

## Original Article

# Immunoregulative effect of Buyang Huanwu decoction in experimental autoimmune encephalomyelitis

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**Abstract:** Buyang Huanwu Decoction (BYHWD), a classic formula, is widely used for treating a variety of disorders, including stroke-induced disability and paralysis in traditional Chinese Medicine. Clinical studies have shown that BYHWD had a therapeutic effect in ameliorating the severity of multiple sclerosis (MS), and reducing the number of relapses. However, its mechanism of action in treating MS remains undefined. In this study, we investigate the therapeutic effects and possible mechanism of action of BYHWD in experimental autoimmune encephalomyelitis (EAE), an animal model that closely imitates many characteristics of MS. Our results showed that BYHWD effectively ameliorated the clinical severity of EAE and CNS inflammatory infiltration. BYHWD also modulated peripheral immune responses. Further, we showed that BYHWD inhibited the expression of TLR-4/Myd88/NF- $\kappa$ B signaling pathway. Taken together, our results demonstrate the therapeutic potential of BYHWD in EAE, accompanied by the inhibition of CNS inflammation and the regulation of immune responses. The therapeutic efficacy of BYHWD will contribute to the development of clinical applications in MS.

**Keywords:** Buyang Huanwu decoction, experimental autoimmune encephalomyelitis (EAE), TLR-4/NF- $\kappa$ B signaling pathway, immunomodulation

## Introduction

Multiple sclerosis (MS) is an immune-mediated chronic inflammatory demyelinating disease of the central nervous system (CNS), destroying the myelin and the axon in variable degrees [1]. The pathological hallmarks of MS are multifocal inflammation, oligodendrocyte loss, demyelination and axonal degeneration [2]. During the development of MS, auto-reactive T cells and macrophages which are stimulated in peripheral immune system, infiltrate into the CNS and produce inflammatory molecules, leading to oligodendrocyte death and axonal damage in the CNS [3, 4].

The etiology and pathogenesis of MS are complex, and the current treatment is still the lack of specificity and effectiveness. In China, Traditional Chinese Medicine (TCM) has some characteristics and advantages in the treatment of

MS, including multiple targets, the overall regulation, synergistic effect and fewer side effects. Buyang Huanwu Decoction (BYHWD), a well-known traditional formula, invigorates the body, enhances blood circulation and meridian circulation, and has been traditionally used in the treatment of stroke and paralysis for centuries [5]. BYHWD has long been used to improve the recovery of the neurological function in patients with stroke by inducing neuroprotective effects against cerebral ischemia/reperfusion (CIR) injury [6-8], and to promote the growth potential during peripheral neural regeneration [9, 10]. Previous genomics study reported that BYHWD protected mice against ischemic stroke by downregulating inflammation, apoptosis, angiogenesis, and blood coagulation-related genes, as well as upregulating neurogenesis and the nervous system's development-related genes [11]. Modern pharmacological studies reveal that some active ingredients of BYHWD exert

neuroprotective effects by inhibiting inflammatory response and oxidative stress.

Experimental autoimmune encephalomyelitis (EAE) is an established model of MS that has been used in drug development [12]. In the present study, we observed the therapeutic potential of BYHWD by oral administration, and explored possible mechanisms of action.

## Materials and methods

### *Animals*

Female C57BL/6 mice (8-10 weeks and 18-20 g) were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). All mice were housed under pathogen-free conditions and kept in a reversed 12:12-h light/dark cycle in a temperature controlled room (25±2°C) for one week prior to experimental manipulation. All animal protocol was performed according to the International Council for Laboratory Animal Science guidelines. The study was conducted by the Council for Laboratory and Ethics Committee of Shanxi Datong University, Datong, China.

### *Herbal materials*

BYHWD consists of the following ingredients: Radix Astragali (120 g), also known as huáng qí, is the root of *Astragalus membranaceus* (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao; Radix Angelicae Sinensis (6 g), also known as dang gui is the root of *Angelica sinensis* (Oliv.) Diels; Radix Paeoniae Rubra (4.5 g), also known as chi shao, is the root of *Paeonia lactiflora* Pall; Rhizoma Chuanxiong (3 g), also known as chuan xiong, is the root of *Ligusticum chuanxiong* Hort.; Semen Persicae (3 g), also known as tao ren, is the dry ripe seed of *Prunus persica* (L.) Batsch; Flos Carthami (3 g), also known as hong hua, is the flower of *Carthamus tinctorius* L.; and Lumbricus (3 g), also known as di long and *Pheretima aspergillum* (perrier) [13]. All the herbs had been extracted according to standard methods outlined in the Chinese Pharmacopoeia [14]. The mixture was decocted to yield a final concentration of 2 g crude drug/ml, which was stored at 4°C for further use [15].

### *Induction and clinical evaluation of EAE*

Mouse myelin oligodendrocyte glycoprotein peptide<sub>35-55</sub> (MOG<sub>35-55</sub>, MEVGW YRSPFSRVVHLY-

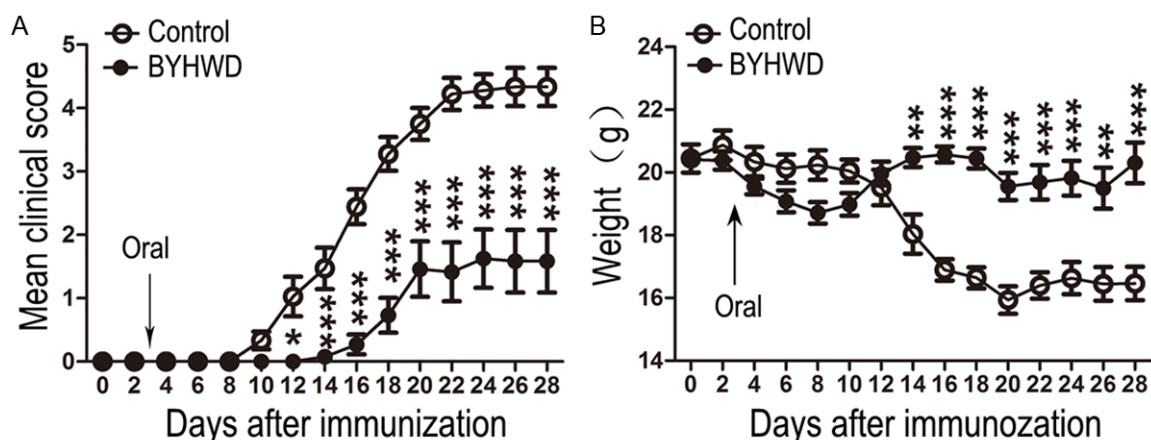
RNGK) was synthesized in an automatic synthesizer (CL Bio-Scientific Company, Xi'an, China). Purity of the peptide was >95% as determined by HPLC. Chronic EAE was induced by subcutaneous immunization on the upper dorsal flanks with 250 µg/mice of MOG<sub>35-55</sub> in Freund's complete adjuvant (Sigma, USA) supplemented with 350 µg/mice of inactivated *Mycobacterium tuberculosis* (strain H37 RA; Difco). Mice were then injected with 300 ng/mice of pertussis toxin (Enzo Life Sciences, USA) via abdominal cavity at the same time of immunization and again 48 h later. Animals were evaluated for clinical score and weight daily (from day 1 to day 28 p.i.) in a blinded fashion by at least two investigators. Clinical score of EAE was graded according to the following criteria: 0. healthy; 1. limp tail; 2. ataxia and/or paresis of hind limbs; 3. paralysis of hind limbs and/or paresis of forelimbs; 4. tetraparalysis; and 5. moribund or death. Once the clinical score of EAE reached 3, we provided special care, i.e., softening the food with water in a dish, adding nutrients such as egg, and putting the dish at the bottom of the cage, making it easy for mice to obtain food, water and nutrition.

### *Administration of BYHWD*

Mice were divided into 2 groups, i.e., BYHWD-treated (BYHWD group) and saline control group (control group) (n = 12 each group). BYHWD was removed from the 4°C refrigerator to 37°C thermal cycling chamber for 20 min, so that BYHWD was suitable to be taken orally and would not hurt the stomach. Mice were taken orally at 1000 µl (100 g/kg) every day on day 3 post-immunization (p.i.) till day 27 p.i. Saline was given to the control group in a similar manner.

### *Histology and immunohistochemistry*

On day 28 p.i., mice were perfused with saline and 4% buffered paraformaldehyde. Spinal cords (lower thoracic-lumbar) were sliced (10 µm), and pathological changes were detected by hematoxylin and eosin (H&E) staining and Luxol Fast Blue (LFB) staining. For immunohistochemistry, non-specific binding was blocked with 1% bovine serum (Sigma, USA) at room-temperature (RT) for 30 min. The sections we-



**Figure 1.** The administration of BYHWD ameliorates the severity of EAE. Chronic EAE was induced in C57BL/6 mice with MOG<sub>35-55</sub> and treated with oral administration of BYHWD and saline (n = 12/each group). A. Mean clinical score; B. Mean body weight. The comparison in each time point was separately analyzed by Mann Whitney U test after nonparametric Kruskal-Wallis test. Differences between two groups were analyzed using Student's t test \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

**Table 1.** The clinical evaluation of EAE mice

Group	n	Mean onset date	Mean maximum score	Cumulative clinical score
Control	12	11.92±1.80	4.12±0.58	58.69±4.34
BYHWD	12	20.70±2.49***	1.05±1.19***	17.90±9.65***

Control vs. BYHWD \*\*\*P<0.001.

re incubated at 4°C overnight with anti-CD4 (Sigma, USA), anti-CD68 (BD Bioscience, USA), and then incubated with corresponding secondary antibodies at RT for 2 h. As a negative control, additional sections were treated similarly, but the primary antibodies were omitted. Results were visualized under fluorescent microscopy by Image-Pro Plus software in a blinded fashion. Quantification was performed on three sections per mouse, and five mice per group were analyzed.

#### Western blot analysis

On day 28 p.i., mice were only perfused with saline, and spinal cords were homogenized with a glass homogenizer using RIPA Lysis Buffer (Beyotime Institute of Biotechnology, PR China) supplemented with protease inhibitors. The homogenates were centrifuged at 12,000×g for 20 min at 4°C, and the supernatants were collected. Protein concentration was measured by BCA kit (Beyotime Institute of Biotechnology, PR China). Equal amounts of protein (30 µg) were loaded onto SDS-polyacrylamide gels and transferred onto a nitrocellu-

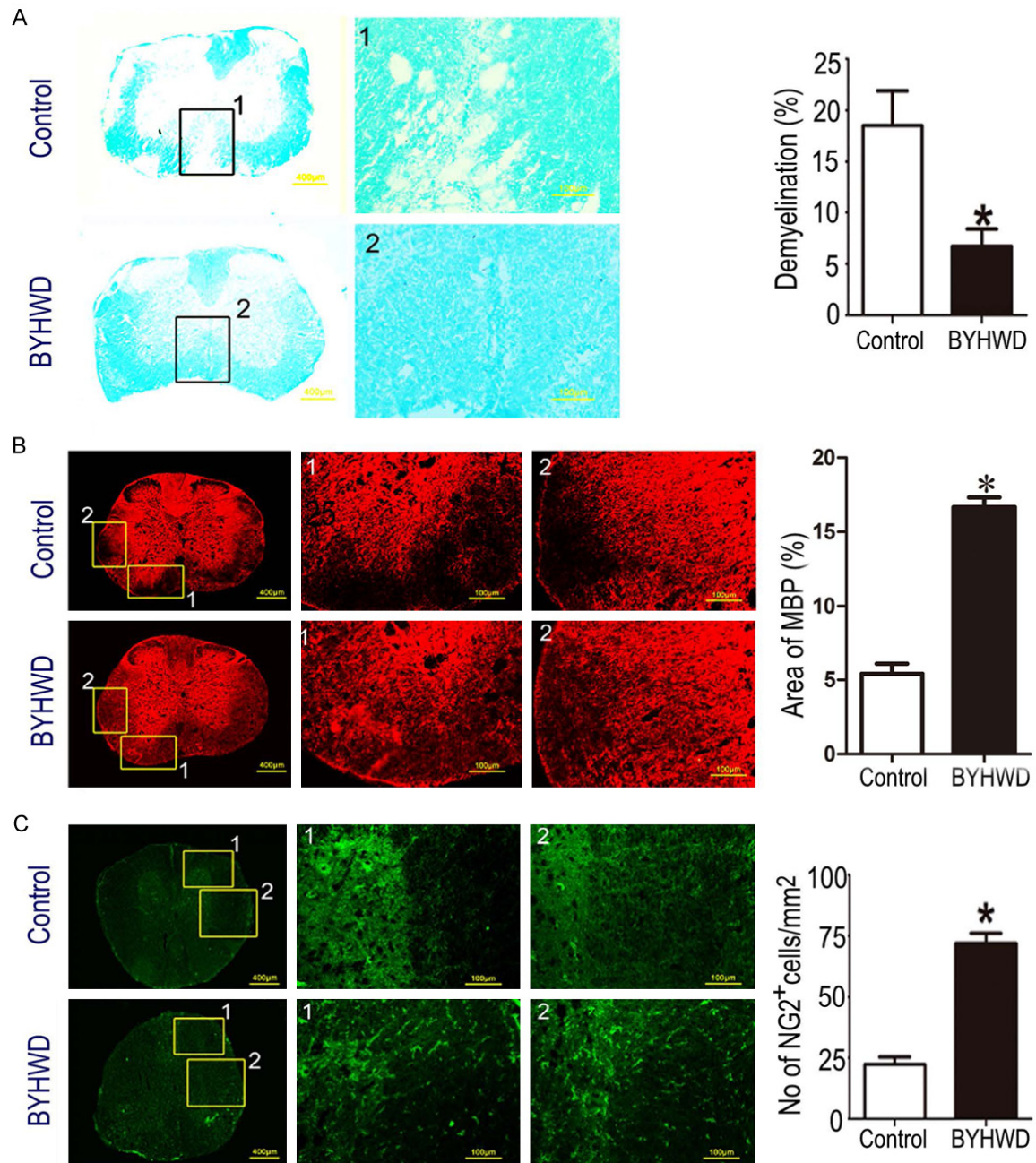
lose membrane (Millipore) after electrophoresis. After blocking with 5% milk at RT for 1 h, the membranes were incubated at 4°C overnight with primary antibodies against TLR4, p-NF-κB (p65), (1:1000, Cell Signaling Technology, USA), Myd88 (1:1000, Abcam, USA), and β-actin (1:10000, Cell Signaling Technology, USA).

In the following day, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:10,000, Earthox LLC, USA) at RT for 1 h. Immunoblots were developed with an enhanced chemiluminescence system (GE Healthcare Life Sciences) and measured using Quantity Software (Bio-Rad, Hercules, CA, USA). β-actin was used as the optical density of internal reference.

#### Flow cytometry analysis

Splenocytes were prepared on day 28 p.i. by pushing spleen through a sterile 70-µm nylon cell strainer (BD, Franklin Lakes, NJ) to generate the suspension of mononuclear cells (MNCs). For intracellular staining, MNCs were stained for 20 min at RT in 0.3% saponin/1% BSA-PBS buffer with the following panel of antibodies: FITC-CD4 and PE-IL-10, PE-CD25, PE-TGF-β, PE-IFN-γ, PE-IL-17 (eBioscience), as well as Alexa Fluor 488-CD11b (BD, USA) and PE-CD206 (eBioscience), PE-IL-12 (PharmLia). At least 10,000 events were collected using flow cytometer (BD Biosciences, USA) and data were analyzed using CellQuest software.





**Figure 2.** Spinal cords from lumbar regions were harvested for histologic and immunohistochemistry staining. A. Luxol Fast Blue; B. MBP; C. NG2. Data represent mean standard error (SEM) from seven or eight mice in each group. Differences between two groups were analyzed using Student's t test. Scale bars, 400  $\mu$ m and 100  $\mu$ m, respectively. \* $P < 0.05$ .

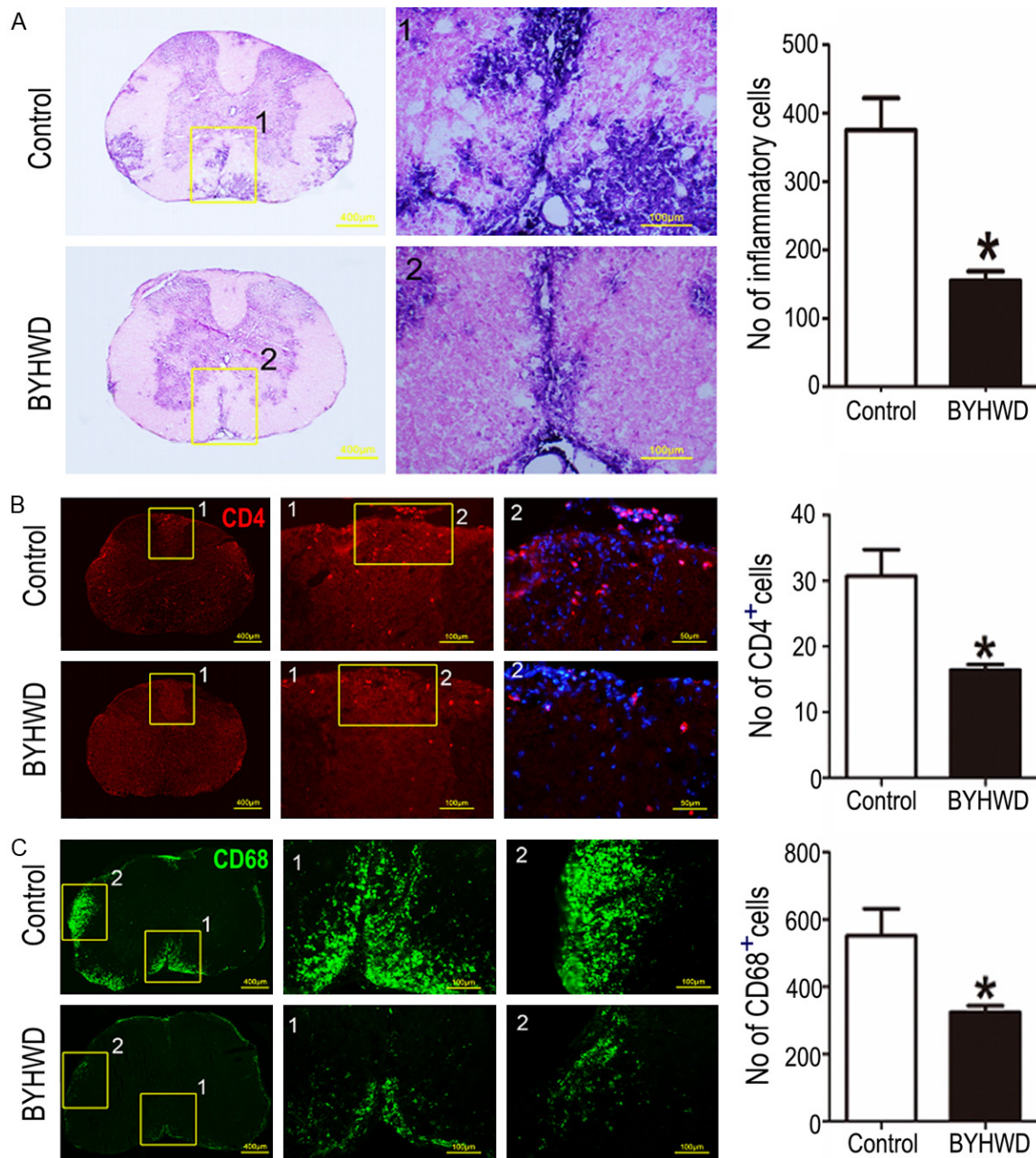
#### Cytokine ELISA assay

On day 28 p.i., mice were sacrificed and spleens were removed under aseptic conditions. Splenic MNCs ( $5 \times 10^6$ /ml) were incubated for 48 h at 37°C in the presence of MOG<sub>35-55</sub> (10  $\mu$ g/ml). Supernatants were harvested and measured for cytokine concentrations of IL-1 $\beta$  (Invitrogen Inc, USA), IL-6, TNF- $\alpha$ , (Pepro tech Inc, USA), by

a sandwich ELISA kits following the manufacturer's instructions. Determinations were performed in duplicate in 3 independent experiments. The results were expressed as pg/ml.

#### Statistical analysis

GraphPad Prism 5 software (Cabit Information Technology Co., Ltd., Shanghai, China) was



**Figure 3.** Spinal cords from lumbar regions were harvested for histologic and immunohistochemistry staining. A. Hematoxylin and Eosin (HE) staining; B. CD4 T cells; C. CD68 macrophages in the white matter. Data represent mean standard error (SEM) from seven or eight mice in each group. Differences between two groups were analyzed using Student's t test. Scale bars, 400 µm and 100 µm, respectively. \*P<0.05.

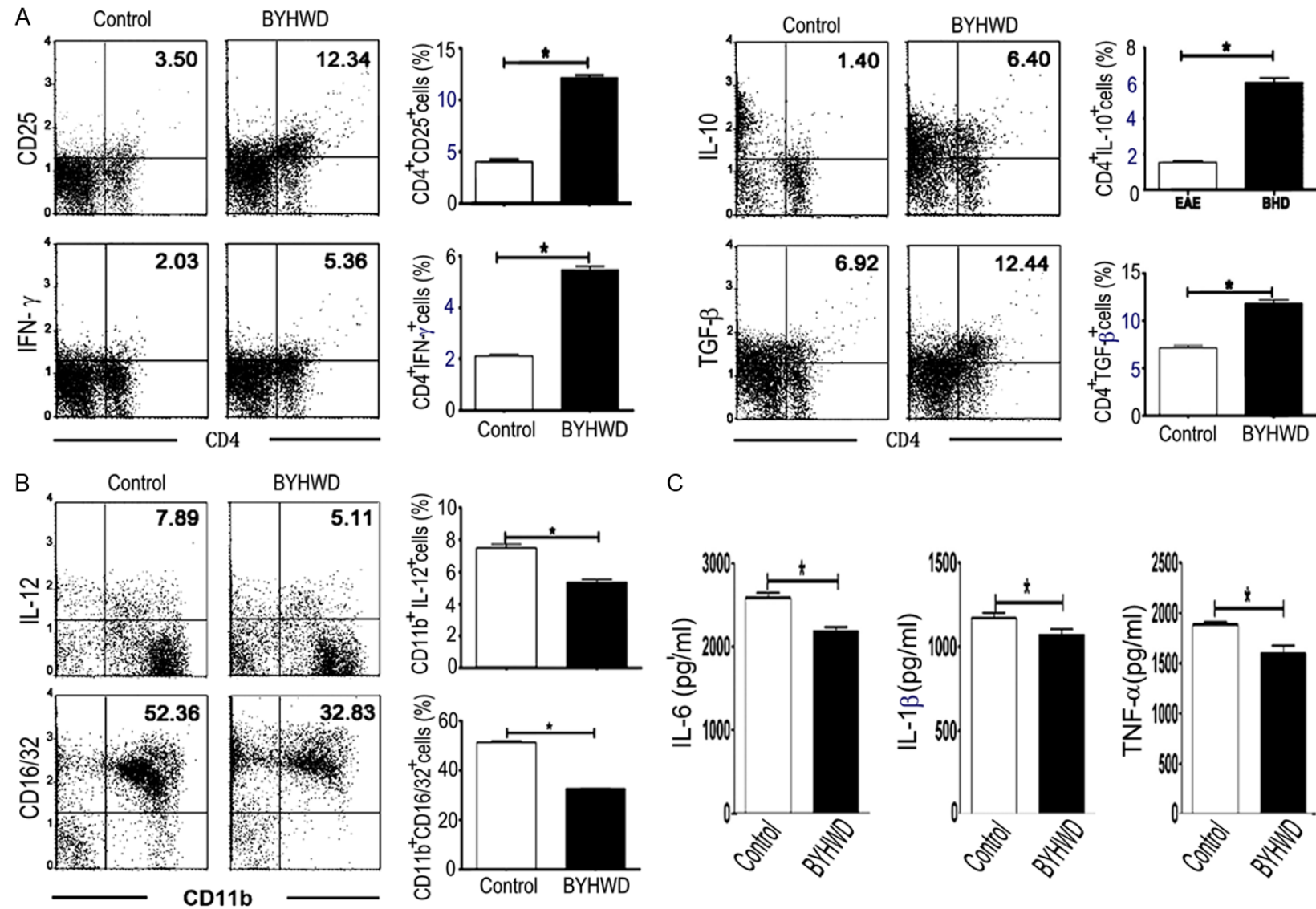
used for statistical analysis. For clinical mean score, nonparametric Kruskal-Wallis test was performed to determine whether an overall statistically significant change existed before using the Mann Whitney U test to analyze the difference between any two groups. Student's t-test was used to compare demyelination, inflammation and others. Significance level is

set at P<0.05. All graphed results were expressed as mean ± standard error of the mean.

## Results

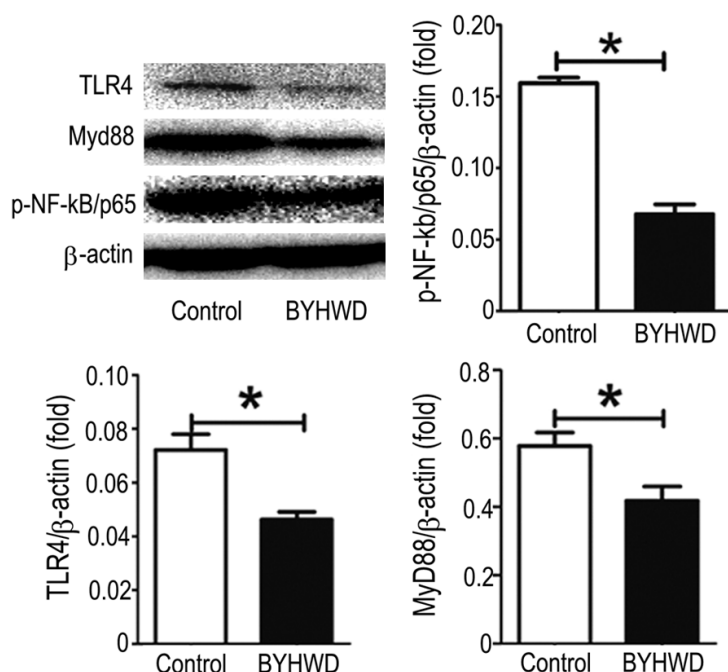
### *BYHWD attenuates the severity of EAE*

In the present study, mice were immunized with MOG<sub>35-55</sub> peptide to develop an EAE model whi-



**Figure 4.** Spleens were harvested for measuring immune and inflammatory responses. A: percentages of CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup>, CD4<sup>+</sup>IL-10<sup>+</sup> and CD4<sup>+</sup>TGF- $\beta$ <sup>+</sup> T cells; B: CD11b<sup>+</sup>CD16/32<sup>+</sup> and CD11b<sup>+</sup>IL-12<sup>+</sup> macrophages; C: production of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in the supernatants of cultured supernatants. Data represent mean standard error (SEM) from seven or eight mice in each group. Differences between two groups were analyzed using Students t test. \*P<0.05.





**Figure 5.** Spinal cords from lumbar regions were harvested and protein was prepared for measuring the expression of TLR4, MyD88 and p-NF-κB/p65 by western blot. Data represent mean standard error (SEM) from seven or eight mice in each group. Differences between two groups were analyzed using Student's t test. \*P<0.05.

ch closely imitates many characteristics of MS. Starting on day 3 p.i., mice received, daily, 1000 μl solution with or without 2 g BYHWD by taking orally for 25 consecutive days. Clinical score and body weight were then monitored from day 0 to 28 p.i. As shown in **Figure 1** and **Table 1**, mice receiving oral saline developed typical EAE, whereas BYHWD-treated mice had significantly lower clinical score and less body weight loss ( $P<0.05$ - $0.001$ ). In the EAE control group, the mean onset date was at day 11.  $92\pm1.80$  p.i., and the mean maximum score was  $4.12\pm0.58$ . The cumulative clinical score was  $58.69\pm4.34$ . However, BYHWD delayed onset (mean onset date =  $20.70\pm2.49$ ,  $P<0.001$  vs. EAE control), reduced maximum clinical score (mean maximum score =  $1.05\pm1.19$ ,  $P<0.001$  vs. EAE control) and cumulative clinical score ( $17.90\pm9.65$ ,  $P<0.001$  vs. EAE control). The results showed that BYHWD significantly alleviated development of EAE.

#### BYHWD reduces demyelination

Longitudinal sections of the spinal cord from lower thoracic-lumbar were examined for demyelination by LFB staining. The results showed

that the administration of BYHWD significantly prevented the demyelination in EAE mice, as compared with control EAE (**Figure 2**,  $P<0.05$ ).

#### BYHWD inhibits inflammatory cell infiltration and inflammatory response

We then evaluated the effect of BYHWD administration on infiltration of inflammatory cells into the spinal cord of EAE mice. As shown in **Figure 3**, extensive inflammatory infiltration was found in spinal cord of control EAE mice by H&E staining, while the administration of BYHWD obviously reduced inflammatory infiltration foci (**Figure 3A**,  $P<0.05$ ). When cell types of CNS infiltration were determined by immunostaining, we found that spinal cord of EAE control mice contained high numbers of CD4<sup>+</sup> T cells (**Figure 3B**) and CD68<sup>+</sup> macrophages (**Figure 3C**), and

the numbers of these cells was significantly reduced in mice treated with BYHWD (all  $P<0.05$ ).

#### BYHWD modulates peripheral immune responses

In our study, we explored whether administration of BYHWD could modulate systemic immune responses in EAE mice. The subsets of CD4<sup>+</sup>CD25<sup>+</sup>, CD4<sup>+</sup>IL-10<sup>+</sup>, CD4<sup>+</sup>IFN-γ<sup>+</sup> and CD4<sup>+</sup>TGF-β<sup>+</sup> T cells as well as M1 markers IL-12<sup>+</sup> and CD16/32<sup>+</sup> in spleen were assessed by flow cytometry. As expected, the percentages of CD4<sup>+</sup> cells expressing IL-10, CD25 and TGF-β were significantly enhanced in mice treated with BYHWD compared with EAE control mice (**Figure 4A**,  $P<0.05$ ). The percentages of CD4<sup>+</sup> cells expressing IFN-γ was also increased in BYHWD-treated mice compared with EAE control mice (**Figure 4A**,  $P<0.05$ ). As shown in **Figure 4B**, BYHWD treatment significantly reduced expression of CD16/32 and IL-12 on F4/80<sup>+</sup> macrophages ( $P<0.05$ ). We next measured the levels of inflammatory cytokines such as IL-6, IL-1β, and TNF-α by ELISA kits. As expected, the treatment of BYHWD sig-

nificantly inhibited the production of IL-6, IL-1 $\beta$ , and TNF- $\alpha$  in spleen (**Figure 4**, all  $P < 0.05$ ).

## *BYHWD inhibits TLR4/Myd88/NF- $\kappa$ B signaling pathway in spinal cord*

TLR4 has been linked to CNS inflammation, and is possibly the major determinant of the susceptibility to inflammation and infection through Myd88-dependent and independent signaling pathways. The modulation of TLRs/Myd88 signaling could be an important adjuvant to current EAE treatments. Thus, we measured the expression of TLR-4 and Myd88 in spinal cord of mice. It is interesting to note that BYHWD treatment resulted in a significant decrease in the expression of TLR4 and Myd88 in spinal cord compared with EAE control mice (**Figure 5**,  $P < 0.05$ ). Given that Myd88 signaling mediates NF- $\kappa$ B pathway, we next determined the activation of NF- $\kappa$ B pathway by western blot. As shown in **Figure 5**, BYHWD treatment effectively inhibited the expression of p-NF- $\kappa$ B (P65) ( $P < 0.05$ ). These results indicate that BYHWD treatment inhibited the activation of TLRs/Myd88/NF- $\kappa$ B signaling pathway.

## **Discussion**

BYHWD, as TCM effective prescription, has been used in the treatment of various disorders for more than 300 years. However, little is known about the therapeutic effect and mechanism of BYHWD on autoimmune diseases such as MS. In the present study, we demonstrate that oral administration of BYHWD has a beneficial effect in EAE mice. Treatment of BYHWD delayed the onset of EAE, attenuated clinical severity, suppressed inflammation and demyelination within the CNS, and inhibited the activation of TLRs/Myd88/NF- $\kappa$ B signaling pathway. So we think that BYHWD is an effective, safe and easily delivered approach (oral) as a long-term therapy, given the lifelong disease course of MS.

In our current study, the results of immunohistochemical staining showed that numbers of CD4 $^+$  T cells and CD68 $^+$  macrophages were increased in spinal cords of EAE mice, and the infiltration of these cells were significantly reduced in mice treated with BYHWD. Based on different phenotypes and functions, macrophages are divided into inflammatory M1 type and anti-inflammatory M2 type. M1 macro-

phages secrete proinflammatory cytokines and chemokines, while M2 macrophages produce inhibitory cytokines IL-10 and/or TGF- $\beta$ . The treatment of BYHWD effectively increased the percentages of CD4 $^+$ CD25 $^+$ , CD4 $^+$ IL-10 $^+$  and TGF- $\beta$  $^+$  regulatory T cells in splenic MNCs. In contrast, the percentages of CD16/32 $^+$  and IL-12 $^+$  on F4/80 $^+$  macrophages were significantly decreased in EAE mice treated with BYHWD. These results support the hypothesis that BYHWD regulates peripheral immune responses by upregulating regulatory T cells and downregulating inflammatory M1 macrophages, which is consistent with the decreased inflammatory cytokine IL-6, IL-1 $\beta$ , and TNF- $\alpha$  in spleen of mice treated with BYHWD. To our knowledge, this is the first report on the anti-inflammatory effects of BYHWD in EAE mice.

Although IFN- $\gamma$  serves as a hallmark of T helper type 1 (Th1) cells, the functions of Th1 cells in EAE is questionable. IFN- $\gamma$ -deficient mice were susceptible to EAE, showed massive inflammatory infiltrates, and had poorer clinical outcomes than control mice [16]. Further study confirmed that monoclonal anti-IFN- $\gamma$  antibodies also exacerbated the clinical symptoms of EAE [17], while exogenous administration of IFN- $\gamma$  decreased Th17 cells in EAE, orchestrating the number and function of Th17 cells [18]. These results suggest that IFN- $\gamma$  may provide a certain degree of immunomodulation against disease progression. Our present study also showed that BYHWD increased the production of IFN- $\gamma$ , which may be related to the improvement of EAE.

The immune system responds to many environmental stimuli by the maturation of antigen presenting cells (APCs) and activation of lymphocytes via cellular receptors such as Toll like receptors (TLRs) [19, 20]. In MS, both infiltrating T cells and resident cells of the CNS express TLRs that are induced to elevate in MS [21, 22], therefore contributing to CNS autoimmunity within the CNS. The resultant transduction of intracellular signals occurs primarily via the activation of the TLR-4 associated adapter molecule myeloid differentiation factor 88 (MyD88). This adaptor initiates a cascade resulting in the activation of nuclear factor  $\kappa$ B (NF- $\kappa$ B) and the production of proinflammatory cytokines [23, 24]. Based on the available literatures on TLRs, the present results support the hypothesis that



BYHWD appears to exert its effect in ameliorating EAE through blockade of the TLR-linked inflammatory signaling pathway. In the current study, we observed that the oral administration of BYHWD significantly reduced the expression of TLR4, MyD88 and p-NF- $\kappa$ B/p65, indicating an inhibition of TLR4/MyD88/NF- $\kappa$ B signaling pathway by BYHWD.

In this study, the reduction of neuroinflammation and the protection of demyelination should be closely related to the inhibition of inflammatory invasion. Of course, we don't know the precise cellular and molecular mechanisms that controls the invasion of inflammatory cells within the CNS. Based on a lot of experimental results, we hypothesize that there are two reasons: 1) BYHWD may protect the integrity of the BBB, thereby preventing the invasion of inflammatory cells within the CNS; 2) BYHWD may inhibit the expression of adhesion molecules or chemotactic factors on the immune or inflammatory cells, reducing the migration of these cells into the CNS. Because BYHWD is a complex, more studies should be taken to uncover the other mechanisms by which BYHWD could improve the EAE.

In conclusion, BYHWD delays the onset and ameliorates the severity of EAE, accompanied by the improvement in myelination and the inhibition of inflammatory responses in the CNS. BYHWD suppresses CNS inflammatory responses possibly through inhibiting TLR-4 and/or NF- $\kappa$ B activation, which may be related to the shift of macrophage/microglia phenotype from M1 to M2. The precise cellular and molecular mechanisms of BYHWD in the treatment of EAE still remains to be determined.

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## Disclosure of conflict of interest

None.

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