

Article

Oral Administration of Myelin Oligodendrocyte Glycoprotein Attenuates Experimental Autoimmune Encephalomyelitis through Induction of Th2/Treg Cells and Suppression of Th1/Th17 Immune Responses

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Abstract: Multiple sclerosis (MS) is a demyelinating autoimmune disorder of the central nervous system (CNS). Experimental autoimmune encephalomyelitis (EAE) has been widely used to determine the pathogenesis of the disease and evaluate new treatment strategies for MS. Therefore, we investigated the efficacy of oral administration of a Myelin Oligodendrocyte Glycoprotein (MOG) in the treatment of EAE. Female C57BL/6 mice were utilized in three groups (Control group: received PBS orally; prevention group: oral administration of MOG35-55 two weeks before EAE induction; treatment group: oral administration of MOG35-55 after EAE induction. MOG administration, both as prevention and treatment, significantly controlled clinical score, weight loss, CNS inflammation, and demyelination, mainly through the modulation of T cell proliferation, reduction of pro-inflammatory cytokines and transcription factors, including TNF- α , IFN- γ , IL-17, T-bet, and ROR- γ t. MOG administration, both as prevention and treatment, also induced anti-inflammatory cytokines and transcription factors, including IL-4, TGF- β , GATA-3, and Foxp3. The results showed that oral administration of MOG, both as prevention and treatment, could efficiently control EAE development. Immunomodulatory mechanisms include the induction of Th2 and Treg cells and the suppression of pro-inflammatory Th1 and Th17 cells.

Keywords: Multiple Sclerosis; Experimental autoimmune encephalomyelitis; Myelin Oligodendrocyte Glycoprotein; Immunomodulatory mechanisms

1. Introduction

Multiple sclerosis (MS) as a prototypical chronic inflammatory disorder of the central nervous system (CNS) is characterized by demyelination with the axonal transaction, which affects more than 2.8 million people worldwide and 29.3 per 100,000 people in Iran (1,2). The multifactorial etiology of MS is mainly caused by the cooperation of hereditary and environmental aspects in a complex manner. The formation of focal plaques around post-capillary venules and disruption of the blood-brain barrier (BBB) are the hallmarks of MS pathogenesis (3). Although the mechanisms of BBB breakdown remain elusive, the production of pro-inflammatory cytokines and chemokines, including TNF- α , IL-1, and IFN- γ by endothelial cells and self-reactive Th1 and Th17 plays a pivotal role in the initiation and perpetuation of CNS inflammation (4,5). Th2/Treg cell-associated cytokines,

such as IL-4, IL-10, and TGF- β have been determined to be accompanied by a diminution of inflammation and recovery of manifestations. However, the abnormalities of Treg cells, such as reduced expression of Foxp3, may lead to the aberrant effector CD4⁺ and CD8⁺ T cells function, that traffic to the CNS parenchyma and cause glial cell stimulation, perivascular demyelination, and axonal damage (6).

Experimental autoimmune encephalomyelitis (EAE) has been widely used for addressing MS pathogenesis and evaluating new treatment strategies for MS. EAE is an excellent model for many features of MS with resembling histopathological, immunological, and clinical properties (7). Induction of EAE is mediated through the activation of myelin-specific CD4⁺ T and CD8⁺ T cells that migrate to the CNS and produce the pro-inflammatory cytokines in response to the recognition of myelin peptides on the local APCs, resulting in oligodendrocyte damage and axonal demyelination (8,9).

Over time, numerous innovative modifying therapies have been developed for MS treatment that mainly focused on the modulation of the immune system through the decrease of Th1 and Th17 activation, induction of Th2 and Treg differentiation, and immunomodulatory cytokines production, such as IL-10 and TGF- β , reduction of BBB permeability, and suppression lymphocyte migration to the CNS (10,11). But none of them is responsible for effectively breaking neuroinflammation and degeneration of disease and only alleviates clinical relapses and inhibits the other lesion formation (12). Moreover, a variety of side effects, including systemic immediate post-injection reaction, increased risk of infections, cytokine storm, gastrointestinal symptoms, thyroid dysfunction, cardiotoxicity, and irregularities in liver enzymes have been reported regarding these therapies (13). However, an improved understanding of the immunological process of the disease led to a suggestion of antigen-specific tolerance induction, using myelin proteins, such as myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG), in both animal models and MS patients (14,15). Oral immunotherapy is an effective method for preventing and treating undesired immune responses that cause a range of illnesses or immune-related problems. However, the underlying cellular and molecular mechanisms are not fully understood. The gut immune system generates immune signals that can modify the systemic immune response pattern. Oral immunotherapy is a promising method for targeting the systemic immune system in autoimmune and inflammatory diseases, such as EAE (16). A recent study showed reduced progression of encephalomyelitis symptoms in MS patients treated with MOG-modified liposomes encapsulating doxorubicin (17). Moreover, the combined administration of MOG35-55 associated with paricalcitol, a synthetic vitamin D receptor activator, controlled disease development and reduced clinical score in EAE (18). It has also demonstrated that transdermal application of a mixture of myelin peptides containing MBP85-99, MOG35-55, and PLP139-155 could ameliorate the severity of disease in relapsing-remitting MS patients (10). The effectiveness of these strategies was associated to reduce the number of Th17 cells and concomitantly Treg cell expansion.

In this study, we evaluated the oral administration of MOG35-55 peptide for the prevention and treatment of EAE in C57BL/6 mice.

2. Results

2.1. Oral administration of MOG inhibits the development of EAE in C57BL/6 mice

Oral administration of endogenous peptides was efficient in treating cell-mediated autoimmune conditions, including rheumatoid arthritis. As a result, MOG was also regarded as a candidate for both prevention and treatment of MS in an animal model. MOG assessment with different administration times in EAE-induced mice was not studied previously. This study aimed to establish the most effective MOG administration strategy for

the amelioration of EAE. The mice were randomly separated into three groups, namely the control, prevention, and treatment groups.

Prevention and treatment with MOG groups demonstrated reduced severity of the disease. MOG administration in both prevention and treatment groups significantly reduced the degree of impairment and paralysis, when compared to the control group. Moreover, the prevention group greatly reduced the disease's symptoms.

The clinical scores (on day 18, maximal score) of the prevention group with MOG (1.9 ± 0.13) and treatment group with MOG (2.5 ± 0.14) were significantly ($p < 0.001$) lower than the control group (on day 17, 4.3 ± 0.19) (Figure 1a and Table 1). Prevention and treatment with MOG also significantly reduced weight loss in EAE mice. The mean body weight of the prevention and treatment groups on day 18 was 20.7 ± 0.14 g ($p < 0.01$) and 19.8 ± 0.14 g ($p < 0.05$), respectively, compared to the control group with 18.8 ± 0.12 g (Figure 1b). As expected, the EAE control mice without any treatment developed clinical symptoms, which reached a maximum score on day 17 post EAE induction. In contrast, EAE mice receiving MOG showed milder symptoms of the disease.

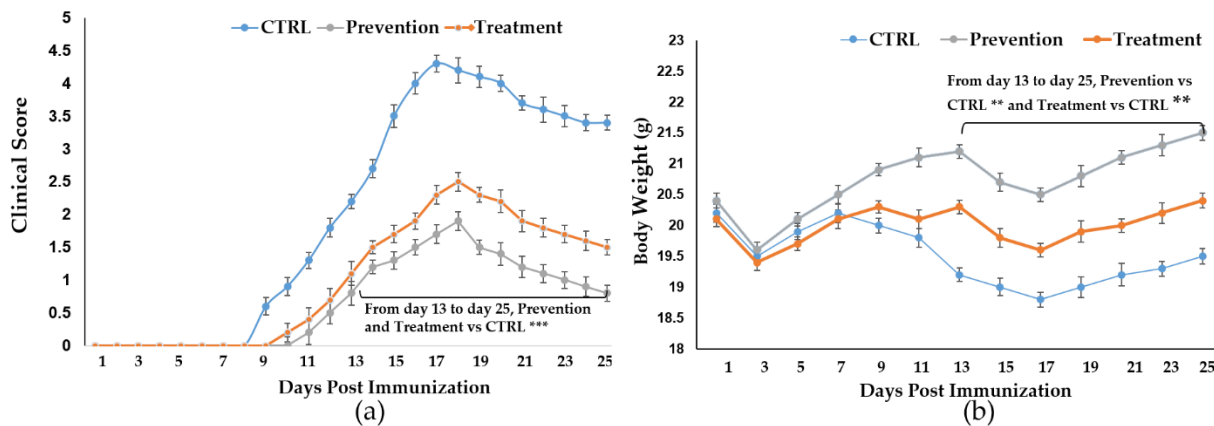


Figure 1. Oral administration of MOG inhibited the development of EAE in C57BL/6 mice. Prevention group received 250 μ g of MOG35-55 orally two weeks before EAE induction until 14 days after EAE induction, every day, and treatment group received MOG35-55 orally after EAE induction until 14 days post-immunization. Mice were monitored for signs of EAE, and the results for all mice, were presented as (a) mean clinical score; and (b) body weight. Results were expressed as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with control group. Mice were divided into three groups: 1. Control group (CTRL); 2. Prevention group with MOG; 3. Treatment group with MOG.

Table 1. Clinical features of EAE in the administration of MOG, both as prevention and treatment

| Group | Day of onset | Maximal score (Score at peak) | Mean score (Last Day) | Cumulative (CDI) | Disease Index |
|-------------------------|---------------------|----------------------------------|--------------------------|-----------------------|------------------|
| CTRL ¹ | 9.5 ± 0.4 | 4.3 ± 0.13 | 3.4 ± 0.11 | 51.2 ± 1.27 | |
| Prevention ² | $11.2 \pm 0.3^{**}$ | $1.9 \pm 0.14^{***}$ | 0.8 ± 0.12 | $17 \pm 0.61^{***}$ | |
| Treatment ³ | $10.6 \pm 0.4^*$ | $2.5 \pm 0.14^{***}$ | $1.5 \pm 0.12^{***}$ | $25.3 \pm 0.78^{***}$ | |

1. CTRL: Control group EAE induced received PBS

2. Prevention: Prevention group with MOG

3. Treatment: Treatment group with MOG

Data were expressed as mean \pm SEM.

All experiment groups compared with CTRL group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

2.2. MOG administration ameliorates CNS inflammation and demyelination

Brain sections ($5\mu\text{m}$ thick) were prepared and stained with hematoxylin and eosin (H&E) for cell infiltration, and Luxol fast blue (LFB) for demyelination. H&E staining revealed a significant reduction of CNS infiltrating leukocytes in the prevention group (1.1 ± 0.13) and the treatment group (1.4 ± 0.16), when compared with the control group (2.7 ± 0.18 , $p < 0.001$; Figure 2a and c). LFB staining showed reduced demyelination in the areas of the brain during the development of disease in the prevention and the treatment groups, respectively (1.2 ± 0.14 , 1.5 ± 0.12), when compared with the control group (2.8 ± 0.17 , $p < 0.001$; Figure 2b and 2c).

Cell infiltration in the brain and demyelination in the prevention and treatment groups notably decreased, when compared to the control group. The findings showed that MOG treatment significantly reduced the inflammatory cell infiltration and demyelination in prevention and treatment groups in comparison with the control group. These results showed that MOG administration offered extensive protection for EAE mice.

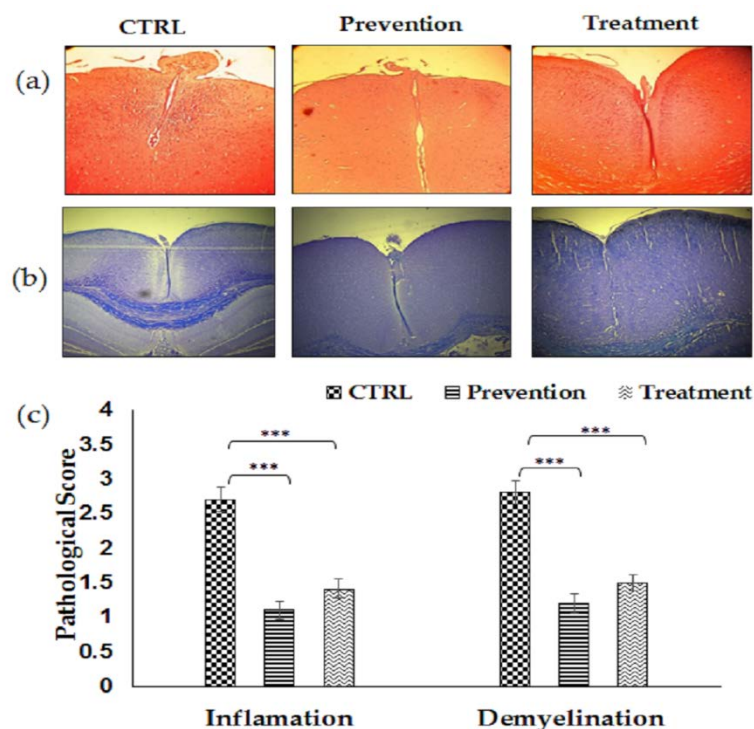


Figure 2. MOG administration ameliorates CNS inflammation and demyelination. Histopathological evaluation of CNS from all the mice was performed. Brain sections from each group were collected on day 25 post-immunization, fixed in paraformaldehyde, and embedded in paraffin. Five μm sections from different regions of the brain from each of the groups were stained with (a) Hematoxylin and eosin, to enumerate infiltrating leukocytes, and (b) Luxol fast blue to assess demyelination. (C) CNS inflammatory foci and infiltrating inflammatory cells were quantified. Pathological scores, including inflammation and demyelination, were analyzed and shown as mean scores of pathological inflammation or demyelination \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, when compared with control group. Mice were divided into three groups: 1. Control group (CTRL); 2. Prevention group with MOG; 3. Treatment group with MOG.

2.3. Oral administration of MOG suppress T cell proliferation

The cell proliferation assay of T-cells extracted from the lymph nodes and spleen was done by a Cell Proliferation ELISA, BrdU (colorimetric) kit. Cultivation of samples was carried out with MOG (20 $\mu\text{g/mL}$) and PHA (20 $\mu\text{g/mL}$) as a positive control. Reduced inflammation demonstrated by histopathological evaluation was further confirmed by suppression of T cell proliferation. Cell proliferation rates of all sample groups with PHA were similar. Splenocytes of the prevention and the treatment groups had significantly lower proliferative capability, when compared with the control group ($p < 0.001$; Figure 3).

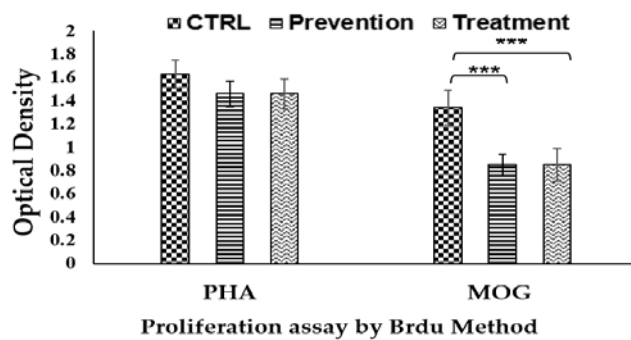


Figure 3. Oral administration of MOG suppresses T-cell proliferation. Splenocytes and lymph nodes were harvested on day 25 post-immunization and cultured in PHA (20 $\mu\text{g/mL}$), as a positive control, or with MOG (20 $\mu\text{g/mL}$) for 72h on 96-well plates. Proliferation responses were tested using a Cell Proliferation ELISA, BrdU (colorimetric) kit (Roche Applied Science, Indianapolis, USA). The proliferation assay was done in triplicates. Data presented as mean optical density \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, when compared with control group. Mice were divided into three groups: 1. Control group (CTRL); 2. Prevention group with MOG; 3. Treatment group with MOG.

2.4. Administration of MOG suppresses pro-inflammatory and enhanced anti-inflammatory cytokines

Cytokine milieu was evaluated in the spleens and lymph nodes of all mouse groups. Cytokine production in lymph nodes and spleens was determined for cytokine expression by culturing 72 hours lymph node cells and splenocytes isolated from mice, and then collecting supernatants.

To determine whether anti-inflammatory cytokines contributed to the suppression of clinical disease observed during administration of MOG, production of IL-4, IL-10, and TGF- β by splenocytes and lymph node cells was examined. IL-4 was found to be produced in significantly larger amounts in both the prevention and the treatment groups, respectively (164 ± 11 , 147 ± 10 , $p < 0.01$), when compared to the control group (79 ± 9 ; Figure 4a). IL-10 (132 ± 12 , 124 ± 11 , $p < 0.01$) and TGF- β (2034 ± 89 , 1984 ± 81 , $p < 0.001$) were significantly upregulated in the prevention and the treatment groups, respectively, when compared to the control group (Figure 4a). These results suggest that administration of MOG, upregulated Th2 and Treg differentiation, and could modulate Th1 and Th17 cytokine secretion.

The prevention and treatment groups produced lower levels of IFN- γ (972 ± 48 , 1094 ± 34 , 3752 ± 63) TNF- α (1123 ± 59 , 1286 ± 64 , 3845 ± 71), and IL-17 (827 ± 47 , 892 ± 56 , 7193 ± 34), when compared to control group, respectively (Figure 4b). These data indicate that prevention and treatment with MOG result in significantly ($p < 0.001$) reduced production of pro-inflammatory cytokines.

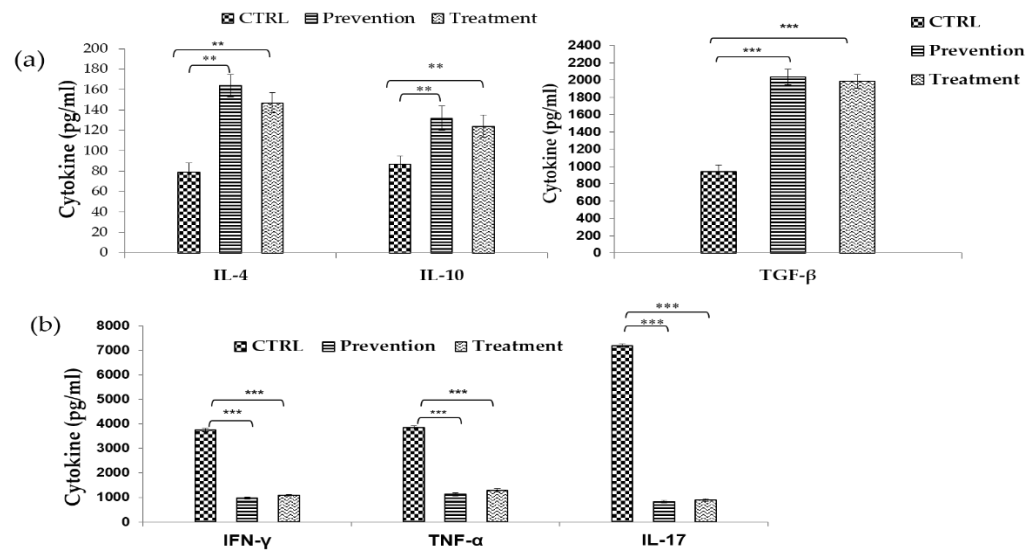


Figure 4. Oral administration of MOG suppressed pro-inflammatory cytokines production and enhanced anti-inflammatory cytokines production in splenocytes and lymph nodes from EAE mice. Splenocytes and lymph nodes from immunized mice from all groups were isolated on day 25 post-immunization and re-stimulated with MOG35–55 (20 µg/mL) for 72 h. Cell culture supernatants were collected and indicated cytokine levels were measured by ELISA. Cytokine assays were conducted in duplicate wells. (a) Anti-inflammatory cytokines IL-4, IL-10, and TGF-β; (b) pro-inflammatory cytokines IFN-γ, TNF-α, and IL-17 released by splenocytes in culture, ± SEM. Results from lymph nodes were similar to splenocytes (not shown). *p<0.05, **p< 0.01, ***p<0.001, when compared with control group. Mice were divided into three groups: 1. Control group (CTRL); 2. Prevention group with MOG; 3. Treatment group with MOG.

2.5. Administration of MOG upregulated expression of cytokines and transcription factors in Th2 and Treg, and downregulated in Th1 and Th17

Quantitative real-time PCR was used to measure the mRNA expression levels of T cell-related cytokines and transcription factors, to assess the infiltration of activated T cells, and define T helper polarization in the CNS microenvironment. The prevention and the treatment groups had lower expression of Th1 and Th17 cytokines and transcription factors (IFN-γ, T-bet, IL-17, ROR-γt), when compared to the control group on day 25 following EAE induction (Figure 5a). Additionally, prevention and treatment groups expressed more Th2 and Treg cytokines and transcription factors (IL-4, GATA3, TGF-β, Foxp3), when compared to the control group (Figure 5b). These alterations in cytokines and transcription factors confirmed cytokine ELISA results taken from peripheral lymphoid organs, including spleen and lymph nodes.

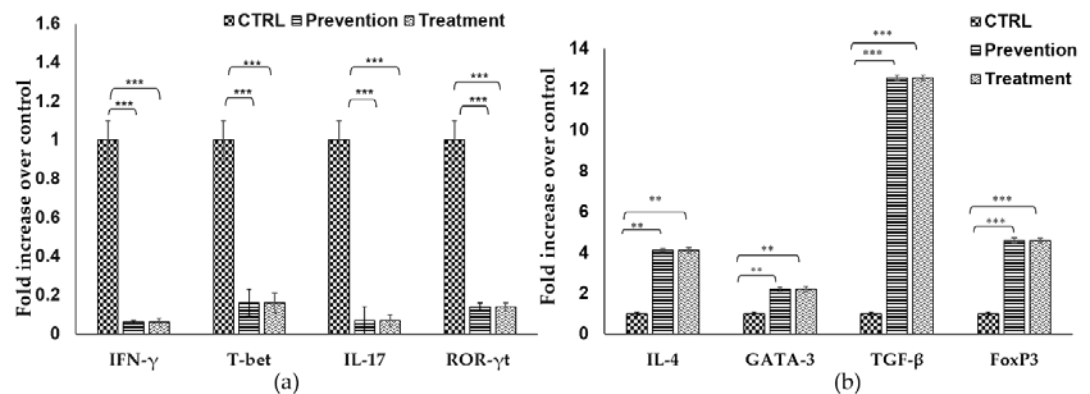


Figure 5. Gene expression of cytokines and transcription factors in CNS. On day 25 post-immunization, brains and spinal cords were collected, and mRNA levels of cytokines and transcription factors were assessed by real-time quantitative PCR. The assay was run in triplicates, fold change expression of genes was determined, and then compared to the control group. (a) Th1- and Th17-related cytokines and transcription factors IFN- γ , T-bet, IL-17, and ROR- γ t; (b) Th2- and Treg-related cytokines and transcription factors IL-4, GATA3, TGF- β and FoxP3. Results were expressed as fold change normalized to the control group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Mice were divided into three groups: 1. Control group (CTRL); 2. Prevention group with MOG; 3. Treatment group with MOG.

3. Discussion

The concept of utilizing oral tolerance to control autoimmune and allergy diseases by administering target antigens orally was a significant breakthrough that was successfully tested in the 1980s. Therefore, Antigen-specific immunotherapy which has been used for allergen immunotherapy, is considered a promising approach to treat autoimmune diseases, such as MS, mainly through induction of Treg, to promote auto-antigen-specific tolerance. Several experiments since then have demonstrated that feeding particular antigens may be utilized to prevent and treat chronic inflammatory disorders in both animal models and clinical settings [24].

Administration of self-myelin proteins, such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), and proteolipid protein (PLP) that are predominantly involved in the pathology of MS, at low concentrations might lead to immune tolerance. However, the selection of the appropriate administration route is a key point for the successful implementation of immune tolerance. Oral immune therapy promoting Treg is one potential mechanism for the suppression of systemic inflammation at target organs via an effect on gut mucosal surfaces [25].

In this study, we evaluated the therapeutic potential of MOG₃₅₋₅₅ peptide administered by oral route for treatment of EAE in C57BL/6 mice. In this context, the therapeutic effect of MOG delivered by oral route before and after EAE induction was compared with non-treated EAE mice as the control group. Administration of myelin peptides through the skin, transdermal, and epicutaneous routes was well tolerated by patients with relapsing-remitting MS and markedly reduced disease activity [10, 18, 26]. However, oral tolerance is one of the most common strategies for the suppression of pathologic auto-reactivity immune responses against self in autoimmune disorders.

Multiple mechanisms are involved in oral tolerance, including induction of Th2 and Treg, along with bystander suppression of Th1 and Th17 [27]. In this sense, our results demonstrated that oral administration of MOG efficiently controlled EAE development. These findings were recently validated; it was demonstrated that administration of MOG

and MOG plus paricalcitol in an epicutaneous route significantly reduced EAE development and ameliorated clinical symptoms [18]. However, considering that immune-mediated destruction of CNS myelin and oligodendrocytes is a cause of self-tolerance disruption and immune attack, we investigated whether our therapeutic strategy resulted in suppressing the immune attack.

As confirmed by previous studies, prevention and treatment with MOG showed neuroprotective properties and ameliorated CNS inflammation and demyelination. Jiang *et al.* revealed that although intraventricular MOG-treated EAE mice showed a normal CNS cell morphology, demyelination and extensive inflammatory infiltrates were observed in the white matter of spinal cords of PBS-treated EAE mice [28]. Moreover, Duraes *et al.* showed that transferring the MOG₃₅₋₅₅-loaded plasmacytoid DC led to the reduction of histological spinal cord inflammatory foci and decreased demyelination [29]. We demonstrated that this protective effect was mediated by suppression of T cell proliferation, as confirmed by *in vitro* results. In the current study, we induced immune tolerance in EAE mice by daily administration of MOG peptide in the oral route. The investigation of cytokine profile and transcription factors associated with T cells revealed a significant decrease in levels of pro-inflammatory cytokines. We found that oral antigen-induced immune tolerance is associated with suppression of ROR- γ t expression and IL-17 production. IL-17 with potent pro-inflammatory effects is expressed by Th17 and has an elevated level in MS. Langrish *et al.* demonstrated that neutralization of IL-17 ameliorated clinical symptoms in MS [30].

Our results were accompanied by a reduction in the levels of IFN- γ and TNF- α , besides a decrease in T-bet expression upon MOG treatment. However, there is conflicting evidence about the role of Th1 and its main cytokine, IFN- γ , in the pathogenesis of MS. Zorzella-Pezavento *et al.* observed that the IFN- γ levels increased in MOG-treated mice [18]. According to the classical model, IFN- γ as a pro-inflammatory cytokine led to induction and progression of both, EAE and MS. Moreover, IFN- γ has an important role in the migration and function of Th17 cells. Further, it has been demonstrated that IFN- γ and IL-17 double-producing T cells are the main effector cells in EAE induced by MOG₃₅₋₅₅ [28, 31]. Despite these results, Voorthuis *et al.* showed that intraventricular injection of IFN- γ in EAE mice relieved the disease symptoms and reduced morbidity and mortality [32]. Moreover, IFN- γ -deficient animals showed an increased incidence of EAE, earlier disease onset, and severe symptoms, when compared to the control mice [33]. However, disease stage-specific properties might be considered for the opposing role of IFN- γ in EAE/MS, as augmentation of pathogenesis during the initiation phase and immunosuppression in the effector phase [34]. Furthermore, IL-4 and IL-10, which are known to modulate IL-17 pathogenicity [35, 36], were also observed in tolerated mice in the current study.

It has been well understood that immune regulation in autoimmune diseases involves both the target organs and the secondary lymphoid tissues. We showed the upregulation of FoxP3 expression, and together with GATA-3, it was associated with an increase of IL-4 and TGF- β expression upon prevention and treatment with MOG. Consistent with our results, McGeachy *et al.* reported a positive correlation between the accumulation of IL-10-producing Treg cells within the CNS and recovery of patients with MS [37]. Moreover, Etesam *et al.* showed a significant diminish in the T-bet/GATA-3 expression ratio in MOG-stimulated peripheral blood mononuclear cells (PBMCs) of MS patients [38]. Our results also revealed that oral administration of MOG resulted in increased expression of TGF- β , a cytokine with the ability to induce Treg cells in autoimmune diseases mediated by Th17 [39]. TGF- β plays a regulatory role mainly through the suppression of Th1 differentiation and IFN- γ production, induction of Treg and Th2 differentiation, and regulation of Th17/Treg balance in MS [40]. In line with it, Zorzella-Pezavento *et al.* showed that TGF- β levels were increased in MOG-treated EAE mice [18].

In conclusion, considering the protective effect of MOG₃₅₋₅₅ peptide when orally administered in EAE mice, our results demonstrated that MOG was highly effective to control EAE development, mainly through the prevention of proliferation and function of

effector T cell subsets and their mediators, along with the induction of Treg cells. Oral immune therapy is an attractive clinical approach to treat autoimmune and inflammatory disorders. It can induce immune modulation without immune suppression, has minimal toxicity, and is easily administered. Targeting the systemic immune system via the gut immune system can serve as an attractive novel therapeutic method for MS. The possibility that this protective effect is being approached was mentioned in previous studies, however, an extension of this knowledge to MS patients needs further preclinical studies.

4. Materials and Methods

4.1. Animals

Female C57BL/6 mice (8-10 weeks old) were obtained from the Royan Institute for Biotechnology (Isfahan, Iran), and kept in specific pathogen-free conditions (at $23\pm 2^{\circ}\text{C}$ with a $50\pm 5\%$ relative humidity and a 12 h light/dark periods). Mice were randomly allocated in cages (maximum of four animals per cage) with sterilized food and fresh water ad libitum.

4.2. EAE induction and Treatment

The mice were subcutaneously injected at the flanks, with $250\mu\text{g}$ synthetic MOG₃₅₋₅₅ (BioBasic, Canada), which was emulsified with Complete Freund's Adjuvant (Sigma Aldrich, St. Louis, MO, USA) containing 4mg/mL of Mycobacterium tuberculosis H37RA (Difco Laboratories, Detroit, MI, USA). Mice also received 250ng Pertussis toxin (Sigma-Aldrich, St. Louis, MO, USA) intraperitoneally, both simultaneously with EAE induction and 48 hours later. Mice were randomly separated into three groups: control, Prevention group, and Treatment group. The control group received PBS (Sigma-Aldrich) orally, the prevention group received $250\mu\text{g}$ of MOG₃₅₋₅₅ orally two weeks before EAE induction and up to 14 days after EAE induction every day, and the treatment group received MOG₃₅₋₅₅ orally after EAE induction and up to 14 days post-immunization.

Daily monitoring of clinical score and body weight were performed up to 25 days according to the following criteria: 0, no detectable symptom; 1, partial limp tail; 2: complete limp tail; 3, hind legs, weakness or abnormal gait; 4, complete hind limb paralysis; 5, complete hind limb paralysis and forelimb paresis; 6, complete hind and foreleg paralysis; 7: moribund or death [19, 20].

Moreover, body weight change was determined based on the weight change at the time of EAE induction until 25 days post-immunization. Mice were as evaluated for incidence, the beginning day of disease, mean score (at the latest day), maximum score (at the peak day), and Cumulative Disease Index (entire disease score over experiment duration). On day 25 post-immunization, mice were euthanized by ketamine/xylazine and bled by cardiac puncture, and then their brains, spleens, spinal cords, and lymph nodes were collected. The blood was allowed to clot and centrifuged, and the sera were collected and kept at -80°C , until the cytokine analysis was performed.

4.3. Histopathology

The mice were euthanized by decapitation, brain tissues were removed, fixed in 4% paraformaldehyde overnight at room temperature, and embedded in paraffin. Subsequently, $5\mu\text{m}$ sections of samples were prepared using standard microtome HM355S (Microm,

Walldorf, Germany) and stained with hematoxylin and eosin (H&E) for valuation of inflammation or Luxol Fast Blue (LFB) for demyelination. All slides were coded and read in a blinded manner [21]. The area of LFB-stained sections of photographed images (Axio-plan 2, Zeiss, Cologne, Germany) was measured by Fiji/ImageJ 1.46j software (NIH, Bethesda, US) for quantitative analysis of demyelination, and the area of demyelination was calculated as a percentage of the white matter area within a given section. Sections were assessed as follows; 0, no inflammation; 1, a few inflammatory cells; 2, presence of perivascular infiltrates; 3, spreading intensity of perivascular cuffing with extension into contiguous tissue [22]. Moreover, demyelination was scored as follows: 0, no demyelination; 1, unique foci (few sections with demyelination); 3, appreciable numbers of pieces with demyelination [23].

4.4. BrdU proliferation assay

Single-cell suspensions of lymph nodes and spleen of mice were prepared under sterile conditions, on day 25 post-immunization. Primed lymphoid populations passed through a 40µm cell strainer followed by ammonium chloride-based erythrocyte lysis (BD Biosciences, Germany). Subsequently, single-cell suspensions were prepared in a complete medium consisting of RPMI-1640 medium, 10% fetal bovine serum (FBS), 100 IU penicillin/ml, and 100µl streptomycin/ml (all reagents given by Sigma, St. Louis, MO). Cells were then located into round-bottom 24-well and 96-well plates respectively 2×10^6 cells and 2×10^5 cells to each well and incubated for 72 hours at 37°C and 5% CO₂. To stimulate the cells, MOG₃₅₋₅₅ peptide (20µg/ml) and PHA (20µg/ml) were added. Throughout the last 24 hours, the cells were cultured in the presence of a BrdU-labeling solution (Roche Applied Sciences). Afterward, proliferation was measured using the Roche Cell Proliferation ELISA BrdU kit, conferring to the manufacturer guidelines. The final steps of this test were picked up in a Stat Fax 2100 Awareness microplate reader (Fisher BioBlock Scientific, Palm City, FL) at 450 nm.

4.5. Cytokine assay

IFN-γ, TNF-α, IL-17, IL-4, IL-10, and TGF-β concentrations in isolated cells were measured using an ELISA kit (Biosciences, San Diego, CA), as directed by the manufacturer. Isolated lymph nodes and splenocytes (2×10^5) were cultivated in full tissue culture media in 24 well round-bottom plates. Moreover, cells were stimulated with 20 µg/ml of MOG and 20 µg/ml of PHA for 72 hours in a 10% CO₂ incubator at 37°C. Subsequently, supernatants were collected and frozen at -70°C until used in enzyme-linked immunosorbent assay (ELISA) assays. Absorbance was measured at 450 nm using the microplate reader (Stat Fax 2100 Awareness, Phoenix, AZ, USA). The amounts of each cytokine in a sample were then estimated from standard curves in parallel using kit-provided recombinant cytokine standards.

4.6. Quantitative Real-time PCR

To evaluate the expression of IFN-γ, T-bet, IL-17, ROR-γt, IL-4, GATA-3, TGF-β, and FoxP3, spinal cord and brain were collected from mice on day 25 post-immunization and RNA was extracted with the TRIzol™ RNA isolation kit (Roche Diagnostics GmbH,

Mannheim, Germany). In turn, cDNA was synthesized using gene-specific Stem-loop reverse transcription and Oligo dT primers, according to the PrimeScript™ RT Kit recommendation (Takara Biotechnology, Otsu, Shiga, Japan). Real-time PCR reactions were performed using SYBR Green Mix (Ampliqon, Odense, Denmark) with appropriate primers (Table 1) by Step One Plus system (Applied Biosystems). For sample normalization, $\beta 2$ microglobulin ($\beta 2m$) was employed as an endogenous control. Relative quantification ($\Delta\Delta CT$) values were determined based on target gene expression adjusted to $\beta 2m$ levels, and the findings were given as fold-change compared to the control.

Table 2. Sequences of primers that were used in the study.

| Genes | Forward | Reverse |
|-----------------|---------------------|------------------------|
| IFN- γ | CCAAGTTTGAGGTCAACA | CTGGCAGAATTATTCTTATTGG |
| T-bet | TGTGGTCCAAGTTCAACC | CATCCTGTAATGGCTTGTG |
| IL-17 | CCTCAGACTACCTCAACC | CCAGATCACAGAGGGATA |
| ROR- γt | GGATGAGATTGCCCTCTA | CCTTGTCGATGAGTCTTG |
| IL-4 | CTGGATTCATCGATAAGC | GATGCTCTTTAGGCTTTC |
| GATA3 | CTGCGGACTCTACCATAA | GTGGTGGTCTGACAGTTC |
| TGF- β | CGGACTACTATGCTAAAGA | CTGTGTGAGATGTCTTTG |
| Foxp3 | CAGAGTTCTTCCACAACA | CATGCGAGTAAACCAATG |
| B2m | CCTGTATGCTATCCAGAA | GTAGCAGTTCAGTATGTTC |

4.7. Statistical analysis

Data were expressed as mean± standard deviation (SD). A one-way non-parametric analysis of variance (ANOVA; Kruskal–Wallis test) followed by a Bonferroni’s post hoc or an unpaired Student’s t-test was used for comparisons of clinical signs between groups. Statistical analysis was accomplished with SPSS software version 21 (Chicago, IL), and $P<0.05$ was considered significant.

4.8. Ethics statement

This study was approved by the ethical committee of Semnan University of Medical Sciences, Semnan, Iran (ID: 1407, IRCT: IR.SEMUMS.REC.1397.122), and was conducted following the Helsinki declaration.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: title; Table S1: title; Video S1: title.

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Informed Consent Statement: Informed consent was obtained from all individual participants for whom identifying information is included in this article.

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