Fasudil-modified macrophages reduce inflammation and regulate the immune response in experimental autoimmune encephalomyelitis

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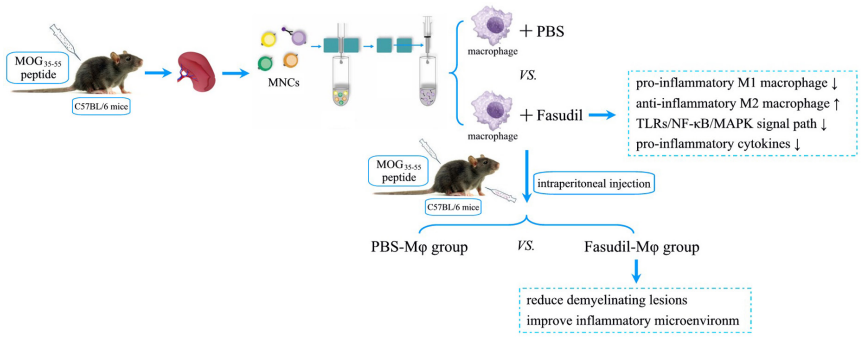
From the Contents

Graphical Abstract

Regulatory mechanism of immune cell polarization and activity in mice with experimental autoimmune encephalomyelitis



Abstract

Multiple sclerosis is characterized by demyelination and neuronal loss caused by inflammatory cell activation and infiltration into the central nervous system. Macrophage polarization plays an important role in the pathogenesis of experimental autoimmune encephalomyelitis, a traditional experimental model of multiple sclerosis. This study investigated the effect of Fasudil on macrophages and examined the therapeutic potential of Fasudil-modified macrophages in experimental autoimmune encephalomyelitis. We found that Fasudil induced the conversion of macrophages from the pro-inflammatory M1 type to the anti-inflammatory M2 type, as shown by reduced expression of inducible nitric oxide synthase/nitric oxide, interleukin-12, and CD16/32 and increased expression of arginase-1, interleukin-10, CD14, and CD206, which was linked to inhibition of Rho kinase activity, decreased expression of toll-like receptors, nuclear factor-κB, and components of the mitogen-activated protein kinase signaling pathway, and generation of the pro-inflammatory cytokines tumor necrosis factor-α, interleukin-1β, and interleukin-6. Crucially, Fasudil-modified macrophages effectively decreased the impact of experimental autoimmune encephalomyelitis, resulting in later onset of disease, lower symptom scores, less weight loss, and reduced demyelination compared with unmodified macrophages. In addition, Fasudil-modified macrophages decreased interleukin-17 expression on $CD4^+$ T cells and CD16/32, inducible nitric oxide synthase, and interleukin-12 expression on $F4/80^+$ macrophages, as well as increasing interleukin-10 expression on $CD4^+$ T cells and arginase-1, CD206, and interleukin-10 expression on $F4/80^+$ macrophages, which improved immune regulation and reduced inflammation. These findings suggest that Fasudil-modified macrophages may help treat experimental autoimmune encephalomyelitis by inducing M2 macrophage polarization and inhibiting the inflammatory response, thereby providing new insight into cell immunotherapy for multiple sclerosis.

Key Words: anti-inflammatory; experimental autoimmune encephalomyelitis; Fasudil; macrophage; multiple sclerosis; pro-inflammatory; Rho kinase

Introduction

Multiple sclerosis (MS) is a neurological illness that is characterized by multifocal inflammation, extensive demyelination, neuronal destruction, and axon loss. Spatially and temporally, MS clinical manifestations are intricate and multifaceted (Liu et al., 2021; Gharagozloo et al., 2022). Experimental autoimmune encephalomyelitis (EAE), the best-studied experimental animal model of MS, mimics many of the clinical symptoms and pathophysiology of MS and provides an excellent experimental platform for investigating disease mechanisms and treatments (Stavropoulos et al., 2021). The immunopathologic mechanism of MS/EAE is thought to be activation of autoreactive immune cells, relocation of activated immunocytes to the central nervous system (CNS), reactivation and amplification of immune inflammatory cells in the CNS, and the release of harmful substances in the CNS leading to nerve damage; the transfer of activated immunocytes to the CNS is believed to initiate MS/EAE paroxysm, and the infiltrating inflammatory cells are dominated by macrophages/microglia or T lymphocytes (Liu et al., 2013; Jafari Rad et al., 2022; Luoqian et al., 2022).

Macrophages are antigen-presenting immune effector cells that are distributed throughout the body and play a key role in the pathogenesis of a number of chronic neuroinflammatory and neurodegenerative diseases, including MS, Parkinson's disease (PD), and Alzheimer's disease (AD) (Prineas and Parratt, 2021). Macrophages are classified as pro-inflammatory M1 type or anti-inflammatory M2 type based on their activating molecules, intracellular signaling molecules, surface markers, released cytokines, and cellular functions (Zhang et al., 2013; Zeng et al., 2022). Pro-inflammatory M1 macrophages are activated by T helper 1 (Th1) cytokines and secrete high levels of pro-inflammatory factors, as well as exhibiting antiproliferative and cytotoxic effects via a range of reactive nitrogen and reactive oxygen species. M1 macrophages are key pathogenic factors in MS/EAE, as they induce inflammatory demyelination and mediate the formation of inflammatory

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microenvironments in the CNS (Zhang et al., 2013; Tang and Le, 2016; Zhao et al., 2022). Anti-inflammatory M2 macrophages are activated by Th2 cytokines, express high levels of anti-inflammatory molecules, decrease immunity, and promote the maturation of oligodendrocyte progenitors by secreting pro-repair factors that promote tissue healing (Chu et al., 2018, 2019; Che et al., 2022). Therefore, pro-inflammatory M1 macrophages are associated with MS/EAE development, while anti-inflammatory M2 macrophages reduced the inflammatory response, myelin loss, and neuron degeneration in EAE (Grassivaro et al., 2020; Barnes et al., 2021).

Numerous investigations have revealed that Rho kinase (ROCK) sensitization is linked to the pathophysiology of a variety of neurological disorders (Chong et al., 2017; Martín-Cámara et al., 2021). The ROCK signaling pathway regulates cytoskeletal rearrangement, which provides energy for T cells and macrophages to move into the CNS. In addition, ROCK degrades the tight junction proteins between endothelial cells, promoting contraction of the endothelial cytoskeleton and increasing the permeability of the blood-brain barrier (BBB), which allows extensive invasion of the CNS by immunocytes (Chen et al., 2018; Wang et al., 2022b). Fasudil, a ROCK inhibitor, has been used to treat subarachnoid hemorrhages since 1995. According to previous studies from our and other groups Fasudil reduces the severity of EAE in mice through the following mechanisms: (1) Fasudil inhibits Th1 and Th17 cytokine release while increasing Th2 cytokine expression; (2) Fasudil promotes macrophage and microglia conversion from a pro-inflammatory type to an anti-inflammatory type; (3) Fasudil inhibits ROCK activity in vascular endothelial cells and improves BBB function; and (4) Fasudil inhibits the adhesion and migration of peripheral immune cells, reduces the inflammatory response, and decreases inflammation in the CNS (Hou et al., 2012; Liu et al., 2013, 2015). We previously showed that intraperitoneal injection of Fasudil (800 μg/mouse) induced significant vasodilation in the feet of EAE mice after 30 and 60 minutes. Another group showed that injection of Fasudil (1600 and 2000 μg/mouse, i.p.) caused nearly 33–67% mortality within 2 hours (Xin et al., 2015). Although Fasudil has substantial therapeutic benefits, its clinical applicability is limited because of its vasodilating effects, its short safety window that precludes long-term use, and the lack of an oral form of the medication (Xin et al., 2015). Thus, researchers are seeking for a novel treatment strategy that is more efficient, safer, and suitable for long-term treatment of neurological disorders. ROCK inhibitors are promising drug candidates for preventing neurodegeneration and stimulating neuroregeneration, as shown in stroke patients as well as animal models of stroke, MS, AD, and PD. We previously demonstrated that Fasudil can reduce EAE severity both early and late in the treatment process, as indicated by decreased CD16/32, iNOS, IL-12, TLR4, and CD40 expression and increased CD206, Arg-1, IL-10, and CD14 expression (Liu et al., 2013). In addition, Fasudil-modified immune cells, for example mononuclear cells (MNCs), administered intraperitoneally or as nasal drops, not only prevent EAE development, but also treat active EAE, decreased CNS inflammation, and reduce demyelination. *In vitro* studies have shown that treating MNCs with Fasudil promotes macrophage conversion from the pro-inflammatory M1 phenotype to the anti-inflammatory M2 phenotype. Furthermore, an *in vivo* study showed that Fasudil-modified MNCs decreased the number of macrophages exhibiting pro-inflammatory M1 subtypes (CD16/32, CCR7, IL-12, and CD8a expression) and increased the number of macrophages exhibiting anti-inflammatory M2 subtypes (CD206, CD200, and CD14 expression) in the spleen (Liu et al., 2015; Guo et al., 2019). However, these studies did not show whether T cells or macrophages mediate the improvements in EAE symptoms seen with Fasudil-modified MNCs, nor could they rule out a potential interaction between T cells and macrophages. Fasudil-treated macrophages and Fasudil have different targets: Fasudil is a ROCK inhibitor, while macrophages may have more extensive immunoregulatory effects. Autologous transfusion of Fasudil-treated cells, compared with direct treatment with Fasudil, is likely to be associated with fewer side effects and a better safety profile. Therefore, in this study we modified immune cells *in vitro* and performed autologous reinfusion to demonstrate, enhanced safety and refine our understanding of the biological properties of Fasudil.

In recent years, innovative biotherapeutic technologies have developed rapidly, and emerging technologies such as cell therapy show great promise for the treatment of malignant tumors, inflammation, autoimmune diseases, and metabolic diseases and in the field of regenerative medicine (Mount et al., 2015). Thus, cell therapy is likely to become a key therapeutic option for CNS disease in the future. The benefits of cell immunotherapy include simple sampling, easy isolation and culture, autologous transplantation, no allograft rejection, low cost, and no moral or ethical concerns (Liu et al., 2015; Ghorbani et al., 2022). Moreover, cell immunotherapy is not associated with tumorigenicity, as immune cells have a short lifespan because of terminal differentiation. The donor sources of cell therapy are divided into autologous cells, allogeneic cells, and xenogeneic cells, and *in vitro* manipulation methods include conventional culture, directed differentiation, gene editing, and genetic modification (Marks et al., 2015; Jiang et al., 2017). There are significant differences in the complexity of different types of cell preparation processes, in the biological characteristics of different cell types *in vivo*, and in the safety risks of cell therapy-based clinical applications. In addition, *in vitro* cell modification and the use of exogenous factors to promote *in vitro* cell growth complicate cell quality control, increasing the risk of using cell therapy for clinical applications (Gou et al., 2019; Locke et al., 2019). The aim of the current study was to explore the effects of Fasudil-modified macrophages on EAE, as well as to investigate the possible mechanism underlying these effects.

Methods

Experimental animals

A higher incidence of EAE and more severe symptoms of EAE in female mice than in male mice indicate that female mice are more sensitive to this condition than male mice are (Voskuhl et al., 1996). Female mice were used in our previous studies (Liu et al., 2013, 2015). Female C57BL/6 mice (8–10 weeks of age and 18–20 g in weight) were purchased from Beijing Huafukang Bioscience Co., Ltd (Beijing, China) (license No. SCXK (jing) 2019-0008) and kept under pathogen-free conditions with a reversed 12/12-hour light/dark cycle in a temperature-controlled room (25 ± 2°C). All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8th ed, National Research Council, 2011) and were approved by the Animal Ethics Committee of Shanxi Datong University (approval No. 2019019) on July 29, 2019. The results are reported in accordance with the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) 2.0 guidelines (Percie du Sert et al., 2020).

Preparation of EAE model and macrophages

To establish the EAE model, mouse myelin oligodendrocyte glycoprotein peptide 35–55 ($MOG_{35–55}$; 6 mg; CL. Bio-Scientific, Xi'an, China; aminophenol sequence MEVGWYRSPFSRVVHLYRNGK) was dissolved in 2 mL normal saline (NS). The peptide solution was then mixed completely with 2 mL Freund's complete adjuvant (Sigma, St Louis, MO, USA) containing 1 mg/mL of *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Inc., Detroit, MI, USA) at a ratio of 1:1 (v/v) to yield an antigen emulsion. Each mouse was injected with 100 μL of the antigen emulsion at four points on both sides of the midline of the spine and with 200 μL of pertussis toxin (300 ng; Enzo Life Sciences, Farmingdale, NY, USA) via the abdominal cavity at the time of immunization and again later to enhance the immunogenic effect.

On day 9 postimmunization (p.i.), EAE mice were anesthetized by an intraperitoneal injection of 2% pentobarbital (40 mg/kg, Sigma), and MNCs were obtained via grinding the spleens and passing the ground tissue through a 40-μm disposable nylon strainer screen (BD Biosciences, San Jose, CA, USA) in medium. The cells (1×10^7) were resuspended in 90 μL of buffer, 10 μL of macrophages magnetic beads (Miltenyi Biotec GmbH, Bergesch Gladbach, Germany, Cat# 130-049-601, RRID: AB_2927377) was added, the solution was mixed thoroughly for about 15 minutes at 4°C and centrifuged at 300 × *g* for about 10 minutes, and the resulting cell suspension was applied to an LS column (Miltenyi Biotec, Cat# 130-042-401) containing a magnetic field. The magnetically bound macrophages were rinsed rapidly from the column and collected.

Intraperitoneal injection of Fasudil-modified macrophages

The macrophages that were isolated as described above were treated with 15 μg/mL of Fasudil (Tianjin Chase Sun Pharmaceutical Co., Tianjin, China) or phosphate-buffered saline (PBS) at 37°C in a 5% CO_2 atmosphere for 72 hours. EAE mice were randomly divided into two groups: the Fasudil-macrophage group (Fasudil-Mϕ) (n = 12) and the PBS-macrophage group (PBS-Mϕ) (n = 12). The treated macrophages were suspended in PBS at a concentration of 2×10^7 cells/200 μL and intraperitoneally injected into EAE mice on days 3 and 11 p.i. Mice were weighed and evaluated for EAE symptoms every other day by at least two investigators who were blinded to the group assignments, according to the following 5-point scale (Benson et al., 2000): 0, normal; 1, limp tail; 2, tail palsy and posterior limb myasthenia; 3, hindlimb paralysis and/or forelimb paresis; 4, limb paralysis and reduced crawling; 5, dying or dead. Mice with a symptom score of 3 exhibited significant motor dysfunction, resulting in difficulty consuming water and food; therefore, we took special care of these mice, for example by providing milk, eggs, softer food, and supplementary nutrients.

Pathological and immunohistochemical staining

On day 28 p.i., EAE mice were anesthetized by intraperitoneal injection of 2% pentobarbital (40 mg/kg). Then, the heart was perfused with 50 mL NS until the liver turned white, followed by perfusion with 4% paraformaldehyde until the tail became stiff. Next, the muscles and connective tissue surrounding the spinal cord were dissected away, and the lumbar enlargement was removed, embedded in optimum cutting temperature medium, and flash frozen in in liquid nitrogen. The spinal cord was then cut into 5-μm-thick sections and stained with hematoxylin and eosin (HE), luxol fast blue (LFB), and immunofluorescent reagents. For HE staining, frozen sections were dried, stained with hematoxylin (Beyotime Institute of Biotechnology, Shanghai, China), rinsed with HCL-alcohol, stained with eosin (Beyotime Institute of Biotechnology), dehydrated in an alcohol gradient (70%, 80%, 95%, 95%, and 100%), cleared with alcohol-xylene, and mounted on slides using neutral gum to seal the edges of the coverslip. After staining, the inflammatory cells appeared as dark blue and the cytoplasm as reddish. Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA) was applied to assess inflammatory infiltration into the spinal cord based on HE staining and the Okuda histopathology score (Okuda et al., 1999): 0, no infiltration of inflammatory corpuscles; 1, infiltrative inflammatory corpuscles around the blood vessel and spinal meninges; 2, slight inflammatory corpuscle invasion of the spinal cord (1–10 cells/field of vision); 3, moderate inflammatory corpuscle invasion of the spinal cord (10–100 cells/field of vision); 4, substantial inflammatory corpuscle invasion of the spinal cord (> 100 cells/field of vision). For LFB staining, the sections were placed in 70% ethanol for 15 minutes, immersed

in solid blue dye (Sigma) in a 57°C incubator for 48 hours, dehydrated in an alcohol gradient, treated with xylene, and mounted on slides using neutral gum to seal the edges of the coverslip. White matter and demyelinated areas visualized by LFB staining were delineated by hand, and Image-Pro Plus software was used to determine the percentage of demyelinated area compared with white matter. For immunofluorescence staining, frozen sections were dried at 37°C, blocked with 1% bovine serum albumin (BSA) (Sigma), incubated with an antibody to the macrophage marker CD68 (1:1000; Bio-Rad, Hercules CA, USA, Cat# MCA1957, RRID: AB_322219) overnight at 4°C, and incubated with the corresponding secondary antibody (Alexa Fluor 555 Conjugate, goat anti-rat, 1:500; Cell Signaling Technology, Boston, MA, USA, Cat# 4417, RRID: AB_10696896) for 2 hours at 37°C. A negative control was included that omitted the primary antibody. The fraction of $CD68^+$ macrophages was computed using Image-Pro Plus software.

Flow cytometry assay

As described above, macrophages were isolated from MNCs from EAE mice using magnetic beads and cultured for 72 hours with 15 μg/mL of Fasudil or PBS at 37°C in a 5% CO_2 atmosphere. They were then incubated in 1% BSA-PBS buffer at room temperature (RT) for 30 minutes with antibodies to markers of the pro-inflammatory M1 macrophage subtype–PE-CD16/32 (BioLegend, San Diego, CA, USA, Cat# 101307, RRID: AB_312806) and PE-CD40 (Thermo Fisher Scientific, Waltham, MA, USA, Cat# 12-0401-82, RRID: AB_465649)–and the anti-inflammatory M2 macrophage subtype–PE-CD206 (Thermo Fisher Scientific, Cat# 12-2061-82, RRID: AB_2637422) and PE-CD14 (Thermo Fisher Scientific, Cat# 12-0141-82, RRID: AB_465563). Intracellular macrophage staining was carried out by treating the cells with 0.3% saponin (Sigma)/1% BSA-PBS buffer at RT for 30 minutes and then incubating with primary antibodies to markers of the pro-inflammatory M1 macrophage subtype–inducible nitric oxide synthase (iNOS) (Enzo Life Sciences, Cat# ADI-KAS-NO001, RRID: AB_10622409) and PE-interleukin (IL)-12 (Thermo Fisher Scientific, Cat# 12-7123-82, RRID: AB_466185)–and markers of the anti-inflammatory M2 macrophage subtype–arginase-1 (Arg-1) (BD Biosciences, Cat# 610708, RRID: AB_398031) and PE-IL-10 (Thermo Fisher Scientific, Cat# 12-7101-82, RRID: AB_466176). Next, the cells were incubated with the corresponding secondary antibodies for iNOS (1:200, Alexa Fluor 555, goat anti-rabbit; Molecular Probes, Eugene, OR, USA, Cat# A-21428, RRID: AB_141784) and Arg-1 (1:200, Alexa Fluor 555, goat anti-mouse; Molecular Probes, Cat# A-21127, RRID: AB_141596). At least 10,000 events were recorded using a flow cytometer (BD Biosciences). Data were analyzed using CellQuest software (BD Biosciences). Results are expressed as mean fluorescence intensity.

On day 28 p.i., the EAE mice were sacrificed, and MNCs were prepared as described above and stained for 30 minutes at RT with the following antibodies diluted in 1% BSA-PBS buffer: the macrophage marker Alexa Fluor 488-anti-F4/80 (Bio-Rad, Cat# MCA497A488, RRID: AB_321210), the pro-inflammatory M1 macrophage subtype marker PE-CD16/32, and the anti-inflammatory M2 macrophage subtype marker PE-CD206. For intracellular staining, the MNCs were stained for 30 minutes at RT with the following antibodies diluted in 0.3% saponin/1% BSA-PBS buffer: the $CD4^+$ T cell marker FITC-CD4 (Thermo Fisher Scientific, Cat# 11-0041-82, RRID: AB_464892), the Th1 marker PE-interferon (IFN)-γ (Thermo Fisher Scientific, Cat# 12-7311-82, RRID: AB_466193), the Th17 marker PE-IL-17 (Thermo Fisher Scientific, Cat# 12-7177-81, RRID: AB_763582), the Th2 marker PE-IL-10 (Thermo Fisher Scientific, Cat# 12-7101-82, RRID: AB_466176), the macrophage marker Alexa Fluor 488-anti-F4/80, the pro-inflammatory M1 macrophage subtype markers PE-IL-12 (Thermo Fisher Scientific, Cat# 12-7123-82, RRID: AB_466185), anti-iNOS, and anti-Arg-1, and the anti-inflammatory M2 macrophage subtype marker PE-IL-10, followed by staining with the corresponding secondary antibodies for iNOS and Arg-1. At least 10,000 events were recorded using a flow cytometer. The data were analyzed using CellQuest software. The results are expressed as the percentage of double-positive cells in a four-quadrant diagram.

Cytokine detection by enzyme-linked immunosorbent assay

Macrophages were isolated from MNCs from EAE mice using magnetic beads and cultured for 72 hours with 15 μg/mL of Fasudil or PBS at 37°C in a 5% CO_2 atmosphere. The cell culture supernatants were collected, and enzyme-linked immunosorbent assay (ELISA) kits were used to detect the inflammatory cytokines IL-6 (PeproTech, Rocky Hill, NJ, USA; Cat# 500-P56-50ug, RRID: AB_147637), tumor necrosis factor (TNF)-α (PeproTech, Cat# 400-14, RRID: AB_2665385), and IL-1β (MultiSciences, Hangzhou, China, Cat# EK201B, RRID: AB_2934104) according to the manufacturer's instructions. Cytokines were quantified in triplicate using calibration curves, and the findings are presented as pg/mL.

Nitrite detection

Macrophages were isolated from inflammatory MNCs using magnetic beads and cultured for 72 hours at 37°C in a 5% CO_2 atmosphere with 15 μg/mL of Fasudil or PBS. Nitric oxide (NO) was quantified using a Griess reaction-based colorimetry assay (Zhang et al., 2013), as follows: cell supernatants (100 μL) and Griess reagent (100 μL) were combined and mixed at RT for 10 minutes. Absorbance at 510 nm was detected using a spectrometer (Thermo Fisher Scientific). Nitrite concentrations were ascertained by comparing values to a calibration curve generated using sodium nitrite (Sigma).

Measurement of Arg-1 activity

Macrophages were isolated from inflammatory MNCs using magnetic beads and cultured for 72 hours with 15 μg/mL of Fasudil or PBS at 37°C in a 5% CO_2 atmosphere. A QuantiChrom™ arginase test kit (BioAssay Systems, San Francisco, NC, USA) was used to detect Arg-1 activity. Briefly, 40 μL of supernatant was mixed with/without (blank control) 5×substrate buffer (10 μL) and incubated for 2 hours at 37°C in 96-well plates; 50 μL of H_2O and 50 μL of 1 mM urea were used as the negative control and to generate the standard curve, respectively. Then, urea (200 μL) and 5×substrate (10 μL) were added to all wells, the plates were incubated at RT for 1 hour, and the optical density was detected at 430 nm. The following formula was used to determine Arg-1 activity:

$(OD_{sample}–OD_{blank}/OD_{standard}–OD_{water}) \times 10.4$ (expressed in U/L).

Western blot assay

Macrophages were isolated from MNCs from EAE mice using magnetic beads and cultured for 72 hours with 15 μg/mL of Fasudil or PBS at 37°C in a 5% CO_2 atmosphere, then homogenized with a microcontent motor-operated tissue homogenizer (Kimble Kontes, Vineland, NJ, USA) in RIPA Lysis Buffer (Beyotime Institute of Biotechnology) supplemented with protease inhibitors on the ice. The homogenates were centrifuged at 12,000 × *g* for 20 minutes, and protein concentrations were measured using a bicinchoninic acid (BCA) protein assay kit (Sangon Biotech, Shanghai, China). Cell extracts (30 μg) were loaded onto sodium dodecyl sulfate-polyacrylamide gels, subjected to electrophoresis, and transferred to a nitrocellulose membrane (Merck Millipore, Tullagreen Carrigtwohill, Cork, Ireland). The membranes were blocked with 5% milk at RT for 1 hour and incubated with primary antibodies overnight at 4°C. After washing in the next day, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 2 hours at RT. Proteins were detected using a Chemiluminescence Kit (Solarbio, Beijing, China, Cat# PE0010), and the bands were visualized with a Molecular Imager ChemiDoc XRS1 System (Bio-Rad). Protein band optical densities were quantified using Image Lab software (Bio-Rad) and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as the internal reference. All measurements were performed by a researcher blinded to the experimental groups.

The antibodies used for western blotting were as follows: mouse anti-ROCK-II (1:1000; BD Biosciences, Cat# 610624, RRID: AB_397956), rabbit anti-myosin light chain phosphatase target subunit 1 (p-MYPT1)-Thr696 (1:1000; Millipore, Tullagreen Carrigtwohill, Cork, Ireland, Cat# ABS45, RRID: AB_10562238), rabbit anti-iNOS (1:1000, Enzo Life Sciences, Cat# ADI-KAS-NO001, RRID: AB_10622409), mouse anti-Arg-1 (1:1000, BD Biosciences, Cat# 610708, RRID: AB_398031), rabbit anti-toll like receptor 2 (TLR-2) (1:1000; Cell Signaling Technology, Cat# 2229, RRID: AB_2204442), rabbit anti-TLR-4 (1:1000, Cell Signaling Technology, Cat# 14358, RRID: AB_2798460), rabbit anti-myeloid differentiation factor 88 (Myd88) (1:1000; Abcam, Cambridge, UK; Cat# ab2064, RRID: AB_302807), rabbit anti-p-Jun N-terminal kinase (p-JNK) (1:1000, Cell Signaling Technology, Cat# 4668, RRID: AB_823588), mouse anti-p-extracellular regulated protein kinases (p-ERK) (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA, Cat# sc-81492, RRID: AB_1125801), mouse anti-p-P38 (1:1000; Santa Cruz Biotechnology, Cat# sc-7973, RRID: AB_670359), rabbit anti-p-nuclear factor kappa B (p-NF-κB) (1:1000, Cell Signaling Technology, Cat# 3037, RRID: AB_2341216), rabbit anti-ribosomal protein S3 (RPS3) (1:1000, Abcam, Cat# ab128995, RRID: AB_11145466), anti-GAPDH (1:10,000, Cell Signaling Technology, Cat# 2118, RRID: AB_561053), anti-mouse IgG HRP-linked secondary antibody (1:10,000, Cell Signaling Technology, Cat# 7076, RRID: AB_330924), and anti-rabbit IgG HRP-linked secondary antibody (1:10,000, Cell Signaling Technology, Cat# 7074, RRID: AB_2099233).

Statistical analysis

The experimental sample size was chosen based on the prior studies from our and other groups (Guo et al., 2019). None of the animals were excluded from the analysis, and the weights and symptom scores of the EAE mice were evaluated by investigators blinded to the group assignments. GraphPad Prism 8.0 software (GraphPad Software Inc., San Diego, CA, USA) was used to perform all of the statistical analyses. Data are expressed as the mean ± standard error of mean (SEM). Student's *t*-test was used to analyze inter-group differences, and Mann-Whitney *U* test was used to analyze differences in clinical scores. $P < 0.05$ was considered to be statistically significant.

Results

Fasudil-modified macrophages ameliorate the severity of EAE in mice

First, we looked at the effect of injecting Fasudil-modified macrophages intraperitoneally into EAE mice on days 3 and 11 p.i. Mice in the control group progressively developed EAE symptoms beginning on day 9 p.i., including signs of depression, anorexia, weight loss, patchy fur, diminished limb and tail muscle strength, and even paralysis over time. However, injection with Fasudil-modified macrophages alleviated these effects. Compared to the PBS-Mϕ group (100%), the rate of EAE morbidity in the Fasudil-Mϕ group (75%) was lower. The mean time of onset was 12.08 ± 1.78 days, and the mean peak clinical score was 3.67 ± 0.91 in the PBS-Mϕ group. Injection with Fasudil-treated macrophages significantly delayed disease onset ($P < 0.01$; **Table 1**) and resulted in a mean reduction in maximum clinical score ($P < 0.05$; **Table 1**). In addition, mice in the Fasudil-Mϕ group exhibited less weight loss than those in the PBS-Mϕ group (**Figure 1B**), which was consistent with the trend toward improved clinical scores in the Fasudil-Mϕ group (**Figure 1A**).

Table 1 | **Clinical symptoms in both groups**

Group	*n*	Morbidity (%)	Mean time of onset (d)	Mean maximum clinical score
PBS-Mϕ	12	100	12.08 ± 1.78	3.67 ± 0.91
Fasudil-Mϕ	12	75	15.11 ± 2.26**	2.08 ± 1.76*

A mouse model of chronic experimental autoimmune encephalomyelitis (EAE) was induced by subcutaneous injection of myelin oligodendrocyte glycoprotein peptide 35–55. Macrophages from EAE mice isolated with magnetic beads were treated with 15 μg/mL of Fasudil at 37°C in 5% CO_2 atmosphere for 72 hours. Fasudil-treated macrophages were suspended in phosphate-buffered saline (PBS) at a concentration of 2×10^7 cells/200 μL and intraperitoneally injected into EAE mice on days 3 and 11 postimmunization. PBS-treated macrophages were used as a control. The mice were randomly divided into two groups: the Fasudil-macrophage group (Fasudil-Mϕ) (n = 12) and the PBS-macrophage group (PBS-Mϕ) (n = 12). The data are shown as mean ± SEM, and statistical significance was determined by Student's t-test. *P < 0.05, **P < 0.01, *vs.* PBS-Mϕ. EAE: Experimental autoimmune encephalomyelits; Mϕ: macrophage.

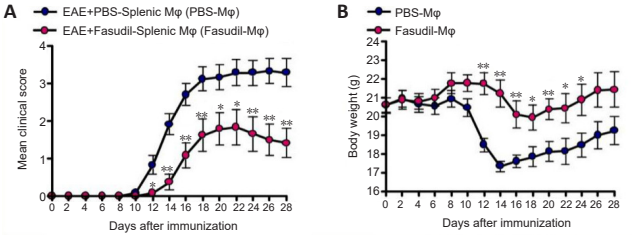

Figure 1 | **Fasudil-modified macrophages ameliorate the severity of EAE in mice.**
A mouse model of chronic EAE was induced by subcutaneous injection of myelin oligodendrocyte glycoprotein peptide 35–55. Macrophages from EAE mice isolated with magnetic beads were treated with 15 μg/mL of Fasudil at 37°C in 5% CO_2 atmosphere for 72 hours. Fasudil-treated macrophages were suspended in PBS at a concentration of 2×10^7 cells/200 μL and intraperitoneally injected into EAE mice on days 3 and 11 postimmunization. PBS-treated macrophages were used as a control. The mice were randomly divided into two groups: the Fasudil-macrophage group (Fasudil-Mϕ) (n=12) and the PBS-macrophage group (PBS-Mϕ) (n = 12). (A) Clinical symptom scores. (B) Body weight. Compared with the PBS-Mϕ group, the Fasudil-Mϕ group exhibited lower symptom scores and less weight loss. The results are presented as the mean ± SEM. *P < 0.05, **P < 0.01 (Mann-Whitney U test for A and Student's t-test for B). EAE: Experimental autoimmune encephalomyelits; Mϕ: macrophage; PBS: phosphate buffer saline.

Fasudil-modified macrophages inhibit inflammation and reduce demyelination in EAE mice

The pathological changes that occur during EAE include demyelinating lesion formation and inflammatory cell infiltration, both of which are intimately correlated with disease progression when they develop in the CNS microenvironment. Thus, we observed neuroinflammation and spinal cord demyelination in the two groups. As shown in **Figure 2A** and **B**, injection with Fasudil-treated macrophages significantly inhibited inflammatory cell infiltration (P < 0.001; **Figure 2A**) and demyelination (P < 0.01; **Figure 2B**) compared with injection with PBS-treated macrophages. Furthermore, immunofluorescence staining showed that, in comparison with the PBS-Mϕ group, infiltration of $CD68^+$ macrophages was significantly decreased in the Fasudil-Mϕ group (P < 0.01; **Figure 2C**).

Fasudil-modified macrophages regulate peripheral T cells and macrophages *in vivo*

EAE pathogenesis begins with activation of the cells that comprise the peripheral immune system, which can then invade the CNS, where they cause inflammation and destroy meylin. We analyzed T cell and macrophage subsets by fluorescence-activated cell sorting to determine the effect of Fasudil-modified macrophages on immunomodulation in EAE mice. The percentage of $CD4^+$ T cells expressing IL-10 (P < 0.01) was clearly elevated, while the percentage of $CD4^+$ T cells expressing IL-17 (P < 0.001) was significantly decreased, in the Fasudil-Mϕ group compared with the PBS-Mϕ group. There was no difference in the percentage of $CD4^+IFN\text{-}\gamma^+$ T cells between the PBS-Mϕ and Fasudil-Mϕ groups (**Figure 3**).

On day 28 p.i., phenotypic analysis showed that Fasudil-modified macrophages had significantly decreased the percentages of F4/80 macrophages expressing the pro-inflammatory M1 subtype markers CD16/32 (P < 0.001), iNOS (P < 0.05), and IL-12 (P < 0.05). In contrast, the percentages of F4/80 macrophages expressing the anti-inflammatory M2 macrophage subtype markers Arg-1 (P < 0.01), IL-10 (P < 0.05), and CD206 (P < 0.01) were significantly increased in the Fasudil-Mϕ group compared with the PBS-Mϕ group (**Figure 4**).

Fasudil suppresses macrophage ROCK activity *in vitro*

Given the clear effects of Fasudil-modified macrophages on EAE mice, we next sought to explore the mechanism underlying these effects. Upregulation of ROCK-II expression can mediate EAE pathogenesis. The ROCK-II substrate

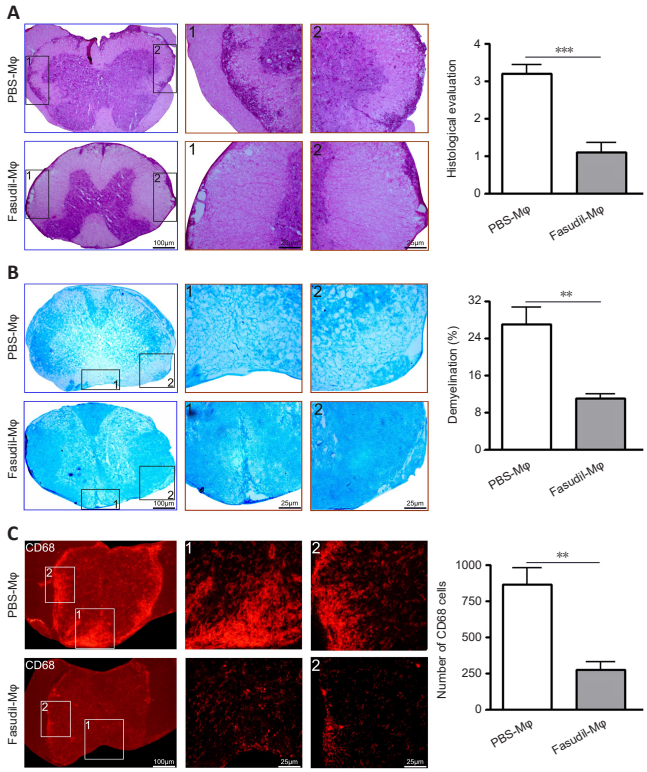

Figure 2 | **Fasudil-modified macrophages decrease inflammation and promote myelination.**
A mouse model of chronic EAE was induced by subcutaneous injection of myelin oligodendrocyte glycoprotein peptde 35–55. Macrophages from EAE mice isolated with magnetic beads were treated with 15 μg/mL of Fasudil at 37°C in 5% CO_2 atmosphere for 72 hours. Fasudil-treated macrophages were suspended in PBS at a concentration of 2×10^7 cells/200 μL and intraperitoneally injected into EAE mice on days 3 and 11 postimmunization. PBS-treated macrophages were used as a control. The mice were randomly divided into two groups: the Fasudil-macrophage group (Fasudil-Mϕ) (n=12) and the PBS-macrophage group (PBS-Mϕ) (n = 12). (A) HE-stained spinal sections. The inflammatory cells are dark blue, and the cytoplasm is reddish. The degree of inflammation was scored using the Okuda system. (B) Spinal cord sections stained with Luxol fast blue (LFB) to detect demyelination. Normal myelin tissue appears blue-green, while demyelinated areas appear lighter or even white. Image-Pro Plus software was used to calculate the area (%) of demyelination and all white matter. (C) Infiltration of $CD68^+$ macrophages (red, stained by Alexa Fluor 555). Fasudil-modified macrophages inhibited the formation of spinal inflammatory foci and the infiltration of $CD68^+$ macrophages, reduced demyelination, and decreased CNS inflammation. The results are presented as the mean ± SEM for six mice from each group. **P < 0.01, ***P < 0.001 (Student's t-test). EAE: experimental autoimmune encephalomyelits; PBS: phosphate buffer saline; Mϕ: macrophage; HE: hematoxylin-eosin; LFB: Luxol fast blue.

is p-MYPT1, which can serves as an indicator of ROCK-II activity. Thus, we assessed the activities of ROCK-II and p-MYPT1 in macrophages collected from EAE mice and treated with Fasudil or PBS by western blot. The results showed that p-MYPT1 and ROCK-II expression levels were increased in macrophages treated with PBS, while treatment with Fasudil significantly inhibited ROCK-II (P < 0.05; **Figure 5**) and p-MYPT1 (P < 0.01; **Figure 5**) expression. These findings suggest that Fasudil inhibits macrophage ROCK activity.

Fasudil promotes macrophage conversion from the pro-inflammatory M1 type to the anti-inflammatory M2 type *in vitro*

The functional state of macrophages–that is, whether they are pro-inflammatory or anti-inflammatory–changes depending on the environment, and the functional status of any given population of macrophages is very heterogeneous. Pro-inflammatory M1 macrophages mainly express iNOS, IL-12, or CD16/32, while anti-inflammatory M2 macrophages mainly express Arg-1, IL-10, or CD206. Phenotypic analysis of macrophages by fluorescence-activated cell sorting showed that the expression levels of CD16/32 (P < 0.01), IL-12 (P < 0.01), and iNOS (P < 0.01) were considerably reduced following Fasudil treatment (**Figure 6**). In addition, Fasudil-modified macrophages showed significantly increased expression of CD206 (P < 0.001), Arg-1 (P < 0.05), IL-10 (P < 0.001), and CD14 (P < 0.05) compared with PBS-treated macrophages (**Figure 6**). These findings indicate that Fasudil may promote conversion of macrophages from the pro-inflammatory M1 subtype to the anti-inflammatory M2 subtype.

iNOS and Arg-1 are considered to be the most reliable markers of macrophage polarization. Therefore, we performed western blotting for these two proteins to quantitatively analyze the effect of Fasudil on macrophage phenotype. Compared with PBS-treated macrophages, Fasudil-modified macrophages

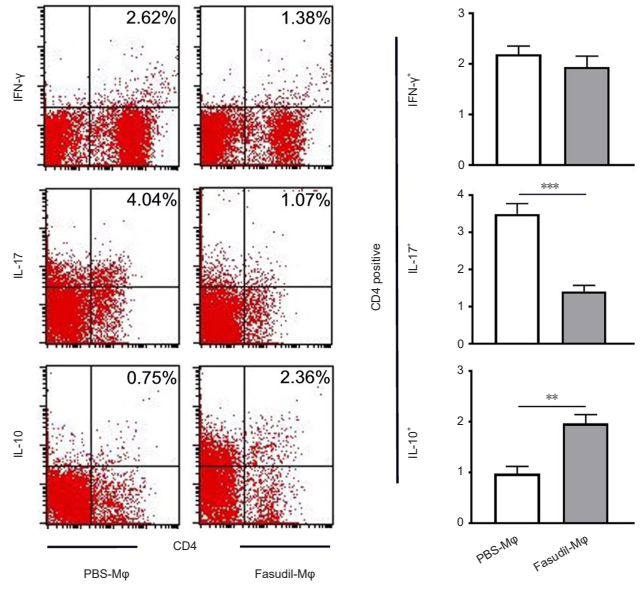

Figure 3 | **Fasudil-modified macrophages affect T cell polarization.**
A mouse model of chronic EAE was induced by subcutaneous injection of myelin oligodendrocyte glycoprotein peptde 35–55. Macrophages from EAE mice isolated with magnetic beads were treated with 15 μg/mL of Fasudil at 37°C in 5% CO_2 atmosphere for 72 hours. Fasudil-treated macrophages were suspended in PBS at a concentration of 2 × 10^7 cells/200 μL and intraperitoneally injected into EAE mice on days 3 and 11 postimmunization. PBS-treated macrophages were used as a control. The mice were randomly divided into two groups: the Fasudil-macrophage group (Fasudil-Mϕ) (*n*=12) and the PBS-macrophage group (PBS-Mϕ) (*n* = 12). On day 28 p.i., splenic MNCs were prepared and stained with anti-CD4 (for $CD4^+$ T cells), IFN-γ (for Th1), IL-17 (for Th17), and IL-10 (for Th2) antibodies, and T cell subsets were analyzed by flow cytometry. Injection of Fasudil-modified macrophages decreased the percentage of $CD4^+IL\text{-}17^+$ T cells and increased the percentage of $CD4^+IL\text{-}10^+$ T cells. Quantitative results are shown for five mice from each group. The results are expressed as the percentage of double positive cells in the four-quadrant diagram. Data are presented as the mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t*-test). EAE: Experimental autoimmune encephalomyelits; IFN-γ: interferon-γ; IL-10:interleukin-10; IL-17:interleukin-17; MNCs: mononuclear cells; Mϕ: macrophage; PBS: phosphate buffer saline.

significantly decreased iNOS ($P < 0.01$) expression and significantly increased Arg-1 ($P < 0.01$) expression in macrophages (**Figure 7A**). Furthermore, chemical and enzymatic assays showed that macrophages treated with Fasudil produced significantly less nitrite ($P < 0.001$), a major end product of NO metabolism, and exhibited significantly increased Arg-1 activity ($P < 0.01$) compared with macrophages treated with PBS, indicating that Fasudil reduced NO generation and enhanced Arg-1 release (**Figure 7B**).

Fasudil reduces TLR-4/Myd88/MAPK signaling in macrophages

TLRs are vital mediators of the neurological damage that occurs in EAE, and their effects mediated by Myd88-dependent and -independent signaling pathways. Therefore, we next assessed the expression of components of the TLRs-Myd88 cytokine axis in macrophages by western blot assay. The results showed that macrophages isolated from EAE mice expressed high levels of TLR-4 ($P < 0.01$) and Myd88 ($P < 0.01$), and that treatment with Fasudil efficiently suppressed expression of these two proteins (**Figure 8A**).

Given that the MAPK signaling pathway is downstream of the TLRs/Myd88 signaling pathway, we then used western blot to look at expression levels of p-P38, p-JNK, and p-ERK, all of which are MAPK signaling pathway components. The results showed that treatment with Fasudil significantly decreased p-JNK ($P < 0.01$) and p-P38 ($P < 0.05$) levels, but had no effect on p-ERK levels, in macrophages compared with treatment with PBS (**Figure 8B**). These findings suggest that the initial target of Fasudil that affects the macrophage-mediated inflammatory response is TLR4 and its downstream molecule MyD88.

Fasudil inhibits release of inflammatory factors by macrophages

Neuroinflammation has been linked to an increased chance of developing EAE. p-NF-κB, a critical transcription factor, has been implicated in numerous immunological reactions and is critical for the production of many inflammatory factors. Thus, we employed western blotting to assess p-NF-κB and RPS3 expression in macrophages and ELISA to detect secretion of IL-1β, IL-6, and TNF-α by macrophages. The results showed that macrophages treated with PBS expressed high levels of p-NF-κB and RPS3 and secreted large amounts of IL-1β, IL-6, and TNF-α, while treatment with Fasudil significantly decreased p-NF-κB ($P < 0.05$; **Figure 9A**) and RPS3 ($P < 0.05$; **Figure 9A**) expression, as well as IL-6 ($P < 0.05$; **Figure 9B**), IL-1β ($P < 0.01$; **Figure 9B**), and TNF-α ($P < 0.001$; **Figure 9B**) secretion. These findings suggest that Fasudil inhibits the release of inflammatory mediators by macrophages.

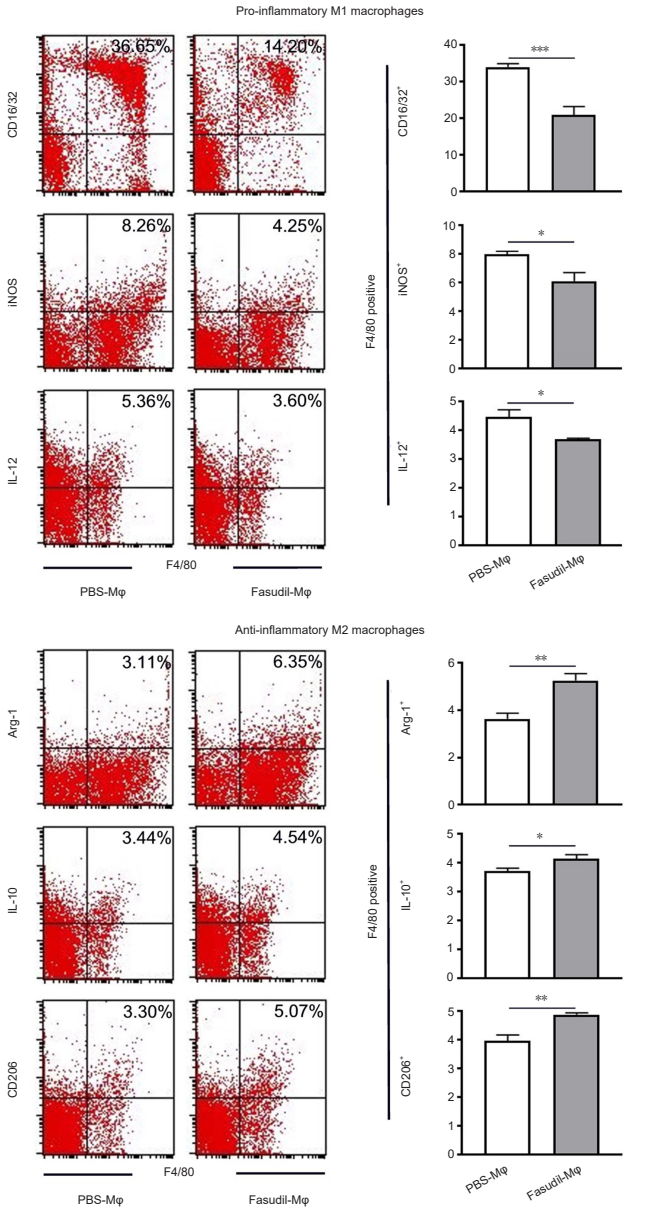

Figure 4 | **Fasudil-modified macrophages affect macrophage polarization.**
A mouse model of chronic EAE was induced by subcutaneous injection of myelin oligodendrocyte glycoprotein peptde 35–55. Macrophages from EAE mice isolated with magnetic beads were treated with 15 μg/mL of Fasudil at 37°C in 5% CO_2 atmosphere for 72 hours. Fasudil-treated macrophages were suspended in PBS at a concentration of 2 × 10^7 cells/200 μL and intraperitoneally injected into EAE mice on days 3 and 11 p.i.. PBS-treated macrophages were used as a control. The mice were randomly divided into two groups: the Fasudil-macrophage group (Fasudil-Mϕ) (*n* = 12) and the PBS-macrophage group (PBS-Mϕ) (*n* = 12). On day 28 p.i., splenic MNCs were prepared and stained with antibodies to F4/80 (for macrophages), CD16/32, iNOS, and IL-12 (for pro-inflammatory M1 macrophages), and CD206, Arg-1, and IL-10 (for anti-inflammatory M2 macrophages), and the macrophage subsets were analyzed by flow cytometry. Injection with Fasudil-modified macrophages reduced the percentages of $CD16/32^+$, $iNOS^+$, and $IL\text{-}12^+$ pro-inflammatory M1 macrophages and increased the percentages of $Arg\text{-}1^+$, $IL\text{-}10^+$, and $CD206^+$ anti-inflammatory M2 macrophages. Quantitative results are shown for five mice from each group. The results are expressed as the percentage of double positive cells in the four-quadrant diagram. Data are presented as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t*-test). Arg-1: Arginase-1; EAE: experimental autoimmune encephalomyelits; IL-10: interleukin-10; IL-12: interleukin-12; iNOS: inducible nitric oxide synthase; MNCs: mononuclear cells; Mϕ: macrophage; PBS: phosphate buffer saline; p.i.: postimmunization.

Discussion

Our data indicate that intraperitoneal injection of Fasudil-modified macrophages postponed EAE onset, decreased disease severity, and reduced demyelination, similar to direct treatment of EAE with Fasudil. As variations in T cell and macrophage subsets play a crucial role in the pathogenesis of EAE, we further observed the effects of Fasudil-modified macrophages on peripheral immune cells in EAE mice. The results confirmed that PBS-treated macrophages amplified peripheral inflammation, leading to CNS damage.

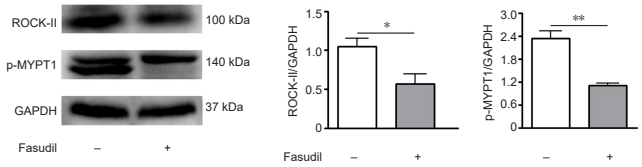

Figure 5 | **Fasudil inhibits macrophage ROCK activity *in vitro*.**
A mouse model of chronic EAE was induced by subcutaneous injection of myelin oligodendrocyte glycoprotein peptde 35–55. Macrophages from EAE mice isolated with magnetic beads were treated with 15 μg/mL of Fasudil at 37°C in 5% CO_2 atmosphere for 72 hours. Protein concentrations of ROCK-II and p-MYPT1 were determined by western blot assay. Fasudil inhibited ROCK-II and p-MYPT1 expression in macrophages. Quantitative results are shown as the mean ± SEM of four to six exemplars for each group. **P* < 0.05, ***P* < 0.01 (Student's *t*-test). EAE: Experimental autoimmune encephalomyelits; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; p-MYPT1: myosin light chain phosphatase target subunit 1 (p-MYPT1)-Thr696; ROCK-II: Rho kinase II.

In contrast, Fasudil-modified macrophages converted pro-inflammatory T cells and macrophages into anti-inflammatory T cells and macrophages that promoted tissue repair and decreased the immune response, thus improving the symptoms of EAE. More significantly, Fasudil-modified macrophages are likely to have fewer side effects than direct treatment with Fasudil, such as vascular enlargement.

ROCK, a previously identified downstream target of Rho, has a relative molecular mass of 160 kDa, a complicated molecular structure, and substantial variation in terms of tissue distribution (Mulherkar and Tolias, 2020). When Rho activates ROCK, ROCK then activates downstream substrates; thus, ROCK functions as a bridge to transmit signals from inside to outside the cell and *vice versa* (Shinozaki et al., 2019; Lu et al., 2021). ROCK has been linked to blood-brain barrier breakdown, immunological cell activation, inflammatory cell infiltration, and the glial cell response in MS/EAE (Yu et al., 2016; Yan et al., 2019). Thus, we investigated macrophage ROCK activity and CNS inflammation. Our findings showed that Fasudil decreased macrophage ROCK-II/p-MYPT1 activity, and intraperitoneal injection of Fasudil-modified macrophages reduced the number of infiltrating inflammatory corpuscles in the CNS, supporting our hypothesis that Fasudil-modified macrophages affect peripheral immune cell migration, aggregation, and release by inhibiting ROCK activity.

Next, we explored the molecular mechanism by which Fasudil-modified macrophages improve EAE symptoms. Macrophages, as vital antigen-presenting cells, can both degrade the myelin sheath and repair damaged tissues during the course of EAE (Alrehaili et al., 2018). Normally, macrophages exist in a highly differentiated resting state. Under pathological conditions, they can exert both inflammatory and anti-inflammatory effects (Cai et al., 2021; Ma et al., 2022). Pro-inflammatory macrophages secrete hyperoxidative stress products and pro-inflammatory factors that cause inflammation and mediate cytotoxicity (Liu et al., 2015; Jiang et al., 2022), while anti-inflammatory macrophages release anti-inflammatory factors that have neuroprotective effects and promote axon regeneration (Wang et al., 2022a). Our flow cytometry data indicated that the expression of pro-inflammatory M1 markers, such as iNOS, IL-12, and CD16/32, was considerably reduced in Fasudil-modified macrophages. On the other hand, the expression of anti-inflammatory M2 markers, including CD206, CD14, Arg-1, and IL-10, was increased in Fasudil-modified macrophages. Oxidative stress is critical in the development of EAE inflammation, mostly because of iNOS-induced production of a large amount of NO, which results in excessive oxidative stress in the body and, ultimately, CNS injury (Nasrnezhad et al., 2021). Activation of pro-inflammatory M1 macrophages is followed by an increase in the production of inflammatory factors like iNOS and a considerable elevation in NO expression. Loss and breakdown of the myelin sheath of nerves is encouraged by the increased NO expression, which also makes blood vessels more permeable, resulting in a significant rise in infiltration of inflammatory cells (Sonar and Lal, 2019; Bibi et al. 2022). In our study we found that Fasudil decreased iNOS expression and NO release, while promoting Arg-1 secretion. Taken together, our findings show that the Fasudil-induced transformation of macrophages from an inflammatory phenotype to an anti-inflammatory phenotype helps control the inflammatory microenvironment, which may open up new avenues for treating neurological illnesses like MS.

A previous study showed that inflammation may be common to all neurodegenerative disorders, resulting in neuronal damage through transmission and amplification effects (Alam et al, 2021). In addition, cell signaling proteins serve as a key link between inflammation and neurodegeneration (Yeung et al., 2018). TLRs are transemembrane proteins that respond to endogenous damage-associated molecular patterns generated by apoptotic cells or damaged tissues, and can also activate the MAPK pathway via the downstream protein Myd88 (Jiang et al., 2020; Wang et al., 2020). MAPKs, a family of threonine and serine kinases, trigger external stimulating factors via a protein phosphorylation cascade, activating microglia and boosting the activity of pro-inflammatory cytokines (Chen et al., 2020; Liu et al., 2020). Macrophage/microglia activation occurs through the TLR-2/4-mediated pathway, and the presence of functional TLR-2/4 is related to an increase in the activity of pro-inflammatory cytokines (Anstadt et al., 2016; Liu et al., 2022). TLR-4 is expressed at high levels in macrophages, and it increases the generation of more inflammatory factors, for example IL-1β, TNF-α, and IL-6. Interestingly, we found that Fasudil decreased TLR-4 expression in and inflammatory cytokine production (iNOS, IL-lβ, and TNF-α) by macrophages. Multiple signal transduction pathways involved in the inflammatory response, apoptosis, synaptic plasticity, and so on, converge on MAPK, which can

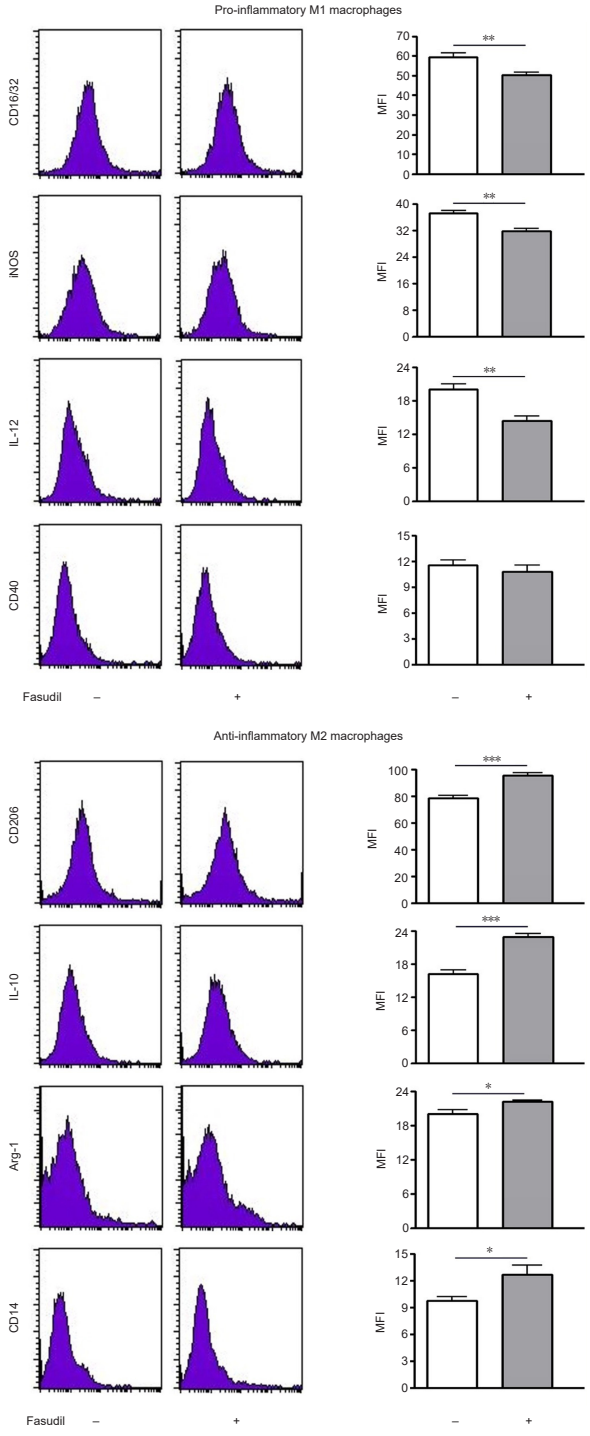

Figure 6 | **Fasudil affects macrophage polarization *in vitro*.**
A mouse model of chronic EAE was induced by subcutaneous injection of myelin oligodendrocyte glycoprotein peptde 35–55. Macrophages from EAE mice isolated with magnetic beads were treated with 15 μg/mL of Fasudil at 37°C in 5% CO_2 atmosphere for 72 hours. Cells were labeled with antibodies to CD16/32, iNOS, IL-12, CD40, CD206, IL-10, Arg-1, and CD14 and analyzed by flow cytometry. Decreased levels of the pro-inflammatory M1 macrophage subtypes CD16/32, iNOS, and IL-12 and increased levels of the anti-inflammatory M2 macrophage subtypes CD206, IL-10, Arg-1, and CD14 were observed in response to Fasudil treatment. Quantitative data are shown for five exemplars from each group and are reported as mean fluorescence intensity (MFI). Data are presented as the mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (Student's *t*-test). Arg-1: Arginase-1; EAE: experimental autoimmune encephalomyelits; IL-10:interleukin-10; IL-12:interleukin-12; iNOS: inducible nitric oxide synthase.

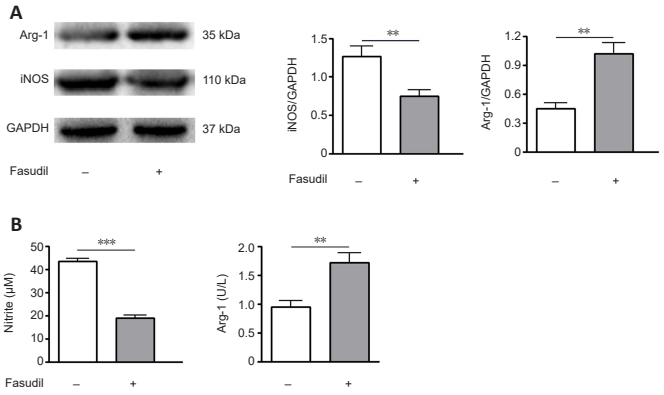

Figure 7 | Effects of Fasudil on iNOS/NO and Arg-1 expression.
A mouse model of chronic EAE was induced by subcutaneous injection of myelin oligodendrocyte glycoprotein peptde 35–55. Macrophages from EAE mice isolated with magnetic beads were treated with 15 μg/mL of Fasudil at 37°C in 5% CO_2 atmosphere for 72 hours. (A) Arg-1 and iNOS expression levels were determined by western blot. (B) The Griess technique was used to determine the NO content of the supernatant, and Arg-1 activity was determined using an Arg-1 assay kit. Fasudil reduced iNOS/NO production by and enhanced Arg-1 release from macrophages. Quantitative data are shown as the mean ± SEM of four to six exemplars from each group. $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t*-test). Arg-1: Arginase-1; EAE: experimental autoimmune encephalomyelits; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; iNOS: inducible nitric oxide synthase.

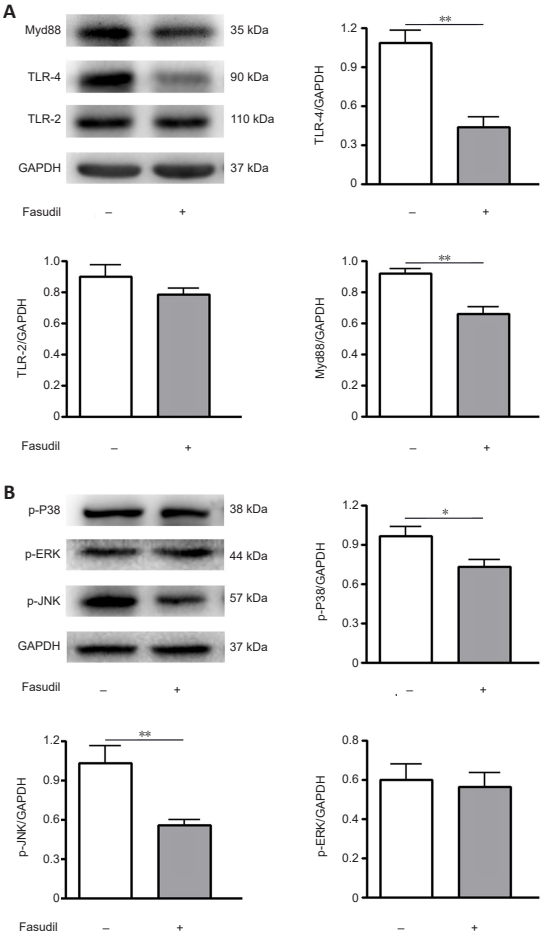

Figure 8 | Fasudil affects the TLRs/Myd88/MAPK signaling pathway in macrophages.
A mouse model of chronic EAE was induced by subcutaneous injection of myelin oligodendrocyte glycoprotein peptde 35–55. Macrophages from EAE mice isolated with magnetic beads were treated with 15 μg/mL of Fasudil at 37°C in 5% CO_2 atmosphere for 72 hours. (A) TLR-2, TLR-4, and Myd88 expression levels were determined by western blot. (B) p-ERK, p-P38, and p-JNK expression levels were determined by western blot. Fasudil suppressed expression of TLR-4, Myd88, p-JNK, and p-P38 by macrophages. Quantitative data are shown as mean ± SEM of four to six exemplars from each group. $^{*}P < 0.05$, $^{**}P < 0.01$ (Student's *t*-test). EAE: Experimental autoimmune encephalomyelits; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; Myd88: myeloid differentiation factor 88; PBS: phosphate buffer saline; p-ERK: p-extracellular regulated protein kinases; p-JNK: p-Jun N-terminalkinase; TLR-2: toll like receptor 2; TLR-4: toll like receptor 4.

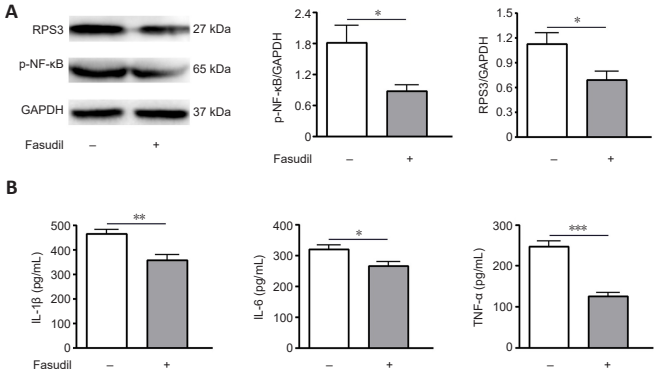

Figure 9 | Fasudil affects macrophage-mediated inflammation.
A mouse model of chronic EAE was induced by subcutaneous injection of myelin oligodendrocyte glycoprotein peptde 35–55. Macrophages from EAE mice isolated with magnetic beads were treated with 15 μg/mL of Fasudil at 37°C in 5% CO_2 atmosphere for 72 hours. p-NF-κB and RPS3 expression levels were determined by western blot. (B) IL-1β, TNF-α, and IL-6 secretion levels were assessed by ELISA. Fasudil suppressed p-NF-κB and RPS3 expression, as well as IL-6, IL-1β, and TNF-α secretion. Quantitative data are shown as mean ± SEM of four to six exemplars from each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t*-test). EAE: Experimental autoimmune encephalomyelits; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; IL-1β: interleukin-1β; IL-6: interleukin-6; p-NF-κB: p-nuclear factor kappa B; RPS3: ribosoma protein S3; TNF-α: tumor necrosis factor-α.

activate and regulate the activity of various intracellular factors in response to extracellular stimuli. MAPK cascades include JNK, ERK, and p38, among which the p-ERK pathway is involved in cell survival, hyperplasia, and motility, while the p-P38 and p-JNK pathways are associated with inflammation and neuronal death (Tian et al., 2014; Birkner et al., 2017; Wang et al., 2017; Yang et al., 2020). Indeed, our study showed that p-ERK, p-P38, and p-JNK expression levels were elevated in macrophages, and that activation of the MAPK signaling pathway greatly enhanced the expression levels of pro-inflammatory factors such as iNOS, COX-2, TNF-α, and IL-lβ. Fasudil efficiently inhibited p-P38 and p-JNK expression, but not p-ERK expression, in macrophages, demonstrating that Fasudil-mediated macrophage polarization and Fasudil-inhibited macrophage inflammation involve both TLR-4-p-P38/p-JNK-dependent and p-ERK-independent signal pathways.

NF-κB is a transcription factor found in almost all cells that is essential for the expression of pro-inflammatory factors by macrophages. In spite of extensive research into the function of NF-κB signaling, it is still unclear how NF-κB selectively activates its targets (Liu et al., 2015; Guo et al., 2019; Nadeem et al., 2022). Ribosomal protein S3 (RPS3) is a component of the 40S ribosomal subunit that transports information between the nucleus and the cytoplasm. RPS3 was recently reported to bind to the p65 subunit of NF-κB, promoting nuclear accumulation of the NF-κB complex (Stanborough et al., 2014; Hodgson et al., 2015). The NF-κB activation signaling cascade precisely regulates RPS3's subcellular localization, and RPS3-p65 binding within the nucleus increases the affinity of NF-κB for its target genes, thereby conferring transcriptional specificity and promoter selectivity and enhancing transcriptional activity (Wier et al., 2015; Li et al., 2020; Rius-Pérez et al., 2020). The results from our study showed that NF-κB and RPS3 are expressed at high levels in macrophages, emphasizing the importance of RPS3/NF-κB in the immunological response mediated by pro-inflammatory macrophages. Additionally, the interplay between NF-κB and RPS3 is critical to immunomodulation, and Fasudil can diminish NF-κB activation by impacting RPS3 expression levels, ultimately limiting inflammatory cytokine release. Macrophages are considered to be key to EAE pathology, and their activity is modulated by the NF-κB/RPS3 signaling pathway. Moreover, Fasudil targets NF-κB and its regulatory protein RPS3 to decrease the inflammatory response of macrophages, as demonstrated by our experimental results. Thus, the NF-κB/RPS3 signaling pathway is a promising target of Fasudil-modified macrophage-based immunotherapy.

This study has some limitations that should be noted. First, we did not track PBS-/Fasudil-modified macrophages *in vivo* after intraperitoneal injection. Second, we did not perform RNA-seq to comprehensively determine the polarization status of macrophages modified by PBS/Fasudil. Third, quantitative analysis of $CD68^{+}$ macrophages and pro-inflammatory M1 subtypes or anti-inflammatory M2 subtypes by immunofluorescence double staining was not performed when exploring pathological changes to the CNS. The main conclusion from this study is that the impact of EAE can be lessened by injection with Fasudil-modified macrophages. Tracking macrophage location and survival time *in vivo*, performing immunofluorescence double staining of macrophages in the CNS to determine their subtypes, and assess macrophage polarization status *in vitro* by RNA-Seq would help more fully explain the mechanism by which Fasudil-modified macrophages improve EAE symptoms. These experiments should be included in future studies.

In conclusion, our data indicate that Fasudil-modified macrophages have a therapeutic effect on EAE, ameliorating clinical severity, alleviating demyelination, and inhibiting the inflammatory response. Fasudil changes the

phenotype of macrophages from pro-inflammatory M1 to anti-inflammatory M2, which is associated with decreased ROCK-II/p-MYPT1 activity, reduced expression of TLRs/MAPK and NF-κB/RPS3 signaling pathway components, and reduced inflammatory cytokine production. In summary, our findings suggest that immuno-cytotherapy might be a novel therapeutic approach for EAE. However, the molecular basis of the effects of Fasudil-modified immune cells on neuroinflammatory disease still requires further exploration.

Author contributions: *CL, BX and CM conceived and designed the experiments. CL did the major part of the experiments. SG helped in in vitro experiments. QW and ZC analyzed the flow cytometry data. MG and RL helped with immune cells experiments. CL performed the analysis and created the figures. CL and SG wrote the paper. BX and CM did the manuscript revision. All authors approved the final version of manuscript.*
Conflicts of interest: *The authors declare that they have no competing and non-financial conflict of interests.*
Data availability statement: *All relevant data are within the paper.*

References

Alam MS, Otsuka S, Wong N, Abbasi A, Gaida MM, Fan Y, Meerzaman D, Ashwell JD (2021) TNF plays a crucial role in inflammation by signaling via T cell TNFR2. Proc Natl Acad Sci U S A 118:e2109972118.

Alrehaili AA, Lee JY, Bakhuraysah MM, Kim MJ, Aui PM, Magee KA, Petratos S (2018) Nogo receptor expression in microglia/macrophages during experimental autoimmune encephalomyelitis progression. Neural Regen Res 13:896-907.

Anstadt EJ, Fujiwara M, Wasko N, Nichols F, Clark RB (2016) TLR tolerance as a treatment for central nervous system autoimmunity. J Immunol 197:2110-2118.

Barnes DA, Galloway DA, Hoener MC, Berry MD, Moore CS (2021) TAAR1 expression in human macrophages and brain tissue: a potential novel facet of MS neuroinflammation. Int J Mol Sci 22:11576.

Benson JM, Campbell KA, Guan Z, Gienapp IE, Stuckman SS, Forsthuber T, Whitacre CC (2000) T-cell activation and receptor downmodulation precede deletion induced by mucosally administered antigen. J Clin Invest 106:1031-1038.

Bibi T, Khan A, Khan AU, Shal B, Ali H, Seo EK, Khan S (2022) Magnolol prevented brain injury through the modulation of Nrf2-dependent oxidative stress and apoptosis in PLP-induced mouse model of multiple sclerosis. Naunyn Schmiedebergs Arch Pharmacol 395:717-733.

Birkner K, Wasser B, Loos J, Plotnikov A, Seger R, Zipp F, Witsch E, Bittner S (2017) The role of ERK signaling in experimental autoimmune encephalomyelitis. Int J Mol Sci 18:1990.

Cai F, Liu S, Lei Y, Jin S, Guo Z, Zhu D, Guo X, Zhao H, Niu X, Xi Y, Wang Z, Chen G (2021) Epigallocatechin-3 gallate regulates macrophage subtypes and immunometabolism to ameliorate experimental autoimmune encephalomyelitis. Cell Immunol 368:104421.

Che J, Li D, Hong W, Wang L, Guo Y, Wu M, Lu J, Tong L, Weng Q, Wang J, Dong X (2022) Discovery of new macrophage M2 polarization modulators as multiple sclerosis treatment agents that enable the inflammation microenvironment remodeling. Eur J Med Chem 243:114732.

Chen FB, Liu ZY, Peng W, Gao ZQ,Ouyang H, Yan TJ,Ding SB, Cai ZK, Zhao B, Mao LJ, Cao ZY (2018) Activation of EphA4 induced by EphrinA1 exacerbates disruption of the blood-brain barrier following cerebral ischemia-reperfusion via the Rho/ROCK signaling pathway. Exp Ther Med 16:2651-2658.

Chen HL, Jia WJ, Li HE, Han H, Li F, Zhang XL, Li JJ, Yuan Y, Wu CY (2020) Scutellarin exerts anti-inflammatory effects in activated microglia/brain macrophage in cerebral ischemia and in activated BV-2 microglia through regulation of MAPKs signaling pathway. Neuromolecular Med 22:264-277.

Chong CM, Ai N, Lee SM (2017) ROCK in CNS: Different roles of isoforms and therapeutic target for neurodegenerative disorders. Curr Drug Targets 18:455-462.

Chu F, Shi MC, Zheng C, Shen DH, Zhu J, Zheng XY, Cui L (2018) The roles of macrophages and microglia in multiple sclerosis and experimental autoimmune encephalomyelitis. J Neuroimmunol 318:1-7.

Chu T, Zhang YP, Tian Z, Ye C, Zhu M, Shields LBE, Kong M, Barnes GN, Shields CB, Cai J (2019) Dynamic response of microglia/macrophage polarization following demyelination in mice. J Neuroinflammation 16:188.

Gharagozloo M, Mace JW, Calabresi PA (2022) Animal models to investigate the effects of inflammation on remyelination in multiple sclerosis. Front Mol Neurosci 15:995477.

Ghorbani MM, Farazmandfar T, Abediankenari S, Hassannia H, Maleki Z, Shahbazi M (2022) Treatment of EAE mice with Treg, G-MDSC and IL-2: a new insight into cell therapy for multiple sclerosis. Immunotherapy 14:789-798.

Gou L, Gao J, Yang H, Gao C (2019) The landscape of CAR T-cell therapy in the United States and China: A comparative analysis.Int J Cancer 144:2043-2050.

Grassivaro F, Menon R, Acquaviva M, Ottoboni L, Ruffini F, Bergamaschi A, Muzio L, Farina C, Martino G (2020) Convergence between microglia and peripheral macrophages phenotype during development and neuroinflammation. J Neurosci 40:784-795.

Guo SD, Liu CY, Yu JW, Chai Z, Wang Q, Mi XT, Song GB, Li YH, Yang PW, Feng L, Xiao BG, Ma CG (2019) Nasal delivery of Fasudil-cocultured immune cells exhibits therapeutic potential in experimental autoimmune encephalomyelitis. CNS Neurosci Ther 25:783-795.

Hodgson A, Wier EM, Fu K, Sun X, Yu H, Zheng W, Sham HP, Johnson K, Bailey S, Vallance BA, Wan F (2015) Metalloprotease NleC suppresses host NF-κB/inflammatory responses by cleaving p65 and interfering with the p65/RPS3 interaction. PLoS Pathog 11:e1004705.

Hou SW, Liu CY, Li YH, Yu JZ, Feng L, Liu YT, Guo MF, Xie Y, Meng J, Zhang HF, Xiao BG, Ma CG (2012) Fasudil ameliorates disease progression in experimental autoimmune encephalomyelitis, acting possibly through antiinflammatory effect. CNS Neurosci Ther 18:909-917.

Jafari Rad M, Navi Z, Heidari AR, Lavi Arab F, Tabasi N, Rastin M, Khadem Rezaiyan M, Moghaddas E, Mahmoudi M (2022) Evaluation of the immunoregulatory effect of Dicrocoelium dendriticum eggs on inflammatory and anti-inflammatory cytokines in EAE model. Parasite Immunol 44:e12942.

Jiang F, Liu X, Cui X, Hu J, Wang L, Xue F, Guo S, Wang X (2022) Circ_0000518 promotes macrophage/microglia M1 polarization via the FUS/CaMKKβ/AMPK pathway to aggravate multiple sclerosis. Neuroscience 490:131-143.

Jiang G, Gong M, Song H, Sun W, Zhao W, Wang L (2020) Tob2 inhibits TLR-induced inflammatory responses by association with TRAF6 and MyD88. J Immunol 205:981-986.

Jiang N, Qiao G, Wang X, Morse MA, Gwin WR, Zhou L, Song Y, Zhao Y, Chen F, Zhou X, Huang L, Hobeika A, Yi X, Xia X, Guan Y, Song J, Ren J, Lyerly HK (2017) Dendritic cell/cytokine-induced killer cell immunotherapy combined with S-1 in patients with advanced pancreatic cancer: a prospective study. Clin Cancer Res 23:5066-5073.

Li Q, Zhou L, Wang L, Li S, Xu G, Gu H, Li D, Liu M, Fang L, Wang Z, Han S, Zheng B (2020) Bcl6 modulates innate immunity by controlling macrophage activity and plays critical role in experimental autoimmune encephalomyelitis. Eur J Immunol 50:525-536.

Liu CY, Li YH, Yu JZ, Feng L, Hou SW, Liu YT, Guo MF, Xie Y, Meng J, Zhang HF, Xiao BG, Ma CG (2013) Targeting the shift from M1 to M2 macrophages in experimental autoimmune encephalomyelitis mice treated with fasudil. PLoS One 8:e54841.

Liu CY, Guo SD, Yu JZ, Li YH, Zhang H, Feng L, Chai Z, Yuan HJ, Yang WF, Feng QJ, Xiao BG, Ma CG (2015) Fasudil mediates cell therapy of EAE by immunomodulating encephalomyelitic T cells and macrophages. Eur J Immunol 45:142-152.

Liu GY, Wu Y, Kong FY, Ma S, Fu LY, Geng J (2021) BMSCs differentiated into neurons, astrocytes and oligodendrocytes alleviated the inflammation and demyelination of EAE mice models. PLoS One 16:e0243014.

Liu X, Zhang X, Niu X, Zhang P, Wang Q, Xue X, Song G, Yu J, Xi G, Song L, Li Y, Ma C (2022) Mdivi-1 modulates macrophage/microglial polarization in mice with eae via the inhibition of the TLR2/4-GSK3β-NF-κB inflammatory signaling axis. Mol Neurobiol 59:1-16.

Liu Z, Yao X, Jiang W, Li W, Zhu S, Liao C, Zou L, Ding R, Chen J (2020) Advanced oxidation protein products induce microglia-mediated neuroinflammation via MAPKs-NF-kappaB signaling pathway and pyroptosis after secondary spinal cord injury. J Neuroinflammation 17:90.

Locke FL, Ghobadi A, Jacobson CA, Miklos DB, Lekakis LJ, Oluwole OO, Lin Y, Braunschweig I, Hill BT, Timmerman JM, Deol A, Reagan PM, Stiff P, Flinn IW, Farooq U, Goy A, McSweeney PA, Munoz J, Siddiqi T, Chavez JC, et al. (2019) Long-term safety and activity of axicabtagene ciloleucel in refractory large B-cell lymphoma (ZUMA-1): a single-arm, multicentre, phase 1-2 trial. Lancet Oncol 20:31-42.

Lu W, Chen Z, Wen J (2021) RhoA/ROCK signaling pathway and astrocytes in ischemic stroke. Metab Brain Dis 36:1101-1108.

Luoqian J, Yang W, Ding X, Tuo QZ, Xiang Z, Zheng Z, Guo YJ, Li L, Guan P, Ayton S, Dong B, Zhang H, Hu H, Lei P (2022) Ferroptosis promotes T-cell activation-induced neurodegeneration in multiple sclerosis. Cell Mol Immunol 19:913-924.

Ma X, Wang S, Li C, Jia X, Wang T, Leng Z, Lu R, Kong X, Zhang J, Li L (2022) Baicalein inhibits the polarization of microglia/macrophages to the M1 phenotype by targeting STAT1 in EAE mice. Int Immunopharmacol 113:109373.

Marks PW, Witten CM, Califf RM (2017) Clarifying stem-cell therapy's benefits and risks. N Engl J Med 376:1007-1009.

Martín-Cámara O, Cores Á, López-Alvarado P, Menéndez JC (2021) Emerging targets in drug discovery against neurodegenerative diseases: Control of synapsis disfunction by the RhoA/ROCK pathway. Eur J Med Chem 225:113742.

Mount NM, Ward SJ, Kefalas P, Hyllner J (2015) Cell-based therapy technology classifications and translational challenges. Philos Trans R Soc Lond B Biol Sci 370:20150017.

Mulherkar S, Tolias KF (2020) RhoA-ROCK signaling as a therapeutic target in traumatic brain injury. Cells 9:245.

Nadeem A, Ahmad SF, Al-Harbi NO, Sarawi W, Attia SM, Alanazi WA, Ibrahim KE, Alsanea S, Alqarni SA, Alfardan AS, Bakheet SA (2022) Acetyl-11-keto-β-boswellic acid improves clinical symptoms through modulation of Nrf2 and NF-κB pathways in SJL/J mouse model of experimental autoimmune encephalomyelitis. Int Immunopharmacol 107:108703.

Nasrnezhad R, Halalkhor S, Sadeghi F, Pourabdolhossein F (2021) Piperine improves experimental autoimmune encephalomyelitis (EAE) in Lewis rats through its neuroprotective, anti-inflammatory, and antioxidant effects. Mol Neurobiol 58:5473-5493.

National Research Council (2011) Guide for the Care and Use of Laboratory Animals, 8th ed. Washington, DC: The National Academies Press.

Okuda Y, Sakoda S, Fujimura H, Saeki Y, Kishimoto T, Yanagihara T (1999) IL-6 plays a crucial role in the induction phase of myelin oligodendrocyte glycoprotein 35-55 induced experimental autoimmune encephalomyelitis. J Neuroimmunol 101:188-196.

Percie du Sert N, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, Browne WJ, Clark A, Cuthill IC, Dirnagl U, Emerson M, Garner P, Holgate ST, Howells DW, Karp NA, Lazic SE, Lidster K, MacCallum CJ, Macleod M, Pearl EJ, et al. (2020) The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. PLoS Biol 18:e3000410.

Prineas JW, Parratt JDE (2021) Multiple sclerosis: microglia, monocytes, and macrophage-mediated demyelination. J Neuropathol Exp Neurol 80:975-996.

Rius-Pérez S, Pérez S, Martí-Andrés P, Monsalve M, Sastre J (2020) Nuclear factor kappa B signaling complexes in acute inflammation. Antioxid Redox Signal 33:145-165.

Shinozaki Y, Danjo Y, Koizumi S (2019) Microglial ROCK is essential for chronic methylmercury-induced neurodegeneration. J Neurochem 151:64-78.

Sonar SA, Lal G (2019) The iNOS activity during an immune response controls the CNS pathology in experimental autoimmune encephalomyelitis. Front Immunol 10:710.

Stanborough T, Niederhauser J, Koch B, Bergler H, Pertschy B (2014) Ribosomal protein S3 interacts with the NF-κB inhibitor IκBα. FEBS Lett 588:659-664.

Stavropoulos F, Georgiou E, Sargiannidou I, Kleopa KA (2021) Dysregulation of blood-brain barrier and exacerbated inflammatory response in Cx47-deficient mice after induction of EAE. Pharmaceuticals (Basel) 14:621.

Tang Y, Le W (2016) Differential roles of M1 and M2 microglia in neurodegenerative diseases. Mol Neurobiol 53:1181-1194.

Tian X, Sui S, Huang J, Bai JP, Ren TS, Zhao QC (2014) Neuroprotective effects of Arctium lappa L. roots against glutamate-induced oxidative stress by inhibiting phosphorylation of p38, JNK and ERK 1/2 MAPKs in PC12 cells. Environ Toxicol Pharmacol 38:189-198.

Voskuhl RR, Pitchekian-Halabi H, MacKenzie-Graham A, McFarland HF, Raine CS (1996) Gender differences in autoimmune demyelinaton in the mouse: implicatons for multple sclerosis. Ann Neurol 39:724-733.

Wang C, Zhou W, Su G, Hu J, Yang P (2022a) Progranulin suppressed autoimmune uveitis and autoimmune neuroinflammation by inhibiting Th1/Th17 cells and promoting treg cells and M2 macrophages. Neurol Neuroimmunol Neuroinflamm 9:e1133.

Wang L, Li B, Quan MY, Li L, Chen Y, Tan GJ, Zhang J, Liu XP, Guo L (2017) Mechanism of oxidative stress p38MAPK-SGK1 signaling axis in experimental autoimmune encephalomyelitis (EAE). Oncotarget 8:42808-42816.

Wang Q, Song LJ, Ding ZB, Chai Z, Yu JZ, Xiao BG, Ma CG (2022b) Advantages of Rho-associated kinases and their inhibitor fasudil for the treatment of neurodegenerative diseases. Neural Regen Res 17:2623-2631.

Wang Y, Zhang S, Li H, Wang H, Zhang T, Hutchinson MR, Yin H, Wang X (2020) Small-molecule modulators of Toll-like receptors. Acc Chem Res 53:1046-1055.

Wier EM, Fu K, Hodgson A, Sun X, Wan F (2015) Caspase-3 cleaved p65 fragment dampens NF-κB-mediated anti-apoptotic transcription by interfering with the p65/RPS3 interaction. FEBS Lett 589:3581-3587.

Xin YL, Yu JZ, Yang XW, Liu CY, Li YH, Feng L, Chai Z, Yang WF, Wang Q, Jiang WJ, Zhang GX, Xiao BG, Ma CG (2015) FSD-C10: A more promising novel ROCK inhibitor than Fasudil for treatment of CNS autoimmunity. Biosci Rep 35:e00247.

Yan Y, Yu J, Gao Y, Kumar G, Guo M, Zhao Y, Fang Q, Zhang H, Yu J, Jiang Y, Zhang HT, Ma CG (2019) Therapeutic potentials of the Rho kinase inhibitor Fasudil in experimental autoimmune encephalomyelitis and the related mechanisms. Metab Brain Dis 34:377-384.

Yang M, Wang Y, Patel G, Xue Q, Singor Njateng GS, Cai S, Cheng G, Kai G (2020). In vitro and in vivo anti-inflammatory effects of different extracts from Epigynum auritum through down-regulation of NF-κB and MAPK signaling pathways. J Ethnopharmacol 261:113105.

Yeung YT, Aziz F, Guerrero-Castilla A, Arguelles S (2018) Signaling pathways in inflammation and anti-inflammatory therapies. Curr Pharm Des 24:1449-1484.

Yu HZ, Zhu BQ, Zhu L, Li S, Wang LM (2022) NR4A1 agonist cytosporone B attenuates neuroinflammation in a mouse model of multiple sclerosis. Neural Regen Res 17:2765-2770.

Yu JW, Li YH, Song GB, Yu JZ, Liu CY, Liu JC, Zhang HF, Yang WF, Wang Q, Yan YP, Xiao BG, Ma CG (2016) Synergistic and superimposed effect of bone marrow-derived mesenchymal stem cells combined with Fasudil in experimental autoimmune encephalomyelitis. J Mol Neurosci 60:486-497.

Yu JZ, Chen C, Zhang Q, Zhao YF, Feng L, Zhang HF, Meng J, Ma CG, Xiao BG (2016) Changes of synapses in experimental autoimmune encephalomyelitis by using Fasudil. Wound Repair Regen 24:317-327.

Zeng Q, Tang N, Ma Y, Guo H, Zhao Y, Tang R, Yan C, Ouyang S, Langdon WY, Yang H, O'Brien MC, Zhang J (2022) Cbl-b restrains priming of pathogenic Th17 cells via the inhibition of IL-6 production by macrophages. iScience 25:105151.

Zhang HF, Li YH, Yu JZ, Guo MF, Meng J, Liu CY, Xie Y, Feng L, Xiao BG, Ma CG (2013) Rho kinase inhibitor fasudil regulates microglia polarization and function. Neuroimmunomodulation 20:313-322.

Zhao X, Di Q, Liu H, Quan J, Ling J, Zhao Z, Xiao Y, Wu H, Wu Z, Song W, An H, Chen W (2022) MEF2C promotes M1 macrophage polarization and Th1 responses. Cell Mol Immunol 19:540-553.

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