Cordycepin Prevents and Ameliorates Experimental Autoimmune Encephalomyelitis by Inhibiting Leukocyte Infiltration and Reducing Neuroinflammation

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Research

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Abstract

Background

Multiple sclerosis (MS) is a neuroinflammatory autoimmune disease characterized by multifocal perivascular infiltration of immune cells in the central nervous system (CNS). Current treatment for MS is unsatisfactory, and we aimed to search for immunomodulatory agents from bioactive constituents of natural origin. Cordycepin (3'-deoxyadenosine), an adenosine analogue initially extracted from the fungus *Cordyceps militarisa*, is one of the candidates that has multiple actions. However, its effect on MS is unknown.

Methods

We first investigated the *in vitro* effects of cordycepin on mouse and human dendritic cells, microglial cells, macrophages, and T cell subsets. Cordycepin was administered to the experimental autoimmune encephalomyelitis (EAE) model at various time points to evaluate the effects in preventive and therapeutic model. Chemokine array and next-generation sequencing (NGS) were applied to identify the key molecules and genes involved in the mechanism.

Results

Cordycepin attenuated the activation of LPS-induced mouse bone marrow-derived dendritic cells (BMDCs) and human monocyte-derived dendritic cells (MoDCs) through the inhibition of the AKT, ERK, NF-κB, and ROS pathways and impaired the migration of BMDCs through the downregulation of adhesion molecules and chemokine receptors *in vitro*. In EAE model, preventive treatment with cordycepin decreased the expression of trafficking factors in the CNS, inhibited the secretion of inflammatory cytokines (IFN-g , IL-6, TNF-a, and IL-17) induced by specific peptides, and attenuated clinical symptoms. A chemokine array indicated that cordycepin treatment reversed the high levels of CCL6, PARRES2, IL-16, CXCL10, and CCL12 in the brain and spinal cord of EAE mice, consistent with the RNA-seq data. Moreover, cordycepin suppressed the release of neuroinflammatory cytokines by activated microglial cells, macrophages, Th17 cells, Tc1 cells, and Th1 cells *in vitro*. Furthermore, cordycepin treatment exerted therapeutic effects on attenuating the clinical disease severity in the early disease onset stage and late disease progression stage.

Conclusions

Our study suggests that cordycepin treatment may not only prevent the occurrence of MS by inhibiting DC activation and migration but also potentially ameliorates the progression of MS by reducing neuroinflammation, which may provide insights into the development of new approaches for the treatment of MS.

Introduction

Multiple sclerosis (MS) is the most prevalent inflammatory autoimmune disease of the central nervous system (CNS). MS is mainly driven by myelin-specific autoreactive T cells that infiltrate the CNS, attack myelinated axons and induce neuroinflammatory responses [1]. The prevalence of MS ranges from 50 to 300 per 100,000 population, with an estimation of 2.3 million people with MS worldwide [2]. Patients diagnosed with MS suffer substantial limitations in their physical activities, cognitive function and quality of life, which leads to socioeconomic burdens. Current treatment for MS includes disease-modifying therapies (DMTs), symptomatic treatment, psychological support, physical and occupational therapies, and lifestyle modifications. Although some DMTs (interferons, glatiramer acetate, teriflunomide, sphingosine 1-phosphate receptor modulators, fumarates, cladribine, natalizumab, ocrelizumab and alemtuzumab) that aim to modify the disease course of MS have been approved, their adverse effects and unsatisfactory efficacy are of concern. Therefore, novel therapeutic agents that slow disease progression and prevent the development of severe clinical disability are still needed [3, 4].

Experimental autoimmune encephalomyelitis (EAE) is a widely used animal model in studies of the pathogenesis and therapeutic interventions for MS [5]. Many studies have clearly established a key role for Th1 and Th17 CD4+ cells in the progression of EAE and MS [6, 7]. In addition to CD4+ T cells, CD8+ T cells that produce IFN-γ, known as Tc1 cells, also appear to play a role in disease progression [8]. However, the priming and activation of autoreactive T cells likely occurs in peripheral lymph nodes, where antigen-presenting cells (APCs), such as dendritic cells (DCs) and macrophages, may present myelin epitopes to naive T cells. Then, following activation and differentiation, autoreactive effector T cells migrate to the CNS and induce inflammation, tissue damage and demyelination [9]. Moreover, T cells are restimulated by APCs in the brains of EAE animals, resulting in disease induction and progression [10]. DCs are the most potent antigen-presenting cells for naive T cells because of their high expression of MHC and costimulatory molecules. In an EAE model, strong Th1 and Th17 responses are induced by CNS antigens, and large numbers of both T cells and DCs have been detected in the CNS under a variety of inflammatory conditions [11-13]. Activated DCs migrate from peripheral tissues to secondary lymphoid organs, which are probably highly arranged by a group of cell surface receptors and chemokines. In EAE models and patients with MS, CCR2, CCR5 and CCR7 are examples of the possible mediators of DC recruitment to the CNS [14-17]. Moreover, some adhesion molecules expressed on DCs, such as ICAM-1, c-type lectin and integrin, enhance cell crossing of the blood-brain barrier (BBB) [18-20]. Some studies have shown that pharmacological modulation of DCs alleviates the clinical severity of EAE [21, 22]. Based on their important roles in the immune system, DCs are a potential therapeutic target for the treatment of MS.

Natural products derived from traditional medicine have historically made a major contribution to pharmacotherapy [23]. According to previous studies, compounds derived from Chinese herbal medicine regulate DC maturation to enhance or suppress immunity [24, 25]. *Cordyceps militaris*, an entomopathogenic fungus, has been widely used by traditional Chinese medicine doctors for thousands of years [26]. It consists of a few different compounds, including cordycepin (3'-deoxyadenosine) [27], polysaccharides, Soya-cerebroside [28], ergosterol analogs, mannitol, and xanthophylls [29]. Among them, cordycepin is a valuable bioactive compound that exerts anti-inflammatory effects. In an MPTP-treated

parkinsonism model, cordycepin mitigated motor disorders and exerted neuroprotective effects by alleviating inflammation and the oxidative stress response [30]. In an LPS-induced acute lung injury model, cordycepin alleviated acute lung injury by blocking the expression of several adhesion molecules, such as ICAM-1 and VCAM-1, the cytokines/chemokines MCP-1, MIP-1α, MIP-2, and KC, and the chemokine receptor CXCR2 [31]. In a CPZ-induced demyelination model, cordycepin rescued motor dysfunction, promoted remyelination, inhibited glial cell activation, decreased the expression of the proinflammatory cytokines IL-1β and IL-6, and increased the levels of the anti-inflammatory cytokine IL-4 [32]. Cordycepin ameliorates long-term neurological deficits and reduces neuronal tissue loss in mice with traumatic brain injury by suppressing neutrophil infiltration, preserving blood-brain barrier integrity and changing microglia/macrophage polarization [33]. However, the mechanism and therapeutic effect of cordycepin treatment on multiple sclerosis remain unknown.

In the present study, cordycepin treatment ameliorated the development and progression of EAE. Cordycepin inhibited LPS-induced dendritic cell activation, impaired dendritic cell migration, and blocked the recruitment of immune cells to the CNS. These processes may be the mechanism by which cordycepin treatment attenuated the clinical symptoms in the preventive EAE model. Moreover, cordycepin treatment decreased cytokine production in activated microglial cells, macrophages, Th17 cells, Tc1 cells, and Th1 cells. The inhibition of systemic inflammation and neuroinflammation may be the mechanism by which cordycepin treatment attenuated the clinical symptoms in the therapeutic EAE model. This study clarified the immunoregulatory effect and mechanism of cordycepin treatment in EAE for the future development of novel therapeutic agents.

Materials And Methods

Reagents

The small molecules cordycepin (3'-deoxyadenosine) and fingolimod (FTY720) were purchased from Sigma-Aldrich (MO, USA). Fingolimod (FTY720) is an FDA-approved immunomodulatory drug for treating multiple sclerosis.

Culture of mouse bone marrow-derived dendritic cells (BMDCs)

BMDCs were harvested as described previously [25, 34]. Bone marrow cells from 8- to 12-week-old C57BL/6 mice (National Laboratory Animal Breeding and Research Center, Taiwan) were cultured in Petri dishes at a density of 2×10^6 cells in 10 ml of complete RPMI 1640 medium (Gibco, NY, USA) supplemented with 20 ng/ml recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF; PeproTech Inc., New Jersey, USA). Another 10 ml of complete RPMI 1640 medium containing 20 ng/ml GM-CSF were added on day 3. The BMDCs were collected from each dish, washed, and counted on day 6.

Culture of human monocyte-derived dendritic cells (MoDCs)

Human MoDCs were harvested as described previously [25]. Human peripheral blood mononuclear cells (PBMCs) were isolated from human blood using Ficoll and Percoll gradient centrifugation. Monocytes were enriched by adherence as follows: 8×10^5 PBMCs per milliliter were seeded in 10 cm dishes containing 10 ml of complete RPMI 1640 medium, incubated for 18 h, and then the suspended cells were removed by washing with RPMI 1640 medium. After washing, monocytes were cultured in 10 ml of complete RPMI 1640 medium supplemented with 100 ng/ml human GM-CSF (PeproTech Inc., New Jersey, USA) and 100 ng/ml human IL-4 (PeproTech Inc., New Jersey, USA). Another 10 ml of complete RPMI 1640 medium containing 100 ng/ml GM-CSF and IL-4 was added on day 3. The MoDCs were collected from each dish, washed, and counted on day 6. The study involving human subjects was approved by the Research Ethics Committee of China Medical University & Hospital, Taiwan (CMUH110-REC2-052).

Measurement of surface markers and cytokines from dendritic cells and macrophages

Mouse BMDCs, human MoDCs or RAW264.7 macrophages were plated at a density of 1×10^6 cells per milliliter in complete RPMI 1640 medium. Then, 0.1 μg/ml lipopolysaccharide (LPS) (Sigma, MO, USA) with or without the indicated concentration of cordycepin (Sigma, USA) were added, and the cells were subsequently incubated for 18 h at 37°C in a 5% CO_2 atmosphere. The cells were incubated with 0.1 μg/ml LPS as a stimulator. After the incubation, the cells were harvested, and fluorescence-labeled mouse/human anti-CD11c, anti-CD40, anti-CD86, anti-mouse I-A^b (MHC II), anti-CCR7 (BD Bioscience, CA, USA), anti-integrin b1, anti-integrin a4, anti-LFA-1, anti-c type lectin and anti-ICAM-1 (Biolegend, CA, USA) monoclonal antibodies were used to stain DC surface markers. The expression of these markers was analyzed using a FACSVerse instrument (BD Bioscience, CA, USA). The supernatants from cells cultured for 18 h were isolated and assayed for mouse/human IL-6, TNF-α and IL-12 levels using ELISA kits (BD Bioscience, CA, USA) according to the manufacturer's protocol.

Western Blotting

Mouse BMDCs (1×10^6 cells/ml) were plated in complete RPMI 1640 medium. Then, 0.1 μg/ml LPS (Sigma, MO, USA) with or without the indicated concentration of cordycepin were added, and the cells were further incubated for 30 min at 37°C in a 5% CO_2 atmosphere. Whole-cell lysates (15 μg per lane) were separated by electrophoresis on 10% sodium dodecyl sulfate–polyacrylamide gels and then analyzed by Western blotting using specific antibodies against AKT/p-AKT, ERK/p-ERK, NFκB/p-NFκB (Cell Signaling Technology, MA, USA), and actin (Proteintech, China). Actin served as a loading control.

Measurement of Reactive Oxygen Species (ROS)

BMDCs (5×10^5/ml) were cultured in the presence of ddH_2O (control) or the indicated concentration of cordycepin for 1 h and then stimulated with LPS (0.1 μg/ml) for 3 h to detect the intracellular ROS level. After stimulation, the media were replaced with fresh medium containing ROS Label buffer (BioVision, CA, USA) and incubated for 20 min at 37°C before thorough washing with PBS. The intracellular ROS level

was assayed using an ROS Detection Assay Kit (BioVision, CA, USA) according to the manufacturer's protocol. The fluorescent signal was detected at Ex/Em= 495/529 nm in end point mode in the presence of compounds and controls.

In vitro migration assay

BMDCs (1×10^6) were treated with 0.1 μg/ml LPS in the presence or absence of 50 μg/ml cordycepin for 18 h prior to the migration studies. After washing and counting, 2×10^5 BMDCs from different groups in 100 μl were transferred to the upper chamber (Millicell cell culture inserts, pore size: 5.0 μm) (Millipore, Germany) of a 24-well plate. The upper chamber contained cells in 100 μl media lacking cytokines and serum. The lower chamber contained 500 μl of complete media (10% FBS) treated with or without CCL21 (250 ng/ml). The upper chamber was removed after 6 h, and the cells that migrated into the lower chamber were counted using flow cytometry and WST-1 staining (Takara Bio, CA, USA).

Experimental autoimmune encephalomyelitis (EAE) model

C57BL/6 mice were obtained from the National Laboratory Animal Breeding and Research Center, Taipei, Taiwan. All mice were housed in a specific pathogen-free room at the Animal Center, China Medical University, Taichung, Taiwan and maintained in accordance with institutional animal care guidelines. The animal protocol was approved by the Animal Care and Use Committee of China Medical University, Taichung, Taiwan (CMUIACUC-2018-144).

Eight- to ten-week-old female C57BL/6 mice were immunized subcutaneously (s.c.) with 200 μg of myelin peptide (MOG_{35-55}) (Kelowna International Scientific INC, Taiwan) and 600 μg of mycobacterium tuberculosis (BD Bioscience, MD, USA) emulsified in 100 μl of PBS and 100 μl of complete Freund's adjuvant (CFA) (BD Bioscience, MD, USA) per mouse, as described previously [35]. Pertussis toxin (PTX; 400 ng; List Biological Laboratories, CA, USA) was administered intraperitoneally (i.p.) on the day of immunization and on day 2. Mice were monitored daily and scored for EAE as follows: 0, no clinical signs; 0.5, partially limp tail; 1, paralyzed tail; 2, hind limb paresis; 2.5, one hind limb paralyzed; 3, both hind limbs paralyzed; 3.5, weakness in forelimbs; 4, forelimbs paralyzed; and 5, moribund or dead animals [35]. Each experimental group contained 4-5 mice. Wild-type (WT; n=3) mice were not administered any treatment, while mice in the control group (CFA; n=3-5) were similarly s.c. immunized with CFA and PTX without MOG. Mean clinical scores on separate days were calculated by adding scores of individual mice and dividing by the number of mice in each group. The statistical analysis was performed using Student's t-test.

Preventive effect of cordycepin treatment on the EAE model

Animals were i.p. injected with 50 mg/kg cordycepin (EAE+CO50; n=6) or saline (EAE+saline; n=6) daily from days 0 to 21 after immunization to assess the preventative effect of cordycepin on EAE. The CFA group (CFA; n=3) was similarly immunized without MOG. Disease scores were recorded daily as mentioned above. On the day of sacrifice (day 21 after immunization), the brains and spinal cords were

harvested from anesthetized mice after an intracardiac infusion with PBS. Subsequently, the brains and spinal cords were fixed with 4% paraformaldehyde. Fixed samples were embedded in paraffin, and cross sections were stained with hematoxylin-eosin (H&E) (service of Rapid Science, Taiwan) to analyze inflammatory cell infiltration.

We also established other experiments to examine the percentages of dendritic cells and T cells in the brains and spleens on day 8 after immunization. Brains and spleens were harvested and stained with a fluorophore-labeled mouse anti-CD45 Ab, anti-CD11c Ab, anti-CD4 Ab, and anti-CD8 Ab (BD Bioscience, CA, USA) and analyzed using flow cytometry. Additionally, splenocytes were restimulated with MOG peptide *ex vivo* to determine the capacity of cytokine production. A total of 1×10^6 spleen cells were cultured in 24-well culture plates in the presence of 10 μg/ml MOG peptide for 3-4 days. The supernatant of cultured cells was collected and assayed for IL-6, TNF-α, IL-17A, and IFN-γ levels (BD Bioscience, CA, USA) using ELISA kits according to the manufacturer's protocol.

Therapeutic effect of cordycepin treatment on the early disease onset stage and late disease progression stage

Cordycepin (n=5) or saline as a vehicle control (n=5) was i.p. administered daily from day 11 to day 22 to evaluate its effect on the early disease onset stage. Cordycepin (n=5), the positive control fingolimod (FTY720; n=5) (Sigma, USA), the vehicle control saline (n=4) and the negative control CFA (n=5) were i.p. administered from day 24 to day 38 to evaluate the effect of cordycepin on the late disease progression stage. Disease scores were recorded daily. Brains were harvested to detect IFN-γ+ CD4+ T cell and IL-17A+ CD4+ T cell populations. Cell surface markers (fluorochrome-conjugated anti-CD45 Ab and anti-CD4 Ab) (BD Biosciences, CA, USA) were labeled first, and intracellular proteins were stained with fluorochrome-conjugated anti-IFN-γ Ab and anti-IL-17 Ab (BD Biosciences, CA, USA) after fixation and permeabilization. The populations of IFN-γ- and IL-17-producing CD4+ T cells in the brain were analyzed using flow cytometry.

Chemokine array

For the *in vivo* evaluation of chemokine production, the brains and spinal cords were harvested from different groups of mice on day 14 after immunization and intracardially perfused with PBS. Blood samples were harvested from mice by cardiac puncture and centrifuged at 12,800 × g for 10 min to collect serum. Proteins were isolated by homogenizing tissues in RIPA buffer (Abcam, Cambridge, UK). Chemokine production was assessed using a Mouse Chemokine Array Kit (R&D Systems, MN, USA) according to the manufacturer's instructions. Array images were analyzed by densitometry using Image Lab Software (Bio-Rad). The data are presented as fold changes in chemokine expression compared to the corresponding positive control spots.

Next-generation sequencing (NGS)

The spinal cords were harvested from cordycepin-treated and untreated EAE mice on day 17 after immunization, and total RNA was extracted from the spinal cord using an RNeasy Micro Kit (Qiagen, Germany). RNA quantity and quality were assessed with a Bioanalyzer 2100 device using the RNA 6000 Nano Kit (Agilent Technologies). Differential gene expression analyses were conducted using RNA-seq quantification according to the Illumina procedure and were performed by Genomics Ltd. (New Taipei City, Taiwan). Differentially expressed genes (DEGs) were calculated using EBSeq, and functional analyses, including KEGG analyses, were performed using clusterProfiler. RNA-seq was performed using the Illumina platform according to the manufacturer's protocols. DEGs were calculated using EBSeq, and functional analyses, including KEGG analyses, were analyzed using clusterProfiler. Both RNA-seq and subsequent analyses were performed by Genomics Ltd. (New Taipei City, Taiwan).

Preparation and stimulation of EOC13.31 microglial cultures

Both LADMAC and EOC13.31 cells were purchased from the Bioresource Collection and Research Center (BCRC; Taiwan). LADMAC cells were cultured in complete Eagle's Minimum Essential Medium (Gibco, NY, USA) supplemented with 10% FBS, and the supernatants were collected to produce conditioned medium. Microglial EOC13.31 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 20% LADMAC conditioned medium in the presence of 5% CO_2 at 37°C. For the quantification of microglial responses, EOC13.31 cells were seeded in a 24-well plate at a density of 1×10^6 cells/ml. Then, the cells were stimulated with IFN-γ (PeproTech, Taiwan) and treated with or without cordycepin. The supernatants from cultured cells were collected after 24 h and assayed for TNF-α levels using an ELISA kit (BD Bioscience, CA, USA) according to the manufacturer's protocol.

Mouse Th1, Tc1, and Th17 polarization and intracellular staining (ICS)

Lymph nodes and spleens from C57BL/6 mice were harvested, and naïve CD4 or CD8 T cells were isolated using magnetic bead-based separation (STEMCELL Technologies, Vancouver, Canada). Isolated $CD4^+$ or $CD8^+$ cells (1×10^5 cells/200 μl) were activated with an anti-CD3 Ab (10 μg/ml) and anti-CD28 Ab (1 μg/ml) in 96-well plates. The following molecules were added to the indicated medium for 4 days to produce each T cell population: Th/Tc1 cells (3 ng/ml IL-12 and 10 μg/ml anti-IL-4 Ab) and Th17 cells (20 ng/ml IL-6, 1.25 ng/ml TGF-β, 20 ng/ml IL-1β, 20 ng/ml IL-23, 10 μg/ml anti-IFN-γ Ab and 10 μg/ml anti-IL-4 Ab). The skewed cells were further incubated with cordycepin for 16 h and restimulated with PMA (50 ng/ml), ionomycin (500 ng/ml), and GolgiStop (BD Biosciences, CA, USA) for 5 h. Cell surface markers (fluorochrome-conjugated anti-CD4 Ab and anti-CD8 Ab) (BD Biosciences, CA, USA) were labeled first, and intracellular proteins were stained with fluorochrome-conjugated antibodies (anti-IFN-γ Ab and anti-IL-17 Ab) (BD Biosciences, CA, USA) after fixation and permeabilization. Flow cytometry was performed using a FACSVerse instrument (BD Biosciences, CA, USA) and analyzed using FlowJo software (Tree Star, OR, USA).

Results

Cordycepin inhibits antigen-presenting cell activation in a dose-dependent manner

DCs are the most potent antigen-presenting cells for naive T cells. Immature DCs residing in the periphery have a strong ability to endocytose antigens and become mature DCs upon exposure to a variety of stimuli [36]. Some studies have investigated whether Chinese herbal medicines inhibit DC maturation to ameliorate autoimmune diseases and inflammatory responses [37-41]. First, we investigated the effect of cordycepin treatment on LPS-stimulated BMDCs. After 18 h, the levels of TNF-α, IL-6 and IL-12 produced by BMDCs were decreased (Fig. 1A). The expression of the costimulatory molecules CD40 and CD86 was also reduced in LPS-stimulated BMDCs in a dose-dependent manner (Fig. 1B). Furthermore, we examined the effect of cordycepin on activated human MoDCs. Monocytes from three donors were enriched and cultured to induce the formation of MoDCs. The secretion of TNF-α and IL-6 by LPS-stimulated human MoDCs was decreased following cordycepin treatment in a dose-dependent manner (Fig. 1C). Additionally, we determined the capacity of BMDCs to capture exogenous antigens after cordycepin treatment, and the uptake of FITC-labeled dextran by BMDCs was examined using flow cytometry. The internalization of FITC-labeled dextran in BMDCs was not significantly different after treatment with various concentrations of cordycepin (Supplementary Figure 1). Moreover, the MAPK, NF-κB and Akt signaling pathways and reactive oxygen species (ROS) have been shown to participate in the activation of DCs [42, 43]. The levels of AKT, ERK, and NF-κB phosphorylation in LPS-stimulated BMDCs were reduced by a 30 min cordycepin treatment in a dose-dependent manner (Fig. 1D). ROS levels were decreased in LPS-induced activated BMDCs after the cordycepin pretreatment (Fig. 1E). Therefore, the maturation of LPS-stimulated DCs was suppressed by cordycepin treatment through the inhibition of the AKT, ERK, and NF-κB signaling pathways and ROS production.

Cordycepin downregulates adhesion molecules and chemokine receptors and impairs cell migration

Activated DCs migrate from peripheral tissues to secondary lymphoid organs, which are probably highly arranged by a group of cell surface receptors and adhesion molecules [44]. According to previous studies, adhesion molecules, such as ICAM-1, c-type lectin, and integrin, expressed on DCs modulate cell crossing of the blood-brain barrier (BBB) [18-20]. We identified that cordycepin treatment suppressed the expression of adhesion molecules (integrin b1, integrin a4, c-type lectin, and ICAM-1) on LPS-stimulated BMDCs (Fig. 2A). Moreover, CCR2, CCR5 and CCR7 are possible mediators of DC recruitment to the CNS in the EAE model and patients with MS [14-17]. Cordycepin treatment reduced the percentage of CCR7+ cells and the fluorescence intensity of CCR7 but not CCR2 in LPS-stimulated BMDCs (Fig. 2B). However, we did not determine whether cordycepin treatment inhibited CCR5 production because LPS does not stimulate CCR5 expression in BMDCs (data not shown). Based on the aforementioned results, DC migration was tested *in vitro* using a transwell migration assay. The lower chamber was loaded with CCL21 (CCR7 ligand), and the upper chamber was loaded with BMDCs to assess migration toward CCL21. Pretreatment with LPS for 18 h increased BMDC migration, which was inhibited by cordycepin cotreatment, as measured using flow cytometry and the WST-1 assay (Fig. 2C). These results suggested that cordycepin treatment impairs dendritic cell migration by downregulating adhesion molecules and chemokine receptors.

Cordycepin ameliorates the clinical severity of EAE in the preventive model

According to the results of the *in vitro* experiments described above, we further investigated whether cordycepin represented a potential immunosuppressive agent in mice with EAE, the murine model of MS. In the preventive model, cordycepin was administered daily beginning on day 0. Cordycepin treatment significantly ameliorated the clinical disease severity (Fig. 3A). Furthermore, we analyzed the level of infiltrated cells in the spinal cord by performing H&E staining on day 21. More infiltrated cells were detected in the spinal cord of EAE mice than in normal mice. Moreover, cordycepin treatment inhibited cell infiltration in the spinal cord of EAE mice (Fig. 3B). Additionally, we established another set of experiments to examine the populations of immune cells in the brains of EAE mice following cordycepin treatment on day 8. DCs, CD4+ T cells and CD8+ T cells infiltrated the CNS of EAE mice and cordycepin treatment blocked EAE-induced DC, CD4+ T cell and CD8+ T cell infiltration (Fig. 3C). In addition, fewer DCs, CD4+ T cells and CD8+ T cells remained in the spleen of EAE mice, and cordycepin treatment significantly reversed changes in the levels of DCs and CD8+ T cells (Fig. 3D). These data suggested that cordycepin treatment blocked immune cell infiltration into local CNS lesions; therefore, more immune cells remained in the systemic immune organ. On the other hand, we determined the specific immune response of the spleen, a systemic immune organ, to the MOG antigen. We used a specific peptide (MOG) to restimulate splenocytes derived from cordycepin-treated or untreated EAE mice, and then the specific peptide-induced production of inflammatory cytokines, such as IFN-g (Th1 and Tc1-associated cytokines), IL-6, TNF-a (Th2-associated cytokines), and IL-17 (Th17-associated cytokines), were determined using ELISAs. Cordycepin treatment decreased the secretion of IL-6, TNF-a, IL-17 and IFN-g, and the levels of IL-6, TNF-a and IFN-g were particularly significantly reduced (Fig. 3E). Based on these results, cordycepin treatment exerts a preventive effect by inhibiting the infiltration of immune cells and may exert a therapeutic effect by attenuating the production of inflammatory cytokines.

Cordycepin inhibits the chemotactic response in the CNS

Chemokines and their receptors are important mediators of the recruitment of immune cells to inflammatory sites. Chemokines are expressed at higher levels in patients with MS and recruit inflammatory cells into the CNS [45]. We used a chemokine array to analyze the changes in trafficking factor and chemokine protein expression in the brain, spinal cord and serum between EAE mice treated with or without cordycepin. Higher CCL6, PARRES2, IL-16, CXCL10, and CCL12 expression was detected in the brain and spinal cord of EAE mice than in normal mice (CFA group). Simultaneously, the high levels of CCL6, PARRES2, IL-16, CXCL10, and CCL12 were reversed in the brain and spinal cord of cordycepin-treated EAE mice (Fig. 4A and 4B). However, these changes were not observed in the serum (Fig. 4C). The fold changes in the levels of the indicated chemokines in the lower panel of Fig. 4A, 4B and 4C showed that cordycepin treatment inhibited CCL6 and CXCL10 production by more than five-fold in the brain and spinal cord. The fold change in total chemokine expression is shown in Supplementary Figure 2. Furthermore, we examined the levels of adhesion molecules (CCR2, CCR5 and CCR7) expressed on infiltrated lymphocytes in the CNS *ex vivo*. Higher levels of adhesion molecules were detected on infiltrated lymphocytes in the spinal cord of EAE mice than in normal mice (CFA group). Moreover, lower

levels of adhesion molecules were expressed on infiltrating lymphocytes in the spinal cord of cordycepin-treated EAE mice than saline-treated EAE mice (Fig. 4D). We next assessed the transcriptomes of the spinal cord in response to cordycepin treatment in EAE mice. We confirmed that cordycepin treatment inhibited the expression of most adhesion molecules, chemokines, and chemokine receptors in the spinal cord of EAE mice (Fig. 5A). The gene expression levels of adhesion molecules (Fig. 5B), chemokine receptors (Fig. 5C), and chemokines (Fig. 5D) were consistent with the protein expression levels measured *in vitro* (Fig. 2) and *in vivo* (Fig. 4). The mRNA and protein expression levels of adhesion molecules, chemokine receptors, and chemokines were inhibited by cordycepin treatment in BMDCs or the CNS of EAE mice. These results suggested that cordycepin treatment reduces chemotactic activity in the CNS.

Cordycepin inhibits neuroinflammation and ameliorates the clinical severity of EAE in a therapeutic model

Macrophages and microglia promote neuroinflammatory and neurodegenerative changes in individuals with MS by releasing inflammatory cytokines and stimulating leukocyte activity and infiltration into the CNS [46]. To investigate the effect of activated microglia on cordycepin treatment, We stimulated mouse EOC13.31 microglial cells with IFN-γ, which mimics the inflammatory environment *in vivo*, to investigate the effect of activated microglia on the changes induced by cordycepin treatment. The increased level of TNF-α induced by IFN-γ stimulation was significantly reduced by cordycepin treatment in a concentration-dependent manner (Fig. 6A). We used LPS to stimulate the murine macrophage-like cell line RAW264.7, and the levels of TNF-α and IL-6 were determined using ELISAs to confirm that cordycepin inhibited inflammatory cytokine production from macrophages. Cordycepin treatment decreased the secretion of TNF-α and IL-6 by LPS-stimulated macrophages in a dose-dependent manner (Fig. 6B). These *in vitro* experiments suggested that cordycepin has the potential to inhibit neuroinflammation in individuals with MS. Moreover, IL-17-producing CD4+ T cells (Th17), IFN-γ-producing CD8+ T cells (Tc1), and IFN-γ-producing CD4+ T cells (Th1) are known to play important roles in the progression of EAE and MS [6, 8]. We treated Th17, Tc1, and Th1 cells with cordycepin *in vitro* to analyze whether it affected proinflammatory cytokine production. Cordycepin treatment reduced the percentages of activated IL-17-producing Th17 cells, IFN-γ-producing Tc1 cells, and IFN-γ-producing Th1 cells (Fig. 6C). Furthermore, we determined whether cordycepin treatment exerted a therapeutic effect on the model of CNS inflammation (morbidity). In the early disease onset stage, cordycepin was administered daily beginning on day 11 after immunization, and the therapeutic effect was statistically significant throughout the period from day 14 to day 18 (Fig. 6D). In the late disease progression stage, cordycepin or fingolimod was administered daily beginning on day 24 after immunization, and both compounds exerted significant therapeutic effects ≥ day 35 after immunization (Fig. 6E). Furthermore, cordycepin and fingolimod treatment reduced the percentages of IL-17-producing T cells and IFN-γ-producing T cells in the brain in the late disease progression stage (Fig. 6F). Therefore, cordycepin treatment not only exerts a preventive effect by blocking immune cell infiltration but also exerts a therapeutic effect by reducing neuroinflammation to treat multiple sclerosis.

Discussion

This study sought to determine the preventive and therapeutic effects of cordycepin and its possible underlying mechanism in regulating immune cell infiltration and neuroinflammation. The small compound cordycepin from *Cordyceps militaris* inhibited LPS-induced dendritic cell activation by inhibiting the AKT, ERK, and NF-κB signaling pathways and ROS production (Fig. 1), impaired dendritic cell migration through the downregulation of adhesion molecules and chemokine receptors (Fig. 2), and blocked recruitment of immune cells to the CNS (Fig. 3) by decreasing the expression of trafficking factors in the CNS (Fig. 4-5). These processes may be the mechanism by which cordycepin treatment attenuated the clinical symptoms in the preventive EAE model. Moreover, cordycepin treatment inhibited the production of proinflammatory cytokines, such as IFN-g , IL-6, TNF-a, and IL-17, in EAE mice treated with a specific peptide (Fig. 3E). We further identified that cordycepin treatment decreased cytokine production in activated microglial cells, macrophages, Th17 cells, Tc1 cells, and Th1 cells (Fig. 6). The inhibition of systemic inflammation and local neuroinflammation may be the mechanism by which cordycepin treatment attenuated the clinical symptoms in the therapeutic EAE model (Fig. 6). Fig. 7 shows a schematic diagram of the possible mechanism of cordycepin treatment in EAE mice.

The early hallmarks of MS pathogenesis are immune cell trafficking into the CNS and degradation of the endothelial BBB. The migration of immune cells to the CNS is a multistep process regulated by the sequential interaction of different adhesion molecules on the BBB and on immune cells. Briefly, circulating T cells roll on the endothelial surface via α4β1-integrin and LFA-1 engagement of endothelial VCAM-1, ICAM-1, and ICAM-2 [47, 48]. Some molecules were previously shown to participate in the recruitment of various subsets of leucocytes to the CNS, including CD4+ T cells (LFA-1 and ALCAM) [49, 50], CD8+ T cells (α4 integrin) [51], Th17 cells (CCR6 and CCL20) [52], and monocytes/dendritic cells (αL integrin, ALCAM, CCR2, CCR5, and CCR7) [14-17, 50, 53]. In addition, several proinflammatory cytokines and chemokines play a role in MS pathogenesis by disrupting the BBB and increasing immune cell trafficking, such as TNF-α, IFN-γ, IL-1, IL-6, IL-16, IL-17, IL-22, MMPs, CXCL9, CXCL10, and CXCL11 [54-58]. In the current treatment of multiple sclerosis, some drugs are mainly used to suppress the immune response. Fingolimod is an agonist of sphingosine-1 phosphate receptors 1, 3, 4, and 5 that sequesters lymphocytes in lymph nodes by inhibiting their egress into lymph [59], reduces the death of human brain microvascular endothelial cells induced by high concentrations of proinflammatory cytokines and downregulates vascular adhesion molecules such as ICAM-1, VCAM-1, and P-selectin on the BBB endothelium [60, 61]. Laquinimod is a derivative of the immunomodulator linomide (quinoline-3-carboxamide) that reduces ICAM-1 and ALCAM mRNA expression in the brain endothelium and blocks the migration of Th1 and Th17 cells across brain endothelial cells [62]. Natalizumab is a humanized monoclonal antibody that selectively blocks the VLA-4-mediated interaction of autoaggressive T cells with endothelial VCAM-1 on the BBB and inhibits T cell entry into the CNS in patients [63]. These clinical medicines targeting immune cell trafficking have proven to be a successful therapy for MS but have side effects and are no longer effective once patients have entered the progressive phase of the disease.

In the present study, cordycepin treatment reduce levels of the integrin b1, integrin a4, c-type lectin, ICAM-1, and CCR7 proteins on activated DCs and impaired their migration *in vitro* (Fig. 2C*)* and *in vivo* (Fig. 3C). Our chemokine array indicated that cordycepin treatment reduced the protein levels of chemokines (CCL6, PARRES2, IL-16, CXCL10, and CCL12) expressed in the brain and spinal cord (Fig. 4) and blocked lymphocyte infiltration into the CNS of EAE mice (Fig. 3B and 3C). In addition to the abovementioned trafficking factors, NGS data showed that cordycepin treatment also significantly inhibited the mRNA expression of other adhesion molecules, cytokines, and their receptors, such as *CxCl14, Xcl1, Ccl24, Ccr9, Cxcr6, Itgb7, Cxcl2, Pecam1, Pvr, Selplg, CxCl12, Ccl4, Cd2, Cxcr3, Pvrl2, Cd226, Cxcr4,* and *Cx3cr1*. Some of these trafficking factors have not been confirmed to be related to multiple sclerosis. Therefore, we will further study the roles of these factors in multiple sclerosis in the future.

Neuroinflammation is one cause of brain injury and involves glial activation and the release of inflammatory mediators, such as cytokines and chemokines. It is considered an event that induces neuronal dysfunction and the progression of neurodegenerative diseases such as MS, Alzheimer's disease and Parkinson's disease. Microglia are macrophage-like cells in the CNS that are activated and secrete various inflammatory cytokines. For example, IL-6 and TNF-α have been identified as the major drug targets for neurodegeneration [64]. Neuroinflammation in MS is caused by lymphocytes invading the central nervous system, accompanied by damage to BBB function and a strong glial response [65]. In the present study, cordycepin treatment reduced cytokine production from not only microglial cells and macrophages but also Th1, Th17, and Tc1 cells *in vitro* (Fig. 6A-C). Either in the early disease onset or late disease progression stage, lymphocytes had already infiltrated into the CNS, and cordycepin treatment still exerted a therapeutic effect (Fig. 6D-E). The therapeutic effect of cordycepin on these stages may be due to the reduction in neuroinflammation.

Cordycepin from *Cordyceps militaris* has been reported to exhibit anti-inflammatory, antioxidant [66] and neuroprotective effects [30]. It was also shown to block the expression of several adhesion molecules, such as ICAM-1 and VCAM-1, and chemokines/receptors, such as MCP-1, MIP-1α, MIP-2, KC, and CXCR2 [31], in different disease models. However, the therapeutic effect and mechanism of cordycepin treatment in multiple sclerosis have not been investigated previously. This study is the first to document the effect and mechanism of cordycepin in an EAE model. However, the 3D structure of cordycepin is similar to adenosine and may participate in certain biochemical reactions of adenosine. Adenosine receptors (ARs) are members of the G protein-coupled receptor family and four AR subtypes have been identified: A1AR, A2aAR, A2bAR, and A3AR [67]. Cordycepin may induce apoptosis in cancer cells by activating adenosine receptors [68, 69]. Further investigations are needed to determine whether cordycepin binds to the various subtypes of adenosine receptors to elicit the effects we observed on the EAE model.

Conclusion

We demonstrated that cordycepin treatment ameliorated EAE by blocking immune cell infiltration in the preventive model and reducing neuroinflammation in the therapeutic model. These findings may provide insights into the development of novel therapeutic agents for the treatment of MS.

Abbreviations

APCs: Antigen-presenting cells

BBB: Blood-brain barrier

BMDCs: Bone marrow-derived dendritic cells

CFA: Complete Freund’s adjuvant

CNS: Central nervous system

DCs: Dendritic cells

DEGs: Differentially expressed genes

DMTs: Disease-modifying therapies

EAE: Experimental autoimmune encephalomyelitis

ICS: Intracellular staining

LPS: Lipopolysaccharide

MoDCs: Monocyte-derived dendritic cells

MS: Multiple sclerosis

NGS: Next-generation sequencing

PBMCs: Peripheral blood mononuclear cells

PTX: Pertussis toxin

ROS: Reactive oxygen species

Declarations

Ethics approval and consent to participate

All mice were housed in a specific pathogen-free room at the Animal Center, China Medical University, Taichung, Taiwan and maintained in accordance with institutional animal care guidelines. The animal protocol was approved by the Animal Care and Use Committee of China Medical University, Taichung, Taiwan (CMUIACUC-2018-144). The study involving human subjects was approved by the Research Ethics Committee of China Medical University & Hospital, Taiwan (CMUH110-REC2-052).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YCS and HRY conceived and supervised the project. YCS and HRY designed the project. CTL, HJL and YCS performed experiments. HJL and YCS analyzed all of the data. YCS and HRY prepared the manuscript. All authors read the manuscript, provided feedback, and approved the final manuscript.

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Figures

Figure 1

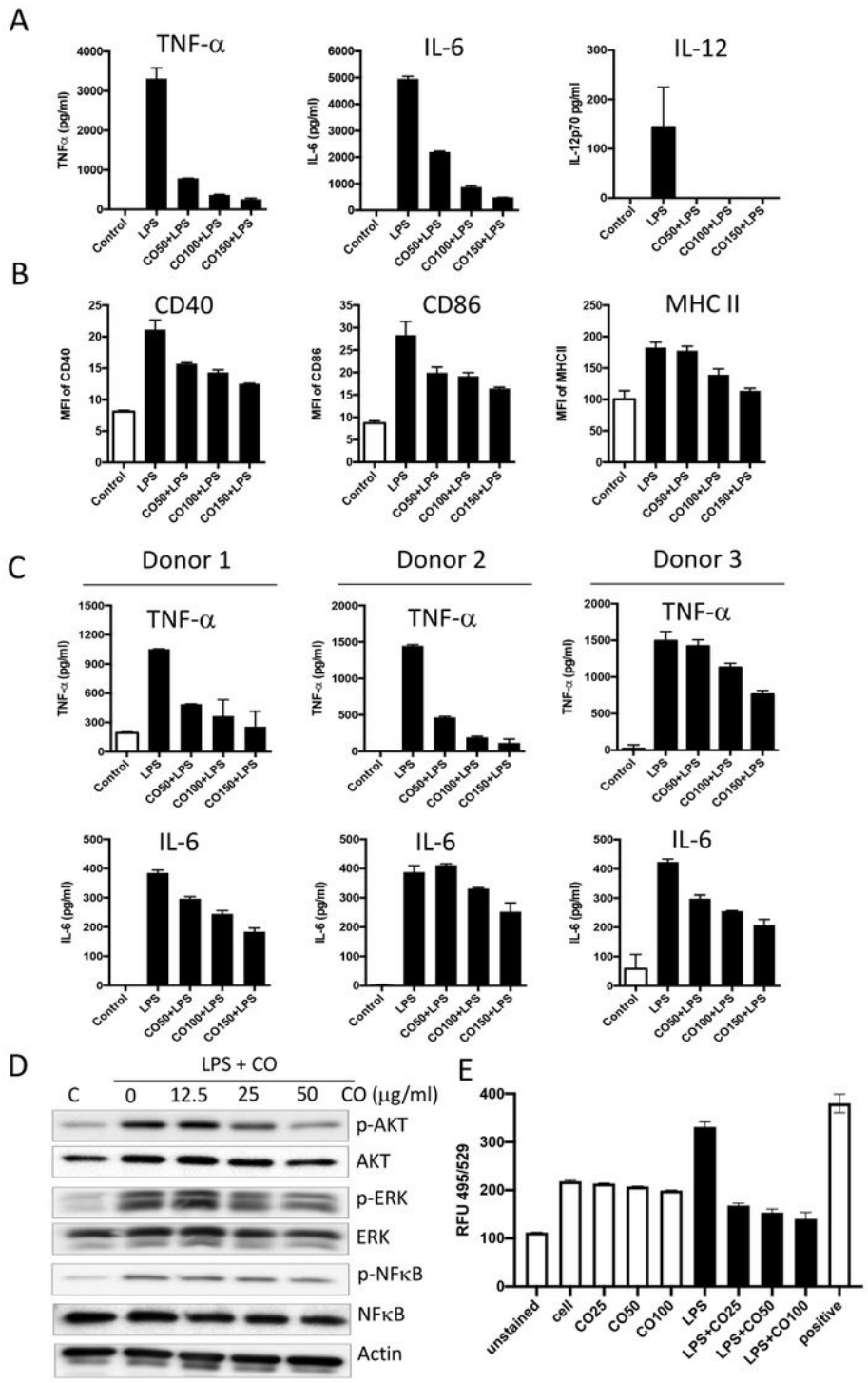

Figure 1

The effects of cordycepin treatment on TLR-induced BMDC activation. (A-B) BMDCs were cotreated with LPS (0.1 μg/mL) in the presence of 50, 100 and 150 μg/mL cordycepin (CO50, CO100 and CO150) for 18 h. The control (ddH2O treatment) was the vehicle. (A) The amounts of TNF-α, IL-6 and IL12 in the culture supernatant were measured using ELISAs. (B) The levels of CD40, CD86 and MHC II on the cell surface were measured using flow cytometry. The results are presented as the mean cytokine levels + SD. (C)

Human MoDCs from three donors were cotreated with LPS (0.1 μg/mL) combined with 50, 100 and 150 μg/mL cordycepin (CO50, CO100 and CO150) for 18 h. The levels of TNF-α and IL-6 in the culture supernatant were measured using ELISAs. The results are presented as the means + SD. (D) BMDCs were cotreated with LPS (0.1 μg/mL) in the presence of 0, 12.5, 25 and 50 μg/mL cordycepin (CO) for 30 min. Levels of AKT/p-AKT, ERK/p-ERK, NFκB/p-NFκB, and actin were determined using Western blotting. (E) BMDCs were pretreated with 25, 50 and 100 μg/mL cordycepin (CO25, CO50 and CO100) for 1 h and then stimulated with/without 0.1 μg/mL LPS for 3 h. ROS generation within the cells was determined by measuring the absorbance at Ex/Em wavelengths of 495/529 nm. An ROS inducer was used as an experimental control according to the manufacturer's protocol.

Figure 2

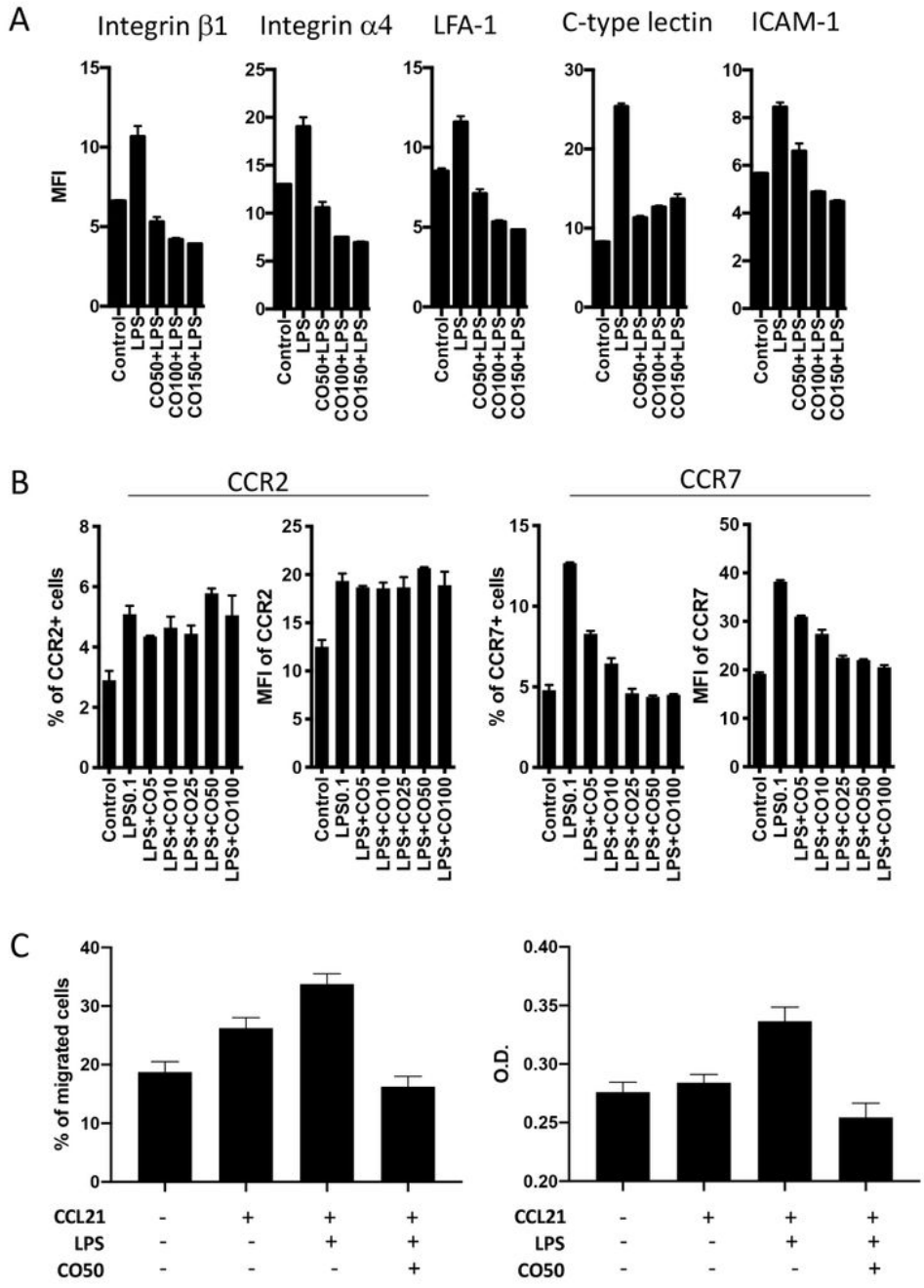

Figure 2

The effects of cordycepin treatment on adhesion molecule production, chemokine receptor expression and migration of BMDCs. BMDCs were cotreated with LPS (0.1 μg/mL) with/without 50, 100, 150 μg/mL cordycepin (CO50, CO100, CO150) for 18 h. The levels of the adhesion molecules (A), integrin β1, integrin α4, c-type lectin, and ICAM-1, as well as the chemokine receptors (B) CCR2 and CCR7 on the cell surface were measured using flow cytometry. Control refers to the vehicle control (ddH2O treatment). (C) In the

transwell migration assay, the upper chamber contained BMDCs from pretreated groups (LPS stimulation with or without cordycepin treatment) in 100 µl of serum-free media, while the lower chamber contained 500 µl of complete media with or without CCL21 (250 ng/ml). The migrated cells were analyzed using flow cytometry (left panel) and the WST-1 assay (right panel). Data are presented as the means + SD.

Figure 3

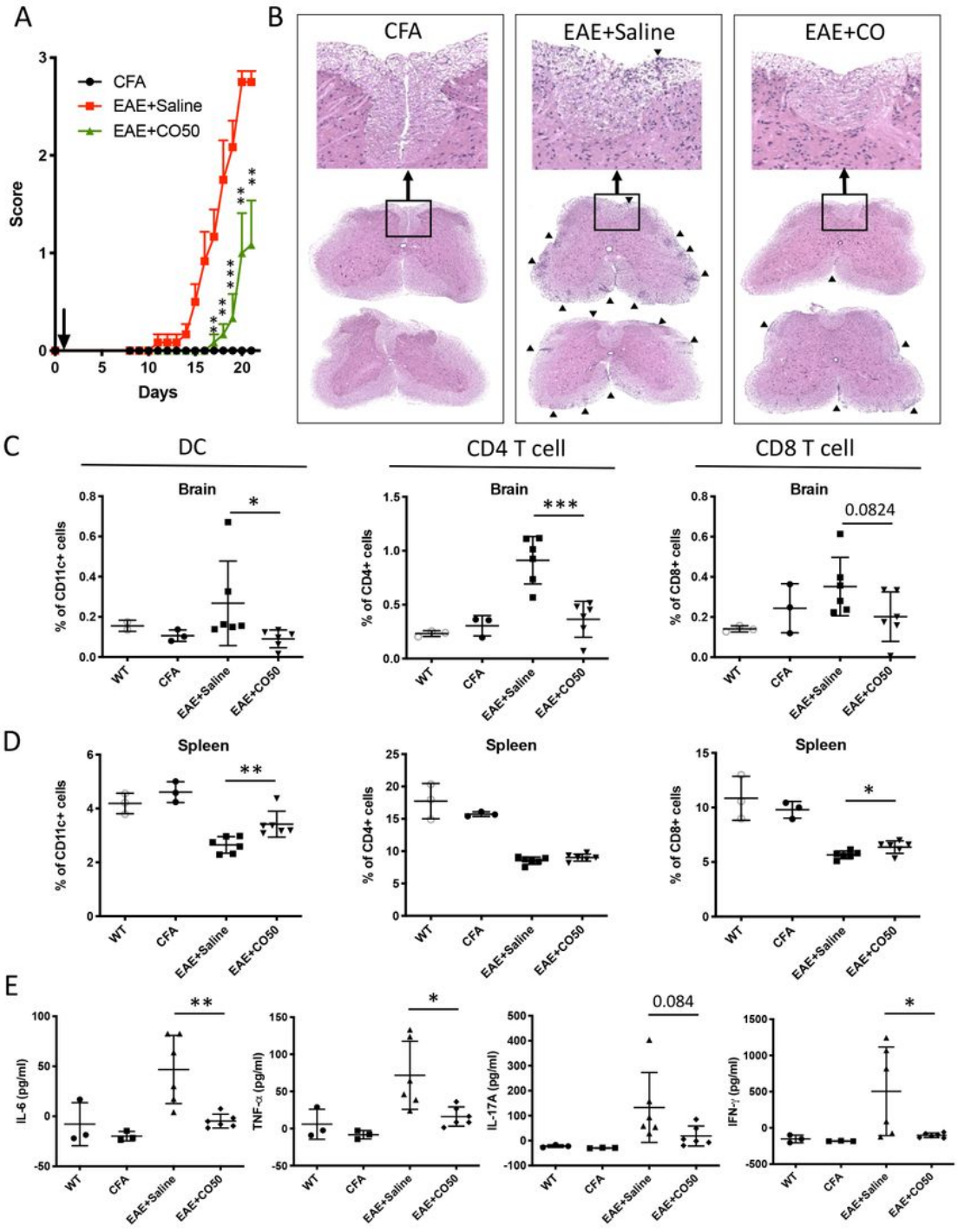

Figure 3

Clinical evaluation of cordycepin treatment in the EAE model. Fifty milligrams/kg cordycepin (EAE+CO50; n=6) or saline (EAE+saline; n=6) were administered daily beginning on day 0. The CFA group (CFA; n=3) was similarly immunized with CFA and PTX without MOG. (A) Disease scores were recorded daily. (B) Mice were then sacrificed on day 21. The infiltrating lymphocytes were analyzed using H&E staining. The black triangles indicate the area infiltrated by leukocytes. In another batch of experiments, populations of DCs, CD4+ T cells and CD8+ T cells in the brain (C) and spleen (D) of the wild-type group (WT; n=3), CFA group (CFA; n=3), and cordycepin-treated (EAE+CO50; n=6) or untreated (EAE+saline; n=6) EAE mice were stained with anti-CD45 Ab, anti-CD11c Ab, anti-CD4 Ab and anti-CD8 Ab followed by a flow cytometry analysis. (E) Splenocytes were restimulated with MOG (10 μg/mL) ex vivo for 3-4 days. The amounts of IL-6, TNF-α, IL-17 and IFN-γ in the culture supernatant were measured using ELISAs. Data obtained on day 3 (IL-6 and TNF-α) and day 4 (IL-17 and IFN-γ) are shown. The results are presented as the means ± SEM and were considered statistically significant when $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

Figure 4

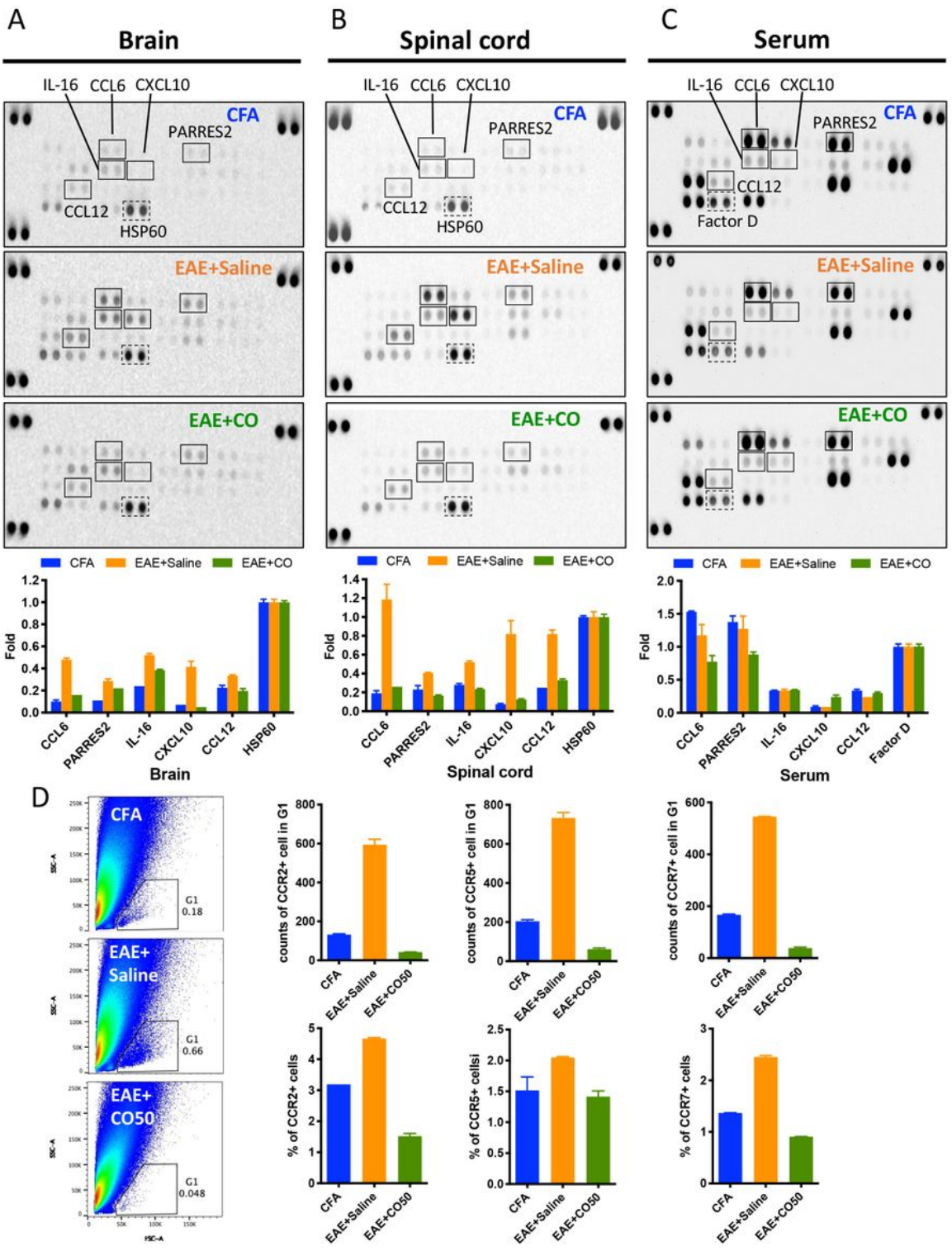

Figure 4

The chemokine expression profiles in the brain, spinal cord, and serum of EAE mice treated with or without cordycepin. EAE mice were administered saline (EAE-Saline) and 50 mg/kg cordycepin (EAE+CO) daily beginning on day 0 after immunization. The CFA group was similarly immunized without MOG as a negative control. Brain (A), spinal cord (B) and serum (C) samples were harvested from different groups on day 14 after immunization. Chemokine production was measured using a Mouse Chemokine Array Kit.

The bar charts show the fold change in the level of the indicated chemokine compared to the corresponding positive control spots. HSP60 was used as a positive control in the brain and spinal cord. Factor D was used as a positive sample control in serum. Data are presented as the means ± SD of two spots. (D) The spinal cords were harvested from different groups of mice on day 14 after immunization. The amount of chemoattractant receptor expressed on infiltrating cells in the spinal cord was determined by staining with an anti-CCR2 Ab, anti-CCR5 Ab, and anti-CCR7 Ab and flow cytometry analysis. The left panels show the gating of infiltrating leukocytes (G1 gate) in the spinal cord. The bar charts show the cell counts and percentages of the indicated cell populations in the spinal cord.

Figure 5

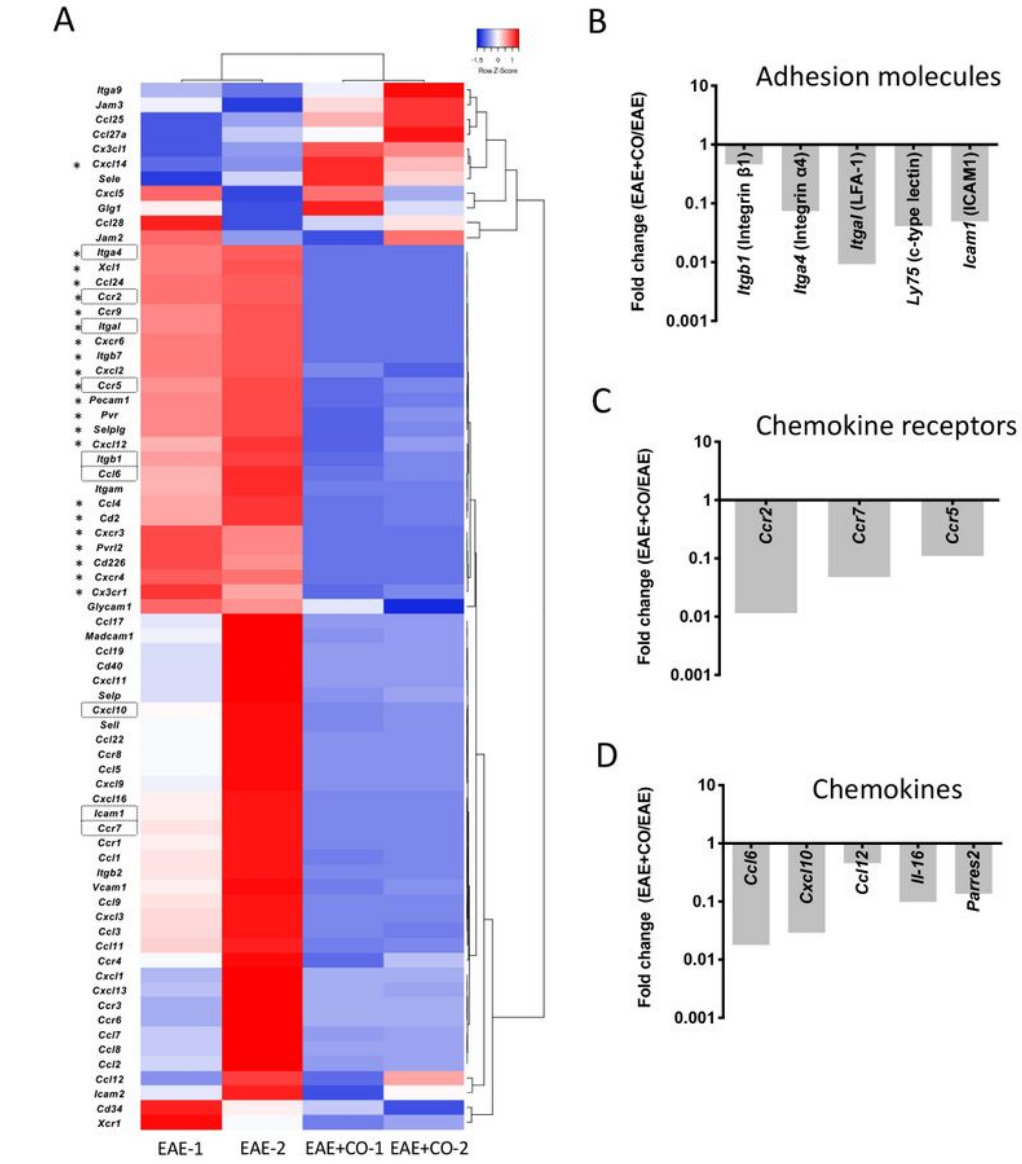

Figure 5

Adhesion molecule, chemokine receptor, chemokine gene expression analysis. (A) Heatmap representation of normalized RNA-Seq expression levels (Z-scores) of adhesion molecule, chemokine, and chemokine receptor genes (selected by KEGG pathway analysis) in spinal cord samples from cordycepin-treated or untreated EAE mice (EAE or EAE+CO) (n=2 mice/group). $p < 0.05$ (*). The mapping grids are colored according to their Z-scores. Bar charts show fold changes in the mRNA expression of the selected

adhesion molecules (B), chemokine receptors (C), and chemokines (D) in the cordycepin-treated group (EAE+CO) compared to the untreated group (EAE).

Figure 6

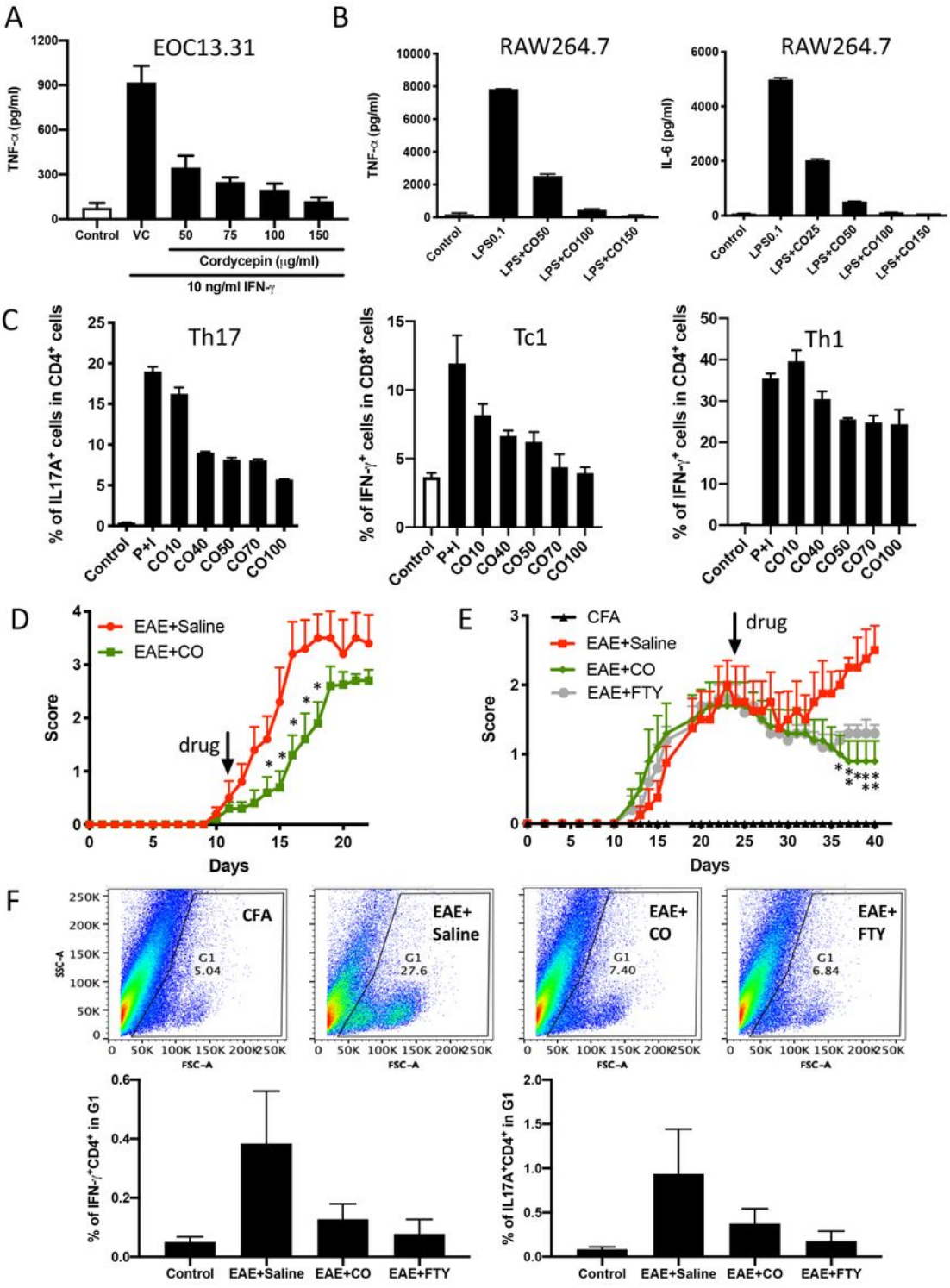

Figure 6

The effects of cordycepin treatment on inflammation and therapeutic efficacy. (A) EOC13.31 microglial cells were cotreated with IFN-γ (10 ng/mL) in the presence and absence of 50, 75, 100, and 150 μg/mL cordycepin for 18 h. The level of TNF-α in the culture supernatant was measured using an ELISA. VC

denotes the vehicle control. (B) RAW264.7 macrophages were cotreated with LPS (0.1 μg/mL) in the presence and absence of 50, 100, and 150 μg/mL cordycepin (CO50, CO100, and CO150) for 18 h. The levels of TNF-α and IL-6 in the culture supernatant were measured using ELISAs. (C) Polarized Th17, Tc1 and Th1 cells were treated with 0, 10, 40, 50, 70 and 100 μg/mL cordycepin (CO10, CO40, CO50, CO70 and CO100) for 16 h and restimulated with PMA and ionomycin (P+I) for 5 h. Cell surface markers and intracellular proteins (IL-17A and IFN-γ) were stained with fluorochrome-conjugated antibodies and analyzed using flow cytometry. The results (A-C) are presented as the means + SD. The data shown are representative of two independent experiments. (D) In the early disease onset stage, 50 mg/kg cordycepin (EAE+CO50; n=5) and saline as a vehicle control (EAE+saline; n=5) were i.p. administered daily from day 11 to day 22. Disease scores were recorded daily. (E) In the late disease progression stage, 50 mg/kg cordycepin (EAE+CO50; n=5), 1 mg/kg fingolimod (EAE+FTY; n=5), saline as a vehicle control (EAE+saline; n=4), and CFA as a negative control (CFA group; n=5) were i.p. administered daily beginning at the peak of disease severity (day 24). Clinical disease scores were examined daily. The results are presented as the means ± SEM and were considered statistically significant results when $p < 0.05$ (*) and $p < 0.01$ (**). (F) On day 38, mice were sacrificed and brains were harvested. The population of CD4+ T cells that produce IFN-γ and IL-17 in the brain was stained with an anti-CD45 Ab, anti-CD4 Ab, anti-IFN-γ Ab, and anti-IL-17 Ab and analyzed using flow cytometry. The upper panels show the percentages of infiltrating leukocytes (G1 gate) in the brain. The bar charts show the percentages of the indicated cell populations in brains. The results are presented as the means + SEM.

Figure 7

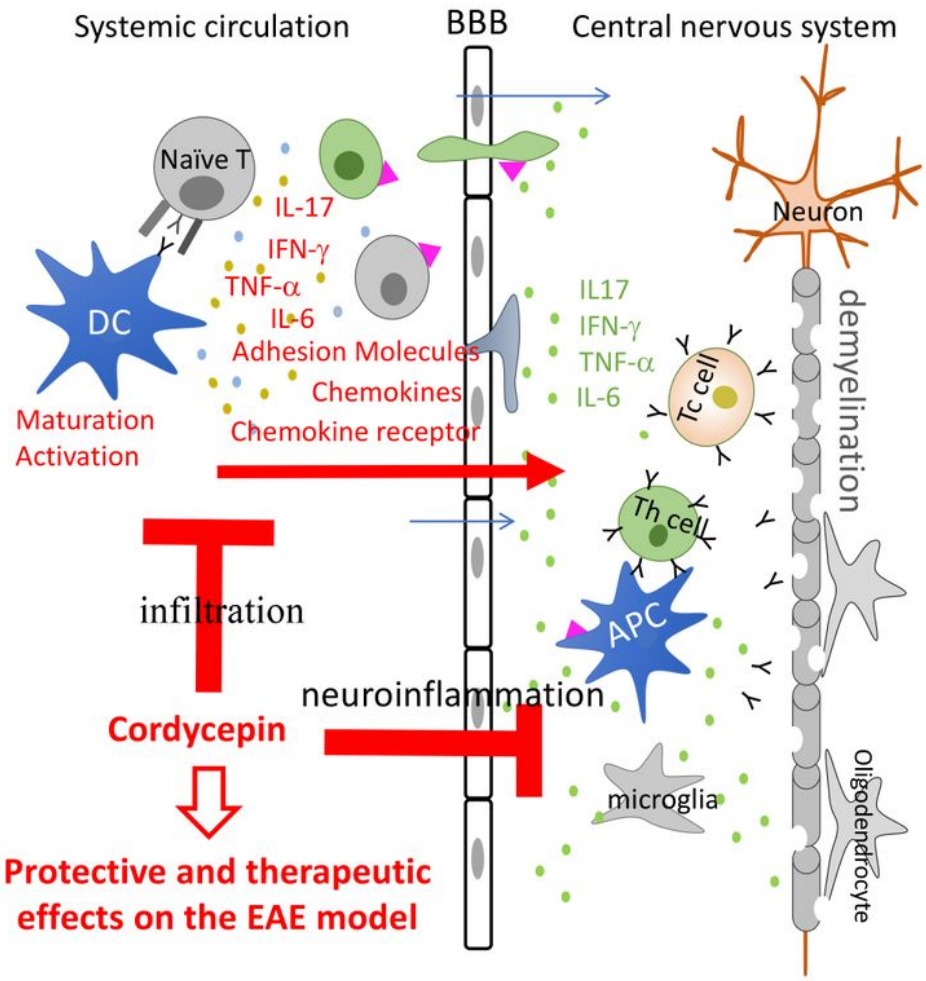

Figure 7

Proposed mechanism of action of cordycepin treatment in EAE. Cordycepin treatment induces preventive effects by inhibiting the activation of DCs, decreasing the production of trafficking factors and blocking the infiltration of immune cells, and exerts therapeutic effects by reducing neuroinflammation in the CNS.

Supplementary Files

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

- supplFig1.pdf
- supplFig2.pdf