

## Novel immunoregulatory properties of EGCG on reducing inflammation in EAE

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### TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Materials and methods
  - 3.1. Mice and reagents
  - 3.2. Induction of EAE
  - 3.3. EGCG treatment and evaluation of EAE
  - 3.4. Histopathological analysis
  - 3.5. LDH cytotoxicity assay
  - 3.6. MOG-specific T cell response
  - 3.7. Measurement of cytokine production
  - 3.8. Isolation of mononuclear cells from mouse CNS tissue
  - 3.9. Flow cytometric analysis
  - 3.10. Immunoblot analysis
  - 3.11. Quantitative real-time polymerase chain reaction (PCR)
  - 3.12. Mouse Th1 and Th17 cell differentiation in vitro
  - 3.13. Cell migration assay
  - 3.14. Statistical analysis
4. Results
  - 4.1. Amelioration of disease severity in EAE by EGCG
  - 4.2. Regulatory effects of EGCG on encephalitogenic T cell responses
  - 4.3. Selective down-regulation of STAT signaling pathway in encephalitogenic T cells by EGCG
  - 4.4. Direct effect of EGCG on Th1 and Th17 cell differentiation
  - 4.5. Reduced encephalitogenic lymphocyte migration by EGCG treatment
  - 4.6. Altered expression of co-stimulatory molecules and reduced function of APCs by EGCG treatment
5. Discussion
6. Acknowledgments
7. References

## 1. ABSTRACT

EGCG is one of the major catechins in green tea. In this study, we investigated the novel regulatory mechanism of EGCG on amelioration of experimental autoimmune encephalomyelitis (EAE). The data showed that EGCG reduced disease severity in EAE by decreasing brain inflammation and demyelination damage, accompanied by decreased encephalitogenic T cell responses and reduced expression of inflammatory cytokines and chemokines. The effect of EGCG was attributable to its selective inhibition of interferon-gamma and interleukin-17 production in CD4<sup>+</sup> T cells, mediated via alteration of the STAT pathway and the transcription factors T-bet and retinoid-related orphan receptor (ROR) gamma/ROR alpha. More important, EGCG has been found novel properties of directly inhibiting Th1 and Th17 cell differentiation in this study. On the other hand, EGCG-treated antigen presenting cells (APC) exhibited reduced co-stimulatory function as a result of altered expression of CD80 and CD86. The results of this study indicate that EGCG is a novel anti-inflammatory agent that could act as a useful drug for the treatment of multiple sclerosis and other neuroinflammatory diseases in the further.

## 2. INTRODUCTION

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) characterized by chronic inflammatory and demyelinating lesion. MS is generally considered to have an autoimmune pathology (1). Experimental autoimmune encephalomyelitis (EAE) has similar clinical, histological, genetic and immunological features to MS, and is thus often used to study the pathogenesis and therapeutic strategies for MS. EAE is known to be mediated by autoantigen-specific T cells and has previously been identified as a Th1-mediated inflammatory autoimmune disease (2). Recent studies revealed that treatment with a neutralizing anti-interleukin (IL)-17 antibody after the onset of EAE significantly reduced disease severity, while IL-17-deficient mice were resistant to the development of EAE (3, 4). Many studies have detected accumulation of Th17 and Th1 cells in the formation and perpetuation of spinal cord lesions (2, 5, 6). These data suggest that both Th17 and Th1 cells play a critical role in the induction and development of EAE. Emerging data suggest that both Th17 and Th1 cells contribute to CNS autoimmunity, albeit through different

mechanisms. A better understanding of the roles of Th17 and Th1 cells in autoimmune inflammation will be helpful in developing new therapeutic approaches (7).

The currently approved therapies for MS are only partly effective, or are often limited by their side effects. Green tea is one of the most popular beverages in the world. It is made by brewing the leaves of *Camellia sinensis* to inactivate polyphenol oxidase. Drinking green tea is associated with a lower incidence of human cancers (8), and subsequent studies have suggested that the polyphenol fractions of green tea play an important function in this effect (9). (-)-Epigallocatechin gallate (EGCG) is a major polyphenolic component of green tea and the most abundant and bioactive compound. It has been shown to lower the incidences of cancers, diabetes, arthritis, inflammatory mediator production and oxidative stress, and to reduce body weight and body fat (10-12). In recent years, research into EGCG has been extended to its immunoregulatory properties. EGCG has previously been shown to have a positive effect in animal models of inflammatory or autoimmune diseases (13-16), such as collagen-induced arthritis (CIA) and concanavalin A-induced hepatitis. EGCG was also found to inhibit the production of pro-inflammatory cytokines such as interferon (IFN)-gamma and tumor necrosis factor (TNF)-alpha in autoimmune diseases (17-19). However, little is known about the mechanisms responsible for the effects of EGCG in MS and EAE. In light of the pharmacological profile of EGCG and its reported immunoregulatory properties, we hypothesized that EGCG might play a beneficial role in the treatment of neuroinflammation.

This study therefore aimed to elucidate the underlying regulatory mechanisms of EGCG. The results of this study will improve our understanding of the pathogenesis of EAE, and also help in the development of future therapeutic strategies for MS.

### 3. MATERIALS AND METHODS

#### 3.1. Mice and reagents

Male C57BL/6 mice, 7 weeks old, were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China) and housed in the animal care facilities of Shanghai JiaoTong University School of Medicine under pathogen-free conditions, according to the Institutional Animal Care and Use Committee guidelines. Myelin oligodendrocyte glycoprotein (MOG) (residues 35-55) peptide (MOG<sub>35-55</sub>), with the sequence Met-Glu-Val-Gly-Trp-Tyr-Arg-Ser-Pro-Phe-Ser-Arg-Val-Val-His-Leu-Tyr-Arg-Asn-Gly-Lys, was supplied by GL Biochem (Shanghai, China) and its purity was greater than 95%. Pertussis toxin was purchased from List Biological Laboratories Inc (Campbell, CA, USA). Incomplete Freund's adjuvant and heat-killed H37Ra, a strain of *Mycobacterium tuberculosis*, were obtained from Difco Laboratories (Franklin Lakes, NJ, USA). EGCG was supplied by Sigma-Aldrich (St. Louis, MO, USA).

#### 3.2. Induction of EAE

Each mouse was subcutaneously immunized with 200 µl inoculum containing 300 µg of MOG<sub>33-35</sub>

peptide in 100 µl phosphate-buffered saline (PBS) and 100 µl complete Freund's adjuvant (CFA) containing 5 mg/ml heat-killed H37Ra. All mice were injected intravenously with 200 ng pertussis toxin in PBS twice at 0 and 2 days post-immunization.

#### 3.3. EGCG treatment and evaluation of EAE

Mice received 300 µg EGCG in 100 µl PBS daily, while the control group was administered PBS alone, as soon as clinical signs of EAE appeared. EAE clinical signs were scored according to the following criteria: 0, normal behavior; 1, limp tail; 2, paraparesis (weakness, incomplete paralysis of one or two hind limbs); 3, paraplegia (complete paralysis of two hind limbs); 4, paraplegia with forelimb weakness or paralysis; 5, moribund state or death. The animal protocol was approved by Shanghai Institute of Immunology.

#### 3.4. Histopathological analysis

Spinal cords from mice transcardially perfused with 4% paraformaldehyde were dissected and postfixed overnight. Paraffin-embedded 5 µm spinal cord sections were stained with hematoxylin and eosin (H&E) or Luxol fast blue and examined by light microscopy.

#### 3.5. LDH cytotoxicity assay

Cytotoxicity assay was performed using a CytoTox 96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI) in accordance with the instructions of the manufacturer. Briefly, splenocytes from EAE mice were cultured in 96-well microplates at  $1 \times 10^5$  cells per well and incubated at 37°C in 5% CO<sub>2</sub>. All wells except for control wells were supplied with culture medium with EGCG at final concentrations of 20, 40 and 60 µM, respectively. After various treatments for 72 h, medium from each well was collected to measure the amount of released LDH (Experimental LDH release). Cells in separate sister wells were exposed to lysis buffer for 30 min at 37°C and media were collected to measure the total amount of cellular LDH (Maximum LDH release). Optical density was measured at a wavelength of 490 nm. The cytotoxicity was calculated using the following equation: cell cytotoxicity (%) = Experimental LDH release (OD<sub>490</sub>) / Maximum LDH release (OD<sub>490</sub>) × 100.

#### 3.6. MOG-specific T cell response

To investigate the *ex vivo* proliferation response, splenocytes isolated from EGCG-treated and PBS-treated (as control) EAE mice were cultured in triplicate in RPMI 1640 with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 µg/ml penicillin/streptomycin, 1 mM sodium pyruvate, 0.5 mM 2-mercaptoethanol, 10 mM HEPES, and 1% non-essential amino acids. Splenocytes were plated at a density of  $5 \times 10^5$  per well in 96-well plates in the presence or absence of MOG<sub>35-55</sub> peptide (20 µg/ml). To assess the function of antigen-presenting cells (APCs), CD11b<sup>+</sup> cells were isolated from EGCG- or PBS-treated mice using antibody-coated magnetic beads, according to the manufacturer's protocol from Miltenyi Biotec (Bergisch Gladbach, Germany). CD4<sup>+</sup> MOG-reactive T cells purified from control EAE mice with a CD4 Negative Isolation Kit (Miltenyi Biotec) were cultured with the above APCs, at a

ratio of 3:1. All cultures were maintained at 37°C in 5% CO<sub>2</sub> for 72 h. Cells were pulsed with 1 µCi of [<sup>3</sup>H]-thymidine during the last 16–18 h of culture before harvest. [<sup>3</sup>H]-Thymidine incorporation was measured as cpm, detected using a MicroBeta beta counter (PerkinElmer, Shelton, CT, USA).

### 3.7. Measurement of cytokine production

Splenocytes isolated from EGCG-treated and PBS-treated EAE mice were cultured in 96-well plates at a density of 1.5×10<sup>6</sup> per well at the presence of MOG<sub>35-55</sub> peptide (20 µg/ml) in complete RPMI 1640 medium. Supernatants were collected after 48 h and diluted for measurement of IFN-γ, IL-4, TNF-α, IL-17, IL-6 and transforming growth factor (TGF)-β using enzyme-linked immunosorbent assay, according to the manufacturer's instructions from R&D Systems (Minneapolis, MN, USA). A standard curve was produced for each plate and used to calculate the absolute concentrations of the indicated cytokines.

### 3.8. Isolation of mononuclear cells from mouse CNS tissue

Mice were perfused with 10 ml PBS via the heart to eliminate peripheral blood. Mononuclear cells from brain and spinal cord were centrifuged by Percoll gradient centrifugation (GE Healthcare, NJ, USA). Mononuclear cells at the interface between the 37% and 70% Percoll gradients were collected and washed by centrifugation with medium.

### 3.9. Flow cytometric analysis

For intracellular cytokine staining, mononuclear cells isolated from CNS tissue were stimulated with 50 ng/ml phorbol myristate acetate (PMA) and 500 ng/ml ionomycin (Sigma-Aldrich) in the presence of GlogiPlug (BD Biosciences, Erembodegem, Belgium) for 5 h. Cell surfaces were stained with fluorochrome-conjugated specific antibody against mouse CD4 from eBioscience (Camarillo, CA, USA). Cells were then washed with fluorescence-activated cell sorting (FACS) buffer (PBS with 2% FBS), fixed and permeabilized with Cytofix/Cytoperm buffer (BD Biosciences), and intracellular cytokines were stained with fluorochrome-conjugated specific antibodies against mouse IL-17 and IFN-γ (eBioscience). In order to analyze the expression of co-stimulatory factors on APCs, cell surfaces were stained with fluorochrome-conjugated specific antibodies against mouse CD11b, CD80 and CD86 (eBioscience). Flow cytometric analysis was performed with a FACSCalibur (BD Bioscience).

### 3.10. Immunoblot analysis

Splenocytes from EGCG-treated or PBS-treated EAE mice were cultured in the presence of MOG<sub>35-55</sub> peptide (20 µg/ml) for 24 h. Cells were directly lysed and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Immunoblot analysis was performed by transfer of proteins onto nitrocellulose membranes (Schleicher & Schuell Microscience, Riviera Beach, USA) using a mini Trans-Blot apparatus (Bio-Rad). After 2 h blocking, the membranes were incubated overnight at 4°C

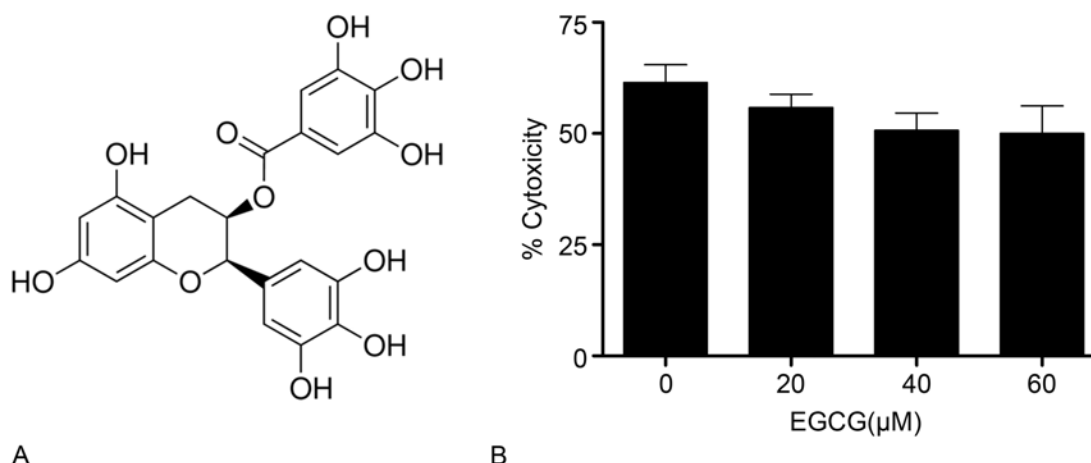
with specific primary mouse antibodies: anti-phospho-STAT3, anti-STAT3, anti-phospho-STAT6, anti-STAT6 (Cell Signaling Technology, Danvers, MA, USA); anti-phospho-STAT4 (Zymed Laboratories, San Francisco, CA, USA); anti-phospho-STAT1, anti-STAT1, anti-STAT4 (BD Biosciences); anti-T-bet (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and mouse β-actin Ab (Sigma-Aldrich, Shanghai, China). After washing, subsequent incubation with appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature, and extensive washing, signals were visualized by ECL substrate (Pierce Chemical, Dallas, Texas, USA).

### 3.11. Quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated from cell pellets using RNeasy Mini Kit (Qiagen, Hilden, Germany), and first-strand cDNA was subsequently synthesized using a Sensiscript RT Kit (Qiagen), according to the manufacturer's instructions. mRNA expression was determined by real-time PCR using SYBR Green Master Mix under standard thermocycler conditions (Applied Biosystems, Mississauga, Ontario, Canada), comprising initial holding at 50°C for 2 min and subsequently at 95°C for 10 min, followed by a two-step PCR program consisting of 95°C for 15 s, and 60°C for 60 s for 40 cycles. Data were collected and quantitatively analyzed on an ABI Prism 7900 Sequence Detection System (Applied Biosystems). The mouse β-actin gene was used as an endogenous control for sample normalization. Results were presented as folds relative to the expression of mouse β-actin. The sequences of PCR primer pairs were as follows: mouse β-actin, forward 5'-TGTCCACCTTCCAGCAGATGT-3' and reverse 5'-AGCTCAGTAACAGTCCGCCTAGA-3'; mouse RANTES, forward 5'-ACACCACTCCCTGCTGCTTT-3' and reverse 5'-GACTGCAAGATTGGAGCACTTG-3'; mouse monocyte chemotactic protein (MCP)-1, forward 5'-AAAAACCTGGATCGGAACCAA-3' and reverse 5'-CGGGTCAACTTCACATTCAAAG-3'; mouse macrophage inflammatory protein (MIP)-1α, forward 5'-CACCTCTGTCTCACCTGCTCAA-3' and reverse 5'-ATGGCGCTGAGAAGACTTGGT-3'; mouse RORα, forward 5'-GCCAAACGCATTGATGGATT-3' and reverse 5'-GCACGGCACATCCTAATAAACA-3'; mouse RORγ, forward 5'-GGAGCTCTGCCAGAAATGACC-3' and reverse 5'-CAAGGCTCGAAACAGCTCCAC-3'; mouse T-bet, forward 5'-CAGCCTGGGGACGCCCTACT-3' and reverse 5'-CCGGGAAAGCCAGCCACCTG-3'.

### 3.12. Mouse Th1 and Th17 cell differentiation *in vitro*

Naïve CD4<sup>+</sup> T cells were isolated from splenocytes of C57BL/6 mice, activated with plate-bound anti-CD3 and soluble anti-CD28 under Th1-polarizing conditions (IL-12, 10 ng/ml; anti-IL-4, 10 µg/ml) or under Th17-polarizing conditions (IL-6, 20 ng/ml; TGF-β, 2 ng/ml; anti-IL-4, 10 µg/ml; anti-IFN-γ, 10 µg/ml) in the absence or presence of different concentrations of EGCG (20 µM and 50 µM). After 4 days of culture, cells were restimulated with 50 ng/ml PMA and 500 ng/ml ionomycin in the presence of GlogiPlug for 5 h. IFN-γ-producing cells and IL-17-producing cells were



**Figure 1.** Chemical structure and cytotoxicity test of EGCG. (A) Chemical structure of EGCG. (B) Cytotoxicity of EGCG was measured by a CytoTox 96 nonradioactive cytotoxicity assay kit. Splenocytes from EAE mice were treated with EGCG at the indicated concentrations for 72 h. Optical density was measured at a wavelength of 490 nm. The cytotoxicity was calculated as described in “Materials and methods”. There is no statistical significant between the EGCG-treated group and untreated group.

analyzed using intracellular staining, as described previously.

### 3.13. Cell migration assay

Cell migration assays were performed in micro-transwells of 5-μm pore size membrane (Millipore, Billerica, MA, USA). Lymphocytes from EAE mice at day 14 post-immunization were cultured with MOG<sub>35-55</sub> peptide *in vitro* for 48 h. Cells were then added at 50,000 cells per well in the upper chambers. Supernatants for attracting cells were derived by culturing splenocytes from EGCG-treated and PBS-treated EAE mice for 48 h, harvesting the supernatants, and adding them to the lower chambers. The cells and supernatants were incubated at 37°C for 3 h. The migratory cells in the lower chambers were counted by light microscopy. The migration rate of the cells was calculated by the following equation: migration rate (%) = number of cells in the lower chambers/50,000.

### 3.14. Statistical analysis

Student's *t*-tests were used to analyze the differences between two groups. One-way ANOVA was initially performed to determine if an overall significant difference existed before using the two-tailed paired or unpaired Student's *t*-test. A value of  $P < 0.05$  was considered statistically significant.

## 4. RESULTS

### 4.1. Amelioration of disease severity in EAE by EGCG

The chemical form of EGCG used in this study was shown as Figure 1A, which was from green tea. It displayed no cytotoxicity toward primary mouse cells *in vitro* at concentration up to 60 μM, compared to control group without EGCG treatment ( $P > 0.05$ ) (Figure 1B).

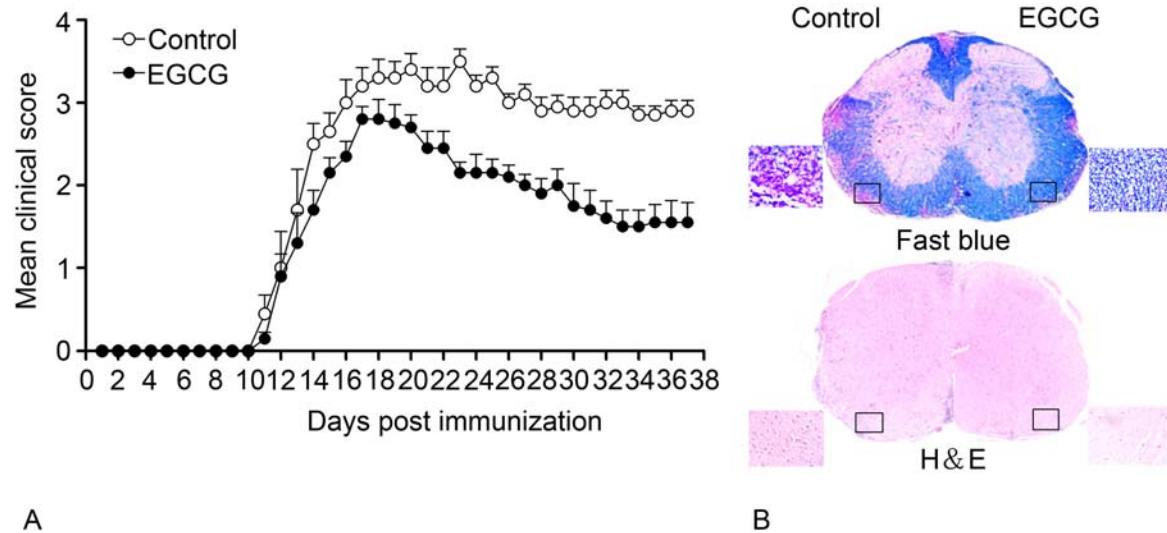
To explore the therapeutic effect of EGCG on EAE, C57BL/6 mice were immunized with MOG<sub>35-55</sub> peptide emulsified in CFA and the occurrence of clinical

signs of EAE was observed. EGCG therapy or vehicle was initiated at the time of disease onset. The severity of EAE was much milder in EGCG-treated mice compared to control mice that received PBS alone (Figure 2A). The effect became visible on day 14 after EGCG treatment and persisted throughout the entire course of EAE. No mice died during the course of EAE. We investigated the relationship between the observed clinical effect of EGCG and the histological analysis. The spinal cords from EGCG-treated and control mice were stained with H&E or Luxol fast blue. Histological evaluation of affected spinal cord lesions from EAE mice treated with EGCG exhibited markedly reduced inflammation and demyelination compared to those from untreated controls (Figure 2B). Collectively, these results suggest that EGCG is effective in amelioration of EAE development at both clinical and histological levels.

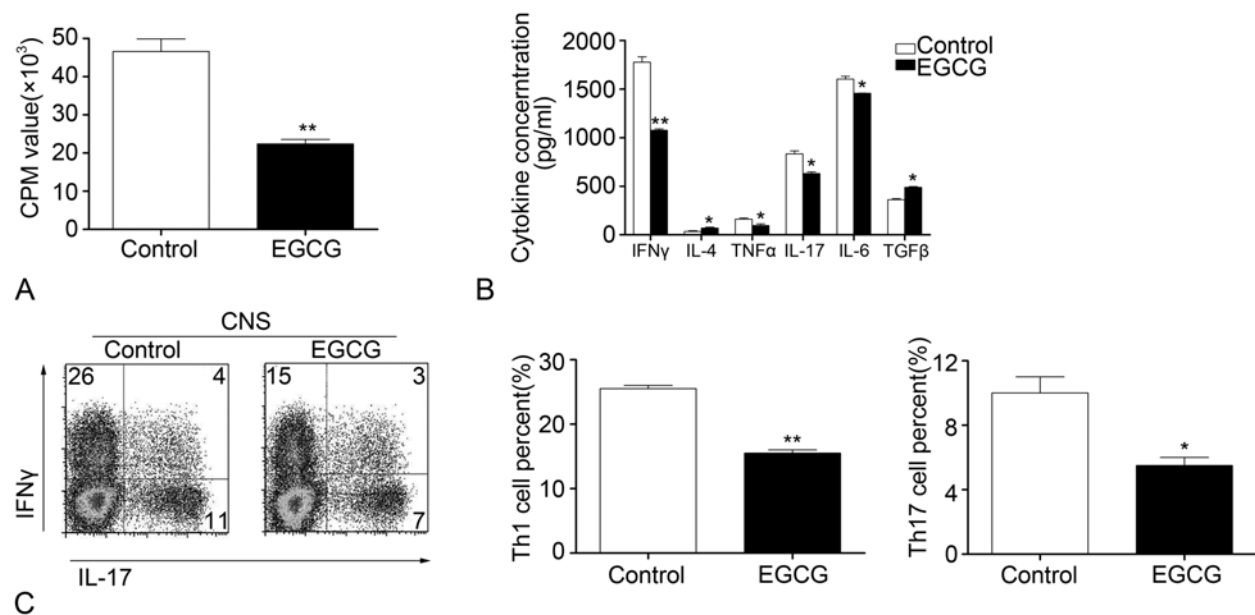
### 4.2. Regulatory effects of EGCG on encephalitogenic T cell responses

MOG-specific autoreactive T cells play a critical role in the development of EAE, and we therefore determined if the therapeutic effect of EGCG on EAE was associated with inhibition of the proliferation of MOG-specific T cells. Splenocytes were isolated from EGCG-treated and control EAE mice and T cell reactivity was analyzed in response to MOG<sub>35-55</sub> peptide challenge. Proliferation of MOG-reactive T cells derived from EGCG-treated EAE mice was significantly inhibited compared to that of untreated controls ( $P < 0.01$ ) (Figure 3A).

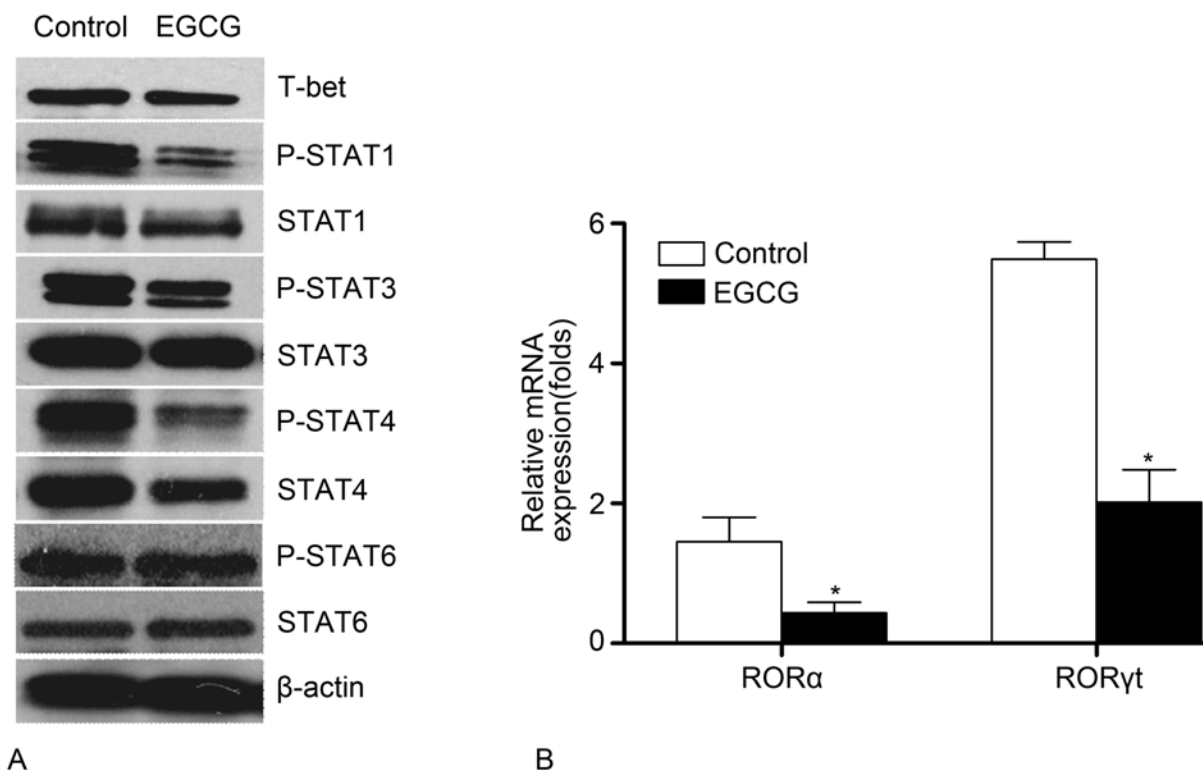
In EAE mice, immune cells are activated and pro-inflammatory or inflammatory cytokines such as IFN-γ are released. We therefore investigated the effects of EGCG treatment on cytokine profiles *in vivo*. We also isolated splenocytes from EGCG-treated and control EAE mice and analyzed the cytokine profiles in response to antigenic challenge. The EGCG-treated profile was characterized by significantly reduced production of IFN-γ, IL-17, IL-6, and TNF-α and increased



**Figure 2.** Clinical course and severity of EAE in mice treated with EGCG. (A) C57BL/6 mice (10 mice per group) were immunized with MOG<sub>35-55</sub> peptide to induce EAE, followed by daily intraperitoneal injections of EGCG or PBS at the time of disease onset. Mice were monitored and scored daily as described in “Materials and methods”. Data are representative of three independent experiments. (B) Histopathology of spinal cord tissue sections from EAE mice treated with EGCG or treated with PBS by fast blue (upper panel) and H&E (lower panel) staining.



**Figure 3.** Encephalitogenic T cell responses and cytokine profiles in response to MOG<sub>35-55</sub> peptide in EAE mice treated with EGCG. (A) Encephalitogenic T cell response was measured as MOG-induced T cell proliferation in EGCG-treated and PBS-treated mice (control mice). Splenocytes isolated from EGCG-treated and control mice at peak disease phase were stimulated with MOG<sub>35-55</sub> peptide (20  $\mu$ g/ml) and examined for *ex vivo* proliferation. Data are presented as mean [<sup>3</sup>H]-thymidine incorporation (cpm  $\pm$  SEM) in triplicate. (B) Splenocytes isolated from EGCG-treated and control mice at peak disease phase were stimulated with MOG<sub>35-55</sub> peptide (20  $\mu$ g/ml). Supernatants were collected from the above-mentioned cultures after 48 h, and concentrations of the indicated cytokines were measured using enzyme-linked immunosorbent assays. Data shown are means  $\pm$  SEM. (C) Mononuclear cells were isolated from brain and spinal cords of EGCG-treated or control mice and stained for intracellular IFN-gamma and IL-17 in CD4<sup>+</sup> T cells. Percentages of Th1 and Th17 cells in the CD4<sup>+</sup> T cell subset from representative subjects were determined by flow cytometry (left panel). Data shown are means  $\pm$  SEM in the right panel. Asterisks represent significant differences between the groups; \**P* < 0.05, \*\**P* < 0.01.



**Figure 4.** Regulation of STAT signaling pathway by EGCG. (A) Splenocytes isolated from EGCG-treated or control mice were cultured with MOG<sub>35-55</sub> peptide at a concentration of 20 μg/ml for 24 h and subjected to immunoblotting analysis for phosphorylated and total STAT1, STAT3, STAT4 and STAT6, as well as for T-bet. β-actin was loaded as a control throughout the experiments. Data are representative of three independent experiments. (B) mRNA levels of RORγt and RORα in the above-mentioned cells were analyzed by real-time PCR. Data shown are means ± SEM. Asterisks represent significant differences between groups; \**P* < 0.05.

production of IL-4 and TGF-β (P < 0.05) (Figure 3B). These results suggest that EGCG treatment may act partly through inhibition of the Th1 response and promotion of Th2 deviation. Reduced production of IFN-γ and IL-17 was also observed in CD4<sup>+</sup> T cells from the brain and spinal cord tissue of EGCG-treated EAE mice, compared to control mice, based on flow cytometric analysis (P < 0.05) (Figure 3C). The results indicate that EGCG treatment was associated with selective inhibition of Th1 and Th17 cells.

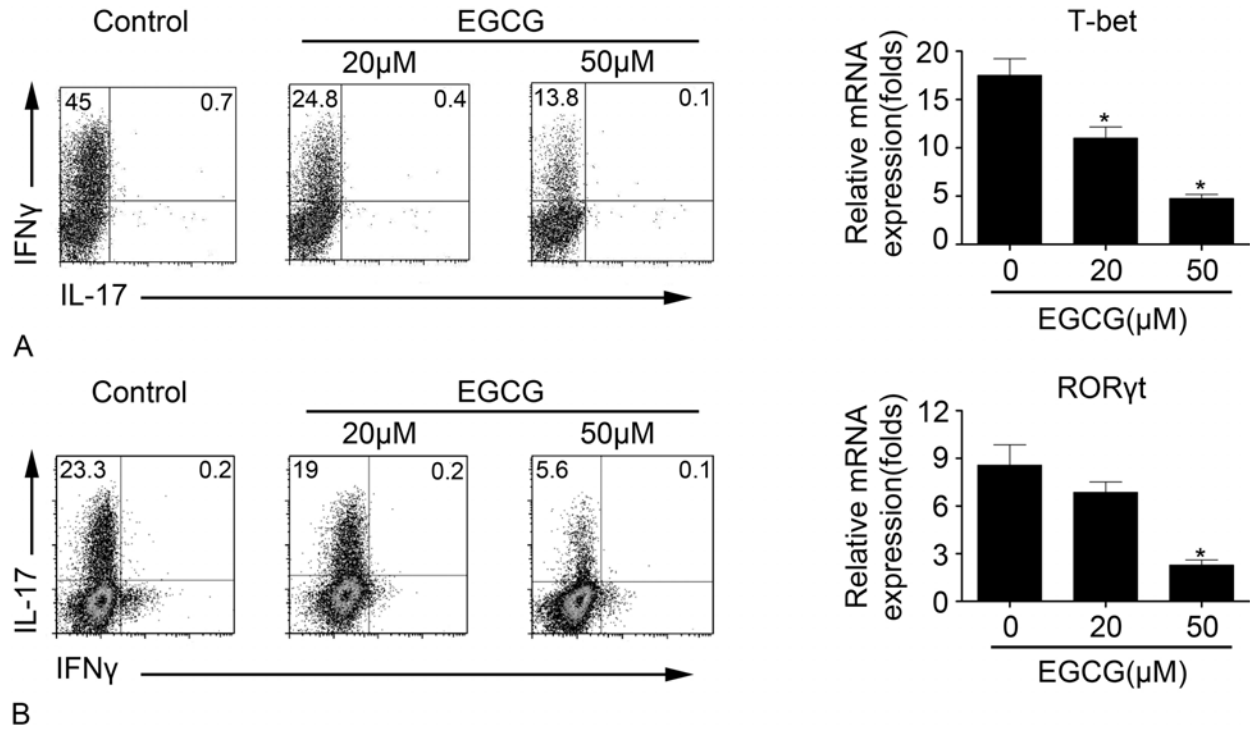
#### 4.3. Selective down-regulation of STAT signaling pathway in encephalitogenic T cells by EGCG

We further investigated the regulatory mechanisms of EGCG to determine its effects on encephalitogenic T cell responses and to identify its target molecules. The JAK/STAT pathway is recognized as one of the major mechanisms by which cytokine receptors transduce intracellular signals. Differentiation and survival of Th17 and Th1 cells are known to be related to the JAK/STAT signaling pathway, and we therefore analyzed the levels of transcription factors associated with the differentiation of Th1 (STAT1, STAT4 and T-bet), Th2 (STAT6) and Th17 (STAT3) by immunoblotting. Splenocytes from EGCG-treated or control mice were cultured in the presence of MOG<sub>35-55</sub> peptide for 24 h and subjected to analyze the key signaling molecules of the

STAT pathway by immunoblot. EGCG treatment specifically inhibited phosphorylation of STAT1, STAT3, and STAT4, as well as the expression levels of T-bet (Figure 4A). The expression of the transcription factors RORγt and RORα is known to be critical for Th17 cell differentiation, and we therefore also analyzed the expression of RORγt and RORα genes by quantitative-PCR. RORγt and RORα expression levels were markedly reduced in MOG-reactive splenocytes derived from EAE mice treated with EGCG, compared to control EAE mice (P < 0.05) (Figure 4B). Collectively, these findings indicate that EGCG plays a role in selectively inhibiting Th17 and Th1 cells by reducing the expression of phosphorylated STAT3 and STAT4, consistent with the expression of RORγt/RORα and T-bet.

#### 4.4. Direct effect of EGCG on Th1 and Th17 cell differentiation

We also determined if EGCG could directly affect Th1 and Th17 cell differentiation. Naïve CD4<sup>+</sup> T cells isolated from splenocytes of C57BL/6 mice were cultured for 4 days under Th1 or Th17-polarizing conditions, respectively, in the presence of anti-CD3 and anti-CD28. FACS revealed high levels of IFN-γ and IL-17 production from CD4<sup>+</sup> T cells under Th1 or Th17-



**Figure 5.** EGCG inhibited mouse Th1 and Th17 cell differentiation *in vitro*. Naïve CD4<sup>+</sup> T cells were cultured in the presence or absence of indicated concentrations of EGCG for 4 days under Th1-polarizing conditions (A) or Th17-polarizing conditions (B). Intracellular staining of IFN-gamma and IL-17 was analyzed in CD4<sup>+</sup> T cells (left panel) and mRNA expression level of T-bet and RORgammat was determined (right panel, means ± SEM). Data are representative of three independent experiments. Asterisks represent significant differences between groups; \**P* < 0.05.

polarizing conditions, respectively. The addition of EGCG markedly inhibited IFN-gamma and IL-17 production in dose-dependent manners (Figure 5A-B, left panel). We subsequently investigated the effects of EGCG on the expression levels of transcription factors required for Th1 and Th17 cell differentiation. T-bet and RORgammat expression levels in CD4<sup>+</sup> T cells were also reduced by EGCG in dose-dependent manners (*P* < 0.05) (Figure 5A-B, right panel). Collectively, these findings suggest that EGCG directly inhibited Th1 and Th17 cell differentiation.

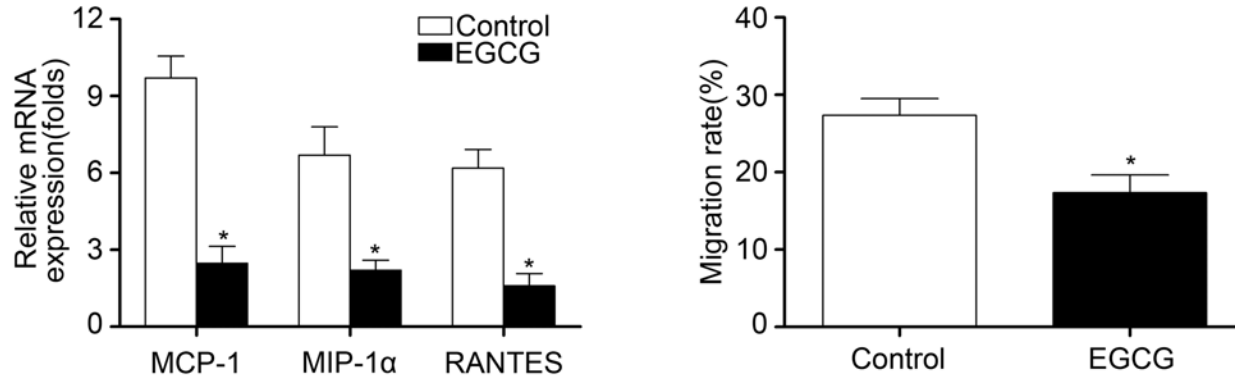
#### 4.5. Reduced encephalitogenic lymphocyte migration by EGCG treatment

Chemokines such as RANTES, MCP-1 and MIP-1α are critical mediators and play important roles in the migration of lymphocytes into CNS lesion *in vivo*. We extracted total RNA from splenocytes from EGCG-treated EAE or control mice and analyzed the mRNA expression levels of the indicated chemokines. As expected, RANTES, MCP-1 and MIP-1α expression levels were significantly reduced in EGCG-treated EAE mice compared to control mice (*P* < 0.05) (Figure 6A). To further investigate the effects of EGCG on encephalitogenic lymphocyte migration, lymphocytes from control EAE mice were cultured in supernatants from EGCG-treated and PBS-treated splenocyte cultures, and the migration rate of MOG-reactive lymphocytes towards EGCG-treated supernatants was markedly reduced compared to control supernatants (*P* < 0.05) (Figure 6B).

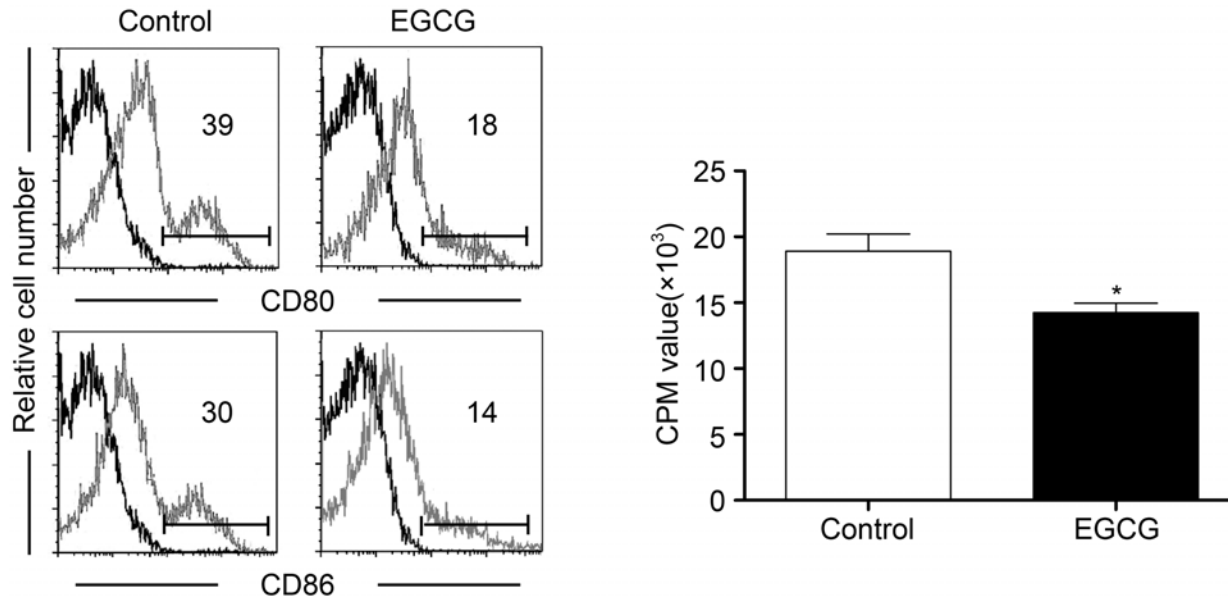
These results suggest that the therapeutic effect of EGCG on EAE may be mediated by the expression of chemokines that attract encephalitogenic lymphocytes and reduce the migration of inflammatory cells into the CNS.

#### 4.6. Altered expression of co-stimulatory molecules and reduced function of APCs by EGCG treatment

T cell activation requires two signals: recognition by T cell receptors of antigenic peptides presented by MHC molecules, and interaction between T cells and co-stimulatory molecules on APCs. We hypothesized that inhibition of the MOG-reactive T cell response in EGCG-treated EAE mice was associated with a reduced antigen-presenting function of APCs. We thus determined if the regulatory properties of EGCG on MOG-reactive T cell responses were attributable in part to its ability to alter the function of APCs. Splenocytes were obtained from EGCG-treated or control EAE mice and the expression of selected co-stimulatory molecules on CD11b<sup>+</sup> cells was examined by FACS. Surface expression of the predominant co-stimulatory molecules on APCs, including CD80 and CD86, was decreased in CD11b<sup>+</sup> cell populations from EGCG-treated EAE mice compared to control mice (Figure 7A). CD11b<sup>+</sup> cells as APCs were also isolated from EGCG-treated or PBS-treated EAE mice, respectively, and co-cultured with purified MOG-reactive CD4<sup>+</sup> T cells from PBS-treated EAE mice. Proliferation of MOG-reactive CD4<sup>+</sup> T cells co-cultured with APCs from EGCG-treated EAE mice was significantly reduced compared to APCs



**Figure 6.** EGCG treatment reduced encephalitogenic lymphocyte migration. (A) Splenocytes isolated from EGCG-treated or control mice were cultured with MOG<sub>35-55</sub> peptide at a concentration of 20 µg/ml for 24 h and subjected to real time PCR analysis to determine mRNA levels of MCP-1, MIP-1α and RANTES. (B) Splenocytes were cultured with MOG<sub>35-55</sub> peptide (20 µg/ml) for 48 h and the culture supernatants were collected for assessing directional migration of encephalitogenic lymphocytes obtained from control EAE mice. Migration assays were performed as described in “Materials and methods”. Data shown are means ± SEM. Asterisks represent significant differences between groups; \**P* < 0.05.



**Figure 7.** Effect of EGCG on CD11b<sup>+</sup> APCs. (A) Splenocytes from EGCG-treated or control mice were stained for CD80 and CD86 (light-colored line) or isotype control (dark-colored line) after MOG<sub>35-55</sub> peptide stimulation and examined by flow cytometry gated on the CD11b<sup>+</sup> cell population. (B) CD11b<sup>+</sup> cells isolated from spleens of EGCG-treated or control mice were cultured with CD4<sup>+</sup> MOG-reactive T cells respectively for 72 h. T cell proliferation was then examined. Data are presented as mean cpm ± SEM of triplicates and are representative of at least three independent experiments with similar results. Asterisks represent significant differences between groups; \**P* < 0.05.

from untreated EAE mice (*P* < 0.05) (Figure 7B). These results indicate that inhibition of the MOG-reactive T cell responses in EGCG-treated EAE mice was attributable to reduction of APC function by EGCG.

## 5. DISCUSSION

EGCG is the major polyphenolic compound in green tea, and possesses strong antioxidant and free radical scavenging

properties (20). The results of this study demonstrated that EGCG effectively ameliorated EAE through its unique anti-inflammatory properties involving pathogenic Th1 and Th17 cells. EGCG suppressed the proliferation of encephalitogenic T cells via alteration of the JAK/STAT pathway, and influencing the expression of pro-inflammatory cytokines and chemotactic factors. EGCG also inhibited the differentiation of Th1 and Th17 cells, but had no effect on the relative numbers of CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (data not shown).



The subset of helper T cells most critical for the pathogenesis of EAE is currently unclear. Previous studies have regarded EAE as a Th1-mediated autoimmune disease, and Th2-mediated responses have thus had beneficial effects on its severity and progression (21, 22). The balance of Th1/Th2-type cytokines might play a substantial role in the regulation of autoimmune diseases. Th17 cells were recently described as playing an important role in the pathogenesis of EAE (23). Mice deficient in either ROR $\gamma$  or T-bet are resistant to EAE induction, supporting the idea that both Th17 and Th1 cells are involved in CNS autoimmunity (24, 25). IFN- $\gamma$ -secreting Th1 cells express the lineage-specific transcription factor T-bet (26), and IL-17-secreting Th17 cells express the lineage-specific transcription factors STAT3 and ROR $\gamma$  (27). The current study demonstrated a novel aspect of the regulatory mechanism of EGCG with respect to Th17 and Th1 cells. The STAT signaling pathway is known to be a major signaling network that regulates T cell differentiation into various functional lineages (28). The current results showed that EGCG treatment inhibited STAT3 and STAT4 phosphorylation in encephalitogenic T cells, altering Th17 and Th1 cell differentiation and the clinical course of EAE. STAT3 has been shown to act as an upstream regulator for ROR $\gamma$ , a transcription regulator of Th17 cell differentiation (29, 30). The demonstrated regulatory properties of EGCG on Th17 and Th1 cell differentiation are consistent with its predicted effect on the STAT signaling pathway. As we know, NF- $\kappa$ B plays an essential role during Th1 and Th17 development. We found EGCG could increase the expression of I $\kappa$ B protein through inhibiting phosphorylated I $\kappa$ B level in previous research (data not shown) and the result is similar to the literature reported by Aktas *et al* (18). In IL-2 signaling pathway, it has been illustrated that EGCG only reduced expression of IL-2R, not affect IL-2 synthesis (31).

Chemokines are small proteins (8–12 kD) that can be divided into four subfamilies (CXC, CC, C, and CX3C) according to the organization of the positionally-conserved cysteine residues (32). They are mainly expressed at inflammatory sites, e.g., the synovium in rheumatoid arthritis and the CNS in MS or EAE (32, 33). RANTES, MCP-1 and MIP-1 $\alpha$  are members of the CC family of chemokines and can be secreted by activated encephalitogenic T cells and macrophages. Encephalitogenic lymphocytes traffic to the CNS and initiate inflammation and destruction of CNS myelin, with consequent neurological impairment (34–36). In order to investigate the effects of EGCG on the factors regulating the traffic of lymphocytes and monocytes in EAE, we studied the expression of RANTES, MIP-1 $\alpha$  and MCP-1 in splenocytes from control and EGCG-treated mice. EGCG administration significantly decreased the expression of the three chemokines in EAE mouse. Functionally directional migration assays indicated that EGCG could suppress the migration of encephalitogenic lymphocytes from EAE mice *in vitro*. These results may provide a basis for the development of EGCG as a therapeutic strategy for preventing autoimmune diseases.

This study also clarified the involvement of APCs

in the immunomodulatory effects of EGCG. APCs are known to be vital for the full activation of T cells by antigen presentation through the tricomplex of MHC/antigen and T cell receptors and the expression of co-stimulatory molecules, such as CD80 and CD86. This study showed that EGCG reduced APC function, correlated with downregulation of the co-stimulatory molecules CD80 and CD86, consequently affecting T cell proliferation. Overall, the results of this study indicated that EGCG had novel immunoregulatory properties to inhibit Th1 and Th17 cell differentiation and to reduce APC function. It provided new evidence supporting EGCG as a potential drug for the treatment of MS and other neuroinflammatory diseases.

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**Abbreviations:** EGCG: (-)-Epigallocatechin gallate; EAE: experimental autoimmune encephalomyelitis; ROR: retinoid-related orphan receptor; APC: antigen presenting cells; MS: multiple sclerosis; CNS: central nervous system; CIA: collagen-induced arthritis; IL: interleukin; IFN: interferon; TNF: tumor necrosis factor; MOG: myelin oligodendrocyte glycoprotein; PBS: phosphate-buffered saline; CFA: complete Freund's adjuvant; H&E: hematoxylin and eosin; FBS: fetal bovine serum; TGF: transforming growth factor; PMA: phorbol myristate acetate; RANTES: regulated upon activation normal T cell expressed and secreted; MCP-1: monocyte chemotactic protein-1; MIP-1alpha: macrophage inflammatory protein-1alpha; FACS: fluorescence-activated cell sorting

**Key Words:** EGCG, Th1, Th17, APC, Experimental Autoimmune Encephalomyelitis

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