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Latent Infection with Cytomegalovirus Is Associated with Poor Memory CD4 Responses to Influenza A Core Proteins in the Elderly

Evelyna Derhovanessian,* Andrea B. Maier,†,‡,§ Karin Hähnel,* Janet E. McElhaney,* Eline P. Slagboom,‡,§ and Graham Pawelec*

Influenza remains a major pathogen in older people. Infection with CMV and the accumulation of late-differentiated T cells associated with it have been implicated in poor Ab responsiveness to influenza vaccination in the elderly, most of whom are CMV positive. However, whether CMV infection also affects memory T cell responses to influenza remains unknown. To investigate this, we assessed T cell responses to influenza A matrix protein and nucleoprotein ex vivo in 166 Dutch individuals (mean age 62.2 y, range 42–82) and validated the results in a second cohort from North America (mean age 73.1 y, range 65–81, n = 28). We found that less than half of the CMV-infected older subjects mounted a CD4 T cell response to influenza Ags, whereas ~80% of uninfected elderly did so. A similar proportion of younger subjects possessed influenza A virus–responsive CD4 T cells, and, interestingly, this was the case whether they were CMV-infected. Thus, the effect of CMV was only seen in the older donors, who may have been exposed to the virus for decades. The percentage of donors with CD8 responses to influenza A virus was lower than those with CD4; this was not influenced by whether the subjects were CMV seropositive or seronegative. CMV-seropositive responders had significantly higher frequencies of late-differentiated CD4 T-cells (CD45RA+/CD27−) compared with CMV-infected nonresponders. These data add to the accumulating evidence that infection with CMV has profound but heterogeneous effects on responses to the products of other viruses and have implications for the design of influenza vaccines, especially in the elderly. The Journal of Immunology, 2014, 193: 3624–3631.

Seasonal epidemics of influenza cause serious illness and death throughout the world. The likely global disease burden is estimated by the World Health Organization at up to 1 billion infections, 3–5 million cases of severe disease, and between 300,000 and 500,000 deaths annually (1). In developed countries, the elderly account for >90% of influenza-related deaths. This is believed to be due to the diminished state of immunity in the elderly (2, 3) generally referred to as “immunosenescence,” as well as the probably related low success rate of prophylactic vaccination (4), still the most effective tool for controlling influenza and influenza-related complications. Immunosenescence is thought to be more marked in the adaptive arm of the immune system and is characterized by reduced numbers and percentages of circulating naive T cells reciprocated by accumulations of late-differentiated memory T cells as well as reduced B cell diversity and function (5). Some of these parameters, especially the accumulation of late-differentiated memory CD4 and CD8 T cells, have been shown to correlate with poor humoral responses of the elderly to influenza vaccination (6–8).

Besides chronological age, a latent infection with CMV has been repeatedly demonstrated to be a major force driving T cells toward a more late-differentiated phenotype, one of the hallmark features of immunosenescence (9, 10). CMV is a ubiquitous herpes virus, the seroprevalence of which rises with age. In many countries, >70% of individuals over the age of 60 are infected by CMV (11). Although usually asymptomatic in immunocompetent carriers, this virus poses a major challenge to the immune system of the host, evidenced by deviation of a very large proportion of memory CD4 and CD8 T cells to maintaining this virus in a latent state in healthy individuals (12). The chronic encounter of the immune system with the virus leads to accumulation of late-differentiated CD8 cells, lacking expression of CD28, a parameter that has been associated with poor humoral and cellular responses to influenza vaccination (7, 8, 13). Accordingly, seropositivity for CMV has been demonstrated to correlate with poor humoral responsiveness of the elderly to influenza vaccination in several studies (6, 14–16).

Humoral immune responses are required to prevent infection; they depend on Ag-specific CD4+ T cell help for antiviral B cell responses, Ab class switch, affinity maturation, and long-lived plasma cell generation. In addition, both CD4 and CD8 T cells, which directly kill and clear already virus-infected cells, are essential for protecting the organism (17). Accordingly, pre-existing
CD4 memory T cells have been found to be associated with disease protection and limiting disease severity in an influenza challenge model in humans (18). Cellular responses are mainly targeted against matrix protein (MP) and nucleoprotein (NP), two core proteins of the virus, which are conserved between strains; hence, memory T cells specific for these proteins can mediate cross-protective immunity, as was demonstrated recently (19). This is especially very important in the context of pandemics and in the absence of neutralizing Abs.

Whether cellular responses to influenza are affected by aging or the presence of a latent infection with CMV has not been explored to date. Given the fact that CMV infection has been associated with poor cellular responses to EBV (20), we aimed to analyze whether this was also the case for influenza. In this study, we demonstrate in two independent cohorts that CD4 responses to influenza core proteins are absent in almost half of the CMV-seropositive elderly, whereas older people not infected with this virus respond as well as the young. Hence, advanced chronological age plays a role in depressed responses to influenza but only in concert with CMV infection. Our data also suggest that contrary to the widely accepted concept, a more late-differentiated CD4 compartment is not detrimental but is associated with better CD4 responses to the influenza virus and, hence by implication, better protection in vivo.

Materials and Methods

Study population

Cryopreserved PBMCs from two different cohorts were included in this study. Cohort 1 consisted of 166 subjects participating in the Leiden Longevity Study. Detailed characteristics of these donors have been published previously (21). They were between the ages of 42 and 82 y with a mean age of 65.8 y and a CMV seropositivity rate of 45.2% overall. Study cohort 2 was recruited in Vancouver, BC, Canada and consisted of 28 donors between the ages of 65 and 81 y with a mean age of 73.1. The CMV prevalence was 75%.

Analysis of T cell responses

T cell responses to different Ags were tested as described previously (22). Briefly, PBMCs were thawed, Fc receptors blocked and dead cells were stained as described above. Cells were then stained with anti-KLRG-1 primary Ab (provided by Prof. Dr. H.-P. 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. After blocking with mouse serum (see above) the following directly-conjugated Abs were added: CD3-PE (Caltag; Invitrogen), CD4-PerCP, CD8-allophycocyanin-H7, CCR7-PE-Cy7 (BD Biosciences, Heidelberg, Germany), CD27-allophycocyanin, CD45RA-Pacific Blue, CD28-Alexa Fluor 700 (BioLegend, San Diego, CA), and CD57-FITC (Immunootech, Freiburg, Germany). After 20-min incubation on ice, cells were washed and analyzed immediately on an LSR II cytometer. For a detailed gating strategy, please see Ref. 21.

CMV serology

CMV serostatus and CMV-specific IgG titers were determined by ELISA, using the CMV-IgG-ELISA PKS assay (Medac, Wedel, Germany), according to the manufacturer’s instructions. IgG specificity for different Ags was determined by means of a recombinant CMV IgG immunoblot (Mikrogen, Neuried, Germany), according to the manufacturer’s instructions.

Analysis of T cell phenotypes

Differentiation phenotypes of T cells were determined in cohort 1 as published previously (21). Briefly, PBMCs were thawed, Fc receptors blocked and dead cells were stained as described above. Cells were then stained with anti-KLRG-1 primary Ab (provided by Prof. Dr. H.-P. 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. After blocking with mouse serum (see above) the following directly-conjugated Abs were added: CD3-PE (Caltag; Invitrogen), CD4-PerCP, CD8-allophycocyanin-H7, CCR7-PE-Cy7 (BD Biosciences, Heidelberg, Germany), CD27-allophycocyanin, CD45RA-Pacific Blue, CD28-Alexa Fluor 700 (BioLegend, San Diego, CA), and CD57-FITC (Immunootech, Freiburg, Germany). After 20-min incubation on ice, cells were washed and analyzed immediately on an LSR II cytometer. For a detailed gating strategy, please see Ref. 21.

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Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5. Differences between groups for the categorical variables were assessed with the χ2 test. Mann–Whitney testing was used for comparison of two independent groups. The p value was set at 0.05.

Results

Ex vivo detection of IAV-specific T cells in cohort 1

We first determined the frequency of CD4 and CD8 T cells responding to a mixture of both NP and MP peptides after an overnight stimulation with peptide pools spanning the whole sequence of these Ags in a cohort of 166 middle-aged and elderly individuals (Fig. 1). Peptide-specific CD4 responses were detected in 129 individuals (77.7%), whereas only 97 had CD8+ IAV-specific T cells (58.4%). Fig. 2A shows representative FACS plots from one donor with a CD4-dominant (donor 1, left) and another donor with a CD8-dominant response (donor 2, right) to these influenza peptides. The frequency of IAV-reactive CD4+ cells ranged between 0.015 and 0.46% of total CD4+ T cells (Fig. 2B) and was generally lower than the frequency of IAV-reactive CD8+ T cells (range: 0.032–1.33%) of total CD8+ T cells (Fig. 2B). In 23 individuals (13.9%), no responses of either CD4 or CD8 T cells could be detected. Comparing these individuals (nonresponders, NR) with the remainder (responders, R) regarding age and CMV seropositivity, two parameters known to impact the functional status of T cells, we observed that NR were slightly but not significantly older (median age 65 versus 60.7 y [p = 0.17]). In addition, NR tended to be more often CMV-infected than R: 13 of 23 (56.5%) versus 62 of 143 (43.3%), respectively, but this was not significant (p = 0.24). Despite a lack of cytokine response after stimulation with influenza Ags, NR did not differ
from R in their CD4 and CD8 T cell production of IFN-γ, TNF, or IL-2 on stimulation with PMA/ionomycin (Fig. 2C).

**CMV seropositivity is associated with lower CD4 T cell responses to IAV Ags only in the elderly**

Having observed a trend toward a possible negative association between age and CMV-seropositivity with memory responses to IAV in vitro, we sought to determine whether there was an additive effect between these parameters. For this, individuals were stratified according to CMV serostatus and age (<65 or >65 y). In the <65 y group, CD4 responses were detected in 52 of 61 (85.2%) of CMV-seronegative and in 41 of 48 (85.4%) of CMV-seropositive donors (Fig. 3A). However, in individuals over the age of 65 y, CMV seropositivity was significantly associated with fewer CD4 responders; while older CMV-seronegative individuals had a similar CD4 response rate compared with the younger donors (23 of 30, 76.7%), only 13 of 27 (48.1%) CMV-seropositive elderly mounted a memory CD4 response to IAV (p = 0.026 compared with CMV-seronegative age-matched donors and p = 0.0006 compared with CMV-matched younger donors; Fig. 3A). IAV-specific CD8 responses were detected in similar proportions of donors in each age group, regardless of CMV infection and with no difference between younger or older donors (Fig. 3B). Thus, neither age nor CMV affected the frequency of individuals capable

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**FIGURE 1.** Gating strategy for detection of peptide-specific T cells. After an overnight incubation period with or without Ags, cells were harvested, washed, and treated with human Ig, Gamunex, and EMA. The cells were then stained for CD3, CD4, and CD8-allophycocyanin-H7 followed by fixation, permeabilization, and intracellular staining for IFN-γ, TNF, and IL-2. For the data analysis, first events acquired during a nonconstant flow of the machine were excluded (A), followed by gating the lymphocyte population based on size and granularity (B). After exclusion of duplets (C and D) and EMA-negative dead cells (E), T cells were gated as CD3⁺ (F), followed by gating of CD4⁺CD8⁻ and CD4⁺CD8⁺ cells (G). Production of each cytokine was then determined in CD4⁺ (H) and CD8⁺ (I) cells separately.
of mounting CD8 T cell responses to influenza core proteins as measured by TNF, IFN-γ, or IL-2 production. The lower proportion of CMV-seropositive elderly individuals mounting a CD4 T cell response to IAV Ags was not due to a lower intrinsic capacity of their T cells to produce IFN-γ, TNF, or IL-2 when stimulated with PMA/ionomycin. On the contrary, CMV-seropositive donors produced significantly higher levels of IFN-γ and TNF in both age groups (Fig. 3C, 3D), whereas neither age nor CMV serostatus had an impact on IL-2 levels (data not shown).

We sought to validate these findings in a second independent cohort of individuals >65 y of age from a different country (Canada). In this cohort, the response rate to MP and NP peptide pools was analyzed separately. The CD4 response rate to MP in CMV-seronegative elderly (six of 85, 7.1%) was similar to that detected in the Dutch cohort. In this cohort too, a significantly lower proportion of individuals mounted a CD4 response (and in this case also a CD8 response against MP) if they were CMV seropositive (Table I). Cellular responses against NP were detected in a much lower frequency of donors and were not influenced by CMV status (Table I).

The frequency of cytokine-producing cells and the amount of cytokine produced by the cells (as determined by mean fluores-

FIGURE 2. Ex-vivo detection of T cells specific for core proteins of the influenza virus. (A) Cryopreserved PBMCs were thawed and were either left untreated (unstimulated control) or stimulated overnight with peptide pools overlapping the whole sequence of influenza A MP and NP. Representative FACS plots from two donors are presented. The cells are from the CD3+ gate. (B) Frequency of influenza-specific CD4 and CD8 cells in 129 and 97 healthy donors, respectively, with detectable CD4 and CD8 responses. (C) Frequency of CD4 (left panel) and CD8 (right panel) T cells producing IFN-γ, TNF, and IL-2 after stimulation with PMA/ionomycin.

FIGURE 3. CMV seropositivity is associated with lower CD4 responses to IAV only in the elderly. The percentage of CMV-seronegative (■) or CMV-seropositive (■) individuals less than or more than the age of 65 mounting a CD4 (A) or a CD8 (B) response to MP and NP proteins, measured as described in the legends to Figs. 1 and 2. IFN-γ and TNF production in CD4 (C) and CD8 (D) cells in response to nonspecific stimulation with PMA/ionomycin. <65 y CMV−: n = 61, <65 y CMV+: n = 48, >65 y CMV−: n = 30, >65 y, CMV+: n = 27. Each symbol represents a single donor tested. Horizontal lines represent the median. *p < 0.05, **p < 0.01, ***p < 0.001.
Table 1. Memory responses to influenza A MP and NP in cohort 2

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<td>Response to NP, n (%)</td>
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<td>CD4 response</td>
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<td>CD8 response</td>
<td>4 (57.1)</td>
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cence intensity) did not differ between CMV-seronegative and CMV-seropositive responders (data not shown). We then analyzed the pattern of cytokine response of IAV-specific T cells. Because of the low number of donors producing IL-2, this cytokine had to be excluded from the polyfunctionality analysis. The majority of IAV-specific CD4 and CD8 T cells produced only IFN-\( \gamma \) but not TNF (Fig. 4A, 4B, middle panels). Neither age nor CMV serostatus had an impact on the proportion of cells producing both TNF and IFN-\( \gamma \) or only one of these cytokines (Fig. 4).

The absence of CD4 response to IAV in CMV-seropositive individuals is not reflected in a lower T cell response to CMV Ags

Next, we sought to determine whether CMV-seropositive NRs to IAV Ags also failed to mount a cellular response to CMV. For this, peptide pools of pp65 and IE-1, two immunodominant Ags of CMV, were used. These experiments were performed in 71 CMV-seropositive individuals from cohort 1. CMV-specific CD4 and/or CD8 responses were detected in 70 (98.6%) donors; the majority of whom (92.8%) mounted both a CD4 and a CD8 response against these Ags, confirming that the absence of CD4 responses against IAV in some CMV-seropositive donors is not due to a general lack of T cell functionality. Next, we stratified the donors according to the presence or absence of IAV-specific CD4 or CD8 T cells and compared the frequency of CMV-specific CD4 and CD8 cells between these individuals. The frequency and also the absolute numbers per unit of blood of CD4 and CD8 T cells specific for CMV did not differ between individuals without a CD4 response to IAV (CD4-NR n = 18, median age 68.9 y, interquartile range 8.2) and those mounting a CD4 response (CD4-R, n = 47, median age 61.3 y, interquartile range 8.6) (Fig. 5). CMV-specific CD4 or CD8 responses were detected at equal levels between individuals with or without a CD8 response to IAV (data not shown).

The absence of a CD4 response to IAV in CMV-seropositive individuals is not associated with lower serum anti-CMV IgG titers, but more CD4-responders have IgG specific for CMV-gB2

Next we sought to determine whether the humoral response to CMV was different between CMV-seropositive individuals who did not have CD4 T cell responses to IAV (CD4-NR) and those who did (CD4-R). Both groups had similar titers of anti-CMV IgG Abs (Fig. 5C). Almost half of the donors in both groups had IgG titers higher than the detection limit of the kit. These donors have been excluded from Fig. 5C. We then characterized the specificity of IgG responses to different CMV Ags. As shown in Fig. 5B, a significantly higher proportion of CD4-R compared with CD4-NR doners had serum IgG reactivity to gB2, one of the main targets of neutralizing Abs against CMV (Fig. 5D).

**CMV-seropositive individuals mounting a CD4 response to IAV possess T cells with a more late-differentiated CD4 phenotype**

It is well established that a higher proportion of peripheral blood CD4 and CD8 T cells from CMV-seropositive than -negative donors has a late-differentiated phenotype, the accumulation of which has been correlated with poor cellular responses to influenza vaccination in the elderly. Thus, we next sought to determine whether a more late-differentiated T cell compartment was associated with poor CD4 responses to influenza core proteins. For this, the frequency of different naive and memory phenotypes was determined according to the surface expression of CD45RA, CCR7, CD27, and CD28. A differentiation index was calculated by dividing the frequency of the most late-differentiated effector memory and effector populations (CD45RA\(^-\)CCR7\(^-\)CD27\(^-\)CD28\(^-\)) by the frequency of naive cells (characterized as CD45RA\(^+\)CCR7\(^+\)CD27\(^+\)CD28\(^+\)). CMV-seropositive individuals were again grouped according to the presence or absence of a CD4 response to IAV as described in the previous section. CD4 T cells from CMV-seropositive compared with the NR (Fig. 6A). This was not due to a lower frequency of naive CD4 cells because the frequency and absolute number of cells with this phenotype was similar between CD4-R and CD4-NR

**FIGURE 4.** The quality of cytokine response to IAV core proteins is similar in young and old CMV-seronegative and CMV-seropositive donors. Frequency of CD4\(^+\) (A) and CD8\(^+\) (B) cells producing only TNF (left panels), only IFN-\( \gamma \) (middle panels), and both TNF and IFN-\( \gamma \) (right panels) was compared between CMV-seronegative and CMV-seropositive donors below or above the age of 65 y. This analysis was limited to those donors with a detectable IAV-specific T cell response. Each dot represents a single donor tested. Horizontal lines represent the median.
In this study, we report that memory CD4 responses to influenza virus core protein MP are compromised in a subset of CMV-seropositive elderly individuals, whereas almost all CMV-seronegative elderly possess influenza-reactive CD4 memory T cells. Unexpectedly and counterintuitively, this was not due to CMV-associated immunosenescence, because individuals lacking a CD4 response had significantly lower levels of late-differentiated effector memory and effector cells in the CD4-R group (Fig. 6B). Consistent with this, cells expressing markers of very late or “terminal” differentiation, sometimes also termed markers of “senescence” (CD57 and KLRG-1), were enriched in the CD4-R group (Fig. 6C, 6D).

Discussion

In this study, we observed significantly higher frequencies and absolute numbers of late-differentiated effector memory and effector cells in the CD4-R group (Fig. 6B). Consistent with this, cells expressing markers of very late or “terminal” differentiation, sometimes also termed markers of “senescence” (CD57 and KLRG-1), were enriched in the CD4-R group (Fig. 6C, 6D). What proportion, if any, of the late-differentiated CD4 T cells observed in our study to be more abundant in CD4-Rs is specific for influenza, could not be tested. We have previously demonstrated in three different cohorts of different ages that late-differentiated CD4 T cells lacking CCR7, CD27, and CD28 and expressing KLRG-1 are directly associated with CMV seropositivity and are almost completely absent in CMV-seronegative donors (6, 22, 26); thus, it is more likely that these cells are specific for CMV and not influenza. However, it has been shown that in CMV-seropositive individuals, especially (but not only) when they are old, CD4 T cells specific for other viruses such as EBV, Varicella Zoster virus, and HSV also have lost the expression of CD27 and CD28 and are significantly more late-differentiated compared with cells of the same specificity in CMV-seronegative donors (27). In fact, the phenotype of CD4 T cells specific for all non-CMV Ags (also the recall Ag purified protein derivative) was very similar to that of CMV-specific CD4+ T cells, suggesting a bystander effect mediated through differentiation-inducing factors secreted as a result of CMV infection (27). It has been reported that CD4+ T-cells reactive to CMV and lacking the expression of CD27 and CD28 have regulatory properties (28). Whether this is true for T cells with the same phenotype specific for other viruses has not been investigated to date but might indicate another immunosuppressive property of CMV by induction of suppressive regulatory T cells specific for other Ags, for example influenza A. Why this negative correlation is only observed in CD4 memory response and not CD8 is not yet clear. It has been recognized for many years that people 65 y and older are at greater risk of serious complications from influenza disease than younger

FIGURE 5. CMV-specific cellular and humoral responses in CD4-Rs and NRs to IAV. PBMCs from CMV-seropositive donors were stimulated overnight with overlapping peptides spanning the whole length of pp65 and IE-1, followed by staining for extracellular markers and intracellular cytokines as described in Fig. 2 legend. Individuals were then grouped according to absence (NR) or presence of a CD4 response (CD4-R) to influenza MP and NP. A positive response was defined by at least a 2-fold increase in the frequency of cells producing at least one cytokine (IFN-γ, TNF, or IL-2) in response to IAV peptides compared with nonstimulated control. The frequency of CD4 (A) and CD8 (B) cells producing either IFN-γ and/or TNF are shown. (C) Anti-CMV IgG titer in CD4-Rs; (D) The IgG reactivity of serum of CMV-seropositive donors was tested against five different CMV Ags pp65, IE-1, CM2, gB1, and gB2 by immunoblotting. The proportion of donors with IgG reactivity to these Ags is shown in individuals without a CD4 response to IAV (CD4-NR) and those harboring IAV-specific CD4 responses. Each dot represents a single donor tested. Horizontal lines represent the median.
adults. Cellular responses to influenza, especially memory CD4 responses, have been shown to correlate with disease protection, particularly in the absence of neutralizing Abs (18). Thus, the lack of memory CD4 responses to IAV in almost half the CMV-seropositive old individuals might help to explain the increased susceptibility of the elderly, the majority of whom is infected with CMV, to influenza-related complications and death. CMV seropositivity has been associated with poor cellular responses to other viruses both in humans (20) and in murine models of CMV infection (29, 30). One explanation for this may be that accumulation of CMV-specific T cells suppresses other memory T cells by filling the “immunological space” and through competition for growth factors or secretion of suppressive factors. Indeed, in our study, the lack of CD4 responses to influenza was not reflected in a poor response to CMV.

One limitation of our study is the lack of information on vaccination history in cohort 1. However, all individuals from the Canadian cohort had at least had one round of influenza vaccination prior to the time of our analysis. This fact and the similar rate of cellular responses observed between the two cohorts suggest that the difference between young and elderly CMV-seropositive donors is most probably not because of different vaccination histories. Another open question is why CD4 responses are affected but CD8 responses are not in these assays. It is well established by many investigators that the distribution of different memory CD8 T cell phenotypes is altered more dramatically than the CD4 subset in CMV-seropositive donors and in the elderly. However, the emergence of late-differentiated CD4* memory T cells, which is observed in only a few CMV-seropositive donors is directly associated with a latent infection with this virus, whereas CD8 T cells of the same phenotype also accumulate in CMV-seronegative donors. This indicates a differential impact of a latent infection with CMV on the development of CD4 and CD8 memory T cells, which in turn might help explain a differential impact of this virus on their function.

These data add to the accumulating evidence that infection with CMV has profound but heterogeneous effects on responses to the products of other viruses and have implications for the design of influenza vaccines especially in the elderly. The perhaps unexpected finding that CMV-associated immunosenescence is not necessarily detrimental to the host also is demonstrated in our recent studies of the very elderly (22, 31), albeit in the CD8 subset, suggesting that the remodeling of the T cell compartment in the face of a latent infection with CMV might not be pathological, but necessarily detrimental to the host also is demonstrated in our recent studies of the very elderly (22, 31), albeit in the CD8 subset, suggesting that the remodeling of the T cell compartment in the face of chronic challenge with CMV. Thus, continued immunosurveillance against persistent CMV is more important for host survival than reserving immune resources for responses to other viruses to which the individual might never be exposed.

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Disclosures
The authors have no financial conflicts of interest.

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