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Research Article

Immune Biomarkers in Renal Transplant Recipients and Long-Term Graft Outcome, a Retrospective Observational Cross-Sectional Indian Study

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Keywords

- Kidney transplantation
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- Immune biomarkers
- Regulatory T cells (Tregs)
- T helper 17 cells (Th17)
- Interleukin 10 (IL 10)
- Interleukin 17 (IL 17)
- Immunosuppressive drugs

Abstract

Background: A major challenge for successful renal transplantation is to develop an efficient regulation to prevent allograft rejection. T helper 17 cells (Th17) (pro-inflammatory) and many regulatory T cells (CD4+CD25+; Tregs) (anti-inflammatory) have opposite functions influencing allograft survival. In contrast, IL-10, produced by multiple cells has potent anti-inflammatory properties. We have examined whether the evaluation of the percentage of Treg cells (Tregs %) in peripheral blood leukocytes (PBLs) as well as serum concentrations of IL-17 and IL-10 levels may correlate with allograft dysfunction and rejection.

Methods: This retrospective study included 57 patients who underwent kidney transplantation at the Nizam's Institute of Medical Sciences (NIMS). All patients were followed up for a minimum of two years because of regular follow-up, allograft dysfunction, and/or allograft rejection. The Tregs% in PBLs and serum concentrations of IL 10 and IL-17 were measured simultaneously by the flow cytometry and sandwich Enzyme-linked immunosorbent assay (ELISA) method, respectively.

Summary: The level of Tregs% was significantly decreased in PBLs of patients during allograft rejection (GR) in comparison to patients with stable transplant (ST) (median 6.25% vs. 5.85%, $p<0.05$). Serum IL-17 concentrations increased significantly in patients with graft rejection than in those with ST. While serum IL-10 levels also increased in GR than that in ST but they were statistically not significant. Furthermore, the Treg% levels, as well as the ratios of Treg/IL-10, Treg/IL-17, and IL-17/IL-10, can predict the long-term graft outcome. Further epigenetic studies are required to understand the variability of IL-10 levels with variable graft function.

INTRODUCTION

Renal transplantation is used to improve the survival as well as the quality of life of patients with end-stage renal disease. A significant barrier to kidney transplantation is the humoral and cellular rejection that is mediated by antibodies, T cells, and innate immune cells [1]. Out of total graft losses, 12% of graft loss is because of acute graft rejection (ACR) [2]. There is evidence that disturbed T-cell homeostasis play's a critical role in the development of acute graft rejection episodes [3]. Tregs are important regulators of immune tolerance and can actively suppress pro-inflammatory T-cell responses [4,5]. Quantitative and/or qualitative deficiencies of Tregs have been associated with the development of organ transplantation rejection [6-9]. Previous studies in animal models have shown that a deficiency in Tregs favours kidney transplantation rejection [6,7]. Human Tregs are not as well characterized as their murine counterparts; in part, this is due to restrictions and limitations of clinical studies. Furthermore, the characterization of Tregs in humans is more complex [10,11].

The main T subsets which are pivotal for this T-cell balance consist of T-helper 17 (Th17) cells and regulatory T cells (Tregs) [3,12]. Imbalanced Th17 and impaired Treg cells have been suggested to be involved in the pathogenesis of allograft rejection [13-15]. However, little is known about the number of Tregs and Th17 cells, and their association with different types of rejection in kidney transplant recipients (KTR) patients. IL-10 is one of the most significant antiinflammation cytokines produced during infectious diseases, cancer, and transplantation [16]. However, the roles of IL-17 and IL-10 in renal transplant recipients have not been clearly elucidated. In this study, we focused on the immune biomarkers (Tregs%, IL 10, and IL 17) in KTRs with variable graft function and their association.

MATERIALS & METHODS

This retrospective cross-sectional observational Indian study included57 kidney transplant recipients (KTR)of both sexes, aged 18-60 years who were transplanted at Nizams Institute of Medical Sciences, Hyderabad, India with a minimum of two years of follow-up. The subjects were KTR, those who presented to the hospital during the study period (from February 2019 to December 2019) either with graft dysfunction and graft rejection or with regular follow-up. The exclusion criteria were subjects with graft dysfunction without graft rejection and those who had not given informed consent. The study was approved by the Institutional ethics committee with review letter No. EC/NIMS/2275/2018 and ESGS No.761/2018. Informed consent was obtained from all the subjects.

After taking informed consent from the recruited subjects, blood samples were collected for estimating the immune biomarkers (Tregs, IL-10, and IL-17). Simultaneously clinical history was taken and corresponding medical records were checked to retrieve the medical data (pre-transplant data, transplant data, and post-transplant data) retrospectively. As per the records, all renal transplants were performed after taking permission from an Institutional competent authority (NIMS) as per the transplant human organ act. All patients received a kidney allograft with a negative lymphocyte cross-match.

For estimation of Tregs%, five milliliters of blood sample were collected in Ethylenediamine tetraacetic acid (EDTA) tube, and estimation was done by flow cytometry (BD FACS CANTO) using fluorochrome-labeled antibodies (anti-CD4 APC, anti-CD25 BV421, and anti-CD45 V-500-C RUO). The reagents for estimating Tregs were purchased from Becton Dickinson (BD) (approx. ₹.2000/test). The samples were processed as per the manufacturer's protocol and the percentage of T cells in blood was determined with the BD FACS Diva software in the Trucount tube. The parent percentage had been considered the final percentage for the analysis. The subpopulations were measured in relation to T cells, in the form of percentages. For estimation of the concentrations of cytokines, five millilitres of blood were collected in plain tubes and were processed to separate the serum. The serum was separated, aliquoted, and kept at -80°C until use. The estimation of cytokine concentration was done by the sandwich enzyme-linked immunosorbent assay (ELISA) method as per the manufacturer's protocol using a corresponding optical density. For cytokine concentration estimation kits were purchased from Krishgen Biosystems (approx. ₹.200/test).

The analysis was done by categorizing all the recruited subjects into two clinical categories. They are subjects with Transplant stable (TS), and with graft rejections (GR). Transplant stable was defined as no complaints were reported after transplantation with a normal serum creatinine level, estimated glomerular filtration rate (eGFR), and no indication for biopsy, protocol biopsies were not considered as indication biopsies. Graft rejection was defined as a rise in serum creatinine of 15% from baseline with an acute cell and/or antibody-mediated rejection (AR) in graft biopsy as per the BANFF 1997 criteria at any time during the follow-up. The immune biomarkers (Tregs%, IL-10, IL-17) were analysed in two clinical categories independently and in relation to the other biomarkers. For statistical analysis of the data Graph pad prism version, 8.4.2 software was used. $p<0.05$ was considered statistically significant.

RESULTS

This retrospective, observational, single-center cohort Indian study evaluated the long-term graft function in renal transplant recipients in relation to the percentage of Tregs, and cytokines concentration IL-10, and IL-17. Fifty-seven kidney transplant patients with a minimum of 2 years follow-up with both gender and aged 18-60 years were included. Both live renal transplant (LRT) (≈58%, n = 29) and deceased donor renal transplant (DDRT) (≈42%, n = 21) recipients were included. The pre-transplant data, transplant data, and post-transplantation data were collected retrospectively from the medical records and by taking the clinical history. The study participants were taking corticosteroids (5-10mg/day) (100%), calcineurin inhibitors (CNIs) (77.17%), mycophenolate mofetil (MMF) (89.5%), and Everolimus (17.56%).TS was observed in 59.64% and GR was seen in 40.3%.Treg% was estimated with the flow cytometry and cytokines concentrations of IL-10, and IL-17 were estimated by the sandwich ELISA method. The analysis of the data was done according to their graft function, they were divided into TS and GR patients. All demographic and clinical parameters are shown in Table 1 and Figure 1.

The Treg% in TS and GR, are shown in Table 2 and Figure 2A respectively. Treg% was significantly lower in patients with GR[median [95% confidence interval (CI) = 5.85(4.8-9.5),P<0.0463 *]than that in TS [Median,95% CI = 6.25(5.7-10.9)]. The ROC analysis of Treg% was performed with the risk of graft rejection in KTR to understand the predictive value, for each predictor variable sensitivity and specificity were calculated (Figure 3). A receiver operating curve (ROC), area under the curve (AUC) of 0.67 was observed with the Tregs% with P-value <0.037.

The IL-10 (pg./ml) concentrations in TS and GR are shown in Table 2 and Figure 2B. The concentration of IL-10 was higher in patients with GR [Median, 95% CI = 2.83 (2.25-4.3)] than that in TS [Median, 95% CI = 2.06 (1.7 – 3.8)] which is statistically not significant (P=0.2688). The ROC analysis of IL-10 was performed with the risk of graft rejection in KTR to understand the predictive value, for each predictor variable sensitivity and specificity were calculated (Figure 4). A ROC AUC of 0.58 was observed with the IL-10 with P-value 0.325.

The IL-17 (pg./ml) concentrations in TS and GR are shown in Table 2 and Figure 2C. The concentration of IL-17 was significantly higher in GR [Median, 95% CI = 147.9 (87.4-220.5), P<0.0381*] than that in TS [Median,95% CI = 67.85(62.54-138.5)].The ROC analysis of IL-17 was performed with the risk of graft rejection in KTR to understand the predictive value, for each predictor variable sensitivity and specificity were calculated (Figure 5). A ROC AUC of 0.63 was observed with the IL-17 with a P-value of 0.138.

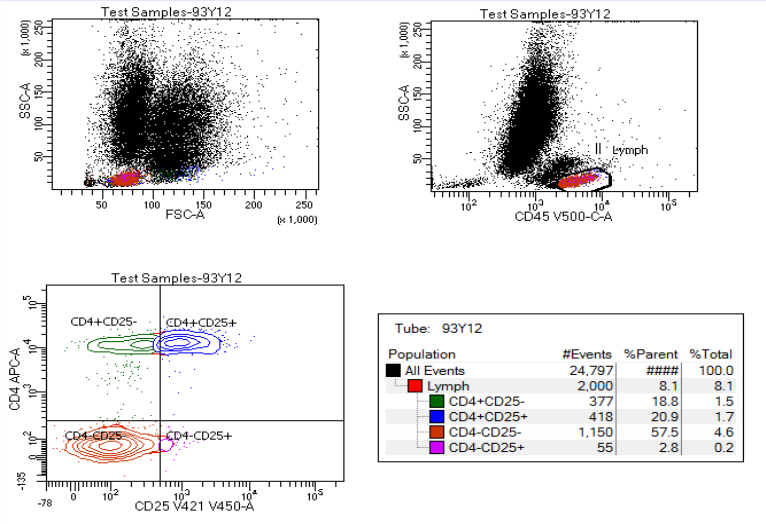

Figure 1 Flow cytometry gating strategies. Lymphocytes were selected based on Forward scatters/side scatters (FSC/SSC). CD25 vs. CD4 dot plot of CD4+CD25+ lymphocytes

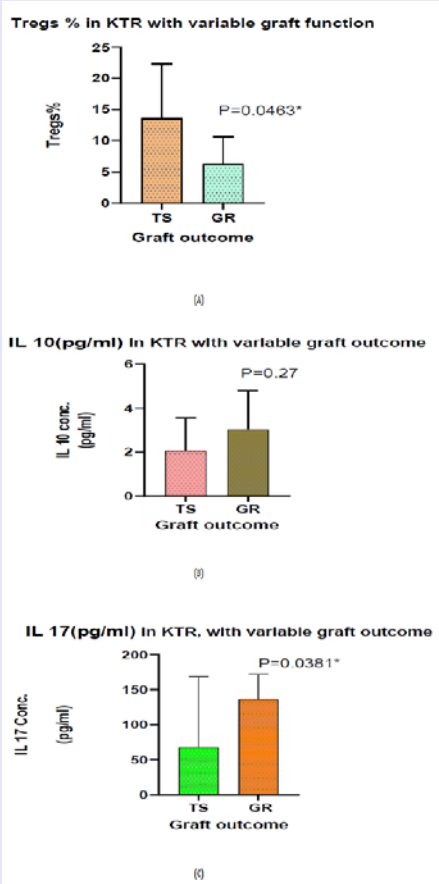

Figure 2 Immune biomarkers in KTR with variable graft function (A) Treg% in KTR with TS and GR (B) IL-10 in KTR with TS and GR (C) IL-17 in KTR with TS and GR. *P<0.05 transplant stable vs. graft rejection.

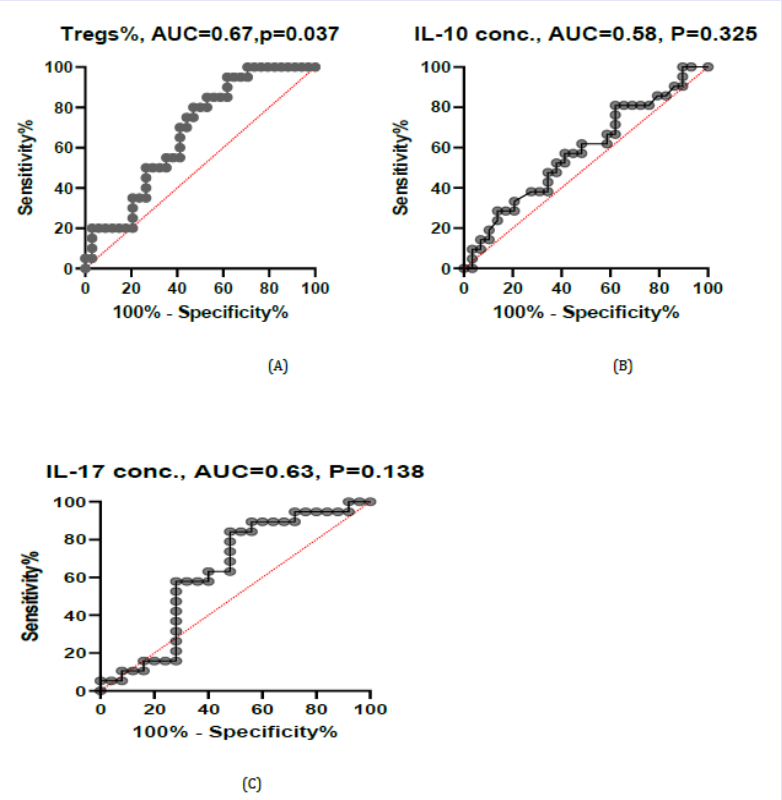

Figure 3 ROC Analysis for the prediction of acute graft rejection with (A). Tregs%, (B) IL-10 and (C) IL-17.

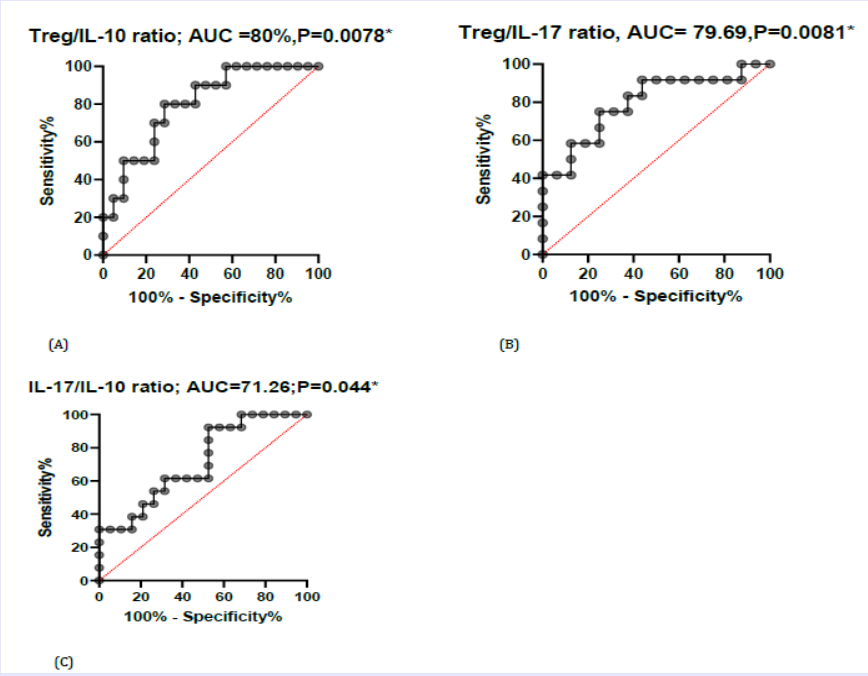

Figure 4 ROC analysis for the prediction of acute graft rejection with (A) Treg/IL-10, (B) Treg/IL-17, and (C) IL-17/IL-10.

Table 1: Demographic and Clinical Characteristics of the study participants

Parameter	Results		
	Transplant stable (n=34)	Graft rejection (n=23)	P value
Total no. of subjects (Male; Female)	30;4	19;4	0.749
Age in yrs. (Mean±SD)	37±10.7	35±9.2	0.487
Body mass index (kg/m^2) (Mean±SD)		18.6±4.2	0.054
Dialysis vintage in months (Mean ± SD)	20.5±3.1 9.32±9.02	11.14±9.27	0.463
Types of Dialysis			
Haemodialysis; N (%)	34(100%)	23(100%)	
Peritoneal dialysis	0%	0%	
Native kidney Disease (NKD)			
CGN; N (%)	15(44.1%)	11(47.82%)	
CIN; N (%)	14 (41.2%)	8(34.8%)	
DN; N (%)	1(2.9%)	0(0%)	
FSGS; N (%)	1(2.9%)	0(0%)	
IgAN; N (%)	2(5.88%)	3(13.04%)	
Others(Nephrosclerosis, MN); N (%)	1(2.9%)	1(4.34%)	
HLA Typing			
Haplo/Diplo match; Nil match (N)	22;12	15;8	0.4626
Type of transplantation			
LRT; DDRT (N)	28;6	20;3	0.7306
S. Cr in mg/dl (Mean ± SD)	1.25±0.344	2.95±1.6	0.0001*
eGFR	69.52±19.55	31.73±17.3	0.0001*
immune suppression Induction			
No induction; N (%)	23(68.42%)	16(69.5%)	
Induction Total; N (%)	11(32.35)	7(30.4%)	
ATG; N (%)	3(27.27%)	1(14.3%)	
ILRB; N (%)	8(72.72%)	6(85.7%)	
Immunosuppression Maintenance			
Wysolone; N (%)	34(100%)	23(100%)	
CNI, MMF; N (%)	23(67.64%)	15(65.21%)	
CNI, Everolimus; N (%)	2(5.88%)	1(4.34%)	
CNI, Azathioprine; N (%)	1 (2.94%)	1(4.34%)	
MMF, Everolimus; N (%)	3(8.82%)	3(13.04)	
CNI; N (%)	2(5.88%)	1(4.34%)	
MMF; N (%)	3(8.82%)	2(8.7%)	
Initial graft function			
IGF; N (%)	27(79.4%)	14(60.8%)	
DGF; N (%)	5(14.7%)	6(26.1%)	
SGF; N (%)	2(5.8%)	2(8.6%)	
Comorbidities			
Diabetes; N (%)	2(5.8%)	1(4.34%)	
Hypertension; N (%)	2(5.8%)	3(13.04%)	
Final graft outcome			
TS; N (%)	34(59.64%)		
GR; N (%)	23(40.3%)		
Types of rejection			
ACR; N (%)		10(43.5%)	
ABMR; N (%)		5(21.7%)	
Combined; N (%)		5(21.7%)	
CR; N (%)		3(13.04%)	
Mortality; N (%)		1(4.35%)	

Abbreviations: CGN, chronic glomerular nephritis; CIN, contrast-induced nephropathy; DN, diabetic nephropathy; FSGS, focal segmental glomerulosclerosis; IgAN, IgA nephropathy; JN, juvenile nephronophthisis; ATG, anti-thymocyte globulin; IL2RB, IL 2 receptor blocker; ACR, acute cellular rejection; ABMR, antibody-mediated rejection; CR, chronic rejection; IGF, immediate graft function; DGF, delayed graft function; SGF, slow graft function; GD, graft dysfunction; GL, graft loss; CNI, calcineurin inhibitors; MMF, mycophenolate mofetil.

The median ratio of Treg/IL-10, Treg/IL-17, and IL 17/ IL 10 in patients with TS vs. GR is 0.08 vs. 0.06, P=0.0197; 3.7 vs. 1.8, P=0.0432; and 98.2 vs. 38.64, P=0.0155 respectively (Table 2). The median ratio of Treg/IL 10 had decreased in patients with GR than that with TS, indicating that Treg% decreased and IL-10 conc. increased in patients with GR compared to TS patients. The median ratio of Treg/Il-17 had decreased in patients with GR than that with TS, indicating that Treg% decreased and IL-17 conc. increased in patients with GR compared to TS patients. The ratio of IL-17/IL-10 had decreased in patients with GR than that with TS, indicating that IL-17 conc. and IL-10 conc. increased in patients with GR compared to TS patients. The ROC analysis of the ratios Treg/IL-10, Treg/IL-17 and IL-17/IL-10 with the risk of graft rejection were shown AUC= 80%, P=<0.0078; AUC= 79.69, P=<0.0081; AUC=71.26, P=<0.044 respectively. The ratios of cytokines in relation to Treg% are better predictors in graft rejection than that of independent markers and the IL-17/IL-10 ratio.

All recruited patients with transplant stable and graft rejection were divided into three categories (from 2-5 years; 6-10 years; >10 years) based on their transplant duration and compared each biomarker in each category of a similar transplant duration. All categories of graft rejections showed a significant decline in the Tregs% compared with the transplant stable (Table 3). With increasing duration of transplant, Treg% had increased with TS up to 10 years of transplantation and later it was maintained.

The IL 10 concentrations showed an increase in their levels in patients with graft rejection than that of transplant stable in all categories though we could not observe a statistically significant difference (Table 3)

The IL 17 concentrations showed an increase in their levels in patients with graft rejection than that of transplant stable in all categories though we could not observe a statistically significant difference (Table 3).

The association of immune biomarkers in KTR with Variable transplant duration and variable graft function is shown in Table 4. With the variable duration of transplantation, the ratios of Treg/IL-10, Treg/ IL-17, and IL-17/IL-10 levels were decreased in GR compared to TS. But it was not universally significant in all variable transplant duration.

DISCUSSION

In this study, we measured and analyzed immune biomarkers (Tregs%, IL 10, and IL17) to estimate the predictive value of graft rejection in KTR independently and in relation to the other biomarkers.

Our data showed that Tregs% was significantly decreased in patients with graft rejection compared to the patients with transplant stable (Figure 2A). Various studies have also demonstrated that the Treg cell population reduces during rejection episodes for both acute and chronic rejection episodes

Table 2: Immune biomarkers and their ratios in KTR with variable graft function.

Subjects	Treg% (%CD4 cells)	IL 10 (pg.//ml)	IL 17 (pg./ml)	The median ratio		
				Treg/IL-10	Treg/IL-17	IL-17/IL-10
TS (n=34)	6.25(5.7-10.9)	2.06 (1.7 – 3.8)	67.85(62.54-138.5)	3.7(3.48-7.7)	0.08(0.14-0.46)	98.2(95.8-229.7)
GR (n=23)	5.85(4.8-9.5)	2.83(2.25-4.3)	147.9 (87.4-220.5)	1.8(1.6-3.4)	0.06(0.044-0.08) *	38.64(36.55-60)
P-Value (TS v/s GR)	0.0463*	0.2688	0.0381*	0.0197*	0.0432*	0.0155*

Abbreviations: TS, transplant stable; GR, graft rejection. Data represented as median (95%CI); The median ratio was shown. *p<0.05 transplant stable vs. graft rejection.

Table 3: Immune biomarkers in KTR with Variable transplant duration and variable graft function.

Variable	Treg%		P value	IL-10 (pg./ml)		P value	IL-17(pg./ml)		P value
Duration of Tx.	**TS**	**GR**		**TS**	**GR**		**TS**	**GR**	
2-5 yrs.	5.36(3.93 – 9.73) (n=14)	3.62(1.7-5.3) (n=9)	P=.0374 *	2.06(1.7-3.7)	4(2.69-6.41)	P=0.231	126.64 (194.6-448)	147.91(83.4-345)	P=0.453
6-10 yrs.	10.65(7.2-16.3) (n=12)	4.83(2.8-7) (n=8)	P=0.0462*	3.67(2.8-4.8)	3.04(1.89-3.51)	P=0.842	271.3(222.23-607)	181(120.3-218)	P=0.679
>10 yrs.	8.76 (6.65-11.7) (n=8)	2.4(0.55-4.5) (n=6)	P=0.0267*	1.11(0.7-2.07)	4.8(0.9-5.84)	P=0.5421	18.01(14.5-31)	96.34(16.5-176.2)	P=0.178

Abbreviations: TS – Transplant stable; GR- Graft Rejection; Tx- Transplantation; Yrs. – Years. *P<0.05 transplant stable vs. graft rejection with similar transplant duration; Data represented as (median,95% CI)

Table 4: Association of immune biomarkers in KTR with Variable transplant duration and variable graft function.

Variable	Treg/IL-10			Treg/IL-17			IL-17/IL-10		
Duration of Tx.	**TS**	**GR**	**P-Value**	**TS**	**GR**	**P-Value**	**TS**	**GR**	**P-Value**
2-5 yrs.	3.5(1.75-7) (n=14)	1.01(0.8-2.8) (n=9)	P=0.234	0.03(0.01-0.065)	0.0097(0.008-0.02)	P=0.0364*	137.85(97.6-243.7)	49.6(23.5-77.88)	P=0.0143*
6-10 yrs.	1.2(0.27-4.27) (n=12)	1.22(0.77-1.74)(n=8)	P=0.875	0.08(0.03-0.16)	0.023(0.01-0.04)	P=0.334	124.8(33.1-285.5)	45.4(38.3-83.9)	P=0.246
>10 yrs.	14.5(5.7-21.8) (n=8)	0.55(0.09-1.01)(n=6)	P=0.0213*	0.61(0.4-0.9)	0.004(0.002-0.03)	P=0.0173*	28.7(25.9-33.4)-	18.03(0.9-36.9)	P=0.234

* p<0.05 transplant stable vs. graft rejection.

[17,18]. The above results are in concordance with the findings from our study. The ROC analysis of our study also showed a 67% prediction of acute graft rejection with the percentage of peripheral blood Tregs%, which had shown a statistically significant predictive value (Figure 3). In Inomata T et al., study renal transplant recipients with low Tregs were reported to have a higher risk for acute cellular rejection [19]. According to Tanya et al., Tregs' immunosuppressive capabilities make them a possible treatment for organ transplant recipients to minimize rejection and prevent negative outcomes [20]. Min Hu et al., stated that in kidney transplants, Treg cells may provide tolerance or reduce immunosuppression [21].

There were increased IL 10 levels observed in patients with GR than that in TS, though they are statistically not significant (Figure 2B). The ROC analysis showed an area under the curve with a 58% prediction of acute graft rejection which was statistically not significant (Figure 4). According to Kapoor A et al**.,** increased expression of IL-10 was observed in patients undergoing acute rejection. Chronic graft rejection and poor graft survival are associated with interstitial fibrosis and tubular atrophy is accompanied by the upregulation of IL-10 [22]. A study by Mahmut Ilker et al., postulated that IL-10 levels increase along with decreased kidney function [23]. Yongsheng et al., stated that there was a significant increase in IL-10 infiltration in the AMR group with high C-X-C motif chemokine 13 (CXCL13) expression [24].

There were increased IL 17 levels observed in patients with GR than that in TS which was statistically significant (p<0.05) (Figure 2C). Similarly, a study by Bagheri M et al., reported that in kidney transplant recipients, the presence of Th1 and Th17 cells was shown to be associated with acute rejection or delayed graft function. In a study by Van Kooten C et al., immunofluorescence showed expression of IL-17 in kidney biopsies from patients suffering from graft rejection, whereas pre-transplant biopsies and normal kidneys were negative for IL-17 expression [25]**.** Similarly, Liang Ma et al., reported there was an increase in the number of Th 17 cells, IL 17 concentration, and lower levels of Tregs levels and IL-10 levels in antibody-mediated rejection (AMR), acute cellular rejection, and chronic rejection groups [26]**.** In contrast, our study showed increased IL-10 levels in GR than in those with TS. The ROC analysis of our study showed that 63% of predictions with acute graft rejection but it is statistically not significant (Figure 4).

Similarly, Corroborative evidence from a study by Mortazavi H et al., showed that an imbalance in Th17/Treg cells is associated

with allograft rejection, and immunosuppressive therapy following transplantation is associated with an improved Th17/Treg balance [27]. Byung HC et al., postulated that Foxp3/IL 17 ratio is a useful indicator in assessing the severity of graft injury, and allograft dysfunction, and predicting the clinical outcome of cell-mediated rejection [28]. A study by Chung BH et al., showed a lower Treg/IL 17 ratio infiltrating ratio is significantly associated with the reduced allograft function and more severe interstitial and tubular injury [29]. A study by Sara Assadiasl et al., reported Th17/Tregs ratio in liver transplant patients with acute rejection was significantly higher than in the stable recipients [30-32]. Similarly in our study, the median ratio of Treg/IL-17 was decreased in patients with GR than in TS. A study by Youssra et al., stated that a significant increase of IL-17A mRNA and protein levels in AR recipients that are genetically controlled highlights the role of this cytokines that can be a useful clinical biomarker to predict early acute renal allograft rejection.

CONCLUSIONS

The Tregs% had decreased significantly in patients with graft rejection than in those with transplant stable in long term. IL-17 concentrations had increased significantly in patients with graft rejection than that in TS. IL-10 concentration levels had also increased in GR than that in TS but they are statistically not significant. The Treg%, as well as the ratios of Treg/IL-10, Treg/IL-17, and IL-17/IL-10, can predict the long-term graft outcome.

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DATA STATEMENT

The research data is confidential

AUTHOR CONTRIBUTIONS

Swarnalatha Guditi and Gangadhar Taduri participated in the research design, edited the manuscript, and interpreted the data. Katyayani Bejugama participated in the research, writing the paper, and data analysis.

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