Umbilical cord mesenchymal stem cells and monosialotetrahexosy 1 ganglioside alleviate neuroinflammation in traumatic brain injury

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Research Article

Keywords: traumatic brain injury, umbilical cord mesenchymal stem cells, monosialotetrahexosy 1 ganglioside, Microglial polarization, neuroinflammation

Posted Date: July 15th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1833517/v1

Abstract

Neuroinflammation and activated microglia play important role in neuron damage in the traumatic brain injury (TBI). In this study, we determined the effect of human umbilical cord mesenchymal stem cells (UMSCs) combined with monosialotetrahexosy 1 ganglioside (GM1) on the neuroinflammation and polarization of microglia in a rat model of TBI, which was established in male rats using a fluid percussion brain injury device. Rats survived until day 7 after TBI were randomly treated with saline (NS), GM1, UMSCs, and GM1 plus UMSCs. Modified neurological severity score (mNSS) was assessed on days 7 and 14, and the brain tissue of the injured region was collected. Immunofluorescence, RT-PCR, and western blot analysis found that neuroinflammatory cytokines and CD163 protein expression levels in injured brain tissues was significantly decreased rats treated with GM1+UMSCs, GM1, or UMSCs were up-regulated compared with saline-treated rats. iNOS protein expression was down-regulated compared with rats treated with saline. The protein expression levels of NE, NF-200, MAP-2 and β-tubulin III were increased in the injured brain tissues from rats treated with GM1 + UMSCs, or GM1 and UMSCs alone compared with those in the rats treated with NS. The protein expression levels in rats treated with GM1 plus UMSCs was most significant on day 7 following UMSC transplantation. The rats treated with GM1 plus UMSCs had the lowest mNSS compared with that in the other groups. These data suggest that UMSCs and GM1 reduce the neuroinflammation and neurodegeneration through coordinating the polarization state of microglia to promote the recovery of neurological functions in the TBI.

Introduction

Traumatic brain injury (TBI) is the main cause of injury and disability. It is generally believed that the direct damage to nerves and brain tissue caused by primary injury is not enough to cause extremely high fatality and disability rates. The uncontrollable cascade inflammatory response in the "secondary injury" amplified, triggers immune-damaging neuroinflammation, and causes extensive and lasting damage, which leads to poor prognosis, is the key link in pathogenesis (1). Neuroinflammation has become a hot spot in the study of TBI intervention and treatment strategies. Umbilical cord mesenchymal stem cells (UMSCs)are a kind of pluripotent stem cells existing in the umbilical cord tissue, which can release various trophic factors and cell repair factors (2), relieve secondary neurodegeneration and neuroinflammation (3), and improve neurological function .Compared with other sources of stem cells, UMSCs have lower immunogenicity, faster self-renewal, stable doubling time, and strong proliferation ability, and are considered to be the first choice for transplantation therapy after brain injury (4). Monosialotetrahexosy1 ganglioside (GM1) is a glycosphingolipid containing sialic acid residues in neuronal cell membranes, which can promote neuronal maturation, differentiation and Synapse formation (5) by binding to neurotrophin receptors. Studies have shown that GM1 can induce UMSCs to differentiate into neuron-like cells and express neuron-specific surface markers in vitro. In our study, to explore the protective effect of GM1 combined with UMSCs transplantation on neurological function in traumatic brain injury rats, we determined the expression level of inhibitory inflammatory cytokines

CD163 and decreases the protein expression levels of inflammatory cytokines iNOS in injured brain tissues in the TBI rat model.

Materials And Methods

Animals. Male Sprague Dawley rats (weight, 270–330 g) were purchased from the Laboratory Animal Center of Hebei Medical University (Hebei, China). All animal experiments were performed in accordance with the Animal Management Regulations of Hebei Medical University [approval no. SYXK (Hebei) 2018-008], and were reviewed and approved by the Ethics Committee of The Second Hospital of Hebei Medical University, ethical approval reference number 2018-R051 (Hebei, China). The study was not preregistered. Simple randomization was performed with the online tool QuickCalcs from GraphPad (URL: https://www.graphpad.com/quickcalcs/). Animals were coded and assigned randomly to different groups for simple randomization (pseudorandomization) by opening: "Random numbers," "Randomly assign subjects to groups," and "Randomly choose a group for each subject " in QuickCalcs.

Cells. The human UMSCs were obtained from Shandong Qilu Cell Therapy Engineering Technology Co., Ltd. (Shandong, China) and cultured as described previously (6), In Brief, under aseptic conditions, the umbilical cord of healthy newborns was moved from term cesarean section to H-DMEM/F12 medium at 4℃, and they were subjected to the following treatment: After blood accumulation in the specimen was removed by rinsing thoroughly with D-Hank's solution and the umbilical artery and umbilical vein were removed, the remaining umbilical cord interstitial tissue was cut into 1–3 mm long tissue pieces. Then, the tissue pieces were digested by 0.2% collagenase II and cultured in H-DMEM/F12 medium containing 2ng/ml epithelial growth factor (EGF), 20% fetal bovine serum (FBS), 25mM L-glutamine (L-Glu), 100U/ml penicillin, and 100U/ml streptomycin (6). Cytomics FC500 flow cytometry (BD Biosciences) was performed to identify UCMSCs by FITC-conjugated antibodies against CD34, CD45, CD90, and CD105.

Reagents. Rabbit anti-CD163, Rabbit anti-iNOS, Rabbit anti-β-actin monoclonal antibody was purchased from Cell Signaling Technology, Inc. Fluorescein isothiocyanate goat anti-rabbit fluorescent secondary antibody was purchased from Invitrogen (Thermo Fisher Scientific, Inc.). Rabbit anti-β-tubulin , rabbit anti-microtubule-associated protein-2 (MAP-2) and rabbit anti-nestin (NE) monoclonal antibodies were purchased from Abcam. Mouse anti-neurofilament 200 (NF-200) monoclonal antibody was purchased from ProteinTech Group, Inc. Rabbit anti-β-actin monoclonal antibody was purchased from Cell Signaling Technology, Inc. Fluorescein isothiocyanate goat anti-rabbit fluorescent secondary antibody was purchased from Invitrogen (Thermo Fisher Scientific, Inc.).

TBI model. The rat fluid percussion brain injury device (MODEL-01B) was used for the TBI model, as previously described (7). Briefly, the Sprague Dawley rats were anesthetized with an intraperitoneal injection of 2% sodium pentobarbital (60 mg/kg), then a circular bone window, 5 mm in diameter was opened using a bench-type dental drill (Shanghai Dental Medical Instrument Factory) on the right-side of the skull 5 mm lateral and 5 mm posterior to the Bregma, with the meninges kept intact. The moderate to

severe TBI model was established by applying an impact pressure of 200 KPa using the fluid percussion device to the brain tissue.

During the entire experiment no animals were replaced. A blind procedure was used in this study. The specific blind procedure was that the experimenter did not know the group of animals during the experiment and during the statistical analysis. In addition, the analysis or experimental group assignment was performed by different people. Animal codes were used for analysis to perform blind quantification. No sample calculation was performed. The values above or below the mean ± 3σwere excluded from the data. The study was exploratory. The rat model of severe TBI was validated. At 3 h after severe TBI in rats, the sensory and motor function of the rats were scored using the mNSS neurological deficit score scale. If the score was greater than 10, the severe TBI model was established successfully. If the mNSS score was greater than 10 points at 3 h after TBI, these rats were included. If the mNSS score was less than 10 points, the rats were excluded.

Experimental grouping. A total of 112 rats survived 7 days after TBI and were randomly divided into groups A to D, with 14 rats in each group. Group A was the GM1 + UMSC group [10 μl injection containing 1×10^6 UMSCs in saline, into the lesion center and 10 μl injection GM1 (50 mg/kg) into the peritoneal cavity once a day for 7 days] ; Group B was the UMSC group (10 μl injection containing 1×10^6 UMSCs in saline into the lesion center and 10 μl saline injected into the peritoneal cavity, 7 days after TBI); Group C was the GM1 group [10 μl saline injected into the lesion center and 10 μl GM1 (50 mg/kg) into the peritoneal cavity, 7 days after TBI]; and Group D was the normal saline (NS) group (10 μl injection saline into the lesion center and 10 μl saline injected into the peritoneal cavity, 7 days after TBI).

Neurological function in the different experimental groups was evaluated using a modified neurological severity score (mNSS) 7 and 14 days following UMSC and/or GM1 treatment. Then, the rats were decapitated to harvest the brain tissue of the injured region following anesthesia with an intraperitoneal injection of 2% sodium pentobarbital (60 mg/kg). The brain tissue was divided into two parts, one was preserved in 4% paraformaldehyde and the other was stored in liquid nitrogen and used within 2 weeks. Immunofluorescence and western blot analysis were performed to detect the protein expression level of microglia polarization-specific markers.

Measures were taken to reduce the number of animals used and reduce animal suffering throughout the experiment. Thrombin (50-1000 units/mL) was locally perfused into the wound during surgery to stop bleeding. After surgery, tramadol (1mg/kg) was injected intramuscularly to relieve pain. Penicillin sodium (15 mg/kg) was injected intraperitoneally once a day for 7 days after surgery to avoid infection. The surgical incision was observed postoperatively. If the wound re-opened, it was treated in a timely manner.

mNSS. At 7 and 14 days following UMSC and/or GM1 treatment (8), mNSS, including functions of movement, sensation, and balance and reflex in each group of the rats with TBI, were assessed in a double-blind method.

ELISA measurement of inflammatory cytokines. At days 7 and 14 after UMSCs transplantation, the rats were sacrificed to collect brain tissue. The levels of IL-6, TGF-β, COX-2 and IDO proteins were detected by ELISA according to the kit instructions. The tissue samples and standards at different concentrations (100 μl/well) were added to the corresponding wells and incubate at 37°C for 90 min. Then, biotinylated antibodies were added into the working solution (100 μl/well) and incubate at 37°C for 60 min and enzyme conjugate working solution (100 μl/well) were added and incubate at 37°C for 30 min. Then, stop solution was added at 100 μl/well. the OD450 values were determined immediately.

Western blotting. The protein expression levels of microglia polarization-specific markers, CD163 and iNOS were detected on days 7 and 14 following UMSC transplantation. After tissue homogenization, the modified Lowry method was used to quantify the protein, which were then separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electrotransfer to a PVDF membrane. The membrane was blocked with 5% skimmed milk in TBS for 2 h at room temperature. Subsequently, the membrane was incubated with the diluted primary antibody overnight at 4˚C, then with the secondary antibody at room temperature for 1 h. The primary antibodies used were anti-CD163 (abcam, ab5392, dilution 1:1000), anti-iNOS (abcam, ab221660, dilution 1:1000), and anti- β-Actin (Cell Signaling Technology, 4970, dilution 1:1000). The target bands were detected with a super ECL western blot detection kit. The images were captured using an Image Quant LAS 4000 gel documentation system and the bands were analyzed using ImageJ (National Institutes of Health) software. The relative expression level of each protein was expressed as the gray value ratio of CD163 and iNOS to that of β-actin.

Immunofluorescence. On days 7 or 14 following UMSC transplantation, the rat brain was removed and fixed with 4% paraformaldehyde. A total of five specimens were collected from the NS, UMSC, GM1 and the UMSCs + GM1 groups, respectively. The brain tissues were frozen, embedded in optimal cutting temperature compound and cut into 10 μm-thick sections. They were washed three times with PBS (5 min each time), then blocked with 10% goat serum for 30 min. Subsequently, the samples were incubated with the primary antibody specific to the target protein in the wet box overnight at 4˚C, followed by incubation with the fluorescently labeled secondary antibody in the dark in the wet box for 30 min at room temperature the next day. The primary antibodies used were anti-CD163 (abcam, ab18207, dilution 1:300), anti-iNOS (abcam, ab5392, dilution 1:300), and anti- β-Actin (Cell Signaling Technology, 4970, dilution 1:300). Lastly, the samples were washed with TBS three times (5 min each time). DAPI was added to stain the nucleus, followed by observation under a fluorescence microscope. The fluorescence signal was quantitatively analyzed using ImageJ (National Institutes of Health) software. A total of five consecutive sections were selected and images of the five random fields of view for each section were captured under a fluorescence microscope. The average number of positive cells in the five sections was calculated, to represent the expression level of CD163 and iNOS in the injured region of the brain. In some of the rats with TBI, UMSCs labeled with PKH26 were injected into the injured site in rats in the day 14 group.

RT-PCR. 7 days and 14 days after UMSCs transplantation, the rat brain injury area was used as the experimental material, and total RNA was extracted, and cDNA was synthesized by reverse transcription. Primers were designed according to the gene sequences of β-actin, CD163 and iNOS published by GenBank (iNOS: F: GTTCCTCAGGCTTGGGTCTT ; R: TGGGGGAACACAGTAATGGC. CD163: F: GACAGACCCAACGGCTTACA; R: GGTCACAAAACTTCAACCGGA). β-actin was set as the internal reference gene, CD163 and iNOS genes were used as the detection target genes, semi-quantitative RT-PCR technology was used, and the software Image J was used to semi-quantitatively analyze the CD163 and iNOS gene expression in the damaged area of each experimental group.

Statistics analysis. All data were reported as mean ± SD. Furthermore, all statistical analyses were implemented using SPSS v21.0 (IBM Corp). One Sample Kolmogorov–Smirnov test was performed to determine normal distribution. In this study, all samples demonstrated normal distribution. Statistical significance was determined using a one-way ANOVA procedure followed by the Bonferroni post hoc test and Student's t-test with 95% confidence. The p values of less than 0.05 were considered to be statistically significant.

Results

Identification of UMSCs

We were able to successfully isolate and culture UC-MSCs from fresh umbilical cord (6). The UMSCs displayed as spindle-shaped cells crawled out of the tissue pieces when the tissue blocks were adhered to the bottom of culture flask within medium for 7–10 days. After culture for 10–14 days, the third passage of UMSCs displayed a shoal and small balls in the middle of the cells (Fig. 1A). UMSCs were identified by harvesting cells at the third passage and analyzed by flow cytometry. These cells were positive for CD105 and CD90 but negative for CD34, and CD45, and HLA-DR (Fig. 1B).

Treatment with UMSCs plus GM1 improves the recovery of neurological function in the TBI rat model. The mNSS in the rats with TBI was analyzed on days 7 and 14 after treatment with NS, UMSCs and/or GM1. The mNSS score was significantly increased in TBI rats compared with normal control rats ($P < 0.001$, Fig. 1C). Compared with that in TBI rats treated with NS, the mNSS obtained from the rats with TBI and treated with UMSCs or GM1 alone was significantly lower on days 7 and 14 ($P < 0.05$). Furthermore, the mNSS in the rats with TBI treated with GM1 plus UMSC was further lower compared with that in the rats with TBI treated with UMSC or GM1 alone ($P < 0.05$) (Fig. 1C). These data suggest that GM1 plus UMSCs improve the recovery of neurological function after TBI.

Treatment with UMSCs, GM1, or UMSCs plus GM1 increased the protein expression levels of inhibitory inflammatory cytokines and decreased the protein expression levels of inflammatory cytokines in injured brain tissues in the TBI rat model.

We determined the expression levels of cytokines which inhibit or promote neuroinflammation in injured brain tissues in the rats with TBI treated with GM1, UMSCs, or GM1 plus UMSCs using ELISA. Treating TBI

rats with GM1 and/or UMSC for days 7 and 14 significantly increased protein and mRNA levels of neuroinflammation inhibitory cytokine (transforming growth factor β (TGF-β) ($P < 0.05$, Fig. 2) but the indoleamine 2,3-dioxygenase (IDO) level had no significant change ($P > 0.05$, Fig. 2). On the other hand, treatment of TBI rats with GM1 and/or UMSC for days 7 and 14 significantly decreased protein and mRNA levels of neuroinflammation promoting cytokines IL-6 and COX-2 ($P < 0.05$, Fig. 2)

In addition, we determined the expression levels of CD163, a neuroinflammation inhibitory cytokine, and iNOS, a neuroinflammation promoting cytokine by using immunostaining, western blot and PCR analysis. On days 7 and 14 after TBI and GM1 and/or UMSC treatment, positive staining of CD163 was significantly increased and iNOS was significantly decreased in the rats with TBI treated with GM1 or UMSCs alone or in combination compared with that in the rats with TBI treated with NS (Fig. 3). Also, the protein expression levels of CD163 and iNOS in the injured tissue in rats with TBI treated with NS, GM1, or UMSCs was analyzed. Western blot and PCR analyses revealed that treatment with GM1 or UMSCs alone or a combination significantly increased the protein and mRNA levels of CD163 while decreased protein and mRNA levels of iNOS in injured brain tissues in rats with TBI compared with those in the NS group ($P < 0.05$, Fig. 4).

Treatment with UMSCs + GM1 increases the protein expression levels of neural markers in injured brain tissues in the TBI rat model. Next, the protein expression levels of the neural markers, NE, NF-200, MAP-2 and β-tubulin , in the injured tissue in rats with TBI treated with NS, GM1 and/or UMSCs was analyzed. On day 7 after treatment, western blot analysis showed that the protein expression levels of NE, NF-200, MAP-2 and β-tubulin were significantly increased in the rats with TBI treated with GM1 or UMSCs alone or in combination compared with those in the NS group (Fig. 5A **and B**). Treatment with GM1 + UMSCs significantly increased the protein expression levels of NE, NF-200, MAP-2 and β-tubulin compared with those in the rats with TBI treated with NS, GM1 or UMSCs alone ($P < 0.05$). On day 14, the protein expression levels of NE and MAP-2 in the rats with TBI treated with GM1 + UMSCs were significantly higher compared with those in the rats with TBI treated with NS, GM1 or UMSCs alone ($P > 0.05$) (Fig. 5C and D). The protein expression levels of NF-200 and β-tubulin did not differ between the rats with TBI treated with NS, GM1 or UMSCs alone or in combination.

Treatment with UMSCs + GM1 increases neuron-like cells in injured brain tissues in the TBI rat model. Subsequently, the protein expression level of neural markers in the injured brain tissues in the rats with TBI treated with GM1 or UMSCs alone, or in combination was determined using immunofluorescent staining. On day 14 after GM1 and/or UMSC treatment, positive staining of NE, NF-200, MAP-2 and β-tubulin was significantly increased in the rats with TBI treated with GM1 or UMSCs alone or in combination compared with that in the rats with TBI treated with NS (Fig. 6). The expression levels of the neural markers in the GM1 + UMSC group were comparable to those in the uninjured site (negative control).

Discussion

Traumatic brain injury (TBI) is closely related to neuroinflammation (9). Studies have shown that immune regulation significantly affects neuroinflammatory responses, inducing an inflammatory cascade involving the activation of peripheral immune cells such as neurons, glial cells, and macrophages (10). Microglia are macrophages widely distributed in the central nervous system, accounting for 10%-15% of the total number of cells in the central nervous system (11). As innate immune cells, microglia are very sensitive to environmental changes in the brain (12), and are the main effector cells of the inflammatory response in the nervous system. It plays a key role in maintaining the homeostasis of brain function and immune defense (13), and its dysfunction is closely related to the occurrence and development of various acute, chronic, and uncontrollable inflammations, Is Traumatic brain injury, ischemic and hemorrhagic brain stroke (14, 15)and other neurological diseases intervention targets.

Microglia function is closely related to its polarization state. Activated microglia have two functional phenotypes, "classically activated" (M1) and "alternatively activated" (M2), depending on their environment and surrounding stimuli. M1-type microglia can induce the activation of iNOS and NF-κB pathways to produce various pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, as well as superoxide, reactive oxygen species and nitric oxide, etc to promote inflammation (16). M2-type microglia can secrete CD163, IL-10, TGF-βand other anti-inflammatory cytokines and neurotrophic factors to inhibit inflammation and promote tissue repair (17). Several studies have confirmed that iNOS is a M1-type microglia/macrophage membrane protein marker, and CD163 is an M2-type microglia/macrophage membrane protein marker (18). INOSs are encoded by genes located in the 17th human autosome 17cen-q11.2 position (19) and are mainly in macrophages, neutrophils, endothelial cells and epithelial cells to catalyze synthesis of nitric oxide (NO). Factors such as lipopolysaccharide, endotoxin, cytokines, inflammatory mediators, injury, ischemia, ultraviolet light activate iNOS-encoding gene to promote iNOS expression for up to several hours. A large amount of NO exerts toxic effects (20). CD163 belongs to the scavenger receptor cysteine-rich (SRCR) family A1 type B group transmembrane protein receptor. In the central nervous system, it is only expressed in perivascular cells and meningeal microglia/macrophages.

Studies have shown that UCMSCs can survive for a long time after allogeneic intracerebral transplantation(21), and the therapeutic effect of MSCs is attributed to their paracrine effect(22, 23). Its role in promoting regeneration is mainly related to the release of various nutritional factors and cell repair factors from its secretory (24, 25). In our experiment, a TBI rat model was created, and UMSCs were transplanted into the penumbra around the injured area by stereotaxic microinjection, and then TBI rats were treated with GM1 by intraperitoneal administration. The effect of GM1 combined with UMSCs on TBI rats was observed. Studies have shown that endogenous neural stem cell (NSCs) began to increase on the 1st day after TBI, reached a peak on the 7th day, and gradually decreased on the 14th, 21st, and 28th days (26). The homing peak of EPCs induced by many cytokines is also around 7 days, at which time EPCs are most involved in damage repair, suggesting that this time point is the best intervention time window to improve the proliferative capacity of endogenous NSCs (27, 28). Therefore, in our study, GM1 + UMSCs combined therapy was given 7 days after TBI. In the preliminary experiment, the microglial polarization state markers iNOS and CD163 were detected by immunofluorescence, RT-PCR and Western Blot 28 days after UMSCs transplantation. The results showed that there was no significant expression in

each group. No statistically significant difference. Therefore, this study focused on the detection of the intervention effect on 7d and 14d after UMSCs transplantation.

Days 7 and 14 after UMSCs transplantation, western blot, RT-PCR and immunofluorescence results showed that CD163 protein expression in GM1 + UMSCs group, GM1 group and UMSCs group was up-regulated compared with NS group, iNOS protein expression was down-regulated compared with NS group, The protein expression in GM1 + UMSCs group was the most significant, and the expression changes was the most obvious at 7d after transplantation, the mNSS score of the GM1 + UMSCs group was the lowest, and the recovery of neurological function was the most obvious. This indicates that GM1 and UMSCs may synergistically reduce the secondary neurodegeneration and neuroinflammation in TBI by regulating the polarization state of microglia. Our previous study showed that GM1 could effectively induce UMSCs to differentiate into neuron-like cells under specific culture conditions (6). The latest research shows that exogenous GM1 can increase GalNAcT synthesis through a "positive feedback" mechanism, thereby promote the differentiation of neural stem cells and produce more GM1 (29). One of the main functions of MSCs for nerve repair is the direct secretion or paracrine secretion of biologically active fibroblast growth factor-2 (FGF2), nerve growth factor, transforming growth factor-b1 (TGFb1) and Brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF)(26)and other cytokines, as well as macrovesicles and vesicles(30). which Providing adaptive conditions for the complex microenvironment of protein replication and secretion (31, 32), reprograming nascent cells, triggering endogenous proliferation and differentiation of neural progenitor cells, and playing a important effects on neuronal survival, differentiation and neurite outgrowth (33–35), conductting immune inflammatory regulation and promotting angiogenesis in ischemic tissue, reducing secondary neurodegeneration and neuroinflammation and promoting repair of damaged areas (36).

In the present study, the expression changes of each protein in the GM1 + UMSCs group were the most significant around day 7 after UMSCs transplantation, and the expression changes were significantly decreased at day 14, and no significant expression was found at 28d. Studies have shown that the incubation period of UMSCs is 12–24 hours, then enters the logarithmic proliferation phase after 2 days, which lasts for 6–8 days. During this period, the cells have vigorous mitosis and the strongest proliferation ability, and then enter the plateau phase and stop the growth. According to the three time points studied by the author, it is suggested that 7d may be the best time window for the intervention effect of GM1 combined with UMSCs on TBI rats, and the effect gradually weakened on the 14th and 28th day. The author believes that the combined treatment of GM1 and UMSCs at 7d after TBI has the best intervention effect at 7d after transplantation. which Promotes differentiating into functional nerve cells, migrating to the injured area, secreting various trophic factors and cell repair factors, and promotes the recovery of nerve function and improves prognosis. The latest research shows that preconditioning secretome in TBI may be a novel and safer method of neural repair (37).

Nestin, a cytoskeletal protein belonging to the VI intermediate filament proteome, regulates cell proliferation, migration, polarity and morphology, and plays an important role in characterizing cell homeostasis and damage repair (38). Therefore, it is considered to be a specific marker of pluripotent

neural stem cells/progenitor cells (39). β-tubulin forms a heterodimer with α-tubulin to participate in the construction of the microtubule system, as well as plays an important role in cell proliferation, division, movement and migration, signal transduction and development, maintenance of cell structure, and the maturation of axons and dendrites (40). It is one of the earliest neuron markers expressed in primitive neuroepithelium and a unique neuron differentiation marker, which is widely used in neurobiological research (40–42). MAP-2 is mainly present in the dendrites and axons of neurons, and is involved in the growth of neurites, transportation of cytoplasmic proteins and neuron shaping, and stabilization of the microtubules and dendrites (43–45). It has been shown that MAP-2 can be used as a neuron-specific marker to reflect the degree of neuronal cell damage (45). NF-200, a neuron-specific intermediate filament protein, constitutes the main component of the cytoskeleton in the neuron cell body and axon (46). It contains a neurofilament heavy chain, which plays a key role in the maturation of neuronal axons (47). NF-200 interacts with actin for maintaining the structure of neurons (48) and is used as a marker for the damage degree of neurons (49).

In the present study, UMSCs were transplanted into the penumbra around the injured region of the brain via a stereotactic microinjection, then GM1 was administered to the rats with TBI via intraperitoneal administration. The protein expression level of the neuron-like cell specific markers, NE, β-tubulin , NF-200 and MAP-2, was observed in the rats with TBI treated with a combination of GM1 and UMSCs. Studies have shown that endogenous neural stem cells start to increase on day 1 following TBI and reaches a peak on day 7, then gradually decreases on days 14, 21 and 28 (26). Gong *et al* (27) found that the numerous cytokines produced following TBI promoted the homing of endothelial progenitor cells (EPCs) and peaked at day 7, when most of the EPCs participated in damage repair, suggesting that this time point was the optimal time window for intervention to improve the proliferation of endogenous neuronal stem cells (28). Therefore, GM1 + UMSCs was administered at day 7 following TBI. In the preliminary experiment, immunofluorescence and western blot analysis were initially performed to detect the neural markers, NE, β-tubulin , NF-200, β-tubulin and MAP-2, on day 28 following UMSC transplantation. However, there was no significant expression in each group and no statistically significant difference. Therefore, the present study focused on the intervention effects on days 7 and 14 following UMSC transplantation.

It was found that GM1 or UMSC treatment reduced the mNSS and increased the protein expression level of NE, NF-200, MAP-2 and β-tubulin in rats with TBI. Furthermore, treatment with GM1 + UMSCs induced a higher increase in these parameters as compared with that in the rats treated with GM1 or UMSCs alone. In addition, it was found that the changes in protein expression were more notable on day 7 compared with that on day 14 following UMSC transplantation. There was the lowest and the most notable recovery of neurological function in the GM1 + UMSCs group. These findings suggest that GM1 and UMSCs may act synergistically to promote the recovery of TBI. Our previous studies have shown that GM1 could effectively induce UMSCs to differentiate into neuron-like cells under specific culture conditions (6). The latest research shows that exogenous GM1 could increase the synthesis of GalNAcT via a "positive feedback" mechanism, thereby promoting the differentiation of neural stem cells and producing more GM1 (29). GM1 could epigenetically promote the differentiation of neural stem cells into

neurons via enhanced histone acetylation (50). The main functions of stem cells in nerve repair is cell replacement therapy (51), that is, to replace damaged and diseased cells via the differentiation and proliferation of stem cells and rebuild neural circuits. We found that the protein expression level of the neuron-like cell markers, NE, NF-200, MAP-2 and β-tubulin , in the GM1 + UMSC group was significantly increased, which might be attributed to UMSC replacement therapy. Of course, the high expression level of the neuron-like cell-related markers may be associated with various cytokines with biological activity via direct or paracrine secretion following UMSC transplantation, which activates endogenous neuronal stem cells for immuno-inflammatory regulation and promotion of angiogenesis in ischemic tissues, thereby promoting the differentiation of neuron-like cells to repair damaged tissues (36). Makri *et al* (52) found that GFAP and NSE were expressed in the rat brain tissue following UMSC transplantation, indicating that UMSCs could survive and differentiate in the injured region of rat brain, and become involved in the repair of the injured region by neuron-like or glial-like cells.

In the present study, the expression of proteins in the GM1 + UMSC group was the most significant on day 7 following UMSC transplantation and was significantly reduced on day 14. No significant difference was found in the protein expression level of NF-200 and β-tubulin ($P > 0.05$), and there was no significant expression of various proteins on day 28 (data not shown). Studies have shown that the culture period of UMSCs was 12–24 h. After day 2, they enter the logarithmic phase, which lasts for 6–8 days. During this period, the cells undergo vigorous mitosis and the strongest proliferation ability. Then, the cells enter the plateau phase and the growth stops. According to the three time points, day 7 might be the optimal time window for the intervention with GM1 combined with UMSCs in rats with TBI. The effect was gradually reduced on days 14 and 28. We hypothesized that the combined treatment of GM1 and UMSCs on day 7 after TBI could be the optimal intervention effect. At this time point, the UMSCs were more notably differentiated into functional nerve cells, migrated to the injury region and replaced damaged cells, more significantly promoted the recovery of nerve function and improved the prognosis. In animal studies, intraperitoneal administration of GM1, at 50 mg/kg, significantly reduced autophagy, neurological dysfunction and ischemic infarction without notable side effects (6). However, in a clinical setting, GM1 is used to treat patients with trauma at 100 mg per day for 2 to 3 weeks. The clinically used dosage is less that that used in rodents. It is not clear if the dose of GM1 at 50 mg/kg is achievable in the clinic and could produce similar effects as shown in rodents. Thus, future studies are warranted to investigate the discrepancy in the dosage of GM1 in rodents and humans.

However, the present study has several limitations. First, it is not clear if the optimal effect of GM1 combined with UMSCs was observed within the 7-day window following TBI. Further verification will be required to determine whether there is a difference in the protein expression level at 24 and 72 h, and 7 days following treatment with a combination of GM1 and UMSCs. Second, related biological approaches were not performed to analyze the signal pathways activated during tissue recovery following TBI. Third, it is not clear whether the beneficial effect of UMSCs and GM1 in combination is specific to TBI or not. The effect of this combination will be investigated on neurodegeneration using rodent models of hypertensive cerebral hemorrhage or glioma. Therefore, additional research is required to confirm its safety and feasibility in clinical application.

Declarations

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (grant no. 81870984), the National Key R&D Program Intergovernmental Cooperation on International Scientific and Technological Innovation of the Ministry of Science and Technology of China (grant no. 2017YFE0110400), the Hebei Natural Science Foundation General Project–Beijing-Tianjin-Hebei Basic Research Cooperation Project (grant no. H2018206675), the Special Project for the Construction of Hebei Province International Science and Technology Cooperation Base (grant no. 193977143D), the Projects on Training of outstanding Clinical Medical Personnel and Basic Research Projects of Hebei Province in 2017 and 2019.

Funding

The present study was funded by the National Natural Science Foundation of China (grant no. 81870984), the National Key R&D Program Intergovernmental Cooperation on International Scientific and Technological Innovation of the Ministry of Science and Technology of China (grant no. 2017YFE0110400), the Hebei Natural Science Foundation General Project–Beijing-Tianjin-Hebei Basic Research Cooperation Project (grant no. H2018206675), the Special Project for the Construction of Hebei Province International Science and Technology Cooperation Base (grant no. 193977143D), the Projects on Training of outstanding Clinical Medical Personnel and Basic Research Projects of Hebei Province in 2017 and 2019.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on a reasonable request. YF, SK, and ZZ confirm the authenticity of all the raw data during this study.

Authors' contributions

Conception and study design, Y.F., W.Q., X.L., and Z.Z.; Data collection, Y.F., W.Q., X.L., Q.J., H.Y., N.Y., J.Z.; Data analysis and interpretation, Y.F., W.Q., X.L., J.Z., and Z.Z.; Collection of materials and samples, N.Y., J.Z. Y.T. and Y.F.; Drafting manuscript, Y.F., W.Q., X.L., and Z.Z.; All the authors have read and approved the final manuscript.

Ethics approval and consent to participate

The experimental procedure was approved by the Animal Ethics Committee of Hebei Medical University (#SYXK (Hebei) 2018-008) and the Ethics Committee of The Second Hospital of Hebei Medical University 2018-R051 .

Patient consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

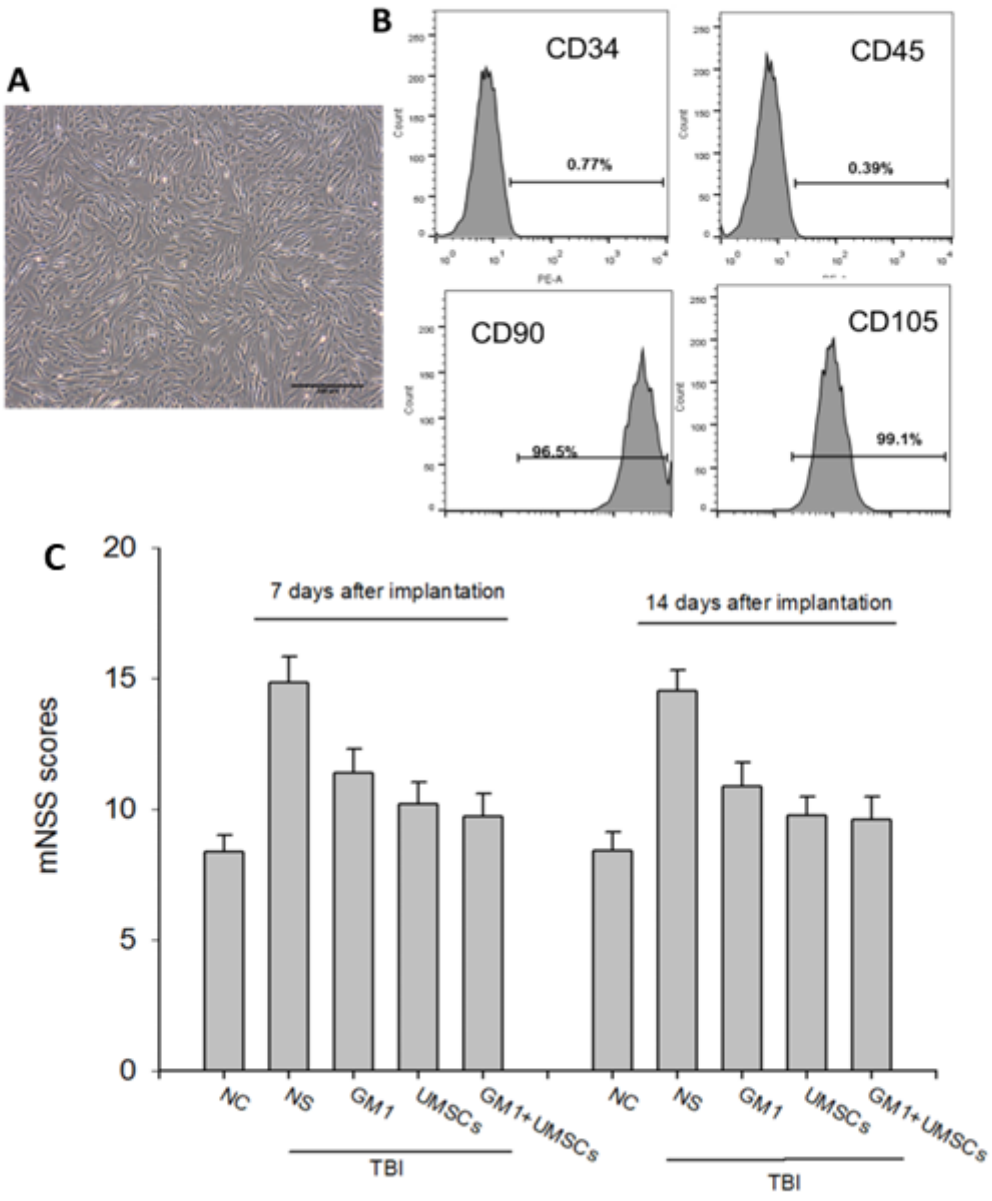

Figure 1

Identification of UMSCs. (A) Morphologic features of cultured UMSCs; (B) surface antigen labels of UMSCs. Treatment with GM1, UMSCs or GM1 plus UMSCs improved neurological function in the TBI rat model. Modified neurological severity score (mNSS) were determined in rats with different treatment. Please note that TBI rats had significant higher mNSS value compared with normal rats. Treatment of TBI rats with GM1, UMSCs or GM1 plus UMSCs significantly reduced mNSS values. Data were shown as mean ± SD and evaluated by one-way ANOVA followed by Tukey's post hoc test. * $P < 0.05$, and *** $P < 0.001$ compared with values in NC group. $P < 0.05$ was considered statistically significant. UMSCs,

umbilical cord mesenchymal stem cells; GM1, monosialotetrahexosy 1 ganglioside; TBI, traumatic brain injury.

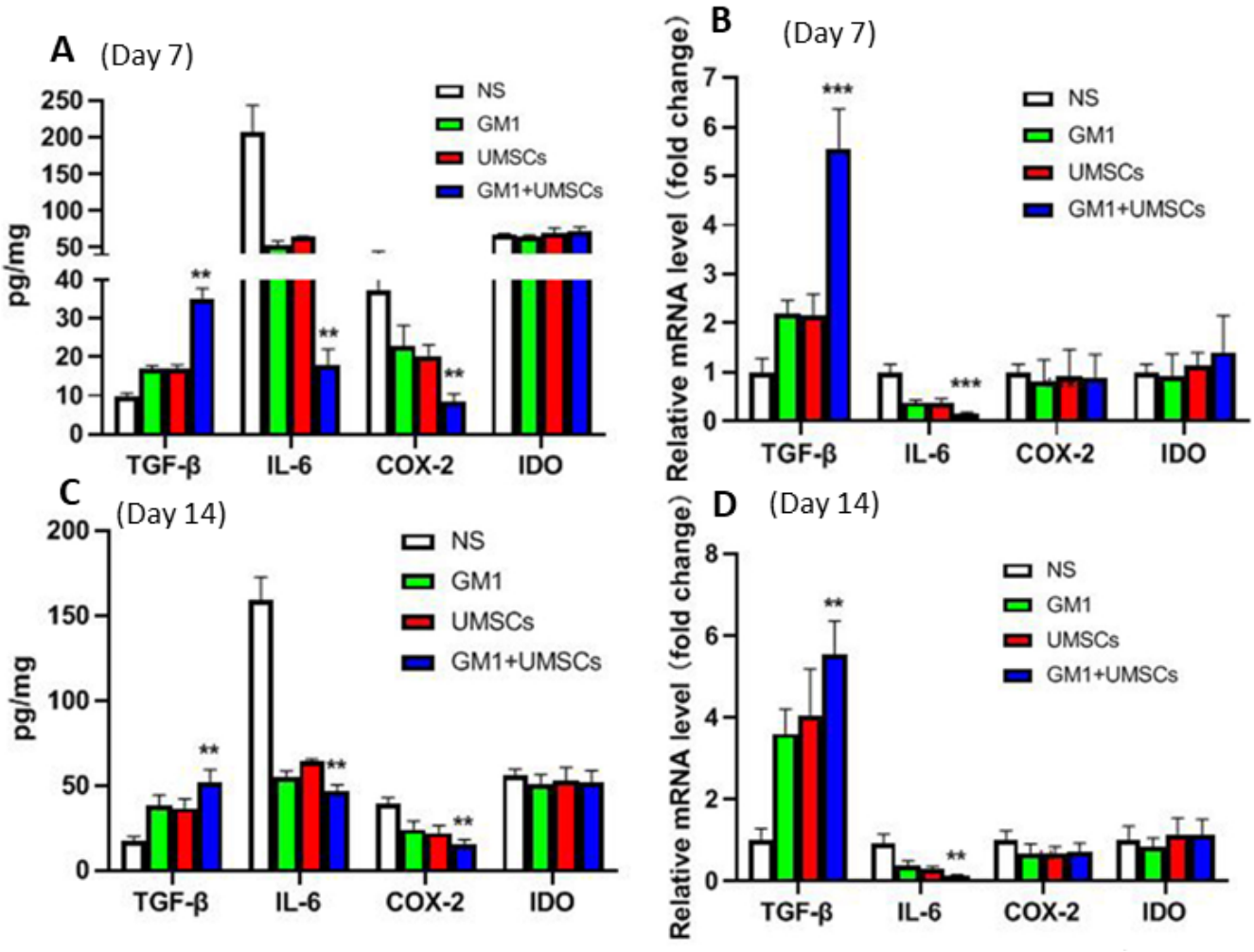

Figure 2

Effect of GM1, UMSCs or GM1 + UMSCs on the expression of neuroinflammation cytokines. Treatment with GM1, UMSCs or GM1 + UMSCs increased the TGP-β and decreased IL-6 and COX-2 in injured brain tissues on days 7 and 14 following treatment in rats with TBI. UMSC, umbilical cord mesenchymal stem cells; GM1, monosialotetrahexosy 1 ganglioside; TBI, traumatic brain injury.

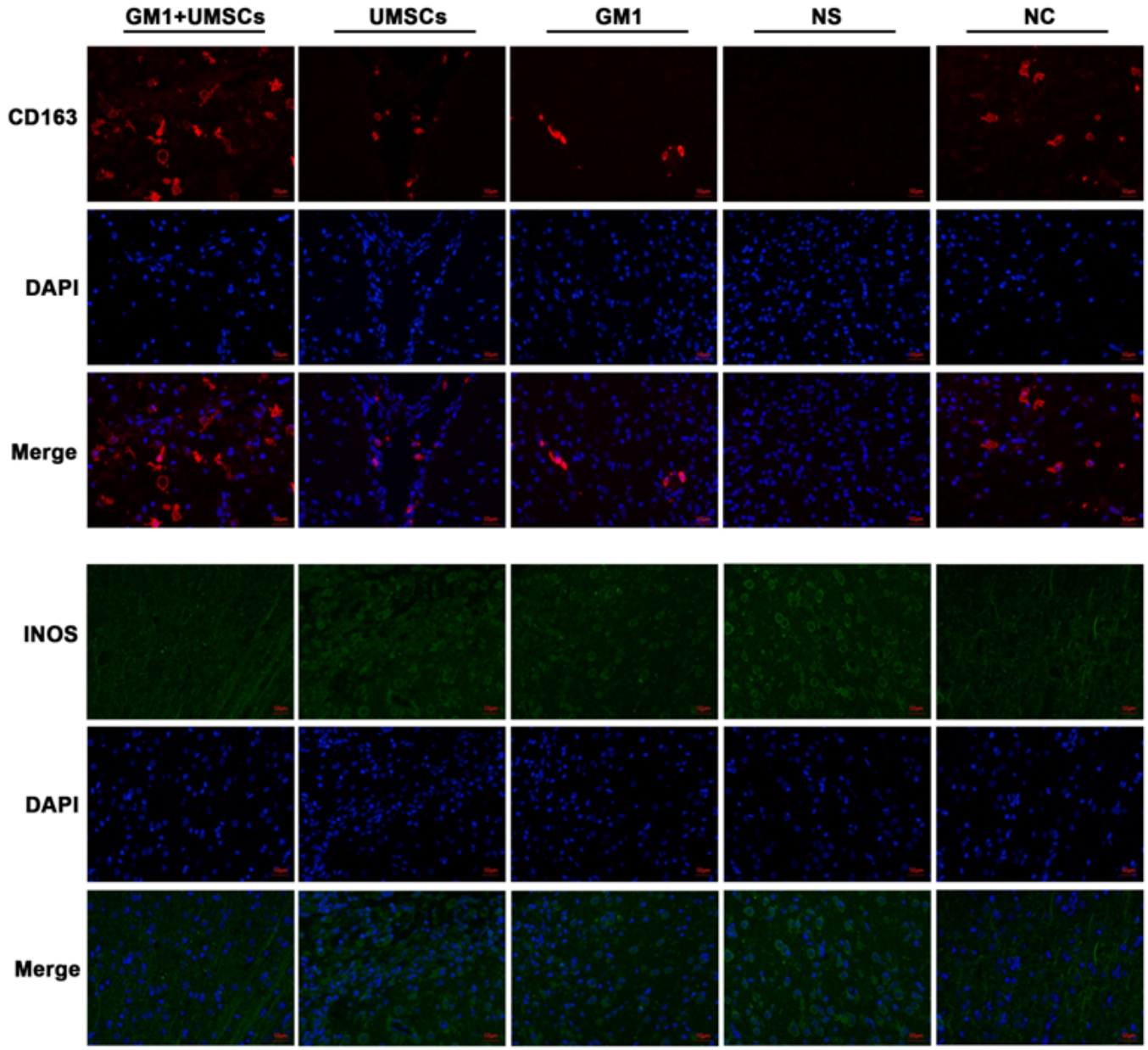

Figure 3

Effect of GM1, UMSCs or GM1 + UMSCs on the expression of neuroinflammation markers. Treatment with GM1, UMSCs or GM1 + UMSCs increased the **number of positive cells** for **CD163** and decreased iNOS in injured brain tissues on days 7 and 14 following treatment in rats with TBI. Please note that **CD163** immunostaining signals were increased **and iNOS** immunostaining signals were decreased on days 7 and 14 following treatment with GM1, UMSCs or GM1 plus UMSCs in TBI rats. UMSC, umbilical cord mesenchymal stem cells; GM1, monosialotetrahexosy 1 ganglioside; TBI, traumatic brain injury.

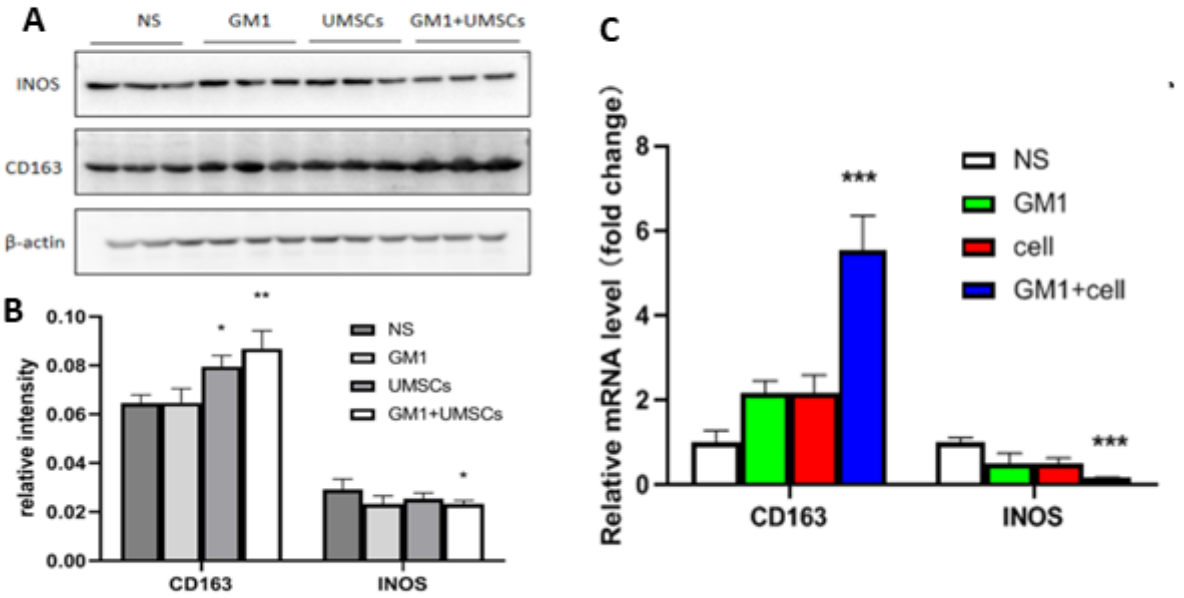

Figure 4

Neuroinflammation marker expression levels were altered in response to treatment with GM1, UMSCs or GM1 plus UMSCs in injured tissue in TBI rats. Expression levels of protein (A and B) and mRNA (C) for CD163 and iNOS in injured brain tissues in control and TBI rats treated with GM1, UMSCs or GM1 plus UMSCs on days 7 and 14 after treatment. Data were shown as mean ± SD and evaluated by one-way ANOVA followed by Tukey's post hoc test. * $P < 0.05$, and ** $P < 0.01$ compared with values in NC group. UMSC, umbilical cord mesenchymal stem cells; GM1, monosialotetrahexosy 1 ganglioside; TBI, traumatic brain injury.

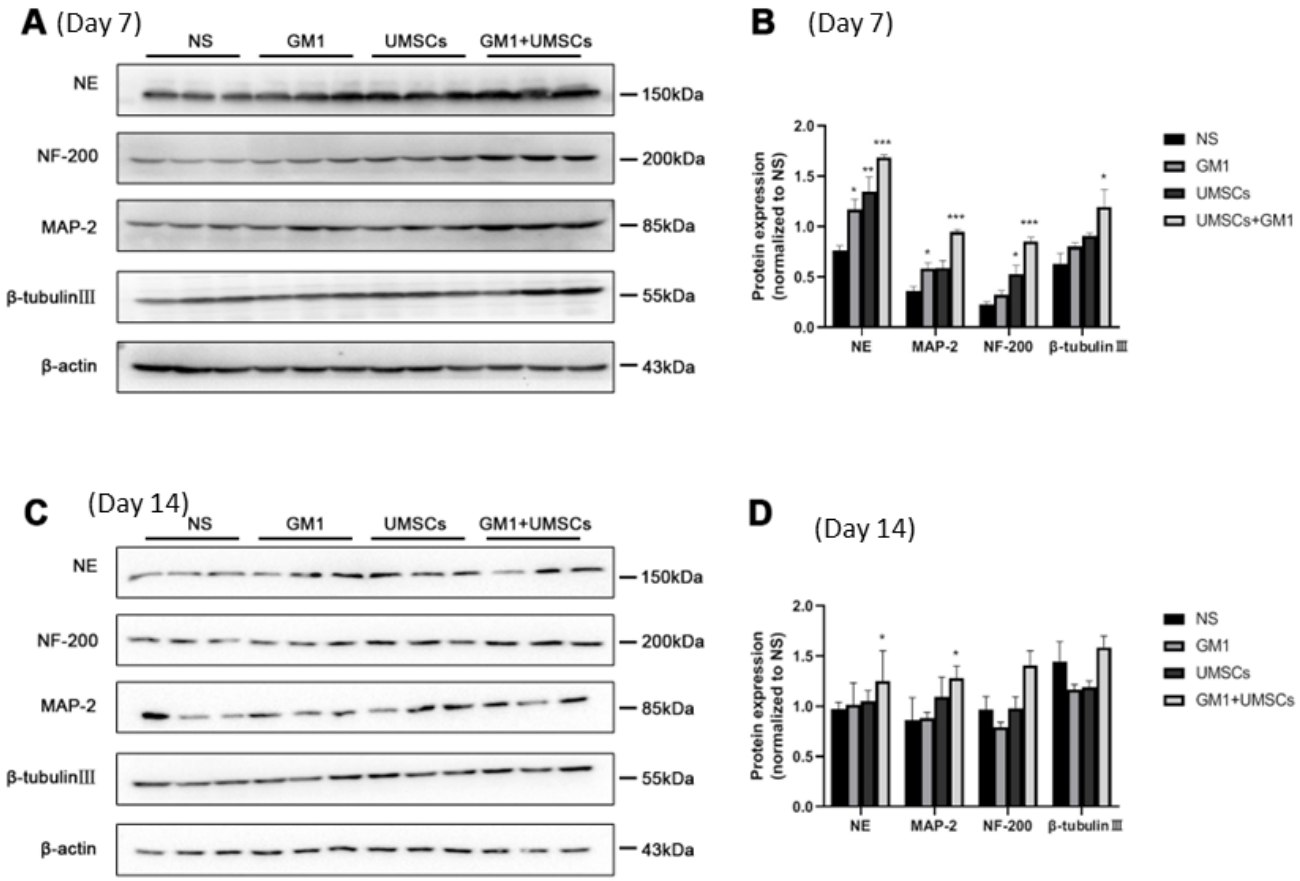

Figure 5

Treatment with GM1, UMSCs or GM1 + UMSCs increases the protein expression levels of the neural markers, NE, β-tublin III, NF-200 and MAP-2 in rats with TBI. Western blot analysis was performed 7 days following TBI to determine the effect of GM1, UMSCs or GM1 + UMSCs on the protein expression levels of the neural markers in the TBI injured tissue. MAP-2, microtubule-associated protein-2; NF-200, neurofilament 200; UMSC, umbilical cord mesenchymal stem cells; GM1, monosialotetrahexosy 1 ganglioside; TBI, traumatic brain injury; NE, nestin. * $P < 0.05$, ** $P < 0.01$; and *** $P < 0.001$ compared with values in NS group. $P < 0.05$ was considered statistically significant.

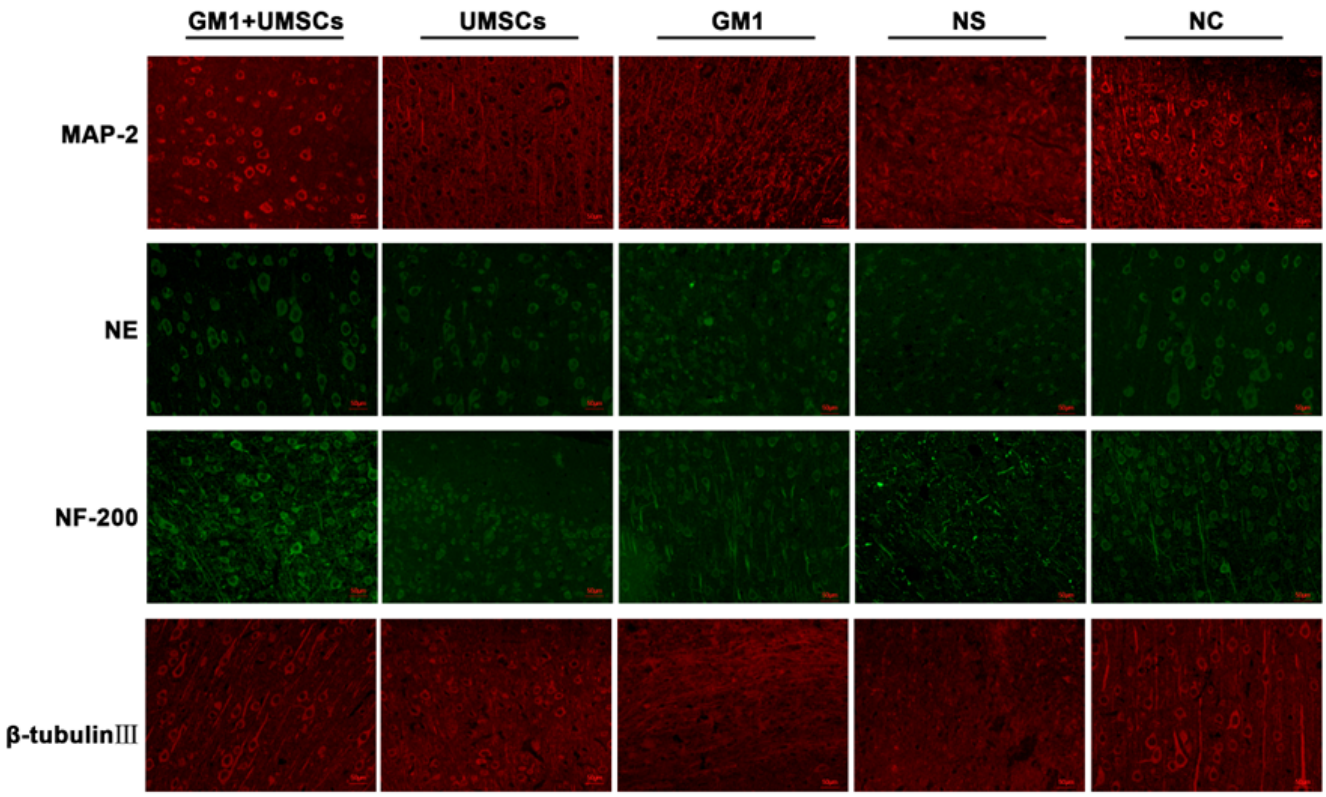

Figure 6

Effect of GM1, UMSCs or GM1 + UMSCs on the expression of neural markers. Treatment with GM1, UMSCs or GM1 + UMSCs increase the number of positive cells for NF-200, MAP-2, NE and β-tubulin on day 7 following treatment in rats with TBI. MAP-2, microtubule-associated protein-2; NF-200, neurofilament 200; UMSC, umbilical cord mesenchymal stem cells; GM1, monosialotetrahexosy 1 ganglioside; TBI, traumatic brain injury; NE, nestin.