Association Between *IL-17A*, *FOXP3*, and *CTLA4* Genes Expression and Severity of Lupus Nephritis

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Introduction. Elevated levels of interleukin 17A (IL-17A) have been found in systemic lupus erythematosus (SLE). Forkhead box protein P3 (FOXP3) activates T-regulation lymphocytes and is a master regulator of cell function. The cytotoxic T-lymphocyte-associated protein 4 (*CTLA4*) gene plays a similar role. We investigated the role of the expression of these genes in SLE patients with and without nephritis.

Methods. The present study was a case-controlled trial including 49 patients with SLE and 26 healthy controls. Gene expressions of *IL-17A*, *FOXP3*, and *CTLA4* were measured by quantitative Real-Time PCR. The relation between lupus nephritis disease activity and *IL-17A*, *FOXP3*, and *CTLA4* gene expression was evaluated.

Results. *IL-17A*, *FOXP3*, and *CTLA4* expression in T-cells were significantly higher in SLE patients than controls ($P < .0001$). When comparing the nephritis group and non- nephritis group with the control group, the expression of the mentioned genes was also higher ($P < .05$). There was no significant difference regarding *IL-17A*, *FOXP3*, and *CTLA4* genes expression in the nephritis group and non- nephritis group ($P > .05$). Nonetheless, there was a low expression of *FOXP3* and *IL-17A* in patients with the higher stages of nephritis ($P < .05$).

Conclusion. Our findings showed that elevated *IL-17A*, *FOXP3*, and *CTLA4* expression significantly correlate with SLE pathophysiology. This study provides new insight into the function of *IL-17A*, *FOXP3*, and *CTLA4* in a disease setting. Heterogeneity of SLE patients is reflected in the multiple abnormalities found in the immune system. Finding such variations can provide targets for better manipulation of the immune system.

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Original Paper

INTRODUCTION

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease that is related to immune system mediators.[1, 2] Lupus nephritis (LN) as the most severe SLE complication, is characterized by immune complex development and renal tubules and glomeruli inflammation, which if not treated can result in renal failure.[3, 4]

Recent studies highlight the importance of the abnormal expression or function of T-helper cells in pathogenesis of SLE. T-helper cells are classified as Th1, Th2, and Th17. Th1 and Th2 cells produce higher amounts of IFNγ and IL4. Th17 cells produce inflammatory cytokines IL17A, IL22,

IL10, and TGF-β and are characterized by *FOXP3* gene expression.[5, 6]

Regulatory T (Treg) cells suppress the activation of T-helper cells. The increased number ofTh17 and reduced Treg cells might promote autoimmune diseases.[7, 8] Though both Th17 and inducible T-reg cells need the similar cytokine, TGF-b1, during the early stage of differentiation, in the presence of pro-inflammatory cytokines IL-1b and IL-6, FoxP3 is down-regulated, and T-cells with transitional phenotype exhibit a set of essential proteins for Th17 development.[9] The Th17/Treg balance may be important in the pathogenesis of SLE and LN, although it needs to be further elucidated.

In addition, IL-2, which is needed for the development and maintenance of FoxP3 expressing Treg cells, has been discovered to inhibit the growth of Th17 cells.[10] Consequently, the counteractive outcomes of IL-2 and IL-6 on Th17 differentiation and Treg in the periphery might interfere with immunoregulatory feedback and promote the persistence of autoimmune inflammation.

Although the imbalance ofTh17 /Treg in LN has been demonstrated in several studies, the relationship of these cells with the severity of LN needs to be more evaluated. Thus, the current study aims to investigate the role of association between *IL-17A*, *FOXP3*, and *CTLA4* genes expression in SLE patients with or without nephritis.

MATERIALS AND METHODS

Study Population and Protocol

This case-control study was conducted from September 2017 to August 2018 in the Lupus clinic of Hafez hospital, a tertiary healthcare center affiliated to Shiraz University of Medical Sciences. We included 49 SLE patients and 26 healthy age and sex-matched controls. Patients with confirmed SLE, who had symptoms of at least one-year duration and fulfilled at least four of the American College of Rheumatology criteria, were enrolled.[11] Fifty patients participated in this study, but after performing test results, data of one patient was not acceptable and removed. SLE patients were divided into two groups; 29 with nephritis and 19 without nephritis. Thirty healthy volunteers participated as controls, but during the study, 4 cases quitted.

We enrolled adult patients with 18 years old of age or more, and patients with acute or chronic renal insufficiency, acute or chronic infection, and co-morbidities like diabetes mellitus, liver disease, and malignancy were excluded. Disease activity categories were defined based on systemic lupus erythematosus disease activity index 2000 (SLEDAI): no activity (SLEDAI: 0), mild activity (SLEDAI: 1 to 5), moderate activity (SLEDAI: 6 to 10), high activity (SLEDAI: 11 to 19), and very high activity (SLEDAI: 20). The LN patients were also categorized into groups I to VI according to pathologic reports of tissue specimens based on the 2003 International Society of Nephrology/ Renal Pathology Society Classification of LN. [12] All patients included in our study had received prednisolone, 44 patients less than 30 mg/d and 5 patients more than 30 mg/d.

The protocol was approved by the Declaration of Helsinki and the local ethic committee. All patients provided written informed consent before inclusion. Fasting blood samples (5 mL) were obtained from all cases and collected into test tubes containing 0.2 mL sodium heparin. Sera were stored at -70 °C. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation. For Th17 analysis, PBMCs were suspended at a density of 2×10^6 cells/ml in a complete culture medium (RPMI 1640 supplemented with 100 U/ mL penicillin and 100 ng/mL streptomycin 2 nM glutamine, 10% heat-inactivated fetal calf serum; all from Gibco, Thermo Fisher Scientific, USA). The cell suspension was transferred to each well of 24-well plates. Cultures were stimulated with Phorbol Myristate Acetate (PMA, 50 ng/mL) plus Ionomycin (1 ng/mL) for 4 h in the presence of Monensin (1.7 ng/mL; all from Sigma Aldrich, St. Louis, USA). The incubator was set at 37 °C under a 5% CO_2 environment.

Total RNA Extraction

Total RNA was extracted from peripheral blood samples by TRIzol™ Reagent (Invitrogen, USA) according to the manufacturer's instructions. Briefly, 5 mL of peripheral blood was transferred in a conical 50 mL tube. After that, 45 ml NH_4Cl lysis solution was added to each tube and incubated at room temperature for 10 minutes. All samples were centrifuged at 600 g to sediment white blood cells (WBCs), and supernatants were discarded. WBCs pellet was washed twice with ice-cold phosphate buffer saline (PBS) and resuspended in 100 µL

PBS. One mL of TRIzol reagent was added to each tube and incubated on ice for further lysing. After three times up and down rotations, 200 µL chloroform was added to each tube. After several times up and down, samples were centrifuged at high speed for 20 minutes at 4 °C. RNA in the upper phase was pelleted by 100% isopropanol. For this purpose, 500 µL of isopropanol was added to each tube that contained approximately 500 µL of previous step supernatant. The RNA pellet was washed with 75% ethanol and eluted in the 40 µL DEPC-treated water. The quantity of RNA was evaluated by the spectrophotometric method. The optical density (OD) of extracted RNA was determined at 260, 280, and 320 nm after making a diluted sample (1:100 in DEPC-treated water). The concentration of the RNA sample was calculated using the following formula:

RNA (µg/mL) = 40 × (OD260-OD320) × dilution factor.

A ratio of OD260/OD280 more than 1.8 was considered as acceptable purity. The quality of extracted RNA samples was also checked by gel electrophoresis. For this purpose, 5 µg of the extracted RNA was mixed with one µL of loading dye (6x), and the mixture was electrophoresed in 1% agarose gel in TAE buffer. Observations of 28S and 18S rRNA bands (2:1 ratio) were considered as intact high-quality RNA. To avoid DNA contamination, RNA was treated with DNase I (Invitrogen-Gibco, Paisley, UK) before cDNA synthesis. To perform this step, 5 µg of total RNA (set to 8 µl with DEPC) were mixed by 2-unit DNase I and DNase I buffer (1 µL of DNase I enzyme and 1 µL of 10X DNase I buffer) and were incubated 30 minutes in 37 °C followed by 10 minutes at 65°C to inactivate DNase enzyme.

Reverse Transcription (cDNA Synthesis)

Synthesis of cDNA was performed from 5 µg of DNase treated total RNA, utilizing the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, USA) according to the kit instructions. Briefly, 5 µg of DNase I treated total RNA (10 µL) and 1 µL oligo dT and 1µL Random Hexamer primer in the final volume of 12 µL was added into 0.5 mL microtube and incubated 5 minutes at 70°C in Dri-Block (Techne, UK). Then tubes were transferred on ice, and 4 µL reaction buffer (5x), 2 µL dNTP (10 mM), 1-unit RNase inhibitor was added to each tube, and tubes were incubated 5 minutes at 37 °C followed by 5 minutes at 25 °C to allow the primer to were annealed. After that, 1 µL reverse transcriptase enzyme (5 units) was added to all tubes, and samples were incubated at 42 °C for 90 minutes in a thermal cycler (Eppendorf, Germany) with a heated lid. Finally, the reverse transcriptase enzyme was inactivated at 70 °C in 10 minutes, and cDNA was kept at -70 °C.

Quantitative Real-Time RT-PCR (qRT-PCR)

The expression and quantity of *IL-17A*, *FOXP3*, *CTLA4*, and 18s rRNA gene transcripts were determined using an ABI 7500 system (Applied Biosystem, Foster City, CA, USA) and utilizing Universal TaqMan PCR Master Mix (Applied Biosystems). As an indication for the target gene expression level, 18s rRNA housekeeping gene expression was used. Specific primers and probes to determine the expression of *IL-17A*, *FOXP3*, *CTLA4*, and 18s rRNA were designed using Primer-Blast online software. Sequences of each primer were listed in Table 1. Each PCR reaction was carried out in a final volume of 20 µL, which contained 0.5 µg of the cDNA product, 150 nM of each primer and probe, and 1x reaction mixture consisting of FastStart DNA polymerase, dNTPs, reaction buffer, and SYBR green I provided by the supplier (Applied Biosystems). Thermal cycling for all genes was set off through denaturation step at 95 °C for 10 min, followed by 50 cycles denaturation at 95 °C for 15 s, annealing at 56 °C for 30 s, and extension at 60 °C for 60 s, respectively (Table 2). Fluorescence acquisition was performed at the final step of each extension step.

Table 1. Sequences of Primer Pairs

Primer Name	Forward Primer 5' Sequence 3'	Reverse Primer 5' Sequence 3'
18s rRNA	GGCGGCACCACCATGTACCC	GGAGGGGCCGGACTCGTCAT
IL-17A	ACCTCAACTCCTGCCACAAT	GCCTTCTTGGGCATGTAAAA
FoxP3	CATGGGCCTTCATGCTATTT	TGATGTACTTGCAGCCTTGC
CTLA4	ACCAGGTGGAGTTCAAGACC	TGGCACAGTCTCACTGTTGA

Table 2. Thermal Cycling Steps for Amplification of Target Genes

Steps	Temperature	Time	Cycle
Initial Denaturation	95 °C	5 min	One time
Cycling Denaturation	95 °C	15 second	50 times
Annealing	56 °C	30 second	
Extension	60 °C	60 second	
Fluorescence Acquisition			

Real-Time PCR Efficiency

To determine the efficiency of Real-Time PCR, logarithmic dilutions of one of the cDNA samples were used in reactions with different primer and probe pairs. Standard curves were obtained for this sample. The Real-Time PCR efficiencies were calculated from the slope of standard curves according to the established equation: Efficiency (E) = [10 $^{(-1/\text{slope})}$].

Results were showed that the efficiency of each reaction was acceptable to continue gene expression calculation. Efficiency of *IL-17A*, *FOXP3*, *CTLA4*, and 18s rRNA was 92.8%, 91.6%, 93.2% and 96.7%, respectively. Finally, the $2^{-\Delta Ct}$ model was used to combine gene quantification and normalization into a single calculation.[13]

Statistical Analysis

Data were checked for normality using Shapiro–Wilk test. Comparisons of the relative expression of *IL-17A*, *FOXP3*, *CTLA4*, and 18s rRNA gene transcripts were evaluated using the Mann-Whitney and Kruskal-Wallis test. Correlation between target genes expression was calculated by spearman's correlation test. The level of statistical significance was set at P value < .05 Data were analyzed in GraphPad Prism for Windows (version 9.0, GraphPad Software Inc. La Jolla, California, USA).

RESULTS

In the present study, 49 SLE patients [46 females (93.9%) and 3 males (6.1%)] were divided into two groups: 29 with nephritis and 20 without nephritis and compared with 26 healthy controls (24 women and 2 men). SLE patients and healthy controls were effectively matched for age and gender. Serum levels of C3 and C4 complement were significantly lower in the nephritis group than controls (P < .001). In addition, patients in the nephritis group showed worse kidney function with lower eGFR and higher serum creatinine and proteinuria compared to patients in the non-nephritis and healthy control groups.

Hemoglobin levels were significantly lower in SLE patients compared to controls, and there was also a significant difference between nephritis and non-nephritis groups (P < .01). Also ESR levels were significantly higher in LN group compared with healthy controls (Table 3).

Twenty-nine renal biopsy specimens with LN were re-evaluated, and LN activity and chronicity indices reported as described in Table 4, Further

Table 3. The Comparison of Demographic, Clinical, and Laboratory Data Between SLE Patients and Healthy Controls

Variables	Healthy Controls (n = 26)	SLE Patients (n = 49)	
		Non-nephritis (n = 20)	Nephritis (n = 29)
Age, y	32.1 ± 9.1	32.3 ± 8.5	31.0 ± 10.1
Sex (Female/Male)	24/2	28/1	18/2
WBC, 10^3/μL	5.8 ± 1.6	6.1 ± 2.3	6.7 ± 1.9
Hemoglobin, g/dL	13.5 ± 1.6	10.6 ± 1.8***	8.6 ± 1.7***, ##
Platelet, 10^3/μL	242.0 ± 92.6	233.0 ± 59.8	221.0 ± 85.9
Serum Creatinine, mg/dL	0.9 ± 0.2	1.1 ± 0.2	2.4 ± 1.9***, ##
C3, mg/dL	136.0 ± 18.9	120.8 ± 32.1	102.5 ± 35.2***
C4, mg/dL	33.8 ± 8.6	22.5 ± 17.8**	16.5 ± 8.9***
ESR, mm/h	14.6 ± 4.5	25.8 ± 15.6	45.3 ± 25.3***, ##
eGFR, mL/min/1.73 m^2	88.6 ± 4.6	55.7 ± 7.3***	46.7 ± 5.4***, ###
Proteinuria, mg/24 h	< 150	180.9 ± 128.2	1025.2 ± 326.1***, ###

Values are expressed as mean ± SD and percentage.
**P < .01 & ***P < .001 represent significant differences with healthy controls.
##P < .01 & ###P < .001 represent significant differences between non-nephritis and nephritis groups.

Table 4. Biopsy Findings, Clinical Details, and Treatment Protocol of SLE Patients

Variables	SLE Patients (%) (n = 49)
Lupus Nephritis Classes	
Non Nephritis (No, %)	20 (41.0)
Nephritis	29 (59.0)
Activity Index (Mean ± SD)	6.9 ± 3.9
Chronicity Index (Mean ± SD)	1.9 ± 1.3
Serum Albumin Levels < 3.5 mg/dL	13 (26.5)
WBC Count / mm^3 < 4000	4 (8.2)
Platelets Count / mm^3 > 100000	27 (55.1)
Serum Creatinine > 2 mg/dL	32 (65.3)
Positive Anti-Nuclear Ab (ANA)	30 (61.2)
Positive Anti dsDNA	24 (49.0)
Low C3 Level	15 (30.6)
Low C4 Level	8 (16.3)
ESR > 30 mm/h	12 (24.5)
Active Protein Loss in Urine	31 (63.3)
Urine Protein Level	
0 to 500 mg/24h	33 (67.3)
500 to 1000 mg/24h	7 (14.3)
1000 mg/24h	9 (18.4)
Immunosuppressive Therapy	
Prednisolone	49 (100.0)
Cyclophosphamide	12 (24.5)
Cellcept	8 (16.3)
Azathioprine	11 (22.4)
Methotrexate	2 (4.1)
Cyclosporin	6 (12.2)
Tacrolimus	1 (2.0)
Hydroxychloroquine	35 (71.4)

laboratory findings and treatment protocols of SLE are also reported in table 4.

Expression of IL-17A Gene

Expression of *IL-17A* gene was dramatically increased in peripheral blood of SLE patients; as it showed 6 logs increases in these patients compared with controls ($P < .001$) (Figure 1A). Our results showed that different nephritis classes and SLE activity index did not play any role in *IL-17A* gene expression; only class V showed a non-significant reduction in *IL-17A* gene expression ($P > .05$).

Although *IL-17A* expression was lower in patients with ESR > 30, this difference was not statistically significant ($P = .058$). *IL-17A* gene expression was decreased in the patients with higher proteinuria rates; as patients with proteinuria between 0-500 mg/24 hours and 500-1000 mg/24 hours expressed 34-fold ($P < .001$), and 20-fold ($P = .049$), respectively higher *IL-17A* transcripts compared with patients with proteinuria > 1000 mg/24 hours (Figure 2).

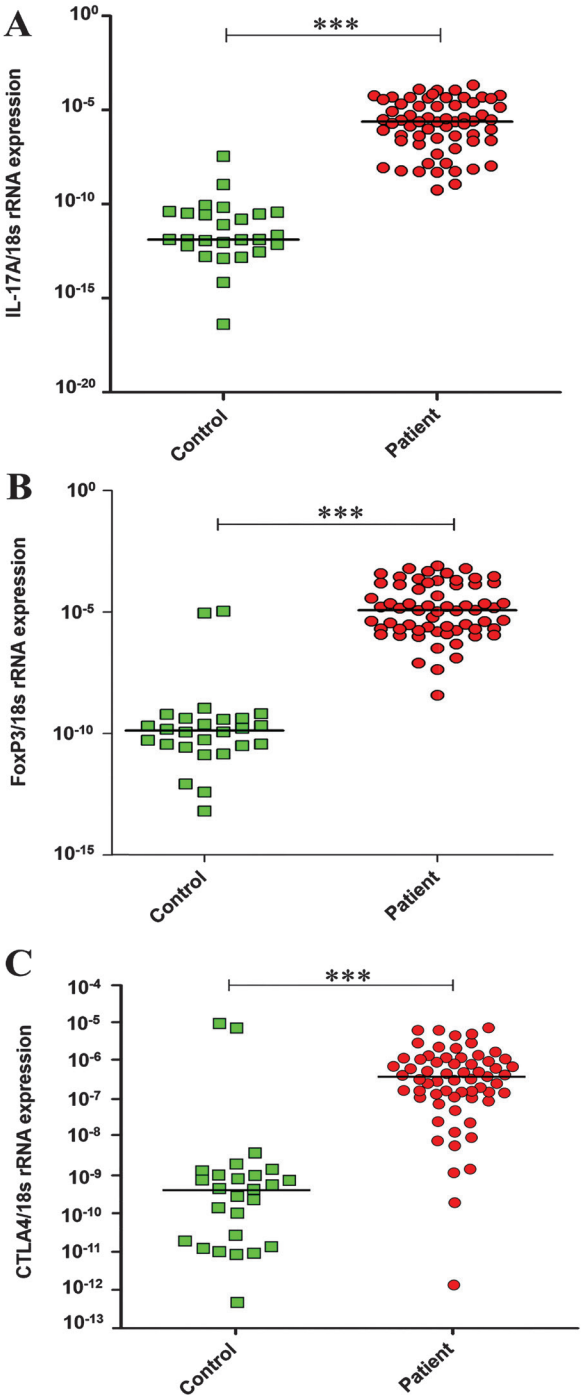

Figure 1. Expression of *IL-17A* (A), FOXP3 (B), and CTLA4 (C) genes increased in SLE patients compared to healthy controls ($P < .001$).

Expression of FOXP3 Gene

Our findings showed that *FOXP3* gene expression increased 5-fold in peripheral blood of SLE patients in comparison with the control group ($P < .001$)

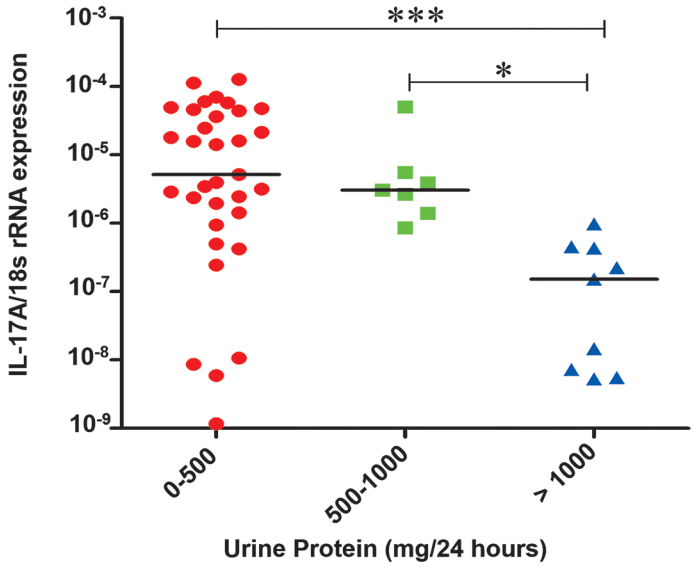

Figure 2. Expression of *IL-17A* gene decreased in SLE patients with more protein loss ($P < .001$).

(Figure 1 B), however, it was expressed similarly in patients with different classes of nephritis ($P > .05$). Patients with SLE disease activity indices 1 to 5 expressed a higher *FOXP3* gene than patients with indices 6 to 10 ($P = .014$) (Figure 3). *FOXP3* gene expression was decreased in peripheral blood of patients with serum albumin level < 3.5 mg/dL and it increased 9-fold in those with serum albumin > 3.5 mg/dL ($P = .014$) (Figure 4).

Expression of CTLA4 Gene

Expression of *CTLA4* gene increased significantly (about 1300-fold higher) in patients with SLE diseases compared with healthy controls ($P < .001$) (Figure 1C). *CTLA4* gene expression was not different between patients with different classes of nephritis and SLE activity index ($P > .05$).

Correlation Between Target Genes Expression

The expression of *FOXP3* and *CTLA* genes were significantly correlated ($P = .035$, R = 0.267, 95% CI: 0.012 to 0.48). In addition, there were positive correlation between *FOXP3* and *IL-17A* gene expression ($P < .0001$, R = 0.798, 95% CI: 0.681 to

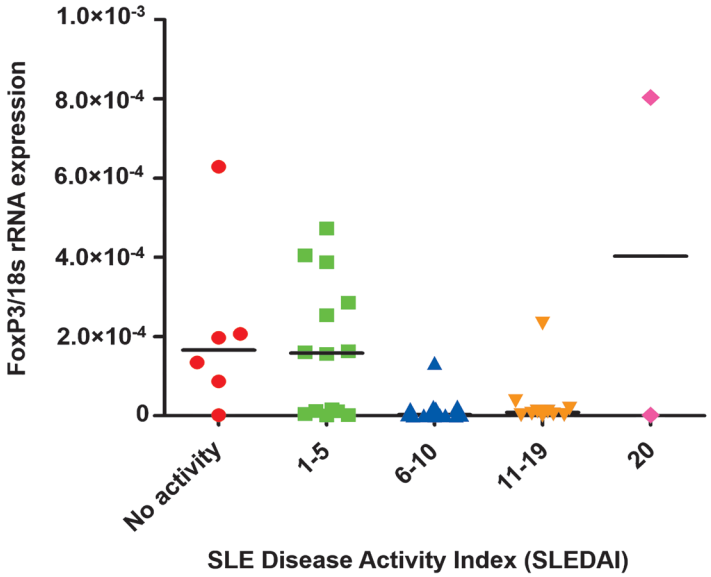

Figure 3. Expression of *FOXP3* gene decreased in SLE patients indices 6-10 compared to indices 1-5 ($P < .05$).

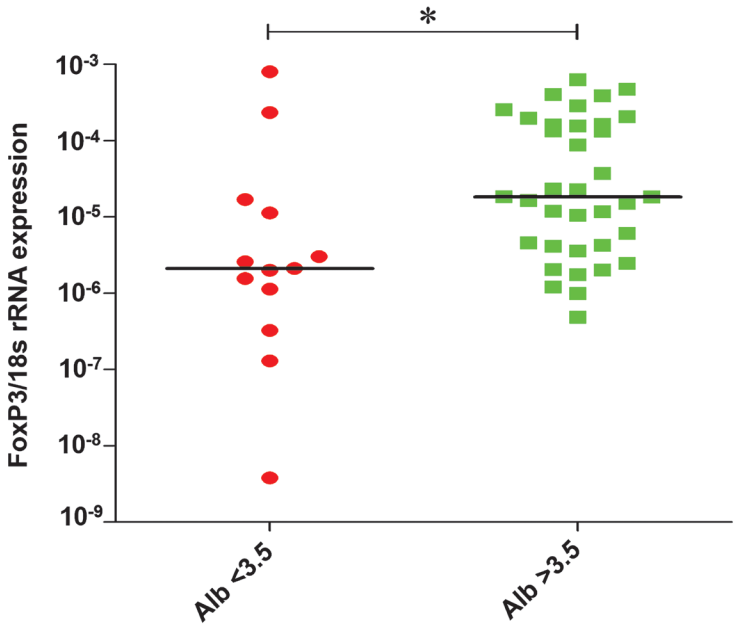

Figure 4. Expression of *FOXP3* gene decreased in SLE patients with lower serum albumin levels ($P < .05$).

0.875). However, *CTLA4* and *IL-17A* genes did not show any significant correlation ($P > .05$) (Figure 5).

DISCUSSION

In general, our findings show that the expression of *IL-17A*, *FOXP3*, and *CTLA4* genes in the peripheral blood cells of SLE patients are increased compared to healthy individuals, and this is somewhat independent of disease activity and sometimes even inversely correlated with it. These findings indicate the complexity of immune function in this disease, which requires more extensive and multifaceted studies.

The deposition of immune complexes in the kidney initiates inflammatory processes leading to LN; however, the pathogenesis of the LN is complex and relatively unknown. Many abnormalities on both effector and regulatory T cells have been described in SLE patients, such as dysregulated intracellular signal transduction, defective apoptosis, and imbalanced cytokine production, which influence B cell function and anomalous autoantibody production. [14-17] Whether effector or regulatory T cell have a specific role in pathogenesis of SLE and its complications, including nephropathy, is still unclear.

Based on previous research data, Th17 and IL-17 have an essential function in SLE promotion and are identified as much higher active state levels than inactive conditions.[18] We observed no correlation between IL-17A gene expression and disease activity irrespective of several reports, which have declared a linear association. One explanation for this finding is that IL-7 might associate with disease severity rather than disease activity.

Th17 cells can produce TNF, IL-2, and IFN-γ.[19] Based on this concept, elevated Th17, and IL-17 levels intensify their infiltration into target organs in patients with SLE.[20] Higher numbers of Th17 in patients with SLE leads to excessive secretion of pro-inflammatory cytokines and chemokines; so that patients with SLE will be continuously be in an inflammatory state, triggering more organ failures, with principally renal and hematological impairments.[21, 22]

However, our findings showed that IL-17A gene expression has an inverse correlation with ESR and proteinuria. Although high expression of this gene has been reported in SLE patients in the present study, it is possible that defects in IL-17A protein synthesis and related signaling pathways have affected gene expression. However, other studies have reported increased Th17 cell numbers and IL-17A levels in sera and renal biopsy of patients with LN.[23, 24]

In agreement with our results, another study showed that IL-17A levels were relatively constant over time and were irrelevant to SLE disease

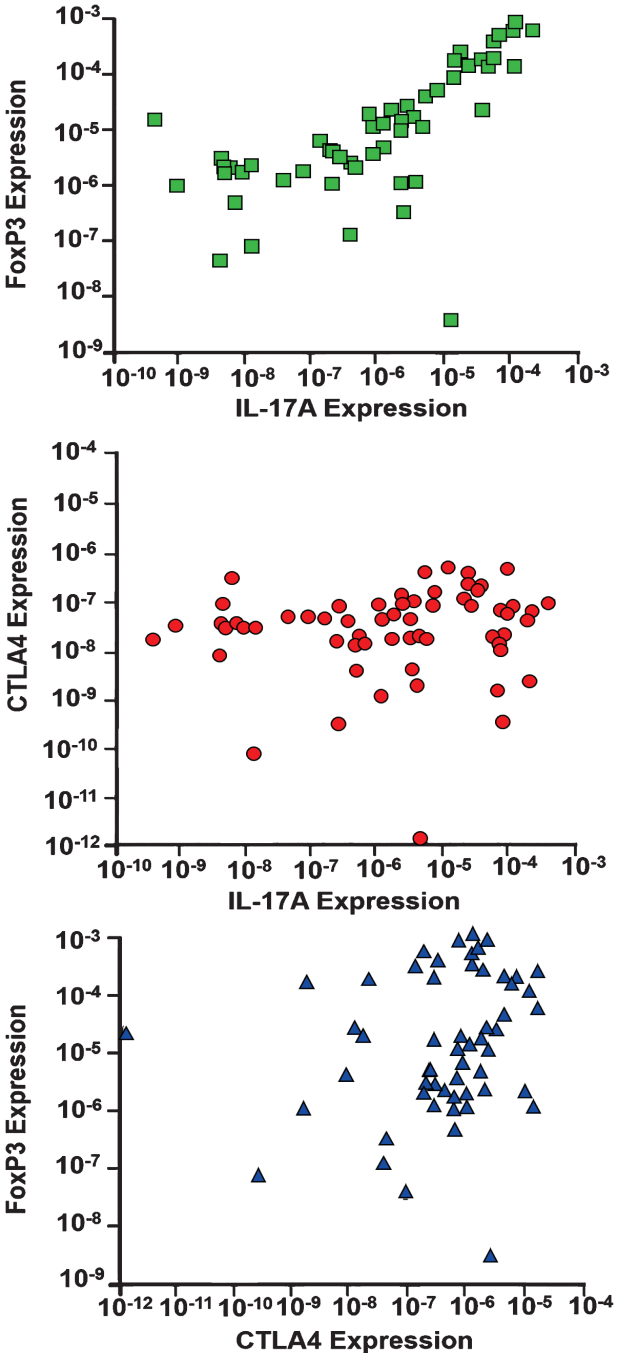

Figure 5. Correlation between *IL-17A*, *FOXP3*, and *CTLA4* genes expression in SLE patients.

activity (SLEDAI-2K) or cumulative damage (SLICC-DI). IL-17A levels are highly correlated with some serologic markers of inflammation.[25] Similar to our work, other studies indicate that IL-17A does not show different characteristics across disease activity and organ damage states.[26, 27] The interplay of cytokines and immune cells across stages of disease activity revealed that other than IL-17A, there are more specific biomarkers which can differentiate between patients with active and inactive disease.[25] Zickert, *et al.* showed that IL-17A levels were reduced seven months after cyclophosphamide-based induction treatment for LN.[24] In the present study including patients with immunosuppressive therapy, there were also changes in *IL-17A* gene expression over a series of developmental stages of the disease.

Regulatory T cells are responsible for the maintenance of tolerance and play a protective role in controlling the unwanted responses, and an imbalance between regulatory and effector T helper 17 cells are involved in the pathogenesis of LN.[15] Previous studies demonstrated no substantial decrease of Treg level in SLE patients.[21] Others provided inconsistent results; some revealed decreased Treg level, others declared its similarity with healthy patients or even elevated Treg level.[28,29] Our results might be explained by other studies that mentioned the decreasing function of Treg in SLE without reduced level in peripheral blood.[30] This conflict might be caused by different isolation methods and various characteristics of studied subjects, considering the lack of consensus for the Treg phenotyping method in humans.[31] This study showed an elevation of *FOXP3* gene expression compared to healthy subjects. The elevation could be caused by mixing activated T-cells during counting, where T-cells can be adaptively converted to Treg.[32] Another reasonable explanation is that Treg might attempt to control the intensified immune response during disease activity.[33]

On the other hand, *FOXP3* expression had an inverse correlation with disease activity in our study. The study by Shakweer *et al.* showed an elevated Foxp3+ expression in non-proliferative LN compared to the proliferative ones.[34] CD4+Foxp3+ T cells without CD25 expression are an important subset of Foxp3+ T cells that have been found regularly in SLE patients. CD4+ Foxp3+ T cells may be distinctive in SLE, but the origin and function of this subset of Treg is mainly unknown.[35]

Besides, IL17A-expressing CD4+ Foxp3+ T cells were also detected in renal tissue of active LN patients. This unique thymic Treg cell subset has two different aspects in SLE pathogenesis; immunosuppressive and pro-inflammatory, although whether this subset is harmful or protective remains unclear.[14]

Our results indicated that increased *FOXP3* expression in SLE patients was not associated with disease activity. It has been recently reported that Treg cells become unstable under specific inflammatory and pathologic conditions and adopt characteristics of effector CD4+ T cells.[36,37] In particular, Foxp3+ Treg cells can differentiate into IL-17-producing Th17-like cells upon receiving appropriate external stimulus.[14]

Recently the abnormal expression of the *CTLA4* gene in autoimmune diseases, including SLE, has shown that CTLA4 negatively regulates T-cell activation, thereby controlling the B cells responses and humoral immunity. However, the underlying mechanism is still not completely understood.[38,39] CTLA4 is an inhibitory cell-surface receptor with a fundamental role for immune regulation and is mainly expressed by Treg and activated CD4+ T-effector cells.[40] CTLA4-Ig is currently approved for treating rheumatoid arthritis, but in SLE, its benefits have been more challenging to establish.[41] A phase II clinical trial showed no overall benefit for this treatment, despite classical reports in favor of individual patients.[42]

Moreover, the gene region 2q33 has been identified as a susceptibility region for SLE in genome-wide scans and is of particular interest because it harbors the genes encoding the *CTLA4* and *CD28* cell surface receptors expressed by T cells.[43,44] Genetic studies linking *CTLA4* with SLE provide an additional rationale to investigate the role of CTLA4 in the pathogenesis of this disease. Indeed, an association between *CTLA4* polymorphisms and lupus has been found in numerous studies worldwide.[45,46] In addition, in agreement with our results, increased *CTLA4* expression has been shown in T cells from patients with SLE.[47]

Similar to our work, another study demonstrated that regardless of the dramatically increased *CTLA4* expression in T responder cells from patients with SLE compared with healthy controls, its ability to regulate lupus T-cell signaling and proliferation was impaired. This defect was associated with excluding CTLA4 from lipid microdomains, which could account for its loss of function in T cells from patients with SLE.[48] The data on dysfunctional CTLA4 in responder T cells indicate a disturbance in the immunoregulatory pathway of lupus patients and provide a potential target for future therapeutic strategy.

In another study similar to our work, increased expression of *CLTA-4* was irrespective of lupus disease activity, perhaps suggesting an intrinsic, possibly genetic defect in CLTA-4 regulation.[49] Our observation that expression of *CTLA4* was increased irrespective of disease activity would be consistent with a causal linkage rather than a disease consequence.

CTLA4 is an important marker of Treg, but the increase in *CTLA4* expression in lupus T cells was confined to the FOXP3 population. Although we found a significant rise in *FOXP3* gene expression in lupus patients in agreement with a recent reports, and there is also a positive correlation between Foxp3 and *CTLA4* gene expression, these could be a further indicator of irregular T-cell activation.[50] Defining the relationship between impaired CTLA4 function and the expanded CD4+Foxp3+ population in lupus patients could be vital in re-establishing tolerance in this disease.

The limitations of this study lie in the fact that our patients were all of the Persian descent and were mainly in a state of moderate disease activity, such that results cannot be extrapolated to cohorts with a different genetic or clinical manifestation. Our results are based on clinical and serological findings and, therefore, cannot confirm the cellular source or causation of effects by IL-17A, FoxP3, and CTLA4, for which further experimental studies will be needed. Lack of evaluation of the expression of these genes before and after receiving the treatment for active LN was another limitation of our investigation. Also, measuring these genes levels in the patients' sera and kidney tissue and investigating their relationships with other interleukins and different immune system pathways would add more value to this study.

CONCLUSION

Our study showd that the expression of three important immune regulatory genes, *IL-17A*, *FOXP3,* and *CTLA4 are* increased in nephritis and non- nephritis SLE patients, compare with controls. It seems that there is a reduced *IL-17A/FOXP3* expression in uncontrolled and more severe LN patients. In SLE patients with active nephritis, despite the increased expression of *FOXP3*, the expression rate of *IL-17* was also increased, which demonstrates the impaired suppressive function of regulatory T cells. Our findings revealed irregular

T-cell CTLA4 biology in lupus patients and provided new insight into CTLA4 function in a disease setting. Finding such variations in the immune system and regulatory pathways can provide targets for better manipulation of the immune system in disease states by immunotherapeutic approaches.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

All authors contributed to the design of the research. Zahiri L., Masjedi F., and Habibagahi M. contributed to the data collection, analysis, and interpretation, also drafted the manuscript. Habibagahi Z. and Malekmakan L. contributed to the draft and revised the manuscript, did the final approval, and accepted responsibility for the overall work.

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