

Original Article

The effects of Langchuangding on the Th17 cell-related transcription factors BATF and ROR γ t in MRL₁pr mice

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Abstract: Th17 cells participate in the occurrence and development of systemic lupus erythematosus (SLE). It was reported that Langchuangding (LCD) could be used in the treatment of SLE. However, the therapeutic effect of LCD on SLE was not reported. MRL₁pr mice were divided into 3 groups, a model group with normal feeding, and prednisone and LCD groups treated with 0.5 ml prednisone or LCD administered through gavage for 70 days. 24 h urinary protein, serum IgG, and anti-dsDNA antibody were measured. Flow cytometry was used to measure the distribution of Th17 cell subsets. ELISA was applied to test the secretions of interleukin-17 (IL-17) and IL-21 in the Th17 cells. The effect of As₂O₃ on the Th17 transcription factors BATF and ROR γ t in PBMCs was assessed using real-time PCR. The prednisone and LCD groups showed significantly elevated 24-hour urinary protein and serum immunoglobulin (IgG) compared with the control group ($P < 0.05$) without differences between the prednisone group and the LCD group ($P > 0.05$). Anti-dsDNA expression, the proportion of Th17 cell subsets, IL-17 and IL-21 levels, and BATF and ROR γ t expressions were significantly decreased in the treatment groups compared to control group. They were more significant in the LCD group compared with the prednisone group ($P < 0.05$). LCD can inhibit Th17 cell-associated transcription factor expression in MRL₁pr mice, which in turn restrains Th17 cell production and the secretion of cytokines and improves the immune pathological process of SLE.

Keywords: LCD, SLE, MRL₁pr mice, Th17 cells, BATF, ROR γ t

Introduction

Systemic lupus erythematosus (SLE) is characterized by inflammatory connective tissue lesions [1, 2]. SLE is more common in young women with an increasing trend due to lifestyle changes and environmental pollution [3]. The incidence of SLE is occult, which is mainly from the skin and mucous membranes. However, mild symptoms can slowly or rapidly change to severe ones, involving the heart, kidneys, and respiratory system, and SLE may cause death due to severe multiple organ damage [4, 5].

The pathogenesis of SLE is related to increased B cells, decreased T lymphocytes, and a large number of autoantibodies [6]. Due to the production of a large number of pathogenic autoantibodies and immune complexes, much damage is caused. In turn, a large number of autoantibodies are generated to form an immune complex to damaged tissues and organs [7, 8].

Immunity, physical factors, genetic and endocrine factors, and other abnormalities are also common predisposing factors for SLE [9]. In the study of autoimmune diseases, Th17 cell subsets play a role in several autoimmune diseases, such as allergic rhinitis, multiple sclerosis, and rheumatoid arthritis [10-12]. Autoimmunity cannot be simply explained by the Th1/Th2 cell paradigm. IL-17 produced by Th17 cells might be involved in the pathogenesis of autoimmune phenomena [13, 14]. Some studies reported that Th17 cells can participate in the occurrence and development of SLE [15, 16] and increased circulating Th17 cells were found in active SLE patients and inactive SLE patients compared with a control group [17]. In addition, circulating Th17 cells were positively correlated with the SLEDAI score [17]. The current treatment methods for SLE include glucocorticoids, immunosuppressive agents, and non-steroidal anti-inflammatory drugs. However, despite the continuous improvement and diversity of treat-

ment methods, the therapeutic effect and prognosis are still not satisfactory [18, 19]. At present, traditional Chinese medicine has obtained promising results in the treatment of SLE. Langchuangding (LCD) is an experience-developed traditional Chinese medicine that can be used to treat SLE [20, 21]. However, whether LCD is better than hormone therapy in SLE treatment and whether it has a regulatory effect on Th17 cell-related transcription factors remains unclear.

Materials and methods

Experimental animals

SLE model MRL₁pr female mice aged 6-8 months and weighing (25 ± 2) g were bought from the Experimental Animal Center of the Chinese Academy of Sciences and fed at the SPF Animal Experimental Center at a temperature of $21 \pm 1^\circ\text{C}$, a relative humidity of 50-70%, and a 12 h day/night cycle. This study was approved by the Ethics Committee of Jiujiang No. 1 People's Hospital (Jiujiang, Jiangxi, China).

Main materials and methods

LCD was purchased from the XXX Hospital Traditional Chinese Medicine Preparations Division. Prednisone was purchased from Zhejiang Xianyi Pharmaceutical Co., Ltd. A total protein detection kit (pyrogallol red-molybdenum complex dyeing method) was purchased from the Shanghai Mingbo Biotechnology Co., Ltd. IL-17 (catalogue number: EK0431) and IL-21 (catalogue number: EK0797). ELISA kits were purchased from Boster (Wuhan, China). PVDF membranes were purchased from Pall Life Sciences (Port Washington, NY, USA). Western blot related chemical reagents were from Beyotime (Nantong, China). ECL reagents were from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Rabbit anti-mouse ds-DNA monoclonal antibody and HRP-conjugated secondary antibody were from Cell Signaling Technology (Danvers, MA, USA). An RNA extraction kit and a reverse transcription kit were purchased from American ABI Corporation (Waltham, Massachusetts, USA). Mouse lymphocyte lysate was purchased from the Tianjin Haoyang Biological Products Co., Ltd (Tianjin, China). The Amp PCR System 2400 DNA Amplification System was purchased from PE Gene Corporation (Foster City, CA, USA). An IMark microplate reader was purchased from

BD. Clean bench was purchased from Suzhou Antai Instrument Co., Ltd (Suzhou, China). A Flow cytometer-EPICS XL was purchased from BECKMAN-COULTER (Brea, CA, USA). An ACL TOP-700 automatic analyzer was purchased from Beckmann (Brea, CA, USA).

Methods

Animal grouping and treatment: MRL₁pr mice were divided into a model group with normal feeding, and prednisone and LCD groups treated with 0.5 ml prednisone or LCD through gavage for 70 days [22]. The LCD suspension (0.025 g/ml) and the prednisone (0.5 mg/ml) were prepared in a 0.9% sodium chloride solution.

24 h urine protein and IgG measurement: 24 h urine protein and serum IgG were analyzed using a total protein detection kit (pyrogallol red-molybdenum complex dyeing method) and an automatic biochemical analyzer.

Anti-dsDNA detection: A RIPA lysis buffer was used to isolate protein from the tissue. After protein quantification, the sample was stored at -20°C . The isolated proteins were separated on 10% SDS-PAGE (separation range 20-80 KD), transferred to a PVDF membrane and blocked with 5% skim milk for 2 h. Next, goat anti-mouse dsDNA (1:1000) and β -actin (1:2000) monoclonal antibodies were added with overnight incubation at 4°C , followed by the addition of a secondary antibody in the dark for 30 min and subsequent exposure and development after the addition of a chemiluminescence reagent for 1 min. The experiment was repeated four times ($n=4$).

Sample collection: 6 ml of blood was extracted via the tail vein for peripheral blood mononuclear cell (PBMCs) isolation. The remaining blood sample was centrifuged at 3000 rpm for 15 min, and the serum was placed in an Ep tube at -20°C for ELISA detection.

Flow cytometry: The PBMCs were centrifuged and resuspended in 1.5 ml of flow-washing solution. Then, the cells were blocked with serum at 4°C for 15 min and incubated with 10 μl of CD4-FITC and IL17-PE at 4°C , in the dark for 30 min. Next, the cells were incubated with a 750 μl fixation mix at room temperature in the dark for 20 min, and then they were centrifuged at 400 g for 5 min. Next, 1.2 ml of a membrane-

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Table 1. Primer sequences

Gene	Forward 5'-3'	Reverse 5'-3'
GAPDH	ACTGCTCTAGGTAGTTG	TAACCACTGCCATGTGGT
BATF	TGACTCTGTAGTAGTT	GTAICTCATCGTAACCATG
ROR γ t	TATCGCCGTCATTAGCCT	TCACTAACTAACCATGTC

Table 2. The influence of LCD on the 24 h urine protein and serum IgG of MRL $_1$ pr mice

Group	24 h urine protein (mg)	IgG (g/L)
Control	3.62 \pm 0.57	14.28 \pm 1.21
Prednisone	0.97 \pm 0.59*	8.98 \pm 0.86*
LCD	0.89 \pm 0.35*	8.18 \pm 0.29*

*P < 0.05, compared with the control.

breaking agent diluted in deionized water was added followed by centrifugation at 400 g for 5 min. Finally, 300 μ l of flow washing solution was added for the flow cytometric analysis.

ELISA: The serum levels of IL-17 and IL-21 were measured using ELISA. A standard curve was established using 50 μ l serially diluted standards. The samples were added to a 96-well plate followed by the addition of 50 μ l of enzyme-labeled reagent for 30 min of incubation at 37°C. Then, 50 μ l of reagent A and reagent B were added for 10 min incubation at 37°C. Finally, 50 μ l stop solution was added and the photometric value (OD value) was determined using a microplate reader to calculate the corresponding concentration.

Real-time PCR: Trizol reagent was used to isolate the mRNA which was reversely transcribed to DNA according to the kit instructions. The primers are shown in **Table 1**. The PCR reaction conditions: 35 cycles of 92°C for 30 s, 58°C for 45 s, and 72°C for 35 s. For the data collection, GAPDH was used as an internal control. The expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot: A RIPA lysis buffer was used to isolate the total protein which was quantified using a BCA assay. The isolated protein was separated on 12% SDS-PAGE, transferred to a PVDF membrane, blocked with 5% milk followed by incubation with a ds-DNA antibody (1:2000 dilution) at 4°C overnight and subsequent incubation with HRP-conjugated secondary antibody at 37°C for 2 h followed by washing and the addition of an ECL reagent for exposing and developing.

Statistical analysis

The data were analyzed using SPSS 19.0 software. The measurement data are shown as the means \pm standard deviations, and the comparisons of difference were assessed using one-way ANOVA. P < 0.05 was considered to be a statistically significant difference.

Results

LCD reduces 24 h urine protein and serum IgG in MRL $_1$ pr mice

It was shown that the 24 h urinary protein and serum IgG in MRL $_1$ pr mice were significantly reduced after LCD or prednisone treatment compared with the control (P < 0.05) without a difference between the LCD and prednisone groups (P > 0.05) (**Table 2**).

LCD decreases dsDNA antibody expression in MRL $_1$ pr mice

Western blot revealed that the anti-dsDNA antibody expression in the MRL $_1$ pr mice was significantly decreased after the LCD or prednisone treatment (P < 0.05). The influence of LCD was significantly stronger than the prednisone (P < 0.05) (**Figure 1**).

LCD decreases Th17 cell subsets distribution in MRL $_1$ pr mice

After treatment with LCD or prednisone, the distribution ratio of Th17 cell subsets in MRL $_1$ pr mice was significantly declined (P < 0.05). The influence of LCD was significantly stronger than the prednisone (P < 0.05) (**Figure 2**).

LCD downregulates Th17 cell-associated transcription factor expression in MRL $_1$ pr mice

After treatment with LCD or prednisone, the expressions of BATF and ROR γ t mRNA were significantly downregulated (P < 0.05). The influence of LCD was significantly stronger than the prednisone (P < 0.05) (**Figure 3**).

LCD inhibits cytokines secretion by Th17 cells in MRL $_1$ pr mice

The effect of LCD on cytokine secreted by the Th17 cells was analyzed using ELISA. It was revealed that after treatment with LCD or prednisone, the IL-17 and IL-21 secretions from the MRL $_1$ pr mice were significantly reduced (P <

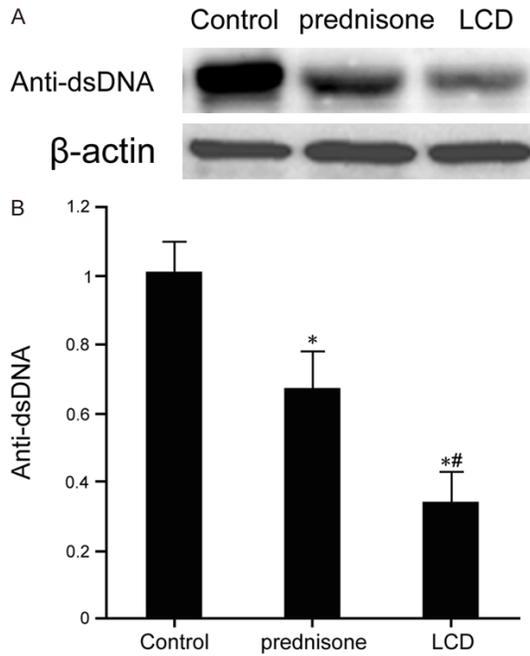


Figure 1. The effect of LCD on dsDNA antibody expression in MRL₁pr mice. A. Western blot analysis of anti-dsDNA expression; B. Anti-ds-DNA expression analysis. *P < 0.05, compared with the control. #P < 0.05, compared with prednisone.

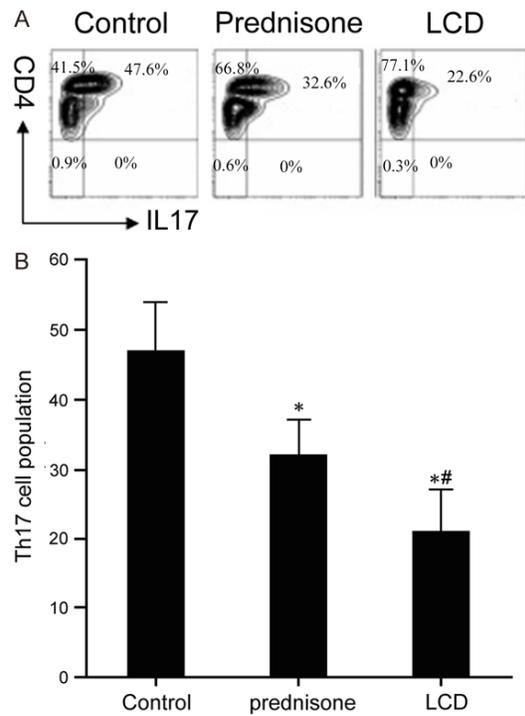


Figure 2. The impact of LCD on the Th17 cell subset distribution in MRL₁pr mice. A. Flow cytometry analysis of Th17 cell subsets distribution; B. Th17 cell subsets distribution analysis. *P < 0.05, compared with the control. #P < 0.05, compared with prednisone.

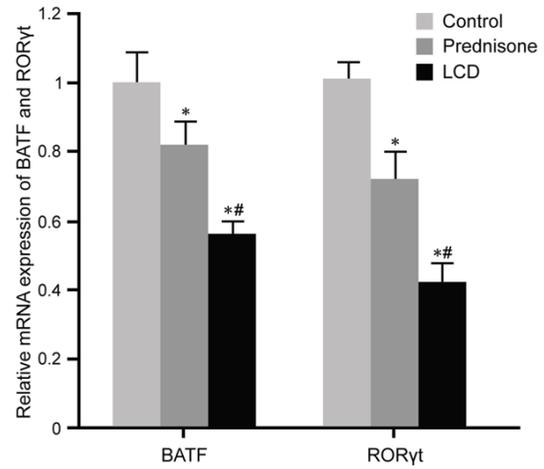


Figure 3. The influence of LCD on the Th17 cell-associated transcription factor in MRL₁pr mice. *P < 0.05, compared with the control. #P < 0.05, compared with prednisone.

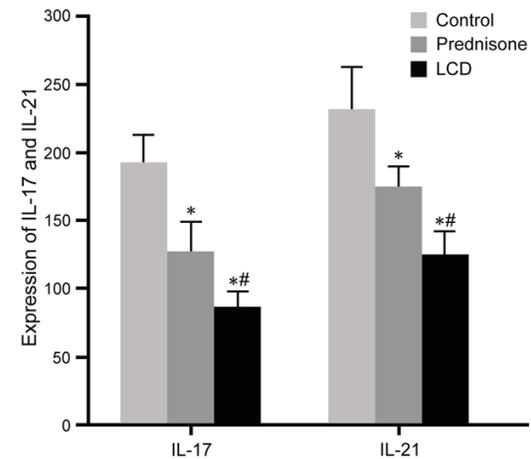


Figure 4. The effect of LCD on cytokines secreted by Th17 cells in MRL₁pr mice. *P < 0.05, compared with the control. #P < 0.05, compared with prednisone.

0.05). The influence of LCD was significantly stronger than prednisone (P < 0.05) (Figure 4).

Discussion

LCD is composed of traditional Chinese medicines, such as Radix Astragali, Lithospermum radix, Radix Rehmanniae, Leonurus, and Cimicifuga, which have been proved to be useful for the treatment of SLE [20]. At present, the Western medicine treatment of SLE mainly uses glucocorticoids, together with other drugs according to the severity. Thus, this study selected corticosteroid prednisone as a positive control to compare the efficacy of LCD and

the traditional treatment on SLE. In this study, MRL₁pr mice were first selected for the experiments, since MRL₁pr mice are considered an SLE animal model [23]. The results confirmed that 24 h urinary protein and serum IgG were significantly reduced in the LCD group and the prednisone group compared with the control, without any difference between the two treatment groups. However, the anti-dsDNA antibody expression in LCD group was significantly lower than it was in the prednisone group. Due to the presence of self-anti-nuclear antibodies such as anti-dsDNA antibody, the immune pathological injury of SLE is aggravated, which in turn attacks multiple organ systems [24]. Therefore, this result suggests that LCD affects the progression of SLE by regulating the immune system.

Furthermore, we conducted a study to explore the mechanism of LCD on SLE. CD4⁺ T lymphocytes participate in the development and progression of SLE. Early studies indicated that CD4⁺ T lymphocytes can differentiate into the helper T cells (Th cells) Th1 and Th2 as well as Th17 and Treg subpopulations [25]. After activation, Th17 cells secrete IL-17, IL-21, and other inflammatory factors to exert biological functions. IL-17 and IL-21 causes the release of colony stimulating factor, chemotaxis, adhesion molecules, recruits inflammatory cells such as macrophages and neutrophils, and results in a large number of infiltrating lymphocytes, leading to the occurrence of autoimmune diseases. Th17 cells involve in the inflammatory lesions of autoimmune diseases, which are part of the immune damage [26, 27]. Therefore, this study verified the effect of LCD on Th17 cell subsets in SLE and revealed that it could reduce the proportion of Th17 cell subsets in MRL₁pr mice and inhibit the secretion of related cytokines, which is superior to prednisone. Further analysis on Th17 cell transcription factors demonstrated that LCD can suppress the expressions of BATF and ROR γ t in MRL₁pr mice and is superior to the prednisone group. The specific transcription factor that differentiates into Th17 cells from the precursor cells is the activation of the expressions of BATF and ROR γ t, which can determine the differentiation and secretion of Th17 cells [28]. Thus, this study indicated that MRL₁pr mice inhibited Th17 cell differentiation and secretion by repressing the expression of relevant transcription factors in Th17 cells. This study pro-

vided the theoretical basis for the use of LCD for SLE treatment, but its related mechanism needs further investigation.

Conclusion

LCD can inhibit Th17 cell-associated transcription factor expression in MRL₁pr mice, which in turn restrains the Th17 cell production and secretion of cytokines, and improves the immune pathological process of SLE.

Disclosure of conflict of interest

None.

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