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

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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+/CCR7−/CD27−/CD28−) 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: 000–000.

Seasomal 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 naïve 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 devolution 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 chronologic 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 62.2 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 and allowed to rest for 8 h in X-Vivo 15 medium (Cambrex) at 1 × 10^6/ml at 37°C. Cells were then stimulated with overlapping peptides covering the whole sequence of NP and/or MP (cohort 1/cohorts 2, respectively) from the influenza A virus (IAV). These PepMixes were purchased from JPT Technologies (Berlin, Germany) and were used at a concentration of 1 μg/ml as recommended by the manufacturer. Cells from CMV-seropositive donors were additionally stimulated with pp65 and IE-1 PepMix. A total of 1 μl/ml GolgiPlug (BD Biosciences, Heidelberg, Germany) was added together with the peptides, and the cultures were incubated for 16 h. This provides a direct ex vivo readout of responding memory cell frequencies without any intervening long-term culture period, which could possibly lead to expansion of naive T cells. Medium alone and 50 ng/ml PMA (Sigma-Aldrich, Munich, Germany) together with 750 ng/ml ionomycin (Merck, Darmstadt, Germany) were used as negative and positive controls, respectively. The cells were then harvested, washed, and treated with human Ig, Gamunex (Bayer, Leverkusen, Germany), and ethidium monoazide (EMA) (Invitrogen, Karlsruhe, Germany) for 10 min on ice to block FcRs and label nonviable cells. The cells were then stained first indirectly for CD3 (OKT3 primary and/or TNF and/or IL-2 was considered as a positive response.

Analysis of T cell phenotypes

Differences in the frequency of responding memory CD4 and CD8 T cells could be detected. Comparing these independent cohorts, the CMV-seropositive donors displayed a lower frequency of responding CD4 T cells compared with their CMV-seronegative counterparts (Fig. 2B) and was generally lower than the frequency of IAV-reactive CD8 T cells (38.4%). 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 remaining (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

![Image of gating strategy for detection of peptide-specific T cells](http://www.jimmunol.org/)

**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 seven, 85.7%) 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 fluorescence intensity) were analyzed by flow cytometry. CMV seropositivity is associated with lower CD4 responses to IAV only in the elderly. The percentage of CMV-seronegative (circles) or CMV-seropositive (squares) 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. *p < 0.05, **p < 0.01, ***p < 0.001.
ence 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-γ 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-γ 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 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 donors 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 had a significantly higher differentiation index in CD4-R 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.
Discussion

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 CD4 T cells expressing the putative senescence markers CD57 and KLRG-1, and a less-differentiated CD4 subset, relative to people mounting a CD4 response to IAV Ags. This is interestingly in contrast to our recent data demonstrating a negative impact of the presence of such late-differentiated CD4 T cells on humoral responses to influenza vaccination (6). However, our in vitro memory T cell response assay may not be reflecting T cell help for Ab formation. It is also consistent with findings that the presence of prevaccination late-differentiated IAV-specific memory T cells correlates with poor Ab responsiveness after vaccination (25). In addition, responsiveness to influenza vaccination is well known to negatively correlate with prevaccination hemagglutination inhibition (HI) titers. In other words, donors who already have high amounts of HI Ab, thus a stronger immune response to the virus, respond less well to vaccination in terms of fold increase in HI titer, the parameter of which is used to estimate vaccine responsiveness. Hence, the presence of late-differentiated CD4 T cells correlating with better cellular responses to IAV could be indicative of higher HI titers and therefore poorer responsiveness to vaccination.

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

![CMV-specific cellular and humoral responses in CD4-Rs and NRs to IAV](image-url)
Cellular responses observed between the two cohorts suggest that the remodeling of the T cell compartment in the face of a latent infection with CMV might not be pathological, but representing an adaptation of the immune system of the host 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.

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 represent an adaptation of the immune system of the host 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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