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Circulating Exosomes Mediate Neurodegeneration Following Hepatic Ischemia-reperfusion Through Inducing Microglial Pyroptosis in the Developing Hippocampus

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Background. Poor neurodevelopmental outcomes after pediatric liver transplantation seriously affect the long-term quality of life of recipients, in whom hepatic ischemia reperfusion (HIR) is considered to play a pivotal role. However, the link between HIR and brain injury remains unclear. Because circulating exosomes are considered as the key mediators of information transmission over long distances, we aimed to assess the role of circulating exosomes in HIR-induced hippocampal injury in young rats. **Methods.** We administered exosomes extracted from the sera of HIR model rats to normal young rats via the tail vein. Western blotting, enzyme-linked immunosorbent assay, histological examination, and real-time quantitative polymerase chain reaction were used to evaluate the role of exosomes in neuronal injury and activation of microglial pyroptosis in the developing hippocampus. Primary microglial cells were cocultured with exosomes to further assess the effect of exosomes on microglia. To further explore the potential mechanism, GW4869 or MCC950 was used to block exosome biogenesis or nod-like receptor family protein 3, respectively. **Results.** Serum-derived exosomes played a crucial role in linking HIR with neuronal degeneration in the developing hippocampus. Microglia were found to be the target cells of ischemia-reperfusion derived exosomes (I/R-exosomes). I/R-exosomes were taken up by microglia and promoted the occurrence of microglial pyroptosis in vivo and in vitro. Moreover, the exosome-induced neuronal injury was alleviated by suppressing the occurrence of pyroptosis in the developing hippocampus. **Conclusions.** Microglial pyroptosis induced by circulating exosomes plays a vital role in developing hippocampal neuron injury during HIR in young rats.

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INTRODUCTION

Pediatric liver transplantation is a lifesaving therapy for children with end-stage liver disease. However, neurological complications after pediatric liver transplantation remain a major challenge.[1] A recent study has indicated that children after liver transplantation have worse cognitive function and lower health-related quality of life compared with their healthy peers.[2] Hepatic ischemia reperfusion (HIR) is an inevitable consequence during liver transplantation. HIR promotes oxidative stress and inflammatory reactions, resulting in damage to the liver and remote organs.[3,4] The hippocampus is a critical brain region that performs an essential function in cognition, memory, and navigation.[5] The hippocampus is a plastic brain structure, especially during the early postnatal period. During this sensitive developmental period, exposure to unfavorable

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Y.W. and L.J. designed the research and edited the article. Y.W., M.W., L.J., J.L., Z.D., and W.H. performed the experiments. Y.S., M.S., and Y.R. provided technical assistance. Y.W. and W.Y. offered guidance of this study. Y.W. and L.J. contributed equally and are co-first authors.

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conditions may produce adverse effects on cognitive development.[6] Previous studies have revealed that HIR induces hippocampal injury and even cognitive impairment.[7-9] Nonetheless, the potential mechanism of brain injury secondary to HIR remains unclear.

Exosomes, nanosized (40–150 nm) small extracellular vesicles, are produced by most cells and widely distributed in various body fluids, including blood.[10,11] Exosomes selectively carry functional components, such as proteins, RNA, and DNA.[12] Exosomes are taken up by neighboring or distant cells and then transfer their components to change the function of target cells.[12,13] Thus, exosomes have been considered pivotal mediators of intercellular communication and information transmission over long-range distances with high targeting ability. Researchers have demonstrated that HIR increases serum extracellular vesicle concentrations and induces subsequent liver damage in mice.[14] Moreover, increasing evidence has confirmed that peripheral circulating exosomes pass through the blood–brain barrier (BBB) and lead to neurological disorders.[15,16] However, it remains unknown whether circulating exosomes link HIR with subsequent brain injury in young rats.

Pyroptosis is a novel inflammatory programmed cell death characterized by the activation of caspase-1 or caspase-4/5/11.[17,18] Canonical pyroptosis is performed by caspase-1, which originates from the activation of pro-caspase-1 under a signaling platform provided by the inflammasome.[19,20] The inflammasome is a cytosolic protein complex that consists of a sensor protein (such as NLRs), an adaptor protein (called apoptosis-associated speck-like proteins containing a caspase recruitment domain [ASC]), and inflammatory caspases.[20] The nod-like receptor family protein 3 (NLRP3) inflammasome has been extensively investigated in the central nervous system (CNS) because it is the most common inflammasome in the CNS and one of the critical factors for various neurological disorders characterized by neuroinflammation.[21] Gasdermin-D, the final executor of pyroptosis, is cleaved by activated inflammatory caspases into the N-terminal p30 fragment, which interacts with membrane lipids and forms pores to induce cell rupture and the subsequent release of interleukin (IL)-18 and IL-1β.[17,18,22] These proinflammatory cytokines in turn lead to further inflammatory cascade reactions, ultimately resulting in injury to healthy neighboring cells and even cell death.[20,23] A previous study has revealed that HIR induces hippocampal pyroptosis in adult rats.[24] However, it is unclear whether pyroptosis occurs in young rats under HIR conditions and whether it is involved in neuronal injury.

Microglia, the resident innate immune cells in the brain, are closely related to neuroinflammation. Similar to macrophages, microglia perform primary immune surveillance and phagocytosis in the CNS, and they produce cytokines, chemokines, and reactive oxygen species, which are pivotal mediators of neuroinflammation.[25,26] Researchers have demonstrated that circulating exosomes target microglia and contribute to enhanced neuroinflammation.[16] Furthermore, emerging studies have shown that inflammasome-induced pyroptosis can occur in microglia, causing deterioration in neurological function.[27-29] However, it remains unclear whether circulating exosomes target microglia and mediate neuroinflammation under HIR conditions.

In the present study, we administered exosomes extracted from the sera of HIR rats to normal young rats or primary microglial cells to explore whether circulating exosomes mediate hippocampal neuron injury by activating microglial pyroptosis during HIR.

METHODS AND MATERIALS

Animals

Healthy Sprague-Dawley rats (male, 2–3 wk) and pregnant Sprague-Dawley rats were bought from the Beijing HFK Bioscience Company (Beijing, China). All the animals were kept under standard conditions of constant temperature (23–24 °C), humidity, and light (12-h light/12-h dark cycle) with free access to food and water. All animal procedures were manipulated according to the National Institute of Health's Guidelines for the Care and Use of Laboratory Animals and were approved by the Ethics Committee of Tianjin First Center Hospital (Tianjin, China).

Primary Microglia Culture

Rat primary microglial cells were obtained from meninges-free brain tissues of postnatal (1- to 3-d-old) Sprague-Dawley rats. The isolated hippocampal tissues were digested and dissociated using 0.25% trypsin solution for 15 min. The digested tissues were centrifuged at 1500 rpm (centrifugal radius, 10 cm) for 5 min. Next, the mixed glial were resuspended in DMEM/F12 medium (10% fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin) and plated onto polylysine-coated cell culture flasks. Microglia reached maturity after 10 to 14 d of mixed culture in humidified air containing 5% CO_2 at 37 °C. The mature microglia cells were separated from mixed glial by shaking at 100 rpm for 2 h at 37 °C. The supernatants were collected and centrifuged at 1500 rpm for 3 min to collect microglia cells. Then microglia were plated onto 6-well cell culture plates at a density of 5×10^5 cells/mL. Two days later, microglia cells were used for the subsequent experiments.

Animal Model

The 70% warm HIR model was built according to the previous description.[9] The details are provided in **Supplemental Content** (**SDC**, http://links.lww.com/TP/C776).

Animal Study Protocol

In total, 229 rats were randomly assigned to appropriate groups for different experimental purposes. The animal experiment included 4 parts as shown in Figure 1C.

To explore the role of exosomes in HIR-related brain injury, S-exosomes or I/R-exosomes were extracted from 2 mL of sera from sham operation rats or hepatic I/R rats at 6 h after reperfusion, dissolved in 100 μL PBS, and injected into normal rats via the tail vein (Figure 1A). In part 1, to evaluate the role of exosomes in hippocampal pyroptosis, normal rats were injected with S-exosomes or I/R-exosomes for 6 h, whereas hepatic I/R rats or sham operation rats were injected with 100 μL of PBS (Figure 1B). In part 2, GW4869 was used to inhibit exosome biogenesis. Rats

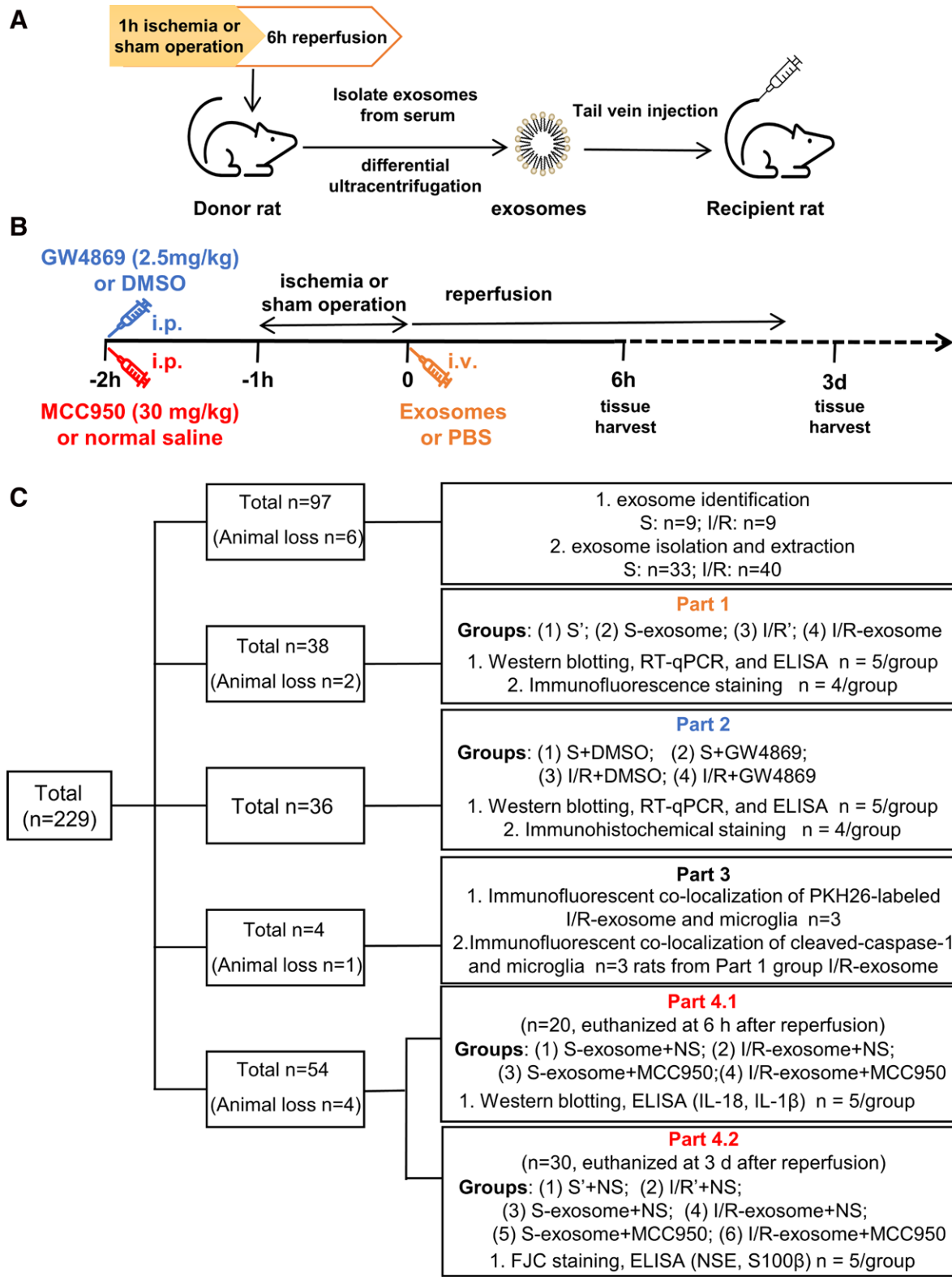

FIGURE 1. Animal experimental procedures and flow diagram. A, Protocol schematics for isolation and administration of exosomes. B, Protocol schematics for drug administration and treatment. C, Protocol schematics for animal groups. Part 1. S' group: sham operation rats treated with PBS; I/R' group: hepatic I/R rats treated with PBS; S-exosome group: normal rats treated with S-exosomes; I/R-exosome group: normal rats treated with I/R-exosomes. Part 2. S + DMSO group: sham operation rats pretreated with DMSO; S + GW4869 group: sham operation rats pretreated with GW4869; I/R + DMSO group: hepatic I/R rats pretreated with DMSO; I/R + GW4869 group: hepatic I/R rats pretreated with GW4869. Part 4. S' + NS group: S' group rats pretreated with NS; I/R' + NS group: I/R' group rats pretreated with NS; S-exosome + NS group: S-exosome group rats pretreated with NS; I/R-exosome + NS group: I/R-exosome group rats pretreated with NS; S-exosome + MCC950 group: S-exosome group rats pretreated with MCC950; I/R-exosome + MCC950 group: I/R-exosome group rats pretreated with MCC950. DMSO, dimethyl sulfoxide; I/R, ischemia–reperfusion.

were randomly divided into 4 groups and pretreated with GW4869 (2.5 mg/kg, MCE, USA) or dimethyl sulfoxide (DMSO) by intraperitoneal injection 1 h before ischemia induction or sham operation (Figure 1B).[30,31]

In part 3, to determine the target cells of I/R-exosomes, I/R-exosomes or PKH26-labeled I/R-exosomes were injected into normal rats. After 6 h, brain tissues were harvested to perform immunofluorescence colocalization experiments.

In part 4, to explore the relationship between exosome-induced microglial pyroptosis and neuronal injury in the hippocampus, rats were randomly divided into 6 groups. MCC950 (30 mg/kg, diluted to 10 mg/mL with normal saline, Selleckchem, USA) or normal saline was intraperitoneally injected into rats 2 h before administration of exosomes (Figure 1B).[24] In part 4.1, hippocampal tissues from 4 groups (20 rats) were harvested at 6 h after reperfusion to detect the level of pyroptosis. In part 4.2, the sera and brain tissues from 6 groups (another 30 rats) were collected to observe neuronal injury at 3 d after reperfusion.

Exosome Isolation, Identification, and Labeling

Exosomes were extracted by differential ultracentrifugation and labeled with PKH26 red fluorescence. The details are provided in **Supplemental Content** (**SDC**, http://links.lww.com/TP/C776).

Laboratory Methods

Details on Western blotting, real-time quantitative polymerase chain reaction, enzyme-linked immunosorbent assay (ELISA), immunofluorescence staining, immunohistochemical staining, and Fluoro-Jade C (FJC) staining are provided in **Supplemental Content** (**SDC**, http://links.lww.com/TP/C776).

Statistical Analysis

All experiments were independently performed in at least triplicate. Data are presented as the mean ± standard error of the mean. The Student *t* test was applied for the comparison of 2 groups. Analysis of variance was used to perform multiple group comparisons. Statistical analyses were performed using GraphPad Prism software. A *P* value of <0.05 was considered statistically significant.

RESULTS

HIR Increases the Concentration of Exosomes in Serum

To observe the alteration of circulating exosomes induced by HIR, we isolated exosomes from the sera of sham operation or HIR rats by ultracentrifugation. Transmission electron microscopy was used to visually confirm the existence of exosomes in the 2 groups. The typical cup-shaped morphology of exosomes was observed (Figure 2A). Subsequently, nanoparticle tracking analysis was performed to identify the size of isolated exosomes. There was no difference between the 2 groups in the diameters of exosomes (Figure 2B); however, the concentration

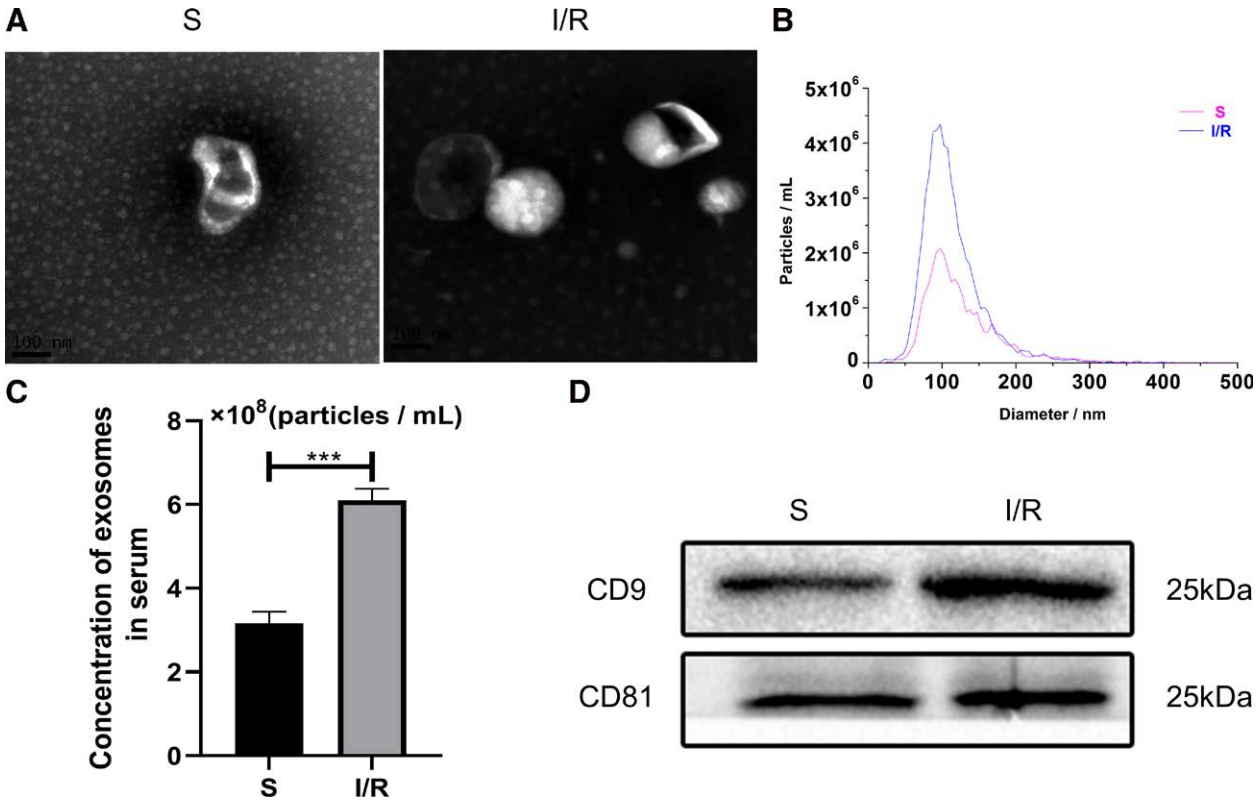

FIGURE 2. Isolation and characterization of serum-derived exosomes. A, Representative photomicrograph of circulating exosomes isolated from sham operation (S) or hepatic–ischemia reperfusion (I/R) rats detected by a transmission electron microscope. Scale bar: 100 nm. n = 3 rats per group. NTA shows the size distribution profiles (B) and the concentration (C) of serum-derived exosomes isolated from 2 groups. n = 3 rats per group. D, Western blotting results of CD81 and CD9 (marker protein of exosomes) for 2 groups. n = 3 rats per group. $^{***}P < 0.0001$. NTA, nanoparticle-tracking analysis.

of serum exosomes was significantly increased in the I/R group (Figure 2C), which indicated that HIR promoted the secretion of exosomes. Western blot analysis further indicated that typical exosome markers, including CD81 and CD9, were enriched in the I/R group (Figure 2D). These observations suggested that HIR significantly promotes the production of circulating exosomes but does not significantly modulate their size.

Serum-derived Exosomes Under HIR Conditions Trigger NLRP3 Inflammasome-induced Pyroptosis in the Developing Hippocampus

A previous study has demonstrated that HIR induces hippocampal pyroptosis, reaching a peak at 6 h after reperfusion in adult rats.[24] To determine whether exosomes trigger hippocampal pyroptosis in young rats, S-exosomes or I/R-exosomes isolated from sham operation or hepatic I/R rats were intravenously transfused into normal rats, and the levels of pyroptosis in hippocampal tissues were detected 6 h later (Figure 1C; part 1). Western blot analysis showed that the protein expression levels of pyroptosis-related markers (NLRP3, ASC, cleaved-caspase-1, and cleaved-GSDMD) were significantly upregulated in both the I/R' group and I/R-exosome group compared with the S' group and S-exosome group, whereas there was no difference between the S' group and S-exosome group (Figure 3A and B). A similar trend was observed in the mRNA expression levels of NLPR3, ASC, and GSDMD (Figure 3C). However, the mRNA level of caspase-1 was not altered among the 4 groups, which suggested that I/R-exosomes promoted the activation but not the generation of caspase-1. Immunofluorescence analysis confirmed that the expression of NLRP3 in the hippocampal CA1 and DG regions was elevated in the I/R' group and the I/R-exosome group (Figure 3D). Furthermore, the ELISA results showed that the levels of IL-1β and IL-18 were increased in the I/R' group and the I/R-exosome group (Figure 3E). These results indicated that I/R-exosomes mediate pyroptosis in the developing hippocampus.

Inhibition of Exosome Biogenesis Attenuates Pyroptosis and Consequential Neuroinflammation in the Hippocampus of HIR Rats

To further validate the role of I/R-exosomes in developing hippocampal pyroptosis, we pretreated rats with GW4869, a chemical inhibitor of exosome biogenesis, before establishing the HIR model (Figure 1C; part 2). As expected, compared with the I/R + DMSO group, the expression of CD9 (an exosomal marker) was decreased in the I/R + GW4869 group (Figure 4A), which indicated that GW4869 treatment effectively reduced the production of circulating exosomes. Western blot analysis showed that GW4869 treatment significantly reduced the expression levels of NLRP3, ASC, cleaved-caspase-1, and cleaved-GSDMD in the hippocampus during HIR (Figure 4A and B). A similar trend was observed in the mRNA levels of NLRP3, ASC, and GSDMD (Figure 4C). In addition, the histological results indicated that the expression of cleaved-caspase-1 in the hippocampal CA1 and DG regions was effectively suppressed by GW4869 treatment during HIR (Figure 4D and E). Moreover, the IL-18 and IL-1β proinflammatory cytokines were detected by ELISA, which demonstrated that GW4869-treated rats exhibited decreased levels of IL-18 and IL-1β in the hippocampus compared with HIR rats (Figure 4F). Collectively, the protective effects of exosome inhibition by GW4869 further confirmed that exosomes are involved in hippocampal pyroptosis during HIR.

I/R-exosomes Are Mainly Taken Up by Microglia in the Hippocampus

To determine the target cells of I/R-exosomes in the hippocampus, we intravenously transfused I/R-exosomes labeled with PKH26 into normal rats (Figure 5A). The brain slices were stained with an anti-Iba-1 antibody (microglial marker), and the cellular uptake of exosomes was observed under a fluorescence microscope. As shown in Figure 5B, the red fluorescent-labeled exosomes primarily colocalized with the green fluorescent-labeled microglia in the hippocampal CA1 and DG regions, which suggested that I/R-exosomes were taken up by microglia.

I/R-exosomes Induce Microglial Pyroptosis in the Hippocampus

Based on the finding that I/R-exosomes were taken up by microglia, immunofluorescence colocalization was performed to verify whether exosome-induced pyroptosis occurs in microglia. After administration of I/R-exosomes to rats for 6 h, we observed that cleaved-caspase-1 labeled by red fluorescence principally colocalized in microglia labeled by green fluorescence (Figure 6A), which indicated that microglia were the major cell type involved in pyroptosis.

I/R-exosomes Contribute to Microglial Pyroptosis In Vitro

To further validate whether I/R-exosomes are taken up by microglia and mediate microglial pyroptosis, primary microglial cells were cocultured with S-exosomes or I/R-exosomes for 6 h in vitro (Figure 7A). First, microglial cells were cocultured with PKH26-labeled exosomes and immunostained with an anti-Iba-1 antibody, which confirmed that I/R-exosomes were taken up by microglia with a higher uptake rate than S-exosomes (Figure 7B and C). Next, the level of pyroptosis induced by I/R-exosomes in microglia was evaluated. To determine the effective concentration of I/R-exosomes, 3 concentrations (10^7/mL, 10^8/mL, and 10^9/mL) of I/R-exosomes were administered to microglial cells for 6 h. Compared with the control group, 10^7/mL I/R-exosomes had no significant influence, but treatment with 10^8/mL and 10^9/mL I/R-exosomes significantly elevated the protein levels of NLRP3, ASC, cleaved-caspase-1, and cleaved-GSDMD (Figure 7D and E). Thus, treatment with 10^8/mL exosomes, a safe and effective concentration, was used in subsequent experiments. Next, the same concentration (10^8/mL) of S-exosomes or I/R-exosomes was administered to microglial cells. As expected, compared with the control group and S-exosome group, the mRNA levels of NLRP3, ASC, and GSDMD were increased in the I/R-exosome group, whereas the mRNA expression of caspase-1 was unchanged (Figure 7F). In addition, the levels of IL-1β and IL-18 in the cell culture supernatant were evaluated by

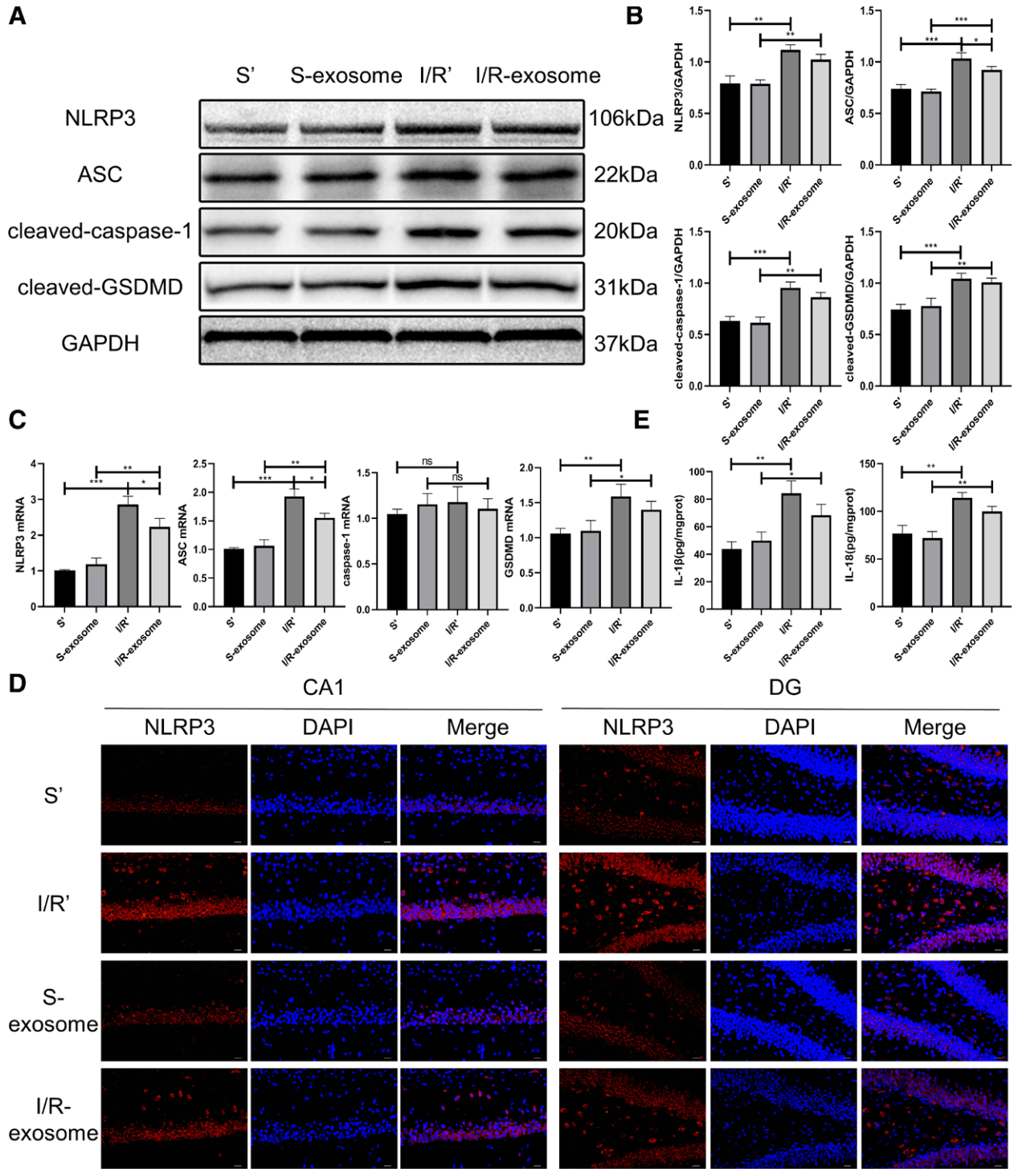

FIGURE 3. Circulating exosomes increase the level of molecules related to pyroptosis in the hippocampus under HIR condition. A and B, Western blotting results of pyroptosis-related proteins (NLRP3, ASC, cleaved-caspase-1, and cleaved-GSDMD) in the developing hippocampus among S', S-exosome, I/R', and I/R-exosome groups. n = 5 per group. C, RT-qPCR results show the mRNA levels of NLRP3, ASC, caspase-1, and GSDMD. n = 5 per group. D, Images of immunofluorescence staining with NLRP3 antibody in the hippocampal CA1 and DG regions among 4 groups (magnification, ×200; scale bars, 20 μm). n = 4 per group. E, ELISA results of IL-1β and IL-18 in the hippocampus among 4 groups. n = 5 per group. $^{ns}P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.0001$. ASC, apoptosis-associated speck-like proteins containing a caspase recruitment domain; ELISA, enzyme-linked immunosorbent assay; GSDMD, gasdermin D; IL, interleukin; I/R, ischemia–reperfusion; NLRP3, nod-like receptor family protein 3; ns, nonsignificant; RT-qPCR, real-time quantitative polymerase chain reaction.

ELISA, which demonstrated that the I/R-exosome group exhibited significantly higher levels of inflammatory cytokines (Figure 7G). Together, these results confirmed that I/R-exosomes target microglia and mediate microglial pyroptosis.

Targeting NLRP3 With MCC950 Alleviates Neuroinflammation and Neuronal Injury Induced by I/R-exosomes in the Developing Hippocampus

To assess the role of exosomes in HIR-related hippocampal neuron injury, which peaks at 3 d after reperfusion

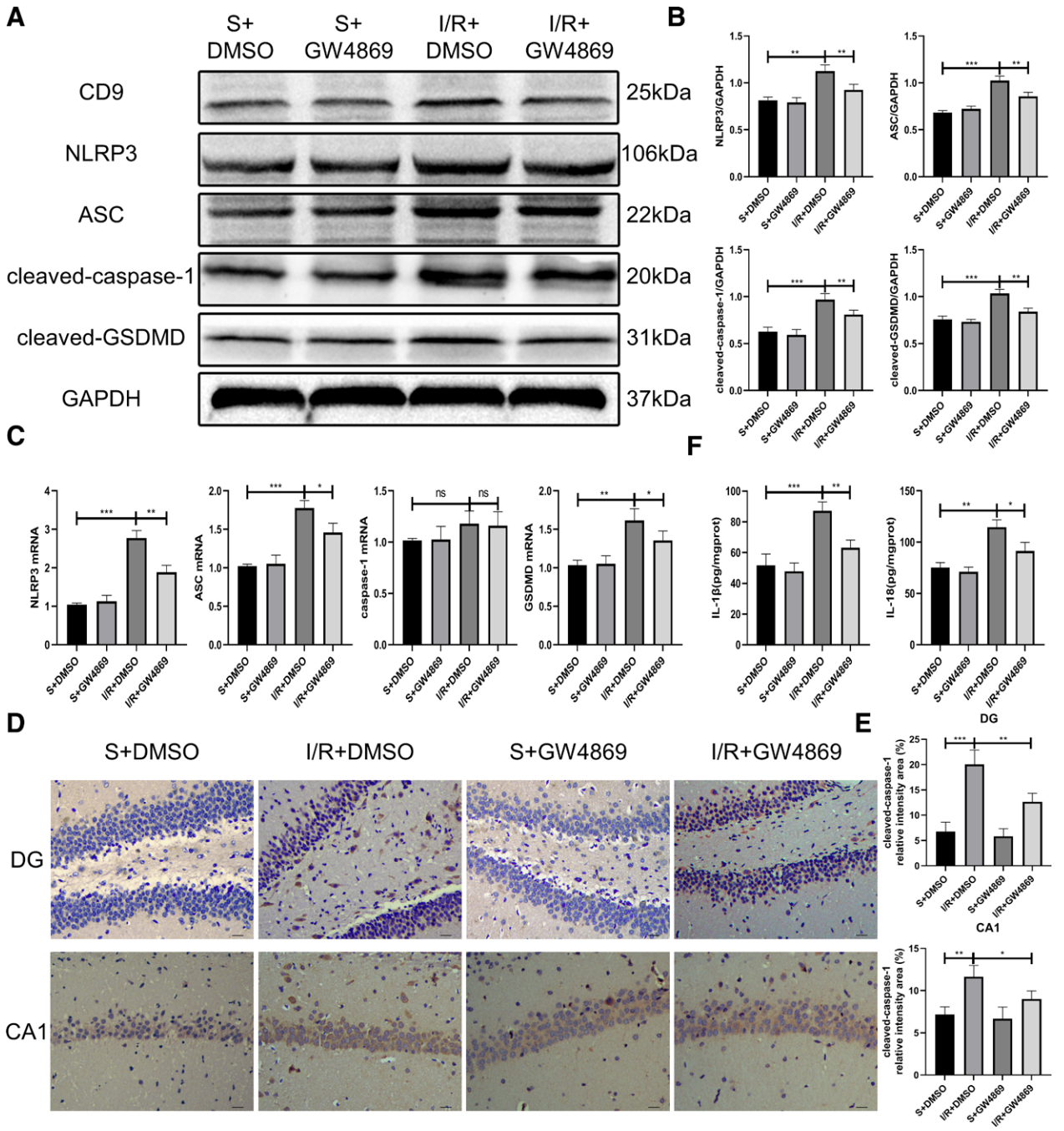

FIGURE 4. GW4869 treatment mitigates HIR-induced pyroptosis and consequential neuroinflammation in the developing hippocampus. A and B, Western blotting results of CD9, NLRP3, ASC, cleaved-caspase-1, and cleaved-GSDMD among 4 groups. n = 5 rats per group. C, RT-qPCR results show the mRNA levels of NLRP3, ASC, caspase-1, and GSDMD. n = 5 rats per group. D, Images of immunohistochemical staining with cleaved-caspase-1 antibody in the hippocampal CA1 and DG regions among 4 groups (magnification, ×200; scale bars, 20 μm). E, Quantitative analysis of immunohistochemical staining. n = 4 rats per group. F, ELISA results of IL-1β and IL-18 in the hippocampus among 4 groups. n = 5 rats per group. $^{ns}P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.0001$. ASC, apoptosis-associated speck-like proteins containing a caspase recruitment domain; ELISA, enzyme-linked immunosorbent assay; GSDMD, gasdermin D; HIR, hepatic ischemia reperfusion; IL, interleukin; NLRP3, nod-like receptor family protein 3; ns, nonsignificant; RT-qPCR, real-time quantitative polymerase chain reaction.

according to our previous research,[7] we administered exosomes to normal rats intravenously and observed neuron degeneration in the hippocampal CA1, DG, and CA3 regions using FJC staining after 3 d (Figure 1C; part 4.2). The green fluorescence represented degenerated neurons. Compared with the S'+NS group or the S-exosome+NS group, neuronal degeneration in all 3 regions of the hippocampus was aggravated in the I/R'+NS group and the I/R-exosome+NS group (Figure 8A and B). In addition, the essential serum biomarkers of brain injury (S100β and NSE) detected by ELISA were also higher in the I/R'+NS group and the I/R-exosome+NS group (Figure 8C). These findings suggested that I/R-exosomes contribute to hippocampal neuron injury.

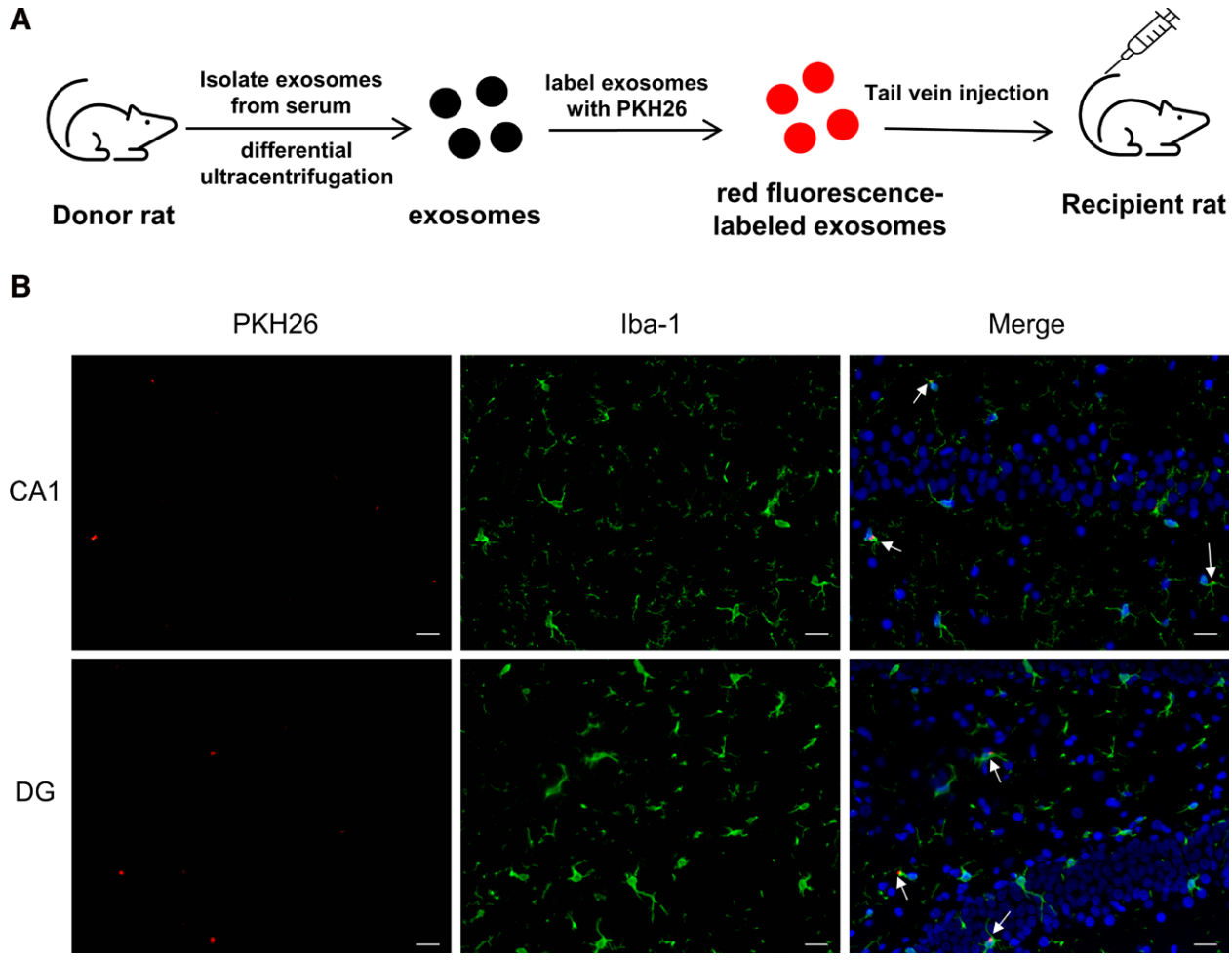

FIGURE 5. I/R-exosomes uptake in the hippocampus. A, Protocol schematic for isolation, label, and infusion of exosome. B, Colocalization of the PKH26-labeled (red) exosomes and microglia in the hippocampal CA1 and DG regions. The slices were stained with anti-Iba-1 antibody (microglia, green) and DAPI (nucleus, blue). White arrowheads indicated PKH26 positive microglia (magnification, ×200; scale bars, 20 μm). n = 3 rats. DAPI, diamidino phenyl indole; I/R, ischemia–reperfusion.

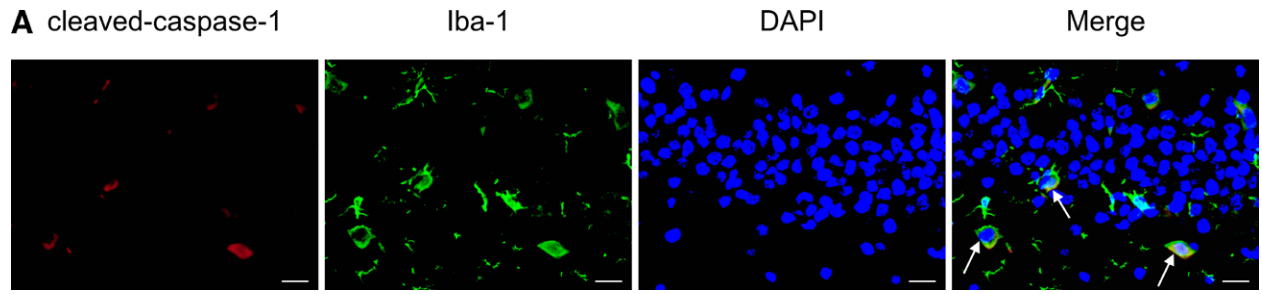

FIGURE 6. I/R-exosomes trigger pyroptosis in microglia. A, Immunofluorescence colocalization of cleaved-caspase-1 and microglia in the hippocampus. At 6 h after injection of I/R-exosome, the brain tissues were stained with anti-cleaved-caspase-1 antibody (red), anti-Iba-1 antibody (microglia, green), and DAPI (nucleus, blue). White arrowheads indicated cleaved-caspase-1 positive microglia (magnification, ×200; scale bars, 20 μm). n = 3 rats. DAPI, diamidino phenyl indole; I/R, ischemia–reperfusion.

Pyroptosis is not only a form of cell death but also a pro-inflammatory process. As previously reported, pyroptosis-mediated sterile inflammation leads to neuronal damage.[28] To examine whether microglial pyroptosis induced by exosomes is responsible for hippocampal neuron injury, we used MCC950, a selective inhibitor of NLRP3, to suppress NLRP3 inflammasome-mediated pyroptosis (Figure 1C; part 4.1). Treatment with MCC950 decreased the protein expression levels of NLRP3, ASC, cleaved-caspase-1, and cleaved-GSDMD as detected by Western blot analysis (Figure 8D and E). Moreover, MCC950 treatment decreased the levels of proinflammatory cytokines (IL-18 and IL-1β) as detected by ELISA at 6 h after the administration of exosomes (Figure 7F), which indicated that MCC950 effectively inhibited pyroptosis-mediated inflammation. Furthermore, FJC staining showed that exosome-induced neuronal degeneration in the hippocampal CA1, CA3, and DG regions was suppressed by pretreatment

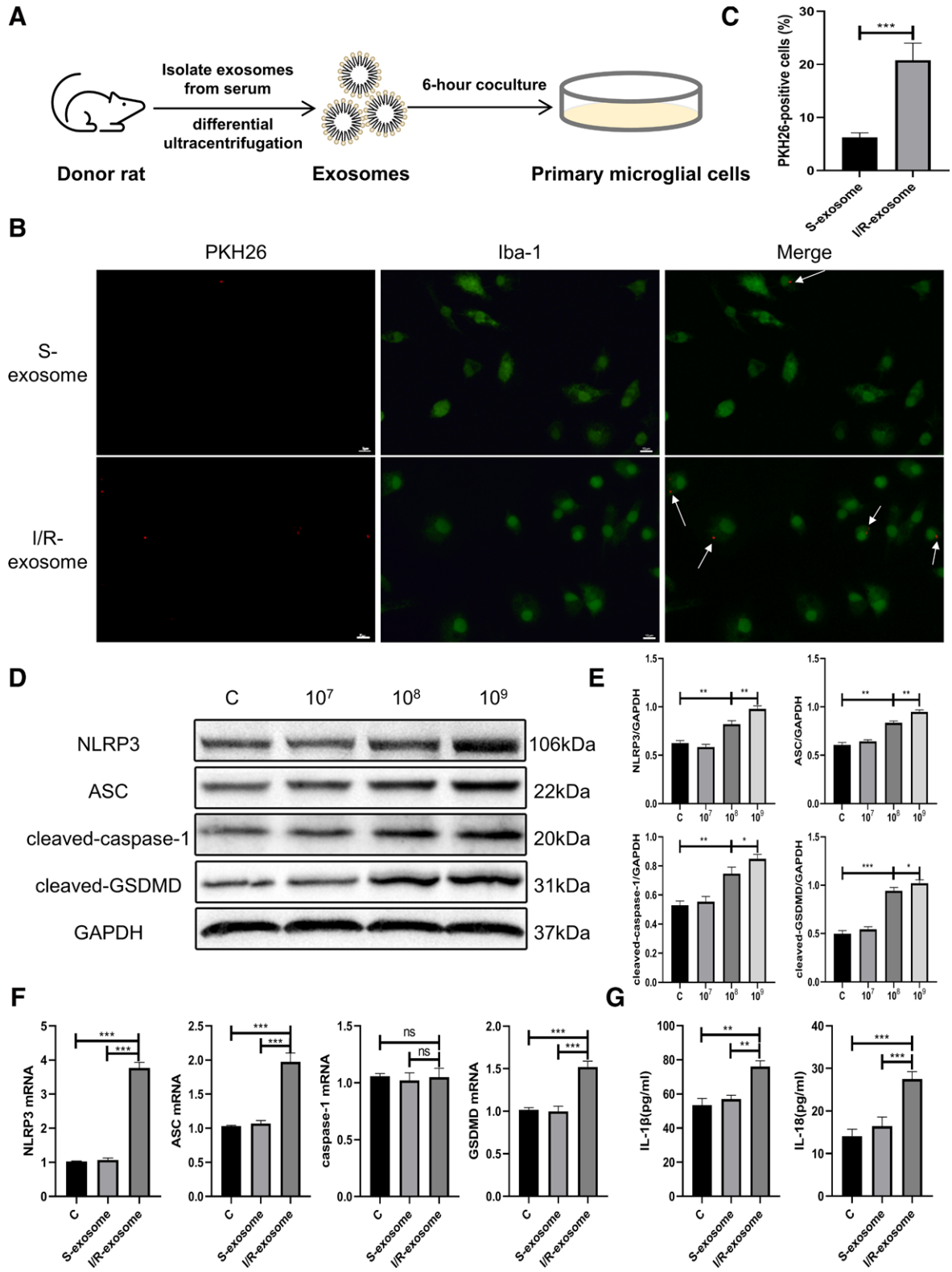

FIGURE 7. I/R-exosomes promote microglial pyroptosis in vitro. A, A schematic diagram of the experimental procedure. B, The PKH26-labeled exosomes (red) were cocultured with primary microglia (green) for the indicated time. The uptake of exosomes by microglia was observed under a fluorescence microscope. White arrowheads indicated PKH26-positive microglia (magnification, ×400; scale bars, 10 μm). C, Quantitative analysis of the percent of PKH26-positive microglia. n = 6 slices per group. D and E, Western blotting results of NLRP3, ASC, cleaved-caspase-1, and cleaved-GSDMD in primary microglia treated with gradient concentrations (10^7/mL, 10^8/mL, 10^9/mL) of I/R-exosomes. n = 5 per group. F, RT-qPCR results showing the mRNA levels of NLRP3, ASC, caspase-1, and GSDMD in microglia among 3 groups. n = 5 per group. G, ELISA results of IL-1β and IL-18 in the cell culture supernatant among 3 groups. n = 5 per group. $^{ns}P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.0001$. ASC, apoptosis-associated speck-like proteins containing a caspase recruitment domain; ELISA, enzyme-linked immunosorbent assay; FJC, Fluoro-Jade C; GSDMD, gasdermin D; IL, interleukin; I/R, ischemia–reperfusion; NLRP3, nod-like receptor family protein 3; RT-qPCR, real-time quantitative polymerase chain reaction.

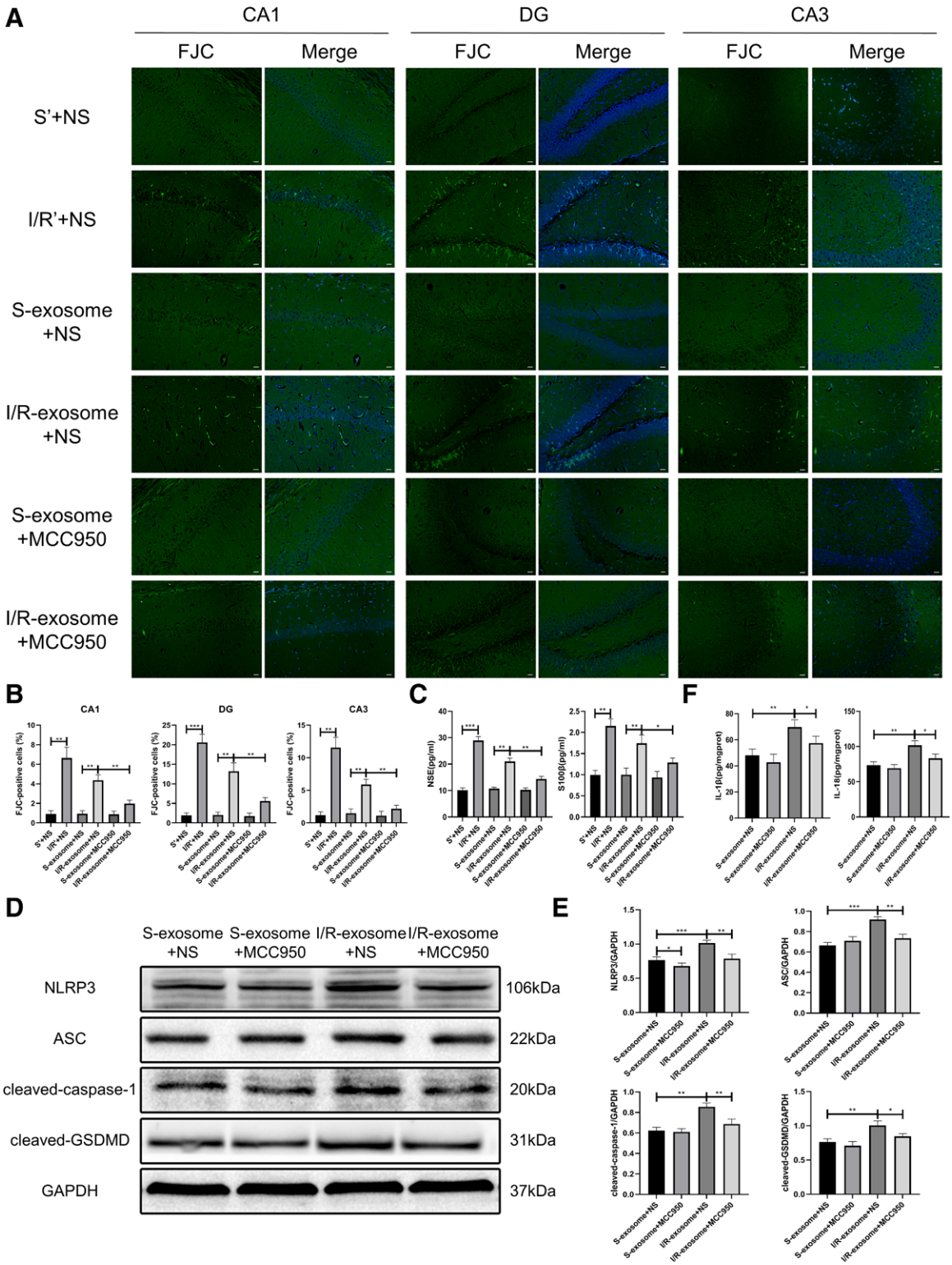

FIGURE 8. The protective effect of MCC950 on exosome-induced pyroptosis and neuronal injury in the developing hippocampus. A, Images showing FJC staining in the hippocampal CA1, CA3, and DG regions. The degenerated neurons were detected by FJC (green; magnification ×200; scale bars, 20 μm). B, Quantitative analysis of the percent of FJC-positive neurons in the hippocampal CA1, CA3, and DG regions. The data were calculated as follows: the number of FJC-positive neurons/the number of total cells × 100%. N = 5 rats per group. C, ELISA results of serum brain damage markers (S100β and NSE). n = 5 rats per group. D and E, Western blotting results of NLRP3, ASC, cleaved-caspase-1, and cleaved-GSDMD in the hippocampus. n = 5 rats per group. F, ELISA results of IL-1β and IL-18 in the hippocampus. n = 5 rats per group. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.0001$. ASC, apoptosis-associated speck-like proteins containing a caspase recruitment domain; ELISA, enzyme-linked immunosorbent assay; FJC, Fluoro-Jade C; GSDMD, gasdermin D; IL, interleukin; NLRP3, nod-like receptor family protein 3.

with MCC950 (Figure 8A and B). In addition, the levels of S100β and NSE displayed a similar decreasing trend (Figure 8C). Collectively, these findings indicated that exosome-induced hippocampal neuron injury is alleviated by suppressing pyroptosis-mediated sterile inflammation.

DISCUSSION

Despite improvements in survival rates after pediatric liver transplantation, neurological complications are still common and associated with lower health-related quality of life for pediatric patients.[1,2] Multiple factors contribute to neurological complications after pediatric liver transplantation, including primary liver diseases, immunosuppressant toxicity, prolonged hospitalization, and surgical procedures.[32,33] One of the prime causes is HIR, which is a common process during liver transplantation and may cause liver and remote multiple organ injury.[3,4] In the present study, we established a 70% hepatic warm I/R young rat model to reproduce the critical pathophysiological process and explore the mechanisms of brain injury, which demonstrated the significance of circulating exosomes in mediating microglial pyroptosis and subsequent neuronal injury in the hippocampus during HIR.

The pathogenesis of HIR injury mainly involves aggravated inflammatory injury and activation of the immune system.[34] A previous clinical trial has shown that the levels of plasma inflammatory cytokines are elevated throughout the hepatic perfusion period in young individuals.[35] During HIR, acute oxidative stress and consequent hepatocellular death cause an inflammatory response and the release of damage-associated molecular patterns (DAMPs). In turn, DAMPs initiate further proinflammatory and immune regulatory responses by activating pattern recognition receptors to exacerbate HIR injury.[36] The median age of pediatric liver transplantation is <2 y, a critical period of nervous system development, especially in the hippocampus, which is easily affected by HIR.[1,2,6] HIR is characterized by sterile systemic inflammation. Thus, neuroinflammation has been recognized as one of the most important causes of HIR-related hippocampal injury and postoperative cognitive impairment.[37,38]

Pyroptosis is a form of lytic programmed cell death that causes a cascade of inflammatory responses. The NLRP3 inflammasome plays a critical role in triggering the innate immune system by recognizing DAMPs.[36] The NLRP3/caspase-1 pathway, which is regarded as the major signaling pathway that leads to pyroptosis, has been extensively studied in multiple neurological disorders characterized by neuroinflammation[21,39] and HIR injury.[40,41] In the present study, we observed elevated expression levels of NLRP3, ASC, cleaved-caspase-1, and GSDMD in the developing hippocampus during HIR, suggesting that HIR mediates hippocampal pyroptosis via the canonical NLRP3 inflammasome pathway.

Exosomes represent an efficient way to transport various biological information and modulate the pathophysiological processes of target cells.[11-13] The function of exosomes depends on their cargo. Previous studies have revealed that some exosomes cross the BBB and induce neuroinflammation as media between the peripheral and CNS immune systems.[15,16,42] In the present study, we found that HIR increased the concentration of circulating exosomes in young rats. To confirm the effect of exosomes released during HIR on hippocampal injury, we administered exosomes derived from 2 mL of sera, nearly the whole sera of 1 rat, to another normal rat via the tail vein. The present data demonstrated that intravenous administration of I/R-exosomes induced hippocampal neuron injury, which indicated that exosomes, as mediators, link HIR with CNS injury, suggesting that exosomes may be a critical therapeutic target of HIR-related brain injury. Moreover, I/R-exosomes also induced the occurrence of pyroptosis in the hippocampus, indicating that exosomes are the major factor for hippocampal pyroptosis during HIR. Therefore, we used GW4869 to inhibit the biogenesis of exosomes, which resulted in a protective effect against hippocampal pyroptosis.

However, the specific alterations in exosomal components that result in hippocampal injury were not clarified in the present study. Differences in exosomal cargoes determine their different performances. Some exosomes are restorative and used to treat organ injury. For example, stem cell-derived exosomes play significant roles in the treatment of many diseases and are an effective alternative to therapeutic stem cells. Moreover, engineered exosomes are widely used for targeted drug delivery.[43]

Exosomes have the ability to target specific cell types.[44] Previous studies have revealed that circulating exosomes can penetrate the BBB and interact with any type of neurocyte.[45-47] The present findings showed that I/R-exosomes were mainly taken up by microglia in the hippocampus. Moreover, primary microglia treated with the same concentration of S-exosomes or I/R-exosomes exhibited a higher uptake of I/R-exosomes in vitro. These findings suggested that microglia may be the major target cells of I/R-exosomes in the CNS.

Microglia are considered to be CNS-resident macrophages, forming the first immune barrier of the brain. The morphology and function of microglia are modulated in accordance with the brain microenvironment.[48] Microglia are the pivotal cell type for any discussion of neuroinflammation. Although pyroptosis has been shown to occur in various CNS cell types under pathological conditions, microglia are still the prominent cell type for pyroptosis.[39,49] The uptake of exosomes by target cells is the precondition for exosomes to alter the biological functions of target cells. Based on the present finding that I/R-exosomes were taken up by microglia, we hypothesized that exosome-induced pyroptosis mainly occurs in microglia. Consistently, the present data demonstrated that cleaved-caspase-1 mainly colocalized in microglia in the hippocampus after the administration of I/R-exosomes. In addition, the ability of I/R-exosomes to induce pyroptosis in microglia was confirmed in vitro. Together, these findings suggested that I/R-exosomes target microglia and mediate microglial pyroptosis.

Neuroinflammation is closely related to the activation of glial cells and the release of proinflammatory mediators.[50] The release of IL-1β and IL-18, along with pyroptosis, accounts for neuroinflammation. Pyroptosis-mediated sterile inflammation in the hippocampus has been widely considered a key factor for cognitive dysfunction and secondary brain injury.[51,52] In the present study, both neuronal injury and microglial pyroptosis in the developing hippocampus were observed. Recent

studies have revealed that pyroptosis-mediated sterile inflammation leads to neuronal damage.[28,53,54] Therefore, neuronal degeneration may be directly triggered by I/R-exosomes themselves or by microglial pyroptosis-mediated sterile inflammation, indicating that it is critical to confirm the relationships among I/R-exosomes, neuronal degeneration, and pyroptosis. In the present study, we targeted NLRP3 with the specific inhibitor, MCC950. In addition to alleviating pyroptosis and the release of IL-18 and IL-1β, the NLRP3 inhibitor also improved neuronal degeneration, suggesting that pyroptosis-mediated inflammation contributes to exosome-induced neuronal degeneration. Thus, the present study elucidated the crosstalk between microglial pyroptosis and neuronal damage.

The present study had several limitations. Most current exosomal isolation techniques face the challenge of low purity.[55] Although we used ultracentrifugation, the most widely used and gold-standard technique for exosomal isolation, we still cannot ignore the potential effect of low purity on the exosomal downstream analysis. Moreover, the present study did not clearly illustrate the differences in functional components between S-exosomes and I/R-exosomes. Thus, the specific molecular mechanism involved in the activation of pyroptosis was not clarified. Future studies are needed to explore the alteration of exosomal protein or nucleic acid cargoes and potential clinical applications. Because plasma exosomes are emerging as a noninvasive biomarker for disease diagnosis and prognosis,[56] future studies will investigate the correlation between circulating exosome profiling and posttransplantation cognitive function in young individuals. In addition, along with further research on the mechanisms of exosome-mediated brain injury during HIR, exosomal engineering technology and modification strategies may allow exosomes to be used in future clinical practice to prevent HIR-related brain injury.

In conclusion, the present study demonstrated that circulating exosomes released during HIR contribute to neuronal injury in the developing hippocampus by mediating microglial pyroptosis. Hence, this pathway may be a potential target for the prevention of brain injury associated with HIR.

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