Journal Homepage: - www.journalijar.com
INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)
Article DOI: 10.21474/IJAR01/1500
DOI URL: http://dx.doi.org/10.21474/IJAR01/1500
ISSN NO. 2320-5407

RESEARCH ARTICLE

T REGULATORY CELL FREQUENCY IN IRAQI RHEUMATOID ARTHRITIS.

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Manuscript Info

Manuscript History

Received: 16 July 2016
Final Accepted: 22 August 2016
Published: September 2016

Key words:-
Rheumatoid arthritis, T-regulatory cells, $CD4^{+}CD25^{+}$.

Abstract

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease leading to the destruction of joint structure and tissue surrounding the joints and degrading their function. It is widely recognized that T-regulatory are important in preventing autoimmune disease and are likely to contribute to the resolution of inflammation and in general, increasing the Treg cell number or enhancing Treg cell suppressive function may prove to be beneficial in the suppression of autoimmune diseases, including arthritis. Treg cells were shown to be protective against arthritis, since the transfer of $CD4^{+}$ $CD25^{+}$ Treg cells inhibited arthritis, while the deletion of $Foxp3^{+}$ Treg cells resulted in exacerbation of the disease.This study planned to determine the frequencies of CD4+CD25+ Treg cells in development and severity of RA in Iraqi patients. In conclusion, reduction of the Treg cells in patients with biological therapy than control group.

Introduction:-

Rheumatoid arthritis (RA) is an autoimmune disease with still uncertain etiopathogenesis, which characterized by chronic joint inflammation and subsequent joint destruction. The abnormal immune response causes inflammation that can damage many joints and organs[1]. As with most autoimmune disease sex preponderance has clearly shown in females with 2.5 times higher than males while locally in Iraq rheumatoid arthritis is obviously not as rare as was previously thought.

Regulatory T (Treg) cells are essential for normal immune surveillance systems, and their dysfunction leads to development of diseases, such as autoimmune disorders. CD4+CD25+ Treg cells are well-known suppressive cells, which express the transcription factor Foxp3, are indispensable for the maintenance of immune self-tolerance and homeostasis by suppressing aberrant or excessive immune response. Regulatory T cells (Treg) are functionally defective in patients with RA. Restoring their function may not only control inflammation but also restore tolerance in these patients. Biologic therapies have been tremendously successful in treating RA. Here we review numerous reports suggesting that these immunomodulatory therapies have an impact on Treg and that this may contribute to their beneficial effects. Better understanding of their mode of action may not only lead to improvements in therapies

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and sustained remission but also enable the development of biomarkers of response, which would be the first steps towards personalized medicine.

There has been significant progress in the understanding of RA pathogenesis over the past few decades, leading to the introduction of several new biologic therapies. These therapies have shed light not only by proving that their targets are important in disease, but also in numerous studies dissecting the immunopathogenesis of RA. One such mechanism that governs immune homeostasis involves the Treg. Treg are important mediators of peripheral immune tolerance, modulating many aspects of the innate and adaptive immune response, including T effector cells, NK cells and antigen-presenting cells. Restoration of tolerance is likely to be Key in curing RA.

Material and Methods:-

Thirty RA patients diagnosed according to the 2010 ACR/EULAR classification criteria[2] were recruited from consultation of Baghdad teaching hospital in medical city. The inclusion criteria in the study were an inadequate response to one disease-modifying anti rheumatic drug and patients were taken anti-TNFα therapy. Thirty age and sex matched apparently healthy subjects were included as a control group.

All patients underwent full medical history taking and clinical examination with special attention to tender joint count (TJC), swollen joint count (SJC) and morning stiffness. Patients who had Paget disease, multiple myeloma, breast cancer, bone metastasis and patients who were receiving biological treatment in the form of TNF-α inhibitors during the last 6 months were excluded from this study. Disease activity score (DAS28) was assessed and considered low (DAS28 ⩾2.6 −<3.2) moderate (⩾3.2–<5.1), high (⩾5.1) and DAS28 <2.6 as remission [3]. Modified Health assessment questionnaire (MHAQ) was calculated; eight activities were rated as 0 = without any difficulty, 1 = with some difficulty, 2 = with much difficulty, and 3 = unable to do. MHAQ scores were divided into categories of mild (MHAQ <1.3), moderate (1.3 < MHAQ < 1.8) and severe (MHAQ >1.8) functional losses[4].

Laboratory evaluation included complete blood count (CBC), erythrocyte sedimentation rate (ESR), Anti-cycliccitrullinated peptide (ACCP) and rheumatoid factor (RF).T-regulatory cells ($CD4^+$ and $CD25^+$ markers) determined by Flowcytometery. The procedure was done according to the manufacturer instruction at two stages, First stage: Staining the specimens, appropriate volume of CD4 FITC-conjugated monoclonal antibody (fluorescein isothiocyanate) was added, and in the another tube added appropriate volume of CD25 PE-conjugated monoclonal antibody (Phycoerythrin) to 100µL of whole blood in a 12x75-mm capped polystyrene test tube. Vortex gently and incubated from 15 to 30 minutes in the dark at room temperature (20^OC-25^OC). Then 2ml of 1X BD FACS lysing solution was added to each tube. the tubes was vortex gently then incubated it in dark at room temperature for 10 minutes after that put the tubes in centrifuge at 300g for 5 minutes, then aspirated the supernatant. 2ml of buffer solution was added to each tube. The tubes was centrifuged at 300g for 5 minutes, then aspirated the supernatant and added 0.5 ml of buffer to each tube, and analyze the samples immediately or added fixative. Second stage: Adding of fixative, 0.5 ml of fixative was added, then vortex the tubes gently and incubated it at 2^OC to 8^OC in dark. The tubes was mixed thoroughly before analysis, stored at 2^OC to 8^OC until analyzed (must be analyzing within 24 hours of staining).

Statistical analysis:

The results were presented as number, mean, and whenever possible as mean ± SD. The data were analyzed using two-tailed, unpaired difference between two means Student's t test, Chi-squared test and Pearson's (rho) correlation test. Statistical analysis was carried on by using Excel 2007 and SPSS version 17 programs taking a probability (p) value of ≤ 0.05 as the lowest limit of significance.

Results:-

The results of anthropometric measurements of this study were showed in Table 1. The body mass index, as an indicator of whole body obesity was significantly higher in RA patients compared with the corresponding value of control subjects, and the measurements of the blood pressure were significant higher systolic and pulse pressures were observed in RA patients (Group II) compared with healthy subjects (Group I).

Table 1:- Blood pressure measurements and Anthropometric measurements.

Blood pressure (mmHg)	Group I (*n*=30)	Group II (*n*=30)	Probability
Systolic	128.0±15.1	138.4±19.6	0.024*
Diastolic	82.8±11.1	85.4±13.0	0.409
Pulse	45.2±10.7	53.1±12.9	0.013*
Mean	97.8±11.5	103.0±14.3	0.124
Weight (kg)	77.0 ±18.2	80.4±16.3	0.446
Height (m)	1.63±0.1	1.58±0.07	0.05*
Body mass index (kg/m^2)	28.85±4.76	32.1±5.9	0.024*

The results are expressed as mean ± SD. * Probability value of significant difference between Group I and Group II. Also the results are expressed as mean ± SD. * probability value of significant difference between Group I and Group II. Pulse pressure is equal to systolic *minus* diastolic; Mean arterial pressure is calculated by the equation: diastolic + ($\frac{1}{3}\times$pulse pressure).

Table 2. Show comparison between patients and control group in Treg cells, ESR, RF, ACCP and HDL. The results appear reduction in Treg cells in patients (Group II) with biological therapy than control group (Group I) which show significant difference ($p< 0.05$). Also Significant higher erythrocyte sedimentation rate (mm/h)

Table 2:- Inflammatory markers levels.

	Group I (*n*=30)	Group II (*n*=30)	Probability
Erythrocyte sedimentation rate (mm/h)	12.7±6.8	31.2±22.6	0.000*
Rheumatoid factor reactivity (No.)	0.0	21	
ACCP Negative Critical Positive	 26 3 1	 29 0 1	
Monocyte/High density lipoprotein-cholesterol (Number of cells per mg)	709.2±254.9	788.6±362.7	0.331
Treg cells (%)	2.62±2.84	0.62±0.52	0.000*

The results are expressed as mean ± SD. * probability value of significant difference between Group I and Group II.

Discussion:-

In the last decade, a growing number of studies underscored the effects of biologic agents on Treg and Th17 cells in RA. Concerning Treg cells, the possible role of tumor necrosis factor (TNF) blockers on this cell subset was initially reported by Ehrenstein et al. in 2004[5]. In fact, they observed that treatment with infliximab, a chimeric monoclonal antibody against TNF, was able to increase the percentage of circulating $CD4^+FoxP3^+$cells and to revert the defective suppressive activity of CD25 high Treg cells[5]. To note, however, the increase of circulating $CD4^+FoxP3^+$ cells induced by infliximab was due to a selective upregulation of the FoxP3 transcription factor in $CD25^-$ rather than CD25 high T cells[6]. Hence, in a coculture system of RA $CD25^-$and CD24 high T cells, the apparent restoration of CD25 high Treg cell suppressive activity following infliximab treatment was an artifact due to increased percentage of suppressive $FoxP3^+$ cells among the $CD25^-$ fraction.

Subsequently, several studies attempted to investigate the effect of other commercially available TNF blockers on RA Treg cells. Concerning the human monoclonal antibody adalimumab, while two studies failed to observe any differences in Treg cell percentage before and after treatment[7, 8], three other groups reported increased percentages of circulating CD25 high $FoxP3^+$ Treg cells either in accordance with[9] or independently from clinical response to adalimumab [10, 11]. Moreover, Treg cells isolated from RA patients with good clinical response to adalimumab appear to exert a more pronounced suppressive activity[9, 11]. Increased FoxP3 expression among $CD4^+$ lymphocytes has been described in patients treated with etanercept, a fusion protein acting as TNF inhibitor[12], but these data were not confirmed in other studies evaluating the in vivo effects of this compound on RA Treg cells[8, 9].

The exact molecular mechanism underlying the possible inhibitory effect exerted by TNF on Treg cells, thus explaining their modulation by TNF blockers, was only recently clarified. Valencia et al., indeed, observed that TNF

is directly responsible for the impaired suppressive activity of RA CD25 high Treg cells, as it determines a consistent reduction of FoxP3 mRNA, required to convey a regulatory activity[13]. This effect appeared to be mediated through TNFRII that is constitutively expressed by Treg cells[13]. More recently, Nie et al. demonstrated that FoxP3 transcriptional activity and Treg cell suppressive function are regulated by TNF-dependent dephosphorylation of the FoxP3 DNA-binding domain (Ser418 in the C-terminal DNA-binding domain)[14, 15]. This abnormal dephosphorylation of FoxP3 in RA Treg cells is due to the ubiquitous enzyme protein phosphatase 1 that is induced by TNF through the IKK–NF-κB pathway. Of interest, treatment of RA patients with TNF blockers decreased protein phosphatase 1 expression, increased FoxP3 phosphorylation, and, in consequence, restored Treg cell suppressive activity.

In conclusion it is widely recognized that Treg are important in preventing autoimmune disease and are likely to contribute to the resolution of inflammation. It is apparent that most biologic therapies appear to have an impact on Treg, and restoration of Treg function has been reported, particularly with anti-TNF antibody therapy. However, restoration of Treg function may not be sufficient if target cells are resistant to suppression. Understanding how biologic therapy affects pathogenic and Treg subsets could lead to the generation of biomarkers of response. For instance an in vitro assay based on Treg induction could be developed to predict which patients will respond to specific therapies. Ultimately, it is likely that a combination approach may be the most successful, involving suppression of the inflammatory response together with optimizing Treg function in order to restore tolerance and cure disease.

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