Exosomes derived from bMSCs inhibit neuroinflammatory after traumatic brain injury by influencing the IL-10/STAT3 signaling pathway

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Research

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Abstract

Exosomes are important membranous vesicles in several physiological and pathological processes. It is postulated that the microRNAs contained in the exosomes play the major roles in these processes. Exosomal microRNAs from bone marrow mesenchymal stem cells (bMSCs) have emerged as promising therapeutic agents against inflammation. However, its specific mechanism has not been clarified. The present study was designed to elucidate the underlying mechanism of bone marrow mesenchymal stem cells derived exosomes on neuroinflammation *in vitro* and *in vivo*.

Methods: *In vitro* co-culture experiments were done to demonstrate the effects of bMSCs and bMSCs-derived exosomes on the polarization state of BV2 microglia cells. *In vivo* experiments were subsequently done to validate the role of exosomes derived from bMSCs in neuro-inflammation after brain injury. MicroRNA sequencing was then done to reveal the potential expanded diversity between bMSCs and exosomes derived from bMSCs. The microRNAs were then screened and quantified by quantitative polymerase chain reaction (qPCR) to identify the most significant miRNAs. Moreover, lentiviral transfection was performed to establish animal models with overexpressed and downregulated microRNA expression levels. The animal models were subsequently used to determine the effects of specific microRNAs on neuroinflammation after TBI and their possible mechanisms of action.

Results: Both bMSCs and exosomes derived from bMSCs promoted the polarization of activated BV2 microglia cells to the anti-inflammatory phenotype. They inhibited the expression of pro-inflammatory cytokines but promoted the expression of anti-inflammatory cytokines. Exosomes derived from bMSCs played a stronger role in regulating the expression of inflammatory factors. *In vivo* animal-based experiments further revealed that the exosomes reduced neuronal apoptosis in the cortical area. They also promoted inhibition of neuroinflammation and transformation of microglia to anti-inflammatory phenotype. In addition, MicroRNA sequencing and subsequent analysis revealed that microRNA-181b was actively involved in the process. Subsequent lentiviral transfection studies revealed that overexpression of miR181b effectively reduced neuronal apoptosis and neuroinflammatory response after traumatic brain injury. Overexpression of microRNA181b also promoted the transformation of microglia to the anti-inflammatory phenotype. These effects were achieved through activation of the IL-10/STAT3 pathway.

Conclusion: Exosome derived from bMSCs promotes the polarization of microglia to anti-inflammatory phenotype and inhibit neuroinflammation both *in vitro* and *in vivo*. They also reduce neuronal apoptosis and protect damaged brain tissue. The microRNA-181b plays a vital role in this process by activating the IL-10/STAT3 pathway. Our study indicates that IL-10/STAT3 pathway may be involved in neuroinflammatory progression and that up-regulation of the microRNA-181b is a potentially new therapeutic approach for neuroinflammation.

Introduction

Microglia are the resident macrophages in the nervous system. They are responsible for regulating the microenvironment of the nervous system, phagocytosis of extracellular debris, and initiation of inflammation. As such, they are considered to be basic immune cells in the central nervous system (CNS) which regulates immune responses and brain function [6]. Microglia are activated in two ways after traumatic brain injury: either “classically activated” M1 phenotype or “alternatively activated” M2 phenotype. The balance between classically and alternatively activated microglial phenotypes influence disease progression in the CNS [22]. M1 phenotype exhibits pro-inflammatory responses and expresses pro-inflammatory cytokines. In contrast, the M2 type exhibits anti-inflammatory effects and produces anti-inflammatory factors. However, there is no absolute boundary between them. There are cells that exhibit the markers of M1 and M2 phenotype at the same time as well as those that express specific M1 and M2 markers. Nonetheless, researchers believe that demonstrating the complex phenotype of microglia in central nervous system based on M1/M2 classification is quite controversial because the two phenotypes exist upon stimulation and may thus reverse to the other phenotype. It is a reversible process of modification. Transformation of its phenotype and function may be a process with temporal and spatial heterogeneity [8, 11, 30].

In the wave of stem cell research at the beginning of the 21st century, mesenchymal stem cells from various tissues were used to treat nerve damage after brain injury or cerebral ischemia. Bone marrow mesenchymal stem cells (MSCs) are capable of repairing and replacing damaged tissues by differentiating into effector cells such as neurons and glial cells. They can also secrete some cytokines to inhibit neuroinflammation and promote nerve repair. Cognizant to this, used of MSCs is considered to be a promising method for treating diseases affecting the central nervous system [5, 20, 43]. In recent years, researchers have found out that the role of repair is through the paracrine effect and the exosomes secreted by MSCs rather than cell replacement [2, 3, 12, 25, 31, 33]. Exosomes are membranous vesicles secreted by different cells and are capable of crossing the blood-brain barrier (BBB) [45]. They play a major role in cell-cell interactions. Exosome vesicles contain many components such as proteins, lipids, and nucleic acids. In recent years, microRNAs contained in exosome vesicles have been found to play important roles in many physiological and pathological processes [2, 3, 25, 27, 32, 37]. Exosomes have been previously reported to display neuroprotective effects in rats after traumatic brain injury (TBI) [9]. Some scholars suggest that exosomes derived from mesenchymal stem cells can inhibit neuroinflammation and promote neural repair by regulating the microglial phenotype [13, 38]. However, the molecular mechanisms involved have not been reported. Moreover, there is still no conclusion of the mechanism involved during exosome regulation of the microglial phenotype after TBI. As such, this study hypothesized that bMSCs derived microRNAs in the exosomes exhibits neuroprotective effects. *In vivo* and *in vitro* experiments, and microRNAs sequencing were thus conducted to explore the microRNAs involved in neuroprotection. Lentivirus was subsequently used to regulate the expression levels of related microRNAs to test the hypothesis and verify the possible downstream mechanisms.

Materials And Methods

Culturing and identification of BV2 and bMSCs cells

BV2 cells were cultured in dulbecco's modified eagle medium (DMEM) high glucose culture medium supplemented with 10% Fetal Bovine Serum (FBS). Lipopolysaccharide (LPS, 1000 ng/ml) was used to activate BV2 cells for 48 hours. On the other hand, BMSCs were cultured in RPMI medium 1640 supplemented with 10% FBS. BV2 and bMSCs cells were subsequently identified through immunostaining using anti-Iba1 and anti-CD44 antibodies, respectively.

Extraction and identification of exosomes

Exosomes were extracted from the cell culture supernatant of bMSCs. The bMSCs were first washed twice with PBS. The culture medium was then replaced with exosome depleted FBS and culturing continued for 48 hours. The resultant supernatant was collected and first centrifuged for 10 minutes at 300 g. It was then transferred to an ultracentrifuge and centrifuged for 30 minutes at 10000 g. Further centrifugation of the supernatant was done at 100000 g for 70 minutes to form an exosome pellet. The supernatant was discarded and the pellet resuspended in 500 ul PBS for use in subsequent experiments. The exosomes were then identified using a transmission electron microscope (TEM). Nanoparticle tracking analysis (NTA) was employed to measure the diameter and particle number of the exosomes. The exosome marker tumor susceptibility gene 101 (TSG101) and heatshockprotein70 (HSP70) were detected through western blotting.

In vitro co-culture experimental design

Activated BV2 cells and bMSCs cells were co-cultured using a transwell system with a 0.4μm pore size. The MG group, the MG+bMSCs group, and the MG + Exo experimental groups were set up. The upper inserts were seeded with DMEM (500 ul/well), bMSCs (0.5×10^5 cells/well) or exosomes derived from bMSCs (6.3×10^9 particles/well) while the lower chambers were seeded with activated BV2 (2×10^5 cells/well). Cell-climbing slices were set at the lower chambers in advance. The transwell plates were then incubated for 48 hours in an incubator set at 37°C and 5% CO_2. Total RNA of BV2 cells was then extracted and the cell-climbing slices harvested for further analysis. The BV2 cells cocultured with DMEM in the MG group served as the negative control.

Cell phenotype analysis

BV2 cells in the lower chambers were collected after 48 hours of co-culturing for flow cytometry analysis. To verify the percentage of M1 and M2 macrophages, the cells were incubated with the following mixture of directly conjugated antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-IBA1 antibodies, allophycocyanin(APC)-conjugated anti-CD86 antibodies, and phycoerythrin (PE)-conjugated anti-CD206 antibodies. They were then fixed with a fixation buffer for 30 minutes at room temperature followed by determination of the proportions of $Iba1^+CD86^+CD206^-$ and $Iba1^+CD86^-CD206^+$ BV2 microglia cells. BV2 cells were evaluated in a flow cytometer (BD FACSCanto™ II Flow cytometer; BD Biosciences) and FlowJo v10.4 software (FlowJo LLC)

MicroRNAs sequencing

MicroRNA sequencing was performed after the exosomes were extracted to compare the different microRNA expression levels between bMSCs and exosomes derived from bMSCs. This analysis was performed by OBiO Technology (Shanghai) Corp., Ltd.

The traumatic brain injury model

A TBI model was made as reported by Wen et al., 2019[35]. A scalp incision was first made to expose the skull. A cranial window (3 mm diameter) was then drilled on the right parietal area of the skull, 2 mm away from the midline. A lateral fluid percussion injury (LFPI) device was finally used to induce TBI. Ketamine (80-100 mg/kg) was used for anesthesia.

Lentiviral transfection

The lentiviruses (1 ul) were injected into each mouse at the exposed cortex through the cranial window using a stereotaxic instrument and a microinjection pump. Lentiviral transfer vectors were used to up-regulate or down-regulate the expression level of microRNA-181b based on the microRNA sequencing. The expression level of microRNA-181b was tested by real-time PCR 7 days after the transfection.

In vivo animal experimental design

C57BL/6 male mice aged between 6 and 8 weeks were used in this study. The *in vivo* experiment was divided into two parts.

In the first part, healthy male C57/BL6 mice were divided into 4 groups: the sham group (sham operation was performed), brain trauma group (TBI model was made, TBI group), normal saline group (TBI model was made and saline was injected through the tail vein, TBI + Saline group), and exosomes group (TBI model was made and exosomes were injected through the tail vein, TBI + Exo group).

In the second part, mice modified by lentiviruses were divided into three groups: the TBI group (TBI model was made in normal mice), TBI-down group (TBI model was made in microRNA down-regulated mice), and TBI-up group (TBI model was made in microRNA up-regulated mice).

The brain tissues were harvested and excised on ice on days 1, 3, and 7 post initiation of brain trauma. Total RNA and proteins of the brain tissues were then extracted for follow-up rt-PCR and Western Blot tests. Brain tissues harvested on day 7 post initiation of TBI were prepared into sections for TUNEL staining and immunofluorescence analysis.

Immunofluorescence analysis

Immunofluorescence analysis was performed based on the classical protocol. Anti-iba1 and anti-CD44 were used to identify BV2 and bMSCs cells, respectively. The cell climbing slices in the *in vitro* experiments were double stained with anti-Arg1 and anti-iNOS followed by DAPI staining. Brain tissues collected in the first part of the animal experiments were prepared into sections and stained with anti-Arg1 and DAPI. Evaluation of the cell-climbing slices and brain sections was done using a confocal

microscope. Data was analyzed by counting the number of Iba1/DAPI, CD44/DAPI, Arg1/DAPI, and iNOS/DAPI double-positive cells with 2 high power fields in each cell climbing slice or brain section.

RNA extraction and real-time PCR

The expression levels of inflammatory factors such as IL-1β, IL-6, TNF-α, IL-10 and TGF-β was quantified by real-time PCR to determine whether exosomes and the micro181b could inhibit neuroinflammation. BV2 cells were collected 48 hours after the coculture experiments. The expression factors were quantified to verify the effect of bMSCs and exosomes derived from bMSCs. The factors were quantified at 1,3, and 7 days post TBI in the *in vivo* experiment. This was done to observe the dynamic changes of related factors in both parts of the animal experiments. Further to this, the expression levels of the candidate microRNAs selected through microRNA sequencing and the preliminary screening results were quantified.

Total RNA from BV2 cells and brain tissues of mice from all groups was extracted using an RNA purification Kit in accordance with the manufacturer's instructions. The concentration of RNA samples was with a Nanodrop® spectrophotometer (Thermo Scientific) and the samples stored at -80°C for preservation. First-strand cDNA synthesis was done through normal reverse transcription for mRNAs analysis. In the same line, a poly(A) tail was added to the microRNAs followed by reverse transcription for microRNA analysis. All primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The primer sequences were listed in Table 1 and 2. U6 expression was used as the internal reference gene to determine microRNAs expression while GAPDH expression served as the internal reference gene for expression of other genes. Gene expression levels were measured and calculated using the $2^{-\Delta\Delta Ct}$ method.

Detection of apoptosis

TUNEL assays were done in the two *in-vivo* experiments to determine whether bMSCs-Exo and microRNA-181b would reduce apoptosis in the damaged cerebral cortex. TUNEL staining was performed using a TUNEL kit in accordance with the manufacturer's instructions. The number of TUNEL positive cells in 2 regions of each brain section were counted to evaluate the effect of exosomes or microRNA-181b through statistical analyses. This process was performed 7 days post TBI.

Western blot analysis

The expression levels of iNOS and Arg1 were detected to determine the phenotype of M1 and M2, respectively in the *in vivo* experiments. In the same line, the expression level of STAT3 as the potential responsible pathway was measured to verify its effect in the second part of animal experiments. Protein extracts were first obtained from the brain tissue samples 7 days post TBI and their concentration determined using a BCA kit. The primary antibody anti-Arg1 was used at 1:1000 dilution, anti-iNOS at 1:500 dilution, anti-STAT3 at 1:200 dilution, anti-α-tubulin at 1:500, and anti-β-actin at 1:500 dilution for western blotting (WB). The gray values were then detected using the Image J software and the value relative to the expression of the internal reference calculated. Statistical analyses based on the relative gray values were then done.

Data analysis

The Statistical Product and Service Solutions (Version 17.0, Chicago, IL, USA) was used for data analyses. Data was reported as mean values ± standard error. Variations between groups were calculated using one-way analysis of variance (ANOVA). In addition, the LSD-t test was employed for group comparisons. P values less than 0.05 ($P < 0.05$) indicated that there were significant differences between groups.

Results

bMSCs and bMSCs derived exosomes induced transformation of microglial polarization towards the anti-inflammatory phenotype

Exosomes were isolated from bMSCs cells by ultracentrifugation. The exosomes were then identified using TEM, nanoparticle tracking analysis (NTA) and western blot analysis. A typical cup-shaped membrane vesicle morphology was observed (Figure 1). The size distribution profiles from the nanoparticle tracking analysis revealed that most of the vesicles had a diameter of ~130 nm. The original concentration of the exosomes was 6.3×10^{10} particles/ml. Western blotting analysis further revealed that exosome markers TSG101 and HSP70 were expressed in the exosomes (Figure 1).

Figure 1:Identification and NTA analysis of bMSCs derived exosomes. TEM results revealed the membrane vesicle had a typical cup-shaped morphology. Scale bar =100 nm. NTA revealed that most vesicles had a diameter of ~130 nm. The original concentration of the exosomes was 6.3×10^{10} particles/ml. Western Blot further revealed that exosome markers TSG101 and HSP70 were expressed in the exosomes.

BV2 microglia cells were activated using LPS (1000ng/ml) and then incubated at 37℃ for 48 hours prior to coculture with bMSCs and exosomes. They were then cocultured with bMSCs and exosomes for 48 hours to facilitate the polarization of activated BV2 cells toward the anti-inflammation type (Figure 2A). This was done to investigate the effects of bMSCs and bMSCs derived exosomes on the polarization phenotype of activated BV2 cells in a co-culture system. The expression of anti-inflammation marker Arg1 increased significantly in the MG+bMSCs and MG+Exo groups, while the expression of pro-inflammation marker iNOS decreased compared to that of the MG group. Flow cytometry analysis was then performed to determine the proportion of pro-inflammatory and anti-inflammatory phenotype cells. The BV2 cells were labelled using FITC-conjugated anti-IBA1 antibodies, APC-conjugated anti-CD86 antibodies, and PE-conjugated anti-CD206 antibodies. The Q1 area indicated Iba-1/CD86 double-positive BV2 cells while the Q3 area indicated IBA-1/CD206 double-positive BV2 cells (Figure 2B). In the same line, activated BV2 cells were transformed to the anti-inflammatory phenotype after 48 hours of co-culturing with bMSCs and exosomes (Figure 2C). Moreover, the exosomes were associated with a stronger anti-inflammatory effect.

Figure 2: Effects of bMSCs and exosomes on the phenotype of activated BV2 microglia.A. Immunofluorescence results of DAPI (blue), $Arg1^+$ (red) and $iNOS^+$ (green) cells. **B, C**. Flow cytometry analysis of the proportion of IBA1/CD86 double-positive and IBA1/CD206 double-positive cells.

bMSCs and exosomes promoted the expression of anti-inflammatory factors

Phenotype transformation is accompanied with functional change. As such, RT-qPCR was used to detect the expression of BV2 cells-related inflammatory factors after 48 hours of co-culture with bMSCs and exosomes. Both bMSCs and exosomes inhibited the expression of pro-inflammatory factors (IL1β, IL6, TNFα) but promoted the expression of anti-inflammatory factors (IL10, TGFβ). The exosomes played a stronger role in regulating the expression of related inflammatory factors (Figure 3).

Figure 3: The expression of inflammatory factors of BV2 cells in the co-culture system.

** Differences between the three groups were all statistically significant (P<0.05).

* The difference between the MG and the MG+Exo group was statistically significant (P<0.05).

Exosomes decreased neuronal apoptosis after induction of TBI in mice.

The lesion area and the neuron apoptosis in each group was determined after 7 days of TBI to detect the effects of exosomes on the nervous system after TBI *in vivo* (Figure 4). The damaged area of the mice in the TBI group and TBI + Saline group were larger compared with those of mice in the TBI + Exo group (Figure 4A & B). Brain sections were then subjected to TUNEL staining followed by quantification of the TUNEL-positive cells. Red staining represented the TUNEL positive cells (indicated by white arrows). The quantities of TUNEL positive cells in the sham group, TBI group, TBI + Saline group, and TBI + Exo group were 0.4 ± 0.55, 42.1 ± 4.30, 40.8 ± 5.26, and 22.2 ± 3.03, respectively. The TBI + Exo group had significantly fewer apoptotic neurons than the TBI group and the TBI+ Saline group (Figure 4C & D).

Figure 4: Exosomes induced reduction of the injured area and neuronal apoptosis. A. Nissl staining of the □) sham group, □) TBI group, □) TBI + Saline group, □) TBI + Exo group. **B.** The measured lesion areas of **A**. **C.** Quantification of D shows the difference of $TUNEL^+$ cells. **D.** TUNEL staining of the four groups. Scale bar = 0.2 mm. *, P<0.05.

Exosomes inhibited inflammation of the brain tissue after TBI

An *in-vivo* experiment was designed in which exosomes were injected through the tail vein followed by induction of the TBI model to examine whether exosomes could inhibit inflammation in the CNS after induction of TBI.The expression levels of Arg1, iNOS, and inflammatory factors were then detected by immunofluorescence, western blotting and real time qPCR (Figure 5). The proportion of $Arg1^+$ cells in the TBI+Exo group was significantly higher than that of the other groups (Figures 5A & B). In the same line, the expression level of Arg1 in the TBI+Exo group was significantly higher than that of the other groups. However, the expression of iNOS in the TBI+Exo group was significantly lower than that of the TBI and

TBI+Saline group (Figure 5C, *P*<0.05). Figure 5D-(, ,) shows the expression levels of the related inflammatory factors after 1 day, 3 days, and 7 days, respectively from the onset of TBI.Evidently, the expression levels of IL-10 and TGF-β in the TBI +Exo group were higher than that of the other two groups on day1, 3, and 7 post TBI. Moreover, the expression levels of IL-1β and TNF-α in the TBI+Exo group were lower than those of the TBI and TBI+Saline groups on day 3 and 7 post TBI. In the same line, injection of exosomes upregulated the expression of STAT3 but inhibited the expression of NFκB (Figure 5D-).

Figure 5: Effects of exosomes on Neuroinflammation. A. Immunofluorescence assay of Arg1 on brain slices 7 days post TBI. Scale bar = 200 um **B.** The proportion of $Arg1^+$ cells in A. (*. P<0.05) **C.** The relative expression of Arg1 and iNOS tested by Western Blotting. (*. P<0.05) **D.** , , and respectively shows the expression levels of inflammatory factors on day 1, 3, and 7 post TBI (*. P<0.05 between TBI+Exo and TBI group; **. P<0.05 between TBI+Exo and the other two groups.).) The different expression levels of NFκB and STAT3. (**.P<0.05 between TBI+Exo and the other two groups).

miRNA-181b was highly expressed in both exosomes and brain tissues

miRNA sequencing of bMSCs cells and exosomes derived from bMSCs cells was done to explore the effective components of exosomes. Sequencing results revealed that there were more than 500 miRNAs highly expressed and more than 300 microRNAs lowly expressed in the exosomes compared to the bMSCs cells. Parts of the differentially expressed microRNAs are shown in the heat map and volcano plot (Figures 6A & B). Based on microRNAs reported in other relevant studies, let-7c, miR-124, miR-21a, and miR-181b were chosen to be intensively studied. The expression levels of these microRNAs in brain tissues of mice in the TBI + Exo group at day 7 post TBI were thus determined. miR-181b was found to be highly expressed in the TBI + Exo group (Figure 6C). This strongly suggested that miR-181b could inhibit neuroinflammation and regulate the phenotype of the microglia by up-regulating the STAT3 related pathway.

Figure 6: Differential expression of microRNAs. A. heat map of differentially expressed microRNAs between bMSCs and exosomes. **B.** volcano plot of the differentially expressed microRNAs (Blue represents low expression while red denotes high expression in exosomes. Gray denotes lack of differences between them.) **C.** The differentially expressed microRNAs *in vivo*. *, *P*<0.05

Up-regulation of microRNA-181b inhibited neuronal apoptosis, reduced neuroinflammation and regulated the phenotype of microglia

Lentiviral vectors were used to obtain different expression levels of miR-181b to verify the role of miR-181b in the neuroinflammation process post TBI. The expression level of miR-181b was detected 7 days after transfection of the mice with the lentivirus. The expression level of miR-181b in the up-regulation and down-regulation group was 2.7 times higher and 0.4 times lower than that of the normal C57BL/6 mice, respectively (Figure 7B). Neuron apoptosis detected in each group at day 7 post TBI revealed that the numbers of TUNEL-positive cells in the three groups were 38.2±3.76, 35.6±4.50, and 24.8±1.94, respectively (Figures 7A & C). Notably, the number of TUNEL-positive cells in the miR-181b up-regulation

group was significantly low than the other two groups. In the same line, the microglia markers Arg1 and iNOS, and the transcriptional regulator STAT3 were detected by Western Blotting (Figure 7D). The TBI-up group had a higher expression level of Arg1 and STAT3, but a lower expression level of iNOS. Inflammatory factors were also detected at day 1, 3, and 7 post TBI (Figure 7 E- I, II & III). The expression level of IL-10 and TGF-β in the TBI-up group was significantly higher than that of the other two groups on the 1st, 3rd, and 7th day post TBI (P<0.05). However, the expression level of IL-1β in the TBI-up group was significantly lower than that of the other two groups on the 1st, 3rd and 7th day post TBI (P<0.05). Similarly, the expression level of TNF-α in the TBI-up group was significantly lower than that of the other two groups on the 3rd and 7th day post TBI (P<0.05). Nonetheless, the expression level of IL-6 was not significantly different among the groups on the 1st, 3rd, and 7th day post TBI. Further analysis of the TBI-up group revealed that the expression levels of IL-10 and TGF-β gradually increased after TBI (Figure 7E-□).

Figure 7: The effect of different expression levels of miR-181b on neuronal apoptosis and neuroinflammation. A. TUNEL staining showing the levels of neuronal apoptosis in the three groups. Scale bar = 0.2 mm. **B.** The expression level of miR-181b in the three groups 7 days after the transfection of lentivirus. **C.** Quantification of TUNEL-positive cells of **A** (*. P<0.05). **D.** The expression of microglia markers Arg1 and iNOS as well as the transcriptional regulator STAT3**. E.** The expression of inflammatory factors on the 1st, 3rd, and 7th day post TBI in the three group (□, □, □) (**. P<0.05 between TBI-up and the other two groups.) The dynamic changes of inflammatory factors in TBI-up group (□). Dpi: days post injury.

Discussion

Microglia plays an important role in regulating neuronal functions such as cell survival, neurogenesis, and neuroinflammation [1, 15, 24, 40]. In healthy brain tissues, microglia can phagocytose cell debris and damaged neurons. Activated microglia can also release various pro-inflammatory and anti-inflammatory cytokines and mediators such as interleukin-1β, interleukin-6, interleukin-10, arginase-1, cyclooxygenase-2 and inducible nitric oxide synthase among others [10, 21]

Exosomes derived from MSC protect various tissues from damage [2, 9, 23, 31, 44] Nonetheless, there are still many unanswered questions regarding their role in the pathophysiological process. Kim reported that exosomes isolated from MSCs could improve the cognitive impairment in TBI mice [9]. However, the molecular signals that mediated interactions between exosomes and neurons to promote neuronal survival were still unclear.

On the other hand, neuroinflammation affects the recovery of nerve function and the survival of neurons. As such, it is an important process in secondary brain injury. Herein, exosomes co-cultured with bMSCs inhibited the pro-inflammatory effect of activated microglia-like BV2 cells. They also played a crucial role in promoting macrophage polarization towards the M2 phenotype. Moreover, the BV2 cells expressed both Arg1 and iNOS at the same time. This result was consistent with that of Pettersen's, Vogel's, and

Wong's reports[19, 34, 36]. In the same line, Italiani reported that human macrophages could be polarized to the M1 phenotype and then mature to the M2 phenotype with continuous changes in culture conditions [7]. Similarly, after exposure to classic M1 activation signals or interferon-γ, M2 macrophages can express M1 specific cytokines and markers thereby transforming to the M1 phenotype [18, 29]. Although there is no universal consensus on the transformation of microglia phenotype, phenotype changes of macrophages induced by external factors are dynamic, and show heterogeneity in space and time. Cognizant to this, the phenotypic definition of macrophages should be interpreted based on specific markers and function. As such, the real situation is by no means as simple as M1/M2.

Zhang *et al.* reported that TBI rats treated with bMSCs exosomes had significantly stronger learning ability than those in other groups when tested using Morris water maze at 34–35 days after injury [41]. In addition, their sensory function and behavioral scores were higher than those of other groups at 14–35 days post TBI. In the same study, the exosome treatment group also had a significant increase in the number of neovascular endothelial cells in the dentate gyrus that effectively reduced neuroinflammation. This was the first *in vivo* study of exosomes reporting their role in treatment of TBI. Herein, the damaged area in the TBI + Exo group was significantly less than that of the TBI and TBI + Saline groups. In addition, the number of TUNEL-positive cells in the damaged area was also significantly reduced after treatment with exosomes. This strongly indicated that the use of exosomes in the acute phase after TBI could reduce brain tissue damage and neuronal apoptosis. In the same line, the neuroinflammatory response in the exosome treatment group was inhibited. The expression of pro-inflammatory factors IL-1β and TNF-α was significantly suppressed while that of the anti-inflammatory factors IL-10 and TGF-β was significantly increased. The expression of M2 type microglia marker Arg1 was also significantly increased in the exosome treatment group.

There are numerous studies on neuroinflammatory signal pathways. On one hand, Man reported that the expression of the Notch1/NFκB pathway protein was significantly increased in the cerebral ischemic stroke models [4]. On the other hand, Zeng reported that hypertonic saline attenuated the expression of pro-inflammatory mediators and down-regulate the Notch signaling pathway [39]. The role of STAT3 signaling pathway during inflammation is controversial. Ryu reported that dasatinib regulated the neuroinflammatory response of LPS-induced microglia and astrocytes by inhibiting the expression of STAT3 and the pro-inflammatory factors [26]. In the same line, Zhang reported that ginkgo biloba extract protected the brain tissues after ischemic stroke by significantly reducing the expression of pro-inflammatory cytokines [42]. These effects may have been achieved by inhibiting the JAK2/STAT3 pathway. However, Staples reported that IL-10 activated the phosphorylation of transcription factor STAT3 through the autocrine feedback. As such, activation of STAT3 could up-regulate the expression of IL-10. The autocrine feedback enhanced the anti-inflammatory effects of IL-10 [28].

The relationship between IL-10 and STAT3 seems to be particularly subtle. IL-10 plays a role in the anti-inflammatory response by binding to its receptor (IL-10R). The Activation of the IL-10/JAK1/STAT3 cascade pathway in turn causes activation of the phosphorylated STAT3 within seconds. Though STAT3 has no anti-inflammatory effects, it can activate many anti-inflammatory effector genes which can inhibit

pro-inflammatory genes at the transcriptional level [16, 17]. Herein, the IL-10 level of the exosomes treatment group was significantly higher than that of the other two groups on the 1st, 3rd and 7th day post TBI. Moreover, the expression of STAT3 in the exosome treatment group was significantly higher than that in the TBI group on the 7th day post TBI. Based on the interaction between IL-10 and STAT3, it can be concluded that IL-10 activates the STAT3 cascade pathway to inhibit neuroinflammation and to promote the transformation of microglia to an anti-inflammatory phenotype.

Recently, researchers have postulated that the therapeutic effect of exosomes depends on large amounts of miRNAs [14, 27]. Cognizant to this, miRNAs sequencing was done to measure the differential expression level of miRNAs between exosomes and bMSCs. Based on microRNAs reported in other relevant studies and the sequencing results, let-7c, miR-124, miR-21a, and miR-181b were screened as those strongly associated with neuroinflammation. Subsequent qPCR results of the brain tissue revealed that the expression of miR-181b in the exosome treatment group was significantly higher than that of the other groups. It was thus concluded that miR-181b is involved in regulation of neuroinflammation as well as regulation of the microglial phenotype.

Further investigations of the effects of miR-181b using lentiviral vectors revealed that there were significantly fewer apoptotic cells in the TBI-up group compared to the other groups. The TBI-up group had significantly higher levels of IL-10 and TGF-β and lower levels of IL-1β and TNF-α in the injured area. Further analysis revealed that the expression levels of IL-10 and TGF-β gradually increased after TBI. Compared with the TBI group, the expression of ARG1 and STAT3 in the TBI-up group increased significantly, while the expression of iNOS decreased. Taken together, these results strongly suggested that mirR-181b could activate the IL-10/STAT3 pathway, promote phenotype transformation of microglia, and inhibit neuroinflammation and neuronal apoptosis.

Nonetheless, this study was limited by several factors. The direct targets of miR181b and the mechanism of how miR-181b activate the IL-10/STAT3 pathway were not identified. The transfection efficacy *in vivo* was limited by the injection dose and the titer of lentivirus. Further studies on miR181b targets and their mechanisms of action are therefore needed to decipher its role in neuroprotection. Modified transfection process was needed as well. Despite these limitations, our current study disclosed that exosomes derived from bMSCs can inhibit neuroinflammation both *in vitro* and *in vivo* as well as depressing the apoptosis after TBI. And microRNA-181b palys as a potential target which may regulate the IL-10/STST3 pathway, and subsequently influence the neuroinflammation post TBI.

Conclusion

Exosomes derived from bMSCs promotes the polarization of microglia to the anti-inflammatory phenotype and inhibit the neuroinflammatory response both *in vitro* and *in vivo*. The miR-181b may plays a vital role in this process by activating the IL-10/STAT3 pathway. The prominent role of microRNAs in neuroprotective offers new potential avenues of research into the treatment of neuroinflammation post TBI.

Abbreviations

bMSCs: bone marrow mesenchymal stem cells; STAT3: signal transducer and activator of transcription 3; CNS: central nervous system; BBB: blood-brain barrier; TBI: traumatic brain injury; DMEM: dulbecco's modified eagle medium; FBS: fetal bovine serum; LPS: Lipopolysaccharide; TEM: transmission electron microscope; NTA: nanoparticle tracking analysis; TSG101: tumor susceptibility gene 101; HSP70: heatshockprotein70; MG: microglia; TUNEL: Terminal deoxynucleotidyl transferase-mediated nick end labeling; TNFα : Tumor Necrosis Factor α; TGFβ: transforming growth factor β;

Declarations

Ethics approval and consent to participate

All animal experiments were approved by the ethics committee of the First Affiliated Hospital, School of Medicine, Zhejiang University and followed the National Institutes of Health guide for the care and use of Laboratory animals.

Consent for publication

Not applicable

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Authors' Contributions

Liang Wen, Yadong Wang, and Xiaofeng Yang conceived and designed the study. Yadong Wang, Peidong Zheng and Mengdi Tu carried out the main experiments and analyzed the data. Yadong Wang drafted this manuscript. We thank Wendong You, Yuanrun Zhu and Junfeng Feng for their useful suggestions for the revision of this paper. We are grateful to Dongfeng Shen and Hao Wang for assistance with our experiments.

Competing Interests

Declarations of interest: none.

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Tables

Table 1 The primer sequences of several mRNAs

GAPDH	F：TGGATTTGGACGCATTGGTC
	R：TTTGCACTGGTACGTGTTGAT
IL-1β	F：GCAACTGTTCCTGAACTCAACT
	R：ATCTTTTGGGGTCCGTCAACT
IL-6	F：TAGTCCTTCCTACCCCAATTTCC
	R：TTGGTCCTTAGCCACTCCTTC
TNF-α	F：GCAGGAGGGACTTCAGGTGA
	R：GCCCCCACTGTCCGTTCT
IL-10	F：CGGCTGAGGCGCTGT
	R：TGCCTTGCTCTTATTTTCACAGG
TGF-β	F：TCTGCATTGCACTTATGCTGA
	R：AAAGGGCGATCTAGTGATGGA
NFκB	F：ATGGCAGACGATGATCCCTAC
	R：TGTTGACAGTGGTATTTCTGGTG
STAT3	F：CAATACCATTGACCTGCCGAT
	R：GAGCGACTCAAACTGCCCT

Table 2 The primer sequences of some miRNAs

U6	F: AGAGAAGATTAGCATGGCCCCTG
mmu-let-7c	F: GCGTGAGGTAGTAGGTTGTATGGTT
mmu-miR-124	F: TAAGGCACGCGGTGAATGC
mmu-miR-21a	F: CGGGTAGCTTATCAGACTGATGTTGA
mmu-miR-181b	F: AACATTCATTGCTGTCGGTGGG
universal reverse primer	R: ATCCAGTGCAGGGTCCGAGG

Figures

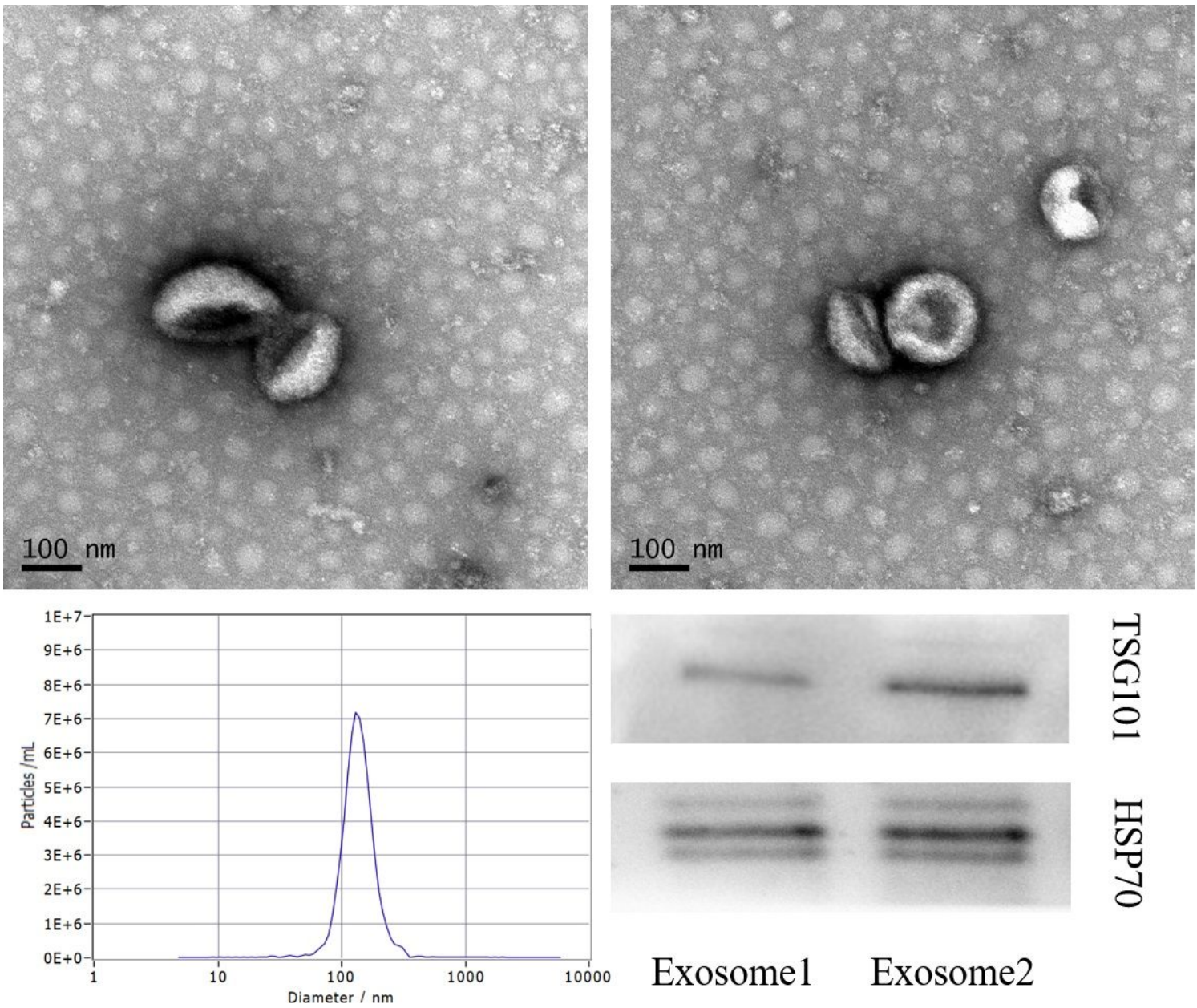

Figure 1

Identification and NTA analysis of bMSCs derived exosomes. TEM results revealed the membrane vesicle had a typical cup-shaped morphology. Scale bar =100 nm. NTA revealed that most vesicles had a diameter of ~130 nm. The original concentration of the exosomes was 6.3×1010 particles/ml. Western Blot further revealed that exosome markers TSG101 and HSP70 were expressed in the exosomes.

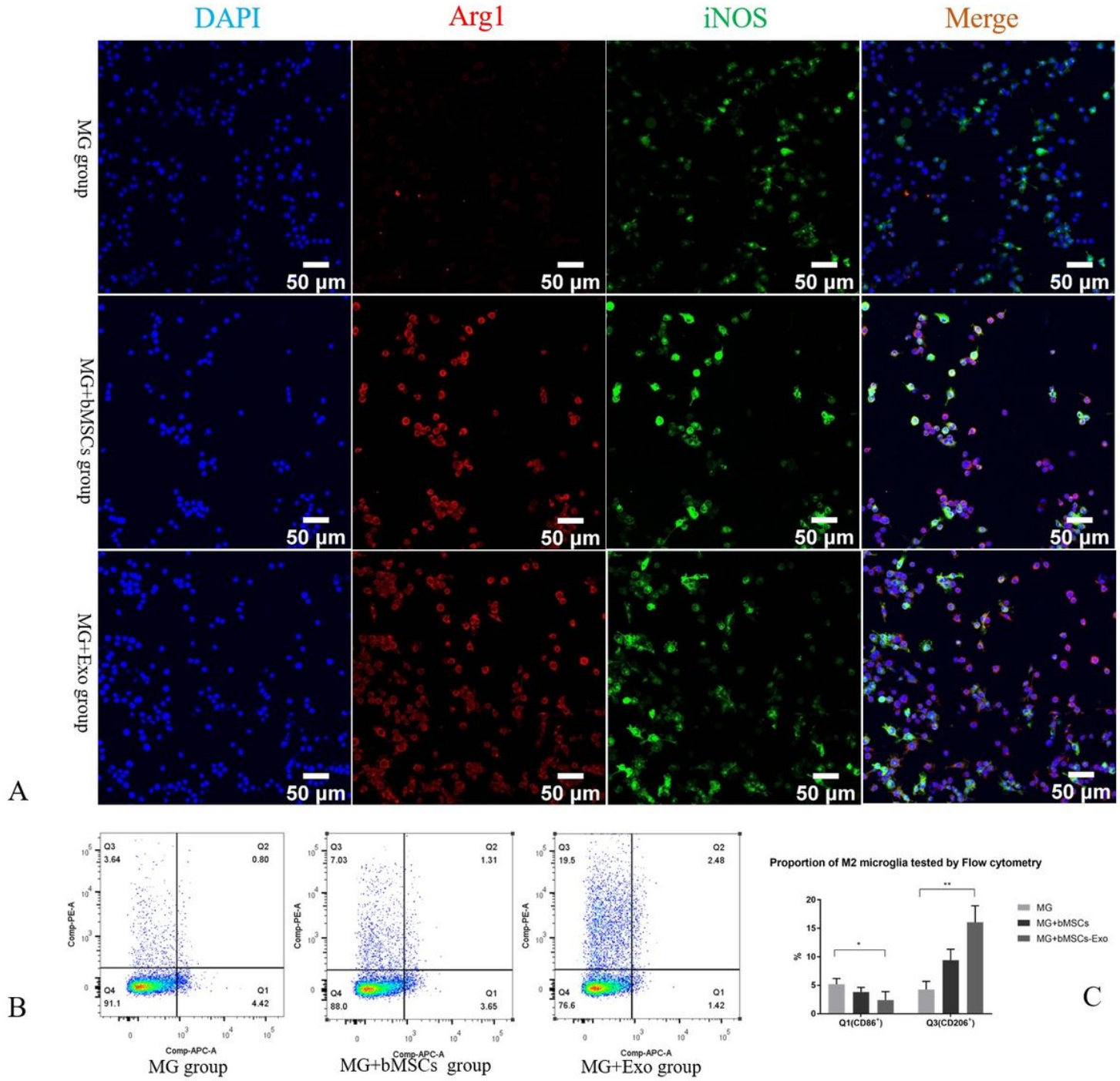

Figure 2

Effects of bMSCs and exosomes on the phenotype of activated BV2 microglia. A. Immunofluorescence results of DAPI (blue), Arg1+ (red) and iNOS+ (green) cells. B, C. Flow cytometry analysis of the proportion of IBA1/CD86 double-positive and IBA1/CD206 double-positive cells.

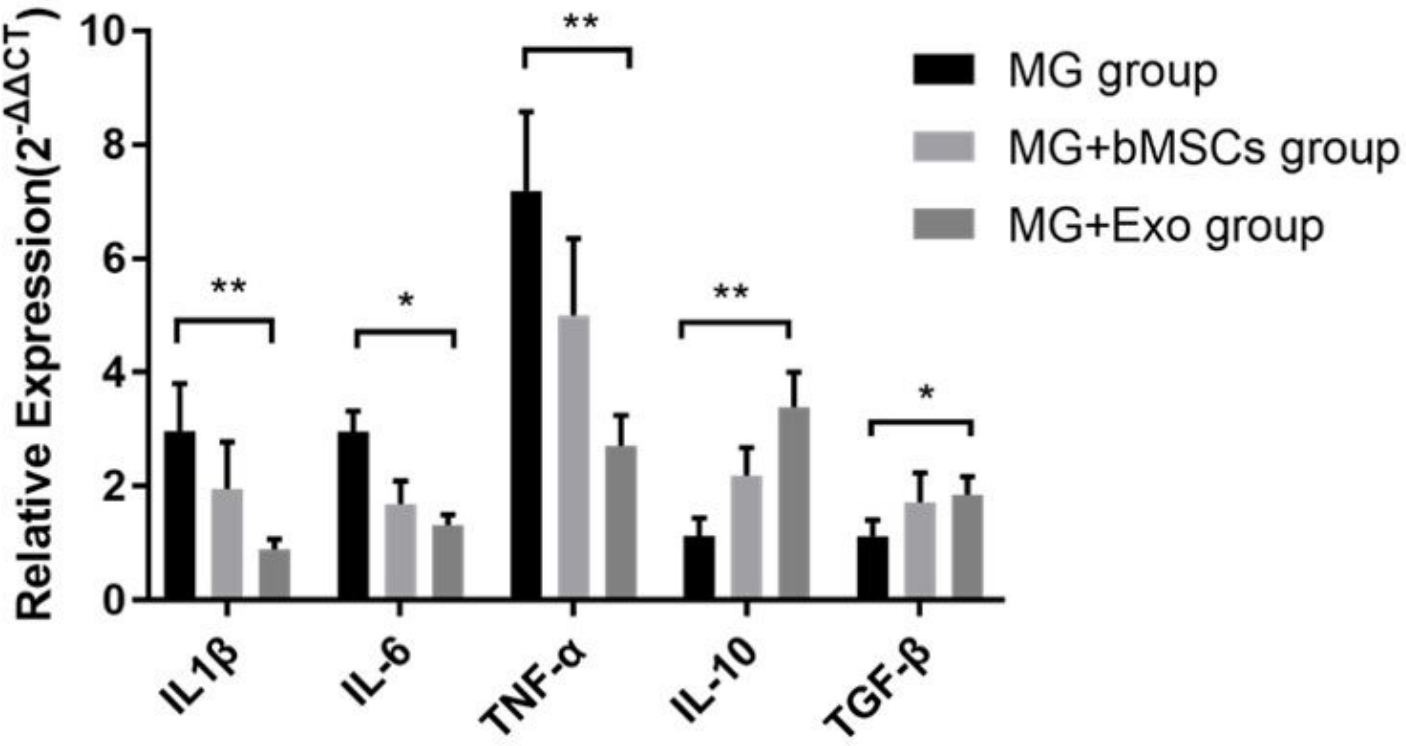

Figure 3

The expression of inflammatory factors of BV2 cells in the co-culture system. ** Differences between the three groups were all statistically significant (P<0.05). * The difference between the MG and the MG+Exo group was statistically significant (P<0.05).

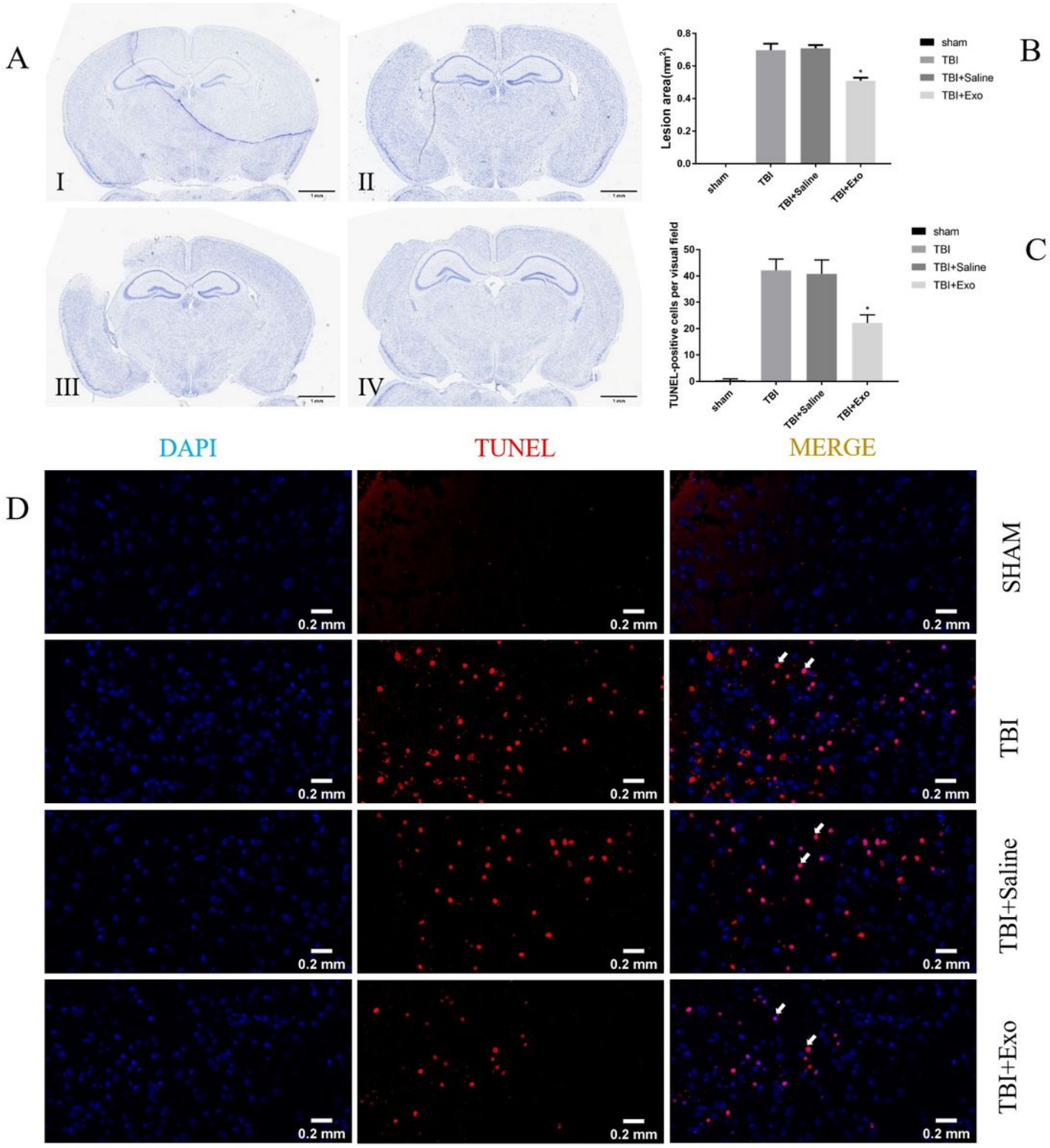

Figure 4

Exosomes induced reduction of the injured area and neuronal apoptosis. A. Nissl staining of the ⍰) sham group, ⍰) TBI group, ⍰) TBI + Saline group, ⍰) TBI + Exo group. B. The measured lesion areas of A. C. Quantification of D shows the difference of TUNEL+ cells. D. TUNEL staining of the four groups. Scale bar = 0.2 mm. *, P<0.05.

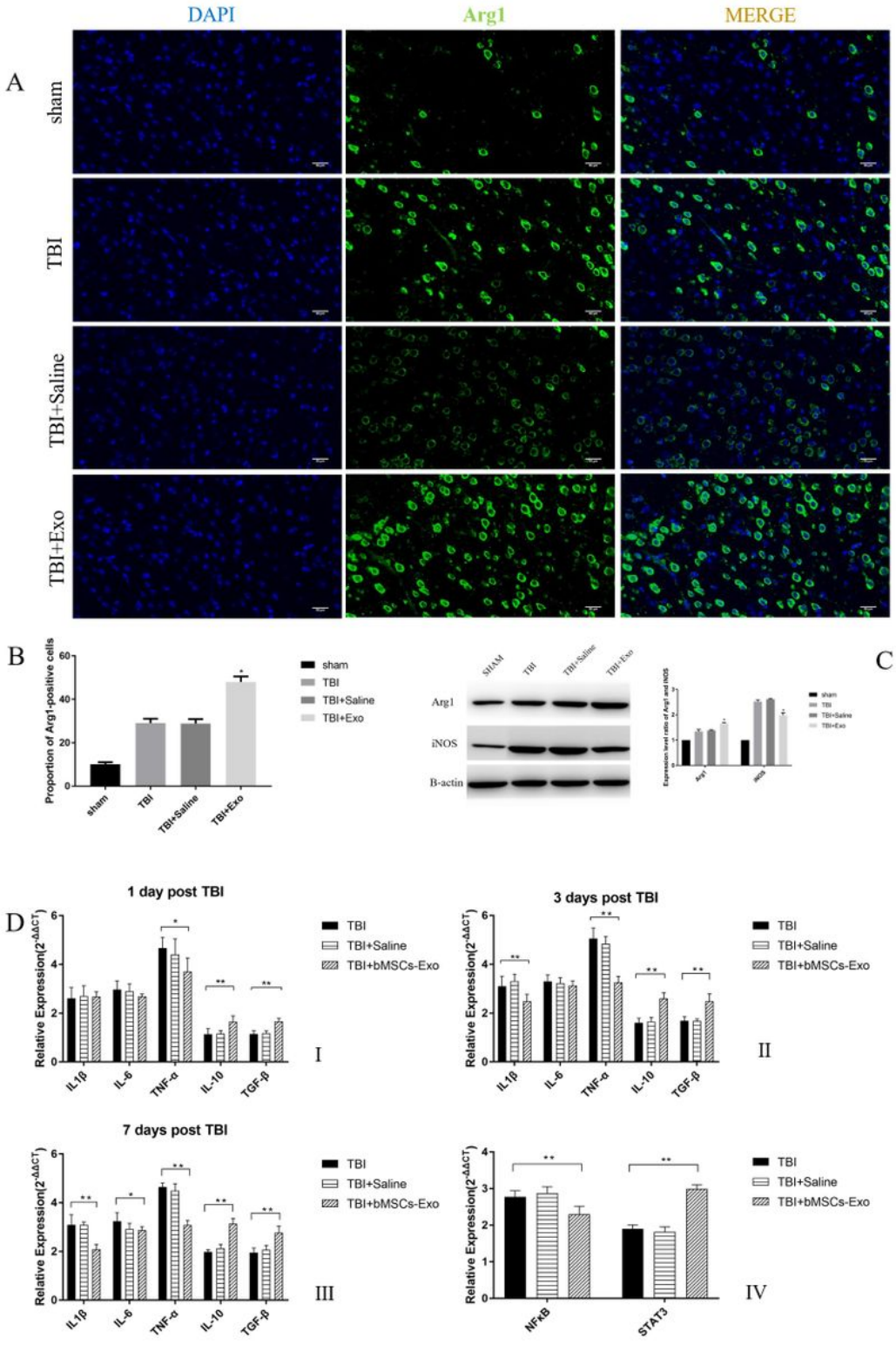

Figure 5

Effects of exosomes on Neuroinflammation. A. Immunofluorescence assay of Arg1 on brain slices 7 days post TBI. Scale bar = 200 um B. The proportion of Arg1+ cells in A. (*. P<0.05) C. The relative expression of Arg1 and iNOS tested by Western Blotting. (*. P<0.05) D. Ⅰ, Ⅱ, and Ⅲ respectively shows the expression levels of inflammatory factors on day 1, 3, and 7 post TBI (*. P<0.05 between TBI+Exo and TBI group; **.

P<0.05 between TBI+Exo and the other two groups.).) The different expression levels of NFκB and STAT3. (**.P<0.05 between TBI+Exo and the other two groups).

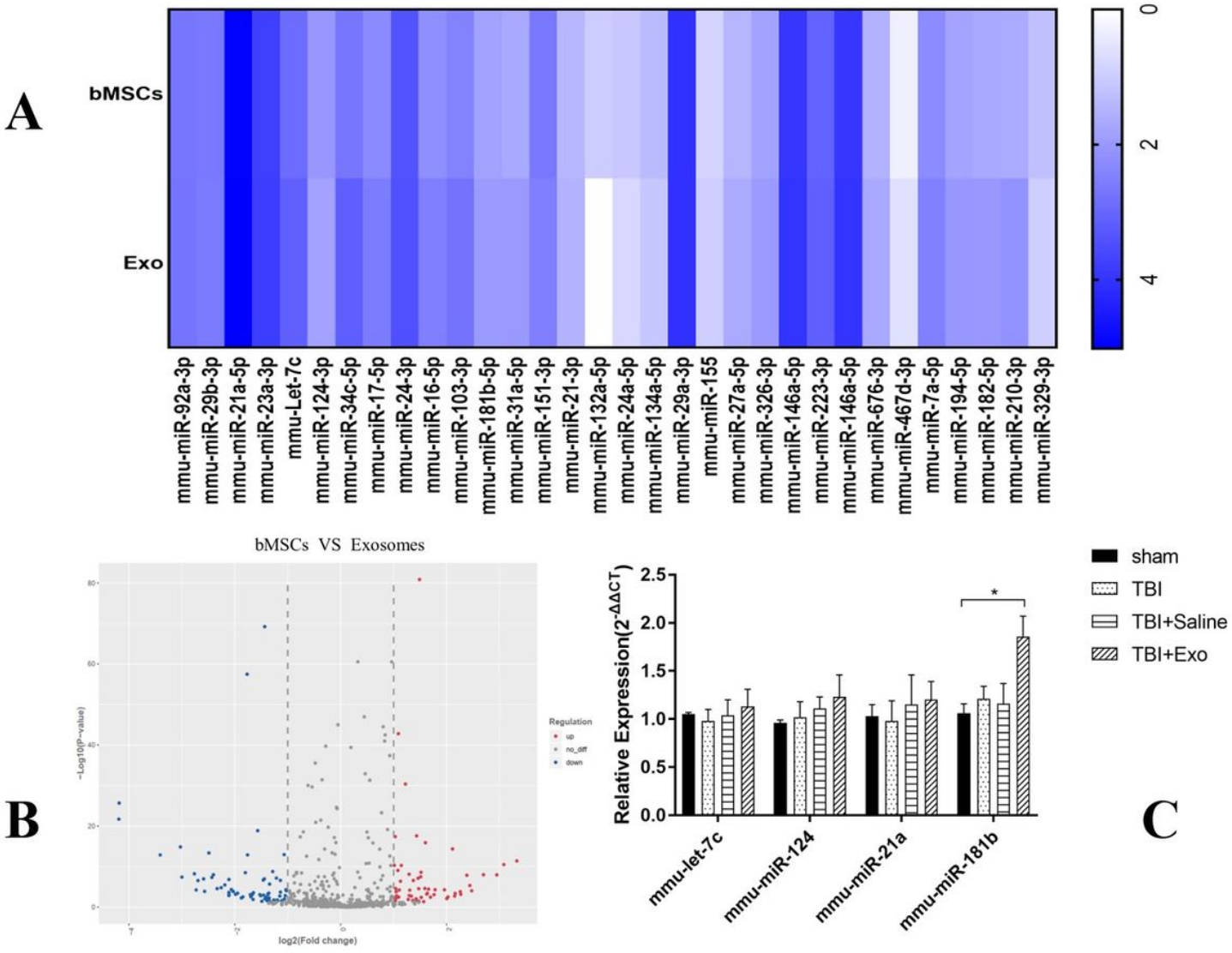

Figure 6

Differential expression of microRNAs. A. heat map of differentially expressed microRNAs between bMSCs and exosomes. B. volcano plot of the differentially expressed microRNAs (Blue represents low expression while red denotes high expression in exosomes. Gray denotes lack of differences between them.) C. The differentially expressed microRNAs in vivo. *, P<0.05

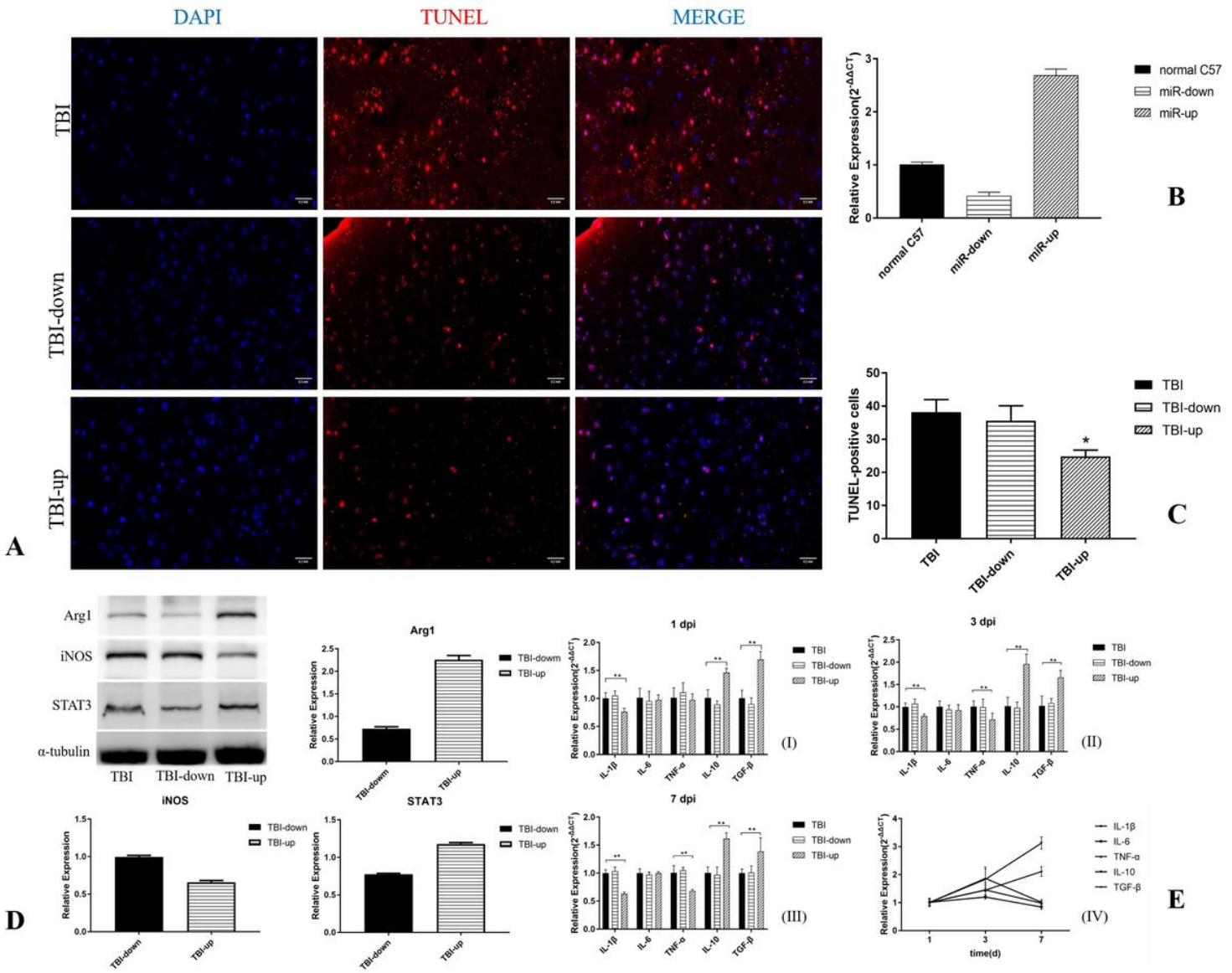

Figure 7

The effect of different expression levels of miR-181b on neuronal apoptosis and neuroinflammation. A. TUNEL staining showing the levels of neuronal apoptosis in the three groups. Scale bar = 0.2 mm. B. The expression level of miR-181b in the three groups 7 days after the transfection of lentivirus. C. Quantification of TUNEL-positive cells of A (*. P<0.05). D. The expression of microglia markers Arg1 and iNOS as well as the transcriptional regulator STAT3. E. The expression of inflammatory factors on the 1st, 3rd, and 7th day post TBI in the three group (□, □, □) (**. P<0.05 between TBI-up and the other two groups.) The dynamic changes of inflammatory factors in TBI-up group (□). Dpi: days post injury.