Life Sciences 233 (2019) 116731

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Down-regulation of taurine-up-regulated gene 1 attenuates inflammation by sponging miR-9-5p via targeting NF-κB1/p50 in multiple sclerosis

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ARTICLE INFO

Keywords:
Multiple sclerosis
TUG1
Inflammation
NF-κB1/p50
MiR-9-5p

ABSTRACT

Aims: Multiple sclerosis (MS) is an inflammatory disease of the central nervous system characterized by widespread inflammation. LncRNA taurine-up-regulated gene 1 (TUG1) has been reported to be involved in multiple biological processes and human diseases. The aim of this study was to investigate the role of lncRNA TUG1 in MS and the underlying mechanism.

Main methods: Experimental autoimmune encephalomyelitis (EAE) was induced in mice by immunization with myelin oligodendrocyte glycoprotein peptide 35–55 (MOG35–55). Lentiviral vectors encoding sh-TUG1 was constructed to silence TUG1 in MOG-EAE mice by intracerebroventricular (ICV) injection. The effect of TUG1 on inflammation in MS was evaluated by real-time PCR, Western blot, ELISA and Hematoxylin-eosin staining. To further study the mechanism of TUG1 in MS, TUG1 knockdown and miR-9-5p overexpression were performed in LPS-induced BV2 cells.

Key findings: Down-regulation of TUG1 improved mice behavior, reduced granulocyte-macrophage colony stimulating factor (GM-CSF) level, decreased the levels of pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin (IL)-6 and IL-17, and increased IL-10 in EAE mice. Notably, TUG1 expression was negatively correlated with miR-9-5p expression, while positively correlated with NF-κB1/p50. Knockdown of TUG1 or enforced expression of miR-9-5p inhibited LPS-induced inflammation in BV2 cells, while these effects were abolished by inhibition of miR-9-5p. We further verified that TUG1 negatively regulated miR-9-5p expression and NF-κB1/p50 is a direct target of miR-9-5p.

Significance: Down-regulation of TUG1 attenuates MS through inhibition of inflammation by sponging miR-9-5p via targeting NF-κB1/p50, suggesting that TUG1 is a potential therapeutic target for MS treatment.

1. Introduction

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS), which is characterized by widespread inflammation, demyelination and axonal loss [1]. MS is the most common disabling neurodegenerative disease in young adult and 80% of patients suffer from a clinical course of relapse and remission [2,3]. The lesions of MS have a predilection for the brain and spinal cord, and optic neuritis is one of the major complications leading to impaired vision [4]. The variation in the prevalence of MS is considerable in the worldwide and a trend of an increasing incidence and prevalence has been reported [3]. However, the etiology and pathogenesis of MS has not been fully elucidated due to the unknown cause and complicated pathophysiologic mechanisms, to which there is no improved diagnostic techniques and efficient treatment so far.

Long non-coding RNAs (lncRNAs) are defined as RNA transcripts with length of over 200 nucleotides that have no protein coding ability, but regulate gene expression in terms of epigenetic, transcriptional and post-transcriptional levels [5]. LncRNAs have been demonstrated to participate in a variety of biological processes including cell apoptosis, proliferation, pluripotency, development and neuronal processes [5,6]. Recent studies illustrated that lncRNAs might play an important role in regulation of activation, differentiation and imbalanced expression of immune cells, indicating lncRNA may be associated with the progression of autoimmune disease [7]. A recent study screened 84 lncRNA involved in autoimmunity and human inflammatory response [8]. Taurine-up-regulated gene 1 (TUG1) is a 7.1-kb lncRNA that initially discovered to be upregulated in response to taurine treatment of developing retinal cells, whose function is associated with the normal development of the retina and nervous system [9]. TUG1 is suggested to

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https://doi.org/10.1016/j.lfs.2019.116731
Received 29 May 2019; Received in revised form 25 July 2019; Accepted 3 August 2019
Available online 05 August 2019
0024-3205/

function variably in different types of diseases and can be a potent therapeutic target for the treatment of human diseases [10,11]. Currently, mounting researchers have shifted the focus to the role of lncRNA TUG1 in autoimmune diseases. Santoro et al. found that lncRNAs participated in the regulation of pro-inflammation and anti-inflammation, and TUG1 was up-regulated in the serum samples from MS patients [8]. However, the role and mechanism of TUG1 in MS is largely unexplored to this day. Investigation of lncRNAs in MS is beneficial to understand the pathogenesis of MS and develop the therapeutic approach to autoimmune diseases.

In the present study, we studied the role of TUG1 in a mouse model of MS. To further explore the underlying mechanism of TUG1 in MS, LPS-induced BV2 cells were used to mimic MS for in vitro study. We demonstrate a TUG1/miR-9-5p/NF-κB1/p50 axis in MS and that down-regulation of TUG1 ameliorates MS through inhibition of inflammation by sponging miR-9-5p via targeting NF-κB1/p50.

2. Materials and methods

2.1. Animal model and treatment

Male C57/BL6 mice aged 6–8 weeks and weighting 22 ± 2 g were purchased from Changsheng Biotechnology Co., LTD., with SCXK (LIAO)2015-0001 and all protocols in this study were approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Zhengzhou University. Experimental autoimmune encephalomyelitis (EAE) mouse model is a widely used animal model for studying MS. Mice were randomly divided into 4 groups (n = 48): (1) control, (2) EAE, (3) EAE + sh-NC, (4) EAE + sh-TUG1. To establish an EAE mouse model, mice were subcutaneously immunized on the back and foot with 200 μl of complete Freund's adjuvant and phosphate buffered saline (PBS) at 1:1, containing 200 μg of myelin oligodendrocyte glycoprotein peptide 35–55 (MOG35-55) (GL Biochem, China) and 4 mg/ml *Mycobacterium tuberculosis*. Forty-eight hours after immunization, the mice were injected with 200 ng of pertussis toxin (Biolead, China) intraperitoneally. While the mice received the injection of PBS were served as control. For the model + sh-TUG1 and model + sh-NC groups, lentiviral vectors encoding sh-TUG1 were constructed to suppress TUG1 expression and that carrying nonsense gene sequence (sh-NC) were used as negative control in this study. Briefly, mice were anesthetized with intraperitoneal injection of pentobarbital (50 mg/kg) and immobilized using stereotaxic instrument followed by exposing the skull for subsequent intracerebroventricular (ICV) injection. Injections were performed into the lateral cerebral ventricle located 1.8 mm lateral and 1 mm dorsal to the bregma. After drilling 1 mm holes in both sides of the skull, the guide cannula was laid at a depth of 2.4 mm bellow the skull surface. Then 5 μl of lentiviral vectors carrying lncRNA TUG1 or 5×10^8 TU/ml of negative controls was injected into bilateral lateral ventricles of mice using Hamilton syringe through the cannula once a week for 4 weeks.

To determine the clinical signs of MS, behavioral performance of rats was scored every 5 days for 30 days after ICV injection as mentioned above. A clinical MS scoring system was established using a standard scale of 0–4 as follows: 0, no clinical sign; 1, limp tail; 2, paralysis of single hind limb; 3, paralysis of both hind limbs; 4, paralysis of trunk. After behavioral study, all rats were sacrificed with 150 mg/kg of pentobarbital by intraperitoneal injection. Part of brain tissues was fixed in 10% formaldehyde and the others, after flash-freezing by liquid nitrogen, were stored at 80 °C for future experiments.

2.2. Cell culture, treatment, infection and transfection

Microglia BV2 cells were purchased from CHI Scientific (USA). They were seeded into 6-well plates at the density of 4×10^5 and cultured in DMEM medium containing 10% fetal bovine serum at 37 °C in a humidified incubator of 5% CO_2 for subsequent experiments.

For LPS treatment, BV2 cells were cultured in the complete culture medium supplemented with 100 ng/ml lipopolysaccharides (LPS) at 37 °C under 5% CO_2 in a humidified incubator for 24 h.

For lentivirus infection, when the cells reached to 70% confluency, the medium was replaced with the fresh culture medium containing 6 μg/ml polybrene (Sigma, USA), followed by addition of the calculated lentivirus sh-NC or sh-TUG1 (WanleiBio, China) at multiplicity *of infection* (*MOI*) of 100. After infection for 72 h, the cells were performed with further transfection or other detections.

For cell transfection, cells were treated with serum-free basal medium with 2 ml per well for 1 h. Then cells were transfected with miR-9-5p mimic, negative control (NC) mimic (GenePharma, China), miR-9-5p inhibitor or NC inhibitor, respectively. Transfections were performed using Lipofectamine 2000 (Invitrogen, USA) according to the manufacture's instruction. Twenty-four hours after transfection, the cells were subjected to LPS treatment or further detections.

2.3. Quantitative Real-time PCR

Total RNA was isolated from cerebral cortex or cell lysates and the concentration was determined. Super M-MLV reverse transcriptase (Bioteke, China) was used to synthesize cDNA by reverse transcription. The real-time PCR reaction system was established using SYBR Green kit (Solarbio, China) according to the manufacture's instruction and then subjected to fluorescent quantitative Polymerase chain reaction using ExicyclerTM 96 (Bioneer, Korea). β-Actin (Bioss Antibodies, China) was measured to normalize the expression of lncRNA TUG1 and p50, and U6 (Sangon Biotech, China) was used as reference for miR-9-5p level. Data were calculated using the $2^{-\Delta\Delta CT}$ method. Real-time PCR primers are listed in Table 1.

2.4. Western blot

Total protein was extracted by RIPA lysis buffer (Beyotime, China) from cerebral cortex or cell lysates, and then quantified with the use of a bicinchoninic acid (BCA) assay kit (Beyotime, China). 40 μg protein was equally loaded, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA). After blocking with 5% (M/V) skimmed milk for 1 h, membranes were incubated with primary antibody including p50 (1:1000, Proteintech) and β-actin (1:500, Bioss Antibodies) at 4 °C overnight. They were then washed with Tris-buffered saline buffer containing Tween-20 (TBST), followed by incubation with appropriate secondary antibody including sheep anti-rabbit IgG-HRP (1:5000, Beyotime) and sheep anti-mouse IgG-HRP (1:5000, Beyotime) at 37 °C for 45 min. The protein bands were visualized by chemiluminescence (ECL) kit (Beyotime, China), and the band density was analyzed using Gel-Pro-Analyzer.

Table 1
Real-time PCR primers.

Name	Sequence	Length(bp)
LncRNA TUG1 Forward	CATCTCACAAGGCTTCAACCA	197
LncRNA TUG1 Reverse	ACCTCAACTCCCACTTCACTA	
P50 Forward	CAACCAAAACAGAGGGGATT	160
P50 Reverse	TTGTGACCAACTGAACGATA	
β-actin Forward	CTGTGCCCATCTACGAGGGCTAT	155
β-actin Reverse	TTTGATGTCACGCACGATTTCC	
miR-9-5p Forward	CGCGCTCTTTGGTTATCTAGCTGTA	62
miR-9-5p Reverse	GTGCAGGGTCCGAGGTATTC	
U6 Forward	CTCGCTTCGGCAGCACA	137
U6 Reverse	GTGCAGGGTCCGAGGTATTC	

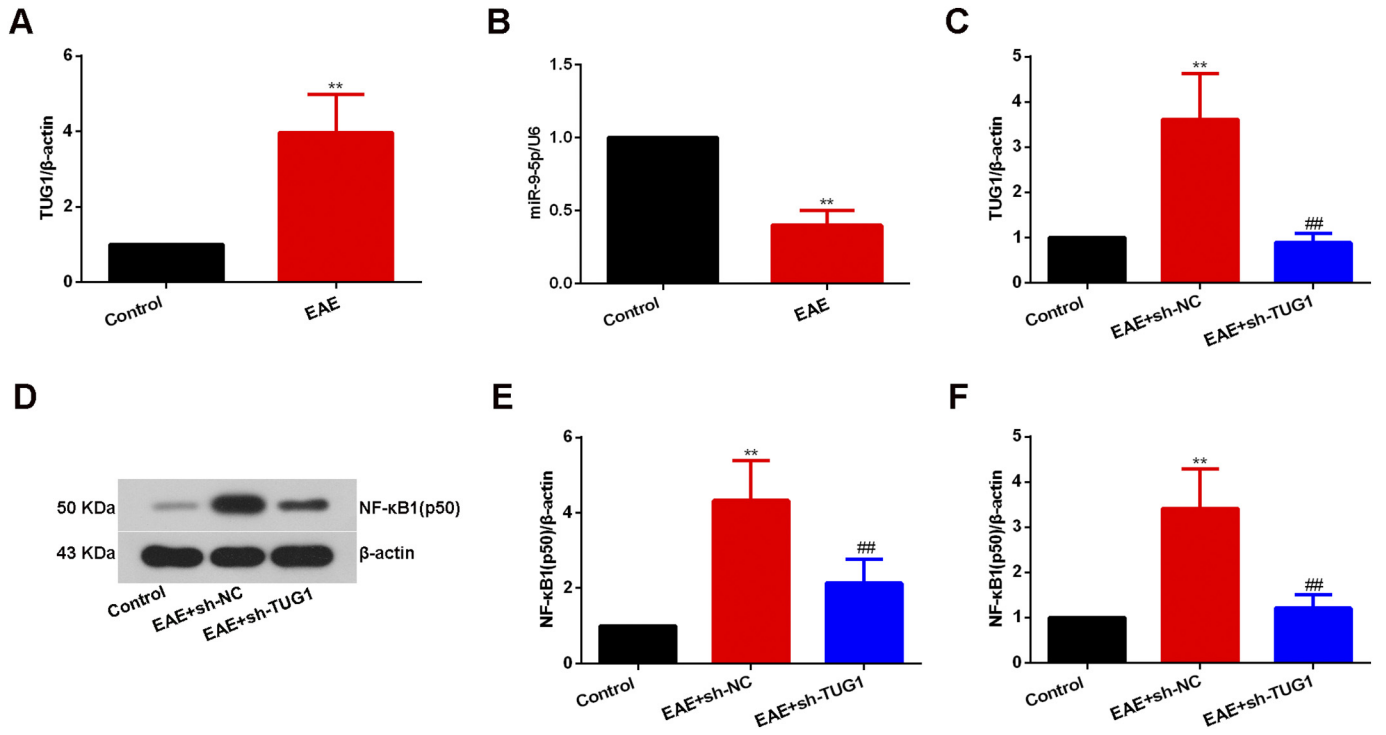


Fig. 1. The expression of TUG1 and NF-κB1/p50 in cerebral cortex of EAE mice. An EAE mouse model was established by immunization with MOG35-55 in this study. (A) Real-time PCR analysis of TUG1 expression in cerebral cortex of control and model mice. (B) Real-time PCR analysis of miR-9-5p expression in cerebral cortex of control and model mice. After intracerebroventricular injection of lentivirus carrying lncRNA TUG1 (sh-TUG1) or negative control (sh-NC) into EAE mice, (C) TUG1 level in cerebral cortex was assessed by real-time PCR. The protein and mRNA expression levels of NF-κB1/p50 were examined by Western blot (D–E) and real-time PCR (F), respectively. Data are presented as means ± SD (n = 6) and analyzed with a *t*-test or one-way analysis of variance followed by Bonferroni's multiple comparisons test. $^{**}p < 0.01$ compared with Control group. $^{\#\#}p < 0.01$ compared with EAE + sh-NC group. EAE, experimental autoimmune encephalomyelitis; TUG1, taurine-up-regulated gene 1.

2.5. Hematoxylin-eosin (H&E) staining

Cerebral cortex were harvested and fixed in 10% formaldehyde. The tissues were then embedded in paraffin and sectioned at 5 μm. After deparaffinization and rehydration, the sections were stained with hematoxylin (Solarbio, China) for 5 min and immersed in ddH2O for 5 min. Then, they were treated with 1% hydrochloric acid for 3 s followed by flushing with tap water for 20 min. After being immersed in ddH2O for 2 min, the sections were subjected to eosin staining (Sangon, China) for 3 min. The staining results were then observed under a microscopy at 200× magnification (Olympus, Japan).

2.6. Enzyme-linked immunosorbent assay (ELISA)

Tissue homogenates or cell culture supernatants were harvested for ELISA assay. The protein concentration was measured using BCA detection kit (Beyotime, China). The expression levels of matrix metalloproteinase-9 (MMP-9), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin (IL)-17, IL-6, IL-10 and granulocyte-macrophage colony stimulating factor (GM-CSF) in tissue homogenates or cell culture supernatants were determined by the commercial ELISA kits according to the manufacture's instruction. The ELISA kit for MMP-9 detection was purchased from Boster Biological Technology Co., Ltd. (Wuhan, China), that for TNF-α , IFN-γ, IL-17 and GM-CSF detections were from MultiSciences (LiankeBio) Co., Ltd. (Hangzhou. China), and that for IL-6 and IL-10 was from Union Biotech (Hangzhou, China).

2.7. Dual luciferase reporter assay

The pmirGLO vectors purchased from Promega (USA) were subcloned by NF-κB1/p50 3′-UTR fragments or negative control sequences, and then co-transfected with miR-9-5p mimic or miR-9-5p mutant (GenePharma, China) into the BV2 cells. Cells were then collected and lysed for luciferase detection 48 h after transfection. Transfections were performed using Lipofectamine 2000 (Invitrogen, USA) according to the manufacture's instruction. To evaluate the binding activity between miR-9-5p and NF-κB1/p50, the relative luciferase activity was calculated according to Firefly and Renilla luciferase activities using commercial detection kit (Promega, USA). Every sample has three duplicates and all detections were performed in triplicate.

The two seed regions of lncRNA TUG1 that binding with miR-9-5p were respectively subcloned into pmirGLO vectors, which termed as pmirGLO-TUG1-seed1-wt and pmirGLO-TUG1-seed2-wt. On the other hand, two mutant vectors containing mutations of miR-9-5p binding sites in the two respective seed regions were constructed, which termed as pmirGLO- TUG1-seed1-mut and pmirGLO- TUG1-seed2-mut. Cells were co-transfected with the constructed pmirGLO vector and miR-9-5p mimic or NC mimic using Lipofectamine 2000 (Invitrogen, USA). The binding activity between lncRNA TUG1 and miR-9-5p was determined through the detection of relative luciferase activity followed the above description.

2.8. Statistical analysis

Data were presented as the mean ± SD and analyzed using a *t*-test or one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test with GraphPad Prism 7.0 software. A $p < 0.05$ was considered statistically significant.

3. Results

3.1. The expression of TUG1 and NF-κB1/p50 in cerebral cortex of EAE mice

An EAE mouse model of MS was established by immunization with MOG35–55 in this study. Real-time PCR analysis showed that the expression level of TUG1 was significantly increased, while miR-9-5p level was decreased in EAE model mice (Fig. 1A–B). After ICV injection of lentivirus carrying sh-TUG1 into EAE mice, the level of TUG1 was remarkably suppressed compared to negative controls that injected with sh-NC (Fig. 1C). Western blotting and real-time PCR analyses showed that the expression of NF-κB1/p50 at both protein (Fig. 1D–E) and mRNA (Fig. 1F) levels were significantly increased in EAE model mice treated with sh-NC, while decreased by TUG1 knockdown.

3.2. ICV injection of lentivirus carrying sh-TUG1attenuates EAE in mice

To study the effect of TUG1 on MS, Lentiviral vectors encoding sh-TUG1 was constructed to suppress TUG1 expression in EAE mice by ICV injection. Lentivirus carrying nonsense gene sequence (sh-NC) was used as negative control. The morphological changes in cerebral cortex were observed by H&E staining (Fig. 2A). Compared to the normal structure displayed in the control rats, there was a mass of inflammatory cell infiltrates with aggregated inflammatory cells and a widespread distribution in cerebral cortex of EAE mice treated with sh-NC lentivirus. While EAE mice injected with sh-TUG1 lentivirus showed reduced inflammatory infiltration and less inflammatory cells with small quantity distribution. Additionally, ELISA was performed to detect the levels of pro-inflammatory cytokines including TNF-α, IL-6, IL-17 and IFN-γ (Fig. 2B–E), encephalitogenic cytokine GM-CSF (Fig. 2F), and anti-inflammatory cytokine IL-10 (Fig. 2G) in cerebral cortex of mice. Compared to control mice, EAE mice treated with sh-NC lentivirus exhibited up-regulated TNF-α, IL-6, IL-17, IFN-γ and GM-CSF levels, and down-regulated IL-10 level. Down-regulation of TUG1 was found to attenuate EAE-induced inflammation in cerebral cortex of EAE mice. The level of MMP9 was increased in EAE mice but decreased following TUG1 knockdown (Fig. 2H). The effects of lncRNA TUG1 on clinical signs in EAE mice were evaluated and the clinical scores were presented in Fig. 2I. No sign was observed in the control rats treated with PBS during 30 days. In comparison, EAE mice injected with sh-NC or sh-TUG1 lentivirus showed obvious higher clinical scores throughout the duration of the examination. However, a significant reduction in clinical scores was observed in EAE rats treated with sh-TUG1 lentivirus compared to those treated with sh-NC following day 10.

3.3. The expression of lncRNA TUG1 and miR-9-5p in LPS-induced BV2 cells

For LPS induction, BV2 cells were treated with 100 ng/ml LPS for 24 h. Real-time PCR analysis showed that LPS treatment induced up-regulation of lncRNA TUG1 (Fig. 3A) and down-regulation of miR-9-5p (Fig. 3B) in BV2 cells. ELISA was performed to assess the levels of TNF-α and IL-6 (Fig. 3C–D). The results showed that both levels were significantly increased in LPS-induced BV2 cells compared with the controls.

3.4. Down-regulation of TUG1 inhibits LPS-induced inflammation in BV2 cells via sponging miR-9-5p

To further explore the role and mechanism of lncRNA TUG1 in LPS-treated BV2 cells, BV2 cells were infected with lentivirus containing sh-TUG1 or sh-NC and then transfected with miR-9-5p inhibitor or NC inhibitor followed by LPS treatment. Real-time PCR was performed to detect the efficiency of lentivirus infection and cell transfection. The expression of TUG1 was significantly suppressed in LPS-treated BV2 cells infected with lentivirus carrying sh-TUG1 (Fig. 4A). The expression of miR-9-5p was significantly elevated by knockdown of TUG1 in LPS-induced BV2 cells, while reduced following transfection with miR-9-5p inhibitor (Fig. 4B). ELISA analysis showed that down-regulation of TUG1 decreased TNF-α and IL-6 levels in LPS-induced BV2 cells, while inhibition of miR-9-5p abolished the suppressive effect on these inflammatory cytokines induced by TUG1 knockdown (Fig. 4C–D). The expression of NF-κB1/p50 at both protein and mRNA level was inhibited by silencing of TUG1, while significantly increased by further depletion of miR-9-5p in BV2 cells with LPS treatment (Fig. 4E–G). Moreover, dural-luciferase assay was performed to determine the specific binding activity between lncRNA TUG1 and miR-9-5p. The two seed regions of TUG1 containing miR-9-5p binding sites and the corresponding mutation sites were displayed in Fig. 4H. The results showed that, in both two TUG1-seed-wt reporter systems, the relative luciferase activity was significantly lower in cells transfected with miR-9-5p mimic than that transfected with NC mimic (Fig. 4I–J). In comparison, no difference was observed in either of TUG1-seed-mut reporter systems. These results indicated that lncRNA TUG1 acted as the miR-9-5p sponge and negatively regulated its expression, and down-regulation of TUG1 inhibited LPS-induced inflammation via up-regulation of miR-9-5p in BV2 cells.

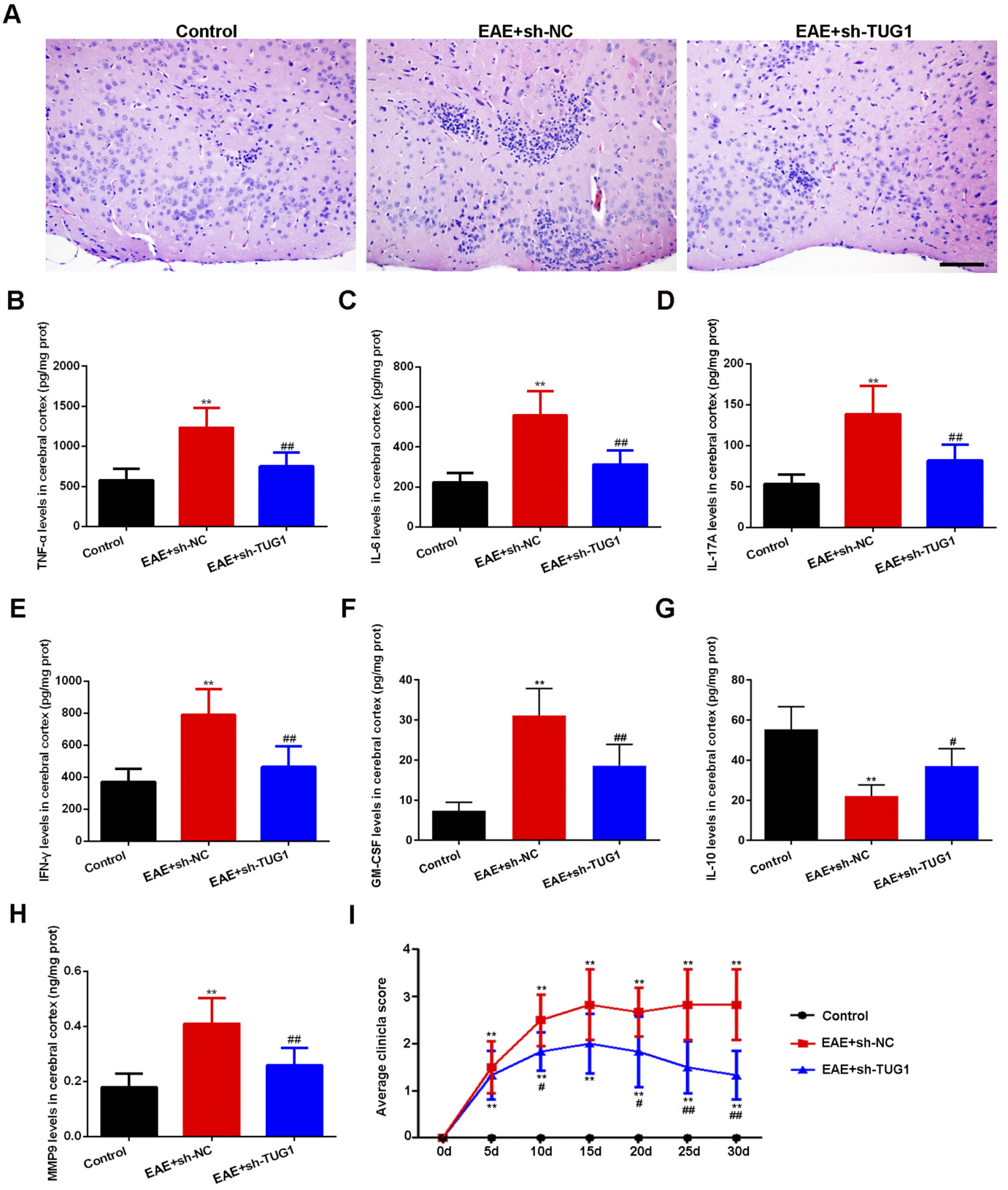
3.5. Enforced expression of miR-9-5p inhibited LPS-induced inflammation in BV2 cells

To study the influence of miR-9-5p in LPS-treated BV cells, miR-9-5p was overexpressed in BV2 cells by transfection with miR-9-5p mimic. Transfection with NC mimic was used as negative control. Twenty four hours after transfection, the cells were treated with 100 ng/ml LPS for 24 h. The transfection efficiency was examined using real-time PCR, and the results showed that miR-9-5p expression was significantly up-regulated in LPS-induced BV2 cells transfected with miR-9-5p mimic compared with negative controls (Fig. 5A). The levels of TNF-α and IL-6 were both lowered in LPS-induced BV2 cells with enforced expression of miR-9-5p (Fig. 5B–C). Real-time PCR and Western blotting analysis showed that the expression of NF-κB1/p50 was decreased by up-regulation of miR-9-5p in LPS-treated BV2 cells (Fig. 5D–F). Further, the binding activity between NF-κB1/p50 and miR-9-5p was assessed by dural-luciferase assay. The sequence of miR-9-5p and NF-κB1/p50 with their binding sites and mutation sites was showed in Fig. 5G. The results showed that the relative luciferase activity was significantly weakened following co-transfection with NF-κB1/p50 report vector and miR-9-5p mimic, suggesting NF-κB1/p50 as a direct target of miR-9-5p (Fig. 5H). These results indicated that miR-9-5p inhibited LPS-induced inflammation in BV2 cells by targeting NF-κB1/p50.

4. Discussion

In the present study, we studied the role of TUG1 in MS using a MOG-EAE mouse model of MS induced by immunization with MOG35-55. Down-regulation of TUG1 mitigated MS by decreasing the levels inflammatory cytokines and improving motor function of EAE mice. Further in vitro study using LPS-induced BV2 cells to mimic MS showed that TUG1 mediated inflammation by sponging miR-9-5p via targeting NF-κB1/p50. Therefore, we demonstrate a mechanism TUG1/miR-9-5p/NF-κB1/p50 in MS and suggest that TUG1 is a candidate therapeutic target for the treatment of MS.

LncRNAs have been reported to be implicated in multiple biological function and dysfunction such as cell proliferation, inflammation, morphogenesis, pluripotency and development, as well as a variety of human diseases including cancer, cardiovascular and neurological disease [12]. Thus, the study of lncRNAs is emerging as the focus for human diseases in recent years. LncRNA TUG1 was initially demonstrated to play essential roles in nervous system and retinal development [13]. Dysregulation of TUG1 is suggested to be strongly correlated



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with pathogenesis of human disease. TUG1 was aberrantly upregulated in neurodegenerative diseases such as Huntington's disease [14], inflammatory diseases such as MS [8], vascular disease such as atherosclerosis [15] and cancers such as glioma [16]. However, the exact function of TUG1 in MS and the underlying mechanism remain largely unidentified. In this study, an EAE mouse model was induced by immunizing mice with MOG35-55 peptide. The level of TUG1 was found to be significantly higher in cerebral cortex of EAE mice than that of

Fig. 2. Intracerebroventricular injection of lentivirus carrying sh-TUG1 ameliorates EAE in mice. To study the role of TUG1 in MS, lentiviral vectors encoding sh-TUG1 was constructed to silence TUG1 in EAE mouse model of MS by intracerebroventricular injection Lentivirus carrying nonsense gene sequence (sh-NC) was used as negative control. (A) H&E staining was performed and observed under a microscopy at 200× magnification to assess the morphological changes in cerebral cortex of mice. ELISA analysis was preformed to examine the levels of pro-inflammatory cytokines including TNF-α (B), IL-6 (C), IL-17 (D) and IFN-γ (E), encephalitogenic cytokine GM-CSF (F) and anti-inflammatory cytokine IL-10 (G). (H) The level of MMP9 in cerebral cortex of mice was detected by ELISA. (I) Following knockdown of TUG1, the clinical signs were scored every 5 days for 30 days to evaluate mice movement. A clinical MS scoring system was established using a standard scale of 0–4 as follows: 0, no clinical sign; 1, limp tail; 2, paralysis of single hind limb; 3, paralysis of both hind limbs; 4, paralysis of trunk. Data are presented as means ± SD (n = 6) and analyzed with one-way analysis of variance followed by Bonferroni's multiple comparisons test. $^{**}p < 0.01$ compared with Control group. $^{\#\#}p < 0.01$ compared with EAE + sh-NC group. Scale bar = 100 μm. MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; TUG1, taurine-up-regulated gene 1; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; IL, interleukin; GM-CSF, granulocyte-macrophage colony stimulating factor.

controls and previous findings has showed that TUG1 is overexpressed in MS patients [8,17], indicating that MOG-EAE could be a mouse model used for studying TUG1's function in MS in vivo. In the present study, EAE mice exhibited abnormal elevations in the levels of encephalitogenic cytokine GM-CSF and inflammatory cytokine TNF-α, IL-6, IL-17 and IFN-γ, reduction in anti-inflammatory cytokine IL-10 as well as impaired motor function. These results indicated EAE-induced inflammation in the brain of mice, and the EAE mouse model in our study was suggested to be established successfully.

The pathology of MS is associated with the peripheral activation of Th1 and Th17 cells, and their subsequent recruitment into CNS [18]. The dysfunction of regulatory T (Treg) cells is one of the reasons that induce the loss of peripheral tolerance and involved in MS pathology as well [19]. To study the effect of TUG1 on inflammatory response, the present study examined the levels of TNF-α, IL-6, IL-17, IFN-γ, GM-CSF and IL-10. They are typical pro-inflammatory or anti-inflammatory cytokines involved in regulation of inflammation, which are crucial contributor to the pathogenesis of autoimmune diseases [20–23]. GM-CSF is known as one of the most important encephalitogenic cytokine involve in CNS autoimmunity [24]. Inhibition of GM-CSF resulted in amelioration of MS progression in EAE mouse model [25]. Elevated Th17 cells have been found in patients with atopic dermatitis, Crohn's disease, rheumatoid arthritis (RA), psoriasis, and multiple sclerosis (MS) [18,26–29]. Experimental model have shown that Th17 cells by producing IL-17A and encephalitogenic cytokine GM-CSF are important pathogenic factor leading to autoimmune disease such as experimental autoimmune encephalomyelitis (EAE) [30–32]. Determination of the cerebral levels of IFN-γ (a Th1 cytokine), IL-17 (a Th17 cytokine), and IL-10 (a Treg cytokine) will help to elucidate the effects of TUG1 silencing on T helper cell subsets in the CNS of EAE mice. We found that cerebral IFN-γ and IL-17 were upregulated in EAE mice, and down-regulated following TUG1 knockdown, while IL-10 behaved in an opposite manner. These data at least suggest that TUG1 silencing partly restores the Th1/Th2/Treg imbalance in the CNS of EAE mice, and thus contributes to the maintenance of immune homeostasis. TUG1 was suggested to promote inflammation [33] and TUG1 knockdown was revealed to reduce inflammatory response and thus ameliorate atherosclerosis [34]. Our results together with previous findings demonstrate that down-regulation of TUG1 inhibits inflammation, which thus contributes to improvement of behavioral performance of EAE mice. Thus,

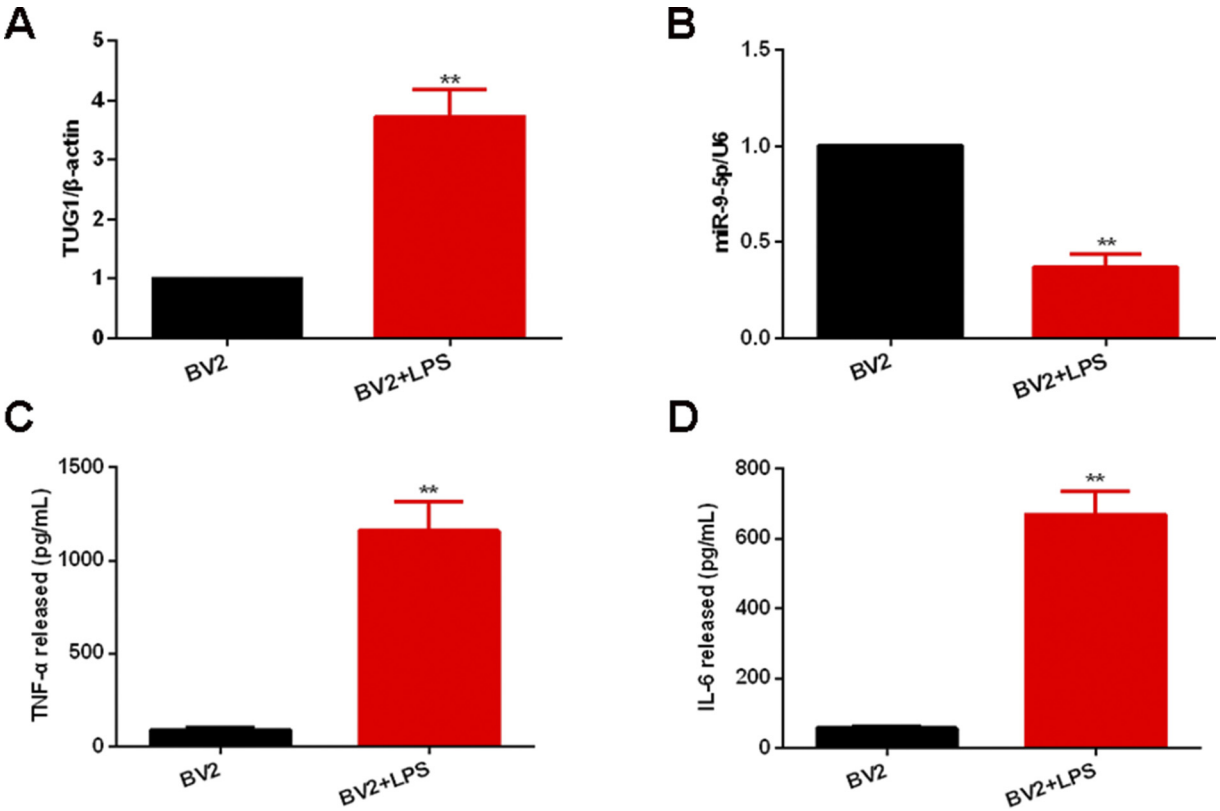

Fig. 3. The expression of lncRNA TUG1 and miR-9-5p in LPS-induced BV2 cells. LPS-induced BV2 cells were used to mimic MS for in vitro study. (A) Real-time PCR analysis of lncRNA TUG1 in BV2 cells with or without LPS treatment. (B) Real-time PCR analysis of miR-9-5p in BV2 cells with or without LPS treatment. The levels of TNF-α (C) and IL-6 (D) in cell medium of each group were examined by ELISA. Data are presented as means ± SD (n = 3) and analyzed with a *t*-test. $^{**}p < 0.01$ compared with BV2 group. TUG1, taurine-up-regulated gene 1; TNF-α, tumor necrosis factor-α; IL, interleukin.

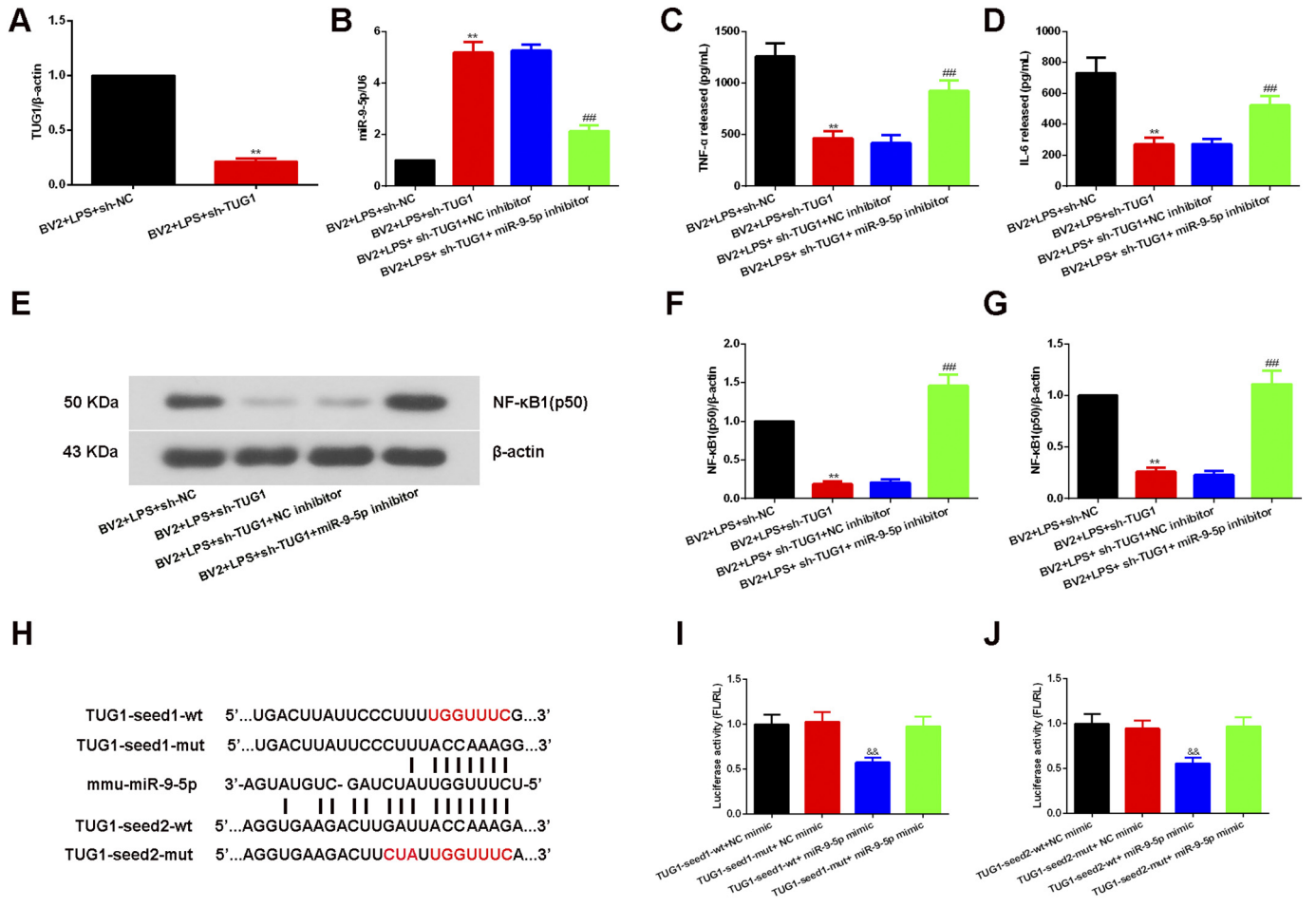

Fig. 4. Down-regulation of TUG1 inhibits LPS-induced inflammation in BV2 cells via sponging miR-9-5p. To study the role and mechanism of lncRNA TUG1 in LPS-treated BV2 cells, BV2 cells were infected with lentivirus carrying sh-TUG1 or negative control (sh-NC) and then transfected with miR-9-5p inhibitor or NC inhibitor, followed by 100 ng/ml LPS treatment. (A) Real-time PCR analysis of lncNRA TUG1 expression level. (B) Real-time PCR analysis of miR-9-5p expression level. The levels of TNF-α (C) and IL-6 (D) in cell medium of each group were examined by ELISA. Western blot (E–F) and real-time PCR (G) was performed to detect NF-κB1/p50 at the protein and mRNA expression levels, respectively. (H) The binding sites of miR-574-5p in two seed regions of TUG1 and the corresponding mutant sequences. (I–J) Dural-luciferase assay was performed to verify the binding activity between lncRNA TUG1 and miR-9-5p. Data are presented as means ± SD (n = 3) and analyzed with a *t*-test or one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. $^{**}p < 0.01$ compared with BV2 + LPS + sh-NC group. $^{\#\#}p < 0.01$ compared with BV2 + LPS + sh-TUG1 + NC inhibitor. $^{\&\&}p < 0.01$ compared with TUG1-seed-mut + miR-9-5p mimic group. TUG1, taurine-up-regulated gene 1; TNF-α, tumor necrosis factor-α; IL, interleukin.

TUG1 could be a potential therapeutic target in MS.

Notably, the expression of TUG1 was found to be negatively correlated with miR-9-5p expression, while positively correlated with NF-κB1/p50. These findings suggest that miR-9-5p and NF-κB1/p50 might be in involved in the biological actions of TUG1 in MS. NF-κB is identified as the first transcription factor that acts as a key regulator of inflammation [35]. NF-κB1/p50, as the member of NF-κB family, is highly expressed at sites of inflammation and regulates the proliferation, activation, and cytokine production of immune responses [36,37]. The coordination of the expression of immune-related genes that regulate the functions of inflammatory cells is necessary for the development of autoimmune disease such as MS. The activation of NF-κB cascade was observed to be increased in patients with MS [36]. A study demonstrated that NF-κB1-deficient mice showed less incidence of EAE induced by MOG, suggesting that NF-κB regulated autoimmune inflammation in the CNS [38]. Imbalance of Treg/Th17 is an important factor that drives autoimmune disease [39]. NF-κB signaling is suggested to play an important role in restricting T cell activation and Th17 differentiation [40,41]. Studies showed that inhibition of NF-κB activation ameliorated inflammatory diseases through suppressing TH17 cell differentiation and enhancing Treg cell differentiation, and thus impaired Treg/Th17 balance [42,43]. In our study, TUG1 knockdown suppressed the expression of NF-κB1/p50 in EAE mice, accompanied with reductions in pro-inflammatory cytokine TNF-α, IL-6, IL-17, IFN-γ and GM-CSF and increase in anti-inflammatory cytokine IL-10. Moreover, NF-κB has been identified to be a key regulator of the production and activity of MMPs, which is associated with various cytokines including TNF-α and IL-17A [44–46]. The level of MMP9 was found increased in EAE mice while decreased following knockdown of TUG1. These results imply that downregulation of TUG1 may attenuate EAE-induced inflammation via restoring Th17/Treg balance through NF-κB signaling pathway. Therefore, NF-κB signaling pathway, which is responsible for the regulation of inflammatory response in the CNS, plays an important role in MS.

MS is known as an autoimmune disease characterized by inflammation and demyelination of the CNS [47], and microglia are the major effectors that regulate inflammatory process in the CNS, which will release inflammatory cytokines to modulate inflammation while it is activated [48]. BV2 cells, which are microglial cells from murine microglia, have been used as a model of microglia in vitro study [49]. To further explore the function and underlying mechanisms of TUG1 in MS, in vitro cell study to mimic MS was performed using LPS-induced BV2 microglia cells. The expression of TUG1 was increased and miR-9-5p was decreased in BV2 cells with LPS treatment. Moreover, the levels

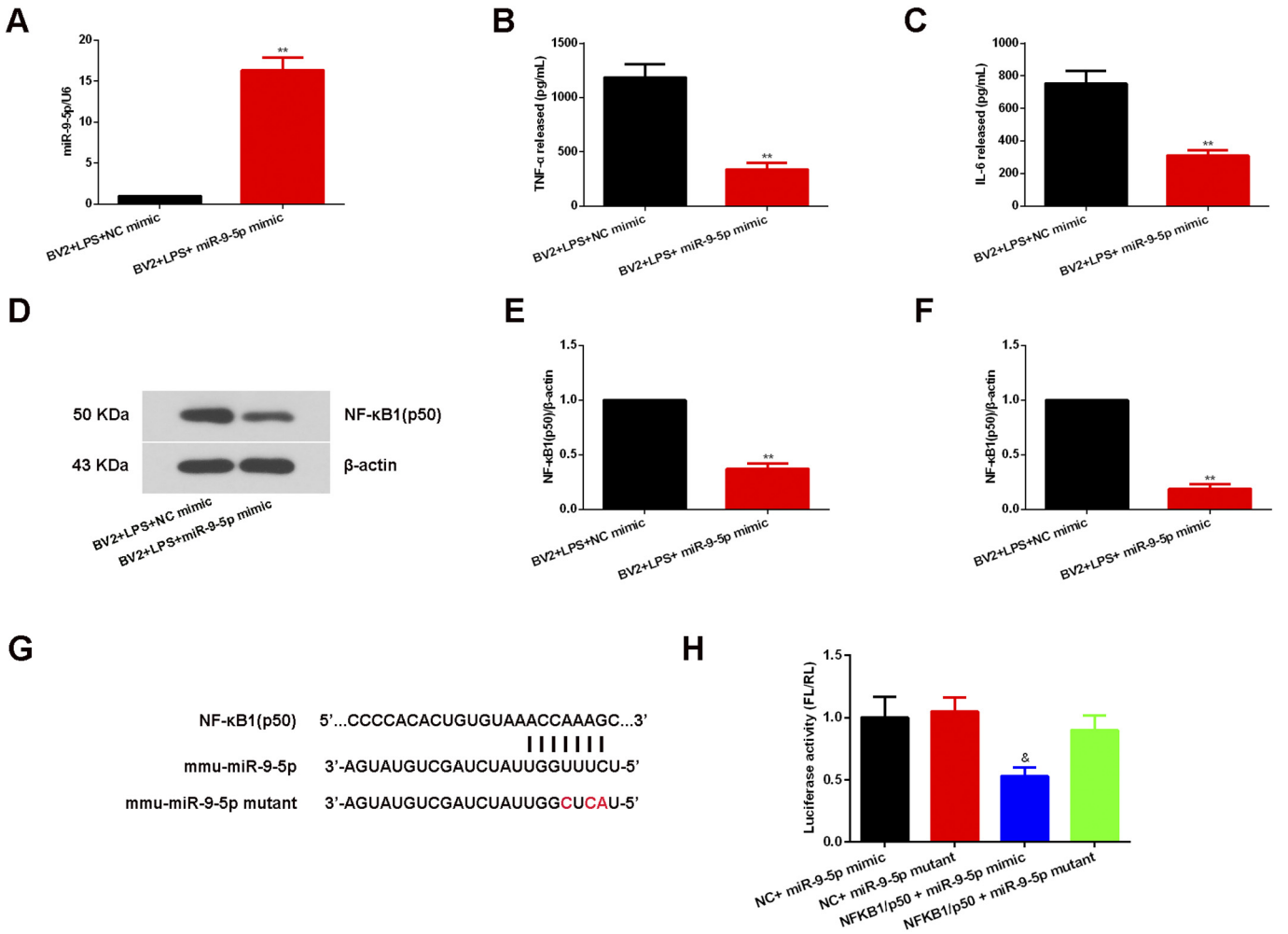

Fig. 5. Enforced expression of miR-9-5p inhibits LPS-induced inflammation in BV2 cells. To study the influence of miR-9-5p in LPS-treated BV cells, BV2 cells were transfected with miR-9-5p mimic or negative control (NC) mimic followed by 100 ng/ml LPS treatment. (A) The expression level of miR-9-5p was detected by real-time PCR. The levels of TNF-α (B) and IL-6 (C) in cell medium of each group were examined using ELISA. The protein (D–E) and mRNA expression (F) levels of NF-κB1/p50 were examined by Western blot and real-time PCR, respectively. (G) The sequence of miR-9-5p and NF-κB1/p50 with their binding sites and mutation sites. (H) The binding activity between miR-9-5p and NF-κB1/p50 was determined by dural-luciferase activity. Data are presented as means ± SD (n = 3) and analyzed with a *t*-test. **p < 0.01 compared with BV2 + LPS + NC mimic. $^{\&}$p < 0.05 compared with NF-κB1/p50 + miR-9-5p mutant. TNF-α, tumor necrosis factor-α; IL, interleukin.

of inflammatory cytokines including TNF-α and IL-6 were significantly up-regulated in LPS-treated BV2 cells, suggesting that LPS treatment induced inflammatory injury in BV2 cells. Further depletion of TUG1 inhibited LPS-induced inflammation in BV2 cells, as evidenced by decreased levels of TNF-α and IL-6. In line with our results, previous studies reported that LPS-induced inflammation was mitigated by up-regulating lncRNA TUG1 [50,51].

Emerging insights into lncRNAs have revealed that they are able to regulate gene expression at the transcriptional, post-transcriptional and epigenetic levels. Some lncRNAs could act as sponges for the miRNAs, which contain miRNA-binding sites, to modulate miRNAs expression and biological functions, and regulate miRNAs-targeted downstream genes as well [52,53]. These lncRNAs therefore are referred as competing endogenous RNA. Recent studies have illustrated the potential role of lncRNA TUG1 and the TUG1-miRNA interaction in relation to pathogenesis of diseases [34]. LncRNA TUG1 was found to sponge miR-9 to promote neurons apoptosis under ischemia [54]. LncRNA TUG1 facilitated osteosarcoma via regulation of POU2F1 expression by sponging miR-9-5p [55]. In the present study, TUG1 was knocked down in LPS-induced BV2 cells to explore the underlying mechanism of its regulatory function in MS. The level of miR-9-5p was significantly increased by down-regulation of TUG1, and inhibition of miR-9-5p abolished the suppression of inflammation induced by TUG1 knockdown. Further, dural-luciferase assay confirmed the interplay of TUG1 and miR-9-5p that TUG1 acted as a miR-9-5p sponge and negatively regulated its expression. In accordance with previous findings, we suggest that lncRNA TUG1 inhibits inflammation by acting as miR-9-5p sponge in MS. Moreover, enforced up-regulation of miR-9-5p inhibited LPS-induced inflammation and suppressed the expression of NF-κB1/p50, and NF-κB1/p50 was verified as a direct target gene of miR-9-5p. Thus, miR-9-5p negatively regulated NF-κB1/p50 expression by directly targeting its 3'UTR. In agreement with our findings, Daohai Qian et al. [56] found that miR-9-5p targeted NF-κB1/p50 gene and inhibited the NF-κB signaling pathway in bone marrow-derived mesenchymal stem cells. Taken together, we suggest that knockdown of TUG1 attenuates MS-induced inflammation via sponging miR-9-5p by down-regulation of NF-κB1/p50.

5. Conclusion

The present study demonstrates that down-regulation of TUG1 ameliorates inflammation in MS by up-regulation of miR-9-5p via targeting NF-κB1/p50 and inhibiting its regulatory function as shown in Fig. 6. These findings suggest a mechanism TUG1/miR-9-5p/NF-κB1/

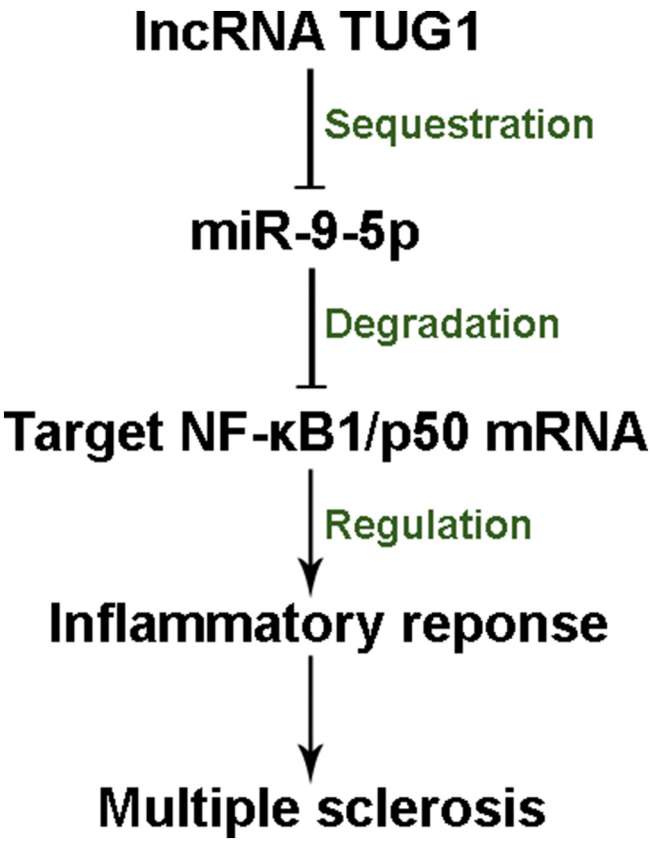

Fig. 6. A schematic diagram of TUG1/miR-9-5p/NF-κB1/p50 axis in multiple sclerosis. Down-regulation of TUG1 could attenuate multiple sclerosis though inhibition of inflammation by up-regulation of miR-9-5p via targeting NF-κB1/p50.

p50 in MS, which may shed a new light on the pathogenesis of MS and provide potential therapeutic targets for the treatment of MS.

Acknowledgement

This study was supported by grants from the National Natural Science Foundation of China (No. 81501109) and the First Affiliated Hospital of Zhengzhou University.

Declaration of competing interest

The authors declare no conflict of interest with the content of this article.

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