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ORIGINAL RESEARCH

MCC950 Alleviates Experimental Autoimmune Neuritis by Inhibiting NLRP3 Inflammasome Activity and Down-Regulating Interleukin-23/Interleukin-17 Axis Expression

Yi Li[1], Xiaocong Li[1], Tao Gu[2], Li Wang[2], Man Song[2], Liping Yang[2], Qinghua Huang[3], Juan Yang[1]

[1]Department of Neurology, General Hospital of Ningxia Medical University, Yinchuan, People's Republic of China; [2]Ningxia Medical University, Yinchuan, People's Republic of China; [3]Emergency department, General Hospital of Ningxia Medical University, Yinchuan, People's Republic of China

Correspondence: Qinghua Huang, Emergency Department, General Hospital of Ningxia Medical University, Yinchuan, People's Republic of China, Email huang17851618 2@qq.com; Juan Yang, Department of Neurology, General Hospital of Ningxia Medical University, Yinchuan, People's Republic of China, Email yangjuan7@163.com

Objective: Guillain-Barré syndrome (GBS), an autoimmune disease involving the peripheral nervous system, is the most common and severe acute paralytic neuropathy. However, the exact pathogenesis remains unclear. The aim of this study was to reveal the role of the NLRP3 inflammasome in regulating the Interleukin-23/Interleukin-17 axis (IL-23/IL-17 axis) in experimental autoimmune neuritis (EAN) and to explore the potential of the NLRP3 inflammasome as a drug target for the treatment of GBS.

Methods: We first evaluated the expression of NLRP3 inflammasome-related genes in peripheral blood mononuclear cells (PBMCs) of GBS patients using real-time quantitative polymerase chain reaction (qPCR). Subsequently, MCC950, a NLRP3 inflammasome inhibitor, was used to detect its therapeutic effect on EAN rats induced by $P2_{57-71}$ peptide immunization. The expression of NLRP3 inflammasome mRNA in the sciatic nerve was detected by qPCR, and the changes of NLRP3 inflammasome and IL-23/IL-17 axis related proteins were evaluated by Western blotting (WB) and immunofluorescence (IF). The effect of MCC950 on EAN peripheral nerve injury and its potential mechanism were evaluated in multiple dimensions through clinical symptom scoring, neuroelectrophysiological examination and IF.

Results: We observed that the expression of NLRP3 inflammasome related genes was increased in the peripheral blood of patients with GBS. In the EAN rat model, inhibition of NLRP3 inflammasome with MCC950 not only alleviated neurological symptoms, decreased peripheral nerve $CD4^+$ T cell and macrophage infiltration, but also ameliorated peripheral nerve conduction disorders and mitigated myelin loss. Mechanically, the potential protective effect of MCC950 on EAN might realized via inhibiting the NLRP3 inflammasome signaling pathway and down-regulating the expression of IL-23/IL-17 axis.

Conclusion: In the study, we demonstrated that NLRP3 inflammasome is involved in the injury of experimental autoimmune neuritis by up-regulating the expression of IL-23/IL-17 axis. This discovery provides strong evidence for the NLRP3 inflammasome as a drug target for GBS.

Keywords: NLRP3 inflammasome, interleukin-23/interleukin-17 axis, experimental autoimmune neuritis, MCC950

Introduction

Guillain-Barré syndrome (GBS) is an inflammatory demyelinating polyradiculoneuropathy with acute or subacute onset, which is related to infection and immune mechanism. It causes motor dysfunction, sensory dysfunction, and autonomic dysfunction. In severe cases, it can involve the intercostal muscles and diaphragm, resulting in dyspnea and require mechanical ventilation. It is characteristic in cerebrospinal fluid and electrophysiological examination.[1] About 1 to 2 people per 100,000 people suffer from this disease each year.[2] So far, plasma exchange or intravenous immunoglobulin (IVIG) are recognized as effective immunotherapy for GBS, but 20% of patients still have varying degrees of mobility disorders, and 5–10% of patients die.[3,4] Therefore, at present, exploring new treatment methods for GBS is a key issue to be addressed urgently.

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Experimental autoimmune neuritis (EAN) simulates the symptoms, signs, pathological process and electrophysiological characteristics of GBS, which is the most commonly used animal experimental model to study the pathogenesis of GBS and develop new therapies.[5] One of the pathophysiological characteristics of EAN is the breakdown of the blood-nerve barrier, with activated T and macrophage cells invading the peripheral nervous system, leading to neuroinflammation and myelin damage.[1] Thus, neuroinflammation exerts an crucial role in the pathogenesis of GBS.

The inflammasome is a key component of the inflammatory response and consists of a receptor protein, which can be a nucleotide-binding domain and leucine-rich repeat (NLR) protein or a member of the AIM2-like receptor (ALR) family, an adaptor protein, namely, apoptosis-associated speck-like protein containing a CARD (ASC), and an effector protein pro-caspase-1.[6] It can be activated by danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs), and exert its biological effects in regulating inflammatory response.[7,8] The NACHT, leucine-rich repeat (LRR), and pyrin domain (PYD)-containing protein 3 (NLRP3) is currently the most widely studied inflammasome, which plays an important role in the progression of inflammatory bowel disease (IBD),[9] rheumatoid arthritis (RA),[10] systemic lupus erythematosus (SLE)[11] and other autoimmune diseases.[12,13] More importantly, NLRP3 inflammasome is also involved in the inflammatory response of central nervous system diseases including ischemic stroke,[14] subarachnoid hemorrhage,[15] Alzheimer's disease (AD),[16] cerebral ischemia/reperfusion injury[17] and multiple sclerosis (MS).[18] Further, it has been shown that blocking the NLRP3 inflammasome with the application of MCC950 prevents the development of neuronal inflammation and improves motor neuron survival and motor axon regeneration after peripheral nerve suture.[19] Recently, it has also been reported that NLRP3 expression was up-regulated in the sciatic nerve of EAN rats induced by $P0_{180\text{-}199}$ peptide, but in this study, NLRP3 inflammasome was not inhibited.[20]

Interleukin-23 (IL-23) is released by both innate and adaptive immune cells. Interleukin-17 (IL-17) is produced primarily by Th17 cells and is a major regulator of adaptive immunity.[21] Th17 cells activated by IL-23 and producing IL-17, have been shown to contribute to the development of autoimmune diseases.[22] Previous studies have highlighted that the IL-23/IL-17 axis plays a critical role in the pathological process of GBS and its animal model (EAN).[23] A study of experimental autoimmune encephalomyelitis (EAE) has shown that inflammasome plays an important role in the Th17-mediated pathological mechanism of EAE.[24] However, little is known about the role of the NLRP3 inflammasome in regulating the IL-23/IL-17 axis in EAN.

In this study, we used MCC950, a potent and highly selective NLRP3 inflammasome inhibitor, to investigate the effect of NLRP3 inflammasome on peripheral nerve injury in EAN rats and to investigate the underlying molecular mechanisms. We found that MCC950 attenuated neuroinflammatory response and neurological impairment in EAN rat model by regulating IL-23/IL-17 axis through inhibiting NLRP3 inflammasome activation. The finding suggests that targeting the NLRP3 inflammasome may be helpful for the treatment of GBS.

Materials and Methods

Patients and Healthy Control Group

Eleven GBS patients who met the diagnostic criteria were recruited from the General Hospital of Ningxia Medical University.[25] Exclusion criteria were a possible diagnosis of acute chronic inflammatory demyelinating polyneuropathy (CIDP), that is, a second exacerbation after 8 weeks of onset, or three or more exacerbations; MS, IBD, RA, type 1 diabetes, malignant tumors and other autoimmune diseases; peripheral blood was not collected for recent infection or during the acute phase (within 3–5 days from symptom onset to admission) and before treatment with IVIG.[26] Eleven healthy volunteers matched for age and gender were included as controls. All research procedures were conducted according to the ethical guidelines formulated by the Ethics Committee of the General Hospital of Ningxia Medical University (Approval No: 2018-318) and complied with the Declaration of Helsinki. Peripheral blood was collected, PBMC was isolated and qPCR was performed with the consent and signed informed consent of patients and healthy volunteers, as shown in Table 1.

Animals

Eighteen female Lewis rats aged 6 to 8 weeks and weighing 160 to 180 g were purchased from Vital River Company in Beijing, China. Rats were kept under standard, specific, pathogen-free conditions, provided with sufficient water and food, on a 12-h light/dark cycle, and randomly assigned to three groups: Sham, EAN, and MCC950 groups, with six rats

Table 1 Demographics of Patients with GBS and Healthy Controls

Characteristic	HCs (n = 11)	GBS (n = 11)
Demographic characteristics		
Male (%)	9 (82)	9 (82)
Age, median (IQR)	46 (36–61)	50 (44–58)
Precedent infection		
Upper respiratory tract infection (%)	N/A	2 (18)
Diarrhea (%)	N/A	1 (9)
Other (%)	N/A	1 (9)
None (%)	N/A	7 (64)
GBS disability scale scores		
1	N/A	3 (27.3)
2	N/A	2 (18.2)
3	N/A	1 (9)
4	N/A	3 (27.3)
Sensory deficits (%)	N/A	2 (18.2)
Result of CSF analysis		
Median protein level (g/liter, IQR)	N/A	0.74 (0.4–0.77)
Increased protein level (%)	N/A	6 (54.5)
Median white-cell count per mm^3 (IQR)	N/A	5 (2–5)

in each group. All the animal protocols adopted in this research have been reviewed and approved by the Ethics Committee of Ningxia Medical University. All animals were cared for in accordance with the guidelines outlined in the "Laboratory Animal-Guideline for ethical review for animal welfare" (IACUC-NYLAC-2022-055).

Induction of EAN and Evaluation of Clinical Scores

Referring to a previous research,[27] using 200 μL of emulsifier, namely: The $P2_{57-81}$ peptide (Hangzhou Dangang Biotechnology Co., China) was dissolved in phosphate buffered saline (PBS), emulsified with an equal volume of incomplete Freund's adjuvant (Sigma-Aldrich, F5506, USA) containing Mycobacterium tuberculosis H37RA (BD Difco, ATCC 25177, USA), and then injected subcutaneously into the tail roots of rats. As a control, the Sham group received a subcutaneous injection of the same amount of emulsifier without $P2_{57-81}$ peptide.

Two researchers assessed the rats for neurological symptoms using the following scales at the same time each day after immunization: 0 (normal state), 1 (decreased tail tension), 2 (partial tail paralysis), 3 (complete tail paralysis or disappearance of the righting reflex), 4 (gait ataxia), 5 (mild hind limb paralysis), 6 (moderate hind limb paralysis), 7 (severe hind limb paralysis), 8 (quadriplegia), 9 (near-death state), and 10 (death).

Treatment Protocol

MCC950 (Purity > 95%, MedChemExpress, HY-12815A, USA) was dissolved in normal saline. Intraperitoneal injection of MCC950 (10 mg/kg)[28] was performed every other day from the day of neurological symptoms (day 10 post-immunization) until neurological symptoms began to recover (day 24 post-immunization). The Sham and EAN groups were given an equal volume of normal saline.

Histopathology

At the end of the experiment, sciatic nerves of rats in each group were collected and cut into 4 μm longitudinal slices after fixation, dehydration and paraffin embedding. Hematoxylin-eosin (HE) (Solarbio Science & Technology, G1005-500ML, China) staining was utilized to detect the infiltration of inflammatory cells in the sciatic nerve. The specific steps are as follows: the sections are deparaffinized and rehydrated, then placed in hematoxylin staining solution for 5min, differentiated for 30s with 1% hydrochloric acid ethanol, rinsed with running water, followed by eosin staining for 3min, then dehydrated with gradient alcohol, transparent with xylene, and sealed with neutral glue. Four sciatic nerve sections

were randomly selected from each group and observed under an Olympus DP80 microscope. The number of inflammatory cell infiltrates per square millimeter was calculated from four random microscopic views.

Next, luxol fast blue (LFB) (Solarbio Science & Technology, G1030-100ML, China) staining was used to evaluate the degree of demyelination of the sciatic nerve. Briefly, the steps of deparaffinized and rehydrated were the same as those previously mentioned for HE staining. The sections were placed in preheated myelin staining solution A overnight at room temperature, washed with 95% ethanol and then rinsed with distilled water, followed by myelin staining solution B for 15s, subsequently, 70% ethanol for 30s and immediately rinsed with distilled water, myelin staining solution C for 30–40s, rinsed with distilled water, and then dehydrated with gradient alcohol, transparent with xylene, and sealed with neutral glue. The sections viewed and photographs were taken under an Olympus DP80 microscope. Demyelination appeared as light blue staining or no blue staining (no staining).

Immunofluorescence Staining

The paraffin sections of sciatic nerve tissue were prepared as above. After being dewaxed, rehydrated and antigen repaired, the sections were incubated with 0.2% Triton-X 100 at room temperature for 30min, and then blocked with 5% goat serum for 60 min. Then, the following antibodies were incubated at 4°C overnight: NLRP3 (1:100, Proteintech, 19771-1-AP, China), ASC (1:200, Bioss, bs-6741R, China), $CD4^+$ T (1:100, Novus, NBP1-19371, USA), CD68 (1:200, Servicebio, GB113109-100, China) and MBP (1:200, Cell Signaling Technology, 78896S, USA). Next, after washing the sections with PBS, they were incubated with goat anti-rabbit IgG-FITC (1:100, Absin, abs20004, China) or Alexa Fluor 594-conjugated IgG (1:100, Jackson ImmunoResearch, 163359, USA) for 1 hour at room temperature under dark conditions, and then stained with 4′,6-diamidino-2-phenylinyl (DAPI). The sections were placed under a fluorescence microscope (Olympus DP80) to observe and collect images, and the Image J software was used for analysis.

qPCR Analysis

Total RNA was extracted from human PBMCs and EAN rat sciatic nerve tissue by Trizol method. To be specific, 1mL Trizol (Theromfisher Scientific, 15596016CN, USA) was added to the extracted PBMC and the ground sciatic nerve tissue, 200μL chloroform was added according to the ratio of 5:1, thoroughly mixed, left for 10min, centrifuged at 12000 rpm at 4°C for 20 min, then the supernatant was aspirated, and the equal volume of isopropanol was added and centrifuged again, followed by washed with 75% ethanol, and finally dissolved in enzyme-free water. Next, the RNA concentration and purity were determined using a NanoDrop2000 microspectrophotometer (Theromfisher Scientific, USA). Subsequently, cDNA synthesis was carried out in accordance with the instructions of the Revert Aid First Strand cDNA Synthesis Kit (Theromfisher Scientific, K1622, USA). Finally, qPCR was performed on a real-time fluorescent quantitative PCR instrument (Qtower3G, Germany) using TB Green Premix Ex Taq II kit (TaKaRa, RR820A, Japan). The reaction conditions were as follows: predenaturation at 95°C 1min for 1 cycle, followed by 40 cycles consisting of 10s of denaturation at 95 °C and 30s of annealing extension at 60 °C.[29,30] The dissolution curve generation conditions: 95°C for 5s, 60°C for 1min, and 95°C for 1min. The primers used are listed in Table 2. GAPDH was used as a reference gene for relative gene expression normalization and quantified analyzed by $2^{-\Delta\Delta Ct}$ method.

Western Blot

The sciatic nerves of rats in each group were lysed in RIPA buffer (keyGEN, KGP250/KGP2100, China) containing protein phosphatase inhibitor mixture, then total protein was extracted by centrifugation, and then protein quantification and denaturation were performed. Proteins were isolated using 10% or 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). It was then transferred to polyvinylidene difluoride membranes (Millipore, USA) and blocked with 5% skim milk powder solution for another 90min. Subsequently, the membranes were incubated overnight at 4 °C with the following primary antibodies: NLRP3 (1:500, Wanleibio, WL02635, China), ASC (1:500, Wanleibio, WL02462, China), cleaved-caspase-1 (1:500, Wanleibio, WL02996a, China), IL-1β (1:500, Wanleibio, WL02635, China), IL-18 (1:500, Wanleibio, WL01127, China), IL-17A (1:500, Affinity, DF6127, China), IL-23 (1:500, Wanleibio, WL01655, China), GAPDH (1:50000, Proteintech, 60004-1-AP, China), β-actin (1:20000, Proteintech, 20536-1-AP, China) and β-tubulin (1:5000, Affinity, AF7011, China). Next, after washing the membranes, the membranes were incubated with horseradish

Table 2 Primers Sequence for qPCR

Gene	Organism	Forward (5′–3′)	Reverse (5′–3′)
NLRP3	Homo sapiens	GGAGAGACCTTTATGAGAAAGCAA	GCTGTCTTCCTGGCATATCACA
ASC	Homo sapiens	AGTTCAAGCTGAAGCTGCTGTCG	CGCCGTAGGTCTCCAGGTAGAAG
Caspase-1	Homo sapiens	CCGAAGGTGATCATCATCCA	CTCCTCAGTCGGCAGCCAAG
GAPDH	Homo sapiens	CAGGAGGCATTGCTGATGAT	GAAGGCTGGGGCTCATTT
NLRP3	Rat	ACCTCAACAGACGCTACACCC	GCCTGTCCCACATCTTAGTCCT
ASC	Rat	ATGGTTTGCTGGATGCTCTGTATGG	AAGGAACAAGTTCTTGCAGGTCAGG
Caspase-1	Rat	AAACACCCACTCGTACACGTCTTG	AGGTCAACATCAGCTCCGACTCTC
IL-1β	Rat	TACATCAGCACCTCTCAAGC	GTCAACTATGTCCCGACCA
IL-18	Rat	CGACCGAACAGCCAACGAATCC	GTCACAGCCAGTCCTCTTACTTCAC
IL-17A	Rat	TGCCTGATGCTGTTGCTGCTAC	GCGTTTGGACACACTGAACTTTGAG
IL-23	Rat	ATACTCCTGTGGCTGTTGCC	GTGCAGAGATTCCGGGAGAG
GAPDH	Rat	GACATGCCGCCTGGAGAAAC	AGCCCAGGATGCCCTTTAGT

peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse secondary antibodies (1:10000, Absin, abs20040ss/abs20001, China) for 60 min at room temperature, and then the protein bands were exposed using an ultrasensitively multi-functional imager, the images were saved and analyzed with Image J software.

Electrophysiologic

At the end of the experiment, the sciatic nerves of rats in each group was examined by electromyography using the Haishen Electromyography (Shanghai, China). To put it simply, the rats were anesthetised to fully expose the sciatic nerve, then, the recording needle electrode was inserted into the gastrocnemius muscle of one side of the hind limb of the rats, the stimulation needle electrode was lightly touched the spine side of the sciatic nerve on the same side and the sciatic nerve notch respectively. The stimulation was given to generate compound muscle action potential, when the waveform was repeated stably for more than three times, the waveform was recorded, and the distance between the two stimulation points was measured and recorded to obtain the conduction velocity, amplitude and end latency of the sciatic nerve.

Statistical Analysis

All experimental data were presented as mean±standard deviation (SD) and processed using GraphPad Prism 9.5.0 software. Differences between the two groups were compared by the two-tailed unpaired Student's *t*-test, and the differences among multiple groups were analyzed using the One-or Two-way ANOVA followed by Tukey's post hoc test. A p less than 0.05 was considered statistically significant.

Results

NLRP3 Inflammasome Expression Increased in PBMC from Patients with GBS

To clarify the potential role of inflammasome-related genes in GBS, we detected the expression of NLRP3 inflammasome-related genes in peripheral blood immune cells of patients with GBS by qPCR. Compared with healthy controls (HC), mRNA expressions of NLRP3, ASC and caspase-1 in PBMCs of GBS patients were up-regulated (Figure 1).

MCC950 Inhibited NLRP3 Inflammasome Activation and Downstream Inflammatory Factor Expression in EAN

Previous studies have demonstrated that MCC950 was able to selectively inhibit NLRP3 inflammasome activity in EAE mice,[28] but its effect on the NLRP3 inflammasome signaling in EAN remains unclear. qPCR analysis showed that MCC950 inhibited the expression of NLRP3 inflammasome (NLRP3, ASC and caspase-1) genes in EAN rats' sciatic nerve (Figure 2A–C), and also decreased the mRNA expression of its downstream inflammatory factors (IL-1β and IL-18) (Figure 2D and E). Next, the protein levels of NLRP3 inflammasome and downstream inflammatory factors in the sciatic nerve were detected using Western blot. The

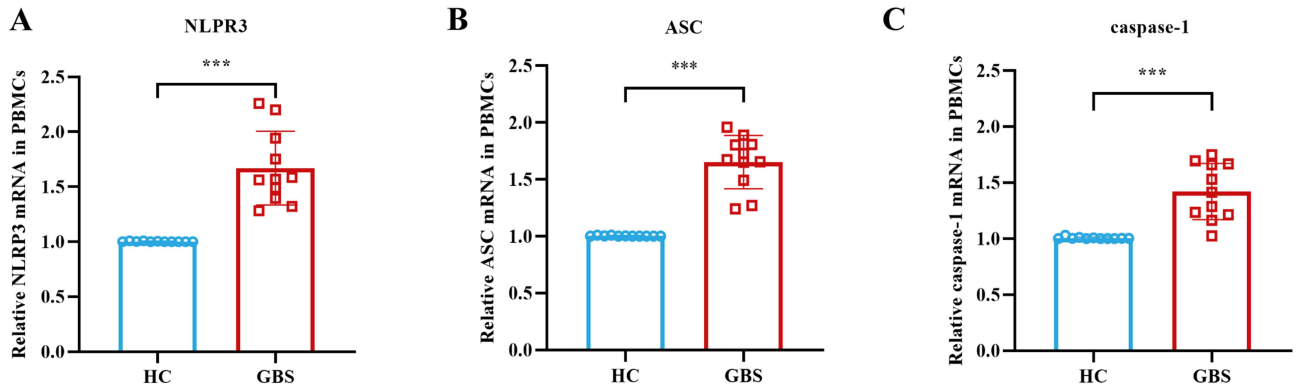

Figure 1 The expression of NLRP3 inflammasome-related genes in the peripheral blood of patients with GBS is increased. mRNA expression levels of NLRP3 (**A**), ASC (**B**) and caspase-1 (**C**) in PBMCs of GBS patients and HC. Result are denoted as mean ± SD. ***P < 0.001.

data showed that NLRP3, ASC, caspase-1 and IL-1β, IL-18 expression were increased in the EAN group compared to the Sham group, but these effects were inhibited by MCC950 treatment compared with the EAN group (Figure 2F–L). Further, we carried out immunofluorescence staining of NLRP3 and ASC. The results displayed that NLRP3 and ASC were rarely expressed in the Sham group, which was increased in the EAN group, while decreased in the MCC950 group, further confirming the results of Western blotting (Figure 3A–D). Collectively, these results indicated that MCC950 treatment inhibited the downstream signaling pathway following NLRP3 inflammasome activation in EAN.

NLRP3 Inflammasome Inhibition by MCC950 Alleviated Neurological Symptoms and Reduced $CD4^+$ T Cell and Macrophage Infiltration in EAN

To examination the role of NLRP3 inflammasome in EAN, the rats were treated with intraperitoneal injection of 10mg/kg MCC950 at the onset of neurological symptoms (day 10 after immunization) every other day until neurological symptoms began to recover (day 24 after immunization), and changes in neurological symptoms were observed every day. We found that MCC950 reduced the severity of EAN (Figure 4A). Correspondingly, the rats that received intraperitoneal injection of MCC950 had an improvement in the cumulative neurologic symptom score (Figure 4B). Furthermore, HE staining was utilized to observe the infiltration of sciatic nerve inflammatory cells in each group in order to determine whether the improvement of neurological symptoms in rats was related to inflammatory response. The results showed an increase in inflammatory cell counts per square millimeter in the EAN group and a decrease in inflammatory cell counts per square millimeter in the MCC950-treated group compared with the Sham group (Figure 4C and D). Next, we evaluated the expression of $CD4^+$ T cells and macrophages in the rat sciatic nerve by immunofluorescence staining. As shown in Figure 4E–H, the expression of $CD4^+$ T cells and CD68-positive macrophages was increased in the sciatic nerve of the EAN group, as expected, was decreased by MCC950 treatment. All data demonstrated that MCC950 treatment ameliorated neurologic symptoms and decreased $CD4^+$ T cell and macrophage infiltration in EAN rats.

NLRP3 Inflammasome Inhibition by MCC950 Improved Peripheral Nerve Conduction Disorders and Attenuated Demyelination in EAN

At the end of the experiment, neuroelectrophysiological studies were performed on bilateral sciatic nerves to determine whether MCC950 had a neuroprotective effect on EAN-induced peripheral nerve injury (Figure 5A). Neuroelectrophysiological examination results reveled that EAN rats manifested reduced nerve conduction velocity and amplitude compared with those in the Sham group, and the inhibition of NLRP3 inflammasome by MCC950 effectively reversed the decline (Figure 5B and C), while there was no difference in end latency between all groups (Figure 5D). Subsequently, LFB staining was applied to observe sciatic nerve myelin loss in each group. We found that sciatic nerve myelin appeared a uniform and consistent blue color in the Sham group, light blue or uncolored in the EAN group, and less severe demyelination in the MCC950 group (Figure 5E). Immediately afterwards, we applied

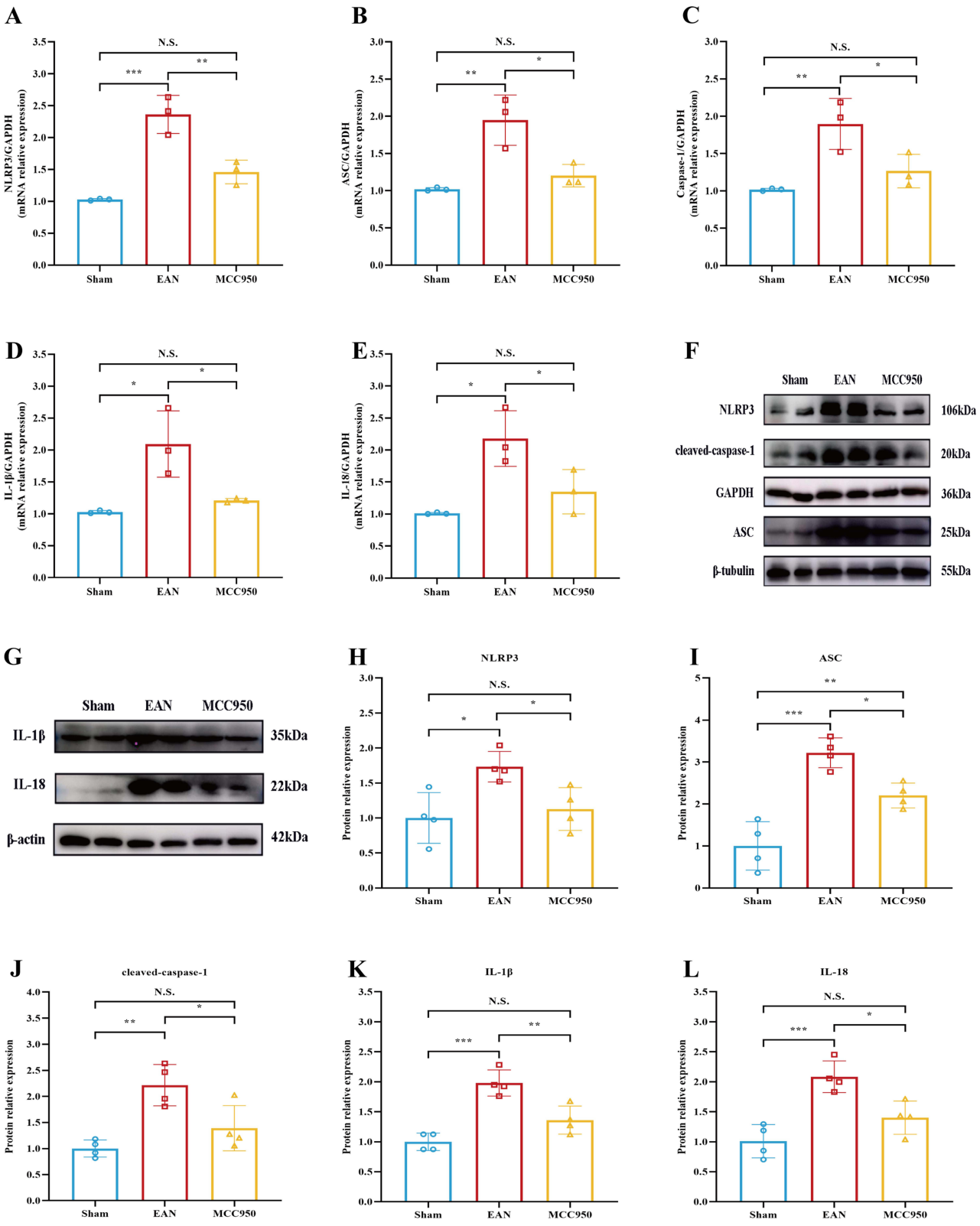

Figure 2 MCC950 inhibited the activation of NLRP3 inflammasome and the expression of downstream inflammatory factors in EAN. (**A–E**) Relative levels of the NLRP3 inflammasome and downstream inflammatory factors mRNA expression (n = 3 rat per group). (**F** and **G**) Representative Western blot bands of the NLRP3 inflammasome and downstream inflammatory factors in the sciatic nerve. (**H–L**) Quantitative analysis of NLRP3 inflammasome and downstream inflammatory factor proteins (n = 4 rats/group). Result are denoted as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 and N.S. denotes Not Significant.

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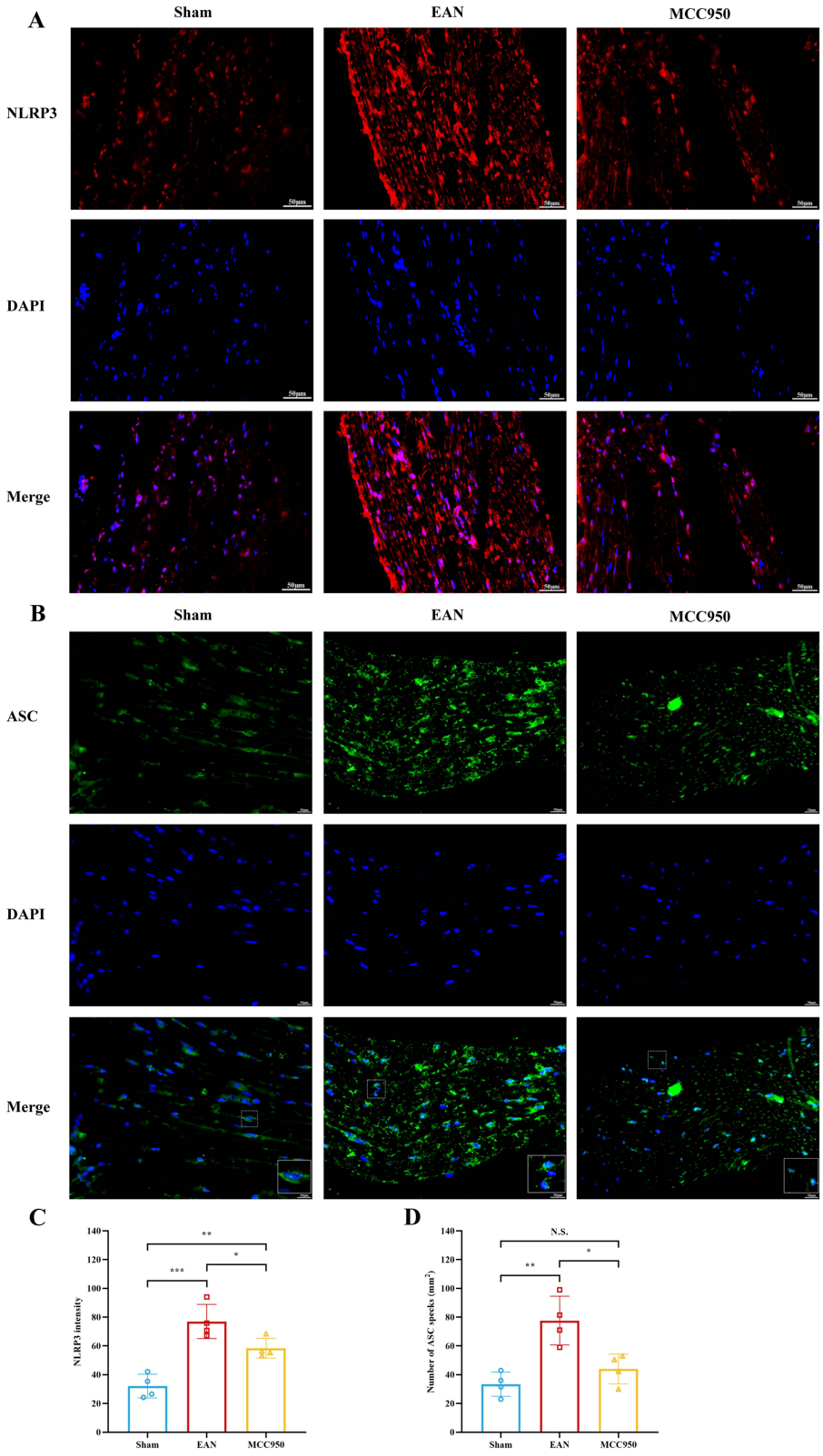

Figure 3 MCC950 reduced NLRP3 inflammasome activation in EAN. (**A**) Representative micrograph of sciatic nerve sections stained with NLPR3 by immunofluorescence. Scale bar = 50 μm. (**B**) Representative micrograph of sciatic nerve sections stained with ASC by immunofluorescence. Scale bar = 50 μm. (**C**) Quantitative analysis of NLRP3 fluorescence intensity (n = 4 rat per group). (**D**) Quantitative analysis of the number of ASC specks (n = 4 rat per group). Result are denoted as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 and N.S. represents Not Significant.

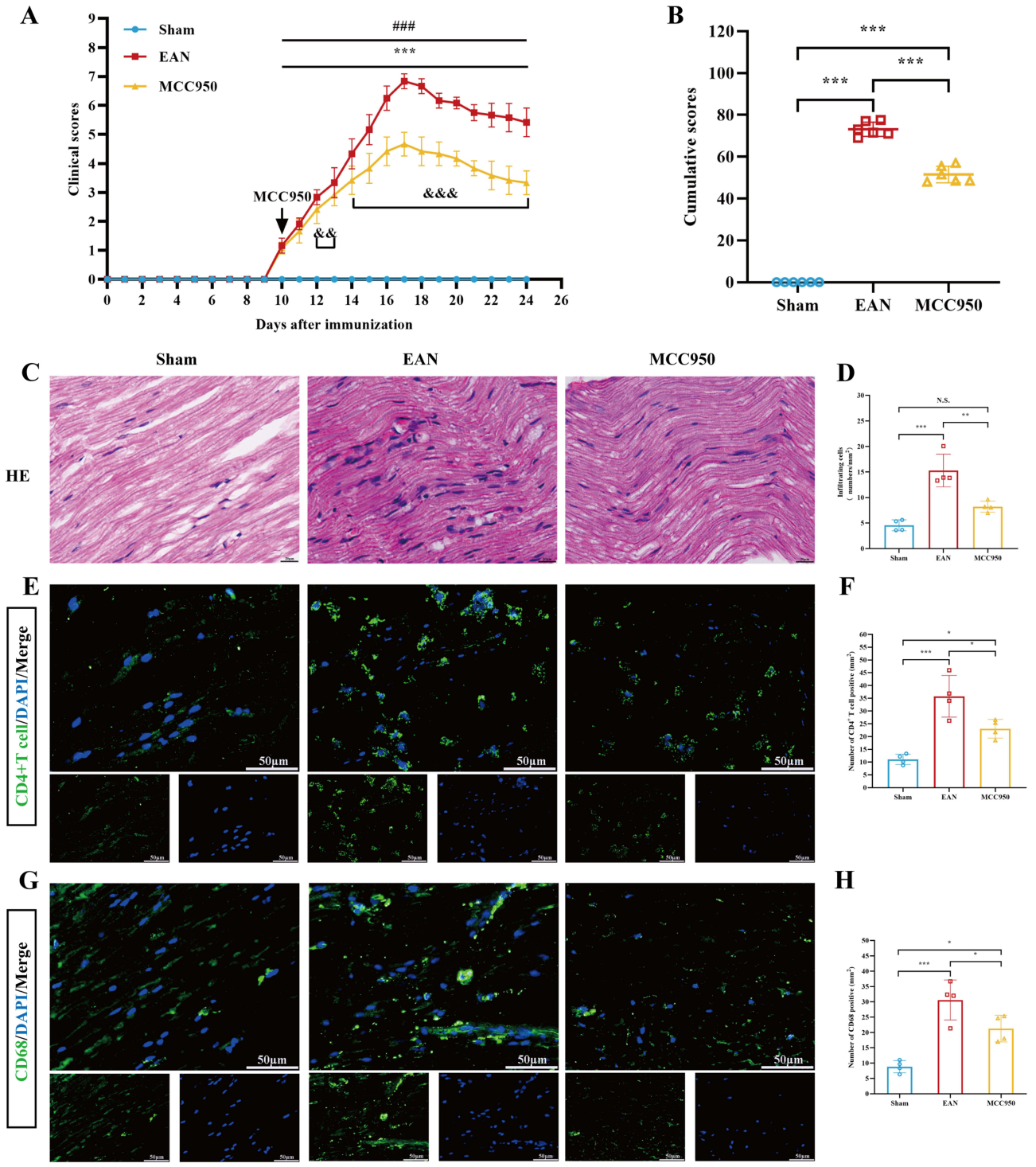

Figure 4 Inhibition of NLRP3 inflammasome by MCC950 mitigated neurological symptoms and decreased $CD4^+$ T cell and macrophage infiltration in EAN. (**A**) The clinical scores of rats in each group from day 0 to day 24 (n = 6 rat per group). (**B**) Cumulative clinical scores for each rat after immunization (n = 6 rat per group). (**C**) Representative images of sciatic nerve sections stained with HE. Scale bar = 20 μm. (**D**) The mean number of inflammatory cells per mm^2 of sciatic nerve section (n = 4 rats/group). (**E**) Representative micrograph of sciatic nerve sections stained with $CD4^+$ T cell by immunofluorescence. Scale bar = 50 μm. (**F**) Quantitative analysis of $CD4^+$ T-positive cells (n = 4 rat per group). (**G**) Representative micrograph of sciatic nerve sections stained with macrophages CD68 by immunofluorescence. Scale bar = 50 μm. (**H**) Quantitative analysis of CD68-positive macrophages (n = 4 rat per group). Result are denoted as mean ± SD. The Sham group versus the EAN group: $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$; The Sham group versus the MCC950 group: $^{\#\#\#}P<0.001$; The EAN group versus the MCC950 group: $^{\&\&}P<0.01$, $^{\&\&\&}P<0.001$.

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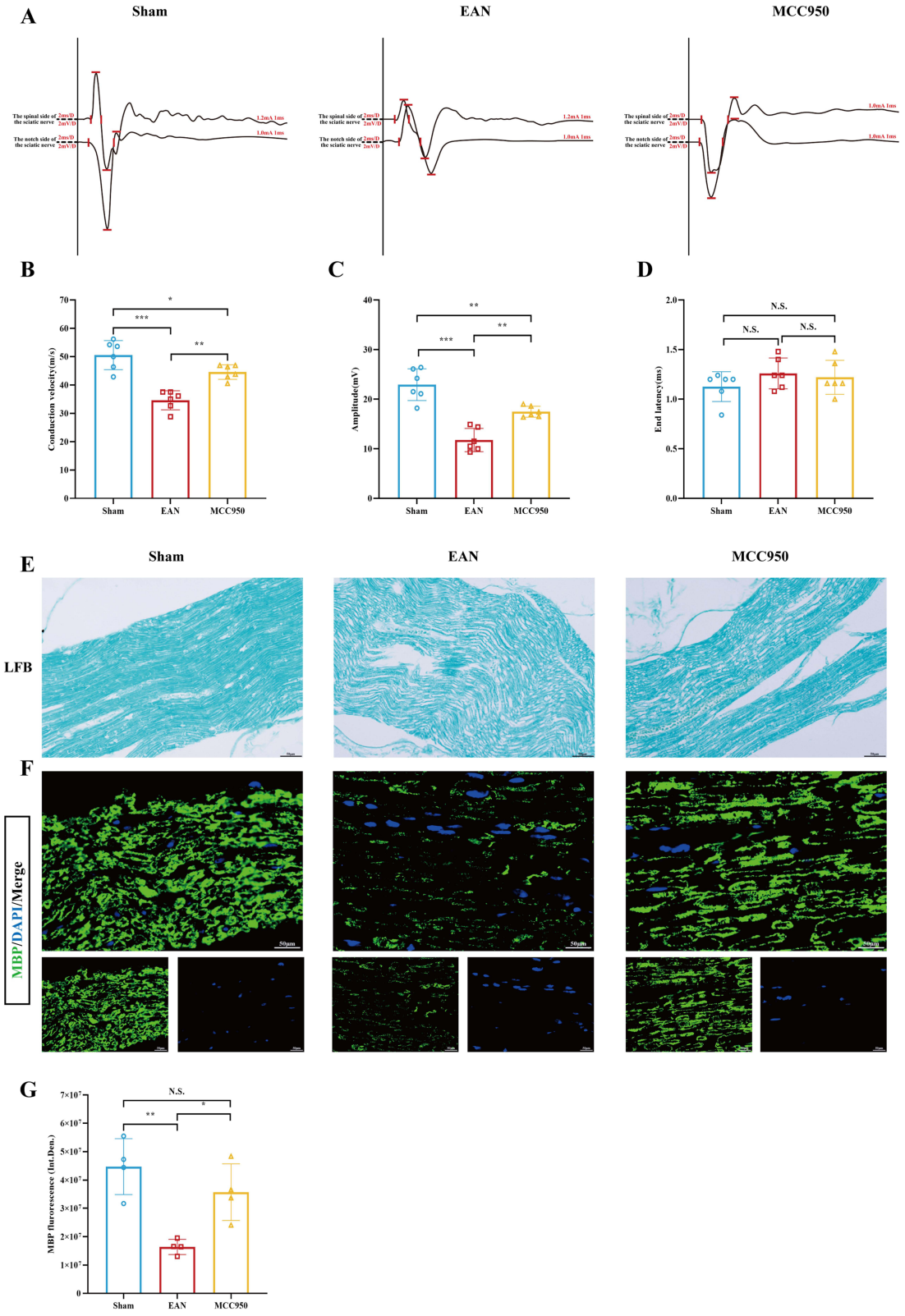

Figure 5 Inhibition of NLRP3 inflammasome by MCC950 ameliorated peripheral nerve conduction deficits and mitigated myelin loss in EAN. (**A**) Representative electrophysiological waveforms of sciatic nerve in each group. (**B**–**D**) Statistical analysis of sciatic nerve conduction velocity, amplitude and end latency in each group (n = 6 rat per group). (**E**) Representative images of sciatic nerve sections stained with LFB. Scale bar = 50 μm. (**F**) Representative micrograph of sciatic nerve sections stained with MBP by immunofluorescence. Scale bar = 50 μm. (**G**) Quantitative analysis of the intensity of MBP fluorescence (n = 4 rat per group). Result are denoted as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 and N.S. represents Not Significant.

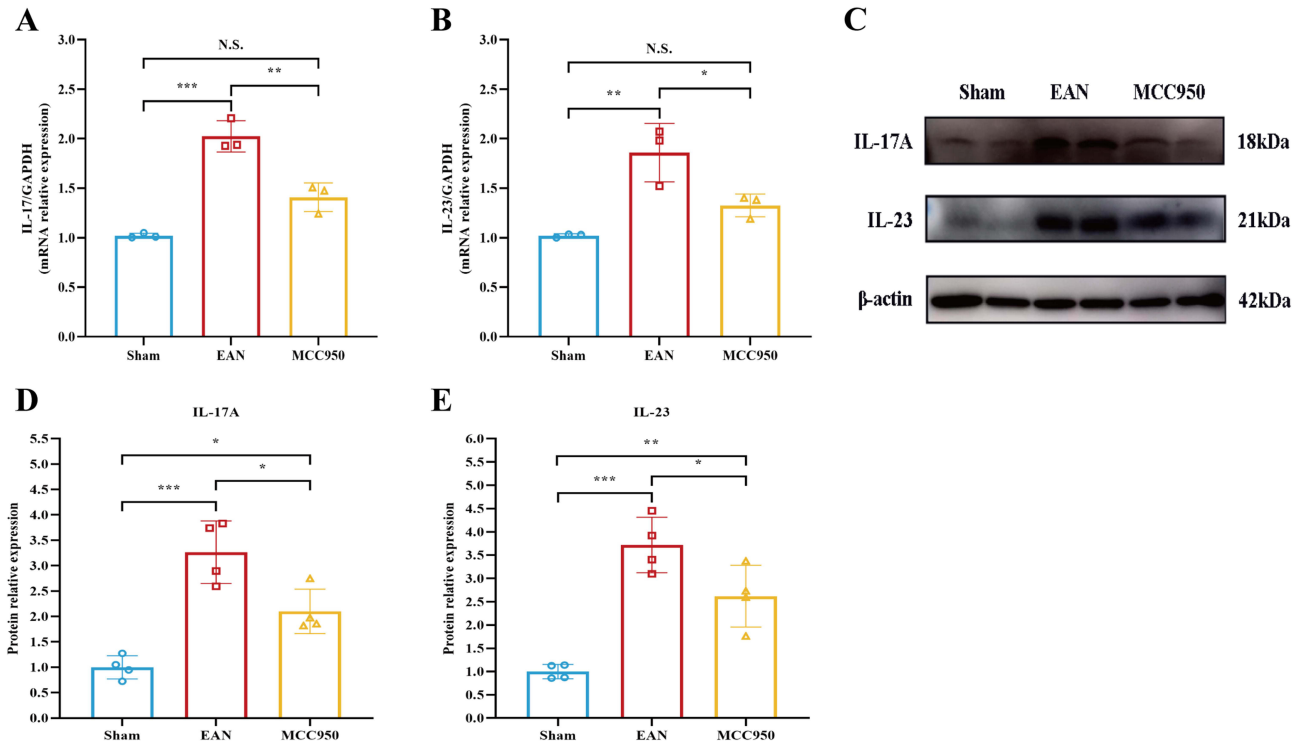

Figure 6 Inhibition of NLRP3 inflammasome by MCC950 declined IL-23/IL-17 axis expression in EAN. (**A** and **B**) Relative levels of the IL-23/IL-17 axis mRNA expression (n = 3 rats/group). (**C**) Representative Western blot bands of the IL-23/IL-17 axis in the sciatic nerve. (**D** and **E**) Quantitative analysis of IL-23/IL-17 axis proteins (n = 4 rats/group). Result are denoted as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 and N.S. represents Not Significant.

immunofluorescence to confirm the expression level of myelin marker MBP in sciatic nerves of each group, which further verified the results of LFB staining (Figure 5F and G). Altogether, these findings suggested that NLRP3-inflammasome inhibition by MCC950 ameliorated peripheral nerve conduction deficits and mitigated myelin loss in EAN.

NLRP3 Inflammasome Inhibition by MCC950 Decreased the Expression of IL-23/IL-17 Axis in EAN

Next, to explore the underlying neuroprotective mechanism behind the inhibition of NLRP3 inflammasome by MCC950, we examined the expression changes of IL-23/IL-17 axis genes in the sciatic nerve using qPCR. The results showed that compared with Sham group, mRNA expression levels of IL-23 and IL-17A were increased in EAN group, while those of MCC950 treatment were down-regulated (Figure 6A and B). More importantly, the expression levels of IL-23 and IL-17A in the sciatic nerves of each group were determined by Western blot, which further verified the results of qPCR (Figure 6C–E). Taken together, these results indicated that the inhibition of NLRP3 inflammasome by MCC950 might exert a neuroprotective effect by regulating the IL-23/IL-17 axis.

Discussion

Despite immunotherapy, some patients with GBS remain suffer from severe disability or death. Although there is evidence that inhibition or regulation of the NLRP3 inflammasome might help protect against neurological impairment, the exact role of the NLRP3 inflammasome in GBS and its possible mechanisms still unclear. Our study found that NLRP3 inflammasome (NLRP3, ASC and caspase-1) mRNA expression levels in the PBMC of GBS patients were higher than those of HCs. Treatment with MCC950 mitigated EAN severity, reduced $CD4^{+}$T cells and macrophages infiltration, improved peripheral nerve conduction dysfunctions and alleviated demyelination, which might be achieved

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by attenuating the sciatic nerve inflammatory cascade triggered by NLRP3 inflammasome activation affecting the expression of the IL-23/IL-17 axis.

Several studies have shown that there are abnormal signs of inflammatory biomarkers in the serum or cerebrospinal fluid of patients with GBS.[26,31–33] Nevertheless, it remains unclear which signaling pathways play a dominant role in triggering the inflammatory response in GBS. The NLRP3 inflammasome, which consists of NLRP3, ASC and caspase-1, has been shown to play a key role in coordinating the inflammatory response process.[34] Specifically, the caspase-1 precursor is cleaved into activated caspase-1 under the activation of the NLRP3 inflammasome, which activates and promotes the release of IL-1β and IL-18, mediating the inflammatory cascade reaction.[35,36] Activation of NLRP3 inflammasome has been found in a study of paclitaxel-induced peripheral neuropathic pain.[37] Furthermore, inhibition of NLRP3 inflammasome has been reported to alleviate neuropathic pain in multiple sclerosis.[38] A recent study showed that the NLRP3 inflammasome is activated when the sciatic nerve is damaged, and NLRP3 inflammasome expression was significantly reduced after knocking down the NLRP3 gene, which was also found to decrease inflammatory response.[39] In our study, we observed elevated mRNA expression of the NLRP3 inflammasome (NLRP3, ASC and caspase-1) in PBMCs of patients with GBS. We also found convincing evidence that the EAN rat model exhibited NLRP3 inflammasome activation and increased expression of downstream inflammatory factors (IL-1β and IL-18), and as expected, inhibition of NLRP3 inflammasome activation was accompanied by a concomitant decrease in IL-1β and IL-18 expression after MCC950 treatment. This suggests that MCC950 attenuates the downstream inflammatory response mediated by the NLRP3 inflammasome.

As indicated by the neurological symptom score, MCC950 treatment delayed the occurrence of neurological symptoms in EAN rats and also alleviated the severity of neurological symptoms in EAN rats. It is well known that the pathological features of EAN are inflammatory cell infiltration and loss of nerve myelin.[5] Inflammatory reaction aggravates peripheral nerve damage, and myelin loss slows down nerve conduction velocity and even decreases amplitude. In the present study, we observed less inflammatory cell counts in the sciatic nerve in the MCC950 treatment group than in the EAN model group. Furthermore, immunofluorescence examination of the expression of $CD4^+$T cells and macrophage marker CD68 revealed that the expression of $CD4^+$ T cells and CD68 was up-regulated in the EAN group, while MCC950 treatment inhibited this trend. In addition, our electrophysiological results showed that the nerve conduction velocity and amplitude were slowed down in the EAN model, while the nerve conduction velocity and amplitude were increased in the MCC950 treatment group, suggesting that MCC950 inhibition of NLRP3 inflammasome could improve the nerve conduction deficit. At the same time, LFB staining of sciatic nerve in EAN model group showed obvious demyelination, which could be alleviated by MCC950 treatment. MBP is known to be a key component of the myelin, essential for maintaining the structure and function of nerve fibers, and is expressed in a variety of neurological diseases.[40,41] In peripheral nerve injury, decreased MBP expression may be closely related to inflammation mediated myelin injury.[42] In the present study, MBP expression was decreased in the EAN group rats, and this trend was reversed by MCC950 treatment, indicating that MCC950 treatment protects against myelin injury. In conclusion, our findings support that MCC950 protects EAN rats from injury by inhibiting the downstream inflammatory response mediated by NLRP3 inflammasome and reducing the infiltration of inflammatory cells, but further studies are needed to elucidate the molecular mechanism behind the neuroprotective effect.

IL-23 is a growth factor that protects against bacterial and fungal infections. A growing body of research confirms that altered expression of IL-23 exacerbates autoimmune diseases, including EAE, collagen-induced arthritis, inflammatory bowel disease, and psoriasis.[43,44] IL-23 has been proven to be an upstream cytokine driving IL-17 production, forming the IL-23/IL-17 axis.[45] MCC950 has been reported to reduce the activation of the IL-23/IL-17 axis by inhibiting the NLRP3 inflammasome signaling pathway to alleviate neural ischemia-reperfusion injury.[46] However, its role in immune peripheral neuropathy such as GBS remains unclear. The results of the present study confirmed that the protein expression of IL-23 and IL-17 in the sciatic nerve of EAN group increased, while the IL-23/IL-17 axis in the sciatic nerve of rats treated with MCC950 decreased. Previous studies have shown that IL-23 production depends in part on the NLRP3 inflammasome signaling pathway.[47] It has also been reported that caspase-1 inhibitors can inhibit the production of IL-17 in EAE, thereby alleviating its clinical symptoms.[48] Based on our results, we speculate that MCC950 may inhibit the activation of NLRP3 inflammasome, reduce the

expression of IL-1β and IL-18, change the expression of IL-23, and then affect the activation of IL-17, thereby alleviate neuroinflammatory response and myelin injury, and ultimately play a protective role in peripheral nerves.

There are several limitations to this study. First, we explored the role of NLRR3 inflammasome in EAN studies using MCC950, actually, a more reliable way would be to use NLRP3 gene knockout rats. Second, our study lacked a positive control group, but not all patients with GBS respond to immunotherapy, so this limitation cannot be avoided in most studies of in vivo experiments. Finally, the pathogenesis of GBS is complex, and this study only focused on one of the signaling pathways, and other potential molecular mechanisms may also play a role.

Conclusion

To summarize, MCC950 may play a neuroprotective role in EAN by inhibiting the activation of NLRP3 inflammasome and reducing the expression of IL-1β and IL-18, affecting the IL-23/IL-17 axis to alleviate peripheral neuroinflammation and myelin injury. These findings provide additional laboratory evidence for potential treatment strategies for GBS.

Data Sharing Statement

Data and analysis can be reasonably requested from the corresponding author.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors have declared that no competing interests exists in this work.

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