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Variability of Immune Biomarkers with the Graft Function in Kidney Transplant Patients in India, an Observational Prospective Cohort Study

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Abstract: In renal transplantation (RT), the major issue is to maintain the immune homeostasis, limiting graft rejection (GR), and promoting transplant tolerance. A total of 70 subjects of chronic kidney disease patients on maintenance haemodialysis, opted for RT and 20 controls were recruited. The Tregs% (CD4+CD25+), concentration of cytokines IL –10 and IL 17 were measured in pre-and post-transplant at a defined timelines with stable graft function (SGF) and with GR for two years, using flow cytometer and sandwich ELISA method. With SGF, Tregs% Baseline [8.5 (6.5–10.7) vs. HCs [14.25 (13–18), $p < 0.01$)], at Baseline vs. six months [11.54 (8.9–15)], $p < 0.001$); At Baseline [3.05 (1.05–5.2) vs. GR 8.5 (6.5–10.7), $p < 0.05$]. Serum IL 10 baseline [3.6 (2.56–4.6) vs. HC (6.4 (4.8-9.8), $p<0.001$]. Serum IL 17 levels at baseline [120 (92 - 176) vs. HC [20.88 (18-55), $p<0.05$], day four vs. baseline [180 (160.5-257.45); $p<0.05$], day 90 vs. baseline [53.3 (48-100), $p< 0.05$] and this was maintained for two years, with GR vs. baseline [190 (105-372); $p<0.05$]. ROC analysis of Tregs% (AUC of 0.758 and a p – value of <0.05), IL-10 (AUC of 0.8 and a p – value of 0.117), IL-17 (AUC of 0.937 and a p – value of <0.05). With SGF, Tregs % increased from 6 months, IL-17 decreased from 3 months, IL-10 did not show changes and continued till two years; with GR, Tregs% decreased from baseline, IL-10 did not show changes, and IL-17 increased due to high inflammation. ROC analysis showed that the Tregs% and IL-17 are better predictors of graft outcome. However, the association between biomarkers with graft function couldn't be evaluated which needs further studies.

Keywords: Renal Transplantation, Immune Biomarkers, Graft Function, Enzyme-linked Immunosorbent Assay, Flow Cytometer

1. Introduction

Dialysis is associated with a lower quality of life and a higher mortality rate than kidney transplantation. Hence, Kidney transplantation is the optimal treatment for end stage renal disease patients [1]. The cellular rejection that happens as a result of renal transplantation and is mediated by antibodies, T cells, and innate immune cells is a substantial obstacle. A tissue biopsy is still the gold standard for evaluating immunologic graft damage, which is a painful procedure and requires hospitalisation of patient [2]. Renal transplant rejection may be influenced by an imbalance between various CD4 (+) T cell subsets and abnormally elevated inflammatory cytokines [3]. In the setting of transplantation, regulatory T cells (Tregs) are crucial for sustaining self-tolerance and for controlling allo-immune responses [4]. To suppress T cells, Tregs release suppressive cytokines such as IL-10, TGF-B, and IL-35. Additionally, Tregs have the ability to prevent target T cells from producing IL-2 mRNA and bind to target cells to trigger cell cycle arrest and death [5]. Several regulatory systems exist in organs to prevent graft rejection, and Tregs play a crucial

role in these systems. As a result of their immunosuppressive capacities, Tregs cells have become a biomarker for predicting different graft outcomes [6]. Recently, it has been discovered that there is a subpopulation of lymphocytes called Th17 cells that produce IL-17 in particular. There is also evidence that T (H) 17 (also known as IL-17) and IL-6 may contribute to organ rejection [7]. Increasing Th17 levels may cause acute rejections or chronic allograft dysfunctions. In allografts, Tregs regulates immune response in order to protect them [7]. Hence, in our study, we included Tregs, IL-17, IL-10 as immune biomarkers with the graft function to understand the role of these biomarkers with graft outcome in kidney transplant recipients.

2. Methods

2.1. Design of the Study

2.1.1. Study Population

70 subjects, both males and females, ranging from 18 to 60 years of age, were recruited and divided into a study cohort (n=50) and healthy control group (n=20). A study cohort included 50 ESRD patients, those who were opted for both live and deceased kidney transplantation from February 2019 to December 2019 at Nizams Institute of Medical Sciences, Hyderabad. (Figure 1).

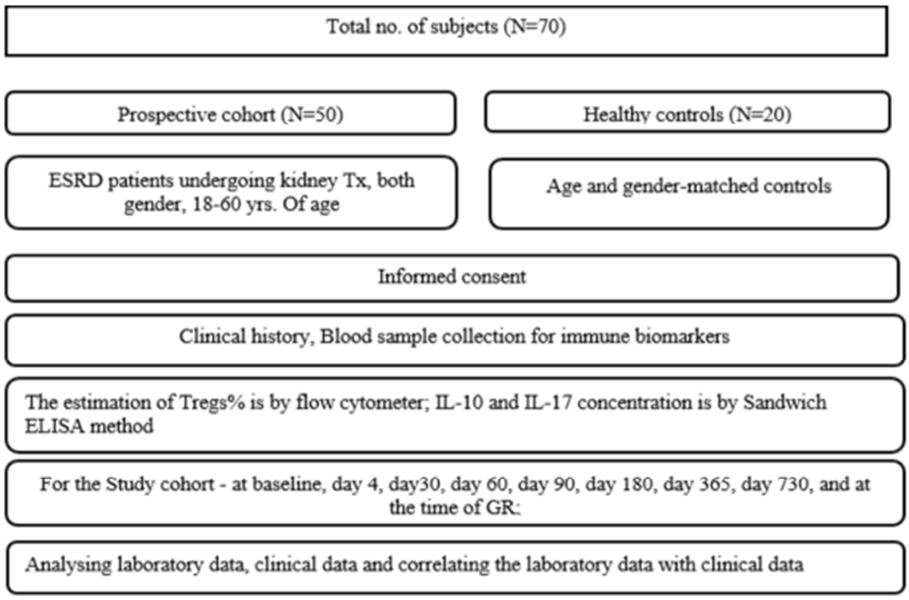

Figure 1. *Showing that experimental design of the study.*

2.1.2. Data Collection Methods & Tools

It is important to note that the transplantation procedure and post-transplant treatment were performed strictly for medical reasons and not for the purposes of this study. The study was commenced after approval from the Institutional ethics committee. All subjects provided informed consent.

Demographic, Laboratory and the clinical data were taken (Table 1).

The prospective study cohort was followed up for two years at defined time points (at baseline, day 4, day 30, day 60, day 90, day 180, day 365, day 730, and at the time of GR). The clinical outcomes considered are the occurrence of graft rejection and stable graft function. Data on episodes of acute cellular rejection based on Banff criteria (2017 guidelines), graft failures, and deaths were recorded.

2.2. Experimental Protocols

The Whole blood samples (3ml) were collected in EDTA vacutainers for estimation of Tregs% and in Plain vacutainers for estimation of IL 10 and IL 17 concentration levels at defined time points in the study cohort. To separate the serum, plain vacutainers were kept at room temperature for 15 min, then centrifugation for 10 min at 2000rpm. The serum was separated, aliquoted, and kept at -80°C until use. Estimation of the immunobiomarkers (IL-10, IL-17) was done according to manufacturer's protocol using ELISA. The ELISA kits of IL 10, and IL 17 were procured from the Krishgen Biosystems.

All the reagents (APC mouse anti-human CD4, BV421 mouse anti-human CD 25, Anti-mouse IgG/-ve cont. (BSA) comp plus, CD45 V 500-C RUO, Lysing solution, Sheath fluid) for the estimation of the Tregs% were procured from the BD and Tregs% was assessed using BD FACS flow cytometer according to the manufacturers protocol.

2.3. Data Analysis

Data analysis was done using Graph Pad Prism. Descriptive analysis of normally distributed variables is reported as median and 95%CI. Paired t-test was performed to get the level of significance in pre and post-transplant time points. An unpaired t-test was performed to get the level of significance in baseline pre-transplant and the healthy control. Level was considered statistically significant when $p<0.05$. With the risk of GR, ROC analysis was performed with

immunobiomarkers. Sensitivity and specificity were calculated for all immunobiomarkers.

3. Results

3.1. Demographic, Laboratory and Clinical Data of Study Participants

This study evaluated the role of immune biomarkers in RT with SGF as well as with acute GR at short-term graft function. Demographic medical and surgical details of study participants are shown in Table 1.

Table 1. *Demographic, laboratory and clinical data of the study participants.*

Variables	Results
Total subjects (Male: Female)	50 (37: 13)
Age in years (mean ± SD)	31.1 ± 9.46
Body mass index (kg/m^2) (mean ± SD)	19.45 ± 2.2
Dialysis vintage (mean ± SD) in months	66 ± 25.45
Baseline creatinine (mean ± SD) mg/dL	1.48 ± 0.6
Baseline eGFR (mean ± SD) in mL/min/1.73 m^2	38.95 ± 11.9
Types of Dialysis	
Hemodialysis; N (%)	50 (100%)
Peritoneal dialysis	0 (0%)
HLA Typing	
Haplo /Diplo match; N (%)	19 (38%)
Nil match; N (%)	31 (62%)
Type of transplantation	
Deceased donors; N (%)	21 (42%)
Live donors; N (%)	29 (58%)
Immunosuppression maintenance	
Wysolone; N (%)	50 (100%)
CNI, MMF; N (%)	50 (100%)
Comorbidities	
Diabetes; N (%)	2 (4%)
Hypertension; N (%)	20 (40%)
Final graft outcome	
Normal graft function; N (%)	25 (50%)
Graft dysfunction	23 (46%)
Graft loss; N (%)	2 (4%)
Types of rejection	
ACR	Nil
ABMR; N (%)	7 (14%)
Combined; N (%)	2 (4%)
Mortality; N (%)	12 (24%)

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.

3.2. Immune Biomarkers in Study Participants

Table 2 displays the Tregs% in HCs and RT at specified time intervals. At baseline, the Tregs% decreased [median (95% (CI) = 8.5 (6.5–10.7)] compared to that in controls [median (95% CI) = 14.25 (13–18)], $p < 0.01$], at fourth POD [median (95% CI) = 5.13 (4.6–7.2)], and at six months [median (95% CI) = 11.54 (8.9–15)], $p < 0.001$)] compared to that in baseline [median (95% CI) = 8.5 (6.5–10.7)], $p < 0.01$]. This was maintained for two years with SGF. With the GR, Tregs% decreased significantly than that of baseline [median (95% CI) = 3.05 (1.05–5.2) vs. 8.5 (6.5–10.7)], $p < 0.05$] (Figure 3). The representative diagram of flow cytometer was shown in Figure 2.

Table 2. *Immune biomarkers in healthy controls and prospective cohorts at different time points.*

Time points	Tregs%	IL-10 conc. (pg. /ml)	IL-17 conc. (pg. /ml)
Healthy Control (N=20)	14.25 (13-18)	6.4 (4.8-9.8)	
Baseline (N=50)	8.5 (6.5 – 10.7) **	3.6 (2.56–4.6) ***	120 (92 - 176) *
Day 4 (N=50)	5.13 (4.6-7.2) **	3.45 (2.5–4.5)	180 (160.5-257.45) *
Day 30 (N= 48)	9.53 (6.6-9.9)	3.52 (2.8 - 4.3)	62.6 (65.2-135)
Day 60 (N=45)	7.6 (6.2-10.2)	4.8 (3–5.4)	68 (66- 134)
Day 90 (N=45)	6.1 (4.82-8.8)	2.43 (2.24–4.6)	53.3 (48-100) *
Day 180 (N=44)	11.54 (8.9-15) ***	1.1 (0.8 - 2) **	35.32 (27.3 – 64) *
Day 365 (N=43)	9.25 (7.5-11)	1.48 (1.3–2.7)	11.46 (10.3-36) **
Day 730 (N=38)	10.86 (9.4-11.6) **	1.92 (0.5 - 4.8)	17.52 (13-21.24) **
GR (N=5)	3.05 (1.05- 5.2) *	4.7 (1.0 -5.6)	190 (105-372) *

*p < 0.05 vs. baseline, **p < 0.01 vs. baseline, ***p < 0.001 vs. baseline; baseline vs. control
Data are presented as the median (95% CI).

With SGF, serum IL 10 decreased in baseline [median (95% CI) = 3.6 (2.56–4.6); p<0.001] compared to HC [median (95% CI) = 6.4 (4.8-9.8)], decreased at six months of RT [median (95% CI) =1.1 (0.8 - 2), p<0.01] than baseline and maintained for two years. (Figure 4 & Table 2), with GR, IL-10 did not show any changes. (Table 2).

Serum IL 17 increased at baseline [median (95% CI) = 120 (92 - 176); p<0.05] compared to HC [median (95% CI) = 20.88 (18-55)], increased on day 4, [median (95% CI) = 180 (160.5-257.45); p<0.05] than that of baseline. With SGF, serum IL-17 decreased on day 90 [median (95% CI) = 53.3 (48-100)], this was maintained for two years. (Table 2, Figure 5) and with GR, IL-17 increased [median (95% CI) = 190 (105-372); p<0.05].

Two years after transplantation, ROC analysis of Tregs% was carried out due to the risk of acute GR. Its results indicated that the Tregs% can be a good indicator of graft outcome, with an Area under the curve (AUC) of 0.758 and a p-value of less than 0.05. (Figure. 6A). AUC of IL-10 showed 0.8, however p – value was not statistically

significant (p = 0.117), indicated that it requires further studies for prediction of graft outcome. (Figure. 6B). AUC of IL-17, showed 0.937 and a p – value of <0.05, suggested that the IL-17 concentration can be utilized for prediction of graft outcome (Figure. 6C).

The correlation of immune biomarkers were assessed using Pearson correlation coefficient. However, we could not evaluate the correlation, further studies are required.

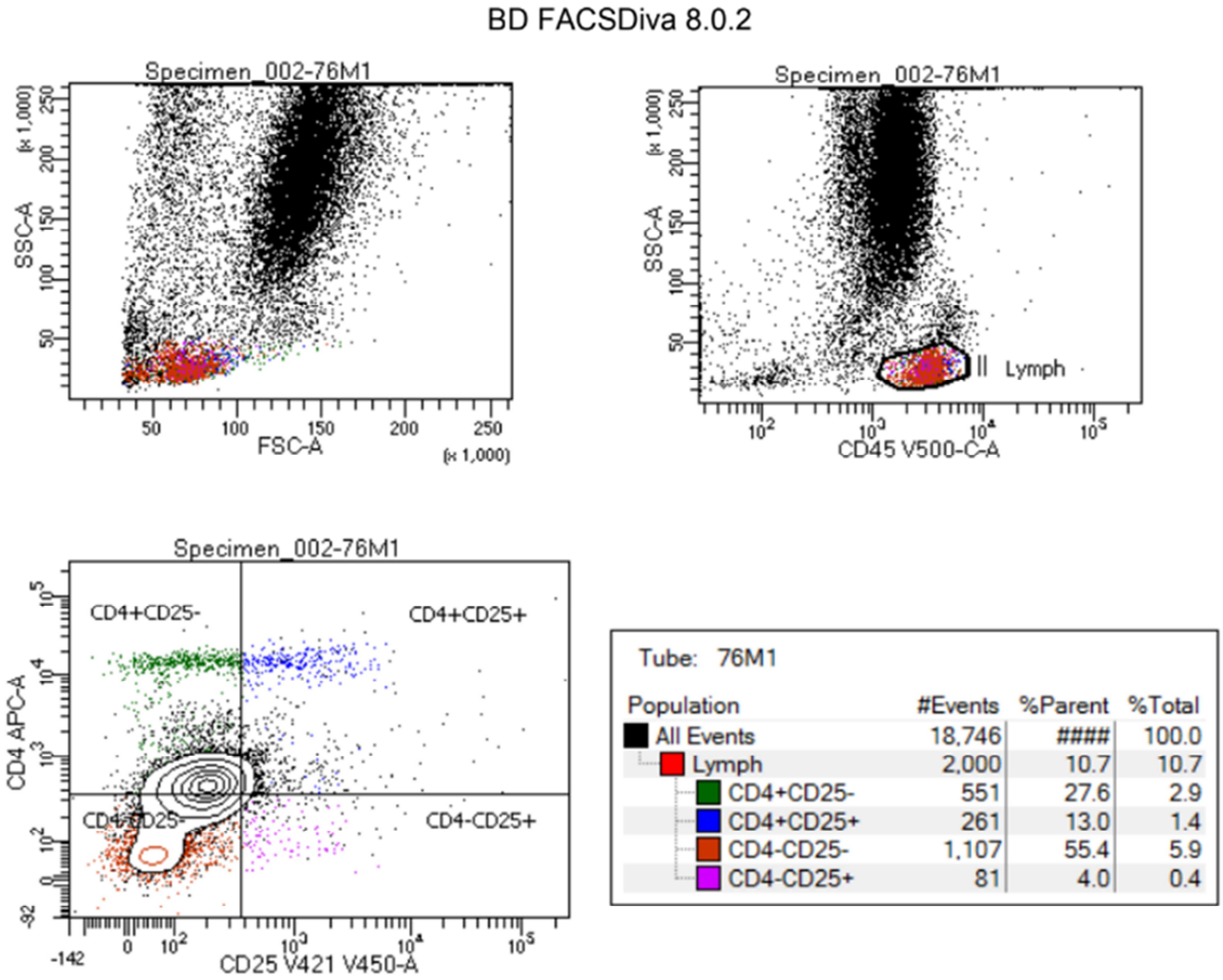

Figure 2. *A sample flow cytometric study of Tregs (CD4+CD25+) diagram; A) lymphocyte population gating schemes based on forward and side scatters; B) lymphocyte CD45 vs. SSA dot plot; and C) lymphocyte CD4 vs. CD25 dot plot.*

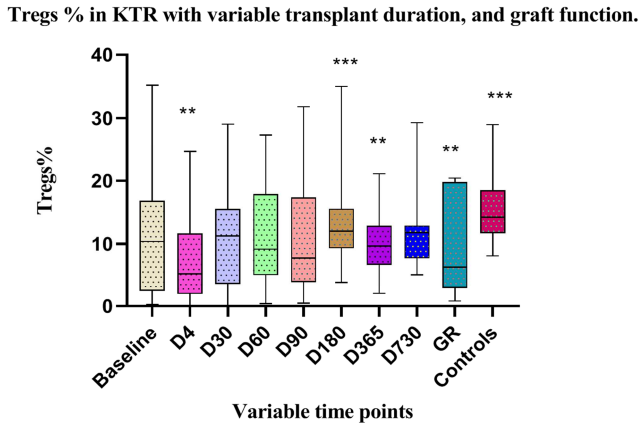

Figure 3. *Tregs% in HC, at baseline, and in KTR up to two years after transplantation are compared. Baseline vs. control; *p 0.05; **p 0.01; ***p 0.001; baseline vs. control. The median (95% CI) is used to present data.*

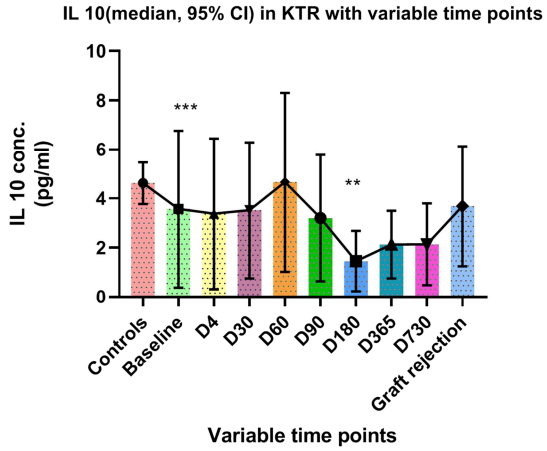

Figure 4. *Serum IL-10 levels in HC, KTR, and baseline samples were compared up to two years after transplantation. ***p 0.001 vs. baseline; baseline vs. control; *p 0.05 vs. baseline; **p 0.01 vs. baseline. The median (95% CI) of the data is displayed.*

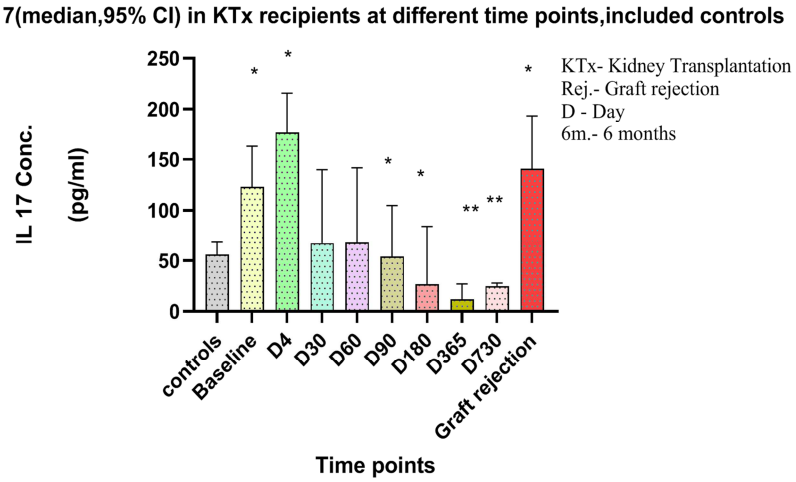

Figure 5. *Serum IL-17 levels in pg./ml in HC, baseline, and KTR samples were compared up to two years after transplantation. *p < 0.05 vs. baseline, **p < 0.01 vs. baseline, ***p < 0.001 vs. baseline; baseline vs. control. The median (95% CI) of the data displayed.*

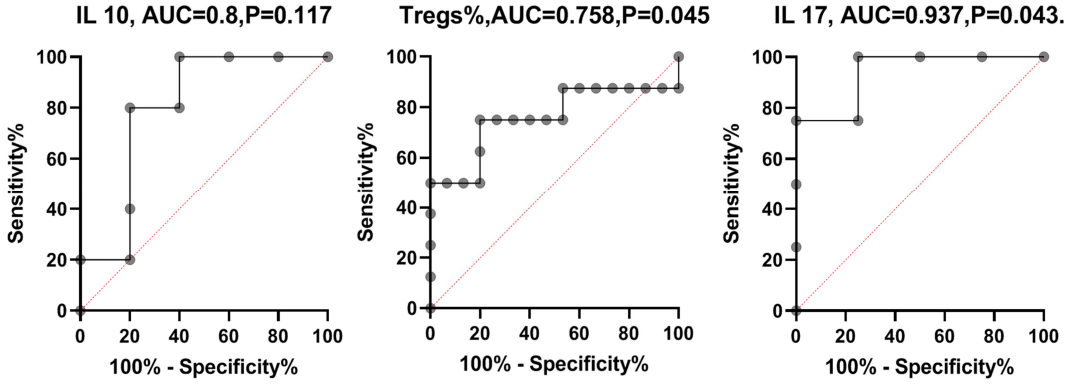

Figure 6. *ROC analysis for the prediction of acute GR with the (A) Tregs% (AUC=0.758), (B) IL-10 concentration (AUC=0.8), (C) IL-17 concentration (AUC=0.937). For all predictor factors, the ROC was displayed with 1-specificity on the X-axis and specificity on the Y-axis. A profile with an AUC of (C) = 0.5 demonstrates no discriminatory capacity, whereas (C) = 1 demonstrates the highest level of discrimination. C is a concordance statistic that is equivalent to AUC.*

4. Discussion

4.1. Tregs%

The Tregs% (CD4+CD25+) was estimated in patients who underwent kidney transplantation. Tregs% decreased during the first week, increased at six months, and continued till two years with SGF. Tregs% after kidney transplantation were lower until six months, and they returned to baseline levels one year after the transplant, according to San Segundo et al. [8]. Early transplant recipients had a lower Tregs% three months after the induction of antibodies, according to Aly et al. [9]. In contrast to our findings, Presser et al. observed that the direct immunosuppressive medication effects that impede Tregs% within the first week following transplantation; Twenty weeks of long-term follow-up revealed that Tregs% remained low. [10]. According to Krajewska et al., immunosuppressant medication may have an impact on Tregs%. [11]. In Chu et al.'s research of renal transplant recipients on various immunosuppressive medications, the calcineurin inhibitor (CNI)-based regimen group exhibited significantly lower Tregs% compared to the sirolimus-based regimen group and the healthy participants' group. [12]. In our study, all renal transplant recipients received triple immunosuppressants (CNI + wysolone + MMF). Within one year following transplantation, the frequency of activated Tregs gradually declined to baseline levels [13]. In our study, Tregs% reduced with GR in comparison to the baseline. The patients with acute GR had lower Tregs% than the SGF [21]. Patients with increased Tregs one year after transplantation had higher graft survival (five-year survival) regardless of proteinuria and renal function, according to a study by David San Segundo et al. They may be employed as prognostic markers. [8]. Acute cellular rejection is more likely to occur in kidney transplant recipients with fewer Tregs, according to Inomata et al. [14]. Numerous studies have also shown that the Tregs% is decreased during rejection episodes for both acute and chronic rejection episodes, which is consistent with our findings. [15, 16]. Our study's ROC analysis also revealed a 75.8% accuracy rate for predicting acute GR from the Tregs% in peripheral blood. An Indian study, by Sharad

et al., revealed that the Tregs frequency in transplant recipients who are on CNIs decreased significantly post-transplant at six to eight weeks, and for up to eight months, this level was maintained. [17].

4.2. Interleukin-10 and Interleukin-17 Levels

We examined IL-10 which is an anti-inflammatory cytokine and IL-17 concentration which is a pro-inflammatory cytokine in our study cohort to understand the role of inflammatory, proinflammatory markers in KTR and their association with Tregs% which was correlated with the clinical outcome. In our study, the study cohort showed increased IL-17 on day four, decreased at three months of transplantation, which was maintained for two years with SGF, with GR, the IL 17 increased. Comparably, a study by Bagheri M et al. found that the presence of Th1 and Th17 cells in kidney transplant recipients is associated with acute rejection or delayed graft function. [18]. In kidney biopsies from GR patients, immunofluorescence revealed the presence of IL-17, whereas normal kidneys and pre-transplant biopsies did not show any signs of IL-17 expression by van kooten et al. [19]. Liang Ma et al. found that the antibody-mediated rejection (AMR), acute cellular rejection, and chronic rejection groups had higher Th 17 cell numbers, higher IL-17 concentrations, and lower levels of Tregs. [3]. In the present study, we found a significant decrease in serum IL-10 at baseline compared to healthy controls. In contrast, Alwahaibi NY et al. have shown elevated levels of IL-10 in hemodialysis patients as compared to healthy subjects [21]. In our study at six months, IL-10 decreased compared to baseline, which was continued till two years. Daniel V et al., reported that lower levels of plasma IL-10 in late post-transplant as compared to early post-transplant [20]. At GR, Serum IL 10 did not show significant changes (Figure 4 & Table 2). According to Kapoor A et al. increased expression of IL-10 was observed in patients undergoing acute rejection. ROC analysis of immune biomarkers suggested that Tregs and IL- 17 can be used as predictors of graft outcome in renal transplant recipients. Further studies are required to understand the role of IL-10 in renal transplant recipients.

4.3. Correlation of Immune Biomarkers

According to Liang Ma et al. the number of Tregs were negatively correlated with the number of Th17 cells and serum IL-10 had showed positive correlation with Tregs in KTR patients. The imbalance between different types of CD4 (+) T cells and deregulated inflammatory cytokines may contribute towards renal transplantation rejection [3]. However, in our study we could not evaluate these correlations, further studies are required.

5. Conclusion

With SGF, Tregs % were higher, IL-17 were lower compared to baseline & GR, IL-10 did not show changes and this had continued till two years; ROC analysis of immune biomarkers suggested that the Tregs% and IL-17 are better predictors of graft outcome. However, the correlation between immune biomarkers with graft function couldn't be evaluated which needs further studies.

6. Recommendations

1. This study recommended that Tregs% and IL-17 can be used as predictor for graft outcome.
2. To evaluate the other immune biomarkers like IL-10 as predictors of graft outcome requires further studies in kidney transplantation.
3. To evaluate the correlation between immunobiomarkers in kidney transplantation with graft outcome requires further studies.

Author Contribution Statement

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.

Conflict of Interest Statement

All the authors do not have any possible conflicts of interest.

Data Availability

Data will be available on request.

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