

# Baicalin reduces the severity of experimental autoimmune encephalomyelitis

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## Abstract

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*Scutellaria baicalensis* Georgi is one of the important medicinal herbs widely used for the treatment of various inflammatory diseases in Asia. Baicalin (BA) is a bioactive anti-inflammatory flavone found abundantly in *Scutellaria baicalensis* Georgi. To explore the therapeutic potential of BA, we examined the effects of systemic administration of the flavone (5 and 10 mg/kg, *ip*) on relapsing/remitting experimental autoimmune encephalomyelitis (EAE) induced by proteolipid protein 139-151 in SJL/J mice, an experimental model of multiple sclerosis. The mice treated with PBS or BA at day -1 and for 3 consecutive days were observed daily for clinical signs of disease up to 60 days after immunization. In the PBS-EAE group, neurological scores were: incidence (100%), mean day of onset ( $8.0 \pm 0.73$ ), peak clinical score ( $3.0 \pm 0.4$ ), and cumulative disease index ( $141.8 \pm 19.4$ ). In the BA-EAE group (5 or 10 mg kg<sup>-1</sup> day<sup>-1</sup>, respectively), incidence (95 or 90%), mean day of onset ( $9.0 \pm 0.80$  or  $9.2 \pm 0.75$ ;  $P = 0.000$ ), peak clinical score ( $2.2 \pm 0.3$  or  $2.0 \pm 0.3$ ;  $P = 0.000$ ), and cumulative disease index ( $75.9 \pm 10.1$  or  $62.9 \pm 8.4$ ;  $P = 0.000$ ) decreased, accompanied by the histopathological findings (decrease of dense mononuclear infiltration surrounding vasculum) for the spinal cord. Additionally, the *in vitro* effects of BA (5, 10, and 25  $\mu$ M) on mononuclear cells collected from popliteal and inguinal lymph nodes of day-10 EAE mice were evaluated using an MTT reduction assay for cell proliferation, and ELISA to measure IFN- $\gamma$  and IL-4 cytokines. Compared with the control group, BA caused an increase in IL-4 (EAE-DMSO:  $3.56 \pm 0.42$  pg/mL vs EAE-BA (5, 10, and 25  $\mu$ M):  $6.03 \pm 1.1$ ,  $7.83 \pm 0.65$ ,  $10.54 \pm 1.13$  pg/mL, respectively;  $P < 0.001$ ); but inhibited IFN- $\gamma$  (EAE-DMSO:  $485.76 \pm 25.13$  pg/mL vs EAE-BA (5, 10, and 25  $\mu$ M):  $87.08 \pm 9.24$ ,  $36.27 \pm 5.44$ ,  $19.18 \pm 2.93$  pg/mL, respectively;  $P < 0.001$ ) and the proliferation of mononuclear cells (EAE-DMSO:  $0.73 \pm 0.021$  vs EAE-BA (5, 10, and 25  $\mu$ M):  $0.41 \pm 0.015$ ,  $0.31 \pm 0.018$ ,  $0.21 \pm 0.11$ , respectively;  $P < 0.001$ ) in a concentration-dependent manner. The results suggest that BA might be effective in the treatment of multiple sclerosis.

### Key words

- Experimental autoimmune encephalomyelitis
- Baicalin
- IFN- $\gamma$
- IL-4
- Neurologic score

## Introduction

Experimental autoimmune encephalomyelitis (EAE) is a CD4<sup>+</sup> T-cell-mediated inflammatory demyelinating disease of the central nervous system (CNS) that is commonly used as a model of multiple sclerosis (MS). The highest incidence of MS onset is between 20 and 40 years. The etiology of MS is unclear, but it is considered to be the result of the perivascular migration of activated T-cells and mononuclear cells into the CNS where they persist and induce an inflammatory cascade that results in injury to previously normal neural tissue (1). Th1 cytokines such as IFN- $\gamma$  and TNF- $\alpha$ , and Th2 cytokines such as IL-4 are believed to play a key role in this process. In view of the wide variety of cytokines and chemokines involved in inflammatory and autoimmune diseases, strategies that modulate the activity of multiple cytokines may prove useful.

*Scutellaria baicalensis* Georgi (Labiatae) is one of the important medicinal herbs for the treatment of cancer, viral diseases and inflammatory diseases in East Asian countries. It has been reported to contain a large number of flavonoids, among them the flavone baicalin (5,6-dihydroxy-4-oxygen-2-phenyl-4H-1-benzopyran-7-beta-D-glucopyranose acid, BA). *Scutellaria radix* is yet another plant rich in BA. BA is known for its anti-inflammatory and smooth muscle relaxant properties and as a constituent of some hepatoprotective herbal mixtures such as *Xiaochahu-tang* (*Sho-saiko-to*), popular in Japan and China (2-4). Previous studies have shown that 100  $\mu$ g/mL BA inhibits by 98% *in vitro* superantigenic staphylococcal exotoxin-stimulated T-cell proliferation and production of IL-1 $\beta$ , TNF- $\alpha$ , IFN- $\gamma$  by peripheral blood mononuclear cells, a Th1-type immune response (5,6).

The present study was designed to examine the efficacy of BA in abrogating EAE induced by proteolipid protein 139-151 (PLP<sub>139-151</sub>) in mice and to elucidate the pos-

sible mechanism involved.

## Material and Methods

### Animals

Female SJL/J mice (8-10 weeks of age; Jackson Laboratory, Bar Harbor, ME, USA) were housed in the Laboratory Animal Center of Nanjing Medical University. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care. The Scientific Committee of the First Affiliated Hospital of Nanjing Medical University approved all experimental protocols that involved the use of animals. All mice were age matched for individual experiments and were housed in groups with a 12-h light/dark cycle. The access to food and water was facilitated for paralyzed mice to prevent dehydration.

### Proteolipid protein peptide and baicalin

Immunodominant mouse PLP<sub>139-151</sub> peptide (HSLGKWLGHDPKF) was synthesized by Genemed synthesis (South San Francisco, CA, USA), purity was assessed by HPLC (>97%). It was initially solubilized in LPS-free phosphate-buffered saline (PBS) to obtain a concentration of 4 mg/mL and stored at -80°C. BA was provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), purity was assessed by HPLC (>95%). Molecular formula: C<sub>21</sub>H<sub>18</sub>O<sub>11</sub>, molecular weight: 446.36, and CAS registry number: 21967-41-9. The structure is shown in Figure 1.

### Induction of experimental autoimmune encephalomyelitis

Mice were immunized subcutaneously in the left flank at three sites with a total of 0.15-mL emulsion containing 150 mg PLP<sub>139-151</sub> and an equal volume of complete

Freund's adjuvant (Sigma-Aldrich, St. Louis, MO, USA). Immediately and 48 h later, 300 ng pertussis toxin (Sigma-Aldrich) was injected subcutaneously. The mice (N = 20, per group) were treated with PBS or BA (5 and 10 mg kg<sup>-1</sup> day<sup>-1</sup>, respectively, in PBS, with pH adjusted to 7.2) beginning on the day -1 of the immunization and for 3 consecutive days. Control mice (N = 12) treated with PBS were injected with complete Freund's adjuvant plus pertussis toxin according to the same schedule (data not shown).

### Clinical evaluation

Mice (N = 20) were observed daily for clinical signs of disease up to 60 days after immunization. Mice were scored according to their clinical severity as follows: grade 0, no abnormality; grade 0.5, partial loss of tail tonicity, assessed by inability to curl the distal end of the tail; grade 1, reduced tail tone of slightly clumsy; grade 2, tail atony, moderately clumsy gait, impaired righting ability, or any combination; grade 3, hind limb weakness of partial paralysis; grade 4, complete hind limb paralysis or fore limb weakness; grade 5, tetraplegia or moribund state; grade 6, death (7). The data were plotted as daily mean clinical score for all animals in a particular treatment group. Several parameters of disease were examined to evaluate the severity of EAE and the efficacy of BA therapy that include mean clinical score, incidence, mean day of onset, peak clinical score, and cumulative disease index (8).

### Histopathology

The lumbar region of the spinal cord was dissected from mice (N = 8, per group) at the peak of clinical disease between 14-20 days after immunization and fixed in 10% formalin. The spinal cords were cut into 4- $\mu$ m thick sections after fixation and embedded in paraffin before sectioning. The sections

were stained with hematoxylin and eosin (Sigma-Aldrich) for histologic analysis. Histologic scores were graded on each cross-section, according to the following criteria: 0, no infiltrate; 1, three or less focal meningeal infiltrates; 2, more than three focal meningeal infiltrates; 3, up to five perivascular infiltrate foci in the parenchyma; 4, five to ten perivascular foci in the parenchyma; 5, more than ten perivascular foci or diffuse infiltration.

### Cell proliferation assay and cytokine analysis

Mice (N = 10, per group) were killed at day 10 after PLP<sub>139-151</sub> sensitization. The popliteal and inguinal lymph nodes (PILN) were removed, dissected and dispersed through a stainless steel wire mesh as described (9). Cells were then washed three times in complete modified Eagle's medium (Flow, Irvine, UK) supplemented with modified RPMI-1640 medium containing 25 mM HEPES, L-glutamine (Gibco, Paisley, UK), 100 U/mL penicillin, 100 mg/mL streptomycin (Gibco), and 10% (v/v) heat-inactivated FCS (Gibco). PILN mononuclear cell (MNC) was adjusted to an appropriate density for analysis.

BA was dissolved in dimethylsulfoxide (DMSO) as 10 mM stock solutions and diluted to 1 mM with culture medium just before use. Blank cells received an equivalent amount of DMSO. The final concentration of DMSO did not exceed 0.2% (v/v).

An MTT (Sigma-Aldrich; 2.5 mg of MTT/

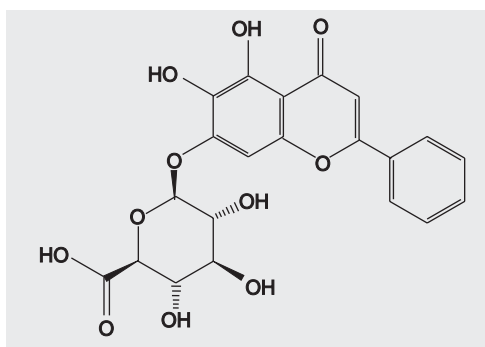


Figure 1. The structure of baicalin.

mL in PBS) assay (10) was performed to measure the proliferation of PILN MNC. Aliquots of 100  $\mu$ L PILN MNC suspensions from EAE and control mice were cultured in triplicates in round-bottomed 96-well polystyrene microtiter plates (Nunc, Copenhagen, Denmark) at a cell density of  $2 \times 10^6$  cells/mL in culture medium. PILN MNC were stimulated for 72 h with PLP<sub>139-151</sub> and treated for 72 h with various concentrations of BA or an equivalent amount of medium containing DMSO at 37°C in 95% humidity and 5% CO<sub>2</sub> (3 wells for each sample). Blank cells from control mice received an equivalent amount of medium containing DMSO. After drug treatments (final concentration: 100  $\mu$ g/mL PLP<sub>139-151</sub>; 5, 10, and 25  $\mu$ M BA) the supernatant was removed, MTT was dissolved in PBS to a concentration of 5 g/L, and 20  $\mu$ L was added to each well of a 96-well plate. The MTT-containing medium was then removed after 4-h incubation, DMSO was added to dissolve the reduced formazan product. Finally, the plate was read at 550 nm with a microplate reader (Bio-Rad 2550, Richmond, CA, USA).

One  $\mu$ g/mL PILN MNC suspensions from EAE and control mice were added to each well of round-bottomed 24-well polystyrene microtiter plates (Nunc) at a cell density of  $5 \times 10^6$  cells/mL in culture medium. Each treatment as described above was performed in triplicate. PILN MNC were stimulated with 100  $\mu$ g/mL PLP<sub>139-151</sub> for 72 h and treated with various concentrations of BA or an equivalent amount of DMSO for 72 h. Culture supernatant was collected and stored at -70°C until use. Cytokines (IFN- $\gamma$  and IL-4) were detected in culture supernatants on the same day with the same reagent using commercially available Mouse ELISA kits (Diacclone Research, Besancon, France) according to manufacturer instructions. Absorbance was read at 450 nm using an enzyme-linked immunosorbent microplate reader (Bio-Rad; Model 2550). Standard curves for each assay were generated and the

concentration of the cytokines in the cell supernatants was determined by extrapolation from the appropriate standard curve. The lower limits of detection for each assay were 15 pg/mL for IFN- $\gamma$  and 3.0 pg/mL for IL-4. The intra-assay coefficient of variation was <5% for IFN- $\gamma$  and IL-4.

#### Statistical analysis

Analyses were performed using one-way ANOVA and Kruskal-Wallis ANOVA with *post hoc* analysis using Dunnett's multiple comparison test, or Fisher's exact test, as appropriate. Results are reported as mean  $\pm$  SD; P values <0.05 were considered to be statistically significant.

## Results

#### Effects of baicalin on the clinical signs of mice

We evaluated the ability of BA (5 and 10 mg/kg, respectively) to affect induction and progression of EAE after immunization with PLP<sub>139-151</sub>. Treatment with BA (5 or 10 mg kg<sup>-1</sup> day<sup>-1</sup>) was initiated prior to immunization (from day -1 to day 1 relative to immunization) for 3 consecutive days. BA (5 or 10 mg kg<sup>-1</sup> day<sup>-1</sup>) led to a statistically significant reduction in EAE clinical score. These effects led to significant clinical improvement and delayed disease progression during the following 60 days of observation, indicating that BA inhibited both development and progression of EAE (Table 1 and Figure 2).

#### Effect of baicalin on inflammation in the spinal cord

The neuropathological changes in EAE and MS are associated with the blood-brain barrier breakdown and infiltration by MNCs. In order to identify the mechanism of therapeutic efficacy in EAE, we studied the effect

of BA on the inflammation in the CNS in EAE model. Spinal cord from BA-treated mouse shows lesser dense mononuclear infiltration surrounding vasculum and edge compared with that from PBS-treated mouse ( $P = 0.000$ ) (Figure 3).

### Effects of baicalin treatment on peripheral T-cell responses

PILN MNCs from SJL/J mice with EAE were analyzed for proliferation and cytokine responses to the immunizing PLP<sub>139-151</sub> peptide. Immune cell responses were assessed just after disease onset (day 10). As shown in Figure 4A, BA inhibited proliferation responses to PLP<sub>139-151</sub> peptide in a concentra-

Table 1. Effect of treatment with baicalin on neurological impairment during experimental autoimmune encephalomyelitis (EAE) in SJL/J female mice.

Group	Incidence (number/total)	Mean day of onset	Peak clinical score	Cumulative disease index
PBS/EAE	20/20 (100%)	8.0 ± 0.73	3.0 ± 0.4	141.8 ± 19.4
BA (5 mg/kg)	19/20 (95%)	9.0 ± 0.80*	2.2 ± 0.3*	75.9 ± 10.1*
BA (10 mg/kg)	18/20 (90%)	9.2 ± 0.75*	2.0 ± 0.3*	62.9 ± 8.4*

Female SJL/J mice were immunized with proteolipid protein 139-151 (day 1) and treated intraperitoneally with baicalin (BA) (5 or 10 mg/kg) (day -1 to day 1) as described in Material and Methods. Incidence of disease evaluated by Fisher's exact test ( $\chi^2 = 2.105$ ,  $P = 0.349$ ). Mean day of onset and clinical score were evaluated by the Kruskal-Wallis test with *post hoc* analysis using Dunnett's multiple comparison test.

\* $P = 0.000$  compared to phosphate-buffered saline (PBS). Cumulative disease index was determined as the sum of daily scores for each mouse in the indicated group compared to PBS and the data were analyzed by ANOVA (\* $P = 0.000$  with *post hoc* analysis using Dunnett's multiple comparison test).

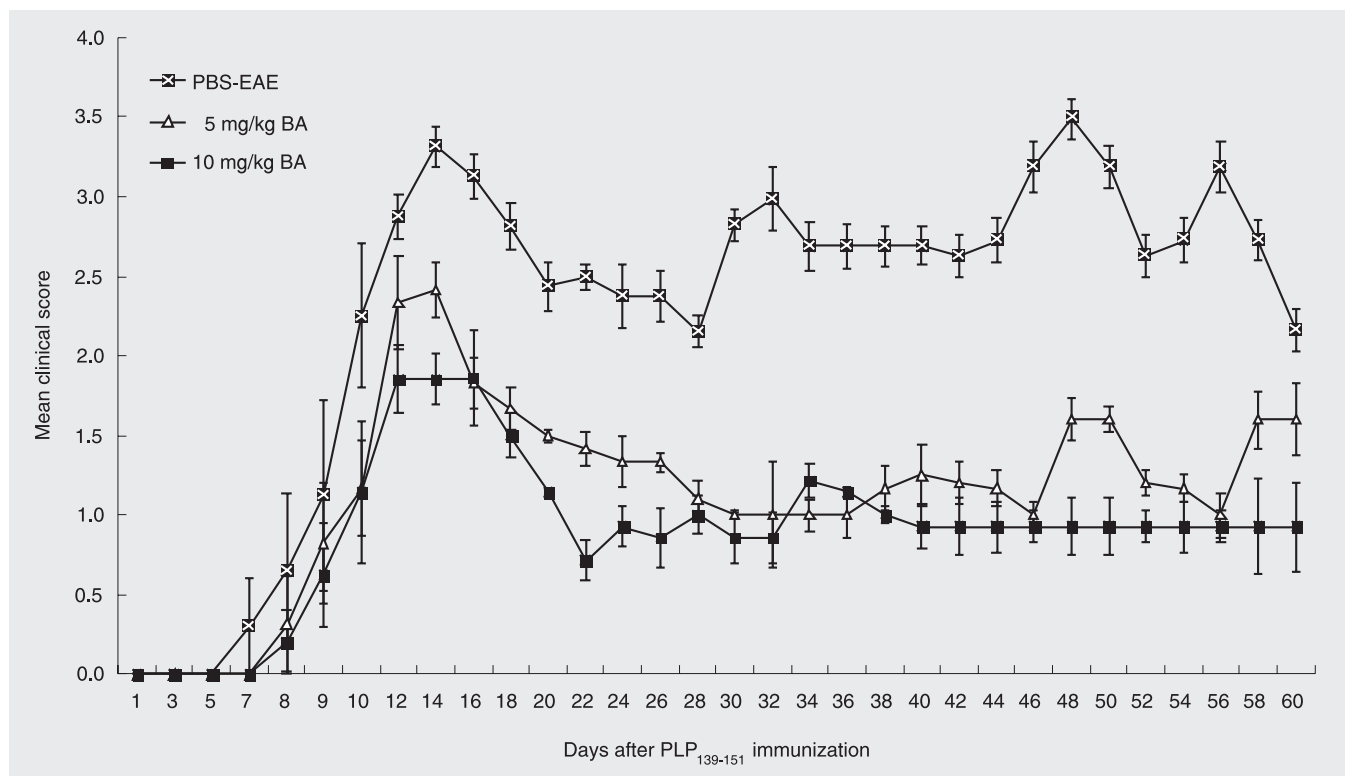


Figure 2. Effect of baicalin treatment on clinical score of experimental autoimmune encephalomyelitis associated disease activity. SJL/J female mice were immunized subcutaneously at three sites in the left flank with a total of 0.15 mL of an emulsion containing 150 mg PLP<sub>139-151</sub> and an equal volume of complete Freund's adjuvant. Immediately and 48 h later, 300 ng pertussis toxin was injected subcutaneously. Starting one day before immunization (day -1), the animals received a different dose of baicalin (5 or 10 mg/kg) by intraperitoneal injection for three consecutive days. Animals were scored daily for clinical symptoms on a 0-6 scale. Mean clinical scores were determined for each treatment group (N = 20). PBS-EAE = phosphate-buffered saline-experimental autoimmune encephalomyelitis; BA = baicalin; PLP<sub>139-151</sub> = proteolipid protein 139-151.

tion-dependent manner. There was a significant reduction in secreted IFN- $\gamma$  and a significant increase in secreted IL-4 (Figure 4B and C).

## Discussion

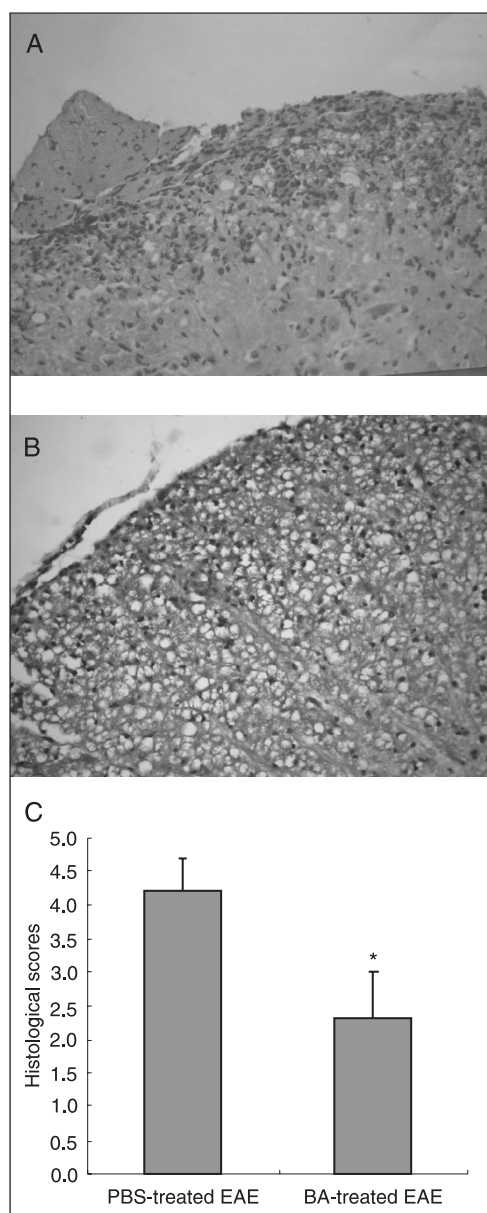
EAE is an autoimmune disease of CNS mediated by CD4<sup>+</sup> T lymphocytes specific for autoantigens of the myelin sheath, including myelin basic protein and proteolipid

protein (11,12). Remitting/relapsing EAE (RR-EAE) induced by active immunization with the immunodominant epitope of PLP<sub>139-151</sub> (13), is characterized by an initial acute phase, followed by a series of remissions and relapses (14). CD4<sup>+</sup> Th1 cells and their proinflammatory cytokines are suspected to be important in the pathogenesis of MS (15), and necessary for the induction of RR-EAE (14,16,17). The cytokine profile and the nature of cellular infiltrate were markedly different during the clinical course of RR-EAE, wherein the maximal expression of the proinflammatory cytokines IFN- $\gamma$  and TNF- $\alpha$  are important mediators for disease induction. Th2 cell clones specific for encephalitogenic peptides are unable to induce the disease and can inhibit Th1 autoimmune clones, presumably by secreting IL-4, IL-10, and TGF- $\beta$  (18-20). Several studies have suggested a significant role for IL-4, but not IL-10, in promoting RR-EAE remission.

Although we did not test directly the ability of BA to ameliorate disease symptoms at the peak of disease, our findings suggest that this drug is capable of suppressing a pre-activated immune system in the late effector phase leading into disease eruption. Since BA can penetrate into brain and spinal cord, we did not test the time point related to the stage of disease progression, in which the encephalitogenic T-cells are initiating infiltration into the CNS and beginning to recruit macrophages and microglia (21). The dose-response experiments carried out in the present study demonstrated that BA is therapeutic at the two doses used (Table 1). BA of 5 mg/kg is no less effective than that of 10 mg/kg. Furthermore, the fact that no reversing trend observed in the amelioration of multiple disease parameters would suggest that the efficacy limit might be at 5 mg/kg (Table 1, Figures 2 and 3).

The pathogenic processes in EAE may greatly rely on the secretion of IFN- $\gamma$  and IL-4, likely to be modulated by BA. *Ex vivo* studies using PILN isolated from EAE animals showed

Figure 3. Inflammation in a section of lumbar spinal cord from experimental autoimmune encephalomyelitis (EAE) + phosphate-buffered saline (PBS) and EAE + baicalin (BA; 10 mg/kg) SJL/J mice. BA reduced inflammation in the spinal cord of EAE. The lumbar region of the spinal cord was dissected from the animals (N = 8 per group) at the peak of clinical disease 14-20 days after immunization and fixed in 10% formalin. The spinal cords were embedded in paraffin and cut into 4- $\mu$ m thick sections. The sections were stained with hematoxylin and eosin. A, Spinal cord from a PBS-treated mouse shows diffuse dense mononuclear infiltration surrounding vasculum and edge. B, Spinal cord from BA-treated mouse shows lesser dense mononuclear infiltration surrounding vasculum. C, The means and SEM of histologic scores of PBS- and BA-treated mice. Each cross-section was examined in each lumbar region of spinal cord. The data are summarized from each mouse in each group. \*P = 0.000 vs PBS-treated EAE (independent-sample *t*-test, N = 8 per group). Magnification: 100X for A and B.



that BA (5, 15, and 25  $\mu\text{M}$ ) inhibits the PILN MNC proliferation, as well as reduced IFN- $\gamma$  production while increasing IL-4 production in a concentration-dependent manner. These studies indicate that BA treatment inhibited a proinflammatory Th1-biased cytokine response as well as promoted an increase of anti-inflammatory cytokine and attenuated clinical disease in the PLP<sub>139-151</sub>-induced model of EAE in SJL/J mice.

The dried roots of *Scutellaria baicalensis* Georgi (common name: *Huangqin* in China) have been widely employed for many centuries. They are effective against staphylococci, cholera, dysentery, pneumococci, and influenza virus. The active components are flavonoids, the major being BA, baicalein and wogonin (22). In our preliminary experiments, although all the three flavonoids manifested similar antioxidant activity, BA was more effective on antigen-specific inhibition of Th1 cells of autoimmune encephalomyelitis. The glycoside of baicalein, BA, is a potent anti-inflammatory and anti-tumor agent (23). BA showed strong anti-inflammatory activity, similar to that of dexamethasone in the treatment of endotoxic lung injury (5) and it could inhibit the binding of a number of chemokines to human leukocytes or cells transfected to express specific chemokine receptors (24) affecting cell migration. Recent studies showed that BA as a potential cancer chemopreventive agent inhibited T-cell proliferation and Th1 cytokines such as TNF- $\alpha$  and IFN- $\gamma$  (25). In the light of our observations and the literature findings that highlight the anti-inflammatory potential of BA, we hypothesize that BA would alter the cytokine production pattern from Th1-type to favorable Th2-type and contribute to the reduced severity of EAE. The results obtained in this study clearly show that BA (5 and 10 mg/kg) treatment effectively reduces the severity of EAE-associated clinical symptoms and neuroinflammation in spinal cord, and further show a differential cytokine response of PILN

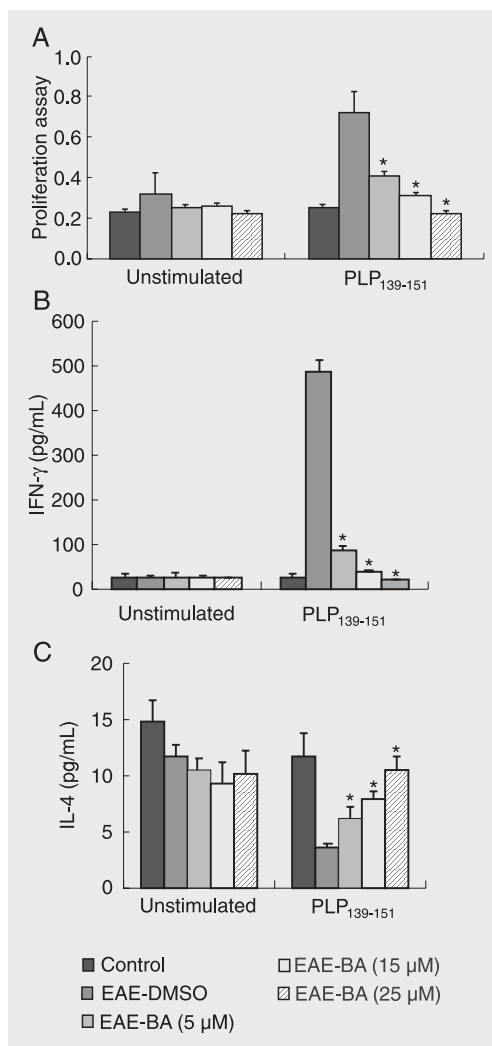


Figure 4. Effect of baicalin (BA) treatment on a proliferation assay (A) and on cytokine levels (B, IFN- $\gamma$  and C, IL-4) *in vitro*. Animals with experimental autoimmune encephalomyelitis (EAE) (N = 10) were analyzed at day 10 after immunization. Popliteal and inguinal lymph node mononuclear cells were stimulated with proteolipid protein 139-151 (PLP<sub>139-151</sub>) and various concentrations of BA (5, 15, and 25  $\mu\text{M}$ ) or dimethylsulfoxide (DMSO) for 72 h. Blank cells from control mice received an equivalent amount of DMSO. The proliferation assay was determined by MTT. The levels of IFN- $\gamma$  and IL-4 were determined by ELISA. \*P < 0.001 vs EAE-DMSO (one-way ANOVA followed by the *post hoc* multiple comparison Dunnett test).

MNCs *ex vivo*, decreasing the proinflammatory Th1 cytokine IFN- $\gamma$  while increasing the anti-inflammatory Th2 cytokine IL-4. These results suggest that BA modulates EAE, at least in part, by suppressing the induction of IFN- $\gamma$  and increasing the induction of IL-4. This finding encourages further investigation on BA in its role as a potential candidate for developing more efficient therapy strategies against multiple sclerosis.

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