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The PD-1/PD-Ls pathway is up-regulated during the suppression of experimental autoimmune encephalomyelitis treated by *Astragalus polysaccharides*

Yu Sun[a], Yuanya Jing[b], Mengwen Huang[b], Jinyun Ma[a], Xiaoyan Peng[a], Jinying Wang[a], Guoling Li[a], Xiaodong Cheng[a,b,*]

[a] *Yue-yang Hospital of Integrative Medicine, Shanghai University of Traditional Chinese Medicine, Shanghai 200437, China*
[b] *School of Life Sciences and Technology, Tongji University, Shanghai 200092, China*

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ABSTRACT

Multiple sclerosis (MS) is an inflammatory demyelinating disease of CNS. *Astragalus polysaccharides* (APS), the main active extract from *astragalus membranaceus* which is a kind of traditional Chinese medicinal herb, is associated with a variety of immunomodulatory activities. We have evaluated the therapeutic effects of APS in the animal model of MS, experimental autoimmune encephalomyelitis (EAE). It was found that APS could effectively alleviate EAE through inhibiting $MOG_{35–55}$-specific T cell proliferation and reducing the expression of proinflammatory cytokines, which is mediated by up-regulating the expression of PD-1/PD-Ls signaling pathway. Our results demonstrated that EAE could be suppressed significantly by APS administration. It indicated that APS might be a potential of developing innovative drug for the therapy of MS.

1. Introduction

Multiple sclerosis (MS) is a chronic autoimmune inflammatory demyelinating disease in the central nervous system (CNS). The main feature of MS includes demyelination, inflammation, gliosis, varying degrees of axonal deformation and progressive neurological dysfunction (Dendrou and Fugger, 2017). The etiology and pathogenesis are not yet clear, and there is no specific effective treatment up to now. Experimental autoimmune encephalomyelitis (EAE) is a commonly used animal model for MS research (Krishnamoorthy and Wekerle, 2009). During the progression of EAE, $CD4^+$T cell-mediated immune response plays a major role (Wan et al., 2018). Inflammation and demyelination in the CNS are associated with infiltrating T cells and local antigen presenting cells (APCs). Infiltrating T cells including Th1 cells and Th17 cells have been shown to have a pathogenic role in EAE (Kroenke and Segal, 2007; Kroenke and Segal, 2011). In addition, Th1 cells promote inflammation within the CNS and facilitate Th17 cell infiltration into EAE lesions (Califano et al., 2014). Programmed death-1 (PD-1) is a novel negative regulatory molecule and a member of B7-CD28 family (Agata et al., 1996). It has been confirmed to have the negative function of regulating T cells (Gibson et al., 2014). This molecule expresses on activated $CD4^+$ and $CD8^+$ T cells. It binds to two ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC), which express on APCs and bring into regulating T cells negatively (Sharpe et al., 2007).

During the recent years, many experiments have been performed on the effects of plant polysaccharides in treating different diseases and lots of results have been obtained. A double blind, multi-center, randomized phase IV study conducted by Wang et al. (2019) showed that the antioxidant and anti-inflammatory effects of Astragalus polysaccharides (APS) could alleviate cancer-related fatigue in these patients. Zhang et al. (2018) found that APS could also improve insulin sensitivity by enhancing glucose uptake. Yu et al. (2018) determined that a new type of Astragalus polysaccharide (APS4) significantly suppressed the proliferation of MGC-803 cells in a dose-dependent manner and induced cell apoptosis. de Oliveira et al. (2018) demonstrated that pectin polysaccharides fraction (RSBAL) could improve the symptoms of gastritis patients by maintaining gastric mucus and GSH levels to exert the gastric defensive mechanisms. In the recent studies, it was reported that persimmon polysaccharides were of anti-coagulant, antioxidant, and immune-stimulatory activities. The studies by Hwang et al. (2018) showed that persimmon polysaccharides exerted anti-osteoporotic effects by inhibiting osteoclast differentiation and improving trabecular bone loss. Acetaminophen induced hepatotoxicity could be relieved by Seabuckthorn berry polysaccharide via activating the Nrf-2/HO-1-SOD-2 signaling pathway (Wang et al., 2018). Zhang et al. (2017) found that angelica polysaccharides significantly inhibited the growth

* Corresponding author at: Yue-yang Hospital of Integrative Medicine, Shanghai University of Traditional Chinese Medicine, Shanghai 200437, China.
E-mail address: chengxiaodong@shutcm.edu.cn (X. Cheng).

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and proliferation of glioma cells and facilitated their apoptosis. Therefore, it is meaningful and significant to further investigate the abundant biological activities of plant polysaccharides in different diseases.

Astragalu spolysaccharides (APS) is an active extract isolated from one kind of traditional Chinese herb *Radix Astragali seu Hedysari*. Our previous studies found that *Astragalus* water extract could effectively ameliorate EAE symptoms and reduce the inflammatory response in the central nervous system (Sun et al., 2013). And APS was proved to have the activities of immunoregulation bidirectionally. In this study, we explored the therapeutic effects and immunological mechanisms of APS on EAE. It showed that APS could effectively ameliorate EAE symptoms and reduce the inflammatory response and demyelination in the CNS. It could inhibit $MOG_{35–55}$-specific T cell proliferation and proinflammatory cytokines secretion. And it also could up-regulate the expression of PD-1, PD-L1 and PD-L2 in both transcriptional and translational levels. It came to the conclusion that APS could effectively suppress EAE by inhibiting $MOG_{35–55}$-specific T cell proliferation and down-regulating cytokines of IFN-γ, TNF-α, IL-2 and IL-17. APS alleviates EAE through activating the PD-1/PD-Ls signaling pathway.

2. Material and methods

2.1. Animals

Female C57BL/6 mice were purchased from Shanghai Experimental Animal Center (Shanghai, China). Mice were housed in pathogen-free conditions. Mice in 8–9 weeks of age are prepared for EAE induction. The experiments were approved and conducted in accordance with the guidelines of the Animal Care Committee of Tongji University.

2.2. Reagents

Astragalus polysaccharide (APS) was purchased from Xi'an West Hongsheng Biotechnology Company (Xi'an, China) with purity 70%. $MOG_{35–55}$ (MEVGWYRSPFSRVVHLYRNGK) was purchased from GL Biochem (Shanghai, China) with purity > 98%. Complete Freund's adjuvant (CFA) was purchased from Sigma-Aldrich. *Mycobacterium tuberculosis* H37Ra (TB) was purchased from BD Biosciences. Pertussis toxin (PT) was purchased from Calbiochem. CCK-8 kit was purchased from Dojindo. Mouse ELISA Kit(IFN-γ, TNF-α, IL-2, IL-17, IL-4, IL-10) and mouse monoclonal antibody of PD-1, PD-L1, PD-L2 was purchased from R&D.

2.3. EAE induction and drug administration

Female C57BL/6 mice in 8–9 weeks of age were immunized subcutaneously with 200 μg $MOG_{35–55}$ in complete Freund's adjuvant containing heat-killed *Mycobacterium tuberculosis* (H37Ra strain; 5 mg/ml). Pertussis toxin 200 ng/mouse in PBS was administered intraperitoneally (i.p.) on day 0 and day 2 post-immunization. Mice were examined daily for disease signs by researchers blinded to experimental conditions and were assigned scores on a 0–5 scale (Stromnes and Goverman, 2006) as following: 0, no clinical signs; 1, paralyzed tail; 2, loss in coordinated movement; hind limb paresis; 3, both hind limbs paralyzed; 4, forelimbs paralyzed; 5, moribund state or death. For drug treatment, APS was given by oral administration 500 mg/(kg.d) since the three different dates, including day −7 before immunization (−7d), day 0 (0d) and day 7 (+7d) post-immunization. Water was given as vehicle control (200 μl/mouse.d).

2.4. Histopathological, immunohistochemistry and immunofluorescent assay

Mice were anesthetized and perfused with 0.9% saline and 4% paraformaldehyde. Spinal cord samples were then saved in 4% (*w/v*) paraformaldehyde overnight. Paraffin-embedded sections were prepared for HE and LFB staining for observation of infiltration of inflammatory cells and demyelination of spinal cord tissue. Frozen sections of spinal cord were stained with anti-PD-1 or PD-Ls antibodies, or with appropriate fluorescent-labeled secondary antibodies.

2.5. T cell proliferation assay

Single cell suspensions of monocytes (MNCs) from the spleen were prepared on day 11 (onset), day 16 (peak) and day 21 (remission) post-immunization. Cells were cultured at a cell density of 2×10^6 cells/well in RPMI 1640 in triplicate wells of 96-well plates. The cells were stimulated by adding 20 μg/ml of $MOG_{35–55}$ in the culture medium. After 68 h of incubation, each well was added 10 μl CCK-8. Cultured to 72 h, the absorbance at 450 nm was measured using a microplate reader.

2.6. Measurement of cytokine production

Single cell suspensions of MNCs from the spleen were prepared on day 11 (onset), day 16 (peak) and day 21 (remission) post-immunization. Cells were cultured at a cell density of 2×10^6 cells/well in RPMI 1640 in triplicate wells of 24-well plates. The cells were stimulated by adding 20 μg/ml of $MOG_{35–55}$ in the culture medium. After 48 h of incubation, supernatants were collected for ELISA assay of IFN-γ, TNF-α, IL-2, IL-17, IL-4 and IL-10 production.

2.7. Real-time PCR

Total RNA were extracted from splenocytes using RNA simple Total RNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. The RNA was used to reverse transcription using Fast Quant RT Kit (Tiangen) to gain cDNA. Real-time PCR was prepared with Super Real PreMix (Tiangen). Expression values were normalized to GAPDH.

2.8. Western blot

Spleen from EAE mice were washed with PBS, added lysis buffer and made tissue homogenates on ice. Protein concentration was determined by the Bradford method. Samples were mixed with SDS-PAGE loading buffer and loaded into SDS-PAGE, and then transferred onto a PVDF membrane (Millipore). Western blotting was performed using the goat anti-PD-1 antibody, goat anti-PD-L1 antibody, goat anti-PD-L2 antibody, and corresponding HRP conjugated secondary antibody.

2.9. Statistical analysis

Student's *t*-test was used to analysis the difference between two groups. Comparisons among multiple groups used one-way ANOVA test. $P < .05$ was considered statistically significant.

3. Results

3.1. The effects of APS on clinical manifestations of EAE mice

The onset of EAE was starting at day 11 post-immunization, the peak was on day 16, and after day 16 EAE mice gradually entered into remission. The groups of “APS-7d”, “APS0d”, and “APS + 7d” which were given drug on the different dates of day −7, day 0 and day +7, showed varying degrees of mitigation in clinical symptoms of EAE mice, the onset and peak dates in the APS-7d group were delayed. It showed that APS had a certain role in the treatment and prevention of EAE mice (Fig. 1A). After the induction of EAE, the body weight in each group was decreased. Since the day 3 post-immunization, body weight began to rise slowly. On day 11 around the onset, there was a significant

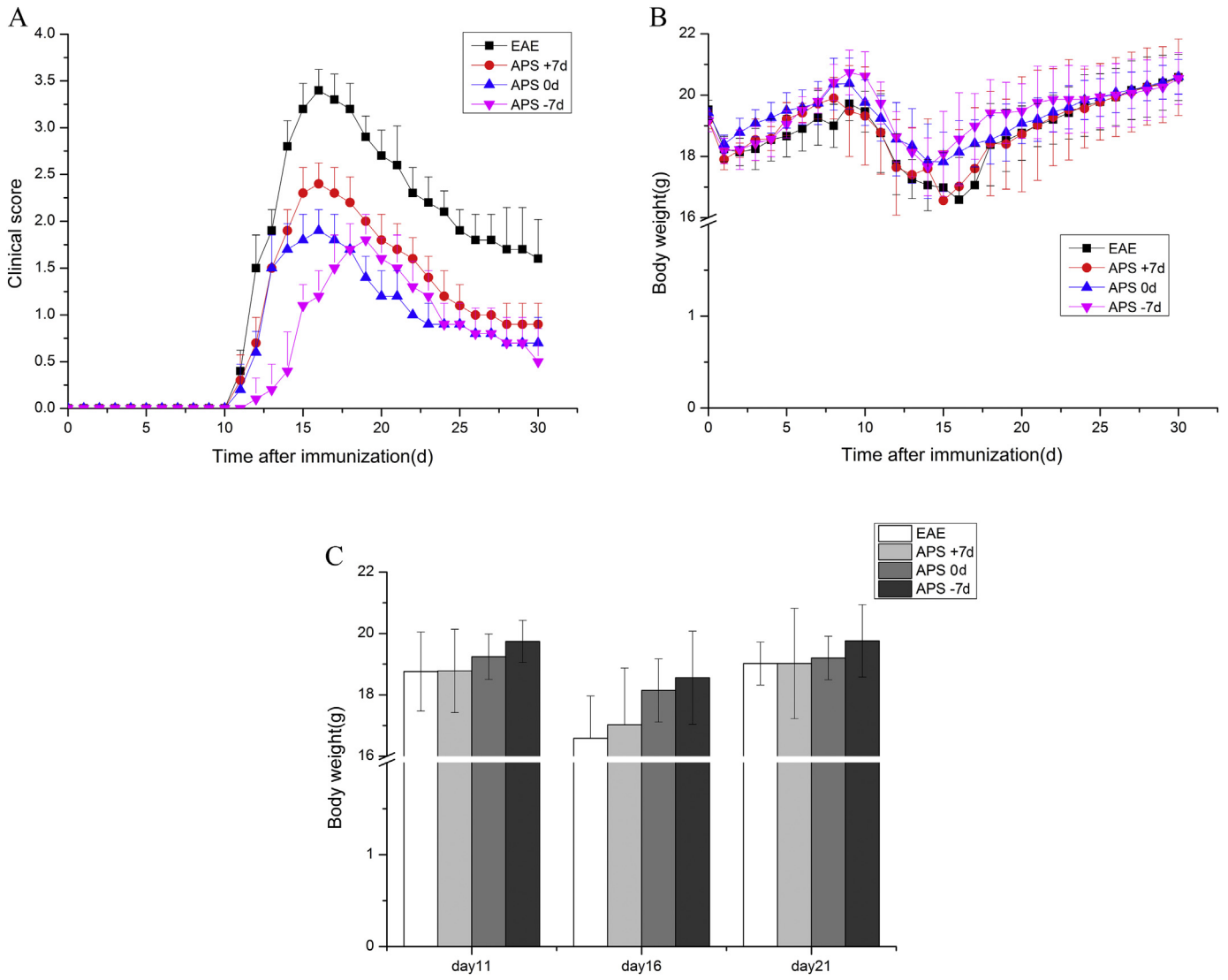


Fig. 1. The effects of APS on clinical signs of EAE mice.

weight loss starting. After the peak date, the body weight gradually rise (Fig. 1B). On day 11, day 16, and day 21, there were no differences among the groups (Fig. 1C).

EAE was induced in female C57BL/6 mice by $MOG_{35–55}$ immunization. Mice were examined daily for disease signs on a 0–5 scale. Data represent the mean clinical scores ± SEM ($n = 10$). For drug treatment, APS was given by oral administration 500 mg/(kg.d) on day −7, day 0 and day +7. Water was given as vehicle control (200 μl/mouse.d). The clinical signs of EAE mice were alleviated in APS treatment groups, especially in the APS-7d group (Fig. 1A).

EAE was induced in female C57BL/6 mice by $MOG_{35–55}$ immunization. Mice were weighed daily. Data represent the mean clinical scores ± SEM ($n = 10$). After the induction of EAE, the body weight in each group was decreased. Since the day 3 post-immunization, body weight began to rise slowly (Fig. 1B). On day 11, day 16, and day 21, there were no differences among the groups (Fig. 1C).

3.2. APS could alleviate inflammation and demyelination in CNS of EAE mice

Obtained spinal cord of mice on day 16, HE staining showed that spinal cord of naïve mice was structural integrity. There was no infiltrating inflammatory cell. While in EAE mice, the white matter area in spinal cord showed a large number of inflammatory cells. In the APS + 7d group and the APS0d group, infiltration of inflammatory cells could also be found. In the APS-7d group, the number of infiltrating inflammatory cells was reduced. It indicated that APS could effectively inhibit the inflammatory response in CNS of EAE mice (Fig. 2A, a-j). The myelin was stained with blue after LFB staining. It was found that the myelin evenly distributed in the spinal cord of naïve mice, while there was varying size of vacuolar demyelination areas in the white matter of spinal cord in the EAE mice. In the APS + 7d group and the APS0d group, vacuolar demyelination could also be observed but the extent of demyelination was decreased. In the APS-7d group, vacuolar demyelination could almost not be found and the myelin distributed in the spinal cord could be detected. It indicated that the severity of demyelination of spinal cord in the APS treated mice was reduced and APS could effectively suppress demyelination in the CNS of EAE mice (Fig. 2B, a-j).

(Fig. 2A) HE staining of spinal cord on day 16; Naïve - a, b; Control - c, d; APS + 7d - e, f; APS0d - g, h; APS-7d - i, j; (a, c, e, g, i × 40; b, d, f, h, j × 100) APS could alleviate inflammation in CNS of EAE mice. (Fig. 2B) LFB staining of spinal cord on day 16; Naïve - a, b; Control - c, d; APS + 7d - e, f; APS0d - g, h; APS-7d - i, j; (a, c, e, g, i × 40; b, d, f, h, j × 100) APS could alleviate demyelination in CNS of EAE mice.

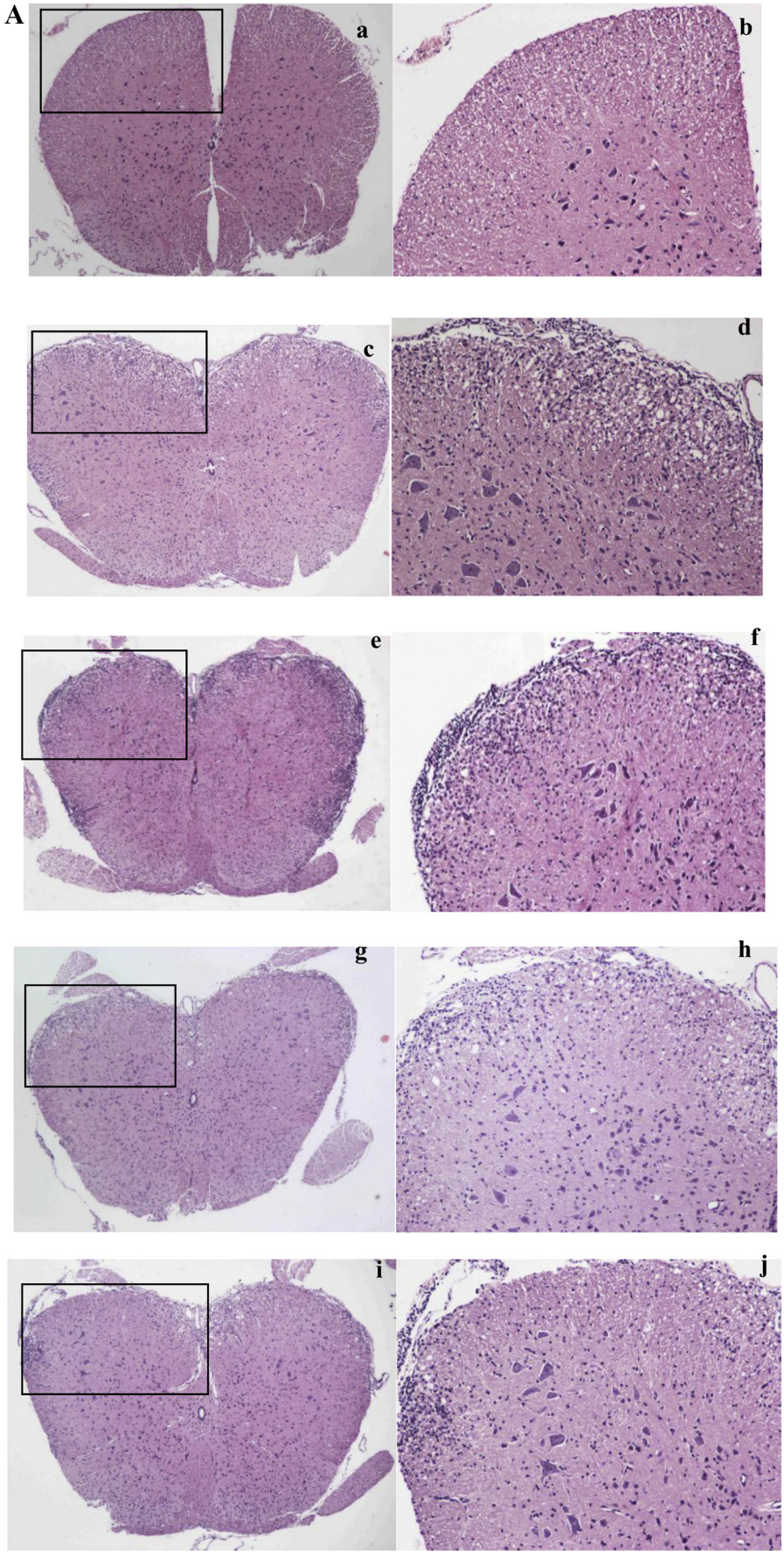


Fig. 2. The effects of APS on alleviating inflammation and demyelination in CNS of EAE mice.

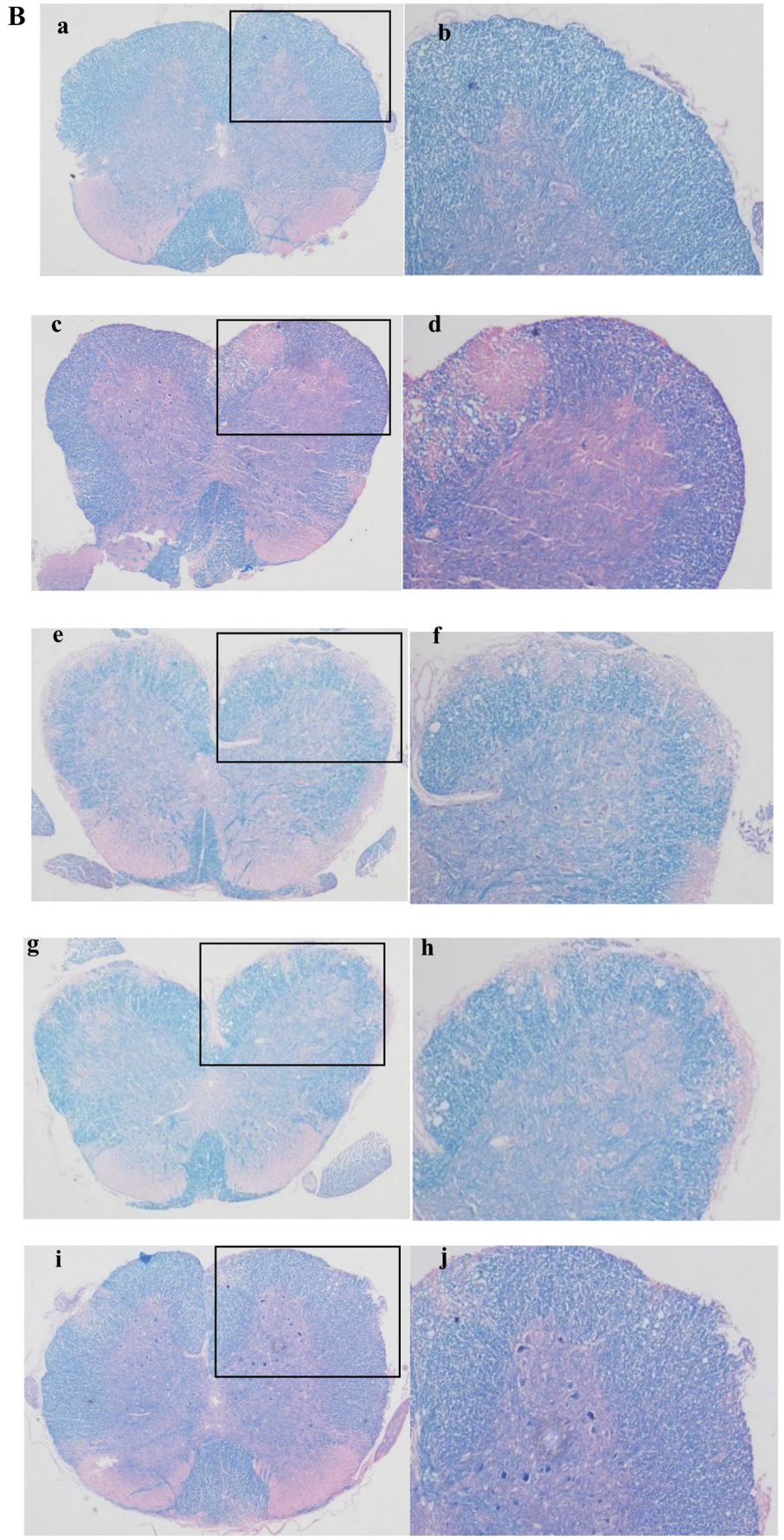


Fig. 2. (*continued*)

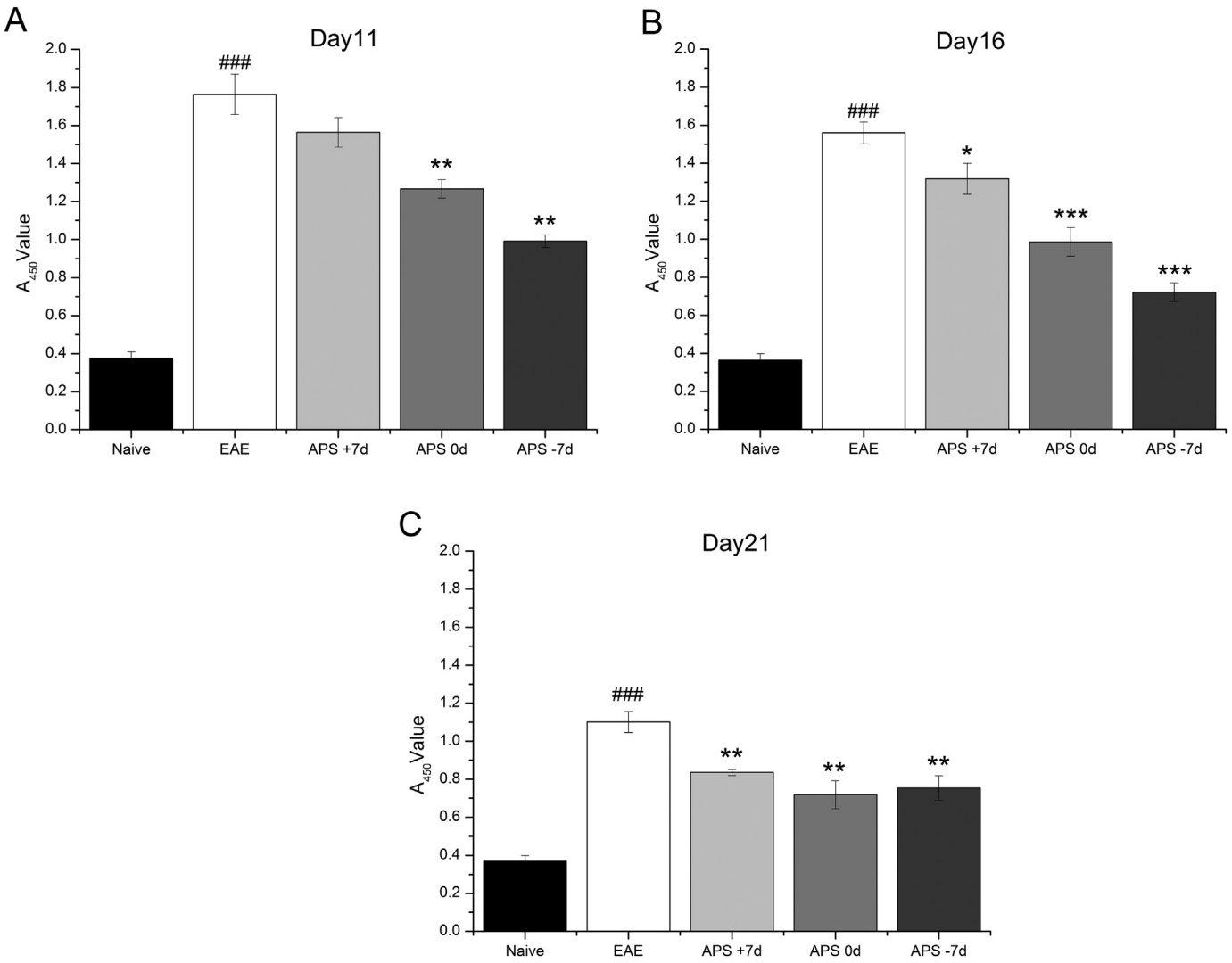


Fig. 3. Comparison of $MOG_{35–55}$-specific T cell proliferation on different courses of EAE.

3.3. APS could inhibit $MOG_{35–55}$-specific T cell proliferation

On different time course, including day 11, day 16 and day 21, the $MOG_{35–55}$-specific T cells proliferated significantly by stimulated with $MOG_{35–55}$ antigen ($P < .001$). Compared with the EAE group, $MOG_{35–55}$-specific T cell proliferation in the APS-7d group and the APS0d group was inhibited obviously on day 11 ($P < .01$), while the APS + 7d group had no significant difference. On day 16 and day 21, $MOG_{35–55}$-specific T cell proliferation in the three APS treatment groups were all effectively suppressed. The effect of APS on EAE was a slow continuous process. On day 21, $MOG_{35–55}$-specific T cell proliferation in the APS-7d group and the APS0d group were almost the same, it may be associated with that the proliferation of T cells was relatively active in peak of the APS-7d group delaying (Fig. 3A-C).

Single cell suspensions of MNCs from the spleen were prepared on day 11 (onset), day 16 (peak) and day 21 (remission) post-immunization. Cells were cultured at a cell density of 2×10^6 cells/well in RPMI 1640 in triplicate wells of 96-well plates. The cells were stimulated by adding 20 μg/ml of $MOG_{35–55}$ in the culture medium. After 68 h of incubation, each well was added 10 μl CCK-8. Cultured to 72 h, the absorbance at 450 nm was measured using a microplate reader. APS could inhibit $MOG_{35–55}$-specific T cell proliferation obviously (Fig. 3A-C). Data represent mean ± SEM ($n = 6$). Compared with the control group, $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$; compared with the naïve group, ##P < .01, ###P < .001.

3.4. APS could affect cytokine secretion produced by $MOG_{35–55}$-specific T cells

With $MOG_{35–55}$ antigen stimulation, the level of IFN-γ, TNF-α, IL-2, IL-17, IL-4 and IL-10 were significantly increased in the control group. APS could suppress IFN-γ, TNF-α, IL-2, IL-17 secretion to different extent in each treatment group, but has no effect on IL-4, IL-10 secretion (Fig. 4A-F). IFN-γ production was significantly decreased in the APS-7d group and in the APS0d group at all time points, but not in the APS + 7d group (Fig. 4A). At different time points, TNF-α level were significantly lower in the APS-7d group. It was decreased significantly in the APS0d group on the peak point, but had no difference at different time points in the APS + 7d group (Fig. 4B). In the APS-7d group, IL-2 secretion was obviously reduced at all time points, and in the APS0d group it was reduced on day 16 and on day 21. Only on day 21, there was a significant reduction of IL-2 secretion in the APS + 7d group (Fig. 4C). Production of IL-17 was significantly decreased in the APS-7d group and in the APS0d group in all time courses, and it was decreased in the APS + 7d group on day 16 and day 21 (Fig. 4D). There was no significant change of IL-4, IL-10 production in each treatment group in all courses. (Fig. 4E-F).

Single cell suspensions of MNCs from the spleen were prepared on

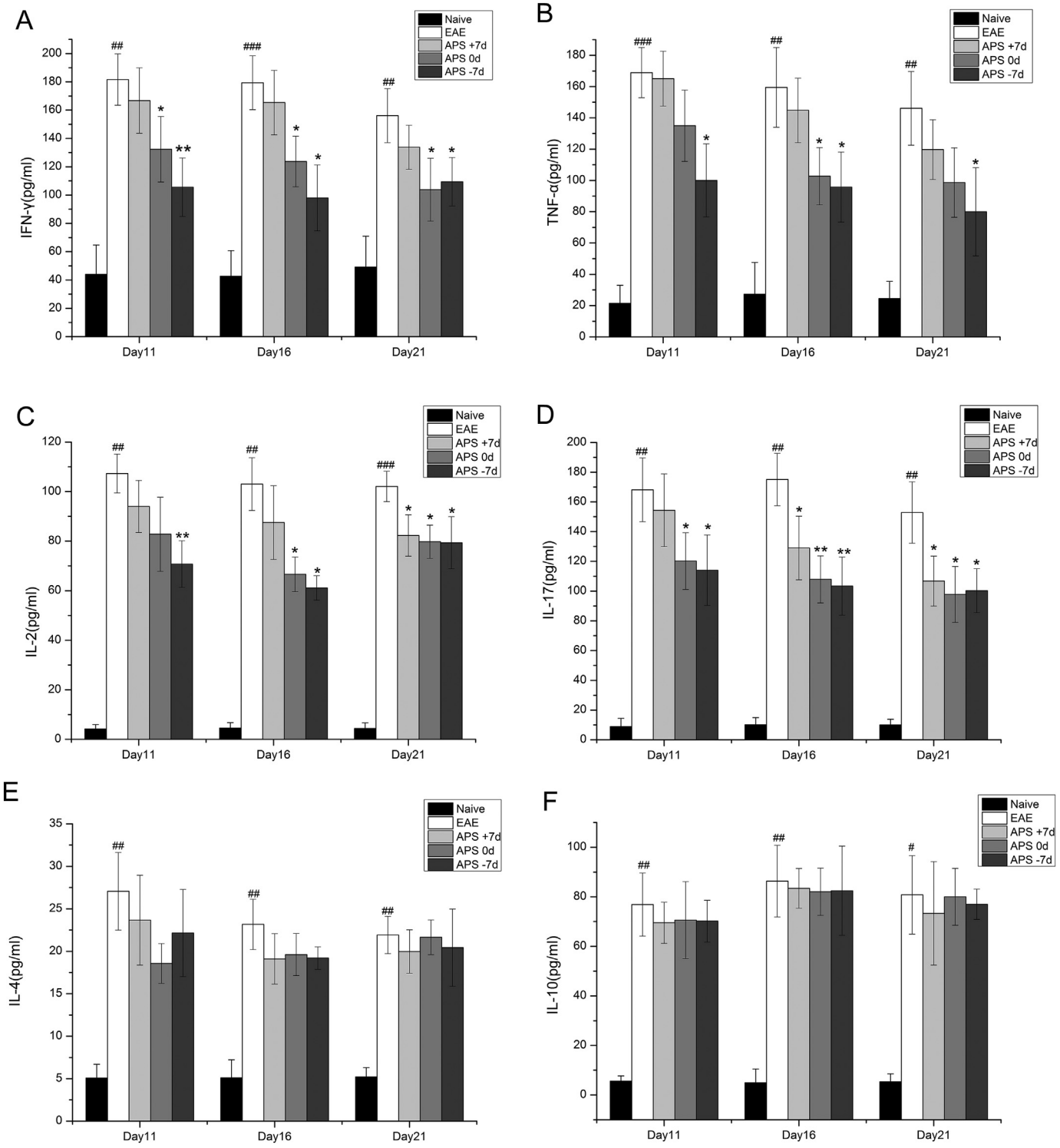


Fig. 4. Levels of cytokine production in different courses of EAE.

day 11 (onset), day 16 (peak) and day 21 (remission) post-immunization. Cells were cultured at a cell density of 2×10^6 cells/well in RPMI 1640 in triplicate wells of 24-well plates. The cells were stimulated by adding 20 μg/ml of $MOG_{35–55}$ in the culture medium. After 48 h of incubation, supernatants were collected for ELISA assay of IFN-γ, TNF-α, IL-2, IL-17, IL-4 and IL-10. APS could suppress IFN-γ, TNF-α, IL-2, IL-17 secretion to different extent in each treatment group (Fig. 4A-D), but have no effect on IL-4, IL-10 secretion (Fig. 4E-F). Data represent mean ± SEM ($n = 6$). Compared with the control group, $^*P < .05$, $^{**}P < .01$; compared with the naïve group, $\#\#P < .01$.

3.5. The impact of APS on PD-1/PD-Ls expression in the spleen of EAE mice

On day 11, compared with the control group, the mRNA expression of PD-1, PD-L1 and PD-L2 molecules were all significantly increased in the APS0d and the APS-7d groups ($P < .05$, $P < .01$), while there is no significant difference of the mRNA expression of PD-1, PD-L1 and PD-L2 molecules in the APS + 7d group. On day 16, compared with the

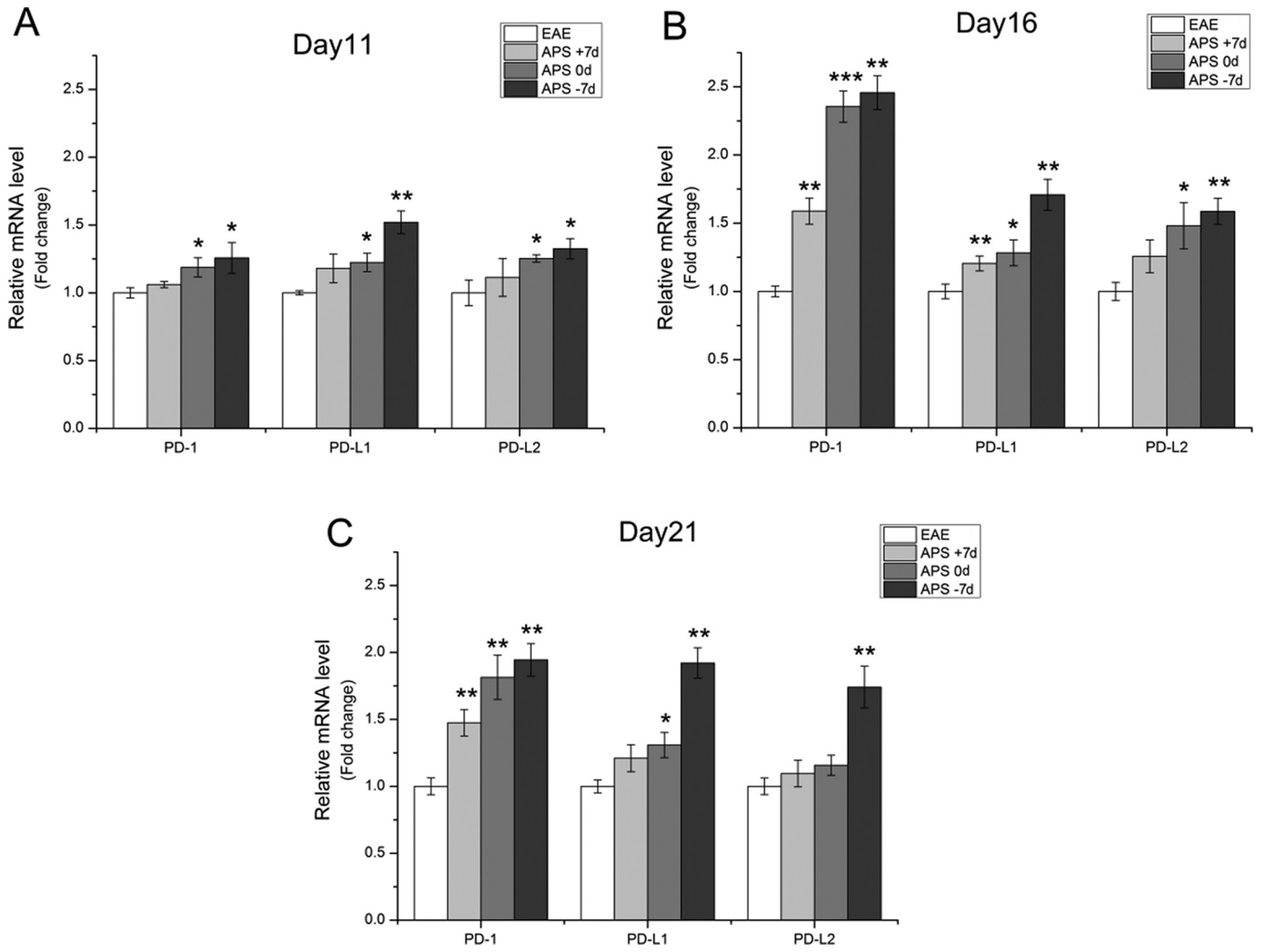


Fig. 5. The mRNA expression of PD-1/PD-Ls molecules in the spleen of each group after the intervention of the APS administration.

control group, the mRNA expression of PD-1 and PD-L1 molecules was all up-regulated in the APS + 7d, the APS0d and the APS-7d groups. PD-L2 was up-regulated in the APS0d and the APS-7d groups ($P < .05$, $P < .01$), but had no changed in the APS + 7d group. On day 21, compared with the control group, PD-1 was all up-regulated in the APS + 7d, the APS0d and the APS-7d groups, PD-L1 was up-regulated in the APS0d and the APS-7d groups ($P < .05$, $P < .01$), while in the APS + 7d there was no significant difference. PD-L2 was increased in the APS-7d group ($P < .01$), while in the APS + 7d and the APS0d groups there was no obvious change. (Fig. 5A-C).

In different time courses, the expression of PD-1/PD-Ls protein was raised. In the APS intervention groups, the expression of PD-1, PD-L1 and PD-L2 protein was increased in mice spleen tissues (Fig. 6A-C). On day 11, compared with the control group, the expression of PD-L1 and PD-L2 protein in the APS0d group was significantly increased ($P < .05$), as well as in the APS-7d group ($P < .01$, $P < .05$). Only in the APS-7d group, the expression of PD-1 protein was significantly increased ($P < .05$), while the expression of PD-1, PD-L1 and PD-L2 protein in the APS + 7d group had no significant difference. It may be related to that the APS administration in the APS + 7d group was later and APS had not played an effective role. On day 16, compared with the control group, the expression of PD-1 and PD-L1 protein in the APS + 7d, the APS0d and the APS-7d groups was significantly increased ($P < .05$, $P < .01$). PD-L2 was significantly increased in the APS-7d and the APS0d group ($P < .01$), there was no difference in the APS + 7d group. On day 21, compared with the control group, the expression of PD-1 protein in the APS-7d, the APS0d, the APS + 7d groups was all significantly increased ($P < .01$). PD-L1 was significantly increased in the APS0d and the APS-7d groups ($P < .05$, $P < .01$). The level of PD-L1 and PD-L2 had no differences in the APS + 7d group (Fig. 6A-C).

Total RNA was extracted from splenocytes. The RNA was used to reverse transcription to gain cDNA. Real-time PCR was prepared with SuperReal PreMix. Expression values were normalized to GAPDH. (Fig. 5A-C) PD-1, PD-L1 and PD-L2 mRNA expression were up-regulated in the APS treatment groups in different time course. Experiments were repeated for three times and similar results were obtained. One representative experiment is showed. Compared with the control group, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$.

Western blotting was performed using the goat anti-PD-1 antibody, goat anti-PD-L1 antibody, goat anti-PD-L2 antibody, and corresponding HRP conjugated secondary antibody. (Fig. 6A-C) In different time courses, the expression of PD-1/PD-Ls protein was raised. In the APS intervention groups especially in the APS-7d group, the expression of PD-1, PD-L1 and PD-L2 protein was increased in the spleen tissues. Experiments were repeated for three times and similar results were obtained. One representative experiment is showed. Compared with the control group, $^{*}P < .05$, $^{**}P < .01$.

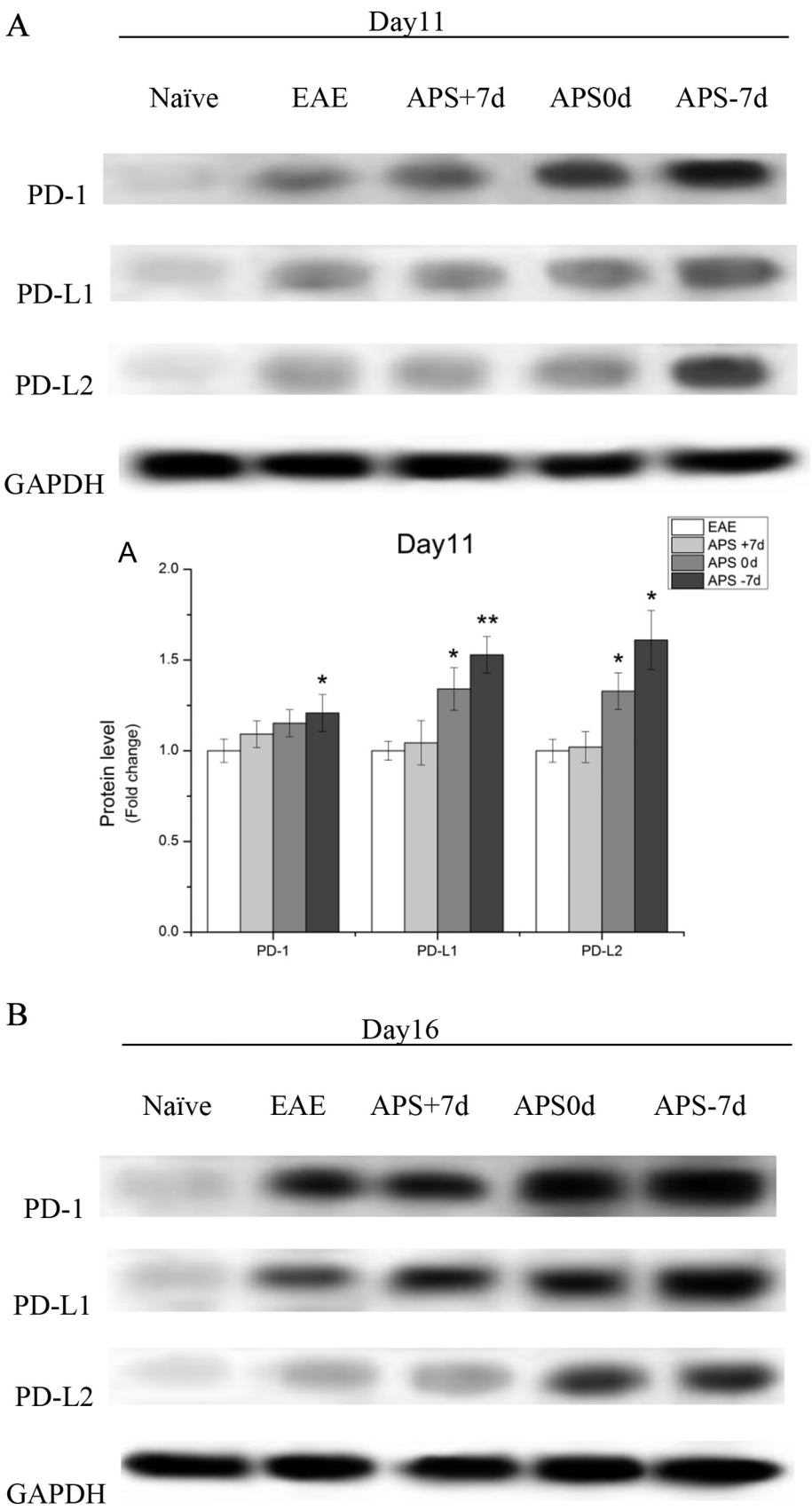


Fig. 6. The expression of PD-1/PD-Ls protein in the spleen of each group after the intervention of the APS administration.

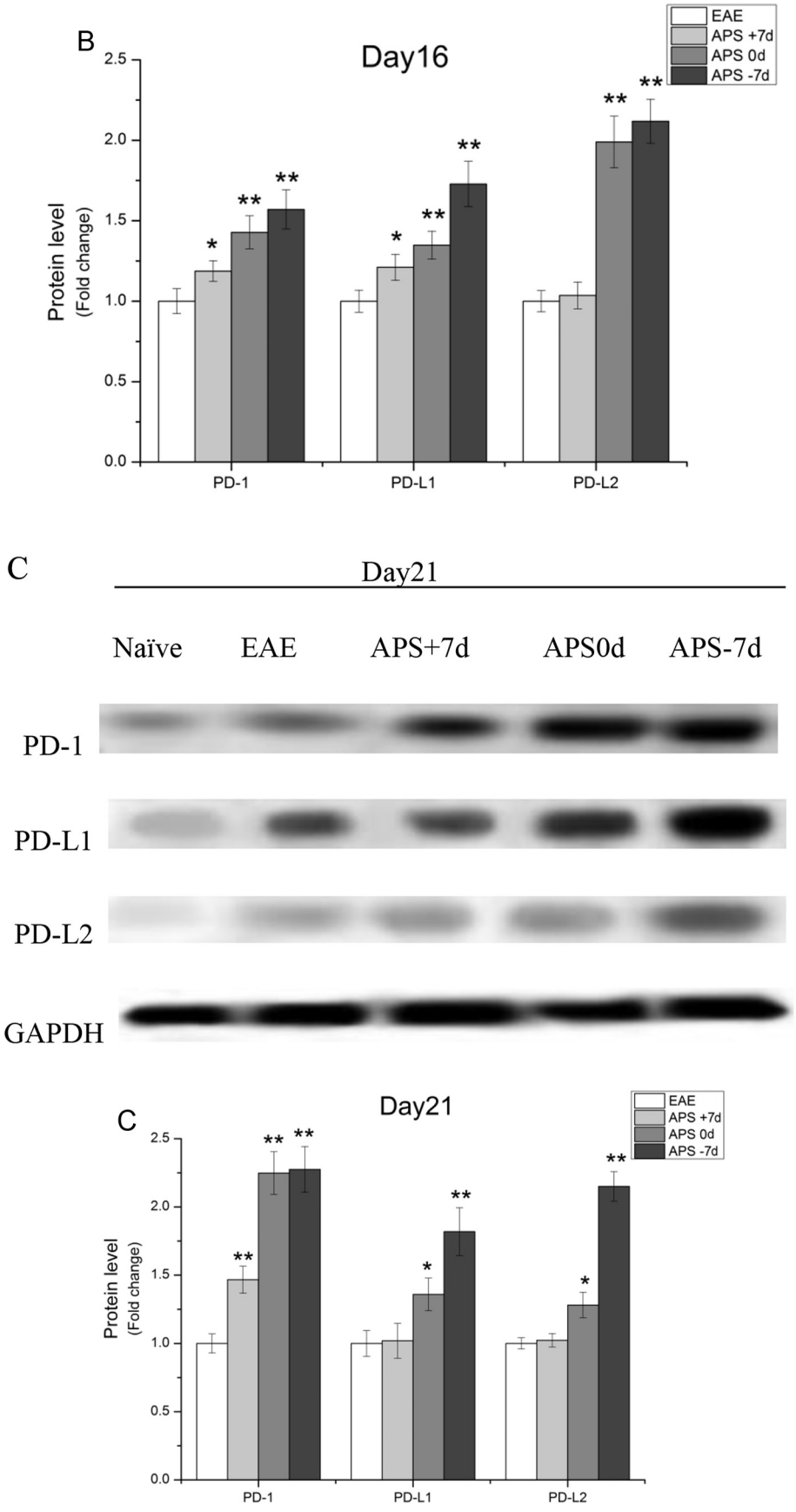
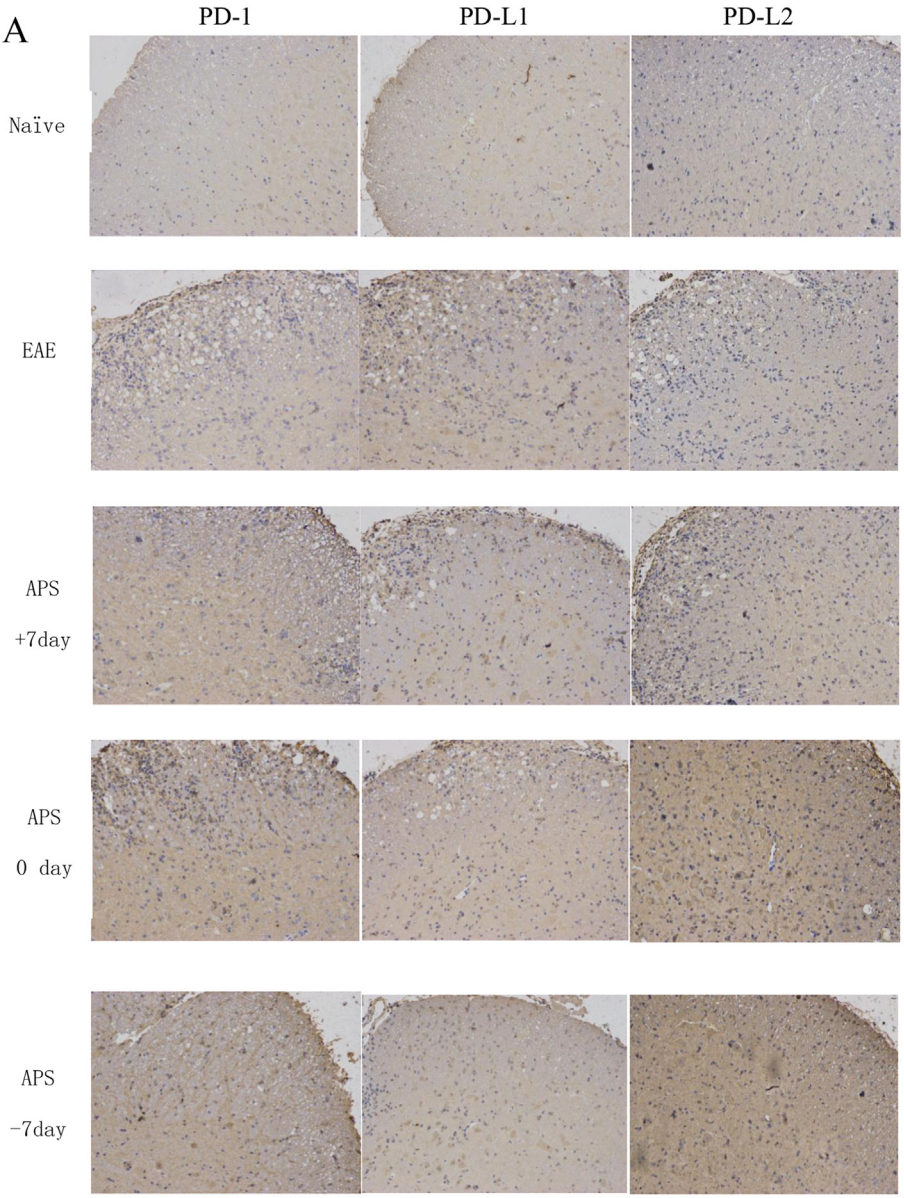


Fig. 6. (*continued*)



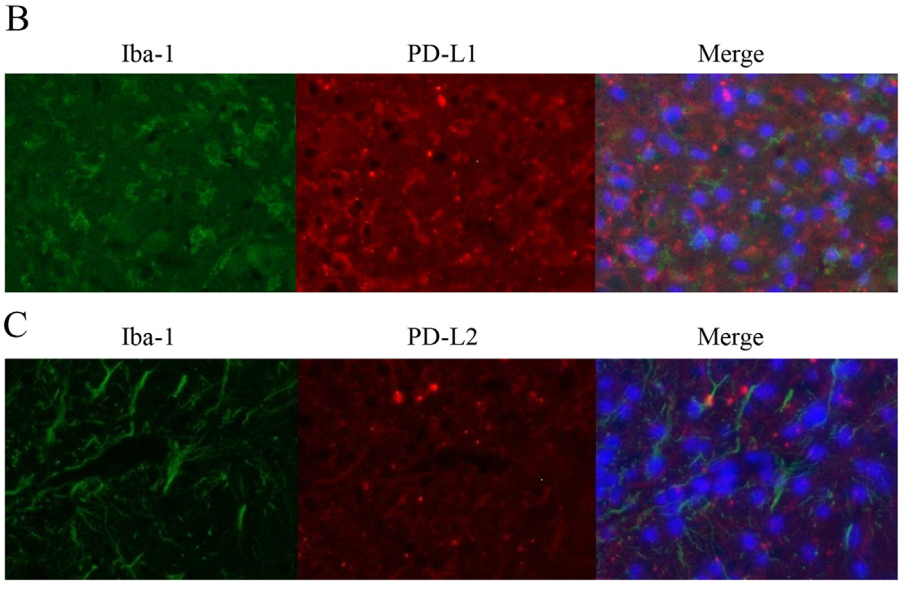


Fig. 7. The effect of APS on PD-1/PD-Ls expression in the spinal cord of EAE mice. (Fig. 7A) Immunohistochemistry of spinal cord for PD-1, PD-L1, PD-L2 detection in the spinal cord (Day 16, Naïve, Control, APS + 7d, APS0d, APS-7d, ×200). The naïve mice expressed small amount of PD-1, PD-L1, PD-L2, while in the spinal cord of EAE mice, PD-1, PD-L1 and PD-L2 were expressed significantly. In the APS intervention groups, PD-1, PD-L1 and PD-L2 were also expressed significantly, especially in the area of demyelination parts. (Fig. 7B-C) Frozen sections of spinal cord were stained with anti-PD-L1 or PD-Ls antibodies, and then with appropriate fluorescent-labeled secondary antibodies. Microglia was co-stained with lectin Iba-1 on day 16. Immunofluorescent of spinal cord for PD-L1, PD-L2 detection on microglia (Day 16, Control group, ×200). Microglia, the resident APC, expressed PD-L1 and PD-L2.

3.6. The effect of APS on PD-1/PD-Ls expression in the spinal cord of EAE mice

PD-1, PD-L1 and PD-L2 expression in the spinal cord of EAE mice were detected by immunohistochemistry technique. The naïve mice expressed small amount of PD-1, PD-L1, PD-L2, while in EAE mice, PD-1, PD-L1 and PD-L2 were expressed significantly. In the APS intervention groups, PD-1, PD-L1 and PD-L2 were also expressed significantly, especially in the area of demyelination parts (Fig. 7A). It was also detected that microglia, the resident APC, expressed PD-L1 and PD-L2 (Fig. 7 B—C).

4. Disscussion

The pathogenesis of EAE/MS, which is associated with autoimmune inflammatory demyelination in the CNS, has been still unclear up to now. Most of researchers believe that predisposing factors cause the autoimmune response in body. Lymphocytes and monocytes in the peripheral blood are activated during the progress of the disease. The activated T cells can express a variety of adhesion molecules and bind with the receptors on vessel wall. Vascular endothelial cells can also express the selectin binding to T cells. Chemokines can promote T cells to enter the CNS. $CD4^+$T cells play a major role in the progression of the immune response (Popescu and Lucchinetti, 2016; Mardiguian et al., 2017). The study found that the anti-myelin autoantibodies play a very important role in the formation of EAE/MS. Myelin oligodendrocyte glycoprotein (MOG) is the membrane protein outermost on the surface of the oligodendrocyte. It is a key component of demyelination in EAE (Peschl et al., 2017). Therefore, MOG was widely used as an antigen for EAE induction to investigate the pathogenesis of MS. We induced EAE with $MOG_{35–55}$ peptide and observed that $MOG_{35–55}$ can stimulate $MOG_{35–55}$- specific T cell proliferation. APS can significantly inhibit the $MOG_{35–55}$-specific T cell proliferation after oral administration. It inhibits IFN-γ, TNF-α, IL-2 and IL-17 secretion but has no effect on IL-4 and IL-10 secretion.

Naïve $CD4^+$T cells differentiate to different T cell subsets, including Th1, Th2, Th17 and Treg in different microenvironment (Bravo et al., 2016; Kraj and Ignatowicz, 2018). Th1 cells mediate cytotoxicity and local inflammatory immune responses and involve in cellular immune response. It is also referred to inflammatory T cells. Th1 cells secrete IFN-γ, TNF-α and IL-2 after activation, which promotes the immune response. Th2 cells secrete IL-4 and IL-10, which can resist the immune function of Th1 cells. And Th2 cells regulate EAE inflammatory response negatively. IFN-γ can induce Th1 cells differentiation, but inhibit Th2 cells differentiation and proliferation. IL-4 induces Th2 cells differentiation, but inhibits the differentiation and function of Th1 cells with IL-13. Dynamic equilibrium between Th1 and Th2 cells regulate the body homeostasis of immune response (Gimsa et al., 2001). Besides Th1 and Th2 cell subsets, $CD4^+$ effector T cell comprises Th17 cells which secrete IL-17. IL-17 has a role in promoting the inflammatory response in EAE. In our study we found that APS can suppress $MOG_{35–55}$-specific T cells to secrete IFN-γ, TNF-α, IL-2 and IL-17, while APS has no effect on IL-4 or IL-10 secretion. We infer that APS primarily alleviates the symptoms of EAE by inhibition of Th1, Th17 cells mediated immune response, while APS has no significant effect on Th2 cells.

PD-1 and PD-Ls are members of the B7-CD28 super family of costimulatory molecules, which act as negative regulators of activated T cells (Khoury and Sayegh, 2004). Programmed death ligands (PD-Ls) express on antigen-presenting cells. It binds to the receptor programmed cell death-1(PD-1) which expresses on T cells and regulates immune response negatively. The two ligands, PD-L1 and PD-L2 are differentially regulated by Th1 and Th2 cells (Zamani and Rezaei, 2016). The expression of PD-Ls on microglia increases in the inflammatory response of EAE (Schreiner et al., 2008; Phares et al., 2009). The dynamic expression in brain tissue is consistent with the same course of the disease. Its role might be associated with the suppressor of T cells and reduction of the inflammation of the CNS destruction.

PD-1 and PD-L1 were up-regulated in CNS in the $MOG_{35–55}$ induced EAE mice. Blocking PD-1 resulted in a more severe infiltration of lymphocytes in CNS.

Blocking PD-1 in vivo led to the amplification of antigen-specific T cell activation and cytokine production (Casacuberta-Serra et al., 2016). Using genetic modification techniques, MOG and PD-L1 both express on embryonic stem cell-derived dendritic cells (DC). Dendritic cells can induce NOD mice reduced $CD4^+$T responses, myelin inflammatory cell infiltration, as well as the severity of EAE (Ikeda et al., 2014). Our previous study (Cheng et al., 2007) found that PD-1/PD-L1 signaling pathway has an important regulatory role in EAE. IL-12 inhibits inflammatory response through PD-1/PD-L1 pathway up-regulation on $CD11b^+$ antigen presenting cells mediated by IFN-γ. These findings suggest that PD-1/PD-L1 pathway plays an important role in the inhibition of EAE disease process and protection of brain tissue from injury. In this study we found that APS could promote the expression of costimulatory molecules PD-1/PD-Ls in spinal cord tissue of EAE mice. It also could up-regulate the expression of protein and mRNA of PD-1, PD-L1 and PD-L2. It revealed that APS alleviated EAE through the PD-1/PD-Ls pathway. Microglia is the main effector cells of the inflammatory lesions in the brain, in addition to phagocytosis, microglial cells have the similar immune phenotype and functional characteristics with antigen-presenting cells (Bose, 2017). There are microglial cells from the resting state quickly turning to the activated state which is showing the waterfall effect in MS/EAE (Fang et al., 2017). Our study found that PD-L1 and PD-L2 expressed on microglia in CNS of EAE mice. We will try to explore the impact of APS on microglia activation in CNS for further study.

Astragalus polysaccharides (APS) is the active extract from *Astragalus membranaceus* (a kind of traditional Chinese medicinal herb), which has a variety of immunomodulatory activities. In the present research, APS can effectively suppress EAE by inhibiting $MOG_{35–55}$-specific T cell proliferation, down-regulating proinflammatory cytokines, up-regulating the costimulatory molecules PD-1/PD-Ls signaling pathway, and leading to inhibition of T cell-mediated immune response. Traditional Chinese medical herb exerts the favourable curative effects in treating autoimmune disease multiple sclerosis without adverse reactions or side-effects. In addition, it plays a crucial role in bidirectionally regulating immune system based on the host conditions and maintaining homeostasis of host immune system. In compare, cytokine interferon-β (IFN-β) exerts the reversed effects, although it was commonly used as a clinical agent. The recurrence could not be completely controlled by interferon therapy. Therefore, promoting the development and clinical application of natural herbal medicine is very promising with a great potential.

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Conflict of interest

The authors have no conflict of interest to declare.

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