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Frequency of CD39+, LAG3+, and CTLA4+ Regulatory T Cells in Two Different Immunosuppressive Protocols in Renal Allograft Recipients (Sirolimus vs Mycophenolate mofetil): A Cohort Report

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ABSTRACT

Background: Impaired renal function is considered as a significant risk factor for cardiovascular events in chronic kidney disease patients. Several immunosuppressive drugs are used in these patients, which necessitates to minimize the drug-related side effects by employing alternative strategies.

Objective: This study aimed to evaluate prospectively the influence of low dose ATG induction therapy with two different protocols (Sirolimus versus Mycophenolate mofetil) on the expression of functional markers (LAG-3, CD39, and intracellular CTLA-4) on conventional Tregs in renal recipients.

Methods: Thirty-eight renal transplant recipients were enrolled in this study. The patients were randomly assigned into two groups, including TMP: Tacrolimus (Tac), Mycophenolate mofetil (MMF), and Prednisolone (n=23); and TSP: Tac, Sirolimus (SRL), and Prednisolone (n=15). The frequency of LAG-3, CD39, and intracellular CTLA-4 on circulating Tregs was analyzed by flow cytometry before and after transplantation.

Results: Analysis of the flow cytometry data showed that the frequency of CD4+CD25+FOXP3+ Tregs increased 4 months post-transplantation compared to pre-transplantation in both groups, although this increase was only significant in TMP group. In TMP treated patients, the frequency of LAG-3+ Tregs and CD39+ Tregs increased, whereas the frequency of intracellular CTLA-4+ Tregs decreased 4 months post-transplantation. In TSP group, while the frequency of CD39+ Tregs increased, the frequency of CTLA-4+ Tregs decreased in post-transplantation compared to pre-transplantation.

Conclusions: it seems that both treatment regimen protocols with a low dose ATG induction therapy may be clinically applicable in kidney transplant recipients.

Keywords: CD39, CTLA4, Kidney transplantation, LAG-3, Treg

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INTRODUCTION

Impaired renal function is a prominent risk factor for cardiovascular diseases, especially diseases in patients suffering from chronic kidney disease (CKD) (1). The risk potentially increased whenever CKD upgraded to end-stage renal disease (ESRD) that requires dialysis or renal transplantation (1). The paucity of kidney donors and high transplant capacity can negatively affect the waiting time before transplantation in many patients (2). Several immunosuppressive drugs are used in recipients of a kidney transplant to avoid chronic rejection of transplantation. However, these treatments may also advance the risk of infections and a variety of malignancies (3). In addition, long-term usage of immunosuppressive agents causes detrimental side effects, which may decrease the survival of patients and grafts (4). Thus, it is necessary to minimize the immunosuppressive drug-related side effects by employing other novel strategies that induce and sustain transplant tolerance. The transplant tolerance can be induced for a long time by regulatory T cells (Tregs), especially $CD4^+CD25^+FOXP3^+$ Tregs. It is a potential strategy extensively investigated nowadays.

Currently, Calcineurin inhibitor (CNI)-based treatments, such as Tacrolimus, (Tac) are abundantly administered after kidney transplantation (5). However, long-term CNI-based treatment has been demonstrated to be accompanied by some side effects e.g., vascular diseases and even nephrotoxicity (6). To minimize the dose of CNI and its toxicity, novel alternative immunosuppressive regimens, including mTOR inhibitors (i.e., Sirolimus (SRL)) and antimetabolites i.e., Mycophenolate mofetil (MMF), are suggested in combination with CNI (7). Some studies have recently shown that SRL can reduce the incidence of acute allograft rejection and expand the Treg populations selectively. In this context, several previous studies have shown that SRL, and by interfering with DNA demethylation, enhances the expression of FoxP3 and selectively improves the functional Tregs both *in vivo* and *in vitro* (8-12).

As a pro-drug of mycophenolic acid, MMF is an inosine monophosphate dehydrogenase inhibitor. Inosine monophosphate dehydrogenase takes part in the *de novo* synthesis of guanosine nucleotides in T lymphocytes, and its activity appears to be necessary for cell proliferation. MMF inhibits clonal expansion of antigen-stimulated T cells (13). In patients with a kidney transplant, particularly following T cell-depleting therapy, T cells exert properties of exhaustion, which is generally defined by decreased proliferative capacity when encountering donor antigen and increased expression of exhaustion markers, including lymphocyte-activation gene-3 (LAG-3), cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), T-cell immunoglobulin mucin-3 (TIM-3), and programmed cell death protein-1 (PD-1) (14).

A recent study has reported that Tac/MMF-containing immunosuppressive regimens may prominently elevate the number of $CD4^+CD25^+FOXP3^+$ Tregs (15). $CD4^+$ Tregs usually express CD25, GITR, CTLA-4, and FOXP3 (16). $CD4^+$ Tregs also express other molecules such as LAG-3 and CD39. LAG-3 is a homolog for CD4, which can bind to major histocompatibility complex-II (MHC-II) on dendritic cells (17). Similar to CTLA-4, LAG-3 may harm proliferation, activation, and the balance of T cells. Moreover, LAG-3 enables Tregs to suppress immune cells (15). CD39, an integral membrane protein that phosphohydrolyzes ATP, plays a critical role in homeostasis and T cells' function. The expression of CD39 by Tregs has been demonstrated (18, 19) and is limited to a subset of memory Tregs (mTregs) (20) that potentially suppress IL-17 synthesis (21). Evaluating the specific markers of Tregs, which allows the monitoring of this cell population and inhibitory function in kidney transplantation recipients, is of great importance. This study aimed to assess the frequency of Tregs in patients receiving two different immunosuppressive protocols

(TMP: Tacrolimus; Tac, Mycophenolate mofetil; MMF, and TSP: Tac, Sirolimus; SRL), and to evaluate the effects of both protocols (TMP and TSP) on the frequency of LAG3, CD39, and intracellular CTLA-4 in peripheral blood $CD25^{+}FOXP3^{+}$ Tregs in kidney transplant recipients before and four months post-transplantation.

MATERIALS AND METHODS

Patients and Study Design

Thirty-eight hospitalized patients (28 males and ten females) were enrolled from September 2016 to August 2019. All the patients were selected based on their first kidney transplantation in Labbafinejad Hospital, Tehran, Iran. This study was performed in compliance with the Helsinki Declaration and approved by the local Ethics Committee of Tehran University of Medical Sciences (IR.TUMS.REC.1395.2827-2016.09.10). All participants received written informed consent before the initiation of the study.

All hospitalized patients received induction therapy with low-dose Anti-thymocyte globulin (ATG) (3 mg/kg) for four consecutive days, and Prednisolone was given at 250 mg for two consecutive days, followed by 1 mg/kg (max 60 mg) for three consecutive days. Tapering of Prednisolone was done daily (15 mg in 14 days, continued 10 mg for 30 days, and finally tapered to 5 mg per day). The patients were randomly categorized into two groups in terms of immunosuppressive protocol: TMP group (n=23): Tacrolimus (Tac), Mycophenolate mofetil (MMF), and TSP group (n=15): Tac, Sirolimus (SRL).

Clinical and laboratory findings were gathered when blood sampling was done. The levels of serum creatinine, blood urine nitrogen (BUN), and uric acid were measured in all the participants by autoanalyzer. Peripheral blood (10ml with EDTA) was obtained from the patients in both groups 24h before immunosuppressive therapy and after four months of post-transplantation.

The beginning Tac oral dose in the TMP group was 0.1 mg/kg per day, and the target through levels was 8-10 ng/ml during the first three months and 5-8 ng/ml thereafter. The MMF dose (360 mg was administered three times per day for seven days) reduced to 720 mg per day. In the TSP group, the initial dose of Tac was 0.08 mg/kg/day, and target levels were 6-7 ng/ml within the first six months and after 4-5 ng/ml. The dose of SRL was 2 mg in the first 96 hours after the surgery continued by a decrement to 1 mg/day to obtain a plasma level of 3-5 ng/ml in the first six months, followed by an increment to obtain 6-8 ng/ml plasma levels.

All the patients received their first kidney transplant from either living unrelated donors or cadavers under the age of 50. Stable graft function, serum creatinine level 1-2 mg/dl (the normal creatinine serum levels are approximately 0.6-1.2 mg/dl in adult males and 0.5-1.1 mg/dl in adult females), negative panel reactive Ab, negative pre-transplant anti-HLA Abs, and absence of histological signs of acute/chronic rejection or infections were considered inclusion criteria. The patients with slow graft function, delayed graft function, opportunistic infections, or malignancies were excluded from the study. The patients with ABO-incompatible and those who had second kidney transplantation or a chance of acute rejection were also excluded. Table 1 summarizes the baseline demographic characteristics of kidney transplant recipients.

Peripheral Blood Mononuclear Cells Isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh whole blood using Ficol-Paque centrifugation (Inno-Train, Germany) and resuspended in RPMI1640 (Gibco Life Technology, Paisley, UK) supplemented with 10% FCS (Gibco). Isolated cells were then counted and aliquoted in liquid nitrogen until flow cytometry examination (22, 23).

Table 1. Patient inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
18-65 years	Slow graft function
Stable graft function	Delayed graft function
Level of serum creatinine between 1 and 2 mg/dl	Opportunistic infections or malignancies
Negative panel reactive antibody	Patients with ABO-incompatible
Negative pre-transplant anti-HLA antibodies	Declined participation during Follow up
Absence of histological signs of acute/chronic rejection or infections	
First kidney transplant	

Assessing Treg Populations Using Flow Cytometry

The evaluation of peripheral blood Tregs ($CD4^+CD25^+FOXP3^+$) frequency as well as $LAG3^+$ Treg, $CD39^+$ Treg, and $CTLA4^+$ Treg populations in the two groups of kidney transplant recipients was performed with multicolor flow cytometry. The viability of PBMCs after thaw was checked using trypan blue dye. High-viability PBMCs were utilized for the flow cytometry test. In brief, the cells were washed two times with phosphate-buffered saline (PBS) containing 0.3% fetal bovine serum and stained with FITC anti-human CD4 antibody (Biolegend/clone: RPA. T4/Host: Mouse), APC anti-human CD39 (BD/ Clone: TU66/Host: Mouse), Alexafloura 647 anti-human LAG-3(BD/Clone: T47-530/ Host: Mouse/) and PE-Cy7 anti-human CD25 (BD/Clone: M-A251/Host: Mouse). After the first incubation at 4 °C for 30 min, PBMCs were also stained for intracellular CTLA-4 and FOXP3. For intracellular staining, cells were fixed and permeabilized using Transcription Factor Buffer Set (BD Transcription factor Buffer set). Eventually, PBMCs were stained with PE anti-human FOXP3 (BD/ Clone: 259D/C7/Host: Mouse) and APC anti-human CTLA-4 (BD/Clone: Bni3/Host: Mouse (antibodies. Staining of cells was also performed with isotype control antibodies (from eBioscience, Biolegend, and BD) to set all gates and verify the specificity of the staining. Data were then acquired using an Attune NxT flow cytometer system and finally analyzed by FlowJo V10 CL software.

Flow Cytometric Gating Strategy

Lymphocytes were gated based on parameters of forwarding/side scatter. Then, $CD4^+$ cells were gated using the FITC signal and proper side scatter (SS) in the lymphocyte gate. The Treg population was gated on $CD4^+$ T cells gate by CD25 and FOXP3 staining using concomitant PE-Cy7 and PE signals, respectively. The frequency of $CTLA4^+$Tregs, $CD39^+$ Tregs (Figure 1.e), and $LAG3^+$ Tregs was assessed on Treg gate ($CD4^+CD25^+FOXP3^+$ lymphocytes). Unstained cells and cells stained with isotype control antibodies were used to detect auto-fluorescence or background staining to justify FLPMT voltages and to find negative gates.

Statistical Analysis

Continuous and categorical variables were represented as median (IQR) and n (%), respectively. To compare the differences between the independent groups, Wilcoxon rank-sum test was used. Also, Wilcoxon signed-rank test was used to compare the differences between before and after transplantations. Statistical analyses were performed with R version 4.1.0 (2021-05-18) and P values less than 0.05 were considered statistically significant.

RESULTS

Demographic and baseline characteristics of recipients in the two TMP and TSP groups are represented in Table 2, whereas Tables 3

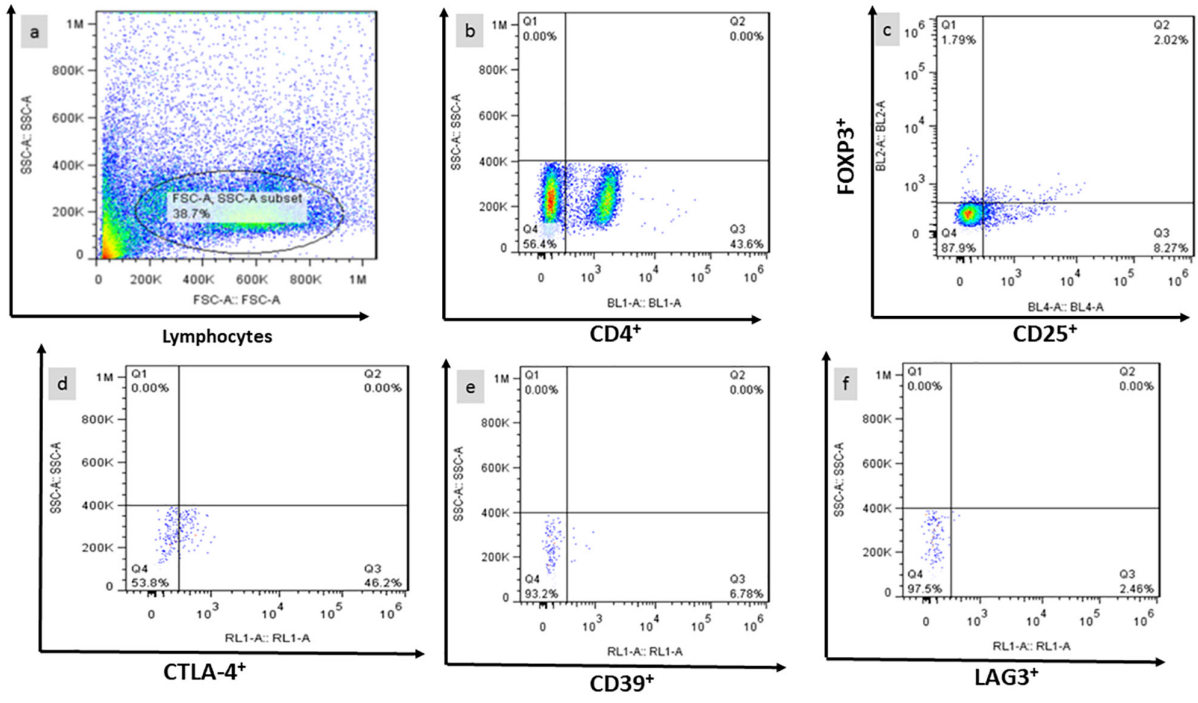

Figure 1. Frequency of $CD4^+CD25^-FOXP3^+$ T cells analyzed by Flow cytometry. Gating strategy for detection of $CD4^+CD25^+FOXP3^+$ Tregs and Tregs expressing CD39, CTLA-4, and LAG-3 in the peripheral blood of kidney graft patient. Gating strategy for lymphocytes (a), $CD4^+$ lymphocytes (b), $CD25^+FOXP3^+$ Tregs (c), Tregs expressing CD39 (d), Tregs expressing CTLA-4 (e), and Tregs expressing LAG-3 (f) were displayed.

Table 2. Demographic and the causual ESRD of renal transplant recipients (N=38) in TMP (N=23) and TSP (N=15) groups.

Variable	N (%)	TMP	TSP
CKD	1 (4.0%)	1 (5.9%)	0 (0%)
Congenital	1 (4.0%)	1 (5.9%)	0 (0%)
Diabetes	3 (12%)	0 (0%)	3 (38%)
Hypertension	10 (40%)	9 (53%)	1 (12%)
IgM nephropathy	1 (4.0%)	0 (0%)	1 (12%)
Lupus	2 (8.0%)	2 (12%)	0 (0%)
Nephrotic syndrome	2 (8.0%)	2 (12%)	0 (0%)
Proteinuria	2 (8.0%)	1 (5.9%)	1 (12%)
Reflux	1 (4.0%)	0 (0%)	1 (12%)
Renal cyst	2 (8.0%)	1 (5.9%)	1 (12%)
Age (years)		33 (28-48)	32 (29-42)
Female	10 (26%)	6 (26%)	4 (27%)
Male	28 (74%)	17 (74%)	11 (73%)
Cadaveric	21 (55%)	14 (61%)	7 (47%)
Unrelated Living	17 (45%)	9 (39%)	8 (53%)

ESRD: end stage renal disease; MMF: Mycophenolate mofetil; TAC: Tacrolimus; SRL: Sirolimus; CKD: chronic kidney disease; N: Number

display their clinical and laboratory findings for the TMP and the TSP groups, respectively. Concerning the age and gender distribution, there was no significant difference between the TMP and the TSP groups. All the recipients had not experienced kidney transplants before the study. Seventeen patients (45%) were transplanted with

Table 3. Paraclinical and laboratory findings for TMP and TSP groups

Protocol	Variable	Transplantation		Unit	P-value
		Before: Median (IQR)	After: Median (IQR)		
TMP (n=23)	GFR	6 (5-7)	65 (52-81)	ml/min	<0.001
	Cr	10.4 (8.0-14.9)	1.3 (1.0-1.7)	mg/dl	<0.001
	BUN	114 (79-129)	36 (26-44)	mg/dl	0.002
	Uric Acid	7.35 (6.73-7.78)	6.05 (4.68-7.45)	mg/dl	0.9
	Ca	9.10 (8.50-9.95)	9.30 (9.05-9.65)	mg/dl	0.2
	P	5.76 (4.99-6.20)	3.90 (3.45-4.37)	mg/dl	0.048
	Na	141 (136-142)	138 (138-140)	mEq/L	0.5
	K	4.60 (4.30-5.23)	4.20 (4.00-4.45)	mEq/L	0.3
	AST	12 (8-15)	18 (16-20)	U/L	0.003
	ALT	15 (11-20)	20 (16-44)	U/L	0.003
	ALP	263 (218-332)	256 (196-294)	U/L	0.2
	WBC	6.50 (5.27-7.90)	5.70 (4.60-7.10)	10^9/L	0.033
	Lym	33 (25-36)	26 (21-31)	%	0.2
	Hb	11.40 (10.45-12.25)	13.50 (12.30- 14.40)	gr/dl	0.004
	RBC	4.01 (3.60- 4.44)	4.75 (4.19- 5.14)	Mil/cumm	0.002
TSP (n=15)	GFR	6 (6-7)	70 (61-78)	ml/min	<0.001
	Cr	8.6 (7.4-10.6)	1.2 (1.0-1.3)	mg/dl	<0.001
	BUN	108 (86-120)	39 (34-40)	mg/dl	0.004
	Uric Acid	6.35 (5.22-7.53)	5.00 (3.55-6.55)	mg/dl	0.4
	Ca	8.45 (8.22-9.10)	9.30 (9.10-9.80)	mg/dl	0.078
	P	6.26 (5.07-7.10)	3.36 (3.00-4.40)	mg/dl	0.039
	Na	138 (135-139)	139 (137-142)	mEq/L	0.3
	K	5.40 (4.95-6.10)	3.98 (3.73-4.52)	mEq/L	0.002
	AST	12 (8-12)	23 (20-27)	U/L	0.009
	ALT	10 (8-14)	35 (22-37)	U/L	0.014
	ALP	227 (160-387)	216 (157-286)	U/L	>0.9
	WBC	5.70 (4.40-8.03)	6.75 (4.10-8.07)	10^9/L	>0.9
	Lym	29 (26-35)	31 (24-36)	%	0.7
	Hb	12.35 (10.95-14.43)	13.15 (12.10-13.97)	gr/dl	0.5
	RBC	4.20 (3.64-4.69)	4.40 (4.29-4.80)	Mil/cumm	0.5

GFR: Glomerular Filtration Rate, Cr: Creatinine, BUN: Blood Urea Nitrogen; Ca: Calcium; P: Phosphate; Na: Sodium; K: Potassium; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; WBC: White Blood Cell; Lym: Lymphocyte; Hb: Hemoglobin; RBC: Red Blood cell IQR: Interquartile range; TMP: Tacrolimus, Mycophenolate mofetil, Prednisolone

a living unrelated donor and 21 (55%) were transplanted with a cadaveric donor. The Renal function was measured by serum creatinine, uric acid levels, BUN, and glomerular filtration rate (GFR) in both TMP and TSP groups. Renal function of the patients improved four months after transplantation. Proportional to baseline values, serum creatinine level decreased with a mean of 1.3 (1.0, 1.7) in the TMP group and 1.2 (1.0, 1.3) in the TSP group four months after transplantation. Although a significant increase was observed in GFR four months post-transplantation in both groups, no significant difference was found in the GFR between the two groups four months after transplantation (Table 3). BUN concentrations significantly decreased in the two groups four months after transplantation when compared with the baseline. Moreover, no significant differences were found in terms of BUN levels between the TMP and the TSP groups (Table 4).

The frequency of circulating $CD4^+$ T cells decreased in both the TMP and the TSP groups. The frequency of CD4 significantly decreased in the TMP group (19% vs 39%, P=0.011) 4 months after transplantation

Table 4. The comparison of paraclinical results between the two groups 4 months post-transplantation

Variable	TMP: Median(IQR)	TSP: Median (IQR)	P value
GFR	65 (52-81)	70 (61-78)	0.6
Cr	1.26 (1.03-1.67)	1.19 (0.99-1.32)	0.3
BUN	36 (26-44)	39 (34-40)	0.6
UA	6.05 (4.68-7.45)	5.00 (3.55-6.55)	0.2
Ca	9.30 (9.05-9.65)	9.30 (9.10-9.80)	0.8
P	3.90 (3.45-4.37)	3.36 (3.00-4.40)	0.4
Na	138 (138-140)	139 (137-142)	0.7
K	4.20 (4.00-4.45)	3.98 (3.73-4.52)	0.3
AST	18 (16-20)	23 (20-27)	0.056
ALT	20 (16-44)	35 (22-37)	>0.9
ALP	256 (196-294)	216 (157-286)	0.7
WBC	5.70 (4.60-7.10)	6.75 (4.10-8.07)	0.5
Lym	26 (21-31)	31 (24-36)	0.6
Hb	13.50 (12.30-14.40)	13.15 (12.10-13.97)	0.7
RBC	4.75 (4.19-5.14)	4.40 (4.29-4.80)	0.5

GFR: Glomerular Filtration Rate, Cr: Creatinine, BUN: Blood Urea Nitrogen; Ca: Calcium; P: Phosphate; Na: Sodium; K: Potassium; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; WBC: White Blood Cell; Lym: Lymphocyte; Hb: Hemoglobin; RBC: Red Blood cell IQR: Interquartile range; TMP: Tacrolimus, Mycophenolate mofetil, Prednisolone; TSP: Tacrolimus, Sirolimus, Prednisolone; The number of patients In TMP group: 23; The number of patients In TSP group: 15

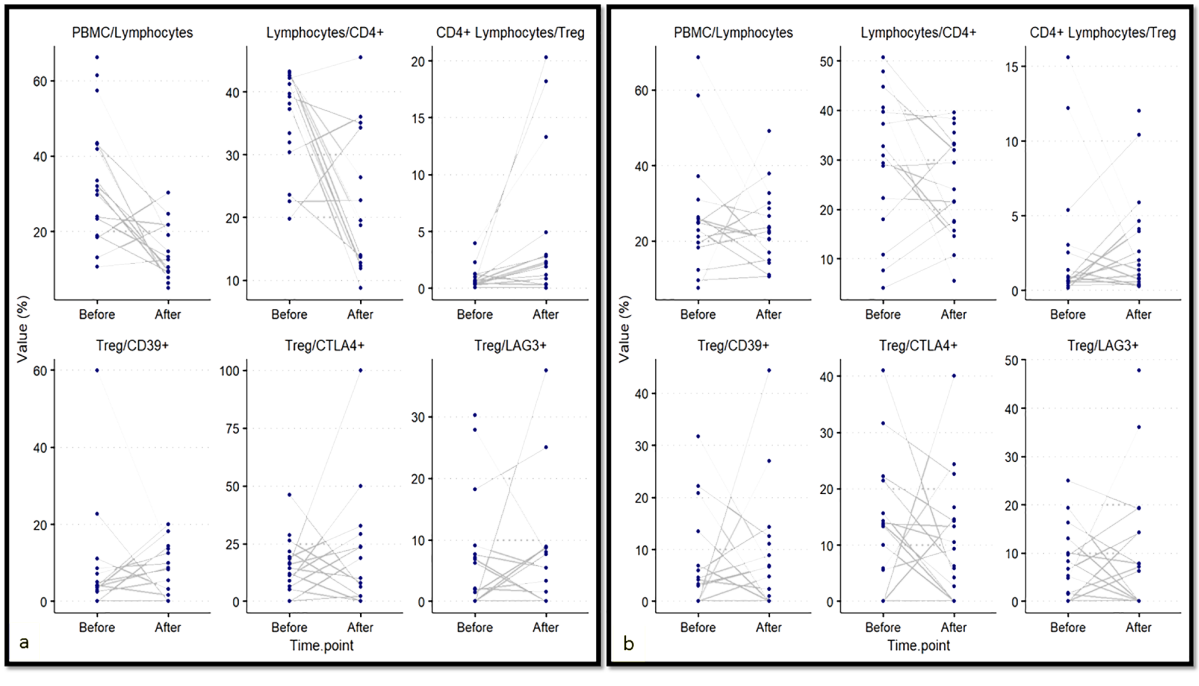

Figure 2. Analysis of peripheral Treg frequency. The frequency of Lym, $CD4^+$, Treg cells, $Treg\_CD39^+$, $Treg\_CTLA\text{-}4^+$, and $Treg\_LAG3^+$ are shown for patients before and 4 months after transplantation in TMP (2a) and TSP (2b) groups. Wilcoxon signed-rank test was used to compare the differences between before and after transplantations.

compared with before transplantation, although the difference was not statistically significant in the TSP group (24% vs 31%, P=0.2) (Figure 2.a, Figure 2.b and Table 5). Furthermore, there were no statistically significant differences in the frequency of $CD4^+$ T cells between the TMP and the TSP groups (P>0.05), following Transplantation (Table 6).

Table 5. Frequency of circulating CD39$^+$/Tregs, CTLA4$^+$/Tregs and LAG3$^+$/ Tregs pre and four months post transplantation in TMP and TSP groups.

Protocol	Variable %	Transplantation		P value
		Before: Median (IQR)	After: Median (IQR)	
TMP	Lym in PBMC	31 (22-43)	13 (9-21)	0.005
	CD4$^+$_Lym	39 (32-42)	19 (13-32)	0.011
	Tregs_CD4$^+$	0.6 (0.4-1.0)	2.2 (0.9-4.4)	0.003
	Tregs_CD39$^+$	4 (3-7)	9 (2-13)	0.6
	Tregs_CTLA4$^+$	15 (8-20)	14 (3-28)	0.5
	Tregs_LAG3$^+$	4 (0-8)	5 (0-9)	0.6
TSP	Lym in PBMC	25 (19-29)	23 (17-29)	0.5
	CD4_Lym	31 (20-40)	24 (17-33)	0.2
	Treg_CD4+	0.8 (0.6-2.8)	1.7 (0.7-4.1)	0.8
	Treg_CD39	4 (0-10)	7 (0-11)	>0.9
	Treg_CTLA4	14 (6-18)	9 (3-15)	0.4
	Treg_LAG3	7 (2-12)	7 (0-14)	>0.9

IQR: Interquartile range; TMP: Tacrolimus, Mycophenolate mofetil, Prednisolone; TSP: Tacrolimus, Sirolimus, Prednisolone; The number of patients

Table 6. Frequency of circulating CD39$^+$/Tregs, CTLA4$^+$/Tregs and LAG3$^+$/Tregs four months after transplantation TMP and TSP groups

Variable%	TMP: Median (IQR)	TSP: Median (IQR)	P value
Lym in PBMC	13 (9-21)	23 (17-29)	0.006
CD4$^+$ _Lym	19 (13-32)	24 (17-33)	0.4
Tregs _CD4$^+$	2.2 (0.9-4.4)	1.7 (0.7-4.1)	0.6
Tregs_CD39$^+$	9 (2-13)	7 (0-11)	0.5
Tregs_CTLA4$^+$	14 (3-28)	9 (3-15)	0.3
Tregs_LAG3$^+$	5 (0-9)	7 (0-14)	>0.9

The number of patients; IQR: Interquartile range; TMP: Tacrolimus, Mycophenolate mofetil, Prednisolone; TSP: Tacrolimus, Sirolimus, Prednisolone

There was a significant increase in the frequency of Tregs at the end of the four-month follow-up in the TMP group compared with before transplantation (2.2% vs 0.6% P=0.003, Table 5, Figure 2. a). Patients in the TSP group also showed an increase in Tregs four months after transplantation compared with before transplantation (1.7 vs 0.8), (Table 5 and Figure 2.b) although the difference did not reach statistically significant (P=0.8). Looking at the frequency of Tregs after transplantations, a higher level of CD4$^+$CD25$^+$FOXP3$^+$ Tregs was found in the TMP group compared with the patients in the TSP group (2.2 vs 1.7%). However, the difference was not statistically significant (Table 6). To further investigate the impact of immunosuppressive drug regimens on the number of CTLA-4$^+$, LAG-3$^+$, and CD39$^+$ Tregs, the frequency of Tregs was counted by flow cytometry in both groups. In this context, as indicated in Figure 2a and Table 5, the frequency of LAG3$^+$ Tregs and CD39$^+$ Tregs increased in the TMP group four months after transplantation in comparison with before transplantation (5% vs 4% and 9% vs 4%, respectively), but the number of CTLA4$^+$ (intracellularly) Tregs reduced four months after transplantation when compared with before transplantation (14% vs 15%). However, the differences were not statistically significant.

In the TSP group, while there was an increase in the frequency of $CD39^+$ Tregs four months after transplantation in comparison with before transplantation (7 vs 4%), the frequency of $CTLA4^+$ Tregs (intracellularly) reduced four months after transplantation in comparison with before transplantation (9% vs 14%). However, the differences observed before and after transplantations did not reach statistically significant (Table 5). The frequency of $LAG3^+$ Tregs did not show any change in the TSP group four months after transplantation in comparison with before transplantation (Table 5). Moreover, no statistically significant differences were found in the frequency of the $LAG3^+$ Tregs, $CD39^+$ Tregs, and $CTLA4^+$ Tregs between the two groups after transplantation (Table 6).

DISCUSSION

Successful long-time survival of kidney allografts concordant with the reduction of complications, in an optimal dose of extended immunosuppressive treatment, is a goal in renal transplantation (24). An immunosuppressive strategy is aimed to reduce the immune responses-associated adverse effects while preserving graft survival (25). In this regard, Immunosuppressive therapy with the mammalian target of rapamycin (mTOR) inhibitor (SRL) and purine biosynthesis inhibitor (e.g., MMF) are being utilized in combination with CNIs to decrease CNI dose and the related nephrotoxicity. In the present study, we have demonstrated that the frequency of peripheral blood $CD4^+CD25^+FOXP3^+$ Tregs elevated in both the TMP and the TSP groups four months after transplantation in comparison with before transplantation. However, the increased $CD4^+CD25^+FOXP3^+$ Tregs level was not statistically significant in the TSP group. Several previous studies have shown that the Tregs can play an effective role in long-term graft survival and the evaluation of peripheral and tissue-infiltrated Tregs may be considered a tolerance biomarker (26, 27). In particular, our findings were consistent with the current report by Jamali et al., which indicated that a combination of immunosuppressive regimens containing Tac/MMF or Tac/SRL may modulate the impacts of Tac on Tregs because the frequency of $CD4^+CD25^+FOXP3^+$ Tregs increased after transplantation (28).

Moreover, the frequency of $CD4^+CD25^+FOXP3^+$ Tregs in the TMP group was significantly higher at the end of the 4-month follow-up compared with before transplantation. Our findings were consistent with others, who showed that the low-dose Tac plus MMF could expand CD4+CD25+FoxP3+ Tregs (28, 29). It has also been reported that the combination of Tac and MMF could enhance the number of $CD4^+CD25^+FoxP3^+$ Tregs in renal transplant recipients compared with the combination of Tac and Everolimus (27). Nonetheless, the equilibrium between effector and regulatory cells can delineate the fate of the transplanted organ (30).

The mTOR inhibitors, such as SRL, and Everolimus are newly emerged immunosuppressive agents that, despite Tac, do not affect Tregs expansion. SRL may significantly reduce the frequency of Tregs in kidney transplant recipients compared with the healthy individuals (31). Sansgundo et al. also explained that the conversion of therapeutic protocol from Tac to SRL could expand the absolute number of Tregs (32). However, it has been reported that the concomitant administration of Tac and SRL could repress the proliferation of alloreactive Th1 and Th17 cells more evidently while maintaining Treg cells (15). Consistent with this finding, our study demonstrated that the combination of SRL with low-dose Tac could induce the expansion of Tregs.

MMF is commonly utilized in combination with CNIs in transplantation, which significantly decreases acute rejection risk. The relationship between MMF and Treg has not been elucidated completely. Demirkiran

et al. demonstrated that the conversion from CNIs to MMF could increase the percent of $CD4^+FoxP3^+$Tregs in liver transplantation (33). However, Lim et al. demonstrated that the expansion of $CD4^+CD25^+$ Tregs is not much comparable in the presence or absence of MMF (34).

Our findings also indicated an increase in the frequency of $CD39^+$ Tregs in the TMP and the TSP groups four months after transplantation compared with before transplantation and this increase was not statistically significant. In particular, several studies suggested that monitoring $CD4^+CD25^{+/-}CD39^+$ T cell subsets may help identify patients with, or at high risk of, renal allograft rejection (35). Similarly, the study by McRae et al. has shown that $D4^+CD25^+CD39^+$ memory Treg (mTreg) and $CD4^+CD25^-CD39^+$ memory T effectors (mTeff) in peripheral blood can be tracked in renal transplant patients (36). They showed that patients with acute T cell-mediated rejection had decreased mTregs and mTeffs cells compared with non-rejecting patients. Interestingly, remaining mTregs in stable transplant patients exhibited more potent suppressive capacity compared with the non-immunosuppressed controls (36).

In the present study, an immunosuppressive regimen containing the TMP in renal allograft recipients showed an increased level of circulating $CD4^+CD25^+FOXP3^+LAG3^+$ Tregs 4 months after transplantation versus before transplantation in contrast to an immunosuppressive regimen containing the TSP in renal allograft recipients whose increase was not statistically significant. Therefore, further studies are needed to conclude the inhibitory effect of $LAG\text{-}3^+$ Tregs in the TMP group. However, similar previous studies have described $CD4^+CD25^+LAG\text{-}3^+$ T cells as a new regulatory T cell population that suppresses T cell proliferation in an IL-10-dependent and FoxP3-independent mechanism, showing that LAG-3 may act as an essential molecule for the maximum inhibitory function of Tregs (37, 38).

We further evaluated the frequency of CTLA-4+ Tregs in both groups. A lower number of CTLA-4+ Tregs was found in PBMCs of the TSP group four months after transplantation when compared with before transplantation. While in the TMP group, a less decrease in CTLA-4+ Tregs was found in Tregs four months after transplantation compared with before transplantation. Our findings suggested that fewer CTLA-4 are stored in the cytoplasm of peripheral blood Treg cells in the TSP grope, which appears to indicate a more stable frequency of surface-CTLA-4+ Treg cells. Moreover, other findings have previously reported that the intracellular form of CTLA-4 molecule markedly increased in $CD4^+CD25^{high}$ T cells in the antibody-mediated chronic rejection (AMCR) recipients. The surface form of CTLA-4 indicated a reverse expression trend in comparison with its intracellular counterpart. It is more expressed in middle-term kidney transplants than in long-term kidney transplants and AMCR. They also reported a decreased surface and an increased intracellular CTLA-4 expression in Tregs of AMCR patients, demonstrating a tendency of AMCR-derived Tregs to compartmentalize CTLA-4 in intracellular storages, with lower surface immunoregulatory activity. Inversely, the elevated surface expression of CTLA-4 in transplanted patients with a good prognosis may correlate with better control over immune responses by functional inhibitory molecules (39).

Looking at the Paraclinical and laboratory findings, a significant increase in GFR 4 months after transplantation was found. Furthermore, BUN and creatinine levels significantly reduced in both groups 4 months after transplantation compared with before transplantation. However, the difference in GFR, BUN, and creatinine was not statistically significant between the two groups. These paraclinical patterns of our study were similar to previous reports described in transplantation studies. In this context, it has been shown that the ratio of Tregs to total T cells three months after

transplantation can predict increased GFR 6 and 12 months after transplantation, showing that the Treg percentage after transplantation positively correlated with better allograft function (24). In particular, a significant low negative and positive correlation was found between the number of CD4+CD25^{high} Treg cells with creatinine and GFR, respectively (40). Our findings are in agreement with previous reports, indicating the positive role of Treg cells in graft survival maintenance.

In conclusion, both the TMP and the TSP protocols can increase the frequency of circulating Tregs, CD39^{+} Tregs, and LAG3 Tregs although not intracellular CTLA-4+ Treg cells. It sounds like Treg cells have critical roles in the maintenance of transplant tolerance along with immunosuppressive agents. Using immunosuppressive drugs in kidney transplantation is like a double-edged sword, the choice of specific drugs, as well as the timing and the adjusting dose, are very important in inducing and maintenance of tolerance mediated by Tregs and better graft survival. Tregs could be considered a possible biomarker of tolerance after transplantation. However, our research is a preliminary study, and further multicentral studies with larger sample sizes and longer follow-ups are necessary.

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