T Regulatory Cells in Rheumatoid Arthritis with Reference to Anti-Citrullinated Peptide Antibody and TNF-alpha Inhibitor Therapy

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T regulatory cells (Tregs) plays an important role in maintaining self-tolerance and preventing autoimmune diseases by inhibiting proliferation and cytokine production of self-reactive T cells. Controversy was reported regarding the frequency of $CD4^+CD25^+$ Tregs in the peripheral circulation of rheumatoid arthritis (RA) patients compared to normal controls. Also, some showed that treatment with TNF-α inhibitor restored the capacity of Tregs. This work aimed to study Tregs in the peripheral blood of RA patients versus control in addition to those on TNF-α inhibitor therapy compared to those who have not received it and to correlate with status of anti-cyclic citrullinated peptide antibody (ACPA). Two groups of RA patients were studied; one on TNF-α inhibitor therapy and the other not. Additionally, age-matched apparently healthy controls were studied. The percentage of CD4+CD25+ T cells in the total lymphocytic cell population was determined by flow cytometry analysis while ACPA concentration was measured by a second-generation peptide-based ELISA. Mean level of Tregs was significantly lower in the studied RA patients compared to the control group. Patients in early disease (0-5 years) had low mean Tregs percentage compared to patients with long duration of disease (>10 years) (*P*=0.044). Patients on TNF-α blocker therapy had elevated Tregs percentage relative to patients on methotrexate (MTX) (*P*=0.022) and other therapies. No effect of gender or age was found on Tregs levels. In RA patients, 85.4% were ACPA seropositive and 65.9% of seropositive patients have concentration of >100U/ml. The mean Treg percentage was significantly lower in ACPA seronegative group compared to the seropositive group (*P*=0.013). In conclusion, the studied RA patients have low Treg, and TNF-α blocker therapy increased its number, compared to other therapies.

Rheumatoid arthritis (RA) is a common autoimmune disease, with a prevalence of 1% among adult population in US and has a comparable prevalence worldwide [1], characterized with chronic inflammation of synovial joints. RA affects people of all ages and races with varying severity related to genetic and environmental factors. RA involves various cells of the immune system with elevated expression of inflammatory cytokines like tumor necrosis factor alpha (TNF-α), certain interleukins (IL), proteinases, and multiple chemokines [1]. Several autoantibodies have been described in RA, but only rheumatoid factor (RF) and antibodies to citrullinated antigens are considered clinically relevant. RF is detectable in 70% to 80% of RA patients but is also detectable in 10% of healthy individuals and in other systemic diseases. Anti-cyclic citrullinated peptide antibodies (ACPAs) are present in about two-thirds of all RA patients but are rare (<2%) in healthy individuals and relatively rare in other inflammatory diseases [2]. ACPAs production is thought to be dependent on genetic background of individual patient.

ACPA positivity has been associated with a more severe destructive disease [3]. Because ACPAs are known as strong predictor of erosive RA, RA is clinically divided into distinct two subgroups, ACPA positive RA and ACPA negative RA [3].

Tregs ($CD4^+CD25^+FOXP3^+$) have a central role in protecting an individual from autoimmunity. Subsequently, several studies have investigated Tregs in human autoimmunity [2]. There is controversy regarding the frequency of $CD4^+CD25^+$ Tregs in peripheral blood of RA patients compared to normal controls, as some studies reported normal levels, some others reported increased levels, whereas a fair number have reported decreased levels [2, 4].

The initiation of the disease modifying antirheumatic drugs (DMARDs) are required for most patients to alter the disease progression. Most commonly used non-biologic DMARDs include methotrexate (MTX), sulfasalazine, hydroxychloroquine and leflunomide. MTX is commonly used as initial DMARD. Biologic agents are therapies used for different diseases, which have been introduced for treatment of RA mainly the resistant type. The available biologic treatments for RA include TNF-α inhibitors (infliximab, etanercept, adalimumab, golimumab, and certolizumab), IL-1 receptor antagonists (anakinra), CTLA-4 immunoglobulin (abatacept), anti-CD20 antibodies (rituximab), and IL-6 inhibitor (tocilizumab) [5]. Currently it is believed that restoration of Treg number and function is the ultimate treatment for autoimmunity. A number of available therapies for RA involve some level of Treg modulation, although none of them were designed as such. TNF-α blockers, anti-IL-6 therapy, CTLA-4 immunoglobulin therapy, and anti-CD3 therapy all affect Treg function [6]. TNF-α blocker therapy (infliximab) is prescribed to the Bahraini RA patients, but no up-to-date data are available that determines whether the therapy causes any modulation to Treg percentage. This article reports findings of a study of T regulatory cells in the peripheral blood of RA patients versus controls. In addition, it compares those on TNF-α inhibitor therapy versus those who have not received it, and to correlate with the status of ACPAs.

Subjects, Materials and Methods

Subjects

This study was a population-based case-control study, enrolling RA patients from the Rheumatology clinic in Salmaniya Medical Complex (SMC), Ministry of Health, Kingdom of Bahrain. Subjects with following conditions were excluded: old age (>75 years), non-Bahraini, malignancies, other autoimmune diseases, genetic disorders (sickle cell anemia, G6PD), pregnancy, allergies, and other rheumatic diseases. Following screening, and patient consent for enrollment, 42 patients were selected, and it was confirmed that these patients satisfied the 1987 revised classification criteria of the American College of Rheumatology (ACR). Additionally, the patients were also selected on basis of therapy. Selected patients were included in three groups based on their therapy: those on TNF-α blocker therapy (Infliximab), patients on MTX, and those on other therapies such as NSAIDs, steroids, and other DMARDs. The control group included 37 age matched subjects with no history of rheumatoid arthritis, chronic diseases, malignancies, autoimmune and genetic diseases, pregnancy, allergies, and rheumatic diseases.

Methods

For detection of Tregs, peripheral blood mononuclear cells (PBMCs) were subjected to CD4 and CD25 cell surface immunoflourscence staining as outlined by the protocol of Biolegend, San Diego, CA. In brief, blood samples on EDTA were treated with lysing solution to get rid of the RBCs, then incubated with florescent stained monoclonal antibodies $CD4^+CD25^{bright}$. The stained PBMCs were analyzed with flow cytometer (Partec PAS, Müster, Germany) within 24 hours. The gate was set at $CD4^+CD25^{bright}$

T cells. ACPA IgG autoantibodies were assayed in the patient and control sera using a semi-quantitative/qualitative second-generation peptide-based enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions [DIASTAT^{TM} Anti-CCP test (Axis-Shield Diagnostics Limited, Dundee, UK)]. To correlate the Tregs percentage to disease duration, the RA patients were divided into three groups according to disease duration: 0-5 years , 6-10 years and long history of the disease (>10 years).

Statistical Analysis

Statistical analyses were performed with Minitab 15.0 and Microsoft Excel 2007. Data were expressed as means ± standard deviation and percentage. The statistical significance; a *P*-value less than 0.05 was considered statistically significant. For ACPA concentration and Tregs analysis, 2 sample t-test was calculated.

Ethical/Research Approval

The research was reviewed and approved by Salmaniya Medical Complex (SMC) research committee. Rheumatoid arthritis patients and apparently healthy controls included in the study were asked to complete and sign an informed consent form agreeing to participate in the study and allow access to their medical records. Patients and controls were informed of the purpose of the study and its implications.

Results

The study included 42 RA patients and 37 age-matched controls that do not have RA nor have any related rheumatic or autoimmune conditions, of Arab Bahraini origin, and did not include other Bahraini ethnicities. Many subjects included in the study were females, with 88.1% in the patient group and 67.6% in the control group. The average disease duration within the patient group was 8 years (SD ± 6.43). The age ranged from 27 to 75 years in the patient group with a mean of 45 years (SD ± 10.53). In the control group, the age range was 22 to 68 years with a mean of 37 years (SD ± 13.12). The levels of Tregs in the peripheral blood were measured for 40 patients and 37 controls.

The percentage of T regulatory cells ($CD4^{+}CD25^{bright}$) was determined in peripheral blood of 40 RA patients and the 37 controls. The percentage of T regulatory cells in patient's blood was significantly lower than that in the control group (P<0.01). The mean percentage of T regulatory cells in RA patients was 0.566±0.338 whereas in the control group was 1.66±0.411 (Figure 1).There was no difference between the percentage of Tregs in female (n=37) and male (n=5) RA patients (P = 0.229). Similarly, there was no relation between the percentage of Tregs and age.

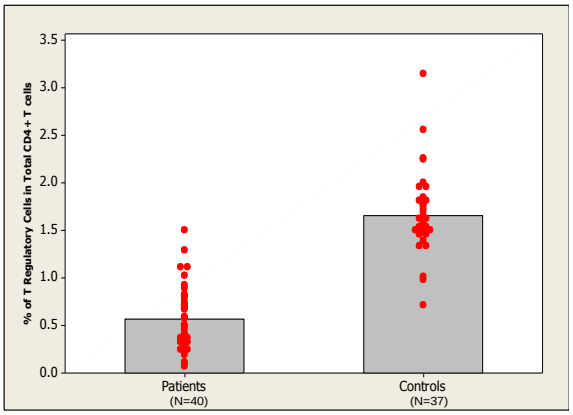

Figure 1. Mean Treg percentage of the peripheral blood of RA patients and the age-matched controls. The mean Treg percentage was significantly lower in patient's group compared to the controls (P<0.01). The standard deviation values were ± 0.338 and ± 0.411 for the patients and controls, respectively. Each circular dot (●) represents an individual subject, the bar represents the mean, and N represents the number of subjects.

The mean percentage of Tregs was 0.695±0.326, 0.380±0.248, and 0.450±0.147 in blood of patients on TNF-α blocker (n=17), MTX (n=7), and other DMARD therapies (n=10) patient groups, respectively. The mean percentage of Tregs in control group was 1.66±0.411 (Figure 2).

The TNF-α blocker therapy patient group showed elevated Treg percentage compared to the patients on MTX (*P*=0.022) and patients on other therapies (*P*=0.014). The MTX patient group showed no difference in their Treg percentage relative to the other therapies group (*P*=0.523). Though the patients on TNF-α blocker therapy had elevated Treg percentage, but the percentage was lower compared to control group (*P*<0.01) (Figure 2).

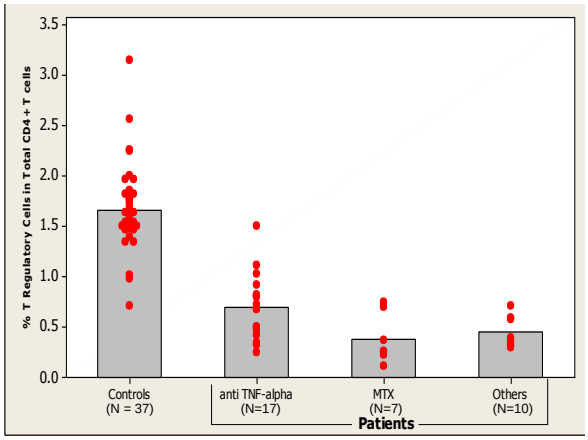

Figure 2. Mean percentage of Tregs in RA patients according to different therapies. The patients on TNF-α blocker therapy have elevated Treg percentage relative to patients on methotrexate (MTX) (*P*=0.022) and other therapies such as NSAIDs, steroids, and other DMARDs apart from MTX (*P*=0.014). The mean percentage of Tregs was not different between patients on MTX and patients on other therapies (p=0.523). The percentage of Tregs was lower for RA patients irrespective of the therapy relative to the controls (*P*<0.01). The standard deviation values were ±0.411, ±0.326, ±0.248, and ±0.147 for controls, TNF-α blocker therapy, MTX, and 'other therapies' patient groups respectively. Each circular dot (●) represents an individual subject, the bar represents the mean, and N represents the number of subjects

The mean Treg percentage was 0.374±0.201, 0.554±0.256, and 0.767±0.469 in blood of patient groups with 0-5, 6-10, and >10 years of disease duration, respectively. The patients early in the disease (0-5 years) had lower Treg percentage compared to patients with long history of the disease (>10 years) (*P*=0.044). However, there was no difference in the mean percentage of Tregs in patient's group with disease duration of 6-10 years compared to patients early in the disease (*P*=0.071) and patients with long history of the disease (> 10 years; *P*=0.239) (Figure 3).

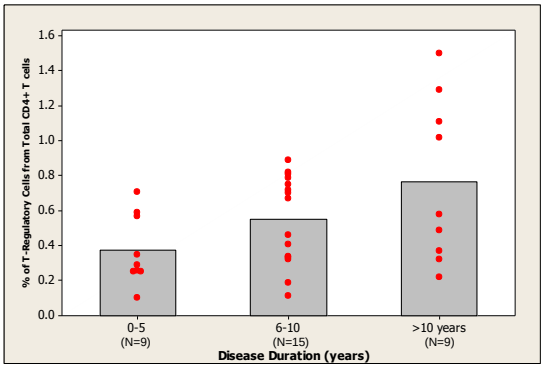

Figure 3. Mean percentage of Tregs in RA patients according to disease duration. The patients early in the disease (0-5 years) have low mean Treg percentage compared to patients with long duration of disease (>10 years) (*P*=0.044). The patients with 6-10 years of disease inception did not differ than patients early in disease (*P*=0.071) and from patients with long duration of disease onset (p=0.239). The standard deviation values were ±0.201, ±0.256, and ±0.469 for 0-5, 6-10, and >10 years of disease duration patient groups respectively. Each circular dot (●) represents an individual subject, the bar represents the mean, and N represents the number of subjects.

C-reactive protein (CRP) and rheumatic factor (RF) concentrations along with erythrocyte sedimentation rate (ESR) values for RA patients were collected from patient medical records in Salmaniya Medical Complex. The CRP concentration ranged from 0.57 to 176 with a mean of 25.48 ± 39.81 mg/L. Of the 42 patients, 28 were RF seropositive, 6 RF seronegative, and 8 had unknown RF status. In seropositive RF patient group, the RF concentration ranged from 20.7 to 1000 IU with a mean of 168.8±236.12 IU. The ESR ranged from 1 to 110 with a mean of 33.44 (SD ± 29.52).

Correlation of Tregs percentage with serological parameters of disease activity including CRP and ESR was made, (table 1). The mean Treg percentage in patient group with lower CRP concentration (0-50 mg/L)

was 0.589±0.352 %, 0.480±0.028 % in patient group with 51-100 mg/L CRP concentration, whereas the mean Treg percentage is 0.277±0.038 % in patients with highly elevated CRP concentration (>100 mg/L). There was no statistical significance in mean Treg percentage when the low CRP concentration group (0-50 mg/L) was compared to group which had CRP concentration of 51-100 mg/L (P=0.099). The Treg percentage was significantly low in RA patients with high CRP concentration (>100 mg/L) compared to patient group with CRP concentration of 0-50 mg/L (P<0.01) and relative to patient group with CRP concentration of 51-100 mg/L (P=0.021).

Table 1. Correlation of Treg percentage with CRP Levels

CRP levels	0-50 mg/L	51-100 mg/L	More than 100 mg/L
Treg percentage	0.589%	0.48%	0.277%

The Treg percentage was significantly low in RA patients with high CRP concentration (more than 100mg/L) when compared with patients with low CRP concentration (0-50mg/L) (P<0.01).

When RA patients with high ESR (>20) (n=23) were compared to low ESR (0-20) (n=18) for mean Treg percentage, there was no significant difference between the two groups.

Based on the ACPA results, and the manufacture kit instructions, the RA patients were divided into three groups: (1) patients ACPA seronegative with concentration of 0-3 U/ml concentration (n=6, 14.6%), (2) patients ACPA seropositive with concentration of 3.1- 99.9 U/ml (n=8, 19.5%), and (3) patients highly ACPA seropositive with concentration >100 U/ml (n=27, 65.9%). Many of the patients were highly ACPA seropositive (n=27/41, 65.9%) (Figure 4). The controls were all ACPA seronegative with mean concentration of 0.844± 0.411 U/ml.

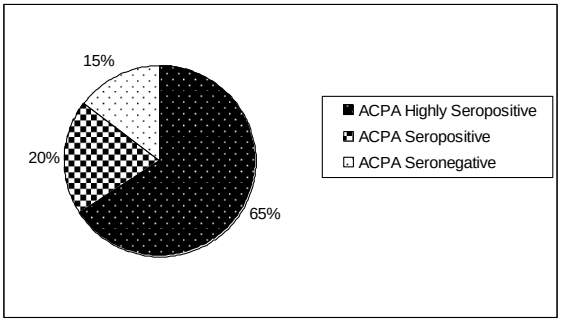

Figure 4. Distribution of RA patients according to ACPA sero-status (total # of RA patients=41). (Highly seropositive, ACPA concentration >100 U/ml; Seropositive, ACPA concentration 3.1-99.9 U/ml; and Seronegative, ACPA concentration 0-3 U/ml).

The mean Treg percentage was 0.298±0.145 in 5 ACPA seronegative patients, 0.641± 0.414 in 8 ACPA seropositive patients, and 0.549±0.253 in 24 highly seropositive ACPA patients. The mean Treg percentage was significantly lower in seronegative ACPA patient group relative to patient group with concentration >100 U/ml (P=0.013). There was no difference in the mean Treg percentage between ACPA patient group with concentration of 4-40 U/ml (P=0.06) or highly seropositive ACPA patient groups (P=0.568) (Figure 5). Furthermore, there was no difference between seronegative and seropositive ACPA RA patients regarding their ESR and CRP data. In addition, no correlation was found between disease duration and ACPA titers.

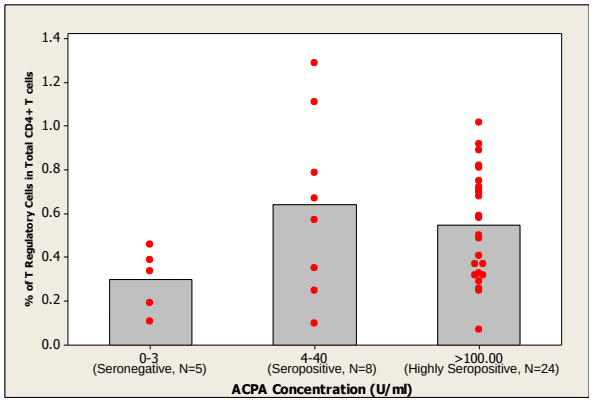

Figure 5. Mean percentage of Tregs according to ACPA concentration in RA patients. The mean Treg percentage was significantly lower in ACPA seronegative group relative to highly seropositive patient group (*P*=0.013), whereas no difference was seen when comparing the serpositive patient group with seronegative group (*P*=0.06) and highly seropositive group (*P*=0.568). The standard deviation values were ±0.145, ±0.414, and ±0.253 for seronegative, seropositive, and highly seropositive ACPA patient groups respectively. Each circular dot (●) represents an individual subject, the bar represents the mean, and N represents the number of subjects.

Discussion

In the current study, we investigated the levels of Tregs in the peripheral blood of RA patients and compared it with age-matched controls. Additionally, the Treg levels were also compared between the RA patients to investigate the effect of immunotherapy on Treg cells. The future of RA therapeutics includes patient-tailored therapy, which will be based on presence or absence of the identified molecular and genetic pathogenic factors [7, 8]. Also, an important target of future RA therapy is the Treg cells. Treg cells play dominant role in suppression of autoimmune diseases [9].

In the literature, previous studies have showed contradictions regarding the frequencies of Tregs in peripheral blood of RA patients when compared to age-matched controls. These contradictions are thought to arise from use of different methodologies to determine the levels of Tregs. Some researchers considered $CD25^{bright}$ cells as Tregs, others have counted total population of $CD25^{+}$ cells and defined them as Tregs [10]. The studies that used total defined Tregs as total CD25+ cells have reported an increase in Treg levels [11], whereas studies that used $CD25^{bright}$ as Tregs have reported a decrease or no change in Treg level relative to study controls [12, 13,14]. In our study, we used $CD25^{bright}$ as Tregs and consistent with aforementioned studies [12-14], we also found a decrease in levels of Tregs relative to the controls.

In our study, we compared the levels of Tregs in RA patients on infliximab, MTX, and other therapies and we found an increase in Treg levels in patients on infliximab therapy compared to patients on MTX and other therapies. Some scientists showed that treatment with anti-TNF-α (infliximab) led to significant rise in the percentage of peripheral blood Tregs in RA patients responding to therapy and this correlated with reduction in CRP [15]. Furthermore, others reported that infliximab therapy gives rise to a $CD4^{+}CD25^{hi}$ $FoxP3^{+}$ Treg cell population, which mediates the suppression via transforming growth factor (TGF-β) and interleukin-10 (IL-10) and lacks CD62L expression, thereby being distinct from natural Treg cells present in normal individuals and patients with active RA [16]. Moreover, it was reported that antagonizing TNF-α with infliximab in human TNF-α transgenic mice, which are a model of strictly TNF-α-dependent arthritis, led to increased Treg frequency and upregulated CTLA-4 [17]; while the infliximab treatment promoted differentiation of $CD62L^{-}$ Treg population in those mice. In another study, they measured the levels of Tregs ($CD4^{+}CD25^{hi}$) in RA patients following other anti-TNF-α blocker therapies (etanercept (ETA) and adalimumab

(ADA)) after 6 and 12 weeks of administration which had no effect on Treg percentage both the bright and the absolute $CD25^+$ [18]. Those reports from the literature support our findings.

In the current study, we also correlated Treg levels in RA patients with different clinical parameters. First, we correlated the levels of Treg cells with disease duration in RA patients. The Treg frequencies increased with disease duration, specifically, the recently diagnosed RA patients had lower levels of Tregs compared to the patients with greater than 10 years on disease onset. This finding is in contradiction with other studies, which have reported no difference in Treg levels with disease duration., neither when comparing the Treg levels ($CD4^+CD25^{hi}$) in the peripheral blood of RA patients with early active RA to patients with stable well controlled RA [12, 19] .

In the current study, we found a significant decrease in Treg levels with increasing CRP concentration, but no association was observed between ESR and Treg levels. A previous study measured Treg ($CD4^+CD25^+CD127^{low}$) frequencies in peripheral blood of RA patients and correlated it with CRP and ESR. They found Treg levels negatively associated with both CRP and ESR [20]. In our study, we only found negative correlation with CRP and no association with ESR, On the other hand, another study reported no association between CRP and Treg levels in peripheral blood [12]. This data contradictions could be explained by the differences of the number of patients included in the mentioned studies together with the different medications taken which have direct effect on the disease activity.

Our finding of no association between sex or age with Treg levels in peripheral blood of RA patients is consistent with other studies, who also reported no change in Treg frequency with age and patient sex [11].

ACPA is a highly specific marker for RA [3]. ACPA can be detected at onset of disease and even prior to the clinical phase and are reported to be good predictors of RA development. Based on ACPA status, RA is classified into two disease subsets, ACPA-positive RA and ACPA-negative RA. We measured the ACPA concentration in RA patients and age-matched controls. In the patient group, seronegative ACPA and seropositive ACPA subjects were found, whereas in the control group, all subjects were ACPA seronegative, which may indicate relative specificity of ACPA autoantibodies for RA.

Following classification of RA patients into ACPA negative and ACPA positive, we compared the levels of Tregs in these two patient groups to determine the effect of ACPA on Treg levels. The levels of Tregs were higher in highly seropositive ACPA RA patient group compared to seronegative ACPA patients. This finding is in contradiction with a study of Chen *et al.*, 2007 [21] which reported negative correlation between ACPA and Treg levels ($CD4^+CD25^{hi}$).

Moreover, our analysis of serological parameters of disease activity (CRP and ESR) between ACPA-positive and ACPA-negative RA patients showed no difference in both of the parameters. This finding is consistent with another study that also reported no difference between ACPA positive and ACPA-negative RA patients regarding CRP and ESR levels [22].

Although the study has limited number of study subjects, it showed that the studied RA patients have a lower Treg percentage in their peripheral blood than the study controls. Other findings implicated that TNF-α blocker therapy, enhanced Treg

levels compared to other therapies. Furthermore, a significant portion of the patients were highly seropositive for ACPAs, which may further amplify the risk for more erosive RA disease.

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