Functional and Developmental Analysis of CD4+CD25+ Regulatory T Cells Under the Influence of Streptococcal M Protein in Rheumatic Heart Disease

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ABSTRUCT:

BACKGROUND:

CD4+CD25+ regulatory T cells are known to suppress the immune response in general, these cells were studied in the presence of streptococcal M protein which has an important role in the pathogenesis of rheumatic heart disease.

OBJECTIVE:

The purpose of this study was to determine the role of streptococcal M protein in naturally occurring CD4+CD25+ regulatory T cells (nTregs) function and development in rheumatic heart disease Iraqi patients.

METHODS:

Streptococcus pyogenes was isolated for subsequent M protein extraction. Also, peripheral blood nTregs and CD4+ T cells were isolated by using Magnetic Cell Separation System (MACS). Tissue culture system for isolated cells was performed in the presence and absence of M protein stimulation. Cell count was performed, also, TNF-α, and IL-4 were determined in culture supernatant using ELISA system.

RESULTS:

It was found a highly significant positive association between the number of the cellular proliferation for both nTregs and CD4+ T cells with or without streptococcal M protein stimulation in isolated cell culture systems ($p < 0.01$), but, there found a highly significant negative correlation between the mean number of nTregs and CD4+ T cells in mixed culture system in the absence of M protein ($r = -0.995$). whereas, in the presence of M protein, there was a positive non-significant association between the mean number of both nTregs and CD4+ T cells ($r = 0.353$) ($p > 0.05$). Results obtained from ELISA test revealed that M protein-stimulated CD4+ T cells produced IL-4 in very little amounts (< 4 pg/ml) in all cultures of samples and there was no significant difference among them. Whereas, TNF-α was produced in higher concentrations in the culture supernatants when compared with IL-4.

CONCLUSION:

Streptococcal M protein has an important role in increasing the proliferation of both CD4+CD25+ regulatory T cells and CD4+ T cells, but the newer generation of CD4+CD25+ regulatory T cells in the presence of M protein has lower suppressive activity against CD4+ T cells.

KEYWORDS: CD4+CD25+ regulatory T cells, CD4+ T cells, magnetic cell separation system, Streptococcus pyogenes, M protein.

INTRODUCTION:

Self-reactive lymphocytes can be dangerous to the body when these lymphocytes attack self and cause autoimmune disease under certain conditions. Hence, the immune system has evolved several mechanisms to prevent this from occurring. The most well-documented mechanisms are the so-called clonal deletion in the thymus, anergy and ignorance. There is evidence that self-reactive T-cells are dominantly suppressed by newly identified T-cells subpopulations called regulatory T-cells, among which naturally occurring CD4+CD25+ regulatory T cells (nTregs) have been the best characterized. nTregs have been the object of intense of this study because their function appears critical in maintaining self tolerance and preventing autoimmune disease. This is illustrated by the finding that their removal leads

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to spontaneous development of autoimmune disease, such as autoimmune gastritis, thyroiditis, and type 1 diabetes and their reconstitution prevents it [1,2,3].

Rheumatic heart disease (RHD) is the most common cardiovascular disease affecting children and young adults in the world and till now it is considered a major public health problem causing about 400000 deaths annually worldwide especially in developing countries. Molecular mimicry between heart tissue proteins and streptococcal antigens, mainly the M protein which is the major component of the streptococcal cell surface and the most important virulence factor, has been proposed as the triggering factor leading to autoimmunity in RHD patients.[4,5,6]

M-protein is one of the best-defined determinants of bacterial virulence with more than 80 serotypes identified. The streptococcal M-protein extends from the surface of the streptococcal cell as an alpha–helical coiled coil dimmer which appears as fibrils on the surface of group A streptococci, and shares structural homology with cardiac myosin and other alpha-helical coiled coil molecules, such as tropomyosin, keratin and laminin. It has been suggested that this homology is responsible for the pathological findings in acute rheumatic carditis.[7]

METHODS:

This study was conducted from October 2006 to September 2007. During the study period, seven blood samples were taken from patients with chronic rheumatic heart disease which were selected from Ibn Al-Bitar Hospital for Cardiac Surgery, and twenty throat swab samples were taken from patients with tonsillitis in AL-Kadhimya Teaching Hospital \ Iraq \ Baghdad. nTregs and CD4+ T cells were isolated from the blood samples by using Magnetic Cell Separation System (MACS) in the presence of nTregs and CD4+ T cells isolation kits (Miltenyi Biotec GmbH, Germany, 2006). Pure colonies of β-hemolytic ***Streptococcus pyogenes*** were isolated from throat swab samples,[8] and M protein was extracted by using nitrous acid,[9] and the extracted M protein was determined by using Lowry Method.[10] Culture systems were performed by using RPMI-1640 with L-glutamine (USBiological, USA), fetal calf serum 10%, and lymphocult -T- HP (Human IL-2) was added to a final concentration of 50 IU/ml.

Isolated peripheral blood naturally occurring CD4+CD25+ regulatory T cells and CD4+ T cells were cultured in isolated and mixed cell culture systems by adding about $2X10^5$/100 µl of cell suspension for each type with and without the addition of extracted streptococcal M protein (5µg/ml) into 10 ml of culturing media in sterilized falcon containers under full sterilized conditions, then, incubated for 7 days at 37ºC in a humidified CO_2 incubator. Before and during the incubation period at day (0, 3, 5, and 7), cells number was detected by using immunoflouresence technique, and viability were detected by using trypan blue stain, then the cell culture suspension was centrifuged at 3000 rpm for 10 minutes. The supernatant for each culture type of each patient was collected in a sterile tube and stored at -20°C for later ELISA TNF-α, and IL-4 cytokines determination test which performed by using human TNF-α, and IL-4 EASIA Kits (BioSource Europe, S.A.).

Statistical Analysis: The statistical analysis was performed by using SPSS 10.01 statistical package for social sciences and also Excell 2003. A *p* value of less than 0.05 ($p < 0.05$) was considered the level of significant.

RESULTS:

In isolated cell cultures, rho values have shown highly significant positive association ($p < 0.01$) between the number of the cellular proliferation for both nTregs and CD4+ T cells with or without M protein stimulation which recorded by immunoflouresence technique through (0-7) days of incubation period at 37°C (Table 1), (Figure1). These results revealed the important role of M protein in enhancing the proliferation of nTregs and CD4+ T cells. However, there was a highly significant negative correlation between the mean number of nTregs and CD4+ T cells in mixed culture system in the absence of M protein ($r = -0.995$) (Figure 2), whereas, a different picture in the presence of M protein which showed a positive non-significant association between the mean number of both nTregs and CD4+ T cells ($r = 0.353$) ($p > 0.05$).

Results obtained from ELISA test (OD value) revealed that M protein- stimulated CD4+ T cells were produced IL-4 in very little amounts and mostly not produced**.** In other words, IL-4 was found < 4 pg/ml in all cultures of samples and there was no significant difference among them (table 2). On the other hand, TNF-α was produced in higher concentrations in the culture supernatants when compared with IL-4. Cultures of patients number 1, 4, 6, and 7, which incubated with nTregs exhibited lower TNF-α

concentrations than non-exposed once, and this strongly reinforce the inhibitory role of nTregs against CD4+ T cells, but patient number 2, and 5 were recorded high TNF-α concentrations in the presence of nTregs (288.790 pg/ml and 742.889 pg/ml respectively) when compared with cultures not exposed to the suppressive effect of nTregs, and highly significant positive association ($p < 0.01$) was found between them. \ Also, TNF-α appeared in lower concentrations (4.556 pg/ml) in CD4+ T cell culture in spite of stimulation with streptococcal M protein.

Table 1: Comparison in the mean percentage of nTregs and CD4+ T cells through (0-7) days of culture incubation period.

I.P. /day	MP stimulation status	Culture type								
		CD4+ T cells \ field No.	nTregs / field No.	Total	CD4+ T cells / nTreg cells					
					CD4+		nTreg		Total	
					No.	(%)	No.	(%)	No.	(%)
0	WM	30	30	60	30	50.00	30	50.00	60	100
	ØM	30	30	60	30	50.00	30	50.00	60	100
3	WM	61	32	93	34	51.52	32	48.48	66	100
	ØM	45	31	73	32	50.79	31	49.21	63	100
5	WM	86	61	145	24	32.88	49	67.12	73	100
	ØM	71	53	124	17	26.56	47	73.44	64	100
7	WM	113	72	179	40	39.22	62	60.78	102	100
	ØM	89	61	143	6	9.23	60	92.31	65	100

I.P. = Incubation period, MP = M Protein.
WM = With M Protein, ØM = Without M Protein.

Table 2: TNF-α and IL-4 concentration level in culture supernatant in different M protein- stimulated culture type.

Patient's No.	Culture type			
	CD4+ T Cells/ MP		CD4+ T Cells/ MP/nTregs	
	TNF-α pg/ml.	IL-4 pg./ml.	TNF-α pg./ml.	IL-4 pg./ml
1	422.556	< 4	152.678	< 4
2	4.556	< 4	288.790	< 4
3	809.772	< 4	879.001	< 4
4	280.576	< 4	51.767	< 4
5	517.600	< 4	742.889	< 4
6	665.446	< 4	167.932	< 4
7	441.330	< 4	25.952	< 4

MP = M Protein.

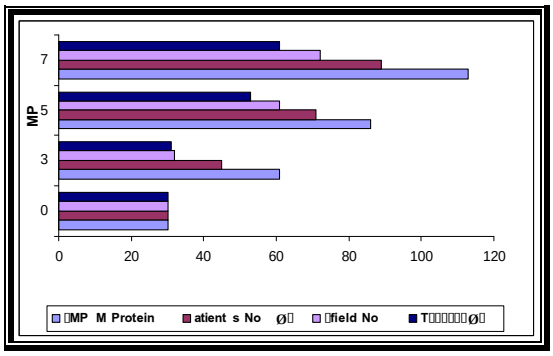

Figure 1:Comparison in the mean number of nTregs and CD4+ T cells with the time of incubation period in the presence/ absence of M protein in isolated cell culture system.

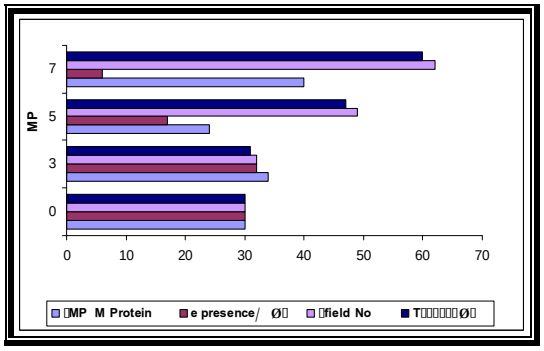

Figure 2:Comparison in the mean number of nTregs and CD4+ T cells with the time of incubation period in the presence/ absence of M protein in mixed cell culture system.

DISCUSSION:

Here, this study describes the proliferation and functional suppression of the human nTregs against streptococcal M protein-activated CD4+ T cells from peripheral blood of chronic rheumatic heart disease patients. The mechanism of action by which nTregs so effectively inhibit proliferation of CD4+ T cells remains unknown. M protein, the major virulence factor of Streptococcus pyogenes group A bacteria that extends from the cell wall membrane displayed a major important role in T cell activation and our results (Table 1), (Figure 1), and (Figure 2) revealed that streptococcal M protein has the ability to activate the proliferation of both CD4+ T cells and nTreg cells at various degrees which may depends on the type of peptide protein which binds with TCR. Previous studies observed that M protein-specific T-cell clones generated from peripheral blood of RHD patients and healthy individuals were cross-reactive with heart proteins. As a result of activation, CD4+ T cells begin to secrete cytokines. Also, these studies showed that the isolation and culturing of nTregs from human peripheral blood appears that these nTregs are hyporesponsive to TCR engagement yet are able to totally inhibit cytokine secretion by co-cultured CD4+ T cells activity, and TCR stimulation is required to induce suppressor function, however, freshly isolated nTregs do not manifest suppression. Several studies suggest that the suppression mediated by nTregs appears to be linked in part to the strength of signal delivered through the TCR[(1)]. In this study the immunoflourescence staining of CD4+CD25+ nTreg cells exhibit significant increases in their numbers when culturing with M protein-cross-reactive CD4+ T cells. Earlier studies reported that 40% of ARF patients and 46% of patients with inactive valvular lesions have proliferative responses to heart homogenates[(12)]. RHD patients may have a higher precursor frequency of heart-reactive T-cells or a pool of memory T cells capable of recognizing specific heart autoantigens. This pool may be further expanded following re-exposure to streptococcal antigens. Alternatively, the enhanced cellular recognition of myocardial antigens following stimulation with opsonized bacteria may be mediated by streptococcal superantigens, including M proteins [(13)] that

stimulate T cells based on the Vβ type and regardless of the antigenic specificity of the T cell receptor. It has been suggested that, through this mode of stimulation, superantigens can potentially activate autoreactive T cells. The link between the stimulation with streptococcal M protein and the development of T cells with the capacity to kill myocardial cell lines have been reported [14,15]. In addition, generated T cell lines to different M protein peptides and demonstrated that several of these clones also recognize heart proteins.[11,15] Also, other study reported that T cell clones derived from heart biopsies from RHD patients recognized M5 protein-derived synthetic peptides. These seemingly conflicting findings raise the possibility that more than one antigen of GAS and/or more than one cross-reactive heart autoantigen is involved in the pathogenesis of RHD.[16] Therefore, further studies examining cellular and humoral immune responses of RHD patients toward specific heart proteins before and after stimulation with specific antigens derived from rheumatogenic strains of GAS will undoubtedly shed light on the mechanism of pathogenesis of this disease.

This study displayed that there was very little or no secretion of IL-4 from CD4+ T cells and correlated with low suppressive activity of nTregs and high TNF-α level in culture supernatant (Table 2). Thus, our results revealed that the impairment of nTreg cells function and the development of heart damage in RHD patients may occur by two pathways, first, TNF-α can inhibit nTreg cells through TNFRII during the acute stage of rheumatic carditis and in the recurrent inflammatory attacks during the chronic stage and second, the low amount of IL-4 in chronic rheumatic carditis will alter the nTreg cells. The modulatory effects of interleukin 4 (IL-4) on the function of nTregs has been explored in many studies. IL-4 was shown to prevent the spontaneous apoptosis and the decline of Foxp3 mRNA which were found to occur during culture of isolated nTregs. It has been reported that IL-4 prevents death of resting T cells. nTregs are known to rapidly die in vitro, and to test whether IL-4 could also preserve viability of nTregs, CD4+CD25+ T cells were incubated in medium with or without IL-4, the percentage of living cells was significantly higher for Tregs incubated with IL-4 compared to nTregs incubated in medium alone. nTregs exposed to IL-4 were more potent in suppression the proliferation of naïve CD4+ T cells and they better inhibited IFN-γ production by CD4+ T cells as compared to nTregs cultured in medium. IL-4 also enhanced membrane IL-2Rα (CD25) expression on nTregs above the levels observed on freshly isolated cells. IL-4-mediated effects on nTregs function persisted in Tregs from Stat6 deficient mice, pointing to a Stat6-independent intracellular transduction pathway. Other studies also suggest that the anti-inflammatory function of IL-4 could partly be mediated by effects on nTregs function. Therefore, any reduction in the production of this cytokine may affect the suppressive function of nTregs against CD4+ T cells.[17,18,19] Thus, more damage will affect the heart valves due to the lack of nTreg cells at the inflammatory sites in the heart tissues. Therefore, autoimmune rheumatic carditis process will depend to a great degree on cellular immunity rather than humoral immune response and very important role for nTreg cells in reversing this autoimmunity will tack place to further future directions towards the prevention of rheumatic carditis. Interestingly, T cells derived from mitral valve lesions did not produce IL-4 when stimulated by cross-reactive M peptides and heart tissue proteins. These results suggest that mimicry between streptococcal antigen and heart tissue proteins, combined with high inflammatory cytokine and low IL-4 production, leads to the development of autoimmune reactions, causing cardiac tissue damage in RHD patients.(20)

CONCLUSION:

Our finding confirmed that CD4+CD25+ nTreg cells play an important role in regulation the autoimmune inflammatory response against the heart, and also this study revealed that streptococcal M protein has the ability to activate both of CD4+ T cells and CD4+CD25+ nTreg cells which enhance these cells to proliferate and produce TNF-α from CD4+ T cells, but it was found that M protein has an inhibitory effect on expanded CD4+CD25+ nTreg cells function, as a result, CD4+CD25+ nTreg cells become unable to suppress the autoreactive CD4+ T cells which play the major role in the development of rheumatic heart damage, and this result may reinforce the role of streptococcal M protein in the pathogenesis of ARF and RHD.

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