

Granzyme B positive Cytotoxic T cells are Associated with Chronic Active Antibody Mediated Rejection.

Gene and protein

expression analysis of

Granzyme B

LN2

using Qiagen RNA mini kit

Reverse Transcriptase (RT)

expression determination

Run Real Time PCR ABI -7500

Machine)

Data was analyzed with 2^^CT

Intragraft Granzyme B protein

Cut section of 3-5um paraffin

following deparaffinisation and

Done heat fixation of tissue section

Done antigen retrieval in Tris-EDTA

Done Granzyme-B staining with

Done HRP conjugated 2ndry

mouse monoclonal primary antibody

Develop color with DAB reagent and

counter stain with Hematoxyline

Analyze Granzyme-B+ cell in

different compartments of graft

expression analysis

embedded section

rehydration

(pH=8.5) buffer

antibody labeling

CABMR

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Introduction

- Chronic antibody mediated rejection respond poorly to conventional steroid therapy and remained one of major cause for graft loss in late post transplant time after transplantation.
- CABMR accounts for 20-60% of graft loss by 5 years
- CABMR is often associated with C4d deposition in peritubular capillaries and donor specific antibody against the either or both class of HLA.
- Clinically, it is associated with proteinuria, hypertension, in serum creatinine level decline in glomerular filtration rate
- Histologically, shows multilayering in peritubular capillary wall, glomerular basement membrane, interstitial fibrosis

Cytotoxic T cell

- Granzyme-B Cytotoxic positive CD3+CD8+ T cell.
- On activation it secrets serine protease Granzyme B, Perforin, lymphotoxin and express surface receptor Fas L.
- Perforin form pore in target cell and disturb osmolitic balance of cell.
- Granzyme cleaves protease, serine procaspase in to active caspase and induce apoptosis in kidney cell.
- Granzyme-B also cleave metalloproteinase IL-1β and generate and inflammatory milliue.
- FasL binds to FasR of target cell and induce apoptosis in them.
- The exact mechanism of immune injury in such conditions are not well established.

AIM

- To study the frequency of cytotoxic T cell (CD3+CD8+GzmB+),in blood, Granzyme-B level in serum, Cell intact Granzyme-B level in PBMCs culture supernatants of peripheral mononuclear cell in patients of SGF and CABMR.
- To determine the Granzyme-B mRNA and Protein expression in allograft biopsy tissue of these patient in these conditions

Material and Methods

Patient recruitment (N=42)

CABMR (N= 32) ✓ PTCBMML (Peritubular) capillary basement membrane multilayering)

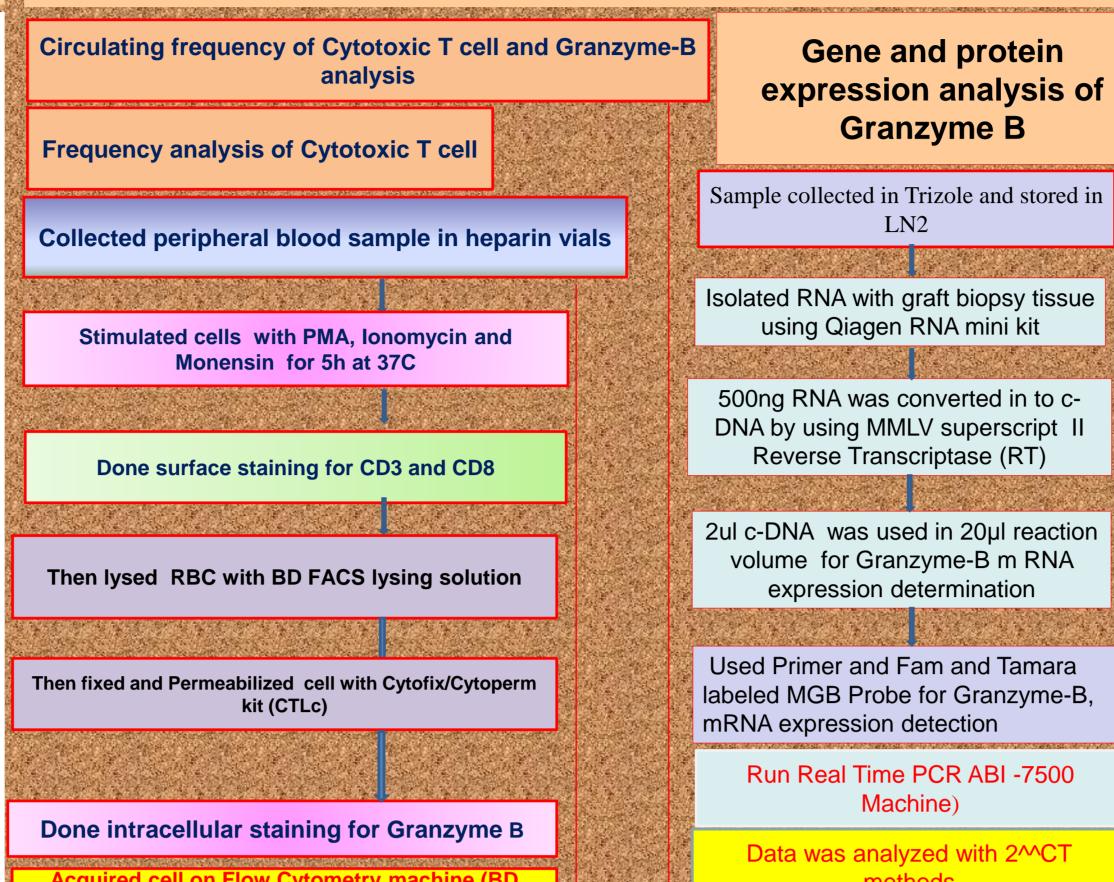
- ✓ C4d+ in peritubular capillary ✓ DSA positive against either
- class of HLA ✓ Proteinuria, >25% rise in
- serum creatinine DSA
- SGF (N=10) √<10% cortical surface
 </p> with evidence of lesions ✓ Stable serum creatinine level in last six month ✓ No proteinuria ✓ Negative C4d staining in PTCs and absence of

All patients were included in study following as per criteria of Banff-2007

Renal transplantation - experimental

Narayan Prasad

Materials and Methods



Isolate PBMCs by overlayering on histopaques (density=1.07g/ml) Culture 1x10^6 mononuclear cell in complete RPMI media by stimulation with PMA(10ng/ml) and Ionomycin (1µg/ml) and Culture for 24 hrs.

Centrifuge at 1500 RPM for 5 minutes and collect serum/ supernatants

> Set ELISA with Biolegend max Granzyme-B ELISA kit and calculate the concentration

Characteristics

Statistical analysis: The means values in both groups were compared with T independent test

The relative change in Granzyme mRNA expression was calculated by 2-^^Ct method

Granzyme-B expression was analyze with Image J software p<0.05 is considered to be significant,

Results

cytoskeleton protein, Table 1 | Demographic and clinical characteristics of patients

SGF (Mean±SD)

<u> </u>	(======================================	· · · · · · · · · · · · · · · · · · ·	
		(Mean±SD)	
Pt. Gender (M: F)	10:0	21:11	0.182
Do. Gender (M: F)	2:8	7:25	0.874
Patient age (Years)	44.36 ± 8.20	37.93±12.78	0.148
Post Tx bx interval (Months)	46.70 ± 17.30	70.18±34.48	0.046
eGFR (mL/min/1.73 m ²)	70.62 ± 22.14	44.60 ± 16.62	< 0.001
Tac level (ng/ml)	4.82 ± 0.98	5.57 ± 1.31	0.102
TLC	8.39 ± 2.12	8.05 ± 5.08	0.839
BUN	25.79 ± 13.3	40.55±11.79	0.002
Baseline creatinine (mg/dl)	0.81 ± 0.47	0.88 ± 0.42	0.644
S. Creatinine (mg/dl)	$1.21 \pm .18$	2.40 ± 0.80	< 0.001
24 hour urine protein (gm)	0.16 ± 0.085	2.94 ± 1.54	< 0.001
HLA mismatch	3.40 ± 0.69	3.15 ± 0.54	0.170
Induction regimen (Basiliximab)	10	32	1.00
Baseline Immunosuppression Tacrolimus+MMF+Pred	10	32	1.00
ESRD cause (MN/HTN/NOS)	6/3/1	20/9/3	0.698

Banff- 2007, Histological injury score of allograft

Characteri SGF(10) C		CABMR(32 P value	C4d staining			
stics)	cd0 (%)	10	0 (0%)	<0.00
	Peritubula	r capillaritis		(100%)		
ptc0 (%)	9(90%)	2 (6.25%) < 0.001	cd1 (%)	0 (0%)	2 (6.25%)	
ptc1 (%)	1(10%)	7 (21.8%)	cd2 (%)	0 (0%)	21 (65.62)	%)
ptc2 (%)	0(0%)	8 (25%)	cd3 (%)	0 (0%)	9 (28.1%)	
ptc3 (%)	0(0%)	15(46.8%)	,	,	al fibrosis	
Glomerulitis		ci0 (%)	10(100%)) 3 (9.3%)	<0.001	
cg0 (%)	9 (90%)	3 (9.32%) <0.001	ci1 (%)	0 (0%)	12(37.5%	١
cg1 (%)	1 (10%)	14(43.7%)	C11 (70)	0 (070)	12(37.370)
			ci2 (%)	0 (0%)	9 (28.1%)	
cg2 (%)	0 (0%)	12(37.5%)		, ,	, ,	
	, ,		ci3 (%)	0 (0%)	8 (26.6%)	
cg3 (%) 0 (0%) 3 (9.32%)		1.0 (0()		hyalinosis		
Interstitial Inf		,	aah0 (%)	,	24 (75%)	<0.001
			aah1 (%)	1 (10%)	7(21.8%)	
ti0 (%)	8 (80%)	2 (6.25%) < 0.001	aah2 (%)	0 (0%)	1 (%)	
ti1 (%)	2 (20%)	14(43.7%)	aah3 (%)	0 (0%)	0 (3.1%)	
				Tubulur	atrophy	
ti2 (%)	0 (0%)	14(43.7%)	t0 (%)	6 (60%)	2 (6.6%)	0.021
(/0)	3 (373)	()	t1 (%)	4 (40%)	7(23.3%)	

t2 (%)

t3 (%)

Results

Table 2. Mean % of CD3+CD8+T cell and CD3+CD8+ Gzm B+ in SGF and CABMR group. Characteristics CCE CARMD D Voluc

Characteristics	SGF	CABMR	P Value
CD3+CD8+Cell%	33.10±4.38	26.80±6.16	0.031
CTLc (CD3+CD8+GzmB)%	27.32±2.7	12.44±1.68	<0.001
A. 768	1024 0.00% 768	0.00%	.65% 13.16%
T O S S S	T 0 512-	H 10 ²	
Gate 2 32.84% 67.16% 10 ⁰ 10 ¹ 10 ² 10 ³ 10 10 10 10 10 10 10 10 10 10 10 10 10	0 ⁴ 10 ⁰ 10 ¹ El	28.01% H 10 ¹ 10 ⁰ 10 ⁰ 10 ⁰	9 47% 10 ¹ 10 ² 10 ³ 10 ⁴ FL4-H
1024 0.00%	1024 0.00%	NI	3.63% 29.92%
B. 768- H-055 512-	768- I	10 ³ H-Z-H	
34 26% 65 72%	256- 57 84 6	42:19% 10 ¹	11.63%

Figure 1. Representative flowcytometric picture of renal transplant in SGF (Panel A) and CABMR (Panel B) group respectively

CD3-Per-Cp Cy5.5

SGF

P value

CABMR

level (pg/ml)

100.82±22.41

177.82±48.66

CABMR

Table 3. Soluble and cell-intact Granzyme-B expression analysis in serum and PBMCs culture supernatants. **Serum soluble Granzyme-B PBMCs** culture supernatants

SGF

CABMR

CD8-APC

Granzyme-B level (pg/ml)

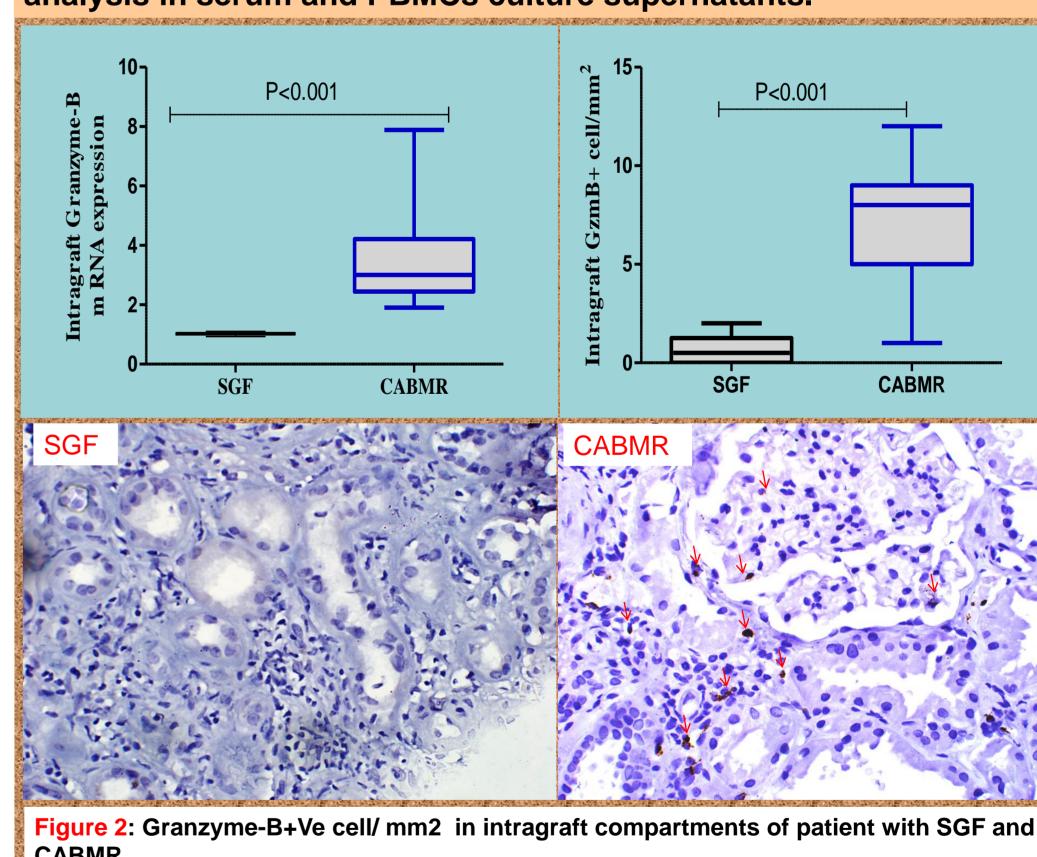
109.41±33.27

82.69±19.88

CABMR

P value	<0.001	P value	0.003
Serum soluble granzymeB (pg/ml) 100 100 100 100 100 100 100 1	P<0.001	PBMCs culture supnt soluble granzym-B (pg/ml) c c c c c c c c c c c c c c c c c c c	P=0.003

Table 4. Intragraft Granzyme-B, mRNA and protein expression analysis in serum and PBMCs culture supernatants.



Conclusions

- ✓ The low frequency of Granzyme-B+ cytotoxic T cell and higher soluble serum Granzyme-B level in CABMR suggest activated cytotoxic T cell releases Granzyme-B in serum.
- ✓ Higher intragraft Granzyme-B, mRNA and protein expression suggest sequestration of CTLc in graft tissue from circulation.
- Granzyme-B dependent allograft injury may be the cause for higher allograft injury score in CABMR group.

Acknowledgments:

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2 (6.25%)

0 (0%)

ti3(%)



15 (50%)

6 (20%)

0 (0%)



