Th1-Th17 Ratio as a New Insight in Rheumatoid Arthritis Disease

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ABSTRACT

The Th17, Th1 and dual Th17/Th1 cells are important players in rheumatoid arthritis (RA) disease. To assess their roles, the frequency and impact of these cells were investigated in patients with different disease activity.

In 14 new cases and 41 established RA patients in comparison with 22 healthy controls, the percentages of Th17, Th1 and dual Th17/Th1 cells were determined by flow-cytometry and their correlations were investigated with disease activity score (DAS28). Moreover, serum levels of IL-6 and IL-17 as inducer and functional cytokines for Th17 were investigated. Finally, serum levels of anti citrullinated protein antibody (ACPA) and rheumatoid factor (RF) were assessed.

Percentage of Th17 cells in RA patients were increased in comparison with healthy controls ($p<0.01$). In correlation with this finding, IL-17 and IL-6 cytokines in RA patients also increased ($p<0.01$). The Th1 cells in RA patients were less than healthy group ($p<0.05$) and showed negative correlation with disease activity (r=-0.328, $p<0.01$). Dual Th17/Th1 cell only in new cases of RA were more than healthy control groups ($p<0.01$). The Th1/Th17 ratio in RA patients is statistically different with healthy control group ($p<0.01$) and it has negative correlation with disease activity (r=-264, $p<0.05$). The levels of ACPA and RF were increased with disease progression.

Decreasing of Th1/Th17 ratio in RA patient suggested a new paradigm in the field of autoimmune disease and indicated that imbalance or plasticity between these subsets can be important in progress, diagnosis and therapy of RA disease.

Keywords: Rheumatoid arthritis; Th1 cells; Th17 cells

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INTRODUCTION

Rheumatoid arthritis (RA) is one of the most common autoimmune-related disorders that affecting 1% of population with persistent inflammation and

symmetrical pattern in joint synovium membrane.[1] Women are affected three times more than men and the peak age of onset being around the fourth and fifth decades of life.[2] Related antigen in the joints induce immune response that can result in inflammatory symptoms and signs such as pain , swelling that ultimately can lead to structural damage in bone and cartilage.[3,4] The anti-citrullinated protein antibody (ACPA) is found in approximately 70-90% of RA patient and is highly specific for RA.[5] Thus, it is valuable in the diagnosis of this disease. Based on presence of ACPA and RF in serum, RA could be subdivided into two distinct subsets; seropositiveand seronegative RA disease.[6] Despite much progression in understanding of the various aspects of disease, the exact immune-pathomechanism of RA remains to be elucidated. Subtle studies showed presence of the great quantity of autoreactive T cells in affected joint as a key component of immune response.[7] The CD4+ T helper (Th) lymphocytes have central role in the development and regulation of immune responses and they subdivided to different subsets of cells based on the distinct profile of cytokines expression and immunological functions. The type 1 Th (Th1) lymphocytes are induced in response to intracellular pathogens and produce high levels of gamma interferon (IFN-γ) and tumor necrosis factor (TNF). The type 2 Th (Th2) lymphocytes produce interleukin (IL)-4, IL-5, and IL-13 and mediate immunity to extracellular pathogens. In addition to their protective functions, Th1 and Th2 cells are involved in the pathogenesis of immune system diseases.[8,9] A new most important subset of CD4+ Th effector cells named as type 17 T helper (Th17), because of their ability to produce IL-17 family cytokines.[10] In RA disease, the Th17 express the chemokine receptors CCR6 and CCR4. The CCL20 as a ligand for CCR6 is expressed at high levels in inflamed joints.[11-13]

Thus Th17 cells infiltrate into the RA synovium. In contrast to their protective role, Th17 are the typical proinflammatory cells, which lead to induction of inflammation and expansion of autoimmune diseases.[14] This finding indicates that neutralization of IL-17 or blocking of its receptor seems to be effective in RA clinical trials.[15] IL-17 enhances inflammation, cellular infiltration, cartilage degradation and bone erosion in RA disease.[16] Several observations indicate that both Th1 and Th17 cells are important drivers of the inflammatory process in autoimmunity. RA had long

been classified as a Th1- mediated disease, but there is little knowledge about Th17 role in RA. Although both Th17 and Th1 classically viewed as distinct lineages, subtle studies in humans have demonstrated that it could shift towards each other.[17,18] Mainly the plasticity of Th17 leads to differentiated cells that produce both IL-17A and IFN-γ cytokines. These IL-17/IFN-γ-double producers' cells named Th17/Th1 cells (also called non-classic Th1 cells) with both Th17 and Th1 cell characteristics. Th17/Th1 cells have been shown to promote inflammatory responses and to contribute in pathogenesis of different autoimmune diseases such as RA.[19] In the present study; we investigated the Th17, Th1 and dual Th17/Th1 cells frequencies and their association with the different stages and disease activity. The ACPA and RF were also evaluated.

PATIENTS AND METHODS

Patients

Peripheral blood was obtained from fifty-five patients (Male/female: 5/50) in Sayad Shirazi hospital (Gorgan) diagnosed as RA based on the revised classification criteria of the American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) 2010.[20] Moreover, twenty-two healthy controls (Male/female: 3/19) were enrolled in this study without positive family history of autoimmune disease. Disease activity was assessed with the 28-joint disease activity score (DAS28) that is a clinical index of RA disease.DAS28 assessed by the swollen and tender joint count and the CRP or ESR test results. Patients were classified as having active or inactive disease on the basis of the DAS28 scoring. Healthy controls were free of chronic pain, cardiovascular complaints, or other chronic inflammatory diseases. Control group were matched with patients for age and sex (Table 1). New cases have not received disease modifying anti rheumatic drugs (DMARDs) or steroids. This study was approved by the ethics committee of the Golestan University of Medical Sciences (No. 31078693122410)), and written informed consent was obtained from all participants after full explanation of procedures and aim of the study.

Assessment of Anti-citrullinated Protein Antibody (ACPA) and Rheumatoid Factor (RF)

Blood samples were centrifuged and sera were divided into aliquots and stored at -70°C until

Table 1. Demographic details of rheumatoid arthritis patients and healthy controls

Characteristics	New caseRA (n=14)	Established RA (n=41)	Healthy controls (n=23)	*p* value
Age (year), mean±SD	50.64±17.72	46.04±13.42	41.78±11.02	0.466
Sex (male/female)	4/10	1/40	3/20	0.093

assessment. Serum ACPA (IgG) was investigated using a commercially available second-generation ELISA kit (Euroimmun, Germany) which was performed according to the manufacturer's instructions. The experiment were done in duplicate (Positive ≥5 U/mL). RF was measured in all sera by the slide latex agglutination test according to the manufacturer's instructions (Omegadiagnostics, UK).

Frequency of Circulating Th17, Th1 and Th17/Th1 Cells

For flow cytometric analysis, 500 μL of dilute sodium-heparinized whole blood of each sample was cultured in 12-well plates containing complete culture medium RPMI 1640 (Gibco, USA) supplemented with 10% FBS (Gibco, USA), 100 U/mL penicillin (Gibco, USA), 100 μg/mL streptomycin (Gibco, USA)for 12 h in the presence of the cell stimulation cocktail (500X) that contained 40.5 μM of phorbol 12-myristate 13-acetate (PMA) and ionomycin (670 μM). The brefeldin A (5.3 mM) and monensin (1 mM) as protein transport inhibitors were also added at 1X. After stimulation, 100 μL activated cells were then aliquoted into a tube and then stained with peridinin-chlorophyll-protein (PerCP)-Cyanine5.5-labeled anti-human CD3 (Biolegend, USA) and fluorescein isothiocyanate (FITC)-labeled anti-human CD8a (Biolegend, USA) according to the manufacturer's instruction. After elimination of erythrocytes using lysis buffer, cells were fixed and permeabilized with 1X intracellular fixation and permeabilization buffer (eBioscience, USA). Then cells stained with phycoerythrin (PE)-labeled anti-human IL-17A (Biolegend, USA) and allophycocyanin (APC)-labeled anti-human IFN-γ (Biolegend, USA). Isotype matched control antibodies were appropriately used to eliminate the background staining. Finally cells fixed in PBS containing 1% paraformaldehyde and analyzed for cytoplasmic cytokines by 4-color flow cytometry FACS-Calibur (Becton–Dickinson Bioscience, USA) followed by analysis with FlowJo (Tree Star, USA). The profile of the CD3$^+$CD8$^-$T cells was defined by their ability to exclusively express IL-17 (Th17 cells), IFN-γ (Th1 cells), or IL-17 plus IFN-γ (Th17/Th1 cells).

Assessment of Serum IL-6 and IL-17Levels

The serum levels of cytokines were determined by commercial available ELISA kits (Biolegend, USA). IL-6 and IL-17 cytokines were determined according to the manufacturer's instructions. The main parameters affecting on the efficacy of ELISA assay were considered and samples were measured in duplicate.[21,22]

Statistical Analysis

Statistical software SPSS 22.0 (IBM Corp., USA) and Graphpad Prism5.04 (GraphPad Software, USA) were used for data analysis and preparation of graphs. One-Way ANOVA and Kruskal-Wallis tests were used to compare the means of multiple samples for parametric and nonparametric, respectively. *p*-values lower than 0.05 were considered as statistically significant. We also evaluated correlation between different type of T cells and activity of disease (DAS28) using Spearman and Pearson correlation tests.

RESULTS

Percentage of Circulating Th17 in Patients with RA

Our results showed that the percentage of circulating Th17 (CD3$^+$CD8$^-$IL17$^+$) cells were elevated in both new case and established RA patients in comparison with healthy controls (*p*<0.01). Moreover, our data indicated that percentage of Th17 cells did not have statistical difference between two RA groups (*p*>0.05) (Figure 1). Moreover, correlation between these cells and DAS28 was not significant(r=0.103, *p*>0.05).
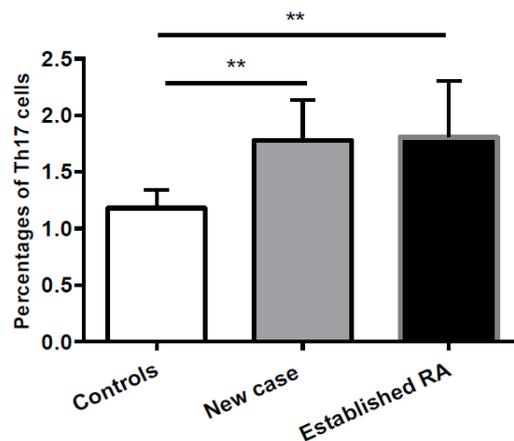
Figure 1. Flow-cytometry analysis of IL-17 expression on gated CD3⁺CD8⁻ T cells. Kruskal-Wallis with Dunn-Bonferroni *post hoc* **test was used to compare the means of samples. Data of each bar demonstrates median±IQR.** *p***-values lower than 0.05 were considered as statistically significant. Results in all three groups showed that frequency of Th17 in new cases and established rheumatoid arthritis patients increased significantly when compared with healthy controls. No statistically difference was seen between new case and established rheumatoid arthritis patients. *****p*<0.01.**

Percentage of Circulating Th1 in Patients with RA

The mean percentage of circulating Th1 (CD3⁺CD8⁻ IFNγ⁺) cells in RA patients have been decreased significantly when compared with healthy control group ($p<0.01$). Moreover, comparison the circulating Th1 between new case and established RA groups indicated that there is not statistically significant difference ($p>0.05$) (Figure 2A). Interestingly, there is

a negative correlation between these cells and DAS28 ($r=-0.353$, $p<0.01$) (Figure 2B).

We also calculated the Th1/Th17 ratio for all samples (Figure3A). In both new case and established RA groups it showed statistically different from healthy control group ($p<0.01$). Moreover, it was revealed that statistically correlated with disease activity in patients groups ($r=-0.264$, $p<0.05$) (Figure 3B).
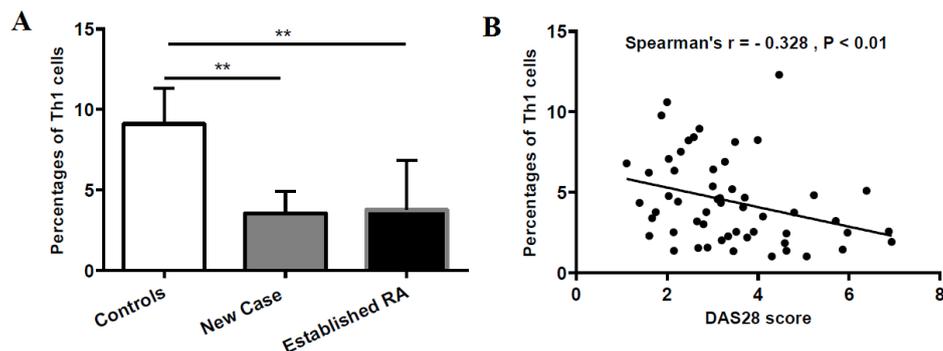

Figure 2. The Th1 and Rheumatoid arthritis (RA) disease: (A) Flow-cytometry analysis of IFN-γ expression on gated CD3⁺CD8⁻ T cells. Kruskal-Wallis with Dunn-Bonferroni *post hoc* **test was used to compare the means of samples. Data of each bar demonstrates median±IQR.** *P***-values lower than 0.05 were considered as statistically significant. Result showed that frequency of Th1 cell in healthy controls significantly more than RA patients. No statistically difference was seen between new case and established RA patients (*****p*<0.01). (B) The correlation between percentages of peripheral blood Th1 cells and disease activity score28 index in RA patients. There is a negative correlation between them (r=-0.328,** *p***<0.01). Correlations were calculated using Spearman's test.**
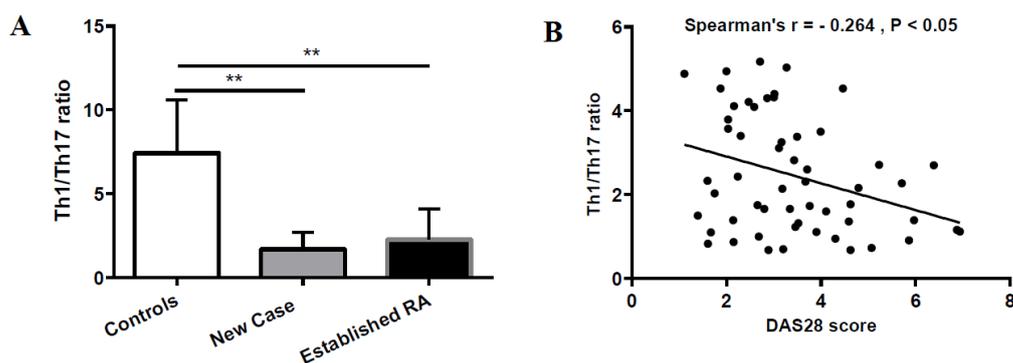
Figure 3. The Th1/Th17 ratio and rheumatoid arthritis disease: (A) the Th1/ Th17 ratio in rheumatoid arthritis patients in comparison to normal subjects. Kruskal-Wallis with Dunn-Bonferroni *post hoc* test was used to compare the means of samples. Data of each bar demonstrates v±IQR. *p*-values lower than 0.05 were considered as statistically significant. Result showed that this ratio in rheumatoid arthritis patients significantly lower than healthy controls. No statistically difference was seen between new case and established RA patients (**p<0.01). (B) Assessment of correlation between the Th1/Th17 ratio with disease activity score28 index in rheumatoid arthritis patients. There is a negative correlation between them (r=-0.264, *p*<0.05). Correlations were calculated using Spearman's test.

Percentages of Circulating Th17/Th1

As shown in Figure 4, the mean percentage of dual Th17/Th1 cells in new case of RA patients is statistically different from healthy controls (*p*<0.01). But in established RA group, it did not show statistically different (*p*>0.05). Our data also revealed that these cells did not have correlation with disease activity (*p*>0.05).

Serum Levels of IL-17 and IL-6 Cytokines

As shown in Fig. 5A and B, mean serum levels of IL-17 and IL-6 cytokines in both new case and established RA patients showed significantly higher than healthy controls (*p*<0.01).But these cytokines in patients with established RA were not significantly different from new cases(*p*>0.05). In RA patients, between serum IL-6 concentrations and percentages of Th1 cells showed negative correlation (r=-0.318, *p*<0.05) (Figure 5C). Moreover, our data indicated that there are significant correlation between mean serum levels of IL-17 and IL-6 (r=0.427, *p*<0.01) (Figure 5D). However, IL-17 and IL-6 did not show significant correlation with ACPA, RF and DAS28 (*p*>0.05).

Percentages of Circulating Th17, Th1 and Th17/Th1 Based on ACPA and RF in RA Patients

Our results showed that ACPA and RF were found in 67.3% and 43.6% of RA patients, respectively (Table 2 and 3).The ACPA antibody could be

detectable in 53.3% of new case and 72.5% of established RA. The results about RF are somewhat similar and it could be detectable in 33.3% of new case and 47.5% of established RA. Moreover, our data indicated that there were no statistical correlations between ACPA, RF and subpopulation of Th cells or DAS28 (*p*>0.05).
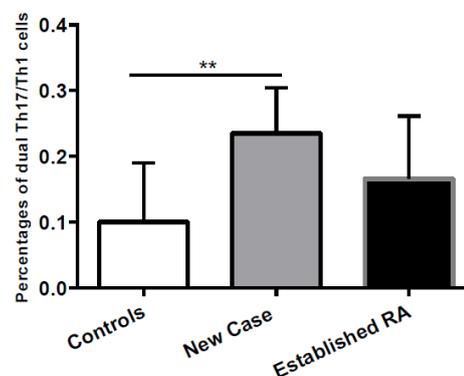

Figure 4. Flow-cytometry analysis of IL-17 and IFN-γ expression on gated CD3⁺CD8⁻ T cells. Kruskal-Wallis with Dunn-Bonferroni *post hoc* test was used to compare the means of samples. Data of each bar demonstrates median±IQR. *P*-values lower than 0.05 were considered as statistically significant. Result showed that frequency of dual Th17/Th1 cells in new case rheumatoid arthritis patients was significantly more than healthy controls. No statistically difference was seen between new case and established RA patients. **p<0.01.
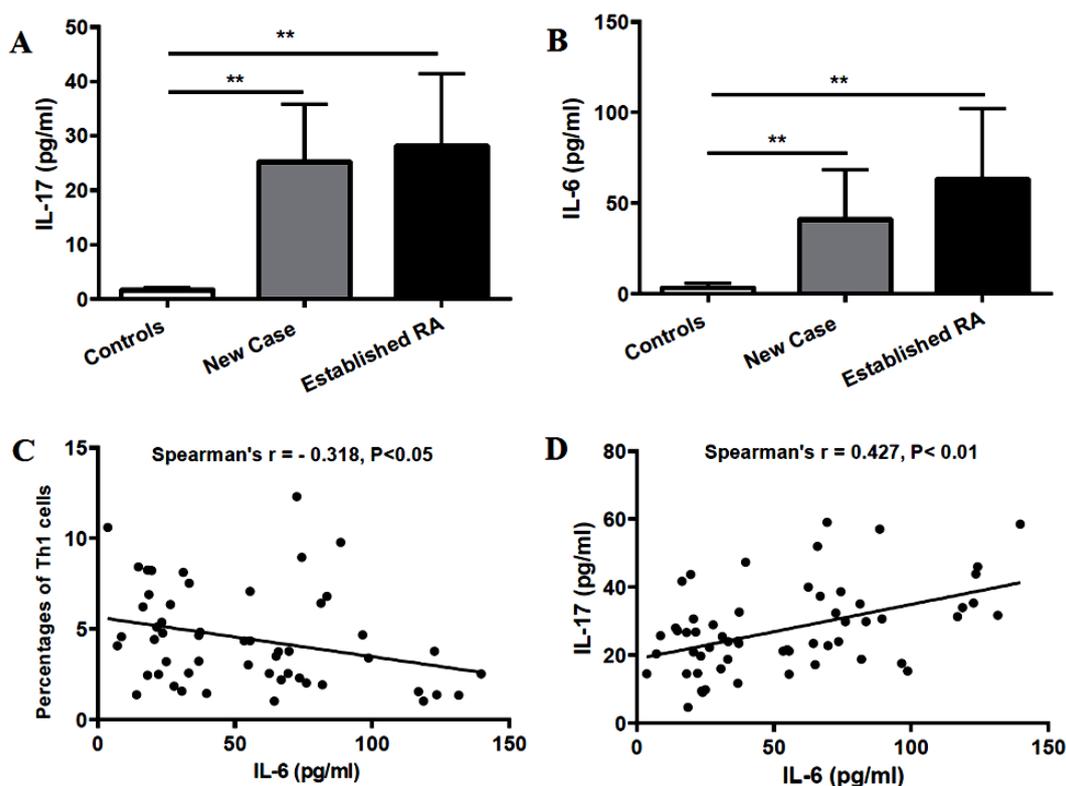
Figure 5. Assessment of IL-17 and IL-6 serum concentrations. Kruskal-Wallis test was used to compare the means of cytokine serum concentration. (A) Concentration of IL-17 as a Th-17 functional cytokine. It was increased in rheumatoid arthritis patients in comparison with healthy subjects ($p<0.01$). (B) Concentration of IL-6 as a Th-1 inhibitor and Th-17 inducer. It was increased in both RA patients groups (**$p<0.01$). (C) Correlation between Th-1 cells and IL-6 serum concentration. There are a negative correlations between them (r=-318, $p< 0.05$). (D) Correlation between IL-17 and IL-6 serum concentration. There are positive correlation between them (r=0.427, $p<0.01$). All Correlations were calculated using Spearman's test**

Table 2. Frequency of anti-citrullinated protein antibody positive and negative among rheumatoid arthritis patients

	ACPA (+)	ACPA (-)	*p* value
New case RA	8 (53.3%)	7 (46.7%)	0.796
Established RA	29 (72.5%)	11 (27.5%)	0.002
Total RA patient	37 (67.3%)	18 (32.7%)	0.010

Table 3. Frequency of rheumatoid Factor positive and negative among rheumatoid arthritis patients

	RF (+)	RF (-)	*p* value
New case RA	5(33.3%)	10(66.7%)	0.197
Established RA	19(47.5%)	21(52.5%)	0.873
Total RA patient	24(43.6%)	31(56.4%)	0.345

DISCUSSION

To investigate the role of Th17, Th1 and dual Th17/Th1 cells in RA, we evaluated the percentage of these cells in RA patients including new and established cases and also healthy controls. Our findings in this study showed that the numbers of Th17 cells were significantly increased in patients with RA in comparison to controls. The percentage was higher in new case of RA than established RA group. It suggested that Th17 cells may contribute in disease onset. This finding supports several reports describing the boost of circulating Th17 cells frequency in RA patient.[23-26] The mechanisms of this augment are not entirely understood, but the higher percentages of Th17 cells in patients with RA suggested that these cells may play a considerable role in the pathogenesis of RA. Previous findings revealed that the development of Th17 cells is dependent to cytokine environment.[27] However, in contrast to our results; some reported that frequency of peripheral blood Th17 cells is decreased in RA patients compared to normal controls. Aerts et al. showed that patients with established RA did not display this elevation in peripheral Th17 cells.[28] Yamada et al showed that the percentages of Th17 cells were not significantly different between RA and healthy controls.[29] The rationale for these discrepancies are not obvious, but it is possible that complexity of Th17 cells biology, limited size of RA subjects and techniques for measurement of Th17 cells contribute in this disagreement. In mentioned studies the focus has been on RA patients with established disease that treated with a broad diversity of treatments. But in this study, the frequency of Th17 cells in new case RA patient group was strongly different from healthy controls. Even so dissimilar with our results in Th17 cells numbers, we detected that the percentage of circulating Th1 cells were reduced significantly in RA patients compared to their controls. But some studies indicated that the percentage of Th1 cells in RA were not significantly different from healthy ones.[25] Potential reasons for these findings may be as a result of the negative effect of Th17 on Th1 cells expansion, or some Th1 cells shifted to Th17 cells. Further studies are clearly needed to determine the mechanism for decreasing Th1 cells in peripheral blood and their effects on outcome of RA disease.

RA had long been classified as a Th1-mediated disease, but there are some information supported that both Th1 and Th17 cells have main role in RA disease. In experimental arthritis models, both Th17 and Th1 cells have been clarified as central contributors in the inflammatory progression.[17,27] There are evidences of inefficiency of monoclonal antibodies against IFN-γ in therapy of most RA patients[30]. Furthermore a number of studies suggested that IFN-γ can even be protective and is a powerful inhibitor of Th17 differentiation.[31] Thus, it seems that Th17 cells are the dominant initiators of inflammation in RA disease. Furthermore we found that conversely to percentages of Th17, frequencies of Th1 in established RA patient is more than new cases (Not significant). Thus, it is possible that during progress of disease, some of the Th17 cells gradually convert to Th1 cells. Recent studies have demonstrated that CD4$^+$Th cells are not completely distinct lineages, and Th17 cells can shift to cells producing both IL-17A and IFN-γ cytokines.[32] Some studies showed that low levels of IFN-γ can be found in joint of early stage of RA disease. But in later stages of RA, expression of this cytokine was detected in CD4$^+$ T cell clones of inflamed synovium.[33,34] However, our results indicated that the balance between Th1 and TH17 characterized by high Th1/ TH17 ratio in healthy state may be broken down in RA disease. These observations indicate that the balance of Th17- and Th1-cell responses is deregulated in RA patients. The immunocyte balance has long been hypothesized as one of the vital factors in immune system function and the Th1/ Th17 imbalance may be a major effect in RA progression.[35]

Also, our results showed that mean percentage of dual Th17/Th1 cells in new case of RA patients were statistically different from healthy controls. Consistent with our data, previous studies also detected a relatively small but significant percentage of Th17/Th1 cells.[36] But Arroyo-Villa et al showed that the percentage of Th17/Th1 cells were significantly lower in early RA patients when compared with healthy controls.[37] Nistala et al., proposed that the Th17 cells may be unstable and shift to Th17/Th1 or Th1 cells in human RA.[38] Because master gene regulator for Th1 differentiation, T-bet, could be activated in Th17 cells, these cells have considerable potential to differentiate to Th17/Th1 or Th1 cells.[39] In this regard, our finding indicated that plasticity between Th1 and Th17 cells are present and these subsets were not fully distinct lineages. Some studies resulted to an association between Th17, Th1 and Th17/Th1 subsets and disease

activity.[40] Our findings showed that only Th1 cells and Th1/Th17 ratio have statistically correlation with disease activity score (DAS28) in RA patients. In correlation with immunophenotyping, the results of cytokine analysis showed that serum concentration of IL-17 was significantly higher in RA patients in comparison with healthy control group, but there is no correlation between percentage of Th17 cells and this cytokine. One reason for this result is that IL-17 produced by different cells such as Th17, CD8+ Tc17, γδ-T cells, NKT cells, neutrophils and mast cells.[10] These findings indicated that serum levels of the IL-17 are not specific indicator for percentage of Th17 cells. Assessment of IL-6 concentration revealed that it was significantly higher in RA patients and there are negative correlation between its concentrations and mean percentages of Th1 cells (Figure 5B and 5C). Previous studies showed that IL-6 can inhibit Th1 cell differentiation by blocking of IFN-γ-signaling.[41]Thus this negative effect can be one of the reasons for Th1 cells decreasing in RA patients. Also our data indicated that there are significant positive correlations between mean serum levels of the IL-17 with mean serum levels of the IL-6 cytokine. In correlation with this finding, other studies indicated that IL-17 cytokine stimulates expression of inflammatory genes, like IL-6. Although our data showed that there are no correlation between percentages of Th17 cells with serum levels of the IL-6, but it is entirely accepted that in human, IL-6 beside other cytokines is important in lineage commitment of not only Th17 cells but alsoIL-17-secreting CD8$^+$Tc cells.[42] Thus, there are positive bidirectional effects between IL-6 and IL-17 in RA. While ACPAs and RF auto antibodies are important in diagnosis of RA, our findings indicated that there are no statistically differences in circulating Th17, Th1 and Th17/Th1 cells between ACPA and RF positive and negative patients.

There are some limitations in this study. First, we only measured the percentages of Th1 cells, Th17 cells and cytokine levels in serum but not in inflamed joints of RA patients. Thus detailed research of the frequency of Th cell subsets and levels of cytokines in the synovial fluids of participants is needed to be carried out in the further studies. Second, in-depth phenotypic and characterization of the functional memory Th17 and Th1 cells will help determine how these T cells may contribute to RA disease. In addition, we also did not study possible effects related to the use of RA drugs.

The balance between different types of T cells plays an important role in outcome of RA disease. Changes in the balance of T helper population especially Th1 / Th17 ratio affect RA disease process. Our findings show that the percentages of Th17 cells were increased in rheumatoid arthritis. In correlation with this finding, serum levels of IL-17 and IL-6 cytokines in RA patients also increased. Plasticity between T helper cells lead to introduce another subclass of T helper named dual Th17/Th1 cells. In new case RA patient, Dual Th17/Th1 cells were significantly more than healthy controls and established RA patients. Moreover, Th1/Th17 ratio in RA patients is statistically lower than healthy controls.

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