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Inflammatory and T Helper 17/ Regulatory T Cells Related Cytokines Balance in Cutaneous Lupus Erythematosus (CLE)

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ABSTRACT

The cutaneous lupus erythematosus (CLE) is a common manifestation among systemic lupus erythematosus (SLE) patients. Malar rash and discoid lupus (DLE) are in the category of acute and chronic CLE, respectively.

The pathogenesis of CLE is multifactorial, and cytokine imbalances contribute to immune dysfunction and the induction of organ damage. Many aspects of cytokine dysregulation are still unclear in SLE and in particular CLE. Therefore, we concurrently measured the inflammatory [Tumor necrosis factor-alpha (TNF-α) and Interleukin (IL)-6)], T helper (Th)-17 (IL-17 and IL-23) and regulatory T cells [Transforming growth factor-beta (TGFβ) and IL-10)]-related cytokines in patients with CLE (patients with malar rash and/or DLE) and compared them with SLE patients and healthy individuals (n=25 in each group, a total of 75 patients). The serum levels of cytokines were assessed by Enzyme-Linked Immunosorbent Assay (ELISA) method.

IL-6 cytokine was significantly higher in SLE, DLE, and malar rash patients compared to those in healthy controls (p=0.025) and in patients with arthralgia (p=0.038), and gastrointestinal involvement (p=0.048). IL-17 was significantly higher in malar rash patients compared to normal individuals (p=0.023), SLE (p=0.008) and DLE patients (p=0.019) and in patients with oropharyngeal ulcer (p=0.05) but, IL-23 was significantly higher only in DLE patients than healthy controls (p=0.019).

In conclusion, inflammatory cytokines such as IL-6 involved in inflammation and differentiation of Th17 cells are probably responsible in part for Th17 activity in CLE. IL-17, IL-23, and IL-6/IL-6R (IL-6 receptor) inhibitors may be good treatments for CLE patients. So targeting these cytokines activity pathways can improve the CLE treatment strategy and may open a novel guideline for SLE and CLE treatment.

Keywords: Cutaneous lupus erythematosus; Inflammatory cytokines; Regulatory T cells; T helper 17

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INTRODUCTION

The systemic lupus erythematosus (SLE) is a group of autoimmune diseases that cause connective tissue

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and vascular inflammation. In lupus, all organs can be affected and the most affected organs are kidneys, joints, skin, heart, and nervous system. Skin involvement generally occurs in 70% of patients and is the second affected organ in this disease.[1-3] The cutaneous lupus erythematosus (CLE) is a common manifestation among SLE patients that may occur at any stage of the disease[2,4,5] and appears in three forms: acute CLE (ACLE), subacute CLE (SCLE), and chronic CLE (CCLE).[1] ACLE is often seen in patients with active systemic disease. Malar, or butterfly rash, is an acute manifestation of SLE and the most common form of ACLE. These rashes appear as flat red patches over both cheeks and nasal bridge that looks like a sunburn.[6] The most common form of CCLE is discoid lupus (DLE). DLE appears as coin-like lesions and it is classified by thickened and red scaly patches that often become visible on the cheeks, ears, and nose.[7] The pathogenesis of CLE is multifactorial. Genetic predisposition, environmental factors, and abnormalities in the innate and adaptive immune responses may be involved in disease induction and progression.[4]

Cytokine imbalances contribute to immune dysfunction and the induction of organ damage in autoimmune diseases.[8]Tumor necrosis factor-alpha (TNF-α) is amplified in the serum and skin of CLE patients and recruits inflammatory cells into the skin by enhancing the production of inflammatory cytokines, chemokines, and adhesion molecules and also triggers B cells to produce antibodies.[9,10] Interleukin (IL)-6 is a main pro-inflammatory cytokine, which is necessary for T helper (Th)-17-cell differentiation and prevention of transforming growth factor (TGF)-β-induced regulatory T cell (Treg)-development.[11] IL-6 is probably responsible in part for Th17 activity in CLE.[12] In previous studies, it has been shown that IL-6 production is higher in patients with active SLE and elevated cytokine level measured in skin lesions, correlates with disease activity.[13]

IL-17 can stimulate T cells and raises production of the inflammatory cytokines, chemokines and autoantibodies.[14] Increase in serum level of IL-17 and in the number of Th17 cells was related to a higher Disease Activity Index (SLEDAI) in SLE patients.[15] Few previous studies showed that Th17 had a central role in the pathogenesis of SCLE and the use of ustekinumab as a therapy for SCLE can successfully treat SCLE patients. Ustekinumab inhibits the contact of both IL-12 and IL-23 with their receptors, therefore hindering both Th1 and Th17 differentiation.[16-18]

Tregs inhibit inflammation and suppress auto-reactive T cells by secreting inhibitory cytokines such as TGF-β and IL-10 or through direct contact. Hence, a reduction in the number or function of Tregs has been described in CLE lesions.[19] Increasing the ratio of proinflammatory Th17 cells to Treg cells plays an important role in the pathogenesis of SLE and correlates with disease activity and specific organ involvement.[20] However, many aspects of cytokine dysregulation are still unclear in SLE[21] and in particular CLE. There are no Food and Drug Administration (FDA) approved therapies for CLE, and understanding the precise mechanisms of CLE lesions will give novel methods for the prevention and treatment of cutaneous lesions.[4]

In this study, we concurrently measured the inflammatory (TNF-α and IL-6), Th-17 (IL-17 and IL-23) and regulatory T cells (TGF-β and IL-10) -related cytokines in the serum of the patients with CLE and compared them with SLE patients and healthy individuals. Promising results relating to therapeutic targeting Th17/Treg cells imbalance may open a novel guideline for SLE and CLE treatment.

PATIENTS AND METHODS

Study Subjects

This case-control study was conducted at the Hafez Hospital (Shiraz, Iran) between September 2017 and October 2018. We included 25 SLE patients without skin involvement (mean age ±SD 39.7±9.9, M/F: 3/22) and 50 patients with cutaneous lupus: 25 with DLE (mean age±SD 33.72±9.88, M/F: 3/22) and 25 with a malar rash (mean age±SD 36.32±10.3, M/F: 4/21). To be qualified, patients had to meet classification criteria for systemic lupus erythematosus according to the ACR criteria[22] and to have an active lupus particular lesion well-matched with DLE. The diagnosis of cutaneous lupus was proved by a rheumatologist and biopsy exam. Among patients, 16(64%) of SLE, 18(72%)of DLE and 19(76%) of patients with a malar rash had arthralgia. Also, one (4%), 2(8%) and 4 (16%) of these patients had GI involvement and 7(28%), 8(32%) and 15(60%) of them had oropharyngeal ulcers respectively. Patients were permitted to use oral steroids and immune suppressors but they should not use topical steroids therapy in the last 4 weeks. Patients were excluded if they had any concomitant cutaneous lesion not credited to lupus or an overlap autoimmune situation. In addition, 25 sera from sex-and age-matched (mean age±SD: 36.5±10.0, M/F: 4/21)

university staffs stored in the Autoimmune Diseases Research Center, Shiraz, Iran were used as normal control samples. Normal control individuals had no infections and inflammatory or autoimmune diseases. The Ethical Committee at the Shiraz University of Medical Sciences (IR.SUMS.REC.1396.S1025) approved this study. Accordingly, the patients were informed and signed the consent form prior to participating in this study. After obtaining informed consent, clinical and laboratory, information of the patients was recorded. Then 5 mL of blood was collected and sera were separated and maintained at -70° C for further use.

Cytokine Assay

The levels of IL-6, IL-10, IL-17, IL-23, TNF-α and TGF-β cytokines in sera were measured by ELISA kits (eBioscience; San Diego, CA) with the sensitivity of 1, 0.5, 4, 15, 4 and 4 pg/mL, respectively. ELISA assay was performed as described by the manufacturer. Briefly, 96-well microplates were coated with 100 µL capture antibody for an overnight at 4°C. The plates were blocked and then 100 µL/well of samples and standards were added for 2 h at room temperature (RT). 100µl of the detection antibody (kit-provided) was added to each well and incubated at RT for 1 h. After washing, avidin-horseradish peroxidase (HRP) and then tetramethylbenzidine (TMB) substrate and stop solution were added, respectively. The absorbance of the reaction was measured at 450 nm with background subtraction at 570 nm using a microplate reader (Biotek, Carson City, NV).

Statistical Analysis

AlldatawereanalyzedbySPSSversion23forWindows (SPSS Inc., Chicago, IL) and according to the normality test (Kolmogorov-Smirnov test), the appropriate parametric (t-test and one-way ANOVA with Tukey post hoc test) or non-parametric (Mann-Whitney U) tests were used. Pearson correlation test was used to assess the correlation between the number of serum cytokines and quantitative parameters. *p*-values<0.05 were considered as significant level. Graphs were drawn by Graph Pad Prism (version 6, La Jolla, CA). All data presented as mean±standard error of the mean (SEM) unless otherwise specified.

RESULTS

Characteristics of the Patients

Demographic and clinical lab data of the patients including levels of white blood cells (WBC), platelets (PLT), erythrocyte sedimentation rate (ESR), and values of serum Cytoplasmic antineutrophil cytoplasmic antibodies (C-ANCA), perinuclear anti-neutrophil cytoplasmic antibodies (P-ANCA), anticardiolipin antibody (ACLA), antinuclear antibody (ANA), C-Reactive Protein (CRP), complement 3(C3) and complement 4(C4) extracted from patients'medical records is shown in Table1.

Table 1. Demographic and clinical parameters of SLE patients

Variables	Total	SLE without skin involvement	SLE with malar rash	Discoid lupus (DLE)	*p*-value
Patients(n)	75	25	25	25	
Age (mean±SD) (years)	34.9±10.7	39.7±9.9	36.32±10.3	33.72±9.88	0.77
Sex:					
Male n (%)	10(13.3)	3(12)	4(16)	3(12)	0.6
Female n (%)	65(86.7)	22(88)	21(84)	22(88)	
SLEDAI (mean±SD)	8.44±7.08	6.45±6.1	10.12±7.6	9.38±8.7	0.21
c-ANCA (median) (IU/mL)	0.14	0.1	0.8	0.1	0.41
p-ANCA (median) (IU/mL)	0.1	0.1	0.17	0.1	0.63
WBC (mean±SD) $\times 10^9$/L	6.18±2.5	6.42±1.8	6.86±2.9	4.51±1.6	0.002
CRP^+ patients n (%)	11(14.6)	4(16)	4(16)	3(12)	0.83
ESR (mean±SD) (mm/hr.)	23.4±19.9	27±22.3	19.57±15.7	24.22±22.04	0.33
ANA (median) (IU/mL)	78.50	83	114	72	0.65
C3(mean±SD) (g/l)	1.15±0.44	1.22±0.4	1.15±0.45	1.08±0.5	0.8
C4(mean±SD) (g/l)	0.23±.12	0.25±0.11	0.23±0.11	0.22±0.14	0.9
ACLA (median) (IU/mL)	3.1	2.6	2.35	6.50	0.04

Data are presented as mean ±SD, median or number (%), SLEDAI: SLE Disease Activity Index, c-ANCAs: Cytoplasmic anti-neutrophil cytoplasmic antibodies, p-ANCA: Perinuclear anti-neutrophil cytoplasmic antibodies, WBC: White blood cells, CRP: C-reactive protein, ESR: Erythrocyte sedimentation rate, ANA: Antinuclear antibody, C3: Complement component 3, C4: Complement component 4, ACLA: Anticardiolipin antibody.

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Serum Levels of Inflammatory Cytokines in Patients and Healthy Individuals

As shown in Figure 1A, the levels of IL-6 were significantly higher in SLE (68.9±24 pg/mL), DLE (59.4±14.6 pg/mL) and malar rash (49.6±8.2 pg/mL) patients compared to those in healthy controls (30.2±3.2 pg/mL) (p=0.02). Additionally, the IL-6 level showed a positive correlation with P. ANCA titer in all patients (R=0.448; p=0.025) (Figure 1B) and it showed a positive correlation with ANA titer in SLE patients without skin involvement (R=0.786; p=0.021) (data not shown). Also, IL-6 level was significantly higher in patients with arthralgia (67±13.6 vs 36.1±5.4 pg/mL; p=0.038) and gastrointestinal (GI) involvement (116.9±82.7 vs 51.5±6.6 pg/mL; p=0.048) (Figure1C, D).

TNF-α, another inflammatory cytokine, did not show any significant difference between groups (Figure 2A). TNF-α showed positive correlation with IL-17 (R=0.58; p=0.0001) and IL-23 levels (R=0.428; p=0.001) (Figure 2B, C) and negative correlation with TGF-β levels (R=-0.439; p=0.044) in all patients (Figure 2D).

Serum Levels of Th-17-related Cytokines in Patients and Healthy Individuals

In the case of T helper 17 (Th17)-related cytokines, IL-17 was significantly higher in malar rash patients (80±12 pg/mL) compared to normal individuals (52±1.9 pg/mL) (p=0.023), SLE (49.2±4.8 pg/mL) (p=0.008) and DLE patients (44.7±1.8 pg/mL) (p=0.019) (Figure 3A). IL-23 was significantly higher only in DLE patients (498.6±86.7 pg/mL) than healthy controls (239.7 ±33.9 pg/mL) (p=0.019) (Figure 3B).

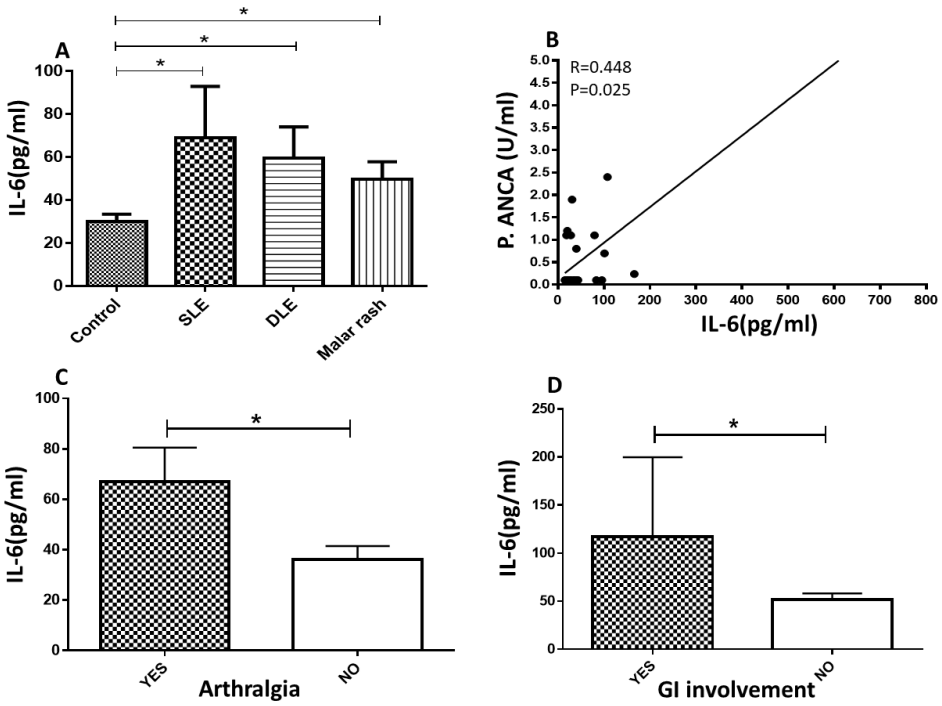

Figure 1. The levels of inflammatory cytokine, IL-6 (pg/mL) in the sera of healthy controls, and patients with SLE, DLE and malar rash (One- way ANOVA with Tukey post hoc test) (A). Correlation of serum IL6 levels with P. ANCA in all patients (Pearson test) (B). IL-6 levels in all patients(SLE, DLE, and malar rash) with and without arthralgia (C), and with and without GI involvement (D)(unpaired T-tests). Data are expressed as the mean ±SEM and *p<0.05 shows significant results. Systemic Lupus Erythematosus (SLE), Discoid lupus (DLE), IL-6: Interleukin-6, GI involvement: Gastro-intestinal involvement, P.ANCA: Perinuclear anti-neutrophil cytoplasmic antibodies.

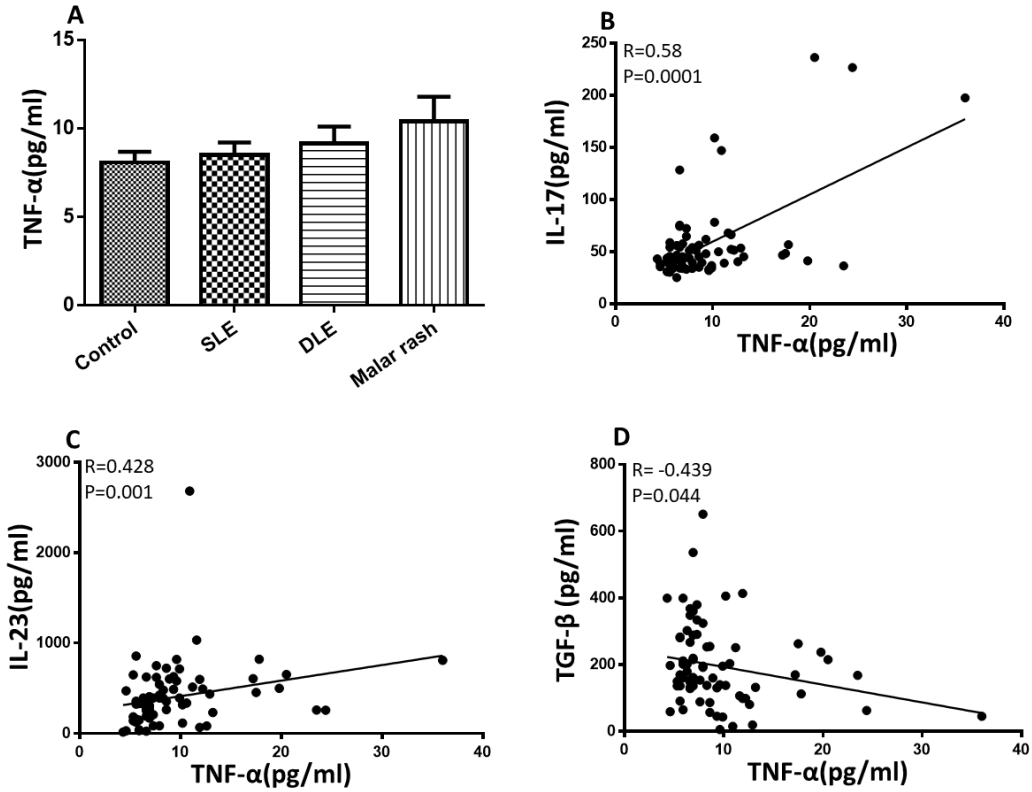

Figure 2. The levels of TNF-α(pg/mL) in the sera of healthy controls, and patients with SLE, DLE and malar rash (One- way ANOVA with Tukey post hoc test). Correlation of serum TNF-α levels with IL-17(B), IL-23 (C) and TGF-β (D) in all patients (Pearson test). Data are expressed as the mean ±SEM and *p<0.05 shows significant results. IL-17: Interleukin-17, TNF-α: Tumor necrosis factor-α, TGF-β: Tumor growth factor-β, IL-23: Interleukin-23.

In the case of IL-17, this cytokine levels had a positive correlation with IL-23(R=0.30; p=0.009) in all patients (Figure3C). IL-17 level were significantly higher in patients with oropharyngeal ulcer (68.1±10 vs 49.2±4 pg/mL; p=0.05) (Figure 3D) and positive anti-nuclear antibody (ANA) (57.7±5 vs 39.9±2.6 pg/mL; p=0.003) (data not shown).

Serum Levels of Regulatory Cytokines in Patients and Healthy Individuals

As shown in Figure 4A, the level of IL-10 was significantly lower in SLE patients (89.4±9.27 pg/mL) compared to healthy controls (131.4±17.4 pg/mL) (p=0.04). TGF-β did not show any significant difference between groups (Figure 4B). However, the levels of TGF-β were significantly lower in all patients (SLE, DLE, and malar rash) with GI involvement compared to patients without GI involvement (119.1±20 vs 203±15.2 pg/mL; p=0.005) (Figure 4C). In DLE patients, TGF-β level showed a positive correlation with IL-10 (R=0.424, p=0.035) (data not shown) and negative correlation with SLEDAI score (R=-0.55, p=0.037) (Figure 4D).

The Ratio of Th-17-related Cytokines to the Anti-inflammatory Cytokine, IL-10 in Patients and Healthy Individuals

As shown in Figure 5A, the ratio of IL-17/IL-10 was significantly higher in patients with malar rash (1±0.18; p=0.015) compared to SLE (0.6±0.06; p=0.04), DLE (0.45±0.04; p=0.003) and healthy individuals (0.55±0.06; p=0.015). There was no difference between IL-17/IL-10 ratio in normal controls and SLE and DLE patients. IL-23/IL-10 ratios were significantly higher in all three groups of patients compared to the normal controls (p=0.05) (Figure 5B).

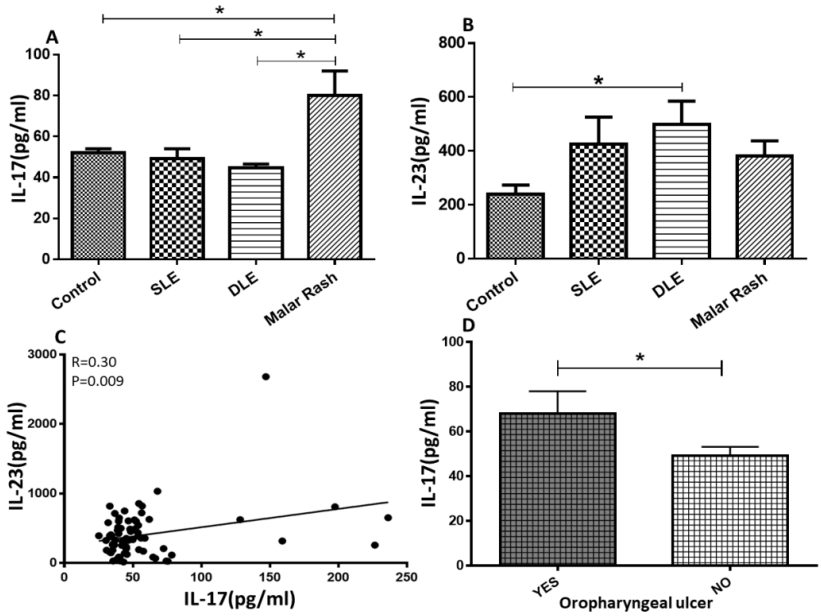

Figure 3. The levels of IL-17 (pg/mL)(A) and IL-23(pg/mL) (B) in the sera of healthy controls, and patients with SLE, DLE and malar rash (One- way ANOVA with Tukey post hoc test). Correlation of IL17 serum levels with IL-23 in all patients (Pearson test) (C), and IL-17 serum levels in patients with and without oropharyngeal ulcer (unpaired T-tests) (D). Data are expressed as the mean ±SEM and*p<0.05 shows significant results. Systemic Lupus Erythematosus (SLE), Discoid lupus (DLE), IL-17: Interleukin-17, IL-23: Interleukin-23.

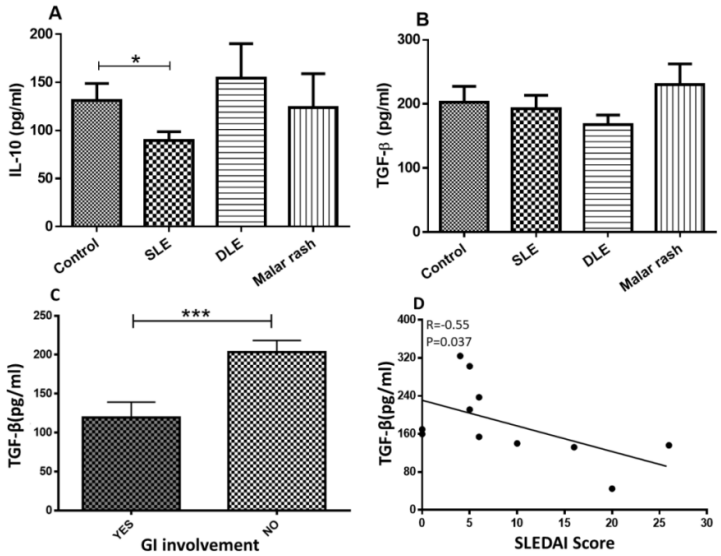

Figure 4. The levels of IL-10 (pg/mL) (A) and TGF-β(pg/mL) (B) in the sera of healthy controls and patients with SLE, DLE and malar rash (One- way ANOVA with Tukey post hoc test). TGF-β levels in patients with GI involvement (unpaired T-test) (C), and Correlation between TGF-β and SLEDAI in the serum of DLE patients (Pearson test)(D). Data are expressed as the mean±SEM and *p<0.05 and*p<0.001 show significant results. Systemic Lupus Erythematosus (SLE), Discoid lupus (DLE), IL-10: Interleukin-10, TGF-β: Tumor growth factor-β, GI: Gastro-intestinal. SLEDAI: SLE Disease Activity Index.**

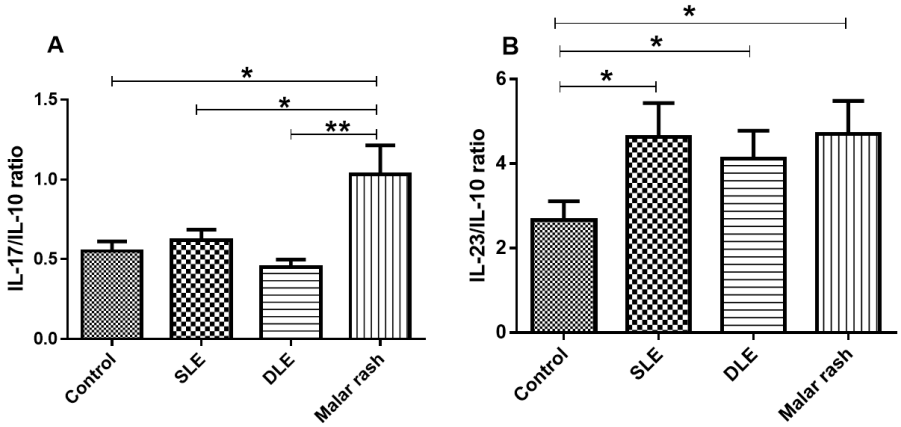

Figure 5.The ratio of Th-17-related cytokines to IL-10 in the sera of healthy controls and patients with SLE, DLE and malar rash. *p*-value was calculated by One- way ANOVA with Tukey post hoc test. Data are expressed as the mean±SEM and *$p<0.05$ shows significant results. Systemic Lupus Erythematosus (SLE), Discoid lupus (DLE), IL-17: Interleukin-17, IL-23: Interleukin-23, IL-10: Interleukin-10.

DISCUSSION

CLE is a common manifestation of SLE. In previous studies, it has been shown that IL-6 production is higher in patients with active SLE [13] and correlated with disease activity and anti-DNA levels[8] but the role for IL-6 in the pathogenesis of CLE, are still unclear.[23] Our results exhibited that the IL-6 level is higher in SLE with or without skin involvement. Therefore, IL-6may be involved in the pathogenesis of cutaneous lupus. We also displayed that inflammatory cytokines such as IL-6 are significantly higher in patients with arthralgia and GI involvement. IL- 6 can sensitize sensory neurons, trigger activation of pain,[24] and therefore induce joint pain. In addition, we revealed higher IL-6 and IL-17 levels in the patient with raised ANCA titer and positive ANA respectively. Previous studies revealed that IL-6 has an important role in ANCA-associated vasculitis[25] and it supports arthritis and joint deformation in SLE patients.[26] IL-6 is probably responsible in part for Th17 activity in CLE.[12] A significant role for IL-6 is the ability to alter the Th17/Treg ratio via Th17-cell differentiation, TGF-β-induced Treg-development inhibition [27], and B-cell hyperactivity and autoantibodies production.[28] Tanasescu et al. reported the elevated expression of IL-17 in the skin of patients with CLE.[29] In our study, we revealed a significant increase in IL-17 only in patients with malar rash. Because IL-17 plays an important role in the acute inflammation,[30] so it seems that IL-17 is responsible for the acute form of CLE. Our data showed that IL-17 levels were significantly higher in patients with oropharyngeal ulcers and ANA positive patients. Although Pisitkun et al. reported that the absence of IL-17 did not reduce ANA titer,[31] Miletic et al. confirmed higher IL-17 concentrations in ANA-positive in comparison to ANA-negative patients with primary Sjögren's syndrome.[32] We have now identified the positive correlation of IL-17 with TNF-α and IL-23 in both SLE and CLE patients, suggesting that these cytokines may function to stimulate the inflammatory process in CLE. We also showed that IL-23 is important in the chronic form of CLE (DLE). Previous studies showed that IL-23 plays a critical role in chronic inflammation and Th17 cell activation, and increased IL-23 production was associated with lupus nephritis, CLE and serositis.[33]

Reduction in the number or function of Tregs has been described in CLE lesions.[19] Our study showed a negative correlation between SLEDAI and TGF-β serum levels in DLE patients. This finding is in agreement with[34] a previous study; showing that the reduction of TGF-β in DLE resulted in defective immune suppression and caused tissue injury in lupus patients.[35] However, many aspects of cytokine dysregulation are still unclear in SLE and in particular CLE.[21] Although we revealed that the level of IL-10 decreased in SLE patients, this cytokine increased non-significantly in DLE. Despite IL-10 is a potent anti-inflammatory cytokine and modulates autoimmune

diseases, it can stimulate humoral immune responses and immunoglobulin production. Therefore, the high IL-10 level in SLE patients may be pathogenic.[36] In this study, IL-17/IL-10 ratio was greater in patients with malar rash compared to other patients and normal controls and IL-23/IL-10 ratios were higher in patients with SLE and CLE compared to normal individuals. Therefore, an increase in the ratio of pro-inflammatory Th17 cells-related cytokines to IL-10 can play an important role in the pathogenesis of SLE and CLE. There are no FDA approved therapies for CLE and treatment with TNF-α inhibitor drugs is accompanied by the appearance of Subacute Cutaneous Lupus Erythematosus (SCLE)-like lesions.[37] From the research that has been performed, it is possible to conclude that inhibitors of IL-6 or IL-6R can be beneficial in the treatment of SLE and DLE. The previous study, in RA disease, showed that monoclonal antibodies targeting the human IL-6R (Tocilizumab and sarilumab) could cause a significant reduction in Th17 and increase in Tregsnumber.[27] Therefore, promising results relating to therapeutic targeting Th17/Treg cells imbalance may open a novel guideline for SLE and CLE treatment. On the other hand, anti-IL-17 and anti-IL-23 treatments may be useful for the treatment of patients with malar rash and DLE respectively.A study showed that targeting IL-23 is more beneficial than IL-17 because anti–IL-23 can partially suppress IL-17A and reduce inflammation, but anti-IL-17A therapy may neutralize all protective roles of IL-17.[38] Ustekinumab is an approved human monoclonal antibody against p40 subunit of IL-23 that is used for the treatment of psoriatic arthritis and Crohn's disease.[38] Thus, it is suggested that the use of IL-23 and IL-6/IL-6R inhibitors may be a good treatment for CLE patients. The limitations of our study were moderate sample size and patients with incomplete clinical lab data.

Because the present study has shown that IL-6 and IL-23/IL-17 axis are involved in the development of cutaneous lupus erythematosus, so treatments that target the inflammatory cytokines and Th17/Treg cells perturbation can improve the CLE treatment strategy by targeting these cytokines activity pathways.

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