Aim of the work: To investigate the frequency of FoxP3+CD4+CD25+high cells (Tregs) in rheumatoid arthritis (RA) patients and their association with clinical and radiological parameters. Also to study the possible relationship between Tregs and TGF-β as an indicator of Treg function, with IL17 as an indicator of Th17 function and with the ratio between them to throw light on the imbalance between these two cytokines in RA.

Patients and methods: Forty RA patients and 20 age and sex matched healthy controls were enrolled in the study. Patients underwent clinical, laboratory and radiographic assessment. The frequency of Tregs was determined by flowcytometry. Serum IL-17 and TGF-β cytokines were analyzed using ELISA.

Results: The frequency of Tregs was significantly decreased among RA patients and with a parallel significant decrease in the mean fluorescence intensity (MFI) of FoxP3. Both IL-17 and TGFβ were significantly increased. Tregs, IL-17 and TGF-β did not correlate with any of the clinical findings, laboratory and radiographic scores. TGFβ:IL-17 ratio dropped from 15.8 ± 9.6 among controls to 5.33 ± 5 among patients with mild-to-moderate activity and 2.45 ± 1.8 among patients with severe activity.
1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by synovial inflammation and hyperplasia, autoantibody production, cartilage and bone destruction, and systemic features, including cardiovascular, pulmonary, psychological, and skeletal disorders [1]. The pathogenesis of RA is multifaceted and involves T cells, B cells and the complex interaction of many pro-inflammatory cytokines [2]. Advances in understanding the pathogenesis of the disease have fostered the development of new therapeutics, with improved outcomes [1].

In RA, it is well known that an imbalance between pro- and anti-inflammatory cytokine activities favors the induction of autoimmunity, chronic inflammation and thereby joint damage. However, it is less clear how cytokines are organized within a regulatory network [3].

A novel hypothesis has been proposed that the T helper 17 (Th17)/Regulatory T-cell (Treg) imbalance, along with Th1/Th2 imbalance, may be responsible for the development and progression of RA [4]. The CD4+ CD25 high regulatory T-cells are among the most important cells in the immune system regulation. They can control autoimmune T cells and B cells, which escape negative selection in the thymus. They block activation and effector functions of autoreactive T cells maintaining peripheral self-tolerance and preventing the development of various inflammatory diseases through direct contact with effector immune cells and the secretion of anti-inflammatory cytokines, such as IL-10 and TGF-β [5,6]. Tregs are divided into several populations. Naturally occurring Tregs, which express the forkhead transcription factor (FoxP3), are found in the thymus and in the peripheral blood, where they account for 5–10% of CD4+ T cells. FoxP3 is responsible for the development of Tregs in the thymus. It is also needed to maintain the suppressive activity of mature peripheral Tregs and is a specific molecular marker for Tregs in the human peripheral blood. Decreased FoxP3 expression causes conversion of Tregs into effector cells [7,8]. Thus, FoxP3 expression is crucial for the identification of Tregs bearing suppressive activity [9].

It has been reported that Tregs regulate the effector function of T-helper cells (i.e. Th1, Th2 and Th17 cells) [10]. Th17 subset of T-helper cells is pro-inflammatory; it plays vital roles in host defense and has been shown to be involved in the pathogenesis of autoimmune and inflammatory diseases primarily by secreting IL-17 which activates numerous cell types involved in the pathogenesis of RA, including synovial fibroblasts, monocytes, macrophages, chondrocytes, and osteoblasts [11,12].

It is therefore clear that Th17 and Treg cells have a functional antagonism, in which Tregs act as immunosuppressive cells and Th17 cells are involved in inducing autoimmunity. Tregs and Th17 effectors arise in a mutually exclusive fashion. TGF-β plays a complex and intertwined role in inflammation, T cell lineage commitment, antibody generation, immune suppression, and tolerance. TGF-β produced in the immune system will suppress the generation of Th17 effector cells and induce FoxP3+ Tregs and thereby maintain self-tolerance. It appears that TGF-β is the mediator of Treg’s potent immune suppression and regulation of cell differentiation [6,13].

Data regarding the number of Tregs in the circulation of RA patients compared to healthy individuals are inconclusive and contradictory which caused recent investigations into Tregs with focus on the balance between Tregs and other proinflammatory T-cell populations such as Th17 cells [14].

The aim of the present study was to investigate the frequency of FoxP3+ CD4+ CD25+ high cells (Tregs) in rheumatoid arthritis (RA) patients and their association with clinical and radiological parameters. Also to study the possible relationship between Tregs and TGF-β as an indicator of Treg function, with IL17 as an indicator of Th17 function and with the ratio between them to throw light on the imbalance between these two cytokines in RA.

2. Patients and methods

2.1. Clinical assessment

The present study is an observational cross-sectional one that included 40 patients with RA fulfilling the new 2010 EULAR/ACR criteria [15]. Twenty age and sex matched healthy subjects were also included in the study and served as the control group. Patients were recruited from inpatient and outpatient clinic of the Internal Medicine Department-Rheumatology division as well as from the outpatient clinic of Physical Medicine, Rheumatology and Rehabilitation Department of Ain Shams University Hospitals. Written consent was obtained from all patients after a full explanation of the study which was approved by the local ethics committee. Patients with other autoimmune disorders or those receiving any biologic therapy were excluded from the study.

Patients underwent detailed history taking, thorough general and musculoskeletal examination. Pain was assessed by a 0–100 mm horizontal visual analogue scale (VAS). 0 indicated no pain while 100 indicated the worst intolerable pain. Measurement of the 28-joint count of tender and swollen joint with calculation of the disease activity score (DAS-28) for each RA patient was done. Patients with score ≤3.2 were classified as having low disease activity, >3.2 to ≤5.1 were classified as having moderately active disease while >5.1 denoted very active disease [16].

Functional disability was evaluated using the Health Assessment Questionnaire (HAQ) to calculate the disability index (DI). The eight categories assessed by the DI are (1)
dressing and grooming, (2) arising, (3) eating, (4) walking, (5) hygiene, (6) reach, (7) grip, and (8) common daily activities. The difficulty during each of these acts was assessed as follows: zero = without any difficulty, one = with some difficulty, two = with much difficulty and three = unable to do, then the sum of the categories scores is calculated and divided by the number of categories. This gives a score in the 0–3 range [17].

2.2. Laboratory assessment

Seven milliliters of venous blood was drawn under aseptic conditions from each subject which were divided into three tubes: an ethylene diamine tetra-acetate (EDTA) tube for CBC and flow cytometric analysis; a plain tube in which blood was left to clot and samples were then centrifuged and sera were separated to be used for assaying CRP, RF, serum IL-17 and serum TGF-β; and finally 2 ml into a sodium citrate tube for ESR.

- Complete blood count (Cell Dyne-2700, Abbott Lab., USA).
- Erythrocyte sedimentation rate (ESR) using Westergren method.
- C-reactive protein (CRP) was assessed by latex agglutination using AVITEX CRP supplied by Omega Diagnostics Ltd.
- Rheumatoid factor (RF) was detected by the kit supplied by Biotec Laboratories based on agglutination test using particles sensitized with human IgG.

2.3. Assessment of T regulatory FoxP3+CD4+CD25high

The frequency of FoxP3+CD4+CD25high was carried out by direct immunofluorescence technique using Coulter EPICS XL Flow cytometry system, which was performed on the peripheral blood of both patients and controls. For each case, 2 tubes were prepared; the test sample and the isotypic control sample. As for the test sample, 50 µL of whole anticoagulated blood was lysed using 1 mL Lysing Solution (Sigma chemical co., St. Louis, MO) followed by washing with phosphate buffer saline (PBS). The cells were stained with combinations of anti-CD25-Phycocerythrin (PE) [eBioscience, USA, clone (BC96)] and anti-CD4-Fluorescein isocyanate (FITC) [eBioscience, USA, clone (RPA-T4)]. Samples were incubated in the dark for 20 min which was followed by washing with PBS. Cell pellet was resuspended in 0.5 mL of freshly prepared fixation/permeabilization working solution (eBioscience, USA) and incubated for 30 min at 4 °C in the dark. This was followed by washing once with PBS then washing with 1 mL of 1× permeabilization buffer (eBioscience, USA). Ten microliters of Phycocerythrin-Cy5 (PE-Cy5)-conjugated anti-humanFoxP3 [eBioscience, USA, clone (PCH101)] was added and incubated for 30 min at 4 °C in dark. After washing with PBS, the cells were resuspended in PBS for analysis. For the isotypic control sample, the same steps were followed except that instead of adding anti-CD25-(PE), PE isotypic control (Beckman Coulter Immunotech, France) was added and instead of adding (PE-Cy5)-conjugated anti-humanFoxP3, PE-Cy5 isotypic control (eBioscience, USA) was added. Data acquisition and analysis were performed on flow cytometry (EPICS XL coulter, France) using SYSTEM II version 3 software with a standard 3-color filter configuration.

Data interpretation: Lymphocytes were gated via their forward and side scatter properties, and T cells were identified based on their expression of CD4. To discriminate between CD25high T-regs and CD25low activated effector-memory T cells, we used CD25 expression on non CD4+ cells as an internal control. They only express intermediate levels of CD25 (CD25low), whereas CD4+ T cells express CD25 with high (CD25high) or intermediate (CD25low) intensities. Only CD4+ cells expressing CD25 with higher intensities than the internal control cells were included in the gate for CD25high cells. The gate for CD25low cells was set to include cells expressing CD25 at levels above those of the isotype control but at lower expression levels than the CD25high cells. The amount of FoxP3 per cell [mean fluorescence intensity (MFI)] was calculated in CD4+CD25high T cell populations.

2.4. Assessment of serum IL-17 and TGF-β

ID ELISA Human IL-17 and TGF-β kits (ID Labs biotechnology Inc, Canada) were used for assessment of serum IL-17 and TGF-β respectively. The method is solid phase sandwich ELISA (Enzyme-linked immunosorbent Assay). The concentration of standards was plotted against their optical densities on a semi-log graph. The concentrations of samples were read from this graph.

2.5. Radiologic assessment

Plain X-rays were done on both hands and wrist joints. Scoring was done using the Modified Steinbrocker scoring method. This includes the wrist and metacarpophalangeal joints of both hands. According to the scoring 0 is normal, 1 reflects juxta-articular osteopenia or soft tissue swelling, 2 denotes the presence of erosions, 3 the presence of erosion and joint space narrowing and 4 total joint destruction, either lysis or ankylosis [18]. Total X-ray scoring was done by adding the score of the 12 joints, where the maximum score is 48.

Musculoskeletal Ultrasound (MSUS) using real time ultrasonography was performed by Philips HDI 200 ultrasound machine with linear 7–12 MHz probe on the wrist and metacarpophalangeal (MCP) joints of the hands for detection of synovitis which is defined as Hypoechoic or anechoic synovial hypertrophy. Semiquantitative scoring systems were used to assess both synovial hypertrophy and erosions; for hypertrophy, the used scoring system ranges from 0 to 3, where 0 = absence of visible synovial hypertrophy, 1 = mild synovial hypertrophic, 2 = moderate synovial hypertrophy with bulge above the line of adjacent bone, 3 = marked synovial hypertrophy with extension to one or more of the diaphyses. The same scoring system was applied to erosions where 0 = absence of erosions, 1 = mild erosions, 2 = moderate erosions, 3 = severe erosions. Total US scoring was done by adding the score of the 12 joints for both hypertrophy and erosions, maximum score was 72 [19,20].

Statistical analysis: Statistical software package “SPSS” version 9.05 was used. The descriptive data for quantitative data were expressed as ranges, mean, standard deviation (SD) and numbers and percentages for qualitative data. Student’s t test was used to compare between two independent
means, Wilcoxon Rank Sum test to compare between two independent groups for nonparametric data, Chi-square test to compare the qualitative data between different groups and Pearson’s correlation coefficient, for relationship between different variables in the same group.

3. Results

The current study included forty RA patients (33 females and 7 males). Their ages ranged from 22 to 49 years with a mean of 39.54 ± 7.67 years. Twenty age and sex matched healthy individuals were included in the study as a control group. The descriptive data of the patients are shown in Table 1.

Radiological evaluation of patients using X-rays according to the modified Steinbrocker scoring method revealed a mean score of 12 ± 2.9. Musculoskeletal US examination and scoring confirmed the presence of bony erosions in all of our patients with a combined ultrasonography score of 13.33 ± 7.26 (hypertrophy score 7.04 ± 4.51, erosions score 6.29 ± 3.01) (Fig. 1).

The distribution of FoxP3+CD4+CD25+ high cells (Tregs) revealed a highly significant decrease in the frequency of Treg cells in RA patients compared to healthy controls (16.86 ± 6.16% and 26.38 ± 14% respectively, p = 0.008) as well as a highly significant decrease in the MFI of FoxP3 on the surface of patient Tregs (2.37 ± 0.73% and 6.88 ± 7.26% respectively, p = 0.000) (Figs. 2 and 3).

We assessed the serum levels of TGF-β1 and IL17 as well as the ratio between them among both RA patients and healthy controls and significant rise in both cytokine levels was found (16.86 ± 6.16% and 26.38 ± 14% respectively, p = 0.008) as well as a highly significant decrease in the MFI of FoxP3 on the surface of patient Tregs (2.37 ± 0.73% and 6.88 ± 7.26% respectively, p = 0.000) (Figs. 2 and 3).

It has been suggested that the balance between Th17 and Treg cells may be abnormal in RA and this imbalance may play a pivotal role in RA pathogenesis because predominant Th17 cells can exert strong pro-inflammatory response by producing IL-17 and impaired Tregs, partly due to the cytokine microenvironment, cannot control this immune response [23].

In this study, flowcytometric analysis in RA patients revealed a highly significant decrease in the frequency of circulating FoxP3CD4+CD25high Tregs as compared to healthy controls (p < 0.001). This result was in agreement with other groups [24–27]. 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. Tregs accumulated in inflamed joints express high levels of surface and intracellular CTLA-4, GITR, OX-40, and FoxP3 [28]. Moreover, Tregs were found to display increased tendency to undergo spontaneous apoptosis in active RA [29,30]. 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 [14]. This decline in regulatory T-cell numbers may pre-dispose to persistent auto-immune diseases including RA [24].

Contrary to our results there are reports of increased Tregs in RA peripheral blood [31,32] but in their research they assessed different CD4+CD25+ T cell subtypes (including CD4+ T-cells expressing low levels of CD25 and those expressing FoxP3 and those not expressing it) which could account for the discrepancy in the results.

In our study we reported lack of correlation between Treg percentages in the blood, with all the clinical findings, laboratory tests and radiographic assessment of RA joints (both by
X-ray and US). Our observation is also concomitant with a previous study which stated that the frequencies of synovial CD4+CD25high T cells in patients with RA was not correlated with clinical parameters such as disease duration, the presence of rheumatoid factor, the level of CRP and the presence of erosions. These data suggest that the presence of...
CD4+CD25+ T cells in the rheumatoid synovium is a function of the disease but unrelated to treatment, clinical course and disease activity [31]. Han et al. have also shown that no relationship exists between the number of CD4+CD25high T cells and RA disease activity [32].

The forkhead box P3 (FoxP3) transcription factor is now considered the most specific marker of Tregs as it is essential for both Treg cell development and function [33]. Assessing MFI of FoxP3 in Tregs showed a highly significant decrease among RA patients than healthy controls (p < 0.001) which agrees with the reports of other groups [34,27]. This decline in FoxP3 expression could explain the depressed suppressive capacity of T-reg cells of RA patients as FoxP3 amplifies and stabilizes molecular features of Treg precursor cells, beneficial to their function and maintenance, and reverses features harmful to Treg cell function. Decreased FoxP3 expression enhances autoimmune responses by weakening the suppressive function of Tregs and converting them into effector cells [35,8,36]. This result was not in agreement with Han et al. who showed that the FoxP3+ cells from RA patients expressed more FoxP3 per cell when compared to healthy controls [32].

The production of IL-17 plays a crucial role in the development of cartilage and bone lesions as 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 metalloproteinase.

![Figure 3](image.png)

**Figure 3** Median percentages of FoxP3CD4+CD25high Tregs (Left) and mean fluorescence intensity (MFI) on Tregs (Right) among control and rheumatoid arthritis cases.

![Figure 4](image.png)

**Figure 4** Comparison between control and cases as regards median values of serum IL-17 and serum TGF-β.

<table>
<thead>
<tr>
<th>Cytokine mean ± SD</th>
<th>Controls (n = 20)</th>
<th>RA patients (n = 40)</th>
<th>Z</th>
<th>p</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL17 (ng/mL)</td>
<td>2.01 ± 2.7</td>
<td>10.9 ± 4.9</td>
<td>-4.27</td>
<td>0.000</td>
<td>HS</td>
</tr>
<tr>
<td>TGF-β1 (ng/mL)</td>
<td>16.4 ± 5.8</td>
<td>35.5 ± 10.7</td>
<td>-4.36</td>
<td>0.000</td>
<td>HS</td>
</tr>
<tr>
<td>TGF: IL17 ratio</td>
<td>15.8 ± 9.6</td>
<td>4.8 ± 4.7</td>
<td>-3.37</td>
<td>0.001</td>
<td>HS</td>
</tr>
</tbody>
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IL17: interleukin-17, TGF: transforming growth factor.
as [37]. Our study revealed a highly significant 5 fold increase in IL-17 serum level among RA patients. These results are in accordance with the findings of other groups [38–43]. Some studies have shown that plasma IL-17 levels in RA patients did not differ from those in OA patients or healthy controls [35,44,45]. Though IL-17 serum level did not correlate with the DAS28 score yet it was significantly higher among severely active patients as compared to patients with moderate activity which agrees with the reports of other groups [41].

The major source of TGF-β1 in the immune system is FoxP3-expressing regulatory T cells, Local TGF-β1 activation seems to be necessary for both immune suppression and Th17 generation [46,47]. TGF-β1 has been shown to induce FoxP3, which is responsible for the suppression activity of Tregs [47].

We reported a significant increase in the serum level of TGF-β1 among rheumatoid patients as compared to controls. Similar findings were reported by other groups [48,49]. TGF-β1 did not correlate with clinical or radiological findings. This disagrees with the findings of Mieliauskaite et al., who reported correlations between TGF-β1 levels and radiologically defined joint damage determined by the Steinbroocker scoring system.

Although TGF-β1 is well known for its immune-suppressive and anti-inflammatory properties, it is also capable of promoting inflammation. TGF-β1 is expressed abundantly in the rheumatoid synovium and is thought to contribute to the progression of inflammation and joint destruction in RA [50].

In the absence of IL-6, TGF-β1 combined with IL-10, promotes the differentiation of naive T-cells to Tregs, which in turn produce IL-10 and TGF-β1, exerting a nonspecific immunosuppressive effect that can inhibit Th1, Th1, and Th2 cells. In mice, in contrast, combination of TGF-β1 with IL-6 induces naive T-cells to differentiate into Th17 cells [8,51].

Among RA patients both cytokines increased in spite of the decrease in the percentage of Tregs. IL-17 increased over five folds while TGF-β1 increased a little over 2 folds tilting the balance of these two cytokines in the favor of inflammation. This could be a result of the compensatory increase in Treg cell function in an attempt to overcome the systemic immune stimulation in RA patients despite their relative deficiency in the peripheral circulation.

Upon comparing the results of our patients according to their activity level as assessed by the DAS28 we found a rise in serum IL-17 level that did not reach statistical significance accompanied by a significant decrease in serum TGF-β1 which resulted in a significant difference in the TGFβ1: IL-17 ratio. The ratio dropped from 15.8 ± 9.6 among controls to 5.33 ± 5.0 among patients with mild to moderate activity and further decreased to 2.45 ± 1.8 among patients with severe activity. This reflects a shift toward a dominance of IL-17 as a pro-inflammatory cytokine secreted by Th17 cells over the suppressive cytokine TGFβ1 secreted by Tregs.

In conclusion, RA patients show decreased frequency of Tregs that is not associated with clinical parameters or with radiological damage. The IL-17 five fold increase and two fold increase in TGF-β1 prove a disturbance in the balance of the cytokine milieu in the favor of inflammation and draw attention to the importance of considering the interplay of the Treg/Th17 Cytokine Axis in the pathogenesis of RA and hence aiming at restoring that balance during treatment.

Conflict of interest

All authors have no conflict of interest.

References


