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C/EBP β mRNA expression is upregulated and positively correlated with the expression of *TNIP1*/*TNFAIP3* in peripheral blood mononuclear cells from patients with systemic lupus erythematosus

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Abstract. CCAAT/enhancer-binding protein β (C/EBP β) has important roles in numerous signaling pathways. The expression of the majority of regulators and target gene products of C/EBP β, including tumor necrosis factor α-induced protein 3 (*TNFAIP3*) and TNFAIP3-interacting protein 1 (*TNIP1*), are upregulated in patients with systemic lupus erythematosus (SLE). The aim of the present study was to investigate whether *C/EBP β* expression is associated with SLE pathogenesis and correlates with *TNIP1* and *TNFAIP3* expression. Quantitative reverse transcription-polymerase chain reaction analysis was used to assess the expression of *C/EBP β*, *TNIP1*, and *TNFAIP3* mRNA in peripheral blood mononuclear cells (PBMC) from 20 patients with SLE and 20 healthy controls. Spearman's rank test was used to determine the correlation between *C/EBP β* expression and SLE disease activity, and that between *C/EBP β* expression and *TNIP1*/*TNFAIP3* expression in PBMCs from patients with SLE. *C/EBP β* mRNA expression was markedly increased in patients with SLE compared with healthy controls. The expression of *C/EBP β* was positively correlated with the SLE disease activity index and negatively correlated with the serum level of complement components C3 and C4. In addition, *C/EBP β* mRNA expression was increased in PBMCs from SLE patients that were positive for antinuclear, anti-Smith and anti-nRNP antibodies, compared with the antibody negative SLE patients. Furthermore, the mRNA expression levels of *C/EBP β* in patients with SLE was positively correlated with *TNIP1* and *TNFAIP3* expression. The results of the current study suggest that the increased expression of *C/EBP β* in PBMCs and the interaction between *C/EBP β* and *TNIP1*/*TNFAIP3* may be involved in the pathogenesis of SLE.

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Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease involving multiple organs and is characterized by chronic inflammation with autoantibody production. The pathogenesis of SLE is complex and involves numerous immune cells and cytokines. B cells are known to have an essential role in autoantibody production in autoimmune disease (1). In addition, T cells have been recognized as a crucial component in the pathogenicity of SLE through their communication with B cells, which helps drive autoantibody production (2). In recent years, the importance of innate immunity in SLE pathogenesis has become increasingly evident, particularly regarding the role of Toll-like receptor (TLR) signaling pathways (3,4). Furthermore, the abnormal expression of certain cytokines and their receptors leads to immune hyperactivity in SLE; thus, cytokines such as interferon (IFN)-α, interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF) are considered to be therapeutic targets for SLE (5).

Previous studies have determined that several cytokine genes are regulated by CCAAT/enhancer-binding protein β [C/EBP β, also known as nuclear factor for IL 6, cysteine rich protein 2, IL-6DBP, liver-enriched activating protein (LAP), NF-M, α1 acid glycoprotein/enhancer-binding protein, or ApC/EBP), which is a member of the C/EBP family of transcription factors (6). Three C/EBP β isoforms have been identified: Liver-enriched activating protein* (LAP*, or C/EBP β1), liver-enriched activating protein (LAP, or C/EBP β2), and liver-enriched inhibitory protein (LIP, or C/EBP β3) (7). C/EBP β is expressed in immune cells, specifically in myelomonocytic cells and macrophages (8-10). C/EBP β has important roles in adipocyte differentiation, breast cancer and the initiation of liver regeneration (11-13). In myelomonocytic cells and monocytes/macrophages, C/EBP β is regulated by a variety of differentiation- and

proliferation-inducing agents, including cytokines and inflammatory substances (10), which mostly increase *C/EBP β* expression. In murine J774.2 macrophage-like cells, *C/EBP β* mRNA and protein expression was induced by TNF, IL-1 and IFN γ (14). Furthermore, the expression of *C/EBP β* mRNA increased markedly during differentiation to a macrophage lineage in M1 mouse myeloid leukemia cells, U937 human histiocytic leukemia cells, HL-60 promyelocytic leukemia cells and human peripheral monocytes (15). Vascular endothelial growth factor (VEGF) reduced the expression levels of the inhibitory isoform of C/EBP β (LIP) in the THP-1 cultured human monocytic leukemia cell line (16). C/EBP β contributes to the regulation of certain inflammatory cytokines, such as TNF, IL-1β, IL-6, IL-10 and IL-12, which have important roles in SLE pathogenesis (10,17-19), and the serum levels of IL-1, IL-6, TNF-α, IFN-γ and VEGF are significantly elevated in SLE patients (20-22). Furthermore, IL-10 shows a positive correlation with C-reactive protein and a negative correlation with complement component C3 in SLE (20).

Single-nucleotide polymorphisms of TNF-α-induced protein 3 (*TNFAIP3*) confer susceptibility to SLE (23). The TNFAIP3 protein, also known as A20, is a potent anti-inflammatory signaling molecule, and TNFAIP3 down-regulation and dysfunction are associated with inflammation in SLE (23). C/EBP β was reported to bind to the promoter of *TNFAIP3* following lipopolysaccharide (LPS) stimulation in RAW264.7 cells (24). However, the levels of C/EBP β and its target gene products were increased in mice with knocked-out TNFAIP3-interacting protein 1 (*TNIP1*), which also confers susceptibility to SLE (25,26).

Although there is a known association between SLE disease and the regulators and target gene products of C/EBP β, the expression level of *C/EBP β* in immune cells from SLE patients is unknown. Furthermore, the association between the expression of *C/EBP β* and the expression of *TNFAIP3* and *TNIP1* in SLE is unclear. Therefore, the present study compared the expression of *C/EBP β* mRNA in peripheral blood mononuclear cells (PBMCs) from patients with SLE and healthy controls, and analyzed the association of *C/EBP β* with *TNFAIP3* and *TNIP1* in order to elucidate the role of *C/EBP β* expression in the pathogenesis of SLE.

Materials and methods

Human subjects. A total of 20 patients with SLE who were diagnosed according to the criteria of the American College of Rheumatology (27) were enrolled in this study. In addition, 20 gender- and age-matched healthy controls without any rheumatological conditions were recruited. Individual disease activity was quantified using the SLE disease activity index (SLEDAI) score (28). All of the blood samples collected from the patients with SLE and healthy controls were used with informed consent and approval from the Ethics Committee of Southwestern Hospital (Chongqing, China). The study was performed in accordance with the 1964 Declaration of Helsinki and its later amendments.

For all of the patients with SLE, routine blood and urine tests were conducted using a hematology analyzer (Shenzhen Mindray Bio-Medical Electronics Co., Ltd., Shenzhen, China) and a urine sediment analyzer (Dirui Industrial Co., Ltd., Changchun, China). The serum levels of complement components C3 and C4 were detected using immunoturbidimetric assays (IMMAGE 800; Beckman Coulter, Inc., Fullerton, CA, USA) according to the manufacturer's instructions. Autoantibodies, including anti-nuclear (ANA), anti-dsDNA, anti-Smith (anti-Sm) and anti-nuclear ribonuclear protein (anti-nRNP) autoantibodies were detected using a EUROLINE test (EUROIMMUN AG, Luebeck, Germany) according to the manufacturer's instructions.

PBMC preparation and RNA extraction. PBMCs were separated by density gradient centrifugation, 1,200 rpm/min 15 min, from peripheral blood anticoagulated with sodium citrate (Tianjin Haoyang Co., Ltd., Tianjin, China). Total RNA was extracted from $5x10^5$ PBMCs using RNAiso Plus (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions, and then quantified by photometrical measurement.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). For each sample, 1 μg RNA was reverse transcribed to cDNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) with gDNA Eraser (Takara Biotechnology Co., Ltd.). The quantity of *C/EBP β*, *TNIP1* and *TNFAIP3* cDNA was then evaluated by qPCR. The expression levels of β-actin mRNA were also determined and served as an internal control. qPCR was performed using a SYBR Green I Real-Time PCR kit (Takara Biotechnology Co., Ltd.) on a Stratagene Mx3000p Real-Time PCR system (Agilent Technologies GmbH, Waldbronn, Germany). The PCR thermal cycling conditions were as follows: 95˚C for 2 min; 50 cycles of 95˚C for 30 sec, 62˚C for 30 sec and 72˚C for 20 sec; and 1 cycle of 95˚C for 1 min, 55˚C for 30 sec and 95˚C for 30 sec. The primers used were as follows: *C/EBP β* forward, 5'-CACAGCGACGACTGCAAGATCC-3' and reverse, 5'-CTTGAACAAGTTCCGCAGGGTG-3'; *TNIP1* forward, 5'-CAGAATGAGTTGCTGAAACA-3' and reverse, 5'-TCTCCTCATCTTTGAATGCT-3'; *TNFAIP3* forward, 5'-GTGTATTTTGGGACTCCAGA-3' and reverse, 5'-ACTTCTGGCAGTATCCTTCA-3'; and β-actin forward, 5'-AGCGAGCATCCCCCAAAGTT-3' and reverse, 5'-GGGCACGAAGGCTCATCATT-3' (Sangon Biotech Co., Ltd., Shanghai, China). A melting-curve analysis was performed to ensure specificity of the PCR products, and all of the PCR products were subjected to electrophoresis in an agarose gel to confirm the presence of a single band of the expected size. The expression levels of *C/EBP β*, *TNIP1*, and *TNFAIP3* were normalized to β-actin and determined using the comparative ($2^{-\Delta\Delta Ct}$) method. The experiment was repeated three times.

Statistical analysis. The relative expression of *C/EBP β*, *TNIP1*, and *TNFAIP3* mRNA for each sample is presented as a mean ± standard deviation. The difference in *C/EBP β* mRNA expression levels between the subject groups was analyzed with the Mann-Whitney test. Correlation analyses were conducted using Spearman's rank test. All analyses were performed using GraphPad Prism software, version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Clinical characteristics of patients with SLE.

Parameter	SLE patients (n=20)	Healthy controls (n=20)
Demographic characteristics		
Female, n (%)	18 (90)	18 (90)
Male, n (%)	2 (10)	2 (10)
Age, years	33.20 (18-54)	32.45 (20-52)
Clinical features		
Arthritis, n (%)	3 (15)	-
Vasculitis, n (%)	2 (10)	-
Rash, n (%)	4 (20)	-
SLEDAI, points	4.70 (0-12)	-
Laboratory measurements		
ANA, n (%)	20 (100)	-
Anti-dsDNA, n (%)	6 (30)	-
Anti-Sm, n (%)	6 (30)	-
Anti-nRNP, n (%)	10 (50)	
C3, g/l	0.66 (0.38-1.36)	-
C4, g/l	0.13 (0.04-0.42)	-
Thrombocytopenia, n (%)	4 (20)	-
Proteinuria, n (%)	3 (15)	-

Values represent the mean (range), except where indicated. Normal C3 range, 0.79-1.32 g/l; normal C4 range, 0.16-0.38 g/l. SLE, systemic lupus erythematosus; SLEDAI, SLE disease activity index; ANA, anti-nuclear antibody; anti-dsDNA, anti-dsDNA antibody; anti-Sm, anti-Smith antibody; anti-nRNP, anti-nuclear ribonuclear protein.

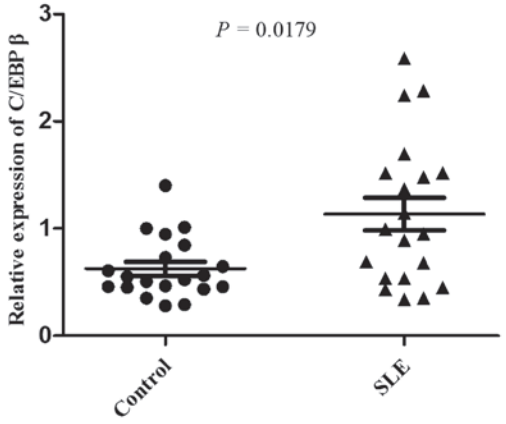

Figure 1. Relative expression of *C/EBP β* mRNA in PBMCs from patients with SLE. The *C/EBP β* mRNA expression levels in PBMCs from patients with SLE (n=20; circles) and healthy controls (n=20; triangles) were determined by reverse transcription-quantitative polymerase chain reaction. C/EBP β, CCAAT/enhancer-binding protein β; PBMC, peripheral blood mononuclear cells; SLE, systemic lupus erythematosus.

Results

Clinical characteristics of patients with SLE. The demographic characteristics, clinical manifestations, and laboratory measurements of the patients with SLE are presented in Table I. The demographic characteristics were not significantly different between the SLE group and the healthy control group. Arthritis, vasculitis and rash were present in 3, 2 and 4 of the 20 patients with SLE, respectively. The mean SLEDAI score was 4.70 (range, 0-12). Anti-ANA, anti-dsDNA, anti-Sm and anti-nRNP autoantibodies were detected in 20, 6, 7 and 10 of the 20 patients with SLE, respectively. Thrombocytopenia and proteinuria were detected in 4 and 3 of the 20 patients with SLE, respectively. The mean level of complement components C3 (0.66 g/l) and C4 (0.13 g/l) in patients with SLE was lower than the controls (C3 normal range, 0.79-1.32 g/l; C4 normal range, 0.16-0.38 g/l).

C/EBP β mRNA expression was elevated in PBMCs from patients with SLE. The expression of *C/EBP β* mRNA in PBMCs was examined in 20 patients with SLE and 20 gender- and age-matched healthy controls using RT-qPCR. The relative mRNA expression levels of *C/EBP β* were significantly higher in PBMCs from patients with SLE (1.1340), as compared with the PBMCs from the healthy controls (0.6256; P=0.0179; Fig. 1).

C/EBP β mRNA expression was elevated in SLE patients that were positive for anti-Sm or anti-nRNP antibodies, or had a high ANA titer. The relative mRNA expression levels of *C/EBP β* were higher in PBMCs from SLE patients with high ANA titer (≤1:160; 1.254), as compared with patients with low ANA titer (≥1:80; 0.4567; P=0.0262). In addition, the relative expression levels of *C/EBP β* mRNA were significantly higher in PBMCs from SLE patients positive for anti-Sm (1.657) or anti-nRNP (1.550) antibodies, as compared with patients negative for anti-Sm (0.8530; P=0.0324) or anti-nRNP (0.7185; P=0.0039) antibodies (Fig. 2).

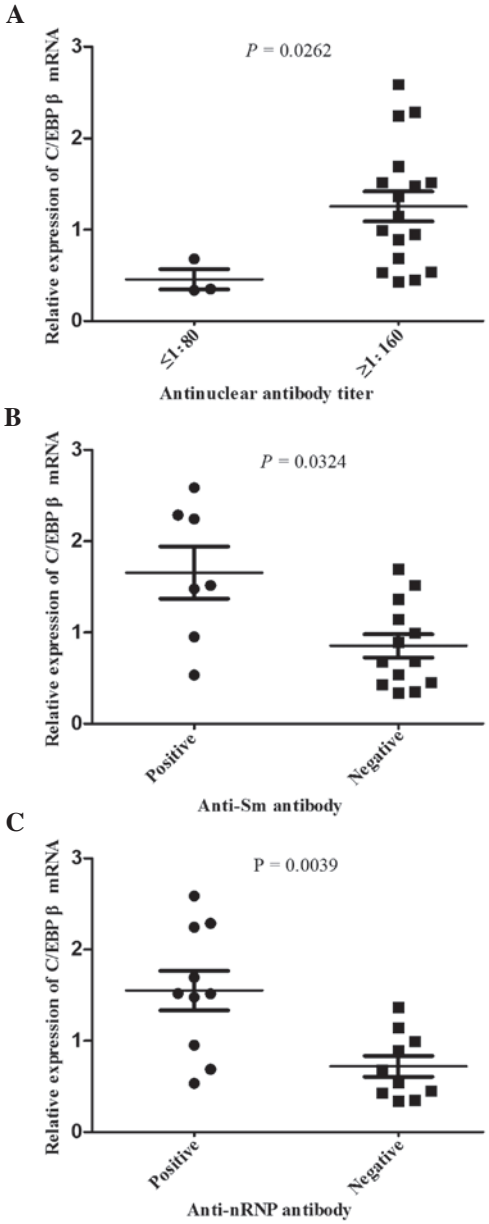

Figure 2. *C/EBP β* relative mRNA expression in PBMCs from SLE patients with various autoantibodies. (A) The difference in *C/EBP β* relative mRNA expression between SLE patients with high ANA titer (≤1:160; n=3) and low ANA (≥1:80; n=17) titer was analyzed with a Mann-Whitney test. (B) The difference in *C/EBP β* relative mRNA expression between patients positive (n=7) and negative (n=13) for anti-Sm antibody, or (C) between patients positive (n=10) and negative (n=10) for anti-nRNP antibody was analyzed with a Mann-Whitney test. SLE, systemic lupus erythematosus; *C/EBP β*, CCAAT/enhancer-binding protein β; PBMC, peripheral blood mononuclear cells; ANA, anti-nuclear antibody; anti-dsDNA, anti-dsDNA antibody; anti-Sm, anti-Smith antibody; anti-nRNP, anti-nuclear ribonuclear protein.

C/EBP β mRNA expression correlates with disease activity in patients with SLE. The association between *C/EBP β* mRNA expression and the demographic characteristics, clinical manifestations and laboratory parameters was analyzed. As shown in Fig. 3, *C/EBP β* expression was positively correlated with the SLEDAI score (r=0.5105; P=0.0215) in the patients with SLE. Furthermore, *C/EBP β* expression was negatively correlated with the serum levels of complement components C3 (r=-0.6341; P=0.0027) and C4 (r=-0.6904; P=0.0008). No statistically significant association was observed between

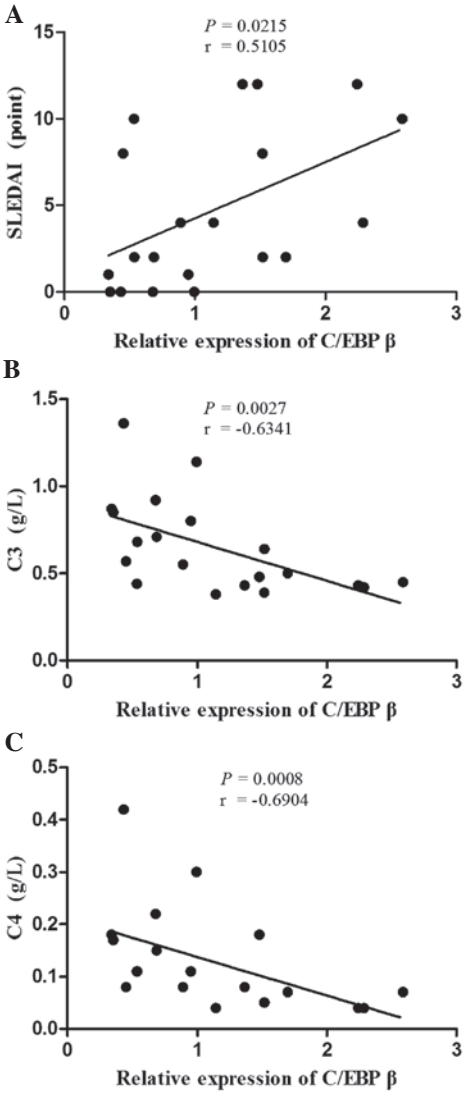

Figure 3. Correlation analysis of *C/EBP β* mRNA expression level with the clinical characteristics of patients with SLE. The association between *C/EBP β* mRNA expression levels, (A) SLEDAI and complement (B) C3 and (C) C4 was analyzed by Spearman's rank test in the patients with SLE (n=20). SLE, systemic lupus erythematosus; C/EBP β, CCAAT/enhancer-binding protein β; SLEDAI, SLE disease activity index.

C/EBP β mRNA expression levels and the other characteristics, clinical manifestations or laboratory parameters in patients with SLE.

C/EBP β mRNA expression is positively correlated with TNIP1 and TNFAIP3 expression in patients with SLE. The expression levels of *TNIP1* and *TNFAIP3* mRNA were determined in the PBMCs from the 20 patients with SLE using RT-qPCR, and the association between *C/EBP β* expression and *TNIP1* and *TNFAIP3* expression was examined. As shown in Fig. 4, *C/EBP β* expression was positively correlated with *TNIP1* expression (r=0.5865; P=0.0086) and *TNFAIP3* expression (r=0.4692; P=0.0369) in the patients with SLE.

Discussion

The results of the present study demonstrated the upregulation of *C/EBP β* expression in patients with SLE, specifically in

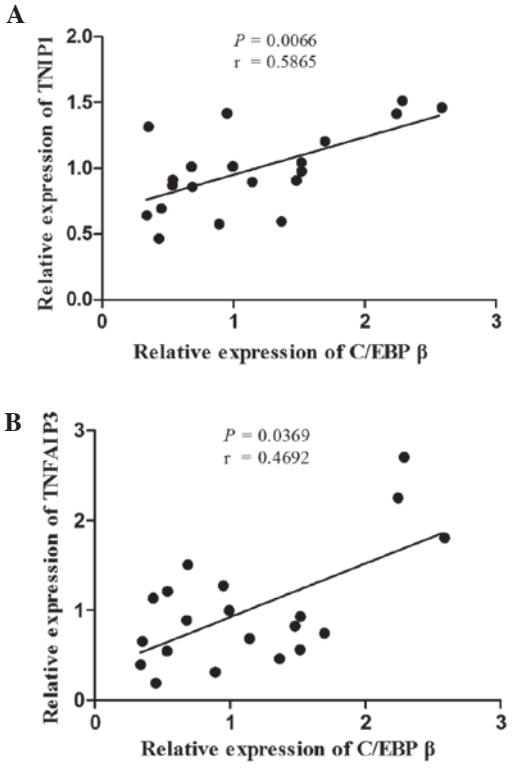

Figure 4. Correlation analysis of *C/EBP β* mRNA expression levels with *TNIP1* and *TNFAIP3* expression in patients with SLE. The association between *C/EBP β* mRNA expression levels and (A) TNIP1 and (B) TNFAIP3 expression was analyzed by Spearman's rank test in patients with SLE (n=20). SLE, systemic lupus erythematosus; C/EBP β, CCAAT/enhancer-binding protein β; TNFAIP3, tumor necrosis factor α-induced protein 3; TNIP1, TNFAIP3-interacting protein 1.

patients with anti-Sm and anti-nRNP antibodies, or high ANA titer, which is specificity with SLE or common in SLE (29). A positive correlation was observed between *C/EBP β* expression and disease activity (SLEDAI score), and a negative correlation between *C/EBP β* expression and the serum levels of complement components C3 and C4, which are considered to be part of the disease activity index. The data also demonstrated a positive correlation between *C/EBP β* and *TNFAIP3*/*TNIP1* expression in patients with SLE. These results suggest that increased expression of *C/EBP β* and interactions between *C/EBP β* and *TNIP1*/*TNFAIP3* may be involved in the pathogenesis of SLE.

SLE is a chronic inflammatory disease associated with the dysfunction of numerous immune cells and cytokines. The TLR signaling pathway has a crucial role as a trigger of inflammation in SLE (30). TLR activation (recognition of CpG-DNA by TLR9 or recognition of LPS by TLR4) leads to the recruitment of TLR signaling complexes involving TNF receptor-associated factor (TRAF)3 and TRAF6 via myeloid differentiation marker 88 (31-33). The signaling complexes activate C/EBP β through the mitogen-activated protein kinase (MAPK) p38 (34,35). As mentioned above, the majority of regulators and target gene products of C/EBP β are abnormally expressed in patients with SLE, and this aberrant expression contributes to the pathogenesis of SLE. Therefore, C/EBP β is thought to be a potential pivotal component of the inflammatory signaling pathway in SLE.

TNFAIP3 and *TNIP1* were identified as SLE susceptibility loci in a genome-wide association study (36). TNFAIP3 is an inhibitor of inflammation, and the deubiquitination of TNFAIP3 in T cells negatively regulates NF-κB, which is crucial for inflammatory and immune responses (37). Furthermore, NF-κB and p38 were observed to regulate the transcription of *TNFAIP3* via C/EBP β in activated macrophages (24). *TNFAIP3* mRNA expression was shown to be reduced in patients with SLE (38). *TNIP1* knockout mice showed an increased expression of *C/EBP β* without changes to NF-κB or MAPK, and developed an inflammatory disease with characteristics similar to human SLE (26). In the present study, the positive correlation between *C/EBP β* and *TNIP1*/*TNFAIP3* mRNA expression in patients with SLE suggests that the interaction between *C/EBP β* and *TNIP1*/*TNFAIP3* may be involved in the pathogenesis of SLE, and may indirectly demonstrate that C/EBP β could regulate TNFAIP3 mRNA expression. However, *TNFAIP3* and *TNIP1* mRNA expression was observed to be reduced in patients with SLE (38,39). Apparent discrepancies between the TNFAIP3 and TNIP1 mRNA expression reported in the current study, and those of earlier studies may be due to the dysfunction of TNFAIP3 and TNIP1 single nucleotide polymorphisms (SNP) in different regions (39,40). The National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov/) website database states that the SNP loci are in introns, exons, promoters, enhancers and other regions. Some polymorphisms may cause reduced expression or activity of anti-inflammatory A20, predisposing individuals to develop SLE (41). Therefore, in SLE the association between *TNFAIP3* and *TNIP1* SNP mutations and expression and the function of its production required further study. Future research we focus on investigating the association between *C/EBP β* and *TNIP1*/*TNFAIP3* in SLE.

In recent years, the development of targeted therapies for SLE has become an important research focus, due to the fact that the available first-line drugs show poor efficacy and lead to certain adverse reactions in patients with SLE. With the exception of belimumab, which is an anti-B-cell-activating factor (anti-BAFF) antibody, the efficacy and safety of the majority of immune cell-targeted therapies for SLE, such as rituximab, ofatumumab, ocrelizumab and veltuzumab (anti-CD20); epratuzumab (anti-CD22); and atacicept (anti-BAFF and a proliferation-inducing ligand) are not as good as expected (42). Therefore, a clear understanding of the pathogenesis of SLE may significantly improve the targeted therapy of SLE. An important step will be defining the function and regulation of *C/EBP β*, a pivotal component of the SLE inflammatory signaling pathway. Additional potential SLE therapeutic targets include *TNF* and *TNFAIP3*, which are closely associated with *C/EBP β* (43,44). However, as *C/EBP β* is also involved in adipocyte differentiation, breast cancer and liver regeneration (11-13), the potential side effects of changes in *C/EBP β* expression resulting from targeted therapy must be assessed.

In conclusion, the present study demonstrated that *C/EBP β* mRNA expression was upregulated and positively correlated with disease activity and the expression of *TNIP1* and *TNFAIP3* mRNA in patients with SLE. These results suggest that increased *C/EBP β* expression and an interaction

between *C/EBP β* and *TNIP1/TNFAIP3* may contribute to the inflammation pathogenesis of SLE. These data also indicate that the influence of *C/EBP β* should be considered during the development of targeted therapies for SLE.

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