Regulatory T Cell Subtypes and TGF-β1 Gene Expression in Chronic Allograft Dysfunction

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ABSTRACT

Background: Regulatory T cells have been suggested to have a protective role against acute rejection in allograft recipients. However, there is little information available about their contribution to chronic rejection process. The role of transforming growth factor-beta 1 (TGF-β1) as a profibrogenic and/or immunoregulatory cytokine in renal allografts is also controversial. **Objectives:** To evaluate the frequency of CD4+CD25+CD127- and CD3+CD8+CD28- regulatory T cells in chronic allograft dysfunction (CAD) and to investigate the expression of TGF-β1 in renal allografts. **Methods:** Thirty biopsy-proven CAD patients were pair-matched with 30 stable graft function patients and a third group of healthy volunteers. Flowcytometry was performed on PBMCs to determine the frequency of CD3+CD8+CD28- and CD4+CD25+CD127- regulatory T cells in lymphocyt population. TGF-β1 gene expression was assessed by Real Time PCR. **Results:** The percentage of CD3+CD8+CD28- Tregs among renal allograft recipients was higher than healthy controls ($p<0.001$) since stable graft patients showed the most rates. The frequency of CD4+CD25+CD127- Tregs was lower in CAD patients than stable recipients ($p=0.024$) and healthy group ($p=0.015$). TGF-β1 gene expression was greater in CAD patients compared to healthy group ($p=0.03$) but there was no significant difference between gene expression of stable graft patients and healthy volunteers. **Conclusion:** The negative association between the frequency of regulatory T cell subtypes and chronic allograft dysfunction proposes these cells as probable candidates for promoting allograft survival. Moreover, despite the immunoregulatory capacity of TGF-β1, it is likely to be implicated in chronic damages of allograft tissue.

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INTRODUCTION

Despite all improvements in controlling acute rejection episodes, chronic rejection remains a considerable obstacle, preventing from reaching appropriate long-term graft survival. Chronic allograft dysfunction or chronic allograft nephropathy (CAN) is the major cause of long-term graft loss in kidney recipients and a prevalent pathologic lesion reported in renal protocol and cause biopsies (1). CAN, as a result of immunological and non-immunological injuries, is described by pathological findings such as interstitial fibrosis, tubular atrophy, glomerulosclerosis, fibrointimal hyperplasia and arteriolar hyalinosis in the presence or absence of clinical findings (2). Recently, according to the last Banff classification, the term CAN was replaced by nonspecific interstitial fibrosis and tubular atrophy (IF/TA) (3).

Over the past decades, many studies have been conducted to explore the impact of immunological factors on IF/TA progress. As the most important subset of regulatory cells in humans, CD4+CD25+ T cells have been considered in the evaluation and manipulation of the immune system in order to reach a favorable tolerance among recipients (4,5). This subset has been characterized by either high expression of forkhead box P3 (FOXP3) transcription factor or low expression of IL-7 receptor α-chain (CD127). Regarding marginal expression of FOXP3 in activated effector T cells, lack of CD127 expression seems to be a more reliable marker to distinguish CD4+CD25+ regulatory T cells from other T cells (6,7). CD4+CD25+ Treg cells regulate immune responses through IL-2 excessive consumption and cell-to-cell contact dependent suppression by glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) and cytotoxic T lymphocyte associated antigen-4 (CTLA-4) signaling. Furthermore, these cells produce immunosuppressive cytokines such as IL-10, IL-35, fibrinogen-like protein 2 and Transforming growth factor beta (TGF-β) (8).

CD8+ T suppressor cells have also been evaluated in number and function among different organs recipients (9-13). CD3+CD8+CD28- T cells, known as a regulatory subset of T cell population, affect antigen presenting cells (APCs) via cell-to-cell contact. These cells recognize dendritic cells presenting allo-specific antigens and inhibit activation of NFkB transcription factor, which results in inhibiting expression of co-stimulatory molecules CD40, CD80, CD86, and adhesion molecules CD54 and CD58 on APCs. Besides, CD3+CD8+CD28- T cells can upregulate inhibitory molecules such as immunoglobulin-like transcript 3, 4 (ILT3, ILT4) on APCs, and consequently render them tolerogenic to T helper cells. There are also some reports of the secretion of inhibitory cytokines (e.g. TGF-β and IL-10) by this subset, which induces tolerogenic environment for effector cells in tissues (14,15). This subset has been introduced as a beneficial part of the immune system, which helps maintain liver, heart, kidney and intestine grafts survival (9-13,16-19).

Several studies have shown a negative correlation between Treg cells and acute rejection episodes (9-12,16). Some groups have found that recipients with higher level of these cells have a satisfactory graft survival with, lower doses of immunosuppressive drugs (17,18). However, their precise impact on long-term allograft outcomes and chronic rejection remains almost undetermined.

Transforming growth factor-β is a pivotal immunoregulatory cytokine, which exerts its suppressive activity by inhibiting IL-2 production from CD4+ T cells and down-regulating transcription factors GATA binding protein-3 (GATA-3) and T-bet. Moreover, TGF-β prevents dendritic cell maturation and activation mainly by

decreasing MHC-II expression. TGF-β has a substantial link with Tregs, because it is not only secreted by Tregs, but also plays a critical role in inducing them in peripheral circulation (19). On the other hand, the profibrogenic capacity of this cytokine is presumed to be responsible for drug-induced and immunological IF/TA progression (20). In the present study we investigated a relationship between regulatory T cells frequencies and different outcomes of allograft. In addition, according to the controversial role of TGF-β in suppressing immune responses and inducing fibrotic lesions we assessed its expression among CAD patients and normal recipients.

MATERIALS AND METHODS

Patients. Enrolled patients were 60 adult renal transplant recipients who had received allograft 6 month to 5 years before sampling. A group of 15 healthy controls were also studied. The protocol was approved by Ethical Committee of Tehran University of Medical Sciences. All patients gave written informed consent prior to inclusion in this study.

Chronic allograft dysfunction patients (n=30) exhibited a progressive deterioration of renal allograft function with 15% or more irreversible rise in creatinine within 1 to 3 months and proteinuria more than 1 gr/24h (21). Consequently they were subjected to cause biopsy and IF/TA pathologic lesions were reported in their biopsies according to last updated Banff classification (2,3). None of these patients had been diagnosed with infectious diseases at the time of sampling.

Stable graft patients (n=30) were selected according to their sex, age and time post-transplant in order to be pair-matched with CAD patients. They were normal in clinical examination, their serum creatinine levels were ≤ 1 mg/dl, they had proteinuria less than 0.5 gr/24h and their Cockcroft creatinine clearance was more than 80 ml/min (21). They had no registered history of acute rejection episodes and no diagnosed viral or bacterial infectious disease within one month before enrollment. Since these patients presented no deterioration of graft function, and protocol biopsies are not routine procedures in our transplantation centers, no biopsy was available for this group.

Healthy volunteers (n=15) consisted of age and sex-matched healthy individuals without any renal or immunological disorders and no family history of chronic kidney diseases. Their serum creatinine levels were ≤1 mg/ml and their Cockcroft creatinine clearance rates were more than 85 ml/min.

PBMC Isolation and Flowcytometry Staining. For CD3+CD8+CD28- Regulatory T cell staining, we used FITC anti-human CD3 and PE anti-human CD8a conjugated antibody cocktail (eBioscience, San Diego, USA) in addition to APC anti-human CD28 (eBioscience, San Diego, USA). CD4+CD25+CD127- T cells were stained by human regulatory T cell staining kit (eBioscience, San Diego, USA) and PE anti-human CD127 (eBioscience, San Diego, USA). FITC conjugated Mouse IgG1, mouse IgG1 isotype control PE and mouse IgG1 isotype control APC (eBioscience, San Diego, USA) were also used as isotype controls.

Isolated peripheral blood mononuclear cells (PBMCs) were adjusted to a concentration of $1x10^6$ cells/ml and incubated with 10 µl of mentioned antibodies for 30 minutes. The cells were washed by PBS centrifugation and resuspended in PBS. Flowcytometry was performed by FACSCalibur (BD FacsCalibur Becton Dickinson, USA) with cell quest pro software.

RNA Isolation and Real Time PCR. RNA was isolated using the High Pure RNA Isolation kit of Roche Diagnostics (Mannheim, Germany) according to manufacturer instructions. RNA quality was assessed by NanoDrop1000 spectrophotometer (Thermo Scientific, USA) and samples with A_{260}/A_{280} ratios = 1.8–2.2, and A_{260}/A_{230} ratios = 2-2.2 were presumed as acceptable. RNA reverse transcription to cDNA was performed using Transcriptor First Strand cDNA Synthesis kit of Roche Diagnostics (Mannheim, Germany). cDNA quality was also evaluated by NanoDrop1000 spectrophotometer (Thermo Scientific, USA) and samples with A_{260}/A_{280} ratios = 1.7-2 were stored in -70°C until use.

Gene expression assay by Applied Biosystem StepOnePlus. The relative gene expression assay was quantified using TaqMan Gene Expression Assay. In the real-time polymerase chain reaction (RT-PCR) procedure, each well contained 10 µl Master mix, 1 µl assay mix (TGF-β ABI, Hs00998133_m1), 7 µl H_2O and 2 µl of diluted sample cDNA of 5 ng/µl, the final reaction volume was 20 µl. The endogenous control was the housekeeping gene β-actin (ABI, Hs99999903_m1). The mixture was incubated for 2 min at 50°C, for 10 min at 95 °C, followed by 45 cycles of 15 sec at 95°C and 60 sec at 60°C. Relative gene expression was calculated with the standard curve method using C_t value for each amplified sample and cDNA from a healthy control. Data was analyzed by Applied Biosystem, StepOne Software v2.1.

Statistics. Data was presented as mean ± SD. The comparison between three groups was performed by non-parametric Kruskal-wallis test. One-way ANOVA test was used for comparison between groups. Correlations between scale numeric variables were analyzed by correlation bivariate and linear regression (SPSS 16.0; SPSS Inc., Chicago, USA). P values less than 0.05 were considered as significant.

RESULTS

Higher Frequency of CD4+CD25+CD127- T Cells Among Stable Graft Patients in Comparison with CAD Group. Detailed clinical and demographic data of the three studied groups are shown in Table 1. The percentage of CD4+CD25+CD127- T cells in renal transplant recipients was lower than healthy controls (p=0.021). Nonetheless, it was higher among stable graft patients than CAD group (p=0.024). The frequency of CD4+CD25+CD127- Tregs in stable group and healthy volunteers did not show any statistical difference (p=0.51; Figure 1 and Table 2).

This is incomplete.

Increased Frequency of CD3+CD8+CD28- Regulatory T cells in Kidney Recipients. Flowcytometric analysis exhibited a significant expansion of CD3+CD8+CD28- regulatory T cells among kidney recipients in comparison with healthy group (p<0.001). Moreover, patients with stable graft function had the highest frequency of CD3+CD8+CD28- T cells in their peripheral blood. The percentage of these cells was diminished in CAD patients compared to recipients with stable graft function (Figure 2 and Table 2).

Table 1. Patients' demographics and clinical data: patients with chronic allograft nephropathy (CAN), patients with stable graft function (SGF). Healthy volunteers (HV)

Group	CAN	SGF	HV
N	30	30	15
Age in year: mean ± SD	39.40 ± 13.28	39.10 ± 12.25	38.33 ± 12.88
Gender ratio (M/F)	19:11	19:11	10:5
Cadaver/living donor	15:15	17:13	
Cockcroft creatinine clearance ml/min: Mean ± SD	34.36 ± 13.06	96.28 ± 14.11	109.03 ± 19.13
Time post-transplantation in months: Mean ± SD	41 ± 17.76	41 ± 17.76	
Anti HLA class I antibodies +	12/30	8/30	2/15
Anti HLA class II antibodies +	4/30	1/30	1/15
Anti HLA class I and II antibodies +	4/30	0/30	0/15
IS protocol:			
CsA, MMF, Steroids	22 (73.3%)	26 (86.7%)	
Tac, Aza, Steroids	5 (16.7%)	3 (10%)	
Rapa, MMF, Steroids	3 (10%)	1 (3.3%)	
Etiology of ESRD:			
Diabetic nephropathy	5 (16.7%)	3 (10%)	
Hypertension	6 (20%)	7 (23.3%)	
Polycystic kidney disease	1 (3.3%)	5 (16.7%)	
Infection	2 (6.7%)	6 (20%)	
Renal stone	5 (16.7%)	2 (6.7%)	
Reflux	5 (16.7%)	2 (6.7%)	
Chronic glomerulonephritis	3 (10%)	0 (0%)	
Drug toxicity	0 (0%)	3 (10%)	
Unknown	3 (10%)	2 (6.7%)	

IS, immunosuppressive; Aza, azathioprine; CsA, cyclosporine A; MMF, mycophenolate mofetil; Tac, Tacrolimus.

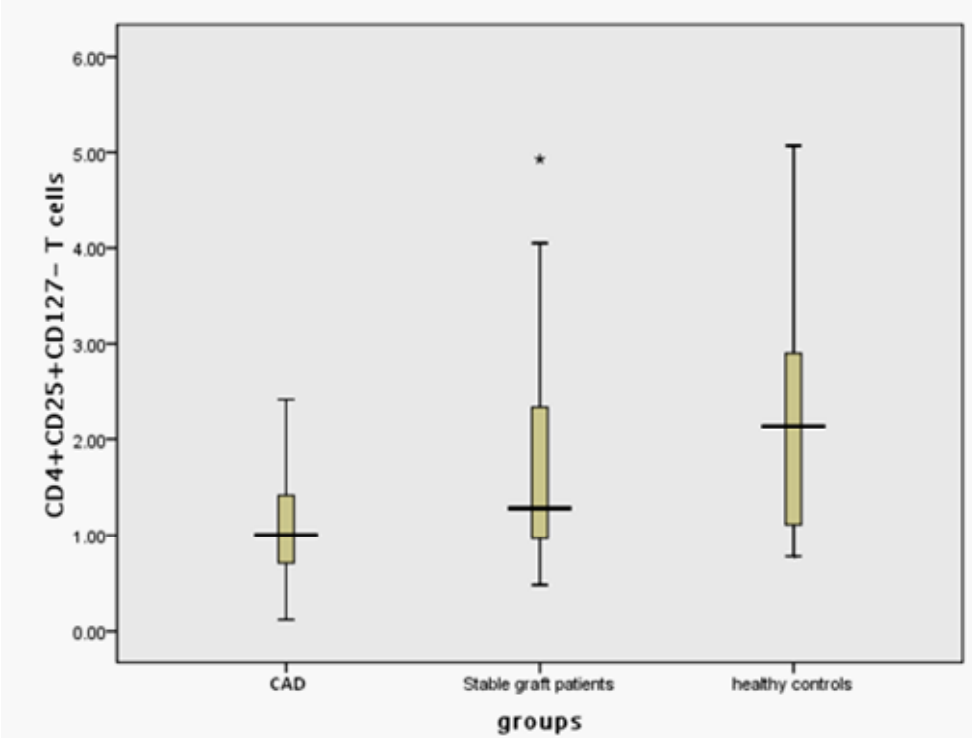

Figure 1. CD4+CD25+CD127-T cells percent in CAD patients, Stable graft patients and healthy controls.

Correlation of IF/TA Pathologic Grades with CD4+CD25+CD127- and CD3+CD8+CD28- T Cells Frequencies. There was a negative association between CD4+CD25+CD127- T cells percentage and pathologic grade of IF/TA (i.e., grades I, II, III). This difference was statistically significant between grades I and III (p=0.005), but not so considerable between grades I and II or grades II and III.

Table 2. Frequency of CD4+CD25+CD127- and CD3+CD8+CD28- T regulatory cells in CAD patients, Stable graft recipients and healthy controls.

	N	CD4+CD25+CD127- T (%)	P Value	CD3+CD8+CD28- T (%)	P Value
CAD patients	30	1.11 ± 0.60		3.90 ± 1.92	
Stable graft patients	30	1.75 ± 1.10	0.024*	5.89 ± 2.48	0.003*
Healthy group	15	2.24 ± 1.31	0.015#	2.95 ± 0.97	0.097#

P.v: P value, *: CAD versus stable graft patients; #: CAD patients versus healthy controls.

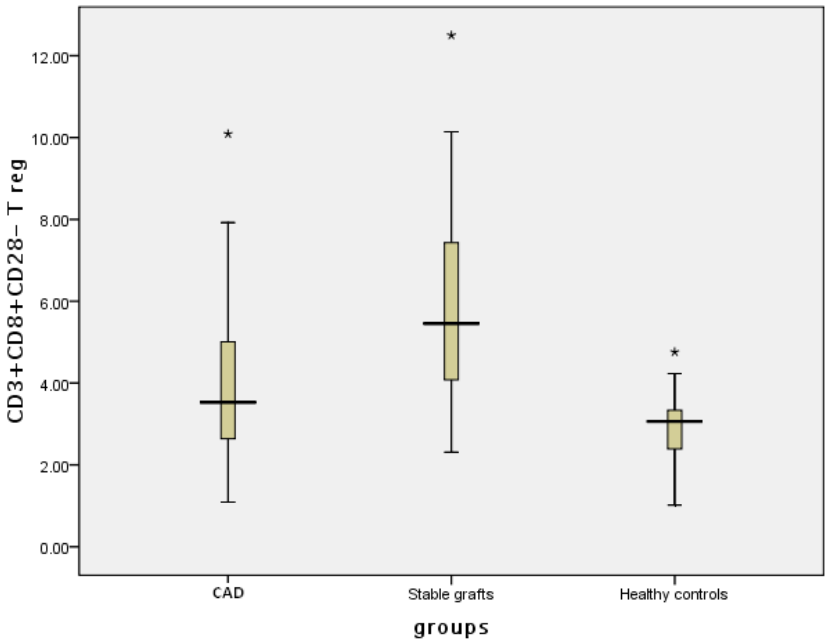

Figure 2. The percentage of CD3+CD8+CD28- T regulatory cells among lymphocytes of CAD patients, stable graft patients and healthy controls.

A similar trend was found in CD3+CD8+CD28- T cells frequency among different grades of CAD patients as the difference between grades I and III was notable (p=0.04; Table 3).

Table 3. Frequency of CD3+CD8+CD28- and CD4+CD25+CD127- T cells in CAD patients with different grades of IF/TA.

	N	CD4+CD25+CD127- T cells (%)	P.v	CD3+CD8+CD28- T cells (%)	P.v
IF/TA I	8	1.54 ± 0.40		5.63 ± 2.71	
IF/TA II	16	1.01 ± 0.67	0.07*	3.56 ± 1.02	0.18*
IF/TA III	6	0.82 ± 0.27	0.005#	2.51 ± 0.73	0.04#

P.v: P value; *: IF/TA grade I vs. II; #: IF/TA grade I vs. III

Correlation of Cockcroft Creatinine Clearance with CD3+CD8+CD28- and CD4+CD25+CD127- T Cells. We observed a positive correlation between Cockcroft creatinine clearance rate and CD3+CD8+CD28- T cells percentage in whole group of recipients including both CAD and stable graft patients (correlation coefficient=0.521, coefficient of determination=0.272, regression p=0.001; Figure 3).

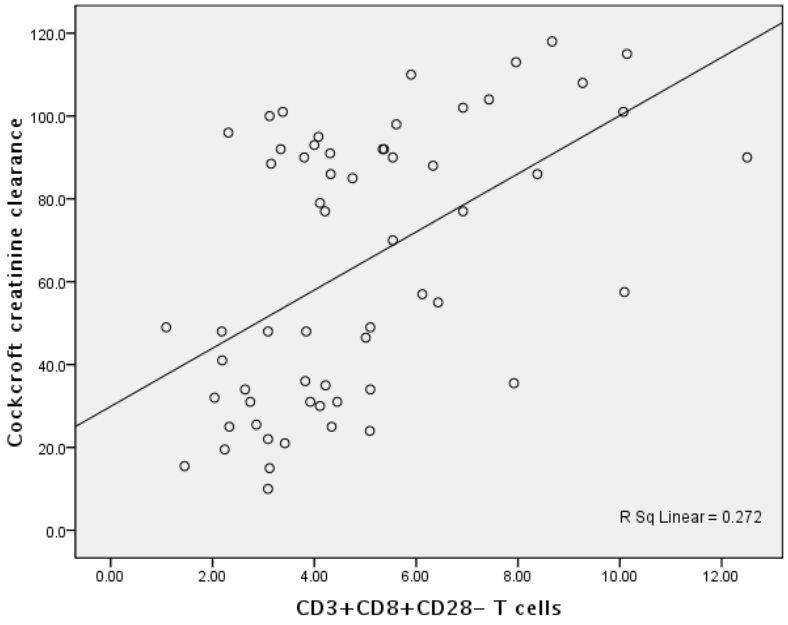

Figure 3. Linear correlation between Cockcroft creatinine clearance rate and CD3+CD8+CD28- T cells.

There was also a weak positive correlation between Cockcroft creatinine clearance rate and CD4+CD25+CD127- T cells frequency in kidney recipients regardless of their clinical condition (correlation coefficient= 0.354, coefficient of determination= 0.125, regression p=0.006; Figure 4).

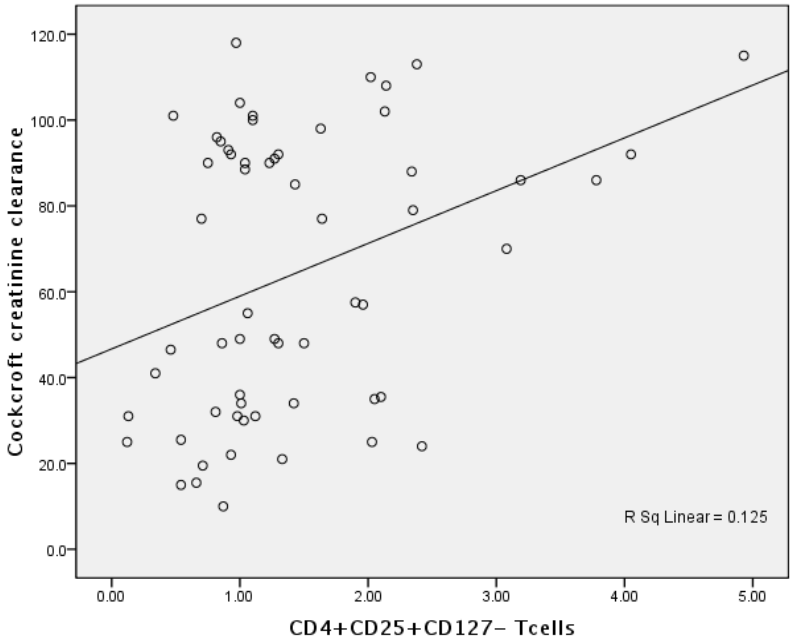

Figure 4. Linear correlation between cockcroft creatinine clearance rate and CD4+CD25+CD127- T cells.

Higher Level of TGF-β1 gene expression in CAD patients. TGF-β1 gene expression in CAD patients was higher than stable recipients and healthy group (p=0.03). This gene was expressed at least in the last group; however, the difference between stable recipients and healthy group was not substantial (p= 0.32; Figure 5).

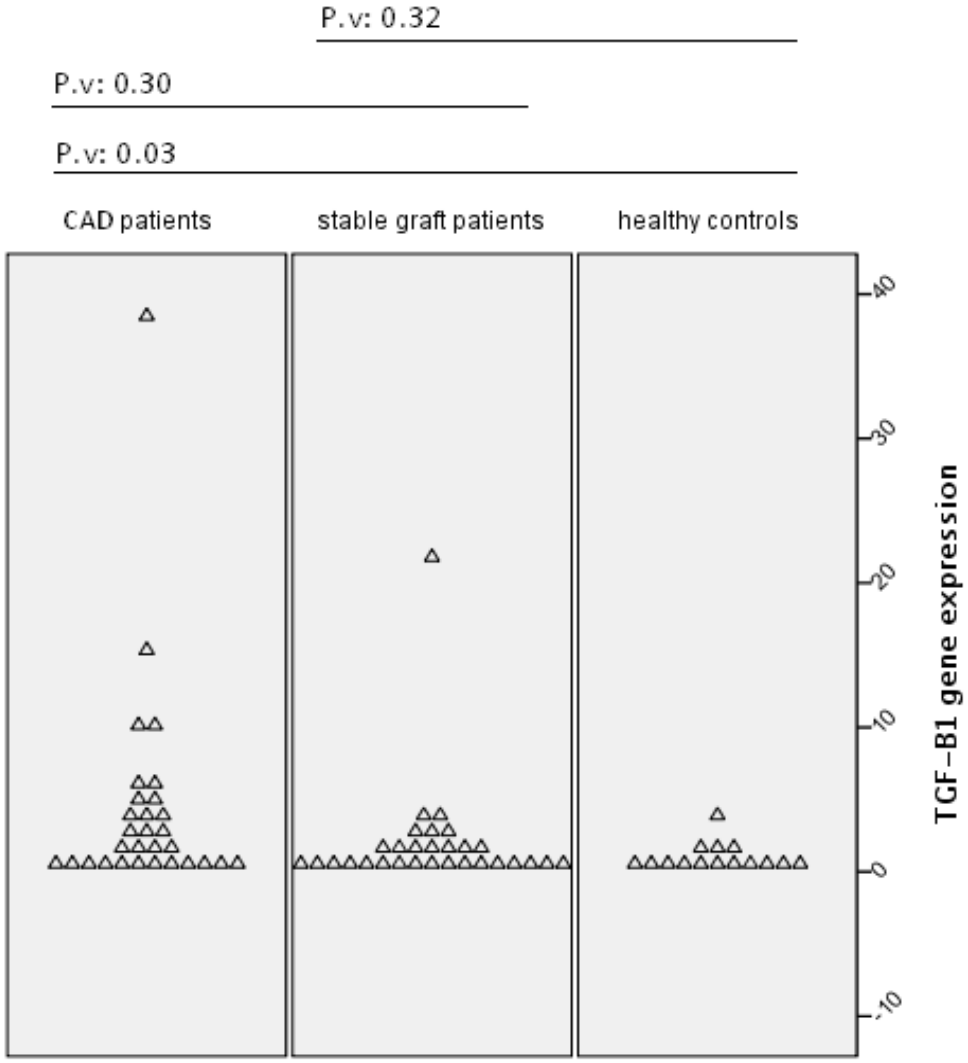

Figure 5. TGF-β1 gene expression in CAD patients, stable graft patients and healthy controls. P.v= p value

TGF-β1 gene expression in three grades of IF/TA. In order to evaluate TGF-β1 gene expression among biopsy-proven CAD patients, we omitted an out-of-range patient, whose gene expression was 38 (rechecked). Without this patient, there was no significant difference between gene expression of IF/TA grades II and III (p=0.92); however, patients of grade I exhibited slightly lower expression of TGF-β1 gene (Table 4).

Table 4. TGF-B1 gene expression in CAD patients with different grades of IF/TA.

	N	TGF-B1 gene expression	P.v
IF/TA I	8	1.94 ± 2.21	
IF/TA II	16	3.47 ± 4.17	0.63*
IF/TA III	5^{Δ}	3.88 ± 3.70	0.65#

Association of TGF-β1 gene expression with CD4+CD25+CD127- and CD3+CD8+CD28- Treg cells. A weak insignificant negative correlation between CD4+CD25+CD127- regulatory T cells frequency and expression of TGF-β1 gene was shown in whole group of recipients (n=60; Pearson correlation coefficient= -0.120 , coefficient of determination= 0.014, regression p value = 0.360; Figure 6).

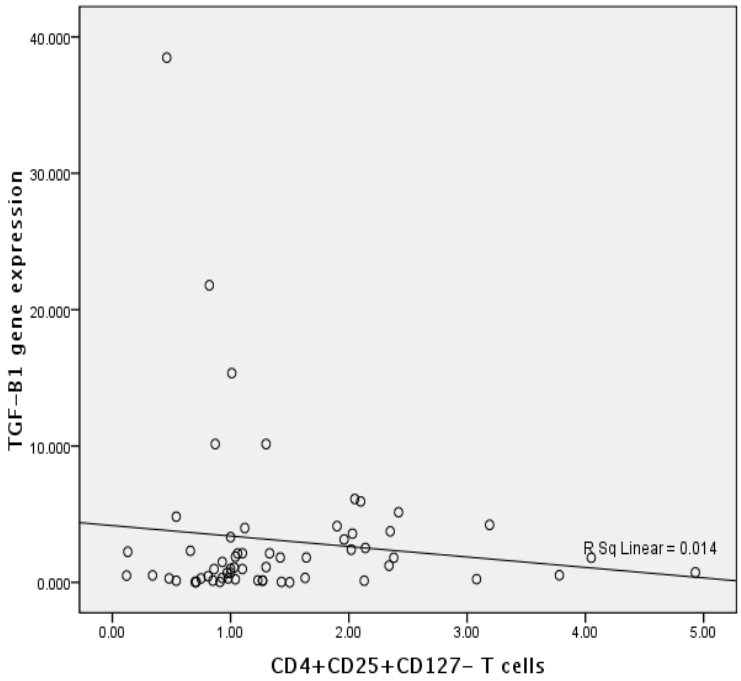

Figure 6. Linear correlation between TGF-B1 gene expression and CD4+CD25+CD127- T cells.

There was also a weak negative association between CD3+CD8+CD28- regulatory T cells frequency and level of TGF-β1 gene expression among kidney recipients (Pearson correlation coefficient= -0.080, coefficient of determination= 0.006, regression p= 0.541; Figure 7).

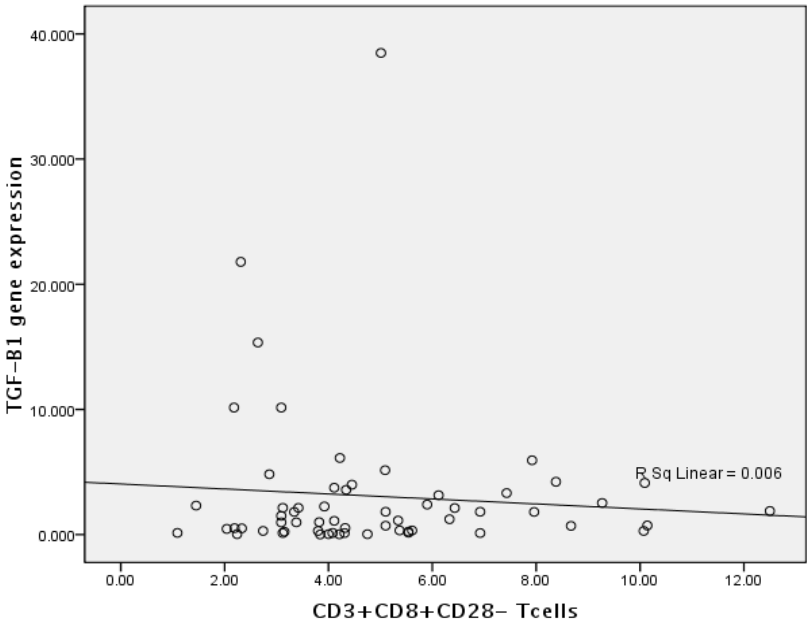

Figure 7. Linear correlation between TGF-B1 gene expression and CD3+CD8+CD28- T cells.

DISCUSSION

Considering chronic rejection as the major obstacle in favorable transplantation outcomes, we investigated some of the major elements of the immune system, influencing donated organ rejection, in order to pave the way for effective clinical manipulations. Recent studies suggest a noticeable role for CD4+CD25+CD127- Tregs in graft survival (22-25) and some investigators are trying to use the potential of this subset to prolong graft survival (26). Alonso-Arias *et al.* found that these cells are lesser in renal recipients than healthy controls but their level correlates positively with creatinine level [22]. On the other hand, Yuan-lin *et al*. have shown considerably higher levels of CD4+CD25+CD127- Treg cells in stable kidney recipients in comparison with healthy group and patients experiencing acute rejection (23); another study showed the frequency of these cells to be much lower among heart transplant recipients in comparison with healthy volunteers. They also reported CD4+CD25+CD127- Treg cells increase within acute rejection episodes (24).

Present study showed significantly diminished level of CD4+CD25+CD127- Treg cells in CAD patients in comparison with stable recipients and healthy controls. The percentage of CD4+CD25+CD127- T cells was highest among healthy individuals, and recipients with stable graft function had approximately the similar percentage. This finding is close to a previous report showing a significantly lower frequency of CD4+CD25+CD45RO+CD127^{low} T cells among solid organ transplant recipients (25). However, despite lower levels of these cells among kidney recipients the difference between recipients and healthy volunteers was not reported statistically significant (27). Similarly, our study showed lower percentage of these cells in CAD patients with advanced grades of IF/TA, suggesting a fairly protective role for CD4+CD25+CD127- Treg cells in transplantation.

CD3+CD8+CD28- T cells, as the other important subtype of regulatory cells have been investigated in liver (9,16,17), heart (10,13), intestine (17) and kidney transplantation recipients (11,12). The role of these cells in preventing acute rejection has already been shown in liver transplantation (16). Our results showed that the percentage of these cells appears to be higher in kidney recipients than healthy individuals; similar findings were reported by Lin Y *et al*. who found a significant expansion of CD8+CD28- T suppressor cells in living donor liver transplant recipients (9,16). In addition, we observed higher frequency of CD3+CD8+CD28- T cells in stable graft group of recipients in comparison with CAD patients. This study also showed lower CD3+CD8+CD28- Tregs frequency in CAD patients with advanced pathologic grades. Although due to small number of enrolled patients our findings are totally conclusive, variations in CD3+CD8+CD28- Tregs percentages between CAD and stable graft patients represents this subset as a potential participant in preventing silent allograft destruction. Neither CD3+CD8+CD28- nor CD4+CD25+CD127- Tregs showed correlation with chronic antibody mediated nephropathy (results not shown).

Our study also represented a positive association between creatinine clearance rate and the percentage of CD3+CD8+CD28- Treg cells among recipients. Bestard *et al*. have also reported a negative correlation between Foxp3+ Tregs presence in renal transplant biopsies and achieving estimated glomerular filtration rate (eGFR) <40 ml/min per 1.73 m^2 in recipients (28). Because of recent attempts to replace invasive diagnostic tests with non-invasive methods for long-term surveillance of allograft function; such correlations could be of clinical and practical significance.

Due to its controversial characteristics, TGF-β1 serves as a double-edged sword in transplantation. The fibrinogenicity of this cytokine along with its accepted relationship with regulatory cells creates difficulties for clinicians to make a conclusive decision on the intensifying or suppressing its expression. The beneficial role of this cytokine in preventing acute rejection has already been shown in many studies (29,30). In contrast, there are some studies, which have shown its detrimental effect on the graft (20,31). Moreover, available data suggest that high producer genotypes of TGF-β1 (32) or its higher level in serum (33) lead to poorer long-term allograft outcomes, whereas down-regulation of its gene has positive effects on transplant (34).

Since expression of TGF-β1 was greater in CAD patients than stable graft patients and healthy controls, our data supports the detrimental effect of TGF-β1 on graft over its immunosuppressive effect. Moreover, its expression among IF/TA grade I patients was lower than advanced grades. In addition, neither CD4+CD25+CD127- nor CD3+CD8+CD28- Tregs showed any meaningful association with TGF-β1 gene expression among recipients, which further weakens the hypothesis of its beneficial participation in graft survival at least in the long term. This finding is in concordance with results of study conducted by Alvarez *et al*. who reported higher levels of TGF-β1 gene expression in chronic renal allograft rejection cases than stable recipients and healthy volunteers (35). Although TGF-β regulates immunological responses at early phases of transplantation, its implication in tissue repair, especially collagen synthesis, might make it damaging in the long term.

In conclusion, present study shows that chronic allograft dysfunction might be associated with the insufficient number of CD4+CD25+CD127- and CD3+CD8+CD28- regulatory T cells and it could be helpful to monitor these cells in evaluating the immune statusof kidney recipients to take appropriate preventive strategies. However, in order to find a cause-effect mechanism between Tregs low percentage and IF/TA

process or vice versa, conducting larger cohort studies is necessary. We also found a positive association between the percentage of Treg cells and Cockcroft creatinine clearance which may help discover a reasonable relationship between clinical and paraclinical data of recipients. TGF-β1 gene was more expressed in patients with IF/TA lesions, suggesting its negative effect on allograft in the long term.

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REFERENCES

1. Nankivell BJ, Borrows RJ, Fung CL, O'Connell PJ, Allen RD, Chapman JR. The natural history of chronic allograft nephropathy. N Engl J Med. 2003; 349:2326-33.
2. Solez K, Colvin RB, Racusen LC, Sis B, Halloran PF, Birk PE, et al. Banff '05 meeting report: differential diagnosis of chronic allograft injury and elimination of chronic allograft nephropathy ('CAN'). Am J Transplant. 2007; 7:518-26.
3. Haas M, Sis B, Racusen LC, Solez K, Glotz D, Colvin RB, et al. Banff 2013 Meeting Report: Inclusion of C4d-Negative Antibody-Mediated Rejection and Antibody-Associated Arterial Lesions. Am J Transplant. 2014; 14: 272-83.
4. Nikoueinejad H, Sharif MR, Amirzargar A, Mirshafiey A, Einollahi B. Regulatory T cells as a therapeutic tool to induce solid-organ transplant tolerance: current clinical experiences. Exp Clin Transplant. 2013; 11:379-87.
5. Battaglia M. potential T regulatory cell therapy in transplantation: how far have we come and how far can we go? Transpl Int. 2010; 23:761-70.
6. Seddiki N, Santner-Nanan B, Martinsone J, Zaunders J, Sasson S, Landay A, et al. expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. J Exp Med. 2006; 203:1693-700.
7. Liu W, Putnam AL, Xu-yu Z, Szot GL, Lee MR, Zhu S, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J Exp Med 2006; 203:1701-11.
8. Miyara M, Sakaguchi S. Natural regulatory T cells: mechanisms of suppression. Trends Mol Med. 2007; 13:108-16.
9. Lin Y, Wang LL, Yan LN, Cai P, Li B, Wen TF, et al. Analysis of CD8+CD28- T-Suppressor Cells in living donor liver transplant recipients. Hepatobiliary Pancreat Dis Int. 2009; 8:241-6.
10. Colovani AI, Mirza M, Vlad G, Wang Su, Ho E, Cortesini R, et al. Regulatory CD8+CD28- T Cells in Heart Transplant Recipients. Hum Immunol. 2003; 64:31-7.
11. Renna-Molajoni E, Cinti P, Ho E, Evangelista B, Peritore D, Poli L, et al. Allospecific human T suppressor cells in kidney transplantation. Transplant Proc. 2001; 33:1129-30.
12. Ranjbar M, Solgi G, Mohammadnia M, Nikbin B, Pourmand G, Ansaripour B, et al. Regulatory T-cell subset analysis and profile of interleukin (IL)-10, IL-17 and interferon-gamma cytokine-producing cells in kidney allograft recipients with donor cells infusion. Clin Exp Nephrol. 2012; 16:636-46.
13. Colovani AI, Mirza M, Vlad G, Wang S, Cortesini R, Suciu-Foca N. Characterization of regulatory CD8+CD28- T cells in heart transplantation recipients. J Heart and Lung Transplant 2003; 22: s106.
14. Manavalan JS, Kim-Schulze S, Scotto L, Naiyer AJ, Vlad G, Colombo PC, et al. Alloantigen specific CD8+CD28-FOXP3+ T suppressor cells induce ILT3+ ILT4+ tolerogenic endothelial cells, inhibiting alloreactivity. Int Immunol. 2004; 16:1055-68.
15. Shalev I, Selzner N, Shyu W, Grant D, Levy G. Role of regulatory T cells in the promotion of transplant tolerance. Liver Transpl. 2012; 18:761-70

16. Lin YX, Yan LN, Li B, Wang L, Wen TF, Zeng Y, et al. A significant expansion of CD8+ CD28- T-suppressor cells in adult-to-adult living donor liver transplant recipients. Transplant proc. 2009; 41:4229-31.
17. Sindhi R, Manavalan JS, Magill A , Suciu-Foca N, Zeevi A. Reduced Immunosuppression in Pediatric Liver-Intestine Transplant Recipients With CD8+CD28- T-Suppressor Cells. Hum Immunol. 2005; 66:252-7.
18. Cortesini R, Renna-Molajoni E, Cinti P, Pretagostini R, Ho E, Rossi P, et al. Tailoring of Immunosuppression in Renal and Liver Allograft Recipients Displaying Donor Specific T-Suppressor Cells. Hum Immunol. 2002; 63:1010-18.
19. Regaterio FS, Howie D, Cobbold SP, Waldman H. TGF-β in transplantation tolerance. Curr Opin Immunol. 2011; 23:660-9.
20. Faust SM, Lu G, Marini BL, Zou W, Gordon D, Iwakura Y, et al. Role of T cell TGF signaling and IL-17 in allograft acceptance and fibrosis associated with chronic rejection. J Immunol. 2009; 183:7297-306.
21. Chapman JR, O'Connell PJ, Nankivell BJ. Chronic renal allograft dysfunction. J Am Soc Nephrol. 2005; 16:3015-26.
22. Alonso-Arias R, Suárez-Alvarez B, López-Vázquez A, Moro MA, Baltar J, Gomez-Huertas E, et al. CD127(low) expression in CD4+CD25(high) T cells as immune biomarker of renal function in transplant patients. Transplantation. 2009; 88:S85-93.
23. Yuan-lin C, Yu-qin Z. CD4+ CD25+ CD127low/- regulatory T cells in the peripheral blood of kidney transplantation patients. J Clin Rehabilitative Tissue Engineering Research 2011; 15: 8209-11
24. Chen M, Mohtize M, Matteï MF, Villemot JP, Kohler C, Faure GC, et al. Reduced levels of both circulating CD4+CD25+CD127$^{low/neg}$ and CD4+CD8^{neg} invariant natural killer regulatory T cells in stable heart transplant recipients. Clin exp immunol. 2011; 163:104-12.
25. Codarri L, Vallotton L, Ciuffereda D, Venetz JP, Garcia M, Hadaya K, et al. Expansion and tissue infiltration of an allospecific CD4+CD25+CD45RO+IL-7Rα^{high} cell population in solid organ transplant recipients. J Exp Med. 2007; 204:1533-41.
26. Trzonkowski P, Bieniaszewska M, Juscinska J, Dobyszuk A, Krzystyniak A, Marek N, et al. First-in-man clinical results of the treatment of patients with graft versus host disease with human ex vivo expanded CD4+CD25+CD127− Tregulatory cells. Clin Immunol. 2009; 133:22-6.
27. Fourtounas C, Dousdampanis P, Sakellaraki P, Rodi M, Georgakopoulos T, Vlachojannis JG, et al. Different immunosuppressive combinations on T-cell regulation in renal transplant recipients. Am J Nephrol. 2010; 32:1-9.
28. Bestard O, Cruzado JM, Rama I, Torras J, Goma M, Seron D, et al. Presence of FoxP3 regulatory T cells predicts outcome of subclinical rejection of renal allograft. J Am Soc Nephrol. 2008; 19:2020-26.
29. Lessan-Pezeshki M, Amirzargar A, Fathi A, Khatami MR, Einollahi B, et al. Value of pretransplantation cytokine profiles for predicting acute rejection in renal transplant recipients. Transplant Proc. 2005; 37:2982-4.
30. Mohammadnia M, Solgi G, Ranjbar M, Shahrestani T, Edalat R, Razavi A, et al. Serum levels of IL-10, IL-17, TGF-β1, and interferon-γ cytokines and expression levels of IL-10 and TGF-β1 genes in renal allograft recipients after donor bone marrow cell infusion. Transplant Proc. 2011; 43:495-9.
31. Morris-stiff G. TGFβ-1 and the development of chronic graft nephropathy: relative roles of gene, mRNA and protein. Ann R Coll Surg Engl. 2005; 87:326-30.
32. Nikolova PN, Ivanova MI, Mihailova SM, Myhailova AP, Baltadjieva DN, Simeonov PL, et al. Cytokine gene polymorphism in kidney transplantation-impact of TGF-beta 1, TNF-alpha and IL-6 on graft outcome. Transpl Immunol. 2008; 18:344-8.
33. Baczkowska T, Perkowska-Ptasinska A, Sadowska A, Lewandowski Z, Nowacka-Cieciura E, Cieciura T, et al. serum TGF-B1 correlates with chronic histopathologic lesions in protocol biopsies of kidney allograft recipients. Transplant proc. 2005; 37:773-5.
34. Crews GM, Erickson L, Pan F, Fisniku O, Jang MS, Wynn C, et al. Down-regulation of TGF-B and VCAM-1 are associated with successful treatment of chronic rejection in rats. Transplant proc. 2005; 37:1926-8.
35. Alvarez CM, Opelz G, Garcia LF, Süsal C. Expression of regulatory T-cell-related molecule genes and clinical outcome in kidney transplant recipients. Transplantation. 2009; 87:857-63.