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Disturbance in Serum Levels of IL-17 and TGF-β1 and in Gene Expression of ROR-γt and FOX-P3 Is Associated with Pathogenicity of Systematic Lupus Erythematosus

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Abstract: To investigate the disturbance in serum levels of interleukin-17 (IL-17) and transforming growth factor-beta1 (TGF-β1) and gene expression of retinoic acid-related orphan receptor-gamma t (ROR-γt) and forkhead box-P3 (FOX-P3) in patients with systemic lupus erythematosus (SLE) and to study their association with disease pathogenicity and activity. Newly diagnosed active patients with SLE (n=88) and healthy volunteers (n=70) were included. Serum IL-17 and TGF-β1 were measured using enzyme-linked immunosorbent assay. Gene-expression profiles of ROR-γt and FOX-P3 were screened using real-time polymerase chain reaction. The IL-17/TGF-β1 and ROR-γt/FOX-P3 levels were also calculated. The mean age of the patients was 30.96±8.25 years; they were 82 women and 6 men. Of the patients, 11.4% manifested mild disease while 88.6% had severe disease. The serum level of TGF-β1 was significantly lower (70.2±34.9 vs. 200.23±124.77 pg/ml), while both IL-17 (614.7±317.5 vs. 279.76±110.65 pg/ml) and IL-17/TGF-β1 (18.5±30.1 vs. 1.66±0.9) levels were significantly higher, in patients than in controls ($p<0.0001$). The gene-expression level of FOX-P3 (0.6±0.8 vs. 13.68±39.35) was reported to be lower, while ROR-γt (3.9±3.5 vs. 1.99±2.09) and ROR-γt/FOX-P3 (18.6±21.1 vs. 7.63±17.19) levels were significantly higher, in patients than in controls ($p<0.0001$). Disturbance in serum levels of IL-17 and TGF-β1 in T helper-17 and T-regulatory

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cells proliferation was highlighted through an imbalance in the gene expression of FOX-P3 and ROR-γt, as both are signature genes for the two cell types, respectively. These findings underscore the critical role of IL-17 and TGF-β1 in SLE development, rendering them potential targets for developing novel immunotherapeutic strategies.

Introduction

Systemic lupus erythematosus (SLE) is a chronic, debilitating, systemic autoimmune disease. Excessive innate immune response contributes to SLE pathogenicity through tissue damage due to extensive production of proinflammatory cytokines, as well as by activation of autoreactive T- and B-cells, which in turn leads to autoantibody production, immune complex deposition, and organ failure (Schinocca et al., 2021). Several key factors are associated with SLE pathogenesis, including cytokine imbalance, oxidative stress, and apoptosis. Interleukin (IL-)17, IL-23, IL-21, and transforming growth factor beta 1 (TGF-β1) have only recently been distinguished for their pathogenic roles in SLE (Metawie et al., 2015; Schinocca et al., 2021).

Cytokines play a complex role in SLE pathogenesis. In patients with active SLE marked by extensive organ damage, IL-17 levels are high. The T helper (Th-)17 cell subset is the main producer of IL-17, wherein the retinoic acid-related orphan receptor gamma t (ROR-γt) is the signature transcriptional factor that primarily controls cell differentiation (Capone and Volpe, 2020). IL-17A and IL-17F both contribute to systemic inflammation associated with bone-cartilage destruction and generalized tissue damage, and the expanded population of Th-17 cells was defined in the peripheral blood of patients with SLE (Robert and Miossec, 2020).

The TGF-β1 isoform is the most potent immunosuppressant mediator that controls cell proliferation and fate via apoptosis. TGF-β1 is a substantial negative regulator of B-cell proliferation and differentiation, thereby dampening the production of immunoglobulins, with a distinctive role in T-cell proliferation, differentiation, and homeostasis (Oh and Li, 2013; Metawei et al., 2015). TGF-β1 production is reduced in patients with SLE, which could predispose them to autoreactive T-cell development and pathogenic autoantibody production (Metawei et al., 2015). A specific subset of TGF-β1-deficient mice showed early mortality, pointing to their crucial role in maintaining tolerance (Oh and Li, 2013). Both TGF-β and the transcription factor forkhead box-P3 (FOX-P3) act synergistically to promote T-reg cell differentiation. Conversely, the presence of TGF-β and IL-6 dampens T-reg cells and enhances Th-17 differentiation by inhibiting FOX-P3 and promoting RORc (Robert and Miossec, 2020).

T-reg cells produce IL-10, IL-35, and TGF-β; additionally, they stimulate IL-2 and TGF-β, which play a protective role in autoimmune diseases by inhibiting effector T-cell activity. However, in SLE, when IL-6 levels increase, T-reg cells are converted into Th-17 cells (Yuliasih et al., 2019). Immunotherapy agents have recorded more successful outcomes in inflammatory joint disorders than in SLE. This may be due to the complexity of the immunological profile of SLE (Becker-Merok et al., 2010).

To understand the abnormalities that lead to SLE development at the cytokine and cellular levels pertaining to the IL-17 and TGF-β1 axes, the aim of the present study was to reveal the immune balance between IL-17 and TGF-β1 and the gene-expression balance between ROR-γt and FOX-P3 in patients with SLE, and their relationship with disease pathogenicity and activity.

Material and Methods

This case-control study was conducted between December 2020 and March 2021. Eighty-eight patients visiting the Rheumatology Unit, Baghdad Teaching Hospital, Iraq, fulfilling the European League Against Rheumatism/American College of Rheumatology (EULAR/ACR) classification criteria for SLE (Petri et al., 2012) and 70 healthy age- and sex-matched controls were included. Participants aged <18 years, those with cancer, and those with other autoimmune and/or infectious diseases were excluded.

Data were collected through direct interviews and blood samples were collected. The SLE Disease Activity Index (SLEDAI) (Bombardier et al., 1992) was also evaluated. Disease activity was scored as follows: ≤ 3, no flare; score 3–12 indicating moderate disease activity; and > 12, severe flare (Gladman et al., 2000).

The study protocol was approved by the institutional review board of the College of Medicine/Al-Nahrain University (Approval No. 202011115). All the participants provided written informed consent.

Quantification of cytokines

Absolute serum levels of IL-17A and TGF-β were determined using the sandwich enzyme-linked immunosorbent assay (ELISA) technique (Human IL-17A and TGF-β kits, Abnova, Korea), according to the manufacturer's instructions. The results were expressed as optical density (OD), read at 450 nm, and calculated according to the OD of standards. As TGF-β is mostly present in an inactive form, activating reagents were used. The minimally detectable rate was 16.5 pg/ml for both IL-17A and TGF-β.

Quantitative mRNA expression

Quantitative mRNA expression of ROR-γt and FOX-P3 in peripheral mononuclear cells (PMNCs) was analysed using reverse-transcription polymerase chain reaction (RT-PCR). Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) vacutainer tubes. Total RNA was extracted (Wizbio Solution, Korea) within 3 h of sample collection. The concentration and purity of the extracted RNA were further verified by measuring the absorption at 260 nm and 260/280 ratio, respectively (Nanodrop spectrophotometer/Thermo scientific). The eluted RNA was subjected to complementary DNA (cDNA) synthesis (Wizbio solutions/Korea) on the same day as the RNA extraction. Cycling conditions were as follows: 25 °C for 10 min, 42 °C for 30 min, and 4 °C for hold. Synthesized cDNA samples were stored

at –20 °C until use. For RT-PCR, glyceraldehyde 3-phosphate dehydrogenase (GADPH) was used as a housekeeping gene. Primers (Applied Biological Materials/ Canada) were as follows:

FOX-P3: forward (TACAGCACGGTATGCAAGCC);
reverse (GCAACCGATCTAGCTCACAGAG).
ROR-γt: forward (AGATTACTACAACCGATCCACCT);
reverse (GGGGACAGAGTTCATGTGGTA).
GADPH: forward (CATGTTCGTCATGGGTGTGAACCA);
reverse (AGTGATGGCATGGACTGTGCTCAT).

Two microliters cDNA, 10 µl mastermix-SYBR Green (KAPA SYBR Fast qPCR [2X]/USA), 2 µl primers were added to the reaction tube and completed to 20 µl RNase free water in an automated thermocycler (Sacace Biotechnologies/Italy) programmed as follows: enzyme activation 95 °C/5 min, denaturation 95 °C/20 s, annealing 60 °C/20 s (40 cycles), and extension at 72 °C for 20 s.

Statistical analysis

It was conducted using the Statistical Package for the Social Sciences (SPSS) software, version 23. Data are presented as mean ± SD (standard deviation), median and interquartile range (IQR), or n (%). The Mann-Whitney test was applied to compare the two groups. The receiver operating characteristic (ROC) curve was used to identify sensitivity and specificity. The area under the curve (AUC) was measured and discrimination considered premium > 0.9, excellent 0.7–0.8, agreeable 0.6–0.7. Statistical significance was set at $p \leq 0.05$.

Results

Clinical and laboratory characteristics of the participants

The age of the patients and controls ranged from to 18 to 50 years. The mean age of the patients was 30.96 ± 8.25 years, F:M 13.6:1, and matched with the control parameters (age 29.97 ± 8.06 years and F:M 10.6:1; p=0.45 and p=0.77, respectively). The body mass index (BMI) was also comparable (26.8 ± 4.38 vs. 26.04 ± 4.01; p=0.26). The controls had normal values of haemoglobin (11.79 ± 1.12 g/dl), platelets (235.7 ± 49.54×10^3/mm^3), erythrocyte sedimentation rate (ESR) (13.62 ± 5.3 mm/h), C-reactive protein (CRP) (1.45 ± 1.03 mg/dl), urea (18.24 ± 5.64 mg/dl), creatinine (0.51 ± 0.19 mg/dl), complement C3: 125.81 ± 19.45 mg/dl, complement C4: 35.71 ± 8.05 mg/dl, negative antinuclear antibodies (ANA) (125.81 ± 19.45 IU/ml), and anti-double-stranded deoxyribonucleic acid (anti-dsDNA) (2.29 ± 2.31 IU/ml). None of the participants were smokers. Ten (11.4%) patients and 2 (2.9%) controls had a family history of SLE (p=0.07). Regarding the SLEDAI, 10/88 (11.4%) patients had mild disease, while 78/88 (88.6%) had severe disease. Patient characteristics are presented in Table 1. The laboratory results are presented in Table 2.

Table 1 – Characteristics of the patients with systemic lupus erythematosus (SLE) and healthy controls

Variable		Patients (n=88)	Control (n=70)	p-value
Age (year), mean ± SD		30.96 ± 8.25	29.97 ± 8.06	0.453
BMI (kg/m^2), mean ± SD		26.80 ± 4.38	26.04 ± 4.01	0.261
Sex, n (%)	female male	82 (93.2%) 6 (6.8%)	64 (91.4%) 6 (8.6%)	0.767
Smoking, n (%)	negative positive	88 (100%) 0 (0.0%)	70 (100%) 0 (0.0%)	1.000
Family history, n (%)	absent present	78 (88.6%) 10 (11.4%)	68 (97.1%) 2 (2.9%)	0.067
Disease activity score, n (%)	no flare mild severe	0 (0.0%) 10 (11.4%) 78 (88.6%)		
Organ involvement				
Mucocutaneous	negative positive	20 (22.7%) 68 (77.3%)		
Musculoskeletal	negative positive	18 (20.5%) 70 (79.5%)		
Renal	negative positive	88 (100%) 0 (0.0%)		
Cardiovascular	negative positive	82 (93.2%) 6 (6.8%)		
Serositis	negative positive	88 (100%) 0 (0.0%)		
Neuropsychiatric	negative positive	52 (59.1%) 36 (40.9%)		
Hematological	negative positive	0 (0.0%) 88 (100%)		
Fever	negative positive	16 (18.2%) 72 (81.8%)		
Hair loss	negative positive	0 (0.0%) 88 (100%)		

BMI – body mass index; SD – standard deviation

Comparison of IL-17 and TGF-β levels between patients with SLE and healthy controls
The medians of IL-17 and TGF-β levels in patients with SLE (n=88) were compared to those of controls (n=70) using the Mann-Whitney test. Sandwich ELISA showed a significantly elevated level of circulating IL-17 in patients with SLE than in healthy controls, with a median of 594.61 pg/ml vs. 319.83 pg/ml ($p<0.001$). TGF-β1 levels

Table 2 – Comparison of laboratory markers between patients with systemic lupus erythematosus (SLE) and healthy controls

Variable	Patients (n=88)			Control (n=70)			p-value
	median	mean ± SD	IQR	median	mean ± SD	IQR	
dsDNA (IU/ml)	80.07	80.36 ± 55.48	77.0	1.89	2.29 ± 2.31	3.0	<0.001
ANA (IU/ml)	19.85	29.93 ± 38.40	37.0	0.32	0.36 ± 0.25	0.0	<0.001
C3 (mg/dl)	36.40	45.01 ± 38.15	36.0	122.45	125.81 ± 19.45	28.0	<0.001
C4 (mg/dl)	11.20	11.52 ± 7.58	8.0	36.00	35.71 ± 8.05	14.0	<0.001
ESR (mm/h)	45.00	54.32 ± 33.10	40.3	12.50	13.62 ± 5.3	7.0	<0.001
CRP (mg/dl)	2.70	2.92 ± 2.47	2.0	1.17	1.45 ± 1.03	2.0	<0.001
Hb (g/dl)	11.15	10.99 ± 1.72	2.0	11.85	11.79 ± 1.12	1.0	0.008
Platelet ($\times 10^3/mm^3$)	144.00	160.56 ± 87.77	106.0	230.00	235.7 ± 49.54	76.0	<0.001
Urea (mg/dl)	27.00	28.12 ± 13.16	19.0	17.50	18.24 ± 5.64	7.0	<0.001
Creatinine (mg/dl)	0.62	0.62 ± 0.24	0.0	0.50	0.51 ± 0.19	0.0	0.005

Hb – hemoglobin; CRP – C-reactive protein; C3 – complement 3; C4 – complement 4; ANA – antinuclear antibody; dsDNA – double-stranded deoxyribonucleic acid; ESR – erythrocyte sedimentation rate; IQR – interquartile range; SD – standard deviation

Table 3 – Comparison between patients with systemic lupus erythematosus (SLE) and healthy controls regarding interleukin-17 (IL-17), transforming growth factor beta 1 (TGF-β1), forkhead box-P3 (FOX-P3), and retinoic acid-related orphan receptor gamma t (ROR-γt)

Variable	Patients (n=88)		Control (n=70)		p-value
	median (IQR)	mean ± SD	median (IQR)	mean ± SD	
TGF-β1 (pg/ml)	76.4 (59.7)	70.2 ± 34.9	168.1 (69.6)	200.23 ± 124.77	<0.001
IL-17A (pg/ml)	594.6 (271.5)	614.7 ± 317.5	319.8 (191.9)	279.76 ± 110.65	<0.001
IL-17A/ TGF-β1	8.70 (14.5)	18.5 ± 30.1	1.60 (1.4)	1.66 ± 0.90	<0.001
FOX-P3*	0.28 (0.57)	0.6 ± 0.8	0.80 (5.1)	13.68 ± 39.35	0.001
ROR-γt*	3.01 (3.14)	3.9 ± 3.5	1.01 (2.8)	1.99 ± 2.09	<0.001
ROR/ FOX-P3	8.82 (18.2)	18.6 ± 21.1	0.90 (6.7)	7.63 ± 17.19	<0.001

IL-17 – interleukin-17; TGF-β1 – transforming growth factor beta 1; FOX-P3 – forkhead box-P3; ROR-γt – retinoic acid-related orphan receptor gamma t; IQR – interquartile range; SD – standard deviation; *the marker was determined by relative gene expression

were lower in patients with SLE than in controls (76.38 pg/ml vs. 168.13 pg/ml) (p<0.001). The IL-17/TGF-β ratio was significantly higher among patients with SLE (8.69), in comparison to that seen in controls (1.61%) (p<0.001). Comparisons of IL-17 and TGF-β levels and mRNA expression of FOX-P3 and ROR-γt between patients and controls are presented in Table 3.

Differential mRNA expression of FOX-P3 and ROR-γt in patients with SLE and healthy controls

The medians of FOX-P3 and ROR-γt of patients with SLE (n=88) were compared with those of controls (n=70) using the Mann-Whitney test. Quantitative measurement of mRNA expression of FOX-P3 using RT-PCR revealed that its relative gene expression in patients with SLE was 0.28 compared to 0.76 in healthy controls, which was significantly lower (p=0.001). However, the gene expression of ROR-γt in patients with SLE was 3.0, which was significantly higher than that in healthy controls (1.0) (p<0.001). Furthermore, the ROR-γt/FOX-P3 ratio was higher in patients with SLE (8.8) than in healthy controls (0.91) (p<0.001). Comparison of IL-17 and TGF-β levels and mRNA expression of FOX-P3 and ROR-γt in patients according to disease activity grade is shown in Table 4.

Table 4 – Comparison between patients with systemic lupus erythematosus (SLE) according to disease activity regarding interleukin-17 (IL-17), transforming growth factor beta 1 (TGF-β1), forkhead box-P3 (FOX-P3) and retinoic acid-related orphan receptor gamma t (ROR-γt)

Variable	Mild activity (n=10)		Severe activity (n=78)		p-value
	median (IQR)	mean ± SD	median (IQR)	mean ± SD	
TGF-β1 (pg/ml)	86.4 (56.3)	81.4 ± 28.8	72.8 (69)	68.8 ± 35.6	0.360
IL-17A (pg/ml)	555.7 (251.7)	582.6 ± 146.8	601.3 (256.7)	618.8 ± 333.5	0.730
IL-17A/ TGF-β1	8.90 (4.5)	7.9 ± 2.9	8.50 (15.4)	19.8 ± 31.7	0.490
FOX-P3*	0.41 (0.9)	0.7 ± 0.8	0.25 (0.5)	0.5 ± 0.8	0.510
ROR-γt*	6.23 (6.5)	7.4 ± 5.7	2.91 (2.6)	3.5 ± 2.9	0.003
ROR/ FOX-P3	17.60 (41.5)	29.7 ± 29.8	8.80 (17)	17.2 ± 19.5	0.150

IL-17 – interleukin-17; TGF-β1 – transforming growth factor beta 1; FOX-P3 – forkhead box-P3; ROR-γt – retinoic acid-related orphan receptor gamma t; IQR – interquartile range; SD – standard deviation; *the marker was determined by relative gene expression

The ROC analysis of IL-17, TGF-β1 and IL-17/TGF-β1 ratio

For differential diagnosis, ROC analysis showed that the AUC for TGF-β1 was 0.988, with 91.4% sensitivity and 97.9% specificity under a 116.7 cut-off value. For IL-17, the AUC was 0.918, with 81.8% sensitivity and 88.6% specificity under a 392.8 cut-off value. For IL-17/TGF-β, the AUC was 0.99, with 90.9% sensitivity and 97.1% specificity under a 3.38 cut-off value (Figure 1).

The ROC analysis of FOX-P3, ROR-γt, and ROR-γt/FOX-P3 ratio

For differential diagnosis, ROC analysis showed that FOX-P3 had an AUC of 0.661, with 54.3% sensitivity and 59.1% specificity, and 0.44 cut-off value. ROR-γt had an AUC of 0.710, with 68.2% sensitivity and 68.6% specificity, under a cut-off value of 2.23. Regarding ROR-γt/FOX-P3, it had an AUC of 0.797, with 72.7% sensitivity and 74.3% specificity, with 5.42 cut-off value (Figure 2).

Discussion

Systemic lupus erythematosus results from global self-tolerance collapse and is characterized by an aberrant innate immune response that contributes to tissue injury via the release of inflammatory mediators (Lee et al., 2016).

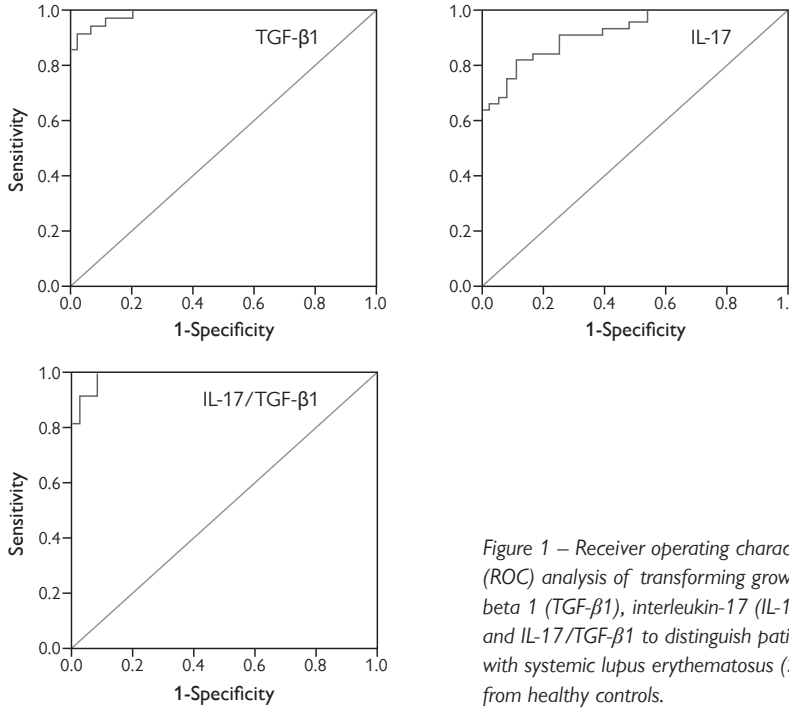

Figure 1 – Receiver operating characteristic (ROC) analysis of transforming growth factor beta 1 (TGF-β1), interleukin-17 (IL-17), and IL-17/TGF-β1 to distinguish patients with systemic lupus erythematosus (SLE) from healthy controls.

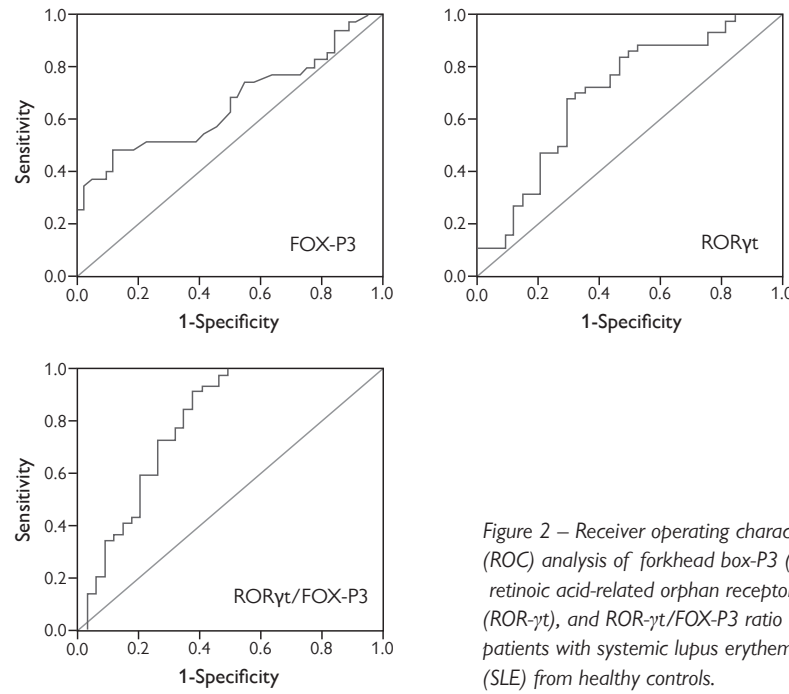

Figure 2 – Receiver operating characteristic (ROC) analysis of forkhead box-P3 (FOX-P3), retinoic acid-related orphan receptor gamma t (ROR-γt), and ROR-γt/FOX-P3 ratio to distinguish patients with systemic lupus erythematosus (SLE) from healthy controls.

The present study pointed to a significant female predominance (1:13.6 male:female ratio), which concurs with previous Iraqi and international studies (Al-Hattab and Al-Waiz, 2004). Notably, none of the patients were smokers, probably because most of them are women, and, by inference, non-smokers, due to pervasive cultural factors in Iraq. The most prevalent clinical manifestations were mucocutaneous (77.3%), musculoskeletal (79.5%), neuropsychiatric (40.9%), cardiovascular (6.8%), and fever (81.8%). None of the patients had renal or serositis involvement, which may be because the cases were newly diagnosed. It is likely that renal manifestations are observed on follow-up. Additionally, the cases were collected from a tertiary rheumatology center, and, possibly, cases with musculoskeletal manifestations were referred to, while those with renal manifestations were referred to the nephrology centers. Regarding the high percentage of neuropsychiatric manifestations, the occurrence of a particular neurological or psychiatric syndrome does not necessarily imply that SLE is the underlying cause, particularly with relatively common syndromes. Certain neurological symptoms may be coincidental, while others may arise from treatment complications or comorbidity. The present study recorded a disturbance in the serum levels of IL-17 and TGF-β1 in patients with SLE in comparison to control levels, and the IL-17/TGF-β ratio was significantly higher. IL-17, TGF-β, and their ratios were not significantly related to disease activity grade. In another study, IL-17 was found to be associated with disease activity (Chen et al., 2010). Concordantly, IL-17 family members are involved in several chronic inflammatory disorders, such as SLE, with an elevated serum concentration of IL-17 (Chen et al., 2010) and the number of Th-17 cells (Xing et al., 2012). Other studies have reported increased circulating levels of IL-17 in patients with SLE, with no association with disease activity (Zhao et al., 2010; Vincent et al., 2013). Conversely, comparable serum levels of IL-17 in patients with SLE and controls without a clear link to disease activity have been reported (Schinocca et al., 2021).

The pathogenic action of IL-17 is an essential attribute of acute inflammation; it is involved in neutrophil infiltration via a pathway distinct from that of IL-1 and TNF-α (Liu et al., 2016). Elevated circulating IL-17 levels have also been recorded in other autoimmune disorders, psoriasis, and ankylosing spondylitis, and its antagonists have been potentially successful (Schinocca et al., 2021). IL-17 is also capable of inducing inflammation through mediators, such as matrix metalloproteinases and nitric oxide, which is being implemented in patients with SLE, it is associated with disease activity and renal injury (Adamidis et al., 2019). The pathologic role of IL-17 in the development of rheumatic diseases such as SLE has led to the emergence of novel anti-IL-17 drugs, such as secukinumab (Rafael-Vidal et al., 2020).

TGF-β1 levels are significantly lower in patients with SLE than in healthy controls and are not associated with disease activity (El Menyawi et al., 2018). Another Egyptian study reported a significantly reduced level of TGF-β1 in patients with SLE in comparison to the control level; in contrast, they emphasized that low plasma

TGF-β1 levels are associated with high disease activity and severe renal damage (Metawie et al., 2015). Abnormal levels of TGF-β have been reported in patients with SLE, associated with both disease activity and renal damage (Becker-Merok et al., 2010). It is speculated that the reduction in serum TGF-β1 is associated with SLE through its role in abrogating T-reg cell function and differentiation (Joshi et al., 2014). Another proposed mechanism for TGF-β implementation in immunoregulation is through the induction of naive $CD4^+$ T cell differentiation into Th-3 cells, which are found to play a remarkable role in inducing peripheral tolerance through elevated TGF-β secretion, with low amounts of IL-10 and IL-4 (Carrier et al., 2007). Patients with SLE who reported reduced plasma TGF-β levels showed autoreactive T-cell activation and differentiation, in addition to pathogenic autoantibody production. Simultaneously, TGF-β1 upregulation may worsen outcomes. Although TGF-β plays a pivotal role in maintaining immune tolerance, direct signalling blockade may result in critical adverse effects, owing to its pleiotropic effect. This represents an obstacle in the development of direct TGF-β1 therapy or neutralizing antibodies (Komai et al., 2018).

Notably, disturbances in IL-17 and TGF-β levels may be driven by polymorphisms in the genes encoding these two cytokines or their receptors, which may culminate in the development of SLE (Hristova et al., 2021).

In a prior *in vivo* study, the IL-17/TGF-β1 ratio was increased in comparison to that seen in the control (Poeranto, 2019). A previous study conducted in India highlighted the association between cytokine imbalance and autoimmune diseases; they recorded an increase in INF-γ/IL-10 in patients with vitiligo in comparison to the ratio in healthy individuals (Ala et al., 2015).

In the present study, the gene-expression level of FOX-P3 was lower in patients than in controls, and the ROR-γt and ROR-γt/FOX-P3 levels were higher. However, ROR-γt levels were significantly higher in patients with mild activity than in those with severe activity. An increase in peripheral ROR-γt gene expression indicates an increase in activation and proliferation of Th-17 cells. As ROR-γt is the master regulator gene in Th-17 cells, it controls differentiation and induces IL-17A and IL-17F expression. Furthermore, naive $CD4^+$ helper cells require ROR-γt to respond to IL-6 and TGF-β, which are required for Th-17 differentiation (Lamb et al., 2021). Concordant with the findings of the current study, an increase in the number of Th-17 cells in patients with SLE in comparison to that seen in controls has been reported, which is significantly correlated with disease activity and renal injury (Narani, 2019).

Th-17 cells are implicated in the pathogenesis of autoimmune diseases not only for their critical role in producing IL-17; these cells also produce IL-21, which has been implicated in autoimmune diseases, including SLE, and its mRNA expression is associated with disease severity (Liu and King, 2013). Th-17 cells produce a wide range of inflammatory mediators, including IL-22, IL-26, IL6, TNF-α, GM-CSF, and chemokines (Schinocca et al., 2021). Th-17 cells profoundly express IL-23 receptors,

thereby conditioning them for IL-23 stimulation, considering that IL-23-induced Th-17 cells are more pathogenic compared with IL-12-induced Th-17 cells (Ouyang et al., 2008).

Reduced FOX-P3 gene expression in patients is also concordant with the results of other studies (Miyara et al., 2005; Álvarez-Rodríguez et al., 2019; Yuliasih et al., 2019). In contrast, other researchers have reported an association between FOX-P3 and disease manifestations (Miyara et al., 2005). FOX-P3 is a master controller of T-reg cell gene expression. The suppressive effect of T-reg cells is mediated by CTLA-4, which is a highly expressed molecule on the surface of T-regs. It is involved in the suppression of T-reg cells through two mechanisms: their interaction with CD80/86 co-stimulatory molecules on antigen-presenting cells (APCs) inhibits their expression. Additionally, CTLA-4 interaction with CD80/86 induces "indoleamine 2,3 dioxygenase", which controls the synthesis of pro-apoptotic metabolites, thereby inhibiting the activation of effector T-cells (Rowshanravan et al., 2018).

Both TGF-β and T-reg cells may be involved in autoimmunity via a B cell-dependent pathway. When autoantigens are expressed at low levels, they are presented by B cells, which produce autoantibodies in addition to various cytokines, primarily TGF-β, which modulates the conversion of $CD4^+$ T cells to T-reg (Rowshanravan et al., 2018). It has been found that IL-2 plays a crucial role in limiting $CD8^+$ T cell activation; however, simultaneously, it shows a potential role in improving T-reg suppression function through STAT5, highlighting the importance of IL-2 agonists as therapeutic agents for SLE, mainly because T-regs extensively express IL-2 receptors (Chinen et al., 2016).

The present study shows molecular evidence for a peripheral Th-17/T-reg (ROR-γt/FOX-P3) imbalance with no association with disease activity. The role of effector/regulatory cell imbalance in SLE pathogenicity was first recorded *in vivo* and further confirmed by others, from T-reg cells isolated from patients with active SLE (Lee et al., 2008). In agreement, it was reported that Th-17/T-reg was higher among patients with SLE with no link to disease activity (Kleczynska et al., 2011). Another study reported a significantly higher Th-17/T-reg ratio in SLE and in patients with primary antiphospholipid syndrome (Álvarez-Rodríguez et al., 2019). Other studies have reported an increase in Th-17/T-reg among patients with SLE; however, the number of Th-17 cells is still within the normal range, which indicates that the imbalance is the primary independent driving factor of SLE, and not Th-17 solely (Kleczynska et al., 2011). A similar study also reported an increase in Th-17/T-regs in patients with SLE and a significant association with disease activity (Yuliasih et al., 2019). It has been speculated that these two cell subsets consort and frustrate each other's activities to maintain immune homeostasis (Yang et al., 2008). The ROR-γt/FOX-P3 ratio could be applied as an indicator of the immune balance of Th-17/T-regs and is anticipated to be an excellent disease target in patients with SLE. In contrast, TGF-β promotes the generation of T-regs by activating FOX-P3

expression. An imbalance in these cytokines is reflected in the Th-17/T-regs (Mitra et al., 2015).

Circulating cytokines (IL-17, TGF-β1, and IL-17/TGF-β1) showed higher sensitivity and specificity than the peripheral molecular markers (ROR-γt, FOX-P3, and ROR-γt/FOX-P3), making them more suitable for SLE diagnosis and monitoring. Patients with SLE demonstrated disturbance in the Th-17 and T-reg axes compared with controls, with no significant contribution to disease activity. Circulatory cytokine levels are more valuable than the molecular signatures of immune cell subsets for SLE diagnosis.

A limitation of the present study is that a larger number of participants should be analysed, including those receiving various treatment regimens. In addition, more extensive cytokine, chemokine, and immune cell profiles, such as B-cell profiles, should be verified to fully recognize the immune mechanisms underlying disease pathogenesis. Assessment of the cytokine profile at the early onset stage and follow-up is warranted.

Conclusion

IL-17/Th-17 and TGF-β1/T-regs axes and their balance are skewed during SLE manifestation; therefore, immune disturbance is substantially implicated in SLE pathogenicity. Identifying the axes that are abnormal in each patient may pave the way for a precise therapeutic target, especially among those who do not respond to conventional therapy.

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