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ORIGINAL ARTICLE

Uremia-associated immunological aging is stably imprinted in the T-cell system and not reversed by kidney transplantation

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aging, end-stage renal disease patients, kidney transplantation, T cells, telomeres, T-cell receptor excision circle.

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Summary

The uremia-induced inflammatory environment in end-stage renal disease (ESRD) patients is associated with premature T-cell aging resulting in a defective T-cell immunity. As kidney transplantation (KTx) reduces the pro-inflammatory environment, we hypothesized that KTx would rejuvenate the aged T-cell system. As aging parameters, we determined in 70 KTx 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-KTx, 3, 6 and 12 months post-KTx. In addition, T-cell function was determined by measuring the proliferative capacity and percentages of cytokine-producing cells. Directly post-KTx, memory T-cell numbers were diminished but restored to pre-KTx 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-KTx 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-KTx. Our findings demonstrate that the uremia-associated aged phenotype is stably imprinted in the T-cell system and not reversed by KTx.

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 [1–4]. In addition, cytotoxic helper T cells (CD4$^+$CD28null T cells) are generated which play a role in the destabilization of atherosclerotic plaques [5–7].

Retention of uremic molecules and cytokines in end-stage renal disease (ESRD) patients are key mechanisms in generating oxidative stress and inflammation [8,9]. This uremia-associated pro-inflammatory condition underlies the impaired T-cell system in ESRD patients by causing premature immunological aging [10,11]. Physiological aging 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 [10,11]. Thymic output can be determined by assessing the content of T-cell receptor excision circles (TRECs), 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

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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 [10]. As changes in T-cell differentiation can be heavily influenced by viral infections, particularly cytomegalovirus (CMV), all the aforementioned hallmarks of immunological aging must be assessed and established [12–14].

Table 1. Clinical and demographic characteristics of patients.

	ESRD patients (n = 70)	Healthy controls (n = 70)
Age in years*	54.2 ± 11.6†	50.5 ± 12.7†
CMV seropositive	45 (64.3%)	39 (55.7%)
Male	44 (62.9%)	32 (45.7%)
Renal replacement therapy*		
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)‡	
Underlying kidney disease	21 (30.0%)	
Hypertensive nephropathy	6 (8.6%)	
Glomerulonephritis	14 (20.0%)	
Diabetic nephropathy	9 (12.9%)	
Polycystic kidney disease	2 (2.8%)	
Reflux nephropathy	18 (25.7%)	
Other/unknown		
Previous KTx*		
1	3 (4.2%)	
2	1 (1.4%)	
Living kidney donation	70 (100%)	
Mismatches HLA class I	2.5 ± 1.03†	
Mismatches HLA class II	1.2 ± 0.71†	
Immunosuppressive medication		
Basiliximab induction therapy§	70 (100%)	
Prednisolone¶	70 (100%)	
MMF	70 (100%)	
Tacrolimus	70 (100%)	
Trough levels**	1.75 ± 1.0†	
MMF (mg/l)	6.07 ± 2.7†	
Tacrolimus (μg/l)	5 (7.0%)	
Patients with delayed graft function		
Patients with rejection (treated with methylprednisolone)	8 (11.4%)	
Patients with CMV reactivation post-KTx	0	
EGFR (ml/min)*	50.0 ± 18.3†	
Serum creatinine concentration (μmol/l)*	136.5 ± 64.3†	

CMV, cytomegalovirus; ESRD, end-stage renal disease; KTx, kidney transplantation; MMF, mycophenolate mofetil.
*At pre-KTx time point.
†Mean with standard deviation.
‡Median with Interquartile range.
§Given at day 0 and day 4 post-KTx.
¶Given the first 3 months post-KTx.
**At 12 months post-KTx time point.

Using the combination of these three T-cell aging parameters, the average immunological age of T cells in ESRD patients is 20–30 years higher than their chronological age [10].

Kidney transplantation (KTx) rapidly reverses the levels of pro-inflammatory proteins and oxidative stress to levels that are comparable to healthy individuals [15]. 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 KTx recipients in received a kidney from a living donor and received basiliximab as induction therapy. Post-KTx the standard triple immunosuppression consisted of prednisolone, mycophenolate mofetil (MMF) and tacrolimus. After 3 months, patients were taken of prednisolone. All CMV-seronegative patients (n = 25) included in this study received a kidney from a CMV-seronegative donor. The CMV-seropositive patients received a kidney from either a CMV-seronegative (n = 13) or CMV-seropositive donor (n = 32). As antiviral prophylaxis, all CMV-seropositve patients received valgancyclovir the first 6 months post-KTx. CMV-seronegative patients who received a kidney from a CMV-seropositive donor were excluded because CMV infection is known to influence the T-cell aging parameters [13,14,16].

Patients and healthy controls (HC) characteristics are listed in Table 1. Eight patients had a biopsy-proven rejection within the first year post-KTx, and all were treated with methylprednisolone. None of the patients received T-cell depletion therapy (i.e. rabbit antithymocyte 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 [10,11,13]. 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).

Peripheral blood mononuclear cells isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples drawn from KTx recipients 1 day before KTx, 3, 6 and 12 post-KTx and from donors 1 day before KTx.

DNA isolation and TREC assay

T-cell receptor excision circle content was assessed using 1×10^6 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′-TCGTGAGAACGGTGAATG AAG-3′ and 5′-CCATGCTGACACCTCTGGTT-3′, 150 nmol/l of hydrolysis probe 5′-(FAM) CACGGTGATG CATAGGCACCTGC-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 (C_t) between replicates of >1.5 necessitated to repeat the PCR experiment. ΔCt was calculated using the formula: *Ct* value TREC PCR−*Ct* value albumin PCR [11,17].

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 subcell 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 [11,18]:

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

In addition, the RTL within FACS-sorted purified naïve or memory $CD4^+$ and $CD8^+$ T cells was assessed. For this purpose, PBMCs (20×10^6) 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 ViaProbe (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%.

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-kidney transplantation (KTx), 3, 6 and 12 months post-KTx of the KTx patients (white bars) and that of the HCs are shown (black bars) ($n = 70$, mean ± SEM). Significant differences between pre-KTx and post-KTx time-points for KTx 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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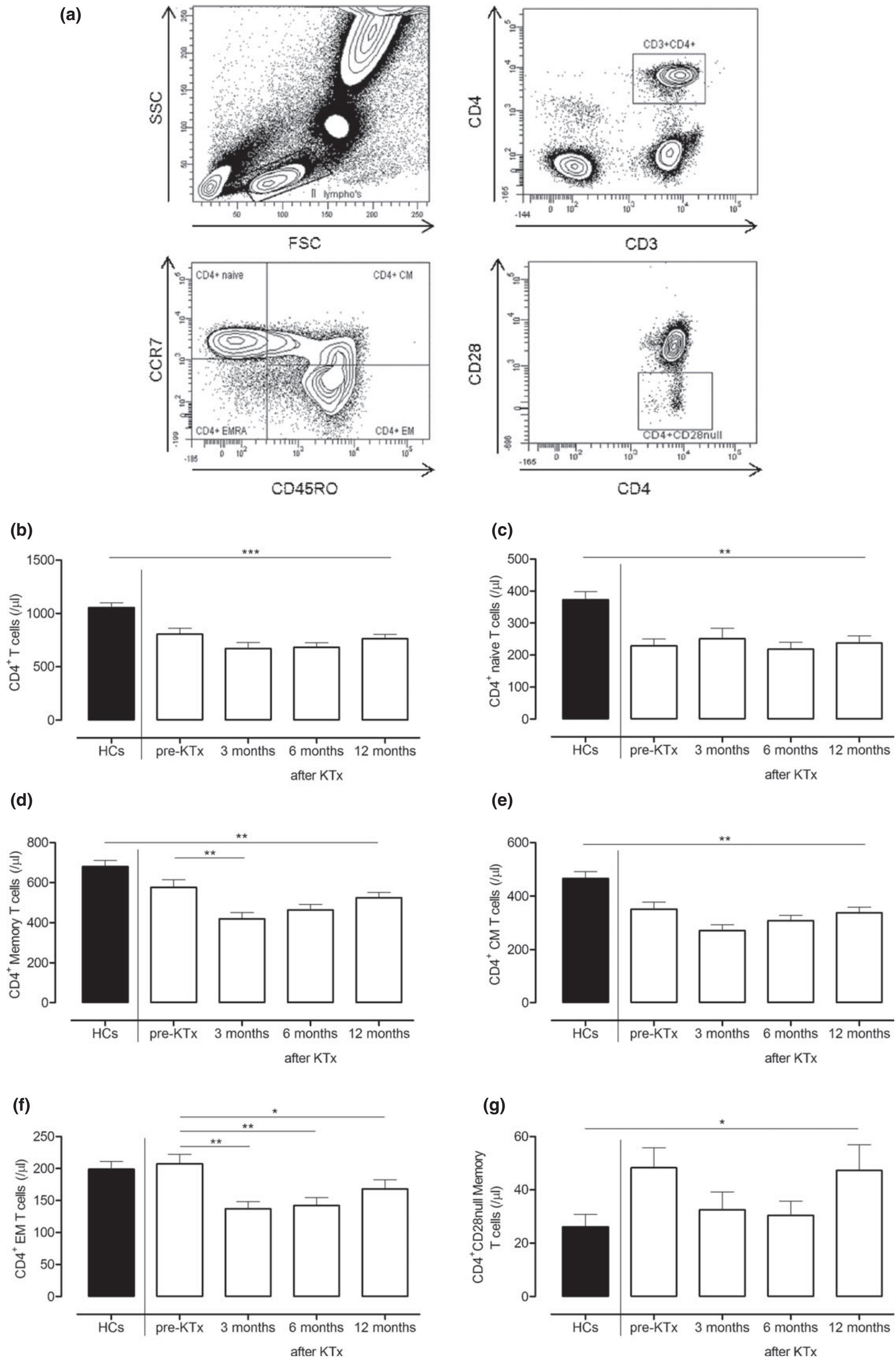
(a)
SSC
FSC
lympho's
CD4
CD3
CD3+CD4+
CCR7
CD45RO
CD4+ naive
CD4+ CM
CD4+ EMRA
CD4+ EM
CD28
CD4
CD4+CD28null
(b)
CD4+ T cells (/µl)
***
(c)
CD4+ naive T cells (/µl)
**
(d)
CD4+ Memory T cells (/µl)
**
**
(e)
CD4+ CM T cells (/µl)
**
(f)
CD4+ EM T cells (/µl)
*
**
**
(g)
CD4+CD28null Memory T cells (/µl)
*
HCs
pre-KTx
3 months
6 months
12 months
after KTx

Analysis of proliferative capacity of T cells

Peripheral blood mononuclear cells were thawed and labeled using PKH26 cell linker kit (Sigma-Aldrich, St. Louis, MO, USA) according manufacturer's instructions. The labeled cells were concentrated to 5×10^5/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 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, ME, USA).

Cytokine-producing T cells

Peripheral blood mononuclear cells (1×10^6/ml) were stimulated anti-CD3/anti-CD28 coated beads (Invitrogen) for 12 h in the presence of the cytokine secretion inhibitor Brefeldin A (1 μl/ml; Golgiplug, BD) to determine frequencies of cytokine-producing cells [19]. The background signal was determined by stimulation in the 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 0.5–1 × 10^6 T cells/measurement) using the FACSCanto II and analyzed using FACS DIVA software version 6.1.2.

Statistical analyses

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

Results

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

The differentiation status of the $CD4^+$ and $CD8^+$ T-cell compartment of KTx recipients were determined at pre-KTx and at 3, 6 and 12 months post-KTx. The 12 months post-KTx value was compared to the value of HCs. In Figs 1a and 2a, typical examples of the gating strategy are depicted. The total number of $CD4^+$ T cells (Fig. 1b) was not affected 12 months post-KTx, and the number remained significantly lower ($P < 0.001$) compared with HCs. Naïve $CD4^+$ T-cell numbers were maintained (Fig. 1c) but memory $CD4^+$ T-cell numbers decreased significantly ($P < 0.01$) 3 and 6 months post-KTx but were restored to pre-KTx level at 12 months post-KTx (Fig. 1d). 12 months post-KTx, both naïve and memory T cells were significantly lower compared with HCs ($P < 0.01$). The CM T-cell numbers did not change post-KTx (Fig. 1e). The EM $CD4^+$ T-cell numbers were significantly ($P < 0.01$) lower 3 and 6 months post-KTx compared with pre-KTx, but also at 12 months post-KTx, the EM $CD4^+$ T-cell numbers were still significantly ($P < 0.05$) lower (Fig. 1f). At 12 months post-KTx, 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 KTx, but were significantly ($P < 0.05$) higher than those of HCs. (Fig. 1g).

The total $CD8^+$ T-cell count decreased significantly 3 months post-KTx (Fig. 2b, $P < 0.01$) due to a decrease

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), central memory (CM) (e), effector memory (EM) (f), effector memory $CD45RA^+$ (EMRA) (g) $CD8^+$ T cells and CD28null within the memory $CD8^+$ T-cell population (h) at pre-kidney transplantation (KTx), 3, 6 and 12 months post-KTx of the KTx patients (white bars) and that of the HCs are shown (black bars) ($n = 70$, mean ± SEM). Significant differences between pre-KTx and post-KTx time-points for KTx 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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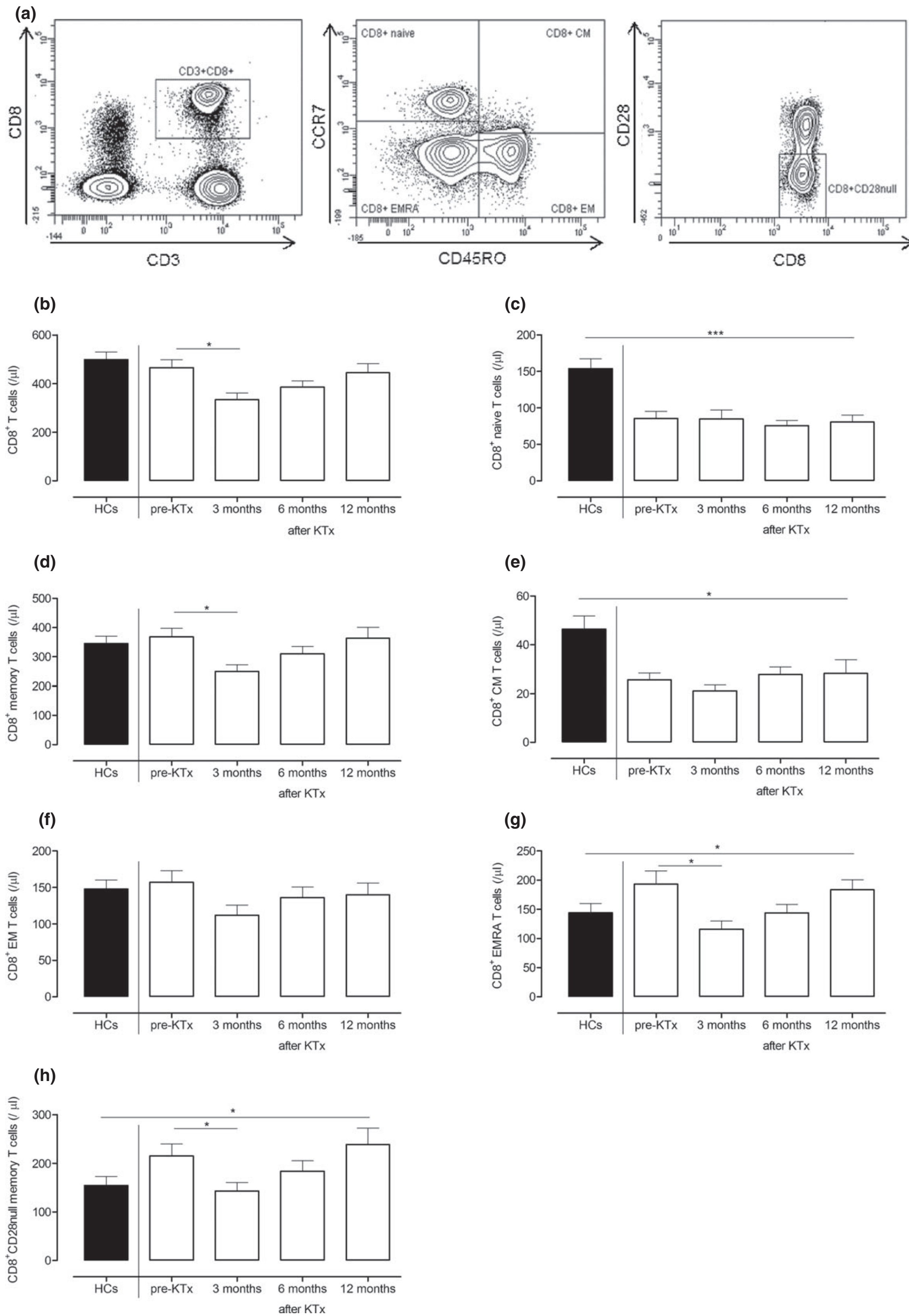

(a)
CD3+CD8+
CD8
CD3
CD8+ naive
CD8+ CM
CD8+ EMRA
CD8+ EM
CCR7
CD45RO
CD28
CD8+CD28null
CD8
(b)
CD8+ T cells (/μl)
HCs
pre-KTx
3 months
6 months
12 months
after KTx
(c)
CD8+ naive T cells (/μl)
(d)
CD8+ memory T cells (/μl)
(e)
CD8+ CM T cells (/μl)
(f)
CD8+ EM T cells (/μl)
(g)
CD8+ EMRA T cells (/μl)
(h)
CD8+CD28null memory T cells (/ μl)

in memory $CD8^+$ T cells (Fig. 2d, $P < 0.001$) with stable naïve $CD8^+$ T-cell numbers (Fig. 2c). Moreover, 12 months post-KTx, 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 with HCs (Fig. 2e). The EM, EMRA $CD8^+$ T cell and $CD8^+$CD28null memory T-cell numbers were significantly ($P < 0.05$) lower 3 months post-KTx (Fig. 2f, g and h) but were restored to pre-KTx values at 1 year post-KTx. Both numbers of EMRA $CD8^+$ T cells and the number of $CD8^+$CD28null memory T cells were significantly higher ($P < 0.05$) compared with those observed in HCs (Fig. 2g and h) at 12 months post-KTx.

No restoration of thymic output within 1 year post-KTx

Thymic output was determined by TREC content analysis (Fig. 3a) and expression of CD31, a marker for RTEs, within the naïve T-cell compartment (Fig. 3b–d). Twelve months post-KTx, thymic output was maintained at a similar level as pre-KTx and the ΔCT remained significantly ($P = 0.009$) higher compared with that observed in HCs, indicative that thymic output was not restored (Fig. 3a). In accordance with this observation, the number of $CD31^+$ naïve $CD4^+$ and $CD8^+$ T cells remained unaltered post-KTx. Consequently, at 12 months post-KTx, the recipients had still significantly lower ($P < 0.001$) numbers of $CD31^+$ naïve $CD4^+$ and $CD8^+$ T cells compared with HCs (Fig. 3c and 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 $CD4^+$ (Fig. 4b) and $CD8^+$ (Fig. 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-KTx when compared to pre-KTx 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 $CD4^+$ ($P = 0.004$, Fig. 4d) and $CD8^+$ T cells ($P = 0.04$, Fig. 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.

T-cell aging parameters after KTx and clinical characteristics

In accordance with previous data [11], no relation was observed between patients who had a remaining kidney function pre-KTx and those who received renal replacement therapy (RRT) pre-KTx. In addition, the outcome of the aging parameters 1 year post-KTx 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-KTx (data not shown).

T-cell function is unchanged 1 year after KTx

T-cell function was analyzed by examining proliferative capacity and frequencies of cytokine-producing cells using flow cytometry. An example of the gating strategy (Fig. 5a) and subsequent analysis of proliferation by MODFIT is shown for unstimulated T cells, tetanus toxoid stimulation and anti-CD3/anti-CD28 stimulation (Fig. 5b).

One year post-KTx, the percentage of proliferating $CD4^+$ and $CD8^+$ T cells in response to both tetanus toxoid (Fig. 5c and d) and anti-CD3/anti-CD28 coated beads (Fig. 5e and f), was similar compared with pre-KTx.

In addition, there was no increase in the frequency of cytokine-producing T cells in response to polyclonal stimulation for $CD4^+$ (Fig. 5g) and $CD8^+$ (Fig. 5h) T cells.

Discussion

In this study, we evaluated whether KTx 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 1 year post-KTx such changes have not occurred.

In accordance with previous studies, the results show that the T-cell system of ESRD patients before KTx 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 1 year post-KTx, although within the year significant changes within the $CD4^+$ and $CD8^+$ memory compartment were observed. Both young and old KTx recipients had a similar course of the aging parameters post-KTx, 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-KTx 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 [20,21]. In addition, CD31 expres-

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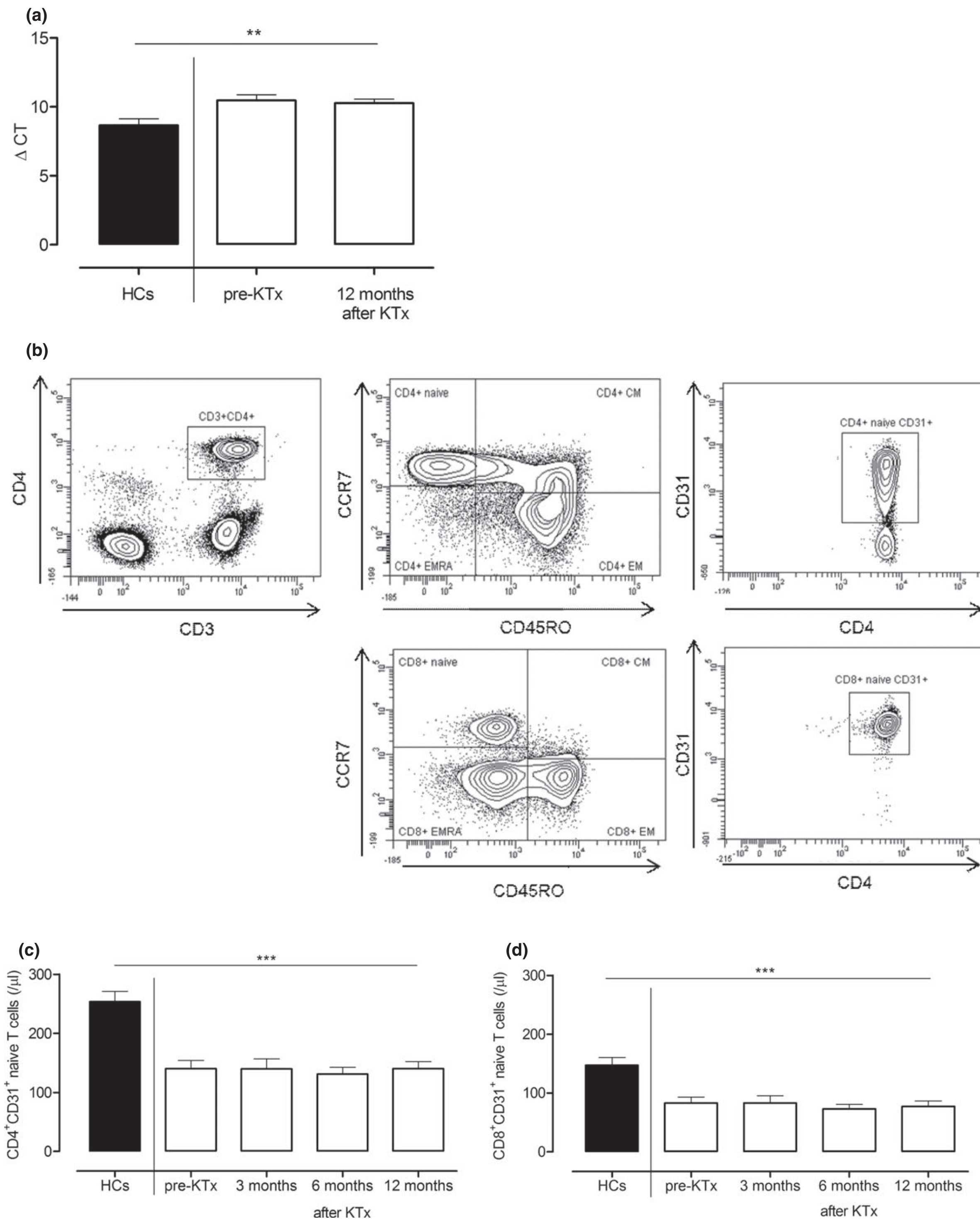


Figure 3 The thymic output was measured by T-cell receptor excision circle (TREC) content and expression of CD31$^+$ by naïve T cells. The ΔCT (difference in number of amplification cycles between TREC and albumin as a control for DNA input; inversely related to TREC content) was calculated pre-kidney transplantation (KTx) and 12 months post-KTx of the KTx recipients (white bars) and pre-KTx 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 flow cytometric example (b). Absolute numbers of CD31$^+$ T cells within the naïve population (n = 70, mean ± SEM), indicating recent thymic emigrants (RTEs) are depicted for the CD4$^+$ (c) and the CD8$^+$ (d) T-cell compartment. Significant differences between pre-KTx and post-KTx time-points for KTx recipients and between patients at 12 months and the HCs were calculated and shown (*P < 0.05, **P < 0.01, ***P < 0.001).

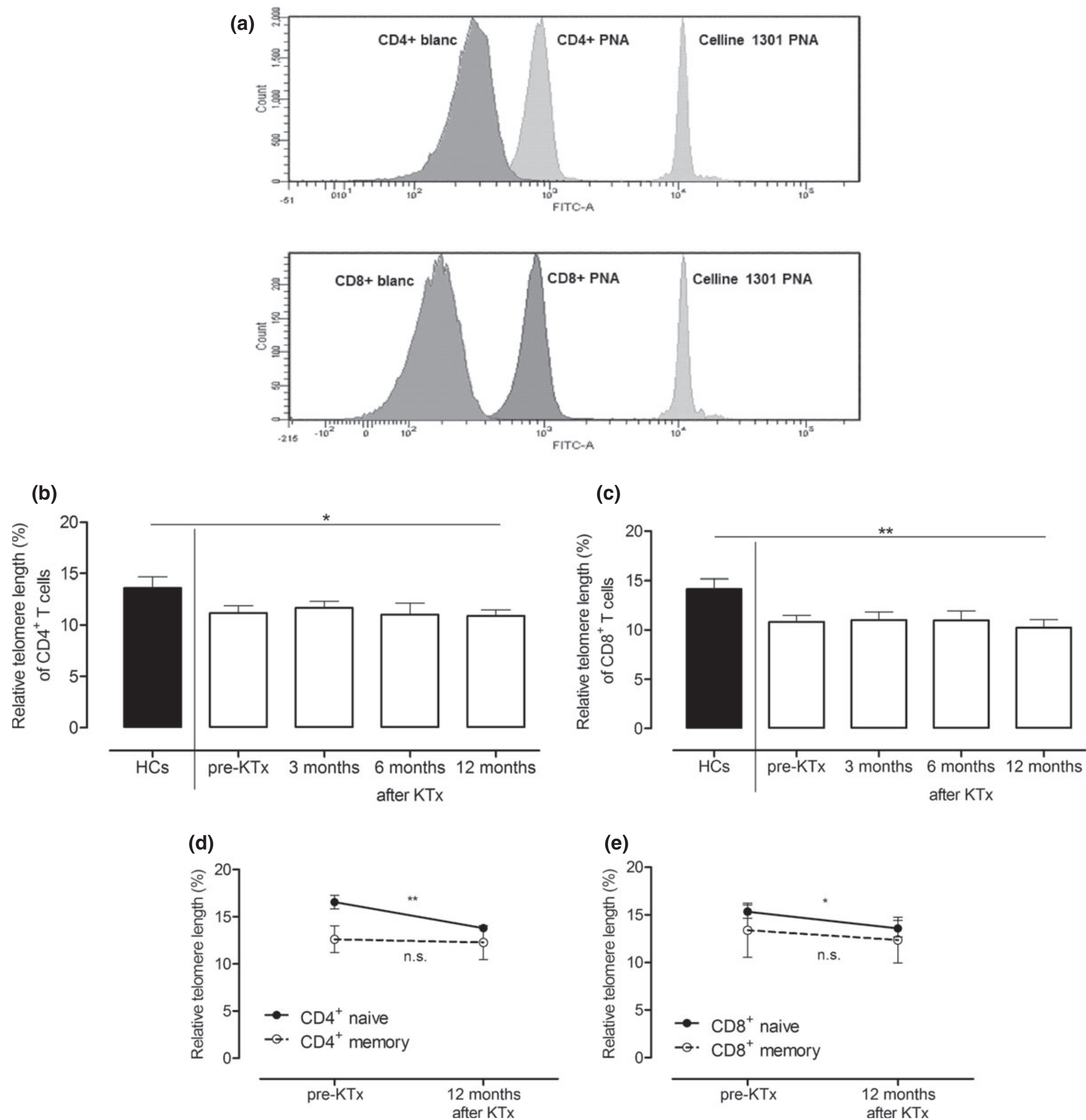
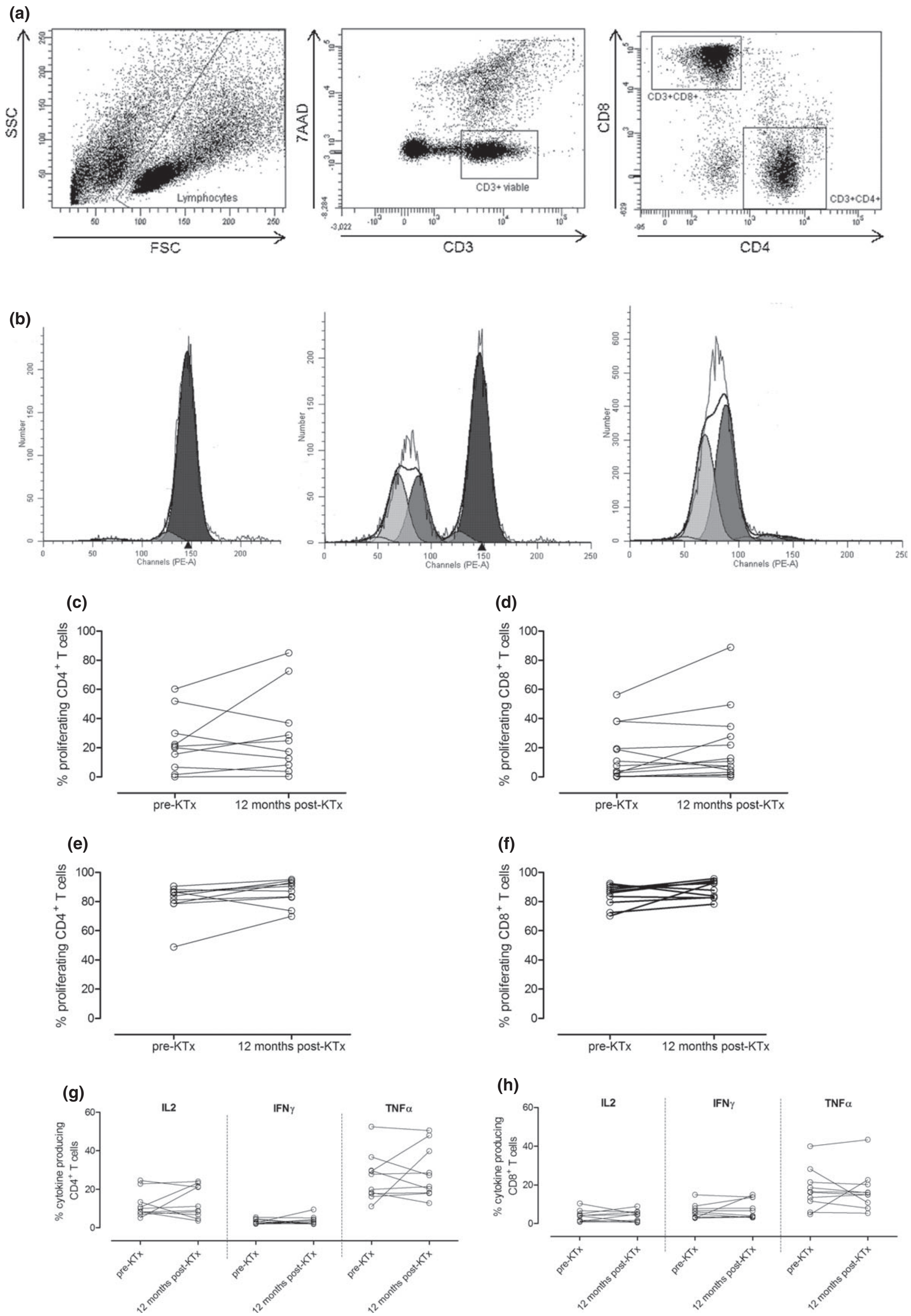


Figure 4 Proliferative history was measured by the relative telomere length. A typical example of the flow cytometric example of the PNA (probe to detect telomeres)-fluorescence intensity, depicted in a histogram, for the cell-line 1301, $CD4^+$ and $CD8^+$ T cells and the negative control (without PNA probe) is depicted (a). The RTL of (b) the $CD4^+$ and (c) $CD8^+$ T-cell compartment was determined pre-kidney transplantation (KTx), 3, 6 and 12 months post-KTx (white bars), and the RTL of the HCs was determined pre-KTx (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-KTx and the post-KTx time-points for KTx recipients was calculated and shown as well as the differences between the HCs and the 12 months post-KTx time-point (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

sion within naïve T cells may not necessarily reflect RTEs as CD31 expression is maintained upon homeostatic proliferation in the 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 [22,23]. 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 aging parameters (TREC, RTL and differentiation status) in KTx recipients pointed toward a prematurely aged T-cell system prior to KTx which is irreversible by KTx.


(a)
SSC
FSC
Lymphocytes
7AAD
CD3
CD3+ viable
CD8
CD4
CD3+CD8+
CD3+CD4+
(b)
Number
Channels (PE-A)
(c)
% proliferating CD4+ T cells
pre-KTx
12 months post-KTx
(d)
% proliferating CD8+ T cells
(e)
(f)
(g)
% cytokine producing CD4+ T cells
IL2
IFNγ
TNFα
(h)
% cytokine producing CD8+ T cells

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-KTx. 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 [24–26].

In humans, a study with myasthenia gravis patients showed that tacrolimus affects the thymic output of mainly $CD8^+$ naïve T cells [27]. 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 with HCs [27]. A previous study by Nickel *et al.* [28]. reported that the percentages CD31-expressing naïve T cells were unchanged post-KTx under standard triple or quadruple IS. However, in contrast to their findings pre-KTx [28], we and others consistently observe a negative effect of uremia on thymic function using both CD31-expressing naïve T cells and TREC content as read-out of thymic output [10]. Here, we observed a stable number of $CD31^+$ naïve T cells and an unchanged TREC content 12 months post-KTx. 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 [29]. One year post-KTx, the T-cell system has returned to the pre-KTx 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 [22,30].

The question remains why immunological aging does not reverse even when adequate graft function is achieved post-KTx. 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 [27,31]. However, a more likely explanation might lie at the epigenetic level. Normal aging is associated with epigenetic changes in hematopoietic stem cells (HSC) resulting in a shift in the balance toward myeloid precursors at the expense of the lymphoid ones [32,33]. 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 with their chronological age [34]. 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 [35].

The persistence of the aged T-cell system post-KTx has several clinical implications as it may increase the risk for infections and malignancies in KTx recipients. For instance, T-cell lymphopenia has been associated with a high risk for infections and malignancies post-KTx [36,37]. Furthermore, the presence of highly differentiated $CD4^+$ T cells lacking CD28 is associated with the risk for a cardiovascular event post-KTx [5–7]. A potential benefit of a relatively unchanged aged T-cell system post-KTx 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 [38–40].

In addition to the unaltered T-cell aging parameters, the T-cell function did also not improve post-KTx. Although we did not have the impression that T-cell function

Figure 5 Proliferative capacity and percentages of cytokine-producing T cells upon T-cell receptor (TCR)-triggering. A typical gating strategy for flow cytometric 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-kidney transplantation (KTx) and at 12 months post-KTx. 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-KTx and 12 months afterward are shown (open symbols, $n = 10$, mean $\pm$ 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-KTx are shown (open symbols, $n = 10$, mean $\pm$ SEM).

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improved following KTx 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 aging is not reversed by KTx and therefore remains a determinant of immune deficiency independent of graft function. Thus, ESRD induces an irreversible imprint on the immune system.

Authorship

RM: performed the experiments, statistical analysis and wrote the manuscript. NL and MB: designed the study and wrote the manuscript. EdW: performed some of the experiments. AL and CB: contributed in writing the manuscript.

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