

## The T<sub>H</sub>17/Treg Imbalance in Rheumatoid Arthritis and Relation to Disease Activity

Taghrid Gaafar<sup>1\*</sup>, Reem Farid<sup>1</sup>, Hala Raafat<sup>2</sup>, Faten Bayoumi<sup>3,4</sup>, Botros Gerges<sup>3</sup> and Dina Rasheed<sup>1</sup>

<sup>1</sup>Department of Clinical Pathology, Faculty of Medicine, Cairo University, Cairo, Egypt

<sup>2</sup>Department of Rheumatology, Faculty of Medicine, Cairo University, Cairo, Egypt

<sup>3</sup>Department of Immunogenetics, National Research Centre, Giza, Egypt

<sup>4</sup>Head of Microbiology and Immunology Department, College of Pharmacy, MSA University, Egypt

\*Corresponding author: Taghrid Gaafar MD, Clinical and Chemical Pathology Department (Stem cell research unit), Faculty of Medicine, Cairo University, Egypt, Tel: +2-01223106418; Fax: +20225269911; E-mail: [tagrid8@yahoo.com](mailto:tagrid8@yahoo.com)

Received date: September 03, 2015; Accepted date: December 28, 2015; Published date: December 31, 2015

Copyright: © 2015 Gaafar T, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

### Abstract

**Objectives:** T regulatory cells (Treg) and proinflammatory T<sub>H</sub>17 cells are newly identified T lymphocyte subsets, which have significant effects on autoimmunity and inflammation. Tregs play key role in the maintenance of self-tolerance. T<sub>H</sub>17/Treg disturbed balance has been reported to contribute to several autoimmune diseases.

Our aim was to assess the Treg/T<sub>H</sub>17 pattern and T<sub>H</sub>17 related cytokines, in peripheral blood of Egyptian rheumatoid arthritis patients, and their relation to disease activity score (DAS). Analyzing IL17A and IL23 as indicators of T<sub>H</sub>17 function, was to study the effect of T<sub>H</sub>17 in RA.

**Methods:** 100 Egyptian rheumatoid arthritis patients, divided into Group I (14), Group II (48), Group III (38), with low <3.2, moderate 3.2 to <5.1 and high >5.1, activity DAS score respectively; and 50 healthy age and sex matched controls were enrolled. Peripheral blood T<sub>H</sub>17 and Treg (CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>) frequencies were analyzed by flowcytometry, and the serum levels of interleukins (IL17A), (IL23) were determined by ELISA.

**Results:** Active RA patients (groups II and III) revealed an obvious increase in peripheral T<sub>H</sub>17 frequencies, and levels of T<sub>H</sub>17-related cytokines, and a significant decrease in Treg (CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>) frequencies in group III, when compared to healthy controls. T<sub>H</sub>17/Treg ratios were positively correlated with serum concentrations of IL17A and IL23 cytokines. Frequencies and levels showed statistical significant correlation with DAS scoring.

**Conclusions:** Our study indicated that T<sub>H</sub>17/Treg balance was disturbed in peripheral blood of RA patients, leading to an increase of proinflammatory cytokines, correlating with DAS, and suggesting an important role in the development of RA.

**Keywords** Tregs; IL-17; IL-23; Rheumatoid arthritis; T<sub>H</sub>17

### Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disorder that may lead to the destruction of joint architecture without effective therapy. Over the past few decades, extensive research has provided data to support the hypotheses that RA is an antigen-driven autoimmune disease [1]. T cells clearly play a central role in the initiation and perpetuation of chronic inflammation prevalent in RA. Besides Th1/Th2 cell populations, two other subpopulations were subsequently identified: regulatory T cells (Treg), producing IL-10 and transforming growth factor (TGF)- $\beta$  [2], and IL-17-producing CD4<sup>+</sup> T cells (T<sub>H</sub>17) [3]. These two subsets play a key role during tolerance and inflammatory responses [4,5]. The role of T<sub>H</sub>17 cells, during development of autoimmune diseases, has largely been described over the past few years [5]. These cell subsets can act at different stages of RA disease, at different sites, and with different intensities, to participate in the complex network of cell-cell interactions that govern the development and chronicity of rheumatoid synovitis.

T helper-type 17 (T<sub>H</sub>17) cells can secrete interleukin (IL17) in humans [6]. IL17 is a pleiotropic cytokine that induces the expression of proinflammatory cytokines and matrix metalloproteases [7], hence participates in tissue inflammation and destruction. It has been demonstrated in many experimental arthritis models, the pathogenic effect of IL17 [8]. On the other hand, regulatory T (Treg) cells are T cell subset, whose peculiar function is to suppress autoreactive lymphocytes. They mediate immune tolerance, and attempt to maintain lymphocyte homeostasis. The two T cell subpopulations different behavior as well as their reciprocal plasticity pointed out the importance of T<sub>H</sub>17/Treg cell disturbed balance in the pathogenesis of RA. As a result, a novel hypothesis has been proposed regarding the pathogenic mechanism of RA, that the T<sub>H</sub>17/Treg imbalance, along with T<sub>H</sub>1/T<sub>H</sub>2 imbalance, may be responsible for the development and progression of RA [9-11]. In addition, the effects of currently employed RA therapeutic strategies on these lymphocyte subpopulations, have been investigated. A particular interest on the possible therapeutic targeting of these cells and their products was developed to overcome the limitation of currently employed biological therapies [12]. Blockade of critical cytokines may result in a shift in this polarization

from T<sub>H</sub>17/T<sub>H</sub>1 phenotypes toward Treg/T<sub>H</sub>2 [13]. Yet, there is controversy in results [14]. Meanwhile, variations in patient response to available anti-RA therapeutics, corroborate the fact that RA is a heterogeneous and complex disease due to various immune pathways involved.

The frequencies of T<sub>H</sub>17 cells were found to increase in peripheral blood mononuclear cells (PBMCs) of RA patients compared to healthy controls [15,16]. An enhanced expression of IL-17 has been observed in the rheumatoid synovium, and synovial fluids of patients with early RA [17].

Few studies have been conducted to evaluate the role of Treg cells in blood of RA patients. Conflicting results have been reported concerning Treg cell proportion in RA peripheral blood. Most studies emphasize the presence of increased numbers of Treg cells in RA synovium [18,19]. Treg cells isolated from the synovial fluid of RA patients appear to be functionally inactive in regards to their ability to suppress pro-inflammatory cytokine production [20].

IL-23 promotes the expansion and survival of T<sub>H</sub>17 cells. IL 23 promotes T<sub>H</sub>17 cells producing tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-17, IL-6, IL-22, and GM-CSF which are associated with the induction of autoimmune inflammation in RA disease [21].

## Aim

To analyze the Serum cytokine Levels (IL17 and IL23), frequency and cell surface phenotype of Treg and T<sub>H</sub>17 cells, in a sample of Egyptian patients with rheumatoid arthritis (RA), compared to healthy control subjects, and to correlate findings with DAS.

## Materials and Methods

This study has been approved by the Research Ethical Committee of Cairo University, Faculty of Medicine (Ethical protocol number: N-68-2012).

## Patients and healthy controls

One hundred RA patients with variable disease activity, fulfilling the revised classification criteria of the American College of Rheumatology [22], were enrolled in this study. They were all chronic rheumatoid arthritis patients, attending the rheumatology outpatient clinic, Cairo University hospital. All had no extra-articular manifestations. They were treated with disease modifying anti-rheumatic drugs [DMARDS] or methotrexate. Patients taking corticosteroids were excluded. Disease activity was estimated according to clinical indices, as well as laboratory markers of inflammation: the erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), rheumatoid factor and calculation of the disease activity score (DAS28). Patients were classified as having active or inactive disease on the basis of the DAS28: 14 patients had low active disease with DAS28 (<3.2), while 48 had moderately active disease (3.2 to <5.1) and 38 patients had high activity disease with DAS28 (>5.1). The main patients' characteristics were summarized in Table 1. Laboratory investigations included rheumatoid factor (RF), erythrocyte sedimentation rate (ESR), complete blood count and liver and kidney functions. According to these clinical and laboratory data, indices of disease activity were calculated such as Ritchie Articular Index and Disease Activity Score. Samples were taken during day time, before administration of any drug, or co-medication.

Fifty healthy controls free of pain, acute and chronic inflammatory diseases and matched with patients for age and sex, were included in the study. Written consent was obtained from all the subjects after full explanation of the procedure.

## Treg (CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>) cells enumeration using flow-cytometry

Small aliquots of whole peripheral blood were labeled with appropriate combinations of monoclonal antibodies (mAb): 10  $\mu$ l of Mouse anti-human CD4 fluorescein conjugated FITC [Becton Dickinson, San Jose, CA, USA, BD] and Mouse anti-human CD25 (IL-2 R alpha) phycoerythrin PE labelled [BD] mAb were incubated for 45 min, followed by red cell lysis. Following surface staining, cells were fixed and permeabilized using BD Fixation/Permeabilization Kit. Cells were resuspended in 250  $\mu$ l of cytofix and incubated for 20 minutes at 4°C then washed. 10  $\mu$ l of polyclonal anti-human/mouse/rat Foxp3- Allophycocyanin, APC conjugated [e Bioscience, USA], were incubated with the cells overnight in the dark. Gating was done on CD4<sup>+</sup> cells first then detection of CD25<sup>+</sup>Foxp3<sup>+</sup> within the population followed. Unstained cells, as isotypic control, were used to exclude autofluorescence. Analysis was performed using a flow cytometer (Beckmann Coulter, FL, USA).

## Quantitation of circulating TH17 cells [23]

T<sub>H</sub>17 cells were quantified by flow cytometry, following staining with FITC conjugated anti-CD4 (BD), and PE conjugated anti-human IL-17A (BD), according to the manufacturers protocol and through the following steps:

**Stimulation of cytokine secreting cells:** 1000  $\mu$ l of the sterile heparinized whole blood were stimulated for 4 hours with the polyclonal activator phorbol myristate acetate (PMA) (50 ng/ml; Sigma -Aldrich) and ionomycin (1  $\mu$ g/ml ; Sigma -Aldrich) and 10  $\mu$ g/ml brefeldin A (Sigma-Aldrich ) to inhibit intracellular protein transport at 37°C in a humidified 5% CO<sub>2</sub> incubator. Activated cultures of blood samples were washed in wash buffer (PBS, 5% fetal bovine serum, 0.1% sodium azide, Merck).

**Staining of cell surface CD4:** Blood sample were stained using 10  $\mu$ l FITC-conjugated anti-CD4 (mouse IgG 1 isotype Sigma- Aldrich, Inc) for 30 min at room temperature. Cells were washed using 1 ml staining Buffer/wash and pellet by centrifugation (250  $\times$  g). 2 ml of FACS lysing solution (Becton Dickinson, Lincoln Park NJ, USA ) were added to blood samples to lyse red cells.

**Fixation and permeabilization of cells using BD Fixation/ Permeabilization Kit:** The cells were thoroughly resuspended in 250  $\mu$ l of BD cytofix/Cytoperm solution per tubes, and incubated for 25 min at 4°C. Cells were washed twice in 1 ml of 1x BD Perm/Wash buffer, which can be used as the wash buffer and as the antibody diluents, and then subjected to intracellular cytokine staining.

**Intracellular IL-17 staining and analysis:** Cells were incubated with PE -conjugated anti-human IL-17A (mouse IgG1 isotype BD) for 30 min at room temperature in the dark. FITC Mouse IgG1 and PE Mouse IgG1 (BD) isotype controls were used as negative controls. After staining, cells were washed and immediately analyzed using flow cytometry. Lymphocytes were gated on the basis of forward and side scatter properties and at least 10000 CD4<sup>+</sup> cells were analyzed. Cell analysis was performed using CYTOMICS FC 500 Flow Cytometer (Beckman coulter, FL, USA) and CXP Software version 2.2.

### Determination of serum IL-17 and IL-23 levels by ELISA

Serum IL17A levels were determined using ELISA Kit (e Biosciences, USA) and IL23 levels were determined using Quantikine human IL23 ELSA kit (R&D Systems, catalog number D2300B according to the manufacturers recommendations). Results were expressed as pg/ml.

### Statistical analysis

All statistical calculations were done using computer programs SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 20 for Microsoft Windows. Data were statistically described in terms of mean ± standard deviation (± SD), frequencies when appropriate. Spearman's test was used for correlation analysis. Student t test was used for comparison of numerical variables between the study groups. P values less than 0.05 were considered statistically significant at the level of 95%.

### Results

The current study included one hundred RA patients (81 females and 19 males). Their ages ranged from 19 to 70 years with a mean of

42.23 ± 12.7 years. Age of disease onset ranged from 19 to 62 years and disease duration ranged from 1 to 28 years. Fifty age and sex matched healthy individuals were included as a control group, their ages ranged from 20 to 60 years with a mean of 37.6 ± 9.8 years. The descriptive data of the patients are shown in Tables 1, 2 and 3.

Groups	Number	Male/Female	Age (years)	
			Range	Mean ± SD
I (Low)	14 (14%)	3/11	19 – 59	36.4 ± 12.3
II (Mod)	48 (48%)	8/40	20 – 66	41.8 ± 14.9
III (High)	38 (38%)	8/30	34 – 70	48.5 ± 11.5

Patients were classified as having active or inactive disease on the basis of the DAS28;  
14 patients had low active disease with a DAS28 <3.2, while 48 had moderately active disease (3.2 < DAS28 <5.1) and 38 patients had high activity disease with a DAS28 >5.1

**Table 1:** Groups of RA patients according to Disease Activity Score.

Laboratory parameters	Controls		Patients	
	Range	Mean ± SD	Range	Mean ± SD
ESR (mm)	5-13	8.3 ± 2.8	5-144	47 ± 34.9
WBC (10 <sup>3</sup> cell/μl)	4.4-6.1	5.1 ± 0.5	3.8-14	6.58 ± 2.3
Haemoglobin (g/dl)	11.5-14.2	12 ± 1.5	7.5-14.1	11.5 ± 2.4
S. Urea (mg/dl)	19-33	26 ± 5.5	15-40	24 ± 6.3
S. Creatinine (mg/dl)	0.45-0.8	0.65 ± 0.1	0.6-1.5	0.65 ± 0.2
ALT (U/L; N <37)	17-28	22 ± 2.6	10-45	23 ± 8.6
AST (U/L; N <37)	18-27	22 ± 3.8	11-43	26 ± 9.9
RF positive	0%		61%	
RF negative	100%		39%	

ESR: Erythrocyte sedimentation rate; WBCs: White Blood Cells; ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; RF: Rheumatoid Factor

**Table 2:** Laboratory investigations for control and patient groups.

Parameter	Range	Mean ± SD	Group (I) Mean ± SD (Range)	Group (II) Mean ± SD (Range)	Group (III) Mean ± SD (Range)
Ritchie articular index	0–26	11.20 ± 9.35	1.80 ± 1.9 (0–5)	9.20 ± 4.9 (0–20)	16.59 ± 7.6 (4–26)
Morning stiffness (hours)	0.25–6	2.49 ± 1.55	1.36 ± 0.7 (0.25–2.5)	1.79 ± 0.94 (0.25–3.5)	3.78 ± 1.8 (0.25–6)
Disease activity score (DAS)	1.13–8.34	5.26 ± 2.64	2.89 ± 0.54 (1.13–3.08)	4.61 ± 0.5 (3.25–5.08)	6.95 ± 0.7 (5.11–8.34)
Rheumatoid factor (IU/ml)	17.4–47.8	32.6 ± 15.20			
Anti-citrullinated peptide antibody	175–275	200 ± 25			
C-reactive protein (mg/L)	18–30	24 ± 6			

**Table 3:** Activity and severity indices of RA patients.

### Frequencies of Treg cells

**Decreased frequencies of Treg cells in peripheral blood of RA patients:** Table 4 shows decreased prevalence of Treg cells (CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>) of highly active RA patients when compared with that of healthy controls (1.01 ± 0.87 and 1.72 ± 1.27%; p<0.001), while there was no obvious divergence observed between low activity and moderate activity RA patients and healthy controls (p=0.739 and p=0.293, respectively). This decrease showed a significant correlation with DAS score of patients group III (r=-0.35, p<0.05) (Figure 1). A weak insignificant correlation was found between percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells and Ritchie Articular Index in patient's group (r=0.104).

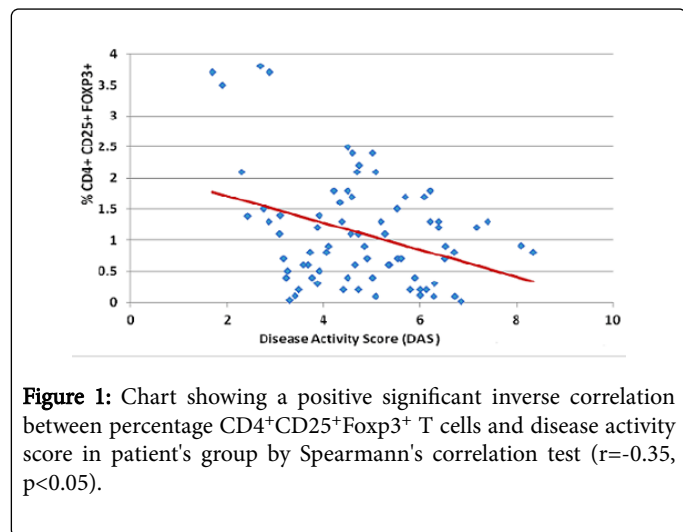
Groups	No.	Percentage of CD4 <sup>+</sup> CD25 <sup>+</sup> FOXP3 <sup>+</sup>	P value	TH 17%	P value
		Mean ± SD		Mean ± SD	
Control	50	1.72 ± 1.27		0.61 ± 0.25	
Group (I)	14	1.80 ± 0.83	>0.05	1.09 ± 0.74	>0.05
Group (II)	48	1.49 ± 1.00	>0.05	1.19 ± 0.34	<0.001*
Group (III)	38	1.01 ± 0.87	<0.001*	1.62 ± 0.73	<0.001*

\*p value = 8.6 × 10<sup>-4</sup> <0.001 Highly Significant.

Th17% p values were 0.07NS for group I; 9 × 10<sup>-4</sup> HS for group II and 7.1 × 10<sup>-4</sup> HS for group III

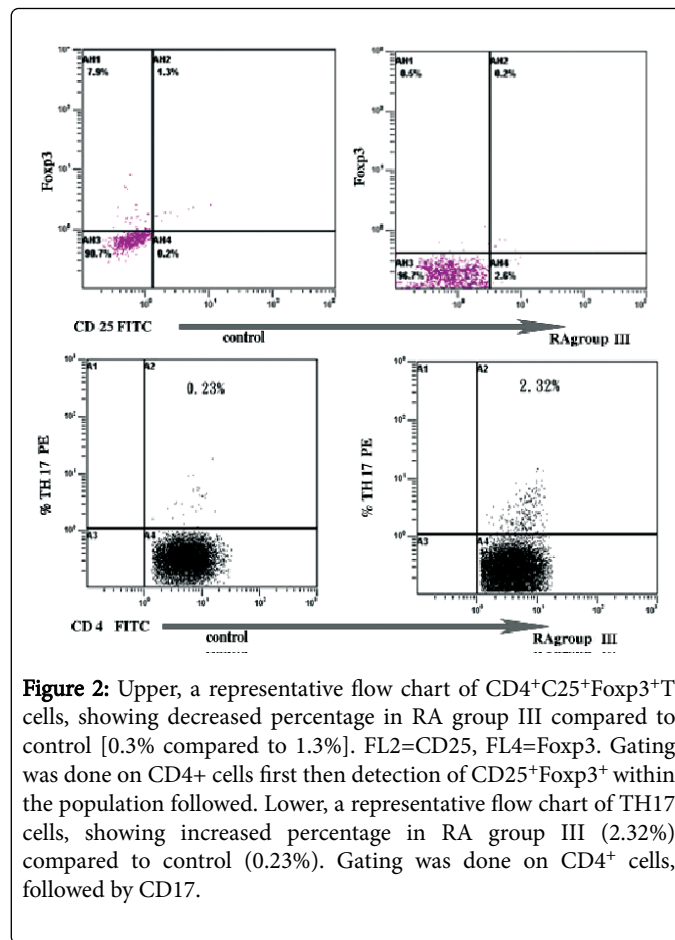
p values of each group are in comparison to control group.

**Table 4:** Frequencies of circulating CD4<sup>+</sup> CD25<sup>+</sup> FOXP3<sup>+</sup>, Th17<sup>+</sup> cells in RA patients and controls.

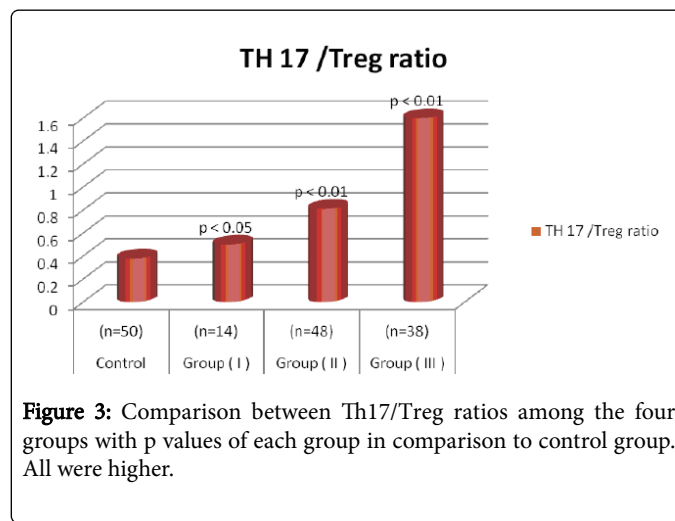


**Figure 1:** Chart showing a positive significant inverse correlation between percentage CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells and disease activity score in patient's group by Spearmann's correlation test (r=-0.35, p<0.05).

**Frequencies of circulating TH17 cells in RA patient:** Representative example of flow cytometry chart of TH17 cells obtained from the PB of one RA patient and from a healthy control is shown in Figure 2. Significantly higher mean frequencies of circulating (CD4<sup>+</sup> IL-17A<sup>+</sup>) TH17 cells were observed in RA patients groups II and III compared to healthy controls (p<0.001, Table 4). Consistently, the number of TH17 cells per volume of RA compared to healthy controls peripheral blood was significantly higher after stimulation with phorbol myristate acetate, ionomycin for 4 h.



**Figure 2:** Upper, a representative flow chart of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>T cells, showing decreased percentage in RA group III compared to control [0.3% compared to 1.3%]. FL2=CD25, FL4=Foxp3. Gating was done on CD4<sup>+</sup> cells first then detection of CD25<sup>+</sup>Foxp3<sup>+</sup> within the population followed. Lower, a representative flow chart of TH17 cells, showing increased percentage in RA group III (2.32%) compared to control (0.23%). Gating was done on CD4<sup>+</sup> cells, followed by CD17.



**Figure 3:** Comparison between Th17/Treg ratios among the four groups with p values of each group in comparison to control group. All were higher.

The significance of lower Treg cells and higher TH17 cells was further explored by calculation of TH17/ Treg ratio of individual RA patients and healthy controls. The TH17/Treg ratio was compared among four groups, as shown in Figure 3. Low activity RA group exhibited higher TH17/Treg ratio (0.51 ± 0.91) than that of healthy controls (0.38 ± 0.4, p<0.05). Moreover, there was a sharp elevation of TH17/Treg ratio in moderately and highly active RA patients (0.87 ± 0.74 and 1.58 ± 0.59, respectively) compared with those of low activity group and healthy controls (both p<0.01). Such results indicate that

there would be a T<sub>H</sub>17/Treg dysbalance in peripheral blood of RA patients, especially in active state.

**Increased TH17-related cytokines in the serum:** Two T-helper cell-related cytokines as components in serum cytokine environment of RA patients and healthy controls were measured using ELISA. As shown in Table 5, RA group I exhibited higher levels of serum IL-17A and IL-23 than those of healthy controls ( $p > 0.05$  NS;  $p < 0.05$  S) respectively but lower than those of RA groups II & III. Active RA group had higher levels of serum IL-17A as well as serum IL-23 when compared to healthy controls (groups II and III  $p < 0.001$  and  $p < 0.05$ ) respectively. Collectively, these results suggest that there would be an increased T<sub>H</sub>1- and T<sub>H</sub>17-related cytokines profile in the serum from active RA patients (groups II and III DAS).

Parameter	Mean ± SD			
	Control	Group (I)	Group (II)	Group (III)
Serum IL-17 level pg/ml	7.96 ± 3.05	11.31 ± 5.08	17.31 ± 5.67	30.70 ± 18.77
Serum IL-23 level pg/ml	277.10 ± 49.04	313.5 ± 40.47	301.08 ± 57.54	402.71 ± 79.36
IL-17 p values: Group I >0.05 NS, Group II < 0.001 H.S., Group III <0.001 H.S in comparison to control group.				
IL-23 p values: Group I <0.05 S., Group II <0.05 S., Group III <0.05 S. in comparison to control group.				

**Table 5:** Serum levels of IL-17 and IL23 in RA patient and controls.

IL-17A showed approximately four-fold increase among patients while IL23 two-fold increase in serum level as compared to controls. The serum level of IL17 and IL23 correlated well with the T<sub>H</sub>17 percentages in the blood of RA patients ( $p = 0.01$ ).

Correlation between the frequencies of circulating T<sub>H</sub>17 cells, serum levels of IL17 and IL23 and disease activity Frequencies of circulating T<sub>H</sub>17 cells in RA patients were positively correlated with DAS28 score ( $r = 0.63$ ,  $p < 0.001$ ), IL17A levels ( $r = 0.48$ ,  $p < 0.05$ ), IL 23 ( $r = 0.67$   $p < 0.001$ ). There is strong direct correlation between DAS28 and inflammatory T<sub>H</sub>17.

**Correlation of T<sub>H</sub>17/Treg ratio with T<sub>H</sub>17-related cytokines in active RA patients:** T<sub>H</sub>17/Treg ratios were positively correlated with serum concentrations of IL17A ( $r = 0.650$ ,  $p < 0.05$ ), IL-23 ( $r = 0.713$ ,  $p < 0.01$ ) of highly active RA patients. These data indicate that the serum environment characterized by increased T<sub>H</sub>17-related cytokines may contribute to the dysbalance of circulating T<sub>H</sub>17/Treg in active RA patients.

## Discussion

Dysregulation in the immune system plays an important role in the pathogenesis of rheumatoid arthritis. The persistent nature of arthritis strengthens the suggestion of immune dysfunction.

In this study, flow cytometric analysis of RA patients peripheral blood, revealed a significant decrease in the frequency of circulating Fopx3CD4<sup>+</sup> CD25<sup>+</sup> Tregs as compared to healthy controls. The low frequency of Treg cells was only observed in group III highly active RA patients, while there was no difference between low activity RA patients and healthy controls, which supported a protective role for Treg cells in periods of inactive RA disease.

This result is in agreement with other groups [20-26]. The exact mechanisms that reduce the level of Tregs are not clear. It was suggested that regulatory cells are recruited to sites of inflammation in an attempt to suppress disease, resulting in a relative reduction in the peripheral blood population [24]. Reports of a higher number of Tregs present in synovial fluid than that in the peripheral blood in RA patients support this explanation. Cell migration to local inflammatory tissue is enhanced by expressing particular chemokine receptors [27]. Moreover, Tregs were found to display increased tendency to undergo spontaneous apoptosis in active RA [28]. Chavele and Ehrenstein proposed that in an inflammatory condition like RA, it is quite possible that Tregs in the presence of the different proinflammatory cytokines will become unstable and convert to pathogenic T-cells [29]. Serum cytokine environment of active RA state is not in favor of the differentiation of Treg cells. This decline in regulatory T-cell numbers may predispose to persistent auto-immune diseases including RA [13]. Contrary to our results there are reports of higher Tregs in peripheral blood of RA patients [30] but in their research they assessed different CD4<sup>+</sup>CD25<sup>+</sup> T cell subtypes (including CD4<sup>+</sup> T cells expressing low levels of CD25 and those expressing Fopx3 and those not expressing it) which could account for the discrepancy in the results. We used CD4<sup>+</sup>CD25<sup>+</sup>Fopx3<sup>+</sup> as specific markers for Treg cells, and our results were similar to the reports using CD25<sup>bright</sup> CD4<sup>+</sup> as specific markers. Among CD4<sup>+</sup>CD25<sup>+</sup> cells, only those expressing large amounts of CD25 (CD4<sup>+</sup>CD25<sup>bright</sup>) exert suppressive effects. Cells expressing intermediate amounts of CD25, in contrast, are effector CD4 cells [31].

It has been documented that the frequencies of T<sub>H</sub>17 cells were higher in PBMCs of RA patients compared to healthy controls [15,16,32]. Our study as well confirmed such finding, especially with the highly and moderately active RA patients. The difference was noted suggesting a potential predominant role of T<sub>H</sub>17 cells in the inflammatory process and chronic progression in RA. Our observations supported the idea that T<sub>H</sub>17 cells contributed to the pathogenesis of RA.

In contrast to our study, Yamada et al. reported that the frequency of T<sub>H</sub>17 cells was neither increased in RA ( $n = 123$ ), nor correlated with the DAS28 disease activity score. In addition, such cells were significantly decreased in the joints compared with PBMC of the same patients, whereas T<sub>H</sub>1 cells were more abundant in the joints than in PBMC in these established RA patients [33]. The reason for this discrepancy is unclear, but may be due to methods for assessing disease activity and detecting T<sub>H</sub>17 cells.

Considering contradictory results of frequency of T<sub>H</sub>17 cells and Treg cells, we calculated the T<sub>H</sub>17/Treg ratio for each sample. Healthy controls exhibited a low T<sub>H</sub>17/Treg ratio, while RA patients, especially active RA patients, have a high T<sub>H</sub>17/Treg ratio. This result indicated that the balance between T<sub>H</sub>17 and Treg characterized by low T<sub>H</sub>17/Treg ratio may be compromised in RA disease. Such T<sub>H</sub>17/Treg imbalance may play a pivotal role in the development of RA, because predominant T<sub>H</sub>17 cells can exert strong pro-inflammatory effects by producing IL-17. The imbalance of TH17/Treg has been also reported in several inflammatory disorders, such as primary biliary cirrhosis and inflammatory bowel disease [34].

We next investigated serum proinflammatory T<sub>H</sub>17 cytokine profile in patients with RA, two related cytokines were measured; IL17 and IL23. IL-17 can promote inflammation by inducing the production of pro-inflammatory mediators, including cytokines, chemokines and other mediators of bone and cartilage destruction such as metalloproteinases [7]. IL-23 promotes the expansion and survival of

T<sub>H</sub>17 cells. IL 23 promotes T<sub>H</sub>17 cells producing tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-17, IL-6, IL-22, and GM-CSF which are associated with the induction of autoimmune inflammation in RA disease [21]. IL-17 showed a four-fold increase among patients while IL23 showed approximately two-fold increase in serum level as compared to controls. The serum level of IL17 and IL23 correlated well with the T<sub>H</sub>17 percentages in the blood of RA patients (p=0.01). Considering higher frequencies of T<sub>H</sub>17 cells and its two related cytokines in active RA in our study, these results are in accordance with the findings of other groups [35-38].

Nevertheless, our results also demonstrated that T<sub>H</sub>17/Treg ratios were positively correlated with serum concentrations of T<sub>H</sub>17 related cytokines, which further confirmed the T<sub>H</sub>17/Treg dysbalance in peripheral blood of RA patient. Skapenko et al., reported that cytokine profile in peripheral blood of active RA patients may provide suitable environment for the differentiation of T<sub>H</sub>1/T<sub>H</sub>17 cells but not suitable for Treg cells [39].

We reported a significant correlation between DAS score and the decrease in frequency of Treg cells on one hand, and the increase in T<sub>H</sub>17 percentages, serum IL17 and serum IL23 of RA patients on the other hand. The lower the frequency of Tregs, the higher the DAS score reflecting higher disease activity. Several studies also investigated possible correlations between IL17A or T<sub>H</sub>17 cell proportion and disease activity. Most studies reported a good correlation with DAS score for T<sub>H</sub>17 and related cytokines, or showed that IL17 was significantly higher among severely active patients, as compared to controls or to patients with inactive disease [35,36,38-40]. Others didn't report any correlation between Treg cell frequencies or cytokines with DAS score [41].

In summary, our results indicated that there would be a T<sub>H</sub>17/Treg imbalance in peripheral blood of RA patients, and such disturbed balance may partly be due to the serum cytokine effects on microenvironment, which correlated with Disease Activity Score, especially with RA group III.

Our results were in line with the study of Niu et al. [42] and Wang et al. [43] who indicated that development of RA is associated with peripheral T<sub>H</sub>17/Treg imbalance, and characterized by continuing generation of T<sub>H</sub>17 cells. T<sub>H</sub>17/Treg balance may be modified to restore homeostasis. The combination of the treatment that manipulates key cytokines with the treatment that target T<sub>H</sub>17/Treg imbalance, may lead the road to development of novel and effective immunotherapy for RA.

## Declaration of interest

Authors declare that they have no conflict of interest.

## Source or Research Support

This work has been partially sponsored by the National Research Center, Cairo.

## Acknowledgement

We would like to thank the National Research Centre and colleagues in Immunology and Rehabilitation Department, Cairo University for their help.

## References

1. Goronzy JJ, Weyand CM (2001) T cell homeostasis and autoreactivity in rheumatoid arthritis. *Curr Dir Autoimmun* 3: 112-132.
2. Ohkura N, Kitagawa Y, Sakaguchi S (2013) Development and maintenance of regulatory T cells. *Immunity* 38: 414-423.
3. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, et al. (2005) Interleukin 17-producing CD4 effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 6: 1123-1132.
4. Nakayamada S, Takahashi H, Kanno Y, O'Shea JJ (2012) Helper T cell diversity and plasticity. *Curr Opin Immunol* 24: 297-302.
5. Boniface K, Blom B, Liu YJ, de Waal Malefyt R (2008) From interleukin-23 to T-helper 17 cells: human T-helper cell differentiation revisited. *Immunol Rev* 226: 132-146.
6. Bettelli E, Oukka M, Kuchroo VK (2007) T(H)-17 cells in the circle of immunity and autoimmunity. *Nat Immunol* 8: 345-350.
7. Agarwal S, Misra R, Aggarwal A (2008) Interleukin 17 levels are increased in juvenile idiopathic arthritis synovial fluid and induce synovial fibroblasts to produce proinflammatory cytokines and matrix metalloproteinases. *J Rheumatol* 35: 515-519.
8. Nakae S, Nambu A, Sudo K, Iwakura Y (2003) Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice. *J Immunol* 171: 6173-6177.
9. Boissier MC, Assier E, Falgarone G, Bessis N (2008) Shifting the imbalance from Th1/Th2 to Th17/treg: the changing rheumatoid arthritis paradigm. *Joint Bone Spine* 75: 373-375.
10. Eisenstein EM, Williams CB (2009) The T(reg)/Th17 cell balance: a new paradigm for autoimmunity. *Pediatr Res* 65: 26R-31R.
11. Wang W, Shao S, Jiao Z, Guo M, Xu H, et al. (2012) The Th17/Treg imbalance and cytokine environment in peripheral blood of patients with rheumatoid arthritis. *Rheumatol Int* 32: 887-893.
12. Ichikawa T, Kageyama Y, Kobayashi H, Kato N, Tsujimura K, et al. (2010) Etanercept treatment reduces the serum levels of interleukin-15 and interferon-gamma inducible protein-10 in patients with rheumatoid arthritis. *Rheumatol Int* 30: 725-730.
13. Alunno A, Manetti M, Caterbi S, Ibba-Manneschi L, Bistoni O, et al. (2015) Altered Immunoregulation in Rheumatoid Arthritis: The Role of Regulatory T Cells and Proinflammatory Th17 Cells and Therapeutic Implications. *Mediators of Inflammation* 2015: 751793.
14. Afzali B, Lombardi G, Lechler RI, Lord GM (2007) The role of T helper 17 (Th17) and regulatory T cells (Treg) in human organ transplantation and autoimmune disease. *Clin Exp Immunol* 148: 32-46.
15. Chen DY, Chen YM, Chen HH, Hsieh CW, Lin CC, et al. (2011) Increasing levels of circulating Th17 cells and interleukin-17 in rheumatoid arthritis patients with an inadequate response to anti-TNF- $\alpha$  therapy. *Arthritis Res Ther* 13: R126.
16. Zhang L, Li Y-g, Li Y-h, Qi L, Liu X-g, et al. (2012) Increased Frequencies of Th22 Cells as well as Th17 Cells in the Peripheral Blood of Patients with Ankylosing Spondylitis and Rheumatoid Arthritis. *PLoS ONE* 7: e31000.
17. Lubberts E (2010) Th17 cytokines and arthritis. *Semin Immunopathol* 32: 43-53.
18. Sarkar S, Fox DA (2007) Regulatory T cell defects in rheumatoid arthritis. *Arthritis Rheum* 56: 710-713.
19. Dejaco C, Duftner C, Klauser A, Schirmer M (2010) Altered T-cell subtypes in spondyloarthritis, rheumatoid arthritis and polymyalgia rheumatica. *Rheumatology International* 30: 297-303.
20. Moradi B, Schnatzer P, Hagmann S, Rosshirt N, Gotterbarm T, et al. (2014) CD4<sup>+</sup> CD25<sup>+</sup>/high CD127 low/ regulatory T cells are enriched in rheumatoid arthritis and osteoarthritis joints—analysis of frequency and phenotype in synovial membrane, synovial fluid and peripheral blood. *Arthritis Research & Therapy* 16: 97.
21. Brennan FM, McInnes IB (2008) Evidence that cytokines play a role in rheumatoid arthritis. *J Clin Invest* 118: 3537-3545.

22. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, et al. (1988) The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 31: 315-324.
23. Chen DY, Chen YM, Lan JL, Lin CC, Chen HH, et al. (2010) Potential role of Th17 cells in the pathogenesis of adult-onset Still's disease. *Rheumatology (Oxford)* 49: 2305-2312.
24. Lawson CA, Brown AK, Bejarano V, Douglas SH, Burgoyne CH, et al. (2006) Early rheumatoid arthritis is associated with a deficit in the CD4+CD25high regulatory T cell population in peripheral blood. *Rheumatology (Oxford)*; 45: 1210-1217.
25. Sempere-Ortells JM, Pe´rez-Garcia V, Marin-Alberca G, Peris- Pertusa A, Benito JM, et al. (2009) Quantification and phenotype of regulatory T cells in rheumatoid arthritis according to disease activity score-28. *Autoimmunity* 42: 636-645.
26. Chen R, Tao Y, Qiu K, Huang W, Huang C, et al. (2012) Association of circulating Treg cells with disease activity in patients with rheumatoid arthritis. *Nan Fang Yi Ke Da Xue Xue Bao* 32: 886-889.
27. Jiao Z, Wang W, Jia R, Li J, You H, et al. (2007) Accumulation of FoxP3-expressing CD4+CD25+ T cells with distinct chemokine receptors in synovial fluid of patients with active rheumatoid arthritis. *Scand J Rheumatol* 36: 428-433.
28. Li N, Ma T, Han J, Zhou J, Wang J, et al. (2014) Increased apoptosis induction in CD4+ CD25+ Foxp3+ T cells contributes to enhanced disease activity in patients with rheumatoid arthritis through IL-10 regulation. *Eur Rev Med Pharmacol Sci* 18: 78-85.
29. Chavele KM, Ehrenstein MR (2011) Regulatory T-cells in systemic lupus erythematosus and rheumatoid arthritis. *FEBS Lett* 585: 3603-3610.
30. Han GM, O'Neil-Andersen NJ, Zurier RB, Lawrence DA (2008) CD4+CD25high T cell numbers are enriched in the peripheral blood of patients with rheumatoid arthritis. *Cell Immunol* 253: 92-101.
31. Wan YY, Flavell RA (2007) Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. *Nature* 445: 766-770.
32. Kim J, Kang S, Kim J, Kwon G, Koo S (2013) Elevated Levels of T Helper 17 Cells Are Associated with Disease Activity in Patients with Rheumatoid Arthritis. *Ann Lab Med* 33: 52-59.
33. Yamada H, Nakashima Y, Okazaki K, Mawatari T, Fukushi JI, et al. (2008) Th1 but not Th17 cells predominate in the joints of patients with rheumatoid arthritis. *Ann Rheum Dis* 67: 1299-1304.
34. Eastaff-Leung N, Mabarrack N, Barbour A, Cummins A, Barry S (2010) Foxp3+ regulatory T cells, Th17 effector cells, and cytokine environment in inflammatory bowel disease. *J Clin Immunol* 30: 80-89.
35. Metawi SA, Abbas D, Kamal MM, Ibrahim MK (2011) Serum and synovial fluid levels of interleukin-17 in correlation with disease activity in patients with RA. *Clin Rheumatol* 30: 1201-1207.
36. Mei Y, Pan F, Gao J, Ge R, Duan Z, et al. (2011) Increased serum IL-17 and IL-23 in the patient with ankylosing spondylitis. *Clin Rheumatol* 30: 269-273.
37. Paradowska-Gorycka A, Grzybowska-Kowalczyk A, Wojtecka-Lukasik E, Maslinski S (2010) IL-23 in the Pathogenesis of Rheumatoid Arthritis. *Scand J Immunol* 71: 134-145.
38. Melis L, Vandooren B, Kruithof E, Jacques P, De Vos M, et al. (2010) Systemic levels of IL-23 are strongly associated with disease activity in rheumatoid arthritis but not spondyloarthritis. *Ann Rheum Dis* 69: 618-623.
39. Skapenko A, Leipe J, Lipsky PE, Schulze-Koops H (2005) The role of the T cell in autoimmune inflammation. *Arthritis Res Ther* 7: S4-14.
40. Miao J, Zhang K, Lv M, Li Q, Zheng Z, et al. (2014) Circulating Th17 and Th1 cells expressing CD161 are associated with disease activity in rheumatoid arthritis. *Scand J Rheumatol* 43: 194-201.
41. Al-Zifzaf D, El Bakry S, Mamdouh R, Shawarbyc L, Abdel A, et al. (2015) FoxP3+T regulatory cells in Rheumatoid arthritis and the imbalance of the of the Treg/TH17 cytokine axis. *The Egyptian Rheumatologist* 37: 7-15.
42. Niu Q, Cai B, Huang ZC, Shi YY, Wang LL (2012) Disturbed Th17/Treg balance in patients with rheumatoid arthritis. *Rheumatol Int* 32: 2731-2736.
43. Wang W, Shao S, Jiao Z, Guo M, Xu H, et al. (2012) The Th17/Treg imbalance and cytokine environment in peripheral blood of patients with rheumatoid arthritis. *Rheumatol Int* 32: 887-893.