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ORIGINAL PAPER

MiR-124 Enriched Exosomes Promoted the M2 Polarization of Microglia and Enhanced Hippocampus Neurogenesis After Traumatic Brain Injury by Inhibiting TLR4 Pathway

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Abstract

MicroRNA-124 (miR-124) is a brain specific miRNA that is highly expressed in microglia. The upregulation of miR-124 contributes to M2 polarization of microglia, which is beneficial to neurogenesis. Exosomes are lipid membrane vesicles that can deliver miR-124 into the brain. However, whether miR-124 enriched exosomes (Exo-miR-124) can regulate the polarization of microglia and affect hippocampus neurogenesis after traumatic brain injury (TBI) is unknown. To clarify this, the Exo-miR-124 was first constructed, and then was intravenously administrated into rats via tail vein with the dose of 3×10^9 particles/each rat at 24 h post TBI. The polarization of microglia in hippocampus was evaluated through measuring the signature genes and cytokines of M1/M2 phenotype by reverse transcription-polymerase chain reaction (RT-PCR) and enzyme-linked immune sorbent assay (ELISA) at 7/14/21/28 days after TBI. Hippocampus neurogenesis was evaluated through detecting the proliferation marker BrdU/SOX2 and differentiation marker BrdU/NeuN by immunofluorescence (IF) at 7 and 28 days after TBI respectively. Neurological function was evaluated by neurological severity score (NSS) and morris water maze (MWM) at 7/14/21/28 and 24–28 days after TBI respectively. To explore the underlying mechanisms, the mRNA expression of TLR4 pathway molecules in hippocampus were measured by RT-PCR, and the polarization of microglia and the activation of TLR4 pathway in BV2 cells were measured after exosome treatment as well. Results demonstrated that Exo-miR-124 treatment promoted the M2 polarization of microglia, enhanced neurogenesis in hippocampus, and improved function recovery after TBI. The M2 polarization effect of Exo-miR-124 was produced through inhibiting TLR4 pathway, which was verified in hippocampus and BV2 microglia. In conclusion, Exo-miR-124 treatment promoted M2 polarization of microglia and improved hippocampal neurogenesis and functional recovery after brain injury, which might be a strategy to improve the outcome of TBI.

Keywords Traumatic brain injury · Exosome · MiR-124 · Microglia · Neurogenesis · TLR4

Introduction

Traumatic brain injury (TBI) is an acute neurotrauma disease induced by the sudden external force, one of the major causes of death and long-term disability worldwide [1]. Until recently, no effective treatment was verified to improve neural structural reparation and functional recovery after TBI in clinic trials [2]. Newly studies suggested that neural stem cells (NSCs) reserved in neurogenic regions such as dentate gyrus (DG) of hippocampus in adult mammalian brain played regenerative and reparative roles after TBI [3], which made the hippocampus neurogenesis to be a research focus. Currently, most of researchers who concentrated on hippocampus neurogenesis after TBI spared their efforts to explore new strategies

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to promote the NSCs proliferation and differentiation into neurons. Neuroinflammation is a natural reaction after TBI that has protective effects on the injured brain in a way, while the excessive inflammatory response often becomes a significant driving force that leads to reduced hippocampus neurogenesis and worse functional recovery [4]. Microglia played key roles in activating and regulating neuroinflammation, which was reported to produce double-edged effects on hippocampus neurogenesis depending on its classical (M1, pro-inflammatory)/alternative (M2, anti-inflammatory) phenotype polarization [4]. M1 microglia secreted pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α which were detrimental to hippocampus neurogenesis [5–7], while M2 microglia secreted anti-inflammatory cytokines such as IL-4, IL-10 and TGF-β which were beneficial to hippocampus neurogenesis [8–10]. Thus, exploring effective strategies to promote the microglia to polarize from M1 to M2 phenotype and inhibit the excessive neuroinflammation might has great significance for the improvement of hippocampus neurogenesis and functional recovery after TBI.

MicroRNAs (miRNAs) are important molecules enriched in the brain that can modulate gene transcription and associated pathway expression in the polarization of microglia [11–13]. Among the known miRNAs, miR-124 is a brain specific miRNA that is highly expressed in microglia. In physiological conditions, miR-124 regulates the function of microglia and plays a key role in its quiescence [14, 15]. In pathological conditions, the downregulation of miR-124 increased neuroinflammation by polarizing microglia into M1 phenotype [16], while the upregulation of miR-124 reduced neuroinflammation by polarizing microglia into M2 phenotype [17, 18]. These studies indicated that miR-124 had the potential to be an effective agent to polarize microglia into the M2 phenotype and thus promote hippocampus neurogenesis after TBI. One promising carrier that can deliver miR-124 into the brain and play its role is exosome, which is an endosomal origin membrane-enclosed small vesicle (30–100 nm) containing proteins, lipids, mRNAs and microRNAs [19]. The advantages of exosome include its ability to cross the (blood brain barrier) BBB which makes the delivery of miR-124 into the brain to be a great possibility, and its bi-lipid membrane which makes the embedded miR-124 be stable and play its role after delivery. Excitingly, latest studies have demonstrated that the administration of MSCs-derived exosomes reduced neuroinflammation, promoted neurogenesis and functional recovery after TBI [20, 21], and miR-124 enriched exosome (Exo-miR-124) promoted the M2 polarization of N9 microglia in vitro and enhanced neurogenesis in vivo [22–24]. However, whether Exo-miR-124 can regulate the polarization of microglia and hippocampus neurogenesis after TBI and the underlying mechanisms are still unknown.

According to the findings of previous studies, the TLR4 signaling pathway might be involved in the mechanisms of Exo-miR-124 regulating the polarization of microglia after TBI. First, the administration of TLR4 activator lipopolysaccharide (LPS) induced inflammatory responses, which resulted from the activation of microglia and inhibited the neurogenesis in DG of hippocampus [5]. Second, LPS stimulation increased the expression of TLR4 downstream inflammatory mediator NF-κB, which ultimately induced the production of inflammatory cytokines IL-6 and TNF-α, that were widely known to disrupt neurogenesis [25, 26]. And, LPS could activate TLR4 signaling pathway in microglia, which further contributed to the depletion of NSCs in DG of hippocampus [27]. Third, newly studies demonstrated that the absence of TLR4 induced the M2 polarization of microglia and alleviated the development of neuroinflammation, which showed potential neuroprotective effects after TBI [28, 29]. Moreover, TLR4 activator LPS down-regulated the expression of miR-124, and miR-124 overexpression was shown to inhibit the LPS-induced microglial activation by targeting TLR4 [12, 16]. Collectively, TLR4 signaling pathway might play a role in the process of Exo-miR-124 regulating the polarization of microglia and affecting hippocampus neurogenesis after TBI.

The purpose of this study was to explore the potential of Exo-miR-124 in regulating the polarization of microglia and further affecting hippocampus neurogenesis after TBI, as well as to reveal the role of TLR4 signaling pathway in this process. The results of our research will provide an underlying strategy to improve the outcome of TBI through delivering miR-124 into the brain by using the exosome.

Materials and Methods

Animals

Male Sprague–Dawley rats (8–10 weeks old, weighing 250–300 g) were purchased from the Experimental Animal Center of Airforce Military Medical University (Fourth Military Medical University) (Xi'an, China). Animals were housed in comfortable temperature (21.0 ± 2 °C), appropriate humidity (50–55%) and a 12 h light/dark cycle (lights on and off at 8:00 am and 8:00 pm), with enough food and water. A total of 180 rats were randomly divided into four groups: sham, TBI, sham + Exo-miR-124 and TBI + Exo-miR-124, with 45 rats in each group. The Airforce Military Medical University (Fourth Military Medical University) Ethic Committee gave permission to the experimental protocols and animal procedures, which were implemented according to the National Experimental Animals Guidelines.

Isolation and Culture of BM-MSCs

BM-MSCs were acquired from femurs and tibias, which were sterilely isolated from rats and washed by DMEM-low glucose (DMEM-LG). The washing medium was first centrifuged at 1500 g for 5 min, and then the sediment was resuspended in 1.073 g/ml Percoll after being washed twice by medium. BM-MSCs were harvested by density gradient centrifugation at 2000 g at last. The isolated BM-MSCs were cultured in DMEM-LG medium containing 20% Fetal Bovine Serum (FBS, Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml amphotericin under the environment of 37 °C, 5% CO_2. The culture medium was replaced 48 h after incubation for the first time and once every 2 days afterwards. BM-MSCs were then used to construct Exo-miR-124 when their confluence was more than 95%.

Construction of Exo-miR-124

Exo-miR-124 was constructed by means of plasmid transfection with BM-MSCs and Exo-Quick exosome isolation. The pSUPER-miR-124 and pSUPER-control plasmid was first constructed by miR-124 and miR-con cloning, and then was transfected into BM-MSCs using Lipofectamine 2000 (Life Technologies, USA) according to manufacturer's instructions. The transfection effect was evaluated by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Later on, the transfected BM-MSCs were cultured in MSC medium using GibcoTM Exosome-Depleted FBS (Thermo Fisher, USA). After 7 days' incubation, exosomes were isolated from supernatants of BM-MSCs culture medium using Exo-Quick exosome isolation kit (System Bioscience, USA). Furthermore, the isolated exosome was identified through measuring the expression of membrane protein CD9, CD63 and CD81 by western blot (WB), and analyzing its shape and size by electron microscope. The expression of miR-124 in Exo-miR-124 and Exo-con was measured by RT-PCR.

BV2 Microglia Culture and Treatment with Exosome

The BV2 microglia were purchased from China Infrastructure of Cell Line Resources (Beijing, China) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin. Then, the cells were seeded onto plates at a density of 1×10^6 cells/well, and incubated in the environment of 37 °C, 5% CO2 and saturated humidity. To investigate the effect of Exo-miR-124 on the polarization of microglia and the activation of TLR4 signaling pathway, BV2 microglia were grown for 4 days and then were randomly divided into four groups: Control, LPS, Control + Exo-miR-124 and LPS + Exo-miR-124. The cells in all groups were incubated in DMEM solution for 24 h. LPS (1 μg/ml) treatment was performed to activate the TLR4 signaling pathway in BV2 microglia, Exo-miR-124 were added into the culture medium to explore their effects on the TLR4 signaling pathway. The Exo-miR-124 were added into BV2 microglia at the concentration of 3×10^9 particles/ml after being washed twice by PBS, which was performed according to the previous study [30]. Then, the expression of miR-124 in BV2 microglia was measured by RT-PCR. The polarization of BV2 microglia was evaluated through measuring the expression of signature genes in M1/M2 microglia by RT-PCR, as well as through measuring the expression of pro-inflammatory and anti-inflammatory cytokines by ELISA. The expression of TLR4 and its downstream molecules were measured by WB.

Establishment of TBI Rat Model

TBI model in this study was established using the controlled cortex injury (CCI) device (Hatteras Instruments, Cary, NC, USA). Rats were first anesthetized by 2% pentobarbital sodium (60 mg/kg) through intraperitoneal injection, and fixed on the stereotaxic frame (Kopf Instruments, Tujunga, CA, USA) to secure the head horizontally by an incisor bar and two lateral ear pins. The sagittal incision and fascia reflection were then performed at the midline of the scalp to expose the skull. Later on, a diameter craniotomy of 4.0 mm on the right hemisphere was performed between the bregma and lambda and 3.0 mm lateral to the sagittal suture, the skull flap was removed as well. After that, the piston rod was used to perform a perpendicular impact brain contusion on the exposed dura with contact surface of 3.0 mm diameter, impact velocity of 3.0 m/s, vertical cortical impact depth of 1.5 mm and contact time of 100.0 ms. At last, the skull flap was restored to the primary position and the scalp was sutured sterilely. Rats in the sham group were only underwent craniotomy but without cortical impact contusion. The heating pad was used to maintain the body temperature of rats at 37.0 ± 0.5 °C during the surgical and recovery period. A total of 92 rats were used for the construction of TBI model, and 8 rats died from the injury.

Administration of Exosome and Drug

The Exo-miR-124 isolated from the supernatants of BM-MSCs culture medium was first dissolved in 0.5 ml phosphate-buffered solution(PBS), and then was intravenously administrated over 5 min via tail vein at 24 h after the establishment of TBI. The dose of exosomes administrated in each rat was 100 μg total exosomal protein or equal to 3×10^9 particles more or less, which was chosen based on the reports of previous studies [21, 31]. For labeling the endogenous NSCs in DG of hippocampus, the thymidine analog bromodeoxyuridine (BrdU) (Sigma-Aldrich, MO, USA) was dissolved in sterile saline solution at a concentration of 10.0 mg/ml. To

assess the proliferation of NSCs, rats received three times i.p. injection of BrdU (100 mg/kg) with an 8 h interval at the 6th day after TBI and were sacrificed at the seventh day. To assess the neuron differentiation of NSCs, rats received i.p. injection of BrdU (100 mg/kg) once per-day for 7 days, starting from 1 day after TBI, and then were sacrificed at 28 days after TBI. The administration protocol of BrdU was chosen based on previous reports [32, 33], which had been conducted in our previous studies as well [34, 35].

RT-PCR

To measure the expression of miR-124 in exosomes, brain and BV2 microglia, total RNA was extracted using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. PrimeScript First-Strand cDNA Synthesis Kit (Takara, Japan) was used for treverse-transcription, and cDNAs PrimeScript RT Master Mix (Takara, Japan) was used for RT-PCR. To detect the level of miR-124, miRNA First-Strand cDNA Synthesis Kit (Tiangen Biotech, China) was used to reverse-transcribe total RNA to cDNA, and miRNA qPCR Detection Kit (SYBR Green, China) was used to perform RT-PCR. All PCR reactions were run in triplicate by ABI PRISM 7000 sequence detection system (Applied Biosystems, USA). The relative level of miR-124 was calculated by the comparative threshold cycle (Ct) method (relative expression levels = $2^{-\Delta Ct}$) and was normalized to the expression of control β-actin or U6 snRNA.

RT-PCR was also performed to evaluate the mRNA expression of TLR4 pathway molecules and the polarization of microglia in hippocampus of rats, as the M1/M2 phenotype of microglia were characterized by the expression of signature genes. The hippocampus was first isolated from the brain at 3/7/14/28 days after TBI (n = 6 in each group/each time point). Tissue samples were then snap-frozen by liquid nitrogen and stored at − 80 °C until use. Subsequently, total RNA was extracted from the tissue samples by Trizol reagent (Takara, Japan) according to manufacturer's instructions, and 1 μg total RNA was used to reverse transcribe first strand cDNAs by a High Capacity cDNA Archive Kit (Applied Biosystems, USA). At last, 50 ng cDNA was used to perform qPCR reactions in triplicate using the ABI PRISM 7,000 sequence detection system (Applied Biosystems, USA). The parameters of qPCR including incubation at 50 °C for 2 min, denaturing at 95 °C for 10 min, 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. The sequences of the PCR primers (M1,M2) including: CD32 (forward primer: 50-AATCCTGCCGTTCCTA CTGATC-30; reverse primer: 50-GTGTCA CCGTGTCTTCCTTGAG-30); IL-1b (forward primer: 50-CAGGCTCCGAGATGAACAAC-30; reverse primer: 50-GGTGGAGAGCTTTCAGCTCA TA-30); CD206 (forward primer: 50-TCTTTGCCTTTC CCAGTCTCC-30; reverse primer: 50-TGACACCCAGCG GAATTTC-30); and Arginase 1 (forward primer: 50-GAA CACGGCAGT GGCTTTAAC-30; reverse primer: 50-TG CTTAGCTCTGTCTGCTTTGC-30). The final results were normalized to the expression of 18S RNA that was assayed using 5 ng of total cDNA.

ELISA

To evaluate the expression of pro-inflammatory and anti-inflammatory cytokines in the hippocampus, the tissues were first isolated from the brain at 3/7/14/28 days after TBI (n = 6 in each group/each time point). Then the hippocampus was pulverized in the homogenizer with liquid nitrogen. Later on, lysis buffer including 1 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 150 mM NaCl, 10 mM Tris pH 8.0 and 5 μl/ml protease inhibitor (Sigma-Aldrich, USA) was added into the lysates and incubated for 1 h at 4 °C. The lysates were further centrifugalized at 3000 rpm for 20 min, and the supernatants were collected to measure the concentration of pro-inflammatory cytokines IL-1β, IL-6, TNF-α and anti-inflammatory cytokines IL-4, IL-10 and TGF-β by ELISA kits (Minneapolis, USA). The experiments were conducted according to manufacturer's instructions.

In order to evaluate the expression of pro-inflammatory and anti-inflammatory cytokines in BV2 microglia, the cell culture medium was gathered at 24 h after the exosome treatment. The concentrations of pro-inflammatory cytokines IL-1β, IL-6, TNF-α and anti-inflammatory cytokines IL-4, IL-10 and TGF-β were measured by ELISA kits (Minneapolis, USA) in accordance with the manufacturer's instructions described above.

Western Blot

Western blot (WB) was performed to explore the effects of Exo-miR-124 on the TLR4 signaling pathway in BV2 microglia after LPS stimulation. Briefly, the exosome treated BV2 cells were collected and lysed by Mammalian Cell Lysis kit (Sigma, USA). After centrifugation at 12,000 rpm for 20 min at 4 °C, the supernatant of lysis was collected for BCA protein assay (Beyotime, China) and protein extraction. A total of 40 μg protein was electrophoresed on the 10% SDS-polyacrylamide gel and transferred to the PVDF membrane (Millipore, USA). Then, the membrane was blocked by 5% nonfat milk in tris-buffered saline with 0.1% Triton X-100 (TBST) for 1 h and incubated overnight at 4 °C with the primary antibodies including TLR4 (Thermo Fisher, USA), MyD88 (Santa Cruz, USA), IRAK1 (Enzo Life Sciences, USA), TRAF6 (Novus Biological, USA), NF-κB p65 (Gene Tex, USA), and β-actin (Proteintech, USA). After 3 time washes in PBS, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG antibody (Cell Signaling Technology, USA) at room temperature

for 1 h. The immunoreactivity was measured by Western Bright Enhanced chemiluminescence reagents (K12045-d20, USA), and the optical density (OD) was analyzed by Gel-Pro Analyzer software (version 6.0, USA).

Immunofluorescence

The immunofluorescence (IF) was performed to evaluate hippocampus neurogenesis by labeling the proliferation marker $BrdU^+/SOX2^+$ and differentiation marker $BrdU^+/NeuN^+$ on NSCs in DG. Rats were first intraperitoneally deep anesthetized by 2% pentobarbital sodium (60 mg/kg) at 7 and 28 days after TBI, then the brain was removed and immersed in 4% paraformaldehyde in PBS (pH 7.4) at 4 °C for 2–4 days (n = 6 in each group/each time point). After that, the fixed brain was dehydrated by alcohol and embedded in paraffin, and 5 μm thick coronal brain sections which contained the complete DG of hippocampus (from bregma − 2.40 mm to − 4.68 mm) were prepared by microtome (Leica, Nussloch, Germany) and dried at 92 °C overnight. For the denaturation of DNA, brain sections were first deparaffinized by alcohol and dimethylbenzene, and then were incubated with citric acid antigen retrieval buffer (pH 6.0) at 95 °C for 15 min. To block nonspecific signals, brain sections were treated by 1% donkey serum albumin and 0.3% Triton X-100 in PBS at room temperature for 30 min. To evaluate the proliferation and differentiation ability of NSCs, brain sections were first incubated with the primary antibodies including sheep anti-BrdU antibody (GeneTex, USA), rabbit anti-rat SOX2 antibody (GeneTex, USA), and mouse anti-rat NeuN antibody (Merck Millipore, USA) in PBS overnight at 4 °C. After being washed 3 times in PBS, the sections were further incubated with the secondary antibodies including Alexa fluor 594-labeled donkey anti-sheep IgG antibody (Molecular Probes, USA), Alexa fluor 488-labeled goat anti-rabbit IgG antibody (Molecular Probes, USA), and Alexa fluor 488-labeled goat anti-mouse IgG antibody (Molecular Probes, USA) in PBS for 1 h at room temperature. At last, after 3 time washes in PBS once again, brain sections were mounted with the anti-fade mounting medium containing 1,4-Diazobicyclo and cover slipped for the further IF assay. The confocal laser scanning microscope (Olympus, Japan) with a FLUO-VIEW image system (Olympus, Japan) and Photoshop 7.0 software (Adobe Systems, USA) was used to observe and capture the images. The hippocampus neurogenesis was analyzed by counting the $BrdU^+/SOX2^+$ and $BrdU^+/NeuN^+$ cells at SGZ of five consecutive visual fields (400x) in each brain section. The number of positive cells in each section was determined by its average number in the five visual fields, and the average number of five sections was taken as the final number of newborn neurons in each rat brain.

Neurological Severity Score Test

Neurological Severity Score (NSS) test was performed to evaluate the neurological function of rats at 7, 14, 21 and 28 days after TBI (n = 6 in each group). According to the description of previous studies, the NSS was consisted of 10 individual parameters for the alertness measurement, balancing examination and motor ability evaluation [36, 37]. The rat would be awarded for one score point if it lacked a tested reflex or failed to accomplish a task. The total score of NSS was graded on a scale of 0–10, with 0 indicated normal status and 10 suggested maximal neurobehavioral deficits. Thus, the higher the accumulated score, the severer the neurobehavioral dysfunction.

Morris Water Maze Test

Morris Water Maze (MWM) test was performed to measure the neurocognitive function of rats at 24–28 days after TBI (n = 6 in each group), and the procedure was conducted according to previous studies [38, 39]. The instrument used in MWM test was a 160 cm in diameter and 50 cm in depth circular tank with a black inner wall, which was filled with water of 30 cm in depth and 25 °C in temperature. The learning ability and memory function of rat was evaluated by conducting the hidden platform trial and probe trial respectively. In the hidden platform trial, the tank was first divided into four equal quadrants, then a black circular platform of 12 cm in diameter was hidden 2 cm under water surface in the center of one quadrant. Later on, the rat was allowed to swim freely in the maze and had a maximum of 120 s to find the platform. The trial would be terminated after being guided onto the platform and remaining on it for 30 s, if the rat failed to reach the platform within 120 s. During this process, the interval time between rat was placed into the water and reached the platform was recorded as escape latency, which was further used to evaluate the learning ability. Each rat received four hidden platform trials per day for four consecutive days from 24 days post-trauma. After that, the hidden platform was removed from the quadrant and the probe test was conducted at 28 days post-trauma. Briefly, the rat was first put into the water and allowed to swim freely in the maze to find the removed platform. Then the route in target quadrant was identified according to the rat's trace in which quadrant the platform was placed previously. The platform crossing time was calculated based on the time of the rat swam over the previous platform location. The neurocognitive function of rat was evaluated according to the parameters in above trails, which was recorded by the tracking system (Dig Beh-MR, Shanghai Auspicious Software Technology Company Limited, China).

Statistical Analyses

Data in the study was expressed as mean values ± standard deviations (M ± SD). The differences between two groups were analyzed by Unpaired *t* test with Welch's correction. The differences among four groups of rats were analyzed by Two-way ANOVA test followed by Tukey honest signifcant difference post hoc testing, results included the weighted average treatment effect comparisons of injury state and exosome administration, and post hoc pairwise comparisons. The differences of NSS and MWM score of rats among four groups were analyzed by repeated measures Two-way ANOVA, with Dunnett multiple comparison post hoc testing. The differences among four BV2 microglia groups were analyzed by Two-way ANOVA test followed by Tukey honest signifcant difference post hoc testing as well. The analysis was performed by SPSS 13.0 software (SPSS Inc., USA), and two-tailed $P < 0.05$ was considered statistically significant. Figures in this manuscript were drawn by the GraphPad Prism 5 software (GraphPad Software, Inc., USA).

Results

Construction and Characterization of Exo-miR-124

To construct Exo-miR-124, the BM-MSCs with high expression of miR-124 were first generated by plasmid transfection, and the transfection effect was evaluated by RT-PCR. Compared to pSUPER-control, pSUPER-miR-124 significantly increased miR-124 expression in BM-MSCs (Fig. 1a). Then exosomes were isolated from the culture supernatants of BM-MSCs, and identified by analyzing their shape and size using electron microscope and measuring the expression of membrane protein CD9, CD63 and CD81 by WB. Images captured by the electron microscope showed that exosomes were round-shaped morphology nanoparticles with a size of 40–100 nm (Fig. 1b). The qNano nanopore-based exosome detection system identified the peak diameter of exosome was 77.1 ± 19.5 nm. WB indicated that the

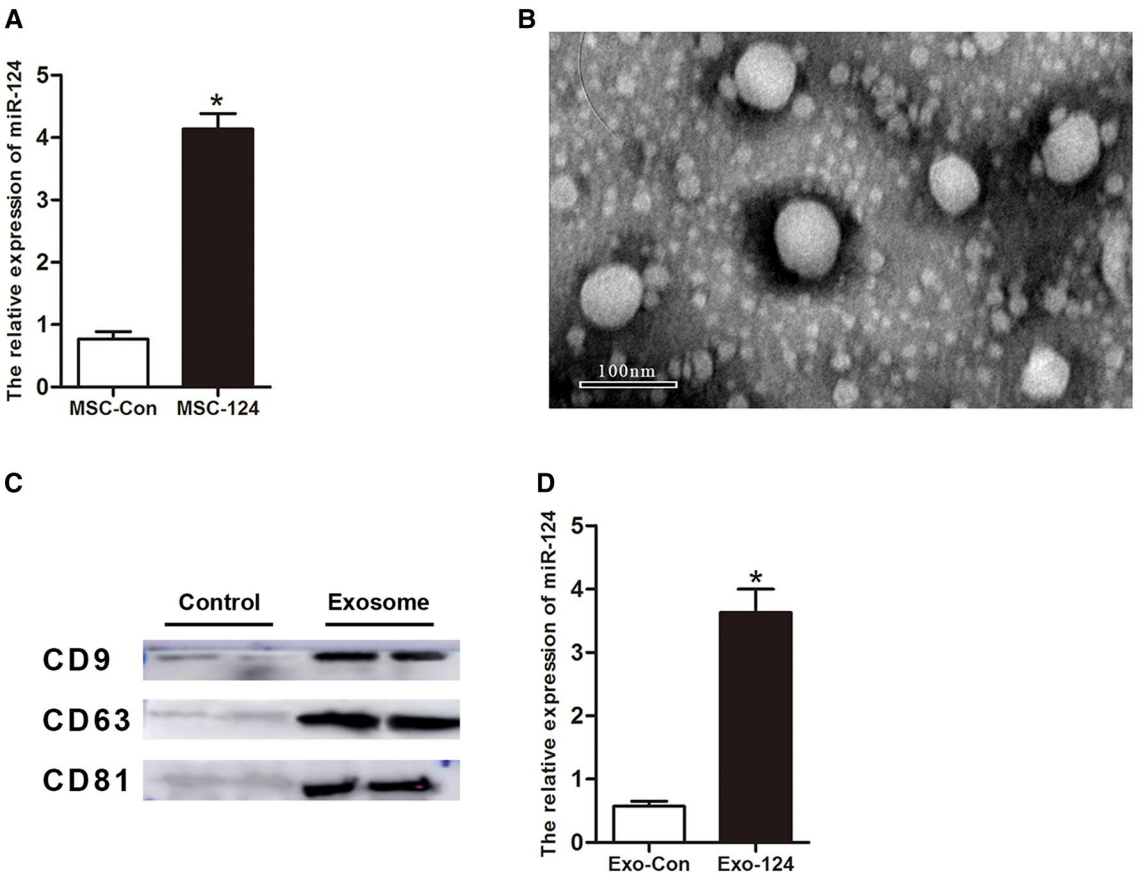


Fig. 1 The construction and identification of Exo-miR-124. **a** The relative expression of miR-124 in MSCs was significantly elevated after transfected with pSUPER-miR-124. **b** The representative electron microscope image of modified exosomes derived from MSCs. **c** The Western blotting analysis of the characteristic marker of exosome CD9, CD63 and CD81. **d** The relative expression of miR-124 in Exo-miR-124 was significantly higher than in the Exo-miR-con. *$P < 0.05$ indicates statistical significance

characteristic biomarkers of exosomes including CD9, CD63 and CD81 were highly expressed on the particles as well (Fig. 1c). These results suggested that the isolated nanoparticles were exosomes. Next, the expression of miR-124 in exosomes was measure by RT-PCR. Compared with exosomes harvested from BM-MSCs transfected with pSUPER-control, the exosomes harvested from BM-MSCs transfected with pSUPER-miR-124 expressed significant higher levels of miR-124 (Fig. 1d). All of these data indicated that the Exo-miR-124 were successfully constructed.

Exo-miR-124 Promoted the M2 Polarization of Microglia in Hippocampus After TBI

The subsequent study was aimed at exploring the effects of Exo-miR-124 on the polarization of microglia in hippocampus after TBI. Exosomes were first injected into rats via tail vein at 24 h post trauma, and the expression of miR-124 in hippocampus was measured by RT-PCR at 3/7/14/28 days after TBI. Exo-miR-124 treatment significantly increased the expression of miR-124 in hippocampus at 3/7/14/28 days after TBI (Fig. 2a–d). Then, RT-PCR was performed to measure the polarization of microglia in hippocampus by detecting the expression of M1 signature genes CD32 and IL-1b and M2 signature genes CD206 and Arginase 1. The expression of pro-inflammatory cytokines IL-1β, IL-6, TNF-α and anti-inflammatory cytokines IL-4, IL-10, TGF-β produced by M1/M2 microglia were measured in hippocampus by ELISA. Results demonstrated that the expression of M1 signature genes CD32 and IL-1b (Fig. 2e–l) and pro-inflammatory cytokines IL-1β, IL-6, TNF-α (Fig. 3a–l) was significantly elevated in hippocampus at 3/7/14/28 days after TBI. Exo-miR-124 treatment significantly reduced the expression of CD32 and IL-1b (Fig. 2e–l), as well as IL-1β, IL-6, TNF-α (Fig. 3a–l) in hippocampus at 3/7/14/28 days after TBI. Furthermore, the expression of CD206, Arginase-1 (Fig. 4a–h) and IL-4, IL-10, TGF-β (Fig. 5a–l) was also significantly elevated in hippocampus at 3/7/14/28 days after TBI. Exo-miR-124 treatment significantly increased the expression of CD206, Arginase-1(Fig. 4a–h), as well as IL-4, IL-10, TGF-β (Fig. 5a–l) in hippocampus at 3/7/14/28 days after TBI. Taken together, these results suggested that Exo-miR-124 treatment increased the expression of miR-124, and further promoted the M2 polarization of microglia and the production of anti-inflammatory cytokines in hippocampus after TBI.

Exo-miR-124 Treatment Enhanced the Hippocampus Neurogenesis After TBI

Above experiments demonstrated that Exo-miR-124 treatment increased the expression of miR-124 and promoted the M2 polarization of microglia in hippocampus after TBI. Together with previous studies reported that the M2 polarization was beneficial to hippocampus neurogenesis [8–10], it is reasonable to assume that Exo-miR-124 treatment could further enhance the hippocampus neurogenesis after TBI. To verify this hypothesis, IF was performed to evaluate the hippocampus neurogenesis by labeling the proliferation marker $BrdU^+$(red)/$SOX2^+$(green) and differentiation marker $BrdU^+$(red)/$NeuN^+$ (green) in SGZ of DG. The results showed that the number of $BrdU^+/SOX2^+$ cells in TBI rats was more than in Sham rats at 7 days post trauma, the Exo-miR-124 treatment further induced more $BrdU^+/SOX2^+$ cells in TBI rats(Fig. 6a). Moreover, the number of $BrdU^+/NeuN^+$ cells in TBI rats was more than in Sham rats at 28 days post trauma, and Exo-miR-124 treatment further induced more $BrdU^+/NeuN^+$ cells in TBI rats as well (Fig. 6b). Collectively, these results indicated that Exo-miR-124 treatment promoted the proliferation and neuronal differentiation of NSCs in hippocampus after TBI.

Exo-miR-124 Treatment Improved the Neurological Function Recovery After TBI

Exo-miR-124 treatment promoted the M2 polarization of microglia and further enhanced the neurogenesis in hippocampus after TBI, which suggested that the neurological function recovery might be improved as well. Herein, NSS and WMW tests were subsequently conducted to measure the neurobehavioral and neurocognitive functions after TBI. In neurobehavioral assessment, the severity score of TBI rats was higher than that of Sham rats (Fig. 7a). Exo-miR-124 treatment notably improved this condition in TBI rats (Fig. 7a). In neurocognitive evaluation, the escape latency to find the hidden platform of TBI rats was longer than that of Sham rats (Fig. 7b). Exo-miR-124 treatment significantly reduced much more the latency in TBI rats (Fig. 7b). The platform crossing time and target quadrant route of TBI rats was less than that of Sham rats (Fig. 7c, d). Exo-miR-124 treatment significantly increased much more the two indexes in TBI rats (Fig. 7c, d). These results indicated that Exo-miR-124 treatment improved the neurobehavioral and neurocognitive recovery after TBI.

Exo-miR-124 Treatment Inhibited the TLR4 Pathway in Hippocampus After TBI

Above experiments demonstrated that Exo-miR-124 promoted the M2 polarization of microglia after TBI. Together with new studies indicated that miR-124 regulated the polarization of microglia by targeting TLR4 signaling pathway [16, 29], we came to the hypothesis that Exo-miR-124 might produce this effect by inhibiting TLR4 pathway. To test this hypothesis, RT-PCR was performed to measure the mRNA expression of TLR4

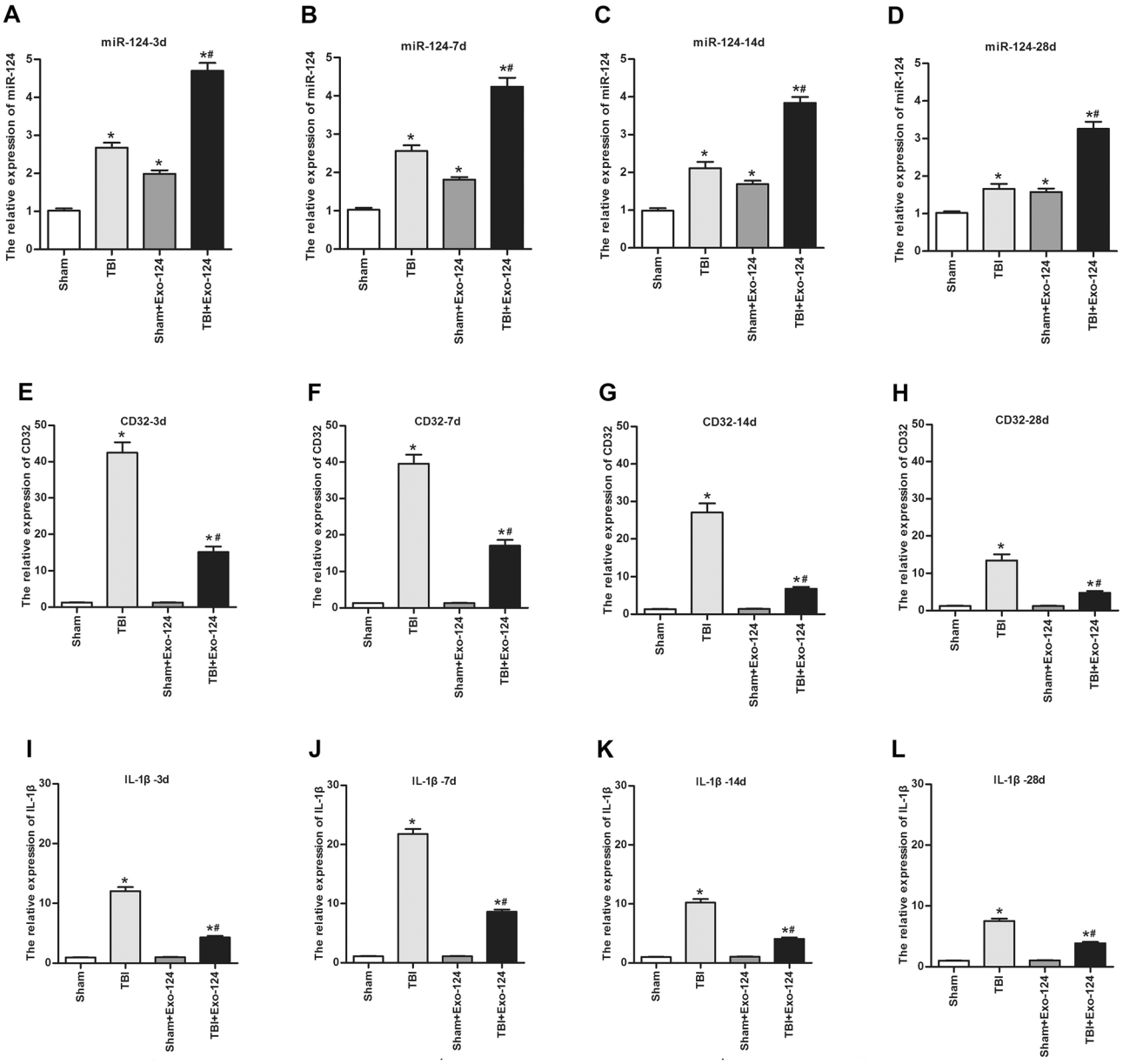


Fig. 2 Exo-miR-124 reduced the expression of M1 microglia signature genes CD32 and IL-1b in hippocampus after TBI. **a**–**d** Exo-miR-124 treatment significantly increased the expression of miR-124 in the hippocampus at 3/7/14/28 days after TBI. **e**–**l** Exo-miR-124 treatment significantly reduced the mRNA expression of CD32 and IL-1β in the hippocampus at 3/7/14/28 days after TBI. n = 6 in each group/each time point. *P < 0.05 versus the sham group, #P < 0.05 versus the TBI group

pathway molecules including MyD88, IRAK1, TRAF6 and NF-κB p65 in hippocampus after TBI. Results demonstrated that the expression of TLR4, MyD88, IRAK1, TRAF6 and NF-κB p65 was significantly elevated in hippocampus at 3/7/14/28 days after TBI. Exo-miR-124 treatment significantly reduced the expression of TLR4, MyD88, IRAK1, TRAF6 and NF-κB p65 in hippocampus at 3/7/14/28 days after TBI. These results indicated that TBI activated the TLR4 signaling pathway in hippocampus, which was inhibited by Exo-miR-124 treatment.

Exo-miR-124 Inhibited TLR4 Pathway and Promoted the M2 Polarization in BV2 Microglia

This in vitro experiment was performed to further investigate whether Exo-miR-124 regulated the polarization of microglia by targeting TLR4 pathway. Exosomes were first incubated with BV2 microglia, the expression of miR-124 in BV2 cells was measured by RT-PCR. Then, LPS was used to stimulate the BV2 microglia by activating TLR4 pathway. The expression of TLR4 and its downstream molecules

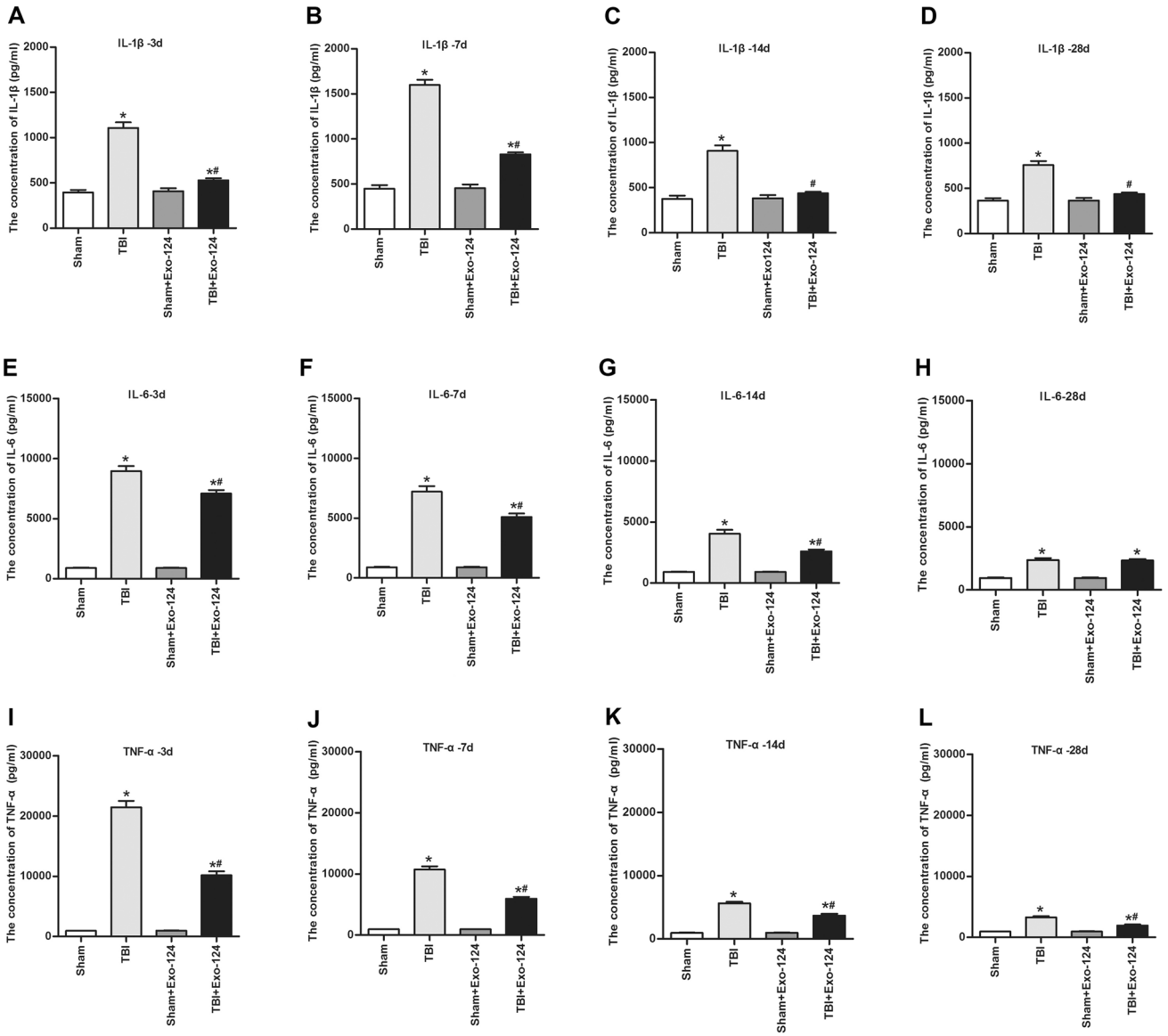


Fig. 3 Exo-miR-124 reduced the production of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α in hippocampus after TBI. **a**–**l** Exo-miR-124 treatment significantly reduced the concentration of IL-1β, IL-6 and TNF-α in the hippocampus at 3/7/14/28 days after TBI. n = 6 in each group/each time point. *P < 0.05 versus the sham group, #P < 0.05 versus the TBI group

MyD88/IRAK1/TRAF6/NF-κB p65 was measured by WB. The polarization of BV2 microglia was measured by detecting the expression of M1 signature genes CD32, IL-1b and M2 signature genes CD206, Arginase 1 via RT-PCR. The level of pro-inflammatory cytokines IL-1β, IL-6, TNF-α and anti-inflammatory cytokines IL-4, IL-10, TGF-β produced by M1/M2 microglia was measured by ELISA. Results showed that Exo-miR-124 treatment significantly increased the expression of miR-124 in BV2 microglia (Fig. 8a). LPS stimulation upregulated the expression of TLR4 and its downstream molecules MyD88/IRAK1/TRAF6/NF-κB p65 in BV2 microglia (Fig. 8b–f). And, Exo-miR-124 treatment reduced the expression of TLR4 pathway molecules (Fig. 8b–f). The expression of M1 signature genes CD32 and IL-1b and pro-inflammatory cytokines IL-1β, IL-6, TNF-α was significantly elevated in BV2 microglia after LPS stimulation (Fig. 9a–e). Exo-miR-124 treatment significantly reduced the expression of CD32, IL-1β and IL-1β, IL-6, TNF-α in BV2 microglia after LPS stimulation (Fig. 9a–e). Moreover, the expression of CD206, Arginase 1 and IL-4, IL-10, TGF-β was also elevated in BV2 microglia after LPS stimulation (Fig. 9f–j). Exo-miR-124 treatment significantly increased the expression of CD206, Arginase 1 and IL-4, IL-10, TGF-β in BV2 microglia after LPS stimulation (Fig. 9f–j). These results suggested that Exo-miR-124 treatment reduced the expression of TLR4

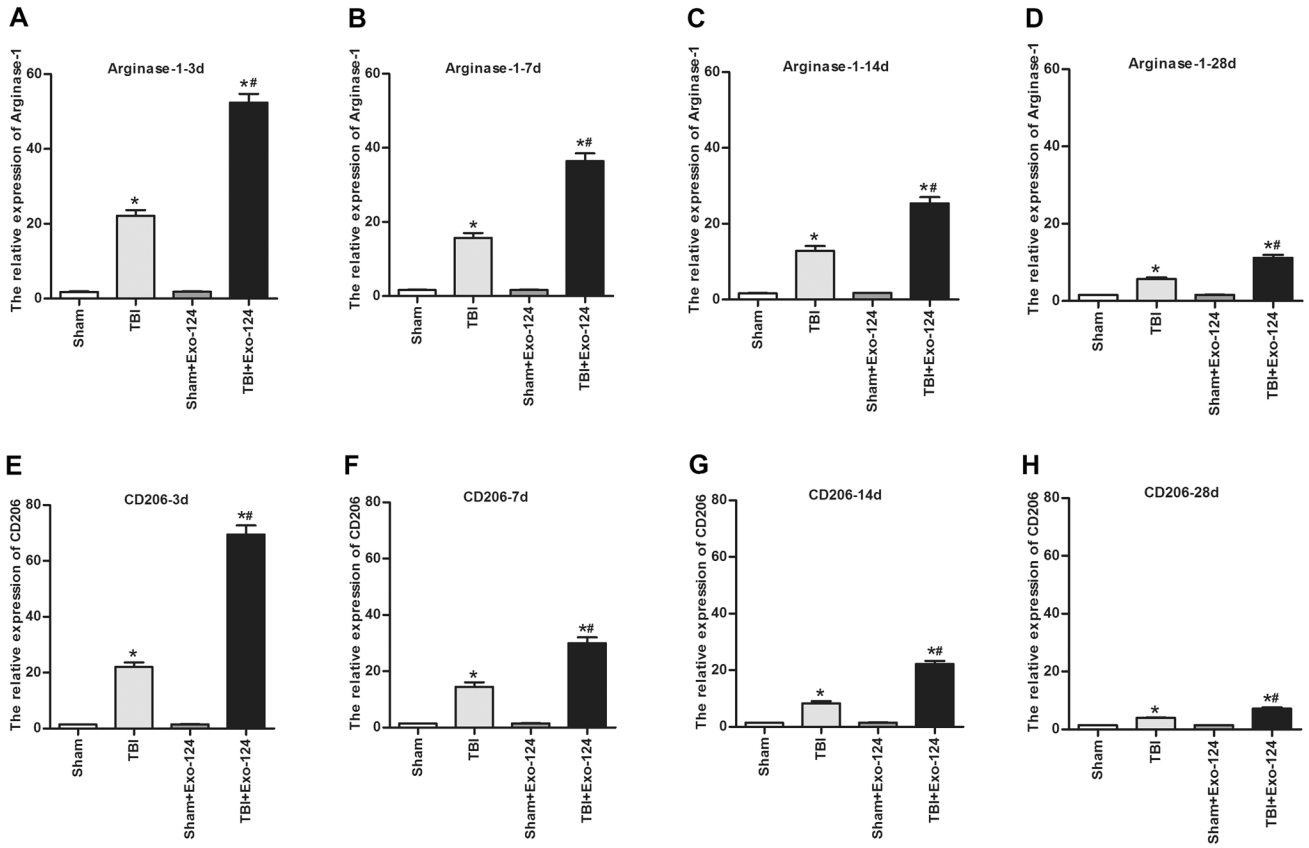


Fig. 4 Exo-miR-124 increased the expression of M2 microglia signature genes CD206 and Arginase-1 in hippocampus after TBI. **a**–**h** Exo-miR-124 treatment significantly increased the mRNA expression of CD206, Arginase-1 in the hippocampus at 3/7/14/28 days after TBI. n = 6 in each group/each time point. *P < 0.05 versus the sham group, #P < 0.05 versus the TBI group

pathway molecules, and promoted the M2 polarization and the anit-inflammatory cytokines production in BV2 microglia after LPS stimulation. Collectively, experiments in this part indicated that Exo-miR-124 treatment promoted the M2 polarization of BV2 microglia by inhibiting TLR4 signaling pathway (Fig. 10).

Discussion

The present study explored a potential approach to regulate the polarization of microglia and further improve the neurogenesis in hippocampus after TBI. The exosome was selected as a tool to deliver miR-124 into the brain based on its prominent advantages such as the nano-size, the ability to transfer microRNA and the capacity to cross BBB. The polarization of microglia was taken as a research focus according to its important role in hippocampus neurogenesis after TBI and its possibility of being modulated by miR-124. Moreover, because the TLR4 pathway was involved in the microglia polarization and could be regulated by miR-124, it was considered as a potential target of Exo-miR-124. Results indicated that Exo-miR-124 promoted the M2 polarization of microglia and neurogenesis in hippocampus and improved function recovery after TBI. The M2 polarization effect of Exo-miR-124 was mediated by inhibiting TLR4 signaling pathway.

Exosomes are endosome-derived membrane nanovesicles (30–100 nm) that are released by cells in all living systems [40]. Exosomes can mediate intercellular communication by transferring proteins, lipids, and genomic molecules such as miRNAs between the origin and target cells [41]. The potential clinical values of exosomes in neurological diseases can be attributed to their low immunogenicity, long half-life in circulation and ability to cross the BBB [42]. Recently, the MSC derived exosome was reported to promote neurogenesis after being intravenously administrated in the TBI rats [20, 21], and it was taken as a novel approach to deliver miRNAs such as miR-17-92 cluster and miR-124 into brain and promote neurogenesis in the stroke model [24, 31]. Therefore, the Exo-miR-124 used in this research was constructed by modifying the expression of miR-124 in BM-MSC, and was isolated from the supernatant of BM-MSC culture medium. In order to transfer miR-124, the modified

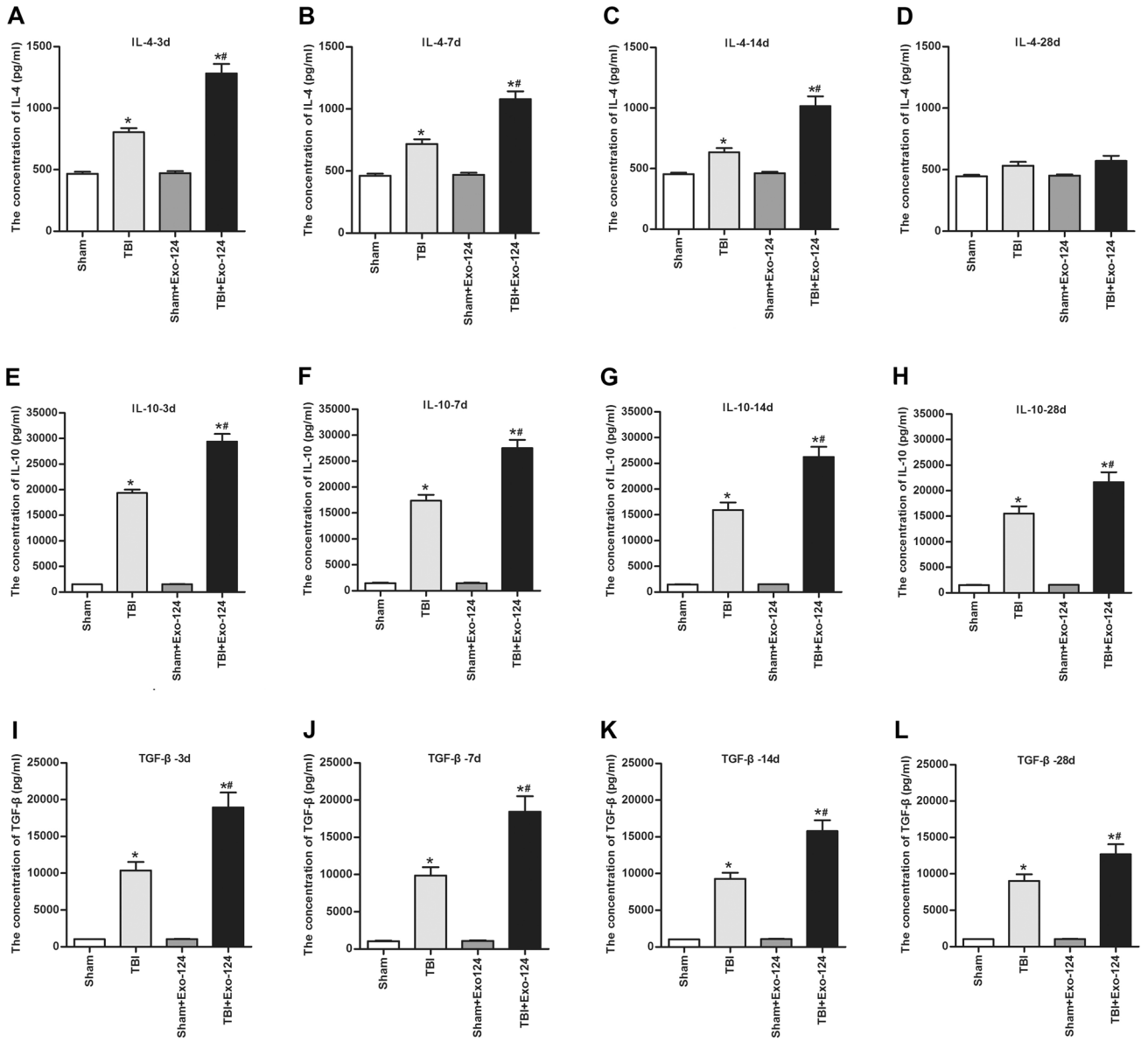


Fig. 5 Exo-miR-124 increased the production of anti-inflammatory cytokines IL-4, IL-10, TGF-β in hippocampus after TBI. **a**–**l** Exo-miR-124 treatment significantly increased the concentration of IL-4, IL-10 and TGF-β in the hippocampus at 3/7/14/28 days after TBI. n = 6 in each group/each time point. *P < 0.05 versus the sham group, #P < 0.05 versus the TBI group

exosomes were then intravenously administrated into the rats and added into the BV2 microglia culture medium. Further measurement showed that the expression of miR-124 in hippocampus and BV2 microglia was notably elevated after the Exo-miR-124 treatment, indicating the exosomes successfully delivered miR-124 in vivo and in vitro.

MiRNAs are important small non-coding RNAs that can modulate the gene expression involved in life stages of growth, development and death at the post-transcriptional level [43]. It has been reported that numerous miRNAs can regulate the activation and polarization of microglia [44]. Among these miRNAs, miR-124 is the most abundantly expressed one in CNS which can regulate the function of microglia in both physiological and pathological conditions [14–18]. Generally, downregulating the expression of miR-124 promoted the M1 polarization of microglia [16], while upregulating the expression of miR-124 promoted the M2 polarization of microglia [17, 18]. Consistent with these studies, the Exo-miR-124 treatment increased the expression of miR-124 in the hippocampus and BV2 microglia, which further promoted the M2 polarization of microglia and the production of anti-inflammatory cytokines after TBI and LPS stimulation respectively. As microglia played an essential role in neuroinflammation after brain injury,

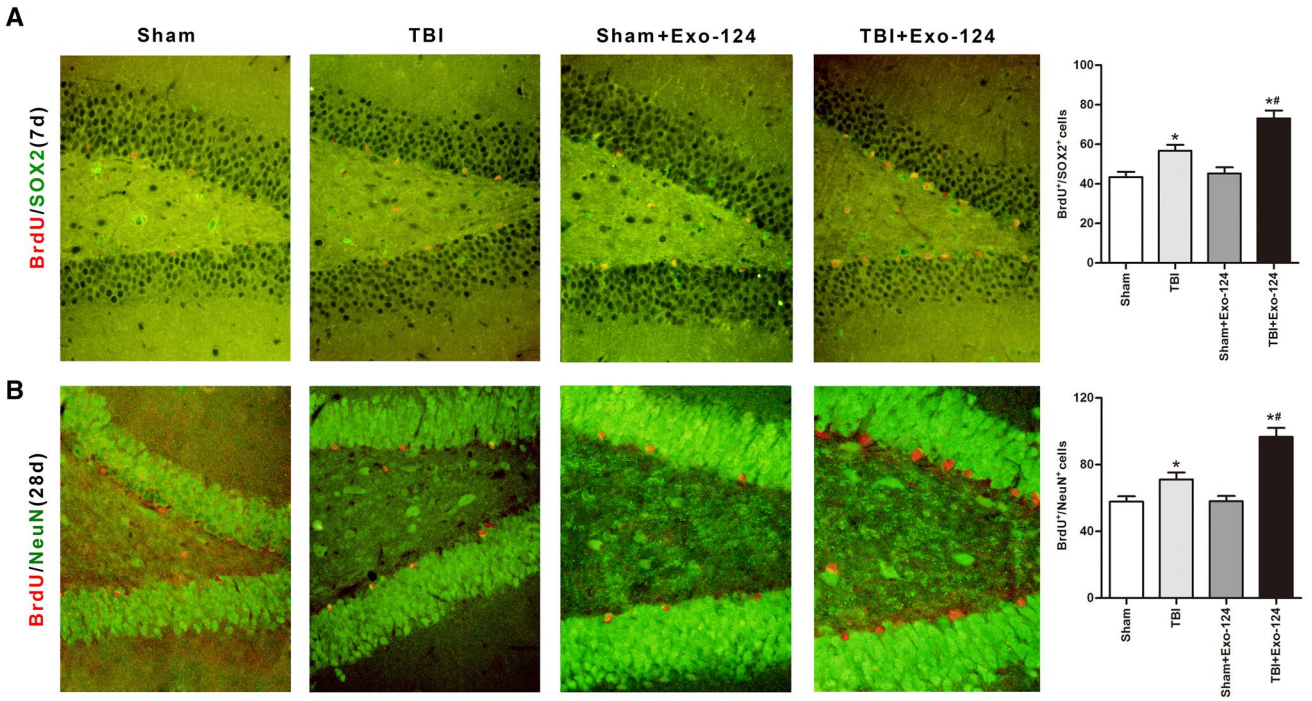


Fig. 6 Exo-miR-124 treatment enhanced the hippocampus neurogenesis after TBI. **a** Representative IF images of hippocampus immunolabeled with BrdU/SOX2 in the four groups at 7 days after TBI. Quantitation analysis showed that TBI induced more $BrdU^+/SOX2^+$ cells, and Exo-miR-124 treatment further induced much more $BrdU^+/SOX2^+$ cells. **b** Representative IF images of hippocampus immunolabeled with BrdU/NeuN in the four groups at 28 days after TBI. Quantitation analysis showed that TBI induced more $BrdU^+/NeuN^+$ cells, Exo-miR-124 treatment further induced much more $BrdU^+/NeuN^+$ cells. n = 6 in each group/each time point. $^*P < 0.05$ versus the sham group, $^{\#}P < 0.05$ versus the TBI group

Exo-miR-124 might be a promising approach to alleviate neuroinflammation and improve the outcome after TBI. Compared with other anti-inflammatory treatments such as Interleukin-1 receptor antagonist (IL1ra) that have been shown to improve outcomes of TBI in the experimental research and clinical trial [45, 46], Exo-miR-124 has both advantages and disadvantages. As for the advantages, Exo-miR-124 may have lower immunogenicity and less pharmacology side-effect than IL1ra that makes it safer. As for the disadvantages, the research about Exo-miR-124 and TBI is just at the beginning, much more work need to be done to clarify the specific mechanism and to explore its potential in clinical application.

The NSCs located in the DG of hippocampus are characterized by self-renew, production of newborn neurons and integration into the damaged neural network following CNS injury, which makes the endogenous neurogenesis be a great potential to replenish neural loss and restore neurological function after TBI [3]. However, the functional disability is often occurred due to the limited capacity of endogenous neurogenesis caused by several detrimental factors after TBI [47]. One important factor is neuroinflammation, which is generally considered to be detrimental to hippocampal neurogenesis after TBI. Actually, neuroinflammation has protective effects on the injured brain in a way, but excessive inflammatory response often becomes a detrimental factor that leads to decreased hippocampus neurogenesis and worse functional recovery after TBI [4]. Thus, exploring effective approach to regulate neuroinflammation might be beneficial for the hippocampus neurogenesis. Microglia are essential to activate and regulate neuroinflammation, they produce dual effects on hippocampus neurogenesis depending on the classical (M1, pro- inflammatory)/alternative (M2, anti-inflammatory) activation [4]. Though the M1/M2 terminology is outdated to some extent, and this paradigm simply divides the activated microglia into M1 and M2 phenotypes, it remains the most frequently used definition to explore the role of microglia. And, as the M1/M2 polarization of microglia has been exactly verified in several brain diseases [13, 18, 29], this classification might be a novel approach to study the function of microglia in hippocampal neurogenesis after TBI. As we known, M1 microglia secrete pro-inflammatory cytokines IL-1β, IL-6, TNF-α that are detrimental to hippocampus neurogenesis [5–7], while M2 microglia secrete anti-inflammatory cytokines IL-4, IL-10, TGF-β that are beneficial to hippocampus neurogenesis [8–10]. In the present study, Exo-miR-124 treatment was demonstrated to promote the M2 polarization of microglia and the production

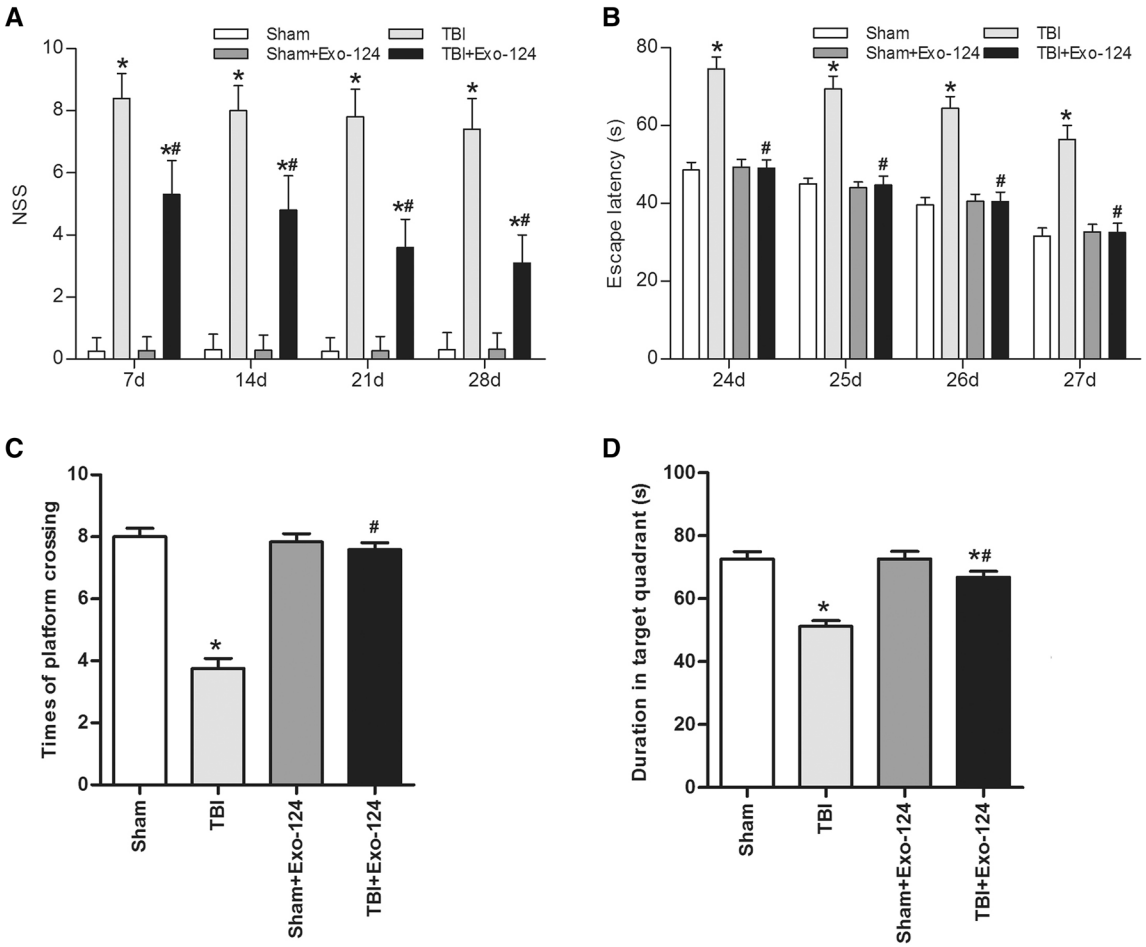
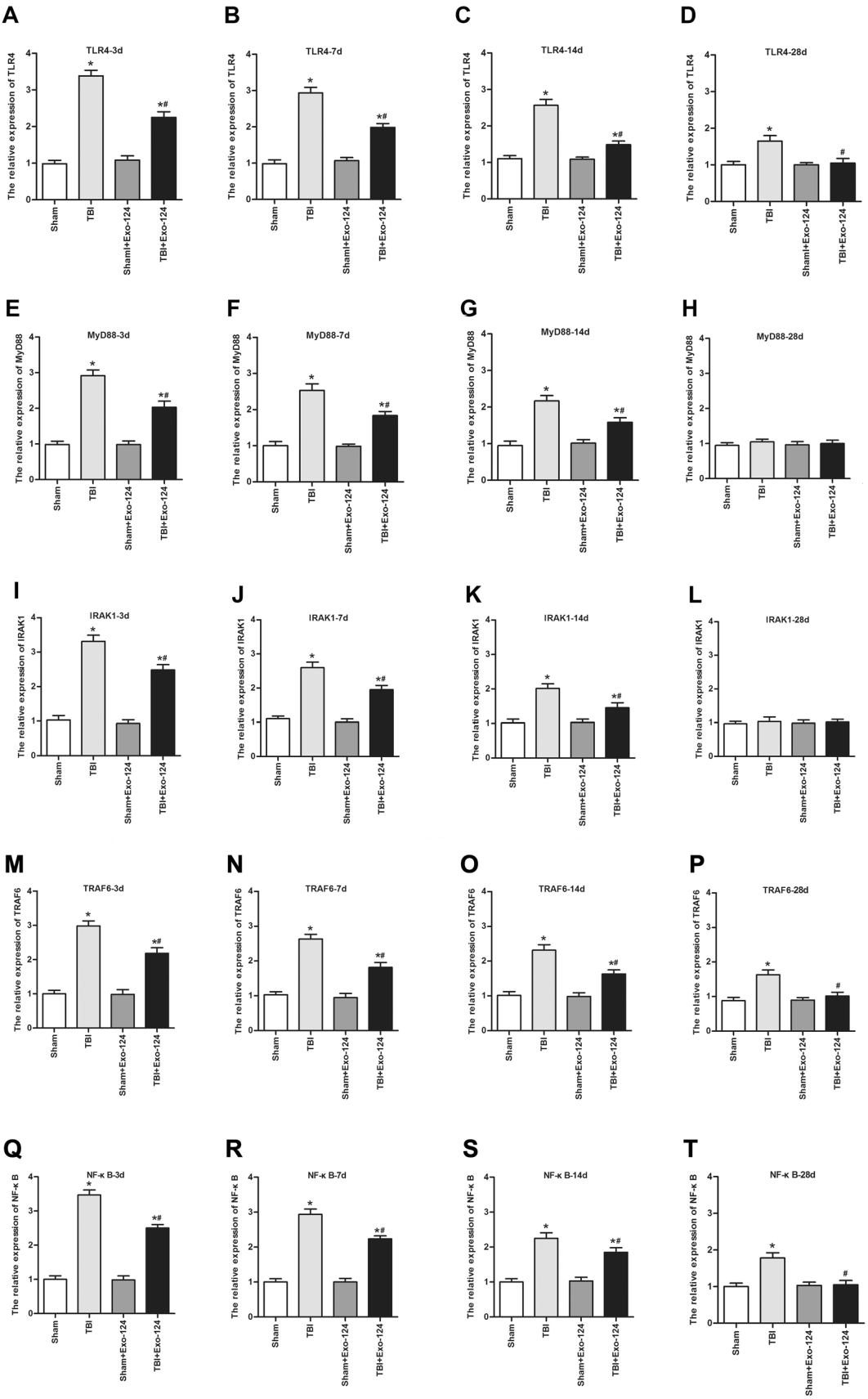


Fig. 7 Exo-miR-124 treatment improved the neurological function recovery after TBI. **a** The NSS score was higher in TBI group than in sham group, and Exo-miR-124 treatment reduced this score. **b** The escape latency was longer in TBI group than in sham group, and Exo-miR-124 treatment shorten this latency. **c, d** Rats in the TBI group presented shorter platform crossing times and less target quadrant route than the sham group, and Exo-miR-124 treatment increased the route and crossing times. n = 6 in each group. $^{*}P < 0.05$ versus the sham group, $^{\#}P < 0.05$ versus the TBI group

of anti-inflammatory cytokines IL-4, IL-10, TGF-β in hippocampus after TBI. And the hippocampus neurogenesis was verified to be enhanced after TBI in subsequent experiments. In a word, these results indicated that Exo-miR-124 treatment promoted the M2 polarization of microglia and further enhanced hippocampus neurogenesis after TBI. Furthermore, the WMW and NSS tests revealed that the neurocognitive and neurobehavioral functions of TBI rats were significantly improved after Exo-miR-124 treatment. Taken together, Exo-miR-124 might be an effective agent to improve the hippocampus neurogenesis and functional recovery after TBI.

Previous studies indicated that TLR4 signaling pathway played key roles in the activation and polarization of microglia and their detrimental effects on hippocampus neurogenesis [25–29]. Moreover, miR-124 promoted the M2 polarization of microglia by negatively regulating the expression of TLR4 and its downstream molecules MyD88, TRAF6, IRAK1 and NF-κB [12, 16]. Together with the findings of this study that Exo-miR-124 treatment promoted the M2 polarization of microglia and further improved hippocampus neurogenesis in TBI rats, the TLR4 pathway was considered to be involved in this process. To verify this hypothesis, the mRNA expression of TLR4 pathway molecules including MyD88, IRAK1, TRAF6 and NF-κB p65 in hippocampus were first measured, results indicated that TBI activated the TLR4 signaling pathway in hippocampus, which was inhibited by Exo-miR-124 treatment. Then, Exo-miR-124 was added into the culture medium of BV2 microglia after LPS stimulation, the expression of TLR4 pathway molecules and the polarization of microglia was measured as well. Results showed that LPS stimulation significantly increased the expression of TLR4 and its downstream molecules MyD88, TRAF6, IRAK1 and NF-κB in BV2 cells, and Exo-miR-124 effectively blocked these effects. Since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NF-κ B-28d
The relative expression of NF-κ B
Sham
TBI
Sham+Exo-124
TBI+Exo-124

◂**Fig. 8** Exo-miR-124 inhibited the TLR4 signaling pathway in hippocampus after TBI. **a**–**t** TBI upregulated the mRNA expression of TLR4 and its downstream molecules MyD88/IRAK1/ TRAF6/ NF-κB p65 in hippocampus. Exo-miR-124 treatment significantly reduced the expression of these proteins in TLR4 signaling pathway. n = 6 in each group/each time point. $^{*}P < 0.05$ versus the sham group, $^{\#}P < 0.05$ versus the TBI group

miR-124 is known to maintain the quiescence of microglia and promote the transition of microglia from M1 (pro-inflammatory) to M2 (anti-inflammatory) phenotype [12, 48], further experiments were performed to investigate the effect of Exo-miR-124 on LPS activated microglia. Results indicated that LPS stimulation significantly increased the expression of M1 signature genes CD32, IL-1b and pro-inflammatory cytokines IL-1β, IL-6, TNF-α in BV2 microglia, while Exo-miR-124 effectively reversed these effects. Concomitantly, the expression of M2 signature genes CD206, Arginase 1 and anti-inflammatory cytokines IL-4, IL-10, TGF-β was substantially increased in Exo-miR-124 treated microglia. These results implied that Exo-miR-124 blocked LPS-mediated microglia activation by promoting the M2 polarization of microglia, and the inhibition of TLR4 signaling pathway was involved in this process. Following the results of in vivo experiments, the conclusion can be deduced that Exo-miR-124 promoted the M2 polarization of microglia in hippocampus by inhibiting the TLR4 signaling pathway.

Based on the advantages of Exo-miR-124 including nano-size, cannot proliferate, low immunogenicity and easy to preserve and transfer, it has the promise to be a new and valuable strategy for the treatment of TBI. Furthermore, the combination of miRNAs and MSC-derived exosomes might be a novel approach for the treatment of TBI, and MSC-derived exosomes have the potential to serve as a non-invasive intervention for the successful delivery of therapeutic agents such as drugs and miRNAs into the brain and treat TBI in the clinical practice. Some researchers even affirmed that exosomes present a novel weapon for the treatment of

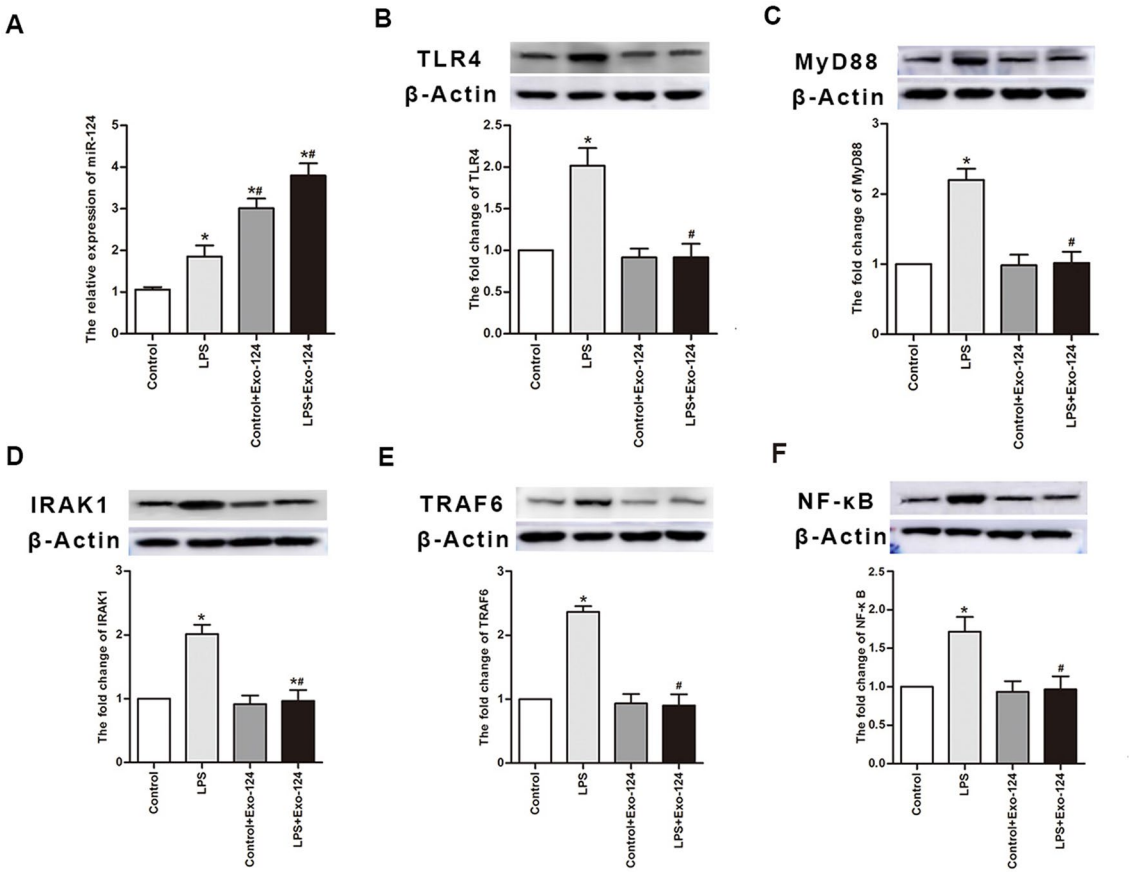

Fig. 9 Exo-miR-124 inhibited the TLR4 signaling pathway in BV2 microglia. **a** Exo-miR-124 treatment significantly increased the expression of miR-124 in BV2 microglia. **b**–**f** LPS stimulation upregulated the expression of TLR4 and its downstream molecules MyD88/IRAK1/ TRAF6/NF-κB p65 in BV2 microglia. Exo-miR-124 treatment significantly reduced the expression of these proteins in TLR4 signaling pathway. The data were acquired from six independent experiments. $^{*}P < 0.05$ versus the control group, $^{\#}P < 0.05$ versus the LPS group

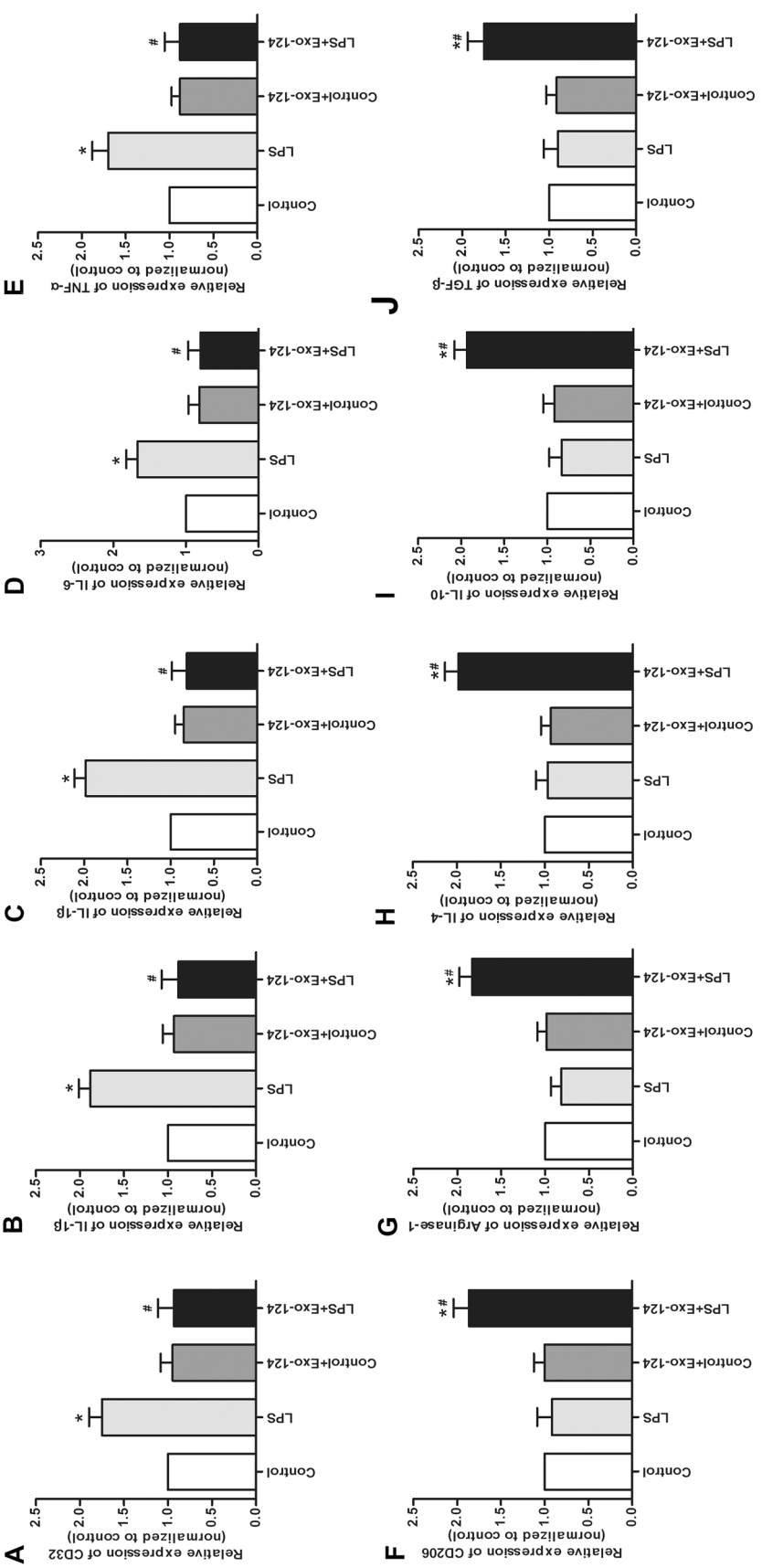

Fig. 10 Exo-miR-124 promoted the M2 polarization of BV2 microglia. **a–e** The expression of M1 signature genes CD32, IL-1b and pro-inflammatory cytokines IL-1β, IL-6, TNF-α was elevated in BV2 microglia after LPS stimulation. Exo-miR-124 treatment significantly reduced the expression of CD32, IL-1b and IL-1β, IL-6, TNF-α in BV2 microglia. **f–j** The expression of CD206, Arginase 1 and IL-4, IL-10, TGF-β was elevated in BV2 microglia after LPS stimulation. Exo-miR-124 treatment significantly increased the expression of CD206, Arginase 1 and IL-4, IL-10, TGF-β in BV2 microglia. The data were acquired from six independent experiments. $^{*}P < 0.05$ versus the control group, $^{\#}P < 0.05$ versus the LPS group

TBI in terms of their many advantages including nano-size, easy administration and the potential drug delivery vehicles across the BBB [49, 50]. It can be envisaged that many of this work will be addressed by growing researches in the field of MSCs-derived exosomes and TBI. Excitingly, a clinical trial about testing the effect of cell-free cord blood derived MSC exosomes on β-cell mass in Type I Diabetes Mellitus among others is ongoing (ClinicalTrials.gov, NCT02138331). In addition, because TBI rarely occurs in isolation and often suffers multiple concomitant peripheral injuries, systematic treatment is very essential for a better outcome. As one of the concomitant peripheral injuries, neuroinflammation has been verified to be capable of modifying the outcomes and pathobiology of TBI [51, 52]. In this regard, Exo-miR-124 not only can reduce neuroinflammation in the brain, but also can alleviate concomitant extracranial inflammatory responses, both which will improve the outcome of TBI.

In conclusion, the present study explored the effects of Exo-miR-124 on the polarization of microglia and the neurogenesis after TBI, and further investigated whether TLR4 pathway played a role in this process. The findings demonstrated that Exo-miR-124 treatment promoted the M2 polarization of microglia and further improved hippocampal neurogenesis and functional recovery after brain injury, the M2 polarization effect was mediated by inhibiting the TLR4 pathway. Thus, the administration of Exo-miR-124 might be a potential approach to promote hippocampus neurogenesis and improve the neurological function recovery after TBI.

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Author Contributions XH designed the experiments and guided the writing of this article. YY, YY, CK and XS were responsible for performing experiments and writing the manuscript. XZ and WB contributed to acquire and analyze the data. Authors included in this article agreed with the final manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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