

# Experimental autoimmune encephalomyelitis inhibited by Huangqi Guizhi Wuwu Decoction by enhancing Th2 cytokine

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## Research

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# Abstract

**Background:** Current modern conventional medicine (MCM) on multiple sclerosis (MS) are non-specific immunosuppressive drugs, which remains many side effects. *Huangqi Guizhi Wuwu decoction* (HQGZWW) is a common formula of Chinese herbal medicine (CHM) has good effects on treatment of MS and its animal model, experimental autoimmune encephalomyelitis (EAE) very well, so it is very important to understand the precise mechanism. Our previous study suggested that CD8<sup>+</sup> autoreactive T cells in EAE had a lower encephalitogenic function but were unique and independent on pathogenic of EAE rather than their CD4<sup>+</sup> counterparts. The aims of current study were to determine the pathological interrelationship between CD4<sup>+</sup> and CD8<sup>+</sup> autoreactive T cells in MS/EAE upon the HQGZWW treatment.

**Methods:** Female C57BL/6 mice (n=8, each group) were induced by myelin oligodendrocyte glycoprotein (MOG)<sub>35-55</sub> peptide, and meantime were treated with distilled water, prednisone, high dose or low dose HQGZWW. At 14 days after immunization, T cells were isolated from the spleen and purified as CD4<sup>+</sup> and CD8<sup>+</sup> T cells by using CD4 and CD8 isolation kits, and then the purity was determined by flow cytometric analysis. These cells were stimulated by MOG<sub>35-55</sub> peptide and applied to proliferation assays. The interferon-gamma (IFN-g), interleukin (IL)-4 and IL-10 secretion of supernatant of cultured CD4<sup>+</sup> and CD8<sup>+</sup> T cells were measured by enzyme-linked immunosorbent assays (ELISA). For adoptive transfer, recipient mice were injected with MOG<sub>35-55</sub>-specific CD8<sup>+</sup> or CD4<sup>+</sup> T cells. EAE clinical course was measured by EAE score at 0-5 scale and spinal cord was examined by staining with hematoxylin and eosin and Luxol fast blue staining.

**Results:** For aEAE, there were significant improvement of EAE score in both HQGZWW high dose and prednisone groups by EAE score and pathological examination of spinal cord. CD8<sup>+</sup> CD3<sup>+</sup> and CD4<sup>+</sup>CD3<sup>+</sup> cells were around 90% pure of total CD3<sup>+</sup> cells after CD8/CD4 bead enrichment in 4 groups, respectively. These cells were stimulated by MOG<sub>35-55</sub> peptide and applied to proliferation assays. There is lower antigen-specific responses of CD8<sup>+</sup> as well as CD4<sup>+</sup> T cells in HQGZWW high dose and prednisone group, compared with HQGZWW low dose and distilled water groups. For cytokine profiles, the CD4<sup>+</sup> and CD8<sup>+</sup> T cell supernatants contained lower levels of IFN-g and higher levels of IL-4 and IL-10 in HQGZWW high dose and prednisone groups compared with HQGZWW low dose and distilled water groups. Finally, HQGZWW had similar effect at high dose level to typical conventional medicine prednisone on tEAE, but no effect at low dose level.

**Conclusion:** Our data suggested that HQGZWW had similar effect at high dose level to typical conventional medicine prednisone on EAE, but no effect at low dose level, and it suggested that the protection role of HQGZWW on EAE might be upon Th2 cytokine secretion profile by either MOG<sub>35-55</sub> specific CD8<sup>+</sup> or CD4<sup>+</sup> T cells.

# Background

As a central nervous system (CNS) autoimmune disease, multiple sclerosis (MS) occurs on middle age, and cause big social and economic problem. <sup>[1]</sup> Current modern conventional medicine (MCM) are focusing on acute relapse, disease-modifying therapies and MS symptoms, <sup>[2]</sup> and the effect has obviously increased, such as  $\beta$ -interferon, glatiramer acetate, mitoxantrone, teriflunamide, dimethyl fumarate, fingolimod, alemtuzumab, natalizumab. <sup>[3]</sup> However there is some serious side-effect, such as progressive multifocal leukoencephalopathy, serious viral and fungal infections, <sup>[4, 5]</sup> due to their universal immunosuppression. <sup>[3]</sup>

To reduce the side effects of hormones and immunosuppressants, more ideal results had been achieved. Complementary and alternative medicines (CAM) was widely use to treat MS or associated symptoms. <sup>[3]</sup> Traditional Chinese medicine (TCM) is one of main form CAM, included Chinese herbal medicine (CHM), acupuncture and other non-medication therapies. <sup>[6]</sup> Current Chinese national medical system is the combination of TCM and MCM complement and cooperation with each other, which has satisfactory results in clinic. <sup>[7]</sup> Face to the dilemma of current situation of MCM treatment, TCM treatment which is based on dialectical treatment provides a new possibility of MS treatment. There are many prescriptions of CHM which treated MS/EAE very well, such as 1) *Buyang Huanwu decoction*, <sup>[8, 9]</sup> 2) *Bushen Yisui Fang*, Formerly known as *Erhuang Fang*, <sup>[3]</sup> 3) *Liuwei Dihuang Pills*, *Zuogui pills* and *Yougui pills*, <sup>[10]</sup> 4) Catalpol, an extraction from the root of *Rehmannia*, <sup>[11, 12]</sup> 5) *Hyungbangpaedok-san*, a classic formula in TCM and named *Jingfangbaidu San*, <sup>[13]</sup> 6) *Huangqi Guizhi Wuwu decoction* (HQGZWW). <sup>[3]</sup> Among them, the mechanism of HQGZWW treatment on MS is the closest to immune-protection mechanism, and it is better than MCM which have big problem due to their universal immune inhibition. However, the precise mechanism of HQGZWW treatment on MS is not clear now. HQGZWW is a common formula of Chinese herbal medicine (CHM) for arthromyodynia, consisting of 5 kinds of herbs: *Astragalus mongholicus*, *Radix Paeoniae Rubra*, *Cassia twig*, *Ginger*, 4 *Fructus Ziziphi Jujubae*. HQGZWW was reported effective in treating MS patients, as well as in experimental autoimmune encephalomyelitis (EAE) model. <sup>[14]</sup>

It is well-known that MS is a T cell-mediated CNS autoimmune disease. MCM on MS are non-specific immunosuppressive drugs, which remains many side effects. <sup>[15]</sup> Typically, studies of MS and EAE have focused on CD4<sup>+</sup> T cells, however, CD8<sup>+</sup> T cells are involved in MS/EAE pathogenesis. <sup>[16]</sup> As we reported previously, myelin oligo-dendrocyte glycoprotein (MOG)<sub>35-55</sub>-specific CD4<sup>+</sup> and CD8<sup>+</sup> autoreactive T cells from B6 EAE mouse were encephalitogenic, and our previous data already showed that CD8<sup>+</sup> T cells were unique and independent on pathogenic of EAE rather than their CD4<sup>+</sup> counterparts. <sup>[17]</sup> However, CD8<sup>+</sup> autoreactive T cells, there was weaker proliferation response, and less interferon-gamma (IFN- $\gamma$ ) secretion and encephalitogenic. <sup>[17]</sup> From the previous clinical and animal studies, it is undoubted that HQGZWW plays a protection role on MS/EAE, however the precise mechanism is not clear. As the first study on the role of MOG<sub>35-55</sub> CD8<sup>+</sup> T cells of MS/EAE treated by HQGZWW, MOG<sub>35-55</sub>-specific CD8<sup>+</sup> T cells were isolated from MOG<sub>35-55</sub> induced aEAE which are treated by different dose of HQGZWW and prednisone, then studied their function such as antigen-specific, cytokine secretion profile, adoptive transfer.

# Methods

## Ethical approval

All the experiments were performed at School of Life Sciences, Central South University, and all experimental steps were examined and approved by the Ethics Committee of Central South University (No. 2019sydw0005). The treatments for animal were in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals. We had taken all steps to minimize the animal's pain and suffering.

## Animals

Specific pathogen-free female C57BL/6 mice (8-10 weeks) were purchased from Hunan Slake Jingda Laboratory Animal Co., Ltd. (Changsha, Hunan, China) (License No. SCXK [Hunan] 2016-0002) and maintained at the Center of Experimental Animal, Xiangya Medical College, Central South University (License No. SCXK [Hunan] 2015-0017). The mice were housed in a single cage with an independent ventilation system at a temperature of 18–22°C and relative humidity of 40%–60% with free access to food and water. The sample size prediction matched the requirements of the experimental design.

## Preparation of HQGZWW

HQGZWW was prepared from the following herbs: *Astragalus mongholicus* 9g, *Radix Paeoniae Rubra* 9g, *Cassia twig* 9g, *Ginger* 18g, *Fructus Ziziphi Jujubae* 4g. All herbs were dry decoction pieces (i.e. traditional Chinese medicines prepared in ready-to-use forms), which were purchased from Honghua Decoction pieces company (Changsha, Hunan, China). All the dry decoction pieces were immersed in distilled water for 30 minutes and boiled for 2 hours. The inspissation of the filtered solution was under reduced pressure at 70°C, and ready for use.

## Active-induced EAE Models (aEAE) and adoptive-transferred EAE (tEAE) models

The mice were randomly divided into four groups (n=8 for each group): (1) distilled water; (2) HQGZWW high dose (no dilution); (3) HQGZWW low dose (1:10 dilution by distilled water); (4) prednisone (6mg/kg). The models were established according our previous studies.<sup>[17]</sup> For active induction of disease, aEAE was induced by subcutaneous injection of 200 µL of an emulsion containing 200 µg MOG<sub>35–55</sub> peptide purified by High Performance Liquid Chromatography (HPLC) with a purity of >95% (amino acids 35–55 of bovine MOG; Sigma-Aldrich, St. Louis, MO, USA) and complete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO, USA) distributed over six sites at the tail base and on the flank. At 0 and 24 hours after

immunization, mice were intraperitoneally injected with pertussis toxin (250 ng per mice, List Biological, Campbell, CA, USA). For adoptive transfer, unless stated otherwise, recipient mice were injected via the caudal vein with  $5 \times 10^6$  MOG<sub>35-55</sub>-specific CD8<sup>+</sup> or CD4<sup>+</sup> enriched T cells prepared as described previously in 0.2 mL phosphate buffer saline (PBS).<sup>[17]</sup>

### **Treatment and EAE score**

The EAE mice were treated with distilled water, prednisone, high dose or low dose HQGZWW by oral gavages, started on 1 hour after immunization once a day for 14 days. EAE was scored by a 0–5 scale as follows: 0, no obvious changes in motor functions of the mouse in comparison with non-immunized mice; 1, limp tail; 2, limp tail and weakness of hind limbs; 3, limp tail and complete paralysis of hind limbs (most common) or limp tail with paralysis of one front and one hind limb; 4, complete hind limb and partial front limb paralysis; 5, death or euthanized because of severe paralysis. <sup>[17, 18]</sup>

### **Preparation of MOG<sub>35-55</sub>-specific T Cells**

The procedure was modified from our previously published protocol.<sup>[17]</sup> At 14 days after immunization, EAE mice were sacrificed by over dose sodium pentobarbital (150mg/kg Sinopharm Chemical Reagent Co., Beijing, China,) and T cells were isolated from the spleen by passage through a nylon wool column (Kisker, Steinfurt, Germany). Then,  $1 \times 10^7$  cells in 2 mL RPMI 1640 medium per well in a six-well plate (Costar; Corning, Corning, NY, USA) were stimulated with 20 µg/mL MOG<sub>35-55</sub> in the presence of  $1 \times 10^7$  mitomycin C (MCE, Monmouth Junction, NJ, USA)-treated syngeneic spleen cells as antigen-presenting cells (APCs). After 2 days, the activated lymphoblasts were isolated by density gradient centrifugation (Lymphocyte Separation, Tianjin, China) and cultured in RPMI 1640 medium containing IL-2 (USCN Co., Wuhan, Hubei, China, 10 ng/mL).

### **Proliferation Assay**

As modified from our previously published protocol,<sup>[17]</sup> CD8 or CD4 enriched T cells from MOG<sub>35-55</sub>-immunized wild-type B6 mice were prepared and seeded at  $4 \times 10^5$  cells/well in 96-well plates. The cells were then cultured at 37°C for 48 hours in 200 µL medium with or without MOG<sub>35-55</sub> in the presence of mitomycin C-treated syngeneic spleen APCs ( $1 \times 10^5$  cells/well). [<sup>3</sup>H] thymidine incorporation during the last 8 hours was assessed by a microplate scintillation counter (Packard; PerkinElmer, Meriden, CT, USA). The proliferative response is expressed as the mean counts per minute (cpm) ± standard deviation (SD) of triplicate determinations.

## Purification of CD4<sup>+</sup> and CD8<sup>+</sup> T Cells

As modified from our previously published protocol,<sup>[17]</sup> purified CD4<sup>+</sup> and CD8<sup>+</sup> T cells were prepared from spleens using CD4 and CD8 isolation kits (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The spleen cells were first incubated in 10  $\mu$ L of CD8 (TIL) MicroBeads and 90  $\mu$ L buffer per  $1 \times 10^8$  cells for 15 minutes in a refrigerator (2–8 °C). Buffer was added to a final volume of 500  $\mu$ L for up to  $5 \times 10^8$  cells. The cells were then separated into bound and unbound cells on a magnetic separator column (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and washed with 15 mL medium, according to the manufacturer's protocol. The flow through fraction containing CD4- or CD8-enriched cells was collected, and the purity of the isolated cell fraction was determined by flow cytometric analysis.

## Flow Cytometry Analysis

As modified from our previously published protocol,<sup>[17]</sup> aliquots of  $2 \times 10^5$  cells were double stained with combinations of APC-, FITC- or PE-conjugated monoclonal antibodies against mouse CD3, CD4, or CD8 (Biolegend, San Diego, CA, USA). Data collection and analysis were performed on a flow cytometer Dxp Athena (Cytek Biosciences Inc., Fremont, CA, USA). The data were analyzed by FlowJo software (FlowJo Co., San Diego, CA, USA).

## Enzyme-linked Immunosorbent Assays

As modified from our previously published protocol,<sup>[17]</sup>  $1 \times 10^6$  cells in 1 mL RPMI 1640 medium per well in a 24-well plate (Costar; Corning, Corning, NY, USA) were stimulated with 20  $\mu$ g/mL MOG<sub>35–55</sub> in the presence of mitomycin C-treated syngeneic spleen cells as APCs. After 24 hours, the interferon-gamma (IFN- $\gamma$ ), interleukin (IL)-4, and IL-10 secretion of supernatant of cultured CD4<sup>+</sup> and CD8<sup>+</sup> T cells were measured by commercially available Enzyme-linked Immunosorbent Assays (ELISA) kits (USCN Co., Wuhan, Hubei, China).

## Histology

As modified from our previously published protocol,<sup>[17]</sup> spinal cord sections were prepared from tissue collected from aEAE animals or tEAE animals that were adoptively transferred with MOG<sub>35–55</sub>-specific CD8 or CD4 enriched T cells from aEAE animals. Spinal cord tissues were fixed in ice-cold 4% paraformaldehyde/methanol and embedded in paraffin before microtome sections (5  $\mu$ m thick) were prepared for staining with hematoxylin and eosin (HE), and Luxol fast blue (LFB) to stain myelin.

## Statistical analyses

The statistical analysis was achieved by SPSS 21.0 statistical software (IBM, Almonk, NY,USA). All data were analyzed by the one-way analysis of variance (ANOVA), followed by LSD test for multiple comparisons. Data were expressed as the mean  $\pm$  standard deviation (SD), including proliferation assay, ELISA, EAE score, flow cytometry. Each experiment was repeated at least three times.  $P$ -value  $\leq 0.05$  was considered as significant.

## Results

### Great improvement of EAE clinical signs in HQGZWW high dose group

To determine the role of HQGZWW in the pathogenesis of EAE, we randomly divided EAE mice into four groups upon four treatments (distilled water, HQGZWW high dose, HQGZWW low dose or prednisone) and then measured the clinical signs using the EAE score assessed by daily checks and histopathology.<sup>[17, 18]</sup> As shown in Figure 1A, from day 0 to day 21st after MOG<sub>35-55</sub> peptide immunization, the EAE score were measured. For the maximum EAE score, compare with distilled water group ( $3.98 \pm 0.04$ ), there were significant improvement of EAE score in both HQGZWW high dose and prednisone groups ( $2.52 \pm 0.09$  and  $2.46 \pm 0.04$ , respectively,  $P = 0.001$ ), however only slight improvement in HQGZWW low dose group ( $3.35 \pm 0.09$ ,  $P = 0.616$ ). Among different treatments, it was shown that HQGZWW high dose group had much better effective on EAE mice than HQGZWW low dose group ( $P = 0.001$ ), and similar to prednisone group ( $P = 0.569$ ).

Compare to pathological examination of spinal cord from naïve mice (HE, Fig.1B and LFB, Fig. 1G), it revealed sever inflammation and demyelination in distilled water group (HE, Fig.1C and LFB, Fig. 1H) or HQGZWW low dose group (HE, Fig.1E and LFB, Fig. 1J); on the another hand, there is slight inflammation and demyelination in HQGZWW high dose group (HE, Fig.1D and LFB, Fig. 1I) or prednisone group (HE, Fig.1F and LFB, Fig. 1K). Our data suggested that HQGZWW had similar effect at high dose level to typical conventional medicine prednisone on aEAE, but no effect at low dose level.

### MOG<sub>35-55</sub> specificity of purified CD8<sup>+</sup> and CD4<sup>+</sup>T Cells

Try to answer the above questions, unfractionated T cells were isolated by CD8 or CD4 kit. As shown in Figure 2, there were around 90% CD8<sup>+</sup> CD3<sup>+</sup> cells in the fraction of T cells in 4 groups after CD8 bead enrichment and around 90% CD4<sup>+</sup>CD3<sup>+</sup> cells in the fraction of T cells after CD4 bead enrichment in 4 groups. These CD8<sup>+</sup>CD3<sup>+</sup> and CD4<sup>+</sup>CD3<sup>+</sup> cells were highly pure and appropriate for the following functional experiments.

The purified CD8<sup>+</sup> and CD4<sup>+</sup> T cells were examined for antigen-specific functions by a proliferation assay. The representative results shown in Figure 3 indicated that the MOG<sub>35-55</sub> peptide had a strong stimulatory effect on both CD8<sup>+</sup> and CD4<sup>+</sup> T cells. Although the purified CD8<sup>+</sup> T cells had a generally lower response to MOG<sub>35-55</sub> than CD4<sup>+</sup> T cells, it was apparent that the response of CD8<sup>+</sup> T cells was not always dependent on CD4<sup>+</sup> T cells. By the way, upon to the highest MOG<sub>35-55</sub> concentration (20mg/mL) as shown in Figure 3A, compare with distilled water group (21711.33 ± 1589.42 cpm), there is lower antigen-specific responses of CD8<sup>+</sup> T cells in HQGZWW high dose and prednisone groups (10510.67 ± 1189.87 cpm and 9575.33 ± 1064.00 cpm, respectively, P = 0.001), but not in when they are compared with HQGZWW low dose group (19665.33 ± 1553.61 cpm, P = 0.104). Among different treatments, it was shown that antigen-specific responses of CD8<sup>+</sup> T cells in HQGZWW high dose group than HQGZWW low dose group (P = 0.001), and similar to prednisone group (P = 0.427).

As shown in Figure 3B, It is shown that similar pattern for CD4<sup>+</sup> T cells proliferation, compare with distilled water group (64557.67 ± 2547.57 cpm), there were significant decrease of EAE score in both HQGZWW high dose, prednisone group and HQGZWW low dose groups (21281.67 ± 1739.08 cpm, 20104.00 ± 1364.82 cpm, and 50799.33 ± 2449.17 cpm, respectively, P = 0.001). Among different treatments, it was shown that antigen-specific responses of CD8<sup>+</sup> T cells in HQGZWW high dose group than HQGZWW low dose group (P = 0.001), and similar to prednisone group (P = 0.509). To sum up, the antigen-response of CD8<sup>+</sup> or CD4<sup>+</sup> T cells in four groups were consistent with the EAE score and histology result in Figure1.

### **Cytokine profiles of MOG<sub>35-55</sub>-specific CD8<sup>+</sup> T and CD4<sup>+</sup> T Cells**

To determine the cytokine profiles of activated CD4<sup>+</sup> and CD8<sup>+</sup> autoreactive T cells, we used ELISAs to measure cytokine levels in the culture supernatants of activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells at 24 hours post-stimulation. As shown in Figure 4A, compare with distilled water group (816.31 ± 42.88 pg/ml), there is much lower IFN-g secretion of CD8<sup>+</sup> T cells supernatant in HQGZWW high dose and prednisone groups (531.66 ± 25.54 pg/ml and 484 ± 36.52 pg/ml, respectively, P = 0.001), and also lower in HQGZWW low dose group (718.33 ± 29.81 pg/ml, P = 0.008). Among different treatments, it was shown that less IFN-g secretion of CD8<sup>+</sup> T cells in HQGZWW high dose group than HQGZWW low dose group (P = 0.001), and similar to prednisone group (P = 0.127).

As shown in Figure 4D, compare with distilled water group (1474.07 ± 90.43 pg/ml), there is much lower IFN-g secretion of CD4<sup>+</sup> T cells supernatant in HQGZWW high dose and prednisone groups (722.66 ± 64.50 pg/ml and 646 ± 64.50 pg/ml, respectively, P = 0.001), and also lower in HQGZWW low dose group (1323.12 ± 79.50 pg/ml, P = 0.04). Among different treatments, it was shown that less IFN-g secretion of CD8<sup>+</sup> T cells in HQGZWW high dose group than HQGZWW low dose group (P = 0.001), and similar to prednisone group (P = 0.249).

On the another hand, as shown in Figure 4B, compare with distilled water group ( $220.66 \pm 56.51$  pg/ml), there is much higher IL-4 secretion of CD8<sup>+</sup> T cells supernatant in HQGZWW high dose and prednisone groups ( $762.33 \pm 78.21$  pg/ml and  $954.06 \pm 75.07$  pg/ml, respectively,  $P = 0.001$ ), but not in HQGZWW low dose group ( $293 \pm 49.03$  pg/ml,  $P = 0.215$ ). Among different treatments, it was shown that more IL-4 secretion of CD8<sup>+</sup> T cells in HQGZWW high dose group than HQGZWW low dose group ( $P = 0.001$ ), and similar to prednisone group ( $P = 0.07$ ).

As shown in Figure 4E, compare with distilled water group ( $324.33 \pm 27.50$  pg/ml), there is much higher IL-4 secretion of CD4<sup>+</sup> T cells supernatant in HQGZWW high dose and prednisone groups ( $620.33 \pm 38.07$  pg/ml and  $707.66 \pm 35.59$  pg/ml, respectively,  $P = 0.001$ ), but not in HQGZWW low dose group ( $355.13 \pm 35.59$  pg/ml,  $P = 0.349$ ). Among different treatments, it was shown that more IL-4 secretion of CD4<sup>+</sup> T cells in HQGZWW high dose group than HQGZWW low dose group ( $P = 0.001$ ), and similar to prednisone group ( $P = 0.221$ ).

Another important Th2 cytokine, IL-10, was also detected. As shown in Figure 4C, compare with distilled water group ( $830.33 \pm 68.24$  pg/ml), there is much higher IL-10 secretion of CD8<sup>+</sup> T cells supernatant in HQGZWW high dose and prednisone groups ( $2048.66 \pm 114.02$  pg/ml and  $2250.01 \pm 67.55$  pg/ml, respectively,  $P = 0.001$ ), but not in HQGZWW low dose group ( $1056.13 \pm 114.89$  pg/ml,  $P = 0.237$ ). Among different treatments, it was shown that more IL-10 secretion of CD8<sup>+</sup> T cells in HQGZWW high dose group than HQGZWW low dose group ( $P = 0.001$ ), and similar to prednisone group ( $P = 0.12$ ).

As shown in Figure 4F, compare with distilled water group ( $853.66 \pm 48.26$  pg/ml), there is much higher IL-10 secretion of CD4<sup>+</sup> T cells supernatant in HQGZWW high dose and prednisone groups ( $2242.33 \pm 156.35$  pg/ml and  $2380.21 \pm 131.73$  pg/ml, respectively,  $P = 0.001$ ), but not in HQGZWW low dose group ( $1055.33 \pm 66.01$  pg/ml,  $P = 0.247$ ). Among different treatments, it was shown that more IL-4 secretion of CD4<sup>+</sup> T cells in HQGZWW high dose group than HQGZWW low dose group ( $P = 0.001$ ), and similar to prednisone group ( $P = 0.271$ ).

In summary, the CD4<sup>+</sup> and CD8<sup>+</sup> T cell supernatants contained lower levels of IFN-g and higher levels of IL-4 and IL-10 in HQGZWW high dose and prednisone groups compared with HQGZWW low dose and distilled water groups. Our data suggested that there is Th2 cytokine profile of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in HQGZWW high dose and prednisone groups, and it was consistent with the EAE score, histological studies in Figure1, and proliferation assay in Figure 3. By the way, in Figure 4, compared to CD4<sup>+</sup> T cells, there is lower IFN-g and similar IL-4 and IL-10 secretion in CD8<sup>+</sup> autoreactive T cells of 4 groups; and it is also consistent with our previous published data in EAE and EAU studies. <sup>[17, 19]</sup>

### **Adoptive transfer of MOG<sub>35-55</sub>-specific CD8<sup>+</sup> T Cells to naïve mice induces tEAE**

To determine the role of MOG<sub>35-55</sub>-specific CD8<sup>+</sup> T cells in the pathogenesis of EAE, we induced the disease in wild-type B6 naïve mice by adoptive transfer of MOG<sub>35-55</sub>-specific T cells from B6 aEAE mice of four groups upon four treatments (distilled water, HQGZWW high dose, HQGZWW low dose or prednisone) and then measured the clinical signs using the EAE score assessed by daily checks and histopathology. [17]

As shown in Figure 1A, from day 0 to day 21st after MOG<sub>35-55</sub> adoptively transferring, the EAE score were measured. For the maximum EAE score, compare with distilled water group (2.95± 0.09), there were significant improvement of EAE score in both HQGZWW high dose and prednisone groups (2.10± 0.10 and 2.03 ± 0.04, respectively, P = 0.001), however only slight improvement in HQGZWW low dose group (2.91 ± 0.07, P = 0.742). Among different treatments, it was shown that HQGZWW high dose group had much better effective on EAE mice than HQGZWW low dose group (P = 0.001), and similar to prednisone group (P = 0.631).

It revealed moderate inflammation and demyelination in distilled water group (HE, Fig.1B and LFB, Fig. 1F) or HQGZWW low dose group (HE, Fig.1D and LFB, Fig. 1H); on the another hand, there is slight inflammation and demyelination in HQGZWW high dose group (HE, Fig.1C and LFB, Fig. 1G) or prednisone group (HE, Fig.1E and LFB, Fig. 1I). Our data suggested that HQGZWW had similar effect at high dose level to typical conventional medicine prednisone on tEAE, but no effect at low dose level.

## Discussion

MS is an immune-mediated CNS disease with inflammation, demyelination, and axonal damage. MS/EAE had been considered as antigen-specific, CD4<sup>+</sup> T helper (Th) cell-dominated diseases for a long time. [20] Cytokines play a critical role in the pathogenesis of MS/EAE, and treatments altered cytokine profiles such as Glatiramer acetate (Copaxone), Interferon- $\beta$ , cyclophosphamide. [21-24] There are two major cytokines secreted by Th cells. Th1-cytokines include IFN- $\gamma$ , IL-2, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), on the another hand, Th2-cytokines consist of IL-4, IL-5, IL-10, and IL-13. [23]

Recently, there are more evidence about CD8<sup>+</sup> T cells involved in MS/EAE pathogenesis. [16, 25, 26] For example, CD8<sup>+</sup> T cells were present in MS tissue at all stages of the disease, and are characterized by their antigenic repertoire in MS and EAE. [16, 25, 26] Cytokine profiles produced by CD8<sup>+</sup> T cells involved in MS/EAE were also studied. [27] Like their CD4 counterparts, pro-inflammatory cytokines are IFN- $\gamma$ , IL-2, and TNF- $\alpha$ , as well as IL-17; [28, 29] and immunosuppressive cytokines are IL-4, IL-10. [30-32] For example, IFN $\gamma$  and TNF $\alpha$  secretion of auto-reactive CD8<sup>+</sup> T-cells was detected in the peripheral blood by responding to their cognate antigen ex vivo. [33] Apoptotic T-cell-associated self-epitopes increased IFN $\gamma$ - and IL-17-producing CD8<sup>+</sup> T-cells in CNS. [34] On another hand, in virus-induced encephalitis models, IL-10-producing CD8<sup>+</sup> T-cells in increased expression of pro-inflammatory cytokines and chemokines, resulted in diminishing disease pathology. [35] As our previous report, it is shown that CD8<sup>+</sup> autoreactive T cells in EAE have a lower encephalitogenic function but are unique and independent on pathogenic of EAE rather

than their CD4<sup>+</sup> counterparts, which might be explained that CD8<sup>+</sup> T cells had a generally lower response, less IFN- $\gamma$  and IL-4 secretion to MOG<sub>35-55</sub> than CD4<sup>+</sup> T cells.<sup>[17]</sup> Our published data were consistent with our previous data in the EAU model and PLP<sub>56-70</sub>-specific CD4<sup>+</sup> autoreactive T cells of EAE in Biozzi AB/H mice.<sup>[19,36]</sup>

The aim of the current study was to determine the precise mechanism of HQGZWW on MS, so we used the same strategy to focus on the pathological interrelationship between CD4 and CD8 autoreactive T cells during different treatments. In Figure 4, our data suggested that there was a Th2 cytokine profile of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in HQGZWW high dose and prednisone groups, not in HQGZWW low dose and distilled water groups. This was consistent with the EAE score, histological studies in Figure 1, proliferation assay in Figure 3, as well as adoptive transfer in Figure 4.

Some reports were published about HQGZWW on EAE models which support our current data. It was shown that HQGZWW could increase the level of IL-35 in peripheral blood in EAE rats.<sup>[37]</sup> Furthermore, *Astragalus mongholicus*, the major herb of HQGZWW, was also shown to have effects on MOG<sub>35-55</sub> T cells in the EAE C57BL/6 mice model, and resulted in reducing EAE score, inhibiting CNS inflammation and MOG<sub>35-55</sub> T cells proliferation, also reducing interferon- $\gamma$ , TNF- $\alpha$ , IL-17 secretion and enhancing IL-4 secretion.<sup>[38]</sup> Recently, it is shown that the major extraction of *Astragalus mongholicus*, *Huangqi* glycoprotein (HQGP) treatment delayed the onset of EAE, attenuated the clinical symptoms and inhibited CD68<sup>+</sup> macrophage infiltration into the CNS, and also downregulated the secretion of nitric oxide, TNF- $\alpha$ , IL-6, and increased the secretion of IFN- $\gamma$ . In addition, HQGP treatment effectively increased the numbers of CD4<sup>+</sup> CD25<sup>+</sup> T cells, CD4<sup>+</sup> IL-10<sup>+</sup> T cells and CD4<sup>+</sup> IFN- $\gamma$ <sup>+</sup> T cells.<sup>[3]</sup> Another study is also done by the same group, and it is shown that HQGP effectively alleviated EAE score and delayed the onset time, decreased the numbers of CD4<sup>+</sup> T cells, CD68<sup>+</sup> macrophages and CD11b<sup>+</sup> cells; further studies showed that HQGP could inhibit the secretion of IL-17, TNF- $\alpha$ , IL-12 and promote the production of IL-4, IL-10, IFN- $\gamma$ .<sup>[39]</sup> Even the treatment of HQGZWW, its major herb, and the extraction of its major herb on CD4<sup>+</sup> T cells, macrophages, and dendritic cells of the EAE model is slightly investigated, however, until now, CD8<sup>+</sup> T cells of MS/EAE by HQGZWW treatment is never studied.

## Conclusions

In summary, our data suggested that HQGZWW had similar effects at high dose to typical conventional medicine prednisone on EAE, but no effects at low dose. It suggested that a protective role of CD8<sup>+</sup> T cells in EAE that were treated with HQGZWW was majorly by enhancing Th2 cytokine. We also noticed that there are more mechanisms like CD8<sup>+</sup> Treg cells which have been recently extensively studied<sup>[16,40]</sup>. It is a good direction for our further investigation on the mechanism of HQGZWW protecting MS/EAE.

## Abbreviations

MCM: modern conventional medicine; MS: multiple sclerosis; HQGZWW: Huangqi Guizhi Wuwu decoction; CHM: Chinese herbal medicine; EAE: experimental autoimmune encephalomyelitis; IFN- $\gamma$  : interferon-gamma; IL: interleukin; ELISA: enzyme-linked immunosorbent assays; CNS: central nervous system; CAM: Complementary and alternative medicines; TCM: Traditional Chinese medicine; MOG: myelin oligo-dendrocyte glycoprotein; aEAE: Active-induced EAE Models; tEAE: adoptive-transferred EAE; HPLC: High Performance Liquid Chromatography; PBS: phosphate buffer saline; APCs: antigen-presenting cells ; HE: hematoxylin and eosin; LFB: Luxol fast blue; ANOVA: one-way analysis of variance; SD: standard deviation; Th: T helper; TNF- $\alpha$ : tumor necrosis factor- $\alpha$ ; HQGP: Huangqi glycoprotein.

## Declarations

- Ethics approval and consent to participate: yes, already in Methods Ethical approval
- Consent to publish: all authors read this manuscript and agreed to publish on Chinese Medicine
- Availability of data and materials: supplementary data was uploaded
- Competing interests: no competing interests
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- Authors' Contributions: Y.P, J.X.Z., G.S, Z.X.C, L.G., S.S.Y., X.D., Z.L.L., Q.Q.L, Z.F.Z. performed the experiment. Y.P. developed the research hypothesis, designed study, collected, analysed, interpreted data, Y.P.and Z.F.Z. wrote and critically revised the manuscript. The final manuscript is an end product of joint writing efforts of all the authors
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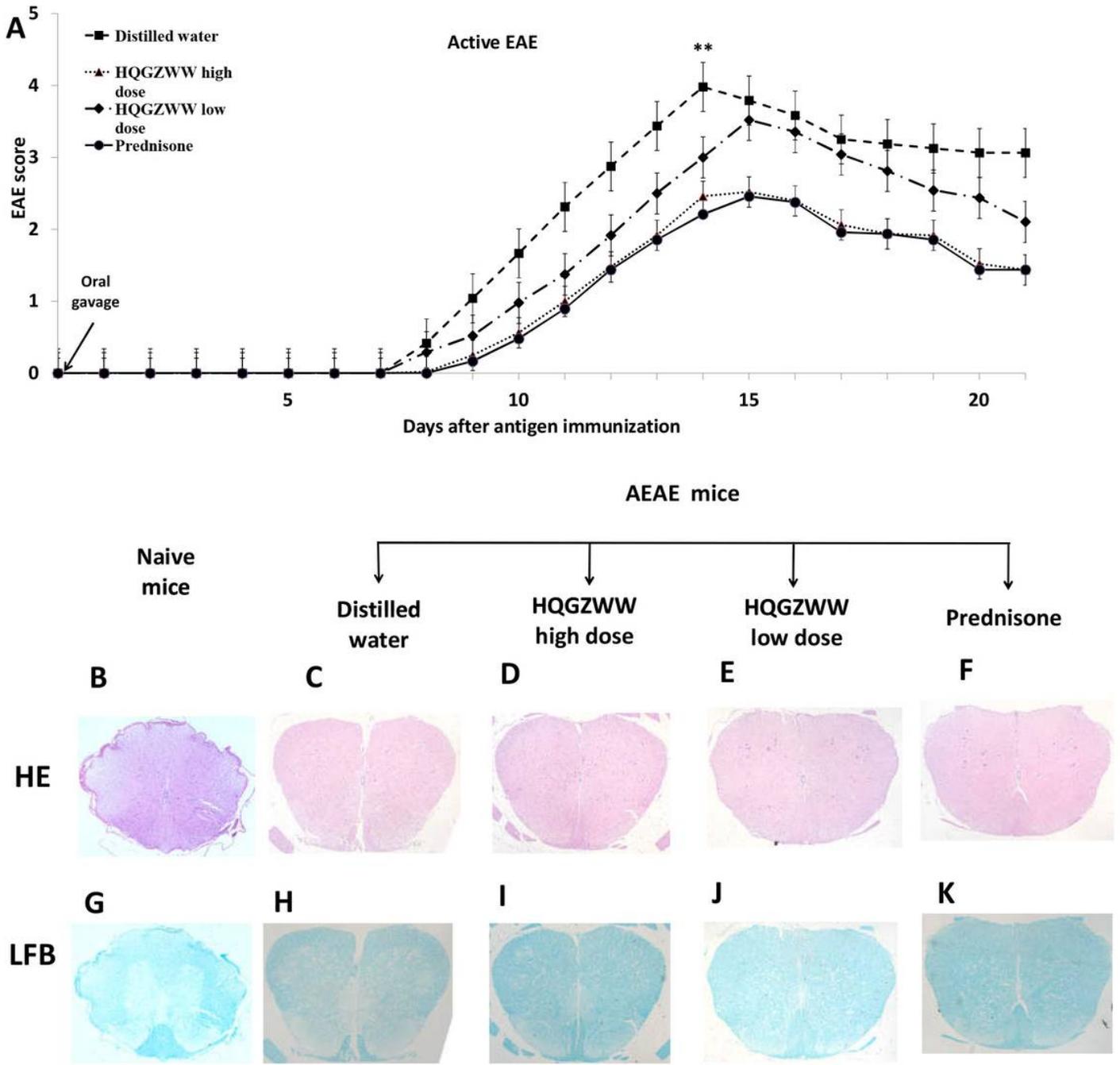
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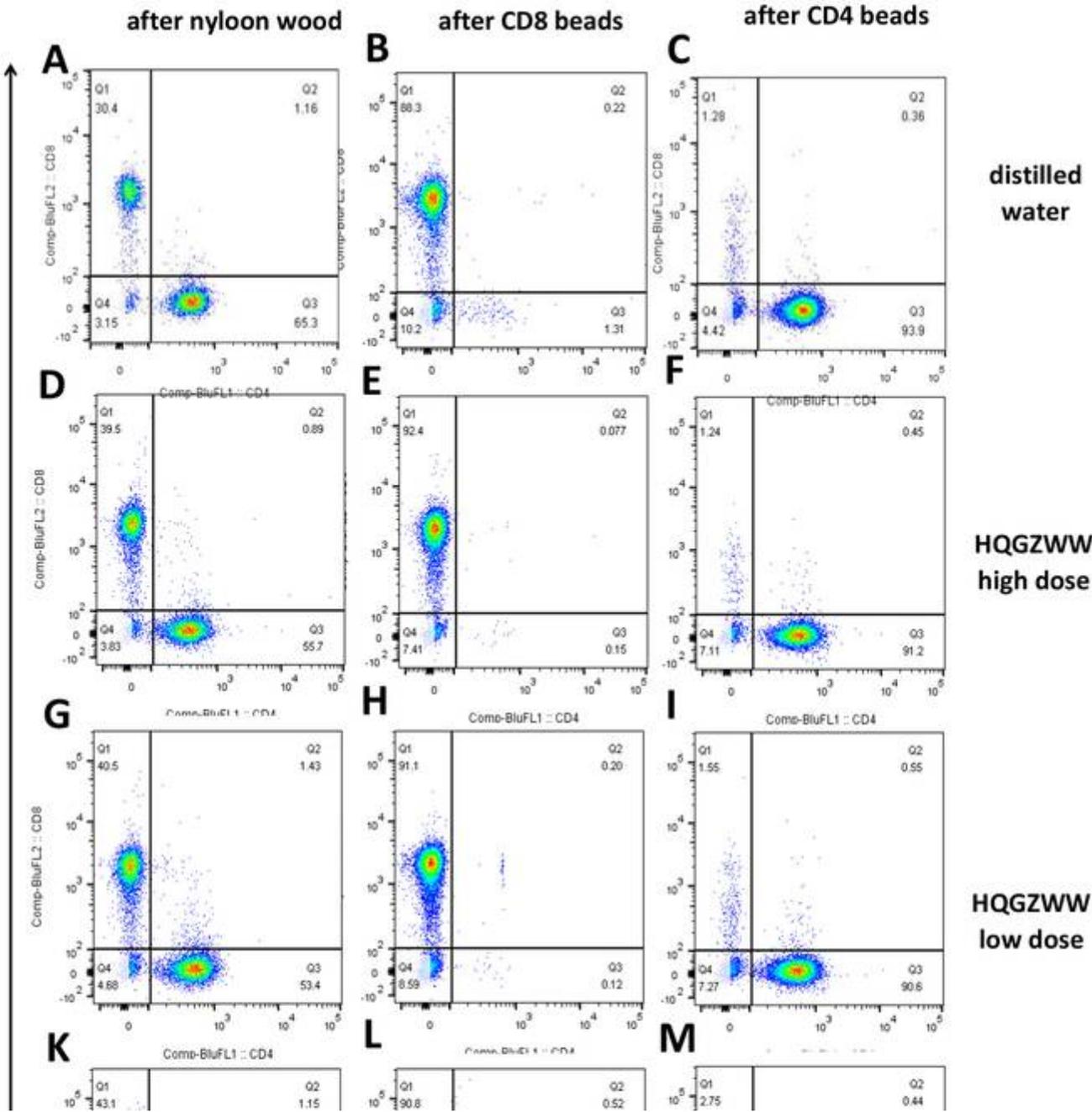
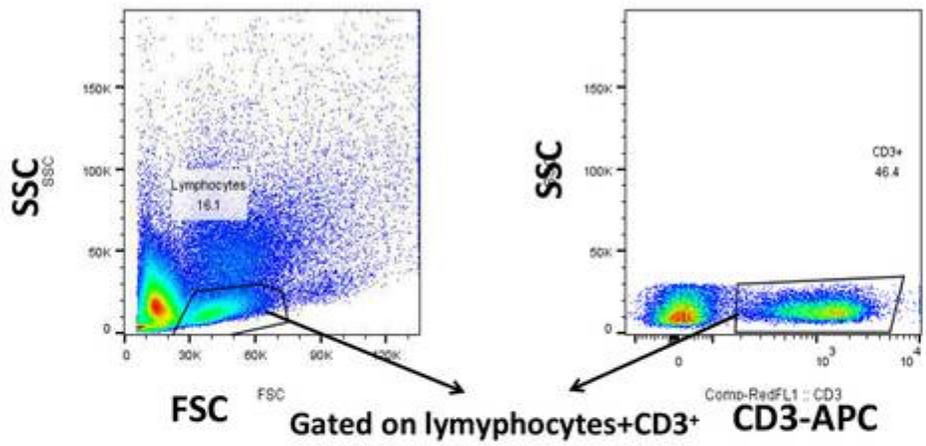
## Figures

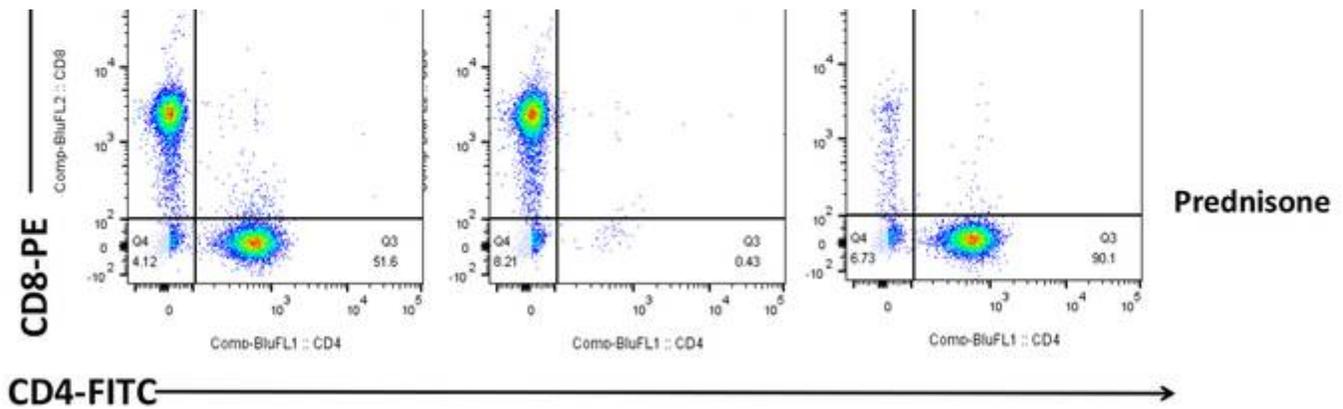


**Figure 1**

Great improvement of EAE clinical course in HQGZWW high dose and prednisone groups. AEAE mice which were induced by an emulsion containing 200 µg MOG35-55 and CFA plus pertussis toxin, were treated with four groups: distilled water, HQGZWW high dose, HQGZWW low dose or prednisone by oral gavages started on 1 hour after immunization once a day for 14 days. EAE was scored on a 0–5 scale, MOG35-55-induced B6 EAE mice, and measurement of clinical signs of EAE score was assessed by daily check and histopathology (Spinal cord, HE&LFB). Naïve mice (1B,1G), distilled water group (1C,1H), HQGZWW high dose group (1D,1I), HQGZWW low dose group (1E,1J) or prednisone group (1F,1K). \*\*:  $P < 0.01$ , (HQGZWW high dose and prednisone groups vs distilled water group); myelin oligodendrocyte

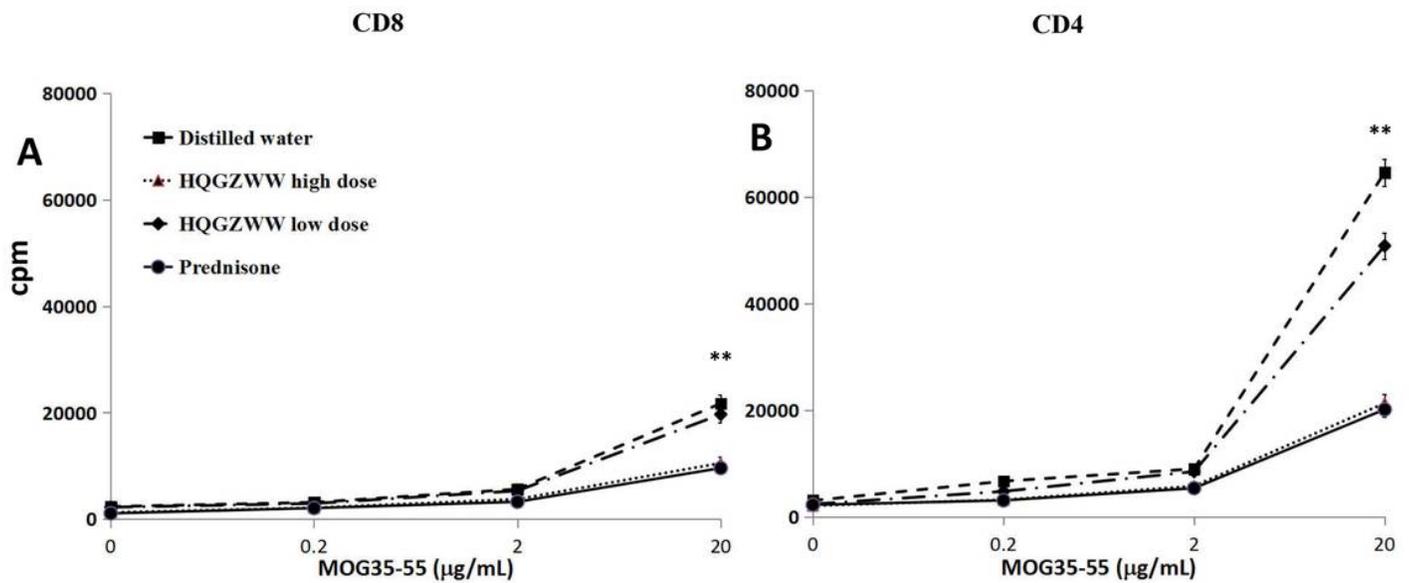
glycoprotein (MOG); antigen presenting cell (APC); standard deviation (SD); Fig. 1B, 1D, 1F&1H (HE, 50X); Fig. 1C, 1E, 1G&1I (LFB, 50X); myelin oligodendrocyte glycoprotein (MOG); standard deviation (SD); hematoxylin and eosin (HE); Luxol fast blue (LFB), Huangqi Guizhi Wuwu Decoction (HQGZWW).





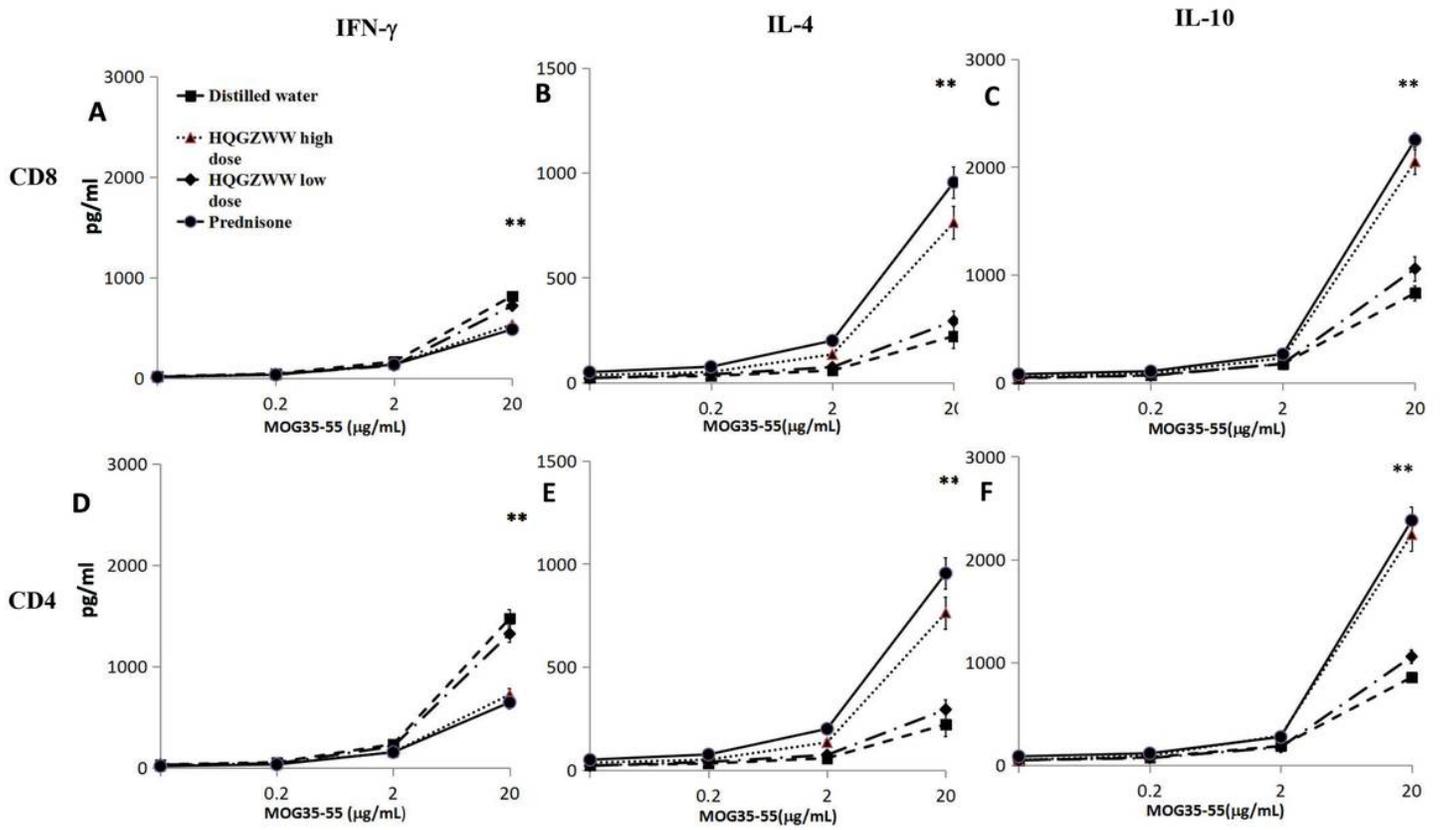
**Figure 2**

Lowest percentage of CD8+ T cells and lowest CD8/CD4 ratio in total CD3+ cells of unfractionated T cells in distilled water group. The phenotype and CD8/CD4 ratio in four groups were studied. T cells were isolated from spleen cells of aEAE mice of 4 groups by passing through a nylon wool column, then purified by CD4 and CD8 isolation kits, finally the purity of the isolated cell fraction was determined by flow cytometric analysis with APC-, FITC- or PE-conjugated anti-CD3, anti-CD4 and anti-CD8 antibodies. \*\*:  $P \leq 0.01$ , (HQGZWW high dose and prednisone and HQGZWW low dose groups vs distilled water group); Side scatter (SSC); Forward scatter (FSC); activated EAE (aEAE).



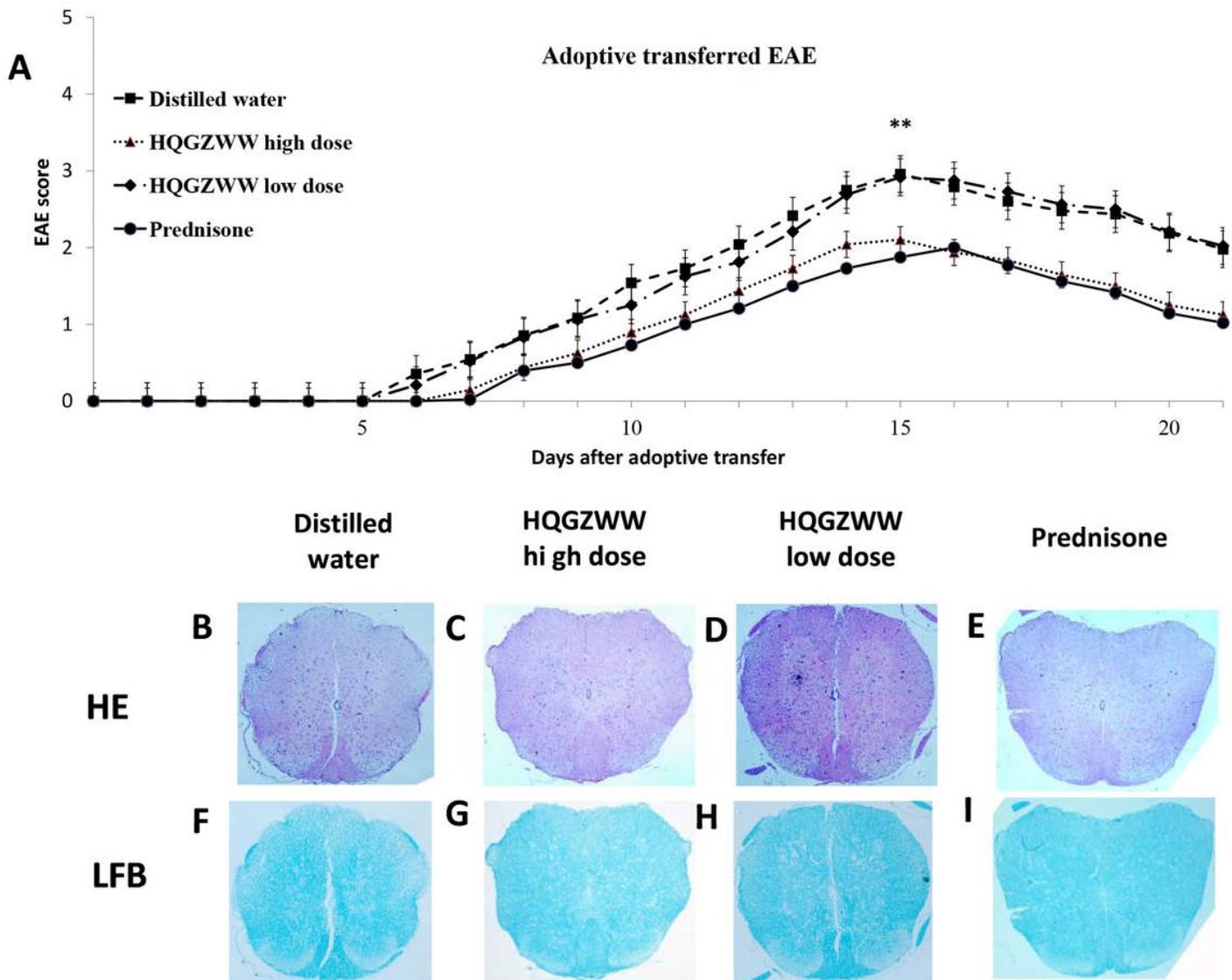
**Figure 3**

MOG35–55 specificity of purified CD8+ and CD4+T Cells. To compare of the response of the purified CD4 and CD8 T cell populations to MOG35-55, CD8 or CD4 enriched T cells from MOG35-55-immunized wild-type B6 mice of 4 groups were prepared and seeded at  $4 \times 10^5$  cells/well in 96-well plates and cultured at  $37^\circ\text{C}$  for 48 hours in a total volume of 200  $\mu\text{L}$  of medium, with or without MOG35-55, in the presence of mytomycin C-treated syngeneic spleen APCs ( $1 \times 10^5$ ), and [3H] thymidine incorporation during the last 8 hours was assessed. The proliferative response is expressed as the mean counts per minute  $\pm$  SD of triplicate determinations. \*\*:  $P \leq 0.01$ , (HQGZWW high dose and prednisone groups vs distilled water group); myelin oligodendrocyte glycoprotein (MOG).



**Figure 4**

Cytokine profiles of MOG35–55-specific CD8+ T and CD4+ T Cells. Cytokine levels in the supernatants of activated CD4+ and CD+8 T cells at 24–48 hours poststimulation in vitro are measured by ELISA. Stimulation means that CD4 enriched T cells from MOG35-55-immunized wild-type B6 mice were prepared and seeded at  $8 \times 10^5$  cells/well in 24-well plates and cultured at 37°C for 24-48 hours in a total volume of 500  $\mu$ L of medium, with or without MOG35-55, in the presence of mytomycin C-treated syngeneic spleen APCs ( $2 \times 10^5$ ). The data are the mean  $\pm$  SD from three separate experiments. \*\*:  $P < 0.01$ , (HQGZWW high dose and prednisone groups vs distilled water group); myelin oligodendrocyte glycoprotein (MOG); antigen presenting cell (APC); standard deviation (SD); IFN- $\gamma$ : interferon-gamma, IL-4: interleukin-4, IL-10: interleukin-10.



**Figure 5**

Adoptive transfer of MOG35–55-specific CD8+ T Cells to naïve mice is able to induce tEAE. Attempts were made to induce disease in wild-type B6 naïve mice by adoptive transfer of MOG35–55-specific CD8+ T Cells from B6 aEAE mice of four groups upon four treatments (distilled water, HQGZWW high dose, HQGZWW low dose or prednisone), and measurement of clinical signs of EAE score was assessed by daily check(A) and histopathology (Spinal cord, HE&LFB, 50X). distilled water group (5B,5F), HQGZWW high dose group (5C,5G), HQGZWW low dose group (5D,5H) or prednisone group (5E,5I). The data are the mean  $\pm$  SD from three separate experiments. aEAE: Activated EAE; H&E: Hematoxylin and eosin; LFB: Luxol fast blue; MOG: Myelin oligodendrocyte glycoprotein; SD: Standard deviation; LFB: Luxol fast blue, Huangqi Guizhi Wuwu Decoction (HQGZWW).

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [figuresplusrawdataforEAEandHQGZWW2020051901.xlsx](#)