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Transitional immature regulatory B cells and regulatory cytokines can discriminate chronic antibody-mediated rejection from stable graft function

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ABSTRACT

Background: The balance between inflammatory and anti-inflammatory responses of the immune system has been demonstrated to determine the fate of transplanted allografts. Here we analyzed $CD19^{+}CD24^{hi}CD38^{hi}$ immature transitional regulatory B (TRB) cells, as well as the gene and protein levels of interleukin (IL)-10 and transforming growth factor (TGF)-β in the three separate groups, include of stable transplanted subjects, chronic antibody-mediated rejection (cAMR) patients, and healthy individuals.

Method: Peripheral blood mononuclear cells (PBMCs) from stable subjects (n = 36), cAMR patients (n = 36) and healthy controls (n = 18) were isolated. Flowcytometry was performed for CD19, CD24, and CD38 surface markers. ELISA and quantitative real-time PCR were performed for IL-10 and TGF-β cytokines.

Result: The percentages of immature TRB cells were significantly decrease in cAMR patients (0.98%) versus stable recipients (2.81%) and healthy subjects (4.03%) ($P = 0.001$ and $P < 0.001$, respectively). Total lymphocytes, circulating B cells, memory and mature subsets of B cells did not show any significant difference between the groups. TGF-β mRNA was 3-fold upregulated in the cAMR group compared to stable patients ($P < 0.001$.), but without significant alteration at the protein level. Also, long-term survival renal transplant recipients had a higher protein but not mRNA levels of IL-10 than short-term survival renal transplant recipients.

Conclusion: It seems that immature TRB cell subpopulation might be a crucial regulator of immune system response and plays an important role in determining the transplantation outcome. Furthermore, immunosuppressive IL-10 and TGF-β cytokines might act as a double sword and can exhibit either pathogenic or protective effects against allograft.

1. Introduction

The global burden of disease studies have reported that incidence, prevalence, disability-adjusted life years (DALY) index, and death due to chronic kidney disease (CKD) have been increased from 1990 to 2016 by 89%, 87%, 63%, and 98%, respectively [1]. The final stage of CKD is an end-stage renal disease (ESRD), and renal replacement therapies (dialysis or transplantation) are common treatment options for patients with ESRD. Owing to better function of kidney (10–29% *vs*. 50%), higher 5-year survival (38.5% *vs*. 85.5%), and lower rate of cardiovascular disease (1% *vs*. 10% in middle-aged population) during transplantation [2], most patients tend to choose predominantly the

Abbreviations: TRB, Transitional regulatory B cell; TGF- β, transforming growth factor beta; IL-10, interleukin 10; cAMR, chronic antibody-mediated rejection; DALY, disability-adjusted life years; CKD, chronic kidney disease; ESRD, end-stage renal disease; GODT, global observatory on donation and transplantation; Breg, regulatory B cell; CTLA-4, Cytotoxic T-lymphocyte-associated protein 4; GITRL, Glucocorticoid-induced TNFR ligand; PDL-1, programmed cell death ligand 1; FasL, Fas ligand; IDO, adenosine, indoleamine 2,3-dioxygenase; Treg, regulatory T cell; BUN, blood urea nitrogen; eGFR, estimated glomerular filtration rate; CKDEPI, chronic kidney disease epidemiology; IVIg, intravenous immunoglobulin; RPMI, Roswell Park Memorial Institute; EDTA, ethylenediaminetetraacetic acid; PBMC, Peripheral blood mononuclear cell; FBS, fetal bovine serum; CBC, complete blood count; WHO, world health organization; ELISA, enzyme-linked immunosorbent assay; cDNA, complementary DNA; MD, mean difference; SMD, standardized mean difference; LTSRTR, long term survival renal transplant recipients; STSRTR, short term survival renal transplant recipients; IFN-γ, interferon gamma

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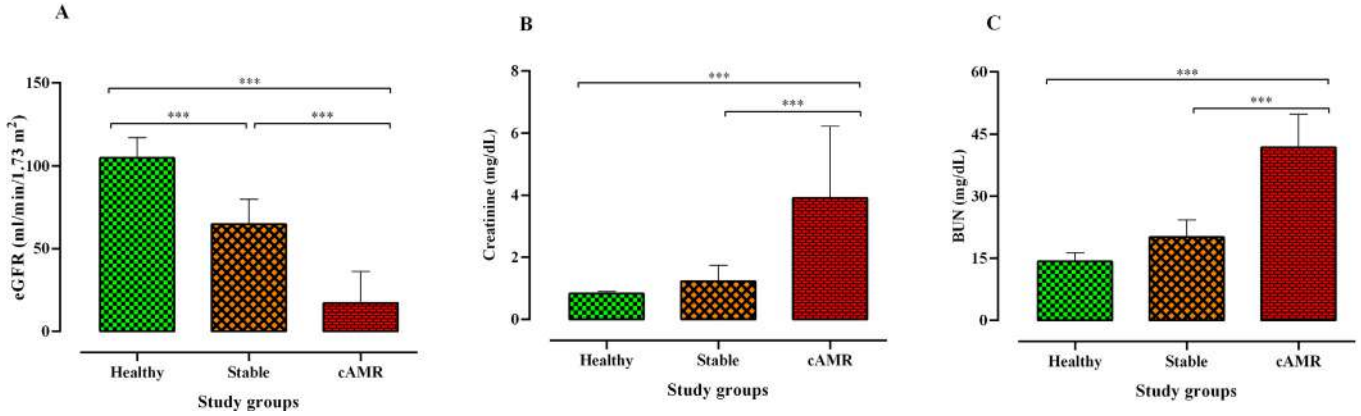


Fig. 1. Comparison of renal function in the study groups. Some parameters that used for evaluating the renal function were the mean of eGFR (A), serum creatinine concentration (B), and BUN (C). cAMR patients showed impaired kidney function, determined by higher serum creatinine and BUN levels and lower eGFR. Bar graphs show median and inter-quartile range (IQR). Healthy controls, n = 18; stable patients, n = 36; cAMR group, n = 36. Inter-group differences were evaluated with analysis of variance (ANOVA). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

transplantation as the treatment option in comparison with dialysis. The last reports of the global observatory on donation and transplantation (GODT) show that renal transplantation has been increased by 6.5% over 2015 [3]. However, despite 1-year graft survival has been increased by nearly 90% and the incidence of acute rejection by one-year post-transplantation has been decreased by 12.2% [4], but chronic allograft rejection has been remained as one of the major barriers to the long-term survival of the transplanted organ. So that the 10-year survival of renal transplant patients falling below 55% and 45% in living and deceased donors, respectively [5].

Although B cells have been found to play a considerable role in the chronic rejection process, but a new category of B cells, termed as regulatory B (Breg) cells, has been identified through studies on autoimmune disease [6], cancers [7,8], and viral infections [9]. These cells mediate regulatory immune responses by various mechanisms, which are classified into interleukin (IL)-10 dependent and IL-10 independent approaches. The IL-10 independent pathway consists of cell to cell contact by Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), Glucocorticoid-induced TNFR ligand (GITRL), programmed cell death ligand 1 (PDL-1), and Fas ligand (FasL) as well as secretion of soluble mediators, such as transforming growth factor (TGF)-β, IL-35, adenosine, indoleamine 2,3-dioxygenase (IDO), etc. [10–14]. Unlike the regulatory T (Treg) cells, Breg cells constitute a heterogeneous subpopulation and do not have the lineage-specific transcription factor or characteristic surface markers. However, a well-characterized phenotype of Breg cells in humans have been suggested by some researchers in the context of transplantation [15–18]. These studies showed that operational tolerant patients (transplant patients with stable graft function, in the absence of immunosuppression for at least one year with normal serum creatinine and albumin) exhibited significant higher transitional regulatory B (TRB) cells, characterize as $CD19^{+}CD24^{hi}CD38^{hi}$ phenotype.

There are increasing shreds of evidence for the regulatory function of B cells in controlling the auto and alloimmune responses. Indeed, the balance between inflammatory and anti-inflammatory responses of the immune system establishes the fate of transplanted allografts. Maintenance of this balance has been mainly attributed to the regulatory arm of the immune system, such as Treg cells and TRB cells as well as soluble mediators like IL-10 and TGF-β. Indeed, a cytokine storm generated by an alloimmune response after transplantation can lead to either allograft rejection or survival based on the type of secreted cytokines. Therefore, the analysis of cytokines production might be a beneficial approach to understand an allograft's fate.

Most previous investigations have focused on regulatory function of Treg cells in solid organ transplantation [19,20] however information about regulatory subsets of B cells is still limited. Moreover, although regulatory cytokines as a major component of anti-donor inflammatory immune response have received much attention, but the results have been inconsistent. On the other hands, the role of regulatory arm of humoral immune system is less known in renal transplant recipients with stable graft function and during the chronic rejection episode. Therefore, the main objective of this study was to assess the $CD19^{+}CD24^{hi}CD38^{hi}$ TRB cells as the representative of cellular arm of humoral mediated immunity (HMI) as well as the protein and mRNA levels of the regulatory cytokines such as IL-10 and TGF-β as the representative of the soluble arm of HMI. Hopefully, approaching with a valid and reliable relationship between the pathologic pattern of chronic graft rejection and the harmful or protective factors of the immune system, like TRB cells and their mechanisms of action, in the blood or urine will contribute to the development of diagnostic tests.

2. Methods and patients

2.1. Study design

In this case-control study, the following groups were recruited: a) stable graft function subjects (n = 36), chronic antibody-mediated rejection patients (cAMR, n = 36), and healthy individuals (n = 18). The study subjects were classified into 3 groups: a) stable graft function under immunosuppressive regimen (tacrolimus or cyclosporine/ cellcept/ prednisolone with or without induction therapy [anti-thymocyte globulin]) and without any clinical and laboratory indication of graft rejection (mean creatinine level: 1.5 mg/dl, estimated glomerular filtration rate (eGFR): 64.49 ml/min/1.73 m^2); b) biopsy-proven chronic antibody-mediated rejection with high creatinine concentration (4.33 mg/dl) and low eGFR (23.27 ml/min/1.73 m^2); c) healthy subjects with a normal kidney function (creatinine: 0.83 mg/dl, eGFR: 106.28 ml/min/1.73 m^2) and without any infectious history for at least 6 months before the sampling. The renal function was evaluated by an eGFR higher than 40 ml/min/1.73 m^2 (Fig. 1A), stability of serum creatinine concentration during six months before the study enrollment (Fig. 1B), and blood urea nitrogen (BUN) (Fig. 1C). The eGFR was calculated by chronic kidney disease epidemiology (CKD-EPI) formula [21]. None of the participants received intravenous immunoglobulin (IVIg) and rituximab. Renal transplanted patients were recruited from individuals who attended to the kidney transplantation unit of three hospitals in Tehran. Informed consent forms obtained from all participants. This research was conducted with the approval of the ethics committee of the Tehran University of Medical Science, Tehran, Iran with registration code of IR.TUMS.MEDICINE.REC.1396.4411.

2.2. Cell isolation

Ten milliliter peripheral blood samples were collected in collection tubes containing ethylenediaminetetraacetic acid (EDTA). Peripheral blood mononuclear cells (PBMCs) were separated by ficoll lymphodex (Inno-train, Germany) density gradient centrifugation and then preserved in a solution containing 90% fetal bovine serum (FBS) (Thermo Fisher Scientific, Gibco, USA) and 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, UK), and then were cryopreserved in liquid nitrogen tank at −196 °C until performing flow cytometry.

2.3. Multi-color flow cytometry

The PBMCs were thawed and freshened in Roswell Park Memorial Institute (RPMI) 1640 medium (Biosera, USA) supplemented with 10% FBS. Then, the cells were washed by the washing buffer containing phosphate-buffered saline (PBS) and 0.1% FBS, and then resuspended in PBS. Subsequently, the cell suspension was stained with anti-human CD24 PE mAb (clone ML5), anti-human CD19 FITC mAb (clone HIB19), anti-human CD38 PE-Cy5 mAb (clone HIT2) (all mAb from Biolegend, San Diego, CA, USA) according to the manufacture's protocol. The appropriate isotype controls (MOPC-21) were utilized for gating purposes. The BD FACSCalibur analyzer and CellQuest software were used for cell analysis. The data analysis was performed by FlowJo software 7.6 (Tree Star, Ashland, OR, USA). We considered $CD19^+$ cells as total B cells, and $CD19^+CD24^{int}CD38^{int}$, $CD19^+CD24^{hi}CD38^{hi}$, and $CD19^+CD24^{hi}CD38^-$ for mature, immature, and memory B cells, respectively (Fig. 2). The absolute number of each subpopulation was calculated based on complete blood count (CBC) parameters obtained at the moment of sampling, and the fraction of each subset was determined by flow cytometry based on the dual-platform method described in the world health organization (WHO) guideline [22].

2.4. Serum isolation and ELISA

Serum samples were obtained after centrifugation of coagulated whole blood samples (1000g/5 min/room temperature) and kept at −70 °C until use. Serum levels of IL-10 and TGF-β were measured by antigen capture sandwich enzyme-linked immunosorbent assay (ELISA) kits (Karmania Pars Gene, Iran) according to the manufacturer's instruction.

2.5. RNA extraction and quantitative real-time PCR

Total RNA was extracted by the GeneJET RNA purification kit (Thermo Fisher Scientific, USA) from fresh whole blood samples according to the manufacture's protocol. After evaluating quantity and purity of extracted RNA by NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA) based on their absorbance at 260/280 and 230/280 nm ratio, RNAs were reversely transcribed with a high-efficiency complementary DND (cDNA) reverse transcription kit (Thermo Fisher Scientific, USA), and stored at −70 °C until use. Quantitative Real-time PCR was performed to determine the mRNA expression level of IL-10 and TGF-β. cDNAs were amplified by StepOne Plus Real-time PCR instrument (ABI, USA). Primer sequences (designed by tag Copenhagen, Denmark) and the PCR program are summarized in Table 1. All these primers are commercially optimized for SYBR Green-based Real-time-PCR reactions (Real Q plus high ROX master mix, Amplicon, USA). The quality of each PCR reaction was confirmed by melt curve analysis for all genes. mRNA expression in each sample was normalized to expression level of the corresponding β-actin housekeeping gene. The changes in the expression levels of IL-10 and TGF-β mRNAs were calculated by the $2^{-\Delta\Delta Ct}$ method, in which $\Delta\Delta Ct$ = [ΔCt (sample) – ΔCt (total sample)].

2.6. Statistical analysis

All statistical analyses were carried out with Stata 13.0 software (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP), and graphical presentation was done using Prism 6.0.1 (GraphPad Software, La Jolla California USA; www.graphpad.com) and MedCalc 18.2.1 (MedCalc Software bv, Ostend, Belgium; https://www.medcalc.org; 2018). Scale and nominal variables were depicted as mean (SD) and median [Q1/Q3] and number (percent), respectively. Data were analyzed using the analysis of variance (ANOVA) test. The means were exhibited as horizontal line in scatter/dot plots. In the bar graphs, the mean and standard deviation (SD) or mean (confidence interval) or median (interquartile range) were expressed. Confounding/covariate effect of demographic/clinical parameters [age, sex, and body mass index (BMI)] in all study groups was assessed and four statistical models were designed, and the final results reported after adjustment for these items. Partial Eta squared ($P\eta^2$) indicates the variance of each variable. If the value of $P\eta^2$ be more than 10%, it regarded as considerable effect and adjusted. For avoiding the alpha error accumulation, Sheffe's correction was done by the post-

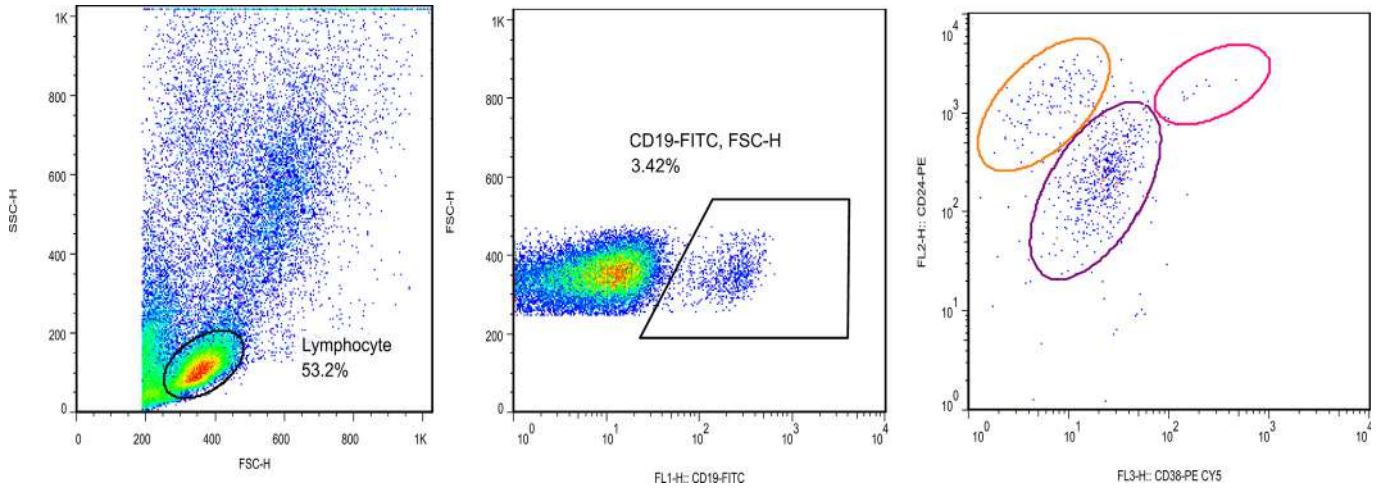


Fig. 2. A typical gating strategy for B cell subpopulations. A. Forward and side scatter gating for lymphocytes. B. Total B cells are displayed as $CD19^+$ cells (trapezoidal area). C. Naïve/mature B cells are depicted as $CD19^+CD24^{int}CD38^{int}$ (green oval), memory B cells as $CD19^+CD24^{hi}CD38^-$ (orange oval), and transitional regulatory B cells as $CD19^+CD24^{hi}CD38^{hi}$ (pink oval). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Primer sequence and PCR program for IL-10, TGF-β and β-actin genes.

Gene	Primer sequence (5′→3′)	Amplicon size	Tm (°C)	Thermo cycling program
TGF-β/F	GACACCAACTATTGCTTCAGCTCC		57.4	95 °C/ 15 s, 40 cycles of 95 °C/ 15 s, 58 °C/ 30 s, 72 °C/ 30 s.
TGF- β/R	CGTGTCCAGGCTCCAAATGTAG	162	56.7	
IL10/F	TGCTGGAGGACTTTAAGGGTTACC		57.4	95 °C/ 15 s, 40 cycles of 95 °C/ 15 s, 58 °C/ 30 s, 72 °C/ 30 s.
IL10/R	TCACAGGGAAGAAATCGATGACAG	190	55.7	
β-actin/F	GACCTCTATGCCAACACAGT		51.8	95 °C/ 15 s, 40 cycles of 95 °C/ 15 s, 58 °C/ 30 s, 72 °C/ 30 s.
β-actin/R	AGTACTTGCGCTCAGGAGGA	139	53.8	

TGF-β, transforming growth factor beta; F, Forward; R, reverse; Tm, melting temperature.

estimation module "pwcompare" in the STATA software. The level of statistical significance was considered as a *P* value less than 0.05. The correlation analysis was performed with Spearman's test. To address the strength of the observed relationship, we calculated the standardized mean difference (SMD) of Cohen's *d*. The following classification was used for the interpretation of Cohen's *d* effect size (Supplementary Table A) [23]. By using Cohen's *d* calculation for this and previous similar studies, we compared to strength and conclusiveness of relationship between TRB cells percentage and renal function, intuitively and exhibited the results in a forest plot.

3. Results

3.1. Basic characteristics of study groups

Demographic characteristics, clinical and laboratory data, and patient's pharmaceutical protocol were shown in Tables 2, 3, Supplementary Table H, and I.

3.2. Distribution of B cell subsets in study groups

All kidney transplant recipients exhibited a remarkable decrease in the percentage of total circulating B cells in comparison to healthy subjects (Fig. 3A). Cohen's *d* of this effect was −0.78 with 95% CI (−1.36, −0.19) that represented a moderate size of the effect for comparison of stable and healthy groups. This means that the two group's means differed by 0.78 SD and mean difference (MD) of −2.32 with 95% confidence interval (CI) of (−4.55, −0.09). In cAMR patients and healthy controls, we found a *d* of −0.90 (−1.49, −0.30) and MD 2.63 (−4.77, −0.48) (Supplementary Table B).

In addition, percentage and number of total lymphocytes had significant decline in the transplant recipients (Fig. 3C, D), therefore the absolute number of B cells was significantly lower in these groups (Fig. 3B).

To further analyze the B cell subpopulations, we used mice anti human CD24 and CD38 antibodies as described in material and methods section. The most striking result to emerge from this analysis was that the percentage of $CD19^{+}CD24^{hi}CD38^{hi}$ TRB cells was much lower in the cAMR group (0.98%) compared to stable patients (2.81%) and healthy controls (4.03%) ($P = 0.001$ and $P < 0.001$, respectively), whereas stable patients presented preserved percentage of TRB cells (similar to healthy individuals) (Fig. 3E). MD between cAMR and stable groups equaled −1.83 with 95% CI (−2.98, −0.68) and Cohen's *d* was −0.94 (−1.42, −0.45) that represented a strong effect size. This means that two group's means differed by 0.94 SD (Supplementary Table C). However, non-significant difference in the absolute number of TRB cells per ml was found between cAMR and stable patients (Fig. 3F). A Cohen's *d* of −0.37 with 95% CI (−0.84, 0.09) indicated that the two group's means differed by 0.37 SD (Supplementary Table D). Interestingly, the percentage and absolute number of other subpopulations (mature and memory B cells) remained unchanged between stable and cAMR patients, but presented a considerable decrease in comparison to healthy volunteers (Supplementary figure 1 A-D). The percentage of TRB cells revealed a negative and positive correlation with creatinine and eGFR levels, respectively (Creatinine: *rho* = -0.46, $P < 0.001$; eGFR: *rho* = 0.43, $P < 0.001$).

Table 2
Demographic characteristics of the subjects.

Variables	Study groups			*P* value
	cAMR (n = 36)	Stable (n = 36)	Healthy (n = 18)	
Age (years)	40.19 (14.45)	45.83 (13.47)	32.11 (10.78)	
	38.00 [28.50; 47.00]	44.00 [33.00; 56.50]	26.50 [26.00; 30.00]	0.003
Sex (women)	9.00 (50.00)	12.00 (33.33)	11 (30.56)	0.356
BMI (kg/m^2)	25.06 (3.35)	25.42 (3.59)	22.79 (2.34)	
	24.02 [21.78; 28.44]	25.30[23.41; 27.22]	22.95 [21.16; 24.34]	0.088
Post- TX months	68.81 (76.76)	55.00 (53.79)	–	0.38
	36.00 [4.00; 129.00]	30.00 [18.00; 75.00]	–	
Donor type:				
Living related	0	3 (8.33)	–	
Living unrelated	9 (25.00)	11 (30.56)		0.095
Deceased	27 (75.00)	22 (61.11)		
Number of TX:				
1	34 (94.44)	31 (86.11)		
2	2 (5.56)	5 (13.89)	–	0.460

Scale and nominal variables were depicted as mean (SD) and median [Q1; Q3] and number (percent), respectively. Inter-group differences were evaluated with analysis of variance (ANOVA). Abbreviations: cAMR, chronic antibody-mediated rejection; BMI, body mass index; BP, blood pressure; TX, transplantation.

3.3. Serum levels and mRNA expression of TGF-β

The serum levels of TGF-β had no statistically significant difference between cAMR and stable patients (14.50 and 14.24 pg/ml, respectively; $P = 0.06$). However, both these groups exhibited a higher concentration of TGF-β than healthy individuals (4.57 pg/ml, *P* values < 0.001; Fig. 4A). Both of stable recipients and cAMR patients showed a strong relationship between kidney function and serum concentration of TGF-β in comparison to healthy subjects (Cohen's *d* 20.25 and 19.71, respectively; Supplementary Table E).

Quantification of mRNA expression in whole blood samples obtained from study groups showed that TGF-β mRNA was significantly upregulated in cAMR patients compared to stable patients and as well as healthy subjects (fold change = 2.71 and 4.59, respectively, and *P* values < 0.001; Fig. 4B). For this comparison, Cohen's *d* was 1.26 with 95% CI (0.75, 1.76), implying that cAMR and stable group's means differed by 1.26 SD and MD of 7.61 with CI 95% (4.02, 11.20) (Supplementary Table F). Moreover, similar to TGF-β protein levels, cAMR patients had a higher levels of TGF-β mRNA expression compared to healthy controls ($P < 0.001$). In contrast to the protein levels, the nearly similar mRNA expression levels of TGF-β was found in stable patients compared to healthy subjects ($P = 0.074$, Fig. 4B).

Table 3
Clinical laboratory data.

Variables	Study groups			*P* value
	cAMR (n = 36)	Stable (n = 36)	Healthy (n = 18)	
FBS (mg/dl)	119.47 (79.09)	113.57 (47.93)	87.64 (12.89)	0.148
	92.00 [84.00; 115.00]	97.00 [87.00; 117.00]	89.00 [80.00; 90.00]	
Triglycerides (mg/dl)	154 (71.70)	162.60 (112.20)	91.21 (55.54)	
	142 [106.00; 200.00]	138 [98.00; 184.00]	86 [55.00; 107.00]	0.044
Cholesterol (mg/dl)	152.89 (32.16)	160.42 (50.10)	149.11 (33.80)	
	152.50 [136.00; 169.50]	152.50 [121.00; 176.50]	148.50 [123.00;158.00]	0.610
HDL (mg/dl)	42.79 (15.79)	48.26 (18.89)	54.61 (12.21)	0.051
	44.50 [32.00; 52.00]	46.00 [34.00; 59.00]	53.00 [48.00; 60.00]	
LDL (mg/dl)	81.29 (28.33)	86.24 (38.12)	71.21 (20.18)	
	81.50 [70.50; 100.00]	77.50 [63.00; 111.00]	67.00 [62.00; 78.00]	0.269
Uric acid (mg/dl)	9.79 (14.95)	6.06 (1.40)	4.12 (0.73)	
	7.30 [6.10; 8.80]	5.80 (5.00; 6.90]	4.00 (3.50; 4.80]	0.091
Urea (mg/dl)	83.03 (37.37)	46.17 (21.39)	30.61 (8.10)	
	89.00 [55.00; 10.50]	43.00 [31.00; 53.00]	30.50 [26.00; 35.00]	< 0.001
BUN (mg/dl)	38.81 (17.55)	22.22 (11.68)	14.30 (3.78)	
	41.80 [25.72; 49.65]	20.10 [14.49; 24.15]	14.26 [12.15; 16.36]	< 0.001
eGFR (ml/min/1.73 m	23.27 (17.08)	64.48 (16.45)	106.28(11.84)	
	16.4 (11.14; 32.07)	62.49 [52.53; 74.76]	109.01 [97.63; 117.17]	< 0.001
Creatinine (mg/dl)	4.33 (2.36)	1.58 (0.87)	0.83 (0.12)	
	3.91 [2.48; 6.19]	1.23 [1.06; 1.69]	0.83 [0.74; 0.90]	< 0.001
WBC (*10^3/μl)	7.40 (3.30)	8.59 (4.67)	7.78 (1.81)	
	6.30 [5.15; 9.20]	7.45 [5.27; 9.35]	8.00 [5.85; 9.13]	0.390
RBC (*10^6/μl)	3.59 (0.72)	4.42 (0.90)	4.84 (0.52)	
	3.50 [3.15; 4.00]	4.45 [3.75; 5.09]	4.87 [4.38; 5.10]	< 0.001
Platelet (*10^3/μl)	193.94 (79.70)	197.36 (73.87)	253.22 (55.63)	
	188.00 [135.00; 238.00]	186.50 [149.00; 217.00]	238.50 [220.00; 298.00]	0.014
Lymphocyte (%)	22.58 (10.21)	20.16 (10.33)	35.46 (5.78)	
	23.50 [15.00; 29.00]	19.50 [14.50; 26.00]	33.00 [32.00; 38.00]	< 0.001

Scale variables were depicted as mean (SD) and median [Q1; Q3]. Inter-group differences were evaluated with analysis of variance (ANOVA). Abbreviations: cAMR, chronic antibody-mediated rejection; FBS, fasting blood sugar; LDL, low density lipoprotein; HDL, high density lipoprotein; BUN, blood urine nitrogen; eGFR, estimated glomerular filtration rate; WBC, white blood cell; RBC, red blood cell.

3.4. Serum levels and mRNA expression of IL-10

We found a significant increase in the serum level of IL-10 in both groups of transplant recipients relative to healthy controls ($P < 0.001$). Cohen's d of these comparisons correspond to very strong relationship between protein levels of IL-10 and renal function (Supplementary Table G). But, cAMR and stable patients showed no significant difference in the serum level of IL-10 ($P = 0.996$, Fig. 5A). Of note, for further analysis, we divided the stable patients into two categories based on years after transplantation; i.e. definite five years graft survival as a cutoff. Stable patients with graft survival greater than five years were considered as long term survival renal transplant recipients (LTSRTRs) and patients with graft survival 1–5 years were regarded as short term survival renal transplant recipients (STSRTRs). Interestingly, the serum level of IL-10 was significantly higher in the LTSRTRs than STSRTRs ($P < 0.001$), cAMR patients ($P = 0.001$), and healthy controls ($P < 0.001$). In other words, LTSRTRs had the highest concentration of IL-10 in comparison to other study groups (Fig. 5B). In contrast to the serum level, mRNA expression levels of IL-10 did not show significant difference among the study groups (Fig. 5C).

Moreover, serum IL-10 levels showed a positive and negative correlation with creatinine and eGFR levels, respectively (Creatinine: $rho = 0.50$, $P < 0.001$; eGFR: $rho = -0.55$, $P < 0.001$). Also, serum levels of IL-10 and TGF-β were positively correlated to each other with rho equal to 0.49.

4. Discussion

A delicate orchestration of the effector functions and endogenous tolerance mechanisms in the course of an immune response determines the fate of a transplanted allograft. So far, maintenance of this balance has been mainly attributed to the regulatory arm of the immune system, such as regulatory cells and soluble mediators like IL-10 and TGF-β. Over the course of past few decades, a number of these factors has attracted the attention of researchers in the field of solid organ transplantation. Recently, a small subpopulation of B cells, called immature TRB cells, seems to play a regulatory role during immune system responses. The main markers introduced for characterization of this group of regulatory cells are $CD19^+$, $CD24^{hi}$ and $CD38^{hi}$ [24]. Previous studies proposed that TRB cells play a crucial role in maintaining of a long-term tolerance as well as in the development of allograft tolerance [25,26].

In this study, it was revealed that stable and healthy control groups had a similar percentage of circulating $CD19^+CD24^{hi}CD38^{hi}$ TRB cells, whereas in cAMR patients these subset of cells was significantly reduced compared to other two groups. Moreover, comparison of cAMR and stable patients demonstrated that there is a strong relationship between the TRB percentage and renal function status. However, mature and memory B cell subpopulations remained unchanged between cAMR and stable patients. These findings suggest that TRB cells, but not memory and mature subsets, might be a vital regulator of the inflammatory immune response, and their insufficiency could be involved in the process of chronic graft rejection. Nonetheless, it should be noted that $CD19^+CD24^{hi}CD38^{hi}$ cells constitute a heterogeneous subpopulation and may contain more than one type of B cells, as others have highlighted previously [24,27]. In addition, some researchers have suggested IL-10 production as the best marker for identification of human Breg cells due to the difficulty and diversity of specific surface markers for this subset [28]. It is worthy to note that IL-10 producing B cells (B-10 cells), which are characterized by surface expression of CD24 and CD38 and also high levels of CD27, are distinguished from TRB cells by expression of CD27 surface marker in humans [15,28]. These findings supported by some other reinvestigations, which also showed that cAMR patients had a striking reduction in TRB cells

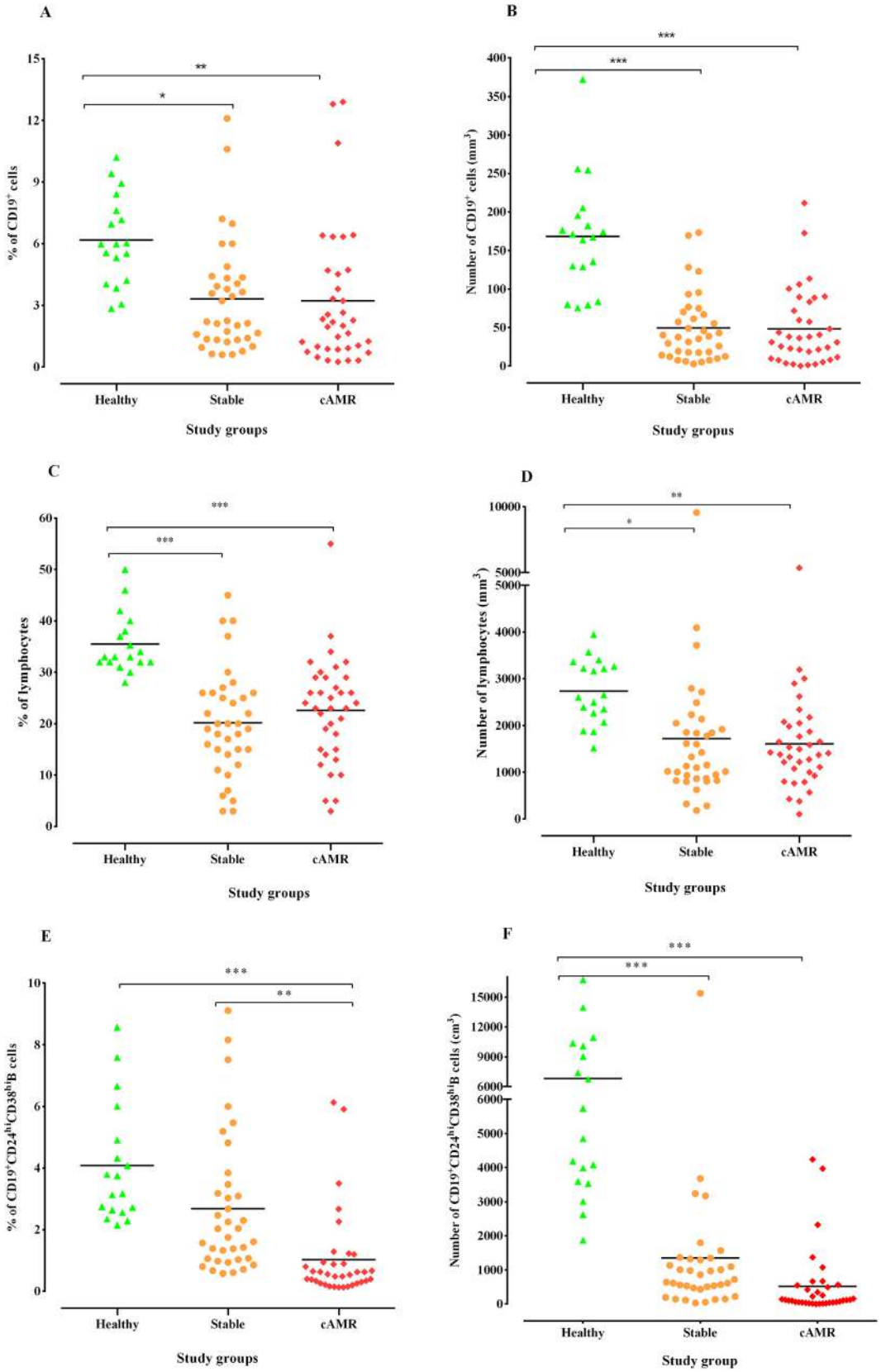


Fig. 3. Quantitative analysis of lymphocytes and total B cells and TRB cells in the study groups. Flowcytometric analysis of peripheral blood mononuclear cells (PBMCs) from healthy subjects (n = 18), stable patients (n = 36), and chronic antibody mediated rejection (cAMR) patients (n = 36). Percentage and absolute number of lymphocytes (A, B), total B cells (C, D), and transitional regulatory B cells (E, F) exhibited as individual values in each study group. The mean percentage of lymphocytes was 35.50%, 20.17%, and 22.58% in healthy subjects, stable patients, and chronic antibody mediated rejection (cAMR) patients, respectively. Inter-group differences were evaluated with analysis of variance (ANOVA). Horizontal lines show mean value (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

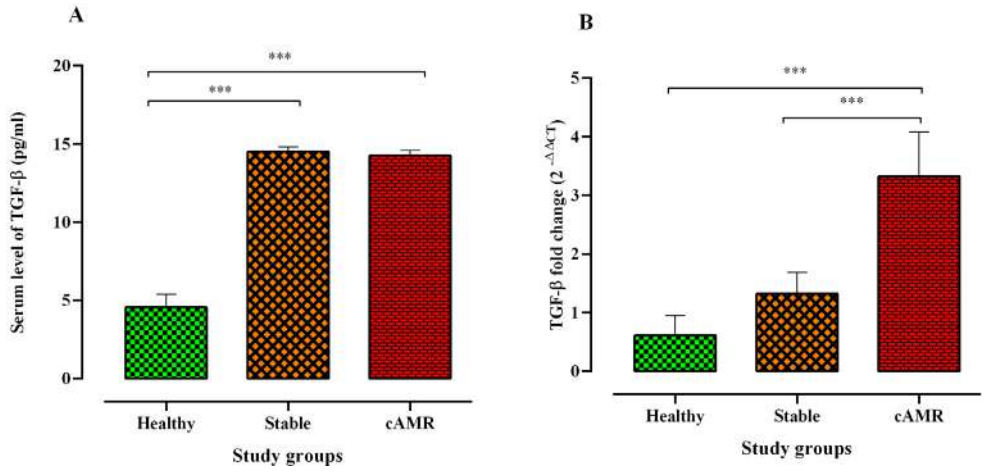


Fig. 4. Serum and relative mRNA expression level of TGF-β in the study groups. A. Serum concentration of TGF-β (pg/ml). The mean (SD) TGF-β serum level was 4.57 (0.51) pg/ml in 18 healthy subjects, 14.50 (0.48) pg/ml in 36 stable patients, and 14.24 (0.48) in 36 chronic antibody mediated rejection (cAMR) patients. B. Relative expression level of TGF-β mRNA in whole blood of stable renal recipients and cAMR patients *vs.* healthy volunteers were 2.71- and 4.59-fold, respectively. Each bar graph and corresponding error bars illustrate mean serum concentration and SD (A) or mean mRNA level and 95% confidence interval (CI) (B). (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

frequency as well as their suppressive activity [29]. However, Silva and colleagues detect no difference between patients with chronic rejection and subjects with stable allograft [30]. Most recently, Lee *et al.* demonstrated that patients with chronic rejection exhibited higher frequency of circulating TRB cells in comparison to patients with stable graft function [31]. Taken together, the evidence from this and other studies highlight regulatory function of TRB cells in the context of transplantation.

Herein, we used Cohen's *d* to determine the strength of the relationship of TRB cells percentage and renal function between the cAMR and stable groups in this study and similar previous studies, intuitively. The findings of our study suggests that there is a strong (d = -0.94) but inconclusive (wide 95% CI) relationship between TRB cells percentage and kidney function (Fig. 6).

Different studies demonstrated a higher frequency of TRB cells in operational tolerance in kidney transplant recipients in comparison to stable groups under immunosuppressive regimen and highlighted the important role of TRB cells in the induction and maintenance of systemic tolerance [17,25,29–31]. In this study, we analyzed the patients according to the subgrouping of stable patients based on transplantation time (less and more than five years), type of calcineurin inhibitor (either tacrolimus or cyclosporine A) in immunosuppressive regimen, and source of allograft (living or deceased). Although the transplant recipients were different with regard to this items, but these inter-group differences did not affect the results (Supplementary figure 2 A-C). Our findings appear to be well supported by other researchers [29,32]. Our results indicated that total B cells was reduced in renal transplant recipients compared to healthy subjects. This reduction can be regarded as an effect of immunosuppressive treatment. In general, this result implies that decrement in the percentage of total B cells in cAMR group accompanied with a decline in the TRB subset, whereas other subsets remained fixed. It is suggested that TRB subsets are an important component of anti-inflammatory alloimmune response and any defect in the number or function of these subsets affect the allograft outcome. Our findings in the current study in the percentage of TRB subpopulations in kidney transplantation appear to be well supported by other researchers [29,32].

We also found that mRNA expression levels of TGF-β was higher in cAMR group relative to stable patients and observed a very strong relationship (Cohen's d = 1.26) with renal function, whereas protein levels of TGF-β were nearly similar in both of study groups. Indeed, although the protein level of this cytokine was not significant, its Cohen's d showed a moderate relationship. Therefore, according to the strength of the relationship, it seems the result of the protein and mRNA levels to be in the same direction. It seems that the most possible explanation of this discrepancy is due to difference of sources used; i.e. protein level of the cytokine was measured in serum, whereas whole blood sample was used for mRNA expression measurement. Indeed, evaluation of mRNA expression level is limited to blood nuclear cells (the white blood cells mRNA), but serum proteins might be produced

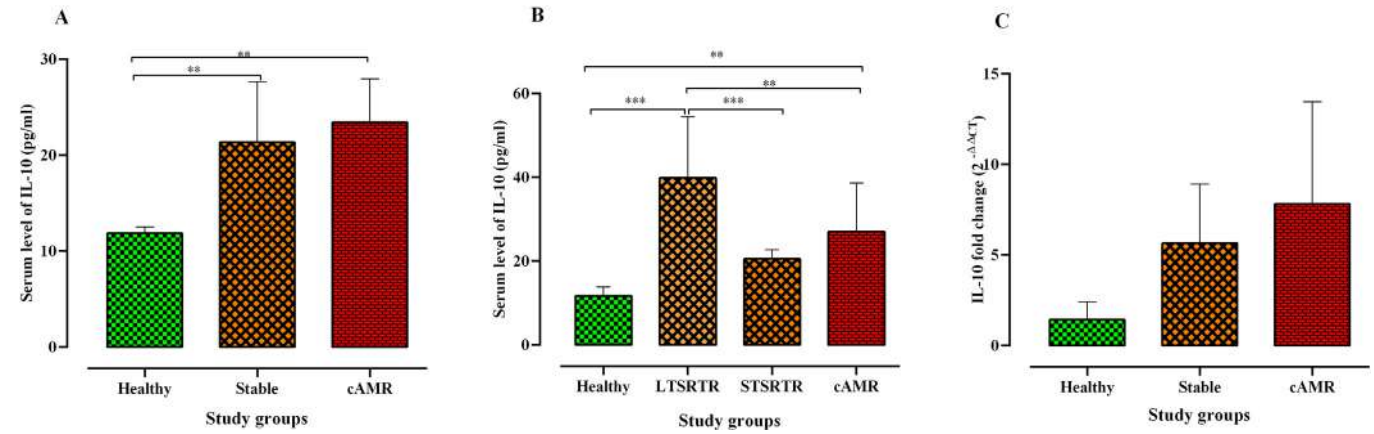
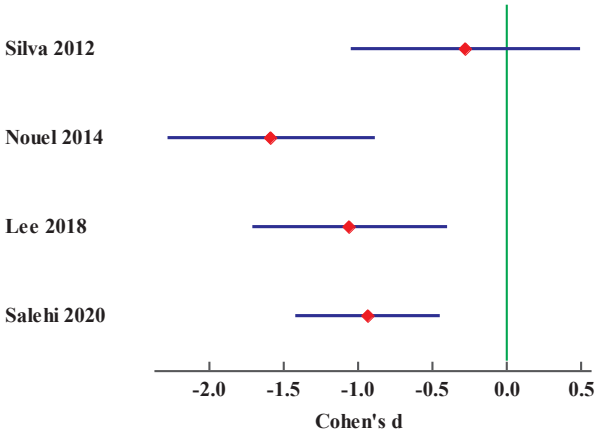


Fig. 5. Serum concentration and relative mRNA expression level of IL-10 in the study groups. A. The mean (SD) IL-10 serum level was 12.16 (1.36) pg/ml in healthy controls (n = 18), 26.98 (12.49) pg/ml in stable patients (n = 36), and 27.01 (11.61) pg/ml in cAMR patients (n = 36). B. Protein level of IL-10 in subgroup of stable patients with short term survival was 20.53 (2.22) pg/ml (n = 24) and stable patients with long term survival was 39.87 (14.64) pg/ml (n = 12). Each bar graph and corresponding error bars illustrate mean serum concentration and SD (A and B) or mean mRNA level and 95% confidence interval (CI) (C). (STSRTR, short term survival renal transplant recipients; LTSRTR, long term survival renal transplant recipients, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).



Study	Cohen's *d*
Silva 2012	-0.28 (-1.05, 0.49)
Nouel 2014	-1.59 (-2.28, -0.89)
Lee 2018	-1.06 (-1.71, -0.40)
Salehi 2020	-0.94 (-1.42, -0.45)

Fig. 6. Association of TRB percent with renal function. The vertical line (green) is known as the line of null zone. This line is located at the value where there is no relationship between TRB percent and renal function. For Cohen's *d*, the null zone value is zero. Each horizontal line represents a study result (here, four studies showed). Each study result consists of two components: a point estimate (red box) that gives a representation of the strength of relationship, and a horizontal line representing the 95% CI of the study result. A wide 95% CI shows an inconclusive relationship. The numbers that have showed in the table represented point and 95% CI for each study. As shown in the forest plot, none of the studies have yielded a conclusive relationship between TRB cells and renal function.

and released to serum by various cells in different sites of the body. In addition, Elnokeety and colleagues reported serum and urinary levels of TGF-β were elevated in the rejection patients [33]. However, Du *et al.* suggested that high serum level of TGF-β could be a positive effect on a long term survival. Also, Lessan-Pezeshki *et al.* reported that the mean concentration of TGF-β in the short time after renal transplantation were higher in stable subjects compared to patients with rejection [34]. A possible explanation for this finding is that TGF-β plays a significant role in the induction of inducible Treg (iTreg) cells and suppression of immune system, and therefore, has a positive effect on graft survival [35]. Indeed, it seems that TGF-β acts as a double-edged sword; on the one hand, it regulates the function of 27% of 49 genes involved in tolerance [36], and it has been shown that operational tolerant recipients expressed higher mRNA levels of TGF-β and its receptor on PBMCs [37]. On the other hands, some studies have shown that TGF-β, in combination with IL-6, led to differentiation of Th-17 cells, and finally resulted in graft rejection episodes [38].

We also reported that both groups of stable recipients and cAMR patients demonstrated similar level of mRNA expression and protein levels of IL-10. However, classification of stable patients into two groups based on years after transplantation exhibited that LTSRTRs had higher levels of serum but not mRNA expression of IL-10. One explanation might be that Treg cells are closely pertinent to the long-lasting graft survival, since they modulate recipient's harmful immune responses against allograft and IL-10 plays an important role in the suppressive function of these cells [39–41]. Therefore, IL-10 might confer a positive function in the long term survival of allograft. IL-10 controls immune responses by various mechanisms, such as inhibition of antigen presenting cells (APCs), downmodulating the inflammatory cytokines, and regulating the growth or differentiation of different cells like B cells, mast cells, endothelial cells, and mesangial cells [42]. In contrast, Karckzveski *et al.* reported that the dominant cytokine pattern in chronic rejection patients was related to Th-2 cells that included IL-10, IL-4 and IL-6, whereas stable group exhibited lower levels of these cytokines and higher levels of interferon (IFN)-γ [43]. This might stem from the fact that Th-2 cells contribute to the initiation and exacerbation immune response in the chronic rejection patients by secretion of IL-10, a B cell growth factor. However, we are aware that this research may have some limitations. The first is only eGFR, serum creatinine, and BUN were used for evaluating the graft function status, and intragraft changes were not assessed. The second is our study results cannot provide any evidence about suppressive mechanisms of TRB cells because we have not examined the factors that involved in its suppressive activity. Further research with respect to mechanisms of TRB cells function will hopefully pave the way toward better understanding of allograft rejection and provide avenues for devising effective therapeutics to increase transplantation survival.

5. Conclusion

Altogether, these findings propose that circulating TRB cells population might be an essential regulator of inflammatory immune response and that insufficiency of these cells might be involved in the allograft rejection. In spite of the positive function of TGF-β in immune regulation in the transplant recipients, it may induce an inflammatory response against allograft, in combination with inflammatory cytokines, and led to graft fibrosis. Besides, it seems that IL-10 exerts its regulatory roles on the graft survival upon years after transplantation. In other words, cytokines known as regulatory mediators might act as a double-edged sword and can exhibit either pathogenic or protective effects against allograft.

CRediT authorship contribution statement

Saeedeh Salehi: Conceptualization, Investigation, Data curation, Formal analysis, Writing - original draft, Writing - review & editing. **Abbas Shahi:** Conceptualization, Investigation, Writing - review & editing. **Shima Afzali:** Conceptualization, Investigation, Writing - review & editing. **Abbas Ali Keshtkar:** Data curation, Formal analysis, Writing - review & editing. **Samad Farashi Bonab:** Investigation, Writing - review & editing. **Tayebeh Soleymanian:** Resources, Writing - review & editing. **Bita Ansaripour:** Investigation, Writing - review & editing. **Ali Akbar Amirzargar:** Supervision, Funding acquisition, Project administration, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2020.106750.

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