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Original Article

Immunologic Parameters for Disease Activity in Rheumatoid Arthritis

Riyadh Mohsen, E[1], Ali, N. H[1 *], Aldaoseri, H. A[1]

1. College of Medicine, University of Basrah, Basrah, Iraq

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Corresponding Author: naael.ali@uobasrah.edu.iq

Abstract

This study aimed to determine the correlation of disease activity of rheumatoid arthritis (RA) with Th17/regulatory T cell (Treg) and Forkhead box protein 3 (Foxp3) cells ratio in patients under therapy with anti-tumor necrosis factor (TNF)-α. Totally, 84 patients with RA and 13 healthy controls were included in this case-control study. The patients were divided into four groups to receive only methotrexate (MTX) (n=25), monotherapy (anti-TNF) (n=18), and combined therapy (MTX+anti-TNF) (n=26); however, one group received no medications (n=15) and was regarded as a positive control. Other 13 healthy controls that were considered negative controls were also enrolled in this study. Patients with RA were attending Basrah General Hospital, Rheumatology Unit, Biological Therapy Center for receiving anti-TNF therapy. Flow cytometry was used for measuring Treg/Foxp3 and Th17 markers, and the DAS-28 score was utilized to measure RA disease activity. Anti-TNF inhibitors (e.g., infliximab and etanercept), as well as other inflammatory and hematological parameters (e.g., erythrocyte sedimentation rate, total white blood cells, lymphocytes, monocytes, and neutrophil counts), were also measured in this study. DAS-28 as a disease activity score was significantly correlated with Th17/Treg/Foxp3 ratio and the Th17 cells count. Statistically, Th17/Treg/Foxp3 ratio was not correlated with body mass index, morning stiffness, and duration of the disease. Th17/Treg/Foxp3 ratio correlated significantly with DAS-28 as an RA disease activity. The lower Treg/Foxp3 frequency led to the higher DAS score reflecting higher disease activity. In the combined therapy group, disease activity was found lower than that in other patient groups indicating the effect of this combination on the relationship between MTX and anti-TNF. This study demonstrated that the main advantage of this combined therapy in RA patients was the reversion of Th17 cell expansion.

Keywords: Anti-TNF; Etanercept; Immunotherapy; Infliximab; Rheumatoid Arthritis; Th17/Treg/Foxp3 ratio

1. Introduction

Rheumatoid arthritis (RA) as a multifactorial origin autoimmune disease is associated with chronic inflammation and articular destruction in the joints. The pathogenesis is determined by induction and progression of abnormal regulatory T cell (Treg) response with a shift towards a Th17 cell response (1). The Treg ($CD4^+CD25^+$) cells play a role in maintaining homeostasis by mediating immune tolerance and suppressing autoreactive lymphocytes. This homeostasis has been found to be altered in active RA (2, 3). Th17 cells are the other players in the pathogenesis of RA, and they secret certain proinflammatory cytokines, such as Interleukin-17A (IL-17A) (4). Both IL-17A and tumor necrosis factor-α (TNF-α) act to induce chemokine and cytokine production from synovial fibroblasts, resulting in the destruction of cartilage (5). Therefore, anti-TNF-α therapy has been found to improve the ability of Treg in regulation and decrease the Th17 cell population (1). This immunomodulatory therapy has faced a number of challenges. A proportion of patients (30%-40%) fail to

respond to anti-TNF therapy that makes them have adverse effects and loss the response as their disease progressively worsens (6-8). The explanation papers for this irresponsiveness were scarce. Recently, Atiqi, Hooijberg (9) concluded that neutralizing anti-drug antibodies (ADA) interfere with the ability of TNF inhibitors (TNFi) to block TNF-α. These blockers are directed against idiotopes inside or outside the TNF-binding fragments of the anti-TNF-α (10). Others revealed that alteration in the Th17/Treg ratio in combination with an imbalance in the Th1/Th2 ratio is responsible for RA diseases activity and progression of RA (11). Immunomodulatory drugs, such as adalimumab and etanercept have no effect on changing the number or phenotype of peripheral blood Tregs in RA patients even in conjunction with methotrexate (MTX) (12).

Forkhead box protein 3 (FoxP3), a transcription factor responsible for the development of Tregs in the thymus, is required to maintain the suppressive ability of Tregs in the human peripheral blood. A decrease in the FoxP3 expression might lead to the conversion of Tregs from regulatory into effectors cells (Th17) (13, 14), which might lead to Th17/Treg cells imbalance. Pro- or anti-inflammatory cytokines production is connected with this imbalance and is relevant for the development and/or progression of the disease (5).

Both Th17 and Treg rely on transforming growth factor-β for their induction; however, when the level of Interleukin-6 (IL-6) is increased, Th17 response is favored (15). Therefore, FoxP3 expression is critical for the identification of Tregs that carry suppressive activity, which is neglected by other similar studies (16). Th17/Treg ratio is used as a biomarker, predictive marker, and progression marker for many autoimmune diseases (4). In this study, it is hypothesized that this ratio correlated with the activity of RA disease, especially among those receiving anti-TNF therapy. The present study aimed to investigate the correlation between Th17/Treg/FoxP3 cells and the RA disease activity in patients under therapy with anti-TNF-α.

2. Material and Methods

2.1. Study Population

A total of 97 participants were included in this case-control study from August 2019 to February 2020. In total, 84 patients with RA attended Basrah General Hospital, Rheumatology Unit, Biological Therapy Center for receiving anti-TNF therapy. The negative control group included 13 healthy individuals. The number, age, and gender of the participants according to groups are shown in table 1 below:

Table 1. Number, age, and gender of the participants participated in this study

Group No.	Character	N=	Age range (years) (median)	Females No. (%)
G1	Methotrexate (MXT) only	25	(20- 79) (52)	18 (72.0)
G2	Monotherapy (anti-TNF)	18	(33- 69) (53)	11 (61.1)
G3	Combined therapy (antiTNF + MXT)	26	(25- 71) (50)	24 (92.3)
G4	Positive control	15	(20- 65) (48)	10 (66.7)
	Total	84	(20 -79) (50)	71 (73.2)
G5	Negative control	13	(25- 67) (43)	8 (61.5)

Information about socio-demographic characteristics and clinical features (body weight, treatment information, clinical presentation, and disease activity) was obtained prior to collect the blood sample from all participants. The clinical parameters were obtained according to the 2010 EULAR/ACR criteria (DAS 28). Patients who received at least six doses of anti-TNF and those whose age was more than 18 years were included in the study. On the other hand, those with other autoimmune or chronic diseases, switching to other drugs, irregular therapy, overlap diseases, and patients receiving corticosteroid drugs, or pregnant women were excluded from the study.

2.2. Sampling and Processing

Blood samples (5 ml) were collected from all participants for flow cytometry and other laboratory investigations.

2.2.1. Flow Cytometry work

BriCyteE6 (Mindray, China) flow cytometry was used for T cells phenotyping which was equipped with dual-laser-based optics. A red diode laser and a blue diode laser were used for exciting 4 to 6 colors of fluorescent lights. Considering Treg ($CD4^+CD25^+Foxp3^+$) cells enumeration flow cytometry, after RBC lysis, 10 µL of mouse anti-human CD4 fluorescein-conjugated with FITC (Becton Dickinson, San Jose, CA, USA, BD) monoclonal antibodies (mAb) and mouse anti-human CD25 (IL-2Rα) phycoerythrin PE labeled (BD) mAbs were incubated for 45 min. Following surface staining, the cells were fixed and permeabilized using the BD Fixation-permeabiliztion kit. The cells were then re-suspended in 250 µL by cytofix and incubated for 20 min at 4°C, and they were then washed. 10 µL of polyclonal anti-human/mouse/rat Foxp3-Allophycocyanin-APC conjugated (e Bioscience, USA)- were incubated with the cells overnight in the dark. Unstained cells, as isotypic control, were used to exclude autofluorescence.

Th17 cells were quantified by recombinant human IgG1 to CD196 (CCR6) and CD194 (CCR4) (Bio-Connect Diagnostics B.V. the Netherlands). According to the product instructions, the recommended antibody dilution for the labeling of cells and subsequent analysis by flow cytometry was 1:50 for up to 106 cells/100 µL. Up to 106 was re-suspended nucleated cells per 98 µL of the buffer. Subsequently, 2 µL was added to the antibody. The mixture was mixed well and incubated for 10 min in the dark in the refrigerator (2-8°C). Attention was made for avoiding the higher temperatures and/or longer incubation times to avoid non-specific cell labeling. The cells were washed by adding 1-2 mL of buffer and centrifuged at 300×g for 10 min. Following that, the supernatant was aspirated completely, and the cell pellet was re-suspended in a suitable amount of buffer and analyzed by flow cytometry. Treg/FoxP3/Th17 ratio was calculated according to the output resulted from flow cytometry.

2.2.1.1. Enzyme Immunoassay for the Qualitative Determination of Antibodies in Serum to Etanercept (Enbrel®) and Infliximab (Remicade®)

Anti-TNF-α inhibitor antibodies (ADA) (e.g., infliximab and etanercept) were measured as well for each participant using the ELISA method by MatriksBiotek® kits. According to manufactures' instructions, the ADA was measured for only three groups of the study (e.g., monotherapy, combined therapy, and control positive groups). Other blood parameters (total white blood cells [WBC], total lymphocytes, neutrophils, monocytes [10^9 cell/L], and erythrocyte sedimentation rate [ESR]) were done for each participant.

2.3. Statistical Analysis

Data were entered and analyzed in SPSS software (version 26). The data in the present study were nonparametric. Therefore, the statistical methods were used in accordance with this type of data. Descriptive statistics were presented as a median for quantitative data, as well as frequencies and percentages (%) according to the types of the variables. The Chi-square and Fishers' Exact tests were also utilized to compare the proportion and frequencies. Kruskal-Wallis test was

employed to compare the median of different study groups. The correlations among variables were investigated using Spearman's test. A p-value less than 0.05 was considered statistically significant.

3. Results

Concerning the socio-demographic characteristics (age, gender, place of residency, and smoking tobacco), there were no significant differences between the patient and control groups in this regard (Table 2).

The clinical features included body mass index (BMI), age at the onset of the disease, duration of the disease, morning stiffness (minutes), family history, history of primary and secondary failure, and DAS-28 among patients and control groups (Table 3). Results showed that only DAS-28 and family history were significantly different between groups with prominence to patients receiving only MTX. Table 4 tabulates the flow cytometry outcomes for all five groups. The Th17, Treg, Treg/Foxp3, and Th17/Treg/Foxp3 ratio markers were differed significantly ($P<0.01$), whereas CD4 marker was not significantly differed among the five groups. Laboratory investigations, including WBC count, ESR, and total lymphocyte count were significantly different among all five groups. Other investigated factors, such as neutrophils and monocytes showed no significant difference (Table 4).

Table 2. Sociodemographic features of Patients and controls

Sociodemographic features		MTX n (25)	Monotherapy n (18)	Combined therapy n (26)	Positive control n (15)	Negative control n (13)	P. value
Age (years)	Median	52.00	53.50	50.00	48.00	53.00	0.360
	(min-max)	(20-79)	(33-69)	(25-71)	(20-65)	(25-67)	
Sex	Male n (%)	7 (28%)	7 (38.9%)	2 (7.3%)	5 (26.8%)	5 (38.5%)	0.073
	Female n (%)	18 (72%)	11 (61.1%)	24 (92.7%)	10 (73.2%)	8 61.5%)	
Residency	Urban n (%)	4 (16%)	5 (27.8%)	8 (30.8%)	3 (20%)	4 (30.8%)	0.724
	Rural n (%)	21 (84%)	13 (66.7%)	18 (69.2%)	12 (80%)	9 (96.2%)	
Smoking	smoking n (%)	3 (12.0%)	3 (16.7%)	5 (19.2%)	3 (20.0%)	0 (0.0%)	0.502
	Nonsmoking n (%)	22 (88.0%)	15 (83.3%)	21 (80.8%)	12 (80.0%)	13 (100%)	

Table 3. Clinical features of patients and controls groups

Features		MTX	Monotherapy	Combined therapy	Positive control	P. value
BMI	n	25	18	26	15	0.228
	Median (min-max)	25.7100 (16.30 - 40.00)	30.4800 (24.34-34.67)	27.6250 (2.50-44.92)	27.4100 (3.00-37.57)	
Age onset of the disease (years)	n	22	18	26	13	0.163
	Median (min-max)	44.00 (15-78)	37.00 (17-59)	30.00 (18-59)	44.00 (14-55)	
Duration of the disease diagnosis (years)	n	22	18	26	13	0.257
	Median (min-max)	1.00 (0.5-30)	0.50 (0.75-13)	2.00 (0.75-30)	2.50 (1-16)	
Morning stiffness (munities)	n	25	18	26	15	0.330
	Median (min-max)	30.00 (0-120)	15.00 (0-120)	22.50 (0-120)	20.00 (0-60)	
DAS-28	n	25	18	26	15	*0.044
	Median (min-max)	4.86 (1-6)	3.61 (2-6)	3.93 (2-7)	3.49 (2-6)	
Family history	Yes n (%)	4 (16%)	4 (22.2%)	11 (42.3%)	1 (6.7%)	*0.041
	No n (%)	21 (84%)	14 (77.8%)	15 (57.7%)	14 (93.3%)	
History of primary and secondary failure	Yes n (%)	0 (0%)	2 (11.1%)	1 (3.8%)	-	0.241
	No n (%)	25 (100%)	16 (88.9%)	25 (96.2%)	15 (100%)	

Table 4. Flow cytometry outcomes for patient and control groups

Marker		MTX N (25)	Monotherapy N (18)	Combined therapy N (26)	Positive control N (15)	Negative control N (13)	Sig. at level of
CD4	**Median (min-max)**	32.200 (15.4-66.7)	31.500 (6.3-60.7)	32.450 (7.4-62.2)	36.300 (18.0-61.8)	29.070 (22.0-41.0)	0.613
Th17	**Median (min-max)**	19.200 (4.3-32.1)	3.100 (0.7-6.7)	4.200 (1.7-14.0)	17.300 (7.1-28.6)	0.900 (0.3-4.5)	**0.01
Treg	**Median (min-max)**	1.5000 (0.54-4.20)	3.7000 (0.26-7.50)	2.3700 (0.69-3.90)	1.8000 (0.90-5.70)	11.6500 (2.10-19.23)	**0.01
Foxp3	**Median (min-max)**	0.7000 (0.03-2.70)	3.6000 (0.20-11.00)	1.8000 (0.06-9.30)	0.7000 (0.03-2.70)	9.2000 (1.92-15.27)	**0.01
Th17/ Foxp3	**Median (min-max)**	20.4300 (2.95-690.00)	0.7050 (0.18-13.50)	2.9100 (0.18-155.00)	28.5700 (7.93-830.00)	0.1100 (0.03-0.61)	**0.01

Correlations of the results between the Th17/Treg/FoxP3 ratio and some clinical features were insignificant in patient groups (Table 5). Table 6 shows the correlations between disease activity and flow cytometry outcomes, including Th17/Treg/FoxP3 ratio. Significantly (at the level of 0.05) a positive correlation is observed between Th17/Treg/Foxp3^{+}T cells ratio and disease activity score in the patient groups by Spearman's correlation test (r=0.231, P=0.034) (Figure 1). This correlation is also found with Th17 (r=0.226, P=0.039) (Figure 2). Treg/FoxP3 was insignificantly correlated with disease activity in patient groups.

Anti-TNFi (ADA) was found among those who received infliximab more than etanercept (Table 7) with a significant difference among monotherapy, combined therapy, and positive control groups (P<0.05).

Table 5. Laboratory investigations for patient and control groups

Features		MTX N (25)	Monotherapy N (18)	Combined therapy N (26)	Positive control N (15)	Negative control N (13)	Sig. at level of
ESR (mm/hr)	**Median (min-max)**	50.00 (5-105)	40.00 (8-95)	31.00 (6-110)	35.00 (5-96)	14.00 (7-20)	0.01
Total WBC (10^9 cell/L)	**Median (min-max)**	7.4300 (1.00-18.00)	8.1000 (4.00-12.00)	7.2500 (4.20-15.00)	7.1500 (3.50-15.07)	8.4000 (4.80-9.90)	0.767
Total Lymphocyte (10^9 cell/L)	**Median (min-max)**	2.5900 (1.11-7.00)	2.3500 (0.90-5.20)	2.6000 (1.52-4.70)	2.0000 (1.40-3.37)	1.3000 (0.32-1.80)	0.01
Neutrophil (10^9 cell/L)	**Median (min-max)**	4.0000 (1.00-11.40)	5.0000 (3.00-8.40)	3.9500 (0.40-11.00)	4.5300 (1.20-12.20)	3.8500 (0.36-6.20)	0.200
Monocytes (10^9 cell/L)	**Median (min-max)**	0.3500 (0.10-1.01)	2.8000 (0.47-9.90)	1.3500 (0.19-5.90)	0.4700 (0.12-0.93)	0.1800 (0.10-0.80)	0.01

Table 6. Spearman's correlation values between Th17/Treg/Foxp3 ratio and clinical features of patients

Parameters	TH17/TregFoxP3	
	P. value	r. value
BMI	0.227	0.133
Age of onset	0.363	0.104
Duration of the disease	0.465	-0.085
Morning stiffness	0.121	-0.171

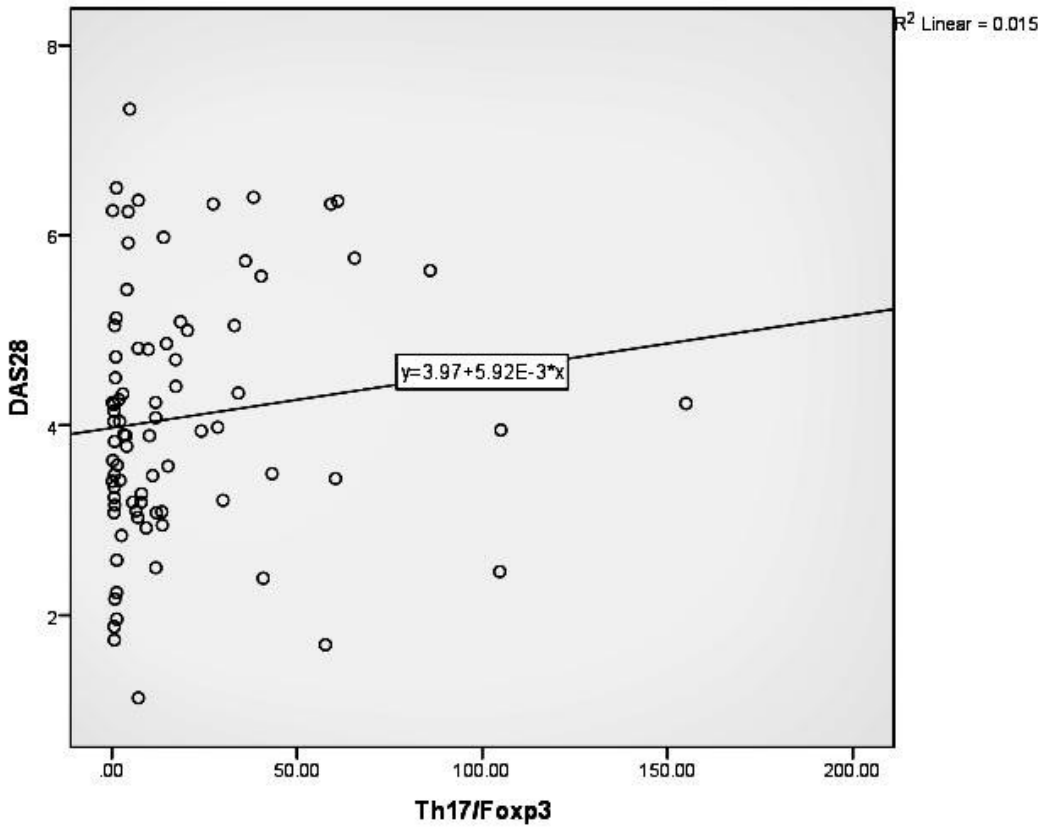

Figure 1. Chart showing a positive significant (at the level of 0.05).
Correlation betweenTh17 /Treg Foxp3+ T cells ratio and disease activity (DAS-28).
Score in patient's groups by Spearmann's correlation test (r= 0.231, P= 0.034)

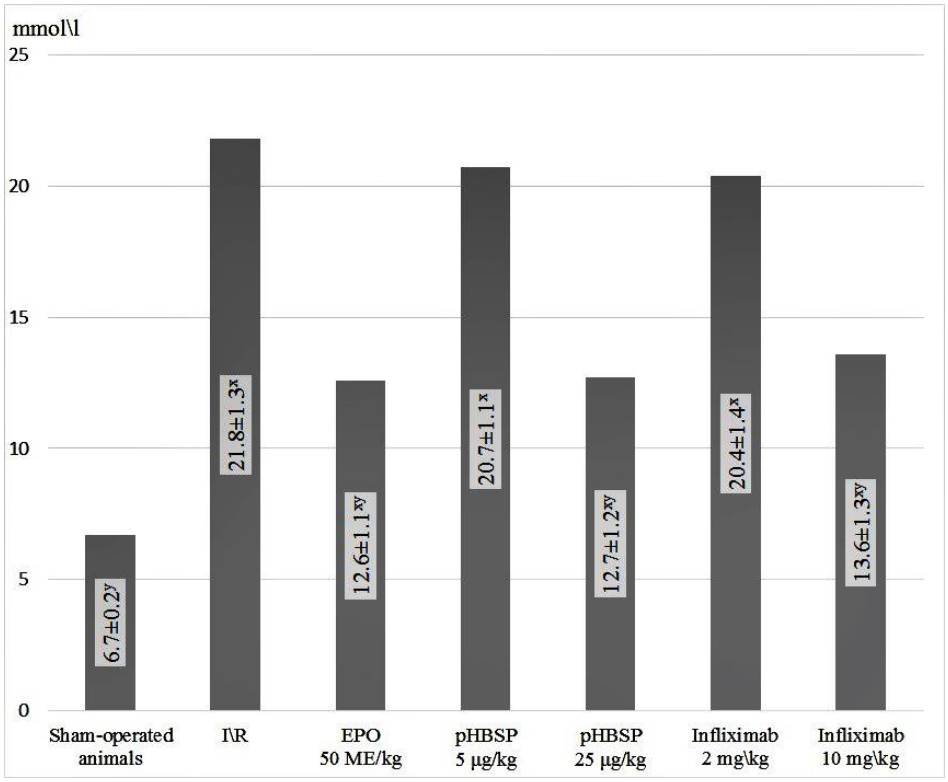

Figure 2. Chart showing a positive significant (at the level of 0.05) correlation betweenTh17 T cells and disease activity score in patient groups by Spearman's correlation test (r=0.226, P=0.039).

Table 7. Spearman's correlation values of DAS-28 and Th17/Treg/Foxp3 ratio with other parameters

Parameters	DAS-28	
	P. value	r. value
BMI	0.665	-0.048
Age of onset	0.523	0.073
Duration of the disease	*0.044	0.071
Morning stiffness	*0.054	0.184
Th17	*0.039	0.226
TregFoxp3	0.109	-0.176
Th17/TregFoxp3 ratio	*0.034	0.231

4. Discussion

The ratio or balance between Th17 and Treg is critically important for pathogenesis, prognosis, and therapy of many autoimmune diseases, including RA (16). In animal models, re-establishing the T effector/Treg ratio can control the autoimmune responses. The published information about the correlation of this ratio as a marker for immunotherapy and its correlation with disease activity was sparse. It is unclear whether the ratio of Th17/Treg in the peripheral blood of RA patients receiving anti-TNF is altered. In the present study, two CD markers (CD 194 and CD 196) were used for the identification of Th17 by flow cytometry. The results revealed a significant increase in the Th17 among RA patients with only MTX treatment, compared to other groups, whereas Tregs ($CD4^+$ 25^+ Foxp3) were significantly increased in the healthy group. In humans, Tregs constitute about 5%-10% of the $CD4^+$T cell population in the peripheral blood of healthy individuals. These cells express high levels of CD25 (IL2Ra). They are also characterized by the expression of the lineage-specific transcription factor-Foxp3, which is pivotal for Treg function and homeostasis (13, 17). Foxp3 was neglected by other researchers (12) so that it might lead to controversial results in Treg function. In the present study, the Treg/Foxp3 accounts for approximately 50% of the total count of Treg in all patient and control groups. Therefore, it is recommended to investigate Treg/Foxp3 for more precision in describing their homeostasis and regulatory activity. This finding is in line with the results of studies conducted by Komatsu, Okamoto (18), who revealed that exTreg cells were subsets of Treg which converted to Th17 in the inflammatory environment in autoimmune arthritis. Therefore, the strengthening of the future resolution and therapy of autoimmune diseases depending on the stabilization of Treg/Foxp3 function at the same time dampen proinflammatory cytokines, such as IL-17. Accordingly, it is reasonable that Th17/Treg/Foxp3 ratio be used as a biomarker in the present study.

One of the important results in the present study was that Th17/Treg Foxp3 ratio correlated significantly with DAS-28, which represented that the lower Treg/Foxp3 frequency led to the higher DAS score reflecting higher disease activity and vice versa. In the combined therapy group, disease activity was found lower than that in other patient groups indicating the effect of this combination between MTX and anti-TNF. It was also demonstrated that the main advantage of this combined therapy in RA patients was the reversion of Th17 cell expansion. Many authorities resulted in unchanged outcomes (7, 19) or a decreased Th17 frequency following treatment with MTX and/or TNFi (11). The latter findings are in accordance with the results of the current study. Anti-TNF therapy with MTX was able to effectively limit the Th17 population only in patients with early disease (6). However, many studies strengthen that the effectiveness of anti-TNF therapy concerning Th17 expansion should be evaluated not earlier than 12 weeks after biologic usage irrespective of the MTX administration (2, 20).

Molecularly, the imbalance between Th17 and Treg cells is supported, and the results show that both Th17 and Treg cells require the same cytokine Transforming Growth Factor-β 1 during the early stage of differentiation. Foxp3 is downregulated when other transcription factors essential for Th17 development, such as RORγ are over-expressed, especially in the presence of IL-1b and IL-6. This last study might strengthen our results. On the other hand, IL-2, which is required for the expansion and maintenance of FoxP3 expressing Treg cells, has been found to inhibit the development of Th17 cells (15). Therefore, IL-2 and IL-6 have counteractive effects on the development and differentiation of Th17 and Treg in the periphery which may hamper immunoregulatory responses and facilitate the persistence of autoimmune inflammation.

Concerning immunogenicity of TNF antagonists, etanercept has lower immunogenicity, compared to infliximab in the present study. These findings may be due to the effect of well-known infliximab which is a

purified, recombinant DNA-derived chimeric human-mouse IgG monoclonal antibody and contains murine heavy (H) and light (L) chain variable regions ligated to genomic human heavy and light chain constant regions, while etanercept is a fusion-protein between a human IgG1 Fc-tail and the TNF-receptor type 2 (9, 21, 22), a fact which makes infliximab more immunogenic than etanercept. Studies on rheumatology patients identified different genetic factors associated with reduced response to infliximab (23). However, patient-specific predictors of infliximab immunogenicity have not been identified so far. That gap has precluded rational tailoring of individualized therapy, namely prescribing combination MTX-infliximab therapy (combined therapy group) to those with a high risk of infliximab immunogenicity.

In conclusion, Th17/Treg/Foxp3 ratio correlated significantly with DAS-28 as an RA disease activity. The lower Treg/Foxp3 frequency led to the higher DAS score reflecting higher disease activity. In the combined therapy group, disease activity was found lower than that in other patient groups indicating the effect of this combination between MTX and anti-TNF. In this study, the main advantage of this combined therapy in RA patients was the reversion of Th17 cell expansion. Moreover, the combination of MTX-infliximab therapy was prescribed to patients with a high risk of infliximab immunogenicity, sparing them from developing antibodies to infliximab, as well as its risks and side effects.

In general, the key massages of the current study are as follows:

1- Th17/Treg/Foxp3 ratio correlated significantly with RA disease activity

2- Th17/Treg/Foxp3 ratio should be considered a biomarker in the prediction and evaluation of patients under the therapy of TNF antagonists.

3- Immunogenicity to infliximab should be tested for RA patients treated with infliximab by checking anti-drug antibodies.

Authors' Contribution

Study concept and design: E. R. M.
Acquisition of data: H. A. A.
Analysis and interpretation of data: N. H. A.
Drafting of the manuscript: N. H. A.
Critical revision of the manuscript for important intellectual content: E. R. M. and N. H. A.
Statistical analysis: H. A. A.
Administrative, technical, and material support: E. R. M., N. H. A. and H. A. A.

Ethics

All instruments applied in this study were calibrated and maintained in accordance with routine quality control procedures overseen by the University of Basrah, Basrah, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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