Correlation of interleukin 6 and transforming growth factor β1 with peripheral blood regulatory T cells in rheumatoid arthritis patients: a potential biomarker

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Abstract

Introduction: *Proinflammatory cytokines and regulatory T cells (Tregs) are considered as important factors involved in autoimmunity development especially in rheumatoid arthritis (RA).*

Aim of the study: *To investigate the frequency of peripheral blood Tregs and related cytokines in RA patients and to determine the possible correlation between Treg percentage and interleukin 6 (IL-6) and transforming growth factor β1 (TGF-β1) as indicators in assessment of Treg function and mechanisms preceding autoimmunity in RA.*

Material and methods: *Thirty-seven Iranian RA patients with a moderate (3.2-5.1) disease activity score (DAS) and the same number of healthy age- and sex-matched individuals were enrolled. Frequency of peripheral blood Tregs (CD4$^+$FoxP3$^+$CD25^{high}) was determined by flow cytometry. Serum levels of IL-6 and TGF-β1 and their expression levels in peripheral blood mononuclear cells (PBMCs) were evaluated by ELISA and Q-PCR, respectively.*

Results: *Rheumatoid arthritis patients showed significantly lower peripheral blood Treg frequencies compared to healthy individuals. Additionally, Treg (%) showed a significant inverse correlation between serum concentrations of IL-6 and mRNA expression of PBMCs, whereas there was no significant correlation between Treg (%) and TGF-β1 levels.*

Conclusions: *The current study revealed that Treg numbers were reduced in peripheral blood of RA patients. This reduction inversely correlated with IL-6 levels, which may lead to persistent autoimmune and inflammatory conditions in RA patients.*

Key words: *rheumatoid arthritis, TGF-β1, IL-6, regulatory T cells.*

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Introduction

Rheumatoid arthritis (RA) is a chronic and systemic autoimmune disease characterized by joint inflammation eventually leads to destruction of cartilage and bone [1]. Although what precisely causes RA is not fully understood [2], studies highlight the potential importance of T cell subsets and cytokines in both driving and suppressing inflammation in RA [3, 4]. Regulatory T cells (Tregs) have an important role in the control of auto-reactivity and the pathogenesis of RA. The particular function of Treg cells (CD4$^+$FoxP3$^+$CD25^{high} cells) is preventing autoimmunity either via the suppression of other autoreactive lymphocytes through cell-cell interactions or via secretion of regulatory cytokines such as transforming growth factor β1 (TGF-β1) [5, 6].

The cytokine network is complex in RA and includes various cytokines presenting in both synovial joints and peripheral blood [7, 8]. Transforming growth factor β1 is one of the cytokines in RA secreted from various cell types. This cytokine regulates proliferation and differentiation of T cell subsets [9] and has a modulatory effect on inflammatory cells [10].

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Furthermore, IL-6 is one of the most prominent inflammatory cytokines, which has pleiotropic activities in RA pathogenesis and its systemic manifestations [11]. Overproduction of IL-6 from several cells results in maturation of B cells, and consequently production of auto-antibodies as well as enhanced migration of inflammatory cells into the joint and also stimulates production of harmful compounds from chondrocytes and other immune cells [12, 13]. Additionally, IL-6 has a pivotal role to determine T cell fate in collaboration with other cytokines [14]. There is cross-talk between TGF-β1 and IL-6 signaling pathways [15]; however, a novel hypothesis has been proposed that they act in a dose-dependent manner, and therefore polarization as well as plasticity of T cells is changed in the mentioned cytokine milieu and may be one of the factors responsible for autoimmunity development [16]. *In vitro* studies showed that IL-6 and TGF-β1 signaling promotes FoxP3 degradation, consequently resulting in imbalance in the population of Treg/Th17 [17].

Related information about Treg frequency in the peripheral blood of RA patients is controversial. Thus the current investigation was carried out to evaluate the frequency of peripheral Tregs (CD4⁺FoxP3⁺CD25ʰⁱᵍʰ) and correlation with serum level and mRNA expression of IL-6 and TGF-β1 between RA patients in comparison with healthy individuals.

Material and methods

Patient selection

In this study, 37 patients with RA and the same number of age- and sex-matched healthy individuals were recruited from the Rheumatology Clinic of Imam Ali in Shahrekord, Iran. In all RA patients, the diagnosis was established with ACR/EULAR diagnostic criteria for RA 2010 [18]. In this cross-sectional study, the patient conditions were all chronic and they were selected as having moderately active disease (3.2-5.1) on the basis of disease activity score 28 (DAS28). Written consent was obtained from all participating individuals prior to sample collection. The study was approved by the ethical committee of Sharekord University. Rheumatoid arthritis patients were treated with disease-modifying anti-rheumatic drugs (DMARDs) and steroids. Mean doses of medications for RA are presented in Table 1.

Table 1. Mean doses of DMARD therapy in RA patients

Medication	Mean dose
Methotrexate (MTX)	7.5-15 mg/wk
Pprednisone (steroid)	5-10 mg/d
Hydroxychloroquine	200-400 mg/d
Sulfasalazine	1-2 g/d

Sample preparation

After taking 6 ml of whole blood with K₃EDTA, the samples were immediately transferred to the laboratory on ice. Small aliquots of fresh whole blood were separated for flow cytometric analysis of Tregs. Then in order to isolate PBMCs, blood was mixed with the same volume of cold PBS. Diluted blood was gently transferred to a sterile tube containing Ficoll Histopaque-1077 (Sigma). The PBMC ring was separated by washing twice with cold PBS by 30 min centrifugation at 400 g, 4°C. Total RNA was quickly isolated from PBMCs using total RNA extraction TRIzol Reagent (Invitrogen). Quality and quantity of RNA were determined by a spectrophotometer (NanoDrop ND-1000, Thermo Scientific) and gel agarose electrophoresis (observing distinct 28S and 18S ribosomal bands).

Real time-PCR

For cDNA synthesis 0.5 μg of total RNA was used for the reverse transcription reaction, which was carried out using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Fermentas) according to the manufacturer's instructions. The quantification of mRNA expression of cytokines was performed using Rotor-Gene TM 6000 (Corbett). Real time-PCR (RT-PCR) reactions were performed in a total volume of 25 μl containing 2 μl of synthesized cDNA as a template, 12.5 μl of 2x Rotor-Gene Probe PCR Master Mix (Qiagen, Germany), 500 nM of each primer and 250 nM of the TaqMan-probe (FAM, TAMRA), 3 μl, and finally DNase/RNase free water was added to make a total volume of 25 μl. The amplification program included a pre-heating step (10 min at 94°C), a denaturation step (94°C for 15 s) and an annealing/extension step (60°C for 60 s). The mRNA expression of

Table 2. Sequences of primers and probes

Gene	Sequence
β-Actin Probe	5'- CCGCCGCCCGTCCACACCCGCC-3'
β-Actin Forward	5'-AGCCTCGCCTTTGCCGA-3'
β-Actin Reverse	5'-CTGGTGCCTGGGGCG-3'
IL-6 Probe	5'- TGTTACTCTTGTTACATGTCTCCTTTCTCAGGGCT -3'
IL-6 Forward	5'- GGTACATCCTCGACGGCATCT -3'
IL-6 Reverse	5'- GTGCCTCTTTGCTGCTTTCAC -3'
TGF-β1 Probe	5'- CTGCTGGCACCCAGCGACTCG -3'
TGF-β1 Forward	5'- CAGCAACAATTCCTGGCGATA -3'
TGF-β1 Reverse	5'- AAGGCGAAAGCCCTCAATTT -3'

Table 3. Laboratory investigations of RA patients and healthy individuals included in this study ($n = 37$)

	RA patients	Healthy individuals	*p*-value
	Mean ±SD	Mean ±SD	
Age	46.9 ±7.6	44.5 ±8.8	NS
WBC count (10^3 cell/µl)	7750 ±2390	5950 ±830	**
Lymphocyte count (10^9/l)	2200 ±630	2050 ±370	*
ESR (mm/h)	45.6 ±23.55	9.8 ±5.35	***
CRP (mg/l)	21.3 ±7.8	< 4	***
Rheumatoid factor (IU/ml)	83.4 ±60.2	–	***
Anti-citrullinated peptide antibody (IU/ml)	60.5 ±43.3	–	***
Disease activity score (DAS28)	4.2 ±0.38	–	–

*NS – not significant; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$*

Table 4. Data and statistical interpretations of investigation

	RA patients	Healthy individuals	*p*-value
	Mean ±SD		
Treg percentage	1.58 ±0.24	1.85 ±0.3	**
Serum IL-6 (pg/ml)	28.1 ±9.1	4.2 ±0.8	***
Serum TGF-β1 (ng/ml)	41.2 ±10.43	29.8 ±8.96	***
mRNA expression in PBMCs (fold change)	Mean ±SEM		
IL-6	1.3 ±0.21	0.57 ±0.21	***
TGF-β1	1.35 ±0.24	2.15 ±0.37	**
Correlation	Correlation coefficient (*r*)		
Tregs (%) and serum IL-6	0.47		**
Tregs (%) and IL-6 expression in PBMCs	0.37		**

**$p < 0.01$, ***$p < 0.001$

cytokines was normalized to β-actin (housekeeping gene) and relative quantification was determined using the $2^{-\Delta\Delta Ct}$ method. The sequence of oligonucleotides of IL-6, TGF-β1 and β-actin primers and probes is shown in Table 2.

Flow cytometry

Antibodies used for Treg cell assessment in flow cytometry were purchased from BD Pharmingen (Becton Dickinson, San Jose, CA, USA, BD); anti-CD4 conjugated to PE-Cy5, anti-CD25 (alpha chain of IL-2 receptor) conjugated to allophycocyanin (APC) and anti-FoxP3 conjugated to phycoerythrin (PE). The staining process was as follows: small aliquots (100 µl) of whole blood containing K_3EDTA were incubated for 30 min with anti-CD4 and anti-CD25, and red blood cells were lysed subsequently. After surface staining, cells were fixed and permeabilized using the Fixation/Permeabilization Kit (BD, Human FoxP3 Buffer Set) for intracellular staining. Briefly, cells were fixed in FoxP3 Buffer A, for 15 minutes at room temperature (RT) and the cells were washed. To permeabilize cells they were incubated for 30 minutes in human FoxP3 Buffer B. Finally, after washing, cells were stained with 10 µl of PE Mouse anti-Human FoxP3. All of the process was protected from light.

Firstly gating was done on CD4$^+$ cells then detection of CD25^{high}, FoxP3$^+$ within the population followed. Isotype controls were used to discriminate unstained cells. Analysis was performed using a flow cytometer (PARTEC, Münster, Germany). The results were analyzed using Flow Jo software version 7.6.1 (Tree Star, Ashland, OR, USA).

Measurement of serum IL-6 and (active) TGF-β1 concentration by ELISA

Serum concentrations of IL-6 and TGF-β1 were determined with an ELISA kit (Quantikine; R&D, Minneapolis, MN). These assays were performed according to methods outlined in the manufacturer's instructions. To activate latent TGF-β1, HCL/NaOH was added to samples in the concentration listed in the instructions. Thereafter, we separately for each plate added 50 and 100 µl of activated TGF-β1 and IL-6 of samples per well, respectively. The plates were incubated for 2 hours at RT. After aspirating and washing each well three times, 100 µl for TGF-β1 and 200 µl for IL-6 conjugate antibody were added to each well and the plates were incubated for 2 hours at RT. We repeated the aspiration/wash three times and added 200 ml of substrate solution to each well and incubated plates away from lights for 30 minutes. Finally, we added stop solution and absorbance in each well was measured using a spectrophotometric plate reader at a wavelength of 450 nanometers (Biohit, BP800, Finland). Serum levels of TGF-β1 and IL-6 were calculated from a linear regression standard curve in logarithm scales.

Statistical analysis

All statistical analyses were done with SPSS version 21 (SPSS Inc, Chicago, Illinois, USA). At first normality tests were used to determine whether a data set is well modeled by a normal distribution. The differences between patients and healthy groups as well as correlation for Treg percentage and cytokine levels were analyzed using the

Mann-Whitney and Spearman test, respectively. Afterwards, p values less than 0.05 were considered significant at the level of the 95% confidence interval. Results for cytokine expression and frequencies of Treg cells were presented using Prism software 6.01 (Graph Pad, La Jolla, California) as means ±standard error of mean (SEM) and standard deviation (SD), respectively.

Results

In this study, 37 patients (32 females and 5 males) with RA and the same number (31 females and 6 males) of healthy individuals were included. The descriptive data, laboratory finding and statistical interpretation of investigation in RA patients and healthy individuals are shown in Tables 3 and 4.

RT-PCR results

Interleukin 6 expression was significantly higher in PBMC specimens of RA patients compared with healthy individuals ($p < 0.001$). The mean ± SEM of IL-6 expression in RA patients and healthy individuals were 1.3 ±0.21 and 0.57 ±0.21, respectively. Consequently, IL-6 mRNA expression in RA patients showed a 2.6-fold difference in comparison with healthy individuals. In addition, TGF-β1 expression was significantly lower in PBMC specimens of RA patients compared with healthy individuals ($p < 0.01$). The mean ± SEM of TGF-β1 expression in RA patients and healthy individuals was 1.35 ±0.24 and 2.15 ±0.37, respectively. Therefore, the TGF-β1 mRNA expression in RA patients showed a 0.63-fold difference in comparison with healthy individuals (Fig. 1A, B).

ELISA results

The mean ± SD of the TGF-β1 in serum of RA patients and healthy individuals was 41.2 ±10.43 and 29.8 ±8.96 ng/ml, respectively. This level was significantly higher in RA patients than in healthy individuals ($p < 0.001$). Additionally, the level of IL-6 was significantly higher in the RA patients than in healthy individuals (28.1 ±9.1 vs. 4.2 ±0.8 pg/ml) ($p < 0.001$) (Fig. 2C, D).

Treg cell frequencies

The frequency of Tregs (CD4$^+$FoxP3$^+$CD25^{high}) was significantly lower ($p < 0.01$) in the RA patients (1.58 ±0.24) compared to healthy individuals (1.85 ±0.3). Also, the percentage of Tregs showed a significant inverse correlation with serum IL-6 ($r = 0.47$, $p < 0.01$) and mRNA IL-6 expression in PBMCs ($r = 0.37$, $p < 0.01$) of RA patients. The inverse correlation was stronger between Treg percentage and serum IL-6 levels (Fig. 1E, F). However, there was no significant correlation between frequency of Tregs and TGF-β1 (data not shown).

Discussion

Deregulation of tolerance mechanisms plays a pivotal role in the pathogenesis of rheumatoid arthritis. Furthermore, the persistent immune dysfunction with systemic inflammation suggests an imbalance in the cytokine secretion and frequency of Tregs.

In this study we investigated the frequency of regulatory T cells, then determined possible relationships between their frequency and cytokine production in moderately active RA patients compared to healthy individuals. At first, flow cytometric results of RA patients revealed a significantly lower percentage of circulating CD4$^+$Foxp3$^+$CD25^{high} Tregs as compared to healthy controls. Similarly, our findings are consistent with most studies, although the authors have presented various conclusions [19-21]. A major differences between studies could be a result of the definition of Tregs in flow cytometric gating patterns [22-24]. However, some studies have suggested that regulatory T cells are recruited to sites of inflammation to modulate disease, resulting in a relative reduction in the population of peripheral blood Tregs. Reports indicating an increased number of Tregs in synovial fluid support this hypothesis [25]. In addition, some studies have shown increased Tregs' spontaneous apoptosis in active RA [26, 27].

Contrary to our findings there are reports indicating higher Treg levels in RA peripheral blood, but they assessed different levels of CD25 T cells, which could account for the variation in the results [20, 28]. Nevertheless, some *ex vivo* studies on CD4$^+$T cells showed that only high CD25 expressing T cells exert inhibitory effects. In contrast, intermediate expressing CD25 T cells are effector cells [29, 30].

We next investigated IL-6 and TGF-β1 in two fractions: serum levels and mRNA expression in PBMCs. We observed significantly higher IL-6 mRNA expression of PBMCs and serum levels in RA patients compared to healthy individuals. Although the level of TGF-β1 was higher in serum, mRNA expression of PBMCs was lower than in the healthy group.

Interleukin 6 is produced predominantly by macrophages and fibroblasts, but the exact source and mechanisms leading to IL-6 increase in the serum of RA patients are still unclear. The effects of IL-6 on disease activity, symptom persistence and inflammation have been formerly established in the pathogenesis of RA [31, 32]. Therefore, several studies have documented that an elevated IL-6 level is in correlation with physical damage, disease activity (DAS) and erythrocyte sedimentation rate (ESR) [11, 33, 34].

Wang *et al.* proposed that an inflammatory environment made Tregs unstable and they are converted to pathological T cells such as Th17 [35]. Nevertheless, our results also demonstrated that Treg percentages showed a positive inverse correlation with serum concentration of
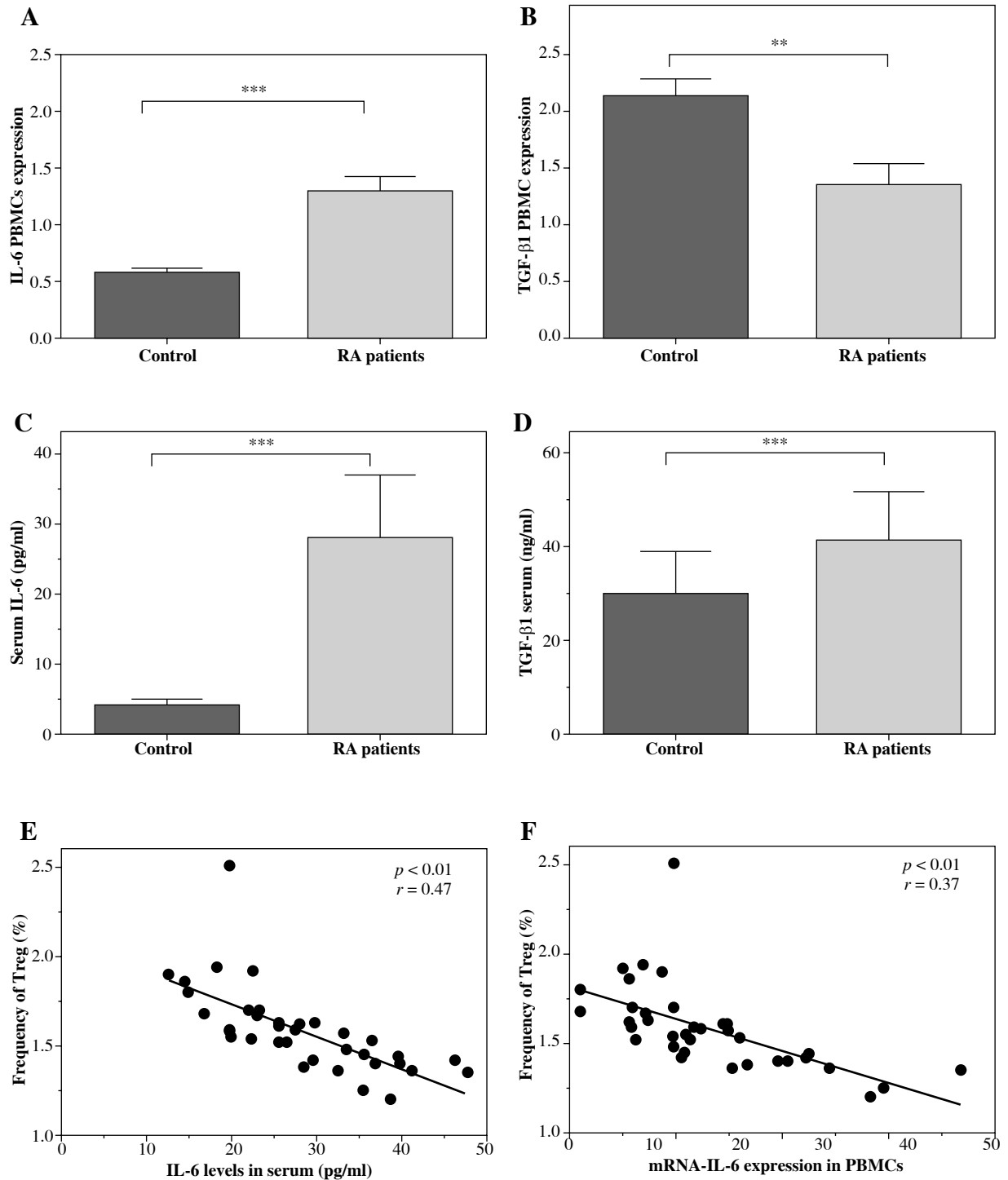
Fig. 1. Level of serum concentration, mRNA expression and the correlation of IL-6 and TGF-β1 in RA patients and healthy controls. IL-6 and TGF-β1 mRNA expression in PBMCs was analyzed by real-time quantitative-PCR (mean ±SEM). The results were normalized to β-actin **(A, B)**. Serum concentrations of IL-6 and TGF-β1 were measured by ELISA. Data are expressed as means ±SD **(C, D)**. Correlation coefficient and regression line of peripheral blood Tregs (%) with IL-6 serum levels **(E)** and mRNA expression in PBMCs **(F)** were represented as scatter plots. Spearman's correlation analysis was performed. There was no significant correlation between Treg and TGF-β1 (not shown)
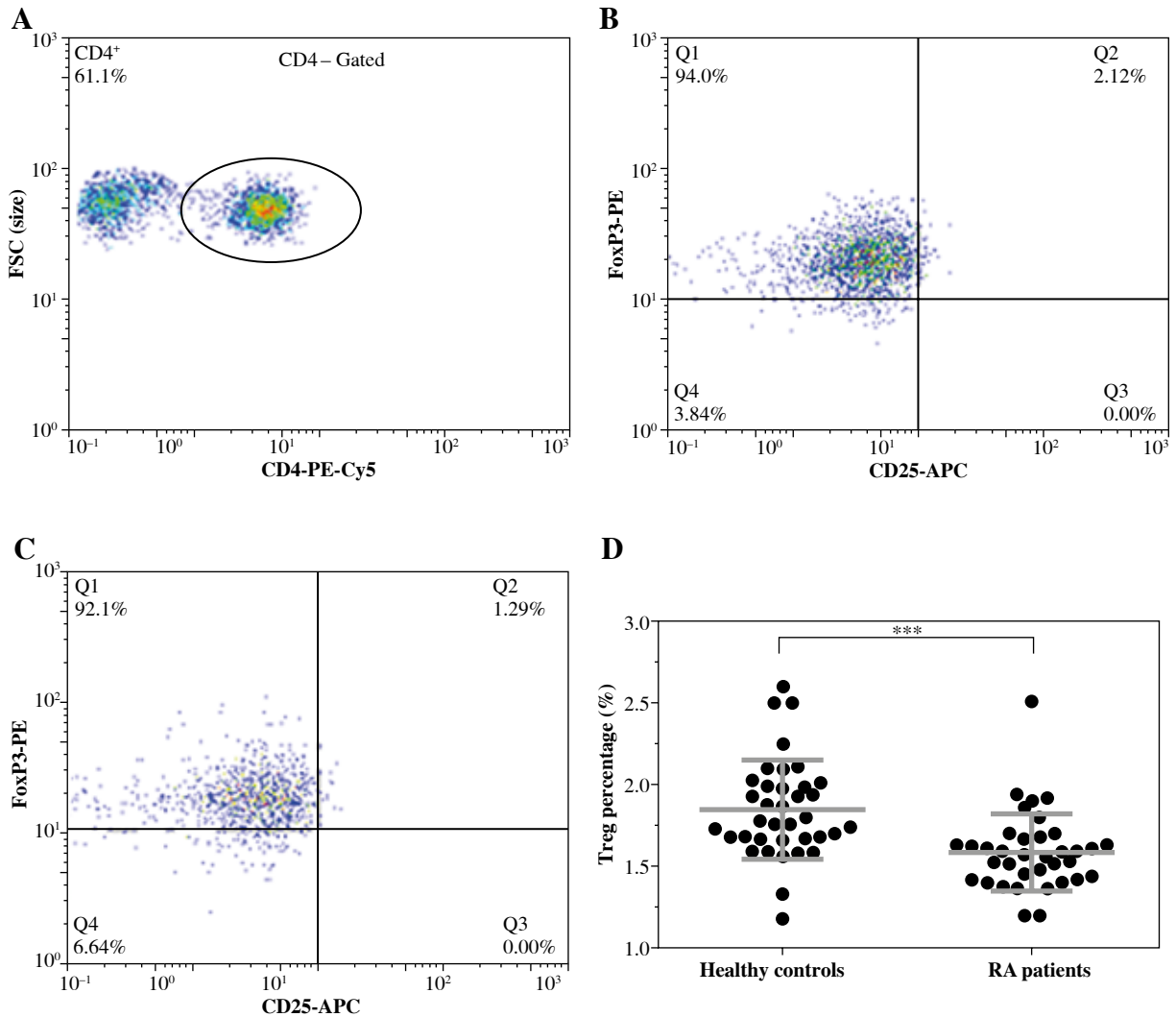
Fig. 2. Flow cytometric analyses of peripheral blood Tregs in RA patients and healthy controls. CD4 positive cells were gated from lymphocyte population (**A**). Intracellular FoxP3 and the expression of surface CD25 were indicated as dot plots (Q2) for healthy controls (**B**) and RA patients (**C**). Data are shown as mean and SD in scatter plot, representing significant difference of Treg (%) between healthy controls and RA patients (**D**)

IL-6 and its mRNA expression in PBMCs. This correlation is more robust in the serum fraction than mRNA expression of PBMCs. In this way, investigations showed that cytokine profile is not appropriate for differentiation and survival of Treg cells in peripheral blood of RA patients, and subsequently it leads to a decrease in Treg numbers. It seems this circumstance may facilitate establishment of the autoimmunity process [36].

Transforming growth factor β1 is one of the most prominent immune suppressor cytokines that is secreted by almost all immune cell lineages, including T, B, dendrite cells, macrophages, as well as fibroblasts [9]. Transforming growth factor β1 negatively regulates the proliferation,

differentiation and death of different cell types; therefore the most critical function of TGF-β1 is prevention of aberrant responses leading to autoimmunity [9, 10]. Our study showed a significantly higher serum level of TGF-β1 in RA patients compared with healthy controls. However, mRNA expression of TGF-β1 was markedly lower in PBMCs of RA patients. Several studies have suggested that elevated TGF-β1 in rheumatoid sera and also synovial fluids may suppress joint inflammation by inhibiting RANTES secretion [37]. In addition, higher TGF-β1 levels in serum and synovial tissue were found in patients with rheumatoid arthritis compared with osteoarthritis patients [38]. On the other hand, some reports showed that TGF-β1 accounts

for increasing the expression of proinflammatory cytokines and metalloproteinase-1 (MMP-1) by synovial fibroblasts and promotes joint destruction by induction of aggrecanase [39]. These contradictory functions of TGF-β1 might depend on dose and site of effect. Some animal experiments have demonstrated that systemic administration of TGF-β1 to mice inhibits collagen-induced arthritis (CIA), whereas its local administration to joints induces synovitis and exacerbates the disease activity. Similarly, blocking endogenous TGF-β1 by systemic injection of anti-TGF-β1 antibody aggravates CIA in mice, whereas the local blockade of TGF-β1 ameliorates continuous inflammation [37, 40].

The Q-PCR results of TGF-β1 expression in PBMCs showed a decrease in RA patients which was scarcely described elsewhere. This finding could be a result of DMARD therapy, systemic inflammatory condition and immune cell infiltration, especially joint involvement. No association was found between serum levels and PBMCs' mRNA expression of TGF-β1 with Treg frequency in RA patients. There are scant studies that have investigated *in vivo* the effect of TGF-β1 on Treg frequency, but numerous *in vitro* studies have shown how TGF-β1 and IL-6 act together to promote the degradation of FOXP3 in Tregs.

Recent clinical studies showed that DMARD therapy would result in a reduction of IL-6 and other pro-inflammatory cytokines such as TNF-α in both serum and synovial fluid, but in spite of DMARD therapy, these values remained higher in patients in comparison to healthy individuals [11]. In addition, *in vitro* studies demonstrated that methotrexate (MTX) induced more apoptosis in T cells than monocytes. Also, other studies have shown that the IL-6 level produced by monocytes was higher than T cells, but there were some recent *in vitro* observations that MTX induced the expression of pro-inflammatory cytokines such as IL-1 and IL-6 in a monocyte cell line; however, they were a small population in PBMCs [41]. Moreover, RA patients on combinational therapy (DMARD therapy plus biologic drugs) showed a smaller increase in pro-inflammatory cytokine levels compared to patients on DMARD therapy alone [42]. In summary, identification of a reliable and accessible biomarker for disease activity, its progression, and monitoring of patients is an attractive therapeutic aim. It seems that the levels of some pro-inflammatory cytokines, for example, IL-6 produced by PBMCs, can be used as a biomarker to predict the status of treatment.

Conclusions

In summary, the present study showed that RA patients have a lower peripheral blood Treg percentage and higher serum levels of IL-6 and TGF-β1 than healthy individuals. We also found increased expression of IL-6 but not of TGF-β1 in PBMCs. Also, our results confirm that IL-6 inversely correlated with Treg frequency in peripheral blood.

Further studies are needed to assay other cytokines and their molecular relationships with Treg frequency.

Limitations

In this study, in order to keep uniform the severity of disease, RA patients with similar activity (moderate group) were included based on DAS28. Nonetheless, this categorization and also the impact of DMARD therapy do not coincide for all cases. Consequently, further research is needed to evaluate more accurately the correlation between Treg percentage and levels of IL-6 and TGF-β in all RA patients with different disease activities. Additionally, in order to avoid influencing the therapy at cytokine levels, early diagnosed RA patients with no treatment should be included in the future studies to more efficiently categorize patients.

The authors declare no conflict of interest.

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