Methylprednisolone modulates the TFR/TFH balance in EAE neuroinflammation through the PI3K/AKT/FoxO1 and PI3K/AKT/mTOR signaling pathways

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Research Article

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Abstract

Background

Methylprednisolone (MP) is a potent glucocorticoid, which can effectively inhibit immune system inflammation and brain tissue damage in MS patients. T Follicular helper (Tfh) cells are a subpopulation of activated CD4+ T cells, while T Follicular regulatory (Tfr) cells, a novel subset of Treg cells, possess specialized abilities to suppress the Tfh-GC response and inhibit antibody production. Dysregulation of either Tfh or Tfr cells has been implicated in the pathogenesis of MS. However, the molecular mechanism underlying the anti-inflammatory effects of MP therapy on experimental autoimmune encephalomyelitis (EAE), a representative model for MS, remains unclear.

Purpose

This study aimed to investigate the effects of MP treatment on EAE and elucidate its possible molecular mechanisms.

Method

We evaluated the effects of MP on disease progression, CNS inflammatory infiltration and myelination, microglia and astrocyte activation, as well as Tfr/Tfh differentiation and related molecules/inflammatory factors in EAE. Additionally, western blotting was used to assess protein abundance associated with the PI3K/AKT pathway.

Results

Our findings demonstrated MP treatment ameliorated clinical symptoms, inflammatory cell infiltration, and myelination. Furthermore, it reduced microglia and astrocyte activation. MP up-regulated the differentiation of Tfr cells and cytokine TGF-β1, while downregulating Tfh cells differentiation and cytokine IL-21, as well as regulating the imbalanced Tfr/Tfh ratio in EAE. The PI3K/AKT/FoxO1 and PI3K/AKT/mTOR pathways were found to be involved in EAE development; however, MP treatment inhibited their activation.

Conclusion

MP demonstrated the ability to reduce neuroinflammation in EAE by regulating the balance between Tfr/Tfh cells via inhibition of the PI3K/ AKT/FoxO1 and PI3K/AKT/mTOR signaling pathway.

1. Introduction
Multiple sclerosis (MS) is one of the most prevalent chronic autoimmune diseases involving the central nervous system (CNS), which leads to focal lesions in the white and gray matter, as well as diffuse neurodegeneration throughout the brain[1]. Experimental autoimmune encephalomyelitis (EAE), induced by myelin oligodendrocyte glycoprotein (MOG), is the most common animal model for the study of MS[2]. The pathological manifestations of EAE consist of inflammatory cell infiltration, demyelination in the white matter of the brain and spinal cord, which are very similar to MS[3]. Also, MS is the most common non-traumatic disabling disease affecting more than 2.5 million people worldwide especially young adults between 20 and 40 years of age. MS implicated women nearly two to three times as often as men[4]. It is disturbed by completely or partially reversible episodes of neurologic symptoms, generally sustaining days or week[5]. The typical clinical presentations of MS include visual loss, limb weakness or sensory disturbances, double vision, ataxia, spasticity, cognitive deficits and so on, depending on the location of lesions[6]. Currently, MS is incurable because no medication could fully prevent or reverse the progressive neurologic deficits[5].

So far, the underlying cause and mechanisms of MS remain unclear[7]. Recently, MS is regarded as a heterogeneous and multifactorial disease caused by multiple genetic and environmental risk factors, including risk alleles (the genes for major histocompatibility complex, interleukin-2 receptor, interleukin-7 receptor), smoking, vitamin D deficiency, Epstein-Barr virus infection, diet and obesity[8]. Except for the particular triggering cause, a dysregulated immune response is a key player in the pathogenesis of MS[9]. Different subsets of CD4+ T cells orchestrate an inflammatory wave to initiate the pathogenesis of MS[10, 11]. In addition to T cells, B cells and innate immunity also participate in the onset of MS. B cells can produce specific oligoclonal band IgG present in the cerebrospinal fluid, which is one of the diagnostic criteria for MS[12]. T follicular helper cells (Tfh) cells, a helper T (Th) cell subset, are essential for helping B cells to become memory B cells in germinal centers (GCs), and can promote antibody-producing plasma cells to produce autoantibodies[13]. Besides, Tfh cells not only express the chemokine receptor CXCR5, inducible T-cell costimulator (ICOS), and programmed death-1 (PD-1), but also secrete cytokines including IL-6, and IL-21[14, 15].

Recently discovered follicular regulatory (Tfr) cells are considered negative regulators for GC responses due to their secretion of anti-inflammatory cytokines including IL-10 and TGF-β1[16]. These dual-functioning subpopulations possess characteristics both from Tfh and regulatory T (Treg) lineages. On one hand, they express typical molecules associated with heritage from Tfh lineage like CXCR5, ICOS, PD-1, and Bcl6. On the other hand, Tfr cells express Foxp3, the master regulator of Treg, as well as other Treg-related molecules such as cytotoxic T-lymphocyte–associated protein 4 (CTLA-4) and CD25[17, 18]. Furthermore, there exists a dynamic equilibrium between Tfr and Tfh cells. Several studies have demonstrated that an imbalance in the ratio of Tfr/Tfh cells may result in massive production of self-reactive autoantibodies, thereby promoting the occurrence of autoimmune disorders such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) and myasthenia gravis (MG)[19–21]. Hence, regulating their balance may confer therapeutic benefits for autoimmune diseases.
Recent findings suggest that the secretion of reactive oxygen species, chemokines, and cytokines by infiltrating macrophages and activated microglia within the central nervous system is known to play a crucial role in the pathogenesis of MS and EAE[22]. In MS pathology, microglia undergo activation leading to polarization into M1 or M2 phenotypes. Notably, an increase in M1 phenotype population contributes to myelin damage along with axonal and neuronal impairment. Conversely, an increase in M2 phenotype population aids disease alleviation[23]. Additionally, astrocytic neuroinflammation is closely associated with MS pathogenesis. Hyperactivated astrocytes induce inflammatory mediator generation. Bidirectional crosstalk between microglia and astrocytes occurs largely through inflammatory mediators. IL-1β secreted by M1 type microglia can activate astrocytes that are harmful in MS[24, 25].

Phosphatidylinositol-3-kinase (PI3K) pathway plays a core role in cell growth, cell cycle, and differentiation, and AKT serves as a direct downstream effector molecule for PI3K signaling cascade. Meanwhile, PI3K/AKT pathway has an essential role in the process and release of pro-inflammatory factors[26]. Forkhead box protein 1 (FoxO1), which is encoded by the FoxO1 gene and belongs to the Forkhead box (fox) family, undergoes regulation primarily via phosphorylation on numerous residues.. FoxO1 and mTOR are key downstream targets of the PI3K/AKT signaling pathway[27]. FoxO1 has been found to control various downstream genes, such as molecules involved in inflammation, molecules responsible for cell adhesion, regulators of B-cells, and modulators of T-regulatory cells[28]. Evidence suggests that targeting the mTOR pathway holds promise as a potential therapeutic avenue for rheumatoid arthritis (RA) and other autoimmune diseases, offering the opportunity to restore Tfr cell functionality and quantity, thereby addressing underlying pathogenic mechanisms. Conversely, baicalin exerts its effects by rebalancing Tfr and Tfh cells through suppression of the mTOR signaling pathway, resulting in amelioration of lupus nephritis symptoms[29]. Methylprednisolone (MP) is a potent glucocorticosteroid which effectively inhibit inflammation and allergic reaction of the immune system[30]. MP can inhibit T cell activation, dampen inflammatory cytokines, decrease immune cell extravasation into CNS to alleviate brain tissue injury in MS patients[31]. In spite of these advances, whether MP could inhibit inflammation through regulating Tfr/Tfh balance under the context of EAE remains unclear. Besides, whether the protective effect of MP is correlated with PI3K/AKT/FoxO1 and PI3K/AKT/mTOR signaling pathways remains largely undefined.

In this study, we aimed to provide insights into the underlying mechanisms of the pathogenesis of EAE.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice of specific pathogen-free (SPF) level, aged 6–8 weeks and weighing 19–21g, were purchased from Jinan Pengyue Laboratory Animal Breeding Co., Ltd. (Jinan, China). Each animal was bred under SPF conditions with a humidity of 60%–80% and an environmental temperature of 22 to 26 °C, and had ad libitum access to food and water. All animals used in the study were treated in
accordance with the guidelines set forth by the Institutional Animal Care and Use Committee of Binzhou Medical University Hospital.

**2.2. Establishment of EAE Model and clinical evaluation**

5mg of MOG35-55 polypeptide (GenScript, New Jersey, USA) was added into 2.5 ml PBS at a final concentration of 2 mg/ml. Then 17.5mg heat-killed mycobacterium tuberculosis (Becton, Dickinson and Company Sparks, USA) was dissolved in 2.5 ml complete Freund's adjuvant (CFA, Sigma-Aldrich, Missouri, USA), the concentration of Mycobacterium tuberculosis was 5 mg/ml. The two solutions were thoroughly mixed at a ratio of 1:1. Then the antigen was fully emulsified on ice to form a water-in-oil emulsion. Each mice was immunized subcutaneously on the back with 0.2 ml polypeptide. On day 0 and day 2 after immunization, mice received intraperitoneal injections of pertussis toxin (PTX, Merck Millipore Corp, USA) at a dose of 300 ng per injection.

Clinical signs were evaluated daily using an established scoring system ranging from no clinical signs to tetraplegia or moribund by EAE

- 0, no clinical signs;
- 0.5, limp distal tail;
- 1, paralyzed or staggering tail;
- 1.5, limp tail and hind limb weakness;
- 2, mild paresis of both hind limbs or severe paralysis of one hind limb;
- 3, complete paralysis of both hind limbs;
- 4, hind limbs and forelimbs paralyzed;
- 5, tetraplegia or moribund by EAE. The clinical signs in between are scored 0.5 point.

**2.3 Treatment of EAE Model**

All C57BL/6 mice were randomized assigned to 3 groups: (A) Control group, normal mice; (B) EAE group, saline solution treated EAE mice; and (C) EAE + MP group, MP treated EAE mice. Treatment with MP (Sinopharm Ronshyn Pharmaceutical Co., Ltd.) began from the 9th day (Early onset of EAE) to the end of the experiment, once every day. The dosage was gradually reduced over 9 days, starting at 100 mg/kg for 3 days, followed by 50 mg/kg for another 3 days, and finally 25 mg/kg for the remaining 3 days. Injections were stopped on the 18th day of the experiment. Control mice received equal volumes of saline solution.

**2.4. Histopathological analysis**

Histopathological examination was performed at the peak (day 19 post-immunization). The spinal cord samples were meticulously dissected from mice, fixed in 4% paraformaldehyde, dehydrated, paraffin wax-embedded, and sectioned (at 6 µm). Subsequently, spinal cord sections were stained not only with hematoxylin and eosin (H&E) (Solebo Biotech, Beijing, China) to evaluate inflammation but also with luxol fast blue (LFB) (Solebo Biotech, Beijing, China) to assess the demyelination. These sections were then scanned and captured with the microscope (Olympus, Japan), and the inflammatory infiltration and demyelination of spinal cord in mice were analyzed in a blinded fashion. The inflammation scale was determined based on the
presence of inflammatory cells: 0 indicated no presence, 1 denoted a few scattered inflammatory cells, 2 represented organized infiltration around blood vessels, and 3 signified extensive perivascular cuffing with extension into the surrounding tissue. Demyelination assessment in the spinal cords followed this scale: 0 indicated absence of demyelination, 1 represented rare focal areas, 2 denoted multiple demyelinated regions, and 3 signified large areas exhibiting demyelination[32, 33].

2.5. Flow cytometry

For cell phenotype analysis, anti CD4-FITC, anti CXCR5-percp-cy5.5, anti Foxp3-PE, anti ICOS-APC, anti PD-1-APC as well as the corresponding IgG isotypes were procured from BioLegend (San Diego, CA, USA).

For cell surface staining, single cells from spleens of EAE mice treated with saline solution or MP were incubated with fluorescent monoclonal antibodies at 4°C for 30 minutes with optimal dilutions. For intracellular staining of cells, a Foxp3 Staining Buffer Set (Invitrogen) was employed to fix and permeabilize the cells following the manufacturer's instructions. (After the incubation period, a double wash was performed on the cells using PBS supplemented with 2% fetal bovine serum.)

Flow cytometry analysis was conducted using a FACS Calibur flow cytometer (Beckman Coulter). The obtained data were analyzed utilizing FlowJo 10.0 software.

2.6. Immunofluorescence staining

The samples were fixed in 4% paraformaldehyde for 30 minutes, and permeabilized with PBS containing 0.4% Triton X-100 (BOSTER, China). Subsequently, the spinal cord/spleen sections were blocked with 5% bovine serum albumin (BSA, BOSTER; China) at room temperature for one hour. Following this, the sections were incubated over-night at 4°C with primary antibodies GFAP (Cell Signaling Technology), Iba-1 (Cell Signaling Technology), CD4 (Santa), CXCR5 (BioLegend), Foxp3 (ABclonal). The secondary antibodies labeled with fluorophores (Cell Signaling Technology) were then incubated at room temperature for one hour. Next, nuclei were stained with DAPI before being captured through the lens of a microscope(Olympus, Japan).

2.7. Western blot analysis

Spinal cord tissues were dissected from each group. For EAE group, the spinal cord samples were carefully dissected on Day 9 post-immunization (the pre-immune of EAE, Pre-EAE group), on Day 19 post-immunization (the peak of EAE, P-EAE group) and frozen immediately in liquid nitrogen. The spinal cord was subjected to protein extraction using RIPA lysis buffer supplemented with phosphatase and protease inhibitors. The concentration of proteins was determined by employing the BCA assay (BOSTER, China). Protein samples were added to each well for electrophoresis separation with SDS-PAGE (80 V, 0.5 h and then 120 V, 1.5 h). The separated protein bands were transferred to 0.45 µm PVDF membranes (Servicebio, China). The membranes were blocked in a solution containing TBST with 5% skim milk for 1 h, and then incubated respectively with anti-PI3K (Cell Signaling Technology, 34050), anti-S473 phospho-AKT (Affinity, AF0016), anti-AKT (Affinity, AF6261), anti-phospho-FoxO1 (Cell Signaling Technology, 9464), anti-FoxO1(Affinity, AF3417), anti-phospho-mTOR (Cell Signaling Technology, 5536), anti-mTOR
(Cell Signaling Technology, 2983), CXCR5 (ABclonal, A8950), Foxp3 (Affinity, AF6544), iCOS (ABclonal, A1811), PD-1 (Cell Signaling Technology, 84651), TGF-β1 (Proteintech, 21898-1-AP), IL-21 (Affinity, DF4818), GFAP (Cell Signaling Technology, 3670), Iba-1 (Cell Signaling Technology, 17198), Arg-1 (Proteintech, 16001-1-AP), iNOS (Proteintech, 18985-1-AP) and GAPDH (BOSTER, BM3874) overnight at 4 °C. The membranes underwent incubation with an anti-rabbit antibody conjugated with HRP (Boster, BA1054) for 1 h. Finally, ECL (Boster, China) was added, and the membranes were detected by chemiluminescence imaging system (LI-COR Biosciences). The band density was quantitatively analyzed through Image J.

2.8. Statistical analysis

The data for each group were presented as the mean ± standard error of the observations (SEM). Statistical analyses were conducted using GraphPad Prism, V8.0 (GraphPad Software Inc., San Diego, USA). To calculate differences between two groups, the statistical analysis involved the utilization of a Student t-test, while for examining comparisons among multiple groups, a one-way analysis of variance (ANOVA) was conducted followed by Bonferroni’s multiple comparison tests. In MOG-EAE experiments, statistical significance in neurological scores was determined using two-way ANOVA. Significant distinctions between or among groups were assessed accordingly: * P < 0.05, ** P < 0.01, and *** P < 0.001.

3. Results

3.1. MP ameliorated the severity of EAE.

To evaluate the therapeutic efficacy of MP on EAE, we examined the clinical score of mice from day 0 to day 19 after immunization. Neurological deficits became apparent on the 9th day after immunization and progressively worsened thereafter. EAE+MP group had reduced clinical scores significantly compared with EAE group (p < 0.05, Figure 1). The EAE+FMT group demonstrated significantly reduced cumulative, maximum, and mean EAE scores in comparison to the EAE group. However, there was no significant difference between the onset days of the EAE and EAE+MP groups (Figure 1).

Figure 1. MP reduced the clinical score of EAE.

(A) Daily fluctuations in clinical scores between the two cohorts throughout the course of the disease. (mean ± SEM; n = 8 each; * P < 0.05, ** P < 0.01, and *** P < 0.001).

(B) Analysis was conducted on the maximal scores, cumulative scores, and mean scores as well as onset day for both the EAE group and MP group. (n = 8 each; * P < 0.05, ** P < 0.01, and *** P < 0.001).

3.2. MP attenuated inflammatory cell infiltration and demyelination in the spinal cord of EAE mice

To evaluate the extent of inflammatory infiltration and remyelination in the lumbar spinal cord of EAE mice, H&E and LFB staining were performed (p < 0.05, Figure 2 (A)). We observed decreased perivascular
and parenchymal mononuclear cell infiltrates within white matter as well as reduced perivascular cuffs in the EAE+MP group compared to the EAE group (p < 0.05, Figure 2(A)). Additionally, LFB staining revealed less extensive demyelination in the spinal cords of mice treated with MP compared to those without treatment (p < 0.05, Figure 2(B)). These findings indicate that MP effectively alleviates inflammatory infiltration and demyelination in a murine model of EAE.

Figure 2. MP reduced the CNS inflammation and demyelination of EAE Mice.

(A) Inflammatory infiltration observed through H&E staining with corresponding inflammation scores.

Demyelination was detected by LFB staining with corresponding demyelination scores.

3.3 MP ameliorated EAE by suppressing the microglia and astrocytes activation

Microglia and astrocyte activation promotes the production of pro-inflammatory cytokines and inflammatory infiltration in the CNS of EAE animals[34]. We evaluated the response of glial fibrillary acidic protein (GFAP), a marker for astrocytes, and ionized-calcium binding adaptor protein (Iba-1), a marker for microglia, through immunofluorescence analysis on lumbar spinal cord sections. The mice in the EAE+MP group exhibited significantly fewer Iba-1-positive microglia compared to those in the P-EAE group (p<0.05, Figure 3(A)). Moreover, there was a decrease in GFAP-positive astrocytes observed in the spinal cord of MP-treated EAE group when compared with P-EAE mice (p<0.05, Figure 3(B)). The Western blot analysis revealed a significant upregulation of GFAP expression in the EAE group compared to both the control and Pre-EAE groups. However, MP treatment reversed the upregulation within EAE group (p<0.05, Figure 3(C)). Additionally, iNOS protein levels showed a consistent trend with Iba-1 among different groups indicating an anti-inflammatory effect but without statistical significance (Figure3(C)). Furthermore, Arg-1 protein levels decreased significantly within the EAE group as compared to control and Pre-EAE groups; however, MP treatment reversed this decline without statistical significance. These findings suggest that MP can ameliorate EAE by suppressing microglial and astrocytic activation in vivo while reducing inflammatory responses and promoting anti-inflammatory responses through inhibiting M1 microglial activity as well as promoting polarization towards M2 phenotype.

Figure 3 MP inhibited microglia and astrocyte activation in EAE mice.

(A)-(B) The expression of the anti-GFAP and anti-Iba-1 protein was detected by immunofluorescence staining in lumbar spinal cords of P-EAE, EAE+MP groups.

(C) The protein expression levels of GFAP, Iba-1, iNOS, and Arg-1 were investigated in lysates from lumbar spinal cords using Western blot analysis. Statistical significance was determined as * P <0.05, ** P < 0.01, and *** P < 0.001

3.4. MP inhibits Tfh cell differentiation, promotes Tfr cell differentiation, and restores the balance of Tfr/Tfh ratio in EAE mice
The Tfr cells were positive for Foxp3 expression while Tfh cells were negative[35, 36]. We determined the location of CD4+CXCR5+Foxp3+ Tfr cells and CD4+CXCR5+Foxp3- Tfh cells in the spleen (Figure 4(A)) using immunofluorescence staining. To further investigate the systemic immunoregulatory effects of MP, we performed flow cytometry analysis on individual spleen cells. Tfh cells were defined as CD4+CXCR5+Foxp3-, and their percentage expanded from an average of 1.22% in Pre-EAE mice to 2.07% in P-EAE mice. However, MP treatment significantly reduced the percentage of Tfh cells to an average of 0.88% (Figure 4(B)), indicating its inhibitory effect on Tfh cell differentiation in EAE mice.

The percentage of CD4+CXCR5+Foxp3+ Tfr cells was lower in both Pre-EAE and P-EAE groups compared to the control group ($p<0.05$, Figure 4(B)). Nevertheless, MP treatment partially restored the percentage of Tfr cells. The ratio of Tfr/Tfh cells was significantly reduced in both Pre-EAE and P-EAE groups compared to the control group but was reversed after treatment with MP ($p<0.05$, figure 4(B)). We observed an increase in the percentage of Tfh cell subtype, identified as CD4+CXCR5+Foxp3-ICOS+, increased in Pre-EAE, reached a maximum at the P-EAE, and decreased after MP treatment($p<0.05$, Figure5(B)). Similar results were obtained when using CD4+CXCR5+Foxp3-PD-1 as another subtype of Tfh cells ($p<0.05$, Figure5(B)). Here, our findings suggest that MP treatment can correct the imbalance between Tfr and Tfh cells by inhibiting the differentiation of Tfh cells and promotes the differentiation of Tfr cells, thereby exerting its systemic immuno-inflammatory regulation effect in EAE mice.

Figure 4: Confocal photomicrographs of Tfr cell localization in spleen characterized by the distinct density of CD4 T cells (red (A)), CXCR5 (blue (B)), Foxp3 (green (C)), and Merge (D) and Tfh cell localization in spinal cord characterized by the distinct density of CD4 T cells (red (A)), CXCR5 (blue (B)), and Merge (D).

Figure 5: Flow cytometry analysis of different subsets of spleen Tfr and Tfh cells in the control, Pre-EAE, P-EAE, and MP groups.

The percentage of CD4+CXCR5+Foxp3+Tfr and CD4+CXCR5+Foxp3-Tfh cells in the spleen of EAE mice in these four groups.

The percentages of CD4+CXCR5+Foxp3-ICOS+, CD4+CXCR5+Foxp3-PD-1+ Tfh cells in the spleen of EAE mice in these four groups.

3.5. MP treatment resulted in the downregulation of Tfh-related molecules and pro-inflammatory factors while promoting the expression of Tfr-related molecules and anti-inflammatory factors.

We detected various cell surface and intracellular markers associated with Tfh in spleen tissues from control, Pre-EAE, P-EAE, and EAE+MP groups using Western Blot, including CXCR5, ICOS, PD-1, as well as IL-21. Compared to the Control and Pre-EAE group, the expression of CXCR5, ICOS, PD-1, and IL-21 were elevated in the EAE group. However, administration of MP effectively prevented the upregulation of CXCR5, ICOS, PD-1, and IL-21 protein expression observed in the EAE group ($p<0.05$, Figure 6). Furthermore, in this study, the level of TGF-β1 protein expression significantly decreased in the EAE group.
compared to that in Control and Pre-EAE groups, however, treatment with MP reversed this reduction specifically within P-EAE Group (p<0.05, Figure 6). Moreover, the expression levels of Foxp3 protein exhibited a consistent correlation with TGF-β1, notably, only the EAE+MP group demonstrated a significantly elevated level compared to the P-EAE group (p<0.05, Figure 6).

Figure 6: The EAE mice were classified into the control group, the Pre-EAE group, the P-EAE group and the EAE+MP group. Representative blots band of CXCR5, PD-1, iCOS, TGF-β1, FoxP3, IL-21 in spinal cord of EAE mice were analyzed.

3.6. MP modulated Tfr/Tfh proliferation in EAE via the PI3K/AKT/FoxO1 and PI3K/AKT/mTOR pathway.

To gain further insight into the molecular mechanism of MP in the treatment of EAE, we quantitatively analyzed the proteins associated with PI3K/AKT/FoxO1 and PI3K/AKT/mTOR pathway. Western blot analysis revealed significantly increased levels of PI3K, phosphorylated AKT (p-AKT), phosphorylated FoxO1 (p-FoxO1), and phosphorylated mTOR (p-mTOR) in the EAE group compared to the control and Pre-EAE groups. However, total AKT protein, total Foxo1 protein, and total mTOR protein did not show significant changes among the three groups. Interestingly, MP treatment could significantly reduce the expression of PI3K, p-AKT, p-FoxO1, and p-mTOR compared to P-EAE group (p<0.05, Figure 7). This result further supports involvement of the PI3K/AKT/FoxO1 and PI3K/AKT/mTOR pathways in EAE occurrence and development while highlighting that MP treatment can inhibit activation of these signaling pathways. Our findings suggest that MP may modulate Tfr/Tfh cell ratio through the PI3K/AKT signaling pathway.

Figure 7. MP relieved EAE through the PI3K/AKT/mTOR and PI3K/AKT/FOXO1 signaling pathways.

Western blotting analysis of total PI3K, p-AKT, total AKT, p-FoxO1, total FoxO1, p-mTOR, and total mTOR.

(B) Quantitative analysis revealed decreased levels of expression for PI3K, p-AKT, p-FoxO1 and p-mTOR in the spinal cord following MP treatment.

Discussion

In the early phase of MS and EAE, activation and infiltration of immune cells into the CNS, lead to disease onset and progression[37]. As an autoimmune disease, immune suppressive therapies have always been the focus for MS.

MP plays a crucial role in the current treatment of MS, with it gradually recognized immune regulatory properties[38]. Despite the development of numerous new drugs in the past decade, high-dose MP pulse therapy remains widely used for treating acute relapses, resulting in symptom amelioration within a few days[39]. The results showed that MP treatment ameliorated the clinical symptoms during the acute phase of EAE mice at 19 dpi (p<0.05, Figure 1). Histological analysis using HE and LFB staining showed that MP treatment reduced inflammatory infiltration and demyelination in the spinal cord of EAE mice (p<0.05, Figure 2).
This finding suggests that MP exerted certain anti-inflammatory and myelin protective effects on EAE mice. In the development of MS, astrocytes and microglia can produce various cytokines and inflammatory mediators, which induce inflammation, demyelination, axonal loss and gliosis[40]. Thus, inhibiting the overactivation of microglia and astrocyte while reducing the secretion of neurotoxic cytokines represent a novel approach for MS treatment. We further investigated the effect of MP on astrocytes and microglia in vivo. Consistent with these reports, WB analysis showed a significant increase in GFAP expression (Figure 3 (A)) and Iba-1 expression (Figure 3 (B)) in EAE group, while treatment with MP significantly reduced their expression levels. These data indicate that MP can inhibit the microglial activation as well as astrocytes reactivity in vivo. Subsequently, upregulation of the inflammatory mediator iNOS (M1 marker) and a decrease in the anti-inflammatory mediator Arg-1 (M2 marker) were observed in the spinal cord of mice with experimental autoimmune encephalomyelitis (EAE), compared to the control and Pre-EAE group (Figure 3(C)). During the progression of EAE, microglia release proinflammatory enzymes (iNOS) which may contribute to CNS damage. After MP treatment, microglia secrete anti-inflammatory enzymes (Arg-1), potentially contributing to resolution of inflammation and tissue repair in the CNS.

To further investigate the clinical significance of our findings, we tested the effect of MP on differentiation of Tfr and Tfh cells in EAE mice. Tfr and Tfh cells are two subsets of lymphocytes with reciprocal regulatory functions involved in autoimmune diseases[41]. PD-1 serves as a key phenotypic marker for Tfh cells by regulating long-term persistence and enhancement of memory B cells and plasma cells through survival and refinement processes at the GC while promoting IL-21 secretion[42]. ICOS is a CD28 family member costimulatory molecule expressed on T cells that upregulates CXCR5 expression on Tfh cell surfaces to promote their differentiation. Both PD-1 and ICOS expression can be used as indicators for active Tfh cell differentiation[2, 18, 26]. In this study, we observed an elevation in the percentage of PD-1 and ICOS in CD4+CXCR5+Foxp3-Tfh cells during EAE progression, however, this increase was reversed following MP therapy. On the other hand, Tfr cells can be distinguished from Tfh cells based on the FoxP3, CD25, and GITR expressions[43, 44], where they inhibit the expansion and activity of Tfh and B cells in GC in a CTLA-4-dependent manner[45]. We presented strong evidence showing a deficiency in Tfr cells from the spleen in untreated EAE and the reversal of this deficiency following MP therapy (Figure 5). Subsequently, our study demonstrated dysregulation of CD4+CXCR5+Foxp3+Tfr and CD4+CXCR5+Foxp3-Tfh cells ratio during the progression of EAE mice. We observed that MP treatment effectively restored the Tfr/Tfh imbalance in the spleen of EAE mice (p<0.05, Figure 4 (B)). Therefore, maintaining a balanced Tfr/Tfh ratio may serve as a potential therapeutic target and a valuable prognostic tool. Furthermore, a negative correlation between the Tfr /Tfh and IL-21 levels, as well as a positive correlation with TGF-β1[46]. In this study, MP treatment inhibited the expression of Tfh-related molecules (PD-1, ICOS) and pro-inflammatory factors (IL-21), while enhancing the expression of Tfr-related molecules (Foxp3) and anti-inflammatory factors (TGF-β1, IL-10) in spinal cord of EAE mice (p<0.05, Figure 6).
The PI3Ks are a group of lipid kinases that exist as heterodimers and can be classified into three classes based on different stimuli[47]. AKT, belonging to the protein kinase AGC subfamily, functions as an indispensable downstream effector of PI3K[48]. The PI3K/AKT pathway has been reported to play an important role in pro-inflammatory factor production and release[49]. Increased activity of the PI3K/AKT pathway has been observed in autoimmune diseases such as rheumatoid arthritis[50]. FoxO1 and mTOR are downstream target proteins regulated by AKT activation. Activation of AKT phosphorylates Foxo1 leading to its translocation from nucleus to cytoplasm which inhibits nuclear membrane transport and transcriptional activity[51]. The PI3K/AKT/mTOR pathway drives differentiation pathways for CD4+T cells through its downstream signaling cascades, where mTOR activation promotes differentiation towards Tfh cells but inhibits generation of Tfr cells[52]. Consistent with the aforementioned reports, our study also observed an increase in phosphorylation of AKT, FoxO1, and mTOR in the spinal cord of EAE mice. In summary, activation of the PI3K/AKT signaling pathway and increased phosphorylation of FoxO1 and mTOR may play important roles in EAE progression. However, after MP treatment, there was a decrease in the phosphorylation level of this pathway ($p<0.05$, Figure 7).

Our findings demonstrate that imbalance of Tfr and Tfh cells and Reactivation of the PI3K/AKT pathway may potentially contribute to the pathogenesis of EAE. Furthermore, MP not only ameliorated inflammatory infiltrates and demyelination but also regulated Tfr/Tfh cell differentiation by preventing AKT, mTOR and FoxO1 from phosphorylating.

**Conclusion**

In light of the above findings, therapeutic efficacy exhibited by MP treatment confirmed a notable decrease in the size of demyelination, inflammation, and infiltration of immune cells, as well as activation of microglia and astrocytes. Moreover, MP upregulated the differentiation of Tfr cells while downregulating Tfh cells in EAE mice as well as imbalanced Tfr/Tfh-type cytokines (TGF-$\beta$1 and IL-21). Mechanistically, MP may regulate Tfr and Tfh cells by inhibiting PI3K/ AKT/FoxO1 and PI3K/AKT/mTOR signaling pathways.

**Declarations**

**Conflicts of Interest**

The authors declare that they have no conflicts of interest.

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**Author Contribution**

N and F wrote the main manuscript text and other authors prepared all figures. All authors reviewed the manuscript.

**References**


**Figures**
Figure 1

MP reduced the clinical score of EAE.

(A) Daily fluctuations in clinical scores between the two cohorts throughout the course of the disease. (mean ±SEM; n = 8 each; * P < 0.05, ** P < 0.01, and *** P < 0.001).
(B) Analysis was conducted on the maximal scores, cumulative scores, and mean scores as well as onset day for both the EAE group and MP group. (n = 8 each; * P < 0.05, ** P < 0.01, and *** P < 0.001).

Figure 2

MP reduced the CNS inflammation and demyelination of EAE Mice.

(A) Inflammatory infiltration observed through H&E staining with corresponding inflammation scores.
(B) Demyelination was detected by LFB staining with corresponding demyelination scores.

**Figure 3**

MP inhibited microglia and astrocyte activation in EAE mice.
(A)-(B) The expression of the anti-GFAP and anti-Iba-1 protein was detected by immunofluorescence staining in lumbar spinal cords of P-EAE, EAE+MP groups.

(C) The protein expression levels of GFAP, Iba-1, iNOS, and Arg-1 were investigated in lysates from lumbar spinal cords using Western blot analysis. Statistical significance was determined as * P < 0.05, ** P < 0.01, and *** P < 0.001.
Figure 4

Confocal photomicrographs of Tfr cell localization in spleen characterized by the distinct density of CD4 T cells (red (A)), CXCR5 (blue (B)), Foxp3 (green (C)), and Merge (D) and Tfh cell localization in spinal cord characterized by the distinct density of CD4 T cells (red (A)), CXCR5 (blue (B)), and Merge (D).

Figure 5
Flow cytometry analysis of different subsets of spleen Tfr and Tfh cells in the control, Pre-EAE, P-EAE, and MP groups.

(A) The percentage of CD4+CXCR5+Foxp3+Tfr and CD4+CXCR5+Foxp3-Tfh cells in the spleen of EAE mice in these four groups.

(B) The percentages of CD4+CXCR5+Foxp3-ICOS+, CD4+CXCR5+Foxp3-PD-1+ Tfh cells in the spleen of EAE mice in these four groups.
Figure 6

The EAE mice were classified into the control group, the Pre-EAE group, the P-EAE group and the EAE+MP group. Representative blots band of CXCR5, PD-1, iCOS, TGF-β1, FoxP3, IL-21 in spinal cord of EAE mice were analyzed.
Figure 7

MP relieved EAE through the PI3K/AKT/mTOR and PI3K/AKT/FOXO1 signaling pathways.

(A) Western blotting analysis of total PI3K, p-AKT, total AKT, p-FoxO1, total FoxO1, p-mTOR, and total mTOR.
(B) Quantitative analysis revealed decreased levels of expression for PI3K, p-AKT, p-FoxO1 and p-mTOR in the spinal cord following MP treatment.