Decreased Interleukin 35 and CD4+EBI3+ T cells in Patients With Active Systemic Lupus Erythematosus

Han Ouyang, MS, Yong-bing Shi, MD, PhD, Zhi-chun Liu, MD, PhD, Zhi Wang, MD, PhD, Sheng Feng, MS, Shu-min Kong, MD, PhD and Ying Lu, MS

Abstract: *Background:* Interleukin 35 (IL-35) is likely to contribute to the development of autoimmune diseases, as the Epstein-Barr virus–induced gene protein 3 (EBI3) is the specificity subunit of IL-35. Nevertheless, until recently, no studies have evaluated its role in systemic lupus erythematosus (SLE) in humans. The objective of this study was to investigate the serum IL-35 level and the percentage of $CD4^+EBI3^+$ T cells in the peripheral blood of patients with SLE and explore the roles of double-positive T cells and IL-35 in the pathogenesis of SLE and the effects of glucocorticoid on these roles. *Methods:* Fifty-five hospitalized patients with SLE were recruited, and 20 volunteers were enrolled as healthy controls. Serum IL-35 levels were measured by enzyme-linked immunosorbent assay, and the percentage of $CD4^+EBI3^+$ T cells was analyzed by flow cytometry. *Results:* The serum IL-35 level and the percentage of $CD4^+EBI3^+$ T cells were significantly decreased in patients with active SLE compared with healthy controls and patients with inactive SLE. The serum IL-35 level and the percentage of $CD4^+EBI3^+$ T cells were negatively correlated with the SLE disease activity index. The percentages of $CD4^+EBI3^+$ T cells and serum IL-35 levels in 10 untreated patients with active SLE were increased at days l, 3, and 7 after the treatment with methylprednisolone ($0.8\ mg\cdot kg^{-1}\cdot d^{-1}$) compared with the percentages before the treatment. *Conclusions:* These results demonstrate that abnormalities in IL-35 and $CD4^+EBI3^+$ T cells may play important roles in the pathogenesis of SLE; the percentage of double-positive T cells and the level of IL-35 are parameters for the evaluation of SLE activity and severity.

Key Indexing Terms: Lupus erythematosus; Systemic; Interleukin 35; EBI3; Glucocorticoid. **[Am J Med Sci 2014;348(2):156–161.]**

Systemic lupus erythematosus (SLE) is an autoimmune disease of unknown origin that affects virtually all organ systems.[1] Previous research has shown that SLE is primarily caused by high levels of autoantibodies that are generated by enhanced apoptosis in conjunction with defective clearance of apoptotic cells and immune complex deposition.[2] Although the exact pathogenesis of SLE remains unclear, many studies have suggested that cytokines are involved in the pathogenesis of SLE.[3–6]

IL-35 is a heterodimeric cytokine consisting of Epstein-Barr virus–induced gene protein 3 (EBI3) and the p35 subunit of IL-12.[7] Both EbI3 and p35 knockout mice show overt autoimmunity and inflammatory disease, suggesting that the EBI3-p35 heterodimer may be an important immunomodulator.[8,9] A recent study found that EBI3 is the specificity subunit of IL-35.[10] A study by Yang et al[11] showed that decreased EBI3 induce IL-17 production and suppressive function of T-regulatory (Treg) cells, and Nieuwenhuis et al[12] further confirmed that EBI3 deficiency enhanced experimental colitis disease, showing that EBI3 plays anti-inflammatory role in experimental colitis. Additional evidence indicates that decreased EBI3 may contribute to the development of autoimmune diseases.

IL-35 is secreted by Treg cells and has been considered to be the characteristic factor of Treg cells.[10,13,14] Treg cells play an important role in controlling immune responses and the maintenance of peripheral immune tolerance, the decrease or a function of them will result in the development of SLE. Besides human Treg cells, placental trophoblast cells, activated dendritic cells and macrophages also express IL-35.[15–18] IL-35 is an important anti-inflammatory cytokine and can efficiently suppress T effector cells (Teff) cell activity and reduce the progression of inflammatory diseases and autoimmune diseases.[16–19]

However, whether the abnormal expression and secretion of IL-35 and EBI3 are present in patients with SLE remain unknown. Therefore, in this study, to better understand its interrelationship and immunopathologic roles in SLE, we investigated the IL-35 level and the percentage of $CD4^+EBI3^+$ T cells in patients with SLE and their relationships with disease manifestations and activity.

METHODS

Subjects

Peripheral blood samples were obtained from 55 patients with SLE (including 34 and 24 patients with active and inactive SLE) in the Department of Nephrology and Rheumatology at the Second Affiliated Hospital of Soochow University, excluding those with renal failure, patients with a current infection were also excluded, and all of them did not have any other autoimmune diseases. Patients with active SLE included recrudescent patients (N = 24) and incipient patients (N = 10); patients with incipient SLE did not receive any immunomodulating medication at the time of analysis. Samples from healthy controls (HCs) were collected from 20 volunteers, none of whom had any rheumatologic diseases. The clinical characteristics and patient demographics are shown in Table 1. The studies and informed consent forms and processes were approved by the participating institutions.

Systemic Lupus Erythematosus

The SLE diagnosis was established by the presence of 4 or more American College of Rheumatology (ACR) diagnostic criteria.[20] The SLE disease activity index (SLEDAI) questionnaire was completed for each patient with SLE. Active SLE was defined as an SLEDAI score >6; patients with SLEDAI score ≤ 6 were classified as inactive.[21]

Sample Processing and Cell Culture

Blood was collected into Vacutainer tubes containing ethylenediaminetetraacetic acid. After collecting peripheral

From the Departments of Nephrology (HOY, YBS, ZW, SF, SMK, YL) and Rheumatology (ZCL), Suzhou, China.

Submitted May 31, 2013; accepted in revised form October 4, 2013.

This study was partially supported by the National Natural Science Foundation of China (Grant No. 81200507).

The authors have no financial or other conflicts of interest to disclose.

Correspondence: Yong-bing Shi, MD, PhD, Department of Nephrology, the Second Affiliated Hospital of Soochow University, 1055 San-Xiang Road, Suzhou 215004, Jiangsu Province, China (E-mail: kdigo2013@gmail.com).

TABLE 1. Baseline characteristics and medication of patients with SLE

Categories	Patients with active SLE	Patients with inactive SLE	Healthy controls
General conditions			
Case number, N	34	21	20
Age, yr	34 ± 14	32 ± 15	33 ± 15
Male:female	2:32	2:19	3:17
SLEDAI, mean ± SD	11.8 ± 0.6	4.7 ± 0.3	0
Duration, mo	12.1 ± 7.4	14.6 ± 8.9	0
Medications, N			
None	10	0	0
Methylprednisolone	23	17	0
Tacrolimus	2	1	0
MMF	4	3	0
LEF	7	5	0
CTX	6	3	0
Hydroxychloroquine	1	1	0

CTX, cyclophosphamide; LEF, leflunomide; MMF, mycophenolate mofetil; SLE, systemic lupus erythematosus; SLEDAI, SLE disease activity index.

blood, serum was first collected and peripheral blood mononuclear cells were then isolated by centrifugation against a density gradient. The enriched cells were collected from the density medium:plasma interface. The sample was washed 2 times with phosphate-buffered saline. For intracellular staining, isolated peripheral blood mononuclear cells (1×10^6 in 1.5 mL of RPMI 1640) were stimulated for 6 hours with phorbol myristate acetate (PMA) (50 ng/mL) and ionomycin (500 ng/mL; both from Sigma-Aldrich, St. Louis, MO). At 4 hours before collection, Brefeldin A (GolgiPlug, 1 μL/mL; BD Pharmingen, Franklin Lakes, NJ) was added to the cell culture, along with PMA and ionomycin. The cells were fixed and permeabilized (Fix/Perm; eBioscience, San Diego, CA) according to the manufacturer's instructions and incubated at room temperature with formaldehyde.

Measurement of Serum IL-35 Levels

Venous blood samples were drawn into pyrogen-free blood collection tubes, immediately immersed in melting ice, and allowed to clot for 1 hour before centrifugation. All serum samples were stored at −80°C until use. All procedures were standardized. Serum IL-35 levels were measured with specific enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN). Each sample was tested in duplicate. The results were expressed as picograms per milliliter, and the detection limit of the assay was 0.5 pg/mL.

Flow Cytometric Analysis

The enriched cells were stained with anti-CD4 Alexa Fluor 488 antibody (eBioscience) to examine their purity. Intracellular staining was performed with anti-EBI3-PE (eBioscience). The percentage of cytokine-secreting $CD4^+EBI3^+$ T cells was determined with flow cytometry using a FACS Calibur instrument (Becton Dickinson, Franklin Lakes, NJ).

Clinical and Laboratory Parameters

Laboratory abnormalities were recorded, including leukopenia, thrombocytopenia, decreased erythrocyte sedimentation rate (ESR), the presence of anti-dsDNA, antinuclear and anti-Sm antibodies; IgA, IgM, IgG, serum levels of C3 and C4 and 24-hour urinary protein were also reviewed. The clinical manifestations of patients with SLE were defined as follows: renal involvement (persistent proteinuria ≥0.5 g/d, the presence of active cellular casts or biopsy evidence of lupus nephritis), arthritis (nonerosive arthritis affecting 2 or more peripheral joints) and nervous system disorder (psychosis, seizure, depression or peripheral neuropathy).

Effects of Glucocorticoids on Serum IL-35 Levels and the Percentage of $CD4^+EBI3^+$ T cells

To investigate the effect of glucocorticoids on serum IL-35 levels and the percentages of $CD4^+EBI3^+$ T cells *in vivo*, 10 patients with active SLE (9 women and 1 man; mean age 33 ± 11 years) who did not receive any immunomodulating medication were enrolled in our study. Blood samples were collected at baseline and at multiple time points (days l, 3 and 7) with post-treatment methylprednisolone ($0.8\ mg \cdot kg^{-1} \cdot d^{-1}$). Serum IL-35 levels were measured using a specific enzyme-linked immunosorbent assay.

TABLE 2. Comparison of serum IL-35 levels and percentages of $CD4^+EBI3^+$ T cells between different groups

	HCs (N = 20)	Active SLE (N = 34)	Inactive SLE (N = 21)	Incipient SLE (N = 10)	Recrudescent SLE (N = 24)
Serum IL-35 level, pg/mL	108.5 ± 13.6[a]	59.5 ± 8.8	93.4 ± 12.7[b]	60.5 ± 12.2	59.1 ± 15.1
$CD4^+EBI3^+$ T cells, %	1.78 ± 0.33[a]	1.07 ± 0.17	1.81 ± 0.29[a]	0.93 ± 0.23	1.04 ± 0.21

[a] Versus patients with active SLE ($P < 0.01$).
[b] Versus patients with active SLE ($P < 0.05$).
SLE, systemic lupus erythematosus.