

Original Article

As₂O₃ inhibits the expression of Th17 cell-related transcription factors in MRL₁pr mice

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Abstract: Systemic lupus erythematosus (SLE) is common in rheumatism. Th17 cells can participate in the occurrence and development of SLE. Arsenic trioxide, also known as As₂O₃, is widely used in the treatment of leukemia and other malignancies. However, the therapeutic effect of As₂O₃ on SLE and Th17 cells has not been reported. MRL₁pr mice were randomly divided into 2 groups, including control group and As₂O₃ group. The mice were intraperitoneally injected with As₂O₃ at 0.4 mg/kg/d every other day for 55 days. Body weight, 24 h urinary protein, serum IgG, and anti-dsDNA antibody were measured. Flow cytometry was used to detect the distribution of Th17 cell subsets. ELISA was applied to test the secretion of interleukin-17 (IL-17) and IL-21 in Th17 cells. The effect of As₂O₃ on the Th17 transcription factor ROR γ t in PBMCs was detected by real-time PCR. In the As₂O₃ group, the expression of 24-hour urinary protein, serum immunoglobulin (IgG), and anti-dsDNA antibody was significantly decreased, while body weight was significantly elevated compared with the control group (P < 0.05). The proportion of Th17 cell subsets in the As₂O₃ group was significantly lower than that in the control group. The serum levels of IL-17 and IL-21 were significantly decreased, whereas the ROR γ t mRNA expression was reduced compared with the control group (P < 0.05). As₂O₃ can inhibit the expression of Th17 cell-associated transcription factor in MRL₁pr mice, which in turn restrains the production of Th17 cells and the secretion of cytokines, and improves the immune pathological process, indicating that As₂O₃ has a therapeutic effect on SLE.

Keywords: As₂O₃, SLE, MRL₁pr mice, Th17 cells, cytokine

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease with inflammatory connective tissue lesions [1, 2]. SLE is more common in women with an increasingly younger trend [3]. The incidence of SLE is obscure, originating mainly from the skin and mucous membranes. However, mild symptoms can be slowly or rapidly changed to more severe forms, involving the heart, kidneys, and respiratory system. It may cause death due to severe multiple organ damage [4, 5]. The pathogenesis of SLE is very complicated, which is related to immune, genetic, and endocrine abnormalities, physical factors, chemical factors, and mental stress. Under the influence of various factors, it leads to imbalance of the immune system [6, 7]. The current treatment methods for SLE contain immunosuppressive agents combined with glu-

cocorticoids, non-steroidal anti-inflammatory drugs, plasma exchange, and immunoglobulin shots [8]. However, despite the continuous improvement and diversity of treatment methods, the therapeutic effect and prognosis are still not satisfactory [9].

The occurrence of SLE is related to an abnormal immune system, which can lead to an increase in B cells, decrease in T lymphocytes, and a large number of autoantibodies, causing immune related organ damage [10]. In the study of autoimmune diseases, Th17 cell subsets play a role in various autoimmune diseases, such as allergic rhinitis, multiple sclerosis, and rheumatoid arthritis [11-13]. Some studies report that Th17 cells can participate in the occurrence and development of SLE [14]. At present, the pathogenesis of SLE is unclear and the therapeutic effect is poor. Therefore, it is urgent to find effective drugs for clinical treat-

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ment of SLE. As₂O₃ has made great progress in the treatment of leukemia and other malignancies [15, 16]. In addition, arsenic compounds have also been demonstrated to be able to inhibit certain immune functions [17, 18], indicating they might play an immune suppressive role in the immune response. However, the role and mechanism of As₂O₃ in SLE are rarely reported. Therefore, this study intends to investigate the therapeutic effect of As₂O₃ on SLE and Th17 cells.

Materials and methods

Experimental animals

SLE model MRL₁pr female mice aged 6-8 months, weighing (20±2) g were purchased from the Experimental Animal Center of the Chinese Academy of Sciences and fed at the SPF Animal Experimental Center. Housing conditions include maintained temperature at 21±1°C, relative humidity at 50-70%, and 12 h day/night cycle.

Main reagents and instruments

Pentobarbital sodium was purchased from Shanghai Zhaohui Pharmaceutical Co., Ltd. As₂O₃ was purchased from Sigma. IL-17 and IL-21 ELISA kits were purchased from R&D. Total protein test kit (pyrogallol red-molybdenum complex dyeing method) was purchased from Shanghai Mingbo Biotechnology Co., Ltd. PVDF membranes were purchased from Pall Life Sciences. Western blot related chemical reagents were purchased from Beyotime. ECL reagents were purchased from Amersham Biosciences. Rabbit anti-mouse ds-DNA monoclonal antibody, goat anti-rabbit BMPR Ia monoclonal antibody, and horseradish peroxidase (HRP) labeled IgG secondary antibody were purchased from Cell Signaling. RNA extraction kit and reverse transcription kit were purchased from American ABI Corporation. Mouse lymphocyte lysate was purchased from Tianjin Haoyang Biological Products Co., Ltd. Microsurgical instruments were purchased from Suzhou Medical Instrument Factory. The Amp PCR System 2400 DNA Amplification System was purchased from PE Gene Corporation. Imark microplate reader was purchased from BD. Clean bench was purchased from Suzhou Suzhou Antai Instrument Co., Ltd. Flow cytometer-EPICS XL was purchased from BECKMAN-COULTER. The

Sonic VCX130 Ultrasonic Cell Disrupter was purchased from Sonic. ACL TOP-700 automatic analyzer was purchased from Beckmann.

Methods

Animal grouping and treatment: MRL₁pr mice were randomly divided into 2 groups, including control group and As₂O₃ group. The mice were intraperitoneally injected with As₂O₃ at 0.4 mg/kg/d every other day for a total of 55 days [19]. Mice in the control group received intraperitoneal injections of the equal volume of vehicle.

Body weight, 24 h urine protein, and IgG measurement: After treatment, the body weight of each mouse was recorded. Twenty-four h urine protein and serum IgG were analyzed using total protein detection kit (pyrogallol red-molybdenum complex dyeing method) and automatic biochemical analyzer.

Western blot: The tissue was mixed with lysate on ice for 15-30 min and sonicated for 5 s × 4 times at 4°C. After being centrifuged at 10,000 × g for 15 min, the supernatant was moved to a new Eppendorf (Ep) tube. After protein quantification, the sample was stored at -20°C. The isolated proteins were electrophoresed using 10% SDS-PAGE (separation range 20-80 KD). The gel was transferred to PVDF membrane by semi-dry transfer method at 160 mA for 1.5 h. The non-specific background was removed by 5% skim milk at room temperature for 2 h. Next, the membrane was incubated in goat anti-mouse dsDNA (1:1,000) and β-actin (1:2,000) monoclonal antibodies at 4°C overnight. After being incubated in secondary antibody in the dark for 30 min, the membrane was imaged using chemiluminescence reagent for 1 min and analyzed by image processing system software and Quantity one software. The experiment was repeated four times (n=4).

Sample collection: Six ml of blood was extracted via the tail vein for peripheral blood mononuclear cells (PBMCs) isolation. The remaining blood sample was centrifuged at 3,000 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. After being centrifuged at 1,000 rpm for 5 min, the cells were blocked with serum at

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Table 1. Primer sequences

Gene	Forward 5'-3'	Reverse 5'-3'
GAPDH	ACTGCTCTAGGTAGTTG	TAACCAGTGCCATGTGGT
RORγt	TAT CGCCGTCATTAGCCT	TCACTAACTAACCATGTC

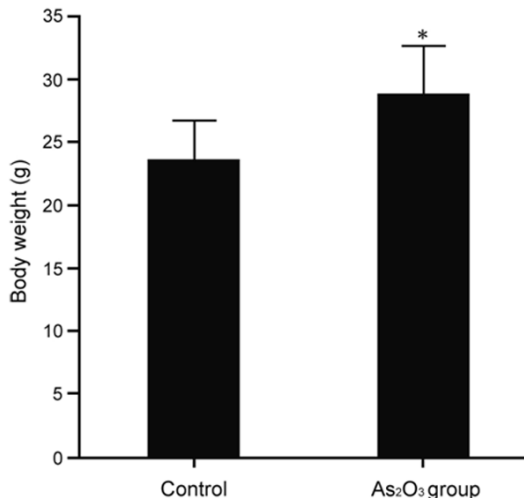


Figure 1. The impact of As₂O₃ on the body weight of MRL_1pr mice compared with control. *P < 0.05.

Table 2. The influence of As₂O₃ on the 24 h urine protein and serum IgG of MRL_1pr mice

Group	24 h urine protein (mg)	IgG (g/L)
Control	3.37±0.06	12.15±1.08
As ₂ O ₃	0.89±0.02*	8.23±0.91*

*P < 0.05, compared with control.

4°C for 15 min. After removing the supernatant, the cells were incubated in 10 µl of CD4-FITC and IL17-PE at 4°C, avoid light for 30 min. Next, the cells were incubated in 750 µl fixation mix at room temperature avoid of light for 20 min, and then centrifuged at 400 g for 5 min. Next, the cells were mixed with 1.2 ml membrane-breaking agent diluted in deionized water and centrifuged at 400 g for 5 min. Finally, the cells were mixed with 300 µl flow washing solution and tested by flow cytometry.

ELISA: The serum levels of Th17 cytokines IL-17 and IL-21 were measured by ELISA. Fifty µl serially diluted standards and samples were added to the 96-well plate. After being washed three times, 50 µl enzyme-labeled reagent was added to each well at 37°C for 30 min. Next, 50 µl reagent A and reagent B were added and incubated at 37°C for 10 min. Finally, 50 µl stop solution was added to each well and the

photometric value (OD value) was measured using a microplate reader to calculate the corresponding concentration.

Real-time PCR: Total mRNA was extracted using Trizol reagent and reverse transcribed to DNA according to the kit instructions. The primers were designed by Primer Premier 6.0 and synthesized by Invitrogen (**Table 1**). The PCR reaction was performed for 35 cycles at 92°C for 30 s, 58°C for 45 s, and 72°C for 35 s. For data collection, GAPDH was used as a reference. The relative expression level was calculated by 2^{-ΔCt} method.

Statistical analysis

All data analyses were performed on SPSS 19.0 software. The measurement data were presented as mean ± standard deviation and compared by one-way ANOVA. P < 0.05 was considered as a statistical difference.

Results

The impact of As₂O₃ on the body weight of MRL_1pr mice

It was found that body weight of mice after As₂O₃ treatment was significantly increased compared with the control group (P < 0.05) (**Figure 1**).

The influence of As₂O₃ on the 24 h urine protein and serum IgG of MRL_1pr mice

It was shown that the 24 h urinary protein and serum IgG levels in MRL_1pr mice were significantly reduced after As₂O₃ treatment compared with the control group (P < 0.05) (**Table 2**).

The effect of As₂O₃ on dsDNA antibody expression in MRL_1pr mice

Western blot was used to analyze the impact of As₂O₃ on dsDNA antibody expression. It was revealed that the anti-dsDNA antibody expression in MRL_1pr mice was significantly decreased after As₂O₃ treatment compared with the control group (P < 0.05) (**Figure 2**).

The impact of As₂O₃ on Th17 cell subset distribution in MRL_1pr mice

Flow cytometry was used to test the effect of As₂O₃ on Th17 cell subset distribution. After treatment with As₂O₃, the distribution ratio of Th17 cell subsets in MRL_1pr mice was significantly declined compared with the control group (P < 0.05) (**Figure 3**).

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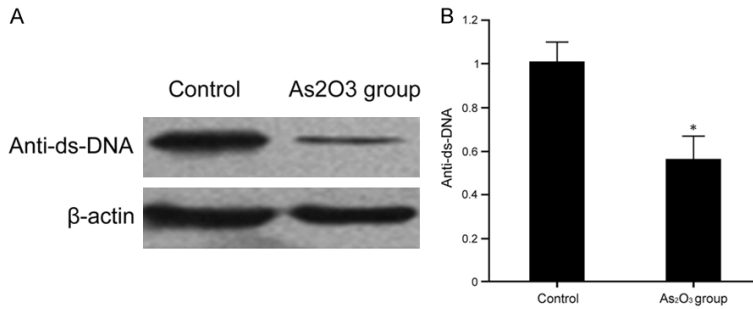


Figure 2. The effect of As₂O₃ on dsDNA antibody expression in MRL₁pr mice. A. Western blot analysis of anti-ds-DNA expression; B. Anti-ds-DNA expression analysis. *P < 0.05, compared with control.

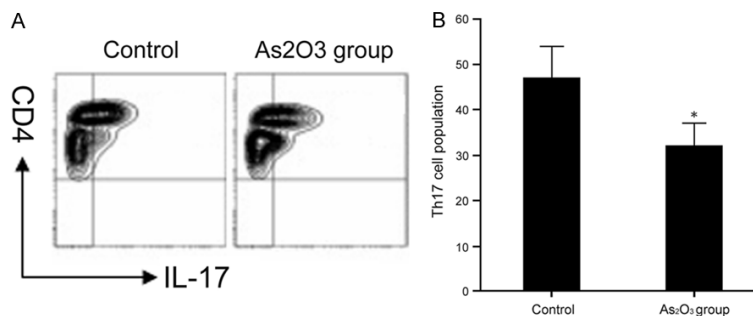


Figure 3. The impact of As₂O₃ on Th17 cell subsets distribution in MRL₁pr mice. A. Flow cytometry analysis of Th17 cell subsets distribution; B. Th17 cell subsets distribution analysis. *P < 0.05, compared with control.

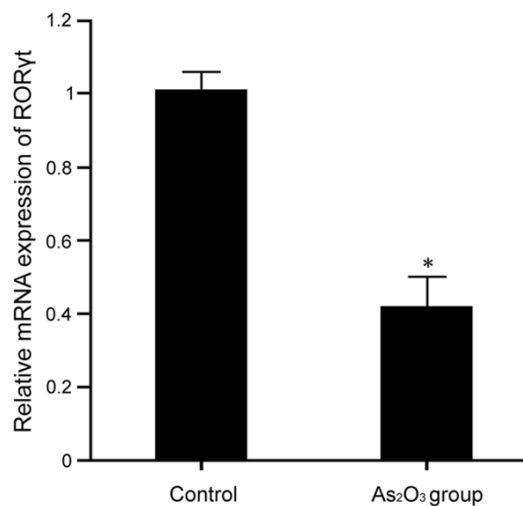


Figure 4. The influence of As₂O₃ on Th17 cell-associated transcription factor in MRL₁pr mice. *P < 0.05, compared with control.

The influence of As₂O₃ on Th17 cell-associated transcription factor in MRL₁pr mice

The effect of As₂O₃ on Th17 cell-associated transcription factors was analyzed by Re-

al-time PCR. It was observed that after treatment with As₂O₃, the expression of RORyt mRNA was significantly downregulated compared with the control group (P < 0.05) (Figure 4).

The effect of As₂O₃ on cytokines secreted by Th17 cells in MRL₁pr mice

The effect of As₂O₃ on cytokines secreted by Th17 cells was analyzed by ELISA. It was revealed that after treatment with As₂O₃, the secretion of cytokines IL-17 and IL-21 in Th17 cells from MRL₁pr mice was significantly decreased compared with the control group (P < 0.05) (Figure 5).

Discussion

CD4⁺ T lymphocytes can differentiate into Th1, Th2, Treg, and Th17 cell subsets. Treg and Th17 are derived from co-precursor cells, which mutually restrict the maintenance of immune balance [20, 21]. Treg cells play an immunosuppressive role to regulate immune tolerance, and thus are immune protective factors that can inhibit the occurrence of autoimmune diseases. Th17 cells are immune damage factors that are involved in inflammatory lesions of autoimmune disease. Imbalance between Th17 cells and Treg cells can lead to autoimmune diseases [22, 23]. Th17 cell abnormalities can be detected in SLE patients [14, 20]. Therefore, regulation of Th17 cells may serve as a potential therapeutic target for treating SLE.

As₂O₃, also known as arsenic, can be used as a clinical drug. It is currently used to treat relapsed acute promyelocytic leukemia after treatment with retinoic acid and has achieved remarkable clinical results [24]. It was found that it can be used to treat autoimmune diseases such as asthma [25]. As₂O₃ can inhibit the proliferation of T cells and B cells, suppress humoral immune responses, and modulate Th1/Th2 cytokines to regulate immune res-

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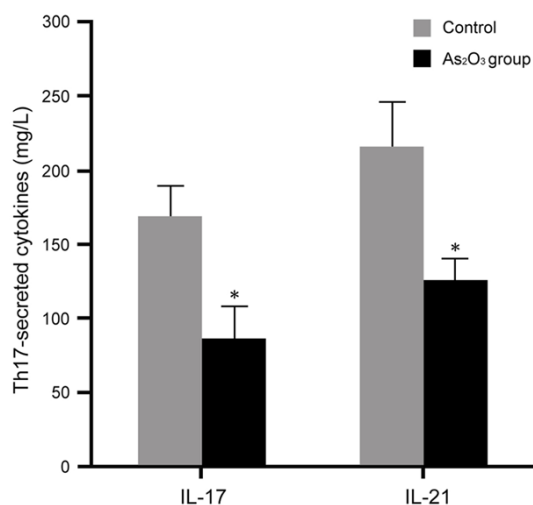


Figure 5. The effect of As₂O₃ on cytokines secreted by Th17 cells in MRL₁pr mice. *P < 0.05, compared with control.

ponses [26]. However, the role of As₂O₃ in SLE has not been fully elucidated. In this study, the MRL₁pr SLE animal model mice was used to test the impact of As₂O₃. It was confirmed that As₂O₃ treatment can reduce 24 h urine protein, serum IgG, and anti-dsDNA antibody expression, and increase body weight, suggesting that As₂O₃ has a therapeutic effect on SLE. However, whether As₂O₃ can regulate Th17 cells participating in SLE has not been reported. Therefore, this study first examined the effect of As₂O₃ on Th17 cell subsets, and confirmed that it could reduce the proportion of Th17 cell subsets in MRL₁pr mice and inhibit their secretion of related cytokines including IL-17 and IL-21. Transcription factors are a key factor in determining the differentiation direction of Th cells. The specific transcription factor that differentiates Th17 cells from precursor cells is ROR γ t, which can determine the differentiation of and secretion from Th17 cells [27]. Therefore, this study explored the impact of As₂O₃ on Th17 cell-related transcription factors, and the results demonstrated that As₂O₃ can inhibit the expression of ROR γ t. It was found that As₂O₃ can restrain Th17 cell differentiation and secretion by inhibiting the expression of Th17 cell-related transcription factors. The relevant mechanism of As₂O₃ in regulating Th17 cell transcription factor in SLE still needs further exploration, which will provide new evidence for understanding the mechanism of SLE.

Conclusion

As₂O₃ can inhibit the expression of Th17 cell-associated transcription factors in the MRL₁pr mouse, which in turn restrains the production of Th17 cells and the secretion of cytokines, improves the immune pathological process: suggesting As₂O₃ might have a therapeutic effect on treating SLE.

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Disclosure of conflict of interest

None.

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