

MYD88-silencing hUCMSC-derived extracellular vesicles alleviate sepsis- associated encephalopathy by modulating neurovascular and microglial inflammation

Ma Fan

Minzu University of China

Wenyu Yang

Minzu University of China

Feier Lei

Minzu University of China

Minghe Liu

Minzu University of China

Ruhui Wang

Minzu University of China

Yi Shen

Minzu University of China

Yi Yu

Minzu University of China

Yijing Zhang

Minzu University of China

Xingmei Shu

Chinese Academy of Sciences

Limeng Zhang

Minzu University of China

Aizhang Tu

Minzu University of China

Jingyan Shen

Minzu University of China

Xinyu Xie

Minzu University of China

Manjun Yang

Hunan Landfar Biotechnology Co. Ltd.

Chuncui Jia

National Institutes for Food and Drug Control

Feng Yan

Hunan Landfar Biotechnology Co. Ltd.

Yang Hu

yang.hu@muc.edu.cn

Minzu University of China

Research Article

Keywords: Sepsis-associated encephalopathy, hUCMSC-derived extracellular vesicles, Cell-free therapy, MYD88, Neurovascular injury, Microglia, Immune regulation

Posted Date: October 26th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10259207/v1>

License:  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

MYD88-silencing hUCMSC-derived extracellular vesicles alleviate sepsis-associated encephalopathy by modulating neurovascular and microglial inflammation

Fan Ma^{a,1}, Wenyu Yang^{a,1}, Feier Lei^{a,1}, Minghe Liu^a, Ruhui Wang^a, Yi Shen^a, Yi Yu^a, Yijing Zhang^a, Xingmei Shu^d, Limeng Zhang^a, Aizhang Tu^a, Jingyan Shen^a, Xinyu Xie^a, Manjun Yang^b, Chuncui Jia^{c,*}, Feng Yan^{b,*}, Yang Hu^{a,*}

^a Key Laboratory of Ecology and Environment in Minority Areas, National Ethnic Affairs Commission, Center on Translational Neuroscience, College of Life and Environmental Sciences, Minzu University of China, Beijing, 100081, China

^b Hunan Provincial Engineering Technology Research Center of Stem Cell Exosome, Hunan Landfar Biotechnology Co., Ltd., Changsha 410205, China

^c National Health Commission Key Laboratory for the Testing and Standardization of Biotechnology Products, Beijing Key Laboratory of Quality Control and Non-clinical Research and Evaluation for Cellular and Gene Therapy Medicinal Products, National Institutes for Food and Drug Control, Beijing, 102629, China

^d State Key Laboratory of Organ Regeneration and Reconstruction, Institute of Zoology, Chinese Academy of Sciences, Beijing, 100101, China

* Corresponding author.

E-mail addresses: jiachuncui@nifdc.org.cn (C. Jia), yanf@landfar.cn (F. Yan), yang.hu@muc.edu.cn (Y. Hu).

¹ These authors contributed equally to this work.

Abstract

Background: Sepsis-associated encephalopathy (SAE) is a common complication of sepsis and is closely associated with persistent neuroinflammation, neurovascular injury and long-term cognitive impairment. Myeloid differentiation primary response 88 (MYD88) is a shared signaling adaptor for the Toll-like receptor family and the interleukin-1 receptor (IL-1R) family, and its expression remains persistently elevated in microglia and vascular endothelial cells in surviving mice after cecal ligation and puncture (CLP).

Methods: hUCMSC-derived EVs were isolated from serum-free conditioned medium, loaded with MYD88-targeting siRNA (siMYD88) or negative-control siRNA by electroporation, and characterized by nanoparticle tracking analysis, transmission electron microscopy, and EV marker profiling. siRNA protection and MYD88 knockdown were evaluated in BV2 cells and primary microglia. Therapeutic efficacy was examined in a CLP survivor model using a rescue intervention initiated 72 h after CLP. Brain delivery, blood-brain barrier permeability, inflammatory and antioxidant signaling, hippocampal ultrastructure, and behavioral outcomes were assessed.

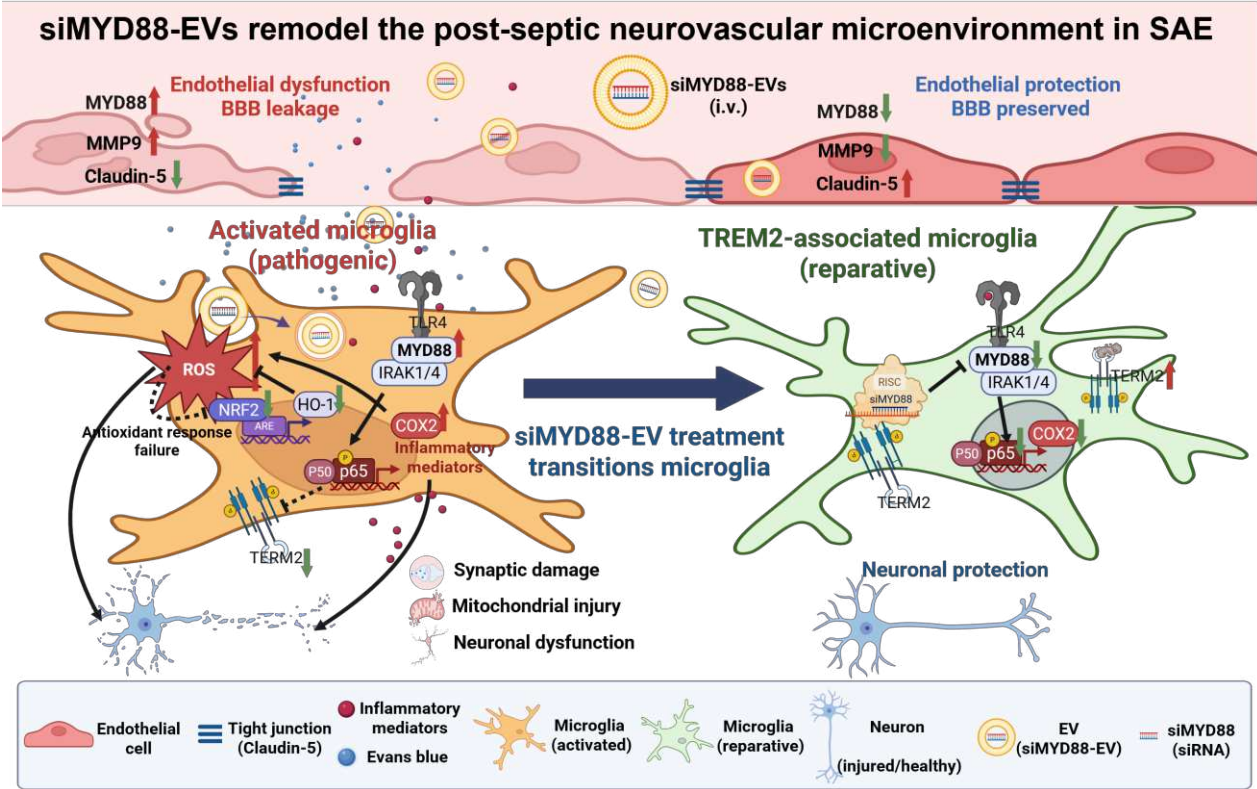
Results: siMYD88-loaded hUCMSC-EVs protected siMYD88 from RNase degradation and mediated functional MYD88 knockdown in microglial cells. After tail-vein administration, siMYD88-EVs entered the brain and were taken up by microglia. Rescue treatment with siMYD88-EVs at 72 h after CLP reduced MYD88 overexpression in vascular endothelial cells and protected against sepsis-induced disruption of blood-brain barrier integrity. siMYD88-EVs also suppressed hippocampal microglial activation, attenuated MYD88/NF- κ B/COX2 inflammatory-redox signaling, and restored NRF2/HO-1-associated antioxidant responses impaired after CLP. MYD88 silencing further enhanced TREM2-associated microglial responses, preserved hippocampal mitochondrial and synaptic ultrastructure, and improved anxiety-like behavior and cognitive deficits after CLP.

Conclusions: These findings indicate that sustained MYD88 signaling is a targetable driver of post-septic neurovascular injury and neuroinflammatory damage, and support engineered EV-mediated MYD88 silencing as a potential cell-free RNA delivery strategy for SAE.

Keywords

Sepsis-associated encephalopathy; hUCMSC-derived extracellular vesicles; Cell-free therapy; MYD88; Neurovascular injury; Microglia; Immune regulation.

Graphical Abstract



Background

Sepsis-associated encephalopathy (SAE) is a diffuse brain dysfunction that occurs during systemic infection without direct evidence of central nervous system invasion [1]. Its clinical spectrum ranges from delirium to coma and is associated with increased mortality, prolonged intensive care unit stay and poor functional recovery [1] [2]. SAE is not limited to the acute phase of sepsis; even after apparent control of systemic infection, many survivors develop persistent cognitive impairment and functional disability that can last for years [3]. Current clinical management mainly focuses on infection control, haemodynamic resuscitation, organ support and sedation optimization [4], and no established brain-protective strategy is available to prevent persistent SAE-related neurocognitive injury [2].

Systemic inflammation is increasingly recognized as an important driver of secondary neuroinflammation in SAE. Circulating cytokines, complement activation products, damage-associated molecular patterns and oxidative mediators can act on the cerebral endothelium and neurovascular unit,

increasing blood-brain barrier permeability and disturbing cerebral microvascular function [5]. Persistent neuroimmune activation is a major biological feature of post-septic brain dysfunction [6]. Clinical data show that cognitive impairment and functional disability can persist for years after sepsis[7][8], while cecal ligation and puncture (CLP) survivor models show sustained central myeloid inflammation and cognitive abnormalities long after the initial insult [6]. Microglia, the principal innate immune cells of the brain, can amplify inflammatory injury by producing pro-inflammatory cytokines, reactive oxygen species (ROS) and reactive nitrogen species (RNS), and by responding to damage-associated molecular patterns (DAMPs) [9] [10]. Excessive microglial activation contributes to aberrant synaptic remodeling, hippocampal dysfunction and cognitive impairment [11]. In parallel, oxidative stress and impaired antioxidant defense aggravate neuronal and cerebrovascular injury, suggesting that inflammatory dysregulation and redox imbalance may jointly sustain SAE pathology [9] [12].

Pattern-recognition receptor pathways provide proximal routes through which infection- and injury-derived signals are converted into inflammatory transcriptional programs [13]. Myeloid differentiation primary response 88 (MYD88) is a central adaptor for most Toll-like receptors and members of the interleukin-1 receptor family [14] [15]. Through downstream NF- κ B and MAPK activation, MYD88 signaling promotes the production of inflammatory mediators [14] [15] [16]. In sepsis, MYD88-dependent signaling may also contribute to mitochondrial stress, ROS generation and oxidative tissue injury [13]. Clinical transcriptomic data have shown increased MYD88, NFKB1 and IL6 expression in neonatal sepsis, with higher MYD88 expression associated with poorer survival [17]. Targeting sustained MYD88/NF- κ B signaling may therefore offer a rational strategy to interrupt inflammatory, oxidative and neurovascular injury after sepsis.

Mesenchymal stem cell-derived extracellular vesicles (MSC-EVs) are cell-free nanocarriers capable of transferring proteins, lipids and nucleic acids to recipient cells [18] [19, 20]. A large body of evidence indicates that unmodified MSC-EVs have a certain ability to protect the body from sepsis-induced organ injury [21]. In acute sepsis, adipose-derived MSC small extracellular vesicles (adipose-derived MSC-sEVs) can downregulate TNF- α expression [22]. MSC-EVs exert protective effects through immunomodulatory and redox-regulatory mechanisms, including activation of NRF2-dependent anti-ferroptotic pathways and miR-146b/IRAK1/NF- κ B-mediated anti-inflammatory signaling [23] [24]. In addition, engineered MSC-

derived exosomes have also shown some potential. MSC-EV-mediated delivery of miR-140-3p can improve CLP-induced cognitive dysfunction by targeting HMGB1 and reducing hippocampal neuroinflammation, NF- κ B activation, inflammasome signaling and pyroptosis [25]. These studies suggest that disease-relevant RNA cargo can be used to redirect EV function and exert greater effects. Among different MSC sources, human umbilical cord-derived mesenchymal stem cells (hUCMSCs) provide a readily expandable cell source for EV engineering. Umbilical cord-derived MSCs have shown immunomodulatory and endothelial-protective effects in preclinical sepsis models, and hUCMSC-EVs have also shown certain anti-inflammatory activity [26] [27]. However, whether hUCMSC-derived EVs can be engineered to silence intracellular targets in microglia and further attenuate persistent post-septic neurovascular injury and neuroinflammation remains unclear. In this study, we generated hUCMSC-derived EVs loaded with MYD88-targeting siRNA and evaluated their therapeutic effects in a CLP survivor model of SAE.

Methods

Animals and ethics statement

Specific pathogen-free male C57BL/6J mice (12 weeks old, 23 $\pm$ 2 g; Vital River, China) were housed under standard conditions with free access to food and water and were acclimatized for 7 days before experiments. All procedures were approved by the Biological and Medical Ethics Committee, Minzu University of China (approval no. ECMUC2024015AA; approved 28 May 2024). The individual mouse was the experimental unit for all in vivo experiments. All animal experiments were conducted and reported in accordance with the ARRIVE guidelines 2.0. A total of 71 adult mice were used across the in vivo experiments: 12 in the MYD88 time-course experiment, 9 in the EV brain-distribution experiment, and 50 in the treatment experiment. Sample sizes were determined on the basis of previous CLP studies, preliminary observations, experimental feasibility and anticipated postoperative mortality; no formal a priori power calculation was performed. Surviving CLP mice were allocated to treatment groups using a computer-generated random-number sequence by an investigator not involved in outcome assessment. Treatment administration was performed with knowledge of group allocation, whereas behavioral assessment, image quantification and data analysis were conducted using coded group labels, with codes disclosed only after analysis. Animals unable to complete behavioral testing because of severe postoperative condition or obvious motor impairment were

prespecified for exclusion; no surviving animal met these criteria. Early deaths and the single later death are reported below. Treatment and testing were conducted in parallel across groups, and the order of behavioral testing and sample analysis was balanced across groups. No single primary outcome was prespecified because this exploratory study assessed complementary neurovascular, molecular, ultrastructural and behavioral outcomes.

CLP model and experimental design

Sepsis-associated encephalopathy was induced by cecal ligation and puncture. Mice were anesthetized with sodium pentobarbital (30 mg/kg, intraperitoneally), and the distal 10% of the cecum was ligated and punctured once with an 18-gauge needle. Sham-operated mice underwent laparotomy and cecal exposure without ligation or puncture. After surgery, mice received pre-warmed sterile saline (40 mL/kg, subcutaneously) and were kept on a heating pad until recovery. A total of 50 mice were used for the in vivo treatment experiment, including 11 sham-operated mice and 39 mice subjected to CLP. At 72 h (day 3) after CLP, 33 mice survived and were randomly assigned to the CLP, CLP+siNC-EVs, and CLP+siMYD88-EVs groups (n=11 per group). One mouse in the CLP group died on day 4 before completion of behavioral testing; therefore, one mouse was randomly excluded from each of the other groups and not included in the behavioral analyses. Behavioral analyses were performed using 10 mice per group that completed the full testing protocol. Beginning on day 3 after CLP, mice in the CLP+siMYD88-EVs and CLP+siNC-EVs groups received siMYD88-EVs or siNC-EVs by tail-vein injection at 2.5×10^{10} particles/kg/day in 100 μ L sterile PBS once daily from day 3 to day 8 after CLP (six injections in total). CLP mice received an equal volume of sterile PBS as vehicle control. Behavioral tests were performed between days 20 and 28, followed by tissue collection. Before tissue collection, mice requiring transcardial perfusion were anesthetized with sodium pentobarbital (30 mg/kg, intraperitoneally), whereas mice used for fresh-tissue collection without perfusion were euthanized with an overdose of sodium pentobarbital (150 mg/kg, intraperitoneally).

EV isolation

Banked human umbilical cord mesenchymal stem cells (hUCMSCs) were supplied at passage 2 by the Nanhua Stem Cell Bank (China). Cells were expanded in Youkang serum-free medium (Youkang, China) under standard conditions, and passage 5 hUCMSCs were used for extracellular vesicle (EV) collection. Conditioned medium was collected when cells reached 70–80% confluence and sequentially centrifuged to remove cells and debris, followed by filtration through a 0.22 µm filter (Millipore, USA). EVs were isolated using the EXODUS H-600 system (Exosome Plus, China) according to the manufacturer's instructions and resuspended in sterile PBS.

EV electroporation and siRNA loading

For siRNA loading, purified EVs were mixed with siMYD88 or siNC and subjected to electroporation under optimized conditions. The protocol was adapted from a previously reported EV electroporation method [28]. Purified hUCMSC-EVs were electroporated with siMYD88 or siNC at a ratio of 1 µg siRNA per 1×10^9 EV particles in 400 µL electroporation buffer using a Gene Pulser Xcell system (Bio-Rad; 400 V, 125 µF, single pulse). After a 10 min incubation on ice, the resulting EVs were termed siMYD88-EVs and siNC-EVs. The siRNA sequences used in this study are listed in Supplementary Table S2.

EV characterization and DiI labeling

EV morphology was examined by transmission electron microscopy (TEM). Briefly, EV suspensions were applied to carbon-coated copper grids, negatively stained with 2% uranyl acetate for 5 min, gently blotted with filter paper, rinsed with distilled water to remove excess stain, air-dried, and imaged using a Hitachi transmission electron microscope (Hitachi, Japan). Particle size distribution and concentration were measured by nanoparticle tracking analysis using a ZetaView system (Particle Metrix, Germany), calibrated with 100 nm microspheres before sample measurement. EV marker proteins, including CD9, CD63, TSG101 and ALIX, together with the negative marker Calnexin, were analyzed by Western blotting. For EV tracing, purified EVs were labeled with the lipophilic fluorescent dye DiI (Beyotime, China). EVs and 5 µM DiI were mixed at a 1:1 volume ratio and incubated at 37 °C for 30 min in the dark, followed by PBS washing and ultracentrifugation to remove unbound dye. For the DiI-only control, sterile PBS was used instead of the EV suspension at the same volume.

Open field test

Mice were placed in the center of a white open-field arena ($40 \times 40 \times 27$ cm) divided into nine equal squares and allowed to explore freely for 5 min. Each mouse was tested once. Locomotor activity was recorded with an overhead camera and analyzed using SMART v3.0 software (Panlab Harvard Apparatus, Spain). Time spent in the central zone and distance traveled in the central zone were quantified. The arena was cleaned with 75% ethanol between trials.

Novel object recognition test

Recognition memory was assessed using the novel object recognition test. On day 1, mice were habituated to an empty arena ($50 \times 50 \times 20$ cm) for 10 min. On day 2, two identical objects were placed in opposite corners and mice were allowed to explore for 10 min. On day 3, one familiar object was replaced with a novel object of similar size and texture but different shape and color. Exploration was defined as sniffing or touching the object. Behavioral data were recorded using a video-tracking system and analyzed using SMART v3.0 software (Panlab Harvard Apparatus, Spain). Recognition index and discrimination index were calculated based on the exploration time of the novel and familiar objects. The arena and objects were cleaned with 75% ethanol between trials.

Barnes maze test

Spatial learning and memory were assessed using a Barnes maze consisting of a rotatable circular platform (75 cm diameter, 18 holes, 10 cm hole diameter), one of which was connected to an escape box. Four visual cues were positioned around the maze, and illumination was set at 400 lumens. Mice underwent habituation (3 min) followed by 6 consecutive days of training and a probe trial 24 h after the final training session. During training, mice were confined in a central start chamber for 1 min and allowed to locate the escape box within 5 min. Mice that failed to locate the target hole were guided to the escape box and kept there for 3 min. Escape latency and error number were recorded using SMART v3.0 software (Panlab Harvard Apparatus, Spain). During the probe trial (2 min), time spent in the target quadrant was analyzed. The platform was randomly rotated between sessions while the target location remained fixed.

RNase protection assay

To assess whether siMYD88 was protected within EVs, siMYD88-EVs or free siMYD88 were incubated with RNase A (100 μ g/mL; Thermo Fisher Scientific, USA) at 37 °C for 30 min. RNase-treated siMYD88-

EVs or free siMYD88 were then applied to BV2 cells for 12 h, and MYD88 mRNA expression was measured by RT-qPCR.

Cell culture and treatment

BV2 microglial cells were kindly provided by Prof. Zengqiang Yuan (Academy of Military Medical Sciences, China) and cultured in high-glucose DMEM (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA) at 37 °C in a humidified incubator containing 5% CO₂. For knockdown validation, BV2 cells were treated with siMYD88-EVs or siNC-EVs, or transfected with siMYD88 or siNC using Chemifect-R (Ruifeng Bio, China) according to the manufacturer's instructions, and collected 12 h after treatment or transfection for RT-qPCR analysis.

Primary microglia were isolated from neonatal C57BL/6 mice (P0-P1; Vital River, China) as previously described. Mixed glial cultures were prepared from cerebral cortices and maintained in DMEM/F12 medium (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, USA) and 1% penicillin-streptomycin. Microglia were harvested by shaking after 10-14 d in vitro and used for subsequent experiments. For EV treatment, cells were seeded in 24-well plates, stimulated with LPS (1 µg/mL; Sigma-Aldrich, USA) for 6 h, and then treated with siMYD88-EVs, siNC-EVs or an equal volume of PBS at a final EV concentration of 1×10^8 particles/mL in 500 µL culture medium for 24 h, unless otherwise indicated.

Evans blue assay

Blood-brain barrier permeability was assessed using the Evans blue (EB) assay. Evans blue dye (2% in PBS; Solarbio, China) was administered by tail-vein injection (4 mL/kg). After circulation for 3 h, mice were transcardially perfused with cold PBS to remove intravascular dye. Brain tissues were collected, weighed, and incubated in formamide (Sigma-Aldrich, USA) at 60 °C for 24 h to extract EB. Samples were centrifuged, and absorbance of the supernatant was measured at 620 nm using a microplate reader (Thermo Fisher Scientific, USA). EB content was quantified using a standard curve and normalized to tissue weight.

Immunofluorescence staining

Brain tissues were fixed in 4% paraformaldehyde (Solarbio, China), dehydrated in 20% and 30% sucrose (Sigma-Aldrich, USA), embedded in OCT compound (Sakura Finetek, USA), and sectioned at 30 µm using a cryostat (Leica, Germany). Sections were washed with PBS, blocked for 20 min at room temperature, and

incubated with primary antibodies overnight at 4 °C in a humidified chamber. After three PBS washes, sections were incubated with appropriate secondary antibodies for 2 h at room temperature in the dark, counterstained with DAPI (Beyotime, China) for 10 min, and mounted with antifade medium (Beyotime, China). Images were acquired using a confocal microscope (Leica, Germany) and analyzed with ImageJ (NIH, USA). For three-dimensional reconstruction, z-stack images were acquired at 0.5 μm intervals and reconstructed using Imaris software (Oxford Instruments, UK). Antibodies used for immunofluorescence staining and Western blotting are listed in Supplementary Table S1.

Western blotting

Brain tissues were lysed in RIPA buffer (Beyotime, China) supplemented with protease and phosphatase inhibitors (Beyotime, China). Equal amounts of protein were separated by SDS–PAGE and transferred to PVDF membranes (Millipore, USA). Membranes were incubated with primary antibodies overnight at 4 °C followed by HRP-conjugated secondary antibodies. Signals were detected using an ECL kit (Thermo Fisher Scientific, USA) with an e-BLOT imaging system (e-BLOT, China) and quantified using ImageJ (NIH, USA).

RT-qPCR

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) and reverse-transcribed using NovoScript® Plus All-in-one 1st Strand cDNA Synthesis SuperMix (Novoprotein, China). Quantitative PCR was performed using a SYBR Green qPCR kit (Novoprotein, China), and relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method. Primer sequences are listed in Supplementary Table S2.

Transmission electron microscopy of hippocampal tissue

Hippocampal CA1 tissue was cut into approximately 1 mm³ blocks, fixed in 2.5% glutaraldehyde, post-fixed with 1% osmium tetroxide, dehydrated through a graded ethanol series, resin-embedded, sectioned into 70-nm ultrathin sections, and stained with uranyl acetate and lead citrate. Sample processing and TEM imaging were performed by Wuhan Servicebio Technology Co., Ltd. (Wuhan, China).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.0 (GraphPad Software, USA). Data are presented as mean ± SEM. Sample sizes and definitions of n are provided in the figure legends. Normality was assessed using the Shapiro–Wilk test. Two-group comparisons were analyzed using unpaired two-tailed

Student's *t*-tests or Welch's *t*-tests, as appropriate. Multiple-group comparisons were analyzed using one-way or two-way ANOVA followed by Tukey's or Dunnett's multiple-comparisons tests. Repeated behavioral measurements were analyzed using two-way repeated-measures ANOVA. Non-parametric data were analyzed using the Mann–Whitney U test or Kruskal–Wallis test. Survival curves were analyzed using the Kaplan–Meier method with log-rank testing. $P < 0.05$ was considered statistically significant.

Results

CLP induces sustained MYD88 upregulation in microglia

To define the post-septic window of myeloid differentiation primary response 88 (MYD88) activation, we examined MYD88 protein expression after cecal ligation and puncture (CLP). CLP surgery was staggered so that sham-operated mice and mice at 28 days, 3 months and 6 months after CLP were sampled at the same terminal age (Figure 1A). Western blotting showed a significant increase in MYD88 protein expression at 28 days after CLP compared with sham controls (Figure 1B,C). MYD88 remained numerically higher at 3 months but was no longer significantly different from sham, and returned close to sham levels by 6 months. Thus, MYD88 elevation was most evident at 28 days after CLP, the time point selected for subsequent intervention studies.

Multiplex immunofluorescence was used to examine the distribution of MYD88 signal at 28 days after CLP. Sham brain sections showed low MYD88 signal, with limited overlap around IBA1-positive microglia. After CLP, MYD88 immunoreactivity increased in IBA1-positive cells and in linear structures with a vessel-like distribution (Figure 1D). These data indicate that post-septic MYD88 elevation was detectable at the whole-tissue level and was spatially associated with microglial and vessel-like signals.

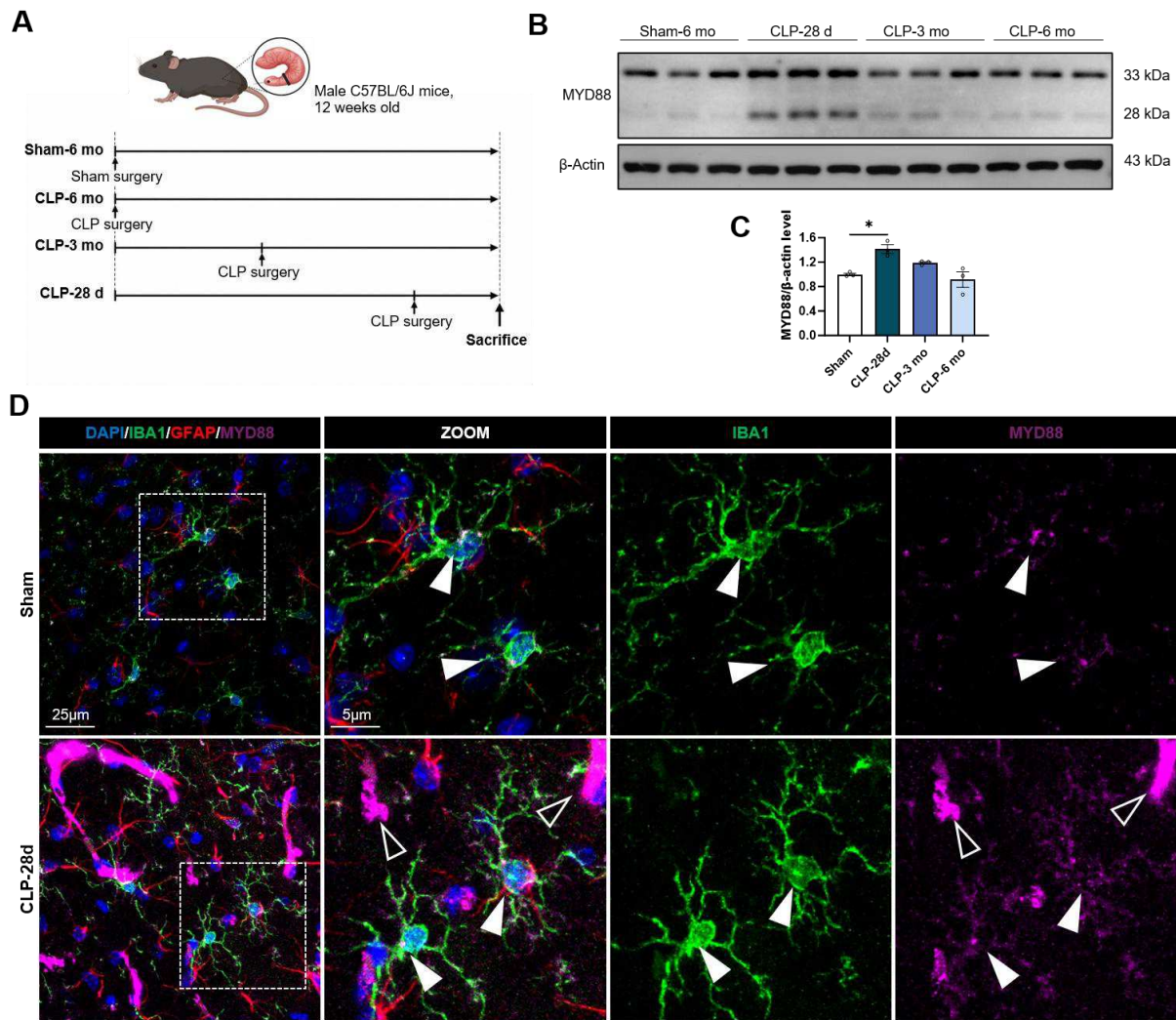


Figure 1. Sustained MYD88 upregulation after CLP and its co-localization with microglia. (A)

Staggered experimental timeline of the CLP model. (B) MYD88 expression in hippocampal tissues at different time points after CLP. (C) MYD88 protein expression normalized to β-actin (Sham=1; n=3; mean ± SEM; one-way ANOVA with Tukey's test; * $P < 0.05$). (D) High-magnification immunofluorescence images showing DAPI, IBA1, GFAP and MYD88 signals in sham and CLP-28d brain sections. Dashed boxes indicate enlarged regions; filled arrowheads indicate MYD88-positive microglia overlapping with IBA1; open arrowheads indicate high-intensity MYD88-positive vessel-like structures.

siMYD88-loaded hUCMSC-EVs mediate MYD88 knockdown and brain delivery

On the basis of the temporal pattern of sustained postoperative MYD88 elevation, we prepared extracellular vesicles (EVs) derived from human umbilical cord mesenchymal stem cells (hUCMSCs) for the delivery of MYD88-targeting small interfering RNA (siRNA). hUCMSCs were maintained in commercial serum-free medium, and passage-5 cells were used for EV collection. EVs were isolated from hUCMSC-conditioned

medium using the EXODUS H-600 negative-pressure oscillation system and were electroporated with siMYD88 or siNC to generate siMYD88-EVs and siNC-EVs, respectively (Figure 2A). Nanoparticle tracking analysis (NTA) showed a main particle peak at 102.8 nm, a median diameter of 104.9 nm, and an original particle concentration of approximately 1.0×10^{10} particles/mL (Figure 2B). Transmission electron microscopy (TEM) showed membrane-bound vesicles of approximately 100 nm in diameter with a saucer-like morphology (Figure 2C). Western blotting showed enrichment of the EV markers ALIX, TSG101, CD9, and CD63, whereas the endoplasmic reticulum marker calnexin was absent from EV preparations (Figure 2D).

We next examined whether EV loading could protect the target-gene knockdown activity of siMYD88 in the BV2 microglial cell line. After 12 h of treatment, siMYD88-EVs reduced MYD88 mRNA expression to a level comparable to that achieved by chemically transfected siMYD88. RNase A pretreatment did not abolish the knockdown effect of siMYD88-EVs, whereas RNase A-treated free siMYD88 failed to produce a comparable effect. These results indicate that EV encapsulation protects siMYD88 from enzymatic degradation and allows functional intracellular regulation in BV2 cells (Figure 2G). We then examined whether siMYD88-EVs exerted a similar knockdown effect in primary microglia under inflammatory stimulation. Primary microglia were stimulated with LPS (1 μ g/mL) for 6 h and then treated with siMYD88-EVs or siNC-EVs for 24 h. Compared with control cells, LPS increased MYD88 expression. siMYD88-EVs markedly reduced the LPS-induced MYD88 signal, whereas siNC-EVs produced only a partial reduction (Figure 2E,F).

To verify whether intravenously administered siMYD88-EVs could mediate brain delivery, EVs were labeled with DiI. DiI-labeled siMYD88-EVs were injected via the tail vein, with blank control and free DiI groups included for comparison. After administration of DiI-labeled siMYD88-EVs, DiI signals were clearly detected in brain sections, and signals were visible within microglial somata, whereas free DiI produced relatively weak signals (Figure 2H,I). These results indicate that, even in healthy mice, at least a fraction of systemically administered siMYD88-loaded hUCMSC-EVs entered the brain and was taken up by microglia.

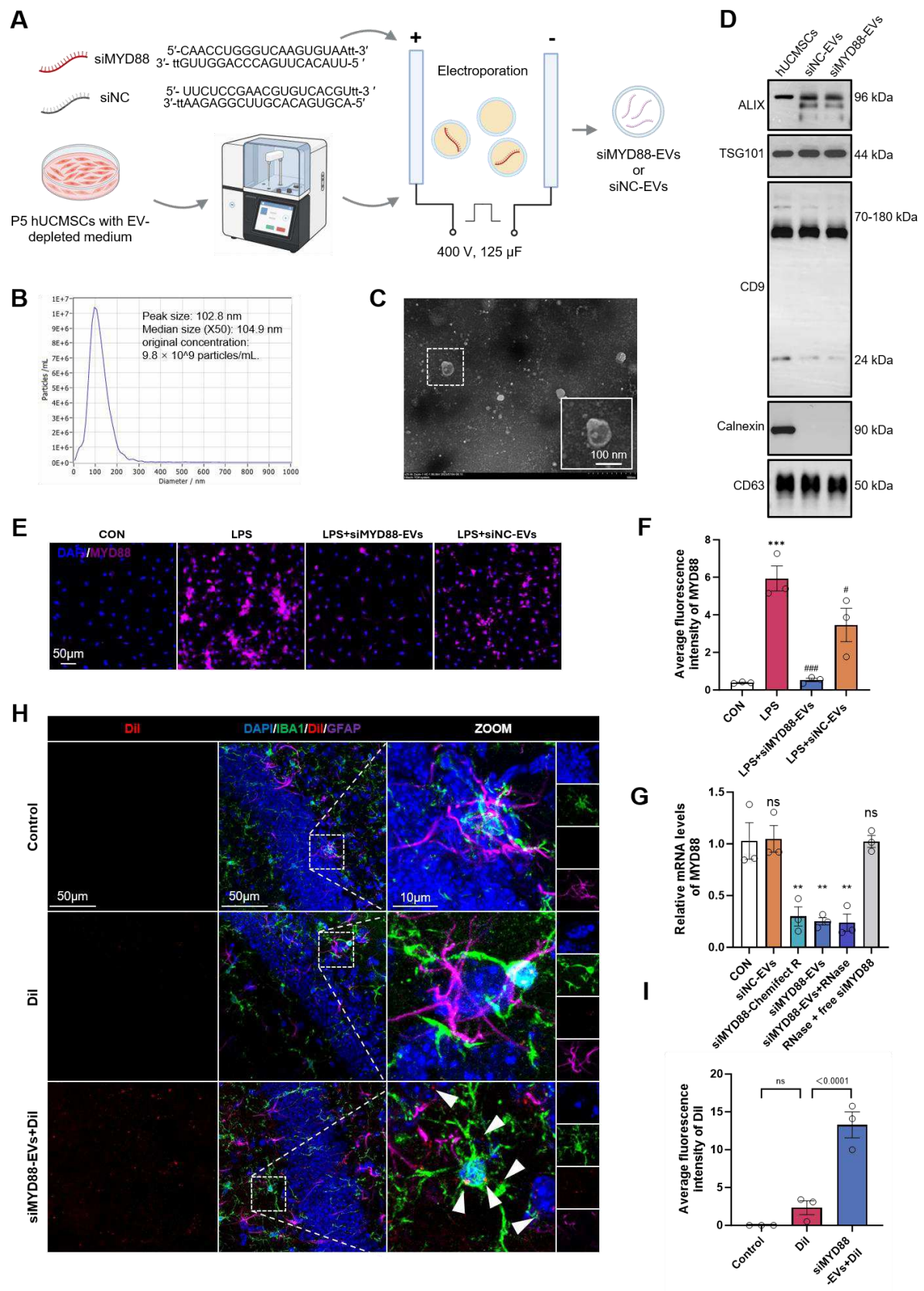


Figure 2. Preparation, characterization, and delivery validation of siMYD88-loaded hUCMSC-derived EVs. (A) Workflow for preparing engineered hUCMSC-derived siMYD88-EVs. (B) Nanoparticle tracking analysis (NTA) profile of isolated hUCMSC-derived EVs. Peak diameter, 102.8 nm; median

diameter, 104.9 nm; original concentration, 9.8×10^9 particles/mL. (C) Representative TEM image of isolated hUCMSC-derived EVs. (D) Western blot analysis of ALIX, TSG101, CD9, CD63, and calnexin in hUCMSCs, siNC-EVs, and siMYD88-EVs. (E) MYD88 immunofluorescence in primary microglia treated with control medium, LPS, LPS+siMYD88-EVs, or LPS+siNC-EVs. (F) Quantification of MYD88 fluorescence intensity in primary microglia. For (F), $n=3$; mean $\pm$ SEM; one-way ANOVA followed by multiple-comparisons test; *** $P < 0.001$ versus control; # $P < 0.05$ and ##### $P < 0.0001$ versus LPS. (G) MYD88 mRNA expression in BV2 cells treated for 12 h with control medium, siNC-EVs, chemically transfected siMYD88, siMYD88-EVs, RNase A-pretreated siMYD88-EVs, or RNase A-treated free siMYD88. For (G), $n=3$; mean $\pm$ SEM; one-way ANOVA followed by multiple-comparisons test; ** $P < 0.01$ versus CON; ns, not significant versus CON. (H) DiI signals in brain sections after tail-vein administration of DiI-labeled siMYD88-EVs. (I) Quantification of DiI fluorescence intensity in brain sections from the control, DiI-only, and DiI-labeled siMYD88-EV groups. For (I), $n=3$; mean $\pm$ SEM; one-way ANOVA followed by Dunnett's test; ns, not significant; exact P values are indicated in the panel.

siMYD88-EVs attenuate post-septic BBB disruption and vascular-associated MYD88 activation

After confirming brain delivery of siMYD88-EVs, we further evaluated whether tail-vein administration of siMYD88-EVs could rescue post-septic neurovascular injury in vivo. Mice underwent 10% CLP on day 0, which caused limited early mortality before treatment allocation (Supplementary Figure 1). Surviving CLP mice were assigned to untreated CLP, CLP+siMYD88-EVs or CLP+siNC-EVs groups. EV treatment was administered once daily from day 3 to day 8 after surgery, followed by behavioral testing from day 20 to day 28 and tissue collection on day 28 (Figure 3A).

Because MYD88 remained elevated during the post-septic phase and showed vessel-like signals in brain sections, we first assessed blood-brain barrier (BBB) permeability using Evans blue staining. At 28 days after surgery, mild Evans blue retention was still visible in coronal brain sections from CLP mice, together with increased Evans blue absorbance in whole-brain homogenates. siMYD88-EV treatment markedly reduced Evans blue extravasation, whereas siNC-EVs produced a partial protective effect (Figure 3B-D).

We then examined whether this vascular protection was accompanied by reduced MYD88 expression within the brain parenchyma. In the prefrontal cortex, sham brain sections showed low basal MYD88 and IBA1 signals, whereas CLP induced strong MYD88 immunoreactivity together with enhanced IBA1-positive

microglial activation. siMYD88-EVs significantly reduced total MYD88 fluorescence, IBA1 fluorescence and MYD88 signal within IBA1-positive areas. Compared with siNC-EVs, siMYD88-EVs produced a stronger reduction in MYD88 expression, consistent with delivery of MYD88-targeting siRNA (Supplementary Figure 2A,B).

Vascular-associated MYD88 signals were further examined by multiplex staining for CD31, CD11B and MYD88, followed by Imaris-based three-dimensional reconstruction and colocalization rendering. CD31 was used to label vascular endothelial structures, whereas CD11B was used to identify myeloid-lineage cells associated with vascular or perivascular compartments. In sham mice, MYD88 signal was sparse within vascular structures and showed more evident colocalization with CD11B-positive myeloid signals. Under physiological conditions, these vascular-associated CD11B-positive signals likely include perivascular myeloid populations, although their precise identity cannot be resolved by CD11B staining alone. After CLP, MYD88 immunoreactivity increased markedly within CD31-positive vascular endothelial structures, forming a continuous endothelial-associated distribution pattern. Because the endothelial compartment became strongly MYD88-positive after CLP, the fluorescence contribution of internal or adjacent CD11B-positive myeloid cells within inflamed vessels was difficult to distinguish. This endothelial-associated MYD88 pattern persisted in CLP+siNC-EV-treated mice but was markedly reduced after siMYD88-EV treatment (Figure 3E).

To determine whether these vascular MYD88 changes were accompanied by molecular evidence of BBB remodeling, we examined MMP9 and claudin-5. CLP increased MMP9 protein expression and reduced claudin-5 abundance, consistent with matrix remodeling and tight-junction disruption. siMYD88-EVs suppressed MMP9 induction and restored claudin-5 expression, whereas siNC-EVs showed weaker or incomplete effects (Figure 3F-H). Thus, siMYD88-EV treatment reduced endothelial-associated MYD88 activation after CLP, improved the MMP9/claudin-5 imbalance and ultimately protected BBB integrity.

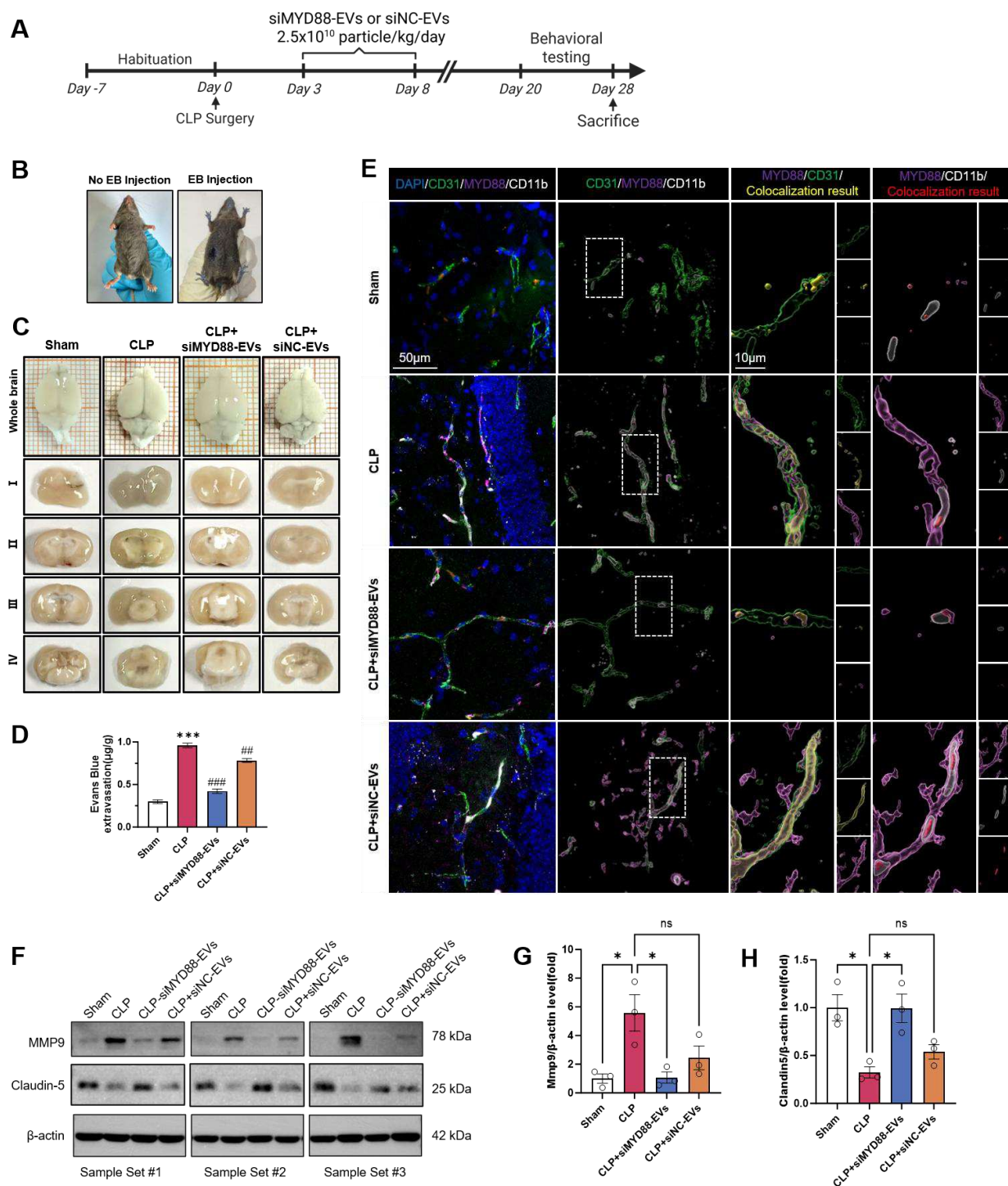


Figure 3. siMYD88-EVs protect BBB integrity and attenuate vascular-associated MYD88

overexpression after CLP. (A) In vivo treatment timeline. Mice underwent 10% CLP on day 0; siMYD88-EVs or siNC-EVs were administered from day 3 to day 8; behavioral testing was performed from day 20 to day 28; tissues were collected on day 28. (B) Gross appearance of mice with or without Evans blue (EB) tail-vein administration for 3 h. (C) Whole-brain and coronal brain-section images from Sham, CLP, CLP+siMYD88-EVs and CLP+siNC-EVs groups. The pale gray-blue staining in CLP mice

indicates EB leakage. (D) EB extravasation in brain tissues collected after PBS perfusion (n=4; mean $\pm$ SEM; one-way ANOVA with multiple-comparisons test; ### $P < 0.001$ versus Sham; ## $P < 0.01$ versus CLP). (E) Multiplex immunofluorescence and colocalization renderings of DAPI, CD31, MYD88 and CD11B in brain sections. CD31 labels vascular endothelial cells; CD11B labels myeloid-lineage cells. Yellow rendering indicates colocalized MYD88 signal within vascular endothelial structures, and white rendering indicates colocalized MYD88 and CD11B signals. Dashed boxes indicate enlarged regions. (F) Western blots of MMP9, claudin-5 and β -actin in hippocampal tissues; three independent biological sample sets are shown. (G,H) MMP9 and claudin-5 protein levels normalized to β -actin (n=3; mean $\pm$ SEM; one-way ANOVA with Tukey's test; * $P < 0.05$, ** $P < 0.01$; ns, not significant).

siMYD88-EVs suppress hippocampal microglial activation and inflammatory-redox injury signaling after CLP

Because blood-brain barrier disruption and neurovascular injury may disturb brain neuroimmune homeostasis, we examined microglial and astrocytic immunofluorescence signals in the hippocampus. In sham mice, IBA1-positive microglia showed relatively low fluorescence intensity across hippocampal subfields, accompanied by weak MYD88 signals. CLP induced broad increases in IBA1 immunoreactivity in the CA1, CA3 and DG regions, together with markedly enhanced MYD88 fluorescence (Figure 4A-C). siMYD88-EV treatment significantly reduced MYD88 fluorescence across the examined hippocampal regions and was accompanied by decreased IBA1 signals. siNC-EVs also reduced IBA1 fluorescence, although the within-group variability was greater (Figure 4B,C).

In contrast, GFAP fluorescence did not show a robust CLP-induced increase across hippocampal subfields, and EV-related changes were modest and region-dependent (Figure 4A,D). These results indicate that siMYD88-EVs mainly suppressed CLP-induced MYD88-associated inflammatory responses in microglia rather than producing a dominant effect on astrocytes.

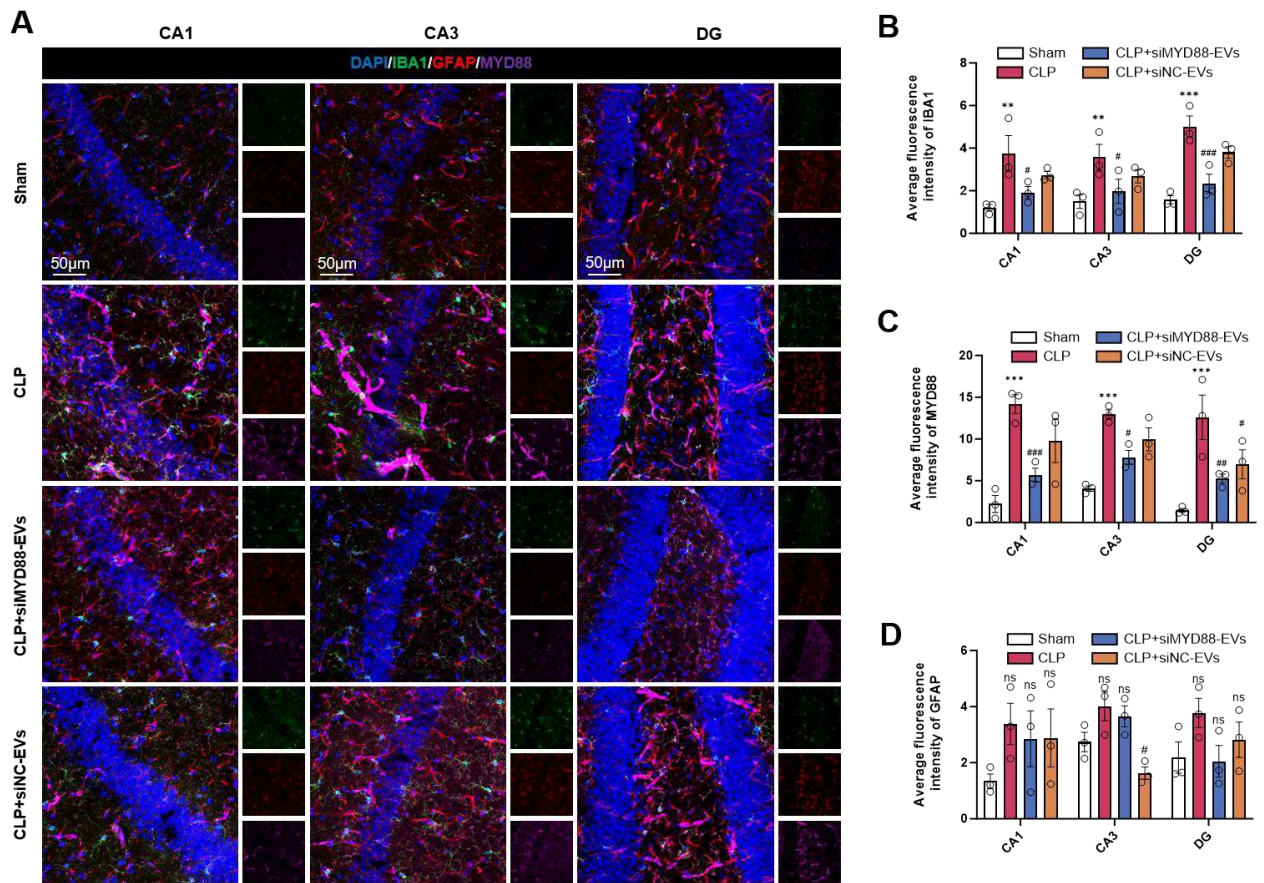


Figure 4. siMYD88-EVs attenuate hippocampal MYD88-associated microglial activation after CLP.

(A) Immunofluorescence images showing DAPI, IBA1, GFAP and MYD88 staining in the hippocampal CA1, CA3 and DG regions from Sham, CLP, CLP+siMYD88-EVs and CLP+siNC-EVs mice. (B) IBA1 fluorescence intensity. (C) MYD88 fluorescence intensity. (D) GFAP fluorescence intensity. For (B-D), $n=3$; mean $\pm$ SEM; one-way ANOVA with Dunnett's test within each hippocampal region. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ versus Sham; # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ versus CLP; ns, not significant.

To further define the position at which MYD88 knockdown acted within the inflammatory cascade, we examined the TLR4-MYD88-NF- κ B signaling axis and downstream effector proteins in brain tissues. As an upstream pattern-recognition receptor that recruits MYD88-dependent signaling after ligand engagement, TLR4 protein abundance did not differ significantly among groups (Figure 5A,B). In contrast, consistent with the preceding results, CLP markedly increased sustained MYD88 protein expression, whereas siMYD88-EV rescue treatment reduced MYD88 to near-sham levels; siNC-EVs did not produce this effect (Figure 5A,C). CLP also increased the phosphorylated NF- κ B p65 signal and total NF- κ B p65 abundance, and these changes were attenuated by siMYD88-EVs (Figure 5A,D). However, because total NF- κ B p65 increased in parallel, the phosphorylated NF- κ B p65/total NF- κ B p65 ratio did not differ significantly among

415 groups (Figure 5A,E). IRAK4 protein abundance, assessed as a proximal kinase in MYD88-dependent
416 signaling, was also not significantly altered by CLP or EV treatment (Supplementary Figure 3A,B). Thus,
417 the principal treatment-responsive change was sustained MYD88/NF- κ B p65 signaling rather than upstream
418 TLR4 abundance or total IRAK4 expression.

419 Because COX2 is a robust downstream effector of NF- κ B signaling and can contribute to oxidative
420 intermediate production and lipid peroxidation-related stress, we examined COX2 as a downstream
421 inflammatory-redox effector. CLP strongly increased COX2 protein expression, whereas siMYD88-EVs
422 suppressed this induction; siNC-EVs did not reduce COX2 to the same extent (Figure 5F,G). We then
423 examined whether this response was accompanied by impaired endogenous antioxidant defense. NRF2, a
424 central regulator of cellular antioxidant responses, was significantly reduced after CLP, and siMYD88-EV
425 treatment restored NRF2 protein expression to near-sham levels; siNC-EVs did not show a significant
426 restorative effect (Figure 5H,I). KEAP1, the canonical negative regulator of NRF2, showed a non-significant
427 increase after CLP alone; however, compared with CLP, both EV treatments significantly reduced KEAP1,
428 with the lowest mean level observed in the siMYD88-EV group (Figure 5H,J). HO-1, a downstream
429 antioxidant effector of NRF2 signaling, was decreased after CLP and recovered most clearly in the
430 siMYD88-EV group, indicating that MYD88 knockdown reactivated NRF2/HO-1-associated antioxidant
431 defense (Figure 5H,K). We also examined the COP1/C/EBP β signaling axis to assess whether MYD88
432 inhibition was accompanied by broader inflammatory transcriptional regulation. CLP reduced the
433 phosphorylated COP1/total COP1 ratio, whereas siMYD88-EVs restored COP1 phosphorylation
434 (Supplementary Figure 4A,B). Total C/EBP β abundance did not differ significantly among groups
435 (Supplementary Figure 4A,C), indicating that the detectable change in this module was mainly associated
436 with COP1 phosphorylation status rather than altered total C/EBP β expression.

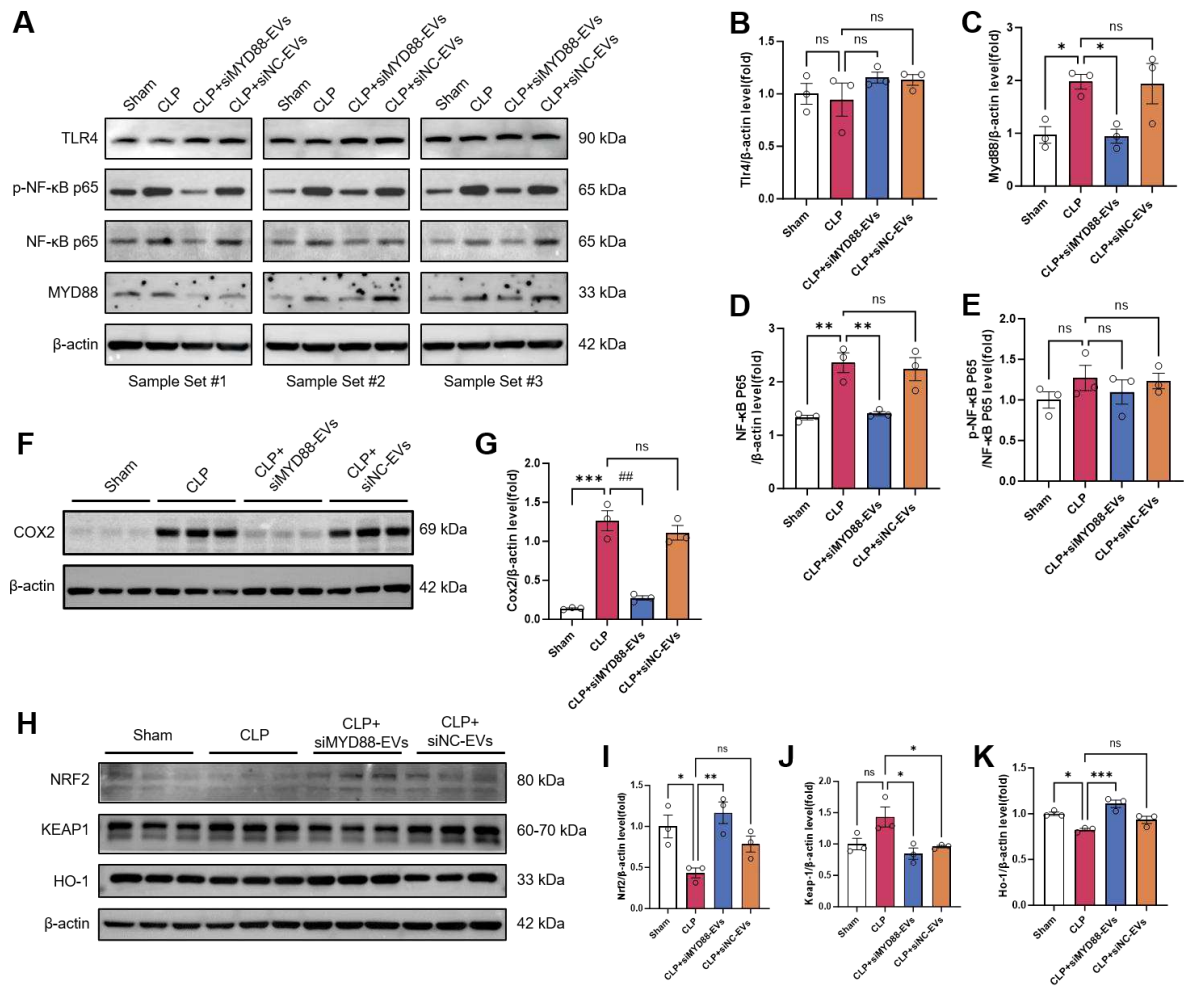


Figure 5. siMYD88-EVs suppress MYD88-NF-κB-COX2 inflammatory signaling and restore antioxidant responses after CLP. (A) Representative Western blots of TLR4, phosphorylated NF-κB p65, total NF-κB p65, MYD88 and β-actin in brain tissues; three independent biological samples are shown per group. (B) Quantification of TLR4 protein expression. (C) Quantification of MYD88 protein expression. (D) Quantification of total NF-κB p65 protein expression. (E) Quantification of the phosphorylated NF-κB p65/total NF-κB p65 ratio. (F) Western blots of COX2 and β-actin. (G) COX2 protein expression. (H) Representative Western blots of NRF2, KEAP1, HO-1 and β-actin. (I-K) NRF2, KEAP1 and HO-1 protein levels normalized to β-actin. For Western blot analyses, $n=3$; mean $\pm$ SEM; one-way ANOVA with Tukey's test. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$; $\#P < 0.05$ and $##P < 0.01$; ns, not significant.

siMYD88-EVs enhance TREM2 expression during post-septic inflammatory resolution

Next, we investigated whether suppression of MYD88 inflammatory signaling was accompanied by changes in microglial homeostatic and repair-associated signals. Triggering receptor expressed on myeloid cells 2 (TREM2), a marker associated with microglial damage sensing, phagocytic adaptation and repair-related

451 responses, was only mildly and non-significantly increased after CLP alone. Notably, the most pronounced
452 increase in TREM2 reactivity emerged after targeted suppression of MYD88-driven inflammation.
453 siMYD88-EV treatment markedly enhanced TREM2 signal while reducing MYD88-dominant inflammatory
454 features, whereas siNC-EVs produced a weaker effect (Figure 6A,C). Western blotting confirmed that
455 TREM2 protein abundance was higher in the siMYD88-EV group than in the CLP and siNC-EV groups
456 (Figure 6B,D).

457 We further reproduced this phenomenon ex vivo. Primary microglia were stimulated with LPS (1 µg/mL)
458 for 6 h and then treated with siMYD88-EVs, siNC-EVs or an equal volume of PBS for 24 h. LPS increased
459 IBA1 and MYD88 signals but reduced TREM2 fluorescence intensity, suggesting that TREM2 expression
460 was suppressed during early inflammatory activation. siMYD88-EVs attenuated IBA1-positive microglial
461 activation, restored TREM2 signal and markedly reduced the MYD88/TREM2 ratio, whereas siNC-EVs had
462 a weaker effect (Figure 6E-H). Together, these in vivo and ex vivo data indicate that MYD88 silencing did
463 not simply suppress microglial activation, but shifted the balance of microglial responses from a
464 TLR4/MYD88/NF-κB-dominant inflammatory state toward a TREM2-associated response.

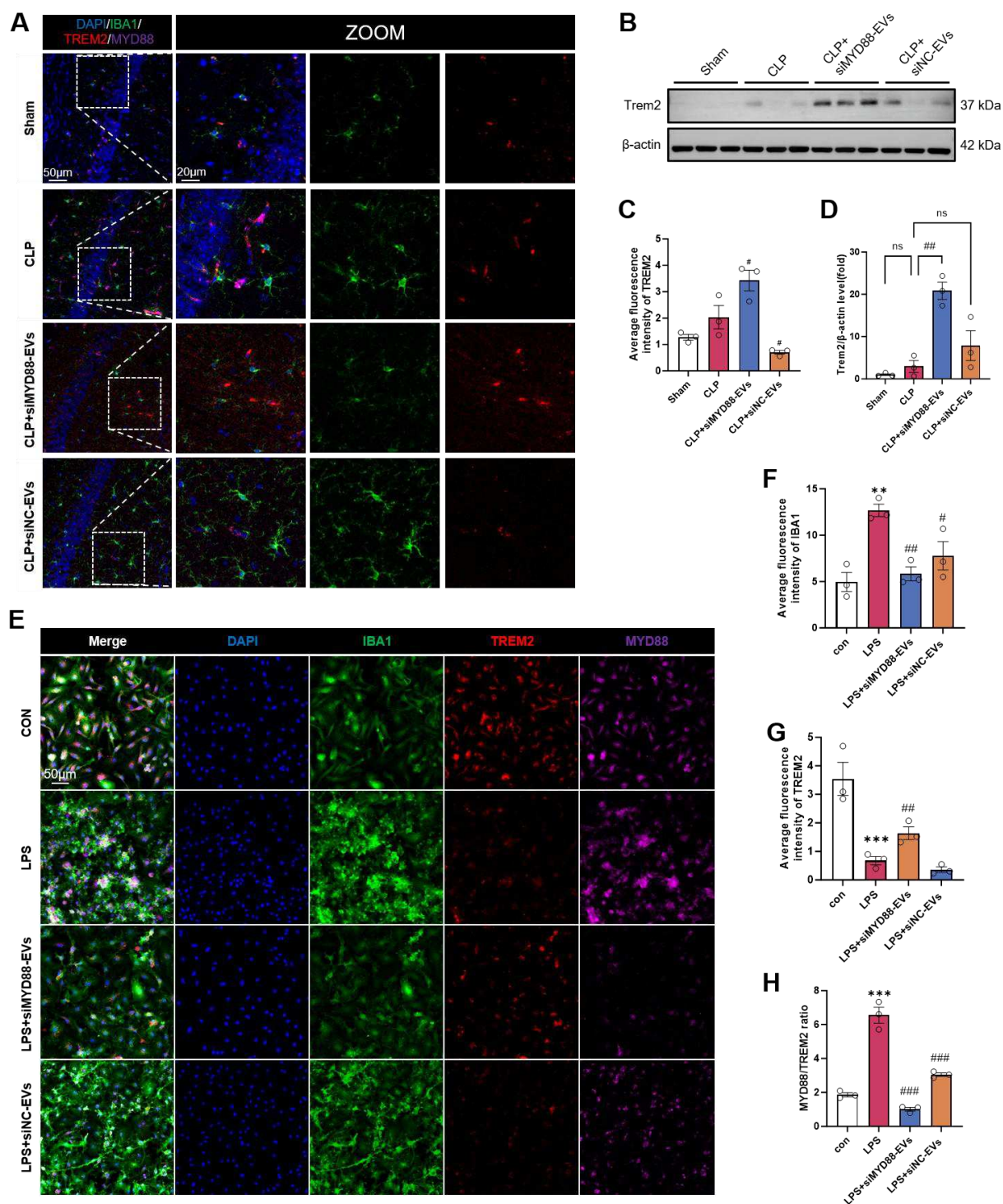


Figure 6. siMYD88-EVs enhance TREM2-associated microglial responses after CLP. (A) Multiplex immunofluorescence images showing DAPI, IBA1, TREM2 and MYD88 staining in brain sections from Sham, CLP, CLP+siMYD88-EVs and CLP+siNC-EVs mice. Dashed boxes indicate enlarged regions. (B) Representative Western blots of TREM2 and β -actin in brain tissues. (C) Mean TREM2 fluorescence intensity in brain sections. (D) TREM2 protein expression normalized to β -actin. For (C,D), $n=3$; mean $\pm$ SEM; one-way ANOVA with Tukey's test; $*P < 0.05$ versus Sham; $###P < 0.01$ versus CLP; ns, not

significant. (E) Immunofluorescence images of primary microglia treated with control medium, LPS, LPS+siMYD88-EVs or LPS+siNC-EVs and stained for DAPI, IBA1, TREM2 and MYD88. (F-H) IBA1 fluorescence intensity, TREM2 fluorescence intensity and MYD88/TREM2 fluorescence intensity ratio (n=3; mean $\pm$ SEM; one-way ANOVA with Dunnett's test; *** P < 0.001 versus control; # P < 0.05, ## P < 0.01 and ### P < 0.001 versus LPS).

siMYD88-EVs preserve hippocampal neuronal mitochondrial and synaptic ultrastructure after CLP

Hippocampal neuronal ultrastructure in the CA1 region further revealed changes consistent with downstream consequences of sustained neuroinflammation and impaired antioxidant defense after CLP, as well as the protective effects of siMYD88-EVs. In sham mice, neuronal mitochondria displayed relatively preserved morphology with intact membranes and organized cristae. CLP mice showed mitochondrial swelling and structural disruption, as highlighted in the enlarged regions and by black arrowheads. siMYD88-EV treatment alleviated these abnormalities, with most mitochondria showing preserved double-membrane structures and organized cristae, whereas siNC-EVs provided only partial protection (Figure 7A). Consistently, CLP increased mitochondrial area and the proportion of damaged mitochondria, both of which were reduced after siMYD88-EV treatment (Figure 7B,C).

Synaptic ultrastructure showed a similar protective pattern. Sham mice exhibited clearly organized synaptic contacts, with identifiable postsynaptic density (PSD) regions and abundant synaptic vesicles. After CLP, synaptic organization was impaired, with reduced vesicle density and a thinner or less distinct PSD. The dotted outlines in the enlarged TEM panels mark PSD regions, and black arrowheads indicate representative synaptic vesicles and PSD-associated structures. siMYD88-EVs improved PSD thickness and restored synaptic vesicle density, whereas siNC-EVs showed a weaker rescue effect (Figure 7A,D,E).

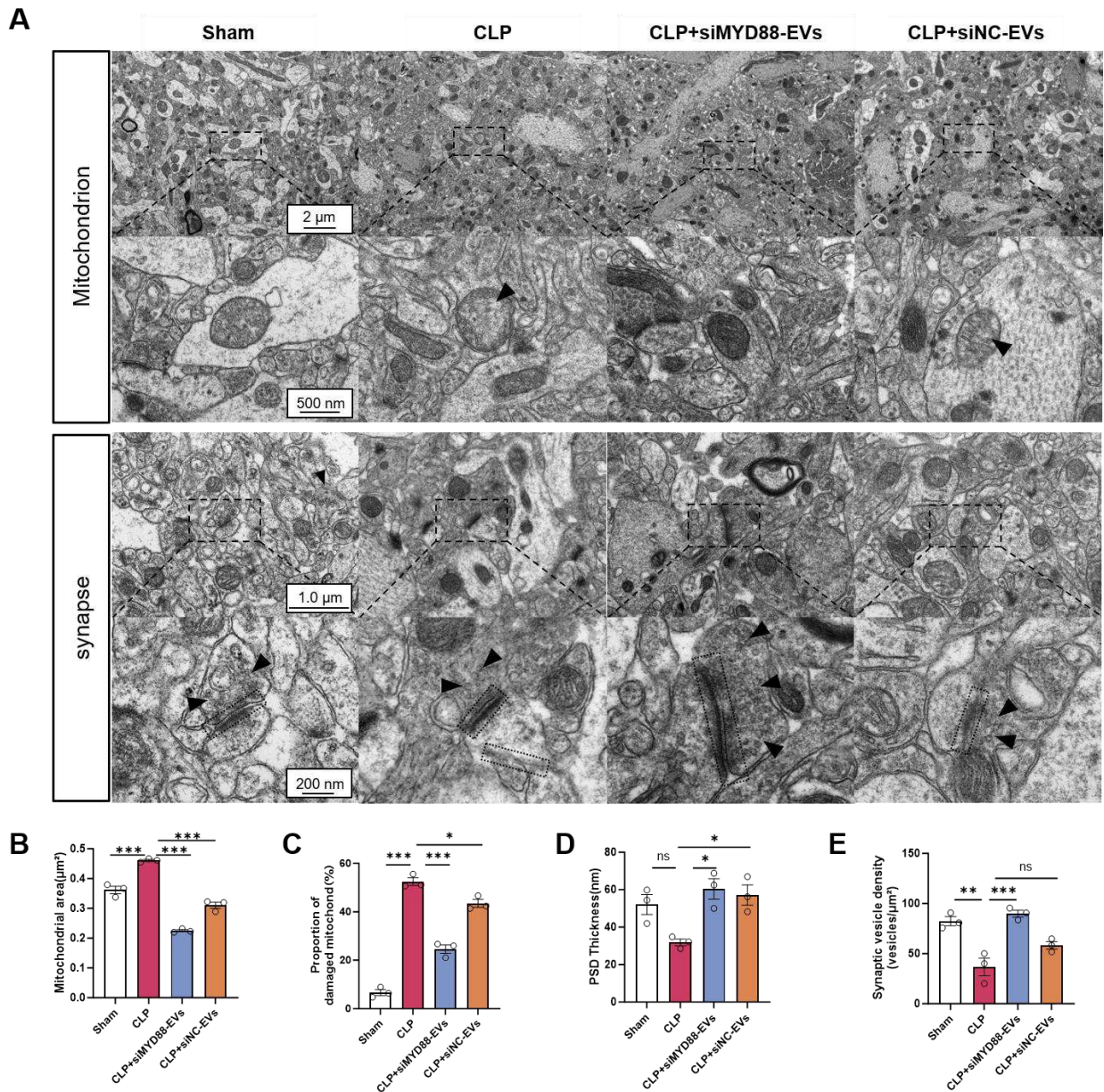


Figure 7. siMYD88-EVs preserve hippocampal mitochondrial and synaptic ultrastructure after CLP.

(A) Transmission electron microscopy images of hippocampal mitochondrial and synaptic ultrastructure from Sham, CLP, CLP+siMYD88-EVs and CLP+siNC-EVs mice. Dashed boxes indicate enlarged regions; black arrowheads indicate mitochondrial or synaptic abnormalities; dotted outlines mark postsynaptic density (PSD) regions. (B) Mitochondrial area. (C) Proportion of damaged mitochondria. (D) PSD thickness. (E) Synaptic vesicle density. For (B-E), $n=3$; mean $\pm$ SEM; one-way ANOVA with Tukey's test. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$; ns, not significant.

siMYD88-EVs rescue post-septic anxiety-like behavior and cognitive deficits

Having shown that siMYD88-EVs limited post-septic neurovascular disruption, inflammatory-redox signaling and hippocampal ultrastructural injury, we next determined whether these protective effects translated into behavioral recovery. Anxiety-like behavior, spatial learning and recognition memory were therefore assessed during the late post-septic phase.

In the open-field test, representative movement heatmaps showed reduced central exploration in CLP mice compared with sham mice (Figure 8A). Total distance traveled did not differ significantly among groups, indicating that the behavioral changes were not mainly caused by gross locomotor impairment (Figure 8B). Compared with sham mice, CLP mice showed marked reductions in both distance traveled in the center area and time spent in the center area, whereas siMYD88-EV intervention significantly increased both measures of central exploration. siNC-EVs also increased center distance but did not significantly increase center time (Figure 8C,D).

Spatial learning and memory were further assessed using the Barnes maze. Representative movement traces during the probe trial showed that sham mice preferentially searched near the target area, whereas CLP mice showed more dispersed search patterns. Mice receiving siMYD88-EVs showed more target-directed searching than untreated CLP mice (Figure 8E,F). During training, CLP mice showed persistently longer escape latency and more errors. The training curves showed shorter escape latency and fewer errors in the siMYD88-EV group than in the CLP group during the later training phase, whereas the siNC-EV group showed less stable improvement (Figure 8G,H). Similarly, in the probe trial, siMYD88-EVs significantly increased the time spent in the target quadrant. siNC-EVs did not significantly improve this measure (Figure 8I).

Novel object recognition was used to assess recognition memory. Compared with sham mice, CLP mice showed reduced exploration of the novel object (Figure 8J,K). Quantification of the discrimination ratio further confirmed impaired recognition memory after CLP. siMYD88-EVs restored the discrimination ratio, whereas siNC-EVs produced only a slight, non-significant increase (Figure 8L).

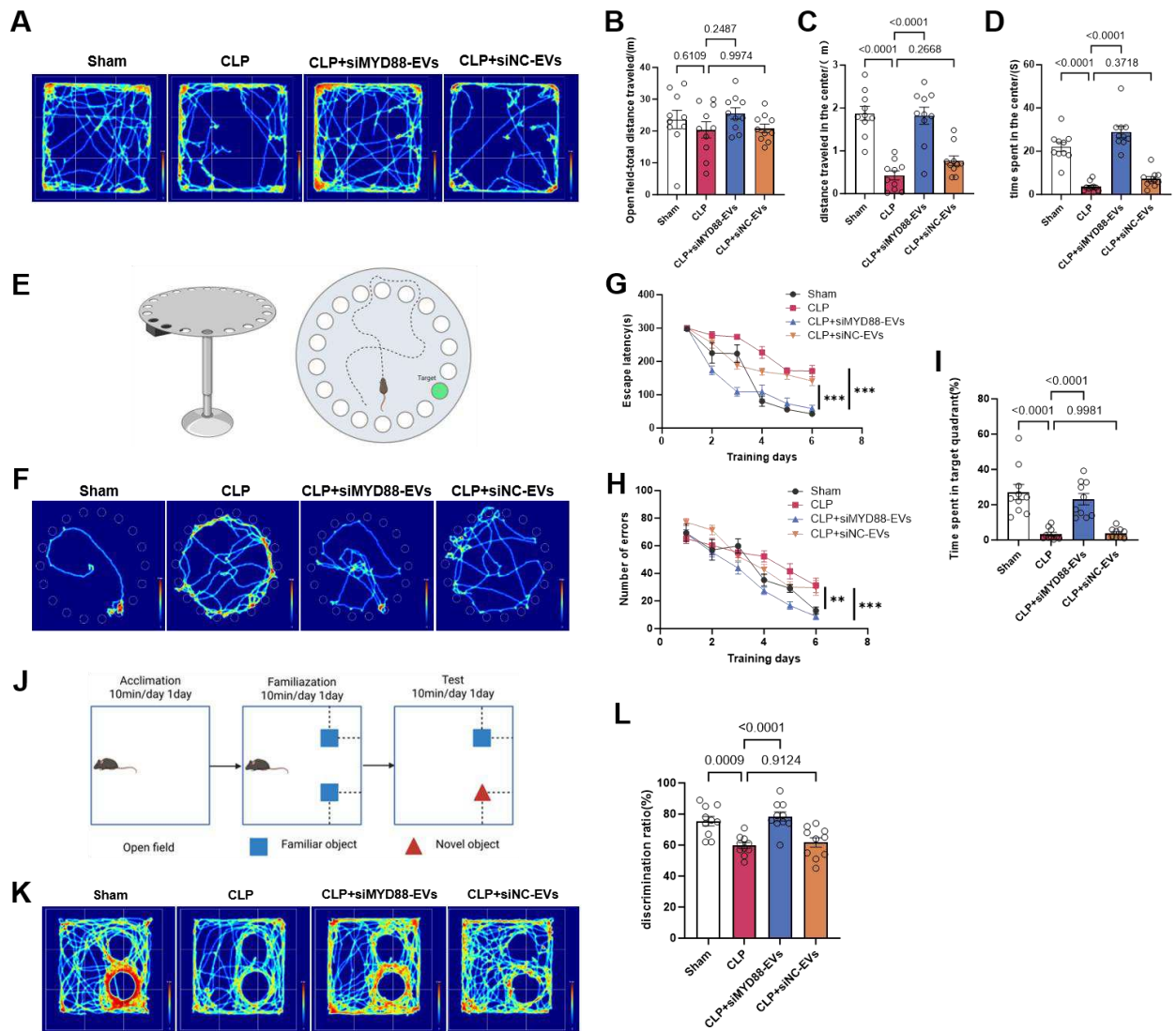


Figure 8. siMYD88-EVs improve post-septic anxiety-like behavior and cognitive deficits. (A) Open-field movement heatmaps. (B) Total distance traveled. (C) Distance traveled in the center area. (D) Time spent in the center area. For (B-D), $n=10$; mean $\pm$ SEM; one-way ANOVA with Dunnett's test using CLP as the reference group; exact P values are indicated. (E) Barnes maze schematic. (F) Movement traces during the Barnes maze probe trial. (G) Escape latency during Barnes maze training. (H) Number of errors during Barnes maze training. For (G,H), $n=10$; mean $\pm$ SEM; two-way repeated-measures ANOVA. (I) Time spent in the target quadrant during the Barnes maze probe trial ($n=10$; mean $\pm$ SEM; one-way ANOVA with Dunnett's test using CLP as the reference group; exact P values are indicated). (J) Novel object recognition schematic. (K) Novel object recognition movement heatmaps. (L) Discrimination ratio ($n=10$; mean $\pm$ SEM; one-way ANOVA with Dunnett's test using CLP as the reference group; exact P values are indicated).

Discussion

In this study, EV treatment was initiated 72 h after CLP as a rescue intervention. This design allowed us to evaluate whether siMYD88-EVs could treat established post-septic neurovascular injury and neuroinflammation after the initial septic insult. Under these stringent conditions, hUCMSC-derived EVs carrying negative-control siRNA showed partial protective effects in several readouts, including Evans blue extravasation, hippocampal microglial activation and open-field behavior. Consistent with previous studies, hUCMSCs and their EVs have been reported to possess intrinsic immunomodulatory, anti-inflammatory and redox-regulatory properties in multiple inflammatory models [27]. hUCMSCs have also been reported to exhibit low immunogenicity even in mice and favorable tolerability in clinical and non-clinical safety studies [28]. Against this background, the additional benefits observed with siMYD88-EVs are more consistent with targeted MYD88 silencing than with the basal activity of the EV carrier alone. Our data suggest that EV-mediated MYD88 silencing attenuates post-septic neurovascular disruption, inflammatory-redox imbalance and hippocampal injury, placing MYD88 as a potential signaling node linking innate immune activation, redox imbalance and neurovascular remodeling in SAE (Figure 9).

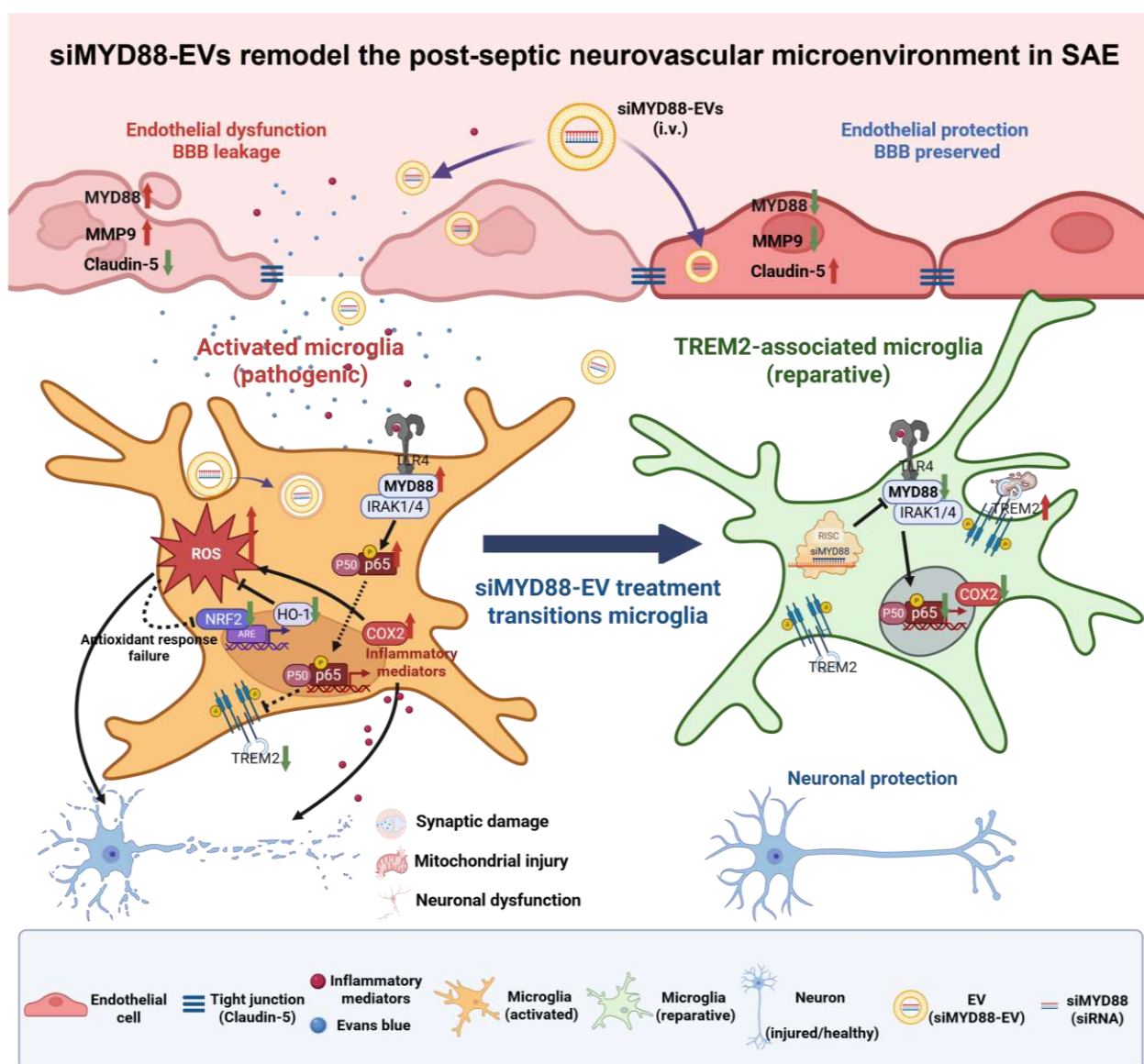


Figure 9. Proposed model of siMYD88-EV-mediated neurovascular protection and microglial rebalancing in SAE. Black arrows and black T-shaped inhibitory lines indicate direct activation and direct inhibition, respectively. Dashed lines indicate indirect effects. Red upward arrows and green downward arrows indicate increased and decreased expression, respectively, in the indicated pathological or treatment state.

MYD88 mediates signaling downstream of the IL-1 receptor family and all Toll-like receptors except TLR3. After receptor engagement, MYD88 nucleates myddosome assembly, bringing together MYD88, IRAK4 and IRAK1/2; within this complex, IRAK4 acts as the proximal kinase that licenses downstream NF- κ B/MAPK activation and inflammatory cytokine production [29]. MYD88 signaling may persist after the acute septic phase through innate immune reprogramming and trained-immunity-like mechanisms, even in the absence of increased upstream TLR4 abundance [30, 31]. In this context, elevated MYD88 may act

less as a transient acute-phase adaptor than as an amplifier of chronic neuroimmune activation, thereby intensifying mitochondrial dysfunction, reactive oxygen and nitrogen species production, lipid peroxidation and oxidative tissue injury [32].

The recovery of NRF2/HO-1 signaling after siMYD88-EV treatment is therefore unlikely to reflect only a nonspecific consequence of reduced inflammation. Activated NF- κ B can directly constrain NRF2-dependent antioxidant transcription: p65 competes with NRF2 for the transcriptional co-activator CBP and promotes HDAC3 recruitment to MafK-associated antioxidant response elements, thereby limiting NRF2/ARE-driven gene expression [33]. This mechanism is consistent with the broader reciprocal antagonism between NF- κ B-driven inflammatory signaling and NRF2-mediated antioxidant defense [34]. Conversely, NRF2 and HO-1 can restrain NF- κ B activation by reducing ROS-dependent inflammatory signaling and suppressing NF- κ B-mediated cytokine transcription [33, 35-37]. Thus, the concomitant reduction of COX2 and recovery of NRF2/HO-1 signaling after MYD88 knockdown may indicate partial release of an inflammation-imposed brake on endogenous antioxidant defense, providing a plausible mechanistic basis for the preservation of mitochondrial and synaptic ultrastructure.

MYD88 silencing appeared to rebalance microglial signaling states rather than induce broad immune suppression. TREM2 regulates microglial homeostasis, phagocytosis, lipid metabolic adaptation and damage-associated responses in a highly context-dependent manner [38-40]. Although acute pro-inflammatory cues can downregulate TREM2 expression, TREM2 is also induced in chronic injury settings in which microglia engage debris clearance, lipid handling and repair-related programs [41]. This temporal context may explain why untreated CLP mice showed only modest TREM2 reactivity despite marked MYD88-associated inflammation, whereas siMYD88-EVs enhanced TREM2-associated responses during post-septic inflammatory resolution. Thus, TREM2 upregulation in our model more likely reflects a recovery-phase microglial response than acute inflammatory activation alone. The COP1-C/EBP β signaling axis was examined as an additional regulatory module linked to inflammatory transcriptional control. siMYD88-EVs restored the phosphorylated COP1/total COP1 ratio without significantly changing total C/EBP β abundance, suggesting that MYD88 silencing may also affect COP1-associated inflammatory regulation. Whether COP1 directly contributes to the anti-inflammatory phenotype will require analyses of C/EBP β ubiquitination, nuclear localization and transcriptional activity.

The neurovascular findings further extend the mechanistic scope of MYD88 signaling after sepsis. CLP-induced MYD88-high structures were not confined to IBA1-positive microglia; a substantial fraction showed a vessel-like pattern and was located within CD31-positive vascular endothelial structures, whereas this pattern was barely detectable in sham brain sections. In the less inflamed sham and siMYD88-EV-treated groups, MYD88 signals associated with CD11B-positive myeloid-lineage cells were more readily resolved, whereas in untreated CLP mice this component was largely obscured by the dominant endothelial-associated MYD88 signal. Taken together, CLP induced a continuous endothelial MYD88-high pattern with signal intensity exceeding that of adjacent intravascular myeloid cells and nearby parenchymal microglia. Because fluorescence area or intensity measurements alone cannot fully resolve the cellular source of this signal, future cell-type-resolved analyses after vascular-associated cell isolation will be needed to define their relative contributions. This interpretation is consistent with evidence that the brain endothelium is an active immune interface during systemic inflammation. Brain endothelial cells express innate immune sensors and respond to peripheral inflammatory cues through Toll-like receptor- and inflammasome-related pathways, thereby influencing blood-brain barrier integrity and neuroimmune communication [42, 43]. Brain endothelial MYD88 signaling has been shown to mediate neuronal and neuroendocrine responses to systemic inflammatory stimuli, including vascular prostaglandin-related transcriptional activation [44]. However, endothelial MYD88 is not universally required for sepsis-induced BBB dysfunction: in an acute CLP model, endothelial MYD88 deletion did not prevent BBB permeabilization or hippocampal inflammatory transcriptional responses [45]. Thus, endothelial MYD88 in our model is best interpreted as a prominent component of persistent post-septic neurovascular inflammation, rather than the sole driver of BBB injury. In our study, persistent Evans blue leakage, MMP9 induction and Claudin-5 loss one month after CLP indicate ongoing BBB remodeling. The ability of siMYD88-EVs to reduce MMP9 and preserve Claudin-5 supports a role for MYD88-dependent vascular inflammation in post-septic neurovascular injury.

Mechanism-directed therapies for long-term cognitive impairment after sepsis remain limited. Current management largely addresses infection control, hemodynamic stabilization, organ support, and sedation strategy, whereas interventions aimed at persistent neuroinflammation and neurovascular injury are lacking. Corticosteroids are used mainly in selected septic shock settings and remain conditionally recommended and clinically debated in ICU guidelines [46]. MYD88 is therapeutically attractive but difficult to target because it is an intracellular adaptor rather than a conventional extracellular receptor or enzyme. EV-mediated siRNA

delivery provides a feasible route for modulating such intracellular signaling. By loading siMYD88 into EVs, this study extends MSC-EVs from a broadly immunomodulatory platform toward a pathway-directed strategy that targets persistent MYD88-dependent inflammatory-redox and neurovascular signaling after sepsis. Although further studies are needed to define cell-specific delivery, long-term safety, and therapeutic windows, these findings support engineered EV-mediated MYD88 silencing as a potential cell-free approach for sepsis-associated encephalopathy.

Conclusions

This study demonstrates that elevated MYD88 signaling in the late post-septic phase is an intervenable target linking neurovascular injury, neuroinflammation and cognitive impairment. Engineered hUCMSC-derived EVs mediated the delivery of MYD88-targeting siRNA to the brain. Rescue intervention with siMYD88-EVs initiated 72 h after CLP almost restored hippocampal neuronal mitochondrial and synaptic ultrastructure, as well as learning and memory performance, to near-normal levels. Mechanistically, these effects were driven by dual inflammatory regulation in vascular endothelial cells and microglia. After inhibition of endothelial MYD88, MMP9, which participates in extracellular matrix degradation, was downregulated, claudin-5 expression was restored, and blood-brain barrier integrity was re-established. In microglia, MYD88 knockdown directly reduced downstream responses of the canonical NF- κ B inflammatory signaling pathway, suppressed oxidative stress, restored NRF2/HO-1 axis function, and was accompanied by enhanced TREM2-associated microglial responses. Taken together, siMYD88-EV-mediated MYD88 silencing may serve as a target-defined, cell-free RNA delivery strategy that retains the inherent advantages of hUCMSC-derived EVs and shows clinical potential for neuroprotection in sepsis.

Abbreviations

BBB: blood-brain barrier; CLP: cecal ligation and puncture; CNS: central nervous system; COX2: cyclooxygenase-2; DAMPs: damage-associated molecular patterns; DiI: 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; EVs: extracellular vesicles; GFAP: glial fibrillary acidic protein; hUCMSCs: human umbilical cord-derived mesenchymal stem cells; IBA1: ionized calcium-binding adaptor molecule 1; IRAK4: interleukin-1 receptor-associated kinase 4; LPS: lipopolysaccharide; MSC-EVs: mesenchymal stem cell-derived extracellular vesicles; MYD88: myeloid differentiation primary response 88; NF- κ B: nuclear factor κ B; NRF2: nuclear factor erythroid 2-related factor 2; PFC: prefrontal cortex;

ROS: reactive oxygen species; SAE: sepsis-associated encephalopathy; siMYD88-EVs: extracellular vesicles loaded with MYD88-targeting small interfering RNA; siNC-EVs: extracellular vesicles loaded with negative-control small interfering RNA; TEM: transmission electron microscopy; TLR4: Toll-like receptor 4; TREM2: triggering receptor expressed on myeloid cells 2.

Declarations

Ethics approval and consent to participate

All animal procedures were approved by the Biological and Medical Ethics Committee, Minzu University of China, under the project title "Pharmacological mechanisms and clinical translation of engineered exosomes targeting neuroinflammation for the treatment of sepsis-associated encephalopathy" (approval no. ECMUC2024015AA; approval date: 28 May 2024). Banked human umbilical cord mesenchymal stem cells were supplied at passage 2 by the Nanhua Stem Cell Bank (China), operated by Hunan Nanhua Biotechnology Co., Ltd. According to documentation provided by the biobank, the original umbilical cord tissue was obtained with written informed consent permitting scientific research, and the biobank was approved under the project title "Biobank of Hunan Nanhua Biotechnology Co., Ltd." by the Ethics Review Committee of Changsha Taihe Hospital (approval no. (2023) 081; approval date: 8 October 2023). The authors did not recruit donors, collect human tissue, or have access to identifiable donor information.

Consent for publication

Not applicable.

Availability of data and materials

The data supporting the findings of this study are provided in the supplementary materials, including the Source Data file and the original full-length uncropped Western blot images. Additional materials are available from the corresponding author upon reasonable request.

Competing interests

The authors have declared that no competing interests exist.

Funding

This project was supported by The Science and Technology Program of Hunan Province (project number 2024JK2139).

Authors' contributions

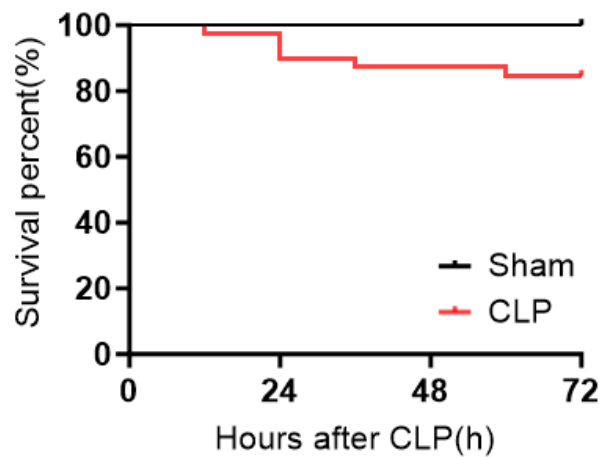
FM: Conceptualization, Data curation, Formal analysis, Investigation, Animal experiments, Molecular experiments, Writing - original draft. WY: Data curation, Formal analysis, Investigation, Cell experiments, Molecular experiments. FL: Data curation, Formal analysis, Investigation, Immunofluorescence experiments, Imaging analysis. ML: Investigation, Formal analysis. RW: Investigation. YS: Data curation, Formal analysis, Statistical analysis, Data visualization. YY: Investigation, qPCR experiments. YZ: Investigation, Animal experiments. XS: Immunofluorescence experiments, Imaging analysis. LZ: Investigation, Cell experiments. AT: Investigation, Extracellular vesicle isolation, Methodology. JS: Investigation, CLP model establishment, Animal model validation. XX: Investigation. MY: Methodology, Resources. CJ: Supervision, Writing - review and editing. FY: Funding acquisition, Resources, Supervision, Writing - review and editing. YH: Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Writing - original draft, Writing - review and editing. All authors read and approved the final manuscript.

Acknowledgements

Figure 9 and the graphical abstract were created with BioRender.com. ChatGPT (OpenAI) was used for language editing and grammatical correction; the authors take full responsibility for the scientific content and interpretations.

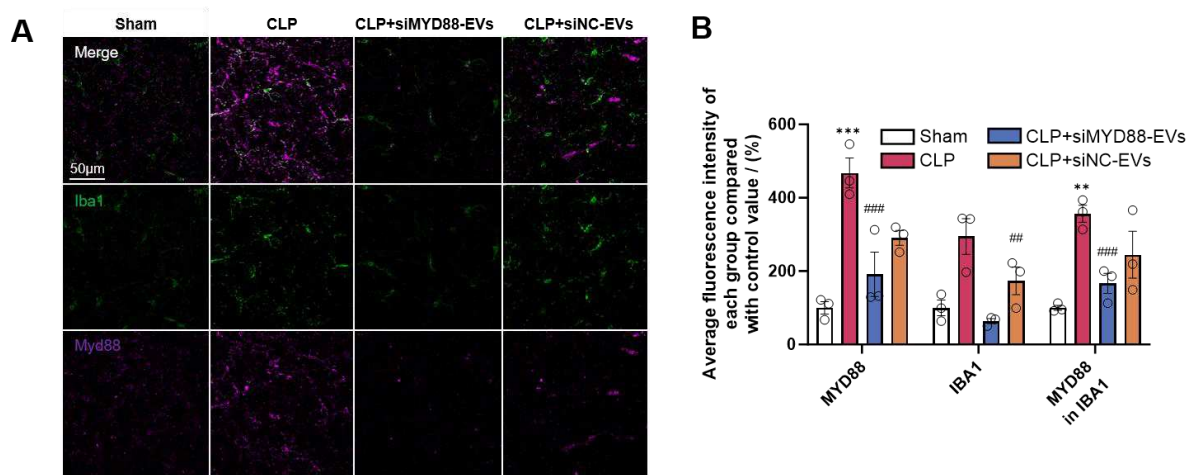
693

694 **Supplementary Figures**

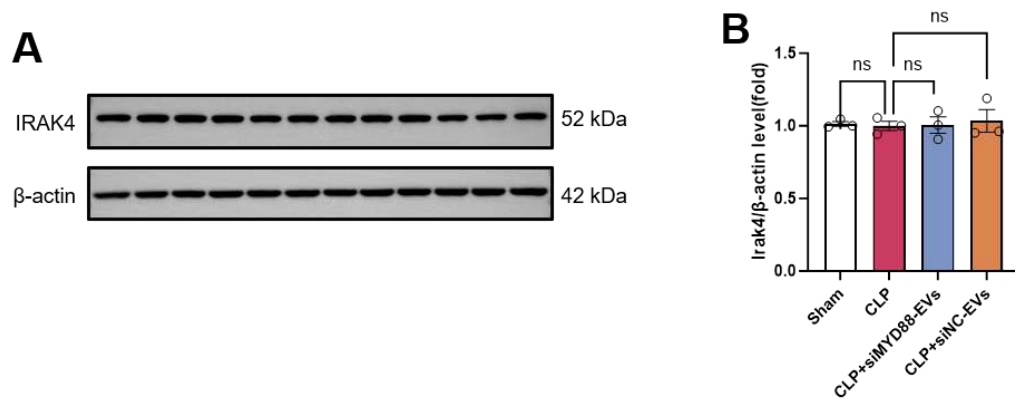


695

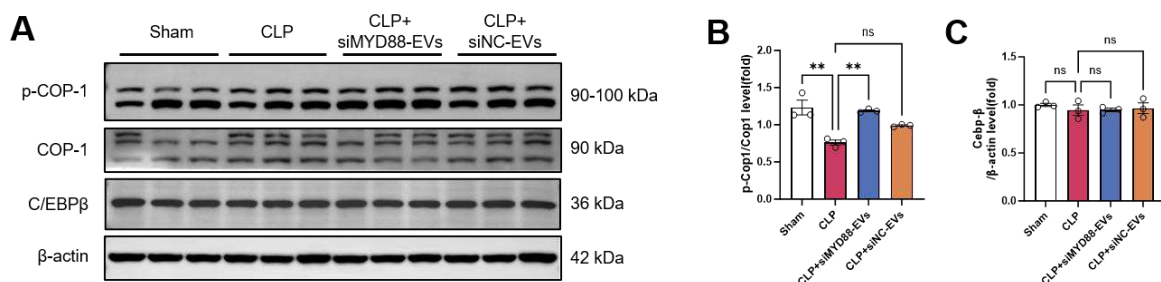
696 **Supplementary Figure 1. Early survival after CLP before EV treatment.** Kaplan-Meier survival curves
697 of sham-operated and CLP mice during the first 72 h after surgery, before EV treatment (Sham, $n=11$; CLP,
698 $n=39$).



Supplementary Figure 2. siMYD88-EVs reduce MYD88 expression and microglial activation in the prefrontal cortex after CLP. (A) Representative immunofluorescence images of IBA1 (green) and MYD88 (magenta) in the prefrontal cortex of Sham, CLP, CLP+siMYD88-EVs and CLP+siNC-EVs mice. Scale bar, 50 μ m. (B) Quantification of total MYD88 fluorescence, IBA1 fluorescence and MYD88 fluorescence within IBA1-positive areas, expressed relative to Sham. Data are shown as mean $\pm$ SEM ($n=3$); one-way ANOVA with Dunnett's multiple-comparisons test; ** $P < 0.01$, *** $P < 0.001$ versus Sham; ## $P < 0.01$, ### $P < 0.001$ versus CLP; *ns*, not significant.



Supplementary Figure 3. IRAK4 expression remains unchanged after CLP and EV treatment. (A) Representative western blots of IRAK4 and β -actin in brain tissue. (B) Densitometric quantification of IRAK4 normalized to β -actin. Data are shown as mean $\pm$ SEM (n=3); one-way ANOVA with Tukey's multiple-comparisons test; ns, not significant.



Supplementary Figure 4. siMYD88-EVs restore COP1 phosphorylation without altering C/EBPβ expression after CLP. (A) Representative western blots of p-COP1, COP1, C/EBPβ and β-actin in brain tissue. (B) p-COP1/COP1 ratio. (C) Densitometric quantification of C/EBPβ normalized to β-actin. Data are shown as mean ± SEM (n=3); one-way ANOVA with Tukey's multiple-comparisons test; **P < 0.01; ns, not significant.

References

- Gofton, T.E. and G.B. Young, *Sepsis-associated encephalopathy*. Nat Rev Neurol, 2012. **8**(10): p. 557-66.
- Manabe, T. and M.T. Heneka, *Cerebral dysfunctions caused by sepsis during ageing*. Nat Rev Immunol, 2022. **22**(7): p. 444-458.
- Iwashyna, T.J., et al., *Long-term cognitive impairment and functional disability among survivors of severe sepsis*. JAMA, 2010. **304**(16): p. 1787-94.
- Evans, L., et al., *Surviving sepsis campaign: international guidelines for management of sepsis and septic shock 2021*. Intensive Care Med, 2021. **47**(11): p. 1181-1247.
- Danielski, L.G., et al., *Brain Barrier Breakdown as a Cause and Consequence of Neuroinflammation in Sepsis*. Mol Neurobiol, 2018. **55**(2): p. 1045-1053.
- Singer, B.H., et al., *Cecal Ligation and Puncture Results in Long-Term Central Nervous System Myeloid Inflammation*. PLoS One, 2016. **11**(2): p. e0149136.
- Irzan, F.I., et al., *The Role of HMGB1 in Infection-Related Cognitive Deficits*. Frontiers in Bioscience (Landmark Edition), 2025. **30**(2): p. 25544.
- Li, J., et al., *Sepsis-associated encephalopathy: Mechanisms, Diagnosis, and Treatments update*. Int J Biol Sci, 2025. **21**(7): p. 3214-3228.

- 735 9. Yan, X., et al., *Central role of microglia in sepsis-associated encephalopathy: From mechanism to*
736 *therapy*. Front Immunol, 2022. **13**: p. 929316.
- 737 10. Ma, M., W. Jiang, and R. Zhou, *DAMPs and DAMP-sensing receptors in inflammation and*
738 *diseases*. Immunity, 2024. **57**(4): p. 752-771.
- 739 11. Yin, X.Y., et al., *HMGB1 mediates synaptic loss and cognitive impairment in an animal model of*
740 *sepsis-associated encephalopathy*. J Neuroinflammation, 2023. **20**(1): p. 69.
- 741 12. Sekino, N., M. Selim, and A. Shehadah, *Sepsis-associated brain injury: underlying mechanisms*
742 *and potential therapeutic strategies for acute and long-term cognitive impairments*. J
743 Neuroinflammation, 2022. **19**(1): p. 101.
- 744 13. Zhang, Y.Y. and B.T. Ning, *Signaling pathways and intervention therapies in sepsis*. Signal
745 Transduct Target Ther, 2021. **6**(1): p. 407.
- 746 14. Kawai, T., et al., *Decoding Toll-like receptors: Recent insights and perspectives in innate*
747 *immunity*. Immunity, 2024. **57**(4): p. 649-673.
- 748 15. Deguine, J. and G.M. Barton, *MyD88: a central player in innate immune signaling*. F1000Prime
749 Rep, 2014. **6**: p. 97.
- 750 16. Wesche, H., et al., *MyD88: an adapter that recruits IRAK to the IL-1 receptor complex*. Immunity,
751 1997. **7**(6): p. 837-47.
- 752 17. AbdAllah, N.B., et al., *MYD88, NFKB1, and IL6 transcripts overexpression are associated with*
753 *poor outcomes and short survival in neonatal sepsis*. Sci Rep, 2021. **11**(1): p. 13374.
- 754 18. Tenchov, R., et al., *Exosomes—Nature's Lipid Nanoparticles, a Rising Star in Drug Delivery and*
755 *Diagnostics*. ACS Nano, 2022. **16**(11): p. 17802-17846.
- 756 19. Sun, Y., et al., *Mesenchymal stem cells-derived exosomes for drug delivery*. Stem Cell Res Ther,
757 2021. **12**(1): p. 561.
- 758 20. Shu, J., et al., *Evaluating the Mechanisms of Action of Mesenchymal Stem Cell-Derived*
759 *Extracellular Vesicles in Ischemic Stroke*. Frontiers in Bioscience (Landmark Edition), 2025.
760 **30**(12): p. 43790.
- 761 21. Yang, S., et al., *Protective properties of extracellular vesicles in sepsis models: a systematic*
762 *review and meta-analysis of preclinical studies*. J Transl Med, 2023. **21**(1): p. 262.

- 763 22. Koutroulis, I., et al., *Mesenchymal stem cell-derived small extracellular vesicles alleviate the*
764 *immunometabolic dysfunction in murine septic encephalopathy*. iScience, 2024. **27**(8): p. 110573.
- 765 23. Shen, K., et al., *miR-125b-5p in adipose derived stem cells exosome alleviates pulmonary*
766 *microvascular endothelial cells ferroptosis via Keap1/Nrf2/GPX4 in sepsis lung injury*. Redox
767 Biol, 2023. **62**: p. 102655.
- 768 24. Zhang, R., et al., *Human umbilical cord mesenchymal stem cell exosomes alleviate sepsis-*
769 *associated acute kidney injury via regulating microRNA-146b expression*. Biotechnol Lett, 2020.
770 **42**(4): p. 669-679.
- 771 25. Ma, Y., et al., *MSC-derived exosomal miR-140-3p improves cognitive dysfunction in sepsis-*
772 *associated encephalopathy by HMGB1 and S-lactoylglutathione metabolism*. Commun Biol, 2024.
773 **7**(1): p. 562.
- 774 26. Li, M., et al., *Clinical investigation on nebulized human umbilical cord MSC-derived extracellular*
775 *vesicles for pulmonary fibrosis treatment*. Signal Transduct Target Ther, 2025. **10**(1): p. 179.
- 776 27. Hum, C., et al., *Efficacy and Safety of Umbilical Cord-Derived Mesenchymal Stromal Cell*
777 *Therapy in Preclinical Models of Sepsis: A Systematic Review and Meta-analysis*. Stem Cells
778 Transl Med, 2024. **13**(4): p. 346-361.
- 779 28. Kalluri, V.S., et al., *Engineered exosomes with Kras(G12D) specific siRNA in pancreatic cancer: a*
780 *phase I study with immunological correlates*. Nat Commun, 2025. **16**(1): p. 8696.
- 781 29. Bryant, C.E., *Rethinking Toll-like receptor signalling*. Curr Opin Immunol, 2024. **91**: p. 102460.
- 782 30. Alexopoulou, L. and M. Irla, *Toll-like receptors (TLRs) in the trained immunity era*. Elife, 2025.
783 **14**: p. e106443.
- 784 31. Netea, M.G., et al., *Defining trained immunity and its role in health and disease*. Nat Rev
785 Immunol, 2020. **20**(6): p. 375-388.
- 786 32. Lingappan, K., *NF- κ B in oxidative stress*. Curr Opin Toxicol, 2018. **7**: p. 81-86.
- 787 33. Gao, W., et al., *Dissecting the crosstalk between Nrf2 and NF- κ B response pathways in drug-*
788 *induced toxicity*. Front Cell Dev Biol, 2021. **9**: p. 809952.
- 789 34. Mao, H., X. Zhao, and S.C. Sun, *NF-kappaB in inflammation and cancer*. Cell Mol Immunol,
790 2025. **22**(8): p. 811-839.

35. Wardyn, J.D., A.H. Ponsford, and C.M. Sanderson, *Dissecting molecular cross-talk between Nrf2 and NF- κ B response pathways*. Biochem Soc Trans, 2015. **43**(4): p. 621-6.
36. Sivandzade, F., et al., *NRF2 and NF- κ B interplay in cerebrovascular and neurodegenerative disorders: molecular mechanisms and possible therapeutic approaches*. Redox Biol, 2019. **21**: p. 101059.
37. Cuadrado, A., *Brain-Protective Mechanisms of Transcription Factor NRF2: Toward a Common Strategy for Neurodegenerative Diseases*. Annu Rev Pharmacol Toxicol, 2022. **62**: p. 255-277.
38. Shi, Q., R.A. Gutierrez, and M.A. Bhat, *Microglia, Trem2, and Neurodegeneration*. Neuroscientist, 2025. **31**(2): p. 159-176.
39. Pocock, J., et al., *Microglia and TREM2*. Neuropharmacology, 2024. **257**: p. 110020.
40. Ulland, T.K. and M. Colonna, *TREM2 - a key player in microglial biology and Alzheimer disease*. Nat Rev Neurol, 2018. **14**(11): p. 667-675.
41. Abdulkhaliq, A.A., et al., *TREM2 in neurodegeneration and diseases*. Mol Psychiatry, 2026.
42. Acioglu, C. and S. Elkabes, *Innate immune sensors and regulators at the blood brain barrier: focus on toll-like receptors and inflammasomes as mediators of neuro-immune crosstalk and inflammation*. J Neuroinflammation, 2025. **22**(1): p. 39.
43. Galea, I., *The blood-brain barrier in systemic infection and inflammation*. Cell Mol Immunol, 2021. **18**(11): p. 2489-2501.
44. Gosselin, D. and S. Rivest, *MyD88 signaling in brain endothelial cells is essential for the neuronal activity and glucocorticoid release during systemic inflammation*. Mol Psychiatry, 2008. **13**(5): p. 480-97.
45. Honig, G., et al., *Blood-Brain Barrier Deterioration and Hippocampal Gene Expression in Polymicrobial Sepsis: An Evaluation of Endothelial MyD88 and the Vagus Nerve*. PLoS One, 2016. **11**(1): p. e0144215.
46. Chaudhuri, D., et al., *2024 Focused Update: Guidelines on Use of Corticosteroids in Sepsis, Acute Respiratory Distress Syndrome, and Community-Acquired Pneumonia*. Crit Care Med, 2024. **52**(5): p. e219-e233.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementarymaterialSCRT.docx](#)
- [siMYD88EVSourceData.xlsx](#)
- [OriginalWesternblotimagesfinal.pdf](#)