Expansion of Highly Differentiated Cytotoxic Terminally Differentiated Effector Memory CD8+ T Cells in a Subset of Clinically Stable Kidney Transplant Recipients: A Potential Marker for Late Graft Dysfunction

Michelle Yap,*†‡ François Boeffard,*†‡ Emmanuel Clave,§ Annaick Pallier,*†‡ Richard Danger,*†‡ Magali Giral,†‡ Jacques Dantal,*†‡ Yohann Foucher,‡ Cécile Guillot-Gueguen,‡ Antoine Toubert,§ Jean-Paul Souillou,*†‡ Sophie Brouard,*†‡ and Nicolas Degauque*†‡

*Institut National de la Santé et de la Recherche Médicale, Unité Mixte de Recherche 1064, Nantes, France; †Centre Hospitalier Universitaire de Nantes, Institut de Transplantation Urologie-Néphrologie, Nantes, France; ‡Faculté de Médecine, Université de Nantes, Nantes, France; §Institut National de la Santé et de la Recherche Médicale, Unité Mixte de Recherche 940, Paris, France; and †Université de Nantes, Equipe d’Accueil 4275 and Labex Transplantex, Nantes, France

ABSTRACT

Despite the effectiveness of immunosuppressive drugs, kidney transplant recipients still face late graft dysfunction. Thus, it is necessary to identify biomarkers to detect the first pathologic events and guide therapeutic target development. Previously, we identified differences in the T-cell receptor Vβ repertoire in patients with stable graft function. In this prospective study, we assessed the long-term effect of CD8+ T-cell differentiation and function in 131 patients who had stable graft function. In 45 of 131 patients, a restriction of TCR Vβ diversity was detected and associated with the expansion of terminally differentiated effector memory (TEMRA; CD45RA+CCR7−CD27−CD28−) CD8+ T cells expressing high levels of perforin, granzyme B, and T-bet. This phenotype positively correlated with the level of CD57 and the ability of CD8+ T cells to secrete TNF-α and IFN-γ. Finally, 47 of 131 patients experienced kidney dysfunction during the median 15-year follow-up period. Using a Cox regression model, we found a 2-fold higher risk (P=0.06) of long-term graft dysfunction in patients who had increased levels of differentiated TEMRA CD8+ T cells at inclusion. Collectively, these results suggest that monitoring the phenotype and function of circulating CD8+ T cells may improve the early identification of at-risk patients.

The major immunologic cause of late kidney graft failure is chronic antibody-mediated rejection (CAMR).1 Its diagnosis relies on renal dysfunction, histologic features, and donor-specific antibodies.2 The biologic mechanisms leading to CAMR are poorly defined. Whereas anti-donor antibodies are identified as a risk factor for graft survival,3,4 preexisting T-cell memory is associated with high incidence and severe rejection episodes,5 and recipients prone to acute rejection have a higher precursor frequency of alloreactive CD8 T cells than nonrejectors.6 Using an experimental model of CAMR,7 we previously reported that a similar T-cell receptor (TCR) Vβ selection of CD8 T cells

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S.B. and N.D. contributed equally to this work.

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Correspondence: Dr. Jean-Paul Souillou, Pavillon Jean Monnet, Hôtel Dieu, CHU de Nantes, 30, Bd Jean Monnet, 44093 Nantes cedex 01, France. Email: jean-paul.souillou@univ-nantes.fr

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can be identified in the blood and graft of recipients.8 This profile was associated with transcript coding for granzyme B (GZM-B) in the graft.9–11 Similar observations made in the blood of CAMR patients reported a restricted TCR Vβ repertoire, an increase in IFN-γ, GZM-B, and perforin-1 (PERF-1) transcripts, and an increase in CD8 T cells.12–14 These observations suggest that alteration in the TCR Vβ repertoire of CD8 T cells may be associated with kidney dysfunction.

We previously reported that different shapes of TCR Vβ repertoire are identified in patients with stable graft function, despite the stringent clinical criteria used to constitute a homogeneous group.14 In this prospective study, we examined CD8 T-cell phenotype and function and the long-term clinical outcome of these patients with stable graft function (n=131) who exhibit different usage of their TCR Vβ repertoire. We found that the restriction of the TCR Vβ repertoire diversity is associated with an increase of highly differentiated terminally differentiated effector memory (TEMRA; CD45RA+CCR7− CD27−CD28−) CD8 T cells, which are characterized by a high expression of cytotoxic molecules, PERF and GZM-B, T-bet, and CD57 and the ability to secrete TNF-α and IFN-γ. During the follow-up period, 47 patients exhibit kidney dysfunction. Using an unsupervised clustering strategy, we report that stable patients who have an increase in highly differentiated TEMRA CD8 T cells have a 2-fold higher risk of long-term graft dysfunction (P=0.06). Additional prospective clinical investigation will be necessary to provide definitive proof of the association between kidney graft dysfunction and the accumulation of highly differentiated TEMRA CD8 T cells.

RESULTS

Study Population

In total, 131 kidney transplant recipients who displayed a stable graft function (Table 1) were enrolled. At the time of inclusion, patients’ TCR Vβ repertoire was analyzed, T-cell phenotype and function were characterized, and signal joint TCR excision circle (sjTREC) levels were measured (Figure 1). With more than 6 years of follow-up, the kidney graft was re-evaluated for graft dysfunction.

Reduction in TCR Vβ Repertoire Diversity Is Associated with an Increase of Highly Differentiated TEMRA (CD45RA−CCR7− CD27−CD28−) CD8 T cells

Of 131 patients (median time post-transplantation=7.78 years, range=5.01–21.66 years), 45 patients exhibited a restricted TCR Vβ repertoire (median time post-transplantation=6.55 years; range=5.11–19.58 years), and 86 patients did not (median time post-transplantation=8.10 years; range=5.01–21.66 years) (Table 1). Patients with a restricted TCR Vβ repertoire were older (P<0.001), transplanted with an older kidney (P=0.002), and more frequently treated with mycophenolate-mofetil (P=0.01) compared with patients with a diverse TCR Vβ repertoire (Table 1). All the other clinical parameters were similar between the two groups. CD8 T cells were classified as naive (CD45RA−CCR7−), central memory (CD45RA− CCR7+), effector memory (EM; CD45RA− CCR7−), or TEMRA (CD45RA+CCR7−).15,16 CD28 and CD27 expressions were also used to identify early (CD27+CD28−), intermediate (CD28−CD27+), and late (CD28−CD27−)16 differentiated cells (Supplemental Figure 1). Patients with a restricted TCR Vβ repertoire exhibit a higher frequency of CD45RA−CCR7− TEMRA CD8 T cells compared with patients with a diverse TCR Vβ repertoire (52.74±2.96% versus 31.39±1.99%; P<0.001) (Figure 2A) and a decrease in CD45RA−CCR7− naïve CD8 T-cell frequency (14.13±1.62% versus 29.31±1.82%; P<0.001) (Figure 2A).

A restricted TCR Vβ repertoire was associated with a marked increase in late differentiated CD27−CD28+ CD8 T cells (55.13±3.14% versus 23.06±2.30%; P<0.001) (Figure 2B). Finally, an increase in CD27−CD28− T cells was associated with a significant decrease in CD27−CD28+ T cells in EM and TEMRA CD8 T cells in restricted TCR Vβ repertoire patients (P<0.001) (Figure 2C). Collectively, the restriction in TCR Vβ diversity was associated with an expansion of TEMRA cells with highly differentiated phenotype.

CD8 T Cells in Patients with Restricted TCR Vβ Repertoire Showed High Cytotoxic Molecule Expression

A significant increase of CD8 T cells expressing either GZM-B only (28.04±3.05%; P=0.007) or GZM-B and PERF (30.61±2.83%; P<0.001) was observed in patients with a restricted TCR Vβ repertoire. Three levels of expression of PERF were observed within CD8 T cells (Figure 3B). CD8 T cells with a restricted TCR Vβ repertoire exhibit a higher expression of PERF compared with patients with a diverse TCR Vβ repertoire (PERF hi: 21.04±2.80% versus 7.84±1.82%; P<0.001) (Figure 3C). The enhanced expression of PERF was associated with an increase in the mean fluorescence intensity of GZM-B (diverse TCR Vβ repertoire [4375±487] versus restricted TCR Vβ repertoire [5809±283]; P=0.008) (Figure 3D). Finally, a positive correlation between the expression of PERF and GZM-B was observed (R2=0.46, P<0.001).

High cytolytic potential can be measured using the expression of CD57.17,18 Patients with restricted TCR Vβ repertoire display a higher frequency of CD57+ CD8 T cells compared with patients with a diverse TCR Vβ repertoire (47.75±3.29% versus 26.83±2.48%; P<0.001) (Figure 3E). The frequency of T-bet low CD8 T cells was similar between patients, patients with a restricted TCR Vβ repertoire exhibited a marked
increase in T-bet<sup>high</sup> CD8<sup>+</sup> T cells (44.05±4.05% versus 25.25±1.88%; P<0.001) (Figure 4A). The expression of T-bet<sup>high</sup> was positively correlated with CD57 expression (R<sup>2</sup>=0.40; P<0.001) (Figure 4B). Most of the T-bet<sup>high</sup> CD8<sup>+</sup> T cells express the effector-associated marker CD57, lose the expression of CD27 and CD28, and are preferentially found within the EM and TEMRA CD8<sup>+</sup> T cell subset (Figure 4C). Altogether, patients with a restricted TCR V<sub>β</sub> repertoire exhibit T-bet<sup>high</sup> CD8<sup>+</sup> T cells with an increased expression of CD57 (67.37±2.34% versus 52.86±2.13%; P=0.001) (Figure 4D) associated with a highly differentiated phenotype of CD27<sup>-</sup>CD28<sup>-</sup> T cells (75.57±2.50% versus 44.76±3.26%; P<0.001) (Figure 4D) and an increase in TEMRA CD45RA<sup>+</sup>CCR7<sup>-</sup> T cells (59.45±3.06% versus 48.00±2.67%; P=0.02) (Figure 4D).

Downregulation of CD127 by CD8<sup>+</sup> T Cells in Patients with Restricted TCR V<sub>β</sub> Repertoire

High expression of CD127 (IL-7R<sub>a</sub>) allows the maintenance of a diverse repertoire of naïve CD8<sup>+</sup> T cells. We report that the level of CD127 expression was lower in patients with a restricted TCR V<sub>β</sub> repertoire (Figure 5, A and B). Whereas the frequency of CD127<sup>dim</sup> CD8<sup>+</sup> T cells with an increased expression of CD57 (67.37±2.34% versus 52.86±2.13%; P<0.001) (Figure 4D) associated with a highly differentiated phenotype of CD27<sup>-</sup>CD28<sup>-</sup> T cells (75.57±2.50% versus 44.76±3.26%; P<0.001) (Figure 4D) and an increase in TEMRA CD45RA<sup>+</sup>CCR7<sup>-</sup> T cells (59.45±3.06% versus 48.00±2.67%; P=0.02) (Figure 4D).
versus 54.22±2.10%; P<0.001 and 75.28±2.90% versus 63.06±2.44%; P=0.006) (Figure 5C).

Collectively, the reduction of TCR Vβ repertoire in kidney transplant recipients is associated with a profound downregulation of CD127 in CD8 T cells with a highly differentiated phenotype.

**CD8 T Cells in Patients with Restricted TCR Vβ Repertoire Exhibit Potent Effector and Cytotoxic Functions**

Restriction of TCR Vβ repertoire diversity was associated with an increase in T-bet+ TNF-α+ CD8 T cells (14.52±1.64% versus 4.29±0.66%; P<0.001) (Figure 6A) and T-bet+ IFN-γ+ CD8 T cells (6.76±1.03% versus 1.86±0.57%; P<0.001) (Figure 6A) after short-term polyclonal stimulation. IFN-γ–secreting CD8 T cells were virtually all enclosed within TNF-α+ CD8 T cells. Polyclonal stimulation results in a fast release of cytotoxic granules as assessed by CD107a upregulation at the cell surface (7.11±2.35% versus 1.69±0.65%; P=0.003) (Figure 6B) in patients with a restricted TCR Vβ repertoire. Collectively, the restriction of TCR Vβ repertoire is associated with the acquisition of potent effector function markers of CD8+ T cells.

**Reduction in Thymic Output Does Not Account for the TCR Vβ Repertoire Alteration**

Given the accumulation of highly differentiated CD8 TEMRA cells in patients with a restricted TCR Vβ repertoire, we asked whether these patients could have a defective thymic T-cell output. A generalized linear model was used to take into account potential confounding factors (recipient age and sex). The difference in sjTREC levels was not statistically significant between the two groups of patients (Supplemental Table 1).

**Increase of Highly Differentiated TEMRA CD8 T Cells Is a Risk Factor for Long-Term Graft Dysfunction**

Finally, we examined the association between accumulation of highly differentiated CD8 T cells and graft function outcome. A set of markers, linked phenotypically and functionally (TEMRA+CD27−CD28−CD57−T-bet+GZM-B+PERF+), allows the segregation of the population into two groups of 54 and 50 patients according to the level of differentiated TEMRA CD8 T cells (Figure 7A). We assessed whether the kidney dysfunction (47 of 131 patients) (Table 2) was associated with markers expressed by CD8 T cells. Graft dysfunction was defined as significant proteinuria greater than 1 g/d (10 patients), an isolated increase of the creatinine level without proteinuria (20 patients), or both increased proteinuria and creatinine levels (17 patients). Only two patients had an isolated episode of proteinuria, six patients had an isolated episode of

**Figure 2.** Reduction in TCR Vβ repertoire diversity is associated with an increase of highly differentiated TEMRA (CD45RA−CCR7−CD27−CD28−) CD8 effector T cells. Expression of (A) CD45RA and CCR7 and (B) CD27 and CD28 was measured in CD8 T cells in PBMCs from stable patients with a diverse (n=71; open circles) or restricted (n=35; filled circles) TCR Vβ repertoire. CD3+CD8+ cells were gated by morphology and viability before being subdivided into (A) central memory (CM), naïve, TEMRA, and EM subsets based on the expression of CD45RA and CCR7 or (B) CD27 and CD28 subsets. (C) Expression of CD27 and CD28 within CM, naïve, TEMRA, and EM subsets. Each dot represents one individual, and the mean and the SEM are displayed.

Comparison of the frequency of each CD8 subset was performed using a Holm multiple comparison test. *P<0.05; **P<0.01; ***P<0.001.
Figure 3. CD8 T cells in patients with restricted TCR Vβ repertoire showed a high expression of cytotoxic molecules (GZM-B and PERF) compared with patients with a polyclonal TCR Vβ repertoire. (A) CD3⁺CD8⁺ cells from patients with a restricted TCR Vβ repertoire (n=35; filled circles) express high amounts of GZM-B with and without the coexpression of PERF compared with cells from diverse TCR Vβ repertoire patients (n=71; open circles). (B) Three levels of PERF expression could be identified. Representative flow cytometry plots.
functional degradation, and six patients displayed concomitant donor-specific antibodies (six were suspicious; i.e., HLA-DQ donor typing not done). Among six patients who were subjected to a biopsy, four patients presented lesions of chronic humoral rejection, one patient had isolated transplant glomerulopathy, and one patient had acute cellular rejection after immunosuppression withdrawal. A biostatistical model was developed to adjust for the different times at which patients were enrolled post-transplantation (Table 1) and followed (median time of follow-up=15.01 years; range=5.60–29.71 years). The probability of late graft dysfunction during the 10 years after the fifth anniversary of transplantation was 41.6% (95% confidence interval, 20.7% to 57.0%) for patients with an increase of highly differentiated TEMRA CD8 T cells, whereas it was only 23.0% (95% confidence interval, 6.5% to 36.5%) for the other group of patients (Figure 7B). Independently from the time post-transplantation, recipient and donor age, recipient and donor sex, number of HLA mismatches above four, induction therapy, plasma renin activity (PRA) at inclusion, and eGFR at inclusion, patients with an increase of highly differentiated TEMRA CD8 T cells had a 1.96-fold higher risk of graft dysfunction during their follow-up and before their 15th graft anniversary (P=0.06) (Table 3). The increased risk of kidney dysfunction was observed preferentially in the first 5 years after inclusion (Figure 7B). At later time points, the increased risk was similar between the two groups.

Collectively, CD8 T-cell monitoring allows for the identification of patients with higher risk of graft dysfunction at a distance from the actual occurrence of kidney dysfunction.

**DISCUSSION**

Using a prospective analysis of a cohort of patients with long-term stable kidney function, we found that highly differentiated TEMRA CD8 T cells accumulate in patients with a restricted TCR Vβ repertoire. Despite exhibiting stable graft function at the time of analysis, patients with reduced TCR Vβ repertoire diversity exhibit an accumulation of highly differentiated TEMRA (CD45RA⁺CCR7⁺CD27⁻CD28⁻) CD8 T cells with all the attributes of cytotoxic and effector cells. Of interest, similar modifications were observed in the CD4 compartment (data not shown). We also report that patients with an increase of highly differentiated TEMRA CD8 T cells have higher risk of graft dysfunction (hazard ratio, 1.96; 95% confidence interval, 0.97–4.05; P=0.06). The P value may be explained by the low sample size (n=131) and incomplete data (truncated and censored observations). Rather than using only the P values, the size of the effect should be used to judge on the clinical importance of a result, because the risk of incorrectly stating that accumulation of highly differentiated TEMRA is a risk factor of graft dysfunction was as low as 6%.

A subgroup of patients with stable graft function was characterized by a restriction of TCR Vβ repertoire diversity and an accumulation of GZM-B⁺PERF⁺ CD8 T cells with high cytotoxic function. The expression of the two cytotoxic molecules was positively correlated and associated with CD57 expression, which identifies high cytotoxic potential cells. Interestingly, increased PERF and GZM-B expression has also been associated with acute rejection. Our data suggest that high expression of these molecules also implicates adverse chronic events. Therefore, CD8 T cells of patients with a restricted TCR Vβ repertoire have a greater potential of lysis. The identification of the potential target will be of great importance. Based on our prior work, viral targets could be excluded, because we showed no link between viral infection and the shape of the TCR Vβ repertoire. Moreover, active inflammatory processes, including viral and bacterial infections, were exclusion criteria in the current study. Anti-cytomegalovirus IgG prevalence was similar in patients with highly different TCR Vβ repertoire typologies.

The accumulation of effector CD8 T cells was strengthened by the enhanced T-bet expression, which is crucial in T11 T-cell commitment and CD8 T-cell differentiation and function. Moreover, T-bet⁺ CD8 T cells expressed the highest levels of PERF and GZM-B and are associated with HIV-specific cytotoxic function. Interestingly, we found that T-bet⁺ expression in CD8 T cells correlated with cytotoxic potential marker CD57 expression (R²=0.40; P<0.001). Moreover, the ability to secrete TNF-α and IFN-γ was restricted to T-bet expressing CD8 T cells, which confirmed the functionality of the TEMRA CD8 T cells within the at-risk patient group.

A profound decrease in CD127 was observed in patients with restricted TCR Vβ repertoire, suggesting that TEMRA CD8 T cells do not rely on IL-7–related homeostasis. CD127low CD8 T cells were preferentially found within the CD45RA⁺CCR7⁺CD27⁻CD28⁻ subsets, a phenotype consistent with the reported prevalence of low levels of CD127 in effector CD8 T cells. Downregulation of CD127 expression in CD8 T cells has been observed in latent viral or chronic viral infections and correlated with CD8 T-cell exhaustion. We found that prolonged exposure to allogeneic antigens does not result in similar exhaustion. Patients with a diverse TCR Vβ repertoire retain high expression of CD127 on CD8 T cells; because these patients also exhibit a high frequency of naïve CD8 T cells, the retention of high expression of IL-7R is in
agreement with the fact that the maintenance of naïve CD8 T cell pool relies on intermittent response to IL-7 and self-TCR. The modulation of IL-7R expression may be caused by a switch in survival factors, leading to either the maintenance of naïve CD8 T cells with diverse TCR Vβ repertoire or the expansion of TEMRA CD8 T cells with restricted TCR Vβ repertoire. TCR Vβ repertoire restriction results in decreased CD127 expression without altering CD8 T-cell effector functions, which was exemplified by the high expression of T-bet, CD57, GZM-B, and PERF by CD127low CD8 T cells. However, low expression of CD127 by TEMRA CD27− CD28− CD8 T cells does not necessarily preclude their ability to respond to IL-7.

Longitudinal monitoring of the CD8 T cells compartment will be needed to document the differentiation from naïve to TEMRA cells. Before transplantation, an increase of TEMRA CD8 T cells and a decrease of naïve CD8 T cells were associated with a lower risk of acute rejection. The reduction of T-cell diversity may reflect a reduction of alloreactive T cells before transplantation. In contrast, we identified that the increase of TEMRA CD8 T cells and the decrease of naïve CD8 T cells were risk factors for long-term graft dysfunction. Moreover, TEMRA CD8 T cells exhibit potent effector functions, including the ability to secrete proinflammatory cytokines and cytotoxic molecules. Whereas at the early stages post-transplantation, the T-cell immunity is controlled by the immunosuppressive drugs, it is possible that there is a gradual leakage in the control of the antidonor immune response, leading to an expansion of TEMRA CD8 T cells. The continuous stimulation of the alloimmune system is evidenced by CD27 and CD28 downregulation. Controlling TEMRA CD8 T-cell function and preventing their deleterious effects will be the next challenge. Targeting the CD28-CD80/86 pathway in patients with CD80/86 antagonists (Belatacept) or using monovalent anti-CD28 mAb may not be effective on TEMRA CD8 T cells given the lack of expression of costimulatory molecules (CD28 and CD27). An interesting candidate could be anti-TNF treatment that decreases the granulysin-expressing CD8+ CCR7− CD45RA− effector memory T-cell population.

Collectively, our study provides new evidence implicating blood CD8 T cells in kidney graft dysfunction. Additional prospective clinical investigation will be necessary to provide definitive proof of the association between kidney graft dysfunction and the accumulation of highly differentiated TEMRA CD8 T cells. Monitoring CD8 T-cell differentiation may be useful for identifying patients at risk of kidney dysfunction. Innovative therapies targeting the CD8 TEMRA compartment may offer new opportunities to prevent and treat kidney dysfunction.

Figure 4. CD8 T cells in patients with restricted TCR Vβ repertoire expressed higher levels of T-bet than patients with diverse TCR Vβ repertoire. (A) Frequency of T-betneg, T-betnull, and T-bethigh CD8 T cells was measured in CD8 T cells in PBMCs from stable patients with a diverse (n=71; open circles) or restricted TR Vβ repertoire (n=35; filled circles). (B) The frequency of CD57+ CD8 T cells was plotted against the frequency of T-bethigh CD8 T cells within all patients. Linear regression was performed to determine statistical significance, and the regression factor is indicated. (C) Phenotype of T-betneg (white bars) versus T-bethigh (black bars) of the whole cohort. (D) Phenotype of T-bethigh CD8 T cells from stable patients with a diverse (n=71; black bars) or restricted TCR Vβ repertoire (n=35; white bars). Comparison of the frequency of the each CD8 subset was performed using a Holm multiple comparison test. *P<0.05; ***P<0.001. CM, central memory.
Figure 5. Downregulation of CD127 by CD8 T cells in patients with restricted TCR Vβ repertoire. (A) Three levels of CD127 expression could be identified. Representative flow cytometry plots showing CD45RA and CCR7 or CD27 and CD28 by CD127high, CD127int, and CD127low CD3+CD8+ cells in stable patients with a diverse (left panel) or restricted TCR Vβ repertoire (right panel). The various CD127 populations (blue) were overlaid onto dot plots (red) of total CD8+ T cells. (B) Frequency of CD127high, CD127int, and CD127low CD3+CD8+ cells in patients with a diverse TCR Vβ repertoire (n=71; open circles) or a restricted TCR Vβ repertoire (n=35; filled circles). Each dot represents one individual, and the mean and the SEM are displayed. (C) Phenotype of CD127low CD8 T cells from stable patients with a diverse (n=71; black bars) or restricted TCR Vβ repertoire (n=35; white bars). The mean and the SEM are displayed. Comparison of the frequency of the each CD8 subset was performed using a Holm multiple comparison test. *P<0.05; **P<0.01; ***P<0.001. CM, central memory.
Comparison of the frequency of each CD8 subset was performed 6 hours, and expression of CD107a was measured on CD8 T cells. were stimulated with plate-bound anti-CD3 anti-CD28.2 mAbs for

**Figure 6.** CD8 T cells in patients with restricted TCR Vβ repertoire exhibit potent effector functions (secretion of proinflammatory cytokines [TNF-α and IFN-γ] and cytotoxic function). (A) PBMCs from patients with a diverse TCR Vβ repertoire \( (n=18) \) or a restricted TCR Vβ repertoire \( (n=16) \) were stimulated with plate-bound anti-CD3 anti-CD28.2 mAbs for 6 hours, and expression of T-bet, IFN-γ, and TNF-α was measured on CD8 T cells. The mean and the SEM are displayed. (B) PBMCs from patients with a diverse TCR Vβ repertoire \( (n=15) \) or a restricted TCR Vβ repertoire \( (n=13) \) were stimulated with plate-bound anti-CD3 anti-CD28.2 mAbs for 6 hours, and expression of CD107a was measured on CD8 T cells. Comparison of the frequency of each CD8 subset was performed using a Holm multiple comparison test. ***P<0.001.

**CONCISE METHODS**

**Subjects and Ethics Statement**
The University Hospital Ethical Committee and the Committee for the Protection of Patients from Biologic Risks approved the study. All kidney transplant recipients gave informed consent; 131 transplant recipients who had received a first and unique kidney transplant from a deceased donor and displayed a stable graft function (Modification of Diet in Renal Disease eGFR>40 ml/min and proteinuria<1 g/24 h) for at least 5 years were enrolled. Patients were prescreened and designated as stable according to an eGFR above 40 ml/min, a stable creatinemia (±25% of the mean value of creatinemia in the year before the inclusion), and a daily proteinuria<1 g/d. Criteria of graft stability were confirmed at 3 months. Graft dysfunction was defined by at least three values of creatinemia above 25% of the basal level within the last 2 years of follow-up and/or a proteinuria>1 g/24 h.

Patients received tacrolimus or cyclosporine A for maintenance therapy with or without mycophenolate mofetil, azathioprine, and/or steroids. All patients were compliant with the medical description and had not undergone an episode of rejection or ongoing infection during the monitoring period. At the inclusion, anticlass I PRA was detected for three patients, and anticlass II PRA was detected for six patients, including two patients who exhibited both anticlass I and II PRA.

**Characterization of the TCR Vβ Repertoire and Identification of Restricted TCR Vβ Repertoire**
The TcLandscape was performed as previously described by combining the complementary determining region 3 length distribution with each normalized amount of Vβ transcript. A capillary sequencer (Applied Biosystems 3730) was used to determine the CDR3 length distribution after amplification of the Vβ genes transcript. The level of Vβ family transcripts was measured by quantitative RT-PCR and normalized by a reference gene (HPRT). TCR Vβ repertoires were stratified into diverse and restricted profiles using the rules recently developed.

**Study Design**
One hundred thirty-one patients were prospectively recruited according to the exclusion/inclusion criteria described above (Figure 1). Patients were classified into two groups according to the alteration of the TCR Vβ repertoire (diverse stable, \( n=86 \); restricted stable, \( n=45 \)). Among 131 patients recruited, frozen PBMCs were available for 106 patients (diverse stable, \( n=71 \); restricted stable, \( n=35 \)) and subjected to phenotypic and functional analyses at the time of inclusion. A fraction of the patients was selected to characterize the transcripts expression of a selected CD8-related genes set (diverse stable, \( n=17 \); restricted stable, \( n=12 \)). The selection of the patients was done to minimize the disparity of the demographic and clinical parameters (time post-transplantation, recipient age and sex, donor age and sex, and HLA mismatches).

**Blood Samples**
PBMCs were separated on a Ficoll gradient layer and frozen in 10% DMSO autologous serum.

**Polychromatic Flow Cytometry**
Cells were analyzed with an LSRII flow cytometer (BD Immunocytometry Systems); \( 2 \times 10^6 \) frozen PBMCs were surface-stained with antibodies specific for CD3 (BW264/56; VioBlue), CD8 (BW135/80; VioGreen), CD45RA (T6D11; APC-Vio770), CCR7 (3D12; PE-Cy7), CD27 (L128; Brilliant Violet 605), and CD28 (CD28.2; PE-CF594). In addition to this core-staining cocktail, different combinations of antibody were used: CD127 (MB15–18C9; PE), CD57 (TB03; FITC), T-bet (O4–46; PE), GZM-B (GB11; Alexa Fluor 700), and PERF (B-D48; PE). Yellow LIVE/DEAD Fixable Dead Cell Stain Kit was used to exclude dead cells from analysis. BD CompBeads stained separately with individual mAbs were used to define the compensation matrix. Data were analyzed using FlowJo Version 9.0.1 (TreeStar). All the
Figure 7. Identification of patients at risk of late kidney dysfunction based on the features of CD8 T cells. (A) Unsupervised clustering of kidney recipients based on CD8 T-cell markers measured by flow cytometry at the time of inclusion. Two groups of patients (referred as left and right CD8) are identified. (B) The cumulative probability of graft dysfunction was assessed in patients with stable graft function according to the shape of the TCR Vβ repertoire (restricted versus diverse TCR Vβ repertoire) and the time post-transplantation after the fifth anniversary of transplantation. At the end of the follow-up, the low remaining number of transplant recipients explained the sudden increase in kidney dysfunction. CM, central memory.
Table 2. Summary of clinical characteristics of patients with a kidney dysfunction

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<th>SD or Percentage</th>
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<td>Time between inclusion and kidney dysfunction (yr)</td>
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<td>Anti-HLA antibodies</td>
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Table 3. Multivariate analysis of the time between the fifth anniversary of transplantation and the time of graft dysfunction using a Cox model adapted to truncated data

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<td>-0.68</td>
<td>0.37</td>
<td>1.96 (0.96–4.05)</td>
<td>0.06</td>
</tr>
<tr>
<td>Recipient age (yr)</td>
<td>-0.03</td>
<td>0.02</td>
<td>0.97 (0.94–1.01)</td>
<td>0.15</td>
</tr>
<tr>
<td>Donor age (yr)</td>
<td>0.04</td>
<td>0.02</td>
<td>1.04 (1.00–1.08)</td>
<td>0.03</td>
</tr>
<tr>
<td>Recipient sex (woman versus man)</td>
<td>-0.90</td>
<td>0.42</td>
<td>0.41 (0.18–0.97)</td>
<td>0.04</td>
</tr>
<tr>
<td>Donor sex (woman versus man)</td>
<td>-1.37</td>
<td>0.50</td>
<td>0.26 (0.09–0.68)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HLA (A+B+DR) mismatches</td>
<td>0.46</td>
<td>0.45</td>
<td>1.58 (0.66–3.81)</td>
<td>0.30</td>
</tr>
<tr>
<td>eGFR level at collection (ml/min)</td>
<td>-0.02</td>
<td>0.01</td>
<td>0.98 (0.95–1.00)</td>
<td>0.06</td>
</tr>
<tr>
<td>PRA at inclusion</td>
<td>-0.28</td>
<td>0.47</td>
<td>0.76 (0.30–1.89)</td>
<td>0.56</td>
</tr>
<tr>
<td>Polyclonal induction therapy (yes versus no)</td>
<td>-0.10</td>
<td>0.40</td>
<td>0.91 (0.42–1.96)</td>
<td>0.80</td>
</tr>
</tbody>
</table>

The confounding variables were all reinforced in the model, and the Wald’s test was used in this model to test for correlations. Patients were classified into two groups according to the clustering based on the expression of phenotypic markers. 95% CI, 95% confidence interval.

Functional Assays

PBMCs were thawed and rested overnight in complete RPMI medium (10% fetal calf serum). Cell concentration was adjusted at 2×10⁶ cells/ml and stimulated for 6 hours with coated anti-CD3 (3 μg/ml) and anti-CD28.2 mAb (5 μg/ml) in a final volume of 1 ml in 24-well flat-bottom plates. When indicated, PE-conjugated anti-CD107a mAb (10 μl/well) was added at the beginning of the culture. After 2 hours, monensin (1 μg/ml; in a well with anti-CD107a mAb) or brefeldin A (5 μg/ml) was added. PBMCs were stained for cell surface markers (CD3, CD8, CD45RA, CCR7, CD27, and CD28), fixed, permeabilized according to the manufacturer’s procedure (eBiosciences), and then, stained for IFN-γ (AlexaFluor 700), TNF-α (FITC), and T-bet (O4–46; PE). A minimum of 1×10⁴ CD3⁺CD8⁺ cells was recorded (median=8×10⁴; range=1×10⁴–2×10⁶).

Quantification of sjTRECs by Real-Time PCR

Genomic DNA was extracted from 5×10⁶ to 10×10⁶ cells stored in TRIzol reagent. Quantification of thymic sjTREC was done by multiplex real-time quantitative PCR. Values were normalized for the genomic copy number using albumin gene quantification. Data were expressed per 150,000 PBMCs.

Statistical Methods

Descriptive Analyses

Quantitative variables were described according to mean and SEM and compared using a t test; the P value was corrected according to Holm’s procedure. Qualitative variables were compared using a Fisher exact test.

Survival Analyses

The outcome was the time between the fifth anniversary of transplantation and the time of graft dysfunction. A nonparametric estimator of survival function for truncated and censored data was used. A Cox model adapted to truncated data was used for additional multivariate analysis. The Wald’s test was used to evaluate the significance of hazard ratios. The model was adjusted on all possible confounding factors according to well established risk factors of renal dysfunction.

sjTREC Analyses

Multivariate analysis was used to evaluate the confounding factors using a generalized linear model with a γ-distribution and inversed link function. The model was adjusted on all possible confounding factors according to well established risk factors of sjTREC modification.

CD8⁺ T-Cell Phenotype and Function Analysis

Analysis and presentation of distributions were performed using Simplified Presentation of Incredibly Complex Evaluations (SPICE version 5.1; http://exon.niaid.nih.gov). Holm multiple comparison test was used to compare the different markers.

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DISCLOSURES
None.

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