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Original Article

Circulating level of regulatory T cells in rheumatic heart disease: An observational study



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ABSTRACT

Background: The regulatory T cell (Treg) is essential for prevention of autoimmunity. In a preliminary study, we showed significant deficiency of Tregs (CD4CD25 T cells) in rheumatic heart disease (RHD) patients (an autoimmune disease), but the markers used could not reliably differentiate Treg from nonregulatory conventional T cells (Tcon). The study aim was to reassess the level of circulatory Tregs by using more specific markers.

Methods: 70 adults of RHD and 35 controls were studied. Patients were subdivided according to the extent of left-sided valvular involvement. 35 patients with significant mitral-valve disease only were enrolled in the univalvular group while 35 patients with significant involvement of both mitral and aortic-valves in the multivalvular group. Circulating Treg cell level was determined by flow-cytometry.

Results: Level of Tregs (CD4+CD25^{med-high}CD127^{low} Foxp3^{high}) in CD4+ T lymphocyte was significantly lower in RHD patients compared to controls (median 0.6% versus 3.2%; $p = 0.001$) with no significant difference in Tcon cells ($p = 0.94$). Within the study group Treg count was significantly lower in patients with multivalvular-disease only (median 0.1% versus 3.2%; $p = 0.001$) with no significant difference in Treg cell count between the univalvular group and control (median 1.9% versus 3.2%, $p = 0.10$).

Conclusion: There is significant deficiency of circulating Tregs in patients of chronic RHD and the deficiency is greater in patients with multivalvular than univalvular involvement.

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1. Introduction

Rheumatic heart disease (RHD) is an autoimmune progressive destructive valvular disorder that occurs as a sequel of acute rheumatic fever in genetically predisposed subjects. Molecular

mimicry between tissue proteins and streptococcal antigens like M protein (the major component and most virulent factor of streptococcal cell surface) leads to autoimmunity.^{1–3} Extensive research over the last 25 years has identified streptococcal antigen primed circulating CD4+ T cells as the major effector cell for immunological damage in RHD.^{4–8} On

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the other hand, in a preliminary observational study, we have reported that T regulatory cells (Tregs), which inhibit the autoreactive effector CD4 cells and protect against tissue injury, are significantly decreased in patients of RHD.⁹ One of the major limitations of our study was that we defined Tregs as CD4+CD25+ cells, but the surface marker CD25 is also expressed by activated nonregulatory T cells.¹⁰ In current literature, Tregs are defined as CD4CD25 positive cells with increased intracellular concentration of the transcription factor Fox P3.¹¹

As Fox P3 is intracellular, it cannot be used to separate human Treg cells for functional studies or to assess in vivo expansion for cellular therapy, thereby limiting its use in human setting. Recently, a new surface marker CD 127 (interleukin 7 receptor) has been reported, which can be used in lieu of Foxp3 to define Tregs (CD127 expression varying inversely with FoxP3 concentration) and differentiate them reliably from nonregulatory T cells.¹²⁻¹⁴ Accordingly, Koreth et al.¹⁵ defined CD4+ cells with moderate to high expression of CD25 and low expression of CD127 (as these cells have high intracellular Foxp3) as Tregs, while CD4 cells with low expression of CD25 and medium to high expression of CD127 as T conventional cells (as these cells have low intracellular Fox P3).^{*} As there have been no studies using these markers in patients of RHD, we decided to use all the four markers, CD4, CD25, CD127, and Fox P3, to reliably differentiate Treg from Tcon cells and assess their levels in patients of RHD. In our study, Tregs were defined as cells, which were CD4+CD25^{med-high}CD127^{low} FoxP3^{high} while Tcon cells as those, which were CD4+CD25^{low}CD127^{med-high} Fox P3.^{low}

Hence, to overcome the limitation of our previous study, we undertook this study to reevaluate the level of circulating Tregs cells in patients of RHD using more specific markers. Secondly, in our preliminary study, circulating Tregs were lower to a greater extent in patients with extensive disease (multivalvular involvement) compared to limited disease (univalvular involvement).⁹ The secondary aim of our present study was to assess the level of circulating Treg, in patients with univalvular (limited) and multivalvular (extensive) disease.

2. Materials and methods

A total of 105 adult subjects were enrolled in the study, which was cross sectional in design. Patients with echocardiographic evidence of RHD were further subdivided according to the extent of left-sided valvular involvement. 35 patients with echocardiographic evidence of significant mitral valve disease only (moderate to severe mitral stenosis and/or moderate to severe mitral regurgitation) were enrolled in 'univalvular' group, while 35 patients with significant involvement (moderate to severe) of both mitral and aortic valves were enrolled in the 'multivalvular' group. The control group comprised of 35 healthy volunteers.

Patients with autoimmune, hematological, or rheumatologic disorders, diabetes mellitus, vasculitis, malignancies, acute or chronic systemic and/or local infection, infective endocarditis, history of rheumatic fever within 2 years, cardiomyopathies, left ventricular ejection fraction (LVEF)

<50%, atrial fibrillation, coronary artery disease; pulmonary, renal, and hepatic disorders were excluded.

The study was approved by the institutional ethics committee at the authors' institution, and all subjects provided their written informed consent voluntarily to participate in the study. None of the subjects enrolled in the study had declined to participate in the study and gave consent to look at health records both in respect of the current study and any further research that may be conducted in relation to it. The subjects also agreed that they do not have any objection to the use of any data or results that arise from this study provided it is used, only for scientific purpose.

2.1. Study procedures

Demographic variables including age and sex were recorded. All patients underwent detailed clinical evaluation to assess their New York Heart Association (NYHA) functional class, severity and extent of valvular disease and exclusion of concomitant conditions like acute rheumatic fever, infective endocarditis, systemic infections, and autoimmune diseases. Electrocardiogram was evaluated to detect rhythm abnormalities and chamber enlargements.

Transthoracic echocardiography (Philips, IE33) was performed in order to evaluate extent and severity of valvular involvement. The extent and severity of disease in mitral and aortic valves were evaluated as already described in our previous study.⁹ All echocardiograms were performed by the same operator to avoid interobserver variability.

Baseline laboratory tests, hemogram, renal function tests, and liver function tests were performed in all patients. Total leukocyte count (TLC) and lymphocyte count were determined by automatic analyzer (Sysmex, XT2000i Singapore). The erythrocyte sedimentation rate (ESR; Westergren method) and C-reactive protein (CRP) levels (CRP-Turbilatex, Spinreact, S.A.U. Spain) were estimated in all cases. Special laboratory tests (antinuclear antibody, rheumatoid arthritis factor) were done in selected patients if clinically indicated to exclude other autoimmune diseases.

2.2. Flow cytometry

5 ml of peripheral venous blood was obtained from patients with RHD and normal healthy volunteers in EDTA tubes. The blood samples were transported to the laboratory (ensuring sterility) at room temperature for isolation of the peripheral blood mononuclear cells (PBMCs). Flow cytometry analysis was done in a blinded manner. PBMCs were isolated by using commercially available lysing solution (FACS lyse solution, BD Biosciences) according to manufacturer's recommendations. The PBMCs were then washed twice with phosphate buffered saline (PBS) (pH 7.4). The isolated PBMCs were incubated with following conjugated antihuman monoclonal antibodies.

CD4 phycoerythrin (PE), CD25 piperidine chlorophyll protein (PerCP), and 127 allophycocyanin (APC) for cell surface markers according to the manufacturer's instructions (Serotec, Oxford, UK). Stained PBMCs were processed with fixation buffer and permeabilizing buffer (Ebioscience Inc., USA) and were incubated with fluorescein isothiocyanate (FITC) conjugated antiFoxp3 (Ebioscience Inc., USA). Cells were then fixed

in 500 μ l of 2% paraformaldehyde in 0.1 M PBS (phosphate buffer solution) and were kept at room temperature in dark for 30 min. After incubation, cells were washed twice in staining buffer and pelleted by centrifugation to resuspend again in PBS for acquisition and analysis on Flow Cytometer. A FACS Calibur TM Flow Cytometer (Becton Dickinson, Mountain View, USA) equipped with a 15 mW argon ion laser and filter

settings for FITC, PE, PerCP, and APC were used in this study. The Flow Cytometer was calibrated and compensated using CaliBRITE™ beads using FACSCOMP software according to the manufacturer's recommendations. Total 10,000 events were acquired for each sample. The compensation standard used was lymphocyte stained with strongly positive single-color monoclonal antibodies for surface markers, e.g., CD4 or CD25

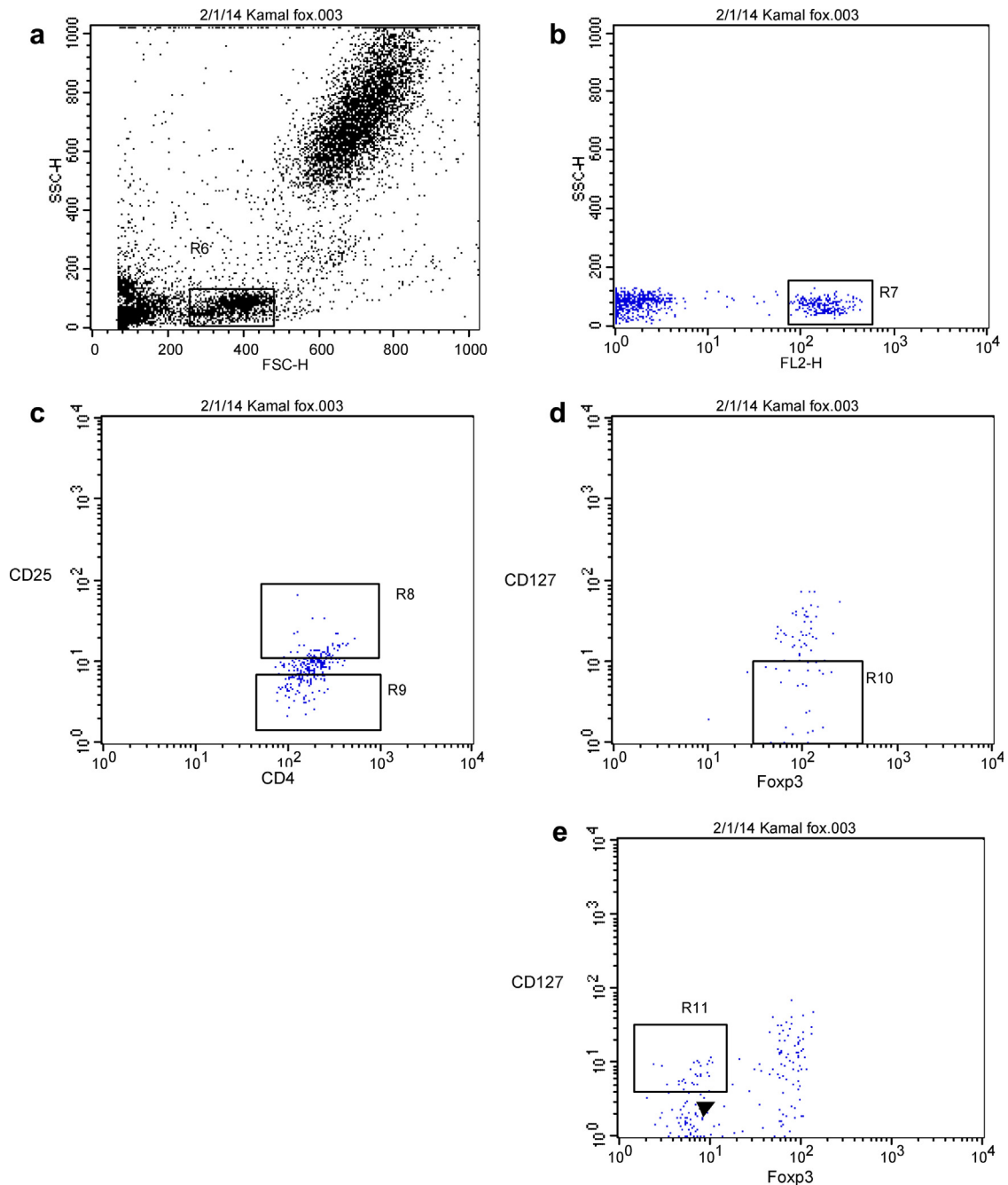


Fig. 1 – (a) Dot plot is showing PBMCs separated from the blood of patients are shown based on their forward scatter (FSC) and side scatter (SSC) properties. The lymphocytes are gated (R6) on the basis of their low FSC and SSC properties. (b) The representative dot plot is showing CD4+ and CD4– lymphocytes taken from gate R6. The high expressing CD4 cells or bright CD4+ are gated with R7 as a population of interest. (c) The dot plot is showing the CD4+ CD25^{med-high} cells (R8) and CD4 +CD25^{low} cells (R9). All cells on this plot were taken from CD4 bright cells (R7). (d) The dot plot is showing Foxp3^{high} and CD127^{low} cells (R10) taken from R8 gate. The R10 gated cells were CD4+CD25^{med-high}Foxp3^{high}CD127^{low} Treg cells. (e) The figure is showing Foxp3^{low} CD127^{med-high} (R11) cells which were taken from R9 gated cells. These cells were having properties of CD4+CD25^{low}Foxp3^{low} CD127^{med-high} and were Tcon cells.

or CD127 and intracellular Foxp3 in separate tubes. For each of the fluorochromes FITC, PE PerCP and APC, appropriate fluorescence minus one (FMO) and relevant isotype controls (Ebiosciences) were used to determine the nonspecific binding of monoclonal antibodies under study. An initial standardization of the technique was done on samples from control subjects. As a result of the differences, in the cell size and granularity, light scattering separates the blood cells into three major populations: lymphocytes, monocytes, and granulocytes. For each analysis, dot plot graphs of forward scatter versus side scatter were drawn, and a lymphocyte region was defined by placing a tight gate (R6) according to cell size and complexity, where debris and monocytes were excluded (Fig. 1a). CD4 T cells were gated by their low side scattered characteristics and also by the high expression of CD4+ (R7), thus excluding CD4 low monocytes and dead cells (Fig. 1b). CD4+ high expression cells were our population of interest to carry forward for further evaluation to narrow down to Treg cells and Tcon cells. Therefore, R8 and R9 gates were then displayed on dot plot to enumerate the percentage of cells, which were CD25^{med-high}CD4+ cells (R8) and CD25^{low} CD4+ cells (R9) (Fig. 1c). Data have been expressed as the percentage of cells, which were CD4+CD25^{med-high}Foxp3^{high}CD127^{low} cells (R10) (Fig. 1d) defined as Treg cells and cells, which were CD4+CD25^{low} Foxp3^{low} CD127^{high} (R11) [Fig. 1e] defined as conventional T cells (Tcon). The percentage of positive cells was recorded by carefully excluding all cellular debris, identifying the number of antibody positive cells. The results have been expressed as the values of the actual percentage of cells positive for CD4+ CD25^{med-high} Foxp3^{high}+CD127^{low} (Treg cells) or CD4+CD25^{low} Foxp3^{low} CD127^{med-high} (Tcon cells) in relation to CD4+ lymphocyte cells (Fig. 1a–e).

2.3. Statistical analysis

Results have been expressed as mean \pm SD for normally distributed data and as median with inter-quartile range for non-normal distributed data and frequencies as percentages. Statistical tests have been performed to find out significant difference if any in Treg and Tcon cell count among different groups. Unpaired Student's t-test has been performed to

compare mean between the patients (univalvular and multivalvular) and controls for normally distributed data and Mann–Whitney U test for skewed distribution. Chi-square/Fisher's exact test was applied for comparing the proportion of qualitative variable between the patients and controls. One-way ANOVA followed by Tukey's test for equal variances and Dunnett's T3 post-hoc tests for unequal variance was applied to compare the mean among the three groups for normally distributed variables; Levene's test was applied to find the homogeneity of variances among the groups. Kruskal–Wallis test followed by Mann–Whitney U test with Bonferroni adjustment was applied for not normally distributed data.

A Spearman correlation analysis was used to evaluate the correlation between ESR and CRP with circulating Treg and Tcon cell count. A p value of <0.05 was considered significant.

3. Results

The demographic profile and baseline characteristics of patients and control are listed in Table 1. There was no significant difference in age or sex ratio between patients of RHD versus controls or between the univalvular and multivalvular group.

In the univalvular group, 22 (71%) patients had severe mitral stenosis and 7 (23%) had moderate mitral stenosis. Mean mitral valve area by planimetry was 0.82 ± 0.15 cm² and Wilkins score was 6.8 ± 1.4 . 25 (71%) patients in this study group had associated mitral regurgitation, of which 12 patients (34%) had mild, 7 (20%) had moderate and 6 (17%) had severe mitral regurgitation. Mild aortic regurgitation was present in 8 (23%) patients. Secondary tricuspid and pulmonary regurgitation due to pulmonary artery hypertension were present in 28 (80%) and 5 (14%) patients, respectively (Table 2).

In the multivalvular group (n = 35), all patients had multivalvular involvement in the form of either moderate or severe mitral and aortic valve disease (Table 3). In this group, secondary tricuspid and pulmonary regurgitation was present in 33 (94%) and 6 (17%) of patients, respectively (Table 3).

The ESR and CRP levels were within normal limits in both the univalvular and multivalvular group with no significant

Table 1 – Baseline characteristics of study population.

Parameter	RHD group (n = 70)	Controls (n = 35)	p-value	Univalvular (n = 35)	Multivalvular (n = 35)	p-value
Age (years)	32.03	29.11 \pm 6.30	0.060	31.09 \pm 8.49	32.97 \pm 10.22	0.355
Gender ratio (M:F)	0.84:1	0.94:1	0.782	0.75:1	0.94:1	0.631
History of RF	34 (75)	0	–	14 (40)	20 (57)	0.185
NYHA class						
I	0	–	–	0	0	–
II	34 (48.6)	–	–	20 (57.1)	14 (40)	0.195
III	30 (42.9)	–	–	14 (40)	16 (45.7)	
IV	6 (8.6)	–	–	1 (2.9)	5 (14.3)	
Hb (g%)	12.56 \pm 1.7	12.87 \pm 1.9	0.379	12.45 \pm 1.9	12.6 \pm 1.5	0.652
ESR (mm/h)	–	–	–	20.37 \pm 21.8	15.74 \pm 11	0.268
CRP (μ g/ml)	–	–	–	2.0 [1.4–3.10]	2.60]	0.264

Values are represented in mean \pm SD, median [inter quartile range], and n (%).

NS, non-significant; RF, rheumatic fever; RHD, rheumatic heart disease. NYHA, New York heart Association; M:F, male:female.

Table 2 – Echocardiographic data of univalvular group.

Parameter	Mitral stenosis	Mitral regurgitation
Valve severity, n		
Absent	1 (3)	
Mild	1 (3)	12 (34)
Moderate	7 (23)	7 (20)
Severe	22 (71)	6 (17)
Wilkins score	6.8 ± 1.4	
Tricuspid regurgitation, n (%)	28 (80)	
Mild aortic regurgitation, n (%)	8 (23)	
Pulmonary regurgitation, n (%)	5 (14)	
PASP (mm Hg)	44.43 ± 20.5	
LA diameter (cm)	5.25 ± 1.38	
LV EF (%)	60 ± 1.64	

Values are mean ± SD.
Values in parentheses are percentages.
LVEF, left ventricular ejection fraction; PASP: pulmonary artery systolic pressure; LA, left atrial.

difference between the two groups (Table 1). While the total cell count was not statistically significant between patients and controls (Table 4), the absolute lymphocyte count (per mm³) was significantly lower in patients of RHD compared to controls (Table 4). The percentage of Tregs in CD4 lymphocytes was significantly lower in patients of RHD compared to controls ($p = 0.001$, Table 4).

The absolute lymphocyte count (per mm³) in the univalvular group was significantly lower compared to the

multivalvular group (2228 ± 816 versus 2662.4 ± 710, $p = 0.030$) or controls ($p = 0.0001$). But there was no significant difference in the absolute lymphocyte count between the multivalvular group and controls ($p = 0.189$). Further on subgroup analysis, only patients with multivalvular disease had significantly lower Treg cells compared to controls ($p = 0.001$; Table 5). The percentage of Treg cells in CD4 lymphocytes was not significantly different in patients with Univalvular versus multivalvular disease ($p = 0.32$) or those with Univalvular disease versus controls ($p = 0.1$).

There was no significant difference in the percentage of Tcon cells in patients with RHD compared to controls ($p = 0.94$). Similarly no difference in Tcon cells compared to controls was seen either in univalvular ($p = 0.84$) or multivalvular groups ($p = 1.0$), or between univalvular and multivalvular groups ($p = 0.34$) (Table 5).

There was no correlation of ESR or CRP with circulating Treg cells or Tcon cells in our study.

4. Discussion

The aim of our present study was to assess the level of circulating Tregs, in adult patients of chronic RHD and also assess the same in patients with extensive disease compared to limited disease.

There are no data available in world literature regarding the frequency of circulating Tregs cells in patients of RHD using the markers we have used to define regulatory cells to compare

Table 3 – Echocardiographic data of multivalvular group.

Parameter	Mitral stenosis	Mitral regurgitation	Aortic stenosis	Aortic regurgitation
Valve severity				
Absent, n (%)	3 (12)	5 (14)	15 (43)	5 (14)
Mild, n (%)	8 (32)	10 (28.6)	3 (9)	4 (11)
Moderate, n (%)	0	10 (28.6)	4 (11)	17 (49)
Severe, n (%)	14 (56)	10 (28.6)	13 (37)	9 (26)
Tricuspid regurgitation, n (%)	33 (94)			
Pulmonary regurgitation, n (%)	6 (17)			
PASP	35.6 ± 11.7			
LA diameter (cm)	4.9 ± 0.94			
LV EF (%)	60 ± 1.84			

Values are mean ± SD.
Values in parentheses are percentages.
LVEF, left ventricular ejection fraction; PASP, pulmonary artery systolic pressure; LA, left atrial.

Table 4 – Total cell count, absolute cell count and subpopulation of T cells in RHD patients and controls.

Parameter	RHD patients (n = 70)	Controls (n = 35)	p-value
TLC (per mm ³)	8245.9 ± 1815	8589 ± 1187	0.249
Absolute lymphocyte count (per mm ³)	2445.9 ± 790	2944.6 ± 552	0.001
CD4+CD25 ^{med-high} CD127 ^{low} (Regulatory cells)/CD4 cells (%)	0.60 [0.04–3.60]	3.2 [1.4–7.50]	0.001
CD4+CD25 ^{low} CD127 ^{med-high} (T conventional) cells/CD 4 cells (%)	0.10 [0.02–1.70]	0.20 [0.0–0.50]	0.948

Values are mean ± SD and median [IQR].
Values in parentheses are percentages.
TLC, total cell count.

Table 5 – Total cell count, absolute cell count and subpopulation of t cells according to valvular involvement.

Parameter	Univalvular	Multivalvular	Controls	Overall p-value	P ₁	P ₂	P ₃
TLC (per mm ³)	8160 ± 2249	8331.6 ± 1270	8589 ± 1187	0.518 ^{\$}	0.971	0.685	0.763
Absolute lymphocyte count (per mm ³)	2228 ± 816	2662.4 ± 710	2944.6 ± 552	0.0002 [†]	0.030	0.0001	0.189
CD4+CD25 ^{med-high} CD127 ^{low} (Regulatory cells)/CD4 cells (%)	1.9 [0.05–3.70]	0.10 [0.04–2.0]	3.2 [1.4–7.5]	0.001 [#]	0.38	0.100	0.0013
CD4+CD25 ^{low} CD127 ^{med-high} (T conventional) cells/CD4 cells (%)	0.07 [0.02–0.30]	0.30 [0.02–3.30]	0.20 [0.0–0.50]	0.220 [#]	0.342	0.843	1.000

Values are mean ± SD, median [IQR].
[†] One-way ANOVA with Tukey's test.
^{\$} Welch test with Dunnett's T3.
[#] Kruskal–Wallis (multiple comparison p-values are Bonferroni adjusted).
P₁ – p value between univalvular and multivalvular.
P₂ – p value between univalvular versus control.
P₃ – p value between multivalvular versus control.

our present results. But like our previous study, the level of circulating Tregs was significantly lower in our overall study population of RHD compared to controls. On subgroup analysis, though the frequency of circulating Tregs was lower than the control group, in both the univalvular and multivalvular group, it achieved statistical significance only in patients with multivalvular disease. But apart from quantitative deficiency, the circulating Tregs may also have been made dysfunctional by the streptococcal antigen as has been shown by *in vitro* studies¹⁶ or the effector cells may be resistant to the inhibitory effect of Tregs as has been shown in other autoimmune diseases like systemic lupus erythematosus.¹⁷ Hence, in our study, in spite of no significant difference in level of circulating Tregs in patients with univalvular (limited) disease compared to controls, functional deficiency of Tregs can explain the damage mediated by autoreactive effector cells in this subset of patients of our study. On the other hand, enhanced apoptosis of the circulating Tregs by proinflammatory cytokines like TNF alpha,¹⁸ which is also raised in patients of RHD,¹⁹ may be responsible for quantitative deficiency and more extensive damage in patients with multivalvular disease. To the best of our knowledge, this is the first study to systematically evaluate the level of Tregs in patients with univalvular and multivalvular disease.

At present, there is no therapeutic measure available to arrest or attenuate the progressive valvular damage by targeting the autoreactive CD4 cells directed against the valvular tissue. The CD4+ effector T cells mediate progressive valvular damage by two mechanisms (1) through regular reactivation precipitated by recurrent streptococcal sore throat [to prevent which intramuscular injection of benzathine penicillin (BPG) every 3 weeks is recommended, efficacy being limited by discrepancy in global supply and quality of BPG as well as low patient compliance to 3 weekly BPG injections].^{20,21} (2) By epitope spreading,^{22,23} a phenomenon where T cells at the site of the disease no longer recognize the original mimicking epitopes but rather recognize epitopes in other proteins of the target organ that continue to perpetuate the disease long after the initiating pathogen has been eliminated. This progressive adverse effect on valves due to either recurrent infections or epitope spreading mediated by

CD4 Effector cells can be effectively inhibited by Tregs as been shown experimentally.¹⁵

Treg cells inhibit Effector T cell function by direct cell to cell contact or by generation of suppressor cytokines like interleukins 10 (IL-10), IL-35, transforming growth factor B (TGF B) or by competing for growth factors like IL-2.^{24–29}

Selective expansion of Tregs by IL- 2 injection (thus restoring the imbalance between Treg and Effector cells) has been shown to arrest and even reverse the tissue damage in various organs in patients of hepatitis C virus induced vasculitis³⁰ and graft versus host disease¹⁴ Our study, using the latest markers to define Tregs, has conclusively shown the presence of Treg cell deficiency in patients of RHD. Hence, in future, studies are needed to see whether correction of Treg cell deficiency can attenuate or arrest the progress of autoimmune valve damage in RHD. This approach may become the future therapeutic modality for patients of RHD.

5. Limitation of the study

We could not assess the suppressive function of the Tregs due to lack of facilities. But from our study results, we feel that functional deficiency of the Tregs was primarily responsible for mediating damage in patients with limited disease. Hence, studies should be conducted to assess the functional deficiency of Tregs in patients of RHD.

6. Conclusion

There is significant deficiency of circulating Tregs in patients of chronic RHD and both qualitative (functional) and quantitative deficiency of the Tregs are responsible for autoimmune damage to the valves by autoreactive T cells.

Conflicts of interest

The authors have none to declare.

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