IJMS
Vol 36, No 2, June 2011

Brief Report

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

Nidhal Abdul-Auhaimen,
Zaman I. L. Al-Kaabi

Abstract

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 in Iraqi patients. 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. Tissue culture for isolated cells was performed in the presence and absence of M protein. Cell count was performed, and tumor necrosis factor alpha (TNF-α) and interleukin-4 (IL-4) were determined in culture supernatant using ELISA system. There was a significant positive correlation ($P<0.01$) between the number of proliferated nTregs and $CD4^+$ T cells in the presence as well as the absence of streptococcal M protein. Moreover, there was a significant negative correlation between the mean number of nTregs and $CD4^+$ T cells in mixed culture system in the absence of M protein ($r=-0.995$). There was also a positive, but not significant ($P>0.05$), association ($r=0.353$) between the mean number of nTregs and $CD4^+$ T cells in the presence of M protein. The M protein stimulated $CD4^+$ T cells to produce IL-4 in very little amount (<4 pg/ml) in all samples. Compared to the production of IL4, TNF-α was produced in higher concentrations in the culture supernatants. The findings of the study indicate that streptococcal M protein has an important role in increasing the proliferation of $CD4^+CD25^+$regulatory T cells and $CD4^+$ T cells. However, $CD4^+CD25^+$ regulatory T cells have lower suppressive activity against $CD4^+$ T cells in the presence of M protein.

Iran J Med Sci 2011; 36(2): 122-127.

Keywords ● Rheumatic heart disease ● $CD4^+CD25^+$ regulatory T cells ● M protein

Department of Microbiology,
Alnahrain College of Medicine
P.O. Box 12444,
Baghdad, Iraq.

Correspondence:
Nidhal Abdul-Auhaimen MSc, PhD,
Department of Microbiology,
Alnahrain College of Medicine
P.O. Box 12444,
Baghdad, Iraq.
Email: dr.nidhalmohammed@yahoo.com
Received: 16 May 2010
Revised: 18 November 2010
Accepted: 23 January 2011

Introduction

Self-reactive lymphocytes can be dangerous to the body when they attack themselves and cause autoimmune diseases 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-cell subpopulations called regulatory T cells (Treg), which play important roles in the maintenance of immune homeostasis and the control of adaptive immune response.[1] Among the reg cells, naturally occurring $CD4^+$ and $CD25^+$ regulatory T cells (nTregs) have been best characterized. Many subsets of Treg cells have been described that can suppress T cell response via different mechanisms. Recent studies showed that $CD69^+$, $CD4^+CD25^-$ T cells are a new subset of $CD4^+$ Treg cells.[2] Naturally occurring Tregs have been the object 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 to spontaneous development of autoimmune disease, such as autoimmune gastritis, thyroiditis, and diabetes type one and their reconstitution prevents such diseases.[3-5]

Rheumatic heart disease (RHD) is the most common cardiovascular disease affecting children and young adults in the world. It is considered a major public health problem causing about 400,000 deaths annually worldwide, especially in developing countries. Molecular mimicry between heart tissue proteins and streptococcal antigens such as the M protein has shown that this protein, which is the major component of the streptococcal cell surface and the most important virulence factor, may act as the triggering factor leading to autoimmunity in RHD patients.[6-8]

M-protein, with more than 80 serotypes being identified, is one of the best-defined determinants of bacterial virulence. The streptococcal M-protein extends from the surface of the streptococcal cells 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 myocarditis.[9] Therefore, the present study was designed to determine the role of M protein extracted from streptococcus in $CD4^+CD25^+$ regulatory T cells (nTregs) and $CD4^+$ T cell activations.

Materials and methods

Seven blood samples were obtained from patients with chronic rheumatic heart disease, who were candidates for cardiac surgery in Ibn Al-Bitar Hospital Baghdad, Iraq. The samples were used for lymphocytes separation using Ficoll (Biochrom) density gradient centrifugation.

T-Cell Separation

T-lymphocyte cells ($CD4^+$) were purified with anti-CD4 magnetic beads and Detach-a-Bead Abs according to the manufacturer's instructions. $CD4^+CD25^+$ cells were purified by MACS (Miltenyi Biotec Miltenyi Biotec GmbH, Germany, 2006). Purity of sorted cells was >97%.

Streptococcus Pyogenes Group a Isolation

Twenty throat swab samples were obtained from patients with tonsillitis in AL-Kadhimya Teaching Hospital Baghdad, Iraq for bacterial isolation using the method of Collee and colleagues.[10]

Extraction of M Protein

Two hundred and fifty ml of brain heart infusion broth was inoculated with the isolated bacteria and then incubated overnight at 37 °C. M protein was extracted using the method of Hafez and colleagues.[11] The protein content of the extracted material was determined using Lowry method.[12]

In Vitro T Cell Proliferation Assay

For *in vitro* T cell proliferative responses, T cells were purified using a MACS Pan-T cell isolation kit (Miltenyi Biotec). Cells were then cultured in 0.2 ml of standard culture medium using RPMI-1640 with L-glutamine (USBiological, USA), fetal calf serum 10%, and lymphocult-T-HP (Human IL-2) at a density of 2×10^5 cells/well in 96-well plates (Costar). Isolated peripheral blood naturally occurring $CD4^+CD25^+$ regulatory T cells and $CD4^+$ T cells were cultured in isolated and mixed cell culture systems with and without the addition of extracted streptococcal M protein. The protein (5 µg/ml) was added under full sterilized conditions, and the plates then, incubated for 7 days at 37 ºC in a humidified CO_2 incubator. Before and during the incubation period on days 0, 3, 5, and 7, the cells number was detected by using immunoflouresence technique, fluorescence microscope and fluorescence labeled anti $CD4^+$ monoclonal antibodies and PE labeled anti $CD25^+$ monoclonal antibodies. The percentage of positive cells was determined using the following equation.

Percentage of positive cells=The number of positive cells /The number of total cells ×100

The cells' viability was detected using trypan blue stain. Then the cell culture suspension was centrifuged at 3000 rpm for 10 minutes.

Cytokine Quantification

Supernatants of *in vitro* differentiated cells were analyzed for cytokines using human tumor necrosis factor-a (TNFa) and interlukin (IL-4) ELISA Kits (Bio-Source Europe, S.A.)

Statistical Analysis

The correlation coefficient (r) was also calculated as a quantitative measure of the association between the mean percentages of $CD4^+$ T cells and $CD4^+CD25^+$ nTreg cells among different study groups.

The correlations between the mean percentages of nTregs and TNF-α with that of the mean percentages of $CD4^+$ T cells were analyzed by using Spearman's rank correlation test. Also, Spearman's rank correlation test was used to analyze the correlation between the mean number of the proliferated cultured nTregs and $CD4^+$ T cells with and without the stimulation of streptococcal M protein in isolated and mixed cultures. The statistical analysis was performed using Statistical Package for Social Sciences (SPSS version 10.01) and Microsoft Excell 2003. A *p* value of less than 0.05 was considered as the level of statistical significance.

Results

In the isolated cell cultures, the values of correlation coefficient showed a highly significant positive correlation (r=0.754, P<0.01) between the number of the cellular proliferation for both nTregs and $CD4^+$ T cells with or without M protein stimulation, which was recorded by immunoflouresence technique on days 0, 3, 5 and 7 of incubation (figure 1).

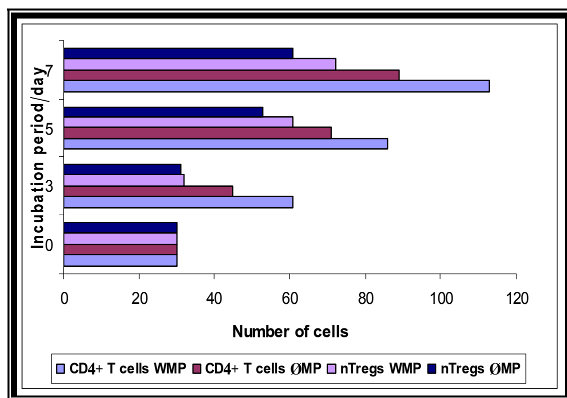

Figure 1: The number of nTregs and $CD4^+$ T cells in the presence and absence of M protein in isolated cell culture system on days 0, 3, 5, and 7 of incubation.

A highly significant negative correlation was found 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), However, there was a positive insignificant correlation between the mean number of nTregs and $CD4^+$ T cells in the presence of M protein which showed (r=0.353) (P>0.05).

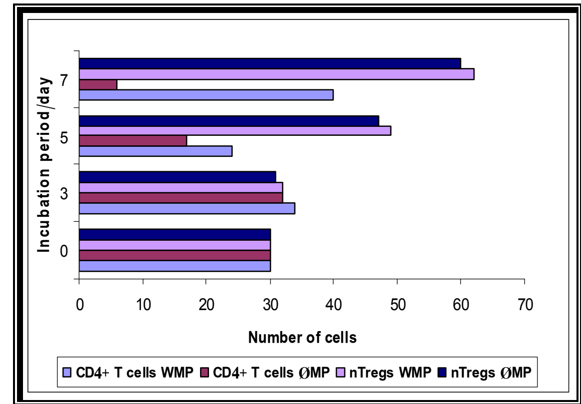

Figure 2: The number of nTregs and $CD4^+$ T cells in the presence and absence of M protein in mixed cell culture system on days 0, 3, 5, and 7 of incubation. .

Results obtained from the ELISA test (optical density values) revealed that there was no significant difference among all cell cultures in terms of IL-4 production (table 1). Tumor necrosis factor-α was produced in higher concentrations in the culture supernatants when compared with IL-4. The cultures of patients number one, 4, 6, and 7, which were incubated with nTregs exhibited lower TNF-α concentrations. However, patients number 2, and 5 showed 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 nTregs, a highly significant positive association (P<0.01) was found between them. Also, in spite of stimulation with streptococcal M protein, TNF-α was produced in a low concentration (4.556 pg/ml) in $CD4^+$ T cell culture.

Discussion

This study describes the proliferation of the human nTregs against streptococcal M protein-activated $CD4^+$ T cells from peripheral blood of patients with chronic RHD. The mechanism of action by which nTregs so effectively inhibit the proliferation of $CD4^+$ T cells is by producing regulatory cytokines. The findings of the study revealed that streptococcal M protein has the ability to activate the proliferation of both $CD4^+$ T cells and nTreg cells to various degrees. Previous studies observed that M protein-specific T-cell clones generated from peripheral blood of patients with chronic RHD and healthy

Table1: The concentrations of TNF-α and IL-4 in supernatants from M protein-stimulated CD4+Tcells and CD4+T cell/naturally-occurring regulatory T cells (nTregs) cultures.

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.

individuals were cross-reactive with heart proteins. As a result of activation, $CD4^+$ T cells begin to secrete cytokines. Also, the results showed that the isolation and culturing of nTregs from human peripheral blood were able to totally inhibit cytokine secretion when co-cultured with $CD4^+$ T cells. The immunoflourescence staining of $CD4^+CD25^+$ nTreg cells showed significant increases in the number of such cells when cultured with M protein activated $CD4^+$ T cells. Patients with RHD may have a higher number of precursors of heart-reactive T cells or a pool of memory T cells capable of recognizing specific heart autoantigens. This pool may further be expanded following re-exposure to streptococcal antigens. The link between the stimulation with streptococcal M protein and the development of T cells with the capacity to kill myocardial cell lines has been reported.[13,14] In addition, it has been demonstrated that after exposure to different M protein, generated T cell lines recognize heart proteins.[11-15] The findings of the resent study may raise the possibility that more than one antigen of group A streptococcus (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 in patients with RHD to 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 the disease.

This study displayed that there was very little or no secretion of IL-4 from $CD4^+$ T cells, and that the low IL-4 secretion was related to low suppressive activity of nTregs. Therefore, any reduction in the production of this cytokine may affect the suppressive function of nTregs against $CD4^+$ T cells leading to more damage to the heart especially the mitral valve. This appeared visible in the significant correlation between positive/negative IL-4 cells and the extent of histopathological abnormalities (odds ratio=4.5, data are not shown). Other studies also suggest that the anti-inflammatory function of IL-4 could partly be mediated by its effects on nTregs function. Therefore, any reduction in the production of this cytokine may affect the suppressive function of nTregs against $CD4^+$ T cells.[17-19] Thus, more damage will affect the heart valves due to the lack of nTreg cells at the inflammatory sites in the heart tissues. Results showed that cultured cells produced high levels of TNF-α in culture supernatant (table 1). These results may correlate with that. TNF-α is the main mediator of inflammatory processes in RF and RHD in response to GAS antigen especially M protein. Many studies suggest that streptococcal M protein interacts with TLR-2 on human peripheral blood monocytes. As a consequence of monocytes activation by M proteins, monocytes express the cytokines IL-6, IL-1β, and TNF-α.[20] Another study,[21] found that TNF-α inhibited the suppressive function of both naturally occurring $CD4^+CD25^+$ Tregs and transforming growth factor–beta1 (TGF-β1)-induced $CD4^+CD25^+$ T-regulatory cells. Thus, our results revealed that the impairment of the function of nTreg cells and the development of heart damage in RHD patients may occur in two pathways. First, TNF-α can inhibit nTreg cells during the acute stage of rheumatic myocarditis and in the recurrent inflammatory attacks during the chronic stage. Secondly, the low amount of IL-4 in chronic rheumatic myocarditis will alter the nTreg cells under certain conditions, or other cytokines like B cell activation factor of the TNF family (BAFF) can play a role. BAFF-expanded $CD4^+CD25^+Foxp3^+$ regulatory

T cells (Tregs) were consistent with an ability to home to inflammatory sites and prevent T cell effector responses.[22] Therefore, autoimmune rheumatic myocarditis process will depend to a great degree on cellular immunity rather than humoral immune response. The very important role for nTreg cells in reversing this autoimmunity will take place to further directions towards the prevention of rheumatic myocarditis.

Conclusion

The findings of this study revealed that streptococcal M protein has the ability to stimulate both of $CD4^+$ T cells and $CD4^+CD25^+$ nTreg cells. It caused the proliferation of both cells and the production of TNF-α from $CD4^+$ T cells. Moreover, the findings show that M protein has an inhibitory effect on expanded $CD4^+CD25^+$ nTreg cells function resulting in the inability of $CD4^+CD25^+$ nTreg cells to suppress the autoreactive $CD4^+$ T cells, which play the major role in the development of rheumatic heart damage. Also, the findings may reinforce the role of streptococcal M protein in the pathogenesis of RHD.

Acknowledgement

The authors would like to thank all the staffs in the Departments of Microbiology and Pathology, College of Medicine, Al-Nahrain University, Ibn Al-Bitar Hospital for Cardiac Surgery, Al-Kadhimya Teaching Hospital, and Laboratory of Health Centre for their assistance in this study.

Conflict of Interest: None declared

References

1 Han Y, Guo Q, Zhang M, Chen Z, Cao X. CD69+ CD4+ CD25- T cells, a new subset of regulatory T cells, suppress T cell proliferation through membrane-bound TGF-beta 1. *J Immunol* 2009; 182: 111-20.
2 Sakaguchi S. Regulatory T cells in the past and for the future. *Eur J Immunol* 2008; 38: 901-37.
3 Shevach EM. CD4+CD25+ suppressor T-cells: more questions than answers. *Nat Rev Immunol* 2002; 2: 389-400.
4 Sakaguchi S. Naturally arising CD4+ regulatory T-cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 2004; 22: 531-62.
5 Maloy KJ, Powrie F. Regulatory T-cells in the control of immune pathology. *Nat Immunol* 2001; 2: 816-22.
6 Carapetis JR, McDonald M, Wilson NJ. Acute rheumatic fever. *Lancet* 2005; 366: 155-68.
7 Robertson KA, Volmink JA, Mayosi BM. Antibiotics for the primary prevention of acute rheumatic fever: a meta-analysis. *BMC Cardiovasc Disord* 2005; 5: 11.
8 Fischetti *VA.* Streptococcal M protein. *Sci Am* 1991; 264: 58-65.
9 Fischetti VA, Pancholi V, Schneewind O. Conservation of a hexapeptide sequence in the anchor region of the surface proteins of gram positive cocci. *Mol Microbiol* 1990; 4: 1603-5.
10 Collee J, Fraser A, Marmion B, Simmons A. Mackie and McCartney, practical medical microbiology. 14 th edition. Longman Singapore Publishers. Churchill Livingstone. Singapore 1996. p. 264-8.
11 Hafez K, El Kholy AM, Facklam RR. *Extraction of group A streptococcal M protein with nitrous acid. J Clin Microbiol* 1981; 14: 530-3.
12 Lowry OH, RosebrougH NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193: 265-75.
13 Pruksakorn S, Currie B, Brandt E, et al. Identification of T cell autoepitopes that cross-react with the C-terminal segment of the M protein of group A streptococci. *Int Immunol* 1994; 6: 1235-44.
14 Meric N, Berkel A. Cellular immunity in children with acute rheumatic fever and rheumatic carditis. *Pediatr Res* 1979; 13: 16-20.
15 Watanabe-Ohnishi R, Aelion J, LeGros L, et al. Characterization of unique human TCR V beta specificities for a family of streptococcal superantigens represented by rheumatogenic serotypes of M protein. *J Immunol* 1994; 152: 2066-73.
16 Dale JB, Beachey EH. Human cytotoxic T lymphocytes evoked by group A streptococcal M proteins. *J Exp Med* 166: 1825-35.
17 Kotb M, Courtney HS, Dale JB, Beachey EH. Cellular and biochemical responses of human T lymphocytes stimulated with streptococcal M proteins. *J Immunol* 1989; 142: 966-70.
18 Maerten P, Shen C, Bullens D, Assche G, et al. Effects of IL-4 on CD4+CD25+ regulatory T cell function. *J Autoimmunity* 2005; 25: 112-20.

19 Thornton AM, Piccirillo CA, Shevach EM. Activation requirements for the induction of CD4+CD25+ T cell suppressor function. *Eur J Immunol* 2004; 34: 366-76.
20 Påhlman LI, Mörgelin M, Eckert J. Streptococcal M protein: A multipotent and powerful inducer of inflammation. *J Immunol* 2006; 177: 1221-8.
21 Valencia X, Stephens G, Goldbach-Mansky R, et al. TNF downmodulates the function of human CD4+CD25+ Tregulatory cells. *Blood* 2006; 108: 253-61.
22 Walters S, Webster KE, Sutherland A, et al. Increased CD4+Foxp3+ T Cells in BAFF-Transgenic Mice Suppress T Cell Effector Responses. *J Immunol* 2009; 182: 793-801.