Patients with Active Rheumatoid Arthritis Have Lower Frequency of nTregs in Peripheral Blood

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ABSTRACT

Background: Patients with rheumatoid arthritis (RA) suffer from wide ranges of autoimmune reactions in joints. The mechanism of which is generally unknown and maybe associated with Treg deregulation. **Objective:** To compare the frequency of nTregs in peripheral blood of patients with active rheumatoid disease with healthy individuals. **Methods:** Twenty five newly diagnosed patients with active RA disease were selected based on the clinical and laboratory criteria before starting their therapies. Treg cells in peripheral blood samples were enumerated by immune staining and flowcytometry analysis. **Results:** Clinical and laboratory results were in favor of active disease in all the studied patients although they showed variations in Disease Activity Score-28 (DAS-28). Compared to the healthy controls, RA patients had significantly lower frequency of $CD4^{+}CD25^{hi}$ or $CD4^{+}CD25^{+}FoxP3^{+}$ natural regulatory T cells. In spite of that, there were no significant differences between patients and healthy controls in respect to the CD4/CD8 ratio. Interestingly, more $CD4^{+}CD25^{-}FoxP3^{+}$ cells were found in peripheral blood of patients. The frequencies of the Tregs did not show strong associations with the DAS-28. **Conclusion:** We showed lower abundance of nTregs in peripheral blood of RA patients which highlights the significance of these cells in RA.

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INTRODUCTION

Rheumatoid arthritis (RA) is a common systemic autoimmune disease with chronic relapsing inflammation primarily in peripheral joints. It is characterized by disturbed immune regulation which induces a progressive cartilage and bone destruction (1). Normally, immune system exploits several mechanisms to maintain self tolerance and suppress auto-reactive T cells. Among different immune tolerance mechanisms naturally occurring regulatory T cells (nTregs) play important roles to do the job by generating balance between pro- and anti-inflammatory mechanisms. Typically, nTregs are described as $CD4^+$ T cells with high levels of CD25 (IL-2 receptor α-chain) expression. nTregs are also characterized by the expression of the lineage-specific transcription factor for khead box protein p3 (FoxP3) although effector T cells up-regulate FoxP3 transiently following activation (2). nTregs demonstrate low expression of CD127 (IL-7 receptor α-chain) in combination with CD25 expression ($CD4^+CD25^{+/high}CD127^{low/-}$) which distinguishes nTregs from early activated $CD4^+CD25^+CD127^+$ conventional effector T cells (3). nTregs execute their suppression through contact dependent and independent mechanisms (2). These cells produce high levels of IL-10 and express several molecules with immunomodulatory effect such as CTLA-4 (CD152) and glucocorticoid induced tumor necrosis factor (GITR) which enable the cells to suppress evolution, maturation or activation of immune effector cells (4-7).

Due to regulatory roles of Tregs in general, any change in the proportion of the cells or any defect in their suppressive function may result in a pathological auto-reactivity. Although the detailed mechanisms of the function of Tregs in the prevention of autoimmunities are poorly understood, many studies could show variations in different subsets of Treg in diseases such as systemic lupus erythematosus (SLE), Behcet disease and rheumatoid arthritis (8-10). However, the available data for most studied diseases have been controversial. While many studies reported the reduction in the frequency of Tregs in RA patients, due to promoted apoptosis in the cells (11), differential epigenetic variations (12-13), or trans-differentiation to Th17 cells (14), others could not show a significant difference between RA patients and healthy individuals (15). Besides, some investigators also tried to show alterations in the function of regulatory T cells rather than cell count where more variations were found among the patients (16). There are other reports which addressed the differences in the availability of Tregs in synovial fluid or synovial membrane but not peripheral blood (17).

Due to all the debates that exist, more studies are needed to analyze the variations about the quantity and quality of Tregs in patients with rheumatoid arthritis. Therefore, in this study we selected 25 new cases of rheumatoid arthritis with active disease before they started their therapies, in particular corticosteroid, and assessed the abundance of some of the T cell populations with proposed regulatory function.

MATERIALS AND METHODS

Patients and Control Volunteers. This study was conducted by selection of 25 patients with rheumatoid arthritis (females: 18, males: 7; mean age: 41.44 years, ranged between 15 years and 65 years), diagnosed on the basis of the American College of Rheumatology (ACR) board of directors and the European League Against Rheumatism

(EULAR) executive committee criteria (18). All the patients were freshly diagnosed for RA disease and had received no prior steroid therapy to minimize possible effect of previous treatments. The patients were interviewed for the disease history (joint pain, morning stiffness and their assessment of the disease status), examined for the clinical signs of rheumatoid arthritis (joint effusion, tenderness, pain and limitation of motion) and extra-articular manifestations. They also were tested for erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and anti-CCP antibody. None of the selected patients had diabetes mellitus, other rheumatologic or autoimmune diseases, ischemic heart disease and active infection at the time of sampling. Patients' global assessment of disease activity (PGA), tender joint count (TJC), and swollen joint count (SJC) were determined. In this way the disease activity score in 28 joints (DAS-28) was calculated and patients where categorized into four groups of remission, mild, moderate and severe disease (19). Additionally 25 sex and age-matched healthy volunteers (females: 19, males: 6; mean age: 39.6 years, ranged between 17 years and 63 years) with no history of autoimmune or metabolic systemic diseases were recruited as healthy control (HC) group. Written informed consents were obtained from all the patients and healthy volunteers after the approval of the study by the ethics committee of the Shiraz University of Medical Sciences.

Blood Sample Processing. Blood samples were taken from the patients by venipuncture after the diagnosis, before starting the treatment. Peripheral blood mononuclear cells (PBMC) were isolated from blood samples by Ficoll density-gradient centrifugation. Isolated cells were re-suspended in PBS with 2% fetal calf serum and viable cells were counted. The purified cells were used for flowcytometry staining or frozen down in liquid nitrogen for later assessment. Plasma fractions of the blood samples were also separated, aliquoted and used for clinical laboratory tests and the rest were snap frozen at -80°C for further analysis. Blood samples donated by healthy controls were processed in the same way.

Flowcytometry Analysis. Frozen PBMCs were thawed, washed and restored during overnight culture. Thawed cells were used for further analysis if contained over 90% viable cell. Thawed or freshly purified PBMCs (1×10^6) blocked before staining and then stained with optimal concentrations of anti-CD4 antibody (PerCP) and anti-CD25 antibody (FITC), for 20 min at 4°C. The stained cells were washed, fixed, permeabilized and stained intracellularly with anti-FoxP3 (PE) antibody according to the manufacturer's instruction. Parallel staining with anti-CD4 (PerCP) and anti-CD8 (FITC) were performed to find out the proportions of the sub-populations in peripheral blood of patients and controls. All antibodies and fixation/permeabilization reagents were purchased from BD Pharmingen, USA. Fluorochrome matched monoclonal antibodies with appropriate isotypes were used as control to assess the background fluorescent and exclude non-specific staining. FACScalibur instrument with CellQuest-pro software (Becton Dickinson, USA) was used to analyze the stained cells. Flowjo software (Tree Star, Inc, USA) was used for data analysis and graphical presentation.

Statistical Analysis. The differences between patients and healthy controls were assessed by Mann-Whitney U test. P values less than 0.05 were considered as statistically significant. The relationship between different values was examined by Spearsman's rank correlation test. All statistical analyses and graphical presentation of the data were performed by Graphpad Prism software version 5 (Graphpad Software, Inc, USA).

RESULTS

Patients and Healthy Controls. In this study we recruited 25 patients with recent diagnosis of rheumatoid arthritis with extreme precautions. Findings for the disease history and physical examinations of the patients including Patients' global assessment of disease activity (PGA), tender joint counts (TJC), and swollen joint counts (SJC) are illustrated in Figure 1. Findings were used to determine the disease activity score of 28 joints, DAS-28. The median disease score among the patients was 6 (ranged between 3 and 7.2). The majority of the screened patients (80%) had high disease activity with disease score over 5.1 while the rest of the patients showed moderate disease activity with DAS-28 between 3.2 and 5.1. Age distribution of the patients did not show any association with DAS-28 data (r^2= -0.292, p= 0.15). Most patients (83%) complained of having more than one hour morning stiffness. Only 44% of the patients demonstrated one or more extra-articular manifestations such as dry mouth, dry eye, rash or subcutaneous nodules.

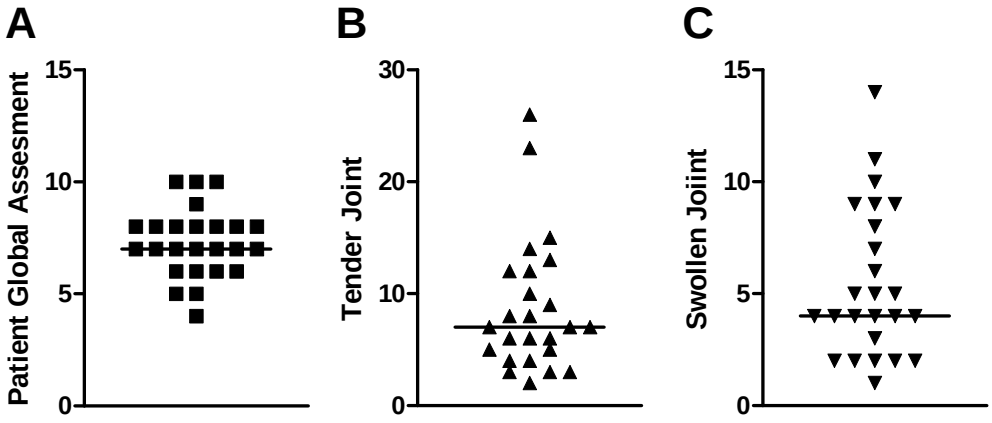

Figure 1. Physical examination data obtained from the studied patients. Physical examination data and history from 25 RA patients: A: patients' global assessment; B: Number of tender joints; C: Number of swollen joints. Short horizontal lines show medians of the values among the screened patient. Data showed the presence of active disease in almost all the patients.

CD4+/CD8+ ratios in peripheral blood of RA patients are similar to healthy controls. Peripheral blood mononuclear cells from the patients and the healthy individuals were assessed for the percentages of CD4+ and CD8+ cells by flowcytometry. Figure 2 shows the distribution of the CD4/CD8 ratios of those findings. The ratios did not show any association to the age of the subjects and there was no significant difference between the two groups.

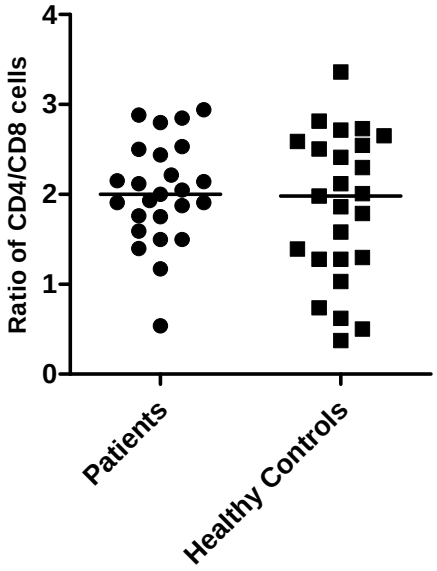

Figure 2. Distribution of CD4/CD8 ratios. Purified PBMCs from blood samples of the patients and the healthy controls were stained with anti-CD4 (PerCp) and anti-CD8 (FITC) antibodies and analyzed by flowcytometry. Frequency of CD4 and CD8 T cells were measured to determine CD4/CD8 ratio. Data showed no significant difference between the two groups ($p>0.01$).

Frequency of nTregs. To estimate the frequency of T cells with putative regulatory role we quantified the frequency of $CD4^{+}CD25^{hi}$, $CD4^{+}CD25^{+}FoxP3^{+}$ and $CD4^{+}CD25^{-}FoxP3^{+}$ cells among T cell populations in the peripheral blood of the patients and healthy participants by flowcytometry. Figure 3 shows typical plots of flowcytometry results for the staining of the target cell populations.

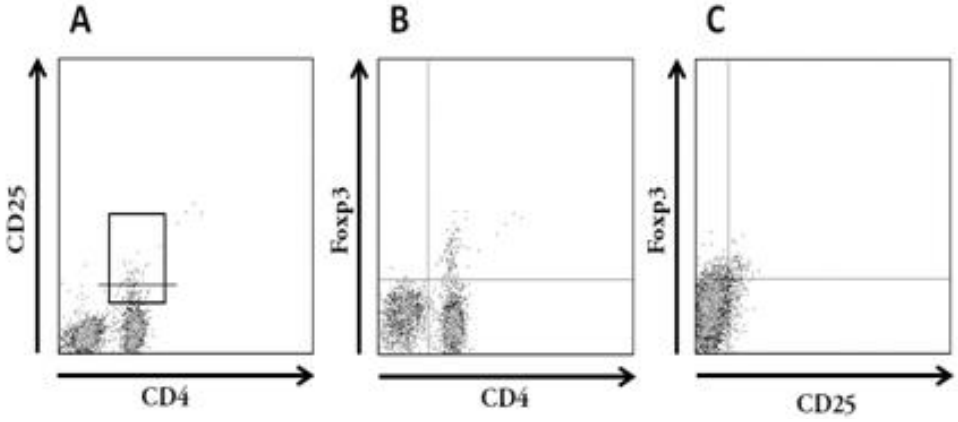

Figure 3. Typical flowcytometry plots of T cells with putative regulatory role. PBMCs were stained with anti-CD4 (PerCp), anti-CD25 (FITC) and anti-FoxP3 (PE) antibodies and analyzed by flowcytometry. Plot A shows $CD4^{+}CD25^{+}$ cells. The rectangle gates $CD25^{+}$ cells while the horizontal line marks the arbitrary level of $CD25^{hi}$ cells; plot B shows $FoxP3^{+}$ cells in $CD4^{+}$ population however FoxP3 expression was not restricted to CD25+ cells and some CD25- cells also had FoxP3 expression (c). These populations were compared between patients and controls.

Figure 4 demonstrates the distribution and median frequencies of the studied cell populations among the patients and healthy controls. The median frequency of $CD4^{+}CD25^{hi}$ population (Figure 4A) in the patients was 1.24% (ranged between 0.1%

and 6.7%). There were significantly more of the cells in the peripheral blood of the healthy controls than the patients (median=3.7%, ranged between 0.6% and 6.4%) (p=0.002). We included intracellular staining of FoxP3 to assess $CD4^+CD25^+FoxP3^+$ T cells frequencies. Once again RA patients had significantly lower frequency of the population in their peripheral blood than healthy individuals (Figure 4B) (p<0.001). The median frequency of $CD4^+CD25^+FoxP3^+$ cells in the peripheral blood of patients and controls were 2.62% (ranged between 0.06% and 5.6%) and 4.0% (ranged between 1.3% and 6.3%), respectively.

The difference in the frequency of $CD4^+CD25^-FoxP3^+$ cells between the patients and the healthy participants was investigated as well. As it was shown in Figure 4C the median frequency of $CD4^+CD25^-FoxP3^+$ cells in the patients was higher than the controls (7.8% vs. 5.1%). However, the difference was not statistically different (p=0.065).

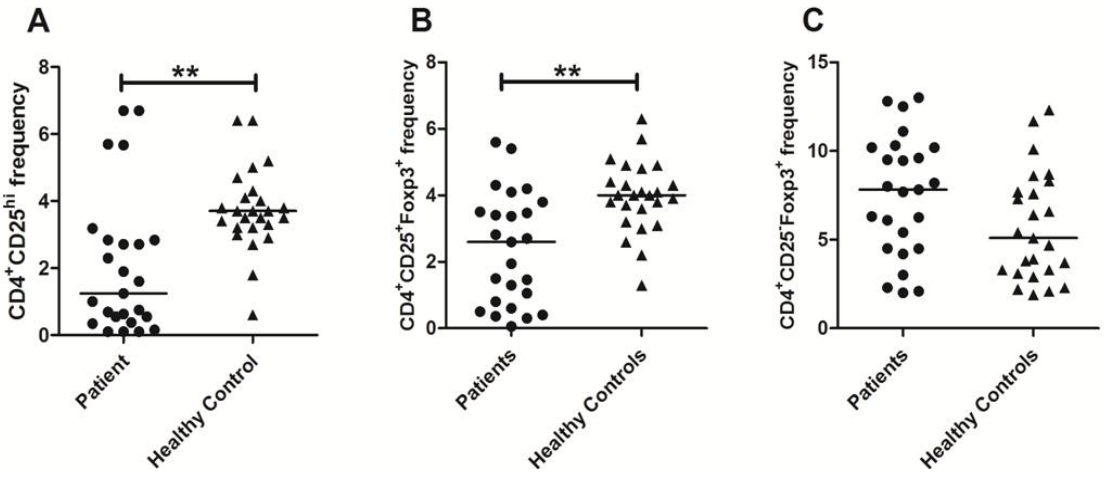

Figure 4. RA patients had different frequency of regulatory T cells in peripheral blood. PBMCs were purified from 25 patients and 25 healthy controls and stained as described in Figure 3. The frequency of $CD4^+CD25^{hi}$ cells (A) and $CD4^+CD25^+FoxP3^+$ cells (B) in the peripheral blood of the studied patients were significantly lower than healthy controls (p=0.0002 and 0.0008, respectively). In spite that, $CD4^+CD25^-FoxP3^+$ cells (C) were present with slightly higher frequency in peripheral blood of the patients; however the difference was not statistically significant (p=0.06). Horizontal lines show median frequencies of the data. The differences of the data were compared by Mann-Whitney U test. p values less than 0.05 were considered as statistically significant. Asterisks show the significance of the differences.

Correlation between the disease activity score and abundance of regulatory T cells. We used the Spearsman's rank correlation test to analyze the relation between the disease activity and the abundance of the studied cell populations. No significant correlation between the frequencies of $CD4^+CD25^{hi}$ population and DAS-28 scores was found (r=-0.042, p=0.84). Similarly, the disease score did not demonstrate a considerable correlation with the frequency of $CD4^+CD25^+FoxP3^+$ cells (r=-0.086, p=0.68) or $CD4^+CD25^-FoxP3^+$ cells (r=0.800, p= 0.70).

DISCUSSION

Previous studies propose several mechanisms of action by nTregs and other suppressive T cells to inhibit self reactivity of immune system (2). However, autoimmune diseases such as systemic lupus erythematosus, Behcet disease and rheumatoid arthritis are the evidences of insufficiency or imperfectness of immune tolerance in those patients. The deficiency could be in quantity and/or quality of mechanisms. In this study we quantified the frequency of T cell populations with putative regulatory roles in peripheral blood of patients with active rheumatoid arthritis and compared that with findings in healthy individuals. To enumerate nTregs, we included CD4+ T cells with high expression of CD25. nTregs also express transcription factor FoxP3 which was revealed by intracellular staining of the cells. We also compared the frequency of CD4+CD25-FoxP3+ cells as reports show regulatory activities of those cells as well (20-21). Although synovial membranes and joints are the target sites of autoreactivity in RA disease, there should be a flux/traffic of both effector and regulatory cells from peripheral blood toward these sites, in addition to what may evolve on site. Therefore, determining the abundance of the cells in peripheral blood of the patients can provide a valuable index which may better reveal the pathogenesis of the disease and generate possible target for therapy too. To minimize the interference of immune suppressive therapies we excluded any patient with history of steroids therapy and only freshly diagnosed patients were studied. The median frequency of $CD4^{+}CD25^{hi}$ cells in the studied patients with active rheumatoid arthritis was significantly lower than findings in the healthy controls. In the same way, there were lower frequencies of CD4+CD25+FoxP3+ cells in the peripheral blood of the patients. Similar findings showing lower frequency of Tregs in the peripheral blood of RA patients have been reported by other investigators (9,22). In that regard Li *et al*. showed that decrease in the number of the cells could be related to defective mechanism for controlling apoptosis in Tregs in RA patients (11). Others like Kim *et al.* also showed lower numbers of the cells in peripheral blood of RA patients however their results had great variations among different subsets of Tregs (9). Other investigators that included more criteria for identification of Tregs showed similar results of decreased frequency of Tregs in peripheral blood of RA patients. Kawashiri *et al.* demonstrated significant decrease of $CD4^{+}CD25^{+/high}CD127^{low/-}$ cells in the peripheral blood of RA patients (23). Contrary to these there are other results from human studies or animal researches that showed increased frequency and absolute cell count of nTregs in peripheral blood (24-25). Results suggested that some of the increased frequency could belong to cells with plasticity toward Th17 cells (26-27). If true, this may explain how the presence of excess numbers of Tregs could not inhibit autoreactivity in the patients. There are other reports which showed no significant change in the frequency of Tregs in peripheral blood of RA patients but demonstrated the enrichment of Tregs at the site of inflammation, i.e. joints and synovial membranes (17,28). It seems the inflamed milieu of joints in RA patients reduces the ability of Tregs to suppress the overwhelming autoreactivity. Some studies also focused on the quality of Tregs rather than quantity of the cells. Tregs isolated from patients with active RA disease showed anergic phenotype after *in vitro* stimulation which caused un-responsiveness of the cells for immune suppression (29). The complexity of findings multiplies by results showing resistance of effector cells against suppression by Tregs (30-31).

The median frequency of $CD4^+CD25^-FoxP3^+$ cells in peripheral blood of the patients that we studied was higher than the healthy individuals. It is believed that this population may act as reservoir for actual regulatory cells. de Parz *et al.* also studied this population in the peripheral blood of patients with active RA disease and showed the increased frequency of the cells (32). In that study the frequency of $CD4^+CD25^-FoxP3^+$ cells demonstrated strong negative correlation with DAS-28. Contrary to that we could not show such strong association. Interestingly, not so many studies could show significant associations between the frequency of regulatory T cells and the disease score. We also could not establish such association. Contradictory reports also show great variations in CD4/CD8 T cell ratio in peripheral blood of RA patients (33-34). In our study no significant difference was found between the patients and the healthy individuals in spite of differences in Treg frequencies. Similar variation exists for the relative frequency of different subsets of T cells i.e. Th1, Th2 or Th17 in the patients (35).

Existing controversies may demonstrate contribution of complex and heterogeneous arrays of mechanisms for selective induction of cell traffic and retention of Tregs in the joints of RA patients. In that regard it was shown that Tregs cells on synovial membrane have activated effector memory phenotype of $CD62L^-CD69^+$ compared to resting central memory phenotype of $CD62L^+CD69^-$ in peripheral blood (17).

All together, present literature shows great heterogeneity regarding the role and function of subsets of Tregs in rheumatoid arthritis. Further studies are needed to reveal function of different subsets of Tregs for the induction, maintenance or cure of rheumatoid arthritis. That needs molecular findings of specific antigens for both effector and regulatory T cells.

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