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Original Article

Modulation of parietal cytokine and chemokine gene profiles by mesenchymal stem cell as a basis for neurotrauma recovery

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KEYWORDS
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Background & purpose: Following traumatic brain injury (TBI), primary mechanical injury to the brain may cause blood-brain-barrier damage followed by secondary injury, ultimately culminating in cell death. We aimed to test whether one injection of mesenchymal stem cells (MSC) derived from the human umbilical cord can modulate brain cytokine and chemokine gene profiles and attenuate neurological injury in rats with TBI.
Methods: One-day post-TBI, the injured rats were treated with one injection of MSC (4×10^6/rat, i.v.). Three days later, immediately after assessment of neurobehavioral function, animals were sacrificed for analysis of neurological injury (evidenced by both brain contusion volume and neurological deficits) and parietal genes encoding 84 cytokines and chemokines in the injured brain by qPCR methods.
Results: Three days post-TBI, rats displayed both neurological injury and upgrade of 11 parietal genes in the ipsilateral brain. One set of 8 parietal genes (e.g., chemokine [C-X-C motif]

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ligand 12, platelet factor 4, interleukin-7, chemokine [C-C motif] ligand (CCL)19, CCL 22, secreted phosphoprotein 1, pro-platelet basic protein 1, and CCL 2) differentially upgraded by TBI was related to pro-inflammatory and/or neurodegenerative processes. Another set of 3 parietal genes up-graded by TBI (e.g., glucose-6-phosphate isomerase, bone morphogenetic protein (BMP) 2, and BMP 4) was related to anti-inflammatory/neuroregenerative events. Administration of MSC attenuated neurological injury, down-regulated these 8 parietal pro-inflammatory genes, and up-regulated these 3 parietal anti-inflammatory genes in the rats with TBI.

Conclusion: Our data suggest that modulation of parietal cytokines and chemokines gene profiles by MSC as a basis for neurotrauma recovery.

Introduction

According to a recent overview of neuroinflammation after traumatic brain injury (TBI), primary mechanical injury to the brain may cause cell membrane disruption, endothelial rupture, and blood—brain barrier disruption immediately after the injury.[1] During the period of 1—3 days following the primary mechanical injury, secondary injury reaction may ensue as ionic imbalance, the release of excitatory amino acids, calcium overload, and mitochondrial dysfunction, ultimately culminating neurodegeneration. Primary and secondary injury lead to the release of cytokines, chemokines, activation of microglia and astrocytes, and infiltration of circulating immune cells. The primary injury is irreversible and amenable only to preventive measures to minimize the extent of damage. The secondary injury leads to a variety of neuroimmune and neuroinflammatory responses aimed to restore the homeostasis of the damaged brain tissues, which, if not controlled, will lead to neurodegeneration as well as neurological dysfunction. Post-traumatic neuroinflammation is characterized by secretion of mediators such as cytokines and chemotactic cytokines (chemokines),[2] and now considered to have both beneficial and detrimental roles. However, it remains unclear whether the occurrence of secondary injury is associated with an upgrade or a downgrade of pro- and/or anti-inflammatory cytokines and chemokines in injured brain tissues.

Mesenchymal stem cells (MSC) can be derived from different sources, including bone marrow, adipose tissue, the umbilical cord or the umbilical cord blood, and placenta.[3] In addition to their multilineage differentiation potential, MSC may exert their regenerative effect via the production of multiple paracrine factors.[4,5] A previous study has demonstrated that systemic transplantation of MSC derived from the human umbilical cord blood improves outcomes of TBI in rats by stimulating angiogenesis and neurogenesis.[6] However, it remains unclear whether systemic administration of MSC derived from the human umbilical cord suppresses the cerebral expression of pro-inflammatory genes and enhances that of anti-inflammatory genes, while reducing neurobehavioral deficits and brain contusion volume.

Therefore, in the present study, we evaluated the therapeutic effects of MSC derived from the human umbilical cord in rats with TBI. We also performed quantitative real-time PCR (qPCR) to determine which cytokines and chemokines genes in cerebral tissue ipsilateral to the trauma were significantly upregulated after TBI. Finally, we examined whether MSC attenuate neurological injury in TBI rats by modulating the brain expression of chemokines and cytokines genes.

Methods

Fluid percussion-induced traumatic brain injury model and sham surgery

The Animal Ethics Review committee of the Chi Mei Medical Center approved all procedures performed in the present study (IACUC104042801). Adult male, specific pathogen-free Sprague Dawley rats (aged 10 weeks and weighing 350—385 g) were obtained from BioLASCo Taiwan Co., Ltd. (Taipei, Taiwan) and housed at the Central Animal Facility of the Chi Mei Medical Center. The animals were housed in groups of four at an ambient temperature of 22 ± 1 °C and under a 12h light—dark cycle, and fed with rat chow pellets and tap water *ad libitum*.

The experimental TBI model was established by fluid percussion injury in anesthetized rats, as detailed previously.[7] Rats were anesthetized with an intraperitoneal injection of a mixture of ketamine hydrochloride (Ketalar, 50 mg/kg; Pfizer, New Taipei City, Taiwan), atropine sulfate (1 mg/kg; Tai Yu Chemical & Pharmaceutical Co. Ltd., Hsinchu, Taiwan), and xylazine hydrochloride (Rompun, 5 mg/kg; Bayer, Leverkusen, Germany). Each rat was placed in a stereotaxic frame and a median incision was made to expose the parietal bone. A 3 mm circular craniotomy was performed 3 mm posterior to bregma and 4 mm right lateral to the sagittal suture, without injuring the dura mater. A modified Luer-lock connector (trauma cannula) with an inner diameter of 2.6 mm was secured in the craniotomy with cyanoacrylate adhesive and dental acrylic. A moderate percussion (2.2 atm) was produced by rapidly injecting a small volume of saline into the closed cranial cavity with a fluid percussion device (VCU Biochemical Engineering, Richmond, VA, USA). The rat was removed from the device, then the acrylic was removed and the incision

was sutured. Temperature, respiratory rate, oxygen saturation, and heart rate were monitored during the surgery with a small-animal Oximeter (Mouse STAT Pulse Oximeter & Heart Rate Monitor Module; Kent Scientific, Torrington, CT, USA). After surgery, the rat was placed in a warm cage and allowed to awaken; the rat was then given free access to food and water. Buprenorphine (0.05 mg/kg every 12 h for 2 days, subcutaneously; Sigma–Aldrich, St. Louis, MO, USA) was used for postoperative analgesia. For sham surgery, animals underwent the same surgical procedures except for fluid percussion injury and received the same postoperative care. Each injured or sham-injured rat was housed individually, and behavioral recovery was closely evaluated immediately following TBI.

HUC-MSC isolation, culture, identification, and preparation

Human umbilical cord (HUC)-derived MSC were obtained from Meridigen Biotech Co., Ltd (Taipei, Taiwan). All donor women were 20–45 years old, negative for syphilis, HIV, CMV, HBs Ag, and HCV, and had no history of infectious diseases or complications during pregnancy. Donors with full-term delivery provided written informed consent. Umbilical cord tissue was harvested under sterile conditions, rinsed with PBS, and cut into $1 \times 1x1\ mm^3$ pieces with scissors. Next, the specimens were digested in a 37 °C incubator. The digestion was terminated with the addition of culture media. Cell suspensions were centrifuged and supernatants were aspirated and the cells resuspended in complete media. The cells were then cultured in a humidified incubator with 5% CO_2 at 37 °C. After 3 days, the culture media was replaced and non-adherent cells removed. HUC-MSC were passaged once they reached 80–90% confluence. For long-term storage, they were suspended in Cryostar® CS10 and stored in a vapor phase liquid nitrogen tank. Flow cytometry analysis revealed that the HUC-MSC were $CD44^+$, $CD73^+$, $CD90^+$, $CD105^+$, $CD34^-$, $CD45^-$, $CD116^-$, and HLA-DR (Fig. 1). Once thawed, the cells were mixed with culture media and plated at a density of 3000 cells/cm^2 in a T175 flask. Once the cells reached 80% confluence, they were trypsinized at 37 °C, and the trypsin neutralized with complete culture media. After the cell suspensions was centrifuged, the supernatant was aspirated and the cell pellet resuspended in normal saline (NS). The final concentration was adjusted to 1×10^6 cells/mL or 4×10^6 cells/mL. Each umbilical cord (35 mL volume) contained 1.2×10^9 cells with viability above 95%.

Study design, experimental groups, and experimental procedures

Phase 1 of the study (Fig. 2A) examined the effects of MSC on neurobehavioral function and brain contusion volume after injury. Fifty rats were randomly allocated to the following five groups: (i) sham + normal saline (NS) ($n = 10$), in which rats underwent sham surgery followed by an intravenous (i.v.) injection of NS (1 mL/rat) at a rate of 1 mL within 5 minutes (via tail vein) 1 day later; (ii) sham + HUC-MSC 4×10^6 ($n = 10$), in which rats underwent sham surgery and received 4×10^6 HUC-MSC/mL/rat i.v.; (iii) TBI + NS ($n = 10$), in which rats were subjected to TBI and received NS (1 mL/rat i.v.); (iv) TBI + HUC-MSC 1×10^6 ($n = 10$), in which rats were subjected to TBI and received 1×10^6 HUC-MSC/mL/rat i.v.; and (v) TBI + HUC-MSC 4×10^6 ($n = 10$), in which rats were subjected to TBI and received 4×10^6 HUC-MSC/mL/rat i.v. MSC or NS were administered to the appropriate groups one day after surgery. Neurobehavioural tests (described below) were performed before and after surgery. At the end of the experiment (28 days after surgery), all rats were sacrificed by injection of sodium pentobarbital (200 mg/kg, i.p.) for evaluation of the brain contusion. Based on a recent review of Anderson et al.,[8] one injection of HUC-MSC or NS was delivered 1 day (or 24 hours) after a TBI by many investigations.

Phase 2 of the study (Fig. 2B) examined the effects of MSC on parietal inflammatory gene expression after injury. Forty rats were randomly allocated to the following four groups: (i) Sham + NS ($n = 10$); (ii) Sham + HUC-MSC 4×10^6 ($n = 10$); (iii) TBI + NS ($n = 9$); and (iv) TBI + HUC-MSC 4×10^6 ($n = 9$). Rats from all groups were killed by an overdose of sodium pentobarbital 3 days after surgery.

Parietal gene expression analysis

The brains from the rats in phase 2 were removed, and the ipsilateral parietal cortex, deep in 2.0 mm, including the site of injury 3.0 mm in diameters was homogenized into supernatant and pellet fractions within 30 min; the sham samples were removed from the same location. Total cortical RNA was isolated from the supernatant portion using Trizol reagent (Invitrogen, Grand Island, NY, USA), treated with DNAse I (QIAGEN SABioscience, Valencia, CA, USA) to remove any trace of DNA, and further purified using an RNeasy Mini Kit (RNeasy Protect Animal Tissue Kit; QIAGEN BABioscience, Valencia, CA, USA). As detailed in our previous report,[9] we performed a quantitative real-time polymerase chain reaction assay (qPCR) to quantify the genes encoding 84 cytokines and chemokines in triplicate in four separate experiments using an Applied Biosystems 7500 RT-PCR system (Applied Biosystems, Foster City, CA, USA). We also determined the ratio of mRNA levels between groups using the Δct method ($2^{-\Delta\Delta ct}$) to calculate the fold change in a comparative clustergram with the Qiagen online web analysis tool.

We generated lists of differentially expressed genes (DEGs) from RT-PCR analysis using a relative expression threshold of more than two-fold between the TBI + NS group and sham + NS group, or the TBI + HUC-MSC 4×10^6group and TBI + NS group. These were used as the input for Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Redwood, CA, USA) to determine the top three canonical pathways enriched in the DEG datasets.

Neurobehavioural tests

Neurobehavioral performance of rats in phase 1 was assessed 1 day before surgery and 1, 7, 14, 21, and 28 days after surgery. We used the modified neurological severity score (mNSS) assay to evaluate motor, sensory, reflex and

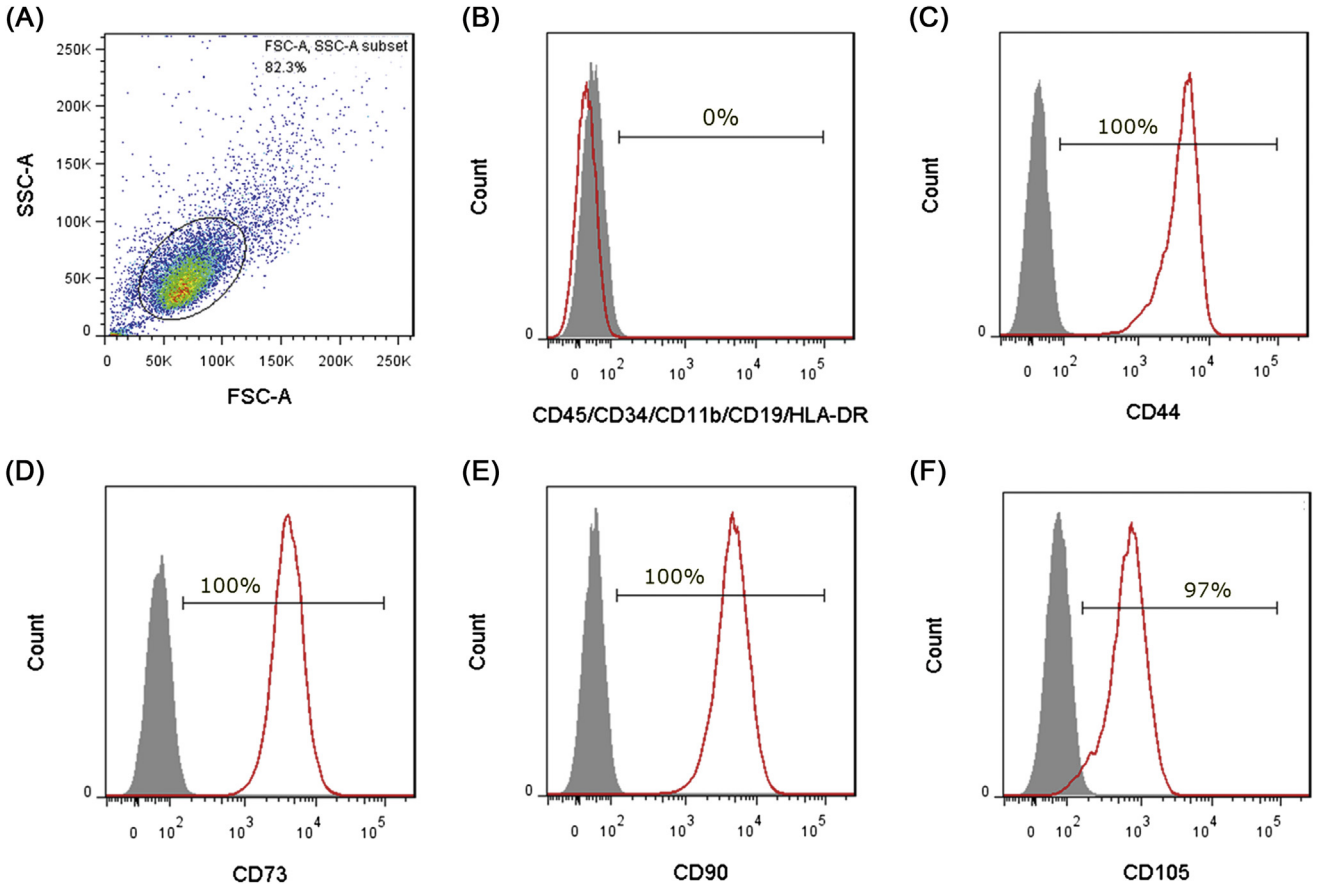

Figure 1 The expression of human umbilical cord-specific CD surface markers was analyzed by flow cytometry. The BD stemflow™ human MSC analysis kit was used to analyze MSC-specific cell surface marker (CD44, CD73, CD105, and CD90).

balance deficits.[10] We also performed inclined-plane tests to measure limb strength,[11] and the adhesive removal test and forelimb placing (foot-fault) test to analyze forepaw sensitivity.[12,13] The cylinder test, also called the spontaneous forelimb-use asymmetry test, was further used to evaluate spontaneous forelimb use.[13] Finally, we used the ladder climbing test to evaluate sensorimotor capacity.[14,15]

Cerebral contusion assessment

Twenty-eight days after surgery, all groups were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and then perfused transcardially with saline. The brains were removed, immersed in cold saline for 5 min, and cut into 2-mm sections with a tissue slicer. Ischemic tissue was revealed by negative 2,3,5-triphenyl tetrazolium chloride (TTC) staining, which labels dehydrogenase-deficient tissue, and measured and summed in each slice using an image analysis system (Image-Pro Plus 4.50.29; Media Cybernetics, Inc., Silver Spring, MD, USA).[16] The brain edema was found to correlate well with injury volume quantified by TTC staining.[17] The following formula was used to calculate the corrected contusion volume (CCV) in mm^3: $CCV = (LT - RNIH) \times d$, where LT is the area of the left hemisphere in mm^2, RNIH is the area of the right non-injured hemisphere (stained) in mm^2, and d is the slice thickness (1 mm).

Statistical analysis

Significant effects identified by a two-way analysis of variance (ANOVA) were followed by either Tukey or Bonferroni *post hoc* tests to analyze the contusion volume and behavioral performance, respectively. All data shown are expressed as the mean ± standard deviation (SD). The threshold of statistical significance was set at $\alpha = 0.05$, in line with standard conventions.

Results

HUC-MSC attenuate brain contusion volume

Compared with the Sham + NS group and Sham +4 × 10^6MSC group, the TBI + NS group had significantly larger cerebral contusion volumes (Fig. 3). Both the TBI+1 × 10^6MSC and TBI+4 × 10^6MSC groups had significantly lower cerebral contusion volumes and contusion areas than did the TBI + NS group (Fig. 3). However, the beneficial effects on cerebral contusion of the higher dose

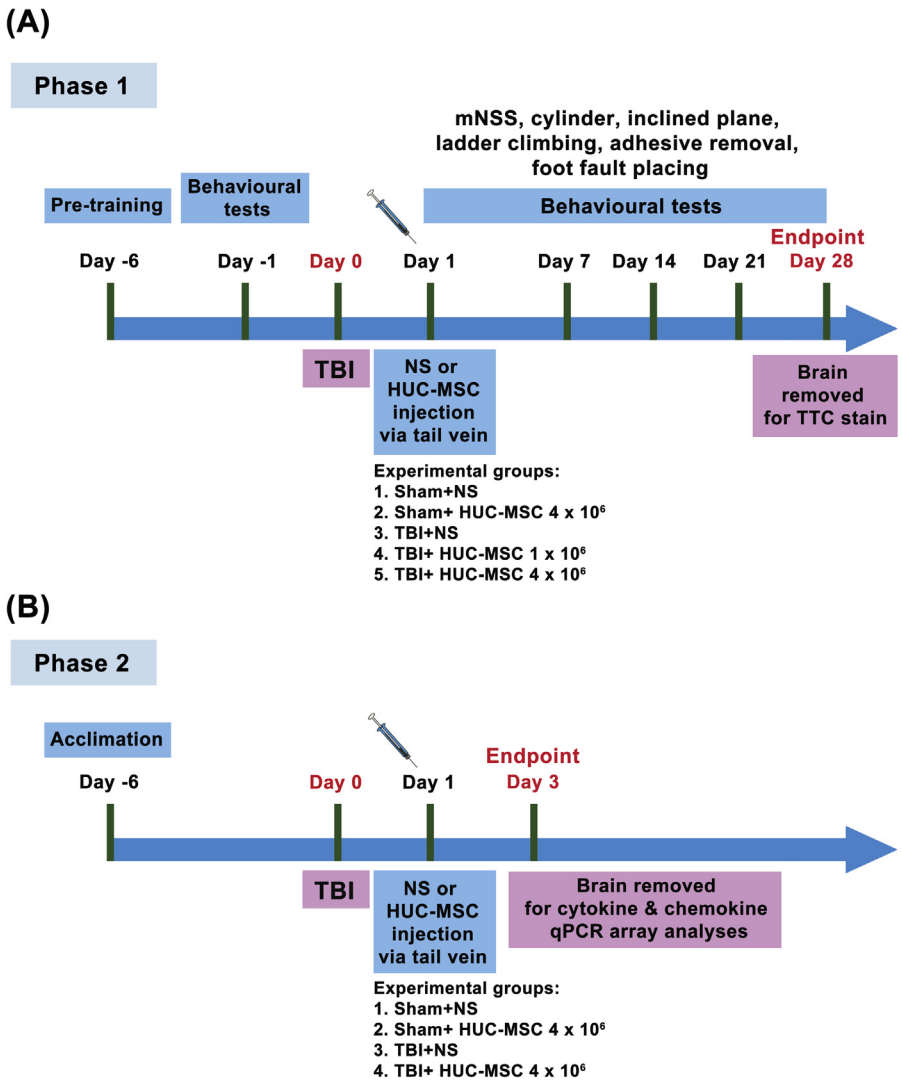

Figure 2 Study design, experimental groups, and experimental procedures for (A) phase 1, and (B) phase 2 of the study. Please see the Materials and Methods section for explanations of group abbreviations.

(4×10^6HUC-MSC) were not superior to those of the lower dose (1×10^6 HUC-MSC). Overall, these results show that intravenous delivery of HUC-MSC successfully reduced TBI-induced cerebral contusion volumes.

HUC-MSC promote functional recovery

Compared with the sham + NS group and sham + HUC-MSC 4×10^6 group, rats from the TBI + NS group reached a lower maximal angle in the inclined plane test; had a higher error ratio in the forelimb placing (foot-fault) test; had a higher mNSS score; took longer to remove the adhesive; and made fewer forelimb placements in the cylinder test (Fig. 4). However, the magnitude of the impairment after TBI was attenuated by treatment with 4×10^6 HUC-MSC (Fig. 4). The results show that treatment with HUC-MSC attenuated TBI-induced behavioral deficits. The beneficial effects of HUC-MSCs persisted up to 28 days post-TBI. In the subsequent experiments, described below, only the 4×10^6HUC-MSC dose was used.

HUC-MSC protect against altered cortical inflammatory gene expression profiles after TBI

We used a qPCR-based array to analyze the expression levels of 84 mRNAs in ipsilateral parietal cortex 3 days after TBI and found 73, 1, 10 out of 84 parietal genes in the ipsilateral brain were up-regulated, down-regulated and unaffected respectively by TBI, compared to sham operation group (Fig. 5). Compared to those of TBI + NS group rats, 11 out of the 84 parietal genes were significantly affected by both TBI and MSC therapy simultaneously (Figs.5 and 6). Among these 11 parietal genes, 8 pro-inflammatory/neurodegenerative genes (e.g., chemokine [C-X-C motif] ligand 12 [CXCL12], platelet factor 4 [PF4], interleukin-7 [IL7], chemokine [C-C motif] ligand 19 [CCL19], chemokine [C-C motif] ligand 22 [CCL22], secreted phosphoprotein 1 [SPP1], pro-platelet basic protein [PPBP], and chemokine (C-C motif) ligand 2 [CCL2]) and 3 anti-inflammatory/neurodegenerative parietal genes (e.g., glucose-6-phosphate isomerase [GPI], bone morphogenetic protein 2 [BMP2], and [BMP4] were

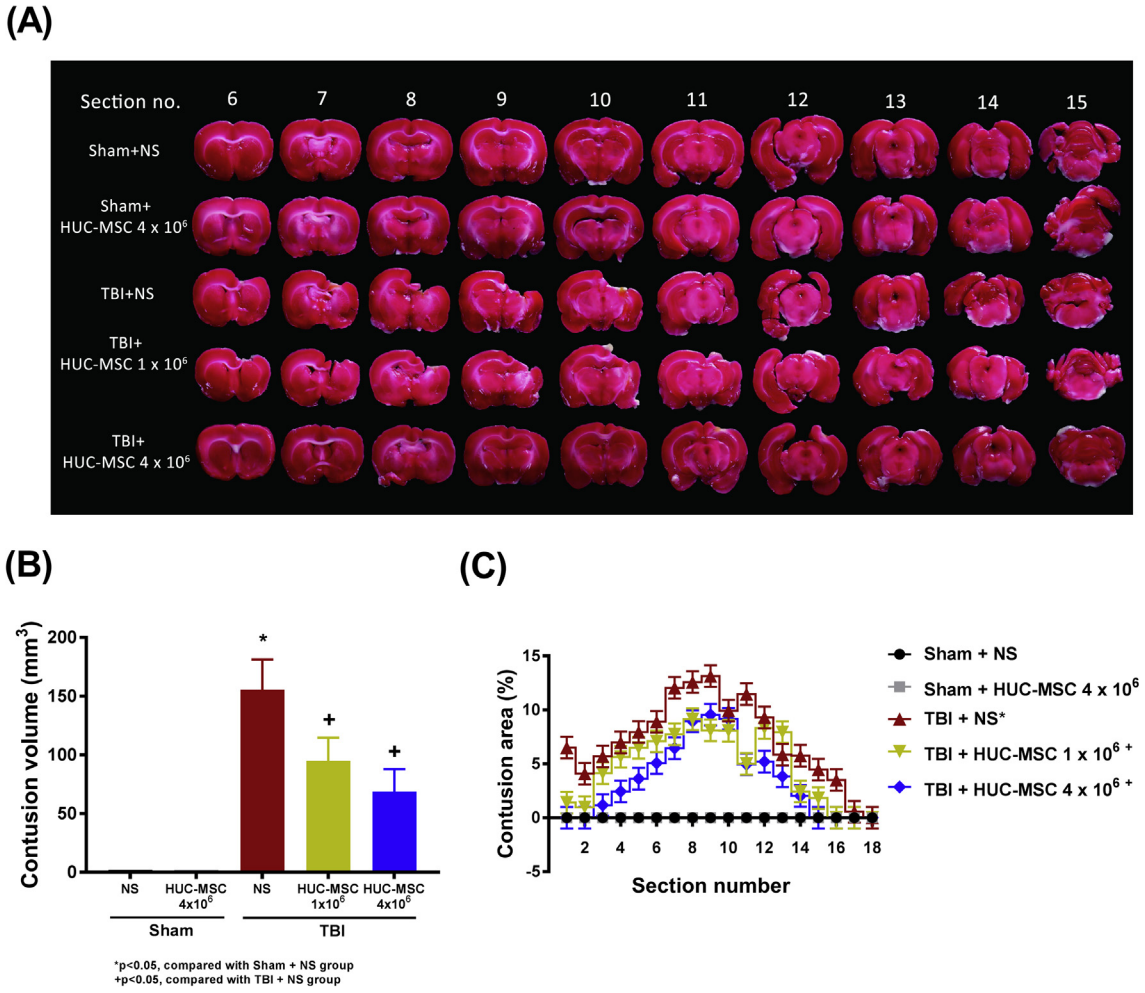

Figure 3 Effect of MSC therapy on cerebral contusion at 28 days post-TBI, as evaluated by TTC staining. (A) Representative TTC-stained brain sections from individual rats from the following groups (top to bottom): sham + NS; sham + HUC-MSC 4×10^6; TBI + NS; TBI + HUC-MSC 1×10^6; and TBI + HUC-MSC 4×10^6. Bar graphs showing (B) contusion volume, and (C) contusion area in different groups of rats at 28 days post-TBI. Contusion volume and area were significantly smaller in TBI + MSC rats than in TBI + NS rats. Values are mean ± SD ($n = 10$ per group). $^*P < 0.05$, TBI + NS vs. sham + NS; $^+P < 0.05$, TBI + HUC-MSC vs. TBI + NS.

significantly up-regulated by MSC therapy (Figs. 5 and 6). Furthermore, MSC therapy significantly suppressed the 8 pro-inflammatory/neurodegenerative genes and simultaneously enhanced the 3 anti-inflammatory/neurodegenerative genes (Table 1).

Bioinformatic analysis of functional pathways and gene networks associated with DEGs

We used the IPA tool to search for the molecular pathways that were affected in parietal cells following TBI. A data set of the eight parietal cell function-associated genes listed above was included in the IPA core analysis, which allowed us to interpret the data set in the context of biological pathways and gene networks. The top three canonical pathways and gene networks in cortical cells that were most significantly affected by TBI were shown in Fig. 7. We found that six parietal genes (BMP2, BMP4, PF4, SPP1, CCL2, and CCL22) were included in the brain inflammation, neuronal proliferation, and cell viability pathways identified by IPA (Fig. 7A). In addition, CCL19 and PPBP genes were included in the IPA-defined tumor necrosis factor activity (Fig. 7B), neurodegeneration and neuro-degeneration (Fig. 7C) pathways, respectively. HUC-MSCs reduced neuroinflammatory and/or neurodegeneration gene expression after TBI (Fig. 7) (see Fig. 8).

Discussion

TBI is associated with a mixture of pro-inflammatory genes and anti-inflammatory genes coding for cytokines and chemokines

In this study, qPCR-based array analyses revealed that 11 out of the 84 parietal cytokines and chemokines genes examined in the ipsilateral parietal tissue were affected by both TBI and MSC. Eight out of 11 parietal mRNAs that up-regulated by TBI (e.g., CXCL12, pf4, IL-7, CCL19, CCL22, SPP1, PPBP, and CCL2) were all suppressed by MSC therapy.

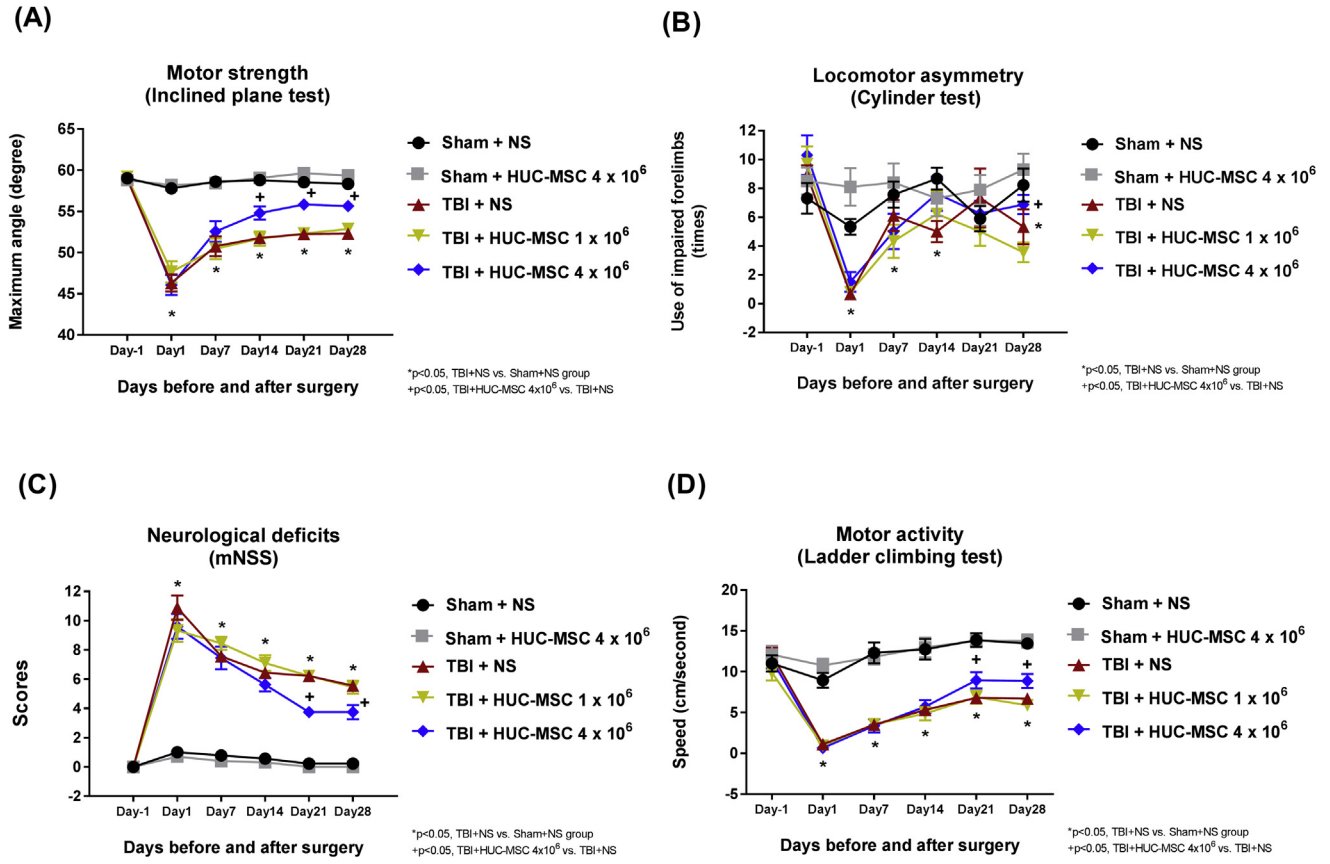

Figure 4 Effect of MSC therapy on functional outcomes in rats with TBI, as evaluated by:(A) the inclined plane test; (B) the cylinder test; (C) the ladder climbing test; and (D) the modified neurological severity score (mNSS). Values are mean ± SD ($n = 10$ per group). $^{*}P < 0.05$, TBI + NS vs. sham + NS; $^{+}P < 0.05$, TBI + HUC-MSC (4×10^6) vs. TBI + NS.

Prior work has shown that all eight parietal genes are associated with pro-inflammatory/neurodegenerative presses. An increased serum concentration of CXCL12, for example, is associated with greater trauma severity in patients following TBI.[17] CXCL12, also known as the stromal cell-derived factor-1 (SDF-1) attracts leukocytes across the blood–brain barrier to the site of injury in rats,[18] and participates in pro-inflammatory and/or neurodegenerative processes.

Platelet factor 4 (PF4), also known as CXCL4, has been detected in the serum of patients suffering from stroke.[19] Overexpression of PF4 attracts microglia or other immune cells to the site of injury.[20] In addition, PF4 exogenously applied to the brain after stroke increases the severity of stroke symptoms in gerbils.[21]

Interleukin-7 (IL-7) stimulates tumor necrosis factor-alpha and TH1 cytokine production in the joints of patients with rheumatoid arthritis.[22] IL-7 enhances proliferation and pro-inflammatory cytokine production of T cells in multiple sclerosis.[23]

CCL19 participates in the development of chronic inflammation and lymphoid neogenesis, guiding B cells and T cells into target organs such as the brain.[24] Microglial overexpression of CCL19 occurs in spinal cord injury.[25] CCL19 attracts leucocytes to injured tissues[26] and is involved in the pathogenesis of TBI.

Likewise, the upregulation of CCL22, SPP1, PPBP and CCL2 are likely to have contributed to TBI pathogenesis. CCL22 (also known as macrophage-derived chemokine) and its receptor, CCR4, recruit inflammatory macrophages in experimentally induced autoimmune brain disease.[27] Secreted phosphoprotein-1 (SPP1), also known as osteopontin (OPN), is implicated as a pro-inflammatory mediator in a variety of neurodegenerative diseases such as multiple sclerosis,[28] Alzheimer's disease[29] and ischemic stroke.[30] SPP1 is secreted by macrophages, activated microglia and astrocytes and is thought to promote macrophage activation and microglial infiltration.[30] Pro-platelet basic protein (PPBP), also known as CXCL7 or neutrophil-activating peptide 2, is secreted by human natural killer cells and can stimulate MSC recruitment.[31] Inhibition of the PPBP receptors CXCR1/2 is protective in models of cerebrovascular disease.[32] Elevated levels of CCL2 (also known as monocyte chemoattractant protein-1)in astrocytes are associated with neuronal death.[33] CCL2 deficiency resulted in reduced lesion size and improved neurological recovery by 28 days post-injury in the weight-drop mouse model of TBI.[34] Therefore, CCL22, SPP1, PPBP, and CCL2 all play a detrimental role in the pathogenesis of TBI.

On the other hand, 3 parietal chemokines and cytokines genes (including GPI, BMP2 and BMP4) upgraded by TBI were all further significantly enhanced by MSC. Evidence

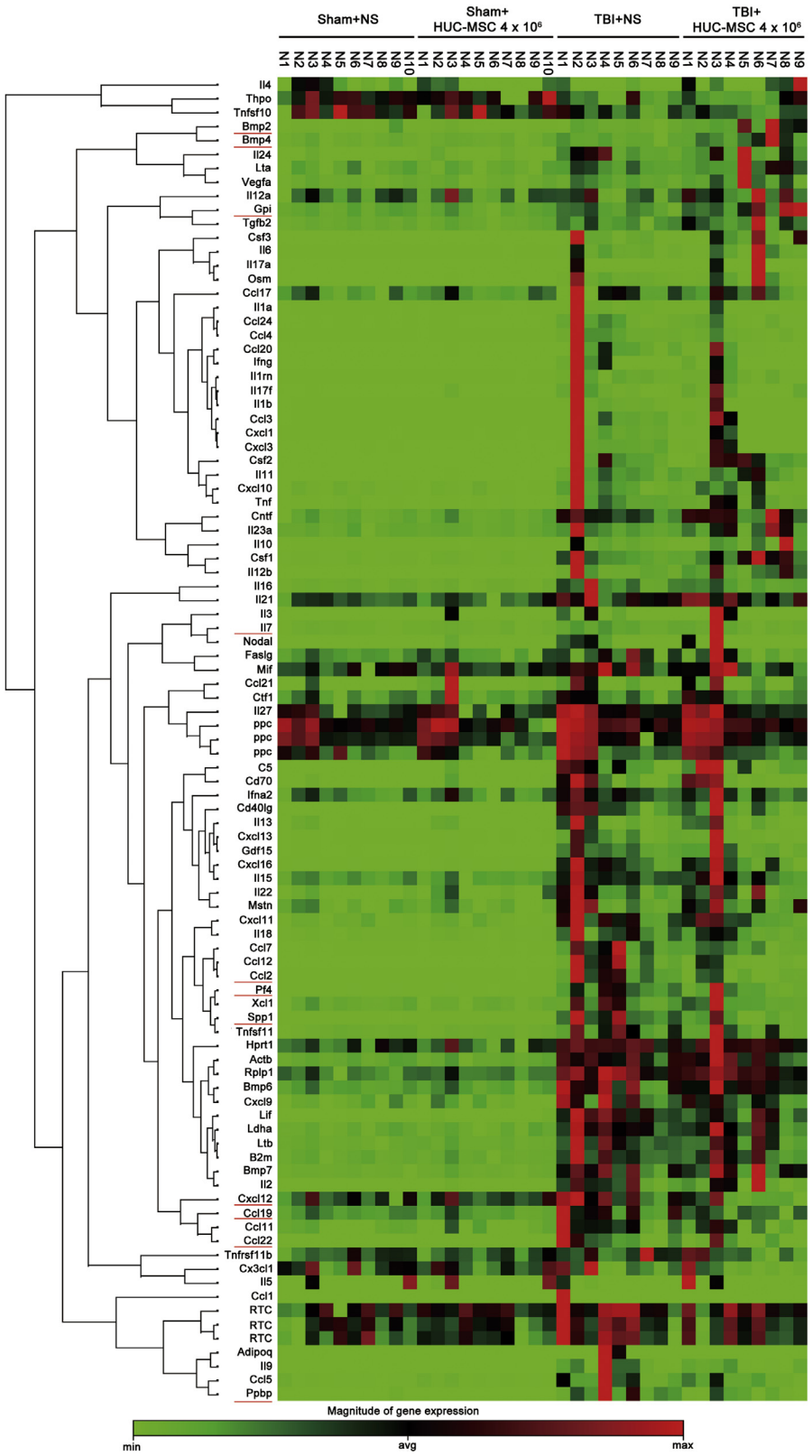

Figure 5 Cluster heat map generated from qPCR-based array analysis of expression levels of 84 mRNAs in frontal cortex ipsilateral to the TBI for different groups of rats. Samples assessed at 3 days after surgery (TBI or sham) are listed in the columns, and the 84 mRNAs are listed in the rows. Rats from the following groups are shown (left-to-right in the columns, 9—10 rats per group): sham + NS; sham + HUC-MSC 4 × 10^6; TBI + NS; and TBI + HUC-MSC 4 × 10^6. Relative mRNA expression is given according to the color scale shown at the bottom: red indicates a higher expression level than average, and green indicates a lower expression level than average. Red lines highlight the rows showing the 11 genes listed in Table 1 on this cluster gram.

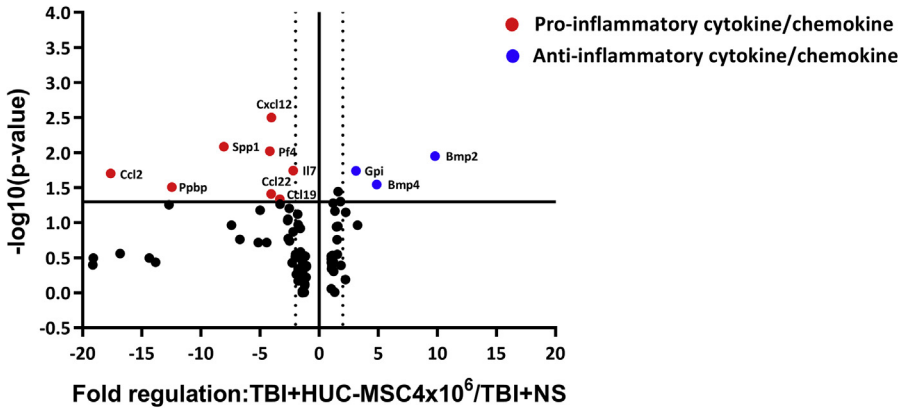

Figure 6 Volcano plot of qPCR notch in rat parietal cortex of fold regulation of TBI + MSC group/TBI + NC group. Both red and blue lines represent p values of 0.05. Red labels, down-regulation at least four-fold, blue, up-regulation at least fourth fold. Red labels represent pro-inflammatory genes and blue labels represent anti-inflammatory genes.

Table 1 The 11 cortical genes determined by qPCR as significantly up- or downregulated in rat ipsilateral frontal cortex 3 days after traumatic brain injury (TBI).

Gene symbol	Fold increase; *p* value[a,b]		Gene name
	TBI + NS	TBI +4 × 10^6 HUC-MSC	
GPI	1.58; 0.032*	3.11; 0.018**	Glucose Phosphate Isomerase
BMP2	2.41; 0.013*	4.89; 0.028**	Bone Morphogenetic Protein-2
BMP4	2.03; 0.25*	9.82; 0.011**	Bone Morphogenetic Protein-4
CXCL12	1.74; 0.065*	−4.04; 0.0032**	C-X-C Motif Chemokine Ligand 12
PF4	1.19; 0.00012*	−4.16; 0.0096**	Platelet Factor 4
IL-7	3.94; 0.0002*	−2.19; 0.018**	Interleukin-7
CCL19	2.16; 0.0065*	−3.32; 0.046**	Chemokine (C-C motif) Ligand 19
CCL22	20.18; 0.0035*	−4.06; 0.039**	Chemokine (C-C motif) Ligand 22
SPP1	11651.45; 0.00055*	−8.05; 0.0083**	Secreted phosphoprotein
PPBP	37.38; 0.0059*	−12.46; 0.031**	Pro-Platelet Basic Protein (also known as CXCL7)
CCL2	700.01; 0.0021*	−17.64; 0.02**	Chemokine (C-C motif) Ligand 2

Data are from 10 control (Sham + NS) and 29 TBI rats. Only significant and annotated transcripts are included. Abbreviations: NS, normal saline; TBI, traumatic brain injury; HUC-MSCs, human umbilical cord-derived $CD34^-$ mesenchymal stem cells.
*TBI + NS vs. Sham + NS; **TBI + HUC-MSC 4 × 10^6 vs. TBI + NS.
[a] The fold increase for each parameter for sham + NS group rats is 1.
[b] The fold increase for each parameter for sham+4 × 10^6 HUC-MSC is insignificantly different from those of sham + NS (Data are not shown here).

has accumulated to indicate that these 3 genes are associated with anti-inflammatory/neuroregenerative events. For example, GPI is an autocrine motility factor[35] or neuroleukin,[36] acting as a neurotrophic factor in the spinal cord and in sensory neurons, and a growth factor in the mouse salivary gland.[37] It is also a potential mediator of neurite outgrowth and protrusions along axons,[38] and a chemoattractant factor for MSC.[39] BMP2 and BMP4, in contrast, play critical roles in the determination of the fate of neural stem cells.[40] These are multipotent progenitor cells that possess the ability to self-renew and generate differential neuronal cell types such as astrocytes, neurons, and oligodendrocytes. Differential expression of BMP2/4 was reported following head injury in rats,[41,42] which could be a mechanism by which head injury induces ectopic verification. In the present study, therefore, upregulation of GPI, BMP2 and BMP4 following treatment with HUC-MSC is likely to have played a protective role in response to TBI. It should be stressed that although the inflammatory response is crucial to the clearance of debris, repair, and regeneration after TBI, dysregulated inflammation can lead to additional acute and chronic brain injury.[1]

HUC-derived MSC attenuate neurological injury by modulating parietal expression of cytokines and chemokines genes in the ipsilateral brain after TBI

Our data showed that HUC-MSC treatment attenuated neurological injury, down-regulated eight parietal pro-inflammatory/neurodegenerative genes (CXCL12, PF4, IL-7, CCL19, CCL22, SPP1, PPBP, and CCL2) and up-regulated three parietal anti-inflammatory/neuroregenerative genes (GPI, BMP2, and BMP4) in the rats with neurotrauma.

Our results appear partially consistent with a recent review suggesting that MSC therapy improves trauma

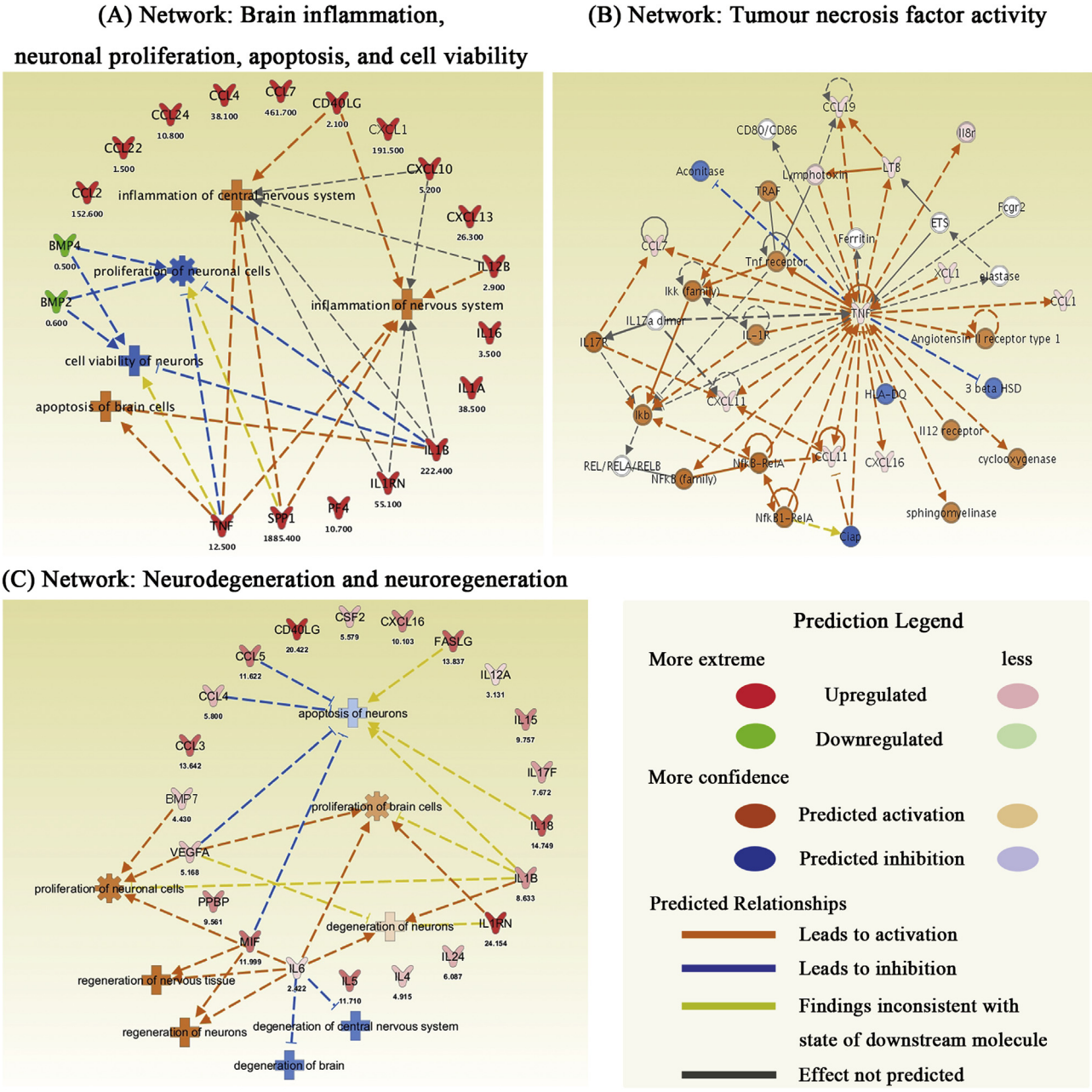

Figure 7 Core molecular networks identified by Ingenuity Pathway Analysis (IPA). Altered genes are involved in significant biological functions associated with:(A) immunological and infectious diseases; (B) cell-to-cell signaling and interaction, cellular movement, and immune cell trafficking; and (C) cellular development, cellular growth, and proliferation. Red notes indicate upregulated genes; green nodes indicate downregulated genes. Blue lines indicate inhibition; orange lines indicate activation.

outcome by inhibiting the activity of the M1 microglial phenotype, which drives pro-inflammatory/neurodegenerative processes but augmenting the activity of the M2 phenotype, which opposes M1 activity by driving anti-inflammatory/neuroregenerative processes.[3] In the present study, the differential expression of cortical genes driving pro- or anti-inflammatory processes following TBI might be accounted for by the activation of microglia or macrophages of the M1 and M2 phenotypes, respectively. This hypothesis is supported by a previous report showing that the macrophages and microglia that respond to TBI in rats comprise a mixture of M1 and M2 phenotypes.[43]

Thus, it appears that MSC therapy exerts its neuroprotective effect in TBI rats by (i) limiting the acute pro-inflammatory response to the level needed for debris clearance; (ii) promoting an anti-inflammatory immune phenotype; and (iii) preventing the development of chronic neuroinflammation and returning to normal function. In line with this, a recent study showed that double-injected human stem cells enhance rehabilitation in TBI mice by

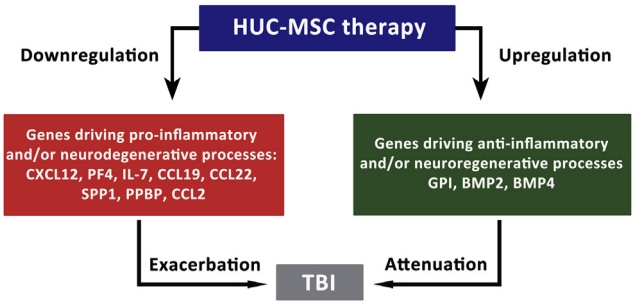

Figure 8 Schematic illustrating how MSC might affect the temporal relationship between pro-inflammatory and/or neurodegenerative processes and anti-inflammatory and/or neuroregenerative events in the ipsilateral parietal cortex after TBI. HUC-MSC suppress the parietal expression of proinflammatory neurodegenerative genes and enhance that of anti-inflammatory/neuroregenerative genes while reducing neurological injury.

enhancing anti-inflammatory and survival signals, and by suppressing pro-inflammatory and oxidative responses.[44] However, the rehabilitation as well as the definition of survival signals may be quite different between various animal models and diseases.

Our present study focuses mainly on the difference between the TBI + NS group rats and the TBI + MSC group rats. After that, the anesthesia or procedures in the experiments may not affect the cytokine or immunological response of the "control" brain. Additionally, our present results are unable to determine whether these genes just expressed in the parietal area are specific to the parietal lobes.

HUC-MSC with $CD34^-$, like human umbilical cord blood-derived MSC with CD^+, improve the outcomes of TBI in rats

In the present study, intravenous administration of 0% human $CD34^+$ cells (in 1×10^6 HUC-MSC or 4×10^6 HUC-MSC) significantly attenuated the severity of both TBI-induced cerebral contusion and neurological deficits in rats. It has been well documented that the MSC obtained from the human umbilical cord tissue were negative for the specific surface antigen CD34, whereas the majority of the MSC obtained from human umbilical cord blood were positive for CD34. Previous results demonstrated that intravenous administration of 95% human $CD34^+$ cells (in 5×10^5 human cord blood lymphocytes and monocytes) were also able to attenuate neurotrauma.[6] Thus, it appears that $CD34^-$ HUC-MSC, like $CD34^+$ HUCB-MSC, improve the outcomes of TBI in rats. However, in vitro results have suggested that $CD34^+$ cells are superior to $CD34^-$ cells in homing to neural ischemic tissue.[22] It has promoted that there is another, still undiscovered, protective cell population within the umbilical cord-derived MSC.[22]

Finally, it should mention that MSC that are transplanted by intracerebral or intravenous routes minimally and selectively migrate to the ischemic bounding sites.[45,46] Considering the small number of MSC in injured brain tissues, the presence of therapeutic neurotrophic factors that are secreted by MSC confers neuroprotection.[3] Therefore, in the present results, we did not provide the results of MSC staining in the injured brain.

Conclusion

Our present results showed that rats with TBI displayed neurological injury as reflected by both cerebral contusion and neurobehavior deficits. In addition, qPCR array analysis revealed that TBI rats upgraded eight pro-inflammatory genes (CXCL12, PF4, IL-7, CCL19, CCL22, SPP1, PPBP and CCL2), and three anti-inflammatory genes (GPI, BMP2 and BMP4) in their parietal cortex of the ipsilateral brain simultaneously. One injection of MSC via the tail vein one-day post-TBI significantly attenuated neurological injury, suppressed the parietal expression of the eight pro-inflammatory genes, and enhanced the parietal expression of the three anti-inflammatory genes in rats with TBI. Thus, it appears that modulation of parietal cytokines and chemokines gene profiles by MSC as a basis for neurotrauma recovery.

Ethical approval

All protocols involving the use of animals were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the Chi Mei Medical Center (IACUC104042801). The research use of human umbilical cord was approved by the Institutional Review Board of the Chi Mei Medical Center (IRB 10405-008).

Statement of human and animal rights

Animal experiments were performed in accordance with international accredited guidelines and were approved by the Institutional Animal Care and Use of the Chi Mei Medical Center, Tainan, Taiwan. The human umbilical cords were approved for research by the Institutional Review Board of the Chi Mei Medical Center and informed donor consent was obtained.

Statement of informed consent

Informed consent for the use of umbilical cord was obtained as described in Statement of Human and Animal Rights.

Conflicts of interest

The used of human umbilical cord-derived mesenchymal stem cells in this study were obtained from Meridigen company. Drs. Willie Lin, Yu-Chin Su, and Yogi Chang-YoHsuan are employees of Meridigen company. The affiliation of Drs. Willie Lin, Yu-Chin Su, and Yogi Chang-Yo Hsuan with Meridigen does not alter the authors' adherence to journal policies on sharing data and materials.

Authors' contributions

C.H.L.: conception and design, provision of study material collection and assembly of data, and manuscript writing; W.L.: provision of study material, and data analysis and interpretation; Y.C.Y.H.: conception and design, and administrative support; Y.C.C: provision of study material, and collection and assembly of data; Y.C.S.: provision of study material, and data collection; C.PC.: conception and design, financial support, and manuscript correspondence; K.C.L.: conception and design, final approval of manuscript, financial support, and manuscript correspondence.

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Abbreviations

ANOVA	Analysis of variance
BMP2	Bone morphogenic protein 2
BMP4	Bone morphogenic protein 4
CCL19	Chemokine (C-C motif) ligand 19
CCL2	Chemokine (C-C motif) ligand 2
CCL22	Chemokine (C-C motif) ligand 22
CCV	Corrected contusion volume
CD	Cluster of differentiation
CXCL12	Chemokine (C-X-C motif) ligand 12
EphA2	Ephrin-type-A receptor 1
GPI	Glucose phosphate isomerase
HLA-DR	Human leukocyte antigen — antigen D related
HUC	Human umbilical cord
IL-7	Interleukin-7
IPA	Ingenuity pathway analysis
LT	left hemisphere
OPN	Osteopontin
PF4	Platelet factor 4
PPBP	Pro-platelet basic protein
qPCR	quantitative real-time polymerase chain reaction
RNIH	right non-injured hemisphere
SD	Standard deviation
SPP 1	Secreted phosphoprotein 1
TBI	Traumatic brain injury
TTC	2,3,5-triphenyl tetrazolium chloride

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jfma.2019.01.008.

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