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MiR-665 inhibits inflammatory response in microglia following spinal cord injury by targeting TREM2

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Abstract. – OBJECTIVE: The purpose of this study was to uncover the role of microRNA-665 (miR-665) in protecting inflammatory response in microglia following spinal cord injury (SCI) and the underlying mechanism.

PATIENTS AND METHODS: The serum levels of miR-665 and TREM2 (triggering receptor expressed on myeloid 2) in SCI patients (n=24) and healthy subjects (n=24) were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Then, the serum levels of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were detected by enzyme-linked immunosorbent assay (ELISA). After lipopolysaccharide (LPS) induction in BV2 cells, the relative levels of miR-665 and TREM2 were detected by qRT-PCR, and relative levels of IL-6 and TNF-α in the culture medium were examined by ELISA. Next, TREM2, the target gene of miR-665, was determined by Dual-Luciferase reporter assay, and the relationship between the expression levels of TREM2 and miR-665 in SCI patients and BV2 cells was analyzed. Finally, the regulatory effects of miR-665 and TREM2 on IL-6 and TNF-α levels in the culture medium of LPS-induced BV2 cells were assessed.

RESULTS: It was found that miR-665 was downregulated in serum of SCI patients and LPS-induced BV2 cells, while TREM2 was upregulated. Silenced miR-665 or overexpressed TREM2 was involved in protecting inflammatory response following SCI. Besides, rescue experiments showed that miR-665 participated in the regulation of inflammatory response following SCI by targeting TREM2.

CONCLUSIONS: MiR-665 inhibits inflammatory response following SCI by targeting TREM2.

Key Words:
Spinal cord injury, MiR-665, TREM2, Inflammatory factors.

Introduction

Spinal cord injury (SCI) is the damage of spinal cord structure and functions, resulting in movement, sensation and autonomic dysfunctions below the level of injury. With the development of economy and society, the incidence of SCI is increasing year by year. Due to its high medical cost, poor prognosis and high disability rate, SCI brings a heavy economic burden to society and families, and eventually develops into a serious social problem[1,2].

Microglia are immune cells of the central nervous system (CNS). As part of the innate immunity of CNS, microglia exert phagocytosis function to remove damaged neurons and cell debris, therefore providing a suitable living environment for neurons[3]. Activated microglia following SCI exert a dual role in regulating the plasticity of neurons and axons. However, overactivated microglia for a long time will also display toxic effects on neurons[4]. Various inflammatory molecules secreted by microglia promote inflammatory cell infiltration and neuronal apoptosis, leading to aggravation of secondary SCI. Pro-inflammatory mediators [i.e., interleukin-1 (IL-1), lL-6, tumor necrosis factor-α (TNF-α), and iNO] produced by microglia can cause neuronal damage[5]. Therefore, it is necessary to properly regulate the activation of microglia and inflammatory response following SCI[6].

MicroRNAs (miRNAs) are non-coding RNAs containing 24 nucleotides that participate in post-transcriptional regulation and have been identified to be vital following SCI. In particular, miR-155 stimulates pro-inflammatory effect on microglia[7]. MiR-155$^{-/-}$ macrophages protect axonal growth and neuronal survival in co-cultured

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dorsal root ganglion neurons[8]. It is suggested that miRNAs may be utilized as therapeutic targets and intervention strategies for SCI treatment[9]. Song et al[10] have reported that miR-124 accelerates the differentiation from BMSCs to neurons by downregulating pyridoxal kinase (PDXK) and thus triggers neuronal repair following SCI.

MiR-665 is discovered to be upregulated in gastric adenocarcinoma, which aggravates tumor progression by activating the FAK pathway by targeting suppressor of cytokine signaling 3 (SOCS3)[11]. Besides, miR-665 is involved in the protection of cardiomyocytes under the stimuli of oxidative stress[12]. Targeting miR-665-3p/ATG4B/autophagy axis can effectively alleviate inflammation and apoptosis caused by intestinal ischemia/reperfusion[13].

In this paper, the role of miR-665 in regulating inflammatory response following SCI and the potential mechanism were explored.

Patients and Methods

Clinical Samples and Cell Culture

Blood samples of SCI patients (n=24) and healthy controls (n=24) in Changhai Hospital, Second Military Medical University from June 2016 to December 2018 were collected. Informed consent was obtained from each subject before this trial. This study was approved by the Ethics Committee of Changhai Hospital, Second Military Medical University. BV2 cells (Cell Bank, Shanghai, China) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), and 1% penicillin/streptomycin. Cells were observed to be adherent under a microscope (Olympus, Tokyo, Japan).

Cell Transfection

The cells were inoculated before they were transfected with 50-100 nM plasmids using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA).

Lipopolysaccharide (LPS) Induction

The cells were prepared into suspension (3×10^5 cells/mL) and 2 mL of suspension was inoculated in each well of a 6-well plate. Four replicates were set in each group. Then, the cells were induced with 0, 1, 5 or 10 μg/mL LPS (Sigma-Aldrich, St. Louis, MO, USA) for 24 h, and finally, 5 μg/mL LPS was selected as the optimal dose.

RNA Extraction and quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Plasma RNA was extracted using the Stabilized Blood-to-CT™ Nucleic Acid Preparation Kit (Ambion, Austin, TX, USA). Triggering receptor expressed on myeloid 2 (TREM2) complementary deoxyribose nucleic acid (cDNA) was obtained using M-MLV kit (Promega, Madison, WI, USA) and subjected to qRT-PCR using LightCycler 480 Probes Master Kit (Roche, Mannheim, Germany). Nest, miR-665 cDNA was yielded by miScript II RT kit and amplified by miScript SYBR Green PCR (Qiagen, Hilden, Germany). With glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 as internal controls, the relative level of the target was calculated using $2^{-\Delta\Delta Ct}$ method. The primer sequences are listed as follows: TREM2: forward: 5'-CCAGCCTGCATACTTGCCA-3', reverse: 5'-GGCAGAGTAGTCTCTTGCCAG-3', miR-665: forward: 5'-GGTCTACAAAGGGAAGC-3', reverse: 5'-TTTGGCACTAGCACATT-3', GAPDH: forward: 5'-TCAAGATCATCAGCAATGCC-3', reverse: 5'-CGATACCAAAGTTGTCATGGA-3', U6: forward: 5'-ATACAGAGAAAGTTAGCACGG-3', reverse: 5'-GGAATGCTTCAAAGAGTTGTG-3'.

Dual-Luciferase Reporter Assay

Luciferase vectors (wild-type and mutant TREM2) were constructed based on the binding sites in 3′-untranslated region (3′-UTR) of TREM2 and miR-665. Cells were co-transfected with TREM2 MUT/TREM2 WT and control/miR-665 mimics, respectively. 24 hours later, the cells were lysed and subjected to Luciferase activity measurement (Promega, Madison, WI, USA).

Western Blot

Cells were lysed for isolating cellular protein and electrophoresed. Protein samples were loaded on polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 h. Thereafter, the membranes were reacted with primary antibody against TREM2 (Abcam, ab86491, Cambridge, MA, USA) and secondary antibody (Beyotime, Nantong, China) for indicated time. Band exposure and grey value analyses were finally conducted.

Enzyme Linked Immunosorbent Assay (ELISA)

Sample preparation: (1) for the serum samples, the blood was naturally coagulated at room temperature for 10-20 min, and centrifuged at 2000-3000 g/min for 20 min. The supernatant was collected; (2) for culture medium, the culture medium was collected, centrifuged at 2000-3000 g/min for 20 min, and diluted in phosphate-buffered saline (PBS) (pH7.2-7.4) at 1×10^6 cells/mL. After freeze-thawing, intracellular components were released. The mixture was centrifuged again, and the supernatant was collected for use. At last, the relative levels of inflammatory markers in prepared samples were determined by ELISA (Abcam, Cambridge, MA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. Data were expressed as mean ± SD (standard deviation). Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). $p<0.05$ considered the difference was statistically significant.

Results

Expression Levels of MiR-665, IL-6, and TNF-α in SCI Patients and LPS-Induced BV2 Cells

QRT-PCR data showed a lower level of miR-665 in SCI patients than healthy controls (Figure 1A). Compared with controls, serum levels of IL-6 (Figure 1B) and TNF-α (Figure 1C) were higher in SCI patients. *In vitro* SCI model was constructed by LPS induction in BV2 cells for 24 h. It was shown that miR-665 was dose-dependently downregulated in BV2 cells (Figure 1D), while IL-6 (Figure 1E) and TNF-α (Figure 1F) were gradually upregulated in culture medium.

TREM2 was the Target Gene of MiR-665

Bioinformatics analysis predicted that TREM2 was the target gene binding to miR-665 (Figure 2A). Subsequently, decreased Luciferase activity in wild-type TREM2 vector after overexpression of miR-665 confirmed the binding between TREM2 and miR-665 (Figure 2B). TREM2 was upregulated in serum of SCI patients (Figure 2C) and displayed a negative correlation to miR-665 level ($R^2=0.2872$, $p=0.007$, Figure 2D). Furthermore, it was found that TREM2 was negatively regulated by miR-665 in BV2 cells (Figure 2E,

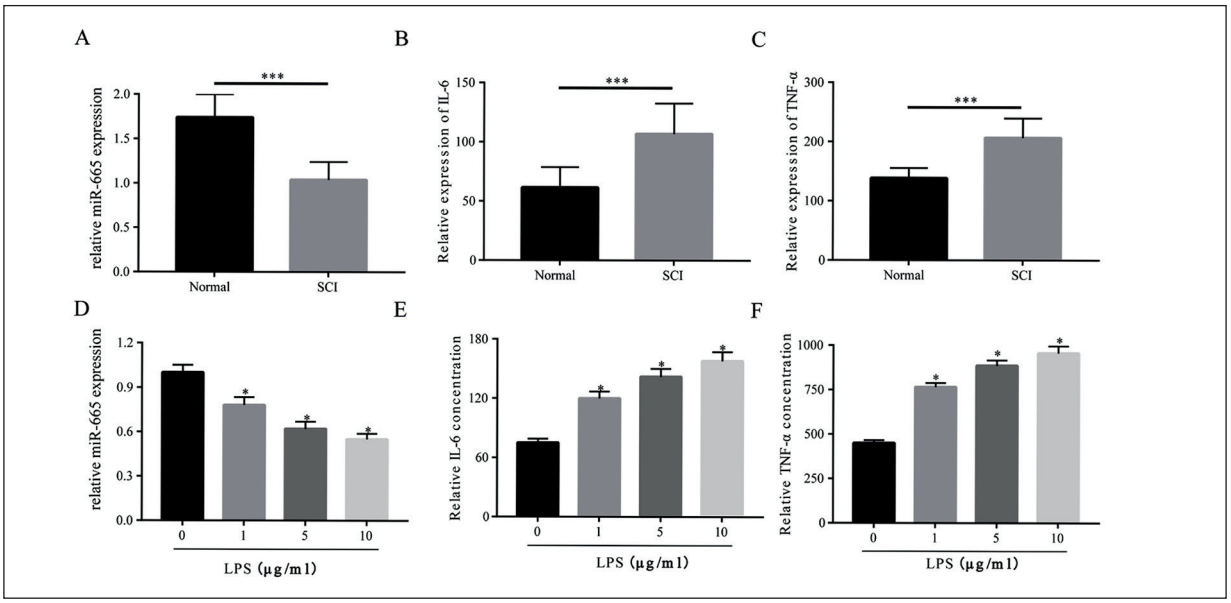

Figure 1. Expression levels of miR-665, IL-6 and TNF-α in SCI patients and LPS-induced BV2 cells. **A,** MiR-665 levels in serum of healthy controls and SCI patients detected by qRT-PCR. **B, C,** IL-6 (**B**) and TNF-α (**C**) levels in serum of healthy controls and SCI patients detected by ELISA. **D,** MiR-665 level in BV2 cells induced with 0, 1, 5 or 10 μg/mL LPS for 24 h. **E, F,** IL-6 (**E**) and TNF-α (**F**) levels culture medium of BV2 cells induced with 0, 1, 5 or 10 μg/mL LPS for 24 h.*$p<0.05$, **$p<0.01$, ***$p<0.001$.

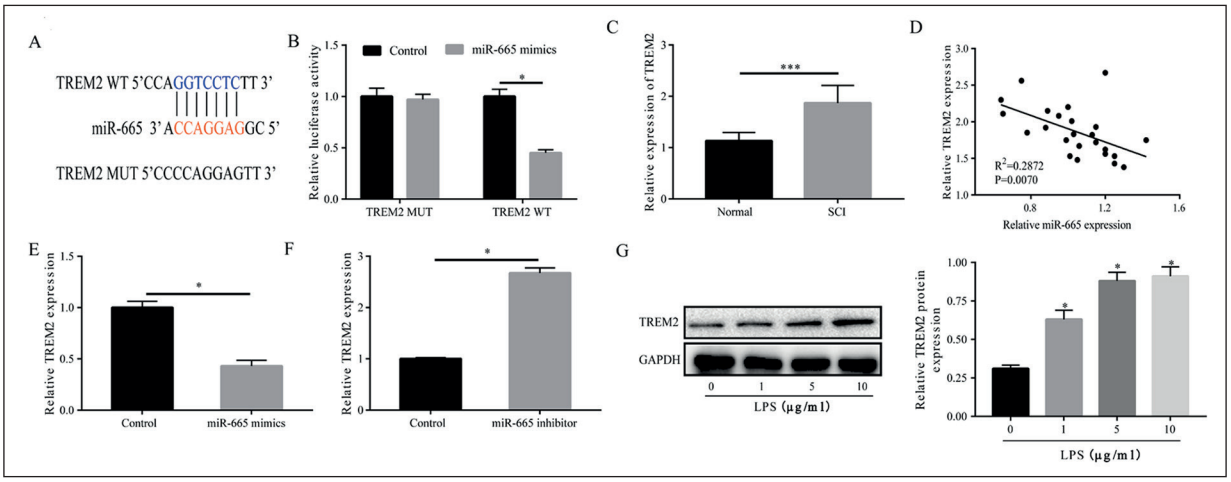

Figure 2. TREM2 is the target gene of miR-665. **A,** Binding sites in 3'UTR of miR-665 and TREM2. **B,** Luciferase activity in BV2 cells co-transfected with TREM2 MUT/TREM2 WT and control/miR-665 mimics. **C,** TREM2 levels in serum of healthy controls and SCI patients detected by qRT-PCR.**D,** A negative correlation between expression levels of miR-665 and TREM2 in SCI patients. **E, F,** TREM2 level in BV2 cells with overexpression (**E**) or knockdown (**F**) of miR-665. **G,** TREM2 level in BV2 cells induced with 0, 1, 5 or 10 μg/mL LPS for 24 h. *p<0.05, **p<0.01, ***p<0.001.

2F). In LPS-induced BV2 cells, TREM2 was dose-dependently upregulated (Figure 2G).

Regulatory Effects of MiR-665 and TREM2 on Inflammatory Response

In the following experiments, 5 μg/mL LPS was selected as the optimal dose to induce BV2 cells. Transfection of miR-665 mimics markedly upregulated miR-665 in LPS-induced BV2 cells (Figure 3A). Overexpression of miR-665 reversed the upregulated IL-6 (Figure 3B) and TNF-α (Figure 3C) in LPS-induced BV2 cells, showing a certain protective role in the inflammatory response. Subsequently, transfection efficacy of si-TREM2 was tested (Figure 3D). Besides, the knockdown of TREM2 also displayed a protec-

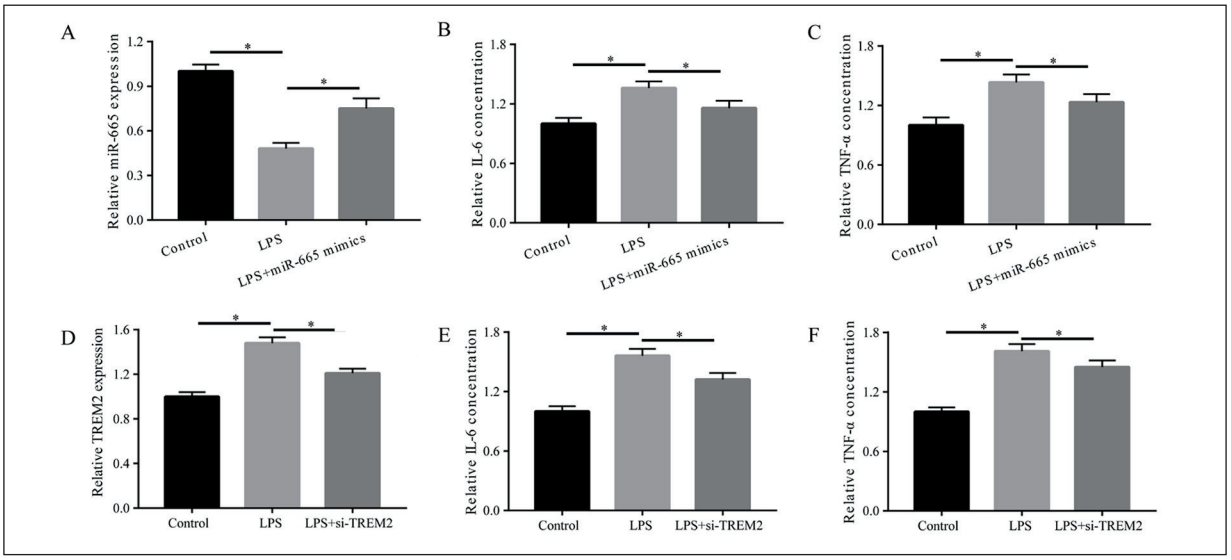

Figure 3. Regulatory effects of miR-665 and TREM2 on inflammatory response. **A,** MiR-665 level in BV2 cells treated with blank control, 5 μg/mL LPS or 5 μg/mL LPS + miR-665 mimics. **B, C,** IL-6 (**B**) and TNF-α (**C**) levels in culture medium of BV2 cells treated with blank control, 5 μg/mL LPS or 5 μg/mL LPS + miR-665 mimics. **D,** TREM2 level in BV2 cells treated with blank control, 5 μg/mL LPS or 5 μg/mL LPS + si-TREM2. **E, F,** IL-6 (**E**) and TNF-α (**F**) levels in culture medium of BV2 cells treated with blank control, 5 μg/mL LPS or 5 μg/mL LPS + si-TREM2. *p<0.05, **p<0.01, ***p<0.001.

tive effect on alleviating inflammatory response (Figure 3E, 3F).

MiR-665 Alleviated Inflammatory Response Through Targeting TREM2

We speculated that TREM2 was involved in the protective function of miR-665 following SCI. Interestingly, the knockdown of miR-665 markedly elevated inflammatory marker levels (Figure 4A, 4B) in LPS-induced BV2 cells, while their increased levels were further reduced by co-transfection of si-TREM2. Similarly, the overexpression of TREM2 aggravated inflammatory response in LPS-induced BV2 cells overexpressing miR-665 (Figure 4C, 4D).

Discussion

LPS is a bacterial endotoxin that produces reactive oxygen species (ROS) and pro-inflammatory cytokines. It is generally used in *in vitro* experiments that activates macrophages and induces post-injury infections[14]. During the process of SCI, excessively activated microglia can produce a variety of microscopic mediators, thereby aggravating the severity of secondary injury[15]. In this paper, BV2 cells were induced with LPS to mimic *in vitro* SCI injury.

IL-6 and TNF-α are two vital pro-inflammatory cytokines activated following SCI[16]. IL-6 is closely related to secondary SCI. In the early stage of SCI, local concentration of IL-6 in the lesion increases. TNF-α is immediately released following inflammatory response and quickly accumulated in the damaged lesion and induces cell apoptosis as a multi-directional growth pro-inflammatory cytokine[17]. A sustained increase in TNF-α at post-SCI triggers death of neurons and oligodendrocytes. *In vivo* experiments have revealed the proinflammatory effect of TNF-α on the acute phase of SCI[18]. The findings of this study demonstrated that miR-665 was downregulated in the serum of SCI patients and LPS-induced BV2 cells. On the contrary, IL-6 and TNF-α were upregulated.

TREM2 (triggering receptor expressed on myeloid 2) is a novel immunoglobulin receptor located on human chromosome 6, and it is mainly expressed in monocytes, macrophages, dendritic cells, and microglia[19]. As a regulator in inflammatory response and phagocytosis of cell debris, TREM2 is upregulated in microglia that protects neurons from damage[20,21]. Here, TREM2 was proven to be the target binding miR-665 and found to be upregulated in serum of SCI patients and LPS-induced BV2 cells. Importantly, TREM2 was involved in the protective effect of miR-665 on inflammatory response following SCI.

Pathological changes following SCI are complicated involving multiple cytokines, inflammatory mediators, and cells. MiRNAs exert vital functions in inflammatory response, motor neu-

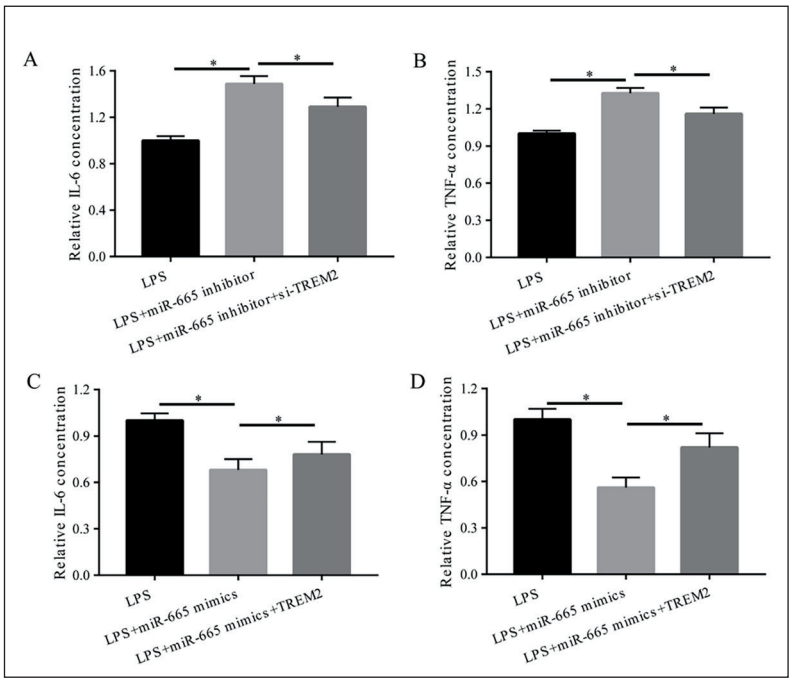

Figure 4. MiR-665 alleviates inflammatory response through targeting TREM2. **A, B,** IL-6 (**A**) and TNF-α (**B**) levels in culture medium of BV2 cells treated with 5 μg/mL LPS, 5 μg/mL LPS + miR-665 inhibitor or 5 μg/mL LPS + miR-665 inhibitor + si-TREM2. **C, D,** IL-6 (**C**) and TNF-α (**D**) levels in culture medium of BV2 cells treated with 5 μg/mL LPS, 5 μg/mL LPS + miR-665 mimics or 5 μg/mL LPS + miR-665 mimics + pcDNA-TREM2. *p<0.05, **p<0.01, ***p<0.001.

ron regeneration, axon growth, removal of ROS and neuronal apoptosis. This study provides a potential biomarker in protecting SCI-induced inflammation.

Conclusions

In summary, miR-665 inhibits inflammatory response following SCI by targeting TREM2, which may be utilized as a therapeutic target.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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References

1) Wang H, Liu X, Zhao Y, Ou L, Zhou Y, Li C, Liu J, Chen Y, Yu H, Wang Q, Han J, Xiang L. Incidence and pattern of traumatic spinal fractures and associated spinal cord injury resulting from motor vehicle collisions in China over 11 years: an observational study. Medicine (Baltimore) 2016; 95: e5220.
2) Bhalala OG, Srikanth M, Kessler JA. The emerging roles of microRNAs in CNS injuries. Nat Rev Neurol 2013; 9: 328-339.
3) Hsieh CL, Koike M, Spusta SC, Niemi EC, Yenari M, Nakamura MC, Seaman WE. A role for TREM2 ligands in the phagocytosis of apoptotic neuronal cells by microglia. J Neurochem 2009; 109: 1144-1156.
4) Gaudet AD, Fonken LK. Glial cells shape pathology and repair after spinal cord injury. Neurotherapeutics 2018; 15: 554-577.
5) Fan H, Tang HB, Shan LQ, Liu SC, Huang DG, Chen X, Chen Z, Yang M, Yin XH, Yang H, Hao DJ. Quercetin prevents necroptosis of oligodendrocytes by inhibiting macrophages/microglia polarization to M1 phenotype after spinal cord injury in rats. J Neuroinflammation 2019; 16: 206.
6) Yang YH, Zhu J. Targeting miR-106-3p facilitates functional recovery via inactivating inflammatory microglia and interfering glial scar component deposition after neural injury. Eur Rev Med Pharmacol Sci 2019; 23: 9000-9008.
7) Xu M, Wang HF, Zhang YY, Zhuang HW. Protection of rats spinal cord ischemia-reperfusion injury by inhibition of miR-497 on inflammation and apoptosis: possible role in pediatrics. Biomed Pharmacother 2016; 81: 337-344.
8) Cardoso AL, Guedes JR, Pereira DAL, Pedroso DLM. MiR-155 modulates microglia-mediated immune response by down-regulating SOCS-1 and promoting cytokine and nitric oxide production. Immunology 2012; 135: 73-88.
9) Yunta M, Nieto-Diaz M, Esteban FJ, Caballero-Lopez M, Navarro-Ruiz R, Reigada D, Pita-Thomas DW, Del AA, Munoz-Galdeano T, Maza RM. MicroRNA dysregulation in the spinal cord following traumatic injury. PLoS One 2012; 7: e34534.
10) Song JL, Zheng W, Chen W, Qian Y, Ouyang YM, Fan CY. Lentivirus-mediated microRNA-124 gene-modified bone marrow mesenchymal stem cell transplantation promotes the repair of spinal cord injury in rats. Exp Mol Med 2017; 49: e332.
11) Tang H, Long Q, Zhuang K, Yan Y, Han K, Guo H, Lu X. MiR-665 promotes the progression of gastric adenocarcinoma via elevating FAK activation through targeting SOCS3 and is negatively regulated by lncRNA MEG3. J Cell Physiol 2019. Doi: 10.1002/jcp.29349.
12) Yu J, Yang W, Wang W, Wang Z, Pu Y, Chen H, Wang F, Qian J. Involvement of miR-665 in protection effect of dexmedetomidine against oxidative stress injury in myocardial cells via CB2 and CK1. Biomed Pharmacother 2019; 115: 108894.
13) Jiang L, Yang F, Zhao Q, Pan L. MicroRNA-665 mediates propofol-induced cell apoptosis in human stem cell-derived neurons. Bioengineered 2019; 10: 493-500.
14) Miyake K. Innate recognition of lipopolysaccharide by Toll-like receptor 4-MD-2. Trends Microbiol 2004; 12: 186-192.
15) Henn A, Lund S, Hedtjarn M, Schrattenholz A, Porzgen P, Leist M. The suitability of BV2 cells as alternative model system for primary microglia cultures or for animal experiments examining brain inflammation. ALTEX 2009; 26: 83-94.
16) Yang L, Blumbergs PC, Jones NR, Manavis J, Sarvestani GT, Ghabriel MN. Early expression and cellular localization of proinflammatory cytokines interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha in human traumatic spinal cord injury. Spine (Phila Pa 1976) 2004; 29: 966-971.
17) Knoblach SM, Fan L, Faden AI. Early neuronal expression of tumor necrosis factor-alpha after experimental brain injury contributes to neurological impairment. J Neuroimmunol 1999; 95: 115-125.
18) Shi LL, Zhang N, Xie XM, Chen YJ, Wang R, Shen L, Zhou JS, Hu JG, Lu HZ. Transcriptome profile of rat genes in injured spinal cord at different stages by RNA-sequencing. BMC Genomics 2017; 18: 173.
19) Bouchon A, Dietrich J, Colonna M. Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. J Immunol 2000; 164: 4991-4995.
20) Hickman SE, El KJ. TREM2 and the neuroimmunology of Alzheimer's disease. Biochem Pharmacol 2014; 88: 495-498.
21) Mazaheri F, Snaidero N, Kleinberger G, Madore C, Daria A, Werner G, Krasemann S, Capell A, Trumbach D, Wurst W, Brunner B, Bultmann S, Tahirovic S, Kerschensteiner M, Misgeld T, Butovsky O, Haass C. TREM2 deficiency impairs chemotaxis and microglial responses to neuronal injury. EMBO Rep 2017; 18: 1186-1198.