Arsenic trioxide ameliorates experimental autoimmune encephalomyelitis in C57BL/6 mice through inducing apoptosis of CD4⁺ T cells

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Abstract

Background: Multiple sclerosis (MS) is an immune-mediated disease of the central nervous system characterized by severe demyelination of white matter. There is no definite cure for MS owing to its complex pathogenesis. Experimental autoimmune encephalomyelitis (EAE) is an ideal animal model for the study of MS. Arsenic trioxide (ATO) is an ancient Chinese medicine used for its therapeutic properties for several autoimmune diseases. It is also used to inhibit acute immune rejection due to its anti-inflammatory and immunosuppressive properties. However, it is unclear whether ATO has a therapeutic effect on EAE, and the underlying mechanisms have not been clearly elucidated. In this study, we attempted to explore the possibility of using ATO to ameliorate EAE in mice.

Methods: ATO (0.5 mg/kg/day) was administered intraperitoneally to EAE mice 10 days post-immunization for 8 days. On day 22 post-immunization, the spinal cord, spleen, and blood were collected to analyze demyelination, inflammation, microglia activation, and proportion of CD4⁺ T cells. *In vitro*, for mechanistic studies, CD4⁺ T cells were sorted from the spleen of naïve C57BL/6 mice and treated with ATO and then used for apoptosis assay, JC-1 staining, transmission electron microscope, and western blotting.

Results: ATO delayed the onset of EAE and alleviated the severity of EAE in mice. Treatment with ATO also attenuated demyelination, alleviated inflammation, reduced microglia activation and decreased the expression of IL-2, IFN- γ , IL-1 β , IL-6, and TNF- α in EAE mice. Moreover, the number and proportion of CD4⁺ T cells in the spinal cord, spleen, and peripheral blood were reduced in ATO-treated EAE mice. Finally, ATO induced CD4⁺ T cells apoptosis through the mitochondrial pathway both *in vitro* and *in vivo*. Additionally, the administration of ATO had no adverse effect on the heart, liver, and kidney function and did not induce apoptosis in the spinal cord.

Conclusions: Overall, our findings indicated that ATO plays a protective role in the initiation and progression of EAE and has the potential to be a novel drug in the treatment of MS.

Background

Multiple sclerosis (MS) is an immune-mediated disease of central nervous system (CNS) that is typically characterized by neuroinflammation, demyelinated white matter, gliosis, and blood-brain barrier damage [1]. MS primarily onset occurs between the ages of 20 and 40 years, and it is the second largest neurological disease that causes disability in the young people [2, 3]. Although the pathogenesis of MS remains unclear, T cells, B cells, and other immune cells are appeared to mediate immune responses in the CNS [1]. To date, the US Food and Drug Administration has approved 15 disease modifying therapies (DMTs) for relapsing MS, all of which could reduce rates of relapse to some extent by exerting immunosuppressive or immunomodulatory effects. However, current drugs are only effective for some MS patients and have no therapeutic action against the secondary progressive MS. Additionally, the long-

term effects of these treatments are associated with complications [4]. Consequently, there is an urgent need to develop a novel and effective therapeutic drug.

Experimental autoimmune encephalomyelitis (EAE) is an ideal animal model for the study of MS that is induced by immunization with myelin oligodendrocyte glycoprotein (MOG₃₅₋₅₅) in C57BL/6 mice [5, 6]. It has been reported that Th1 cells influence the initiation of EAE pathogenesis [7]. When the local cytokine environment contains IL-12 and IFN- γ , naïve CD4⁺ T cells can be selectively induced to express T-bet and further differentiate into Th1 cells to activate macrophages contributing to cellular immunity by secreting IFN- γ , IL-2, and TNF- α [8].

Arsenic is a metalloid element that exists widely in nature in different forms and oxidation states. The primary arsenides found in air, soil, sediment, and water are inorganic arsenic such as arsenite and arsenate. While organic arsenic are found in seafood, including arsenobetaine and arsenosugars. In general, inorganic arsenic is more toxic than organic arsenic [9]. The trivalent arsenic is the most toxic and often interacts with thiol-containing enzymes which suppresses critical biochemical reactions, while the pentavalent arsenic has less toxicity but usually replaces the phosphate group in some metabolic pathways [10]. Organic arsenic shows mild toxicity, and even some with a higher degree of alkylation, such as arsenobetaine and arsenosugars, it is almost non-toxic. Additionally, arsenic-based products have been used as feed additives [11] or pesticides [12] for decades.

Arsenic trioxide (ATO), an ancient Chinese medicine, used to treat several diseases [13]. Studies reported that ATO can treat syphilis and trypanosomiasis, which both damage the CNS [14, 15]. In the 19th century, ATO was successfully approved as the frontline agent for the treatment of acute promyelocytic leukemia (APL) [16]. In APL patients, ATO promotes the degradation of the promyelocytic leukemia protein/retinoic acid receptor- α fusion protein that drives the growth of APL cells, which leads to apoptosis and partial differentiation of APL cells [17]. Studies have also shown that ATO exerts therapeutic effects against various solid tumor cells such as breast cancer, ovarian cancer, hepatoma, prostate cancer, pancreatic cancer, and gastric cancer [13]. Although the exact underlying mechanisms are not entirely understood, ATO may induce apoptosis, promote cell differentiation, suppress cell growth, and inhibit angiogenesis in many different tumor cell lines [13].

Recently, it has been shown that ATO is a novel and efficacious therapeutic drug in the treatment of autoimmune diseases, such as asthma [18], and human lupus-like syndrome [19]. A recent study suggested that ATO suppressed acute graft-versus-host disease in mice [20]. Our previous work demonstrated that ATO attenuated acute rejection and prolonged graft survival in heart [21] and islet [22] transplantation models. These findings indicate that ATO elicits anti-inflammatory and immunosuppressive effects. However, it is not clear whether ATO shows therapeutic effects in EAE.

In this study, we assessed the therapeutic effects of ATO in EAE mice by evaluating differences in the clinical symptoms, histology and microglial activation in the spinal cord, inflammatory factor expression,

and proportion of CD4⁺ T cells between ATO-treated and non-treated mice. Additionally, we also investigated the underlying mechanism of the ameliorating effects of ATO in EAE mice.

Methods

Mice

Female C57BL/6 mice (6-8 weeks old, 20 ± 2 g) were purchased from Vitallihua Experimental Animal Co., Ltd. (Beijing, China). All mice were housed in a specific pathogen-free facility. All experiments in this study were approved and performed in accordance with the guidelines of the Animal Ethics Committee of Xiamen University (Approval ID: XDYX2015008).

ATO

Arsenious acid and sodium chloride injection was purchased from Harbin Medical University Pharmaceutical Co., Ltd. (Harbin, China) with an ATO concentration of is 1 mg/mL. A working solution was prepared for *in vitro* and *in vivo* experiments by diluting the injection in normal saline.

EAE induction and ATO treatment

To induce EAE, female C57BL/6 mice were immunized with 200 µg of MOG₃₅₋₅₅ peptide (BAM Biotech Co., Ltd., Xiamen, China) in complete Freund's adjuvant (Sigma, MO, USA) supplemented with 2.5 mg/mL H37RA (Cohesion Biosciences, CA, England). Pertussis toxin (500 ng; List Biological Laboratories Inc., CA, USA) was administered intraperitoneally on the day of immunization and 48 h later. The clinical symptoms were scored as follows: 0, normal; 1, tail paralysis; 2, partial hindlimbs paralysis; 3, complete hindlimbs paralysis; and 4, complete hindlimbs paralysis and partial forelimbs weakness. Animals were randomly divided into three groups (10 mice/group): control, no treatment; EAE, MOG treatment; and EAE + ATO (MOG combined ATO treatment). Starting at 10 days post-immunization, mice in the EAE + ATO group were intraperitoneally injected with ATO (0.5 mg/kg/day) for 8 days. On day 22 post-immunization, mice were sacrificed and peripheral blood, spinal cord, and spleen were collected and used for further experiments.

Histopathology, Immunohistochemistry, and Immunofluorescence

The spinal cord was dissected and fixed with ice-cold 4% paraformaldehyde overnight at 4 °C, embedded in paraffin, cut into 5 µm slices, and stained with luxol fast blue (LFB) and hematoxylin and eosin (HE). LFB-stained sections were scored for demyelination as follows: 0, none; 1, rare foci; 2, a few areas of demyelination; 3, one to two large areas of demyelination; and 4, extensive demyelination. Representative examples of LFB stained histological sections illustrating the different demyelination scores were presented in supplementary figure 1. HE-stained sections were also scored for inflammation as follows: 0, none; 1, a few scattered inflammatory cells; 2, perivascular infiltrates; 3, extensive perivascular cuffing with extension into adjacent parenchyma; and 4, extensive cell infiltration in the white matter [23].

Additionally, the sections were subjected to indirect immunostaining. The primary antibodies were used as follows: anti-CD4 (1:70, Servicebio, Wuhan, China), anti-MBP (1:50, Boster, Wuhan, China), and anti-Iba-1 (1:200, Servicebio). Staining was quantified using the HALO™ image analysis software (Indica Labs, NM, USA). Briefly, in the HALO analysis software, we set the measurement target area for each slice. For analysis of CD4⁺ cells in the spinal cord, Indica Labs-Multiplex IHC module was used to set Stain 1 as negative cells (blue nuclei) and Stain 2 as positive cells (brown granule). For analysis of Iba-1⁺ cells in the spinal cord, Indica Labs-HighPlex FL module was used to identify positive cells (red cytoplasm) and negative cells (blue nuclei). After the setup was completed, the software automatically calculated the number of positive and total cells on the section. The percentage of positive cells in total cells was calculated from nine different sections of the spinal cord.

Proinflammatory cytokine detection

Blood was collected from the cavernous sinus in the posterior eye orbit and kept at room temperature for 30 min. After centrifugation at 4,000 rpm for 20 min, the serum was transferred into a new tube and stored at -80 °C. The concentration of IFN-γ in serum was measured using a commercial V-PLEX proinflammatory panel 1 kit (MSD, NJ, USA).

Flow cytometry

Red blood cells were removed to obtain peripheral and spleen lymphocytes. The cells were incubated in anti-CD4-FITC (Biolegend, CA, USA) at 4 °C for 30 min. The IgG-FITC isotype antibody (Biolegend) was used as the negative control. The stained cells were examined by flow cytometry with Beckman Cytoflex S (CA, USA). All data from flow cytometry were processed using FlowJo software V.10.

Assessment of liver and kidney function parameters

ALT (alanine aminotransferase), AST (aspartic aminotransferase), creatinine, and urea in serum were measured through mindray automated biochemical analyzer (Mindray Bio-medical Electronics Co., Ltd., Shenzhen, China) using standard diagnostic kits and analytical grade reagent (Mindray, Shenzhen, China) according to the manufacturer's instructions.

Apoptosis

Apoptosis of CD4⁺ T cells was detected with the Annexin V-FITC/PI Detection Kit (Meilunbio, Dalian, China). Briefly, after washing with PBS, cells were stained with Annexin V-FITC and PI for 15 min at room temperature. Subsequently, cells were detected by flow cytometry with Beckman Cytoflex S. Apoptosis signals of spinal cords were examined with the Apoptosis Detection Kit (Servicebio).

JC-1 staining

CD4⁺ T cells were sorted from the spleen of naïve C57BL/6 mice with the Mouse CD4⁺ T cell Isolation Kit (Miltenyi, Bergisch Gladbach, Germany), seeded in 12-well plates, and treated with 5 µg/mL ConA alone or

5 µg/mL ConA + ATO (1, and 2 µM) for 24 h. JC-1 staining was performed to monitor the change of mitochondrial membrane potential. Briefly, the CD4⁺ T cells were incubated with 1640 RPMI medium contained 10 mg/mL JC-1 probe (Sigma) for 30 min at 37 °C. After washing three times with PBS, the stained cells were examined by flow cytometry with Beckman Cytoflex S.

Transmission electron microscope (TEM)

CD4⁺ T cells were cultured and treated as described above. After washing with PBS, the cells were fixed with 2.5% glutaraldehyde overnight at 4 °C. The following day they were fixed again with 1% osmium tetroxide for 2.5 h at room temperature. Subsequently, the cells were embedded after dehydration. Ultra-thin sections were counterstained with uranyl acetate for 30 min and lead citrate for 30 s and observed by a TEM (HT7800, Hitachi).

Caspase 3 Activity Assays

Total protein was extracted from CD4⁺ T cells to measure Caspase 3 activity with the Caspase 3 Activity Assay kit (Applygen, Beijing, China). Briefly, BCA assay was used to determine the protein concentrations; then, 10 µL of Caspase 3 substrate was incubated with 10 µL of the total protein (30 µg) in a final volume of 100 µL for 3 h at 37 °C. The absorbance of *p*-nitroanilide was measured using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 405 nm in turn to calculate the Caspase 3 activity.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from spinal cords or spleen with Trizol (TansGen, Beijing, China). Reverse transcription was performed using the cDNA Synthesis SuperMix for qPCR kit (TansGen). The mRNA expression was quantified using qPCR SuperMix kit (TansGen) and normalized to β-actin. The relative mRNA expression of genes was calculated using the $2^{-\Delta\Delta C_t}$ method. $\Delta\Delta C_t = (C_{T, target} - \text{mean } C_{T, \beta\text{-actin}})_{\text{treated sample}} - (\text{mean } C_{T, target} - \text{mean } C_{T, \beta\text{-actin}})_{\text{control sample}}$. The control sample is the spinal cord from naïve C57BL/6 mice and used as the calibrator. The sequences of primers are as follows: IL-1β, forward 5'-TCGCAGCAGCACATCAACAAGAG-3', reverse 5'-TGCTCATGTCCTCATCCTGGAAGG-3'; IL-2, forward 5'-GGAGCAGCTGTTGATGGACCTAC-3, reverse 5'-AATCCAGAACATGCCGAGAG-3'; IL-6, forward 5'-TGGGACTGATGCTGGTGACA-3', reverse 5'-ACAGGTCTGTTGGGAGTGGT-3'; IFN-γ, forward 5'-CGGCACAGTCATTGAAAGCCTA-3', reverse 5'-GTTGCTGATGGCCTGATTGTC-3'; TNF-α, forward 5'-GCCTCTTCTCATTCCTGCTTGTGG-3', reverse 5'-GTGGTTTGTGAGTGTGAGGGTCTG-3'; MBP, forward 5'-GCTCTGGCAAGGACTCACACAC-3', reverse 5'-TGGAGGTGGTGTTCGAGGTGTC-3'; β-actin, forward 5'-CATCCGTAAAGACCTCTATGCCAAC-3', reverse 5'-ATGGAGCCACCGATCCACA-3'.

Western blot

Spinal cords were homogenized and CD4⁺ T cells were lysed with cold RIPA buffer (Proteintech, IL, USA) supplemented with a protease inhibitor cocktail (Sigma). After centrifugation at 4 °C for 15 min, the supernatants were collected and used for western blot analysis. The following antibodies were used: anti-

Bax (1:1,000, Proteintech, IL, USA), anti-MBP (1:1,000, Proteintech), anti-cleaved Caspase 3 (1:1,000, Affinity, OH, USA), anti-cleaved Caspase 9 (1:1,000, Affinity), anti-Bcl-2 (1:1,000, Proteintech), anti-cleaved PARP (1:1,000, Affinity), anti- β -actin (1:5,000, Bioworld, MN, USA).

Statistical analysis

Data were analyzed with GraphPad Prism 6 software (La Jolla, CA, USA) and represented as means $\pm$ SD of three separate experiments. Clinical scores, demyelination scores, and inflammation scores were compared using the Kruskal–Wallis test. One-way ANOVA was used for multiple comparison in the rest of assays. A p value ≤ 0.05 was considered to be statistically significant.

Results

ATO ameliorated EAE progression in mice

We first explored whether ATO had a protective role on MOG₃₅₋₅₅-induced EAE mice. Twenty-two days after immunization, EAE mice exhibited severe clinical signs with flaccid tail and complete paralysis of the hindlimbs; however, ATO-treated EAE mice only showed tail paralysis (Fig. 1a). EAE clinical score data showed that the onset of symptoms was lower in ATO-treated mice (day 22) as compared with that in EAE mice (day 18), and the maximal score (1.5) and mean score (1.02 ± 0.04) were lower in ATO-treated mice than the maximal (3.0) and mean score (1.86 ± 0.39) in EAE mice. (Fig. 1b). Additionally, weight loss was lower in ATO-treated mice than that in EAE mice (Fig. 1c). These observations suggest that ATO could effectively alleviate the severity of EAE in mice.

ATO alleviated demyelination in the spinal cord of EAE mice

To further confirm the therapeutic effects of ATO on EAE mice, we measured demyelination in spinal cord using LFB staining. The results showed that the area of demyelination in the spinal cord of EAE mice was large than that in ATO-treated EAE mice (Fig. 2a, c). Similarly, treatment with ATO significantly increased the expression of MBP, a structural protein of myelin, compared to that in the EAE group. (Fig. 2b, d-f). Overall, these findings suggest that ATO alleviated myelin damage associated with the progression of EAE.

ATO reduced inflammation in the spinal cord of EAE mice

Since EAE is an autoimmune disease that is associated with severe neuroinflammation, we investigated whether ATO could decrease inflammatory cell infiltration, microglia activation, and inflammatory cytokine levels. Our results showed that EAE mice exhibited the extent of inflammatory cell infiltration in the spinal cord, whereas ATO-treated EAE mice showed only mild infiltration (Fig. 3a, c). Additionally, microglia activation (as evidenced by Iba-1 expression) in the spinal cord was dramatically reduced in ATO-treated EAE mice as compared with that in EAE mice (Fig. 3b, d). Moreover, treatment with ATO decreased the concentration of IFN- γ in serum of EAE mice (Fig. 3e). The expression of inflammatory

cytokines, such as IL-2, IFN- γ , IL-1 β , IL-6, and TNF- α , was decreased in the spinal cord of ATO-treated EAE mice as compared with that in EAE mice (Fig. 3f). Therefore, ATO appeared to reduce inflammatory cell infiltration and microglia activation during EAE progression.

ATO reduced the number and proportion of CD4⁺ T cells in EAE mice by inducing apoptosis

Since CD4⁺ T cells-mediated neuroinflammation is considered to result in the initiation of EAE, we evaluated changes of CD4⁺ T cells in the spinal cord, spleen, and blood. EAE induction led to the prominent infiltration of CD4⁺ T cells in the spinal cord; however, this effect was counteracted following treatment with ATO (Fig. 4a, b). Similarly, treatment with ATO significantly reduced the proportion of CD4⁺ T cells in the spleen (Fig. 4c, d) and peripheral blood (Fig. 4e, f) as compared with that in EAE mice.

Studies have reported that ATO induced apoptosis in a variety of cells, including T cells. Therefore, we investigated if the ATO-reduced proportion and population of CD4⁺ T cells in EAE mice was related to apoptosis induction. The ratio of apoptotic CD4⁺ T cells in spleen was significantly increased in ATO-treated EAE mice as compared with that in EAE mice (Fig. 5a, b). Additionally, the amount of TUNEL positive signals was higher in EAE mice than that in ATO-treated EAE mice (Fig. 5c, d), showing that ATO decreased apoptosis in the spinal cord of EAE mice. Collectively, these results indicated that the decrease in CD4⁺ T cells in ATO-treated EAE mice may be attributed to apoptosis induction.

ATO induced CD4⁺ apoptosis T cells through mitochondrial pathway

To investigate the underlying mechanism of ATO-induced apoptosis *in vitro*, CD4⁺ T cells were isolated from naïve C57BL/6 mice and cultured for 24 h. As shown in Fig. 6a, c, ATO significantly increased the ratio of apoptotic cells in a dose-dependent manner. The mitochondrial membrane potential was high in activated CD4⁺ T cells, whereas ATO treatment diminished the J-aggregates and increased the J-monomers (Fig. 6b, d). Moreover, transmission electron microscopic examination showed that mitochondria swelling, decrease or disappearance of mitochondria crista, and vacuolization were observed in ATO-treated groups (Fig. 6e). Meanwhile, the level of pro-apoptotic proteins, such as cleaved-Caspase 3, Bax, cleaved-Caspase 9, and cleaved-PARP was significantly increased in ATO-treated EAE mice; however, ATO decreased Bcl-2 expression (Fig. 6f, g). Moreover, consistent to the results observed *in vitro*, ATO treatment significantly increased the level of pro-apoptotic proteins in the spleen of EAE mice, while decreased Bcl-2 expression (Fig. 6h, i). These data indicated that ATO induced CD4⁺ T cells apoptosis through the mitochondrial pathway both *in vitro* and *in vivo*.

No toxicity signs were evident in ATO-treated mice

To determine whether the administration of ATO had toxic effects on mice, we analyzed the functions of liver and kidney after consecutive intraperitoneal injections of ATO for 20 days. The results of HE staining showed that there were no obvious abnormalities in the heart, liver, and kidney in mice after ATO treatment as compared with control mice (Fig. 7a). Consistently, no significant differences in the levels of

ALT, AST, creatinine, and urea were observed between ATO-treated mice and control mice, even at a dose of 1 mg/kg/day (Fig. 7b). Moreover, treatment with ATO did not result in evident TUNEL positive signals in the spinal cord (Fig. 7c). These results suggested that ATO had no adverse effect on liver and kidney function at a dose of 0.5 or 1 mg/kg/day and did not induce apoptosis in the spinal cord.

Discussion

Despite recent advances achieved with DMTs for the treatment of MS patients, the overall therapeutic effect against MS has not yet reached an ideal state, developing new and safe drugs for the treatment of MS remains important. EAE is a classic mouse model used for the study of MS. Studies reported that ATO demonstrates anti-inflammatory or immunosuppressive effects in a variety of disease settings, including leukemia [16], asthma [18], human lupus-like syndrome [19], and graft-versus-host disease [20]. However, it remains unclear whether ATO has a therapeutic effect on EAE. To the best of our knowledge, this is the first to attempt to explore the possibility of using ATO to ameliorate EAE in mice. Our results suggested that ATO delayed the onset of EAE, alleviated the clinical signs and severity of EAE in mice, reduced neuroinflammation, and attenuated demyelination.

Autoreactive CD4⁺ T cells play a major role in the initiation and orchestration of EAE [24]. The activated CD4⁺ T cells migrate from the periphery to the CNS, where the cascade of inflammatory reaction is initiated by secreting cytokines and chemokines. Therefore, apoptosis induction of CD4⁺ T cells may contribute to ameliorating EAE. Studies reported that drugs such as glatiramer acetate [25] and interferon- β [26], treat MS by inducing the peripheral T cells apoptosis. Similarly, results from our study demonstrated that ATO induced CD4⁺ T cells apoptosis in the spleen. Cell apoptosis could be triggered by the mitochondria pathway, the endoplasmic reticulum stress pathway, and the death receptor-mediated extrinsic pathway. Among these pathways, the mitochondria pathway can be activated by caspase cascades and Bcl-2 family members in the mitochondria [27]. Other studies have reported that ATO-induced apoptosis is attributed to the downregulation of Bcl-2, the upregulation of Bax, and reduction of the mitochondrial membrane potential [28]. Results from our study support these findings and demonstrate that ATO induced alterations in the protein level of Bcl-2 and Bax and decreased the mitochondrial membrane potential in CD4⁺ T cells. Additionally, our results suggest that ATO decreased the proportion of CD4⁺ T cells in the spleen and peripheral blood, which is probably due to apoptosis induction; thus, the population of CD4⁺ T cells that infiltrated into the CNS was also reduced. Indeed, we found that treatment with ATO decreased the number of CD4⁺ T cells infiltration into the white matter. Therefore, these data indicate that ATO treatment could induce CD4⁺ T cells apoptosis through the mitochondrial pathway, thus inhibiting the infiltration of CD4⁺ T cells into the CNS, thereby delay the onset of EAE and effectively ameliorate the severity of EAE in mice.

Some pro-inflammatory cytokines secreted by activated CD4⁺ T cells in the CNS could attract various immune cells into the CNS, which gradually aggravates CNS demyelination. Thus, inhibition of the release of some proinflammatory cytokines is an effective strategy to attenuate EAE clinical symptoms.

IFN- γ and IL-6 can induce the expression of chemokines and adhesion molecules, which recruit the migration of leukocytes into the CNS [29, 30]. In the CNS, IL-1 β can recruit and activate lymphocytes through acting on astrocytes and CNS endothelial cells [31]. TNF- α produced by infiltrated macrophages can exacerbate the severity of EAE through promoting inflammatory infiltrates and disrupting the blood-brain barrier [32]. Our data demonstrated that treatment with ATO decreased the mRNA level of inflammatory cytokines in the spinal cord and reduced the concentration of IFN- γ in the serum of EAE mice. Consistent with our findings, studies have reported that ATO reduced inflammation in splenocytes of MRL/*lpr* mice and human lupus peripheral blood mononuclear cells by reducing the expression level of the INF- γ [33]. Collectively, the therapeutic effects of ATO in our study may be partially due to lower expression of proinflammatory cytokines, such as IL-2, IFN- γ , IL-1 β , IL-6, and TNF- α .

Excessive activation of microglia induces neuroaxonal injury [34]. As the resident macrophages of the CNS, microglia activation is secondary to infiltrating CD4⁺ T cells and a hallmark of demyelinated lesions [35, 36]. Despite the role of microglia in autoimmune diseases such as in EAE remains undefined, studies reported that microglia activation result in damage of myelin, axonal and neuron, which are probably mediated by expressing high levels of oxidative stress and iNOS as well as excessively releasing some cytotoxic mediators [37, 38]. In this study, a treatment with ATO reduced the expression of Iba-1 indicating that ATO decreased microglial activation, which also suggests that ATO alleviated demyelination in the spinal cord of EAE mice. Consequently, part of the therapeutic effects of ATO in EAE mice may be due to its ability to reduce the proportion of peripheral CD4⁺ T cells and microglia activation, and ultimately inhibit demyelination.

The clinical application of ATO may be limited by its adverse effects on healthy tissues, including cardiotoxicity, hepatotoxicity, and nephrotoxicity. However, a 10-year follow-up study suggested that none of the side effects was severe enough to discontinue treatment. Their observations suggested that the long-term use of ATO in APL patients was safe and not associated with any major side effects [39]. Moreover, studies from Lo-Coco et al. and Burnett et al. shown that the hepatotoxicity is usually reversible and could be successfully managed with the temporary cessation or a decrease of ATO; there have been no reports of fatal hepatic failure in clinical trials [40, 41]. Furthermore, increasing evidence indicated that multiple drugs could be used for inhibition cardiotoxicity induced by ATO, such as salvianolic acid A, omega-3 fatty acid, sorbus pohuashanensis, resveratrol, genistein, and metallothionein [42]. ATO-mediated cardiotoxicity could be reduced by combination with other drugs. Therefore, the toxic side effects of ATO as a clinical drug are relatively controllable. Zheng et al. [43] and our previous work [44, 45] suggested that in the mice allogeneic heart transplantation model, when the dose of ATO was less than 5 mg/kg/day, no abnormalities were observed in the liver, kidney, and lung. Zhang et al. reported that treatment with 1 mg/kg/day ATO for 14 days did not cause damage to the hearts of the mice [46]. These findings suggested that ATO had no toxic side effects on the heart, liver, kidney, and lungs at doses less than 5 mg/kg/day in animal models.

The therapeutic effect of ATO on nervous system diseases such as EAE and the reported ATO-induced neurotoxicity appears to be contradictory, which warrants further investigation. However, in fact, the

therapeutic effect and toxic side effects of ATO are closely associated with the administrated dose [47]. Récher et al. shown that mice intraperitoneally injected with ATO (5 mg/kg/day) for two consecutive weeks (5 days per week) had a blood ATO concentration of 0.23 μM after two weeks of treatment [48]. Lu et al. found that 1 μM ATO did not induce neuronal cell apoptosis *in vitro* [49]. Thus, we believe that an ATO dose lower than used in the study of Récher et al., that is, 0.5 mg/kg/day, will not result in neurotoxicity in mice. Indeed, we did not observe any TUNEL positive signaling in the spinal cord after mice were administrated with 0.5 or 1 mg/kg/day ATO for 20 days. Therefore, in a certain concentration range, ATO has no adverse effects on CNS.

In this study, mice in the EAE + ATO group were intraperitoneally injected with ATO at a dose of 0.5 mg/kg/day for 8 days based on the following considerations. In a murine model of asthma, treatment with 2.5 mg/kg/day of ATO for 7 days alleviates airway hyperresponsivity and eosinophilia [18]. In MRL/*lpr* mice, treatment with 5 mg/kg/day of ATO for 2 months inhibited autoreactive lymphocytes and blocked the progression of autoimmune diseases [19]. In mice allogeneic islet transplantation model, treatment with 1 mg/kg/day of ATO twice per day significantly prolonged the survival of islet allograft [22]. A recent study suggested that ATO (1 mg/kg/day) suppressed acute graft-versus-host disease in mice [20]. Based on the above, 2 doses of ATO (0.5 and 1 mg/kg) were studied to determine the optimal dose in pre-experiment. Treatment with ATO (0.5 or 1 mg/kg) for 8 days had similar effect in ameliorating EAE. Since 0.5 mg/kg was the lowest therapeutic dosage of ATO against EAE, it was used for subsequent experiments. Furthermore, treatment with 0.5 or 1 mg/kg ATO did not result in adverse to the heart, liver, and kidney of mice. Therefore, the dose of ATO used in this study is safe and effective.

The successful application of ATO in mice EAE model may contribute to treating other immune diseases such as MS. Several comorbidities often occur in MS patients, such as cancer and autoimmune diseases, which limit the choice of DMTs and reduce the quality of life [4]. Thus, the development of drugs that possess both anti-tumor and anti-autoimmunity properties may also benefit MS patients. At this point, ATO may manage these comorbidities due to its antineoplastic [50-53] and anti-inflammatory [20, 22, 54] properties, thereby improving the efficiency of DMTs therapy. Additionally, as a traditional Chinese medicine, ATO has been used for thousands of years [55] and is the first-line drug for the treatment of APL in clinical settings [16]. The new use of old drug is an effective way to develop new drugs. However, it should be noted that although existing findings confirmed that ATO could effectively ameliorate symptoms of EAE in MOG-induced EAE mice, due to the lack of human trial data, whether ATO could be used for the treatment of MS remains to be further studied.

Although our results suggest that ATO has a good therapeutic effect on EAE in mice, there are some limitations in the present study. Despite the pathogenesis of MS is not fully elucidated, CD4^+ T cells, CD8^+ T cells, B cells, and other immune cells are likely involved [1]. Since EAE is initiated and orchestrated by autoreactive CD4^+ T cells, we focused on the CD4^+ T cells *in vivo* and *in vitro* experiments. Therefore, it is necessary to use other suitable animal models to explore the role of CD8^+ T cells, B cells, and other immune cells in the curative effect of ATO on EAE in future work.

Conclusion

To the best of our knowledge, our study is the first to suggest that ATO ameliorated EAE in C57BL/6 mice by inducing CD4⁺ T cells apoptosis via the mitochondrial pathway. Moreover, the administration of ATO did not cause adverse health effects in mice. Our findings may facilitate the clinical application of ATO for the treatment of MS or other autoimmune diseases.

Declarations

Ethics approval and consent to participate

All animals and experiments in this study were approved by the Animal Ethics Committee of Xiamen University.

Consent for publication

Not applicable

Availability of data and materials

All data used in this study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

Z.Q. and J.X. made contributions to experimental design, data analysis, manuscript revision, and funding. K.A. performed the experiments, analyzed the data, and drafted the article. M.X., J.Z. and S.Y. performed the experiments.

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Abbreviations

MS, multiple sclerosis; ATO, arsenic trioxide; EAE, experimental autoimmune encephalomyelitis; LFB, luxol fast blue; TEM, transmission electron microscope; qRT-PCR, quantitative real-time PCR; CNS, central nervous system; Th, T helper; FITC, fluorescein isothiocyanate; PBS, phosphate buffered saline; MBP, myelin basic protein; TUNEL, TdT-mediated dUTP nick end labeling; IL-1 β , interleukin-1 β ; MOG, myelin oligodendrocyte glycoprotein; IL-2, interleukin-2; CFA, Complete Freund's Adjuvant; IL-6, interleukin-6; HE, hematoxylin and eosin; IFN- γ , interferon gamma; TNF- α , tumor necrosis factor- α ; PI, Propidium Iodide; ANOVA, analysis of variance. SD, standard deviation; ALT, alanine aminotransferase; AST, aspartic aminotransferase; DMTs, disease modifying therapies.

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Figures

Figure 1

ATO ameliorates EAE progression in mice. (a) Representative images show behavioral symptoms of EAE mice in individual groups. Black arrow presents limp tail, and red arrow presents hind limb paralysis. The clinical scores (b) and changes in body weight (c) were recorded. $n = 10$ mice/group. Data are representative of three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$ the EAE group vs. the control group; $+p < 0.05$, $++p < 0.01$, and $+++p < 0.001$ the EAE+ATO group vs. the control group; $\#p < 0.05$ and $##p < 0.01$ the EAE+ATO group vs. the EAE group.

Figure 2

ATO alleviates demyelination in the spinal cord of EAE mice. Representative LFB staining sections (a) and demyelination score (c) of spinal cord ($n = 9$). Areas enclosed within black squares are magnified on the right. (b) Representative immunofluorescent staining sections of MBP in spinal cord. Areas enclosed within green squares are magnified on the right. (d) Mean fluorescence intensity analysis of MBP in b ($n =$

3). (e) The mRNA level of MBP in spinal cord was quantified by qRT-PCR (n = 3). (f) The protein level of MBP in spinal cord was analyzed by western blot. Data are representative of three independent experiments. *p < 0.05, ***p < 0.001, and ****p < 0.0001 vs. the control group; #p < 0.05 and ###p < 0.001 the EAE+ATO group vs. the EAE group.

Figure 3

ATO reduces inflammation in the spinal cord of EAE mice. (a) Representative HE staining sections of spinal cord. Areas enclosed within black squares are magnified on the right. (c) Analysis of spinal cord infiltrates in a (n = 9). (b) Iba-1 immunofluorescent staining in spinal cord. Areas enclosed within green squares are magnified on the right. (d) Analysis of Iba-1+ cells in b (n = 3). (e) Concentration of IFN- γ in the serum was measured with the V-PLEX proinflammatory panel 1 kit (n = 3). (f) The mRNA levels of inflammatory cytokines in the spinal cord were determined through qRT-PCR (n = 3). Data are representative of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 vs. the control group; #p < 0.05, ##p < 0.01, and ###p < 0.001 the EAE+ATO group vs. the EAE group.

Figure 4

ATO reduces the number and proportion of CD4⁺ T cells in EAE mice. (a) Immunohistochemical staining of CD4 was performed in spinal cord. (b) Analysis the infiltration of CD4⁺ T cells in a (n = 3). The percentage of CD4⁺ T cells in spleen (c) and peripheral blood (e) were determined by flow cytometry, respectively. (d, f) Analysis of CD4⁺ T cells in c, e (n = 3). Data are representative of three independent experiments. **p < 0.01 and ****p < 0.0001 vs. the control group; #p < 0.05, ##p < 0.01, and ####p < 0.0001 the EAE+ATO group vs. the EAE group.

Figure 5

ATO induces CD4⁺ T cells apoptosis in EAE mice. (a, b) CD4⁺ T cells were isolated from the spleen with the Mouse CD4⁺ T cell Isolation Kit. Annexin-V/PI staining was performed to analyze the apoptosis of CD4⁺ T cells (n = 3). (c) Representative TUNEL staining sections of spinal cord. Boxes show magnified areas. (d) Analysis of TUNEL positive cells in c (n = 3). Data are representative of three independent experiments. *p < 0.05 vs. the control group; #p < 0.05 the EAE+ATO group vs. the EAE group.

Figure 6

ATO induces CD4⁺ T cells apoptosis through the mitochondrial pathway. CD4⁺ T cells were sorted from the spleen of normal mice and cultured with Con A or ATO (0, 1, and 2 μ M) for 24 h. (a, c) Annexin V/PI was performed to evaluate the apoptosis of CD4⁺ T cell (n = 3). (b, d) The mitochondrial membrane potential was monitored by loading with JC-1 probe. J-aggregates indicates high membrane potential, and J-monomers indicates low membrane potential (n = 3). (e) The observation of mitochondrial

ultrastructure by TEM. Boxes show magnified areas. (f) The protein level of Bax, Bcl-2, cleaved-Caspase 9, and cleaved-PARP was analyzed by western blot. (h) On day 22 post-immunization, CD4⁺ T cells isolated from the spleen were used to extract total protein. The protein level of Bax, Bcl-2, cleaved-Caspase 9, and cleaved-PARP in CD4⁺ T cells was detected by western blot. (g, i) The activity of Caspase 3 was determined with the Caspase 3 Activity Assay kit (n = 3). Data are representative of three independent experiments. *p < 0.05, ***p < 0.001, and ****p < 0.0001 vs. the activated or control group; #p < 0.05 the EAE+ATO group vs. the EAE group.

Figure 7

No toxicity signs were observed in ATO-treated mice. Normal mice were administered intraperitoneal injections of normal saline, ATO (0.5 mg/kg/day) or ATO (1 mg/kg/day) for 20 days. (a) Heart, liver, and kidney were collected for HE staining. Scale bar = 50 µm. (b) The levels of ALT, AST, creatinine, and urea in the serum were measured by an automated biochemical analyzer. (c) Representative TUNEL staining sections of the spinal cord. Scale bar = 100 µm. n = 5 mice/group. Data are representative of three independent experiments. ALT, alanine aminotransferase; AST, aspartic aminotransferase.

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