

Analysis of changes in joint function and peripheral blood mononuclear cells in patients with systemic lupus erythematosus and intervention effects of different drugs

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Abstract. – **OBJECTIVE:** To investigate the therapeutic effect of drug therapy with cyclophosphamide and leflunomide on the joint function damage of patients with systemic lupus erythematosus (SLE) and its regulatory effects on expression levels of programmed death receptor 1, Notch signaling pathway genes and interferon-inducible protein 10 in peripheral blood mononuclear cells.

PATIENTS AND METHODS: A total of 60 patients with SLE were randomly divided into two groups. They were treated with cyclophosphamide and leflunomide, respectively. The number of painful joints, joint tenderness index, joint swelling index and erythrocyte sedimentation rate of patients before and after treatment were evaluated, and the peripheral blood was collected from patients in the two groups; the peripheral blood mononuclear cells were extracted.

RESULTS: We observed that the number of painful joints, joint tenderness index and joint swelling index in cyclophosphamide group were decreased after treatment ($p<0.05$), and the erythrocyte sedimentation rate was significantly decreased ($p<0.05$). The number of painful joints, joint tenderness index and joint swelling index in leflunomide group were decreased after treatment ($p<0.05$), and the erythrocyte sedimentation rate was significantly decreased ($p<0.05$). The comparisons of changes in joint functions and erythrocyte sedimentation rates between cyclophosphamide group and leflunomide group after drug therapy showed that the curative effect in leflunomide group was superior to that in cyclophosphamide group ($p<0.05$). The positive expression rate of peripheral blood mononuclear cell Notch1 in leflunomide group after treatment was significantly decreased, and the curative effect was superior to that in cyclophosphamide group ($p<0.05$). The comparisons of changes in programmed death receptor 1 of lymphocytes and interferon-inducible

protein 10 between cyclophosphamide group and leflunomide group after drug therapy showed that the curative effect in leflunomide group was superior to that in cyclophosphamide group ($p<0.05$). The comparison of positive expression rate of nuclear factor- κ B (NF- κ B) in peripheral blood mononuclear cells between the two groups after treatment showed that the curative effect in leflunomide group was superior to that in cyclophosphamide group ($p<0.05$). There were positive correlations of the expression level of programmed death receptor 1 of peripheral blood lymphocytes in SLE patients with double-stranded DNA (ds-DNA) and SLE disease activity index ($p<0.05$). There were positive correlations of the expression level of peripheral interferon-inducible protein 10 in SLE patients with ds-DNA and SLE disease activity index ($p<0.05$).

CONCLUSIONS: This study proved that both leflunomide and cyclophosphamide have therapeutic effects on the joint functions and immune dysfunction of peripheral blood mononuclear cells of SLE patients; however, and the effect of leflunomide is better. There are positive correlations of SLE disease activity index with the Notch signaling pathway genes, programmed death receptor 1 and interferon-inducible protein 10 in peripheral blood mononuclear cells, suggesting that these factors are related to the immune dysfunction of peripheral blood mononuclear cells.

Key Words:

Systemic lupus erythematosus, Joint, Mononuclear cells, Immunization, Leflunomide, Cyclophosphamide.

Introduction

Systemic lupus erythematosus (SLE) is a kind of autoimmune disease involving multiple organs

and systems with a variety of autoantibodies^{1,2}. A large number of pathogenic autoantibodies and immune complexes in the body cause the tissue damage, so its clinical manifestation is damage to various systems and organs, such as skin, joints, serosa, heart, kidney, central nervous system and blood system. The exact pathogenesis of the disease is still not clear, but most scholars believe that SLE is caused by the interactive effects of genetic factors and environmental factors, and a variety of cytokine networks and signal transduction pathways participate in the regulation in the whole process³⁻⁵. Notch signaling pathways are widely found on the surface of a variety of stem cells, lymphocytes and mononuclear cells, etc. Studies have shown that⁶⁻⁸ interferon-inducible protein 10 is expressed by monocytes and endothelial cells under the induction of interferon γ and tumor necrosis factor, which can chemotactically activate T cells and mononuclear cells and participate in the delayed type hypersensitivity. Programmed death receptor 1 is a kind of important immunosuppressive molecule, and the immune regulation with it as the target is of great significance in autoimmune diseases. This work aimed to investigate the therapeutic effect of drug therapy with cyclophosphamide and leflunomide on the joint function damage of SLE patients and its regulatory effects on expression levels of programmed death receptor 1, Notch signaling pathway genes and interferon-inducible protein 10 in peripheral blood mononuclear cells.

Patients and Methods

Patients

A total of 60 SLE patients treated in the Department of Rheumatology and Immunology of our hospital from July 2015 to June 2016 were selected. There were 14 males and 46 females aged 23-65 years. This study was approved by the Ethics Committee of the Affiliated Jiangyin Hospital of Southeast University. Signed written informed consents were obtained from all participants before the study.

Inclusion criteria: (1) patients meeting the above diagnostic criteria; (2) patients aged 20-65 years old in either gender; (3) SLE disease activity index (SLEDAI): 0-4 points: basically no activity; 5-9 points: mild activity; 10-14 points: moderate activity; 15 points and above: severe activity; (4) patients without receiving the drug therapy with immunosuppressive effect, such as corticosteroid

hormone, in the past 1 month; (5) patients who signed the informed consent. Exclusion criteria: (1) patients aged below 20 or above 65 years old; (2) patients complicated with various tumors; (3) patients complicated with other immune diseases; (4) patients complicated with inflammation or fever or who could not cooperate.

A total of 60 SLE patients were randomly divided into two groups and treated with cyclophosphamide and leflunomide, respectively. Specific treatment regimen: two groups of patients were treated with intravenous injection of methylprednisolone at a dose of $1.0 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ for 4 consecutive weeks, and the dosage was halved every 2 weeks. Cyclophosphamide group, besides hormone, was treated with intravenous injection of cyclophosphamide ($0.2 \text{ g}/\text{time}$) for 2-3 times a week; leflunomide group, besides hormone, was treated with leflunomide at a load dosage of 50 mg/day in the first 3 days and a maintenance dose of 20 mg/day after that.

Disease Evaluation and Detection Methods

The number of painful joints, joint tenderness index, joint swelling index and erythrocyte sedimentation rate of patients in the two groups before and after drug therapy were evaluated.

The peripheral blood was collected from patients in the two groups, and the peripheral blood mononuclear cells were extracted. The expressions of programmed death receptor 1 in peripheral blood mononuclear cells in patients before and after drug therapy were detected via the flow cytometry, and the expression levels of peripheral Notch signaling pathway genes (Notch1 and NF- κ B), interferon-inducible protein 10, and antinuclear antibodies and double-stranded DNA (ds-DNA) in serum were detected using the enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence technique.

Statistical Analysis

In this study, Statistical Product and Service Solutions 20.0 software (SPSS IBM, Armonk, NY, USA) was used for statistical analysis and processing of data. Measurement data were presented as mean \pm standard deviation ($\bar{x} \pm s$); enumeration data were presented as %; and χ^2 -test was used for intergroup comparison. Spearman correlation coefficient was used to describe the correlation of programmed death receptor 1 in peripheral blood mononuclear cells in SLE patients. $p < 0.05$ suggested that the difference was statistically significant.

Table I. Comparisons of general situations between the two groups.

| Group | n | Gender | | Average age (years old) | Course of disease (months) |
|------------------------|----|--------|--------|-------------------------|----------------------------|
| | | Male | Female | | |
| Cyclophosphamide group | 30 | 8 | 22 | 44.6±10.7 ^a | 34.4±15.6 ^a |
| Leflunomide group | 30 | 6 | 24 | 42.1±11.2 ^a | 32.7±16.1 ^a |
| χ^2 or <i>t</i> | | | 0.373 | 0.884 ^b | 0.415 ^b |
| <i>p</i> | | | 0.542 | 0.380 | 0.679 |

Note: a: Data were presented as ($\bar{x}\pm s$); b: *t*-value.

Results

Comparisons of General Situations Between the Two Groups

In cyclophosphamide group, there were 8 males and 22 females aged 23-65 years with an average of (44.6±10.7) years old; the course of disease was (6.5-66.4) months with an average of (34.4±15.6) months. In leflunomide group, there were 6 males and 24 females aged 22-65 years with an average of (42.1±11.2) years old, and the course of disease was (7.3-71.2) months with an average of (32.7±16.1) months. The comparisons of general situations between the two groups showed that there were no statistically significant differences in the gender ratio, age, and course of disease ($p>0.05$) (Table I).

Comparisons of Changes in joint Functions and Erythrocyte Sedimentation Rates of Patients in the Two Groups Before and After Drug Therapy

In this study, the changes in joint functions and erythrocyte sedimentation rates in cyclophosphamide group and leflunomide group were measured. The results were as follows: after treatment, the number of painful joints in cyclophosphamide group (n=30) was decreased from (12.8±4.7) on average to (4.6±2.4): $p<0.05$; the joint tenderness index was decreased from (12.9±4.8) on average to (4.3±1.6), and the difference was statistically significant ($p<0.05$); the joint swelling index was decreased from (7.5±3.3) on average to (3.1±1.6): $p<0.05$; the erythrocyte sedimentation rate was decreased from (71.8±15.7) mm/hr on average to (42.1±10.9) mm/hr: $p<0.05$. After treatment, the number of painful joints in leflunomide group was decreased from (13.1±4.9) on average to (2.1±1.4): $p<0.05$; the joint tenderness index was decreased from (12.5±5.8) on average to

(2.3±1.6): $p<0.05$; the joint swelling index was decreased from (7.2±4.3) on average to (1.1±0.6): $p<0.05$; the erythrocyte sedimentation rate was decreased from (75.2±16.3) mm/hr on average to (32.4± 8.5) mm/hr: $p<0.05$. The comparisons of changes in joint function and erythrocyte sedimentation rate between cyclophosphamide group and leflunomide group after drug therapy showed that the curative effect in leflunomide group was superior to that in cyclophosphamide group: $p<0.05$ (Table II).

Comparison of Notch1 Expression Levels in Peripheral Blood Mononuclear Cells

In this work, the expression levels of Notch1 in peripheral blood mononuclear cells in cyclophosphamide group and leflunomide group were detected. The detection results were as follows: in cyclophosphamide group (n=30), the positive expression rate of Notch1 in peripheral blood mononuclear cells after drug therapy was decreased from 80.0% (24/30) to 66.7% (20/30), and the difference was not statistically significant ($p>0.05$). In leflunomide group (n=30), the positive expression rate of Notch1 in peripheral blood mononuclear cells after drug therapy was decreased from 76.7% (23/30) to 40.0% (12/30): $p<0.05$. The comparison of positive expression rate of Notch1 in peripheral blood mononuclear cells after drug therapy between cyclophosphamide group and leflunomide group showed that the curative effect in leflunomide group was superior to that in cyclophosphamide group: $\chi^2=4.3439$, $p<0.05$ (Table III).

Changes in the Expressions of Programmed Death Receptor 1 and Interferon-Inducible Protein 10 in Peripheral Blood Mononuclear Cells

In this study, the expressions of programmed death receptor 1 and interferon-inducible pro-

Table II. Comparisons of changes in joint functions and erythrocyte sedimentation rates of patients in the two groups before and after drug therapy.

| Group | n | Number of painful joints | Joint tenderness index | Joint swelling index | Erythrocyte sedimentation rate (mm/hr) |
|------------------------|----|--------------------------|------------------------|-----------------------|--|
| Cyclophosphamide group | 30 | | | | |
| Before treatment | | 12.8±4.7 | 12.9±4.8 | 7.5±3.3 | 71.8±15.7 |
| After treatment | | 4.6±2.4 ^a | 4.3±1.6 ^a | 3.1±1.6 ^a | 42.1±10.9 ^a |
| Leflunomide group | 30 | | | | |
| Before treatment | | 13.1±4.9 | 12.5±5.8 | 7.2±4.3 | 75.2±16.3 |
| After treatment | | 2.1±1.4 ^{ab} | 2.3±1.6 ^{ab} | 1.1±0.6 ^{ab} | 32.4± 8.5 ^{ab} |

a: Compared with this group before treatment, $p < 0.05$; b: Comparison of results between the two groups after treatment, $p < 0.05$.

Table III. Notch1 expressions in peripheral blood mononuclear cells in patients in the two groups after drug therapy [n (%)].

| Group | n | Positive expression of Notch1 | |
|------------------------|----|-------------------------------|-----------------|
| | | Before treatment | After treatment |
| Cyclophosphamide group | 30 | 24 (80.0) | 20 (66.7) |
| Leflunomide group | 30 | 23 (76.7) | 12 (40.0) |
| χ^2 | | 0.0982 | 4.2857 |
| p | | 0.7540 | 0.0384 |

tein 10 in peripheral blood mononuclear cell in cyclophosphamide group and leflunomide group were measured. The results were as follows: in cyclophosphamide group ($n=30$) after drug therapy, the expression of programmed death receptor 1 of granulocytes was decreased from (22.5±14.9)% on average to (17.8±10.4)%, and the difference was not statistically significant ($p > 0.05$); the expression of programmed death receptor 1 of lymphocytes was decreased from (21.4±8.3)% on average to (12.1±4.8)%: $p < 0.05$; the expression of interferon-inducible protein 10 was decreased from (307.1±100.3) kU/L on average to (237.1±52.6) kU/L: $p < 0.05$. In leflunomide group ($n=30$) after drug therapy, the expression of programmed death receptor 1 of granulocytes was decreased from (23.7±14.5)% on average to (16.1±9.4)%: $p > 0.05$; the expression of programmed death receptor 1 of lymphocytes was decreased from (22.9±8.5)% to (9.3±3.2)%: $p < 0.05$; the expression of interferon-inducible protein 10 was decreased from (300.5±94.7) kU/L to (189.4±50.2) kU/L: $p < 0.05$. The comparisons of changes in programmed death receptor 1 of lymphocytes and interferon-inducible protein 10 after drug therapy between cyclophosphamide group and

leflunomide group showed that the curative effect in leflunomide group was superior to that in cyclophosphamide group, and the difference was statistically significant ($p < 0.05$). The results are shown in Table IV.

Changes in Expression of NF- κ B in Peripheral Blood Mononuclear Cells

In this study, the expression levels of NF- κ B in peripheral blood mononuclear cells in cyclophosphamide group and leflunomide group were detected. The detection results were as follows: in cyclophosphamide group ($n=30$), the positive expression rate of NF- κ B in peripheral blood mononuclear cells after drug therapy was decreased from 76.7% (23/30) to 56.7% (17/30), and the difference was not statistically significant ($p > 0.05$). In leflunomide group ($n=30$), the positive expression rate of NF- κ B in peripheral blood mononuclear cells after drug therapy was decreased from 70.0% (21/30) to 30.0% (9/30): $p < 0.05$. The comparison of positive expression rate of NF- κ B after drug therapy between cyclophosphamide group and leflunomide group showed that the curative effect in leflunomide group was superior to that in cyclophosphamide group $\chi^2=4.3439$, $p < 0.05$. The results are shown in Table V.

Table V. Changes in expression of NF-κB in peripheral blood mononuclear cells in the two groups [n (%)].

| Group | n | Positive expression of NF-κB | |
|------------------------|----|------------------------------|-----------------|
| | | Before treatment | After treatment |
| Cyclophosphamide group | 30 | 23 (76.7) | 17 (56.7) |
| Leflunomide group | 30 | 21 (70.0) | 9 (30.0) |
| χ^2 | | 0.3409 | 4.3439 |
| p | | 0.5593 | 0.0371 |

Table VI. Relevant research on the programmed death receptor 1 in peripheral blood mononuclear cells of SLE patients.

| Item | Programmed death receptor 1 of granulocytes | Programmed death receptor 1 of lymphocytes | Interferon-inducible protein 10 |
|------------------------|---|--|---------------------------------|
| ds-DNA | -0.057 | 0.411 ^a | 0.582 ^a |
| SLEDAI | -0.066 | 0.425 ^a | 0.423 ^a |
| Antinuclear antibodies | -0.092 | 0.086 | 0.083 |

a: $p < 0.05$.

Relevant Research on the Programmed Death Receptor 1 in Peripheral blood Mononuclear Cells of SLE Patients

The expression level of the programmed death receptor 1 in peripheral blood lymphocytes in SLE patients was positively correlated with ds-DNA, and the Spearman rank correlation coefficient r_s was 0.411 ($p < 0.05$). The expression level of the programmed death receptor 1 in peripheral blood lymphocytes in SLE patients was positively correlated with SLEDAI, and

the Spearman rank correlation coefficient r_s was 0.425 ($p < 0.05$). The expression level of interferon-inducible protein 10 in peripheral blood was positively correlated with ds-DNA, and the Spearman rank correlation coefficient r_s was 0.582 ($p < 0.05$). The expression level of interferon-inducible protein 10 in peripheral blood was positively correlated with SLEDAI, and the Spearman rank correlation coefficient r_s was 0.423 ($p < 0.05$). The results are shown in Table VI and Figures 1-4.

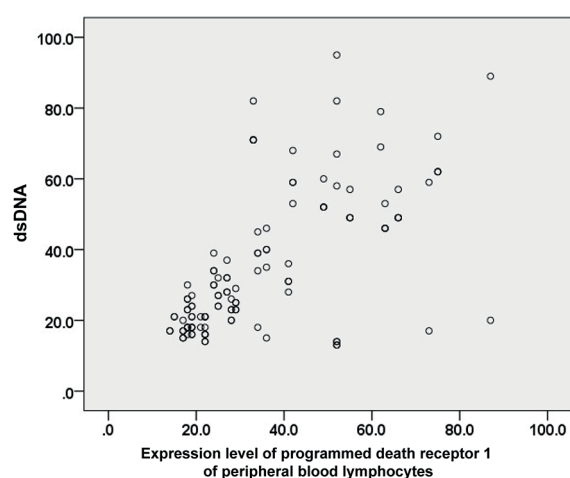


Figure 1. Scatter diagram of correlation between the expression level of programmed death receptor 1 of peripheral blood lymphocytes and ds-DNA.

Discussion

SLE is a typical multi-system autoimmune disease. It is characterized by the production of autoantibody, hyperactivity of T cells and B cells, immune complex deposition and multiple organ damage (lupus nephritis, coronary artery disease and osteoporosis)^{9,10}. SLE often occurs in women of childbearing age, and 20% patients suffer from it since the childhood. The pathogenesis of SLE is affected by multiple factors, including genetic, hormonal and environmental factors¹¹. A variety of genes has been found to be related to the pathogenesis of SLE. Peripheral blood mononuclear cells refer to cells with single nuclei in peripheral blood, including lymphocytes and monocytes, in which lymphocytes account for about 95%.

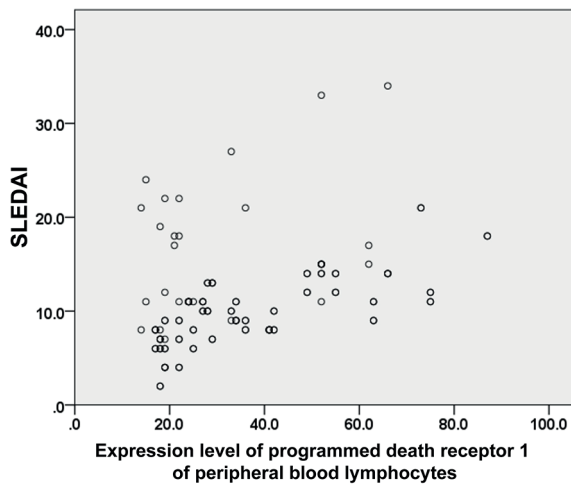


Figure 2. Scatter diagram of correlation between the expression level of programmed death receptor 1 of peripheral blood lymphocytes and SLEDAI.

Programmed death-1 (PD-1) is located in the chromosome 2q37 region. The latest study lists PD-1 as an important factor of SLE susceptibility. PD-1 molecules with tyrosine inhibitory motifs are immunosuppressive receptors of CD28/B7 family. In peripheral blood, CD4⁺/CD8⁺ T cells, NKT cells, B cells and mononuclear cells are activated to express PD-1¹². PD-1 regulates the immune tolerance of T cells and B cells by inducing the activation of cell death or autoimmune defense response¹³⁻¹⁵. And mice lacking PD-1 may suffer from SLE-like diseases¹⁶. PD-1 belongs to the co-signal molecule, in the SLE, which is abnormally expressed in SLE, rheumatoid arthritis, ankylosing spondylitis and other autoimmune diseases and gastric cancer, colon cancer and other malignant tumors. PD-1/PD-L pathways include the PD-1 receptor and its PD-L1 and PD-L2 ligands. PD-1 receptors are expressed in the activated T cells and B cells as well as bone marrow cells. PD-1 is widely expressed in T cells compared to the specific expression of other members of CD28 family. And its expression is much more extensive in PD-L than PD-L2. PD-L1 and PD-1 are expressed in CD4⁺ and CD25⁺ T cells, but it is not clear whether they can affect the functions of these regulatory T cells⁵.

Leflunomide reduces the proliferation of T cells and B cells by inhibiting dihydroorotate dehydrogenase (DHODH), leading to the decline in DNA and RNA synthesis and cell proliferation¹⁷. In lupus mouse models, leflunomide reversed the inhibition of T cell responses in healthy mice,

suggesting that leflunomide has the potential to treat SLE¹⁸. The study also found that leflunomide reduced the number of autoantibodies and immune complex deposits on the glomeruli¹⁹. In the prospective multicenter observational study²⁰, it was proved by biopsy that after patients with proliferative LN were treated with leflunomide or cyclophosphamide accompanied by prednisone, the kidney parameters and SLEDAI were significantly improved. Serum creatinine was decreased in both groups.

We observed that changes in programmed death-1 and interferon-inducible protein 10 in leflunomide group after drug therapy were superior to those in cyclophosphamide group ($p < 0.05$). The comparison of positive expression rate of NF- κ B in peripheral blood mononuclear cells between the two groups after treatment showed that the curative effect in leflunomide group was superior to that in cyclophosphamide group ($p < 0.05$). There were positive correlations of the expression level of programmed death receptor 1 of peripheral blood lymphocytes in SLE patients with ds-DNA and SLEDAI ($p < 0.05$). There were positive correlations of the expression level of peripheral interferon-inducible protein 10 in SLE patients with ds-DNA and SLEDAI ($p < 0.05$).

Conclusions

We showed that both leflunomide and cyclophosphamide have therapeutic effects on the joint functions and immune dysfunction of

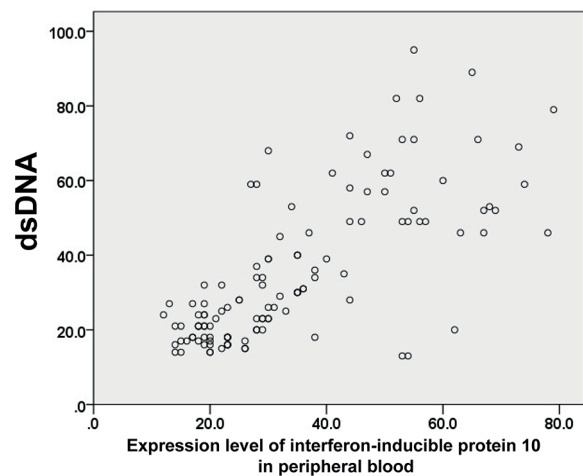


Figure 3. Scatter diagram of correlation between the expression level of interferon-inducible protein 10 in peripheral blood and ds-DNA.

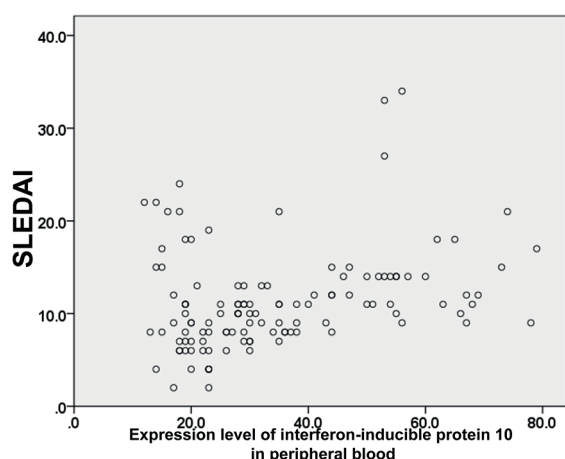


Figure 4. Scatter diagram of correlation between the expression level of interferon-inducible protein 10 in peripheral blood and SLEDAI.

peripheral blood mononuclear cells of SLE patients, and the effect of leflunomide is better. There are positive correlations of SLEDAI with the Notch signaling pathway genes, programmed death receptor 1 and interferon-inducible protein 10 in peripheral blood mononuclear cells, suggesting that these factors are related to the immune dysfunction of peripheral blood mononuclear cells.

Conflict of interest

The authors declare no conflicts of interest.

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