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Sirolimus vs mycophenolate moftile in Tacrolimus based therapy following induction with Antithymocyte globulin promotes regulatory T cell expansion and inhibits RORγt and T-bet expression in kidney transplantation

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ABSTRACT

Background: Accumulating evidence suggests that regulatory T cells (Tregs) have a crucial role in immune tolerance and long-term graft survival. However, the influence of immunosuppressive drugs on the level of Tregs has not been fully understood. Therefore we prospectively compare the effect of two different calcineurin inhibitor (CNI)-based immunosuppression protocols on Tregs frequencies and expression of regulatory and effector T cell-related genes in renal transplant recipients.

Methods: The study included 24 renal transplant recipients who received induction therapy (Antithymocyte globulin) and were on triple immunosuppressive therapy so that one group was on Tacrolimus (Tac), mycophenolate moftile (MMF) and prednisolone (P) whereas another group was on Tac, Sirolimus (SRL) and P. The frequency of circulating Treg cells was analyzed by flow cytometry before and 4 months after transplantation. Also, the mRNA expression of FOXP3, T-bet, GATA3 and RORγt was examined by quantitative RT-PCR before and 4 months after transplantation.

Results: Compared to baseline, the frequency of $CD4^+$ $CD25^+$ $FOXP3^+$ Treg cells was significantly increased in the all patients following transplantation. Patients who received Tac/MMF had significantly higher $CD4^+$ $CD25^+$ $FOXP3^+$ Treg cells compared to patients who received Tac/SRL. There was no a significant difference in the frequency of $CD3^+CD8^+$ $CD28^-$ Tregs between two different calcineurin inhibitor (CNI)-based immunosuppression protocols. FOXP3 mRNA levels in the patients who received Tac/MMF were increased 4 months after transplantation and the expression was significantly higher than patients who received Tac/SRL. On the other hand, T-bet and RORγt expression levels were significantly lower in the Tac/SRL group in comparison to Tac/MMF group. We did not observe any significant difference in GATA3 mRNA level between the two groups.

Conclusions: Our results suggest that although Tac/MMF-containing immunosuppressive regimen could significantly increase the frequency of $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs, unlike to Tac/SRL-containing regimen, it could not significantly decrease the expression levels of RORγt and T-bet.

1. Introduction

Due to recent advances in immunosuppressive drugs, transplantation is still known as a standard treatment for end-stage renal disease (ESRD) [1]. However, these drugs cannot only prevent the chronic rejection of the transplantation, but also prolonged use of these drugs may increase the risk of various types of malignancies and infections [2]. Therefore, avoiding long-term immunosuppression with the goal of achieving immunological tolerance can be considered as a final solution for long-term survival of allograft [3]. There is scattered evidence that

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allograft tolerance is often accompanied with a specialized population of regulatory T lymphocytes (Treg) [4]. Currently, most calcineurin inhibitor (CNI)-based immunosuppression treatment regimens like Tacrolimus (Tac) are commonly used after kidney transplantation so that it has been shown that these immunosuppression treatment regimens can significantly prevent acute rejection [5]. However long-term treatment with CNI is associated with adverse side effects such as nephrotoxicity and vascular disease [6].Therefore, an alternative immunosuppressive treatment regimen containing mTOR inhibitors (e.g. Sirolimus (SRL)) and antimetabolite (e.g. Mycophenolate Mofetil (MMF)) are used in combination with CNI to minimize the CNI dose and its related toxicity [7]. Recently, many studies have shown that sirolimus cannot only prevent acute graft rejection and but also selectively expand Treg populations [8–11]. Various transplant models have also revealed that MMF does not only interfere with the function of Treg cells but also it has a positive effect on the process of tolerance induction [12–14]. $CD4^+$ T cells play an important role in transplant rejection as well as induction of transplant tolerance. In the transplantation scenario, both of T helper1 (Th1) and T helper17 (Th17) cells and their related cytokines, such as IFN-γ and IL-17, can cause transplant rejection while Treg cells prolong the allograft survival. However it has been shown that T helper2 (Th2) cells and their related cytokines have different effects in this scenario [15].Treg cells are a specific subset of T cells that can suppress both humoral and cellular immune responses [16]. Recent studies suggest that $CD4^+$ $CD25^+$ Treg cells, which constitute about 5 to 10 percent of $CD4^+$ T cell in humans and mice, play a key role in tolerance induction against self and non-self antigens. The transcription factor FOXP3 (forkhead box P3) is expressed predominantly in $CD4^+$ $CD25^+$ Tregs and it has a potent inhibitory function [17,18]. It has been reported that $CD4^+$ $CD25^+$ $FOXP3^+$ Treg cells are capable of inducing and maintaining tolerance in the transplantation due to the suppression of immune responses against allograft antigens [19]. The suppressor T cells (Ts) are another subtype of Treg cells which were identified as $CD8^+$ $CD28^-$ phenotype. These suppressor T cells induce immunological tolerance by different mechanisms compared to $CD4^+$ $CD25^+$ Treg cells [20,21]. It has been shown that the expansion of $CD8^+$ $CD28^-$ Tregs was associated with the reduced incidence of acute or chronic graft rejection. In addition, in patients who have received a minimum therapeutic dose of immunosuppressive drug, kidney function was maintained because of the presence of $CD8^+$ $CD28^-$ Treg cells in their peripheral blood [22].

Considering the central role of Tregs in the induction of tolerance and the irreversible effect of immunosuppressive agents on allograft survival, the aim of our study was to examine the frequency of ($CD4^+$ $CD25^+$ $FOXP3^+$ and $CD8^+$ $CD28^-$) Tregs and also compare the expression of Foxp3, T-bet, GATA-3 and RORγt genes in peripheral blood of kidney transplant recipients who received immunosuppressive regimens either as Tac/MMF or Tac/SRL before transplantation and 4 months after transplantation. The results of the present study suggest the role of these two protocols in immune tolerance and Tregs expansion.

2. Materials and methods

2.1. Patients and study design

This study was comprised 24 first kidney transplant recipients [15 male and 9 female] which were referred to Labbafinejad Hospital, Tehran, Iran during May 2016 to August 2017. The study was approved by the Local Institutional Ethical Committee of the Tehran University of Medical Sciences and informed consent was taken by the patient prior to the study. Peripheral blood was collected before immunosuppressive therapy and 16 weeks after transplantation and immunosuppressive treatment. At the time of transplantation, patients were randomly assigned to two groups based on immunosuppressive protocol: (1) Tac/MMF group: Tacrolimus (Tac), Mycophenolate mofetil (MMF) and prednisolone (n = 14), and (2) Tac/SRL group: Tacrolimus, Sirolimus (SRL) and prednisolone (n = 10).

All recipients received induction therapy with anti-thymocyte globulin (ATG) (3 mg/kg) for 4 days and received prednisolone 250 mg for 2 days followed by 1 mg/kg (max 60 mg) for 3 days and 5 mg daily. The dose was gradually tapered to 15 mg in 14 days and 10 mg dose was then continued for up to 30 days to reach 5 mg per day thereafter.

2.1.1. Tac/MMF group

The initial oral dose of TAC was 0.1 mg/kg/day, and target trough levels were 8–10 ng/ml during the first 3 months and 5–8 ng/ml afterward. The dose of MMF (360 mg was administered 3 times/day for 7 days) and then was reduced to 720 mg/day.

2.1.2. Tac/SRL group

The initial dose of TAC was 0.08 mg/kg/day, and target trough levels were 6–7 ng/ml during the first 6 months and 4–5 ng/ml afterward. The dose of Sirolimus was 2 mg for 96 h during the surgery followed by a reduction to 1 mg/day to reach a plasma level of 3–5 ng/ml in the first 6 months followed by an increase to reach a plasma level of 6–8 ng/ml.

2.2. PBMC isolation

The blood specimens were collected in EDTA sterile tubes. The peripheral blood mononuclear cells (PBMCs) were isolated from whole blood with Ficoll gradient (Inno-Train, Germany) according to the manufacturer's instructions. PBMCs were frozen in a cryoprotective media containing 10% dimethyl sulfoxide (DMSO) and 90% fetal bovine serum (FBS) and stored in a liquid nitrogen tank until further analysis.

2.3. Flow cytometric analysis

For analysis of Tregs abundance, PBMCs were thawed and the viability of cells was checked with trypan blue dye exclusion method. Only PBMCs with viability greater than 90% were used for flow cytometry assay. Next, the cells (PBMCs) were washed twice with phosphate-buffered saline (PBS) containing 0.3% fetal bovine serum. To determine cell surface markers, the cells were first stained with the following fluorochrome-conjugated monoclonal antibodies (all from eBioscience, San Diego, CA): Anti-CD3-PE-Cyanine 5.5, Anti-CD8a-FITC, Anti-CD28-PE, Anti-CD4-FITC and Anti-CD25-PE. After primary incubation (30 min at 4 °C), cells were washed. For intracellular staining of FoxP3 (anti-FOXP3-PE-Cyanine5.5, eBioscince), the cells were firstly fixed and permeabilized with Foxp3/Transcription Factor Staining Buffer Set (eBiosciences, eBioscience, San Diego, CA). Staining with isotype control antibodies [FITC- and PE-conjugated IgG1 K isotype controls and PE-Cyanine-conjugated IgG2a K isotype control (all from eBioscience)] was used to set gates and confirm staining specificity. Flow cytometric data were obtained using a FACSCalibur flow cytometer system and analyzed by CellQuest software (BD Biosciences).

2.4. Flow cytometric gating strategy

As seen in Fig. 1, the lymphocytes were first gated based on forward scatter/sideward scatter parameters. Next, $CD4^+$ cells were gated based on FITC signal and appropriate sideward scatter (SS) in the lymphocyte gate. $CD4^+$ cells were then evaluated for the expression of CD25 and FOXP3 based on the presence of a concomitant PE and PE-Cyanine5.5 signals (Fig. 2). For flow cytometric analysis of CD8 and CD28 expression, after gating of CD3-positive events, cells were characterized by CD8 and CD28 co-expression (Fig. 3). Unstained control was used to detect autofluorescence or background staining, to adjust FL PMT voltages and to set up negative gates.

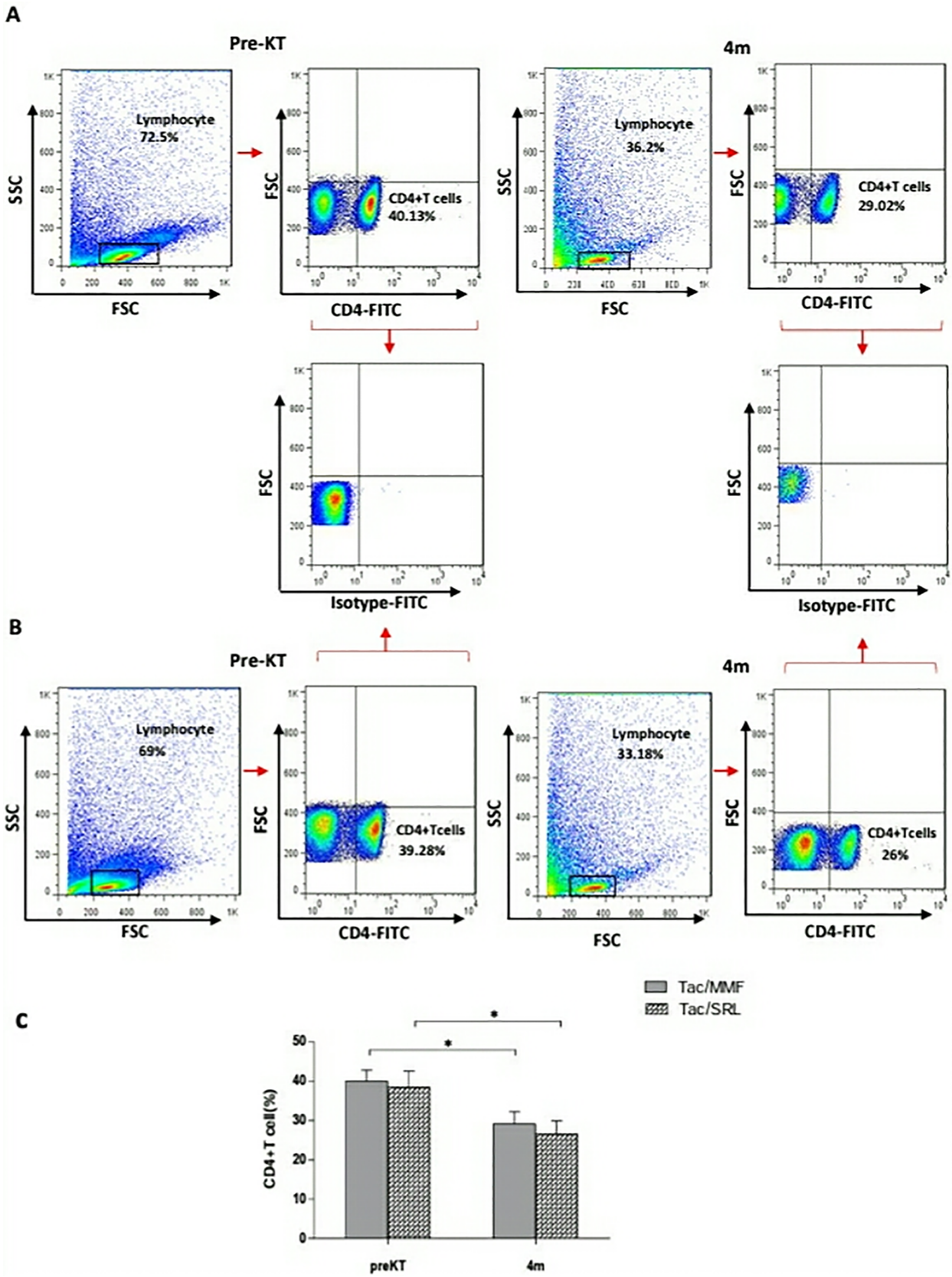

Fig. 1. Frequency of $CD4^+$ T cells in 24 renal transplant recipients taking Tacrolimus (Tac) and Mycophenolate mofetil (MMF) or sirolimus (SRL) at 4 months (4 m) after transplantation compared to pre-transplantation (PreKT). The frequency of $CD4^+$ T cells was measured by flowcytometry. A: representative a FACS plot of recipients in Tac/MMF group before and after transplantation. B: representative a FACS plot of recipients in Tac/SRL group before and after transplantation. C: Collective analysis of result of two groups receiving different drugs. Bar shows median. *P < 0.05 for 4 m vs. PreKT.

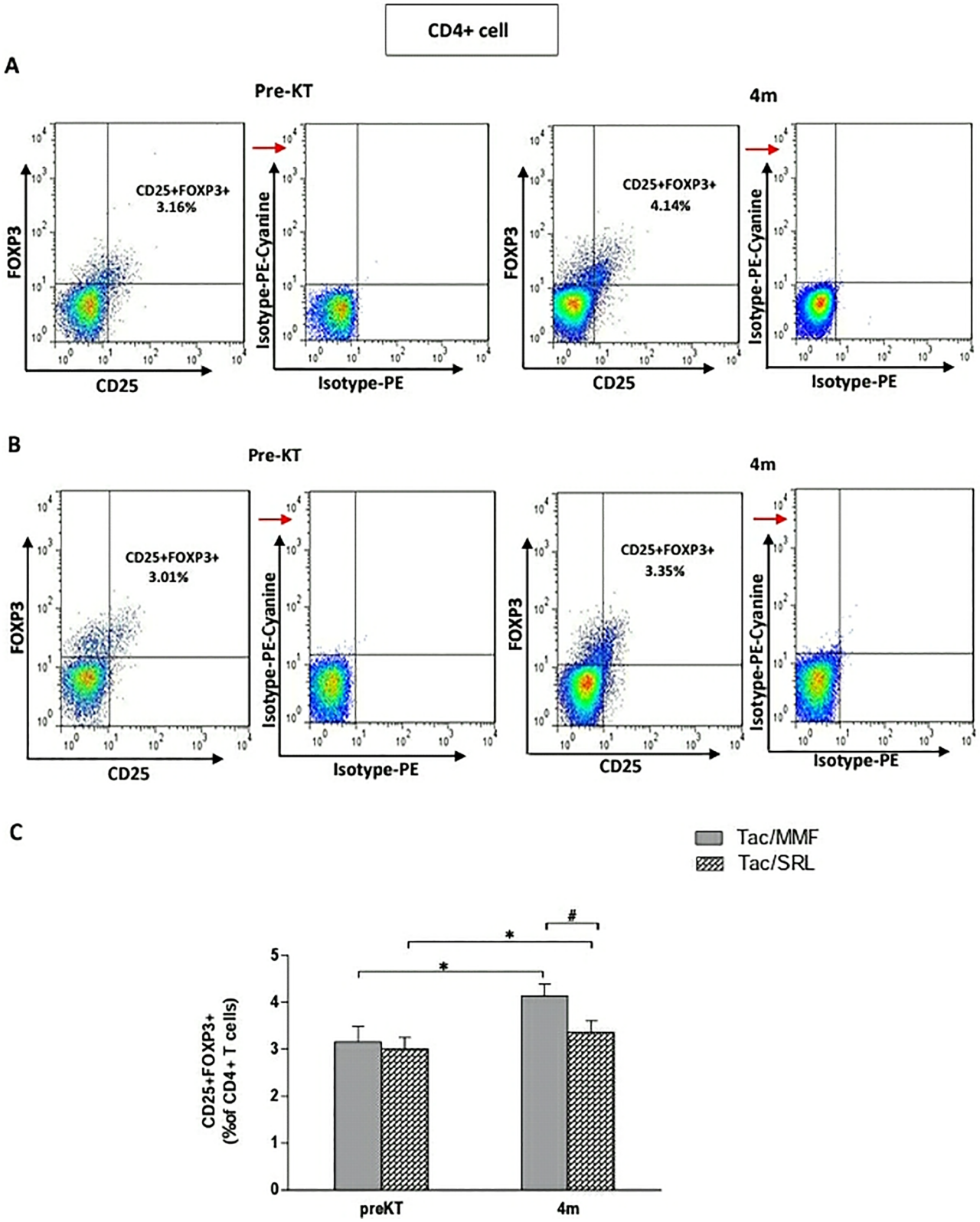

Fig. 2. Frequency of $CD4^+CD25^+FOXP3^+$ Treg cells in 24 renal transplant recipients taking Tacrolimus (Tac) and Mycophenolate mofetil (MMF) or sirolimus (SRL) at 4 months (4 m) after transplantation compared to before transplantation (PreKT). The frequency of $CD4^+$ $CD25^+$ $FOXP3^+$ Treg cells was measured by flowcytometry. A: representative a FACS plot of recipients in Tac/MMF group before and after transplantation. B: representative a FACS plot of recipients in Tac/SRL group before and after transplantation. C: collective analysis of result of two groups receiving different drugs. Bar shows median. *$P < 0.05$ for 4 m vs. PreKT. $^{\#}P < 0.05$ for Tac/MMF group vs. Tac/SRL group.

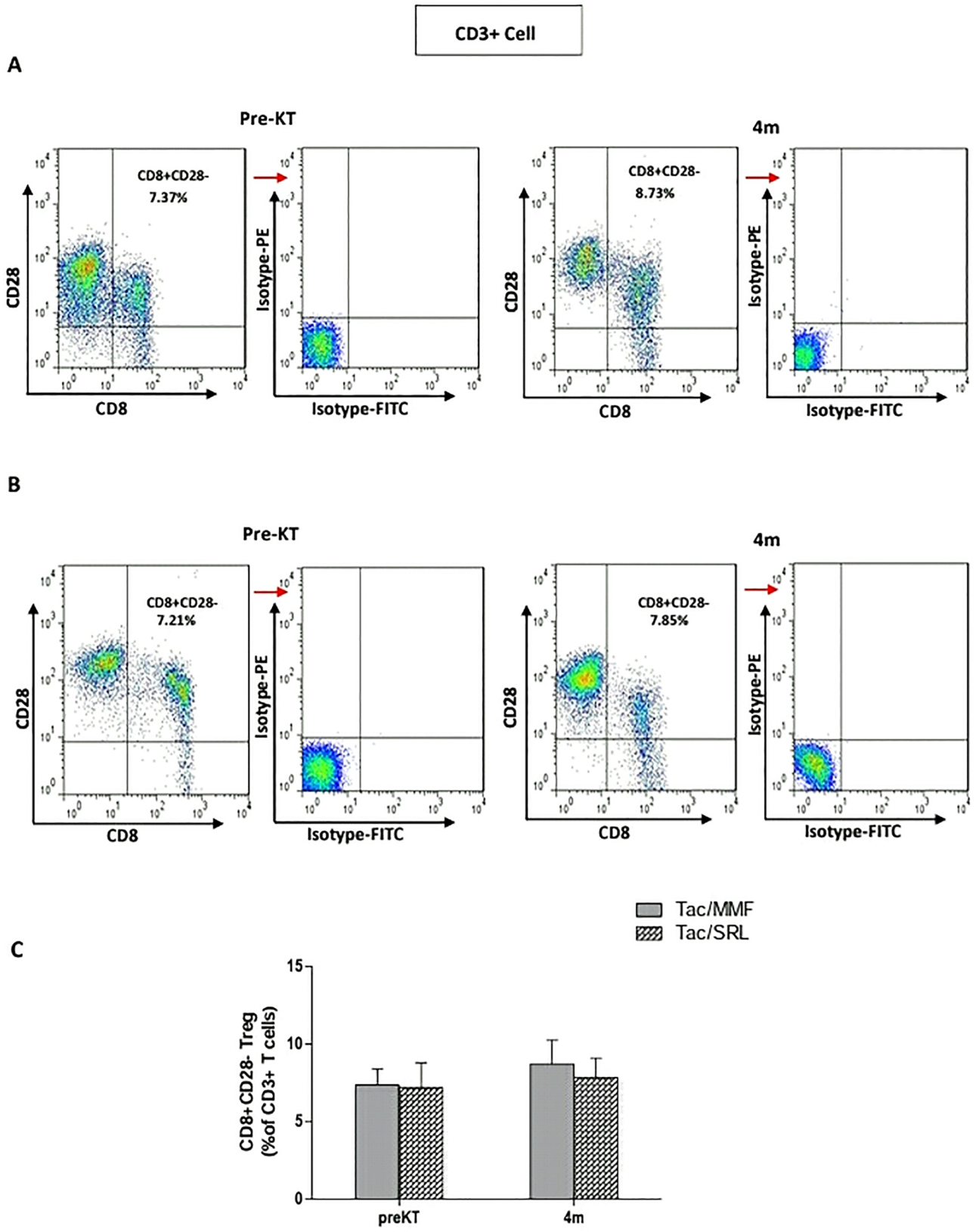

Fig. 3. Frequency of $CD3^+CD8^+CD28^-$ Treg cells in 24 renal transplant recipients taking Tacrolimus (Tac) and Mycophenolate mofetil (MMF) or Sirolimus (SRL) at 4 months (4 m) after transplantation compared to before transplantation (PreKT). The frequency of $CD3^+CD8^+CD28^-$ Treg cells was measured by flowcytometry. A: representative a FACS plot of recipients inTac/MMF group before and after transplantation. B: representative a FACS blot of recipients in Tac/SRL group before and after transplantation. C: collective analysis of result of two groups receiving different drugs. Bar shows median.

2.5. Quantitative mRNA analysis by real-time PCR

Total RNA was extracted from PBMCs (5×10^6 cells) using the TRI Reagent RNA Isolation Reagent (Sigma-Aldrich T9424) according to manufacturer's instructions. The quantity and purity of the RNA [the rate of A260 and A280 absorbance (A260/A280 ratio)] was measured by Nanodrop ND-1000 (Thermo Fisher) device. DNA template was removed by the addition of RNase-free DNase I (CinnaClon, Iran) before reverse transcription. In brief, reverse transcription was performed using High Capacity cDNA Reverse Transcription kit (Cat no. 4368814, Applied Biosystems, Foster City, CA). Synthesized cDNA was stored at −70 °C until assayed. Absolute quantitative determination of genes was performed using a Step OnePlus™ Real-Time PCR system (Applied Biosystems). Expression of T-bet (Hs00894392_m1), GATA-3 (Hs00231122_m1), RORγt (Hs01076112_m1) and FOXP3 (Hs0108534_m1) mRNAs was quantified using the TaqMan Gene Expression Assay according to the manufacturer's instructions (Applied Biosystems). Gene expression was normalized to 18s rRNA (Hs99999901_s1, Applied Biosystems) as an endogenous control. In this study, pretransplant samples were taken as control samples. The analyses of amplified products were performed by the relative quantification method 2-ΔΔct.

2.6. Statistical analysis

Data were analyzed using SPSS 16.0 software. The comparison of value before transplantation and four months after transplantation was performed using a Paired *t*-test and the comparison between groups was also made using an unpaired *t*-test. Values are presented as the mean (± SD) and P-value < 0.05 was considered statistically significant.

3. Results

3.1. Basic characteristics of renal recipients

Table1 represents demographic data and baseline characteristics of recipients whereas Table 2 shows their clinical and biochemical data. There was no significant difference in age and gender distribution between the two groups. All patients were first kidney transplants. Twelve patients (50%) were living un-related donor transplants and 12 (50%) were cadaveric donor transplants. Renal function improvement was observed as early as 4 months after transplantation in the two groups. Relative to baseline value, serum creatinine was decreased with a mean of 1.71 ± 0.89 in Tac/MMF group and 1.25 ± 0.42 in the Tac/SRL group 4 months after transplantation. The glomerular filtration rate (GFR) measurement also revealed that there was a significant increase in GFR four months after transplantation. There was no significant difference in GFR rate between the two groups (table 2). BUN levels were also significantly decreased in two groups after transplantation however there was no significant difference in the BUN level between two groups.

Table 1
Demographic and baseline characteristics of renal transplant recipients.

Parameter	Groups	
	Tac/MMF	Tac/SRL
N	14	10
Gender (M:F)	9:5	6:4
Age (year)		
Original renal disease, n (%)	34 ± 11.26	32 ± 7.05
Proteinuria	2 (14.28%)	2 (20%)
Hypertension	4 (28.57%)	2 (20%)
Renal cyst	0	1 (10%)
Lupus	1 (7.14%)	0
Diabetes	1 (7.14%)	2 (20%)
Nephrotic syndrome	2 (14.28%)	0
IgM nephropathy	0	1 (10%)
Reflux	0	1 (10%)
Congenital	1(7.14%)	0
Unknown	3 (21.42%)	1 (10%)
Donor type, n (%)		
Living unrelated	7 (50%)	5 (50%)
Cadaveric	7 (50%)	5 (50%)

Data are expressed as the number of subjects or mean ± SD.
Tac, Tacrolimus. MMF, mycophenolate moftile. SRL, sirolimus.

3.2. Frequency of total $CD4^+$ T cells in the renal recipients

As shown in Fig. 1, the frequency of $CD4^+$ T cells in MMF group was significantly decreased 4 months after transplantation (29.10 ± 11.70%) where compared with pre-transplantation (39.92 ± 10.49%, P = 0.02) (Fig. 1A). Likewise, there was a significant decrease in the abundance of $CD4^+$ T cells in Tac/SRL group 4 months after transplantation (26.61 ± 10.11%) where compared with pre-transplantation (38.46 ± 12.57%, p = 0.04) (Fig. 1B). However, there was no significant difference in the frequency of $CD4^+$ T cells between Tac/MMF and Tac/SRL group (P > 0.05) (Fig. 1C).

3.3. Frequency of $CD4^+CD25^+FOXP3^+$ Treg in the renal recipients

We next examined the abundance of $CD4^+CD25^+$ $FOXP3^+$ T subsets in the two studied groups before and 4 months after transplantation. The frequency of $CD4^+CD25^+FOXP3^+$ Tregs was 3.16 ± 1.25% before transplantation, however it was significantly increased to 4.14 ± 0.93% (P = 0.02) 4 months after transplantation, in the Tac/MMF group (Fig. 2A). Similar to the Tac/MMF group, in the Tac/SRL group, the frequency of $CD4^+CD25^+FOXP3^+$ Tregs was significantly higher 4 months after transplantation (3.36 ± 0.81%) compared with pre-transplantation (3.01 ± 0.75%, P = 0.04) (Fig. 2B). However, patients receiving Tac/MMF showed higher level of $CD4^+CD25^+FOXP3^+$ Tregs (4.14 ± 0.93%) compared with patients on Tac/SRL (3.36 ± 0.81%, P = 0.04) (Fig. 2C).

3.4. Frequency of $CD3^+CD8^+CD28^-$ Treg in recipients

$CD8^+CD28^-$ T cells are considered as regulatory cells. These cells have been recently drawn much attention however their role and frequency are controversial in the solid- organ transplantation. Thus, we analyzed the frequency of $CD3^+CD8^+CD28^-$ Tregs in peripheral blood of all recipients. Before transplantation, the frequency of $CD3^+CD8^+CD28^-$ Tregs was 7.37 ± 3.59% and it was not significantly changed 4 months (8.37 ± 5.75%, P = 0.4) after transplantation in the Tac/MMF group (Fig. 3A). In the Tac/SRL group, There was also no significant change in the frequency of $CD3^+CD8^+CD28^-$ Tregs 4 months after transplantation (7.85 ± 3.94%) when compared with pre-transplantation (7.21 ± 5.04%, P = 0.7) (Fig. 3B). There was no significant difference in the abundance of $CD3^+CD8^+CD28^-$ Tregs between two groups receiving different drugs (P = 0.6) (Fig. 3C).

3.5. Expression of specific transcription factors in $CD4^+$ T cell subsets in the renal recipients

To further explore the effect of immunosuppressive drug regimens on T cell subsets, we quantified gene expression of Treg and T helper –specific transcription factors including FOXP3, T-bet, GATA3 and RORγt in the kidney transplant recipients. FOXP3 mRNA levels were increased 4 months after transplantation compared to pre-transplantation in all recipients. However, the expression of FOXP3 mRNA was significantly higher in Tac/MMF group compared to Tac/SRL group (P = 0.01). In contrast, the mRNA level of T-bet and GATA3 was reduced 4 months after transplantation compared with pre-

Table2
Clinical and biochemical data (mean ± SD) of renal transplant patients.

Parameter	Groups			
	Tac/MMF		Tac/SRL	
	Baseline	4 m	Baseline	4 m
Creatinine (mg/dL)	20 ± 5.37	1.71 ± 0.89*	9.77 ± 4.10	1.25 ± 0.42*
GFR (mL/min)	6.84 ± 2.85	53.47 ± 21.46*	7.19 ± 2.26	68.79 ± 17.19*
BUN	109 ± 28.25	51.09 ± 55.21*	104 ± 24.82	39 ± 11.67*
UA	6.81 ± 1.30	6.88 ± 2.07	6.60 ± 1.33	5.62 ± 2.28
AST	10.50 ± 4.90	21.30 ± 9.23*	9.87 ± 4.48	22.87 ± 4.88*
ALT	12.20 ± 5.73	37.7 ± 22.15*	11.87 ± 5.74	30.25 ± 15.67*
ALP	276 ± 102.57	259 ± 59.47	269 ± 130.46	245 ± 147.44
Ca	9.36 ± 0.58	9.47 ± 0.62	8.67 ± 0.55	9.30 ± 0.44
Na	142 ± 2.82	139 ± 515	138 ± 3.33	138 ± 2.47
P	5.85 ± 1.91	3.98 ± 0.46	6.05 ± 1.70	4.06 ± 1.01
K	4.58 ± 0.95	6.91 ± 7.72	5.18 ± 0.77	4.07 ± 0.57

GFR, Glomerular filtration rate. BUN, Blood urea nitrogen. UA, Uric acid.
AST, Aspartate aminotransferase. ALT, Alanine aminotransferase.
ALK, Alkaline phosphatase. Ca, Calcium. Na, Sodium.
P, phosphorus. K, Potassium.
* Before versus after transplantation was significant ($p < 0.05$).

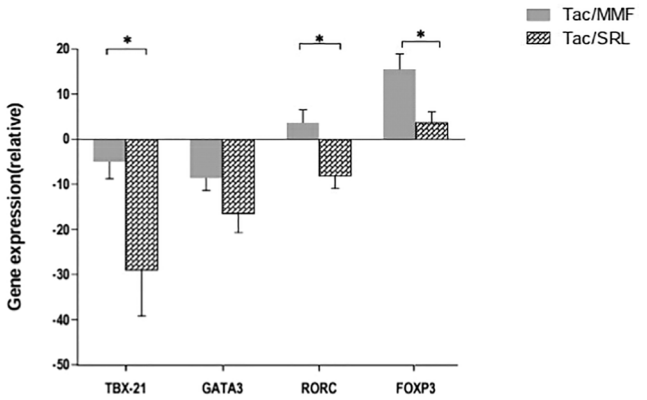

Fig. 4. The expression levels of FOXP3, T-bet, GATA3 and RORγt which are associated with Treg and Th1/Th2/Th17 cells were quantified by real-time PCR, in Tacrolimus (Tac)/Mycophenolate mofetil (MMF) compared to Tacrolimus (Tac)/sirolimus (SRL) group. Bar shows median. *P < 0.05 Tac/MMF group vs. Tac/SRL group.

transplantation. As presented in Fig. 4, Tac/SRL group showed a significantly lower level of T-bet mRNA expression than Tac/MMF group (P = 0.04). However, there was no significant difference in GATA3 mRNA expression between two groups (P = 0.1). Although in Tac/MMF group, the expression of RORγt was not only suppressed 4 months after transplantation compared with pre-transplantation, but also the expression was increased. In contrast, in Tac/SRL group the expression of RORγt was decreased 4 months after transplantation compared to pre-transplantation. The alterations of RORγt gene expression in the two treatment groups was significantly different (P = 0.01) (Fig. 4).

4. Discussion

A successful renal transplantation is credited with the continuous examination of effective factors in transplant rejection as well as investigation of related toxicity and the function of immunosuppressive drugs. Allotransplantation responses create an inflammatory microenvironment in transplant and outcome is depending on how the immunosuppressive drugs administer T lymphocytes. Most of the immunosuppressive drugs are selected based on their ability to control the activity of T lymphocytes and to maintain the function of allograft [23]. Calcineurin inhibitors such as Tacrolimus interfere with the signaling pathway of both effector T cells and Tregs by suppressing IL-2 transcription [24]. Nowadays, therapeutic protocols are extensively based on the CNI minimization. Hence, in order to reduce the dose of CNI and thereby to minimize its related adverse side effects, the use of newer immunosuppressive drugs can be effective [25]. In the present study, in order to reduce the risk of transplant rejection, anti- thymocyte globulin (ATG) was used for induction therapy. Lopez et al. have reported that ATG can expand $CD4^+$ $CD25^+$ $FOXP3^+$ regulatory T cells and maintain their regulatory functions in MLR assay using peripheral blood cells [26]. Our results showed that a combination immunosuppressive regimen containing Tac/MMF or Tac/SRL can modulate the effects of Tacrolimus on Tregs since the frequency of $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs was increased in two groups four months after transplantation compared to pre-transplantation.

mTOR inhibitors such as Sirolimus and Everolimus are new immunosuppressive drugs that, unlike Tacrolimus, do not interfere with the expansion of Treg cells. Chu and colleagues have demonstrated that CNIs could significantly decrease the percentage of Treg cells in kidney transplantation recipients compared with healthy subjects, while sirolimus could not change the percentage of Tregs [27]. Sansgundo et al. also reported that conversion of therapeutic protocol from Tacrolimus to sirolimus could increase the absolute number of Treg cells [28]. However, it has been shown that the concomitant use of Tacrolimus and sirolimus can suppress the proliferation of alloreactive Th1 and Th17 cells more efficiently, while maintaining Treg cells [15]. In line with above study, our study showed that combination of sirolimus with low dose of Tacrolimus can induce expansion of Tregs. MMF is commonly used in combination with calcineurin inhibitors in the transplantation, and it significantly reduces the risk of acute rejection. The relationship between MMF and Treg is not yet well understood. It has been shown that combined treatment with MMF with vitamin D3 can increase the frequency of $CD4^+$ $CD25^+$ regulatory cells in a mouse model of islet allograft transplantation [13]. In the liver transplantation, Demirkiran et al. showed that conversion from Calcineurin inhibitors to MMF can increase the frequency of $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs [12]. Inversely, Lim et al. reported that expansion of $CD4^+$ $CD25^+$ Treg cells is not much different in presence or absence of MMF [29]. Our results showed that frequency of $CD4^+$ $CD25^+$ $FOXP3^+$ regulatory T cells in Tac/MMF group is significantly higher than another group. Confirming our findings, Wang et al. exhibited that low-dose Tacrolimus plus MMF can expand $CD4^+$ $CD25^+$ $FOXP3^+$ regulatory cells [30]. In another study, it has been reported that, in the kidney transplantation recipients, combination of Tacrolimus and MMF can increase the

frequency of $CD4^+$ $CD25^+$ $FOXP3^+$ T cells in comparison with combination of Tacrolimus and Everlimus [31].

$CD3^+$ $CD8^+$ $CD28^-$ T cells are another subset of regulatory cells that have recently attracted more attention and their role is described in various transplant organs. $CD8^+$ $CD28^-$ T cells have shown that they can suppress alloimmune and autoimmune reactions in many animal models and their abundance can be considered as a prognostic biomarker for transplanted organ. It has been observed that, in the heart transplantation recipients, the frequency of $CD8^+$ $CD28^-$ cells is higher than normal subjects [20]. In the liver transplantation patients, it has been shown that combination of Tacrolimus and MMF can expand $CD3^+$ $CD8^+$ $CD28^-$ cells [32]. Conversely, Korecka-polac et al. showed that cyclosporine A and rapamycin can suppress the expansion of $CD8^+$ $CD28^-$ regulatory T cells [33]. In the numerous studies, it has been reported that the frequency of $CD3^+$ $CD8^+$ $CD28^-$ cells is increased in the liver transplantation and their higher percentage is associated with a better prognosis. However, unlike liver transplantation, frequency of these cells in the different studies focusing on the kidney transplantation is variable. Such variation may be related to different time periods in each study since percentage of these cells varies in the short and long term, and it seems that the frequency of these cells is increased over time. Our study showed that abundance of $CD3^+$ $CD8^+$ $CD28^-$ cells are increased after transplantation, this finding in agreement with previous studies. Nevertheless, we did not find a relationship between frequency of $CD3^+$ $CD8^+$ $CD28^-$ regulatory cells and immunosuppressive drugs. Keeping the balance between the function of effector cells and regulatory cells is a complex phenomenon that determines the allograft survival outcome. Tolerance and rejection are two extreme ends of a scale that can be tipped in either direction so that multiple factors can play a role in influencing the course of these two events. New studies are more paid attention to plasticity of Th17 cells and their balance with Treg cells in vivo. It should be noted that, Th17 and Treg cells are developed from the same precursor and their development is depend on the cytokine signals [34]. The imbalance of Th17/Treg cell populations in peripheral blood has been suggested to be a strong predictor of renal transplantation rejection [35]. Various and specific transcription factors are associated with functional evolution of different T cell subsets they are including T-bet (Th1), GATA3 (Th2), RORγt (Th17) and foxp3 (Treg) [36]. In our study, the expression of both FOXP3 and RORγt were increased in Tac/MMF group. FOXP3 has a regulatory function, but RORγt is seemed to be elevated inflammatory settings. The co-expression of FOXP3 and RORγt is controversial, so that we showed that in the Tac/SRL group, the expression of FOXP3 was increased however the expression of RORγt was decreased. In respect to gene expression pattern of these two genes, it can be found out that the Tac/MMF protocol has failed to establish a balance between inflammatory and regulatory responses, while in the Tac/SRL group the balance is tipped toward regulatory status. Kopf et al. have reported that sirolimus not only increases FOXP3 expression but also blunts the differentiation of the naïve T cells into Th17, and consequently, it creates a more stable phenotype of $CD4^+$ Treg cells [37]. It has been shown that Th1 cells have a role in inflammatory transplantation process, but the role of Th2 cells are not yet fully understood [38]. The ratio of Th1 to Th2 is very important in the prediction of allograft survival. In the present study, both therapeutic protocols could suppress expression of both T-bet and GATA3 genes four months after transplantation compared to pre-transplantation. Moreover, the Tac/SRL group could repress T-bet gene expression more efficiently and this is in favor of the allograft survival. Li et al. demonstrated that conversion from CNI to the sirolimus reduces frequency of Th1 and Th17cells [39].

A balance between activated effector and regulatory T cells is a complicated mechanism. Such balance is necessary for proper immune responses that determine allograft outcome. Tolerance and rejection are two distinct sides of immune system reaction to alloantigens expressed by the graft. To date, many therapeutic strategies have been developed to introduce a more effective and appropriate treatment regimen and to promote allograft – specific immune tolerance aiming to prevent pro inflamatory responses and chronic rejection. In conclusion, our data demonstrated that although, in the Tac/MMF group, the frequency of $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs was significantly increased; the expression of RORγt, a transcription factor that directs the differentiation program of proinflamatory Th17 cells, was not decreased whereas the Tac/SRL group could increase the frequency of Tregs and could significantly decrease the expression of RORγt. Thus, it seems that Tac/MMF protocol is not effective in achieving immunological balance between protective and pathogenic immune responses. It is suggested that considering the balance between alloreactivity and tolerance, utilizing Tac/SRL-containing immunosuppressive regimen may have a better performance in keeping balance and improving renal function.

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Conflict of interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humimm.2018.12.010.

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