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Original Article

ERβ-selective agonist alleviates inflammation in a multiple sclerosis model via regulation of MHC II in microglia

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Abstract: Multiple sclerosis (MS) is an autoimmune, demyelinating, and neurodegenerative disease of the central nervous system (CNS) that affects 2-2.5 million people worldwide. Although the etiology of MS is not well known, MS is widely considered to be an autoimmune disease. Currently approved MS drugs reduce relapse rates but fail to reverse or prevent neurodegeneration and disability progression. Increasing evidence indicates that microglia and major histocompatibility complex class II (MHC II) expression in these cells play important roles in the pathophysiology of MS. For a T cell to contribute to CNS pathogenesis, it must be reactivated by antigen-presenting cells within the CNS parenchyma. Susceptibility to MS is associated with MHC II genes, suggesting that presentation of antigens on MHC II plays an important role in CD4+ T-cell reactivation and disease initiation. An ERβ-selective agonist was previously reported to suppress reactivation of T cells invading the spinal cord, thereby reducing the severity of symptoms and decreasing mortality in the first 2 weeks after disease onset. However, the mechanism by which the expression of MHC II in microglia is regulated by ERβ-selective agonists is still unclear. Therefore, we hypothesize that ERβ-selective agonists inhibit MHC II expression in microglia via inhibition of class II trans-activator (CIITA) expression by a mechanism involving inhibition of the translocation of IFNγ regulatory factor (IRF-1) to the nucleus, thereby inhibiting the inflammatory response and symptoms in the MS model.

Keywords: Multiple sclerosis, experimental autoimmune encephalomyelitis, major histocompatibility complex class II, ERβ, microglia

Introduction

MS is an autoimmune, demyelinating, and neurodegenerative disease of the central nervous system (CNS) that affects millions of people worldwide [1]. MS comprises a blood-brain-barrier (BBB) disruption accompanied by an activation of macrophages/microglia as well as T- and B-cell infiltration into the CNS, ultimately resulting in demyelination and degeneration of neurons [2]. Although the etiology of MS is not well known, MS is widely considered to be an autoimmune disease, and immune cells are thought to play important roles in the initiation and progression of MS. Currently approved MS drugs reduce relapse rates but fail to reverse or prevent neurodegeneration and disability progression. Most immunosuppressive drugs used for treating MS patients have certain side effects. Many MS patients in South Scotland who were treated with recombinant interferon beta were recently reported to experience thrombotic microangiopathy [3]. Therefore, many new drugs are being investigated and developed, and better therapeutic effects and fewer side effects are becoming the primary goals in the treatment of MS.

Recent genome-wide association studies (GWASs) have provided strong support for the classification of MS as an autoimmune disease by identifying >50 susceptibility loci associated

with MS, of which the vast majority represent genes with immune cell function [4]. For over 40 years, linkage studies in MS have shown that the strongest genetic influence by far is genes encoding MHC II molecules, potentially indicating a role for CD4+ T cells in MS and suggesting that presentation of antigens such as myelin proteolipid protein (PLP) and myelin basic protein (*MBP*) on MHC II plays an important role in CD4+ T-cell reactivation and disease initiation [4].

Microglia, the resident innate immune cells of the CNS, have been shown to be the main antigen-presenting cells of the CNS [5, 6]. These cells normally express low levels of MHC II proteins; however, in inflammatory or neurodegenerative conditions, activated microglia highly upregulate MHC II and costimulatory molecules [7]. MHC II is abundantly expressed in microglia and may serve as a pivotal mediator of CD4+ T-cell reactivation during the course of EAE induction [8]. Microglia also assist in the reactivation of T cells once they enter the CNS parenchyma. For a T cell to contribute to CNS pathogenesis, it must be reactivated by antigen-presenting cells within the CNS parenchyma. Macrophages/microglia have been observed to upregulate MHC II molecules when they are activated [9]. These observations suggest that activated microglia are capable of reactivating primed T cells entering the CNS.

Nuclear receptors are ligand-activated *transcription factors* that regulate gene expression by interacting with specific DNA sequences. Targets of 13% of drugs approved by the FDA are nuclear receptors [10]. Recent studies have examined the effect of one nuclear receptor, ERβ, on the CNS, and a large amount of research has investigated its role in cancer. The ERβ-selective agonist LY3201 has been shown to suppress reactivation of T cells invading the spinal cord in EAE mice, reducing the severity of symptoms and decreasing mortality in the first 2 weeks after disease onset [11]. Increasing evidence indicates a central role for microglial MHC II in the activation of adaptive immune responses in EAE and suggests that MHC II signaling may be a target of neuroprotective therapies for the disease [8, 9, 11].

MHC II molecules are cell-surface glycoproteins that present antigenic peptides to CD4+ T lymphocytes of the adaptive immune system [12]. MHC II expression is primarily regulated at the level of transcription by several well-studied elements within the MHC II proximal promoter, which bind a series of ubiquitously expressed transcription factors to form a basal enhanceosome complex [13-15]. The regulation of MHC II protein expression is under the control of a complex cytokine network that requires the coordinated action of multiple transcription factors and coactivators. Class II transactivator (CIITA) is the master regulator of MHC II and is critical for the initiation of adaptive immune responses [16]. CIITA-deficient mice lack inducible MHC II expression and have sparse constitutive MHC II expression on subsets of thymic stromal cells [17]. IFNγ regulatory factor-1 (IRF-1), a transcription factor essential for CIITA expression, must translocate to the nucleus and bind to the promoter IV of CIITA and thus regulate MHC II production [18].

Several molecules, including chemokines and steroid hormones, are known as ligands that regulate microglia activation status and mediate signaling via different nuclear receptors [19]. ER ligands, through ERα or ERβ, can inhibit NF-κB transcriptional activity and significantly inhibit inflammatory responses in microglia [20]. In this respect, stimulation of ERβ with synthetic ligands efficiently reduces microglial activation and thereby reduces EAE symptoms [11, 21]. Activation of ERβ decreases NO production and iNOS expression in response to LPS stimulation of BV-2 microglia in which only ERβ is expressed [22]. However, Vegeto *et al*. identified ERα as the receptor modulating microglial activity [23]. There remain differences in opinion among laboratories as to whether ERα or ERβ is responsible for the anti-inflammatory effects of estrogen on microglia [24]. However, we have clear evidence that ERβ is the unique estrogen receptor expressed in microglia [11]. The action of ERβ selective agonists on both microglia and T cells suggests that these drugs may be potential candidates for therapeutic intervention in MS and perhaps other neurodegenerative diseases in which both cell types play crucial roles in the pathogenesis.

Many MS models have been developed. However, the classic and most widely used animal model for MS is EAE. No single model replicates the full spectrum of inflammatory mechanisms

and neurodegeneration seen in MS, just as individual patients manifest only a subset of the diverse features of the disease. Because EAE is initiated by immunization with autoantigens presented to MHC II-restricted CD4+ T cells, the model is ideally suited to study MHC II in microglia and its role in the reactivation of CD4+ T cells [25].

In the present study, we used an ERβ-selective agonist diarylpropionitrile (DPN) in the EAE mouse model to demonstrate that DPN can inhibit MHC II expression in microglia via inhibition of *CIITA* expression by a mechanism involving inhibition of the translocation of IRF-1 to the nucleus, thereby inhibiting the inflammatory response and symptoms in the MS model. These findings may contribute to the development or screening of highly ERβ-selective agonists to treat MS.

Methods and results

Animals, EAE model, and tissue preparation

Thirty 8-week-old C57BL/6 female mice were divided randomly into three groups: (1) normal (n = 10), (2) EAE+NS (n = 10), and (3) EAE+DPN (Tocris Bioscience) (n = 10). Mice from the second and third groups were immunized with s.c. injections of 50 μL of a 1 mg/mL solution of PLP (PLP139-151, Hooke Kit™ PLP139-151/CFA Emulsion PTX, No. EK-21201) in equal volumes of 0.9% sodium chloride (*normal saline* or *NS*) and complete Freund's adjuvant containing the Mycobacterium tuberculosis strain H37RA into the shoulders and flanks. Then, 200 ng of pertussis toxin was given in an i.p. injection on the day of PLP injection and again 2 days later. Mice from the second group and third group were administered s.c. injections of NS and DPN at 8 mg/kg per 48 h, respectively, beginning at EAE postinduction day 0 and continuing every other day until day 42 (end of experiment) [11, 26]. Mice were killed on day 42 with 60 mg/kg pentobarbital (Mebunat; Orion Pharma) and transcardially perfused with saline followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). All spinal cords were dissected and postfixed in the same fixative overnight at 4°C. After fixation, lumbar spinal cords were processed for paraffin sections (5 μm). All experiments were approved by the Ethics Committee of Chongqing Medical University.

HE staining, immunohistochemistry, and immunofluorescence

Paraffin sections were deparaffinized in xylene and rehydrated through graded alcohol. Spinal sections were used for hematoxylin and eosin staining.

Paraffin sections were deparaffinized in xylene, rehydrated through graded alcohol, and processed for antigen retrieval by boiling in 10 mM citrate buffer (pH 6.0) for 12-15 min in the PreTreatment module. Sections were incubated in 0.3% H_2O_2 in 50% methanol for 30 min at room temperature to quench endogenous peroxidase. Sections were then incubated in 3% BSA for 30 min to block nonspecific binding, after which a biotin-blocking system (Dako) was used to block endogenous biotin. Sections were then incubated with anti-Iba1 (1:400; Abcam) at 4°C after blocking nonspecific binding in 3% BSA. BSA replaced primary antibodies in negative controls. After the sections were washed, they were incubated with an HRP polymer kit (GHP-516; Biocare Medical) for 30 min at room temperature, followed by 3,3'-diaminobenzidine tetrahydrochloride as the chromogen. For cell counting, we took every fifth slice from 25 consecutive slices, that is, five slices from each mouse. For immunofluorescence, sections were incubated overnight with anti-MBP (1:500; Abcam) and anti-NF-200 (1:500; Abcam) at 4°C after blocking nonspecific binding in 3% BSA. Primary antibodies were detected with secondary antibodies. Sections were later counterstained with DAPI (Vector Laboratories) to label nuclei. Iba1+, NF-200+ and MBP+ staining intensities were quantified using Image Pro Plus 6.0.

The dorsal funiculus of the spinal cords was removed and then postfixed at least 1 day for the next steps as previously reported. A magnification of 5000 was obtained using transmission electron microscopy. The ratio of axon diameter to total fiber diameter (g-ratio) was measured by dividing the circumference of an axon without myelin by the circumference of the same axon including myelin [27].

Primary microglial cultures

Microglial cells were isolated from P1-P3 C57BL/6 mice. Briefly, brains were dissected, minced, and trypsinized with 0.25% trypsin-EDTA

for 20 min at 37°C. The reaction was stopped by the addition of horse serum, and the tissue was triturated with a Pasteur pipette and consecutively filtered through 70- and 45-μm pore size nylon cell strainers. Cells were resuspended in Dulbecco's-modified Eagle's medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin and plated in 80-mm^2 tissue culture flasks. After 10 days, the flasks were gently shaken for 1 h, and the medium was harvested and centrifuged for 10 min at 1000×g for microglial cell collection. The microglia were resuspended in the medium described above and plated in either 12-well plates at a density of 750,000 cells/well or 8-chamber slides at a density of 150,000 cells/chamber. Primary microglia were divided into three groups: (1) normal (PBS-treated microglia), (2) PLP-treated microglia, and (3) PLP and DPN-treated microglia. Cells from the second group were treated with 50 μg/mL PLP for 6 h. However, cells from the third group were treated with 50 μg/mL PLP for 6 h after being treated with 25 nM DPN for 6 h.

Immunocytochemistry

Primary microglial cells were treated as described above. Cells were fixed with cold 100% methanol for 10 min followed by a 10-min incubation in 10% normal goat serum to block nonspecific binding. Cells were stained with anti-Iba1 (1:400; Abcam) and anti-MHC II (1:200; Abcam) at a 1:500 dilution for 1 h at room temperature followed by a 30-min incubation. Primary antibodies were detected with corresponding secondary antibodies. Sections were later counterstained with DAPI (Vector Laboratories) to label nuclei. Iba1+ and MHC II+ staining intensities were quantified using Image Pro Plus 6.0.

Western blot analysis

Whole-cell extract was prepared by lysing the cells in 2× lysis buffer (20 mM Tris, 2 mM EDTA, 1 mM Na3V04, 2 mM dithiothreitol, 2% SDS, 20% glycerol). Proteins (5-20 μg/lane) were separated by 10% SDS-PAGE and transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). Membranes were blocked in 5% nonfat milk for 1 h at room temperature followed by incubation with primary antibodies. The MHC II antibody (Santa Cruz Biotechnology, Santa Cruz, CA), IRF-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and CIITA antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were used at a 1:500 dilution overnight at 4°C. After washing, the membranes were incubated with appropriate secondary horseradish peroxidase-conjugated antibodies for 1 h at room temperature followed by detection with chemiluminescence.

Nuclear extract preparation

Primary microglial cells were treated as described above. Nuclear extracts were prepared using a NucBuster protein extraction kit (71183; Novagen) according to the manufacturer's protocol. Nuclear proteins were then separated by 10% SDS-PAGE and analyzed by Western blot.

Cytokine assay

Mice were deeply anesthetized; their spleens were removed and dissociated through a 70-μM cell strainer, and their red blood cells were hypotonically lysed. CD4 T cells were positively selected using the FlowComp CD4 isolation kit according to manufacturer's protocols (Life Technologies, Invitrogen). Isolated CD4 T cells were cocultured with primary microglia for 60 h in RPMI supplemented with 10% heat-inactive FBS, 1% penicillin/streptomycin, 1% sodium pyruvate, 1% nonessential amino acids, 1% L-glutamine, and 0.1% β-mercaptoethanol during the 60-h coculture. Immediately following coculture, conditioned medium was removed and frozen at -80°C for multiplex ELISA analysis.

Flow cytometry

Flow cytometric analysis was performed on a BD FACSCalibur flow cytometer (BD Biosciences). Spleens were harvested, and the cells were surface stained with an antibody against CD4 (IMG-5922A, Novus Biologicals). Then, the cells were washed, fixed, and permeabilized with Cytofix/Cytoperm buffer (BD Biosciences), and intracellular cytokines were stained with Abs against IL17A and IFN-γ. Data were collected and analyzed using CellQuest Pro software (BD Biosciences). Flow cytometry data were acquired with a FACS Canto II (BD Biosciences) and were analyzed with FlowJo software (Tree star, Ashland, OR, USA).

ChIP-qPCR (Fold Enrichment Method)

Primary microglia were cross-linked with 1% formaldehyde at room temperature for 10 min, washed twice with ice-cold PBS, collected in 1 ml of PBS (3×10^7 cells per tube) and centrifuged in a bench-top microfuge for 5 min at 5000 r.p.m. Cells were resuspended in 1 ml of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8) plus protease inhibitors (aprotinin, leupeptin and pepstatin), incubated on ice for 10 min and sonicated to an average size of 500 bp. A 100-µl aliquot of sonicated chromatin (3×10^6 cell equivalents) was used per immunoprecipitation. Chromatin was diluted in 1 ml of buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl pH 8) and pre-cleared with 2 µg of sheared salmon sperm DNA and protein A-sepharose (Sigma) (45 µl of 50% slurry in 10 mM Tris-HCl pH 8, 1 mM EDTA) for 2 h at 4°C. Immunoprecipitation (IP) was performed overnight at 4°C with no antibody, the anti-IRF1 antibody (Abcam) or the anti-CIITA antibody (Santa Cruz). A 45-µl aliquot of protein A-sepharose, 2 µg of salmon sperm DNA and 45 µl of yeast tRNA were added per IP and incubated for 1 h. Precipitates were washed sequentially for 10 min in 1× TSEI (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8, 150 mM NaCl), 4× TSEII (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8, 500 mM NaCl), 1× buffer III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8) and 3× TE (10 mM Tris-HCl pH 8, 1 mM EDTA). Samples were extracted twice with 250 µl of elution buffer (1% SDS, 0.1 M $NaHCO_3$) and heated at 65°C overnight to reverse cross-links, and DNA fragments were purified with a QIAEX II Gel Extraction Kit. A 3-µl aliquot from a total of 50 µl was used in the PCR. CIITA promoter IV-specific primers were 5'-TTGGACTGAGTTGGAGAG-3' and 5'-GTGACCTTGAGCAAGTAG-3'. MHC II promoter primers were as follows: 5'-TCCAATGAACGGAGTATCTTGTGT-3' and 5'-TGAGATGACGCATCTGTTGCT-3'.

Data analysis

All in vitro experiments were repeated at least four times. Data are expressed as the mean ± SD. Statistical comparisons were performed using one-way ANOVA. $P < 0.05$ was considered to indicate statistical significance.

Results

The tissue damage and microglial activation in the spinal cord were alleviated by DPN treatment

The white matter in the spinal cord of the EAE model treated with NS (normal saline) was full of holes that were occupied by axons (**Figure 1B**). After treatment with DPN, the holes in the white matter were reduced (**Figure 1C**). The structure of the spinal cord was obviously in better condition after DPN treatment but was still worse than that of the normal group (**Figure 1A-C**). Focal lesions (in the dotted circle) were found in the EAE+NS group (**Figure 1E** and **1H**), characterized by loss of both axons and the surrounding myelin. However, the damage was ameliorated by DPN treatment (**Figure 1F** and **1I**).

Demyelination is one of the major histopathologic hallmarks in EAE and MS. The axons of normal mice had normal morphology, and the myelin sheaths were tightly packed (**Figure 1J**). The EAE+NS mice exhibited myelin and axonal loss (**Figure 1K**), but the loss of myelin was more severe than the loss of axons (**Figure 1G**). After DPN treatment, fewer myelin sheaths wrapped around the axons (**Figure 1L**).

Microglia, the resident innate immune cells of the CNS, were activated in the EAE model. In the normal group, the microglia were in a resting state (**Figure 1M**). However, in the EAE+NS mice, the microglia were activated, with many of them in an amoeboid form (**Figure 1N**). No microglia in the amoeboid form were observed in the spinal cord of the EAE model mice treated with DPN.

Microglial activation and MHC II expression in vitro were reduced by DPN

To confirm the findings that DPN can reduce microglial activation in vivo, primary microglia were treated with DPN before being treated with PLP. The group treated with PLP alone exhibited significant microglial activation (**Figure 2B** and **2E**) and more amoeboid microglia compared to the normal group (**Figure 2A** and **2D**). When the microglia were pretreated with DPN for 6 h, PLP did not cause microglial activation as strong (**Figure 2C** and **2F**) as that observed in the PLP group (**Figure 2B** and **2E**).

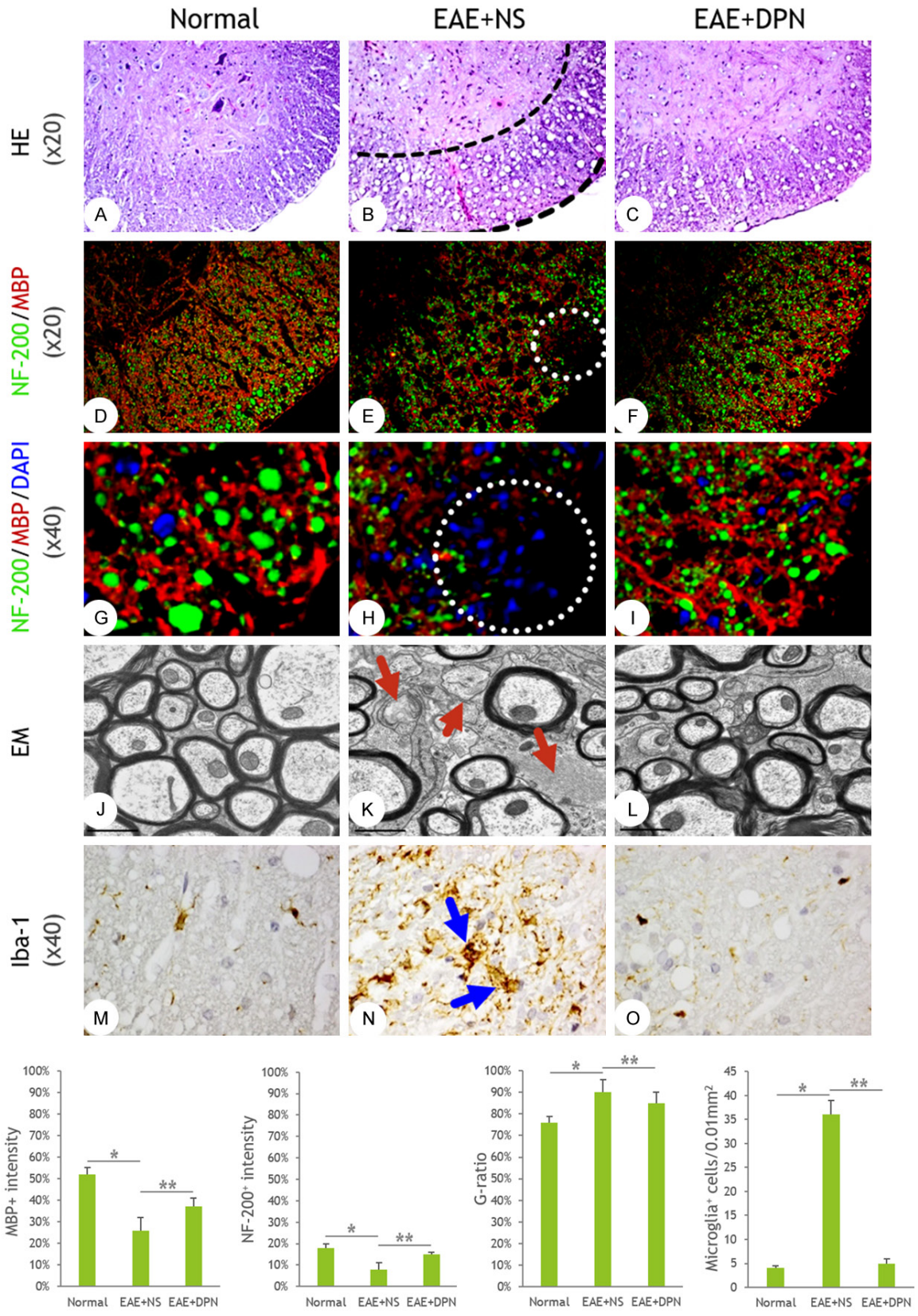

Figure 1. Tissue damage and microglial activation in the spinal cord. When compared to the normal group (A, D, G, J), morphology damage was observed in the EAE+NS group (B, E, H, K), such as loss of axons and myelin (area between dotted lines in B, dotted circle area in E and H, and area pointed by red arrows in K). However, the dam-

age was ameliorated in the DPN treatment group (C, F, I, L), with less myelin and axon loss. When compared to the normal group (M), many activated microglia in the amoeboid form (blue arrow in N) were observed in the EAE+NS group but not in the DPN treatment group (O). *P < 0.05,**P < 0.001.

Microglia are the main antigen-presenting cells of the CNS that normally express low levels of MHC II proteins. To determine the expression of MHC II after DPN treatment, we treated primary microglia with DPN for 6 h before treatment with PLP. In the control group, MHC II expression on the surface of microglia was weak (**Figure 2G** and **2J**). However, after treatment with PLP (**Figure 2H** and **2K**), MHC II expression increased significantly compared to the expression in the normal group. In the group treated with DPN (**Figure 1I** and **1L**), MHC expression was significantly reduced compared to the expression in the PLP group (**Figure 2H** and **2K**).

MHC II expression was regulated by DPN via the IRF-1/CIITA pathway

CIITA is the master regulator of MHC II expression. To determine the expression of MHC II, IRF-1, and CIITA, whole proteins were extracted. CIITA and MHC II expression was significantly decreased in primary microglia treated with DPN (**Figure 3A** and **3B**) when compared to the expression after PLP treatment alone. Moreover, CIITA expression was positively regulated by IRF-1. Furthermore, IRF-1 expression was significantly reduced in the PLP+DPN group compared to that in the group given PLP treatment alone (**Figure 3A** and **3B**).

However, IRF-1, which is essential for CIITA expression, must translocate to the nucleus and bind to promoter IV of CIITA and thus regulate MHC II production. Therefore, we extracted nuclear proteins and separated them by 10% SDS-PAGE. In the PLP treatment group, more IRF-1 translocated into the nucleus than in the normal group. However, in the PLP+DPN treatment group, much less IRF-1 translocated into the nucleus than in the PLP treatment group (**Figure 3C** and **3D**).

To determine whether DPN inhibits MHC II production by regulating the interaction between IRF-1 and the CIITA promoter and regulating the interaction between CIITA and the MHC II promoter in the nucleus, ChIP-qPCR (Fold Enrichment Method) experiments were performed using antibodies recognizing IRF-1 and CIITA. The immunoprecipitated DNA was then subjected to qPCR. After treatment with PLP alone, the relative enrichment of IRF-1 at the CIITA promoter was significantly increased compared to that in the normal group (**Figure 3E**). However, DPN treatment reduced the relative enrichment (**Figure 3E**). Furthermore, the relative enrichment of CIITA at the MHC II promoter increased significantly after treatment with PLP alone when compared to that in the normal group (**Figure 3F**). However, DPN treatment reduced the relative enrichment (**Figure 3F**).

Activation of CD4+ T cells was alleviated by DPN

In the MS and EAE model, CD4+ T cells infiltrated the central nervous system, producing a large number of cytokines and promoting central injury and myelin loss. To determine the effect of DPN on CD4+ T cells in EAE mice, flow cytometry analysis was used. The ability of CD4+ T cells to secrete IL17A (**Figure 4A** and **4B**) and IFNγ (**Figure 4C** and **4D**) was significantly lower in the DPN group than in the EAE group given NS alone.

To validate our findings in vivo, primary microglia from C57BL/6 mice were stimulated by PLP or PLP+DNP and then cocultured in CD3-coated plates with CD4+ T cells derived from the spleen of C57BL/6 mice. Cytokines from the supernatant of cocultured cells were detected by ELISA. The results showed that the concentrations of IL17A and IFNγ were significantly reduced in the DPN treatment group compared to those in the PLP group (**Figure 4E** and **4F**).

Discussion

Multiple sclerosis and its primary animal model, experimental autoimmune encephalomyelitis, are immune-mediated inflammatory diseases of the CNS characterized by demyelination, axonal loss and a heterogeneous clinical course. The most common presenting form of MS is relapsing-remitting, which affects approximately 85% of newly diagnosed patients [28]. Many disease-modifying therapies are currently

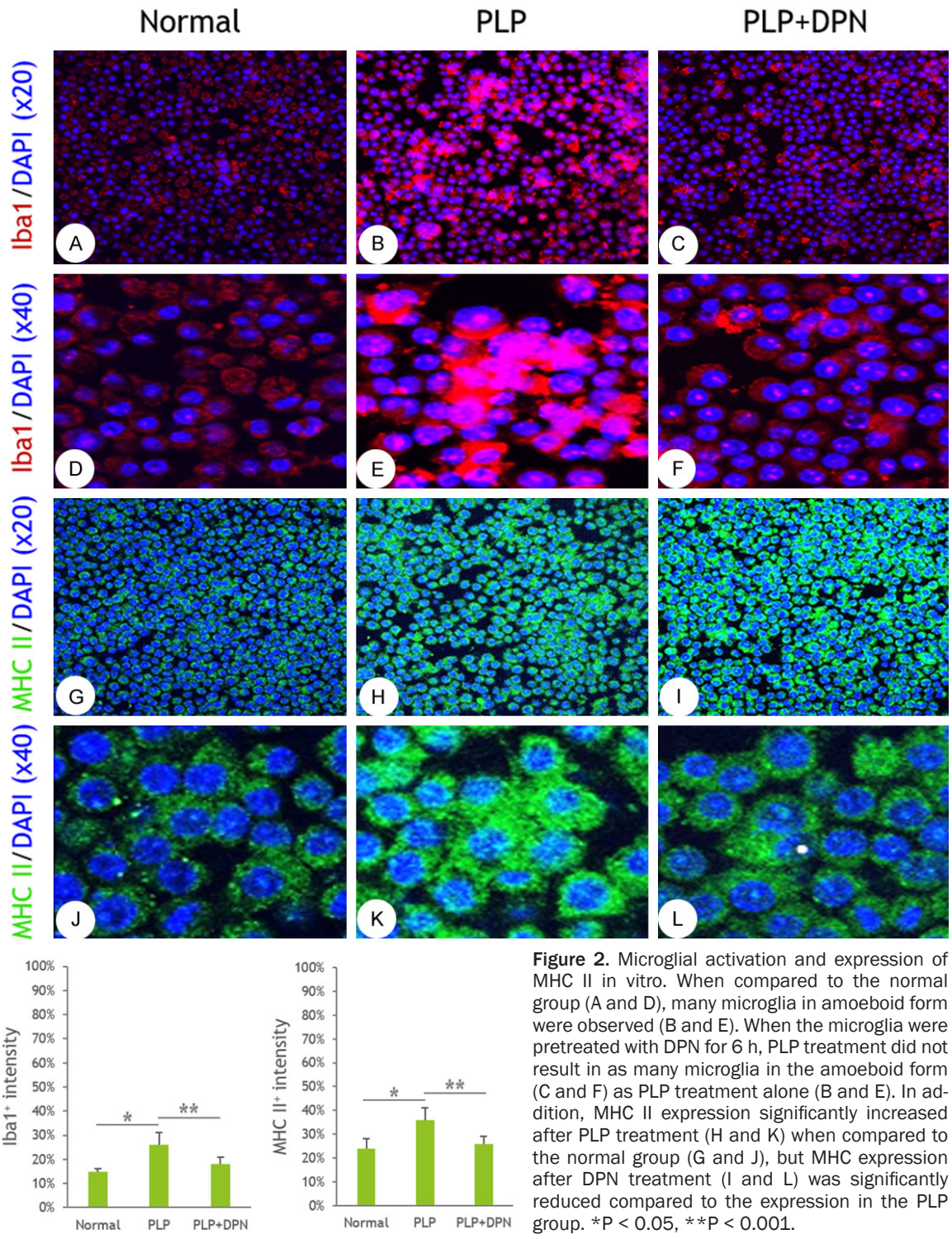

Figure 2. Microglial activation and expression of MHC II in vitro. When compared to the normal group (A and D), many microglia in amoeboid form were observed (B and E). When the microglia were pretreated with DPN for 6 h, PLP treatment did not result in as many microglia in the amoeboid form (C and F) as PLP treatment alone (B and E). In addition, MHC II expression significantly increased after PLP treatment (H and K) when compared to the normal group (G and J), but MHC expression after DPN treatment (I and L) was significantly reduced compared to the expression in the PLP group. *P < 0.05, **P < 0.001.

licensed for relapsing-remitting multiple sclerosis, but none of these have provided evidence of effectiveness in secondary progressive multiple sclerosis. Our previous findings suggest that the ERβ-selective agonist could suppress reactivation of T cells invading the spinal cord in EAE mice, thereby reducing the severity of symptoms and decreasing mortality in the first 2 weeks after disease onset [11].

In this study, consistent with previous findings, extensive demyelination was observed, charac-

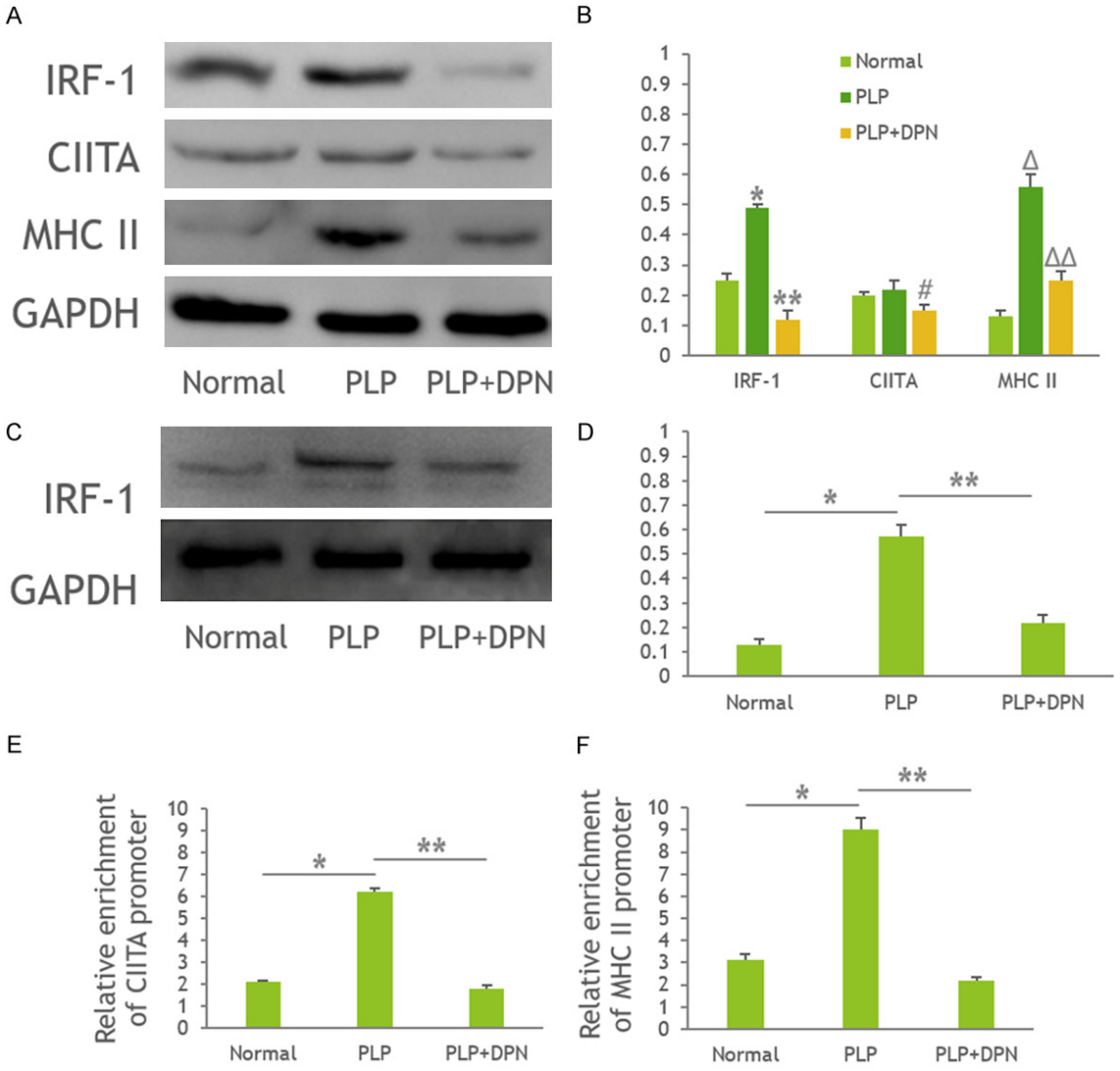

Figure 3. The expression of MHC II via the IRF-1/CIITA pathway. In the whole-cell extract, IRF-1 and CIITA expression was increased in the PLP group but significantly decreased in the primary microglia treated with DPN compared to that in the PLP group (A and B). In the nuclear protein extract, the PLP group exhibited more nuclear translocated IRF-1 than the normal group. However, the PLP+DPN treatment group exhibited less IRF-1 translocation into the nucleus than the PLP-treated group (C and D). The relative enrichment of both IRF-1 at the CIITA promoter (E) and CIITA at the MHC II promoter (F) were significantly greater in the PLP-treated group than in the normal group. However, DPN treatment reduced the relative enrichment (E and F). *P and #P< 0.05. **P, ΔP and ΔΔP<0.001.

terized by axon and myelin loss. Findings from transmission electron microscopy further discovered a great reduction in myelin sheaths. Axonal loss and gray matter damage have been regarded as the leading causes of irreversible neurological disability in progressive stages [28]. Demyelination and degeneration of neurons in MS is well known to be caused by a BBB disruption accompanied by an activation of macrophages/microglia as well as T- and B-cell infiltration into the CNS [19]. Our findings clearly show that microglia are activated in the amoeboid form in the EAE model or after treatment with PLP. However, treatment with an estrogen receptor β (ERβ)-selective agonist, DPN, ameliorated microglial activation and lessened the demyelination. Activated microglia induce the production of cytokines, nitric oxide, and reactive oxygen species, which in turn lead to neuronal mitochondrial dysfunction, energy failure and increased levels of intracellular calcium and sodium. Acidosis and

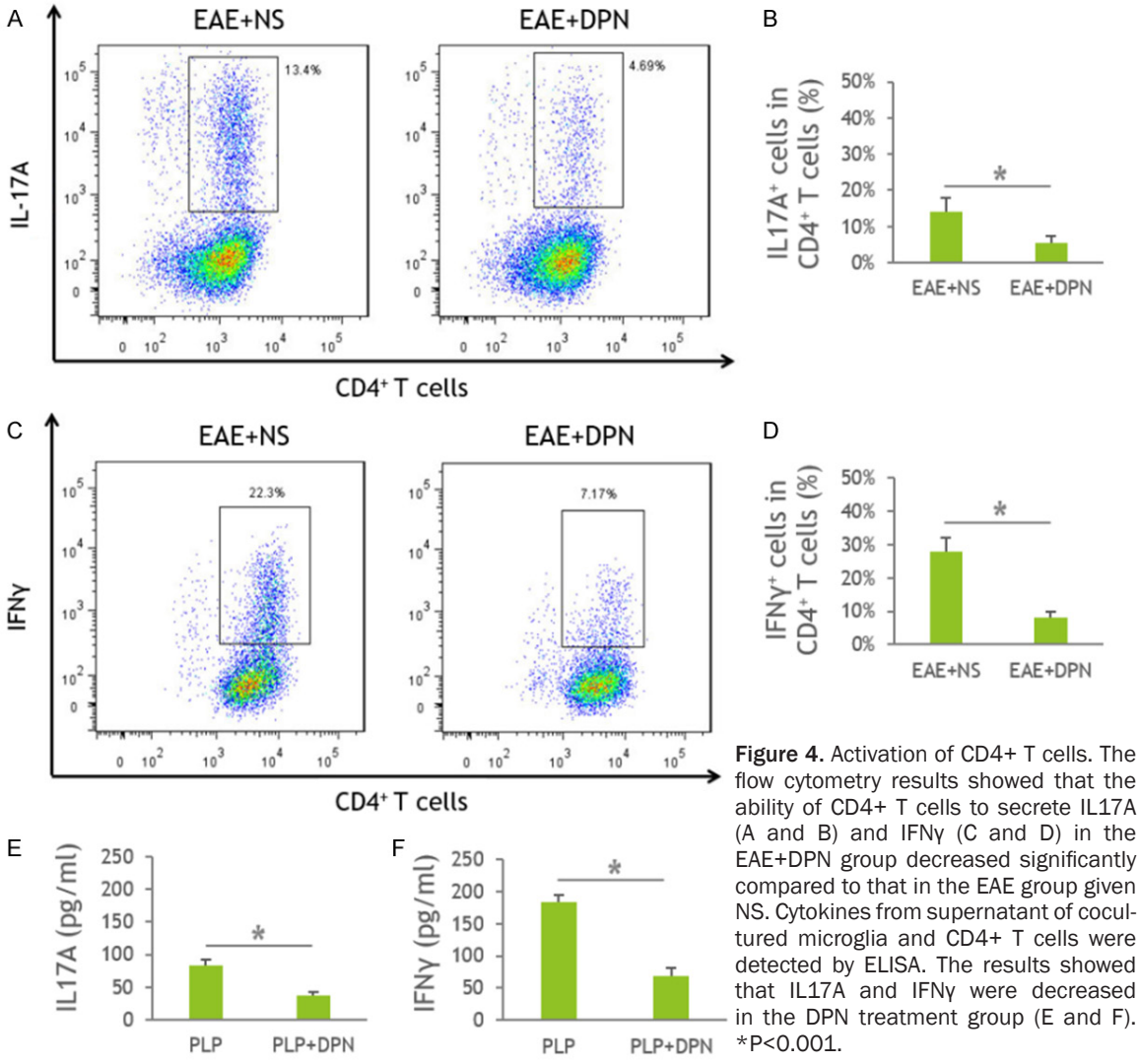

Figure 4. Activation of CD4+ T cells. The flow cytometry results showed that the ability of CD4+ T cells to secrete IL17A (A and B) and IFNγ (C and D) in the EAE+DPN group decreased significantly compared to that in the EAE group given NS. Cytokines from supernatant of cocultured microglia and CD4+ T cells were detected by ELISA. The results showed that IL17A and IFNγ were decreased in the DPN treatment group (E and F). *P<0.001.

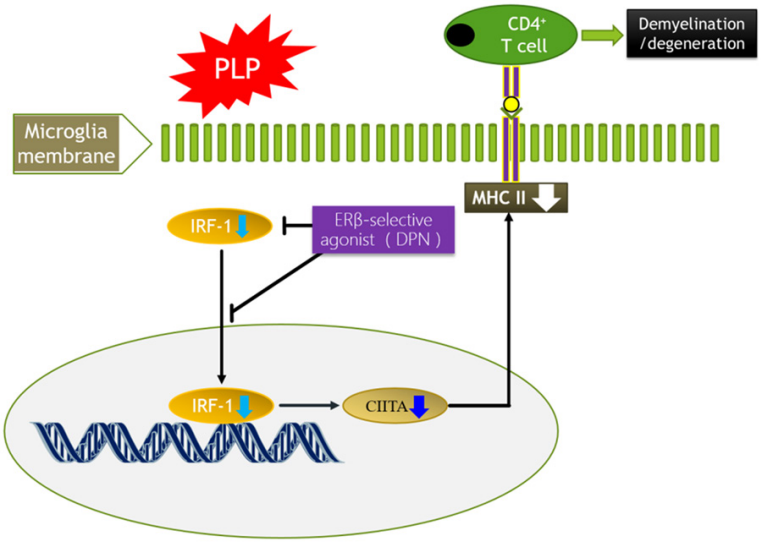

Figure 5. Schematic model depicting the regulation of MHC II by DPN.

glutamate-mediated excitotoxicity contribute to an increased intracellular level of calcium and, ultimately, apoptosis of oligodendrocytes and degeneration of axons and neuronal death [29].

Microglia, the main antigen-presenting cells of the CNS [5, 6], normally express low levels of MHC II proteins. However, in inflammatory or neurodegenerativeconditions,activatedmicroglia highly upregulate MHC II, which is consistent with our findings [7]. MHC II may serve as a pivotal mediator of CD4+ T-cell reactivation during the

course of EAE induction [8]. After treatment with DPN, the expression of MHC II on activated microglia is reduced, which suggests that ERβ-selective agonists may be candidates to reduce the inflammation in MS by targeting MHC II on microglia. Because MHC II expression in microglia is a requisite for T-cell reactivation, understanding the mechanism whereby ERβ-specific ligands repress MHC II expression and reactivation of T cells may elucidate critical pathways that can be targeted to treat EAE or MS.

Many genetic, environmental and endogenous factors are believed to be important elements driving inflammation, demyelination, and ultimately neurodegeneration in MS [30]. With the destruction of myelin, new myelin peptides are released, including myelin proteolipid protein and myelin basic protein. MHC II in activated microglia presents those peptides to the CD4+ T cells that can recognize the initiated myelin antigen and subsequently become reactivated. Therefore, MHC II signaling may be a target of neuroprotective therapies for the disease.

MHC II expression is primarily regulated by class II transactivator at the MHC II proximal promoter, which binds a series of ubiquitously expressed transcription factors to form a basal enhanceosome complex [13, 16]. In addition, CIITA expression is regulated by a transcription factor, IRF-1, which must translocate to the nucleus, bind to promoter IV of CIITA and thus regulate MHC II production [18]. We found that IRF-1, CIITA, and MHC II expression was increased after PLP treatment alone but decreased significantly in the primary microglia treated with DPN. Meanwhile, IRF-1 in the nucleus was highly increased after PLP treatment, but nuclear translocation was reduced by DPN treatment. Our findings suggest that DPN can inhibit MHC II expression in microglia via inhibition of *CIITA* expression by a mechanism involving inhibition of IRF-1 translocation to the nucleus. Our further ChIP-qPCR assay confirmed that DPN inhibits MHC II production by not only regulating the interaction between IRF-1 and the CIITA promoter but also regulating the interaction between CIITA and the MHC II promoter in the nucleus.

Some research found that stimulation of ERβ with synthetic ligands efficiently reduced microglial activation and thereby reduced EAE symptoms [11, 21]. Reactivation of CD4+ T lymphocytes by MHC II molecules could also be reduced because of the reduction in MHC II expression on the microglia [12]. Reactivated CD4+ T cells infiltrate the central nervous system, produce a large number of cytokines and promote central injury and myelin loss in MS and EAE. The flow cytometry results showed that the ability of CD4+ T cells to secrete IL17A and IFNγ was inhibited by DPN. The reduction in IL17A and IFNγ in the supernatant of cocultured cells after treatment with DPN supports the in vivo results. The role of IL17A and IFNγ in EAE has been studied in recent decades. Prophylactic anti-IL17A prevents acute disease of the EAE model and relapse and is associated with reduced clinical and functional severity [31]. Inhibition of human and mouse T cell IFNγ by myeloid differentiation factor 88 could ameliorate clinical manifestations of EAE in mice [32]. Therefore, inhibition of the secretion of IL17A and IFNγ by T cells may contribute to the repression of inflammation in MS and EAE.

Conclusions

Overall, our study shows that the ERβ-selective agonist DPN inhibits MHC II expression in microglia via inhibition of class II transactivator expression by a mechanism involving inhibition of IFNγ regulatory factor expression and translocation to the nucleus, which finally inhibits the inflammatory response and symptoms in MS model, as depicted in the following schematic model (**Figure 5**). This findings can help us to the development or screening of highly ERβ-selective agonists to treat MS by targeting MHC II on microglia.

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Disclosure of conflict of interest

None.

Abbreviations

MHC II, major histocompatibility complex class II; MS, Multiple Sclerosis; EAE, experimental autoimmune encephalomyelitis; IRF-1, IFNγ regulatory factor-1; CIITA, Class II Transactivator; ERβ, estrogen receptor β; CNS, central nervous system; BBB, blood-brain-barrier; GWASs, genome-wide association studies; PLP, proteolipid protein; *MBP,* myelin basic protein; DPN, diarylpropionitrile; *NS, normal saline* (0.9% sodium chloride).

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References

[1] Moore SM, Khalaj AJ, Kumar S, Winchester Z, Yoon J, Yoo T, Martinez-Torres L, Yasui N, Katzenellenbogen JA, Tiwari-Woodruff SK. Multiple functional therapeutic effects of the estrogen receptor β agonist indazole-Cl in a mouse model of multiple sclerosis. Proc Natl Acad Sci U S A 2014; 111: 18061-18066.

[2] Goldmann T, Prinz M. Role of microglia in CNS autoimmunity. Clin Dev Immunol 2013; 2013: 208093.

[3] Hunt D, Kavanagh D, Drummond I, Weller B, Bellamy C, Overell J, Evans S, Jackson A, Chandran S. Thrombotic microangiopathy associated with interferon beta. N Engl J Med 2014; 370: 1270-1271.

[4] International Multiple Sclerosis Genetics Consortium; Wellcome Trust Case Control Consortium 2, Sawcer S, Hellenthal G, Pirinen M, Spencer CC, Patsopoulos NA, Moutsianas L, Dilthey A, Su Z, Freeman C, Hunt SE, Edkins S, Gray E, Booth DR, Potter SC, Goris A, Band G, Oturai AB, Strange A, Saarela J, Bellenguez C, Fontaine B, Gillman M, Hemmer B, Gwilliam R, Zipp F, Jayakumar A, Martin R, Leslie S, Hawkins S, Giannoulatou E, D'alfonso S, Blackburn H, Martinelli Boneschi F, Liddle J, Harbo HF, Perez ML, Spurkland A, Waller MJ, Mycko MP, Ricketts M, Comabella M, Hammond N, Kockum I, McCann OT, Ban M, Whittaker P, Kemppinen A, Weston P, Hawkins C, Widaa S, Zajicek J, Dronov S, Robertson N, Bumpstead SJ, Barcellos LF, Ravindrarajah R, Abraham R, Alfredsson L, Ardlie K, Aubin C, Baker A, Baker K, Baranzini SE, Bergamaschi L, Bergamaschi R, Bernstein A, Berthele A, Boggild M, Bradfield JP, Brassat D, Broadley SA, Buck D, Butzkueven H, Capra R, Carroll WM, Cavalla P, Celius EG, Cepok S, Chiavacci R, Clerget-Darpoux F, Clysters K, Comi G, Cossburn M, Cournu-Rebeix I, Cox MB, Cozen W, Cree BA, Cross AH, Cusi D, Daly MJ, Davis E, de Bakker PI, Debouverie M, D'hooghe MB, Dixon K, Dobosi R, Dubois B, Ellinghaus D, Elovaara I, Esposito F, Fontenille C, Foote S, Franke A, Galimberti D, Ghezzi A, Glessner J, Gomez R, Gout O, Graham C, Grant SF, Guerini FR, Hakonarson H, Hall P, Hamsten A, Hartung HP, Heard RN, Heath S, Hobart J, Hoshi M, Infante-Duarte C, Ingram G, Ingram W, Islam T, Jagodic M, Kabesch M, Kermode AG, Kilpatrick TJ, Kim C, Klopp N, Koivisto K, Larsson M, Lathrop M, Lechner-Scott JS, Leone MA, Leppä V, Liljedahl U, Bomfim IL, Lincoln RR, Link J, Liu J, Lorentzen AR, Lupoli S, Macciardi F, Mack T, Marriott M, Martinelli V, Mason D, McCauley JL, Mentch F, Mero IL, Mihalova T, Montalban X, Mottershead J, Myhr KM, Naldi P, Ollier W, Page A, Palotie A, Pelletier J, Piccio L, Pickersgill T, Piehl F, Pobywajlo S, Quach HL, Ramsay PP, Reunanen M, Reynolds R, Rioux JD, Rodegher M, Roesner S, Rubio JP, Rückert IM, Salvetti M, Salvi E, Santaniello A, Schaefer CA, Schreiber S, Schulze C, Scott RJ, Sellebjerg F, Selmaj KW, Sexton D, Shen L, Simms-Acuna B, Skidmore S, Sleiman PM, Smestad C, Sørensen PS, Søndergaard HB, Stankovich J, Strange RC, Sulonen AM, Sundqvist E, Syvänen AC, Taddeo F, Taylor B, Blackwell JM, Tienari P, Bramon E, Tourbah A, Brown MA, Tronczynska E, Casas JP, Tubridy N, Corvin A, Vickery J, Jankowski J, Villoslada P, Markus HS, Wang K, Mathew CG, Wason J, Palmer CN, Wichmann HE, Plomin R, Willoughby E, Rautanen A, Winkelmann J, Wittig M, Trembath RC, Yaouanq J, Viswanathan AC, Zhang H, Wood NW, Zuvich R, Deloukas P, Langford C, Duncanson A, Oksenberg JR, Pericak-Vance MA, Haines JL, Olsson T, Hillert J, Ivinson AJ, De Jager PL, Peltonen L, Stewart GJ, Hafler DA, Hauser SL, McVean G, Donnelly P, Compston A. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. Nature 2011; 476: 214-219.

[5] Aloisi F. Immune function of microglia. Glia 2001; 36: 165-179.

[6] O'Keefe GM, Nguyen VT, Benveniste EN. Regulation and function of class II major histocompatibility complex, CD40, and B7 expression in macrophages and microglia: implications in neurological diseases. J Neurovirol 2002; 8: 496-512.

[7] Kreutzberg GW. Microglia: a sensor for pathological events in the CNS. Trends Neurosci 1996; 19: 312-318.

[8] Neumann H, Misgeld T, Matsumuro K, Wekerle H. Neurotrophins inhibit major histocompatibil-

ity class II inducibility of microglia: involvement of the p75 neurotrophin receptor. Proc Natl Acad Sci U S A 1998; 95: 5779-5784.
[9] Rawji KS, Yong VW. The benefits and detriments of macrophages/microglia in models of multiple sclerosis. Clin Dev Immunol 2013; 2013: 948976.
[10] Overington JP, Al-Lazikani B, Hopkins AL. How many drug targets are there? Nat Rev Drug Discov 2006; 5: 993-6.
[11] Wu WF, Tan XJ, Dai YB, Krishnan V, Warner M, Gustafsson JÅ. Targeting estrogen receptor β in microglia and T cells to treat experimental autoimmune encephalomyelitis. Proc Natl Acad Sci U S A 2013; 110: 3543-3548.
[12] Gerloni M, Zanetti M. CD4 T cells in tumor immunity. Springer Semin Immunopathol 2005; 27: 37-48.
[13] Nagarajan UM, Louis-Plence P, DeSandro A, Nilsen R, Bushey A, Boss JM. RFX-B is the gene responsible for the most common cause of the bare lymphocyte syndrome, an MHC class II immunodeficiency. Immunity 1999; 10: 153-162.
[14] Steimle V, Durand B, Barras E, Zufferey M, Hadam MR, Mach B, Reith W. A novel DNA-binding regulatory factor is mutated in primary MHC class II deficiency (bare lymphocyte syndrome). Genes Dev 1995; 9: 1021-1032.
[15] Masternak K, Barras E, Zufferey M, Conrad B, Corthals G, Aebersold R, Sanchez JC, Hochstrasser DF, Mach B, Reith W. A gene encoding a novel RFX-associated transactivator is mutated in the majority of MHC class II deficiency patients. Nat Genet 1998; 20: 273-277.
[16] Harton JA, Ting JP. Class II transactivator: mastering the art of major histocompatibility complex expression. Mol Cell Biol 2000; 20: 6185-6194.
[17] Chang CH, Guerder S, Hong SC, van Ewijk W, Flavell RA. Mice lacking the MHC class II transactivator (CIITA) show tissue-specific impairment of MHC class II expression. Immunity 1996; 4: 167-178.
[18] Muhlethaler-Mottet A, Di Berardino W, Otten LA, Mach B. Activation of the MHC class II transactivator CIITA by interferon-gamma requires cooperativeinteraction between Stat1 and USF-1. Immunity 1998; 8: 157-166.
[19] Yin J, Valin KL, Dixon ML, Leavenworth JW. The role of microglia and macrophages in CNS homeostasis, autoimmunity, and cancer. J Immunol Res 2017; 2017: 5150678.
[20] Chadwick CC, Chippari S, Matelan E, Borges-Marcucci L, Eckert AM, Keith JC Jr, Albert LM, Leathurby Y, Harris HA, Bhat RA, Ashwell M, Trybulski E, Winneker RC, Adelman SJ, Steffan RJ, Harnish DC. Identification of pathway-selective estrogen receptor ligands that inhibit NF-kappaB transcriptional activity. Proc Natl Acad Sci U S A 2005; 102: 2543-2548.
[21] Saijo K, Collier JG, Li AC, Katzenellenbogen JA, Glass CK. An ADIOL-ERβ-CtBP transrepression pathway negatively regulates microglia-mediated inflammation. Cell 2011; 145: 584-595.
[22] Baker AE, Brautigam VM, Watters JJ. Estrogen modulates microglial inflammatory mediator production via interactions with estrogen receptor beta. Endocrinology 2004; 145: 5021-5032.
[23] Vegeto E, Belcredito S, Ghisletti S, Meda C, Etteri S, Maggi A. The endogenous estrogen status regulates microglia reactivity in animal models of neuroinflammation. Endocrinology 2006; 147: 2263-2272.
[24] Gold SM, Sasidhar MV, Morales LB, Du S, Sicotte NL, Tiwari-Woodruff SK, Voskuhl RR. Estrogen treatment decreases matrix metalloproteinase (MMP)-9 in autoimmune demyelinating disease through estrogen receptor alpha (ERalpha). Lab Invest 2009; 89: 1076-1083.
[25] Robinson AP, Harp CT, Noronha A, Miller SD. The experimental autoimmune encephalomyelitis (EAE) model of MS: utility for understanding disease pathophysiology and treatment. Handb Clin Neurol 2014; 122: 173-189.
[26] Khalaj AJ, Yoon J, Nakai J, Winchester Z, Moore SM, Yoo T, Martinez-Torres L, Kumar S, Itoh N, Tiwari-Woodruff SK. Estrogen receptor (ER) β expression in oligodendrocytes is required for attenuation of clinical disease by an ERβ ligand. Proc Natl Acad Sci U S A 2013; 110: 19125-19130.
[27] Moore S, Khalaj AJ, Yoon J, Patel R, Hannsun G, Yoo T, Sasidhar M, Martinez-Torres L, Hayardeny L, Tiwari-Woodruff SK. Therapeutic laquinimod treatment decreases inflammation, initiates axon remyelination, and improves motor deficit in a mouse model of multiple sclerosis. Brain Behav 2013; 3: 664-682.
[28] De Angelis F, Plantone D, Chataway J. Pharmacotherapy in secondary progressive multiple sclerosis: an overview. CNS Drugs 2018; 32: 499-526.
[29] Ciccarelli O, Barkhof F, Bodini B, De Stefano N, Golay X, Nicolay K, Pelletier D, Pouwels PJ, Smith SA, Wheeler-Kingshott CA, Stankoff B, Yousry T, Miller DH. Pathogenesis of multiple sclerosis: insights from molecular and metabolic imaging. Lancet Neurol 2014; 13: 807-822.
[30] Kawachi I, Lassmann H. Neurodegeneration in multiple sclerosis and neuromyelitis optica. J Neurol Neurosurg Psychiatry 2017; 88: 137-145.
[31] Mardiguian S, Serres S, Ladds E, Campbell SJ, Wilainam P, McFadyen C, McAteer M, Choud-

hury RP, Smith P, Saunders F, Watt G, Sibson NR, Anthony DC. Anti-IL17A treatment reduces clinical score and VCAM-1 expression detected by in vivo magnetic resonance imaging in chronic relapsing EAE ABH mice. Am J Pathol 2013; 182: 2071-2081.

[32] Dishon S, Cohen SJ, Cohen IR, Nussbaum G. Inhibition of myeloid differentiation factor 88 reduces human and mouse T-cell interleukin-17 and IFNγ production and ameliorates experimental autoimmune encephalomyelitis induced in mice. Front Immunol 2017; 8: 615.