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ORIGINAL PAPER

MSCs-Derived Exosomes Attenuate Acute Brain Injury and Inhibit Microglial Inflammation by Reversing CysLT2R-ERK1/2 Mediated Microglia M1 Polarization

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Abstract

Inflammatory responses play a major role in the pathophysiology of cerebral ischemia. Mesenchymal stem cell-derived exosomes (MSC-exos) have important anti-inflammatory effects on the treatment of organ injury. This study aimed to determine the anti-inflammatory effect and furtherly investigate the potential mechanism of MSC-exos on acute cerebral ischemia. MSC-exos were isolated by ultracentrifugation, characterized by transmission electron microscopy and FACS. Rats subjected to middle cerebral artery occlusion/reperfusion (MCAO/R) surgery were administered MSC-exos through the tail vein. In vitro, microglia exposed to oxygen- and glucose-deprivation (OGD) and leukotrienes were used to study the protective mechanism of exosomes against ischemia/reperfusion-induced inflammation. The intake of exosomes into microglia was visualized through immunofluorescence staining. The results showed that MSC-exos treatment significantly improved motor, learning and memory abilities of MCAO/R rats 7 days later. The production of pro-inflammatory factors decreased, while the anti-inflammatory cytokines and neurotrophic factors increased both in the cortex and hippocampus of ischemic hemisphere as well as in the culture supernatant of microglia treated with OGD and NMLTC4. MSC-exos treatment also significantly inhibited M1 microglia polarization and increased M2 microglia cells. Furthermore, western blot analysis demonstrated that $CysLT_2R$ expression and ERK1/2 phosphorylation were downregulated both in vivo and in vitro. Thus, MSC-exos attenuated brain injury and inhibited microglial inflammation by reversing $CysLT_2R$-ERK1/2 mediated microglia M1 polarization.

Keywords MSCs · Exosome · M1 microglia polarization · $CysLT_2R$ · Inflammation

Abbreviations

BDNF	Brain-derived neurotrophic factor
CysLTs	Cysteinyl leukotrienes
CysLT1R	Cysteinyl leukotrienes receptor 1
DMSO	Dimethyl sulfoxide
ERK	Extracellular regulated protein kinases
GDNF	Glial cell-derived neurotrophic factor
IFN	Interferon
IGF	Insulin like growth factor
IL	Interleukin
LT	Leukotriene
MSC-exos	MSCs-derived exosomes
MCAO	Middle cerebral artery occlusion
NMLTC4	N-methyl leukotriene C4
OGD	Oxygen–glucose-deprivation
TEM	Transmission electron microscopy
TGF	Transforming growth factor
TNF	Tumor necrosis factor

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Introduction

Microglia are the main inherent immune cells in the brain and play a central role in regulating the immune and inflammatory responses after various injuries. Brain injuries trigger microglia activation within minutes after cerebral ischemia [1]. Persistent microglia activation and M1 polarization finally lead to severe cerebral inflammation [2]. As known to us all,

microglia have two different phenotypes. During the course of cerebral ischemia, they function completely contrary to each other. M1 microglia aggravate ischemic injury to the brain by releasing pro-inflammatory and neurotoxic factors, highly expressing IL-1β, TNF-α, IFN-γ, and CD86. However, M2 microglia protect nervous tissues by secreting anti-inflammatory and neurotrophic mediators to promote brain repairment. Its polarization markers include Arg-1, CD206, TGF-β, and IL-10 [3]. Therefore, microglia M1/M2 polarization is closely associated with the prognosis of brain injuries. To reach a good balance between M1 and M2 phenotypes may represent a prospective therapeutic strategy for ischemic injury.

Cysteinyl leukotrienes (CysLTs) are largely produced with the decomposition of necrotic cells. As potent inflammatory mediators, they accelerated the development of several central nervous diseases, including epilepsy, depression, cerebral ischemia, and brain trauma. Up to now, $CysLT_1R$ and $CysLT_2R$ are two well-studied CysLT receptors to induce inflammatory responses [4]. N-methyl LTC4 (NMLTC4) was a selective agonist of $CysLT_2R$ and LTD4 was a non-selective agonist of $CysLT_1R$ and $CysLT_2R$ [5]. It has been reported that $CysLT_1R$ has no effect on early microglia activation and proliferation. While $CysLT_2R$ is mainly related to both post-ischemic inflammation in vivo and in vitro. HAMI3379, a selective antagonist of $CysLT_2R$, protects neurons against ischemic injury through inhibiting IL-1β, IFN-γ, TNF-α expression and ameliorating microglia activation [6].

Recently mesenchymal stem cells (MSCs) transplantation has been widely investigated in the treatment of various organ injuries for their immunosuppressive and anti-inflammatory properties. MSCs-derived exosomes are membranous structures that come from the cytoplasm, characterized by the expression of specific marker proteins from the tetraspanin superfamily such as CD9, CD63, and CD81. Exosomes are one of the main effectors of paracrine function. These markers are inevitable in the formation and transportation of exosomes within the cells as well as the recognition of target cells, which represent a novel cell-to-cell interaction mode and play an important role in regulating signal pathways [7]. They deliver their content, such as protein, mRNA and miRNA to recipient cells and regulate occurrence, progression and prognosis of many diseases. In the current study, we evaluated the effect of MSC-exos on OGD and CysLTs induced microglia activation as well as middle cerebral artery occlusion (MCAO) induced cerebral ischemia via $CysLT_2R$-ERK1/2 mediated pathway.

Materials and Methods

Animals

Male SD rats (Slac, Shanghai, China) were kept in a clean room with controlled temperature (23 ± 1 ℃). All rats had access to enough food and water except for the 8 h fasting before the operation. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals and the guidelines of the Ethics Committee of Hangzhou Medical College. Every effort was made to minimize animal suffering and to reduce the number of animals used. Anesthetization was induced with pentobarbital sodium (30 mg/kg) through intraperitoneal injection.

MSCs Exosomes Isolation and Characterization

MSCs were isolated from SD rats by the whole bone marrow adherence method and cultured [8]. When MSCs were nearly 80 ~ 90% confluence, they were washed with PBS, and cultured in FBS-free L-DMEM for 48 h. Then the supernatant was collected and subject to sequential centrifugation: 300 $g \times 10$ min, 2000 $g \times 10$ min, 10,000 $g \times 30$ min, and 100,000 $g \times 120$ min. The precipitated exosomes were reserved at − 80 ℃.

For transmission electron microscopy (TEM), exosomes were fixed with 1% glutaraldehyde and a drop was loaded onto a formvar/carbon-coated grid negatively stained with 3% aqueous phosphotungstic acid for 1 min and observed under TEM (Hitachi, Tokyo, Japan, SU-8010).

For flow cytometry analysis of exosomes, MSC-exos were mixed with 3 μm aldehyde/sulfate latex beads (Invitrogen, Batch Num: 979383) for 15 min with continuous rotation, then diluted to 0.4 ml with PBS and left overnight at 4 ℃. The next day 1 M glycine in PBS containing 2% BSA was added into the mixture to stop the reaction. Beads coated with exosomes were washed twice with PBS with 2% BSA and incubated with antibodies CD63-FITC (Abcam, Lot: GR320523-9), CD81-PE (Invitrogen, Cat: MA5-17941) at 37 ℃ for 30 min. After being washed the beads were resuspended in 200 μL PBS with 0.5% BSA for detection on a BD FACSalibur. The protein concentrations of MSC-exos were determined with the Bradford protein quantification kit (Beyotime Biotech, Nanjing, China).

MCAO Rats Preparation and MSC-Exos Therapy

MCAO surgery was performed on male SD rats (with bodyweight 270 ± 10 g). Under anaesthetization with pentobarbital sodium, the right cerebral artery was occluded for 90 min using a nylon filament that was coated with silica gel at one end (Jialing, Guangzhou, China). The filament was carefully withdrawn 90 min after MCAO. In sham-operated rats, the same procedure was done without advancing of the filament into the MCA. The average mortality was about 10 ~ 15%. MSC-exos (200 μL/rat) were administered through the tail vein 2 h after reperfusion. The sham and MCAO/R groups were given an equal volume of normal saline (n = 8 in each

group). Rats were sacrificed 7 days after ischemia and the brain tissues were separated.

Measurement of Neurological Deficit

According to Longa's method [9], the neurological severity scores (NSS) were assessed at 1 h, 1 days, 3 days, and 7 days after ischemia (n = 8) by investigators blind to the groups, which implied motor, sensory, reflex and balance abilities. The motor behavior and memory function were also assessed by the shuttle box test. In the boxes, light, a sound from the loudspeaker or an electric shock from the floor grid incentivized the rats to move to the opposite end of the box. The latency and frequency of active avoidance were recorded for further analysis.

OGD Treatment and MTT Assay of Microglia

To simulate an in vivo environment of MCAO, microglia cell line BV-2 (Bioleaf, Shanghai, China) cultured in glucose-free Earle's balanced salt solution (EBSS), was placed in an incubator with 95% N_2, 5% CO_2 for 1–5 h, then under normal condition for an additional 24 h. MTT assay aimed to find an appropriate OGD time with no cell activity reduction. In the following experiments, microglia cells were firstly subject to OGD for the given time, then incubated with NMLTC4 or LTD4 (0.1 nM/mL). Meanwhile, MSC-exos (5 μL/mL, L-Exos group; 10 μL/mL, H-Exos group) were furtherly added into the culture medium to explore the effect of MSC-exos on microglia polarization phenotypes and inflammation status. In order to confirm whether MSC-exos entered microglia, MSC-exos labeled with CD81-PE monoclonal antibody (Invitrogen, Cat: MA5-17941) were used to incubate with microglia cells.

Inflammatory Factors Detection

Cytokines production was detected in the hippocampus and cortex homogenate of the ischemic hemisphere as well as the supernatant collected from microglia culture medium 24 h after treatment with cysLTs (0.1 nM/mL) and MSC-exos by commercial ELISA kits according to the instructions (Elisa, Biotech, China). The content of NO was detected through the Griess method (Beyotime, Nanjing, China).

Multiple Immunofluorescence Staining

Multiple indirect immunofluorescence staining was performed on microglia cells seeded on coverslips and frozen sections of brains. The antibodies were as follows: Iba-1 (Gene Tex, Cat: GTX100042), CD86 (BD, Cat: 551396), CD206 (Origene, Cat: TA326270S), FITC (Biowestern, Cat: ISH8004-1), Cy3 (Beyotime, Lot: 032218180628). At last, nuclei were labeled with DAPI (Beyotime, Cat: C1005) for 15 min in the dark. The fluorescence was observed under fluorescence microscopy (Zeiss Scope A1, Germany).

Flow Cytometric Analysis

Microglia were harvested and incubated with CD86, CD206 (two characteristic makers of different microglial phenotypes) and fluorescence antibodies. Flow cytometric analysis (Cytoflex, Beckman Coulter) was performed to quantify the percentage of M1/M2 microglia.

Western Blotting

Protein was isolated from cultured microglia, cortex and hippocampus tissues of the ischemic hemisphere, respectively. Samples were separated using the standard SDS–polyacrylamide gel electrophoresis. Bands were visualized with enhanced chemiluminescence and images were captured with the image analysis system (Bio-Rad, CA, USA). Antibodies rabbit anti-cysLT_2R (1:1000, Bioworld, Cat: BS2614), p-ERK1/2 (1:2000, Huabio, Cat: ET1610-13) and ERK1/2 (1:5000, Huabio, Cat: ET1601-29) were used for detection.

Statistical Analysis

Data were reported as mean ± SD and analyzed with GraphPad Prism (GraphPad 6.0, San Diego, CA). All the data were checked for normality and variance homogeneity by F test or Bartlett's test. The comparison between two groups was determined by an independent sample *t-test*. Significant differences among multiple groups were determined by one-way analysis of variances (ANOVA) followed by Tukey's post hoc test. The repeated-measures analysis was conducted on the behavior scores. $P \leq 0.05$ was considered statistically significant.

Results

Characterization of MSC-Exos

Under the transmission electronic microscope, MSC-exos presented widespread, derangement distribution, and conglobation in some areas. They were membrane-encapsulated vesicles with round or cup-like shapes. As MSC-exos were isolated through ultracentrifugation and 0.22 μm filtration, these vesicles exhibited typical exosome morphology with a dimension varying from 30 to 150 nm (Fig. 1a). FACS indicated MSC-exos isolated through ultracentrifugation were of certain purity. The CD63 and CD81 positive rates were 30.84% and 43.75%, respectively (Fig. 1b). They were

characteristic protein markers of exosomes. According to the Bradford quantification, the protein concentration of the MSC-exos was 0.6034 mg/mL.

MSC-Exos Improved Neurological Deficit

In this study, we applied NSS to assess the neurological function of MCAO rats after MSC-exos treatment. MCAO rats exhibited severe behavior deficits after ischemia. In the following days, there was a persistent improvement in both MCAO/R group and MSC-exos treated group (0.4 mg/kg) in a time-dependent manner. The difference between the two groups increased progressively over time. On the 3rd ($F_{(2,21)}=171.5$, $P<0.05$) and 7th day ($F_{(2,21)}=95.15$, $P<0.001$), there was significant difference in NSS scores between the Exo group and the MCAO/R group (Fig. 1c).

Frequency and latency of active avoidance are the most important indicators of the shuttle box experiment. Compared with the sham group, the frequency of active avoidance responses of MCAO/R groups was significantly reduced ($F_{(2,21)}=48.02$, $P<0.001$), and latency increased ($F_{(2,21)}=20.43$, $P<0.001$, Fig. 1d). After MSC-exos therapy, there was a markedly increase in frequency ($P<0.05$) and a decrease in latency of active avoidance ($P<0.01$). It showed a great improvement in fear memory and locomotor ability, which was completely consistent with NSS.

Microglia Activity After OGD and Phagocytosis of MSC-Exos

Microglia activity changed in different OGD period. MTT showed that OGD more than 4 h greatly decreased cell activity and promoted cell death ($t=8.924$, $P<0.001$, Fig. 2a). We selected 2 h as the optimal OGD time for the subsequent experiments to investigate the effect of MSC-exos on microglia in vitro.

After incubation with MSC-exos labeled with CD81-PE antibody, immunofluorescence staining showed unevenly distributed orange fluorescence spots. They all closely surrounded the microglia cell nuclei (Fig. 2b). It demonstrated that MSC-exos had been efficiently taken into microglia cells. Whether NMLTC4 ($t=9.009$, $P<0.001$) or LTD4

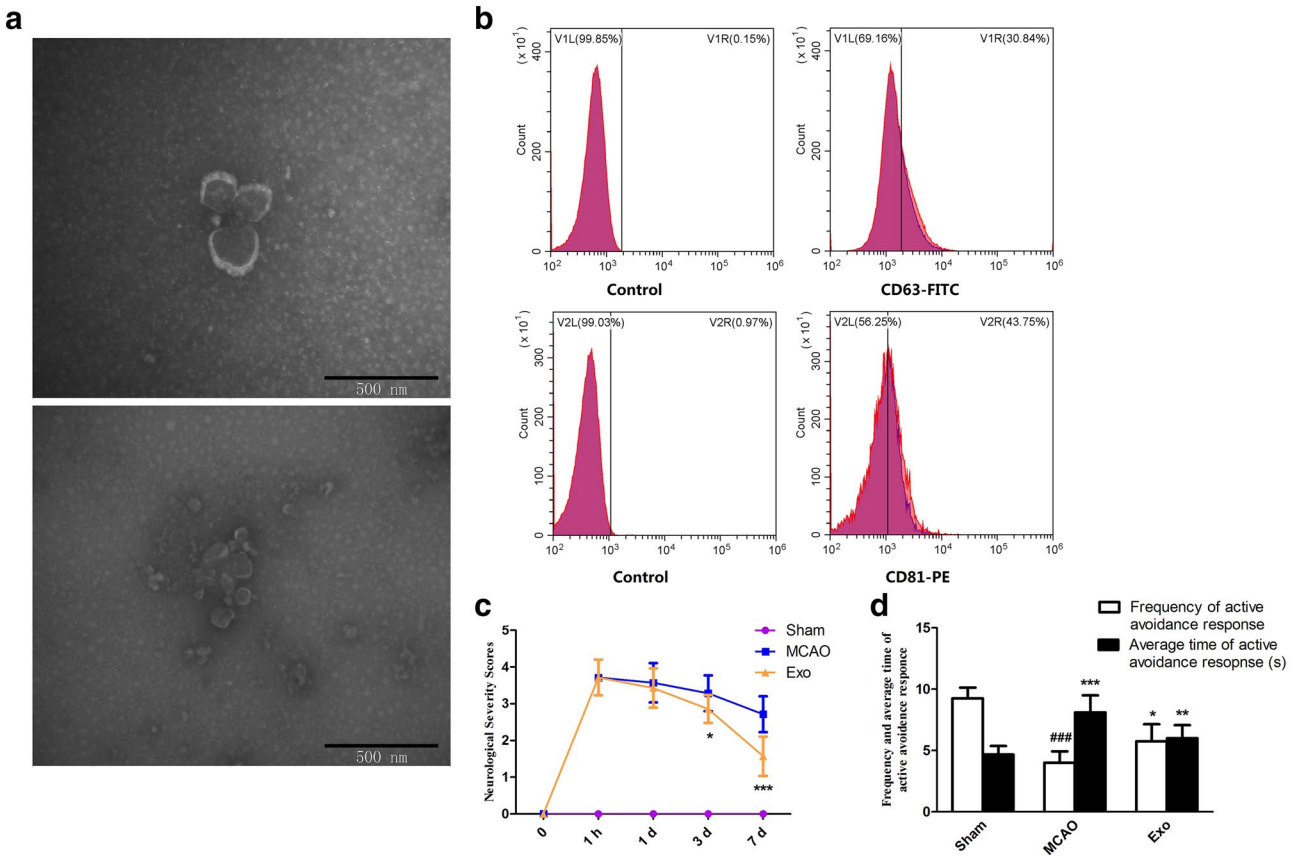

Fig. 1 **a** Morphological observation of exosomes under transmission electron microscopy. **b** FACS analysis of exosome surface markers CD63 and CD81. NSS assessment (**c**) and shuttle box avoidance test (**d**) of MCAO rats after MSC-exos treatment (n = 8). Data were expressed as mean ± SD, $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *vs* sham; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs* MCAO. NSS was analyzed by repeated-measures method and data of shuttle box test by one-way ANOVA

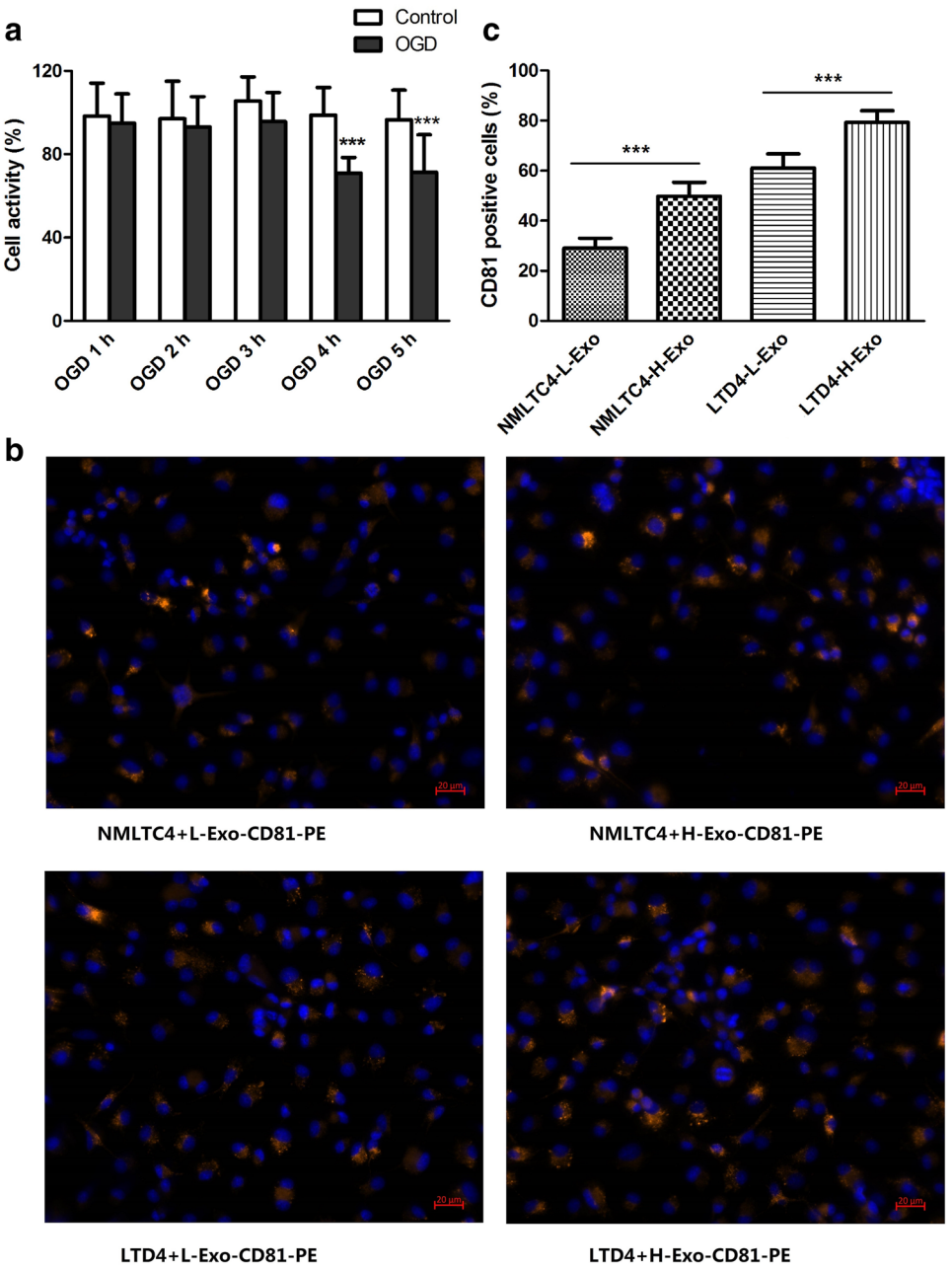

Fig. 2 **a** MTT assay of microglia activity after different OGD period (n = 24). **b**, **c** Immunofluorescence staining of microglia after incubation with MSC-exos labeled with CD81-PE antibody (n = 8). Data were expressed as mean ± SD, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, analyzed by *t-test*

(t = 7.455, $P < 0.001$) pre-treatment, the number of CD81 positive cells was much higher in H-Exo group than L-Exo (Fig. 2c). Besides, LTD4 triggered phagocytosis more extensively than NMLTC4.

MSC-Exos Mitigated MCAO and cysLTs Induced Microglia M1 Polarization

Microglia cells subject to OGD for 2 h were treated with NMLTC4/LTD4 (0.1 nM/mL) and MSC-exos (5 μg/mL and 10 μg/mL, respectively) for 24 h, then harvested for flow cytometric analysis (Fig. 3). The results demonstrated that there was a sharp rise in CD86+ microglia after treatment with NMLTC4 ($F_{(3,32)} = 548.7$, $P < 0.001$) or LTD4 ($F_{(3,32)} = 1433$, $P < 0.001$) compared with the control. But MSC-exos co-incubation only significantly reversed NMLTC4 induced M1 microglia conversion in a concentration dependent manner ($F_{(3,32)} = 548.7$, $P < 0.001$). As for the surface marker of the M2 phenotype, CD206, there was statistical significance ($F_{(3,32)} = 129.8$, $P < 0.001$) only in the NMLTC4-H-Exo group. Therefore, exosomes from mesenchymal stem cells significantly mitigated microglial M1 polarization induced by OGD and NMLTC4 treatment and promoted microglial conversion into M2 phenotype.

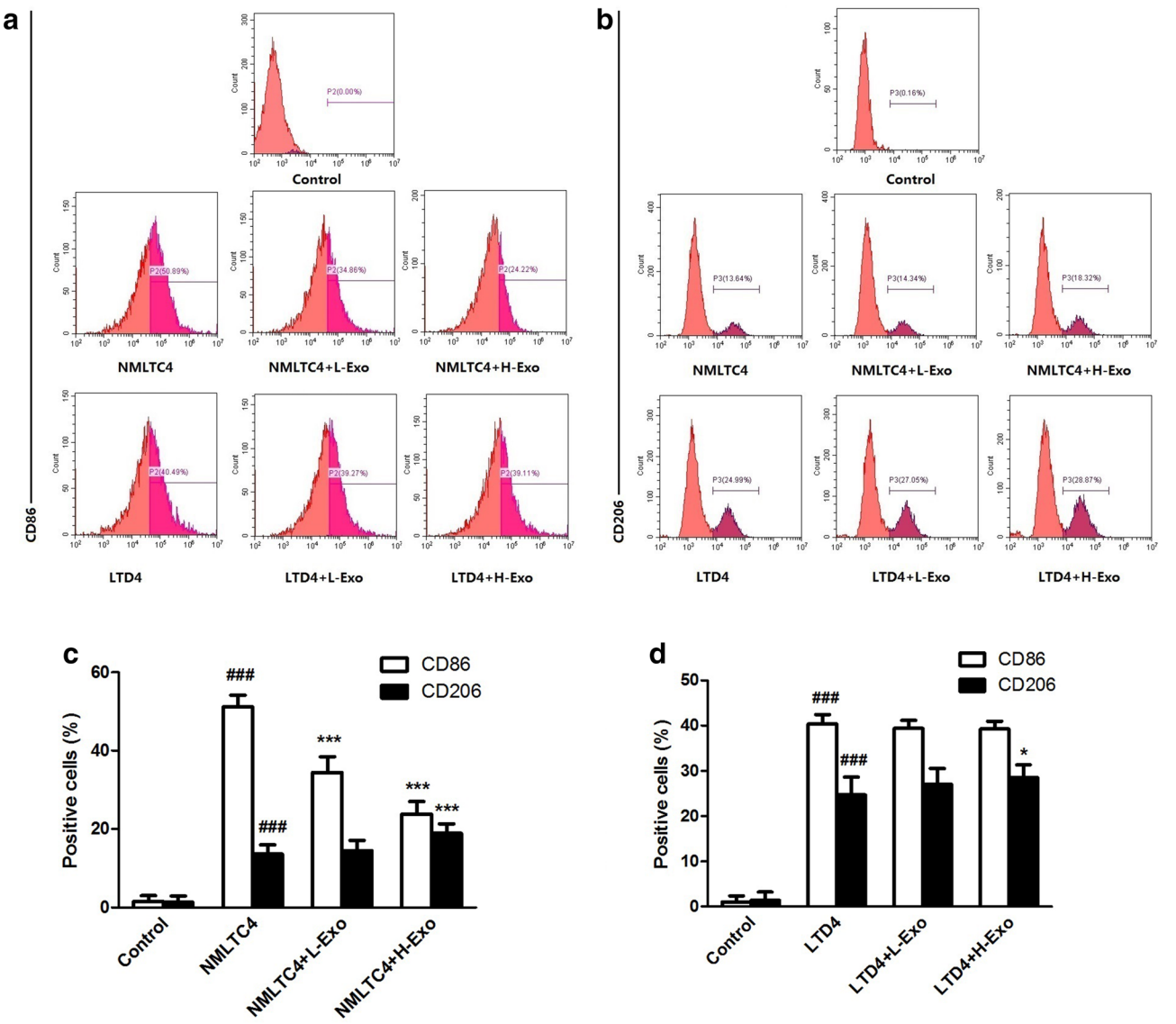

Fig. 3 Flow cytometric analysis of M1/M2 microglia (n = 3, each sample tested in triplicate). Data were expressed as mean ± SD, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* control; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* NMLTC4/LTD4 group, analyzed by one-way ANOVA

Immunofluorescence further illustrated the effect of exosomes on microglia phenotype after OGD and NMLTC4 treatment as well as in the brain tissues 7 days after MCAO/R (Fig. 4). Exos co-incubation reduced of CD86 expression in BV-2 cells induced by OGD and NMLTC4 treatment ($F_{(3,16)} = 99.09$, $P < 0.001$). The higher the exosome concentration was, the less the CD86 + antigen expressed. It was in complete consistency with that obtained through flow cytometry. The expression of CD206 in BV-2 was absolutely contrary to CD86 ($F_{(3,16)} = 167$, $P < 0.001$). In the brain tissue around the infarction site, there was more CD206 ($F_{(2,21)} = 54.20$, $P < 0.01$) and less CD86 ($F_{(2,21)} = 2018$, $P < 0.001$) expression in microglia in the Exos group than in the MCAO.

MSC-Exos Affected MCAO and NMLTC4 Induced Cytokines Secretion

In the culture supernatant of microglia after treatment with OGD and NMLTC4, the production of pro-inflammatory mediators such as NO ($F_{(3,32)} = 18.9$, $P < 0.001$), IL-1β ($F_{(3,32)} = 8.9$, $P < 0.01$), IL-12 ($F_{(3,32)} = 25.63$, $P < 0.05$) and TNF-α ($F_{(3,32)} = 16.23$, $P < 0.001$) was greatly increased in the model group than in the control. However, they were significantly decreased in the MSC-exos co-incubation groups, especially in the H-Exo group ($P < 0.05$). On the contrary, the secretion of anti-inflammatory and neurotrophic factors, including TGF-β ($F_{(3,32)} = 23.37$, $P < 0.001$), IL-10 ($F_{(3,32)} = 22.42$, $P < 0.001$) and BDNF ($F_{(3,32)} = 19.75$,

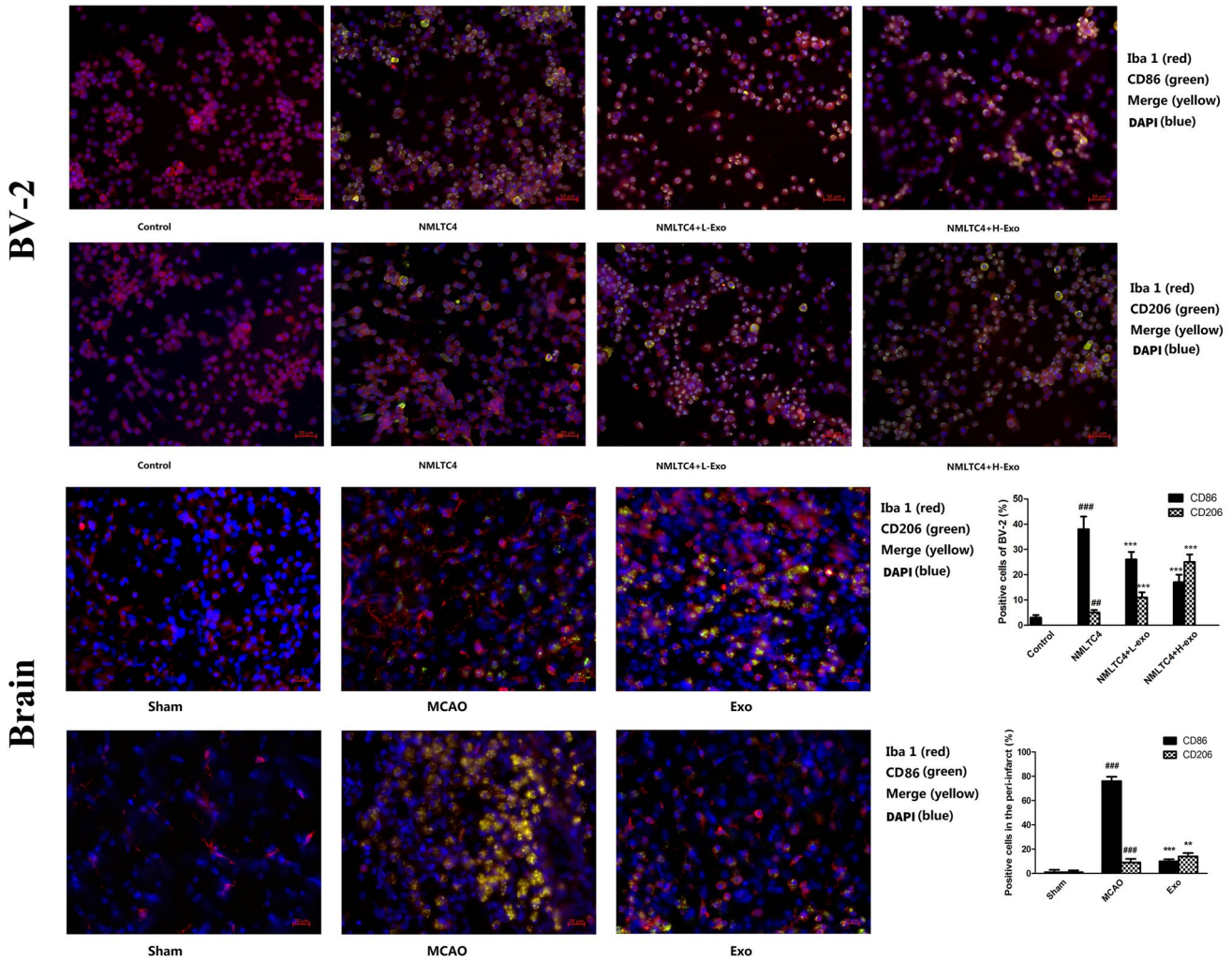

Fig. 4 Immunofluorescence staining of M1 microglia in vitro (n = 5) and in vivo (n = 4, each sample examined twice). Data were expressed as mean ± SD, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* sham/control, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* MCAO/NMLTC4 group, analyzed by one-way ANOVA

$P < 0.001$), was remarkably upregulated in the MSC-exos groups. Their expression level rose with the increase of MSC-exos concentration. Interestingly, there was no statistical difference in GDNF content among these groups ($F_{(3,32)} = 0.4271$, $P > 0.05$, Fig. 5a).

In the hippocampus of right hemisphere, MSC-exos significantly reversed the increased expression of NO ($F_{(2,33)} = 248.9$, $P < 0.001$), IL-1β ($F_{(2,33)} = 9.059$, $P < 0.01$), TNF-α ($F_{(2,33)} = 37.64$, $P < 0.001$) and IL-12 ($F_{(2,33)} = 12.96$, $P < 0.01$) induced by MCAO/R injury. Meanwhile, MSC-exos evidently increased the secretion of IL-10 ($F_{(2,33)} = 21.3$, $P < 0.001$), TGF-β ($F_{(2,33)} = 87.28$, $P < 0.001$) and BDNF($F_{(2,33)} = 12.07$, $P < 0.01$), but there was no distinct change on GDNF level (Fig. 5b). The trend of these cytokines expression was generally consistent with that in vitro. In the cortex of right hemisphere, there was no significant difference in the content of NO ($F_{(2,33)} = 19.24$, $P > 0.05$), IL-1β ($F_{(2,33)} = 1.516$, $P > 0.05$), IL-10 ($F_{(2,33)} = 0.6516$, $P > 0.05$), IL-12 ($F_{(2,33)} = 1.294$, $P > 0.05$) and GDNF ($F_{(2,33)} = 0.08491$, $P > 0.05$) after MSC-exo therapy. Only TNF-α remarkably decreased after MSC-exos therapy compared with the ischemia–reperfusion group ($F_{(2,33)} = 6.329$, $P < 0.01$). On the contrary, TGF-β ($F_{(2,33)} = 72.96$, $P < 0.001$) and BDNF ($F_{(2,33)} = 169.3$, $P < 0.001$) significantly increased after ischemia reperfusion and furtherly increased after MSC-exos injection ($P < 0.01$, Fig. 5b).

MSC-Exos Suppressed cysLT$_2$R Expression and ERK1/2 Phosphorylation

It is well known that NMLTC4 is the selective receptor agonist of cysLT$_2$R. MSC-exos significantly inhibited NMLTC4 induced microglia activation and inflammatory

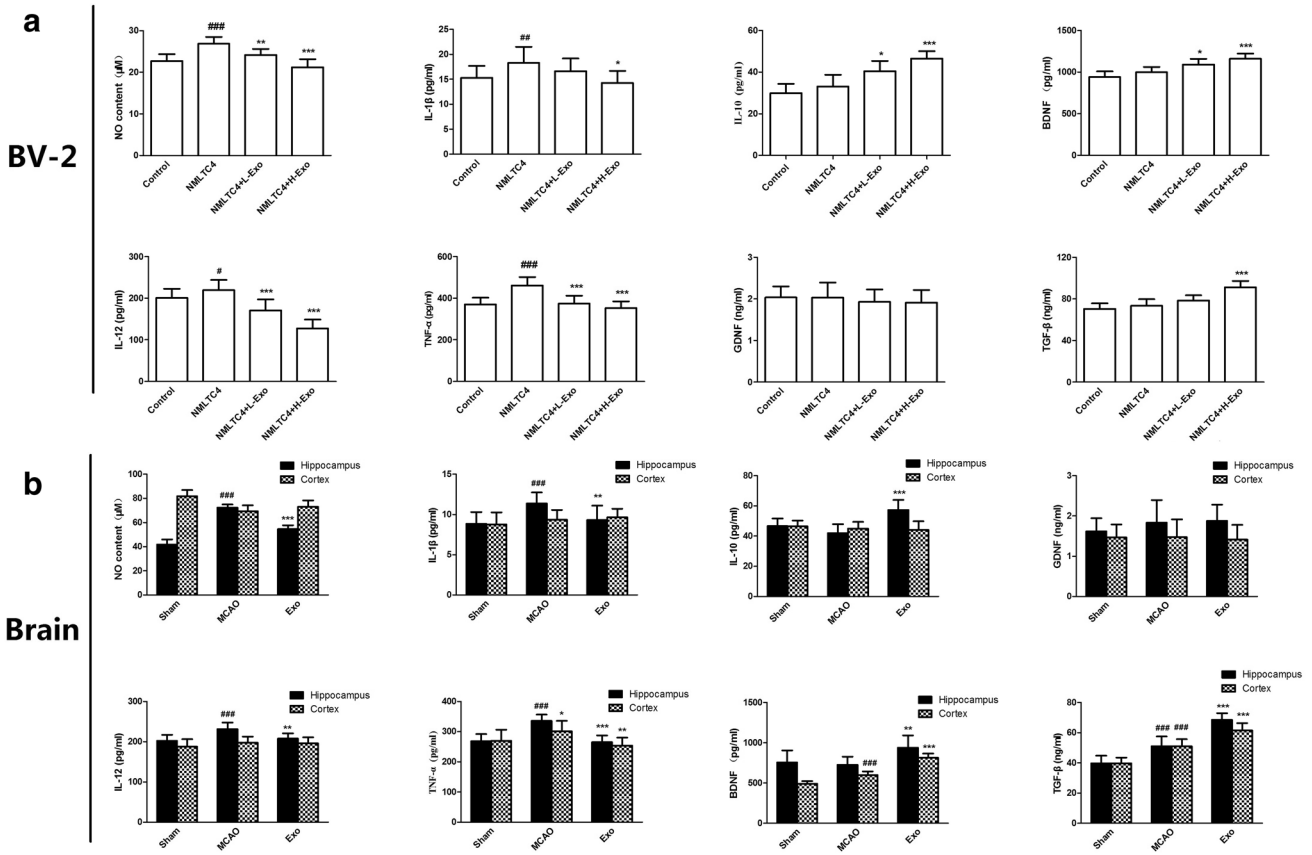

Fig. 5 ELISA of cytokines in the cell supernatant (**a**, n = 3) and the brain tissues (**b**, n = 4). Each sample was tested in triplicate. Data were expressed as mean ± SD, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* control/sham; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* NMLTC4/MCAO, analyzed by one-way ANOVA

cytokines secretion. Here we made a further investigation on the effect of MSC-exos on microglial cysLT$_2$R expression. As the results showed (Fig. 6a), cysLT$_2$R expression in NMLTC4 treated microglia was more than twice the level of the control (($F_{(5,48)} = 36.03$, $P < 0.001$). MSC-exos co-incubation greatly reversed the up-regulated expression of cysLT$_2$R ($P < 0.001$). In the NMLTC4 + H-Exo group, it was decreased nearly to the level of the control. In MCAO/R rats (Fig. 6b, c), MSC-exos significantly reduced cysLT$_2$R expression both in the hippocampus ($F_{(2,33)} = 157.4$, $P < 0.001$) and cortex of the ischemic side ($F_{(2,33)} = 47.37$, $P < 0.001$). The results in vivo were in good agreement with that in vitro.

ERK1/2 is the key mitogen kinase in the ERK signaling pathway involved in inflammation. We found that in rats, MSC-exos therapy obviously decreased the p-ERK1/2(42 KD) ($F_{(2,33)} = 34.70$, $P < 0.001$) and p-ERK1/2 (44 KD) ($F_{(2,33)} = 4.603$, $P < 0.05$) production induced by MCAO in the cortex. ERK1/2 (42 KD/44 KD) phosphorylation also significantly decreased ($F_{(2,33)} = 21.54$, $P < 0.001$; $F_{(2,33)} = 8.401$, $P < 0.01$, respectively). Only the phosphorylation of ERK1/2(44kd) was of statistical significance in the hippocampus ($F_{(2,33)} = 11.62$, $P < 0.001$). All these changes were entirely consistent with that of cysLT$_2$R and inflammatory factors expression (Fig. 6b, c). In vitro, ERK1/2 (42 KD/44 KD) phosphorylation was dramatically diminished in high MSC-exos concentration group ($F_{(3,32)} = 47.37$, $P < 0.001$; $F_{(3,32)} = 39.15$, $P < 0.0\ 01$, respectively. Figure 6a). In the low MSC-exos concentration group, there was a marked rise in ERK1/2 (42 KD) phosphorylation ($F_{(3,32)} = 47.37$, $P < 0.01$).The content of p-ERK1/2 (42 KD) was much less than p-ERK1/2 (44 KD).

Discussion

In the present study, we administered MSC-exos to MCAO rats through tail vein injection at 2 h after ischemia/reperfusion at the dose of 120 μg /rat. Our results showed MSC-exos significantly reduced neurological severity score, improved spatial learning and memory ability. Both In vivo and in vitro experiments demonstrated the effect of MSC-exos might be CysLT$_2$R dependent. MSC-exo markedly inhibited the expression of CysLT$_2$R in the MCAO injured

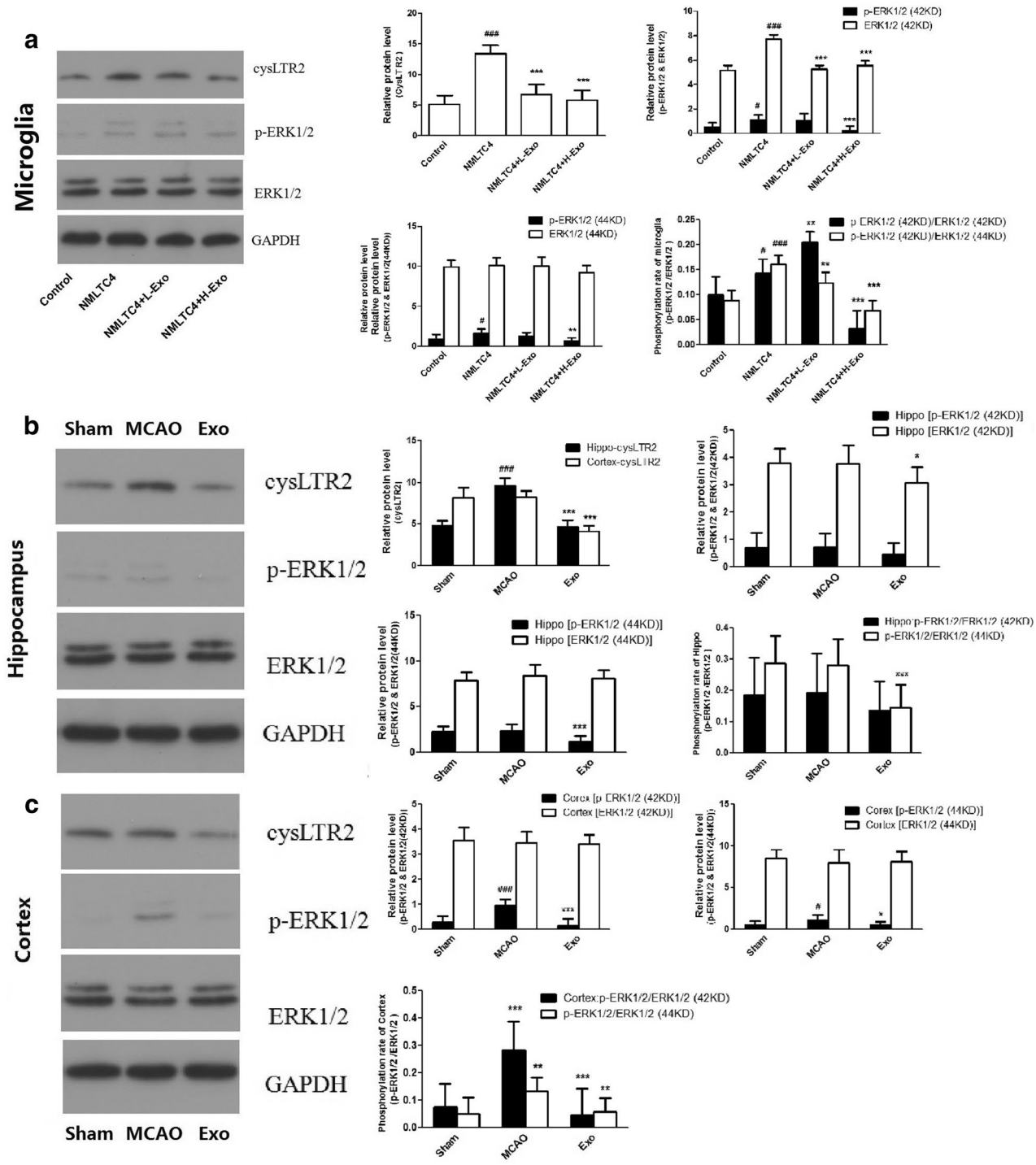

Fig. 6 Western-blot analysis of cysLT$_2$R and ERK1/2 expression in microglia (**a**, n = 3) and the brain tissues (**b**, n = 4). Each sample was tested in triplicate. Data were expressed as mean ± SD, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* control/sham, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* NMLTC4/MCAO, analyzed by one-way ANOVA

brain and NMLTC4 treated microglia, modulated the balance between M1 and M2 microglia, decreased pro-inflammatory cytokines secretion, increased anti-inflammatory and neurotrophic factors production.

Inflammation is widely implicated in the pathogenesis of various central nervous system disorders, such as cerebral ischemia, intracerebral hemorrhage, brain tumor, epilepsy, and Parkinson's disease [10, 11], resulting in the

secondary insult to the brain. CysLTs are potent inflammatory mediators, including LTC4, LTD4, LTE4. $CysLT_2R$ is highly expressed in hypertrophic microglia in the ischemic core zone of rats with MCAO/R [12]. Our results showed that MSC-exos significantly decreased $CysLT_2R$ expression in microglia after OGD and CysLTs treatment and in focal cerebral ischemia rats. They had a much stronger reversing effect on NMLTC4 treated M1 microglia polarization than LTD4. Therefore, MSC-exos have a stronger antagonistic effect against $CysLT_2R$ than other CysLTs receptors to inhibit cerebral inflammation.

Microglia activation is one of the characteristics of central nervous system inflammation. Microglia have two completely opposite functions with different phenotypes: M1 / M2 paradigm [13]. Generally, the former predominates at the injury site and the latter promotes repairment, so it is of clinically benefit to switch microglia phenotype from cytotoxic to neuroprotective by drug treatment or genetic modification. In our experiment in vitro, MSC-exos were effectively internalized by microglia, which promoted the transformation of microglia from M1 into M2 phenotype. A downregulation of pro-inflammatory factors accompanied by M2 microglia activation with an upregulation of CD206 + in vivo and in vitro was clearly proved. The improvement in NSS and shuttle box test showed the central nervous system had benefited from the anti-inflammatory property of MSC-exos.

MSC-exos are important components taking part in the paracrine effects of MSCs in brain injuries. MSC-exos are small vesicles budding off the plasma membrane of MSCs with size between 30 and 150 nm [14]. They could pass through the blood–brain barrier in various cerebral diseases [15, 16]. Exosomes express several specific cell surface markers, such as tetraspanins CD9, CD63, and CD81, which give them a high affinity for target tissues [17]. They could be selectively taken up by brain microglia [15, 16]. Besides, exosomes provide a protective and controlled internal microenvironment, allowing the content to travel long distance within tissues without degradation until harvested by recipient cells [18]. In vivo, we injected MSC-exos through the tail vein far from the ischemic brain. The plasma membrane and cell affinity of exosomes facilitated the transportation in the blood and transferring to microglia in the brain greatly. As confirmed in immunofluorescence staining of BV-2, exosomes were really distributed round microglia nuclei after treatment with OGD and NMLTC4. They specifically acted on their target cells via either ligand-receptor signaling pathways or internalization by phagocytosis, endocytosis, and direct membrane fusion [19, 20].

Exosomes played a connective role in intercellular communication. When exosomes released their content (proteins, mRNA, non-coding RNAs) inside recipient cells, they may change their biological properties and functions [20]. MiRNAs, marker signatures in MSC-exos, regulate gene expression through inhibiting mRNA transcription, accelerating mRNA degradation or disturbing protein translation. Up to now, mi-223 and miR-146a have been two well-studied anti-inflammatory miRNAs in MSCs exosomes [21, 22]. MicroRNA-27a targeting TLR4 negatively modulates inflammation [23]. MiRNA-21 activating TLR7 promotes neurotoxicity [24]. We are interested in which miRNAs in MSC-exos inhibit $CysLT_2R$ expression and will make further study. In the downstream of $CysLT_2R$, the expression and phosphorylation of ERK1/2 were remarkably downregulated, which resulted in the decreased secretion of pro-inflammatory factors and increased production of anti-inflammatory and neurotrophic factors. They protected the brain from neuroinflammation synergistically. With more and more studies carried out on the profiles and specific functions of miRNAs in MSC-exos, the cell-free therapy mediated by exosomes will make further development in the field of inflammation.

Conclusion

MSC-exos significantly improved cerebral inflammatory response and neurological syndrome after acute cerebral ischemic injury through tail intravenous injection. 120 μg / rat MSC-exos within 2 h after ischemia is an effective dose and time in vivo. MSC-exos remarkably inhibited microglial inflammation by reversing $CysLT_2R$-ERK1/2 mediated microglial M1 polarization and promoting M2 polarization. For acute ischemic injury, MSC-exos may be a potential therapeutic choice to improve nervous system functions through suppressing neuroinflammation.

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Author Contributions YZ was the main performer throughout the experiments, YG participated in the cell experiment, GX carried out the animal experiment, GY took part in the content and methods design, DL was responsible for the conception, interpretation, and editing of the whole study. All authors approved the final paper.

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Compliance with Ethical Standards

Conflict of interest Authors have declared no conflict of interest.

Ethical Approval Experiments were approved by the Institutional Animal Care and Use Committee of Hangzhou Medical College (2017, No163). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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