

Baicalein inhibits the Th1 / Th17-mediated inflammatory response by targeting STAT1/3 in EAE mice

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Abstract

Helper T cells (Th) play a crucial role in the pathogenesis of multiple sclerosis (MS) and experimental encephalomyelitis (EAE). Th1/17 cells contribute to neuroinflammation through inflammatory infiltration and cytokine secretion, however, effective therapeutic interventions are currently lacking. Baicalein (BAI), the principal component of *Scutellaria baicalensis*, is a dietary supplement that has demonstrated efficacy in the fields of anti-inflammatory, neuroprotection, and immunomodulation. The activation the JAK-STAT signalling pathway in Th1/17 cells may play a pivotal role in the initiation and progression of inflammation associated with MS/EAE. Therefore, this study aimed to investigate whether BAI could mitigate the inflammatory response of Th1/17 cells by modulating JAK-STAT signaling. Flow cytometry, quantitative real-time PCR and western blotting were used to detect the ratio of Th1 and Th17 cells, the expression of related cytokines in EAE model mice treated with BAI, as well as the expression of proteins related to the JAK/STAT signaling pathway. The findings demonstrated that BAI exhibited therapeutic efficacy in EAE mice by significantly reducing the proportion of Th1 and Th17 cell populations along with the expression levels of relevant inflammatory cytokines. Moreover, transcription factors T-bet and ROR- γ t showed significant downregulation in response to BAI treatment. This effect may be attributed to BAI's ability to inhibit STAT1/3 phosphorylation. Subsequently, molecular docking, pull-down and immunofluorescence experiments confirmed that BAI bound to STAT1/3, and caused p-STAT1/3 to remain in the cytoplasm, hindering JAK/STAT signal transduction and thus inhibiting the inflammatory response of the Th1/17 cells.

1. Introduction

Multiple sclerosis (MS) is an autoimmune inflammation of the central nervous system (CNS) characterized by infiltration of inflammatory cells and demyelination [1, 2]. Experimental autoimmune encephalomyelitis (EAE) serves as a reliable animal model for studying MS[3]. Numerous studies have consistently demonstrated the pivotal roles of Th1 and Th17 cells in the pathogenesis of MS/EAE [4]. The transcription factor STAT1 (Signal transducer and activator of transcription 1) plays a crucial role in the differentiation process of Th1 cells and promotes the production of the iconic cytokine IFN- γ . Activated STAT3 stimulates IL-17 production in Th17 cells. These two cytokines play an important role in promoting CNS inflammation in MS/EAE patients. Many novel drugs have been developed targeting the self-reactive CD4⁺ T cells involved in MS/EAE. However, their therapeutic efficacy remains suboptimal and is often accompanied by adverse effects[5, 6].

Diets abundant in phenolic compounds are acknowledged to confer health benefits, encompassing the mitigation of chronic ailments such as Alzheimer's disease, Parkinson's disease, diabetes, cardiovascular disease and inflammation[7, 8]. Flavonoids are secondary metabolites of polyphenols that are found in fruits, vegetables, grains and other foods and drinks[9]. BAI (5,6,7-trihydroxyflavone, C₁₅H₁₀O₅) (Fig. 1A) is a natural flavonoid derived from *Scutellaria baicalensis*. It is widely utilized as a dietary supplement in both Eastern and Western diets [10]. Numerous studies have demonstrated the multifaceted effects of BAI on regulating peripheral inflammatory responses associated with obesity, diabetes, respiratory and

cardiovascular diseases. Additionally, it has been shown to modulate neuroinflammation in neurodegenerative diseases and encephalomyelitis[11]. Previous studies have demonstrated that BAI exerts its anti-inflammatory effect by inhibiting the activation of the JAK/STAT signalling pathway [12]. However, the precise molecular and signalling mechanisms underlying this anti-inflammatory effect of BAI remain to be further elucidated. Moreover, STAT1 and STAT3 are recognized as pivotal transcription factors in immune and inflammatory pathways[13], regulating numerous genes associated with immunity and acute inflammation.

Therefore, the objective of this study was to investigate whether BAI exerts its anti-inflammatory effect by inhibiting STAT1/3 signaling and attenuating Th1/Th17-mediated inflammation in EAE mice. After BAI intervention in EAE mice, it was confirmed that BAI reduced the incidence of EAE and alleviated the inflammatory reactions and demyelination. Furthermore, BAI suppressed the proportion of Th1 and Th17 cells in vivo. Subsequently, we investigated the impact of BAI on the JAK-STAT signaling pathway through both in vivo and in vitro experiments. Our findings demonstrated that BAI inhibited STAT1 and STAT3 activation, thereby reducing the production of inflammatory cytokines. However, no significant effect on JAK1/2 phosphorylation was observed upon treatment with BAI. Additional experiments revealed that BAI bound to STAT1/3 and impeded their nuclear translocation. This study suggests that by inhibiting the STAT1/3 signaling pathway, BAI exerts an anti-inflammatory effect which ultimately reduces the inflammatory response of Th1/Th17 cells in EAE mice.

2. Materials and methods

2.1. Experimental animals

SPF female C57BL/6 mice (6–8 weeks, 16–18 g) were purchased from Sibef (Beijing) Biotechnology Co., Ltd. Their certificate number was SCXK (Beijing) 2019-0010, and they were raised in the Experimental Animal Center of Qingdao University. All mice were maintained under specific pathogen-free conditions at a temperature of 23 °C with a relative humidity of 60% and subjected to a 12-hour light-dark cycle. Food and drink were provided freely, and clean padding was changed every day.

2.2 Drugs

The BAI (MB7311, purity $\geq$ 95%, MB6699, purity > 98%) was procured from Dalian Meilun Biological Co., Ltd. The molecular structure is illustrated in Fig. 1A. The dexamethasone (DEX) was purchased from Beyotime (ST1258). Sodium carboxymethyl cellulose (C8621) was acquired from Solarbio. Myelin oligodendrocyte glycoprotein (MOG)_{35–55} peptide sequence MEVGWYRSPFSRVVHLYRNGK was synthesized by Sheng Gong Biotech Shanghai Co., Ltd. Complete Freund's adjuvant (CFA)(SLBW1457) was purchased from Sigma–Aldrich (St. Louis, USA). Pertussis toxin (PTX) (180243A1) was purchased from List Biological Laboratories (Campbell, USA). HRP goat anti-rabbit antibody (GB23303) was obtained from Servicebio Biotechnology Co., Ltd. Anti-fluorescence quenching solution (P0126) and

haematoxylin eosin (HE) staining kit (C0105S) were purchased from Beyotime. Luxol Fast Blue myelin staining solution (LFB) (eosin method) (G3240) was purchased from Solarbio.

2.3 Induction and grouping of EAE

Twenty healthy C57BL/6 female mice were randomly divided into four groups: control group (n = 5), EAE model group (n = 5), EAE + BAI treatment group (n = 5) and the EAE + DEX treatment group (n = 5). The mice were immunized with a mixture of MOG₃₅₋₅₅ peptide (200 µg) and CFA, which was ultrasonically emulsified. On the day of immunization, each EAE mouse received intraperitoneal injections of PTX diluent (0.1 ml, containing 250 ng) on Day 0 and Day 2, while the control mice received an equivalent volume of saline administered intraperitoneally (Fig. 1B).

2.4 BAI administration and clinical observation

The BAI and DEX were individually mixed with a 0.5% solution of sodium carboxymethyl cellulose. Starting from the third day, the EAE + BAI group received BAI (150 mg/kg), the EAE + DEX group received DEX (0.5 mg/kg), and the EAE model group received an equal volume of 0.5% carboxymethyl cellulose sodium solution by gavage once daily from Day3 to Day21(Fig. 1B). Starting from the 0th day of immunization, the body weights and neurological function scores of mice in each group were recorded. The neurological function score was evaluated using Kono's5 method[14]: those whose symptoms were between the two criteria scored 0.5 points, as shown in Table 1.

Table 1
Kono's5 scoring criteria

Score	Scoring standard
0	Not any clinical symptoms
1	The tail is drooping, weak, and has a slightly abnormal gait.
2	Bilateral hindlimbs are weak and can recover after passive turning over
3	Bilateral hindlimb paralysis, can not recover after passive turning over but can move a little after stimulation.
4	Bilateral hindlimb paralysis, accompanied by forelimb paralysis or decreased muscle strength
5	A near-death state or death.

2.5 Histopathology

On the 21st day, mice were sacrificed, and their spinal cords were fixed in a 4% (w/v) paraformaldehyde solution at 4°C for 24 hours, followed by overnight immersion in 30% sucrose at 4°C. The tissues were subsequently frozen and embedded in OCT compound (Sakura, 4583). Slices of 6 µm thickness were prepared for histological examination using HE staining and LFB staining. Inflammatory cell infiltration and demyelination were assessed under a light microscope (EclipseE200, Nikon). Inflammation was

scored as follows: no inflammatory infiltration (0 points), slight inflammatory cell infiltration (1 point), small amount of inflammatory infiltration (2 points), or large number of inflammatory cell infiltration (3 points). Demyelination was scored as follows: no observed demyelination (0 points), rare present demyelination (1 point), demyelination confined to few areas only (2 points), or extensive demyelination across a large area (3 points) [15].

2.6 Flow cytometry

The spleens of mice from each experimental group were treated with erythrocyte lysate and subsequently aseptically ground to obtain a single-cell suspension. The cells were then quantified and seeded at a density of 1×10^6 cells per well in a 96-well plate, followed by stimulation with MOG₃₅₋₅₅ (10 µg/ml) supplemented with Brefeldin A (BFA) (420601, Biolegend). The positive control was stimulated with Phorbol myristate acetate (PMA, 50 ng/ml) + ionomycin (500 ng/ml) for 11 hours. On the following day, surface marker staining was performed by incubating the cells with APC-anti-mouse CD3 (100222, Biolegend) and FITC-anti-mouse CD4 (100406, Biolegend) for 30 minutes at 4°C. After fixation and permeabilization, intracellular staining of the cells was conducted using PE-anti-mouse IFN-γ (505808, Biolegend), APC-anti-mouse IL-17A (506915, Biolegend), and PE anti-mouse IL-4 (504103, Biolegend) antibodies in darkness at 4°C for 1 hour. Data acquisition was carried out using CytoFLEX flow cytometer system (Beckman Coulter Eurocentre SA), followed by analysis using FlowJo software.

2.7 Quantitative Real-Time PCR

On the 21st day, total RNA was extracted from the spinal cords and spleen cells of mice in each treatment group using a MolPure® cell/tissue total RNA kit (19221ES50, Yeasen, Shanghai). Subsequently, cDNA synthesis was performed by reverse-transcribing 1 µg of total RNA according to the manufacturer's instructions provided with the cDNA Kit (22106 Tolobio). Real-time fluorescence quantitative polymerase chain reaction (RT-qPCR) was performed using cDNA and a 2 × Q3 SYBR qPCR Master Mix kit (22204, TOLOBIO), followed by detection on an ABI QuantStudio3 rapid real-time polymerase chain reaction system. The primer sequences used in the RT-qPCR assay are presented in Table 2.

Table 2
Sequences of Primers Used in the RT-qPCR Assay

Gene Name	Sequence
IFN- γ	FWD: 5'-TCAAGTGGCATAGATGTGGAAGA-3' REV: 5'-TGCTGATGGCCTGATTGTCT-3'
IL-17A	FWD: 5'-ACTACCTCAACCGTTCCACG-3' REV: 5'-TTCCCTCCGCATTGACACAG-3'
TNF- α	FWD: 5'-CATCTTCTCAAAATTCGAGTGACAA-3' REV: 5'-TGGGAGTAGACAAGGTACAACCC-3'
T-bet	FWD: 5'-AGCAAGGACGGCGAATGTT-3' REV: 5'-GTGGACATATAAGCGGTTCCC-3'
ROR- γ t	FWD: 5'-GACCCACACCTCACAAATTGA-3' REV: 5'-AGTAGGCCACATTACACTGCT-3'
GAPDH	FWD: 5'-AGGTCGGTGTGAACGGATTTG-3' REV: 5'-TGTAGACCATGTAGTTGAGGTCA-3'

2.8 Western blotting assay

30 mg spinal cord from different group of mice in 300ul RIPA (P0013B, Beyotime, China) plus a mixture of protease inhibitor (GRF101, EpiZyme) and phosphatase inhibitor (GRF102, EpiZyme). The mice in each treatment group were sacrificed and their spleen cells were taken aseptically and ground, and a red blood cell lysate was used to make a single cell suspension. A cell count was carried out and the cell concentration was diluted to 1×10^7 /ml. 700ul of RIPA containing protease inhibitor and phosphatase inhibitor were added to 1×10^7 cells, and the cell lysate was collected. The protein concentration was determined by a BCA assay (ZJ101). The protein was separated by 12.5% SDS-PAGE (PG113, EpiZyme) gel electrophoresis and transferred onto PVDF membranes (Millipore). After being sealed with 5% skim milk powder for 2 hours, STAT1 (1:1500, bs-1317R, Bioss), p-STAT1 (Tyr701) (1:1000, 340797, ZENBIO), STAT3 (1:1500, 251219, ZENBIO), p-STAT3 (1:9000, 381552, ZENBIO), JAK1 (1:1500, 310108, ZENBIO), p-JAK1 (Tyr1034 + Tyr1035) (1:1500, bs-3238R, Bioss), JAK2 (1:1000, R24775, ZENBIO, China), p-JAK2 (Tyr1007/1008) (1:1000, 381556, ZENBIO), IFN- γ (1:1500, bs-0480R, Bioss) and TNF- α (1:1000, 346654, ZENBIO) antibodies were added and incubated overnight at 4 °C. After washing out the primary antibody with TBST solution, the bands were incubated with secondary antibodies (1:10000, LF102, EpiZyme) for two hours. Finally, the bands were visualized using a gel imager.

2.9 Molecular Docking

The 3D structure of the BAI SDF format was downloaded from the PubChem database according to BAI CAS. The three-dimensional structure of the STAT1 protein (PDB ID: 7NUF) and the three-dimensional structure of the STAT3 protein (PDB ID: 6NUQ) were obtained from the PDB database. The interaction mode of the docking results was analysed by PyMOL2.3.0 and Discovery Studio2019 or MOE.

2.10 Pull-down assay

A biotin-labelled baicalein (BIO-BAI) probe (Fig. 7B) was synthesized by hydroxyl carboxylation using Scutellarein(4',5,6,7-Tetrahydroxyflavone) as the raw material. For specific labelling methods, please refer to our previous research [16]. C57BL/6 mice were euthanized, and aseptic grinding of the spleen was performed to prepare a single-cell suspension. After stimulation with IFN- γ (10 ng/ml) for 8 hours, the cells were subsequently lysed and incubated with or without BIO-BAI (50 μ m) for 2 hours. The mixture was then centrifuged at 12000 rpm at 4°C for 20 minutes. The supernatant was further incubated with BeyoMag™ streptavidin magnetic beads (P2151, Beyotime, China) on a rotary blender (BE-1100, Kylin-bell) at room temperature for 1 hour. The beads were washed four times before elution by adding 100 μ l of 1 $\times$ SDS PAGE loading buffer. The expression of the collected proteins was examined by Western blotting.

2.11 Immunocytochemistry

After dewaxing, dehydrating, antigen retrieval, and treatment with 3% BSA to minimize nonspecific fluorescence, the spinal cord tissue was incubated overnight at 4°C with primary antibodies: rabbit anti-IFN- γ (1:200, bs-0480R, Bioss), rabbit anti-IL-17A (1:200, GB11110, Servicebio). Subsequently, the primary antibody was washed off and a fluorescent secondary antibody labeled with FITC (1:500 dilution; A0562, Beyotime, China) was applied as the secondary antibody for an incubation period of 50 minutes at room temperature. DAPI staining was used for nuclear visualization.

The splenocytes of C57BL/6 mice were quantified and inoculation into a 6-well plate at a density of 1×10^7 cells per well. To activate the STAT1 or STAT3 signaling pathway, spleen cells were stimulated with IFN- γ (10 ng/ml) or IL-6 (100 ng/ml) (315-05, PeproTech) for 8 hours, respectively, BIO-BAI (50 μ m) was added to the culture medium to observe its effects on the expression of p-STAT1 or p-STAT3 in spleen cells. The fixed splenocyte smears were permeabilized using 0.1% Triton X-100 after fixation with 4% paraformaldehyde. Subsequently, nonspecific fluorescence was blocked with 10% goat serum. Incubation overnight at 4°C was performed using rabbit anti-p-STAT1 (Tyr701) (1:200, 340797, ZENBIO China) or rabbit anti-p-STAT3 (Tyr705) (1:200, 381552, ZENBIO China). Cy3-labeled streptavidin was used to detect biotin-labelled baicalein (BIO-BAI). After washing off the primary antibody, incubation at 37°C for 1 h was carried out with a fluorescent secondary antibody labeled with FITC (1:500, A0562, Beyotime China). Cell images were acquired using an inverted fluorescence microscope (BX3-CBH, OLYMPUS).

2.12. Statistical analysis

All data are expressed as the mean $\pm$ SD. The data were analysed by GraphPad 7.0 software. One-way ANOVA and unpaired t-test were used to compare at least three independent duplicates between the

experimental groups and the control group. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns: there was no significant difference.

3. Results

3.1 BAI effectively reduced both the clinical symptoms and pathological changes in EAE mice

To assess the impact of BAI on EAE in mice, we established the C57BL/6 EAE model using the MOG₃₅₋₅₅ peptide and initiated continuous intragastrically administration of BAI or DEX from day 3 to day 21 post-modeling (Fig. 1B). Daily observations were conducted for changes in body weight and nerve scores. The results showed that mice in the EAE group exhibited clinical symptoms starting on day 12 post-immunization. Initial manifestations included dull hair color, tail paralysis, reduced mobility, and weight loss, reaching their peak on day 18. Mice receiving BAI gavage intervention experienced a delayed onset of symptoms until day 15, characterized by weak tails or dragging movements close to the ground but still capable of being lifted. No disease manifestation was observed in the dexamethasone (DEX) treatment group (Figs. 2A and B).

The clinical manifestations of EAE primarily involve inflammatory infiltration. To further investigate the effect of BAI on CNS inflammation, mice were examined on day 21 post-immunization to assess spinal cord tissue inflammatory changes using HE staining and demyelination using LFB staining. As illustrated in Fig. 2C, compared to the control group, the EAE model group exhibited diffuse infiltration of inflammatory cells within the spinal cord. Both BAI treatment and DEX treatment ameliorated the severity of these lesions. Furthermore, significant demyelination was observed in the spinal cords of the EAE model group compared to the control group. Results from LFB staining demonstrated that both BAI and DEX reduced spinal demyelination in mice (Fig. 2D). The scores for both inflammation and demyelination were significantly lower in both the EAE + BAI group and EAE + DEX group when compared to untreated EAE mice. The findings suggest that BAI effectively mitigates the clinical symptoms of EAE, while also reducing inflammatory infiltration and demyelination.

3.2 The administration of BAI led to a reduction in the proportion of Th1 and Th17 cells

CD4⁺T cells are important sources of IFN- γ , TNF- α and IL-17A, which play key roles in the disease progression of EAE mice [1, 3]. Therefore, we evaluated the effect of BAI on the proportions of Th1 and Th17 cells. On the 21st day post-immunization, splenocytes were collected from each group of EAE mice and analyzed for the expression of IFN- γ and IL-17A in CD4⁺ T cells using flow cytometry. The results demonstrated a significantly higher proportion of Th1 (CD3⁺CD4⁺IFN- γ ⁺) and Th17 (CD3⁺CD4⁺IL-17A⁺) cells in the splenocytes from the EAE model group compared to that of the control group. The proportion of Th1 and Th17 cells in the EAE + BAI group was significantly lower than that in the EAE model group. These findings indicate that BAI administration effectively reduced the levels of both Th1 and Th17 cells in EAE mice (Fig. 3).

3.3 The administration of BAI resulted in a reduction of Th1 and Th17 related inflammatory cytokine expression in the spinal cord of EAE mice

The aforementioned results demonstrated a significant reduction in the incidence and inflammatory infiltration of EAE following BAI intervention. Numerous studies have indicated that autoimmune T cells, including Th1 and Th17 cells, play an important role in promoting inflammation within the central nervous system during the development of MS/EAE by secreting various proinflammatory cytokines [16, 17]. Th1 cells primarily produce IFN- γ , TNF- α and IL-2, while Th17 cells produce IL-17 and IL-22[18]. These factors contribute significantly to inflammatory and autoimmune responses. In order to further investigate the anti-inflammatory effects of BAI on the onset of EAE in mice, we examined the transcriptional levels of three proinflammatory cytokines(IFN- γ , TNF- α and IL-17A) in the spinal cords of EAE mice, as well as transcription factors T-bet and ROR γ t, which play key roles in Th1 and Th17 differentiation, respectively.

The results showed that the mRNA expression levels of IFN- γ , TNF- α and IL-17 in the spinal cord of the EAE group were significantly up-regulated compared with the control group, while the mRNA expression levels of the three cytokines in the spinal cord of the BAI treated mice were significantly lower than those of the EAE model group (Fig. 4A). At the same time, the levels of T-bet related to Th1 cell differentiation and ROR- γ t mRNA related to Th17 differentiation in the spinal cord of EAE mice were significantly increased. BAI intervention could significantly down-regulate the expression level of T-bet mRNA, but there was no significant difference between the expression level of ROR- γ t mRNA and that of EAE model group (Fig. 4A). At the same time, we observed a significant increase in the protein expression levels of IFN- γ and TNF- α in the spinal cords of the EAE model group. After BAI treatment, there was a notable decrease in the expression of these two inflammatory factors, which was consistent with the findings from the EAE + DEX group (Fig. 4B). Additionally, immunofluorescence results demonstrated that compared to the model group, the BAI group exhibited significantly lower expression levels of IFN- γ and IL-17A in the spinal cord (Fig. 4C).

3.4 STAT1/3 signalling pathways were involved in the inhibition of Th1 and Th17 cells by BAI

Activation of the JAK/STAT pathway plays a key role in the differentiation and inflammatory cytokine expression in Th cells[19]. Therefore, we studied whether BAI regulates the inflammatory response of Th cells by inhibiting the JAK/STAT signalling pathway. The results showed that the protein levels of p-STAT1 and p-STAT3 in the spleen cells of the EAE model mice were significantly increased. After the BAI treatment, the protein levels of p-STAT1 and p-STAT3 were significantly decreased, but the phosphorylation levels of JAK1 and JAK2 in each group were not significantly different (Fig. 5A). Further studies showed that in the spinal cords of the mice in each group, the phosphorylation levels of STAT1 and STAT3 in the model group increased, while those in the EAE + BAI group decreased significantly, which was consistent with the results in the EAE + DEX group (Fig. 5B). In addition, we also detected the iconic transcription factors of Th1/17 cells. In the splenocytes of the EAE model mice, the mRNA levels of

T-bet and ROR- γ t increased significantly. After the BAI treatment, the mRNA expression levels of T-bet and ROR- γ t decreased significantly, and the EAE + DEX group was consistent with these results (Fig. 5C). In summary, BAI may inhibit the inflammatory reaction of Th1 and Th17 cells by targeting STAT1/3.

3.5 BAI inhibited the nuclear translocation of p-STAT1/3 by targeting STAT1/3

To further explore how BAI suppresses the function of p-stat1/3, we used a molecular docking technique to predict the binding energy of BAI and STAT1/3. Molecular docking reliably occurs when the binding energy is less than -5.0 kcal/mol[20]. The structural and functional fragments of STAT protein are mainly composed of the following domains (Fig. 6A): the N-terminal domain, the coiled-coil domain, the DNA binding domain, the SH3 domain, the SH2 domain and the transactivation activation domain (TAD). The SH2 domain, a C-terminal transcriptional domain with highly conserved phosphorylated tyrosine (Y) and serine (S) residues, is necessary for activating STAT [21]. Therefore, the SH2 domains of STAT1 and 3 were selected for molecular docking with BAI. The results showed that the SH2 binding energy of BAI with STAT1 was -8.0 kcal/mol, which indicates a good binding effect. BAI interacted with the STAT1 protein mainly through the formation of hydrogen bonds and hydrophobic forces with GLU559 and hydrophobic interactions with LYS567, LEU564, LYS636 and GLU609. The SH2 binding energy of BAI and STAT3 was -5.67 kcal/mol, which predicted that they had a binding effect. Glu612, Lys531, Glu530, Ser590, Glu592, Gly536, Lys591, Val537, and Ile589 all played important roles in the binding of BAI and STAT3 (Fig. 6C). To verify the predicted results of the molecular docking, we synthesized a BIO-BAI probe (Fig. 6B) for pull-down experiments [15]. We found that STAT1, p-STAT1, STAT3 and p-STAT3 could be isolated from splenocyte lysates by adding the BIO-BAI probe. Meanwhile, it could not be isolated from the splenocyte lysate without the BIO-BAI probe. After incubation with BAI and splenocyte lysate for 1 h, the BIO-BAI probe and splenocyte lysate were added and incubated for 1 h, and the protein expression of STAT1, p-STAT1, STAT3 and p-STAT3 decreased (Fig. 6D). Therefore, the interaction between BAI and STAT1/3 was further confirmed.

To verify how the binding of BAI and STAT1/3 affects JAK-STAT signalling, we used Cy3-labelled streptavidin (streptavidin-specific binding biotin) to locate the intracellular BIO-BAI probe in an immunofluorescence assay. The results showed that BIO-BAI, p-STAT1 or p-STAT3 were co-located in the cytoplasm, which further confirmed that BAI can directly bind to STAT1/3. Moreover, under inactive conditions (not stimulated by IFN- γ /IL-6), a small amount of p-STAT1 or p-STAT3 was mainly present in the cytoplasm. After IFN- γ stimulation, both p-STAT1 and p-STAT3 were localized in the nucleus. However, the BIO-BAI group showed that BIO-BAI and p-STAT1 or p-STAT3 were mainly co-localized in the cytoplasm, close to the cell membrane (Fig. 7), which confirmed that BAI can inhibit the nuclear translocation of p-STAT1 and p-STAT3. In summary, after STAT1/3 bound to BAI, BAI blocked p-STAT1/3 signal transduction of p-STAT1/3 by inhibiting its nuclear import.

4. Discussion

MS is a demyelinating disease of the central nervous system that threatens human health and often occurs in young adults, especially in women. In the EAE model, similar to MS, inflammatory cells destroy part of the nervous system, resulting in a variety of clinical symptoms[22]. Our study showed that treatment with BAI significantly alleviated the clinical symptoms of EAE mice (Figs. 2A and B). In addition, the BAI treatment significantly reduced the typical pathological changes of inflammatory cell infiltration and demyelination in the spinal cord of the EAE mice (Figs. 2C and D). These results further confirm the therapeutic effect of BAI on EAE mice.

In MS/EAE, inflammatory cell infiltration is an important cause of CNS inflammation, in which Th cells play a key role in promoting inflammation [23]. Peripheral self-reactive CD4⁺ T cells are activated, migrate through the blood-brain barrier and enter the CNS parenchyma. Our results showed that the infiltration of inflammatory cells in the spinal cord of the mice treated with BAI was significantly less than that of the EAE model group (Fig. 2C). We further found that BAI downregulated the proportion of Th1 and Th17 cells in the EAE mice (Fig. 3). Infiltrating T cells produce inflammatory cytokines, such as IFN- γ and TNF- α produced by Th1 cells and IL-17A produced by Th17 cells, these cytokines play an important role in promoting inflammation[24, 25], resulting in myelin destruction and axon loss. In our study, the levels of IFN- γ , TNF- α , IL-17A and T-bet mRNA in the spinal cords of the mice in the EAE model group were significantly increased, while the levels of IFN- γ , TNF- α , IL-17A and T-bet mRNA in the EAE + BAI group were significantly decreased, However, the mRNA expression level of ROR- γ t in EAE group with no significant statistical differences between the treatment groups(Figure 4A). Additionally, the levels of IFN- γ and TNF- α protein in the spinal cords of the mice in the EAE model group were significantly increased, and the levels of IFN- γ and TNF- α protein decreased significantly after the BAI treatment Figure 4B . The immunofluorescence results also showed that the expression of IFN- γ and IL-17A in the spinal cord of BAI group was significantly lower than that of the model group Figure 4C . T-bet and RoR- γ t are key transcription factors for Th1 and Th17 cells, respectively. The expression levels of these transcription factors are related to the release of proinflammatory cytokines, including IFN- γ and IL-17A[26, 27]. Our results showed that the expression levels of T-bet and ROR- γ t mRNA in the splenocytes of the EAE model group were significantly increased, while the BAI treatment decreased the expression of T-bet and RoR- γ t mRNA in the spleens of the EAE mice (Fig. 5C). Taken together, BAI inhibited Th1 and Th17 inflammatory responses in the EAE mice by reducing cytokine secretion.

Previous studies have found that the cytokine secretion of T cell subsets is regulated by the JAK-STAT pathway. STAT1 is a signal-transduction molecule that promotes Th1 cell secretion of IFN- γ and TNF- α , and STAT3 is a signal-transduction molecule by which Th17 cells promote IL-17A secretion[28]. Previous studies from our group have shown that BAI can inhibit the polarization of macrophages to M1 by targeting STAT1 signal transduction [12, 29]. In the molecular structure of STAT1 and STAT3, the SH2 domain is a conserved region related to phosphorylation and activation [30]. In this study, we also found that BAI not only inhibited STAT1 phosphorylation in T cells but also inhibited STAT3 phosphorylation (Figs. 5A and B). Therefore, we hypothesise that BAI may directly bind to the SH2 domain of STAT1/3 through molecular docking, thus affecting the signal transduction of STAT1/3. We used BIO-BAI to verify

whether the two molecules were directly bound (Fig. 6B). Through pull-down and immunofluorescence experiments, it was preliminarily confirmed that BAI can bind to STAT1/3 (Figs. 6C and D). Because the phosphorylation of STAT proteins on some residues (such as serine and threonine) is necessary for their nuclear translocation and transcriptional activities[31], we examined the nuclear distribution of STAT1/3 to confirm that BAI inhibited the activation of STAT1/3. Our results showed that after 8 hours of IFN- γ /IL-6 stimulation, p-STAT1/3 entered the nucleus, while the BAI treatment prevented p-STAT1/3 from entering the nucleus and remained in the cytoplasm, thus losing its signal transduction function (Fig. 7). Therefore, BAI inhibited the secretion of inflammatory cytokines in Th1/17 cells by targeting STAT1/3, which further improved the clinical condition of the EAE mice, thus providing a potential dietary supplement for the clinical treatment of MS in the acute inflammatory phase.

In conclusion, our study showed that BAI can alleviate the clinical symptoms of EAE mice and improve the inflammatory infiltration of the nervous system in EAE mice. Moreover, this anti-inflammatory effect occurred through targeting STAT1/3 thus inhibiting the inflammatory response of Th1/17 cells. These results further suggest that BAI can be used as a dietary supplement to alleviate the inflammatory response in the acute phase of MS inflammation. In addition, this study provides a road map to further examine the anti-inflammatory mechanism of other flavonoids.

Abbreviations

Abbreviations	Full name
Th	Helper T cells
MS	Multiple sclerosis
EAE	Experimental encephalomyelitis
BAI	Baicalein
CNS	Central nervous system
DEX	Dexamethasone
MOG	Myelin oligodendrocyte glycoprotein
PTX	Pertussis toxin
HE	Haematoxylin eosin
LFB	Luxol Fast Blue
TAD	Transactivation activation domain
Y	Tyrosine
S	Serine

Declarations

CRediT authorship contribution statement

Xiaoran Ma: Investigation, Formal analysis, Writing - original draft, Writing-review & editing. Tiantian Wang: Investigation, Formal analysis, Writing-original draft, Writing-review & editing. Chenglin Li: Investigation. Xihui Jia: Investigation. Huimin Tong: Investigation. Meng Liu: Investigation. Shuang Wang: Conceptualization, Methodology. Ling Li: Conceptualization, Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics approval and Consent to participate

The experimental protocol of our study was performed in accordance with the Guide for the Care and Use of Laboratory Animals and approved by Ethics Committee of Medical College of Qingdao University (approval no.:2022504).

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Figures

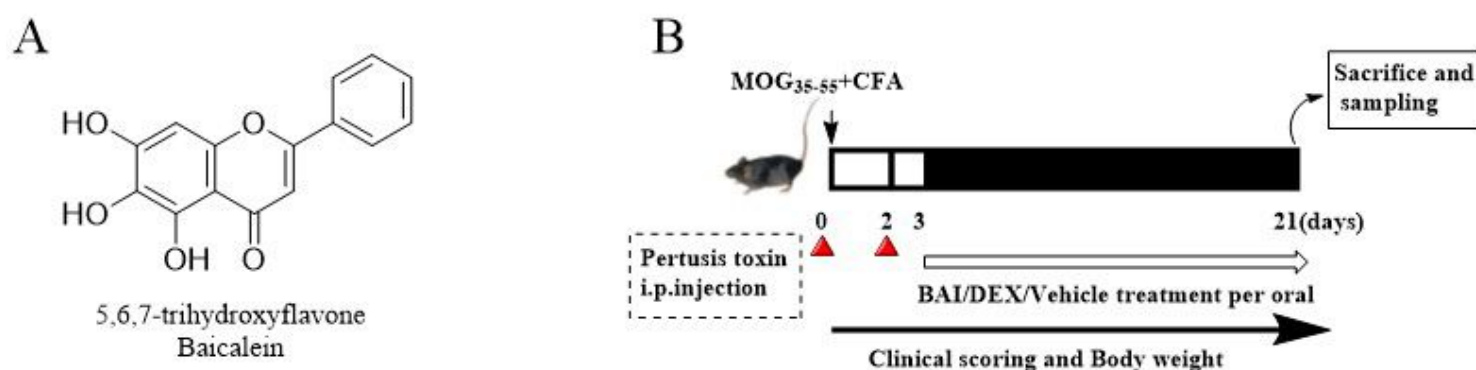


Figure 1

The molecular structure of BAI and the flow chart of the administration of the experimental model. (A) The molecular structural formula of BAI. (B) Flow chart of the administration of the experimental model.

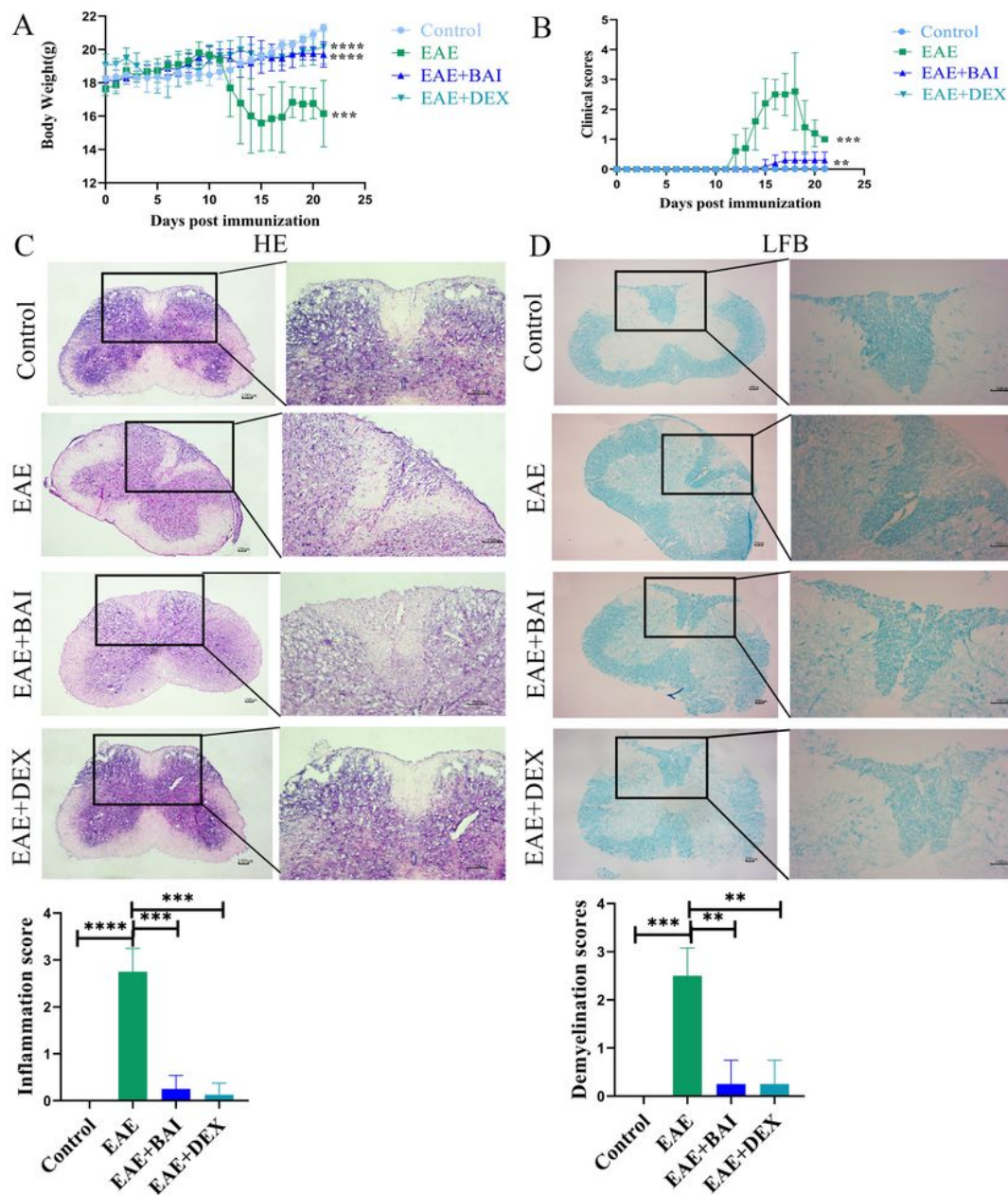


Figure 2

BAI effectively reduced clinical symptoms and pathological changes in the EAE mice. The C57BL/6 mice were immunized with CFA+MOG35-55 peptide to induce EAE. Beginning on the 3rd day after immunization, BAI, DEX or 0.5% sodium carboxymethyl solution was administered intragastrically every day until the 21st day. (A) Body weight. (B) Neurological function scores. (C) HE staining and inflammation scores of the spinal cords from the mice of each group. (D) LFB staining and demyelination

scores from the spinal cord of the mice in each group. Scale bar, 100 μ m. One-way ANOVA and unpaired t-test were used for pairwise comparisons, and all data are expressed as the mean $\pm$ SD. ns, $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. The experiment was repeated 3 times.

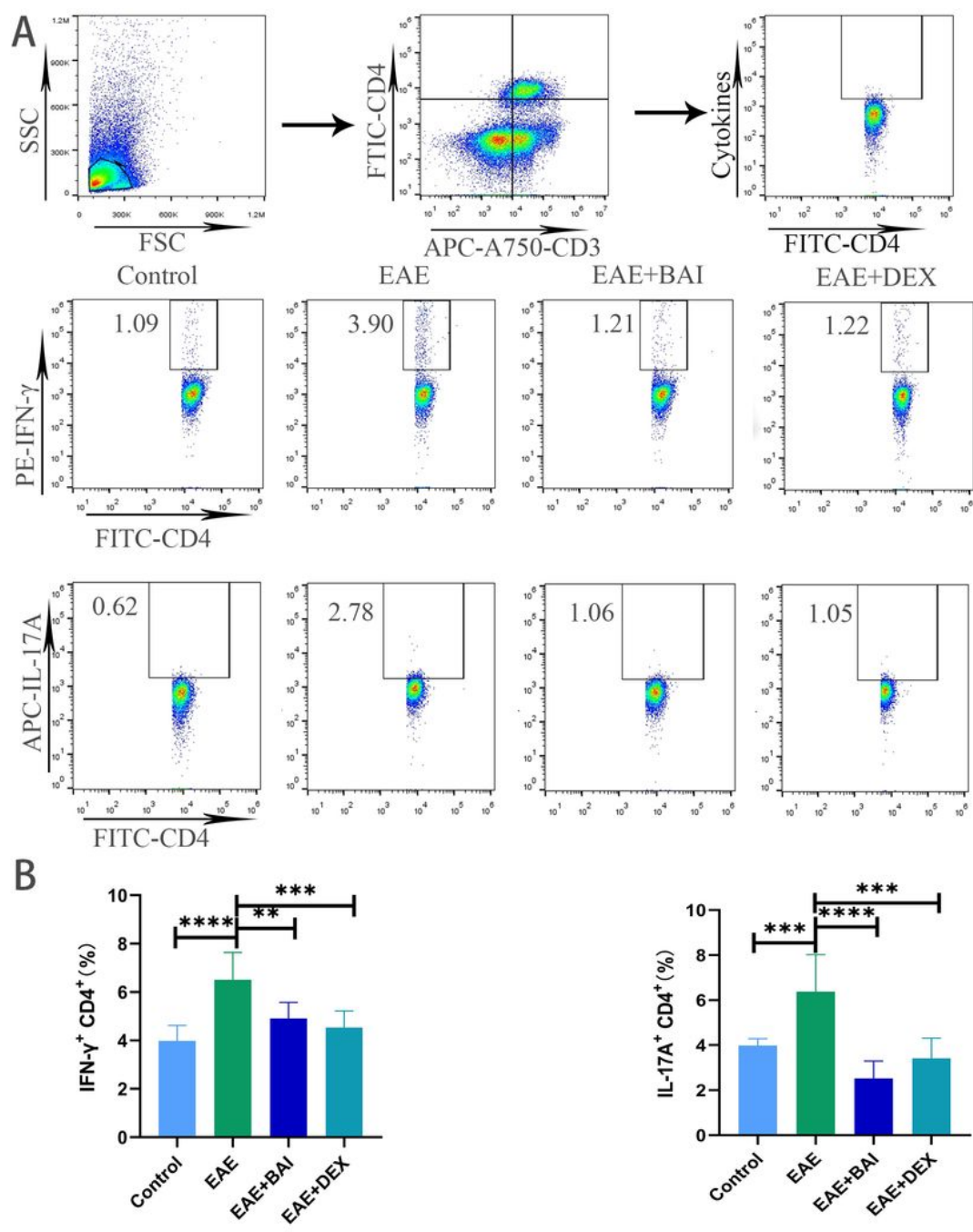


Figure 3

Effect of BAI on the percentage of Th1/Th17 subsets in the splenocytes of the EAE mice. On the 21st day after immunization, the splenocytes from each group were stimulated with 10 µg/ml MOG₃₅₋₅₅ for 24 hours. Th1 and Th17 Th2 cell subsets were detected by IFN-γ and IL-17A intracellular staining. (A) IFN-γ and IL-17A accounted for the percentage of CD4⁺ positive cells. (B) Statistical analysis of the percentages of IFN-γ and IL-17A in CD4⁺ positive cells. One-way ANOVA and unpaired t-test were used for pairwise comparisons, and all data are expressed as the mean ± SD. Ns, $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. The experiment was repeated 9 times.

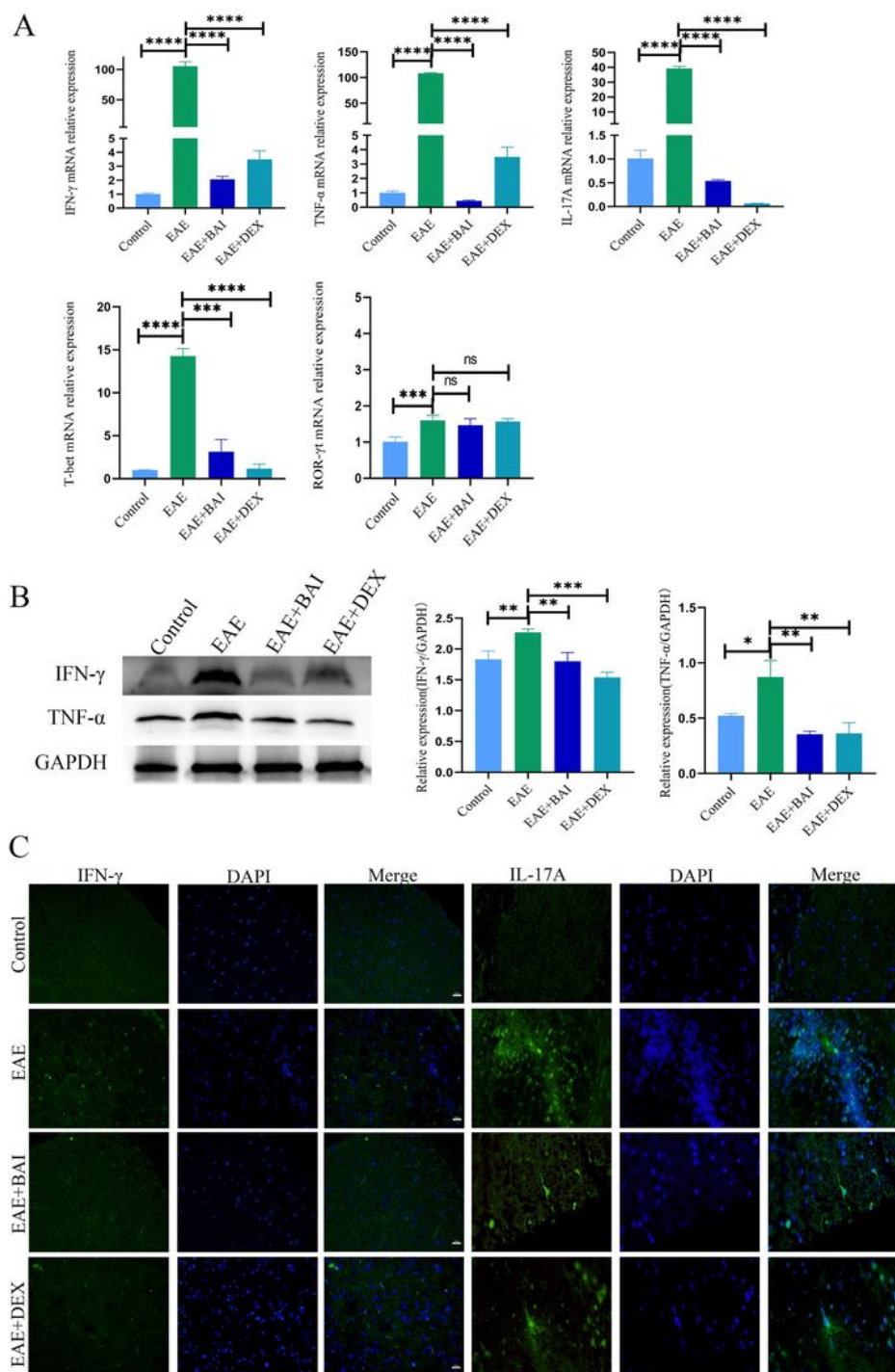


Figure 4

BAI could reduce the expression of inflammatory cytokines in the spinal cord of EAE mice. The mice were killed on Day21, and the spinal cord of each group was collected for western blot test and RT-qPCR. A The mRNA expressions of TNF- α , IL-17A, IFN- γ , T-bet and ROR- γ t in the spinal cord of mice in each group were statistically analyzed. B The protein expression of IFN- γ and TNF- α in the spinal cord of each group, and the protein expression of IFN- γ and TNF- α in each group were statistically analyzed. C

Immunofluorescence was used to detect the expression of IFN- γ and IL-17A in spinal cord tissue of mice in each group. One-way ANOVA and unpaired t-test were used for pairwise comparison, and all data were expressed as Mean $\pm$ SD. ns, $P > 0.05$ * $P < 0.05$ ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. The experiment was repeated 3 times.

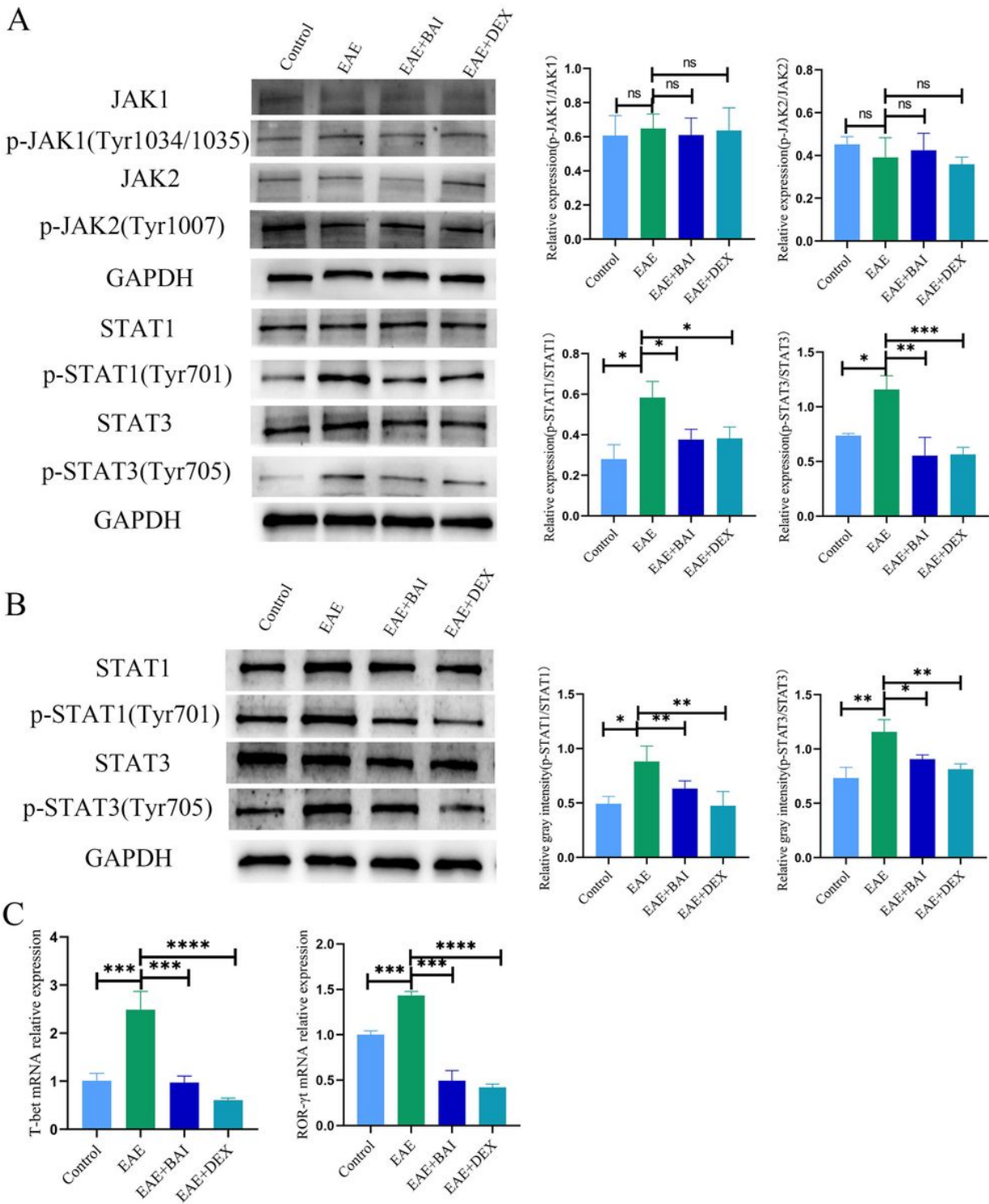


Figure 5

BAI inhibited the inflammatory response of Th1 and Th17 cells by targeting STAT1/3. The mice were sacrificed on Day 21, and the monocytes of the spinal cords and spleens from mice in each group were collected. (A) The protein expression levels of JAK1, p-JAK1, JAK2, p-JAK2, STAT1, p-STAT1, STAT3 and p-STAT3 in the splenocytes of the mice in each group were analysed statistically. (B) The protein expression levels and statistical analysis of STAT1, p-STAT1, STAT3 and p-STAT3 in the spinal cords of the mice in each group. (C) The mRNA expression levels of T-bet and ROR- γ t in the splenocytes of the mice in each group. One-way ANOVA and unpaired t-test were used for pairwise comparisons, and all data are expressed as the mean $\pm$ SD. ns, $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. The experiment was repeated 3 times.

Figure 6

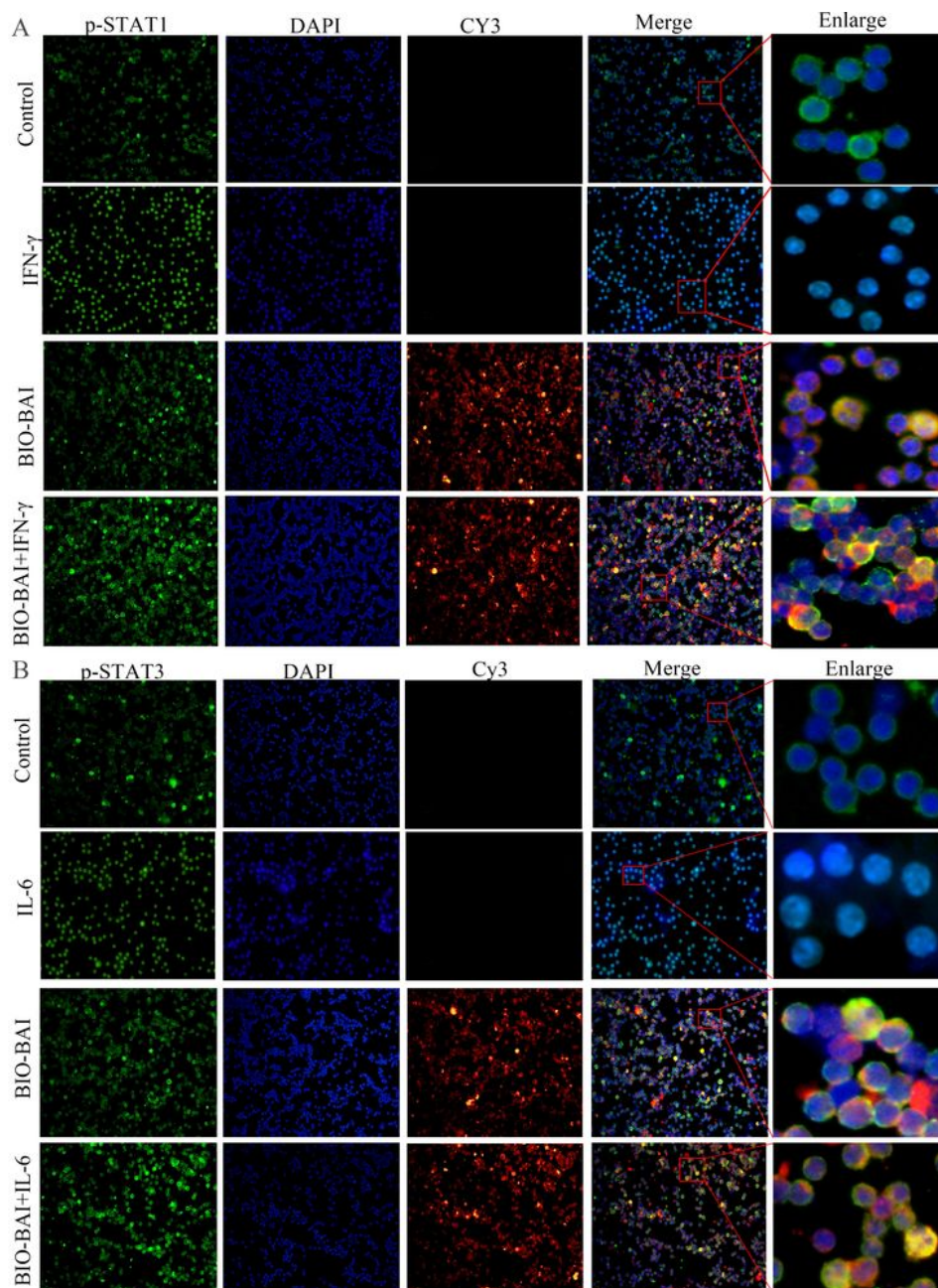


Figure 7

BAI inhibited p-STAT1/3 nuclear translocation. A single-cell suspension was prepared from the spleens of the healthy C57BL/6 mice and treated with/without IFN- γ (10 ng/ml)/IL-6 (100 ng/ml) and/or BIO-BAI (50 μ m) for 8 hours. (A and B) Immunofluorescence was used to detect the localization of BIO-BAI and p-STAT1/3.