UREMIA-ASSOCIATED T-CELL AGEING WITH REGARD TO KIDNEY TRANSPLANTATION

Rudolfus Willem Johannes Meijers

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It is possible to fail in many ways...
while to succeed is possible only in one way.

Aristoteles

TABLE OF CONTENTS

1

GENERAL INTRODUCTION AND OUTLINE

INTRODUCTION

The kidneys are essential for the removal of water soluble waste products from the blood which are derived from oral intake or produced during metabolism. Furthermore they have an important role in maintaining a homeostatic balance in the blood pressure, the pH of the blood and are a source of essential hormones like erythropoietin and active vitamin D. In the Netherlands, one million people are suffering from chronic kidney disease (CKD) (1) which is highly associated with hypertension, diabetes, glomerulonephritis, obesity, cardiovascular diseases and ageing. (2) Based on the degree of kidney function (as measured by glomerular filtration rate (GFR)), CKD can be classified into five stages. (2) Individuals in stage 5 have a kidney function which is so poor that renal replacement therapy (RRT) is usually necessary. Stage 5 CKD is known as end-stage renal disease (ESRD). (1) The two most common types of RRT are hemodialysis (HD) and peritoneal dialysis (PD). (3) However, kidney transplantation (KT) is the best treatment to regain renal function and to improve quality of life. (4)

IMMUNE STATUS OF ESRD PATIENTS

Loss of renal function causes retention of uremic toxins and cytokines which are key mechanisms in generating oxidative stress and inflammation (Figure 1). (5, 6) This creates a pro-inflammatory environment in which immune and non-immune cells respond by expression of pattern recognition receptors (PPRs) which are normally involved in removal of pathogens but are also expressed during cellular stress. (7) The expression of PPRs is upregulated in response to the secretion of reactive oxygen species (ROS) which is a product of oxidative stress. (6, 8) Normally, activated immune cells secrete ROS in response to invading pathogens. ROS is able to promote apoptosis, proliferation and inflammation by interference with signal transduction pathways and transcription factors but can also directly interact with deoxyribonucleic acid (DNA) and cause damage. (6) The cells of the immune system respond by producing even more pro-inflammatory cytokines.

Moreover, oxidative stress results in the production of advanced glycation end products (AGEs), which are recognized by the receptor for AGE (RAGE) that is expressed in response to inflammation by almost all cell types. (9) Activation of the RAGE pathway results in activation of the pro-inflammatory transcription factor nuclear factor κB (NFκB) which leads to an inflammatory response. (9) In addition, oxidized low-density lipoprotein (LDL) might contribute to dysfunction of T cells, which are members of the adaptive immune system. LDL might interact with the T-cell receptor (TCR) present on T cells which results in stimulating activation-induced T-cell apoptosis. (10)

The pro-inflammatory milieu in ESRD patients is strongly associated with a decreased immunity. For instance, the uremia-induced activation of T cells results a higher expression of cytokine receptors (including interleukin (IL)-2 and tumor necrosis factor (TNF) receptors) but a subsequent decreased responsiveness of activated cells

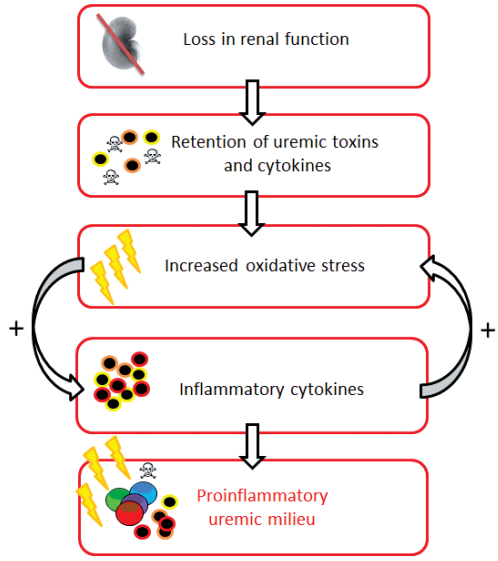

Figure 1: The cause of the pro-inflammatory environment in ESRD patients. Loss in renal function causes retention of uremic toxins and cytokines, leading to increased oxidative stress. This may trigger increased production of inflammatory cytokines by immune cells which will fuel the generation of oxidative stress. Both contribute to the pro-inflammatory uremic milieu in ESRD patients.

to exogenous IL-2 and TNF-α, a phenomenon known as tachyphylaxis. (11, 12) In addition, the activation-induced apoptosis of particular the naïve antigen-inexperienced T-cell population (13) in combination with the expansion of pro-inflammatory (antigen-experienced memory) T cells play an important role in uremia-associated immune dysfunction of ESRD patients. This decreased immunity results in an increased prevalence of infections, which may further contribute to the systemic inflammatory milieu. (14)

The typical uremia-associated changes in the peripheral T cell compartment are reminiscent of age-related changes in healthy individuals. Therefore, it was hypothesized that the mechanism by which a chronic inflammatory milieu affects the T-cell immune compartment can be explained by the concept of premature immunological ageing. Recently, this has been proven and it was concluded that the T-cell age of ESRD patients is approximately 20 to 30 years older compared to an age-matched healthy individual. (15).

THE CONCEPT OF T-CELL AGEING

Physiological ageing of a healthy individual is accompanied by ageing of the T-cell compartment comprising of phenotypical and functional changes. Hematopoietic stem cells (HSCs) which are generated in the bone marrow, give rise to myeloid as well as lymphoid progenitor cells. (16) T cells are generated from the latter. With increasing age, HSCs are skewed towards myeloid-generating subsets at the expense

of lymphoid-generating HSCs, resulting in a lower number of progenitor T cells. These progenitor T cells are further "educated" in the thymus in which naïve T cells will form specific receptors on their cell surface known as T-cell receptors (TCRs). With increasing age, the thymus involutes (17, 18) which already starts at birth and is accelerated during adolescence. (19) This process involves a decrease in functional tissue in combination with a loss of tissue organization with the net outcome that numbers of naïve T cells leaving the thymus, known as recent thymic emigrants (RTEs) are reduced.

RTEs can be identified by their high content of T-cell excision circles (TRECs). During the formation of the T-cell receptor (TCR) in the thymus, DNA sequences in the TCR loci are deleted and circularized into episomal DNA molecules, so called single joint TCR excision circles (TREC), a process known as TCR rearrangement. (20) This TREC remains in the newly formed naïve T cells leaving the thymus. Upon replication of these cells in the periphery, the TREC is only transferred to one daughter cell resulting in a reduction of TRECs in the naïve daughter T cells. Next to the TREC content, the expression of CD31 can be used to distinguish naïve T-cells who are leaving the thymus (known as recent thymic emigrants (RTEs)) and naïve T-cells who are homeostatically driven peripheral expanded. (21, 22). CD31, known as platelet endothelial cell adhesion molecule (PECAM-1) is expressed by RTEs but is lost after after TCR-triggering the expression. (21, 22)

Measuring attrition of telomeres as a marker for the proliferative history of T cells, can be used as a second approach to assess a T-cell age. (23) A telomere is a region of repetitive nucleotides which is located at the end of each chromosome and prevents chromosomal instability during proliferation. (24) However, a decline in telomere length is observed after many cell divisions and has been linked to an increased risk for tumor development and to ageing of a cell. (23) A commonly used method to assess a telomere length is the fluorescent *in situ* hybridization (FISH) method. (25, 26) During this procedure a labeled peptide nucleic acid (PNA) probe binds to the telomere repeats which can be read-out by fluorescence measurements using a flow cytometry (flow FISH). (25) By measuring a cell-line as internal control a relative telomere length (RTL) can be calculated. Inclusion of antibodies in this method makes it possible to analyze the telomere length in different T-cell populations (i.e. $CD4^+$ and $CD8^+$ T cells). (25) The enzyme telomerase protects the telomere by elongation of the end of a chromosome. (27) This might influence the data obtained from the telomere length analysis and measuring the activity of telomerase may give additional information on the telomere shortening. (28)

Assessing the differentiation status of the T-cell compartment by immunophenotyping can be used as a third ageing parameter. After encountering and activation by an antigen presented by an antigen-presenting cell (APC), a naïve T cell will proliferate and differentiate into a memory T cell. During physiological ageing the population of memory T cells will increase at the expense of the naïve population. Based on the expression of the chemokine (C-C motif) receptor 7 (CCR7), enabling cells to migrate to secondary lymphoid organs, and CD45RO, an isoform of the leukocyte common antigen

expressed on memory T cells, a distinction within the memory T-cell compartment can be made. The different memory T-cell subsets include central memory (CM) ($CCR7^+$ and $CD45RO^+$), able to home to secondary lymph nodes and producing mainly interleukin (IL)-2 which is necessary for the proliferation of T cells, effector memory (EM) ($CCR7^-$ and $CD45RO^+$), able to migrate to peripheral tissues exerting direct effector functions and terminally differentiated effector memory $CD45RA^+$ (EMRA) ($CCR7^-$ and $CD45RO^-$), which exert cytotoxic activities and are highly susceptible to apoptosis. (29) Moreover, these terminally differentiated cells often lose the expression of the co-stimulatory molecule CD28 which makes them less dependent on co-stimulation to become activated. (30)

Analyses of the T-cell compartment based on these ageing parameters revealed that the immunological T-cell age of ESRD patients is increased by 20 years compared to their chronological age. (15) Compared to an age-matched healthy individual, ESRD-patients have lymphopenic naïve T-cell number due to a decreased thymic output and more differentiated T-cell compartment with loss of CD28-expression on the cell-surface. Furthermore, the circulating T cells have an overall decline in their telomere length (Figure 2). (15)

THE ROLE OF CYTOMEGALOVIRUS

Cytomegalovirs (CMV) is a member of the β-herpesviruses with a high prevalence among individuals (40%-90%, depending on the socio-economic and ethnic background). (31) An acute or persistant infection is under healthy conditions asymptomatic. (31) CMV-seropositivity has a profound effect on circulating T cells as approximately 10% of these cells are CMV-antigen specific in CMV-seropositive individuals. (31) In addition, CMV has been implicated in immunological ageing as it was found that CMV-seropositive individuals have a more differentiated memory T-cell compartment with a loss of CD28-expression and a decreased CD4/CD8 ratio. (32, 33) Furthermore CMV-seropositive individuals had decline in their T-cell telomere length, indicating an increased proliferative history of T cells. (34) Because of the high prevalence of CMV-seropositivity in ESRD patients (30%-100%) (35), CMV-seropositivity must be taken into account in the assessment of T-cell ageing. In immunocompromised patients, like KT-recipients, a CMV-infection increases the morbidity and mortality of recipients. (36) For that reason CMV-seropositive kindey transplant recipients are treated with anti-viral prophylaxis.

CLINICAL CONSEQUENCES OF THE AGED IMMUNE SYSTEM FOR ESRD PATIENTS

The uremia-associated dysfunctional immune system has a substantial clinical impact leading to an increased morbidity and mortality. Briefly, due to the premature age related T-cell dysfunction, ESRD-patients have a higher risk for infections (37), an increased susceptibility for malignancies and a severe risk for cardiovascular diseases.

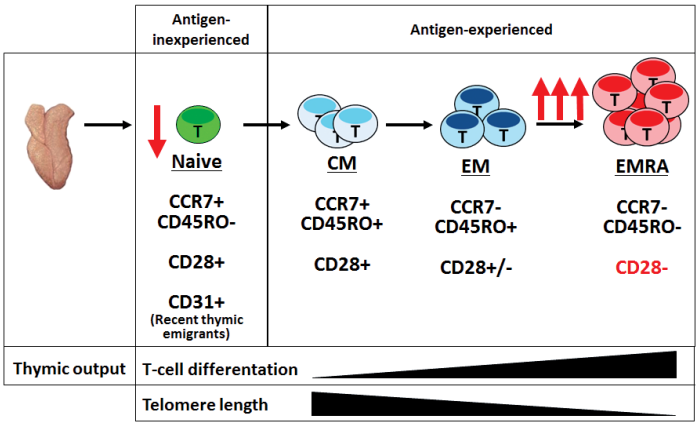

Figure 2: Schematic preview of the T-cell immune system in ESRD patients. The T-cell compartment can be dissected into a naïve antigen-inexperienced population and into an memory antigen-experienced T-cell population. Moreover, based on the expression of CCR7 and CD45RO, the memory T-cell population can be dissected into a central memory (CM), effector memory (EM), and effector memory $CD45RA^+$ (EMRA) population. ESRD patients have a reduced thymic output of naïve T-cells resulting in a lower number of naïve T-cells. Furthermore, they have a higher T-cell differentiation status resulting in an increased number of EMRA T cells which often do not express co-stimulatory molecule CD28. These cells have a higher proliferative history resulting in a reduction of their telomere length.

Low numbers of especially $CD4^+$ T cells are associated with a higher risk for opportunistic infections. (38) Furthermore, by hepatitis-B virus (HBV) vaccination it was shown that the formation of antigen-specific $CD4^+$ T cells is severely impaired in ESRD patients which was associated with low frequencies of HBV-specific antibodies. (39) The increased susceptibility to apoptosis of T cells (15) might result in a loss of antigen-specific T cells and the inability to maintain protective antibody titers after T-cell dependent vaccinations (40, 41) or after a natural infection (42, 43).

The impaired production of IL-2 in combination with an inadequate T-cell proliferative capacity results insufficient T-cell responses. (11, 39, 44) This in combination with a lymphopenic T-cell number leads to inadequate T-cell responses directed to viruses or other pathogens and a decreased tumor surveillance which significantly increases the risk for virus-associated tumors. (45, 46)

The high numbers cytotoxic $CD4^+CD28$null T cells are highly associated with the increased risk for cardiovascular diseases. (47-50) These cytotoxic cells are present in unstable atherosclerotic plaques and are associated with an increased risk for recurrence of both acute coronary events and ischemic stroke resulting in a higher mortality rate. (49)

As KT is the best therapeutic option to ameliorate renal function, the clinical relevance of the uremia-induced premature aged T-cell immunity in the context of KT might be of importance. T cells play an important role in allograft rejection and many of the immunosuppressive medications are targeting T cells to prevent/minimize reactivity towards the allograft. (51) Currently, the immunosuppressive medication is

standard with no consideration for individual variations in the degree of defective T-cell mediated immunity (covering both phenotypic as well as functional aspects). It is likely, that patients with a higher degree of defective T-cell mediated immunity will be over-immunosuppressed. Over-immunosuppression increases the risk for infections and cancer after KT. (52) This raises the need for personalized immunosuppression that can only be realized upon availability of reliable parameters allowing for patient-tailored immunosuppression. Assessing the degree of the uremia-associated dysfunctional T-cell immunity using T-cell ageing parameters and its relation to acute rejection and infections after KT might facilitate personalized immunosuppression.

THE AIM AND OUTLINE OF THIS THESIS

As the uremia-associated premature T-cell ageing results in a decreased immunity in ESRD patients, this might be of clinical relevance in the context of kidney transplantation with respect to the risk for allograft rejection and infections. Assessment of the T-cell age might be a tool to identify patients with a high risk for allograft rejection and to facilitate individualization of immunosuppressive regimens to prevent over-immunosuppression and its associated clinical complications. Therefore **the aim of this thesis** is to establish the degree of ESRD-related premature T-cell ageing by identifying possible factors which influences the immunological T-cell age. Furthermore a possible association between the degree of T-cell ageing and the risk for allograft rejection post-KT is examined.

In **chapter 1** the concept of T-cell ageing in ESRD patients is introduced and its clinical implications in the field of transplantation are explained. **Chapter 2** describes the effects of uremia and RRT on the T-cell ageing parameters used to establish an immunological T-cell age. From studies in healthy individuals it is known that CMV leaves a fingerprint on the T-cell system and has an influence on the ageing parameters. Hence, in **chapter 3** we examine if CMV has an additional effect on the immunological T-cell age in ESRD patients. Kidney transplantation reduces the pro-inflammatory milieu by improving renal function. The effect of kidney transplantation on T-cell ageing parameters is examined in **chapter 4**. Since T cells play an important role in allograft rejection, **chapter 5** examines if establishing the degree of premature T-cell ageing can be used as a predictive value for the risk for AR within the first 3 months post-KT. **Chapter 6** describes the effects of a primary CMV-infection on peripheral T cells under immunosuppression and valganciclovir prophylaxis in CMV-seronegative recipients receiving a kidney from a CMV-seropositive donor. **Chapter 7** presents a review in which the concept of uremia-associated age related changes of T cells is highlighted with the focus on the assessment of an immunological T-cell age, its clinical implications and possible therapeutic options for ESRD patients. Finally, in **chapter 8** all the results obtained in this thesis are summarized and discussed.

2

UREMIA CAUSES PREMATURE AGEING OF THE T-CELL COMPARTMENT IN END-STAGE RENAL DISEASE PATIENTS

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ABSTRACT

Background End-stage renal disease (ESRD) patients treated with renal replacement therapy (RRT) have premature immunologically aged T cells which may underlie uremia-associated immune dysfunction. The aim of this study was to investigate whether uremia was able to induce premature ageing of the T-cell compartment. For this purpose, we examined the degree of premature immunological T-cell ageing by examining the T-cell differentiation status, thymic output via T-cell receptor excision circle (TREC) content and proliferative history via relative telomere length in ESRD patients not on RRT.

Results Compared to healthy controls, these patients already had a lower TREC content and an increased T-cell differentiation accompanied by shorter telomeres. RRT was able to enhance $CD8^+$ T-cell differentiation and to reduce $CD8^+$ T-cell telomere length in young dialysis patients. An increased differentiation status of memory $CD4^+$ T cells was also noted in young dialysis patients.

Conclusion Based on these results we can conclude that uremia already causes premature immunological ageing of the T-cell system and RRT further increases immunological ageing of the $CD8^+$ T-cell compartment in particular in young ESRD patients.

BACKGROUND

Loss of renal function is related to impaired function of the T-cell-mediated immune system. Changes in T-cell subsets and function may underlie this effect. (13, 15) Clinical consequences of this T-cell-mediated immune dysfunction are a reduced efficiency of vaccination (39, 53), an enhanced susceptibility for infectious diseases (54) and an enchanced risk for developing auto-immune diseases and tumors. (55)

T cells leave the thymus as naïve cells. Upon encountering of antigens presented by antigen presenting cells, naive T cells will differentiate into effector T cells and eventually only a fraction of these will develop into memory T cells. The expression of (chemokine (C-C motif) receptor CCR7 and CD45RO can be used to distinguish between the different T-cell subsets, i.e. naïve ($CD45RO^-CCR7^+$), central memory (CM, $CD45RO^+$ and $CCR7^+$, able to home into lymphoid tissues), effector memory (EM, $CD45RO^+$ and $CCR7^-$, exerting direct effector functions) and the more terminally differentiated effector memory $CD45RA^+$ (EMRA, $CD45RO^-CCR7^-$, high in effector function) subset. (13, 56, 57) In addition, the loss of cell surface CD28 expression identifies more differentiated T cells. (58)

During ageing in healthy individuals, the thymic output of new naïve T cells reduces due to the involution of the thymus. Absolute T-cell numbers are largely conserved by homeostatic proliferation of both naïve and memory T cells but eventually this leads to a reduced population of naïve T cells and a relatively preserved population of memory T cells. (19) Elderly individuals have a marked decrease in naïve T cells, a decline in CD4/CD8 ratio and a relative increase in the number of differentiated memory T cells lacking CD28. (19, 33, 59)

The thymic output of new naïve T cells can be determined by measuring the T-cell receptor excision circles (TRECs). (20) These TRECs are small circular DNA episomes that are formed during rearrangement of the T cell receptor (TCR) genes in T cells that are present in the thymus. These TRECs are not replicated and therefore diluted with every cell division. Another hallmark of ageing is the reduction in telomere length. (60, 61) Telomeres are small DNA sequences located at the end of a chromosome and with increasing age they become shorter due to the inability of telomerase to elongate these DNA sequences upon division. Together, TREC content and telomere length reflect the thymic output and replicative history of T cells and may provide a valuable tool to estimate the immunological age of the T cells within an individual.

Using these ageing parameters, the T-cell system of a dialysis patient shows severe T-cell ageing and resembles that of a 20-30 year older healthy individual. (15) This premature ageing of the T-cell system probably underlies the uremia-associated immune defect in dialysis patients. However, it is not known whether the T-cell system of ESRD patients not on renal replacement therapy (RRT) shows a similar degree of immunological ageing and to what extent this is influenced by RRT.

In this cross-sectional study, we have analyzed the T-cell ageing parameters in ESRD patients who are not on RRT and compared this to healthy individuals on one hand, and patients treated with RRT (i.e. hemodialysis or peritoneal dialysis) on the other hand.

RESULTS

T-cell ageing parameters in hemodialysis and peritoneal dialysis patients

Initially, T-cell numbers and ageing parameters of ESRD patients treated with hemodialysis were compared to peritoneal dialysis patients but no statistically significant differences were observed. Therefore, the data of these two RRT groups were combined for comparison with the data of ESRD patients not on RRT (non-RRT group). The clinical and demographic characteristics of patients and healthy controls are shown in Table 1. Compared to the old ESRD patient group, the young patients showed a different distribution of underlying kidney diseases (less frequently hypertensive nephropathy and more frequently reflux nephropathy). Moreover, the young RRT group had a longer history of dialysis treatment than the old RRT group. However, type of underlying kidney disease and dialysis vintage were not significantly associated with any of the ageing parameters measured.

$CD4^+$ T-cell differentiation is increased in ESRD patients but marginally affected by RRT

The young and old non-RRT groups had significant lower numbers of $CD4^+$ T cells when compared to age-matched HC. In the young non- RRT group, a lower absolute number of memory T cells and comparable numbers of naïve T cells were observed when compared to that of age-matched HC. Especially in the old non-RRT group, the $CD4^+$ T cells were shifted towards the memory phenotype with significant less naïve T cells when compared to age-matched HC (Figure 1A). The $CD4^+$ memory compartment of this non-RRT group contained significantly ($p<0.001$) lower numbers of central memory T cells, resulting in a relative increase of more differentiated effector-memory T cells when compared to that of age-matched HC (Figure 1B). No differences were observed if RRT patients were compared to age-matched non-RRT patients, with respect to the composition of the total $CD4^+$ T cell population (Figure 1A) and differentiation of memory $CD4^+$ T cells (Figure 1B).

Elderly, but not young, non-RRT patients had a more differentiated memory phenotype when compared to that of age-matched HC (Figure 1C) based on the percentage of CD28null memory T cells (7.69±1.46% versus 3.83±0.49%, $p<0.05$). RRT only resulted in a significant ($p<0.05$) higher percentage of $CD4^+$CD28null memory T cells in the young patient group (Figure 1C).

$CD8^+$ T-cell differentiation is increased and significantly different in the young RRT patients

Compared to the $CD4^+$ T-cell compartment, the number and differentiation of circulating $CD8^+$ T cells was more affected in non-RRT patients. On average, absolute numbers of $CD8^+$ T cells were lower compared to age-matched HC which was largely attributable to a significant decrease in memory T cells in young non-RRT patients,

Table 1: Study population characteristics

	End-stage renal disease patients not receiving renal replacement therapy		End-stage renal disease patients receiving renal replacement therapy		Healthy controls	
Group	**Young**	**Old**	**Young**	**Old**	**Young**	**Old**
Number of individuals	22	33	49	49	55	65
Age in Years	33.5 ± 9.6*	64.4 ± 6.3*	35.7± 8.5*	63.1± 8.7*	38.4 ± 7.9*	63.5 ± 7.5*
Male	77.3%	60.6%	55.1%	71.4%	49.1%	38.5%
CMV positive	54.5%	51.2%	59.2%	53.1%	50.9%	49.2%
Hemodialysis			25	32		
Peritoneal dialysis			24	17		
Duration of RRT (years)			4.30 (0.1 - 22)**	2.76 (0.2 - 18)**		
Underlying kidney disease						
- Hypertensive nephropathy	13.6%	27.2%	20.8%	30.6%		
- Primary glomerulopathy	31.8%	18.2%	18.8%	18.4%		
- Diabetic nephropathy	0%	21.2%	2.1%	8.2%		
- Polycystic kidney disease	9.1%	9.0%	0%	2.0%		
- Reflux nephropathy	18.2%	3.0%	14.6%	4.1%		
- Other	9.1%	15.2%	20.9%	8.2%		
- Unknown	18.2%	6.0%	22.9%	28.6%		

* Data are given in means with standard deviation.
*** Data are given in means with range*

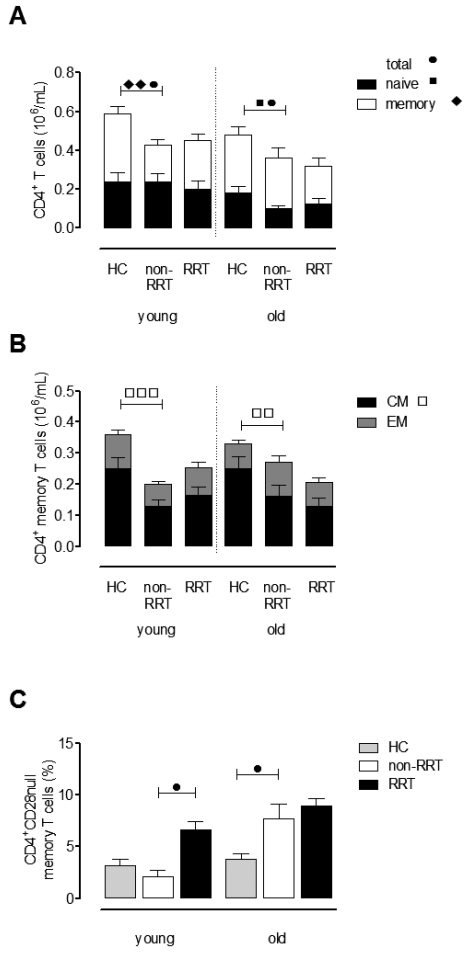

Figure 1: CD4+ T-cell phenotype and differentiation status. Using a whole blood staining, the phenotype and differentiation status of CD4+ T cells were determined in HC and ESRD patients not on RRT or receiving RRT. The absolute number of CD4+ T cells of young (age: <50 years) and old (age: ≥50 years) HC and these ESRD patients was dissected into a naïve (closed bars) and memory (open bars) compartment (A). Next, the composition of the memory compartment (CM in black and EM in grey bars) of CD4+ T cells is given for young and old HC and ESRD patients on RRT or not (B). In addition, we also determined the percentage memory CD4+ T cells lacking CD28 (i.e. CD28null) on their cell surface as another marker for T-cell differentiation for HC (grey bars) and ESRD patients not on RRT (white bars) or receiving RRT (black bars) (C). Bars represent the means + SEM and statistically significant differences between the groups are shown (one symbol: $p<0.05$, two symbols: $p<0.001$, three symbols: $p<0.0001$).

and decreased naïve T-cell numbers in the old non-RRT group (Figure 2A). Comparing old non-RRT patients to age-matched RRT patients revealed a slight but significantly ($p<0.05$) decreased number of naïve and memory T cells (Figure 2A).

The CD8+ memory T-cell compartment of the young and old non-RRT groups consisted of significantly ($p<0.01$) less CM when compared to age-matched HC,

resulting in relatively more differentiated CD8⁺ T cells with a EM/EMRA phenotype (Figure 2B). The influence of RRT was only observed within the young RRT group. These younger patients receiving RRT showed an increased differentiation of the memory CD8⁺ T-cell compartment, reflected by an increase in EMRA T cell numbers ($p<0.01$, Figure 2B) compared to non-RRT patients. This finding was in accordance

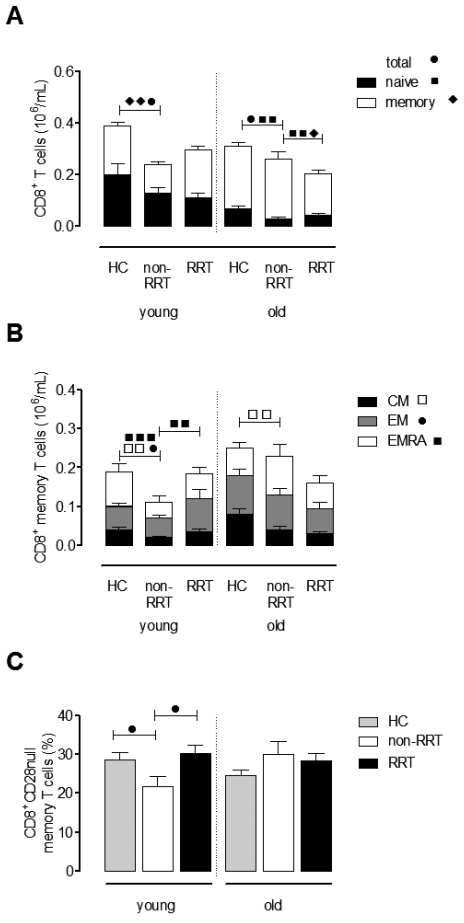

Figure 2: CD8⁺ T-cell phenotype and differentiation status. Using a whole blood staining, the phenotype and differentiation status of CD8⁺ T cells were determined in HC and ESRD patients not on RRT or receiving RRT. The absolute number of CD8⁺ T cells of young (age: <50 years) and old (age: ≥50 years) HC and these ESRD patients was dissected into a naïve (closed bars) and memory (open bars) compartment (A). Next, the composition of the memory compartment (CM in black, EM in grey and EMRA in white bars) of CD8⁺ T cells is given for young and old HC and ESRD patients on RRT or not (B). In addition, we also determined the percentage memory CD8⁺ T cells lacking CD28 (i.e. CD28null) on their cell surface as another marker for T-cell differentiation for HC (grey bars) and ESRD patients not on RRT (white bars) or receiving RRT (black bars) (C). Bars represent the means + SEM and statistically significant differences between the groups are shown (one symbol: $p<0.05$, two symbols: $p<0.001$, three symbols: $p<0.0001$).

with the increased percentage of memory CD8+ T cells lacking CD28 on their cell surface as compared to the group of young non-RRT patients ($p<0.05$, Figure 2C).

Thymic output of T cells and relative telomere length of CD4+ and CD8+ T cells

In young and old non-RRT patients a lower thymic T-cell output was shown by the significant lower TREC content of T cells compared to age-related HC (Figure 3A, $p<0.05$) for comparison of the two lines. Using linear regression analysis for both HC as well as non-RRT patients, an average immunological age for a non-RRT patient with a calendar age of 40 years was estimated. For this purpose, the value for thymic output (delta Ct, TREC content) of this 40 year old patient was calculated using the formula for the regression line of non-RRT patients. This value was then plotted in the formula for the regression line of HC and resulted in an average calendar age of 46.6 years. The immunological age of the patient's T cells using thymic output was thus increased with approximately 7 years (Figure 3A, red lines indicate the discrepancy between calendar ages of a non-RRT patient and a HC (i.e. the immunological age of the non-RRT patient), respectively). However, the TREC content was equally low for non-RRT and RRT patients (Figure 3B).

The relative telomere length (RTL) of CD4+ (Figure 4A) as well as CD8+ T cells (Figure 4C) decreased in both HC and non-RRT patients with increasing age, although these patients had or tended to have shorter telomeres within both CD4+ T cells ($p<0.05$, Figure 4A) and CD8+ T cells ($P=0.07$, Figure 4C) when compared to age-matched HC. Using regression analysis for these ageing parameters, as described above, the immunological age of a 40-year old non-RRT patient amounted to approximately 60 years. A significant ($p<0.05$) lower RTL for CD8+ (11.17±0.74% versus 15.15±1.73%, Figure 4D), was found in the young RRT group when compared to the non-RRT group. No differences in RTL were observed for the CD4+ T cells between RRT and non-RRT groups (Figure 4B).

DISCUSSION

The results of this study show that based on several immunological parameters, ESRD without RRT is associated with premature immunological ageing of the T-cell system. The added effect of RRT on this phenomenon is remarkably small and was predominantly limited to the CD8+ T-cell compartment in young ESRD patients.

Most studies on the immune system of patients with renal failure have been performed in chronic ESRD patients treated with RRT, mainly hemodialysis. Lymphopenia and signs of T-cell activation have been reported in a lot of the studies. (62, 63) In-depth analysis of T-cell subsets showed that lymphopenia is particularly prominent in the naïve T cell subset which showed a progressive decline in numbers as the stage of chronic kidney disease increased. (13, 64). In a recent study, we showed that decreased numbers of naïve T cells in hemodialysis patients is related to decreased

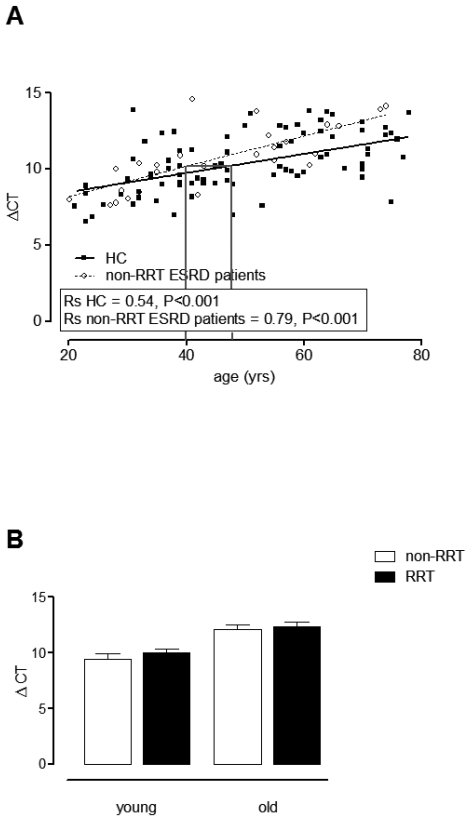

Figure 3: Quantification of the TREC content by quantitative PCR. The threshold cycle (Ct) is the number of amplification cycles needed to detect the TRECs and is a relative measure, inversely related with the concentration of TRECs. Control for DNA input was done by performing a quantitative PCR (qPCR) for albumin and the difference between the Ct for TRECs and the Ct for albumin was calculated (ΔCt). The ΔCt, indicative for TREC content, was determined of HC (straight line) and ESRD patients not on RRT (dotted line) and lines were compared using a linear regression analysis ($p<0.05$ for the difference between lines for ESRD patients not on RRT and HC). The red lines in (A) mark the discrepancy between the calendar age (i.e. 40 years) of a non-RRT patient and the immunological age, by extrapolation of the value for the deltaCT (TREC content) to the HC regression line. In addition, Spearmans Rho correlation coefficients (Rs) were calculated for HC and ESRD patients not on RRT to determine the strength of the association between TREC content (calculated as ΔCt) and age. Age is depicted on the X-axis whereas on the Y-axis the ΔCt value for TREC content is displayed. Next, the effect of RRT on the TREC content (B) was analyzed by dissecting the ESRD patients into a young (<50 years) and old (≥50 years) group and either not (white bars) or receiving (black bars) RRT. Bars represent means + SEM. Statistically significant differences between the groups are shown.

thymic output of naïve T cells with increased but insufficient homeostatic proliferation in the periphery. (35) Memory T cells were in general more differentiated probably due to increased proliferation, given the decrease in relative telomere length. Similar findings were now observed for ESRD patients not on dialysis, indicating that loss of

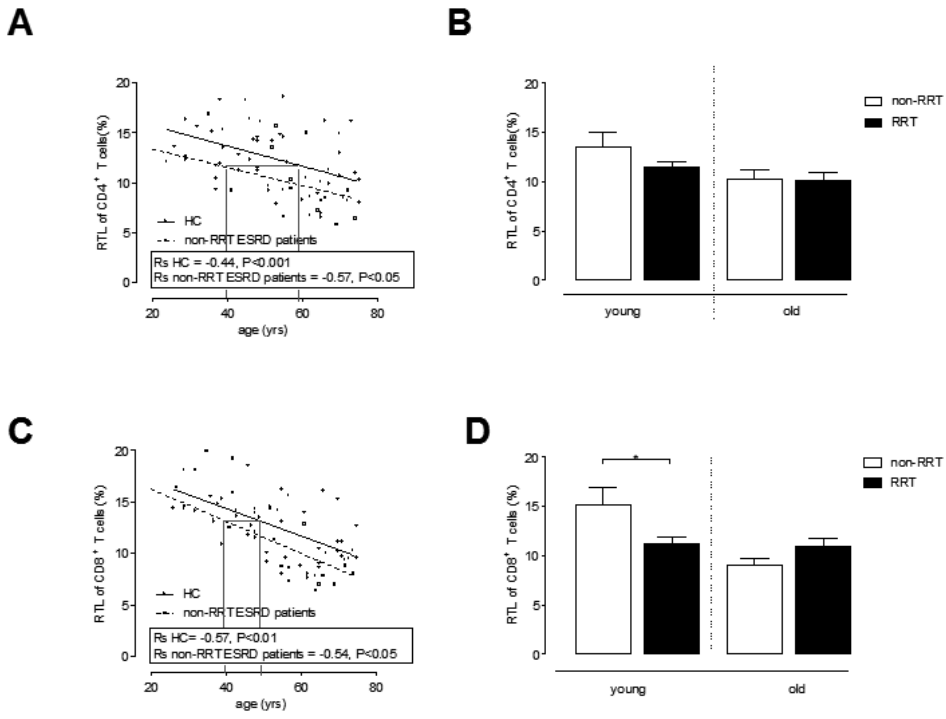

Figure 4: Relative telomere length of CD4⁺ and CD8⁺ T cells. The relative telomere length (RTL) of the CD4⁺ (A) and CD8⁺ (C) T cells is determined of HC (straight line) and ESRD patients not on RRT (dotted line) and lines were compared using a linear regression analysis i.e. $p<0.05$ and $p=0.07$ when comparing the differences between HC and non-RRT patients with respect to CD4⁺ RTL and CD8⁺ RTL respectively). The red lines in A and C highlight the discrepancy between the calendar age (i.e. 40 years) of a non-RRT patient and the immunological age, by extrapolation of the value for the CD4⁺ and CD8⁺ RTL to the HC regression line, respectively. In addition, Spearmans Rho correlation coefficients (Rs) were calculated for HC and ESRD patients not on RRT to determine the strength of the association between RTL and age. Age is depicted on the X-axis and the RTL on the Y-axis. The effect of RRT on the RTL of CD4⁺ (B) as well as CD8⁺ (D) T cells was analyzed by dissecting the ESRD patients into a young (<50 years) and old (≥50 years) group and either not receiving RRT (white bars) or receiving RRT (black bars). Bars represent means + SEM. Statistical differences between the groups are shown (* $p<0.05$).

renal function is the most dominant factor for decreased thymic output of naïve T cells and increased differentiation/proliferation of memory T cells. In healthy individuals these changes are observed in the elderly and therefore considered as a physiological process of immunological ageing of the T-cell system. In comparison to the CD8⁺ T cells, the CD4⁺ T-cell system in healthy individuals remains relatively unaffected by age until the seventh or eighth decade (19, 59, 65-68). However, patients with ESRD not on RRT already showed all characteristics of immunological ageing (lower thymic output, shorter telomeres) of their T-cell system, approximately 10-20 years ahead of their calendar age. The reasons for premature T-cell ageing in patients with chronic

renal failure are not known but a relative lack of the T cell growth factor IL-7 has been documented and may be important. (13, 15) In animal models it was clear that sudden loss of renal function causes involution of the thymus and other lymphoid organs confirming a direct relationship between kidney function and lymphopoiesis. (69) Lymphopenia may trigger increased homeostatic proliferative responses, (70) not only of the circulating naïve T-cell compartment but also of memory T cells thereby inducing differentiation and loss of telomere length. However this concept is hypothetical and has not been tested yet in ESRD patients.

In general, immunological ageing of T cells (e.g. increased numbers of CD28null T cells) has been associated with decreased T-cell immunity. Maintenance and generation of a number of antibody responses seems critically dependent on the presence of antigen-specific $CD4^+$ T cells. (39, 44) Any major disturbances of the T-cell system will therefore affect the humoral immune response as well. ESRD-related premature immunological T-cell ageing may therefore underlie the well-established uremia-associated cellular and humoral immune deficiency in ESRD patients. (15)

In a previous study, it was shown that loss of naïve T cells and increased memory T-cell differentiation progresses with increasing stage of chronic kidney disease but with little difference between ESRD patients with or without RRT. (13, 64) Also on the level of T cell chemokine receptor expression, which is indicative for functional capacities of T cells few differences were observed between T cells of ESRD patient with or without RRT. These findings are remarkable as most patients on dialysis have little to no residual renal function and as such are metabolically more affected than ESRD patient not on dialysis. The results in this study are largely in accordance with these data and show that patients with RRT do not have an altered thymic output of naïve T cells and total numbers of naïve T cells. Summarizing the present data, it appears that the maximum effect of loss of renal function on the T-cell immune system is reached at the level of ESRD. Whether RRT prevents further immunological ageing or not is difficult to assess and cannot be inferred from our data.

However, the $CD4^+$ and $CD8^+$ memory T cells in young dialysis patients showed more differentiation and in the $CD8^+$ T cells this was accompanied by a decrease in RTL. Thus, particularly memory $CD8^+$ T cells in younger patients show a history of more proliferation without the presence of decreased thymic output. This finding indicates that in younger dialysis patients, on top of ESRD-related immunological ageing, other factors drive the proliferation of memory T cells. The dialysis vintage of younger patients was on average higher than the old group suggesting a role for duration of RRT and loss of telomere length. However, we could not find an independent statistically significant relation between duration of RRT and any of the immunological parameters measured. In addition, the type of underlying kidney diseases was not related to any parameter of immunological ageing. Another possible scenario may be that RRT actually improves homeostatic proliferation of memory T cells in the young but not in the elderly patients. However, this should result in a relative increase in memory T cell numbers in the young

RRT patients compared to ESRD patients without RRT which was not observed. The lack of such a finding would argue against this explanation.

A limitation of the present study is the cross-sectional design which may obscure subtle changes in immunological T-cell ageing after patients with ESRD have started RRT. However, the large number of patients included in this study adds to the reliability of the results.

In conclusion, severe loss of renal function leading to ESRD is a very potent inducer of premature immunological T-cell ageing of both the $CD4^+$ and $CD8^+$ T cells. Renal replacement therapy is associated with a small increase of memory T-cell ageing in patient <50 years of age, particularly in the $CD8^+$ T cell subset. Further research is needed to establish the pathophysiology of ESRD-related T-cell ageing and whether this can be reversed by e.g. interleukin-7 therapy (71, 72) or kidney transplantation.

METHODS

Study Population

ESRD patients were defined by a glomerular filtration rate (GFR) of ≤ 15 ml/min and were either not on RRT or treated with hemodialysis or peritoneal dialysis. Patients having an infection, malignancy, autoimmune disease or a history of immunosuppressive drugs (including previous kidney transplantations) were excluded. Healthy individuals were included as controls. They were matched for age and cytomegalovirus (CMV) positivity, as these are well-known factors affecting the composition of the T-cell compartment (15, 35). The clinical and demographic characteristics of the ESRD patients and healthy controls (HC) are shown in Table 1. 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.

Differentiation status of circulating T cells

T cell phenotype and differentiation status was analyzed as described previously (15, 35). Briefly, whole blood was stained with AmCyan labeled anti-CD3 (BD Biosciences, Erembodegem, Belgium) in combination with pacific blue labeled anti-CD4 (BD) or allophycocyanin Cy7 (APC-Cy7) labeled anti-CD8 (BD) to identify $CD4^+$ or $CD8^+$ T cells that are further dissected into four different subsets based on the expression of CCR7 and CD45RO using fluorescein isothiocyanate (FITC) labeled anti-CCR7 (R&D systems, Uithoorn, The Netherlands) and allophycocyanin (APC) labeled anti-CD45RO (BD). Naive T cells are $CCR7^+$ and $CD45RO^-$, Central memory (CM) cells are $CCR7^+$ and $CD45RO^+$, Effector memory (EM) cells are $CCR7^-$ and $CD45RO^+$ and EMRA cells are $CCR7^-$ and $CD45RO^-$.

T-cell differentiation is associated with loss of CD28-expression on cell surface. Percentages of $CD28^-$ (or CD28null) T cells within the T-cell subsets were determined by staining with peridinin chlorophyll-Cy5.5 (PerCP-Cy5.5) labeled anti-CD28 (BD).

PBMC isolation

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples by Ficoll gradient centrifugation. In hemodialysis patients, the blood samples were drawn before a hemodialysis session (15). Two million PBMC were snap-frozen for the TREC assay and the rest of the PBMC were frozen in liquid nitrogen with a minimum amount of 10×10^6 cells per vial for further experiments.

DNA isolation and TREC assay

TREC content was assessed using the snap-frozen PBMC. Briefly, DNA was isolated according to manufacturer's instructions (Qiagen Isolation kit, Qiagen, Venlo, the Netherlands). Subsequently, TREC content was determined using quantitative PCR. For this purpose, a combination of two primers and a hydrolysis probe specific for the so-called δREC (TCRD)-ψJα (TCRA) TREC (sjTREC) were employed. TaqMan quantitative PCR was performed on 50 ng DNA in a 25 µl reaction mixture containing 700 nmol/l of each primer 5'-TCGTGAGAACGGTGAATGAAG-3' and 5'-CCATGCTGACACCTCTGGTT-3', 150 nmol/l of hydrolysis probe 5'-(FAM) CACGGTGATGCATAGGCACCTGC-3' (TAMRA), and 12.5 µl 2× TaqMan Universal PCR Master Mix (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Quantification of the DNA amount in each sample was performed using a quantitative PCR of the single-copy albumin gene. All reactions were performed in duplicate, unless a threshold cycle (Ct) difference between replicates of >1.5 necessitated to repeat the PCR experiment. ΔCt was calculated by using the formula: Ct value TREC PCR – Ct value albumin PCR. (20)

Telomere length assay

Flow fluorescent *in situ* hybridization was performed to determine the relative telomere length of $CD4^+$ and $CD8^+$ T cells. For this purpose, the frozen PBMC were thawed and stained with either CD4-biotin (Beckman-Coulter, BV, Woerden, The Netherlands) or CD8-biotin (Biolegend, Europe BV, Uithoorn, the Netherlands) followed by staining with streptavidin-Cy5 (Biolegend). The PBMC were fixed and permeabilized (Invitrogen Life Technologies, Bleiswijk, The Netherlands) before the relative telomere length (RTL) was determined using the telomere PNA-kit/FITC (Zebra Bioscience BV, Enschede, The Netherlands). The sub cell line 1301 of CCRF-CEM, which is known to have long telomeres, was used to calculate the relative telomere length (RTL) of the $CD4^+$ and $CD8^+$ T cells. (15, 73)

$$\text{RTL} = \frac{\text{(median fluorescence FITC sample cells with probe - median FL1 sample cells without probe) x DNA index of control (=2) cells x 100}}{\text{(median fluorescence FITC control cells with probe - median FL1 control cells without probe) x DNA index of sample (=1) cells}}$$

Statistical analysis

Patients not on RRT were compared to healthy controls on one hand and to patients receiving hemodialysis or peritoneal dialysis on the other hand using the Mann-Whitney test. For the TREC content and the RTL, a linear regression model was used to compare patients not on RRT to healthy controls. In addition, Spearman Rho correlation coefficients (Rs) were calculated to determine the strength of the association between the different ageing parameters and age for HC as well as ESRD patients not on RRT. All statistical tests were performed two-sided and a p-value of <0.05, was considered significant.

Disclosure

All the authors declared no competing interests. This study was funded by the Dutch Kidney Foundation (KSPB.10.12)

3

CYTOMEGALOVIRUS CONTRIBUTES PARTLY TO URAEMIA-ASSOCIATED PREMATURE IMMUNOLOGICAL AGEING OF THE T-CELL COMPARTMENT

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SUMMARY

Cytomegalovirus (CMV) infection has been implicated in accelerated T-cell ageing. End-stage renal disease (ESRD) patients have a severely immunologically aged T-cell compartment but also a high prevalence of CMV infection. We investigated whether CMV-infection contributes to T-cell ageing in ESRD patients.

We determined the thymic output by the T-cell receptor excision circle (TREC) content and percentage of $CD31^+$ naïve T cells. The proliferative history of the T-cell compartment by determination of the relative telomere length (RTL) and the T-cell differentiation status was determined by immunophenotyping.

It appeared that CMV infection did not affect thymic output but reduced RTL of $CD8^+$ T cells in ESRD patients. Moreover, increased T-cell differentiation was observed with higher percentages of $CD57^+$ and CD28null $CD4^+$ and $CD8^+$ memory T cells. These CD28null T cells had significantly shorter telomeres compared to $CD28^+$ T cells.

Therefore we concluded that CMV infection does not affect the decreased thymic output but increases T-cell differentiation as observed in ESRD-related premature T-cell ageing.

INTRODUCTION

Due to the progressive involution of thymic tissue, ageing of the T-cell compartment in healthy individuals is associated with decreased numbers of circulating naive T cells. This coincides with an increased differentiation status and proliferative history of memory T cells. The process of immunological T-cell ageing is related to an age-related decline in cellular immunity resulting in reduced vaccination efficacy, enhanced susceptibility for infectious diseases and a higher risk for the development of tumors. (39, 53, 55, 74, 75) Higher numbers of differentiated $CD4^+$ T-cells have also been associated with a higher prevalence and severity of atherosclerotic disease (47, 76-78). We recently documented that patients with end-stage renal disease (ESRD) have a profound prematurely aged T-cell system which is believed to be caused by the uremia-induced pro-inflammatory conditions (15, 79) The immunological age was determined using three parameters: thymic output of newly formed T cells, the differentiation profile of T cells and their relative telomere length. The thymic function can be measured by T-cell receptor excision circles (TREC), which are small circular DNA episomes created in T-cell precursors that are formed in the thymus during rearrangement of T-cell receptor (TCR) genes (20) and the expression of CD31 on naïve T cells (15). Based on these parameters, the average immunological age of T cells in ESRD patients is 20-30 years higher than that of healthy individuals (15).

Because infection with cytomegalovirus (CMV) has a profound effect on the circulating T-cell compartment in healthy individuals, CMV has been implicated in immunological ageing. For instance, CMV-infected individuals (CMV-seropositive) have a more differentiated memory T-cell compartment, a decreased CD4/CD8 ratio, an expansion of $CD4^+$ and $CD8^+$ T-cells lacking CD28 but expressing CD57 (33, 35, 76, 80) and a reduction in their T-cell telomere length, indicating an increased proliferative history of the T cells (34). These effects of CMV on the T-cell compartment are relevant, as a large population of healthy individuals is infected with CMV (31). The prevalence ranges between 30%-100%, increases with age and is dependent on an individuals socio-economic and ethnic background (77).

Over 70% of ESRD patients are CMV-seropositive and we have previously shown that a seropositive CMV status is associated with an increased differentiation status of the T cells as determined by phenotyping of the T-cell compartment (35, 76). However, no information is available on other parameters of immunological ageing such as TREC content, recent thymic emigrants and telomere length in relation to CMV-serostatus in ESRD patients.

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

MATERIALS AND METHODS

Study Population

ESRD patients, defined by an estimated glomerular filtration rate of ≤ 15 ml/min/m2 by the MDRD formula, and with or without dialyses, were included. Patients were excluded if they had an infection, malignancy, autoimmune disease or a history of immunosuppressive drugs (including previous kidney transplantations). CMV-seronegative and CMV-seropositive ESRD patients were age- and sex-matched as well as matched for all or not receiving renal replacement therapy (hemodialysis or peritoneal dialysis). Patients receiving anti-viral therapy (i.e.) were excluded from the study. Their clinical characteristics are shown in Table 1. All individuals included gave informed consent and the study was approved by the local medical ethical committee (METC number: 2012-022). It was conducted according to the principles of the Declaration of Helsinki and in compliance with the regulations of International Conference on Harmonization/Good Clinical Practice.

CMV serology

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

PBMCs isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples drawn from clinically stable ESRD patients on the day of visiting the outpatient clinic. (15) Two million PBMCs were snap-frozen for the TREC assay; the remaining cells were frozen in liquid nitrogen with a minimum amount of 10×10^6 cells per vial for further experiments.

DNA isolation and TREC assay

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

Table 1: Study Population characteristics

	CMV-seronegative ESRD patients		CMV-seropositive ESRD patients	
	Young	**Old**	**Young**	**old**
Number of individuals	38	38	42	41
Age in Years	33.7 ± 9.4*	61.3 ± 7.7*	36.9 ± 7.8*	64.6 ± 8.4*
Male	69.4%	78.2%	54.7%	61.6%
CMV titer (AU/ml)	-	-	70.9 ± 5.04*	75.8 ± 2.92*
Underlying kidney disease				
Hypertensive nephropathy	6 (15.8%)	9 (23.7%)	10 (23.8%)	14 (34.1%)
Primary glomerulopathy	9 (23.7%)	9 (23.7%)	10 (23.8%)	5 (12.2%)
Diabetic nephropathy	0 (0%)	3 (7.9%)	1 (2.4%)	3 (7.3%)
Polycystic kidney disease	0 (0%)	2 (5.3%)	2 (4.8%)	2 (4.9%)
Reflux nephropathy	8 (21.1%)	2 (5.3%)	4 (9.5%)	3 (7.3%)
Other	8 (21.1%)	7 (18.4%)	9 (21.4%)	6 (14.6%)
Unknown	7 (18.4%)	6 (15.8%)	6 (14.3%)	8 (19.5%)

*Data are given in means with standard deviation

(TAMRA), and 12.5 µl 2× TaqMan Universal PCR Master Mix (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). Quantification of the DNA amount in each sample was performed using a quantitative PCR of the single-copy albumin gene. All reactions were performed in duplicate, unless a threshold cycle (Ct) difference between replicates of >1.5 necessitated to repeat the PCR experiment. ΔCt was calculated by using the formula: Ct value TREC PCR – Ct value albumin PCR (15, 20).

Telomere Length Assay

Flow fluorescent *in situ* hybridization was performed to determine the telomere length of $CD4^+$ and $CD8^+$ T cells. The isolated PBMCs were stained with either CD4-biotin (Beckman-Coulter, BV, Woerden, the Netherlands) or CD8-biotin (Biolegend, Europe BV, Uithoorn, the Netherlands) followed by staining with streptavidin-Cy5 (Biolegend). The PBMCs were fixed and permeabilized (Invitrogen Life Technologies, Bleiswijk, the Netherlands) and then by using the telomere PNA-kit/FITC (Zebra Bioscience BV, Enschede, the Netherlands) we determined the relative telomere length. The sub cell-line 1301 of CCRF-CEM, of which is known to have long telomeres, was used to calculate the relative telomere length (RTL) of the $CD4^+$ and $CD8^+$ T-cells using the following formula: (25)

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

In addition, PBMCs of 5 elderly CMV-seropositive ESRD patients were sorted into a purified $CD28^+$ or CD28null $CD4^+$ or $CD8^+$ T-cell fraction to examine whether the relative telomere length differed in these sorted T cell fractions. For this purpose, PBMCs ($20x10^6$) were stained with AmCyan labeled anti-CD3 (BD, Erembodegem, Belgium), pacific blue labeled anti-CD4 (BD), allophycocyanin (APC) labeled anti-CD8 (BD), phycoerythrin (PE) labeled anti-CD28 (BD) and with 7AAD (BD). Sorting was performed on a FACSAria II SORP (BD). All fractions had a purity of more than 95%.

Telomerase activity assay

The activity of the telomerase enzyme was measured in 5 CMV- seropositive and 5 age-matched CMV-seronegative ESRD patients using the TRAPeze® XL Telomerase detection kit (Millipore, Temecula, CA, USA) according to manufacturer's instructions. Briefly, PBMCs ($20x10^6$) were sorted into a purified and viable $CD4^+$ and $CD8^+$ T-cell fraction (according to sort protocol briefly described under Telomere Length Assay).

The sorted T-cell fractions (all with a purity of more than 95%) were stimulated with anti-CD3/CD28 beads (25 µl/1 ml, Invitrogen) for 3 days at 37°C in a 96-wells plate (Greiner bio-one, Alphen aan den Rijn, the Netherlands). Next, cells were resuspended in CHAPS lysis buffer (provided in the kit) and cell extractions were made (10 µg-750 µg). Protein levels were determined by using the Bio-Rad protein assay (Bio-Rad, München, Germany).

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

Differentiation status of T cells

A whole blood staining was performed to determine the T-cell differentiation status (15, 35, 79). Briefly, whole blood was stained with AmCyan labeled anti-CD3 (BD) in combination with pacific blue labeled anti-CD4 (BD) and allophycocyanin Cy7 (APC-Cy7) labeled anti-CD8 (BD). The T cells are defined as $CD4^+$ or $CD8^+$ and further defined into four different subsets based on the expression of CCR7 and CD45RO. In supplementary Figure 1, a typical example of the gating strategy is depicted. Naive T cells are defined as $CCR7^+$ and $CD45RO^-$, Central memory (CM) cells as $CCR7^+$ and $CD45RO^+$, Effector memory (EM) cells as $CCR7^-$ and $CD45RO^+$ and EMRA cells as $CCR7^-$ and $CD45RO^-$. Expression was determined by staining with fluorescein isothiocyanate (FITC) labeled anti-CCR7 (R&D systems, Uithoorn, The Netherlands) and APC labeled anti-CD45RO (BD). T-cell differentiation is associated with loss of CD28 expression on the cell surface. The ratio $CD28^+/CD28^-$ (or CD28null) T cells within the T-cell subsets were determined by staining with peridinin chlorophyll-Cy5.5 (PerCP-Cy5.5) labeled anti-CD28 (BD) and the ratio $CD57^-/CD57^+$ was determined by staining with APC labeled anti-CD57 (Biolegend). To determine the thymic output of naïve T cells, the percentage $CD31^+$ naïve T cells were determined by staining with PE labeled anti-CD31 (Biolegend) (15, 35, 79).

Ki67 staining of T cells

To quantify the percentage of dividing cells, we stained the cells intracellular with FITC labeled anti-Ki67 after fixation and permeabilization (IntraSure Kit, BD). Ki67 is a nuclear antigen which is selectively expressed in cells that are in the G-M stage of cell division. The frequency of Ki67$^+$ cells was determined in the total CD4$^+$ and CD8$^+$ T-cell population.

Statistical analyses

Differences between CMV-seropositive and CMV-seronegative young (age < 50 years) and elderly (age ≥ 50 years) ESRD patients were analysed using the Mann-Whitney U-test. For TREC content and RTL, a linear regression model was used. In addition, Spearman's Rho correlation coefficients (Rs) were calculated to determine the strength of the association between TREC content or RTL with age for CMV-seropositive and CMV-seronegative ESRD patients. A paired-T test was done to calculate significant differences in RTL between CD28$^+$ T cells and CD28null T cells. All statistical tests were performed two-sided, whilst a p-value of <0.05 was considered significant.

RESULTS

CMV infection does not influence thymic output of naïve T cells

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

In addition, no significant differences were observed when considering absolute numbers (cells/μl, mean±SEM) of CD31 expressing naïve T-CD4$^+$ (young: CMV-seropositive: 151.9±24.42 vs. CMV-seronegative: 173.6±27.04 and old: CMV-seropositive: 121.4±14.32 vs. CMV seronegative: 137.2±13.38) and CD8$^+$ (young: CMV-seropositive: 127.6±15.36 vs. CMV-seronegative: 101.6±15.62 and old: CMV-seropositive: 55.21±5.11 vs. CMV seronegative: 78.8±7.74) T-cells.

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

With increasing age, the RTL of CD4$^+$ (Figure 2A) and CD8$^+$ (Figure 2B) T cells declines in both CMV-seropositive and CMV-seronegative ESRD patients. CMV did not contribute significantly to telomere attrition within CD4$^+$ T cells (p=0.2, Figure 2A) but the RTL of the CD8$^+$ T cells was significantly lower in patients with a latent CMV infection (p=0.04) (Figure 2B).

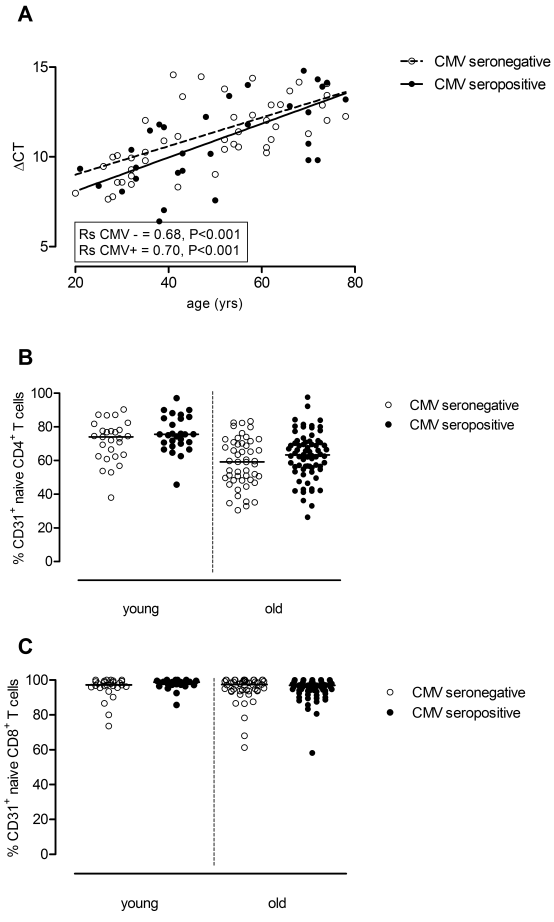

Figure 1: Quantification of TREC content by real-time quantitative PCR and CD31 expression by naïve T cells to measure thymic output. The thymic output was measured by two parameters. First, the TREC content was determined (A). The threshold cycle (Ct) is the number of amplification cycles needed to detect the TRECs and is a relative measure, inversely related with the concentration of TRECs. Control for DNA input was done by performing a quantitative PCR (qPCR) for albumin and the difference between the Ct for TRECs and the Ct for albumin was calculated. The X-axis represents the age and on the Y-axis the ΔCt value is depicted. The ΔCt values of CMV-seropositive ESRD patients (closed symbols) are represented by the closed line; that of CMV-seronegative ESRD patients (open symbols) by the dashed line. Next the patients were separated based on their age (young: < 50 years and old: ≥ 50 years) and the percentage of $CD31^+$ naïve $CD4^+$ (B) as well as $CD8^+$ (C) T cells were determined as a marker for thymic output. Statistical differences between groups are shown, i.e. one symbol: $p<0.05$, two symbols: $p<0.01$, three symbols: $p<0.001$) and medians are shown. Individual data-points are shown for CMV-seropositive patients (closed symbols) and CMV-seronegative patients (open symbols).

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

Upon dissection of CMV-seropositive as well as CMV-seronegative ESRD patients into a younger (<50 years) and an older (≥50 years) population, no differences were observed in RTL for the CD4⁺ T cells between CMV-seropositive and CMV-seronegative age-matched groups (Figure 2C). Younger CMV-seropositive ESRD patients had significant ($p<0.05$) shorter telomeres within their CD8⁺ T-cell compartment (mean RTL±SEM; 11.19±0.83%) when compared to CMV-seronegative age-matched counterparts (13.28±0.75%).

Next, we examined if CMV seropositivity is associated with activity of the telomerase enzyme in the CD4⁺ and CD8⁺ T-cell compartment. Telomerase activity (expressed in TPG units) was similar between CMV-seronegative and CMV-seropositive patients for the CD4⁺ T cells (mean TPG±SEM; CMV-seronegative: 0.54±0.004 vs. CMV-seropositive: 0.55±0.006) and CD8⁺ T cells (CMV-seronegative: 0.55±0.002 vs. CMV-seropositive: 0.55±0.002).

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

The significantly lower CD4⁺ naïve/memory ratio ($p<0.05$), indicated a shift towards the memory phenotype within the CD4⁺ T-cell compartment of CMV-seropositive patients (Figure 3A). Dissection of the memory CD4⁺ T cells into CM and EM did not

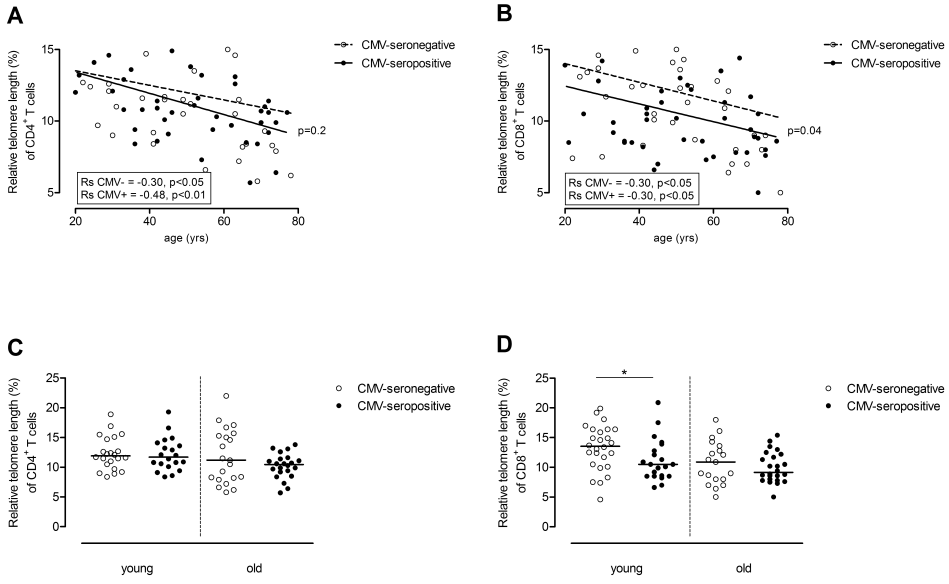

Figure 2: RTL of circulating CD4⁺ and CD8⁺ T cells. The RTL was determined for CD4⁺ (A) and CD8⁺ (B) T cells of CMV-seropositive (closed symbols, closed line) and CMV-seronegative (open symbols, dashed line) ESRD patients. Next, patients were separated based on their age in a young (age < 50 years) and an old (age ≥ 50 years) group and the RTL of CD4⁺ (C) and CD8⁺ T cells (D) are shown for each group. Significant differences between groups are shown (one symbol: $p<0.05$, two symbols: $p<0.01$, three symbols: $p<0.001$). Individual data-points with medians are depicted.

show significant CMV-associated differences (data not shown). Next, we determined the differentiation status by examining the loss in CD28 expression and increase in CD57 expression. CMV-infected ESRD patients had on average a significant lower $CD28^+/CD28^-$ ($p<0.01$) (Figure 3B) and $CD57^-/CD57^+$ ratio (Figure 3C) within their $CD4^+$ T-cell compartment ($p<0.01$ (young) and $p<0.001$ (elderly), respectively), indicative

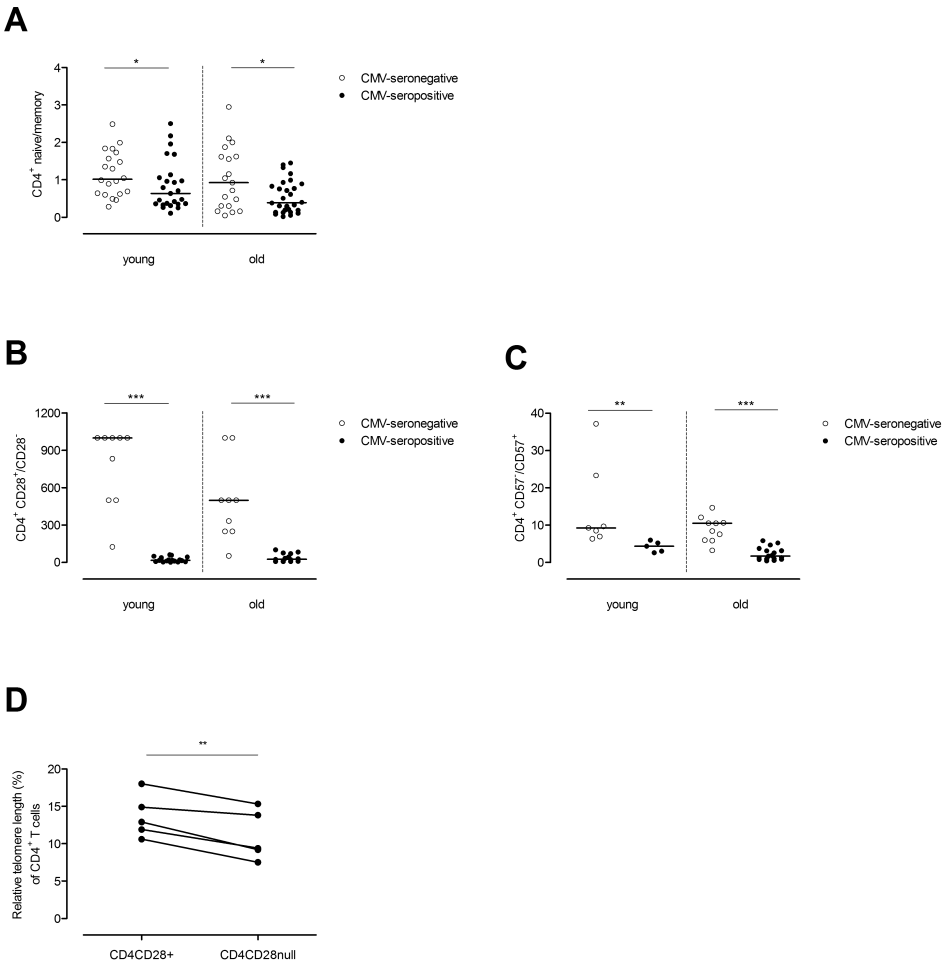

Figure 3: $CD4^+$ T-cell phenotype and differentiation status and the correlation with the RTL. The ratio $CD4^+$ naïve/memory T cells were determined for CMV-seronegative and CMV-seropositive ESRD patients (A). Moreover, the ratio $CD4^+CD28^+/CD28^-$ (B) and $CD57^-/CD57^+$ (C) were calculated to determine the differentiation status of the $CD4^+$ T-cell compartment. ESRD patients were dissected into a young (age <50 years) and an old (age ≥ 50 years) group. Individual dots are shown with the median for the CMV-seronegative patient population (open symbols) and the CMV-seropositive patients (closed symbols). Significant differences between groups are shown (one symbol: $p<0.05$, two symbols: $p<0.01$, three symbols: $p<0.001$). Next, the RTL was determined within a sorted $CD28^+$ and CD28null $CD4^+$ T cell population of 5 CMV-seropositive ESRD patients (D). Individual data points are shown and significant differences between the two T-cell fractions are shown (two symbols: $p<0.01$).

of CMV-induced increased differentiation of $CD4^+$ T cells. Moreover, we determined the percentages of highly differentiated (i.e. having a senescent phenotype) CD28null $CD57^+$ T cells within the $CD4^+$ T-cell compartment for CMV-seropositive and age-matched CMV-seronegative ESRD patient populations. CMV- seropositive ESRD patients had significantly higher percentages of these cells in their circulation than age-matched CMV-seronegative ESRD patients (mean±SEM; young CMV seropositive: 9.90%±3.48 vs. young CMV seronegative: 0.42%±0.23, $p<0.01$ and old CMV seropositive: 26.62%±4.27 vs. old CMV seronegative: 5.78%±3.52, $p<0.001$).

We also compared the RTL of sorted $CD4^+CD28$null to that of $CD4^+CD28^+$ (purity>95%) T cells and found that the $CD4^+$CD28null T cells had significantly shorter telomeres ($p<0.01$) compared to the $CD4^+CD28^+$ T cells (Figure 3D).

CMV affected the $CD8^+$ T-cell compartment more profoundly than the $CD4^+$ T-cell compartment. CMV-seropositive ESRD patients had a significantly ($p<0.05$) lower CD8 naive/memory ratio (Figure 4A), due to a higher number of memory $CD8^+$ T cells consisting of a large population of terminally differentiated $CD8^+$ EMRA T cells (absolute numbers: CMV-seronegative: $0.03x10^6$, CMV-seropositive: $0.12x10^6$, $p<0.05$). This was reflected by the significantly lower $CD28^+/CD28^-$ (Figure 4B) ($p<0.001$) and $CD57^-/CD57^+$ ratio (Figure 4C) ($p<0.01$ (young) and $p<0.001$ (elderly), respectively). Similar as observed for the $CD4^+$ T-cell compartment, a significant higher proportion of $CD8^+$ T cells had a senescent phenotype in CMV-seropositive ESRD patients when compared to their age-matched CMV-seronegative counterparts (young CMV-seropositive: 50.56%±3.77 vs. young CMV-seronegative: 15.56%±4.99, $p<0.01$ and old CMV-seropositive: 47.15%±4.09 vs. old CMV-seronegative: 27.94%±5.16, $p<0.05$).

Also for the $CD8^+$ T cells we determined the RTL in CD28null and $CD28^+$ T-cell sorted populations. The $CD8^+$CD28null T cells had significantly shorter ($p<0.01$) telomeres than the $CD8^+CD28^+$ T cells (Figure 4D).

In an attempt to explain the additional telomere attrition induced by CMV, we determined whether CMV-infection induced an increase in the proliferation of $CD4^+$ as well as $CD8^+$ T cells by determining the percentage of $Ki67^+$ T cells (i.e. percentage of T cells actually dividing). No significant differences were observed in the percentage $Ki67^+$ $CD4^+$ or $CD8^+$ T cells. ($CD4^+Ki67^+$ T cells; CMV seronegative: 2.09%±0.68 CMV seropositive: 1.33%±0.52 and $CD8^+Ki67^+$ T cells; CMV seronegative: 1.99%±0.60 CMV seropositive: 1.34% ±0.25).

DISCUSSION

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

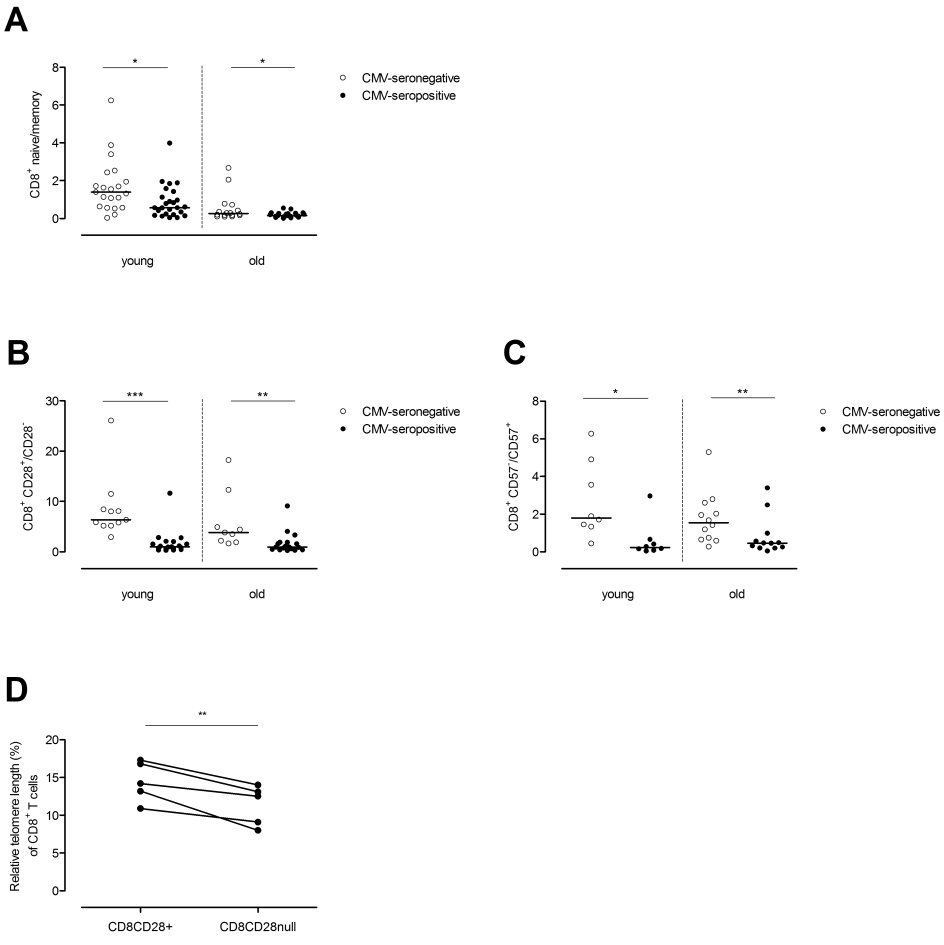

Figure 4: $CD8^+$ T-cell phenotype and differentiation status and the correlation with the RTL. The ratio $CD8^+$ naïve/memory T cells were determined in CMV-seronegative and CMV-seropositive ESRD patients. (A). Moreover, the ratio $CD8^+CD28^+/CD28^-$ (B) and $CD57^-/CD57^+$ (C) were calculated to determine the differentiation status of the $CD8^+$ T-cell compartment. ESRD patients were dissected into a young (age <50 years) and an old (age ≥ 50 years) group. Individual dots are shown with the median for the CMV-seronegative patient population (open symbols) and the CMV-seropositive patients (closed symbols). Significant differences between groups are shown (one symbol: $p<0.05$, two symbols: $p<0.01$, three symbols: $p<0.001$). Next, we determined the RTL within a sorted $CD28^+$ and CD28null $CD8^+$ T-cell population from 5 elderly CMV-seropositive ESRD patients (D). Individual data points are shown and significant differences between the two T-cell fractions are shown (two symbols: $p<0.01$).

In a previous study (15), we observed that the characteristics of the peripheral T-cell system of ESRD patients are very similar to healthy individuals with a chronological age that is on average 20-30 years older. One of the salient findings in ESRD patients and elderly healthy individuals was a decreased number of circulating naïve T cells. (15)

In humans, the thymus is the single organ involved in naive T-cell generation. Ageing results in involution of the thymus with subsequent decrease in TREC content within the circulating T-cell population. Because TREC content is reliably and linearly related with age, measuring the TREC content in blood can be used as a tool for age determination for forensic purposes. (20) In both ESRD patients and elderly healthy individuals a decreased thymic output of naïve T cells based upon TREC analysis was observed. Next to the TREC content, an alternative technique to identify recent thymic emigrants is to measure the CD31 expression on naïve T cells (21), which corroborate with the findings of the TREC content. In addition, activation and increased numbers of proliferating Ki67$^+$ naïve T cells were observed. Homeostatic proliferation occurs in response to this decreased thymic output to maintain the naïve T-cell compartment. Our findings do not support a role for CMV in the decreased output of naïve T cells or their peripheral proliferation in the periphery, as both the TREC content and the percentage of CD31$^+$ and Ki67$^+$ cells were not affected by CMV-serostatus. This also suggests that the expansion and differentiation of memory T cells in CMV-seropositive patients does not change the number or homeostatic proliferation of naïve T cells. This may have been expected, since it is assumed that increased turnover of this compartment would also accelerate the turnover of naïve T cells.

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

CMV-infection induces a shift within both CD4$^+$ and CD8$^+$ T cells towards the memory compartment. Specifically, the increase of CD28null T cells within the CD4$^+$ and CD8$^+$ T-cell compartment is highly associated with a previous CMV infection. (35, 81, 82) However, CD8$^+$ memory T cells contain far more CD28null as well as CD57$^+$

T cells when compared to $CD4^+$ T cells. These differentiated T cells are known to have short telomeres (34, 56), which we could confirm for ESRD patients in this study. The CD57 expressing cells are predominantly found within the CD28 negative memory T cells implying that most of the senescent cells are located within this memory fraction and are found to be higher in CMV-seropositive ESRD patients.

As we did not detect an increase in the number of $Ki67^+$ T cells in the CMV-seropositive patients, we could not establish a higher turnover of memory T cells. This might suggest that after initial expansion of this cell population shortly after CMV infection (83), these cells will enter a more exhausted state during chronic latency of the virus. This results in a loss of the capacity to proliferate accompanied by an increased resistance to apoptosis. (84)

Like ESRD patients, individuals infected with Human Immunodeficiency Virus (HIV) have T-cell deficiencies which resembles premature T-cell ageing which is likely caused by continuous triggering of the immune system by the virus. (85) Although the mechanism of creating a prematurely aged T-cell compartment for both diseases is different, the end-result on T cells is similar (i.e. higher number of differentiated cells with a loss in CD28 expression, shorter telomeres and a lower number of naïve T cells) resulting in similar clinical outcomes like a higher risk for infections, development of cancer and cardiovascular diseases. (86)

In HIV-infected individuals, CMV causes an increase in EMRA $CD8^+CD28$null T cells expressing CD57. These highly differentiated cells are positive for the effector cytotoxins perforin and granzyme B. (87, 88) Next to that, in HIV patients it was found that strong anti-CMV T-cell responses results in a lower number of naïve T cells for the CD4 T-cell compartment. (88) These CMV effects found in HIV patients are in line with CMV effects in ESRD patients.

We have previously postulated that the prematurely aged T cell-system in ESRD patients contributes to clinically relevant complications, such as increased infection risk, decreased vaccination response, and a highly increased risk for cardiovascular diseases (8, 37, 45, 47, 53, 55). Given their cardio-toxic features, the pro-inflammatory and highly cytotoxic $CD4^+CD28$null T cells in ESRD patients can be important for later complications (77). A number of earlier reports have also shown the relation between CMV-serostatus, the expansion of CD28null T cells and the increased risk for atherosclerosis in ESRD patients. (47, 76-78) In addition, increased numbers of highly differentiated $CD8^+$ T-cell numbers and a loss of CD28 expression were associated with less allograft rejection after kidney transplantation. Given the results of this study, it seems unlikely that primary immune responses which involve the naïve T-cell compartment or $CD4^+$ T cell-dependent immune responses in ESRD patients will be affected by their CMV-serostatus. At present, such an association has not been reported and CMV-serostatus does not seem to affect the vaccination response in children. (89, 90)

In healthy elderly individuals, CMV-seropositivity leads to an expansion of effector $CD8^+$ T cells which are $CD8^+CD28nullCD57^+$. These CMV-specific T cells were found

to be oligoclonal and can constitute to up to one-quarter of the total $CD8^+$ T-cell compartment in elderly which makes cells unable to respond to other pathogens. (91) Moreover, these highly differentiated cells have shorter telomeres and are associated with an increased risk for the development of coronary heart diseases. (92)

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

Disclosure

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4

UREMIA-ASSOCIATED IMMUNOLOGICAL AGING IS STABLY IMPRINTED IN THE T-CELL SYSTEM AND NOT REVERSED BY KIDNEY TRANSPLANTATION

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ABSTRACT

The uremia-induced inflammatory environment in end-stage renal disease (ESRD) patients is associated with premature T-cell ageing resulting in a defective T-cell immunity. As kidney transplantation (KT) reduces the pro-inflammatory environment, we hypothesized that KT would rejuvenate the aged T-cell system. As ageing parameters, we determined in 70 KT recipients the differentiation status by immunophenotyping, thymic output by the T-cell receptor excision circle (TREC)-content together with $CD31^+$ naïve T-cell numbers and the relative telomere length (RTL) as a measure for proliferative history at pre-KT, 3,6 and 12 months post-KT. In addition, T-cell function was determined by measuring the proliferative capacity and percentages of cytokine producing cells. Directly post-KT, memory T-cell numbers were diminished but restored to pre-KT values at 12 months, except for $CD4^+$EM T cells. The RTL of (memory) $CD4^+$ and $CD8^+$ T cells did not change. In contrast, TREC-content and $CD31^+$ naïve T-cell numbers were stable post-KT although the RTL of naïve $CD4^+$ and $CD8^+$ T cells decreased implying homeostatic proliferation of naïve cells, in response to a temporary decrease in memory cells. The T-cell function was not improved post-KT. Our findings demonstrate that the uremia-associated aged-phenotype is stably imprinted in the T-cell system and not reversed by KT.

INTRODUCTION

Loss in renal function is strongly associated with a defective T-cell mediated immunity, which underlies a poor vaccination response, increased susceptibility for infections and an increased prevalence of virus-associated cancers. (39-41, 53) In addition, cytotoxic helper T cells ($CD4^+CD28$null T cells) are generated which play a role in the destabilization of atherosclerotic plaques (47, 76, 78).

Retention of uremic molecules and cytokines in end-stage renal disease (ESRD) patients are key mechanisms in generating oxidative stress and inflammation. (5, 6) This uremia-associated pro-inflammatory condition underlies the impaired T-cell system in ESRD patients by causing premature immunological ageing. (15, 79) Physiological ageing of the T-cell system is associated with a progressive decrease in newly formed T cells from the thymus (thymic output), a decrease in relative telomere length (RTL) of T cells and a more differentiated memory T-cell compartment. (15, 79) Thymic output can be determined by assessing the content of T-cell receptor excision circles (TREC), which are small circular DNA episomes that are formed during rearrangement of T-cell receptor (TCR) genes in the thymus. Telomeres are small DNA repeats, which are located at the end of each chromosome and protect against chromosomal damage after repeated cell division. With increasing age, the numerous cell divisions lead to progressive telomere erosion with measurable shortening of the average length.

The differentiation status of the T-cell compartment can be accurately determined by the differential expression of cell surface markers. (15) Since changes in T-cell differentiation can be heavily influenced by viral infections, particularly cytomegalovirus (CMV), all the aforementioned hallmarks of immunological ageing must be assessed and established. (35, 81, 93)

By using the combination of these three T-cell ageing parameters, the average immunological age of T cells in ESRD patients is 20-30 years higher than their chronological age (15).

Kidney transplantation (KT) rapidly reverses the levels of pro-inflammatory proteins and oxidative stress to levels that are comparable to healthy individuals. (94) Given the general flexibility of the immune system, we tested the hypothesis that relieving the pro-inflammatory conditions could lead to reconstitution of the thymic output and normalization of T-cell differentiation status, thereby rejuvenating the aged T-cell system.

MATERIALS AND METHODS

Study Population

In this study, all KT recipients in received a kidney from a living donor and received basiliximab as induction therapy. Post-KT the standard triple immunosuppression consisted of prednisolone, mycophenolate mofetil (MMF) and tacrolimus. After three months, patients were taken of prednisolone. All CMV-seronegative patients (n=25)

Table 1: Clinical and demographic characteristics of patients

	ESRD patients (n=70)	Healthy Controls (n=70)
Age in years [e]	54.2 ± 11.6 [a]	50.5 ± 12.7 [a]
CMV seropositive	45 (64.3%)	39 (55.7%)
Male	44 (62.9%)	32 (45.7%)
Renal replacement therapy [e]		
- no renal replacement therapy	29 (41.4%)	
- Patients on hemodialysis	31 (44.3%)	
- Patients on peritoneal dialysis	10 (14.3%)	
Time on dialysis	2.0 years (0.1-2.3) [b]	
Underlying kidney disease		
- Hypertensive nephropathy	21 (30.0%)	
- Glomerulonephritis	6 (8.6%)	
- Diabetic nephropathy	14 (20.0%)	
- Polycystic kidney disease	9 (12.9%)	
- Reflux nephropathy	2 (2.8%)	
- Other/unknown	18 (25.7%)	
Previous KT [e]		
- 1	3 (4.2%)	
- 2	1 (1.4%)	
Living kidney donation	70 (100%)	
Mismatches HLA class I	2.5 ± 1.03 [a]	
Mismatches HLA class II	1.2 ± 0.71 [a]	
Immunosuppressive medication		
- Basiliximab induction therapy [c]	70 (100%)	
- Prednisolone [d]	70 (100%)	
- MMF	70 (100%)	
- Tacrolimus	70 (100%)	
Trough levels [f]		
- MMF (mg/l)	1.75 ± 1.0 [a]	
- Tacrolimus (µg/l)	6.07 ± 2.7 [a]	
Patients with delayed graft function	5 (7.0%)	
Patients with rejection (treated with methylprednisolone)	8 (11.4%)	
Patients with CMV reactivation post-KT	0	
eGFR (ml/min) [e]	50.0 ± 18.3 [a]	
Serum creatinine concentration (µmol/l) [e]	136.5 ± 64.3 [a]	

[a] = Mean with standard deviation, [b] = Median with Interquartile range, [c] =Given at day 0 and day 4 post-KT, [d] = Given the first 3 months post-KT, [e] = At pre-KT time point, [f] = At 12 months post-KT time point.

included in this study received a kidney from a CMV-seronegative donor. The CMV-seropositive patients received a kidney either from a CMV-seronegative (n=13) or CMV-seropositive donor (n=32). As antiviral prophylaxis, all CMV-seropositive patients received valganciclovir the first 6 months post-KT. CMV-seronegative patients who received a kidney from a CMV-seropositive donor were excluded because CMV-infection is known to influence the T-cell ageing parameters. (34, 35, 93).

Patients and healthy controls (HC) characteristics are listed in table 1. Eight patients had a biopsy-proven rejection within the first year post-KT and all were treated with methylprednisolone. None of the patients received T-cell depletion therapy (i.e. rabbit anti-thymocyte globulin (rATG) or alemtuzumab). The study was approved by the local ethical committee (METC number: 2010-080; EudraCT-No: 2010-019398-14) and it was conducted according to the principles of Declaration of Helsinki and in compliance with International Conference on Harmonisation/Good Clinical Practice regulations.

Differentiation status of T cells

A whole blood staining was performed to determine the T-cell differentiation status (15, 35, 79). Briefly, samples were stained with AmCyan labeled anti-CD3 (BD, Erembodegem, Belgium) in combination with pacific blue labeled anti-CD4 (BD) and allophycocyanin Cy7 (APC-Cy7) labeled anti-CD8 (BD). The T cells are defined as $CD4^+$ or $CD8^+$ and further dissected using fluorescein isothiocyanate (FITC) labeled anti-CCR7 (R&D Systems, Uithoorn, The Netherlands) and APC labeled-CD45RO (BD) into naive ($CCR7^+CD45RO^-$), central memory (CM) ($CCR7^+CD45RO^+$), effector memory (EM) ($CCR7^-CD45RO^+$) and effector memory $CD45RA^+$ (EMRA) ($CCR7^-CD45RO^-$). Numbers of CD28null memory T-cells were determined by staining with peridinin chlorophyll-Cy5.5 (PerCP-Cy5.5) labeled anti-CD28 (BD). $CD31^+$ naïve T-cell numbers, marked as recent thymic emigrants (RTEs), were analyzed following staining with phycoerythrin (PE)-labeled anti-CD31 (Biolegend, Europe BV, Uithoorn, the Netherlands). Samples were measured on the FACSCanto II (BD) and analyzed using FACS Diva software version 6.1.2 (BD).

PBMCs isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples drawn from KT recipients one day before KT, 3, 6 and 12 post-KT and from donors one day before KT.

DNA isolation and TREC assay

TREC content was assessed using $1x10^6$ snap-frozen PBMCs. Briefly, DNA was isolated according to manufacturer's instructions (Qiagen Isolation kit, Qiagen, Venlo, the Netherlands). Subsequently, TREC content was determined using quantitative PCR for which we used a combination of two primers and a hydrolysis probe specific for

the so-called δREC(TCRD)-ψJα(TCRA) TREC (sjTREC). TaqMan quantitative PCR was performed on 50 ng DNA in a 25 µl reaction mixture containing 700 nmol/l of each primer 5'-TCGTGAGAACGGTGAATGAAG-3' and 5'-CCATGCTGACACCTCTGGTT-3', 150 nmol/l of hydrolysis probe 5'-(FAM) CACGGTGATGCATAGGCACCTGC-3' (TAMRA), and 12.5 µl 2× TaqMan Universal PCR Master Mix (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). Quantification of the DNA amount in each sample was performed using a quantitative PCR of the single-copy albumin gene. All reactions were performed in duplicate, unless a difference in cycle threshold (Ct) between replicates of >1.5 necessitated to repeat the PCR experiment. ΔCt was calculated by using the formula: Ct value TREC PCR – Ct value albumin PCR. (20, 79)

Telomere Length Assay

Flow fluorescent in situ hybridization was performed to determine the RTL. The isolated PBMCs were stained with either CD4-biotin (Beckman-Coulter, BV, Woerden, the Netherlands) or CD8-biotin (Biolegend) followed by staining with streptavidin-Cy5 (Biolegend). The PBMCs were fixed and permeabilized (Invitrogen Life Technologies, Bleiswijk, the Netherlands) and the RTL was determined using the telomere PNA-kit/FITC (Dako BV, Heverlee, Belgium). The sub cell line 1301 of CCRF-CEM known for its long telomeres, served as an internal positive control. After acquisition of the samples on the FACSCanto II (BD) and analysis using FACS Diva software version 6.1.2 (BD), the RTL was calculated through the next formula (25, 79):

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

In addition, the RTL within FACS-sorted purified naïve or memory $CD4^+$ and $CD8^+$ T-cells was assessed. For this purpose, PBMCs ($20x10^6$) were stained with AmCyan labeled anti-CD3, pacific blue labeled anti-CD4, APC-Cy7 labeled anti-CD8, PE-Cy7 labeled CCR7 (BD) and APC labeled CD45RO (BD) and using live/dead marker Via Probe (7-aminoactinomycin D (7AAD) viable cells were selected. Cell sorting was performed on a FACSAria II SORP (BD). All fractions had a purity of more than 95%.

Analysis of proliferative capacity of T cells

PBMCs were thawed and labeled using PKH26 cell linker kit (Sigma-Aldrich, St. Louis, USA) according manufacturer's instructions. The labeled cells were concentrated to $5x10^5$/mL in culture medium (consisting of RPMI-1640, glutamax, P/S and 10% heat-inactivated pooled human serum) and 100 µL was transferred to a 96-well plate (Greiner bio-one, Alphen aan den Rijn, the Netherlands) and stimulated in triplicates using either

or not anti-CD3/anti-CD28 coated beads (1 bead: 1 cell; Invitrogen) as a positive and negative control, respectively. To study the proliferative response to recall antigens, cells were stimulated with tetanus toxoid (37.5 lf/mL; NVI, Bilthoven, the Netherlands). Following a 6-day stimulation, cells were harvested and wells pooled and stained using AmCyan labeled anti-CD3 (BD), PacBlue labeled anti-CD4 (BD), APC labeled anti-CD8 (BD). A discrimination between live and dead cells was made using the 7-AAD marker (BD). Percentages of dividing $CD4^+$ and $CD8^+$ T cells were determined by analysis of samples on the FACSCanto II (BD) and using FACS Diva software version 6.1.2 (BD) or ModFit LT software (Verity Software House Inc, Topsham, USA).

Cytokine producing T cells

PBMCs ($1x10^6$/mL) were stimulated anti-CD3/anti-CD28 coated beads (Invitrogen) for 12 hours in presence of the cytokine secretion inhibitor Brefeldin A (1 µL/mL; Golgiplug, BD) to determine frequencies of cytokine producing cells. (95) The background signal was determined by stimulation in absence of these beads. Following this stimulation, cells were harvested and the cell surface stained using AmCyan labeled anti-CD3, PerCP labeled anti-CD4, APC-Cy7 labeled anti-CD8. After staining cells were fixed/lysed with FACS lysing solution (BD) and permeabilized with FACS permeabilizing solution 2 (BD). An intracellular staining was performed with APC labeled anti-IL-2 (BD), FITC labeled anti-IFNγ (BD) and PE labeled anti-TNFα (BD) followed by addition of 1% formaldehyde (Scharlau, Sentmenat, Spain). Percentages of cytokine producing cells were determined by measuring the samples (acquiring 0.5-$1x10^6$ T cells/measurement) using the FACSCanto II and analyzed using FACS Diva software version 6.1.2.

Statistical analyses

Analyses within KT patients over time and to the HCs were done using the repeated measurement statistical test, i.e. the parametric ANOVA followed by the post-hoc analysis Bonferroni or as non-parametric counterpart the Friedman test with Dunns multiple comparisons as post-hoc test were used. P-values <0.05 for two sides were considered statistically significant. Pre-KT values were compared to values at different time points after KT and statistically analyzed with a paired-t-test.

RESULTS

The aged T-cell phenotype is unchanged 12 months post-KT

The differentiation status of the $CD4^+$ and $CD8^+$ T-cell compartment of KT recipients were determined at pre-KT and at 3, 6 and 12 months post-KT. The 12 months post-KT value was compared to the value of HCs. In Figure 1A and 2A, typical examples of the gating strategy are depicted. The total number of $CD4^+$ T cells (Figure 1B) was not affected 12 months post-KT and the number remained significant lower ($p<0.001$)

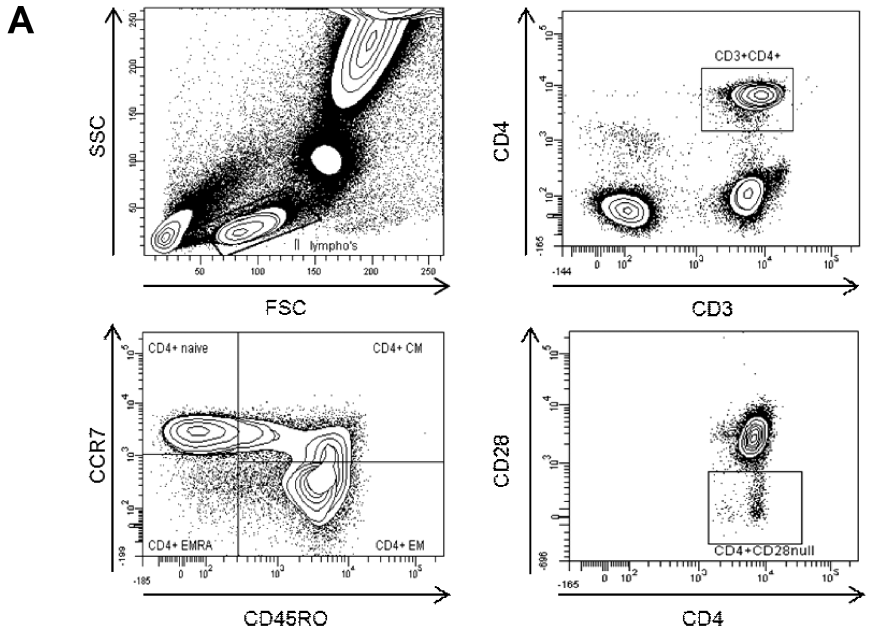
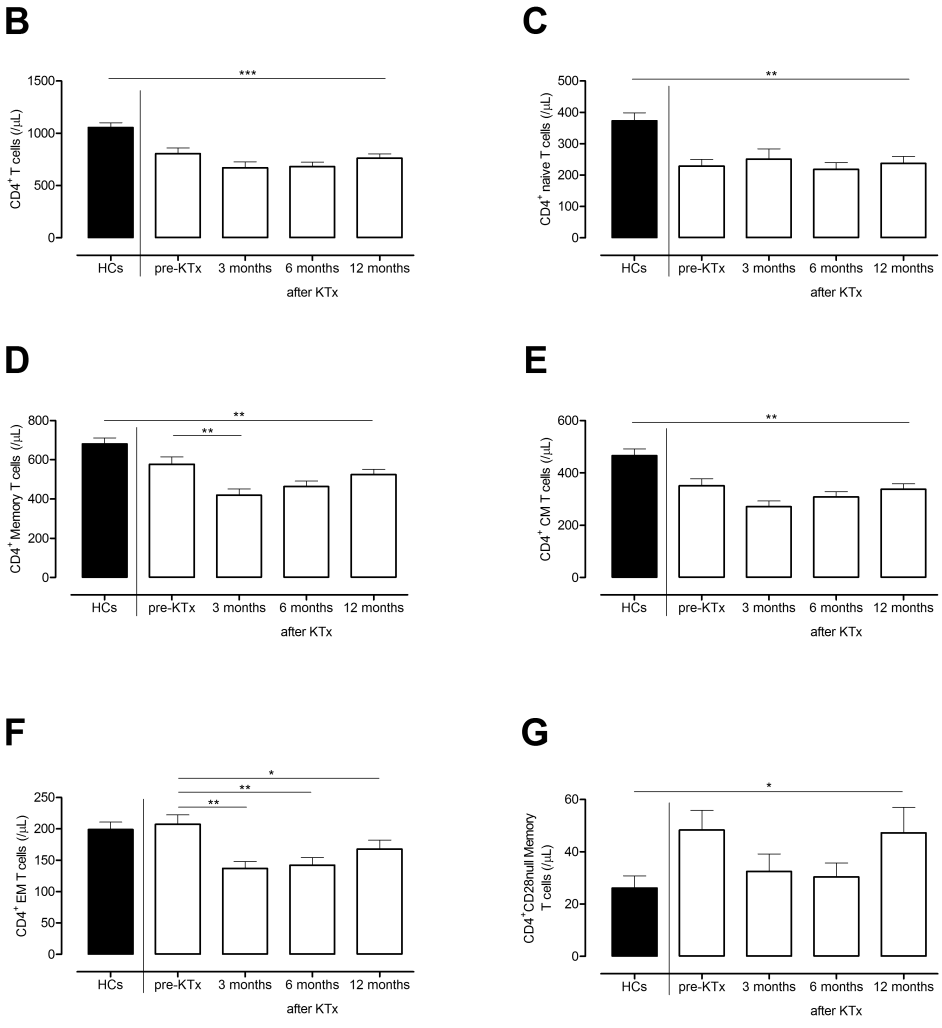


Figure 1: CD4⁺ T-cell differentiation status. First, the gating strategy to examine the CD4⁺ T-cell differentiation status is shown (A). Upon selection of the lymphocytes based on forward/sideward characteristics, the CD3⁺CD4⁺ T cells were selected. These CD4⁺ T cells were dissected into subsets using CD45RO and CCR7. Within the memory CD4⁺ T cells we finally examined absolute numbers of CD28null T cells. Absolute numbers of total (B), naïve (C), memory (D), central memory (CM; E), effector memory (EM; F) CD4⁺ T cells and CD28null within the memory CD4⁺ T-cell population (G) at pre-KT, 3, 6 and 12 months post-KT of the KT patients (white bars) and that of the HCs are shown (black bars) (n=70, mean ± SEM). Significant differences between pre-KT and post-KT time-points for KT recipients and between patients at 12 months and the HCs were calculated and shown (* p<0.05, ** p<0.01, *** p<0.001)

compared to HCs. Naïve CD4⁺ T-cell numbers were maintained (Figure 1C) but memory CD4⁺ T-cell numbers decreased significantly (p<0.01) 3 and 6 months post-KT but were restored to pre-KT level at 12 months post-KT (Figure 1D). 12 months post-KT, both naïve and memory T-cells were significant lower compared to HCs (p<0.01). The CM T-cell numbers did not change post-KT (Figure 1E). The EM CD4⁺ T-cell numbers were significantly (p<0.01) lower 3 and 6 months post-KT compared to pre-KT but also at 12 months post-KT the EM CD4⁺ T-cell numbers were still significantly (p<0.05) lower (Figure 1F). At 12 months post-KT both subsets (CM: p<0.01, EM p<0.05) were significantly lower than that of HCs. The memory CD4⁺CD28null T-cell numbers were not affected by KT, but were significantly (p<0.05) higher than those of HCs. (Figure 1G).

The total CD8⁺ T-cell count decreased significantly 3 months post-KT (Figure 2B, p<0.01) due to a decrease in memory CD8⁺ T cells (Figure 2D, p<0.001) with stable naïve CD8⁺ T-cell numbers (Figure 2C). Moreover, 12 months post-KT, naïve CD8⁺ T-cell numbers were still significantly (p<0.001) lower when compared to HCs. The CM CD8⁺



T-cell numbers remained lower ($p<0.05$) compared to HCs (Figure 2E). The EM, EMRA $CD8^+$ T-cell and $CD8^+CD28$null memory T-cell numbers were significantly ($p<0.05$) lower 3 months post-KT (Figure 2F,G+H) but were restored to pre-KT values at one year post-KT. Both numbers of EMRA $CD8^+$ T-cells and the number of $CD8^+CD28$null memory T-cells were significantly higher ($p<0.05$) compared to those observed in HCs (Figure 2G and 2H) at 12 months post-KT.

No restoration of thymic output within one year post-KT

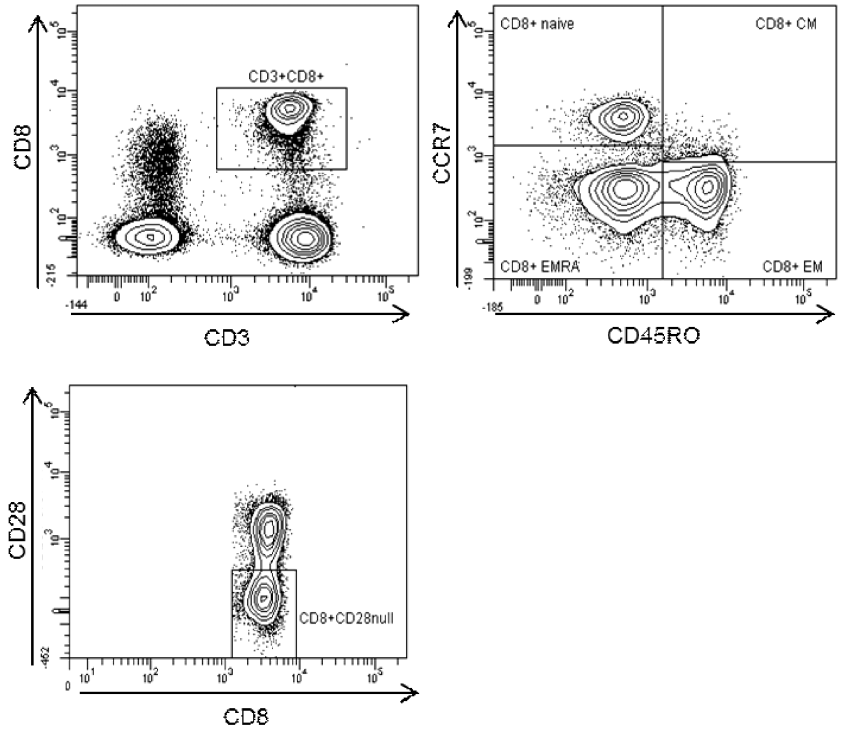
Thymic output was determined by TREC content analysis (Figure 3A) and expression of CD31, a marker for RTEs, within the naïve T-cell compartment (Figure 3B-D). Twelve months post-KT, thymic output was maintained at a similar level as pre-KT and the ΔCT

remained significantly (p=0.009) higher compared to that observed in HCs, indicative that thymic output was not restored (Figure 3A). In accordance with this observation, the number of CD31$^+$ naïve CD4$^+$ and CD8$^+$ T cells remained unaltered post-KT. Consequently, at 12 months post-KT the recipients had still significant lower (p<0.001) numbers of CD31$^+$ naïve CD4$^+$ and CD8$^+$ T cells compared to HCs (Figure 3 C+D).

Relative telomere length of CD4$^+$ and CD8$^+$ T cells is maintained in memory T cells

As a marker for proliferative history, the RTL of CD4$^+$ (Figure 4B) and CD8$^+$ (Figure 4C) T-cells were determined. Figure 4A shows a typical example of the flow-cytometric analysis. For both T-cell compartments, the RTL was not different post-KT when compared to pre-KT and remained significantly lower (CD4$^+$: p=0.05, CD8$^+$: p=0.008) when compared to HCs. Next the RTL of purified naïve and memory T-cell fractions were determined. Remarkably, a significant decline in the RTL for the naïve CD4$^+$ (p=0.004, Figure 4D) and CD8$^+$ T cells (p=0.04, Figure 4E) was observed in contrast to the relatively stable RTL within memory T cells. Collectively, these data point to increased homeostatic proliferation in the naïve CD4$^+$ and CD8$^+$ T-cell compartments.

A



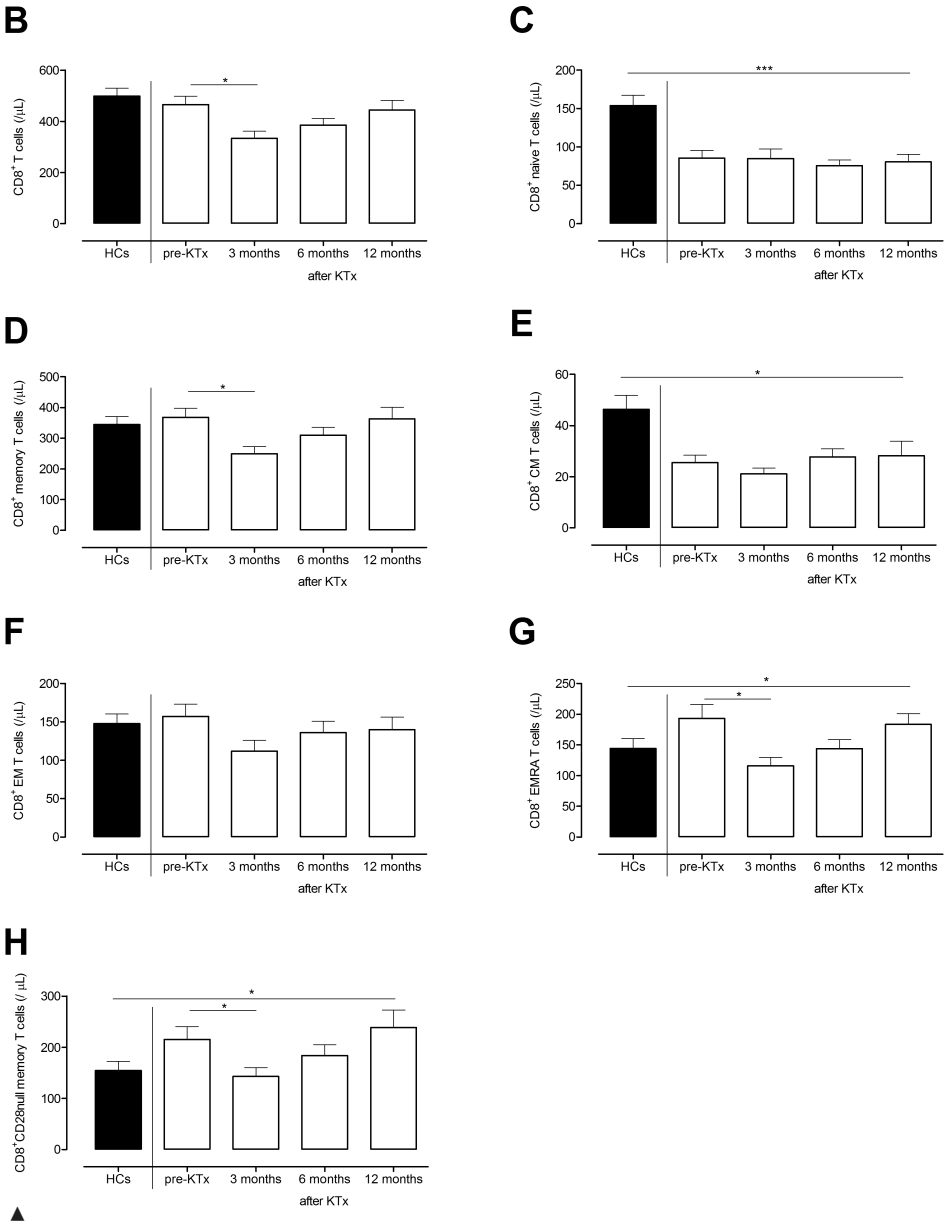
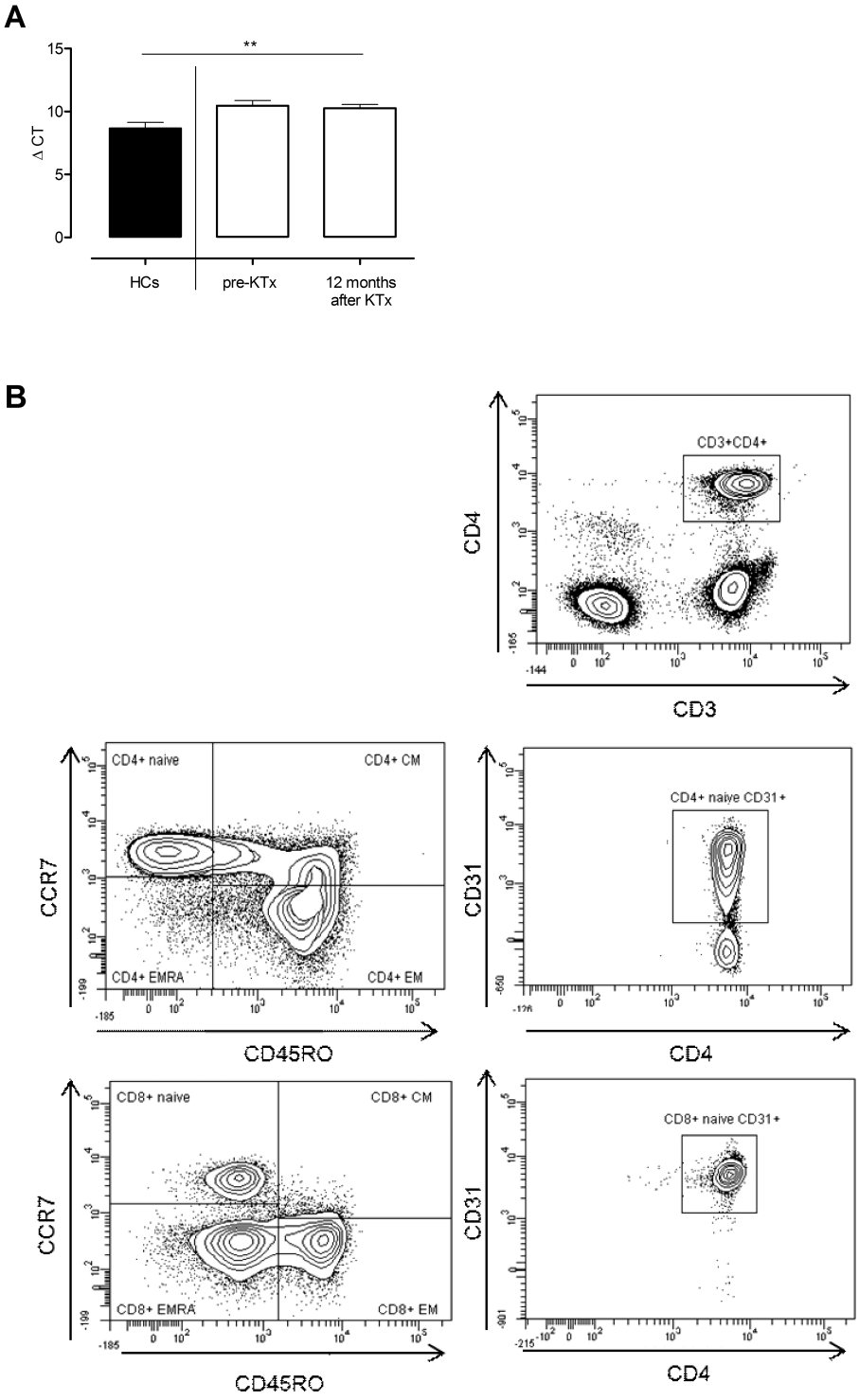


Figure 2: CD8+ T-cell differentiation status. The CD8+ T-cell differentiation status was determined by FACS analysis similar as performed for CD4+ T cells. After selecting CD3+CD8+ T cells, expression of CD45RO and CCR7 was measured to distinguish the different subsets (i.e. naïve, CM, EM and EMRA) within the CD8+ T cells. Staining for CD28 was used to select the CD28null CD8+ T cells within the memory population (A). Absolute numbers of total (B), naïve (C), memory (D), CM (E), EM (F), EMRA (G) CD8+ T cells and CD28null within the memory CD8+ T-cell population (H) at pre-KT, 3, 6 and 12 months post-KT of the KT patients (white bars) and that of the HCs are shown (black bars) (n=70, mean ± SEM). Significant differences between pre-KT and post-KT time-points for KT recipients and between patients at 12 months and the HCs were calculated and shown (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)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CCR7
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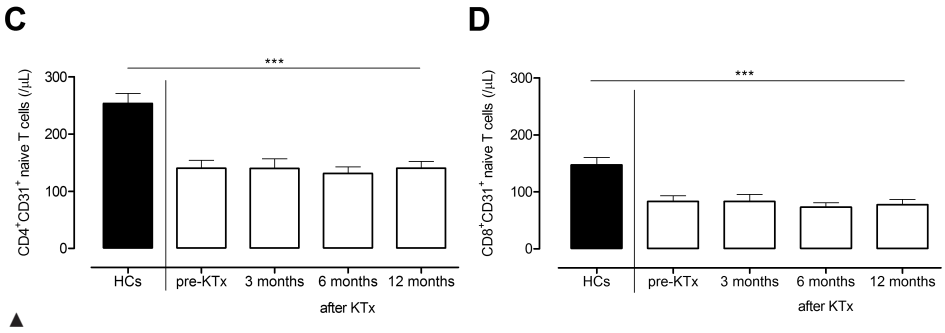


◄ **Figure 3:** The thymic output was measured by TREC content and expression of CD31⁺ by naïve T cells. The ΔCT (difference in number of amplification cycles between TREC and albumin as a control for DNA input ; inversely related to TREC content) was calculated pre-KT and 12 months post-KT of the KT recipients (white bars) and pre-KT of the HCs (black bars) and is depicted (n=10; mean ± SEM) (A). In addition, within naïve CD4⁺ and CD8⁺ T cells, expression of CD31 was determined as shown in this typical flowcytometric example (B). Absolute numbers of CD31⁺ T cells within the naïve population (n=70, mean ± SEM), indicating RTEs are depicted for the CD4⁺ (C) and the CD8⁺ (D) T-cell compartment. Significant differences between pre-KT and post-KT time-points for KT recipients and between patients at 12 months and the HCs were calculated and shown (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)

T-cell ageing parameters after KT and clinical characteristics

In accordance with previous data (79), no relation was observed between patients who had a remaining kidney function pre-KT and those who received renal replacement therapy (RRT) pre-KT. In addition, the outcome of the ageing parameters one year post-KT did not differ between the two patients groups (data not shown).

Also the patients age, kidney transplant function (both serum creatinine concentration and eGFR) and trough levels of MMF and tacrolimus, were not significantly associated with a different course of thymic output, RTL and differentiation status within the first year post-KT (data not shown).

T-cell function is unchanged one year after KT

T-cell function was analyzed by examining proliferative capacity and frequencies of cytokine producing cells using flowcytometry. An example of the gating strategy (Figure 5A) and subsequent analysis of proliferation by ModFit is shown for unstimulated T cells, tetanus toxoid stimulation and anti-CD3/anti-CD28-stimulation (Figure 5B).

One year post-KT, the percentage of proliferating CD4⁺ and CD8⁺ T cells in response to both tetanus toxoid (Figure 5C and D) and anti-CD3/anti-CD28 coated-beads (Figure 5 E and F), was similar compared to pre-KT.

In addition, there was no increase in the frequency of cytokine producing T cells in response to polyclonal stimulation for CD4⁺ (Figure 5G) and CD8⁺ (Figure 5H) T cells.

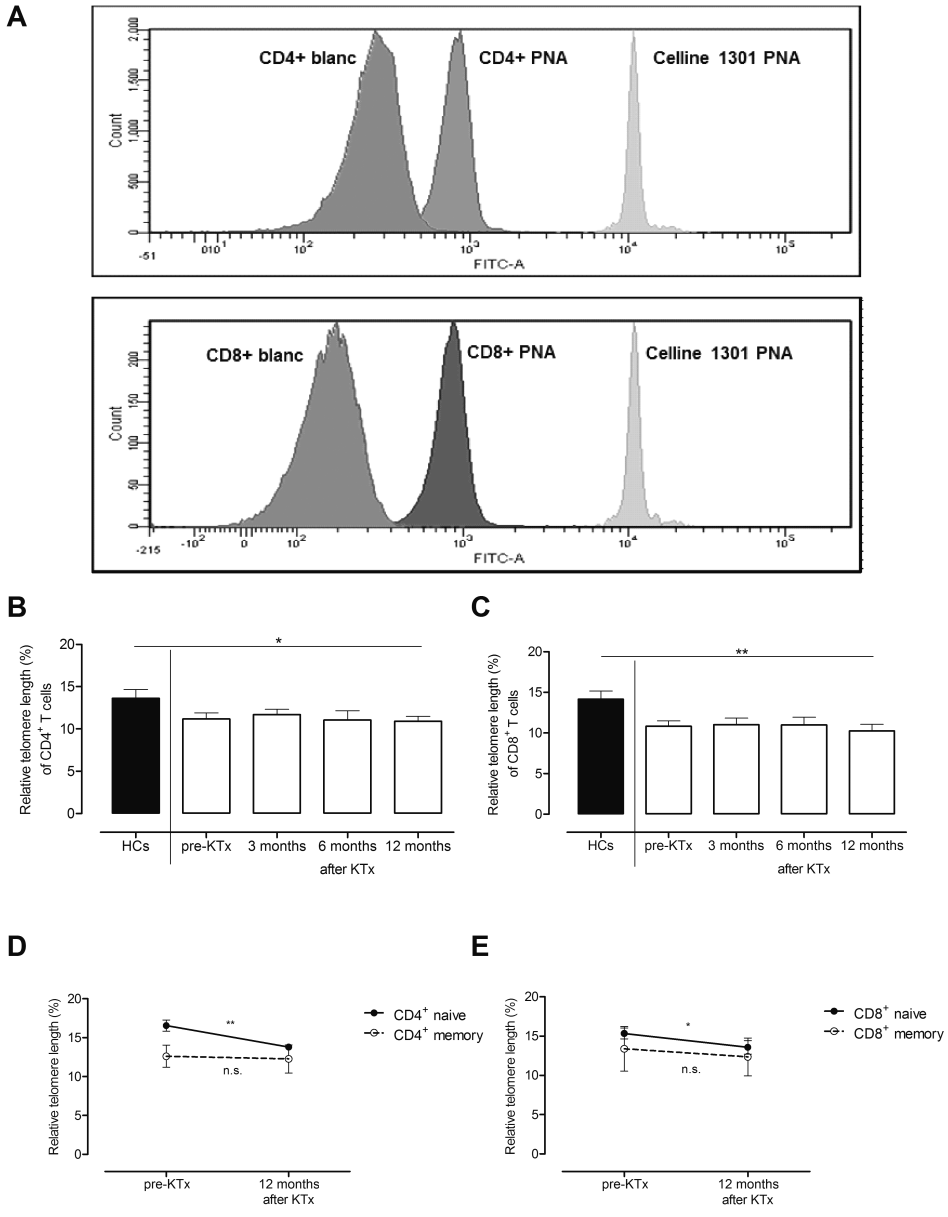


Figure 4: Proliferative history was measured by the relative telomere length. A typical example of the flowcytometric example of the PNA (probe to detect telomeres)-fluorescence intensity, depicted in a histogram, for the cell-line 1301, $CD4^+$ and $CD8^+$ T cells and the negative control (without PNA-probe) is depicted (A). The RTL of (B) the $CD4^+$ and (C) $CD8^+$ T-cell compartment were determined pre-KT, 3, 6 and 12 months post-KT (white bars) and the RTL of the HCs was determined pre-KT (black bars). (n=10, mean ± SEM). The RTL was also determined within a sorted naïve (straight line, n=5) and memory fraction (hatched line, n=5) of the $CD4^+$ (D) and $CD8^+$ (E) T-cell population. Significant differences between pre-KT and the post-KT time-points for KT recipients was calculated and shown as well as the differences between the HCs and the 12 months post-KT time-point. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)

DISCUSSION

In this study we evaluated whether KT is able to rejuvenate the aged T-cell system through reconstitution of thymic output and normalization of the T-cell differentiation status. The results of this study demonstrate that one year post-KT such changes have not occurred.

In accordance with previous studies, the results show that the T-cell system of ESRD patients before KT is significantly different from HC as the thymic output is lower, RTL is shortened and T cells are more differentiated. This prematurely aged T-cell phenotype was essentially unchanged at one year post-KT, although within the year significant changes within the $CD4^+$ and $CD8^+$ memory compartment were observed. Both young and old KT recipients had a similar course of the ageing parameters post-KT and results were similar for patients with or without a history of dialysis before transplantation and independent of kidney transplant function.

The unchanged numbers of CD31-expressing naïve T cells and TREC content post-KT indicate that the thymic output remains stable and does not revert to that observed in HC. Both approaches chosen to determine thymic output have their limitations. First, the use of CD31 to identify RTEs may be a better marker for thymic output of $CD4^+$ rather than $CD8^+$ RTEs. (22, 96) In addition, CD31-expression within naïve T cells may not necessarily reflect RTEs as CD31 expression is maintained upon homeostatic proliferation in presence of cytokines like IL-7. However, our group as well as others have demonstrated an association between percentages of both CD31-expressing naïve $CD4^+$ as well as $CD8^+$ T cells and age (97, 98). Furthermore, we analyzed the TREC content within PBMCs which makes it formally incorrect to draw conclusions about the thymic output as the composition of the T-cell compartment within PBMCs influences the detection of TRECs. However, combining the T-cell ageing parameters (TREC, relative telomere length and differentiation status) in KT recipients pointed towards a prematurely aged T-cell system prior to KT which is irreversible by KT.

Although the trough levels of MMF and tacrolimus did not correlate with the TREC content nor the numbers of $CD31^+$ naïve T cells, we cannot rule out the possibility that immunosuppressive (IS) drugs prevent a possible restoration of thymic function post-KT. Several animal studies have reported IS drugs to reduce the size of thymic tissue or numbers of thymocytes via affecting different developmental stages of thymocytes. (99-101)

In humans, a study with myasthenia gravis patients showed that tacrolimus affects the thymic output of mainly $CD8^+$ naïve T cells. (102) However, tacrolimus only reduced TREC levels in the thymomatous patient-group of myasthenia gravis patients which makes it difficult to comparable the outcomes of this study with the thymic output of ESRD patients that had already a decreased thymic output compared to HCs. (102) A previous study by Nickel et al. (103) reported that the percentages CD31-expressing naïve T cells were unchanged post-KT under standard triple or quadruple IS. However, in contrast to their findings pre-KT (103), we and others consistently observe a

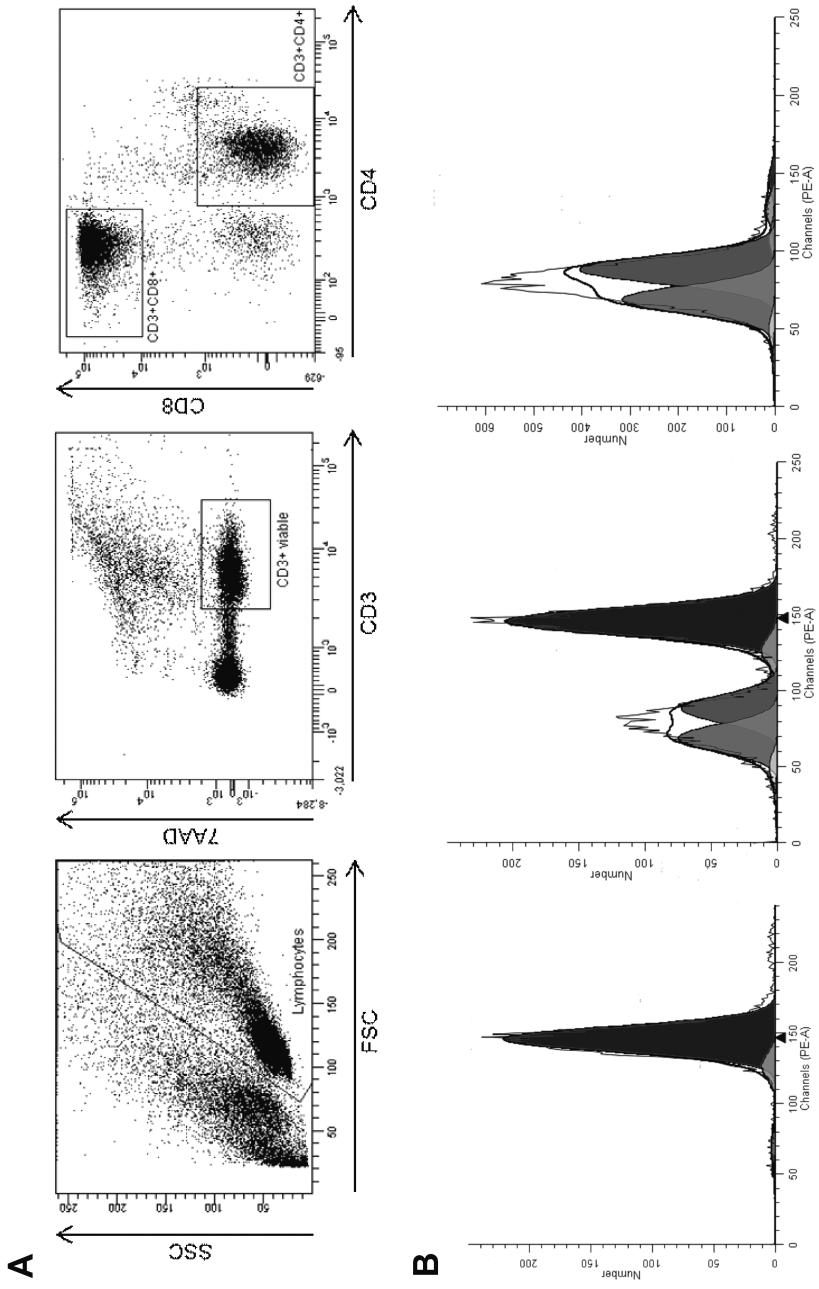
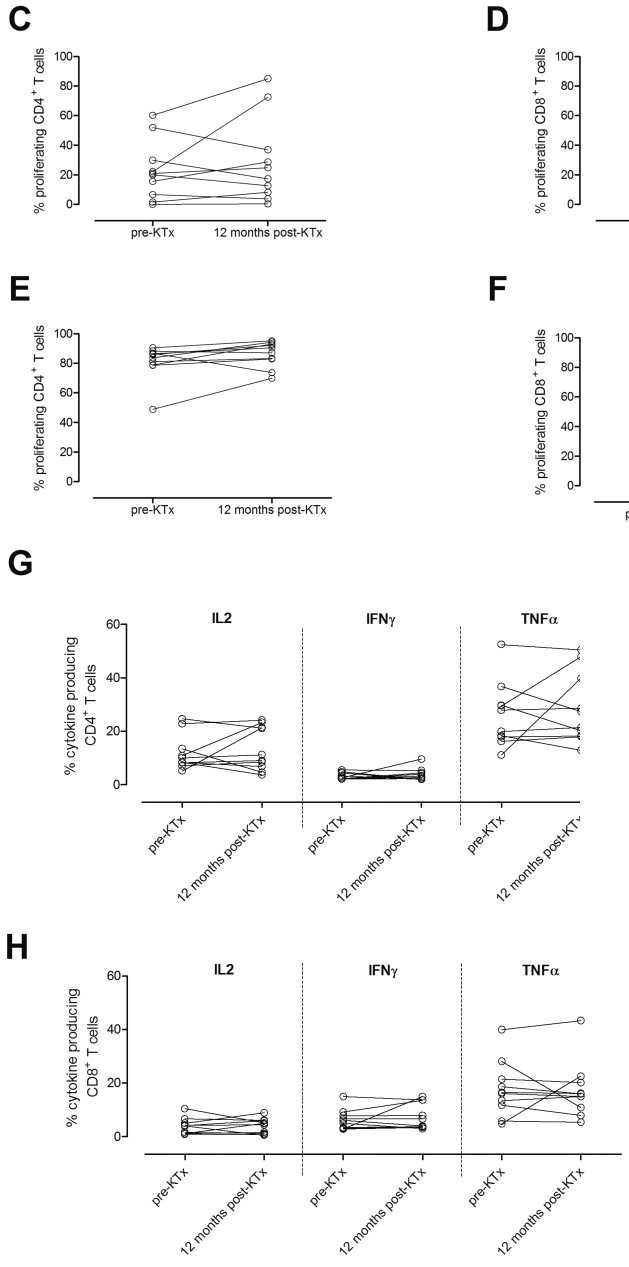


Figure 5: Proliferative capacity and percentages of cytokine producing T-cells upon TCR-triggering. A typical gating strategy for flowcytometric analysis of proliferation is depicted (A). Briefly, live cells were selected followed by the selection of viable $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells. Using ModFit software proliferative responses were analyzed. Histograms depicted are representative examples of background (left histogram), tetanus toxoid-induced (middle histogram) and anti-CD3/ anti-CD28-induced (right histogram) proliferation (B). The percentage proliferating $CD4^+$ (C) and ►



► $CD8^+$ (D) T cells following tetanus toxoid stimulation were determined pre-KT and at 12 months post-KT. As a positive control we examined the proliferative capacity upon stimulation by anti-CD3/anti-CD28 beads of the $CD4^+$ (E) and $CD8^+$ (F) T-cell compartment. Individual values at pre-KT and 12 months afterwards are shown (open symbols, n=10, mean ± SEM). In addition, we examined percentages of cytokine producing $CD4^+$ (G) and $CD8^+$ (H) T cells upon stimulation with anti-CD3/anti-CD28 beads. Percentages of IL2, IFN-γ and TNF-α producing cells were determined and individual values at prior to and 12 months post-KT are shown (open symbols, n=10, mean ± SEM).

negative effect of uremia on thymic function using both CD31-expressing naïve T cells as well as TREC content as read-out of thymic output. (15) Here we observed a stable number of $CD31^+$ naïve T cells and an unchanged TREC content 12 months post-KT. In combination with the decreasing RTL of naïve T cells, these observations imply that the numbers of naïve T cells are maintained by both proliferation in response to homeostatic cytokines like IL-7 (indicated by unchanged $CD31^+$ naïve T cell numbers) as well as to low affinity TCR interaction with antigen presenting cells presenting self-antigens (unchanged $CD31^-$ naïve T-cell numbers (data not shown)). The naïve T-cell proliferative response can be considered as a homeostatic response to the decrease of (memory) T cells in the circulating pool. The latter is most likely caused by the combination of IS drugs which are known to affect in particular the activated memory T cells. (51) One year post-KT, the T-cell system has returned to the pre-KT status at the expense of attrition of telomeres in naïve T cells. This finding of T-cell system reconstitution by homeostatic naïve T-cell proliferation is in accordance with data obtained after complete depletion of T cells by agents like rATG or alemtuzumab. Even in these extreme situations, repopulation of T cells did not result from an enhanced thymic output, but resulted from homeostatic proliferation. (97, 104)

The question remains why immunological ageing does not reverse even when adequate graft function is achieved post-KT. As stated above, we cannot formally rule out that IS drugs prevent the restoration of thymic output, telomere length and reversal of memory T-cell differentiation status. (102, 105) However, a more likely explanation might lie at the epigenetic level. Normal ageing is associated with, epigenetic changes in hematopoietic stem cells (HSC) resulting in a shift in the balance towards myeloid precursors at the expense of the lymphoid ones. (106, 107) In addition, the memory T cells are more differentiated and pro-inflammatory subsets arise both within the T cell and monocyte population. The uremia-associated pro-inflammatory milieu in ESRD patients causes major epigenetic changes, which may result in an aged T-cell system by on average 20 years compared to their chronological age. (13) In addition, there are other striking similarities with the immune system in the very old healthy individuals as there is an overall decrease in cells belonging to the lymphoid cell lineage, pro-inflammatory subsets of T cells and monocytes are increased while myeloid cell numbers are unaffected. The findings in this study point to the possibility that uremia might induce irreversible epigenetic changes at the level of HSC. (108)

The persistence of the aged T-cell system post-KT has several clinical implications as it may increase the risk for infections and malignancies in KT recipients. For instance, T-cell lymphopenia has been associated with a high risk for infections and malignancies post-KT. (38, 109) Furthermore, the presence of highly differentiated $CD4^+$ T cells lacking CD28 is associated with the risk for a cardiovascular event post-KT. (47, 76, 78) A potential benefit of a relatively unchanged aged T-cell system post-KT may be the persistence of high numbers of terminally differentiated $CD8^+$ T cells lacking CD28 which is associated with less kidney allograft rejection and long-term graft survival. (110-112)

In addition to the unaltered T-cell ageing parameters, the T-cell function did also not improve post-KT. Although we did not have the impression that T-cell function improved following KT in our patient population, more research is required enlarging the number the patients studied in this respect. In conclusion, our findings demonstrate that uremia-associated immunological ageing is not reversed by KT and therefore remains a determinant of immune deficiency independent of graft function. Thus, ESRD induces an irreversible imprint on the immune system.

Disclosure

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5

LOSS OF CD28 ON PERIPHERAL T CELLS DECREASES THE RISK FOR EARLY ACUTE REJECTION AFTER KIDNEY TRANSPLANTATION

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ABSTRACT

Background End-stage renal disease (ESRD) patients have a dysfunctional, prematurely aged peripheral T-cell system. Here we hypothesized that the degree of premature T-cell ageing before kidney transplantation (KT) predicts the risk for early acute allograft rejection (EAR).

Methods 222 living donor KT-recipients were prospectively analyzed. EAR was defined as biopsy proven acute allograft rejection within 3 months post-KT. The differentiation status of circulating T cells, the relative telomere length (RTL) and the number of $CD31^+$ naïve T cells were determined as T-cell ageing parameters.

Results Of the 222 patients analyzed, 30 (14%) developed an EAR. The donor age and the historic panel reactive antibody (PRA) score were significantly higher and the number of related donor KT was significantly lower in the EAR group. EAR-patients showed lower $CD4^+CD28$null T-cell numbers and the same trend was observed for $CD8^+CD28$null. No differences regarding the other ageing parameters were found. A multivariate Cox regression analysis showed that higher $CD4^+CD28$null T-cell numbers was associated with a lower risk for EAR. In vitro, a significantly lower percentage of alloreactive T cells was observed within the CD28null T-cell fractions.

Conclusion Immunological ageing-related expansion of highly differentiated CD28null T cells is associated with a lower risk for EAR.

INTRODUCTION

Loss of renal function leads to retention of uremic molecules and cytokines, which creates oxidative stress and inflammation. (6) The resulting pro-inflammatory uremic environment underlies the dysfunctional T-cell immunity of end-stage renal disease (ESRD) patients. (8) The major changes in the peripheral T-cell composition are T-lymphopenia, increased T-cell differentiation and loss of telomere length, the latter indicating a history of enhanced T-cell replication. (113)

The T-lymphopenia is largely due to a loss of naive (antigen-inexperienced) T cells, which show signs of increased activation and are more prone to apoptosis. (113) This loss of circulating naive T cells runs in parallel with a decrease in newly formed naive T cells, known as recent thymic emigrants (RTEs, indicating a premature involution of the thymus). In combination with an expanded, more differentiated memory T-cell compartment, this leads to a relatively large decrease in the percentage of circulating naive T cells. (13, 113) The highly differentiated memory T cells are characterized by a loss of the co-stimulatory molecule CD28, making them less dependent on co-stimulation to become activated. (114) Moreover, these cells are known to have a reduced telomere length due to their numerous cell divisions. (76, 113, 115)

The uremia-associated changes in the composition of the peripheral T-cell compartment resemble the physiological changes in the ageing immune system of elderly healthy individuals, (33, 66, 79) which leads to the concept of ESRD-related premature immunological ageing. This was confirmed when a combined analysis of the thymic output, differentiation status and the telomere length of T cells in ESRD patients was performed and the results were compared to healthy individuals over a wide age range. (113) A consistent pattern of premature immunological ageing was observed with a discrepancy of 15-20 years between the immunological age of T cells of ESRD patients compared to their chronological age. (113, 116) This prematurely aged T-cell system of ESRD patients offers at least a partial explanation for the increased susceptibility to infections (37), reduced vaccination response (39-41, 53), increased prevalence of malignancies (45, 117) and may also be a non-classical risk factor for cardiovascular diseases (47-50)

A prematurely aged T-cell system leading to impaired T-cell immunity may also reduce the risk for acute rejection after kidney transplantation, but this has not been systematically studied. In addition, most studies that have assessed the circulating T-cell compartment in relation to acute rejection have only demonstrated percentages of cells. (110, 112) This can lead to erroneous conclusions given the complex changes in all T-cell subsets and for example expansion of memory T cells may be interpreted as a reduction in the number of naive T cells and vice versa.

In this study, we hypothesized that the degree of premature T-cell ageing, based on the absolute number of differentiated T cells, thymic output and telomere length, prior to kidney transplantation (KT) is associated with the risk for early graft rejection (EAR) in kidney transplant recipients.

MATERIALS AND METHODS

Study population

All patients undergoing a living-donor KT in the period from 1 November 2010 to 1 October 2013 were considered for participation in this study. This study included all ESRD patients with various causes of chronic kidney disease (CKD) (Table 1).

All patients received induction therapy with basiliximab (Simulect®, Novartis; 20 mg i.v. on day 0 and day 4), tacrolimus (Prograf®, Astellas Pharma; aiming for predose concentrations of 10-15 ng/mL in weeks 1-2, 8-12 ng/mL in weeks 3-4, and 5-10 ng/mL, thereafter), mycophenolate mofetil (Cellcept®, Roche; starting dose of 1 g b.i.d., aiming for predose concentrations of 1.5 – 3.0 mg/L), and glucocorticoids. All patients received 50 mg prednisolone b.i.d. intravenously on days 0-3. Thereafter, 20 mg oral prednisolone was started and subsequently tapered to 5 mg at month 3.

Clinical variables such as age at the time of transplantation, gender, CMV-seropositivity, anti-CMV IgG titer, human leukocyte antigen (HLA) class I and class II mismatches, current and historical panel reactive antibody (PRA) score, warm ischemia time (WIT), number of previous kidney transplantations, preemptive KT (defined as receiving a kidney before the start of renal replacement therapy (RRT)) and related KT (defined as receiving a kidney from a genetically related donor) were determined and shown in table 1. The HLA-typing was assessed according to the international standards (American Society for Histocompatibility and Immunogenetics/the European Federation for Immunogenetics) using serologic and DNA-based techniques. The PRAs were determined at the laboratory of the blood bank in Leiden, the Netherlands.

We defined EAR as the development of biopsy-proven acute allograft rejection according to the Banff criteria (118) within 3 months after KT.

All patients participated in a randomized-controlled clinical trial with the primary aim to study the efficacy of a genotype-based approach to tacrolimus dosing (Dutch trial registry number NTR 2226; http://www.trialregister.nl/trialreg/index.asp). All patients gave written informed consent to participate in the clinical trial, as well as for the sub-study, which is presented here. The study was approved by the Medical Ethical Committee of the Erasmus MC (MEC number 2010-080, EudraCT 2010-018917-30). This study was conducted in accordance with the Declaration of Helsinki.

PBMCs isolation

By using Ficoll-Paque Plus (GE healthcare, Uppsala, Sweden), peripheral blood mononuclear cells (PBMCs) were isolated from heparin blood samples drawn from KT-recipients the day before KT. The isolated PBMCs were stored at -150°C with a minimum amount of 10×10^6 cells per vial for further experiments.

Absolute numbers of $CD4^+$ and $CD8^+$ T cells and T-cell differentiation status by FACS analysis

To determine the absolute numbers of the different lymphocyte populations from blood, a Trucount staining was done. In this protocol, 20 µl of the 6-color TBNK reagent (BD Multitest™, BD, Erembodegem, Belgium) was used in combination with a BD Trucount™ tube (BD) and 50 µl of EDTA blood. This tube contains a number of beads (i.e. bead count; lot-specific) and enables calculation of absolute numbers of cells per µl of blood. The 6-color TBNK reagent contains phycoerythrin (PE) labeled anti-CD45, AmCyan labeled anti-CD19, PE-Cy7 labeled anti-CD3, Peridinin chlorophyll (PerCP) labeled anti-CD4, fluorescein isothiocyanate (FITC) labeled anti-$CD8^+$ and allophycocyanin-Cy7 (APC-Cy7) labeled anti-CD16/CD56.

In addition, a whole blood staining was performed to determine the T-cell differentiation status. (113, 119) 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 their expression of CCR7 and CD45RO after staining 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 $CD45RA^+$ (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). Recent thymic emigrants (RTEs) were identified by the expression of CD31 within the naive T-cell pool upon staining with PE-labeled anti-CD31 (Biolegend, Europe BV, Uithoorn, the Netherlands). Samples were measured at the FACSCanto II (BD) acquiring at least $5x10^5$ lymphocytes and analyzed using FACS Diva software version 6.1.2 (BD). (113, 119)

Telomere Length Assay

Flow fluorescent in situ hybridization (flow-FISH) was performed to determine the relative telomere length (RTL) of $CD4^+$ and $CD8^+$ T cells. PBMCs were isolated and stained with either CD4-biotin (Beckman-Coulter, BV, Woerden, the Netherlands) or CD8-biotin (Biolegend) followed by staining with streptavidin-Cy5 (Biolegend). The PBMCs were fixed and permeabilized (Invitrogen Life Technologies, Bleiswijk, the Netherlands) and by using the telomere FITC-labeled PNA-kit (DakoCytomation, Heverle, Belgium) the telomere length was determined. The sub cell-line 1301 of CCRF-CEM known for its long telomeres, served as an internal positive control. After acquisition of the samples on the FACSCanto II (BD) and analysis using FACS Diva software version 6.1.2 (BD), the RTL of the $CD4^+$ and $CD8^+$ T cells was calculated through the next formula (25, 119):

$$RTL = \frac{\text{(median FL1 sample cells with probe - median FL1 sample cells without probe) x DNA index of control (=2) cells x 100}}{\text{(median FL1 control cells with probe - median FL1 control cells without x DNA index of sample (=1)) cells}}$$

Cytokine producing alloantigen-stimulated T cells

To determine frequencies of alloantigen-specific (cytokine-producing) T cells prior to transplantation, we used the CD137 multi-parameter flowcytometric assay as published recently. (120) PBMCs of a kidney transplant recipient were stimulated in presence of co-stimulation (αCD49d, 1 µg/mL; BD) with or without T cell-depleted (>98% pure) donor PBMCs at a 1($5x10^6$):1($5x10^6$) ratio for 24 hours of which the last 12 hours were in presence of Brefeldin A (Golgiplug, BD) and monensin (Golgistop, BD). Subsequently, the cell surface was stained using AmCyan-labeled anti-CD3 (BD), APC-Cy7-labeled anti-CD8 (BD) and PerCP-Cy5.5-labeled anti-CD28 (BD) in order to visualize where the cytokine producing T cells are located. Following fixation and permeabilization, CD137 and cytokines were stained intracellular using APC-labeled anti-CD137 (BD), PE-labeled anti-interferon (IFN)-γ (BD) and FITC-labeled anti-interleukin (IL)-2 (BD). At least 0.5-$1x10^6$ viable $CD3^+$ T cells were acquired on the FACS Canto II. Alloantigen-specific cytokine producing T cells were corrected for the cytokine signal observed in the absence of donor T-cell-depleted PBMC stimulation. Samples were measured on the FACSCanto II (BD) and analyzed using FACS Diva software version 6.1.2 (BD).

Statistical analysis

All variables are presented as medians with interquartile ranges. The difference between continuous variables was analyzed with the Mann–Whitney U test. The difference between categorical variables was analyzed either with the Pearson's chi-squared test or with the Fisher's exact test depending on the expected values in any of the cells of a contingency table. The latter was used when the expected values were lower than 5 in any of the cells. For the assessment of an association between clinical/immunological variables and the presence of EAR, the Cox proportional hazards model was used. The significance level (p-value) was two tailed and 0.05 was used for all analyses. Statistical analyses were performed using SPSS® version 21.0 for Windows® (SPSS Inc., IL, USA). T-cell subset graphs were created using GraphPad Prism 5 (CA, USA).

RESULTS

Patient characteristics

We enrolled 222 consecutive patients who received a kidney transplant from a living donor. Patient characteristics are shown in Table 1. Of the 222 patients analyzed,

Table 1: Patients Characteristics

KT Patients (*n* = 222)	No Early Rejection (*n* =192) (86%)	Early Rejection (*n* = 30) (14%)	p
Age recipient	57 (46 – 64)	55 (47 – 63)	0.60
Age Donor	52 (40 – 62)	58 (50 – 65)	**0.024**
Male gender recipient	118 (61%)	14 (63%)	0.84
Male gender donor	94 (49%)	13 (43%)	0.57
CMV seropositivity recipient	118 (61%)	15 (50%)	0.23
CMV serostatus donor/recipient			
-/-	42 (22%)	10 (33%)	0.17
-/+	38 (20%)	5 (17%)	0.69
+/-	32 (17%)	5 (17%)	1.00
+/+	80 (42%)	10 (33%)	0.39
Anti-CMV IgG titer recipient (AU/mL)	65 (42 – 105)	58 (47 – 86)	0.75
Mismatch HLA class I	2 (2 – 3)	3 (2 – 3)	0.15
Mismatch HLA class II	1 (1 – 2)	1 (1 – 2)	0.31
Mismatch HLA class I and II	4 (3 – 5)	4 (3 – 5)	0.11
PRA current (%)	0 (0 – 4)	0 (0 – 4)	0.52
PRA historic (%)	4 (0 – 4)	4 (0 – 29)	**0.039**
Amount of KT	1 (1 – 1)	1 (1 – 1)	0.63
Warm ischemia time	20 (16 – 24)	21 (16 – 25)	0.69
Cause of CKD			
Nephroclerosis/atherosclerosis/hypertension	44 (23%)	7 (23%)	0.96
Primary glomerulopathies	26 (14%)	4 (13%)	1.00
Diabetes	41 (21%)	2 (7%)	0.06
Urinary tract infections/stones	5 (3%)	1 (3%)	0.59
Reflux nephropathy	9 (5%)	1 (0%)	1.00
Polycystic Kidney Disease	33 (17%)	7 (23%)	0.42
Other	26 (14%)	5 (17%)	0.58
Unknown	8 (4%)	3 (10%)	0.17
Pre-emptive KT	75 (39%)	17 (57%)	0.07
Genetically-related KT	82 (43%)	6 (20%)	**0.018**

Data are presented as medians (interquartile range).

30 (14%) had an EAR. The median age of the patients was 57 years and the median donor age was 53 years. The donors were significantly older in the EAR group with a median age of 58 years compared to a median age of 52 years in the group without EAR (p=0.024). The majority of the patients (92%) received a donor kidney for the first time, 14 patients (6%) for the second time and three patients (1%) for the third

time. Of the 30 patients who developed EAR, 11 (37%) received T cell depletion therapy consisting of either alemtuzumab subcutaneously or rabbit anti-thymocyte globulin (rATG) intravenously. Four patients had a transplantectomy within the first 3 months after transplantation. Two of these patients had a therapy-resistant cellular acute rejection within the first week after KT. The other two patients were in the no EAR group and the graft was removed due to vascular problems that occurred during the transplantation procedure.

The historical PRA score was significantly higher in the EAR group compared with the no EAR group (p=0.039). The relative number of genetically related KT was significantly lower in the EAR group (20% vs 43%, p=0.018). Other potential risk factors for acute rejection, like number of HLA mismatches or previous kidney transplantation, did not associate with EAR in this patient group.

Patients with no EAR have a higher number of CD4$^+$CD28null T cells prior to KT

The CD4$^+$ T-cell differentiation status of both patient groups was determined prior KT. In Figure 1A and 2A typical examples of the gating strategy are depicted for the flowcytometric analysis of the CD4$^+$ and CD8$^+$ T-cell population respectively. No significant differences were found in the number of CD4$^+$ T cells (Figure 1B) between the EAR group and no EAR group. Moreover, the number of naive, memory, CM and EM (Figure 1C-1F) was not significantly different. Interestingly, compared to the no EAR group, the EAR group had significant lower number of CD28null T cells within the CD4$^+$ T-compartment (21 cells/µl vs. 7 cells/µl, p<0.01, Figure 1G).

The total number of CD8$^+$ was not significantly different between the two patient groups (Figure 2B). The number of naive, memory and CM, EM and EMRA (Figure 2C-G) CD8$^+$ T cells did also not show any significant differences between the two groups. Furthermore, the EAR group tended to have a lower number of CD28null CD8$^+$ T cells (p=0.08, Figure 1H) compared to the no EAR group.

No differences in relative telomere length and RTEs between the EAR group and no EAR group prior to KT

As a marker for the proliferative history, the RTL of the CD4$^+$ and CD8$^+$ T cells was determined. For both T-cell subsets no significant differences were found between the EAR group and the no EAR group regarding the RTL (Table 2).

The number and percentages of RTEs were identified by the expression of CD31 within the naive T-cell pool. No significant differences for CD4$^+$ or CD8$^+$ T cells were found between the two groups prior to KT (table 2).

Table 2: RTL and RTEs before kidney transplantation in patients with or without rejection within the first 3 months

KT Patients (n = 222)	No Early Rejection (n =192) (86%)	Early Rejection (n = 30) (14%)	P
RTL of $CD4^+$ T cells	12.1 (9.1 – 15.0)	11.5 (10.0 – 13.3)	0.90
RTL of $CD8^+$ T cells	11.4 (9.2 – 15.3)	11.1 (10.4 – 14.4)	0.83
$CD31^+CD4^+$ naive T-cell numbers (/µl)	106.5 (56.7 – 207.4)	104.3 (77.0 – 192.9)	0.99
$CD31^+$ within $CD4^+$ naive T-cells (%)	66.4 (55.1 – 75.1)	62.2 (53.7 – 76.8)	0.69
$CD31^+CD8^+$ naive T-cell numbers (/µl)	55.4 (24.1 – 566.3)	65.0 (12.8 – 203.9)	0.80
$CD31^+$ within $CD8^+$ naive T-cells (%)	97.7 (94.4 – 98.9)	97.9 (93.9 – 99.5)	0.41

Donor age, historical PRA, a related kidney donation and absolute numbers of $CD4^+CD28$null T cells are related with the risk for EAR

The results of the univariate Cox regression analysis of the patient characteristics are presented in Table 3A. This analysis showed that having an older donor was associated with a higher risk for EAR (HR: 1.04, p=0.011). Besides this, a higher historical PRA score was also associated with a higher risk for EAR (HR: 1.02, p=0.001). Receiving a donor kidney from a relative reduced the risk for EAR (HR: 0.36, p=0.025).

The univariate Cox regression analysis of the T-cell subsets showed that a higher number of absolute $CD4^+CD28$null T cells, i.e. having a more differentiated $CD4^+$ T-cell compartment, was associated with a lower risk for EAR (HR: 0.65, p=0.025). In contrast, the number of $CD8^+CD28$null T cells was not associated with the risk for EAR (HR: 0.98, p=0.420).

A multivariate Cox regression analysis was performed with the three aforementioned clinical characteristics (i.e. donor age, historic PRA and a related kidney donation) as covariates (Table 3B). In accordance with the univariate analysis, a higher absolute number of $CD4^+CD28$null T cells was associated with a lower risk for EAR (HR: 0.66,

Table 3A: Hazard rations for the clinical characteristics in relation to early acute allograft rejection (univariate analysis)

	HR	95% CI	P
Age donor	1.04	1.01 – 1.07	0.011
PRA historic	1.02	1.01 – 1.03	0.001
Genetically related KT	0.36	0.15 – 0.88	0.025
CD4 positive CD28null T cells	0.65	0.45 – 0.95	0.025
CD8 positive CD28null T cells	0.98	0.94 – 1.03	0.420

*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$. CI: confidence interval, HR: hazard ratio. The CD4 positive and the CD8 positive CD28null cells are presented with steps of 20 cells/µL. Data are presented as medians (interquartile range).

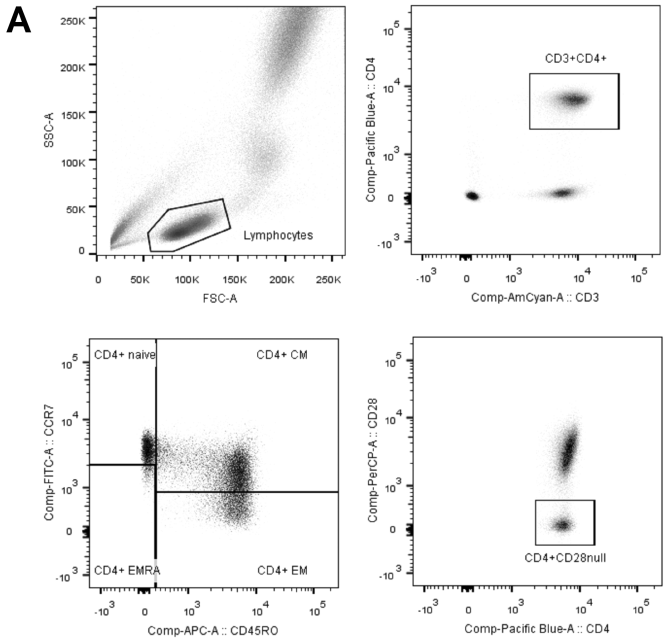


Figure 1: CD4⁺ T-cell differentiation status prior to KT. The CD4⁺ T-cell differentiation status was determined by flowcytometry. An example of the gating strategy is shown in A. Briefly, lymphocytes were identified based on the forward/sideward characteristics followed by the selection of the CD3⁺CD4⁺ T cells. These CD4⁺ T cells were dissected into subsets using CCR7 and CD45RO. Furthermore, the number of CD28null cells was examined within the CD4⁺ T-cell population (A). Absolute numbers of total (B), naive (C), memory (D), CM (E), EM (F) and CD28null (G) CD4⁺ T cells are shown for the no EAR (white boxplot, n=192) and EAR (grey boxplot, n=30) group of patients. Significant differences were calculated and shown (*p<0.05, **p<0.01, **p<0.001).

p=0.029). Again, no association could be observed between the number CD8⁺CD28null T cells and the risk for EAR (HR: 0.99, p=0.419).

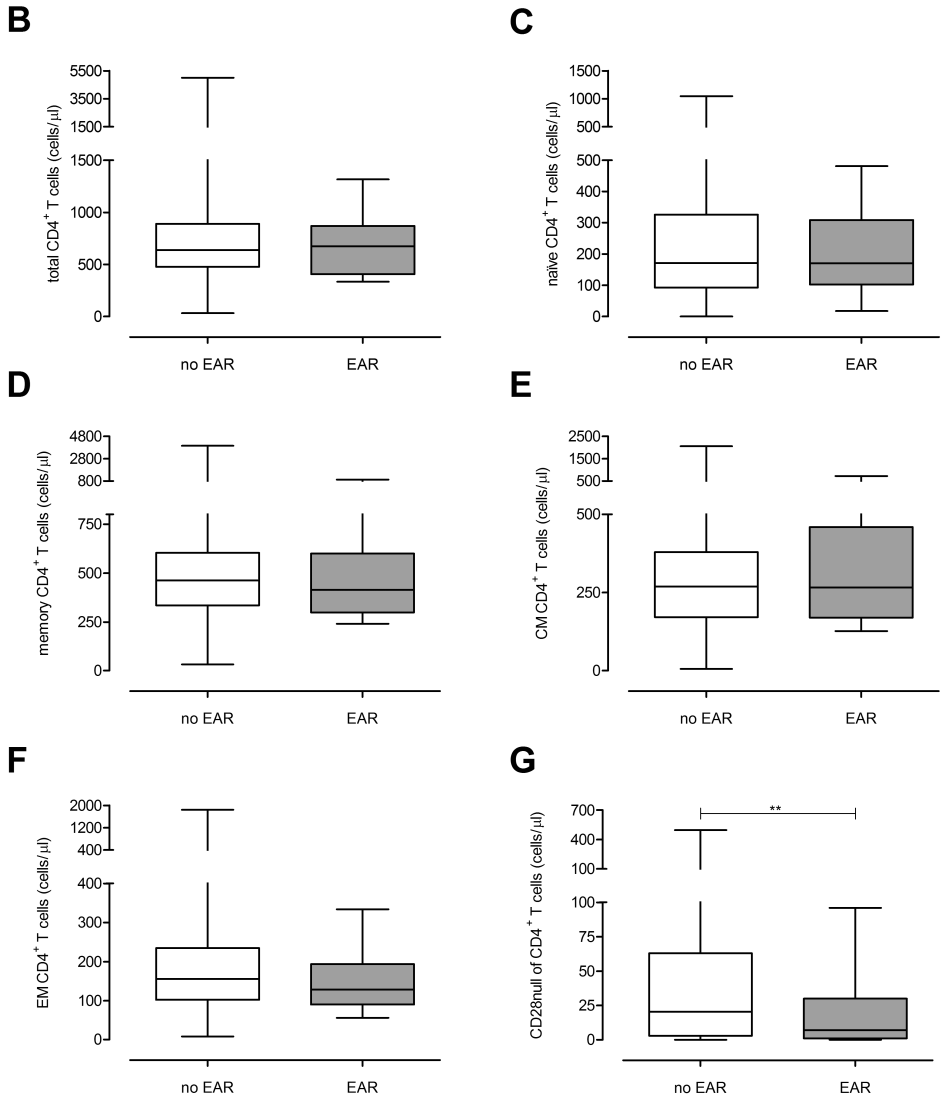
Alloantigen-specific T-cells predominantly co-express CD28

In Figure 3A a representative example of the gating strategy is shown of the dissection of alloantigen-specific (CD137⁺ and CD137⁺ IL-2⁺ or IFN-γ⁺) CD4⁺ T cells into a

Table 3A: HRs for the T-cell parameters in relation to early acute allograft rejection (multivariate analysis)

	HR	95% CI
CD4 positive CD28null T cells	0.66	0.45 – 0.96
CD8 positive CD28null T cells	0.99	0.95 – 1.02

*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$. CI: confidence interval, HR: hazard ratio. The CD4 positive and the CD8 positive CD28null cells are presented with steps of 20 cells/µL. **Covariates:** age donor, PRA historic, related KT.



CD28null- or CD28+ T-cell compartment. A similar FACS analysis was performed for the CD8+ T-cell population.

First the frequency of alloantigen-specific CD137-expressing T cells was determined in the CD4+ T-cell compartment and these were mainly CD28+ (i.e. 72.4% vs 27.6%, $p<0.001$, Figure 3B) In addition, the CD28+ T cells contained more IL-2 (86.9% vs 13.1%, $p<0.001$) and IFN-γ (i.e. 65.1% vs. 34.9%, $p<0.001$) producing alloantigen-specific T cells compared to the CD28null fraction (Figure 3C+D) upon alloantigen-stimulation.

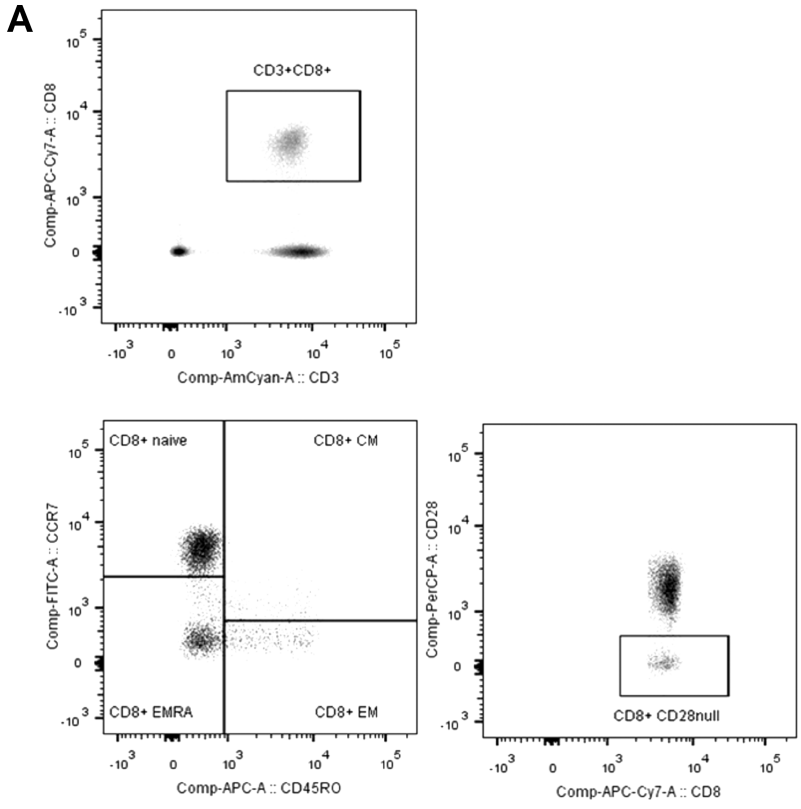
Similar results were found for the CD8+ T cells. Upon alloantigen-stimulation, the CD137+CD8+ T-cells were mainly located within the CD28+ T cells (i.e. 60.4% vs 39.6%,

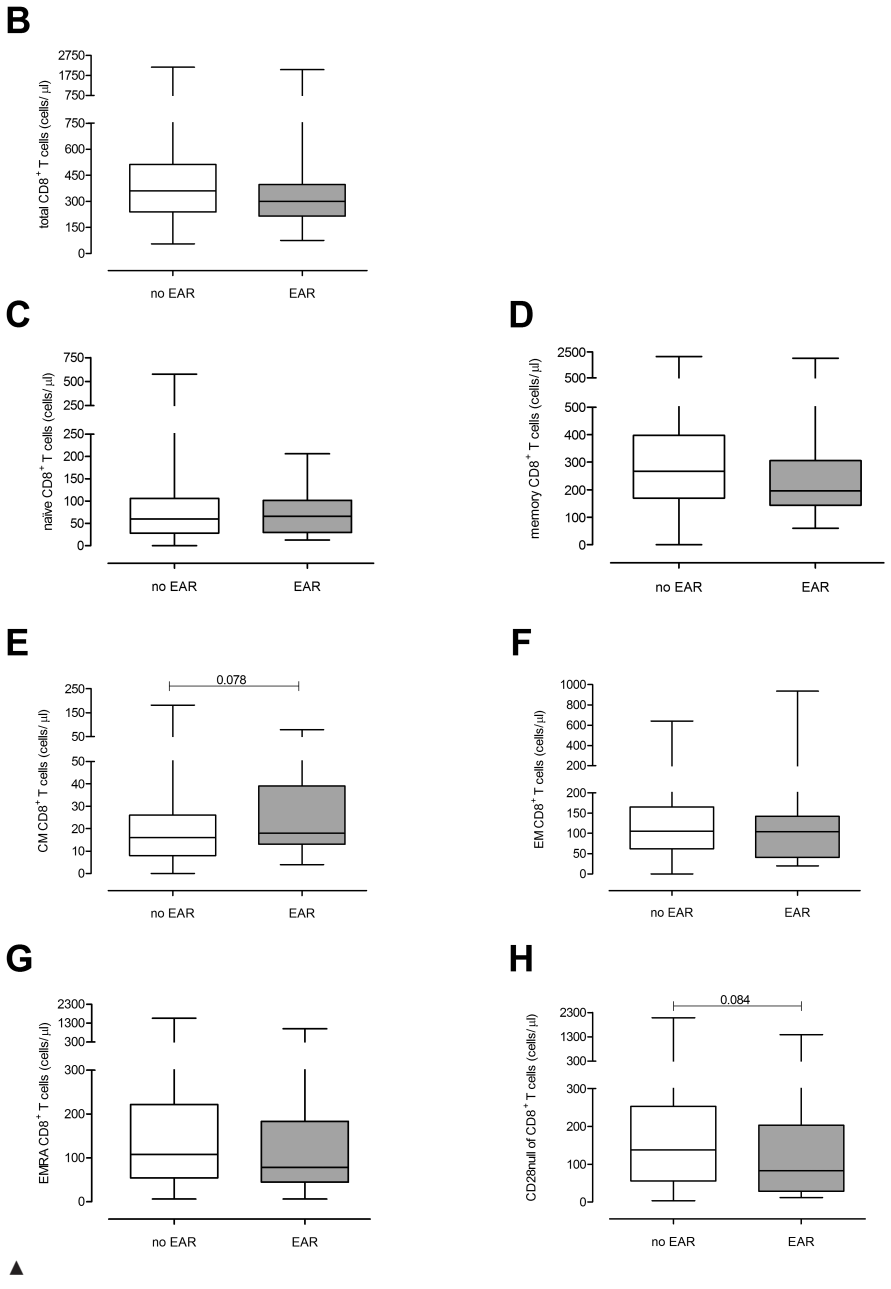
p<0.001, Figure 3E). A higher proportion of these cells were also able to produce more IL-2 compared to their CD28null counterparts (i.e. 72.4% vs 27.6%, p<0.001) and IFN-γ (i.e. 57.4% vs 42.6%, p<0.01) (Figure 3F+G).

DISCUSSION

In this study we analyzed within a large homogenous cohort of patients whether the degree of premature T-cell ageing prior to KT is associated with the risk for EAR post-KT. Of the three T-cell ageing parameters (thymic output, differentiation status and telomere length) used for the assessment of an immunological T-cell age, only the differentiation status was associated with the risk for EAR. A higher number of CD28null T cells, mainly within the $CD4^+$ T-cell population, is associated with a lower risk for EAR. The number of RTEs or the relative telomere length of $CD4^+$ and $CD8^+$ T cells were both not associated with the risk for rejection.

CD28null T cells are predominantly located within the (antigen-experienced) memory population and in particular within the more differentiated T cells. (121) Loss of CD28 on the cell surface of ($CD4^+$) T cells is one of the features of T-cell ageing as CD28null





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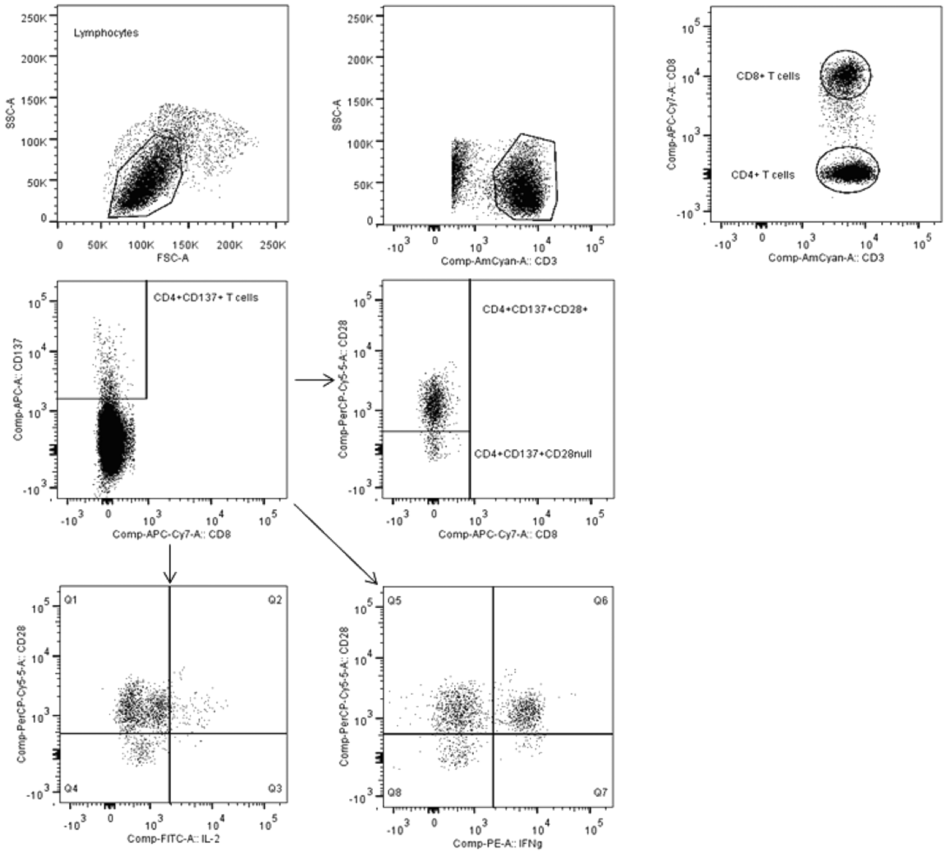
◄ **Figure 2:** CD8⁺ T-cell differentiation status prior to KT. Like for the CD4⁺, also the CD8⁺ T-cell differentiation status was determined by flowcytometry. An example of the gating strategy is shown (A) were the $CD3^{+}CD8^{+}$ were selected from the lymphocytes. The $CD8^{+}$ T cells were dissected into subsets using CCR7 and CD45RO and the number of CD28null cells was examined within the $CD8^{+}$ T-cell population (A). Absolute numbers of total (B), naive (C), memory (D), CM (E), EM (F), EMRA (G) and CD28null (H) $CD8^{+}$ T cells are shown for the no EAR (white boxplot, n=192) and EAR (grey boxplot, n=30) group of patients. Significant differences were calculated and shown (*p<0.05, **p<0.01, **p<0.001).

A



T cells are present at a low frequency and rarely found in young individuals. Moreover, $CD4^+CD28$null T cells are highly associated with seropositivity for cytomegalovirus (CMV). (58, 93, 122) However, in this study we did not observe a significant difference with respect to CMV-seropositivity between the EAR group and no EAR group.

The majority of CD28null T cells lack the expression of CCR7. This is important for homing to secondary lymphoid organs (78) where (naive) T cells are activated by antigen-presenting cells presenting alloantigens in a direct or indirect manner. In addition to the lack of CD28, they are less able to provide co-stimulation through the CD40L-CD40 pathway contributing to defective helper function. (123)

A characteristic feature of $CD4^+CD28$null T cells is their restricted T-cell receptor profile (111, 124) compared to the $CD28^+$ T-cell population. This may compromise their reactivity to foreign antigens like for example alloantigens. Thus theoretically lower numbers of $CD28^+$ T cells might result in lower alloreactivity. This hypothesis is supported by our finding that IL-2 and IFN-γ producing alloantigen-specific T cells

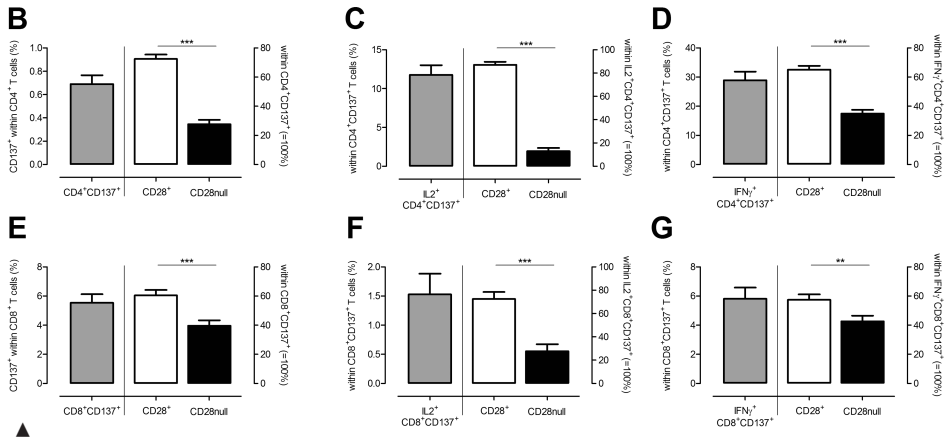


Figure 3: Cytokine producing alloantigen-stimulated T cells. The frequency of alloantigen-stimulated T cells was determined based on the expression of $CD137^+$ by flowcytometry in KT-recipients (n=40) prior to transplantation. First, an example of the gating strategy was shown. (A) Briefly, lymphocytes were identified based on the forward/sideward characteristics. Next the $CD3^+CD4^+$ (i.e. $CD8^-$) and $CD3^+CD8^+$ were selected and within these, the $CD137^+$ were selected as shown for the $CD4^+$ population. These cells were divided into a $CD28^+$ and CD28null population. Furthermore the frequency of IL-2^+ and IFN-γ^+ $CD137^+CD4^+$ was determined and also dissected into a $CD28^+$ and CD28null subset. A similar approach was applied for the $CD8^+$ T-cell compartment. First the frequency of $CD137^+$ cells within $CD4^+$ T-cell population was determined (grey bar) in ESRD patients. These cells were divided into a $CD28^+$ (white bar) and a CD28null subset (black bar) (B). Next the frequency of IL-2^+ cells within the $CD137^+CD4^+$ was determined (grey bar). Furthermore, these cells were divided into a $CD28^+$ (white bar) and a CD28null subset (black bar) (C). Also the frequency of IFN-γ^+ $CD137^+CD4^+$ T cells was determined (grey bar) and also these cells were divided into a $CD28^+$ (white bar) and a CD28null subset (black bar) (D). Next to the $CD4^+$, the frequency of $CD137^+$ cells within $CD8^+$ T-cell population was determined (grey bar) and divided into a $CD28^+$ (white bar) and a CD28null subset (black bar) (E). Within these $CD137^+CD8^+$ T cells, the frequency of IL-2 (F) and IFN-γ^+ (G) was determined (grey bars) and divided into a $CD28^+$ (white bars) and a CD28null subset (black bars) (F+G). Bars represents means and SEM. Significant differences were calculated and shown (*$p<0.05$, **$p<0.01$, **$p<0.001$).

were predominately located within the $CD28^+$ T-cell population, both within the $CD4^+$ and the $CD8^+$ T-cell compartment. In addition, in liver transplant recipients, lower frequencies of $CD4^+CD28^+$ T cells were found in allograft rejecting patients. (125)

The finding that high CD28null T-cell numbers are associated with a lower risk for allograft rejection is in line with an earlier small study in which these cells were shown to have an exhausted phenotype. (112) In a recent study, a lower frequency of $CD4^+$CD28null T cells was also observed in relation to acute rejection within the first year after kidney transplantation but statistical significance was lost in the multivariate analysis. (110) The patient population in that study was much more heterogeneous, including post-mortal kidney transplantations, more re-transplantations and a higher number of CMV-seropositive patients which could in part explain the difference in findings between both studies. (110)

Compared to the $CD28^+$ T-cell population, the CD28null T cells are known to have shorter telomeres. (93) The fact that we could not detect a correlation between the overall telomere length and the risk for EAR, might be explained by the relatively low frequency of CD28null T cells within the $CD4^+$ T-cell population. In line with our findings, a study by Oetting et al. (126) could also not find an association between the RTL and the risk for acute rejection. (126)

In this study we could not find an association between RTEs and the risk for EAR based on the expression of CD31. Since the thymus involutes rapidly after puberty, the contribution of the thymus to maintain the (naive) T-cell pool is relatively small in older individuals. (19) Maintaining adequate numbers of naive T cells upon ageing mainly relies on homeostatic proliferation either through homeostatic cytokines like IL-7 or low-affinity T-cell receptor interactions with self-antigens being presented by antigen-presenting cells (127). Since the total number of naive T cells is not different between the two groups of patients, it is likely that the degree of homeostatic proliferation is similar. These findings suggest that the naive T-cell compartment is not of significant importance for alloreactivity within the first three months after KT and that the memory T-cell compartment is more relevant. (128)

As the T-cell ageing parameters do not change post-KT (119), it is likely that at the time of rejection the composition of T cells including the frequency of CD28null T cells is similar to the pre-KT value. This means that allograft rejection risk assessment based on T-cell ageing prior to KT probably resembles the T-cell age prior to time of rejection.

In conclusion, the T-cell ageing-related expansion of highly differentiated $CD4^+CD28$null T cells in ESRD patients is associated with a lower risk for EAR. This may be related to a significantly lower percentage of alloreactive T cells within the CD28null T cell fraction.

Disclosure

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6

PRIMARY CYTOMEGALOVIRUS INFECTION SIGNIFICANTLY IMPACTS CIRCULATING T CELLS IN KIDNEY TRANSPLANT RECIPIENTS

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ABSTRACT

Cytomegalovirus (CMV)-infection profoundly affects the T-cell compartment and is associated with alterations in T-cell ageing parameters and generation of cytotoxic $CD4^+CD28$null T cells. Hence, the effect of a primary CMV-infection post-kidney transplantation (KT) on the peripheral T-cell compartment was examined. As ageing parameters, we determined the T-cell differentiation status, T-cell receptor excision circle (TREC)-content, $CD31^+$ naïve T-cell numbers and relative telomere length (RTL) pre-KT and 12 months post-KT. CMV-seronegative KT-recipients, receiving a kidney from a CMV-seropositive donor (D+/R-) were compared to D+/R+ KT-recipients. Eleven out of the 22 included D+/R- KT-recipients had a CMV-viremia post-KT. They developed CMV-specific $CD4^+$ and $CD8^+$ T cells and their T-cell compartment shifted towards a more differentiated memory phenotype with expansion of $CD4^+CD28$null and $CD8^+CD28$null cells. One year post-KT, the $CD8^+$ T-cell count was almost doubled compared to non-viremic D+/R- and D+/R+ KT-recipients. In addition, the RTL of the $CD8^+$ was significantly lower and both the TREC-content and $CD31^+$ naïve T-cell numbers significantly decreased. Moreover, primary CMV infection was associated with a negative impact on glomerular filtration rate. In conclusion, a primary CMV-infection has a substantial impact on numbers and phenotype of peripheral T cells and may negatively affect renal allograft function.

INTRODUCTION

Cytomegalovirus (CMV)-infection may markedly alter the peripheral T-cell system both in healthy individuals and end-stage renal disease (ESRD)-patients. CMV-seroposivity, is associated with alterations in parameters related to ageing of T cells such as reduced telomere length and the induction of differentiated $CD4^+$ and $CD8^+$ T cells. (33, 35, 80, 93) A characteristic feature of these differentiated T cells in CMV-seropositive individuals is the higher number of $CD4^+$ as well as $CD8^+$ T cells lacking the surface expression of CD28, a co-stimulatory molecule that is lost late in the course of T-cell differentiation. (66, 114) In particular, the appearance of significant numbers of circulating $CD4^+$CD28null T cells is related to CMV-seropositivity and ESRD further promotes their expansion (76, 129). CMV-infection also contributes to the uremia-associated premature immunological ageing of ESRD patients as it is associated with reduced telomere length of $CD8^+$ T cells (93).

An aged T-cell system is associated with an impaired immune response and contributes to a decreased vaccination response, greater susceptibility to infections and virus-associated cancers (37, 39, 40, 45, 53). The $CD4^+$CD28null T cells are highly cytotoxic and associated with an increased risk of atherosclerotic event (47, 78), while the presence of highly differentiated $CD8^+$ T cells impairs renal graft function on the long term (130). Therefore, primary CMV-infection is of considerable interest, given the potential negative impact on the T-cell immune system, the risk for an atherosclerotic event and loss of renal allograft function.

The impact of CMV-infection post-kidney transplantation (KT) has been studied in patients suffering from CMV-disease but no detailed analysis of its effect on T-cell ageing has been performed. Besides, virtually all of the published data are presented in percentages of T-cell subsets while absolute numbers might be more informative. (131) In addition, anti-viral prophylaxis with valganciclovir in the first 3-6 months post-KT is part of the standard of care in many transplantation centres. This policy has reduced the frequency of symptomatic CMV-infections and may also attenuate the effect of CMV on circulating T cells and graft survival. (132-134)

Recently, we published that the uremia-associated premature immunological ageing is stably imprinted in the T-cell system and not reversible by KT, but the CMV-seropositive donor/CMV-seronegative recipient (D+/R-) combinations, which are at risk for primary CMV-infection were not included. (119) In this study, we examined the effect of a primary CMV-infection post-KT under immunosuppression and anti-viral prophylaxis on circulating T cells with emphasis on T-cell ageing parameters and the association with allograft function in a group of stable KT-recipients.

MATERIALS AND METHODS

Study population

From December 2010 to September 2013 patients receiving a kidney graft from a living donor were included in a study on the effect of KT on immunological ageing parameters (n=211) (119). From this cohort, only stable patients without rejection within the first year post-KT were analyzed to exclude possible confounding effects of anti-rejection therapy. Twenty-two D+/R- KT-recipients were included and 22 age-matched CMV-seropositive donor/CMV-seropositive acceptor (D+/R+) KT-recipients, without CMV reactivation post-KT, served as controls. Immunological parameters and CMV-specific T cells were assessed prior to KT and 12 months post-KT. The modification of diet in renal disease (MDRD) equation was used for GFR assessment.

All patients received induction therapy with basiliximab (Simulect®, Novartis) and following KT the standard triple immunosuppression consisted of tacrolimus (Prograf®, Astellas Pharma), mycophenolate mofetil (Cellcept®, Roche) and prednisolone (the first 3 months post-KT). Anti-viral prophylaxis, i.e. valganciclovir 450 mg q.d., was given (in most of the patients) the first 6 months post-KT and if necessary adjusted for impairment of renal function. Patient characteristics are listed in table 1. A CMV-viremia was assessed every other month in recipients of a D+/R- combination.

All patients participated in a randomized-controlled clinical trial with the primary aim to study the efficacy of a genotype-based approach to tacrolimus dosing (Dutch trial registry number NTR 2226; http://www.trialregister.nl/trialreg/index.asp). All patients gave written informed consent to participate in the clinical trial, as well as for the sub-study, which is presented here. The study was approved by the Medical Ethical Committee of the Erasmus MC (MEC number 2010-080, EudraCT 2010-018917-30).

Age- and CMV-serostatus matched healthy controls (HCs) were included to examine effects of CMV on circulating T cells in the healthy population.

Detection of CMV-viremia and 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. Diagnosis of a CMV-viremic episode was based on the presence of copies of CMV DNA in blood and established by a quantitative polymerase chain reaction (qPCR) at the department of Virology at the Erasmus MC.

Detection of CMV-specific T cells

From D+/R+ (n=8), D+/R- viremic (n=8) and non-viremic patients (n=7), PBMCs were thawed and stimulated ($5x10^6$ PBMCs) in the presence of co-stimulation CD49d (1 µg/mL; BD) without and with overlapping peptide pools covering the whole pp65

Table 1: Patients characteristics

	D+/R+ (n=22)	D+/R- no CMV viremia (n=11)	D+/R- CMV viremia (n=11)
Age in years [C]	52.8±11.7 [A]	50.0±19.8 [A]	50.0±17.3 [A]
Male	14 (63.6%)	7 (63.7%)	9 (81.8%)
CMV IgG titer (AU/ml)	61.5(9-389) [B,C]	0 (0-39) [B,D]	18.0 (7-49) [B,D]
Time point of CMV virema post-KT (months)			5.0 (1-12) [B]
Renal replacement therapy [C]			
- Pre-emptive transplantation	8 (36.4%)	9 (81.8%) [G]	3 (27.3%) [H]
- Patients on hemodialysis	10 (45.5%)	1 (9.1%) [I]	5 (45.5%)
- Patients on peritoneal dialysis	4 (18.2%)	1 (9.1%)	3 (27.3%)
Time on dialysis (in years)	1.5 (0.2-6.0) [B]	2.25 (1.5-3.0) [B]	1.0 (0.5-3.0) [B]
Underlying kidney disease [C]			
- Hypertensive nephropathy	7 (31.8%)	4 (36.4%)	1 (9.1%)
- Glomerulonephritis	1 (4.5%)	0	1 (9.1%)
- Diabetic nephropathy	5 (22.7%)	1 (9.1%)	3 (27.3%)
- Polycystic kidney disease	4 (18.2%)	1 (9.1%)	0
- Reflux nephropathy	0	0	0
- Other/unknown	5 (22.7%)	5 (45.5%)	6 (54.5%)
Previous KT [C]	0	0	0
Mismatches HLA class I	2.2±1.15 [A]	1.9±1.30 [A]	2.9±1.04 [A]
Mismatches HLA class II	1.1±0.73 [A]	1.4±0.67 [A]	1.1±0.70 [A]
Immunosuppressive medication			
- Basiliximab induction therapy [E]	22 (100%)	11 (100%)	11 (100%)
- Prednisolone [F]	22 (100%)	11 (100%)	11 (100%)
- MMF	22 (100%)	11 (100%)	11 (100%)
- Tacrolimus	22 (100%)	11 (100%)	11 (100%)
Donor age in years [C]	48.7±13.2 [A]	56.0±12.12 [A]	57.5±17.6 [A]

[A] = mean ± standard deviation, [B] = median with range, [C] = at pre-KT, [D] = at 12 months post-KT, [E] = Given at day 0 and day 4 post-KT, [F] = Given the first 3 months post-KT, [G]=significantly different compared to D+/R+ (p=0.03), [H]=significantly different compared to D+/R- no CMV viremia (p=0.03), [I]=significantly different compared to D+/R+ (p=0.05).

and IE-1 protein of CMV (1 µg/ml; PepTivator-CMV pp65 and IE-1; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) for 12 hours in the presence of Brefeldin A (Golgiplug; BD Pharmingen). This enables detection of CMV-specific $CD4^+$ as well as $CD8^+$ T cells as these can be identified by *de novo* expression of CD137. (120, 135) To test the capability to respond, $1x10^6$ PBMCs were stimulated with the combination of phorbol 12-myristate 13-acetate (PMA) (50 ng/ml; Sigma Aldrich, St Louis, MO, USA) and ionomycin (1 µg/ml; Sigma Aldrich) as a positive control.

Subsequently, a surface staining was performed to identify the T-cell subsets using AmCyan labeled anti-CD3 (BD, Erembodegem, Belgium), Pacific Blue (PB) labeled anti-CD4 (BD), allophycocyanin-Cy7 (APC-Cy7) labeled anti-CD8 (BD), peridinin chlorophyll-Cy5.5 (PerCP-Cy5.5) labeled anti-CD28 (BD) and phycoerythrin-Cy7 (PE)-Cy7 labeled anti-CD45RO (BD). A sub fraction was characterized as CD45ROnullCD28$^+$ T cells and the rest was considered to be memory T cells. (39).

Following fixation and permeabilization, cells were stained intracellular using APC-labeled anti-CD137 and PE-labeled anti-IFNγ. 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 negative control (background signal) was subtracted from the percentage of CD137$^+$ T cells after CMV-peptide stimulation to calculate the net signal which are shown in the results.

Absolute numbers of CD4$^+$ and CD8$^+$ T cells and T-cell differentiation status by FACS analysis

To determine the absolute T-cell numbers from blood, a Trucount staining was done as described by Bouvy et al (97). In addition, a whole blood staining was performed to determine the T-cell differentiation status as described in previous studies. (113, 119)

TREC assay and Telomere length assay

TREC was assessed using snap-frozen PBMCs (2×10^6). DNA was isolated according to manufacturer's instructions (Qiagen Isolation kit, Qiagen, Venlo, the Netherlands). Subsequently, TREC-content was determined using qPCR as described previously. (20, 119)

Flow fluorescent *in situ* hybridization (flow-FISH) was performed to determine the relative telomere length (RTL) of CD4$^+$ and CD8$^+$ T cells also described previously (25, 119). Both assays were performed in a smaller group of patients due to absence of materials.

Statistical analyses

Statistical analyses were performed using GraphPad Prism version 5.01. For the analyses of effects of CMV on T-cell parameters in patients prior to KT, and HCs (table 2), the non-parametric Mann-Whitney test was performed. Differences between pre-KT and 12 months afterwards within one group of KT-recipients were analyzed using the Wilcoxon signed rank test. *P*-values <0.05 for two sides were considered statistically significant. For all comparisons between groups and/or between different time points (Figure 4 and 7B), a non-parametric ANOVA including post hoc analysis was used. SPSS version 21.0 was used to perform a univariate regression analysis to test which clinical parameters or donor characteristics influenced renal allograft function. All data mentioned throughout the text represent the median and range.

RESULTS

T-cell differentiation, telomere length and thymic output in CMV-seronegative and -seropositive recipients prior to KT

Table 2 summarizes the data of the CMV-seropositive and CMV-seronegative KT recipients prior to KT (i.e. R+/R-) and HCs with respect to the various T-cell parameters. Thymic function is reflected by the presence of RTEs which are T cells that have recently migrated from the thymus into the periphery. RTEs were identified as $CD31^+$ naïve T cells (22) and their relative number was also estimated by the TREC-content, circular DNA remnants which are formed during T-cell receptor rearrangement in the thymus and diluted upon peripheral T-cell proliferation.

A striking difference between R+ and R- patient groups is the significant expansion of the total $CD8^+$ T-cell population ($p=0.005$) in R+ patients, which is caused by an almost doubled number of memory T cells. In both $CD4^+$ and $CD8^+$ T cell populations a more differentiated memory T-cell profile was observed in the R+ patients. The R+ patient group had significant higher numbers of $CD4^+$ EM ($p=0.028$) and CD28null ($p<0.001$) memory $CD4^+$ T cells and an expanded memory $CD8^+$ T-cell compartment ($p=0.020$) with a significant higher number of EMRA ($p<0.001$) and CD28null ($p<0.001$) T-cell populations. A CMV-infection in HCs had a similar major impact on numbers and the differentiation status of the circulating T cells (table 2).

The thymic function showed no differences between the R- and R+ patient groups. The RTL of $CD8^+$ T cells from R+ patients was significantly lower pre-KT ($p=0.046$).

Primary CMV-infection in KT-recipients

Eleven of the 22 D+/R- KT-recipients developed a CMV-viremia post-KT based on the presence of copies of CMV DNA in the circulation. Five KT-recipients developed a CMV-viremic episode during valganciclovir prophylaxis, the other 6 KT-recipients after prophylaxis was stopped. One patient developed CMV colitis and another patient suffered from hemophagocytic lymphohistiocytosis. At 12 months, the T-cell characteristics were similar between D+/R- patients who experienced a CMV-viremia during prophylaxis and those who had a CMV-viremia after prophylaxis and therefore they were pooled.

All these KT-recipients had detectable anti-CMV IgG antibodies within one year post-KT and are identified throughout the manuscript as CMV-viremic D+/R- KT-recipients, whereas the other 11 are grouped and identified as CMV non-viremic KT-recipients. Only 3 of these non-viremic D+/R- KT-recipients seroconverted but no copies of CMV DNA were detected the first year post-KT.

Prior to KT, no differences were observed between viremic and non-viremic D+/R- patients regarding the T-cell ageing parameters (table S1).

Table 2: Effects of CMV on T-cell parameters in healthy controls and kidney transplant recipients pre-KT

	Healthy controls		Kidney transplant recipients	
	CMV-seronegative (n=22)	**CMV-seropositive (n=22)**	**CMV-seronegative (n=22)**	**CMV-seropositive (n=22)**
Total $CD4^+$ T-cell numbers (/µl)	1002 (470-2202)	1056 (348-1891)	698 (33-1492)	639 (247-1692)
naive $CD4^+$ T-cell numbers (/µl)	400 (84-721)	323 (140-721)	188 (33-760)	108 (14-738)
memory $CD4^+$ T-cell numbers (/µl)	630 (268-528)	671 (208-1284)	402 (33-749)	528 (233-1208)
CM $CD4^+$ T-cell numbers (/µl)	411 (155-1311)	441 (105-1103)	268 (6-552)	271 (35-631)
EM $CD4^+$ T-cell numbers (/µl)	154 (63-440)	255 (11-548)	130 (26-362)	188 (15-559)*
$CD28^{null}CD4^+$ memory T-cell numbers (/µl)	2.0 (0-50)	19 (0-234)***	2 (0-20)	49 (0-162)***
$CD31^+CD4^+$ naive T-cell numbers (/µl)	224 (50-792)	216 (100-461)	97 (22-548)	67 (9-485)
Total $CD8^+$ T-cell numbers (/µl)	370 (171-920)	543 (103-1338)*	233 (89-1250)	490 (207-1450)**
naive $CD8^+$ T-cell numbers (/µl)	94 (18-315)	144 (16-363)	82 (15-505)	108 (14-738)
memory $CD8^+$ T-cell numbers (/µl)	220 (81-631)	386 (169-975)***	195 (28-1052)	397 (194-1324)***
CM $CD8^+$ T-cell numbers (/µl)	26 (15-168)	27 (5-77)	24 (4-98)	15 (1-62)
EM $CD8^+$ T-cell numbers (/µl)	139 (49-3681)	145 (28-516)	79 (10-477)	132 (30-378)*
EMRA $CD8^+$ T-cell numbers (/µl)	57 (13-293)**	166 (9-588)**	56 (11-533)	222 (55-931)***
$CD28^{null}CD8^+$ memory T-cell numbers (/µl)	60 (13-296)	218 (13-644)**	48 (9-652)	226 (59-1104)***
$CD31^+CD8^+$ naive T-cell numbers (/µl)	93 (18-308)	139 (16-336)	97 (13-503)	51 (8-256)
TREC content (ΔCT)	10.80 (7.66-13.85)	9.91 (6.54-13.81)	9.22 (5.37-13.65)	8.91 (7.64-12.50)
RTL of $CD4^+$ T cells (%)	12.00 (6.5-14.60)	11.9 (6.9-21.7)	10.80 (4.70-21.30)	9.70 (4.30-23.10)
RTL of $CD8^+$ T cells (%)	12.60 (8.0-16.10)	10.3 (6.9-12.10)	13.00 (6.30-21.20)	9.25 (5.10-20.80)*

Healthy controls were age- and CMV-serostatus matched to the kidney transplant recipients. Data represents median (range), significant differences between CMV-seropositive and CMV-seronegative individuals are shown *p<0.05, **p<0.01, ***p<0.001

Development of CMV-specific CD137-expressing T cells after primary CMV-infection

Only in CMV-seropositive patients a CMV-specific T-cell response was observed. Prior to KT the median (range) percentage and numbers of $CD137^+CD4^+$ T cells was 0.64% (0.21-1.21) and 4.21 cells/µl (1.07-20.49) in the CMV-seropositive patients. For the $CD8^+$ T-cell population this was 1.01% (0.08-3.02) and 2.55 cells/µl (0.49-30.06). The percentage and number of IFN-γ^+CD137^+ cells within the $CD4^+$ and $CD8^+$ T-cell population were 0.46% (0.03-0.88) and 0.41% (0.10-14.81), and 2.51 cells/µl (0.10-4.37) cells/µl and 1.49 cells/µl (0.35-18.72), respectively.

The percentages $CD137^+$ T cells were significantly increased in the CMV-viremic D+/R- KT-recipients at 12 months post-KT compared to pre-KT (Figure 1 B-C, $CD4^+$: p=0.008, $CD8^+$: p=0.008). Similar observations were done for IFNγ-producing $CD137^+CD4^+$ (Figure 1D, p=0.016) and $CD137^+CD8^+$ cells (Figure 1E, p=0.008). Remarkably, one year post-KT the frequency of CMV-specific (IFNγ-producing) $CD4^+$ T cells was significantly lower (p=0.008) in the D+/R+ patients compared to pre-KT (Figure 1B and D). In the non-viremic D+/R- KT-recipients no increase was seen in the frequency of CMV-reactive T cells. (Figure 1B-E).

One year post-KT, CMV-specific (IFNγ-)$CD137^+$ T-cell numbers of CMV-viremic D+/R- KT-recipients were comparable to that of CMV-seropositive recipients (Figure 1F-I).

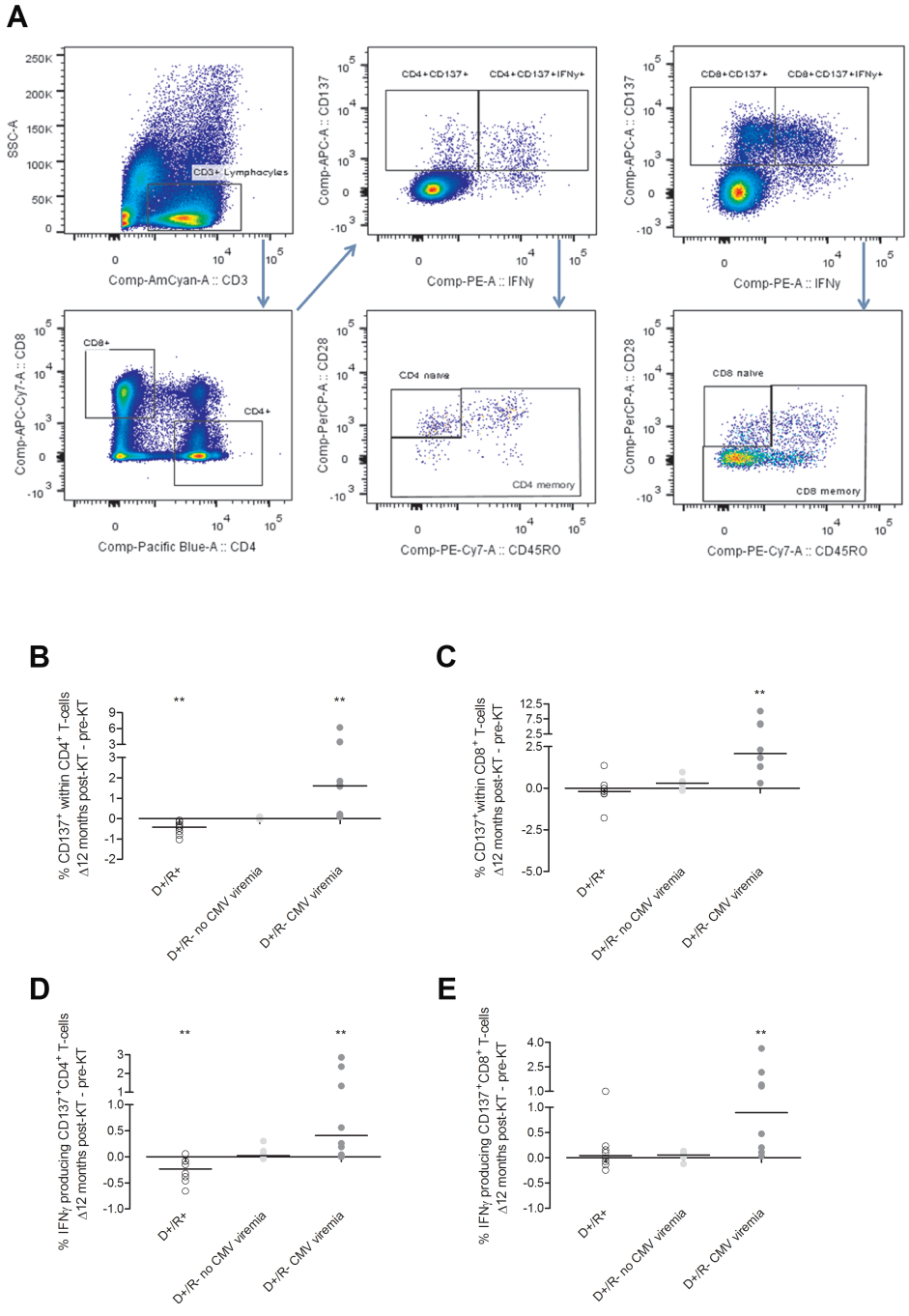
One year post-KT, the distribution of the $CD137^+CD4^+$ and $CD137^+CD8^+$ T cells, either IFNγ^+ or not, over the different subsets was similar for the D+/R+ group and the viremic D+R- group. The CMV-specific CD4 T cells were mainly located within the $CD28^+$ memory T cells while the CMV-specific $CD8^+$ T cells were mostly CD28null (Figure 1F-I).

Impact of primary CMV-infection on T-cell differentiation status

For the D+/R+ KT-recipients, the composition of the $CD4^+$ compartment was not different one year post-KT compared to pre-KT (Figure 2 B-G). The CMV-viremic D+/R- patients had a lower number of $CD4^+$ T cells 12 months post-KT (p=0.014, Figure 2B) and naïve $CD4^+$ T cells (p=0.01, Figure 2C). Specifically the CMV-viremic and to a lesser extent the non-viremic D+/R- patients showed a remarkable increase in $CD28nullCD4^+$ memory T cells (non-viremic: p=0.022, viremic: p=0.014) (Figure 2G). No differences regarding the 12 month values of $CD4^+CD28null$ T cells were found between the non- and serconverted non-viremic patients (Figure S1F).

Interestingly, at 12 months post-KT compared to the D+/R+ patients, the viremic D+/R- patients had a reduced number of $CD4^+$ T cells (i.e. 708 (248-1775) vs. 476 cells/µl (52-1213), p=0.032) due to a significant lower number of memory (i.e. 510 (165-1142) vs. 391 (50-561) cells/µl, p=0.017) and EM (i.e. 189 (54-547) vs. 121 (19-200) cells/µl, p=0.014).

The $CD8^+$ T-cell compartment of CMV-viremic D+/R- patients had a striking expansion of the absolute number of $CD8^+$ T cells (Figure 3B, p=0.027) and memory $CD8^+$ T cells (Figure 3D, p=0.014) due to the expansion of EMRA T cells (Figure 3G, p=0.018).


A
SSC-A
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CD4+CD137+
CD4+CD137+IFNγ+
Comp-APC-A :: CD137
Comp-PE-A :: IFNγ
CD8+CD137+
CD8+CD137+IFNγ+
Comp-APC-A :: CD137
Comp-PE-A :: IFNγ
Comp-APC-Cy7-A :: CD8
CD8+
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B
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C
% CD137+within CD8+ T-cells
Δ12 months post-KT - pre-KT
D
% IFNγ producing CD137+CD4+ T-cells
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D+/R+
D+/R- no CMV viremia
D+/R- CMV viremia
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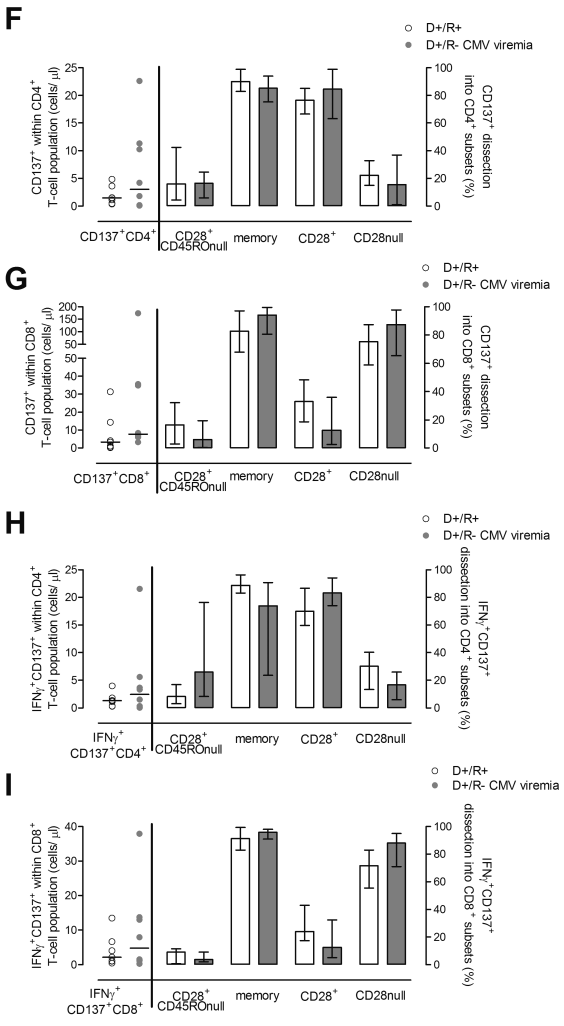


Figure 1: Differences in percentage of CMV-specific T cells between pre-KT and 12 months post-KT and the distribution into T-cell subsets. First an example of the gating strategy is shown (A). Upon selection of the $CD3^+$ lymphocytes, the $CD3^+CD4^+$ and the $CD3^+CD8^+$ T cells were selected in which the fraction of $(IFN\gamma^+)CD137^+$ cells was determined. These cells were dissected into a $CD28^+CD45RO$null, memory , total $CD28^+$ and total CD28null T–cell fraction. The differences in percentages of CMV-specific, $CD137^+$ T cells between 12 months post-KT and pre-KT was calculated and shown as Δ12 months post-KT – pre-KT for the D+/R+ patients (n=8, open dots), D+/R- CMV non-viremic patients (n=7, light grey dots) and D+/R- CMV-viremic patients (n=8, dark grey dots). The Δ12 months post-KT – pre-KT of the percentage $CD137^+$ within $CD4^+$ (B) and $CD8^+$ (C) T cells and the percentage $IFN\gamma^+CD137^+$ within $CD4^+$ (D) and $CD8^+$ (E) T cells was determined. Next, the absolute number of CMV-specific $CD137^+$ T cells of the D+/R+ patients (white dots) and D+/R- CMV-viremic (grey dots) patients at 12 months post-KT within the $CD4^+$ (F) and $CD8^+$ (G) are dissected into a naïve, memory, total $CD28^+$ and total CD28null T-cell subset (D+/R+: white bars, D+/R- CMV-viremic: grey bars). The similar approach was taken for the percentage of $IFN\gamma^+$ $CD137^+$ $CD4^+$ (H) and $CD8^+$ (I) T cells. Medians are shown and bars represents the interquartile range. Significant differences between pre-KT and 12 months post-KT were calculated and shown (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

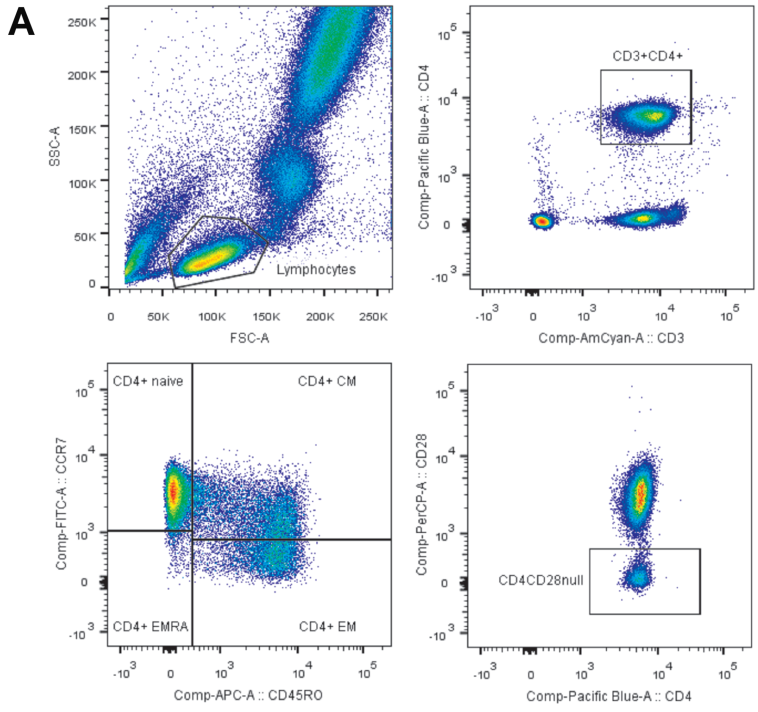
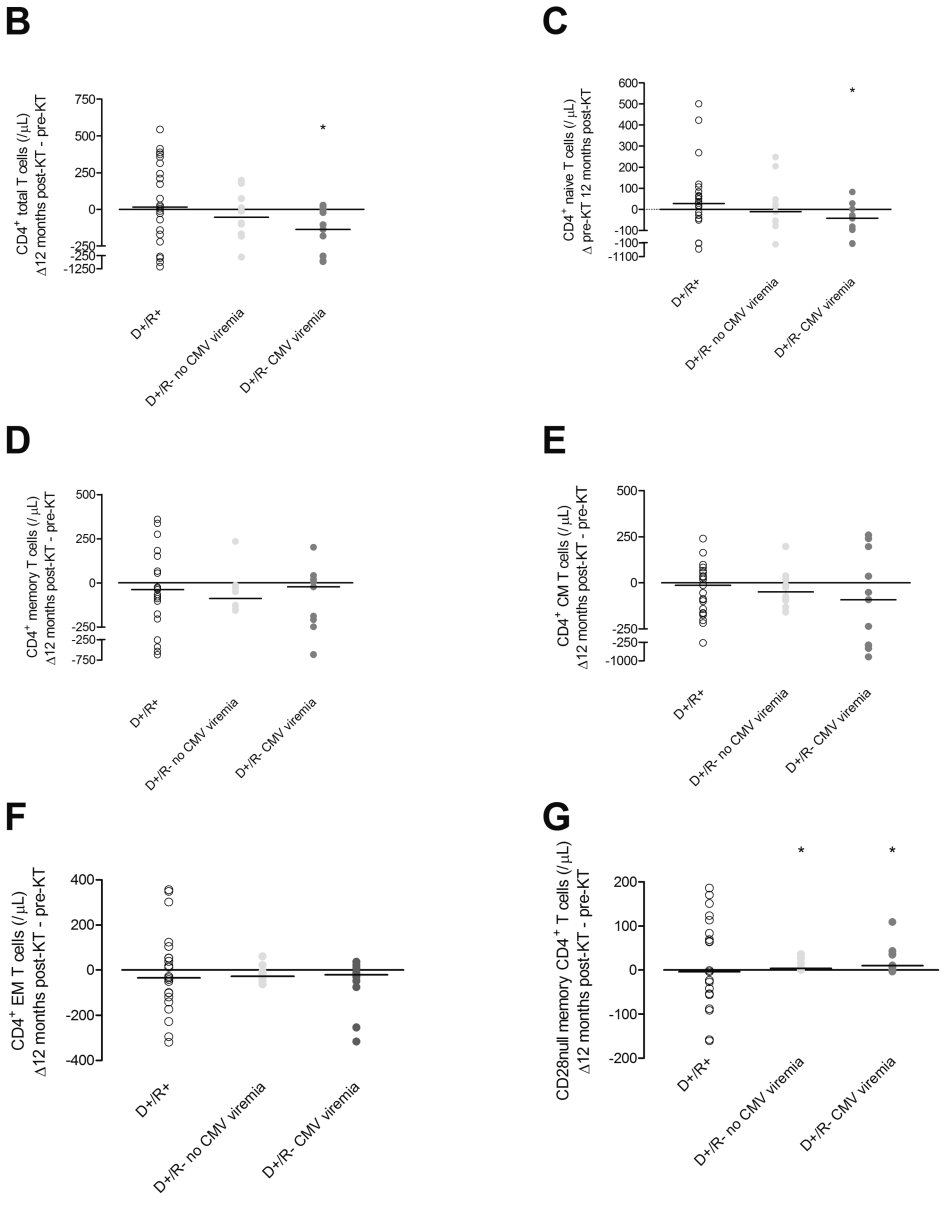


Figure 2: Differences in the differentiation status of the CD4⁺ T cells between pre-KT and 12 months post-KT. First an example of the gating strategy is shown (A). The lymphocytes were identified based on the forward/sideward characteristics followed by the selection of the CD3⁺CD4⁺ T cells. These CD4⁺ T cells were dissected into subsets using CCR7 and CD45RO. In addition, the number of CD28null T cells was examined within the CD4⁺ T-cell population. The differences in CD4⁺ T-cell numbers between pre-KT and 12 months post-KT was calculated for the different T-cell subsets by comparing 12 months post-KT to pre-KT (Δ12 months post-KT – pre-KT). The Δ12 months post-KT – pre-KT value of the total number of CD4⁺ (B), naïve (C), memory (D), CM (E), EM (F) and CD28null within memory CD4⁺ (G) was calculated for the D+/R+ patients (open dots, n=22), D+/R- CMV non-viremic patients (light grey dots, n=11) and D+/R- CMV-viremic patients (dark grey dots, n=11). Medians are shown and significant differences between the pre-KT and 12 months post-KT were calculated and shown (* p<0.05, ** p<0.01, *** p<0.001).

Moreover, the CMV-viremic D+/R- patients had an expansion of CD8⁺CD28null memory T cells (Figure 3H, p=0.004). The number of all CD8⁺ T-cell subsets in the D+/R+ group remained unchanged (Figure 3C-G). At 12 months post-KT, the numbers of these differentiated T-cell populations in viremic D+/R- patients had reached similar values as in the D+/R+ patient group. For the non-viremic D+/R- KT-recipients, the composition of the CD8⁺ was not different post-KT except for an increase in EM CD8⁺ T cells (Figure 3F, p=0.040).

Interestingly, at 12 months the non-viremic patients who seroconverted (n=3) had an expansion of the (memory) CD8⁺ T cells as compared to the non-seroconverted (n=8, p<0.05 Figure S1G and S1I).



CMV-specific T cells and highly differentiated T cells develop following a CMV-viremic episode in D+/R- patients

Following a CMV-viremia (t=0), the viremic D+/R- patients developed (IFNγ^+)CD137$^+$ T cells within both CD4$^+$ (Figure 4A and C) and CD8$^+$ (Figure 4B and D) T cells. In addition, the increase in highly differentiated CD4$^+$CD28null (Figure 4E), CD8$^+$ EMRA (Figure 4F) and CD8$^+$CD28null (Figure 4G) peripheral T cells occurred also following a CMV-viremic episode.

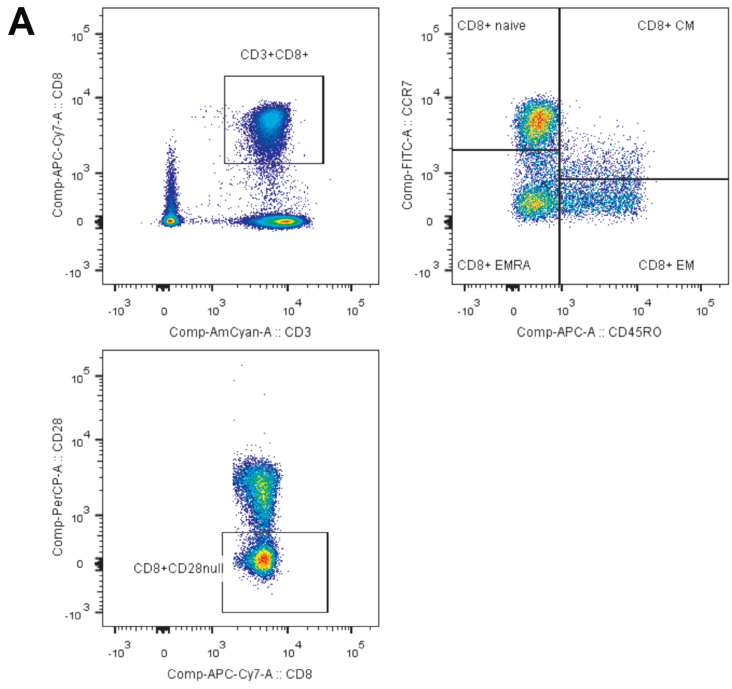


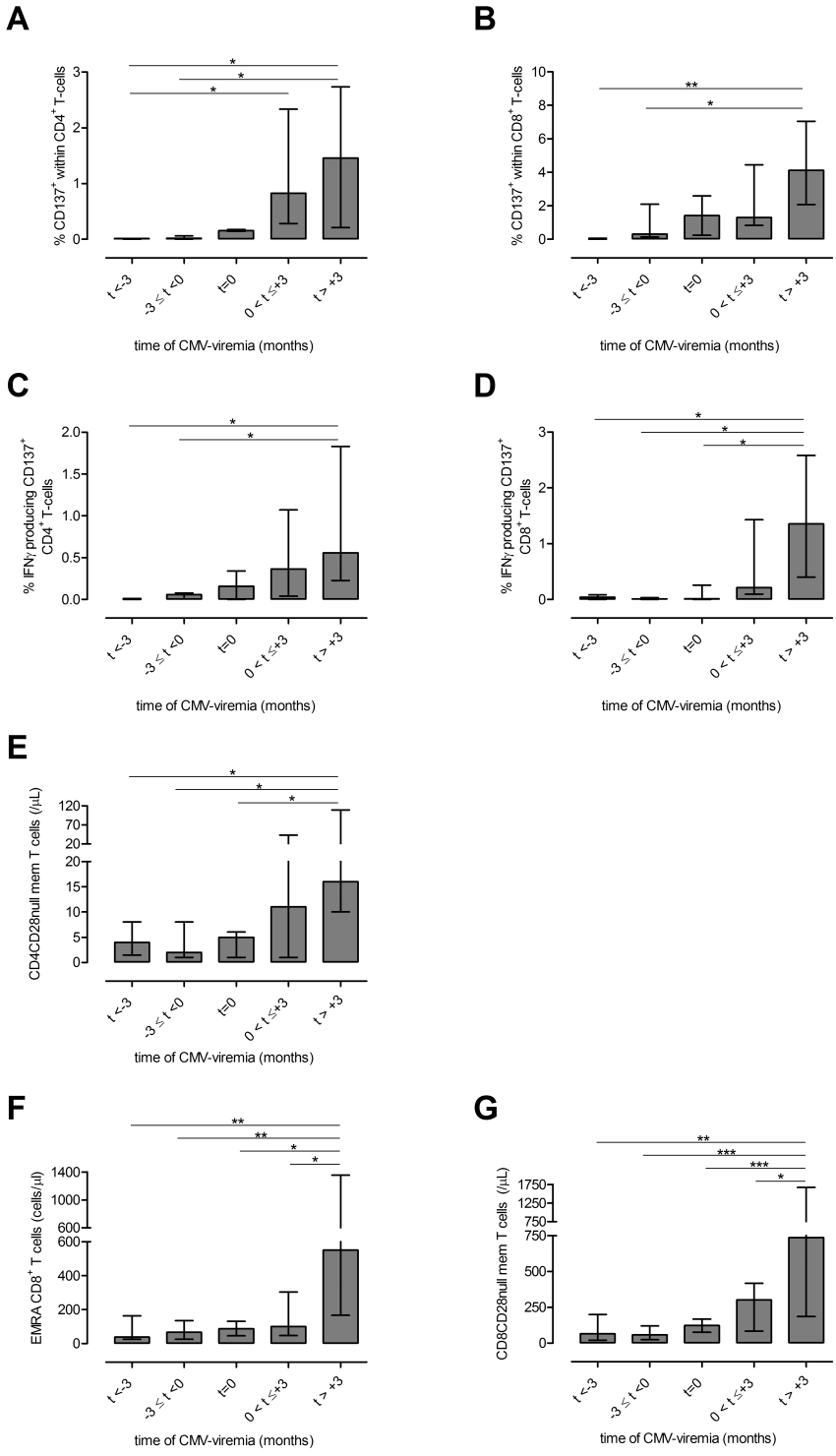
Figure 3: Differences in the differentiation status of the CD8⁺ T cells between pre-KT and 12 months post-KT. An example of the gating strategy is shown (A) were the CD3⁺CD8⁺ were selected from the lymphocytes. The CD8⁺ T cells were dissected into subsets using CCR7 and CD45RO and the number of CD28null T cells was examined within the CD8⁺ T-cell population. The differences in CD8⁺ T-cell numbers between pre-KT and 12 months post-KT was calculated for the different T-cell subsets by comparing 12 months post-KT to pre-KT (Δ12 months post-KT – pre-KT). The Δ12 months post-KT – pre-KT value of the total number of CD8⁺ (B), naïve (C), memory (D), CM (E), EM (F), EMRA (G) and CD28null within memory CD8⁺ (H) was calculated for the D+/R+ patients (open dots, n=22), D+/R- CMV non-viremic patients (light grey dots, n=11) and D+/R- CMV-viremic patients (dark grey dots, n=11). Medians are shown and significant differences between the pre-KT and 12 months post-KT were calculated and shown (* p<0.05, ** p<0.01, *** p<0.001).

The individual reconstitution of relevant T-cell subsets (CD8⁺ EMRA, CD4⁺ and CD8⁺CD28null) is shown in Figure S2. Panel A reveals the patterns of those developing a viremia under prophylaxis and panel B that of patients having a viremic episode after prophylaxis. The relevant T-cell subsets are expanding following a viremia in most of the patients.

Lower number of recent thymic emigrants after CMV-viremia

The TREC-content was significantly decreased in the CMV-viremic (p=0.008), but not in the non-viremic D+/R- or D+/R+ KT-recipients at 12 months post-KT (Figure 5A). In addition, the number of CD31-expressing naïve CD4⁺ (Figure 5C) as well as naive CD8⁺ (Figure 5D) T cells was significantly lowered (i.e. p=0.010 and p=0.048 respectively) in D+/R- KT-recipients.

B
CD8+ total cells (/µL)
Δ12 months post-KT - pre-KT
3000
500
500
250
0
-250
-500
-750
*
D+/R+
D+/R- no CMV viremia
D+/R- CMV viremia
C
CD8+ naive T cells (/µL)
Δ12 months post-KT - pre-KT
300
200
100
0
-100
-100
-600
D+/R+
D+/R- no CMV viremia
D+/R- CMV viremia
D
CD8+ memory T cells (/µL)
Δ12 months post-KT - pre-KT
3000
500
500
250
0
-250
-500
-750
*
D+/R+
D+/R- no CMV viremia
D+/R- CMV viremia
E
CD8+ CM T cells (/µL)
Δ12 months post-KT - pre-KT
400
75
75
50
25
0
-25
-50
-75
D+/R+
D+/R- no CMV viremia
D+/R- CMV viremia
F
CD8+ EM T cells (/µL)
Δ12 months post-KT - pre-KT
600
100
100
0
-100
-200
-300
-400
*
D+/R+
D+/R- no CMV viremia
D+/R- CMV viremia
G
CD8+ EMRA T cells (/µL)
Δ12 months post-KT - pre-KT
3000
500
500
250
0
-250
-500
-750
*
D+/R+
D+/R- no CMV viremia
D+/R- CMV viremia
H
CD28null memory CD8+ T cells (/µL)
Δ12 months post-KT - pre-KT
3000
500
500
250
0
-250
-500
-750
**
D+/R+
D+/R- no CMV viremia
D+/R- CMV viremia


A
% CD137+ within CD4+ T-cells
B
% CD137+ within CD8+ T-cells
C
% IFNγ producing CD137+ CD4+ T-cells
D
% IFNγ producing CD137+ CD8+ T-cells
E
CD4CD28null mem T cells (/µL)
F
EMRA CD8+ T cells (cells/µl)
G
CD8CD28null mem T cells (/µL)
t <-3
-3 ≤ t <0
t=0
0 < t ≤+3
t > +3
time of CMV-viremia (months)

Relative telomere length of the CD8$^+$ T cells declines in D+/R- patients within one year post-KT

The RTL for the CD4$^+$ T cells was similar in all patient groups pre-KT (Table 2) and did not change significantly post-transplantation (Figure 6A). For the CD8$^+$ T cells, the RTL in the D+/R- CMV-viremic patients was significantly (p=0.027) lower one year post-KT compared to the pre-KT value whereas the RTL of the D+/R+ and non-viremic D+/R- remained unaffected (Figure 6B).

Renal allograft function is decreased in D+/R- viremic patients one year post-KT

At 3 months post-KT, the estimated glomerular filtration rate (eGFR) was not different between the groups but at one year post-KT the CMV-viremic D+/R- patients had a significant drop in their eGFR (Figure 7A, p=0.039), which was significantly lower than the eGFR of D+/R+ at one year post-KT (Figure 7A, p=0.030). A univariate regression analysis revealed that only a primary CMV-infection and a CMV-viremia post-KT, were significantly contributing to the observed variation for the eGFR (Table 3).

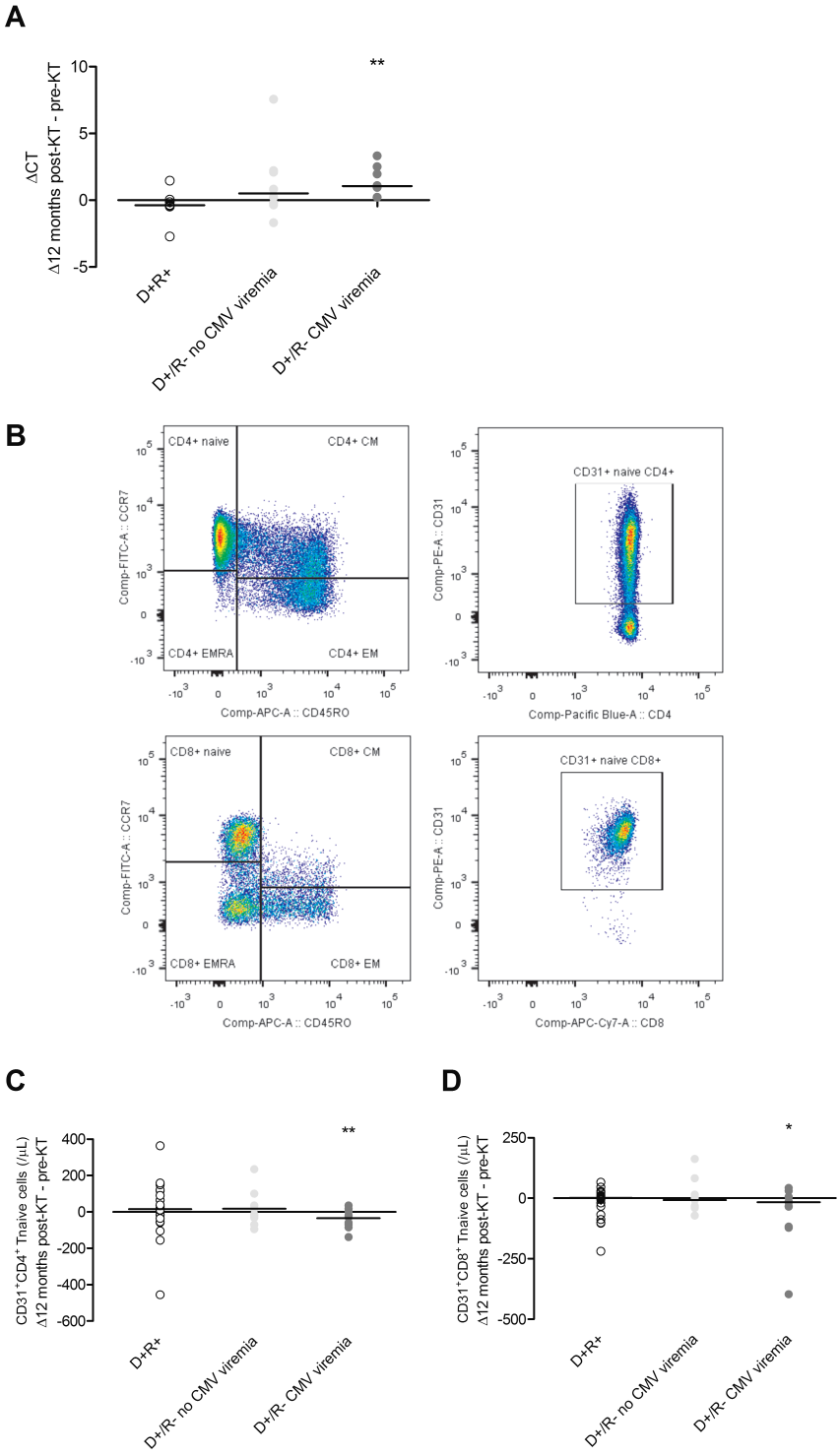
Moreover, the eGFR of the D+/R- CMV-viremic patients was at the moment of a CMV-viremic episode significantly (p<0.01) lower compared to the eGFR 3 months

Tabel 3: Univariate analyses of the effect of clinical parameters on the eGFR one year post-KT

Parameter	β	SE	P
Male gender	0.120	0.004	0.40
Age	-0.060	0.143	0.70
Donor age	-0.163	0.129	0.29
Primary CMV infection	-0.315	0.005	0.037
CMV viremia post-KT	-0.322	0.004	0.033
HLA MM I	-0.060	0.011	0.70
HLA MM II	-0.184	0.006	0.23
Warm ischemia time	-0.015	0.068	0.93

β: regression coefficient. SE: standard error

◄ **Figure 4:** CMV-induced effects on the phenotype of peripheral T cells following CMV-viremia. The longitudinal effects of CMV-viremia (t=0) on the development of CMV-specific T cells and differentiation status of the peripheral T cells in the D+/R- CMV-viremic patients are depicted. First the percentage CD137$^+$ within CD4$^+$ (A) and CD8$^+$ (B) T cells and the percentage IFNγ$^+$CD137$^+$ within CD4$^+$ (C) and CD8$^+$ (D) T cells are shown. Furthermore, absolute numbers of CD4$^+$CD28null (E), EMRA CD8$^+$ (F), CD8$^+$CD28null (G) T cells are shown at more than 3 months pre-CMV viremia (t<-3), within 3 months pre-CMV viremia (-3≤t<0), at the moment of CMV-viremia (t=0), within 3 months after CMV-viremia (0<t≤+3), and more than 3 months post-CMV viremia (t>+3). Bars represents median and interquartile range (IQR) and significant differences were calculated and shown (* p<0.05, ** p<0.01, *** p<0.001).


A
ΔCT
Δ12 months post-KT - pre-KT
10
5
0
-5
**
D+R+
D+/R- no CMV viremia
D+/R- CMV viremia
B
CD4+ naive
CD4+ CM
CD4+ EMRA
CD4+ EM
Comp-FITC-A :: CCR7
Comp-APC-A :: CD45RO
CD31+ naive CD4+
Comp-PE-A :: CD31
Comp-Pacific Blue-A :: CD4
CD8+ naive
CD8+ CM
CD8+ EMRA
CD8+ EM
CD31+ naive CD8+
Comp-APC-Cy7-A :: CD8
C
CD31+CD4+ Tnaive cells (/µL)
Δ12 months post-KT - pre-KT
400
200
0
-200
-400
-600
**
D+R+
D+/R- no CMV viremia
D+/R- CMV viremia
D
CD31+CD8+ Tnaive cells (/µL)
Δ12 months post-KT - pre-KT
250
0
-250
-500
*
D+R+
D+/R- no CMV viremia
D+/R- CMV viremia

before a CMV-viremic episode (Figure 7B). Following the viremic episode, the eGFR only partly restored and was still significantly lower (Figure 7B, $p<0.05$) compared to pre-CMV viremia. The eGFR of the non-viremic D+/R- patients, matched in time post-KT with the viremic group, did not change significantly (Figure 7B).

DISCUSSION

The results of this study show that a primary CMV-infection under immunosuppressive conditions with antiviral prophylaxis significantly affects circulating T-cell numbers and differentiation status, and the T-cell ageing parameters like RTL and thymic output. In addition, it had negative impact on renal allograft function.

In ESRD patients pre-KT, CMV-latency is associated with an increased differentiation of circulating T cells with attrition of telomeres of $CD8^+$ T cells which aggravates the uremia-associated premature immunological ageing. (33, 35, 80, 93) Primary CMV-infection in the first year post-KT induces T-cell changes that are in accordance with data from cross-sectional studies on CMV-latency. The primary CMV-infection leads to a doubling of the $CD8^+$ T-cell population which is largely attributable to a substantial increase in differentiated CD28null and EMRA $CD8^+$ T cells. The presence of EMRA $CD8^+$ T cells has recently been associated with late graft dysfunction (130) and expansion may therefore be of clinical significance.

Within the $CD4^+$ T cells the expansion of $CD4^+$CD28null T cells is the footprint left behind by CMV-infection and the generation of these cells was found in both CMV-viremic and non-viremic D+/R- patients. The appearance of $CD4^+$CD28null cells, which are cytotoxic for endothelial cells, may explain a recent finding that primary CMV-infection post-KT is associated with an increase in cardiovascular events. (136) In contrast, primary CMV-infection does not lead to an EM $CD4^+$ T-cell expansion as is observed in R+ recipients pre-KT but instead leads to a decrease in naïve and memory $CD4^+$ T-cell numbers.

A decrease in EM $CD4^+$ T cells post-KT was observed previously (119) indicating that the use of immunosuppression might prevent expansion of EM $CD4^+$ T cells. The decrease in total $CD4^+$ T cells may be a transient phenomenon, as $CD4^+$ T-cell lymphopenia is not associated with CMV-latency in healthy individuals or ESRD-patients pre- and post-KT. (119)

◄ **Figure 5:** TREC-content and numbers of CD31-expressing naïve T cells following KT. The ΔCT (difference in Threshold cycle (Ct) number between TREC qPCR and albumin qPCR as a control for DNA input; inversely related to TREC-content) was calculated and the differences in ΔCT between 12 months post-KT to pre-KT were calculated (Δ12 months post-KT – pre-KT) and depicted for D+/R+ (n=8, open dots), CMV non-viremic D+/R- (n=8, light grey dots) and CMV-viremic D+/R- (n=8, dark grey dots) KT-recipients (A). In addition to the TREC content, the number of $CD31^+$ T cells were identified within the naïve $CD4^+$ or $CD8^+$ T-cell compartment as shown in the flow-cytometric example. (B) The Δ12 months post-KT – pre-KT in absolute number of CD31-expressing naïve $CD4^+$ (C) and naïve $CD8^+$ (D) T cells, pre-KT and 12 months of the D+/R+ (open dots, n=22), D+/R- no viremia (light grey dots, n=11) and D+/R- CMV-viremic patients (dark grey dots, n=11) are depicted. Medians are shown and significant differences between the pre-KT and 12 months post-KT were calculated and shown (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

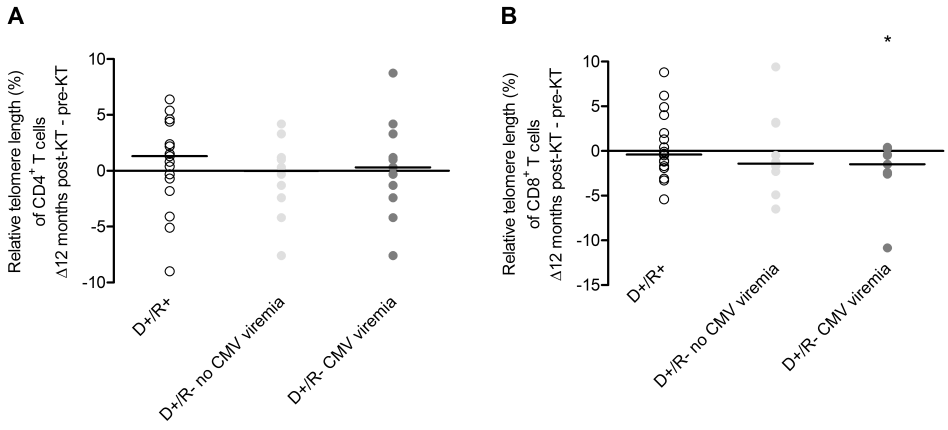


Figure 6: RTL of CD4+ and CD8+ T cells following KT. The difference in RTL between 12m post-KT and pre-KT was calculated (Δ12 months post-KT – pre-KT) and shown for the CD4+ (A) and CD8+ (B) T cells of the D+/R+ (n=20, open dots), CMV non-viremic D+/R- (n=10, light grey dots) and CMV-viremic D+/R- (n=10, dark grey dots) KT-recipients. Medians are shown and significant differences between the pre-KT and 12 months post-KT were calculated and shown (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

The (CD137+) CMV-specific T-cell response, revealed that IFNγ-producing CMV-specific T cells were predominantly located in the memory CD4+CD28+ and in the memory CD8+CD28null T-cell population. The relatively high frequency of CD45ROnullCD28+ CMV-specific T cells might be early branched off memory T-cells which are located in the CD45ROnullCD28+ fraction. (137) These T cells needs to be further characterized by using other markers (i.e. CXCR3 and CCR4) to distinguish them.

In our study, the loss of CD28-expression within the CD4+ T-cell population, specific for CMV-infected individuals (58), cannot be explained by the rise in CMV-specific T cells. Similarly, the CD8+CD28null T-cell expansion is much larger than can be explained by the increase in CMV-specific T cells. These differences might be explained by the fact that IE-1 and pp65 overlapping peptide pools do not represent the total repertoire of CMV-antigenic specificities. Recently, a paper by Suessmuth et al. demonstrated by deep-sequencing of the T-cell receptor that the observed expansion of CD8+ T-cell subsets is most likely all CMV-specific. (138) We cannot rule out that bystander activation of T cells and induction of heat shock proteins (HSP), especially HSP-60 and HSP-70, specific T cells also contribute to this expansion as shown by others. (139)

Overall, the quality and quantity of the CMV-specific T-cell response was strong after primary CMV-infection and the distribution over the different T-cell subsets was similar to the CMV-seropositive KT-recipients. The D+/R+ KT-recipients showed a reduction in the frequency of CMV-specific IFNγ-producing CD4+ cells compared pre-KT which is in line with the study by Abate et al. who found a temporary reduction in CMV-specific cells directly post-KT. (140) The underlying mechanism is unknown but valganciclovir may lower the viral antigenic pressure leading to a declining numbers of CMV-reactive T cells.

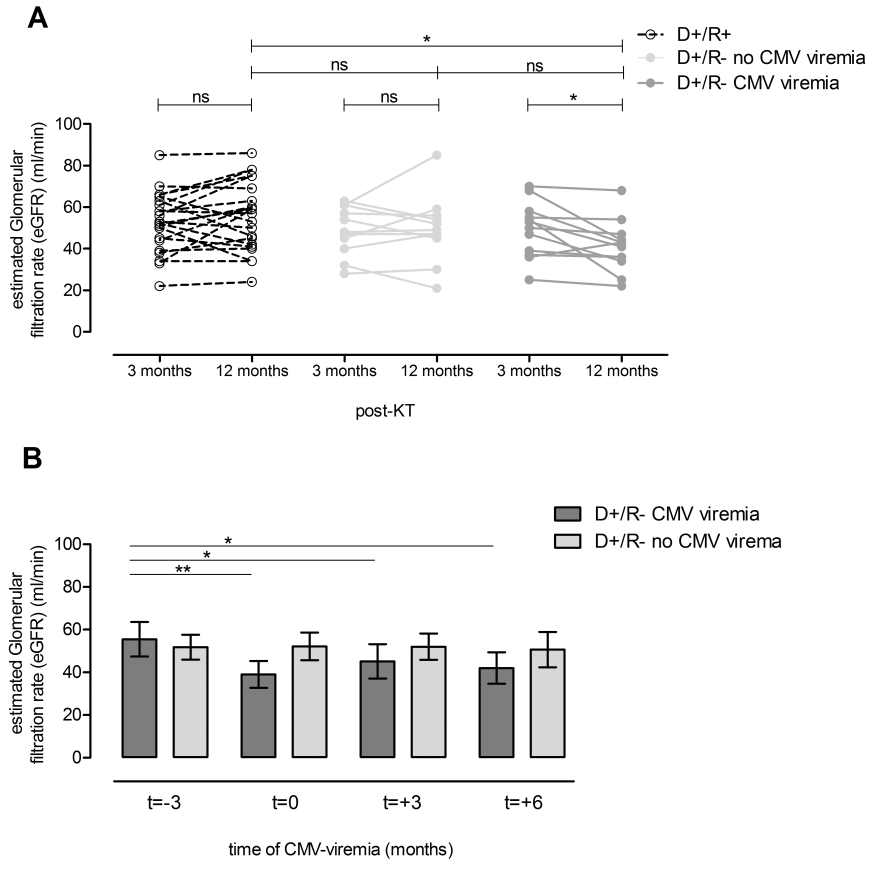


Figure 7: Renal allograft function post-KT. The renal allograft function was analyzed by measuring the eGFR. The eGFR of 12 months post-KT was compared with the eGFR at 3 months post-KT for each KT recipients within the D+/R+ (n=22, open dots and dashed lines), CMV non-viremic D+/R- (n=11, light grey dots and lines) and CMV-viremic D+/R- (n=11, dark grey dots and lines) patient groups (A). Significant differences between the groups and between the 3 and 12 month post-KT time points were calculated and shown (* $p<0.05$). Next, the longitudinal effects of CMV-viremia were depicted for the eGFR in D+/R- viremic patients (dark grey bars) and as a control, the eGFR of non-viremic D+/R- patients, matched for time after transplantation, were included (light grey bars). Bars represents mean and SEM. Differences between time points were calculated and shown (* $p<0.05$, ** $p<0.01$).

Recently we showed that $CD4^+CD28^{null}$ and $CD8^+CD28^{null}$ have shorter telomeres when compared the $CD28^+$ counterparts (93). The effects observed for the CMV-viremic D+/R- KT-recipients with respect to the T-cell differentiation were reflected in telomere attrition of ($CD8^+$) T cells which is in accordance with findings of van de Berg et al. (34). However, we found an unaltered RTL of the $CD4^+$ T cells, which fits with the relatively unchanged differentiation status after primary CMV-infection. Therefore, primary CMV-infection accelerates T-cell ageing of predominantly the $CD8^+$ T cells.

TREC-content and numbers of $CD31^+$ naïve T cells, were significantly decreased after a primary CMV-infection. This is in accordance with observations done in mice where thymic involution occurred upon CMV-infection. (141) In humans, such a correlation has not been described but seems unlikely as CMV-latency is not associated with lower TREC-content or decreased number of CD31-expressing naïve T cells. (93) An alternative explanation may be decreased hematopoiesis of progenitor T cells caused by an active CMV-infection, leading to temporarily lower RTE-numbers (142). The clinical consequences of a temporarily decreased generation of naïve T cells are unknown.

Besides these T-cell effects, an active CMV-infection appears to affect renal allograft function, which could not be explained by differences in clinical characteristics. This finding confirmed previous observations by Erdbruegger et al. (143) In our study, only stable KT-recipients without an acute rejection episode were selected. Others found a higher incidence of allograft rejection in recipients presenting with CMV-disease (133, 144) and introduction of anti-CMV prophylaxis may have increased graft survival in solid organ transplant (SOT)-recipients. (145) However, others failed to find such association. (146) Because of our patient selection, the negative impact of primary CMV-infection cannot be explained by acute rejection. As $CD4^+CD28$null T cells are associated with vasculopathy (47, 50, 76, 78) it is possible that this may be a mechanism by which allograft function is compromised after CMV-infection, but at present this explanation remains speculative and needs confirmation in larger cohorts.

As has been reported before, CMV-specific T cells and seroconversion does not occur in all D+/R- patients implying that not all CMV-seropositive donor kidneys were able to transmit CMV. (147) Three recipients were CMV-seroconverted without a preceding episode of detectable viremia. Except for expansion of $CD8^+$ T cells, they lack other CMV-related T-cell changes indicating that only a vigorous anti-CMV response results in remarkable changes.

Due to absence of materials we were able to assess the frequency of CMV-specific T cells and the T-cell ageing parameters in a smaller group of patients, which is a limitation of this study. Summarizing our findings, it is clear that viremic primary CMV-infections post-KT profoundly affects the circulating T-cell compartment. Increased numbers of terminally differentiated $CD8^+$ T cells with attrition of telomeres in combination with decreased numbers of RTEs mimic premature immunological aging and may contribute to late graft dysfunction. In addition, the generation of cardiotoxic $CD4^+CD28$null T cells is of interest as these cells may be involved in an increased risk for cardiovascular events and reduced allograft function.

Disclosure

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7

T-CELL AGEING IN END-STAGE RENAL DISEASE PATIENTS: ASSESSMENT AND CLINICAL RELEVANCE

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ABSTRACT

End-stage renal disease (ESRD) patients have a defective T-cell-mediated immune system which is related to excessive premature ageing of the T-cell compartment. This is likely to be caused by the uremia-associated pro-inflammatory milieu, created by loss of renal function. Therefore, ESRD patients are highly susceptible for infections, have an increased risk for virus-associated cancers, respond poorly to vaccination and have an increased risk for atherosclerotic diseases. Three ageing parameters can be used to assess an immunological T-cell age. First, thymic output can be determined by assessing the T-cell receptor excision circles (TREC)-content together with CD31 expression within the naïve T cells. Second, the telomere length of T cells and third the T-cell differentiation status are also indicators of T-cell ageing. Analyses based on these parameters in ESRD patients revealed that the immunological T-cell age is increased by on average 20 years compared to the chronological age. After kidney transplantation (KT) the aged T-cell phenotype persist although the pro-inflammatory milieu is diminished. This might be explained by epigenetic modifications at hematopoietic stem cells level. Assessment of an immunological T-cell age could be an important tool to identify KT recipients who are at risk for allograft rejection or to prevent over-immunosuppression.

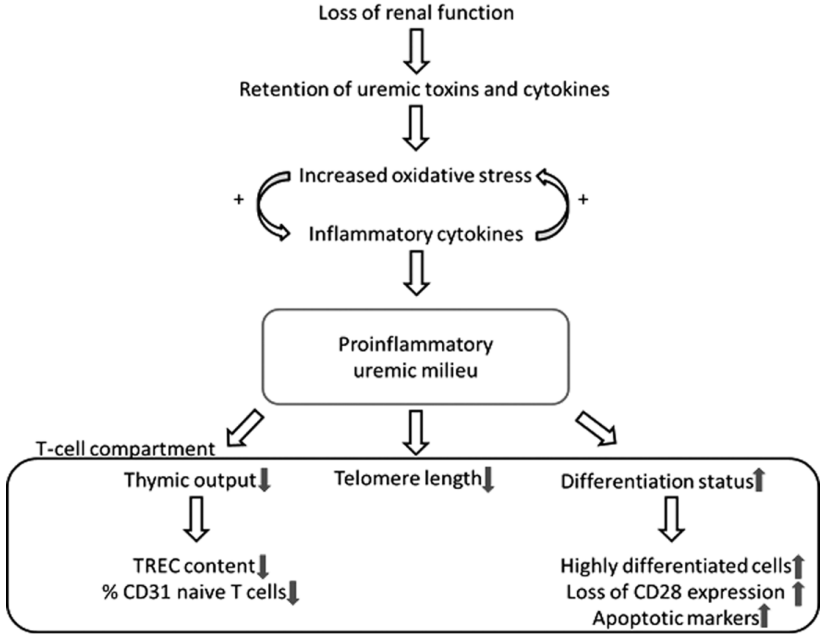
INTRODUCTION

Loss of renal function is strongly associated with a defective immune system which is known as uremia-associated immune deficiency. (8, 79, 148) Retention of uremic molecules and cytokines in end-stage renal disease (ESRD) patients are key mechanisms in generating oxidative stress and inflammation. (5, 6, 79) This creates a pro-inflammatory environment in which both the innate (first line of defense, a-specific) (8, 149-151) as well as the adaptive (specific) immune system are affected (Figure 1). (8, 152, 153)

T cells, members of the adaptive immune system, are the best-studied immune cells in ESRD patients and in the field of transplantation they are the main target of immunosuppressive medication. (51) The uremia-associated pro-inflammatory milieu causes T-cell defects associated with premature T-cell ageing when compared to healthy age-matched individuals (Figure 1). (15) Analysis of the T-cell compartment in ESRD patients revealed that the immunological age of T cells is increased by 20 years compared to their chronological age (Figure 1). (15)

The dysfunctional immune system of ESRD patients has a substantial clinical impact on both the morbidity and mortality of ESRD patients. Patients are highly susceptible for infections (37, 154), have an increased risk for virus-associated cancers (45), respond poorly to vaccination (53) and have an increased risk for atherosclerotic diseases. (47, 78)

In this review, the concept of uremia-associated age-related changes of T cells is highlighted focusing on the assessment of an immunological T-cell age, clinical implications and possible therapeutic options for ESRD patients.



The concept of T-cell ageing

With normal healthy ageing, the T-cell immune system ages as well (27). Hematopoietic stem cells (HSCs), generated in the bone marrow, give rise to myeloid as well as lymphoid progenitor cells. (16) T cells are generated from the latter. With increasing age, HSCs are skewed towards myeloid-generating subsets at the expense of lymphoid-generating HSCs, resulting in a lower number of progenitor T cells. These progenitor T cells are further "educated" in the thymus in which naïve T cells will form specific receptors on their cell surface known as T-cell receptors (TCRs). With increasing age, the thymus involutes. (17, 18) This process involves a decrease in tissue in combination with a loss of tissue organization with the net outcome that numbers of naïve T cells leaving the thymus, known as recent thymic emigrants (RTEs) are reduced. Involution of the thymus starts at birth and is accelerated during adolescence. (19)

This explains the lymphopenic number in naïve T cells with increasing age. Despite the fact that the naïve T-cell pool can also be maintained by homeostatic proliferation in which TCR triggering in combination with the cytokines Interleukin (IL)-7 and IL-15 expand T cells, (155) the net effect is a diminished number of naïve T cells and the number of memory T cells in the peripheral blood of elderly individuals is preserved. (156) A relatively expanded number of naïve T cells by homeostatic proliferation results in a T-cell pool with a restricted TCR repertoire. (117, 155) A diverse TCR repertoire is a necessary prerequisite for an adequate and effective T-cell response towards newly encountered antigens. (157)

After encountering and activation by an antigen, a naïve T cell will proliferate and become a memory T cell. During physiological ageing the population of antigen-experienced memory T cells will increase and the majority of these cells will become highly differentiated. These cells are known to have an increase in pro-apoptotic markers (158) and loss in co-stimulatory molecule CD28. (66, 114) CD28 plays an important role in the activation of T cells and a loss of CD28 can result in insufficient activation, shorter replicative lifespan and a higher toxicity. (114) Furthermore, highly differentiated cells are known to have a reduction in their telomere length. (159)

A telomere is a region of repetitive nucleotides which is located at the end of each chromosome and prevents chromosomal instability. Loss of telomere length has been linked to an increased risk for tumor development and to T-cell ageing. (23, 160)

Assessing an immunological T-cell age

A global assessment of the immunological age of the T-cell system can be performed by the analysis of three ageing parameters. During the formation of the T-cell receptor (TCR) in the thymus, DNA sequences in the TCR loci are deleted and circularized into episomal DNA molecules, so called single joint TCR excision circles (TREC), a process known as TCR rearrangement. (20) This TREC remains in the newly formed naïve T cells leaving the thymus. Upon replication of these cells in the periphery, the TREC is only transferred to one daughter cell resulting in a reduction of TRECs in the naïve daughter

T cells. With an increasing age, the number of RTEs containing a TREC declines log linearity due to a lower thymic output of RTEs and an increase in proliferation of naïve T cells. The TREC content can be determined using a quantitative polymerase chain reaction (qPCR) method normalized to the single-copy albumin gene. (20, 161) Next to the TREC content, these RTEs can be detected by measuring the expression of CD31 within the naïve T-cell pool. (21, 22) In addition to the thymic output of T cells, the diversity of the TCR repertoire can be analyzed by sequencing in order to determine the loss of TCR specificities within the T-cell population and to assess the percentage of oligoclonal T cells. (157, 162) Recently, a novel TREC assay in which the TCR diversity was combined with the TREC content to get quantitative insight into intra-thymic and post-thymic proliferative capacity of T cells and its alterations upon ageing. (163)

As a second parameter for the assessment of an immunological T-cell age, the T-cell telomere length can be determined as a measurement for the proliferative history of a T-cell population. (24) A decline in telomere length is highly associated with an increased proliferative history. A commonly used method to assess a relative telomere length (RTL) is the fluorescent *in situ* hybridization (FISH) method. (25, 26) During this procedure a labeled peptide nucleic acid (PNA) probe binds to the telomere repeats which can be read-out by fluorescent microscopy or by fluorescence measurements using a flow cytometry (flow FISH). The RTL can be calculated by relating the intensity of the bound PNA probe to that of a T-cell lymphoblastic leukemia (1301 CCRF-CEM) cell-line, known for its long telomeres, as an internal control. (25) Inclusion of antibodies in this method makes it possible to analyze the telomere length in different T-cell populations (i.e. $CD4^+$ and $CD8^+$ T cells). (25, 79) A limitation of this assay is the temperature (82°C) which is required for DNA annealing which makes the use of stable fluorochromes necessary. (25, 26) Quantum dots (nanoparticles) were found to be highly fluorescent, bind to antibodies and have a much better temperature stability. Quantum dots conjugated with antibodies directed to T-cell antigens were found to retain most of their fluorescence following the annealing step. The use of quantum dots can be a solution for the limitations in antibody use in the flow-FISH procedure and allows to assess a telomere length in different T-cell subsets within one assay. (26)

In addition to the telomere length, the activity of the telomerase can be measured. Telomerase is responsible for maintaining telomere length and the cellular replicative potential and an impaired activity of telomerase results attrition of telomeres. (27) Measuring the activity of telomerase gives additional information on the telomere shortening. This assay is based on the capacity of a test sample to amplify a telomere template. (28)

The differentiation status of the T-cell compartment can be used as a third parameter to assess an immunological age. The increase in highly differentiated memory cells with increasing age can be determined by analysis of the phenotype of circulating T-cells using multicolor flowcytometry. Based on the expression of the chemokine (C-C motif) receptor 7 (CCR7), enabling cells to migrate to secondary lymphoid organs, and CD45RO, an isoform of the leukocyte common antigen expressed on memory T

cells, a distinction within the memory T-cell compartment can be made. The different memory T-cell subsets include Central Memory (CM) ($CCR7^+$ and $CD45RO^+$), able to home to secondary lymph nodes and producing mainly IL-2 which is necessary for the proliferation of T cells, Effector Memory (EM) ($CCR7^-$ and $CD45RO^+$), able to migrate to peripheral tissues exerting direct effector functions and terminally differentiated effector memory $CD45RA^+$ (EMRA) ($CCR7^-$ and $CD45RO^-$), which exert cytotoxic activities and are highly susceptibility to apoptosis. (29) Moreover, these terminally differentiated cells often lose the expression CD28 which makes them less dependent on co-stimulation to become activated. (30) In addition, CD57 can be measured as a marker for highly differentiated memory T cells. (15, 93) CD95 (FAS) and CD279 (known as programmed death receptor-1 (PD-1)) are both commonly used as pro-apoptotic markers. (15, 158, 164)

The aged T-cell system in ESRD patients

Based on the analyses of the T-cell ageing parameters, i.e. assessment of TREC- content, relative telomere length and differentiation status we showed that the immunological age of ESRD patients is advanced by 20 years compared to their calendar age. (15) As compared to an age-matched healthy control, ESRD patients had a lower thymic output of naïve T cells, a decline in the T-cell telomere length and an increase in the differentiation status towards the terminally differentiated memory phenotype with a large number of CD28-negative (or CD28null) T cells (Figure 1). (15) Progressive loss of renal function was highly correlated with a lack of IL-7, a loss of naïve T cells and an increase in terminally differentiated $CD8^+$ T cells (13).

The effects of renal replacement therapy (RRT) on the T-cell ageing parameters seemed to be small and were limited to the $CD8^+$ T-cell compartment of young ESRD patients. (79) The type of RRT did not influence the ageing parameters since both hemodialysis (HD) and peritoneal dialysis (PD) patients showed signs of an aged T-cell compartment. (79) Moreover, the duration of dialysis did not seem to influence the ageing parameters. (165) Furthermore, the type of underlying kidney disease was not related to any parameter of immunological ageing (79) indicating that the loss of renal function is the dominant factor for a decreased thymic output of naïve T cells and increased differentiation/proliferation of memory T cells.

Cytomegalovirus (CMV) is known to affect the T-cell compartment which closely resembles ageing. (35, 80, 91, 93) Infection with the virus results in chronic latency and the effects of CMV on the T-cell compartment are relevant, since approximately 70% of the ESRD patients is infected with CMV. (35) In these patients, CMV was associated with an increased number of highly differentiated $CD4^+$ and $CD8^+$ T cells and a relatively small decline in $CD8^+$ T-cell telomere length. (35, 76, 93) The effects were restricted to the memory T-cell compartment since the thymic output of T cells was not affected. Therefore we concluded that CMV only affects the differentiation status of circulating T cells. (35, 76, 93)

Clinical implications of an aged T-cell compartment

The uremia-associated prematurely aged T-cell immune system has a substantial clinical impact leading to an increased morbidity and mortality. ESRD patients are highly susceptible for infections which might further contribute to the pro-inflammatory milieu. For instance periodontitis, which is common in patients with chronic kidney disease (CKD), often leads to inflammation. (166)

T cells of ESRD patients have an impaired production of IL-2 and the inadequate T-cell proliferative capacity results insufficient T-cell responses. (11, 39, 44) This in combination with low numbers of T cells results into inadequate T-cell responses directed to viruses and a decreased tumor surveillance which significantly increases the risk for virus-associated tumors. (45, 46) Next to IL-2, in hemodialysis (HD) patients it was found that activated T cells have impaired responses to tumor necrosis factor (TNF)-α, implying a state of tachyphylaxis. (12)

Following vaccination against hepatitis B, the formation of antigen-specific $CD4^+$ EM T cells is severely impaired in ESRD patients. (39) The poor development of IL-2 producing $CD4^+$ EM T cells in patients with ESRD was strongly associated with a low generation of antibodies towards hepatitis B antigens. (39) The inability to maintain protective antibody titers after T-cell dependent vaccinations (40, 41) or after a natural infection (42, 43) might be caused by a loss of antigen-specific T cells as a result of their increased susceptibility for apoptosis. (15, 164)

Furthermore, the loss in TCR diversity of naïve T cells due to a lower number of RTEs but an increase in proliferated naïve T cells is linked to a decreased efficiency of vaccination but also to an increased susceptibility for infections and cancers. (117, 167)

$CD4^+$ T cells lacking CD28 expression, are found to be highly cytotoxic as they produce large amounts of interferon (IFN)-γ and TNF-α and release granzyme-B and perforin upon activation. In several studies (47, 50) it is shown that these cytotoxic cells are present in unstable atherosclerotic plaques and are associated with an increased risk for recurrence of both acute coronary events and ischemic stroke resulting in a higher mortality rate. (49) As confirmed in ESRD patients, high numbers $CD4^+CD28$null T cells is strongly associated with a history of cardiovascular diseases. (47, 48, 50, 78)

$CD8^+CD28$null T cells contain a subpopulation of cells possessing immunosuppressive capacities (168, 169) and has therefore been linked to a decreased vaccination responsiveness of healthy individuals. (170) These immunosuppressive capacities also suggest that these cells could be important in preventing allograft rejection after kidney transplantation (KT). Indeed, we recently demonstrated that patients with an expanded population of highly differentiated (EMRA) $CD8^+CD28$null T cells had a lower risk for allograft rejection after KT. (110) Another explanations might be that $CD8^+CD28$null T cells represents clonal expansions of particular antigen-specific $CD8^+$ T cells that compete for immunologic space which is associated with reduction of T-cell diversity. (124) This might affect the diversity of alloreactive T-cells as well. Next to these highly differentiated $CD8^+$ T cells in KT recipients, a

high proportion of highly differentiated $CD4^+$ T cells was also linked to a lower risk for allograft rejection. (112)

Premature T-cell ageing and Kidney transplantation

After kidney transplantation (KT) the levels of pro-inflammatory proteins and oxidative stress decrease rapidly to levels that are comparable to healthy individuals. (94) Despite this, the uremia- associated prematurely aged T-cell immune system existed after KT. (119)

Immunosuppressive treatment affected the number of highly differentiated cells directly post-KT. However after tapering the immunosuppressive medication, these highly differentiated T-cell numbers were restored to pre-KT values. Furthermore, the telomere length of the T-cell compartment did not change and thymic function was not improved the first year post-KT. (119) Even after T-cell depleting immunosuppressive therapy (i.e. rabbit antithymocyte globulin (rATG)) T cells are repopulating by homeostatic proliferation instead of a higher thymic output of naïve T cells. (97, 103) Therefore, the uremia-associated immunological ageing seems stably imprinted in the T-cell system and not reversible by KT. (119)

Normal ageing is associated with, epigenetic changes in HSCs resulting in a shift in the balance towards myeloid precursors at the expense of the lymphoid ones (106, 107). Healthy ageing results in genetic alterations affecting T cells at developmental stages leading to phenotypic as well as functional changes. (171) In ESRD patients, uremia is able to cause epigenetic changes (108). Young et al. 2012 found that methylation of the KLOTHO gene is initiated by oxidative stress in ESRD patients. (172) KLOTHO deficient mice created a syndrome that resembles human ageing. (173) Although KT reverses the uremic pro-inflammatory environment (94) it is unable to induce changes at the epigenetic level. The persistence of the aged T-cell phenotype post-KT has several clinical implications as it may increase the risk for infections, malignancies and cardiovascular diseases in KT recipients. T-cell lymphopenia has been associated with a high risk for infections and malignancies post-KT. (38, 109)

Due to ageing of the T-cell compartment, elderly patients are more vulnerable for drugs toxicity, infections and malignancies caused by over-immunosuppression. In these patients, the incidence of virus-associated cancers is even higher post-KT as it is pre-KT. (46, 174) Over-immunosuppression might be prevented after mapping the T-cell immune system of the transplant recipient (112, 175) as T cells are the main target of immunosuppressive medication. (51) A study of Ducloux et al. in 2010 (52) showed that prolonged $CD4^+$ T-cell lymphopenia after severe T-cell depletion by rATG is associated with an increased risk for infections and mortality post-KT. High TREC values implying for a "younger" T-cell compartment pre-KT, is associated with a better reconstitution of T-cell numbers after rATG and lower risk for infections and cancer post-KT. (52)

Therapeutic options to reverse T-cell ageing

As mentioned earlier, RRT did not reduce T-cell ageing since no major differences between patients on dialysis and pre-dialysis patients with respect to the T-cell ageing parameters were observed. (79) Adequately targeting the presence of the pro-inflammatory environment in ESRD patients by KT (94) did not successfully reverse the aged T-cell immune system. (119)

Another method to reduce the level of oxidative stress and inflammation in ESRD patients is targeting the transcription factor Nuclear factor-erythroid-2-related factor 2 (Nrf2) which is an important regulator of genes encoding antioxidant and detoxifying molecules. (176) Treatment with bardoxolone methyl, which is an activator of Nrf2 may attenuate T-cell ageing in ESRD patients. (176) However, treatment is restricted due to the increased risk of cardiovascular diseases after treatment with bardoxolone. (177)

Another therapeutic option that might be able to improve T-cell function in ESRD patient is treatment with IL-7, a key cytokine for homeostatic proliferation of naïve T cells, that is reduced in patients causing a depletion of naïve T-cell pool. (13, 72) The first human studies, in which IL-7 was administered, are promising since an increased naïve T-cell pool with a broader TCR repertoire diversity was found. (162, 178) At present, IL-7 administration has not been tested in patients with ESRD.

CONCLUSION

Progressive loss of renal function creates a pro-inflammatory milieu which is highly associated with a dysfunctional immune system. This is a logical explanation for the increased vulnerability for infections, poor vaccination responses, high risk for malignancies and high risk for atherosclerotic diseases. Analysis of the T-cell system showed that ESRD patients have a prematurely aged T-cell compartment resulting in an impaired function. ESRD patients have a lower thymic output of naïve T cells, T cells have shorter telomeres and the T-cell compartment is shifted towards more differentiated T cells.

Therapeutic options to minimize morbidity and decrease mortality by improving or even fully reversing the aged T-cell phenotype are warranted. Although improvement of renal function by adequate renal replacement therapy in the form of KT, which drastically decreases the uremia-associated pro-inflammatory milieu, the prematurely aged T-cell phenotype appeared to be irreversible. Therefore the aged T-cell immune system remains an important determinant of the dysfunctional immune system post-KT. More research is necessary to fully understand the uremia-associated premature T-cell ageing phenomenon, also at earlier developmental stages of T cells, to be able to successfully intervene and increase the life-span of ESRD patients.

Until today, all KT recipients receive the same standard immunosuppressive therapy to prevent allograft rejection. Recently it was shown that the effect of calcineurin-

inhibitors and rapamycin on peripheral blood mononuclear cells (PBMCs) was different between young and elderly individuals. (105) Assessing an immunological T-cell age using T-cell ageing parameters as described in this review, may guide clinicians in decision-making with respect to transplanting an ESRD patient or not, adjusting immunosuppression following KT to minimize its long-term-associated adverse events.

8

SUMMARY AND DISCUSSION

SUMMARY

The research described in this thesis focused on the premature aged T-cell compartment in end-stage renal disease (ESRD) patients, in the context of kidney transplantation (KT). Loss of renal function induces a pro-inflammatory environment which is strongly associated with immune deficiency. It is assumed that retention of uremic toxins and cytokines are underlying the oxidative stress and inflammation in ESRD patients. This causes chronic activation of the immune system, including T cells which results in a lymphopenic T-cell number and a dysfunctional T-cell immunity. The typical uremia-associated changes in the peripheral T-cell compartment are reminiscent of age-related changes in healthy individuals. Recently, it has been demonstrated that the biological age of the peripheral T-cell system of ESRD patients is approximately 20 to 30 years older compared to an age-matched healthy individual. This prematurely aged immune system offers at least a partial explanation for the increased susceptibility for infections, reduced vaccination response, increased prevalence of malignancies and also constitutes a non-classical risk factor for cardiovascular diseases.

KT is considered the best option to regain renal function for ESRD patients. However, immunosuppressive medication is needed to prevent rejection of the graft but introduces an increased risk for infections and cancers post-KT. Currently, immune suppressive medication is targeted on through levels of calcineurin inhibitors, mycophenolate mofetil (MMF) and fixed doses of steroids. However, over- and under immunosuppression is frequently observed. The degree of uremia-associated immunological ageing could be an important parameter in the assessment of the amount of immunosuppression needed, thereby leading to a strategy of personalized immune suppression. The clinical relevance of T-cell ageing in the field of allograft rejection and infections is examined in this thesis.

The concept of immunological ageing is explained in **chapter 1** where the subject of this thesis is introduced. The immunological T-cell age could be determined by three ageing parameters: thymic output of naive T cells, the T-cell differentiation status and the T-cell telomere length. This was explained in more detail in this chapter.

In **Chapter 2** the contribution of uremia and renal replacement therapy (RRT) on premature T-cell ageing in ESRD patients were studied. ESRD patients not on dialysis were compared with age-matched healthy individuals to examine the contribution of uremia to premature T-cell ageing. In addition, these patients not on RRT were compared to patients receiving RRT (hemodialysis (HD) or peritoneal dialysis (PD)) to examine a possible effect caused by RRT. Uremia already induced premature T-cell ageing. Patients not on dialysis had a lower thymic function, a more differentiated T-cell compartment and a reduction in their T-cell telomere length compared to age-matched healthy controls. The additional effect of RRT was remarkably small and only observed within the $CD8^+$ T-cell compartment of young (age: <50 years old) ESRD-patients. In conclusion, uremia already caused premature immunological ageing of

the T-cell system and RRT further increases immunological ageing of the $CD8^+$ T-cell compartment in particular in young ESRD patients.

In healthy individuals, cytomegalovirus (CMV)-latency has been implicated to accelerate T-cell ageing. ESRD-patients have a high prevalence of CMV. For that reason, a possible additional effect on T-cell ageing caused by CMV was examined in **chapter 3**. CMV did not affect the thymic output of naive T cells. The relative telomere length (RTL) of $CD8^+$ T-cells in CMV-seropositive ESRD patients appeared to be significantly shorter than that of an age-matched CMV-seronegative patient. Furthermore, the $CD4^+$ and $CD8^+$ T-cell compartments appeared to be more differentiated in CMV-seropositive patients. In addition, these patients had a higher frequency of memory T cells lacking the expression of co-stimulatory molecule CD28 but gaining expression of CD57 which is a marker for differentiation. These CD28null T cells had shorter telomeres compared to CD28-expressing T cells. In conclusion, CMV-latency partly affected the T-cell age of particularly the $CD8^+$ T-cell compartment in ESRD patients.

Chapter 4 describes the effect of KT on premature T-cell ageing. As KT reduces the uremia-associated pro-inflammatory environment by improving renal function, reversal of premature immunological ageing by KT was examined. Kidney transplant recipients were followed for one year after transplantation with respect to the T-cell ageing parameters. Directly after transplantation, patients had a decline in the absolute number of (differentiated) memory T cells which might be due to the high dose of immunosuppression. However, these differentiated cells returned to pre-KT values upon tapering of immunosuppression. Within one year post-KT, no improvement in the number of recent thymic emigrants (RTEs) as well as the telomere length for both the $CD4^+$ and $CD8^+$ T-cell compartment was observed. In addition, T-cell function was determined by measuring the proliferative capacity and percentages of cytokine producing cells upon T-cell receptor (TCR) triggering. Post-KT, the T-cell function remained unaltered compared to pre-KT. It was concluded that KT is not able to reverse premature T-cell ageing.

Next in **chapter 5** it was hypothesized that an aged T-cell compartment prior to KT is associated with a lower risk for AR post-KT. In this prospective study the pre-KT values of patients experiencing an early allograft rejection (EAR) within 3 months post-KT were compared to KT-recipients who did not. No differences were found regarding the numbers of RTEs and RTL between the two patient groups. EAR patients had a significant lower number of $CD4^+$CD28null T cells compared those who did not. The same trend was found for the $CD8^+$CD28null T cells. These results showed that an expansion of differentiated $CD4^+$CD28null T cells was associated with a reduced risk for early allograft rejection post-KT.

The patients that were analyzed in chapter 4 were either CMV-seropositive or CMV-seronegative pre-KT as well as post-KT. In **chapter 6** the T-cell ageing parameters were studied in CMV-seronegative patients receiving a kidney from a CMV-seropositive donor. These patients were exposed to CMV under immunosuppressive and anti-viral

(valganciclovir) prophylaxis. Based on the presence of viral (CMV) DNA in blood (CMV viremia), these patients were divided into a CMV-viremic and CMV non-viremic group. All of the CMV-viremic patients developed CMV-specific T cells and immunoglobulins specific for CMV (anti-CMV IgG). At one year post-KT, these patients had an enhanced T-cell age as judged from analysis of T-cell ageing parameters. The $CD8^+$ T-cell compartment was almost doubled due to an expansion of the EMRA $CD8^+$ T cells. Furthermore, the CMV-viremic patients had an increased number of CD28null T cells within the $CD4^+$ and $CD8^+$ T-cell compartment. Interestingly, based on the TREC content and percentages of CD31-expressing naive T cells, these patients had a reduced thymic function one year post-KT compared to pre-KT. Next to these effects on T cells, a CMV viremia is negatively associated with renal allograft function one year post-KT. The CMV non-viremic patients did not show these differences compared to pre-KT values, except for the increase in $CD4^+$CD28null T-cells. To conclude, an active CMV-infection post-KT induces T-cell ageing and is negatively associated with allograft function.

In the review of **chapter 7** the concept of premature T-cell ageing in ESRD patients is explained with the focus on assessment of a T-cell age, clinical consequences and possible therapeutic options. Next to the explanation of the different T-cell ageing parameters that were used in this study to assess a T-cell age, the limitations of these methods are explained. Having a prematurely aged immune system increases the morbidity and mortality rate. The increment in risk for infections and malignancies, cardiovascular diseases and poor vaccination response can be partly explained by premature T-cell ageing. Reducing the pro-inflammatory milieu by RRT or by KT seems to be ineffective in reversing the T-cell age. There are indications that epigenetic modifications at the stem cell level is the underlying cause. More research is necessary to fully understand the uremia-associated premature T-cell ageing phenomenon, also at earlier developmental stages of T cells, to be able to successfully intervene and increase the life-span of ESRD patients.

DISCUSSION

The overall aim of the described research was to examine the clinical relevance of premature T-cell ageing with respect to the risk for allograft rejection after KT. The first aim was to unravel premature T-cell ageing in ESRD patients prior to KT by identifying factors that had a possible influence on T-cell ageing. In 2011 our group published the first evidence that the T-cell compartment of ESRD patients is aged by approximately 20-30 years compared their chronological age using age-matched healthy individuals as a reference group. (15) The ESRD patient population in this study were mainly on hemodialysis (HD) (15) and also in earlier studies where a lymphopenic number in naive T cells was found in patient with ESRD, patients received mostly HD. (13) Therefore, the first aim of this thesis was to examine if ESRD patients not on RRT have the same the

degree of immunological T-cell ageing and to what extent this in influenced by RRT (HD and PD). No differences regarding the T-cell ageing parameters were found between the patient groups, indicating that loss of renal function is the predominant factor for the loss in thymic output, increased differentiation status and loss of telomere length of circulating T cells. Furthermore it appeared that the underlying kidney disease as well as the duration of dialysis both did not influence the ageing parameters.

The lymphopenic number of naive T cells might be one of the key findings. (13) There are indications that a lack in IL-7, necessary for maintaining the naive T cell pool, might be of importance. (13) This in combination with thymic involution and chronic antigen stimulation is most likely the cause of low numbers of naive T cells. (85) In addition, it was shown that the naive T cells of ESRD patients are susceptible to activation-induced apoptosis. (15) From animal models it is known that loss of renal function is the cause for involution of the thymus as well as other lymphoid organs showing a causal relationship between kidney function and lymphopoiesis. (69) Lymphopenia might induce homeostatic proliferation of both naive as well as memory T cells causing an expansion of terminally differentiated cells and a loss in telomere length. (70) However, this has not yet been tested in ESRD patients and remains speculative.

Although that there are strong indications that uremia is the cause of premature immunological ageing, targeting the pro-inflammatory milieu by RRT or more adequately by KT did not successfully reverse the prematurely aged T-cell immune system. (94) The lymphopenic numbers of naive T-cells, thymic function as well as RTL were not improved within the first year post-KT. This was independent of the achieved renal allograft function post-KT. A hypothesis for this might lie at the level of epigenetics. DNA modification by epigenetics results in alterations in gene accessibility for transcription. (179) This is an important cellular control mechanism and is influenced by many environmental factors. (179) Normal ageing is associated with epigenetic changes at hematopoietic stem cell (HSC) level and causes a shift towards production of myeloid lineage cells at the expanse of lymphoid cells. (106, 107) Uremia was found to be an inducer of epigenetic changes in ESRD patients. (108) Especially the KLOTHO gene gained interest since oxidative stress can initiate methylation of the promotor region of this gene resulting in down regulation. (172) In addition, KLOTHO deficient mice created a syndrome that has similarities of human ageing. (173)

Another explanation might be that treatment with immunosuppressive medication influences the function and composition of the immune system post-KT. For instance, several animal studies have reported immunosuppressive drugs to reduce the size of thymic tissue or numbers of thymocytes by affecting different developmental stages of thymocytes. (99-101) Therefore, immunosuppression post-KT may have a role in the lack of restoration of the aged T-cell immune system despite reversal of the uremia–associated inflammation and oxidative stress.

After total T-cell depletion by rATG or alemtuzumab, repopulation of T cells occurs by an increased homeostatic proliferation instead of an enhanced thymic output.

(97, 104) The irreversibility of T-cell ageing after KT, might contribute to a severe risk of cardiovascular events (48), infections and malignancies (52) in kidney transplant recipients post-KT. This implies that T-cell ageing post-KT remains an important determinant of the dysfunctional immunity post-KT. Treatment of T-cell ageing might be warranted to alleviate these increased risk.

As premature ageing cannot be reversed by diminishing the pro-inflammatory milieu after improving kidney function, other therapeutic options can be taken into consideration. Activation of Nuclear factor-erythroid-2-related factor 2 (Nrf2), which is a transcription factor involved in the regulation of genes encoding antioxidant and detoxifying molecules (176), by bardoxolone methyl might attenuate T-cell ageing. (176) However, due to its risk for cardiovascular diseases, treatment is restricted. (177) Another therapeutic option might be treatment with IL-7. Levels of IL-7, important for the homeostatic proliferation of naive T-cells, are diminished in ESRD patients contributing to the depletion of naive T-cells. (13, 72) IL-7 administration has not yet been tested in ESRD patients, but first human studies showed that IL-7 administration increases the naive T-cell pool with a broader TCR repertoire. (162, 178) So far, no clinical studies are known targeting ESRD related premature T-cell ageing.

An important aim of this thesis was to examine whether the degree of T-cell ageing prior to KT predicts the risk for acute rejection. Our results demonstrated that the loss of CD28 expression especially within the $CD4^+$ T-cell compartment, which is an important feature of ageing (66, 114) is associated with a lower risk for rejection within 3 months post-KT, i.e. early acute rejection (EAR). Especially within the $CD4^+$ T-cell compartment, the CD28null T cells are associated with a lower risk for rejection within three months post-KT. This is in line with other studies who found a similar association. (111, 112) However a beneficial effect of $CD4^+$CD28null T cells with respect to allograft rejection is contradictory in literature. (180) In 2010, our group found an association between CD28null T cells and allograft rejection mainly for the $CD8^+$ T-cell population in a different, more heterogeneous, cohort of patients. (110)

No association was found between the RTEs (i.e. CD31-expressing naïve T cells) and the risk for EAR. After puberty, the thymus involutes rapidly and the contribution of the thymus to the (naïve) T-cell pool is relatively small and mainly relies on homeostatic proliferation in older individuals. (19, 127) Our findings indicate that the naive T-cell compartment does not contribute to alloreactivity within the first three months post-KT and that the memory T-cell compartment is more relevant in that respect. (128) Next to the thymic output, no association was found between RTL and EAR. The fact that the RTL of the $CD4^+$ T-cell population is not correlated with the risk for EAR, might be due to the low percentages of CD28null T cells within the $CD4^+$ T-cell compartment as they are the cells containing the shortest telomeres.

The lower risk for EAR due to higher numbers of $CD4^+$CD28null T cells might be explained by the fact that these cells are known to have a restricted T-cell receptor profile compared to the $CD28^+$ counterparts. (111, 124) Recently we showed that

alloreactive T cells, based on the expression of CD137$^+$, were predominantly located within the CD28$^+$ T-cell population (120). Theoretically this suggest that a lower number of CD28$^+$ T cells results in lower alloreactivity. A mice study showed that CD8$^+$CD28null T cells contain immunosuppressive capacities (181) and first indications with human cells are found *in vitro*. (182) However, our group could not ascribe immunosuppressive properties to CD8$^+$CD28null T cells (110) and the alloreactive properties of CD4$^+$CD28null T cells are currently under investigation in our lab.

The findings of this thesis suggest that high numbers of peripheral CD28null T cells is beneficial with respect to acute rejection. Future studies testing the alloreactive potential of these CD28null T cells are warranted. This in combination with ageing of other immune cells is necessary for a broader interpretation, before risk-assessment for allograft rejection based on CD28null can be used in the clinic. Furthermore, treatment of ESRD patients might be a difficult task since high numbers of CD4$^+$CD28null T cells are found to be associated with lower risk for allograft rejection on one hand, but on the other hand they are found to be cytotoxic leading to cardiovascular diseases. (47-50)

CMV-seropositivity is strongly associated with CD28null T cells as infection results in expansion of these cells as observed in healthy individuals. (65, 81, 82) In ESRD patients, CMV-latency results in even higher numbers of CD28null T cells on top of the uremia-induced numbers of CD28null T cells. Due to the cytotoxicity of these cells, CMV is highly associated with the risk for atherosclerotic diseases in ESRD patients. (77) In addition to expansion of terminally differentiated (EMRA) CD8$^+$ T cells, the RTL of the CD8$^+$ T-cell compartment was significantly shorter compared to CMV-seronegative ESRD patients. The effects of CMV in ESRD patients were restricted to the expansion of highly differentiated T cells as it did not influence thymic output of T cells.

CMV-seronegative patients receiving a kidney from a CMV-seropositive donor (D+/R-) that are primary infected under immunosuppressive and antiviral treatment, developed these characteristic CMV-effects with respect to the T-cell differentiation status as observed pre-KT. Especially the CMV-viremic D+/R- patients had an expansion of (terminally) differentiated T cells with a loss in CD28 expression and a reduction in the CD8$^+$ telomere length compared to pre-KT. Interestingly, these CMV-viremic patients had a lower thymic output of naive T cells judging from the TREC content and CD31-expressing naive T cells, which could not be detected pre-KT. An explanation may be a decreased hematopoiesis of progenitor T cells caused by an active CMV-infection, leading to temporarily lower RTE-numbers. (142) Perhaps the use of immunosuppression may contribute to difference in thymic output pre-KT and one year post-KT in CMV-infected patients.

Next to these T-cell effects, an active CMV-infection directly post-KT affects graft function in kidney transplant recipients. Within one year post-KT these cells appear in both viremic as well as non-viremic CMV-primary infected patients. As CD4$^+$CD28null T cells are associated with vasculopathy (47, 50, 76, 78) it is possible that this contributes to the mechanism by which allograft function is compromised after CMV-infection, but at present this explanation remains speculative. Several studies found a higher

incidence of allograft rejection in patients with CMV disease. (133, 144) In addition, introduction of anti-CMV prophylaxis may have increased graft survival in solid organ transplant (SOT) recipients. (145) Others failed to find such association. (146) Next to these graft-effects, active replication of CMV post-KT is strongly associated with an increased risk of atherosclerotic events. (136)

Interestingly, not all CMV-seronegative patients who received a kidney from a CMV-seropositive donor generated CMV-specific T cells or detectable antibodies against CMV. This might implicate that not all CMV-seropositive donor kidneys were able to transmit CMV. (147) Some of the patients in our study had seroconversion but a CMV-viremic episode was absent. Based on the T-cell ageing parameters of the recipient prior to KT, we were unable to identify these high-risk patients for developing a CMV-viremia post-KT. The CMV non-viremic recipients lacked the characteristic CMV-related T-cell changes post-KT indicating that only a vigorous anti-CMV response results in remarkable changes in the T-cell compartment.

Throughout the different studies described in this thesis, T-cell ageing was assessed based on three ageing parameters. In addition to the thymic output, the diversity of the TCR repertoire can be analyzed to determine the presence of a skewed TCR. (157, 162) Recently, a novel TREC assay in which the TCR diversity was combined with the TREC content to get quantitative insight into intra-thymic and post-thymic proliferative capacity of T cells and its alterations upon ageing. (163) In addition to the telomere length, measuring the activity of the enzyme telomerase might provide more information. Telomerase is responsible for maintaining the telomere length and cellular replicative potential. An impaired activity of the enzyme results in telomere length attrition. (183) Measuring the activity of telomerase might provide more information on telomere shortening.

As immunological T-cell ageing varies from individual to individual, it is important to realize that the degree of T-cell ageing is not similar for each ESRD patient. The underlying mechanism for this variation is unknown, but it might be that some ESRD patients are better protected against T-cell ageing than others. Another explanation might be that there are other unidentified factors influencing T-cell ageing. Therefore it might be of importance to identify the actual cause at the origin of T-cell ageing which might lie at the stem cell level.

Since T cells are found to be key players in allograft rejection and are targeted by many immunosuppressive medications, (51) the focus of this thesis lays with this immune cell type. However, ESRD affect cells of both the innate as well the adaptive immune system. (8, 184) In general, a combination of activation of cells and decreased immune function is observed. For instance, pro-inflammatory monocytes, characterized as $CD14^{high}CD16^{+}$, are next to $CD4^{+}CD28$null T cells highly associated with atherosclerotic diseases as they can rupture atherosclerotic plaques. (185) High numbers of this cell population was found after KT and is associated with an increased prevalence of vascular disease in kidney transplant recipients after transplantation. (186, 187) The increment in mortality and morbidity in ESRD patients might be due

to an interplay between different immune cells. In addition, allograft rejection is a process in which other types of immune cells are involved. Therefore, assessing the degree of premature ageing of T cells in combination with other immune cells might be of importance to identify high-risk patients for rejection.

One of the goals of this research is to treat KT-recipients with a dosage of immunosuppression that is adjusted to the condition of the immune system. Until today, all patients are treated with the same dose immunosuppression without consideration for the fitness of the immune system or the age of the recipient. From literature it is known that elderly patients have an increased morbidity and mortality rate post-KT as immunosuppression is a strong risk factor for infections and cancers in these patients. (188, 189) For this reason, personalized immunosuppression adjusted on the immune status might be a solution to prevent such clinical complications. Recently it was showed that the effect of calcineurin-inhibitors and rapamycin on peripheral blood mononuclear cells (PBMCs) was different between young and elderly individuals. (105) Others showed that a reduction of immunosuppression increases graft and patient survival in elderly patients. (190)

A first association between ageing and the risk for rejection is described in this thesis. However, allograft rejection is a complex process in which both immunological as well as clinical aspects are involved. This and the whole concept of uremia-associated T-cell ageing must be fully understood before the contribution of ageing to personalized immunosuppression can be assessed.

CONCLUSION

The described research in this thesis aimed to examine whether T-cell ageing is of clinical relevance in the context of kidney transplantation with respect to allograft rejection. It was concluded that uraemia is responsible causing premature immunological ageing of the T-cell system. Prior to KT, the effects caused by RRT on T-cell ageing are remarkably small and CMV-latency significantly affects the differentiation status of $CD8^+$ T cells. Unfortunately, KT it is not able to reverse this uraemia-associated premature aged T-cell compartment. For this reason, the dysfunctional immune system is of importance post-KT as others have shown an association with opportunistic infections and increased risk of cancer. More research is necessary to examine whether other therapeutic treatment options are possible for ESRD patients. On the other side, features of T-cell ageing are associated with a lower risk for allograft rejection.

The findings of this thesis contributed to a better understanding of ESRD-related T-cell ageing by identifying factors that are able to influence ageing of the T-cell system. More research to the underlying mechanism of this premature aged T-cell system is warranted before further implementations into the clinic can be realized. Assessing a peripheral T-cell age might be a way to realize personalized immunosuppression. The findings as described in this thesis is a first step in this process.

9

DUTCH SUMMARY (SAMENVATTING)

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De onderzoeken die in dit proefschrift beschreven zijn, zijn gericht op de vroegtijdige verouderde T-cel afweer, aanwezig bij patiënten met eindstadium nierfalen, in de context van niertransplantatie. Door een verlies van nierfunctie ontstaat een pro-inflammatoir milieu welke sterk geassocieerd is met een verzwakt immuunsysteem. Verondersteld wordt dat het behoud van uremische toxines en cytokines de onderliggende oorzaak is van het ontstaan van oxidatieve stress en inflammatie. Dit resulteert in chronische activatie van het immuunsysteem, waaronder T-cellen wat uiteindelijk leidt tot een lager aantal en een verminderde functie van deze cellen. Deze bevindingen gaven aanleiding om te veronderstellen dat uremie een vroegtijdige veroudering van het T-cel system kan induceren. Recentelijk is aangetoond dat het T-cel immuunsysteem in patiënten met eindstadium nierfalen vroegtijdig verouderd is met ongeveer 20-30 jaar vergeleken de kalenderleeftijd van deze patiënten. Dit verouderd immuunsysteem ligt ten grondslag aan het een verhoogde risico op infecties en maligniteiten het ontwikkelen van hart- en vaatziekten en daarnaast een verminderde bescherming na vaccinatie.

Voor patiënten met eindstadium nierfalen is een niertransplantatie de beste remedie om de nierfunctie te verbeteren. Immunosuppressieve medicatie is noodzakelijk om afstoting van het transplantaat te voorkomen maar brengt een verhoogd risico op infecties en maligniteiten met zich mee na niertransplantatie. Momenteel de immunosuppressieve behandeling gebaseerd op spiegels van calcineurines, mycofenolaat mofetil (MMF) en een vaste dosis steroïden. Desondanks wordt er regelmatig een over- of ondermaat van immunosuppressiva in patiënten waargenomen. Het bepalen van de mate van vroegtijdige T-cel veroudering in niertransplantatie patiënten zou patiënten die een verhoogd risico op afstoting in kaart kunnen brengen en zo een bijdrage kunnen leveren aan deze individualisatie van afweer-onderdrukkende medicijnen. In dit proefschrift is de klinische relevantie van T-cel veroudering op het gebied van afstoting en infecties na transplantatie onderzocht.

Het concept van immunologische veroudering wordt uitgelegd in **hoofdstuk 1**. De immunologische leeftijd kan worden bepaald aan de hand van drie verouderingsparameters: uitstoot van naïeve T-cellen door de thymus, de differentiatie status van het T-cel compartiment en ten slotte door bepaling van de telomeerlengte van T-cellen. Dit wordt breder uitgelegd in dit hoofdstuk en het onderwerp wordt in dit hoofdstuk verder toegelicht.

In het onderzoek beschreven in **hoofdstuk 2** is gekeken naar de bijdrage van uremie en nierfunctie-vervangende therapie in de vorm van dialyse (hemodialyse en peritoneaal dialyse) op vervroegde T-cel veroudering bij patiënten met eindstadium nierfalen. Het effect van uremie werd in kaart gebracht door de T-cel verouderingsparameters van patiënten met eindstadium nierfalen die niet dialyseren te vergelijken met leeftijd-gekoppelde gezonde controles. Daarnaast zijn dialyse patiënten vergeleken met leeftijd-gekoppelde patiënten die niet dialyseren om de bijdrage van dialyse op vroegtijdige T-cel veroudering

in kaart te brengen. Uremie was in staat om vroegtijdige T-cel veroudering te veroorzaken. Patiënten die nog geen nierfunctie-vervangende therapie ontvangen, hadden namelijk al een verminderde functie van de thymus en een meer doorgedifferentieerd T-cel systeem met kortere telomeren. Het toegevoegde effect van dialyse was niet groot en beperkte zich tot slechts kleine verschillen in het $CD8^+$ T-cel compartiment en dan alleen in jonge (<50 jaar) patiënten. Op basis van deze resultaten werd geconcludeerd dat uremie T-cel veroudering induceert en dat nierfunctie vervangende therapie alleen veroudering versterkt van het $CD8^+$ T-cel compartiment in jonge patiënten.

Onderzoeken met gezonde individuen lieten zien dat de aanwezigheid van cytomegalovirus (CMV), veroudering van T-cellen kan versnellen. In patiënten met eindstadium nierfalen is de prevalentie van CMV hoog, en in **hoofdstuk 3** is een mogelijk additioneel effect van CMV op het verouderd T-cel systeem in patiënten met eindstadium nierfalen onderzocht. Daaruit is gebleken dat CMV geen invloed heeft op de productie van nieuwe naïeve T-cellen uit de thymus. De relatieve telomeerlengte van $CD8^+$ T-cellen in een CMV-seropositieve patiënt bleek significant korter te zijn dan die van een leeftijd-gekoppelde CMV-seronegatieve patiënt. Daarnaast bleek dat CMV-seropositieve patiënten zowel meer doorgedifferentieerde $CD4^+$ als $CD8^+$ T-cellen hebben. CMV-seropositieve patiënten bleken een hogere frequentie van geheugen T-cellen te hebben die het co-stimulatoire molecuul CD28 niet tot expressie brengen, ook wel CD28nul T-cellen genoemd. Daarnaast bleken deze cellen positief te zijn voor CD57, een additionele marker voor T-cel differentiatie. Bovendien, hadden deze doorgedifferentieerde CD28nul T-cellen kortere telomeren dan de $CD28^+$ T-cel. Concluderend toonden deze bevindingen aan dat CMV gedeeltelijk de T-cel leeftijd beïnvloedt van met name de $CD8^+$ T-cellen in patiënten met eindstadium nierfalen.

Hoofdstuk 4 beschrijft het effect van niertransplantatie op T-cel veroudering. De geteste hypothese luidde, dat door het verbeteren van de nierfunctie met behulp van een nieuwe nier en het verdwijnen van het pro-inflammatoire milieu in niertransplantatie patiënten er een verjonging van de T-cel zou optreden. In deze longitudinale studie werden niertransplantatie patiënten tot een jaar na transplantatie gevolgd en klinische alsook T-cel parameters gemonitord. Direct na transplantatie daalden de absolute aantallen (doorgedifferentieerde) geheugen T-cellen, wellicht als gevolg van de intensieve immunosuppressieve medicijnen. Echter na afname van de hoeveelheid immunosuppressieve medicijnen bleken deze cellen weer terug te keren tot waarden aanwezig voor transplantatie. Er werd geen verhoogde productie van naïeve T-cellen door de thymus als mede geen verbetering van de telomeerlengte van zowel de $CD4^+$ als de $CD8^+$ T-cellen waargenomen binnen 1 jaar na transplantatie. Naast het bestuderen van deze T-cel verouderingsparameters is de T-cel functie bepaald door de proliferatieve capaciteit van en cytokine producerende T-cellen na een T-cel specifieke stimulatie in kaart te brengen. Ook de functie van de T-cellen bleef ongewijzigd na transplantatie wanneer vergeleken met pre-transplantatie waarden. Op basis van deze

resultaten hebben we geconcludeerd dat niertransplantatie niet in staat was om de vervroegde T-cel veroudering te herstellen.

In **hoofdstuk 5** is onderzocht of de mate van T-cel veroudering voor niertransplantatie kan worden gebruikt om het risico op een afstoting na niertransplantatie in te schatten. In deze prospectieve studie werden de waarden van de T-cel verouderingsparameters voorafgaand aan de niertransplantatie vergeleken tussen patiënten die binnen 3 maanden een afstoting doormaakten en die patiënten die geen afstoting na transplantatie hadden. Er werden geen verschillen gezien met betrekking tot het aantal naïeve T-cellen afkomstig uit de thymus en de relatieve telomeerlengte van de T-cellen. Patiënten met een afstoting in de eerste 3 maanden na transplantatie hadden significant minder aantal $CD4^+CD28nul$ T-cellen dan patiënten die geen rejectie hadden na transplantatie. Dezelfde trend werd gevonden voor de $CD8^+$ T-cellen. Deze bevindingen toonden aan dat expansie van ver doorgedifferentieerde CD28nul T-cellen geassocieerd is met een lager risico op vroege afstoting na transplantatie.

De niertransplantatiepatiënten die bestudeerd zijn in hoofdstuk 4, waren patiënten die of CMV-seropositief dan wel CMV-seronegatief waren en dat bleven na transplantatie. In **hoofdstuk 6** zijn de T-cel verouderingsparameters bestudeerd bij niertransplantatie patiënten die voor de transplantatie CMV-seronegatief waren en een donornier hebben gekregen van een CMV-seropositieve donor. Zij werden dus onder immunosuppressieve medicijnen en anti-virale (valganciclovir) profylaxe voor het eerst blootgesteld aan CMV direct na niertransplantatie. Deze patiëntengroep werd opgesplitst op basis van de aanwezigheid van viraal (CMV) DNA in het bloed (CMV-viremie) en alleen bij CMV-viremische patiënten werd T-cel veroudering geïnduceerd na transplantatie. Deze patiënten ontwikkelden CMV-specifieke T-cellen en immunoglobulinen specifiek voor CMV (anti-CMV IgG). Daarnaast hadden deze patiënten een verdubbeling van hun $CD8^+$ T-cel compartiment met name door een expansie van EMRA $CD8^+$ T-cellen en een toename van CD28nul geheugen $CD4^+$ en $CD8^+$ T-cellen. Opmerkelijk is dat in deze CMV-viremische patiënten de thymusfunctie, gemeten aan de hand van de TREC-content en het percentage $CD31^+$ naïeve T-cellen, 1 jaar na transplantatie verminderd was. Deze T-cel effecten van een primaire CMV-infectie na transplantatie gingen gepaard met een verminderde functie van het transplantaat. In de niet CMV-viremische patiënten bleven deze effecten op T-cellen grotendeels uit. Deze patiënten hadden alleen een toename van $CD4^+CD28nul$ T-cellen na transplantatie. Concluderend tonen de resultaten van deze studie aan dat een actieve CMV infectie na transplantatie veroudering van perifere T-cellen induceert en negatief geassocieerd is met nierfunctie van het transplantaat.

In het review van **hoofdstuk 7** wordt het concept van vervroegde T-cel veroudering bij patiënten met eindstadium nierfalen behandeld met speciale aandacht voor het bepalen van een immunologische leeftijd van T-cellen, het klinisch belang en de eventuele mogelijkheden voor behandeling. Naast de uitleg van de verschillende

T-cel verouderingsparameters die in het onderzoek gebruikt worden voor het bepalen van een T-cel leeftijd worden de beperkingen van deze bepalingen behandeld. Het verouderd immuunsysteem is geassocieerd met een verhoogde mortaliteit en morbiditeit van patiënten met eindstadium nierfalen. Het verhoogde risico op infecties, maligniteiten en hart- en vaatziekten kunnen worden toegeschreven aan het verouderd T-cel immuunsysteem. Dit roept de noodzaak op voor behandeling van het verouderd T-cel immuunsysteem. Ondanks verlaging van het pro-inflammatoire milieu door middel van dialyse of niertransplantatie, blijkt het vroegtijdige verouderen van het T-cel immuunsysteem onomkeerbaar. Er zijn aanwijzingen dat epigenetische veranderingen in voorloper cellen van deze T-cellen hieraan ten grondslag liggen. Meer onderzoek is noodzakelijk om een definitieve conclusie te kunnen trekken met betrekking tot of het bepalen van de mate van vroegtijdige T-cel veroudering kan worden gebruikt voor individualisatie van het immunosuppressieve regime van niertransplantatie patiënten.

10

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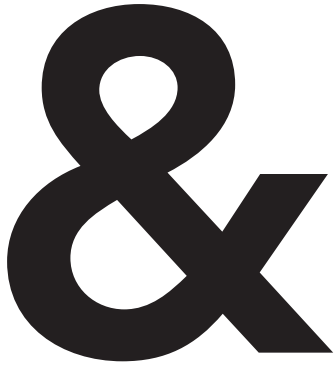

ADDENDUM

ACKNOWLEDGEMENTS
(DANKWOORD)
LIST OF PUBLICATIONS
PHD PORTFOLIO
CURRICULUM VITAE

ACKNOWLEDGEMENT (DANKWOORD)

Ten slotte het veelvuldig gelezen stuk van een proefschrift, het dankwoord. Vier jaar promotieonderzoek is niet iets wat je alleen doet. Alle ondersteuning maar ook de kritische noten hebben geleid naar dit proefschrift. Allen dank daarvoor. Graag wil ik een paar mensen persoonlijk bedanken.

Mijn promotor **Prof. Dr. Carla C. Baan**, dank dat ik de afgelopen vier jaar op jouw lab heb mogen werken. Jouw inzicht in het doen van onderzoek maar ook kritische vragen welke ook hebben bij gedragen aan dit proefschrift. Benoemd worden tot hoogleraar is een bekroning op je carrière waar velen jaloers op mogen zijn. Succes in de toekomstige wetenschap als professor!

Mijn copromotoren. **Dr. Michiel G.H. Betjes,** Dank dat ik mocht werken aan jouw onderzoek. Ik heb ontzettend veel van je geleerd. Van het klinische aspect tot het schijven tot zulke ó zo lastige discussies. Jouw strakke tijdsplanning die er mede voor gezorgd heeft dat dit proefschrift binnen vier jaar klaar is. Ik heb het altijd zeer gewaardeerd dat ik naar vele internationale congressen mocht om mijn kennis te verbreden waarvoor heel veel dank. **Dr. Nicolle H.R. Litjens**, dank voor al jouw hulp. Ik had het mailtje getiteld: "heb je twee minuten?" nog niet verstuurd of je stond naast me. Ik kon je altijd storen als ik ergens mee zat, maar ook vooral als er leuke resultaten waren gevonden. Jou gedrevenheid en enthousiasme voor onderzoek bewonder ik. Ik hoop dat ik niet tegen ben gevallen als eerste OIO, en dat er vele zullen volgen. Heel veel geluk voor de toekomst!

Mijn paranimfen. Lieve **Elly,** het boekje is klaar, het kaftje is gedrukt! Jouw (Quick and dirty?) bijdrage is groots geweest en mede hierdoor is dit proefschrift binnen vier jaar af. Vele kopjes koffie, dansschoentjes, en (jouw) tubes handcrème zijn er versleten. Jouw nuchtere blik op de wereld was een inspiratie voor me. Ik hoop dat we elkaar nog vaak zullen zien, al is het maar op de fiets in het nabije pittoreske Ostrecht! Lieve **Karin**, veel hebben we gedeeld. Of het nou een kleine frustratie was of dat ik iets belangrijks kwijt moest, je was een luisterend oor en dat heb ik zeer gewaardeerd! Altijd wist je dingen goed te relativeren, iets wat ik bewonder. Hoop nog veel van je te blijven horen zowel op onderzoeksgebied, maar ook zeker persoonlijk. Ik ben blij dat jullie mijn paranimfen zijn!

Mijn (oud) AIO collega's. Lieve **Anne,** onze vriendschap die we tijdens ons promotieonderzoek opgebouwd hebben is me heel veel waard. Ik heb de afgelopen jaren met niemand op het lab zoveel gelachen als met jou. Beide zijn we ons er van bewust dat promoveren geen kattenpis is…..'s-Morgens even de shortie bekijken met een intensje, even onze favoriete series doorspreken en tenslotte in de middag nog een vriezeruitje….. Mijn boekje is af, en weet ook zeker dat jou boekje binnenkort afkomt. Hoop nog lang contact te mogen houden met je en wens je alle succes toe in de kliniek en ook zeker in jouw toekomst met Ralph!

Anja, jij zorgde altijd voor een goede sfeer op onze oude AIO-kamer. Je verjaardag of zelfs terugkomen van vakantie was een feestje, omdat jij er dan wel voor gezorgd had dat alles mooi versierd was! Het was heel gezellig om met jou samen te mogen onderzoeken. Danke fur die kinderüberasschungs!

Marieke, aan het begin van mijn promotie ging ik door een moeilijke tijd, maar jouw steun (helaas door ervaring) is me altijd bijgebleven. Ik heb bewondering voor het feit dat je weet wat je wilt en voor je doelen gaat. Succes met je gezin in het (verre) Groningse!

Martijn, ook jij zorgde altijd voor de gezelligheid binnen het lab. Van een koffiemomentje tot een gezellige borrel na het werk. Je was een gezellige en fijne collega maar ook kamergenoot tijdens congressen. Alle succes met je opleiding tot klinisch chemicus!

Gretchen, jouw reddingsactie van "the eye of the tiger" zal ik nooit vergeten! Daarom ben ik ervan overtuigd dat je een succesvolle dokter zal worden. Succes met de afronding van je onderzoek. Ben benieuwd naar jouw (hello kidney) proefschrift.

The triple-A-team. **Ling**, as a second member of our ageing team I'll pass the baton to you. Keep smil(ling) as you always do and do not lose your focus ;), this will eventually lead you to a successful thesis! En onze (special) **Burç,** onze sparkling unicorn rainbow heavenly heavily significant data hebben zeker bijgedragen aan dit eindresultaat! Dank voor al je (her)analyses en als opvolger wens ik je alle succes! In het geval je volleybal carrière nog niet verpest is, kom ik graag nog een keertje met je volleyballen.

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De postdocs. **Marcella**, moltes gràcies per tots els bons moments! I'am proud on our succes at the bruggenloop even though you kicked my a*s! Unfortunatelly, it never came to the paella. But what has not take place yet (death sparrow), is yet to come.

Nicole, ook jou kon ik altijd storen. Even bijpraten over van alles! Zal de gezellige etentjes en uitstapjes in San Francisco niet snel vergeten.

Martin. Ook jij was een fijne kamergenoot. Een gezet kopje koffie in de ochtend zal nooit meer zo goed smaken. Dank voor al je hulp en inspiraties voor het onderzoek.

De analisten van het transplantatie lab, **Mariska** bedankt voor je hulp met de telomeren! Mede door jou werden het er al snel (telo)minder. **Annemiek, Frieda, Jeroen, Joke, Marjolein, Rens, Ruben, Ronella, Sander, Tanja, Thea, Thierry en Wenda** Bedankt voor het verven van alle mozaïek-samples. Jullie zijn stuk voor stuk kunstenaars! Dank voor alle gezellige momenten en overheerlijke traktaties.

De afdeling immunologie. Beste **Ton**, dank voor al je hulp. Je was altijd zeer geïnteresseerd in dit onderzoek en ook altijd in voor een praatje. Leuk dat je in mijn

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Franny, ik vond het erg gezellig om met je te mogen samenwerken. Weet zeker dat jou boekje (prof.) beautiful wordt. Wens je alle succes toe in de afronding van jou promotie!

De D-vleugel**, Beste Prof. Dr. Willem Weimar,** mede dankzij uw bijdrage ligt hier mijn proefschrift. Dank voor uw steun in dit onderzoek. **Saida,** bedankt voor alle lekkere kopjes koffies bij de besprekingen met Michiel en hulp bij belangrijke administratieve zaken. **Alle Nefrologen,** dank voor al jullie hulp bij dit onderzoek. Van het includeren van patiënten tot het aandragen van nieuwe ideeën tijdens een werkbespreking. **Dennis**, dank voor jou betrokkenheid aan mijn promotieonderzoek!

Lieve **pap** (10-11-11), helaas kan je dit niet meer meemaken maar ik weet zeker dat je ongelofelijk trots op mij bent. Zal nooit vergeten hoe blij en trots was toen ik met dit promotieonderzoek begon of toen ik naar een eerste congres mocht om mijn werk te presenteren. Dit eindresultaat is ook voor jou, je bent altijd bij me!

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Woerden, Maart 2015

LIST OF PUBLICATIONS

A killer on the road: circulating CD4(+)CD28null T cells as cardiovascular risk factor in ESRD patients.
Betjes MG, **Meijers RW**, de Wit LE, Litjens NH.
J Nephrol. 2012 Mar-Apr;25(2):183-91. doi: 10.5301/jn.5000057. Review.

Terminally differentiated CD8+ Temra cells are associated with the risk for acute kidney allograft rejection.
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Uremia causes premature ageing of the T cell compartment in end-stage renal disease patients.
Meijers RW, Litjens NH, de Wit EA, Langerak AW, van der Spek A, Baan CC, Weimar W, Betjes MG.
Immun Ageing. 2012 Sep 12;9(1):19. doi: 10.1186/1742-4933-9-19.

Cytomegalovirus contributes partly to uraemia-associated premature immunological ageing of the T cell compartment.
Meijers RW, Litjens NH, de Wit EA, Langerak AW, van der Spek A, Baan CC, Weimar W, Betjes MG.
Clin Exp Immunol. 2013 Dec;174(3):424-32. doi: 10.1111/cei.12188.

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Betjes MG, **Meijers RW**, Litjens NH.
Blood Purif. 2013;36(3-4):173-8. doi: 10.1159/000356084. Epub 2013 Dec 20. Review.

Uremia-associated immunological aging is stably imprinted in the T-cell system and not reversed by kidney transplantation.
Meijers RW, Litjens NH, de Wit EA, Langerak AW, Baan CC, Betjes MG.
Transpl Int. 2014 Dec;27(12):1272-84. doi: 10.1111/tri.12416. Epub 2014 Sep 30.

T-cell ageing in End-stage renal Disease patients: Assessment and Clinical Relevance
Meijers RW, Betjes MG, Baan CC, Litjens NH.
World J Nephrol. 2014 Nov 6;3(4):268-76. doi: 10.5527/wjn.v3.i4.268. Review.

Primary cytomegalovirus infection significantly impacts circulating T cells and renal allograft function
Meijers RW, Litjens NH, Hesselink DA, Langerak AW, Baan CC, Betjes MG.
American Journal of Transplantation. 2015; xx(xx):xx-xx

Loss of CD28 on peripheral T cells decreases the risk for early acute rejection after kidney transplantation
Meijers RW*, Dedeoglu B*, Klepper M, Hesselink DA, Baan CC, Litjens NH, Betjes MG.
Submitted Transplant Int. may 2015
*: Both authors equally share first authorship

PHD PORTFOLIO

Name PhD student:	Ruud Meijers
Erasmus MC department:	Internal Medicine, Section Nephrology and Transplantation
Research school:	Postgraduate School Molecular Medicine
PhD period:	May 2011 - May 2015
Promotor:	Prof. dr. Carla C. Baan
Copromotor:	Dr. Michiel G.H. Betjes and Dr. Nicolle H.R. Litjens

General Courses

– Biostatistical Methods I: Basic Principles	2011
– (Neuro)- Immunology	2012
– English Biomedical Writing and Communication	2013
– Molecular Immunology	2013
– Stalingshygiëne deskundigheidsniveau 5B	2014

Participation and presentations at (inter)national conferences

48th Dutch Society of Immunology (NVVI) annual meeting, Noordwijkerhout, The Netherlands	2011	Posters (2x)
"Wetenschapsdagen" (annual two-day meeting of the Department of Internal Medicine), Antwerp, Belgium	2012	Poster
24th "Boot" congress (Dutch Transplantation Society), Maastricht, The Netherlands	2012	Presentation (2x)
Dutch Society for Nephrology (NND), Veldhoven, The Netherlands	2012	Posters (2x)
3rd European Congress of Immunology, Glasgow, United Kingdom	2012	Poster
Wetenschapsdagen" (annual two-day meeting of the Department of Internal Medicine), Antwerp, Belgium	2013	Presentation
25th "Boot" congress (Dutch Transplantation Society), Duiven, The Netherlands	2013	Presentation + laptop
Dutch Society for Nephrology (NND), Veldhoven, The Netherlands	2013	Posters (2x)
13th American Transplant Congress (ATC), Seattle, WA, USA	2013	Posters (2x)
16th European Society for Organ Transplantation (ESOT), Vienna, Austria	2013	Presentations (2x)
49th Dutch Society of Immunology (NVVI) annual meeting, Noordwijkerhout, The Netherlands	2013	Posters (2x)
"Wetenschapsdagen" (annual two-day meeting of the Department of Internal Medicine), Antwerp, Belgium	2014	Poster
18th Molmed Day, Rotterdam, The Netherlands	2014	Poster
26th "Boot" congress (Dutch Transplantation Society), Leiden, The Netherlands	2014	Presentation
Dutch Society for Nephrology (NND), Veldhoven, The Netherlands	2014	Presentation
3rd International Summer Frontiers Symposium, Nijmegen, The Netherlands	2014	Poster

World Transplant Congres (WTC), San Francisco, CA, USA	2014	Poster
5th International Workshop on CMV and Immunosenescence, Amsterdam, The Netherlands	2014	Presentation*
50th Dutch Society of Immunology (NVVI) annual meeting, Kaatsheuvel, The Netherlands	2015	Poster
"Wetenschapsdagen" (annual two-day meeting of the Department of Internal Medicine), Antwerp, Belgium	2015	Poster
27th "Boot" congress (Dutch Transplantation Society), Bournemouth, United Kingdom	2015	Poster
14th American Transplant Congress (ATC), Philadelphia, PA, USA	2015	Poster
17th European Society for Organ Transplantation (ESOT), Brussels, Belgium	2015	Presentation*

*presented by Dr. N.H.R. Litjens

Travel grants and Awards

Travel grant EUR trust fund	2013
Poster with Distinction, ATC, Seattle, WA, USA	2013
Poster with Distinction, ATC, Seattle, WA, USA	2013
Bootbeurs NTV (Annual Meeting NTV), Leiden, The Netherlands	2014
Travel grant EUR trust fund	2014
NVVI Travel grant by the Dutch Society of Immunology	2014
Travel grant EUR trust fund	2015

Memberships

Dutch Society of Immunology (Nederlandse Vereninging voor Immunology, NVVI)
Dutch Transplantation Society (Nederlandse Transplantatie Vereniging, NTV)

Teaching activities

Supervision of students writing a review in Keuzeonderwijs Transplantatie geneeskunde	2014

CURRICULUM VITAE

Ruud Meijers werd op 2 September 1987 geboren in Tiel. In 2004 behaalde hij zijn HAVO diploma aan het A. Roland Holst College in Hilversum. Daarna volgde hij de HLO-opleiding Life Sciences, met als specialisatie Microbiologie aan de Hogeschool Utrecht te Utrecht welke hij succesvol afrondde in 2008. Aansluitend begon hij aan zijn master Biomedical Sciences aan de Vrije Universiteit in Amsterdam waarin hij zich specialiseerde op het gebied van Infection and Immunity. Zijn diploma behaalde hij in Augustus 2010. Eind Mei 2011 is Ruud begonnen als promovendus bij de afdeling Inwendige Geneeskunde, sectie Nefrologie en Transplantatie onder leiding van promotor prof. Dr. Carla C. Baan en copromotoren Dr. Michiel G.H. Betjes en Dr. Nicolle H.R. Litjens waar hij onderzoek doet naar vroegtijdige veroudering van T-cellen in patiënten met eindstadium nierfalen met betrekking tot niertransplantatie. In 2011 vestigde Ruud zich samen met Femke in Woerden en zij gaven elkaar het Ja-woord op 5 oktober 2012.