

Relationship between T lymphocyte subsets and cortisol in systemic lupus erythematosus

Shah D¹, Kiran R¹, Wanchu A², Bhatnagar A¹

Department of Biochemistry¹, Basic Medical Science Block, Panjab University, Chandigarh, India, Department of Internal Medicine², Postgraduate Institute of Medical Education and Research, Chandigarh, India.

Abstract

Background: Systemic Lupus Erythematosus (SLE) is a complex chronic immunological disease characterized by increased B cell activity and altered T cell function.

Objective: To investigate relationship between T lymphocyte subsets and cortisol with the disease activity of systemic lupus erythematosus patients in North India.

Materials and methods: The percentage of CD4⁺ and CD8⁺ T cells in the lymphocyte of SLE patients and healthy controls were determined by flow cytometry. Serum cortisol of SLE patients and healthy controls was determined by enzyme-linked immunosorbent assay (ELISA).

Results: A significant decrease in the percentage of CD4⁺ T cells and increase in the percentage of CD8⁺ T cells were found in patients with SLE compared to the healthy controls. Decrease in the ratio of CD4⁺/CD8⁺ T cell and low level of serum cortisol were found in the patients with SLE. The ratio of CD4⁺/CD8⁺ T cell was inversely correlated with systemic lupus erythematosus disease activity index (SLEDAI) score and erythrocyte sedimentation rate (ESR). A positive correlation was observed between CD8⁺ T cells and SLEDAI score. Furthermore, CD8⁺ T cells were positively correlated with ESR in the patients with SLE.

Conclusion: The results showed that low level of cortisol and high percentage of CD8⁺ T cells in the lymphocytes could be actively involved in the pathogenesis of SLE.

Key words: CD4⁺/CD8⁺ T cell ratio, cortisol, systemic lupus erythematosus, T-cell activation

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease of unknown aetiology, characterised by arthritis, cutaneous rash, vasculitis, involvement of central nervous system, renal and cardiopulmonary manifestations¹. Due to loss of self-tolerance there is persistence of autoreactive B and T lymphocyte in SLE². This leads to both systemic and organ-specific autoimmunity. The activation of autoreactive T lymphocytes leads to the abnormalities of CD4⁺ and CD8⁺ T cells, their numbers and also engenders the autoantibody production characteristic of SLE^{3,4,5}. Previous studies have found either normal or increased or decreased levels of CD8⁺ T cells and a decrease in CD4⁺ T cells, with a consequent decrease of the CD4⁺/CD8⁺ T cell ratio in SLE patients^{5,6,7}. However, the mechanism of the induction of CD4⁺ T cell abnormality and its relationship with CD8⁺ T cell and clinical features of SLE is still not clear. The circulating lymphocyte ratios have been known to be markedly affected by corticosteroids, an anti-inflammatory hormone, in patients with SLE⁸. The reduced level of cortisol has been found to increase inflammation in the patients with SLE⁹. However, role of cortisol in relation

to the T lymphocytes subsets and pathogenesis of SLE is yet to be investigated.

In the present study, the distribution of T lymphocytes subset and its correlation with cortisol and disease activity has been evaluated in SLE patients.

Materials and methods

Subjects

Patients were selected among individuals attending out-patient Department of Internal Medicine at Postgraduate Institute of Medical Education and Research, Chandigarh, India. The study included 30 patients with SLE (27 females, 3 males) with mean age of 27.50 ± 7.48 years and the control group consisted of 30 healthy volunteers (27 females, 3 males) with mean age of 26.73 ± 5.37 years. SLE was diagnosed

Correspondence

Dr. Archana Bhatnagar
Department of Biochemistry, Basic Medical Science Building
Panjab University, Chandigarh-160014 (India)
E-mail: bhatnagar.archana@gmail.com

using the 1982 American Rheumatism Association revised criteria¹⁰. Disease activity was determined by using SLE Disease Activity Index (SLEDAI) score¹¹. The study protocol was approved by the Institute Ethics Committee, Postgraduate Institute of Medical Education and Research, Chandigarh, India and informed consent was obtained from all the patients and controls (healthy subjects). All the patients enrolled in the present study were non-smoker and non-alcoholic.

Blood Samples

Venous blood samples were obtained from patients and healthy controls. Samples were collected into plain and heparinised vacutainers (Becton Dickinson, USA). Samples from plain vacutainers were left to clot, serum was separated, aliquoted and used for estimation of cortisol. Heparinised blood samples were used for the isolation of peripheral blood mononuclear cells (PBMC) for the determination of percentage of CD4⁺ and CD8⁺ T cells.

Determination of CD4⁺ and CD8⁺ T cells

Peripheral blood mononuclear cells (PBMC) were freshly isolated from heparinised venous blood by Ficoll-Hypaque (Sigma-Aldrich, USA) density gradient centrifugation (400 x g) for 30 minutes. After washing with isotonic phosphate buffered saline solution, cells were enumerated. The cell suspension was adjusted to the concentration of 1×10^6 /ml in RPMI 1640 (Sigma Aldrich, USA), supplemented with 10% heat-inactivated fetal calf serum (Sigma Aldrich, USA). Twenty microlitres of mouse anti-human anti-CD4-FITC (BD Bioscience) and 20µl of mouse anti-human anti-CD8-APC (Immunostep, Spain) were added and incubated in the dark at 4°C for one hour. After washing twice with PBS, cells were fixed with 1% paraformaldehyde. Negative controls were performed simultaneously using FITC-labelled mouse anti-human IgG1 mAb (BD Bioscience) and APC-labelled mouse anti-human IgG1 mAb (BD Bioscience). Cells were acquired and analysis was performed on FACS-Calibur (Becton Dickinson, San Jose, CA, USA) using Cell Quest software (TreeStar, San Carlos, CA, USA).

Determination of serum cortisol

Serum cortisol level was measured by a commercially available enzyme linked immunosorbent assay (ELISA) (Immuno-Technology & Steroid Lab, India). Assay was performed according to manufacturer's instruction. The sensitivity of the kit was 0.28 µg/dl. Briefly, serum samples and standards were incubated in the anti-cortisol antibody coated wells with horse radish peroxidase-cortisol (HRP-cortisol) conjugate. After incubation, the liquid contents of the wells were decanted and the wells were washed with wash buffer to remove the unbound enzyme conjugates. The bound enzyme activity was measured by developing coloured product from colourless substrate (TMB/H₂O₂ solution)

after incubation at 450 nm. The levels of serum cortisol were determined by comparison with that of standard curve of cortisol.

Statistical analysis

Statistically significant differences were determined using the Student's paired t-test. Correlation analyses were performed using two-tailed Spearman's rank correlation. Analyses were performed using GraphPad Prism v.5.00.288 for Windows (GraphPad Software, San Diego, CA). *P* values less than 0.05 were considered significant.

Results

The study included 30 patients with SLE (27 females, 3 males) with mean age of 27.50 ± 7.48 years and the control group consisted of 30 healthy volunteers (27 females, 3 males) with mean age of 26.73 ± 5.37 years. Disease activity was determined by using SLE Disease Activity Index (SLEDAI) score (maximum score of 105)¹¹: Mild score <10; Moderate score 10-20; Severe score >20. Three patients had moderate SLEDAI score while the rest 27 had a SLEDAI score >20 indicating severity of SLE in the present study. The mean SLEDAI Score of the patients with SLE were 38.90 ± 3.31 . The levels of ESR, C3 and C4 were 50.63 ± 22.25 (0 -20 mm/hr), 56.93 ± 18.62 (50-120 mg/dl) and 27.39 ± 12.934 (20-50 mg/dl) respectively. Out of 16 patients showing low serum levels of C3 and C4, 5 of them had nephritis. The demographic and clinical characteristics of SLE patients and healthy controls are summarized in the Table 1 and Table 2 respectively.

CD4⁺ and CD8⁺ T cells in PBMC

The percentage of T lymphocyte subsets in the PBMC of SLE patients and healthy controls are shown in the Table 3. A significant decrease in the percentage of CD4⁺ T cells was found in patients with SLE as compared to healthy controls (*P*<0.001) while an increase in the percentage of CD8⁺ T-cells was found in patients with SLE as compared to healthy controls (*P*<0.001). Furthermore, a considerable decrease in CD4⁺/CD8⁺ T cells ratio was found in patients with SLE compared to the healthy controls (*P*<0.001).

Serum cortisol

To appraise the role of cortisol in the activation of T cells, the serum cortisol level was determined. A noteworthy decrease in the level of serum cortisol was found in patients with SLE as compared to healthy controls (*P*<0.001).

Correlation studies

To evaluate the relation of T cells and low serum cortisol in the pathogenesis of SLE, correlations among CD4⁺, CD8⁺ T cells, serum cortisol, C3, C4, ESR and SLEDAI score was studied. As shown in Table 4, negative correlation was observed between CD4⁺ T cells (*r* = -

0.3931, $P < 0.05$) (Fig 2a) and CD4⁺/CD8⁺ T cell ratio ($r = -0.5594$, $P < 0.01$) (Fig 2c) with SLEDAI score in the patient with SLE. Positive correlations was observed between CD8⁺ T cells and SLEDAI score ($r = 0.4748$, $P < 0.01$) (Fig 2b) and between CD8⁺ T cells and ESR

($r = 0.5926$, $P < 0.001$) (Fig 2e) in patients with SLE. A negative correlation between CD8⁺ T cells and C4 ($r = -0.3631$, $P < 0.05$) (Fig 2d) was found to exist. The CD4⁺/CD8⁺ T cell ratio showed a negative correction with ESR ($r = -0.4687$, $P < 0.01$) (Fig 2f).

Table 1: Demographic characteristics of patients with systemic lupus erythematosus (SLE) and controls

	SLE patients	Controls
Number (n)	30	30
Female/male	27/3	27/3
Age (yrs)	27.5 ± 7.48	26.73 ± 5.37
Duration of disease	2.17 ± 1.60	NA
ESR (mm/hr)	50.63 ± 22.25	NA
C3 (mg/dl)	56.93 ± 18.62	NA
C4 (mg/dl)	27.39 ± 12.934	NA
SLEDAI Score	38.90 ± 3.31	NA

Values are expressed as Mean ± SD, NA: not applicable

Table 2: Symptoms in patients with SLE

Symptoms	Patients (n=30)	%
Arthralgia	26	86.66
Oral ulcers	16	53.33
Skin lesion	22	73.33
Photosensitivity	17	56.66
Lymphadenopathy	4	13.13
Arthritis	14	46.66
Hematological involvement	10	33.33
Renal involvement	8	26.66
CNS involment	3	10
Serositis	2	6

Table 3: Percentage CD4⁺ T cells, percentage CD8⁺ T cells, ratio CD4⁺/CD8⁺ T cells and serum cortisol in patients with systemic lupus erythematosus (SLE) and the controls.

	Controls	SLE Patients
%CD4 ⁺ T cells	40.290 ± 5.819	27.084 ± 6.002*
%CD8 ⁺ T cells	25.035 ± 3.429	36.179 ± 6.495*
%CD4 ⁺ /CD8 ⁺ T cell	1.617 ± 0.172	0.755 ± 0.124*
Cortisol (mg/dl)	18.583 ± 4.725	11.183 ± 4.742*

Values are expressed as Mean ± SD

*Significantly different at ($P < 0.001$)

Table 4: Correlation of T lymphocytes subset with SLE disease activity index (SLEDAI) score, complement protein (C4) and Erythrocyte sedimentation coefficient rate (ESR) in patients with systemic lupus erythematosus (SLE)

Parameters	Spearman rank correlation coefficient (r)
CD4 ⁺ T cells with SLEDAI Score	-0.3931*
CD8 ⁺ T cells with SLEDAI Score	0.4748**
CD4 ⁺ /CD8 ⁺ T cell with SLEDAI Score	-0.5594**
CD8 ⁺ T cells with C4 protein	-0.3631*
CD8 ⁺ T cells with ESR	0.5926***
CD4 ⁺ /CD8 ⁺ T cell with ESR	-0.4687**

Values are expressed as spearman coefficient (r).

* significantly different at ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$)

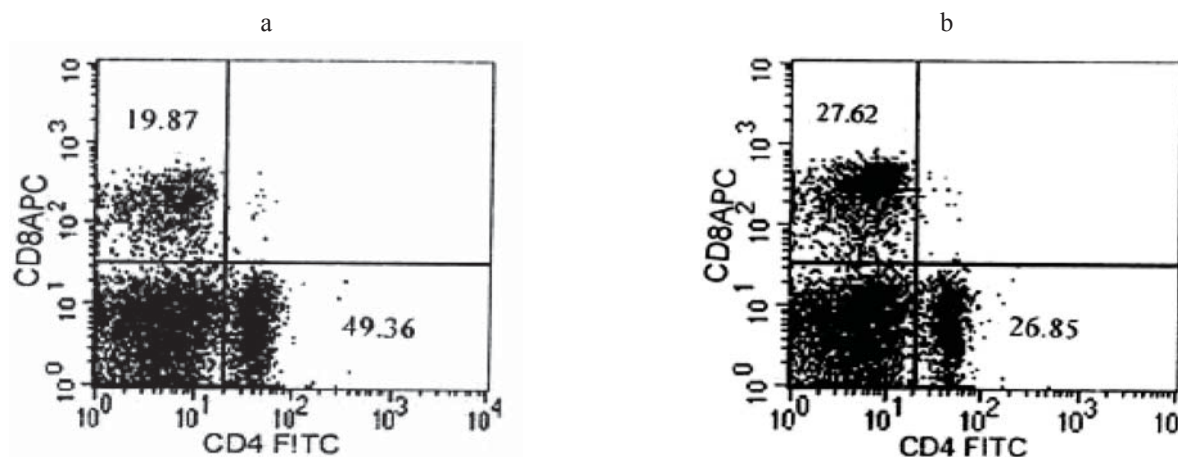
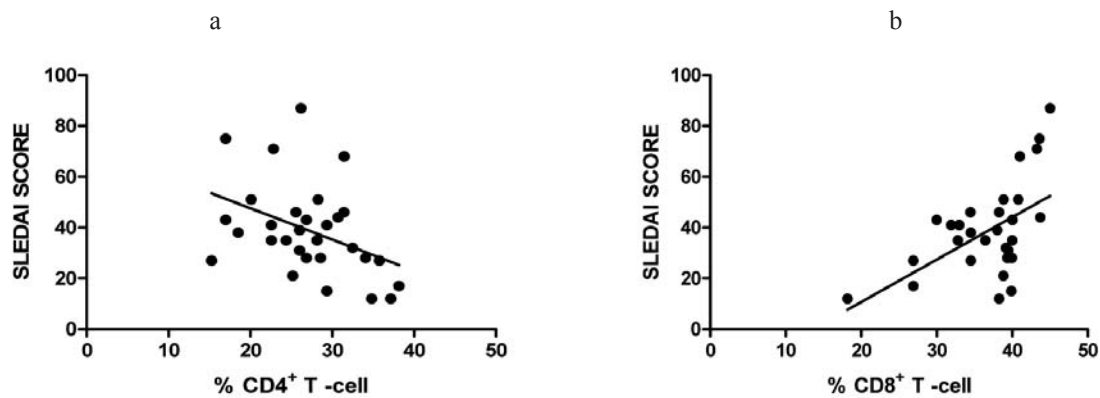


Fig 1: FACS analysis of percentage CD4⁺ T cells and CD8⁺ T cells in the PMBC. The example shown is representative of 30 separate experiments. (a) a healthy control, (b) an SLE patient. Two colour staining protocol was used to assess for the % expression of CD4⁺ and CD8⁺ T cells in the lymphocyte gate.



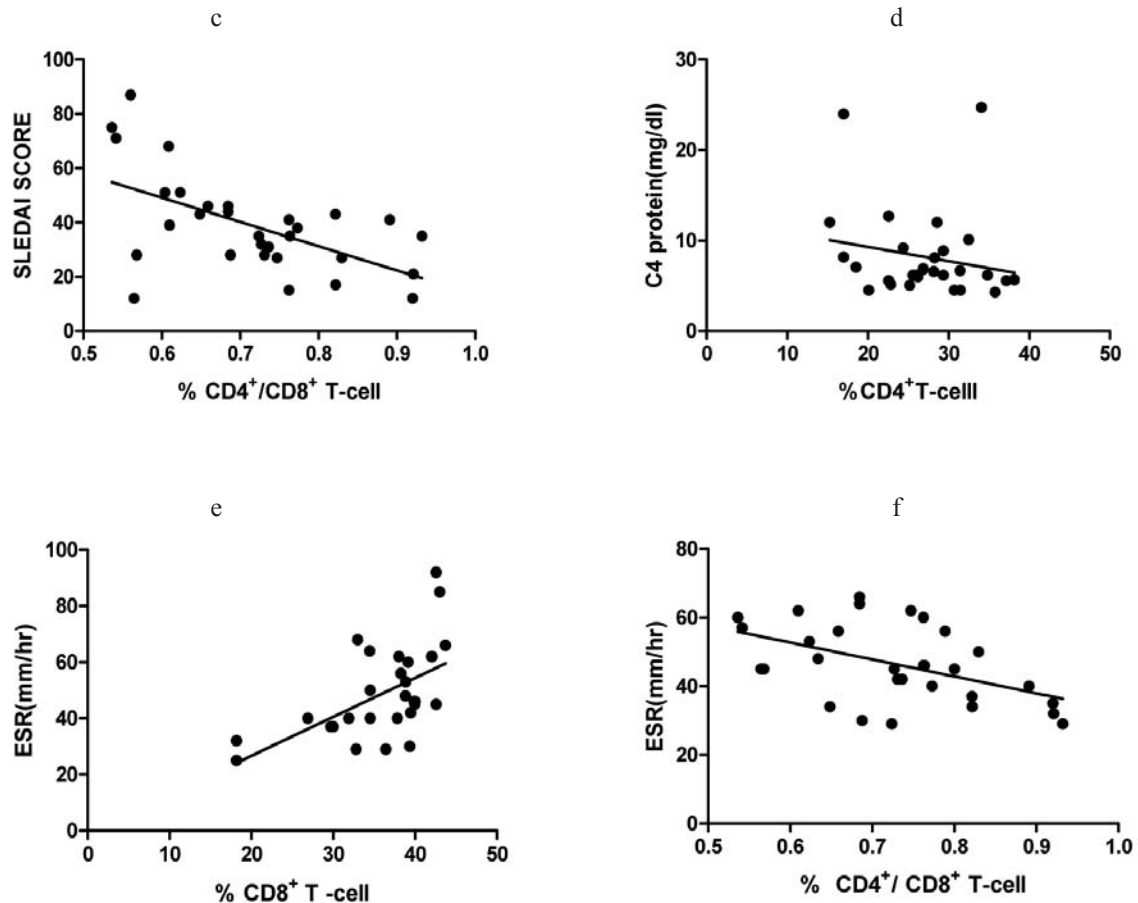


Fig 2: Correlations study between various parameters in the patients with SLE a) correlation between percentage of CD4⁺ T cells and systemic lupus erythematosus disease activity index (SLEDAI) score (Spearman's rank correlation coefficient (r) = - 0.3931, P < 0.05) b) correlation between percentage of CD8⁺ T cells and SLEDAI score (r = 0.4748, P < 0.01) c) correlation between percentage of CD4⁺/CD8⁺ T cell ratio and SLEDAI score (r = 0.4817, P < 0.01) d) correlation between percentage of CD8⁺ T cells and C4 (r = -0.3631, P < 0.05) e) correlation between percentage of CD8⁺ T cells and ESR (r = 0.5926, P < 0.001) f) correlation between percentage of CD4⁺/CD8⁺ T cell ratio and ESR (r = -0.4687, P < 0.01).

Discussion

Systemic lupus erythematosus is a chronic inflammatory disease with unknown aetiology. Immunoregulatory abnormality is considered as one of the universal factors in the pathogenesis of SLE. The most common immunoregulatory abnormalities in SLE are deficiency in suppressor cell activity, hyperactive B cells, defective helper T cells, CD4⁺ T cells deficient in IL-2 production, impaired T-cell activation and responsiveness to antigens^{12, 13, 14}. Different groups have reported variable results about T cells subsets in SLE patients. For example active SLE patients have been found with increased, decreased or normal CD4⁺ T cell count^{15,16}. Abnormalities of CD4⁺ T cells may play an important role in the induction of SLE⁸. The most consistent finding in SLE is impairment of CD8⁺ T cell functions^{15,17}. Studies have reported a relative reduction of CD8⁺ T cells and an increase in the ratio of CD4⁺/

CD8⁺ T cell in patients with SLE^{18, 19}. However, other studies have found either a normal or an increased level of CD8⁺ T cells and a diminution of CD4⁺ T cells with a consequent decrease in the CD4⁺/CD8⁺ T cell ratio in patients with active SLE. This was especially true in patients with severe lupus nephritis^{6, 7}. The present study showed, an increment in CD8⁺ T cell numbers and a decline in CD4⁺ T cells, with a consequent reduction in CD4⁺/CD8⁺ T cell ratio in patients with SLE (Table 3). Similar result has been reported in SLE patients^{19,20}. McInerney *et al*¹⁵ showed that 45% of the SLE patient population had markedly depressed CD4 T cell levels and significantly low CD4/CD8 T cell ratios while Matsushita *et al*²⁰ showed an increase in CD8⁺ T cell population and decline in CD4⁺ T cell numbers in patients with SLE. Patients with decreased CD4⁺/CD8⁺ T cells ratio, had arthritis, rash and increased serological

activation. Six of the patients with diminished CD4⁺/CD8⁺ T cell ratio had involvement of kidney (nephritis). Previous studies by Bakke *et al*⁵, Smolen *et al*⁶ and McInerney *et al*⁷ had similar results.

The CD8⁺ T cells were positively correlated with SLEDAI score and negatively correlated with C4 in the patients with SLE. An inverse correlation was obtained between CD4⁺/CD8⁺ T cell ratio and SLEDAI score in patients with SLE. Additionally, CD4⁺/CD8⁺ T cells ratio was also inversely correlated with ESR in SLE patients. No such correlations were found in healthy controls (data not shown). These results suggest that an activation of T cells strongly occurs in CD8⁺ T cells rather than CD4⁺ T cells. The actual cause for lowering of CD4⁺/CD8⁺ T cell ratio in patients with SLE is still unclear. It is suggested that activated CD8⁺ T cells, and its derived interleukin (IL-16) promotes the activation and anergy of CD4⁺ T cells as well as their cell death, resulting in reduced CD4⁺/CD8⁺ T cell ratio in SLE patients²⁰. A negative correlation of CD4⁺/CD8⁺ T cells ratio and a positive correlation of CD8⁺ T cells with SLEDAI score and ESR suggest that activated CD8⁺ T cells are involved in the pathogenesis of SLE. Linker-Israeli *et al*.^{21,22} showed that the removal of HLA-DR+ CD8⁺ T cells and loss of the factors derived from CD8⁺ T cells lead to the normalization of IL-2 production in the CD4⁺ T cells in SLE patients. This also supports the hypothesis that the activated CD8⁺ T cell is involved in the pathogenesis of SLE. The levels of complement proteins C3 and C4 concentration have also been found to be low in 16 patients with SLE which is similar to the study of Hussain *et al*²³. A significant negative correlation was found between CD8⁺ T cells and C4 in the patients with SLE. This result suggests that activated CD8⁺ T cells may be involved in the alteration of complementary pathway in SLE patients.

The circulating lymphocyte has been markedly affected by corticosteroids in patients with SLE^{8,13}. The low serum levels of cortisol is thought to have a proinflammatory role²⁴ and proved to be a therapeutic alternative in SLE²⁵. Zietz *et al*²⁶ reported that serum cortisol is reduced relative to the degree of systemic inflammation in SLE and condition become severe when the disease persists over a long period of time (over weeks). In the present study, a significantly reduced level of cortisol was found in SLE patients compared to controls. This result is supported by the study of Straub *et al*. in SLE patients²⁷. Straub *et al* demonstrated that low level of serum cortisol is not due to renal clearance in patients with SLE. Inadequate production of cortisol may be due to inflammation induced reduction of adrenal steroidogenesis²⁸. Judd *et al*²⁸ demonstrated in adrenocortical cells that proinflammatory cytokines such as a tumour necrosis factor (TNF) inhibit important

enzyme steps of steroidogenesis. No significant correlation was found to exist between cortisol and T lymphocyte subsets in SLE patients and healthy controls. However, the correlation between cortisol and SLEDAI score was found to be negative, though not very significant in patients with SLE. This relation suggests that low level of cortisol may be involved in the disease activity in SLE.

Conclusion

The present study and those reported by other workers suggest that changes of T cell phenotype and CD8⁺ T cells activation may play an important role in the induction of autoimmunity in SLE. Altered T lymphocyte subsets and low cortisol affect clinical features of disease. Improvement of CD4⁺/CD8⁺ T cell ratio may indicate control of disease, while abnormal CD4⁺/CD8⁺ T cell ratio suggest deterioration or relapse in the clinical features of SLE.

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