Gene expression of cytokines (TNF-α, IFN-γ), serum profiles of IL-17 and IL-23 in paediatric systemic lupus erythematosus

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PAPER

Gene expression of cytokines (TNF-α, IFN-γ), serum profiles of IL-17 and IL-23 in paediatric systemic lupus erythematosus

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Objective: Paediatric systemic lupus erythematosus (pSLE) exhibits an aggressive clinical phenotype and severe complications commonly renal involvement. This could be reflective of the ongoing chronic pro-inflammatory cytokine milieu. We examined relative gene expression of tumour necrosis factor-alpha (TNF-α), interferon-γ (IFN-γ) and serum levels of interleukin-17 (IL-17) and IL-23 and their association with SLEDAI (SLE disease activity index) score and organ manifestations in pSLE. **Methods:** We enrolled 40 pSLE patients (age 5–16 years, on treatment) and 20 age-matched healthy controls. Relative gene expression levels of IFN-γ and TNF-α in the peripheral blood were determined by quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR). β actin gene was used for normalization of gene expression. Serum levels of IL-17 and IL-23 were determined by solid phase sandwich ELISA. Statistical analysis were carried out for comparing (Mann-Whitney U test) and correlating data (Univariate, multivariate analysis and Pearson correlation test) with SLEDAI scores and clinical manifestations. **Results:** Over-expression of TNF-α and IFN-γ was found in 90% (36/40) and 80% (32/40) of pSLE patients, respectively. The relative gene expression of TNF-α and IFN-γ were significantly correlated with renal manifestations ($p < 0.05$). Further, relative expression of IFN-γ gene correlated significantly with skin manifestations and SLEDAI ($p < 0.05$). Serum levels of IL-17 (766.95 ± 357.83 pg/ml) and IL-23 (135.4 ± 54.23 pg/ml) in pSLE were significantly higher than in controls (IL-17, 172.7 ± 39.19 pg/ml and IL-23, 21.15 ± 10.99 pg/ml) ($p < 0.05$). Patients with cutaneous ($p = 0.002$) and haematological involvement ($p = 0.003$) had high serum IL-17 levels. Serum IL-17 levels correlated with SLEDAI ($r = 0.447$; $p < 0.05$). **Conclusions:** In this preliminary study, we observed a persistent, strong pro-inflammatory cytokine milieu in pSLE patients which reflects ongoing inflammatory damage in different organs. The gene expression profile of these cytokines may be used for assessing organ involvement in pSLE. IL-17 may also serve as a prognostic marker in pSLE. However, longitudinal studies on treatment of naïve patients are required to corroborate these findings. *Lupus* (2012) **21,** 1105–1112.

Key word: Paediatric systemic lupus erythematosus; tumour necrosis factor-α; interferon-γ; interleukin-17; interleukin-23; SLE disease activity index

Introduction

Systemic lupus erythematosus (SLE) is a complex, multi-system autoimmune disease of unknown aetiology. It is characterized by various immunological abnormalities. Cytokines play a determinant role in development of SLE.[1] Both type I (e.g. IFN-α) and type II interferon (e.g. IFN-γ) have been implicated in the immunopathogenesis of SLE.[2,3] In murine lupus models, a deficiency in either IFN-γ or the IFN-γ receptor (IFN-γ R) totally abates the disease process,[4] whereas in human SLE, the role of IFN-γ is not very clear. While high serum levels of TNF-α and its soluble receptors have been found in SLE, immunomodulation by TNF-α blocking agents has not been useful in such patients.[5] This implies that TNF-α can exert both immunoregulatory and proinflammatory roles as shown in some experimental models of lupus.[6] Thus, trying to modulate cytokines for therapeutics in pSLE remains a challenge.

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IL-17, a pleiotropic proinflammatory cytokine, is produced largely by a unique CD4 + T-helper (Th) subset called Th17 cells.[7,8] The balance of Th17 and Th1 cell responses is also dysregulated in SLE patients.[8] IL-23, a novel heterodimeric cytokine, is crucial for the pathogenic CD4 + T cell population via the production of IL-17 and IL-22 in SLE patients.[9]

Paediatric-onset SLE has a clinically different phenotype than the adult onset disease. For instance, pSLE patients have increased involvement of the kidney and central nervous system.[10] This could be due to an exaggerated proinflammatory cytokine milieu in pSLE. Gene expression studies have provided insight into the genetic mechanisms of SLE pathogenesis and ensuing knowledge has yielded possible therapeutic targets.[2,11] These studies also aid in differentiating patients with active SLE disease from those with inactive disease.[12,13] However, no study to date has measured gene expression levels of TNF-α and IFN-γ and their association with SLEDAI with respect to paediatric lupus.[5,14–16] In the index study, we have analysed relative gene expression of TNF-α and IFN-γ by highly sensitive real-time reverse transcriptase polymerase chain reaction (RT-PCR). We also determined the serum IL-17 and IL-23 levels of pSLE patients. We correlated all these parameters with SLEDAI and clinical manifestations.

Materials and methods

Subjects

Forty consecutive pSLE patients (5–16 years of age) were recruited from the Division of Allergy Immunology, Advanced Paediatrics Centre, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. The diagnosis of SLE patients was based on the American College of Rheumatology (ACR) criteria for classification.[17] Twenty healthy age-matched volunteer children were recruited as controls with the consent of their parents from families from high socioeconomic backgrounds. They had no past history of fever, rash or joint pain at the time of sampling. On examination, their antinuclear antibody and dsDNA antibody profile were negative, and haemograms were also within the normal range. This study was carried out between March 2007 and April 2011. All patients were positive for antinuclear antibody. Patients with overlap syndrome were excluded from the study. All patients were on treatment using standard protocols incorporating glucocorticoids and immunosuppressive agents (e.g. cyclophosphamide and azathioprine). Patient information in regard to demographic data, cumulative clinical features, serological profile and medications were retrieved from medical records. Disease activity was evaluated according to the SLE Disease Activity Index (SLEDAI) score. A score of more than or equal to 10 was considered as active.[18] A patient was said to have renal involvement if there was significant proteinuria (>0.5 g/day), serum albumin <35 g/l, active urinary sediments and/or renal biopsy consistent with lupus nephritis. Renal biopsy was interpreted according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS) classification criteria.[19] Haematological involvement was defined as presence of autoimmune haemolytic anaemia, leucopaenia (white blood cell count $<3.0 \times 10^9$/l), or thrombocytopaenia (platelet count $<150 \times 10^9$/l). Skin involvement included rash, photosensitivity and alopaecia. The study protocol was approved by the Institute Ethics Committee. All parents/guardians of pSLE patients and controls gave written informed consent.

RNA isolation and cDNA synthesis

Total RNA from fresh whole blood was extracted by RNeasy Mini kit (QIAGEN, Hilden, Germany). RNA was treated with DNase I (Invitrogen, San Diego, CA, USA) to avoid any DNA contamination. Approximately 0.25 μg of total RNA was reverse transcribed to complementary DNA (cDNA). First strand cDNA was synthesized using random hexamer primer and Moloney murine leukaemia virus (M-MuLV) reverse transcriptase in a 20 μl reaction volume. Reagents were obtained from RevertAid™ first-strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania). RNA yield and purity was assessed prior to cDNA synthesis by measuring spectrophotometrical absorbance at 260 nm and 280 nm. The integrity of isolated RNA was then verified by denaturing agarose gel electrophoresis followed by ethidium bromide staining. RNA bands on gel were visualized under ultraviolet light. Presence of both 18 S and 28 S ribosomal RNA (rRNA) bands confirmed good quality of RNA. All RNA and cDNA samples were stored at −70°C before use.

Quantification of mRNA

A real-time RT-PCR assay was used to quantify target gene transcripts using β-actin as an endogenous control. Primer sequences used for reverse

Table 1 Demographics and clinical parameters of paediatric lupus patients and control groups

Variables	*pSLE (N = 40)*[a]	*Controls (N = 20)*	p *value*[b]
Age (years)			
Median	12	13	0.213
Range	5–16	10–15	
Sex (Female/male)	34/6	15/5	0.326
Disease duration (months)			
Median	8	NA	
Range	1–96	NA	
SLEDAI score			
Median	15	NA	
Range	4–26		
Clinical manifestations[a]			
Cutaneous	25 (62.5)	NA	
skin biopsy	13 (32.5)	NA	
Renal	24 (60)	NA	
Renal Biopsy	10 (25)	NA	
Haematological	18 (45)	NA	
Oral ulcers	12 (30)	NA	
Musculoskeletal	12 (30)	NA	
Neurological	10 (25)	NA	
Medications used[a]			
I. Prednisolone	11(27.5)		
II. Prednisolone and anti-malarials (chloroquine and hydroxychloroquine)	19 (47.5)		
III. Immunosuppressive therapy[c]	10 (25)		

SLEDAI: Systemic lupus erythematosus disease activity index; NA: not applicable; [a]values are represented as either median and range or number (percentage): *N* (%); [b]$p \leq 0.05$ as significant; [c](methotrexate, azathioprine, mycophenolate mofetil, cyclosporine and cyclophosphamide).

transcriptase reaction of TNF-α, IFN-γ and β actin were as follows:

Forward primer, 5' CAA GAG ATG GCC ACG GCT GCT 3'; (β actin)
Reverse primer, 5' TCC TTC TGC ATC CTG TCG GCA 3'; (β actin)
Forward primer, 5'-GAG TGA CAA GCC TGT AGC CCA TGT TGT AGC-3'; (TNF-α)
Reverse primer, 5'-GCA ATG ATC CCA AAG TAG ACCTGC CCA GAC T-3'; (TNF-α)[20]
Forward primer, 5' CTA ATT ATT CGG TAA CTG ACT TGA 3'; (IFN-γ)
Reverse primer, 5' ACA GTT CAG GCC ATC ACA TTG GA 3'; (IFN-γ)[21]

PCR amplification was performed with 2X SYBR Green PCR Master Mix (Roche Diagnostics, Manheim, Germany) with 300 nM of primers (Sigma Aldrich Chemicals Pvt. Ltd. Bangalore, Karnataka, India), 16 nanogram of cDNA and nuclease-free water according to the manufacturer's protocol (QIAGEN). PCR conditions included an initial activation at 95°C for five minutes, denaturation at 95°C for one minute, annealing at 58–62°C for 30 seconds and extension at 72°C for 34 seconds followed by 40–50 cycles of repeating. The mRNA levels were measured by a Light Cycler 480 machine (Roche Diagnostics, Manheim, Germany). mRNA levels were expressed as threshold cycle (CT). Comparative CT method was used for analysis. For relative quantification, the expression target genes were normalized by expression of β actin gene. The amount of target was calculated by $2^{-\Delta\Delta CT}$. This method allows the calculation of relative quantities of targets without the necessity of setting up a standard curve for each assay.[22] Results were expressed as relative quantity to the calibrator as normalization ratio where all the other samples are expressed in terms of their fold difference to the calibrator or control. Reactions were run in triplicate for each sample.

Enzyme-linked immunosorbent assay (ELISA)

Quantitative determination of serum TNF-α, IL-17 and IL-23 levels in pSLE and healthy controls was carried out. TNF-α, IL-17 A and IL-23 solid phase sandwich ELISA kit (Diaclone, Besançon city, France) was used. The sensitivity, minimum detectable dose of TNF-α was 5 pg/ml, IL-17 A was 3 pg/ml and for IL-23 was 20 pg/ml.

Statistical analysis

The mRNA levels were expressed as log transformation. Categorical data were analysed using the two-tailed Fisher exact test and continuous variables (gene expression levels of TNF-α and IFN-γ, levels of IL-17 and IL-23) were compared between disease and healthy controls using the two-tailed Mann-Whitney U test. Univariate analysis (chi-square test) was carried out to learn the association of gene expression levels with SLEDAI. The correlations between levels of IL-17 and IL-23 and SLEDAI were established by the Pearson correlation test. Multivariate analysis was performed using a linear regression model. Results were analysed with Statistical Package for the Social Sciences Software (SPSS) version 12.0. A *p* value less than 0.05 was considered significant.

Results

Demographic and clinical parameters

Demographic and clinical parameters of pSLE patients and healthy controls are shown in Table 1. Age median was 12 years. Female to

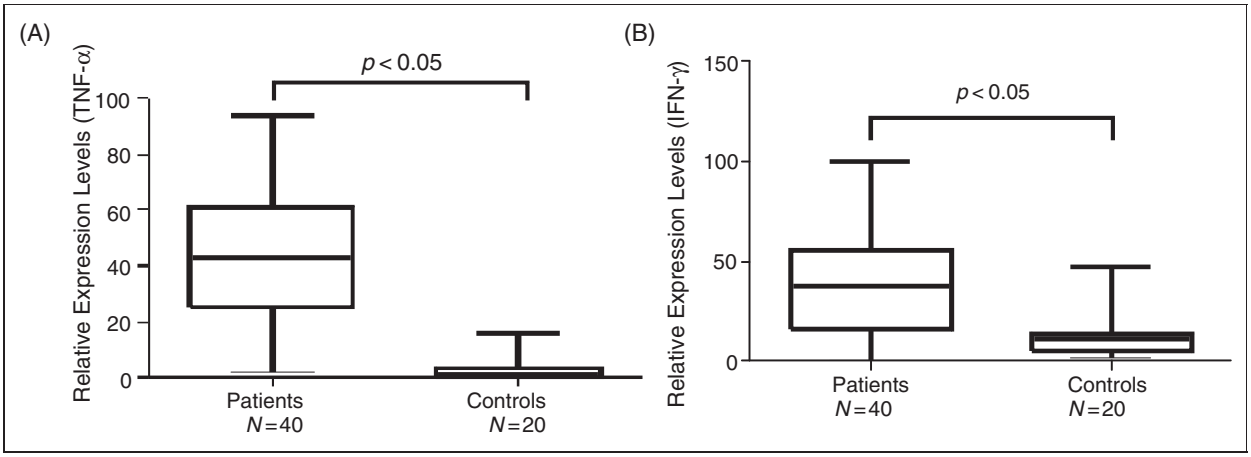

Figure 1 Comparison of gene expressions in peripheral blood from 20 healthy controls and 40 pSLE patients. Relative expression levels of genes TNF-α and IFN-γ are shown in Figure A and Figure B, respectively. Horizontal lines within boxes show medians and vertical lines show standard errors ($p \leq 0.05$).
pSLE: paediatric systemic lupus erythematosus; TNF-α: tumour necrosis factor-alpha; IFN- γ: interferon-γ.

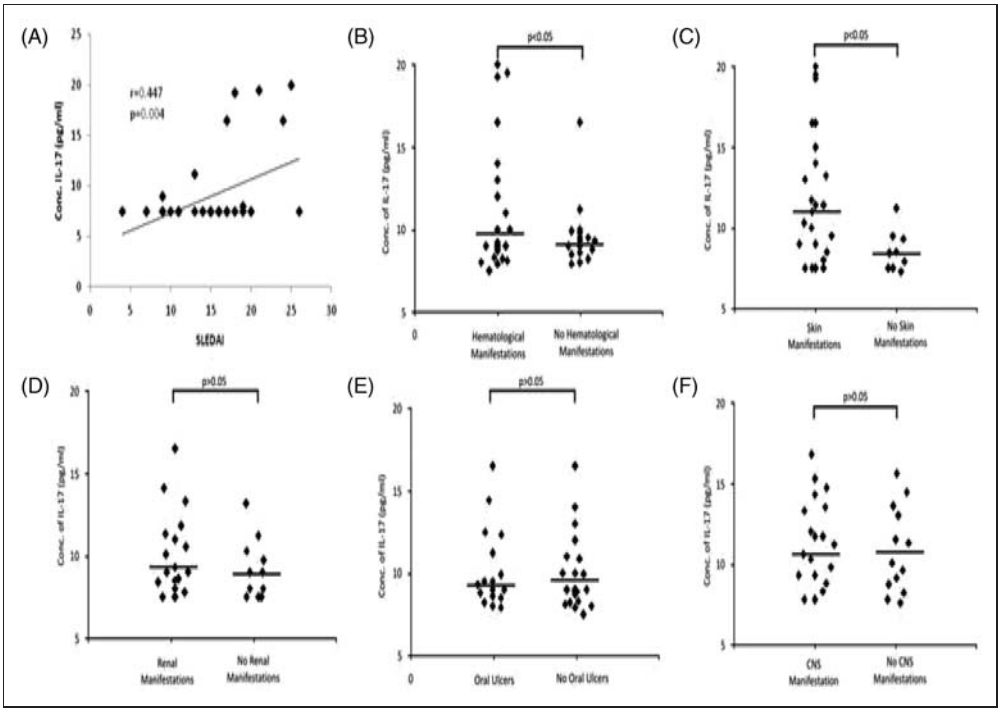

Figure 2 Correlation analysis of IL-17, SLEDAI and clinical involvements in 40 pSLE patients (A to F). Straight line graph between pro-inflammatory IL-17 and SLEDAI showing significant correlation between cytokine IL-17 and SLEDAI in patients ($p \leq 0.01$), Here, Pearson correlation test was used for analysis. The serum levels of IL-17 were compared between patients who were positive and negative for the following: haematological (B); skin (C); renal (D); oral (E); central nervous system (CNS) manifestations (F). Mann-Whitney test was used to analyse these data. Each symbol represents an individual sample and horizontal lines show median values.
IL-17: interleukin-17; pSLE: paediatric systemic lupus erythematosus.

male ratio was 5.7. SLEDAI score median was 15 (with a range of 4–26). Since our data does not show normal distribution or follow Gaussian curves, we provided median and ranges in demographic variables.

Increased expression of TNF-α, IFN-γ genes in peripheral blood in pSLE patients

Over-expression of TNF-α and IFN-γ was found in 90% (36/40) and 80% (32/40) of pSLE

Table 2A Multivariate analysis using linear regression model showing correlations between cytokine (TNF-α) gene expression levels and SLEDAI, clinical manifestations, IL-17 and IL-23 cytokine levels

				95% CI	
		p *value*	*Regression coefficient (B)*	*Lower Bound*	*Upper Bound*
TNF-α Gene expression levels (dependent variable)	SLEDAI	0.164	−0.096	−174.45	32.075
	Renal	0.021[a]	−1131.171	−2075.65	−186.69
	Skin	0.078	−41.98	−64.28	−8.17
	Arthritis	0.096	−283.330	−635.23	−300.99
	CNS	0.068	−182.15	−432.43	−321.00
	Haematological	0.084	−16.558	−437.23	−438.22
	Oral	0.62	−82.15	−143.43	−324.43
	IL-23	0.001[b]	1.199	0.59	1.81
	IL-17	0.052	115.852	0.65	5.98

TNF-α: tumour necrosis factor-alpha; SLEDAI: Systemic lupus erythematosus disease activity index; IL-17: interleukin-17; IL-23: interleukin-23; IFN- γ: interferon-γ; CI: confidence interval.
[a]Correlation is significant at the 0.05 level (two-tailed); [b]correlation is significant at the 0.01 level (two-tailed).

Table 2B Multivariate analysis using linear regression model showing associations between cytokine (IFN-γ) gene expression levels and SLEDAI, clinical manifestations, IL-17 and IL-23 cytokine levels

				95% CI	
		p *value*	*Regression coefficient (B)*	*Lower bound*	*Upper bound*
IFN-γ Gene expression levels (dependent variable)	SLEDAI	0.01[a]	−1.650	−2.95	−0.43
	Renal	0.021[a]	−0.24.3	−31.90	−2.19
	Skin	0.029[a]	−0.297	−34.28	−1.17
	Arthritis	0.052	−10.68	−135.23	−100.99
	CNS	0.061	−26.87	−92.43	−71.00
	Haematological	0.075	−12.91	−37.23	−18.22
	Oral	0.052	−5.216	−43.43	−24.43
	IL-23	0.055	−1.416	−0.03	0.00
	IL-17	0.35	−1.695	0.75	1.98

IFN- γ: interferon-γ; SLEDAI: Systemic lupus erythematosus disease activity index; IL-17: interleukin-17; IL-23: interleukin-23; CI: confidence interval.
[a]Correlation is significant at the 0.05 level (2-tailed); [b]Correlation is significant at the 0.01 level (2-tailed).

patients, respectively. As shown in Figures 1A and 1B, the expression levels of both TNF-α and IFN-γ genes in pSLE patients were significantly higher than those in the normal controls with $p = 0.002$ and $p = 0.003$, respectively.

Relative expression of IFN-γ and TNF-α related with SLEDAI, organ manifestations and IL-23

Using univariate and multivariate linear regression model (Tables 2A and 2B), we found TNF-α gene expression levels significantly correlated with renal manifestations, and IL-23 levels and IFN-γ gene expression levels with SLEDAI, renal and cutaneous manifestations. We did not observe any correlation of TNF-α expression with scores of SLEDAI and other clinical characteristics of SLE such as musculoskeletal and haematological manifestation (data not shown).

Table 3 Comparison of concentrations of cytokines in pSLE patients and healthy controls. [a]Correlation is significant at the 0.01 level (two-tailed)

[a]*Cytokines*		*Concentration in pSLE (pg/ml)*	*Concentration in controls (pg/ml)*	p *value*[a]
IL-17 (serum)	Mean ± SD	766.95 ± 357.82	172.7 ± 39.19	0.002
	Median	7.5	7.5	
	Range	7.5–20	0–7.5	
IL-23 (serum)	Mean ± SD	135.4 ± 54.23	21.15 ± 10.99	0.003
	Median	375	375	
	Range	375–420	0–375	
TNF-α (serum)	Mean ± SD	28.5 ± 15.3	15.22 ± 10	0.665
	Median	15	10	
	Range	10–48	7–20	

pSLE: paediatric systemic lupus erythematosus; IL-17: interleukin-17; IL-23: interleukin-23; TNF-α: tumour necrosis factor-alpha; SD: standard deviation.

Elevated serum levels of pro-inflammatory IL-17 & IL-23 in p SLE

Levels of both cytokines IL-17 and IL-23 were significantly elevated in pSLE patients as compared to controls ($p<0.05$) (Table 3).

Levels of IL-17 were significantly associated with SLEDAI, cutaneous and haematological manifestations in pSLE

We determined correlations of serum levels of IL-17 and IL-23 with SLEDAI scores and clinical characteristics of pSLE using Pearson correlation test. IL-17 in sera of patients was found to be statistically significantly correlated with SLEDAI, cutaneous & haematological manifestations ($p< 0.05$) (Figure 2).

Discussion

Reliable immunoserological biomarkers and a better understanding of pathogenesis of paediatric lupus are the felt need in our setup. We report for the first time in an Indian paediatric lupus cohort, IFN-γ and TNF-α gene expression levels in peripheral blood and serum profiles of TNF-α, IL-17,

IL-23 and their association with disease activity and organ involvement.

In the index study, IFN-γ gene expression levels were significantly higher and correlated with SLEDAI, skin and renal involvement. Several studies have previously pointed a possible role of IFN in the pathogenesis of SLE. Hooks et al.[23] for the first time reported, almost three decades ago, elevated serum titres of IFN-γ in autoimmune diseases including SLE, arthritis and scleroderma. These findings were subsequently confirmed mainly in SLE.[24] Caposio et al. have shown the over-expression of interferon inducible gene MNDA (myeloid nuclear differentiation antigen) in leukocytes and another interferon inducible gene (IFI16) in the epidermis and dermis layers of skin in lupus patients.[25] IFI16 causes inflammation via up-regulation of adhesion molecules and chemokines.[25] Karonitsch et al.[26] demonstrated an increased expression of IFN-γ signalling molecule signal transducer and activator of transcription-1 (STAT-1) and its correlation with disease activity. Hargai et al.[27] too observed an increased expression of IFN-γ in peripheral blood T cells of SLE patients after stimulation with anti-CD3 and anti-CD28 monoclonal antibodies. Bennett et al.,[28] using microarrays, have confirmed over-expression of IFN-inducible genes in the peripheral blood mononuclear cells (PBMCs) of pSLE patients. Moreover, SLE patients with higher expression of IFN-related genes had been reported earlier to have significantly more severe clinical manifestations involving the kidneys, haematopoietic cells and central nervous system.[2] Thus, our study also reiterates a dominant role of IFNs in pathogenesis and in determining dermal and renal manifestations in pSLE.

We also found elevated gene expression levels of TNF-α and correlation with renal involvement. Several earlier studies revealed an elevated expression of TNF-α in peripheral blood and in the kidneys of SLE patients.[29] Zhu et al. investigated the in situ renal expression of TNF-α and the TNF-α adapter proteins involved in the TNF-α signal pathway in PBMCs of SLE patients and their relationship with disease activity.[16] Various in vivo and in vitro studies[30] have also shown TNF-α to exacerbate the inflammatory response by activating transcription of proinflammatory gene targets such as IL-1β, IL-6 and IL-8.[31] Therefore, our study also suggests that increased gene expression of TNF-α may lead to inflammation and secondary tissue destruction.

However, when serum TNF-α level was measured by ELISA, no significant difference was found between the patients and controls. We also estimated the levels of TNF-α in PBMCs culture supernatants stimulated by concanavalin A (for T-cell proliferation) by flow cytometry (multiplex bead array based cytokine assay) and ELISA, and no difference in patients vs. controls was observed (data not shown). Low levels of secreted TNF-α in our cohort might be explained partly by medication that the patients received. On the other hand, the hurdles in measuring the levels of certain molecules (such as TNF-α) could be due to some practical (technical) aspects associated with the short half-life and ex vivo instability of specific molecules during collection and storage of samples. The short half-life of TNF-α has been pointed out by various researchers.[40] Gattorno et al. found similar findings and reported serum concentrations of TNF-α fell within the normal range in patients with both SLE and Henoch-Schönlein purpura (HSP) irrespective of disease activity.[32] Besides this, in literature it had been shown that the balance between TNF and TNF-soluble inhibitors (TNF-soluble receptors 75Kd and TNF-soluble receptor 550 Kd) is altered in favour of the inhibitors in active lupus: this provides support for the idea that low TNF activity is associated with increased disease activity in lupus as in our cohort of active paediatric lupus disease.[33] For further studies, it would be useful to correlate TNF-α gene expression levels, TNF-α serum levels and their polymorphism in treatment of naive patients. TNF-α blocking agents, however, have been found to be more effective in rheumatoid arthritis[34] than in SLE.[30] Our study favours the concept that TNF gene expression levels, serum levels and serum TNF receptors must be taken into consideration when selecting pSLE subjects for anti-TNF therapy.

In addition, serum IL-17 and IL-23 levels were also significantly elevated and IL-17 levels were correlated with cutaneous, haematological manifestations and SLEDAI score. Many studies showed higher IL-17 levels in SLE, whereas others did not report the same.[35,36] Differences in findings could be attributed to the variations in principle and sensitivity of the ELISA tests in various studies. Using immunohistochemistry, Tanasescu et al. reported over-expression of IL-17 in skin lesions of discoid lupus erythematosus, subcutaneous lupus erythematosus. Th17 lymphocyte population may be implicated in cutaneous and visceral lesions of patients with SLE.[37] Thus, it is likely that Th17 cell regulation is disturbed in SLE. There might be infiltration and increasing local production of IL-17 in various organs. We also observed serum IL-23 levels significantly

correlated with TNF-α expression levels. The likely significance of increased IL-23 levels in autoimmune responses is supported by a recent report which describes that IL-23 activates pathogenic Th1 and Th17 cells via the induction of downstream Th1 chemokine (CXCL10) and inflammatory cytokine IL-17.[9] Additionally, IFN-γ and TNF-α have been shown to promote expansion of Th17 cells through generation of an inflammatory milieu.[38] Thus, our findings provide the proof of dysregulation of the IL-17/IL-23 axis in pSLE.

In conclusion, in our paediatric lupus cohort, a strong proinflammatory cytokine milieu persists, which is reflective of the ongoing inflammatory damage in different organs. The understanding should pave the way for potential therapeutic agents targeting cytokines (such as anti-Th17[39]) to improve the outcome in pSLE. Our findings need to be corroborated in longitudinal studies involving larger cohorts of pSLE, preferably in the treatment of naïve patients.

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Conflict of interest

None declared.

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