Quantification of CD4+ CD25+ Regulatory T Cells in Peripheral Blood of Patients with Systemic Lupus Erythematosus and Rheumatoid Arthritis

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Abstract: Recent animal studies have shown that regulatory T cells play a crucial role in the suppression of the immune response and that depletion of this subset of T cells might lead to development of autoimmune diseases. The aim of this work was to quantify regulatory T cells (CD4+ CD25+) in the peripheral blood of Omani patients with Systemic Lupus Erythematosus (SLE) and Rheumatoid arthritis (RA) and correlate these findings with the disease activity of the patients. Thirty patients with SLE, 30 patients with RA and 25 healthy volunteers were enrolled in this study. Patients were divided into highly active or low active groups, depending on the disease activity. Flow cytometer was used to quantify CD4+ CD25+ T cells in the peripheral blood mononuclear cells (PBMC). We found that both highly active SLE (0.242 ± 0.3) and RA (0.56 ± 0.29) patients had significantly ($p<0.001$) lower levels of CD4+CD25^{bright} T cells than did normal controls (1.74 ± 0.47%) or patients with low disease activity (SLE=1.54 ± 0.33, RA=1.829 ± 0.76). The decreased number of CD4+CD25^{bright} T cells during disease activity was restored in remitting phase of SLE patients. This data provides further evidence supporting the hypothesis of defect of regulatory T cells in SLE and RA patients; which may have an important implication in the context of the control of the inflammation and development of autoimmunity.

Key Words: CD25, Regulatory T cells, SLE, RA.

INTRODUCTION

The main function of the immune system is to protect the individual from pathogens and one of its primary characters is the ability to distinguish between self and non-self and between antigens encountered in harmful and non-harmful manner. In the thymus, potentially self-reactive T cells are deleted, resulting in the generation of a peripheral T-cell repertoire that is largely self-tolerant. Despite this, some self-reactive T cells are present in most individuals. Nevertheless, autoimmune diseases only occur occasionally, which suggests that autoreactive T cells are controlled in the periphery [1, 2].

Systemic Lupus Erythematosus (SLE) is a chronic systemic autoimmune disease. Like other related illnesses its etiology is still unknown. Multiple defects in the immune system of patients with this condition have been described; these include B cells over activity, defects in lymphoid activation process, and aberrant cytokine production [3, 4].

Rheumatoid arthritis (RA) is a systemic inflammatory disease, characterized by chronic synovial inflammation resulting in cartilage and bone damage, eventually leading to joint destruction [5]. Several different cell types and their mediators are involved in the tissue-destructive inflammation, e.g., T cells, B cells, monocyte/macrophages, and pro-inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-1 (IL-1) [5-7]. The fact that the inflammatory process in RA is chronic suggests that immune regulation in the joint is disturbed. This disturbed regulation may be caused by an excessive inflammatory response together with a deficiency in the mechanisms that control the immune response [5].

In 1995 Sakaguchi and colleagues showed that a minor population (approximately 5-10%) of CD4+ T cells was crucial for control of autoreactive T cells *in vivo*. These cells co-expressed the Interleukin-2 (IL-2) receptor α chain (CD25) and CD4, thus named CD4+ CD25+ T cells or CD25+Treg (regulatory T cells) [8]. Subsequent *in vitro* studies by several groups confirmed that CD4+CD25+ T cells play a crucial role in suppression of the immune response [9-11].

The mechanisms used by regulatory T cells to suppress immune response are still unresolved. However, a number of hypotheses have been suggested: these cells can kill autoreactive cells by several mechanisms including: inactivate APC or T cells *via* CTLA-4 and release or express molecules (such as perforin and TGF-β) to suppress auto-reactive cells [2, 12, 13].

Several studies of CD4+CD25+ T cells have been conducted in SLE patients, presenting conflicting results. A recent study done by Azab *et al.* showed that the levels of CD4+CD25+ % T cells in SLE patients were higher than in the control group, but it was statistically insignificant [14]. Also Suárez *et al.* [15] found no significant difference of CD4+CD25+ % T cells between SLE patients and control

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group. On the other hand, several studies reported a decrease in the percentage of CD4+CD25+ T cells in SLE patients [16, 17].

Similarly discrepant data were reported in RA patients; Lawson *et al.* reported deficit in CD4+ $CD25^{bright}$ regulatory cells in peripheral blood of early active RA compared to patients with reactive arthritis or control group [18]. However, Minami *et al.* reported that the percentage of CD4+CD25+ T cells was higher in RA patients than controls, but it was not statistically significant [19]. Other reports showed increased number of these cells in RA synovial fluid but not in peripheral blood [20-22].

Studies of regulatory T cells are limited in the middle east and no study has been conducted in the gulf states. The aim of this work was to quantify the CD4+CD25+ T cells mainly CD4+$CD25^{bright}$ T cells in Omani Arab patients with SLE and RA during diseases activity and remission periods.

SUBJECTS AND METHODS

Subjects: Thirty patients (25 females and 5 males, mean age 44 ± 16) with SLE, 30 patients with RA (25 females and 5 males, age 45 ± 15), and 25 healthy volunteers (20 female and 5 male, mean age 35 ± 7) were enrolled in this study. Patients attended the Rheumatology Clinic at Sultan Qaboos University Hospital (SQUH), and all receiving immunosuppressive drugs. The patients fulfilled the American College of Rheumatology (ACR) criteria for SLE [23] or RA [24]. Diseases activity for SLE was assessed using an SLE disease activities index (SLEDAI) [25] and the disease activity for RA was determined using DAS score [26].

Both SLE and RA patients were divided into two groups; a group with low activity of the disease and another group with highly active disease. The SLEDAI score for low active SLE patients was between 0-8 points; whereas, for the highly active SLE patients were above 8 points. Similarly, low active RA must have a DAS score less than 2.6; whereas, highly active RA patients must have DAS score of equal or above 5.4 points. Demographic information and disease manifestations of SLE and RA patients were shown in Tables **1** and **2** respectively.

Informed consent was obtained from each subject. The Study was approved by the Medical Research and Ethics Committee (MREC) at the College of Medicine, Sultan Qaboos University (SQU).

Peripheral Blood Mononuclear Cell (PBMC) Preparation

Peripheral venous blood was drawn from each subject in heparin-containing vacutainer tubes. PBMC were isolated by density centrifugation on Ficoll-Hypaque (Sigma, USA) Buffy coats were harvested, cells were washed (centrifuged at 300g for 5 minutes) in Hanks balanced salt solution (HBSS), (GIBCO, Paisley, UK) and maintained in RPMI-1640 (GIBCO) medium supplemented with 10% heat-inactivated fetal calf serum (FCS, Sigma), 2mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. This medium will be defined as complete medium.

Phenotype Analysis

CD4+ CD25+ T cells were measured by flow cytometry (FacsCaliber, Becton Dickinson USA) method, as described previously [16, 17] with minor modification. Briefly PBMC were adjusted to 4-5 $x10^6$ cell/ml. One hundred µl of cell suspension was simultaneously incubated with 20 µl of peridinin chlorophyll protein (PerCP)-conjugated anti-CD3, flourescein isothiocynate (FITC)-conjugated anti-CD4, and Phycoerythrin (PE)-conjugated anti-CD25 (Becton Dickson, San Jose CA, USA) in the fridge (at 2-8 ^oC) for 30 minutes. Cells incubated with FITC and PE-conjugated mouse IgG were used as isotype control. Ten thousands events were collected, and CD3+ cells were selected and gated for CD4 and CD25 expression. Analysis was performed on a FacsCaliber flow cytometer using CellQuest software (Becton Dickinson). Results are expressed as the percentages of cells of the total population.

Table 1. Demographic Information and Disease Manifestations of SLE Patients

Total SLE Patients	N=30
Female/Male	25/5
Age (mean ± SD)	44 ± 16
Clinical manifestation, n (%)	
Skin (malar rash, vasculitis, discoid lupus)	10 (33%)
Renal	22 (73%)
Neurological	8 (27%)
Cardiac	5 (22.7%)
Respiratory	10 (33%)
Haematological	22 (73%)
SLEDAI score	
Range	0-30
Mean ± SD	15 ± 12

Table 2. Demographic Information and Disease Manifestations of RA Patients

Total RA Patients	N=30
Female/male	25/5
Age (mean ± SD)	45 ± 15
Clinical manifestation	
Erosions, n (%)	22 (73%)
Extra-articular feature n (%)	
Nodules	10 (33%)
Vasculitis	1 (3%)
Neuropathy	1 (3%)
Other	3 (10%)
DAS score	
Range	0-8
Mean	4.7

Statistical Analysis

SPSS software program was used for the data analysis; Descriptive values of variable were expressed as the mean ± SD. All variables were normally distributed and analyzed by

student t-test. P value less than 0.05 was considered statistically significant.

RESULTS

As shown in Fig. (**1**), there is a variation in the percentages of CD4+CD25+ T cells, in both normal controls and patients with SLE or RA. The mean percentages of total CD4+CD25+ T cells were lower in low active SLE (10.50 ± 5.99), highly active SLE (9.9 ± 2.13) and highly active RA (10.42 ± 2) patients, compared to healthy control (12.93 ± 5.8), but statistically insignificant. Whereas the mean percentages of CD4+CD25+ T cells in low active RA group (15.48 ± 5.74) were slightly higher than controls.

If we defined those CD4+CD25+ T cells with fluorescence intensity of CD25 expression exceeding 100 as CD4+ $CD25^{bright}$ T cells, as previously described [17], we found that both highly active SLE (0.242 ± 0.3) and RA (0.56 ± 0.29) patients had significantly ($p < 0.001$) lower levels of $CD4+CD25^{bright}$ T cells than normal controls (1.74 ± 0.47%) or the groups with low activity (SLE=1.54 ± 0.33, RA=1.829 ± 0.76). low active SLE group had also decreased levels of $CD4+CD25^{bright}$ T cells compared to normal controls; but did not reach a statistical significance (Fig. **2**).

$CD4+CD25^{bright}$ regulatory T cells were measured in seven patients with highly active SLE and measured again after 3-8 months when patients were in remission period; we found that the decreased number of $CD4+CD25^{bright}$ T cells was restored in remitting phase (Fig. **3**).

DISCUSSION

In the present study, we have reported that highly active SLE or RA (but not patients with low activity) patients exhibited significantly low level $CD4+CD25^{bright}$ regulatory T cells compared to healthy controls. This is in agreement with previous studies done by several other investigators, enrolling different ethnic groups of patients [16, 17]. Low active

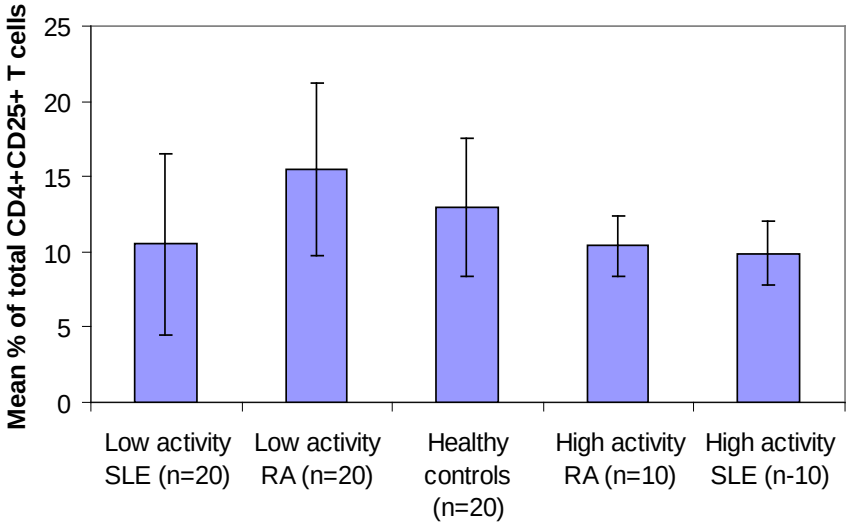

Fig. (1). Statistically insignificant decrease in the percentages of total CD4+ CD25+ T cells in peripheral blood (PB) from Systemic Lupus Erythematosus patients with low activity (SLEDAI=0-8), active SLE (SLEDAI >8) and rheumatoid arthritis (RA) patients with high activity (DAS score ≥5.4) compared to healthy controls. A slight increase in CD4+ CD25+ T cells in PB of RA patients with low diseases activity (DAS score <2.6) was observed. Values are the mean ± standard deviation (SD).

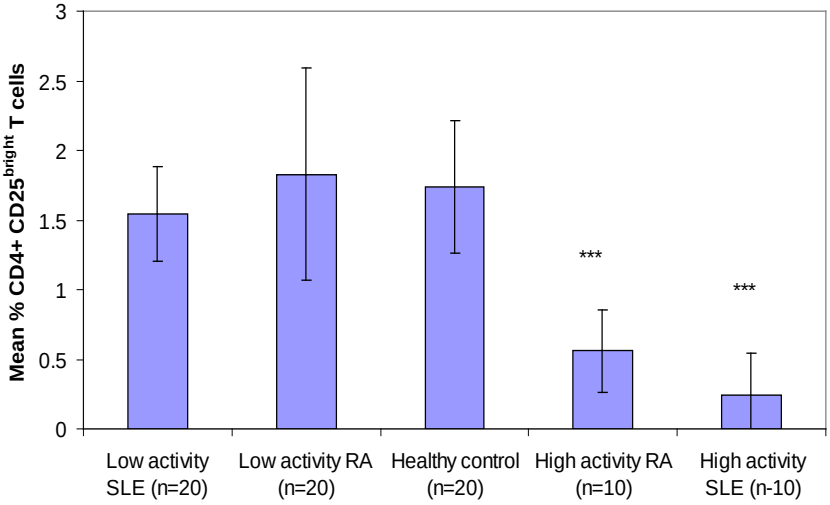

Fig. (2). Percentages of CD4+ $CD25^{bright}$ T cells in each clinical group. Highly active Systemic Lupus Erythematosus (SLE, SLEDAI >8 points) and rheumatoid arthritis (RA, DAS ≥5.4) patients exhibit a significant reduction ($p<0.001$) in the CD4+ $CD25^{bright}$ T cells compared to patients with low activity or healthy control. (***= high significant differences).

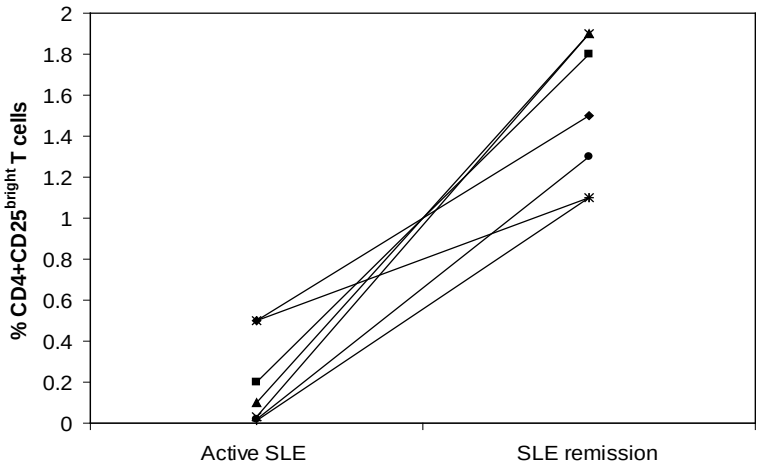

Fig. (3). The decreased number of CD4+ $CD25^{bright}$ T cells during disease activity was restored in remission period of seven patients with Systemic Lupus Erythematosus (SLE).

SLE group also showed low level of CD4+$CD25^{bright}$ T cells but statistically insignificant. This probably a reflection of using low sample size (n=20); larger sample size may offer a significant difference. The mean (± SD) percentages of CD4+$CD25^{bright}$ as well as the total CD4+ CD25+ T cells are in line with the published "normal" values [9, 16, 17].

CD4+CD25+ T cells have been investigated in several autoimmune diseases, presenting conflicting results in most cases [14-22]. These discrepancies may be attributed to the differences in the selection of patients (active or inactive diseases) or probably due to technical difficulties in CD4+ CD25+ T cells phenotypic characterization. Because CD25 is transiently up-regulated in T cells after activation, circulating CD4+CD25+ T cells make a heterogeneous population [27]. Furthermore, patients treated with corticosteroids, alone or with other immunosuppressive drugs, had a significantly higher level of CD4+CD25+ than the group of patients not on corticosteroids treatment and the control group [14]. Our groups of patients were all in immunosuppressive drugs including corticosteroid. We did not correlate the type of medications given to patients with levels of regulatory T cells, because our aim was to correlate the diseases activity with levels of regulatory T cells.

The exact mechanisms that reduce the level of CD4+ CD25+ T cells in SLE patient are not clear. However, the following hypotheses have been suggested: Cytokine profile in SLE or RA patients may have a suppressive function towards those cells (e.g. TNF-α) [11]. Moreover, patients with autoimmune diseases may release lymphocytes-specific cytotoxic antibodies [28].

It has been previously shown that only those cells expressing high levels of CD25 ($CD25^{bright}$) efficiently suppress proliferative responses, thus being considered true regulatory T cells [9]. However, FOXP3 (a forkhead/winged helix family member) has been shown to play an important role in controlling the development and functions of regulatory T cells [29]. In humans, FOXP3 is predominantly expressed in CD4+CD25+ cells, especially in the CD4+ $CD25^{bright}$ cells with regulatory functions [32-34]. Therefore, FOXP3 appears to be a specific marker for regulatory T cells and can be a potential therapeutic target for autoimmune diseases [30-32]. Hence, both of these markers (CD25, FOXP3) should be used for quantification of regulatory T cells in health and disease.

The use of regulatory T cells for the re-establishment of the peripheral tolerance is a novel approach for the treatment of autoimmune diseases. Administration of regulatory T cells may be one way to achieve this goal [33]. However, regulatory T cells are difficult to expand *in vitro* and evidences from animal models that polyclonal regulatory T cells can treat autoimmune diseases are still controversial. For example, adoptive transfer of CD4+ CD25+ regulatory T cells has been shown to cure murine colitis after disease onset in SCID mice [34]. Nevertheless, such transfer of these regulatory T cells has failed in experimental diabetes [35] and arthritis [36]. It is certainly, too early to speculate whether and how cellular therapy with regulatory T cells have therapeutic potential in autoimmune diseases until we have reliable knowledge about the function of these cells in diseases.

In summary, this work suggests that Omani patients with highly active SLE or RA exhibit a significant reduction in the CD4+ $CD25^{bright}$ regulatory T cells subset, corresponding to data reported previously with different ethnic group. This significant defect in regulatory T cells not presents during disease remission. This data provides further evidence supporting the hypothesis of defect of regulatory T cells in SLE and RA patients; and may have an important implication in the context of the control of the inflammation and development of autoimmunity.

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