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# Cytomegalovirus contributes partly to uraemia-associated premature immunological ageing of the T cell compartment

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## Summary

**Cytomegalovirus (CMV) infection has been implicated in accelerated T cell ageing. End-stage renal disease (ESRD) patients have a severely immunologically aged T cell compartment but also a high prevalence of CMV infection. We investigated whether CMV infection contributes to T cell ageing in ESRD patients. We determined the thymic output by the T cell receptor excision circle (TREC) content and percentage of CD31+ naïve T cells. The proliferative history of the T cell compartment by determination of the relative telomere length (RTL) and the T cell differentiation status was determined by immunophenotyping. It appeared that CMV infection did not affect thymic output but reduced RTL of CD8+ T cells in ESRD patients. Moreover, increased T cell differentiation was observed with higher percentages of CD57+ and CD28null CD4+ and CD8+ memory T cells. These CD28null T cells had significantly shorter telomeres compared to CD28+ T cells. Therefore we concluded that CMV infection does not affect the decreased thymic output but increases T cell differentiation as observed in ESRD-related premature T cell ageing.**

**Keywords:** ageing, cytomegalovirus, end-stage renal disease, T lymphocytes, telomeres

## Introduction

Due to the progressive involution of thymic tissue, ageing of the T cell compartment in healthy individuals is associated with decreased numbers of circulating naive T cells. This coincides with an increased differentiation status and proliferative history of memory T cells. The process of immunological T cell ageing is related to an age-related decline in cellular immunity, resulting in reduced vaccination efficacy, enhanced susceptibility for infectious diseases and a higher risk for the development of tumours [1–5]. Higher numbers of differentiated $CD4^+$ T cells have also been associated with a higher prevalence and severity of atherosclerotic disease [6–9]. We recently documented that patients with end-stage renal disease (ESRD) have a profound prematurely aged T cell system which is believed to be caused by the uraemia-induced proinflammatory conditions [10,11]. The immunological age was determined using three parameters: thymic output of newly formed T cells, the differentiation profile of T cells and their relative telomere length. The thymic function can be measured by T cell receptor excision circles (TREC), which are small circular DNA episomes created in T cell precursors that are formed in the thymus during rearrangement of T cell receptor (TCR) genes [12] and the expression of CD31 on naive T cells [10]. Based on these parameters, the average immunological age of T cells in ESRD patients is 20–30 years higher than that of healthy individuals [10].

Because infection with cytomegalovirus (CMV) has a profound effect on the circulating T cell compartment in healthy individuals, CMV has been implicated in immunological ageing. For instance, CMV-infected individuals (CMV-seropositive) have a more differentiated memory T cell compartment, a decreased CD4/CD8 ratio, an expansion of $CD4^+$ and $CD8^+$ T cells lacking CD28 but expressing CD57 [7,13–15] and a reduction in their T cell telomere length, indicating an increased proliferative history of the T cells [16]. These effects of CMV on the T cell compartment are relevant, as a large population of healthy individuals is infected with CMV [17]. The prevalence ranges between 30 and 100%, increases with age and is dependent upon an individual's socio-economic and ethnic background [8].

More than 70% of ESRD patients are CMV-seropositive, and we have shown previously that a seropositive CMV

  

**Table 1.** Study population characteristics

| | CMV-seronegative ESRD patients | | CMV-seropositive ESRD patients | |
|---|---|---|---|---|
| | Young | Old | Young | Old |
| Number of individuals | 38 | 38 | 42 | 41 |
| Age (years) | 33·7 ± 9·4† | 61·3 ± 7·7† | 36·9 ± 7·8† | 64·6 ± 8·4† |
| Male | 69·4% | 78·2% | 54·7% | 61·6% |
| CMV titre (AU/ml) | – | – | 70·9 ± 5·04† | 75·8 ± 2·92† |
| Underlying kidney disease | | | | |
| Hypertensive nephropathy | 6 (15·8%) | 9 (23·7%) | 10 (23·8%) | 14 (34·1%) |
| Primary glomerulopathy | 9 (23·7%) | 9 (23·7%) | 10 (23·8%) | 5 (12·2%) |
| Diabetic nephropathy | 0 (0%) | 3 (7·9%) | 1 (2·4%) | 3 (7·3%) |
| Polycystic kidney disease | 0 (0%) | 2 (5·3%) | 2 (4·8%) | 2 (4·9%) |
| Reflux nephropathy | 8 (21·1%) | 2 (5·3%) | 4 (9·5%) | 3 (7·3%) |
| Other | 8 (21·1%) | 7 (18·4%) | 9 (21·4%) | 6 (14·6%) |
| Unknown | 7 (18·4%) | 6 (15·8%) | 6 (14·3%) | 8 (19·5%) |

†Data are given in means with standard deviation. CMV: cytomegalovirus; ESRD: end-stage renal disease.

status is associated with an increased differentiation status of the T cells as determined by phenotyping of the T cell compartment [7,14]. However, no information is available on other parameters of immunological ageing, such as TREC content, recent thymic emigrants and telomere length, in relation to CMV serostatus in ESRD patients.

In this study we tested the hypothesis that CMV infection in ESRD patients may play an important role in all aspects of premature immunological ageing of the T cell compartment.

## Materials and methods

### Study population

ESRD patients, defined by an estimated glomerular filtration rate of ≤15 ml/min/$m^2$ by the Modification of Diet in Renal Disease (MDRD) formula, and with or without dialyses, were included. Patients were excluded if they had an infection, malignancy, autoimmune disease or a history of immunosuppressive drugs (including previous kidney transplantations). CMV-seronegative and -seropositive ESRD patients were age- and sex-matched as well as matched for all or not receiving renal replacement therapy (haemodialysis or peritoneal dialysis). Patients receiving anti-viral therapy (i.e. valganciclovir) were excluded from the study. Their clinical characteristics are shown in Table 1. All individuals included gave informed consent and the study was approved by the local medical ethical committee (METC number: 2012-022). It was conducted according to the principles of the Declaration of Helsinki and in compliance with the regulations of International Conference on Harmonization/Good Clinical Practice.

### CMV serology

At the diagnostic department of Virology (Erasmus Medical Center, Rotterdam, the Netherlands), serum immunoglobulin (Ig)G antibodies to CMV were measured with an enzyme immunoassay (Biomerieux, Vidas, Lyon, France) and expressed as arbitrary units/ml (AU/ml). In line with the manufacturer's guidelines, an outcome of 6 AU/ml was considered positive with respect to the presence of CMV-specific IgG antibodies. In addition, the absence of serum antibodies IgM to CMV as well as a negative polymerase chain reaction (PCR) for CMV (no detectable level of viral DNA), both determined at the diagnostic department of Virology, served only to include CMV-seropositive patients with a latent CMV infection.

### PBMCs isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples drawn from clinically stable ESRD patients on the day of visiting the out-patient clinic [10]. Two million PBMCs were snap-frozen for the TREC assay; the remaining cells were frozen in liquid nitrogen with a minimum amount of $10 \times 10^6$ cells per vial for further experiments.

### DNA isolation and TREC assay

TREC content was assessed using snap-frozen PBMCs. Briefly, DNA was isolated according to the manufacturer's instructions (Qiagen Isolation kit; Qiagen, Venlo, the Netherlands). Subsequently, TREC content was determined using quantitative PCR. A combination of two primers and a hydrolysis probe specific for the so-called δREC(TCRD)-ψJα(TCRA) TREC (sjTREC) was employed. TaqMan quantitative PCR was performed on 50 ng DNA in a 25-µl reaction mixture containing 700 nmol/l of each primer, 5′-TCGTGAGAACGGTGAATGAAG-3′ and 5′-CCATGCTG ACACCTCTGGTT-3′, 150 nmol/l of hydrolysis probe 5′-(FAM) CACGGTGATGCATAGGCACCTGC-3′ (TAMRA), and 12·5 µl ×2 TaqMan Universal PCR Master Mix (Applied

Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). Quantification of the DNA amount in each sample was performed using a quantitative PCR of the single-copy albumin gene. All reactions were performed in duplicate, unless a threshold cycle (Ct) difference between replicates of >1·5 necessitated repeating the PCR experiment. ΔCt was calculated by using the formula: Ct value TREC PCR – Ct value albumin PCR [10,12].

### Telomere length assay

Flow fluorescence *in-situ* hybridization was performed to determine the telomere length of $CD4^+$ and $CD8^+$ T cells. The isolated PBMCs were stained with either CD4-biotin (Beckman-Coulter, BV, Woerden, the Netherlands) or CD8-biotin (Biolegend, Europe BV, Uithoorn, the Netherlands) followed by staining with streptavidin-cyanin 5 (Cy5) (Biolegend). The PBMCs were fixed and permeabilized (Invitrogen Life Technologies, Bleiswijk, the Netherlands) and then, using the telomere PNA-kit/fluorescein isothiocyanate (FITC) (Zebra Bioscience BV, Enschede, the Netherlands), we determined the relative telomere length. The subcell-line 1301 of CCRF-CEM, which is known to have long telomeres, was used to calculate the relative telomere length (RTL) of the $CD4^+$ and $CD8^+$ T cells using the following formula [18]:

$$\mathrm{RTL} = \frac{\begin{array}{c}(\text{median FL1 sample cells with probe} - \\ \text{median FL1 sample cells without probe}) \times \\ \text{DNA index of control } (=2) \text{ cells} \times 100\end{array}}{\begin{array}{c}(\text{median FL1 control cells with probe} - \\ \text{median FL1 control cells without probe}) \times \\ \text{DNA index of sample } (=1) \text{ cells}\end{array}}$$

In addition, PBMCs of five elderly CMV-seropositive ESRD patients were sorted into a purified $CD28^+$ or $CD28^{null}$ $CD4^+$ or $CD8^+$ T cell fraction to examine whether or not the relative telomere length differed in these sorted T cell fractions. For this purpose, PBMCs ($20 \times 10^6$) were stained with AmCyan-labelled anti-CD3 (BD Biosciences, Erembodegem, Belgium), Pacific Blue-labelled anti-CD4 (BD Biosciences), allophycocyanin (APC)-labelled anti-CD8 (BD Biosciences), phycoerythrin (PE)-labelled anti-CD28 (BD Biosciences) and with 7-aminoactinomycin D (7AAD) (BD Biosciences). Sorting was performed on a FACSAria II SORP (BD Biosciences). All fractions had a purity of more than 95%.

### Telomerase activity assay

The activity of the telomerase enzyme was measured in five CMV-seropositive and five age-matched CMV-seronegative ESRD patients using the TRAPeze® XL telomerase detection kit (Millipore, Temecula, CA, USA), according to the manufacturer's instructions. Briefly, PBMCs ($20 \times 10^6$) were sorted into purified and viable $CD4^+$ and $CD8^+$ T cell fractions (according to the sort protocol described briefly under Telomere length assay).

The sorted T cell fractions (all with a purity of more than 95%) were stimulated with anti-CD3/CD28 beads (25 μl/1 ml; Invitrogen Life Technologies) for 3 days at 37°C. Next, cells were resuspended in CHAPS lysis buffer (provided in the kit) and cell extractions were made (10–750 μg). Protein levels were determined by using the Bio-Rad protein assay (Bio-Rad, München, Germany).

This assay is based on the capacity of a test sample to amplify a telomere template. The activity is expressed in total product-generated (TPG) units, which is calculated using the TSR8 standard curve (provided in the kit).

### Differentiation status of T cells

A whole blood staining was performed to determine the T cell differentiation status [10,11,14]. Briefly, whole blood was stained with AmCyan-labelled anti-CD3 (BD Biosciences) in combination with Pacific Blue-labelled anti-CD4 (BD Biosciences) and APC-Cy70-labelled anti-CD8 (BD Biosciences). The T cells are defined as $CD4^+$ or $CD8^+$ and defined further into four different subsets based on the expression of CCR7 and CD45RO. In Supporting information Fig. S1, a typical example of the gating strategy is depicted. Naive T cells are defined as $CCR7^+$ and $CD45RO^-$, central memory (CM) cells as $CCR7^+$ and $CD45RO^+$, effector memory (EM) cells such as $CCR7^-$ and $CD45RO^+$ and EMRA cells such as $CCR7^-$ and $CD45RO^-$. Expression was determined by staining with FITC-labelled anti-CCR7 (R&D Systems, Uithoorn, the Netherlands) and APC-labelled anti-CD45RO (BD Biosciences). T cell differentiation is associated with loss of CD28 expression on the cell surface. The ratio $CD28^+/CD28^-$ (or $CD28^{null}$) T cells within the T cell subsets were determined by staining with peridinin chlorophyll-Cy5·5 (PerCP-Cy5·5)-labelled anti-CD28 (BD Biosciences) and the ratio $CD57^-/CD57^+$ was determined by staining with APC-labelled anti-CD57 (Biolegend). To determine the thymic output of naive T cells, the percentage of $CD31^+$ naive T cells was determined by staining with PE-labelled anti-CD31 (Biolegend) [10,11,14].

### Ki-67 staining of T cells

To quantify the percentage of dividing cells, we stained the cells intracellularly with FITC-labelled anti-Ki-67 after fixation and permeabilization (IntraSure Kit; BD Biosciences). Ki-67 is a nuclear antigen which is expressed selectively in cells that are in the G-M stage of cell division. The frequency of $Ki\text{-}67^+$ cells was determined in the total $CD4^+$ and $CD8^+$ T cell population.

### Statistical analyses

Differences between CMV-seropositive and CMV-seronegative young (age < 50 years) and elderly (age ≥ 50 years) ESRD patients were analysed using the Mann–Whitney *U*-test. For TREC content and RTL, a linear regression model was used. In addition, Spearman's rho correlation coefficients (*R*s) were calculated to determine the strength of the association between TREC content or RTL with age for CMV-seropositive and CMV-seronegative ESRD patients. A paired *t*-test was performed to calculate significant differences in RTL between $CD28^+$ T cells and $CD28^{null}$ T cells. All statistical tests were performed two-sided, while a *P*-value of <0·05 was considered significant.

## Results

### CMV infection does not influence thymic output of naive T cells

Both CMV-seropositive and -seronegative ESRD patients showed a decrease (reflected by an increase ΔCt) in TREC content with increasing age (Fig. 1). The loss of TREC content was similar in both patient groups; comparison of the two lines showed that there were no significant differences in thymic output of naive T cells. (Fig. 1a). In accordance with this finding, no significant differences in percentages of $CD31^+$ naive T cells (recent thymic emigrants) were detected between the CMV-seropositive and -seronegative patients for the $CD4^+$ (Fig. 1b) and $CD8^+$ T cell compartments (Fig. 1c).

In addition, no significant differences were observed when considering absolute numbers [cells/μl, mean ± standard error of the mean (s.e.m.)] of CD31 expressing naive T-$CD4^+$ (young: CMV-seropositive: 151·9 ± 24·42 *versus* CMV-seronegative: 173·6 ± 27·04 and old: CMV-seropositive: 121·4 ± 14·32 *versus* CMV seronegative: 137·2 ± 13·38) and $CD8^+$ (young: CMV-seropositive: 127·6 ± 15·36 *versus* CMV-seronegative: 101·6 ± 15·62 and old: CMV-seropositive: 55·21 ± 5·11 *versus* CMV seronegative: 78·8 ± 7·74) T cells.

### CMV infection contributes to telomere attrition of $CD8^+$ but not $CD4^+$ T cells, but does not affect the activity of the telomerase enzyme

With increasing age, the RTL of $CD4^+$ (Fig. 2a) and $CD8^+$ (Fig. 2b) T cells declines in both CMV-seropositive and -seronegative ESRD patients. CMV did not contribute significantly to telomere attrition within $CD4^+$ T cells ($P = 0·2$, Fig. 2a), but the RTL of the $CD8^+$ T cells was significantly lower in patients with a latent CMV infection ($P = 0·04$) (Fig. 2b).

Using linear regression analysis for chronological age and the RTL of the $CD8^+$ T cells, we were able to estimate the effect of CMV infection on the immunological age of an ESRD patient. For example, the average RTL of a CMV-infected ESRD patient with a chronological age of 40 years was similar to the average RTL of a 60-year-old CMV-seronegative patient.

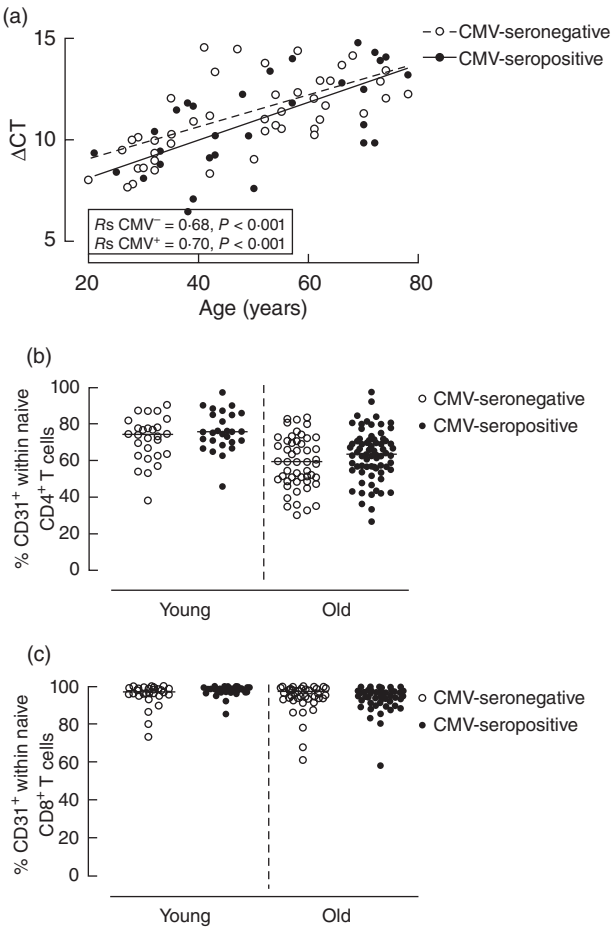


**Fig. 1.** Quantification of T cell receptor excision circle (TREC) content by real-time quantitative polymerase chain reaction (qPCR) and CD31 expression by naive T cells to measure thymic output. The thymic output was measured by two parameters. First, the TREC content was determined (a). The threshold cycle (Ct) is the number of amplification cycles needed to detect the TRECs and is a relative measure, related inversely with the concentration of TRECs. Control for DNA input was conducted by performing a qPCR for albumin and the difference between the Ct for TRECs and the Ct for albumin was calculated. The *x*-axis represents the age and on the *y*-axis the ΔCt value is depicted. The ΔCt values of cytomegalovirus (CMV)-seropositive end-stage renal disease (ESRD) patients (closed symbols) are represented by the closed line; that of CMV-seronegative ESRD patients (open symbols) by the dashed line. Next the patients were separated based on their age (young: <50 years and old: ≥50 years) and the percentage of $CD31^+$ naive $CD4^+$ (b) as well as $CD8^+$ (c) T cells were determined as a marker for thymic output. Statistical differences between groups are shown, i.e. one symbol: $P < 0·05$; two symbols: $P < 0·01$; three symbols: $P < 0·001$) and medians are shown. Individual data-points are shown for CMV-seropositive patients (closed symbols) and CMV-seronegative patients (open symbols).

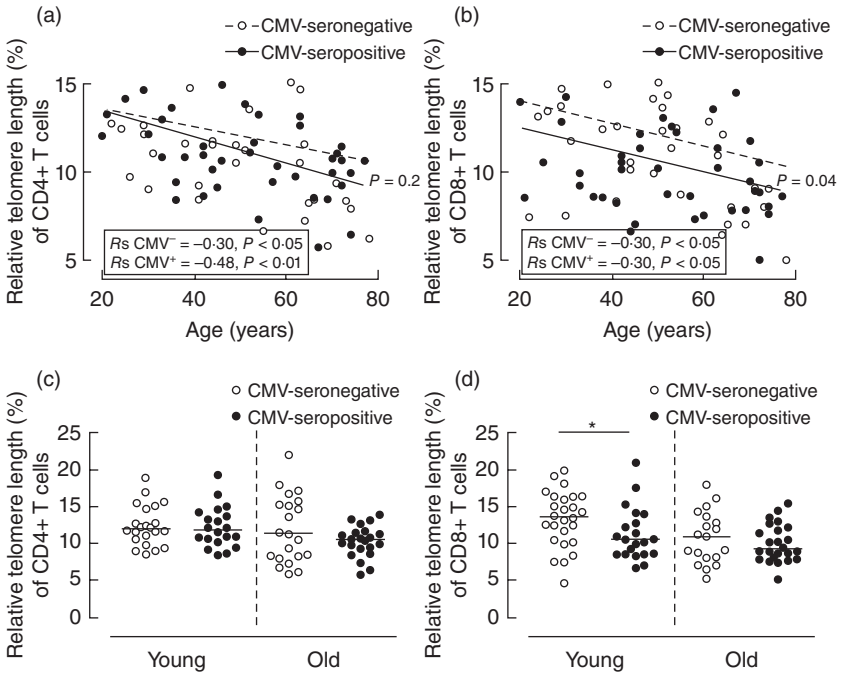


**Fig. 2.** Relative telomere length (RTL) of circulating CD4$^+$ and CD8$^+$ T cells. The RTL was determined for CD4$^+$ (a) and CD8$^+$ (b) T cells of cytomegalovirus (CMV)-seropositive (closed symbols, closed line) and CMV-seronegative (open symbols, dashed line) end-stage renal disease (ESRD) patients. Next, patients were separated based on their age in a young (age < 50 years) and an old (age ≥ 50 years) group and the RTL of CD4$^+$ (c) and CD8$^+$ T cells (d) are shown for each group. Significant differences between groups are shown (one symbol: $P < 0{\cdot}05$; two symbols: $P < 0{\cdot}01$; three symbols: $P < 0{\cdot}001$). Individual data-points with medians are depicted.

Upon dissection of CMV-seropositive as well as CMV-seronegative ESRD patients into a younger (<50 years) and an older (≥50 years) population, no differences were observed in RTL for the CD4$^+$ T cells between CMV-seropositive and -seronegative age-matched groups (Fig. 2c). Younger CMV-seropositive ESRD patients had significantly ($P < 0{\cdot}05$) shorter telomeres within their CD8$^+$ T cell compartment (mean RTL ± s.e.m.; 11·19 ± 0·83%) when compared to CMV-seronegative age-matched counterparts (13·28 ± 0·75%).

Next, we examined if CMV seropositivity is associated with activity of the telomerase enzyme in the CD4$^+$ and CD8$^+$ T cell compartment. Telomerase activity (expressed in TPG units) was similar between CMV-seronegative and CMV-seropositive patients for the CD4$^+$ T cells (mean TPG ± s.e.m.; CMV-seronegative: 0·54 ± 0·004 *versus* CMV-seropositive: 0·55 ± 0·006) and CD8$^+$ T cells (CMV-seronegative: 0·55 ± 0·002 *versus* CMV-seropositive: 0·55 ± 0·002).

## CMV-related changes in differentiation status of T cells and relation to RTL

The significantly lower CD4$^+$ naive/memory ratio ($P < 0{\cdot}05$) indicated a shift towards the memory phenotype within the CD4$^+$ T cell compartment of CMV-seropositive patients (Fig. 3a). Dissection of the memory CD4$^+$ T cells into CM and EM did not show significant CMV-associated differences (data not shown). Next, we determined the differentiation status by examining the loss in CD28 expression and increase in CD57 expression. CMV-infected ESRD patients had, on average, a significantly lower CD28$^+$/CD28$^-$ ($P < 0{\cdot}01$) (Fig. 3b) and CD57$^-$/CD57$^+$ ratio (Fig. 3c) within their CD4$^+$ T cell compartment [$P < 0{\cdot}01$ (young) and $P < 0{\cdot}001$ (elderly), respectively], indicative of CMV-induced increased differentiation of CD4$^+$ T cells. Moreover, we determined the percentages of highly differentiated (i.e. having a senescent phenotype) CD28$^{null}$ CD57$^+$ T cells within the CD4$^+$ T cell compartment for CMV-seropositive and age-matched CMV-seronegative ESRD patient populations. CMV-seropositive ESRD patients had significantly higher percentages of these cells in their circulation than age-matched CMV-seronegative ESRD patients (mean ± s.e.m.; young CMV seropositive: 9·90% ± 3·48 *versus* young CMV seronegative: 0·42% ± 0·23, $P < 0{\cdot}01$ and old CMV seropositive: 26·62% ± 4·27 *versus* old CMV seronegative: 5·78% ± 3·52, $P < 0{\cdot}001$).

We also compared the RTL of sorted CD4$^+$CD28$^{null}$ to that of CD4$^+$CD28$^+$ (purity > 95%) T cells and found that the CD4$^+$CD28$^{null}$ T cells had significantly shorter telomeres ($P < 0{\cdot}01$) compared to the CD4$^+$CD28$^+$ T cells (Fig. 3d).

CMV affected the CD8$^+$ T cell compartment more profoundly than the CD4$^+$ T cell compartment. CMV-seropositive ESRD patients had a significantly ($P < 0{\cdot}05$) lower CD8 naive/memory ratio (Fig. 4a), due to a higher number of memory CD8$^+$ T cells consisting of a large population of terminally differentiated CD8$^+$ EMRA T cells (absolute numbers: CMV-seronegative: $0{\cdot}03 \times 10^6$, CMV-seropositive: $0{\cdot}12 \times 10^6$, $P < 0{\cdot}05$). This was reflected by the significantly lower CD28$^+$/CD28$^-$ (Fig. 4b) ($P < 0{\cdot}001$) and CD57$^-$/CD57$^+$ ratio (Fig. 4c) [$P < 0{\cdot}01$ (young) and $P < 0{\cdot}001$ (elderly), respectively]. Similarly, as observed for the CD4$^+$ T cell compartment, a significantly higher proportion of CD8$^+$ T cells had a senescent phenotype in

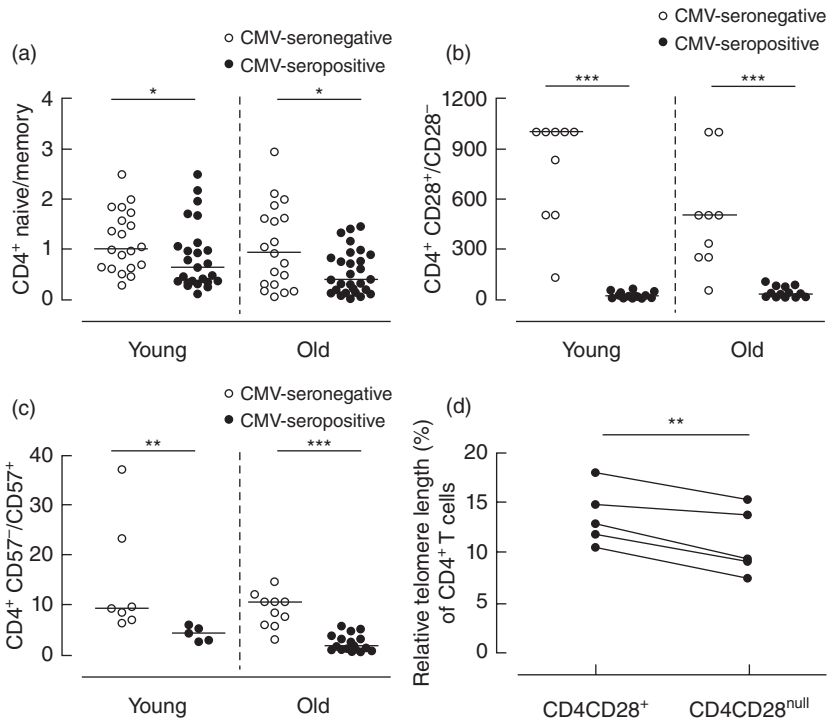


**Fig. 3.** $CD4^+$ T cell phenotype and differentiation status and the correlation with the relative telomere length (RTL). The ratio $CD4^+$ naive/memory T cells were determined for cytomegalovirus (CMV)-seronegative and CMV-seropositive end-stage renal disease (ESRD) patients (a). Moreover, the ratio $CD4^+CD28^+/CD28^-$ (b) and $CD57^-/CD57^+$ (c) were calculated to determine the differentiation status of the $CD4^+$ T cell compartment. ESRD patients were dissected into a young (age < 50 years) and an old (age ≥ 50 years) group. Individual dots are shown with the median for the CMV-seronegative patient population (open symbols) and the CMV-seropositive patients (closed symbols). Significant differences between groups are shown (one symbol: $P < 0{\cdot}05$; two symbols: $P < 0{\cdot}01$; three symbols: $P < 0{\cdot}001$). Next, the RTL was determined within a sorted $CD28^+$ and $CD28^{null}$ $CD4^+$ T cell population of 5 CMV-seropositive ESRD patients (d). Individual data points are shown and significant differences between the two T cell fractions are shown (two symbols: $P < 0{\cdot}01$).

CMV-seropositive ESRD patients when compared to their age-matched CMV-seronegative counterparts (young CMV-seropositive: 50·56% ± 3·77 *versus* young CMV-seronegative: 15·56% ± 4·99, $P < 0{\cdot}01$ and old CMV-seropositive: 47·15% ± 4·09 *versus* old CMV-seronegative: 27·94% ± 5·16, $P < 0{\cdot}05$).

Also, for the $CD8^+$ T cells we determined the RTL in $CD28^{null}$ and $CD28^+$ T cell-sorted populations. The $CD8^+CD28^{null}$ T cells had significantly shorter ($P < 0{\cdot}01$) telomeres than the $CD8^+CD28^+$ T cells (Fig. 4d).

In an attempt to explain the additional telomere attrition induced by CMV, we determined whether CMV infection

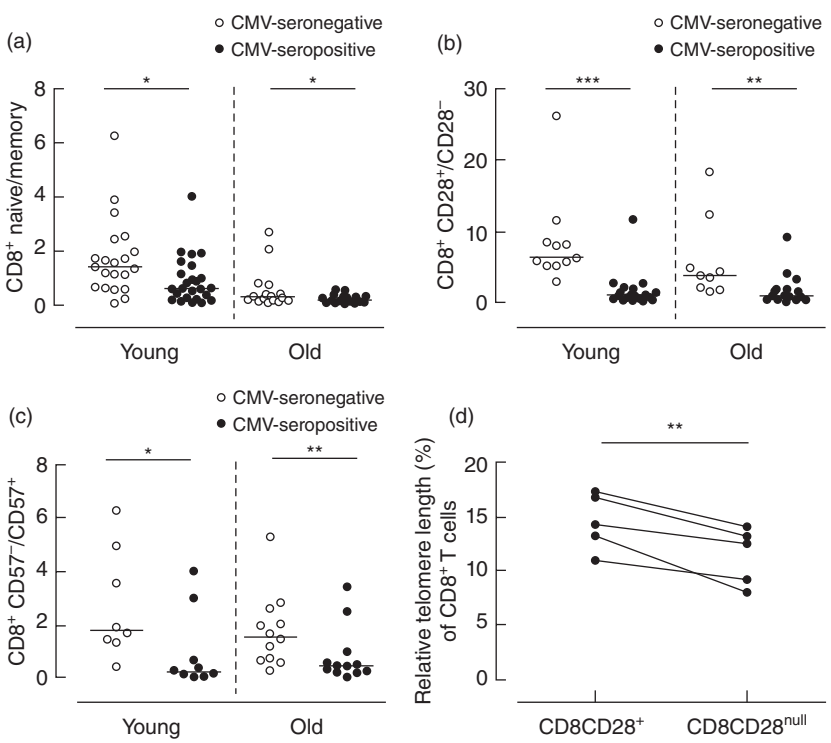


**Fig. 4.** $CD8^+$ T cell phenotype and differentiation status and the correlation with the relative telomere length (RTL). The ratio $CD8^+$ naive/memory T cells were determined in cytomegalovirus (CMV)-seronegative and CMV-seropositive end-stage renal disease (ESRD) patients. (a). Moreover, the ratio $CD8^+CD28^+/CD28^-$ (b) and $CD57^-/CD57^+$ (c) were calculated to determine the differentiation status of the $CD8^+$ T cell compartment. ESRD patients were dissected into a young (age < 50 years) and an old (age ≥ 50 years) group. Individual dots are shown with the median for the CMV-seronegative patient population (open symbols) and the CMV-seropositive patients (closed symbols). Significant differences between groups are shown (one symbol: $P < 0{\cdot}05$; two symbols: $P < 0{\cdot}01$; three symbols: $P < 0{\cdot}001$). Next, we determined the RTL within a sorted $CD28^+$ and $CD28^{null}$ $CD8^+$ T cell population from five elderly CMV-seropositive ESRD patients (d). Individual data points are shown and significant differences between the two T cell fractions are shown (two symbols: $P < 0{\cdot}01$).

induced an increase in the proliferation of $CD4^+$ as well as $CD8^+$ T cells by determining the percentage of Ki-$67^+$ T cells (i.e. the percentage of T cells actually dividing). No significant differences were observed in the percentage of Ki-$67^+$ $CD4^+$ or $CD8^+$ T cells ($CD4^+$Ki-$67^+$ T cells; CMV-seronegative: 2·09% ± 0·68 CMV-seropositive: 1·33% ± 0·52 and $CD8^+$Ki-$67^+$ T cells; CMV-seronegative: 1·99% ± 0·60 CMV-seropositive: 1·34% ± 0·25).

## Discussion

The results of this study show that CMV-seropositivity is associated with more differentiated memory $CD4^+$ and $CD8^+$ T cell compartments. These highly differentiated T cells show loss of CD28 expression, increased expression of CD57 and shorter telomeres. CMV did not affect the thymic output of new naive T cells, and therefore CMV-seropositivity impacts only partly upon the ESRD-related immunological ageing of the T cell system.

In a previous study [10], we observed that the characteristics of the peripheral T cell system of ESRD patients are very similar to healthy individuals with a chronological age that is, on average, 20–30 years older. One of the salient findings in ESRD patients and elderly healthy individuals was a decreased number of circulating naive T cells [10].

In humans, the thymus is the single organ involved in naive T cell generation. Ageing results in involution of the thymus with subsequent decrease in TREC content within the circulating T cell population. Because TREC content is related reliably and linearly with age, measuring the TREC content in blood can be used as a tool for age determination for forensic purposes [12]. In both ESRD patients and elderly healthy individuals a decreased thymic output of naive T cells based upon TREC analysis was observed. Next to the TREC content, an alternative technique to identify recent thymic emigrants is to measure the CD31 expression on naive T cells [19], which corroborates the findings of the TREC content. In addition, activation and increased numbers of proliferating Ki-$67^+$ naive T cells were observed. Homeostatic proliferation occurs in response to this decreased thymic output to maintain the naive T cell compartment. Our findings do not support a role for CMV in the decreased output of naive T cells or their peripheral proliferation in the periphery, as both the TREC content and the percentage of $CD31^+$ and Ki-$67^+$ cells were not affected by CMV serostatus. This also suggests that the expansion and differentiation of memory T cells in CMV-seropositive patients does not change the number or homeostatic proliferation of naive T cells. This may have been expected, as it is assumed that increased turnover of this compartment would also accelerate the turnover of naive T cells.

Another parameter to assess the immunological age of T cells is to determine the telomere length of $CD4^+$ and $CD8^+$ T cells, which is indicative of the proliferative history of the cells. Similarly to TREC content, overall there is a clear inverse correlation between RTL and age in both healthy individuals and ESRD patients. However, the $CD8^+$ T cells of CMV-infected ESRD patients have substantially shorter telomeres than age-matched CMV-seronegative ESRD patients, resulting in an immunological age difference of almost 20 years. This finding indicates a higher burden by CMV on $CD8^+$ T cells of ESRD patients during ageing. We could not detect this CMV-related effect in RTL for the $CD4^+$ T cells. The absence of additional CMV-induced telomere attrition within total $CD4^+$ T cells in ESRD patients in contrast to that within total $CD8^+$ T cells can therefore be explained by the difference in differentiation status of the T cell compartment. To examine whether the telomere shortage in $CD8^+$ T cells is caused by a possible inhibitory effect on the activity of the telomerase enzyme (responsible for extending the telomere length), we analysed the activity of this enzyme in both $CD8^+$ and $CD4^+$ T cell populations. No differences were found between the CMV-seronegative and CMV-seropositive patients, indicating that altered telomerase activity is not a probable cause for the decreased RTL in CD8 T cells of CMV-seropositive ESRD patients. This indicates that the shorter telomeres for the $CD8^+$ T cell compartment is caused by the higher proliferation and differentiation status in CMV-seropositive patients.

CMV infection induces a shift within both $CD4^+$ and $CD8^+$ T cells towards the memory compartment. Specifically, the increase of $CD28^{null}$ T cells within the $CD4^+$ and $CD8^+$ T cell compartment is highly associated with a previous CMV infection [14,20,21]. However, $CD8^+$ memory T cells contain far more $CD28^{null}$ as well as $CD57^+$ T cells when compared to $CD4^+$ T cells. These differentiated T cells are known to have short telomeres [16,22], which we could confirm for ESRD patients in this study. The CD57-expressing cells are found predominantly within the CD2-negative memory T cells, implying that most of the senescent cells are located within this memory fraction and are found to be higher in CMV-seropositive ESRD patients.

As we did not detect an increase in the number of Ki-$67^+$ T cells in the CMV-seropositive patients, we could not establish a higher turnover of memory T cells. This might suggest that, after initial expansion of this cell population shortly after CMV infection [23], these cells will enter a more exhausted state during chronic latency of the virus. This results in a loss of capacity to proliferate accompanied by an increased resistance to apoptosis [24].

Like ESRD patients, individuals infected with human immunodeficiency virus (HIV) have T cell deficiencies which resemble premature T cell ageing, caused probably by continuous triggering of the immune system by the virus [25]. Although the mechanism of creating a prematurely aged T cell compartment for both diseases is different, the end result on T cells is similar (i.e. higher number of differentiated cells with a loss in CD28 expression,

shorter telomeres and a lower number of naive T cells), resulting in similar clinical outcomes such as a higher risk for infections, development of cancer and cardiovascular diseases [26].

In HIV-infected individuals, CMV causes an increase in EMRA $CD8^+CD28^{null}$ T cells expressing CD57. These highly differentiated cells are positive for the effector cytotoxins perforin and granzyme B [27,28]. In HIV patients it was found that strong anti-CMV T cell responses result in a lower number of naive T cells for the CD4 T cell compartment [28]. These CMV effects found in HIV patients are in line with CMV effects in ESRD patients.

We have postulated previously that the prematurely aged T cell system in ESRD patients contributes to clinically relevant complications, such as increased infection risk, decreased vaccination response and a highly increased risk for cardiovascular diseases [2,5,6,29–31]. Given their cardiotoxic features, the proinflammatory and highly cytotoxic $CD4^+CD28^{null}$ T cells in ESRD patients can be important for later complications [8]. A number of earlier reports have also shown the relation between CMV serostatus, the expansion of $CD28^{null}$ T cells and the increased risk for atherosclerosis in ESRD patients [6–9]. In addition, increased numbers of highly differentiated $CD8^+$ T cell numbers and a loss of CD28 expression were associated with less allograft rejection after kidney transplantation. Given the results of this study, it seems unlikely that primary immune responses which involve the naive T cell compartment or $CD4^+$ T cell-dependent immune responses in ESRD patients will be affected by their CMV serostatus. At present, such an association has not been reported and CMV serostatus does not seem to affect the vaccination response in children [32,33].

In healthy elderly individuals, CMV seropositivity leads to an expansion of effector $CD8^+$ T cells which are $CD8^+CD28^{null}CD57^+$. These CMV-specific T cells were found to be oligoclonal and can constitute to up to one-quarter of the total $CD8^+$ T cell compartment in elderly which makes cells unable to respond to other pathogens [34]. Moreover, these highly differentiated cells have shorter telomeres and are associated with an increased risk for the development of coronary heart diseases [35].

In conclusion, CMV-positive serostatus is associated with an increased differentiation status of memory T cells and telomere attrition of $CD8^+$ T cells but does not explain the premature T cell ageing associated with the uraemic environment.

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## Disclosure

All authors declare no financial or commercial interests.

## Author contributions

R. Meijers performed the experiments, statistical analysis and wrote the manuscript. N. Litjens designed the study and wrote the manuscript. E. de Wit performed the experiments. A. Langerak contributed to writing the manuscript. A van der Spek performed some of the experiments. C. Baan contributed to writing the manuscript. W. Weimar contributed to writing the manuscript and provided patient data. M. Betjes designed the study and wrote the manuscript.

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## Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's website:

**Fig. S1.** Gating strategy of the $CD4^+$ and $CD8^+$ T cell subsets. From whole blood we first selected for lymphocytes (a); we then selected the $CD3^+$ lymphocytes (T cells) (b) and made a distinction between the $CD4^+$ and $CD8^+$ T cells (c). On the basis of CCR7 and CD45RO, we divided the different subsets [naive, effector memory (EM), central memory (CM) and end-stage renal disease (EMRA)] for the $CD4^+$ (d) and CD8 (e) T cell compartments.