Fasudil-Triggered Phagocytosis of Myelin Debris Promoted Meylin Regeneration via the Activation of TREM2/DAP12 Signaling Pathway in Cuprizone-Induced Mice

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Research Article

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Abstract

The inflammation and demyelination of the central nervous system (CNS) are mainly involved in multiple sclerosis (MS), in which the disorder of myelin regeneration leads to continual neurologic impairment. Fasudil, one of the ROCK inhibitors, has been shown protective functions in some models of demyelinating diseases. In this study, Fasudil treatment ameliorated the behavioral performance and myelin loss in CPZ-fed mice. Here, we demonstrated a new role of Fasudil, which triggered microglia to uptake myelin debris in both cell and animal experiments. This increased phagocytosis was associated with the polarization of M2 microglia. Furthermore, we found that Fasudil enhanced the expression of triggering receptor expressed on myeloid cells 2 (TREM2) and DNAX-activating protein of 12 kDa (DAP12), which regulated microglial phagocytosis and M2 polarization. The silence of TREM2 effectively blocked Fasudil-triggered phagocytic capacity, suggesting that Fasudil-triggered phagocytosis depends on TREM2 signaling pathway. Based on these evidences that TREM2 regulates microglial M2 polarization and phagocytosis, future studies targeted Fasudil as a therapy for demyelinating and neurodegenerative diseases are warranted.

Introduction

Demyelinating disease is a kind of immune-mediated diseases in central nervous system (CNS), mainly characterized by multifocal inflammatory demyelination, including multiple sclerosis (MS) and neuromyelitis optica (NMO) (Höftberger et al. 2017). In recent years, the incidence rate of demyelinating diseases has been increasing worldwide, which seriously affects the life quality and physical/mental health of patients (Dobson et al. 2019; Hor et al. 2020). Although the etiology of demyelinating diseases is still unclear, its histological feature mainly includes T/B cells infiltration, microglial activation, neuroinflammation, oligodendrocytes (OLs) death, subsequent demyelination and neuronal death (Reich et al. 2018). In terms of MS, with the development of disease, the treatment becomes increasingly complex. At present, disease-modifying therapies can reduce the frequency of relapse and the severity of the MS (Bross et al. 2020). Considering that some new drugs may be associated with potentially serious but rare adverse events, it is necessary to maximize the benefitial profile and minimize the risk to patients.

At present, the available drugs for MS are predominantly immune regulation, which do not directly target demyelination. Myelin regeneration is regarded as a critical approach for the therapy of demyelinating disorders, since the increase of myelin regeneration contributes to the recovery of neuronal function and clinical symptoms (Plemel et al. 2017; Luchetti et al. 2018). Recently, studies have been interested in myelin regeneration, which is a process of myelination around the axons and has been recorded in animal models and MS patients (Lubetzki et al. 2020; He et al. 2021). Therefore, myelin regeneration is a crucial way to treating MS, since myelin is an important element to protect myelin-axon unit (Stadelmann et al. 2019). For this reason, myelin regeneration from OLs is the main mechanism of natural repair against demyelination.

Myelin regeneration is the natural regeneration to counter demyelination (Franklin et al. 2008), but the reasons for the disorder or incompleteness of myelin regeneration during MS are not fully elucidated (Goldschmidt et al. 2009). The process of myelination is subjected to both positive and negative regulation (Plemel et al. 2017). The failure of remyelination can be segregated into at least two distinct stages: the mobilization of OPCs in the lesions is impaired, and/or the differentiation/maturation of OLs is obstructed (Plemel et al. 2017). In progressive MS patients, although OPCs are present in the demyelinating lesion, mature OLs are almost completely deficient (Chang et al. 2002; Kuhlmann et al. 2008). Therefore, the reason for the failure of remyelination may be partially due to the lack of migration/proliferation of OPCs, and more likely to the failure of OPC differentiation, especially due to the imbalance between inhibitory and stimulant signals in the lesion area.

Furthermore, the failure of remyelination may also be associated with the presence of myelin debris in demyelinating regions, which significantly obstruct the formation of mature OLs (Lloyd et al. 2019). The accumulation of degraded debris not only causes inflammatory response, but also impedes the restoration of myelin sheath in the CNS. Studies show that microglial phagocytosis is beneficial for tissue repair and play an critical role in the progress of demyelinating disorders (Neumann et al. 2009; Pinto et al. 2020). Therefore, skewing strategies to enhance the phagocytosis of microglia offer a promising alternative in the future treatment of MS.

Fasudil, a potent ROCK inhibitor, has been shown its beneficial effects in demyelinating diseases possibly through different mechanisms, including anti-inflammation, anti-oxidation, anti-apoptosis and so on (Yan et al. 2019). In this study, we evaluated the therapeutic effectiveness of Fasudil in CPZ-fed mice by microglial phagocytosis of myelin debris and explored the underlying cellular and molecular mechanisms of the action.

Materials And Methods

Animals

Male C57BL/6 mice (9–10 weeks), weighing 21–22 g, were obtained from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). Mice were fed in pathogen-free conditions with constant temperature, humidity and light/dark cycle for seven days before experimental manipulation.

Then mice were divided into three groups (N = 12/group) as follows: normal diet group, CPZ diet plus saline treatment group (CPZ + NS) and CPZ diet plus Fasudil treatment group (CPZ + Fasudil). Mice were fed with 0.2% (w/w) CPZ diet for 6 weeks and weighed every other day. After fed with a standard diet for 4 weeks, mice were intraperitoneally injected with Fasudil (40 mg/kg/d) or saline for consecutive 2 weeks.

Behavioral evaluation

It has been showed that CPZ-induced mice have the abnormalities of anxiety- and depression-like behaviors (Sen et al. 2019; Mohamed et al. 2019). Therefore, elevated plus maze (EPM), and forced swimming (FS) tests were used respectively to measure anxiety and depression of mice (Lister et al. 1990; Naserzadeh et al. 2019). EPM test: each mouse were carefully put into the center of the plus-maze and the total distance in open arm was recorded during the limited time (10 min). FS test: each mouse was put into a cylinder filled with a depth of 20 cm water (25 ± 1℃). The mean swimming speed was calculated by SMART V3.0 software.

Tissue processing

Half of mice underwent transcardial infusion using saline and 4% paraformaldehyde (PFA). Then brains were dehydrated using graded sucrose solutions and embedded with OCT. Brain coronal sections were sliced for fluorescent myelin and immunofluorescence staining. The other half of the mice was perfused with saline and brains were stored at -80℃ for subsequent expriments.

Fluorescent myelin staining

Brain sections were rehydrated with 0.2% TRITON X-100 and the FluoroMyelin Green staining solution (Thermo, USA) was prepared. Then, the sections were flooded with staining solution at RT for 20 min. After washed three times, the brain sections were observed under microscopy and quantitatively measured by Image-Pro Plus 6.0 software.

Immunofluorescent staining

After blocking with 1% BSA/PBS, brain slides were added with anti-Iba1 (1:200, BD Bioscience, USA), anti-MBP (1:500, Abcam, USA), anti-iNOS (1:300, Abcam, USA), anti-Arg-1 (1:300, Gene Tex, USA), anti-TREM2 (1:150, Santa Cruz Biotechnology, USA), anti-DAP12 (1:300, Abcam, USA), anti-NG2 (1:500, Millipore, Germany) and anti-Ki67 (1:200, BD Pharmingen, USA). Then, sections were added with secondary antibodies and analyzed under fluorescent microscopy.

Microglial culture

The BV2 mouse microglia obtained from ShenKe Biological Technology Co., Ltd. and cultured in complete medium (Dulbecco's Modified Eagle Medium with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin) at a constant incubator. BV2 microglia were incubated for further experiments.

Purification and labeling of myelin debris

Myelin debris was extracted as previously reported (He et al. 2019). Briefly, mice brain was homogenized in sucrose and washed with PBS. Then the pellet was obtained using gradient density centrifugation and labeled in carboxyfluorescein succinimidyl ester (CFSE) solution. After centrifugated, fluorescein-labeled myelin debris (FMD) was washed with PBS and resuspended to 100 mg/ml.

Transfection

BV2 cells were cultured in 24-well plates overnight. According to the instructions of Lipofectamine 2000 transfection reagent (Thermo, USA), 1 μl Lipofectamin 2000 was added into 50 μl Opti-MEM (Gibco, USA) in each well and incubated for 5 min. Then appropriate amounts of normal control (NC), TREM2-siRNA1, TREM2-siRNA2 and TREM2-siRNA3 were diluted respectively with 50 μl Opti-MEM for 5 min. The two mixtures were mixed thoroughly for 20 min and added to the the wells containing the basic medium. After 6 h intervention, BV2 cells were replaced with complete medium for additional culturing 48 h. The sequences of siRNAs were NC siRNA (FWD: UUCUCCGAACGAGUCACGUTT; REV: ACGUGACUCGUUCGGAGAATT); TREM2 siRNA1 (FWD: GAUGCUGGAGA UCUCUGGGTT; REV: CCCAGAGAUCUCCAGCAU CTT); TREM2 siRNA2 (FWD: GGAGGUACGUGAGAGAAUUTT; REV: AAUUCU CUCACGUACCUCCTT); TREM2 siRNA3 (FWD: CCUUGCUGGAACCGUCACCAUTT; REV: AUGGUGACGGUUC CAGCAAGGTT). The relative expression of TREM2 was determined by real time-PCR (RT-PCR).

Flow cytometry analysis

1) Phagocytic assay of BV2 cells after Fasudil intervention. To determine whether Fasudil promotes the microglial phagocytosis of FMD, BV2 microglia were plated in 24-well plates and groups were set as follows: PBS group (PBS), FMD plus PBS group (Myelin + PBS) and FMD plus Fasudil group (Myelin + Fasudil). Myelin + PBS and Myelin + Fasudil groups were incubated with 5 mg/ml FMD. According to our previous study, Myelin + Fasudil group was intervented with 15 μg/ml Fasudil (Ding et al. 2021). FMD + Microglia were analyzed by flow cytometry.

2) Phenotype analysis of BV2 cells. PBS group (PBS), Fasudil group (Fasudil), FMD plus PBS group (Myelin + PBS) and FMD plus Fasudil group (Myelin + Fasudil) were set for the subsequent experiments. After BV2 microglia were cultured in 24-well plates and 10 cm culture dishes, Myelin + PBS and Myelin + Fasudil groups were incubated with FMD. Fasudil and Myelin + Fasudil groups were added with 15 μg/ml Fasudil. BV2 microglia in four groups were obtained after 48 h culture.

Then, cultured BV2 cells were fixed with 4% PFA and stained in 1% BSA/PBS buffer with fluorescently-labeled antibodies (eBioscience, USA) as follows: PE-CD206, PE-IL-12, PE-iNOS, APC-CD16/32, APC-IL-10 and APC-Arg-1. Cells were collected and analyzed using flow cytometry. Data were processed with FlowJo V10 software and results were presented as mean fluorescence intensity (MFI).

3) Phagocytic analysis of BV2 cells after TREM2 siRNA interference. Based on the screening of TREM2-siRNA1 that significantly interferes with the expression of TREM2, BV2 microglia were cultured in 24-well plates and groups were set as follows: normal + FMD group, TREM2-siRNA1 + FMD group, normal + FMD + Fasudil group and TREM2-siRNA1 + FMD + Fasudil group. After BV2 microglia were cultured in 24-well plates for 12 h, TREM2-siRNA1 + FMD and TREM2-siRNA1 + FMD + Fasudil groups were transfected with TREM2-siRNA1 for 6 h. After all groups were incubated with FMD, normal + FMD + Fasudil and TREM2-siRNA1 + FMD + Fasudil groups were added with 15 μg/ml Fasudil. After 48 h intervention, microglial phagocytosis were analyzed by flow cytometry.

Phagocytic assay by fluorescence reader and microscopy

BV2 microglia were cultured in 24-well plates and intervented with 5 mg/ml FMD for 48 h. Unphagocytosed FMD was washed away, and level of phagocytosis was analyzed under fluorescence microscopy and multifunctional Microporous Plate Reader using fluorescence excited light (485 nm).

Immunocytochemistry

After cultured and intervened in 24-well plates with coverslips, BV2 microglia in four groups was fixed with 4% PFA and incubated with 1% BSA/PBS. Then these cells were added with the following antibodies: anti-iNOS, anti-Arg-1, anti-TREM2 and anti-DAP12 at 4℃ overnight, followed by secondary antibodies for 1.5 h. Data were analyzed by Image-Pro Plus 6.0 software.

RT-PCR

The total RNA of BV2 microglia were isolated by RNA prep Pure Cell Kit (Tiangen, China). Total yield of RNA were measured by NanoDrop One (Thermo, USA). After cDNA was obtained, quantitative RT-PCR was operated on the CFX96 RT-PCR System (Bio-Rad, USA). Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal reference. The primers were presented as follows: iNOS (FWD: CAGGGAGAACAGTACATGAACAC; REV: TTGGATACACTGCTACAGGGA); Arg-1 (FWD: CATATCTGCCAAAGACATCGTG; REV: GACATCAAAGCTCAGGTGA ATC); TREM2 (FWD: TCATGTACTTATGACGCCTTGA; REV: GAGGTTCTTCAGA GTGATGGTG); GAPDH (FWD: TGTGTCCGTCGTGGATCTGA; REV: TTGCTGTTG AAGTCG CAGGAG). Differences of gene expression in cells between the groups was described using the results of $2^{-\triangle\triangle Ct}$.

Western blot analysis

The protein of brains and BV2 microglia was obtained by RIPA lysis buffer. Then concentration was measured by BCA protein determination method. After separated by SDS-PAGE, the extracts transferred onto PVDF membranes (Millipore, USA). Subsequently, the membranes were added overnight with the following antibodies: rabbit anti-iNOS (1:1000), rabbit anti-Arg-1 (1:600), mouse anti-TREM2 (1:800), rabbit anti-DAP12 (1:1000), mouse anti-NG2 (1:900), rabbit anti-β-actin (1:1000) and rabbit anti-GAPDH (1:1000). Then, the membranes were incubated with HRP-conjugated antibody for 2 h. Immunoblots were obtained and analyzed using Quantity Software (Bio Rad, USA). β-actin and GAPDH were used respectively as an internal control.

Data analysis

All experiments were replicated three times, and Graphpad Prism 8.0 software was used for statistical analysis. Significance of the results was analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons. Data were expressed as the mean ± SD. The statistically significant effects are indicated by asterisks ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$).

Results

Fasudil ameliorated behavior disorder and protected the myelin sheath in CPZ-induced mice

It is well-known that the CPZ-induced demyelinating mice has been widely used to study myelin regeneration in the CNS. The selective loss of OLs can be observed after 2-week CPZ feeding, and obvious demyelination found at six weeks (An et al. 2020). The design of animal experiment and change of body weight were showed in Fig. 1a and b. The weight of CPZ-diet mice was lower than that of normal-fed mice, but there was no difference compared with Fasudil-treated mice.

Some clinical manifestations, such as anxiety- and depression-like behaviors, have hinted the demyelinating lesions in the brain. In this study, EPM and FS tests were performed to analyze these behavior disorders. The results demonstrated that mice fed with CPZ for 6 weeks showed more anxiety and depression than those of normal-fed mice (Fig. 1c, $P < 0.0001$, respectively). However, those behavioral abnormalities were improved effectively by Fasudil intervention (Fig. 1c, $P < 0.0001$ and $P < 0.001$, respectively).

Histopathologically, fluorescent myelin staining was used to observe the severity of myelin sheath injury. The results revealed that the loss of myelin sheath in the corpus callosum and striatum of CPZ diet mice was more than those of normal-fed mice (Fig. 1d, $P < 0.0001$). Fasudil intervention obviously increased the intensity of fluorescent myelin staining (Fig. 1d, $P < 0.01$). These results suggested that CPZ diet mice showed demyelination, which was obviously reversed by Fasudil intervention.

Fasudil enhanced the phagocytosis of myelin debris by microglia

Myelin debris which is formed after demyelination prevented OPC recruitment and differentiation from remyelinating OLs (Neumann et al. 2009). Therefore, removing myelin debris is particularly important for myelin regeneration. Firstly, we observed that some MBP staining was co-located with $Iba1^+$ microglia in CPZ-induced mice (Fig. 2a), and Fasudil intervention increased $Iba1^+$ microglia co-staining MBP compared with CPZ diet mice (Fig. 2a, $P < 0.05$), indicating that microglia can phagocytize myelin debris and Fasudil may promote microglial phagocytosis. Secondly, in the following in vitro cell experiments, our results showed that the fluorescence intensity of phagocytic debris in BV2 microglia increased significantly by fluorescence scanning of microplate reader after Fasudil intervention compared with the PBS treatment group (Fig. 2b, $P < 0.01$). The co-localization of FMD and BV2 cells under phase contrast of fluorescence microscope was shown in enlarged image (Fig. 2b). Finally, the analysis from flow cytometry further showed that the $CFSE^+$ cells representing debris phagocytosis were significantly increased after Fasudil intervention (Fig. 2c, $P < 0.0001$). In short, these results indicated that Fasudil intervention significantly promoted the clearance of toxic debris by microglia.

The phagocytosis was accompanied by microglial M2 polarization

The enhancement of microglial phagocytosis is related to M2 phenotypic transformation (Li et al. 2019). In order to further confirm whether Fasudil triggers the microglial M1 phenotype toward M2 phenotype under myelin debris phagocytosis, we analyzed the M1 markers (CD16/32, iNOS) and the M2 markers (CD206, Arg-1) in vitro experiments. The results demonstrated that the expression of iNOS mRNA/protein and Arg-1 mRNA was increased after myelin debris stimulation, especially the expression of iNOS mRNA/protein (Fig. 3a and b, $P < 0.01$, respectively). Fasudil intervention downregulated the expression of iNOS mRNA (Fig. 3a, $P < 0.05$), but obviously increased the expression of Arg-1 mRNA (Fig. 3a, $P < 0.01$). At the same time, myelin debris stimulation increased the expression of iNOS protein (Fig. 3b, $P < 0.01$), which was effectively inhibited by Fasudil intervention (Fig. 3b, $P < 0.01$). Conversely, the addition of Fasudil induced the expression of Arg-1 protein (Fig. 3b, $P < 0.01$).

Immunocytochemistry staining showed that FMD induced the expression of $iNOS^+$ on BV2 cells, while Fasudil inhibited its expression (Fig. 3c). In contrast, Fasudil obviously upregulated the expression of $Arg\text{-}1^+$ on BV2 cells after FMD stimulation (Fig. 3d). We also analyzed the phenotype of M1 markers (CD16/32, iNOS) and M2 markers (CD206, Arg-1) by flow cytometry. The results showed that myelin debris enhanced CD16/32 and iNOS (Fig. 3e, $P < 0.0001$, respectively), and inhibited CD206 and Arg-1 (Fig. 3e, $P < 0.05$ and $P < 0.01$, respectively). Fasudil effectively inhibited CD16/32 and iNOS (Fig. 3e, $P < 0.0001$, respectively), and upregulated CD206 and Arg-1 (Fig. 3e, $P < 0.0001$, respectively).

The next question is whether BV2 cells phagocytizing myelin debris are associated with M2 polarization. We analyzed the M1 phenotype (CD16/32, iNOS, IL-12) and M2 phenotype (CD206, Arg-1, IL-10) by gating $CFSE^-$ and $CFSE^+$ cells. The results revealed that compared with $CFSE^-$ cells, $CFSE^+$ cells showed lower expression of CD16/32, iNOS and IL-12 (Fig. 4a, $P < 0.001$, $P < 0.0001$ and $P < 0.0001$, respectively), but upregulated the expression of CD206, Arg-1 and IL-10 (Fig. 4b, $P < 0.001$, $P < 0.01$, $P < 0.0001$, respectively). In short, these results indicate that Fasudil promotes BV2 cells to engulf FMD, accompanied by the microglial M2 polarization.

In CPZ-induced demyelinating model, immunofluorescent staining was used to observe the $iba1^+iNOS^+$ and $Iba1^+Arg\text{-}1^+$ microglia in the corpus callosum. $Iba1^+iNOS^+$ cells in CPZ + NS mice were higher than that in control mice, which was effectively inhibited by Fasudil intervention (Fig. 5a). Conversely, Fasudil treatment enhanced $Iba1^+Arg\text{-}1^+$ cells (Fig. 5b). Similarly, western blot analysis showed that CPZ feeding increased the expression of iNOS (Fig. 5c, $P < 0.01$) and declined Arg-1 (Fig. 5c, $P < 0.05$), which was inhibited and elevated separately by Fasudil intervention (Fig. 5c, $P < 0.05$ and $P < 0.001$, respectively).

Fasudil-triggered phagocytosis of myelin debris was dependent on TREM2 signaling pathway

Recent studies has reported that microglial TREM2/DAP12 pathway participates in engulfment of myelin debris and inhibits neuroinflammation of CNS (Konishi et al. 2018; Cignarella et al. 2020). Using immunocytochemistry staining, we observed the expression of TREM2 and DAP12 in vitro experiments. The results revealed that, compared with Myelin + PBS group, the expression of TREM2 and DAP12 was significantly upregulated in Myelin + Fasudil group (Fig. 6a and b). To further confirm whether Fasudil-

triggered phagocytosis of myelin debris is dependent on TREM2 signaling pathway, we downregulated TREM2 via transduction with the TREM2-siRNA vector in vitro. We screened 3 TREM2-siRNA vectors and used TREM2-siRNA1 to perform the following experiments (Fig. 6c). As shown in Fig. 6d, TREM2 knockdown inhibited the engulfment of FMD induced by Fasudil treatment ($P < 0.0001$). In brief, these results indicated that microglial clearance of myelin debris activated by Fasudil is mediated through TREM2/DAP12 signaling pathway.

Next, we need to confirm whether Fasudil intervention can induce the expression of TREM2 and DAP12 in CPZ demyelinating model. Consistent with in vitro results, the immunofluorescent staining revealed that $TREM2^+Iba1^+$ and $DAP12^+Iba1^+$ cells in the corpus callosum of CPZ + Fasudil mice was elevated than that in control and CPZ + NS mice (Fig. 7a and b). Similarly, western blot assay also defined that Fasudil treatment upregulated the expression of TREM2 and DAP12 compared with that of CPZ diet mice (Fig. 7c, $P < 0.0001$, respectively).

Fasudil facilitated the generation of OPCs in CPZ-fed mice

The mobilization and maturation of OPCs are two key steps during myelin regeneration. After the phagocytosis of myelin debris by microglia, we wondered how about the OPCs in the demyelinating area? The results of following experiments showed that compared with CPZ + NS mice, Fasudil treatment upregulated the $NG2^+$ OPCs (Fig. 8a), which expressed Ki67 (Fig. 8b), indicating that these OPCs are proliferating. Western blot assay also suggested that Fasudil treatment upregulated the expression of NG2 protein compared with CPZ-fed mice (Fig. 8c, $P < 0.01$).

Discussion

Myelin debris, a toxic product aggregated in the lesion areas, triggers inflammatory responses in the demyelinating brain of experimental models and MS patients (Clarner et al. 2012). Some studies have demonstrated that myelin debris not only upregulates the expression of inflammatory moleculars (TNF-α, IL-1β, IL-6), but also downregulates the production of anti-inflammatory mediators (IL-4 and TGF-β) (Sun et al. 2010; Yang et al. 2011). In addition, some inhibitory factors (NogoA, oligodendrocyte-myelin glycoprotein and myelin-associated glycoprotein) in myelin debris hinders axonal regeneration and further activates the immune system to cause myelin degeneration (McKerracher et al. 1994; Chen et al. 2000). Abundant studies have shown that the accumulation of myelin debris delay the efficiency of myelin regeneration (Kotter et al. 2006; Lampron et al. 2015), in which myelin debris particularly impedes OPC differentiation, indicating that the phagocytosis of myelin debris is a precondition before myelin regeneration can be started (Shields et al. 1999; Kotter et al. 2006).

The functions of microglia in demyelinating disorders, such as MS and its animal model, is still controversial. Studies have shown that microglia encompass a large amount of harmful factors, such as releasing proteases, inflammatory cytokines and free radicals, as well as promoting T lymphocyte reactivity in the CNS, resulting in the death of neurons and OPCs (Li et al. 2017; Voet et al. 2019).

However, previous studies have indicated that microglia have beneficial effect on neurodegenerative diseases, particularly on the recovery of experimental autoimmune encephalomeylitis (EAE) progression (Du et al. 2017; Yan et al. 2019). Numerous reports hold that microglia plays a crucial role in phagocytizing myelin debris. In demyelinating models, the disfunction of microglial phagocytosis slows the elimination of toxic debris and postpones the process of myelin regeneration (Ruckh et al. 2012; Voss et al. 2012; Marteyn et al. 2016). TREM2, a lipid sensor on microglia, binds to myelin debris and promotes microglial phagocytosis (Poliani et al. 2015). All these observations suggest that myelin debris should be inhibitory to myelin regeneration. Therefore, the elimination of myelin debris plays a beneficial role in axonal remyelination.

Fasudil is an intracellular calcium antagonist and the only clinically approved ROCK inhibitor up to now. Our studies have demonstrated that Fasudil possesses multiple functions, including anti-inflammation, immunomodulation, microglial M2 polarization, synaptogenesis and promoting secretion of neurotrophic factors in the CNS (Yan et al. 2019; Ding et al. 2021). Here, our results indicated that Fasudil enhanced microglial engulfment of myelin debris in cell and animal experiments, accompanied by the upregulation of TREM2/DAP12 and polarization of M2 microglia. It is speculated that Fasudil intervention can improve behavioral abnormality and myelin loss in CPZ-induced mice, which may be related to accelerating phagocytosis and clearance of myelin debris.

In this study, Fasudil effectively upregulated the expression of TREM2 and DAP12 on microglia. TREM2, a phagocytic associated receptor, is involved in microglial clearance of apoptotic neurons, toxic debris and β-amyloid (Fu et al. 2014; Cignarella et al. 2020). DAP12, a signaling adapter protein that pairs with TREM2, is essential for mediation of TREM2 signaling (Yao et al. 2019). The blockade of TREM2 during the progression of EAE led to disease deterioration with more inflammatory reactivity and demyelination in the CNS (Piccio et al. 2007). In CPZ-induced demyelinating mice, the aggregation of myelin debris and axonal injury was aggravated in TREM2 knock-out mice (Cantoni et al. 2015; Poliani et al. 2015). Recent study showed that TREM2 activation on microglia promoted the elimination of myelin debris in the CNS of CPZ-induced mice, resulting in the increase of OPCs and the formation of OLs in lesion areas (Cignarella et al. 2020). Additionally, previous studies indicated that TREM2 not only mediated myelin uptake, but also promoted debris degradation through the phagolysosomal pathway (Cantoni et al. 2015). As a TREM2-mediated signal is conducive to regulation of microglia for debris clearance, it is thought to be required for myelin regeneration (Lampron et al. 2015).

Besides, TREM2 plays an critical role in microglial M2 polarization. Knockdown of TREM2 in BV2 cells impeded M2 polarization and resulted in the cascade amplification of M1 microglial inflammatory responses; Conversely, overexpression of TREM2 strengthened M2 polarization and alleviated microglia-mediated inflammation (Zhang et al. 2018). TREM2 esiRNA decreased the secretion of protective cytokines and the expression of M2 markers, indicating that TREM2 regulated microglial M2 polarization (He et al. 2020). In accordance with the above results, Fasudil induced the upregulation of TREM2/DAP12 in microglia, which, on one hand, could regulate the engulfment of myelin debris, and on the other hand, induce the polarization of M2 microglia. Consequently, Fasudil intervention enhanced the proliferation of

OPCs and accelerated the differentiation of OLs, which may be related to the activation of TREM2/DAP12 signaling pathway, resulting in the increase of myelin debris clearance by M2-polarized microglia.

Undoubtedly, we need more investigations to further explore the above-mentioned findings in the following study. Firstly, the biological effects of microglia had not been dynamically observed after the phagocytosis of myelin debris. Secondly, we only found the high expression of TREM2 and DAP12, but did not understand how Fasudil affect this signaling pathway. Thirdly, we did not clarify whether there is an intrinsic relationship between the upregulation of phagocytic receptors and microglia polarization to M2 phenotype.

In conclusion, we demonstrated that Fasudil ameliorated the behavioral performance and myelin loss in CPZ-induced mice, enhanced the phagocytosis of myelin debris and polarization of M2 microglia, which depends on TREM2/DAP12 signaling pathway. As targeting microglial phagocytosis and M2 polarization, TREM2/DAP12 signaling pathway is upregulated by Fasudil, which should be related to the improvement of microenvironment in the brain. Therefore, future studies on the possibility to use Fasudil as a therapeutic for myelin regeneration are warranted.

Declarations

Author contributions

ZBD, QXH and LJS designed the study, carried out the tests. BGX and CGM conceived the study, participated in its design and coordination and helped draft the manuscript. QW, GYH and GGC participated in its design and revised the manuscript. CGM revised and finalized the manuscript. YQL, ZC and JZY participated in the statistical analysis. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflicts of interest

The authors declared that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethics approval

The animal study was reviewed and approved by Laboratory and Ethics Committee of Shanxi University of Chinese Medicine.

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Figures

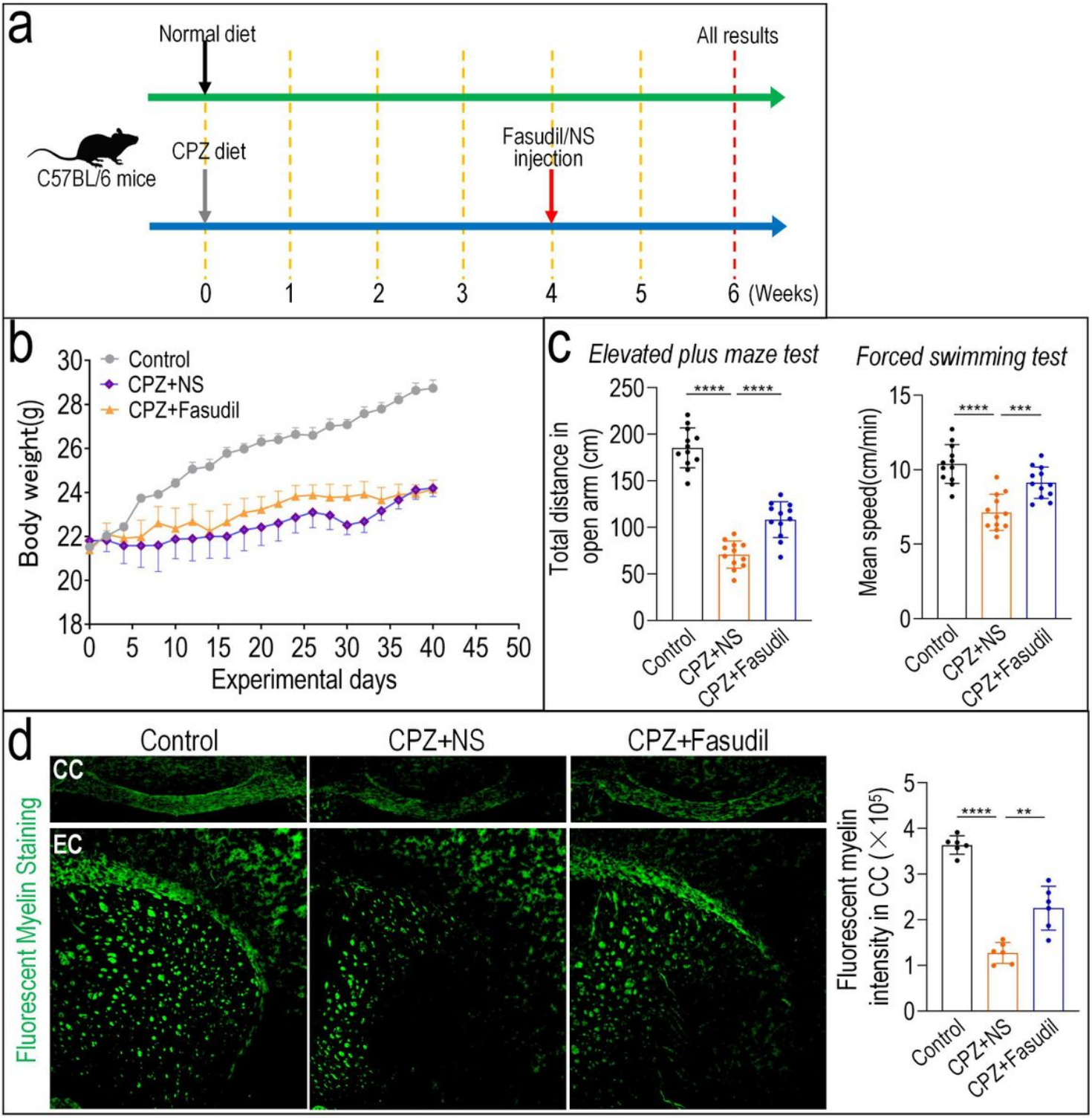

Figure 1

Fasudil ameliorated behavioral performance and reduced myelin loss. (a) The design scheme of experimental protocol, (b) body weight change, (c) Anxiety- and depression-like behavior by EPM and FST tests, (d) pathological evaluation of myelin loss by fluorescent myelin staining. Quantitative results are mean±SD. **P<0.01, ***P<0.001, ****P<0.0001.

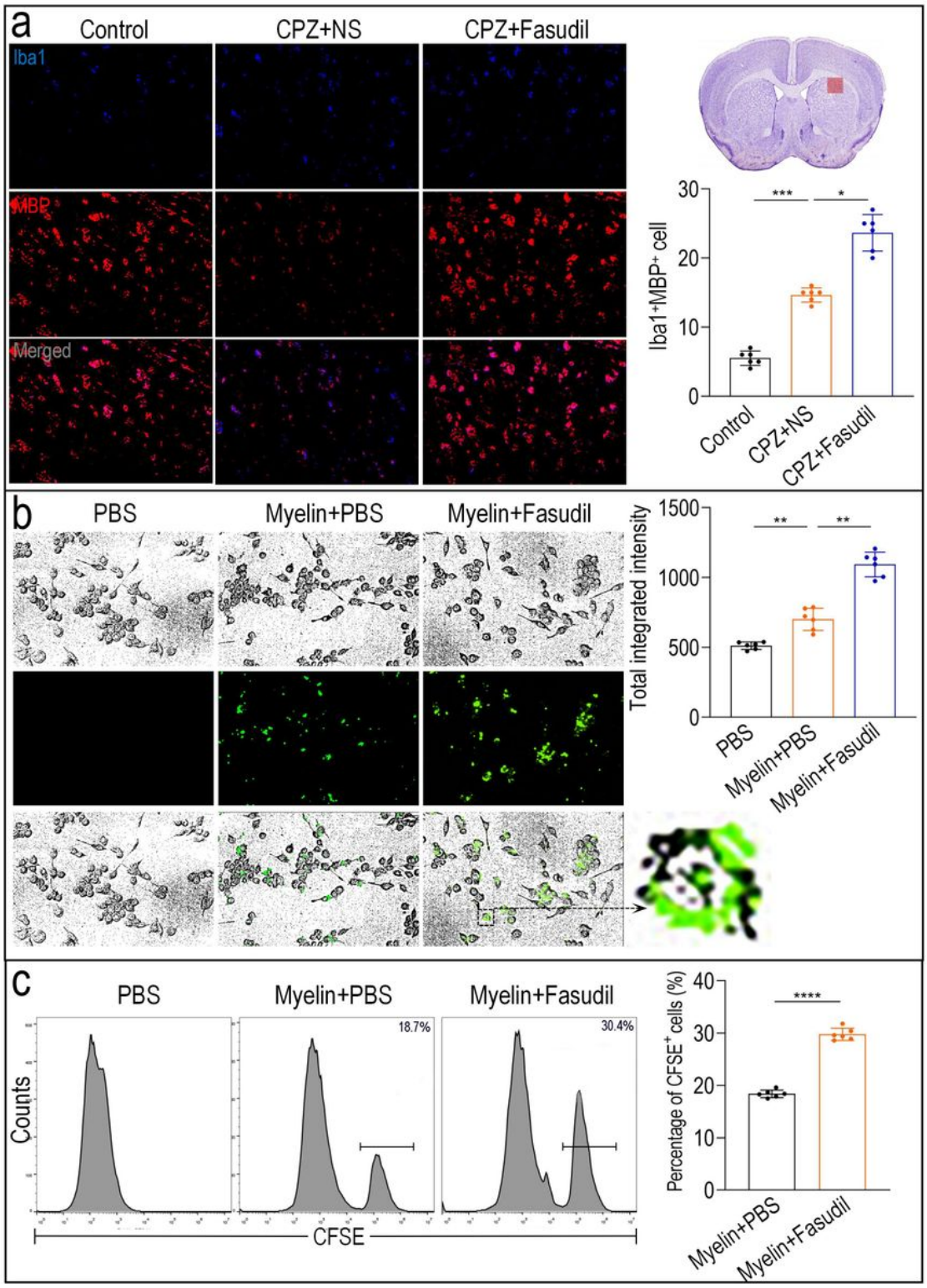

Figure 2

Fasudil induced microglia to uptake myelin debris in vivo and in vitro. (a) Iba1+MBP+ microglia in striatum by double immunofluorescence staining, (b) the phagocytic capacity of BV2 cells was observed by fluorescence microscope after 48 h of Fasudil treatment (left), quantitative analysis of phagocytic intensity was obtained by Multifunctional Microporous Plate Reader (right), (c) the phagocytic capacity of

BV2 cells was analyzed by flow cytometry after 48 h of Fasudil incubation. Quantitative results are mean±SD. *P<0.01, **P<0.01, ***P<0.001, ****P<0.0001.

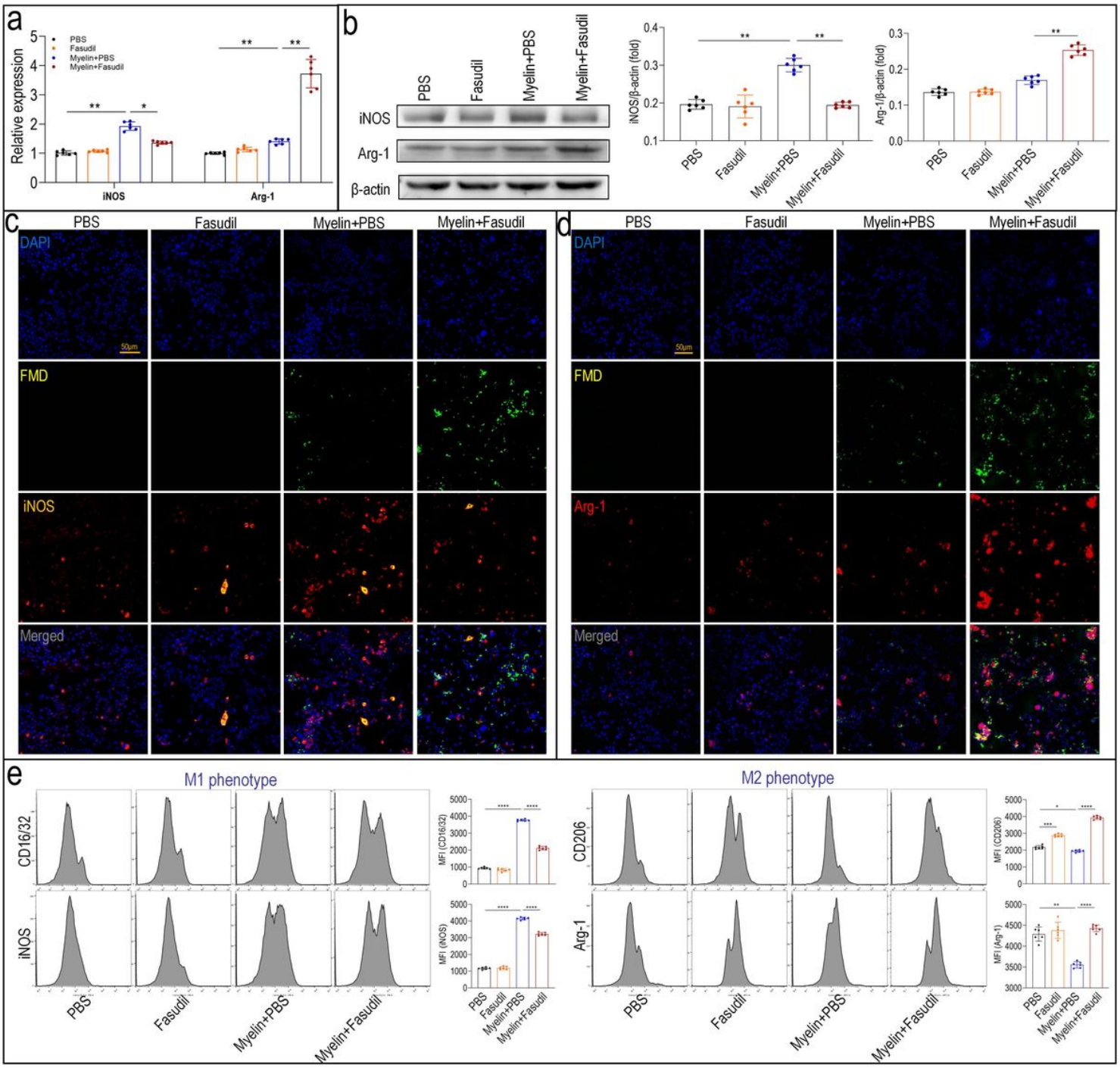

Figure 3

Fasudil influenced the expression of iNOS/Arg-1 in myelin debris-induced BV2 cells. BV2 cells were intervened with FMD plus Fasudil for 48 h. (a) The expression of iNOS and Arg-1 were analyzed by RT-PCR, (b) western blot assay, (c and d) immunocytochemistry staining, (e) Fasudil promoted the polarization of BV2 microglia towards M2 phenotype. Cells were stained with microglial M1 markers (CD16/32, iNOS) as well as M2 markers (CD206, Arg-1) and further analyzed using flow cytometry. Quantitative results are mean±SD. *P<0.05, **P<0.01, ****P<0.0001.

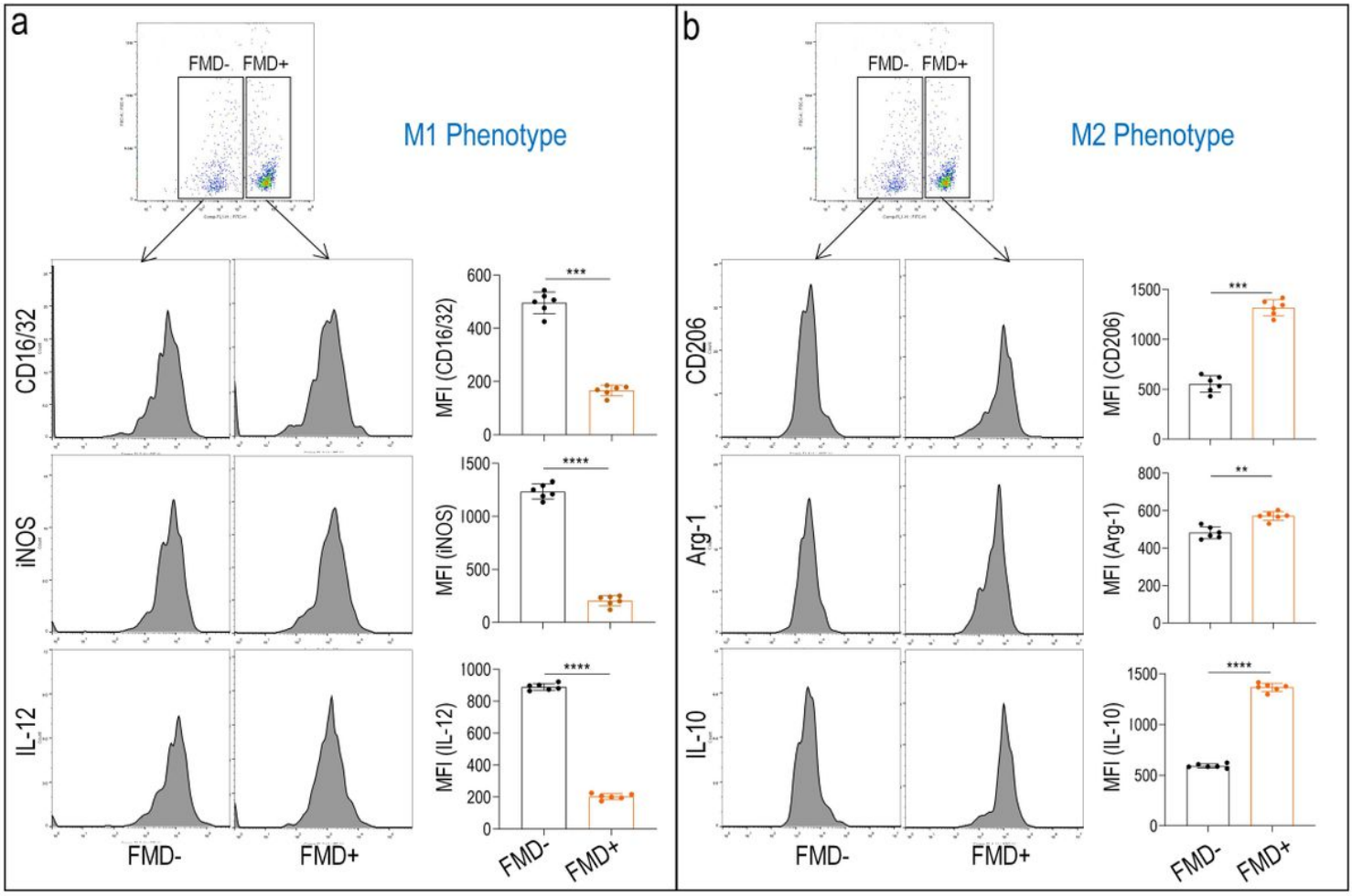

Figure 4

Fasudil enhanced phagocytosis by polarizing microglia in vitro. BV2 microglia were stimulated with 5 mg/ml FMD in the absence/presence of 15 μg/ml Fasudil for 48 h. Then cells were stained respectively with microglial M1 markers (CD16/32, iNOS and IL-12) and M2 markers (CD206, Arg-1 and IL-10). (a) Non-phagocytic and phagocytic M1 microglia were analyzed using flow cytometry, (b) non-phagocytic and phagocytic M2 microglia were analyzed using flow cytometry. Quantitative results are mean±SD. **P<0.01, ***P<0.001, ****P<0.0001.

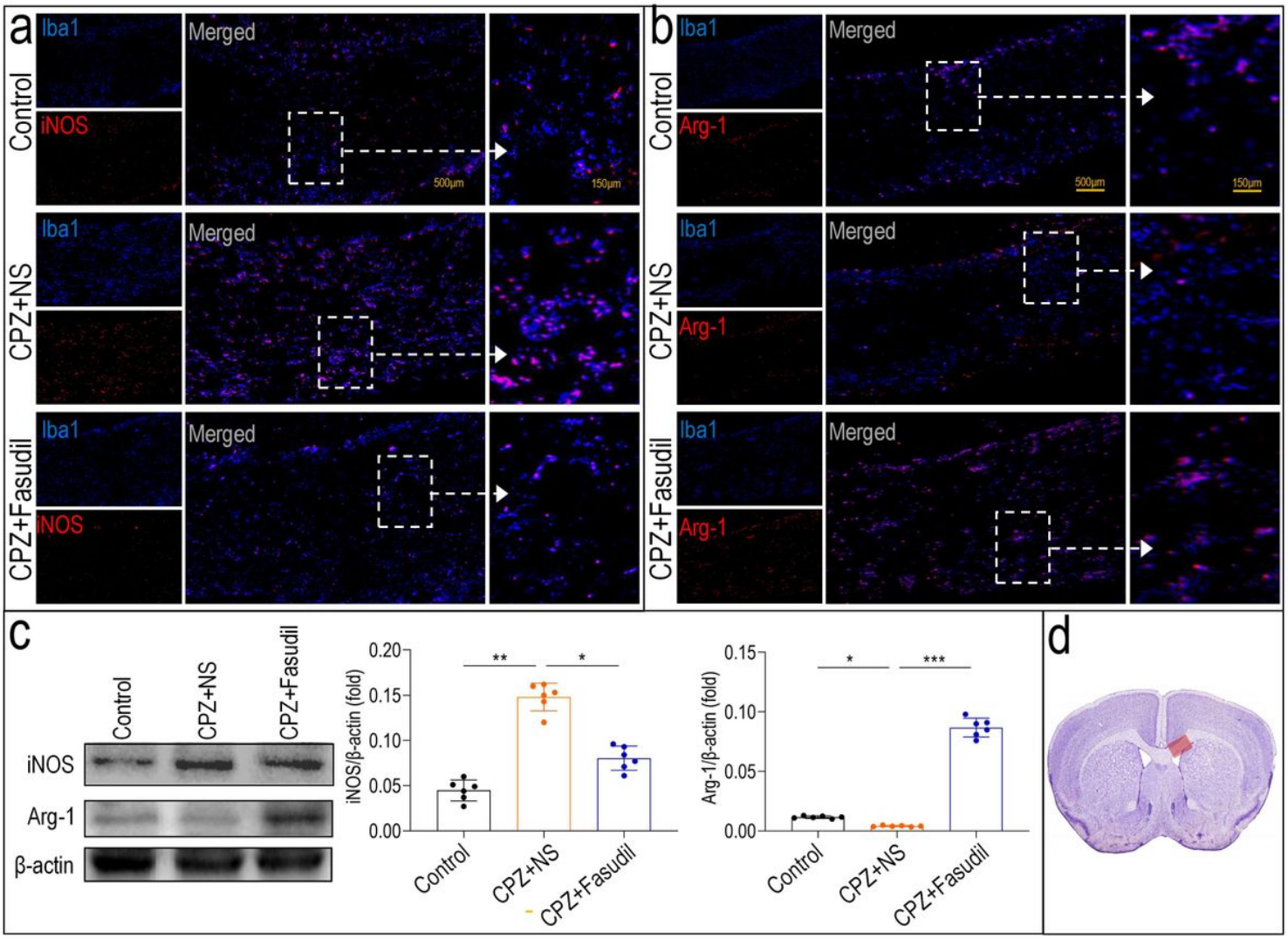

Figure 5

Fasudil shifted M1 to M2 phenotype in CPZ-fed mice. (a) Immunofluorescence staining with anti-Iba1 and anti-iNOS in the regions of corpus callosum, (b) immunofluorescence staining with anti-Iba1 and anti-Arg-1 in the regions of corpus callosum, (c) the expression of Arg-1 and iNOS protein in the extract of brains by western blot, (d) ideograph of mouse brain for the observation. Quantitative results are mean±SD. *P<0.05, **P<0.01, ***P<0.001.

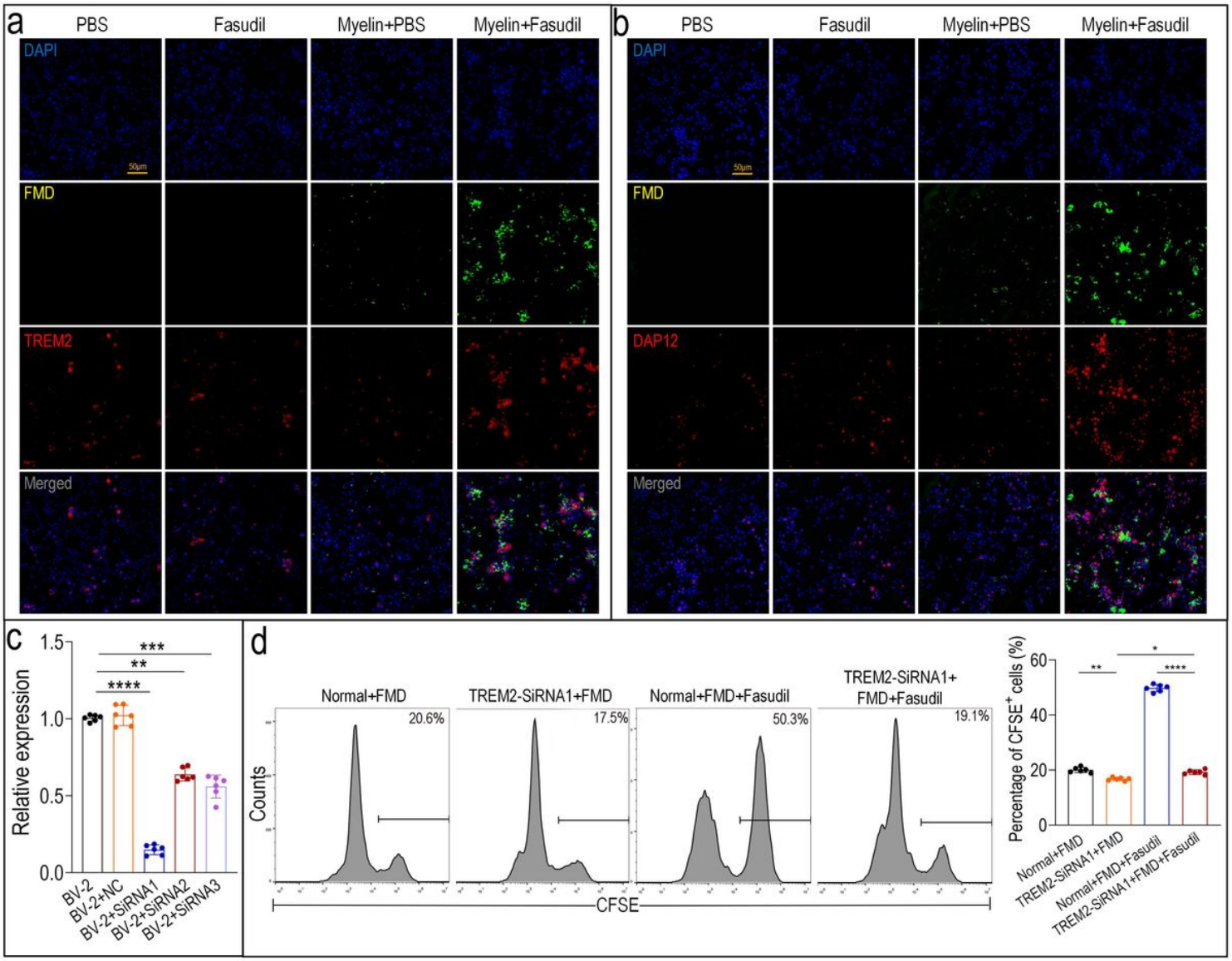

Figure 6

Fasudil enhanced BV2 cells to remove FMD by activating the TREM2/DAP12 pathway. (a) Immunocytochemistry staining with anti-TREM2 and DAPI in the BV2 cells, (b) immunocytochemistry staining with anti-DAP12 and DAPI in the BV2 cells, (c) relative expression of TREM2 mRNA in BV2 cells interfered with TREM2-siRNA vectors, (d) the engulfment of FMD in microglia interfered with TREM2-siRNA were evaluated by flow cytometry. Quantitative results are mean±SD. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

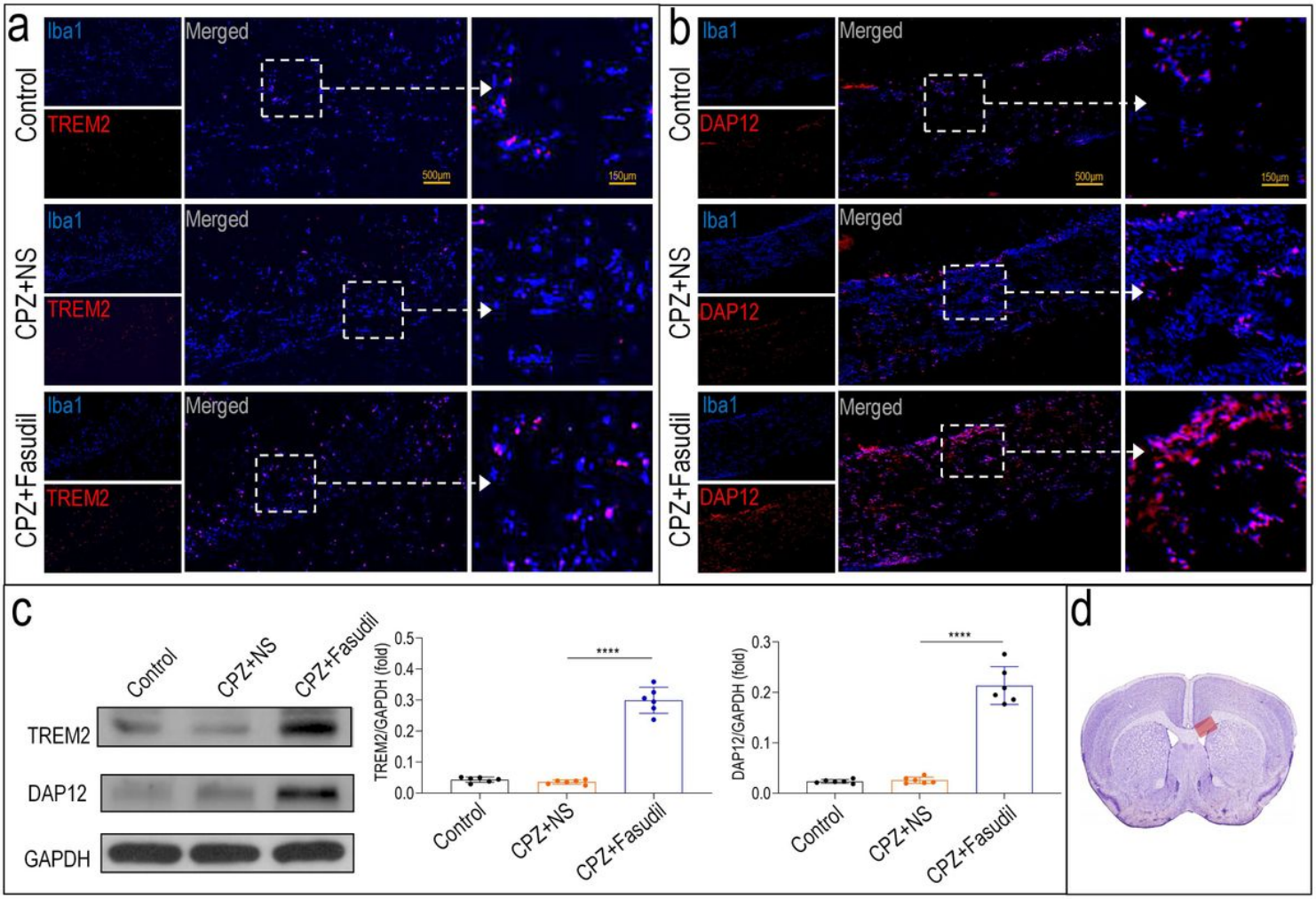

Figure 7

Fasudil activated TREM2/DAP12 pathway in CPZ-induced demyelinating mice. (a) Immunofluorescence staining with anti-Iba1 and anti-TREM2 in the regions of corpus callosum, (b) immunofluorescence staining with anti-Iba1 and anti-DAP12 in the regions of corpus callosum, (c) expression of TREM2/DAP12 protein in the extract of brains by western blot, (d) ideograph of mouse brain for the observation. Quantitative results are mean±SD. ****P<0.0001.

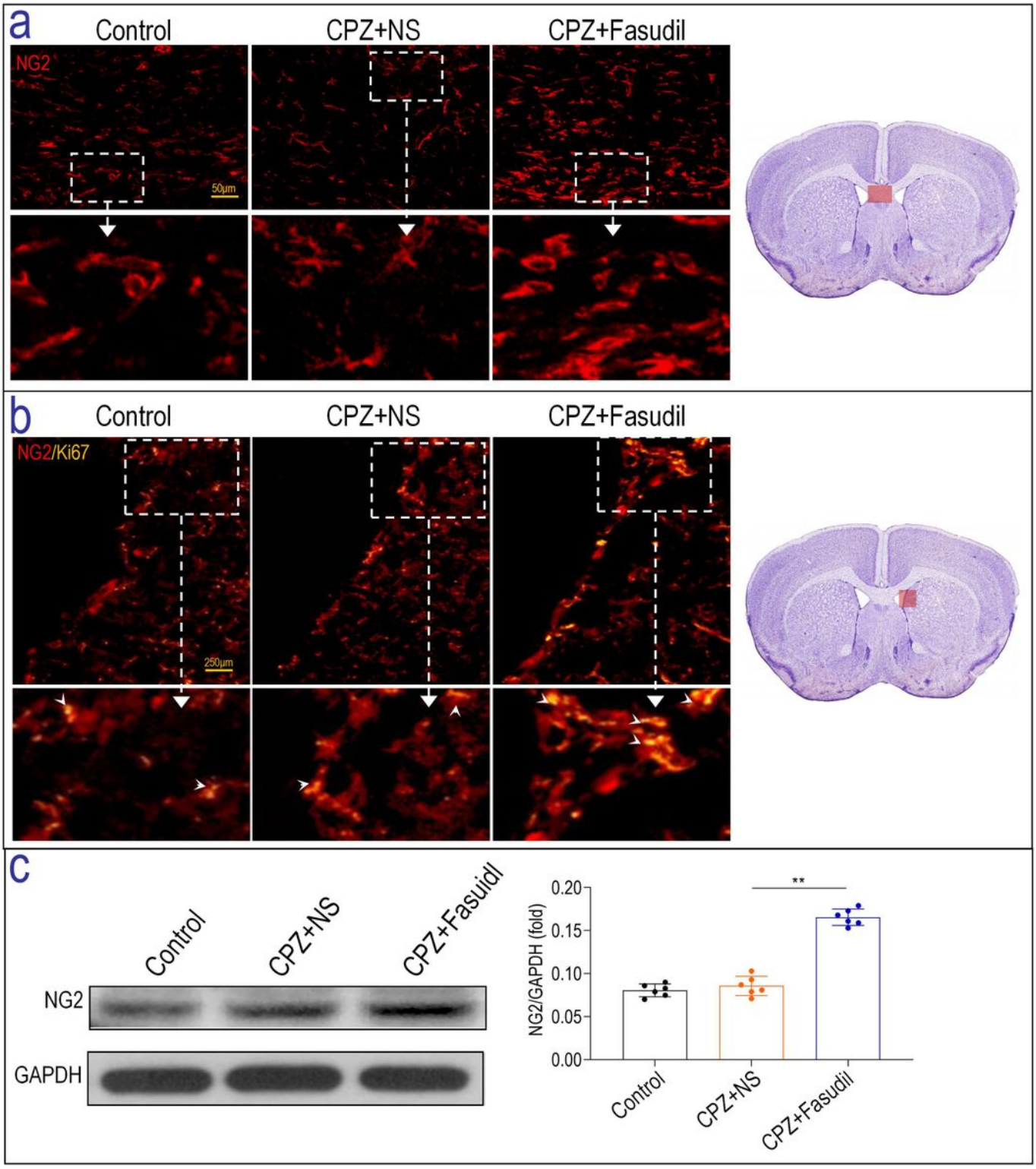

Figure 8

Fasudil induced regeneration of OPCs. (a) Immunofluorescence staining with anti-NG2 in the corpus callosum, (b) immunofluorescence staining with anti-NG2 and anti-Ki67 around the lateral ventricle, (c) the expression of NG2 protein in extract of brains by western blot. Quantitative results are mean±SD. **P<0.01.