Immunophenotyping of Granzyme-B Expressing Lymphocyte Subset in Renal Allograft Recipients with Chronic Allograft Dysfunction

B. Yadav[1], N. Prasad[1]*,
V. Agarwal[2], V. Agrawal[3],
M. Jain3[3]

[1]Department of Nephrology and Renal Transplantation, Sanjay Gandhi Postgraduate Institute of Medical Sciences Lucknow, India
[2]Department of Clinical Immunology, Sanjay Gandhi Postgraduate Institute of Medical Sciences Lucknow, India
[3]Department of Pathology, Sanjay Gandhi Postgraduate Institute of Medical Sciences Lucknow, India

ABSTRACT

Background: Granzyme-B is a serine proteinase expressed and released mainly by the cytotoxic T and NK cell. Granules intact Granzyme-B is directly delivered into the target cell, while extracellular Granzyme-B, released in serum leads to nonspecific cleavages of extracellular matrix molecules like vitronectin, collagen, TGF-β, IL-1 and invites systemic inflammation, tissue remodeling and fibrosis leading to the development of chronic renal allograft dysfunction.

Objective: We aimed to immunophenotype the Granzyme-B positive T-lymphocyte subset and Granzyme-B role in the development of chronic renal allograft dysfunction.

Methods: We have analyzed the Granzyme-B$^+$CD8$^+$T/CD8^{neg} and Granzyme-B$^+$CD3$^+$/CD3^{neg} cell subset by the flowcytometry and serum Granzyme-B level by the enzyme-linked immunosorbent assay.

Results: We have found that the frequency of Granzyme-B$^+$ CD8^{neg} CD3$^+$T cell, Granzyme-B$^+$ CD8^{low}CD3$^+$T cell and Granzyme-B$^+$ CD8^{high} CD3$^+$T cell subset was significantly lower and serum Granzyme-B level was significantly higher in CAD group. The frequency of CD3$^+$T, CD3^{neg} lymphocyte, CD8^{neg} CD3$^+$T, CD8^{low}CD3$^+$T, CD8^{high} CD3$^+$T, Granzyme-B$^+$CD3^{neg}CD8^{neg} lymphocyte was similar between the group. The frequency of CD3$^+$CD8^{neg}Gzm-B$^+$ cell was negatively correlated with serum creatinine and CD3$^+$CD8^{high}Gzm-B$^+$ cell was negatively correlated with serum Granzyme-B level. Similarly, Serum Granzyme-B level was positively correlated with serum creatinine, urine proteinuria and negatively with eGFR.

Conclusion: The circulating frequency of Granzyme-B$^+$ CD8^{neg} CD3$^+$T cell, Granzyme-B$^+$ CD8^{low}CD3$^+$T cell and Granzyme-B$^+$ CD8^{high} CD3$^+$T cell subsets were significantly lower and serum Granzyme-B level was significantly higher in renal allograft recipients with CAD.

KEYWORDS: Stable graft function; Chronic renal allograft dysfunction; Cytotoxic T lymphocyte; Granzyme-B

INTRODUCTION

Renal transplant is a preferred modality of choice over dialysis. However, transplanted graft always remains as verge of rejection [1]. Over the period of post-transplantation, immune cell and drug toxicity exerts regressive effect on transplanted allograft leading chronic allograft dysfunction [2]. Cytotoxic T-cell secretes inflammatory cytokines, cytotoxic molecules and poses a negative impact on graft function [3, 4].

Granzyme-B is a serine protease expressed mainly by the CD8$^+$, CD4$^+$ cytotoxic T cell and NK cell [5, 6]. However, recent evidence suggest that Granzyme-B is expressed by other cell like basophils, mast cell, B cell, regulatory

*Correspondence: Narayan Prasad, MD
Department of Nephrology and Renal Transplantation,
Sanjay Gandhi Post Graduate Institute of Medical Sciences
Lucknow, 226014, India
ORCID: 0000-0001-9801-0474
E-mail: narayan.nephro@gmail.com*

Table 1: Demographic and clinical characteristics of renal allograft recipient patients.

Characteristic		SGF (n=15)	CAD (n=20)	P-value
Pt. age (years)		45.20 ± 8.00	37.50 ± 9.70	0.016
Donor age (years)		50.86 ± 6.30	51.55 ± 9.26	0.80
Pt. Gender M:F		14:1	16:4	0.26
Donor Gender M:F		4:11	6:14	0.82
Blood urea nitrogen (mg/dl)		26.97 ± 11.23	37.41 ± 11.54	0.011
Baseline creatinine (mg/dl)		0.92 ± 0.41	0.92 ± 0.48	0.960
Serum creatinine (mg/dl)		1.58 ± 0.61	2.43 ± 0.60	<0.001
Urine proteinuria (g/24hrs)		0.38 ± 0.42	1.75 ± 1.15	<0.001
Tacrolimus level (ng/ml)		4.48 ± 1.03	5.35 ± 1.30	0.042
eGFR (mL/min/1.73 m^2)		59.34 ± 24.55	44.40 ± 12.09	0.018
Pt. TLC (X103)		8.9 ± 2.60	7.27 ± 4.70	0.230
Pt. Hbs (gm/dl)		12.02 ± 2.21	10.82 ± 1.42	0.061
Native Kidney Disease	Chronic interstitial nephritis	3	3	0.330
	Diabetes mellitus	4	3	
	DM+CGN	0	2	
	FSGS	5	6	
	HTN	3	4	
	Not specified	0	2	
Post-transplant interval (month)		48.80±26.11	58.50±20.11	0.220
HLA Crossmatch Score	2	2	1	0.590
	3	7	12	
	4	6	7	
Induction Regimen	Basiliximab	3	2	0.470
	Anti-thymocyte globulin	4	9	
	No induction	8	9	
MMF+Pred	+Cyclosporin	0	3	0.110
	+Tacrolimus	15	17	

Footnote: Pt.: Patients, DM+CGN: Diabetes Mellitus + Crescentic glomerulonephritis, FSGS: Focal segmental glomeruloscelerosis, HTN: Hypertension, MMF: Mycophenolate Mofetil, Pred: Prednisolone

Table 2: Correlation between Granzyme-B+ subset and Kidney function markers.

Characteristics	Serum creatinine	Urine protein	eGFR	Serum Granzyme-B level
CD3$^+$CD8^{neg}Gzm-B$^+$	r=-0.388 p=0.021	r=-0.214 p=0.21	r=0.25 p=0.14	r=-0.243 p=0.16
CD3$^+$CD8^{low}Gzm-B$^+$	r=0.018 p=0.910	r=-0.105 p=0.055	r=-0.096 p=0.58	r=-0.324 p=0.05
CD3$^+$CD8^{high}Gzm-B$^+$	r=0.065 p=0.700	r=-0.29 p=0.08	r=-0.32 p=0.06	r=-0.428 p=0.010
CD3^{neg}CD8^{neg}Gzm-B$^+$ lymphocyte	r=0.250 p=0.140	r=-0.32 p=0.062	r=0.10 p=0.54	r=0.296 p=0.84
Serum Granzyme-B level (pg/ml)	r=0.346 p=0.046	r=0.585 p<0.001	r=-0.315 p=0.06	r=1.00 p=1.00

T cell, and keratinocytes [7-9]. The intracellular granules intact Granzyme-B is delivered directly into the target cell by a perforin dependent mechanism. However, the extracellular Granzyme-B is released in serum and systematically affects different cellular function nonspecifically. Granzyme-B, cleaves extracellular matrix molecules like vitronectin, collagen, fibronectin induces, tissue remodeling and fibrosis [9]. Cleavage of extracellular matrix releases cytokines, profibrotic molecules, which exacerbates the fibrosis and inflammations leading to chronic allograft dysfunction.

Recipient's antigen presenting cell (APC) recognizes allograft peptide and present it to naïve T cell, leading their tutoring and differentiation either in CD4$^+$Th cell or cytotoxic CD8$^+$T cell depending upon local cytokine milieu and nature of antigen presented to naïve T cell for their maturation [10, 11]. MHC-II restricted APC led to CD4$^+$T helper cell differentiation and cytokines secretion, which further directs other immune cell for their maturation and function, while MHC-I loaded peptide direct naïve T cell to differentiate into cytotoxic CD8$^+$T cell.

CD4$^+$T cell secrets cytokines, which help other cell for their maturation and function. However, Granzyme-B containing CD4$^+$Tcell shows regulatory and cytotoxic activity and CD8$^+$ cytotoxic T cell secrets cytolytic molecule Granzymes, lymphotoxin, perforins and express surface molecules like Fas, which mediates apoptosis in target cell and associated with allograft rejection [12]. However, very little is known about Granzyme-B positive CD4$^+$T and CD3-Tcell and how lymphocyte mediate allograft dysfunction in renal transplant patient.

Therefore, in the current study, we aimed to immunophenotype the Granzyme-B positive lymphocyte subset and Granzyme-B role in development of chronic renal allograft dysfunction.

MATERIALS AND METHODS

In this study, we recruited total thirty-five (n=35) renal allograft recipients. Twenty patients had histology proven chronic renal allograft dysfunction. Chronic allograft dysfunction was defined by clinical, histomorphological evidences of allograft dysfunction, i.e >25% rise in serum creatinine from the baseline, significant increase in proteinuria, histological evidences of allograft injuries as per Banff criteria 2007 [13]. Fifteen subjects of stable graft function were recruited. Stable graft function was defined as stable serum creatinine level in last six-month, insignificant proteinuria, <10% cortical surface area showing evidence of interstitial fibrosis and tubular atrophy on histology. A consent form was obtained from each subject before collection of samples. Ethical approval code for the study was (IEC.2012-117-PhD-63).
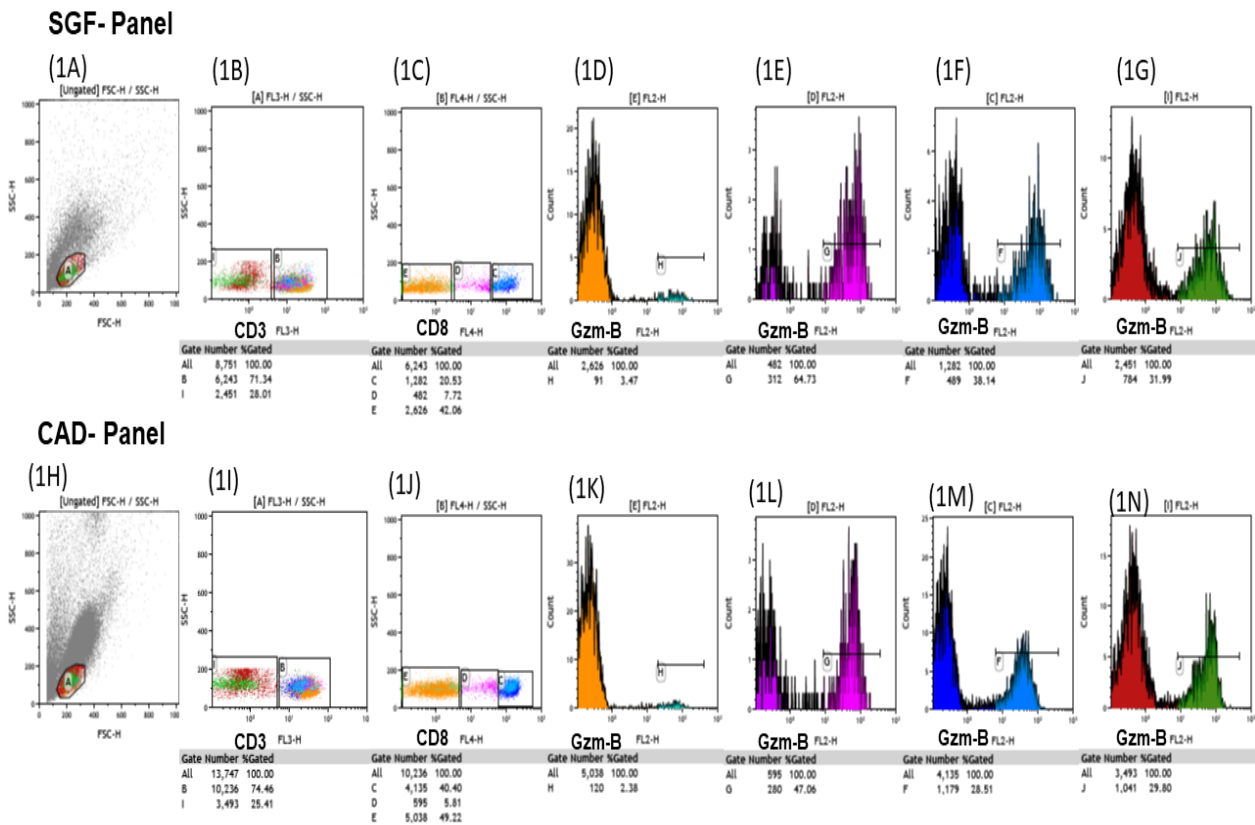
Figure 1: Representative Flow cytometry image of different lymphocyte subset in CAD and SGF patients.

Patients associated demographic and clinical profiles such as age, sex, induction used, maintenance immunosuppressive regimen, native kidney disease, etc. were collected from the electronic medical record of patients.

Blood Sample Collection and Cell Stimulation

One mL blood samples were collected in heparinized vials and 4 ml in plain vial for serum separation. One ml blood samples were diluted with complete RPMI media (1:1 ratio) supplemented with 10% FBS and PBMCs were stimulated with Phorbol 12-myristate 13-acetate (20 ng/ml; Sigma Aldrich, St. Louis, USA) and Ionomycin (1 µg/ml; Sigma Aldrich, St. Louis, USA) for 5 hours and 2µM Monensin (BD Biosciences, San Diego, CA, USA) was added as protein transport inhibitors in last 2 hours of stimulation. Cell was incubated in 5%CO$_2$ at 37°C, at 95% humidity for 5 hours.

Plain vial was centrifuged at room temperature at 1500 RPM for 5 minutes. Serum was separated and stored in -20°C till ELISA setting for Granzyme-B analysis.

Cellular Staining for Flowcytometry Analysis

For the surface and intracellular staining, 100µL of stimulated whole blood was used. Surface staining was performed with 5µl of Peridinin chlorophyll cyanine 5.5 (Percp-Cy5.5) conjugated monoclonal antihuman-CD3 and 10µl of Allophycocyanine (APC) conjugated monoclonal anti-human-CD8 antibody following incubation at room temperature (RT) for 30 minutes in the dark. After RBC lysis with RBC lysis buffer for 13 minutes, the cells were washed and resuspended in cytofix/cytoperm solution followed by intracellular staining with 5µl of Phycoerythrin
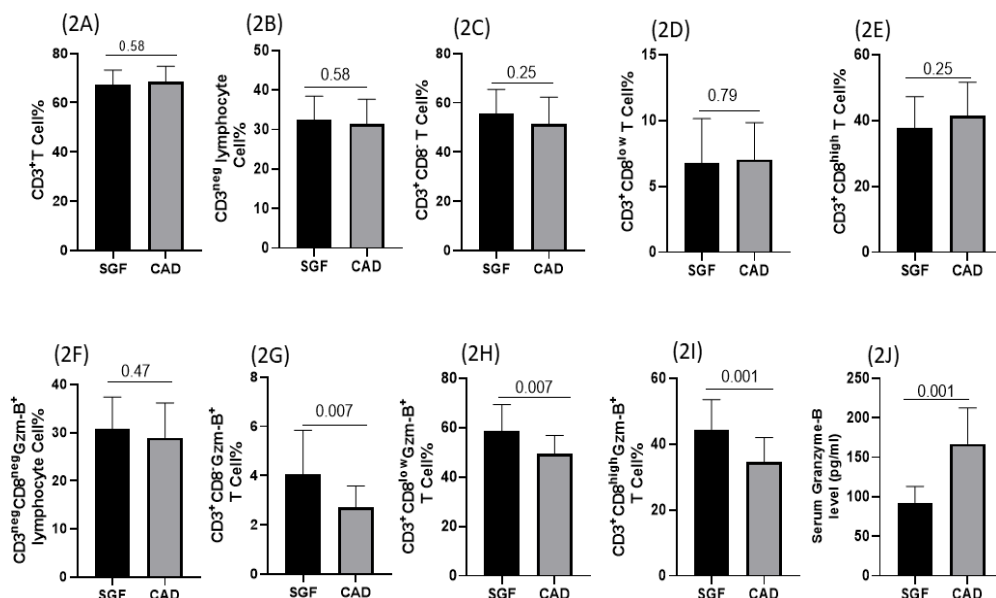
Figure 2: Bar diagram showing % frequency of different lymphocyte subsets. Data were expressed as mean ± SD.

(PE) conjugated mouse monoclonal anti-human Granzyme-B for 30 minutes by incubation in the dark at RT. In case of CD8 and Granzyme-B, a fluorochrome conjugated isotype antibody was used to nullify any false positive gating. All reagents and antibodies were purchased from BD Bioscience (BD Pharmingen). A 10,000 lymphocyte gated cells were immediately acquired by a Facs caliber machine. Cells were analyzed using the Kaluza version 2.1 software. Gating approach was the first lymphocyte cells (Gate-A) in FSC and SSC-H plots were gated, in lymphocyte gate, $CD3^+T$ cells were analyzed (cells in Gate-B). In Gate-B, $CD8^{high}$ (Cells in Gate-C); $CD8^{low}$ (Cells in Gate-D); and $CD8^{neg}$ (Cells in Gate-E) were analyzed. Further, in Gate-C, Granzyme-B^{+ve} cell (Cells in Gate-F); In Gate D, Granzyme-B^{+ve} cell (Cells in Gate-G); In Gate E, Granzyme-B^{+ve} cell (Cell in Gate-H); were analyzed. In another gate (Gate-I) CD-3^{neg} cell were analyzed. In Gate-I, Granzyme-B^{+ve} cell (Cell in Gate-J) were also analyzed.

Granzyme-B Level Analysis
Serum Granzyme-B level was measured by the ELISA using Biolegend Max human Granzyme-B ELISA kit.

Statistical Analysis
Data was analyzed by SPSS version 20. Graph were plotted with GraphPad 8.0. Continuous variables were analyzed by T-independent test and categorical variables by Chi square test. Pearson correlation was performed between lymphocyte subset and renal function parameters. P value<0.05 was considered to be significant.

RESULTS

Demographic Characteristics of Recipients
Patients age and estimated glomerular filtration was significantly lower and serum creatinine, urine proteinuria, blood urea nitrogen was significantly higher in chronic allograft dysfunction group (Table 1).

Granzyme-B Positive Lymphocyte Subset Profiles
Frequency of Granzyme-B$^+$ CD8^{neg} CD3$^+$T cell (cell in gate-H and bar graph 2G), Granzyme-B$^+$ CD8^{low}CD3$^+$T (cell in gate-G and bar graph 2H) cell and Granzyme-B$^+$ CD8^{high}

CD3$^+$T (cell in gate-C and bar graph 2I) cell subset was significantly lower in CAD group. While the frequency of CD3$^+$T (cell in gate-B, bar graph 2A), CD3^{neg} lymphocyte (cell in gate-I and bar graph 2B), CD8^{neg} CD3$^+$T (cell in gate-E and bar graph 2C), CD8^{low} CD3$^+$T (cell in gate-D and bar graph 2D), CD8^{high} CD3$^+$T (cell in gate-C and bar graph 2E), Granzyme-B$^+$CD3^{neg}CD8^{neg} (cell in gate-J and bar graph 2B) was similar between the group. However, of it remarkably 30% of cell were Granzyme-B positive, which may be released in serum any time, which may make graft function worse. Representative flow cytometry Fig 1A-N and Fig 2A-I. Serum granzyme-B level was also significantly higher in CAD group (Fig 2J), suggesting a discharge of Granzyme-B.

Correlation between Granzyme-B$^+$cell and Kidney Function

CD3$^+$CD8^{neg}Gzm-B$^+$ cell was negatively correlated with serum creatinine and CD3$^+$CD8^{high}Gzm-B$^+$ cell was negatively correlated with serum Granzyme-B level. Similarly, Serum Granzyme-B level was positively correlated with serum creatinine, urine proteinuria and negatively with eGFR (Table 2).

DISCUSSION

In this study, we have profiled the Granzyme-B$^+$ lymphocyte cell subsets. We found that the frequency of Granzyme-B$^+$ CD8^{neg} CD3$^+$T (possibly CD4$^+$ cytotoxic T cell), Granzyme-B$^+$CD8^{low}CD3$^+$T and Granzyme-B$^+$CD8^{high}CD3$^+$T cell was significantly lower in CAD group and serum Granzyme-B level was significantly higher in CAD patients. Further, the frequency of Granzyme-B$^+$CD8^{neg}CD3$^+$T cell was negatively correlated with the serum creatinine and CD3$^+$CD8^{high}Gzm-B$^+$ frequency was negatively correlated with the circulating serum Granzyme-B level. This suggesting a pathogenic mechanism of Granzyme-B containing lymphocyte subset that releases Granzyme-B in serum. An encounter of recipient immune cell with allograft peptide leads to activation and synthesis of Granzyme-B in cytotoxic T cell. Studies shows, Granzyme-B

positive lymphocyte cell mediates allograft dysfunction by releasing Granzyme-B in serum and serve as markers of acute and chronic rejection [3, 14]. Lymphocyte comprises T, B and NK cell. CD8$^+$ subset is well recognized for inflammatory cytokine IFN-γ, lymphotoxin, Granzyme-B and perforin secretion [15], However, recent studies shows together with helper property, Granzyme-B$^+$CD4$^+$T cell, also play a role in many disease and remain less regulated compared to CD8$^+$T cell subset [5, 10]. In our finding, we noticed a decreased Granzyme-B$^+$ CD8^{neg} CD3$^+$T cell subset in CAD patients. CD4$^+$ T cell subsets had negative impact on graft function as observed in our previous studies [16, 17]. It has been seen that activated Treg cell (CD4$^+$T cell subset) express and releases Granzyme-B and induces apoptosis in targeted and in itself, this may be one of the reason for lower Treg in chronic renal allograft recipient patient [18], allowing inflammatory cell propagation. However, we did not used specific markers for CD4$^+$Treg cell. Natural killer (NK) cell and Natural killer T cell (NKT) are other two subset, which secrets Granzyme-B and reported to be linked with allograft dysfunction. NKT cell play a regulatory role and reported to be linked with maintaining stable graft function, whereas increased NK cell proportion promotes acute cellular rejection in renal allograft dysfunction [19]. In our finding we noticed a significant decreased frequency of Granzyme-B$^+$CD8^{neg} CD3$^+$T (possibly CD4$^+$T and NKT cell subset) cell. Although, NKT comprises only 0.1% of total T cell. Furthermore, we noticed approximately, 30% of CD3-ve cell (possibly B and NK) cell were Granzyme-B$^+$, suggesting a role of non T cell involvement in Granzyme-B secretion. Role of NK cell in allograft rejection is well appreciated [20]. Furthermore, their occur two subsets in CD8$^+$T cell, CD8^{low} and CD8^{high}. In our analysis approximately, 50-60% CD8^{low} CD3$^+$Tcell were Granzyme-B positive, while in 30-40% of CD8^{high}CD3$^+$T cell were Granzyme-B positive and increased serum Granzyme-B, suggesting granzyme-B secretion by these subsets.

Mechanistically, cytotoxic-T cell delivers Granzyme-B directly into target cell via per-

forin dependent mechanism and induces the apoptosis and fibrosis. However, the extracellular Granzyme-B may affect systematically distant resident cell and damages other cell nonspecifically. Allograft recipient patients usually experiences extrarenal complications like new onset of diabetes after transplant, respiratory and cardiac complications [21, 22]. Further, allograft fibrosis is a common event leading to development of allograft dysfunction. A genetic Granzyme-B deficiency protect mice from developing cardiac hypertrophy, inflammation, accumulation of extracellular matrix and fibrosis [23].

Additionally, Granzyme-B cleaves the endothelial junctional protein and disrupt the endothelial cell barrier allows leakage of circulatory components in extracellular space [23]. Cleavage of extracellular matrix additionally releases damage associated molecular pattern molecules (DAMPs), which additionally, increases the inflammatory cascade. An elevated level of extracellular Granzyme-B in serum was associated with many inflammatory diseases such as rheumatoid arthritis, cardiovascular inflammation [7, 24, 25]. Additionally, Granzyme-B, activates many proprotein like IL-18, TGF-β, IL-1β, which may further aggravate the inflammation many fold. IL-18 and IL-1β are potent inflammatory molecules and TGF-β is a strong profibrotic molecules mediate SMAD dependent fibrosis pathway.

Additionally, Granzyme-B also damage mitochondrial membrane bilayer potential and induces reactive oxygen species formation, which may additionally damage renal allograft [15, 26]. An antioxidant may be an effective therapy in attenuating the development of chronic allograft dysfunction in renal transplant recipient patients.

Although some potential limitation of our study is that we did not used specific markers for clearly distinguishing Granzyme-B containing NK, NKT and CD4+T cell subsets. A marker specific Granzyme-B+ cell analysis will be required to completely delineate the role of specific subset. However, this study still giving a worthful approximate estimate about the involvement of different Granzyme-B containing lymphocyte subset and the mechanism how, these cells may mediate the CAD.

In conclusion, the circulating frequency of Granzyme-B+ CD8neg CD3+T cell, Granzyme-B+ CD8low CD3+T cell and Granzyme-B+ CD8high CD3+T cell subsets were significantly lower and serum Granzyme-B level was significantly higher in renal allograft recipients with CAD. An inhibition of Granzyme-B synthesis and its secretion may be a therapeutic strategy in preventing CAD progression.

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CONFLICTS OF INTEREST: None declared.

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