Dynamic Changes of Regulatory T Cell and Dendritic Cell Subsets in Stable Kidney Transplant Patients

A Prospective Analysis

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Introduction. We aimed to identify immune status of the stable kidney allografts from the point of some cellular changes that may occur after transplantation.
Materials and Methods. This study considered 57 patients with no rejection during the 6 months after transplantation. Flow cytometric frequencies of circulatory CD4+CD25+FoxP3+ and CD8+CD28- regulatory T cells (Treg) as well as myeloid dendritic cells type 1 (MDC1) and type 2 (MDC2) and plasmacytoid dendritic cells (PDC) were measured before transplantation and 2 weeks and 1, 3, and 6 months after transplantation. Using adjusted model of repeated measure analysis, we assessed the influence of different parameters on different cell subsets.
Results. The mean number of Tregs and PDCs decreased 2 weeks after transplantation and then increased as they reached their values before transplantation within a few months after transplantation. The mean MDC1s increased during 2 weeks and then decreased to its before-transplantation values within 6 months. The frequency of Tregs (r = 0.90) and MDC1s (r = 0.75) at month 3 could strongly predict their frequencies at month 6. Different variables including family relationship between donor and recipient, glomerular filtration rate, and human leukocyte antigen antibody mismatch did not change the frequency of different cell subsets during the time.
Conclusions. The dynamism and circulatory changes in the frequency of Tregs and PDCs are opposite to MDCs after kidney transplantation. We describe these changes in a group of patients with stable graft; however, our study does not render any idea in patients with unstable or rejecting grafts.

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INTRODUCTION

Survival upon kidney transplantation depends mainly on its function during the first year posttransplantation.[1,2] Clearly, serial biopsy remains as the best way to detect graft status, but it has not favored both the patient and the physician due to being aggressive and having a 0.5% rate of side effects.[3] It is also associated with sampling errors and interpretation biases.[4] Apart from serial biopsies, glomerular filtration rate (GFR) and serum creatinine as well as creatinine clearance provide no information about the immune status of the organ recipient; therefore, they cannot be used to predict the recovery of the graft or the development of nonrejecting grafts.[4] It seems that biomarkers can help us to better evaluate of the immune status

in transplant recipients noninvasively before the transplanted organ is damaged, and to individualize day-to-day immunosuppressive therapy and patient management care.[5-11]

Regulatory T cells (Treg) and dendritic cells (DC) have definite effects on the result of transplantation.[12-18] Regulatory T cells affect the outcome of transplantation via the suppression of CD4+ and CD8+ alloreactive T cells.[19] There are growing reports demonstrating that patients with kidney transplantation suffering from chronic (and sometimes acute) rejection have a diminished Treg phenotype or function compared to tolerant or nonrejecting patients.[20-26] Dendritic cells, as the main antigen-presenting cells, play an important role in immune response or tolerance.[27] Based on the subgrouping and maturity status, these cells can potentially stimulate or inhibit the allo-antigen-specific T cells.[28,29]

This prospective multicenter study aimed to investigate the dynamism of variation in a set of cellular biomarkers in stable kidney transplant recipients. In line with this aim, we examined the dynamic changes of some interacting immune cells, including Treg and DC subsets, during a 6-month observation period under standard immunosuppressive therapies, and investigated whether they were predictable or there could be a clinical utility for their monitoring post transplantation. Furthermore, we explored the influence of various variables including GFR, familial relationship between donors and recipients, and human leukocyte antigen (HLA) antibody mismatch on Treg and DC levels.

MATERIALS AND METHODS

Study Population

In this prospective study, we enrolled a total of 57 consecutive Iranian adult kidney transplant recipients from living donors. The study took place at 2 referral hospitals of Baqyiatallah and Shahid Labbafinejad in Tehran, Iran, between July 2010 and July 2011. Any adult candidate of first kidney transplantation from a living donor was enrolled. The patients excluded from the study were those whose creatinine remained more than 25% above the baseline in spite of receiving high doses of immunosuppressant, ie, pulse therapy with corticosteroids and polyclonal or monoclonal antibodies, persistent cytomegalovirus or BK infection, any active infection, nonimmunologic acute kidney injury, or any acute illness leading to hospitalization.

Study Design

Peripheral blood samples were obtained 5 times (before transplantation, 2 weeks, and 1, 3, and 6 months after transplantation) to measure the frequency of different subsets of Tregs and DCs. Demographic variables of age and sex and clinical characteristics of all patients including percent panel-reactive antibody values and type of immunosuppression therapy were examined. This study was approved by the Ethics Committee of Tehran University of Medical Sciences. A written informed consent was obtained from the participants as well as an additional signed agreement whenever a blood sample was obtained.

Clinical Parameters

The induction as well as maintenance immunosuppression protocol for all recipients was composed of adjusted doses of calcineurin inhibitors (cyclosporine A or tacrolimus), mycophenolate mofetil, and steroids. The type of underlying disease leading to end-stage renal disease, the age and sex of donors and recipients, the number of HLA mismatches, episode of cytomegalovirus and BK infections, graft function according to serum creatinine levels and GFR based on Cockroft-Gault formula, and serum cyclosporine concentrations according to the radioimmunoassay method were recorded for all patients.

Flow Cytometric Analysis

Regulatory T cell analysis. Fresh peripheral blood mononuclear cells were separated from 2 mL of anticoagulated blood by Ficoll-Hypaque (Lymphodex, inno TRAin, Germany) density gradient centrifugation. Natural CD4+CD25+FoxP3+ Tregs were detected in one tube by staining with a cocktail of anti-human surface CD4+-FITC/CD25+-PE, and intracellular FoxP3-PE-Cy5 according to the manufacturer's instructions (eBioscience, San Diego, CA, USA). Inducible CD8+CD28- Tregs were detected in another tube by staining with antihuman CD3-PE, CD8a-PE-Cy5, and CD28-FITC. All antibodies and their isotype-matched controls were purchased from eBioscience (San Diego, CA, USA). The percentages of CD4+CD25+ FoxP3+ and

CD8+CD28- cells were analyzed by a 3-color flow-cytometry using BD FACScalibur flow-cytometry (BD, San Jose, CA, USA). Lymphocytes were gated on the basis of light scattering properties and CD3+ characteristics and at least 10 000 events were obtained for each patient sample.

Dendritic cells analysis. Fresh whole blood DCs were stained by a human specific blood DC enumeration kit according to the manufacturer instructions (Miltenyi Biotec Inc, CA, USA). Plasmacytoid DCs (PDC), myeloid DCs type 1 (MDC1), and myeloid DCs type 2 (MDC2) were detected in one tube by staining with CD303 (BDCA-2)-FITC, CD1c (BDCA-1)-PE, and CD141 (BDCA-3)-APC, respectively. Finally, cells were analyzed by 4-color flow-cytometry using BD FACScalibur flow-cytometry (BD, San Jose, CA, USA) for enumeration of PDCs, MDC1s, and MDC2s and at least 100 000 events were obtained for each patient sample.

Statistical Analysis

The data were expressed as mean ± standard deviation. Based on the distribution of data, we performed parametric and nonparametric tests. The paired *t* test was used to compare mean values of cell frequencies at different time points posttransplantation with that before transplantation. Correlation of flow cytometric measures at different times were tested by the Pearson correlation coefficient. We performed repeated measure analysis of variance to compare flow cytometric data at different time points. A *P* value less than .05 was regarded significant. All analyses were done by the SPSS software (Statistical Package for the Social Sciences, version 16.0, SPSS Inc, Chicago, Ill, USA).

RESULTS

Patients

Of a total of 90 patients, 2 patients died during the first month of transplantation because of cardiovascular events. There were 8 cases with persistent unexplained elevation of creatinine or failure to return to baseline in spite of high dose of intravenous corticosteroids or antithymocyte globulin (all of them were confirmed to have acute rejection by biopsy), 3 cases with serious prolonged pyelonephritis due to stent infection, 2 cases of nonimmunologic acute kidney disease due to urinary obstruction and severe gastroenteritis, 1 case of serious prolonged respiratory infection, 1 case of BK infection, and 16 cases of prolonged polymerase chain reaction-based cytomegalovirus infection who remained positive during 2 consecutive sampling. Eventually, 57 patients composing our stable graft group finalized the study.

There were no important clinical events in these patients except in 12 patients who experienced a rise in serum creatinine which came back to baseline level after pulse therapy with corticosteroids without any need to biopsy and 16 patients who experienced a diagnosis of CMV infection, which was successfully treated with ganciclovir before subsequent sampling. Any sampling simultaneous to these events was postponed to the end of the treatment course. Comparison of different cell subsets between patients with and without serum creatinine elevation (12 versus 45 patients) and with and without posttransplant history of cytomegalovirus infection (16 versus 41 patients) showed no significance (data not shown). Characteristics of the patients are shown in Table 1.

Regulatory T Cells and Dendritic Cells After Transplantation

Table 2 and Figures 1 to 5 show the trends of the changes in the frequency of CD4+CD25+FoxP3+

Table 1. Baseline Characteristics

Characteristic	Value
Mean age, y	44.42 ± 12.42
Sex	
Male	32 (59.1)
Female	25 (43.9)
Underlying disease	
Hypertension	21 (36.8)
Diabetes mellitus	9 (15.8)
Nephrolithiasis	3 (5.3)
Hypertension and nephrolithiasis	7 (12.4)
Diabetes mellitus and hypertension	3 (5.3)
Glomerulonephritis	2 (3.6)
Polycystic kidney disease	2 (3.6)
Nephrotic syndrome	1 (1.8)
Unknown	9 (15.8)
Serum creatinine, mg/dL	
Before transplantation	6.88 ± 2.62
Week 2	1.55 ± 0.74
Month 1	1.69 ± 1.03
Month 3	1.63 ± 0.87
Month 6	1.52 ± 0.74
HLA mismatch	4.75 ± 1.12

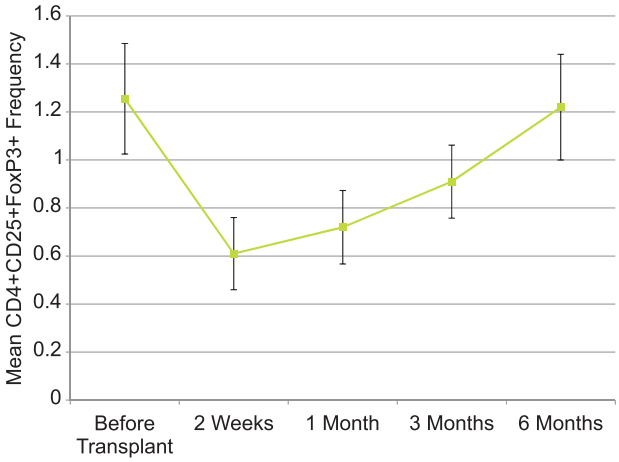

Figure 1. Changes in the frequencies of CD4+CD25+FoxP3+ cells at different times. Data are expressed as mean and 95% confidence interval.

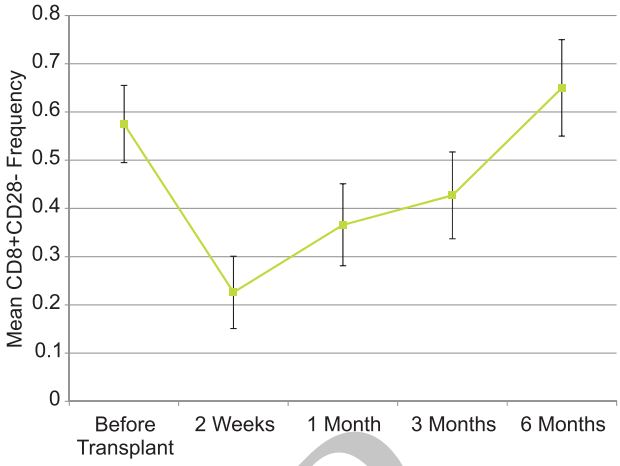

Figure 2. Changes in the frequencies of CD8+CD28- cells in non-rejection group at different times. Data are expressed as mean and 95% confidence interval.

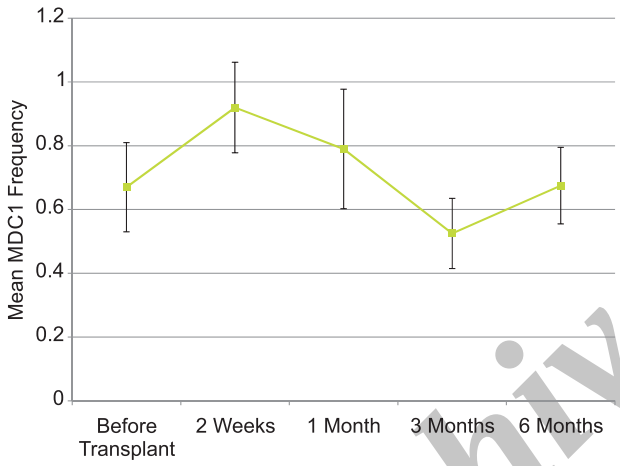

Figure 3. Changes in the frequencies of MDC1 subset at different times. Data are expressed as mean and 95% confidence interval.

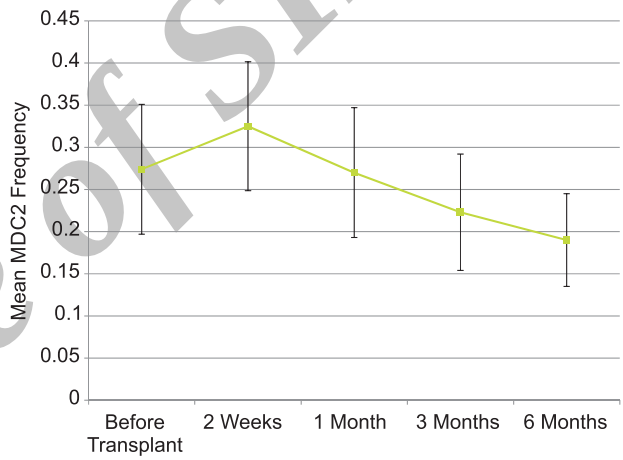

Figure 4. Changes in the frequency of MDC2 subset at different times. Data are expressed as mean and 95% confidence interval.

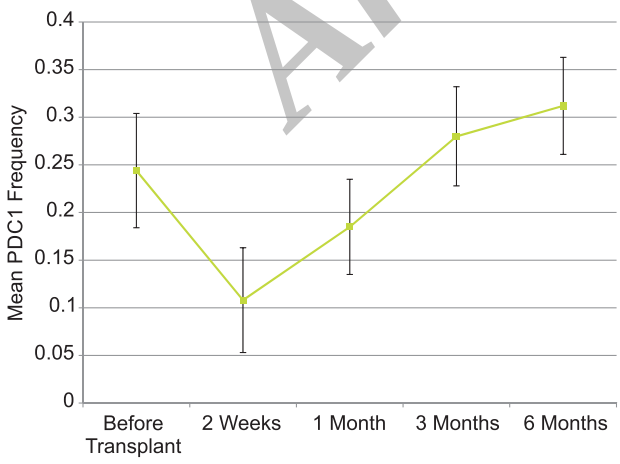

Figure 5. Changes in the frequencies of PDC1 subset at different times. Data are expressed as mean and 95% confidence interval.

cells, CD8+CD28- cells, MDC1s, MDC2s and PDCs. Mean of CD4+CD25+FoxP3+ cells, CD8+CD28- cells and PDCs decreased 2 weeks after transplantation and then gradually increased. The MDC1 and MDC2 subsets had a different trend in which their mean number increased 2 weeks after transplantation and then gradually decreased during the following months.

Repeated Measures Analysis

Adjusting for sex and age, we used repeated measure analysis to assess the influence of different parameters including family relationship between donors and recipients, GFR, and HLA mismatch on the frequency of CD4+CD25+FoxP3+ cells, CD8+CD28- cells, MDC1s, MDC2s, and PDCs

Table 2. Frequency of Regulatory T Cells and Dendritic Cells at Different Time-points Compared to the Values Before Transplantation*

Time Cell	Before Transplant	2 Weeks	1 Month	3 Months	6 Months
CD4+C25+FoxP3+	1.27 ± 0.89 ...	0.61 ± 0.55 *P* < .001	0.71 ± 0.61 *P* < .001	0.91 ± 0.67 *P* < .001	1.19 ± 0.79 *P* = .34
CD8+CD28-	0.58 ± 0.34 ...	0.24 ± 0.28 *P* < .001	0.37 ± 0.33 *P* < .001	0.44 ± 0.40 *P* = .02	0.40 ± 0.05 *P* = .03
MDC1	0.66 ± 0.56 ...	0.92 ± 0.74 *P* < .001	0.80 ± 0.72 *P* = .12	0.52 ± 0.52 *P* = .04	0.68 ± 0.71 *P* = .71
MDC2	0.27 ± 0.27 ...	0.32 ± 0.3 *P* = .21	0.27 ± 0.25 *P* = .98	0.23 ± 0.26 *P* = .37	0.20 ± 0.20 *P* = .04
PDC	0.25 ± 0.27 ...	0.10 ± 0.13 *P* < .001	0.19 ± 0.2 *P* = .16	0.30 ± 0.23 *P* = .33	0.31 ± 0.23 *P* = .02

**P* values are indicative of comparisons with the baseline (before transplantation) values.

during the observational period. None of the above parameters caused an interaction in the frequency of different cell subsets at different time points. It means that there were not any meaningful differences in the marginal mean of cell frequencies according to each parameter. In other words, different variables can not cause any significant alteration in the frequency of different cells during the time (Table 3).

Association of Cell Frequencies at Different Time Points

Table 4 shows the correlation of cell frequencies at different time-points. The most correlation is seen in the case of CD4+CD25+FoxP3+ cells at months 1, 3, and 6 after transplantation. It means that the frequency of such cells at month 1 could strongly predict the frequency of them at month 3 (r = 0.85), and the frequency of such cells at month 3 could strongly predict the frequency of them at month 6 (r = 0.90). In the case of CD8+CD28- cells, the correlation coefficient at different times was not as much as that in CD4+CD25+FoxP3+ cells. The most evident correlation was seen in months 1 and 3 (r = 0.72) as well as months 3 and 6 (r = 0.71) after transplantation.

The number of MDC1 cells showed greater correlation coefficient before transplantation and the 6th month after transplantation (r = 0.81). The number of MDC2s showed greater correlation in months 1 and 3 (r = 0.62) as well as 1 and 6 (r = 0.61) after transplantation. Like MDC2s, PDCs showed no high correlation in which the most correlation was found 1 and 3 months after transplantation (r = 0.67).

DISCUSSION

Our longitudinal cohort had some advantages among which the nature of chosen follow-up interval measurements was in such a way that led us to monitor all probable changes in the frequency of different cells. Also, it lacked some drawbacks seen in other similar conducted studies, ie, they were done either only via retrospective analysis, had a limited number of patients, did not consider the integrity of counteracting cells as we did, or had a shorter follow-up period.

Along with few other studies on CD4+CD25+ FoxP3+ and CD8+CD28- Tregs,[30,31] here, we monitored the changes of Treg frequencies in nonrejecting patients under the same standard and conventional immunosuppression at different times after transplantation. We showed that the Treg subsets were not numerically stable populations and their circulating levels could be different at different time points after transplantation. In

Table 3. Adjusted Repeated Measure Analysis, Comparing the Effects of Different Variables on Different Cell Subsets

Cell Variable	*P*		
	Familial Relationship (Yes versus No)	HLA mismatch (< 5 versus ≤ 5)	Glomerular Filtration Rate (< 60 versus ≤ 60)
CD+CD25+Foxp3+	.59	.33	.98
CD8+CD28-	.14	.96	.79
MDC1	.30	.05	.05
MDC2	.25	.49	.58
PDC	.94	.22	.25

Table 4. Correlation of Regulatory T Cells and Dendritic Cells Frequencies Between Different Times of the Study*

Cell	Before Transplant	2 Weeks	1 Month	3 Months
CD4+CD25+FoxP3+				
2 weeks	0.658 (< .001)	...	...	...
1 month	0.529 (< .001)	0.782 (< .001)	...	...
3 months	0.593 (< .001)	0.733 (< .001)	0.851 (< .001)	...
6 months	0.720 (< .001)	0.768 (< .001)	0.791 (< .001)	0.905 (< .001)
CD8+CD28-				
2 weeks	0.469 (< .001)	...	...	...
1 month	0.492 (< .001)	0.695 (< .001)	...	...
3 months	0.362 (.005)	0.521 (< .001)	0.720 (< .001)	...
6 months	0.612 (< .001)	0.434 (< .001)	0.558 (< .001)	0.707 (< .001)
MDC1				
2 weeks	0.790 (< .001)	...	...	...
1 month	0.573 (< .001)	0.78 (< .001)	...	...
3 months	0.566 (< .001)	0.717 (< .001)	0.729 (< .001)	...
6 months	0.806 (< .001)	0.754 (< .001)	0.655 (< .001)	0.758 (< .001)
MDC2				
2 weeks	0.470 (< .001)	...	...	...
1 month	0.191 (.15)	0.598 (< .001)	...	...
3 months	0.073 (.59)	0.165 (.22)	0.623 (< .001)	...
6 months	0.396 (.002)	0.388 (.003)	0.611 (< .001)	0.516 (< .001)
PDC				
2 weeks	0.510 (< .001)	...	...	...
1 month	0.198 (.14)	0.668 (< .001)	...	...
3 months	0.080 (.55)	0.405 (.002)	0.670 (< .001)	...
6 months	0.632 (< .001)	0.595 (< .001)	0.427 (< .001)	0.572 (< .001)

*Values are Pearson correlation coefficients (*P* values).

line with other studies,[32] we showed that Tregs decreased during the 2 weeks after transplantation. Such an early reduction could be due to high-dose administration of immunosuppressants, namely cyclosporine A, through induction therapy. Cyclosporine A inhibits the proliferation as well as function of Tregs probably through acting on DCs.[33-35] After 2 weeks, there is a clear trend toward rising Treg levels over time concurrent with reduction of immunosuppressive drugs, a concept which states that the number of Tregs could be modulated by the immunosuppressive regimen.[36,37] CD4+CD25+FoxP3+ cells recover within 6 months after transplantation. Such recovery might play a role in the stability of the grafts in our patients, especially considering that the suppressive activity of the CD4+CD25highFOXP3+ cells may not be influenced by presence or absence of immunosuppression.[38] Moreover, the maintenance dose of corticosteroids and mycophenolic acid can possibly improve the suppressive activity and survival of Tregs.[32,39,40]

We also longitudinally investigated 3 DC-subset (MDC1, MDC2, and PDC) frequencies via a precise flow cytometric method in kidney transplant recipients on standard immunosuppression. We excluded dead cells from the analysis that might significantly distort the analysis of these rare leukocyte subsets. In line with Tregs, PDC cells decreased 2 weeks after transplantation and then recovered quickly during one month after transplantation. Fangmann and colleagues,[41] Womer and colleagues,[42,43] and Hesselink and associates[44] also demonstrated that peripheral blood PDCs dramatically decrease immediately after kidney transplantation and afterwards they reached normal levels until the end of the 28-day and 4-month observation periods.[41-43] The reduction in PDC subset levels seen in our transplant recipients during the first 2 weeks may be related to the high doses of immunosuppressants, namely prednisolone,[45-47] received during the perioperative period. Afterwards, it is likely that the fewer prescribed doses of the immunosuppressants lead to more number of PDCs. It is noteworthy that increase of Tregs concurrently with PDCs after transplantation is in part due to PDC-mediated generation of CD4+CD25+FoxP3+ and CD8+ Tregs.[48-51]

Unlike PDCs, the MDC1 levels of our patients were higher 2 weeks after transplantation compared to those before transplantation. This may suggest a severe inflammatory state in newly transplanted patients. It seems that this inflammation counteracts the anti-inflammatory effects of immunosuppressive drugs. A concept which is supported in later time points by observing the changes of DC subsets. Less inflammation in later time points may lead to less number of MDC1s.

Our patients had numerically similar counts of MDC2s in early months after transplantation as well as before transplantation. Our 6-month follow-up showed a tendency to decrease the frequency of MDC2s until it reaches a significant level compared to baseline (P = .04). As reported by Hackstein and coworkers, we may consider this trend to be continuous at later time points because of significant negative impact of prednisolone as a maintenance immunosuppressive drug in our patients.[46]

The limitation of this study is that we were not able to provide a biopsy-proven acute rejection group taken from our main group, in which we could longitudinally follow to obtain serial cellular measurements like our nonrejection group.

CONCLUSIONS

We described some peripheral blood immune cell frequencies in stable kidney transplant recipients in a 6-month observational period using the noninvasive method of flow cytometry. Comparison of such frequencies with those in unstable or rejecting patients may differentially identify nonrejecting patients who likely will not develop acute rejection after transplantation. Further studies should monitor such measures in patients with unstable grafts and also continue the observational period and address the chronic changes of regulatory and inflammatory cells of the transplanted patients.

CONFLICT OF INTEREST

None declared.

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