Lymph node and circulating T cell characteristics are strongly correlated in end-stage renal disease patients, but highly differentiated T cells reside within the circulation

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Summary

Ageing is associated with changes in the peripheral T cell immune system, which can be influenced significantly by latent cytomegalovirus (CMV) infection. To what extent changes in circulating T cell populations correlate with T cell composition of the lymph node (LN) is unclear, but is crucial for a comprehensive understanding of the T cell system. T cells from peripheral blood (PB) and LN of end-stage renal disease patients were analysed for frequency of recent thymic emigrants using CD31 expression and T cell receptor excision circle content, relative telomere length and expression of differentiation markers. Compared with PB, LN contained relatively more CD4$^+$ than CD8$^+$ T cells ($P < 0.001$). The percentage of naive and central memory CD4$^+$ and CD8$^+$ T cells and thymic output parameters showed a strong linear correlation between PB and LN. Highly differentiated CD28^{null} T cells, being CD27$^-$, CD57$^+$ or programmed death 1 (PD-1$^+$), were found almost exclusively in the circulation but not in LN. An age-related decline in naive CD4$^+$ and CD8$^+$ T cell frequency was observed ($P = 0.035$ and $P = 0.002$, respectively) within LN, concomitant with an increase in central memory CD8$^+$ T cells ($P = 0.033$). Latent CMV infection increased dramatically the frequency of circulating terminally differentiated T cells, but did not alter T cell composition and ageing parameters of LN significantly. Overall T cell composition and measures of thymic function in PB and LN are correlated strongly. However, highly differentiated CD28^{null} T cells, which may comprise a large part of circulating T cells in CMV-seropositive individuals, are found almost exclusively within the circulation.

Keywords: ageing, end-stage renal disease, lymph node, peripheral blood, T cells

Introduction

One of the dominant aspects of the ageing process in the T cell immune system is a decreased thymic output of newly formed T cells, named recent thymic emigrants (RTE). This is evident from a decrease in circulating naive T cells expressing CD31, and by a decrease in T cell receptor excision circles (TREC) content [1–5]. Furthermore, an increase in age leads to a decline in the frequency of circulating naive T cells, while the memory T cell compartment may contain more terminally differentiated T cells [6,7]. This progressive T cell differentiation is also marked by the loss of the co-stimulatory molecule CD28 on the surface of memory T cells [8], which enables these cells to become activated without the need for co-stimulation. Next to the loss of CD28, the loss of CD27 and the expression of CD57 and programmed death 1 (PD-1) are indicators of terminal T cell differentiation [9–11]. Lastly, the increased number of rounds of T cell proliferation and differentiation reduces the telomere length of T cells [12,13].

Cytomegalovirus (CMV) infection needs to be taken into account when the T cell compartment is analysed, as this infection may cause a significant imprint on the differentiation status of circulating T cells [8,14]. In general, it leads to an expansion of late-differentiated CD4$^+$ and CD8$^+$ T cells and an inverted CD4 : CD8 T cell ratio [15]. Furthermore, CMV infection may lead to a reduction in telomere length of T cells [16].

The impact of an aged circulating T cell population has been studied, for example, in relation to the risk for infection, decreased vaccination response and cardiovascular events [17–19]. In addition, the presence of highly differentiated CD28^{null} T cells in the peripheral blood has gained considerable interest in the field of solid organ transplantation, as they may be instrumental in co-stimulatory blockade-resistant rejection and long-term allograft (dys)function [17,20–23]. Analysis of the T cell population in lymph nodes (LNs) might prove to be of additional/higher value, as immune responses are initiated in secondary lymphoid organs. Until now, little is known about how changes in circulatory T cells relate to changes in the LN, and only a few studies have investigated the effect of ageing in secondary lymphoid organs [24,25].

In this study, we investigated the relation between T cell parameters of the LN and PB compartments in end-stage renal disease (ESRD) patients. More specifically, ageing parameters such as thymic output, relative telomere length (RTL), terminal differentiation markers and the influence of CMV infection on these were studied. Our results show that overall T cell composition and measures of thymic function are correlated strongly between PB and LN. However, highly differentiated CD28^{null} T cells, which may comprise a large part of circulating T cells in CMV-seropositive individuals, are virtually absent in LNs.

Materials and methods

Study population

Patients prior to kidney transplantation in the period from August 2015 to March 2016 were eligible for participation. All patients gave written informed consent to participate in this study. The study was approved by the Medical Ethical Committee of the Erasmus MC (MEC number 2015-301) and was conducted in accordance with the Declaration of Helsinki and the Declaration of Istanbul.

Clinical variables were assessed as shown in Table 1, including age, gender, CMV-seropositivity, underlying cause of renal failure, dialysis prior to transplantation and dialysis vintage (defined as time on dialysis since the start of any type of dialysis).

PBMC isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples by using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). Blood was obtained from renal transplant recipients 1 day prior to renal transplantation (RT). The isolated PBMCs were stored at −150°C with a minimum amount of 10 × 10^6 cells per vial. The median frequency of lymphocytes within heparinized blood amounted to 21·9% (17·7–26·6%) and

Table 1. Patient characteristics

	RT patients ($n = 38$)
Age	58 (45–65)
Male	24 (63%)
CMV-seropositivity	28 (74%)
Underlying disease	
Nephrosclerosis/atherosclerosis/ hypertension	11 (29%)
Primary glomerulopathies	5 (13%)
Diabetes	7 (18%)
Urinary tract infections/stones	0 (0%)
Reflux nephropathy	0 (0%)
Polycystic kidney disease	8 (21%)
Other	3 (8%)
Unknown	4 (11%)
Dialysis prior to transplantation	23 (61%)
Dialysis vintage (years)	2.4 (0·9–3·3)

Data are presented as median (interquartile range) or as number (percentage). RT = renal transplantation; CMV = cytomegalovirus.

following density gradient centrifugation this frequency amounted to 72·6% (64·7–76·7%).

LNMC isolation

Iliac LNs were isolated from tissue, which was obtained as waste material from renal transplant recipients at the time of implantation of the renal allograft. Upon arrival of the material at the laboratory, excessive fat tissue around the LNs was removed. Next, the LNs were minced and subsequently mashed through a 70-μm sieve to obtain a cell suspension. Then, the cell suspension was centrifuged several times to remove platelets and remaining fat tissue. The isolated lymph node mononuclear cells (LNMCs) were stored at −150°C with a minimum amount of 10 × 10^6 cells per vial. The median yield of a lymph node was 20 × 10^6 cells. The median frequency of lymphocytes within LNMNCs amounted to 94·8% (92·0–96·3%).

T cell differentiation status and absolute numbers of T cell subsets

T cell differentiation status was determined through whole blood staining and on freshly isolated LNMCs, which was based on the study by Sallusto *et al.* [26], as described in detail previously [27]. These cell samples were measured on the fluorescence activated cell sorter (FACS)Canto II (BD Biosciences, San Jose, CA, USA) and analysed with FACS Diva software version 6.1.2 (BD Biosciences). Briefly, differential cell surface expression of CD45RO (pan-T cell marker for memory T cells) and CCR7 (a chemokine receptor involved in homing of T cells to various secondary lymphoid organs such as lymph nodes) was used to distinguish naive from different memory T cell populations. Memory T cells differentiate into central memory (CM) T cells (CD45RO$^+$CCR7$^+$ T cells, able to access the lymphoid

tissue), effector memory (EM) T cells (CD45RO$^+$CCR7$^-$ T cells) and EM CD45RA$^+$ (EMRA) T cells (highly differentiated T cells re-expressing CD45RA but CCR7$^-$) [26]. A typical example of the gating strategy is shown in Supporting information, Fig. S1.

In addition to this definition of memory T cells, the expression of CD28, CD27, CD57 and PD-1 was used to divide the T cell compartment in early or late differentiated T cells. A typical example of the gating strategy is shown in Supporting information, Fig. S2. Essentially, the naive and CM T cells are early differentiated T cells and still express the co-stimulatory molecules CD28 and CD27. EM and EMRA T cells may progressively lose CD28 and subsequently CD27 expression and gain expression of CD57 and PD-1 [9–11,28]. Next to this, examining expression of CD28 together with CD27 is of additional value, as the order of loss of these molecules differs between CD4$^+$ and CD8$^+$ T cells (i.e. CD4$^+$ T cells first lose CD27 before losing CD28, while for the CD8$^+$ T cells this is the other way around) [29–31]. Early differentiated T cells were defined as CD28$^+$CD27$^+$, CD28$^+$PD-1$^-$ and CD28$^+$CD57$^-$ T cells. T cells that were CD28^{null}CD27$^-$, CD28^{null}PD-1$^+$ and CD28^{null}CD57$^+$ were defined as late differentiated memory T cells. This measurement was performed on the Navios flow cytometer (Beckman Coulter, Brea, CA, USA) and the obtained data were analysed with Kaluza software version 1.3 (Beckman Coulter). The T cells were analysed with DuraClone IM T cell subsets tubes (Beckman Coulter), which were coated with allophycocyanin (SAPC) Alexa Fluor 750 (APC-A750) anti-CD3, APC-labelled anti-CD4, Alexa Fluor 700 (A700)-labelled anti-CD8, fluorescein isothiocyanate (FITC)-labelled anti-CD45RA, phycoerythrin (PE)-labelled anti-CCR7, phycoerythrin Texas red (ECD)-labelled anti-CD28, phycoerythrin-cyanine 7 (PE-Cy7)-labelled anti-CD27, phycoerythrin-cyanine 5.5 (PE-Cy5.5-labelled anti-PD1, pacific blue (PacB)-labelled anti-CD57 and krome orange (KO)-labelled anti-CD45.

Relative telomere length

To determine the RTL of CD4$^+$ and CD8$^+$ T cells, flow fluorescence in-situ hybridization was performed on thawed PBMCs and LNMCs, as described in detail previously [27].

Assesment of recent thymic emigrants using CD31 and TREC content

CD31$^+$ naive T cells were assessed by flow cytometry as a measure of recent thymic emigrants (RTE), as decribed previously [32]. TREC content was determined using 1 × 10^6 snap-frozen PBMCs and LNMCs. DNA was isolated from these snap-frozen samples and the TREC content, depicted by ΔCT (which is related inversely to the TREC content), was determined using quantitative polymerase chain reaction (PCR) as described previously [33].

Statistical analysis

All variables are presented as medians with interquartile ranges. The differences between paired samples (i.e. PB and LN T cell ageing parameters of the same ESRD patients) were analysed using the Wilcoxon signed-rank test. Differences between continuous variables from two independent groups (i.e. CMV-seropositive versus CMV-seronegative ESRD patients) were assessed with the Mann–Whitney U-test. Correlations between PB and LN were calculated using Spearman's rank correlation. Relationships between age and the different T cell ageing parameters were assessed using a linear regression analysis. The significance level (P-value) was two-tailed and an α of 0·05 was used for all analyses. Statistical analyses were performed using SPSS version 21.0 for Windows® (SPSS Inc., Chicago, IL, USA) and GraphPad Prism version 5 (San Diego, CA, USA). Figures were created with GraphPad Prism 5.

Results

Patient characteristics

Patient characteristics ($n = 38$) are shown in Table 1. The median patient age was 58 years. Most of the patients were CMV immunoglobulin (Ig)G$^+$ (74%). The major cause of ESRD was nephrosclerosis/atherosclerosis/hypertension (29%), followed by polycystic kidney disease (21%), which together accounted for half the cases.

CD4$^+$ T cell composition of the lymph node and peripheral blood

The median frequency of CD4$^+$ T cells was significantly higher in LN samples compared with the PB ($P < 0·001$) (Fig. 1a), but the relative distribution of CD4$^+$ naive T cells was similar (Fig. 1b). However, compared with PB, the LN contained higher frequencies of CD4$^+$ CM T cells ($P < 0·001$) (Fig. 1c) and lower frequencies of CD4$^+$ EM T cells and EM CD45RA$^+$ (EMRA) T cells ($P < 0·001$ and $P = 0·026$, respectively) (Fig. 1d,e). Except for the frequency of CD4$^+$ EMRA T cells, frequencies of CD4$^+$ T cells, CD4$^+$ naive T cells, CD4$^+$ CM T cells and CD4$^+$ EM T cells showed a remarkably good correlation between PB and LN (Fig. 1).

Next, the early versus late T cell differentiation was analysed by measuring the expression of CD27, PD-1 and CD57 on CD28$^+$ and CD28^{null} T cells (Table 2). Within the CD4$^+$ T cell population, the frequency of CD28^{null} T cells was significantly lower in LN compared with PB (1·0%, range 0·6–1·6% versus 1·8%, range 0·7–8·8; $P = 0·001$) and late memory T cell subpopulations, either defined as CD28^{null}CD27$^-$ T cells, CD28^{null}PD-1$^+$ T cells or CD28^{null}CD57$^+$ T cells, were virtually absent in LN ($P < 0·001$ for all comparisons). Instead, the percentage of CD4$^+$ T cells with full expression of CD28 and CD27
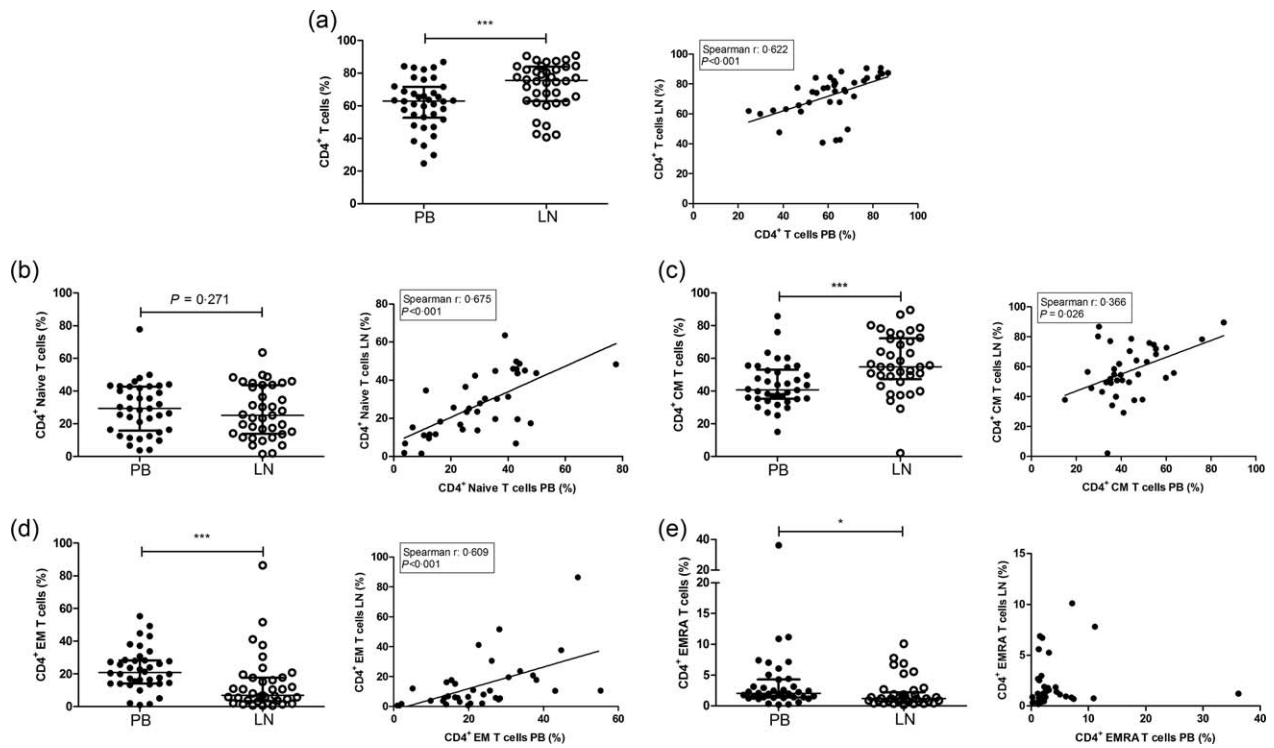
Fig. 1. CD4$^+$ T cell subsets in the peripheral blood (PB) and the lymph node (LN) and the correlation between PB and LN. The frequencies and correlations of (a) CD4$^+$ T cells within CD3$^+$ T cells, (b) CD4$^+$ naive T cells within CD4$^+$ T cells, (c) CD4$^+$ central memory (CM) T cells within CD4$^+$ T cells, (d) CD4$^+$ effector memory (EM) T cells within CD4$^+$ T cells and (e) CD4$^+$ effector memory CD45RA$^+$ (EMRA) T cells within CD4$^+$ T cells are represented. Frequencies of cells are depicted as individual percentages as well as median and interquartile range (left plots). A Spearman's rho was shown only when a significant correlation was present; $n = 38$ for peripheral blood and $n = 38/37$ for lymph node samples. Significant differences were calculated and shown (*$P < 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).

(CD28$^+$CD27$^+$) was significantly higher in the LN (Table 2).

In summary, the composition of the CD4$^+$ T cell compartment in PB and LN are highly inter-related, except for the absence of late differentiated T cells, which almost exclusively appear in PB.

CD8$^+$ T cell composition of the lymph node and peripheral blood

The frequency of CD8$^+$ T cells was significantly lower in LN than in PB with a relative higher proportion of naive T cells ($P < 0.001$ for both variables) (Fig. 2a,b). The CD8$^+$ memory T cell population in LN contained relatively more CM T cells ($P < 0.001$) (Fig. 2c) and fewer highly differentiated CD8$^+$ EMRA T cells ($P < 0.001$) (Fig. 2e).

All CD8$^+$ T cell subsets, except for EM T cells, were correlated positively between the two different compartments (Fig. 2). Specifically, the frequency of total CD8$^+$ T cells, CD8$^+$ naive T cells and CD8$^+$ CM T cells showed a very close correlation.

Differentiated CD8$^+$ CD28^{null} T cells were significantly less frequent in LN than in PB (10·6%, range 7·5–15·0% *versus* 41·5%, range 23·8–64·0%; $P < 0.001$).

The expression of CD27, PD-1 and CD57 in relation to CD28 expression showed similar results within the CD8$^+$ T cell population as those obtained for the CD4$^+$ T cells (Table 2). The frequencies of late differentiated T cells

Table 2. CD27, programmed death 1 (PD-1) and CD57 expression on CD28^{null} T cells in the peripheral blood and lymph node

	PB ($n = 38$)	LN ($n = 33$)	P
CD4$^+$ T cells			
CD28$^+$CD27$^+$ (%)	87·4 (81·5–94·3)	93·8 (91·6–96·1)	**< 0·001**
CD28^{null}CD27$^-$ (%)	1·1 (0·2–7·5)	0·3 (0·1–0·6)	**< 0·001**
CD28$^+$PD–1$^-$ (%)	78·5 (71·0–84·0)	74·2 (65·8–81·6)	0·077
CD28^{null}PD–1$^+$ (%)	1·0 (0·3–7·3)	0·4 (0·3–0·9)	**0·004**
CD28$^+$CD57$^-$ (%)	96·7 (90·1–98·7)	97·4 (96·0–98·2)	0·063
CD28^{null}CD57$^+$ (%)	0·8 (0·1–5·9)	0·1 (0·0–0·2)	**< 0·001**
CD8$^+$ T cells			
CD28$^+$CD27$^+$ (%)	54·8 (36·7–73·7)	89·8 (84·1–92·6)	**< 0·001**
CD28^{null}CD27$^-$ (%)	28·7 (10·8–49·3)	1·9 (1·0–2·6)	**< 0·001**
CD28$^+$PD–1$^-$ (%)	43·1 (25·9–57·5)	63·2 (49·5–75·1)	**< 0·001**
CD28^{null}PD–1$^+$ (%)	12·4 (6·9–18·4)	2·4 (1·6–5·1)	**< 0·001**
CD28$^+$CD57$^-$ (%)	56·2 (37·5–73·9)	87·3 (80·2–89·7)	**< 0·001**
CD28^{null}CD57$^+$ (%)	26·6 (13·4–39·1)	1·5 (1·0–2·3)	**< 0·001**

Data are presented as medians (interquartile range). PB = peripheral blood; LN = lymph node.
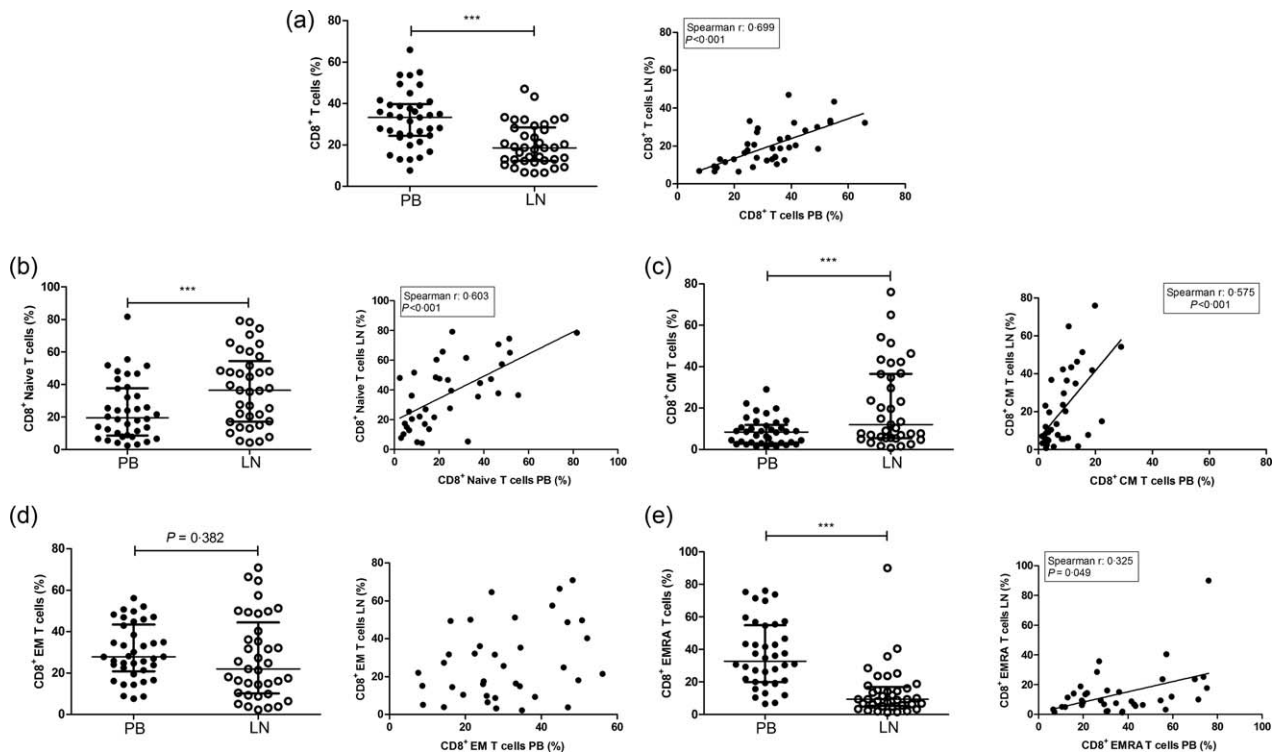
Fig. 2. CD8^{+} T cell subsets in the peripheral blood (PB) and the lymph node (LN) and the correlation between PB and LN. The frequencies and correlations of (a) CD8^{+} T cells within CD3^{+} T cells, (b) CD8^{+} naive T cells within CD8^{+} T cells, (c) CD8^{+} central memory (CM) T cells within CD8^{+} T cells, (d) CD8^{+} effector memory (EM) T cells within CD8^{+} T cells and (e) CD8^{+} effector memory CD45RA^{+} (EMRA) T cells within CD8^{+} T cells are represented. Frequencies of cells are depicted as individual percentages as well as median and interquartile range (left plots). A Spearman's rho was shown only when a significant correlation was present; $n = 38$ for peripheral blood and $n = 38/37$ for lymph node samples. Significant differences were calculated and shown (*$P < 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).

(CD8^{+}CD28[null] T cells lacking CD27^{−}, expressing PD-1 or CD57) were high in PB, but few of these cells were found in LN ($P < 0.001$). In contrast, LN contained significantly more T cells expressing CD28 and CD27.

In summary, similar to the CD4^{+} T cells, the composition of the CD8^{+} T cell compartment of PB and LN was highly inter-related, but late differentiated CD8^{+}CD28[null] T cells were confined to the circulating CD8^{+} T cell pool.

Recent thymic emigrants and RTL in the lymph node and peripheral blood

Frequencies of CD31^{+} naive T cells were significantly lower in LN than in PB for both CD4^{+} and CD8^{+} T cells ($P < 0.001$ and $P = 0.008$, respectively) (Fig. 3,b). Frequencies of CD31^{+}CD4^{+} and CD31^{+}CD8^{+} naive T cells in PB and LN were correlated highly ($P < 0.001$ for both variables) (Fig. 3a,b). Furthermore, the TREC content in the lymphocyte population, which is another measure of the number of RTE, was significantly higher (i.e. lower ΔCT) in the LN ($P = 0.002$) (Fig. 3c) compared with PB. The TREC content also showed a significant positive correlation between PB and LN (Fig. 3c). Combining both parameters, a significant negative correlation between the frequency of

CD31^{+}CD4^{+} and CD8^{+} naive T cells and ΔCT in both the PB and LN was observed (Supporting information, Fig. S3). Interestingly, the RTL of both CD4^{+} as well as CD8^{+} T cells was similar between PB and LN (Fig. 3d,e).

Age-related changes of T cell characteristics in the lymph node

Figure 4 shows the T cell characteristics that were associated significantly with age. A negative association between age and frequency of CD4^{+} naive T cells was observed (B = −0·42, $P = 0.035$) (Fig. 4a), while the frequencies of CD4^{+} memory T cells increased with age (B = 0·42, $P = 0.035$) (Fig. 4b). The expression of CD27, PD-1 and CD57 in combination with CD28 expression within the CD4^{+} T cell population did not show any associations with age (data not shown).

Similarly, frequencies of naïve CD8^{+} T cells were associated negatively with age (B = −0.82, $P = 0.002$) (Fig. 4c), whereas those of CD8^{+} memory T cells revealed a positive association with age (B = 0·59, $P = 0.023$) (Fig. 4d). Next to this, also the frequency of CD8^{+} CM T cells was associated positively with age (B = 0.40, $P = 0.033$) (Fig. 4e). Within the CD8^{+} T cell population, frequencies of early
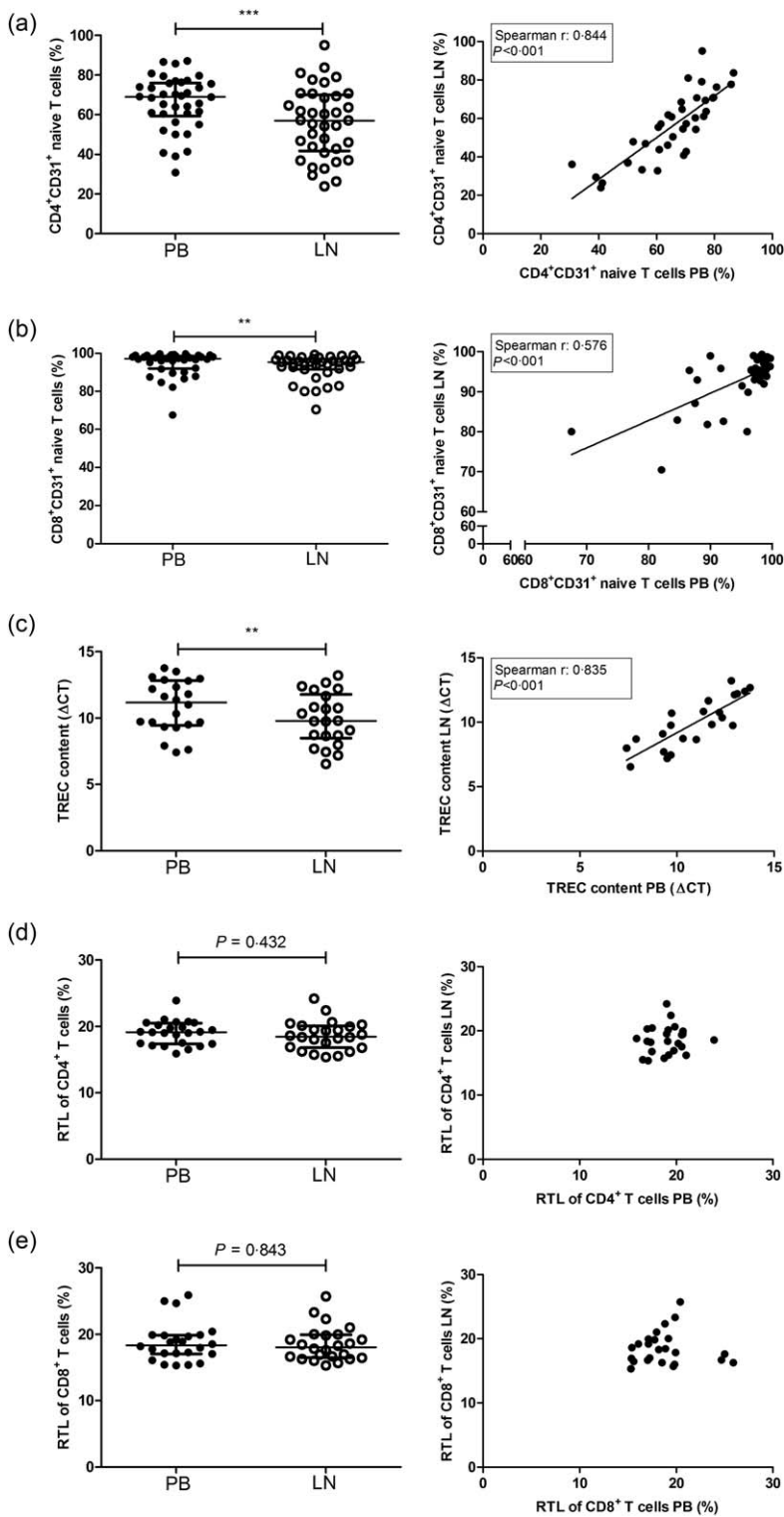
Fig. 3. CD31^{+} naive T cell frequency, T cell receptor excision circles (TREC) content and relative telomere length (RTL) in the peripheral blood (PB) and lymph node (LN) with correlations. The frequencies and correlations of (a) CD4^{+}CD31^{+} naive T cells, (b) CD8^{+}CD31^{+} naive T cells, (c) TREC content, (d) CD4^{+} RTL and (e) CD8^{+} RTL for both the peripheral blood and the lymph node are represented. Frequencies of CD4^{+} and CD8^{+}CD31^{+} naive T cells and RTL are depicted as percentages as well as median and interquartile range. The ΔCT is related inversely to the TREC content; $n = 38$ for peripheral blood and $n = 37$ for lymph node samples with regard to the analysis of CD4^{+}CD31^{+} naive T cells and CD8^{+}CD31^{+} naive T cells; $n = 22$ for analysis of the TREC content and $n = 24$ for the analysis of RTL in both T cell subsets. Significant differences were calculated and shown (*$P < 0.05$; **$P \leq 0.1$; ***$P \leq 0.001$).

differentiated T cells, i.e. CD28^{+}CD27^{+} and CD28^{+}PD1^{−} T cells were decreased with increasing age (B = −0·20, $P = 0.028$ and B = −0·50, $P = 0.043$, respectively).

Assessment of thymic output also revealed age-associated changes within the LN. Frequencies of CD4^{+} RTE decreased with age (B = −0·62, $P = 0.004$) (Fig. 4f), as did the TREC content (i.e. higher ΔCT) (B = 0·09, $O = 0.003$) (Fig. 4g). Conversely, the RTL of both the CD4^{+} and the CD8^{+} T cells in the LN showed no association with age (data not shown).
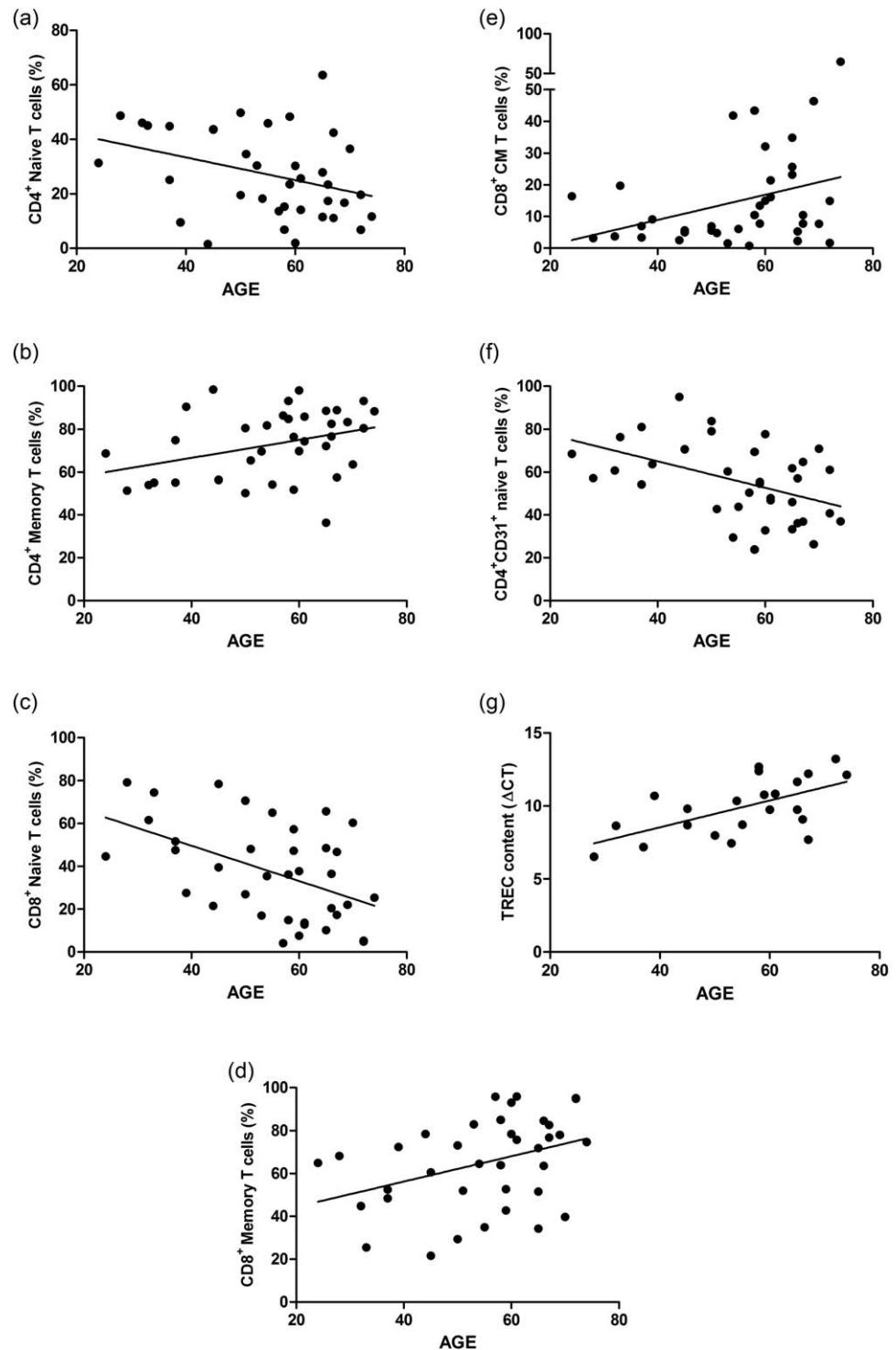
Fig. 4. Effect of age on T cell ageing parameters in the lymph node (LN). Linear regression analysis between age and (a) $CD4^+$ naive T cells, (b) $CD4^+$ memory T cells, (c) $CD8^+$ naive T cells, (d) $CD8^+$ memory T cells, (e) $CD8^+$ central memory (CM) T cells and (f) $CD4^+CD31^+$ naive T cells and (g) T cell receptor excision circles (TREC) content are represented. Frequencies of cells are depicted with percentages and the TREC content is depicted with ΔCT. Only significant associations are shown.

CMV infection does not change lymph node T cell characteristics

In accordance with previous studies [8,14,15], CMV infection altered the peripheral T cell compartment leading to relatively more $CD8^+$ T cells, as well as increased frequencies of $CD4^+CD28^{null}$ and $CD8^+CD28^{null}$ T cells showing decreased expression of CD27 and increased expression of PD-1 and CD57 (Tables 3 and 4).

However, CMV infection did not affect the composition of $CD4^+$ (Table 3) and $CD8^+$ T cells (Table 4) within LN.

In addition, no association was seen between CMV infection and thymic output parameters in LN, with the

Table 3. CD4$^+$ T cell differentiation status in lymph node and peripheral blood for CMV$^+$ and CMV$^-$ patients

	n	CMV$^+$	n	CMV$^-$	P
Lymph node					
CD4$^+$ T cells (%)	28	74·3 (62·5–80·1)	10	83·0 (68·6–87·0)	0·094
CD4$^+$ naive T cells (%)	27	30·2 (13·6–43·7)	10	18·9 (12·3–30·7)	0·313
CD4$^+$ memory T cells (%)	27	69·8 (56·3–86·4)	10	81·1 (69·3–87·7)	0·313
CD4$^+$ CM T cells (%)	27	54·8 (43·1–68·3)	10	63·8 (49·5–76·5)	0·383
CD4$^+$ EM T cells (%)	27	10·5 (4·6–17·8)	10	4·7 (1·9–22·4)	0·494
CD4$^+$ EMRA T cells (%)	27	1·1 (0·8–1·9)	10	1·8 (0·6–2·6)	0·620
CD4$^+$CD28^{null} T cells (%)	27	1·1 (0·7–1·8)	10	0·7 (0·3–1·1)	0·078
CD4$^+$CD28$^+$CD27$^+$ (%)	23	93·9 (92·2–97·3)	10	92·4 (89·9–96·0)	0·248
CD4$^+$CD28^{null}CD27$^-$ (%)	23	0·3 (0·1–0·6)	10	0·4 (0·1–0·5)	0·739
CD4$^+$CD28$^+$PD–1$^-$ (%)	23	73·9 (66·8–78·2)	10	79·7 (64·4–84·6)	0·281
CD4$^+$CD28^{null}PD–1$^+$ (%)	23	0·4 (0·3–1·0)	10	0·6 (0·3–0·9)	0·681
CD4$^+$CD28$^+$CD57$^-$ (%)	23	97·1 (96·6–98·3)	10	97·6 (93·4–98·2)	0·652
CD4$^+$CD28^{null}CD57$^+$ (%)	23	0·1 (0·0–0·2)	10	0·0 (0·0–0·2)	0·246
Peripheral blood					
CD4$^+$ T cells (%)	28	60·3 (48·8–64·8)	10	76·7 (68·5–83·7)	**< 0·001**
CD4$^+$ naive T cells (%)	28	28·9 (13·1–42·2)	10	32·4 (19·9–42·8)	0·703
CD4$^+$ memory T cells (%)	28	71·1 (57·8–86·9)	10	67·6 (57·2–80·2)	0·703
CD4$^+$ CM T cells (%)	28	39·5 (35·3–50·9)	10	44·0 (33·8–54·8)	0·655
CD4$^+$ EM T cells (%)	28	22·5 (15·7–30·1)	10	17·3 (13·8–25·1)	0·380
CD4$^+$ EMRA T cells (%)	28	2·0 (1·3–4·9)	10	2·1 (1·4–3·3)	0·934
CD4$^+$CD28^{null} T cells (%)	28	5·6 (1·3–11·5)	10	0·6 (0·3–0·8)	**< 0·001**
CD4$^+$CD28$^+$CD27$^+$ (%)	28	83·9 (77·8–92·7)	10	94·2 (89·7–9·5)	**0·012**
CD4$^+$CD28^{null}CD27$^-$ (%)	28	3·9 (0·7–9·2)	10	0·1 (0·0–0·2)	**< 0·001**
CD4$^+$CD28$^+$PD–1$^-$ (%)	28	77·7 (71·0–82·8)	10	81·4 (71·1–89·0)	0·214
CD4$^+$CD28^{null}PD–1$^+$ (%)	28	2·3 (0·6–9·0)	10	0·1 (0·0–0·3)	**< 0·001**
CD4$^+$CD28$^+$CD57$^-$ (%)	28	93·9 (88·7–97·4)	10	98·9 (97·8–99·4)	**< 0·001**
CD4$^+$CD28^{null}CD57$^+$ (%)	28	2·7 (0·6–6·5)	10	0·0 (0·0–0·2)	**< 0·001**

Data are presented as medians (interquartile range). CMV = cytomegalovirus; CM = central memory; EM = effector memory; EMRA = effector memory CD45RA$^+$.

frequencies of CD4$^+$CD31$^+$ and CD8$^+$CD31$^+$ naive T cells and the TREC content being similar between CMV$^+$ and CMV$^-$ patients (data not shown). Similarly, no effect of CMV on RTL of CD4$^+$ and CD8$^+$ T cells within LN was observed (data not shown).

In summary, despite a major effect of CMV infection on peripheral blood T cell composition, this was not paralleled by similar effects within LN.

Discussion

In this study we investigated whether the ageing parameters of the circulating T cell population, correlates with the T cell characteristics of LN and the effect of CMV on PB and LN composition. Our main conclusions are that the T cell composition and measures of thymic function in PB and LN are inter-related, but that the highly differentiated CD28^{null} T cells, which may comprise a large part of circulating T cells in CMV-seropositive individuals, are found almost exclusively within the circulation. This study is the first in which a pairwise and a more in depth comparison was performed between PB and LN for different T cell ageing parameters in ESRD patients. Next to the different T

cell subsets, we included analyses of other differentiation markers such as CD27, PD-1 and CD57 in combination with CD28. Furthermore, we also analysed the TREC content and RTL of the T cells in both the lymph node and the peripheral blood.

In previous studies it was shown that the PB compartment of ESRD patients displayed distinct changes that resemble the T cell ageing process in elderly healthy individuals. It is associated with loss of naive T cells, an inverted CD4 : CD8 ratio and an increase in highly differentiated T cells, lacking the co-stimulatory molecule CD28 [27,33–36]. The T cell composition of LNs of the same patients showed a higher frequency of CD4$^+$ T cells and a lower frequency of CD8$^+$ T cells compared with PB. The high frequency of CD4$^+$ T cells was also found in the lymphoid tissues of deceased paediatric and young adult organ donors in a study by Thome *et al.* [37]. Furthermore, there was a lower frequency of highly differentiated T cells within LN samples in contrast to PB. This finding was supported further by the other T cell memory differentiation markers (CD28, CD27, PD-1 and CD57). It is known that CD4$^+$ CD28^{null} are more differentiated than CD8$^+$ CD28^{null}, as the latter can still contain CD27 in contrast to

Table 4. CD8$^+$ T cell differentiation status in lymph node and peripheral blood for CMV$^+$ and CMV$^-$ patients

	n	CMV$^+$	n	CMV$^-$	P
Lymph node					
CD8$^+$ T cells (%)	28	19·0 (13·2–29·5)	10	13·0 (8·7–22·9)	0·159
CD8$^+$ naive T cells (%)	27	39·5 (20·5–51·6)	10	28·5 (11·0–58·4)	0·281
CD8$^+$ memory T cells (%)	27	60·5 (48·4–79·6)	10	71·5 (41·6–89·1)	0·281
CD8$^+$ CM T cells (%)	27	12·0 (5·6–36·5)	10	11·3 (4·6–44·2)	0·959
CD8$^+$ EM T cells (%)	27	21·9 (9·9–36·1)	10	26·5 (14·3–53·7)	0·442
CD8$^+$ EMRA T cells (%)	27	8·8 (5·0–17·7)	10	12·7 (7·4–19·2)	0·330
CD8$^+$CD28^{null} T cells (%)	27	10·7 (7·4–16·7)	10	9·1 (6·8–13·4)	0·365
CD8$^+$CD28$^+$CD27$^+$ (%)	23	90·9 (83·3–92·7)	10	86·5 (84·1–91·4)	0·357
CD8$^+$CD28^{null}CD27$^-$ (%)	23	1·9 (1·2–3·4)	10	1·4 (0·8–2·5)	0·240
CD8$^+$CD28$^+$PD–1$^-$ (%)	23	63·2 (50·2–74·8)	10	63·7 (46·4–76·0)	0·953
CD8$^+$CD28^{null}PD–1$^+$ (%)	23	2·2 (1·5–5·4)	10	3·5 (2·1–5·3)	0·240
CD8$^+$CD28$^+$CD57$^-$ (%)	23	87·3 (81·6–90·1)	10	87·4 (73·6–89·3)	0·681
CD8$^+$CD28^{null}CD57$^+$ (%)	23	1·6 (1·1–2·7)	10	1·3 (1·0–1·8)	0·248
Peripheral blood					
CD8$^+$ T cells (%)	28	35·5 (27·9–41·4)	10	18·3 (13·1–27·3)	**0·002**
CD8$^+$ naive T cells (%)	27	18·7 (7·7–25·8)	10	32·5 (14·2–49·0)	0·123
CD8$^+$ memory T cells (%)	27	81·3 (74·2–92·2)	10	67·5 (51·0–85·8)	0·123
CD8$^+$ CM T cells (%)	27	5·9 (2·6–9·4)	10	14·7 (7·5–19·7)	**0·006**
CD8$^+$ EM T cells (%)	27	27·0 (16·3–37·5)	10	27·9 (24·8–49·2)	0·159
CD8$^+$ EMRA T cells (%)	27	42·2 (29·6–57·1)	10	19·7 (12·8–26·4)	**< 0·001**
CD8$^+$CD28^{null} T cells (%)	27	47·0 (35·8–69·1)	10	22·2 (15·1–32·2)	**< 0·001**
CD8$^+$CD28$^+$CD27$^+$ (%)	28	47·9 (29·5–62·1)	10	76·1 (70·3–85·0)	**< 0·001**
CD8$^+$CD28^{null}CD27$^-$ (%)	28	35·6 (26·6–57·9)	10	7·4 (3·7–11·0)	**< 0·001**
CD8$^+$CD28$^+$PD–1$^-$ (%)	28	38·7 (24·8–49·2)	10	57·8 (40·6–69·9)	**0·026**
CD8$^+$CD28^{null}PD–1$^+$ (%)	28	13·1 (6·9–18·7)	10	10·3 (6·9–19·5)	0·804
CD8$^+$CD28$^+$CD57$^-$ (%)	28	49·7 (30·6–63·2)	10	77·3 (66·9–84·0)	**< 0·001**
CD8$^+$CD28^{null}CD57$^+$ (%)	28	30·5 (21·3–49·1)	10	11·6 (7·9–14·9)	**< 0·001**

Data are presented as medians (interquartile range). CMV = cytomegalovirus; CM = central memory; EM = effector memory; EMRA = effector memory CD45RA$^+$.

the CD4$^+$ T cell fraction [29–31]. However, both the CD8$^+$CD28^{null} T cells as well as the CD8$^+$CD28^{null}CD27$^-$ T cells were more frequent in PB. Even though a positive correlation existed between LN and PB samples for the majority of the different T cell subset frequencies, the highly differentiated T cells (i.e. CD28^{null} T cells) were far more abundant in PB. The low number of highly differentiated T cells in LN might be explained by the lack of co-expression of CCR7, which T cells use for homing to secondary lymphoid organs [21].

The parameters for thymic output showed similar results for both the CD31 and the TREC assay. The results indicate a remarkably high correlation between the data obtained in the circulation and within LN. In addition, CD31$^+$ T cells and TREC content in LN T cell population showed a relation with the age of the individual as we and others have described previously for circulating T cells [3,5,27,33,38]. Taken together, it can be concluded that the loss of thymic function with increasing age is also reflected in the secondary lymphoid tissue. Primary immune responses (e.g. vaccination) are initiated within LN. The intricate relation between the amount of naive T cells in PB and LN

therefore probably explains why the number of circulating naive T cells is associated with the response to vaccination [39].

In this study we did not find any differences regarding the RTL between PB and LN. This could be explained by the depth of the analysis of RTL. We took the CD4$^+$ and CD8$^+$ T cell population as a whole, instead of focusing on the different T cell subsets. Because of this, differences in RTL due to differences in frequencies of subsets might be overlooked. Furthermore, we cannot exclude fully that the isolation procedure resulted in a shift in T cell subsets, possibly influencing the analysis of the RTL.

Age-related changes were observed in both CD4$^+$ and CD8$^+$ T cell compartments within the lymph node. The frequency of naive T cells declined, while the frequency of memory T cells increased. Other studies also showed an age-related decline in naive T cells within LNs of healthy individuals and from deceased organ donors [24,37,40]. Furthermore, a study by Thome *et al.* found that EM T cells were less abundant in paediatric lymphoid tissues, while these were more frequent in young adult lymphoid tissues

[37]. Next to this, the TREC content also decreased with increasing age in the LN, supporting the T cell ageing process in LN. These results indicate that age-related changes in T cell composition are similar for PB and LN, with the exception of the late differentiated T cells, which appear to remain outside the LN.

CMV infection changes the composition of circulating T cells dramatically and almost doubles the total number of CD8^{+} T cells, in particular with a late differentiation phenotype [8,14,15]. However, the T cell differentiation status within the LNs did not differ between CMV^{+} and CMV^{−} patients. In a study by Remmerswaal *et al.*, the frequency of CMV-specific CD8^{+} T cells was significantly lower in the LN compared with the PB, which is in line with our findings [41]. Next to this, a study by Havenith *et al.* also showed the absence of highly differentiated CD4^{+}CD28[null]CD27^{−} T cells in the lymph node, which can appear due to a CMV infection [42]. These findings suggest that a latent CMV infection does not have a significant impact on T cells within LN, while the opposite is true for PB T cells.

In conclusion, the T cell composition in PB is related strongly to T cell composition of the LN, while the presence of highly differentiated CD28[null] T cells is more exclusive to the PB compartment. In addition, CMV changed the T cell differentiation status in PB drastically, while no significant alterations were observed within LN with regard to T cell differentiation and ageing parameters. This study provides a new perspective on the T cell immune system, especially of those with ESRD. PB seems to harbour the majority of terminally differentiated T cells, while LNs do not harbour this specific subset of T cells. These findings suggest that during an immune response the late differentiated T cells access the tissue and exert their immune function directly. This is consistent with the observation that many of these cells carry the chemokine receptor CX3CR1 for the endothelium-derived chemokine fractalkine [41]. Fractalkine is considered a major player in inflammatory responses, recruiting leucocytes into the inflamed tissue [43–45]. These late differentiated T cells then can respond to IL-15 produced by the inflamed parenchyma, proliferate and exert their cytotoxic function [46–48]. Whether the T cell characteristics in the LN might provide a better predictive model for clinical outcomes, such as the development of (co-stimulatory blockade-resistant) rejection and infection after, for example, kidney transplantation is subject to further investigation.

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Author contributions

B. D., N. H. R. L., M. K., W. V., D. R. and L. H. performed experiments; B. D., N. H. R. L. and D. R. analysed results and made the figures; A. E. de W., M. G. H. B. and N. H. R. L. designed the research; B. D., A. E. de W., L. H., A. W. L., F. J. D. M. K., W. V., D. R., C. C. B., N. H. R. L. and M. G. H. B. wrote the paper.

Disclosure

The authors declare no competing financial and commercial interests.

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Supporting information

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

Fig. S1. Gating strategy of the T cell subsets. First, lymphocytes were selected based on the forward- and side-scatter, and CD3$^+$ T cells were selected from these lymphocytes. The CD3$^+$ T cells were then divided further into CD4$^+$ and CD8$^+$ T cell fractions. Each population was dissected into different subsets using CCR7 and CD45RO. After this division, CD4$^+$ (only this is shown) and further CD8$^+$ naive T cells were analysed for CD31 expression. Besides this, total CD4$^+$ (only this is shown) and total CD8$^+$ T cells were analysed for frequencies of cells lacking CD28 expression.

Fig. S2. Gating strategy of the T cell differentiation markers. First, CD45$^+$ leucocytes were gated. From these cells, lymphocytes were selected based on the forward- and side-scatter; CD3$^+$ T cells were selected from these lymphocytes. The CD3$^+$ T cells were then divided further into CD4$^+$ and CD8$^+$ T cell fractions. Each fraction was analysed further for the expression of CD27, programmed death 1 (PD-1) or CD57 in combination with CD28 (only the CD8$^+$ T cell fraction is shown). From these analyses only the least (CD28$^+$CD27$^+$, CD28$^+$PD-1$^-$ and CD28$^+$CD57$^-$) and most differentiated (CD28^{null}CD27$^-$, CD28^{null}PD-1$^+$ and CD28^{null}CD57$^+$) T cells were selected (indicated within the black frames).

Fig. S3. Correlation between CD31$^+$ naive T cells and the T cell receptor excision circles (TREC) content in peripheral blood (PB) and the lymph node. The Spearman's rho correlation analysis is shown between CD4$^+$CD31$^+$ naive T cells within the PB and the TREC content within the PB (a), between CD8$^+$CD31$^+$ naive T cells within the PB and the TREC content within the PB (b), between CD4$^+$CD31$^+$ naive T cells within the LN and the TREC content within the LN (c) and between CD8$^+$CD31$^+$ naive T cells within the LN and the TREC content within the LN (d). Frequencies of cells are depicted on the *y*-axis, and the ΔCT which is related inversely to the TREC content is depicted on the *x*-axis.