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Clinical and immunological features of very long-term survivors with a single renal transplant

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Summary

The aim of this study was to analyze the clinical and immunological features of the 56 still alive patients at our institution harboring a functional first renal transplant since more than 30 years. The mean post-transplant graft survival in all patients was 35.4 ± 3.1 years, the mean serum creatinine concentration was 128.7 ± 7 μmol/l, and the mean urinary protein concentration was 0.6 ± 0.5 g/l. Fifty-one percent of the patients had experienced cancer involving the skin (46.1%) and/or other tissues (28%). Hepatocarcinoma was diagnosed in 11% of the patients with chronic viral hepatitis B and/or C (48%). The 5-year patient survival rate (considered after the 30th transplantation anniversary) was 27% in patients presenting a tumor versus 87% in those tumor-free ($P < 0.0001$). The thymic output, the proportions of the memory and naïve T cell subsets, and the frequencies of EBV- and CMV-reactive, IFN-γ-producing T cells did not differ from those observed in more recently transplanted patients. These results suggest that the impact of chronic immunosuppression on some immune functions does not worsen over time and that the observed high prevalence of cancer in these patients may be related to the synergistic effects of decreased immunosurveillance and the time required for carcinogenesis.

Introduction

Sixty years after the first renal transplantation was performed in a human, considerable progress has been made in the care of patients regarding organ preservation, surgical procedures, intensive care and immunosuppression and in infectious, cardiovascular and cancer disease management. Consequently, a group of transplant patients has emerged that is characterized by patients who received a kidney transplant several decades ago and for which medical care is challenging. Such patients received a kidney transplant at a time when the medical care was very different from current practices regarding donor and recipient selection as well as complications, management and follow-up. These patients constitute a unique population for whom few data have been published regarding the clinical, biological and immunological characteristics [1–3]. The renal transplantation program began at the Necker Hospital in Paris, France, in 1952 [4], which allowed us to select a patient population who still have their first kidney graft that was transplanted at least 30 years ago. The aim of this study was to investigate particular features of this unique population.

Patients and methods

Patients and data collection

The recording of patient data began in January 2008. Patients who received their first and only renal transplant before January 1978 and who were alive at least 30 years after the transplantation were included in this cohort study. Between 1959 and 1978, 499 kidney transplantations

were performed at our institution. Forty patients out of 499 (8%) were identified as lost of follow-up and 226 (45%) patients returned to dialysis (Fig. 1). Graft and patient survival of the whole population transplanted before January 1978 are showed in Fig. S1. Among this population, 56 patients who were still alive and who had received their kidney transplant before January 1978, who will be hereafter referred to as Tx30 patients, were included and evaluated. The mean time from transplantation was 35.4 ± 3.1 years (minimum: 30 years; maximum: 42.5 years). Five out of 56 (9%) Tx30 patients changed the transplant center during the follow-up.

The clinical, histological and biological data regarding the donors and recipients at the time of transplantation and 30 years afterwards were individually collected from the medical records. Anti HLA antibodies were tested with the Luminex® technique. In addition, the immune parameters of thirteen Tx30 patients were evaluated during the last visit at our institution, between 2009 and 2010. The control group subjects were randomly selected between August 2009 and August 2010 for a transversal comparison of immunological features. The control groups included the following: stable renal graft recipients who were receiving conventional immunosuppressive therapy (steroids/tacrolimus/MMF) 1–3 years after transplantation, in whom immunophenotyping (Tx ≤ 3a; n = 12, mean age: 45.1 years) or antiviral T cell responses (Tx ≤ 3b, n = 20, mean age: 47.5 years) were analyzed. Mean steroids doses in the Tx < 3a and Tx < 3b populations were 8.1 ± 2 mg/day and 10.2 ± 1 mg/day, respectively. Mean mycophenolate mofetil doses in the Tx < 3a and Tx < 3b populations were 1.2 ± 0.4 g/day and 1.4 ± 0.5 g/day, respectively. Mean tacrolimus doses in the Tx < 3a and Tx < 3b populations were 9.1 ± 3 mg and 12.1 ± 2 mg, with through levels of 8.2 ± 2 ng/ml and 10.1 ± 1.2 ng/ml, respectively. Informed consent was obtained from the participating subjects for the collection of biological samples that were used for the follow-up immunological studies according to institutional guidelines.

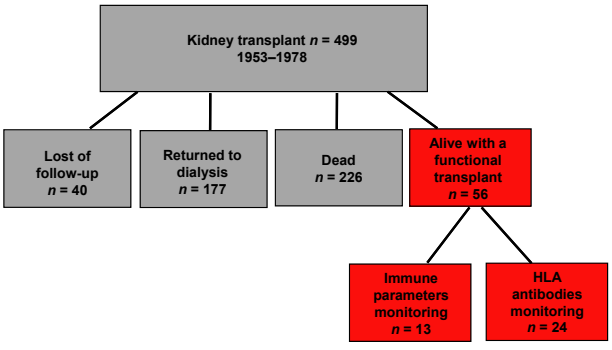

Figure 1 Flow chart in 499 kidney transplant patients.

Flow cytometry

Counting and phenotyping of lymphocyte subsets were performed on fresh whole blood. Absolute lymphocyte counts were determined using MultiTest™ CD3-APC/CD8-perCP/CD45-PE/CD4-FITC (SK3, 2D1, SK1 and SK7 clones respectively) antibody mix with Trucount beads according to manufacturer's instructions with a FACSCalibur cytometer (BD Biosciences, Le Pont de Claix, France). Immunophenotyping of T and B cells was also performed on a FACSCalibur cytometer and the results were analyzed using CellQuest software (BD Biosciences). The following antibodies (clones) were purchased from BD Biosciences: CD3-FITC (clone SK7), CD4-perCP (SK3), CD8-perCP (SK1), CD31-PE (L133-1), CD45RA-FITC (L48), CD45RA-APC (MEM56), CD45RO-PE (UCHL-1), CD57-FITC (HNK1), CD19-PE (SJ25C1), CD20-perCP (L27), CD38-APC (HB7), IgD-FITC (IA6-2), CD27-PE (L128). CCR7-APC (clone 150503) antibody was purchased from R&D. $CD4^+$ and $CD8^+$ T cells were defined as the following subsets according to the expression of CD45RA and CCR7: naïve cells ($CD45RA^+CCR7^+$), recent thymic emigrants (RTEs, $CD45RA^+CCR7^+CD31^+$), effector memory cells (TEMs; $CD45RA^-CCR7^-$), and terminally differentiated memory cells (TEMRA; $CD45RA^+CCR7^-$). The $CD8^+$ T cell subsets were defined as follows according to the expression of CD45RA, CCR7 and CD57: naïve cells ($CD45RA^+CCR7^+$), effector memory cells (TEMs; $CD45RA^-CCR7^-$), and terminally differentiated memory cells (TEMRA; $CD45RA^+CCR7^-$). The expression of CD57 on $CD8^+$ T cells was also analyzed. The subsets of $CD19^+$ B cells were defined into the following phenotypes according to the expression of IgD, CD38 and CD27: naïve ($IgD^+CD38^-CD27^{-/low}$), memory (BMEM; $IgD^-CD38^-CD27^{+/low}$), transitional (IgD^+, $CD38^{++}$, $CD27^-$), and plasmablasts (IgD^-CD38^{+++}). The results were compared to the normal range values that were established in our laboratory using a reference control population (Table 1). The reproducibility of immunophenotyping has been evaluated for each analyzed T or B cell subset assessed, using ten different whole blood samples analyzed in triplicate. The average coefficient of variation is 3.7% (minimal CV for frequent subsets: 0.1%; maximal CV for rare subsets: 18.0%). The laboratory of clinical immunology of Necker hospital participates in an external quality control evaluation program (UK NEQAS, Sheffield, UK) for leucocyte immunophenotyping.

Elispot

The EBV- and CMV-specific $CD4^+$ and $CD8^+$ T-cell responses were investigated using an IFN-γ ELISPOT

assay that was performed on cryopreserved PBMCs. Briefly, 96-well PVDF plates (Millipore, Saint-Quentin-en-Yvelines, France) were coated overnight with an anti-IFN-γ antibody (U-CyTech, Utrecht, the Netherlands). The plates were subsequently blocked with RPMI-10% fetal calf serum, and the peptide pools were added to triplicate wells. The PBMCs were seeded at 3×10^5 cells/well and were cultured for 20 h. The PBMCs were then removed, and IFN-γ secretion was visualized using a biotinylated anti-IFN-γ detection antibody (U-CyTech), streptavidin-horseradish peroxidase and the AEC substrate, yielding red spots. Spots were counted using an AID reader (Strassberg, Germany), and means of triplicate wells were calculated. The overlapping 15-mer peptide pools of viral proteins (EBV: BZLF1, EBNA1, EBNA3A, EBNA3B, EBNA3C, LMP2; CMV: pp65, IE-1, IE-2) were purchased from ProImmune (Oxford, UK) and were used in the assay at a final concentration of 2 μg/ml. In each assay, a negative control (non stimulated cells, RPMI only) and a positive control (PHA-stimulated cells) for T cell responsiveness were included. A test was considered meaningful if the number of SFC obtained with PHA-stimulated cells was superior to the mean SFC number + 5 standard deviations (SD) found in the three wells with non stimulated cells. All readouts were expressed as spot-forming cells (SFC)/10^6 PBMCs after subtraction of the background mean value obtained from wells with non-stimulated cells. For responses to viral antigens, a cut-off for positive responses to viral antigens was calculated after substraction of the background, defined as $3 \times$ SD found in the three wells with non-stimulated cells. The intra-assay coefficient of variation of the assay is 20%.

Table 1. T and B cell subsets in a reference healthy control population.

	Reference values
	Median (range)
$CD4^+$ T cells	
Naïve	40.4% (31.2–63.8)
RTE	30.3% (18.7–54.2)
TEM	23.9% (12.7–36.7)
TEMRA	1.7% (0.4–8.7)
$CD8^+$ T cells	
Naïve	28.6% (14.3–70.8)
TEM	25.9% (14.3–70.8)
TEMRA	16.4% (4.3–53.2)
TEMRA $CD57^+$	12.2% (3.9–40.2)
B cells	
Naïve	66.5% (60.7–83.04)
Memory	22.7% (6.3–28.9)
Transitional	4.5% (2.3–11.7)
Plasma-like	0.3% (0.1–0.7)

Median, range, mean and standard error values are indicated for each subset. RTE: recent thymic emigrants; TEM: effector/memory T cells; TEMRA: terminally differentiated T cells; BMEM: memory B cells; BTRANS: transitional B cells; Plasm.: plasmablasts.

Statistical analysis

The continuous data are expressed as the mean ± SD or the median (range) and were compared using the Mann-Whitney test. The categorical data are expressed as percentages and were compared using Fisher's exact test. The survival curves were generated using the Kaplan and Meier method and were compared using the log-rank test. The statistical analyses were performed using Prism® software. *P* values of less than 0.05 were considered to be significant.

Results

Tx30 patients have a low risk of graft failure

The characteristics of the donors and the Tx30 patients are listed in Table 2. The Tx30 patients strikingly shared certain characteristics, including a young age of the donor and recipient (27.6 ± 10.3 years and 27.1 ± 7.3 years, respectively), a living donor in 23/56 (44%) of the cases and a mean cold ischemia time of 16.5 ± 8.5 h for cadaveric donors. The main cause of chronic renal failure was chronic glomerulonephritis (38/56 cases, 68%), including

Table 2. Patients and allografts characteristics.

Characteristics	Value (*n* = 56)
Donor age (year)	27.6 ± 10.3
Recipient age (year)	27.1 ± 7.3
Recipient sex ratio (F/M)	0.35
Dialysis time (months)	17.1 ± 14.6
Living donor (n, %)	23 (44%)
HLA mismatch (mean)	3.9 ± 11.5
Nephrectomy (n, %)	(40) 71%
Cold ischemia time (hours)	
Cadaveric donors	16.4 ± 8.5
Living donors	0.7 ± 0.2
Initial nephropathy (n, %)	
GNC	38 (68%)
TICN	8 (14%)
Urological	6 (10%)
Other	4 (8%)
Immunosuppressive regimen (day 0)	
Anti-lymphocytes globulins	1 (1.5%)
Azathioprine+corticosteroids	51 (91%)
Immunosuppressive regimen (30 years)	
Azathioprine alone	8 (14%)
Prednisone alone	15 (26%)
Prednisone and azathioprine	20 (39%)
Calcineurin inhibitors	2 (3%)
Mycophenolate mofetil	5 (9%)
None	2 (3%)
Biopsy proven acute rejection episodes	27 (48%)

membranous nephropathy, IgA nephropathy and Alport syndrome which reflects an epidemiology that is different from that of today.

Regarding the surgical procedures, end-to-end uretero-ureteral anastomosis was performed in 91% of the cases. A nephrectomy was systematically performed in 40/56 patients (71%) before the transplantation. This nephrectomy, homolateral to the transplant, was performed in order to use the remnant ureter for the uretero-ureteral anastomosis before it has been noticed that it was possible to let the kidney in situ with the cuted ureter ligated. As an initial immunosuppressive regimen, 51/56 patients (91%) received azathioprine (2 mg/kg) and a high dose (5 mg/kg) prednisone in combination, and only five patients (9%) were treated with azathioprine alone. Induction with anti-lymphocyte globulins was performed in only one case.

Tx30 patients display slight histological changes and nearly normal kidney function

Systematic allograft biopsies were performed in 34/56 patients (60%) after 10 years. The renal histology was classified as normal in 11 patients (32%), mild-moderate arteriolopathy was observed in 11 patients (32%), and non-specific lesions were observed in four patients (11%). Four biopsy samples (11%) displayed recurrent nephropathy: one exhibited IgA nephropathy, and three indicated dense deposit disease. Interestingly, for the three samples of dense deposit disease, the diagnosis of the recurrence was made as soon as 6 months after the transplantation. Among the patients with recurrent nephropathy, mean last creatininemia was 98 ± 24 μmol/l, and proteinuria was 0.3 ± 0.4 g/24 h.

Twenty-seven patients out of 56 (48%) experienced biopsy-proven acute rejection episodes (BPAR) 9.2 ± 24 months after transplantation and were treated with high-dose steroids. Of note, one BPAR episode occurred 10 years after transplantation. Of note, HLA antibodies (systematic monitoring) were found in 6 out of the 24 patients in which they were tested and all were directed against class II HLA.

The mean serum creatinine level was stable over the time, although after 30 years, the variance increased, which suggests that a proportion of renal allografts had begun to fail (Fig. 2a). Thirty years after transplantation, the mean proteinuria level was low, 0.6 ± 0.5 g/l, and the majority of the patients had a level <0.5 g/l (Fig. 2b). Twenty-six out of 56 patients (46%) were on renin-angiotensin system blockade therapy and we found no correlation with proteinuria. The death-censored graft loss rate was 20% at 5 years (i.e., 5 years after the 30th anniversary, Fig. 3a), which is similar to that of the age-related general transplant population [5], but the causes of failure were not identified. Death-censored graft survival was

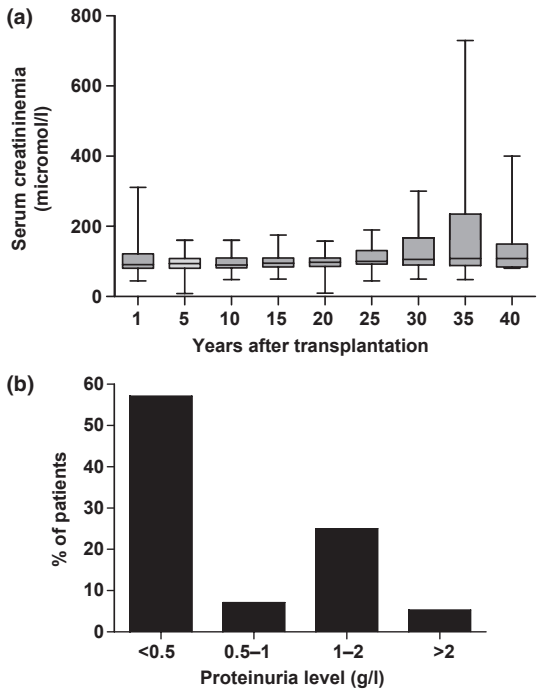

Figure 2 Allograft function in Tx30 patients. (a) Mean serum creatinine levels and standard deviation (whisker boxes). (b) Percentages of Tx30 patients with the proteinuria levels of <0.5 g/l, 0.5–1 g/l, 1–2 g/l and >2 g/l.

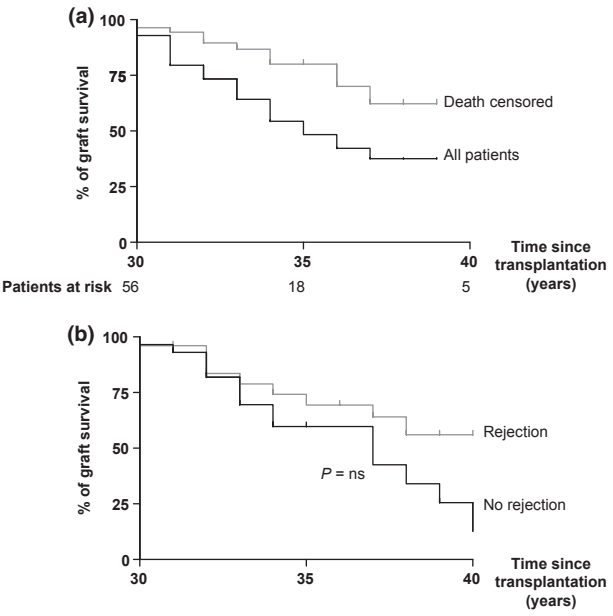

Figure 3 Allograft survival in Tx30 patients. (a) Post-transplant graft survival of all Tx30 patients (black) and death-censored (grey). (b) Post-transplant, death censored, graft survival, in Tx30 patients without (black) or with (grey) rejection.

not influenced by the occurrence of BPAR (Fig. 3b). Only two patients out 56 (3%) received calcineurin inhibitors (CNIs) 30 years after transplantation. Two patients (3%) did not receive any immunosuppressive drugs, and the vast majority of patients (65%) received corticosteroids, which were administered alone in 28% of the cases or in association with azathioprine in 37% of the cases. Regarding the patients without immunosuppression, one died in 1999 from sepsis and the other lost its graft in 2000 (32 years after transplantation). The mean ± SD dose of corticosteroids was 9.1 ± 2.1 mg/day. Together, our data suggest that renal function was stable over time in the Tx30 patients and that death-censored graft survival after the 30th year was not altered compared to the age-related general transplant population [5].

Tx30 patients experience a high mortality due to cancers

Nineteen out of the 56 Tx30 patients (33%) died during the follow-up, after the 30 years mark. A majority (51%) of the 56 Tx30 patients had a cancer 30 years after transplantation and 40% of the deaths were attributed to cancer; the remaining causes were cardiovascular diseases (20%) and infection (Table 3). A diagnosis of cutaneous cancer was made in 26/56 patients (46.1%), and the 5-year patient survival rate was 52.6% vs. 70.3% for unaffected patients (P = ns, Fig. 4a). Sixteen patients out of 56 (28%) had a non-cutaneous cancer (three lymphomas, two esophageal cancers, two breast cancers, three hepatocarcinoma and eight colonic cancers). The 5-year survival rate of these patients was 27% vs. 87% for unaffected patients ($P < 0.0001$, Fig. 4a). Twenty-seven patients (48.2%) had viral hepatitis B and/or a C, which was associated with cirrhosis in 14% of the cases and with an hepatocarcinoma in 11.1% of the cases. However, patients with hepatitis had the same survival than the others (Fig. 4b). Overall, Tx30 patients constitute a population at very high risk for cancer, which may impact their survival.

Table 3. Co morbidities at the end of the observation period.

Characteristics	Value (n = 56)
Cancer	
All types	29 (51%)
Cutaneous	26 (46.1%)
Non cutaneous	16 (28%)
Hip or knee replacement	15 (34%)
Chronic viral hepatitis	
All types	27 (48%)
Hepatitis B	14 (25%)
Hepatitis C	13 (23%)
Hepatitis B and C	4 (7%)
Hepatocarcinoma	3 (11%)
Cardiovascular complications	
Hypertension	32 (57%)
Ischemic cardiovascular event	17 (30%)
NODAT	5 (9%)
Dyslipidemia	27 (48%)

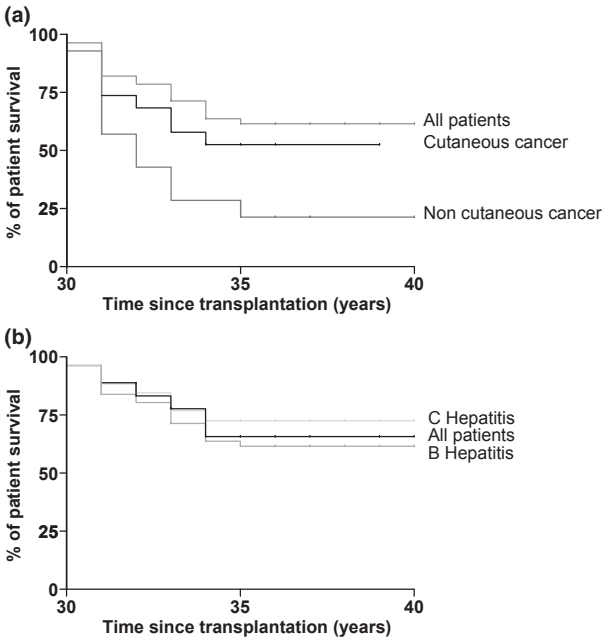

Figure 4 Tx30 patient survival. (a) Post-transplant survival of all Tx30 patients (light grey) and Tx30 patients with cutaneous (black) or non-cutaneous (dark grey) cancer. (b) Post-transplant survival of all Tx30 patients (black) and Tx30 patients with hepatitis C (light grey) or B (dark grey).

T and B lymphocytes subsets of Tx30 patients are similar to those of recently transplanted patients

We were able to perform B- and T-lymphocyte immunophenotyping in 13 Tx30 patients (mean age: 62.7 years) and to compare the results with a group of patients who were receiving full immunosuppression 1–3 years after transplantation (Tx ≤ 3a, n = 12 mean age: 45.1 years). No significant difference in the proportions of the various T cell subsets was observed between Tx30 and Tx ≤ 3a patients (Fig. 5). In particular, RTE were found in similar proportions among $CD4^+$ T cells [median and range values Tx30: 12.1% (3.7–36.5%) and Tx ≤ 3a: 15.9% (6.5–49.6%)], which indicates the persistence of thymic export in older Tx30 patients, although their proportions and absolute numbers were reduced as compared to healthy controls (%RTE Tx30 versus HC, $P = 0.036$; Tx < 3a versus HC, $P = 0.0185$, Table 4). Both groups of patients exhibited low proportions of naïve $CD4^+$ [Tx30: 29.8% (7.6–54.8%), Tx ≤ 3a: 23.5% (12.8–59.6%) versus

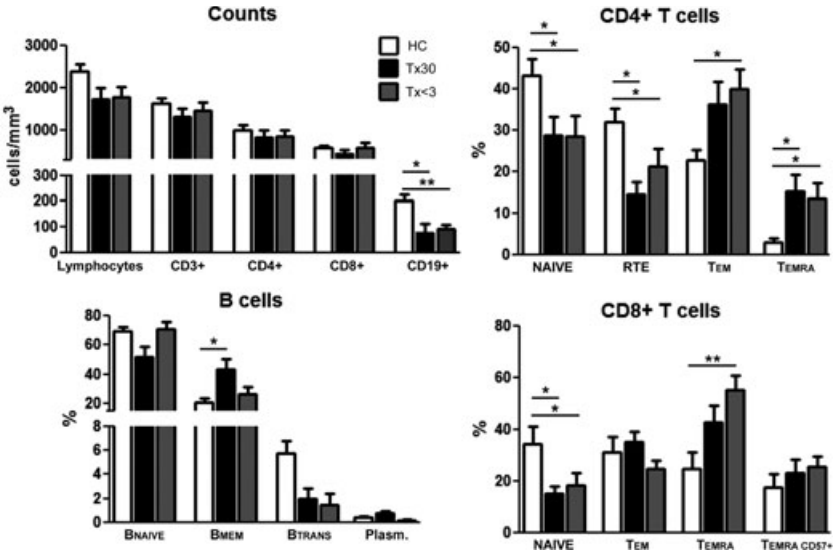

Figure 5 Lymphocyte subsets in Tx30 patients versus patients less than 3 years (Tx ≤ 3a) after transplantation who were receiving full conventional immunosuppression. Lymphocyte counts are expressed as numbers per mm^3 of whole blood. Healthy controls (open bars), Tx30 patients (black bars) and Tx ≤ 3a patients (open bars). RTE: recent thymic emigrants ($CD45RA^+CCR7^+CD31^+$); TEM: Effector/memory T cells ($CD45RA^-CCR7^-$); TEMRA: terminally differentiated T cells ($CD45RA^+CCR7^-$); BMEM: memory B cells ($IgD^-CD38^-CD27^{+/low}$); BTRANS: transitional B cells (IgD^+, $CD38^{++}$, $CD27^-$); Plasm.: plasmablasts (IgD^-CD38^{+++}). $^*P < 0.05$; $^{**}P < 0.01$.

controls 40.4% (31.2–63.8%); $P = 0.0209$ and $P = 0.0435$, respectively] and naïve $CD8^+$ T cells [Tx30: 16.0% (2.5–32.3); Tx ≤ 3a: 12.5% (0.4–56.5) versus controls: 28.6% (14.23–70.8%); $P = 0.020$ and $P = 0.070$, respectively] and expansion of terminally differentiated memory $CD8^+$ T cells (TEMRA) [Tx30: 41.0% (4.7–72.3), Tx ≤ 3a: 59.3% (7.3–80.6) versus controls: 16.4% (4.3–53.2%); $P = 0.0817$ and 0.009 respectively]. As previously reported [6,7], most of the TEMRA $CD8^+$ T cells expressed CD57, and several Tx30 and Tx ≤ 3a patients exhibited proportions of these cells of up to 60% among $CD8^+$ T lymphocytes. When comparing absolute numbers of $CD8^+$ T cells, only the naïve $CD8^+$ subset was found significantly lower in Tx30 patients than healthy controls (Table 4).

Significantly lower numbers of B cells were found in Tx30 and Tx ≤ 3a patients than in healthy controls [median (range): Tx30, 43/mm^3 (7–469) and Tx ≤ 3a, 72/mm^3 (20–507) versus HC 203/mm^3 (71–364); $P = 0.0157$ and $P = 0.0029$ respectively]. Among B cells, slightly higher proportions and counts of plasmablasts were observed in the Tx30 patients [median: 0.5% (0.0–2.8)] than in the Tx ≤ 3a patients [0.0% (0.0–0.8), $P = 0.0142$, and Table 4]. In addition, the Tx30 patients showed a tendency toward lower frequencies and numbers of naïve B cells than Tx ≤ 3a patients [Tx30: 56.8% (15.2–88.2); Tx ≤ 3 70.7% (47.2–85.8); $P = 0.05$, and Table 4] and higher proportions of memory B cells than Tx ≤ 3a patients and healthy controls [Tx30: 34.1% (7.1–87.5); Tx ≤ 3a: 26% (6.7–52.1), $P > 0.05$; healthy controls: 22.7% (6.3–28.9), $P = 0.0135$, Fig. 5]. However, absolute numbers of memory B cells were in fact lower in Tx30 and Tx ≤ 3a patients than in healthy controls (Table 4). Transitional B cells were found significantly decreased in both groups of patients as compared to healthy controls (Fig. 5 and Table 4).

The anti-EBV and -CMV T cell responses of the Tx30 patients do not differ from those of fully immunosuppressed patients

The basal level of replication of the latent viruses EBV and CMV is tightly controlled by T cells and is largely impacted by immunosuppressive therapy in transplant

Table 4. T and B cell counts in Tx30, Tx ≤ 3a patients and healthy controls.

	Healthy controls	Tx30	Tx ≤ 3a
Lymphocytes			
Total	2358 (1280–3351)	1473 (544–3292)	1512 (231–2876)
$CD4^+$ T cells			
Total	852 (498–1673)	805 (177–2293)	911 (56–1547)
Naïve	266 (205–722)	174 (48–761)	218 (13–833)
RTE	258 (142–453)**	115 (7–546)**	148 (4–693)
TEM	162 (113–331)	283 (48–807)	260 (16–587)
TEMRA	11 (3–121)	94 (3–482)	47 (2–644)
$CD8^+$ T cells			
Total	501 (276–1287)	281 (114–1048)	554 (56–1201)
Naïve	160 (79–347)**	45 (5–159)**	56 (0–393)
TEM	130 (49–911)	125 (31–257)	103 (4–351)
TEMRA	99 (22–370)	101 (12–748)	240 (0–667)
TEMRA $CD57^+$	76 (11–198)	58 (10–518)	116 (21–443)
B cells			
Total	203 (71–364)	43 (7–469)	72 (20–507)
Naïve	141 (50–302)**	24 (1–414)**	60 (12–100)**
Memory	48 (10–60)*	20 (3–42)*	15 (4–56)
Transitional	8 (4–31)**	0 (0–47)**	0 (0–3)***
Plasma-like	0.7 (0.1–1.6)	0.2 (0.0–2.3)*	0.0 (0.0–1.1)*

Median, range, mean values are indicated for each subset. RTE: recent thymic emigrants; TEM: effector/memory T cells; TEMRA: terminally differentiated T cells; BMEM: memory B cells; BTRANS: transitional B cells; Plasm.: plasmablasts. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

patients. To estimate the immune competence of Tx30 patients, we analyzed the patients' anti-CMV and -EBV T cell responses using an IFN-γ ELISPOT assay and compared the responses to a second subgroup of EBV^+ and CMV^+ Tx ≤ 3b patients ($n = 20$ mean age = 47.5 years) that was receiving full conventional immunosuppression. The frequencies of the anti-EBV and -CMV T cell responses were similar between both groups of patients (Fig. 6). Particularly high numbers of CMV pp65-specific T cells were found in the Tx30 (median: 971 SFC/10^6 PBMCs, range: 220–1610) and Tx ≤ 3b (median: 1500 SFC/10^6 PBMCs, range: 7–2022) patients. A lower, but significant, frequency of IE-1-specific reactivity was observed in both groups (Tx30: median: 90 SFC/10^6 PBMCs, range: 27–820; Tx ≤ 3b: median: 258 SFC/10^6 PBMCs, range: 42–1663; $P = 0.23$). These data suggest that the anti-EBV and -CMV T cell responses of Tx30 patients were not different, on average, from those of fully immunosuppressed patients.

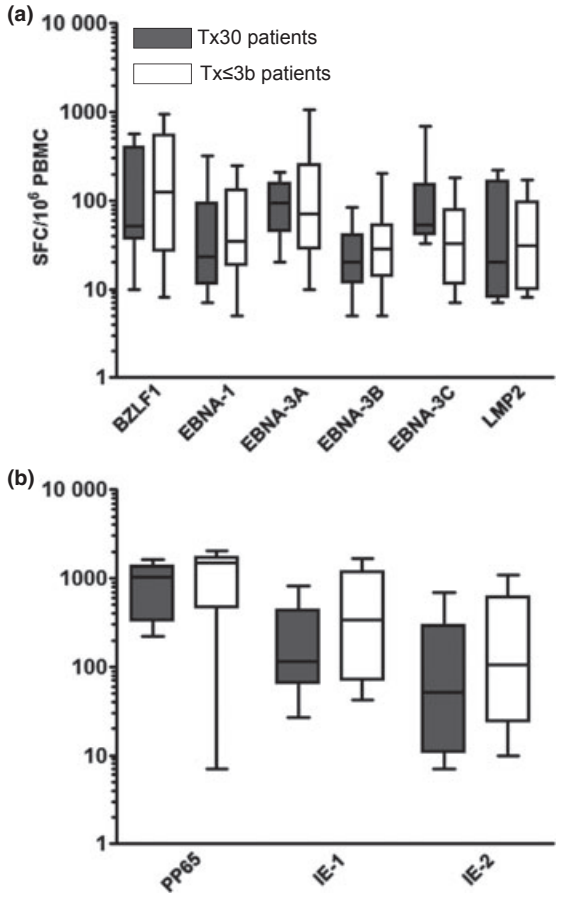

Figure 6 Anti-EBV and -CMV T cell responses. The frequency of IFN-γ-producing T cells in response to EBV (left panel) and CMV (right panel) in Tx30 patients (grey bars) and patients 1–3 years after transplantation receiving full conventional immunosuppression (Tx ≤ 3b, open bars) is shown. The numbers of specific T cells are expressed as spot forming cells (SFCs) per 10^6 PBMCs. Minimal, maximal and median frequencies (whisker boxes) are represented for each viral peptide pool tested. BZLF1: EBV immediate-early Protein; EBNA: Epstein–Barr nuclear antigen; LMP: latent membrane antigen; IE: immediate early antigen.

Discussion

Progress in the care of renal transplant patients and a better understanding of the mechanisms of allograft injury has allowed an impressive increase in graft and patient survival over the past several decades. Such improvements have generated new challenges because of the emergence of a growing population of patients who maintain their transplant for a very long time, and it is important to have a detailed knowledge of the peculiarities of this population. Here, we present the results of a study that is the first to describe the clinical and immunological characteristics as well as the outcomes of a large cohort of patients who received a first kidney transplant and who lived at least 30 years after transplantation.

The characteristics of the Tx30 patients we described here reflect a bias toward a population of patients with a low risk of graft failure. Indeed, factors that have been identified to influence long-term graft function and survival were favorable in these patients; the duration of dialysis and the cold ischemia times were shorter, the proportion of living donors (44%) was high which is known to yield better results. Such a bias is inherent to the retrospective nature of this study, and our results must be interpreted accordingly.

The good functional results of the Tx30 patients should be interpreted taking into account the fact that most of the patients were transplanted before the beginning of the CNI era. The absence of CNI use probably translated into a prevention of chronic nephrotoxicity together with a high incidence (nearing 50%) of BPAR (Biopsy Proven Acute Rejection), which is in accordance with the incidence that was observed before the introduction of CNIs as a modern immunosuppressive therapy [8]. This finding is of importance because the concept of CNIs leading to chronic nephrotoxicity has actually been challenged by studies demonstrating that immune injury is the leading cause of graft damage and loss and that CNI nephrotoxicity has a minor, if any, impact on chronic allograft nephropathy [9–13]. Because of the lack of control patients matched for the time since transplantation (such a population does not exist because the widespread use of CNIs started at the beginning of the 1980s), one can only speculate that avoiding CNI use could contribute to very long-term graft survival in the absence of tubulointerstitial fibrosis.

Our results suggest that the diagnosis of cancer 30 years after renal transplantation is extremely frequent and is associated with an elevated mortality. Forty-six percent of the patients suffered from a cutaneous cancer, and 28% had a non-cutaneous cancer, including solid tumors and lymphomas. This prevalence is in accordance with what has traditionally been reported and confirms that the incidence of cancer increases with the time since transplantation [14,15]. Moreover, the prognosis of cancer appears to worsen with time since transplantation because we found that the survival of the Tx30 patients after a solid cancer was lower than that which is generally reported [16] and that cancer represents 40% of the causes of death in the Tx30 population. Of note, azathioprine, which is known to have oncogenic properties [17] and was used in a large proportion of Tx30 patients, may have favored carcinogenesis in this subset of patients. We found that chronic viral hepatitis does not influence survival 30 years after transplantation, which is in accordance with previous reports regarding hepatitis B [18], whereas chronic hepatitis C is known to negatively impact survival [19]. This discrepancy suggests that Tx30 patients with hepatitis C constitute a subgroup of patients for whom health status is not affected by the virus for unknown virological or immunological reasons.

The long-term graft survival associated with a high prevalence of cancer in transplanted patients is believed to be the result of a global impairment of immune functions, which leads to a simultaneous depression of anti-donor and anti-tumor immune reactivity. However, it remains unclear whether the burden of chronic therapeutic immunosuppression acts in a cumulative manner through accelerated immune senescence [20,21] or, alternatively, whether the high frequency of cancer in long-term organ transplant recipients simply reflects the increasing statistical risk for oncogenic events to occur while the level of immune impairment remains stable over time. In other words, the question of whether an organ transplant recipient is more immunocompromised at 30 than at 3 years post-transplant is still unresolved. To address this issue, we assessed the lymphocyte compartments, thymic output and antiviral immune responses in long- versus short-term renal transplant recipients. The evidence of preserved thymic function in the long-term recipients argues against accelerated immune senescence. This observation is also consistent with the low level of exposure of Tx30 patients to immunosuppressive drugs that are known to impair thymic export such as calcineurin inhibitors [22]. In addition, Tx30 patients who received a mild immunosuppressive regimen did not differ from more recently transplanted and younger patients who were receiving full conventional immunosuppression in terms of their anti-EBV and anti-CMV responses. Both types of patients displayed high frequencies of IFN-γ-producing antiviral T cells, which would suggest that the T cell control over latent viruses is not impaired. However, the particularly high frequency of CMV (pp65) reactivity together with the increased proportion of memory T cells and TEMRA $CD57^+$ $CD8^+$ T cells, which have been described in other groups of transplanted patients [7], most likely reflects a restriction of the T cell repertoire toward chronically stimulating antigens such as CMV. The heavy burden of CMV on the immune system might indeed occur at the expense of other duties, such as cancer immunosurveillance. This situation is highly reminiscent of the proposed role of CMV in immune senescence in subjects aged >85 years, in whom the incidence and prevalence of cancer are also increased [23,24]. However, a demonstration of the negative impact of CMV-specific $CD8^+$ T cell expansions on anti-tumoral responses is still missing both in transplanted patients and in elderly subjects [24–26], as well in our study.

Long-term survival of kidney allograft in Tx30 patients may be related to relative immunosuppression and/or to the development in some cases of tolerance towards alloantigens. Biomarkers of operational tolerance have recently been identified in kidney transplanted patients [27,28]. In addition to a transcriptional B cell signature in peripheral blood, higher numbers of peripheral B cells with increased B cell/T cell ratio and increased frequencies of $IgD^+CD38^{+/-}$ (naïve/activated) and transitional B cells were found the hallmark of drug-free operationally tolerant patients, as compared to stable immunosuppressed kidney graft recipients. These features were not observed in most Tx30 patients. However, one patient displayed increased numbers of B cells, and particularly high numbers of transitional and naïve B cells (maximal range values of B cell subsets for Tx30 group in Table 4). Gene expression studies were not performed in this study. We thus cannot exclude that some Tx30 patients might indeed have developed allograft tolerance.

Taken together, these results indicate that Tx30 patients share some immunological features with more recently transplanted patients receiving full dose immunosuppression. This suggests either that modern immunosuppressive strategies lead in a few years to an impairment of immune functions equivalent to that induced by 30 years of milder immunosuppression, which is unlikely, or that the impact of chronic therapeutic immunosuppression on immune functions, at least those analyzed in this study (i.e. anti-EBV and -CMV T cell responses), does not worsen over time. The high incidence of cancer in Tx30 patients thus most likely results from the synergistic effects of decreased immunosurveillance, which is primarily related to chronic immunosuppression, and the time

required for carcinogenesis. Further immunological studies in this subset of patients could help to decipher the respective role of these factors and the physiological immune senescence on graft survival, the control of CMV infection and the development of cancer.

Authorship

LB: collected data. NP: wrote the paper. ZC and DA: provided helpful comments. HK: was involved in patients management. CL: designed the study. SC: designed the study, performed immunological analysis and wrote the paper.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Graft and patients survivals in the initial population.

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References

1. Miyamoto N, Ishida H, Furusawa M, *et al.* Immunological status in three patients, thirty years after living related renal transplantation: antibody production in long-term survivors. *Transpl Immunol* 2008; **18**: 368.
2. Matas AJ, Gillingham KJ, Humar A, *et al.* 2202 kidney transplant recipients with 10 years of graft function: what happens next? *Am J Transplant* 2008; **8**: 2410.
3. El-Agroudy AE, Bakr MA, Hassan NA, *et al.* Characteristics of long-term live-donor renal allograft survivors. *Am J Nephrol* 2003; **23**: 165.
4. Legendre C, Kreis H. A tribute to Jean Hamburger's contribution to organ transplantation. *Am J Transplant* 2010; **10**: 2392.
5. OPTN. Organ Procurement and Transplantation Network. 2011, Available at: http://optn.transplant.hrsa.gov/.
6. Legendre CM, Guttmann RD, Hou SK, Jean R. Two-color immunofluorescence and flow cytometry analysis of lymphocytes in long-term renal allotransplant recipients: identification of a major Leu-7+/Leu-3+ subpopulation. *J Immunol* 1985; **135**: 1061.
7. Focosi D, Bestagno M, Burrone O, Petrini M. CD57+ T lymphocytes and functional immune deficiency. *J Leukoc Biol* 2010; **87**: 107.
8. Calne RY, Wood AJ. Cyclosporin in cadaveric renal transplantation: 3-year follow-up of a European multicentre trial. *Lancet* 1985; **2**: 549.
9. El-Zoghby ZM, Stegall MD, Lager DJ, *et al.* Identifying specific causes of kidney allograft loss. *Am J Transplant* 2009; **9**: 527.
10. Gourishankar S, Leduc R, Connett J, *et al.* Pathological and clinical characterization of the 'troubled transplant': data from the DeKAF study. *Am J Transplant* 2010; **10**: 324.
11. Matas AJ. Chronic progressive calcineurin nephrotoxicity: an overstated concept. *Am J Transplant* 2011; **11**: 687.
12. Snanoudj R, Royal V, Elie C, *et al.* Specificity of histological markers of long-term CNI nephrotoxicity in kidney-transplant recipients under low-dose cyclosporine therapy. *Am J Transplant* 2011; **11**: 2635.
13. Pinto JR, Arellano Torres EM, Franco A, *et al.* Sirolimus monotherapy as maintenance immunosuppression: a multicenter experience. *Transpl Int* 2010; **23**: 307.
14. Gaya SB, Rees AJ, Lechler RI, Williams G, Mason PD. Malignant disease in patients with long-term renal transplants. *Transplantation* 1995; **59**: 1705.
15. Slavis SA, Novick AC, Steinmuller DR, *et al.* Outcome of renal transplantation in patients with a functioning graft for 20 years or more. *J Urol* 1990; **144**: 20.
16. Howard RJ, Patton PR, Reed AI, *et al.* The changing causes of graft loss and death after kidney transplantation. *Transplantation* 2002; **73**: 1923.
17. O'Donovan P, Perrett CM, Zhang X, *et al.* Azathioprine and UVA light generate mutagenic oxidative DNA damage. *Science* 2005; **309**: 1871.
18. Fornairon S, Pol S, Legendre C, *et al.* The long-term virologic and pathologic impact of renal transplantation on chronic hepatitis B virus infection. *Transplantation* 1996; **62**: 297.
19. Legendre C, Garrigue V, Le Bihan C, *et al.* Harmful long-term impact of hepatitis C virus infection in kidney transplant recipients. *Transplantation* 1998; **65**: 667.
20. Li P, Tian C, Ge N, *et al.* Premature senescence of T cells in long-term survivors of renal transplantation. *Biochem Biophys Res Commun* 2011; **407**: 599.
21. Velasquez SY, Arias LF, Garcia LF, Alvarez CM. T cell receptor beta chain (TCR-Vbeta) repertoire of circulating CD4(+) CD25(-), CD4(+) CD25(low) and CD4(+) CD25(high) T cells in patients with long-term renal allograft survival. *Transpl Int* 2010; **23**: 54.

22. Chau LA, Rohekar S, Wang JJ, *et al.* Thymic re-entry of mature activated T cells and increased negative selection in vascularized allograft recipients. *Clin Exp Immunol* 2002; **127**: 43.
23. Pawelec G, Akbar A, Beverley P, *et al.* Immunosenescence and Cytomegalovirus: where do we stand after a decade? *Immun Ageing* 2010; **7**: 13.
24. Fulop T, Kotb R, Fortin CF, Pawelec G, de Angelis F, Larbi A. Potential role of immunosenescence in cancer development. *Ann N Y Acad Sci* 2010; **1197**: 158.
25. Baron C, Forconi C, Lebranchu Y. Revisiting the effects of CMV on long-term transplant outcome. *Curr Opin Organ Transplant* 2010; **15**: 492.
26. Pawelec G, Derhovanessian E. Role of CMV in immune senescence. *Virus Res* 2011; **157**: 175.
27. Brouard S, Le Bars A, Dufay A, *et al.* Identification of a gene expression profile associated with operational tolerance among a selected group of stable kidney transplant patients. *Transpl Int* 2011; **24**: 536.
28. Sagoo P, Perucha E, Sawitzki B, *et al.* Development of a cross-platform biomarker signature to detect renal transplant tolerance in humans. *J Clin Invest* 2010; **120**: 1848.