New insights in the T-cell immune system of end-stage renal disease patients

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CHAPTER 1

General introduction and outline of the thesis

INTRODUCTION

End-stage renal disease (ESRD) is a public health problem. The incidence of ESRD is increasing worldwide at an annual growth rate of 8%, far in excess of the population growth rate of 1.3% (1). The number of elderly ESRD patients (defined as aged > 65 years) keeps growing rapidly. According to recent data from the Dutch renal replacement system (REgistratie NIerfunktievervanging NEderland, RENINE), the number of elderly ESRD patients receiving renal replacement therapy (RRT, i.e., hemodialysis and peritoneal dialysis) almost doubled from 2005 to 2015 (https://www.renine.nl/). As a group, the elderly have a higher prevalence of comorbidities including serious infections, which reduce life expectancy and impaired quality of life (2, 3). In the United states, 10% of patients of 65–79 years of age die within the first 3 months after dialysis initiation and the 1-year survival rate after dialysis initiation for all patients of at least 80 years is 54% (2). RRT also includes kidney transplantation (KT) which offers patients a better 5-year life expectancy than dialysis in elderly ESRD patients (4). All kidney transplant recipients including these elderly patients are on standard immunosuppression to prevent rejection of the transplanted organ (5).

Immune status of ESRD patients

Loss of renal function causes retention of uremic toxins and cytokines, leading to increased oxidative stress and inflammatory cytokines (6). This contributes to the pro-inflammatory environment in ESRD patients (7), which is marked by chronic immune deficiency and systemic inflammation (8). Immune deficiency leads to a decreased vaccination efficacy (9-12), an increased susceptibility for infection (3, 13, 14) and a higher risk for developing tumors (15-18) while systemic inflammation contributes to atherosclerosis and cardiovascular disease (1, 19, 20). Together these abnormalities account for the large proportion of morbidity and mortality in ESRD patients (21, 22).

T cells represent a major component of adaptive immune system and play a central part in cell-mediated immunity. T cells develop after clonal T cell receptor (TCR) selection in the thymus. Upon emigrating the thymus, naive T cells carry specific TCR that recognise epitopes. In response to corresponding antigens, multiple signal pathways induce naive T cells to proliferate and differentiate into effector cells. The majority of effector cells migrate to peripheral tissues and inflamed sites to facilitate destruction of infected targets (23, 24). Following antigen clearance, most of the effector cells die and only a small pool of T cells ultimately remains as long-lived memory T cells (25). Recently, the concept of premature immunological ageing has proposed/introduced to explain the uremia-associated defective T cell-mediated immune system (26, 27). The immunological age of ESRD patients is increased by 20-30 years compared with the chronological age-matched healthy individuals (HI), based on less thymic output, altered T cell composition towards a more differentiated

phenotype and shorter telomeres (28). A proper in depth understanding of the uremia-induced defective T cell-mediated immune system is warranted to facilitate individualized immunosuppressive regimens to prevent over-immunosuppression and its associated complications.

The ageing immune system

Ageing is associated with a decline in immune function and affects various cells types of both the innate and adaptive immune system(29). The decline in T cell number and function appear to be key features of immune cell ageing. This is due to a decreased capacity of aged hematopoietic stem cells to generate lymphoid progenitor cells, from which T cells originate, and an age-related atrophy of the thymus, in which T cells develop further (30-32). During ageing, naive T cell number, T cell receptor Vβ -repertoire diversity, expression of co-stimulatory molecules and proliferative capacity of T cells decrease (33-36); in contrast, numbers of memory and effector T cells increase, clones of effector T cells expand (33, 34) and especially an upregulation of the low-grade chronic systemic pro-inflammation occurs (37). It is characterized by raised levels of pro-inflammatory cytokines interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF) (38). This age-related inflammation ("inflammageing") is supposed to be caused by a cumulative lifetime exposure to antigenic load and environmental free radicals (39). This induces inflammatory responses, tissue damage and production of reactive oxygen species. Then consequent oxidative damage as well as an additional release of pro-inflammatory cytokines occurs (40). This results in a vicious cycle, driving immune system remodeling and favoring a chronic pro-inflammatory state (37). Age-related changes of the immune system contribute to the increased susceptibility of elderly persons to infectious diseases, a decline in vaccination efficacy, and an increased risk for development of autoimmune diseases and cancer (41-45).

Traditional T-cell ageing parameters

Several assays have already been used to assess/evaluate T-cell ageing. Below the main features of T-cell ageing are described as well as their corresponding assays for evaluating these T-cell ageing characteristics.

Thymic output

One of the most important features of ageing of T cells is a consequence of involution of the thymus, leading to a decline in functional thymic tissue with increasing age. As a result of thymic involution, thymic output declines with increasing age (46-48). T cell receptor excision circle (TREC), is a product formed upon TCR gene rearrangement during intrathymic T cell maturation. These TRECs are not replicated during T cell proliferation and only passed on to one daughter cell (49). TREC content can be measured using qPCR (50). Another method for evaluating thymic output is by determining frequencies of recent thymic emigrants within the circulation by flow cytometry. CD31 (platelet/endothelial cell adhesion molecule-1, PECAM-1) is expressed on a variety of cell types, including T cells (51). Naive T cells that have

recently left the thymus co-express CD31 and possess the highest TREC content (52, 53). With increasing age, a decrease in absolute counts and frequencies of CD31-expressing naive T cells is observed (46, 47). Thus measuring both TREC-content and frequencies or absolute numbers of CD31-expressing naive T cells can be used to quantify thymic output (48, 54, 55).

T cell differentiation status

Another characteristic feature of T-cell ageing is the shift occurring from naive to memory T cells, the T cell differentiation status can be assessed by flow cytometry. As shown in Fig. 1, elderly people display a decline in naive T cell number in peripheral blood; in contrast, the proportion of highly differentiated memory T cells increases markedly (56-58). Naive and memory T cells within the peripheral blood can be distinguished based on CD45RO (isoform of CD45 expressed by memory T cells) and CCR7 (C-C motif receptor 7, a chemokine receptor facilitating T cells homing to lymph nodes) expression (Fig. 1). Naive T cells are $CD45RO^{-}CCR7^{+}$. Central memory (CM) T cells ($CD45RO^{+}$ $CCR7^{+}$) have the potential to home to secondary lymphoid tissues, produce high amounts of interleukin (IL)-2 but low levels of other effector cytokines (e.g. IL-4, IL-5 and IFNγ) (59); while effector memory (EM) T cells have rapid effector function and potential to home to peripheral tissues, produce high levels of IL-4 and IL-5 ($CD4^{+}$ T cells only), and/or IFN-γ (both $CD4^{+}$ and $CD8^{+}$ T cells), and have granules containing perforin and granzyme B for immediate cytotoxicity (part of $CD4^{+}$ T cells and $CD8^{+}$ T cells) (59). Terminally differentiated effector memory $CD45RA^{+}$ (EMRA, $CD45RO^{-}CC7^{-}$) T cells are generally negative for CD28 (a T cell co-stimulatory molecule that with increasing differentiation of T cells gets lost from the cell surface) (60). $CD4^{+}$ $CD28^{-}$ T cells are highly cytotoxic as they secrete large amounts of IFN-γ, and possess perforin- and granzyme B-containing granules, (61, 62). Both $CD4^{+}CD28^{-}$ and $CD8^{+}CD28^{-}$ T cells represent a heterogeneous population

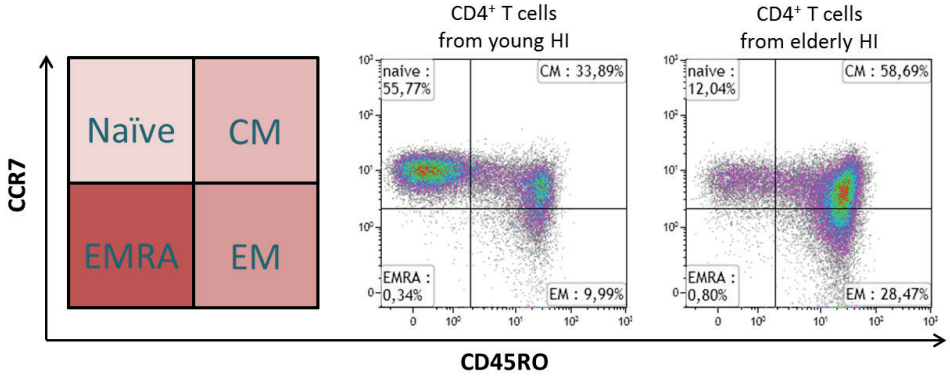

Fig. 1. Age-related effects on $CD4^{+}$ T cell subsets from healthy individuals (HI). Naive, central memory (CM), effector memory (EM), terminally differentiated effector memory (EMRA) can be distinguished based on differential expression of CD45RO and CCR7. A shift from naive T cell subset towards memory subset occurs during ageing.

composed of various functionally competing (cytotoxic and immunosuppressive) subsets (63). The cytotoxic $CD8^+CD28^-$ T cells also produce high amounts of IFN-γ and tumor necrosis factor-α (TNF-α) (64), while immunosuppressive $CD8^+CD28^-$ T cell subsets secrete IL-10 (65). Accumulation of $CD28^-$ T cells may be related to anti-viral immunity directed to for example cytomegalovirus (CMV) (66, 67), or self-antigen directed immune responses (68, 69).

Telomere length

Loss of telomere length, associated with proliferative history of T cells, occurs with increasing age and can be visualized using flow cytometry. Telomere length differs between naive and memory T cells (70). Telomeres are the repetitive nucleotides at the ends of chromosomes, which protects the end of the chromosome from deterioration or from fusion with neighboring chromosomes (70). In the absence of a compensatory mechanism, dividing cells undergo gradual telomere erosion until a critical degree of shortening results in chromosomal abnormalities and cell death or senescence (71). Assessment of telomere length can be used as a reflection of replicative history. Elderly individuals with shorter telomeres have a much higher rate of mortality than those with longer telomeres (72).

New approaches to evaluate the T cell immune status in more detail

In recent years, new assays have become available, allowing for a more detailed analysis of the T cell immune status, evaluating phenotypic aspects, like TCR -repertoire diversity as well as functional characteristics (signaling pathways) upon TCR-mediated activation of T cells and relating this to T-cell ageing.

T cell receptor -repertoire diversity

A diverse (polyclonal) T cell receptor (TCR) -repertoire capable of recognizing a broad range of foreign antigens is key to an effective T-cell-mediated immune response (73). Contraction of TCR Vβ-repertoire has been reported to occur during ageing (74). Naive T cells expressing CD31 possess the broadest TCR -repertoire (i.e. polyclonal TCR -repertoire) (75). Memory T cells that develop upon encountering of an antigen have a repertoire that is skewed towards particular specificities (76) (Fig. 2).

Most TCRs consist of an α and a β chain and each chain is composed of a variable (V) region and a constant (C) region. In the thymus, the V region of the TCR α and β chain is generated by random gene rearrangement of variable (V) and joining (J) genes or V, diversity (D) and J genes, respectively. The TCR Vβ-repertoire can be assessed using several approaches such as gene scan spectratyping via a DNA based PCR (77), Vβ family phenotyping by flow cytometry (78, 79), and clonal diversity via next generation sequencing (NGS) (80). Gene scan spectratyping of the TCR Vβ-repertoire is a qualitative measurement. Both flow cytometry and NGS allow for a more quantitative evaluation of TCR Vβ-repertoire. As NGS is relatively

labor-intensive and requires sorting of highly pure T cells or their subsets, many researchers prefer to use flow cytometry. Flow cytometry allows for measuring percentages of TCR Vβ families at the T cell subset level obviating the need for cell sorting. Tracking alloreactive TCR-repertoire prior to and following KT may provide a good biomarker predicting rejection and drug related side-effects leading to adapt the immunosuppressive regimen, prevent graft dysfunction, and improved graft survival (81).

The TCR Vβ-repertoire diversity might also be affected by chronic antigenic stimulation (82-84). For example, CMV latency may also induce contraction of the TCR Vβ-repertoire as it results in a vast expansion of CMV-specific T cells exceeding 4% of CD8+ T cells in immunocompetent donors (85) and these anti-CMV T cells clones are stably maintained for 5 years (86) (Fig. 2).

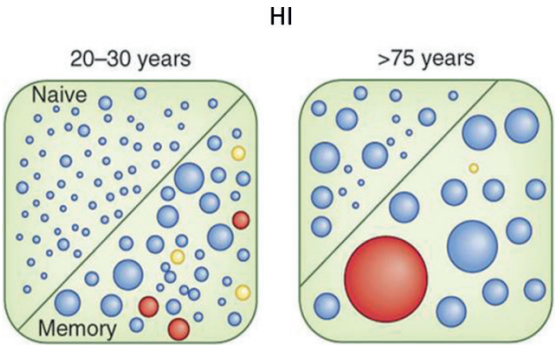

Fig. 2. Age-related effects on TCR -repertoire diversity in healthy individuals (HI). The TCR -repertoire of naive and memory T cells changes with age (circle size indicates clonal size). A diverse naive repertoire has been established in the young adult (left). The naive compartment decreases in size and TCR diversity in elderly life (right). T cell clones specific for CMV (red circles) dominate the repertoire in the elderly and contribute to the contraction of diversity in the memory compartment. Adapted from Jörg J Goronzy & Cornelia M Weyand Nature Immunology 2013.

MAPK pathway during T cell activation (signal transduction)

T-cell ageing is associated with defective signaling pathways (87, 88). The mitogen-activated protein kinase (MAPK) pathway is one of the major pathways induced upon TCR stimulation (89) (Fig. 3). Activation of MAPK is mediated by phosphorylation of MAPK and downregulated by MAPK phosphatase resulting in inactive MAPK (90). In particular, the extracellular signal-regulated kinase (ERK) pathway is one of the important MAPK pathways. ERK activity controls the positive feedback loop in the TCR-induced activation cascade (91, 92), reduces sensitivity of cells to apoptosis and promotes T cell proliferation (93). Reduced ERK activity affects TCR-mediated signal strength, T cell activation and IL-2 production (94, 95). Dual-specificity phosphate (DUSP) 6 is a cytoplasmic phosphatase with substrate

specificity to dephosphorylate pERK and decreased phosphorylation of ERK can be overcome by inhibiting DUSP6 (96). Recently it has been shown that decreased ERK phosphorylation in naive CD4+ T cells from elderly HI was associated with a lower sensitivity to TCR-mediated signals and more time to build up the required signaling strength compared to those from young HI (91). DUSP6 is one of the important regulators of the TCR activation threshold that controls the initial ERK phosphorylation and expression of DUSP6 increases with age (91) (Fig. 4). P38 is another pivotal protein in the MAPK pathway (97). Highly differentiated $CD27^{-}CD28^{-}CD4^{+}$ T cells have higher phosphorylation of P38 compared with CD27+CD28+ and $CD27^{-}CD28^{+}CD4^{+}$ T cells and this could be driven by intracellular changes such as DNA-damage (98, 99). Phosphorylation of signaling proteins upon T cell activation may be used to monitor the immune activation status in KT patients prior to and following transplantation (100).

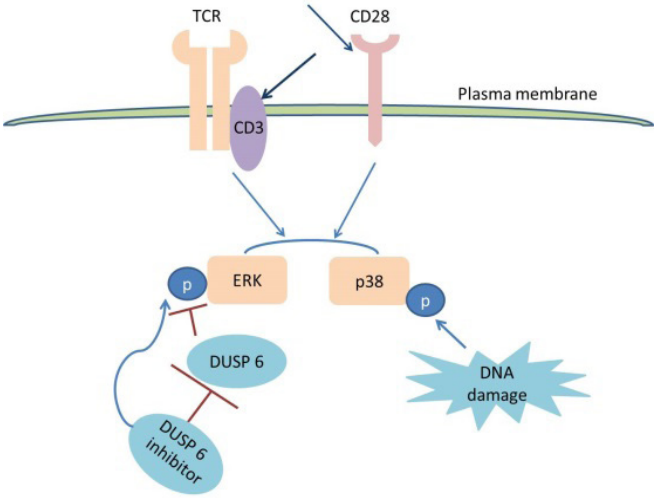

Fig. 3. Mitogen-activated protein kinase (MAPK) pathway after T cell activation. Anti-CD3 (T cell receptor) and anti-CD28 (co-stimulator) stimulation promotes a number of signaling cascades including MAPK. Extracellular signal-regulated kinase (ERK) and P38 belong to MAPK pathway and their activation is mediated by phosphorylation. Dual specificity phosphate (DUSP6) is a phosphatase that inhibits ERK activation. DNA damage can also cause p38 phosphorylation especially in highly differentiated T cells.

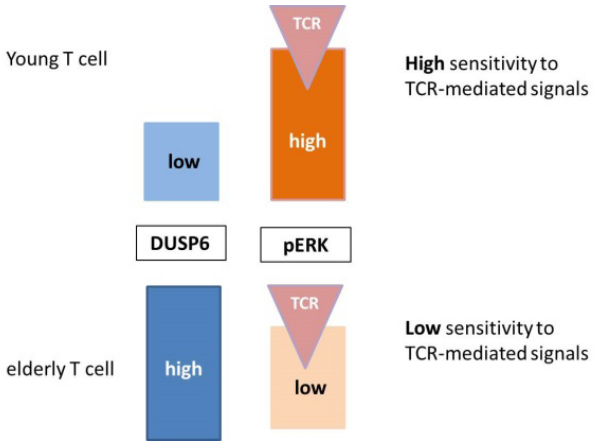

Fig. 4. T cell receptor (TCR) desensitization in the elderly. Phosphorylated ERK (pERK) is associated with TCR sensitivity to TCR-mediated signals. DUSP6 expression increases with age, resulting in a decline in pERK and desensitization of TCR-mediated activation. Adapted from Jörg J Goronzy & Cornelia M Weyand Nature Immunology 2013.

Cytomegalovirus infection in ESRD patients

Depending on ethnicity and social-economic background, 65-100% of elderly ESRD patients are CMV seropositive (101, 102). CMV may have a substantial impact on the composition and function of circulating T cells, resembling features of ageing of the immune system. Studies have shown that CMV significantly expands the number of circulating $CD8^+$ T cells by almost twofold (103), promotes emergence of highly differentiated T cell subsets (104) and may decrease T cell telomere length (105) in immune competent individuals. In young to middle-aged ESRD patients, the additional effects of CMV latency on T cell ageing parameters were modest and mainly confined to $CD8^+$ T cells (106).

An infection with CMV is also one of the most common complications after kidney transplantation. In kidney transplant recipients, CMV infection and disease have been reported in 8-31% and 8% respectively (107), posing a critical challenge on both graft and patient survival (108, 109). In clinical practice, anti-CMV immunoglobulin (Ig) G is used for immune-risk stratification. CMV IgG-seronegative ESRD patients receiving kidneys from CMV IgG-seropositive donors are at high risk to develop CMV infection, but still a considerable proportion (30%-40%) of this group does not experience a CMV infection (110). CMV-specific T cell responses play an important role in controlling viral infection. Furthermore, the production of neutralizing antibodies by CMV specific B-cells/plasma blasts depends on adequate help from CMV-specific $CD4^+$ T cells (111-113). Effective cytotoxic $CD8^+$ T cell (CTL) responses also contribute to control CMV infections (114, 115).

The aim and outline of this thesis

Uremia-associated premature T-cell ageing contributes to a defective T cell-mediated immune system in ESRD patients. A comprehensive more in depth evaluation of defective T cell-mediated immune system, taking functional aspects into account, in ESRD patients is crucial to identify patients at risk for infections, virus-related cancers, and decreased vaccination efficacy and allograft rejection. In this thesis, a more detailed assessment of the defective T-cell mediated immune system, studying both phenotypic as well as functional aspects, in ESRD patients is described. Special emphasis is put on the increasing population of elderly ESRD patients.

In chapter 1, the concept of several T-cell ageing parameters and the role of CMV infection in ESRD patients are introduced. Studies with respect to the defective T-cell mediated immune system in the rapidly growing elderly ESRD population are limited, hence in **chapter 2**, we evaluate the effect of uremia and CMV on T cells focusing on elderly ESRD patients to test whether the uremia-induced premature ageing remains in the elderly population. Although the composition of the circulating T cells may be profoundly altered in ESRD patients, it is not known whether this invariably leads to a change in TCR Vβ- repertoire diversity, therefore, in **chapter 3**, we measure the TCR Vβ-repertoire diversity in a qualitative way using DNA-based spectratyping in ESRD patients, also assessing the contribution of CMV and age on TCR Vβ-repertoire diversity. For a more detailed view of this ESRD-related effect on TCR Vβ- repertoire diversity, a quantitative measure is needed of the TCR Vβ-repertoire within T cell subsets, also taking into account effects of age and CMV. Therefore, we used a flow cytometry-based approach and antibodies to 24 Vβ families as described in **chapter 4**. Upon unraveling the uremia-associated changes of T cell phenotype and DNA, described in previous chapters, we are also interested in the uremia-associated effects on more downstream events upon TCR-mediated activation of T cells (signal transduction pathways). **In chapter 5**, we examine the MAPK pathway including ERK and P38 phosphorylation following TCR stimulation and the role of DUSP6 inhibition on ERK phosphorylation in ESRD patients. Assessing CMV-specific T cell-immunity may allow for a more accurate characterization of the immune risk for a CMV-infection in ESRD patients before transplantation. I**n chapter 6,** CMV-specific T cell-immunity is determined in CMV IgG-seronegative renal transplant recipients before transplantation and clinical relevance with respect to CMV viremia after transplantation is assessed. Finally in **chapter 7**, we summarize and discuss in depth the work of this thesis.

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CHAPTER 2

Latency for cytomegalovirus significantly impacts T cell ageing in elderly end-stage renal disease patients

Ling Huang[1], Anton W. Langerak[2], Carla C. Baan[1], Nicolle H.R. Litjens[1] and Michiel G.H. Betjes[1].

[1]Department of Internal Medicine, Section Nephrology and Transplantation, Erasmus University Medical Center, Rotterdam, the Netherlands.
[2]Department of Immunology, Erasmus University Medical Center, Rotterdam, the Netherlands.

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ABSTRACT

The number of elderly patients with end-stage renal disease (ESRD) has significantly increased over the last decade. Elderly ESRD patients are vulnerable to infectious complications because of an aged immune system. Additional immunological ageing effects may be derived from the uremic environment and cytomegalovirus (CMV) latency. Elderly patients may be affected by these factors in particular, but data in this age group are limited. To assess the degree of immunological ageing and proliferative capacity of T lymphocytes, 49 elderly ESRD patients (defined as aged ≥65 years) on the renal transplantation waiting list were recruited and compared to 44 elderly HI, matched for age and CMV serostatus. CMV latency was associated with more highly differentiated $CD4^+$ and $CD8^+$ T cells in both elderly HI and patients. Elderly CMV-seropositive ESRD patients showed a substantial reduction in the number of naive $CD4^+$ and $CD8^+$ T cells compared with age- and CMV-serostatus-matched HI. Elderly ESRD patients also showed significantly decreased numbers of central memory $CD4^+$ and $CD8^+$ T cells compared with HI, independent of CMV serostatus. In addition, thymic output and relative telomere length of both $CD4^+$ and $CD8^+$ T cells were decreased in CMV-seropositive ESRD patients compared with HI. The proliferative capacity of T cells was similar for patients and HI. Elderly ESRD patients have an advanced aged T cell compartment when compared to age-matched healthy controls, which is mainly driven by CMV latency.

INTRODUCTION

The number of elderly patients (defined as aged ≥65 years) suffering from end-stage renal disease (ESRD) keeps growing rapidly (1). In the USA, during 1994-2004 patients aged more than 75 years increased by 67% compared to 24% for those aged between 5 and 74 years (2). According to recent data from the Dutch renal replacement system (REgistratie NIerfunktievervanging NEderland, RENINE), the number of elderly ESRD patients (aged >65 years) receiving renal replacement therapy (RRT) almost doubled from 2005 to 2015 (https://www.renine.nl/). Importantly, elderly ESRD patients are at high risk of developing serious infections (2-4) and show a poor response to vaccination (5, 6). Also after successful kidney transplantation, elderly ESRD patients are more susceptible to infectious complications (7, 8). T cells are key players in the immune response to foreign antigens, such as those encountered during an infection and after vaccination.

With advanced ageing, the T-cell mediated immune system undergoes dramatic changes (9) and loss of renal function is associated with a defective T-cell mediated immune system(10). We have demonstrated previously that ESRD-related defects in T cell-mediated immunity may be related to premature T-cell ageing, as assessment of T cell receptor excision circle (TREC) content, T cell differentiation status and relative telomere length revealed a discrepancy of 15-20 years between the immunological age of the patients' T cells and their chronological age(11, 12).

Cytomegalovirus (CMV) may have a substantial impact on the composition and function of circulating T cells. Recent studies have shown that CMV latency expands the number of circulating CD8 T cells significantly by almost twofold (13), promotes the emergence of highly differentiated T cell subsets (14) and may decrease T cell telomere length (15) in immune competent individuals. Depending on the ethnicity, 65-100% of all elderly ESRD patients are CMV-seropositive (16, 17). In young to middle-aged ESRD patients, the additional effects of CMV latency on T cell ageing parameters are modest and confined mainly to the $CD8^+$ T cells (18).

However, little is known with respect to the impact of ESRD and CMV latency on the immunological age of the peripheral T cell compartment in elderly (≥65 years of age) ESRD patients. In this study, we show that CMV latency appears to be a dominant factor for the observed advanced immunological ageing of T cells from elderly ESRD patients as compared to healthy age-matched individuals.

MATERIALS AND METHODS

Study population

Forty-nine stable elderly (defined as ≥65 years) ESRD patients, defined as having a glomerular filtration rate of ≤15 ml/min with or without renal replacement therapy and 44 elderly healthy individuals (HI) were included (study population characteristics are described in Table 1) from 1st November 2010 to 1st October 2013 at the outpatient clinic. Patients with any clinical or laboratory evidence of acute bacterial or viral infection, malignancy, immunosuppressive drugs treatment within 28 days prior to transplantation (except glucocorticoids) were excluded. Lithium-heparinized blood was drawn of ESRD patients and healthy kidney donors. All individuals included gave informed consent and the local medical ethical committee approved the study (METC number: 2012–022), which was conducted according to the principles of Declaration of Helsinki and in compliance with International Conference on Harmonization/Good Clinical Practice regulations.

Table 1. Clinical and demographic characteristics of patients with end-stage renal disease (ESRD) and healthy individuals (HI)

	ESRD patients	HI	*P*-value
Number of individuals	49	44	
Age(years; median with range)	68; 65–79	70; 65–89	n.s.
Male (%)	69.4	45.5	0.022
CMV-IgG serostatus (% seropositive)	59.2	63.6	n.s.
Patients on dialysis (%)	55.1		
Haemodialysis (%)	76.9		
Peritoneal dialysis (%)	19.2		
Haemodialysis followed peritoneal dialysis(%)	3.8		
Patients with renal transplant history (%)	2.0		
Underlying kidney disease			
Nephrosclerosis/atherosclerosis/ hypertensive nephropathy	28.6		
Primary glomerulopathy	10.2		
Diabetic nephropathy	28.6		
Reflux nephropathy	8.1		
Polycystic kidney disease	18.4		
Other	6.1		

CMV = cytomegalovirus; Ig = immunoglobulin; n.s. = not significant.

Circulating T cell numbers and their differentiation status

Freshly drawn peripheral blood samples from 49 ESRD patients and 44 HI were stained and acquired on a fluorescence activated cell sorter (FACS) Canto II flow cytometer (BD Biosciences, Erembodegem, Belgium) to determine both absolute numbers and frequencies of the different T cell subsets, as described previously (11, 19) . Data were analyzed using FACS Diva software version 6.1.2 (BD Biosciences).

PBMCs isolation, cell culture and proliferation analysis

PBMCs from 11 elderly ESRD patients and 11 elderly CMV serostatus-matched HI were isolated from peripheral blood, as described previously (20). These PBMCs (responder cells) were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE), according to manufacturer's instructions (Thermo Fisher scientific, Waltham, MA, USA), and then co-cultured in triplicate at 5×10^4/well with allogeneic PBMCs, autologous PBMCs (both irradiated at 40 gray) at a 1:1 ratio or with 5 µg/ml phytohaemagglutinin (PHA) (Sigma-Aldrich, St. Louis, MO, USA) as a positive control for 6 days. Culture medium consisted of RPMI 1640 with GlutaMAX, 10% heat-inactivated pooled human serum and 1% penicillin and streptomycin. After 6 days, PBMCs were harvested, pooled, washed and stained with AmCyan-labeled CD3 (BD Pharmingen, Erembodegem, Belgium), Pacific Blue-labeled CD4 (BD), allophycocyanin-cyanin 7 (APC-Cy7)-labeled CD8 (BD); phycoerythin (PE)-labeled CD28 (BD), APC-labeled CD45RO (BD), and PE-Cy7-labeled CCR7 (R&D systems, Uithoorn, the Netherlands) antibodies, and a live-dead marker ViaProbe (7-aminoactinomycin D, BD). Data were acquired on a FACSCanto II flow cytometer (BD). Percentages of proliferating cells were analyzed by Kaluza® software (Beckman Coulter, Brea, CA, USA). Kinetics of proliferation and precursor frequencies (PF), the latter defined as the proportion of cells present in the original sample being able to respond to the stimulus, were analyzed by Modfit LT® software (Verity Software House, Topsham, ME, USA).

DNA isolation and TREC analysis

DNA was isolated from PMBCs by QIAamp DNA Mini QIAcube Kit, according to manufacturer's instructions (Qiagen, Hiden, Germany). TREC content was measured by TaqMan quantitative PCR as previously described (21). The ΔCt was calculated by subtracting the Ct value for the albumin PCR from that of the TREC PCR. One/ΔCt was used to describe the TREC content of a sample. A Ct value greater than 41 for the TREC PCR was interpreted as the sample having an undetectable TREC content.

Telomere length assay

Flow fluorescent *in-situ* hybridization was performed to determine the relative telomere length (RTL) of $CD4^+$ and $CD8^+$ T cells, as described previously (11, 19).

Statistical analyses

Statistical analyses were performed using SPSS version 20 (IBM, Chicago, IL, USA) and GraphPad Prism version 6 (GraphPad Software, La Jolla, CA, USA). Categorical variables were compared using the χ^2 test or Fisher's exact test. Continuous variables were compared using *t*-test or Mann-Whitney *U*-test. All reported *P*-values are two-sided and were considered statistically significant when $P<0.05$.

RESULTS

Both CMV and ESRD accelerate the ageing phenotype of T cells

The demographic and clinical characteristics of the study population are given in Table 1. Forty-nine ESRD patients (aged 65–79 years) and 44 age-matched HI (aged 65-89 years) were recruited in this study. The elderly ESRD patients consisted of a higher proportion (69.4%, P=0.02) of males compared to the HI (45.5%). Approximately half of the ESRD patients received RRT. CMV-immunoglobulin (Ig) G seropositivity was present in 59.2% of elderly ESRD patients and in 63.6% of HI in this study.

The effect of CMV on absolute T cell numbers and composition was confined mainly to the memory compartment. Elderly CMV seropositive ESRD patients, but not HI, had significantly more total memory $CD4^+$ T cells compared to their CMV seronegative counterparts (Fig. 1a). Within the $CD4^+$ memory compartment, CMV seropositivity was associated with increased numbers of EM (Fig. 1e) and $CD4^+CD28^-$ T cells in elderly ESRD patients (Fig. 1g). The association of CMV seropositivity with higher numbers of $CD4^+CD28^-$ T cells was also observed in elderly HI (Fig. 1g). Elderly CMV seropositive ESRD patients had lower numbers of total (Fig. 1a) and naive (Fig. 1b)

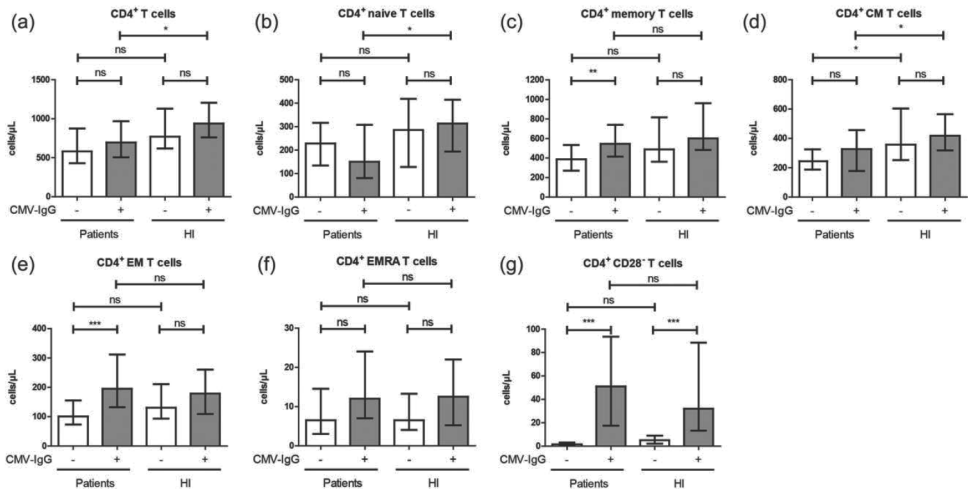

Fig. 1. Absolute numbers of circulating $CD4^+$ T cell subsets in elderly healthy individuals (HI) and elderly end-stage renal disease (ESRD) patients. Numbers of (a) $CD4^+$, (b) $CD4^+$ naive, (c) $CD4^+$ memory, (d) $CD4^+$ central memory (CM), (e) $CD4^+$ effector memory (EM) and (f) $CD4^+$ highly differentiated effector memory (EMRA) and (g) $CD28^-$ $CD4^+$ T cells in HI [n = 45; n = 16 cytomegalovirus (CMV) seronegative and n = 29 CMV seropositive] and ESRD patients (n = 49; n = 20 CMV seronegative and n = 29 CMV seropositive) was determined and dissected for CMV serostatus. Data are given as median with interquartile range. The open bars represent the CMV seronegative individuals and the closed bars represent CMV seropositive ones. P-value: * <0.05; ns: not significant.

CD4⁺ T cells than CMV seropositive HI. Moreover, central memory (CM) CD4⁺ T cells were lower in elderly ESRD patients compared to HI, irrespective of their CMV serostatus (Fig. 1d). Frequencies of T cell subsets also indicated CMV latency and ESRD to induce a more differentiated T cell compartment. CMV seropositivity was associated with lower percentages of CD4⁺ T cells (Supporting information, Fig. S1a) and higher percentages of CD4⁺CD28⁻ T cells (Supporting information, Fig. S1g). Percentages of naive CD4⁺ T cells were lower in CMV seropositive compared with CMV seronegative ESRD patients (Supporting information, Fig. S1b) and within the memory compartment, higher percentages of EM were observed in CMV seropositive ESRD patients compared with CMV seronegative patients (Supporting information, Fig. S1e). In agreement with the absolute number of T cells, the differences of T cell differentiation analyzed as percentage between elderly ESRD patients and HI were observed in the CMV seropositive group. Within CMV seropositive group, ESRD patients had lower percentages of naive CD4⁺ T cells (Supporting information, Fig. S1b) and higher percentages of EM CD4⁺ T cells compared to HI (Supporting information, Fig. S1e).

Within the CD8⁺ T cell compartment, CMV latency induced a strong increase in total numbers of CD8⁺ T cells of both elderly ESRD patients as well as HI. The median CD8⁺ T cell number in CMV seropositive ESRD patients amounted to

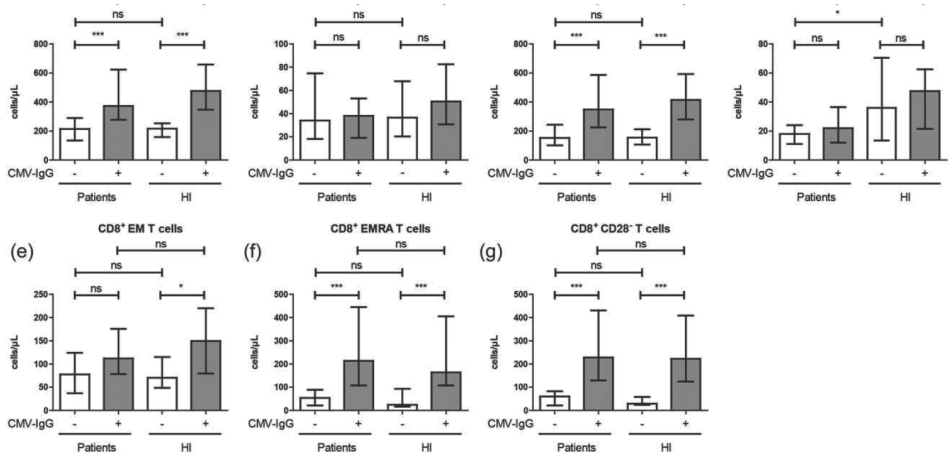

Fig. 2. Absolute numbers of circulating of CD8⁺ T cell subsets in elderly healthy individuals (HI) and elderly end-stage renal disease (ESRD) patients. Numbers of (a) CD8⁺, (b) CD8⁺ naive, (c) CD8⁺ memory, (d) CD8⁺ central memory (CM), (e) CD8⁺ effector memory (EM) and (f) CD8⁺ highly differentiated effector memory (EMRA) and (g) CD28⁻ CD8⁺ T cells in HI [*n* = 45; *n* = 16 cytomegalovirus (CMV) seronegative and *n* = 29 CMV seropositive] and ESRD patients (*n* = 49; *n* = 20 CMV seronegative and *n* = 29 CMV seropositive) were determined and dissected for CMV serostatus. Data are given as median with interquartile range. The open bars represent the CMV seronegative individuals and the closed bars represent of CMV seropositive ones. *P*-value: * <0.05; **< 0.01; ns: not significant.

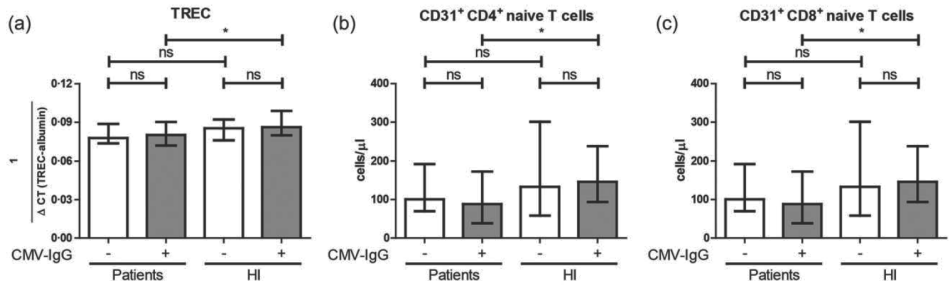

Fig. 3. T cell receptor excision circle (TREC) content and CD31- expressing naive $CD4^+$ and $CD8^+$ T cells in elderly healthy individuals (HI) and end- stage renal disease (ESRD) patients. The (a) TREC content (HI: *n* = 39; ESRD patients: *n* = 43) and absolute number of CD31-expressing naive (b) $CD4^+$ and (c) CD8+ T cells (HI: *n* = 44; ESRD patients: *n* = 49) in elderly HI and ESRD patients was determined and dissected for cytomegalovirus (CMV) serostatus (open bars represent CMV seronegative and closed bars represent CMV seropositive individuals). Data are given as median with interquartile range. *P*-value: *<0.05; ns: not significant.

373 cells/µl, which was a >1.5-fold increase compared to the median of $CD8^+$ in CMV seronegative patients (214 cells/µl, *P*<0.001) (Fig. 2a). A more than two-fold increase in numbers of $CD8^+$ T cells was noted for CMV seropositive HI compared to CMV seronegative HI (476 cells/µl *versus* 217 cells/µl, *P*<0.001) (Fig. 2a). The increase in $CD8^+$ T cells induced by CMV was due mainly to an increase in memory $CD8^+$ T cells in both elderly ESRD patients and HI (Fig. 2c). Within the memory compartment, the number of EM was significantly higher in CMV seropositive HI compared to CMV seronegative HI, and a similar trend was found in patients (Fig. 2e). Increased numbers of highly differentiated T cell subsets including EMRA and $CD8^+CD28^-$ were observed in both elderly ESRD patients and HI (Fig. 2f,g). Similar to the $CD4^+$ T cell compartment, elderly CMV seropositive ESRD patients also had significant lower numbers of naive $CD8^+$ T cells when compared to CMV serostatus-matched HI. In addition, also within the $CD8^+$ T cell compartment, lower numbers of CM $CD8^+$ T cells were observed, irrespective of CMV serostatus when comparing elderly ESRD patients to HI. Comparison of frequencies of $CD8^+$ T cell subsets, revealed CMV seropositivity to be associated with higher frequencies of total $CD8^+$, EMRA and $CD28^-CD8^+$ T cells (Supporting information, Fig.S2a,f,g, respectively) and lower frequencies of CM $CD8^+$ T cells (Supporting information, Fig. S2d). In addition, percentages of naive $CD8^+$ T cells were lower in CMV seropositive compared with CMV seronegative ESRD patients (Supporting information, Fig. S2b). Furthermore, higher frequencies of total memory (Supporting information, Fig. S2c), and within this lower frequencies of EM $CD8^+$ T cells (Supporting information, Fig. S2e) were noted. Within the CMV seropositive group, ESRD patients had higher percentages of total, EMRA and $CD28^-CD8^+$ T cells than HI (Supporting information, Fig.2a, f, g, respectively) and lower frequencies of CM $CD8^+$ T cells (Supporting information, Fig.

S2d). Both RRT (Supporting information, Table S1) and gender (data not shown) did not affect numbers of circulating T cell subsets in these elderly ESRD patients.

Lower thymic output in CMV seropositive elderly ESRD patients

TREC content was comparable for CMV seronegative and CMV seropositive ESRD patients or HI (Fig. 3a), confirming the idea that CMV effects are more limited to the memory compartment. Interestingly, in 4.6% and 10.2% of the elderly ESRD patients and healthy individuals respectively, no DNA encoding for TREC was detected in the PCR assay, which could be related to the lower contribution of the thymus to the naive T cell pool at this age. Of note, in those cases in which DNA encoding for TRECs was detected, a significant (P = 0.046) decrease was observed for TREC content in elderly CMV seropositive but not CMV seronegative ESRD patients compared to CMV serostatus-matched HI (Fig. 3a). In agreement with this, a lower number of recent thymic emigrants, defined as CD31-expressing naive CD4$^+$ (Fig. 3b: 88 cells/µl *versus* 146 cells/µl) and CD8$^+$ T cells (Fig. 3c: 35 cells/µl *versus* 48 cells/µl), was observed in elderly CMV seropositive ESRD patients compared to CMV serostatus-matched HI. Thymic output as measured by TREC content and CD31-expressing naive T cells was not influenced by RRT (Supporting information, Table S1) and gender (data not shown).

Enhanced telomere attrition in CMV-seropositive elderly ESRD patients

RTL was not significantly different comparing CMV seropositive ESRD patients or HI to their CMV seronegative counterparts. CMV seropositive, but not seronegative, elderly ESRD patients had shorter telomeres within CD4$^+$ (Fig. 4a, P<0.001) and CD8$^+$ (Fig. 4b, P<0.001) T cells than CMV serostatus-matched HI. The median RTL of CMV seropositive ESRD patients amounted to 9.0% and 9.1% for CD4$^+$ and CD8$^+$

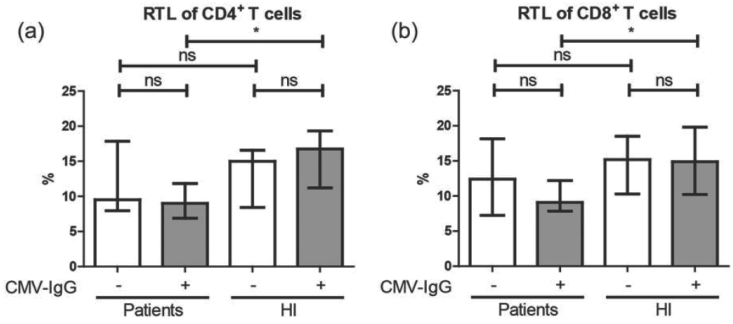

Fig. 4. Relative telomere length (RTL) of CD4$^+$ and CD8$^+$ T cells in elderly healthy individuals (HI) and elderly end-stage renal disease (ESRD) patients. The RTL of (a) CD4$^+$ and (b) CD8$^+$ T cells was determined in circulating T cells of HI [n=36; n=14 cytomegalovirus (CMV) seronegative and n=22 CMV seropositive) and ESRD patients (n=28; n=12 CMV-seronegative and n=16 CMV-seropositive) The open bars represent the CMV-seronegative individuals and the closed bars represent CMV-seropositive ones. Data are given as median with interquartile range. P-value: ***<0.001; NS: not significant.

T cells and values observed in CMV serostatus-matched HI were 16.8% and 14.9% for $CD4^+$ and $CD8^+$ T cells, respectively. No significant difference in RTL of $CD4^+$ or $CD8^+$ was observed between patients with RRT and without RRT (Supporting information, Table S1). The RTL of $CD4^+$ or $CD8^+$ was not significantly influenced by gender in our elderly population (data not shown).

Proliferation characteristics of T cells from elderly ESRD patients and elderly HI are not different

The proliferative capacity as in percentages of proliferating $CD4^+$ and $CD8^+$ T cells in response to an allogeneic stimulus (Fig. 5a, b), as well as a polyclonal stimulus (PHA; Supporting information, Fig. S3a,b) was equal between elderly ESRD patients and elderly HI. In addition, a similar precursor frequency (PF) of $CD4^+$ and $CD8^+$ T cells able to respond to alloantigen (Fig. 5c,d) or a polyclonal stimulus (PHA, Supporting information 3c,d) was observed between elderly patients and HI. Moreover, no differences were observed with respect to proliferation kinetics of $CD4^+$ (Fig. 5e) and $CD8^+$ (Fig. 5f) T cells in response to alloantigen-stimulation. CMV did not influence significantly the capacity of T cells to respond to allogeneic or polyclonal stimulation in both elderly ESRD patients and HI (Supporting information, Table S2).

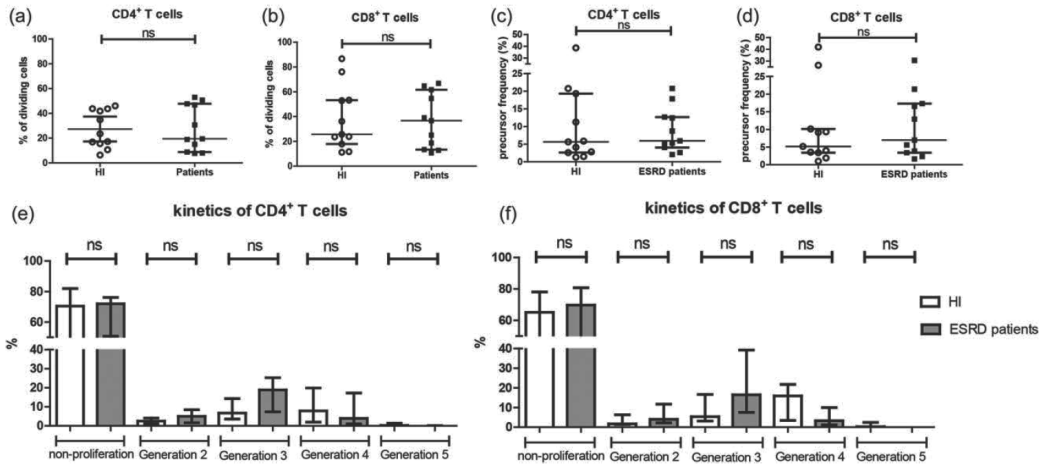

Fig. 5. Proliferation of $CD4^+$ and $CD8^+$ T cells in response to allogeneic stimulation in elderly healthy individuals (HI) and elderly end-stage renal disease (ESRD) patients. Percentage of (a) dividing $CD4^+$ and (b) $CD8^+$ T cells in response to alloantigens. Precursor frequency (%) of (c) $CD4^+$and (d) $CD8^+$ T cells in response to alloantigens. Proliferation kinetics of (e) $CD4^+$ and (f) $CD8^+$ T cells in response to alloantigens. Peripheral blood mononuclear cells (PBMCs) isolated from 11 HI and 11 ESRD patients [five cytomegalovirus (CMV) seronegativity and six CMV seropositivity] were used as response cells and the irradiated PBMCs from the third part were used as the allostimulation cells. Data are given as individual values (a-d) and median with interquartile range (a-f); open symbols/bars represent HI and closed symbols/bars represent the ESRD patients; ns: not significant.

DISCUSSION

The main observation of this study is that CMV latency is a dominant factor for increased ageing of the peripheral T cells in elderly ESRD patients, outweighing the known premature T cell ageing effects of renal failure itself. In our previous study, premature ageing of peripheral T cells was demonstrated in ESRD patients but did not consider CMV latency and did not focus on elderly (≥ 65 years of age) patients (11). The influence of CMV latency *versus* uremia on T cell ageing was investigated in another cohort of young to middle-aged ESRD patients and showed a modest effect consisting of increased T cell differentiation status, in particular higher percentages of CD28-negative T cells, and reduced telomere length of CD8-positive T cells(18). The current study focused on the elderly ESRD patients and identified specific additive effects of ESRD and in particular CMV latency on the ageing of the T cell system in the elderly population.

CMV latency is recognized increasingly as a significant factor for accelerated T-cell ageing (22), and as such may add to the increased risk for infections (23) as well as cardiovascular disease (24) in the healthy elderly. In elderly ESRD patients, the risk of cardiovascular disease events and death (16, 25-27) or infections (28) is even more increased. Studies in the very healthy elderly demonstrated an immune risk phenotype (IRP) for increased mortality defined by an inverted CD4/CD8 ratio and increased number of $CD28^{-}CD8^{+}$ T cells (29), which was associated with CMV seropositivity (13, 30).

Our data indicate that CMV latency in combination with ESRD in the elderly is particular harmful to the T cell system, as numbers of naive T cells are also affected negatively, as well as the known ageing effects on memory T cells. The decline in number of naive T cells is a key feature associated with loss of renal function, and in particular ESRD (11, 31). Naive T cells that have recently left the thymus contain TRECs and express mainly CD31 [Platelet and Endothelial Cell Adhesion Molecule 1 (PECAM-1)] (32). TRECs were not detectable in several elderly healthy individuals or ESRD patients, suggestive of a low thymic output in the elderly population. This is in agreement with the observation generated in healthy individuals that a large part of the functional thymic tissue has been lost by the age of 50 years (33). Aside from the thymus contributing to the naive T cell pool, homeostatic proliferation of the remaining naive T cells is able to maintain the naive T cell pool (34). Homeostatic proliferation of naive T cells may occur in response to homeostatic cytokines like for example IL-7 (35) or in response to low- affinity self-antigens(36-38). The decline in naive T cells induced by ESRD in elderly might also be the result of defects in homeostatic proliferation since plasma levels of IL-7 were lower in ESRD patients compared to healthy individuals (31). Moreover, the decline in naive T cells could also result from differentiation towards memory T cells. The memory compartment in the ESRD patients is more differentiated, i.e. containing fewer CM T cells (31, 39).

Naive, but also CM, T cells are essential for generating a robust immune response (3, 4) and naive T cells contain a more diverse T cell receptor (TCR) Vβ repertoire compared to memory T cells (40), allowing for a better response to newly encountered antigens such as vaccination antigens. Low *in-vivo* numbers of naive $CD4^+$ recent thymic emigrants correlated well with reduced acute responsiveness and altered long-term persistence of human cellular immunity to yellow fever vaccination in the elderly population (41). The underlying mechanism for the reduction in naive and CM T cells in the peripheral blood is not yet clear, but may involve increased apoptosis (39, 42-44) or enhanced proliferation to more differentiated T cell subsets (11). During ageing, naive and CM T cells have been linked to increased sensitivity of tumor necrosis factor (TNF)-α-induced apoptosis (45, 46). Elevated concentrations of serum or plasma TNF-α is associated strongly with progressive loss of renal function (47, 48). TNF-α induced apoptosis may be an explanation of loss of naive and CM in elderly ESRD patients. CMV may reactivate in healthy individuals and more frequently in the elderly (49). This may be caused by an age-related decrease in T cell-mediated control of CMV reactivation as, e.g. INF-γ secretion in response to CMV peptide stimulation is decreased in very elderly individuals (50). Data in ESRD patients on CMV reactivation are largely absent, but the frequent presence of anti-CMV IgM titers in dialysis patients suggests that CMV reactivation is not a rare event (51), and may even lead to CMV disease (52, 53). In addition, anti-CMV IgG titers are increased in elderly ESRD patients but not in healthy elderly individuals, which may also be interpreted as the result of frequent CMV reactivation (54).

A plausible hypothesis could be that ESRD-related premature T cell ageing contributes to a decrease in anti-viral T cell immunity, which allows for more frequent CMV reactivation. Reactivation is controlled at the expense of expanded populations of $CD28^{null}$ T cells with reduced telomere length (18), fewer naive T cells and a narrowed TCR repertoire (55). The expansion of $CD4CD28^{null}$ T cells adds to the increased risk for atherosclerotic disease, while the overall antigen-specific T cell response is weakened further by the loss of T cell diversity.

Unexpectedly, we were unable to attribute the phenotypical defects to specific or more general functional deficits using CFSE dilution as a read-out for proliferation of PBMC in response to an allo-antigen or polyclonal stimulus. This indicates that the overall proliferative potential of T cells is not severely affected in the elderly ESRD patient. However, the stimuli used in our proliferation assay do not allow for measuring the potential of T cells to initiate responses to newly encountered antigens that are more dependent on a diverse T cell repertoire. In addition, the use of CMV-seropositive donors as allogeneic stimuli might result in activation of CMV-specific T cells in CMV-seropositive responders in addition to the alloreactive T cells (56, 57). This might be an explanation for the trend in higher frequencies of proliferating T cells as well as precursor frequencies comparing CMV-seropositive responders to their negative counterparts (Supporting information, Table S2). By using high-throughput sequencing of the diversity of the TCR -repertoire (40, 58) this

might provide a more supportive functional read-out.

In conclusion, CMV latency is a dominant factor for accelerated T cell ageing in elderly ESRD patients, and therefore should be taken into consideration to evaluate the risk of mortality, infection and response to vaccination in this patient population.

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Conflict of Interest
The authors of this manuscript have no financial or commercial conflicts of interest to disclose.

SUPPORTING INFORMATION

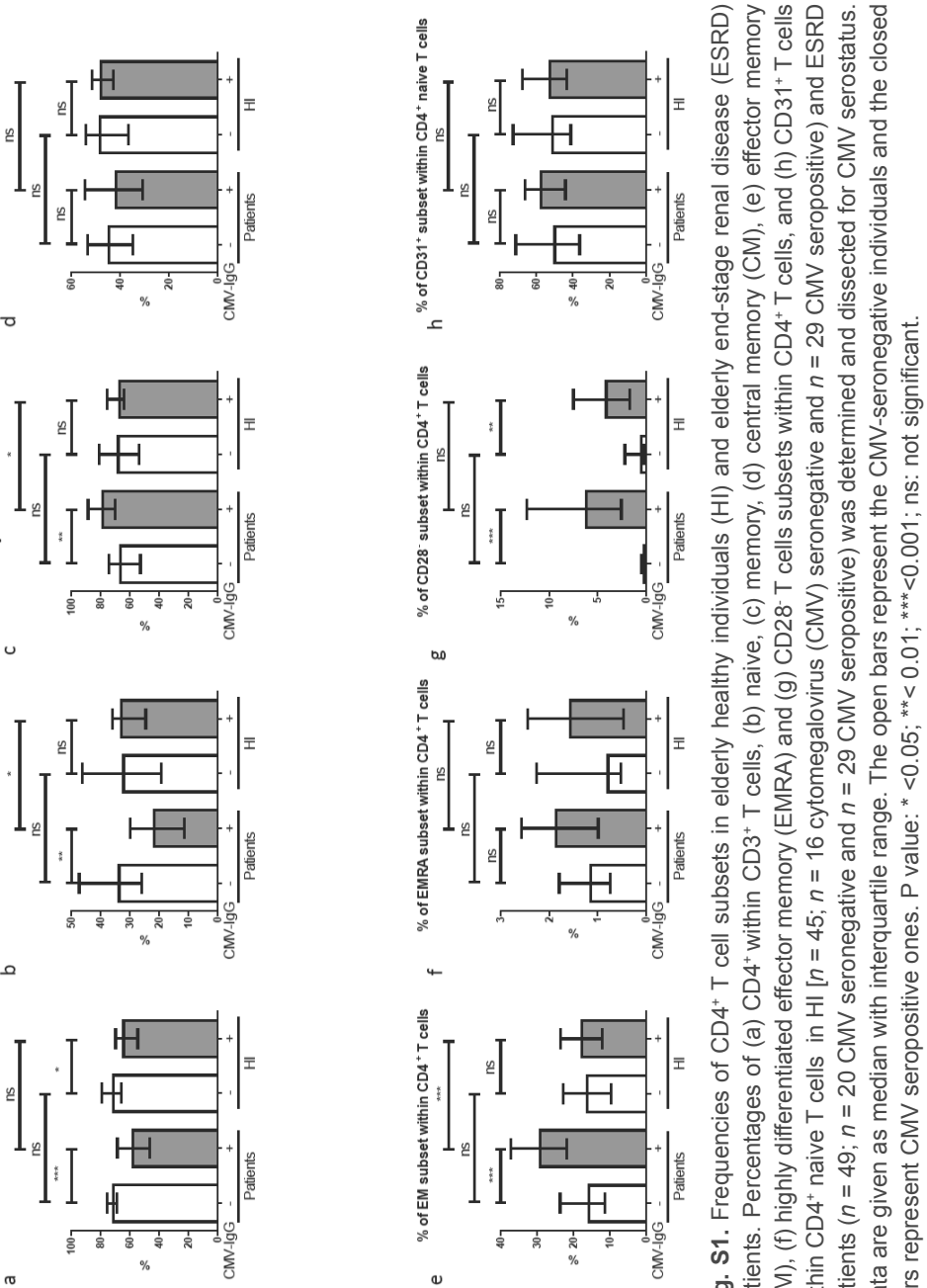

Fig. S1. Frequencies of $CD4^+$ T cell subsets in elderly healthy individuals (HI) and elderly end-stage renal disease (ESRD) patients. Percentages of (a) $CD4^+$ within $CD3^+$ T cells, (b) naive, (c) memory, (d) central memory (CM), (e) effector memory (EM), (f) highly differentiated effector memory (EMRA) and (g) $CD28^-$ T cells subsets within $CD4^+$ T cells, and (h) $CD31^+$ T cells within $CD4^+$ naive T cells in HI [n = 45; n = 16 cytomegalovirus (CMV) seronegative and n = 29 CMV seropositive) and ESRD patients (n = 49; n = 20 CMV seronegative and n = 29 CMV seropositive) was determined and dissected for CMV serostatus. Data are given as median with interquartile range. The open bars represent the CMV-seronegative individuals and the closed bars represent CMV seropositive ones. P value: * <0.05; ** < 0.01; *** <0.001; ns: not significant.

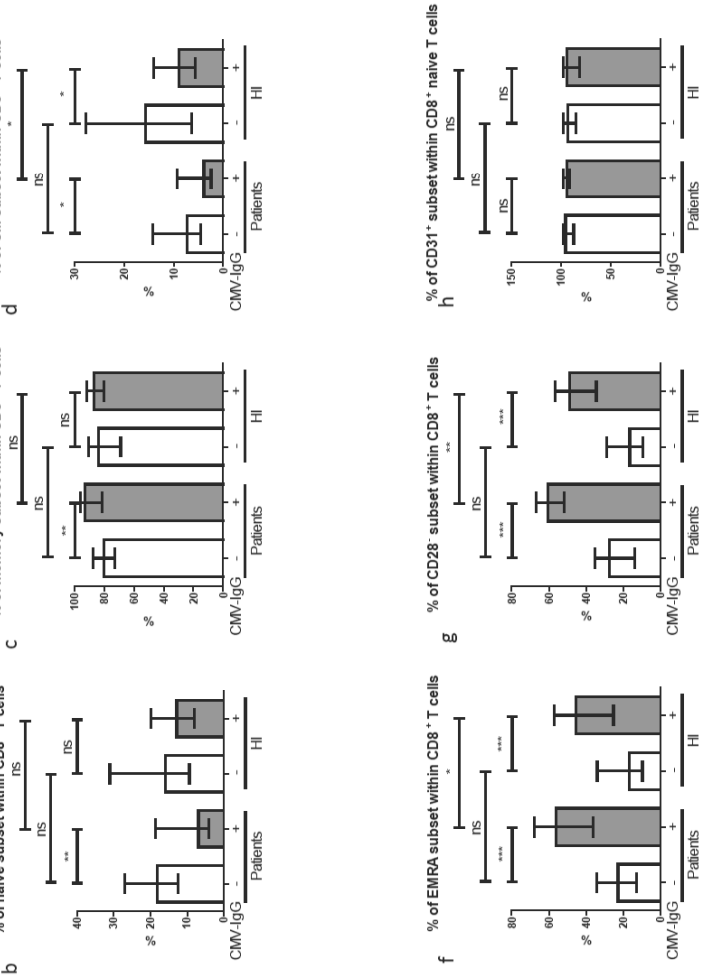

Fig. S2. Frequencies of $CD8^+$ T cell subsets in elderly healthy individuals (HI) and elderly end-stage renal disease (ESRD) patients. Percentages of (a) $CD8^+$ within $CD3^+$ T cells, (b) naive, (c) memory, (d) central memory (CM), (e) effector memory (EM), (f) highly differentiated effector memory (EMRA) and (g) $CD28^-$ T cells subsets within $CD8^+$ T cells, and (h) $CD31^+$ T cells within $CD8^+$ naive T cells in HI [n= 45; n = 16 cytomegalovirus (CMV) seronegative and n = 29 CMV seropositive) and ESRD patients (n = 49; n = 20 CMV seronegative and n = 29 CMV seropositive) was determined and dissected for CMV serostatus. Data are given as median with interquartile range. The open bars represent the CMV seronegative individuals and the closed bars represent CMV seropositive ones. P-value: * <0.05; ** < 0.01; *** <0.001; ns: not significant.

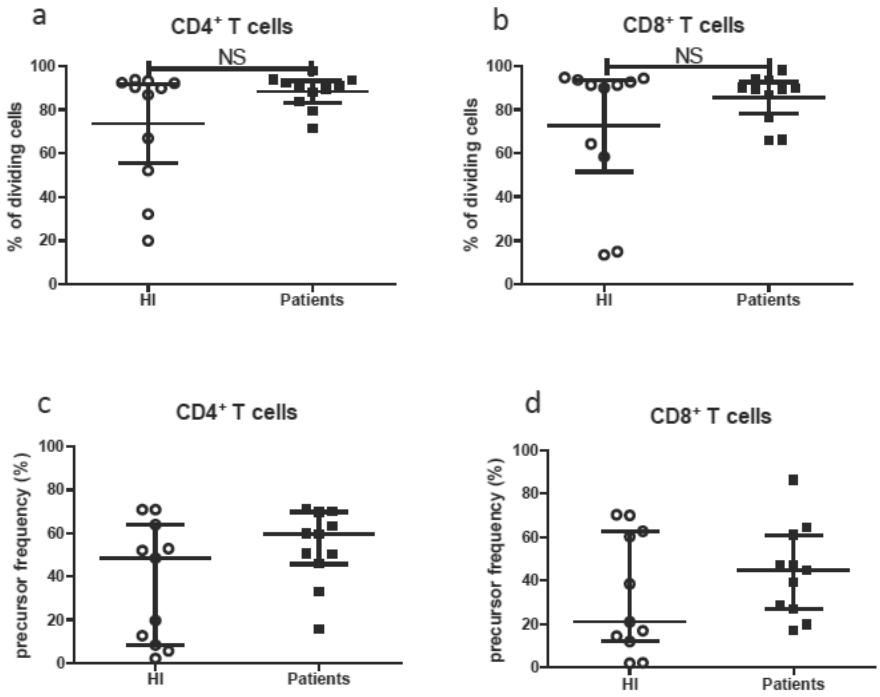

Fig. S3. Proliferation of $CD4^+$ and $CD8^+$ T cells in response to phytohaemagglutinin (PHA) stimulation in elderly healthy individuals (HI) and elderly end-stage renal disease (ESRD) patients. Percentage of (a) dividing $CD4^+$ and (b) $CD8^+$ T cells in response to PHA. Precursor frequency (%) of (c) $CD4^+$ and (d) $CD8^+$ T cells in response to PHA. Peripheral blood mononuclear cells (PBMCs) isolated from 11 HI and 11 ESRD patients [five cytomegalovirus (CMV) seronegativity and six CMV seropositivity] were used as response cells. Data are given as individual values; open symbols/bars represent HI and closed symbols/bars represent the ESRD patients. NS: not significant

Table S1.: T cell ageing parameters of elderly end-stage renal disease (ESRD) patients with and without renal replacement therapy

T-cell ageing parameters	Patients with RRT	Patients without RRT
T cell differentiation status (cells/µL)		
$CD3^+$	950 (684-1649)[a]	1195 (888-1367)
$CD4^+$	560 (439-894)	695 (528- 876)
naive $CD4^+$	166 (84-306)	158 (97-322)
memory $CD4^+$	423 (292-671)	475 (341- 604)
CM $CD4^+$	283 (160- 390)	295 (192- 469)
EM $CD4^+$	169 (103- 227)	128 (95- 239)
EMRA $CD4^+$	12 (6-26)	8 (4 -14)
$CD28^-$ $CD4^+$	21 (2-58)	9 (2 -84)
$CD8^+$	297 (184- 433)	308 (233- 608)
naive $CD8^+$	38 (19- 61)	33 (18- 66)
memory $CD8^+$	243 (117- 380)	251 (173- 565)
CM $CD8^+$	20 (10-24)	19 (11- 36)
EM $CD8^+$	108 (58-180)	91 (36- 146)
EMRA $CD8^+$	108 (45-214)	167 (60- 417)
CD28- $CD8^+$	135 (51 -243)	129 (65- 373)
Proliferative history (RTL in %)		
$CD4^+$	8.90 (7.75-13.63)	9.00 (7.00-11.80)
$CD8^+$	10.35 (7.85-17.10)	9.10 (7.85-14.93)
Thymic output		
TREC (1 / delta CT value)	0.078 (0.072-0.089)	0.079 (0.071-0.088)
$CD31^+$ naive $CD4^+$	100 (44-179)	88 (52-193)
$CD31^+$ naive $CD8^+$	37 (18-59)	32 (14-59)

[a] Numbers were given as median and interquartile range.

Table S2.: Proliferation of $CD4^+$ and $CD8^+$ T cells in response to allogeneic and phytohaemagglutinin (PHA) stimulation in elderly healthy individuals (HI) and elderly end-stage renal disease (ESRD) patients dissected for cytomegalovirus (CMV) serostatus

	patient			**HI**		
	CMV-IgG (+)	CMV-IgG (-)	*P*-value	CMV-IgG (+)	CMV-IgG (-)	*P*-value
allogeneic response						
CD4+						
dividing cells (%)	25.1 (18.0-48.3)[a]	8.8 (7.8-49.2)	ns	42.8 (16.6- 44.4)	17.2 (8.5-29.2)	ns
precursor frequency (%)	7.3 (5.0-14.5)	5.4 (2.3-15.3)	ns	7.7 (2.3-19.7)	5.6 (2.1-22.3)	ns
CD8+						
dividing cells (%)	37.9 (13.2-57.7)	25.1 (14.7-63.2)	ns	38.3 (16.4- 61.7)	25.7 (17.6-56.2)	ns
precursor frequency (%)	10.0 (2.2-20.0)	5.6 (3.6-19.4)	ns	7.3 (2.8-18.1)	4.1 (2.7- 17.8)	ns
PHA response						
CD4+						
dividing cells (%)	92.7 (83.8-96.3)	93.3 (87.1-97.8)	ns	82.5 (30.8-96.2)	91.8 (71.5-96.0)	ns
precursor frequency (%)	50.4 (38.4-60.5)	69.8 (46.6-70.8)	ns	14.0 (4.8-55.0)	52.8 (30.6-70.9)	ns
CD8+						
dividing cells (%)	91.4 (74.2-94.6)	90.7 (77.3-98.1)	ns	80.8 (15.3-97.3)	91.7 (74.8-95.0)	ns
precursor frequency (%)	36.6 (25.2-50.4)	47.0 (28.1-75.5)	ns	14.4 (2.1-46.4)	60.3 (17.7-66.4)	ns

[a] Data were given as the median and interquartile range. ns: not significant

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CHAPTER 3

End-stage Renal Disease Patients have a skewed T Cell Receptor Vβ -repertoire

Ling Huang[1], Anton W. Langerak[2], Ingrid L.M. Wolvers-Tettero[2], Ruud W.J. Meijers[1], Carla C. Baan[1], Nicolle H.R. Litjens[1] and Michiel G.H. Betjes[1].

[1]Department of Internal Medicine, Section Nephrology and Transplantation, Erasmus University Medical Center, Rotterdam, the Netherlands.
[2]Department of Immunology, Erasmus University Medical Center, Rotterdam, the Netherlands.

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ABSTRACT

Background: End-stage renal disease (ESRD) is associated with defective T-cell mediated immunity. A diverse T-cell receptor (TCR) Vβ -repertoire is central to effective T-cell mediated immune responses to foreign antigens. In this study, the effect of ESRD on TCR Vβ-repertoire was assessed.

Results: A higher proportion of ESRD patients (68.9%) had a skewed TCR Vβ-repertoire compared to age and cytomegalovirus (CMV) – IgG serostatus matched healthy individuals (31.4%, $P<0.001$). Age, CMV serostatus and ESRD were independently associated with an increase in shifting of the TCR Vβ-repertoire. More differentiated $CD8^+$ T cells were observed in young ESRD patients with a shifted TCR Vβ-repertoire. CD31-expressing naive T cells and relative telomere length of T cells were not significantly related to TCR Vβ skewing.

Conclusions: ESRD significantly skewed the TCR Vβ-repertoire particularly in the elderly population, which may contribute to the uremia-associated defect in T-cell mediated immunity.

BACKGROUND

Patients suffering from end-stage renal disease (ESRD) have an impaired T-cell mediated immune system, characterized by an increased susceptibility for infections(1), a decreased response to vaccination(2-4) and a heightened risk for virus-associated cancers(5). Loss of renal function is associated with a severe depletion of naive T cells and a shift to more differentiated memory T cells(6). An advanced decline in thymic output and attrition of telomeres was noted in both $CD4^+$ as well as $CD8^+$ T cells of ESRD patients(7, 8). These uremia-induced effects on T cells closely resemble premature T-cell aging and revealed a discrepancy of 15-20 years between the patient's immunological age of their T cells and their chronological age(7).

A diverse (polyclonal) T cell receptor (TCR) -repertoire capable of recognizing a broad range of foreign antigens is key to an effective T-cell-mediated immune response(9). Naive T cells migrating from the thymus into the circulation, carrying CD31 antigen on their cell surface(10), possess the broadest TCR -repertoire (i.e. polyclonal TCR -repertoire)(11). The memory T cells that develop upon encountering of an antigen have a repertoire that is being skewed towards particular specificities (12) but this does not automatically imply a loss of TCR diversity. Similarly, although the composition of the circulating T cells may be profoundly altered in ESRD patients, it is not known whether this invariably leads to an oligoclonal TCR -repertoire reflecting a limited capacity to respond to novel encountered antigens.

Most TCRs consist of an α and a β chain and each chain is composed of a variable (V) region and a constant (C) region. In the thymus, the V region of the TCR α and β chain is generated by random gene rearrangement of variable (V) and joining (J) genes or V, diversity (D) and J genes, respectively. Contraction of TCR Vβ-repertoire has been reported to occur during aging, starting from roughly 600×10^3 clonal types detected per 10^6 T cells in childhood and declining by 5×10^3 clones per year(13). The TCR Vβ-repertoire diversity might also be affected by chronic antigenic stimulation(14-16). For example, cytomegalovirus (CMV) latency may also induce contraction of the TCR Vβ-repertoire as it results in a vast expansion of CMV specific T cell-exceeding 4% of $CD8^+$ T cells in immunocompetent donors (17) and these anti-CMV T cells clones were stably maintained for 5 years(18). CMV-IgG seropositive ESRD patients have shorter telomeres within $CD8^+$ T cells and an increased T cell differentiation status with higher percentages of $CD57^+$ and $CD28^-$ $CD4^+$ and $CD8^+$ memory T cells(19). Both CMV latency and ESRD, alone or in combination, may profoundly alter the composition of the peripheral T cell compartment(19, 20).

We hypothesized that ESRD may decrease the TCR Vβ-repertoire diversity and that CMV latency could further add to this loss of diversity. In this study, we therefore assessed the TCR Vβ-repertoire diversity of ESRD patients relative to age- and CMV-IgG serostatus- matched healthy individuals (HI). The TCR Vβ-repertoire

diversity was evaluated by a qualitative multiplex DNA-based PCR of TCR Vβ-Jβ gene rearrangements, originally designed to diagnose lymphoproliferations(21). The generated Genescan profile was used to distinguish between a Gaussian distributed (diverse or polyclonal) and a more or less skewed (narrowed or even monoclonally expanded) TCR Vβ-repertoire.

Subjects and Methods

Study population

Forty-five stable adult ESRD patients, defined as a glomerular filtration rate of ≤15 ml/min with or without hemodialysis, on the waiting list for the first kidney transplantation and 51 healthy kidney donors were included (Table 1) from 1st November 2010 to 1st October 2013 in nephrology department of Erasmus medical center. Patients with any clinical or laboratory evidence of acute bacterial or viral infection, malignancy, previous kidney transplantations, immunosuppressive drugs treatment within 28 days prior to transplantation (except glucocorticoids) were excluded. Age, CMV-IgG serostatus and total T-cell number in the peripheral blood were matched between these two groups. Lithium-heparinized blood was drawn prior to transplantation of ESRD patients and healthy kidney donors. All individuals included gave informed consent and the local medical ethical committee approved the study (METC number: 2012–022). It was conducted according to the principles of Declaration of Helsinki and in compliance with International Conference on Harmonization/Good Clinical Practice regulations.

Circulating T cell numbers and their differentiation status

Freshly drawn peripheral blood samples from 45 ESRD patients and 51 healthy individuals (HI) were stained and acquired on a FACSCanto II flow cytometer (BD Biosciences, Erembodegem, Belgium) as described previously (7, 35) to determine both frequencies and absolute numbers of the different T cell subsets as well as their differentiation status. Data were analyzed using FACS Diva software version 6.1.2 (BD Biosciences). Detailed methods can be found in the Supplementary Methods section.

PBMCs isolation, DNA isolation and TCR Vβ-repertoire analysis

PBMCs from 45 ESRD patients and 51 healthy individuals (HI) were isolated from peripheral blood as described previously (3). One million PBMCs were snap-frozen for DNA isolation and the remaining were frozen at 10 million per vial until further use. DNA was isolated according to manufacturer's instructions (QIAamp DNA Mini QIAcube Kit). The TCR Vβ gene repertoire was measured by the multiplex TCR Vβ-Jβ gene PCR as developed and approved by the European consortium of 45 laboratories (BIOMED-2 Concerted Action BMH4-CT98-3936) (21). The term skewed TCR Vβ-repertoire was used when the TCR Vβ showed an oligoclonal pattern with one or more clonal peak(s) on the Genescan profile and the term non-skewed Vβ -repertoire was used when the spectratype of TCR Vβ-repertoire on the Genescan profile showed a Gaussian distribution (21).

Table 1 Clinical and biological characteristics of the study population

	Healthy individuals	ESRD Patients	*P* value
Number of individuals	51	45	
Age (young/eldely) (years; mean ± SD)	33.1 ± 7.6 / 68.0 ± 3.0	32.9 ± 9.2 / 68.3 ± 3.5	NS/NS [a]
Sex (% male)	41.2	68.9	P < 0.05
CMV IgG serostatus (% pos)	52.9	51.1	NS
T cells number in peripheral blood (10^6/µl; mean ± SD)	1274 ± 304	1164 ± 260	NS
Renal replacement therapy (number; %)		44.4	
Duration of renal replacement therapy (months; median/range)		13 / 3-68	
Hemodialysis (%)		80.0	
Peritoneal dialysis (%)		15.0	
Peritoneal dialysis followed hemodialysis (%)		5.0	
Underlying kidney disease			
Nephrosclerosis/atherosclerosis/hypertensive nephropathy (%)		17.8	
Primary glomerulopathy (%)		6.7	
Diabetic nephropathy (%)		20.0	
Reflux nephropathy (%)		17.8	
Polycystic kidney disease (%)		20.0	
Other (%)		15.6	
Unknown (%)		2.2	

Abbreviations: ESRD, end-stage renal disease; *CMV,* cytomegalovirus; *NS*, not significant
[a]Age categories of young (≤45 years) and elderly (≥65 years) were used

Sorting of T cell subsets

Cryopreserved PBMCs were thawed, washed and resuspended in PBS. The PBMCs from 10 elderly patients (of which 50% were CMV-seropositive) and 10 elderly healthy individuals (of which 50% were CMV-seropositive) were stained and subsequently sorted into T cells subsets i.e. $CD4^+$ naive/memory and $CD8^+$ naive/ memory using flow cytometry based cell-sorting (BD FACSAria™ II SORP, BD). A typical example of the gating strategy to dissect the different T cell subsets prior to sorting as well as the analysis of the purity of the sorted samples was given in Fig.S. 3. The purity of all T cell subsets was over 95%. The details for flow cytometry-based cell-sorting of T cell subsets are given in the Supplementary Methods section.

Telomere length assay

Flow fluorescent in situ hybridization was performed to determine the relative

telomere length (RTL) of T cells as described previously (7, 35). Detailed information is given in the Supplementary Methods section.

Statistical analyses

Statistical analyses were performed using SPSS 20 (IBM, Chicago, IL, USA) and GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). Categorical variables were compared using the chi-square test or Fisher's exact test. Continuous variables were compared using Mann-Whitney U-test. Binary logistic regression was used to estimate the probability that skewing of TCR repertoire was present given the values of explanatory variables. All reported P-values are two-sided and were considered statistically significant when $P<0.05$.

RESULTS

Study population characteristics

The demographic and clinical characteristics of the study population are given in Table 1. Approximately 45% of ESRD patients received renal replacement therapy with the median dialysis time of 13 months. Twenty-one patients were within the young group (age 19 – 45 years) and 24 patients belonged to the elderly group (age 65 – 77 years).

ESRD is associated with a skewed TCR Vβ-repertoire

A TCR Vβ-repertoire was defined as non-skewed one when the spectratype of TCR Vβ-repertoire on the Genescan profile showed a Gaussian distribution (Fig.1a, left profile) or a skewed one showing an oligoclonal pattern with one (Fig. 1a middle profile) or more clonal (Fig. 1a, right profile) peak(s) on the Genescan profile. A skewed (or oligoclonal) TCR Vβ-repertoire was present in a larger ($P<0.001$) proportion (68.9%) of the ESRD patients compared to HI (31.4%) (Fig. 1b). Further dividing the results into single or multiple (oligo)clonal peaks did not lead to significantly different percentages in skewed TCR Vβ-repertoire between the ESRD patients and HI (Fig. 1c). Percentages of individuals with multiple (oligo)clonal peaks in the skewed TCR Vβ-repertoire amounted to 51.6 % and 43.7% in ESRD patients and HI, respectively ($P>0.05$). In conclusion, ESRD is associated with skewing of the TCR Vβ-repertoire.

Both Aging and CMV serostatus affect the TCR Vβ-repertoire diversity in ESRD patients

Dissecting the ESRD and healthy study population into a young and elderly group, revealed significantly more ($P<0.001$) skewing within the elderly ESRD patients compared to the age-matched HI, proportions were 87.5% versus 32.0%, respectively (Fig. 2a). In addition, an age-related skewing in TCR Vβ-repertoire was observed for ESRD patients ($P < 0.01$), but not for healthy individuals (Fig. 2a). Moreover, like for the overall study population, no differences were observed between the composition of multiple and single oligoclonal peaks in young and elderly ESRD patients with a skewed TCR Vβ-repertoire. (Fig. 2b).

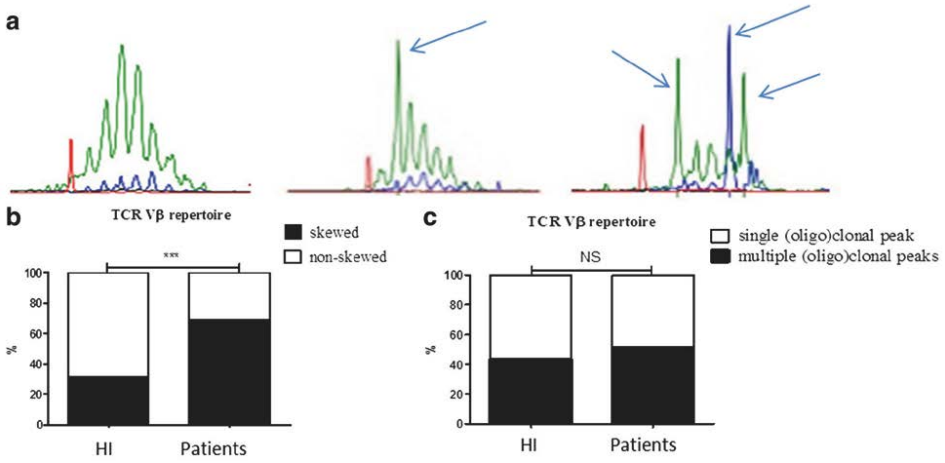

Fig. 1. Skewed and non-skewed TCR Vβ-repertoire distribution in healthy individuals (HI) and end- stage renal disease (ESRD) patients. The different genescan profiles obtained following spectratyping of the DNA-based multiplexed TCR Vβ PCR samples are depicted in **a**. This reaction consists of 23 Vβ primers and 9 Jβ primers. The green profile represents the 6 Jβ primers (Jβ1.1, Jβ1.2, Jβ1.3, Jβ1.4, Jβ1.5, Jβ1.6), whereas the blue line represents the 3 Jβ primers (Jβ2.2, Jβ2.6, and Jβ2.7) in this multiplex PCR. The red profile represents the internal size standard. The left profile shows a non-skewed (polyclonal) pattern, the middle one a skewed pattern with a single (oligo)clonal peak [size of the oligoclonal product is 253 nucleotides (nt)] whereas the right panel depicts a skewed pattern with multiple (oligo) clonal peaks (sizes of the oligoclonal products are 252, 261 and 264 nt, respectively). The arrows indicate oligoclonal peaks within the genescan profile. **b** Frequency of ESRD patients (*n* = 45) and age-matched HI (*n* = 51) with a skewed (closed squares) and non-skewed (open squares) TCR Vβ-repertoire. **c** Frequency of single (open squares) and multiple (oligo)clonal peaks (closed squares) within a skewed TCR Vβ-repertoire of ESRD patients (*n* = 31) and HI (*n* = 16). *P* value: * <0.05; **< 0.01; ***< 0.001; NS: not significant.

We then looked at the effect of CMV IgG seropositivity on TCR Vβ-repertoire skewing. A skewed TCR Vβ -repertoire was observed in 41.7% and 16.7% of CMV-IgG seropositive and CMV-IgG seronegative HI, respectively. Approximately, 91.3% of CMV-IgG seropositive ESRD patients had a skewed TCR Vβ -repertoire whereas skewing of the TCR Vβ-repertoire occurred in 45.5% of CMV-IgG seronegative ESRD patients. CMV latency skewed ($P < 0.01$) the TCR Vβ-repertoire in both HI as well as ESRD patients (Fig. 2c). Data was further analyzed taking the effect of aging into account. In young CMV-IgG seropositive and negative patients, percentage of patients with a skewed TCR Vβ repertoire amounted to 80.0% and 18.2%, whereas in the elderly percentages were 100% and 72.7%, respectively (Fig. 2d). A similar trend occurred in the healthy population, only reaching significance ($P < 0.01$) in the

Table 2 The variables related to a skewed TCR Vβ-repertoire

	Non-skewed TCR Vβ	Skewed TCR Vβ	Odds ratio	P value
Age (years, median)	41	65	3.1[a]	<0.05
% of CMV seropositivity	30.7	74.5	13.8[b]	<0.05
% of ESRD patients	28.6	70.0	10.2[c]	<0.05
% of patients on renal replacement therapy	35.8	48.4		NS
Underlying kidney disease				
Nephrosclerosis/atherosclerosis/ hypertensive nephropathy	1	7		NS
Primary glomerulopathy	0	3		NS
Diabetic nephropathy	2	7		NS
Reflux nephropathy	4	4		NS
Polycystic kidney disease	2	7		NS
Other	5	2		
Unknown	0	1		

Abbreviations: *TCR*, T cell receptor; *NS*, not significant.

[a] means the odds of a skewed TCR Vβ in elderly population is 3.1 times higher than in the young population.

[b] means the odds of a skewed TCR Vβ in CMV- IgG seropositive population is 13.8 times higher than in the CMV- IgG seronegative population.

[C] means the odds of a skewed TCR Vβ in patients is 10.2 times higher than in the healthy individuals.

Table 3 Frequency of elderly individuals with skewed and non-skewed TCR Vβ-repertoire in the sorted T cells subsets

T cell subsets	HI			ESRD patients		
	Skewed	Non-skewed	*P* value	Skewed	Non-skewed	*P* value
CD4 naive	0[a]	10	NS	0	10	NS
CD4 Memory	2	8		3	7	
CD8 Naive	3	7	NS	3	7	<0.05
CD8 Memory	7	3		9	1	

Abbreviations: TCR, *T cell receptor -repertoire*; *ESRD*, end-stage renal disease; *HI*, healthy individuals; *NS*, not significant.

[a]Data are shown as the numbers of individuals

elderly group (Fig. 2d). Although CMV latency significantly added to the skewing of the TCR Vβ-repertoire in ESRD patients, the effect of ESRD on the TCR Vβ-repertoire was present in both CMV-seropositive as well as CMV-seronegative elderly individuals. The percentage of elderly patients with a skewed TCR Vβ-repertoire was significantly higher when compared to the CMV-status matched elderly HI ($P < 0.05$) (Fig. 2d).

ESRD, aging, and CMV latency influenced the TCR Vβ-repertoire diversity independently

To test the influence of several factors such as ESRD, aging, CMV latency, and gender (as the percentage of males was significantly higher in the patients compared to HI) on skewing of the TCR Vβ-repertoire, a binary logistic regression model was used. Except for gender ($P > 0.05$), ESRD [$P < 0.05$, odds ratio (OR) = 4.8], age ($P < 0.05$, OR = 2.3) and CMV-IgG seropositivity ($P < 0.05$, OR = 6.7) were significantly

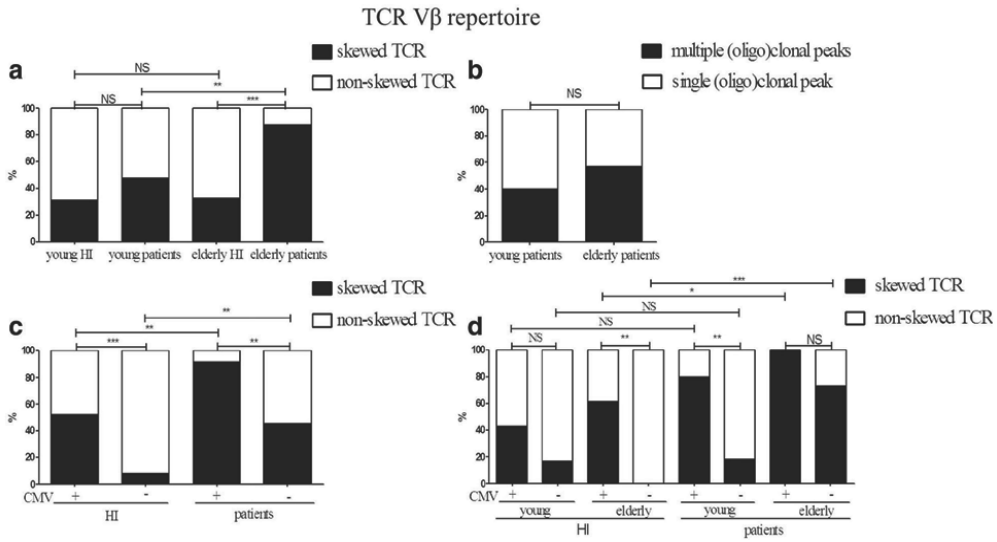

Fig. 2. Skewed and non-skewed TCR Vβ-repertoire distribution in healthy individuals (HI) and end-stage renal disease (ESRD) patients dissecting the population into subgroups according to age and CMV-IgG serostatus. **a** Frequency of a skewed TCR Vβ-repertoire in young (n = 21) and elderly (n = 24) ESRD patients compared to age- matched HI (young, n = 26 and elderly, n = 25). **b** Frequency of multiple (oligo) clonal peaks within a skewed TCR Vβ-repertoire of young (n = 10) ESRD patients compared to elderly ESRD patients (n = 21). **c** Frequency of a skewed TCR Vβ-repertoire in CMV-IgG seropositive (n = 23) and seronegative (n = 22) ESRD patients, compared to CMV-IgG seropositive (n = 27) and seronegative (n = 24) healthy individuals. **d** Frequency of a skewed TCR Vβ-repertoire in young ESRD patients with CMV- IgG seropositive status (n = 10) or CMV-IgG seronegative status (n = 11) and elderly ESRD patients with CMV-IgG seropositive status (n = 13) or CMV-IgG seronegative status (n = 11), compared to age-matched CMV-IgG seropositive (young n = 14, elderly n = 13) and CMV IgG seronegative (young n = 12, elderly n = 12) HI. The closed squares represent skewed or multiple oligo(clonal) peaks within skewed TCR Vβ-repertoires whereas the open squares represent non-skewed or single (oligo)clonal peaks within skewed TCR Vβ-repertoires, respectively. P value: * <0.05; **< 0.01; ***< 0.001; NS: not significant.

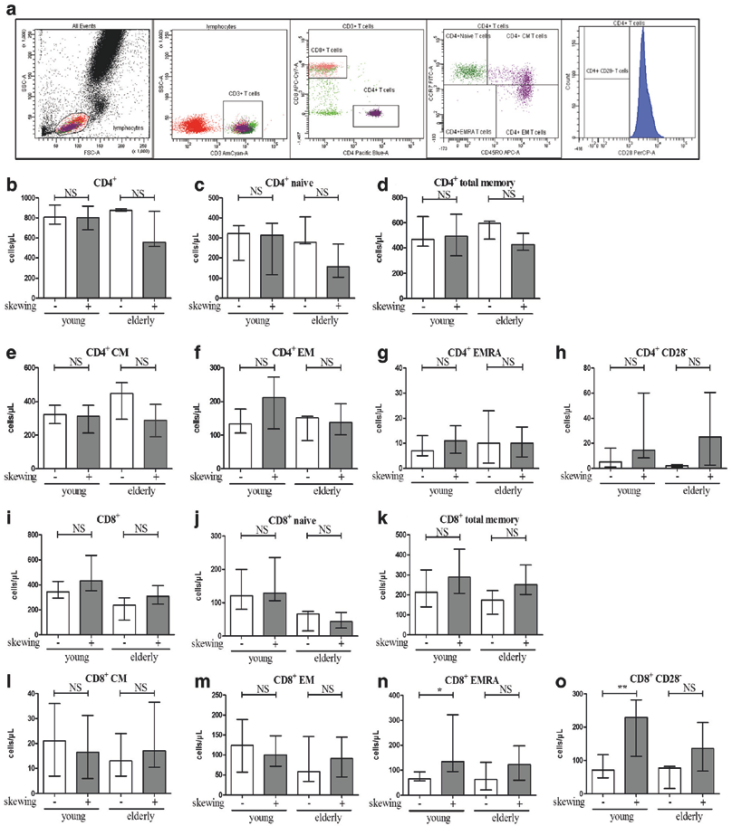

Fig. 3. Absolute number of circulating $CD4^+$ and $CD8^+$ T cell subsets in young (*n* = 10) and elderly end-stage renal disease (ESRD) patients (*n* = 21) with a skewed TCR Vβ-repertoire compared to age-matched ESRD patients without a skewed TCR Vβ-repertoire (young *n*=11; elderly *n*=3). A typical example of the gating strategy for dissection of the different T cell subsets by flow cytometry following a whole blood staining protocol is given in **a**. Briefly, lymphocytes were identified based on the forward/sideward characteristics followed by the selection of $CD3^+$ T cells. These $CD3^+$ T cells were then dissected into $CD4^+$ and $CD8^+$ T cells. CCR7 and CD45RO were used to identify the $CD4^+$ T cells subsets. Furthermore, $CD28^-$ T cells were examined within the total $CD4^+$ population. A similar gating strategy was employed for determination of absolute numbers of $CD8^+$ T cell subsets and the differentiation status. The number of **b** $CD4^+$, **c** $CD4^+$ naive, **d** $CD4^+$ total memory, **e** $CD4^+$ central memory (CM), **f** $CD4^+$ effector memory (EM), **g** $CD4^+$ highly differentiated effector T cells (EMRA), **h** $CD4^+CD28^-$ T cells, as well as **i** $CD8^+$, **j** $CD8^+$ naive, **k** $CD8^+$ total memory, **l** $CD8^+$ central memory (CM), **m** $CD8^+$ effector memory (EM), **n** $CD8^+$ highly differentiated effector T cells (EMRA) and $CD8^+CD28^-$ T cells are shown. Data are given as median with interquartile range. The open bars represent ESRD patients with a non-skewed TCR Vβ-repertoire and closed bars correspond to ESRD patients with a skewed TCR Vβ-repertoire. P value: *<0.05; **<0.01; ***<0.001; NS: not significant.

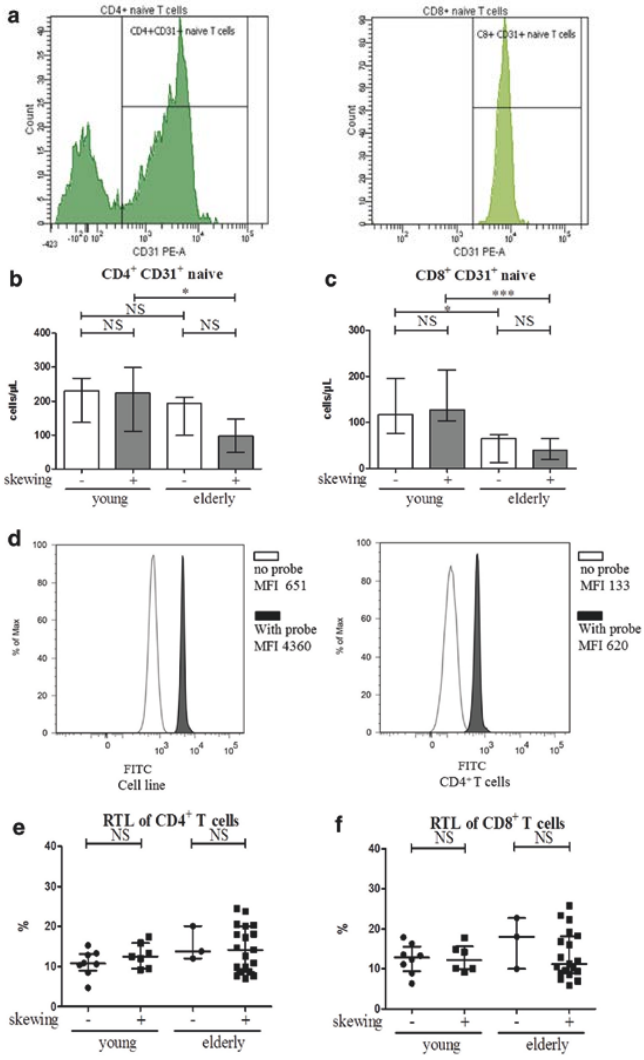

Fig. 4 Absolute number of CD31-expressing naive CD4⁺ and CD8⁺ T cells and relative telomere length (RTL) of CD4⁺ and CD8⁺ T cells in end-stage renal disease (ESRD) patients with skewing in TCR Vβ-repertoire compared with age-matched ESRD patients without skewing in TCR Vβ-repertoire. A typical flow cytometric example of the analysis of CD31-expression within naive CD4⁺ (*left histogram*) and CD8⁺ (*right histogram*) T cells (identified as depicted within Fig.3A) is given in **a**. The number of CD31⁺ **b** CD4⁺ and **c** CD8⁺ naive T cells for young and old ESRD patients with a non- (*open bars*; young *n* = 11, elderly *n* = 3) and skewed (*closed bars*; young *n* = 10, elderly *n* = 21) TCR Vβ-repertoire are given. In **d**, a typical flow cytometric example is depicted of the analysis to allow for the determination of relative telomere length (RTL). Histograms showing the median fluorescence intensity (MFI) of the FITC-channel of samples incubated without (*unfilled peak*) and with the FITC-labelled PNA-probe (*filled peak*) are shown for the CCRF-CEM 1301 subcell-line (*left panel*) and CD4⁺ (*right panel*), respectively. The RTL of **e** CD4⁺ and **f** CD8⁺ T cells are shown. Triangles represent the RTL of ESRD patients with a non-skewed TCR Vβ-repertoire (young *n* = 8, elderly *n* = 3) and squares correspond to ESRD patients with a skewed TCR Vβ-repertoire (young *n* = 6, elderly *n* = 19). Data are given as median with interquartile range. *<0.05; **<0.01; ***<0.001; NS: not significant.

associated with skewing of TCR Vβ-repertoire. A multiple binary logistic regression model was used to further analyze whether the effects on TCR Vβ-repertoire skewing were independent. ESRD ($P < 0.05$, OR =10.2), age ($P < 0.05$, OR =3.1) and CMV-IgG seropositivity ($P < 0.05$, OR =13.8) remained significant, indicating these factors to independently contribute to skewing of the TCR Vβ-repertoire. CMV-IgG seropositivity contributed mostly to the skewing of the TCR Vβ-repertoire followed by ESRD and age (Table 2). In the ESRD patients, neither renal replacement therapy (RRT) (P>0.05) nor underlying kidney disease ($P > 0.05$) were associated with skewing of the TCR Vβ-repertoire (Table 2).

Skewed TCR Vβ-repertoires are presented predominantly within the $CD8^+$ memory T cell subset and is associated with more differentiated T cells

In order to determine whether skewing of TCR Vβ was associated with specific T cell subsets, $CD4^+/CD8^+$ naive and $CD4^+/CD8^+$ memory T cells from 10 elderly healthy individuals (of which 50% were CMV seropositive) as well as 10 elderly ESRD patients (also containing 50% CMV-seropositive individuals) were FACS-sorted. Skewing of the TCR Vβ-repertoire significantly occurred in $CD8^+$, but not $CD4^+$, memory T cells in elderly ESRD patients only (Table 3). Within the CD8 memory T cells, a skewed TCR Vβ-repertoire was observed in 5 CMV-seropositive and 4 CMV-seronegative elderly ESRD patients.

The ratio of $CD4^+/CD8^+$ T cells was significantly lower in young and elderly ESRD patients with a skewed TCR Vβ-repertoire compared to age-matched ESRD patients without skewed TCR Vβ-repertoire, respectively (young: 1.73 versus 2.38 $P < 0.05$; elderly: 2.30 versus 4.71, $P < 0.05$). Next, we determined the association between having a skewed TCR Vβ-repertoire and T cell subsets as well as the differentiation status of T cells. In Fig. 3a, a typical flow cytometric example of the gating strategy for dissecting the different $CD4^+$ T-cell subsets including naive, central memory (CM), effector memory (EM), highly differentiated effector memory (EMRA) as well as for determining the differentiation status of the T-cell compartment by analysis of $CD28^-$ T cells is given. A similar approach was also employed for $CD8^+$ T cells. Young ESRD patients with a skewed TCR Vβ-repertoire had increased numbers of more differentiated memory T cells, i.e. EMRA (Fig. 3n) as well as $CD28^-CD8^+$ T cells (Fig. 3o). The number of individuals without skewing in the elderly ESRD patients was limited (only 3 out of 24), which might explain why the comparison between non-skewed and skewed ESRD patients did not reach significance for $CD8^+$ EMRA or $CD8^+$ $CD28^-$ T cells but similar trends were observed (Fig.3n & 3o). Approximately 80% of the young and 62% of the elderly ESRD patients, that had a skewed TCR Vβ-repertoire, were CMV-seropositive, confirming the previously described effects of CMV-latency on the T cell compartment. Moreover, the skewed TCR Vβ-repertoire was correlated with more differentiated $CD8^+$ T cells in the healthy population as well. In the elderly healthy individuals, those with a skewed TCR Vβ-repertoire had more $CD8^+$ T cells, more $CD8^+$ memory, $CD8^+$ EMRA and $CD8^+CD28^-$ than the individuals without skewed TCR Vβ-repertoire (Fig. S1 H, J, M, and N). Within young

healthy individuals, the numbers of $CD8^+$ memory (MEM), $CD8^+$ effector memory (EM) and $CD8^+CD28^-$ T cells were significantly higher in the individuals with skewed TCR repertoire than the individuals with a polyclonal TCR Vβ-repertoire (Fig. S1 J, L, N). The proportions of CMV-IgG seropositive individuals within the HI with a skewed TCR repertoire amounted to 75% and 100% in young and elderly HI, respectively.

Number of $CD31^+$ naive T cells and relative telomere length (RTL) are not correlated to a skewed TCR Vβ-repertoire

Thymic output, assessed by number of CD31-expressing naive $CD4^+$ and $CD8^+$ T cells (Fig. 4a), was not different for ESRD patients or healthy individuals with and without a skewed TCR Vβ-repertoire. Remarkably, only elderly patients and HI with a skewed TCR Vβ-repertoire showed an age-related decline in $CD31^+$ naive $CD4^+$ T cells (Fig. 4b and Fig. S2A). An age- related decline in $CD31^+$ naive $CD8^+$ T cells was observed in both patients and HI (Fig. 4c and Fig. S2B). Another hallmark of T cell ageing is attrition of telomeres and a typical example of the flow cytometric analysis of telomere length is shown in Fig. 4d. Assessing the association of this parameter to skewing of the TCR Vβ-repertoire only revealed a trend to shorter telomeres in $CD8^+$ (Fig. 4f) T cells of elderly ESRD patients with a skewed TCR Vβ -repertoire compared to those without. The RTL of $CD4^+$ /$CD8^+$ T cells were not significantly different between the healthy individuals with and without a skewed TCR -repertoire either (Fig. S2C&D).

Discussion

The main observation of this study is that ESRD independently skews the TCR Vβ-repertoire. In combination with an older age and CMV seropositivity, this leads to a skewed TCR -repertoire in almost all elderly ESRD patients. This finding extends the knowledge that loss of renal function is associated with significant changes in several T-cell (aging) parameters such as thymic output, T-cell differentiation status and proliferative history as analyzed by relative telomere length analysis(6, 7). In contrast with these studies that demonstrate effects of ESRD on various T-cell parameters over a broad age range, skewing of the TCR Vβ-repertoire by ESRD is limited to the elderly, which indicates that young ESRD patients are still able to maintain a relatively diverse TCR Vβ-repertoire.

In our study, we tested all TCR Vβ gene families, in a multiplex DNA-based approach and considered different variables possibly influencing TCR Vβ-repertoire diversity, like age(13, 22), CMV-serostatus (17), gender (23), ESRD, RRT and underlying kidney disease (24, 25). Although CMV was the strongest factor amongst the examined variables, affecting both the young and elderly ESRD patients as well as elderly HI, ESRD seems to introduce skewing in both the CMV-seronegative as well as CMV-seropositive elderly group. We only observed an age-related effect on TCR Vβ skewing in our ESRD patient population, but aging alone does not seem to significantly compromise TCR Vβ skewing. It is also reported that the TCR-Vβ -repertoire of $CD8^+$ T cells was already skewed in young CMV-seropositive HI and

did not modulate further with age(26). Also, Sunder-Plassmann *et al.* (27) showed ESRD patients receiving hemodialysis treatment to have a skewed TCR Vβ-repertoire, with an impressive increase of TCR Vβ6.7 and a massive deletion of the TCR Vβ8 in peripheral blood lymphocytes compared to healthy controls. In our study, we were unable to find a significant association between RRT and skewing of the TCR Vβ-repertoire. This is in accordance with our previous findings, which did show a significant effect of ESRD on ageing of the peripheral T cells but hardly any differences between patients with or without RRT (8). Gender also did not affect TCR Vβ skewing and in agreement with the research by Sunder-Plassmann *et al.* (27), we did not find a significant contribution of underlying kidney disease with respect to skewing of the TCR Vβ-repertoire. This might indicate that the chronic inflammatory milieu (28) in which the T cells circulate and the significant changes in the peripheral T cell compartment (6) as a consequence of the loss of renal function causes oligoclonality of the TCR -repertoire.

A skewed TCR Vβ-repertoire is associated with significant changes in composition of peripheral T cells and related to a highly differentiated pattern. A skewed TCR Vβ-repertoire in HI was correlated to an increased number of highly differentiated memory $CD8^+$ T cells. This might be due to the fact that most of the healthy individuals with a skewed TCR Vβ-repertoire were CMV-seropositive. CMV latency introduces significant changes in the peripheral T cell compartment, in particular affecting $CD8^+$ T cells (29) and is known to introduce skewing of the TCR Vβ-repertoire as a result of expansion of CMV-specific T cell clones in the HI (17). In refractory cytopenia patients, TCR Vβ skewing correlates with an expansion of effector $CD8^+$ T cells(30). A similar correlation between a skewed TCR Vβ-repertoire and an increased number of highly differentiated memory $CD8^+$ T cells was also found in young ESRD patients and tended to be significant in the elderly patients. Also within ESRD patients, a large proportion was CMV-IgG seropositive, highlighting the possibility that skewing of TCR Vβ-repertoire may be likewise related to clonal expansions of antigen-specific T cells directed to CMV or expansion of other TCR-specificities, e.g. TCR Vβ 6.7 as reported before (27).

CD31-expressing naive T cells have undergone a lower number of cell divisions(10) and have a broader TCR Vβ-repertoire than those lacking CD31(11). Some clones could be lost during naive T cell homeostatic proliferation (31), as a massive deletion of the TCR Vβ8 was observed in peripheral blood lymphocytes of hemodialysis patients(27). However, the numbers of CD31- expressing naive T cells were not significantly different between the patients with skewed and non-skewed TCR Vβ-repertoire. In addition, the skewed TCR Vβ-repertoire was predominantly observed in the sorted $CD8^+$ memory compartment and not in naive T cells. This implies that some memory T-cell clones proliferate more efficiently in response to the ESRD associated pro-inflammatory environment or antigenic stimulation such as CMV. Clonal expanded $CD8^+$ T cells are also observed in the peripheral blood of multiple sclerosis patients. Based on the current findings, we plan to conduct a more in

depth analysis of the TCR Vβ-repertoire including both the diversity (the number of specific clones) and the relative abundance of particular clones (the number of reads per clone). The clinical implications of having a skewed or less diverse TCR Vβ-repertoire prior to kidney transplantation are not yet explored. However, analysis of TCR Vβ-repertoire diversity following transplantation has been the subject of several studies (32, 33). Miqueu *et al.* (34) reported a skewed -repertoire to be related to an increased risk of humoral chronic rejection after kidney transplantation. It might be of interest to investigate the consequences of a skewed TCR Vβ-repertoire before kidney transplantation with respect to clinical outcomes following kidney transplantation like rejection episodes or to examine whether this TCR Vβ-repertoire would change after kidney transplantation.

CONCLUSIONS

ESRD significantly and independently skews the TCR Vβ-repertoire in the elderly individuals and this skewed TCR Vβ pattern is associated with more differentiated T cells subsets. Assessing the TCR Vβ-repertoire diversity in more detail will increase our knowledge with respect to the defective T-cell mediated immunity observed in ESRD patients and will allow to evaluate its clinical relevance with respect to transplantation.

Declarations
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Competing interests
The authors declare that they have no competing interests.

SUPPORTING INFORMATION

Circulating T cell numbers and their differentiation status

Absolute numbers of T cells were obtained using BD TruCount ™ tubes. Briefly, 20 µl of the 6-color TBNK reagent (BD Multitest™, BD Bioscience) was used in combination with a Trucount™ tube (BD Biosciences) and 50 µl of EDTA blood. This tube contains a fixed number of beads (i.e. bead count; lot-specific) which enables calculation of absolute numbers of cells per µl of blood. The 6-color TBNK reagent contains fluorescein isothiocyanate (FITC)-labeled anti-CD3, phycoerythrin (PE) labeled anti-CD16/CD56, peridinin chlorophyll protein **(**PerCP) - cyanine dye (CY) ™5.5-labeled anti-CD45, PE-Cy™7- labeled anti-CD4, allophycocyanin (APC) - labeled anti-CD19, APC-Cy7 labeled anti-CD8.

Analysis of the T cell differentiation status was performed using flow-cytometry following the whole blood staining protocol. Briefly, whole blood was stained with AmCyan-labeled anti-CD3 (BD) in combination with pacific blue (PB)-labeled anti-CD4 (BD) and APC-Cy7-labeled anti-CD8 (BD). T cells were defined as $CD4^+$ or $CD8^+$ and further defined into four different subsets based on the expression of CCR7 and CD45RO using FITC- labeled anti-CCR7 (R&D systems, Uithoorn, The Netherlands) and APC-labeled anti-CD45RO (BD). Naive T cells were identified as $CCR7^+$ and $CD45RO^-$, central memory (CM) cells as $CCR7^+$ and $CD45RO^+$, effector memory (EM) cells as $CCR7^-$ and $CD45RO^+$ and the highly differentiated effector memory (EMRA) cells as $CCR7^-$ and $CD45RO^-$. T-cell differentiation is associated with loss of CD28 expression on the cell surface. Numbers of $CD28^-$ (or CD28null) T cells within the T-cell subsets were determined by staining with PerCP-Cy5.5-labeled anti-CD28 (BD). Thymic output was analyzed by determining numbers of CD31-expressing cells within the naive T-cell pool upon staining with PE-labeled anti-CD31 (Biolegend, Europe BV, Uithoorn, the Netherlands). Samples were measured on the FACSCanto II (BD) acquiring at least $5x10^4$ T lymphocytes.

Sorting pure T cell subsets

PBMCs were stained with AmCyan-labeled CD3 (BD Pharmingen, Erembodegem, Belgium), Pacific Blue-labeled CD4 (BD), APC-Cy7- labeled CD8 (BD); APC-labeled CD45RO (BD), PE- CY7-labeled CCR7 (R&D systems, Uithoorn, The Netherlands) and a live-dead marker ViaProbe (7-Aminoactinomycin D, BD) was included. After staining, the cells were washed and resuspended at 20-25x106/mL and sorted into $CD4^+$ and $CD8^+$ Naive/memory T cells (BD FACSAria™ II SORP, BD). Naive T cells were identified as $CCR7^+$ and $CD45RO^-$ and the remaining CD4+ or CD8+ T cells were collected as memory T cells.

Telomere length assay

Briefly, PBMCs were thawed and stained with either anti-CD4-biotin (Beckman-Coulter, BV, Woerden, the Netherlands) or anti-CD8-biotin (Biolegend) followed by staining with streptavidin-Cy5 (Biolegend). After fixation and permeabilization, the

telomere length of T cells was determined using the telomere PNA-kit/FITC (Dako BV, Heverlee, Belgium) according to manufacturer's instructions. The subcell line 1301 of CCRF-CEM, known for its long telomeres was treated in a similar way and used as an internal control. The relative telomere length was calculated using the following formula, comparing the median fluorescence intensity (MFI) of the FITC signal of the samples to that of the sub-cell line.

$$\textbf{RTL} = \frac{(\text{MFI of sample cells with probe} - \text{MFI of sample cells without probe}) \text{x DNA index of control cells } (=2)}{(\text{MFI of cell line with probe} - \text{MFI of cell line without probe}) \text{ x DNA index of sample cells}(=1)} \; *100\%$$

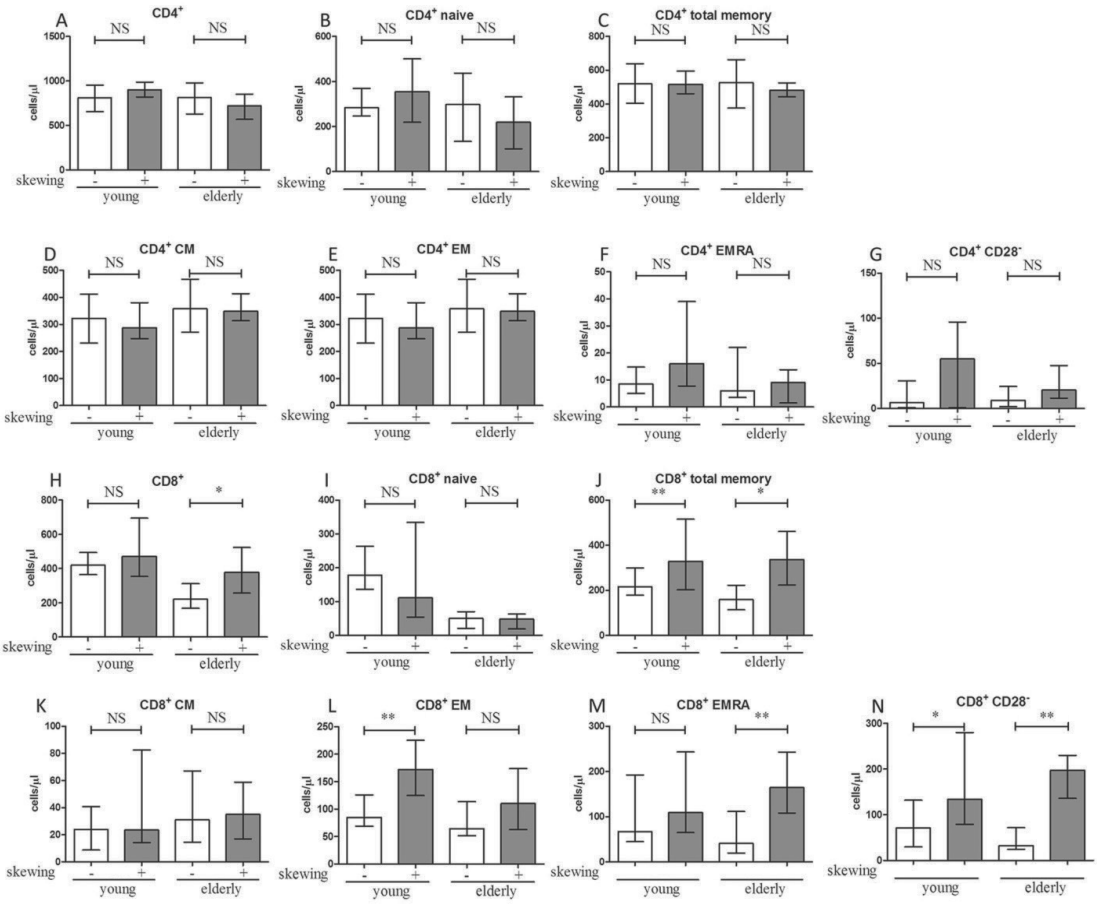

Fig. S1. Absolute number of circulating CD4⁺ and CD8⁺ T cells in young (*n*= 8) and elderly healthy individuals (HI, *n* = 8) with skewing in TCR Vβ-repertoire compared to age-matched HI without a skewing in TCR Vβ-repertoire (young *n* = 18; elderly *n* = 17). The number of (**A**) CD4⁺, (**B**) CD4⁺ naive, (**C**) CD4⁺ total memory, (**D**) CD4⁺ central memory (CM), (**E**) CD4⁺effector memory (EM), (**F**) CD4⁺ highly differentiated effector T cells (EMRA), (**G**) CD4⁺CD28⁻ T cells, as well as (**H**) CD8⁺, (**I**) CD8⁺ naive, (**J**) CD8⁺ total memory, (**K**) CD8⁺ central memory (CM), (**L**) CD8⁺ effector memory (EM), (**M**) CD8⁺ highly differentiated effector T cells (EMRA) and (**N**) CD8⁺ CD28⁻ T cells are shown. Data are given in median with interquartile range. Open bars represent HI with a non-skewed TCR Vβ-repertoire and closed bars correspond to HI with a skewed TCR Vβ-repertoire. *<0.05, **<0.01, ***<0.001; NS: not significant.

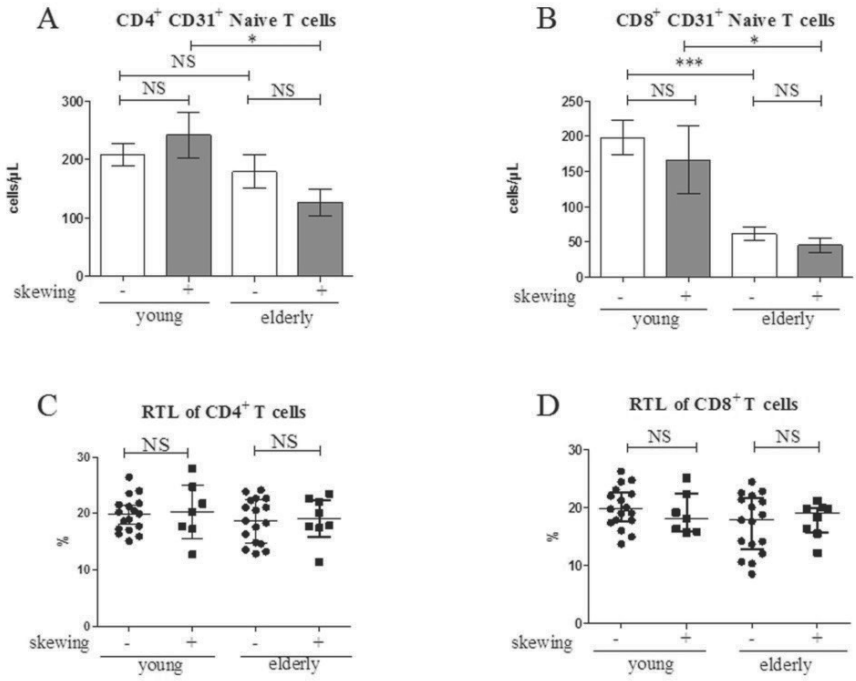

Fig S2. Absolute number of CD31-expressing naive and relative telomere length (RTL) of $CD4^+$ and $CD8^+$ T cells of healthy individuals (HI) with skewing in TCR Vβ-repertoire compared to age-match HI without skewing in TCR Vβ-repertoire. Number of (**A**) $CD31^+CD4^+$ naive and (**B**) $CD31^+CD8^+$ naive T cells, in addition with RTL of (**C**) $CD4^+$ and (**D**) $CD8^+$ T cells are shown. Data are given in median with interquartile range. Open bars represent absolute number of CD31- expressing naive T cells of HI with a non-skewed TCR Vβ-repertoire (young *n* = 18; elderly *n* = 17) and closed bars correspond patients with a skewed TCR Vβ-repertoire (young *n* = 8, elderly *n* = 8). Triangles represent the RTL of patients with a non-skewed TCR Vβ-repertoire (young *n* = 17, *n* = 17) and squares correspond patients with a skewed TCR Vβ-repertoire (young *n* = 7, elderly *n* = 8). *<0.05; **<0.01; ***<0.001; NS: not significant

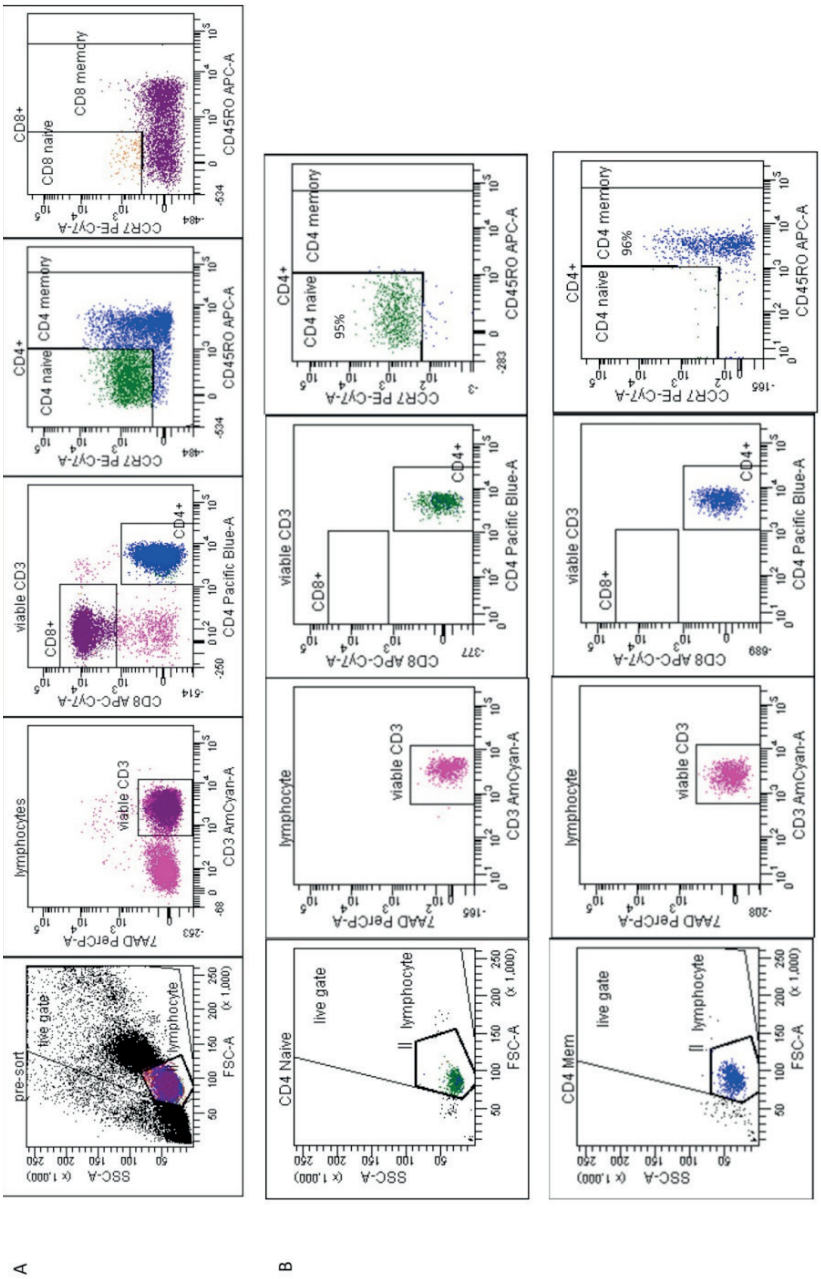

Fig. S3 (**A**) A typical example of the gating strategy to sisect the different T cell subsets prior to sorting and (**B**) the analysis of the purity of CD4⁺ naive/memory sorted samples.

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CHAPTER 4

End-Stage Renal Disease Causes Skewing In The TCR Vβ-Repertoire primarily within $CD8^+$ T-Cell Subsets

L. Huang[1], M.G.H. Betjes[1], M. Klepper[1], A.W. Langerak[2], C.C. Baan[1], N.H.R. Litjens[1*]

Erasmus MC, University Medical Center Rotterdam, Department of Internal Medicine, Nephrology and Transplantation[1], Rotterdam, Netherlands and Erasmus MC, University Medical Center Rotterdam, Department of Immunology[2], Rotterdam, Netherlands.

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ABSTRACT

A broad T cell receptor (TCR-)-repertoire is required for an effective immune response. TCR-repertoire diversity declines with age. End-stage renal disease (ESRD) patients have a prematurely aged T-cell system which is associated with defective T-cell mediated immunity. Recently, we showed that ESRD may significantly skew the TCR Vβ-repertoire. Here we assessed the impact of ESRD on the TCR Vβ-repertoire within different T-cell subsets using a multi-parameter flow-cytometry-based assay, controlling for effects of ageing and CMV latency.

Percentages of 24 different TCR Vβ-families were tested in circulating naive and memory T cell subsets of 10 ESRD patients and 10 age- and CMV-serostatus-matched healthy individuals (HI). The Gini-index, a parameter used in economics to describe the distribution of income, was calculated to determine the extent of skewing at the subset level taking into account frequencies of all 24 TCR Vβ-families. In addition, using HI as our reference population, the differential impact of ESRD was assessed on clonal expansion at the level of a TCR Vβ-family.

$CD8^+$, but not $CD4^+$, T-cell differentiation was associated with higher Gini-TCR indices. Gini-TCR indices were already significantly higher for different $CD8^+$ memory T-cell subsets of young ESRD patients compared to their age-matched HI. ESRD induced expansions of not one TCR Vβ-family in particular and expansions were predominantly observed within the $CD8^+$ T cell compartment. All ESRD patients had expanded TCR Vβ-families within total $CD8^+$ T cells and the median (IQ range) number of expanded TCR Vβ-families/patient amounted to 2 (1-4). Interestingly, ESRD also induced clonal expansions of TCR Vβ-families within naive $CD8^+$ T cells as 8 out of 10 patients had expanded TCR Vβ-families. The median (IQ range) number of expanded families/patient amounted to 1 (1-1) within naive $CD8^+$ T cells.

In conclusion, loss of renal function skews the TCR Vβ-repertoire already in young patients by inducing expansions of different TCR Vβ-families within the various T cell subsets, primarily affecting the $CD8^+$ T cell compartment. This skewed TCR Vβ-repertoire may be associated with a less broad and diverse T-cell-mediated immunity.

INTRODUCTION

End-stage renal disease (ESRD) patients have a decreased vaccination efficacy (1-4), an increased susceptibility for infection (5-7) and a higher risk for the development of tumors (8-11). Loss of renal function is associated with a prematurely aged T-cell system (12), most likely caused by the uremia-induced pro-inflammatory environment(13). These uremia-induced effects on T cells are expressed as a decline in thymic output, a severe depletion of naive T-cell compartment, a shift to more highly differentiated memory T-cell subsets, attrition of T-cell telomeres (14) and a defective TCR-induced ERK phosphorylation (15).

A broad T cell receptor (TCR) -repertoire capable of recognizing a wide range of foreign antigens is crucial for adequate T-cell mediated immune responses (16). Most TCRs consist of an α and β chain and each chain is composed of a variable (V) and a constant (C) region (17). The TCR Vβ-repertoire can be assessed using several approaches such as gene scan spectratyping via a DNA-based PCR (18), Vβ-family phenotyping by flow-cytometry (19-21), and assessment of clonal diversity via next generation sequencing (NGS) (22, 23). Gene scan spectratyping of the TCR Vβ-repertoire is at best a semi-quantitative measurement. Both flow-cytometry and NGS result in a more accurate quantitative assessment of the TCR Vβ-repertoire. As NGS is more labor-intensive and sorting of highly pure T cells or their subsets is required, many researchers prefer to use flow-cytometry. Flow-cytometry allows for measuring percentages of TCR Vβ-families at the T cell-subset level obviating the need for cell sorting.

We recently examined the TCR Vβ-repertoire in ESRD patients using multiplex DNA-based spectratyping. We showed ESRD to significantly and independently skew the TCR Vβ-repertoire in older individuals and this skewing was predominantly present within the $CD8^+$ memory T-cell compartment (24). However, details of this skewed TCR Vβ-repertoire in ESRD patients are still lacking and quantitative data related to the impact of ESRD on TCR Vβ-repertoire in the various T-cell populations is rare.

During ageing the TCR Vβ-repertoire has been reported to contract (25). Ageing is associated with a decline in the naive T-cell compartment which possess the broadest TCR-repertoire (26), and a shift towards memory T cells, developing upon encountering of an antigen and having a skewed repertoire towards particular specificities (27, 28). The prevalence of CMV-seropositivity is high amongst ESRD patients, varying from 30-100%, depending on socio-economic and ethnic background (29). CMV latency profoundly affects circulating T cells resembling features of ageing, including increased frequencies of more differentiated memory T cells (30, 31) and loss of telomere length (32). CMV latency may also induce contraction of the TCR Vβ-repertoire as it induces expansion of CMV-specific T cells immunocompetent donors (33) and these CMV-specific clones are stably maintained

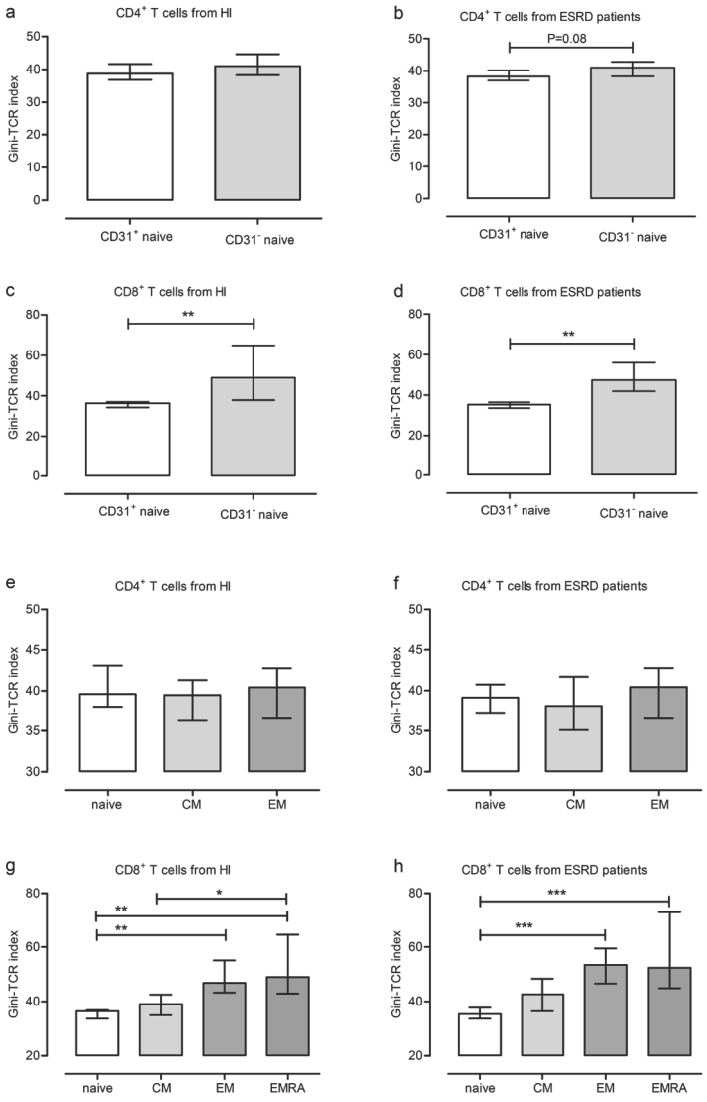

Fig. 1. Gini-TCR indices for different T cell subsets**.** The different Gini-TCR indices are depicted for HI (a, c, e and g) and ESRD patients (b, d, f and h). First, the Gini-TCR indices for RTE ($CD31^+$ naive) and $CD31^-$ naive $CD4^+$ (a and b) and $CD8^+$ T (c and d) cells are given, respectively. Next, the differentiation-associated effects on Gini-TCR indices are depicted for $CD4^+$ (e and f) and $CD8^+$ (g and h) T cell subsets, including naive, central memory (CM), effector memory (EM) and terminally differentiated effector memory (EMRA) T cells. *; ** and *** reflect P-values <0.05; 0.01 and 0.001, respectively. Data from 10 HI and 10 ESRD patients are given as median with interquartile range.

for 5 years (34). Thus TCR Vβ-repertoire diversity may be affected by various factors.

In this study, we assessed the TCR Vβ-repertoire diversity within different T-cell subsets in ESRD patients using a flow-cytometry-based taking into account the effects of ageing and CMV latency.

MATERIALS AND METHODS

Study population

A cohort of ten stable ESRD patients, either younger individuals (*n*=5, age<45 years) or older individuals (*n*=5, age≥65 years) with an oligoclonal TCR Vβ-repertoire, as determined by DNA-based spectratyping earlier (24) were studied in more detail at the T-cell subset level using a flow-cytometry based assay for TCR Vβ-repertoire analysis. Patients having a glomerular filtration rate below 15 mL/min and either or not receiving receiving renal replacement therapy were included. Patients were excluded from the study when having a bacterial or viral infection, malignancy, a previous transplantation or taking immunosuppressive medication (except for glucocorticoids). The patient data are compared to those generated from ten age- and CMV-matched healthy individuals (HI) with a polyclonal TCR Vβ-repertoire, as determined by DNA-based spectratyping (24). Lithium-heparinized blood was drawn from ESRD patients and HI. Written informed consent was obtained from all individuals included. The study was approved by the local medical ethical committee (METC number: 2012–022) and conducted according to the principles of Declaration of Helsinki and in compliance with International Conference on Harmonization/Good Clinical Practice regulations.

Sample preparation

Peripheral blood mononuclear cells (PBMCs) were isolated from 35 mL of lithium-heparinized blood by density centrifugation as described previously (38) and then frozen at $10x10^6$ PBMC per vial at -190°C until further use.

Cryopreserved PBMCs (1 vial of $10x10^6$ PBMCs) were thawed, counted, washed and resuspended in Isoflow™ Sheath Fluid (Beckman Coulter B.V., Woerden, Netherlands). The PBMCs were stained with Brilliant Violet (BV) 510 labeled anti-CD3 (BioLegend, Uithoorn, Netherlands), Alexa Fluor (AF)700 labeled anti-CD4 (Beckman Coulter B.V.) and Allophycocyanin (APC)-Cy7 labeled anti-CD8 (BioLegend) to identify $CD4^+$ and $CD8^+$ within $CD3^+$T cells. ECD labeled anti-CD45RO (Beckman Coulter B.V.), PE-Cy7 labeled anti-CCR7 (BD, Erembodegem, Belgium), V450 labeled anti-CD31 (BD; clone WM59), Peridinin chlorophyll-A protein (PerCP)-Cy5.5 labeled anti-CD28 (BD) and APC labeled anti-CD57 (BioLegend) as well as fluorescence minus one controls (FMOs) were used to appropriately identify the different T-cell subsets (illustrated in **Fig. S1b-d in Supplementary Material**). As shown in **Fig. 1b**, CCR7 and CD45RO are used to distinguish the different naive and memory T-cell subsets, i.e. naive ($CD45RO^-CCR7^+$), central memory

Table 1 TCR Vβ-families in tube A-H

Tube	Vβ family	Fluorochrome	Clone
A	Vβ 5.3	PE	3D11
	Vβ 3	FITC	CH92
	Vβ 7.1	PE+FITC	ZOE
B	Vβ 9	PE	FIN9
	Vβ 16	FITC	TAMAYA1.2
	Vβ 17	PE+FITC	E17.5F3
C	Vβ 18	PE	BA62.6
	Vβ 20	FITC	ELL1.4
	Vβ 5.1	PE+FITC	IMMU157
D	Vβ 13.1	PE	IMMU222
	Vβ 8	FITC	56C5.2
	Vβ 13.6	PE+FITC	JU74.3
E	Vβ 5.2	PE	36213
	Vβ 12	FITC	VER2.32
	Vβ 2	PE+FITC	MPB2D5
F	Vβ 23	PE	AF23
	Vβ 21.3	FITC	IG125
	Vβ 1	PE+FITC	BL37.2
G	Vβ 11	PE	C21
	Vβ 14	FITC	CAS1.1.3
	Vβ 22	PE+FITC	IMMU546
H	Vβ 13.2	PE	H132
	Vβ 7.2	FITC	ZIZOU4
	Vβ 4	PE+FITC	WJF24

Detailed information with respect to the different TCR Vβ-family antibodies in tube A-H, labels and clones (IOTest® Beta Mark TCR V β-repertoire kit, Beckman Coulter) .

(CM, $CD45RO^+CCR7^+$), effector memory (EM, $CD45RO^+CCR7^-$) and terminally differentiated effector memory $CD45RA^+$ subsets (EMRA, $CD45RO^-CCR7^-$). CD31-expression within naive T cells (**Fig.S1c**) identifies T cells that recently have left the thymus, also referred to as recent thymic emigrants (RTE) (39). Loss of CD28 ($CD28^-$ T cells) and gain of CD57 ($CD57^+$ T cells) expression is observed in relation to increased replicative history (40, 41) and allows for identification of more differentiated T cells (**Fig.S1d**).

Subsequently, the cell suspension was divided into 8 tubes (100 µL/tube) labeled A-H, corresponding to the different antibody cocktails to stain for the 24 TCR Vβ-families (IOTest® Beta Mark TCR V beta repertoire kit, Beckman Coulter B.V). Each cocktail contains antibodies directed to 3 different Vβ-families, i.e one is fluorescein isothiocyanate (FITC-), one is PE-labeled and one is labeled with both FITC and PE. **Table 1** shows the description of the antibodies directed to the different TCR Vβ-families in tube A to H. A typical example of the proportions of several TCR Vβ-

families within CD3+ T cells from tube A, tube B and tube C is depicted in **Fig.S1a, the last 3 plots**.

The samples were measured on a Navios flow cytometer (10-color configuration; Beckman Coulter B.V.) and at least 0.5 million CD3+ T cells were acquired for each tube. Data were analyzed by Kaluza™ software (Beckman Coulter B.V.). The number of events acquired for a specific T-cell subset needed to be more than 100 to allow for reliable analysis of frequencies of TCR Vβ-families within this population. The only subset that did not meet this criterion was the EMRA population within the CD4+ T cells.

Gini-TCR index and calculation of expanded TCR Vβ-families

The Gini index is used to describe the distribution of income in economic statistics. As the distribution of TCR Vβ-families shows similarities to that of income, the Gini index can also be applied in TCR Vβ-repertoire analysis by flow-cytometry. It has already been used in TCR-sequencing studies (42, 43), and was recently also introduced as an accurate and reliable way for analyzing TCR Vβ-repertoire data obtained by flow-cytometry (44). The TCR (Vβ)-Gini index with scores ranging from low to high indicates TCR Vβ-families from equal distribution (broad repertoire; i.e. low score) to unequal distribution (skewed repertoire; i.e. high score). A Microsoft excel file allowing for automatic calculation of the Gini-TCR index using percentages

Table 2 Demographic and clinical characteristics of the study population

	Healthy individuals	ESRD patients	P-value
Number of individuals	10	10	
Age (years;range)			
young group (<45 years)	36 (26-42)	28 (20-29)	0.06
elderly group (>65 years)	68 (65-73)	70 (65-73)	0.92
Sex (% male)	60	40	0.66
CMV IgG serostatus (% pos)	60	60	1.00
RRT (%)	n.a.	70	n.a.
Duration of RRT (months; median/rang)		20 (7 - 68)	
Hemodialysis (%)		85.7	
Peritoneal dialysis (%)		14.3	
Underlying kidney disease			
Primary glomerulopathy (%)		20	
Diabetic nephropathy (%)		30	
Reflux nephropathy (%)		10	
Polycystic kidney disease (%)		20	
Lupus nephritis (%)		10	
Unknown (%)		10	

ESRD: end-stage renal disease; CMV: cytomegalovirus; RRT: renal replacement therapy; n.a.: not applicable

of 24 TCR-Vβ families is provided in the **supporting file** (44).

An expansion in a TCR Vβ-family induced by ESRD is defined as a frequency above the mean percentage + 2 times the standard deviation (SD) of a certain TCR Vβ-family obtained using the HI as reference population. By using this approach, finding an expansion by chance is lower than 2.5%.

Statistical analyses

Gini-TCR indices or median number of expanded TCR Vβ-families/individual between two different T-cell subsets within individuals were compared with Wilcoxon signed rank test and Friedman test followed by Dunn's Multiple Comparison T test was used for comparing more than two different T-cell subsets. Trend analyses were performed using two-way ANOVA, comparing different subsets between individuals or $CD4^+$ and $CD8^+$ T cells. In addition, the effect of ESRD with respect to numbers of expanded TCR Vβ-families within different T cell subsets is done using Fisher's exact test. Two-sided p values <0.05 were considered statistically significant. All statistical analyses were performed with GraphPad Prism 5.

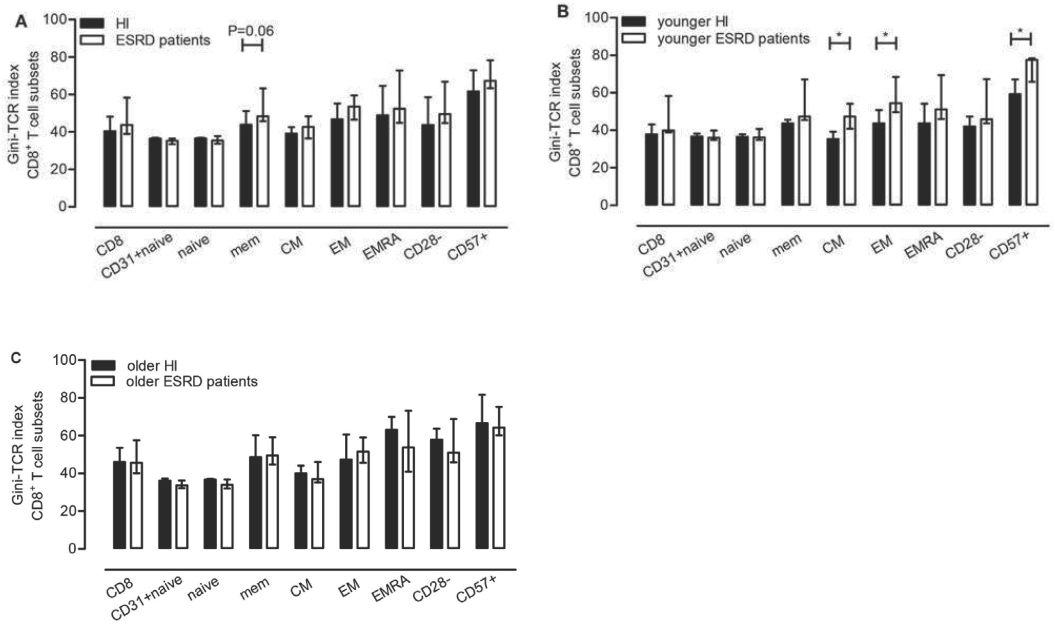

Fig. 2. Effect of ESRD on Gini-TCR indices of $CD8^+$ T-cell subsets. In a) the median and IQ range of the Gini-TCR indices for HI (N=10) and ESRD patients (N=10) for different $CD8^+$ T-cell subsets is depicted whereas in b) and c) those for the young and older group (N=5) are given, respectively. P value: * < 0.05.

RESULTS

Study population

Detailed information of the study population is given in **Table 2**. Ten ESRD patients (5 younger individuals: age 20-29 years & 5 older individuals: age 65-73 years) and 10 age-matched HI (5 younger individuals age 26-42 years & 5 older individuals: age 65-73 years) were recruited into this study. Sixty percent of the ESRD and HI study population is CMV-seropositive. Seven out of 10 ESRD patients received renal replacement therapy (RRT).

Gini-TCR indices increase with T-cell differentiation

Naive T cells expressing CD31 are considered to be recent thymic emigrants and the least-differentiated T-cell subset. In ESRD patients, CD31-expressing naive T cells tended to or have a lower Gini-TCR index when compared to their $CD31^-$ counterparts within $CD4^+$ (**Fig. 1b**) and $CD8^+$ T cells (**Fig. 1d**), respectively. For HI, a significant lower Gini-TCR index was only observed for CD31-expressing naive $CD8^+$ (**Fig. 1c**) but not $CD4^+$ (**Fig. 1a**) T cells when compared to $CD31^-$ naive T cells. Furthermore, a T-cell differentiation-associated increase in Gini-TCR indices was noted for $CD8^+$ (**Fig. 1g and 1h**), but not $CD4^+$ (**Fig. 1e & 1f**), T cells. The median value (IQ range) increased significantly ($P<0.001$) from 36.5 (33.7-37) and 35.5 (33.8-37.9) in naive T cells to 49 (43-64.6) and 52.4 (44.9-72.8) in the highly differentiated effector memory (EMRA) $CD8^+$ T cells for HI and ESRD patients, respectively.

ESRD patients have increased Gini-TCR indices within memory $CD8^+$ T cell subsets

We next analyzed the influence of ESRD, ageing and CMV latency on skewing of the TCR Vβ-repertoire by comparing Gini-TCR indices for different T-cell subsets including total $CD3^+$ T cells, as well as naive, $CD31^+$ naive, total memory (MEM), CM, EM, EMRA, $CD28^-$ and $CD57^+$ populations within both the $CD4^+$ and $CD8^+$ T cell subsets. ESRD effects with respect to Gini-TCR indices were limited to the $CD8^+$ T cell compartment as it tended to induce higher Gini-TCR indices ($P=0.06$) in $CD8^+$ memory T cells when compared to HI (**Fig. 2a**). The median (IQ range) value for Gini-TCR index in $CD8^+$ memory T cells amounted to 48.4 (45.8-63.3) and 43.8 (41.1-51.2) for ESRD patients and HI, respectively. Younger (**Fig. 2b**), but not older (**Fig. 2c**), ESRD patients had significantly higher Gini-TCR indices within the $CD8^+$ CM ($P<0.05$), EM ($P<0.05$) and $CD57^+$ T cell compartment when compared to age-matched HI. The median (IQ range) for Gini-TCR in $CD8^+$ CM, EM and $CD57^+$ T cells amounted to 47.4 (40.8-54.2) versus 35.3 (34.0-39.2), 54.4 (49.6-68.5) versus 43.7 (42.4-50.8) and 77.6 (65.8-78.4) versus 59.2 (57.2-67.1) for younger ESRD patients versus younger HI.

The following results, describing **Supplementary Table 1** and **2** need to be interpreted with caution as the P-values were not adjusted for the number of parameters compared.

Table 3 Effect of ESRD on expansions of TCR Vβ-families

		# of ESRD patients with/without expansions	Median # of expanded TCR Vβ-families (IQ range)	Total # of expanded TCR Vβ-families
$CD3^+$		7/3	1 (0-2)	15
$CD4^+$		5/5	1 (0-2)	10
	$CD31^+$ naive	6/4	1 (0-1)	9
	naive	3/7	0 (0-1)	5
	MEM	9/1	1 (1-3)	16
	CM	6/4	1 (0-2)	11
	EM	6/4	1 (0-2)	13
	EMRA	7/3	2 (0-2)	15
	$CD28^-$	9/1	1 (1-1)	12
	$CD57^+$	9/1	1 (0-2)	11
$CD8^+$		10/0	2 (1-4)	26
	$CD31^+$ naive	7/3	1 (0-2)	15
	naive	8/2	1 (1-1)	12
	MEM	10/0	2 (2-4)	28
	CM	7/3	2 (0-3)	20
	EM	9/1	4 (2-4)	32
	EMRA	10/0	2 (1-3)	26
	$CD28^-$	10/0	3 (1-3)	28
	$CD57^+$	9/1	3 (1-4)	25

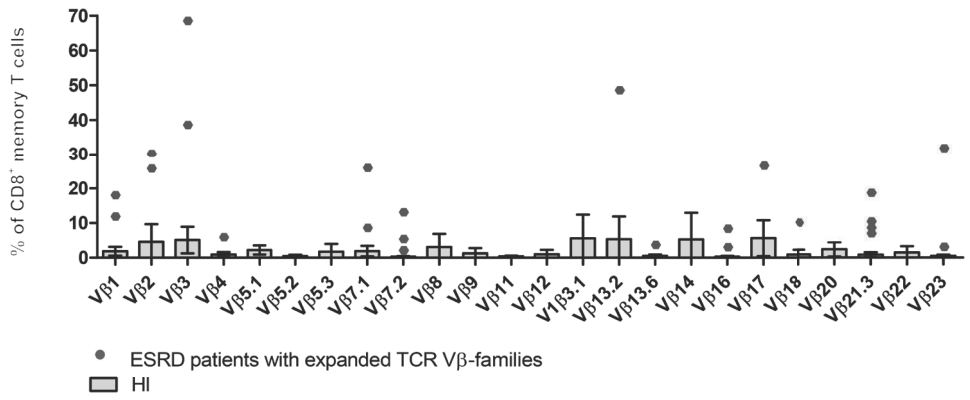

Fig. 3. Expansions of TCR Vβ-families within $CD8^+$ memory T cells from ESRD patients. Boxes and error bars represent the mean and 95% confidence interval (mean ± 2SD) of 24 TCR-Vβ families from 10 healthy individuals (HI). Red dots correspond to expanded TCR Vβ-families from ESRD patients (frequencies> mean+2SD from HI).

Ageing effects were not visible when comparing Gini-TCR indices for the different T-cell subsets between younger and older ESRD patients (**Supplementary Table 1**). In HI, ageing effects were confined to the $CD8^+$ T cell compartment and an ageing-related increasing trend in Gini-TCR index was observed for $CD8^+$ CM T cells (P=0.06), as the median (IQ range) for Gini-TCR amounted to 40.1 (39.1-44.1) in older HI versus 35.3 (34.0-39.2) in younger HI. An increased Gini-TCR index (P=0.03) was observed for older HI, within $CD8^+CD28^-$ T cells (**Supplementary Table 1**). The median (IQ range) value for Gini-TCR in $CD8^+CD28^-$ T cells amounted to 42.0 (40.6-47.4) versus 57.8 (43.7-63.7) for younger and older HI, respectively.

CMV latency did not significantly affect Gini-TCR indices apart from a CMV-related increasing trend within $CD4^+CD57^+$ T cells (P=0.07) of HI, but not ESRD patients, i.e. the median (IQ range) Gini-TCR index amounted to 67.0 (57.4-72.2) for CMV-seropositive HI versus 45.6 (35.5-58.0) for CMV-seronegative ones (**Supplementary Table 2**). No differences were observed when comparing CMV-seronegative and CMV-seropositive ESRD patients to their CMV-serostatus matched HI with respect to Gini-TCR indices for the different T-cell subsets (data not shown).

Clonal expansions of TCR Vβ-families in different T-cell subsets

Apart from characterizing the impact of ESRD on Gini-TCR indices for the different T-cell subsets, we also evaluated the impact of ESRD on clonal expansions of TCR Vβ-families by comparing frequencies to the average+2SD obtained using HI as reference population. **Fig. 3** shows a typical example of expanded TCR Vβ-families within $CD8^+$ memory T cells of ESRD patients.

Clonal expansions of TCR Vβ-families were observed within the $CD3^+$ T cells in 7 out of 10 ESRD patients (**Table 3**). Half versus all of the ESRD patients showed expanded TCR Vβ-families within $CD4^+$ and $CD8^+$ T cells (P<0.05), respectively. The median (IQ range) number of expanded TCR Vβ-families per patient amounted to 1 (0-2) and 2 (1-4) families for $CD4^+$ and $CD8^+$ T cells (P<0.05), respectively (**Fig. 4a**). Interestingly, expansions were also detected within the naive T cell compartment, as 3 out of 10 and 8 out of 10 patients had expansions of TCR Vβ-families within the naive $CD4^+$ and $CD8^+$ T cell compartment, respectively (**Table 3**). Most clonal expansions were observed within the (more differentiated) memory $CD8^+$ T cell subsets (**Fig. 4a**). The median (IQ range) number of expanded TCR Vβ-families amounted to 1 (1-1) versus 2 (2-4) for naive and memory $CD8^+$ T cells, respectively (P<0.05). Moreover, ESRD affected different TCR Vβ-families as illustrated in **Fig. 3** for $CD8^+$ memory T cells. ESRD induced expansions within both younger (**Fig. 4b**) as well as older (**Fig. 4c**) ESRD patients when compared to their age-matched HI.

Discussion

The main finding of this study is that the multiparameter flow-cytometry-based approach for evaluating the skewed TCR Vβ-repertoire diversity in ESRD patients

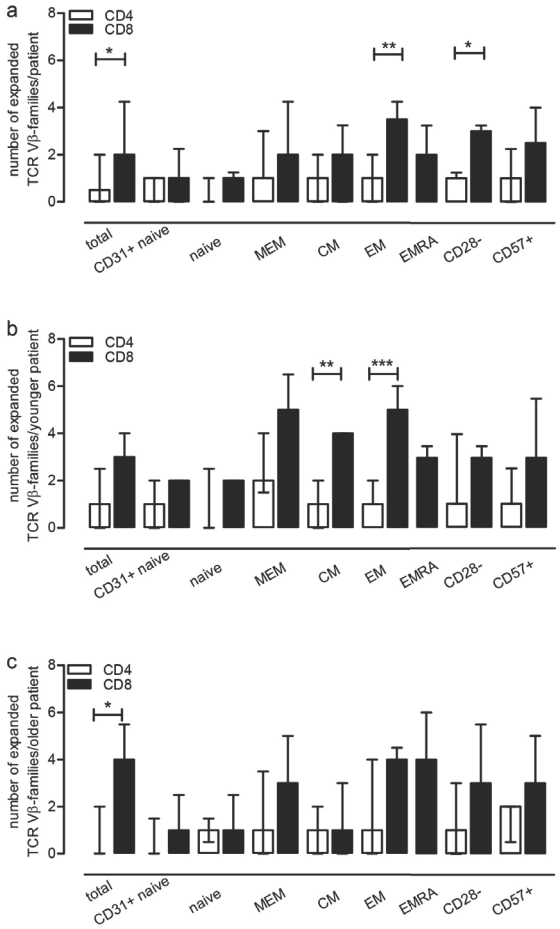

Fig. 4. ESRD effect on TCR Vβ-families within T-cell subsets. Using HI (N=10) as a reference population, we evaluated the number of TCR Vβ-families that were expanded per patient for a T-cell subset (frequency of a TCR Vβ-family exceeding the mean+2SD value obtained for HI) (a). In b and c, the median (IQ range) of expansions per young and older patient for a T-cell subset is depicted using the young (N=5) and older HI (N=5) as a reference, respectively. The open bars represent the median and IQ range for the different $CD4^+$ T-cell subsets, whereas the closed bars represent that for the different $CD8^+$ T-cell subsets.

showed that TCR skewing can be observed primarily in $CD8^+$ T-cell subsets, including naive T cells . However, higher Gini-TCR indices, indicative for an enhanced TCR Vβ-repertoire skewing, were specifically associated with more differentiated $CD8^+$, but not $CD4^+$, T-cell subsets in both ESRD patients and HI.

Our previous data showed that ESRD may lead to a skewed TCR Vβ-repertoire as assessed by DNA spectratyping, providing at best semi-quantitative information about TCR Vβ-clonality (24). The current study provided more quantitative details with respect to this skewed TCR Vβ-repertoire at the T cell subset level using the Gini-TCR index as a tool for calculating skewness (44) and evaluating number/type of expanded TCR Vβ-families. Higher Gini-TCR indices are indicative of a more skewed TCR Vβ-repertoire. The current study confirmed several of our previous findings. Increased skewing of the TCR Vβ-repertoire was observed for more differentiated $CD8^+$, but not $CD4^+$, T cells, corresponding to our spectratyping data as well as findings described by others (27, 45). In addition to the Gini-TCR index, we calculated the number of TCR Vβ-families per patient that were expanded beyond the mean+2SD values of HI. This approach yielded similar results as the Gini-index but gives detailed information at the individual patient level for the different T-cell subsets. For instance some patients have a large number of expanded TCR Vβ-families while others show only a few. Moreover, ESRD did not seem to affect one TCR Vβ-family in particular, indicative of expansions of different clonal origin. Altogether, using both Gini-TCR indices as well as the number of TCR Vβ-family expansions, revealed skewing to mainly occur within the $CD8^+$ and in particular within the $CD8^+$ memory T-cell subset similar to what was observed before using DNA-based spectratyping on sorted T-cell subsets (24).

The commercially available flow-cytometry-based assay, used to characterize the TCR Vβ-repertoire, is composed of 24 different TCR Vβ-antibodies covering about 70% of the normal human TCR Vβ-repertoire (brochure Beckman Coulter). Evaluating other TCR Vβ-families as well as TCR Vα and TCR Vγ/Vδ families may contribute to a better understanding of the whole TCR-repertoire. In this respect, $\gamma\delta^+$ T cells account for approximately 8% of $CD3^+$ peripheral blood T cells and around 6% of $\gamma\delta^+$ T cells were observed within $CD3^+CD8^+$, but not $CD4^+$ T cells in HI (19). As frequencies of $\gamma\delta^+$ T cells may also vary amongst individuals, it might be more accurate to evaluate the TCR Vβ-repertoire not within total $CD3^+$, like performed in the current study, but within $\alpha\beta^+$ $CD3^+$ T cells.

Interestingly, using this multi-parameter flow-cytometry based approach, we were also able to detect expanded TCR Vβ-families within the naive T-cell compartment. This characteristic has, to our knowledge never been described for ESRD patients. Uremia induces a pro-inflammatory environment significantly affecting T-cell mediated immunity characterized by increased risk for infections (5) and decreased vaccination efficacy (1, 46). We have observed that progressive loss of renal function is accompanied by a severe depletion of the naive T-cell compartment and a relative shift towards more differentiated memory T cells (47). Naive T cells employ a mechanism referred to as homeostatic proliferation in order to maintain the naive T-cell pool that is not replenished by newly developed naive T cells from the thymus due to thymic involution. Homeostatic proliferation occurs in response to homeostatic cytokines, e.g. IL-7, or low affinity self antigens presented by antigen-

presenting cells (48). This mechanism has been described to be associated with a decline in TCR Vβ-repertoire diversity within naive T cells with increasing age (27, 49). ESRD enhanced homeostatic proliferation of naive T cells to a similar extent as observed in older HI (12) and as a consequence of this compensatory mechanism, loss of TCR Vβ-repertoire diversity may also be induced by ESRD within naive $CD8^+$ and/or $CD4^+$ T cells.

Naive T cells are required to mount adequate immune responses to newly encountered antigens (50, 51). ESRD patients, with a severely depleted naive T-cell compartment (47), are hampered in inducing adequate protection to for example HBV vaccination as a result of defective generation of antigen-specific memory T cells (46). The ESRD-associated defects in T-cell composition as well as function, reminiscent of ageing-associated T-cell defects, led to the concept of premature T-cell ageing introduced in 2011 (12). ESRD patients have a T-cell compartment that is aged by 15-20 years compared to their chronologic age, using age-matched HI as a reference. Consistent with premature T-cell ageing (12), we observed ESRD-associated increases in Gini-TCR indices and TCR Vβ-expansions to occur already at young age.

Ageing is known to affect TCR Vβ-repertoire diversity towards a more skewed pattern, starting from roughly 600×10^3 clonotypes detected per 10^6 T cells in childhood, declining by 5×10^3 clonotypes per year (25). Age-related effects were limited within our cohort of HI and this may be a consequence of the selection procedure applied. We did select HI with a polyclonal (i.e. non-skewed) TCR Vβ-repertoire using DNA-based spectratyping(24), to ensure a relatively standard healthy population to be used as reference for comparison to ESRD patients. The ESRD patient population however only consisted of individuals with an oligoclonal (skewed) TCR Vβ-repertoire. This might have resulted in an underestimation of the effect of ageing on TCR Vβ-families. Likewise, our selection procedure may also explain the minimal effects of CMV in both cohorts.

CMV latency is known to introduce skewing of the TCR Vβ-repertoire induced by expanded CMV-specific T cell clones in both HI (33-35) and ESRD patients (24). CMV latency may result in a vast and long-lasting expansion of CMV-specific T cells (33, 34). Moreover, CMV latency has additional effects mainly on circulating $CD8^+$ T cells of ESRD patients (52).

ESRD, ageing, and CMV all influenced the TCR Vβ-repertoire diversity to a different extent (24), however, because of different factors present in the environment, they all have their specific effect on clonotype selection. Even though these findings need to be verified in a larger cohort without preselection using DNA-based spectratyping of TCR Vβ-repertoire, our study already shed some light on this altered TCR Vβ-repertoire at the T-cell subset level in particular with respect to ESRD.

Relating TCR-repertoire data to functional capacities of T cells is warranted to increase knowledge on uremia-induced T-cell defects in ESRD patients. Moreover, tracking TCR clones in whole blood or tissue infiltrates may provide additional information on antigen specificity important for diagnosis of infection and allograft rejection after transplantation (53-55).

In conclusion, ESRD is associated with a skewed TCR Vβ-repertoire as a result of variable TCR Vβ-family expansions, but not one in particular. ESRD, ageing and CMV latency exert their effects by influencing different TCR Vβ-families. This altered repertoire may be associated with a less broad and less diverse T-cell mediated immunity.

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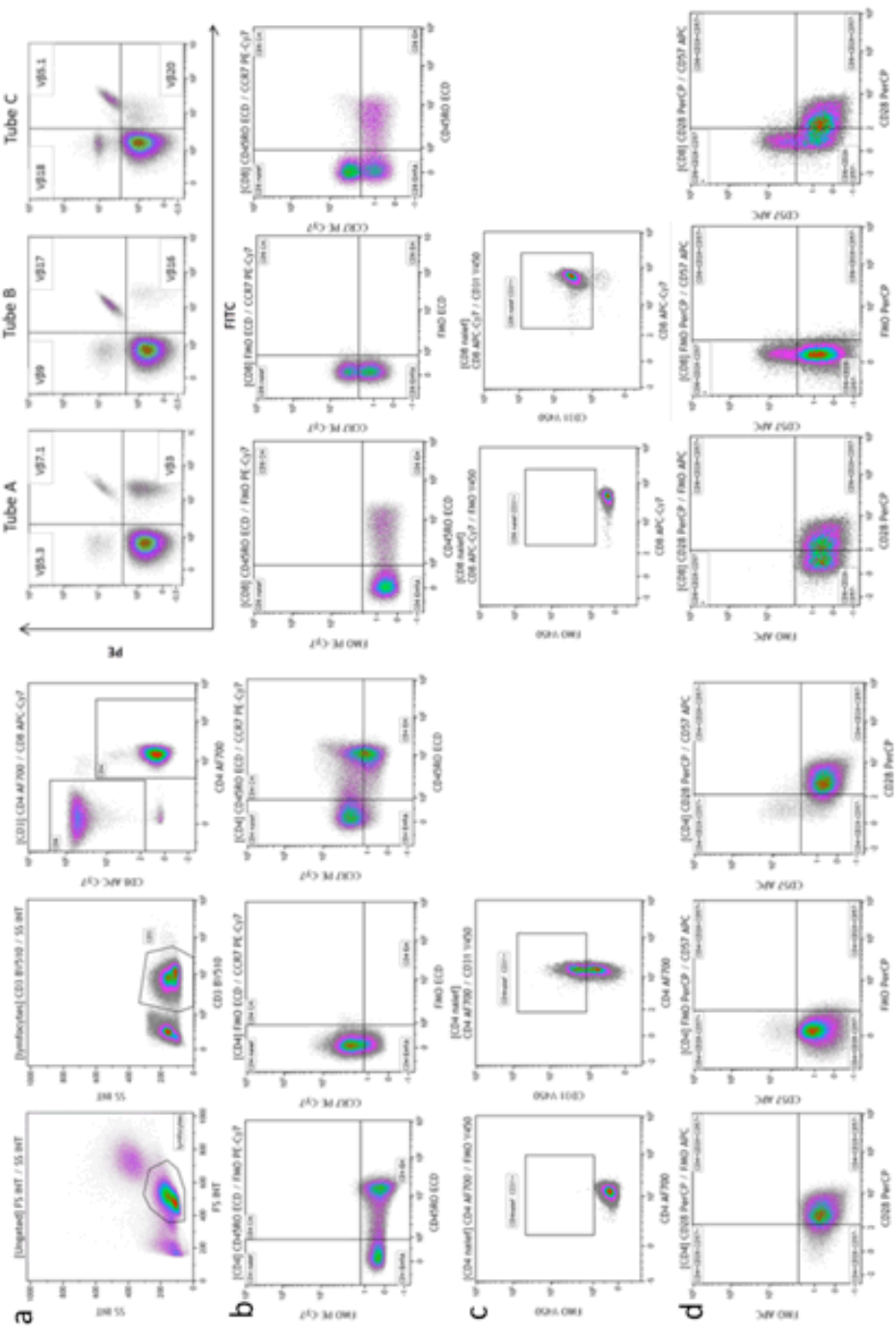
Disclosures
The authors declare that they have no competing interests.

SUPPORTING INFORMATION

Fig. S1 (right) Gating Strategy multi-parameter flow-cytometric analysis TCR Vβ-repertoire. Briefly, lymphocytes were identified based on the forward/sideward characteristics (a, first plot) followed by the selection of CD3+ T cells (a, second plot). These T cells were then dissected into CD4+ and CD8+ T cell (a, third plot). Furthermore, a typical flow-cytometric result for CD3+ T cells with respect to tube A-C, each containing 3 different Vβ-families (identified by PE^+,PE^+FITC^+ and $FITC^+$), is depicted in the last three plots of (a). A similar approach is also followed for tubes D-H and all subsets. Fluorescence minus one controls (FMOs) from the base for all subsequent gating performed to identify the different populations within CD4+ and CD8+ T cells. CCR7 and CD45RO were used to identify naïve and different memory T-cell subsets (b, first and latter 3 plots for $CD4^+$ and $CD8^+$ T cells, respectively). Loss of CD28 and gain of CD57 expression were used for identification of highly differentiated T cells (d, first and latter 3 plots for $CD4^+$ and $CD8^+$ T cells, respectively).



TCR family	%	arrange % of all 24 Vbeta families from low to high
Vb1	2,92	0,36
Vb2	7,6	0,67
Vb3	3,21	0,72
Vb4	2,04	1,07
Vb5.1	5,57	1,28
Vb5.2	0,67	1,58
Vb5.3	1,28	2,04
Vb7.1	2,59	2,06
Vb7.2	0,36	2,12
Vb8	4,61	2,38
Vb9	3,58	2,59
Vb11	5,46	2,61
Vb12	3,24	2,92
Vb13.1	2,98	2,98
Vb13.2	3,49	3,21
Vb13.6	1,58	3,24
Vb14	2,61	3,49
Vb16	1,07	3,58
Vb17	4,54	3,64
Vb18	2,38	4,54
Vb20	2,12	4,61
Vb21.3	2,06	5,46
Vb22	3,64	5,57
Vb23	0,72	7,6

% TCR Vbeta usage *	Ranked (corrected to 100%)	Cumulative	Lorentz Surface Calculation
0,36	0,51	0,51	2,13
0,67	0,95	1,46	4,12
0,72	1,02	2,49	8,24
1,07	1,52	4,01	13,54
1,28	1,82	5,83	20,50
1,58	2,25	8,08	28,97
2,04	2,90	10,98	39,70
2,06	2,93	13,91	51,85
2,12	3,01	16,92	64,23
2,38	3,38	20,31	77,56
2,59	3,68	23,99	92,29
2,61	3,71	27,70	107,69
2,92	4,15	31,85	124,08
2,98	4,24	36,09	141,56
3,21	4,56	40,66	159,89
3,24	4,61	45,26	179,00
3,49	4,96	50,23	198,94
3,58	5,09	55,32	219,89
3,64	5,18	60,49	241,28
4,54	6,46	66,95	265,51
4,61	6,56	73,51	292,62
5,46	7,76	81,27	322,45
5,57	7,92	89,19	355,13
7,6	10,81	100,00	394,15
	Sum	***70,32***	***3405,33***
	100% Correction Factor	***1,42***	***1594,67***
	Gini-TCR skewing index		***31,89***

Supplementary file

Template for calculating the Gini-TCR index using % of TCR Vb-families within $CD3^+$ T cells of patient 1

**copy/paste arranged % of 24 TCR Vbeta families*

Supplementary Table 1 Effect of age on Gini-TCR indices

	ESRD patients			HI		
	Young (N=5) median (IQ range)	Old (N=5) median (IQ range)	P-value	Young (N=5) median (IQ range)	Old (N=5) median (IQ range)	P-value
$CD3^+$	37.9 (34.6-42.8)	35.6 (33.9-42.3)	0.55	37.1 (35.8-38.8)	38.4 (35.5-40.1)	0.69
$CD4^+$	41.2 (37.0-42.9)	37.9 (34.1-40.4)	0.25	41.3 (37.1-42.2)	36.7 (35.9-42.0)	0.69
$CD31^+$ naive	38.8 (37.5-43.6)	37.8 (34.1-39.5)	0.42	39.8 (36.8-42.8)	38.4 (36.7-41.8)	0.69
naive	40.0 (37.8-43.9)	39.1 (34.7-40.0)	0.25	41.5 (37.9-43.7)	38.6 (37.7-43.3)	0.84
MEM	41.8 (37.3-45.1)	39.2 (36.3-40.7)	0.25	41.6 (38.2-42.5)	40.0 (34.8-41.9)	0.55
CM	41.0 (36.5-44.4)	35.3 (33.8-40.2)	0.15	39.6 (36.5-41.6)	39.4 (35.3-41.6)	1.00
EM	42.5 (37.9-46.9)	38.0 (35.7-42.1)	0.25	43.1 (37.9-43.7)	43.2 (34.4-44.2)	1.00
$CD28^-$	46.3 (43.3-52.6)	42.0 (40.0-43.8)	0.10	46.9 (43.4-56.2)	44.5 (43.0-48.6)	0.55
$CD57^+$	60.3 (55.1-75.5)	49.6 (40.5-57.4)	0.06	64.5 (45.6-73.6)	61.9 (34.2-68.1)	0.55
$CD8^+$	39.9 (38.8-58.3)	45.6 (40.1-57.4)	0.84	37.9 (34.2-43.1)	46.1 (40.2-53.6)	0.10
$CD31^+$ naive	36.1 (34.9-39.9)	33.6 (32.2-36.2)	0.15	36.6 (34.2-38.2)	36.2 (33.8-37.2)	0.84
naive	36.2 (34.8-40.7)	33.9 (32.0-36.7)	0.15	36.4 (33.2-37.9)	36.7 (34.1-37.1)	0.55
MEM	47.3 (45.5-67.1)	49.5(44.6-59.1)	0.69	43.6 (40.3-45.6)	48.6 (41.1-60.3)	0.42
CM	47.4 (40.8-54.2)	36.9 (35.2-46.1)	0.15	35.3 (34.0-39.2)	40.1 (39.1-44.1)	0.06
EM	54.4 (49.6-68.5)	51.5 (45.6-59.0)	0.31	43.7 (42.4-50.8)	47.3 (44.9-60.6)	0.31
EMRA	51.1 (45.9-69.4)	53.8 (40.9-73.2)	1.00	43.7 (40.4-54.2)	63.1 (46.0-70.0)	0.10
$CD28^-$	45.9 (43.7-67.3)	51.0 (45.9-68.8)	0.55	42.0 (40.6-47.4)	57.8 (43.7-63.7)	0.03
$CD57^+$	77.6 (65.8-78.4)	64.3 (60.1-75.2)	0.31	59.2 (57.2-67.1)	66.6 (54.6-81.6)	0.69

Median (IQ range) of Gini-TCR indices for the different T-cell subsets were compared between young (N=5) and old (N=5) using the non-parametric Mann-Whitney test. P-values<0.05 were considered statistically significant.

Supplementary table 2 Effect of CMV on Gini-TCR indices

	ESRD patients			HI		
	CMVneg (N=4) median (IQ range)	CMVpos (N=6) median (IQ range)	P-value	CMVneg (N=4) median (IQ range)	CMVpos (N=6) median (IQ range)	P-value
$CD3^+$	35.9 (33.9-44.0)	37.6 (34.8-41.0)	0.61	37.8 (35.8-40.6)	37.6 (35.6-38.6)	0.61
$CD4^+$	38.4 (33.4-43.6)	38.7 (37.2-41.3)	0.76	40.3 (37.0-43.8)	39.8 (36.1-41.4)	0.48
$CD31^+$ naive	38.2 (33.1-45.4)	38.3 (37.5-39.2)	0.91	41.2 (37.4-44.8)	38.8 (36.1-40.0)	0.48
naive	38.7 (33.7-45.4)	39.2 (38.0-40.2)	1.00	41.7 (38.3-45.7)	39.6 (37.3-41.7)	0.48
MEM	39.3 (35.9-43.7)	39.7 (38.0-42.4)	0.91	40.4 (37.2-43.1)	40.2 (36.3-42.0)	0.76
CM	37.8 (33.4-42.8)	38.1 (35.7-42.2)	0.61	39.0 (36.6-42.8)	39.5 (35.6-40.4)	0.61
EM	39.6 (35.1-43.1)	40.4 (37.6-44.6)	0.76	40.7 (36.1-44.9)	43.1 (37.2-43.3)	0.76
$CD28^-$	43.7 (39.6-49.8)	43.8 (41.3-48.3)	0.91	45.4 (43.3-46.7)	47.0 (43.4-53.7)	0.26
$CD57^+$	47.7 (39.1-56.6)	62.1 (50.6-75.3)	0.11	45.6 (35.5-58.0)	67.0 (57.4-72.2)	0.07
$CD8^+$	40.5 (38.5-54.2)	49.8 (39.5-58.9)	0.48	40.4 (35.4-44.9)	42.9 (37.1-53.3)	0.61
$CD31^+$ naive	34.6 (31.7-41.7)	35.4 (34.1-36.4)	0.91	35.6 (34.1-37.4)	36.5 (34.1-37.5)	0.91
naive	34.7 (31.4-41.8)	35.5 (34.0-37.9)	0.76	35.4 (33.4-36.8)	36.7 (33.7-37.7)	0.48
MEM	46.9 (43.8-61.6)	52.8 (46.3-63.6)	0.48	43.0 (41.3-47.4)	45.5 (40.5-59.6)	0.76
CM	47.6 (38.1-49.6)	40.0 (36.5-47.7)	0.61	40.7 (34.9-42.7)	37.6 (35.1-41.4)	0.91
EM	55.9 (47.7-60.2)	53.0 (46.4-63.2)	0.76	46.9 (43.7-52.0)	45.8 (43.1-60.1)	0.91
EMRA	46.3 (38.8-66.7)	59.7 (49.4-73.0)	0.35	48.3 (42.1-60.2)	51.8 (42.7-69.4)	0.76
$CD28^-$	45.9 (42.6-63.3)	58.6 (45.7-67.6)	0.35	43.0 (40.0-54.4)	47.6 (42.7-62.3)	0.48
$CD57^+$	67.4 (62.0-75.3)	71.9 (63.1-79.5)	0.76	57.7 (52.6-64.7)	67.1 (58.1-81.2)	0.17

Median (IQ range) of Gini-TCR indices for the different T-cell subsets were compared between CMV-seronegative (CMVneg; N=4) and CMV-seropositive (CMVpos; N=6) using the non-parametric Mann-Whitney test.
P-values<0.05 were considered statistically significant.

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CHAPTER 5

pERK-dependent defective TCR-mediated activation of $CD4^+$ T cells in end-stage renal disease patients

Ling. Huang, Nicolle. H. R. Litjens, Nynke. M. Kannegieter, Mariska. Klepper, Carla. C. Baan, Michiel. G. H. Betjes.

Department of Internal Medicine, Section Nephrology and Transplantation, Erasmus University Medical Center, Rotterdam, the Netherlands.

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ABSTRACT

Background

Patients with end-stage renal disease (ESRD) have an impaired immune response with a prematurely aged T-cell system. Mitogen-activated protein kinases (MAPKs) including extracellular signal-regulated kinase (ERK) and p38, regulate diverse cellular programs by transferring extracellular signals into an intracellular response. T cell receptor (TCR)-induced phosphorylation of ERK (pERK) may show an age-associated decline, which can be reversed by inhibiting dual specific phosphatase (DUSP) 6, a cytoplasmic phosphatase with substrate specificity to dephosphorylate pERK. The aim of this study was to assess whether ESRD affects TCR-mediated signaling and explore possibilities for intervening in ESRD-associated defective T-cell mediated immunity.

Results

An age-associated decline in TCR-induced pERK-levels was observed in the different $CD4^+$ ($P<0.05$), but not $CD8^+$, T-cell subsets from healthy individuals (HI). Interestingly, pERK-levels of $CD4^+$ T-cell subsets from young ESRD patients were in between young and elderly HI. A differentiation-associated decline in TCR-induced ERK and p38 phosphorylation was observed in T cells, although TCR-induced p38 phosphorylation was not significantly affected by age and/or ESRD. Frequencies of TCR-induced CD69-expressing $CD4^+$ T cells declined with age and were positively associated with pERK. In addition, an age-associated tendency of increased expression of DUSP6 was observed in $CD4^+$ T cells of HI and DUSP6 expression in young ESRD patients was similar to old HI. Inhibition of DUSP6 significantly increased TCR-induced pERK-levels of $CD4^+$ T cells in young and elderly ESRD patients, and elderly HI.

Conclusions

TCR-mediated phosphorylation of ERK is affected in young ESRD patients consistent with the concept of premature immunological T cell ageing. Inhibition of DUSP6 specific for pERK might be a potential intervention enhancing T-cell mediated immunity in ESRD patients.

BACKGROUND

ESRD patients have a defective T-cell mediated immune system that is clinically characterized by an increased risk of a variety of infections (1, 2) and impaired response of vaccination (3-7). Infections are the second leading cause of mortality following cardiovascular disease and a major cause of morbidity in ESRD patients(8).

Uremia-associated T-cell defects closely resemble premature immunological T-cell ageing (9). ESRD patients have a discrepancy of 15-20 years between the immunological T-cell age and their chronological age (10). Declined thymic output, more differentiated memory T cells, T cells lacking co-stimulatory molecules like CD28, skewed T cell receptor (TCR) Vβ repertoire diversity and shorter telomere length are observed in ESRD patients compared to age-matched healthy individuals (HI) (11).

TCR-induced signaling mediates clonal (positive or negative) selection of thymocytes in the thymus and initiates T cell immune responses in the periphery, consisting of T cell proliferation and differentiation (12). The mitogen-activated protein kinase (MAPK) pathway is one of the major pathways induced upon TCR stimulation (13). Activation of MAPK is mediated by phosphorylation of MAPK and downregulated by MAPK phosphatase resulting in inactive MAPK (14). In particular, the extracellular signal-regulated kinase (ERK) pathway is one of the important MAPK pathways. Phosphorylation of ERK can reduce sensitivity of cells to apoptosis and promote cell proliferation (15). ERK activity controls the positive feedback loop in the TCR-induced activation cascade and reduced ERK activity affects signal strength and activation of T cells (16, 17). Reduced phosphorylation of ERK is associated with decreased interleukin-2 (IL-2) production (18) and vice versa (19). Dual specific phosphatases (DUSPs) represents a family of phosphatases that dephosphorylate phosphor-threonine and phosphor-tyrosine residues on MAPK and that are pivotal regulators of MAPK activities. DUSP6 is a cytoplasmic phosphatase with substrate specificity to dephosphorylate pERK (20). Ageing is also associated with defective signaling pathways (21, 22). Recently it was shown that decreased phosphorylation of ERK in naive CD4+ T cells from elderly HI was associated with more time to build up the required signaling strength following stimulation compared to those from young HI. This decreased phosphorylation of ERK can be overcome by inhibiting DUSP6 (16).

P38 is another pivotal protein in the MAPK pathway (23) and of interest with respect to age-related changes is T cell activation. Most stimuli, including engagement of TCR, costimulatory receptors, inflammation, stress, growth factors, as well as DNA damage induce phosphorylation of p38 by various pathways (24, 25). Although phosphorylation of ERK and p38 from T cells share some upstream molecules after triggering of TCR, such as phosphorylation of CD3 zeta-chain associated protein

kinase of 70 kDa (ZAP70) (26), they each have their unique upstream MAPK kinases (MKKs) (14). Highly differentiated $CD4^+$ T cells lacking expression of CD28 are accumulated in elderly healthy individuals (27), patients with ESRD (28), following chronic viral infection (29), and also in patients suffering from autoimmune disease (30). Senescent $CD27^-CD28^-CD4^+$ T cells employ an MKK-independent mechanism for phosphorylating p38 and depend on 5' adenosine monophosphate-activated protein kinase (AMPK) and transforming growth factor-β-activated protein kinase 1(TAK1)-binding protein 1(TAB1) *ex vivo* (31).

Little is known as to how MAPK signaling pathways in ESRD patients. Understanding MAPK signaling in ESRD patients may increase knowledge about mechanisms of uremia-associated impaired T-cell mediated immunity and offer possibilities for intervention. Here, we demonstrate that TCR-induced phosphorylation of ERK, and not p38, in $CD4^+$ T cells decreases with age and T cell differentiation. This pathway is specifically affected in young ESRD patients and at the level of elderly healthy individuals, compatible with the concept of premature immunological T cell ageing in patients with renal failure. In addition, inhibition of DUSP6 may offer a potential intervention for improving T-cell mediated immunity in ESRD patients.

METHODS

Study population

In line with our previous studies, young and elderly patients groups were defined based on their chronological age (32, 33). Twenty-four stable ESRD patients, defined as having a glomerular filtration rate of ≤15 ml/min with or without renal replacement therapy (RRT; i.e. dialysis) and 24 HI were included (Study population characteristics are described in Table 1) at the outpatient clinic. Patients with any clinical or laboratory evidence of acute bacterial or viral infection, malignancy, immunosuppressive drug treatment within 28 days prior to transplantation (except for glucocorticoids) were excluded. Lithium-heparinized blood was drawn of ESRD patients and healthy kidney donors. All individuals included gave informed consent and the local medical ethical committee approved the study (METC number: 2012–022). It was conducted according to the principles of Declaration of Helsinki and in compliance with International Conference on Harmonization/Good Clinical Practice regulations.

PBMCs preparation

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood from HI and ESRD patients as described previously (34) and then cryopreserved for further analysis.

Phosphorylation-specific flow cytometry

PBMCs were stained with eFluor-450-labeled anti-CD7 (eBioscience, Vienna, Austria), allophycocyanin-Cy7 (APC-Cy7)-labeled anti-CD8 (BD, Erembodegem,

Table 1 Clinical characteristics of the study population

	HI	ESRD patients	P value
Number of individuals	24	24	
Age groups (years; mean ± SD)			ns
young	29,4±5,6	34,6±8,0	
elderly	70,5±5,8	70,8±4,2	
Sex (% male)	50	79,2	ns
CMV IgG serostatus (% pos)	62,5	62,5	ns
RRT (number;%)		11; (45,8%)	
Duration of RRT in months (median with range)		22 (1—37)	
Hemodialysis (number)		7	
Peritoneal dialysis (number)		4	
Underlying kidney disease (number ; %)			
atherosclerosis/hypertensive nephropathy		8; (33%)	
primary glomerulopathy		4; (17%)	
Diabetic nephropathy		6; (25%)	
congenital disorder		3; (13%)	
others		2; (8%)	
unknown		1; (4%)	

Belgium), Brilliant Violet (BV)-510-labeled anti-CD16 (BD) and fluorescein isothiocyanate (FITC)-labeled anti-CCR7 (R&D system, Uithoorn, the Netherlands) for 30 min at room temperature. Then 1 million PBMCs/50µl were prepared for stimulation by labeling cells with 20 µg/ml mouse anti-human CD3 (BD) and mouse anti-human CD28 (BD) each on ice for 20 min, followed by an incubation with goat-anti mouse IgG (BD) for cross-linking on ice for 20 min. Stimulation was initiated by transferring cells to a 37 °C water bath for 10 minutes. Cells were fixed using Cytofix (BD) at 37 °C for 10 minutes and then permeabilized in 70% methanol at -20°C for 30 min. Subsequently, cells were stained with peridinin chlorophyll (PerCP)-labeled anti-CD4 (BD), phycoerythrin (PE)-Cy7-labeled anti-CD45RO (BioLegend, Uithoorn, Netherlands), PE-labeled anti-phospho-p38MAPK (pT180/pY182) (BD), and Alexa Fluor (AF) 647-labeled anti-phospho-ERK1/2 (pThr202/pTyr204) (BD). Phosphorylation was measured on a BD FACSCanto II flow cytometer (BD) and data were analyzed by Kaluza™ software (Beckman Coulter, Woerden, Netherlands). Median fluorescence intensity (MFI) of phosphorylated ERK or p38, generated by Kaluza™, were multiplied by 256 to make them comparable to the data analyzed by FACS Diva software (linear value instead of log-transformed value) (BD). The MFI obtained for anti-CD3/anti-CD28-stimulation were corrected by subtracting the MFI of the unstimulated condition.

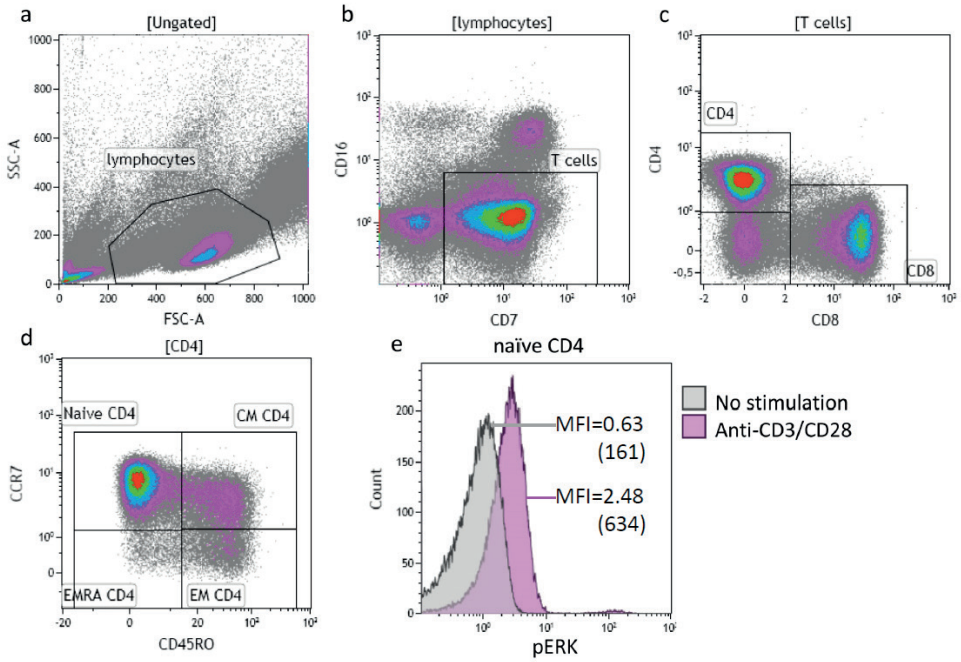

Fig. 1 Typical example of the gating strategy for analysis of phosphorylation of ERK (pERK) in naive $CD4^+$ T cell subsets. Briefly, (**a**) lymphocytes were identified based on the forward/sideward characteristics followed by (**b**) the selection of $CD7^+$ $CD16^-$ T cells. **c** These T cells were then dissected into $CD4^+$ and $CD8^+$ T cells. **d** CCR7 and CD45RO were used to identify naive and different memory subsets within $CD4^+$ T cells. Furthermore, (**e**) pERK was measured in naive $CD4^+$ T cells without and with CD3/CD28 stimulation, and median fluorescence intensities (MFI) were shown (values multiplied by 256 in brackets). A similar gating strategy was employed for phosphorylation of ERK and p38 in all T cells subsets

CD69 and IL-2 measurement

PBMCs were either not or stimulated with anti-CD3/anti-CD28 T-cell expander beads (Invitrogen Dynal, Oslo, Norway) at different ratios, i.e. 1 cell / 0.1 bead, 1 cell / 0.5 bead, 1 cell / 1 bead for 6 hours in human culture medium (HCM; RPMI-1640 with GlutaMAX, 10% heat-inactivated pooled human serum and 1% penicillin and streptomycin) (Lonza, Breda, Netherlands) with Golgistop (BD). Then cells were stained with AmCyan-labeled anti-CD3 (BD), Pacific Blue-labeled anti-CD4 (BD), APC-Cyanin 7 (APC-Cy7)-labeled anti-CD8 (BD), APC-labeled anti-CD45RO (BD) and PE-Cy7-labeled anti-CCR7 (R&D Systems) antibodies and a live–dead marker ViaProbe (7-aminoactinomycin D; 7AAD; BD). Upon fixation with FACS lysing

solution (BD) and permeabilization using FACS permeabilizing solution 2 (BD), cells were stained intracellular using PE-labeled anti-CD69 (BD) and FITC-labeled anti-IL-2 (BD). Percentages CD69-expressing and IL-2 producing CD4+ T cell subsets were evaluated upon measuring the samples on a BD FACSCanto II flow cytometer (BD). Data were analyzed by Kaluza™ software (Beckman Coulter).

DUSP6/1 inhibition

PBMCs were pre-incubated in HCM including 50 μM (E)-2-benzylidene-3-(cyclohexylamino)-2, 3-dihydro-1 H-inden-1-one (BCI) (Merck – Millipore, Amsterdam, Netherlands) at 37°C for 1h. BCI has been shown to be an inhibitor of DUSP6 and DUSP1 activity. PBMCs were subsequently washed 3 times and then stimulated by CD3/CD28 antibodies (as described previously) for 10 mins and then MFI of pERK of BCI-pretreated T cells was measured by phosphorylation-specific flow cytometry as described previously.

DUSP6/1 measurement

PBMCs were stained with AmCyan-labeled anti-CD3 (BD), Pacific Blue-labeled anti-CD4 (BD), APC-Cy7-labeled anti-CD8 (Biolegend); APC-labeled anti-CD45RO (BD) and PE-Cy7-labeled anti-CCR7 (R&D Systems) antibodies and 7-AAD for 30 min at 4 °C. Upon fixation and permeabilization using Fix/Perm buffer (eBioscience), 1% bovine serum albumin (Zwijndrecht, Netherlands) was used to block Fc receptors. Then cells were further stained with AF647-labeled anti-DUSP6 (Santa Cruz Biotechnology, Heidelberg, Germany) and PE-labeled anti-DUSP1 (Santa Cruz Biotechnology) for 30 min at 4 °C. MFI of DUSP6 and DUSP1 was measured on a BD FACSCanto II flow cytometer (BD) and data were analyzed using FACS Diva software version 6.1.2 (BD).

Statistical analyses

Data were analyzed by Graphpad Prism 6 (GraphPad Software, CA, USA). Comparison between two groups (non-parametric data) were using Mann Whitney test. Comparison in multiple groups were using Friedman test followed by Dunn's Multiple Comparison T test or repeated ANOVA test followed by Bonferroni's multiple comparison test. Comparison between DUSP6-treated and non-treated conditions was done by Paired T- test. All reported *P*-values are two-sided and were considered statistically significant when $P<0.05$.

RESULTS

Study population

The demographic and clinical characteristics of the study population are given in Table 1. Twelve patients were within the young group (age 22-44 years) and 12 patients belonged to the elderly group (age 66-78 years). Age- and cytomegalovirus (CMV)-matched HI, i.e. 13 young (age 21- 40 years) and 11 elderly (age 65- 74 years) HI were included for comparison. Approximately half of the ESRD patients received RRT (dialysis) with a median dialysis time of 22 months.

Decreased ERK phosphorylation in young ESRD patients

A typical flow cytometric example for analysis of ERK phosphorylation is given in Fig. 1. First, we compared phosphorylation of ERK and p38 between young and elderly HI or young and elderly ESRD patients. A significant age-related lower TCR-mediated phosphorylation of ERK was observed within all $CD4^+$ T cell subsets from HI (Fig. 2a-d). For example, the median of MFI value of $CD4^+$ phosphorylated ERK (pERK) was 658 in young HI, which was significantly higher than 535 in elderly HI (p=0.015) (Fig. 2a). This trend consistently existed between young and elderly HI when we compared MFI value of pERK in $CD4^+$ naive (median 722 vs. 612, p=0.022) (Fig. 2b), CM (median 666 vs. 489, p=0.021) (Fig. 2c) and EM subsets (517 vs. 364, p=0.018) (Fig. 2d). Due to the almost absent EMRA subset within the $CD4^+$ T cells, phosphorylation of ERK and p38 within this subset was not evaluated.

However, no significant differences in expression levels of pERK in total $CD4^+$ T cells or the subsets were found comparing young and elderly ESRD patients (Fig. 2a-c & d). For example, the median (interquartile range) MFI value of $CD4^+$ pERK in young patients was 613 (490- 664) and 541 (413- 801) in elderly patients (p=0.51). The median MFI value for $CD4^+$ pERK in young patients was in between the MFI values of young HI [658 (485- 1212)] and elderly HI [535 (305- 620)] but did not significantly differ from either group (respectively p=0.13 and p=0.06). This age-associated decline in pERK was not found for $CD8^+$ T cell subsets (Fig. 2e-h & i). In addition, an age-related decline in TCR-mediated phosphorylation of p38, was absent in $CD4^+$ T cells (Fig. 3a-c & d) as well as $CD8^+$ T cells (Fig. 3e-h & i) of both HI and ESRD patients. In conclusion, ESRD patients have a defective ERK, but not p38, phosphorylation in $CD4^+$ T cells, that is independent of age and at a level similar to aged HI.

Phosphorylation of ERK is associated with T-cell differentiation status

Next, we compared phosphorylation of ERK and p38 within different T cell subsets to assess whether differentiation-associated effects exist in the study groups. In all groups, a gradual decrease in TCR-induced phosphorylation capacity was seen with increasing $CD4^+$ T cell differentiation. Phosphorylation of ERK was highest in naive $CD4^+$ T cells of young HI, followed by that in the CM and EM subsets of the memory compartment (Fig. 4a). Median MFI dropped from 722 to 666 and 517 in the naive,

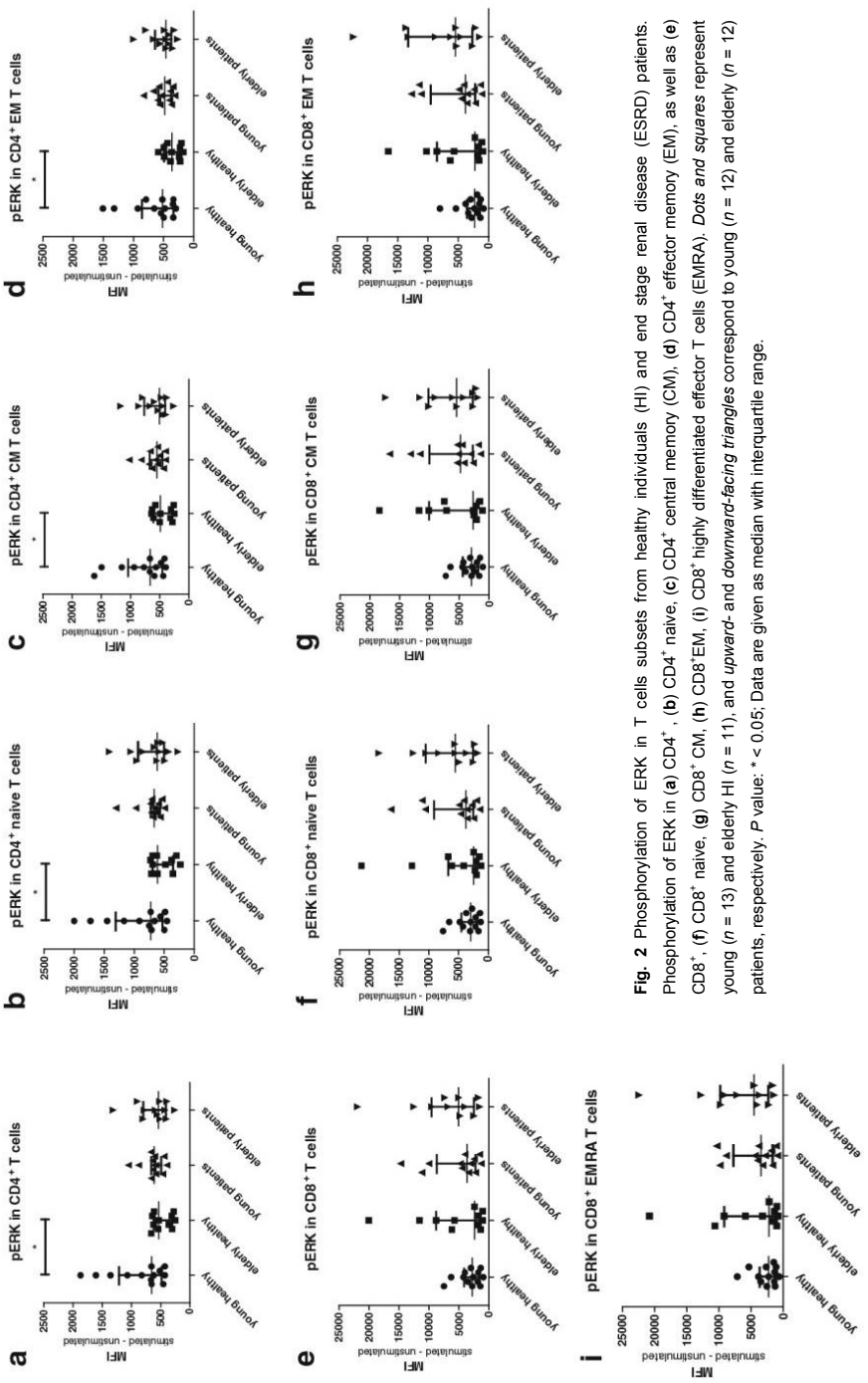

Fig. 2 Phosphorylation of ERK in T cells subsets from healthy individuals (HI) and end stage renal disease (ESRD) patients. Phosphorylation of ERK in (**a**) $CD4^+$, (**b**) $CD4^+$ naive, (**c**) $CD4^+$ central memory (CM), (**d**) $CD4^+$ effector memory (EM), as well as (**e**) $CD8^+$, (**f**) $CD8^+$ naive, (**g**) $CD8^+$ CM, (**h**) $CD8^+$EM, (**i**) $CD8^+$ highly differentiated effector T cells (EMRA). *Dots and squares* represent young (n = 13) and elderly HI (n = 11), and *upward-* and *downward-facing triangles* correspond to young (n = 12) and elderly (n = 12) patients, respectively. *P* value: * < 0.05; Data are given as median with interquartile range.

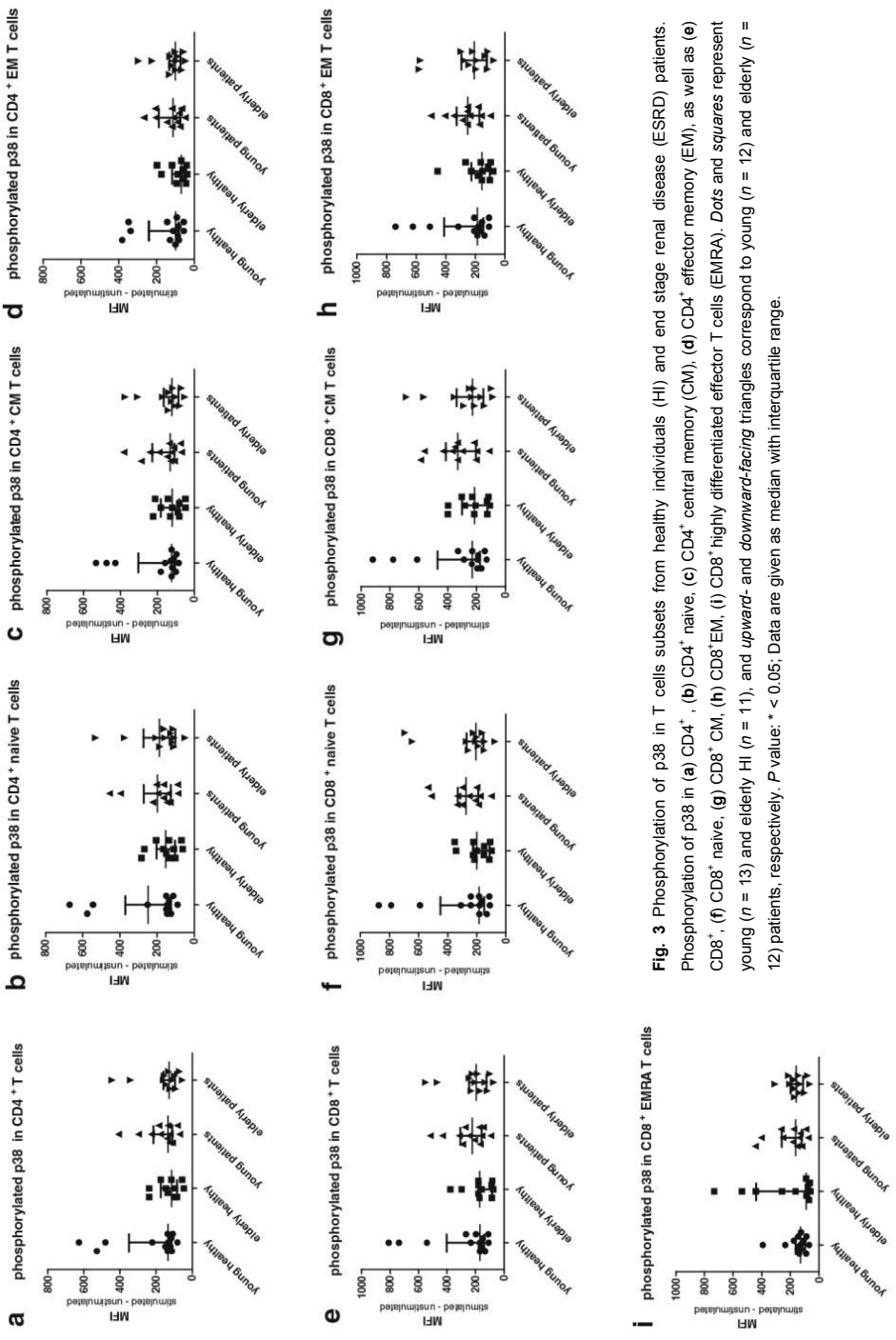

Fig. 3 Phosphorylation of p38 in T cells subsets from healthy individuals (HI) and end stage renal disease (ESRD) patients. Phosphorylation of p38 in (**a**) $CD4^+$, (**b**) $CD4^+$ naive, (**c**) $CD4^+$ central memory (CM), (**d**) $CD4^+$ effector memory (EM), as well as (**e**) $CD8^+$, (**f**) $CD8^+$ naive, (**g**) $CD8^+$ CM, (**h**) $CD8^+$EM, (**i**) $CD8^+$highly differentiated effector T cells (EMRA). *Dots* and *squares* represent young (n = 13) and elderly HI (n = 11), and *upward-* and *downward-facing* triangles correspond to young (n = 12) and elderly (n = 12) patients, respectively. *P* value: * < 0.05; Data are given as median with interquartile range.

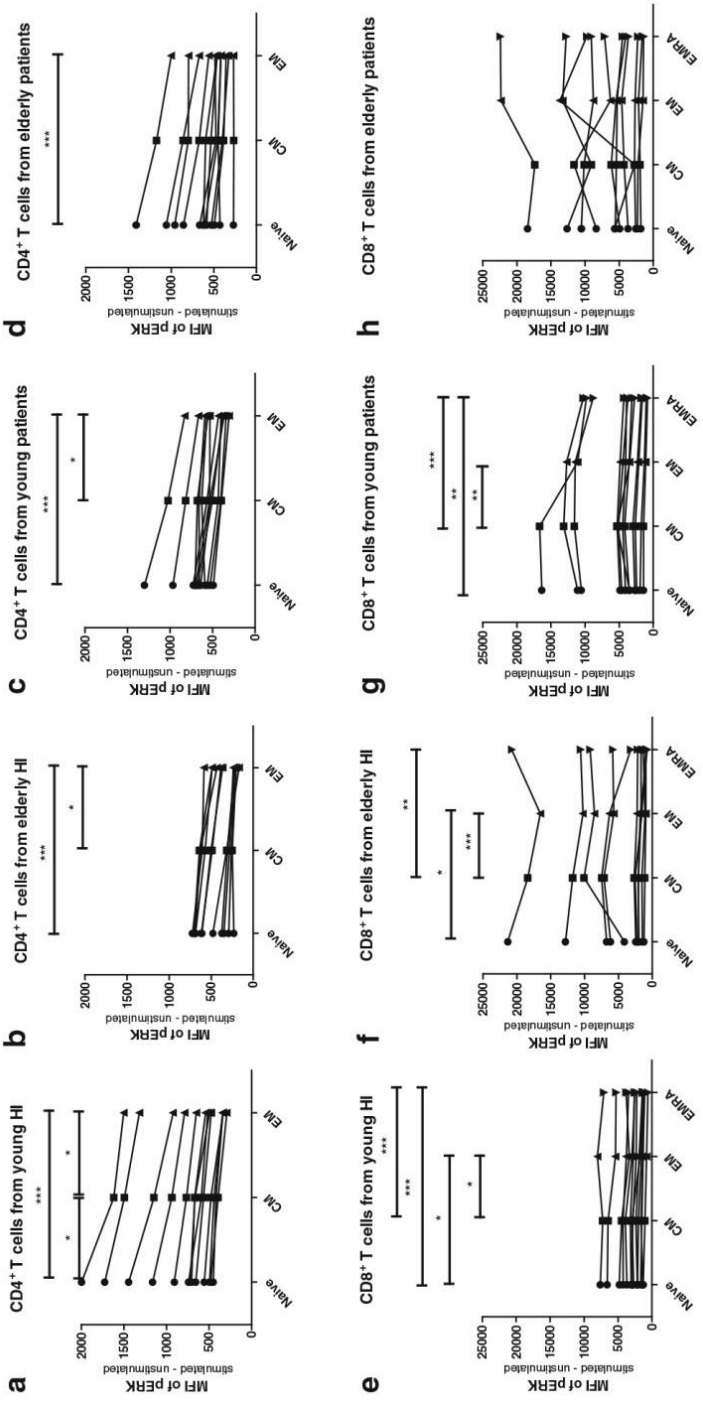

Fig. 4 Phosphorylation of ERK according to T cell differentiation status in healthy individuals (HI) and end stage renal disease (ESRD) patients. Phosphorylation of ERK in $CD4^+$ T cells from (**a**) young HI ($n = 13$), (**b**) elderly HI ($n = 11$), (**c**) young patients ($n = 12$), and (**d**) elderly patients ($n = 12$), as well as $CD8^+$ T cells from (**e**) young HI, (**f**) elderly HI, (**g**) young patients, and (**h**) elderly patients. *Dots, squares, upward- and downward-facing triangles* represent naive, central memory (CM), effector memory (EM) and highly differentiated effector T cells (EMRA) T cells, respectively. *P* value: *<0.05; ** < 0.01; *** < 0.001; Data are given as median with interquartile range.

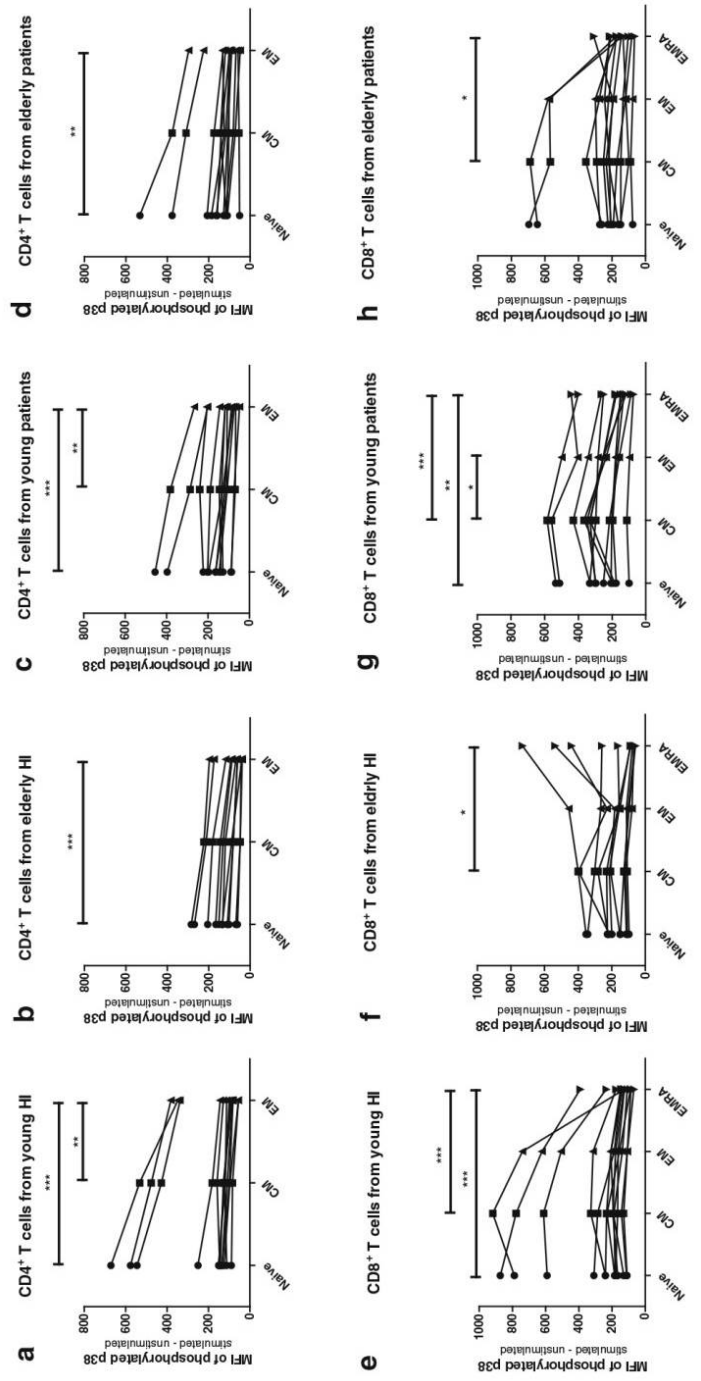

Fig. 5 Phosphorylation of p38 according to T cell differentiation status in healthy individuals (HI) and end stage renal disease (ESRD) patients. Phosphorylation of ERK in $CD4^+$ T cells from (**a**) young HI (n = 13), (**b**) elderly HI (n = 11), (**c**) young patients (n = 12), and (**d**) elderly patients (n = 12), as well as $CD8^+$ T cells from (**e**) young HI, (**f**) elderly HI, (**g**) young patients, and (**h**) elderly patients. *Dots, squares, upward- and downward-facing triangles* represent naive, central memory (CM), effector memory (EM) and highly differentiated effector T cells (EMRA) T cells, respectively. *P* value: *<0.05; ** < 0.01; *** < 0.001; Data are given as median with interquartile range.

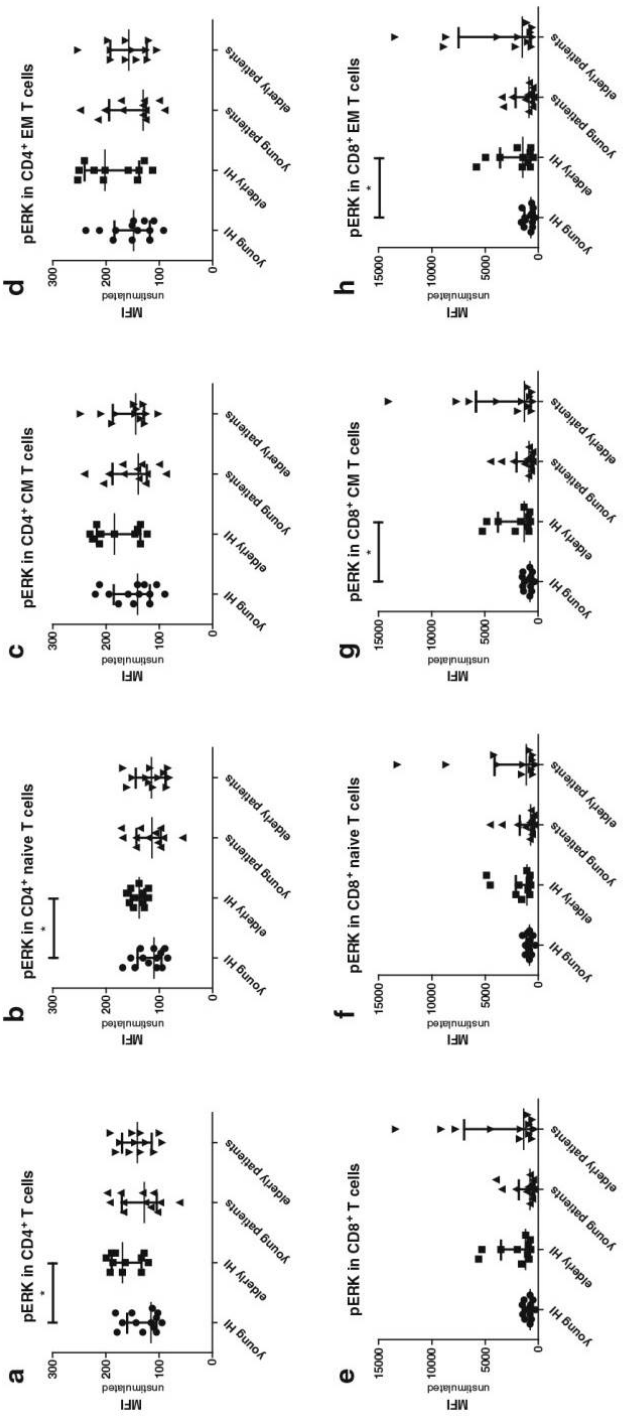

Fig. 6 Auto-phosphorylation (baseline) of ERK in T cells subsets from healthy individuals (HI) and end stage renal disease (ESRD) patients. Phosphorylation of ERK in (**a**) $CD4^+$, (**b**) $CD4^+$ naive, (**c**) $CD4^+$ central memory (CM), (**d**) $CD4^+$ effector memory (EM), as well as (**e**) $CD8^+$, (**f**) $CD8^+$ naive, (**g**) $CD8^+$ CM, (**h**) $CD8^+$EM, (**i**) $CD8^+$ highly differentiated effector T cells (EMRA). *Dots* and *squares* represent young (n = 13) and elderly HI (n = 11), and upward- and downward-facing triangles correspond to young (n = 12) and elderly patients (n = 12), respectively. P value: * < 0.05; Data are given as median with interquartile range.

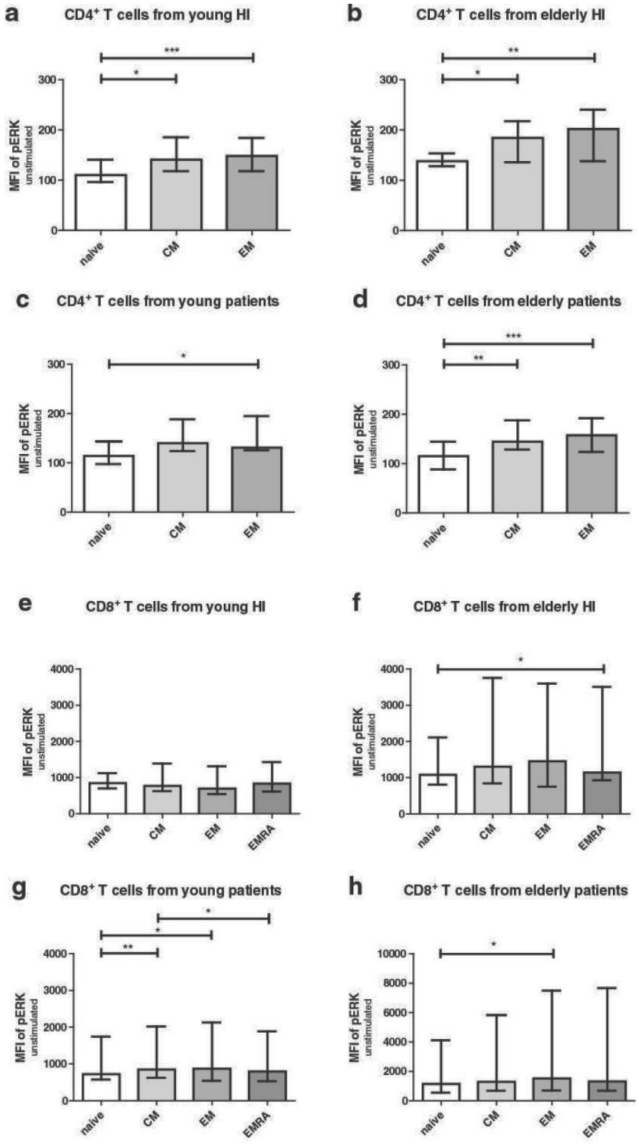

Fig. 7 Auto-phosphorylation (baseline) of ERK according to T cell differentiation status in healthy individuals (HI) and end stage renal disease (ESRD) patients. Phosphorylation of ERK in $CD4^+$ T cells from (**a**) young HI (*n* = 13), (**b**) elderly HI (*n* = 11), (**c**) young patients (*n* = 12), and (**d**) elderly patients (*n* = 12), as well as $CD8^+$ T cells from (**e**) young HI, (**f**) elderly HI, (**g**) young patients, and (**h**) elderly patients. *Blank bars* and *bars with light grey to dark grey* represent naive, central memory (CM), effector memory (EM) and highly differentiated effector T cells (EMRA) T cells, respectively. *P* value: *<0.05; ** < 0.01; *** < 0.001; Data are given as median with interquartile range.

CM and EM T cell subset, respectively. Interestingly, in elderly HI as well as both groups of ESRD patients (Fig. 4b, c & d), pERK levels were still highest within naive CD4+ T cells compared to the more differentiated EM T cell subset, but the difference with that observed within CM T cells disappeared. ERK phosphorylation within CM is higher than that within the EM compartment in young and elderly HI (Fig. 4a & b), as well as in young patients (Fig. 4c), but not in elderly patients (Fig. 4d). Differences for the various CD8+ T-cell subsets with respect to TCR-mediated phosphorylation of ERK between naive and CM compartment, or between EM and EMRA were less outspoken and not significantly different in HI (Fig. 4e & f) and patients (Fig. 4g & h). Similar to ERK, phosphorylation of p38 showed a similar trend to decrease with increasing differentiation status but no significant decline in phosphorylation of p38 from naive to CM in CD4+ in HI and patients (Fig. 5a-c & d). In CD8+ T cells, p38 phosphorylation was decreased in highly differentiated EMRA compared to CM in HI and patients (Fig. 5e-g & h). In conclusion, a differentiation-associated decrease in anti-CD3/CD28-induced phosphorylation of ERK and p38 in T cells was present in HI and patients.

Auto-phosphorylation (baseline) of ERK was associated with ageing in HI and increased according to differentiation status in CD4+ T cells

Baseline phosphorylation of ERK (i.e. auto-phosphorylation) was significantly lower for total and naive CD4+T cells in young HI compared with elderly HI, however, this trend was not observed in patients (Fig. 6a & b). Baseline levels of pERK were significantly lower in the CD8+ memory compartment (CM, EM and EMRA) when comparing young to elderly HI, but no age-related differences were observed for patients (Fig. 6g, h & i). Baseline phosphorylation of ERK was lower in the naive CD4+ T cells when compared to CM and/or EM in HI (Fig. 7 a & b) and patients (Fig. 7c & d). Naive CD8+ T cells also had lower baseline pERK when compared to EMRA in elderly HI (Fig. 7f), CM and EM in young patients (Fig. 7g), and EM in elderly patients (Fig. 7h). Naive CD4+ T cells had lower baseline phosphorylated p38 compared to CM or EM in elderly HI (Additional file 1: Fig. S1b), and lower p38 phosphorylation compared to EM in young patients (Additional file 1: Fig. S1c). In CD8+ T cells, p38 auto-phosphorylation in naive compartment was significantly lower than that of EMRA in young and elderly HI (Additional file 1: Fig. S1e & f), but this trend was not observed in the patient population (Additional file 1: Fig. S1g & h). To summarize, an age- as well as differentiation-related increase in baseline levels of pERK was observed for CD4+ T cells.

Neither CMV-serostatus nor RRT significantly influenced ERK or p38 phosphorylation

CMV-seropositivity may promote immunological T-cell ageing and was therefore analyzed for its association with MAPK pathway activation. However, ERK or p38 phosphorylation within T cells showed no significant difference comparing CMV-seropositive individuals to their CMV- seronegative counterparts in healthy individuals and ESRD patients respectively (Additional file 1: Fig. S2 & S3). Moreover, in ESRD

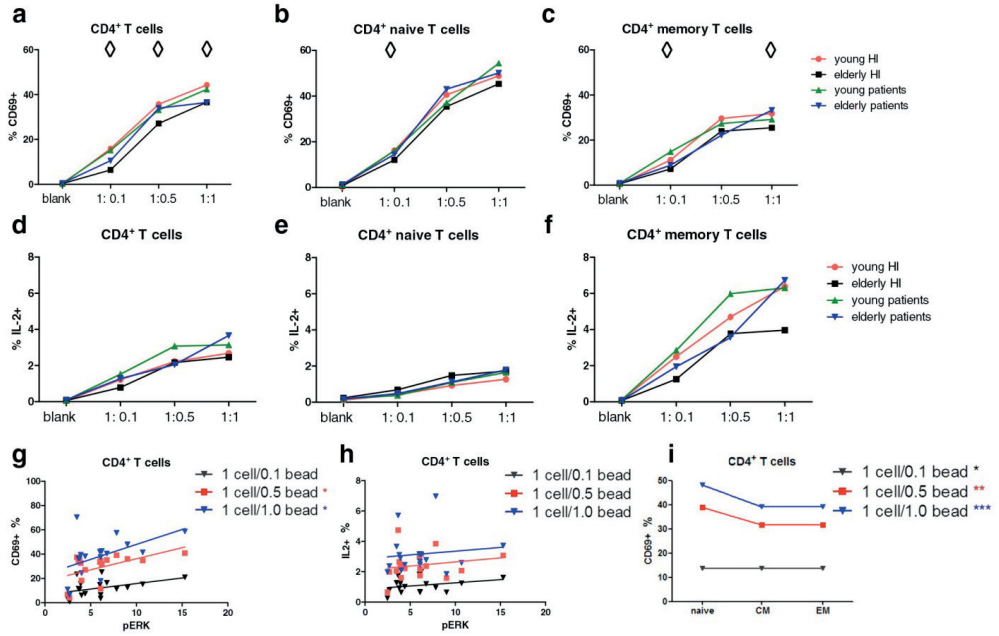

Fig. 8 Percentages of CD69-expressing and IL-2 producing CD4⁺ T cell subsets in healthy individuals (HI) and end-stage renal disease (ESRD) patients. Percentages of CD69⁺ CD4⁺T cells were shown from healthy individuals (HI) (young *n* = 4, elderly *n* = 5) and end-stage renal disease (ESRD) patients (young *n* = 4, elderly *n* = 5) for (**a**) total, (**b**) naive, (**c**) memory CD4⁺ T cells, and frequencies of IL-2 expression were given for (**d**) total, (**e**) naive, (**f**) memory CD4⁺ T cells. *Red dots* and *orange squares* represent young and elderly HI, and *blue upward-* and *purple downward-facing triangles* correspond to young and elderly patients, respectively. The association between (**g**) percentages of CD69⁺ or (**h**) IL-2⁺ CD4⁺ T cells and ERK phosphorylation (depicted as fold increase by dividing the MFI of stimulated cells by that of unstimulated cells) is depicted. (**i**) The differentiation-associated relation between percentages of CD69⁺ and different CD4⁺ T cell subsets (naive, CM, EM) is shown. Black and blue downward-facing triangles represent 1 cell/0.1 bead and 1 cell/1 bead stimulation, and red squares correspond to 1cell/0.5 bead stimulation. ◊ represents a significant difference in the percentage of CD69⁺ T cells calculated for each specific CD4⁺ T cell subset when comparing young with elderly HI, or when comparing young with elderly patients. * represents a significant difference in the percentage of CD69⁺ T cells comparing CD4⁺ subsets or CD8⁺ subsets within each study group. *P* value: ◊ <0.05;* < 0.05; **< 0.01; ***<0.001. Data are given as medians.

patients, ERK or p38 phosphorylation was not significantly different between the patients receiving RRT (i.e. dialysis) and those without RRT (Additional file 1: Fig. S4 & S5). In conclusion, neither CMV-serostatus nor RRT significantly influenced TCR-stimulation induced phosphorylation of ERK or p38.

Age-related decline in anti-CD3/CD28-induced CD69-expressing CD4+ T cells positively associated with phosphorylation of ERK

A typical flow cytometric example for analysis of frequencies of CD69-expressing and IL-2 producing CD4+ T cells after CD3/CD28 stimulation was given in Additional file 1: Fig. S6. Higher frequencies of CD69-expressing CD4+ T cells were observed in young HI compared to that in elderly HI when stimulated with different ratios of anti-CD3/CD28 beads to cells (Fig. 8a). This age-related decline in CD69-expressing cells was observed for naive CD4+ T cells in the healthy population at a ratio of 1:0.1 (Fig. 8b) and at 1:0.1 and 1:1 ratios for memory CD4+ T cells (Fig. 8c). Percentages of IL-2 producing CD4+ T cells did not reveal an age-associated decline in our study population (Fig. 8d, e & f). Percentages of CD69-expressing CD4+ T cells following stimulation with anti-CD3/CD28 beads (at ratios 1:0.5 and 1:1) were associated with ERK phosphorylation, presented as the fold increase in MFI dividing the MFI from stimulated samples by that of unstimulated samples (Fig. 8g). Percentages of IL-2 producing CD4+ T cells were not associated with pERK (Fig. 8h). In addition, frequencies of CD69-expressing CD4+ T cells were significantly higher in the naive subset compared to CM or EM (Fig. 8i). In short, an age-related decline in anti-CD3/CD28-induced percentages of CD69-expressing CD4+ T cells was observed and percentages of CD69-expressing CD4+ T cells were positively associated with phosphorylation of ERK.

BCI promoted TCR-mediated phosphorylation of ERK

BCI is known to inhibit DUSP6 but also decrease levels of DUSP1 (product document, Merck – Millipore). BCI did not significantly enhance phosphorylation of ERK in young HI; in contrast, in elderly HI and both young and elderly ESRD patients, the pERK level was significantly upregulated in all CD4+ T cell subsets pretreated with BCI compared to that without (Fig. 9a-c & d). The average fold increase in pERK levels in naive CD4+ T cell subsets were 3.8, 2.5, and 2.1 in elderly HI, young and elderly ESRD patients, respectively. This was not observed for CD8+ T cells (Additional file 1: Fig. S7). In short, BCI promoted TCR-mediated phosphorylation of ERK in CD4+ T cells of both elderly HI as well as young and old ESRD patients.

DUSP6 and DUSP1 expression in CD4+ T cells

In an attempt to unravel whether effects of BCI on pERK levels were mediated by interfering with DUSP6 and/or DUSP1, we measured DUSP6 and DUSP1 levels within CD4+ T cells in a small fraction of our study cohort. Due to limited availability of materials only 10 HI (4 young and 6 elderly) and 9 ESRD patients (4 young and 5 elderly) could be included. A typical flow cytometric example for analysis of DUSP6 and DUSP1 is given in Additional file 1: Fig. S8. An age-associated trend

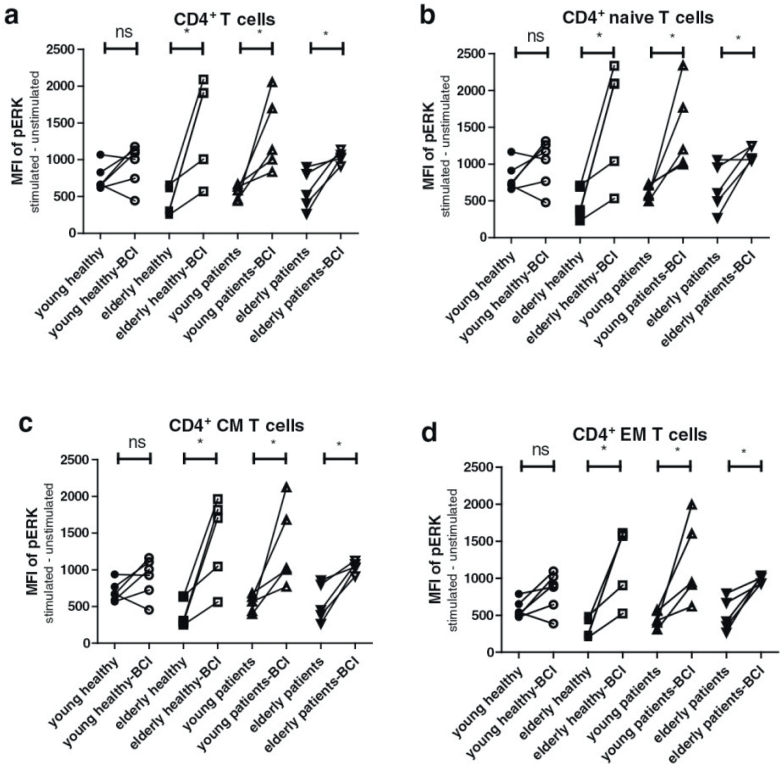

Fig. 9 Phosphorylation of ERK in CD4⁺ T cell subsets without and with BCI treatment from healthy individuals (HI) and end-stage renal disease (ESRD) patients. Phosphorylation of ERK for BCI-pretreated or not BCI-pretreated cells is given for different CD4⁺ T cell subsets: (**a**) total, (**b**) naive, (**c**) central memory (CM) and (**d**) effector memory (EM) of HI (young *n* = 5; elderly *n* = 5) and ESRD patients (young *n* = 5; elderly *n* = 5). *Dots* and *squares* represent young and elderly HI, *upward- and downward-facing triangles* correspond to young and elderly patients, respectively. *P* value: *<0.05; Data are given as individual values.

of increased levels of DUSP6 was noted for total and naive CD4⁺ T cells in HI (Fig. 10a & b). The opposite was present comparing young to old ESRD patients (Fig. 10a, b & c)**.** Levels of DUSP6 in young ESRD patients were similar to old HI. Like observed for pERK, a significant differentiation-associated increase in DUSP6 levels was observed (Fig. 10d). Interestingly, DUSP1 expression in CD4⁺ T cells was quite comparable between both young and elderly HI and patients (Fig. 10e, f, & g). To conclude, an age-related tendency of increased levels of DUSP6, but not DUSP1, was observed for HI. Moreover, DUSP6 expression was significantly associated with T-cell differentiation status in CD4⁺ T cells.

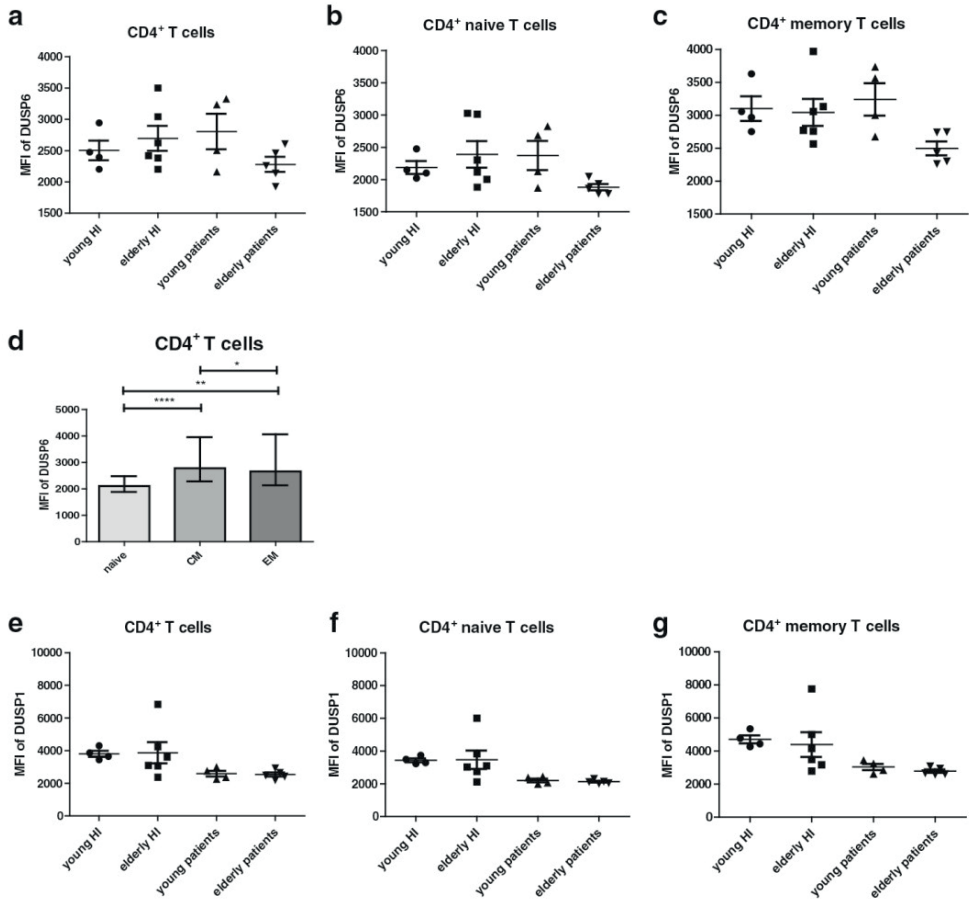

Fig. 10 DUSP6 and DUSP1 expression in CD4+ T cell subsets from healthy individuals (HI) and end-stage renal disease (ESRD) patients. DUSP6 expression in (**a**) total, (**b**) naive and (**c**) memory CD4+ T cells; (**d**) Differentiation-associated DUSP6 expression in CD4+ T cells ; DUSP1 expression in (**e**) total, (**f**) naive and (**g**) memory CD4+ T cells; *Dots* and *squares* represent young (*n* = 4) and elderly HI (*n* = 6), and *upward-* and *downward-facing triangles* correspond to young (*n* = 4) and elderly (*n* = 5) patients, respectively. *P* value: * < 0.05; **< 0.01; ***<0.001; Data are given as (individual values and) medians with interquartile ranges.

DISCUSSION

The main observation of this study was that TCR-mediated phosphorylation of ERK in $CD4^+$ T cells of young patients was in between young and old HI. Phosphorylation of ERK decreased in highly differentiated T-cell subsets compared to naive T cells. This defective TCR-mediated phosphorylation was specific as it could be restored by addition of a DUSP6 inhibitor. TCR-induced p38 phosphorylation was comparable between ESRD patients and HI.

Beyond midlife, the immune system shows age-related features and its defensive capabilities becomes impaired (35). The uremia-associated inflammatory environment present in ESRD patients accelerates this age-related immune senescence process. In addition to declined thymic output, accumulation of highly differentiated T cells, short telomere length (10, 33, 36) and narrowed TCR Vβ -repertoire diversity (32), this study indicates that young ESRD patients also have a defective $CD4^+$ TCR activation judging from the reduced capacity to phosphorylate ERK upon TCR-triggering. ERK activity is critical for TCR threshold calibration, as it controls positive feedback loops in TCR-induced activation (17). Reduced ERK activity impairs TCR signal strength and activation, and favors T cells with higher affinity to antigen to be activated, leading to a contracted immune response to a given antigen (16). The ERK phosphorylation upregulation of early activation marker CD69 on T cells ensures a proper inducing activation of T cells in the lymph node (37), and also play an important role in T cell proliferation (38) and IL-2 production (39, 40). In addition, ERK activation impacts cellular apoptosis as it inhibits Fas-mediated apoptosis in T cells (41). Evaluating ERK phosphorylation is a valuable tool to study more upstream molecules in the defective T-cell mediated immune system from ESRD patients. T cells from rheumatoid arthritis (RA) patients exhibit several defects which can also be viewed as premature immunological ageing (42). However, ERK phosphorylation in $CD4^+$ T cells of RA patients selectively increased (43). This increased ERK activation lowers the TCR threshold in T cells of RA patients to respond to self-antigens, which may partly explain the adaptive immune system of RA patients to exhibit abnormalities that go beyond the local inflammatory response in the synovium (44).

DUSP6 is a cytoplasmic phosphatase with substrate specificity for phosphorylated ERK. In elderly individuals, silencing of DUSP6 increased the expression of T cell activation markers, such as CD69 and CD25, IL-2 production as well as proliferative response (16). Inhibition of DUSP6 could be a potential intervention to increase $CD4^+$ TCR-sensitivity by enhancing ERK phosphorylation in ESRD patients. BCI (an inhibitor of DUSP6 and 1) enhanced TCR-induced pERK in $CD4^+$ T cells from elderly HI, young and elderly ESRD patients, but not young HI, implying a role for DUSP6 and/or DUSP1 in regulation of ERK phosphorylation. Based on the age- as well as differentiation-related expression of DUSP6, but not DUSP1, in our HI, a potential role for DUSP6 may be present in defective TCR-induced ERK phosphorylation,

especially in elderly HI and young patients. This needs to be confirmed in a larger cohort. Furthermore, use of siRNA specific for DUSP6 is required to draw a more definite conclusion with respect to the role of DUSP6 in defective TCR-induced phosphorylation of ERK in ESRD patients. The lack of age-related effects on pERK in $CD8^+$ T cells of ESRD patients and HI, the latter confirming observations done by another study (16), as well as absence of effects of BCI on pERK levels in $CD8^+$ T cells, indicates a different role for DUSP6 in $CD8^+$ T cells compared to $CD4^+$ T cells. We did not observe an association between DUSP6 expression and ERK phosphorylation in $CD4^+$ T cells in ESRD patients. This could be due to the small cohort size or imply other DUSPs (e.g. 2, 4 or 5) (45-49) or upstream signaling molecules to contribute to this defective TCR-induced ERK phosphorylation in ESRD patients.

ERK over-phosphorylation might be as bad as defective ERK phosphorylation. ERK over-activation from kidney cells occurs in the physiologic setting in some chronic kidney diseases, such as compensatory kidney hypertrophy and in pathologic conditions for example glomerular disease(50). Increased ERK phosphorylation in T cells predisposes for autoimmunity for example rheumatoid arthritis (43). Over-expression of DUSP6 is also reported to impair T-cell function in chronic viral infections such as hepatitis C virus infection (51). Therefore, more research is warranted evaluating inhibition of DUSP in the setting of defective T-cell mediated immunity in ESRD patients.

We analyzed the effect of latency for CMV as it represents chronic antigenic stimulation of T cells, but ERK- or p38-activation of $CD4^+$ or $CD8^+$ T cells was not different between the CMV-IgG seropositive population and CMV-IgG seronegative population following CD3/CD28 stimulation. Highly differentiated memory $CD4^+$ and $CD8^+$ T cells may accumulate in CMV seropositive individuals and are functional CMV-specific T cells (29, 52, 53). The results of our study show that non-specific TCR stimulation does not identify a defect p38 and ERK signaling associated with CMV seropositivity. In accordance with the results of a previous study, uremia is the major determinant affecting MAPK pathway parameters in ESRD patients and not RRT (9, 11, 33).

In the present study, we induced phosphorylation of p38 in T cells via triggering CD3 (54) and CD28 (26). Lack of CD28 may only partly explain the decreased p38 activation in more differentiated $CD4^+$ T cells. In addition to that, senescent human $CD27^-CD28^-$ $CD4^+$ T cells lack several essential upstream components including ZAP70 and the loss of TCR signaling machinery in those cells was associated with a defective calcium influx (31), which may indicate the decreased response of TCR-mediated activation in the more differentiated T cells. Interestingly, in contrast to the p38 activation following CD3/CD28 stimulation, baseline levels (spontaneous phosphorylation) of p38 increased during T cells differentiation (55). This might be caused by DNA damage in these more differentiated T cells and mediated by TAB1 (MKK-independent molecule), a key molecule involved in this auto-phosphorylation (31).

Conclusion

We have described for the first time a uremia-mediated defect in TCR-induced phosphorylation of ERK which may contribute to the impaired T-cell mediated immune response in ESRD patients. Inhibition of DUSP6 specific for pERK can restore defective p-ERK-mediated activation of $CD4^+$ T cells in ESRD patients.

Acknowledgements:

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Conflict of Interest

The authors of this manuscript have no financial or commercial conflicts of interest to disclose.

SUPPORTING INFORMATION

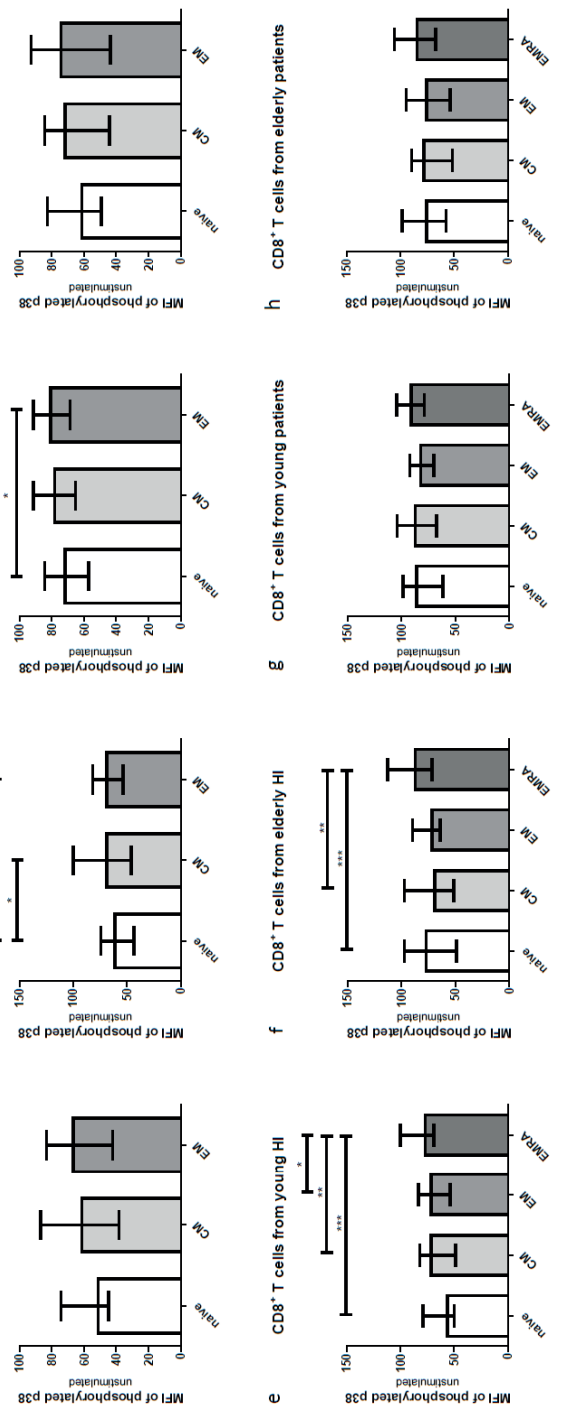

Fig. S1. Auto-phosphorylation (baseline) of p38 according to T cell differentiation status in healthy individuals (HI) and end stage renal disease (ESRD) patients. Phosphorylation of ERK in $CD4^+$ T cells from (a) young HI (n=13), (b) elderly HI (n=11), (c) young patients (n=12), and (d) elderly patients (n=12), as well as $CD8^+$ T cells from (e) young HI, (f) elderly HI, (g) young patients, and (h) elderly patients. Blank bars and bars with light grey to dark grey represent naive, central memory (CM), effector memory (EM) and highly differentiated effector T cells (EMRA) T cells, respectively. P value: *<0.05; ** < 0.01; *** < 0.001; Data are given as median with interquartile range.

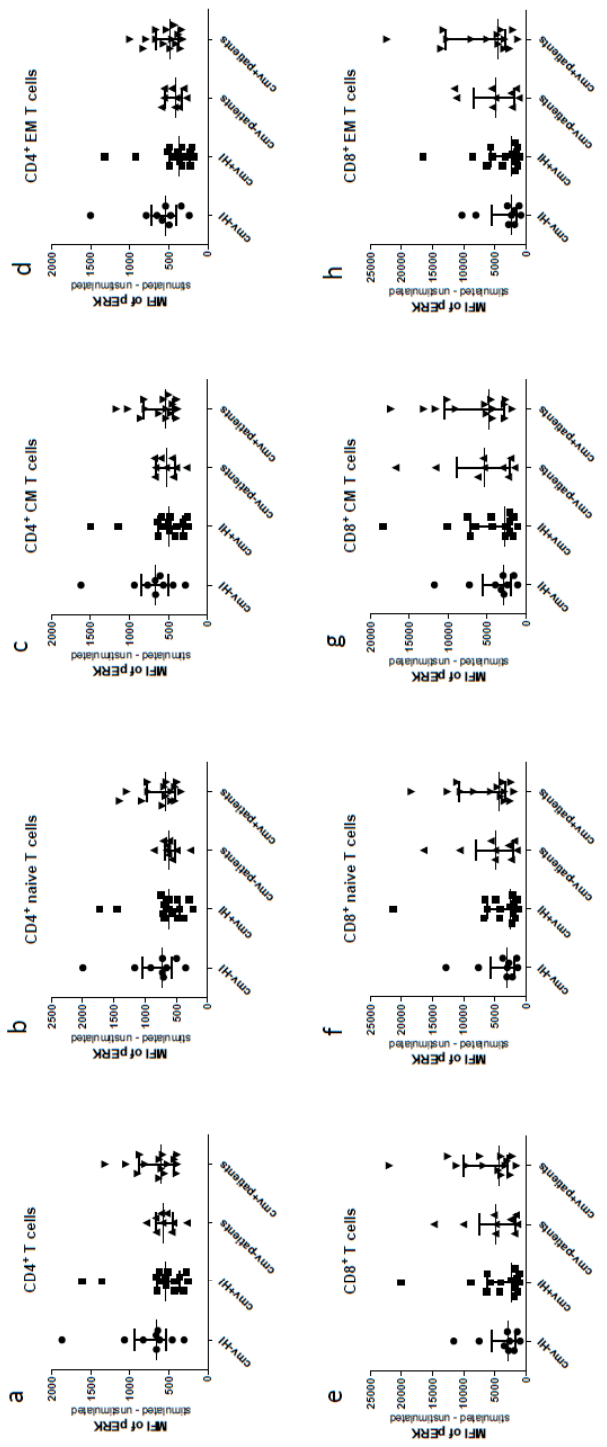

Fig. S2. CMV-effects on phosphorylation of ERK in T cells subsets of healthy individuals (HI) and end stage renal disease (ESRD) patients. Phosphorylation of ERK (a) $CD4^+$, (b) $CD4^+$ naive, (c) $CD4^+$ central memory (CM), (d) $CD4^+$ effector memory (EM), as well as (e) $CD8^+$, (f) $CD8^+$ naive, (g) $CD8^+$ CM, (h) $CD8^+$EM, (i) $CD8^+$highly differentiated effector T cells (EMRA). Dots and squares represent CMV seropositive (n=15) and negative (n=9) HI, upward- and downward-facing triangles correspond to CMV seropositive (n=15) and negative (n=9) patients, respectively. P value: * < 0.05; Data are given as individual values and medians with interquartile ranges .

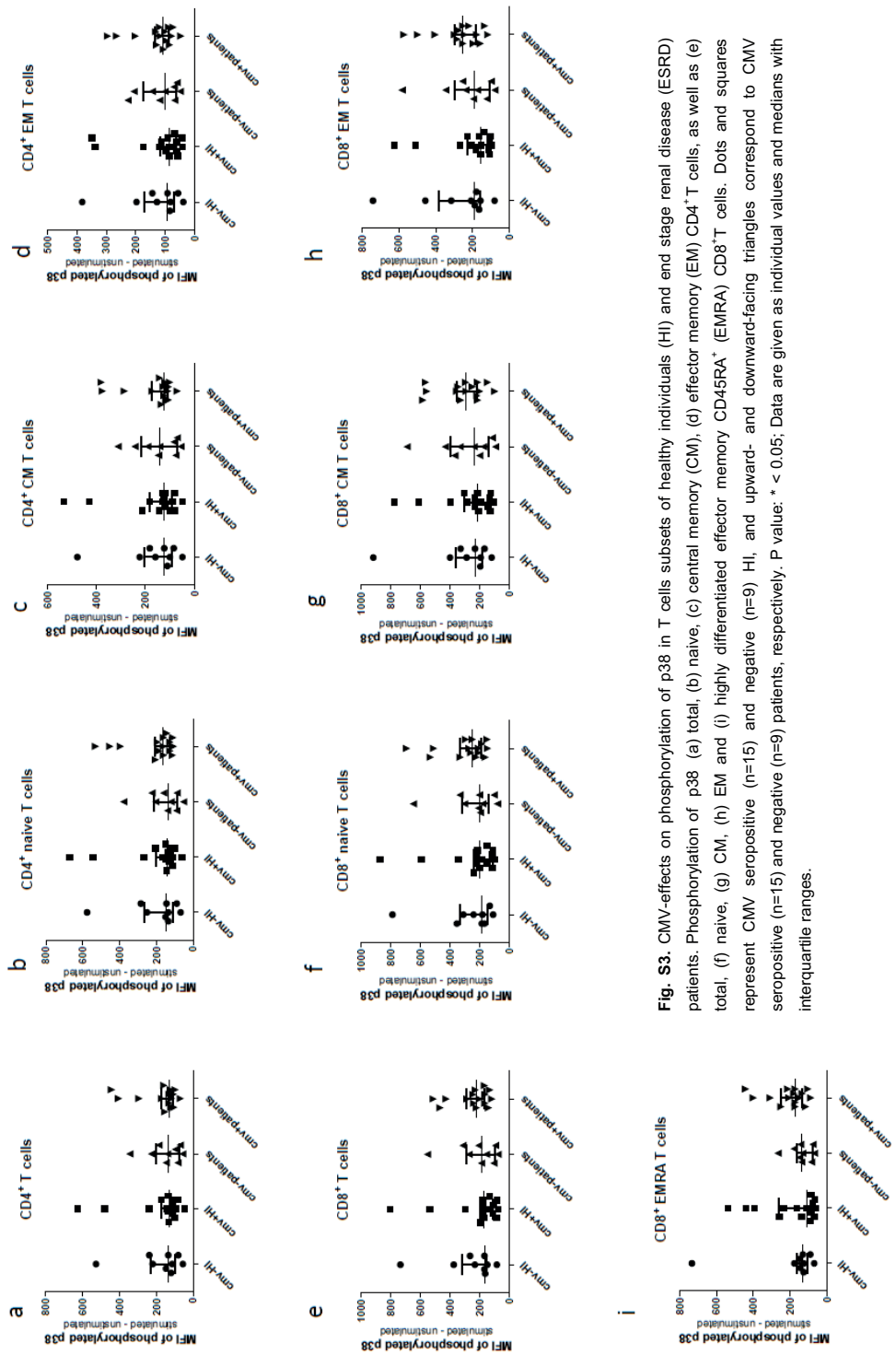

Fig. S3. CMV-effects on phosphorylation of p38 in T cells subsets of healthy individuals (HI) and end stage renal disease (ESRD) patients. Phosphorylation of p38 (a) total, (b) naive, (c) central memory (CM), (d) effector memory (EM) CD4+ T cells, as well as (e) total, (f) naive, (g) CM, (h) EM and (i) highly differentiated effector memory CD45RA+ (EMRA) CD8+T cells. Dots and squares represent CMV seropositive (n=15) and negative (n=9) HI, and upward- and downward-facing triangles correspond to CMV seropositive (n=15) and negative (n=9) patients, respectively. P value: * < 0.05; Data are given as individual values and medians with interquartile ranges.

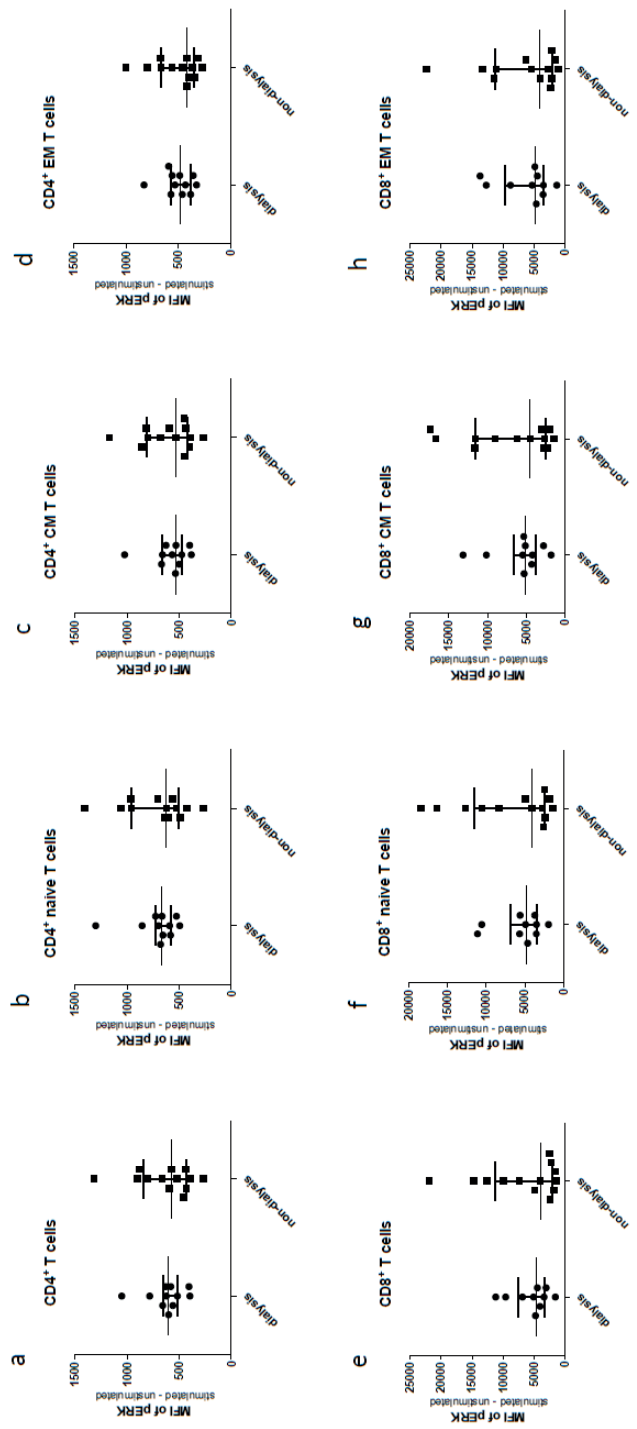

Fig. S4. Effects of renal replacement therapy (RRT) on phosphorylation of ERK in T cells subsets of end stage renal disease (ESRD) patients with and without dialysis. Phosphorylation of ERK in (a) total, (b) naive, (c) central memory (CM), (d) effector memory (EM) $CD4^+$ T cells, as well as (e) total, (f) naive, (g) CM, (h) EM and (i) highly differentiated effector memory $CD45RA^+$(EMRA) $CD8^+$ T cells. Dots and squares represent patients with (n=11) and without dialysis (n=13), respectively. P value: * < 0.05; Data are given as medians with interquartile ranges.

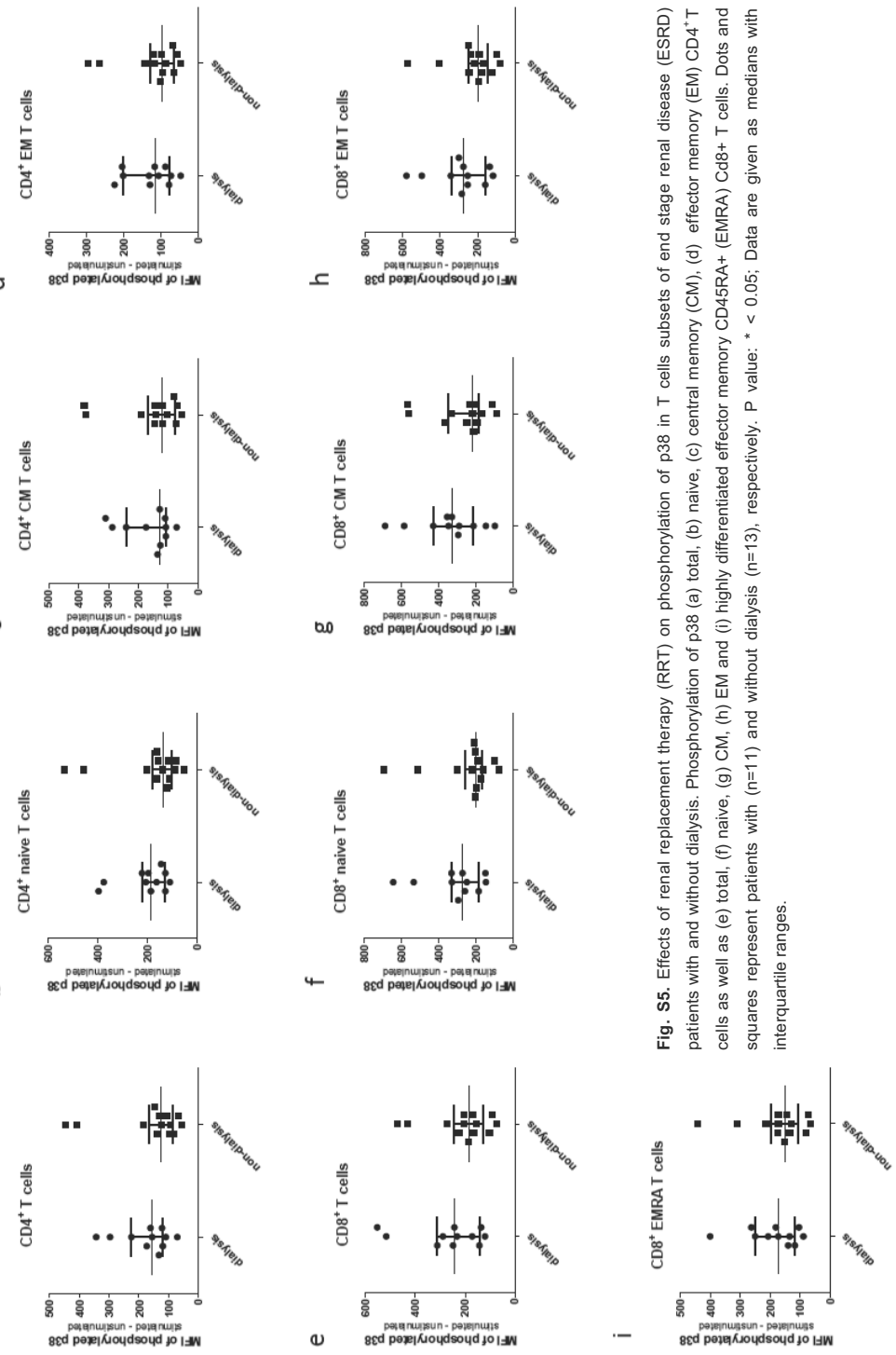

Fig. S5. Effects of renal replacement therapy (RRT) on phosphorylation of p38 in T cells subsets of end stage renal disease (ESRD) patients with and without dialysis. Phosphorylation of p38 (a) total, (b) naive, (c) central memory (CM), (d) effector memory (EM) CD4+T cells as well as (e) total, (f) naive, (g) CM, (h) EM and (i) highly differentiated effector memory CD45RA+ (EMRA) Cd8+ T cells. Dots and squares represent patients with (n=11) and without dialysis (n=13), respectively. P value: * < 0.05; Data are given as medians with interquartile ranges.

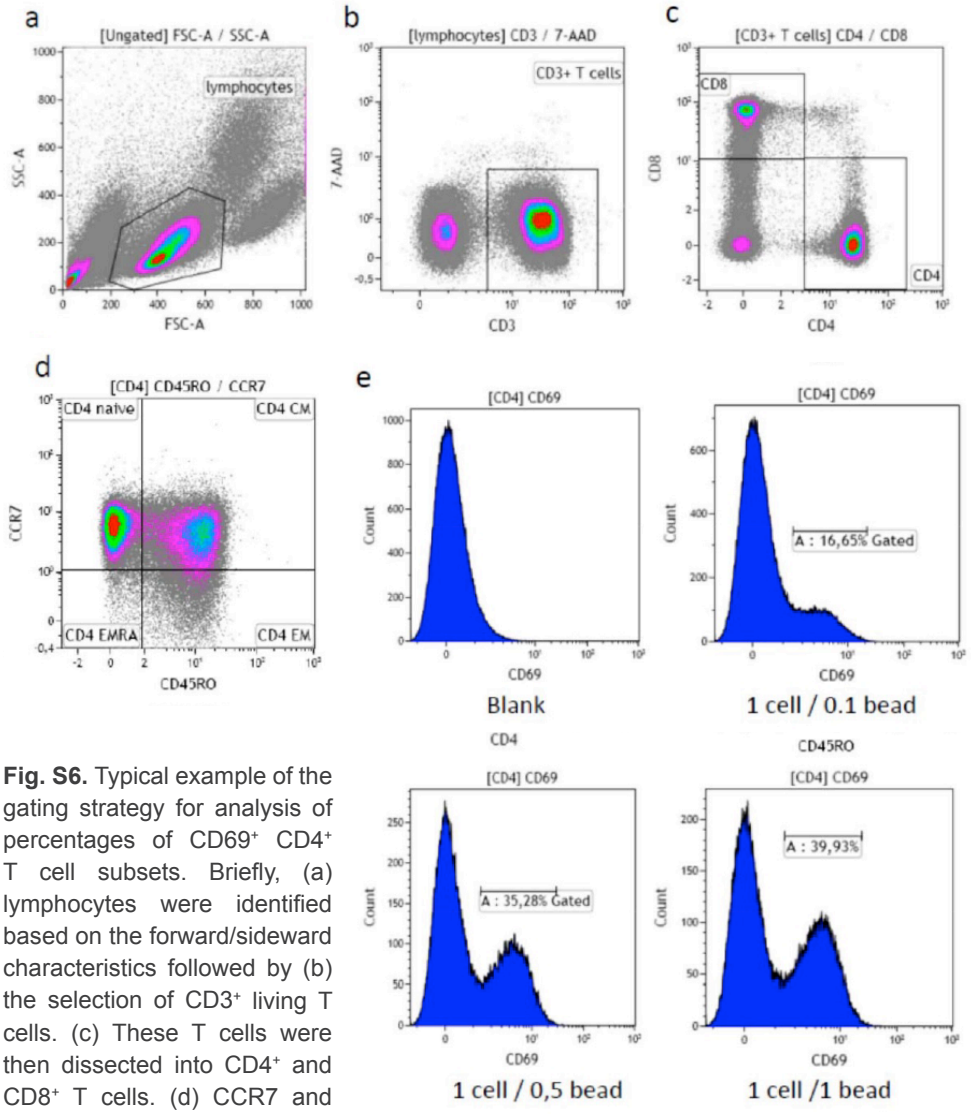

Fig. S6. Typical example of the gating strategy for analysis of percentages of CD69$^+$ CD4$^+$ T cell subsets. Briefly, (a) lymphocytes were identified based on the forward/sideward characteristics followed by (b) the selection of CD3$^+$ living T cells. (c) These T cells were then dissected into CD4$^+$ and CD8$^+$ T cells. (d) CCR7 and CD45RO were used to identify naive and different memory subsets within CD4$^+$ T cells. Furthermore, (e) percentages of CD69$^+$ were measured for CD4$^+$ T cells stimulated or not with different ratios of anti-anti-CD3/CD28 beads (blank control, 1 cell /0.1 bead, 1 cell /0.5 bead, 1 cell/1 bead). A similar gating strategy was employed for analysis of CD69$^+$ and IL2$^+$ the different CD4$^+$ T cells subsets.

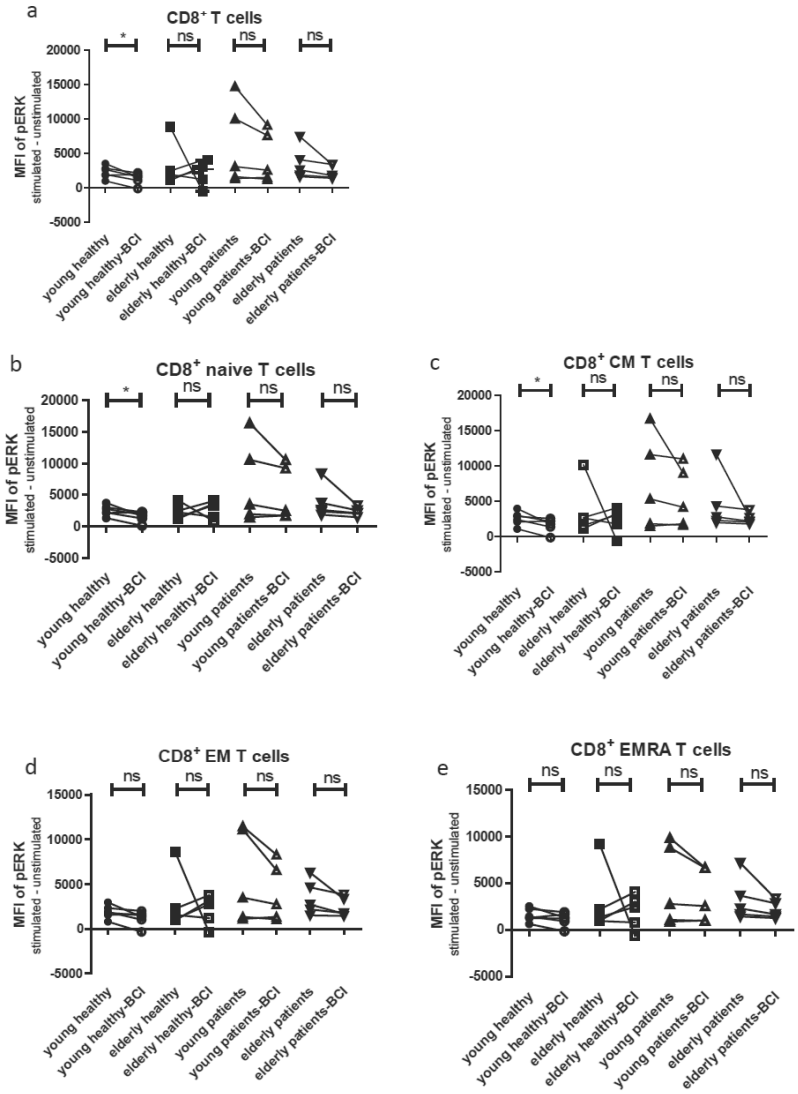

Fig. S7. Phosphorylation of ERK in CD8+ T cell subsets without and with BCI treatment from healthy individuals (HI) and end-stage renal disease (ESRD) patients. Phosphorylation of ERK for BCI-pretreated or not BCI-pretreated cells is given for different CD4+ T cell subsets: (a) total, (b) naive, (c) central memory (CM) and (d) effector memory (EM) of HI (young n=5; elderly n=5) and ESRD patients (young n=5; elderly n=5). Dots and squares represent young and elderly HI, upward- and downward-facing triangles correspond to young and elderly patients, respectively. P value: *<0.05; Data are given as individual values.

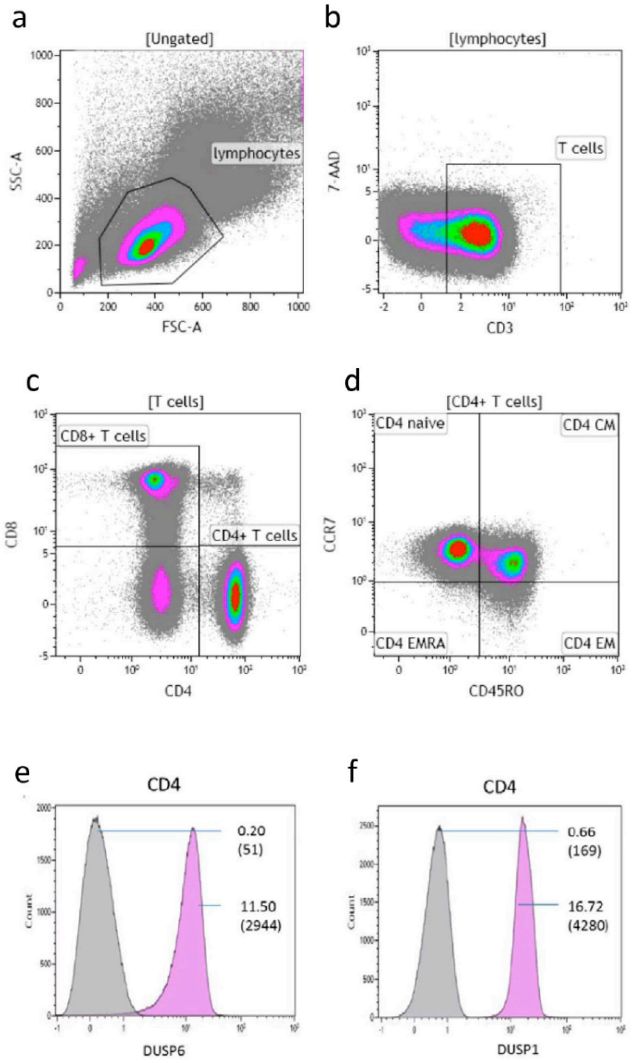

Fig. S8. Typical example of the gating strategy for analysis of DUSP6 and DUSP1 expression in CD4$^+$ T cell subsets. Briefly, (a) lymphocytes were identified based on the forward/sideward characteristics followed by (b) the selection of CD3$^+$ living T cells. (c)These T cells were then dissected into CD4$^+$ and CD8$^+$ T cells. (d) CCR7 and CD45RO were used to identify naive and different memory subsets within CD4$^+$ T cells. Furthermore, (e) DUSP6 or (f) DUSP1 expression was measured in CD4$^+$ T cells and median fluorescence intensities (MFI) were evaluated (values multiplied by 256 in brackets). Grey peaks represent staining without the specific antibodies (fluorescence minus one, FMO), and pink peaks correspond to staining with antibodies directed to DUSP6 or DUSP1. A similar gating strategy was employed for analysis of DUSP6 and DUSP1 expression in all CD4$^+$ T cells subsets.

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CHAPTER 6

Protective Cytomegalovirus (CMV)-Specific T-Cell Immunity Is Frequent in Kidney Transplant Patients without Serum Anti-CMV Antibodies

Nicolle H. R. Litjens[1], Ling Huang[1], Burç Dedeoglu[1], Ruud W. J. Meijers[1], Jaap. Kwekkeboom[2], Michiel G. H. Betjes[1]

Erasmus MC, University Medical Center Rotterdam, Department of Internal Medicine, Nephrology and Transplantation[1] and Department of Gastroenterology and Hepatology[2], Rotterdam, The Netherlands

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ABSTRACT

The absence of anti-CMV IgG is used to classify pre-transplant patients as naive for CMV infection (CMV^{neg} patients). This study assessed whether pre-transplant CMV-specific T-cell immunity exists in CMV^{neg} patients and whether it protects against CMV-infection after kidney transplantation. The results show that CMV-specific $CD137^{+}IFN\gamma^{+}CD4^{+}$ and $CD137^{+}IFN\gamma^{+}CD8^{+}$ memory T cells were present in 46% and 39% of CMV^{neg} patients (n=28) although at much lower frequencies compared to CMV^{pos} patients (median 0.01% versus 0.58% for $CD4^{+}$ and 0.05% versus 0.64% for $CD8^{+}$ T cells) with a less differentiated CD28-expressing phenotype. In line with these data, CMV-specific proliferative $CD4^{+}$ and $CD8^{+}$ T cells were observed in CMV^{neg} patients, which significantly correlated with the frequency of CMV-specific T cells. CMV-specific IgG-antibody secreting cells (ASC) could be detected at low frequency in 36% of CMV^{neg} patients (1 versus 45 ASC/105 cells in CMV^{pos} patients). CMV^{neg} patients with pretransplant CMV-specific $CD137^{+}IFN\gamma^{+}CD4^{+}$ T cells had a lower risk to develop CMV-viremia after transplantation with a CMV^{pos} donor kidney (relative risk: 0.43, p=0.03). In conclusion, a solitary CMV-specific T-cell response without detectable anti-CMV antibodies is frequent and clinically relevant as it is associated with protection to CMV-infection following transplantation with a kidney from a CMV^{pos} donor.

INTRODUCTION

Kidney transplant (KT-) recipients are at increased risk for infections following transplantation, one of the major threats being cytomegalovirus (CMV). A primary infection or reactivation with CMV may cause a viremia and can lead to severe CMV disease with organ involvement (1). Patients at increased risk for an infection with CMV either receive prophylactic or pre-emptive anti-viral therapy (valganciclovir) following kidney transplantation (2). Classification of the risk for a CMV-infection prior to KT is based on the presence of anti-CMV immunoglobulin (Ig)G in the patient in combination with the CMV-serostatus of the kidney donor. Immunity to viruses, for example CMV, is dependent on adequate help from CMV-specific $CD4^+$ T cells enabling production of neutralizing antibodies by CMV-specific B-cells/plasma blasts and effective cytotoxic $CD8^+$ T cell (CTL) responses (3-5). Absence of neutralizing antibodies does not necessarily imply that the antigen was not encountered before as cellular immunity might still be present. Furthermore, it is known that in end-stage renal disease (ESRD) patients protective humoral immunity is not maintained and often not achieved (6, 7). Therefore, several groups have proposed to include an evaluation of cellular immunity against CMV in the risk assessment strategy to more accurately assess sensitization prior to transplantation (8-10). Data are scarce as to whether the presence of limited cellular immunity protects from CMV-viremia after kidney transplantation and no detailed insights into which T-cell subsets are involved in just-sufficient protection are available, as cellular immunity is mainly assessed by ELISpot (11).

T-cell differentiation can be assessed by using expression of CD45RO or CD45RA and the lymph node homing chemokine receptor CCR7 which is able to identify naive T cells and different memory T cell subsets, i.e. central memory (CM), effector memory (EM) and finally terminally differentiated EM $CD45RA^+$ (EMRA)(12). This is accompanied by loss of the co-stimulatory marker CD28. Particularly, an effective CMV-specific T cell response yields many highly differentiated CD28null T cells (13, 14). As we have previously shown, this T-cell response adds to immunological T-cell ageing observed in the circulation of ESRD patients, resulting in a shift towards more differentiated memory T cells and enhanced telomere attrition (14, 15). Whether presence of limited cellular immunity to CMV impacts T-cell ageing characteristics in ESRD patients is not known.

The aim of this study is to assess CMV-specific T-cell immunity including the differentiation stage of CMV-specific T cells, and to determine its clinical relevance with respect to protection from CMV-viremia following transplantation in a cohort of CMV-seronegative patients. Furthermore, the impact on T-cell ageing parameters was evaluated.

MATERIAL AND METHODS

Study population

In a cohort of stable CMV-seronegative and, as a control, age- and gender-matched CMV-seropositive recipients of a kidney from a CMV-seropositive donor (D+/R- and D+/R+, respectively), CMV-specific immunity was assessed prior to transplantation and linked to CMV-viremia following KT. Most of the patients (86%) received induction therapy with basiliximab (Simulect®, Novartis). The standard triple immunosuppression given following transplantation consisted of tacrolimus (Prograf®, Astellas Pharma), mycophenolate mofetil (Cellcept®, Roche) and prednisolone (the first 3 months post-KT), the first given to 93% and the latter two to all of the patients. The first six months following transplantation, anti-viral prophylaxis with valganciclovir 450 mg q.d. was given and if necessary adjusted for impairment of renal function. Patient characteristics are listed in Table 1. All patients gave written informed consent to participate in this study. The study was approved by the Medical Ethical Committee of the Erasmus MC (METC number 2010-080) and conducted in accordance with the Declaration of Helsinki and the Declaration of Istanbul.

Detection of CMV-viremia and CMV-specific antibodies

Serum immunoglobulin G (IgG) antibodies to CMV (expressed as arbitrary units/mL (AU/mL)) were measured with an enzyme immune assay (Biomerieux, VIDAS, Lyon, FRANCE). An outcome of ≥6 AU/mL was considered positive. Patients were monitored at a 3 months interval during the first year following transplantation with respect to presence of CMV DNA. Diagnosis of a CMV-viremic episode was based on the presence of copies (expressed in international units/mL) of CMV DNA in blood and established by a quantitative polymerase chain reaction (qPCR) at the department of Virology at the Erasmus MC. An outcome of >50 international units/mL was indicative for a CMV-viremia.

Isolation of peripheral mononuclear cells (PBMCs)

Prior to KT, PBMCs were isolated as described in detail before (16) from heparinized blood samples drawn from CMV-seronegative and CMV-seropositive patients and stored at 10 million PBMCs per vial at -150^{o}C until further use.

Detection of CMV-specific CD137-expressing cytokine producing T cells

PBMCs of 28 CMV-seronegative and 14 CMV-seropositive patients were thawed, allowed to rest for 8 hours at 37^{o}C and stimulated (5x10^{6} PBMCs/mL) in RPMI-1640 containing glutamax (GibcoBRL, Paisley, Scotland) supplemented with 100 IU/mL penicillin, 100 ug/mL streptomycin and 10% heat-inactivated pooled human serum, further referred to as standard culture medium. Stimulation was performed in polystyrene tubes (BD Pharmingen, Erembodegem, Belgium) in the presence of co-stimulation CD49d (1 µg/mL; BD) without (background) or with a mixture of overlapping peptide pools covering the whole pp65 and IE-1 protein of CMV (1 µg/ml; PepTivator-CMV pp65 and IE-1; Miltenyi Biotec GmbH, Bergisch Gladbach,

Germany) and Brefeldin A (Golgiplug; BD Pharmingen) for 12 hours. This intracellular cytokine staining assay facilitates detailed characterization of CMV-specific $CD4^+$ as well as $CD8^+$ T cells as these can be identified by *de novo* expression of CD137 in combination with effector molecules (17). As a positive control, PBMC of 10 CMV-seronegative and 5 CMV-seropositive patients were stimulated with the combination of phorbol myristate acetate (PMA; 50 ng/mL; Sigma Aldrich, St. Louis, MO, USA) and ionomycin (1 µg/mL; Sigma Aldrich) and treated as described above.

Subsequently, a surface staining was performed to identify naive ($CD45RO^-CCR7^+$) and memory T cell subsets (12). Central memory (CM) T cells are $CD45RO^+CCR7^+$, effector memory (EM) $CD45RO^+CCR7^-$ and terminally differentiated effector memory (EMRA) $CD45RO^-CCR7^-$. In addition, less and more differentiated T cell subsets were also identified by CD28 (i.e. less differentiated being $CD28^+$ and more differentiated, lacking CD28, referred to as CD28null). The following monoclonal antibodies were used: Brilliant Violet (BV)-510 labeled anti-CD4 (Biolegend Europe BV, Uithoorn, The Netherlands), Pacific Blue (PB) labeled anti-CD45RO (Biolegend), allophycocyanin-Cy7 (APC-Cy7) labeled anti-CD8 (BD, Erembodegem, Belgium), peridinin chlorophyll-Cy5.5 (PerCP-Cy5.5) labeled anti-CD28 (BD) and phycoerythrin-Cy7 (PE)-Cy7 labeled anti-CCR7 (BD). Following fixation and permeabilization, cells were stained intracellular using APC-labeled anti-CD137 (BD) and PE-labeled anti-IFNγ (BD Pharmingen). IL-2 producing cells were only evaluated in a fraction of the patients tested, i.e. 12 CMV-seronegative and 6 CMV-seropositive patients by co-staining intracellular using fluorescein isothiocyanate (FITC)-labeled anti-IL-2 (BD). Samples were measured on the FACSCanto II (BD Pharmingen), aiming for 0.5-1x10^6 of T cells to be acquired, and analyzed using FACSDiva software version 6·1.2 (BD). The gating strategy for identifying CMV-specific $CD137^+CD4^+$T cells within the different subsets and in combination with cytokine production are shown in **Fig. S1**, a similar approach was followed for $CD8^+$ T cells. The median (IQ range) background of CD137-expressing $CD4^+$ T cells of all samples amounted to 0.05% (0.03%-0.07%) whereas that of CD137-expressing $CD8^+$ T cells was higher, amounting to 0.44% (0.23%-1.02%). The median background value for $CD137^+IFN\gamma^+$ $CD4^+$ and $CD8^+$ and $CD137^+IL\text{-}2^+$ $CD4^+$ T cells of all samples were 0.01% (0.01%-0.02%), 0.04% (0%-0.09%) and 0.01% (0.01%-0.01%), respectively. Most of the background signal within $CD4^+$ T cells was observed in cells co-expressing CD28 and of a CM/EM phenotype whereas that observed for $CD8^+$ T cells were predominantly lacking CD28 and of the EM/EMRA-phenotype. Since frequencies obtained for the various parameters differed considerably amongst patients, we subtracted the unstimulated value per patient from that after CMV-peptide stimulation to calculate the net signal as shown in the results. A positive detectable CMV-specific response was identified if the net response was over 0. Only detectable $CD4^+$ and $CD8^+$ $CD137^+$ CMV-specific T cell responses were analyzed in more detail with respect to cytokine production and phenotypic aspects.

Detection of CMV-specific proliferating T cells

PBMC of 12 CMV-seronegative and 6 CMV-seropositive patients, were thawed and labeled with carboxyfluorescein diacetate succinimidyl ester according to manufacturer's instruction (CFSE; Molecular Probes®, the Netherlands) and subsequently stimulated in triplicate in standard culture medium at $5x10^4$/well (96 wells-round bottom-shaped plate) without or with CMV-lysate (30 µg/mL; Microbix Biosystems Inc, Ontario, Canada) or with a mixture of overlapping peptide pools covering the whole pp65 and IE-1 protein of CMV (both at a final concentration of 1 µg/ml; PepTivator-CMV pp65 and IE-1; Miltenyi Biotec). Stimulation with CMV-lysate might allow for characterization of the total pool of CMV-specific $CD4^+$ T cells as the overlapping peptide pools cover only 2 (albeit dominant) peptides of the CMV protein. Proliferation of CMV-specific $CD8^+$ T cells was only analyzed following stimulation with overlapping peptide pools of pp65 and IE-1 and not whole CMV-lysate due to their restriction with respect to length (number of amino acids) of the peptides presented in the context of HLA class I. Following 6 days, cells were harvested, stained using monoclonal antibodies directed against CD3, CD4, CD8, CD28 (again to identify less-differentiated versus more-differentiated subsets amongst proliferating T cells) and dead cells were excluded using 7-aminoactinomycin D (7-AAD). Samples were measured on the FACSCanto II (BD) and analyzed using FACS Diva software version 6.1.2 (BD), percentages of CMV-specific proliferating T cells were calculated by subtracting percentages of T cells proliferating in absence of these stimuli. The median (IQ range) of proliferating $CD4^+$ and $CD8^+$ T cells in absence of a stimulus amounted to 0.83% (0.50%-2.48%) and 1.42% (0.69%-3.36%), respectively. A positive detectable response was identified if the net response was over 0.

Total and CMV-specific IgG antibody secreting cells

PBMCs of 11 CMV-seronegative and, as a positive control, 4 CMV-seropositive patients were thawed and subsequently stimulated at a density of $2x10^6$/mL for 5 days with R848 and recombinant IL-2, according to manufacturer's instruction (U-Cytech BV, Utrecht, The Netherlands). On day 4, the wells of a 96-well plate were coated overnight at 4°C according to manufacturer's instruction. Coating consisted of either an anti-human IgG antibody, for enumeration of total IgG antibody secreting cells (ASC) or CMV-lysate (30 µg/mL Microbix Biosystems Inc, Ontario, Canada), for enumeration of CMV-specific IgG ASC. Following stimulation, cells were harvested, counted and added to the coated ELISpot plate at different concentrations, each in triplicate. After a 7 hour incubation (37°C, 5% CO_2), cells were lysed and debris washed away using PBS/0.05% Tween-20. Subsequently, the wells of the ELISpot plate were incubated for one hour at 37°C with a biotinylated anti-human IgG (detection) antibody and upon washing followed by an incubation with phi-labeled anti-biotin antibody (GABA) for one hour at 37°C. Finally ASC were visualized, upon a washing procedure, using an activation solution (U-Cytech BV) resulting in a silver precipitate upon incubation in the dark at room temperature. Color development was stopped using di-ionized water and spots were counted using an ELISpot reader (Bioreader®-600V, BIO-SYS GmbH, Karben, Germany). CMV-IgG ASC are

expressed as number/10^5 cells as well as a frequency of total IgG ASC.

In addition to analyzing ASC, a sample prior to and following stimulation was analyzed for B cell blast formation by flow cytometry . The following panel of monoclonal antibodies was used: BV510-labeled anti-CD19 (Biolegend), BV421-labeled anti-CD38 (BD) and PE-Cy7-labeled anti-CD27 (eBioscience). Plasma blasts were $CD19^+CD38^{high}CD27^+$.

Absolute numbers of $CD4^+$ and $CD8^+$ T cells and T-cell differentiation status by flow cytometry
Absolute numbers of T-cell subsets (18) and T-cell differentiation status (19) were determined using a whole blood staining as described in detail before.

Telomere length assay
Relative telomere length of $CD4^+$ and $CD8^+$ T cells were determined by Flow fluorescent *in situ* hybridization (flow-FISH) as described previously (15, 20).

Statistical analyses
Statistical analyses were performed using GraphPad Prism version 5.01. Non-parametric Mann-Whitney U-test or Kruskall-Wallis test followed by a post-hoc analysis (Dunns multiple comparison test). Categorical variables were compared using the Fisher's exact test. The non-parametric Spearman rank correlation coefficient (Spearmans Rho, Rs) was used to evaluate associations between various parameters. P-values<0.05 for two sides were considered statistically significant.

RESULTS

Study population characteristics
The demographic and clinical characteristics of the study population are given in **Table 1**. Twenty-eight CMV-seronegative and 14 age- and gender-matched CMV-seropositive patients were included in this study. Both groups were well-matched with no significant differences in clinical characteristics. Approximately half of the CMV-seronegative versus none of the CMV-seropositive patients experienced a CMV viremia within 12 months after transplantation (P<0.01).

CMV-seronegative patients frequently have CMV-specific T cells
CMV-specific $CD4^+$ and $CD8^+$ T cells, identified by expression of CD137 upon stimulation with a peptide pool of the 2 immunodominant proteins pp65 and IE-1 ($CD137^+CD4^+$ and $CD137^+CD8^+$) were present in 16 out of 28 CMV-seronegative patients. Median (IQ range) values of the positive responses amounted to 0.03% (0.01%-0.11%) and 0.10% (0.03%-0.18%) for $CD4^+$ and $CD8^+$ T cells, respectively (**Fig. 1A,C, black dots**). A positive correlation was observed between the $CD4^+$ and $CD8^+$ level of CMV-specific T-cell response in CMV-seronegative patients (Rs=0.50, P<0.05). The percentages of CMV-specific CD137-positive $CD4^+$ and $CD8^+$ T cells

Table 1 Demographic and clinical characteristics of study population

	D+/R+ (n=14)	D+/R- (n=28)	P-value
Age in years [A,B]	50 (19-63)	47.5 (21-76)	NS
Male	50% (7)	68% (19)	NS
CMV IgG titer (AU/ml)	47.5 (9-354)		
CMV viremia		46% (13)	
Time point of CMV viremia post-KT (months)		5 (2-12)	
Renal replacement therapy [B]			NS
-Pre-emptive transplantation	43% (6)	43% (12)	
-Patients on dialysis	57% (8)	57% (16)	
Underlying kidney disease [B]			NS
- Nephrosclerosis/atherosclerosis/ hypertension	14% (2)	25% (7)	
- Primary glomerulopathies	36% (5)	29% (8)	
- Diabetes	21% (3)	11% (3)	
- Urinary tract infections/stones		3% (1)	
- Reflux nephropathy		3% (1)	
- Polycystic Kidney Disease		7% (2)	
- Other/unknown	29% (4)	22% (6)	
Previous KT [B]	1	1	NS
Mismatches HLA class I [A]	2 (1-4)	2 (0-4)	NS
Mismatches HLA class II[A]	1 (0-2)	1 (0-2)	NS
Immunosuppressive medication			
-Basiliximab induction therapy [C]	79% (11)	89% (25)	NS
- Prednisolone [D]	100% (14)	100% (28)	NS
- MMF	100% (14)	100% (28)	NS
- Tacrolimus	93% (13)	93% (26)	NS
- switch Tacrolimus->Everolimus[F]	7% (1)	7% (2)	NS
Donor age in years [A,B]	42 (27-66)	57 (29-72)	<0.01

[A] = median (min-max), [B]= at pre-KT,

[C] = Given at day 0 and day 4 post-KT, [D] = Given the first 3 months post-KT,

F= 6 months post-KT

NS=not significant

(present in 14 and 11 out of 14 CMV-seropositive patients, respectively) were significantly (P<0.001) higher in CMV-seropositive patients and amounted to 0.61% (0.11%-1.04%) and 1.45% (0.44%-8.69%) (**Fig. 1A,C**, **open dots**), respectively.

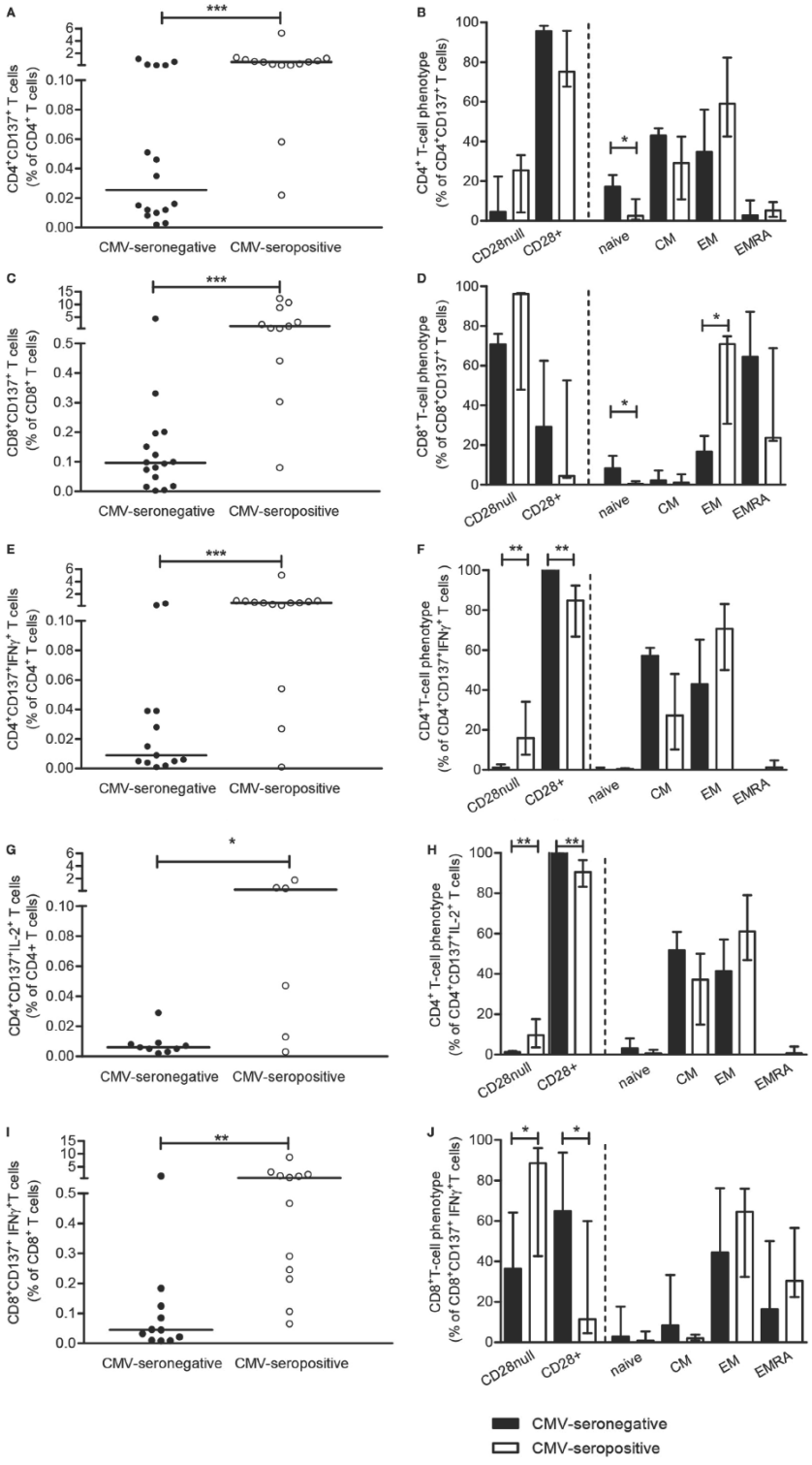
CMV-specific $CD137^+CD4^+$ and $CD137^+CD8^+$ T cells were predominantly of the memory phenotype in both CMV-seropositive as well as CMV-seronegative patients. However, significantly higher percentages (P<0.05) of $CD137^+CD4^+$ and $CD137^+CD8^+$ T cells in CMV-seronegative patients had a naive phenotype when compared to CMV-seropositive patients (**Fig. 1,D, respectively**). No differences were observed when comparing the maximal capacity of T cells to express CD137 between CMV-seronegative and CMV-seropositive patients upon PMA/ionomycin-stimulation (**Fig. S2 A and B**).

CMV-specific T cells in CMV-seronegative patients produce IFNγ and IL-2

Next, the presence and distribution of IFNγ and IL-2 producing CMV-specific T cells was studied. In 13 out of 28 CMV-seronegative patients, CMV-specific $IFN\gamma^+CD137^+CD4^+$T cells were present and median (IQ range) frequencies amounted to 0.01% (0.01%-0.04%) versus 0.58% (0.16%-0.87%) for CMV-seropositive patients, that all had CMV-specific $IFN\gamma^+CD137^+CD4^+$T cells (**Fig. 1E**, P<0.001). In CMV-seronegative patients, approximately 37% of the CMV-specific CD137-expressing $CD4^+$T cells produced IFNγ compared to 69% in CMV-seropositive patients (P<0.05). IFNγ producing CMV-specific $CD137^+CD4^+$ T cells all co-expressed CD28 and similar frequencies were present within the CM and EM subset of CMV-seronegative patients. In CMV-seropositive patients a slightly more differentiated phenotype was observed as 16% (P<0.01) of these cells lacked CD28 and 75% were classified as EM T cells (**Fig. 1F**).

In 12 CMV-seronegative and 6 CMV-seropositive patients frequencies of IL-2 producing $CD4^+$ T cells specific for CMV were evaluated as we have previously shown these to be associated with anti-CMV IgG titers (6). CMV-specific $CD137^+CD4^+$ $IL-2^+$ T cells were detected in 9 out of 12 (75%) of CMV-seronegative versus all of the CMV-seropositive patients and median (IQ range) frequencies amounted to 0.01% (0.01%-0.01%) and 0.26% (0.01%-0.87%) for CMV-seronegative and CMV-seropositive patients (P<0.05; **Fig. 1G**). Forty percent of CMV-specific CD137-expressing $CD4^+$ T cells of CMV-seronegative patients produced IL-2 compared to 37% in CMV-seropositive patients. In CMV-seronegative patients almost all IL-2 producing cells co-expressed CD28, in contrast to the presence of CD28null CMV-specific IL-2 producing cells (10%) in the CMV-seropositive group (P<0.01; **Fig. 1H**).

IFN-γ producing $CD137^+CD8^+$ T cells were detected in 11 out of 28 (39%) CMV-seronegative and 11 out of 14 (79%) CMV-seropositive patients. Median (IQ range) frequencies amounted to 0.05% (0.01%-0.12%) and 0.64% (0.22%-1.90%) within the total $CD8^+$ T cell population for CMV-seronegative and CMV-seropositive patients, respectively (**Fig. 1I**, P<0.01). Only 9% of CD137-expressing $CD8^+$ T cells produced


A
B
C
D
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G
H
I
J
CD4+CD137+ T cells (% of CD4+ T cells)
CD4+ T-cell phenotype (% of CD4+CD137+ T cells)
CD8+CD137+ T cells (% of CD8+ T cells)
CD8+ T-cell phenotype (% of CD8+CD137+ T cells)
CD4+CD137+IFNγ+ T cells (% of CD4+ T cells)
CD4+ T-cell phenotype (% of CD4+CD137+IFNγ+ T cells)
CD4+CD137+IL-2+ T cells (% of CD4+ T cells)
CD4+ T-cell phenotype (% of CD4+CD137+IL-2+ T cells)
CD8+CD137+ IFNγ+T cells (% of CD8+ T cells)
CD8+T-cell phenotype (% of CD8+CD137+ IFNγ+T cells)
CMV-seronegative
CMV-seropositive
CD28null
CD28+
naive
CM
EM
EMRA

Fig. 1 (left) Cytomegalovirus **(**CMV)-specific CD137-expressing and cytokine producing T cells. Peripheral mononuclear cells of patients were stimulated for 12-hours in presence of brefeldin A and αCD49d alone or with a mixture of ppp65 and IE-1 overlapping peptide pools. Subsequently, cells are cell surface and intracellular stained to determine frequencies and phenotypic characteristics of CMV-specific CD137-expressing T cells as well as those producing cytokines. CMV-specific CD137-expressing $CD4^+$ **(A)** and $CD8^+$ **(C)** T cells are depicted dissected for $CD4^+$ **(B)** or $CD8^+$ **(D)** T cells co-expressing or lacking CD28 and the different naive and memory T cell subsets. A similar approach is followed for CMV-specific CD137-expressing IFN-γ and IL-2 producing $CD4^+$ [(**E, G)** and dissection in (**F, H**) and $CD8^+$ **(I)** and dissection in panel **(J)**] T cells, respectively. Closed and open symbols/bars (medians of) represent CMV-seronegative and CMV-seropositive patients, respectively. * P<0.05; ** P<0.01, *** P<0.001

IFNγ in CMV-seronegative versus 36% of CMV-seropositive patients (P<0.01). The differentiation status of these cells was similar for both groups and most $IFN\gamma^+$ $CD137^+CD8^+$ T cells were found within the EM and highly differentiated EMRA subsets (**Fig. 1J**). In CMV-seronegative patients, 65% of CMV-specific IFNγ producing cells co-expressed CD28, in contrast to the presence of 89% CD28null IFNγ producing cells in CMV-seropositive patients (P<0.05; **Fig. 1J**). The frequency of CMV-specific $CD137^+CD8^+$ T cells producing IL-2 was low, i.e.median and IQ range amounted to: 0.001% (0.001%-0.001%) and 0.024% (0.01%-0.05%) of the total $CD8^+$ T cells for CMV-seronegative and CMV-seropositive patients, respectively. As less than 5% of CD137-expressing $CD8^+$ T cells produced IL-2, no further dissection into T-cell subsets was done.

The CMV-specific $CD137^+$ $CD4^+$ and $CD8^+$ T cells with a naive phenotype, present at higher frequencies in CMV-seronegative patients (**Fig. 1B & 1D**), did not produce IL-2 (**Fig. 1H**) or IFNγ (**Fig. 1F & 1J**).

Collectively, these data show that in a substantial fraction of CMV-seronegative patients cytokine producing cells are present within CMV-specific CD137-expressing T cells, although at lower frequenciesSimilar to CMV-seropositive patients, these are mainly of the memory phenotype albeit less differentiated as they are more $CD28^+$. No differences were observed when comparing the maximal capacity of T cells to express CD137 (**Fig. S2A and B**) and produce cytokines between CMV-seronegative and CMV-seropositive patients (**Fig. S2C, D and E**).

CMV-specific T cell proliferation in CMV-seronegative patients

In addition to measuring the capacity to exert effector function by producing cytokines, we evaluated CMV-specific T cell proliferation by using both CMV-lysate and the peptide pool of the immunodominant proteins pp65 and IE-1 **(Fig. 2A** and **2B**, typical flowcytometric example). CMV-lysate-induced proliferation was observed in 10 out of 12 CMV-seronegative KT-recipients for $CD4^+$ T cells, respectively. Median (IQ range) percentages of proliferating $CD4^+$ T cells amounted to 2.2.55% (0.88%-7.09%) (**Fig.**

2C). Proliferation in response to the mixture of pp65- and IE-1-overlapping peptide pools was observed in a smaller proportion of CMV-seronegative patients, i.e. 7 and 3 out of 12 KT-recipients for $CD4^+$ and $CD8^+$ T cells (median and IQ range: 0.35% and 0.09%-0.64% (**Fig. 2C**) and 0.33% and 0.19%-1.00% (**Fig. 2E**), respectively). $CD4^+$ T cells of CMV-seropositive patients proliferated vigorously to CMV-lysate. The median percentage of proliferating $CD4^+$ T cells was 36% (**Fig. 2C**). Proliferation in response to the mixture of pp65- and IE-1-overlapping peptide pools amounted to 6.34% (3.47%-24.32%) and 9.64% (4.26%-43.63%) for $CD4^+$ and $CD8^+$ T cells, respectively (**Fig. 2C and E**). In CMV-seronegative patients, most (>97%) of the proliferating $CD4^+$ T cells co-expressed CD28, indicative for a less-differentiated phenotype. In CMV-seropositive patients approximately 10% of all proliferating $CD4^+$ T cells were $CD28^{null}$ **(Fig. 2D)**. This difference in differentiation status was even more pronounced in proliferating $CD8^+$ T cells as 78% of these cells in CMV-seronegative patients expressed CD28 compared to approximately 34% of CMV-seropositive patients ($P<0.05$) (**Fig. 2F**). Frequencies of CMV-specific IFNγ-producing $CD137^+$ $CD4^+$ T cells and IL-2 producing $CD137^+$ $CD4^+$ T cells were positively correlated with percentages of proliferating $CD4^+$ T cells (for IFNγ:Rs=0.63; P=0.02 (CMV-lysate) and Rs=0.71; P=0.02 (pp65/IE-1 peptides), for IL-2:Rs=0.62; P=0.02 (CMV-lysate) and Rs=0.74; P=0.01 (pp65/IE-1peptides)).

Low frequency of CMV-specific IgG ASC in CMV-seronegative patients

Next we evaluated whether CMV-specific memory B cells could be detected in the circulation of CMV-seronegative patients. The frequency of plasma blasts within the circulation is very low but can be induced from B cells upon polyclonal stimulation. The total B cell number and responses may be negatively affected in ESRD patients and therefore we first documented that this protocol can indeed induce plasma blasts. As shown in **Fig. 3A**, plasma blasts were induced ($CD27^+CD38^{high}CD19^+$, from 1.4% to 48.1%) following a 5-day polyclonal stimulation. Next, total IgG and CMV-IgG ASC were quantified by ELISpot. CMV-IgG ASC were detected above background in 4 out of 11 (36%) of CMV-seronegative patients and net median (IQ range) CMV-specific IgG ASC amounted to only $1/10^5$ ($1\text{-}10/10^5$), i.e. 0.08% (0.02%-0.14%) of total IgG ASC (**Fig. 3B and C**). All CMV-seropositive patients had detectable CMV-IgG ASC (median 45 cells/10^5 ($7\text{-}114/10^5$), 0.38% (0.11%-1.70%) of total IgG ASC). Interestingly, CMV-specific IFNγ- as well as IL-2-producing $CD137^+CD4^+$ T cells of the total patient group (CMV-seropositive as well as CMV-seronegative patients) were positively correlated with numbers of CMV-specific IgG ASC (Rs=0.52; $P<0.05$ and Rs=0.53; $P<0.05$, respectively).

CMV-specific $CD137^+$ IFNγ $^+$ $CD4^+$ T cell responses protect against CMV-viremia

The CMV-specific T-cell response in CMV-seronegative patients was evaluated for clinical relevance by relating it to the occurrence of CMV viremia after transplantation. In 13 out of 28 (46%) CMV-seronegative patients a CMV-viremia developed during the first year following transplantation with a CMV-seropositive donor kidney. CMV-seronegative patients with CMV-specific $CD137^+IFN\gamma^+CD4^+$ T cells prior to

Table 2 Effects of CMV-immunity on T-cell parameters prior to transplantation

	CMV^{pos} ESRD patients (N=14)	CMV^{neg} ESRD patients without CMV-specific $CD137^+IFN\gamma^+CD4^+$ T cells (N=15)	CMV^{neg} ESRD patients with CMV-specific $CD137^+IFN\gamma^+CD4^+$ T cells (N=13)	P-value*
T cells	1184 (887-1519)	1039 (636-1360)	1057 (750-1340)	ns
$CD4^+$ T cells	629 (444-786)	714 (373-916)	639 (451-821)	ns
$CD4^+$ T_{naive} cells	110 (73-173)	213 (119-531)	226 (163-354)	ns
$CD4^+$ T_{MEM} cells	535 (292-641)	385 (185-512)	434 (255-641)	ns
$CD4^+$ T_{CM} cells	263 (174-389)	227 (137-366)	283 (176-363)	ns
$CD4^+$ T_{EM} cells	185 (106-301)	116 (100-148)	108 (80-273)	ns
$CD4^+$ T_{EMRA} cells	7 (5-21)	11 (4-18)	8 (3-13)	ns
$CD4^+CD28^{null}$ T cells	29 (3-65)	3 (2-5)	3 (1-20)	ns
$CD4^+CD31^+$ T_{naive} cells	63 (43-123)	170 (61-264)	144 (91-222)	ns
$CD8^+$ T cells	465 (316-618)	257 (168-445)	240 (213-469)	ns
$CD8^+$ T_{naive} cells	84 (39-120)	104 (59-198)	84 (44-179)	ns
$CD8^+$ T_{MEM} cells	349 (271-528)	140 (87-277)	191 (167-246)	ns
$CD8^+$ T_{CM} cells	15 (9-88)	16 (7-30)	25 (13-44)	ns
$CD8^+$ T_{EM} cells	110 (59-144)	85 (46-188)	83 (53-122)	ns
$CD8^+$ T_{EMRA} cells	151 (85-287)	37 (25-69)	41 (18-76)	ns
$CD8^+CD28^{null}$ T cells	228 (85-276)	37 (24-86)	58 (23-67)	ns
$CD8^+CD31^+$ T_{naive} cells	79 (39-118)	98 (57-175)	76 (41-175)	ns
$CD4^+$ RTL	10.20 (9.2-15.2)	11.2 (9.1-17.8)	12.1 (9.4-14.1)	ns
$CD8^+$ RTL	10.85 (7.7-14)	13 (9.7-21.2)	11.4 (9.1-16)	ns

Cell numbers are given in cells/µLblood; data represent median (interquartile range);
* when comparing CMVneg ESRD patients without to those with CMV-specific CD137+IFNγ+CD4+ T cells.
MEM, memory; CM, central memory; EM, effector memory; EMRA, terminally differentiated effector memory (CD45RA+); RTL, relative telomere length given as a %

transplantation had a lower risk to develop a CMV-viremia after transplantation with a CMV-seropositive donor kidney compared to those without (relative risk: 0.43, p=0.03). No differences in percentages of CMV-specific IFNγ producing $CD137^+CD4^+$ T cells were observed between CMV-seronegative patients without and those with a CMV-viremia (data not shown).

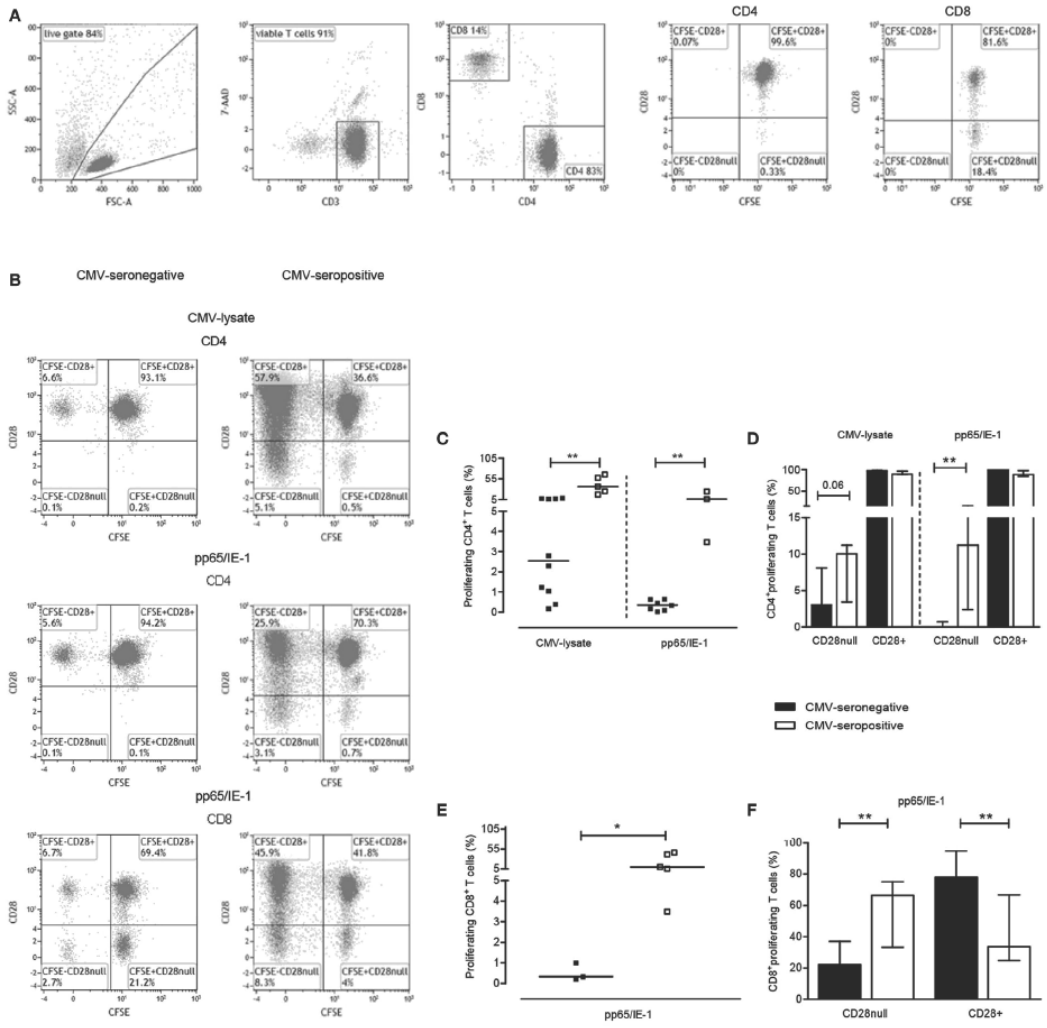

Fig. 2 Cytomegalovirus (CMV)-specific T-cell proliferation. A typical example of the gating strategy for analysis of CMV-specific T cell proliferation is shown. Briefly, based on forward/sideward characteristics live cells are gated and depicted in a dot plot to further select living (7AAD-negative) $CD3^+$ T cells **(A)**. These were then further dissected into $CD4^+$ and $CD8^+$ T cells **(A)**. $CD4^+$ and $CD8^+$ T cells are finally plotted to visualize proliferation (CFSE-dilution) and CD28 expression and a typical example for background proliferation within CD4+ and CD8+ T cells is shown in the last two plots in panel **A**. In panel **B**, typical (detectable) proliferative responses of $CD4^+$ and $CD8^+$ T cells to CMV-lysate ($CD4^+$ T cells alone; upper graphs) or a mixture of overlapping peptides for pp65/IE-1 (both $CD4^+$ and $CD8^+$ T cells; middle and lower graphs, respectively) are depicted for a CMV-seronegative (left panel) and

CMV-seropositive patient (right panel). Percentages of CMV-specific proliferating $CD4^+$ and $CD8^+$ T cells, corrected for frequencies of proliferating $CD4^+$ or $CD8^+$ T cells in absence of a stimulus, are depicted in panels **(C,E)**, respectively. A dissection of uncorrected CMV lysate- or peptide-induced proliferating T cells (set to 100%) into those lacking or expressing CD28 are shown in panels **(D,F)** for $CD4^+$ and $CD8^+$ T cells, respectively. CMV-seronegative (with a detectable response) and CMV-seropositive patients are shown in closed and open symbols, respectively, and bars represent medians and interquartile range of the patient groups. * P<0.05; ** P<0.01

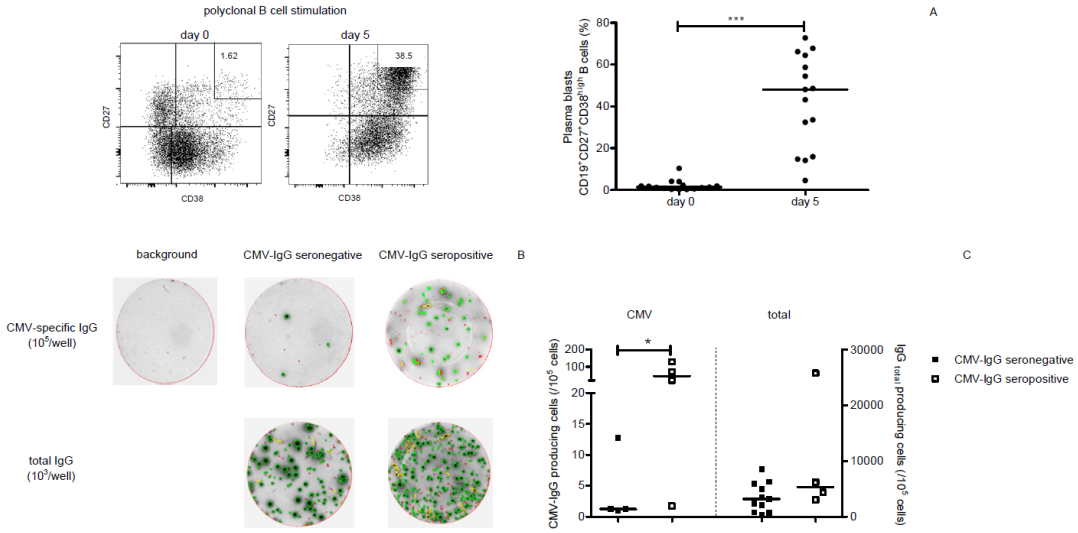

Fig. 3 Cytomegalovirus (CMV)-specific B-cell responses. A B cell ELISpot assay was performed in order to enumerate CMV-specific and total IgG antibody secreting cells (ASC). In panel **(A)**, a typical flow cytometric example is given of B cell blasts, identified as $CD19^+CD27^+CD38^{high}$ cells, prior to an following a 5-day stimulation with R848 and recombinant IL-2 on the left side. On the right side, the individual and median frequencies of B cell blasts are depicted prior to stimulation and following a 5-day stimulation for 15 KT-recipients (11 CMV-seronegative and 4 CMV-seropositive patients). Following B-cell stimulation, cells were transferred at different numbers/well to wells of a 96-well B-ELISpot plate coated with CMV-lysate or an anti-human IgG antibody or not coated (containing PBS; background IgG producing cells). In panel **(B)**, representative examples are shown for background, CMV-specific and total IgG ASC as determined within CMV-seropositive and CMV-seronegative patients. In panel **(C)**, individual and median (detectable) CMV-specific and total IgG ASC, corrected for background (number of spots in absence of a coating antibody) for CMV-seropositive (open symbols) and CMV-seronegative (closed symbols) patients are depicted. * P<0.05; *** P<0.001

CMV-associated T-cell defects mimicking T-cell ageing are not present in CMV-seronegative patients with CMV-specific T cells

CMV latency leaves a clear fingerprint on the T cell immune system. The CMV-associated changes in the circulating T cell compartment of CMV-seropositive ESRD patients comprise of a reduced telomere length, significant expansion of the $CD8^+$ T cell pool and increased differentiation status of the memory T cells, notably the induction of CD28null T cells in both $CD4^+$ and $CD8^+$ T cells (21, 22). However, such changes were not observed when comparing CMV-seronegative patients with and without detectable CMV-specific CD137-expressing IFNγ producing $CD4^+$ T cells (**Table 2**).

DISCUSSION

In the present study, we evaluated the presence of CMV-specific cellular and humoral immunity in a cohort of CMV-seronegative patients and assessed the clinical relevance with respect to the risk for a CMV-viremia after kidney transplantation. Data revealed the presence of a low frequency of CMV-specific T-cells in over half of these patients. Interestingly, in particular IFNγ producing CMV-specific $CD4^+$ T cells were associated with protection from CMV-viremia following transplantation with a kidney from a CMV-seropositive donor. Thus a protective anti-CMV cellular immunity may exist in the absence of serum anti-CMV IgG.

Presence of cellular immunity in the absence of protective antibodies is not uncommon. Possible explanations for this disassociation may be that either cellular immunity is inefficient to induce adequate humoral immunity (6, 7) or that protective antibodies are not adequately maintained. Both these mechanisms may hold true for patients with end-stage renal disease. It is known that T-cell dependent vaccines (e.g. HBsAg, tetanus toxoid, diphtheria) are poor inducers of protective antibody titers in these patients and antibody titers are poorly maintained (23-25). A detailed analysis of the dynamics and quality of the antigen-specific T cell response after vaccination with HBsAg in ESRD patients showed a poor generation of memory $CD4^+$ T cells (6, 7). In particular, the presence of antigen-specific $CD4^+$ EM T cells correlated significantly with the titers of anti-HBsAg and a similar relation could be shown for anti-CMV IgG. The results of this study also showed a statistically significant relation between the presence of IL-2 or IFNγ producing CMV-specific $CD4^+$ T cells and anti-CMV IgG ASC. Therefore, a weak induction of a CMV-specific T cell response may underlie the inadequate generation of an anti-CMV humoral response. In addition, it could still be possible that a low but detectable anti-CMV humoral response was generated initially but not adequately maintained.

Although there may be concerns associated with reliably measuring low frequencies of CMV-specific T cells, the multi-parameter CD137 flow cytometric assay as described previously(17), allows for a detailed characterization of total pool of antigen-specific T cells which is related with other parameters analyzed within this

paper and is associated with clinical outcome, i.e CMV-viremia. The detailed analysis of the CMV-specific response within T cell subsets revealed that CMV-specific memory $CD4^+$ and $CD8^+$ T cells were less differentiated in the CMV-seronegative patients than in CMV-positive patients. This was evident by relatively more $CD28^+$ memory T cells and less CMV-specific T cells producing effector cytokines like IFNγ and IL-2. Of interest was the detection of a CMV-specific $CD137^+$ response in phenotypically naive T cells that did not produce cytokines. These cells may be part of so-called early branched off memory T cells that are phenotypically naive T cells but are in fact memory T cells in an early developmental stage of which some are able to exert effector function (26). Again this finding supports the concept of a less well-differentiated CMV-specific T-cell response. In this respect, a paper by Redeker et al. (27) nicely illustrated, in a mouse model, the large inter-individual variations in the height of the CMV-specific T cell responses to be dependent on the initial viral load which also influences the extent of memory T-cell inflation and phenotype of CMV-specific T cells. CMV-infection generally leads to long-lasting substantial changes in circulating T cells, even more so in ESRD patients(13, 28-31). In particular, highly differentiated EMRA T cells lacking CD28 T cells are expanded in the $CD8^+$ T cell population and to a lesser extent in the $CD4^+$ T cell population (13-15). This typical footprint of CMV-infection in the composition of circulating T cells was not found in the CMV-specific T cells of CMV-seronegative patients. This indicates that these patients have encountered CMV but only mounted a limited T cell response. The most likely explanation is low-level of exposure to viral antigens that may not result in the specific imprint induced by high viral antigen exposure which results in sustained CMV-specific IgG serum titers (32, 33). Furthermore, a proportion of CMV-seropositive patients, had no or low frequencies of CMV-specific $CD8^+$ T cells but had CMV-specific $CD4^+$ T cells as well as CMV-IgG producing cells prior to transplantation, that were sufficient to prevent from a CMV reactivation. This was in contrast with a paper by Tey et al. that described hematopoietic stem cell transplant recipients, a different group of immunocompromised patients, deficient in reconstituting CMV-specific immunity to have higher viral loads (34). Our data are remarkably consistent with a recent study by de Lucia et al. (11) that identified CMV-specific T cell responses in 30% of CMV seronegative individuals. However, their data were generated by ELISpot and therefore lack a more in-depth analysis of the T cell subsets involved. They also claim the detection of CMV-specific memory B cells but frequencies were unfortunately not reported.

In conclusion, assessment of anti-CMV-IgG does not seem to suffice for properly identifying CMV-naive individuals, as a significant proportion has a weak cellular CMV-specific response, which is associated with protection against CMV-viremia after transplantation with a CMV-seropositive donor kidney. We therefore propose to include assessment of cellular immunity in the risk assessment prior to kidney transplantation. Furthermore, monitoring of CMV-specific cellular immunity following transplantation, i.e. at the end of anti-CMV prophylaxis might be useful for further identifying patients at risk for a CMV viremia, requiring additional anti-viral therapy or

adjustment of immunosuppressive medication (35).

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Disclosures
The authors have no conflicts of interest to disclose.

SUPPORTING INFORMATION

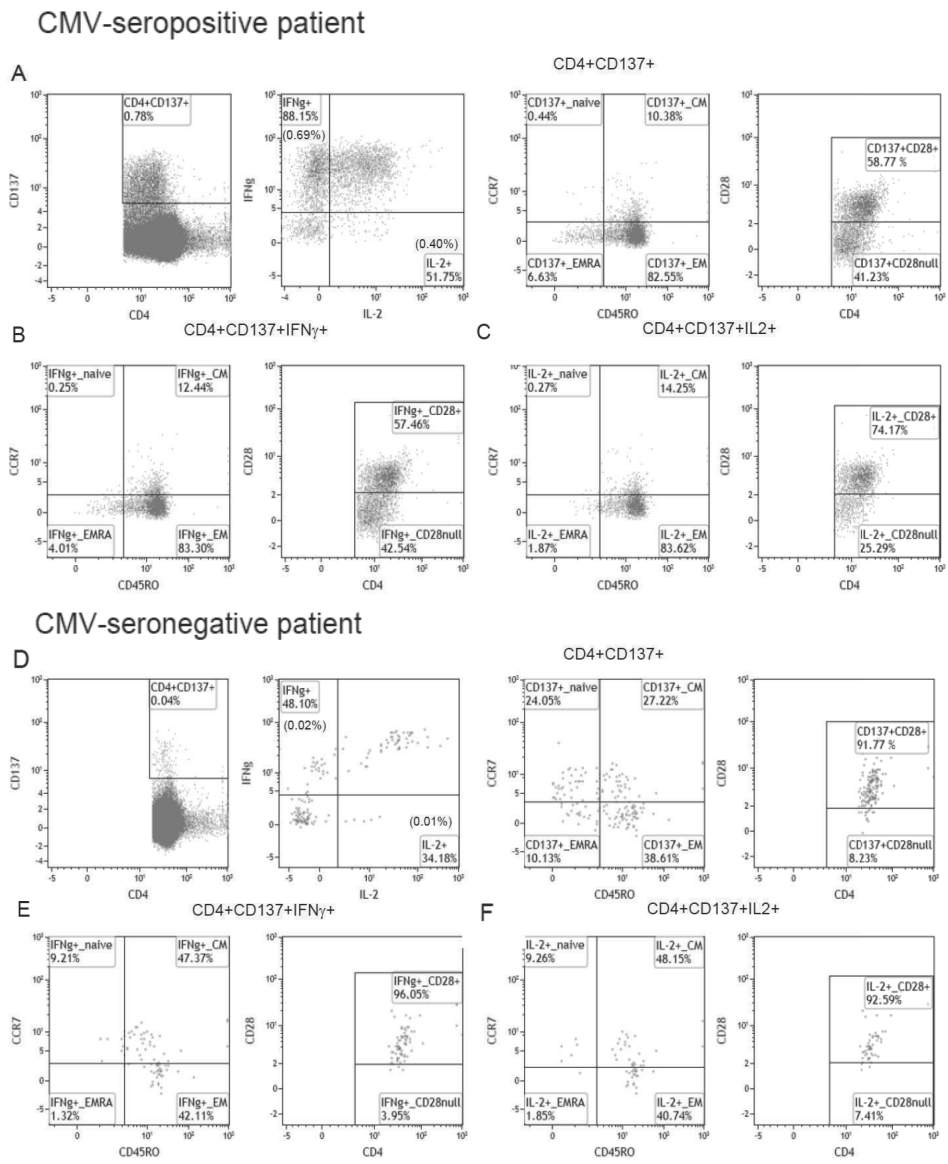

Fig. S1 Typical gating strategy for CMV-specific CD137-expressing and cytokine producing $CD4^+$ T cells. A typical flow-cytometric example is depicted for analysis of CMV-specific CD137-expressing and cytokine producing $CD4^+$ T cells for a CMV-seropositive **(A-C)** and CMV-seronegative **(D-F)** patient. Briefly, based on forward/sideward characteristics live cells

are gated and depicted in a dot plot to further select $CD4^+$ and $CD8^+$ T cells. The CMV-specific CD137-expressing $CD4^+$ T cells are selected [**(A,D)**, first plot] as well as the IFNγ and IL-2 cells within CD137-expressing $CD4^+$ T cells [**(A, D)**, second plot]. CD137-expressing $CD4^+$ T cells are subsequently dissected into naive and different memory T cell subsets [**(A, D)**, third plot] using CCR7 and CD45RO expression ($CCR7^+CD45RO^-$: naive, $CCR7^+CD45RO^+$: CM, $CCR7^-CD45RO^+$: EM and $CCR7^-CD45RO^-$: EMRA) or $CD28^+$ and CD28null T cells [**(A, D)**, fourth plot]. IFNγ **(B, E)** as well as IL-2 **(C, F)** producing CD137-expressing $CD4^+$ T cells were analyzed in a similar way. Furthermore, the same approach was also applied for analysis of CMV-specific CD137-expressing and cytokine producing $CD8^+$ T cells (not shown). Percentages of CD137-expressing $CD4^+$ T cells are of total $CD4^+$ T cells and percentages of $cytokine^+$ within $CD137^+$ $CD4^+$ T cells are given as a proportion of $CD137^+$ $CD4^+$ T cells (set to 100%) and in brackets the frequency of $CD137^+$ $cytokine^+$ within total $CD4^+$ T cells is depicted. The dissection with respect to a certain T-cell phenotype is done by setting the % of $CD137^+$ or $CD137^+cytokine^+$ $CD4^+$ T cells to 100%

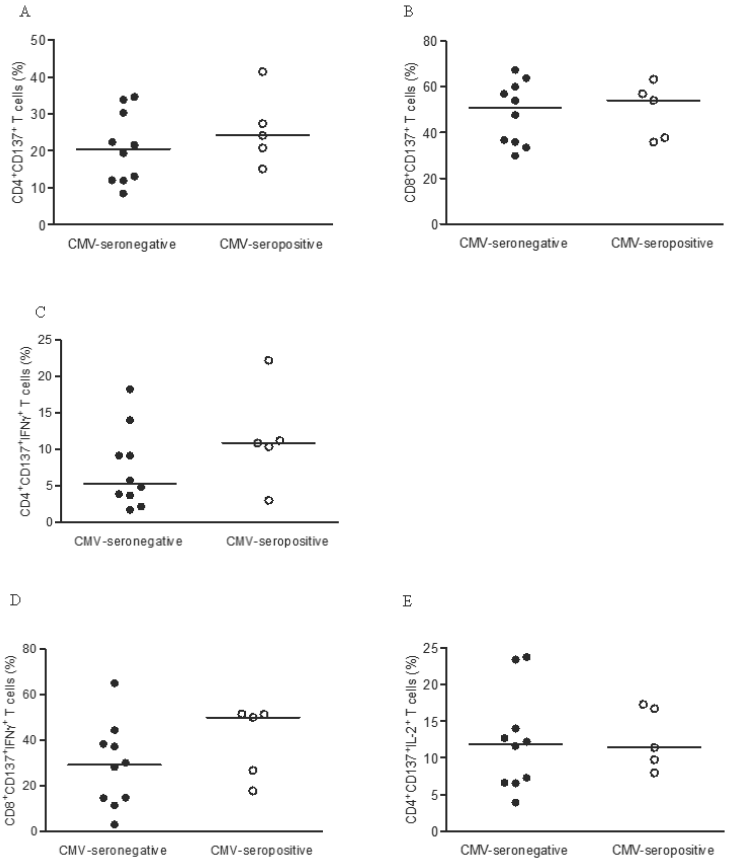

Fig. S2 CD137-expressing and cytokine producing T cells upon stimulation with PMA and ionomycin. Peripheral mononuclear cells of patients were stimulated for 12-hours in presence of brefeldin A and αCD49d alone or with a mixture of PMA and ionomycin. Subsequently, cells are cell-surface and intracellular stained to determine the maximal capacity of T cells to express CD137 and produce cytokines. PMA/ionomycin-induced CD137-expressing $CD4^+$ **(A)** and $CD8^+$ **(B)** T cells, corrected for background (αCD49d only), are depicted as a percentage of total $CD4^+$ or $CD8^+$ T cells. A similar approach is followed for PMA/ionomycin-induced CD137-expressing IFN-g and IL-2 producing $CD4^+$ **(C,E)** and CD137-expressing IFN-g $CD8^+$ T cells **(D)**. Closed and open symbols/bars represent CMV-seronegative and CMV-seropositive patients, respectively.

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CHAPTER 7

Summary and discussion

SUMMARY

The number of patients suffering ESRD, especially the proportion of elderly patients keeps growing rapidly (1). ESRD is accompanied by an impaired T cell immune system (2). This uremia-associated immune deficiency is reflected by a poor vaccination efficacy (3-6), an increased susceptibility for infections (7-9) and a high risk for developing tumors (10-13). Uremia-associated immune deficiency thereby contributes to the increased morbidity and mortality observed in ESRD patients (14, 15). Previous studies have shown that ESRD-associated changes in peripheral T cells are compatible with the concept of premature immunological ageing. This means that T cell characteristics of ESRD patients are on average similar to those of healthy individuals (HI) who are 15-20 years older over a wide range of age (20-80 years). The impact of premature immunological ageing and factors influencing this phenomenon in the elderly ESRD patients (>65 years) is of particular interest given the increase in elderly ESRD patients in recent years. One of the major factors that needs to be taken into account is a previous infection with cytomegalovirus (CMV). CMV is a highly prevalent herpes virus which after primary infection (usually at an early age) establishes latency in the host after leaving a clear foot print in the circulating T-cell population.

Individuals with CMV latency can be easily detected by the presence of anti-CMV antibodies (CMV-seropositive) and the substantial impact on the composition and function of circulating T cells. This CMV-related impact includes changes in T-cell composition which are associated with T-cell ageing. The aim of this thesis is to improve our understanding of the ESRD-associated defective T-cell mediated immune system by evaluating functional in addition to phenotypic aspects, thereby taking into account the impact of CMV. A better understanding is needed, particularly in elderly ESRD patients to facilitate personalized immunosuppression thereby contributing to a better patient outcome after transplantation.

ESRD, the immune status of ESRD patients, the concept of premature ageing, different T-cell ageing parameters and the role of CMV infection are introduced in **chapter 1**. We briefly highlight the traditional parameters for evaluating T-cell ageing and describe relatively new promising tools for evaluating the immune status of an ESRD patient in more detail taking into account phenotypic (TCR Vβ-repertoire diversity) as well as functional (signaling pathways) aspects upon TCR-mediated activation of T cells.

The effects of ESRD and CMV on circulating T cells of elderly ESRD patients were studied in **chapter 2**. CMV latency is associated with enhanced T-cell differentiation in both elderly patients and age-matched HI. The effects of ESRD on T cells mainly exist within the CMV seropositive population. CMV seropositive elderly patients had less thymic output, a reduction in numbers of naive and CM T cells, and their

T cells had shorter telomeres compared with CMV seropositive HI. Most of these ageing parameters were comparable between elderly CMV seronegative patients and elderly CMV seronegative HI except for a lower number of CM T cells observed in elderly CMV seronegative patients. These findings indicate that the premature immunological ageing effect of ESRD in the elderly ESRD patients is very limited in CMV-seronegative patients. However, CMV latency drives to a significant extent the ageing of the peripheral T cell compartment in elderly ESRD patients.

A diverse (polyclonal) TCR Vβ-repertoire capable of recognizing a broad range of foreign antigens is key to an effective T-cell mediated immune response (16) and is known to decrease with ageing (17). The TCR Vβ-repertoire diversity was assessed in ESRD patients by qualitative multiplex DNA-based spectratyping in **Chapter 3;** We found ESRD to skew the TCR Vβ-repertoire and this skewed TCR Vβ-repertoire was mainly observed within isolated $CD8^+$ memory T cells. Using a multiple binary logistic regression model, CMV seropositivity was found to contribute the most to skewing of the TCR Vβ-repertoire followed by ESRD and age. Based on these findings, this skewed TCR Vβ-repertoire was further examined in more detail. In **Chapter 4**, we investigated TCR Vβ-repertoire within T-cell subsets in ESRD patients using a quantitative flow cytometry-based approach, which covers 24 different TCR Vβ-families. The Gini-index, a parameter used in economics to describe the distribution of income, was calculated to determine the extent of skewing. In this study, using age- and CMV serostatus-matched HI as a reference population, we found young ESRD patients to already have significantly higher Gini-TCR indices for different $CD8^+$ memory T cell subsets. ESRD induced expansions of not one TCR Vβ-family in particular and expansions were predominantly observed within the $CD8^+$ T cell compartment. Interestingly, clonal expansions were already observed within naive $CD8^+$ T cells. This skewed TCR Vβ-repertoire may be associated with a less broad and diverse T-cell mediated immunity in ESRD patients.

Important signal transduction pathways engaged upon T-cell activation include ERK and p38, both belonging to the MAPK family, which are known to be affected in the elderly HI. In **Chapter 5**, we studied effects of ESRD on more upstream signaling pathways of TCR-mediated T-cell activation. Young HI had a significantly higher TCR-induced pERK in total $CD4^+$ T cells and subsets compared to elderly HI. Interestingly, this age-associated decline in phosphorylation of ERK was not found in ESRD patients and pERK-levels from young ESRD patients were in between young and elderly HI. The phosphorylation level of ERK was positively associated with frequency of CD69-expressing $CD4^+$ T cells upon TCR-induced activation of T cells. Increased levels of DUSP6 might be one of the mechanism contributing to this defective phosphorylation of ERK as inhibition of DUSP6 significantly increased the pERK level in elderly HI and both young as well as old ESRD patients. In addition, we found a higher baseline level of p38 in the highly differentiated T cells compared with naive T cells, which may contribute to the uremia related defective T-cell immunity.

CMV IgG-seronegative (CMV^{neg}) patients receiving kidneys from CMV IgG-seropositive (CMV^{pos}) donors are at high risk to develop a CMV infection, but still a considerable proportion (30%-40%) of this group does not experience a CMV infection(18). Therefore, we hypothesized that CMV-specific cellular immunity may exist in a fraction of CMV^{neg} patients that may prevent from a CMV infection in CMV^{neg} recipients receiving kidneys from CMV^{pos} donor. In **Chapter 6**, we showed that low frequencies of CMV-specific T cells frequently exist in part of the CMV^{neg} patients and that these cells are of a less differentiated memory phenotype when compared to those from CMV^{pos} patients. CMV-specific IgG-antibody secreting cells could also be detected at low frequencies in a fraction of CMV^{neg} patients. Interestingly, pre-transplant CMV-specific $CD137^{+}IFN\gamma^{+}CD4^{+}$ T cells in CMV^{neg} recipients may protect against CMV viremia after transplantation with a CMV^{pos} donor kidney.

DISCUSSION

The overall aim of this thesis was to improve our understanding of the T-cell mediated immune system in elderly ESRD patients before kidney transplantation by evaluating both phenotypic as well as functional aspects. A more in depth characterization of the T-cell mediated immunity might allow for a proper risk assessment with respect to rejection and facilitate individualized immunosuppressive regimes.
Proper immunosuppressive therapy is essential for a successful kidney transplantation. The ideal protocol is to effectively prevent acute and chronic allograft rejection and also avoid the immunosuppressive drug related adverse effects. Elderly ESRD patients may have a lower risk for rejection compared to young patients (19-22) due to immunosenescence, and be suitable for minimizing immunosuppressive drugs (23). A re-evaluation of current immunosuppressive regimes is warranted especially as the proportion of elderly ESRD patients has increased over the last decades. However, the calendar age may not be similar to the immunological T cell age. Previous research has demonstrated that the uremic environment present in ESRD patients induces premature T-cell ageing and a discrepancy of 15-20 years between the patient's calendar age and immunological age of T cells was noted. A more in-depth analysis of the impact of uremia-associated premature T-cell ageing in elderly ESRD patients is lacking but of significant interest as in these patients the highest impact of T-cell ageing on immune function may be expected. Therefore, a comprehensive assessment of T-cell immunity needs to be established in elderly ESRD patients.

T-cells can be characterized by several (ageing) parameters, including thymic output, phenotyping (assessing both T-cell numbers and differentiation status), telomere length, diversity of TCR repertoire and activation of TCR-induced signaling pathways.

CMV has a substantial impact on the composition and function of circulating T cells. Recent studies have shown that CMV latency expands the number of circulating

$CD8^+$ T cells significantly by almost twofold (24), promotes the emergence of highly differentiated T-cell subsets (25), induces T cell telomere attrition (26), and contraction of the TCR Vβ-repertoire in immunocompetent individuals. This vast expansion of CMV specific T cells may exceed 4% of $CD8^+$ T cells (27) and these anti-CMV T cells clones are stably maintained for 5 years (28).

The effects of ESRD on T cells mainly exist within elderly CMV seropositive patients compared with elderly CMV seropositive HI, as elderly patients had less thymic output, a reduction in numbers of naive and CM T cells, and T cells had shorter telomeres. Increased levels of oxidative stress and inflammatory cytokines caused by retention of uremic toxins (29, 30), together with cumulative exposure to antigens and environmental free radicals during ageing(31), contribute to the pro-inflammatory environment and advanced ageing in elderly ESRD patients. Elevated concentrations of serum or plasma TNF-α are associated strongly with progressive loss of renal function (32) and this TNF-α-induced apoptosis may be an explanation of the loss of naive and CM in elderly ESRD patients (33, 34). In addition, CMV latency leads to a dramatic accumulation of $CD28^-$ T cells in both elderly HI and patients. Higher frequencies of $CD28^-$ T cells are present within $CD8^+$ T cells (up to 20-30%) compared to $CD4^+$ T cells in the peripheral blood (35-37). $CD4^+CD28^-$ T cells are probably involved in destabilizing atherosclerotic plaques and are related with cardiovascular disease in ESRD patients (38). $CD8^+CD28^-$ T cells represent highly cytotoxic T cells producing IFN-γ but no IL-4 and very little IL-2 upon responding to CMV antigen (39). Both uremia and CMV accelerate the ageing process in elderly patients, however, since most differences of ageing parameters between elderly patients and elderly HI are observed within the CMV seropositive population but not CMV seronegative population, CMV infection is a driver of ageing in the elderly ESRD patients. Following a primary CMV infection, CMV becomes latent and keeps challenging the host immune system. Compared with immunocompetent HI, immunocompromised individuals, such as ESRD patients, are supposed to have more highly differentiated T cells responding to CMV virus to keep control over the virus. With increasing age, the CMV seronegative ESRD patients and HI converge in their T cell ageing characteristics when assessed by traditional ageing markers. We speculated ESRD may influence the ageing process in elderly patients in other aspects, then the new T cell ageing parameters were further tested in the elderly ESRD patients.

A diverse TCR Vβ-repertoire is crucial for an effective T-cell mediated immune response. Naive T cells migrating from the thymus into the circulation possess the broadest TCR repertoire (40). The memory T cells that develop upon encountering of an antigen have a repertoire that is being skewed towards particular specificities (41). Ageing induces reduction of naive compartment and relatively expands memory compartment, it also caused the skewing of the TCR Vβ-repertoire composition in elderly population. The TCR Vβ-repertoire can be assessed using several approaches such as gene scan spectratyping (42), flow-cytometry (43-45), and next generation

sequencing (NGS) (46, 47). We used the multiplex DNA-based spectratyping which is a semi-quantitative assessment to have an overview of the TCR Vβ-repertoire in our patient population. Since elderly ESRD patients with CMV seropositivity had less thymic output and a reduction in numbers of naive and CM T cells compared with CMV seropositive HI, it was no surprise that we found CMV-seropositive ESRD patients to have a skewed TCR Vβ-repertoire compared with CMV-seropositive HI. Interesting, the skewed TCR Vβ-repertoire is also observed in CMV-seronegative elderly ESRD patients when compared with CMV-seronegative elderly HI, whilst having similar numbers of naive T cells . This indicates TCR Vβ clonal selection, several clones may be expanded and some clones may have- shrunken meanwhile, in elderly ESRD patients. Then we used a flow cytometry-based approach to evaluate TCR Vβ-repertoire diversity, which results in a more accurate quantitative measurement, assessing percentages of TCR Vβ-families at the T-cell subset level obviating the need for cell sorting. We found ESRD to induce skewing already within naive T cells and but a skewed repertoire was mainly present in highly differentiated memory $CD8^+$ T cells. ESRD did not affect one TCR Vβ-family in particular, indicative of expansions of different clonal origin, that may be induced by different underlying kidney diseases. Moreover, CMV latency induced a vast expansion of CMV-specific T cells, which may also alter the TCR Vβ-repertoire . This skewed TCR Vβ-repertoire composed of selective TCR clones , causes a quick response against specific antigens (e.g. CMV), but in the meantime, the narrowed TCR Vβ-repertoire diversity compromises the possibility to respond to a newly encountered antigen(48).

Studying signaling pathways in immune cells has been a ‘hot topic’ in the last several years. The MAPK pathway including ERK and p38 is one of the major pathways induced upon TCR-stimulation(49). Recently, ERK activity through phosphorylation is reported to be associated with ageing. Elderly HI showed decreased TCR-induced ERK phosphorylation from naive $CD4^+$ T cells compared with young HI (50). Low ERK activity impairs TCR-induced activation, increases sensitivity of cells to apoptosis and reduces cell proliferation (51). As expected, TCR-mediated phosphorylation of ERK in $CD4^+$ T cells of young patients was in between young and old HI (52). This reduced ERK phosphorylation downregulates the early T-cell activation marker CD69 (52) and may be related to a defective activation of T cells in lymph nodes (53). Increased levels of DUSP6 might be one of the factors responsible for this defective phosphorylation of ERK (50). Inhibition of DUSP6 can restore TCR-induced ERK phosphorylation in elderly HI and ESRD patients. Increasing ERK phosphorylation could be a potential target to restore TCR-mediated immune responses and increase for example the vaccination efficiency. We found the baseline of p38 phosphorylation to be positively associated with the differentiation status. The canonical pathway of p38 phosphorylation is mediated by MAPK kinases (MKKs) upon triggering of the TCR (54). However, highly differentiated $CD27^-CD28^-CD4^+$ T cells, accumulated in elderly HI, have an unique MKK-independent mechanism to phosphorylate p38 via 5’ adenosine monophosphate activated protein kinase (AMPK) and transforming growth factor-β-activated protein kinase1 (TAK1)-binding protein 1 (TAB1) (55).

Sestrin increases the baseline of p38 phosphorylation of $CD27^{-}CD28^{-}CD4^{+}$ T cells through AMPK pathway. Silencing of AMPK, TAB1 or sestrin enhances T-cell mediated immunity in elderly HI (56). Therefore, the uremia-mediated defect in TCR-induced phosphorylation of ERK and high baseline phosphorylation of p38, may contribute to the impaired T-cell mediated immune response in ESRD patients.

CMV infection is one of the most common infections in the ESRD patients (57), which usually dramatically changes the T cells composition and function. Interestingly, our data reveal a considerable percentage (>50%) of CMV seronegative ESRD patients have a low frequency of CMV-specific T cells, which is in agreement with others(30%) (58). This emphasizes that the initial viral load of CMV is of importance, since low viral load may lead to CMV-specific cellular immunity to prevent the CMV viremia, but this may not suffice to induce adequate humoral immune responses (59, 60) or to maintain protective antibodies (61). CMV vaccination may have the similar effect to induce cellular response as a low viral load of CMV (62, 63). It may have little impact on the T-cell ageing parameters but protect ESRD patients from CMV infection. CMV vaccination could be essential to prevent excessive ageing in the elderly ESRD patients if they were vaccinated at an early age before ESRD and CMV infection.

We have shown ESRD patients to have a prematurely aged T-cell mediated immune system by assessing several ageing parameters. Ageing and CMV latency may have an additional effect on this defective T-cell mediated immunity. We propose ESRD patients with a low number of naive T cells, high percentage of highly differentiated memory T cell subsets, T cells with short telomeres, skewed TCR Vβ-repertoire, low TCR-induced ERK phosphorylation and high baseline of p38 phosphorylation to represent the low risk group with respect to rejection and potential candidates for lowering immune suppressive drugs. How to define the threshold for each parameter requests a large cohort to be investigated. CMV vaccination at an early age before ESRD and CMV infection maybe a practical strategy to prevent the advanced ageing in the elderly ESRD patients. In fact, the allograft immune response is a complicated process, which may be influenced by the recipients immune system, quality of donor kidney, surgery procedure, dynamic effect of immunosuppressive drugs and so on. It might not be possible to find one specific biomarker to correctly predict the clinical outcomes after renal transplantation such as acute and chronic rejections. However, a comprehensive and careful assessment of the T-cell immune state taking into account the different aspects as mentioned above can contribute to establish an immune profile. Based on those profiles, we would gain more knowledge about how to identify patients with low risk of rejection. Then it would be very interesting to investigate if those low risk patients could benefit from low immunosuppressive drugs treatment.

CONCLUSIONS

In this thesis, we demonstrated that the defective T cell immune system from ESRD patients can be evaluated in more depth using relatively new phenotypic and functional in addition to the traditional T-cell ageing parameters. A declined number of naive, more highly differentiated memory T cells, reduced telomere length, skewed TCR Vβ-repertoire, declined TCR-induced ERK phosphorylation and elevated baseline of p38 phosphorylation are important features of a defective T-cell mediated immunity in ESRD patients. Ageing and CMV infection accelerate this premature T-cell ageing process. In addition, we demonstrate the role of CMV-specific cellular immunity in preventing CMV infection after kidney transplantation. Comprised T-cell mediated immunity seems to be a double-edged sword for ESRD patients. A defective T-cell mediated immune system in ESRD patients, especially elderly ESRD patients, may contribute to a lower vaccination efficacy, more severe infections but on the other hand reduce the risk for allograft rejection after transplantation. Overall, the work described in this thesis provides new insights for evaluating T cell immunity in ESRD patients. Those insights may allow identification of ESRD patients at low risk of a rejection and allow development of strategies for individualizing immunosuppressive regimes.

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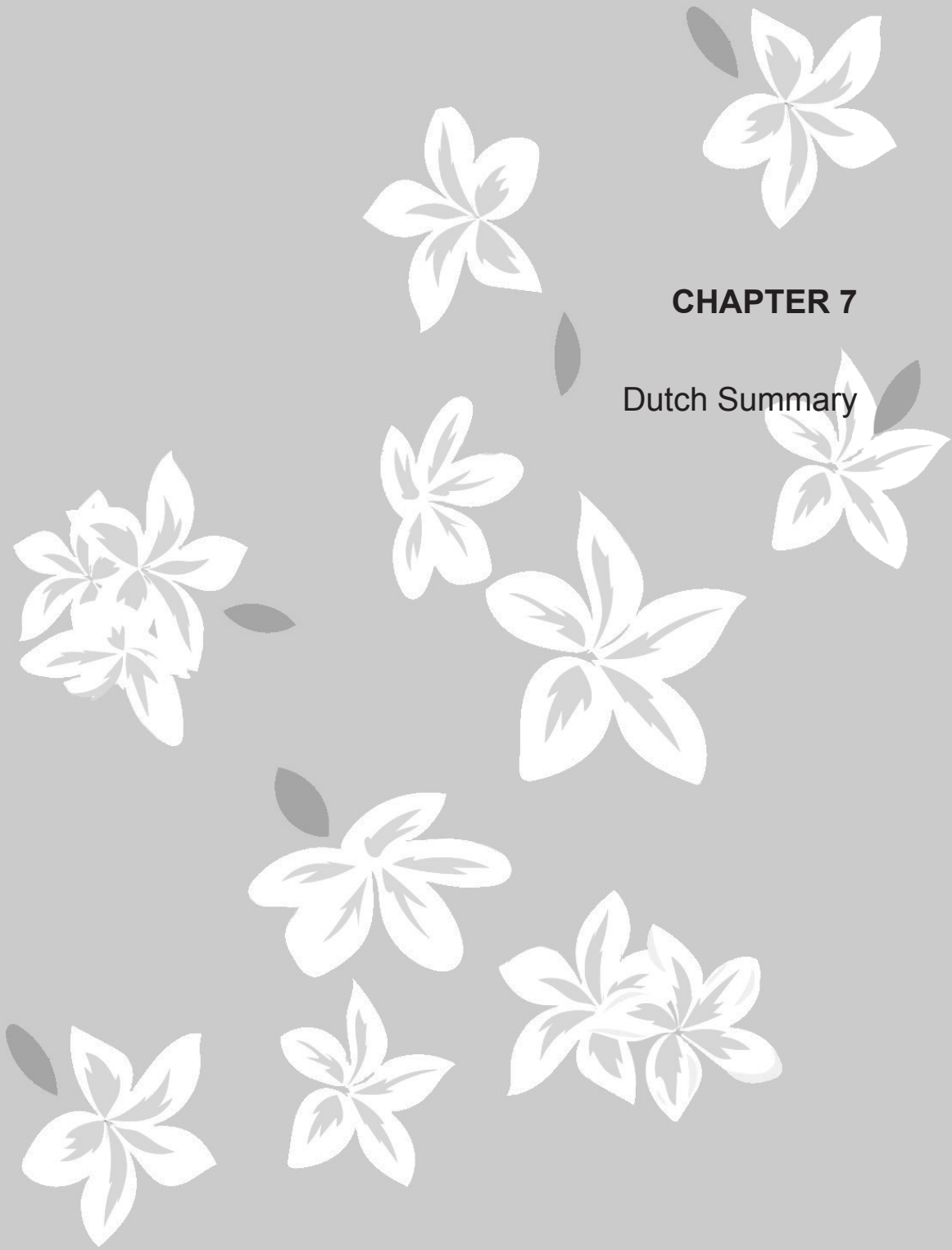

CHAPTER 7

Dutch Summary

De belangrijkste functie van de nier is het verwijderen van afvalstoffen en overtollig vloeistof uit het lichaam. Verschillende aandoeningen waaronder bijvoorbeeld hoge bloeddruk of suikerziekte kunnen leiden tot chronisch verlies van nierfunctie. Het chronisch verlies van nierfunctie kan onderverdeeld worden in 5 stadia, op basis van de overgebleven functie van de nier. Het laatste stadium wordt ook wel eindstadium nierfalen genoemd. De overgebleven nierfunctie is dan minder dan 10 procent van de normale nier capaciteit, wat betekent dat de nier niet meer goed functioneert.

Behandeling van patiënten met eindstadium nierfalen bestaat uit dialyse (hemodialyse or peritoneale dialyse) of niertransplantatie. De voorkeur gaat uit naar niertransplantatie vanwege het feit dat dit leidt tot een betere kwaliteit van leven en een hoger overlevingspercentage. Voor een succesvolle behandeling is er maar één nier nodig. Deze kan gedoneerd worden door een levende of overleden donor. In het algemeen, is het gebruik van een nier van een levende donor beter dan van een overleden donor. Voor een succesvolle niertransplantatie is het levenslang gebruik van afweer onderdrukkende medicijnen van belang om de kans op afstoting van de nier te minimaliseren en te blijven leven. Veel van deze medicijnen zijn gericht tegen T cellen, bepaalde cellen van ons afweersysteem, die een belangrijke rol spelen bij het behoud van het orgaan. Langdurig gebruik van deze afweer onderdrukkende medicijnen gaat echter gepaard met bijwerkingen zoals een verhoogde kans op infecties, ontwikkeling van kanker en veel van deze medicijnen zijn uiteindelijk weer toxisch voor de donornier.

Bijna elke niertransplantatie ontvanger krijgt dezelfde afweer onderdrukkende medicatie voorgeschreven maar er zitten grote verschillen tussen de diverse ontvangers. Zo is de proportie oudere (>65 jaar) patiënten met eindstadium nierfalen sterk toegenomen in de afgelopen jaren. Met het toenemen van de leeftijd verandert echter ook ons T cel afweersysteem. Bovendien hebben wij recent laten zien dat verlies van nierfunctie gepaard gaat met een vervroegde veroudering van het T cel afweersysteem. Het T cel afweersysteem van een jonge patiënt met eindstadium nierfalen is vergelijkbaar aan dat van een 15-20 jaar oudere gezond individu. Er is dus een discrepantie tussen de kalenderleeftijd en de immunologische leeftijd van T cellen. Om de juiste dosering/type afweer onderdrukkende medicatie vast te stellen voor de individuele patiënt is het in kaart brengen van factoren die mogelijk een rol spelen bij o.a. afstoting van een orgaan van belang. Een van deze factoren betreft een zo goed mogelijke karakterisatie van het T-cel gemedieerde afweersysteem.
Het voornaamste doel van dit proefschrift was dan ook om een beter begrip te hebben van de conditie van het T-cel gemedieerde afweersysteem van patiënten met eindstadium nierfalen vóór transplantatie door zowel fenotypische als functionele eigenschappen te bestuderen met daarbij speciale aandacht voor de oudere nierpatiënt.

In **hoofdstuk 1**, wordt het concept premature T cel veroudering geïntroduceerd en passeren de verschillende traditionele en nieuwere (waaronder ook functionele)

parameters om de conditie van T cellen te bepalen in patiënten met eindstadium nierfalen, de revue. De traditionele parameters betreffen het bepalen van de functie van de thymus, T cel differentiatie status, telomeerlengte van T cellen. De functie van de thymus kan worden bestudeerd door de frequentie naïeve T-cellen die net uit de thymus komen en/of de hoeveelheid T cel receptor excisie cirkels te bepalen. Het bepalen van de T cel differentiatie status kan worden gedaan door naast het aantal antigen-onervaren (naïeve) en ervaren (geheugen) T cellen ook eigenschappen die verder onderscheid maken tussen minder en meer gedifferentieerde T cellen te bestuderen. Telomeren zijn repetitieve nucleotiden aan het einde van chromosomen, welke bij elke deling korter worden totdat de cel dood gaat of verouderd. Als nieuwe parameters zal ook diversiteit van het T cel receptor repertoire en belangrijke moleculen bij signaaloverdracht na activatie van de T cel receptor bestudeerd worden. Verlies van nierfunctie, een cytomegalovirus (CMV) infectie en veroudering hebben allemaal invloed op de immunologische leeftijd van patiënten met eindstadium nierfalen, daarom dient rekening te worden gehouden met deze factoren bij het beoordelen van de conditie van het T-cel afweersysteem.

In **hoofdstuk 2**, werden de traditionele alsook een functionele T cel parameter bestudeerd in oudere nierpatiënten. Oudere nierpatiënten hadden een verder gevorderd verouderd T cel afweersysteem wanneer vergeleken met leeftijds-gematchde gezonde individuen voor wat betreft de meeste traditionele parameters. Dit werd met name gezien wanneer de CMV seropositieve individuen werden vergeleken. De T-cel proliferatie capaciteit was niet verschillend tussen patiënten met eindstadium nierfalen en leeftijds- en CMV serostatus-gematchde gezonde individuen.

In **hoofdstuk 3 en 4** werd door middel van een aantal methoden de diversiteit van het T-cel receptor (TCR) repertoire bepaald in patiënten met eindstadium nierfalen en leeftijds- en CMV serostatus-gematchde gezonde controles. Een brede diversiteit is cruciaal voor het ontwikkelen van een effectieve T-cel gemedieerde respons. Patiënten met eindstadium nierfalen hadden een minder divers TCR repertoire dan leeftijds- en CMV serostatus-gematchde gezonde individuen. Een meer gedetailleerde analyse liet zien dat deze gereduceerde diversiteit al zichtbaar was in de naïeve T cellen door een toegenomen aantal expansies van bepaalde TCR receptor families. Echter ook de geheugen en met name de $CD8^+$ T cellen hadden een minder divers TCR repertoire.

Nadat de TCR een antigen tegenkomt, worden een aantal signalen in de cel doorgegeven door middel van activatie (o.a. fosforylatie) van aantal moleculen zodat de T cel verder geactiveerd raakt. Extracellular signal–regulated kinase (ERK) en p38 zijn twee cruciale moleculen voor deze TCR-gemedieerde activatie van T cellen. In **hoofdstuk 5** lieten wij zien dat in patiënten met eindstadium nierfalen de TCR-gemedieerde ERK activatie verminderd was, terwijl het basis niveau van p38 activatie juist was gestegen. Deze verminderde activatie van ERK werd waarschijnlijk

veroorzaakt door een hogere expressie van duale specifieke fosfatasen 6 (DUSP6). Remming van DUSP6 herstelde de activatie (fosforylatie) van ERK. Een CMV infectie is één van de meest voorkomende infecties in niertransplantatie patiënten en ook een factor die de kans op acute afstoting kan vergroten.

Ontvangers die geen CMV antilichamen hebben in het bloed en een orgaan ontvangen van een donor welke een CMV infectie heeft doorgemaakt, maken deel uit van de hoog risico groep en hebben een grotere kans op het ontwikkelen van een CMV infectie na transplantatie. In een deel van deze patiënten is echter wel een cellulaire respons tegen CMV detecteerbaar. In **hoofdstuk 6** werd deze cellulaire respons gericht tegen CMV gekarakteriseerd en het belang voor het ontwikkelen van een CMV infectie na transplantatie bestudeerd. Patiënten zonder CMV-specifieke antilichamen maar met een lage, minder gedifferentieerde, CMV-specifieke cellulaire (T-cel) respons hebben minder kans op het ontwikkelen van een CMV-infectie na transplantatie dan wanneer deze niet meetbaar was.

In feite is de reactie op een transplantaat een gecompliceerd proces, welke kan worden beïnvloed door het immuunsysteem van de ontvanger, de kwaliteit van de donornier, de chirurgische procedure, het dynamische effect van immunosuppressiva en enz. Het is misschien niet mogelijk om één specifieke biomarker te vinden die klinische uitkomstmaten na een niertransplantatie, zoals acute en chronische rejectie, correct kan voorspellen. Een uitgebreide en zorgvuldige beoordeling van de conditie van de T cel door bepaling van de hierboven genoemde aspecten kan echter bijdragen aan het vaststellen van een immunologisch profiel van patiënten (op verschillende tijdstippen). Gebaseerd op deze profielen zouden wij meer informatie kunnen inwinnen met betrekking tot het identificeren van patiënten met bijvoorbeeld een laagrisico op afstoting. Daarnaast zou het interessant zijn om te onderzoeken of deze laag risico patiënten profijt zouden hebben van een lagere dosis van of andere afweer onderdrukkende medicijnen.

Concluderend, we hebben aangetoond dat een verzwakt T-cel afweersysteem van patiënten met eindstadium nierfalen kan worden aangetoond op basis van verschillende verouderingsparameters. Deze patiënten hebben minder naïeve T-cellen en meer gedifferentieerde geheugen T-cellen, kortere telomeren, een minder divers TCR repertoire, minder TCR–gemedieerde fosforylatie (activatie) van ERK en hogere p38 baseline activatie. Veroudering en CMV infectie versnellen deze vroegtijdige verouderingsprocessen. Daarnaast laten wij de rol van CMV-specifieke cellulaire responsen in afwezigheid van CMV-specifieke antilichamen zien bij het verhinderen van een CMV infectie na niertransplantatie met een CMV positieve donornier.

Een verzwakte T-cel gemedieerde afweer lijkt een tweesnijdend zwaard te zijn voor patiënten met eindstadium nierfalen. Verminderde T-cel gemedieerde afweer in patiënten met eindstadium nierfalen, in het bijzonder in ouderen, draagt bij aan

een lage vaccinatie effectiviteit en een verhoogd risico op infecties. Desondanks hebben deze patiënten een laag risico op het krijgen van een afstoting. Over het algemeen biedt het werk dat in dit proefschrift beschreven wordt nieuwe inzichten voor het evalueren van de T-cel afweer bij patiënten met eindstadium nierfalen. Deze inzichten kunnen bijdragen aan de ontwikkeling van een meer gepersonaliseerd afweer onderdrukkende regime na transplantatie en zo de lange termijn bijwerkingen van afweer onderdrukkende medicijnen minimaliseren.

APPENDICES

Curriculum Vitae
List of publications
PhD portfolio
Dankwoord / Acknowledgements

CURRICULUM VITAE

Ling Huang was born in Hancheng, Shaanxi Province, China, on June 9th 1984. From 2002, she started her medical training at Medicine School of Xi'an Jiaotong University. During 2007 to 2009, she did training in Kidney Transplantation department under the supervision of Prof. dr. Wujun Xue and obtained her clinical master degree. In 2009, she started work as a resident in Intensive Care Unit in the Second Affiliated Hospital of Xi'an Jiaotong University for 1 year and 9 months. In August 2011, she came to the Netherlands and joined the 2-year research master program, Infection and Immunity, at Erasmus Medical center (Erasmus MC). Her master thesis was done under the supervision of Prof. dr. T van Gelder and Dr. N. M. Shurker. In May 2013, she was awarded the China Scholarship Council (CSC) scholarship. With the supporting of this scholarship, she conduct her PhD fellowship from October 2013, at Nephrology and Transplantation Division, Internal Medicine department, Erasmus MC, under the supervision of Prof. dr. C.C. Baan, Dr. M.G.H. Betjes and Dr. N.H.R. Litjens. Her PhD project is as the respect to investigate ageing parameters in End-stage renal disease patients and presented in this thesis. After her PHD period, she is going to conduct the training to be a nephrologist at the Seventh Affiliated Hospital, Sun Yat-sen University, in Shenzhen, China.

LIST OF PUBLICATION

Huang L, Langerak AW, Wolvers-Tettero IL, Meijers RW, Baan CC, Litjens NH et al. End-stage renal disease patients have a skewed T cell receptor Vbeta repertoire. Immun Ageing 2015;12:28.

Huang L, Langerak AW, Baan CC, Litjens NH, Betjes MG. Latency for cytomegalovirus impacts T cell ageing significantly in elderly end-stage renal disease patients. Clin Exp Immunol 2016;186(2):239-248.

Huang L, Betjes MGH, Klepper M, Langerak AW, Baan CC, Litjens NHR. End-Stage Renal Disease Causes Skewing in the TCR Vbeta-Repertoire Primarily within CD8(+) T Cell Subsets. Front Immunol 2017;8:1826.

Huang L, Litjens NHR, Kannegieter NM, Klepper M, Baan CC, Betjes MGH. pERK-dependent defective TCR-mediated activation of CD4(+) T cells in end-stage renal disease patients. Immun Ageing 2017;14:14.

Litjens NHR, **Huang L,** Dedeoglu B, Meijers RWJ, Kwekkeboom J, Betjes MGH. Protective Cytomegalovirus (CMV)-Specific T-Cell Immunity Is Frequent in Kidney Transplant Patients without Serum Anti-CMV Antibodies. Front Immunol 2017;8:1137.

Dedeoglu B, de Weerd AE, **Huang L**, Langerak AW, Dor FJ, Klepper M et al. Lymph node and circulating T cell characteristics are strongly correlated in end-stage renal disease patients, but highly differentiated T cells reside within the circulation. Clin Exp Immunol 2017;188(2):299-310.

PHD PORTFOLIO

Name PHD student	Ling Huang
Erasmus MC department	Internal Medicine, Nephrology and Transplantation Division
PhD period	Oct 2013- Jan 2018 (including 4 months maternity leave)
Promoter	Prof. Dr. C.C. Baan
Co-promotor	Dr. M.G.H. Betjes and Dr. N.H.R. Litjens

General Courses

Biomedical Research	2014
Stralingsbescherming, deskundigheidsniveau 5B	2014
Biomedical English Writing	2014
Research Integrity	2015
Biostatistical Methods I: Basic Principles	2015

(Inter)national Conferences

Dutch Society for Immunology (NVVI) congress, Noordwijkerhout, the Netherlands	2013	attended
Science Day, Dep. of Internal Medicine Erasmus MC, Antwerp, Belgium	2014-2017	poster for each year
Bootcongres, Dutch Transplantation Society (NTV), Leiden, the Netherlands	2014	oral
Molecular Medicine Day, Rotterdam, the Netherlands	2014 & 2016	poster for both year
Dutch Nephrology Days, Dutch Society for Nephrology (NND), Veldhoven, the Netherlands	2014	poster
Joint British and NTV congress, Bournemouth,UK	2015	poster
CMV and Immunosenescence, Amsterdam, the Netherlands	2015	attended
Word Transplant Congress (WTC), San Francisco, USA	2015	poster
European Society for Organ Transplantation(ESOT) congress, Brussels, Belgium	2015	Brief Oral
Bootcongres, Groningen, the Netherlands	2016	poster
ATC, Boston, USA	2016	poster
Joint British Society for Immunology (BSI) and NVVI congress, Liverpool, UK	2016	poster
Bootcongres, Zeist, the Netherlands	2017	poster
ATC, Chicago, USA	2017	Poster
ESOT,Barcelona, Spain	2017	poster & brief oral

PHD PORTFOLIO (continued)

Scientific Awards	
China Scholarship Council (CSC) Scholarship	2013
Travel Grants	
Erasmus Trustfonds grant	2015
Membership	
Dutch Transplantation Society (Nederlandse Transplantatie Vereniging, NTV)	2014- present
Dutch Society for Immunology (Nederlandse Vereniging voor Immunologie, NVVI)	2014- present

Prof. dr. C.C. Baan, dear Carla, thank you very much for supervising my research projects This thesis cannot be completed without your help. I hope my experience here would encourage future students with CSC scholarship to pursue their research at our department and would facilitate future collaboration between Erasmus MC and Chinese hospital.

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Diya, thanks for being my paranymph, and a good friend. We have such deep mutual trust as if you are my sister, with whom I can share almost everything. Thanks for your understanding, caring and support, which made my happiness double and the sadness half. You are such an excellent researcher with ambitions. Good luck with your thesis and defence! I wish you all the bests in your career and loads of happiness with **David**. You and David are always more than welcome to visit us in Shenzhen.

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