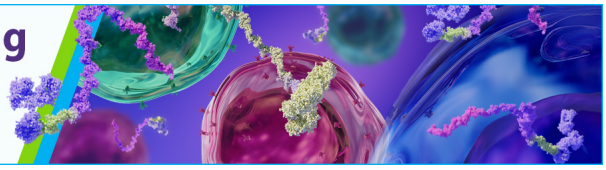

The Journal of Immunology

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J Immunol 2005; 175:8218-8225; ;
doi: 10.4049/jimmunol.175.12.8218
http://www.jimmunol.org/content/175/12/8218

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Cytomegalovirus-Specific CD4$^+$ T Cells in Healthy Carriers Are Continuously Driven to Replicative Exhaustion[1]

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Repeated antigenic encounter drives proliferation and differentiation of memory T cell pools. An important question is whether certain specific T cells may be driven eventually to exhaustion in elderly individuals since the human life expectancy is increasing. We found that CMV-specific CD4$^+$ T cells were significantly expanded in healthy young and old carriers compared with purified protein derivative-, varicella zoster virus-, EBV-, and HSV-specific populations. These CMV-specific CD4$^+$ T cells exhibited a late differentiated phenotype since they were largely CD27 and CD28 negative and had shorter telomeres. Interestingly, in elderly CMV-seropositive subjects, CD4$^+$ T cells of different specificities were significantly more differentiated than the same cells in CMV-seronegative individuals. This suggested the involvement of bystander-secreted, differentiation-inducing factors during CMV infection. One candidate was IFN-α, which induced loss of costimulatory receptors and inhibited telomerase in activated CD4$^+$ T cells and was secreted at high levels by CMV-stimulated plasmacytoid dendritic cells (PDC). The CMV-specific CD4$^+$ T cells in elderly subjects had severely restricted replicative capacity. This is the first description of a human memory T cell population that is susceptible to being lost through end-stage differentiation due to the combined effects of lifelong virus reactivation in the presence of bystander differentiation-inducing factors. *The Journal of Immunology,* **2005, 175: 8218–8225.**

Elderly individuals often experience reactivation of latent organisms such as varicella zoster virus (VZV)[3] (1), and there have been rare case reports of EBV (2), mycobacteria (3), and CMV (4) reactivation. Because these subjects were immune to such agents in their youth, it suggests that some populations of memory T cells may be lost before others during aging (5). Thymic involution occurs (6, 7), thus immunity must be maintained by turnover of existing populations of cells (8). However, the total size of the immune system changes little over time because homeostatic mechanisms are engaged after episodes of immune stimulation (9). Hence, while the quantity of T lymphocytes may remain stable over the lifetime of an individual, their functional quality and proportionate representation within the T cell pool may be dramatically altered. The key questions that remain to be addressed are first, which human memory T cell pools differentiate most rapidly, and second, does this have any impact on the health of elderly subjects.

CMV is a β-herpes virus with a prevalence of between 60 and 90% worldwide, most often acquired during an asymptomatic primary infection in early childhood, after which the virus establishes lifelong persistence (10). The carriage of CMV has long been considered harmless to individuals with a functional immune system (4, 11). However, recent longitudinal studies have defined an immune risk phenotype (IRP) that is predictive of significantly decreased 2- and 4-year survival of patients above the age of 80 years (12, 13). The IRP is comprised of a cluster of immune parameters, including CMV seropositivity, a CD4:CD8 T cell ratio of <1, increased proportions of highly differentiated CD8$^+$CD28$^-$ T cells, the presence of CD8$^+$ T cell clonal expansions, and elevated serum levels of proinflammatory cytokines (12, 13). Recent studies have shown that the majority of highly differentiated oligoclonal CD8$^+$ T cell populations that are found in elderly individuals (14) are specific for CMV (15–17). Thus, CMV may have a more insidious effect on the immune system than previously appreciated; however, it is still unclear how the various immune changes that comprise the IRP are linked and why CMV appears to reduce survival in old age.

Although most studies on the T cell response to CMV infection have focused on the CD8$^+$ T cell population, the limited data available suggests that CMV-specific CD4$^+$ T cells are also more differentiated than EBV-specific populations as defined by surface phenotype (18, 19). However, it is unclear if they show signs of functional differentiation. Previous studies have shown that differentiation related to changes in telomere length in CD4 cells also relate to functional differentiation of these cells in terms of cytokine production (20). To clarify these issues, we have modified previous technology (21) to investigate telomere erosion as a functional marker of T cell differentiation in CMV-specific CD4$^+$ T cells (22, 23). Telomeres are repeating hexameric sequence of nucleotides at the ends of chromosomes that shorten by 50–100 bp/division in the absence of compensatory signals from the enzyme telomerase (24–27). The key points are that highly differentiated T cells have short telomeres, lose the capacity to express telomerase

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Received for publication June 9, 2005. Accepted for publication September 27, 2005.

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[1] This work was funded by the Henry Smith's Charity (to J.M.F.), the Biotechnology and Biological Sciences Research Council Experimental Research into Aging Initiative (to M.V.-S.), Research into Ageing (to J.E.C. and S.E.J.), the Sir Jules Thorn Charitable Trust (to K.E.B.), Dermatrust (to K.E.B. and P.J.D.), and the Edward Jenner Institute for Vaccine Research (to P.J.D.).

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[3] Abbreviations used in this paper: VZV, varicella zoster virus; IRP, immune risk phenotype; PPD, purified protein derivative; flow-FISH, flow cytometric detection of fluorescence in situ hybridization; PDC, plasmacytoid dendritic cell; PD, population doubling; SEB, staphylococcal enterotoxin B; TPG, total product generated.

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activity, and have considerably reduced capacity to divide (27, 28). Our recent studies showing that IFN-α directly inhibits telomerase activity and accelerates telomere erosion of human memory $CD4^+$ T cells in vivo (22) raised the possibility that this cytokine, that is known to be secreted in response to CMV in vitro (29, 30) and in vivo (31), may contribute to the accelerated differentiation of CMV-specific T cells.

In this study, we show CMV infection drives $CD4^+$ T cells toward inexorable end-stage differentiation, which may lead eventually to the loss of functional CMV-specific memory $CD4^+$ T cells in elderly subjects. The loss of functional CMV-specific memory leading to either direct or indirect pathological effects of the virus (4) may be one possible explanation of why CMV infection has a negative impact on survival of elderly individuals.

Materials and Methods

Volunteers

Heparinized blood was collected from young (mean age, 28 years; range, 22–40 years; $n = 30$) or elderly (mean age, 81 years; range, 71–94 years; $n = 37$) donors, with approval from the Ethics Committee of the Royal Free Hospital. Young donors were recruited from healthy staff members. Elderly volunteers were recruited from the Outpatients Department at the Royal Free Hospital and from the general public; all elderly subjects were mobile, did not have any cognitive impairment, were not suffering from acute or chronic illness, and were not on medication known to affect the immune system. Serum IgG titers for CMV, VZV, EBV, and HSV were determined by the routine hospital screening service. The percentages of individuals that tested positive for serum IgG for the viruses were as follows: young group—CMV 52%, VZV 90%, HSV 71%, and EBV 100%; and elderly group—CMV 66%, VZV 95%, HSV 72%, and EBV 100%. The majority (90%) of young individuals had been bacillus Calmette-Guérin vaccinated, compared with only 3% of the elderly group. None of the individuals tested had a previous history of tuberculosis infection. PBMC were prepared by density centrifugation on Ficoll-Paque (Amersham Biosciences).

Intracellular cytokine staining

The following Ags were used to stimulate PBMC before intracellular cytokine staining: 10 μg/ml purified protein derivative (PPD; Statens Serum Institute), 1/25 dilution VZV-infected cell lysate, 1/200 dilution EBV-infected B cell lysate, and 1/50 dilution HSV-infected cell lysate (all from East Coast Biologics). CMV-infected cell lysate (used at 1/10 dilution) was prepared by infecting human embryonic lung fibroblasts with the Towne strain of CMV (European Collection of Animal Cell Cultures) at a multiplicity of infection of 2. After 5 days, the infected cells were lysed by repeated freeze-thaw cycles. PBMC were left unstimulated or stimulated with antigenic lysates for 15 h at 37°C in a humidified CO_2 atmosphere, with 5 μg/ml brefeldin A (Sigma-Aldrich) added after 2 h. The cells were surface stained with CD4-PerCP, CD27-PE, and CD28-FITC and then fixed, permeabilized (Fix & Perm Cell Permeabilization kit; Caltag Laboratories), and stained with IFN-γ-allophycocyanin. Uninfected cell lysates did not induce any IFN-γ secretion. Four-parameter flow cytometric analysis was performed on a FACSCalibur (BD Biosciences) using CellQuest software. There was concordance between seropositivity and a positive IFN-γ response in the blood for CMV, EBV, and HSV. However, particularly in the elderly group, not all individuals that were seropositive (95%) for VZV had a blood response (50%). In the case of PPD, in young individuals 81% had a blood response compared with 68% with a tuberculin skin response, while in the elderly 71% had a blood response compared with only 4% responding in the skin and this is the subject of ongoing studies.

Measurement of telomere length by flow cytometric detection of fluorescence in situ hybridization (flow-FISH)

To measure telomere length of Ag-specific $CD4^+$ T cells, we developed a three-color flow-FISH technique, which was modified from the original methodology described by Lansdorp et al. (21). After overnight stimulation with Ag as described above, samples were stained using CD4-biotin (Immunotech) and streptavidin-Cy3 (Cedarlane Laboratories), fixed, permeabilized, and stained with IFN-γ-FITC (BD Biosciences). Cells were washed once in hybridization buffer (70% formamide, 20 mM Tris, 150 mM NaCl, and 1% BSA) and then incubated at 82°C for 10 min with 0.75 μg/ml Cy5-conjugated telomeric (CCCTAA) peptide nucleic acid probe (Applied Biosystems). After rapid cooling on ice, the samples were hybridized for 1 h at room temperature in the dark, washed twice each in posthybridization buffer (70% formamide, 10 mM Tris, 150 mM NaCl, 0.1% BSA, and 0.1% Tween 20) and PBSA, and analyzed by flow cytometry. Samples were analyzed with and without probe to control for differences in background fluorescence between samples. To ensure consistency of the results between experiments, two cryopreserved PBMC samples with known telomere fluorescence were used as standards. Results were obtained as median fluorescence intensity values, which could then be converted to telomere length in kilobases using a standard curve. The standard curve was constructed using 30 samples of varying telomere length analyzed both by flow-FISH and telomeric restriction fragment analysis. Telomere length in sorted $CD4^+$ T cell subsets was measured as described previously (22).

Telomerase assays

Telomerase activity was measured using the telomeric repeat amplification protocol (TRAPeze Telomerase Detection kit; Serologicals) according to the manufacturer's instructions. Cell extracts were prepared from snap-frozen cell samples, where the volume of the cell extract was adjusted so as to yield 500/250 $Ki67^+$ T cells per reaction as described previously (22). Absolute numbers of proliferating T cells were enumerated by using TruCOUNT tubes with Tritest and also staining for CD3 and intracellular Ki67 (all BD Biosciences). The negative controls included a sample where no cell extract was added. Extracts from a telomerase producing tumor cell line was used as a positive control. In addition, an eight-repeat telomeric PCR template was also include among the controls for the calculation of telomerase activity.

Culture of Ag-specific cell lines

PBMC were stimulated with either PPD (1 μg/ml) or CMV-infected cell lysate (1/100 dilution). Ag-specific cell lines were maintained in HyQ RPMI 1640 (HyClone) containing 10% human AB serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (all Sigma-Aldrich) with 5 ng/ml IL-2 added every 2–3 days. Cell lines were restimulated every 10–14 days with Ag-pulsed irradiated autologous PBMC. Absolute cell numbers were enumerated during and after each cycle of expansion using TruCOUNT tubes, and population doublings (PD) were calculated using the equation: PD = log (number of cells counted after expansion) − log (number of cells seeded)/$\log_2$. Cell lines were free of mycoplasma tested for using the MycoAlert Mycoplasma Detection kit (Cambrex).

Measurement of IFN-α

PBMC or sorted cell subsets were cultured overnight at a concentration of 10^6 cells/ml with medium alone, 1/10 dilution of CMV-infected cell lysate or 10 μg/ml PPD, after which culture supernatants were harvested and stored at −80°C. IFN-α was measured in the cell culture supernatants using an IFN-α ELISA kit that detects human IFN-αA, α2, αA/D, αD, αK, and α4b (PBL Biomedical Laboratories).

Cell sorting of $CD4^+$ T cell subsets

PBMC were first depleted of $CD45RA^+$ cells by labeling with CD45RA-conjugated microbeads and eluting through an AS depletion column (Miltenyi Biotec). The remaining cells were then labeled with CD4-PerCP, CD28-PE (BD Biosciences), and CD27-FITC (Serotec) and sorted using a MoFlo cell sorter (DakoCytomation) to obtain subsets of $CD4^+$ T cells. The purity and absolute number of $CD4^+$ T cell subsets was assessed by flow cytometry using TruCOUNT tubes, and purity was always >98%.

Cell sorting of plasmacytoid dendritic cells (PDC)

PDC were identified by flow cytometry after staining with CD123-PE (BD Pharmingen) and BDCA-2 (Miltenyi Biotec). PDC were either depleted from PBMC or enriched using the BDCA-4 isolation kit (Miltenyi Biotec) according to the manufacturers instructions and magnetic bead isolation over an AS column (Miltenyi Biotec).

Statistical analysis

We used the Student's *t* test, the Mann-Whitney *U* test, or the Wilcoxon-matched pairs test to analyze the results.

Results

Expanded populations of CMV-specific CD4$^+$ T cells in young and elderly subjects

Although it is known that CMV-specific CD8 T cells accumulate with age (32), it is not clear if the same applies to the CMV-specific CD4$^+$ T cell population. We identified Ag-specific CD4$^+$ T cells using a system of antigenic stimulation in the presence of Brefeldin A followed by flow-cytometric detection of the specific cells that synthesize IFN-γ. In both young and old subjects, CMV-specific CD4$^+$ T cells were only observed in individuals who were seropositive for CMV (Fig. 1, *A* and *B*). This was also the case for the other Ags tested in this study (data not shown). We compared the frequency of CD4 T cells in young and old individuals that were specific for the persistent herpes viruses CMV, HSV (herpes simplex virus), EBV, and VZV and the recall Ag PPD (tuberculin-purified protein derivative) (Fig. 1, *C* and *D*). Not all individuals responded to all of the Ags, and for each individual, only positive responses to the various Ags are shown. Although the absolute numbers of CD4$^+$ T cells in young and old subjects were not significantly different from each other (data not shown), the frequency of CMV-specific CD4$^+$ T cells was significantly greater than that of the other Ags within each age group (p values indicated in Fig. 1, *C* and *D*). Furthermore, there was a significant accumulation of CMV-specific CD4$^+$ T cells ($p = 0.02$) and a decrease in the frequency of VZV-specific CD4 T cells ($p = 0.02$) in the old compared with the young groups.

We also found that the TCR Vβ repertoire of CMV-specific CD4$^+$ T cells like the CMV-specific CD8$^+$ T cell population is highly restricted in both young and old subjects (data not shown). Unlike CMV-specific CD8$^+$ T cells (8), these oligoclonal CMV-specific CD4$^+$ T cells are functional as they secrete IFN-γ. Since we use a functional readout to detect Ag-specific cells, it was not possible to analyze the characteristics of putative nonfunctional CMV-specific CD4$^+$ T cell populations.

The differentiation status of CMV-specific CD4$^+$ T cells

The high frequency of CMV-specific CD4$^+$ T cells suggested that they had proliferated extensively in vivo. Previous studies have suggested that that while all naive CD4$^+$ T cells express the co-stimulatory receptors CD27 and CD28, upon repeated cycles of stimulation, these cells progressively lose first CD27 followed by CD28 (33). Highly differentiated CD4$^+$T cells do not express either CD27 or CD28 (18). We first investigated the CD27/CD28 status of specific CD4$^+$ populations that synthesize IFN-γ after stimulation with the various Ags. All of these cells, irrespective of Ag used for stimulation, expressed CD45RO (data not shown). The total CD4$^+$ T cell population in young ($p < 0.006$) and elderly ($p < 0.0001$) CMV-seropositive subjects contained significantly greater numbers of highly differentiated CD27$^-$CD28$^-$ T cells than CMV-seronegative groups (Fig. 2*A*), and the collated data are shown in Fig. 2, *C* and *D*. This was mainly due to the highly differentiated CMV-specific CD4$^+$ T cells that were present (representative plots in Fig. 2*B*; collated data in Fig. 2, *C* and *D*). In young CMV-seropositive subjects, there were significantly more CD27$^-$CD28$^-$ CD4$^+$ T cells within the CMV-specific pool compared with PPD ($p < 0.0001$)-, VZV ($p < 0.0004$)-, EBV ($p < 0.04$)-, and HSV-specific cells ($p < 0.02$; Fig. 2*C*). In the elderly CMV-seropositive group, CMV-specific CD4$^+$ T cells were also more differentiated compared with PPD ($p < 0.0001$) and VZV-specific cells ($p < 0.0002$) and also the total CD4$^+$ T cell pool ($p < 0.001$) but were no longer different from the EBV- and HSV-specific populations (Fig. 2*D*).

We found that CMV-specific CD4$^+$ T cells were more differentiated in the elderly cohort compared with the young group, and there were significantly more CD27$^-$CD28$^-$ cells present within these populations ($p < 0.01$; Fig. 2, *C* and *D*). This indicates continuous differentiation of these specific cells during lifelong viral reactivation. However, we made the unexpected observation that in elderly individuals, their CMV status had a profound impact on the differentiation state of non-CMV- specific populations of CD4$^+$ T cells (Fig. 2*D*). In CMV-seropositive elderly subjects, the PPD ($p < 0.005$)-, VZV ($p < 0.016$)-, EBV ($p < 0.016$)-, and HSV-specific ($p < 0.05$) T cells were more differentiated on the basis of loss of CD27 and CD28 costimulatory molecules than the same populations in elderly individuals who were CMV seronegative (Fig. 2*D*). A similar trend was observed in young subjects but was not as marked as in the elderly group (Fig. 2*C*). These results indicate that CMV infection appeared to have a nonspecific effect on the rate of differentiation of other Ag-specific CD4$^+$ T cells.

CMV-specific CD4$^+$ T cells have short telomeres

We sought additional evidence that CMV-specific CD4$^+$ T cells were highly differentiated. Because highly differentiated T cells have shorter telomeres than less differentiated populations (5, 34),

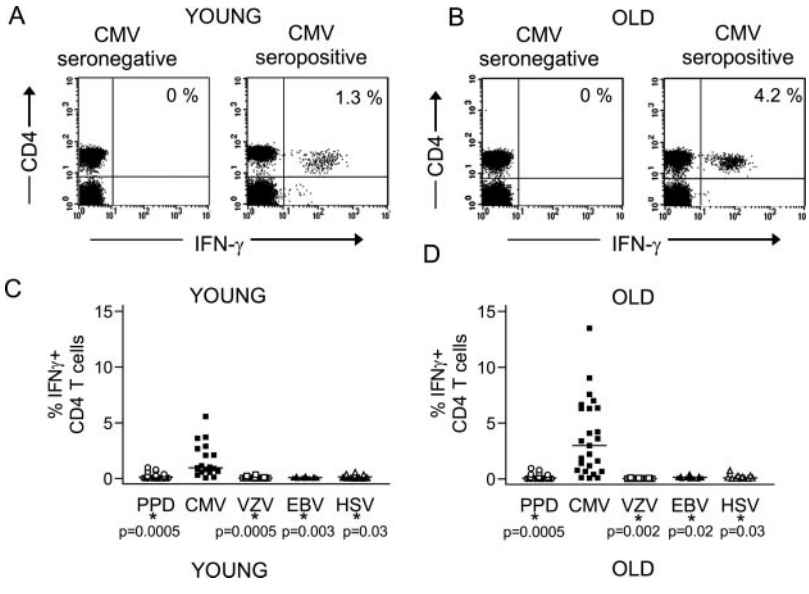

FIGURE 1. High frequency of CMV-specific CD4$^+$ T cells. PBMC from young (*A*) and elderly (*B*) individuals who were either seropositive or seronegative for CMV were stimulated with CMV lysate, and the percentage of IFN-γ secreting, Ag-specific CD4$^+$ T cells was analyzed by flow cytometry. The frequency of CD4$^+$ T cells that were specific for PPD, CMV, VZV, EBV, and HSV were determined in young (*C*) and old (*D*) individuals who were seropositive for these agents by the same methods. Significantly increased frequency of CMV-specific CD4$^+$ T cells relative to the other Ags in each age group is indicated by an asterisk (Wilcoxon rank test).

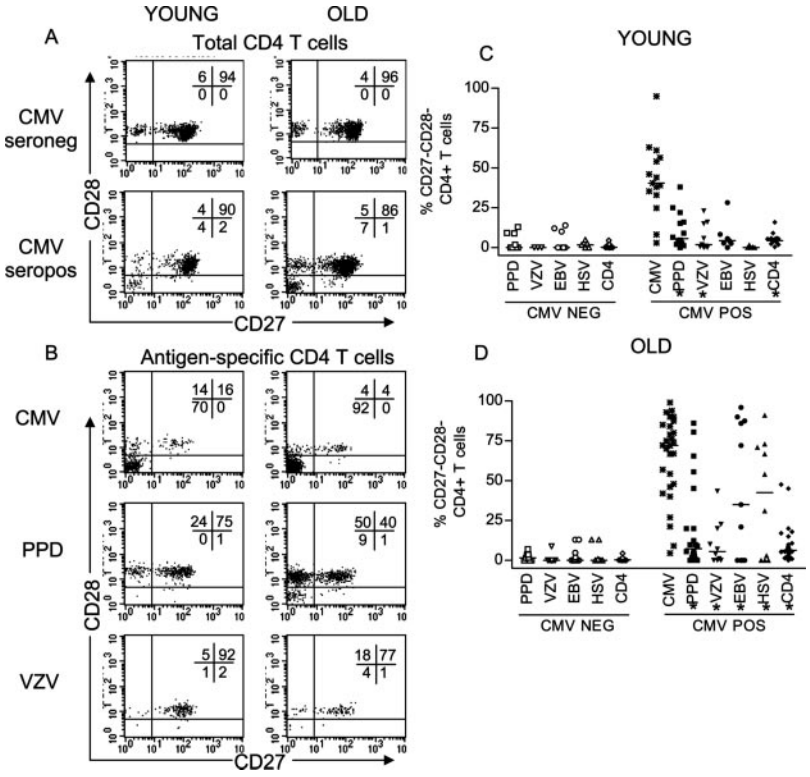

FIGURE 2. Phenotype of different Ag-specific CD4$^+$ T cells. PBMC were stimulated with PPD, CMV, VZV, HSV, or EBV Ags, and the phenotype of the Ag-specific CD4$^+$ T cells was assessed by flow cytometry after staining for CD4, IFN-γ, CD27, and CD28. The phenotype of total CD4$^+$ T cells (*A*) or Ag-specific CD4$^+$ T cells (*B*) from representative young and old subjects who were CMV seronegative or seropositive is shown. The percentage of Ag-specific CD4$^+$ T cells with a CD27$^-$CD28$^-$ phenotype in cohorts of CMV seropositive or seronegative young and old individuals is shown in *C* and *D*, respectively. An asterisk indicates significant differences between CMV-seropositive and -seronegative individuals in each age group (Wilcoxon paired test).

we modified previously described flow-FISH techniques (21) and developed a three-color flow-FISH technique for investigating telomere length in IFN-γ-secreting, Ag-specific CD4$^+$ T cells. An example of flow-FISH analysis is shown for PBMC stimulated with either CMV (Fig. 3*A*) or PPD (Fig. 3*B*), where cells gated on the basis of CD4 and IFN-γ expression were then analyzed as histograms for the intensity of staining with the telomeric probe.

The telomere lengths in total CD4$^+$ T cells as well as Ag-specific CD4$^+$ T cells were determined in 14 young (Fig. 3*C*) and 16 elderly (Fig. 3*D*) CMV-seropositive individuals, all of whom had CMV-specific CD4$^+$ T cells in PBMC preparations. However, not all these individuals had detectable numbers of specific T cells for the other Ags tested. CMV-specific CD4$^+$ T cells in young subjects exhibited significantly shorter telomeres compared with other CD4$^+$ T cells (CMV vs total CD4$^+$, $p < 0.0001$; PPD, $p = 0.0001$; VZV, $p = 0.02$; EBV, $p = 0.01$; HSV, $p = 0.052$; and staphylococcal enterotoxin B (SEB), $p = 0.0006$). Similar results were also found in elderly subjects (Fig. 3*D*), and CMV-specific CD4$^+$ T cells had significantly shorter telomeres compared with total CD4$^+$ cells ($p < 0.0001$), PPD ($p < 0.0001$), and SEB-specific ($p = 0.0008$) CD4$^+$ T cells.

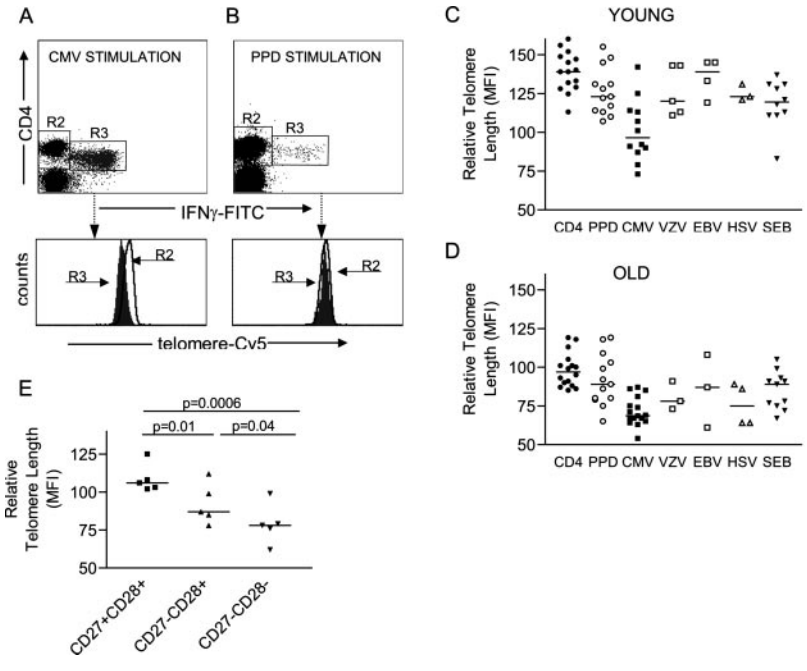

FIGURE 3. Measurement of telomere length in Ag-specific CD4$^+$ T cells by flow-FISH. PBMC were stimulated with various Ags, followed by staining for CD4 and intracellular IFN-γ and hybridization to a Cy-5 conjugated telomeric probe. The cells were analyzed by flow cytometry, as shown for a representative example of CMV (*A*) or PPD (*B*) stimulation, where telomere length was determined in populations gated on the basis of CD4 (R2) and IFN-γ (R3) expression. The relative telomere length (median fluorescence intensity (MFI)) in total or Ag-specific CD4 T cells is shown for young (*C*) or old (*D*) individuals. Values of *p* were determined using a paired Student's *t* test. The relative telomere length of CD4$^+$ T cell subsets sorted on the basis of CD27 and CD28 expression was measured by flow-FISH (*E*).

Ag-specific CD4$^+$ T cells in the young age group were shown to have significantly longer telomeres than the corresponding cells in old individuals (CMV, SEB, $p < 0.0001$; VZV, $p = 0.004$; PPD, $p = 0.002$; EBV, $p = 0.01$; and HSV, $p = 0.02$). We also found that there is a progressive decrease in telomere length during differentiation from a CD4$^+$CD28$^+$ CD27$^+$ to CD4$^+$CD28$^+$ CD27$^-$ to CD4$^+$CD28$^-$CD27$^-$ T cell phenotype, indicating a direct link between accumulation of CD4$^+$CD28$^-$CD27$^-$ T cells and the short telomeres found in the CMV-specific population (Fig. 3*E*). These results confirm that CMV-specific CD4$^+$ T cells exhibit a late differentiated phenotype compared with other specific populations in young individuals and that this difference is maintained into old age.

CMV-induced IFN-α secretion by PDC accelerates phenotypic differentiation and telomere erosion in CD4$^+$ T cells

Our results suggest that CMV-specific CD4$^+$ T cells differentiate more rapidly than other populations of memory T cells. Although this may be due to the extent of CMV reactivation relative to other agents in vivo, the fact that there is also accelerated differentiation of non-CMV-specific T cells in CMV-seropositive individuals suggests that CMV may induce nonspecific factors that affect the rate of CD4$^+$ T cell differentiation. Our previous studies show that IFN-α can accelerate the loss of costimulatory molecules in CD8$^+$ T cells (35) and also inhibit telomerase activity in memory CD4$^+$ T cell populations in vivo (22) and is therefore a candidate for such a factor. PDC are known to produce high levels if IFN-α in response to CMV (30), and we next investigated if production of these cytokines by PDC during stimulation of PBMC by CMV lysates in vitro could have a role in accelerating CD4$^+$ T cell differentiation.

PDC were identified and isolated using the markers CD123 and BDCA-2 from whole PBMC populations (Fig. 4*A*). As shown in Fig. 4*B*, the incubation of unfractionated PBMC overnight with CMV-infected cell lysate resulted in the production of substantial levels of IFN-α (900 IU/ml) while a similar treatment with PPD did not. The CMV-induced IFN-α was produced by PDC since depletion of this population abrogated the IFN-α induction upon stimulation with CMV lysate that was restored by adding back the purified PDC population (Fig. 4, *A* and *B*). We next determined if we could induce PPD-specific CD4$^+$ T cells that were relatively undifferentiated to resemble highly differentiated CMV-specific CD4$^+$ populations by including IFN-α in the cultures during PPD stimulation. We found that 50 IU/ml IFN-α, which was substantially less than the concentration produced by CMV-stimulated PDC in vitro, induced the loss of both CD27 and CD28 in PPD-specific CD4$^+$ T cells during specific stimulation in vitro (Fig. 4*C*). By 21 days, 25% of the cells had a CD27$^-$CD28$^-$ phenotype, compared with 3% in the absence of IFN-α.

To investigate directly whether CMV-induced IFN-α could accelerate telomere loss by inhibiting telomerase activity, we stimulated PPD-specific CD4$^+$ T cells with PPD-pulsed, irradiated APC in the presence of medium alone, supernatants of PBMC that were stimulated with CMV lysate (diluted 1/4, containing ~200 IU/ml IFN-α), or control supernatants of PBMC stimulated with PPD (containing no IFN-α) (Fig. 4*D*). Telomerase activity in 500 proliferating Ki67$^+$ T cells per sample was assessed 4 days after stimulation. As shown in Fig. 4*D*, PPD stimulation induced telomerase activity in T cells (*lane 1*; 46 total product generated (TPG) units) that was significantly inhibited by the presence supernatant containing CMV-induced IFN (*lane 2*; 18 TPG units). The addition of the control supernatant, which contained no IFN-α, had no significant effect on telomerase activity (Fig. 4*D*, *lane 5*; 36 TPG units). The inhibitory effect of CMV supernatant on telomerase activity was abrogated by the addition of a neutralizing anti-IFN-α Ab (Fig. 4*D*, *lane 3*; 41 TPG units). Furthermore, the addition of anti-IFN-α Ab to stimulated cells in the presence of the control supernatant had no effect on telomerase activity (Fig. 4*D*, *lane 6*; 43 TPG units). The addition of an irrelevant isotype control Ab had no effect on telomerase activity (data not shown) (22). These results collectively suggest that CMV may activate both T-specific T cells and PDCs and that PDC-secreted IFN may be responsible in part for accelerating the differentiation of CMV-specific CD4$^+$ T cells. In addition, our results suggest that such cytokines may also accelerate the differentiation of non-CMV-specific CD4$^+$ T cell populations in a bystander fashion. This may explain the greater differentiation of non CMV-specific T cells in CMV-seropositive subjects.

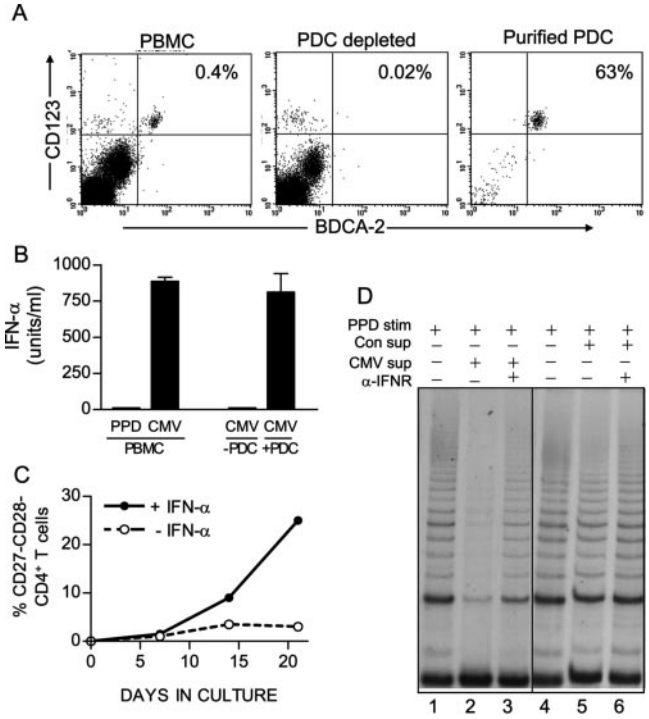

FIGURE 4. The effect of CMV-induced IFN-α on the phenotype and telomerase activity of Ag-specific CD4$^+$ T cells. PDC were depleted from PBMC, and the positive fraction was retained. The purity of the fractions was assessed by staining with CD123 and BDCA-2 (*A*). Total PBMC or PDC-depleted PBMC (−PDC) or the depleted fraction with purified PDC added back (+PDC) were stimulated for 24 h with PPD or CMV Ags, and IFN-α (units/milliliter) in the cell culture supernatants was measured by ELISA ($n = 3$) (*B*). A PPD-specific CD4 T cell line was cultured in the presence (+IFN-α) or in the absence (−IFN-α) of IFN-α (100 IU/ml) and before each restimulation the expression of CD27 and CD28 was determined. The loss of both CD27 and CD28 expression (*y*-axis) over time is shown in *C*. The results shown are representative of two separate experiments. *D*, The effect of CMV-induced IFN-α on the telomerase activity of a PPD-specific CD4 T cell line was assessed. A PPD-specific CD4 T cell line was restimulated using irradiated PPD-pulsed APC ("PPD stim") in the presence or in the absence of either a control ("Con sup") or CMV supernatant ("CMV sup") such as that generated in *A*. A neutralizing Ab specific for the type I IFNR (α-IFNR) was added to block the effects of IFN-α. Telomerase activity was measured by TNF-related activation protein assay after 4 days. The results shown are representative of three experiments.

CMV-specific cells have reduced telomerase activity and replicative capacity

Our results suggested that CMV-specific CD4$^+$ T cells may have reduced capacity to induce telomerase and that this may be one

reason for their short telomeres. To investigate this further, we measured the ability of highly differentiated $CD4^+CD27^-CD28^-$ T cells to up-regulate this enzyme after stimulation. These cells had markedly reduced ability to induce telomerase compared with other less differentiated subsets after polyclonal TCR stimulation (Fig. 5, *A* and *B*). This suggested that CMV-specific $CD4^+$ T cells that are largely $CD27^-CD28^-$ have limited telomerase inducibility. To confirm this directly, we stimulated $CD4^+$ T cells in individuals that had populations of CMV-, PPD-, tetanus-, VZV-, and SEB-specific cells with the respective Ags and measured the level of telomerase induction after stimulation (Fig. 5*C*). To normalize the telomerase activity relative to the extent of proliferation induced by the different Ags, we extracted the enzyme from an equivalent number of proliferating cells after simulation by the different Ags. After 4 days of stimulation, proliferating CMV-specific $CD4^+$ T cells expressed very little telomerase relative to other Ag-specific $CD4^+$ T cell populations (Fig. 5*C*). These results suggest that CMV-specific $CD4^+$ T cell populations are susceptible to replicative senescence upon repeated stimulation since they have short telomeres and have lost their ability to induce telomerase after activation.

We next investigated the replicative potential of highly differentiated CMV-specific $CD4^+$ T cells. $CD4^+$ T cells from young and elderly individuals that contained CMV- and PPD-specific cells were stimulated repeatedly with either CMV lysate or PPD together with exogenous IL-2 in 10-day restimulation cycles (Fig. 5*D*). The representative results of one of four experiments for young and one of two for elderly subjects are shown. PD were calculated over the culture period and in both young and old individuals, and both CMV- and PPD-specific $CD4^+$ cells from elderly individuals showed reduced capacity to expand compared with the same populations in young subjects (Fig. 5*D*). In addition, CMV-specific cells in both age groups had reduced replicative capacity compared with PPD-specific T cells and reached growth arrest earlier (Fig. 5*D*). Because replicative senescence in T cells is linked directly to the extent of telomere erosion (5, 36), a functional consequence of the extreme differentiation of CMV-specific $CD4^+$ T cells is their restricted capacity for expansion after specific stimulation.

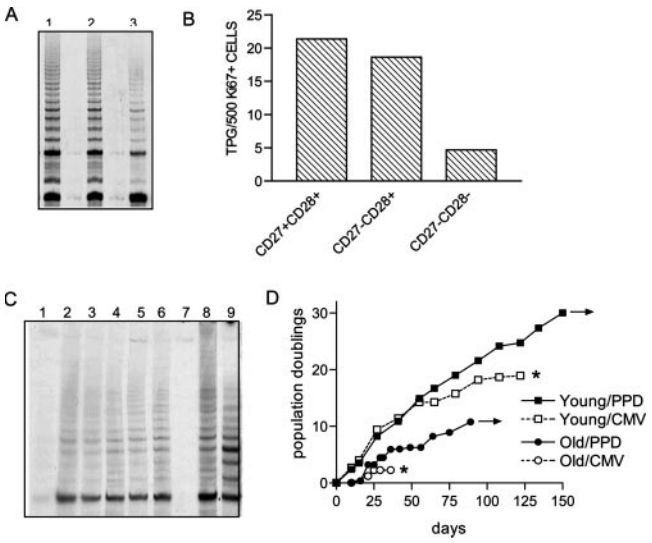

FIGURE 5. Replicative capacity and telomerase inducibility of CMV-specific $CD4^+$ T cells. $CD4^+$ T cells were sorted into $CD27^+CD28^+$ (*lane 1*), $CD27^-CD28^+$ (*lane 2*), and $CD27^-CD28^-$ (*lane 3*) subsets and stimulated with anti-CD3 and -APC, and telomerase activity was measured by TNF-related activation protein assay on day 4 (*A*). The analyzed data are shown graphically as TPG units measured in 500 $Ki67^+CD4^+$ T cells (*B*). Data are representative of three separate experiments. PBMC from a young individual were stimulated with CMV (*lane 1*), PPD (*lane 2*), tetanus (*lane 3*), VZV (*lane 4*), and SEB (*lane 5*) Ags or anti-CD3 (*lane 6*). Results for negative (*lane 7*) and positive (*lane 8*) PCR controls and a telomerase-positive cell line (*lane 9*) are shown (*C*). Telomerase activity was measured on day 4 by TNF-related activation protein assay. Telomerase activity is proportional to the density of the ladder indicated by an arrow. Long-term CMV- and PPD-specific $CD4^+$ T cell cultures were established from young and old donors, and PD (*y*-axis) were calculated after each restimulation (*D*). (*, The point of replicative senescence beyond which cultures did not expand; →, the point at which cultures that continued to expand were cryopreserved). Data shown are representative of four young and two old donors.

Discussion

An emerging consensus is that CMV has a negative impact on immunity in elderly humans and is central in the development of the IRP that predicts early mortality in these individuals (12, 13). It is not known why or how CMV infection exerts this deleterious effect and how the control of immunity to this virus changes during aging. Using new technology to assess the extent of telomere erosion as an indicator of replicative history, we show that CMV-specific $CD4^+$ T cells in old and young subjects have undergone more extensive cell division compared with other Ag-specific $CD4^+$ T cell populations and find that one consequence of this is that these cells are susceptible to be lost as a result of replicative senescence.

The late differentiation phenotype exhibited by CMV-specific $CD4^+$ T cells may result both from persistent reactivation of specific $CD4^+$ T cells and because CMV and vial TLR also activate PDC to secrete differentiation-inducing cytokines such as IFN-α (30, 37). We found that IFN-α secreted by PDC that were stimulated by CMV lysate could inhibit telomerase activity and also induce the loss of costimulatory molecules by Ag-activated $CD4^+$ T cells in vitro. This effect is not restricted to $CD4^+$ T cells, and this cytokine also accelerates the phenotypic differentiation of activated $CD8^+$ T cells in vitro (35). An interesting observation was that in CMV-seropositive subjects, $CD4^+$ T cells that were specific for an array of non-CMV Ags were more likely to have lost expression of costimulatory molecules. Because PDC are found in T cell areas of lymph nodes (38), local CMV reactivation and high IFN-α secretion induction may exert bystander effects on the differentiation of T cells of any specificity that are stimulated locally (39).

IFN-α may not be the only cytokine involved in accelerating T cell differentiation during CMV reactivation. TNF-α, another cytokine that is induced at high concentration by CMV (40), has also been shown to down-regulate CD28 expression by disrupting transcription factor assembly (41). The mechanism of telomerase inhibition by IFN-α may occur in part at the transcriptional level (42) and is the subject of ongoing studies. Additional support for the role of IFN-α in the acceleration of T cell differentiation comes from a study showing that hepatitis C virus-infected individuals on IFN-α therapy displayed increased proportions of $CD8^+$ T cells with a $CD28^-$ phenotype (43). The effect of CMV infection on the secretion of proinflammatory cytokines such as TNF-α is a potential link between the observed raised levels of proinflammatory cytokines and extreme T cell differentiation, which are part of the IRP (12, 13, 44, 45).

The demonstration that there is substantial telomere erosion and loss of replicative capacity of CMV-specific T cells has to be reconciled with the observation that there is an increase rather than a decrease in the number of CMV-specific T cells during aging (16, 17, 32). Clonal expansions within the $CD8^+$ T cell pool have been described in elderly subjects (14, 46), and these cells have been

shown recently to be CMV specific (17, 47). We now show that there are expansions of CMV-specific CD4$^+$ T cells in these individuals. A large proportion of CMV-specific CD8$^+$ T cells have a suboptimal function suggesting that the clonal populations that accumulate may be inefficient at controlling the virus (8). We were not able to address this in the present study because we used a functional assay (IFN-γ secretion) to measure Ag-specific T cells. The dysfunctional CMV-specific CD8$^+$ T cells that accumulate in elderly subjects may arise as a result of clonal evolution within CMV-specific T cell populations during lifelong persistent infection. It has been proposed that clones with the highest avidity and/or functional capacity that are present early in the response are lost and replaced by other cells with lower avidity (48–50). A possible reason for the clonal accumulation may be because a greater number of inefficient CMV-specific T cells are required for virus control in elderly subjects since the most efficient populations may have been exhausted through persistent virus reactivation. The presence of CD8$^+$ T cell clones in elderly individuals may also be related to the resistance of these cells to apoptosis (51, 52). However, our results indicate that oligoclonal populations of functional CMV-specific CD4$^+$ T cells have low Bcl-2 and are susceptible to death (our unpublished observations). Taken together, these data suggest a model where telomere erosion in highly differentiated CMV-specific T cells drives clonal evolution so that those with low avidity and suboptimal functional capacity accumulate in elderly subjects (5).

All these observations point to the key question of why CMV infection and the IRP are associated with decreased survival of elderly individuals. Although extreme differentiation, telomere erosion, and replicative senescence of specific T cells may shape the CMV-specific T cell repertoire, the IRP is not due to loss of CMV-specific immunity because pathological CMV reactivation has not been reported in elderly subjects. However, patients with dyskeratosis congenita who have a defect in the telomerase gene or a telomerase-associated protein dyskerin experience CMV pneumonitis (53). This suggests that lack of telomerase activity in CMV-specific T cells may eventually lead to the premature loss of immunity to this virus and suggests that this requires further study in elderly subjects.

Another explanation for the detrimental effect of CMV infection in old age is that expanded suboptimal clones of CMV-specific T cells may smother other smaller memory T cell populations through competition for space or growth factors (5). Because the memory repertoire in elderly individuals cannot be efficiently replenished by naive thymic precursors, reactivation of silent viruses such as VZV, which causes shingles in elderly subjects (1), may occur when the overall frequency of virus-specific T cells falls below a certain threshold (5). Our observation that CMV-specific CD4$^+$ T cells are increased significantly while VZV-specific populations are significantly decreased in elderly subjects supports this possibility. Furthermore, CMV seropositivity is correlated with reduced EBV-specific T cell responses in old age (54). Conclusive evidence that clonal expansions of specific T cells may compromise the response to other Ags by shrinking the remaining T cell repertoire was provided by a recent study in mice (55). It was observed that reduced T cell diversity and a functional inability to mobilize parts of the T cell repertoire was induced by expanded T cell clones in vivo (55). This suggests that the negative impact of CMV seropositivity and the IRP on survival in the elderly may be due in part to congestion of the immune system with clonally expanded CMV-specific T cells with suboptimal function that hinder the normal function of other memory populations.

The accumulating data highlighting the detrimental effect of CMV infection, especially in elderly individuals, suggests that it may be important to consider ways of controlling CMV replication either with vaccines or with specific drugs (56). A crucial question that remains to be answered is when should such therapy be initiated to impart maximum benefit for elderly subjects.

Acknowledgments

We thank Professors Peter Beverley, Paul Griffiths, and Vince Emery for valuable discussions. We thank C. McGregor for collection of blood samples and T. Tams and L. L. Belaramani for technical help. We are also grateful to all the blood donors who took part in this study.

Disclosures

The authors have no financial conflict of interest.

References

1. Arvin, A. M. 1996. Varicella-zoster virus. *Clin. Microbiol. Rev.* 9: 361–381.
2. Scott, B. J., D. C. Powers, J. E. Johnson, and J. E. Morley. 1994. Seroepidemiological evidence of Epstein-Barr virus reactivation in a veterans' nursing home. *Serodiagn. Immunother. Infect. Dis.* 6: 87–92.
3. Nagami, P. H., and T. T. Yoshikawa. 1983. Tuberculosis in the geriatric patient. *J. Am. Geriatr. Soc.* 31: 356–363.
4. McVoy, M. A., and S. P. Adler. 1989. Immunologic evidence for frequent age-related cytomegalovirus reactivation in seropositive immunocompetent individuals. *J. Infect. Dis.* 160: 1–10.
5. Akbar, A. N., P. C. Beverley, and M. Salmon. 2004. Will telomere erosion lead to a loss of T cell memory? *Nat. Rev. Immunol.* 4: 737–743.
6. Linton, P. J., and K. Dorshkind. 2004. Age-related changes in lymphocyte development and function. *Nat. Immunol.* 5: 133–139.
7. Haynes, B. F., M. L. Markert, G. D. Sempowski, D. D. Patel, and L. P. Hale. 2000. The role of the thymus in immune reconstitution in aging, bone marrow transplantation, and HIV-1 infection. *Annu. Rev. Immunol.* 18: 529–560.
8. Pawelec, G., A. Akbar, C. Caruso, R. Effros, B. Grubeck-Loebenstein, and A. Wikby. 2004. Is immunosenescence infectious? *Trends Immunol.* 25: 406–410.
9. Akbar, A. N., J. M. Lord, and M. Salmon. 2000. IFN-α and IFN-β: a link between immune memory and chronic inflammation. *Immunol. Today* 21: 337–342.
10. Ho, M. 1990. Epidemiology of cytomegalovirus infections. *Rev. Infect. Dis.* 12(Suppl. 7): S701–S710.
11. Ling, P. D., J. A. Lednicky, W. A. Keitel, D. G. Poston, Z. S. White, R. Peng, Z. Liu, S. K. Mehta, D. L. Pierson, C. M. Rooney, et al. 2003. The dynamics of herpesvirus and polyomavirus reactivation and shedding in healthy adults: a 14-month longitudinal study. *J. Infect. Dis.* 187: 1571–1580.
12. Wikby, A., B. Johansson, J. Olsson, S. Lofgren, B. O. Nilsson, and F. Ferguson. 2002. Expansions of peripheral blood CD8 T lymphocyte subpopulations and an association with cytomegalovirus seropositivity in the elderly: the Swedish NONA immune study. *Exp. Gerontol.* 37: 445–453.
13. Olsson, J., A. Wikby, B. Johansson, S. Lofgren, B. O. Nilsson, and F. G. Ferguson. 2000. Age-related change in peripheral blood T lymphocyte subpopulations and cytomegalovirus infection in the very old: the Swedish longitudinal OCTO immune study. *Mech. Ageing Dev.* 121: 187–201.
14. Posnett, D. N., R. Sinha, S. Kabak, and C. Russo. 1994. Clonal populations of T cells in normal elderly humans: the T cell equivalent to "benign monoclonal gammapathy." *J. Exp. Med.* 179: 609–618.
15. Looney, R. J., A. Falsey, D. Campbell, A. Torres, J. Kolassa, C. Brower, R. McCann, M. Menegus, K. McCormick, M. Frampton, et al. 1999. Role of cytomegalovirus in the T cell changes seen in elderly individuals. *Clin. Immunol.* 90: 213–219.
16. Ouyang, Q., W. M. Wagner, A. Wikby, S. Walter, G. Aubert, A. I. Dodi, P. Travers, and G. Pawelec. 2003. Large numbers of dysfunctional CD8$^+$ T lymphocytes bearing receptors for a single dominant CMV epitope in the very old. *J. Clin. Immunol.* 23: 247–257.
17. Khan, N., N. Shariff, M. Cobbold, R. Bruton, J. A. Ainsworth, A. J. Sinclair, L. Nayak, and P. A. Moss. 2002. Cytomegalovirus seropositivity drives the CD8 T cell repertoire toward greater clonality in healthy elderly individuals. *J. Immunol.* 169: 1984–1992.
18. Amyes, E., C. Hatton, D. Montamat-Sicotte, N. Gudgeon, A. B. Rickinson, A. J. McMichael, and M. F. Callan. 2003. Characterization of the CD4$^+$ T cell response to Epstein-Barr virus during primary and persistent infection. *J. Exp. Med.* 198: 903–911.
19. Lucas, M., A. L. Vargas-Cuero, G. M. Lauer, E. Barnes, C. B. Willberg, N. Semmo, B. D. Walker, R. Phillips, and P. Klenerman. 2004. Pervasive influence of hepatitis C virus on the phenotype of antiviral CD8$^+$ T cells. *J. Immunol.* 172: 1744–1753.
20. Sallusto, F., J. Geginat, and A. Lanzavecchia. 2004. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu. Rev. Immunol.* 22: 745–763.
21. Rufer, N., W. Dragowska, G. Thornbury, E. Roosnek, and P. M. Lansdorp. 1998. Telomere length dynamics in human lymphocyte subpopulations measured by flow cytometry. *Nat. Biotechnol.* 16: 743–747.
22. Reed, J. R., M. Vukmanovic-Stejic, J. M. Fletcher, M. V. Soares, J. E. Cook, C. H. Orteu, S. E. Jackson, K. E. Birch, G. R. Foster, M. Salmon, et al. 2004. Telomere erosion in memory T cells induced by telomerase inhibition at the site of antigenic challenge in vivo. *J. Exp. Med.* 199: 1433–1443.

23. Soares, M. V., F. J. Plunkett, C. S. Verbeke, J. E. Cook, J. M. Faint, L. L. Belaramani, J. M. Fletcher, N. Hammerschmitt, M. Rustin, W. Bergler, et al. 2004. Integration of apoptosis and telomere erosion in virus-specific CD8$^+$ T cells from blood and tonsils during primary infection. *Blood* 103: 162–167.
24. Harley, C. B., A. B. Futcher, and C. W. Greider. 1990. Telomeres shorten during ageing of human fibroblasts. *Nature* 345: 458–460.
25. Maini, M. K., M. V. Soares, C. F. Zilch, A. N. Akbar, and P. C. Beverley. 1999. Virus-induced CD8$^+$ T cell clonal expansion is associated with telomerase up-regulation and telomere length preservation: a mechanism for rescue from replicative senescence. *J. Immunol.* 162: 4521–4526.
26. Bodnar, A. G., N. W. Kim, R. B. Effros, and C. P. Chiu. 1996. Mechanism of telomerase induction during T cell activation. *Exp. Cell Res.* 228: 58–64.
27. Valenzuela, H. F., and R. B. Effros. 2002. Divergent telomerase and CD28 expression patterns in human CD4 and CD8 T cells following repeated encounters with the same antigenic stimulus. *Clin. Immunol.* 105: 117–125.
28. Effros, R. B., R. Allsopp, C. P. Chiu, M. A. Hausner, K. Hirji, L. Wang, C. B. Harley, B. Villeponteau, M. D. West, and J. V. Giorgi. 1996. Shortened telomeres in the expanded CD28$^-$CD8$^+$ cell subset in HIV disease implicate replicative senescence in HIV pathogenesis. *AIDS* 10: F17–F22.
29. Chehimi, J., S. E. Starr, H. Kawashima, D. S. Miller, G. Trinchieri, B. Perussia, and S. Bandyopadhyay. 1989. Dendritic cells and IFN-α-producing cells are two functionally distinct non-B, non-monocytic HLA-DR$^+$ cell subsets in human peripheral blood. *Immunology* 68: 488–490.
30. Feldman, S. B., M. Ferraro, H. M. Zheng, N. Patel, S. Gould-Fogerite, and P. Fitzgerald-Bocarsly. 1994. Viral induction of low frequency interferon α producing cells. *Virology* 204: 1–7.
31. Dalod, M., T. P. Salazar-Mather, L. Malmgaard, C. Lewis, C. Asselin-Paturel, F. Briere, G. Trinchieri, and C. A. Biron. 2002. Interferon α/β and interleukin 12 responses to viral infections: pathways regulating dendritic cell cytokine expression in vivo. *J Exp. Med.* 195: 517–528.
32. Ouyang, Q., W. M. Wagner, W. Zheng, A. Wikby, E. J. Remarque, and G. Pawelec. 2004. Dysfunctional CMV-specific CD8$^+$ T cells accumulate in the elderly. *Exp. Gerontol.* 39: 607–613.
33. van Leeuwen, E. M., E. B. Remmerswaal, M. T. Vossen, A. T. Rowshani, P. M. Wertheim-van Dillen, R. A. van Lier, and I. J. Ten Berge. 2004. Emergence of a CD4$^+$CD28$^-$granzyme B$^+$, cytomegalovirus-specific T cell subset after recovery of primary cytomegalovirus infection. *J. Immunol.* 173: 1834–1841.
34. Weng, N. P., B. L. Levine, C. H. June, and R. J. Hodes. 1995. Human naive and memory T lymphocytes differ in telomeric length and replicative potential. *Proc. Natl. Acad. Sci. USA* 92: 11091–11094.
35. Borthwick, N. J., M. Lowdell, M. Salmon, and A. N. Akbar. 2000. Loss of CD28 expression on CD8$^+$ T cells is induced by IL-2 receptor γ chain signalling cytokines and type I IFN, and increases susceptibility to activation-induced apoptosis. *Int. Immunol.* 12: 1005–1013.
36. Hodes, R. J., K. S. Hathcock, and N. P. Weng. 2002. Telomeres in T and B cells. *Nat. Rev. Immunol.* 2: 699–706.
37. Krug, A., A. R. French, W. Barchet, J. A. Fischer, A. Dzionek, J. T. Pingel, M. M. Orihuela, S. Akira, W. M. Yokoyama, and M. Colonna. 2004. TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. *Immunity* 21: 107–119.
38. Colonna, M., G. Trinchieri, and Y. J. Liu. 2004. Plasmacytoid dendritic cells in immunity. *Nat. Immunol.* 5: 1219–1226.
39. Cella, M., D. Jarrossay, F. Facchetti, O. Alebardi, H. Nakajima, A. Lanzavecchia, and M. Colonna. 1999. Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nat. Med.* 5: 919–923.
40. Geist, L. J., H. A. Hopkins, L. Y. Dai, B. He, M. M. Monick, and G. W. Hunninghake. 1997. Cytomegalovirus modulates transcription factors necessary for the activation of the tumor necrosis factor-α promoter. *Am. J. Respir. Cell Mol. Biol.* 16: 31–37.
41. Lewis, D. E., M. Merched-Sauvage, J. J. Goronzy, C. M. Weyand, and A. N. Vallejo. 2004. Tumor necrosis factor α and CD80 modulate CD28 expression through a similar mechanism of T cell receptor-independent inhibition of transcription. *J Biol. Chem.* 279: 29130–29138.
42. Xu, D., S. Erickson, M. Szeps, A. Gruber, O. Sangfelt, S. Einhorn, P. Pisa, and D. Grander. 2000. Interferon α down-regulates telomerase reverse transcriptase and telomerase activity in human malignant and nonmalignant hematopoietic cells. *Blood* 96: 4313–4318.
43. Manfras, B. J., H. Weidenbach, K. H. Beckh, P. Kern, P. Moller, G. Adler, T. Mertens, and B. O. Boehm. 2004. Oligoclonal CD8$^+$ T cell expansion in patients with chronic hepatitis C is associated with liver pathology and poor response to interferon α therapy. *J. Clin. Immunol.* 24: 258–271.
44. Franceschi, C., M. Bonafe, S. Valensin, F. Olivieri, M. De Luca, E. Ottaviani, and G. De Benedictis. 2000. Inflamm-aging: an evolutionary perspective on immunosenescence. *Ann. NY Acad. Sci.* 908: 244–254.
45. Wikby, A., F. Ferguson, R. Forsey, J. Thompson, J. Strindhall, S. Lofgren, B. O. Nilsson, J. Ernerudh, G. Pawelec, and B. Johansson. 2005. An immune risk phenotype, cognitive impairment, and survival in very late life: impact of allostatic load in Swedish octogenarian and nonagenarian humans. *J. Gerontol. A Biol. Sci. Med. Sci.* 60: 556–565.
46. Schwab, R., P. Szabo, J. S. Manavalan, M. E. Weksler, D. N. Posnett, C. Pannetier, P. Kourilsky, and J. Even. 1997. Expanded CD4$^+$ and CD8$^+$ T cell clones in elderly humans. *J. Immunol.* 158: 4493–4499.
47. Weekes, M. P., M. R. Wills, J. G. Sissons, and A. J. Carmichael. 2004. Long-term stable expanded human CD4$^+$ T cell clones specific for human cytomegalovirus are distributed in both CD45RA^{high} and CD45RO^{high} populations. *J. Immunol.* 173: 5843–5851.
48. Annels, N. E., M. F. Callan, L. Tan, and A. B. Rickinson. 2000. Changing patterns of dominant TCR usage with maturation of an EBV-specific cytotoxic T cell response. *J. Immunol.* 165: 4831–4841.
49. Davenport, M. P., C. Fazou, A. J. McMichael, and M. F. Callan. 2002. Clonal selection, clonal senescence, and clonal succession: the evolution of the T cell response to infection with a persistent virus. *J. Immunol.* 168: 3309–3317.
50. Lin, M. Y., L. K. Selin, and R. M. Welsh. 2000. Evolution of the CD8 T cell repertoire during infections. *Microbes Infect.* 2: 1025–1039.
51. Posnett, D. N., J. W. Edinger, J. S. Manavalan, C. Irwin, and G. Marodon. 1999. Differentiation of human CD8 T cells: implications for in vivo persistence of CD8$^+$. *Int. Immunol.* 11: 229–241.
52. Spaulding, C., W. Guo, and R. B. Effros. 1999. Resistance to apoptosis in human CD8$^+$ T cells that reach replicative senescence after multiple rounds of antigen-specific proliferation. *Exp. Gerontol.* 34: 633–644.
53. Dokal, I. 2000. Dyskeratosis congenita in all its forms. *Br. J. Haematol.* 110: 768–779.
54. Khan, N., A. Hislop, N. Gudgeon, M. Cobbold, R. Khanna, L. Nayak, A. B. Rickinson, and P. A. Moss. 2004. Herpesvirus-specific CD8 T cell Immunity in old age: cytomegalovirus impairs the response to a coresident EBV infection. *J. Immunol.* 173: 7481–7489.
55. Messaoudi, I., J. Lemaoult, J. A. Guevara-Patino, B. M. Metzner, and J. Nikolich-Zugich. 2004. Age-related CD8 T cell clonal expansions constrict CD8 T cell repertoire and have the potential to impair immune defense. *J. Exp. Med.* 200: 1347–1358.
56. Griffiths, P. D., J. E. Feinberg, J. Fry, C. Sabin, L. Dix, D. Gor, A. Ansari, and V. C. Emery. 1998. The effect of valaciclovir on cytomegalovirus viremia and viruria detected by polymerase chain reaction in patients with advanced human immunodeficiency virus disease: AIDS Clinical Trials Group Protocol 204/Glaxo Wellcome 123-014 International CMV Prophylaxis Study Group. *J. Infect. Dis.* 177: 57–64.