Systemic Lupus Erythmatosus is Associated with Disturbed Cytokine Milieu and Increased TNF-Related Apoptosis-Induced Ligand Levels

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Systemic lupus erythematosus (SLE) is a prototypic systemic autoimmune disease. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is cytotoxic to a wide variety of transformed cells, but not to most normal cells. This study measures serum levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6 and 10 and soluble TRAIL (sTRAIL) in patients with SLE and assesses their relation to severity of the disease. The study included 70 SLE patients and 20 healthy controls. Patients were diagnosed according to criteria proposed by the American Rheumatism Association for classification of SLE and disease activity was scored using the British Isles Lupus Assessment Group (BILAG-2004). All study participants were subjected to estimation of TNF-α, IL-6, IL-10 and sTRAIL using ELISA. Results revealed that mean disease duration was 6.5±1.5 years, mean BILAG score was 18.2±12.1, while 15 patients (21.4%) had quiescent disease. Blood levels of C3 and C4 and leucocytic count showed progressive decrease, while serum C-reactive protein and anti-double strand DNA antibodies levels showed marked increase with increased disease activity. Five patients (7.1%) were neutropenic. Serum levels of sTRAIL and IL-6 were significantly (*P*>0.05) higher in patients (1113.5±294 ng/ml and 60±21.5 ng/ml, respectively) than controls (354.7±47.2 ng/ml and 15.6±3.3 ng/ml, respectively) and in patients had active (1157±317 ng/ml and 64.3±20.7 ng/ml, respectively) versus patients had quiescent disease (965.4±115 ng/ml and 45.4±18 ng/ml, respectively). Serum levels of TNF-α were significantly (*P*>0.05) higher in patients (2.4±0.7 ng/ml) especially those with active (2.8±2 ng/ml) disease compared to controls (1.45±0.9 ng/ml). Patients with quiescent disease showed non-significantly higher TNF-α level (1.52±0.5 ng/ml) as compared to control, but significantly lower than patients with active disease. Serum levels of IL-10 were significantly lower in total patients (2.4±0.7 ng/ml) and patients with active disease (2.33±0.7) as compared to control (2.61±0.6 ng/ml) with a non-significantly (*P*>0.05) higher levels in patients with quiescent disease (2.61±0.6 ng/ml) than patients with active disease. Estimated serum sTRAIL, TNF-α and IL-6 levels showed positive significant correlation with calculated BILAG activity score, while estimated serum IL-10 levels showed negative significant correlation with activity score. In conclusion, SLE is associated with disturbed levels of serum cytokines and sTRAIL. These disturbances may underlie pathogenesis and/or activation of SLE as BILAG-2004 numeric scoring system significantly correlated with estimated levels of serum cytokines and sTRAIL.

Systemic lupus erythematosus (SLE) is a clinically heterogeneous, human systemic autoimmune disease (Li *et al.,* 2016) characterized by dysregulation of cellular immune responses, reactivity to various endogenous antigens (Banchereau *et al.,* 2016), autoantibody formation (Li *et al.,* 2016) and loss of tolerance to nucleic acids (Banchereau *et al.,* 2016). The precise pathogenic mechanisms for the development of SLE remain partly unexplained, but environmental initiating elements and genetic background play crucial roles in its pathogenesis (Sestak *et al.,* 2011).

Tumor necrosis factor-α (TNF-α) is the most widely studied pleiotropic cytokine of the TNF superfamily (Mukhopadhyay *et al.,* 2016). TNF-α is a key mediator of inflammation and plays a crucial role during the early phase of host's defense against infections (Khera *et al.,* 2010). TNF-α is implicated in the pathogenesis of many chronic inflammatory diseases (Valesini *et al.,* 2007). Moreover, persistent production of TNF-α occurs in many autoimmune

inflammatory diseases and this is associated with significant tissue damage (Khera *et al.,* 2010).

The proinflammatory cytokine interleukin-6 (IL-6) is a key player in innate immunity. It binds to membrane-bound IL-6 receptor or to soluble IL-6 receptor, which is present in the plasma (Axmann *et al.,* 2009). IL-6/sIL-6R complexes can bind to cells without membrane-bound IL-6R (Calabrese & Rose-John, 2014), thus IL-6 can act on many different cell types even if they do not express membrane-bound IL-6R, such as immune cells (Axmann *et al.,* 2009).

The B cells can negatively regulate immune responses by directly inhibiting CD4+ T cells and by inducing regulatory T cells through production of IL-10 (DiLillo *et al.,* 2010). IL-10 is a multifunctional cytokine secreted by a variety of cells. It inhibits activation of monocyte/macrophage system, inhibits synthesis of inflammatory cytokines and also promotes the proliferation and maturation of non-monocyte-dependent T cell, stimulating proliferation of antigen-specific B cell (Tian *et al.,* 2014). Abnormally increased IL-10 synthesis contribute to the spontaneous hyperactivity of the B cell compartment resulting in autoantibody production by committed plasma cells, circulating immune complexes formation, and eventually in tissue and organ damage (Peng *et al.,* 2013).

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily, which induces apoptosis through engagement of death receptors (Tabasi *et al.,* 2015). TRAIL is a transmembrane (type II) glycoprotein with its extracellular domain is homologous to that of other TNF superfamily members and shows a homotrimeric subunit structure. TRAIL exists physiologically in a biologically active soluble homotrimeric form (Pitti *et al.,* 1996).

TRAIL can interact with five receptors, but it triggers apoptosis through interaction with the death receptors TRAIL-R1 and -R2 that contain a cytoplasmic death domain and can induce apoptosis through the activation of caspases upon ligation with a TRAIL (Baetu & Hiscott, 2002). Binding of TRAIL to its receptors can also activate the transcription factor nuclear factor κB, which is known to control cell proliferation (Tan *et al.,* 1982).

The current prospective study aimed to evaluate serum levels of TNF-α, IL-6 and IL-10 and soluble TNF-related apoptosis-inducing ligand (sTRAIL) concentrations and their relation to disease severity in SLE patients.

Materials & Methods

The current study was conducted at Aljedaani group of hospitals under supervision of Ibn Sina National College (K.S.A.) from Jan 2015 till March 2016.The study protocol was approved by Local Ethical Committee. The study included 70 patients with SLE diagnosed according to the criteria proposed by the American Rheumatism Association for the classification of SLE. Patients underwent clinical and laboratory evaluation and disease activity was assessed using the British Isles Lupus Assessment Group (BILAG)-2004 index (Yee *et al*., 2007) this consisted of evaluation of eight systems (general, mucocutaneous, neuropsychiatric, musculoskeletal, cardiorespiratory, vasculitis, renal and haematology (Isenberg & Gordon, 2000). Each system is assigned a disease activity grade, ranging from A to E. Grade A represents very active disease requiring immunosuppressive drugs and/or prednisolone dose of >20mg daily (or equivalent). Grade B represents moderate disease activity requiring a lower dose of corticosteroids, anti-malarials or NSAIDs. Grade C indicates mild disease requiring symptomatic therapy, whereas Grade D implies no current disease activity but the system had previously been affected. Grade E indicates no current or previous disease activity (Yee *et al.,* 2008). To obtain a global score, BILAG component scores was assigned numerical values with A=12, B=5, C=1, D=0 and E=0 (Cresswell *et al.,* 2009, Yee *et al.,* 2010).

SLE patients underwent laboratory assessment of serum levels of C3, C4 and C-reactive protein (CRP) and leucocyte, neutrophil, monocyte and lymphocyte

counts. Patients whose absolute blood neutrophil counts were below 1.5 x 10^3/µl were diagnosed as having neutropenia (Palmblad *et al.,* 2001). Twenty age and sex-matched healthy volunteers and had no history of autoimmune disorders or other inflammatory diseases, without recent (3 month) acute or lasting chronic infection with viruses or bacteria were included in the study as control group.

Blood samples were collected from all study participants by sterile veinpuncture, allowed to clot naturally, the serum was separated, placed in pyrogen-free Eppendorf tubes and stored at -20°C until assayed for estimation of:

Serum IL-10 was measured with an ELISA kit from (Quantikine, USA). The lowest detectable value for IL-10 was 3 pg/ml; normal range from 3.9 pg/ml to 24 pg/ml. The sensitivity is <5 pg/ml; range: 12.5-400 pg/ml (Poll, 1996).

Serum IL-6 was measured with an ELISA kit from (Quantikine, USA). IL-6 values in fresh serum of healthy individuals are <20 pg/ml. Kit sensitivity at 0.7 pg/ml; assay range is 3.1-300 pg/ml; cross-reactivity: <0.5% cross-reactivity observed with available related molecules, <50% cross-species reactivity observed with species tested (Gaines-Das & Poole, 1993).

Serum TNF-α was measured with an ELISA kit from (RayBio®). TNF-α values in fresh serum samples of healthy individuals are <10 pg/ml. The sensitivity is down to <10 pg/ml; range: 25-800 pg/ml (De Kossodo *et al.,* 1995).

Serum sTRAIL was measured with an ELISA kit from Diaclone, Besançon, France. The minimum detectable level was 64 pg/ml; sensitivity is down to 7.87 pg/ml; range: 15.6-1,000 pg/ml. Cross-reactivity: <0.5% cross-reactivity was observed with available related molecules (Ashkenazi *et al.,* 1999).

Serum anti-dsDNA was measured with an ELISA kit from Alpha Diagnostics, USA. Anti-dsDNA values in healthy individuals are less than 25 IU/m**.** Sensitivity equals 0.42 IU/ml; range: 12.5-200 IU/ml (Hook *et al.,* 1979).

Statistical Analysis

Obtained data were presented as mean±SD, median values, numbers and ratios. Results were analyzed using One-way ANOVA with post-hoc Tukey HSD Test and Chi-square test (X^2 test). Possible relationships were investigated using Spearman linear regression. Statistical analysis was conducted using the SPSS (Version 15, 2006) for Windows statistical package. *P* value <0.05 was considered statistically significant.

Results

The study included 70 patients; 12 males and 58 females with mean age of 42.9±11.1; range: 23-64 years. Details of demographic data of studied patients and controls are shown in table 1.

Mean duration of disease was 6.5±1.5; range: 3-9 years with majority of patients (52.9%) had disease since 3-5 years. Mean BILAG score of studied patients was 18.2±12.1; 9-38. Fifteen patients (21.4%) had quiescent disease activity, while 55 patients had active disease with varied grades of activity. Details of disease-related data are shown in table 2.

Systematic activity evaluation showed that general, musculocutaneous and hematological activity manifestations are the most frequently detected. Details of activity grades and mean score for activity of each system are shown in table 3.

Table 1. Demographic data of SLE patients and controls.

Findings			Controls	Patients	*P* value
Age (years)	Strata	<30	4 (20%)	13 (18.6%)	NS
		30-39	7 (35%)	11 (15.7%)	
		40-49	7 (35%)	24 (34.3%)	
		50-59	2 (10%)	20 (28.5%)	
		>60	0	2 (2.9%)	
	Mean		38.4±7.6	42.9±11.1	NS
Gender	Males		4 (20%)	12 (17.1%)	NS
	Females		16 (80%)	58 (82.9%)	
Body weight (kg)			80.5±17.9	82.9±15.1	NS
Body height (cm)			169.8±2.6	169.9±3.6	NS
*Body mass index (kg/m^2)	Strata	<18.5	0	7 (10%)	NS
		18.5-24.99	9 (45%)	10 (14.3%)	
		25-29.99	1 (5%)	32 (45.7%)	
		30-34.99	8 (40%)	13 (18.6%)	
		≥35	2 (10%)	8 (11.4%)	
	Mean		27.9±6.2	28.7±5.2	NS

Data are presented as numbers and mean±SD; percentages are in parenthesis; NS: Non-significant difference
P >0.05 is not significant (NS).

Table 2. Disease related data of SLE patients.

Data			Findings
Duration of disease (years)	Strata	3-5	37 (52.9%)
		6-7	15 (21.4%)
		≥8	18 (25.7%)
	Mean		6.5±1.5
BILAG-2004 activity score	Strata	<10	19 (27.1%)
		10-19	25 (35.7)
		20-29	13 (18.6%)
		≥30	13 (18.6%)
	Mean		18.2±12.1
	Frequency of disease activity	Active	55 (77.6%)
		Quiescent	15 (21.4%)

Data are presented as numbers and mean±SD; percentages are in parenthesis.

Table 3. SLE patients' distribution according to Activity grade and clinical categories.

Activity grade System	Grade A	Grade B	Grade C	Grade D	Total score
General	9 (12.3%)	17 (24.3%)	27 (38.6%)	17 (24.3%)	3.1±3.9
Mucocutanous	8 (11.4%)	19 (27.1%)	25 (35.7%)	18 (25.7%)	3.2±3.8
Nervous system	4 (5.7%)	7 (10%)	3 (4.3%)	56 (80%)	1.2±3.1
Muscloskeletal	9 (12.9%)	11 (15.7%)	16 (22.9%)	34 (48.5%)	2.6±4
Cardio-respiratory	3 (4.3%)	10 (14.3%)	15 (21.4%)	42 (60%)	1.4±2.8
Vasculitis	5 (7.1%)	9 (12.9%)	13 (18.6%)	43 (61.4%)	1.7±3.3
Renal	6 (8.6%)	7 (10%)	16 (22.9%)	41 (58.5%)	1.8±3.5
Hematological	11 (15.7%)	14 (20%)	17 (24.3%)	18 (25.7%)	3.1±4.3

Data are presented as numbers; percentages are in parenthesis; Grade A: very active disease (score=12); Grade B: moderate disease activity (score=5); Grade C: mild disease activity (score=1); Grade D implies no current disease activity (score=0).

Mean blood levels of C3 and C4 showed progressive decrease with increased disease activity, while both serum CRP and anti-dsDNA antibodies levels showed progressive increase with disease activity. Both total and differential leucocytic counts were significantly lower in patients with higher BILAG scores compared to those with lower scores and 5 patients (7.1%) were neutropenic. The differences in levels of estimated laboratory parameters, graduated according to BILAG score, showed significant ($p<0.05$) difference between patients had high versus those had low BILAG score. Details of laboratory findings are shown in table 4.

Table 4, Laboratory investigations in SLE patients categorized according to disease activity using BILAG-2004 score.

	BILAG score			
	<10 (n=22)	>10-20 (n=6)	>20-30 (n=15)	>30 (n=7)
C3 (g/l)	0.95±0.1	0.97±0.1	0.82±0.1*†	0.73±0.1*†
C4 (g/l)	0.14±0.02	0.14±0.02	0.11±0.003*†	0.10±0.003*†
CRP (mg/l)	3.6±0.4	9.65±15.3	34.8±4.3*†	37±5.1*†
Leucocytic count (10^3/µl)	5.35±0.5	5.22±0.5	4.52±0.3*†	4.35±0.4*†
Neutrophil count (10^3/µl)	3.56±0.8	3.5±1.1	3.39±0.4	3.05±0.8*
Monocyte count (10^3/µl)	0.45±0.1	0.4±0.1	0.3±0.03*	0.26±0.03*
Lymphocyte count (10^3/µl)	1.02±0.2	1.08±0.1	0.88±0.1*†	0.76±0.1*†
Anti-dsDNA antibodies (IU/ml)	20±4.3	32.8±38.8	145.6±30.3*†	176.4±32.6*†

Data are presented as numbers and mean±SD; *: significant difference versus patients with BILAG score of <10; †: significant difference versus patients with BILAG score of >10-20; *P*<0.05 is significant

Mean serum levels of TRAIL and IL-6 were significantly ($p<0.05$) higher in patients compared to controls and in patients had active disease compared to those had quiescent disease. Mean serum levels of TNF-α were significantly ($p<0.05$) higher in patients as a total and in patients with active disease compared to controls. Mean serum levels of TNF-α were non-significantly ($p>0.05$) higher in patients with quiescent disease compared to control, but were significantly ($p<0.05$) lower compared to those had active disease. On contrary, estimated serum levels of IL-10 were significantly ($p<0.05$) lower in patients as total and categorized according to activity compared to control with non-significantly ($p>0.05$) higher level in patients with quiescent disease compared to patients with active disease. Details of serum levels of studied parameters are shown in table 5.

Table 5. Serum levels of cytokines in SLE patients according to disease activity.

	Controls	Patients		
		Active SLE	Quiescent SLE	Total
Serum TRAIL (ng/ml)	354.7±47.2	1157±317*	965.4±115*†	1113.5±294*
Serum TNF-α (ng/ml)	1.45±0.9	2.8±2*	1.52±0.5†	2.4±0.7*
Serum IL-6 (ng/ml)	15.6±3.3	64.3±20.7*	45.4±18*†	60±21.5*
Serum IL-10 (ng/ml)	3.4±0.4	2.33±0.7*	2.61±0.6*	2.4±0.7*

Data are presented as mean±SD; *: significant difference versus control levels; †: significant difference versus active SLE; *P*<0.05 is significant.

Estimated serum levels of TRAIL, TNF-α and IL-6 showed positive significant correlation with calculated BILAG activity score, while estimated serum IL-10 levels showed negative significant correlation with activity score. Details of correlations are shown in table 6.

Table 6. Correlation coefficient "r" between estimated levels of studied cytokines and BILAG activity score.

	"r"	*P* value
Serum TRAIL (ng/ml)	0.354	0.003
Serum TNF-α (ng/ml)	0.509	0.0006
Serum IL-6 (ng/ml)	0.392	0.001
Serum IL-10 (ng/ml)	-0.564	0.0004

r: Pearson's correlation coefficient. *P* <0.05 is significant.

Discussion

Evaluation of activity status of studied SLE patients relied on BILAG-2004 scoring system that depended on change in therapy between two consecutive visits as a measure for change in disease activity instead of the classic BILAG scoring system depended on changes of disease manifestations. In line with the choice of BILAG-2004 scoring system,

multiple studies recommended it as a system that is sensitive to change and is suitable for use in longitudinal studies of SLE (Cresswell *et al.,* 2006; Yee *et al.,* 2009 & 2010). Moreover, Zhou & Jiang (2012) documented that BILAG-2004 and SLEDAI-2000 scoring systems can be used to assess renal disease activity of patients with lupus nephritis (LN), but the BILAG-2004 scoring system can provide more reliable and comprehensive assessment. Recently, Murphy *et al.* (2016) concluded that the BILAG-2004 index is the only transitional index that grades clinical features as being new, the same, worse or improving and incorporates severity in the scoring.

Preliminary laboratory investigations defined significantly lower leucocytic counts in patients with active compared to those with quiescent disease and 5 patients were neutropenic. These findings point to a possibility of increased apoptosis of leucocytes and neutrophils that may underlie the development of SLE and/or its sequela. In line with these findings; Midgley & Beresford (2011) experimentally reported increased neutrophil apoptosis induced by SLE serum compared with control serum and attributed this to increased surface expression of nuclear antigens. Bouts *et al.* (2012) suggested a role of both apoptosis and neutrophil extracellular traps (NET) released by dying neutrophils in the pathogenesis of SLE, and suggested that both processes may interact with each other. Also, Yu and Su (2013) reported an imbalance between formation and clearance of NET in SLE patients and suggested that this imbalance may play a prominent role in the perpetuation of autoimmunity and the exacerbation of disease, as well as in the induction of end-organ manifestations. Recently, Mahajan *et al.* (2016) documented that the accumulation of cell remnants represents an initiating event of the etiology, while the subsequent generation of autoantibodies against nuclear antigens including NET results in the perpetuation of inflammation and tissue damage in patients with SLE.

In support of the suggestion that apoptosis may underlie pathogenesis and activity of SLE, the current study reported significantly higher serum sTRAIL in patients than in controls and in patients with active disease compared to those had quiescent disease. These findings go in hand with Komatsuda *et al.* (2007) who reported that mean expression of TRAIL mRNA in peripheral blood mononuclear cells and serum sTRAIL concentration in SLE patients were significantly higher than in healthy controls and suggested an important role for TRAIL in the pathogenesis of SLE. Similarly, Ezzat *et al.* (2013) reported that serum TRAIL concentrations were higher in juvenile SLE patients than in controls, in patients with positive anti-dsDNA antibodies than in those without, in patients with classes III and IV nephritis compared to classes I and II nephritis patients and in patients with neutropenia than those without neutropenia. Also, El-Karaksy *et al.* (2013) detected significantly higher expression levels of TRAIL mRNAs in SLE patients than in controls with a statistically significant association between TRAIL mRNA expression and SLE activity. Recently, Inoue *et al.* (2016) detected polymorphisms in genes encoding apoptosis regulatory factors and disturbed serum levels of ligands in patients with autoimmune diseases and suggested that these polymorphisms may be associated with immune dysregulation.

The current study also reported disturbed cytokine milieu in SLE patients manifested as significantly higher serum TNF-α and IL-6 with concomitantly significantly lower serum IL-10 levels in patients compared to controls and in patients with active disease compared to those had quiescent disease. Moreover,

serum levels of cytokines showed significant correlation with disease activity scores.

These data supported that previously reported in literature; where Qiu *et al.* (2013) reported significantly increased serum levels of IL-6, TNF-receptor (TNFR), TNF-α and anti-dsDNA antibody in SLE patients compared with healthy controls and Mangale *et al.* (2013) found SLE patients frequently have high circulating TNF-α levels and suggested a role for TNF-α in SLE pathogenesis. Also, Cigni *et al.* (2014) detected significantly higher serum IL-1, IL-6 and TNF-α in SLE patients than in healthy controls and concluded that proinflammatory cytokines are important in SLE pathogenesis. Experimentally, Mikołajczyk *et al.* (2014) found apoptotic cell-dependent induction of lipopolysaccharide stimulated production of anti-inflammatory cytokine IL-10 by peripheral blood mononuclear cells was blunted in SLE. Moreover, Munroe *et al.* (2014) reported that the levels of proinflammatory adaptive cytokines and shed TNFR are elevated prior to disease flare, while the levels of regulatory mediators are elevated during periods of stable disease and alterations in the balance between inflammatory and regulatory mediators may help identify patients at risk of disease flare and help decipher the pathogenic mechanisms of SLE.

Multiple recent studies are in line with the reported results and supported the role of inflammatory cytokines in SLE disease pathogenesis wherein Patel *et al.* (2016) reported significantly higher concentration of soluble TNFR2 in SLE patients compared to healthy controls and of TNFR1 in active LN compared to inactive LN. Also, Ferreira *et al.* (2016) documented that plasma levels of sTNFR1 and sTNFR2 showed a positive correlation with SLEDAI score and TNF-α and sTNFR1 levels with the Systemic Lupus International Collaborating Clinics score, and patients with current nephritis and patients with anti-ds-DNA antibodies presented higher sTNFR1 and sTNFR2 levels. Moreover, Heinemann *et al.* (2016) demonstrated that compared to healthy control, the percentages of IL-10-producing B cells was significantly decreased in SLE patients, in particular those with LN, thus reflecting an impaired regulatory function. Talaat *et al.* (2016) indicated that both GG genotype and G allele of IL-6 (-174G/C) could be considered as risk factors for SLE and the polymorphisms at IL-10 (-1082 G/G and AA) may play a role in SLE susceptibility in Egyptian patients. Also, Shaltout *et al.* (2016) suggested that IL6 and IL23 may play role in SLE pathogenesis through their effect on double negative T cells and anti ds-DNA.

In conclusion, Systemic lupus erythmatosus is associated with disturbed levels of serum cytokines and sTRAIL in favor of inflammation and apoptosis. These disturbances may underlie pathogenesis and/or activation of SLE. BILAG-2004 numeric scoring system is reliable for SLE activity grading and was significantly correlated with estimated levels of serum cytokines and sTRAIL.

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