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Circulating-Peripheral Blood Naturally Occurring CD4+CD25+ Regulatory T Cells and CD4+ T Cells in Chronic Rheumatic Heart Disease

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Abstract

Background The development of autoimmune disease involves a breakdown in the mechanisms that control T cell tolerance to self antigens, these mechanisms are many and complex, and they integrate as immunoregulation. Among the cells that might be responsible for this regulation is a specific type of T cells which has the ability to downregulate the differentiation of helper cells or antigen specific effector cells. The main subset of these suppressor T cells is the naturally occurring CD4+CD25+ regulatory T cells (n Tregs) which are the most important and they derived as a functionally mature population from the thymus.

Objective The purpose of this study was to determine the correlation between the numbers of circulating CD4+CD25+ regulatory T cells (nTregs) and CD4+ T cells in chronic rheumatic heart disease patients.

Methods Peripheral blood samples were taken from 48 Iraqi patients with chronic rheumatic heart disease (CRHD). Lymphocytes were isolated from the peripheral blood, nTregs and CD4+ T cells; also, cell numbers were detected by using immunofluorescence technique.

Results In general, nTregs were found in lower numbers in the peripheral blood of CRHD patients in different study groups than in healthy control group, whereas, CD4+ T cells were found in higher numbers in some of patients than controls. Also, our results revealed that there was a significant negative correlation between naturally occurring CD4+CD25+ regulatory T cells and CD4+ T cells in all study groups.

Conclusions Our finding confirmed that there is a significant correlation between circulating nTregs and CD4+ T cells in chronic rheumatic heart disease.

Key words CD4+CD25+ regulatory T cells, CD4+ T cells, chronic rheumatic heart disease.

Introduction

An organism's immune system is designed to maintain a balance between responsiveness to infectious agents and maintenance of self-tolerance. An unresponsive immune system may result in pathogen invasion; in contrast, a hypersensitive immune reaction may result in damaging inflammatory responses [(1)]. Thus, the development of autoimmune disease involves a breakdown in the mechanisms that control T cell tolerance to self-antigens [(2)]; these mechanisms are many and complex, and they collectively known as immuno-regulation.

Among the cells that might be responsible for this regulation are a specific type of T cells which has the ability to downregulate the differentiation of helper cells or antigen-specific effector cells [(3)]. The main subset of these suppressor T cells are the

naturally occurring CD4+CD25+ regulatory T cells (nTregs) which are the most important and they are derived as a functionally mature population from the thymus.

Other regulatory T cells are the type 1 regulatory (Tr1) cells and Th3 cells [4,5]. A better understanding of the mechanisms underlying the induction and functions of T regulatory cells in controlling the immune system is critical in view of a future cellular therapy to modulate immune-mediated pathologies. nTreg cells were first defined in 1995 by Sakaguchi and colleagues, who showed that the passive transfer of T cells lacking in the nTregs subset into a thymic nude mice resulted in the spontaneous development of various T cell-mediated autoimmune diseases. These cells appear to be capable of suppressing a wide variety of immune cells, consisting of those from both the innate and adaptive immune systems [6].

Rheumatic heart disease (RHD) is an autoimmune most severe sequel of group A streptococcal upper respiratory tract infection complicated by rheumatic fever (RF) [7]. It describes a group of acute (short-term) and chronic (long-term) heart disorders and many of its features in the chronic stage are a result of fibrosis occurring during the healing of the acute lesion [8]. Adaptive immune responses are characterized by the capacity to recognize and remember pathogen-specific antigens. When a cognate antigen is encountered, lymphocytes become activated, undergo clonal proliferation and acquire effector functions that enable the activated cells to eliminate the intruder. However, in the acute phase of streptococcal pharingitis, streptococcal antigens (especially M protein) act as the promoter of T cell activation. It is assumed that the streptococcal antigens are initially taken up and processed by antigen-presenting cells, predominantly macrophages, which present the antigens in the context of MHC II molecules to CD4-positive T cells [9]. CD4+ T cells are the major effectors of heart tissue lesions, and *Streptococcus*-primed T cells are able to recognize heart proteins by molecular mimicry. These T cells show a degenerate pattern of antigen recognition (streptococcal antigens and autoantigens) [10].

Naturally occurring CD4+CD25+ regulatory T cells, which comprise approximately 5-10% of peripheral CD4+ T cells, are a central component of active immune suppression [11] and populate the periphery as long-lived cells to control autoimmunity and regulate ongoing immune responses [12,13]. Here, in this study we try to determine the numbers of both nTregs and CD4+ T cells in the peripheral blood of chronic rheumatic heart disease patients to highlights the correlation between them.

Methods

This study was conducted from October 2006 to September 2007. Blood samples were taken from 48 patients with chronic rheumatic heart disease in Ibn Al-Bitar Hospital for Cardiac Surgery, Baghdad – Iraq. All patients were divided according to the positive or negative history of rheumatic fever (PHORF and NHORF), PHORF patients were subdivided according to the frequency of rheumatic fever, and according to the period of medication treatment into single attack under continuous medication (SA^{UCM}), single attack without continuous medication (SA^{WCM}), high risk under continuous medication (HR^{UCM}), and high risk without continuous medication (HR^{WCM}). Lymphocytes isolation was performed by using Ficoll method [14], nTregs and CD4+ T cells, also cell numbers were detected by using immunofluorescence staining technique [15] in the presence of Mouse anti- Human CD4 (FITC), CD25 (PE) Proteins, Dual Color (USBiological , USA)

and immuno-fluorescence microscopic slides (Biomerieux, USA). Fluorescence microscope was used with 40 X-magnification lenses at 490 nm to examine the slides immediately or 1-3 days later as a maximal duration. Detection of positive cells by observing a dot-like apple green fluorescent colored light (FITC) and red fluorescent colored light (PE) on the surface of nTregs. Whereas, CD4+ T cells were detected by observing only a dot-like apple green fluorescent colored light (FITC) on the surface of positive cells.

Statistical Analysis

The percentage of both nTregs and CD4+ T cells was measured by counting the number of positive cells/field in 5 to 10 microscopic fields to the total lymphocyte count as follows:

The percentage of positive cells = *the number of positive cells / the number of total cells X 100*.

The correlation coefficient (*r*) was calculated as a quantitative descriptive to the association between the mean percentage of CD4+ T cells and CD4+CD25+ nTreg cells among different study groups. All statistical analysis was performed with the SPSS 10.01 statistical package for social sciences and also Excel 2003. A *p* value of less than 0.05 ($p < 0.05$) was considered significant.

Results

The percent of naturally occurring CD4+CD25+ cells was evaluated by immunofluorescence staining technique and the results shown in (Table 1 & Figure 1) displayed the difference in the mean percentage among all groups under study. High risk groups (HR^{UCM} and HR^{WCM}) displayed lower CD4+CD25+ regulatory T cells expression than single attack groups. In HR^{WCM} patients, lower percent of nTregs was recorded (1.45%) when compared with SA^{WCM} group (1.56%) and this difference was also found between groups of continuous medication (HR^{UCM} and SA^{UCM}) (3.75% and 4.12% respectively). These results refer to the difference between groups of continuous medical care which displayed higher numbers of nTregs than patients of intermittent medical therapy.

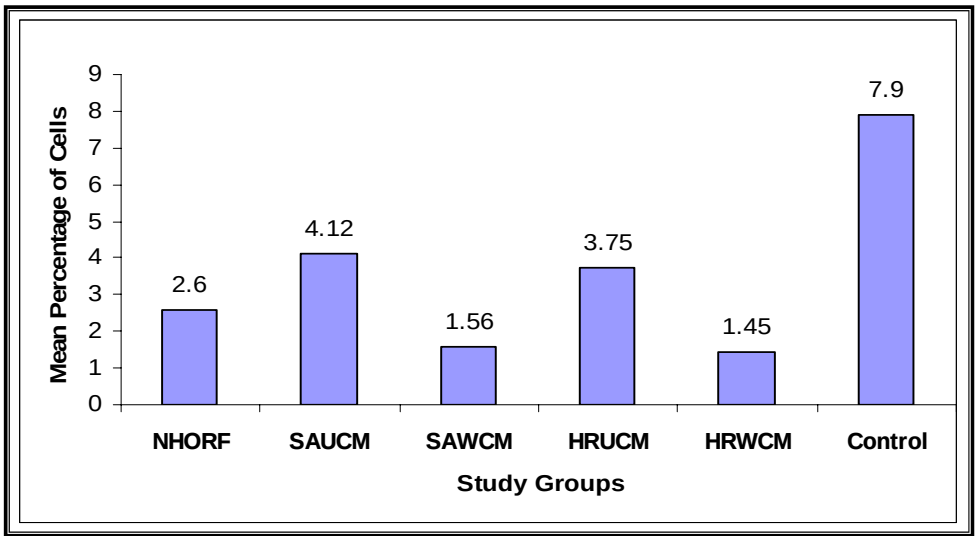

Figure1: Mean percentage of peripheral blood nTreg cells among different study groups

Negative history (NH) group had high mean percentage (2.6%) than both HR^{WCM} and SA^{WCM} groups. In general, CD4+ CD25+ nTregs were found in lower numbers in the

peripheral blood of CRHD patients in different study groups than in healthy control group (7.9%).

Table 1: Comparison in the mean percentage of peripheral blood CD4+CD25+ regulatory T cells and CD4+ T cells among different study population groups

Group type		Patients		Lymph No.	CD4+ & nTregs	No.	Mean percent ± SD*	Min.*	Max.*
		No.	(%)						
NHORF		14	20.59	43.79	CD4+	23.43	53.16±4.710	46.42	59.57
					nTregs	0.59	2.6±0.718	1.42	4.61
PHORF	SA	5	7.35	38.8	CD4+	19.4	49.75±3.707	43.33	52.38
					nTregs	0.8	4.12±0.389	3.64	4.62
		18	26.47	48.11	CD4+	27.28	56.11±5.706	45.45	65.22
					nTregs	0.41	1.56±0.398	1	2
	HR	4	5.88	37.25	CD4+	19.5	51.72±5.536	44.82	57.5
					nTregs	0.7	3.75±0.954	2.6	4.62
		7	10.29	57.86	CD4+	38.14	65.89±4.298	63.33	68.33
					nTregs	0.54	1.45±0.539	1	2.1
Control		20	29.41	31.25	CD4+	15.55	49.82±2.721	45.83	55.55
					nTregs	1.24	7.9±1.303	5.71	10

*SD = Standard Deviation,*Min. = Minimum, *Max. = Maximum.

In this study, approximately one-half of the circulating apparently healthy human peripheral blood lymphcytes express CD4 (49.82%), and we showed that CD4+ T cells were found in higher numbers in the peripheral blood of some CRHD patients than in controls (Figure 2), and, there was a difference in the mean percentage of CD4+ T cells expression among different study groups (see table 1). From the total lymphocyte count, CD4+ T cells represents (65.89%) in HR^{WCM} group which was higher than that in SA^{WCM} patients (56.11%). Lower numbers were recorded in groups under continuous medication (UCM); HR^{UCM} and SA^{UCM} (51.72 and 49.75% respectively) when compared with patients without continuous medication (WCM). CD4 expression in patients with negative history was 53.16%, which is considered higher than that in SA^{WCM} patients but lower than HR^{WCM} group. Some of CRHD patients had normal CD4+ T cells numbers and we found that the total lymphocyte count and CD4+ T cells percentage in patient number (7-negative history), (3, 4-SA^{UCM}), (1, 4, 6-SA^{WCM}), and (1, 4-HR^{UCM}) were (28 and 46.42%), (30, 43.33%; 21, 52.38%), (22, 45.45%; 37, 54.05%; 32, 62.5%), and (29, 44.82%; 36, 50%) respectively.

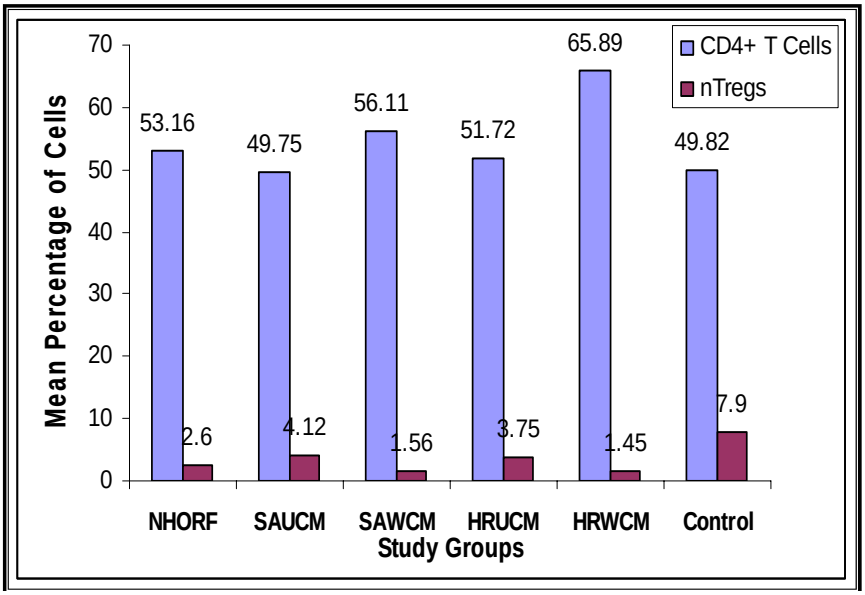

Figure 2: Mean percentage of peripheral blood CD4+CD25+ nTregs and CD4+ T cells among different study groups

According to the results above, spearman's correlation test revealed a significant negative correlation between naturally occurring CD4+CD25+ regulatory T cells and CD4+ T cells in all study groups. The groups SA^{UCM}, SA^{WCM}, and HR^{UCM} exhibited high significant difference in the mean percentage of these cells ($p < 0.05$). Also there was a highly significant negative correlation between nTregs and CD4+ T cells ($p < 0.01$) in both negative history group and patients of HRWCM (Table 2). Figure 3 shows immunofluorescence staining (dual color) of CD4+CD25+ nTreg and CD4+ T cells in the peripheral blood of CRHD patients.

Table 2: The difference in the mean percentage of nTregs and CD4+ T cells expression among different study groups

Group type		Patients		Correlation Coefficient (r =)	*P* value
		No.	(%)		
NHORF		14	20.59	- 0.831	$P < 0.01$ **
PHORF	SA^{UCM}	5	7.35	- 0.921	$P < 0.05$ *
	SA^{WCM}	18	26.47	- 0.483	$P < 0.05$ *
	HR^{UCM}	4	5.88	- 0.973	$P < 0.05$ *
	HR^{WCM}	7	10.29	- 0.892	$P < 0.01$ **

*Significant, **Highly significant.

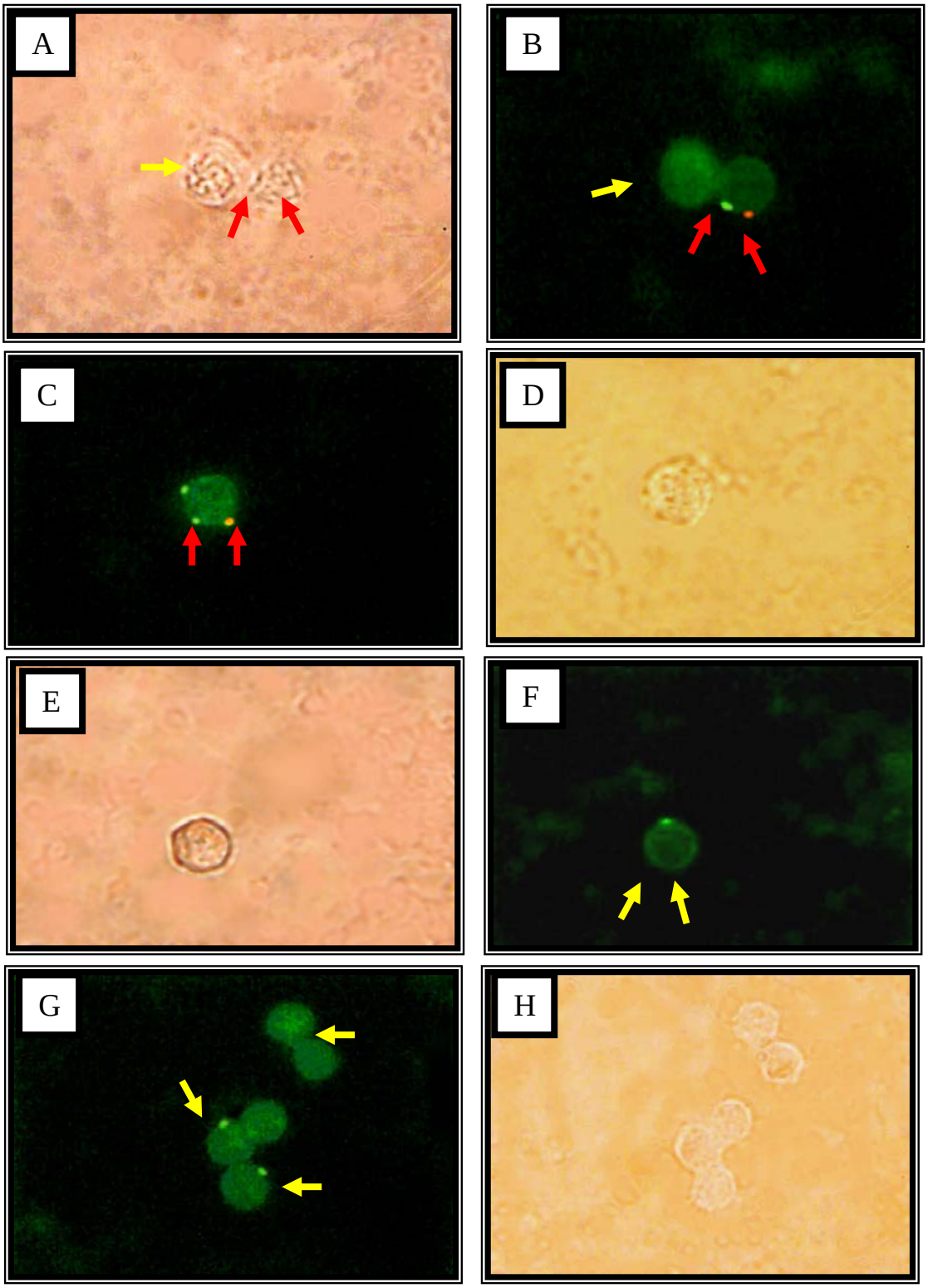

Figure 3: Immunofluoresence staining for circulating human CD4+CD25+ nTregs and CD4+ T cells in chronic rheumatic heart disease patients using mouse anti-human CD4- (FITC), CD25 (PE) proteins, dual color. A & B show CD4+ CD25+ nTregs (red arrows) and CD4+ T cells (yellow arrows). C & D indicate CD4+ CD25+ nTreg cells. E, F, G, & H show CD4+ T cells. Pictures in black color are under UV light of the immunofluoresent microscope, whereas, light pictures are under the light microscope. Microscopic magnification power: X1000.

Discussion

CD4+CD25+ nTregs represent a population of T cells that are highly specialized for suppression of immune responses and are known to play a critical role in the maintenance of peripheral self-tolerance. Approximately one-half of the circulating human peripheral blood lymphocytes express CD4, and of these roughly 10% express the IL-2 growth factor receptor α-chain, CD25, and only those which express high levels of CD25 exhibit suppressive activity in vitro,[16] but, why nTregs cannot eradicate the massive autoimmune response in vivo?

In the present work, circulating CD4+CD25+ regulatory T cells appear in lower numbers in patients with chronic rheumatic carditis than normal persons suggesting an important role for these regulatory T cells in controlling a post-infectious autoimmune disease. Our results (Table 1) show that HR^{WCM} had displayed low prevalence of nTreg cells (1.45%) than SA^{WCM}, negative history and groups of medical care, whereas, patients of SA^{UCM} group had recorded high CD4+CD25+ nTreg cell numbers (4.12%) when compared with HR^{UCM} and patients without continuous medication. Continuous inflammatory state during acute and chronic rheumatic carditis in addition to recurrent attacks of acute rheumatic fever due to exposure to a group A *Streptococcus pyogenes* antigens may lead to reduce frequency of the CD4+CD25+ nTreg cells in the peripheral blood of patients which may occur as a result of an active recruitment of regulatory T cells from circulation to the site of inflammation as a strategy of the immune system to fight an ongoing inflammation. This is consistent with the findings in animal models of chronic inflammatory colitis [17]. A previous study showed that patients with chromosome 22q11.2 deletion syndrome with developmental thymic hypoplasia had markedly fewer CD4+ CD25+ nTreg cells in infancy. That study suggested that patients with chromosome 22q11.2 deletion syndrome had a relatively pure quantitative defect in T-cell production and inclusively CD4+CD25+ nTreg cells which found in fewer numbers in infants, in addition to that, the study suggested that regulation of nTreg-cell production early in life, in humans, is directly related to thymic capacity, and this phenomenon could play a role in the predisposition to autoimmune disease in patients with chromosome 22q11.2 deletion syndrome [18] which may be related with the lower numbers of nTregs in the present CRHD patients if this syndrome is considered one of the causes of autoimmunity in our study.

Nevertheless, we found some of patients had nTregs numbers near to the normal values as in patients with continuous medication, patients number 1, 2, and 3-SA^{UCM} who had 4.17, 4.34, and 4.62 % , and patients number 1, and 4-HR^{UCM} who had 4.62, and 4.44% respectively, and also patient number 7-negative history who had 4.61% of nTregs.

Therefore, many factors may interfere with nTregs function to make them inactive and abrogate their suppressive function. One of these factors believed to play an important role in inhibiting the functional activity of nTreg cells is the tumor necrosis factor alpha. Also, toll-like receptors (TLRs) are primary sensors of both innate and adaptive immune systems and play a pivotal role in the response against structurally conserved components of pathogens. Many researchers found that toll-like receptor-2 signaling in T cells had distinct effects on effectors and nTreg cells. In addition to that, they showed that bacterial lipoprotein (BLP), together with anti-CD3 antibody [T cell receptor (TCR) activation], induced proliferation of both CD4+CD25+ nTregs and CD4+CD25-

(effector) T cells in the absence of antigen-presenting cells. The expanded nTregs showed a transient loss of suppressive activity and suppression of the induction of Foxp3 mRNA in Tregs at the first 8-15 hours after T cell receptor activation [19]. One explanation for the infection-dependent induction of TLR2 might be related to the presence of Gram-positive *Streptococcus pyogenes* [20], therefore, the presence of streptococcal M protein which is known to bind with TLR-2 may be play an important role in loss of naturally occurring CD4+CD25+ nTreg cells their suppressive function.

According to our results, there was no correlation between disease severity and the low prevalence of CD4+CD25+ nTreg cells when compared with patient's clinical and physical parameters because the severity of disease may return to more than one factor that affect the disease activity, among them, number of acute rheumatic fever attacks, duration of disease, treatment, host susceptibility to suppressed autoimmunity, and the host susceptibility to the formation of fibrosis after tissue damage. Peripheral blood CD4+ T cells appeared in high numbers in CRHD patients when compared with controls. For several factors that make patients of HR^{WCM} group more exposed to group A streptococci infection and recurrent attacks of acute rheumatic fever, HR^{WCM} group displayed the highest percentage of CD4+ T cells (65.89%) than all groups under study which confirm the continuous inflammatory state even in the chronic stage of RHD. No significant difference was recorded in the mean percentage of CD4+ T cells among negative history, SA^{WCM}, HR^{WCM}, and also in SA^{UCM}, and HR^{UCM} groups when compared with others. The main cause of increasing the number of CD4+ T cells in some of patients with continuous medical therapy [SA^{UCM}-patient number 1: 51.06% , 2: 50% , 4: 52.38% and 5: 52%] and [HR^{UCM}-patient number 2: 57.5 %, 3: 54.54%, and 4: 50 %] may be referred to the bacterial resistance for penicillin. Long-term management is known to involve regular penicillin prophylaxis in high-risk patients, to prevent further episodes of rheumatic fever [21]. *Streptococcus pyogenes* has been shown to be resistant to penicillin, but because penicillin is inexpensive and available in most countries, it remains the drug of choice for treating group A streptococcal infections [22,23]. Also, other study found that streptococci are becoming increasingly resistant to penicillin and other β-lactams, owing to a decreased β-lactams affinity of their membrane-bound penicillin binding proteins [24] .Thus, more attacks of acute rheumatic fever due to penicillin resistant *Streptococcus pyogenes* A bacteria will affect the heart and lead to increase in the inflammatory response in which CD4+ T cells play the major role. Although CD4+ T cells displayed high numbers in some of patients with medical care, but this study found that other patients had lower values nearest to the normal range [SA^{UCM}-patient number 3: 43.33 %], and [HR^{UCM}-patient number 1: 44.82%], and this result may confirm the immunosuppressive role of naturally occurring CD4+CD25+ nTreg cells (which was found in high numbers in these medical care groups) against autoreactive CD4+ T cells.

In this regard, there was a significant correlation between CD4+CD25+ nTreg cells and CD4+ T cells expression in SA^{UCM}, SA^{WCM}, HR^{UCM} groups ($p< 0.05$), and highly significant correlation was recorded in the negative history and HR^{WCM} patients ($p< 0.01$). These results reveal the important role of CD4+CD25+ nTreg cells in autoreactive CD4+ T cells suppression leading to reverse the autoimmune reaction against the heart. At the same time these results strongly explain the role of CD4+ T cells in increasing the severity of

disease which is considered the main enhancer of acute and chronic rheumatic heart damage.

CD4+CD25+ regulatory T cells which has a critical role in immune suppression and reversing the autoimmunity were found in very lower numbers, and this may explain to a certain degree why the autoimmune-inflammatory process still active in rheumatic heart disease.

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