The Egyptian Rheumatologist xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

The Egyptian Rheumatologist

journal homepage: www.elsevier.com/locate/ejr

CD4$^+$Foxp3$^+$ T cells, interleukin-35 (IL-35) and IL-10 in systemic lupus erythematosus patients: Relation to disease activity

Maha Bassiouny [a], Ahmed Sonbol [a], Hend Eissa [b], Amira El-Shanawany [c,*], Alaa Labeeb [c]

[a] Clinical Pathology Department, Faculty of Medicine-Menoufia University, Egypt
[b] Clinical Pathology Department, Shebin El-Kom Teaching Hospital, Egypt
[c] Rheumatology, Physical Medicine and Rehabilitation Department, Faculty of Medicine, Menoufia University, Egypt

ARTICLE INFO

Article history:
Received 19 August 2018
Accepted 19 August 2018
Available online xxxx

Keywords:
SLE
CD4$^+$Foxp3$^+$ T cells
IL-35
IL-10
Disease activity

ABSTRACT

Aim of the work: To evaluate three subtypes of CD4$^+$Foxp3$^+$ T cells, interleukin-35 (IL-35) and IL-10 in systemic lupus eryhtematosus (SLE) patients and study their relation to disease activity.
Patients and methods: Fifty SLE patients were included and divided according to the SLE disease activity index (SLEDAI) into 2 equal groups with activity or in remission. Twenty healthy subjects were included as controls. All subjects underwent flow cytometric analysis of CD4, CD25, CXCR5 and Foxp3 expression on T cells. Serum IL-35 and IL-10 levels were measured by ELISA.
Results: Patients were 46 females and 4 males with a mean age of 38.0 ± 10.0 years, disease duration of 9.2 ± 6.0 years. The mean SLEDAI was 6.8 ± 3.7 in active ones. SLE patients especially those with activity had significantly reduced percents of CD4$^+$CD25$^+$Foxp3$^+$ and CD4$^+$CXCR5$^+$Foxp3$^+$ T cells, but increased percents of CD4$^+$CD25$^-$Foxp3$^+$ T cells. This was accompanied by significant higher levels of serum IL-35 and IL-10 ($p < 0.0001$). The SLEDAI in active patients significantly correlated with CD4$^+$CD25$^-$Foxp3$^+$ T cell percent, serum IL-35 and IL-10 levels ($p < 0.05$) and inversely with the CD4$^+$CD25$^+$Foxp3$^+$ and CD4$^+$CXCR5$^+$Foxp3$^+$ T cell percents ($p < 0.05$). At cut-off values of 3.29% for CD4$^+$CD25$^+$Foxp3$^+$ T cell, 7.62% for CD4$^+$CD25$^-$Foxp3$^+$ T cell, 1.77% for CD4$^+$CXCR5$^+$Foxp3$^+$ T cell, 22.04 pg/ml for IL-35 level and 30.51 pg/ml for serum IL-10 level were found to be highly sensitive and specific for detecting lupus activity.
Conclusion: CD4$^+$Foxp3$^+$ T cells, IL-35 and IL-10 showed high sensitivity and specificity for detecting SLE activity and may be considered as potentially promising therapeutic targets.

1. Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by activation of the innate immune system and activation of T and B lymphocytes, leading to production of autoantibodies, immune complex deposits and complement-mediated tissue damage [1]. Indeed, proinflammatory CD4$^+$ T cells, particularly follicular helper T (Tfh) cells, can help in producing autoantibodies by activated B cells, but other anti-inflammatory CD4$^+$ T cells, particularly regulatory T cells (Tregs) may inhibit the pathogenesis of SLE directly and indirectly [2].

High affinity antibody secreting plasma cells and long-lived memory B cells are generated in germinal centers (GCs). B cells undergo somatic hypermutation and isotype switching at these sites. Somatic hypermutation may result in the generation of autoantibodies and is under the stringent control of the Tfh cells. The latter cells are CD4$^+$CXCR5$^+$ as they express high levels of CXC Motif Chemokine Receptor-5 (CXCR5), which enables them to gain access into the follicles. The CD4$^+$CXCR5$^+$ cells are expanded in SLE patients and their numbers correlate with increased circulating GC B-cells [3].

The Tregs, a subset of CD4$^+$ T cells, are important regulators of immune tolerance and can suppress inflammatory T cell responses. Quantitative or qualitiative Treg defects are thought to contribute to SLE pathogenesis. They are CD4$^+$CD25$^+$Foxp3$^+$ characterized by high expression of CD25 (interleukin-2 receptor-α) and forkhead box P3 family transcription factor (Foxp3), a highly specific intracellular marker molecule, essential for their development and function [2]. Recently novel Treg subpopulations including CD4$^+$CD25$^-$Foxp3$^+$, CD4$^+$CD25^{low}Foxp3$^+$and CD4$^+$CXCR5$^+$Foxp3$^+$ were revealed [4]. CD4$^+$CD25$^-$Foxp3$^+$ do not express CD25 surface molecules yet share phenotypic characteristics with CD4$^+$CD25$^+$Foxp3$^+$ - and convey lower, but still considerable, suppression of T cell

Peer review under responsibility of Egyptian Society of Rheumatic Diseases.
* Corresponding author at: Faculty of Medicine, Menoufia University, Shiben El-Koom, Menoufia, Egypt.
E-mail address: amira.elshanawany@med.menofia.edu.eg (A. El-Shanawany).

https://doi.org/10.1016/j.ejr.2018.08.001
1110-1164/

Please cite this article in press as: Bassiouny M et al. CD4$^+$Foxp3$^+$ T cells, interleukin-35 (IL-35) and IL-10 in systemic lupus erythematosus patients: Relation to disease activity. The Egyptian Rheumatologist (2018), https://doi.org/10.1016/j.ejr.2018.08.001

proliferation [5]. The $CD4^+CXCR5^+Foxp3^+$ Tregs, similar to Tfh cells, express CXCR5 and localize to GCs. They have lately been identified as important regulators of the humoral immune response as they suppress B-cell antibody production and thereby limit the magnitude of the GC response. Whether $CXCR5^+$ Tregs are involved in defective GC reactions in SLE patients remains to be elucidated [3]. In Egyptian patients with rheumatoid arthritis (RA), expansion of certain $CD4^+$ T cell subsets has been implicated in the disease pathogenesis [6]. Moreover, although $CD4^+CD25^+Foxp3^+$ and $CD4^+CD25^+Foxp3^-$ were increased, their balance was more critical and related to disease activity reinforcing that Foxp3 expression might be an effective therapeutic target [7]. A disturbance in the cytokine milieu balance in favor of inflammation drew attention to the significance of the Treg/cytokine interplay aiming at restoring that balance during treatment [8].

The Foxp3 represents the most specific marker molecule for T cells with suppressive/regulatory capacity (Treg) and in an Egyptian study; its gene expression was found to be increased especially in active SLE patients and tended to correlate with the disease activity [9]. Many cytokines have been assessed in Egyptian SLE patients as promising biomarkers of the disease [10,11]. An imbalance of T-helper-cell subsets and Tregs cytokines including interleukin-10 (IL-10) is suggested to contribute to the pathogenesis of Egyptian SLE patients and was related to the disease activity [12].

Interleukin-35 (IL-35) has been identified as the newest member of the IL-12 family cytokines. It is a distinct cytokine with dimeric immunosuppressive/anti-inflammatory action. It is produced primarily by $CD4^+Foxp3^+$Tregs [13]. In contrast to the proinflammatory effect of other cytokines of the IL-12 family, IL-35 potently inhibits the effector $CD4^+$ T cells through the expansion of Tregs and IL-10 production [14]. IL-10 is produced, to a lesser extent, by $CD4^+Foxp3^+$ T cells. Its actions on immune cells include the inhibition of macrophage and T cell activation and of pro-inflammatory cytokine production [15]. However, in SLE, IL-10 increases B cell survival, proliferation, differentiation and antibody production, and decreases auto-reactive B-cell apoptosis by increasing Bcl-2 expression, resulting in increased autoantibody production [16]. In a previous Egyptian study, impaired production of Tregs in SLE patients was found to play a reciprocal role with certain cytokines including IL-10 and transforming growth factor-β1 and to affect the disease activity recommending that Tregs cells should be targeted to find potentially effective novel therapies [17].

This work aimed at evaluation of three types of $CD4^+Foxp3^+$ T cells, IL-35 and IL-10 in SLE patients and to study their relation to disease activity.

2. Patients and methods

This mono-center hospital-based cross-sectional study included 50 SLE patients fulfilling the Systemic Lupus International Collaborating Clinics (SLICC) classification criteria [18] and 20 age and gender matched healthy controls. They were selected from Menoufia University Hospitals, between May 2016 and November

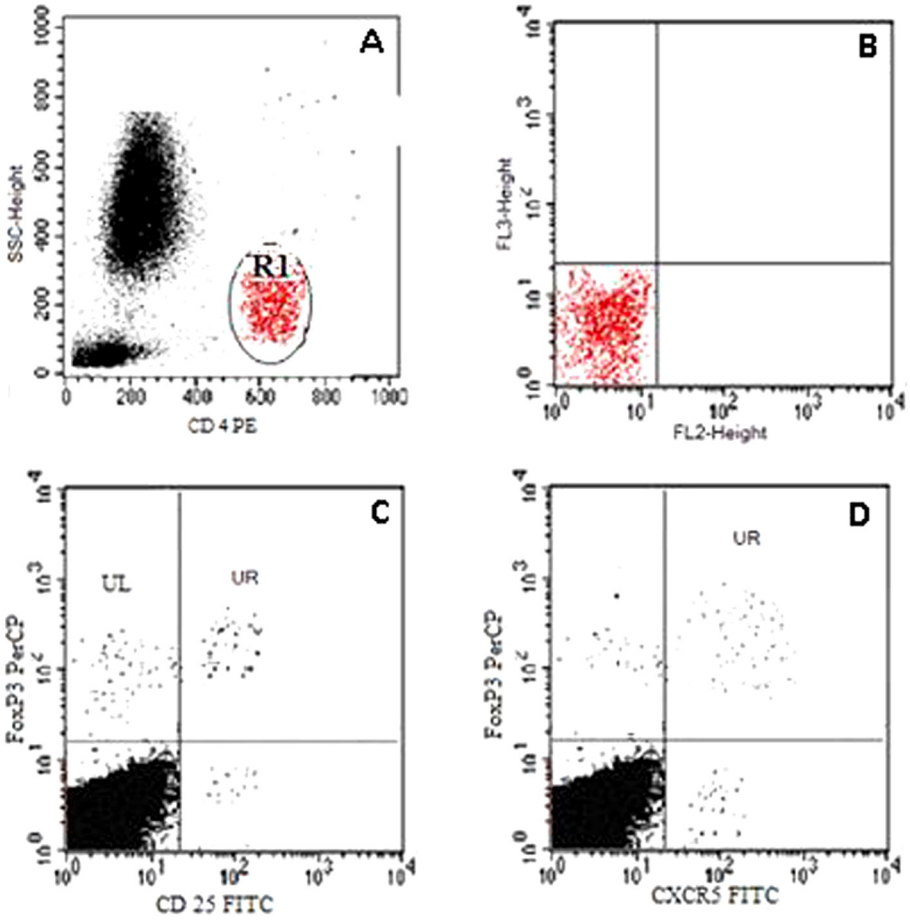

Fig. 1. Flowcytometric charts indicating gating on different $CD4^+Foxp3^+$ T cells in a patient with systemic lupus erythematosus. **A.** Gating on $CD4^+$ T cells (CD 4 against side scattering); **B.** Auto unstained control sample; **C.** Stained sample, the UR region represents $CD4^+CD25^+Foxp3^+$ T cells; **D.** Stained sample, the UR region represents $CD4^+CXCR5^+Foxp3^+$ T cells.

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2017. Patients were excluded if pregnant or had diabetes mellitus, thyroid disease, malignancy or another autoimmune disease. Clinical assessment was independent from the laboratory assessment. Disease activity was evaluated using the SLE disease activity index (SLEDAI) [19]. Patients were divided in to 2 equal groups of activity and remission. The study was approved by the Ethical Research Committee and Institutional Review Board of Faculty of Medicine, Menoufia University, Egypt (Approval number: 28/20160508). A written informed consent was obtained from each subject.

Ten ml Blood sample was collected from each subject; 4 ml were added to an EDTA-contained sterile tube for the determination of complete blood count (CBC) and cluster of differentiation (CD) markers, 2 ml was added to Westergren tube for assessment of the 1st hour erythrocyte sedimentation rate (ESR) and 4 ml were added to a sterile plain tube and left to clot at 37 °C and rapidly centrifuged at 4000 rpm for 10 min. Assessment of serum levels of C-reactive protein (CRP), complement components 3 and 4 (C3 and C4), creatinine, anti-nuclear anti-body (ANA) by indirect immunofluorescence assay, IL-35 and IL-10 by enzyme-linked immunosorbent assay (ELISA) was performed.

Flow cytometry analysis (FCAS): Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation using Ficoll-Paque Plus. PBMCs at 5×10^5/tube were stained in duplicate with PE-anti-CD4/FITC-anti-CXCR5 and PE-anti-CD4/FITC-anti-CD25 for 30 min, fixed and permeabilized using the permeabilization solution. This was followed by intracellular staining with PerCP-anti-Foxp3. After being washed with phosphate buffered saline, the numbers $CD4^+CD25^+Foxp3^+$, $CD4^+CD25^-Foxp3^+$ and $CD4^+CXCR5^+Foxp3^+$ T cells were determined by a flow cytometer (FACS Caliber, BD immune cytometry system, San Jose, CA, USA). A representative flow cytometry gating strategy for the identification of different Tregs is shown in Fig. 1.

Determination of serum IL-35 and IL-10 levels was performed using ELISA (human Elabscience Biotechnology Co., Ltd kit, USA) according to the manufacturer's instructions.

Statistical analysis: The Statistical Package for the Social Sciences (SPSS) software version 20.0 was used. For comparison between the three groups, ANOVA test, Kruskal Wallis test and chi-squared test were used. Bivariate correlations analyses between the studied parameters also were considered. Significance of the obtained results was judged at the 5% level. Receiver operating characteristic (ROC) curve was constructed at different cut-off values for percents of $CD4^+CD25^+Foxp3^+$, $CD4^+CD25^-Foxp3^+$ and $CD4^+CXCR5^+Foxp3^+$ T cells, serum IL-35 and IL-10 levels.

3. Results

The patients were 46 females and 4 males with an age range of 18–54 years (38.0 ± 10.0 years). The controls were matched for gender (19 females and 1 male) and age (19–51 years; 33.2 ± 9.7 years). The disease duration had a range of 1–24 years (9.2 ± 6.0 years). All patients were positive for ANA. Compared to control, patients in remission showed a significant increase in the ESR and CRP ($p < 0.001$). Active patients had significant hypocomplementemia, lymphopenia and elevated creatinine compared to those in remission ($p < 0.001$) (Fig. 2). The SLEDAI score of the active patients ranged 1–13 (6.8 ± 3.7).

Of note, the patients received oral prednisone and traditional synthetic disease modifying drugs (hydroxychloroquine, azathioprine, methotrexate and leflunomide). No biological drugs were given.

Patients in remission showed significant reduction in the frequency of $CD4^+CD25^+Foxp3^+$ T cells (95% CI; 3.8–5.9%) and $CD4^+CXCR5^+Foxp3^+$ T cells (95% CI; 2.1–3.7%) when compared to the

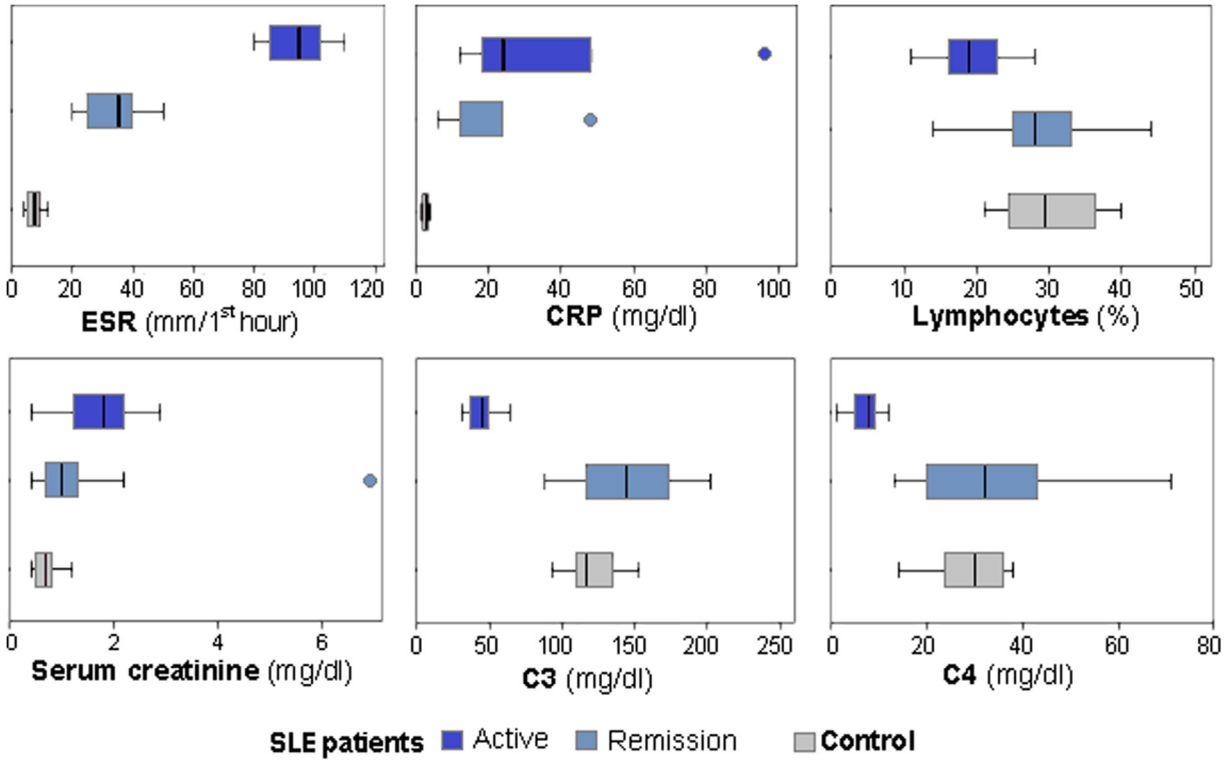

Fig. 2. Comparison of laboratory parameters (erythrocyte sedimentation rate 'ESR', C-reactive protein 'CRP', lymphocye percentage, serum creatinine and complements 'C3 and C4') in systemic lupus eryhtematosus patients (active and in remission) as well as in control.

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Table 1
Comparison of $CD4^+Foxp3^+$ T cells, serum interleukin-35 and IL-10 levels in systemic lupus erythematosus patients (active and in remission) and control.

Studied parameter mean ± SD (range)	SLE patients (n = 50)		Control (n = 20)	p
	Remission (n = 25)	Active (n = 25)		
$CD4^+CD25^+Foxp3^+$ (%)	4.9 ± 0.5 (4.1–6.0)	1.6 ± 0.5 (0.9–2.5)	7.5 ± 0.7 (6.5–8.7)	<0.0001
$CD4^+CD25^-Foxp3^+$ (%)	6.1 ± 0.7 (4.9–7.5)	9.4 ± 1.2 (7.8–11.5)	2.7 ± 0.6 (1.8–3.6)	<0.0001
$CD4^+CXCR5^+Foxp3^+$ (%)	2.9 ± 0.4 (2.2–3.5)	1.1 ± 0.2 (0.8–1.3)	4.95 ± 0.8 (3.9–6.1)	<0.0001
Interleukin-35 (pg/ml)	16.4 ± 5.5 (7.3–23.5)	32.5 ± 7.5 (20.2–44.7)	4.11 ± 2.15 (1.0–8.0)	<0.0001
Interleukin-10 (pg/ml)	22.9 ± 5.6 (13.8–32.8)	47.9 ± 12.0 (29.7–71.4)	6.2 ± 2.2 (2.6–10.3)	<0.0001

SLE: Systemic lupus erythematosus. Bold values are significant at $p < 0.05$.

Table 2
Correlation between systemic lupus erythematosus disease activity index (SLEDAI) and percents of $CD4^+Foxp3^+$ T cells, serum interleukin-35 and IL-10 levels in the active SLE patients.

Studied parameter	SLEDAI in SLE patients (n = 50)	
	r	(p)
$CD4^+CD25^+Foxp3^+$ (%)	−0.45	(0.005)
$CD4^+CD25^-Foxp3^+$ (%)	0.56	(0.004)
$CD4^+CXCR5^+Foxp3^+$ (%)	−0.52	(0.008)
Interleukin-35 (pg/ml)	0.45	(0.02)
Interleukin-10 (pg/ml)	0.51	(0.01)

SLE: Systemic lupus erythematosus, SLEDAI: SLE disease activity index. Bold values are significant at $p < 0.05$.

control ($p < 0.0001$). Lower cell frequencies of both were evident in the active patients (95% CI; 0.6–2.6%) and (95% CI; 0.7–1.4%) respectively ($p < 0.0001$). The remission group showed a significant increase in the frequency of $CD4^+CD25^-Foxp3^+$ T cells (95% CI; 4.7–7.6%) compared to the control ($p < 0.0001$) being higher in the active patients (95% CI; 7–1.2%) ($p < 0.0001$). There was significant increase of serum levels of IL-35 (95% CI; 5.5–27.4 pg/ml) and IL-10 (95% CI; 11.6–34.0 pg/ml) in the remission patients when compared to the control ($p < 0.0001$) and was even higher in the active patients (95% CI; 17.6–47.5 pg/ml) and (95% CI; 23.7–72.0 pg/ml) ($p < 0.0001$) respectively (Table 1).

Comparison of the T cell subtypes frequency and serum cytokines levels did not show any obvious gender differences in the lupus patients.

The SLEDAI in the active patients significantly correlated with the $CD4^+CD25^-Foxp3^+$ T cell percent, serum IL-35 and IL-10 levels ($p < 0.05$) and inversely with the $CD4^+CD25^+Foxp3^+$ and $CD4^+$-$CXCR5^+Foxp3^+$ T cell percents (Table 2).

ROC curve analysis using cut-off values of $CD4^+CD25^+Foxp3^+$ T cell percent (3.29%), $CD4^+CD25^-Foxp3^+$ T cell percent (7.62%), $CD4^+CXCR5^+Foxp3^+$ T cell percent (1.77%), serum IL-35 level (22.04 pg/ml) and serum IL-10 level (30.51 pg/ml) were found to be highly sensitive and specific for detection of lupus activity (Table 3).

4. Discussion

The immune cells of patients with SLE display many abnormalities, including; reduced T cell cytotoxicity, abnormal function and numbers of $CD4^+$T cells, abnormal activation of B cells, and alterations in cytokine biosynthesis [20]. The active SLE patients had significant lymphopenia, hypocomplementemia and increased serum creatinine level. All patients had a positive ANA. Lymphopenia was found to be associated with increased disease activity and organ damage indices [21]. The pathophysiological mechanisms are complex, involving lymphocytotoxic antibodies, excess of apoptosis, increased susceptibility of T cells to complement mediated cytolysis, as well as lymphopoiesis impairment and lymphocyte sequestration [22]. In addition, serum creatinine level, C3 and C4 levels and anti-dsDNA titers, along with creatinine clearance, urine protein level, and urine sediment are the traditional biomarkers used to follow the onset, course, and severity of lupus nephritis [23].

The present study revealed that the $CD4^+CD25^+Foxp3^+$ T cells were significantly reduced in SLE patients especially in those with disease activity; further verified by the inverse relation with the SLEDAI. This was in agreement with the findings in previous studies [2,24]. On the other hand, an unchanged number of circulating $CD4^+$Tregs expressing CD25 molecules and/or Foxp3 has been reported [25]. Furthermore, on the contrary, higher number of these cells in SLE patients has also been presented [26,27]. The global depletion of $CD4^+CD25^+Foxp3^+$ Tregs may be associated with their hypersensitivity to Fas-induced apoptosis. However, the mechanisms responsible for the exacerbated susceptibility to apoptosis of human lupus $CD4^+CD25^+Foxp3^+$ Tregs still need to be clarified [28].

The current work revealed that SLE patients in remission showed a significant increase in the numbers of $CD4^+CD25^-Foxp3^+$ T cells compared to the control and with a more increase in active patients and a significant relation to the SLEDAI. Increased proportions of $CD4^+CD25^-Foxp3^+$ Tregs are observed in particular in SLE patients, a finding that has widely been confirmed [29,30]. This disagrees with a single study that showed an inverse relation between $CD4^+CD25^-Foxp3^+$ T cells and clinical disease activity [25]. The increased frequency of $CD4^+CD25^-Foxp3^+$ Tregs in SLE

Table 3
Receiver operating characteristics (ROC) curve analysis for detecting disease activity of systemic lupus erythematosus patients.

Studied parameter	Disease activity in SLE patients (n = 50)						
	AUC	Cut-off	Sensitivity	Specificity	PPV	NPV	Accuracy
$CD4^+CD25^+Foxp3^+$ (%)	1.00	3.92	100%	100%	100%	100%	100%
$CD4^+CD25^-Foxp3^+$ (%)	1.00	7.62	100%	100%	100%	100%	100%
$CD4^+CXCR5^+Foxp3^+$ (%)	1.00	1.77	100%	100%	100%	100%	100%
Interleukin-35 (pg/ml)	0.97	22.04	92%	84%	85%	91%	88%
Interleukin-10 (pg/ml)	0.99	30.51	96%	92%	92%	96%	94%

SLE: Systemic lupus erythematosus, AUC: area under the curve, PPV: positive predictive value, NPV: negative predictive value.

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patients may compensate for the loss of $CD4^+CD25^+Foxp3^+$ Tregs in active SLE. However, this compensation may not be enough to regulate the autoimmune response, as SLE patients have altered relative ratios of $CD4^+CD25^+Foxp3^+$ Tregs and $CD25^{low}Foxp3^+$ T cells versus effector T cells [28].

In this study, the numbers of circulating $CD4^+CXCR5^+Foxp3^+$ T cells showed a significant reduction in the remission patients in comparison with control, with more decrease in active patients, also, a significant inverse relationship with the SLEDAI in active cases. This was previously reported by *Ma and colleagues* [2]. These cells are decreased in active and new onset SLE, seemingly allowing for the activation of B cells. The action of $CD4^+CXCR5^+Foxp3^+$-Tregs is to gain access into the GCs (through the CXCR5) and directly suppress the B cells that undergo hypermutation and isotype switch at those sites [4].

There was a significant increase of the serum levels of IL-35 in SLE patients being higher in active cases. The serum IL-35 levels were significantly associated with the SLEDAI. Similarly, IL-35 levels were reported to be significantly higher in active SLE patients [31,32]. On the contrary, a significant decrease of serum level of IL-35 in patients with active SLE has been reported [33]. The above results may imply the potential immunological role of anti-inflammatory cytokine IL-35 receptors forming a biochemical basis for the development of potential therapeutic target of IL-35 [32].

Serum IL-10 was significantly increased SLE patients and correlated with the disease activity index. This agrees with the results found in other studies [34,35] and disagrees with another [36]. In addition, no difference in serum IL-10 levels between active and inactive SLE patients has been reported [37]. On the other hand, in another study, serum IL-10 in SLE patients was significantly lower than that in healthy control [2]. The ability of IL-10 to enhance B-cell survival, proliferation, differentiation and antibody production, as well as, inhibiting auto-reactive B-cell apoptosis, may contribute to elevated anti-dsDNA titers in SLE patients [16].

The $CD4^+CD25^+Foxp3^+$, $CD4^+CD25^-Foxp3^+$ and $CD4^+CXCR5^+$-$Foxp3^+$ T cells were found to be highly sensitive and specific for detection of lupus activity. Keeping-up with the current results, a previous prospective study found that $CD4^+CD25^+Foxp3^+$ Tregs alterations reflected changes in SLEDAI with high sensitivity and concluded that these cells may be a reliable biomarker for the assessment of disease activity in SLE by longitudinal measurements [38]. A previous study on a smaller number of Egyptian SLE patients pointed to the possible relation of $CD4^+CD25^{high}Foxp3^+$ regulatory T cells in active SLE patients [17]. The value of Tregs as activity biomarkers has been demonstrated and may help in assessing disease status in controversial circumstances. Their potential to be used for therapeutic purposes, either by direct adoptive transfer or by approaches aiming to increase their numbers, is quite promising in the SLE [4].

Serum IL-35 and IL-10 levels were found to be highly sensitive and specific for detection of lupus activity. According to our knowledge, no previous studies have analyzed the sensitivity of serum IL-35 or IL-10 levels as potential biomarkers of lupus activity in Egyptian patients. Nevertheless, two longitudinal studies have suggested serum IL-10 level as potential biomarker of lupus activity based on its significant elevation in active SLE patients and on the strong correlation with disease activity as measured by SLEDAI [39,40].

Additional prospective studies with larger sample sizes will be necessary to confirm our findings. Moreover, demographic and clinical characteristics of the patients, grading of disease activity and effects of medications need to be considered in future research work.

In conclusion, $CD4^+Foxp3^+$ T cells along with IL-35 and IL-10 showed high sensitivity and specificity for detecting SLE activity. They could be used for monitoring disease activity of SLE patients and may be helpful for therapeutic purposes.

Conflicts of interests

None

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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