td>
<td>48 (62.3)</td>
<td>.016</td>
</tr>
<tr>
<td>CD4+/CD3, median (IQR) 64.3 (45–80.11)</td>
<td>45.3 (29.5–78)</td>
<td>53.7 (39–74.5)</td>
<td>.24</td>
<td></td>
</tr>
<tr>
<td>N/CD4 24.5 (10.9–37.8)</td>
<td>34.4 (24.3–46.2)</td>
<td>55.7 (40.3–64.9)</td>
<td>&lt;.001</td>
<td></td>
</tr>
<tr>
<td>EM3/CD4 6.04 (2.9–8.7)</td>
<td>1.49 (0.24–5.3)</td>
<td>0.27 (0.04–1.04)</td>
<td>&lt;.001</td>
<td></td>
</tr>
<tr>
<td>E/CD4 0.62 (0.19–4.6)</td>
<td>0.4 (0.03–1.51)</td>
<td>0.08 (0.01–0.44)</td>
<td>.003</td>
<td></td>
</tr>
<tr>
<td>CD8+/CD3, median (IQR) 39.5 (24.8–46.1)</td>
<td>11.6 (7.2–18.3)</td>
<td>5.49 (3.58–8.15)</td>
<td>&lt;.001</td>
<td></td>
</tr>
<tr>
<td>N/CD8 0.78 (0.44–2.58)</td>
<td>3.67 (1.6–8.4)</td>
<td>10.4 (4.9–22.3)</td>
<td>&lt;.001</td>
<td></td>
</tr>
<tr>
<td>EM3/CD8 5.55 (2–16.4)</td>
<td>3.36 (1.8–8.79)</td>
<td>2.98 (1.34–8.7)</td>
<td>.276</td>
<td></td>
</tr>
<tr>
<td>E/CD8 50.4 (37.8–60.3)</td>
<td>30.7 (16–42.1)</td>
<td>14.8 (8.3–26.4)</td>
<td>&lt;.001</td>
<td></td>
</tr>
</tbody>
</table>

**Note:** CM = central memory; CMV = cytomegalovirus; E = effector; EM3 = effector memory 3; IgG = immunoglobulin G; N = naïve; R = CD4/CD8 ratio.
22.6% and 20.2% in the 1 < R < 5 group, respectively. In the CMV-seronegative high-ratio group, 32% and 28.6% had SPPB or ADL impairment compared with 24% and 23.3% in the 1 < R < 5 group. No significant differences were found with respect to GDS-15 or MMSE score between people with an R > 5 and the 1 < R < 5 group, regardless of CMV infection (Supplementary Figure S5). No significant differences for physical or mental impairment were found

Figure 2. Expression dot plots (median and interquartile range) of late-stage effector (CD45RA+CCR7−CD27−CD28−) T cells (A) and late-differentiation marker CD57 (B), relative to cytomegalovirus serostatus. p value from Kruskal–Wallis test to compare between three phenotypes.
Figure 3. Proportion of older cytomegalovirus (CMV)-seropositive individuals with a high (upper) or very high (lower) anti-CMV IgG reactivity against five major structural proteins, relative to CD4:8 ratio phenotypes. The CMV IgG serostatus was determined by means of a recombinant CMV IgG immunoblot using six different structural epitopes (IE-1, p150, CM2, pp65, gB1, and gB2). Based on the scored level, very high reactivity (+++) and high reactivity (++) were defined (**p < .05, *p < .10—p for trend test).

Figure 4. Percentage of physical impairment according to their CD4:8 ratio phenotype and cytomegalovirus serostatus. (chi-square test, *p < .05), SPPB: short physical performance battery; ADL: activities of daily living.
between the $R < 1$ group and the $1 < R < 5$ group (Figure 4 and Supplementary Figure S5).

After correcting for age and gender, the probability of having a low SPPB or ADL score was 2.42- or 2.9-fold higher in the seropositive $R > 5$ group than in the seropositive $R < 5$ group, respectively (Table 3). These odds ratios remained significant after further correction for the batch number, number of cardiovascular comorbidities, and number of noncardiovascular comorbidities.

**Age-Specific Prevalence of $R > 5$ in the Belgian Population**

To estimate the age-specific prevalence of the different CD4:8 ratio phenotypes, the CD4:8 ratios of 235 elderly and 25 younger individuals of the BELFRAIL study were pooled with the identically measured CD4:8 ratios of 54 donors (range: 20–81 years) from a clinical influenza vaccine trial in Antwerp, Belgium. The study design, methods, recruitment of participants, and characteristics of this study population were previously described in detail by Derhovanessian and coworkers (25). The CD4:8 ratio significantly increased with 10-year age categories (Kruskal–Wallis, $p = .002$) and the $R > 5$ phenotype was not prevalent before the age of 50 years (Figure 5). Eighteen individuals had a CD4:8 ratio above 10, with very low CD8 (median: 3.46; IQR: 1.57–5.37) T-cell proportions compared with CD4 T cells (median: 50.9; IQR: 37.1–71.8; Supplementary Figure S3 and Table 2).

**DISCUSSION**

To the best of our knowledge, here we have defined for the first time a large naive T-cell-dominated subgroup of elderly people with a CD4:8 ratio $> 5$, which we have shown to be associated with lower anti-CMV IgG reactivities and a higher proportion of those with physical impairment in CMV-seropositive individuals. This was not the case for cognitive function.

**Prevalence of CD4:8 Ratio Spectrum in Older Persons**

With decreasing CD4:8 ratio, there was merely an increase in CD8+ T-cell proportion compared with no change in the CD4+ T cells. This is in line with the CD8+ T-cell accumulation in the 15% of people with an inverted

| Table 3. Odds Ratios of Multivariate Logistic Regression Analysis Between Physical Functioning and High CD4:8 Ratio Phenotype, Stratified for CMV Infection |
|---------------------------------|------------------|------------------|------------------|
|                                 | Unadjusted Model | Model 1           | Model 2           |
|                                 | OR (CI)          | C-stat           | $p$ value         | OR (CI)          | C-stat           | $p$ value         | OR (CI)          | C-stat           | $p$ value         |
| CMV+                            |                  |                  |                  |                  |                  |                  |                  |                  |                  |
| SPPB impairment                 | 2.17 (1.03–4.57) | 0.58             | .042             | 2.42 (1.12–5.26) | 0.71             | .052             | 2.33 (1.05–5.18) | 0.75             | .038             |
| Age categories                  |                  |                  |                  |                  |                  |                  |                  |                  |                  |
| Gender                          | 2.41 (1.52–3.81) | .032             | <.001            | 2.24 (1.37–3.66) | .032             | .001             | 1.05 (0.52–2.15) | .638             | .674             |
| Batch                           | 0.68 (0.29–1.6)  | .374             |                  | 0.68 (0.32–0.99) | .374             |                  | 0.98 (0.91–1.06) | .374             |                  |
| Number of CV                    |                  |                  |                  |                  |                  |                  |                  |                  |                  |
| Number of non-CV                |                  |                  |                  |                  |                  |                  |                  |                  |                  |
| ADL impairment                  | 2.67 (1.27–5.8)  | 0.61             | .013             | 2.67 (1.27–5.8)  | 0.61             | .013             | 1.96 (1.05–3.67) | .024             | .016             |
| Age categories                  | 2.65 (1.26–5.77) | .69              | .009             | 2.65 (1.26–5.77) | .69              | .009             | 1.96 (1.05–3.67) | .024             | .016             |
| Gender                          | 0.95 (0.42–2.66) | .901             |                  | 0.95 (0.42–2.66) | .901             |                  | 0.98 (0.91–1.06) | .901             |                  |
| Batch                           |                  |                  |                  |                  |                  |                  |                  |                  |                  |
| Number of CV                    |                  |                  |                  |                  |                  |                  |                  |                  |                  |
| Number of non-CV                |                  |                  |                  |                  |                  |                  |                  |                  |                  |
| CMV−                            |                  |                  |                  |                  |                  |                  |                  |                  |                  |
| SPPB impairment                 | 1.36 (0.44–4.26) | 0.54             | .595             | 1.69 (0.48–5.88) | 0.75             | .412             | 1.41 (0.38–5.24) | 0.78             | .610             |
| Age categories                  | 2.82 (1.16–6.84) | .022             |                  | 2.82 (1.16–6.84) | .022             |                  | 2.87 (1.16–6.98) | .02             |                  |
| Gender                          | 1.48 (0.41–5.37) | .555             |                  | 1.48 (0.41–5.37) | .555             |                  | 1.83 (0.47–7.18) | .386             |                  |
| Batch                           |                  |                  |                  |                  |                  |                  |                  |                  |                  |
| Number of CV                    |                  |                  |                  |                  |                  |                  |                  |                  |                  |
| Number of non-CV                |                  |                  |                  |                  |                  |                  |                  |                  |                  |
| ADL impairment                  | 1.02 (0.33–3.18) | 0.50             | .970             | 1.22 (0.36–4.15) | 0.74             | .750             | 1.01 (0.29–3.5)  | 0.77             | .984             |
| Age categories                  | 2.68 (1.16–6.21) | .022             |                  | 2.68 (1.16–6.21) | .022             |                  | 2.69 (1.2–6.02)  | .016             |                  |
| Gender                          | 1.05 (0.3–3.73)  | .938             |                  | 1.05 (0.3–3.73)  | .938             |                  | 1.2 (0.31–4.64)  | .792             |                  |
| Batch                           |                  |                  |                  |                  |                  |                  |                  |                  |                  |
| Number of CV                    |                  |                  |                  |                  |                  |                  |                  |                  |                  |
| Number of non-CV                |                  |                  |                  |                  |                  |                  |                  |                  |                  |

**Notes:** ADL = activities of daily living; CI = confidence interval; CMV = cytomegalovirus; C-stat = C-statistics; CV = cardiovascular; OR = odds ratio; SPPB = short physical performance battery. Model 1: age categories and gender. Model 2: age categories, gender, batch, number of cardiovascular diseases, and number of noncardiovascular diseases.
CD4:8 ratio in the OCTO//NONA studies (8). In the present cohort, only 7.2% of the elderly people (and one younger individual) had an inverted CD4:8 ratio, which might be due to the slightly higher age of the BELFRAIL participants. Indeed, in the Leiden 85+ cohort, only 2% of individuals aged 89 years showed an CD4:8 ratio <1 compared with 20% in those between 70 and 81 years of age (12).

We observed a group of almost 33% with a CD4:8 ratio >5, not represented at all in the younger individuals who had a maximum CD4:8 ratio of 3.23. Pooling the CD4:8 ratios with the CD4:8 ratios from 54 donors from a trial in Antwerp gave us a rough idea of the age-specific prevalence of the R > 5 phenotype in the Belgian population. This showed that the R > 5 phenotype and CD:8 ratio significantly increased with age (Figure 5 and Supplementary Table S2), rather than decreased, suggesting that the R > 5 is a distinct phenotype for older persons after the age of 50 years. Likewise, Strindhall and coworkers (11) showed that in surviving OCTO//NONA participants older than 100 years, no inverted CD4:8 ratio had been recorded from the age of 85 on and found the average CD4:8 ratios to increase with age. This increase with age in CD4:8 ratio was also observed in the Japanese population (26). Although this finding was not emphasized, the NONA study in fact recorded a distinct subgroup of individuals with a CD4:8 ratio between 4 and 11 (8). The finding of increasing prevalence of the high CD4:8 ratio phenotype should be confirmed in additional populations in other countries to understand whether this other extreme end of the CD4:8 ratio distribution becomes relevant in late life. Additionally, because our groups younger than 80 years consisted of only 25 and 54 donors from another community-dwelling study, future studies in different age groups are needed to establish whether the high ratio phenotype is indeed absent in younger individuals and if so, at what age it emerges.

Despite the immunosuppressed status of those with CD4:8 ratios above 10, no differences whatsoever were found in terms of available patient characteristics (age, comorbidity patterns, CMV serostatus, anti-inflammatory medication, infectious antibiotics prescriptions, etc.). Of course, we cannot completely rule out the possibility of nondiagnosed diseases (such as subclinical cancer or HIV) or of nonregistered medication use by the general practitioner. Nevertheless, our very heterogeneous population is a representative sample of the general oldest old. The nature and cause of this immunosuppressed status is certainly an important topic for future research.

The Relation Between CD4 and CD8 Remodeling, CMV Serostatus, and CD4:8 Ratio Phenotypes

CMV infection was present in the majority of the elderly people and clearly associated with a lower CD4:8 ratio and expansion of the late-differentiated CD8+ and CD4+ T-cell compartment in our community-dwelling very elderly people, as has been previously reported in other populations (4,27). We did not observe a difference in the proportion of naive CD8+ and CD4+ T cells between the CMV-seropositive and CMV-seronegative individuals, which is probably the result of age-related exhaustion (12). Consistent with the recent study of Turner and coworkers (28), the accumulation of late-stage CD8+ and CD4+ T cells was also seen in the three CMV-seropositive young individuals, although to a lesser extent, as all ratios and T-cell subsets were significantly different between the young and old groups.

BELFRAIL participants in the R > 5 group had the highest proportion of naive CD8+ and CD4+ T cells, independent of CMV serostatus. In the case of CD4+ cells, the proportion of naive cells was almost as high as in the younger participants. Unfortunately, there is no evidence on whether these naive T cells are of (preserved) thymic origin despite the advanced age or whether they are peripherally produced, which should be further clarified in the future to better understand this phenotype and the origin of these cells. A large difference in the distribution of E T cell differentiation subsets was noticed in the CMV-seropositive CD4:8 ratio phenotypes compared with the CMV-seronegative participants (Figure 4). This indicates a lesser or almost-absent CMV-associated expansion of the late-differentiated CD8+ and CD4+ T-cell compartment in those with a high CD4:8 ratio compared with higher frequencies of these cells in CMV-seropositive donors with lower CD4:8 ratios.

Figure 5. Crude prevalence of CD4:8 ratio spectrum with age. Pooled CD4:8 ratio data from BELFRAIL study and a Belgian community-dwelling clinical trial on influenza vaccination. The CD4:8 ratio significantly increased with 10-year age categories (Kruskal–Wallis, p = .002).
Relation Between CD4:8 Ratio and Impaired Physical Functioning With Regard to CMV Serostatus

We showed that impaired physical dependency (assessed by ADL) and performance (SPPB) were significantly more frequent in those elderly people with a high CD4:8 ratio. After stratification and correcting for possible confounders, we showed that only the CMV-seropositive individuals with a high CD4:8 ratio included a higher percentage of persons with an impaired physical status. This contrasted with similar percentages over the CMV-seronegative CD4:8 ratio subgroups. This impairment may be related to their naive cell–dominated T-cell compartment, with fewer effector memory cells available for controlling CMV (eg, in case of reactivation or superinfection), leading to a stronger inflammatory response upon uncontrolled viral reactivations. This strong inflammatory response needed to suppress an episode of CMV reactivation may be more extensive than the inflammatory response necessary to prevent reactivation through the CMV effector T-cell-mediated immunosurveillance. It has been shown that inflammation affects muscle mass and strength and subsequently physical functioning (29,30). Therefore, the more excessive inflammation needed to counter reactivation may explain why physical functioning is more affected in the group with high CD4:8 ratio. Confirming our hypothesis, we also showed that the $R > 5$ group had significantly lower proportions of individuals with high to very high anti-CMV IgG reactivity or a weaker humoral immunosurveillance against certain major viral proteins.

At first sight, these results are in contrast to a study of immunity in people living in a Spanish nursing home. Moro-Garcia and coworkers (13) showed that poorly functioning elders, having a low ADL score, had a decreased CD4:8 ratio. However, the lower number and younger age of the Spanish individuals, and particularly the fact that they were institutionalized, could explain these differences. In fact, our data are quite in line with the results from a different Spanish study on community-dwelling participants (the OCTABAIX immune study), which showed that the subgroup with poor health status did not have a lower CD4:8 ratio in individuals aged 85 years (14). Together, these studies emphasize the importance of comparing different populations under different conditions, rather than expecting to find a global “one-pattern-fits-all” set of biomarkers for immunity, aging, and physical performance. It remains to be seen whether survival will also be associated with an abnormally high CD4:8 ratio in our study. Nevertheless, our findings here are in line with data from the community-dwelling Leiden 85+ study, showing that a lower proportion of naive CD8+ T cells presages a survival advantage at 8-year follow-up from the baseline age of 89 years (12). An inverted CD4:8 ratio, a hallmark of the IRP predicting all-cause mortality in other studies (31), was not associated with physical dysfunction in CMV-seropositive individuals. As the case may be, the small number of individuals with a $R < 1$ have efficiently adapted to repeating CMV immunosurveillance, with high anti-CMV IgG reactivity, and this retention of memory cells specific for previously encountered antigens may provide a survival advantage in this particular population. This survival effect could also have been reflected in our cohort as no association was shown between CMV seropositivity or IgG titers and functional impairment in the BELFRAIL study at baseline (16), which could be due to the heterogeneity in CMV immunosurveillance. Thus, dysbalanced immunity or the nature of immunosurveillance against CMV may be crucial for healthy ageing, at least in the very elderly people. The time of primary infection and the dynamics of the infection though may importantly determine the effects of CMV.

Although cognitive functioning and depression has been previously linked to CMV infection or immune status (15,32), no associations whatsoever were found with cognitive functioning (MMSE) or suspected depression (GDS-15) in the present study. This could partly be due to the fact that patients were selected at baseline for having a MMSE $> 15$. Nevertheless, several patients exhibited mental decline between baseline and the clinical research associate visit at T1 (Figure 1). On the other hand, different methodologies for measuring physical and mental ability and decline can give rise to a great amount of variability. Thus, a more detailed and standardized approach is necessary to confirm these relationships.

Strengths and Limitations

The main strength of the present study is the large sample of community-dwelling very elderly people and information on associated clinical variables on functional outcome, which has rarely been investigated. These data, together with the immune parameters established here, will be crucial for assessing important variables in health and mortality over the foreseen follow-up period. An important limitation of this study is the low number of only three CMV-seropositive younger individuals and two seronegative donors with an inverted ratio. However, the main thrust of this study remains the comparison of heterogeneous groups of elderly individuals with longitudinal follow-up, rather than contrasting old with young in a cross-sectional study. Furthermore, we do not know whether these late-differentiated T cells are CMV-specific clones or when persons became infected with CMV. The final outcome and implications of the immune parameters and CMV status measured here will become apparent over the next few years. But more comprehensive studies incorporating inflammatory markers in accordance with the natural history of CMV infection in oldest old participants will increase our understanding of the biological and clinical relevance of this phenotype.

Conclusions

We studied a rarely investigated immune phenotype in the very elderly people, characterized by a CD4:8 ratio >5.
and retention of a large naive T-cell compartment, which was associated with lower anti-CMV IgG reactivities and a higher proportion of physical impairment in participants infected with CMV. These data are consistent with the rather counterintuitive findings from the Leiden 85+ study, indicating that preservation of naive-phenotype cells is not associated with a survival advantage at advanced age. This suggests not only CMV infection, but also the nature of CMV immunosurveillance, is crucial in the association with functional impairment in the very elderly people.

SUPPLEMENTARY MATERIAL

Supplementary material can be found at: http://biomedgerontology.oxfordjournals.org/

FINANCING

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