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Regulatory T Cells and Their Molecular Markers in Peripheral Blood of the Patients with Systemic Lupus Erythematosus*

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Summary：$CD4^+CD25^+$ regulatory T cells (Tregs) and the expression of their molecular markers (GITR, Foxp3) in peripheral blood of the patients with systemic lupus erythematosus (SLE) were investigated in order to reveal the pathogenesis of SLE on the cellular and molecular levels. The level of Tregs in peripheral blood was detected by flow cytometry. The expression levels of GITR and Foxp3 mRNA in peripheral blood mononuclear cells (PBMCs) were assayed by reverse transcriptase-polymerase chain reaction (RT-PCR). The level of IL-6 in the plasma was measured by ELISA. Comparisons were made among 3 groups: the active SLE group, the inactive SLE group, and normal control group. The level of Tregs in the active SLE group and the inactive SLE group was significantly lower than in the normal control group (P<0.01). The level of Tregs in the active group was lower than in the inactive group with the difference being not significant (P>0.05). The level of Tregs in SLE patients was significantly negatively correlated with the disease active index in SLE (SLEDAI) (r=—0.81, P<0.01). The expression levels of GITR mRNA in PBMCs of the active SLE group and the inactive SLE group were significantly higher than in the normal control group (P<0.05), and those of Foxp3 mRNA in SLE patients of both active and inactive SLE groups were significantly lower than in the normal control group (P<0.05). There was no significant difference in the expression of GITR and Foxp3 mRNA between the active SLE group and inactive SLE group (P>0.05). The plasma levels of IL-6 in both the inactive SLE group and active SLE group were significantly higher than in the normal control group (P<0.01). The plasma level of IL-6 in the active SLE group was significantly increased as compared with that in the inactive SLE group (P<0.05), and the plasma level of IL-6 in SLE was significantly positively correlated with SLEDAI scores (r=0.58, P<0.01) and significantly negatively correlated with the ratio of $CD4^+CD25^+$ cells/$CD4^+$ cells (r=-0.389, P<0.05). It was concluded that the levels of Tregs and Foxp3 mRNA in peripheral blood of SLE patients were decreased and the levels of GITR mRNA and plasma IL-6 were increased. The Tregs and their molecular markers GITR, Foxp3 as well as the plasma IL-6 might play an important role in the pathogenesis of SLE.
Key words：systemic lupus erythematosus; regulatory T cells; Foxp3; GITR; interleukin-6

Systemic lupus erythematosus (SLE) is a common autoimmune disease characterized by abnormal T cells and B cells activation, cytokines disorders and the massive production of auto-antibodies. Studies[1, 2] have shown the existence of $CD4^+CD25^+$ T-regulatory cells (Tregs) abnormality in the peripheral blood of the SLE patients without clarifying the specific mechanisms. In this study, flow cytometry was use to detect the expression of $CD4^+CD25^+$ Tregs in the peripheral blood samples from the patients with SLE. The expression levels of GITR and Foxp3 mRNA in peripheral blood mononuclear cells (PBMCs) were assayed by reverse transcriptase- polymerase chain reaction (RT-PCR), and the levels of IL-6 in the plasma was measured by ELISA in order to investigate their roles in the pathogenesis of SLE.

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1 MATERIALS AND METHODS

1.1 Clinical Data of SLE Patients

The enrolled 38 patients with SLE were from the out-patient clinic and the ward of rheumatology of our hospital. All patients met the ACR (American College of Rheumatology) 1997 criteria for SLE with a female:male ratio of 35∶3. The mean age of the patients was 31.5±11.2 years old (range 15–59) with a mean disease duration of 3.3±1.4 years (range 2 months–19 years). According to the disease active index in SLE (SLEDAI) score[3], the patients were classified into two groups: the active SLE group (n=20) and the inactive SLE group (n=18). Sixteen sex- and age-matched healthy volunteers served as the normal control group.

1.2 Reagents and Equipments

FACSCalibur flow cytometer (Becton Dickinson Co. Ltd., USA), enzyme-labeling instrument (Dragon Co. Ltd., Germany), mouse anti-human CD4-FITC, mouse anti-human CD25-FITC, FITC-conjugated mouse IgG2b,

PE-conjugated mouse IgG1a and IL-6 ELISA kit (Jingmei Bio-tech Co. Ltd., China), oligodT20 primer, reverse transcriptase MMLV, Rnase inhibitor, dNTPs (TOYOBO Co. Ltd., Japan), Taq DNA polymerase (Tianwei Bio-tech Co. Ltd., China), TRIzol (Dongsheng Bio-tech Co. Ltd., China), lymphocyte separating medium (Haoyang Bio-tech Co. Ltd., China), GoodView dye (Saibaisheng Co. Ltd., China), hypothermic centrifugal machine (Hettich Co. Ltd., Germany), PCR amplifier (Biometra Co. Ltd., Germany), gel imaging system (BioRad Co. Ltd., USA), Quantity one image analysis software and nucleic acid/protein u.v analyzer (Beckman Co. Ltd., USA).

1.3 Methods

1.3.1 Detection of the $CD4^+CD25^+$ Tregs in Peripheral Blood Samples by Flow Cytometry Peripheral venous blood samples (5 mL) were drawn under sterile condition from normal controls and SLE patients with heparin anticoagulation. 100 μL whole blood sample was used for flow cytometry. The samples were centrifuged and 1 mL serum was taken out for the measurement of IL-6 levels. The rest of the samples were utilized to separate peripheral blood mononuclear cells (PBMCs) for the purpose of RT-PCR assay.

In 2 tubes with 50 μL whole blood sample each, one tube was added with anti-CD4-FITC (10 μL) and anti-CD-25-PE (10 μL), and the other was added with IgG 2b-FITC (10 μL) and IgG1a-PE (10 μL). After depositing the samples for 20 min at 4°C with avoidance of light, erythrocyte lysate (1 mL) was added. After another 6 min at room temperature with avoidance of light, the solution was centrifuged and the nucleated cells were obtained. The cells were eluted with PBS solution (2 mL) twice. After fixation with formalin (0.4 mL) for 20 min at 4°C, the cells were ready to be analyzed by flow cytometry. All the data were collected by FACSCalibur flow cytometer. 20000 cells per sample were assayed and the data were analyzed by CELL Quest Software.

1.3.2 Assay of the Expression Levels of GITR and Foxp3 mRNA in PBMCs by RT-PCR PBMCs were collected using lymphocyte separating medium. Total RNA was extracted by Trizol method and then reversely transcribed to cDNA. The GITR gene, FoxP3 gene sequence as well as internal reference β-actin gene sequence available from GeneBank was used for the purpose of primer design. Primers were designed using the Primer Premier5 software and synthesized by Shanghai Shenggong Bio-tech Co. Ltd. (China) (table 1).

Table 1 Sequences of the primers and size of the product for GITR, FoxP3 and β-actin genes

Gene symbol	Sequence of primer (5'-3')	Size of products (bp)
GITR	F: CATAGTGCTGTTCCGAGTGG R: GTGCGTTGTGGGTCTTGTT	295
Foxp3	F: CTCAAGCACTGCCAGGCGGACC R: CAGCGGATGAGCGTGGCGTAGG	401
β-actin	F: GGCATCGTGATGGACTCCG R: CTGGAAGGTGGACAGCGA	594

F: Forward; R: Reverse

PCR reaction conditions for GITR were as follows: pre-denaturation at 94°C for 5 min, denaturation at 94°C for 55 s, annealing at 54°C for 50 s, extension at 72°C for 1 min, 30 cycles, and final extension at 72°C for 10 min.

PCR reaction conditions for FOXP3 were almost the same as those of GITR except for the temperature and time duration for annealing were 60°C and 50 s respectively.

The amplified target gene was subsequently transferred to agarose gel (1%) electrophoresis with standard molecular weight Marker II as control. The results were observed under ultra-violet lamp and scanned into computers. Density scan was performed for target bands and internal reference band on a gel imaging/analysis system with the relative density values of GITR mRNA, Foxp3 mRNA (target products) and internal reference β-Actin mRNA output by automatic software. The ratio of target product/β-Actin was used as the index to evaluate the expression levels.

1.3.3 Measurement of the Plasma IL-6 Level by ELISA The plasma IL-6 levels in both SLE patients and normal controls were measured by ELISA according to the manufacturing instructions.

1.4 Statistical Analysis

With SPSS 12.0 statistical software, independent *t* test was used to detect the difference between two groups; Single factor analysis of variance was utilized to do the comparison among groups. Spearman correlation analysis was used to detect any correlation of our data.

2 RESULTS

2.1 $CD4^+CD25^+$ Tregs Levels in Peripheral Blood

The Tregs levels in the active SLE group and the inactive SLE group were both significantly lower than those in the normal control group ($P<0.01$). The ratio of $CD4^+CD25^+$ Tregs/$CD4^+$ cells in both SLE groups were significantly lower than that in the normal control group ($P<0.01$). The Tregs level in the active SLE group was lower than that in the inactive SLE group with the difference being not significant ($P>0.05$, table 2).

Table 2 The $CD4^+CD25^+$ Tregs/$CD4^+$ cell ratio (%) of peripheral blood in SLE patients and normal controls

Groups	*n*	$CD4^+CD25^+$ Tregs	$CD4^+$ cells	$CD4^+CD25^+$ Tregs/ $CD4^+$ cells
SLE	38	4.78±3.00*	30.52±9.22	14.22±7.44*
Active SLE	20	4.24±3.13*	27.78±7.87	12.92±7.09*
Inactive SLE	18	5.66±2.67*	33.74±9.84	15.75±7.75*
Normal control	16	16.25±3.19	29.08±6.04	53.90±4.70

*$P<0.01$ as compared with normal control group

2.2 The Expression Levels of GITR and Foxp3 mRNA in PBMCs

The expression of GITR mRNA in PBMCs of the SLE patients was higher than in the normal controls with the differences being significant between active SLE group and normal control group as well as between inactive SLE group and normal control group ($P<0.05$). However, the expression of Foxp3 mRNA in SLE patients was significantly lower than that in the normal

control group (P<0.05). The expression of GITR and Foxp3 mRNA had no significant difference between active and inactive SLE groups (P>0.05, table 3, fig. 1).

Table 3 The expression levels of GITR and Foxp3 mRNA in PBMCs of SLE patients and normal controls

Groups	n	GITR mRNA expression	Foxp3 mRNA expression
SLE patients	38	0.81±0.47*	0.26±0.15*
Active SLE	20	0.83±0.53*	0.24±0.19*
Inactive SLE	18	0.80±0.42*	0.27±0.11*
Normal control	16	0.48±0.20	0.37±0.18

*P<0.01 as compared with normal control group

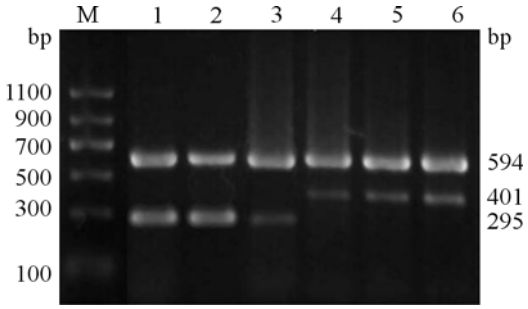

Fig. 1 The electrophoresis of RT-PCR products of GITR and Foxp3
1, 4: Active SLE group; 2, 5: Inactive SLE group; 3, 6: Normal control group; M: Marker; β-Actin: 594 bp; GITR: 295 bp; Foxp3: 401 bp

2.3 Plasma IL-6 Levels

The plasma levels of IL-6 in both the inactive SLE group (30.25±1.78 pg/mL) and active SLE group (46.85±3.13 pg/mL) were significantly higher than in normal control group (P<0.01), and those in active SLE group were significantly higher than in inactive SLE group (P<0.01).

2.4 The Correlation Analysis between Tregs, GITP, Foxp3, Plasma IL-6 and SLEDAI Scores

The Tregs level in SLE patients was significantly negatively correlated with SLEDAI scores (r=–0.81, P<0.01). The Tregs level was also significantly positively correlated with Foxp3 mRNA level (r=0.78, P<0.01). The plasma level of IL-6 in SLE patients was significantly positively correlated with SLEDAI scores (r=0.58, P<0.01) and significantly negatively correlated with the ratio of $CD4^+CD25^+$ cells/ $CD4^+$ cells (r=–0.389, P<0.05). There were no significant correlations between the plasma IL-6 level and Tregs level, or the molecular markers (GITR mRNA and Foxp3 mRNA) (P>0.05).

3 DISCUSSION

$CD4^+CD25^+$ Tregs, which play a vital role in the homeostasis and immunotolerance, belong to a specific T cell sub-group with unique immuno-regulatory function. The insufficiency or dysfunction of Tregs closely correlates with the development of autoimmune and inflammatory diseases as well as the interruption of the homeostasis[4-6]. Crispin *et al*[1] studied the Tregs of SLE patients and suggested a significant reduction of $CD4^+CD25^+$ Tregs in active SLE patients compared to healthy controls and inactive patients. La Cava *et al*[2] demonstrated that the production of autoantibodies by B cells in SLE patients could be interrupted via induction of Tregs and the Tregs could inhibit the production of dsDNA antibodies by B cells via cell contact inhibition induced by membrane bound TGF-β and GITR molecules. Our study showed that the Tregs level in SLE patients was significantly lower than normal controls, and there was significantly negative correlation between Tregs level and SLEDAI scores (r=–0.81, P<0.01), suggesting an important role of Tregs in the pathogenesis of SLE.

To further investigate the contribution of Tregs to the pathogenesis of SLE, the expression levels of GITR and Foxp3 mRNA were detected in PBMCs. GITR is a member of glucocorticoid-induced TNF receptor family which is mainly expressed in $CD4^+CD25^+$ Tregs. GITR specific antibody or removal of T cells with high GITR expression can induce organ-specific autoimmune diseases in normal mice, indicating the vital role of GITR in mediating the immunosuppressive function of Tregs[7]. Recent studies showed that GITR was a co-stimulatory molecules and the activation of GITR/GITRL pathway could stimulate the proliferation of effector T-lymphocytes and partially reverse the immunosuppressive function of $CD4^+CD25^+$ Tregs[8]. The co-stimulation of the T cells through GITR signaling pathway might function via the induction of MAPK and NF-kB activation[9]. Our results showed that the GITR mRNA level in SLE patients was significantly higher than in the normal control group (P<0.05), which suggested that the abnormally high level of GITR expression might be one of the crucial factors which precipitate the onset of SLE.

Transcription regulator FoxP3 is one of the specific markers of Tregs and mainly expressed in $CD4^+$ T-lymphocytes of the lymphoid tissues. It is almost absent in $CD8^+$ T cells and B cells. FoxP3, which is necessary for the function of Tregs, binds to DNA via its forkhead helix at the C-terminal and regulates the transcription of many genes. It is considered as an important 'switch' in the development of Tregs[10]. The results of this study revealed that the Foxp3 mRNA level in active SLE patients was significantly lower than in the normal control (P<0.05) and also lower than that in inactive SLE group despite a lack of significant difference (P>0.05). It was also found that Foxp3 mRNA level was positively correlated with $CD4^+CD25^+$ Tregs without a significant correlation with SLEDAI scores, which was consistent with those reported by Lee *et al*[11].

Studies have suggested an important role of IL-6 in the induction of the differentiation of Tregs[12, 13]. Under the independent action of TGF-β, activated incipient $CD4^+$ T cells differentiate into $CD4^+CD25^+$ Tregs. After the addition of IL-6, these $CD4^+$ T cells would not differentiate into $CD4^+CD25^+$ Tregs, but the anti-IL-6 antibody added during the induction phase would precipitate the differentiation towards $CD4^+CD25^+$ Tregs[14]. IL-6 inhibits both the quantity and the function of peripheral Tregs[15]. This study revealed that the plasma IL-6 level in SLE patients was significantly increased, and signifi-

cantly positively correlated with SLEDAI scores and significantly negatively correlated with the ratio of $CD4^+CD25^+$ cells/ $CD4^+$ cells. All these results suggested IL-6 might be an important influencing factor concerning the differentiation of $CD4^+CD25^+$ Tregs in the peripheral blood of SLE patients.

In general, $CD4^+CD25^+$ Tregs as well as their molecular markers (GITR, Foxp3) are the crucial factors influencing the pathogenesis of SLE. IL-6 also plays a pathogenetic role in SLE and has a negative correlation with $CD4^+CD25^+$ Tregs. Our study provided evidence for further investigation on the pathogenesis of SLE and possible targets for new therapies.

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