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Regulatory T-cell Number in Peripheral Blood at 1 Year Posttransplant as Predictor of Long-term Kidney Graft Survival

David San Segundo, PhD,[1] Luis H. Galván-Espinoza, MD,[2] Emilio Rodrigo, MD, PhD,[2] Juan Irure, BSc,[1] Juan C. Ruiz, MD, PhD,[2] Gema Fernández-Fresnedo, MD, PhD,[2] Laura Riesco, BSc,[1] Jairo Bada, MD,[2] Lara Belmar, MD,[2] and Marcos Lopez-Hoyos, MD, PhD[1]

Background. Regulatory T (Treg) cells play a role in limiting kidney transplant rejection and can potentially promote long-term transplant tolerance. There are no large prospective studies demonstrating the utility of peripheral blood Treg cells as biomarkers for long-term graft outcome in kidney transplantation. The aim of our study was to analyze the influence of the absolute number of peripheral blood Treg cells after transplantation on long-term death-censored graft survival. **Methods.** We monitored the absolute numbers of Treg cells by flow cytometry in nonfrozen samples of peripheral blood in 133 kidney transplant recipients, who were prospectively followed up to 2 years after transplantation. Death-censored graft survival was determined retrospectively in January 2017. **Results.** The mean time of clinical follow-up was 7.4 ± 2.9 years and 24.1% patients suffered death-censored graft loss (DCGL). Patients with high Treg cells 1 year after transplantation and above the median value (14.57 cells/mm^3), showed better death-censored graft survival (5-year survival, 92.5% vs 81.4%, Log-rank P = .030). One-year Treg cells showed a receiver operating characteristic - area under curve of 63.1% (95% confidence interval, 52.9–73.2%, P = 0.026) for predicting DCGL. After multivariate Cox regression analysis, an increased number of peripheral blood Treg cells was a protective factor for DCGL (hazard ratio, 0.961, 95% confidence interval, 0.924–0.998, P = 0.041), irrespectively of 1-year proteinuria and renal function. **Conclusions.** Peripheral blood absolute numbers of Treg cells 1 year after kidney transplantation predict a better long-term graft outcome and may be used as prognostic biomarkers.

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A progressive reduction in acute rejection rates has led to an improvement of kidney graft survival (KGS) throughout the first year, but long-term graft attrition rates remain stable beyond this point.[1] Despite the good results in KGS during the first year, this poor long-term outcome should be improved.[2] Moreover, the use of immunosuppressive drugs provokes an increase in infection and cancer risks, and subsequently, mortality. Thus, the need for individualization strategies in the immunosuppressant treatment is the goal in order to avoid these adverse effects on the kidney transplant recipient (KTR).[3] The reasons for the scarce improvement in KGS are at present under research and the focus is on noninvasive biomarkers as a tool to modulate the immunosuppressant dosing and to predict the survival of the transplanted graft.[4]

The results of in vivo and in vitro studies have suggested an important role of regulatory T (Treg) cells in the field of organ transplantation due to their capacity to suppress effector

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[1]Immunology Service, University Hospital Marqués de Valdecilla-IDIVAL, Santander, Spain.

[2]Nephrology Service, University Hospital Marqués de Valdecilla-IDIVAL, Santander, Spain.

D.S.S. and L.H.G.-E. contributed equally to the authorship of the article.

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Correspondence: Marcos Lopez Hoyos, MD, PhD, University Hospital Marqués de Valdecilla. Immunology Service. B Tower, -1 Floor. Avenida Valdecilla s/n. 39008, Santander, Cantabria, Spain. (marcos.lopez@scsalud.es).

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immune responses.[4,5] Treg cells have been studied in vivo in biopsies of KTRs with promising results, showing a picture of local immune status. However, this invasive procedure could lead to some risks for the graft and is limited by sampling error and inter-observer variability.[6] Noninvasive studies analyzing the Treg cell-associated gene expression in urine might offer a safer means of improving the prediction of the outcome in renal transplants, although it has not been translated into clinical routine.[7] An intermediate option could be to measure Treg cell numbers in peripheral blood, being both minimally invasive and ready to perform. Several studies have related a lower Treg cell level and Treg cell-related mRNA in peripheral blood samples with chronic graft injury in cross-sectional studies,[8-15] but the association of Treg cells with a better kidney transplant outcome has not been consistently found in all the studies.[16,17]

Due to their recent description, there are no long-term prospective studies in KTRs, which monitor peripheral blood Treg cell levels and their association with graft outcome. In 2012, we reported the relationship between a high peripheral blood Treg cell level at 12 months posttransplantation and a long-term better graft survival with a mean follow-up of 62 months in 90 KTRs.[18] In the current study, we show the extension results of the complete cohort consisting of 133 kidney transplants where the Treg cells were prospectively measured in peripheral blood at 6 and 12 months between 2005 and 2011, and where the patients were followed up until January 2017. Importantly, the levels of Treg cells in this study were measured at the same laboratory and in fresh samples. This is of special importance because several works have pointed at a loss of Treg cell phenotype markers after freezing.[19]

METHODS

Patients

A total of 133 consecutive KTR operated in our hospital between 2005 and 2011 were included in the study. The study was approved by the ethics committee of the hospital; all the patients were informed about the study and gave their written consent. The main demographic, clinical, and immunologic parameters are depicted in Table 1. The patients were monitored before transplant and at 6, 12, and 24 months postkidney transplantation. The diagnosis of acute rejection was biopsy-proven. Death-censored graft loss (DCGL) was defined as a return to dialysis therapy or re-transplantation. The causes of DCGL are summarized in **Table S1,** http://links.lww.com/TXD/A179. The immunosuppression was maintained based on trough levels from 4 months posttransplant.

Flow Cytometry

We identified Treg cells as $CD4^+CD25^+CD127^{-/low}Foxp3^+$ cells. More than 95% of $CD4^+CD25^{high}Foxp3^+$ were $CD127^{-/low}$. Peripheral blood effector and regulatory subpopulations were quantified by flow cytometry, as previously described.[20,21] All samples were processed from fresh blood within the first 4 hours after their extraction, and acquired in a FACScalibur (BD Biosciences, San Jose, CA) before 2008, using FACSCanto II (BD Biosciences) thereafter. Daily BD FACS Comp and weekly CST QC (BD Biosciences) were used on FACScalibur and FACSCanto II, respectively, to monitor cytometers performance. The acquisition protocols with both cytometers were validated (**Figures S1,** http://links.lww.com/TXD/A180 **and S2,** http://links.lww.com/TXD/A181), and the software for flow cytometry analysis was FACS Diva (BD Biosciences).

Statistics

The Treg cell levels were nonparametrically distributed as determined by the Kolmogorov-Smirnov fit test. A comparison of Treg cells at different timepoints was assessed by the Wilcoxon paired rank test. Correlations of Treg cell levels at different times were assessed by the Spearman correlation. Graft survival was tested by the Kaplan-Meier survival test. Univariate and multivariate Cox regression analyses and

TABLE 1.

Clinical, demographical, and immunological variables of KTRs

Clinical, demographic, and immunological variables	DCGL (32)	No DCGL (101)	*P*
Donor age: mean ± SD, y	53.3 ± 16.5	51.4 ± 15.6	.54
Recipient age: mean ± SD, y	54.3 ± 13.6	51.4 ± 11.9	.25
Recipient sex (n), F/M	7/25	27/74	.58
HLA-A/-B/-DR matches, mean	0.56/0.50/0.70	0.61/0.43/0.65	.66/.56/.65
Sensitized, n (%)	8 (25)	20 (19.8)	.58
Peak panel reactive of antibodies, mean ± SD	4.91 ± 14.07	3.37 ± 10.01	.50
Current panel reactive of antibodies, mean ± SD	3.21 ± 9.15	0.51 ± 2.57	.11
Delayed graft function, n (%)	7 (5.2)	20 (15)	.80
First year acute rejection, n (%)	16 (50)	9 (9)	.12
Basal IS			
Induction (CD25R-Ab/TG)	13/4	29/4	pNS
CsA/Tac/mTORi	0/28/5	6/94/3	.18/.26/.02*
MMF	32	99	.57
Steroids	32	101	—
IS at 1 y post-Tx			
CsA/Tac/mTORi	0/30/5	2/86/16	.57/.17/.98
MMF/AZA	28/0	98/1	.06/.76
Steroids	7	28	.51

AZA, azathioprine; CsA, cyclosporine; F, female; IS, immunosuppression; M, male; MMF, mycophenolate mofetil; mTORi, mammalian target of rapamycin inhibitors;SD, standard deviation; Tac, tacrolimus; TG, thymoglobulin; Tx, transplant.

receiver operating characteristic curve analysis were performed to establish the robust nature of associations. Statistical analysis was performed by using SPSS version 15.0 (SPSS Inc., Chicago, IL).

RESULTS

All patients had a minimum follow-up of 5 years, the mean follow-up being 7.4 ± 2.9 years, and the graft lost rate in our cohort was 30.8%. Peripheral blood Treg cell levels decreased significantly at 6 months ($P = .0005$) with partial recovery from basal levels at 1 year posttransplantation: median and interquartile range pretransplantation, at 6 months and 1 year posttransplantation were 16.95 [8.54–29.38] cells/mm^3, 11.12 [5.41–20.69] cells/mm^3, and 13.07 [6.62–23.28] cells/mm^3, respectively. Peripheral blood Treg cell levels at 12 and 24 months remained unchanged from levels at 6 months after kidney transplantation (Figure 1).

We assessed the impact of Treg cell levels before transplantation, then at 6 months and 12 months posttransplantation on the risk of DCGL, and analyzed it as a continuous variable. A protective association of Treg cell levels at 12 months postkidney transplant for DCGL was found (Figure 2; hazard ratio [HR], 0.957; 95% confidence interval [CI], 0.922-0.993; $P = .019$), but neither pretransplantation nor 6 months Treg cell numbers showed any relationship to long-term graft survival. Receiver operating characteristic-area under curve of Treg cells at 12 months for predicting DCGL was 63.1% (95% CI, 52.9–73.2%; $P = .026$). The best cutoff value of Treg cells at 12 months posttransplantation to discriminate kidney transplant patients at risk for DCGL was 14.57 Treg cells/mm^3, showing a sensitivity and specificity of 51.5% and 75%, respectively, for DCGL (Figure 3).

Therefore, we did not find an optimal value of Treg cells that would allow us to stratify patients properly, even more so because the Treg cell levels were nonnormally distributed. We decided to stratify the patients based on 12-month Treg cell level tertiles. Those patients with Treg cell levels at 12 months which were >19.51 cells/mm^3 were considered "High Treg cell group," whereas those with Treg cell <7.63 cells/mm^3 were included in "Low Treg cell group." Patients in the high Treg cell group had better death-censored graft survival, although this did not reach statistical significance (Log-rank $P = .146$) (Figure 4).

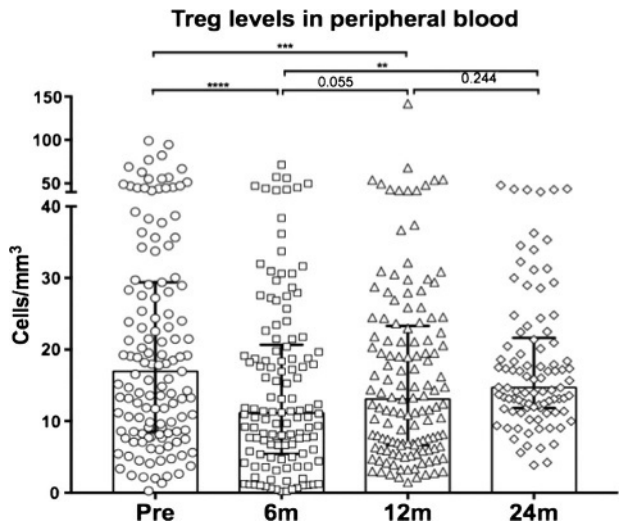


FIGURE 1. Regulatory T (Treg) cell levels in kidney transplant recipients (KTRs). (A) The comparison of the number of Treg cell/mm^3 in peripheral blood of KTRs is depicted and median and interquartile range are shown at different time points (pretransplant, open circles; 6 months posttransplant, open squares; 12 months posttransplant, open triangles; and 24 months posttransplant, open diamonds). Treg cell changes were assessed by the Wilcoxon paired rank test and levels of significance were indicated as $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$.

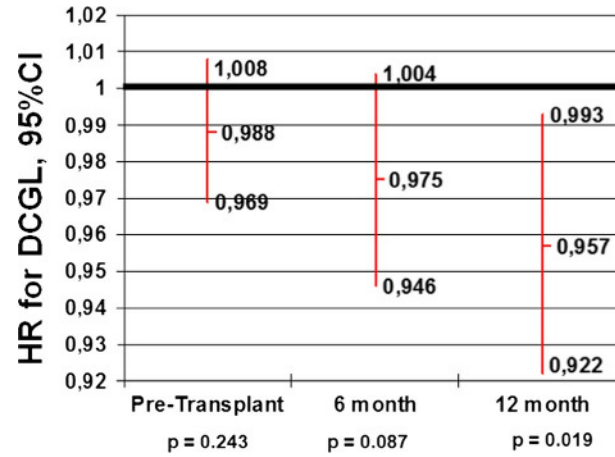


FIGURE 2. Association of early posttransplant Regulatory T (Treg) cell levels and risk of death-censored graft loss (DCGL). The Treg cell levels before transplant, at 6 and 12 months postkidney transplantation were assessed for risk of DCGL by univariate Cox regression analysis. Treg cell levels were analyzed as a continuous variable.

To assess the independent role of Treg cell levels at 12 months postkidney transplantation in DCGL, a multivariate Cox regression model was performed with variables classically involved in DCGL. Among them, we included age at transplant, cold ischemia time, antibody production during the first year, donor age, delayed graft function, biopsy-proven acute rejection with first year, serum creatinine at first year, proteinuria at first year, and absolute numbers of Treg cells at first year (Table 2). A high number of Treg cells in peripheral blood at 12 months after transplantation was a protective factor for DCGL, irrespectively of proteinuria or creatinine at 12 months (HR, 0.961; 95% CI, 0.924–0.998; $P = .041$). However, such an association did not reach statistical significance when considering the percentages of Treg cells (**Table S2,** http://links.lww.com/TXD/A182).

DISCUSSION

The function of Treg cells controlling alloimmune response both in vivo and in vitro has led to propose their role as potential biomarkers of graft outcome.[4,22] Some studies have focused on the presence of Treg cells in kidney biopsies,[7,23] but the identification of the Foxp3$^+$ regulatory cells within the allograft cannot help to differentiate between cytotoxic or tolerogenic infiltrates[24] and the risk of side effects after biopsy discourages its use in monitoring the renal graft. On the contrary, the quantification of Treg cells in peripheral blood is readily accessible for most laboratories with limited drawbacks by using adequate Treg cell markers.[4]

Hence, different authors have reported that patients with chronic allograft nephropathy showed a lower number of peripheral blood Treg cells than those with stable graft

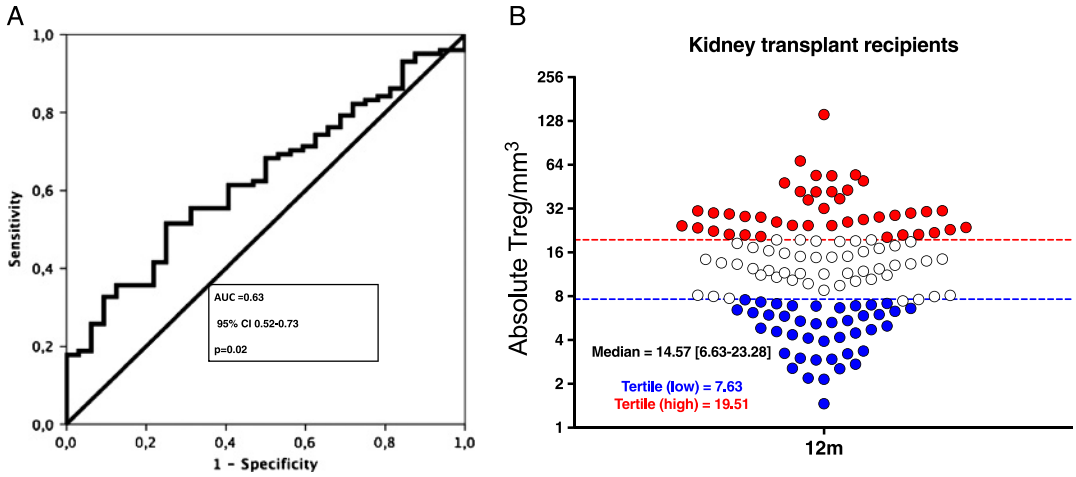
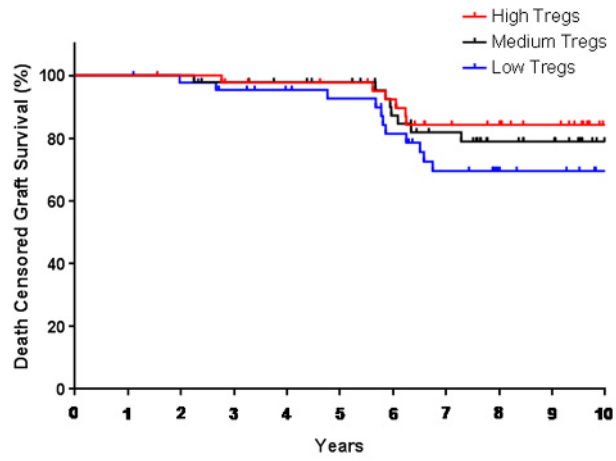


FIGURE 3. (A) Receiver operational curve analysis of regulatory T (Treg) cell levels at 12 months posttransplant and death-censored graft loss (DCGL). The area under curve (AUC) is 63.1%. A cutoff of 14.57 Treg cells/mm^3 in peripheral blood discriminates between DCGL and non-DCGL after kidney transplantation with a sensitivity and a specificity of 51.5% and 75%, respectively. (B) Tertile distribution of Treg cell levels at 12-month postkidney transplantation. Red circles in first tertile and blue circles in third tertile are depicted. White circles represent the second tertile.

function.[8,11-15] Conversely, a higher number of peripheral blood Treg cells has been reported as a marker of operational tolerance in kidney and liver transplant studies, although this finding has not been confirmed in all studies.[11,14,25] Moreover, Lin et al[26] have reported a positive linear relationship between the percentage of Treg cells in peripheral blood lymphocytes and the estimated glomerular filtration rate in kidney transplant patients. All these studies were cross-sectional, performed at different points after transplantation, and they included a low number of patients. The main finding of our current study is that for the first time we have reported an independent relationship between 1 year after transplantation peripheral blood Treg cell number and long-term KGS. In a preliminary study, we reported the relationship between a high peripheral blood Treg cell level at 12 months posttransplantation and a long-term better graft survival with a shorter follow-up in 90 KTRs by only univariate analysis.[18] In the current study, we presented the data with a longer follow-up, including up to 133 patients and carrying out a multivariate study. We did not find an optimal cutoff value of Treg cells that could help to differentiate those KTRs who were going to have a worse graft outcome, but we can conclude that patients with a higher number of circulating Treg cells at 12 month will have a better long-term graft outcome. Besides, this relationship was independent of other variables, which were strongly related with graft outcome, such as renal function and proteinuria. Although we cannot conclude a causal link between the number of Treg cells and a good graft outcome with our design, this finding meets some criteria for the establishment of a cause and effect relationship, such as coherence with known facts, biological plausibility and temporal and dose-response relationships. Despite all this, we have not specifically addressed the relationship between Treg cell number and the causes of graft loss due to their heterogeneous



High Tregs group	43	43	43	41	40	39	36	29	27	21	11
Medium Tregs group	46	46	46	45	43	41	34	29	21	18	7
Low Tregs group	44	44	43	40	37	35	30	24	19	17	13

FIGURE 4. Regulatory T (Treg) cell level stratification and death-censored graft survival. The kidney transplant recipients (KTRs) were stratified based on tertiles of Treg cell levels at 12 months postkidney transplantation. Above first tertile, high Treg cell levels (19.51 Treg cells/mm^3) are shown in red; below third tertile, low Treg cell levels (7.63 Treg cells/mm^3) are shown in blue; and second tertile medium Treg cell levels are shown in black. No statistically significant differences were found among the 3 tertiles by the Kaplan-Meier analysis. The lower table shows the number of patients at risk in each group at the given timepoints.

TABLE 2.

Univariate and multivariate assessments of DCGL after kidney transplantation

Parameters	Univariate	HR (95% CI)	Multivariate	HR (95% CI)
12-month proteinuria	<0.001	1.001 (1.00-1.001)	<0.001	1.001 (1.00-1.001)
12-month serum creatinine	<0.001	3.49 (2.11-5.774)	0.004	2.37 (1.32-4.25)
12-month Treg cell numbers	0.019	0.957 (0.922-0.993)	0.040	0.96 (0.924-0.998)
Recipient age	0.143	1.023 (0.992-1.055)	0.39	1.014 (0.982-1.048)
12-month AR	0.231	1.603 (0.741-3.469)		
Donor age	0.279	1.013 (0.99-1.036)		
DGF	0.830	1.096 (0.474-2.536)		
Cold ischemia	0.841	1.006 (0.949-1.067)		

AR, acute rejection; CI, confidence interval; DCGL, death-censored graft loss; DGF, delayed graft function; HR, hazard ratio; Treg, regulatory T.

etiology. As reported in **Table S1,** http://links.lww.com/TXD/A179, of 23 patients with a biopsy-proven cause of graft loss, an alloimmune etiology was only diagnosed in 11 patients who suffered antibody-mediated rejection, whereas glomerulonephritis recurrence was established in 4 patients, with nonspecific interstitial fibrosis and tubular atrophy was diagnosed in 5 patients. Of note, this distribution of causes of allograft loss is similar to that previously reported.[27]

Importantly, our study was performed with fresh peripheral blood samples. One of the reasons for the differences among studies addressing the quantification of Treg cells in peripheral blood has to do with cryopreservation. Several studies showed that freezing induces a loss of Treg cell phenotype, decreasing the number of cells with such a phenotype.[19] Nonetheless, those cells with Treg cell phenotype maintained their suppressive function after thawing.[28] The importance of a standardized protocol is crucial to having reproducible results in monitoring Treg cells, as well as the use of fresh but not cryopreserved samples.[21]

Our study also confirms previous suggestions about the optimal moment to determine peripheral blood Treg cell measurement as a biomarker to estimate long-term graft outcome.[4] This issue is important because peripheral blood Treg cell numbers are not stable, and they change from pretransplant values throughout the evolution of the transplant. Our group and other authors described a reduction of Treg cell levels at 6 months postkidney transplantation from pretransplant values,[29,30] and this finding was also confirmed in multicenter studies.[21] After this reduction, the absolute count of Treg cells at 12 months of kidney transplantation recover the pretransplant levels.[29] In the current study, Figure 2 shows Treg cell numbers related with the risk of DCGL at 12 months, but not at pretransplant or at 6 months. Hence, their measurement at this point could be useful as a potential biomarker of further long-term kidney graft outcome. The use of circulating Treg cell number at 24 months after transplantation did not add any advantages in the present study. Besides, we consider it important enough not to measure frequencies but absolute numbers of Treg cells, because the statistical significance is lost when considering the Treg cell frequencies in peripheral blood (**Table S2,** http://links.lww.com/TXD/A182). On the other hand, it should be taken into account that the patients suffering from acute rejection would have received higher immunosuppression, which could interfere with Treg cell numbers. In the univariate analysis excluding those patients with acute rejection at 1 year of transplantation, the Treg cell levels were not significantly correlated with DCGL, but they did show a tendency (**Table S3,** http://links.lww.com/TXD/A183). The use of immunosuppression may induce a decrease in the absolute number of lymphocytes with reflection in the numbers of T-cell subsets. However, it cannot have consequences in the frequencies of those subsets, as demonstrated by other authors.[31,32]

Obviously, the percentages and number of Treg cells may be affected by immunosuppressive drugs. It is known that calcineurin inhibitors have a deleterious effect on Treg cells, whereas mammalian target of rapamycin (mTOR) inhibitors do not[20,33] and that there is an inverse correlation between the circulating Treg cells and tacrolimus blood levels.[29] It is possible that our data are limited, because 21 of the patients in our cohort experienced mTOR inhibition therapy at some moment after transplantation and this could bias the results. Indeed, when only the patients in calcineurin inhibitor therapy were selected, but never those with mTOR inhibitors, the statistical significance of the influence of Treg cells at 1 year after transplantation was missed, although there was a trend (**Table S4,** http://links.lww.com/TXD/A184). Regarding induction therapy, thymoglobulin preserves circulating Treg cells,[34] whereas anti-CD25 monoclonal antibodies cause a transient loss of peripheral blood Treg cells[35] with a phenotypic shift of Treg cells from the CD25(+) to the CD25(−) compartment that does not seem to be associated with functional consequences.[36] Due to the sample size, we did not find any relationship between the type of induction therapy, the type of maintenance immunosuppressive drugs or the immunosuppressive blood levels and the number of peripheral blood Treg cells (**Tables S4–S6,** http://links.lww.com/TXD/A184, http://links.lww.com/TXD/A185, http://links.lww.com/TXD/A186). Nonetheless, we could be tempted to conclude that, to have a better long-term graft outcome, we should select immunosuppressive drugs that provide a higher number of Treg cells at 12 months posttransplantation, such as thymoglobulin induction or mTOR inhibitors. Indeed, those patients who received induction therapy almost doubled the number of Treg cells at 12 month posttransplantation, and a high number of circulating Treg cells 12-month posttransplantation showed a trend to protect from DCGL in multivariate analysis, although it did not reach statistical significance (**Table S5**). This conclusion cannot be made without carrying out prospective randomized studies in humans, relating immunosuppressive treatment, the number of regulatory cells at 12 months and the long-term posttransplant evolution, similar to that in animal models.[37]

To conclude, we demonstrated that KTRs who have higher levels of peripheral blood Treg cells at 12 months would have a better long-term graft survival and this association was independent of other 1-year variables, such as proteinuria and renal function. Immunosuppressive treatments or interventions which are able to maintain high Treg cell levels in peripheral blood at 12 months postkidney transplantation could potentially protect from long-term DCGL. One limitation of the present study is that it was conducted in 1 center, whereas multicenter studies should be addressed to better define cutoff values prior to using Treg cell levels at 12 months as a biomarker of DCGL. However, it has the advantage of being addressed in the clinical routine with a well-established flow cytometry protocol on fresh samples.

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