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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 patents 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.

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 CD4cells 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