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PD-L1 is increased in the spinal cord and infiltrating lymphocytes in experimental allergic encephalomyelitis

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Research Highlights

(1) Different from previous studies that subcutaneously injected mice at several points on the back, this study used a novel method to immunize mice, a single subcutaneous injection into the mouse armpit. The injection into this area allows easy transport of antigen and is conducive to eliciting immunological reactions. In addition, single-point injection can avoid the tolerance of sensitized animals to antigens and accordingly reduce morbidity.
(2) This study is characterized by the fact that, the established models of experimental allergic encephalomyelitis are similar to multiple sclerosis in humans, as detected by hematoxylin-eosin staining and Luxol fast-blue staining.
(3) The immunohistochemical detection results showed that, PD-L1 molecule expression was creased in spinal cord, while flow cytometry and western blot analysis revealed that PD-L1 molecule expression was also increased in splenocytes. This evidence provides the basement for exploring the role of PD-L1 in multiple sclerosis.

Abstract

Experimental allergic encephalomyelitis is a mouse model of human multiple sclerosis with similar pathology and pathogenesis. Th1 cells play an important role in the pathogenesis of experimental allergic encephalomyelitis. This study determined the potential effect of programmed cell death 1 ligand 1 in the pathogenesis of experimental allergic encephalomyelitis induced by injecting myelin oligodendrocyte glycoprotein, complete Freund's adjuvant and Bordetella pertussis toxin into C57BL/6J mice. Experimental allergic encephalomyelitis mice developed disease and showed inflammatory changes in the central nervous system by hematoxylin-eosin staining of spinal cord pathological sections, demyelination by Luxol fast-blue staining and clinical manifestations. The expression of programmed cell death 1 ligand 1 in mice was detected by immunohistochemistry, flow cytometry and western blot analysis. The expression of programmed cell death 1 ligand 1 in the spinal cord and splenocytes of mice was significantly increased compared with normal mice. Our findings suggest the involvement of programmed cell death 1 ligand 1 in the pathogenesis of experimental allergic encephalomyelitis and suggest this should be studied in multiple sclerosis.

Key Words

neural regeneration; experimental allergic encephalomyelitis; multiple sclerosis; animal models; autoimmune disease; costimulatory signal; costimulatory molecule; programmed cell death 1 ligand 1; B7-CD28 superfamily; grants-supported paper; neuroregeneration

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INTRODUCTION

Multiple sclerosis is an autoimmune disease characterized by chronic inflammatory demyelination in the central nervous system. Cellular immunity, humoral immunity and complement are involved in this process. However, disease etiology and pathogenesis remain unclear, and there is no effective treatment. According to the T cell activation dual signal hypothesis, at least two signals are required for T lymphocyte activation and antigen-specific T cell immune responses, namely the specific signal provided by MHC-peptide complexes and non-specific costimulatory signal provided by antigen presenting cells (APC) surface molecules. The costimulatory signal pathway provides a positive signal to enhance and maintain T cell immune responses, and a negative signal to reduce T cell immune responses[1-2]. The negative signals may limit, weaken, and/or terminate T cell immune responses, thus they play a critical role in the regulation of T cell immune tolerance and autoimmunity.

Recent studies have focused on the costimulatory signals for T cell activation[3]. Costimulatory molecules are divided into the tumor necrosis factor superfamily and immunoglobulin superfamily. Among them, B7-CD28 molecules, members of the immunoglobulin superfamily, provide costimulatory signals that regulate T cell activation and tolerance. Programmed death-1 molecule belongs to the immunoglobulin CD28 superfamily and functions as a cell surface inhibitory receptor. It is inductively expressed in activated T cells, B cells and monocytes. Programmed death-1 is also a type I transmembrane glycoprotein containing immunoreceptor tyrosine-based inhibitory motifs, and binds with its corresponding ligands to induce inhibitory signals. Programmed death- 1 recognizes two ligands, PD-L1 and PD-L2. *In vitro* studies showed that PD-1/PD-L1 and PD-1/PD-L2 pathway-mediated costimulatory signals inhibit T cell activation[4-5]. Some studies suggest the PD-1/PD-L1 pathway plays an important role in regulating the severity of experimental allergic encephalomyelitis, a mouse model of multiple sclerosis[6]. The pathogenesis of experimental allergic encephalomyelitis is mediated by T cell Th1-type inflammatory factors and interleukin-17[7-9]. Th1-type inflammatory responses were significantly enhanced when the PD-1/PD-L1 pathway was blocked[4]. Therefore, we speculated that the PD-1/PD-L1 pathway plays an important role in the inhibition of Th1-type autoimmune diseases and we investigated the role PD-L1 in experimental allergic encephalomyelitis.

PD-L1 is a negative costimulatory molecule in the B7 family, first discovered in a placental cDNA library[1]. PD-L1 is constitutively expressed in murine B cells, T cells, dendritic cells, macrophages and bone marrow mesenchymal stem cells[2, 10-11]. PD-L1 expression is also observed in bone marrow-derived mast cells and other lymphocytes. The expression level is upregulated in activated lymphocytes and is lower in humans compared with mice[5]. PD-L1 is expressed in splenocytes at the active phase of autoimmune disease[12-13]. PD-L1 molecule binds with inhibitory receptor PD-1 on the surface of activated T cells to exert immunosuppressive effects on T cells, such as inhibiting T cell activation and proliferation and interleukin-2 production, leading to cell cycle arrest[14]. The PD-L1/PD-1 pathway suppressed early T cell activation[15]. *In vitro* studies demonstrated the PD-L1/PD-1 pathway inhibited T cell activation, cytokine production, and cytotoxic T lymphocyte activation[4]. Animals with resistance to experimental allergic encephalomyelitis were more susceptible and clinical symptoms were significantly aggravated after PD-L1 gene knockout[4, 16]. Carreno ***et al*** [16] demonstrated that 129S4/SvJae mice, that are resistant to experimental allergic encephalomyelitis, became sensitive to experimental allergic encephalomyelitis when they were deficient in PD-L1. Injection of anti-PD-L1 monoclonal antibody at the early period of experimental allergic encephalomyelitis inhibited disease progression[15]. PD-L1 expression is scarce in the central nervous system of normal rats, but is significantly increased in experimental allergic encephal-

omyelitis animals[17]. Latchman *et al* [18] demonstrated that the PD-L1/PD-1 signal pathway had a negative regulatory role during the acute phase of experimental allergic encephalomyelitis and that PD-L1 was highly expressed in inflammatory infiltrating cells, vascular endothelial cells, and microglia and astrocytes of brain tissue from MOG35-55-induced C57BL/6J mice.

PD-L1 is an important molecule in the susceptibility, severity and pathogenesis of experimental allergic encephalomyelitis, thus it might be a potential treatment target for multiple sclerosis. In this study, we measured negative costimulatory molecule PD-L1 expression in the spinal cord tissue and spleen $CD4^+$ T cells of experimental allergic encephalomyelitis mice by immunohistochemistry, flow cytometry and western blot analysis. The aim of this study was to determine the potential effect of PD-L1 in the pathogenesis of experimental allergic encephalomyelitis and to determine whether it would be useful for human multiple sclerosis disease surveillance and treatment.

RESULTS

Quantitative analysis of experimental animals

Thirty mice were randomly divided into a model group (n = 20) and control group (n = 10). In the model group, experimental allergic encephalomyelitis was established by injecting myelin oligodendrocyte glycoprotein (MOG35-55), complete Freund's adjuvant and Bordetella pertussis toxin into C57BL/6J mice. The control group was administered PBS injection into the armpit. Three mice failed to develop experimental allergic encephalomyelitis and one mouse died after the onset, so 16 mice in the model group and ten mice in the control group were used in the study analysis.

Appearance of experimental mice

The model mice began to show reduced movement, piloerection and tail weakness at 10 days after disease induction, followed by poor appetite, arched back, and hind limb or forelimb weakness. The clinical symptoms peaked at 12–14 days post-induction and some mice were contaminated by urinary and fecal incontinence, and some became moribund. Clinical symptoms were alleviated at 16–19 days, and reoccurred in two mice at 20–22 days, and was restored at 23 days. Afterwards, disease progression gradually stabilized, and limb paralysis developed into spasmodic paralysis, which was sustained for > 60 days. In the control group, no mice developed disease symptoms. The neurological function score of both groups is shown in Figure 1. At 10 days after immunization, the model group showed significantly lower scores than the control group (P < 0.01).

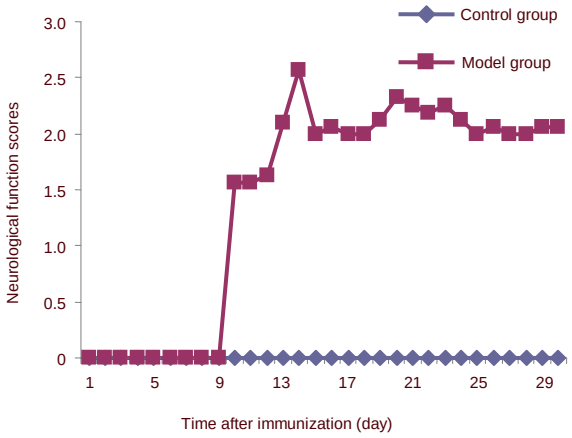

Figure 1 Neurological function decreased in experimental allergic encephalomyelitis mice.

There were 16 mice in the model group and 10 mice in the control group. Higher neurological scores indicated more severe neurological function deficit. The neurological function score of the model mice was significantly increased at 10 days after immunization, suggesting that neurological functions were worse than the controlled mice.

Changes of spinal cord pathological sections

Hematoxylin-eosin staining showed a large number of inflammatory cells infiltrated around blood vessels in the spinal cord of model mice at 2 days after onset, consisting mainly of lymphocytes. Massive inflammatory cell infiltration was observed below the spinal pia mater. In the control group, spinal cord sections showed no significant pathological changes (Figure 2). Luxol fast-blue staining showed that demyelination had occurred in the spinal cord of model mice at 2 days after onset, while it was intact in the control group (Figure 3).

PD-L1 immunoreactivity in the spinal cord of experimental allergic encephalomyelitis mice

Immunohistochemical staining showed that PD-L1 immunoreactivity was present in infiltrating inflammatory cells around blood vessels in the spinal cord of model mice. In the control group, no inflammatory cell infiltration or PD-L1 immunoreactivity was observed (Figure 4).

PD-L1 expression in splenic $CD4^+$ T cells of experimental allergic encephalomyelitis mice

Flow cytometry and western blot analysis showed that PD-L1 expression in splenic $CD4^+$ T cells of experimental allergic encephalomyelitis mice was significantly increased compared with the control group (Figures 5, 6).

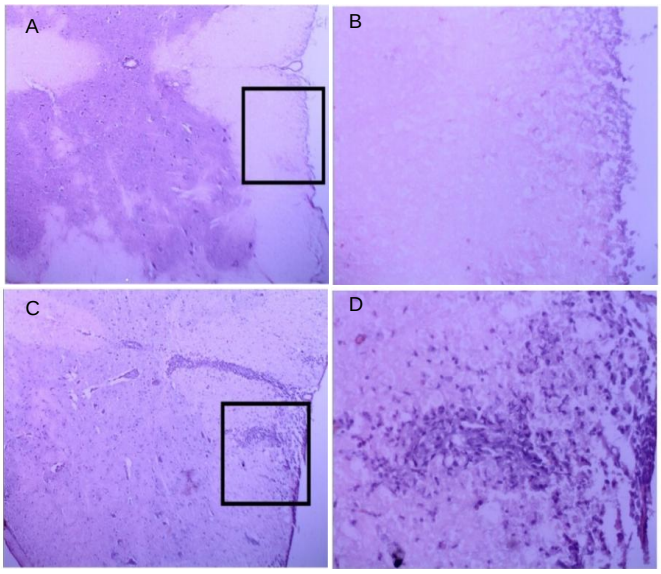

Figure 2 Pathological changes of spinal cord in experimental allergic encephalomyelitis mice (hematoxylin-eosin staining).

(A, B) Control group (A: × 100, B: × 400); (C, D) Model group at 2 days after onset (C: × 100, D: × 400). (B, D) Higher magnification views of boxed areas in A, C. In the experimental allergic encephalomyelitis mice, a large number of inflammatory cell infiltration was observed around a blood vessel in spinal cord tissue, consisting mainly of lymphocytes, and was also apparent below the spinal pia mater. No significant pathological changes were observed in spinal cord tissue from the control group.

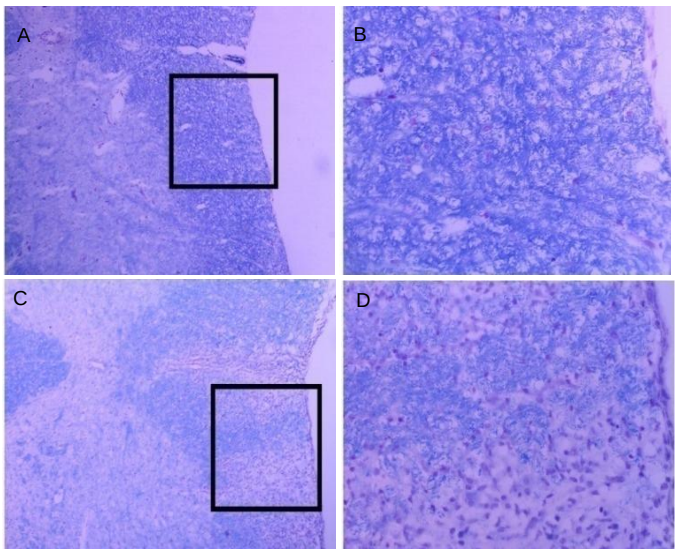

Figure 3 Myelin morphology of spinal cord from experimental allergic encephalomyelitis mice (Luxol fast-blue staining).

(A, B) In the control group, the myelin sheath was complete (A: × 100, B: × 400). (C, D) In the model group, spinal cord demyelination and inflammatory cell infiltration were seen at 2 days after onset (C: × 100, D: × 400). (B, D) Higher magnification views of boxed areas in A, C. In the experimental allergic encephalomyelitis mice, the myelin sheath disintegrated and was lost in the spinal cord of model mice, while it remained intact without in the control group.

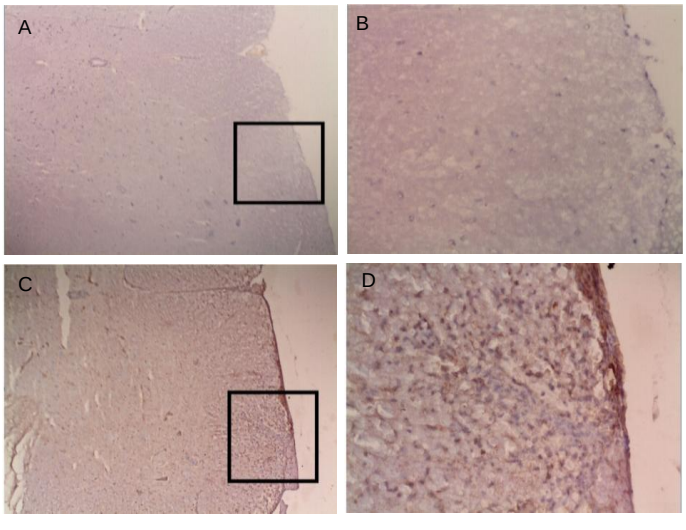

Figure 4 PD-L1 immunoreactivity in the spinal cord of experimental allergic encephalomyelitis mice.

(A, B) Control group (A: × 100, B: × 400). (C, D) Model group at 2 days after onset (C: × 100, D: × 400). (B, D) Higher magnification views of boxed areas in A, C. In the experimental allergic encephalomyelitis mice, brown staining on infiltrating inflammatory cells indicated PD-L1 expression. In the control group, no inflammatory cells were present and PD-L1 molecule immunoreactivity was not observed.

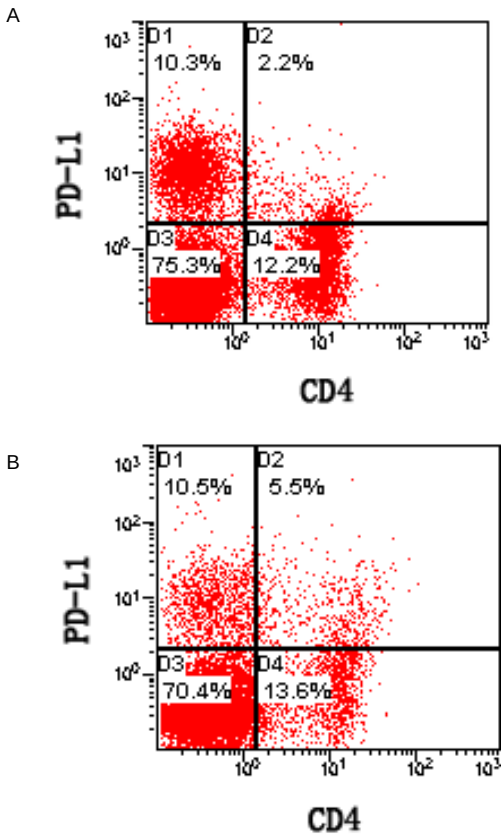

Figure 5 PD-L1 expression in splenic $CD4^+$ T cells from experimental allergic encephalomyelitis mice (flow cytometry).

(A) Control group; (B) model group. At 2 days after onset, PD-L1 expression in the splenic $CD4^+$ T cells of model mice was significantly increased compared with the control group. The left upper quadrant (D1) represents the ratio of PD-L1-positive $CD4^-$ T cells, the right upper quadrant (D2) represents the ratio of PD-L1-positive $CD4^+$ T cells, the left lower quadrant (D3) represents the ratio of PD-L1-negative $CD4^-$ T cells, and the right lower quadrant (D4) represents the ratio of PD-L1-negative $CD4^+$ T cells.

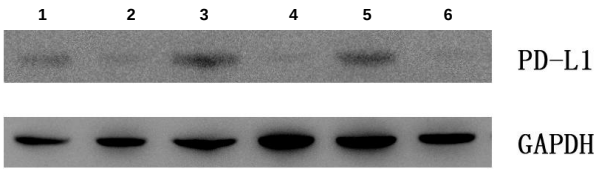

Figure 6 PD-L1 expression in splenic $CD4^+$ T cells from experimental allergic encephalomyelitis mice (western blot analysis).

(1, 3, 5) Protein samples extracted from the splenocytes of three different mice in the model group. (2, 4, 6) Protein samples extracted from the splenocytes of three different mice in the control group. GAPDH served as an internal reference. At 2 days after onset, PD-L1 expression in the splenic $CD4^+$ T cells from model mice was significantly increased compared with the control group.

DISCUSSION

Experimental allergic encephalomyelitis is a commonly used model of human multiple sclerosis because it has similar clinical symptoms, pathological processes and abnormal immune responses[7]. In this study, we utilized female C57BL/6J mice aged 8–10 weeks as experimental animals, and induced experimental allergic encephalomyelitis by immunizing them with MOG35-55 in complete Freund;s adjuvant.

Complete Freund's adjuvant is emulsified with MOG35-55 peptide to prepare a water-in-oil antigen emulsion. Different from previous studies that subcutaneously injected mice at several points on the back, this study used a novel method to immunize mice, consisting of a single subcutaneous injection into the mouse armpit. As the armpit contains abundant lymphoid tissue, injection into this area allows easy transport of antigen and is conducive to eliciting immunological reactions. In addition, single-point injection can avoid the tolerance of sensitized animals to antigens and accordingly reduce morbidity. This is an innovation of this study. Model mice developed clinical symptoms of experimental allergic encephalomyelitis at 10 days after antigen-induced immunization, including slow movement, piloerection, tail weakness, poor appetite, arched back, hind leg weakness, complete paralysis, incontinence, and death. These clinical symptoms are consistent with previous studies of experimental allergic encephalomyelitis and are similar to multiple sclerosis in humans[19]. Mice in the control group showed no symptoms.

Pathological staining revealed inflammatory cell infiltration was present below the spinal pia mater in the spinal cord of model mice, showing sleeve-like changes. Luxol fast-blue myelin staining demonstrated demyelination around the infiltrating inflammatory cells. These pathological characteristics are similar to pathological changes in the central nervous system of multiple sclerosis patients. The success rate of modeling was 85% in this study, similar to previous studies[19].

Experimental allergic encephalomyelitis is a $CD4^+$ Th1 cells-mediated autoimmune disease of the central nervous system and is a useful animal model for multiple sclerosis. This model has been used to study the regulatory effect of the PD-L1/PD-1 pathway in the pathogenesis of experimental allergic encephalomyelitis. Growing evidence[4, 14, 20] suggests that blocking the PD-1/PD-L1 pathway causes significant enhancement of

Th1-type inflammatory responses, thus, the PD-1/PD-L1 pathway might have an important role in inhibiting Th1-type autoimmune diseases. The PD-L1/PD-1 pathway is highly involved in the early activation of T cells[5]. Carreno *et al* [16, 21] found that PD-L1$^{-/-}$ mice were more susceptible to experimental allergic encephalomyelitis compared with wild-type mice, showing aggravated clinical symptoms. *In vitro* experiments showed that secretion of interferon-γ, tumor necrosis factor-α, interleukin-6 and interleukin-17 in pokeweed mitoglu-stimulated T cells was significantly increased compared with wild-type mice. These findings suggest the involvement of the PD-L1/PD-1 pathway in experimental allergic encephalomyelitis pathogenesis. Alan *et al* [22] showed that PD-L1 monoclonal antibody intervention aggravated the symptoms of experimental allergic encephalomyelitis animals, which also support the above conclusion.

Because the spleen is a peripheral immune organ composed of T cells, B cells and other immune cells, we utilized flow cytometry and western blot analysis to detect PD-L1 expression on the surface of mouse spleen lymphocytes. The results showed that spleen CD4$^+$ T cells in the model mice expressed significantly higher levels of PD-L1 than the control mice, consistent with a previous study[13]. Therefore, we speculate that detecting PD-L1 expression in peripheral blood lymphocytes of patients with multiple sclerosis may assist the evaluation and prediction of disease progression, remission, relapse and the treatment efficacy of multiple sclerosis.

PD-L1 is mainly expressed on immune cells, but also on vascular endothelial cells, pancreatic islet cells, astrocytes in the central nervous system, as well as placenta and cornea and other peripheral parenchymal cells[23-24], PD-L1 expression in immune-privileged organs such as brain, placenta and cornea suggests it might down-regulate the immune response of autoreactive T and B cells in peripheral tissues, thereby maintaining immune tolerance of peripheral organs, a mechanism associated with the inhibition of T cells-mediated immune damage to peripheral organs[14]. For example, PD-L1 is highly expressed in human placental syncytiotrophoblasts and the expression levels increase at 3 months of pregnancy and fluctuate in association with the blood oxygen concentration[25-26]. When the oxygen concentration decreased, the expression level sharply decreased and functioned to promote maternal-fetal immune tolerance[25-26]. The survival rate of fetuses in PD-L1-deficient females is significantly decreased[25-26]. Under physiological conditions, the blood-brain barrier controls and regulates proteins, nutrients and cells, which are transferred from peripheral tissues to the central nervous system[27-28]. The blood- brain barrier restricts brain-derived antigens from entering lymphoid organs, thus reducing the production of histocompatibility complexes in brain. The microenvironment of the brain promotes the apoptosis of inflammatory cells invading the brain, and the central nervous system is considered an immune-privileged organ. Recent studies addressing central nervous system infection, cancer and autoimmune disease[29] showed that immune responses could occur in the central nervous system. When inflammatory responses occur, many immune cells such as microglia cells and lymphocytes become activated and infiltrate the central nervous system[30]. In multiple sclerosis patients, the blood-brain barrier is damaged, resulting in peripheral immune cell infiltration to the central nervous system[31]. Multiple sclerosis lesions are thought to be caused by macrophages and T cells such as CD4$^+$ T cells, although increasing attention has been paid to the involvement of CD8$^+$ T cells[32-34]. The number of CD8$^+$ T cells was markedly higher than CD4$^+$ T cells in the brain parenchyma of multiple sclerosis patients[35-40].

Previous studies[18, 41] induced experimental allergic encephalomyelitis in wild-type and PD-L1$^{-/-}$ C57BL/6J mice using MOG35-55 and showed that PD-L1$^{-/-}$ C57BL/6J mice developed early onset and rapid progression of clinical symptoms. The number of myelin-specific T cells in the central nervous system was significantly higher than in wild-type mice, and the symptoms were still visible at late stage of disease. An explanation for this is that after PD-L1/PD-1 signaling pathways are eliminated, negative synergistic signaling pathways that mediate T cells and other cells associated with the central nervous system are also inhibited. Furthermore, immunohistochemistry analysis demonstrated higher PD-L1 expression levels in the central nervous system of patients with multiple sclerosis compared with controls. Wiesemann *et al* [42-43] found that PD-L1/PD-1 pathway molecule expression in immune cells was significantly increased after interferon-β treatment in multiple sclerosis patients. This suggested the PD-L1/PD-1 pathway plays an important role in human central nervous system autoimmune disease. Recently Dong *et al* [44] found PD-L1 autoimmune antibodies in the serum of patients with rheumatoid arthritis, indicating that blocking the inhibition of PD-L1/PD-1 signaling pathways on immune responses by autoimmune antibodies might be an underlying mechanism of autoimmune diseases. Pittet *et al* [34, 45] demonstrated increased PD-L1 expression in vascular endothelial cells of the central nervous system of multiple sclerosis patients with inflammatory responses. In addi-

tion, antibodies blocking PD-L1 significantly increased the infiltration of $CD4^+$ T cells and $CD8^+$ T cells in the central nervous system, and PD-L1 expression in white matter and around blood vessels was also upregulated. These changes were associated with a lack of PD-1 receptor on the surface of $CD8^+$ T cells. Thus, we speculate that blood-brain barrier destruction in multiple sclerosis patients results in the decline of its ability to control cells into the central nervous system and the high expression of PD-L1. However, PD-1 receptor-deficient $CD8^+$ T cells preferably cross the blood-brain barrier and enter the spinal cord parenchyma. Ortler *et al* [17] also demonstrated that PD-L1 down-regulated T cell-mediated inflammatory responses, thereby controlling immune destruction in the central nervous system of patients with multiple sclerosis. More recently, the PD-L1/PD-1 pathway was shown to be a potential mechanism for regulating APC and T cell interactions by affecting virus-induced demyelination and autoimmune responses[46]. Trabattoni *et al* [47] proposed the importance of the PD-1/PD-L1 pathway on the regulation of immune functions in multiple sclerosis patients. Thus, monitoring these proteins may provide diagnostic and therapeutic targets.

In this study, spinal cord lesions of experimental allergic encephalomyelitis mice at acute onset phase were analyzed using immunohistochemical staining of PD-L1. Our findings showed that PD-L1 expression in the spinal cord of model mice was significantly increased compared with the control group, consistent with previous studies[41-48]. Flow cytometry and western blot analysis showed that PD-L1 expression in splenic $CD4^+$ T cells of model mice was significantly increased compared with the control group. Further study is needed to investigate the role of PD-L1 molecules in disease development, remission, relapse and treatment of human multiple sclerosis.

MATERIALS AND METHODS

Design

A group controlled study.

Time and setting

Experiments were performed from October 2008 to December 2009 at the Institute of Clinical Immunology, Soochow University, and Red Cross Blood Center of Suzhou City, China.

Materials

Thirty clean female C57BL/6J mice, aged 6–8 weeks, were provided by Shanghai Experimental Animal Center, Chinese Academy of Sciences (license number SCXK (Hu) 2007-0005). All mice were housed in the Experimental Animal Center of Suzhou University (license number SYXK (Jiangsu) 2007-0035) for 2 weeks, in a specific pathogen free level, at 18–22°C and 50–60% humidity, with access to food and water *ad libitum*. The ammonia concentration was less than 20 ppm and ventilation rate reached 10–20 times/h.

Methods

Establishment of experimental allergic encephalomyelitis

Experimental allergic encephalomyelitis was induced using myelin oligodendrocyte glycoprotein (MOG35-55; GL Biochem (Shanghai) Ltd., Shanghai, China), complete Freund's adjuvant (Sigma, St. Louis, MO, USA) and Bordetella pertussis toxin (Biosource Corporation, San Diego, CA, USA) injected into C57BL/6J mice. Complete Freund's adjuvant (10 mg/mL) was completely emulsified with MOG35-55 peptide (6 mg/mL) on ice, to prepare a water-in-oil antigen emulsion. The emulsion (100 μL) was then subcutaneously injected into single point in the armpit to immunize mice. On the day of immunization and 24 hours after immunization, mice were injected with Bordetella pertussis toxin (0.2 mg/mL) 100 μL into the tai veins. The control group was injected with an equal volume of PBS (Sigma). There was a 30 day period from the purchase of mice to the establishment of disease. The success of disease induction was defined as the clinical symptoms of slow movement, piloerection, and tail weakness.

Clinical observation and neurological function score

All mice were clinically observed and evaluated for neurological function scores at 9:00 daily after immunization, for 60 days. Neurological function were scored on a 5-point scale[7]: 0 point: no clinical symptoms; 0.5 point: partial tail paralysis; 1 point: complete tail paralysis and dragging; 2 points: tail paralysis and ataxia; 2.5 points: tail paralysis and unilateral hind limb paralysis; 3 points: tail paralysis and paralysis of both hind limbs; 3.5 points: tail paralysis, paralysis of both hind limbs and forelimb weakness; 4 points: tail paralysis, paralysis of both hind limbs and forelimb paralysis; 5 points: incontinence, moribund state, unresponsive to outside or death. Higher neurological scores indicated more severe clinical symptoms.

Hematoxylin-eosin staining of mouse spinal cord

Mice in two groups were euthanized at 2 days after disease onset and hearts were perfused with PBS imme-

diately. Spinal cord was harvested and fixed in 4% paraformaldehyde for 6–8 hours, dehydrated in 30% sucrose at 4°C overnight, embedded using optimal cutting temperature compound and sliced. Then spinal cord tissue was sliced into frozen sections of 5 μm thickness, dried and fixed in formalin. After washes, sections were stained with hematoxylin, rinsed with running water for 20 minutes and stained with 0.5% eosin for 10 seconds. The stained sections were dehydrated in 95% alcohol and 100% alcohol, xylene transparency, and neutral gum mounting. Sections were observed under an optical microscope (Olympus, Tokyo, Japan).

Luxol fast-blue myelin staining

Spinal cord frozen slices were placed in an anhydrous ethanol:chloroform solution (1:1) for 5 minutes, then in 95% ethanol for 5 minutes, and stained with 0.1% Luxol fast-blue overnight at 56°C. Slices were transferred to 95% ethanol solution for 5 minutes and 70% ethanol solution for 3 minutes, rinsed with double distilled water for 3 minutes, then differentiated with 0.05% lithium carbonate solution for 5 minutes and with 70% ethanol solution for 30 seconds. After double distilled water washing, slices were differentiated in 70% ethanol solution for 3 minutes and stained with 0.5% eosin for 1 minute, followed by counterstaining in 0.1% cresyl violet solution for 30 seconds, conventional dehydration, transparency, and neutral gum mounting. Between each step, sections were rinsed with double distilled water. Sections were observed under an optical microscope (Olympus).

Immunohistochemical detection of PD-L1 expression in spinal cord

Frozen sections were fixed in acetone at room temperature after drying and 3% H_2O_2 was applied to eliminate endogenous peroxidase. Then sections were blocked using 10% normal calf serum and incubated with rabbit anti-mouse PD-L1 monoclonal antibody (1:100; Santa Cruze Biotechnology, Santa Cruz, California, USA) at 37°C for 1–2 hours and with biotin-labeled anti-rabbit secondary antibody (1:100; Wuhan Boster Biological Company, Wuhan, Hubei Province, China). Sections were developed with 3,3'-diaminobenzidine, counterstained with hematoxylin, mounted with neutral gum, and observed under an optical microscope.

Flow cytometry for PD-L1 expression in splenic $CD4^+$ T cells

At 2 days after disease onset, mice from both groups were intraperitoneally anesthetized using 1% sodium pentobarbital (100 μL) and the spleen was harvested and grinded through a 100 mesh sieve. Cell suspension was centrifuged at 1 000 r/min for 5 minutes, with a centrifugal radius of 10 cm. After the supernatant was removed, slices were lysated with erythrocyte lysate solution 0.5 mL on ice for 2 minutes, neutralized using PBS 15 mL and centrifuged at 1 000 r/min for 5 minutes, with a centrifugal radius of 10 cm. Then slices were rinsed with PBS three times after the supernatant was removed. An equal volume of PBS was taken as a negative control. All slices were incubated with anti-mouse CD4-FITC and PD-L1-PE antibody (1:100; eBioscience Corporation, San Diego, CA, USA) at 4°C for 30 minutes, rinsed with PBS and detected by flow cytometry (Beckman Corporation, Bremen, Germany).

Western blot analysis of PD-L1 expression in splenic $CD4^+$ T cells

At 2 days after the onset, equal amounts of mouse splenocytes from the two groups were selected and added to protein loading buffer, denatured at boiling water for 10 minutes and centrifuged at 10 000 r/min for 2 minutes (centrifugal radius 4 cm). The supernatant was transferring to EP tube and stored in –20°C refrigerator. Then samples were subject to electrophoresis at 4°C, 0.2 A current, transferred to a membrane for 90 minutes, and blocked with TTBS containing 5% skim milk for 1 hour at room temperature. The membranes were incubated with rabbit anti-mouse PD-L1 monoclonal antibody (1:500; Santa Cruze Biotechnology) and hybridized with specific antigen bands; incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (1:100; Boster Biotechnology) and hybridized at room temperature for 1 hour. Between each incubation step, membranes were rinsed with TTBS four times for 10 minutes each. Afterwards the membranes were added to SuperSignal west Pico Chemiluminescent Substrate (Thermo; Santa Cruze Biotechnology) for chemiluminescence detection, while GAPDH served as an internal control. Images were determined using western blot fluorescence imaging analyzer (Fujifilm, Tokyo, Japan).

Statistical analysis

The data were expressed as the mean and analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA). Differences among groups were compared with one-way analysis of variance. A $P < 0.05$ value was considered significantly difference.

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