International Immunopharmacology 70 (2019) 284–294

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

The therapeutic potential of ginkgolide K in experimental autoimmune encephalomyelitis via peripheral immunomodulation

Wen-Bo Yu[a], Qing Wang[b], Sheng Chen[a], Liang Cao[c], Jie Tang[a], Cun-Gen Ma[b], Wei Xiao[c], Bao-Guo Xiao[a,*]

[a] Department of Neurology, Huashan Hospital, State Key Laboratory of Medical Neurobiology, Fudan University, Shanghai, China
[b] 2011 Collaborative Innovation Center/Research Center of Neurobiology, University of Shanxi Traditional Chinese Medicine, Taiyuan, Shanxi, China
[c] State Key Laboratory of New-tech for Chinese Medicine Pharmaceutical Process, Lianyungang, Jiangsu, China

ARTICLE INFO

Keywords:
Ginkgolide K
Experimental autoimmune encephalomyelitis
Th17/Treg balance
M1/M2 polarization
Cell migration

ABSTRACT

Multiple sclerosis is a T cell-mediated inflammatory, demyelinating disease of the central nervous system, accompanied by neuronal degeneration. Based on the anti-inflammatory effects of Ginkgolide K (GK), a platelet activating factor antagonist, we explored the possible application of GK in the treatment of MS. The results showed that GK effectively ameliorated the severity of experimental autoimmune encephalomyelitis. The intervention of GK inhibited the infiltration of inflammatory cells and demyelination in the spinal cord. At the same time, the expression of the inflammation-related molecules TLR4, NF-κB, and COX2 in the spinal cord was significantly lower in the GK-treated mice, indicating that GK intervention can inhibit the inflammatory microenvironment of the spinal cord in EAE mice. In mouse spleen lymphocytes, GK increased the proportion of regulatory T cells (Treg) and reduced the proportion of T helper 17 cells (Th17), modifying the imbalance between Th17/Treg cells. Additionally, GK shifted macrophage/microglia polarization from M1 to M2 cell type. Importantly, GK inhibited the expression of chemotactic molecules CCL-2, CCL-3 and CCL-5, thereby limiting the migration of inflammatory cells to the spinal cord. Our results provide the possibility that GK may be a promising naturally small molecule compound for the future treatment of MS.

1. Introduction

Multiple sclerosis (MS) is a T cell-mediated, chronic progressive inflammatory disease of the central nervous system (CNS) that attacks the myelin sheath and the myelinated axons, resulting in physical disability [1]. Because the cause of MS is not fully understood, the exploration of treatment is still challenging. As is known, the immune system begins to perceive myelin as an invader (antigen) and to generate reactive T cells, resulting in the demyelination in the CNS of MS patients. Thus, the current treatment for MS with immunomodulatory drugs mainly includes interferon beta (IFN-β), glatiramer acetate, natalizumab, ritiximab, fingolimod, teriflunomide, and dimethyl fumarate, which are effective as the subcutaneous injection and oral medication used for RRMS for over decades. Besides injection site reaction, IFN-β is only valid in 30–50% of MS patients, and after prolonged use of IFN-β, some patients exhibited reduced responses to IFN-β due to the production of IFN-β-neutralizing antibodies [2]. Natalizumab can decrease disease activity and prevent disability progression in patients with relapsing-remitting MS (RRMS), but the treatment is associated with an increased risk of fatal progressive multifocal leukoencephalopathy [3,4]. Fingolimod decreases Th17 cells in the peripheral blood and inhibits lymphocyte egress from the lymph node, thus decreasing the reactive lymphocytes invading the CNS [5]. Teriflunomide leads to a cytostatic effect on proliferating T and B lymphocytes by disrupting DNA synthesis [5]. Dimethyl fumarate decreases the Th1/Th17 T cells and increases Th2 T cells [5]. In the clinical trials, most common adverse effects of these immunomodulatory drugs include headache, liver enzyme abnormalities, viral infections, diarrhea, alopecia, nausea, cough, vomiting, pruritus and papular eruption [5]. In addition, their long term safety data should be continued to be monitored in the future. So far, although a variety of FDA-approved drugs are available for MS, we are still far from curing MS.

Ginkgo biloba, extracted from *Ginkgo biloba*, has broad spectrum pharmacological properties, including neuroprotection, anticancer, cardiac protection and stress relief, as well as against ischemic stroke, Alzheimer's disease [6,7] and psychiatric disorders [8]. Ginkgolide B (GB)

* Corresponding author at: Department of Neurology, Huashan Hospital, State Key Laboratory of Medical Neurobiology, Fudan University, No.12 Wulumuqi Middle Road, Shanghai 200040, China.
E-mail address: bgxiao@shmu.edu.cn (B.-G. Xiao).

https://doi.org/10.1016/j.intimp.2019.02.035
Received 8 January 2019; Received in revised form 14 February 2019; Accepted 20 February 2019
Available online 06 March 2019
1567-5769/

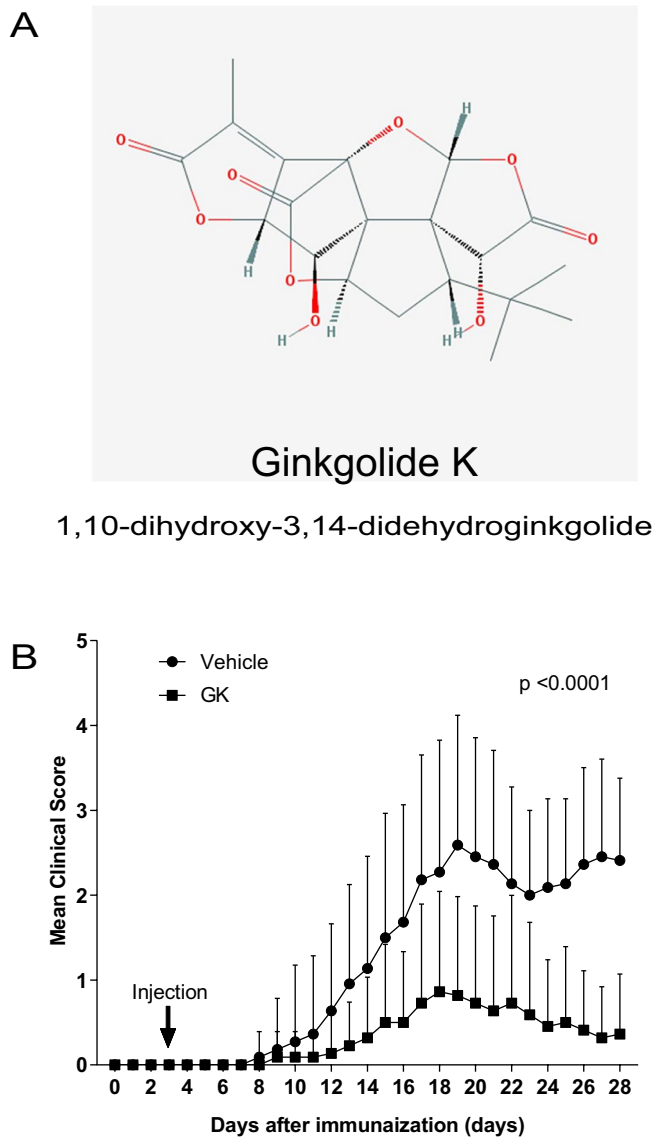


Fig. 1. GK ameliorated the severity of EAE. Mice were immunized with $MOG_{35–55}$ and received with GK by i.p. injection from day 3 to 28 p.i. (A) The chemical substructure of GK. (B) EAE clinical course of a representative experiment (dots represent the mean clinical scores, error bars are SD, n = 10 mice/group, two pooled independent experiments; $p < 0.0001$ by two-way ANOVA).

is the primary active monomer of ginkgolide that inhibits the activity of platelet-activating factor (PAF) by binding to PAF receptors on the platelet membrane [9]. GB exhibits protective effects on myocardial ischemia/reperfusion dysfunction [10], cerebral ischemic injury and neurological deficits in middle cerebral artery occlusion (MCAO) in rats [11]. GB also protects human umbilical vein endothelial cells [12] and inhibits Signal transducer and activator of transcription 1 (STAT1) expression to relieve spinal cord injury efficiently [13]. GB treatment significantly reduced intracranial pressure and decreased the percentage of lactic acid/pyruvate in patients with nontraumatic severe acute hemorrhagic stroke [14]. Thus, GB seems to enhance tissue protection in several diseases through different mechanisms. However, GB treatment does not affect the development of experimental autoimmune encephalomyelitis (EAE), a model of MS, despite having a protective effect for postsynaptic density protein 95 (PSD95)-positive synapse in the hippocampi of mice [15]. Also, an open-labeled pilot study of MS also showed that GB was not an effective treatment for acute exacerbation of MS [16].

Ginkgolide K (GK, Fig. 1A) is a novel naturally small molecule compound isolated from *Ginkgo biloba* [17], and also acts as a PAF antagonist, attracting much attention in recent years due to its potential neuroprotection in several in vitro and in vivo models. In the MCAO model, the pretreatment with GK diminished the infarction volume and brain water content, and improved neurological deficit score [18]. GK pretreatment promoted astrocyte proliferation and migration after oxygen-glucose deprivation (OGD) via inducing protective autophagy, indicating that GK might be a potential agent for cerebral ischemia/reperfusion injury [19]. GK also exhibited neuroprotection against PC12 and A53T cells through the degradation of unfolded protein [20]. It has been reported that GK confers neuroprotection against glutamate cytotoxicity and oxidative stress in PC12 cells [21,22]. GK also protected the heart against endoplasmic reticulum stress injury by activating the inositol-requiring enzyme 1alpha/X box-binding protein-1 pathway [23]. Although GK has shown neuroprotective effects in several models, its precise targets are still unclear. Up to now, there has been no study about the effect of GK in MS or EAE.

Although disease-modifying therapies commonly used in MS are effective and relatively safe, current treatment options are associated with various limitations including widespread and severe adverse effects. Recently, herbal therapies represent a promising therapeutic approach for MS [24]. Therefore, it is still necessary to find new immunomodulatory drugs from natural plants in the treatment of MS. In this study, we tried to observe the therapeutic potential of GK in EAE and explored its possible cellular and molecular mechanisms, especially as a peripheral immunomodulatory.

2. Material and methods

2.1. Animals

Female C57/B6 mice (8–10 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All mice were housed under pathogen-free conditions and received food and water ad libitum. They were kept under a reversed 12:12 h light/dark cycle in a temperature-controlled room (25 ± 2 °C) for one week before the experimental manipulation. The study was approved by the Ethics Committee of Fudan University (Approval No. 20171542A493), and the experiments were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health of the United States.

2.2. EAE induction and treatment

Myelin oligodendrocyte glycoprotein peptide 35–55 ($MOG_{35–55}$) was obtained from CL. Bio-Scientific Company (Xian, China). EAE was induced by subcutaneous immunization (4 loci/100 μL/mouse) in the flanks with 250 μg of $MOG_{35–55}$ incomplete Freund's adjuvant (CFA, Sigma, USA) (including 350 μg/mouse of M. tuberculosis H37Ra, BD Difco, USA). The mice were intraperitoneally (i.p.) injected with 300 ng of pertussis toxin (List Biological Laboratories Inc., USA) at the same time of immunization and 48 h after immunization. According to the literature from other research groups, mice were i.p. injected with GK (7.5 mg/kg) or vehicle (35% PEG400 + 5% ethanol + 60% saline) from day 3 post-immunization (p.i.) to day 27 p.i. into 150 μg/mouse/day [18,23,25]. The clinical scores were evaluated according to the following criteria: 0, no signs of disease; 1, loss of tone in the tail; 2, ataxia or paresis of hind limbs; 3, paralysis of hind limbs or paresis of forelimbs; 4, tetraplegia; and 5, moribund or dead. Two investigators were blinded to the treatments during the experiment.

2.3. Flow cytometry analysis

On day 28 p.i., the spleen was obtained under aseptic conditions, and splenic mononuclear cells (MNCs) were isolated. The MNCs were stimulated for 5 h with 2 μl/ml Leukocyte Activation Cocktail with BD

GolgiPlug™ (BD Biosciences, San Diego, CA, USA). Then, MNCs were stained in 1% BSA-PBS buffer or 0.3% saponin/1% BSA-PBS buffer for 30 min at room temperature (RT), with the following fluorescent-labeled antibodies: FITC-anti-CD4 and PE-anti-CD25; FITC-anti-CD4 and PE-anti-IFN-γ; FITC-anti-CD4 and PE-anti-IL-17; as well as FITC-anti-CD11b and PE-anti-CD16/32; FITC-anti-CD11b and PE-anti-CD206; FITC-anti-CD11b and PE-anti-IL-10; FITC-anti-CD11b and PE-anti-IL-12; PE-Mouse IgG2a Isotype Control and FITC-Mouse IgG2a Isotype Control (BD Biosciences, San Diego, CA, USA). Data were acquired on a FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA) or an Attune™ NxT flow cytometer (Thermo Fisher Scientific, Waltham, MA, USA). We first established FSC and SSC to exclude most of the dead cells, then used homotypic control to determine the location of negative cells, and finally analyzed the proportion of double positive cells. The raw data were analyzed using BD CellQuest (BD Biosciences, San Diego, CA, USA) or FlowjoX10 (FlowJo, LLC, Ashland, Oregon, USA).

2.4. Histology and immunohistochemistry

Mice were anesthetized and perfused with saline and 4% paraformaldehyde on day 28 p.i. The lumbar enlargement of the spinal cord was frozen in liquid nitrogen. Cryostat sections were cut at 10 μm, and hematoxylin and eosin (H&E) and Luxol fast blue staining were detected for histology. The slices were treated with 1% BSA to block nonspecific sites and were permeabilized with 0.1% Triton X-100 in PBS for 30 min. These slices were then incubated overnight at 4 °C with anti-IBA1 (1:1000, Millipore, USA). As a negative control, additional sections were treated similarly, but the primary antibodies were omitted. Then, sections were incubated with Alexa Fluor 488 secondary antibody (1:1000, Invitrogen, USA). All the slices were examined by using a fluorescence light microscope (Olympus BX60, PA, USA).

2.5. Western blot analysis

Tissues from spinal cords or Splenic MNCs were homogenized on ice with T-Per tissue protein extraction (Thermo Scientific, USA) containing Halt Phosphatase Inhibitor Cocktail (1:100, Thermo Scientific, USA) and Halt Protease Inhibitor Cocktail (1:100, Thermo Scientific, USA). Protein concentrations were determined by a Pierce BCA Protein Assay Kit (Thermo Scientific, USA). Protein extracts (20 μg) were separated by SDS-PAGE and transferred onto nitrocellulose blotting membranes (GE Healthcare Life Science, Germany). We pooled four animals in each group together for western blot, and the data obtained were expressed by mean ± SEM. All experiments were repeated three times. Then, the membranes were incubated with primary antibodies, including anti-p-NF-κB/p65, anti-TLR4, anti-Arginase-1, anti-IBA1 (1:1000, Abcam, USA), anti-COX2 (1:1000, BD Bioscience, USA), anti-GAPDH (1:500, Cell Signaling, USA), and anti-iNOS (1:500, BD Biosciences, San Diego, CA, USA) for 16 h at 4 °C. Bands were visualized by HRP-conjugated secondary antibodies and chemiluminescence (ECL) kit under the ChemiDoc XRS$^+$ system (Bio-Rad Laboratories, USA). The band intensity was measured and normalized to loading control. The expression of the target protein was presented as the fold changes relative to the vehicle.

2.6. Quantitative PCR (qPCR)

Splenic MNCs were lysed in RNAiso Plus (Takara Bio, USA), and total RNA was isolated by phenol-chloroform extraction. The total RNA yield and concentration were measured by using Synergy™ H1 (BioTek Instruments, USA). cDNA was then generated using the Prime Script™ RT Master Mix (Takara Bio, USA), according to the manufacturer's instruction. Quantitative real-time PCR was performed by using SYBR® Premix Ex Taq™ (Takara, China) on the Cobas z 480 real-time PCR instrument (Roche Diagnostics, USA). The primers used in this study are as follows:

	Forward primer	Reverse primer
Actin	GAGACCTCAACACCCCAG	CATCACAATGCCTGTGGTAC
Tbet	CCATTCCTGTCCTTCACCGT	CCTGTAATGCTTGTGGGCT
Foxp3	GTCTGGAATGGGTGTCCAGG	AGCGTGGGAAGGTGCAGAG
Rorc	AGCTGCGACTGGAGGACCTT	CCCGTGAAAAGAGGTTGGTG
Ccl2	GAGTAGGCTGGAGAGCTACAAGAG	AGGTAGTGGATGCATTAGCTTCAG
Ccl5	GAGGATTCCTGCAGAGGATCAAGACAG	TCCAAAGAGTTGATGTACTCCCGAACC
Ccl3	CAATTCATCGTTGACTATT	CAGTGATGTATTCTTGGA
Cxcl10	TCCTTGTCCTCCCTAGCTCA	ATAACCCCTTGGGAAGATGG
Cd86	CCTGGTCATTAGAGACCTGAGG	TGCTGCCATAACTTACGGTAGA
Fcgr3	TTTGGACACCCAGATGTTTCAG	GTCTTCCTTGAGCACCTGGATC
Nos2	CCCTTCAATGGTTGGTACATGG	ACATTGATCTCCGTGACAGCC
Il1b	TACATCAGCACCTCACAAGCA	CCAGCCCATACTTTAGGAAGA
Tgfb1	AGGACCTGGGTTGGAAGTGG	AGTTGGCATGGTAGCCCTTG
Mrc1	CTCTGTTCAGCTATTGGACGC	CGGAATTTCTGGGATTCAGCTTC
Il10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
Retnla	CCCTTCTCATCTGCATCTC	CAGTAGCAGTCATCCCAGCA
Il4	ACTCTAGTGTTCTCATGGAGCTGC	AAAGCATGGTGGCTCAGTACTACG

Cycle conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s, and the melt curve was 95–65-95 °C (20 °C/s-20 °C/s-0.1 °C/s). Changes in mRNA levels were quantified using the $2^{-\Delta\Delta CT}$ method using *Actin* mRNA as a control.

2.7. Enzyme-linked immunosorbent assay (ELISA)

According to the process mentioned above (Western blot analysis), the extract of splenic MNCs was carried out. Then, the levels of IL-1β, IL-6, TNF-α, INF-γ, IL-4, and IL-10 in the extract of splenic MNCs were measured by ELISA kits (eBioscience, USA) following the manufacturer's instructions. A standard curve was run for each microwell plate. According to the standard curve, the concentration of cytokine was determined.

2.8. CFSE proliferation assay

On day 9 p.i., the splenic MNCs were isolated and resuspended in prewarmed PBS with 0.1% BSA at a final concentration of 1×10^6cells/ml and were labeled with 5 nM carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes™, Thermo Fisher Scientific, Waltham, MA, USA) for 5 min at 37 °C, 5% CO_2. Then, an equal amount of FBS quenched the reaction. The cells were washed and plated in 96-well round-bottom plates at a concentration of 2×10^5 cells/well. The CFSE-labeled cells were then incubated in the presence of $MOG_{35–55}$ (10 μg/ml) for 3 days and analyzed for proliferation index by using an Attune™ NxT flow cytometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.9. Transwell assay

On day 9 p.i., splenic MNCs were obtained and cultured in the

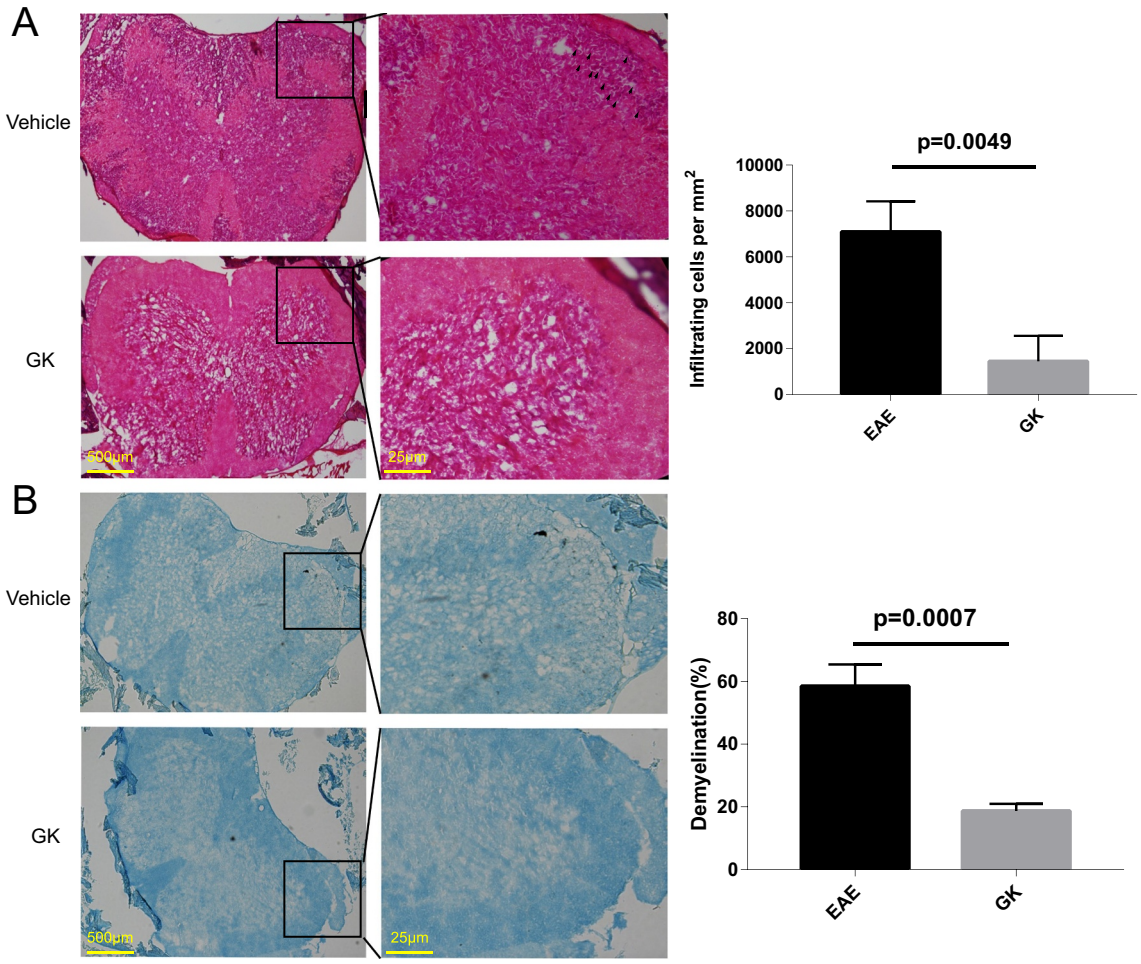


Fig. 2. GK inhibited the infiltration of inflammatory cells and demyelination in the spinal cord of EAE mice. Mice were immunized with $MOG_{35–55}$ and received with GK by i.p. injection from day 3 to 28 p.i. On day 28 p.i., mice were euthanized, and the spinal cord was collected for histological analysis. To evaluate infiltration in the spinal cord, 10 μm frozen spinal cord sections were stained with hematoxylin and eosin: inflammatory cells(arrow) (A). The numbers of infiltrating cells were counted in ImageJ. To visualize demyelination, 10 μm frozen spinal cord sections were stained with Luxol fast blue (B). The percentage of demyelination (demyelination area in total white matter) was quantified using thresholding in ImageJ. Results are shown as mean ± SEM, n = 4, and differences were analyzed using Student's *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

presence of GK (10 μg/ml and 50 μg/ml) and MOG35-55 (10 μg/ml). After 48 h, splenic MNCs were centrifuged. Then the resuspension of the cells (1×10^5/50 μl) was added on top of the transwell membrane in the upper chamber, and 200 μl of complete cell culture was added to the lower chamber. After 24 h, migrated cells in the bottom well were counted under a microscope (Olympus BX60, PA, USA).

2.10. Statistical analysis

Raw data were analyzed by Prism version 7.0 (GraphPad Software, USA). Unpaired two-tailed Student's *t*-tests or a one-way ANOVA followed by Tukey's multiple comparisons test were used to assess the statistical significance. The severity scores of EAE were analyzed by two-way analysis of variance (ANOVA). Statistical significance is reported as not significant (n.s.), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

3. Results

3.1. GK ameliorated the severity of EAE

Clinical scores and pathological changes are critical indicators of treatment effectiveness in EAE. In this study, GK significantly lowered the EAE clinical scores and reduced the severity of the disease (Fig. 1B, $p < 0.0001$). Consistent with the severity of the clinical score, the H&E and Luxol fast blue staining of the spinal cord showed that GK reduced inflammatory infiltration and demyelination in the spinal cord of mice compared with control mice (Fig. 2A and B, $p < 0.01$ and $p < 0.001$, respectively).

3.2. GK improved the inflammatory microenvironment in the spinal cord of EAE mice

Compared with the EAE control mice, the administration of GK significantly decreased the expression of inflammatory molecules toll-like receptor 4 (TLR-4), cyclooxygenase-2 (COX-2), and phosphorylated nuclear factor kappa-light-chain-enhancer of activated B cells/p65 subunit (p-NF-κB/p65) in the spinal cord (Fig. 3A, $p < 0.05$ and $p < 0.0001$, respectively). This finding indicated that the administration of GK inhibited the development of inflammation in the spinal cord of EAE mice.

Ionized calcium-binding adapter molecule 1 (IBA-1) appears to be an excellent marker for detecting microglial activation that was upregulated in activated microglia under immune responses in the CNS [26,27]. By both immunohistochemistry and western blot, we also found that GK clearly prevented the upregulation of Iba-1 in the spinal cord compared with EAE control mice (Fig. 3B $p < 0.01$). These data suggested that microglia are activated in EAE mice, but GK intervention significantly prevented the activation of microglia in the spinal cord of EAE mice.

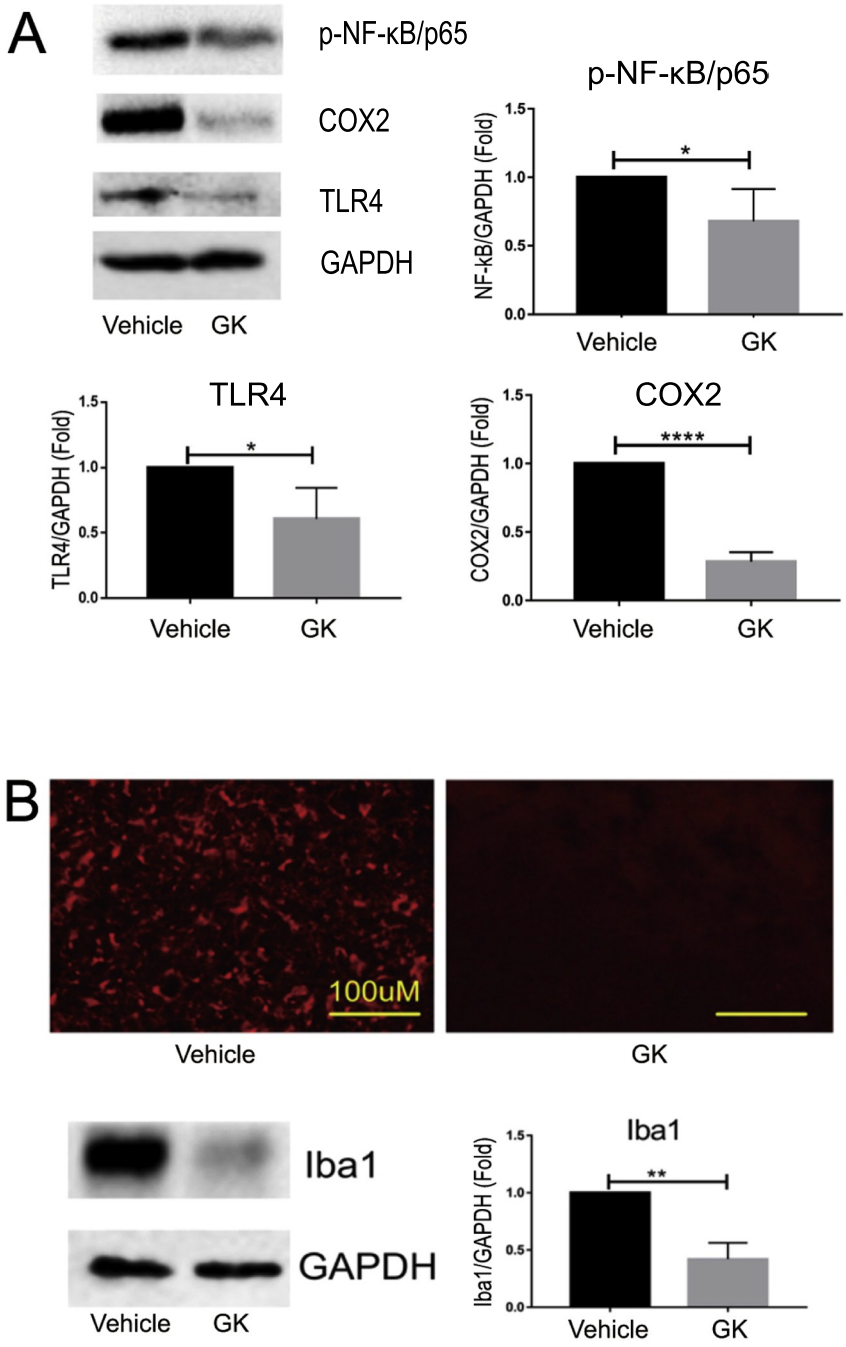


Fig. 3. GK inhibited the inflammatory molecules in the spinal cord of EAE mice. Mice were immunized with $MOG_{35–55}$ and received with GK by i.p. Injection from day 3 to 28 p.i. On day 28 p.i., spinal cords were obtained, and the expression of TLR-4, COX-2 and p-NF-κB/p65 (A), the expression of IBA-1 (B) were measured by western blot or immunohistochemistry. Results are shown as mean ± SEM, n = 4, and differences were analyzed using Student's *t*-test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3.3. GK modified peripheral T cell subsets

The results from the spinal cord showed that there were almost no infiltrating cells in the spinal cord of EAE mice that were treated with GK, compared with EAE control mice. This finding suggested that GK intervention prevented the generation of the inflammatory microenvironment in the spinal cord, possibly through direct peripheral immune regulation. We analyzed lymphocyte subsets of splenic MNCs on day 28 p.i by flow cytometry, qPCR, and ELISA. The results showed that the proportion of $CD4^+CD25^{high}$ T cells in GK-treated mice was significantly higher than that in the EAE control mice (Fig. 4A, $p < 0.001$). The levels of interleukin 4 (IL-4) and interleukin 10 (IL-10) in the extracts of splenic MNCs were also elevated in GK-treated mice compared with those in the EAE control mice (Fig. 4B, $p < 0.05$ and $p = 0.056$, respectively). In contrast, the administration of GK reduced the percentage of $CD4^+IFN\text{-}\gamma^+$ and $CD4^+IL\text{-}17^+$ T cell subgroups compared with the EAE control mice (Fig. 4A, $p < 0.01$ and $p < 0.001$, respectively). The concentration of interleukin **17** (IL-17) in the extracts of splenic MNCs was also reduced in GK-treated mice by ELISA (Fig. 4B, $p < 0.01$).

Forkhead box P3 (Foxp3) is not only a marker for regulatory T cells (Tregs) but also is critically required for their suppressive capacity [28,29]. Retinoid-related orphan receptor r-c (RORC), the primary transcription factor that controls T helper cells 17 (Th17) cell

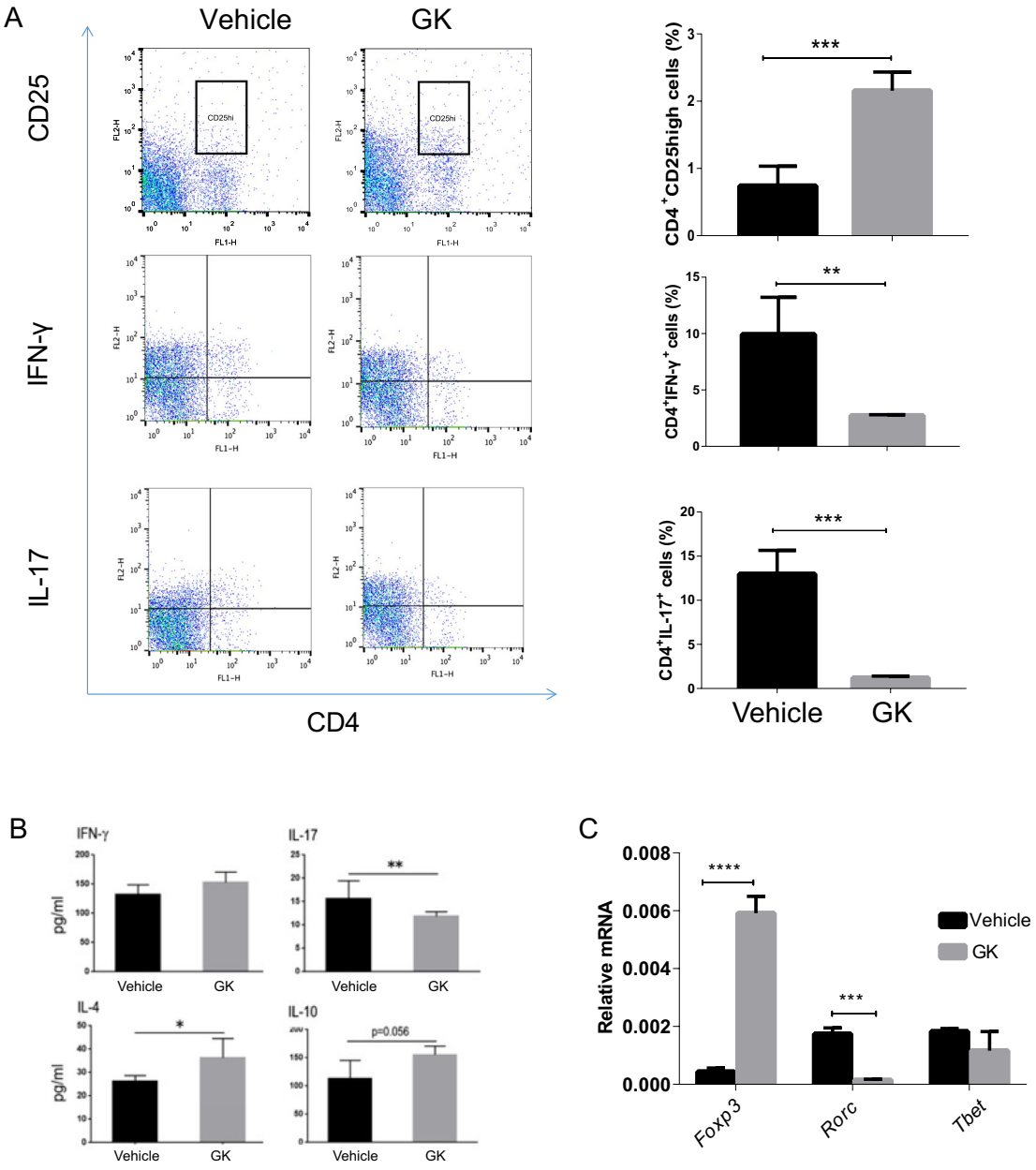


Fig. 4. GK modified the rebalance of T cell subsets in the spleen of EAE mice. Mice were immunized with $MOG_{35–55F}$ and received with GK by i.p. Injection from day 3 to 28 p.i. On day 28 p.i., spleens were obtained, and the percentages of $CD4^+CD25^{high}$, $CD4^+IFN\text{-}\gamma^+$ and $CD4^+IL\text{-}17^+$ T cells were detected by flow cytometry (A). The levels of IFN-γ, IL-17, IL-4 and IL-10 in were detected by ELISA (B), and the expression of *Foxp3*, *Rorc*, and *Tbet* mRNA was measured by qPCR (C). Results are shown as mean ± SEM, n = 4, and differences were analyzed using Student's *t*-test. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

differentiation, cooperates with other transcription factors to induce IL-17 expression [30]. As expected, the administration of GK upregulated the levels of *Foxp3* mRNA and downregulated the levels of *Rorc* mRNA in the splenic MNCs (Fig. 4C, $p < 0.0001$ and $p < 0.001$ respectively), which was consistent with the results of the T cell subsets from flow cytometry. These results demonstrated that GK inhibited inflammatory T helper cells 1 (Th1) and Th17 subsets and upregulated Tregs and T helper cells 2 (Th2) T cell subsets, modifying the imbalance of Th1/Th17 and Th2/Tregs that occurs in EAE.

3.4. GK shifted the polarization of M1 to M2 macrophages

A large number of studies demonstrated that TLR4, NF-kB, and COX-2 are essential signal transduction elements for M1 microglial polarization, contributing to the generation of an inflammatory microenvironment and the development of EAE. Inducible NO synthase (iNOS) and arginase-1 (Arg-1) are two the most specific markers for M1 and M2 microglia/macrophage. We detected the expression of iNOS and Arg-1 in the spinal cord by western blot. Compared to the control of EAE mice, GK downregulated the expression of iNOS and upregulated the expression of Arg-1 (Fig. 5A, $p < 0.0001$ and $p < 0.05$ respectively).

In the spinal cord, downregulation of iNOS and upregulation of Arg-1 expression were observed in GK-treated mice. Next, we investigated whether GK can influence the phenotype of peripheral macrophages by flow cytometer. The results showed that the expression of CD206 and IL-10 (well-known M2 macrophage markers) was significantly increased in splenic $CD11b^+$ macrophages of EAE mice treated with GK (Fig. 5B $p < 0.01$, respectively). However, the treatment of GK did not influence the percentage of $CD16/32^+CD11b^+$ or $IL\text{-}12^+CD11b^+$ cells (well-known M1 macrophage markers) compared with that in EAE

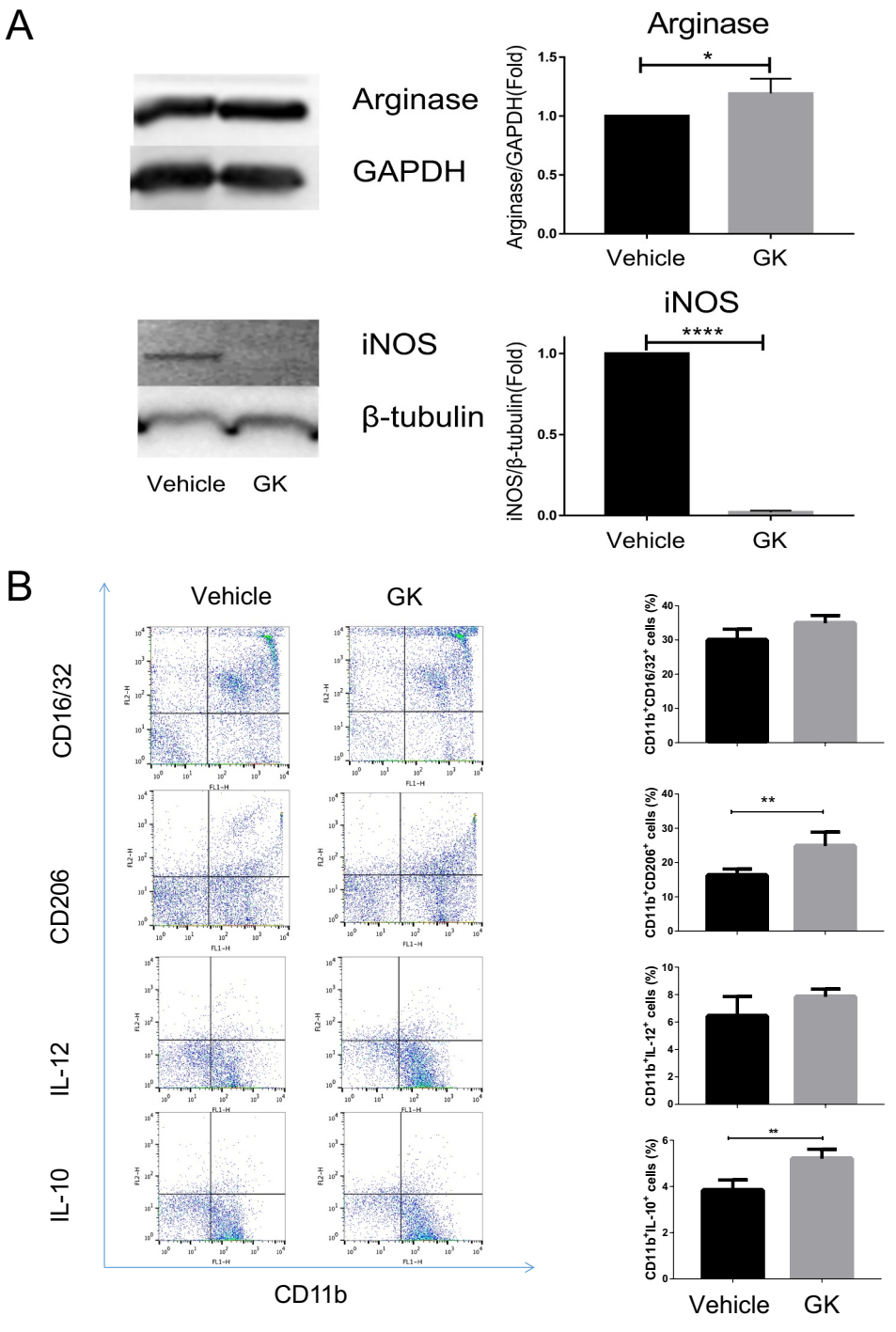
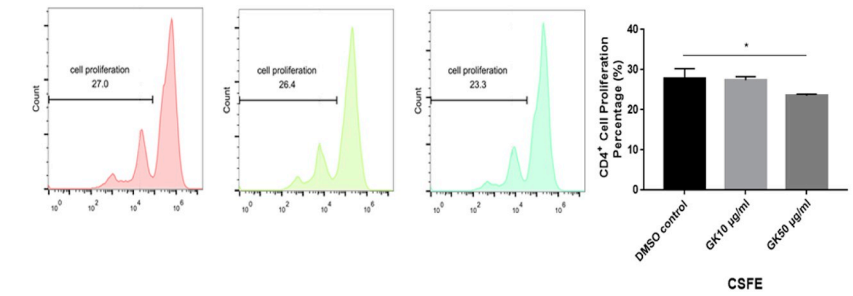


Fig. 5. GK shifted inflammatory M1 to anti-inflammatory M2 macrophages in the spleen of EAE mice. Mice were immunized with MOG_{35-55} and received with GK by i.p. injection from day 3 to 28 p.i. On day 28 p.i., spleens were obtained, the expression of arginase and iNOS (A) were measured by western blot. The percentages of $CD11b^+CD16/32^+$, $CD11b^+CD206^+$, $CD11b^+IL\text{-}12^+$ and $CD11b^+IL\text{-}10^+$ cells were detected by flow cytometry (B). The expression of M1 *Cxcl10*, *Cd86*, *Fcgr3*, *Nos2*, *Il1b* mRNA and M2 *Tgfb1*, *Mrc1*, *Ccl2*, *Il10*, *Retnla*, *Il4* mRNA was measured by qPCR (C). Results are shown as mean ± SEM, n = 4, and differences were analyzed using Student's *t*-test. $^*p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.



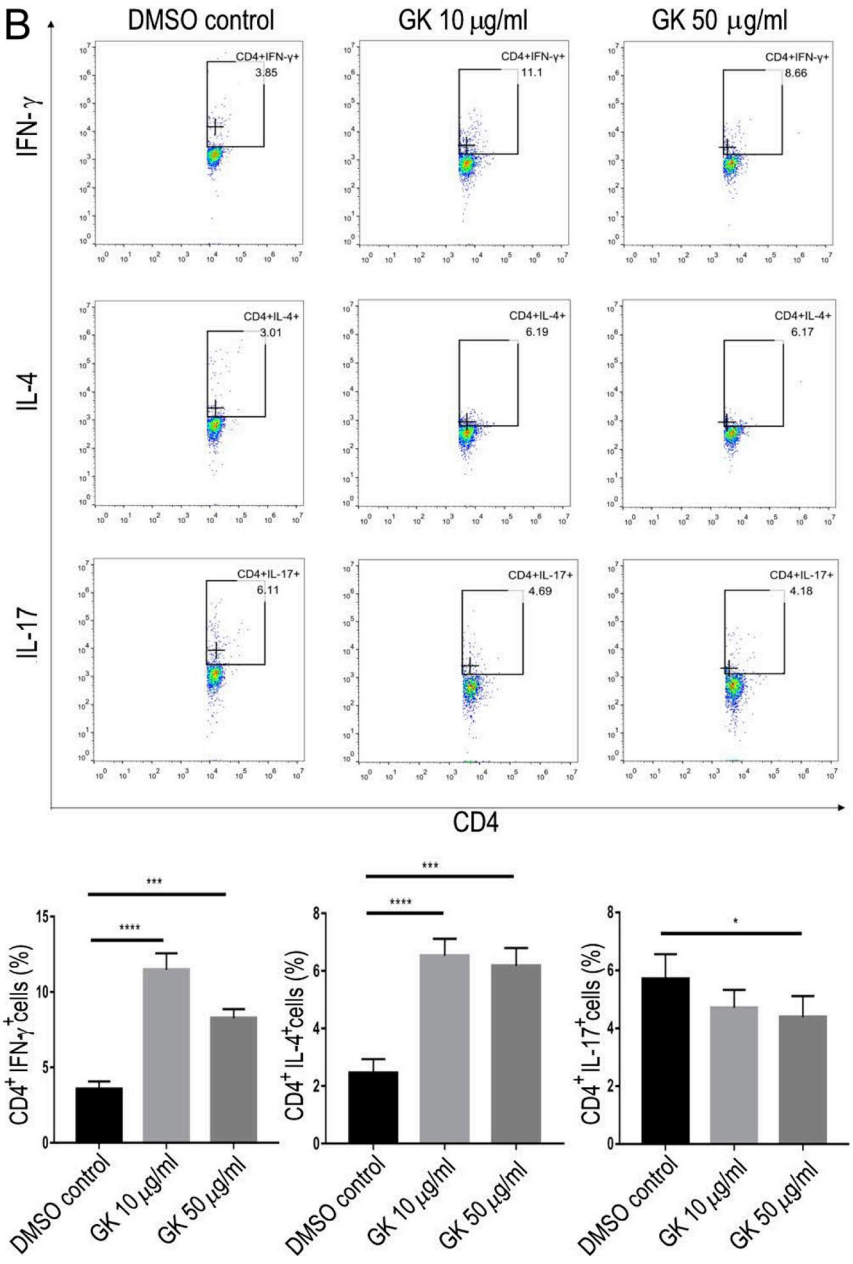


Fig. 6. GK inhibited the proliferation of reactive T cells and regulated T cell subsets in encephalomyelitic cells in vitro. Mice were immunized with MOG_{35-55}, and spleens were obtained on day 9 p.i. Splenic MNCs were prepared and treated with GK (10 μg/ml and 50 μg/ml) in the presence of MOG (10 μg/ml) for 24 h at 37 °C. The proliferation of reactive $CD4^+$ T cells was detected by CFSE labeling (A). The proportion of $CD4^+IFN\text{-}\gamma^+$, $CD4^+IL\text{-}4^+$ and $CD4^+IL\text{-}17^+$ T cells, gated on $CD4^+$ T cells, were detected by flow cytometry (B). Quantitative results are mean ± SEM, n = 4, and analyzed from three independent experiments with similar results. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

control mice. We further measured the expression of the M1 molecule C-X-C motif chemokine 10 (*Cxcl10*), T-lymphocyte activation antigen CD86 (*Cd86*), Low-affinity immunoglobulin gamma Fc region receptor III (*Fcgr3*), Nitric oxide synthase, inducible (*Nos2*) and Interleukin-1 beta(*Il1b*) mRNA, as well as the expression of M2 molecule transforming growth factor beta1 (*Tgfb1*), Macrophage mannose receptor 1 (*Mrc1*), C-C motif chemokine 2 (*Ccl2*), Interleukin-10 (*Il10*), rental resistin-like alpha (*Retnla*) and Interleukin-4 (*Il4*) mRNA in splenic MNCs by qPCR. The results showed that GK inhibited the expression of *Fcgr3*, *Nos2*, and *Il1b* mRNA and induced the expression of *Ccl2*, *Il10* and *Retnla* mRNA in splenic MNCs (Fig. 5C, $p < 0.05$ respectively). Taken together with the data from Western blot, flow cytometry, and qPCR, it is clear that GK shifts the polarization of M1 to M2 microglia/macrophages in EAE mice.

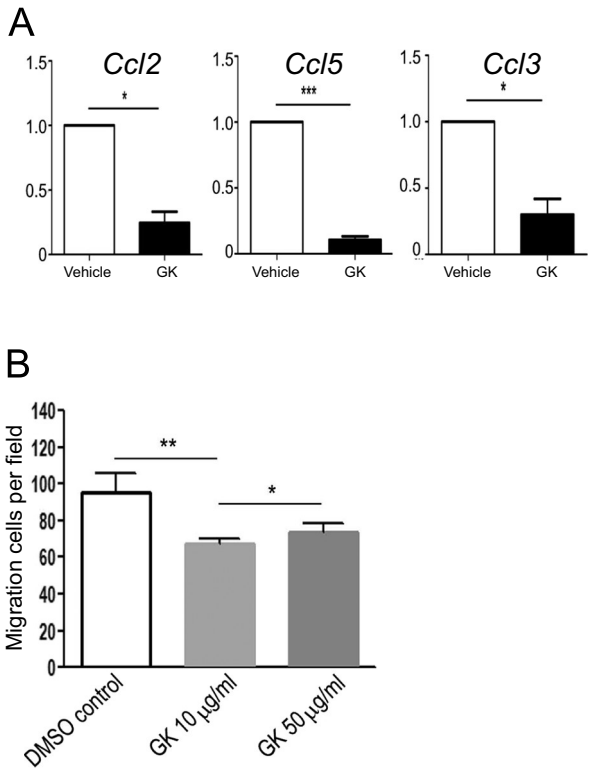


Fig. 7. GK inhibited the expression of chemokines and declined the migration of cells. Mice were immunized with MOG35-55, and spleens were obtained on day 28 p.i. Splenic MNCs were prepared, and the expression of *Ccl2, Ccl3,* and *Ccl5* mRNA was detected by qPCR. Results are shown as mean ± SEM of four mice (A). Mice were immunized with MOG_{35-55}, and spleens were obtained on day 9 p.i. Splenic MNCs were prepared and treated with GK (10μg/ml and 50μg/ml) in the presence of MOG (10μg/ml) for 24 h at 37 °C. The migration of cells was detected by Transwell assay(B). Quantitative results are mean ± SEM, n = 4, and analyzed from three independent experiments with similar results. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3.5. GK inhibited the proliferation and migration of peripheral immune cells in vitro

To better understand the mechanisms for GK-inhibited the infiltration of inflammatory cells in the spinal cord of EAE mice, we further carried out in vitro experiments with splenic MNCs on day 9 p.i., based on this consideration that splenic MNCs at this point can induce EAE by adoptive transfer of MOG-specific $CD4^+$ T cells. As shown in Fig. 6A, GK (50 μg/ml) inhibited the proliferation of reactive $CD4^+$ T cells by CFSE staining (Fig. 6A, $p < 0.05$). The results from flow cytometry showed that GK (10 μg/ml and 50 μg/ml) increased the proportion of $CD4^+IFN\text{-}\gamma^+$ and $CD4^+IL\text{-}4^+$ T cells and decreased the proportion of $CD4^+IL\text{-}17^+$ T cells (Fig. 6B, $p < 0.05$, $p < 0.001$ and $p < 0.0001$, respectively).

To further study why peripheral immune cells did not migrate into the spinal cord in GK-treated mice, we also explored the expression of CCL-2, C-C motif chemokine 3 (CCL-3) and C-C motif chemokine 5 (CCL-5), which affect the adhesion, chemotaxis, and migration of peripheral immune cells. The treatment of GK inhibited the expression of *Ccl2*, *Ccl3* and *Ccl5* mRNA in splenic MNCs compared with EAE control mice (Fig. 7A, $p < 0.05$ and $p < 0.001$, respectively). As expected, the treatment with GK reduced the migratory ability of splenic MNCs from EAE mice on day 9 p.i., (Fig. 7B $p < 0.05$ and $p < 0.01$, respectively), which may be related to the inhibition of chemotactic factors in splenic MNCs.

4. Discussion

In this study, we firstly found that GK effectively reduced the severity of EAE and demyelination of the spinal cord. GK also decreased the infiltration of inflammatory cells and the inflammatory microenvironment in the spinal cord. A further investigation demonstrated that the mechanism of GK action in the treatment of EAE might include the following: 1) GK modified the imbalance between Th1/Th17 and Th2/Tregs; 2) GK shifted inflammatory M1 microglia/macrophages to the M2 phenotype; and 3) GK reduced the migration of immune cells in the spleen, possibly by inhibiting chemotactic factors of immune cells.

Considering our results in EAE, the target of GK may be the peripheral immune system rather than the central immune system. By using flow cytometry, qPCR and ELISA, we found that the proportion of $CD4^+$ $CD25^{high}$ Tregs with Foxp3 mRNA levels significantly increased, indicating that GK may promote the differentiation of Tregs in the splenic MNCs. Dysregulated $Foxp3^+$ Tregs function resulted in uncontrolled immune activation and autoimmunity [31]. Because of the maintenance of peripheral immune tolerance, Tregs are thought to play a critical role in the treatment of MS and EAE [32–34]. Therefore, the strategy inducing Tregs is an area of great importance for MS therapeutics.

At the same time, we also found that GK significantly reduced the proportion of $CD4^+IL\text{-}17^+$ Th17 cells with *Rorc* mRNA levels, triggering the incidence and progression of inflammatory reactions and autoimmune diseases, such as MS [35–38]. Therefore, the inhibition of pathogenic Th17 cells is associated with improvement in the immune dysregulation and may provide a rationale for targeting Th17 cells in EAE. A Th17/Treg imbalance is associated with various autoimmune and inflammatory diseases, including MS/EAE [39]. An approach to suppress Th17 cell differentiation and to correct Th17/Treg imbalances could be an effective treatment for MS/EAE.

Macrophage polarization is the process by which macrophages are transformed into M1 (proinflammatory macrophages) or M2 (anti-inflammatory macrophages) under different microenvironment [40,41]. Long-term M1 macrophages are detrimental, while M2 macrophages are responsible for tissue repair [42]. Our results showed that GK promoted polarization of M1 macrophages toward M2 phenotype in splenic MNCs. IL-17A neutralization alleviated inflammation and facilitated tissue repair by modulating macrophage polarization toward the M2 phenotype [43], finding a compelling linkage between the IL-17 and M1 macrophages [44]. Additionally, GK enhanced Th2 cytokines, such as IL-4 and IL-10, that in turn polarize the M2 phenotype [45,46]. We speculated that GK could convert the imbalance of Th17/Treg occurring in EAE, which contributes to the polarization of M1 to M2.

Splenic MNCs obtained from mice of EAE on day 9 post-immunization can induce EAE model by passive transfer, which is considered as encephalomyelitic cells. In the spinal cord of mice treated with GK, we hardly observed the infiltration of inflammatory cells. Therefore, the inflammatory response in the spinal cord was low, the damage to the tissue was small, and the symptom of EAE was mild. It is assumed that GK may affect cell migration by inhibiting cell migration-related molecules in encephalomyelitic cells. Our results demonstrated that GK strongly inhibited expression of chemotactic factors, such as CCL-2, CCL-3, and CCL-5, in encephalomyelitic immune cells, thereby prohibiting the migration of inflammatory cells into the spinal cords of EAE mice.

Besides, chemokines have proinflammatory abilities that induce extravasation of leukocytes, which regulates the migration and infiltration of monocytes, dendritic cells and T cells [47,48], participating in the generation and development of EAE through monocytes/macrophages activation and recruitment [49]. A previous study observed a significant increase in the amount of leukocyte rolling and adhesion on days 7, 14 and 21 after EAE induction compared to control mice [50]. CCL-2 dramatically increased during the relapsing phase of chronic relapsing EAE, and CCL-2 antibodies effectively mitigated the severity of disease [51,52]. CCL-3 and CCL-5 were also efficient chemoattractants for Th1 cells [53]. Similarly, the administration of anti-CCL-3 and anti-CCL-5 neutralizing antibodies resulted in almost complete suppression of autoimmune diseases via the inhibition of leukocyte

adhesion to the pathological microvasculature and reduction of inflammatory cell recruitment [50,54]. Based on our results, GK dramatically inhibited the levels of *Ccl2*, *Ccl3*, and *Ccl5* mRNA in encephalomyelitic immune cells, partly explaining why inflammatory cells and inflammatory microenvironment are limited in the spinal cords of EAE mice.

5. Conclusion

GK, a naturally monomeric compound from *Ginkgo biloba*, effectively relieved the severity of EAE, possibly through several coordination mechanisms: 1)the site of GK action may mainly be the peripheral immunity and not in the spinal cords; 2) GK modified the imbalance between Th17 and Treg cells; 3) GK shifted macrophage/microglia polarization from M1 to M2 cell type; and 4) GK inhibited the expression of chemotactic molecules, thereby limiting the migration of inflammatory cells to the spinal cord.

Competing interests

None of the authors have any potential financial conflicts of interest related to this manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (grant numbers 81371414), the National Major Scientific and Technological Special Project for Significant New Drug Development (grant numbers 2013ZX09402203), and Chinese Medicine Research Project of Foundation of Shanghai Municipal Commission of Health and Family Planning (2018JP001).

Acknowledgments

We thank Dr. WanJian for the critical review of the manuscript.

References

[1] C. Stadelmann, C. Wegner, W. Bruck, Inflammation, demyelination, and degeneration - recent insights from MS pathology, Biochim. Biophys. Acta 1812 (2011) 275–282.
[2] J.M. Fletcher, S.J. Lalor, C.M. Sweeney, N. Tubridy, K.H.G. Mills, T cells in multiple sclerosis and experimental autoimmune encephalomyelitis, Clin. Exp. Immunol. 162 (2010) 1–11.
[3] S.L. Hauser, J.R. Chan, J.R. Oksenberg, Multiple sclerosis: prospects and promise, Ann. Neurol. 74 (2013) 317–327.
[4] J. Oh, P.A. Calabresi, Emerging injectable therapies for multiple sclerosis, Lancet Neurol. 12 (2013) 1115–1126.
[5] Oz. TaSKapilioGLu, Recent advances in the treatment for multiple sclerosis; current new drugs specific for multiple sclerosis., Noro Psikiyatr. Ars. 55 (2018) S15–S20.
[6] B. Ahlemeyer, J. Krieglstein, Neuroprotective effects of Ginkgo biloba extract, Cell. Mol. Life Sci. 60 (9) (2003) 1179–1792.
[7] Y. Luo, J.V. Smith, V. Paramasivam, A. Burdick, K.J. Curry, J.P. Buford, I. Khan, W.J. Netzer, H. Xu, P. Butko, Inhibition of amyloid-beta aggregation and caspase-3 activation by the Ginkgo biloba extract EGb761, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 12197–12202.
[8] P. Curtis-Prior, D. Vere, P. Fray, Therapeutic value of Ginkgo biloba in reducing symptoms of decline in mental function, J. Pharm. Pharmacol. 51 (1999) 535–541.
[9] K.F. Chung, G. Dent, M. McCusker, P. Guinot, C.P. Page, P.J. Barnes, Effect of a ginkgolide mixture (BN 52063) in antagonising skin and platelet responses to platelet activating factor in man, Lancet 1 (1987) 248–251.
[10] H.X. Pei, R. Hua, C.X. Guan, X. Fang, Ginkgolide B reduces the degradation of membrane phospholipids to prevent ischemia/reperfusion myocardial injury in rats, Pharmacology 96 (2015) 233–239.
[11] Z.M. Shu, X.D. Shu, H.Q. Li, Y. Sun, H. Shan, X.Y. Sun, R.H. Du, M. Lu, M. Xiao, J.H. Ding, G. Hu, Ginkgolide B protects against ischemic stroke via modulating microglia polarization in mice, CNS Neurosci Ther 22 (2016) 729–739.
[12] T. Zhou, W.T. You, Z.C. Ma, Q.D. Liang, H.L. Tan, C.R. Xiao, X.L. Tang, B.L. Zhang, Y.G. Wang, Y. Gao, Ginkgolide B protects human umbilical vein endothelial cells against xenobiotic injuries via PXR activation, Acta Pharmacol. Sin. 37 (2016) 177–186.
[13] J.L. Zheng, B.S. Li, X.C. Cao, W.K. Zhuo, G. Zhang, Alleviation of spinal cord injury by Ginkgolide B via the inhibition of STAT1 expression, Genet. Mol. Res. 15 (2016).
[14] C.L. Chi, D.F. Shen, P.J. Wang, H.L. Li, L. Zhang, Effect of ginkgolide B on brain metabolism and tissue oxygenation in severe haemorrhagic stroke, Int. J. Clin. Exp. Med. 8 (2015) 3522–3529.
[15] M.J. Bellizzi, J.S. Geathers, K.C. Allan, H.A. Gelbard, Platelet-activating factor receptors mediate excitatory postsynaptic hippocampal injury in experimental autoimmune encephalomyelitis, J. Neurosci. 36 (4) (2016) 1336–1346.
[16] B. Brochet, P. Guinot, J.M. Orgogozo, C. Confavreux, L. Rumbach, V. Lavergne, Double blind placebo controlled multicentre study of ginkgolide B in treatment of acute exacerbations of multiple sclerosis, J. Neurol. Neurosurg. Psychiatry 58 (3) (1995) 360–362.
[17] C. Yuan, J. Pan, X. Hu, Determination of the derivative from ginkgolide B, Nat. Prod. Res. 22 (2008) 1333–1338.
[18] S. Ma, H. Yin, L. Chen, H. Liu, M. Zhao, X. Zhang, Neuroprotective effect of ginkgolide K against acute ischemic stroke on middle cerebral ischemia occlusion in rats, J. Nat. Med. 66 (1) (2012) 25–31.
[19] M.M. Chen, W. Zou, M.M. Chen, L. Cao, J. Ding, W. Xiao, G. Hu, Ginkgolide K promotes angiogenesis in a middle cerebral artery occlusion mouse model via activating JAK2/STAT3 pathway, Eur. J. Pharmacol. 833 (2018) 221–229.
[20] W. Yu, S. Chen, L. Cao, J. Tang, W. Xiao, B. Xiao, Ginkgolide K promotes the clearance of A53T mutation alpha-synuclein in SH-SY5Y cells, Cell Biol. Toxicol. 34 (2018) 291–303.
[21] S. Ma, H. Liu, H. Jiao, L. Wang, L. Chen, J. Liang, M. Zhao, X. Zhang, Neuroprotective effect of ginkgolide K on glutamate-induced cytotoxicity in PC 12 cells via inhibition of ROS generation and Ca 2+ influx, Neurotoxicology 33 (2012) 59–69.
[22] S. Ma, X. Liu, Q. Xun, X. Zhang, Neuroprotective effect of Ginkgolide K against H2O2-induced PC12 cell cytotoxicity by ameliorating mitochondrial dysfunction and oxidative stress, Biol. Pharm. Bull. 37 (2) (2014) 217–225.
[23] S. Wang, Z. Wang, Q. Fan, J. Guo, G. Galli, G. Du, X. Wang, W. Xiao, Ginkgolide K protects the heart against endoplasmic reticulum stress injury by activating the inositol-requiring enzyme 1alpha/X box-binding protein-1 pathway, Br. J. Pharmacol. 173 (2016) 2402–2418.
[24] S. Mojaverrostami, M.N. Bojnordi, M. Ghasemi-Kasman, M.A. Ebrahimzadeh, H.G. Hamidabadi, A review of herbal therapy in multiple sclerosis, Adv. Pharm. Bull. 8 (2018) 575–590.
[25] X. Zhou, H.-Y. Wang, B. Wu, C.-Y. Cheng, W. Xiao, Z.-Z. Wang, Y.-Y. Yang, P. Li, H. Yang, Ginkgolide K attenuates neuronal injury after ischemic stroke by inhibiting mitochondrial fission and GSK-3β-dependent increases in mitochondrial membrane permeability, Oncotarget 8 (2017) 44682–44693.
[26] D.M. Norden, P.J. Trojanowski, E. Villanueva, E. Navarro, J.P. Godbout, Sequential activation of microglia and astrocyte cytokine expression precedes increased Iba-1 or GFAP immunoreactivity following systemic immune challenge, Glia 64 (2016) 300–316.
[27] B.H. Ton, Q. Chen, G. Gaina, C. Tucureanu, A. Georgescu, C. Strungaru, M.L. Flonta, D. Sah, V. Ristoiu, Activation profile of dorsal root ganglia Iba-1 (+) macrophages varies with the type of lesion in rats, Acta Histochem. 115 (2013) 840–850.
[28] J.D. Fontenot, M.A. Gavin, A.Y. Rudensky, Foxp3 programs the development and function of CD4+CD25+ regulatory T cells, Nat. Immunol. 4 (2003) 330–336.
[29] S. Hori, T. Nomura, S. Sakaguchi, Control of regulatory T cell development by the transcription factor Foxp3, Science (80-.). 299 (2003) 1057–1061.
[30] K. Hirahara, K. Ghoreschi, A. Laurence, X.P. Yang, Y. Kanno, J.J. O'Shea, Signal transduction pathways and transcriptional regulation in Th17 cell differentiation, Cytokine Growth Factor Rev. 21 (2010) 425–434.
[31] J. Do, D. Kim, S. Kim, A. Valentin-Torres, N. Dvorina, E. Jang, V. Nagarajavel, T.M. DeSilva, X. Li, A.H. Ting, D.A.A. Vignali, S.A. Stohlman, W.M. Baldwin 3rd, B. Min, Treg-specific IL-27Ralpha deletion uncovers a key role for IL-27 in Treg function to control autoimmunity, Proc. Natl. Acad. Sci. U. S. A. 114 (2017) 10190–10195.
[32] E. Bettelli, Y. Carrier, W. Gao, T. Korn, T.B. Strom, M. Oukka, H.L. Weiner, V.K. Kuchroo, Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells, Nature 441 (2006) 235–238.
[33] A. Ephrem, S. Chamat, C. Miquel, S. Fisson, L. Mouthon, G. Caligiuri, S. Delignat, S. Elluru, J. Bayry, S. Lacroix-Desmazes, J.L. Cohen, B.L. Salomon, M.D. Kazatchkine, S.V. Kaveri, N. Misra, Expansion of CD4+CD25+ regulatory T cells by intravenous immunoglobulin: a critical factor in controlling experimental autoimmune encephalomyelitis, Blood 111 (2008) 715–722.
[34] M. Piedavent-Salomon, A. Willing, J.B. Engler, K. Steinbach, S. Bauer, B. Eggert, F. Ufer, N. Kursawe, S. Wehrmann, J. Jager, S. Reinhardt, M.A. Friese, Multiple sclerosis associated genetic variants of CD226 impair regulatory T cell function, Brain 138 (2015) 3263–3274.
[35] S. Huber, N. Gagliani, E. Esplugues, W. O'Connor Jr., F.J. Huber, A. Chaudhry, M. Kamanaka, Y. Kobayashi, C.J. Booth, A.Y. Rudensky, M.G. Roncarolo, M. Battaglia, R.A. Flavell, Th17 cells express interleukin-10 receptor and are controlled by Foxp3(−) and Foxp3+ regulatory CD4+ T cells in an interleukin-10-dependent manner, Immunity. 34 (2011) 554–565.
[36] J. Ramstein, C.E. Broos, L.J. Simpson, K.M. Ansel, S.A. Sun, M.E. Ho, P.G. Woodruff, N.R. Bhakta, L. Christian, C.P. Nguyen, B.J. Antalek, B.S. Benn, R.W. Hendriks, B. van den Blink, M. Kool, L.L. Koth, IFN-gamma-producing T-helper 17.1 cells are increased in sarcoidosis and are more prevalent than T-helper type 1 cells, Am. J. Respir. Crit. Care Med. 193 (2016) 1281–1291.
[37] M.S. Sundrud, S.B. Koralov, M. Feuerer, D.P. Calado, A.E. Kozhaya, A. Rhule-Smith, R.E. Lefebvre, D. Unutmaz, R. Mazitschek, H. Waldner, M. Whitman, T. Keller, A. Rao, Halofuginone inhibits TH17 cell differentiation by activating the amino acid starvation response, Science (80-.) 324 (2009) 1334–1338.
[38] J.F. Zambrano-Zaragoza, E.J. Romo-Martinez, J. Duran-Avelar Mde, N. Garcia-Magallanes, N. Vibanco-Perez, Th17 cells in autoimmune and infectious diseases, Int J Inflam. 2014 (2014) 651503.

[39] M. Noack, P. Miossec, Th17 and regulatory T cell balance in autoimmune and inflammatory diseases, Autoimmun. Rev. 13 (2014) 668–677.
[40] T.A. Hamilton, C. Zhao, P.G. Pavicic Jr., S. Datta, Myeloid colony-stimulating factors as regulators of macrophage polarization, Front. Immunol. 5 (2014) 554.
[41] A. Mantovani, S. Sozzani, M. Locati, P. Allavena, A. Sica, Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes, Trends Immunol. 23 (2002) 549–555.
[42] M. Benoit, B. Desnues, J.L. Mege, Macrophage polarization in bacterial infections, J. Immunol. 181 (2008) 3733–3739.
[43] Y. Zhu, W. Tan, A.M. Demetriades, Y. Cai, Y. Gao, A. Sui, Q. Lu, X. Shen, C. Jiang, B. Xie, X. Sun, Interleukin-17A neutralization alleviated ocular neovascularization by promoting M2 and mitigating M1 macrophage polarization, Immunology 147 (2016) 414–428.
[44] Q. Zhang, I. Atsuta, S. Liu, C. Chen, S. Shi, S. Shi, A.D. Le, IL-17-mediated M1/M2 macrophage alteration contributes to pathogenesis of bisphosphonate-related osteonecrosis of the jaws, Clin. Cancer Res. 19 (2013) 3176–3188.
[45] M. Jung, Y. Ma, R.P. Iyer, K.Y. DeLeon-Pennell, A. Yabluchanskiy, M.R. Garrett, M.L. Lindsey, IL-10 improves cardiac remodeling after myocardial infarction by stimulating M2 macrophage polarization and fibroblast activation, Basic Res. Cardiol. 112 (2017) 33.
[46] F.O. Martinez, L. Helming, S. Gordon, Alternative activation of macrophages: an immunologic functional perspective, Annu. Rev. Immunol. 27 (2009) 451–483.
[47] L. Izikson, R.S. Klein, I.F. Charo, H.L. Weiner, A.D. Luster, Resistance to experimental autoimmune encephalomyelitis in mice lacking the CC chemokine receptor (CCR)2, J. Exp. Med. 192 (2000) 1075–1080.
[48] D.J. Mahad, R.M. Ransohoff, The role of MCP-1 (CCL2) and CCR2 in multiple sclerosis and experimental autoimmune encephalomyelitis (EAE), Semin. Immunol. 15 (2003) 23–32.
[49] T.J. Schall, K.B. Bacon, Chemokines, leukocyte trafficking, and inflammation, Curr. Opin. Immunol. 6 (1994) 865–873.
[50] A.C. dos Santos, M.M. Barsante, R.M. Arantes, C.C. Bernard, M.M. Teixeira, J. Carvalho-Tavares, CCL2 and CCL5 mediate leukocyte adhesion in experimental autoimmune encephalomyelitis–an intravital microscopy study, J. Neuroimmunol. 162 (2005) 122–129.
[51] C. McManus, J.W. Berman, F.M. Brett, H. Staunton, M. Farrell, C.F. Brosnan, MCP-1, MCP-2 and MCP-3 expression in multiple sclerosis lesions: an immunohistochemical and in situ hybridization study, J. Neuroimmunol. 86 (1998) 20–29.
[52] J.E. Simpson, J. Newcombe, M.L. Cuzner, M.N. Woodroofe, Expression of monocyte chemoattractant protein-1 and other beta-chemokines by resident glia and inflammatory cells in multiple sclerosis lesions, J. Neuroimmunol. 84 (1998) 238–249.
[53] J.T. Siveke, A. Hamann, T helper 1 and T helper 2 cells respond differentially to chemokines, J. Immunol. 160 (1998) 550–554.
[54] M. Manczak, S. Jiang, B. Orzechowska, G. Adamus, Crucial role of CCL3/MIP-1alpha in the recurrence of autoimmune anterior uveitis induced with myelin basic protein in Lewis rats, J. Autoimmun. 18 (2002) 259–270.