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Original Research

HMGB1 Mediates Microglia-Astrocyte/Neuron Crosstalk and Pyroptosis by the TLR4/NF-κB Pathway in Multiple Sclerosis

Yang Feng[1], Liang Wang[2], Zhuofeng Mao[2], Weiping Wang[2,*]

[1]Neurology Department, The First Hospital of Hebei Medical University, 050017 Shijiazhuang, Hebei, China
[2]Neurology Department, The Second Hospital of Hebei Medical University, 050000 Shijiazhuang, Hebei, China
*Correspondence: wangweiping@hebmu.edu.cn (Weiping Wang)

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Abstract

Background: Multiple sclerosis (MS) is characterized as a chronic inflammatory autoimmune disorder affecting the central nervous system (CNS). Prior research has explored the involvement of pyroptosis and high mobility group box 1 (HMGB1) in the pathophysiology of MS. Nevertheless, the underlying pathogenic mechanisms and their interactions have yet to be fully elucidated. **Methods**: Myelin oligodendrocyte glycoprotein (MOG)35-55-treated mice and BV-2 microglial cells were utilized as a model for MS. Subsequently, these subjects were transfected with lentiviral vectors that express short hairpin RNA targeting HMGB1. HT-22 cells and Ma-c cells were exposed to conditioned medium (CM) derived from BV-2 cells following treatment. The levels of HMGB1, tumor necrosis factor (TNF)-α, and interleukin-1β (IL-1β) were quantified using enzyme-linked immunosorbent assay (ELISA). Additionally, western blot (WB) analysis was performed to further elucidate the mechanisms involved. **Results**: Mice treated with MOG35-55 (experimental autoimmune encephalomyelitis, EAE) exhibited reduced body weights and significant nerve function impairment ($p < 0.001$), accompanied by increased activation of microglia within the CNS ($p < 0.05$). Additionally, the secretion of HMGB1 was found to be upregulated in the MS cell model ($p < 0.05$), and CM from these cells induced the release of pro-inflammatory cytokines in HT-22 and Ma-c cell lines ($p < 0.001$). Notably, the modulation of HMGB1 and NOD-like receptor family pyrin domain containing 3 (NLRP3) expression was shown to mitigate the release of pro-inflammatory cytokines ($p < 0.01$), TUNEL-positive cells ($p < 0.01$) in both HT-22 cells and Ma-c cells, which were induced by CM from BV-2 cells treated with MOG35-55. Furthermore, WB analysis indicated that the suppression of HMGB1 expression can inhibit the activation of the toll-like receptor 4 (TLR4)/nuclear factor-kappa B (NF-κB) signaling pathway, as well as pyroptosis in EAE mice and HT-22/ Ma-c cells exposed to CM from BV-2 cells ($p < 0.05$). **Conclusion**: HMGB1 has the potential to act as a promoter of MS through the activation of TLR4/NF-κB signaling pathway and the induction of pyroptosis in microglial and other cells. Consequently, the modulation of HMGB1 may represent a novel therapeutic strategy for the management of MS.

Keywords: multiple sclerosis; HMGB1; inflammation; pyroptosis; neuroimmune interaction

1. Introduction

Multiple sclerosis (MS) is a chronic, autoimmune-mediated and demyelinating disorder characterized by a neuroinflammatory process that impacts the central nervous system (CNS). It is estimated that over 2.1 million individuals globally are affected by MS, which stands as the primary cause of neurological disability among young and middle-aged adults [1]. Pathologically, MS is marked by extensive demyelinated lesions in the brain, accompanied by the infiltration of inflammatory immune cells, including microglia and macrophages. Additionally, there is notable proliferation of astrocytes and the synthesis of glial fibers [2]. Although the exact etiology of MS remains elusive, numerous studies indicate that neural network dysfunction and the activation of pyroptosis may play significant roles in the pathogenesis of the disease [3–6].

Recent research has increasingly focused on the interactions between oligodendrocytes and neurons, as well as between microglia and astrocytes, due to their distinct yet complementary roles in various biological processes, including apoptosis, inflammation, neurodegeneration, and myelination [4]. Microglia, which represent approximately 10% of all cells in the CNS, serve as immune cells and facilitate tissue repair within the brain. However, in the context of MS, microglial activation occurs at an early stage, resulting in the production of significant quantities of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin-1β (IL-1β) [7,8]. Subsequently, activated microglia can trigger the transformation of astrocytes into highly reactive and neurotoxic cells in MS [9,10]. Furthermore, these cytokines may exacerbate mitochondrial damage in neurons [11]. Collectively, findings position microglia as pivotal players in the pathogenesis of MS, underscoring the necessity for a deeper understanding of neuroimmune interactions to inform therapeutic strategies for this condition.

Pyroptosis represents a novel form of programmed cell death, distinguished by the release of pro-inflammatory intracellular components alongside cellular demise. In the canonical pyroptotic pathway, the activation of caspase-1

is initiated by NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasomes, which subsequently leads to the cleavage of gasdermin-D (GSDMD). This cleavage results in the formation of an N-terminal fragment with the capacity to create membrane pores. Furthermore, IL-1β and IL-18 are activated by caspase-1 and subsequently released into the extracellular environment through the GSDMD pore [12]. Research has indicated that patients diagnosed with MS exhibit increased levels of IL-1β and GSDMD-N in peripheral blood mononuclear cells and the post-mortem brain tissue [13]. In the context of experimental autoimmune encephalomyelitis (EAE), an animal model for MS, the inhibitors targeting the NLRP3 inflammasome have demonstrated the ability to provide protection against neuroinflammation and demyelination [14]. Consequently, there is a pressing need to investigate the molecular mechanisms that regulate pyroptosis in the context of MS.

High Mobility Group Box 1 (HMGB1) is a highly conserved chromatin-associated protein extensively expressed in neuronal cells. The HMGB1/toll-like receptor 4 (TLR4)/nuclear factor-kappa B (NF-κB) signaling pathway has been recognized as a pivotal mediator in inflammatory responses [15,16]. Nonetheless, the underlying mechanisms and regulatory aspects of this pathway in pyroptosis and the interactions among various cell types in MS remain inadequately elucidated. Consequently, a comprehensive understanding of HMGB1's role could yield significant therapeutic insights for MS.

Our objective in this investigation was to investigate the role of HMGB1 in the inflammatory immune response and pyroptosis associated with MS through the use of EAE mice and conditioned medium (CM) obtained from activated BV-2 cells.

2. Materials and Methods

2.1 Cell Culture and Use of BV-2 Conditioned Medium

The BV-2 murine microglia cell line (CL-0493, Punuosai Biology, Wuhan, China), HT-22 mouse neuroblastoma cell line (IM-M038, Yimo Biology, Changsha, China), and C8D1A mouse cerebellar immortalized astrocytes (Ma-c, CL-0506, Punuosai Biology, Wuhan, China) were maintained in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, ThermoFisher Scientific, Waltham, MA, USA) and 1% penicillin-streptomycin. Cultures were incubated at 37 °C in a humidified atmosphere with 5% CO_2. The aforementioned cell lines were subcultured upon reaching 80–90% confluency. All cell lines were validated using short tandem repeat (STR) profiling and were tested for mycoplasma contamination, confirming that they were free of any contamination.

Two distinct types of CM were obtained: BV-2 cells were subjected to treatment with myelin oligodendrocyte glycoprotein (MOG35-55, MedChemExpress, MCE, Denville, NJ, USA) for a duration of 24 hours [17]. Additionally, BV-2 cells were treated with MOG35-55 for 24 hours following infection with the sh-HMGB1 lentivirus (MOG35-55+ sh-HMGB1). Subsequently, the CM was sterilized by filtration through 0.22 µm syringe filters and administered to HT-22 cells and Ma-c cells for 24 hours.

2.2 Animal Preparation

A total of twelve female C57/BL6 mice, each weighing 18–20 g and aged 6–8 weeks, were procured from Charles River (Beijing) Laboratory (Beijing, China). The mice were accommodated in groups within a standard animal facility, with a maximum of three individuals per cage, and their body weight was monitored daily. The animal study received approval from the Ethics Committee of The Second Hospital of Hebei Medical University (No. 2024-R004), and all the procedures were conducted in accordance with the guidelines for the use of live animals established by the National Institutes of Health.

2.3 EAE Induction and Neurobehavioral Assessment

As previously outlined [18], the EAE model was established through subcutaneous administration of myelin oligodendrocyte glycoprotein (MOG35-55, MCE, USA) at two distinct sites. In summary, the emulsion utilized (100 µL) consisted of 300 µg of MOG35-55 and 400 µg of Mycobacterium tuberculosis H37Ra (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) mixed with Complete Freund's adjuvant (MCE, Denville, NJ, USA). Additionally, on day 0 and on day 2, an intraperitoneal injection of 200 µL of pertussis toxin (300 ng; Sigma, Saint-Louis, MO, USA) was administered to augment the immunogenic response. The body weight of the mice was monitored daily, and the severity of disease was assessed by two trained investigators using a standardized assessment scale, which included the following criteria: 0, no symptoms; 1, tail paralysis; 2, hind limb paralysis; 3, hind limb paraplegia and incontinence; 4, quadriplegia; and 5, moribund state. The investigators were professionally trained and remained blinded to both the treatment administered and the genetic background of each mouse.

2.4 Lentiviral Constructs and Infection

The PLKO-U6-EGFP-P2A-PURO vector was employed to generate short hairpin RNA (shRNA) targeting HMGB1 (sh-HMGB1) and NLRP3 (sh-NLRP3), which were synthesized by Sangon (Shanghai, China). The lentiviral infection was conducted in accordance with established protocols. Specifically, BV-2 cells were exposed to lentiviral particles at a multiplicity of infection (MOI) of 20 for a duration of 16 hours. Following this incubation period, the viral particles were removed, and the BV-2 cells were subjected to selection in a culture medium supplemented with 5 µg/mL puromycin. The efficiency of the infection was assessed through the expression of green fluorescent protein three days post-infection. Concurrently,

HT-22 and Ma-c cells were also infected with the NLRP3 shRNA lentivirus using the aforementioned procedure. In the *in vivo* experiments, the viruses, specifically 1×10^7 infectious units of either sh-HMGB1 or a nontargeting control virus (sh-NC), were administered into the lateral cerebral ventricle. The administration was performed at coordinates 2.0 mm posterior and 1.5 mm lateral to the bregma, at a depth of 2.5 mm, employing stereotaxic methodologies. Furthermore, the EAE mice were stratified into four distinct groups (n = 3 per group): the control group (Control), the EAE group (Model), the sh-HMGB1 + Model group, and the sh-NC + Model group. At the end of the study, which lasted 14 days, the animals in each group were euthanized using 50 mg/kg of 2% pentobarbital sodium, after which the spinal cord and brain tissues were collected for subsequent analysis. The primers utilized for knockdown are detailed in **Supplementary Table 1**.

2.5 Cell Viability Assay of BV-2 Cells

Cell viability was assessed utilizing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, following the manufacturer's protocol (Beyotime, Taicang, China). Briefly, cells were incubated with the MTT labeling reagent for a duration of three hours, after which MTT solvent was added to each well. The absorbance (570 nm) was subsequently assessed utilizing a Multiskan FC Microplate Reader (Thermo Fisher Scientific, Waltham, MA, USA).

2.6 Tissue Preparation

The animals in each group were euthanized using 50 mg/kg of 2% pentobarbital sodium. The brains and spinal cords were preserved at –80 °C following fixation in cold 4% paraformaldehyde or rapid freezing in liquid nitrogen. For the immunohistochemical examination, half of the brain specimens were embedded in paraffin and cut at 4–10 µm for further processing.

2.7 Hematoxylin-Eosin (HE) Staining and Immunohistochemistry

Sections embedded in paraffin were routinely subjected to staining with HE, followed by a dewaxing and hydration process utilizing graded alcohol concentrations (100%, 95%, 90%, 80%, and 70%). The sections were subsequently stained with hematoxylin for a duration of 2 minutes, followed by eosin staining for 3 minutes, prior to examination under a Nikon ECLIPSE TS100 bright-field microscope (Nikon, Minato City, Japan). In the context of immunohistochemistry, the paraffin sections underwent treatment with 0.3% H_2O_2 and subsequently underwent blocking with Dual Endogenous Enzyme Block (S2003, DAKO, Glostrup, Denmark). The sections were then incubated with primary antibodies overnight at 4 °C, specifically Rabbit anti-CD11b (1:250, ab133357, Abcam, Cambridge, MA, USA) and Rabbit anti-CD68 (1:250, 97778, Cell Signaling Technology (CST), Danvers, MA, USA). The sections were then incubated with a biotinylated anti-rabbit IgG antibody (1:200, 31820, Thermo Scientific, Waltham, MA, USA) for one hour at room temperature, followed by avidin-biotin complex (Vector Laboratories, Burlingame, CA, USA). Finally, the stained tissue sections were observed and photographed using an Olympus BX51 microscope (Olympus, Tokyo, Japan).

2.8 Cytometry for CD11b and CD68

BV-2 cells were incubated under previously specified conditions and subsequently washed twice with cold phosphate-buffered saline (PBS). Following this, the cells were subjected to trypsinization and centrifugation. The resulting cell suspension was subsequently stained with anti-CD11b and anti-CD68 antibodies (ab8878 and ab283654, Abcam, Cambridge, MA, USA) at 4 °C for one hour. Flow cytometry analysis was performed utilizing a flow cytometer (FC500 Beckman Coulter, Brea, CA, USA) and analyzed by the FlowJo software (version 6.0, TreeStar, Ashland, OR, USA).

2.9 Enzyme-Linked Immunosorbent Assay (ELISA)

BV-2 cells were subjected to washing with PBS and subsequently cultured with MOG35-55. Following a 24-hour treatment period, the supernatants were harvested, and the concentration of HMGB1 was quantified utilizing a commercially available mouse HMGB1 ELISA kit (Beyotime, Taicang, China). Additionally, supernatants from HT-22 and Ma-c cells were collected after incubation with CM. The concentrations of TNF-α and IL-1β were assessed using commercially available mouse TNF-α ELISA kit (PT512, Beyotime, Taicang, China) and mouse IL-1β ELISA kits (E-EL-M0037, elabscience, Wuhan, China), respectively. All assays were conducted and analyzed in accordance with the manufacturer's protocols.

2.10 TUNEL Assay

Prior to conducting fluorescent imaging, cells were cultured in 35-mm confocal dishes with glass bottoms (FCFC020, Beyotime, Taicang, China). To assess the integrity of the plasma membrane, HT-22 cells and Ma-c cells were analyzed using the TUNEL method, following the manufacturer's instructions (C1086, Beyotime, Taicang, China). In brief, the cells were fixed using 4% paraformaldehyde (P0099, Beyotime, Taicang, China) and permeabilized with 0.25% Triton-X 100 (93443, Sigma, Saint Louis, MO, USA) for 20 minutes. The cells were then incubated in the TUNEL reaction mixture for 60 minutes and DAPI staining solution (C1006, Beyotime, Taicang, China) for 10 minutes at 37 °C in a dark environment. Following staining, images of the cells were captured utilizing a Leica microscope (SP8, Wetzlar, Germany), and the number of apoptotic cells was quantified in at least five randomly selected microscopic fields for each sample using

ImageJ software (version 1.48; National Institute of Health, Bethesda, MD, USA).

2.11 Protein Extraction and Western Blot (WB) Analysis

Tissue and cell extracts were subjected to lysis using cold RIPA buffer (P0013B, Beyotime, Shanghai, China) supplemented with Phenylmethanesulfonyl fluoride (PMSF), as well as protease and phosphatase inhibitors (P0013K, ST2573, and P1045, Beyotime, Taicang, China) at a temperature of 4 °C. An equivalent quantity of protein was separated using a 4–20% SDS-PAGE system (P2012 and P2014, NCM Biotech, Suzhou, China) and subsequently blotted onto polyvinylidene difluoride (PVDF) membranes (IPVH00010, Millipore, Bedford, MA, USA). The membranes were then blocked with a 5% skim milk solution (1172GR500, BioFroxx, Guangzhou, China) and incubated overnight at 4 °C with specific primary antibodies (refer to **Supplementary Table 2**). Then, the membranes were incubated at room temperature for one hour with either mouse or rabbit monoclonal secondary antibodies (as detailed in **Supplementary Table 2**). The protein bands were visualized using an ECL kit (P10300, NCM Biotech, Suzhou, China) and captured with a gel imager (JP-K600, Jiapeng Technology, Shanghai, China), with subsequent quantification performed using ImageJ software (version 1.48; National Institute of Health, Bethesda, MD, USA).

2.12 Statistical Analysis

All experiments were performed a minimum of three times, and the results are expressed as mean plus or minus standard error of the mean (M ± SEM). Group comparisons were evaluated using an unpaired two-tailed Student's *t*-test for two groups, or one-way analysis of variance (ANOVA) with Tukey's post hoc multiple comparison test for multiple groups. Data analysis was conducted using GraphPad Prism version 9.1 (GraphPad Prism, La Jolla, CA, USA), with a p-value of <0.05 deemed statistically significant.

3. Results

3.1 The Reduction of HMGB1 Expression May Mitigate the Weight Loss, Neurological Deficits, and Immune Infiltration in EAE Mice

In EAE mice, the body weights were notably lower in comparison to the control group ($p < 0.001$, Fig. 1A). However, a decrease in weight loss was observed in the sh-HMGB1 + Model group following the interference of HMGB1 expression ($p < 0.001$, Fig. 1A). Furthermore, the assessment of neurological function indicated an increase in scores ($p < 0.001$, Fig. 1B), peaking at approximately 4 on day 17 in the Model group, while the scores in the sh-HMGB1 + Model group were notably reduced ($p < 0.01$, Fig. 1B). Histological analysis via HE staining revealed a substantial presence of inflammatory cells within the brain tissue of EAE mice; however, treatment with sh-HMGB1 alleviated tissue inflammation (Fig. 1C). Comparable findings were also noted in the spinal cord samples (Fig. 1C).

3.2 The Interference of HMGB1 Expression has the Potential to Suppress Microglial Activation and Pyroptosis Through the HMGB1/TLR4/NF-κB Signaling Pathway

To examine the modifications in microglial cells, we conducted immunohistochemical analyses utilizing CD11b and CD68 as microglial markers in the brain and spinal cord tissues. The findings indicated an increase in the population of CD11b+ and CD68+ cells within the brain tissue of EAE mice ($p < 0.001$). Conversely, treatment with sh-HMGB1 resulted in a notable decrease in the number of CD11b+ ($p < 0.05$) and CD68+ ($p < 0.001$) cells in the brains of EAE mice (Fig. 2A). These observations were consistent with the results obtained from spinal cord samples. A critical function of microglia involves the activation of the inflammasome and the process of pyroptosis [19,20]. Consequently, we assessed the expression of proteins associated with pyroptosis and the HMGB1/TLR4/NF-κB signaling pathway through WB. The results revealed a significant elevation in the expression of HMGB1, TLR4, p-IKB (a protein related to NF-κB), NLRP3, cleaved caspase-1, and GSDMD-N in EAE mice ($p < 0.001$). Notably, HMGB1 inhibition led to a substantial reduction in the aforementioned protein levels (Fig. 2B). In detail, the protein expression levels of HMGB1 ($p < 0.001$), TLR4 ($p < 0.01$), p-IKB ($p < 0.001$), NLRP3 ($p < 0.001$), cleaved caspase-1 ($p < 0.001$), and GSDMD-N ($p < 0.01$) were significantly reduced in the sh-HMGB1 + Model group in comparison to the sh-NC + Model group.

3.3 Establishment of MS Model in BV-2 Cells

BV-2 cells that were treated with MOG35-55 were utilized as a cellular model for MS [17]. Initially, BV-2 cells were cultured with specified concentrations of MOG35-55. The findings revealed that both cell viability and the release of HMGB1 increased in correlation with higher concentrations of MOG35-55 ($p < 0.05$) (Fig. 3A–C). Meanwhile, flow cytometry analysis of CD11b and CD68 demonstrated an elevated number of BV-2 cells in response to escalating levels of MOG35-55 ($p < 0.001$) (Fig. 3D). Ultimately, the optimal concentration of MOG35-55 was established at 25 μg/mL, which exhibited a robust capacity for activating BV-2 cells while maintaining a favorable safety profile.

3.4 Pyroptosis was Induced in Neurons and Astrocytes Following Incubation With CM Derived From BV-2 Cells That Had Been Treated With MOG35-55

To investigate the interactions between neurons/astrocytes and microglia in MS, CM from BV-2 cells subjected to various treatments were utilized to incubate with HT-22 and Ma-c cells. The treatments included: (A) BV-2 cells treated with MOG35-55 (cellular model); (B) cell model transfected with sh-HMGB1, and (C) HT-22 and Ma-c cells transfected with sh-NLRP3 and exposed to CM from the cellular model. A comparison of

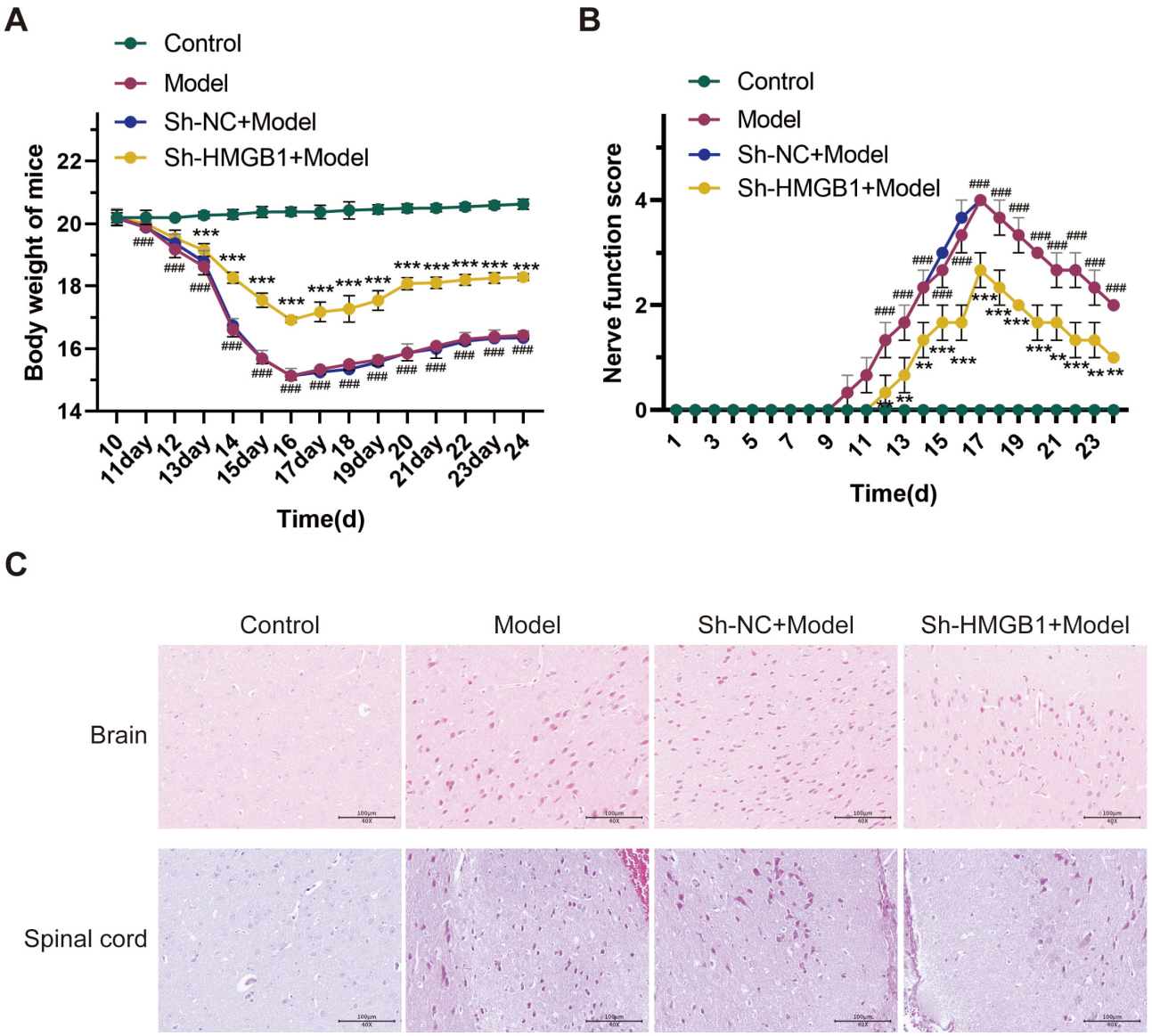

Fig. 1. The interference of HMGB1 expression attenuated the weight loss (A), neurologic deficit (B), and immune infiltration (C) in EAE mice. The groups were as follows: Control: untreated mice (blank control). Model: EAE mice. sh-HMGB1 + Model group: EAE mice with sh-HMGB1 treatment. sh-NC + Model group: EAE mice with vehicle sham treatment (negative control). Data are represented as M ± SEM. $^{###}$ $p < 0.001$, a comparison between the model group and the control group; ** $p < 0.01$, *** $p < 0.001$, a comparison between the sh-HMGB1 + Model group and the sh-NC + Model group. (The specifics of the statistical findings are available in **Supplementary Table 3**). Representative images of the brain and spinal cord tissue stained using HE (C). Calibration bars = 100 μm. HMGB1, High Mobility Group Box 1; EAE, experimental autoimmune encephalomyelitis; sh-NC, shRNA Nontargeting Control; M, Mean; SEM, Standard Error of the Mean; HE, Hematoxylin-Eosin.

group A with group B revealed a reduction in the release of TNF-α (both $p < 0.001$) and IL-1β (both $p < 0.001$) in HT-22 cells (Fig. 4A) and Ma-c cells (Fig. 4B) in group B. In comparison to group A, the results of TUNEL staining indicated a significant decrease in TUNEL-positive cells in HT-22 and Ma-c cells in group B (both $p < 0.01$). This finding implies that the modulation of HMGB1 expression in BV-2 cells resulted in a notable decrease in pyroptosis in both HT-22 ($p < 0.01$) (Fig. 4C) and Ma-c cells ($p < 0.01$) (Fig. 4D), which were induced by CM from BV-2 cells treated with MOG35-55. Furthermore, WB analysis demonstrated a marked decrease in the expression of TLR4 (both $p < 0.001$), p-IKB ($p < 0.01$ & $p < 0.001$), NLRP3 ($p < 0.01$ & $p < 0.001$), cleaved caspase-1 (both $p < 0.001$), and GSDMD-N (both $p < 0.001$) in HT-22 cells (Fig. 4E) and Ma-c cells (Fig. 4F) in groups B, in comparison to group A. Conversely, a comparative analysis between group A and group C demonstrated a significant reduction in the secretion of TNF-α (both $p < 0.001$) and IL-1β (both $p < 0.001$) in HT-22 (Fig. 4A) and Ma-c

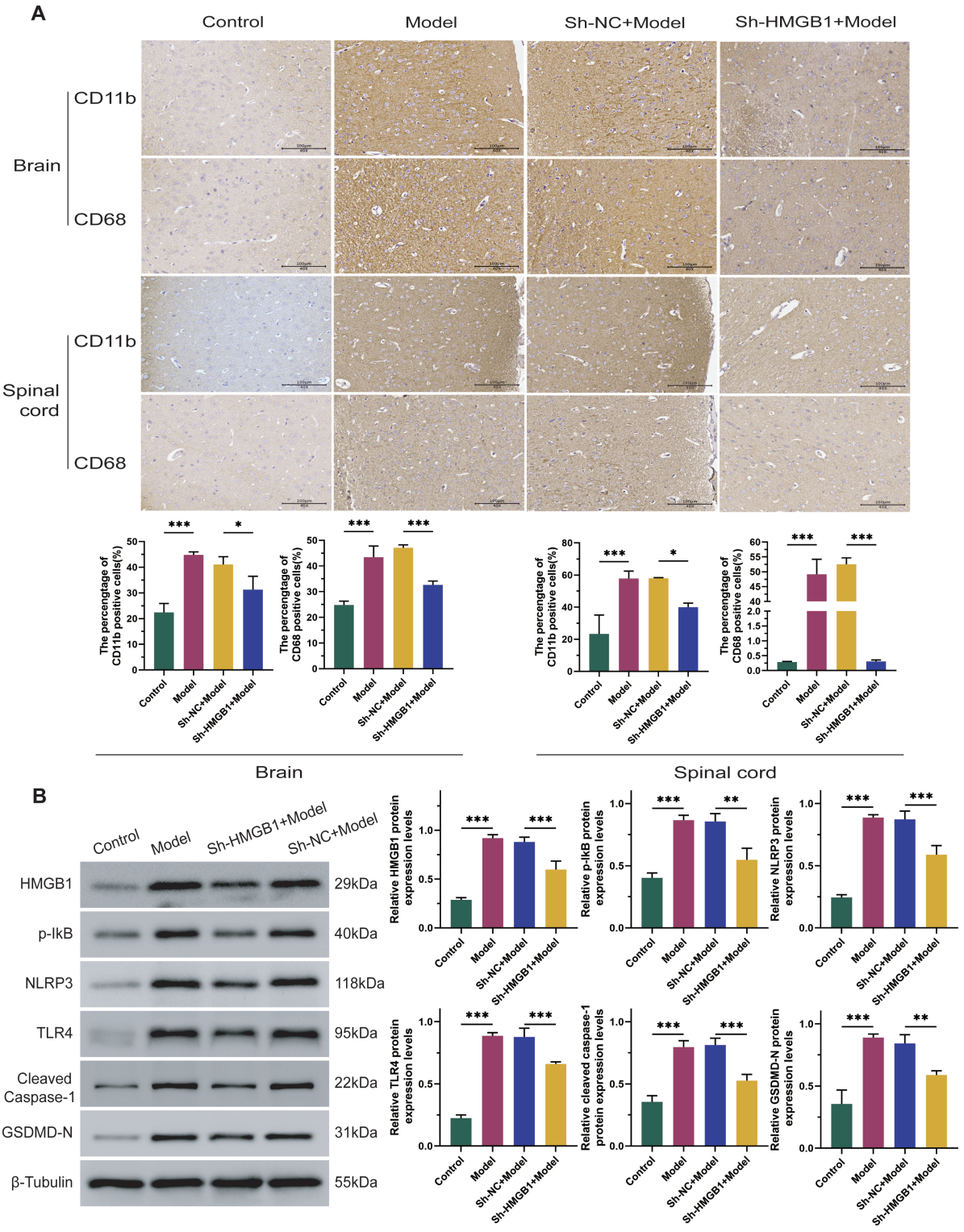

Fig. 2. HMGB1 was upregulated in EAE mice, and interference with HMGB1 expression attenuated the activation of microglia. (A) The expression of pyroptosis and HMGB1/TLR4/NF-κB pathway. Representative images of $CD11b^+$ and $CD68^+$ cells in the brain and spinal cord tissue (A). Calibration bars = 100 μm. Western blot analysis of HMGB1, TLR4, p-IKB, NLRP3, cleaved caspase1, GSDMD-N (B). The groups were as follows: control: untreated mice (blank control). Model: EAE mice. sh-NC + Model group: EAE mice with vehicle sham treatment (negative control). sh-HMGB1 + Model group: EAE mice with sh-HMGB1 treatment. Data are represented as mean ± SEM. Statistical tests were performed by one-way ANOVA. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. TLR4, toll-like receptor 4; NF-κB, nuclear factor-kappa B; p-IKB, phosphorylation of NF-κB inhibitor protein; NLRP3, NOD-like receptor family pyrin domain containing 3; GSDMD-N, GSDMD N-terminal; ANOVA, analysis of variance.

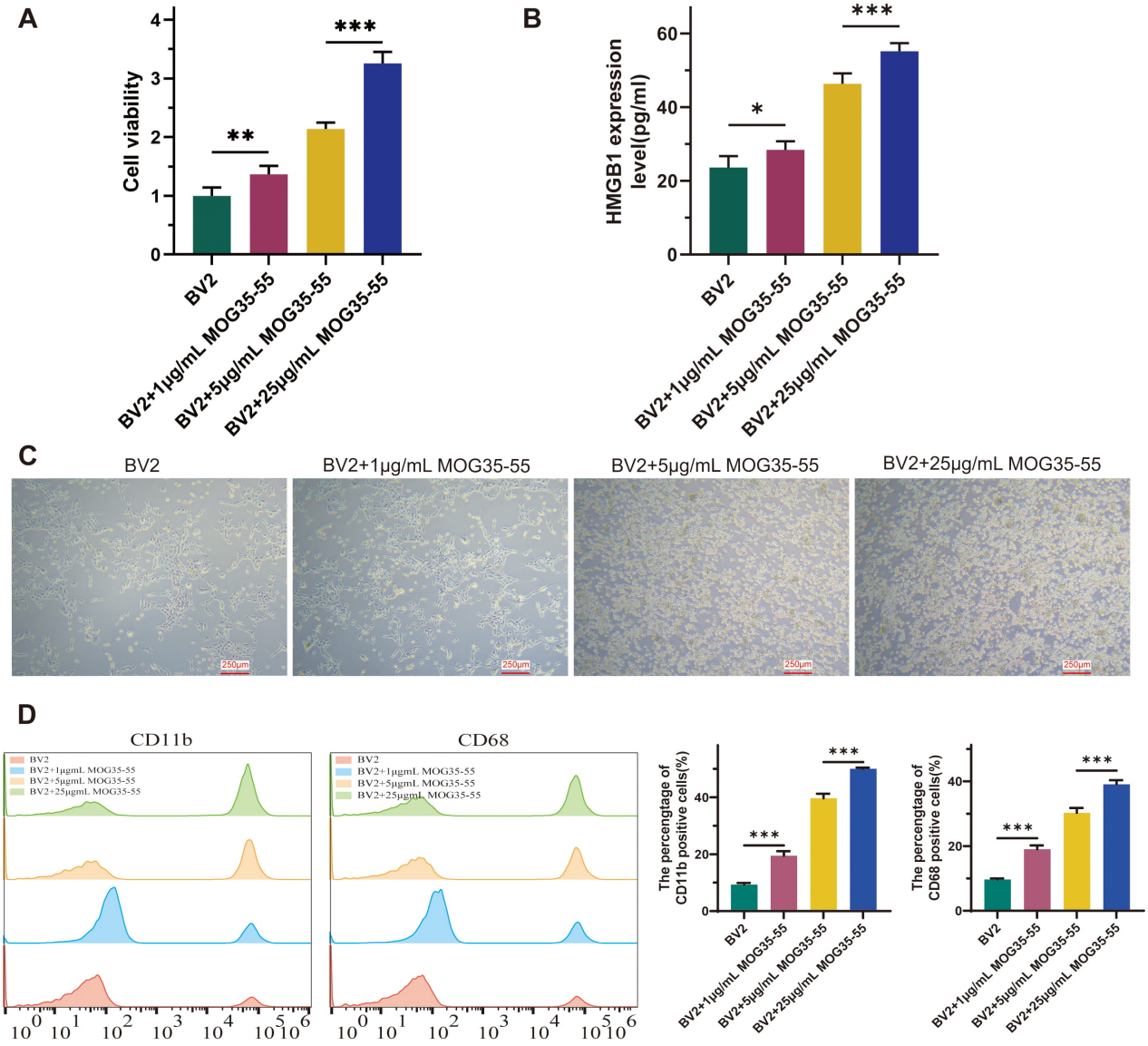

Fig. 3. The activation of BV-2 cells and the release of HMGB1 were MOG35-55 concentration-dependent. The BV-2 cells were cultured in designated concentrations of MOG35-55. MTT analysis of BV-2 cell viability (A). ELISA analysis of the release of HMGB1 (B). Representative images of the cell morphology (C). Cytometry analysis for $CD11b^{+}$ and $CD68^{+}$ cells (D). Calibration bars = 250 μm. Data are represented as mean ± SEM. Statistical tests were performed by one-way ANOVA. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. MOG, myelin oligodendrocyte glycoprotein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; ELISA, enzyme-linked immunosorbent assay.

cells (Fig. 4B) within group C. Additionally, the results from TUNEL staining revealed a notable decrease in the number of TUNEL-positive cells in HT-22 ($p < 0.01$) (Fig. 4C) and Ma-c ($p < 0.05$) (Fig. 4D) cells in group C. Furthermore, WB analysis demonstrated a marked decrease in the expression of NLRP3 ($p < 0.05$ & $p < 0.01$), cleaved caspase-1 (both $p < 0.01$), and GSDMD-N ($p < 0.001$ & $p < 0.01$) in HT-22 (Fig. 4E) and Ma-c cells (Fig. 4F) in groups C, in comparison to group A. However, no significant difference was observed in the protein expression levels of TLR4 and p-IKB between groups A and C (both $p > 0.05$) (Fig. 4E,F).

4. Discussion

Although the molecular mechanisms underlying MS remain incompletely elucidated, intercellular communication, immune dysregulation, and inflammation are widely acknowledged as central contributors to its pathogenesis. Recent reviews have highlighted the involvement of several key signaling pathways—including TNF-α, TGF-β, NF-κB, and Wnt—mediated by exosomal contents such as miRNAs and HMGB1, which offer novel insights into both the underlying mechanisms and potential therapeutic strategies for MS [21]. In this study, we observed that EAE

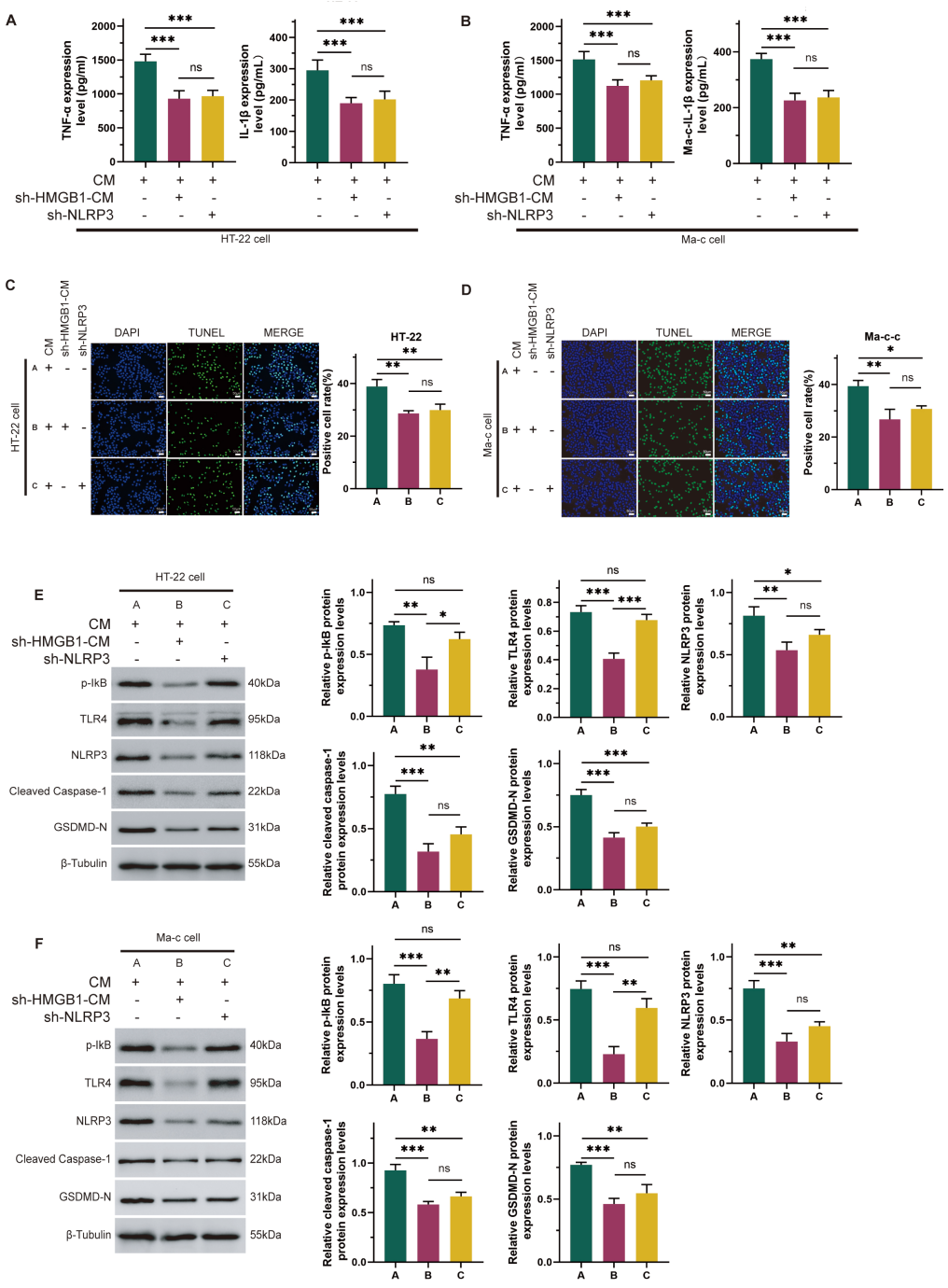

Fig. 4. CM from MOG35-55-treated BV-2 cells induces pyroptosis in HT-22 and Ma-c cells, which is alleviated by HMGB1 knockdown or NLRP3 silencing. Pyroptosis was upregulated in HT-22 and Ma-c cells after treatment with CM from BV-2 cells. However, interference with HMGB1 expression inhibited the release of TNF-α and IL-1β (A,B), reduced cell death (C,D) and suppressed the expression of pyroptosis-related proteins and activation of the TLR4/NF-κB pathway (E,F). Group A: HT-22/Ma-c cells incubated with CM from the MS cellular model (positive control); group B: HT-22/Ma-c cells incubated with CM from the MS cellular model treated with sh-HMGB1; group C: HT-22/Ma-c cells treated with sh-NLRP3 and incubated with CM from the MS cellular model. ELISA analysis of the release of TNF-α and IL-1β (A,B). TUNEL assay analysis of HT-22 cell death (C,D). Western blot analysis of TLR4, p-IKB, NLRP3, cleaved caspase-1, and GSDMD-N expression (E,F) (The results of WB statistical analyses are available in **Supplementary Table 4**). Calibration bars = 50 μm. Data are represented as mean ± SEM. Statistical tests were performed by one-way ANOVA. ns, not significant, $p > 0.05$, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. CM, conditioned medium; TNF-α, tumor necrosis factor-α; IL-1β, interleukin 1β; TUNEL, the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labelling; MS, multiple sclerosis.

mice displayed increased concentrations of HMGB1 within the CNS. The downregulation of HMGB1 may serve to inhibit microglial activation and pyroptosis, thereby mitigating the inflammatory response and enhancing neurological function. Furthermore, we noted an increase in HMGB1 release in the MS cell model, which was associated with neuronal and astrocytic cell death and pyroptosis. Consequently, HMGB1 may act as a fundamental triggering factor in MS and represents a potential therapeutic target for intervention.

Among a variety of contributing factors, persistent activation of microglia is recognized as a significant driver of MS [22]. Research has demonstrated that microglial activation can lead to the formation of the NLRP3 inflammasome and enhance GSDMD-mediated pyroptosis [23], which is regulated by HMGB1 in cases of neonatal hypoxic-ischemic brain injury [24]. Therefore, it is imperative to investigate the roles of HMGB1 and pyroptosis within the pathophysiological mechanisms of MS. Recent studies have identified HMGB1 as a potent pro-inflammatory mediator that facilitates the M1 polarization of microglia [24,25]. Furthermore, it has been observed that there is an elevation of HMGB1 and TLR4 in the cerebrospinal fluid and white matter regions of MS patients [26]. In the context of cardiac ischemia/reperfusion injury, TLR4 may regulate NF-κB expression, thereby amplifying the inflammatory response and initiating pyroptosis through the activation of the NLRP3 inflammasome [27]. Our current investigation revealed that microglia were activated, and that the HMGB1/TLR4/NF-κB signaling pathways, along with proteins associated with pyroptosis, were upregulated in EAE mice. Additionally, the downregulation of HMGB1 was found to reverse the increase in TLR4/NF-κB signaling pathway proteins and pyroptosis-related protein levels in the EAE mice.

Conversely, HMGB1 can be released into the CNS by activated microglia and necrotic cells during MS. Recent studies indicate that serum concentrations of HMGB1 are significantly higher in MS patients [28–30]. Additionally, a notable positive correlation has been identified between HMGB1 levels and both physical and psychological well-being in these patients [29]. Furthermore, research has demonstrated that extracellular HMGB1 may induce an overexpression of NF-κB-regulated genes and facilitate NF-κB nuclear translocation in oligodendrocyte progenitor cells [31]. Consequently, extracellular HMGB1 may serve as a critical mediator in triggering the inflammatory cascade within the CNS by interacting with TLR4 and activating the HMGB1/TLR4/NF-κB signaling pathways. However, to our knowledge, no studies have specifically investigated the role of HMGB1 in the interactions between microglia and neurons or astrocytes in the context of MS. In our investigation, we found that CM from BV-2 cells treated with MOG35-55 resulted in cell death, the secretion of inflammatory cytokines, and pyroptosis in HT-22 and Ma-c cells. Notably, the downregulation of HMGB1 was found to mitigate these inflammatory responses. Therefore, it is plausible that HMGB1 may function as a central hub for neuroimmune interactions and facilitate communication between microglia and astrocytes. Furthermore, recent research has underscored the significant involvement of NLRP3 inflammasome activation and pyroptosis in microglia in the pathogenesis of MS [32]. In addition, the inhibition of NLRP3 through selective small molecules [33], exosomal miR-23b-3p [34], and ketogenic diets [6] has been shown to exert inhibitory effects on neuroinflammation and pyroptosis, potentially alleviating central neuropathic pain associated with MS. Our study further demonstrated that the suppression of HMGB1 expression in microglia could inhibit pyroptosis and reduce the release of TNF-α and IL-1β from neurons and astrocytes via the HMGB1/TLR4/NF-κB signaling pathways. Thus, HMGB1 inhibitors may represent novel therapeutic strategies for MS and NLRP3-related inflammatory diseases.

To date, novel pharmacological agents targeting HMGB1 have been utilized in therapeutic research, including anti-HMGB1 antibodies, the HMGB1 A box (a recognized inhibitor of HMGB1) [35], ethyl pyruvate (EP) [36], and chloroquine [37]. Additionally, certain medications have shown promising results in treating neurological disorders primarily through the inhibition of HMGB1 in microglial cells. Zhan Zhang *et al.* [38] have reported that pregabalin may alleviate microglial activation and neuronal damage by modulating the HMGB1-TLR2/TLR4/RAGE signaling pathway in cases of radiation-induced brain injury. Similarly, Bo Wang *et al.* [39] have demonstrated that minocycline can mitigate depressive-like behaviors by inhibiting the release of HMGB1 from microglia and neurons. Consequently, based on our research findings and the conclusions drawn from other studies, the downregulation of HMGB1 expression in microglia may represent a novel therapeutic approach for MS. However, the current literature assessing HMGB1-targeting agents in the context of EAE and MS patients remains limited [40–42]. Furthermore, there is a significant lack of studies documenting the clinical outcomes associated with the administration of HMGB1 neutralizing agents in MS patients. As a result, the involvement of HMGB1 in MS necessitates further investigation, although it faces certain challenges that may impede its future clinical application. Previous studies have identified two isoforms of HMGB1, fully reduced HMGB1 (fr-HMGB1) and disulfide HMGB1 (ds-HMGB1), both of which are believed to interact with receptors and contribute to pro-inflammatory processes, but another isoform of HMGB1, fully oxidized HMGB1 (oxHMGB1), is regarded as inert [43–46]. The coexistence of these distinct HMGB1 isoforms within the extracellular matrix complicates the determination of the specific functions of individual antagonists. Moreover, the pharmacological inhibition of HMGB1 at inappropriate times may hinder

tissue repair rather than diminish inflammation [47], underscoring a potential risk associated with the use of anti-HMGB1 therapies within the CNS.

As indicated above, increased serum HMGB1 is associated with raised depression levels and poor sleep in MS patients [29]. Poor sleep and depression are commonly associated with decreased pineal and local melatonin production [48], with melatonin increasing sirtuin-1 to deacetylate HMGB1 and decreasing its cytoplasmic translocation, thereby decreasing HMGB1 levels and capacity to induce the TLR4/NF-κB pathway [49]. The suppression of pineal and local melatonin production, commonly observed in MS, may therefore be an important upstream factor in the HMGB1 rise in MS. This includes MS microglia, where the induction of the microglia melatonergic pathway and the autocrine effects of melatonin shift microglia from a pro-inflammatory M1-like phenotype to a pro-phagocytic M2-like phenotype [50]. The beneficial effects of exogenous melatonin on knee muscle strength, manual dexterity, static postural balance, mood, and cognition in MS patients would support this [51]. It is also important to note that alterations in the gut microbiome have long been associated with MS pathogenesis and pathophysiology, including the suppression of the gut short-chain fatty acid, butyrate [52]. Butyrate is an epigenetic regulator and, like melatonin, suppresses HMGB1 [53], whilst the loss of pineal melatonin will contribute to the increased gut permeability and gut dysbiosis evident in MS [54]. Pineal melatonin loss at night may be intimately linked to the pathogenesis of a host of diverse medical conditions [55], partly mediated by an alteration in how biomedical processes are dampened and reset at night, including via alterations in the gut microbiome and its products. The interactions of such circadian and systemic factors in a night-time pathogenesis of MS will be important to determine in future research.

In summary, the results of the present study suggest that the downregulation of HMGB1 expression may alleviate the symptoms observed in EAE mice by reducing microglial activation and pyroptosis within the CNS. Additionally, exposure to MOG35-55 was found to trigger the active release of HMGB1 from BV-2 cells. The supernatant derived from BV-2 cells cultured with MOG35-55 was shown to promote the secretion of TNF-α and IL-1β, and induce the expression of pyroptosis-related proteins in HT-22 and Ma-c cells. However, our study had some limitations. First, the present study primarily relied on *in vitro* cellular models and the EAE mouse model, which, while widely used, may not fully recapitulate the complex immunopathological features of human MS. Validation of these findings in human tissue samples or clinical settings is necessary to assess their translational relevance. Second, we did not employ direct inhibitors targeting extracellular HMGB1, leaving the immediate effects of extracellular HMGB1 blockade to be further investigated. A comprehensive exploration of the diverse roles of HMGB1 at various stages of the disease may enhance therapeutic strategies for MS. Although we observed the activation of pyroptotic pathways and pro-inflammatory cytokine secretion following HMGB1 release, our study did not evaluate upstream signaling events such as pattern recognition receptor engagement or downstream cell-specific responses within the broader neuroimmune environment. Future studies should incorporate primary cell cultures or animal models that better mimic *in vivo* conditions to further investigate the mechanism of HMGB1 in MS pathogenesis.

5. Conclusion

Our findings indicate that HMGB1 serves as a facilitator of weight loss and neurological impairments in EAE mice. The down-regulation of HMGB1 expression has the potential to significantly alleviate these symptoms by modulating microglial activation and pyroptosis. Furthermore, the release of HMGB1 into the extracellular environment by microglia contributes to the inflammatory response and pyroptosis in both neurons and astrocytes. These results underscore the role of HMGB1 as a promoter of pyroptosis and a mediator in the inflammatory immune response. Consequently, the modulation of HMGB1 expression may represent a promising therapeutic target in the treatment of MS.

Abbreviations

MS, Multiple sclerosis; CNS, Central nervous system; IL-1β, interleukin 1β; EAE, Experimental autoimmune encephalomyelitis; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling; HMGB1, High mobility group box 1; SDS-PAGE, Sodium Dodecyl Sulfate-Polyacrylamide; TLR4, Toll-like receptor 4; NF-κB, Nuclear factor-kappa B; CM, Conditioned medium; DMEM, Dulbecco's Modified Eagle's Medium; MOG35-55, Myelin Oligodendrocyte Glycoprotein 35-55; MOI, Multiplicity of infection; NLRP3, NOD-like receptor family pyrin domain containing 3; HE, Haematoxylin-Eosin; PBS, phosphate-buffered saline; ELISA, Enzyme-Linked Immunosorbent Assay; WB, Western Blot; PVDF, Polyvinylidene Difluoride; GSDMD-N, N-terminus of GSDMD; DAPI, 4$'$,6$'$-diamidino-2-phenylindole; EP, Ethyl Pyruvate.

Availability of Data and Materials

The datasets involved in the present study can be provided under reasonable request from the corresponding author.

Author Contributions

YF: Conceptualization, Formal analysis, Investigation, Methodology, Software, Writing — original draft; LW: Investigation, Methodology; ZM: Investigation, Methodology; WW: Conceptualization, Funding acquisi-

tion, Project administration, Supervision, Writing. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The animal study was approved by the Ethic Committee of The Second Hospital of Hebei Medical University (No. 2024-R004), and all the procedures were conducted under the guidelines for the use of live animals of the National Institute of Health.

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Conflict of Interest

The authors declare no conflict of interest.

Declaration of AI and AI-assisted Technologies in the Writing Process

The authors used ChatGPT only to improve the language of the Introduction and Discussion section, with an emphasis on improving clarity and correcting grammar. The scientific content was written entirely independently by the authors, without the use of AI tools. We hope that this clarification will resolve any issues with the use of AI in our submitted manuscript. The authors read the full article after using the AI tool and is responsible for the article.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL37838.

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