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Mdivi-1 Modulates Macrophage/Microglial Polarization in Mice with EAE via the Inhibition of the TLR2/4-GSK3β-NF-κB Inflammatory Signaling Axis

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Abstract

Macrophage/microglial modulation plays a critical role in the pathogenesis of multiple sclerosis (MS), which is an inflammatory disorder of the central nervous system. Dynamin-related protein 1 is a cytoplasmic molecule that regulates mitochondrial fission. It has been proven that mitochondrial fission inhibitor 1 (Mdivi-1), a small molecule inhibitor of Drp1, can relieve experimental autoimmune encephalomyelitis (EAE), a preclinical animal model of MS. Whether macrophages/microglia are involved in the pathological process of Mdivi-1-treated EAE remains to be determined. Here, we studied the anti-inflammatory effect of Mdivi-1 on mice with oligodendrocyte glycoprotein peptide$_{35\text{-}55}$ (MOG$_{35\text{-}55}$)–induced EAE. We found that Drp1 phosphorylation at serine 616 in macrophages/microglia was decreased with Mdivi-1 treatment, which was accompanied by decreased antigen presentation capacity of the macrophages/microglia in the EAE mouse spinal cord. The Mdivi-1 treatment caused macrophage/microglia to produce low levels of proinflammatory molecules, such as CD16/32, iNOS, and TNF-α, and high levels of anti-inflammatory molecules, such as CD206, IL-10, and Arginase-1, suggesting that Mdivi-1 promoted the macrophage/microglia shift from the inflammatory M1 phenotype to the anti-inflammatory M2 phenotype. Moreover, Mdivi-1 was able to downregulate the expression of TRL2, TRL4, GSK-3β, and phosphorylated NF-κB-p65 and prevent NF-κB-mediated IL-1β and IL-6 production. In conclusion, these results indicate that Mdivi-1 significantly alleviates inflammation in mice with EAE by promoting M2 polarization by inhibiting TLR2/4- and GSK3β-mediated NF-κB activation.

Keywords Experimental autoimmune encephalomyelitis · Mdivi-1 · Macrophage/microglial polarization · NF-κB

Introduction

Multiple sclerosis (MS) is a chronic inflammatory and neurodegenerative disease, and its main pathological features include demyelination, axonal loss, reactive gliosis, and inflammatory lesions in the central nervous system (CNS) [1]. Experimental autoimmune encephalomyelitis (EAE) has been well used as an MS animal model and shares many clinical characteristics of MS patients, so it is an effective tool for understanding the inflammatory response through the disease process. In the EAE model, stimulated myelin-specific T cells are activated in the periphery and then infiltrate into the CNS across the blood–brain barrier (BBB). They are restimulated by antigen-presenting cells (APCs), promoting inflammatory response initiation and eventually demyelination and axonal damage [2].

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Macrophage/microglia play critical roles in the inflammatory process as important innate immune cells. They promote T cell activation as APCs and are considered key mediators in the course of MS [3]. In the periphery, macrophages prevent microorganisms from invading as the initial line of defense, clean apoptotic cells, release cytokines and chemokines, and stimulate T cell proliferation [4]. In the CNS, there are two main macrophages. One is resident microglia derived from the embryonic yolk sac, and the other is infiltrated monocyte-derived macrophages, which are generally believed to develop from bone marrow progenitors. The function of microglia is similar to that of macrophages and can be supplemented by macrophages resident from tissue [5]. In response to various stimuli, macrophage/microglia present two phenotypes: the proinflammatory phenotype M1 and the anti-inflammatory phenotype M2. M1 macrophage/microglia rapidly respond to infection, while M2 macrophage/microglia contribute to the repair of lesions. Conversion of the M1 to M2 phenotype may provide effective treatments for inflammatory diseases, such as MS [6].

Mitochondrial fission/fusion is a dynamic process and plays an important role in normal cellular functions in response to metabolism and environmental stress. Mitochondrial fission leads to fragmentation of mitochondria, which produces more reactive oxygen species (ROS) and promotes mitophagy, while fusion between mitochondria decreases mutations in mtDNA and maximizes oxidative capacity [7]. Inhibiting the fission of mitochondria may improve cell longevity and is thought of as a survival mechanism in neurodegenerative diseases [8]. The conserved GTPase Dnm1/Drp1 is a major protein that regulates mitochondrial fission. When Dnm1/Drp1 is phosphorylated, it translocates from the cytosol to the outer mitochondrial membrane (OMM), polymerizes at construction sites, and splits mitochondria [9]. Inhibition of Drp1 is neuroprotective and slows the progression of neurodegenerative disorders, such as Alzheimer's disease (AD) [10]. Mitochondrial fission inhibitor 1 (Mdivi-1) has been widely reported to prevent Dnm1/Drp1 assembly on the surface of mitochondria to suppress mitochondrial fission and Bax/Bak-mediated apoptosis [11]. It can cross the BBB as a hydrophobic molecule and plays cytoprotective roles in different types of cells, such as neurons [12]. In addition to being an inhibitor of Drp1, Mdivi-1 is also involved in various biological processes, such as ROS production, ATP release, and mitochondrial membrane potential modulation [13]. Our previous study showed that Mdivi-1 suppressed EAE by modulating the immune response [14]. However, the exact therapeutic mechanism of Mdivi-1 in these diseases remains unclear.

Here, we further examined the anti-inflammatory function of Mdivi-1 in EAE mice. Our data indicated that Mdivi-1 treatment decreases inflammatory response in the spinal cords of EAE mice, which is accompanied by a decreased antigen presentation capability, and promotes macrophage/microglial M2 polarization *in vivo* and *in vitro*, which may be related to the inhibition of TLR2/4- and GSK-3β-mediated NF-κB inactivation.

Methods

Animals

Female C57BL/6 mice were purchased directly from Beijing HFK Bioscience Co., Ltd. Eight- to ten-week-old mice were randomly divided into two groups (5 mice in each group) and housed in the same cages. The mice were kept in pathogen-free conditions at constant temperature, illumination, and humidity and provided food and water throughout the experimental process. The cages were changed weekly, and the experiment was performed three times. This study was supported by the Institutional Animal Care and Use Committee at Shanxi Datong University.

EAE Induction and Mdivi-1 Treatment

As described previously [14], an emulsion mixture of myelin oligodendrocyte glycoprotein (200 μg) ($MOG_{35–55}$, Genscript, NJ, USA) was prepared in an equal volume of complete Freund's adjuvant (CFA, Sigma, St. Louis, MO, USA), and *Mycobacterium tuberculosis* H37Ra killed by heat was added at a concentration of 10 mg/ml (Difco, Detroit, MI, USA). Each female C57BL/6 mouse was injected subcutaneously at two points. After immunization, 200 ng pertussis toxin (Sigma, St. Louis, MO, USA) were followed injected. Two days later, the mice were given the same dose of pertussis toxin. Mice were injected intraperitoneally with Mdivi-1(25 mg/kg; 0.1% DMSO) (Sigma, St. Louis, MO, USA) or vehicle (0.1% DMSO) starting from Day 3 postimmunization (p.i.) to Day 27 p.i. The clinical evaluation of EAE was performed daily using a standard clinical score. Paralysis was monitored daily on five scales: (1) limp tail; (2) hind limb weakness; (3) paralysis of hind limb; (4) tetraparalysis; (5) moribund/dead.

Spleen Mononuclear Cell Isolation

Spleen mononuclear cell suspensions were isolated from mice with EAE at 28 p.i. as previously described [15]. Briefly, mice with EAE were euthanized after anesthetization, and then the mice were perfused with cold saline. Spleens were removed and pooled in IMDM containing penicillin (100 U), streptomycin (10 μg/mL), L-glutamine (0.3 mg/mL), β-mercaptoethanol (55 μM), and 10% heat-inactivated fetal bovine serum (FBS) (all from Thermo Fisher Scientific, Carlsbad, CA). Spleens were mechanically disrupted and filtered with a 70 μm filter. The generated cells were incubated for 1 min with RBC lysis buffer (Sigma, St. Louis, MO, USA) for lysing the red blood cells and then washed with IMDM.

Histology and Immunohistochemistry

The mice were pooled with saline and 4% paraformaldehyde (PFA), and then the spinal cords were separated and fixed with 2% PFA for 4 hours (h). The fixed spinal cords were dehydrated using gradient sucrose, and then paraffin or OCT embedding was performed. Consecutive 20 μm paraffin sections from six mice per group were collected, and Luxol Fast Blue (LFB) staining was used to assess pathological changes. For immunohistochemistry, 10 μm OCT slices from nine mice per group were blocked with 1% bovine serum for 2 h at room temperature, and then incubated with the indicated primary antibodies at 4 ℃ overnight. Antibodies were purchased from BD Biosciences (GM-CSF, Arginase-1, CD16/32, CD11b, CD45, CD4, and MHC II), Abcam (IBa-1, Ki67, TNF-α, iNOS, IL-10, IL-1β, IL-6), Cell Signaling (pDrp1 (Ser616), p-NF-κB-p65, GSK-3β), Biolegend (CD68 and CD206), Thermo Fisher Scientific (TLR2, TLR4), Santa Cruz (p38), and Bioss (CD86). The next day, slices were incubated with the associated secondary antibody for 2 h at room temperature after washing with PBS for 30 min. Slices were then washed with PBS. The negative control was prepared similarly with the exception of omitting the primary antibodies. The results were captured with a confocal microscope (CLSM, Olympus, Tokyo, Japan). Total white matter in LFB and color photograph was outlined. The rate of pixel area of demyelination and positive expression in the total white matter of the spinal cord were qualified by Image-Pro Plus 6 software. At least two sections per mouse were subjected to histological analyses.

SIM-A9 Microglial Polarization

SIM-A9 immortalized microglial cells were purchased from ShenKe Biological Technology Co., Ltd. The cells were grown in DMEM/F-12 medium supplemented with 5% FBS (all from Gibco, Waltham MA, USA) and cultured at 37 °C in a cell incubator (ESCO, Singapore) with 95% air and 5% CO_2. A total of 5×10^4 SIM-A9 cells were plated per well in a 24-well plate. Cells were allowed to attach to the culture dish for 2 h. Lipopolysaccharide (LPS) (Sigma, St. Louis, MO, USA) was added or not at 2.5 ng/mL for 12 h. Then, cells not treated with LPS and 2.5 ng/mL LPS-treated cells were incubated with 0.1% DMSO or 10 μM Mdivi-1 for 24 h. Finally, the collected cells were used for further immunostaining and flow cytometry analysis.

Flow Cytometry Analysis

For spleen mononuclear cells and SIM-A9 microglial cells, one million cells in FACS tubes were induced with 50 ng/mL phorbol ester (PMA) and 500 ng/mL ionomycin in the presence of GolgiPlug (1 μg/10^6 cells) (all from Sigma, St. Louis, MO, USA) for 4 h at 37 °C. Cells were washed with PBS containing 1% FBS, and then immunostaining was performed. For the staining of proteins expressed on the cell surface, cells were labeled with fluorochrome-labeled antibodies in 100 μL PBS containing 1% FBS for 20 min at 4 °C, such as CD45 (BD Biosciences, NJ, USA), CD11b (BD Biosciences, NJ, USA), CD16/32 (BD Biosciences, NJ, USA), and CD206 (Biolegend, San Diego, CA, USA). For the staining of molecules present inside the cell, Fix and Perm cell permeabilization reagents (Thermo Fisher Scientific, Carlsbad, CA) were used for cell fixation and permeation. Intracellular cytokines were labeled with antibodies against TNF-α (Biolegend, San Diego, CA, USA), IL-10 (BD Biosciences, USA), iNOS (Santa Cruz, Dallas, TX, USA), and Arginase-1 (Santa Cruz, Dallas, TX, USA) in 100 μL permeable buffer overnight at 4 °C. To set up the instrument and compensation, single staining of the cell surface antibodies or intracellular antibodies was performed, and an unstained cell sample was also used. The unstained cell sample did not contain antibodies and was treated in the same way as the stained samples. The results were obtained on a ZE5 Cell Analyzer (BioRad, USA), and analyzed using FlowJo Software.

Statistical Analysis

Data were analyzed as the mean SEM. The nonparametric Kruskal–Wallis test was first used for clinical mean score analysis, and then the comparison between any two sets of data was analyzed via the Mann–Whitney U-test. Student's *t*-test was performed for comparisons between groups. All data were analyzed via GraphPad Prism software. When a p value was less than 0.05, the difference was considered statistically significant.

Results

Mdivi-1 Decreased the Phosphorylation of Drp1 (p-Drp1; Ser616) in IBa-1$^+$ Macrophages/Microglia

As reported previously [14], mice with EAE were injected daily with 25 mg/kg Mdivi-1 or 0.1% DMSO from Day 3 p.i. to 27 p.i. Reduced severity of EAE was observed in the mice treated with Mdivi-1, which showed a remarkable decrease in clinical scores and reduced spinal cord demyelination relative to those of the control group (Fig S1a-b). Mdivi-1 is widely reported to inhibit Drp1-dependent mitochondrial fission, and the phosphorylation of serine 616 promotes Drp1 translocation to the OMM and subsequent scissor mitochondria [9]. Macrophages/microglia play critical roles in the progression of MS. To verify the effect of Mdivi-1 on macrophages/microglia in the spinal cords of

EAE mice, the change in p-Drp1 at serine 616 upon Mdivi-1 treatment was characterized by coimmunostaining of p-Drp1 with IBa-1 in the white matter of EAE mouse spinal cords. The results showed that the number of Ser616$^+$IBa-1$^+$ macrophages/microglia was lower in the spinal cords of Mdivi-1-treated mice with EAE than those of the DMSO-treated mice (Fig. 1).

Mdivi-1 Suppresses Macrophage/Microglial Activation in the Spinal Cords of Mice with EAE

To further explore the effect of Mdivi-1 on macrophages/microglia, the activation of macrophages/microglia in the spinal cords of mice with EAE was investigated. The expression of CD68 was examined via immunofluorescence as an activated macrophage/microglia marker. The results revealed that fewer CD68$^+$ macrophages/microglia were present in the white matter of spinal cords of mice with EAE treated with Mdivi-1 than in DMSO-treated mice with EAE (Fig. 2a). CD11b is a common marker of macrophages and microglia. However, macrophages show higher CD45 expression, whereas microglia express lower CD45 expression. To further quantify the infiltrated macrophages from the peripheral system, a combination of CD11b and CD45 labeling was performed. As shown in Fig. 2b, the high yellow signals were calculated as markers of macrophages. We found that Mdivi-1-treated mice with EAE had fewer macrophages in the white matter of the spinal cord than DMSO-treated EAE mice. These data demonstrated that Mdivi-1 effectively inhibited macrophage/microglia activation and decreased infiltrated macrophages in the spinal cords of EAE mice.

Mdivi-1 Inhibited the Antigen Presentation Capacity of Macrophages/Microglia

Macrophages/microglia play critical roles in activating the adaptive immune response as APCs. The activation of APCs is commonly associated with increased levels of MHC II and cell surface markers such as CD86. To evaluate the effect of Mdivi-1 on the antigen presentation capacity of macrophages/microglia after activation, immunofluorescent staining for CD86 and MHC II in macrophages/microglia from spinal cords was conducted. The data showed that the coexpression of CD86 and IBa-1 was dramatically lower in Mdivi-1-treated mice with EAE than in the DMSO control group (Fig. 3a), and Mdivi-1 suppressed the expression of MHC II in IBa-1$^+$ cells in the white matter of EAE mouse spinal cords (Fig. 3b). We also used Ki67, a T cell activation marker, to quantify proliferating CD4$^+$ T cells [16]. Consistent with the downregulated antigen presentation capacity, the immunostaining results indicated that the high densities of Ki67$^+$ CD4$^+$ cells in the white matter of the spinal cord of DMSO-treated mice with EAE were significantly reduced by Mdivi-1 administration (Fig. 3c). Activated CD4$^+$ T cells mediate many aspects of autoimmune inflammation, and accumulating evidence demonstrates that GM-CSF is produced locally at sites of inflammation to modulate macrophage function as an important cytokine [17]. Thus, we assessed the level of GM-CSF in CD4$^+$ T cells in spinal cords of mice with EAE by immunofluorescence. GM-CSF-expressing CD4$^+$ T cells were observed in the white matter of EAE mouse spinal chords, but were significantly reduced by Mdivi-1 treatment (Fig. 3d). These findings indicated that Mdivi-1 inhibited the antigen presentation capacity of macrophages/microglia and suppressed the inflammatory response in the spinal cords of EAE mice.

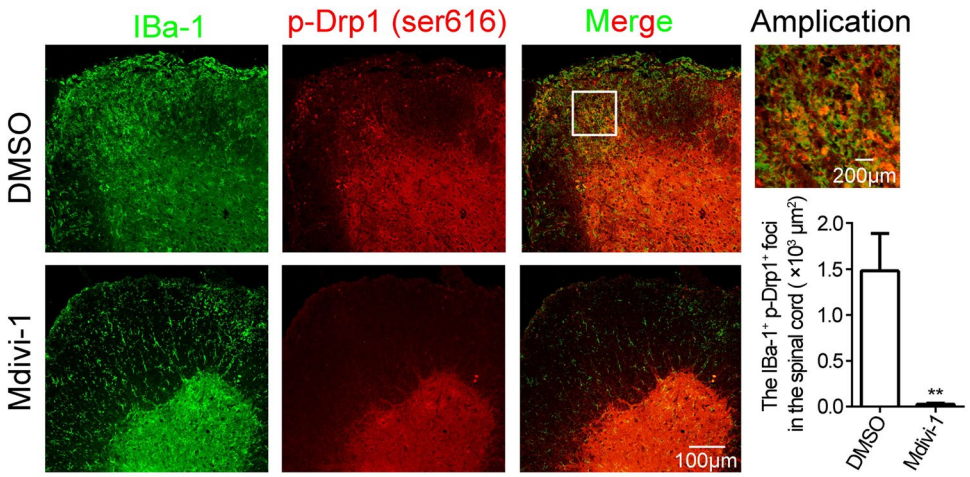

Fig. 1 Mdivi-1 inhibits Drp1 phosphorylation at serine 616 in IBa-1$^+$ microglia. On Day 28 p.i., the lumbar cords were separated from mice with EAE treated with Mdivi-1 or DMSO under anesthesia. Coimmunostaining of lumbar cords for p-Drp1 at serine 616 and IBa-1 was performed. Representative images of p-Drp1 at serine 616 distribution in IBa-1$^+$ cells are shown, and the IBa-1$^+$ser616$^+$ signal was quantified. $N = 5–8$. Mean ± SEM. ** $p < 0.01$. One representative of two experiments is shown

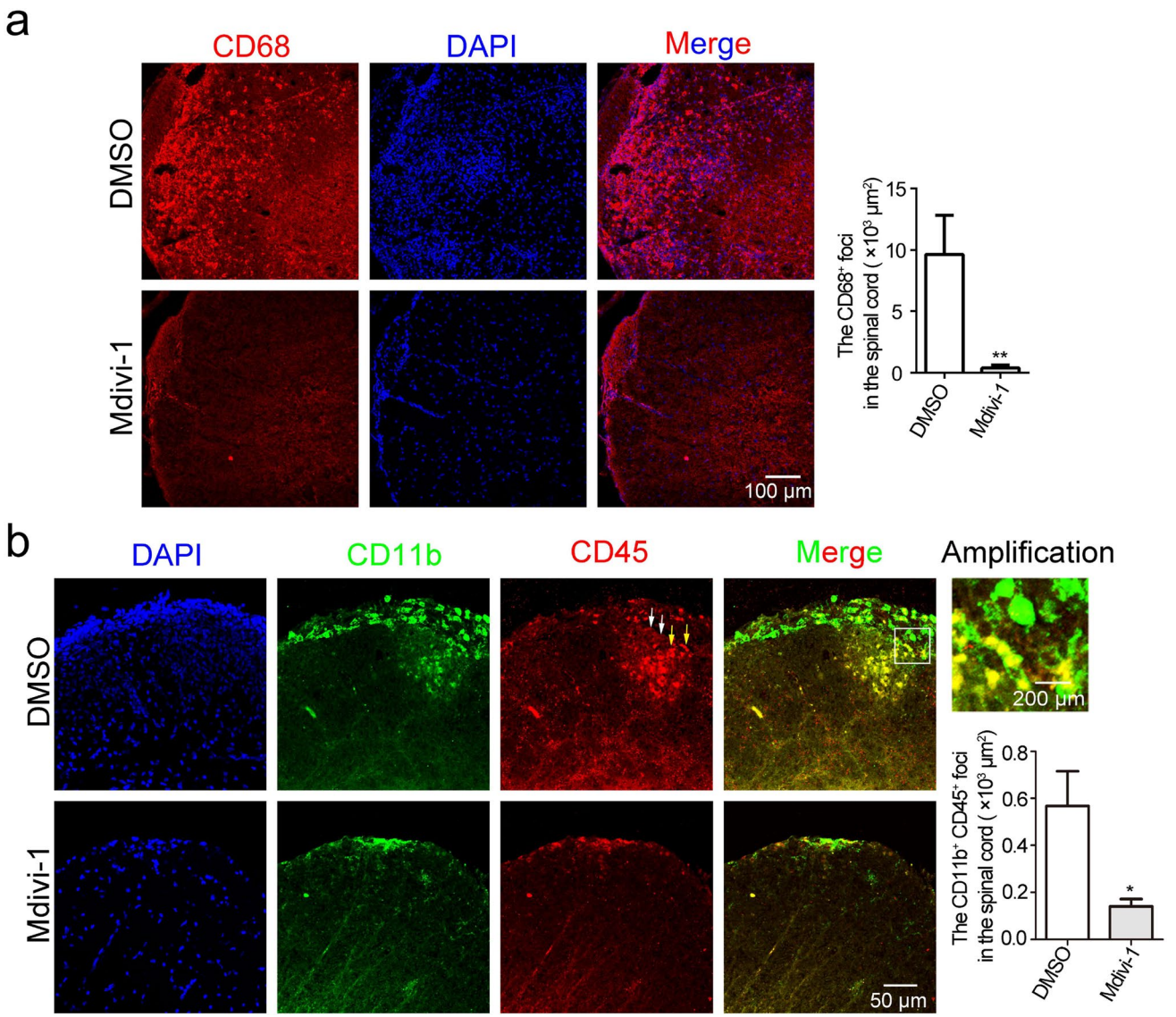

Fig. 2 Mdivi-1 suppresses macrophage/microglial activation in the spinal cords of EAE mice. On Day 28 p.i., the lumbar cords were separated from mice with EAE treated with Mdivi-1 or DMSO under anesthesia. CD68, CD45, and CD11b in lumbar cords were stained. Representative images of $CD68^+$ (**a**) and $CD11b^+$ cells with high CD45 expression (**b**) are shown, and the red signal of $CD68^+$ (**a**) and the high yellow signal of $CD11b^+$ $CD45^+$ (**b**) were quantified. White arrow indicates $CD11b^+$ $CD45^{low}$ cells. Yellow arrow indicates $CD11b^+$ $CD45^{high}$ cells. DAPI was used for nucleus staining. $N = 5–8$. Mean ± SEM. * $p < 0.05$. ** $p < 0.01$. One representative of two experiments is shown

Mdivi-1 Shifted the M1 Phenotype to the M2 Phenotype in the Spinal Cords of Mice with EAE

Macrophages/microglia play two key roles in the response to inflammation: the classically activated (M1) and the alternative activated (M2) phenotypes. It is hypothesized that promoting macrophage shift from M1 to M2 phenotype is a therapeutic strategy for MS. To evaluate the level of macrophage/microglia polarization, we analyzed the macrophage/microglia phenotype by coimmunostaining of M1 markers CD16/32, TNF-α, and iNOS as well as the M2 markers CD206, IL-10, and Arginase-1 with IBa-1 in Mdivi-1-treated mice spinal cords. The results showed that Mdivi-1 treatment markedly decreased the foci of $CD16/32^+$ $IBa\text{-}1^+$, $TNF\text{-}\alpha^+$ $IBa\text{-}1^+$, and $iNOS^+$ $IBa\text{-}1^+$ in the white matter of EAE mouse spinal cords compared to those of DMSO-treated mice with EAE (Fig. 4a–c). In contrast, the foci of $CD206^+$ $IBa\text{-}1^+$, $IL\text{-}10^+$ $IBa\text{-}1^+$, and

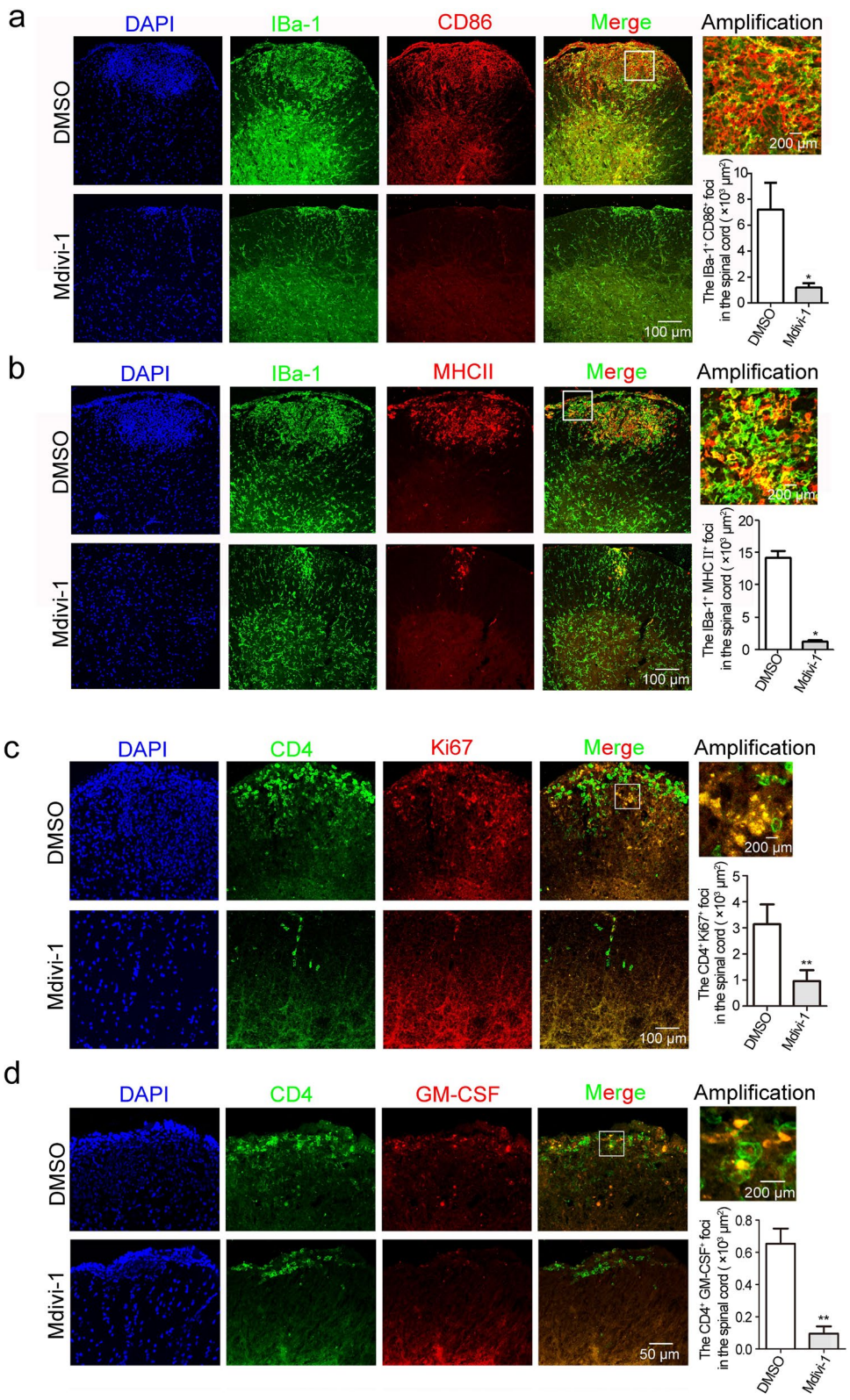

Fig. 3 Mdivi-1 inhibits the antigen presentation capacity of macrophages/microglia. On Day 28 p.i., the lumbar cords were separated from mice with EAE treated with Mdivi-1 or DMSO under anesthesia. Immunostaining of lumbar cords for CD86, MHC II, IBa-1, Ki67, GM-CSF, and CD4 was performed. Representative images of $CD86^+$ (**a**) and MHC II^+ (**b**) in IBa-1^+ cells, and $Ki67^+$ (**c**) and GM-CSF^+ (**d**) in $CD4^+$ cells are shown. The pixel areas of $CD86^+$ IBa-1^+ (**a**), MHC II^+ IBa-1^+ (**b**), $Ki67^+$ $CD4^+$ (**c**), and GM-CSF^+ $CD4^+$ (**d**) in the white matter were quantified. DAPI was used for nucleus staining. $N = 5–8$. Mean ± SEM. * $p < 0.05$. ** $p < 0.01$. One representative of two experiments is shown

Arginase-1^+ IBa-1^+ were significantly increased in the white matter of Mdivi-1-treated mouse spinal cords compared with those without Mdivi-1 treatment (Fig. 4d–f), emphasizing the anti-inflammatory role of Mdivi-1 in the spinal cord by promoting M2 microglia polarization and attenuating the M1 phenotype.

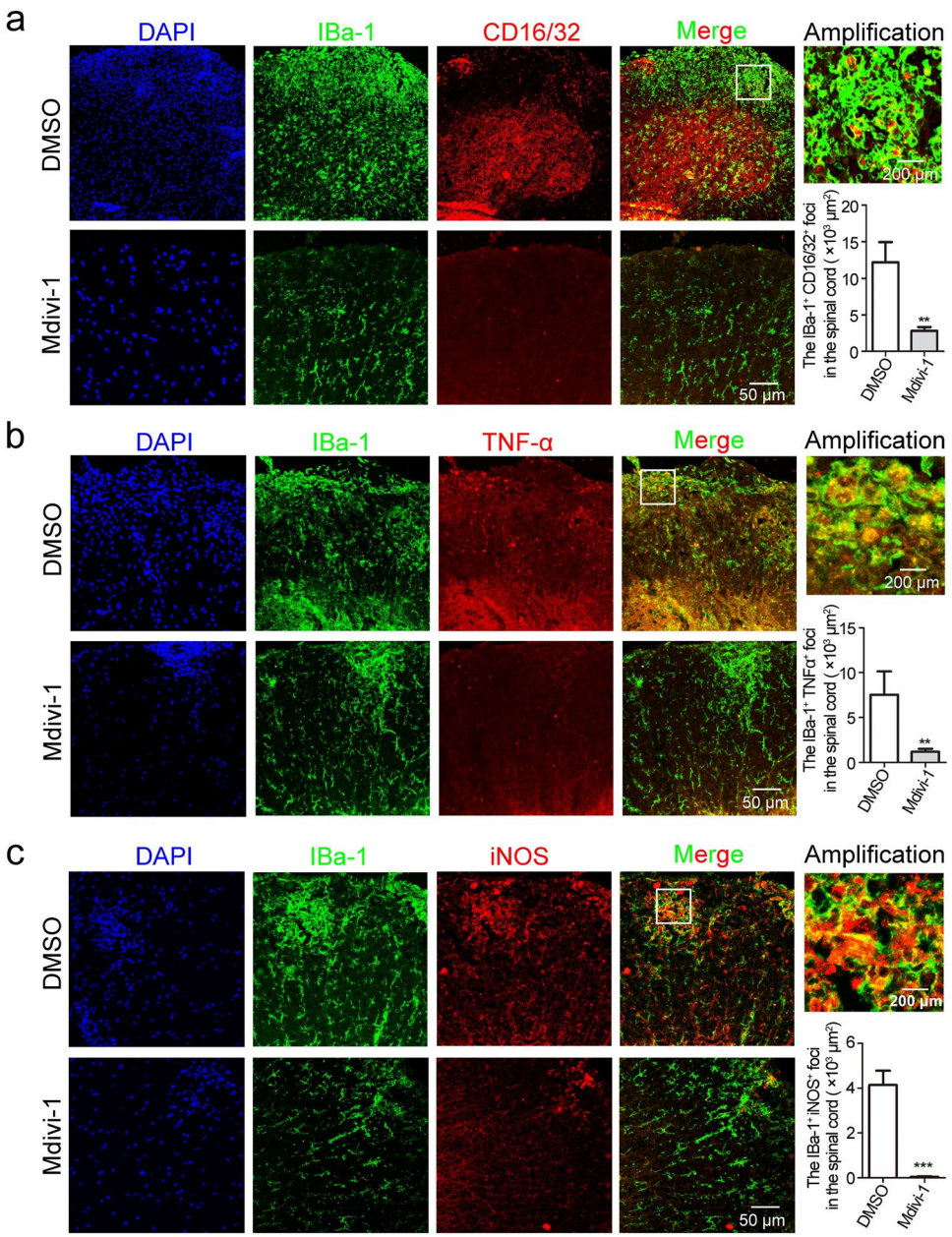

Fig. 4 Mdivi-1 inhibits the M1 phenotype and promotes the M2 phenotype of microglia in the spinal cords of EAE mice. Expression of M1 markers such as CD16/32 (**a**), TNF-α (**b**), and iNOS (**c**) or M2 markers such as CD206 (**d**), IL-10 (**e**), and Arginase-1 (**f**) in IBa-1$^+$ cells in the spinal cords of mice with EAE was determined by double immunohistochemical staining, and the pixel area of double-positive cells was computed. Representative images show the distribution of M1 and M2 markers in IBa-1$^+$ cells. DAPI was used for nucleus staining. $N = 5–8$. Mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. One representative of two experiments is shown

Mdivi-1 Promotes Macrophage M2 Polarization in the Spleen of EAE Mice

Next, we also assessed whether Mdivi-1 affected macrophage polarization in the spleen of EAE mice. CD11b$^+$ cells were gated on CD45$^+$ cells (Fig. 5a), and then the signals of CD16/32, TNF-α, Arginase-1, and IL-10 in CD11b$^+$ macrophages were detected by flow cytometry. The data showed that the percentage of CD11b$^+$ cells expressing M1 markers (CD16/32 and TNF-α) in mice with EAE treated with Mdivi-1 was decreased significantly compared with that in the control group (Fig. 5b). However, the percentage of CD11b$^+$ cells expressing M2 markers (Arginase-1 and IL-10) in Mdivi-1-treated mice with EAE was markedly elevated compared with that in the control group (Fig. 5c). These data suggested that Mdivi-1 treatment facilitated the macrophage phenotypic switch from M1 to M2 in the spleen of EAE mice.

Mdivi-1 Promotes M2 Polarization of SIM-A9 Microglial Cells *In Vitro*

To further determine the influence of Mdivi-1 on M1/M2 polarization *in vitro*, SIM-A9 microglial cells were stimulated with or without 2.5 ng/mL LPS for 12 h and cultured supplemented with 10 μM Mdivi-1 for 24 h. Then, flow cytometry was used to assess

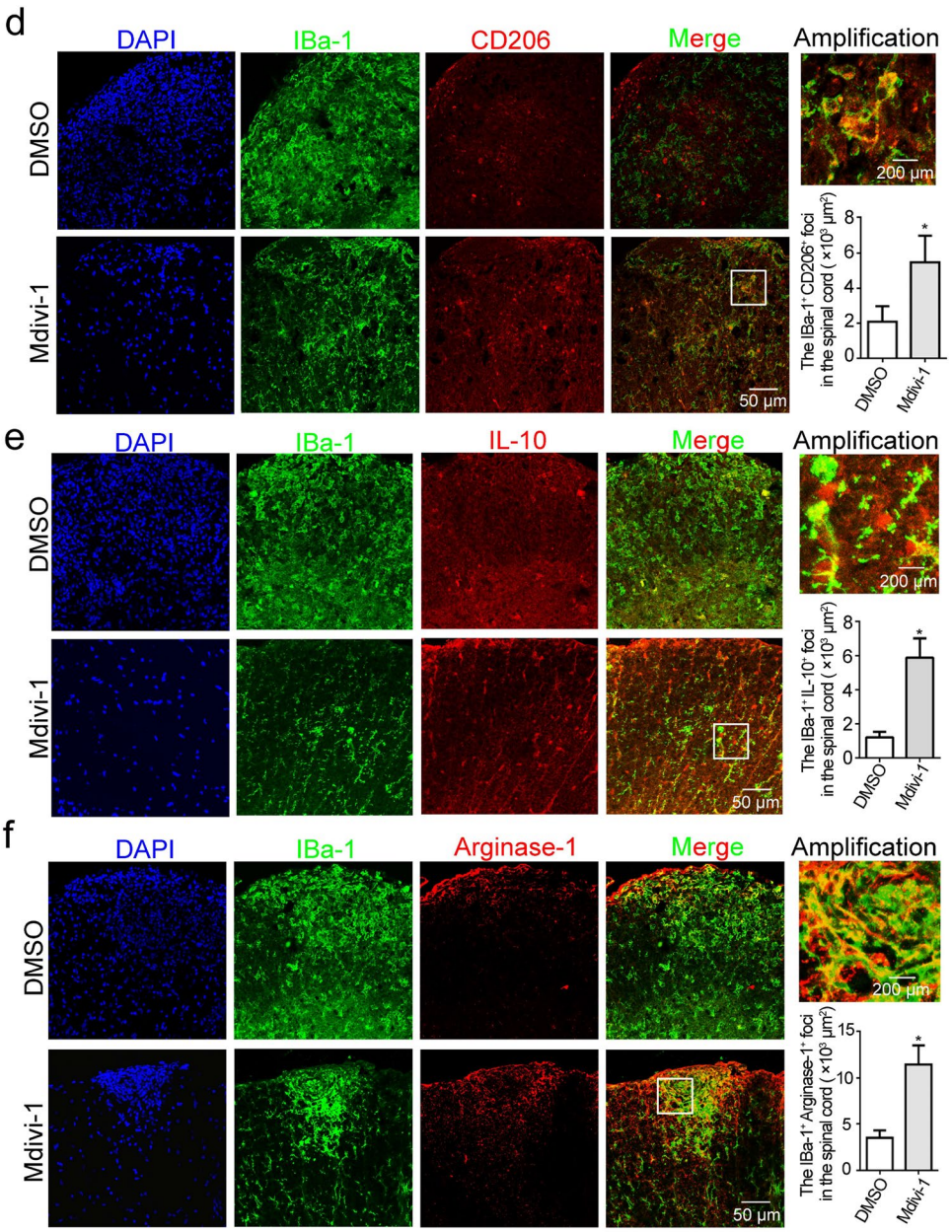

Fig. 4 (continued)

the levels of specific biomarkers of the M1and M2 phenotypes. The results revealed that Mdivi-1 significantly decreased LPS-induced gene expression of iNOS and TNF-α in CD16/32$^+$ M1 cells (Fig. 6a–b). However, Mdivi-1 treatment strikingly increased the levels of IL-10 and Arginase-1 in CD206$^+$ M2 cells both with and without LPS stimulation (Fig. 6c–d). These findings add significant weight to the idea that administration of Mdivi-1 facilitates the M1 to M2 phenotype shift.

Mdivi-1 Suppresses the TLR2/4-GSK-3β-NF-κB Pathway to Reduce Inflammatory Responses in EAE Mice

To explore the mechanism by which Mdivi-1 is involved in the anti-inflammatory response, the possible signaling pathways were subsequently determined. The NF-κB pathway plays an important role in the inflammatory microenvironment, and the p65:p50 dimers are the most abundant form in the canonical NF-κB pathway, and can be activated

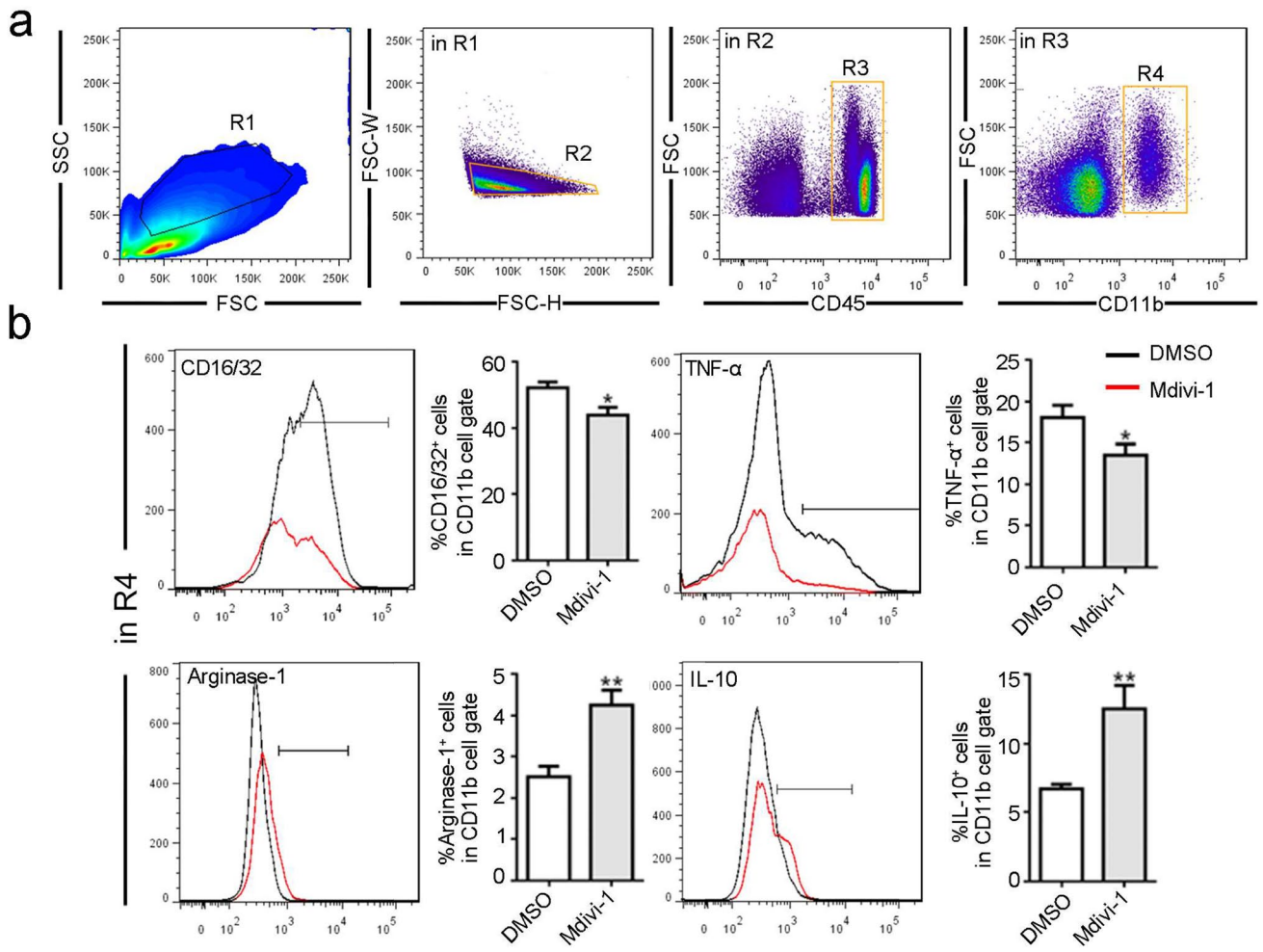

Fig. 5 Mdivi-1 shifts inflammatory macrophages into anti-inflammatory macrophages in the spleen of EAE mice. Mice with EAE treated with Mdivi-1 or DMSO were euthanized on Day 28 p.i. A single spleen mononuclear cell was isolated, and flow cytometry analysis was performed. **a** Representative dot plots illustrating the source of $CD11b^+$ cells in the peripheral spleen system. First, dead cells and debris were excluded using FSC/SSC gating. Subsequently, single cells were gated on an FSC-W/FSC-H pot. Leukocytes were further gated by CD45 expression, and mononuclear cells were defined as $CD11b^+$ $CD45^+$. **b** Upper panel: representative flow plot of the M1 markers CD16/32 and TNF-α in the $CD11b^+$ cells and quantification of the CD16/32 and TNF-α in $CD11b^+$ population. Lower panel: Representative flow plot of M2 markers Arginase-1 and IL-10 in the $CD11b^+$ cells and quantification of the Arginase-1 and IL-10 in the $CD11b^+$ population. $N = 5–8$. Mean ± SEM. * $p < 0.05$, ** $p < 0.01$. One representative of three experiments is shown

by proinflammatory cytokines and pathogens [18]. We first measured the level of NF-κB p65 phosphorylation (p-NF-κB-p65) in macrophages/microglia in the spinal cord by immunostaining. The result showed that the foci of p-NF-κB-$p65^+$ IBa-1^+ were decreased in the white matter of EAE mouse spinal cords treated with Mdivi-1 (Fig. 7a). Then, we analyzed the expression of the proinflammatory cytokines mediated by the p65 subunit-dependent NF-κB pathway, such as IL-1β and IL-6. As expected, Mdivi-1 caused the expression of IL-1β and IL-6 in IBa-1^+ cells to decrease in the white matter of EAE mouse spinal cords compared with the control group (Fig. 7b–c). Considering that Toll-like receptor 2/4 (TLR2/4) can activate the downstream NF-kB pathway as the cell surface receptors [19], the coexpression of TLR2/4 and IBa-1 in the spinal cord was measured by immunostaining. As shown in Fig. 7d–e, $TLR2^+$ IBa-1^+ and $TLR4^+$ IBa-1^+ foci were abundant in the white matter of DMSO-treated EAE mouse spinal cords. In contrast, the expression of TLR2/4 was strongly inhibited by Mdivi-1 in macrophages/microglia. Next, we further investigated the mechanisms involved in Mdivi-1-inhibited NF-κB activation. Previous studies have reported that glycogen synthase kinase (GSK-3β) positively regulates in NF-κB activation by regulating the phosphorylation of p65 at serine 468 [20]. Moreover, several studies have demonstrated that inhibition of GSK-3β can attenuate LPS-induced inflammatory reactions [21–23]. Therefore, we detected the expression of GSK-3β in macrophages/microglia in the spinal cords of

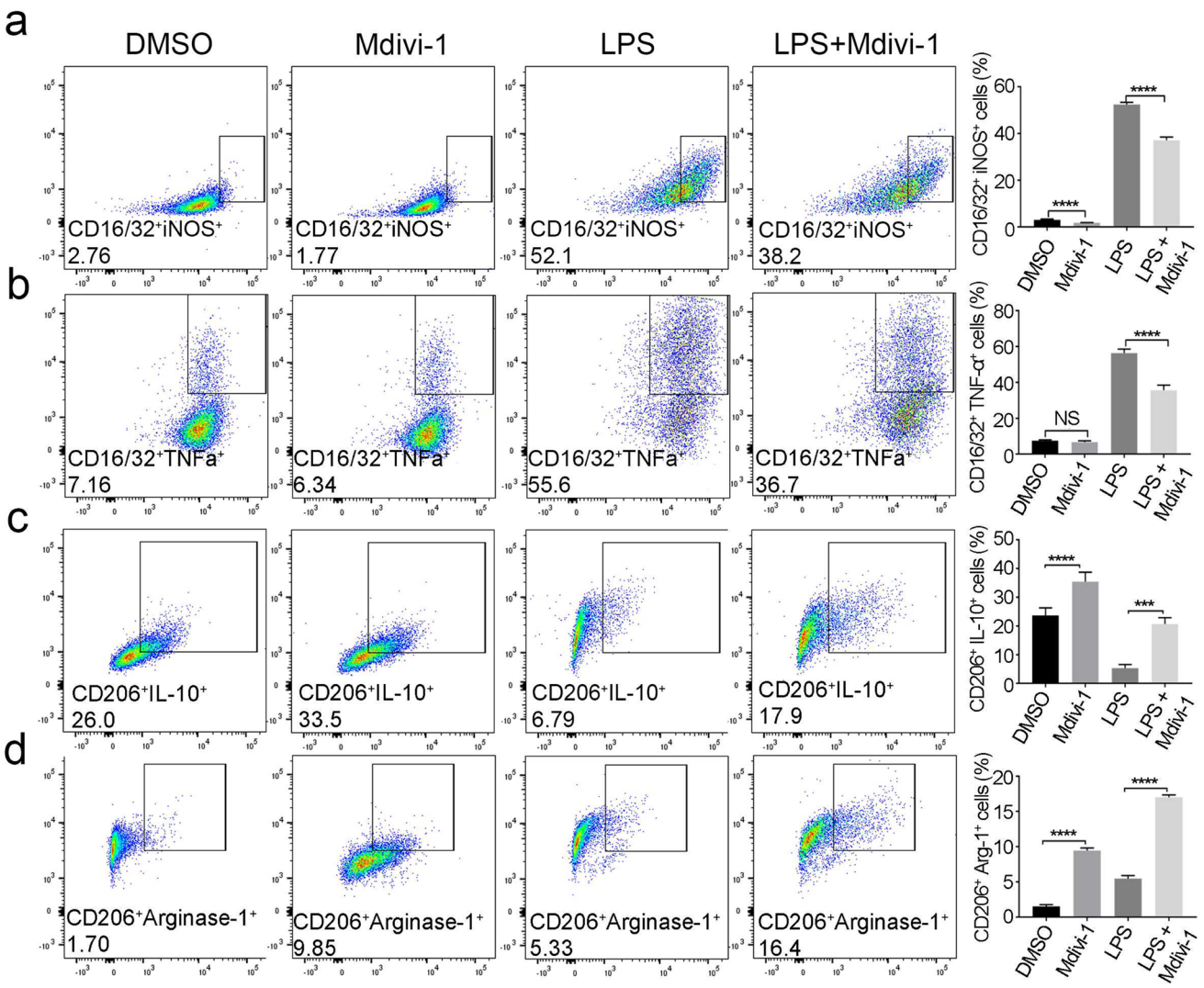

Fig. 6 Mdivi-1 inhibits the M1 phenotype and promotes the M2 phenotype in SIM-A9 microglial cells. SIM-A9 microglial cells were induced with or without 2.5 ng/mL LPS for 12 h and then treated with or without 10 μM Mdivi-1 for 24 h. Flow cytometry analysis of $CD16/32^+$ $iNOS^+$ (**a**), $CD16/32^+$ TNF-α^+ (**b**), $CD206^+$ IL-10^+ (**c**), and $CD206^+$ Arginase-1^+ (**d**) cells and relative quantitative analysis of $CD16/32^+$ $iNOS^+$ (**a**), $CD16/32^+$ TNF-α^+ (**b**), $CD206^+$ IL-10^+ (**c**), and $CD206^+$ Arginase-1^+ (**d**). $N = 5–8$. Mean ± SEM. *** $p < 0.001$, **** $p < 0.0001$. One representative of three experiments is shown

mice with EAE. The results showed that Mdivi-1 treatment strongly lowered the level of GSK-3β in macrophages/microglia in the white matter of spinal cords of mice with EAE (Fig. 7f), suggesting that GSK-3β-mediated promotion of the NF-κB pathway was dampened by Mdivi-1 in the EAE-induced inflammatory response.

Discussion

Mitochondria play central roles in cellular metabolism, antiviral response, and virus stress responses as organelles for ATP production. It is highly dynamic and undergoes fission and fusion processes to regulate mitophagy, apoptosis, and cell proliferation. Thus, mitochondrial dysfunction is involved in a series of diseases, such as neurodegenerative, metabolic, neuromuscular, and cardiovascular diseases [24]. Drp1, a conserved GTPase in the dynamin family, mediates mitochondrial fission and is thought to be a potential molecule for therapies for associated disorders. Mdivi-1 was discovered as the first specific inhibitor of Drp1 and has shown therapeutic promise in animal models of neurodegenerative diseases, such as Parkinson's Disease (PD), AD, and MS [8, 14, 25–27].

Consistent with our previous research [14], Mdivi-1 treatment delays the onset of EAE and alleviates the severity of EAE. While our previous work dedicated that Mdivi-1 played a role in T cell polarization in EAE mice,

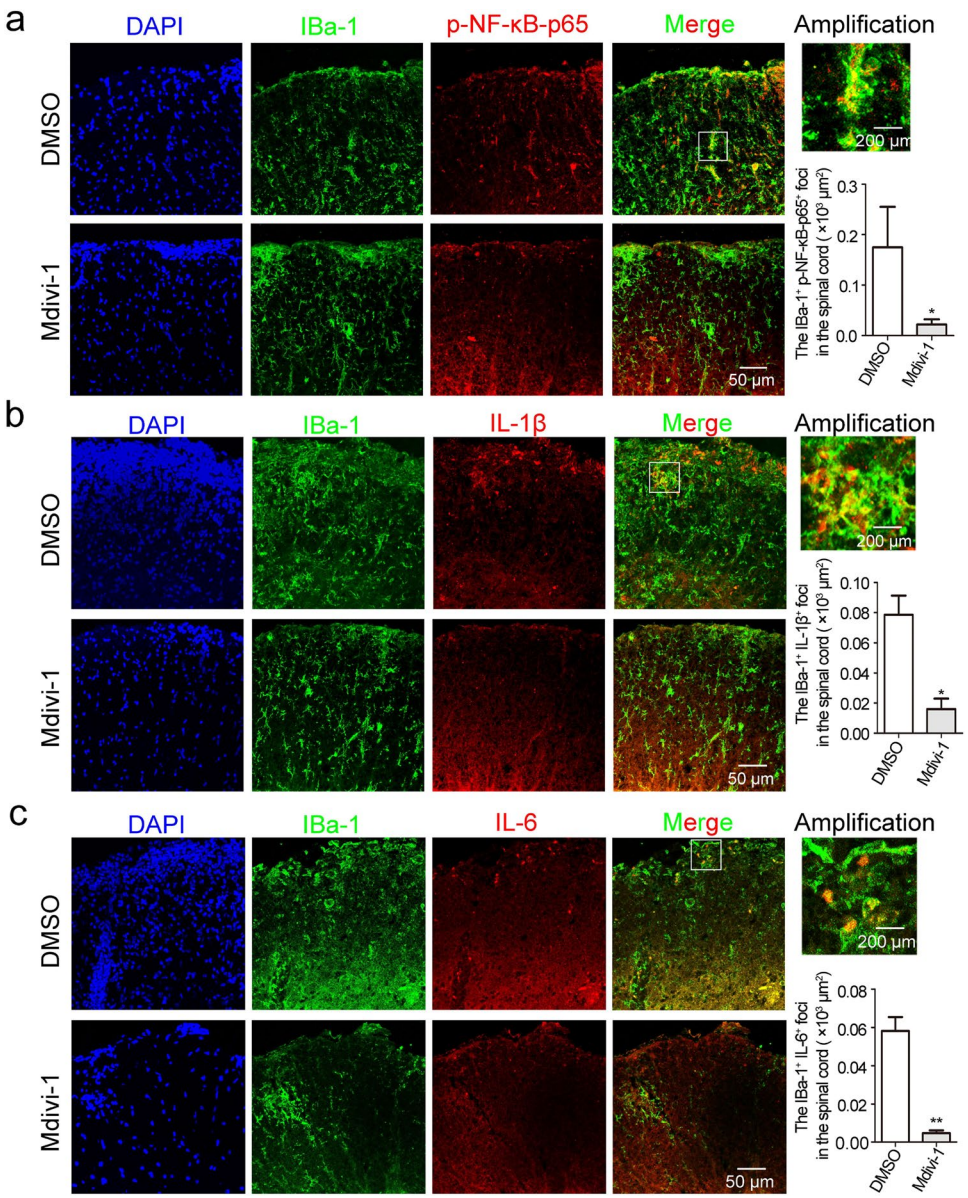

Fig. 7 Mdivi-1 inhibits the TRL2/4- and GSK-3β-mediated NF-κB pathways. On Day 28 p.i., the lumbar cords were separated from mice with EAE treated with Mdivi-1 or DMSO under anesthesia. Immunostaining of p-NF-κB-p65, IL-1β, IL-6, TLR2, TLR4, GSK-3β, and IBa-1 was performed. The foci of IBa-1$^+$p-NF-κB-p65$^+$ (**a**), IBa-1$^+$IL-1β$^+$(**b**), IBa-1$^+$IL-6$^+$(**c**), IBa-1$^+$TLR2$^+$(**d**), IBa-1$^+$ TLR4$^+$ (**e**), and IBa-1$^+$ GSK-3β$^+$ (**f**) were quantified. DAPI was used for nucleus staining. $N = 5–8$, Mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. One representative of two experiments is shown

the effect of Mdivi-1 on macrophage/microglial polarization in EAE remains poorly understood.

MS/EAE is an autoimmune and neuroinflammatory disease in which major pathological characteristics are demyelinated plaques and astrocytic scars resulting from local inflammatory lesions [28]. In the EAE model, myelin antigens are presented by APCs to native T cells and prime the immune reaction in the peripheral system, and then activated cells infiltrate into the CNS across the BBB. The predominant immune cells are macrophages and microglia, followed by CD4$^+$ T cells [5, 29]. Once inside, both infiltrated and resident macrophages/microglia are activated, release cytokines, and restimulate T cells, leading to disease progression. During MS pathology, APCs play pivotal roles in multiple stages. Macrophages/microglia are main the APCs and highly express MHC II, CD80, CD86, and CD40 molecules once activated [30, 31]. It has been reported that the elimination of perivascular macrophages attenuates the clinical signs of EAE, suggesting the potential roles of macrophages in the pathogenesis of EAE [32]. Our results showed that Mdivi-1 inhibited p-Drp1 at serine 616 in IBa-1$^+$ cells in the spinal cords of EAE mice, suggesting that Mdivi-1 plays the roles in macrophages/microglia in the spinal cord, which may be associated with the regulation of mitochondrial dynamics. In macrophages/microglia from the EAE mouse spinal cord, the levels of MHC II and CD86 were reduced in the Mdivi-1-treated group compared to those in the DMSO-treated group, and proliferative CD4$^+$ T cells in the spinal cord were decreased with Mdivi-1 administration. It is likely that the decrease in APC activation and

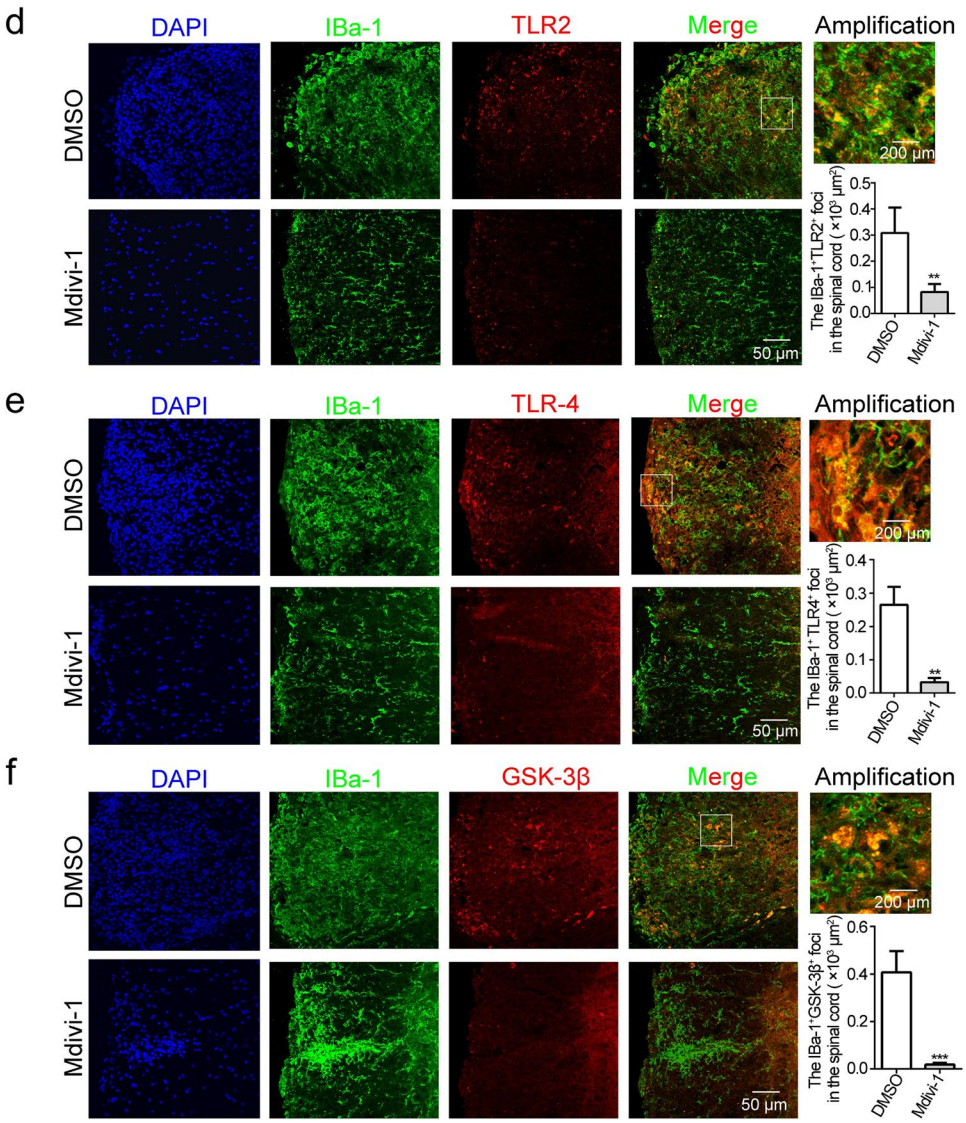

Fig. 7 (continued)

the T cell response contributes to a lower inflammatory response in the spinal cord of Mdivi-1-treated EAE mice, as lower expression of CD68 and GM-CSF was shown.

In response to different microenvironments, both macrophages and microglia are activated and present remarkable plasticity. At present, most studies divide macrophages/microglia into M1 and M2 phenotypes [20]. The cell surface markers expressed in macrophages/microglia are recognized by antibodies to assess their activation, including CD11b, IBa1, CD68, and glycoprotein F4/80. M1 macrophages/microglia secrete proinflammatory cytokines and immunoglobulin Fc receptors, such as IL-1β, IL-6, TNF-α, iNOS, and CD16/32, whereas the M2 phenotype is generally characterized by upregulation of Arginase-1, IL-10, CD206, and CD163 [33]. Due to the prominent roles of the M2 phenotype in tissue repair, promoting the conversion of macrophages/microglia from M1 to M2 may have potential as a therapeutic strategy for neuroinflammatory diseases. The results of our *in vivo* data indicated that Mdivi-1 treatment polarized macrophages/microglia from the M1 phenotype to the M2 phenotype in both the spinal cord and spleen of EAE mice. Meanwhile, Mdivi-1 also promoted the transformation of SIM-A9 microglia to an anti-inflammatory phenotype, as shown by decreased levels of the M1 marker CD16/32 and increased levels of the M2 marker CD206, accompanied by decreased secretion of iNOS and TNF-α and enhanced release of IL-10 and Arginase-1. These results may explain why Mdivi-1 ameliorated the severity of EAE in mice. Previous studies have shown that M2 polarization within microglia and macrophages derived from peripheral tissues are essential during remyelination and contribute to neuroprotective effects [34, 35]. The drug lenalidomide prevents the progression of EAE by promoting macrophage M2 polarization and differentiation of proinflammatory T cells

in both the peripheral lymph system and CNS [36]. Fasudil ameliorates the severity of mice with EAE as a Rho kinase inhibitor and promotes the shift of BV-2 microglia from the M1 to M2 phenotype [37]. In summary, coinciding with past studies, we did observe that macrophages/microglia were polarized toward the anti-inflammatory M2 phenotype both in the peripheral spleen system and spinal cord after Mdivi-1 treatment, which was confirmed by the *in vitro* data from SIM-A9 microglial cells, resulting in the reduced severity of EAE by Mdivi-1 administration.

Based on these observed data in EAE mice, we questioned whether Mdivi-1 treatment converts macrophage/microglia polarization from M1 to M2 via the NF-κB pathway, which releases proinflammatory cytokines leading to neurotoxicity and is considered the major signaling pathway in response to inflammation [38]. Our data showed that Mdivi-1 decreased the expression of p-NF-κB-p65 in the white matter of EAE mouse spinal cords, which was correlated with significantly decreased production of proinflammatory cytokines, such as IL-1β and IL-6. Because TLR2/4 are the main receptors that stimulate the NF-κB signaling pathway, we observed that the expression of TLR2 and TLR4 was significantly inhibited in IBa-1$^+$ cells treated with Mdivi-1. GSK-3β, as a key regulator of glycogen metabolism, plays a central role in multiple signaling pathways, including the NF-κB signaling pathway [39]. Inhibition of GSK3β activity leads to a dramatic decrease in NF-κB activity [40]. Here, we observed that the expression of GSK3β was dramatically decreased in IBa-1$^+$ cells in the white matter of Mdivi-1-treated EAE mouse spinal cords, suggesting that Mdivi-1 ameliorated the inflammatory reaction in mice with EAE through the TLR2/4-GSK3β-NF-κB pathway but did not exclude other pathways involved in the mechanism of alleviation of mice with EAE by Mdivi-1. For example, MAPKs, similar to the NF-κB pathway, are another downstream pathway regulated by LPS-induced TLR2/4 signaling, including ERK, p38, and JNK [41]. Previous studies have reported that p38 is needed for the activation of the NF-κB pathway, and the TLR-ASK1-p38 pathway may serve as a promising target for MS therapy [42, 43]. Therefore, we further investigated whether p38 regulation by Mdivi-1 confers a neuroprotective effect in EAE mice. The results showed that Mdivi-1 markedly decreased p38 expression in IBa-1$^+$ cells in the white matter of EAE mouse spinal cords compared with the control group (Fig. S2). Taken together, these findings imply that TLR2/4-activated GSK3β-NF-κB-p65 pathway is targeted by Mdivi-1 to decrease neuroinflammation

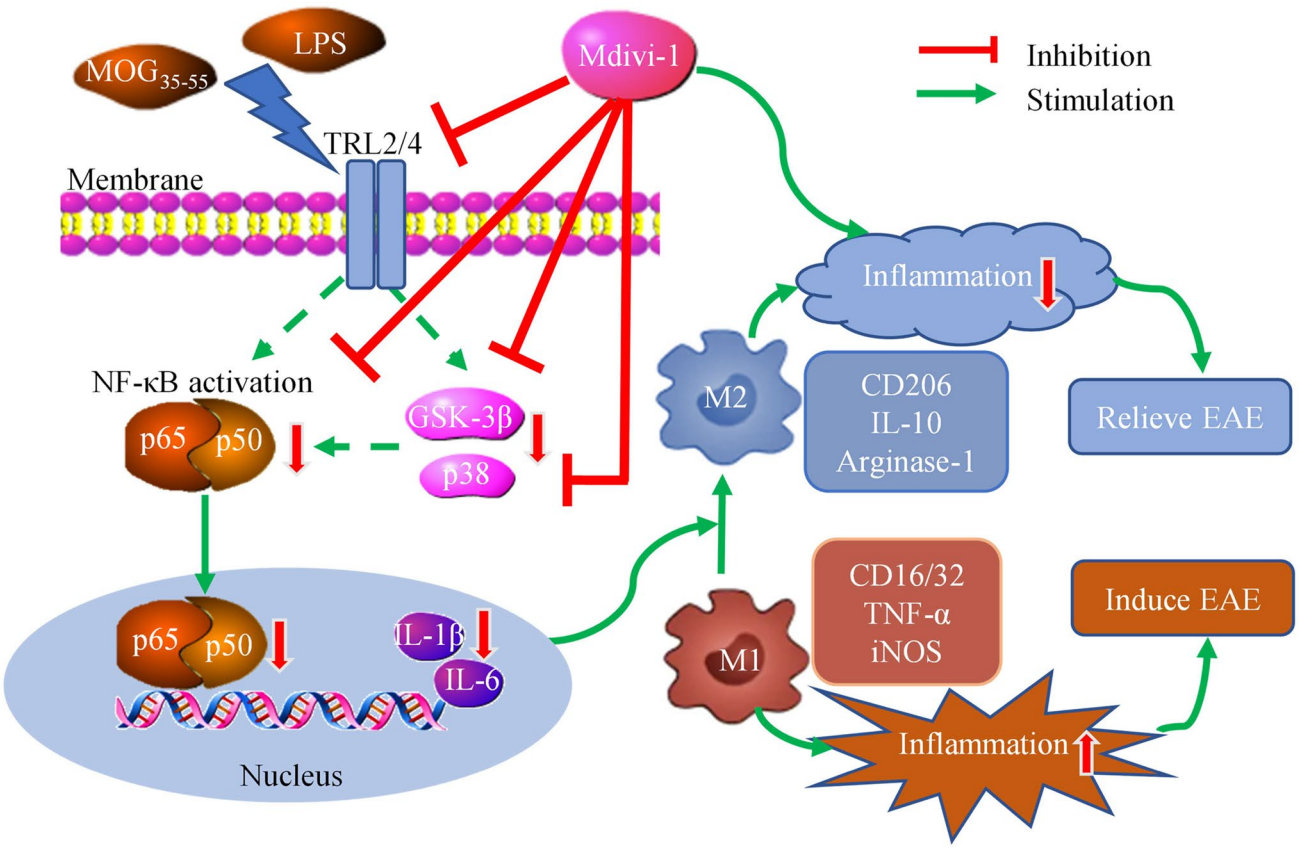

Fig. 8 Schematic diagram of Mdivi-1 promoted M2 polarization through the TLR2/4-GSK-3β-NF-κB pathway in EAE mice

during MS pathogenesis; meanwhile, Mdivi-1 can downregulate the level of p38 to alleviate the severity of EAE in mice (Fig. 8).

Increasing evidence has suggested that the macrophage/microglia phenotype is regulated by cellular metabolism. While M1 macrophages/microglia show enhanced glycolysis and the pentose phosphate pathway (PPP), increased ROS production, and accumulated succinate, M2 macrophages/microglia are characterized by efficient oxidative phosphorylation and reduced PPP [44, 45]. Mitochondria play a critical role in regulating metabolic dynamics as a center of metabolism. Mitochondrial fission regulated by the Drp1 protein leads to increased ROS production [46], while mitochondrial fusion facilitates oxidative phosphorylation of the electron transport chain [47]. However, little is known about mitochondrial fusion/fission contributing to the metabolic reprogramming of macrophages/microglia. It has been reported that aerobic glycolysis is reduced in bone marrow–derived macrophages after LPS stimulation through the inhibition of the phosphorylation of Drp1 by Mdivi-1 [7]. Our data showed that Mdivi-1 promoted M2 polarization in EAE mice, but it was not clear whether this effect depended on the inhibition of pDrp1 at Ser616 by Mdivi-1, including changes in mitochondrial and metabolic dynamics. In addition, Mdivi-1 plays multiple functions in other pathways. For example, some studies indicated that Mdivi-1 inhibited mitochondrial complex I and decreased ROS production [48]. A recent study showed that Mdivi-1 blocks peroxynitrite-induced Drp1 assembly, leading to a decrease in mitophagy and attenuating EAE pathology [49]. Thus, Mdivi-1 may reduce EAE severity through multiple pathways, and the exact mechanisms need further research.

Next, we will further explore the effect of mitochondrial dynamics on the polarization and antigen presentation capacity of macrophages/microglia. Moreover, dendritic cells (DCs) are the main immune cells that induce the peripheral immune response and are involved in the T cell response in the CNS system. It is also involved in immune infiltration in EAE [50]. Our results showed that Mdivi-1 suppressed the activation and antigen presentation capacity of macrophages/microglia, and further decreased activated $CD4^+$ T cells and the inflammatory response in the spinal cord of EAE mice, but we did not determine the effect of DCs on $CD4^+$ T cell activation as an APC with Mdivi-1 treatment. Future work using the CD11c marker could evaluate whether Mdivi-1 affects the antigen presentation capacity of DC cells in EAE progression, which is also an interesting issue.

In conclusion, the present data showed that Mdivi-1 facilitated the polarization of macrophages/microglia from the M1 to M2 phenotype and decreased the antigen presentation capacity of macrophages/microglia, leading to a reduced inflammatory response in the spinal cords of EAE mice, which was regulated by preventing TLR2/4-activated GSK3β-NF-κB-p65 signaling pathways.

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Author Contribution XQL and XJZ participated in the design of the study, conducted the majority of the experiments, analyzed the results, and wrote most of the manuscript. XJN and QW carried out the clinical score analysis of mice with EAE. PJZ, XHX, and JZY helped with the design and data analysis in flow cytometry experiments; GBS, GPX, and LJS helped with the confocal photomicrographs; YHL and CGM contributed to the supervision of the study.

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Availability of Data and Material The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code Availability Not applicable.

Declarations

Ethics Approval Animal experimental protocols have been approved by the Institutional Animal Care and Use Committees of Shanxi Datong University. No human study was performed.

Consent to Participate Informed consent was obtained from all individual participants included in the study.

Consent for Publication The participant has consented to the submission of the case report to the journal.

Conflict of Interest The authors declare no competing interests.

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